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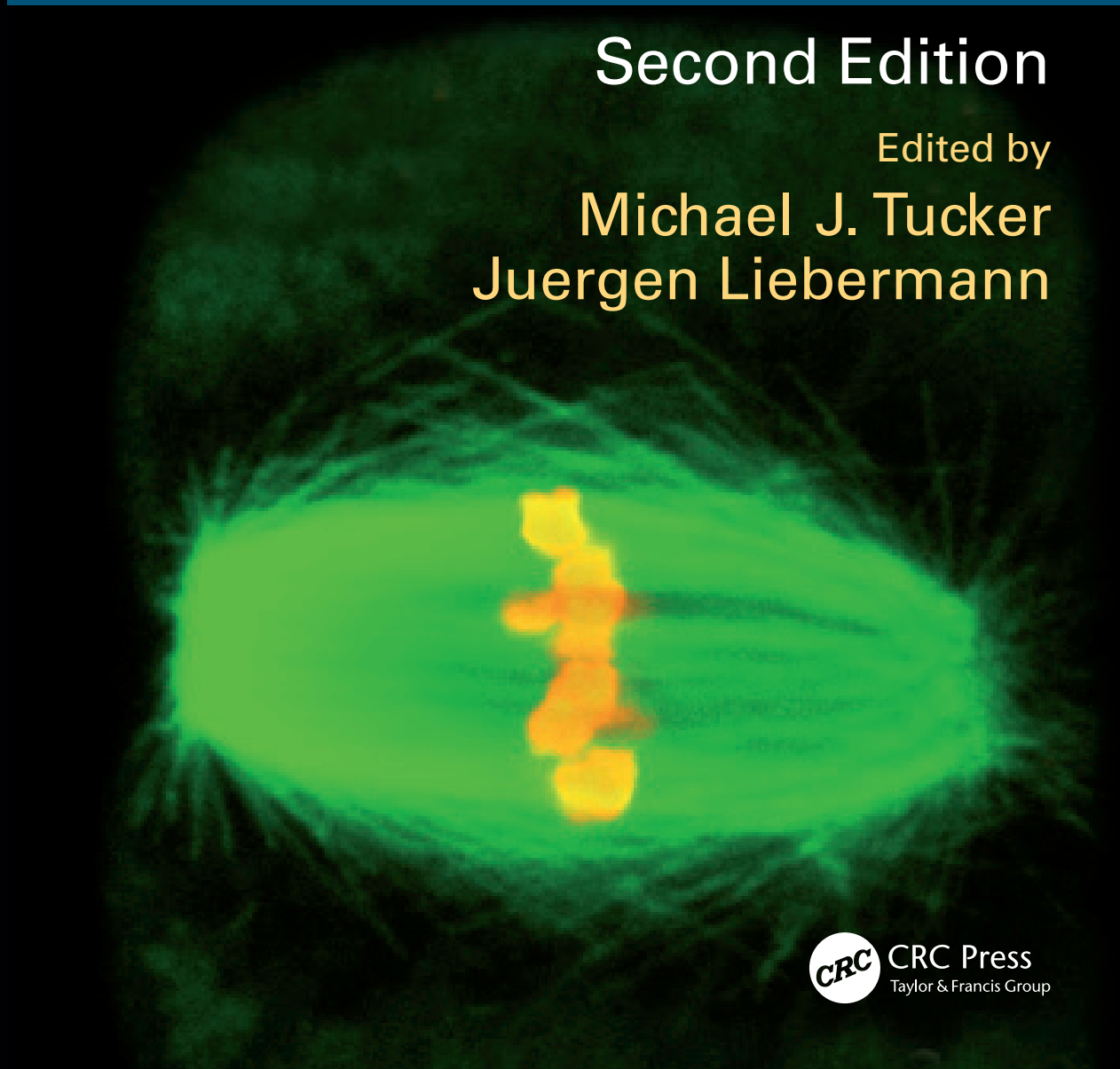
Vitrification in Assisted Reproduction

Second Edition

Edited by

Michael J. Tucker
Juergen Liebermann



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Michael J. Tucker wishes to recognize his friends and family whose love and support helped make this new edition happen, especially the indefatigable Juergen Liebermann, and of course, his wonderful wife Megan and children Harry, Nick, Isabella, and Eliza, who had to suffer through all his complaints!

Juergen Liebermann gives most sincere thanks to his wife Maike and his sons and daughter Richard, Lennart Martin, Tobias Georg, Alexander Johannes, and Franziska Sophie for providing unfailing support and encouragement to bring this work to reality.

Contents

Foreword

In vitro fertilization (IVF) is, in the majority of cases, the most direct and effective solution to the current epidemic of infertility surreptitiously plaguing modern societies and wreaking havoc with their economies, while causing the grief of unwanted childlessness to ruin people's lives. Of course, IVF also poses a host of problems, but many of these can now be resolved with vitrification.

For example, most women will have more than one embryo, and before clinical use of vitrification, slow freezing of surplus embryos was problematic, yielding only inconsistent survival and pregnancy rates. Consequently, there was a strong temptation to transfer more than one or two fresh embryos to increase chances of pregnancy—thus elevating the risk of a high order of multiple pregnancies, which, as an unintended consequence, has been IVF's biggest problem. However, with the more recent introduction of vitrification technology, we are able to cryopreserve extra embryos that are not transferred, and to assure patients that this process will neither damage their embryos nor necessarily lower their ultimate cumulative pregnancy rate. Indeed, clinical experience is now showing that pregnancy rates from "frozen/thawed" embryos may be even higher than those from "fresh" embryos.

The benefits of vitrification also extend beyond embryo cryopreservation, being applicable to both oocytes and ovarian tissue freezing, and including even the whole ovary. "Egg freezing" had always been somewhat of an unfulfilled dream, whereas, at least with embryo slow freezing (the norm for two decades), clinical outcomes following thawing yielded adequate survival much of the time, but still gave a distinctly lower pregnancy rate than did transfer of fresh embryos. Slow freezing of oocytes was never able to provide clinically reliable outcomes, and remained largely sidelined as a routine procedure. The reasons are severalfold: (1) the mature oocyte is the biggest cell in the body and contains a high proportion of water, making it difficult to remove enough water during slow freezing to avoid ice crystal formation; (2) the mature oocyte, with chromosomes precariously aligned on the metaphase II spindle, is extremely sensitive to damage from ice crystal formation and may easily be disrupted; (3) the oocyte is also exquisitely vulnerable to mild chilling, which may cause significant compromise or even complete degeneration. So application of an "ultra-rapid freeze" approach with vitrification allows a consistent solution to all these problems, and has ushered in a new era of successful "egg freezing." This has kickstarted the "donor egg bank" industry, has enabled easy use of vitrification to cryostore eggs if a husband is unable to provide sperm on the day of an IVF egg retrieval, and, perhaps most importantly and of greatest media interest, has addressed the "ticking biological clock" haunting the reproductive potential of women.

The major cause of the current infertility epidemic is that women are putting off childbearing until they are older, because of better opportunities for career and education. Many women today do not consider having a baby until their mid-thirties or even later, and, by then, over 20% are infertile; by age 40, the vast majority are infertile simply because their eggs have aged significantly. However, cancer patients who have had their eggs or ovary frozen before undergoing sterilizing chemotherapy and radiation do not have to worry about this widespread concern, because they have young eggs or ovary safely in the freezer. With vitrification, we can now achieve, in all women, that dream of safely preserving fertility in youth to protect them from the biological clock's relentless progress.

Innovating vitrification in the 1980s, and then perfecting it so that it would be more reliable in the early 2000s, was not an easy journey for its clinical pioneers, such as Rall and Fahy in the mid-1980s in the United States, and Kuwayama in Argentina and Japan in the 1990s, and up to the present. IVF clinicians were reluctant to leave the comfortable space of old ideas and the use of "slow freezing," and often ridiculed vitrification; after all, it seemed too easy, when in fact it was very difficult, simply because it has to be just exactly right to achieve success. It took much experimentation with many protocols; and having found the right one, it was most important not to tamper with it and not to deviate from the details. Cryobiology can be a very empirical science, with much painstaking trial and error (of course, based on scientifically derived postulates) over many years until you finally have the knowledge to get it right. It is not an easy procedure, with numerous points in the vitrification process of just one oocyte, and if a step is inadvertently not carried out perfectly, it spells disaster for that oocyte.

The purpose of this book, therefore, is to provide readers with all the information they will need to perfect vitrification in their own laboratories. Dr. Michael Tucker

and Dr. Juergen Liebermann include every major scientist who has worked on vitrification in recent times, and describe how these individuals brought us closer to this clinical breakthrough. In addition, readers will understand the controversies and pitfalls of the process, and how to resolve and avoid such issues, allowing them to carry out a perfect job in their own IVF lab and make the dream of preserving fertility a clinical reality.

Dr. Sherman Silber
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Preface

Assisted reproductive technology (ART) has been evolving for more than 30 years, and cryopreservation has become one of the keystones in such clinical infertility treatment. However, the true "explosion" in many embryologists' minds in recognizing the true value of cryopreservation occurred with the appearance of vitrification techniques in the mid-1980s. Very gradually since that time, vitrification has established itself as "the cryopreservation technique of choice," through consistency and predictability in terms of the quality of the cell survival following the vitrified "state of suspended animation." Using a vitrification approach, oocyte freezing has become a reality and routine, not to mention embryo vitrification, which in turn provides outcomes comparable to those achieved with fresh transferred embryos. Furthermore, vitrification has opened the doors for new areas in the field of ART such as oocyte banking, preimplantion diagnostics at the blastocyst stage, and notably it has helped to fuel the burgeoning adoption of elective single-embryo transfers. This final area of impact is probably the most important development, because it defines "quality of ART" in a new light—the delivery of a healthy single baby in each IVF cycle.

The success of cryopreservation in benefiting the very specialized field of ART has arisen only after much effort and is nothing short of a small miracle, given that it relies on such an intensive cooperation between science and clinical application. The development of gamete and embryo vitrification specifically represents the triumphant culmination of several decades of frustrating exploration with "slow freeze" technology. The first edition of this book, published in 2007, was subtitled: "A user manual and trouble-shooting guide." This second edition, being published more than 7 years later, has evolved into a more comprehensive book now containing chapters reporting the successful application of vitrification from research up to the level of its use in routine clinical therapy. We as editors of this book are excited, pleased, and grateful to have chapters contributed by scientists and clinical embryologists who have been involved in the development of vitrification at the very highest level. The list of contributors demonstrates a global collaboration of professionals who have given their time, energy, and intellect to share their scientific observations and clinical experiences with the future readership of this book and beyond.

Our destiny working in the field of IVF every single day is based on strong values, and one of these is to fulfill the dreams of our patients in creating healthy families for them; vitrification plays an increasingly essential role in achieving this goal.

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1 Overview of biological vitrification

Gregory M. Fahy

INTRODUCTION

Since 1984, vitrification has been used with rapidly expanding frequency to accomplish cryopreservation in a way that was not previously possible (Figure 1.1). The expected number of new citations of vitrification to appear in PubMed from the time the previous edition of the present volume was published to the end of 2014 is projected to be in the vicinity of 1600–1700 papers, if not more. It is not possible for a short review to encompass all of the findings in this rapidly expanding literature, or even just all of the findings in the realm of reproductive cryobiology, but it is possible to place this research into a broader perspective, and that is the goal of the present chapter. It is worth noting, however, that the general popularity of vitrification was originally based on its efficacy for preserving embryos,[1] and that a major portion of the continuing interest in this technique is sustained by reproductive cryobiologists. There is now a vast literature describing the present use of vitrification for the routine cryopreservation of human[2,3] and animal[3] embryos, and for more experimental but increasingly routine cryopreservation of ovarian tissue,[4,5] ova,[6–8] and even small whole ovaries.[9]

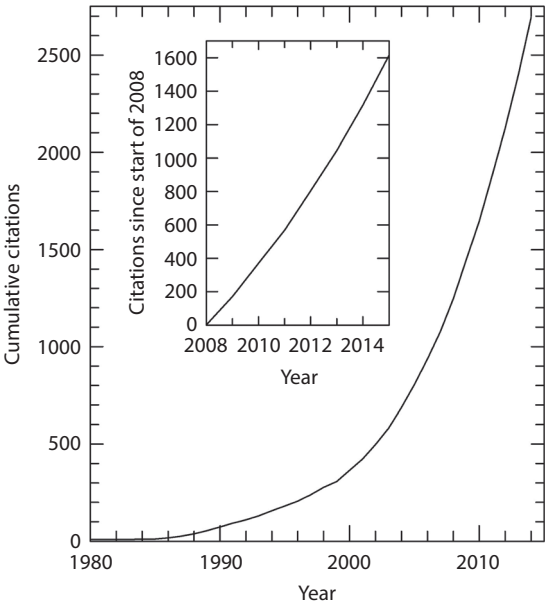

Figure 1.1 **Cumulative citations of vitrification as a method of cryopreservation in PubMed since 1980 (main figure), and since the beginning of 2008 (inset). (Modified from earlier versions Fahy GM, Rall WF. Vitrification: An overview. In Tucker MJ, Liebermann J (eds.),** ***Assisted Reproduction: A User's Manual and Troubleshooting Guide.*** **Informa Healthcare: London, 2007. p. 1–20; With kind permission from Springer Science+Business Media:** ***Cryopreservation and Freeze Drying Protocols,*** **Principles of cryopreservation by vitrification, 2015, Fahy GM, Wowk B. Wolkers WF, Oldenhof H (eds.); Fahy GM, Wowk B, Wu J.** ***Rejuvenation Res*** **2006;9:279–91.)**

In this chapter, an overview is provided concerning the precedent for artificial vitrification that is represented by examples of vitrification or the potential for vitrification in the natural world, the advantages and disadvantages of vitrification, the different eras of research in applied approaches to vitrification, the general physical principles governing vitrification and devitrification, and basic methodological and theoretical considerations for maximizing biological viability while carrying out vitrification procedures. It is hoped that these perspectives will assist investigators in their design of more successful methods based on a better understanding of the physical and biological principles of vitrification.

VITRIFICATION UNDER NATURAL CONDITIONS

Despite concerns over global warming, the record for the lowest temperature ever measured on Earth, previously standing at −89.3°C,[10] was recently bested by over 5 degrees at −94.7°C in eastern Antarctica.[11] The lowest temperature recorded so far in Alaska, −79.8°C[11], is just below the sublimation temperature of dry ice. More commonly, the lowest environmental temperatures encountered are between −30 and −60°C.[10,12] By comparison, glass transition temperatures (T_Gs) higher than −50°C have frequently been measured in living organisms[13–17] and have been documented in freeze-concentrated mammalian tissue.[18] Furthermore, "poikilohydric" species, whose water content declines with declining ambient humidity, can experience apparent cytoplasmic vitrification in connection with partial drying at both low and high temperatures,[10,17,19–23] in the most extreme cases even allowing survival in nature under high-temperature conditions.[24,25] In view of such observations, many have considered it plausible that many species may overwinter in a partly or completely vitrified state,[13,14] and even that this is a more common natural strategy for survival than freeze tolerance.[10,23] Such a possibility derives further support from observations in plants by investigators

such as Sakai, who has concluded[20]: "the most appropriate interpretation is that completely hardy plants form aqueous glasses intracellularly."

A particularly interesting and well-documented example of a complex organism that is in principle able to survive vitrification in nature is that of the Alaskan red flat bark beetle, *Cucujus clavipes puniceus*.[12] No larvae showed exotherms (freezing events) when cooled to −150°C in a differential scanning calorimeter (DSC) in the laboratory. Small larvae showed T_Gs ranging from −58°C to −76°C, which is consistent with the possibility of vitrification under natural conditions, at least for those larvae with the higher T_Gs. Two large larvae had a secondary T_G at −96°C or −98°C, but still had primary T_Gs of −76°C and −75°C, respectively. Furthermore, despite technical problems, some larvae survived cooling to −100°C, which is below the lowest T_G of even the large larvae. In the field setting, about 50% of larvae failed to freeze when cooled to about −60°C to −70°C, and about 50% also survived after cooling to and warming from the mean whole body T_G (−71°C), suggesting that survival is related to successful ice avoidance and even vitrification. Of particular interest in the case of this organism is the fact that the larvae employed large concentrations of glycerol (up to 6.5 M in the cited study, and up to 10 M in a separate study[26]), which mimics the use of high concentrations of permeating cryoprotectants in artificial vitrification and makes these beetles some of the closest natural counterparts to laboratory vitrification.

There is a particularly striking example of convergent evolution that illustrates, for organized tissues in both the plant and the animal kingdoms, the basic desirability of avoiding invasion by ice. In the terrestrial frog, ice forms first in relatively tolerant peripheral tissues, such as between the skin and the muscle, as well as in the abdominal cavity.[27] Because freezing takes place very slowly and with shallow thermal gradients in an animal that is rather small in size and contains empty cavities, there is time for water to migrate from vital structures such as the brain and the heart to the growing ice crystals in these benign locations rather than freezing *in situ*. The liver, heart, brain, intestines, and kidneys can actually be observed to lose volume as ice masses increase in volume,[27] very much akin to the way single cells shrink in response to growing extracellular ice rather than freezing intracellularly, presumably surviving as a result. A plant counterpart to this ice avoidance strategy is provided by the silver fir, *Abies sachalinensis*. In the fir tree, ice forms in a tolerant area adjacent to the apical meristem and is prevented from growing into the meristem by a diffusion barrier that allows water to diffuse through it but blocks the growth of ice. This results in the movement of meristem water from the meristem to the growing ice crystal, preventing death of the shrinking meristem (A. Sakai, personal communication). Other plants appear to have evolved essentially the same mechanism.[28] Although neither of these remarkable examples involves vitrification per se, they do both point to the desirability of avoiding ice formation in organized tissues, which provides additional though indirect support to the strategy of avoiding ice formation entirely by means of vitrification.

ADVANTAGES AND DISADVANTAGES OF VITRIFICATION VERSUS FREEZING

Vitrification has been pursued by many for three reasons. The first is that it can be quick and simple in comparison to freezing, and requires less expensive and complicated equipment. The second it that it transcends the need to cool and warm at identified and rigidly controlled optimal rates to maximize survival[29–31] and avoids the generally imperfect[30,32,33] compromise between intracellular ice formation and excessive dehydration injury that is a common feature of freezing protocols, thus leading to both greater convenience and frequently improved or at least equal results compared to freezing. The third impetus in many cases is the ability to use vitrification to attain cooling rates that are sufficient to "outrun" chilling injury[34–38] without paying the penalty of intracellular ice formation at high cooling rates.

The motivation behind the events that led to the post-1984 explosion of interest in vitrification was the advantage of avoiding mechanical injury to organized tissues from ice.[1,39–41] Both organized tissues[42–48] and cells in suspension[30,47] can be injured from the growth of ice either during cooling or as a result of recrystallization on warming. This concern is particularly pertinent to the cryopreservation of gonadal tissue, as well as to the preservation of tissues of the reproductive tract,[5,49,50] although the influence of ice formation is most powerful in systems that require vascular support after transplantation.[43,49]

The primary disadvantage of vitrification is the need for high concentrations of cryoprotectant, which complicates the avoidance of osmotic damage and the avoidance of cryoprotectant toxicity. Despite this disadvantage, many successful vitrification protocols have been developed, and many methods for avoiding toxic and osmotic injury have been demonstrated. These issues are discussed in more detail below.

Another disadvantage can be fracturing of the vitrified medium, resulting in such lesions as cleavage of the zona pellucida[51] or macroscopic damage to larger three-dimensional structures.[52] This problem can be solved by storing at temperatures that are intermediate between those of the glass transition and liquid nitrogen[53] or by careful cooling and warming protocols that avoid sufficient stress development to result in fracture formation.[51]

Another disadvantage of vitrification is that it usually results in nucleation of unfrozen freezable water at low temperatures, which may lead to the freezing of this water upon warming, with attendant injury. This injury can in principle be avoided by sufficiently rapid warming, but warming may need to be more rapid than is required

after most freezing protocols, in which there is more concern over the recrystallization of previously formed ice than the formation of additional ice, much of which may in principle be intracellular.

Certain vitrification protocols entail an additional disadvantage, at least in principle, which is contamination resulting from the use of open containers or containerless methods of vitrification.[54] However, these methods seem certain to be superseded by equally effective methods that employ closed containers.[55]

THE DIFFERENT EPOCHS OF RESEARCH ON CRYOPRESERVATION BY VITRIFICATION

The Era of Rapid Cooling with Little to No (Deliberate) Intracellular Cryoprotection

Vitrification of water was originally contemplated as being primarily a matter of attaining the required cooling rate.[56–60] Inspired[61] in part by Tammann's observation[62] that rapid cooling enabled the vitrification of 38% of all organic liquids investigated and Tammann's theory that it should be possible to vitrify any liquid through the use of sufficiently high cooling rates, as well as by observations of Moran[63] and Hardy[64] indicating that it might be possible to vitrify gelatin gels, the first serious work on vitrifying living systems was launched by Father Basile J. Luyet in a classic paper published in 1937.[65] As Luyet said in explaining the idea of vitrification in this paper, "The essential problem of the vitrification technique consists of … obtaining a cooling velocity sufficient to prevent the formation of crystals." As reviewed in more detail elsewhere,[66] Luyet and his associates published many papers from 1937 to 1958 in pursuit of the goal of vitrification using a variety of rapid cooling techniques. Most of this literature was undoubtedly published in *Biodynamica*, a journal established by Luyet in October of 1934, which began as a journal dedicated to discovering the nature of life,[67] but transformed fairly rapidly into the world's first journal dedicated to cryobiology, with a heavy emphasis on vitrification.

Although cryoprotection had been described even before Luyet's time,[68] the use of cryoprotectants to minimize freezing injury was not generally known until 1949[69] or generally understood until 1953[70] (although freeze concentration as a mechanism of cryoinjury was elucidated much earlier[71–73]). Therefore, the use of cryoprotectants and/or drying by Luyet and his colleagues to facilitate vitrification was not initially intended to slow the rate of crystallization or limit the maximum possible amount of crystallization of water as it is today, but was merely intended to dehydrate the system just before cooling to minimize the amount of water requiring vitrification.[74–76] Use of cryoprotectants for dehydration by exosmosis rather than for intracellular uptake was presumably based simply on the understanding that smaller objects, and thus smaller amounts of water, are easier to vitrify than larger ones. Little consideration was given to osmotic damage in such procedures, either during the exposure phase to the cryoprotectants or during cryoprotectant removal. Despite these major limitations, it is remarkable how successful some of Luyet's experiments actually were.[74,75]

The partial successes achieved by Luyet, both in terms of biological recovery and in terms of visual observations that suggested successful vitrification, were encouraging enough to sustain research into this approach to cryopreservation until 1958. However, in 1954, Audrey Smith strongly questioned Luyet's interpretations and rationales, pointing out both that survival after cooling and warming was not necessarily proof of vitrification, and that transparency rather than opacity after cooling or during warming was not necessarily proof of vitrification and the avoidance of devitrification.[61] Four years later, Luyet and Rapatz[77] and Meryman[78] presented the results of their investigations of Smith's criticisms of Luyet's evidence for vitrification. The results were undoubtedly disappointing for Luyet.

The first study[77] showed that thin films of gelatin gel subjected to Luyet's vitrification procedure contained "evanescent spherulites," ice crystals that, while thin and undetectable by the naked eye, were nonetheless quite real and prominent under light microscopic examination through crossed polarizing filters. The second study[78] showed X-ray diffraction patterns in similar gels, another verification of the presence of ice. However, only two diffraction peaks were seen, which was interpreted as indicating incompletely formed crystals. Dowell et al.[79] later concluded that the ice formed was cubic ice, but evidently cubic ice does not exist,[80] and the patterns seen by Meryman are actually consistent with ordinary, freshly nucleated but nonrecrystallized ice.[80] Therefore, the results of both investigations were in agreement in showing that Luyet's methods could not be relied upon to capture living systems in the vitreous state.

The Era of Rapid Cooling with Minimal but Deliberate Cryoprotection

Attempted cryopreservation by vitrification ended with the Luyet/Meryman experiments of 1958 and did not resume until being re-introduced exactly 20 years later, but with a significant difference. Pierre Boutron was the first to actively propose that cryoprotectants be used in higher than usual concentrations to effect vitrification, or at least partial vitrification at high cooling rates.[81] There was considerable irony in this, because Boutron's proposal was based in large part on observations made by Luyet and his colleagues after Luyet had abandoned the idea of vitrification. The latter observations indicated that, in high concentrations, cryoprotectant solutions really could be vitrified, and in the highest concentrations, could even be vitrified at low cooling rates.[82–88] Presumably, Luyet, like Boutron, assumed after 1958 that high intracellular concentrations of cryoprotectants could never be tolerated by living cells, and so never pursued this approach. This is particularly ironic because

Rapatz and Luyet actually achieved the first clear-cut success in vitrifying living cells in 1968 by cooling red blood cells in glycerol, and showing that their successful preservation (lack of hemolysis and integrity in the electron microscope) could be obtained coincident with the disappearance of both intracellular and extracellular ice in freeze-fracture/freeze-etch electron microscope images,[89] although they never related this achievement to Luyet's original decades-long quest to accomplish the very same thing, but in a very different way.

The difference between Luyet and Boutron was that Boutron was prepared to look for a way to circumvent cryoprotectant toxicity and achieve vitrification using a combination of two approaches. First, he began a systematic series of studies on cryoprotectant mixtures[81,90–94] in the hope of finding some mixtures that enabled vitrification at particularly low, and therefore perhaps nontoxic, concentrations. Second, he studied in great detail the previously unmapped kinetics of ice crystallization as a function of the interaction between concentration and cooling rate,[81,95,96] and achieved survival of red blood cells by vitrification in 1984,[97] and more clearly in 1988.[98] Boutron pioneered the use of differential scanning calorimetry and its marriage with x-ray crystallography as a means of verifying, quantifying, and characterizing vitrification and the ice that forms when vitrification is incomplete, as well as mathematical analysis of crystallization under borderline conditions and quantitative analysis of devitrification.[81,99] These were all extraordinary contributions, and Boutron made additional contributions by succeeding in his quest to find cryoprotectants that could vitrify water at remarkably low concentrations, the two outstanding examples being first propylene glycol (PG),[100] and later, 2,3-butanediol.[93]

Boutron's work, however, while describing the physics of ice in great detail and truly re-introducing the idea of vitrification in a way that could actually be definitively achieved in practice, did not inspire much enthusiasm for practical application, probably for several reasons. First, Boutron was focused on the study of cryoprotectant solutions that are hard to recover from the vitrified state without seemingly overwhelming problems from devitrification, a nominally lethal event. Second, the methods of vitrification that he did provide were only applied successfully to red blood cells, and then only obscurely, as they were buried in data on the effects of freezing in 1984[97] and 1988,[98] and did not actually improve survival in those systems over what was achieved without vitrification. Although the red cell papers, remarkably, showed that cells can survive devitrification (see the in-depth analysis below), this observation was only possible using warming rates that would be discouraging except for academic purposes, since clinical red cell freezing involved volumes that were far too large to warm at rates in excess of 1000°C/min. In addition, red blood cells are not nucleated, so the generality of Boutron's methods was not clear, and red cells were not in immediate need of better cryopreservation at the time. In addition, Boutron's experiments were complex, difficult to understand, and largely theoretical. Therefore, although working at the edge of the glass-forming concentration range, as Boutron often did, has become almost a way of life in more recent years, at the time it did not inspire general interest in cryopreservation by complete vitrification, a goal that, in Boutron's early papers, seemed to be envisioned mostly as a desirable dream[81] that could only be attained by finding new cryoprotectant systems that permitted vitrification at low concentrations (which is now seen as a largely counterproductive approach[101]).

Not long after Boutron revived interest in the amorphous state, however, Eric James began working on ways to vitrify parasitic worms, using necessarily (due to observed apparent toxicity) marginal concentrations of cryoprotectant (methanol[102] or ethylene glycol [EG][103,104]) for vitrification in combination with high cooling rates, and achieving some degree of success. Although he explicitly referred to the Luyet approach, and not to Boutron, he clearly recognized the need for intracellular uptake of the cryoprotectant to facilitate intracellular vitrification,[104] and in this respect, proceeded more in accord with Boutron's approach than with Luyet's concepts.

The Era of High Concentrations and Relaxed Cooling and Warming Rates

A practical universal approach to vitrification was first proposed by Fahy in 1981[39,41] as a possible general solution to the difficult problem of organ cryopreservation.[1,39–41,105–108] The inspiration to pursue vitrification stemmed from disappointing experiments with transplanted dog kidneys,[109] which appeared to be viable after freezing and thawing when tested *in vitro* (good vasoconstriction response to infused pressors after being frozen at −30°C for 1 week, confirming similar results obtained slightly earlier in rabbits[110]), but passed whole blood and turned blue from vascular stasis shortly after transplantation, which was interpreted as reflecting mechanical injury from ice, reminiscent of the injury seen by electron microscopy in frozen-thawed kidney slices.[42,111] Inspired by unpublished results of Rajotte and McGann that suggested the possibility of deep supercooling as a means of relatively short-term organ preservation,[109] Fahy attempted deep supercooling but found that the supercooled state was insufficiently stable for reliable week-long storage periods.[112,113] But the thought then occurred that perhaps this instability problem could be solved simply by continuing to cool all the way to below the T_G, and this was the genesis of the idea of large-system, low-cooling rate vitrification,[113] which simultaneously, of course, opened the door to the ready vitrification of smaller systems as well.

The idea of using high concentrations of cryoprotectants to allow vitrification of whole organs could have and should have been not only proposed, but also achieved

long before 1981. In 1965, John Farrant[114] was able to recover excellent contractile function in whole guinea pig uteri after cooling them to and rewarming them from –79°C, using what is now called the "liquidus tracking" method,[115] which he invented, to entirely avoid freezing. But he was unaware of the fact that he could have cooled his uteri to below T_G and vitrified them successfully, because the revelation that high concentrations of cryoprotectant vitrify when sufficiently cooled did not begin to be published until the end of 1966.[116] Similarly, Huggins, who came close to the same idea at the same time in attempting to explain red cell cryoprotection,[117] did not contemplate extending cooling to below T_G. However, when Elford and Walter applied Farrant's method to smooth muscle in 1972 and achieved similarly outstanding results,[118] they too did not mention the possibility of using their modified method to achieve vitrification, even though the last paper on the subject of high-concentration physical vitrification to appear in *Biodynamica* had by then been published 2 years previously.[88] A case can also be made for another lost opportunity to propose vitrification on the part of the same Gabriel L. ("Lou") Rapatz who had, with Luyet, demonstrated successful vitrification of red blood cells in 1968.[89] In 1970, he reported[119] at the annual meeting of the Society for Cryobiology in Los Angeles his first results indicating the possibility of recovering good function in at least some frog hearts after cooling them to dry ice temperature using 11 M (68.3% w/v, a vitrifiable concentration) EG and a "liquidus tracking" approach analogous to that of Farrant. He reported elsewhere the formation of ice in at least some of his hearts,[120] but did not directly suggest that the hearts that showed good recovery might have escaped ice formation, although he did try, unsuccessfully, to improve the recovery rate by increasing the exposure time to EG.[121] Interestingly, he reported from the podium, but did not record in his published abstract,[119] that plunging the hearts into liquid nitrogen and then back into a dry ice-temperature bath caused the hearts to "shatter," which also suggests glass-like behavior in these hearts. Later, Rapatz refined his methods and reported near-normal recovery of frog hearts cooled to dry ice temperature using 10 M versus 11 M EG, and showed a video of these hearts contracting normally after rewarming, yet still never suggested that they might have been vitrifiable.[122]

Fahy, in contrast, focused on vitrification and on the need to obviate cryoprotectant toxicity in order to approach the goal of vitrification,[62–66] in this effort combining the use of putative "toxicity neutralizers"[39,123]; Boutron's 1,2-propanediol[39,100,109]; the use of elevated hydrostatic pressure[39,40,109]; nonpenetrating cryoprotectants to enable reduction of intracellular permeating cryoprotectant concentrations[107]; mild osmotic dehydration to facilitate intracellular vitrification, reduce toxicity, and facilitate cryoprotectant washout[107]; exponential addition and washout of cryoprotectants[107,124]; introduction of the highest cryoprotectant concentrations at reduced temperatures[1,65]; and restriction of concentrations of cryoprotectant to the minimum levels needed to vitrify at the cooling rates applicable to organs, as determined both empirically and on theoretical grounds.[61,63,64] By 1984, the first full and generally available description of the concept of high-concentration, low-cooling rate vitrification was provided, together with a demonstration that it was possible to design solutions that could vitrify at a cooling rate of only 10°C/min and yet could permit 90% recovery of the renal cortical K^+/Na^+ ratio.[107] This paper also demonstrated that the fracturing that had dissuaded Rapatz could be avoided, even in a whole vitrified rabbit kidney weighing far more than a frog heart.

The final step in demonstrating the intrinsic workability of this general approach was to establish its efficacy for the cryopreservation of at least one convincing model system. In fact, two such proofs of principle rapidly became available. The first came as a result of a collaboration between Fahy and William F. Rall, and the second was provided by an independent parallel investigation led by Tsuneo Takahashi. Both studies began at about the same time and in the same laboratory at the American Red Cross.

Rall, after being exposed to an explanation of the idea of complete (intracellular and extracellular) vitrification,[106] was attracted to it as a result of his previous experience with the freezing of mouse embryos, which had led him to realize that their survival was dependent on intracellular vitrification,[125–127] and he and his colleagues had even shown, in 1984, that the unfrozen medium in which the embryos were embedded in the frozen state just before rapid cooling to lower temperatures was sufficiently concentrated to vitrify.[128] Rall and colleagues recognized that this phenomenon was not unique to embryos, and concluded: "Other cryopreservation methods that employ a protective additive and rapid cooling from an intermediate subzero temperature may rely on the ability of the residual liquid to form a metastable glass during rapid cooling."[128]

These observations convinced Rall that complete vitrification should be feasible, and he relocated from the United Kingdom to Bethesda, Maryland, to join forces with Fahy and apply his expertise with embryos to prove the feasibility of the new method. The effort was a true collaboration in which Fahy provided the solution and some of the cooling methods, and Rall provided the protocol for adding and removing the vitrification solution, and both worked equally hard, and interactively, on the manuscript. The result, which is well known, showed that mouse embryos could indeed be vitrified and rewarmed with equal success over a range of cooling and warming rates as long as the warming was rapid enough to prevent devitrification.[1] The published paper coined the term "vitrification solution," a bit of new jargon that was originated by Rall during discussions of how to present the

data in the paper. Before long, it was confirmed that vitrified mouse embryos were able to develop to term after transfer to female hosts,[129] and the race to find even better vitrification solutions was begun.

The second proof of principle, by Takahashi et al.,[130] has received much less attention, but is also robust. These authors showed, using both DSC and freeze-fracture/freeze-etch electron microscopy, that essentially the same solution used for vitrification by Rall and Fahy, as well as human monocytes embedded in it, indeed vitrified when cooled, although some small ice crystals, which had no apparent effect on viability or cell function, were seen in some organelles. Rewarming allowed >90% recovery of both cell numbers and all measured cell functions provided devitrification was not permitted, recovery that was not different from the recovery of monocytes treated with the vitrification solution but not vitrified. Devitrification, which was prominent at warming rates of less than 80°C/min and minimal though still present at 80°C/min, resulted in both extracellular and intracellular ice and caused major injury to the monocytes. Avoidance of cryoprotectant toxicity required addition of the vitrification solution at 0°C rather than at room temperature.

The era of high-concentration, relaxed-rate vitrification continues to the present day, particularly for systems that cannot be cooled or warmed at high rates.[131,132] However, the success of this method opened up many opportunities for vitrification under more marginal and ambiguous circumstances, thus leading to the next, and also still-continuing, era of vitrification research.

The Era of Marginal or Accelerated-Rate Vitrification and Rapid Freezing

Not long after the onset of the relaxed-rate era of vitrification research, much innovative work was undertaken to adapt the idea of complete vitrification to a variety of living systems. In most cases, adaptation was necessary given difficulties in directly transferring the mouse embryo methods to other systems. There were three main drivers of the direction of this adaptation. First, problems encountered with cryoprotectant toxicity suggested the need to use lower concentrations and to substitute higher rates of cooling and warming for some of the cryoprotectant, a tradeoff that was generally possible for reproductive preparations such as oocytes and embryos. Second, lower cryoprotectant concentrations also simplified the avoidance of osmotic damage and the procedures for adding and removing the cryoprotectants. But perhaps most definitively, and urgently, there was the necessity for avoiding chilling injury.

In the same year the Rall and Fahy paper was published in *Nature*, the National Science Foundation convened a meeting to discuss the cryopreservation of *Drosophila* embryos and selected Peter Mazur and Peter Steponkus as the cryobiologists to lead the project forward.[133] Both laboratories soon learned that due to the very rapid chilling injury seen in this species, the only path forward that was not doomed to failure was vitrification at high cooling rates, with very rapid warming. Five years later, Steponkus et al. reported that using what he referred to as a modified version of the Rall and Fahy method (which employed 8.5 M EG[37] rather than the VS1 solution of Rall and Fahy) and cooling at ~400°C/s on a copper grid immersed in liquid nitrogen slush yielded over 18% egg development into larvae and 3% egg development into fertile adults.[36] Two years after this, Mazur et al. achieved a hatching rate of 68% and a rate of development to fertile adults of hatched flies of 40% using essentially the same vitrification procedure, but modifying noncryobiological aspects of the procedure (the precise embryo age at the time of vitrification, for example), and incidentally cooling at 100,000°C/min.[37]

In 1996, Martino, Songasen, and Leibo,[34] taking a hint from the *Drosophila* work, described perhaps the first similar attempt to "outrace" chilling injury in a mammalian system (bovine oocytes) using a combination of rapid cooling on copper electron microscopy grids and, in this case, either 5.5 M EG + 1 M sucrose or 4 M EG + 0.5 M sucrose. Encouraging and similar results were obtained with both vitrification solutions even though the latter, when cooled in straws, was found not to vitrify, and the authors remarked that even if extracellular ice were to form, it might not matter.

The Martino et al. paper, with one foot in the stable vitrification tradition and another in the tradition of unstable vitrification[134] harkening back to the work of James and the early work of Boutron, may have re-established a precedent for pushing the cryoprotectant concentration as low as possible, thus essentially launching the next era in vitrification research. Since Martino et al., an avalanche of studies, which continues to this day, has proceeded to develop methods that differ in detail but are united in seeking rapid cooling and warming rates, usually in combination with concentrations of cryoprotectant that are, as in the Martino et al. study, marginal in their ability to support vitrification and especially to prevent devitrification. This work, which is of unquestioned value and has frequently produced very good results, is ably described in the chapters that follow, and needs no additional summarization here.

However, there is an extreme version of this form of attempted vitrification that does deserve a brief comment, and that is the idea that vitrification can be achieved, with recovery of viability, even in the complete absence of cryoprotectants, an idea that is essentially the same as that of Luyet rather than being derived from the other traditions described above. This idea has been championed specifically for spermatozoa on the premise that their low water content[135,136] may make the use of permeating cryoprotectants unnecessary for vitrification.[136–140] However, these claims, while plausible, at least for the intracellular compartment if not the extracellular one, remain

unsubstantiated, as reviewed in detail elsewhere,[134] and contradictory information exists.[141] Therefore, additional development of this line of research is still needed before definitive conclusions can be drawn. Freeze-fracture methods have been used to substantiate vitrification at least since 1968,[89] and there is no obvious reason that this technique could not be applied to rapidly cooled sperm in order to enable direct observation of either ice or vitrified cytoplasm.

Although they are of practical value, it is necessary to point out that many accelerated-rate methods of vitrification tend to obscure understanding of the underlying physical basis of the preservation attained, essentially blurring the meaning of what a vitrification procedure actually is. Rapid freezing is not the same thing as vitrification, although the word "vitrification" is sometimes used to describe methods that are in all likelihood really a form of rapid freezing in the presence of higher-than-traditional cryoprotectant concentrations, which may moderate the biological consequences of intracellular ice formation. The confusion that arises from attempting to achieve vitrification under marginal or even beyond the marginal limits of the method provides ample justification for the brief review of the physics of vitrification in the next section, which may enable all of the various approaches to vitrification, "one-way" vitrification (see below), or rapid freezing to be placed into a clearer physical context.

PHYSICAL ASPECTS OF VITRIFICATION

In this section, a general framework for understanding the physics of cryopreservation by vitrification is provided, including the role of cryoprotectants, the effects of the cooling rate, the effects of the warming rate, projected rates of biological deterioration as a function of temperature, and the thermodynamic necessity of vitrification. More in-depth discussions of the physics underlying biological vitrification are available,[66,142–145] and the reader is invited to consult them to obtain a deeper understanding of specific issues.

Concentration Dependence of Vitrification

The fundamental relationships between vitrification and the use of cryoprotectants are summarized in Figure 1.2, using as an example a supplemented phase diagram for EG composed from data taken from many sources.[85,87,88] The temperatures noted for the events in this figure pertain to moderate cooling and warming rates. The nonthermodynamic temperatures are cooling and warming rate dependent, but the qualitative relationships shown are pertinent to all but the most extreme (and in some cases, unattainable) cooling and warming rates, and the level of detail shown in Figure 1.2 has been worked out only using relatively low cooling and warming rates.

The curve labels in Figure 1.2 indicate thermodynamic melting points (T_Ms), T_Gs, homogeneous nucleation temperatures (T_Hs), and devitrification temperatures (T_Ds), all of which are a function of the concentration of cryoprotectant and all of which behave in the same qualitative way for all major low-molecular mass cryoprotectants.[107] The interplay between the events denoted by these curves enables the definition of different regions

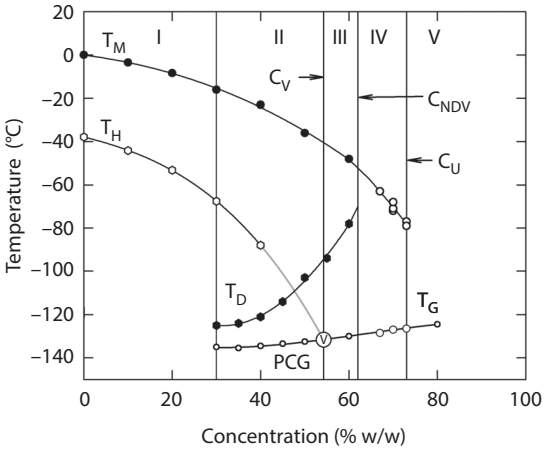

Figure 1.2 **Relationships between the melting point, T_M, the glass transition temperature, T_G, the homogeneous nucleation (ice self-nucleation) temperature, T_H, and the devitrification temperature, T_D, observed when small samples of ethylene glycol–water mixtures are cooled and warmed at moderate rates. Although not indicated in the diagram, ice nucleation is likely in Region I above T_H due to the unavoidable presence of heterogeneous nucleating agents, whereas both heterogeneous and homogeneous nucleation become less effective at higher concentrations, permitting the glass transition to be observed on cooling and the conversion of the amorphous state to the frozen state to be observed on warming (at T_D). In Region II, living systems can be vitrified, but are heavily nucleated. In Region III, nucleation is much less severe because $T_H < T_G$, which enables T_D to move upward and eventually vanish. Region IV is a concentration range in which samples can be vitrified at very low cooling rates and warmed slowly without devitrification, yet in which pre-existing ice, as exists during the slow freezing of a lower concentration, can continue to grow during slow cooling. The white points on the T_M line, which were associated with the T_Gs noted by the large points on the T_G curve, represent inferred T_Ms of concentrations generated by maximum freeze-concentration, the most extreme of which defines the boundary between Regions IV and V. C_V: lowest concentration for vitrification without homogeneous nucleation; C_{NDV}: lowest concentration leading to no devitrification; C_U: maximum concentration attainable by freeze-concentration; PCG: partially crystallized glass. (Data from of Fahy GM et al., *Cryobiology* 1984;21:407–26.)**

of concentration dependence of vitrification-related phenomena that have significant relevance for attempted cryopreservation by vitrification.

In Region I of this diagram, the dominant limiting factor for vitrification is homogeneous nucleation, or the self-nucleation of water, which is a very rapid process in dilute solutions and is hard to "outrun" by rapid cooling.[146] Above T_H, ice nucleation is also possible due to the presence of heterogeneous nucleating agents, which, at low concentrations of cryoprotectant, tend to cause freezing between T_M and T_H, but, as the upper concentration limit of Region I is approached, become less and less likely to manifest themselves. The property of being below T_M without freezing is known as supercooling, and vitrification is made possible by the ability of aqueous solutions, especially at concentrations above those of Region I, to supercool all the way to T_G. In Region I, by contrast, even rapidly cooled small systems tend to become opaque on cooling due to ice formation, and only cooling rates on the order of 10^5–10^6°C/second[58] (almost 10^7–10^8°C/min) are sufficient to suppress ice formation in pure water or dilute aqueous solutions, which renders at least the lower concentration portion of Region I off limits for vitrification, except under the most extreme conditions (see below).

In Region II, the kinetics of homogeneous nucleation are significantly impeded by the presence of cryoprotectant, and T_H becomes harder to define (gray line). In most examples available, T_H is not seen when T_G is seen, but in the case of the EG–water system, remarkably, T_H can be seen at 40% w/w solute, even though T_G can first be detected at 30% w/w EG. Nevertheless, in Region II, homogeneous nucleation likely becomes distributed over a much broader range of temperatures as concentration rises, with the T_H curve then defining the upper boundary, rather than the precise temperature of this event. As T_H curve approaches the curve for the T_G, increasing viscosity undoubtedly has a further significant effect on homogeneous nucleation, but the details have not been explored to date, and other subtleties may actually facilitate nucleation, as discussed in more detail below. For our present purposes, it is sufficient to note that the ability to cool small systems without massive homogeneous nucleation enables the detection of the T_G curve for the first time in Region II. This thus enables vitrification of the great majority of the solution in Region II. However, since T_G is below T_H, the glass formed is undoubtedly heavily nucleated,[145,147] and this is indicated by the appearance of the T_D curve (curve showing crystallization upon warming, which reflects the growth of previously formed nuclei as well as the growth of any additional nuclei formed during warming, which will also form mostly below the T_H line), as well as by the fact that the T_D curve can be very close to T_G despite the extreme viscosity of solutions near T_G. The ability to crystallize a detectable amount of water at such low temperatures is a reflection of the high nucleation density, which means that even a small amount of growth of an astronomical number of nuclei[148] will evolve appreciable heat.

Glasses formed at concentrations for which $T_H > T_G$ are referred to as being "doubly unstable,"[146,149] or as being "partially crystallized glasses (PCG in Figure 1.2)."[145] Although vitrification is clearly incomplete in these circumstances, the nuclei formed, since they predominantly form at temperatures too low to enable crystal growth (temperature optima for nucleation are very much below the temperature optima for ice crystal growth in good vitrification solutions[66,144,150]), tend to be submicroscopic[147,151] and uniform in size,[142] and will generally not be damaging to living cells unless they are able to grow to damaging sizes[151,152] during warming. The uniformity of crystal sizes will also tend to inhibit recrystallization, another potentially favorable factor for survival.[41] As indicated by the low temperatures of detectable devitrification, however, the warming rates required for successful recovery tend to be quite high, and low warming rates are usually lethal.[153] Even when warming leads to survival, however, this does not necessarily mean that devitrification has been avoided (see below), and for that reason, methods that employ vitrification in Region II are often likely to represent what has been called "one-way vitrification,"[112,134] in which vitrification is enabled on cooling but the amorphous state is not fully maintained during rewarming. High survival rates after "one-way vitrification" are feasible, in general, for systems that can be warmed at more than about 1000°C/min, the same warming rate that enables appreciable survival after extensive intracellular ice formation using no or low concentrations of cryoprotectant.[134,154]

The boundary between Region II and Region III is defined by the intersection of the T_H curve with the T_G curve (circle marked with a V), which in principle allows vitrification without homogeneous nucleation at this concentration and above, and in practice, 8-mL samples cooled at about 10°C/min show no visible ice crystals when the concentration is just sufficient for T_H to coincide with T_G in multiple cryoprotectant solutions, and at both ambient and elevated pressures.[40,107] This enables the definition, for any given circumstance, of a specific concentration needed to vitrify (CNV or C_V).

It is interesting that devitrification is seen in Region III even though homogeneous nucleation is not expected. The natural explanation for this is heterogeneous nucleation, and although high-temperature heterogeneous nucleators are not plentiful, weak heterogeneous nucleators that would only act at temperatures too low to enable ice growth are expected to be more plentiful.[155] However, cryoprotectants also tend to inactivate heterogeneous nucleators. A different explanation for the observed devitrification is suggested by the observation of a consistent coincidence between the onset of nucleation and the onset of the glass transition in two significantly different vitrification solutions.[144,150] It is therefore suggested here that nucleation is

catalyzed by the lack of mobility of water near the glass transition, where local rotation and limited diffusion[156] sufficient to slowly but irreversibly reorient water into an ice embryo can still take place, without the high-velocity collisions between the water molecules within the nascent cluster that would normally destabilize the growing ice embryo at higher temperatures, where kinetic energy is more abundant. Beyond these sources of nucleation, it is also possible to observe the continuation of the T_H curve below T_G in some systems.[157]

Despite the persistence of devitrification in Region III, as the concentration is further raised, devitrification takes place at higher and higher temperatures until, at some concentration (the concentration permitting no devitrification, or C_{NDV} in Figure 1.2), devitrification is no longer seen. The latter concentration defines the boundary between Region III and Region IV. Region IV would be ideal theoretically, since systems could be cooled and warmed even at low rates without any danger of ice formation, but given the increasingly overwhelming problems of cryoprotectant toxicity when concentrations are raised to such levels, Region III is generally the preferred concentration range for vitrification when the warming rates required for success in Region II cannot be attained.

Above Region IV is Region V, in which ice cannot grow even when it is already present in the solution. The boundary between Region IV and Region V defines the upper limit of freeze-concentration possible after devitrification; that is, the maximum possible concentration of cryoprotectant that can be generated by freezing[84,150] (the "unfreezable" concentration, or C_U in Figure 1.2).

The Effect of Cooling Rate on the Concentration of Cryoprotectant Needed for Vitrification

Between T_M and either T_H or T_G, the state of the system is determined primarily by the competing effects of the rates of nucleation and crystal growth on the one hand, and the effects of the cooling rate (and warming rate) on the other. The rates of ice nucleation and growth are also dependent upon both the solute in question and its concentration.

The kinetics of ice formation under conditions of interest to the cryobiologist were partly mapped by Luyet and his colleagues, but it was not until Boutron that mathematical generalizations of the net rate of ice development as a function of cooling rate were made available.[81] Boutron's final equation[95]

$$-\ln(1 - x^{1/3}) + 0.5 \ln(1 + x^{1/3} + x^{2/3}) + \sqrt{3}\,\mathrm{arctg}\left(\frac{\sqrt{3}\,x^{1/3}}{2 + x^{1/3}}\right) = \frac{k4}{|v|}$$

related the fraction of crystallizable water frozen, x, to the cooling rate (v) and a constant, k4, which can be regarded as the cooling rate sufficient to reduce x to 3.6% of the maximum amount of ice that can form at low cooling rates.[158] Between x = 1 (maximal ice formation) and x = 0 (no ice formation), x as calculated by the above equation varies in a sigmoidal fashion with the log of the cooling rate.

When x is small, the above equation can be greatly simplified. For example,[158] for $x = 10^{-6}$,

$$v = 33.33\ k4.$$

To calculate the cooling rate required to establish an absolute mass fraction of ice in the solution of interest, rather than a mass that is a particular fraction of the total amount of ice that can form in that solution, Boutron's equation can be simplified for the limiting case of low x by substituting in the definition of x, which is $x = q/q_{max}$ (where q represents the amount of heat evolved as a result of freezing and q_{max} is the amount of heat evolved by freezing at low cooling rates).[158] For example, the cooling rate required to reduce the absolute amount of ice to 0.2% of the total solution mass is[158,159]

$$v = \frac{k4}{(3[0.2/q_{max}]^{(1/3)})},$$

where q_{max} in this case is the maximum mass fraction of the solution that can freeze at low cooling rates.

Boutron and Baudot et al., using the latter definition of the critical cooling rate, tabulated the critical cooling rates of several cryoprotectant–water solutions at several different concentrations. Most of these results are summarized in Figure 1.3, and are related to Bruggeller and Mayer's[58] estimates for the cooling rates needed to vitrify pure water. The unity of the results, and their agreement with projections[113,160] as to the critical cooling rates of solutions intermediate in concentration between those of pure water and those tabulated by Boutron, Baudot, and colleagues, allows the "boundary conditions" shown in Figure 1.3 to be used to evaluate the difficulty of vitrifying lower-concentration solutions, as well as claims of success with the latter endeavor. However, the effects of carrier solutions, nonpenetrating cryoprotectants (npCPAs), and the osmolytes normally present within cells are not taken into account in this diagram. Mapping the influence of these additional solutes remains an important task for future research.

The Warming Rate Needed After Vitrification

As mentioned above, survival after "one-way" vitrification, especially in Region II of the phase diagram of Figure 1.2, indicates that some degree of devitrification, or even complete devitrification, followed by incomplete recrystallization can be tolerated by living cells. Extensive research has been devoted to defining the warming rate

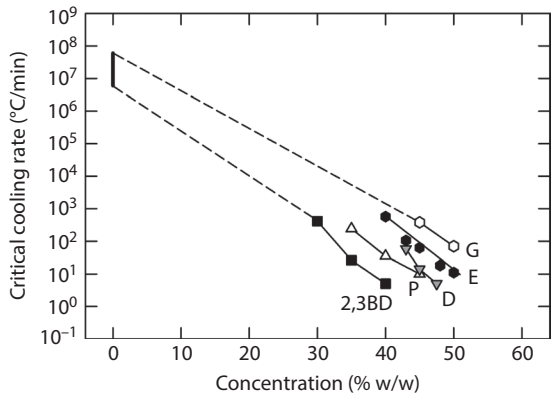

Figure 1.3 **Cooling rates required to vitrify all but 0.2% of the mass of 0%–50% w/w cryoprotectant–water solutions. Data on cryoprotectant solutions are from Baudot and Odagescu[229] and Baudot et al.[159] The heavy vertical line at 0% cryoprotectant represents Bruggeller and Mayer's[58] estimated cooling rate range for the vitrification of pure water (10^5–10^6°C/second). The cooling rates projected by Toner,[160] as presented by Fahy and Rall,[113] to be required for concentrations between 0% and the concentrations tabulated by Baudot and colleagues all fall within the zone between the dashed lines. The curve labels designate glycerol (G), ethylene glycol (E), dimethylsulfoxide (D), propylene glycol (P), and 2,3-butanediol (2,3BD). (Based on Fahy GM, Rall WF. Vitrification: An overview. In Tucker MJ, Liebermann J (eds.), *Assisted Reproduction: A User's Manual and Troubleshooting Guide*. Informa Healthcare: London, 2007. p. 1–20.)**

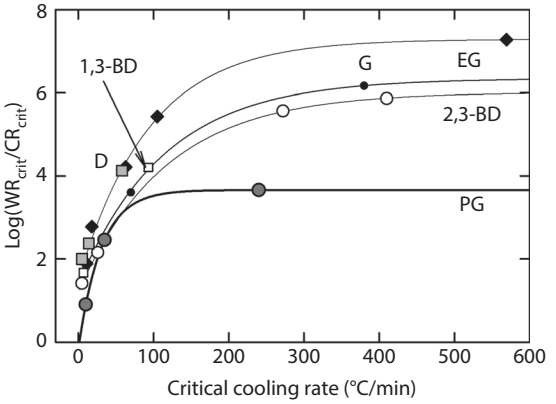

Figure 1.4 **Relationship between the critical warming rate (WR_{crit}) and the critical cooling rate (CR_{crit}) for six different cryoprotectant–water systems (EG = ethylene glycol; G = glycerol; 1,3-BD = 1,3-butanediol; 2,3-BD = levo-2,3-butanediol; PG = propylene glycol; and D = dimethylsulfoxide). The log of the ratio of the WR_{crit} to the CR_{crit} rises exponentially to an apparent maximum, as CR_{crit} rises to greater than 200–500°C/min or so. The function used for curve fitting is $\log([WR_{crit}]/CR_{crit}]) = y_0 + a(1 - e^{-bx})$, where x is CR_{crit}. The parameters found are given in Table 1.1. A separate fit for D was not attempted, but the fit for EG appeared to fit the data for D adequately. There were insufficient data (two data points) to fit the G results separately, but G and 1,3-BD appeared to behave in the same fashion at overlapping rates, so the two G data points and two nearby 1,3-BD data points were pooled to allow a lumped fit through the four available grouped data points. (Data from Boutron P. *Cryobiology* 1993;30:86–97; Baudot A, Alger L, Boutron P. *Cryobiology* 2000;40:151–8; Baudot A, Odagescu V. *Cryobiology* 2004;48:283–94.)**

and temperature dependence[99] (but, to a far lesser degree, the actual extent[161,162]) of devitrification under a variety of conditions, but almost no research has been done to define the extent of devitrification that is sufficient to limit survival.

To begin to place this issue into perspective, Figure 1.4 plots the log of the ratio between the critical warming rate (the warming rate needed to prevent ice formation during warming; parameters found are listed in Table 1.1) and the critical cooling rate as defined in Figure 1.3. As the critical cooling rate increases, the critical warming rate increases far faster, but seems to reach a maximum ratio to the critical cooling rate that can range from just under 10^4 to just over 10^7 times the critical cooling rate, depending on the chosen cryoprotectant.

Using this relationship, the critical warming rate can be calculated for any known critical cooling rate that was associated with the use of a particular cryoprotectant for a particular vitrification experiment in which the vitrified living system survived after warming, thus allowing a comparison to be made between the warming rate associated with survival and the warming rate associated with the absence of ice on warming. A few examples of such relationships are available and are analyzed in Figure 1.5.

Table 1.1 Parameters Found in Figure 1.4

Cryoprotectants	y_0	a	b
EG and D	1.4659	5.8246	0.0107
G and 1,3-BD	1.3668	4.9799	0.0088189
2,3-BD	1.2023	4.8140	0.0085981
PG	−0.1792	3.8417	0.0331

Cells vitrified in PG

Boutron and Arnaud[97] cooled red cells in 30% PG at 4000°C/min, which exceeds the 1200°C/min critical cooling rate for this solution, and rewarmed them at

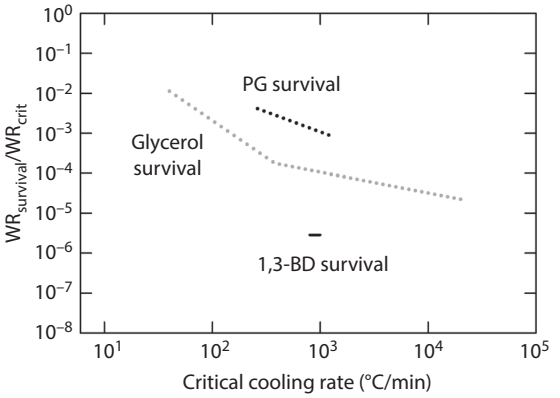

Figure 1.5 **Warming rates yielding survival after vitrification, expressed as a fraction of the critical warming rates (WR_{crit}) for the same concentrations of pure cryoprotectant in water. Line labels refer to the cryoprotectants discussed in the text. The critical cooling rate for 30% 1,3-butanediol (1,3-BD) is not known, so for the purposes of graphical representation, the results for this solution are plotted at 1000°C/min. Since the WR_{crit} could not be calculated from a known critical cooling rate for this solution, the WR_{crit} value was conservatively assigned the same value as the WR_{crit} for the 35% w/w 1,3-BD solution for graphical representation purposes. The survival data do not represent a boundary between survival and nonsurvival, but simply provide specific illustrative examples available from the literature. (PG = propylene glycol.)**

5000°C/min and obtained survival. Red cells cooled in 35% PG at rates above the 240°C/min critical cooling rate for this solution, and then rewarmed at 5000°C/min, also survived. Using the PG curve fit of Figure 1.4, the warming rates giving survival were 9×10^{-4} and 4.5×10^{-3} times as high as the critical warming rates for these solutions.

Cells vitrified in glycerol

Nei[163] found that, according to his electron microscopic observations, 30% (presumably v/v or ~35% w/w) glycerol cooled at 10^5°C/min, but not at 10^4°C/min, appeared to be ice-free, and warming red cells vitrified under these conditions at unspecified warming rates allowed survival. If we generously assume that the critical cooling rate is only 2×10^4°C/min, and that the warming rate was as high as 10^6°C/min, however, the warming rate allowing survival was still ~5 logs less than the critical warming rate. Rapatz and Luyet[89] mixed one volume of 8.6 M glycerol with one volume of packed red cells, producing perhaps a 5.3 M final glycerol concentration[154] (or 44.3% w/w) prior to cooling at a rate that was not specified but was sufficient to avoid both intracellular and extracellular ice formation according to electron microscopic observations, and implied survival of the red cells upon rapid warming. Assuming the critical cooling rate was the same as for 45% w/w glycerol and that a warming rate of 10^5°C/min was achieved, the achieved warming rate would still be 4 logs short of the critical warming rate. Finally, Lehn-Jensen and Rall[127] showed survival of cattle embryos frozen in glycerol to a solute concentration of about 51.4% w/w,[154] and then plunged to lower temperatures and rewarmed at 250°C/min. If one assumes that, based on a critical cooling rate for 50% w/w glycerol of 70°C/min, the critical cooling rate of the Lehn-Jensen and Rall solution was 35°C/min, then survival was obtained after warming at less than 2% of the rate needed to suppress devitrification.

Cells vitrified in other cryoprotectants

Mehl and Boutron[98] vitrified red blood cells in 30% and 35% w/w 1,3-butanediol and obtained survival after warming at 5000°C/min, even though the critical warming rates for these solutions are estimated to be over 100,000 times higher, even for the 35% w/w solution. Rall et al.,[128] in experiments involving the freezing of mouse embryos in a dimethylsulfoxide solution along lines similar to those discussed above, also obtained survival, suggesting innocuous devitrification.[125,154] By this time, there are undoubtedly vast numbers of other examples that could be analyzed, including for cells of reproductive interest,[153] but the point is clear that survival after vitrification is not dependent, in most systems, on the complete avoidance of devitrification.

The explanation for survival after devitrification is undoubtedly multifaceted. Studies of the survival of intracellular ice formation have linked death to the attainment of intracellular ice crystal sizes of 400 nm or above,[151,152] which requires finite time to occur.[161,164] In addition, the higher the warming rate, the less the total amount of ice developed,[161,162,165] even though devitrification is not prevented. Another important factor comes from the fact that vitrification solutions used for living cells contain more than cryoprotectant and water, and the contribution of carrier solution solutes to both vitrification[166,167] and the suppression of devitrification[112,113,167] is considerable. The inclusion of polymers is particularly effective at slowing devitrification,[168] which suggests that natural intracellular polymers will have a similar effect, consistent with evidence that erythrocyte cytoplasm is more resistant to ice formation than the extracellular milieu.[89,163] Unfortunately, the magnitude of these effects and systematic ways of factoring them into predicted results remain to be adequately elucidated, although it appears that, as a rule of thumb, a given percentage by weight sugar is roughly equivalent to the same weight percentage of cryoprotectant for suppressing devitrification.[112]

A negative contributor to survival of devitrification has been observed in slowly frozen embryos that are killed during slow warming by an invisible event that follows devitrification, but precedes recrystallization.[125]

In this case and many others, protection at lower temperatures and susceptibility to injury at higher temperatures has been attributed to the conversion of cubic to hexagonal ice on warming.[169] Even though cubic ice is not currently considered to exist,[80] the correlation might still have some validity, since the X-ray pattern suggestive of cubic ice still represents an incomplete hexagonal ice lattice,[80] whose perfection upon warming might then enable more destructive events to take place. Alternatively, an invisible change could simply include increased cryoprotectant toxicity in the frozen state,[170] driven by freeze-concentration following devitrification.

In summary, there is still too little understanding of the boundary conditions for survival versus nonsurvival of living cells during devitrification, and this is an area deserving of much further study, for both basic and applied reasons. Beyond this, the tolerability of devitrification in organized tissues introduces additional issues of great applied and theoretical interest, which are as yet barely addressed.[131,171]

Safe Storage Times in the Vitreous or Near-Vitreous State

It would be of great interest to know how biological deterioration rates scale with temperature in nonfreezing systems near T_G, in part to enable a rational choice of storage temperature. Because glasses are unable to relieve developed thermal stress and are fragile particularly during warming, they are at risk of fracturing, particularly when cooled and warmed at higher rates, and this risk increases as temperature decreases below T_G. In addition, liquid nitrogen poses safety risks of various kinds, both to the stored samples and to banking personnel, and vapor storage would be a desirable alternative provided safe vapor storage temperatures could be defined and maintained. Limited empirical data suggest that storage just below T_G is safe,[53] but a better theoretical understanding of rates of change near T_G would be reassuring, particularly given that nucleation is known to be able to continue even below T_G.[142,144,150,157]

It was suggested by Fahy and Rall[113] that rates of biological change ought to scale with molecular mobility, which can be related to the viscosity of the solution. The viscosity of a glass-forming liquid can be fit, for example, to the Vogel–Tammann–Fulcher (VTF) equation

$$\eta(T_2) = \eta(T_1)\exp\left(\frac{B}{[T_2 - T_0]}\right)$$

where $\eta(T_1)$ is a known reference viscosity at temperature T_1, $\eta(T_2)$ is the viscosity at temperature T_2, and B and T_0 are constants. The VTF equation can be used down to approximately T_G, below which viscosity shows Arrhenius behavior rather than super-exponential behavior.

We can relate viscosity to the time required for a given amount of diffusion by using the Stokes–Einstein equation, which, for temperatures above T_G, relates diffusion coefficients to viscosity by

$$D = \frac{kT}{6\pi\eta(T)}$$

where $\eta(T)$ is the viscosity at temperature T, D is the diffusion coefficient of the substance, and k is a constant. Since the time t required for a substance to diffuse a distance d is equivalent to $d^2/6D$, we can combine the above relationships to obtain

$$t = t_1\left(\frac{T_1}{T}\right)\exp\left(B\left[\frac{1}{(T - T_0)} - \frac{1}{(T_1 - T_0)}\right]\right),$$

where t is the time required to diffuse distance d at temperature T and t_1 is the time required to diffuse the same distance at reference temperature T_1.

Values of B and T_0 have been determined for a variety of biologically relevant solutions, including the M22 vitrification solution[172,173] used to vitrify and transplant a rabbit kidney, with subsequent permanent survival of the rabbit after excision of the nonvitrified contralateral kidney,[171] based on measured viscosity data and the assumption that the viscosity at T_G equals 10^{13} poise.[174] Using these parameters and the assumption that t_1 is 1 minute at –22°C (a minimal time to accumulate renal injury during M22 perfusion at –22°C), storage times equivalent to t_1 at different temperatures have been calculated, and are given in Table 1.2. Although the results at specific temperatures differ, remarkably, by four orders of magnitude between the different solutions (driven in part by a combination of different assumed T_Gs as well as different initial viscosities at –22°C), they suggest that, even in the worst case, storage of living systems not far below T_G should be safe.

The times calculated in Table 1.2 assume instantaneous cooling to the tabulated temperatures, whereas it will be necessary to integrate the time- and temperature-dependent total amount of diffusion during cooling to various temperatures, as a function of the cooling rates actually contemplated, in order to correct the estimated safe storage times. The fact that many systems survive vitrification well, however, including embryos cooled at only 20°C/min, suggests that, in most cases, damage accumulation during cooling to and warming from T_G will not be a significant factor for permissible storage times below T_G. The effect of nucleation near T_G on safe storage times will also require additional analysis. For the time being, the predictions of Table 1.2 and similar calculations remain entirely theoretical, and await experimental testing.

Table 1.2 Projected Safe Storage Times in the Amorphous State[a]

	Solution			
Constants	**M22[b]**	**DAP_{10}[c]**	**DMSO[c]**	
B	1112	1187.1	906.9	
T_0	−155.4	−154.5	−159.3	
T_G	−123.3	−124	−135	
Temperature	**Time Equivalents**			**Time Units**
−22°C	1	1	1	Min
−80°C	13.2	23.2	2.7	Hours
−90°C	5.5	12.0	0.62	Days
−100°C	18.0	53.3	0.85	Weeks
−105°C	31.2	114	0.83	Months
−110°C	30.5	145	0.39	Years
−115°C	652	4379	3.18	Years
−120°C	32,837	352,276	44.4	Years
−121°C	82,374	990,388	81.7	Years

[a] Temperatures expressed in degrees Celsius, but T_1/T calculated from degrees Kelvin.

[b] Data source: Mullen SF, Fahy GM. Fundamental aspects of vitrification as a method of reproductive cell, tissue, and organ cryopreservation. In Donnez J, Kim S (eds.), *Principles & Practice of Fertility Preservation*. Cambridge University Press: Cambridge, 2011. p. 145–63.

[c] Data source: Fahy GM, Rall WF. Vitrification: An overview. In Tucker MJ, Liebermann J (eds.), *Assisted Reproduction: A User's Manual and Troubleshooting Guide.* Informa Healthcare: London, 2007. p. 1–20, with error in decimal place for the B value for DAP_{10} corrected; DAP_{10} refers to 40% w/v DAP_{10} (10% w/v 1,2-propanediol plus 30% w/v "DA", a 1:1 mole ratio of dimethylsulfoxide and acetamide) + 6% PEG (polyethylene glycol, Mr 6000); DMSO refers to 50% DMSO in a carrier containing 2.7% w/v dextran and 2.25% glucose.

The Thermodynamic Necessity of Vitrification

During the cooling of vitrifiable liquids above T_G, their greater molecular mobility in comparison to frozen systems causes them to lose entropy more rapidly than frozen systems. Since the vitrifiable liquid does not freeze, it was noticed by Kauzmann[175] that a physically impossible situation (Kauzmann's paradox) would arise if the liquid did not become immobilized with cooling: namely, its entropy would eventually, at what is now called the Kauzmann temperature (T_K), become less than the entropy of the corresponding frozen system. This observation can be taken to indicate that the energy needed to drive liquid-like motions is effectively removed by cooling prior to reaching T_K, thus providing a thermodynamic explanation for the kinetic phenomenon known as the glass transition. In fact, T_K is quantitatively almost identical[176] to the T_0 of the VTF equation (see above), which, according to the VTF equation, would be the temperature at which viscosity approaches infinity (a complete loss of liquid-like properties, as required to avoid Kauzmann's paradox).

BIOLOGICAL CONSIDERATIONS FOR SUCCESSFUL VITRIFICATION

What Is Cryoprotectant Toxicity?

New insights are beginning to develop into the nature of cryoprotectant toxicity under conditions of cryoprotectant exposure that are relevant to actual cryopreservation protocols. One of the more interesting developments is an expression profiling study involving precision-cut rat liver slices,[177] one interpretation of which is that a significant driver of injury may be protein denaturation.[134,178] This overall conclusion is consistent with evidence that the toxic effects of vitrification solutions are correlated with the strength of interaction between the permeating cryoprotectants and water,[101] with stronger interactions potentially causing injury by affecting protein hydration and hence stability. Interactions between cryoprotectants and water could account for changes in temperature-dependent actin and microtubule polymerization and aggregation, which has been a factor in the destabilization of the meiotic spindle.[179] Although individual cryoprotectants show toxic effects that occur at concentrations lower than those required to denature proteins,[180,181] vitrification solutions consisting of mixtures of individual cryoprotectants exhibit toxicity at much higher total concentrations, suggesting that the use of mixtures overcomes toxic mechanisms that are specific to individual agents and replaces them with denaturation associated with high aggregate concentrations.[134,178] If denaturation is indeed a significant factor in vitrification solution toxicity, there are likely to be several effective interventions to lessen its effects.

Methods of Preventing Injury from Cryoprotectant Exposure

It is now possible to list at least 14 different methods for controlling apparent cryoprotectant toxicity,[134] which are recapitulated here with additional notes about applicability to manual pipetting methods for oocytes or embryos:

1. Avoid osmotic injury.
2. Use mixtures of permeating cryoprotectants (pCPAs; mutual dilution effect).
3. Include npCPAs to enable the use of less pCPA.[107]
4. Keep temperatures as low as possible without exacerbating Method 1 above or inducing intolerable chilling injury, and be aware of and avoid transient warming during procedures such as pipetting.
5. Select an appropriate carrier solution.
6. In the case of oocytes exposed to 1,2-propanediol, use lower concentrations of calcium in the carrier solution.[182]

7. Keep exposure time to the cryoprotectant as low as possible, and do not allow differences in pipetting times to create exposure time differences between different specimens in the same batch.
8. Employ cryoprotectant toxicity neutralization.[181]
9. Do not use more cryoprotectant than necessary (use care in defining the C_V), but be aware of and correct for the fact that pipetting from one solution to another contaminates the new solution with the old.
10. Employ ice blockers[5,183,184] to assist with Method 9, including emerging novel ice blockers[185–194] as they become available.
11. Employ 3-methoxy-1,2-propanediol.[134,195]
12. Preferentially use weakly glass-forming pCPAs.[101]
13. Minimize the "cost function" of cryoprotectant exposure.[196,197]
14. For perfused organs, use newly emerging perfusion techniques.[131]

What Is Chilling Injury?

A recent DNA microarray study of chilling injury in rat liver slices[177] suggests[134,178] that chilling injury in the presence of vitrification solutions can be an extension of cryoprotectant toxicity, with many changes being similar to but intensified versions of changes seen with cryoprotectant exposure alone.[177] A suggested possibility[134,178] is that protein destabilization by cryoprotectants may summate with protein destabilization by cooling,[198–200] leading to an increase in injury associated with cooling per se. This is a departure from, but is not incompatible with, prior observations linking chilling injury, especially at higher temperatures, to membrane lipid phase transitions,[201–208] and is supported by the finding that chilling stimulates heat shock protein production,[209] which appears to be protective,[209] as well as "cold shock" proteins.[210,211] Protein changes are certainly associated with meiotic spindle disassembly and mis-reassembly, which contribute to chilling injury in OOCYTES,[179,212] and may be exacerbated by cryoprotectants.[213]

Methods of Preventing Chilling Injury

Chilling injury has been blocked by changing the membrane lipid composition,[202,214–216] by using antifreeze proteins to modify membrane lipid phase behavior[217,218] and permeability,[217] by prior heat shock,[209] to some extent by lipid segregation or removal methods,[207,219] by tonicity adjustment,[172,220] and by cooling more rapidly than chilling injury can induce damage.[35–37]

Methods of Preventing Osmotic Injury

Osmotic injury is damage caused by the excessive movement of water across cell membranes, resulting in cell contraction or expansion beyond the safe "osmotic limits" of the cell.[221] An extensive discussion of osmosis and the principles of its control during vitrification procedures is available elsewhere,[134] but by using appropriate computer modelling,[196,197,222–227] limited concentration steps, optimized temperatures (which may be higher at lower concentrations than at higher ones[1]), and osmotic buffers, osmotic injury can generally be avoided. Although the importance of osmosis may seem obvious, it is frequently overlooked.[228]

SUMMARY AND CONCLUSIONS

The cryobiologist is beginning to catch up to nature in the ability to preserve increasingly complex systems at low temperatures without ice crystal damage. The modern success of vitrification as a method of cryopreservation for living systems in general, and for cells and tissues related to reproductive functions in particular, is the result of a decades-long process of gradually improving understanding of both the physical and biological requirements of this approach to cryopreservation. Despite many basic misunderstandings, false starts, and failures along the way, the intrinsic feasibility of cryopreservation by vitrification is now clear, and applications are still continuing explosive growth three decades after the first modern wave of enthusiasm for vitrification was launched. A variety of new methods for avoiding injury, increasing safety, and improving ease of use continues to be established, and this can be expected to continue. A hopeful new development for even more forward momentum for the field is just beginning to dawn in the form of new examinations of the molecular biological effects of vitrification solutions and chilling injury, an enterprise that seems likely to bring with it new remedies for old problems and the ability to address new problems that were previously beyond reach. At the same time, large areas of very basic investigation have hardly even begun in, for example, defining the storage stability of vitrified or near-vitrified biological systems near T_G, the contribution of intracellular biomolecules and surfaces to vitrification tendency, the limits of use of npCPAs for vitrification, the nature, rate, and extent of nucleation in concentrated solutions both above and below T_G, the limits of tolerance of living systems to devitrification, the mechanisms by which devitrification kills cells and damages intercellular structures, and "cost function" approaches to reducing cryoprotectant toxicity in many systems. In conclusion, vitrification remains a dynamic and productive area of research in cryobiology, and is likely to remain so for the foreseeable future.

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2 Vitrification of oocytes and embryos: Finally a recognized technique, but still a source of concern and debate

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Even though vitrification was initiated as a viable cryopreservation technique 30 years ago,[1,2] we have had to wait more than 20 years before a shift away from conventional controlled-rate slow freezing (SF) toward vitrification occurred. It is now recognized worldwide that vitrification is more effective than the standard SF technique for cryopreservation of oocytes and embryos at different stages of development,[3–11] resulting in a doubling of pregnancy rates.[3,4] The implementation of this efficient technique gives us the opportunity to change the strategy for embryo transfer and to extend the indications for cryopreservation of oocytes and embryos.

In the past, oocyte cryopreservation (SF or vitrification) was the only viable option for preserving the fertility of women who can be predicted to undergo premature ovarian failure due to pathological or iatrogenic alterations. In addition, to overcome legal, ethical, and religious restrictions, or in cases of absence or delayed semen production, oocyte cryopreservation has been utilized; and also for medical reasons (e.g., polycystic ovarian syndrome and ovarian hyper-stimulation syndrome), oocyte cryopreservation may be the only alternative. However, for other indications such as oocyte banking or family planning, it was not considered reasonable to propose cryopreservation of oocytes. The patients were informed that cryopreservation was still in an area of active research and that one could not guarantee the success of the procedure for the long-term future. Owing to the remarkable improvement in the efficiency of vitrification techniques as an alternative to the classical SF procedure, the proportion of births following the transfer of cryopreserved oocytes has risen substantially. Vitrification is no longer recognized as an experimental method, and the proportion of vitrification cycles for banking purposes as a replacement for surplus embryo storage and for social reasons is on the increase.

The same tendency is also true for embryos. If we look back 15 years, a patient had to undergo several oocyte retrievals to achieve one pregnancy. As a result of our accomplishments regarding a single blastocyst transfer in combination with the very efficient aseptic vitrification technique on day 5 or 6,[12–15] we are now able to maximize cumulative pregnancy rates.[16] From a single oocyte retrieval, a woman may conceive several babies after the transfer of one vitrified blastocyst at a time.

At present, there is a growing trend toward a "vitrify-all" strategy after *in vitro* fertilization (IVF) with a single embryo transfer of a warmed embryo in a subsequent cycle.[16,17] There have been reports of improved implantation and pregnancy rates with frozen embryo transfer as compared to fresh autologous embryo transfer, suggesting superior endometrial receptivity in the absence of ovarian stimulation.[17,18] Clinical outcomes of vitrification/warming cycles support the expectation that transfer of an embryo into a more "physiologic environment" will result in improved pregnancy rates.[19,20] In line with this, recent data also support a potential decrease of both maternal and perinatal morbidity in cryo-cycles.[21] Moreover, vitrification cycles are a valuable option when blastocysts originate from *in vitro* maturation cycles.[12,22]

With the final objective of increasing pregnancy rates, an efficient, standardized, reliable, and harmless vitrification protocol is mandatory. Vitrification procedures consist of several steps. Some of them generate debate. In this review, several phases of the procedure that have aroused controversy are discussed. For example: (a) which blastocysts should be selected before and after vitrification? (b) Is it necessary to collapse the blastocoele? (c) Are concerns about the use of high concentrations of cryoprotectant solutions (CPsol) justified? (d) Is it possible to vitrify using reduced cooling conditions and achieve aseptic vitrification ? (e) Is the warming process more important than the cooling one? (f) And finally, there are concerns about the stability of vitrified biological materials over time.

PRINCIPLE, DESCRIPTION, AND QUESTIONS THAT ARISE DURING THE DIFFERENT PHASES OF A VITRIFICATION PROCEDURE

Vitrification and warming of embryos (blastocysts) consist of several steps (Figure 2.1):

Step 1: Selection of blastocysts before vitrification with or without the artificial blastocoele collapsing.
Step 2: Exposure of blastocysts to the CPsol.
Step 3: Loading on the carrier device and plunging it in liquid nitrogen (LN_2).
Step 4: Storage in LN_2 containers.
Step 5: The warming process.
Step 6: Rehydration and removal of the intracellular cryoprotectant (CP).
Step 7: Selection of warmed blastocysts before embryo transfer.

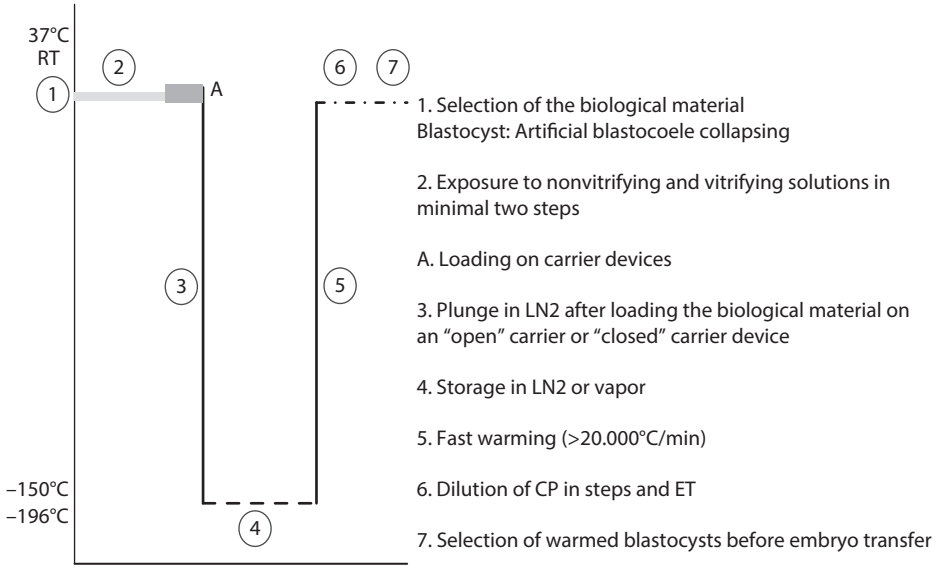

Figure 2.1 **Chronology of the steps in a vitrification/warming protocol. (CP = cryoprotectant; ET = embryo transfer; LN_2 = liquid nitrogen; RT = room temperature.)**

Even though the basic principle for vitrifying biological material is quite similar according to different protocols, some questions are still a matter of debate. We will present our opinions about several of the major questions involved, with special reference to the vitrification of blastocysts.

STEP 1: SELECTION OF BLASTOCYSTS BEFORE VITRIFICATION WITH OR WITHOUT THE ARTIFICIAL BLASTOCOELE COLLAPSING

Selection of Blastocysts before Vitrification: Should We Discard Poor-Quality Embryos?

A pivotal question, especially in autologous cycles, is the selection of blastocysts before vitrification. Which ones should be vitrified and which ones discarded? This raises the question of how to define a good-quality blastocyst: the one that looks morphologically normal or the one that actually implants?

Grading of blastocyst morphology incorporates assessment of the degree of expansion of the blastocyst and the quality of the inner cell mass (ICM) and trophectoderm (TE).[23,24] Several studies have shown that the TE is determined to be significantly related to the rate of ongoing pregnancies and miscarriages. By contrast, neither the ICM nor blastocyst expansion was statistically significantly related.[25,26] In a more recent article, Ahlström et al.[27] analyzed which pre-freeze morphological parameters can be used to predict live birth outcomes after vitrified/warmed blastocyst transfer cycles. They stated that blastocoele expansion and TE grade were identified as the most significant pre-freeze morphological predictors of live birth.

According to our experience, we suggest that, in principle, one will be mainly presented with four groups of blastocysts on day 5:

1. Good-quality blastocysts defined by ICM and TE classified as A and/or B, accompanied by a degree of expansion of 6 (Figure 2.2a), 5 (Figure 2.2b), 4 (Figure 2.2c,d), 3 (Figure 2.2e) or 2 according to the morphological scoring criteria of Gardner and colleagues.[28]
2. The second group is composed of embryos with an ICM and TE classified as B and C, with a degree of expansion of 5, 4, 3, or 2 (Figure 2.3a,b,c).
3. The third group encompasses early blastocysts of good and nontop quality (Figure 2.3d,e).
4. The fourth group includes embryos with delayed blastulation as defined by no signs of cavitation on day 5, plus embryos that show an arrested development on day 3 (Figure 2.3f).

Interestingly, according to the vast majority of data published on cryo-cycles, predominantly blastocysts that fulfill the criteria of the first group are selected for vitrification, and more rarely embryos from the second group. Those that fall into the third and fourth groups are commonly not selected. Thus, clinical results always appear outstanding at first glance. However, if all of the patients who had no embryo transfer and those where no surplus embryos were cryopreserved due to poor morphology would be included in the data, then the overall success rates would look rather more disappointing. But

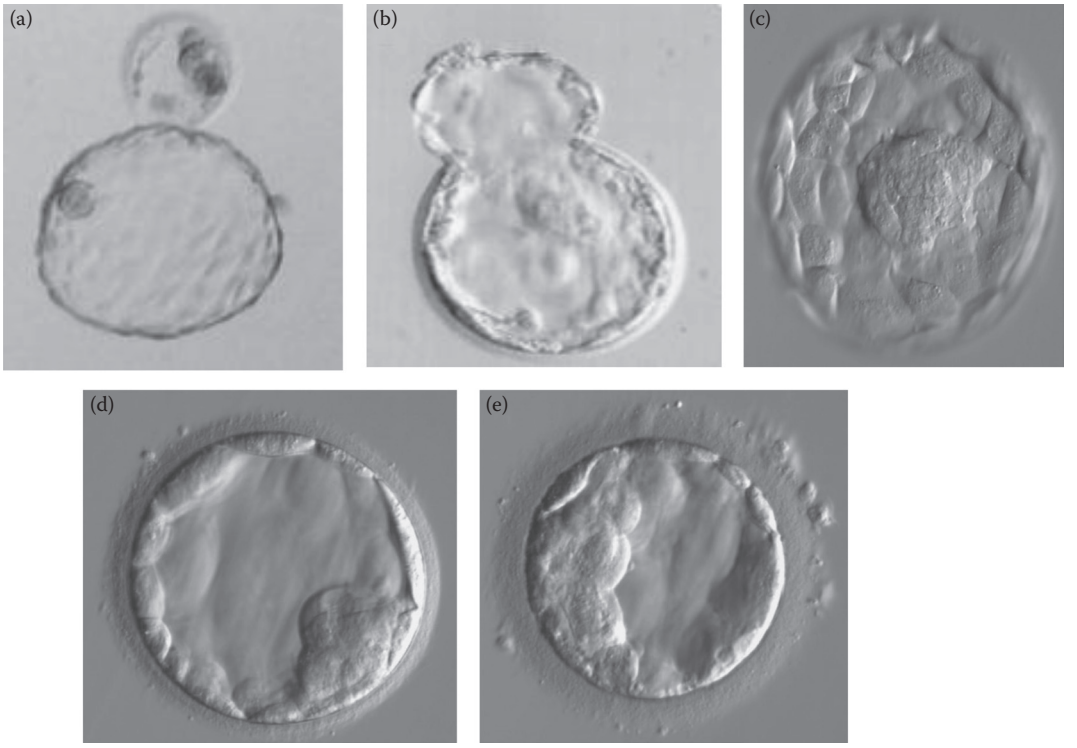

Figure 2.2 **Good-quality blastocysts with optimal inner cell masses and trophectoderm formation and a high degree of expansion.**

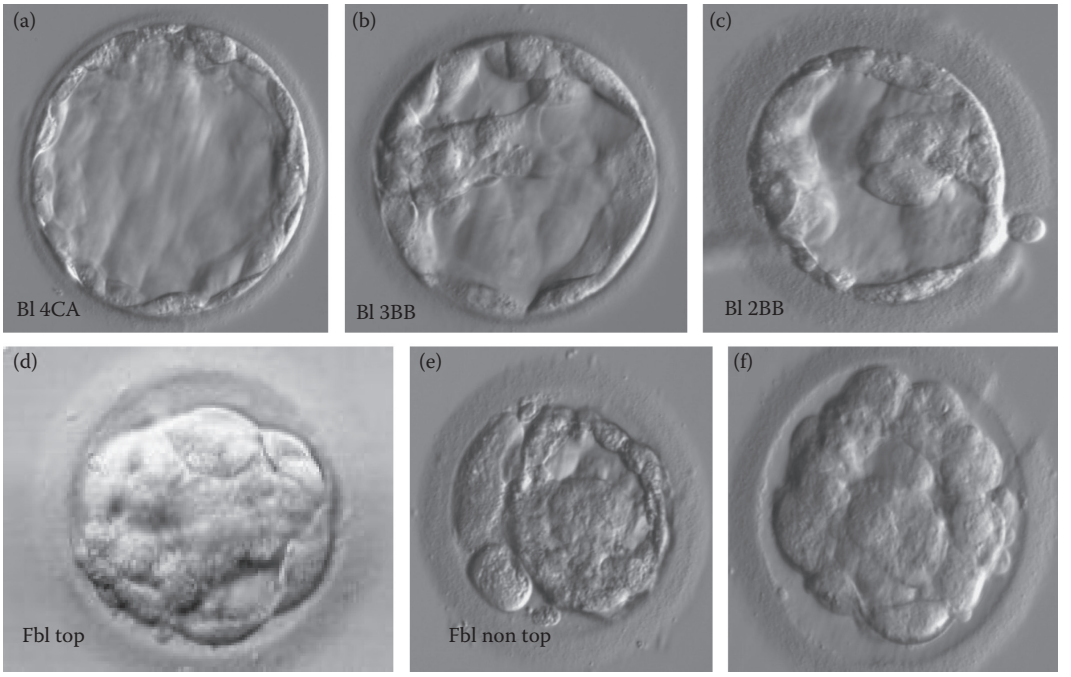

Figure 2.3 **Poor-quality blastocysts with poor inner cell masses and trophectoderm formation as well as a low grade of expansion.**

Table 2.1 Clinical Outcomes after Vitrification/Warming of Blastocysts according to the Morphological Quality

Blastocyst quality before vitrification		Figure	Cycles	Survival rate (%)	Birth rate (%)
i	AA, AB, BA	Figure 2.2c	311	87	30
ii	BB	Figure 2.3b	237	85	32
ii	BC, AC, CA, CB	Figure 2.3a	88	88	35
i	AA, AB, BA	Figure 2.2e	125	89	29
ii	BB	Figure 2.3b,c	203	90	31
ii	BC, AC, CA, CB		66	92	23
iii	Top-grading	Figure 2.3d	44	86	25
iii	Nontop-grading	Figure 2.3e	34	77	22
iv	Blastocysts delayed in development	Figure 2.3f	29	98	37

do we have to present only excellent results, knowing that if we took the patients' point of view, their advice might be totally different?

According to our experience (Table 2.1), we still observe birth rates of 22%–25% when early blastocysts are vitrified and transferred in a cryo-cycle. For those from the fourth group, an extra culture period to day 6 is a valuable option, instead of discarding them according to tangible criteria. Interestingly, we observe a higher birth rate after vitrification of poor-quality embryos on day 5 or 6 when compared to fresh embryo transfers of similar quality.

In the case of slow-blastulating embryos on day 5 (Figure 2.3f), there is a benefit to vitrifying them instead of performing fresh embryo transfer. A total of 488 fresh transfers with slow-blastulating embryos on day 5 were analyzed. Clinical outcomes were compared: (i) with 82 cryo-cycles, after day 5 vitrification and warming 24 hours before cryo-embryo transfer; and (ii) after prolonged culture of slow-blastulating embryos for 24 hours that resulted in 58 cycles with day 6 blastocyst vitrification. Birth rates after a day 5 fresh single embryo transfer with top early blastocysts (mean female age: 37.5 years) were 26.1%, with nontop early blastocysts (mean age: 37.1 years) at 6.5%. In cryo-cycles (mean age: 38.2 years) with vitrified day 5 early blastocysts/morulae, warmed 24 hours prior to embryo transfer (ET), birth rates were 18.3%. When vitrification was done on day 6, the birth rate with top-grade blastocysts derived from slow embryos on day 5 was 42.9%. Nontop-grading blastocysts vitrified on day 6 showed birth rates of 18.3%. Better synchronization between the embryo stage and endometrium is suggested to be mainly responsible for this outcome.

Acceptable results were also obtained after vitrification of blastocysts that originated from embryos that were not selected for fresh ET and underwent cryopreservation on day 3 because of their poor quality. Fifty blastocyst warming cycles resulted in a 76% survival rate, 44% clinical pregnancy rate, and 39% implantation rate.[29]

So, is it really an option to reject those blastocysts for transfer or vitrification? It may be that we discard too many of these poor-morphology embryos in order to "beautify" the implantation rates.

It is worth mentioning that with current technology, it is difficult to select the right embryos even in a routine IVF laboratory. Of course, when good-morphology embryos are present, they will be selected for transfer or for cryopreservation. However, there is still no clear method or algorithm that will improve this selection. But the more difficult task at present is how to select from a cohort of poor-morphology embryos the one that may implant after fresh ET or cryopreservation. Again, what should we do: keep everything, or deselect all?

Following the results of our data, it should be mandatory to cryopreserve all supernumerary blastocysts of moderate to good quality in order to increase the cumulative pregnancy rate. With regard to this, it is very important to achieve a reproducible outcome, especially in terms of survival after warming independent of the quality, to allow high success rates after vitrified/warmed blastocyst transfer.

Is It Necessary to Collapse the Blastocoele?

With respect to blastocyst quality and survival, the question arises as to whether artificial shrinkage or collapsing should be mandatory (Table 2.2). Since the size of the blastocoele corresponds to the amount of water in this cavity, larger blastocysts might show reduced cryosurvival potential due to ice crystal formation during the cooling step and ice recrystallization (devitrification) during the warming step.

In order to reduce the likelihood of ice crystal formation, artificial shrinkage or collapsing of expanded blastocysts has been suggested using either micropipettes[30] or laser pulses.[31]

However, with use of vitrification solutions containing higher sucrose concentrations of 0.75 M instead of 0.5 M, we observed that artificial shrinkage of the blastocoelic cavity is less necessary for preventing injury from intracellular ice, because of sufficient dehydration during the exposure to the vitrification solutions. As another strategy, Zech et al.[32] showed the benefit of opening the zona

Table 2.2 Impact of Artificial Collapsing before Vitrification Using Different Types of Carrier Devices and Vitrification Media

Concentration nVS	Concentration Sucrose VS	Carrier devices	Artificial collapsing	Survival (%)
20%	40% 0.3 M	Open	No	20–30
3 min	30 s	Closed	Yes	78
10%–20%	40% 0.75 M	Open	No	92
4–5 min	60 s	Closed	Yes	90
15%	30% 0.5 M	Open	No	82
8–12 min	60–90 s	Closed	No	64
			Yes	88

Note:
nVS, nonvitrification solutions:
20%: 10%–10% EG/DMSO;
15%: 7.5%–7.5% EG/DMSO.
VS, vitrification solutions:
40%: 20%–20% EG/DMSO;
30%: 15%–15% EG/DMSO.

pellucida some hours before the vitrification process. They observed that blastocysts with a larger blastocoelic cavity survived vitrification better when they were partially or completely hatched, even with a short exposure to CP solutions.

Concentration of CP and Cooling/Warming Speed: Two Parameters to Control in Order to Achieve a Vitrified State

When the temperature decreases, liquid water can be converted either to a solid crystal or to a solid amorphous glass when the supercooled water is dropped instantaneously below the glass transition temperature (T_G). It is simply the ability of being able to prevent ice crystals from forming inside the cell (which can happen during the cooling as well as the warming process) that will determine the viability of the embryos after cryopreservation. The physical process by which a superviscous liquid solution remains supercooled during transition through the crystalline phase and reaches the solid glassy state when it is cooled below its T_G is called vitrification.[1] According to this definition, with the application of the vitrification process, ice crystal formation is still possible either in the intracellular or in the extracellular spaces, but solidification of a superviscous liquid solution that remains supercooled during the cooling process will obviate this possibility (Figure 2.4).

The fundamental issue in all vitrification methods is achieving and maintaining conditions within the cells that guarantee an amorphous state throughout the cooling, as well as during the warming process. Independent of the carrier device that determines the cooling and/or

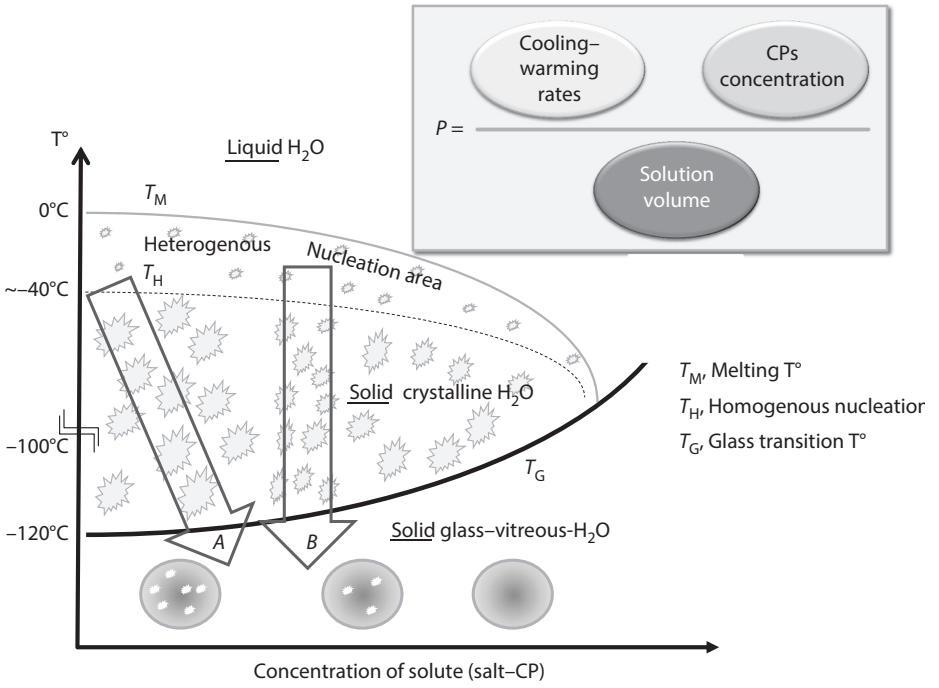

Figure 2.4 **Phase diagram presenting the different phases of water according to the temperature. The equation represents the factors that influence the probability of achieving the vitrified state. The arrows represent the cooling speed and the size of ice crystal formation that are associated. Arrow A represents a less fast cooling than arrow B. (CP = cryoprotectant.)**

the warming rate, the key to success, in order to achieve a "glass-like" state, depends on an optimal balance between the speed of cooling, rewarming (time and temperature), and the optimal cell dehydration and penetration of CP when the cells are exposed to concentrated hypertonic solutions.[33]

STEP 2: EXPOSURE OF BLASTOCYSTS TO HIGH CONCENTRATIONS OF CP SOLUTIONS—WHAT IS THE FINAL INTRACELLULAR CONCENTRATION OF CP?

There is no doubt that all CPs are toxic and that each CP has its own intrinsic toxicity that induces biophysical and biochemical interaction with the cellular structures. This toxicity is related to the type of CP, the incubation temperatures, and the concentrations. During the SF process using 1–2 propanediol, an extracellular and intracellular accumulation of 1–2 M propanediol is generated by long exposure times before freezing and by the formation of extracellular ice crystals. Could this explain why Pinborg et al.[34] reported an increased birth weight of children born after SF embryo transfer using 1–2 propanediol as the CP? It is reported that 1–2 propanediol displays higher genotoxicity and induces DNA damage *in vitro*, leading to chromosomal mutations.[35,36] In addition, cytotoxic effects should not be underestimated.[37]

In the majority of vitrification solutions, ethylene glycol is associated with dimethyl sulfoxide (DMSO). DMSO was shown to alter DNA methylation, changing the epigenetic profile of cells.[38] There are, in fact, plenty of reports with controversial conclusions related to the toxicity of CPs. Therefore, the use of CPs cannot be easily dismissed as a nonissue. What is clear is that the use of very high concentrations of CPs may be a strong argument against the increasing use of vitrification.

The total concentrations required to obtain a vitreous state are usually between 6 and 8 molal and are very near the tolerable limits for cells. As a consequence, fear about exposing gametes and embryos to high amounts of CP solutions that exceed three- to four-fold of those applied in SF has been the central part of a debate initiated by advocates of the SF procedure, where lower concentrations of penetrating CPs are used. This point of the debate is extensively described in Chapter 3.

Concisely, we have found that only a small percentage of the CP present in the vitrification solutions enters through the cell membrane. We have found that the intracellular concentration of cryoprotectant (ICCP) in mouse zygotes during the vitrification procedure prior to plunging them in LN_2 is approximately equal to 2.14 M, and is three-fold below the concentration in the vitrification solution that surrounds the cell.[39] Furthermore, contrary to common belief, it was observed that the intracellular concentration of CP in vitrified zygotes is even lower than after SF, despite working with higher amounts of CPs in the solutions.

Consequently, we may state that although SF has been the standard cryopreservation method for more than 25 years, few practitioners were aware that cell survival was principally a result of the presence of an intracellular vitrified state. This vitrified state is a consequence of the prolonged effect of the solution during cooling, and is a reflection of a very high concentration of CP. Thus, we may suggest that a drop in the survival after SF is probably more related to osmotic shock after warming than to mechanical injuries due to the formation of intracellular ice crystals. To conclude, we may state that SF is nothing more than another way of performing vitrification.

STEP 3: LOADING THE CARRIER DEVICE AND PLUNGING INTO LN_2: CAN WE FORESEE AN END TO THE "OPEN" SYSTEM?

A summary of the open and closed vitrification devices with their specific volume and cooling/warming rates will be discussed in Chapter 6.

Open Systems

It has been postulated that ultra-rapid cooling and warming rates (as high as 20,000–30,000°C/min) are obligatory during the vitrification process in order to reduce the risk of intracellular crystal formation and the concomitant damage to the cell structures.[40] In order to achieve ultra-rapid cooling rates, very small volumes of vitrification solution of less than 1 µL are deposited on open carrier devices (e.g., Cryotop, Vitriplug, Cryoloop, and copper electron microscopy grids), which are directly plunged into LN_2.[41–43]

The advantage of such an approach is that blastocysts are exposed in two steps to increasing concentrations of CP. This is only for a short period of time, but nevertheless it is long enough to permit the extraction of the intracellular water while limiting the amount of CP permeating into cells, thus limiting osmotic stress.

One drawback of the "open" carrier devices is that the blastocysts are directly exposed to LN_2 during the cooling process, as well as during the whole storage time. This is not in concordance with medical directives for the storage of biological tissues that are put in place to minimize the risk of cross-contamination from infected material, and also from the LN_2 itself, which is not sterile. In spite of the existing theoretical risk of cross-contamination by bacteria, viruses, or fungi during cooling as well as during storage in LN_2, the clinical significance is widely debated.[44,45] Retrospective investigations in which commercial LN_2 cryotanks were examined after 35 continuous years of service revealed various bacterial and fungal contaminations in the LN_2 detritus, but reassuringly, many of the identified bacteria isolated were universal environmental microorganisms and were rare opportunistic pathogens of low significance to producing disease in humans or animals.[46] In addition, the potential probability of a toxic

effect with the reactive chemical compounds present in LN_2 raises safety concerns.[47]

Various methods for sterilizing LN_2 have been proposed or are under development, including ceramic filters[48] or ultraviolet light, with subsequent hermetically sealed cryostorage[49,50]; or even by using LN_2 vapor for storage.[51]

Although the probability of an impairment of cellular structures by contact with LN_2 is still being discussed, this potential risk is important, and the ongoing discussion indicates that the storage system, especially long term, should be revised. Even the standard storage conditions with refilling of the tanks pose a hazard, when oxygen from the surrounding air condenses and mixes with LN_2 during the regular opening of the dewar tank for routine refilling, or indeed whenever straws are added or withdrawn. Although it is generally assumed that thermally driven reactions do not occur in cells at –196°C, it has been reported that in the case of irradiation of an LN_2/oxygen mixture, a synthesis of oxygen radicals resulting from ozone formation and decomposition cannot be discounted; and this may even be enhanced by the catalytic effect of nitrogen. Mouse oocytes have shown impaired survival, fertilization rates, and embryonic development after prolonged contact with LN_2.[47]

Closed Systems

The European Directives (2004/23/EC)[52] and the Food and Drug Administration (FDA) directives on tissue and cell storage[53] dictate adherence to certain safety regulations, ensuring that gametes and embryos are protected from any possible contamination with pathogens and attempting to prevent them from any harmful physical conditions during cryo-storage. To comply with the EU directive, a valuable option consists of switching from an open vitrification carrier device to a protocol that entails complete isolation of the biological samples from LN_2 during both the cooling process and storage by hermetically isolating the embryos from LN_2 in the dewar tanks.

A huge difference in cooling/warming rates exists between vitrification protocols with open (>25,000°C/min) or closed (<2000°C/min) carrier devices. This reduction in the cooling rate is responsible for the ongoing debate, as the cooling rate is widely believed to be an important factor in the success of vitrification protocols. However, several studies have shown that vitrification of oocytes,[54] zygotes,[19] and blastocysts in closed carriers can achieve good results in clinical studies.[12,13,15,55,56] Taking into account that the reported survival rates after aseptic vitrification correspond with data observed in open devices, the functional aspect of embryos/gametes frozen with the different devices remains to be investigated.

In a recent study, Chatzimeletiou et al.[57] investigated the effects of aseptic vitrification on the cytoskeleton and development of human blastocysts by analyzing survival rates and spindle and chromosome configurations by fluorescence and confocal laser scanning microscopy. Even though there was a significantly higher incidence of abnormal spindles in the vitrified group compared with the fresh group, the high survival rate following warming and the large proportion of normal spindle/chromosome configurations suggest that aseptic vitrification of blastocysts on day 5 in carrier tools sealed into a container does not adversely affect the development of human embryos and the ability of spindles to form and continue normal cell division.

There is no direct evidence of cryopreserved human oocytes/embryos becoming contaminated during cooling and cryostorage.[58] However, given the available information from bovine embryo studies and potential concerns with long-term storage in open devices, it would appear prudent to avoid such direct contact with LN_2 during vitrification and subsequent cryo-storage, by using closed devices.

STEP 4: STORAGE IN LN_2 CONTAINERS: FOR HOW LONG IS THIS POSSIBLE?

Since the goal of cryopreservation is to preserve cells and tissue in a solid state for long periods, the stability of vitrified biological materials over time is an important issue, and certainly is still a matter of debate when considering the thermodynamic aspect. The instability phenomenon under the T_G is of particular interest, because the considerable molecular mobility that persists near and under the T_G makes the stability of such storage temperatures unclear. In fact, crystalline solids are more stable because there is usually the energy of crystallization that is released upon crystallization with interactions between molecules. By contrast, the conversion to a glass-like state during vitrification is not accompanied by exothermic heat of fusion, but is accompanied by changes in the heat capacity of the sample.[59] The glass transition can be understood on a molecular level as a loss of translational and rotational degrees of freedom over a particular measurement timescale, but still leaves bond vibration within a fixed molecular structure.[60]

Although diffusion is practically nonexistent below the T_G, small local movements of molecules related to relaxation have consequences for cryobiology.[61] This is a result that occurs during the forming of amorphous solids when substances are cooled so quickly that their molecules move too slowly to orient themselves. It is commonly admitted in the field of thermodynamic cryobiology that once the glass state is formed, it can be aged. Aged amorphous materials show decreased physical and chemical reactivity compared to un-aged materials.

Although many thousands of children have already been born after blastocyst vitrification, many aspects of this technique remain to be elucidated. New applications, such as fertility preservation, lead to long storage times of vitrified gametes or embryos. However, it remains to be determined whether these vitrified gametes and embryos

are stable over time. In the assisted reproductive technology field, little is known about the risks of prolonged storage of cryopreserved cells, given that vitrification is the solidification of a fluid without the formation of crystalline structures—a physically disorganized and therefore potentially unstable system. This raises the question that if this state changes over time, will this significantly impair the cryo-survival and implantation potential of vitrified gametes and embryos? Subsequently, any potential impact on the health of the newborn remains largely unknown.

A study by Wirleitner et al.[62] included the transfer of blastocysts that had been vitrified aseptically with the Vitrisafe® device, and showed that the storage of vitrified blastocysts in aseptic conditions did not impair blastocyst viability. The survival rate after warming during the first year of storage was 83.0%, compared with 83.1% after 5–6 years of storage (no significant difference); there were also no decreased pregnancy rates, as the clinical pregnancy rate after 1 year of storage was 40.0% versus 38.5% after 6 years (no significant difference). In addition, no increase in the neonatal malformation rate over time was observed.

STEP 5: IS THE WARMING PROCESS MORE IMPORTANT THAN THE COOLING ONE?

Since the rate of cooling engendered a hot debate, it is surprising that little emphasis was put on the warming procedure. However, it has become clear that the warming rate might play a greater role in establishing cryosurvival after vitrification than the cooling rate. Seki and Mazur[63,64] have shown that mouse MII oocytes survived well in reduced cooling conditions, as long as they were warmed as rapidly as possible.

According to the theoretical definition of vitrification and the probability of obtaining a vitreous state with the increasing concentration of CP, we may expect complete intracellularly vitrified states (Figure 2.5, situation cell 3). Even without the presence of small nuclei of ice crystals, the warming rate is crucial in order to achieve high survival rates. When absolute intracellular vitrification is completed (Figure 2.5: situation cell 3), a slow rise in the temperature above the T_G will induce first a direct devitrification (Figure 2.5A and B), with the formation of plenty of small intracellular crystals that will reorganize (Figure 2.5C and D) with a further rise in temperature.[65] In contrast to what happens during the cooling process,

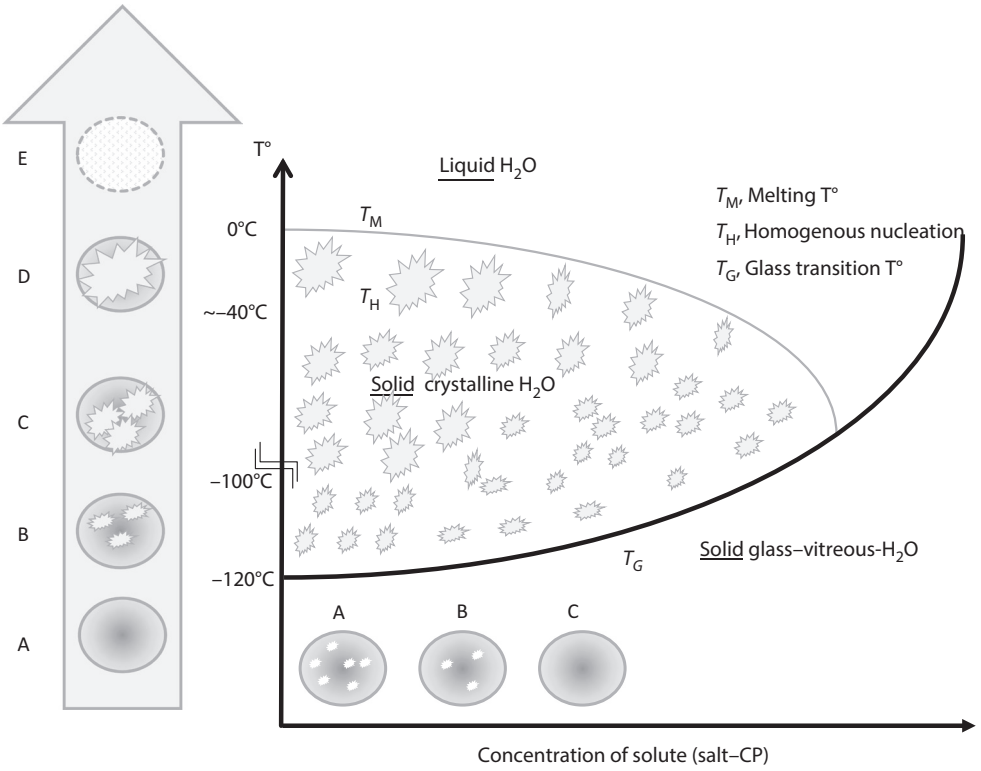

Figure 2.5 **Devitrification and ice re-crystallization during warming procedures that are too slow. (A) Vitrified—maintain a fully amorphous intracellular state. (B) Devitrification may occur when the temperature crosses the T_G. (C) Freezing—crystallization step. (D) Re-crystallization to a damaging size. (E) Thawing and cell lysis. (CP = cryoprotectant.)**

in which the sample is in a supercooled condition after crossing the melting temperature (T_M), the sample above the T_G will start rapidly to develop ice crystals.

We have shown that the intracellular concentration of CP is substantially lower than that present in the vitrification solutions.[39] So the question is—even though good survival rates are obtained after vitrification, are fully vitrified states obtained intracellularly? It has been demonstrated that the faster the cooling rate, the smaller the size of the ice crystals that will be formed[66] (Figure 2.4: arrow B vs A). According to the previous study, Seki and Mazur believe that the crystal size determines the rate of warming, and consequently the survival rate.[65] Therefore, the primary cause of injury or cell death in the vitrification procedure is ice re-crystallization (devitrification) during warming, and not failure to vitrify during cooling.[63,64] If very small intracellular ice crystals are present in the intracellular compartment, they will be innocuous during the cooling process (Figure 2.5: situation cells 1 and 2), but they may become lethal according to the warming rate. In the case of warming being too slow, there is sufficient time for the aggregation of many small-sized crystals into larger ones[65] (Figure 2.5B–D). The oocytes or embryos will then not survive the re-crystallization that occurs during the warming process, as the growing ice crystals will reach a lethal size. Therefore, to compensate for the greater driving force, one needs to warm more rapidly to avoid re-crystallization.

It is well known that for any given concentration of CP, the critical warming rate is much higher than the critical cooling rate.[67] Consequently, the minimal concentration of CP to prevent crystallization during warming must be higher than during cooling. This means that it might be easier to maintain a vitrified state during the cooling process than during the warming process for the same concentration of CP. If the warming rate is reduced by using a device that isolates the drop containing the embryos from the LN_2, then higher intracellular concentrations of CP are needed in order to reduce the likelihood of re-crystallization. However, these higher concentrations of CP might be toxic to the cells.

Hence, in order not to increase the concentration of CP above the toxic level, the biological material has to be warmed extremely rapidly. Consequently, most open or closed devices that are used produce very high warming rates exceeding 20,000°C/min. Mechanistically, this has resulted in the confounding of whether the cooling rate or the warming rate is relatively more important.

STEP 6: WHY IS DILUTION OF THE CP REQUIRED?

During warming, water re-enters the cells and CPs are washed out. This has to be performed in a controlled way in order to avoid cellular damage. The influx of water being too rapid is circumvented by a stepwise exposure to solutions containing reducing sucrose concentrations.[39]

STEP 7: SELECTION OF WARMED BLASTOCYSTS BEFORE ET: WHICH ONE TO SELECT?

Though some post-thaw morphological predictors have been investigated in SF of blastocysts (e.g., immediate re-expansion[68,69] or 24-hour survival), no such data have yet been published for vitrified blastocysts. It has been suggested that as a result of the presence and size of the blastocoelic cavity, vitrified warmed blastocysts experience several morphologic changes and become collapsed during cryopreservation. Thus, it is more difficult to score a vitrified blastocyst after warming than a fresh one.[69] Several factors (unrelated to the vitrification process) are known to directly influence the fate of a cryopreserved blastocyst after thawing/warming and transfer. It is important to realize that the survival rates reported in the literature are not easily comparable, due to the fact that some embryologists focus on immediate cryo-survival, while others suggest an additional waiting period of 24 hours, for example, to facilitate control of growth. Differences in implantation rates may also be attributed to the fact that not all working groups apply assisted hatching to the thawed blastocysts (whilst shrunk), though this has been found to improve outcomes.[40] Re-expansion of the blastocoele (and consequently the blastocyst) after thawing is expected within 24 hours.[68,69] However, immediate re-expansion (e.g., within the first 2 hours after warming) has not been used for prognostic purposes for vitrified blastocysts. Since in SF, approximately half of the frozen blastocysts re-expanded immediately after 2–4 hours in culture,[69] it has been reported that when using vitrification, a higher rate of re-expansion is observed.[70]

Even if it can be assumed that all viable blastocysts will re-expand after several more hours, any delay in this process could be the manifestation of altered osmotic and/or metabolic conditions. This is comparable to the situation found during blastocoele development, when water enters the blastocoelic cavity via tight junctions either by diffusing passively or being pumped actively. To conclude, then, a fast re-expansion post-warming is the best indicator of blastocyst viability.

CONCLUSIONS

It is now proven that aseptic vitrification with the closed devices such as the Vitrisafe® carrier can be effective for oocytes, zygotes, and blastocysts at different stages of development and quality. Vitrification has to be adapted according to the cooling/warming conditions. Higher levels of intra- and extra-cellular CPs are needed to keep the solution in a vitrified state in the event that there is a reduced cooling as well as warming rate. However, the fear about exposure of biological material to higher concentrations of CPs does not appear justified. Even though a lower cooling rate exists with aseptic vitrification, it remains an effective procedure if higher warming rates are achieved. It is our advice that this strategy of "better safe than sorry" has no negative impact on the viability of vitrified blastocysts over

a period of 6 years. Critical warming rates are typically two or more orders of magnitude greater than critical cooling rates. Collapsing of the blastocoele is not really necessary and has to be adapted to the rate of dehydration that occurs in the vitrification solution. Finally, the speed of re-expansion after warming is a good sign of viability.

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3 Intracellular concentration of cryoprotectant during vitrification and slow-freezing cryopreservation procedures

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INTRODUCTION

To reduce the likelihood of intracellular ice crystal formation, which has detrimental effects on cell organelles and membranes, vitrification (VIT) was first introduced as an alternative to slow freezing (SLF) to cryopreserve embryos and gametes 30 years ago.

It is now well documented that compared to SLF, VIT is a very effective procedure to cryopreserve MII oocytes,[1–6] zygotes,[7,8] and embryos at different stages of development.[9–14] Following the reports of an efficient VIT technique, a change in both the strategy for embryo transfer and an expansion of indications for cryopreservation of oocytes and embryos is occurring. For example, the "freeze-all" strategy, in combination with single-embryo transfer and preimplantation genetic screening (PGS) on day 5 or 6 will likely become the standard procedure to increase the cumulative birth rate. Furthermore, at present, in addition to fertility preservation, to avoid legal, ethical, or religious restrictions, oocyte cryopreservation is now commonly applied for oocyte banking and for women who want to preserve their oocytes for social reasons.

VIT: REASONS FOR LACK OF ENTHUSIASM A DECADE AGO

In spite of the first announcement of successful VIT in the mammalian model in 1985,[15] we had to wait for more than two decades of SLF use before the full acceptance of VIT in the assisted reproduction technology field.

How can this unwillingness to move to VIT be explained, despite the up-to-date publications being reassuring in terms of obstetric and perinatal outcomes? One part of the answer is directly related to the methodology of VIT that is totally different when compared to SLF in term of cryoprotectant (CP) concentration.

A fear about exposing gametes and embryos to such high amounts of CPs that exceed by three- to four-fold those found in SLF (CP solutions [CPsol] of 1.5 M) was the central part of a debate initiated by advocates of the SLF procedure in which lower concentrations of penetrating CPs are used.

It is well accepted that all CPs are potentially toxic. The impact of the CPs used on the safety of VIT has always been a major scientific concern. Although the up-to-date publications were reassuring in terms of obstetric and perinatal outcomes,[16–20] further studies were necessary to prove the safety of the procedure and to exclude any negative impacts on the development of vitrified warmed embryos.

EXPOSURE OF OOCYTES OR EMBRYOS TO HIGHER CONCENTRATIONS OF CPS: WHY AND WHEN?

The central part of this debate concerns first the use of solutions containing high concentrations of CP, and furthermore the reasons why in specific situations we have to increase the time of exposure to penetrating CPsol.

Why Use Such High Concentrations of CPs in the Last Solution before Plunging in Liquid Nitrogen?

General principle

Before the oocytes or embryos are immersed in liquid nitrogen (LN_2), they are exposed to CPsol in order to increase the intra- and extra-cellular viscosity to a level such that the liquid water molecules will solidify so quickly that they will not have time to rearrange themselves into a crystalline structure when they have to cross the crystalline phase. This means that before plunging the biological material in LN_2, the intracellular compartment has to be conditioned to allow the emergence and maintenance of a vitreous state.[21]

To achieve this objective, in nearly all VIT methods, the biological materials are exposed to a minimum of two steps of gradually increasing concentrations of non-vitrifying solution (nVS) and vitrifying solution (VS).[8,11] Usually, oocytes or embryos are first exposed to two nVS (nVS1 and nVS2) containing cell-penetrating CPs (2.3–3.2 M).[1,8,11] In the last step, just before being plunged in LN_2, they are exposed for a short time (45–90 seconds) to a VS containing very high concentrations of penetrating (4.8–6.4 M)[1,8,11] and nonpenetrating (0.5–0.75 M) CPs.

The objectives of exposure to the nVS and VS

The objectives of exposing the biological material to the VS that contains nonpermeable CPs (sucrose [SUC] and trehalose) with low or high molecular weights (Ficoll) are two-fold.

The first is to create an intracellular vitrifiable state due to the dehydration of the cells in contact with the SUC that concentrates the intracellular solutions of salts, (glyco)proteins, and also CPs that have previously penetrated the cell in the course of exposure to the nVS. The cytoplasm contains various intrinsic macromolecules and salts that are concentrated during the final dehydration step, so favoring the amorphous state. This generates an intracellular environment that promotes the maintenance of a vitreous state when cells are rapidly plunged into LN_2 or warmed.

The second aim of exposing the cells to such high concentrations of CPsol is to create a vitrifying extracellular sheath to avoid ice crystal formation, which may have detrimental effects. In fact, the extracellular vitrifiable state is obtained by the high concentration of CPs in the VS that encapsulates the embryo in a vitrifying sheath.

In Which Situation Do We Have to Increase the Exposure Time to the CPsol?

VIT is the conversion of a superviscous, supercooled liquid into a glassy state when it is cooled below its glass transition temperature (T_G).[21] The cooling and warming rates, the volume of the drop containing the biological material, and the exposure of the biological material to high concentrations of CPsol are conditions supporting the maintenance of an intra- and extra-cellular environment during the transition from a liquid to a solid glass-like state, and in reverse when warming back to a liquid state.

During the steps of exposure to the different nVS, a certain amount of CP enters the cells. This may take 3–15 minutes according to the type of CP used and the cooling rate, which in turn depends on the carrier device. The time of exposure to the nVS at a defined temperature is of utmost importance and determines the amount of intracellular CP.

The duration of exposure to the permeable CPs is determined by several biophysical factors, such as the membrane properties (cellular permeability to water and CP), the type and concentration of CP, the surface/volume ratio of the cells, and the rate of cooling and warming.[22–25] The nVS is exclusively composed of permeable CPs (e.g., dimethyl sulfoxide [DMSO], ethylene glycol, 1,2-propanediol [PROH], and glycerol).

ASSESSMENT OF THE INTRACELLULAR CONCENTRATION OF CP

The questions regarding the use of high concentrations of CP, or increasing the intracellular concentration in relation to the type of device used, were the origins of a debate. In this context, the question was: how high is the intracellular concentration of CP (ICCP) during the VIT and SLF cryopreservation procedures?

In order to find an answer for those who are concerned about the high concentration of CPs during the VIT process, we determined:

- The ICCP at the end of the exposure steps to the CPsol.
- Which of the techniques—SLF or VIT—result in the highest ICCP.

Determination of the ICCP by Cellular Volume Monitoring

Strategy and method for ascertaining the ICCP

The ICCP was determined at the end of the exposure steps to nVS1, nVS2, and VS using cell volume measurements.

Cells adapt their intracellular compartment, by volume modification, in reaction to osmotic modifications in their environment. Exploiting this mechanism, we determined the final ICCP after the incubation steps in the VIT solutions by exposing the cells to defined molarities of SUC solutions. No changes in cell volume, and therefore no movement of water through the cell membrane, indicates equivalent intra- and extra-cellular osmotic pressures on both sides of the membrane. Knowing the osmolarity of the SUC solution consequently allows determination of the ICCP. The ICCP was then compared with the one in the VS.

Mouse zygotes were exposed to nVS1, nVS2, and VS (Figure 3.1b) before placing them in solutions with various SUC molarities. The observations were performed under an inverted microscope (IX51; Olympus) equipped with a camera (Olympus DP50), and software that records the pictures (AnalySIS version 3.2; Olympus) (Figure 3.1a). In order to maintain the zygote at the same focal plane during the transit from one CPsol to the next, a holding pipette fixed on a micromanipulator was installed (Figure 3.1b).

Results and arguments for reduced ICCP

Using a cinematographic recording system, we estimated the degree of dehydration and penetration of CPs inside the cells during the exposure to nVS1, nVS2, and VS (Figure 3.1c).

The cells in our VIT protocol were exposed for short periods to nVS1 and nVS2, and did not reach complete equilibration. Hence, cells were exposed for a short period to the VS containing SUC, and an intracellular vitrifying state was obtained after dehydration and exposure to concentrations of the previously entered nVS1 and nVS2 (Figure 3.1c, steps v and vi).

The cinematographic analysis also allowed us to determine the SUC concentration in which no modification of the cell volume was observed, allowing inference of the ICCP.

After the steps in the nVS and VS, zygotes were exposed to increased solutions of SUC of increasing molarity (Figure 3.2). We have observed that the volume variations decreased when increasing the SUC molarity. This decrease in amplitude of swelling is the result of reduced inflow of water. Movements of water across cell membranes occur easily and rapidly, and continue until intracellular and

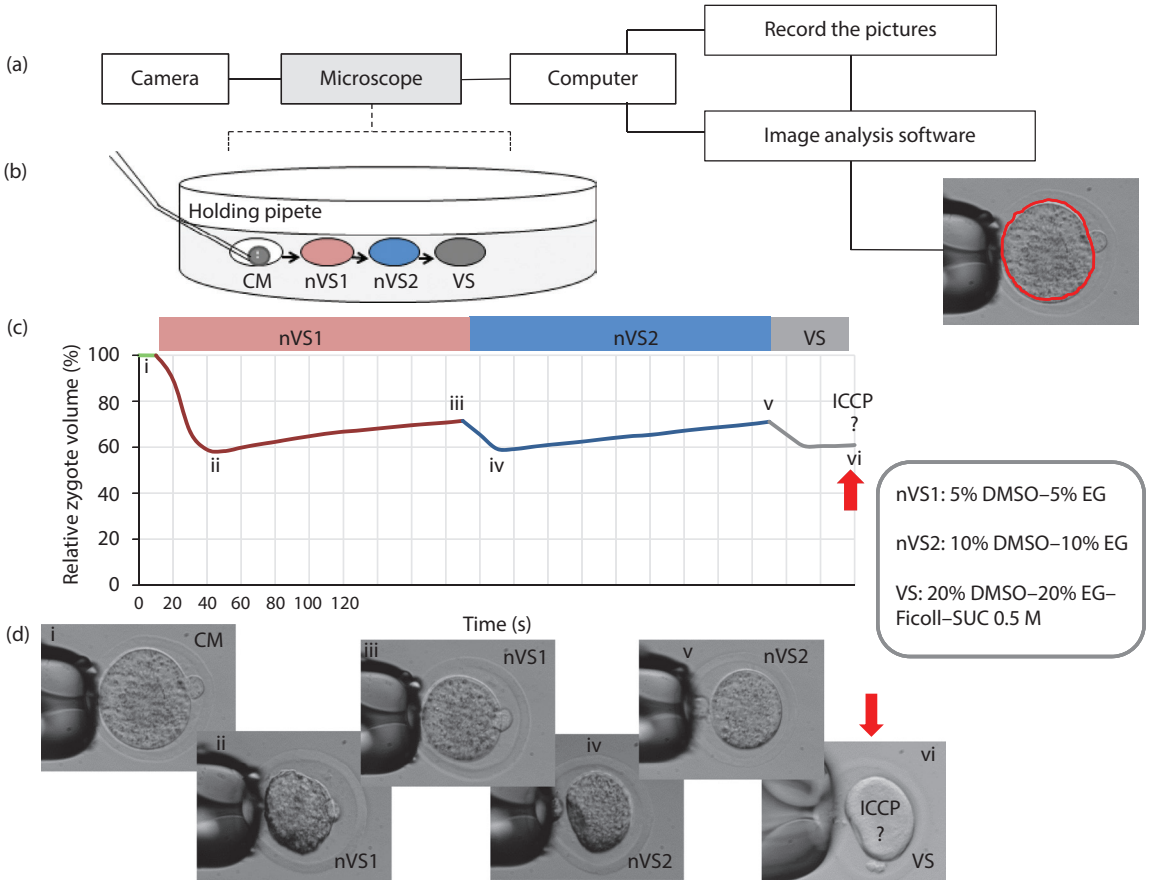

Figure 3.1 **Quantitative assessment of the ICCP by cinematographic analysis. (a) Technical design for the recording and measurement of the zygote. (b) Method used to monitor the change in volume of the zygote during exposure to nVS1, nVS2, VS, and various molarities of SUC (holding pipette used in order to maintain the zygote in the same focal plane from one droplet to the next). (c) Evolution of the relative volume of one zygote through the various CP solutions. (d) Pictures of the zygotes at different time points: (i) the culture medium; (ii) at the end of the dehydration step in nVS1; (iii) before transferring in nVS2; (iv) at the end of the dehydration step in nVS2; (v) before transferring in VS; (vi) arrow indicates the time point at which the ICCP was evaluated. (CM = culture medium; DMSO = dimethyl sulfoxide; EG = ethylene glycol, ICCP = intracellular concentration of cryoprotectant; nVS = Non-vitrifying solution; SUC = sucrose; VS = vitrifying solution.)**

extracellular tonicities are identical. When incubated in 2.14 M SUC solution, only minimal changes in cell volume occurred, indicating an iso-osmotic situation between the intracellular and extracellular compartments. During VIT, therefore, this intracellular osmolarity approaches 2.14 M before plunging in LN_2.

Comparison of the ICCP after SLF or VIT

In a second study, we evaluated in which of the cryopreservation techniques (VIT versus SLF) the final ICCP was higher (Figure 3.3).

It is accepted that the higher the ICCP, the lower the probability of (re)crystallization during the warming step, and the higher the probability of overswelling of the cell due to the fast entrance of water. Based on this assumption, two protocols were designed allowing comparison of the survival rates of mouse zygotes after SLF on the one hand, and VIT on the other, with (i) various warming rates; and (ii) various concentrations of SUC in the warming dilution medium (Figure 3.3).

Comparison of survival rates after VIT or SLF relative to the warming rate

- *Principle.* It is well known that the higher the ICCP, the lower the probability of recrystallization and cell lysis when reduced warming rates are applied.

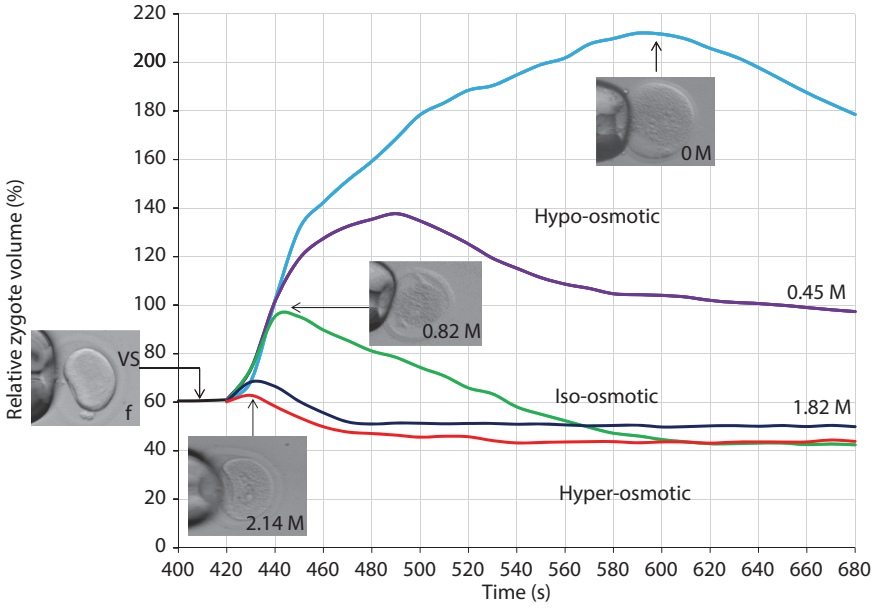

Figure 3.2 **Evolution of the relative zygote volume with exposure to solutions of different concentrations of sucrose (0, 0.45, 0.82, 1.82, and 2.14 M), following the exposure steps to nonvitrifying solution 1, nonvitrifying solution 2, and VS. The volume of the picture at 2.14 M is almost the same as the volume of the picture in (f), indicating an iso-osmotic situation between the intracellular and extracellular compartments. (VS = vitrifying solution.)**

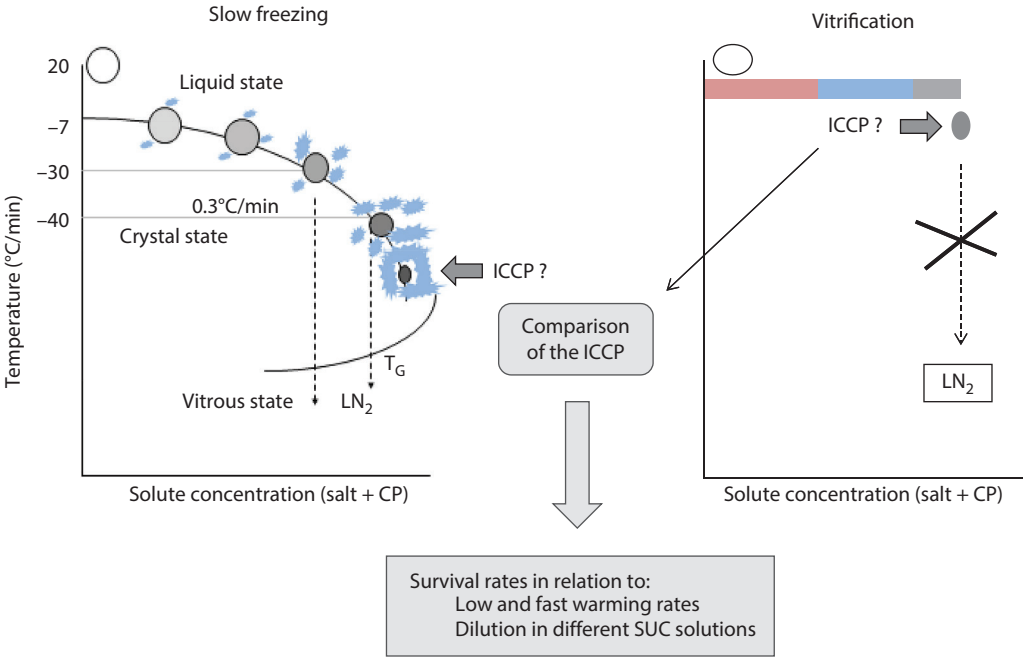

Figure 3.3 **Experimental design for the comparison of the ICCP when VIT and SLF cryopreservation procedures were performed. (ICCP ? = time point where the ICCPs were compared; CP = cryoprotectant; ICCP = intracellular concentration of cryoprotectant; LN_2 = liquid nitrogen; SLF = slow freezing; SUC = sucrose; T_G = glass transition temperature; VIT = vitrification.)**

- *Design of the experiment.* According to this assumption, an experiment was conducted comparing the rate of zygote survival after fast and slow warming following either SLF or VIT. The design of the experiment is explained in Figure 3.4a.
- *Results and arguments for low ICCP after VIT versus SLF.* We observed that the survival rates after SLF is poorly affected by the warming rate, suggesting that the high ICCP prevents intracellular crystallization (devitrification).

After VIT, however, only 10% of the zygotes survived when the warming process took place in air (slow warming). By contrast, a high warming speed (higher than 20,000°C/min) after VIT prevented cell lysis (Figure 3.4b).

This is a physical argument indicating that the ICCP is higher after SLF than VIT (Figure 3.4c). This is due to the fact that in the VIT process, the ICCP is too low to prevent recrystallization during slow warming when the temperature exceeds the T_G (~−120°C). If the warming speed is not fast enough, the supercooled liquid is rapidly transformed into ice crystals in the interval between the T_G and the melting temperature.

Comparison of survival rates after VIT or SLF in relation to dilution in various SUC concentrations

- *Principle.* The higher the ICCP, the higher the SUC concentration necessary to minimize overswelling of the cell due to the fast inflow of water through the cell membrane.
- *Design of the experiment.* In this experiment, the percentages of lysed zygotes were evaluated in various SUC-containing warming solutions. This was performed either after VIT or SLF. The design of the experiment is described in Figure 3.5a.
- *Results and arguments for low ICCP after VIT versus SLF.* A significantly higher rate of lysed zygotes was observed after SLF than after VIT.

Immediately after warming, SLF embryos presented obvious signs of overswelling when dilution was performed in culture medium or in solutions with low concentrations of SUC (Figure 3.5b).

The higher the ICCP, the higher must be the SUC concentration in the warming medium to minimize the overswelling of the cells.

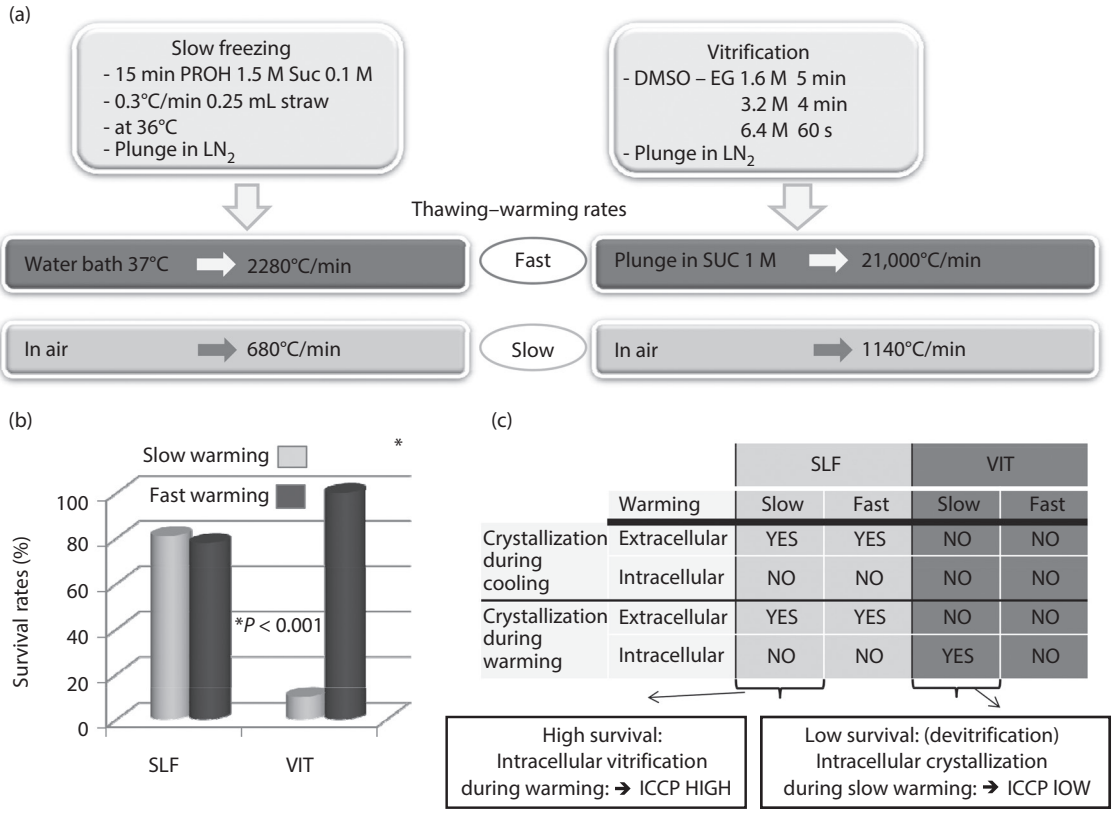

Figure 3.4 **Comparison of survival rates after VIT or SLF in relation to the warming rate. (a) Design of the experiment. (b) Results. (c) Arguments for low ICCP after VIT. (ICCP = intracellular concentration of cryoprotectant; SLF = slow freezing; VIT = vitrification.)**

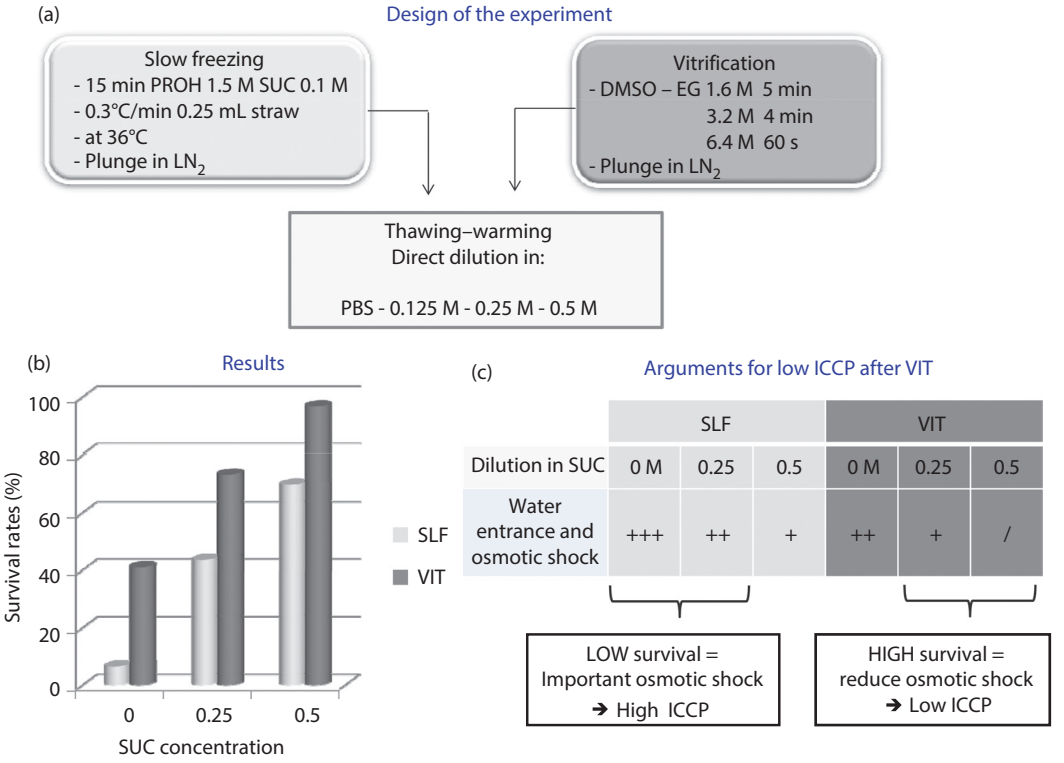

Figure 3.5 **Comparison of survival rates after VIT or SLF in relation to dilution in various SUC concentrations after the warming step. (a) Design of the experiment. (b) Results. (c) Arguments for low ICCP after VIT. (ICCP = intracellular concentration of cryoprotectant; PBS = phosphate buffer saline; SLF = slow freezing; VIT = vitrification.)**

This higher rate of lysed zygotes in low concentrations of SUC can be explained by the too rapid and finally lethal inflow of water into the cells. This is a tangible argument for the presence of a higher intracellular osmolarity in SLF, when compared to that present after VIT of cells (Figure 3.5c).

GENERAL CONCLUSIONS

Is it justified to be anxious about the use of high concentrations of CPs and to favor SLF?

From two studies using mouse zygotes in conjunction with cinematographic analysis, we can conclude that the ICCP during VIT is a lot lower (2.14 M) than the CP concentrations present in the VS solution (>6 M).[26] We have clearly shown that the ICCPs in the vitrified zygotes are, in contrast to common belief, even lower than those observed after a SLF procedure. From all of these experiments, we can conclude that with our VIT procedure, the ICCP that is reached after VIT of mice zygotes is not as high as it was previously thought. As a consequence, we can demystify the problem of the high level of CPs inside the cell.

The CPs are only part of the intracellular agents inhibiting crystallization, so the true ICCP is even lower than 2.14 M. This deduced low-level ICCP explains why VIT gives better results than SLF; although high extracellular concentrations of CPs are needed to allow VIT, only a fraction of them enter the cell, thereby reducing their toxicity. It is probable that the problems linked to SLF are more likely to be due to osmotic shock during the warming–dilution process than due to ice crystal formation, as it was previously thought.

It has become obvious that warming speeds play an even more important role in cell survival after VIT than the cooling rates.[27–29] As critical warming rates are higher than critical cooling rates, the minimal concentration of CPs for preventing recrystallization during warming must be higher than during cooling. This means that it might be easier to maintain a vitrified state during cooling than during the warming process, given the same concentration of CPs.

During SLF, when cooling is applied at a rate of 0.3°C/min, cells are equilibrated with a low concentration of a penetrating CP whose concentration increases throughout the cooling process, as a consequence of the increasing formation of extracellular ice crystals.

Knowing that a cell survives a cryopreservation procedure only if the development of intracellular ice

crystals is avoided, survival after SLF reflects the presence of an intracellular vitrified state. SLF has been the standard cryopreservation method for more than 25 years, without us being fully aware of the presence of a vitrified intracellular state obtained with a very high ICCP.

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4 Importance of cooling versus warming rates in vitrification techniques

Shinsuke Seki

INTRODUCTION

In the cryopreservation of oocytes/embryos, it is known that the formation of more than a trace amount of ice in the cell is lethal. Strategies developed to prevent intracellular ice formation (IIF) are slow freezing[1] and vitrification.[2] More recently, because of its simplicity and high survival, vitrification has become used more widely.

When cells are vitrified with a minimum degree of supercooling (i.e., in near equilibrium), no ice should form in the cell. In most cases, however, the degree of supercooling is not minimal, thus invisible minute ice crystals will form in cells during vitrification. The minute crystals are innocuous as long as they remain minute. During warming, however, they can recrystallize into larger lethal crystals, as a result of differences in surface free energy.[3] In the present article, intracellular ice means recrystallized lethal ice.

With a few exceptions,[4,5] the literature on vitrification has almost wholly emphasized the role of the cooling rate on preventing IIF. As a strategy to increase the cooling rate, ultra-rapid vitrification was developed, in which small tools, such as electron microscope grids,[6] open pulled straws,[7] Cryoloops,[8] and Cryotops,[9] are used. By using these devices, it is possible to achieve very high cooling rates (>10,000°C/min) by manipulating oocytes/embryos with a very small volume (~0.1 μL) of vitrification solution. Given the higher cooling/warming rates, cells are able to survive during this greater supercooling. Ultra-rapid vitrification was first developed for the cryopreservation of bovine oocytes to circumvent chilling injury,[6] but now it is mainly used to prevent IIF in less permeable cells like oocytes and blastocysts; the plasma membrane of blastocysts is quite permeable, but the whole embryo is less permeable because blastocysts have different compartments, as described by Edashige and Kasai in this book (Chapter 5).

The literature has also emphasized the role of the concentration of vitrification solution; that is, the vitrification solution in which cells are suspended must have a high concentration of cryoprotective solutes, especially cell-permeating cryoprotectants. Therefore, it is believed that vitrification requires both the cooling rate and the concentration of cell-permeating cryoprotectant(s) to be quite high for prevention of IIF.

However, my findings with the vitrification of mouse oocytes are not in accordance with this belief. My hypothesis is that high survival of vitrified mouse oocytes/embryos is derived more from rapid warming than rapid cooling and a high concentration of cryoprotectants. In this chapter, I will describe the results that support my hypothesis.

OBSERVATION OF IIF IN MOUSE OOCYTES

Since IIF is lethal, the most important factor determining the success of cryopreservation is whether intracellular ice forms or not during cryopreservation. The only way to avoid IIF is to vitrify cells; that is, to convert cell water into a glass rather than ice. Despite the importance of IIF, the mechanism by which IIF is induced was not understood in detail. At first, I would like to show how IIF is induced.[3,10,11]

Mouse oocytes at metaphase II (oocytes) were suspended in 1.5 M ethylene glycol/phosphate buffered saline (PBS), placed on linkam BCS196 cryostat, and cooled to −25°C at 50°C/min. At this temperature, extracellular ice had been induced, but intracellular ice was not formed. Samples were held there for 0, 5, 10, 20, 30, or 40 minutes, then cooled to −70°C at 50°C/min, and warmed to 20°C moderately (10°C/min). The temperature of the cryostage was controlled with a programmable freezer. By changing the holding time at −25°C, the water content of the cells was varied, because oocytes are dehydrated very slowly (water permeability, Lp being 1.82×10^{-3} μm/min/atm) at such a low temperature (−25°C). The morphological appearance of oocytes during cooling and warming was observed by taking pictures at fixed times, and IIF was evidenced by blackening of the cells.

When the holding time was 0–10 minutes, the water content was more than 50%, and IIF was induced at below −40°C.[11] This shows that IIF is induced during cooling when the water content is high.

When the holding time was 10–30 minutes, the water content was 20%–50% and IIF was not induced during cooling. During warming, however, IIF was induced at between −56°C and −46°C.[11] This shows that IIF is more likely to form during warming than during cooling in cells with 20%–50% water.

When the holding time was 40 minutes, the water content was less than 20% and IIF was induced neither during cooling nor warming, even when both cooling and warming rates were low.[11] This shows that IIF is not induced during cooling/warming if cells are dehydrated sufficiently. Recently, Jin et al.[12] and Mochida et al.[13] reported near-equilibrium vitrification, in which vitrified mouse embryos actually survived slow warming.

HOW CAN INTRACELLULAR ICE BE PREVENTED FROM FORMING IN MOUSE OOCYTES?

The results of the experiments described above show that IIF is not induced during cooling to –70°C when the water content is less than 50%. As it is not difficult to dehydrate cells to less than 50% in vitrification, I assumed that the cooling rate would not need to be increased to avoid IIF if cells were dehydrated enough, whereas the warming rate would need to be rapid to avoid IIF by recrystallization. Firstly, I examined the effect of the cooling rate and warming rate on the survival of oocytes vitrified with a solution containing a lower concentration of cryoprotectants,[14] in which ice is more likely to form.

Mouse oocytes were suspended in a vitrification solution containing 10% ethylene glycol, 10% acetamide, 24% Ficoll, and 0.4 M sucrose (EAFS10/10),[15] cooled to –196°C with plastic cryostraws at rates ranging from 37°C/min to 1827°C/min, and warmed at rates ranging from 137°C/min to 2950°C/min between –70°C and –35°C. Survival was assessed morphologically by osmotic responsiveness. I used EAFS10/10 for the experiments, but this vitrification solution contains acetamide, which would be quite toxic to oocytes for practical use.

When vitrified oocytes were warmed at the lowest rate (137°C/min), the survival rate was close to 0%, regardless of the cooling rate. When they were warmed at a higher rate (2950°C/min), however, the survival rate was more than 80% over a wide range of cooling rates (187–1827°C/min), showing that the warming rate is a more dominant effect over the cooling rate.

Then, mouse oocytes were vitrified with Cryotops, to apply higher rates of cooling/warming.[16] When oocytes were cooled at 95, 880, and 69,000°C/min, then warmed at the highest rate attainable (117,500°C/min), similar results to those with cryostraws were obtained. Morphological survival exceeded 80%, even when the prior cooling rate was as low as 880°C/min. These results also show that the warming rate is a more dominant effect over the cooling rate, probably by eliminating time for the growth of minute intracellular ice crystals through recrystallization.

Cryobiologists think that if cells could be cooled and warmed extremely rapidly, thus eliminating the time for IIF, then the cells would survive even if the cryopreservation medium did not contain cryoprotectant, at least theoretically. When the cooling and warming rates are high, therefore, cells should survive after cryopreservation, even when using a lower concentration of cryoprotectants. Therefore, I examined the effect of decreasing cryoprotectant concentration on the survival of vitrified oocytes.[17]

Mouse oocytes were suspended in various tonicities (1.0×, 0.75×, or 0.5×) of EAFS10/10, loaded in cryostraws or on Cryotops, cooled at rates ranging from 37°C to 69,000°C/min, vitrified at –196°C, and warmed rapidly (2170°C/min, 2950°C/min, or 117,500°C/min). Survival was assessed morphologically, or functionally by observation of cleavage to the two-cell stage after *in vitro* fertilization (IVF).

With full-strength (1.0×) EAFS10/10, morphological survival rates were more than 65%, regardless of the cooling rate, if the warming rate was 2950°C/min, but the rates dropped gradually to less than 56% and 45% when the tonicity of EAFS10/10 was decreased to 0.75× and 0.5×, respectively.

If the warming rate was decreased only slightly (2170°C/min), the drop in the survival with diluted EAFS10/10 became exaggerated, such that with 0.5× EAFS10/10, the survival rate approached 0%. When oocytes were warmed at the highest rate (117,500°C/min), on the other hand, morphological survival rates were 80%–90%, regardless of the tonicity of EAFS10/10 (1.0×, 0.75×, or 0.5×) and the cooling rate (95°C/min, 880°C/min, or 69,000°C/min). These results show that the warming rate definitely has a more dominant effect over both the concentration of cryoprotectants and the cooling rate.

Even when oocytes were cooled and warmed at the highest cooling rates, functional survival rates decreased slightly from 81% to 67% by decreasing the tonicity of EAFS from 1.0× to 0.5×. When cooled at 880°C/min, functional survival rates were 54%–73%; while with the slowest cooling rate (95°C/min), the rates decreased yet further (29%–49%). Therefore, the cooling rate affects the functional survival of the oocytes, although the effect is smaller than that of the warming rate.

Furthermore, a similar experiment was conducted with embryos: eight-cell mouse embryos were vitrified using Cryotops with 1.0×, 0.75×, 0.5×, or 0.33× EAFS10/10.[18] They were cooled at various rates ranging from 95°C/min to 69,000°C/min, and warmed rapidly (117,500°C/min). Survival was assessed by the ability to develop to expanded blastocysts in culture. The survival rates of embryos vitrified with 1.0× to 0.5× EAFS10/10 were more than 80%, even if the cooling rate was moderate (880°C/min). Even when the tonicity of EAFS was reduced to 0.33×, 40% of the embryos survived.

In conclusion, rapid warming has a more dominant effect over rapid cooling and a high concentration of cryoprotectants in the vitrification of mouse oocytes. The results obtained in oocytes should probably be applicable to embryos as well.

APPLICATION OF THESE STUDIES TO THE ART

The importance of the warming rate should have practical implications for successful cryopreservation of oocytes/embryos. Given that the cooling rate does not need to be very high, it is therefore not necessary to apply high cooling rates by direct contact of cells with liquid nitrogen, which may not be aseptic. As a consequence, oocytes/embryos may possibly be cryopreserved within a closed carrier (e.g., cryostraws); and yet vitrified oocytes/embryos do appear to need to be warmed rapidly.

When a solution is vitrified, the liquid phase turns to solid during cooling, and the solid phase turns to liquid during warming at the glass transition temperature (T_G), which is at around −130°C. During the phase transition, the volume slightly changes. If a solution is cooled/warmed rapidly, the solution contains both liquid and solid compartments for a moment, and fracture planes form between the two phases. If cells are situated in the fracture plane, they are physically dissected.[19] During cooling and warming, therefore, it is necessary to pass through the T_G moderately to prevent fracture damage, unless the sample volume is small enough (0.1 μL) to form fracture planes. My results suggest that moderate cooling can be applied at any temperature, but moderate warming should be applied only at around −130°C (below −80°C).

In addition, vitrification with a lower concentration of cryoprotectants may well be possible to reduce the toxicity of the vitrification solution used.

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5 The movement of water and cryoprotectants in mammalian oocytes and embryos: Membrane permeability and aquaporins

Keisuke Edashige and Magosaburo Kasai

INTRODUCTION

During the cryopreservation of cells, intracellular ice formation is the most common cause of cell death. To prevent this in oocytes/embryos, cytoplasm must be vitrified using cell-permeating cryoprotectants, which can cause other major types of cell injury; for example, chemical toxicity of cryoprotectant during loading and osmotic swelling during removal of permeated cryoprotectant.[1] Especially in vitrification, the potential toxicity of the final vitrification solution is high, because of the high concentration of cryoprotectants needed to achieve the vitrified state. Therefore, the exposure time of oocytes/embryos to the vitrification solution has to be limited. However, short exposure times can cause insufficient permeation by the cryoprotectant and insufficient dehydration/concentration of the cytoplasm, resulting in intracellular ice formation. To prevent these major injuries during vitrification, rapid movement of water and cryoprotectants is preferable.

Here, we summarize the movement of water and cryoprotectants at 20–25°C across the plasma membrane of metaphase II stage oocytes and embryos at various developmental stages in the mouse, bovine, pig, and human systems.

THE MOVEMENT OF WATER ACROSS THE PLASMA MEMBRANE OF MAMMALIAN OOCYTES AND EMBRYOS IN A HYPERTONIC SUCROSE SOLUTION

In most types of cells, water moves slowly across the plasma membrane by simple diffusion via the lipid bilayer. However, the plasma membrane of some cells—for example, human red blood cells and cells in renal proximal tubules—is extremely permeable to water. In the 1990s, small intrinsic membrane proteins that act as water channels, called aquaporins, were discovered and characterized.[2] Therefore, water is able to move across the plasma membrane of oocytes/embryos slowly by simple diffusion, or rapidly by facilitated diffusion through aquaporins.

The pathway for the movement of water across the plasma membrane can be deduced from the permeability to water, and its temperature dependency expressed as Arrhenius activation energy (E_a). In general, lower permeability to water with a higher E_a value for the permeability is suggestive of the movement of water by simple diffusion—that is, via a channel-independent process—whereas higher permeability to water with a lower E_a value is suggestive of the movement of water by facilitated diffusion via channel processes. Verkman et al.[3] suggested that low permeability to water with an E_a value higher than 10 kcal/mol is suggestive of water movement principally by simple diffusion, whereas permeability higher than 4.5 μm/min/atm with an E_a value lower than 6 kcal/mol is suggestive of water movement principally via channels. The existence of water channels in embryos can be inferred by Verkman et al.'s criteria, by the presence of mRNA for water channels, by detection of channel proteins, and also by the decreased water permeability in embryos when the expression of such channel proteins is suppressed.

In mouse oocytes, the permeability to water in a sucrose solution is low at 20–25°C (0.4–1.0 μm/min/atm), and its E_a value is higher (11–15 kcal/mol) than 10 kcal/mol (Figure 5.1).[4–11] Applying Verkman et al.'s criteria to mouse oocytes, this implies that water moves slowly by simple diffusion, and that channels do not play a role in the movement of water (Figure 5.2a).[12]

In mouse embryos at the early (one- to four-cell) stages, the permeability to water is low (0.4–0.7 μm/min/atm) and the E_a value is high (12–13 kcal/mol),[4,9,11] similar to oocytes, although these cells do express a small amount of mRNAs for aquaporins and aquaporin proteins.[13–15] In one- to four-cell embryos, therefore, water seems to move across the plasma membrane principally by simple diffusion, and perhaps minimally by channel processes.

In mouse morulae and blastocysts, on the other hand, water permeability is high (3.1–4.5 μm/min/atm) and the E_a value is low (5.1–6.3 kcal/mol) (Figure 5.1),[11] suggesting that the movement of water is principally dependent on facilitated diffusion via channel processes. Among aquaporin families, aquaporin 3[16] is expressed abundantly in mouse morulae[11,14] and blastocysts.[14] In mouse blastocysts, mRNA of aquaporin 3 is much more abundantly expressed than the mRNA of other aquaporins.[17] Furthermore, by suppressing the expression of aquaporin 3 in mouse morulae/blastocysts, the high permeability to water markedly decreases.[18] In mouse morulae/blastocysts, therefore, water moves rapidly principally through aquaporin 3 (Figure 5.2b).[12]

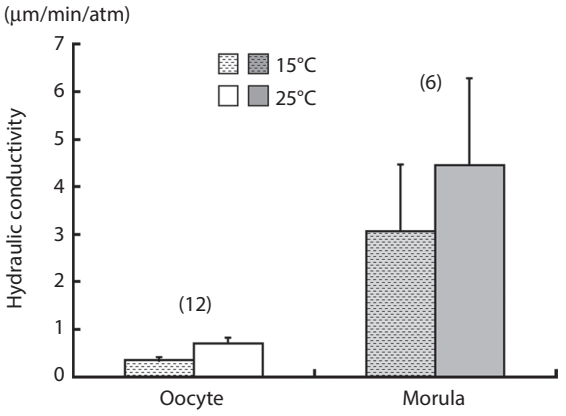

Figure 5.1 **Permeability to water (hydraulic conductivity, L_p) of mouse oocytes and morulae in a hypertonic solution containing sucrose at 15 and 25°C. L_p values were determined from the measurement of the shrinkage of oocytes and morulae in a modified phosphate-buffered saline containing 0.5 Osm/kg sucrose (0.8 Osm/kg in total) for 5 minutes at 15 and 25°C. Values in parentheses are the activation energies for the permeability (kcal/mol). (Data from Edashige K et al.** ***Biol Reprod*** **2006;74:625–32.)**

In bovine oocytes, the permeability to water is low (1.8 μm/min/atm),[19] although higher than that of mouse oocytes (0.4–1.0 μm/min/atm), and the E_a value for the permeability is high (9 kcal/mol), although slightly lower than the 10 kcal/mol level for mouse oocytes. Furthermore, the permeability to water of bovine oocytes is significantly higher than that of bovine morulae when the expression of aquaporin 3 is suppressed (0.6 μm/min/atm). In bovine embryos at the 16-cell stage, similar results to those as with bovine oocytes are reported.[19] In bovine oocytes and 16-cell embryos, therefore, water moves across the plasma membrane principally by simple diffusion, although the channel pathway via aquaporins can also partially be involved in water movement.

In bovine morulae and blastocysts, on the other hand, the permeability to water is high (3 μm/min/atm), although slightly lower when compared to the mouse (4.5 μm/min/atm), and the E_a value for the permeability is much lower (3 kcal/mol) than 6 kcal/mol.[19] Furthermore, the suppression of the expression of aquaporin 3 in bovine morulae markedly decreases the permeability to water. Thus, in bovine morulae/blastocysts, water moves across the plasma membrane principally via the channel pathway through aquaporin 3, as in mouse morulae/blastocysts.

In pig oocytes, the permeability to water is low (1.0 μm/min/atm) and the E_a value is quite high (19 kcal/mol), as in mouse oocytes.[20] In pig morulae, the permeability to water remains low, unlike in mouse and bovine morulae, probably because pig embryos become compacted at the four-cell stage. In pig oocytes and morulae, therefore, water seems to move across the plasma membrane principally by simple diffusion.

In pig blastocysts, the permeability to water increases at the unexpanded stage, and further increases at the expanded stage (3.4 μm/min/atm).[20] The E_a value for the permeability to water in expanded blastocysts (7 kcal/mol) is close to 6 kcal/mol, as seen in the mouse. Therefore, water seems to move across the plasma membrane of pig expanded blastocysts principally via channel pathways. Since pig expanded blastocysts express mRNA of aquaporin 3 abundantly,[20] aquaporin 3 seems to be responsible for the high permeability to water of pig expanded blastocysts, as in mouse and bovine morulae/blastocysts. In pig embryos, therefore, the expression of aquaporin 3 apparently increases at a slightly later stage.

In human oocytes, the permeability to water in hypertonic solutions containing a nonpermeating solute is also low (0.4–1.0 mm/min/atm),[5,21,22] and the E_a value is high (9–11 kcal/mol).[5,22] In human oocytes, therefore, water seems to move across the plasma membrane slowly principally by simple diffusion, as in animal oocytes.

In human embryos, the permeability to water has not been reported, but it would be reasonable to presume that permeability is essentially similar to that in embryos from other experimental models.

In mammalian oocytes and embryos at early stages, therefore, water moves across the plasma membrane slowly principally by simple diffusion. In embryos at later stages, on the other hand, water moves rapidly principally by facilitated diffusion through aquaporin 3, although

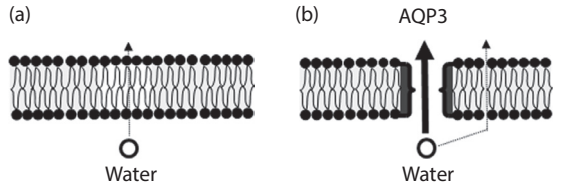

Figure 5.2 **Schematic representation of the pathway for the movement of water across the plasma membrane of mouse oocytes and morulae in a hypertonic solution containing sucrose. Solid lines indicate the movement of water across the plasma membrane by facilitated diffusion via channel pathways, whereas dotted lines indicate the movement of water across the plasma membrane by simple diffusion. (a) The movement of water in oocytes. (b) The movement of water in morulae. (AQP3 = aquaporin 3.) (Modified from Kasai M, Edashige K.** ***Fertility Cryopreservation.*** **Cambridge University Press: Cambridge, 2010. p. 16–23.)**

the developmental stage at which the pathway changes may shift slightly in some species.

THE MOVEMENT OF CELL-PERMEATING CRYOPROTECTANTS ACROSS THE PLASMA MEMBRANE OF MAMMALIAN OOCYTES AND EMBRYOS

Water channels occur in two groups: one is highly selective for water, and the other transports not only water but also neutral solutes with a smaller molecular weight, such as cell-permeating cryoprotectants.[2] Therefore, cell-permeating cryoprotectants will move across the plasma membrane either slowly by simple diffusion, or rapidly by facilitated diffusion through aquaporins.

Although no report is available for quantitative evaluation of the movement of cell-permeating cryoprotectants, it would be reasonable to deduce the pathway of their movement from the permeability and its E_a value, based on the case of permeability to water; low permeability to cryoprotectant with a relatively high E_a value is suggestive of the movement of cryoprotectant principally by simple diffusion, whereas an increase in permeability with a decrease in the E_a value for the permeability is suggestive of movement principally by facilitated diffusion via channels. Among the major permeating cryoprotectants are glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), acetamide, and propylene glycol.

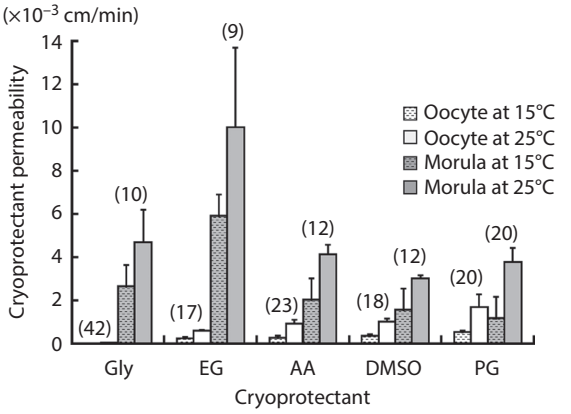

Figure 5.3 **The permeability to cell-permeating cryoprotectants (P_S) of mouse oocytes and morulae and the activation energy for the permeability. P_S values were determined from the measurement of the shrinkage and swelling of oocytes and morulae in a modified phosphate-buffered saline containing 10% v/v glycerol (Gly), 8% v/v ethylene glycol (EG), 1.5 M acetamide (AA), 9.5% v/v dimethyl sulfoxide (DMSO), or 10% v/v propylene glycol (PG) for 5–20 minutes at 15 and 25°C. Values in parentheses indicate the values of the activation energies for the permeability (kcal/mol). (Data from Edashige K et al. *Biol Reprod* 2007;77:365–75.)**

Glycerol

In mouse oocytes and embryos at early developmental stages, the permeability to glycerol is quite low (0.01–0.02×10^{-3} cm/min),[11,23–25] and the E_a value is remarkably high (42 kcal/mol) (Figure 5.3).[11,18] In mouse oocytes and embryos at early stages, therefore, glycerol would move slowly across the plasma membrane by simple diffusion.

In mouse morulae, on the other hand, the permeability to glycerol is markedly higher ($4–5 \times 10^{-3}$ cm/min) than that of mouse oocytes ($0.01–0.02 \times 10^{-3}$ cm/min), and the E_a value for the permeability (10 kcal/mol) is lower than that of mouse oocytes (42 kcal/mol).[11] Furthermore, the high permeability is remarkably decreased by suppressing the expression of aquaporin 3.[18] In mouse morulae (and probably blastocysts), therefore, glycerol moves rapidly across the plasma membrane by facilitated diffusion via the channel pathway through aquaporin 3 (Figure 5.4a).[12]

In bovine oocytes/embryos, essentially similar results to those in mouse oocytes/embryos have been reported.[19]

In pig oocytes, the permeability to glycerol is low (0.02×10^{-3} cm/min), and the E_a value is high (27 kcal/mol), as with mouse oocytes.[20] In pig morulae, the permeability to glycerol remains low (0.05×10^{-3} cm/min),[20] unlike in mouse morulae. So in pig oocytes and morulae, glycerol seems to move slowly by simple diffusion.

In pig blastocysts, the permeability to glycerol increases at the unexpanded stage (0.3×10^{-3} cm/min), then further increases at the expanded stage (0.6×10^{-3} cm/min), similar to the permeability to water.[20] The E_a value for the permeability to glycerol of expanded pig blastocysts is much lower (10 kcal/mol) than that of oocytes (27 kcal/mol). Furthermore, pig expanded blastocysts express mRNA of aquaporin 3 abundantly.[20] Thus, in expanded pig blastocysts, glycerol seems to move rapidly principally by facilitated diffusion through aquaporin 3.

In human oocytes, no data are available for the permeability to glycerol; the permeability of oocytes to glycerol (MW 92) would be too low as a permeating cryoprotectant for practical use.

Ethylene Glycol

In mouse oocytes, the permeability to ethylene glycol is low (0.6×10^{-3} cm/min) and the E_a value is high (17 kcal/mol) (Figure 5.3),[18] similar to the permeability to glycerol. Therefore, ethylene glycol will move through mouse oocytes (and probably embryos at early stages of development) principally by simple diffusion.

In mouse morulae, on the other hand, the permeability to ethylene glycol is extremely high (10×10^{-3} cm/min), and the E_a value is lower (9 kcal/mol) than that in mouse oocytes (17 kcal/mol).[18] Furthermore, the suppression of the expression of aquaporin 3 in mouse morulae markedly decreases the high permeability to ethylene glycol, while exogenous expression of aquaporin 3 in mouse

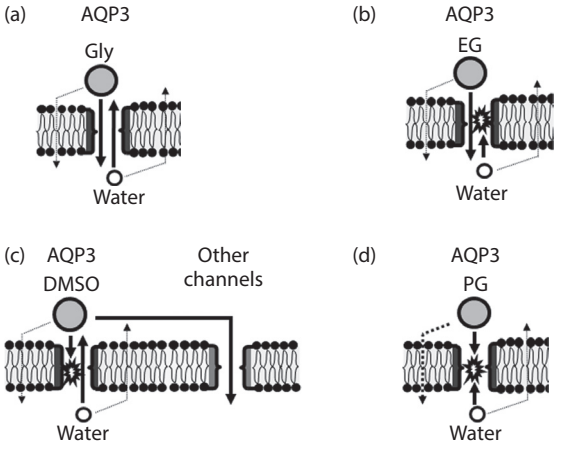

Figure 5.4 **Schematic representation of the pathway for the movement of cell-permeating cryoprotectants and water in the presence of cryoprotectants across the plasma membrane of mouse oocytes and morulae. Solid lines indicate the movement of water and cryoprotectants across the plasma membrane by facilitated diffusion via channel pathways, whereas dotted lines indicate the movement of water and cryoprotectants by simple diffusion. (a) The movement of water and glycerol (Gly). (b) The movement of water and ethylene glycol (EG). (c) The movement of water and dimethyl sulfoxide (DMSO). (d) The movement of water and propylene glycol (PG). "Other channels" refers to DMSO-permeable channels. Open circles represent water molecules; shaded circles represent cryoprotectant molecules. (AQP3 = aquaporin 3.) (Modified from Kasai M, Edashige K.** ***Fertility Cryopreservation*****. Cambridge University Press: Cambridge, 2010. p. 16–23.)**

oocytes (which do not express aquaporin 3) increases their permeability to ethylene glycol.[18] In mouse morulae (and probably blastocysts), therefore, ethylene glycol moves principally via the channel pathway through aquaporin 3 (Figure 5.4b).[12]

In bovine oocytes, the permeability to ethylene glycol is essentially similar to that in mouse oocytes.[19] However, the permeability is higher (3.5×10^{-3} cm/min) than that in mouse oocytes (0.6×10^{-3} cm/min), and the E_a value is slightly lower (14 kcal/mol) than that in mouse oocytes (17 kcal/mol). Since bovine oocytes express a small amount of water channels on the plasma membrane as described above for the permeability to water in the presence of sucrose, ethylene glycol would move through bovine oocytes mainly by simple diffusion, and partially via channel processes through water channels.

In bovine morulae, the permeability to ethylene glycol is essentially similar to that in mouse morulae.[19]

In pig oocytes/embryos, essentially similar results to those in mouse oocytes/embryos are reported, although the permeability to ethylene glycol does not increase at the morula stage but at the blastocyst stage,[20] as with the permeability to glycerol.

In human oocytes, the permeability to ethylene glycol (1.2×10^{-3} cm/min) is low and the E_a value is high (15 kcal/mol),[22] as in mouse oocytes, suggesting that ethylene glycol moves slowly principally by simple diffusion.

Dimethyl Sulfoxide

In mouse oocytes, the permeability to DMSO is low (1.0×10^{-3}cm/min), and its E_a value is high (18 kcal/mol)[18] (Figure 5.3), suggesting that DMSO moves slowly by simple diffusion.

In mouse morulae, on the other hand, the permeability is higher (3.0×10^{-3} cm/min) than that in oocytes (1.0×10^{-3} cm/min), and the E_a value is lower (12 kcal/mol) than that in mouse oocytes (18 kcal/mol), suggesting that DMSO principally moves via channels.[18] However, the suppression of aquaporin 3 in mouse morulae does not decrease the permeability to DMSO, and the exogenous expression of aquaporin 3 in mouse oocytes does not increase the permeability to DMSO.[18] These results show that aquaporin 3 is not involved in the channel pathway for the movement of DMSO in mouse morulae. Therefore, DMSO must move through mouse morulae principally via channels other than aquaporin 3 (Figure 5.4c).[12]

In bovine oocytes, the permeability to DMSO is relatively low (1.5×10^{-3} cm/min) and the E_a value is high (13 kcal/mol), suggesting that DMSO moves slowly by simple diffusion.[19]

In bovine morulae, the permeability to DMSO remains low (1.7×10^{-3} cm/min),[19] while the E_a value is high (21 kcal/mol), which is actually higher than that in bovine oocytes (13 kcal/mol). In bovine blastocysts, no reports on the movement of DMSO are available, but it would be reasonable to assume that the movement of DMSO is essentially similar to that in bovine morulae.

In bovine oocytes/embryos, therefore, DMSO seems to move across the plasma membrane principally by simple diffusion regardless of the developmental stage.

In pig oocytes/embryos, similar results to those in mouse oocytes/embryos are reported.[20] However, the permeability to DMSO increases marginally at the expanded blastocyst.

In human oocytes, the permeability to DMSO is low (1.6×10^{-3} cm/min), as in mouse oocytes (1.0×10^{-3} cm/min),[22] suggesting that DMSO moves slowly principally by simple diffusion.

Acetamide

In mouse oocytes/embryos, the movement of acetamide is essentially similar to that of DMSO (Figure 5.3).[18] Therefore, acetamide would also move through mouse oocytes by simple diffusion and through mouse morulae by facilitated diffusion via channels other than aquaporin 3 (Figure 5.4c).

In bovine, pig, and human oocytes/embryos, no data are available for the movement of acetamide; in any event, acetamide may be too toxic for practical use.

Propylene Glycol

In mouse oocytes, the permeability to propylene glycol is relatively low (1.7×10^{-3} cm/min), and the E_a value is high (20 kcal/mol) (Figure 5.3),[18] suggesting that propylene glycol also moves through mouse oocytes by simple diffusion. However, the permeability of mouse oocytes to propylene glycol is higher (1.7×10^{-3} cm/min) than the permeabilities to other cryoprotectants ($0.6–1.0 \times 10^{-3}$ cm/min). This would be due to the molecular configuration of propylene glycol; propylene glycol is a more hydrophobic molecule than other permeating cryoprotectants. Therefore, propylene glycol would move across the plasma membrane by simple diffusion more rapidly.

In mouse morulae, on the other hand, the permeability to propylene glycol is more than twice as high (3.8×10^{-3} cm/min) as that in mouse oocytes (1.7×10^{-3} cm/min),[18] although the reason for this is not clear. The permeability to propylene glycol (3.8×10^{-3} cm/min) is similar to the permeabilities to glycerol ($4–5 \times 10^{-3}$ cm/min) and DMSO (3.0×10^{-3} cm/min) in mouse morulae, in which water moves principally via the channel pathway. However, the E_a value for the permeability to propylene glycol remains high (20 kcal/mol), unlike the E_a values for the permeability to glycerol and DMSO. Furthermore, the suppression of aquaporin 3 in mouse morulae and the exogenous expression of aquaporin 3 in mouse oocytes do not affect the permeability to propylene glycol.[18] These results suggest that the relatively high permeability to propylene glycol in mouse morulae does not rely on channel processes, but on simple diffusion (Figure 5.4d).[12] From these results, it is speculated that in mouse morulae, the movement of propylene glycol interacts with water, which hinders each other's movement through aquaporin, as described below for the permeability to water in the presence of propylene glycol.

In bovine oocytes/embryos and pig oocytes/embryos, similar results to those as in mouse oocytes/embryos are reported.[19,20]

In human oocytes, the permeability to propylene glycol (2.2×10^{-3} cm/min)[22] is similar to that in mouse oocytes (1.7×10^{-3} cm/min), suggesting that propylene glycol moves slowly principally by simple diffusion.

In mammalian oocytes/embryos, therefore, propylene glycol seems to move across the plasma membrane principally by simple diffusion but relatively rapidly, regardless of the expression of water/cryoprotectant channels.

SUMMARY

In mammalian oocytes and embryos at early developmental stages, cell-permeating cryoprotectants move across the plasma membrane principally by simple diffusion, like water in sucrose solution. In embryos at later developmental stages, glycerol and ethylene glycol move principally by facilitated diffusion through aquaporin 3, DMSO and acetamide move principally via channels other than aquaporin 3 in many species, and propylene glycol moves by simple diffusion but relatively rapidly.

THE MOVEMENT OF WATER ACROSS THE PLASMA MEMBRANE OF MAMMALIAN OOCYTES AND EMBRYOS IN THE PRESENCE OF A CELL-PERMEATING CRYOPROTECTANT

The permeability to water in oocytes/embryos can be assessed in a hypertonic solution containing a nonpermeating solute; for example, sucrose, as described above. In cryopreservation, however, oocytes/embryos are suspended in a solution also containing a cell-permeating cryoprotectant(s). In this case, the movement of water is not always the same as that in a solution containing just a nonpermeating solute, because water will interact with the permeating cryoprotectant. The pathway for the movement of water in the presence of a permeating cryoprotectant may be deduced from the permeability to water and its E_a value, as with the movement of the cryoprotectant.

In the Presence of Glycerol

In mouse oocytes, the permeability to water in the presence of glycerol is low (0.5–0.6 μm/min/atm) and the E_a value is high (14 kcal/mol) (Figure 5.5),[18] suggesting that water moves across the plasma membrane slowly by simple diffusion.

In mouse morulae, on the other hand, the permeability to water in the presence of glycerol is higher (2.2 μm/min/atm) than that in oocytes (0.5–0.6 μm/min/atm), and the E_a value for the permeability is lower (9 kcal/mol) than that in oocytes (14 kcal/mol).[11,18] Furthermore, the suppression of the expression of aquaporin 3 in mouse morulae significantly decreases the permeability to water.[18] In mouse morulae, therefore, water in the presence of glycerol moves rapidly across the plasma membrane principally via the channel pathway through aquaporin 3 (Figure 5.4a).[12] This movement is essentially similar to the movement of water in the presence of sucrose.

In bovine oocytes/embryos, the movement of water in the presence of glycerol is essentially similar to that in mouse oocytes/embryos.[19]

In pig oocytes, the permeability to water in the presence of glycerol is low (0.7 μm/min/atm) and the E_a value is high (17 kcal/mol).[20] In pig morulae, the permeability to water in the presence of glycerol remains low (0.5 μm/min/atm), unlike in mouse morulae. In pig oocytes/morulae, therefore, water seems to move across the plasma membrane slowly by simple diffusion.

In pig blastocysts, the permeability to water in the presence of glycerol increases at the unexpanded stage and further increases at the expanded stage (1.2–2.0 μm/min/atm),[20] as with the permeability to water in the presence

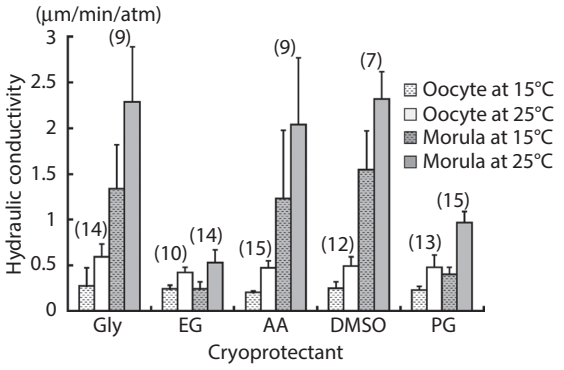

Figure 5.5 **The permeability to water (hydraulic conductivity [L_P]) of mouse oocytes and morulae in the presence of a cryoprotectant, and the activation energy for the permeability. L_P values were obtained when the permeability to cell-permeating cryoprotectant values were estimated in Figure 5.3. Values in parentheses are the activation energies for the permeability (kcal/mol). (AA = acetamide; DMSO = dimethyl sulfoxide; EG = ethylene glycol; Gly = glycerol; PG = propylene glycol.)**

of sucrose. The E_a value for the permeability in expanded blastocysts is much lower (5 kcal/mol) than that in pig oocytes (17 kcal/mol). Furthermore, pig expanded blastocysts express the mRNA of aquaporin 3 abundantly.[20] In pig expanded blastocysts, therefore, water in the presence of glycerol seems to move principally via the channel pathway through aquaporin 3 rapidly.

In human oocytes, the permeability to water in the presence of glycerol has not been reported, but it would be reasonable to presume that the permeability is essentially similar to that in animal oocytes.

In mammalian oocytes/embryos, therefore, the presence of glycerol does not affect the movement of water markedly.

In the Presence of Ethylene Glycol

In mouse oocytes, the permeability to water in the presence of ethylene glycol is low (0.4 μm/min/atm) and the E_a value is high (10 kcal/mol) (Figure 5.5),[18] suggesting that water moves across the plasma membrane slowly by simple diffusion.

In mouse morulae, on the other hand, the movement of water in the presence of ethylene glycol is different from that in the presence of sucrose; the permeability is low (0.5 μm/min/atm) and the E_a value is quite high (14 kcal/mol),[18] regardless of marked expression of aquaporin 3 at this stage. Furthermore, exogenous expression of aquaporin 3 in mouse oocytes increases the permeability to ethylene glycol, but not to water.[18] Therefore, water in the presence of ethylene glycol seems to move slowly by simple diffusion, regardless of the expression of aquaporin 3. Therefore, it is speculated that the movement of water is hindered by the movement of ethylene glycol through aquaporin 3, as a result of strong interactions between water and ethylene glycol (Figure 5.4b).[12]

In bovine oocytes/embryos and in pig oocytes/embryos, the movement of water in the presence of ethylene glycol is essentially similar to that in mouse oocytes/embryos,[19,20] although the permeability does not increase at the morula stage, but at the blastocyst stage in pig embryos.[20]

In human oocytes, the permeability to water in the presence of ethylene glycol is low (0.8 μm/min/atm) and the E_a value is high (11 kcal/mol),[22] as in mouse oocytes, suggesting that water in the presence of ethylene glycol moves across the plasma membrane slowly principally by simple diffusion.

In the Presence of DMSO

In mouse oocytes, the permeability to water in the presence of DMSO is low (0.5×10^{-3} μm/min/atm) and the E_a value is high (12 kcal/mol) (Figure 5.5),[18] suggesting that water moves slowly by simple diffusion.

In mouse morulae, on the other hand, the permeability is higher (2.3×10^{-3} μm/min/atm) than that in oocytes (0.5×10^{-3} μm/min/atm), and the E_a value for the permeability is lower (7 kcal/mol) than that in oocytes (12 kcal/mol).[18] Furthermore, the suppression of the expression of aquaporin 3 in mouse morulae decreases the permeability to water, and exogenous expression of aquaporin 3 in mouse oocytes increases the permeability to water.[18] In mouse morulae, therefore, water in the presence of DMSO moves via the channel pathway through aquaporin 3.

In mouse oocytes and morulae, therefore, the movement of water in the presence of DMSO is essentially similar to that in the presence of sucrose (Figure 5.4c).[18]

In bovine oocytes, the permeability to water in the presence of DMSO is relatively low (1.2 μm/min/atm) and the E_a value is high (11 kcal/mol), suggesting that water principally moves slowly by simple diffusion.[19]

In bovine morulae, the permeability remains low (1.4×10^{-3} μm/min/atm), although the E_a value is markedly lower (2 kcal/mol) than that in bovine oocytes (11 kcal/mol). Furthermore, the suppression of the expression of aquaporin 3 in bovine morulae significantly decreases the permeability to water to a value lower than that in intact bovine oocytes, and exogenous expression of bovine aquaporin 3 in mouse oocytes increases the permeability to water, although the permeability to DMSO does not increase.[19] In bovine morulae, therefore, water in the presence of DMSO seems to move principally via the channel pathway through aquaporin 3, although the increase in the permeability is marginal.

In pig oocytes/embryos, essentially similar results to those in mouse oocytes/embryos are reported, although

the increase in the permeability to water in the presence of DMSO is observed not at the morula stage, but at the expanded blastocyst stage.[20]

In human oocytes, the permeability to water in the presence of DMSO is low (0.6 μm/min/atm), as with mouse oocytes,[22] suggesting that water in the presence of DMSO moves across the plasma membrane slowly principally by simple diffusion.

In mammalian oocytes/embryos, therefore, water in the presence of DMSO moves through oocytes slowly by simple diffusion, and moves through morulae by facilitated diffusion through aquaporin 3 rapidly in mouse morulae and pig expanded blastocysts, or relatively slowly in bovine morulae.

In the Presence of Acetamide

The movement of water in the presence of acetamide (Figure 5.4c) is essentially similar to that in the presence of DMSO in the mouse.[18] Therefore, water in the presence of acetamide moves through oocytes slowly by simple diffusion, and through morulae/blastocysts rapidly by facilitated diffusion through aquaporin 3.

In the Presence of Propylene Glycol

In mouse oocytes, the permeability to water in the presence of propylene glycol is low (0.5 μm/min/atm) and the E_a value is high (13 kcal/mol) (Figure 5.5),[18] suggesting that water moves principally by simple diffusion.

In mouse morulae, the permeability to water in the presence of propylene glycol is low (1.0 μm/min/atm), although higher than that in oocytes (0.5 μm/min/atm), and the E_a value for the permeability is quite high (15 kcal/mol) (Figure 5.5).[18] Furthermore, the suppression of the expression of aquaporin 3 in mouse morulae and the exogenous expression of aquaporin 3 in mouse oocytes do not affect the permeability to water. In mouse morulae, therefore, water in the presence of propylene glycol presumably moves across the plasma membrane principally by simple diffusion, regardless of the abundant expression of aquaporin 3, unlike water in the presence of other cryoprotectants. From these results, it is speculated that water and propylene glycol interact and hinder each other's movement through aquaporin 3 (Figure 5.4d).[12]

In bovine oocytes/embryos and in pig oocytes/embryos, the movement of water in the presence of propylene glycol is essentially similar to that in mouse oocytes/embryos.[19,20]

In human oocytes, the permeability to water in the presence of propylene glycol is low (1.1 μm/min/atm),[22] as with mouse oocytes, suggesting that water in the presence of propylene glycol moves across the plasma membrane slowly principally by simple diffusion.

In the presence of propylene glycol, therefore, water appears to move through mammalian oocytes/embryos slowly principally by simple diffusion, regardless of the expression of water/cryoprotectant channels.

MEMBRANE PERMEABILITY AND SUITABLE CONDITIONS FOR VITRIFICATION OF OOCYTES/EMBRYOS

In vitrification, the time and temperature for exposure of oocytes/embryos to the vitrification solution are important, because oocytes/embryos could easily be injured by the toxicity of a high concentration of the cryoprotectant. Exposure of embryos to cryoprotectant solutions at a high temperature needs to be avoided, because cryoprotectants are more toxic at higher temperatures.

In order to design protocols suitable for the vitrification of mammalian oocytes/embryos, it would be valuable to consider the pathway for the movement of water and cell-permeating cryoprotectants for each stage of development. Our assessment of the movement of water and cell-permeating cryoprotectants in oocytes/embryos shows that the pattern of the movement is rather more stage specific than species specific, although some species-specific cases exist. Therefore, cryopreservation protocols developed for oocytes/embryos in one species should be applicable to protocols of various species at similar stages in terms of permeability.

In oocytes and embryos at early cleavage stages (including pig morulae), water and cryoprotectants principally move across the plasma membrane slowly by simple diffusion. To prevent injury from the toxicity of cryoprotectants in vitrification, a stepwise treatment of oocytes/embryos is effective; oocytes and embryos at early stages are suspended first in a solution containing a lower concentration of cryoprotectant for permeation, and then in a vitrification solution for a short time to maximize the cellular concentration by rapid dehydration. In oocytes and embryos at early cleavage stages, it is preferable to handle them at a moderate temperature (room temperature) to optimize the movement of water and cryoprotectant during loading and during removal of cryoprotectant, because they move slowly by simple diffusion, and their movement is greatly affected by temperature.

In morulae (in many species), water and cryoprotectants move across the plasma membrane rapidly by facilitated diffusion via channels. It is known that the expression of aquaporin 3 (and DMSO/acetamide channels in some species) markedly increases at later stages of embryonic development. Facilitated diffusion not only increases membrane permeability, but also decreases the temperature dependency of the permeability. Therefore, morulae can be handled at a lower temperature (e.g., 20°C) without a large decrease in the permeability.

In blastocysts, water and cryoprotectants also move across the plasma membrane via channels rapidly, but optimal diffusion into the inner cell mass and trophectoderm can be problematic, and so affect the most suitable conditions for vitrification. Notably, the inner cell mass faces not the outside of the blastocyst, but abuts the trophectoderm or the blastocoele. Therefore, water and cryoprotectants do not move across the plasma membrane

of the inner cell mass directly from/to the solution in which blastocysts are suspended, but indirectly through the trophectodermal cells or the blastocoelic fluid. Furthermore, in blastocysts with a larger amount of water, most of the water in the blastocoele has to be removed before the cryopreservation process. At the same time, the cryoprotectant has to permeate the blastocoele via the trophectodermal cells. To minimize the toxic effect of the cell-permeating cryoprotectant whilst promoting permeation and dehydration, stepwise treatment is more effective, as with oocytes, for preventing ice formation in the blastocoele. Consequently, puncturing the blastocoele to collapse it is the most effective strategy for promoting both permeation of cryoprotectant and dehydration.

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6 Open versus closed systems

Mark G. Larman and Pierre Vanderzwalmen

INTRODUCTION

Vitrification is now a part of nearly every *in vitro* fertilization (IVF) cycle. Gametes and embryos are, therefore, stored in liquid nitrogen (LN_2) for a number of years and possibly decades, especially when considering donor cryo-banks and gamete freezing for social reasons or fertility preservation. Many cryopreserved samples within IVF are stored in containers that are not hermetically sealed, which is not in concordance with medical directives for the storage of biological tissues. Such directives have been put in place to minimize the risk of cross-contamination from infected material and also the LN_2 itself, which is not sterile.

This chapter will discuss the potential concerns with the storage of gametes and embryos in LN_2 and how those risks can be minimized. The most obvious solution is to prevent contact with LN_2 completely via the use of closed vitrification devices. Therefore, different closed devices and their clinical comparison with open devices will be discussed.

LN_2 AND CONCERNS OF CONTAMINATION

The risk of contamination during storage in LN_2 has been of concern for a number of years. In a 1976 study, glass vials containing vesicular stomatitis were breached during storage causing contamination of the LN_2.[1] The risk of actual cross-contamination during cryo-storage of biological material was first clinically reported following the transmission of hepatitis B from cryo-stored bone marrow.[2] Microbial transmission should also be considered, given that ice sediments from LN_2 tanks were found to contain both bacterial and fungal contaminations that are capable of causing illness.[3] Bacterial and fungal species were also found in LN_2 used to store bovine embryos and semen.[4]

As there is the potential, during cryo-storage, to contaminate germplasm, it is not too surprising that viral and microbial transmissions have been investigated. Cross-contamination with bacteria between samples was first demonstrated with semen pellets, and it occurred within 2 hours.[5] Spiking LN_2 with different viruses and microbes demonstrated that bovine embryos could also be contaminated.[4,6] As expected, using sealed containers prevented contamination, suggesting that this should be routine and mandatory. One study, albeit with relatively limited numbers, performed viral screening on spent culture media and LN_2 used to vitrify oocytes and embryos from infected women.[7] No viral sequences were detected, suggesting the risk of cross-contamination is low. Safer cryopreservation methods would, however, avoid any of the possible contaminations mentioned above.

MINIMIZING CONTAMINATION DURING LN_2 STORAGE

There are several steps that can be taken to minimize the risk of contamination during cryo-storage.[8] Cryopreserved germplasm should be quarantined until it is deemed free of infectious entities, which will minimize any risk of cross-contamination. Semen is particularly susceptible to a high microbial load. Therefore, semen samples should always be stored separately from oocytes and embryos, although washing has been shown to significantly reduce or remove viruses and bacteria.[9] The zona pellucida does offer some protection to oocytes and embryos. As with semen, multiple washing is very effective in removing microbial and viral pathogens.[10] However, it is now very common for the zona pellucida to be breached because of intracytoplasmic sperm injection (ICSI), biopsy, and assisted hatching, which may make the oocytes and embryos more susceptible to contamination.

It is difficult to sterilize large quantities of LN_2 and also impossible to maintain that sterility in IVF clinics. Cryotanks and dry shippers should undergo periodic decontamination with a solution that does not react with the lining, and then rinsed with sterile water.[8] Even though LN_2 is not generally provided sterile to the clinic, most contamination is probably introduced during opening, distribution to storage vessels, and contaminated samples being stored in open containers. Sterile filtration of LN_2 at the outlet is feasible using a 0.22 µm filter.[11] Ultraviolet (UV) radiation is a further alternative for LN_2 sterilization. Microbial sterilization of small volumes (500 mL) of LN_2 is possible with UV radiation.[12] UV-sterilized LN_2 can also be used to wash devices to remove any external microbial contamination.[13] Filtration and UV irradiation of LN_2 can therefore offer protection against bacterial and fungal contamination. Another option consists of storing the carrier containing the biological material in LN_2 vapor.[14,15] It is, however, still unclear whether LN_2 vapor completely eliminates the potential for contamination.[16,17] Furthermore, one of the drawbacks of this form of storage is the need for careful monitoring of temperatures in different parts of the container and ensuring they remain below the glass transition temperature (–130°C).[18]

In summary, even though it is possible to sterilize LN_2, the methods do not afford complete viral elimination, may be impractical or prohibitively expensive, and most importantly do not prevent subsequent cross-contamination during LN_2 storage. It must also be noted that the IVF laboratory is not a sterile environment, so the sterility of the LN_2 cannot be guaranteed over time. There is no direct evidence of cryopreserved human oocytes/embryos becoming contaminated during cryo-storage, and subsequently transmitting disease or causing infection. Given the available information from bovine embryo studies, however, and potential concerns with long-term storage in open devices, it would appear prudent to avoid such direct contact with LN_2 during vitrification and subsequent storage by using closed devices. The remainder of this chapter will discuss such closed devices and their efficacies with regard to clinical comparisons with open systems.

CLINICAL COMPARISONS OF OPEN AND CLOSED VITRIFICATION DEVICES: CAN WE FORESEE THE END OF THE "OPEN" SYSTEM?

The principal of using a closed system, which would eliminate the risk of contamination, is not new. Kuleshova and Shaw sealed an open pulled straw in an outer straw to provide a closed storage system.[19] This device was successful at vitrifying mouse embryos[19,20] and human pronuclear oocytes.[21] There is, however, a significant difference in cooling rate between vitrification protocols with open (>25,000°C/min) and closed (<2000°C/min) devices. This difference in cooling rates is responsible for a continued debate, as it is widely believed to be the most important factor for achieving successful vitrification.[8] This now appears not necessarily to be the case. Peter Mazur's group has determined the functional relationship between cooling and warming rates and survival of mouse oocytes.[22–24] It was found that mouse oocyte survival was more negatively affected by slower warming rather than slower cooling rates. The rationale behind the critical importance of the warming rate may be that, although small ice nucleation events might occur with slower cooling rates, the warming rate must be fast enough to prevent them from aggregating and forming the larger, damaging ice crystals.

Despite the slower cooling rates, closed devices allow equivalent warming rates to open devices, and there is now growing evidence that closed systems can be as effective as their open counterparts for the vitrification of both human oocytes[25–29] and embryos.[26,30–40] A summary of the open and closed vitrification devices with their approximate volumes and cooling/warming rates is presented in Table 6.1.

When the Cryotip was compared to the Cryotop, the survival and pregnancy rates with human blastocysts were comparable between the two devices.[31] One study, however, has reported unacceptably low recovery with the Cryotip,[41] and another showed more ultra-structural damage of human oocytes with the Cryotip compared to the Cryotop.[42] The Cryotip as well as the Cryopette are very fine straws based on the principle of the open pulled straw. It is true that different users of such devices observe problems in terms of recovery. Furthermore, with such

Table 6.1 A Review of the Main Open and Closed Vitrification Devices

Device		Volume (µL)	Cooling rate (°C/min)	Warming rate (°C/min)
Open devices				
Cryotop	Flat strip	<0.1	23,000	>25,000
Hemi-straw	Small gutter	0.3	>20,000	>25,000
Cryoloop	Nylon loop	<0.1	>20,000	>25,000
Cryoleaf	Flat strip	1	23,000	>25,000
Cryolock	Flat strip	1	>20,000	>25,000
Vitri-inga	Hole	1	20,000	>25,000
Open pulled straw	Mini-straw	1	16,700	<20,000
Fiber plug	Hook	>1	10,000	>25,000
Closed devices				
0.25-mL straw	Straw	25–100	<2500	~1300
Vitrisafe	Small gutter	0.3	1300	>25,000
HSV	Small gutter	0.5	2000	>25,000
Rapid-i	Hole	0.05	1200	>25,000
Cryotip	Mini-straw	1	12,000	<20,000
Cryopette	Mini-straw	1	23,700	<20,000
Ultravit	Quartz glass microcapillary	0.5	Unpublished	Unpublished

devices, the warming rate is lower compared to devices in which the vitrification medium (containing the oocytes/embryos) comes in direct contact with the warming solution. These lower warming rates could explain the observed negative effect on the cytoskeleton after using the Cryotip.[42]

The Rapid-i uses supercooled air and is the most tested closed vitrification device. It was developed using mouse embryos and is capable of vitrifying mouse pronuclear oocytes with a 100% survival rate. The subsequent embryo development, cell number, and embryo viability following embryo transfer were not affected when compared to sibling nonvitrified embryos.[43] The Rapid-i has been compared to two open systems for human embryo vitrification. The results of vitrifying day 3 embryos and blastocysts using the Rapid-i were compared to an open system (Cryoloop).[36] For day 3 embryos, the survival rates for the Rapid-i and Cryoloop were 99%. The implantation rates were 37% and 35% and the clinical pregnancy rates were 47% and 49%, respectively. For blastocysts, the survival rates for the Rapid-i and Cryoloop were 97% and 91%, respectively. The Rapid-i supported a trend to higher implantation (49%) and clinical pregnancy (59%) rates than the Cryoloop (38% and 46%, respectively).

The Rapid-i has also been compared to the Cryotop.[35] The first comparison in this study used zygotes previously cryopreserved at the pronuclear stage. After rewarming, embryo development was assessed. There were no differences between the Rapid-i and the Cryotop in terms of survival (100% and 97%, respectively), blastulation, good blastocyst rates, or mean cell numbers (Table 6.2). To investigate the influence of the vitrification method on apoptosis, blastocysts were vitrified–warmed and compared to nonvitrified blastocysts. There was no difference in the proportion of dead cells between the three treatments. Lastly, the two devices were compared clinically. A total of 263 high-grade blastocysts were randomly assigned to vitrification using either the Rapid-i or the Cryotop. The survival rates for both devices were the same (97%). Single-blastocyst transfer was performed after warming, and the implantation and ongoing pregnancy rates were similar for the Rapid-i and the Cryotop (Table 6.3).

Table 6.2 Two-to Four-Cell Embryos Vitrified with the Rapid-i or Cryotop

	Rapid-i (n = 34)	**Cryotop (n = 32)**
Blastulation rate at 120 h (%)	68	56
Good blastocysts (%)	47	41
Mean cell number	137 ± 14	138 ± 18

Source: Modified from Hashimoto S et al. *J Assist Reprod Genet* 2013;30:371–6.

Note: The subsequent blastulation rate and percentage of good-quality blastocysts are shown with the mean cell number in each blastocyst.

Table 6.3 Clinical Results Following Blastocyst Vitrification Using Either the Rapid-i or Cryotop

	Rapid-i (n = 100)	**Cryotop (n = 163)**
Implantation rate (%)	54	53
Ongoing pregnancy rate (%)	45	47

Source: Modified from Hashimoto S et al. *J Assist Reprod Genet* 2013;30:371–6.

As with embryos, it appears that human oocytes can also be vitrified with the Rapid-i. Sibling *in vitro*-matured human oocytes were vitrified using either the Rapid-i or the Cryotop.[28] The survival rates were 92% and 90%, respectively. Currently, the Rapid-i is being evaluated in a clinical trial for donor oocyte vitrification. Over 500 oocytes have been vitrified and warmed with a survival rate of 94%. Following ICSI, the fertilization rate was 76%. Blastocyst transfer resulted in a 49% ongoing pregnancy rate,[29] with 40 healthy live births now recorded.

A prospective, randomized study was performed by Papatheodorou et al.[25] to validate the effectiveness of using the Vitrisafe device[32] in both an open and closed method for oocyte cryopreservation. Sibling oocytes donated from the same donor were randomly and equally assigned to closed or open vitrification groups. A total of 75 vitrification–warming cycles were performed in each group. Apart from the survival rate (82.9% versus 91.0%, $P < 0.05$) in favor of the open system, no statistically significant differences were observed in clinical pregnancy (36.0% and 28.0%) and live birth (36.0% and 24.0%) rates between the closed and open groups, respectively.

In another prospective randomized study, Panagiotidis et al. analyzed the outcome of vitrified blastocysts being randomly allocated to either an open hemi-straw or closed Vitrisafe device.[37] There were no statistically significant differences in the parameters measured: embryo survival rate (84.1% and 82.1%, respectively), clinical pregnancy rate (45.9% and 42.4%, respectively), implantation rate (25.6% and 24.5%, respectively), cycle cancellation rate (6.7% and 8.4%, respectively), and live birth rate (41.2% and 40.9%, respectively).

In a recent study, Chatzimeletiou et al. investigated the effects of aseptic vitrification with Vitrisafe on the cytoskeleton, chromosome alignment, and development of human blastocysts.[44] Even though there was a significantly higher incidence of abnormal spindles in the vitrified group compared with the fresh group, the high survival rate following warming and the large proportion of normal spindle/chromosome configurations suggests that aseptic vitrification at the blastocyst stage does not adversely affect the development of human embryos and the ability of spindles to form and continue normal cell divisions.

It also appears that long-term storage of embryos in closed systems is not an issue. Mouse zygotes stored for 2 years with the Rapid-i have equivalent embryo development and viability compared to nonvitrified zygotes.[43] The study of Wirleitner et al. included the transfer of blastocysts that had been vitrified for different time periods.[45] The data demonstrated that long-term storage of vitrified blastocysts using the Vitrisafe device did not impair blastocyst viability. The survival rate after warming during the first year of storage was 83.0% compared with 83.1% after 5–6 years of storage. The clinical pregnancy rate after 1 year of storage was 40.0% versus 38.5% after 6 years. Furthermore, no increase in the malformation rate over time was observed.

CONCLUSIONS

There are concerns with regard to contamination from LN_2 when using open systems that use direct contact with LN_2 during vitrification and subsequent long-term storage. A European Parliament directive (EU Tissues and Cells Directive 2004/23/EC), revised in 2006 (Commission Directive 2006/86/EC), has defined medical safety requirements and the use of closed systems for the cryopreservation of human cells.[46,47] There is now evidence that closed vitrification devices are as efficacious as open devices. Therefore, to prevent any contact with LN_2 during the procedure and subsequent storage, the most straightforward solution is to employ a closed device.

CONFLICT OF INTERESTS

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7 Automatic vitrification: Development of the Gavi system

Tammie K. Roy, Susanna Brandi, Cara K. Bradley, and Teija T. Peura

THE CHALLENGES OF VITRIFICATION AND *IN VITRO* FERTILIZATION (IVF) AUTOMATION

The cryopreservation of embryos during assisted reproduction technologies (ARTs) is an essential practice for clinics to maximize cumulative pregnancy rates. The most effective method of cryopreserving embryos is vitrification, which typically uses high concentrations of cryoprotectants and ultra-rapid cooling to preserve cells in a glass-like (vitreous) state without detrimental ice crystal formation.[1] When successfully executed, vitrification produces extremely high embryo recovery rates, in excess of 90%, and pregnancy, live birth, and neonatal outcomes equivalent to (or better than) those from fresh transfers.[2–4] However, vitrification is a manual, time-consuming, and high-skill procedure, and outcomes can vary greatly between operators and ART clinics.[5] Multiple vitrification devices and protocols are currently in use, which vary greatly in the constitution of cryoprotectants and conditions of embryo exposure, the rate of cooling and subsequent warming, and whether or not the embryo comes into direct contact with liquid nitrogen.[6,7] The numerous vitrification variables involved, including actual temperatures during the process, the exact duration of embryo exposure to solutions, and the diffusion gradient of solutions at the embryo level, make it very difficult to standardize the procedure when performed manually.

The recognized need for standardization, as well as the high-labor and high-skill demands of vitrification, could potentially be addressed by automation. However, automation of processes involving fluid exchange and embryos presents many challenges. The most significant of these is how to secure small and fragile embryos, with highly variable physical characteristics depending on developmental stage, during automated fluid movement. Here, we describe our approach to this problem and its application in an automated vitrification instrument, which forms the basis of the Gavi system.

DEVELOPMENT OF AN AUTOMATED VITRIFICATION SYSTEM

The Beginning: A Partnership between Embryologists and Engineers

In 1984, the first human baby was born from a frozen-thawed embryo cryopreserved by slow freezing. For the next 20 years, this method was used by ART clinics worldwide, and this greatly improved cumulative pregnancy rates from a single ovarian stimulation cycle. By the mid-2000s, clinical evidence of improved ART outcomes from embryos cryopreserved by vitrification as compared with the traditional method of slow freezing was accumulating.[8,9] Genea (previously known as Sydney IVF), a leading Australian IVF clinic,[2] also performed testing of the two methods, with embryo recovery and pregnancy outcomes clearly favoring vitrification (unpublished data). As a result, vitrification became the sole method for embryo cryopreservation in Genea clinics during 2006. While the clinical benefits of embryo vitrification for our patients were clearly apparent,[2] the labor-intensive and high-skill nature of the procedure provided challenges for our clinic and made it unattractive or not feasible for many other ART clinics.

In 2009, Genea decided to address the drawbacks of vitrification through automation of the procedure. However, we understood that our strength lay in our fertility and vitrification expertise, and that to be successful in this endeavor, we would need to collaborate with additional skill sets. Therefore, Genea established a partnership with Planet Innovation (Melbourne, Australia), a technology innovation and development company with commercially focused product developers. Teams of embryologists, scientists, and engineers, located at both Genea and Planet Innovation primary sites, were formed to collaboratively develop and optimize various aspects of an automated vitrification system.

The Requirements of an Automated Vitrification System

Prior to attempting to automate vitrification, in-depth "voice-of-customer" studies to understand ART clinics' requirements for an automated vitrification system were performed. Several key needs were identified, with one of the most important being that the recovery and survival rates of embryos processed using an automated system had to be comparable to current commercially available devices. Another key requirement was that the system had to be closed to prevent the embryo and vitrification solution coming into contact with liquid nitrogen. This avoids potential contamination with pathogens that have been shown to survive in liquid nitrogen,[10] and is essential for ART clinics in countries of the European Union to conform with their regulatory requirements.[11] At Genea, the vitrification device used was the Cryotop®, an open system that is arguably the gold-standard for embryo vitrification, and therefore, the decision was made to develop a

closed system with outcomes comparable to the Cryotop® method. Furthermore, the system had to be single use, simple to use, and vitrify multiple embryos simultaneously, ideally all blastocysts from a given patient in a single run. Additionally, the system would ideally be able to process oocytes, cleavage-stage embryos, and blastocysts.

The Greatest Challenge and the Pod Solution

The first and greatest hurdle in developing an automated vitrification system was to solve the problem of how to secure embryos during automated fluid exchange. An additional challenge was that the device used to hold the embryo during fluid exchange ideally would be used as the vitrification and embryo storage device, thus negating the need for embryo handling after automated equilibration. Numerous concepts were brainstormed, including a mesh, a restricted tube, and an open microfluidic channel (Figure 7.1). Subsequently, nine different "concept demonstrator prototypes" were developed by rapid prototyping and their potential evaluated using a "fail fast" approach. Most prototypes were rapidly eliminated including the initial favorites, the mesh, which resulted in embryo disintegration, and the restricted tube, from which the embryo could not be consistently retrieved. Instead, a most unexpected candidate became the most promising concept: an open microfluidic channel. Subsequently renamed the Pod, this approach holds the embryo in place during fluid exchange not by using a physical barrier, but by fluid dynamics[12]; the embryo resides in a microfluidic channel that restricts the movement of the embryo but allows the passage of solutions (for visualization of fluid dynamics).[13]

To fulfill the requirements of a closed system, the Pod was designed for a "lid" to be applied after equilibration to fully enclose the embryo within the Pod. Following investigation of a number of options, it was decided to use a heat-sealing technique to apply the lid. In order to prevent transfer of heat to the embryo during this process, various engineering concepts were introduced into the Pod to minimize energy transfer from the seal location (top of the Pod) to the embryo located in the channel divot. As such, the embryo does not experience temperatures beyond physiological levels after sealing if transferred to liquid nitrogen within the recommended time.

The Pod evolved significantly over the development process to address the many requirements for functionality and usability, and to date, there have been over 15 main (over 40 total) Pod prototypes. The first "test bed" Pods, designed for proof of principle of the fluid dynamic channel, were made by manually molding pre-existing thin concave plastic. Subsequently, numerous concept demonstrator prototypes were manufactured by injection molding using a single-cavity tool. Design modifications were incorporated in later alpha and beta models, including the refinement of the microfluidics channel and the addition of a handle and label area for embryo identification Pods (Figure 7.2). Additionally, a metallic metal insert was added to the beta Pod to allow easy and secure placement into the cassette, as well as removal from the cassette both in and out of liquid nitrogen. Here, considerable effort went into injection molding technology to achieve the thin wall section and co-molding the plastic with an inert metallic insert.

The Gavi System

The Gavi system performs automatic equilibration for closed-system vitrification on up to four embryos at a time. This occurs in Pods using the benchtop Gavi

The Road to Market: The Product Development Process

The project resulting in the Gavi instrument and consumables was executed following a tightly structured product development process using international standards for the design and development of medical devices. The process consisted of stage-gated project phases, with the first phase defining the initial customer needs and product requirements, development of product concepts, and their testing. The second phase contained further refinement of the core technology and feasibility testing of the overall system utilizing concept demonstrator prototype units. The third phase locked in the final design and verified that all product requirements were met utilizing more refined pre-verification prototype units (alpha and beta), while the fourth phase (currently in progress) undertakes the final validation of the complete system to ascertain whether the product functions as intended, utilizing pre-production units and production units.

Both science and engineering aspects were addressed and tested at all phases. All engineering aspects of the work were undertaken by Planet Innovation, and all science aspects by Genea Biomedx. From the science aspects, Gavi vitrification outcomes were always benchmarked against the Cryotop® system in regard to equivalent recovery of embryos, initial survival, and further *in vitro* embryonic development. Engineering aspects of the Gavi system included a variety of variables, such as testing of electronic systems, instrument operating software, fluidic exchange protocol software, precision pipetting and liquid handler unit functions, temperature control, movable platform and sealing unit operations, and many others. Furthermore, a significant amount of science-related testing was undertaken to ensure the safety and efficacy of the system and its parts. This included stability testing of Gavi consumables such as Pods and pipette tips using accelerated (heat and light induced) and real-time aging, as well as the effect of sterilization by irradiation and simulated transportation. Biocompatibility and sterility testing were part of all development phases, as well as final product verification and validation.

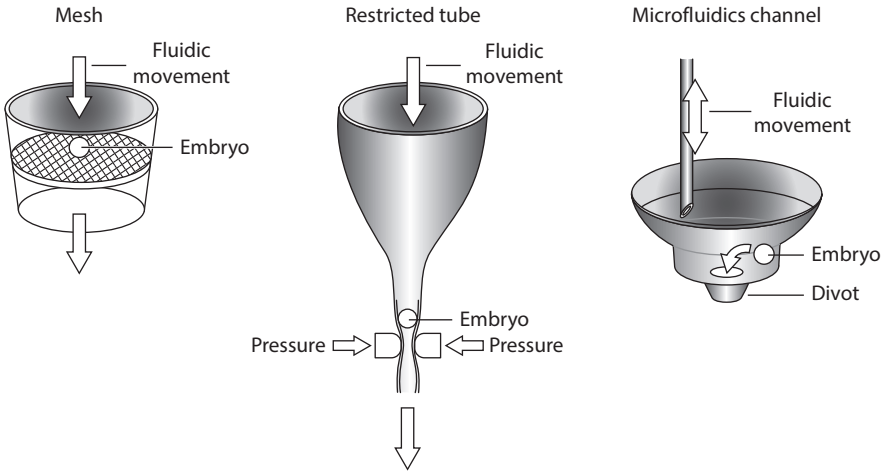

Figure 7.1 **Examples of concepts investigated to secure the embryo during automated fluid exchange.**

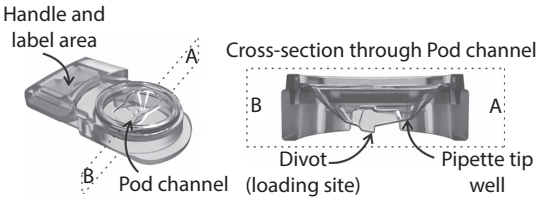

Figure 7.2 **Gavi Pod. Computer-aided design drawings showing Pod (left) and a cross-section through the Pod microfluidic channel (right).**

instrument, which contains high-precision liquid handling pipettes and sealing units with vertical movement capabilities. At the base of the instrument is the carriage, located on a horizontal moving platform. The carriage has fine temperature control with heating and cooling capabilities that ensure very little temperature variation during the protocol execution. Within the carriage are allocated sites for required consumables for automated vitrification, including Pods and vitrification solutions. The instrument contains an inbuilt computer with sophisticated software that controls all aspects of the instrument, including spatial and temporal movements. The various steps of the equilibration protocol are executed by coordination of the platform movement along the horizontal axis with the high-precision pipettes, and sealing units along the vertical axis.

The procedure to operate the Gavi system is simple and the key skill set required is embryo handling (Figure 7.3). Preparation of the Gavi instrument involves placing single-use consumables "medium cartridge" (containing the vitrification solutions) and "tip and seal" (containing a sterile pipette tip and lid seal) on the operating tray, which is then loaded into the Gavi instrument on the carriage to allow the media to equilibrate to the platform temperature. Next, Pods are placed into the "cassette," followed by a single embryo being loaded into each Pod. The cassette is then loaded into the appropriate position of the

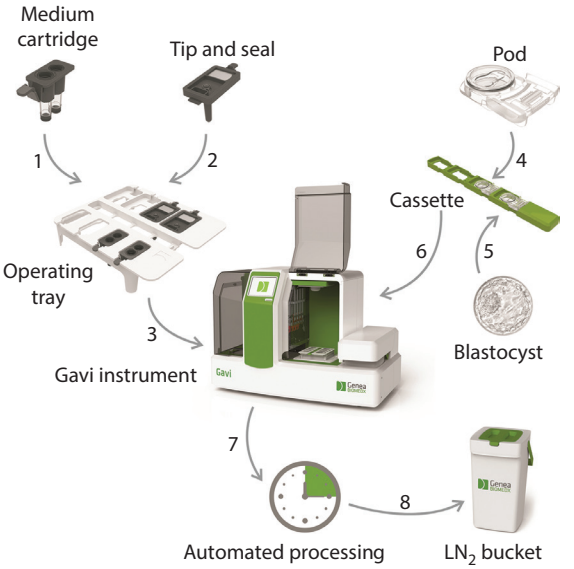

Figure 7.3 **Procedure to vitrify embryos using the Gavi system. Steps to operate the Gavi system: (1) load the "medium cartridge" into the "operating tray"; (2) load the "tip and seal" into the operating tray; (3) load the operating tray into the Gavi instrument; (4) load the Pod into the "cassette"; (5) load a single embryo into the Pod; (6) load the cassette into the operating tray in the Gavi instrument; (7) start the protocol; (8) upon completion of the protocol, immerse the cassette in the LN_2 bucket. (LN_2 = liquid nitrogen.)**

operating tray located within the instrument. The number of Pods to be processed is indicated via the software interface before the "start" button is pressed. Automated equilibration of the embryos then commences, running an approximately 18-minute (human) blastocyst protocol. Once the protocol is complete, the cassette is removed from the operating tray and manually dunked in the "liquid nitrogen bucket," also located on the Gavi instrument. The liquid nitrogen bucket is then used to transport the embryos, now contained in fully closed Pods, to long-term liquid nitrogen storage.

Optimization of Gavi Protocols

Simultaneous to the development of the engineering aspects of the Gavi system, there has been a large amount of research into protocol optimization. This involved in-depth experimentation with the many different variables in the equilibration process, including the temperature, time, volume, media concentration, and aspiration/dispense speed of the equilibrations steps; an example of this can be seen in Table 7.1, which revealed that an exposure time of 60 seconds, but not 90 seconds, in vitrification solution 2 for mouse zygotes produced equivalent *in vitro* development outcomes post-warming compared to the manual Cryotop® method. In many cases, modification of a single variable elicited the need for further refinement and optimization of other variables. Through this lengthy and time-consuming process, we have generated a standard protocol for all blastocyst types, including fully hatched blastocysts (see Roy et al., 2014, Table III and Table IV[13]). The fact that we can achieve equivalent *in vitro* cryopreservation outcomes to that of Cryotop® controlled for both mouse (non-hatched) blastocysts and fully hatched blastocysts using the same protocol on the Gavi system challenges the notion that vitrification needs to be personalized depending on an embryo's (visual) response to vitrification solutions.

Getting the Pod Just Right

During development, extensive testing of multiple Pod prototypes for vitrification and engineering outcomes was performed to ensure optimal design and functionality. The main requirements considered to ensure good vitrification outcomes using the Pod were

- Securing the embryo during fluid exchange without mechanical stress or toxicity
- That the embryo would at all times be covered by solution
- A high rate of cooling and warming
- Good optics for embryo visualization
- Ease of handling
- Consistent successful sealing (and subsequently opening) to create a closed system
- Withstanding pressures of greater than 1 bar, a requirement due to extreme temperature changes from vitrification and warming

Often, these functional requirements conflicted with each other. For example, the width of the Pod plastic had to be thin enough to ensure high cooling and warming rates, but thick enough to ensure Pod endurance under pressure. The material used to manufacture the Pod also provided challenges; for example, one plastic we trialed had excellent optical properties but was difficult to seal reliably, while another plastic was ideal for both but was extremely hydrophobic and irreparably disrupted the fluid dynamics.

As a consequence of conflicting functional requirements, many acceptable compromises had to be navigated. This included Pod cooling and warming rates being approximately 14,100 and 11,200°C/min, respectively, using the final alpha Pod prototype,[13] and approximately 11,400 and 8600°C/min, respectively, (unpublished data) using the final beta Pod prototype. The reason for this decrease in Pod cooling and warming rates in successive prototypes reflects minor revisions to the Pod design, materials, and manufacture, which were essential for ensuring reliable and consistent vitrification outcomes. Critically, extensive *in vitro* testing using mouse embryos with beta Pods revealed that these changes did not compromise the vitrification outcomes achieved with the system (unpublished data), and in fact, our cooling rate

Table 7.1 Example of Optimization of the Gavi System

				≥Expanding blastocyst	Fully hatched blastocyst
Treatment	**Number**	**Recovered**	**Survived**	**Day 5**	**Day 7**
Cryotop®	68	68 (100%)	67 (98.5%)*	54 (79.4%)*	39 (57.4%)
Gavi (V2 60 s)	60	58 (96.7%)	51 (87.9%)*	44 (75.9%)	33 (56.9%)
Gavi (V2 90 s)	64	62 (96.9%)	54 (87.1%)*	37 (59.7%)*	29 (46.8%)

Note: The effect of time spent in vitrification solution 2 (V2) on mouse F1 C57Bl/6J × CBA zygotes processed using the alpha Gavi system. Embryos were incubated in V2 for either 60 or 90 seconds and the results were compared with the manual Cryotop® method. Embryos were considered to have survived if all cells were intact at the completion of warming.

* Significant difference ($p < 0.05$) between test groups.

far exceeds that of other commercially available closed-system vitrification devices.[14,15]

OUTCOMES OF THE GAVI SYSTEM

Vitrification of Mouse Blastocysts

As reported previously,[13] using the alpha model Gavi system, we achieved *in vitro* vitrification outcomes for mouse zygotes, cleavage-stage embryos, and blastocysts (including fully hatched blastocysts) that were comparable to Cryotop® controls. Using Gavi pre-production units, we have processed a total of 2876 mouse blastocysts, of which we have recovered 99.9% of embryos after vitrification–warming. Embryo survival after warming was 94.9% for blastocysts processed with the most recent Gavi protocol, and was comparable to the 96.0% obtained using the Cryotop® method (Table 7.2). There were fewer mouse embryos considered to be re-expanded 2 hours after warming (defined as occupying 90% or greater of its original volume) using the Gavi system as compared with Cryotop® controls. However, the lower re-expansion rate for embryos processed with the Gavi system was not indicative of poor survival, as the developmental potential of the embryos remained intact; there was no difference in *in vitro* embryo development at 24 hours (day 6) and 48 hours (day 7) after warming between Gavi- and Cryotop®-processed embryos. This includes development to fully hatched blastocysts by day 6 being 31.6% for embryos processed with the Gavi system as compared with 30.8% for embryos processed using the manual Cryotop® method.

Vitrification of Human Blastocysts

Encouraged by the positive outcomes for mouse embryos processed using Gavi pre-production units, we have commenced testing of Gavi production units using human embryos excess to reproductive needs and donated for research. These embryos are blastocysts that have either been cryopreserved by slow freezing or vitrification, and are warmed with appropriate protocols for the cryopreservation method, before being vitrified using the Gavi system or the Cryotop® method. To date, the recovery of 18 human embryos processed with Gavi production units is 100% (Table 7.3). The proportion of embryos considered to have survived after warming was 94.4%, which is comparable to the 86.4% achieved with the Cryotop® system. The proportion of embryos considered to have re-expanded

Table 7.2 Evaluation of the Gavi System Using Mouse Blastocysts

					Fully hatched blastocyst	
Treatment	**Number**	**Recovered**	**Survived**	**Re-expanded**	**Day 6**	**Day 7**
Cryotop®	273	273 (100%)	262 (96.0%)	211 (77.3%)*	84 (30.8%)	112 (41.0%)
Gavi	336	335 (99.7%)	318 (94.9%)	225 (67.2%)*	121 (36.1%)	160 (47.8%)

Note: Outcomes for Quackenbush Swiss mouse blastocysts vitrified after automated processing with Gavi pre-production units as compared with the manual Cryotop® method. Embryos were considered to have survived if 75% or more cells were intact at the completion of warming. Embryos were considered to have re-expanded if occupying 90% or greater of their original volume 2 hours after warming.

* Significant difference ($p < 0.05$) between test groups.

Table 7.3 Evaluation of the Gavi System Using Human Blastocysts

					24 h	
Treatment	**Number**	**Recovered**	**Survived**	**Re-expanded**	**Fully hatched blastocyst**	**Hatching/expanding blastocyst**
Cryotop®	22	22 (100%)	19 (86.4%)	13/19 (68.4%)*	2 (9.1%)	15 (68.2%)
Gavi	18	18 (100%)	17 (94.4%)	9 (50.0%)	5 (27.8%)	11 (61.1%)

Note: Outcomes for human blastocysts vitrified after automated processing with Gavi production units as compared with the manual Cryotop® method. Embryos were donated for research via an informed consent process (National Health and Medical Research Council [NHMRC] license 309718) and were either slow frozen (50%) or vitrified blastocysts (50%) that the patients had deemed as excess to their reproductive needs. Only embryos that were grade I or grade II[2] prior to Gavi (50% expanding blastocysts or greater) or Cryotop® (59% expanding blastocysts or greater) vitrification were used. Embryos were considered to have survived Gavi or Cryotop® vitrification if 75% or more cells were intact at the completion of warming. Embryos were considered to have re-expanded if occupying 90% or greater of their original volume 2 hours after warming. Embryo classifications at 24 hours were restricted to grade I and grade II[2] embryos.

* Not all embryos were assessed for re-expansion.

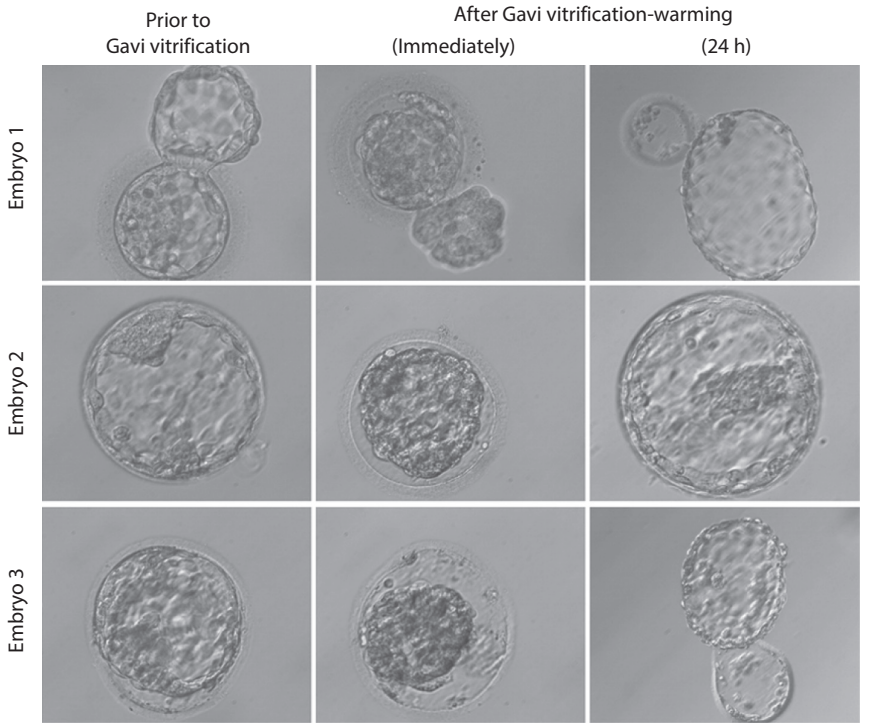

Figure 7.4 **Examples of human blastocysts vitrified using the Gavi system. Embryos were donated for research via an informed consent process (NHMRC license 309718) and were either slow frozen (embryo 2) or vitrified blastocysts (embryo 1 and embryo 3) that the patients had deemed as excess to their reproductive needs. Embryo 1 was a pre-implantation genetic diagnosis (PGD)-biopsied hatching blastocyst. Embryo 2 and embryo 3 were expanded blastocysts. All images were taken at 200× magnification, with the exception of the images of embryo 1 and embryo 3 at 24 hours after Gavi vitrification–warming, which were taken at 100× magnification.**

2 hours after warming was slightly lower for the Gavi system as compared with Cryotop® controls (50.0% and 68.4%, respectively), and is consistent with the mouse blastocyst data. Furthermore, *in vitro* embryo development after vitrification–warming was comparable between Gavi- and Cryotop®-processed embryos; at 24 hours post-warming, 88.9% of Gavi-processed embryos were grade I or grade II[2] expanding blastocysts or greater, as compared with 77.3% for Cryotop® controls (Figure 7.4).

THE BENEFITS OF THE GAVI SYSTEM

The Gavi system provides many benefits to vitrification users compared with manual vitrification. A major advantage of the system is the ability to control and standardize vitrification variables, a very difficult feat if performed manually, thus allowing the procedure to be performed the same way each time. The Gavi system also reduces the constant need for monitoring individual operator outcomes, something that is critical even after a user achieves training requirements. The key skill set required for operation of the Gavi system is basic embryo handling, and during the development of the system, even very junior technicians achieved similar outcomes to senior embryologists (unpublished data). In fact, the greatest challenge when training new staff for this project has been mastering the manual Cryotop® method, and given that this is our vitrification benchmark, stringent certification criteria were necessary before considering new users' Cryotop® results. However, we do not believe that the Gavi system reduces the need for skilled embryologists in the clinic, although it does provide valuable time to embryologists for other work; when considering the "hands-on" time to vitrify four human blastocysts, the Gavi system takes an estimated 36 minutes less than the manual Cryotop® method.[13]

Another advantage of the Gavi system for ART clinics struggling with the challenges of manual vitrification, and even more so for clinics currently performing slow freezing, consists of the *in vitro* vitrification outcomes achieved with the system. The recovery rate alone, being 99.9% using mouse blastocysts with Gavi pre-production units, is outstanding, particularly given it being a closed system. It is difficult to know how this compares with other vitrification devices in a clinical setting, given that

embryo recovery rates are usually not stated and can vary greatly depending on the device used and operator skill. Furthermore, the Gavi system achieves equivalent embryo survival and subsequent *in vitro* development to that of the gold-standard Cryotop® system, with which we have proven expertise.[2]

THE FUTURE OF THE GAVI SYSTEM

Toward Clinical Use

Despite the extremely promising *in vitro* results using the Gavi system on mouse embryos and donated human blastocysts reported here and elsewhere,[13] the ultimate test for determining its success will be in the clinic. When the Gavi system passes the requirements for scientific and engineering validation, as well as a number of other strict criteria, including toxicity studies on the plastic consumables and stability testing of the vitrification solutions, the system will be used to vitrify human blastocysts in a number of Genea ART clinics. This clinical usage, which will include analysis of neonatal outcomes, is expected to commence in 2015, and will be monitored very closely to ensure patient outcomes are not compromised in any way.

The Gavi system (with the blastocyst protocol) is expected to gain regulatory clearance and consequently should be commercially available to ART clinics in late 2015. The long time frame to develop the Gavi system (7 years) reflects the challenging nature of developing an automated vitrification system, the very stringent criteria set for success, and the large amount of testing required before clinical usage and regulatory clearance. Furthermore, research and development of the Gavi system will be ongoing and will include optimization of protocols for cleavage-stage embryos and oocytes. These will be incorporated into the Gavi system by software updates. Additionally, it is anticipated that future versions of the Gavi system may automatically place equilibrated sealed Pods into liquid nitrogen, thus removing the current requirement for manual transfer.

Potential Application to Other IVF Processes

While the Gavi system has the potential to revolutionize and standardize embryo cryopreservation, the system may have applications for assisted conception procedures beyond that of vitrification. As the Pod solves the most difficult issue of how to hold the embryo in place during automated fluid exchange, it may be possible to use this new technology in other areas to minimize embryo handling and thus stress and the potential for embryo loss. We are also applying our knowledge and experience from developing the Gavi system to finding solutions to other challenges and inefficiencies in the ART clinic. This includes combining advanced embryo incubator technology with time-lapse monitoring of embryo development, which may assist in the identification of embryos with the highest pregnancy potential.[16] These technologies will provide another step toward ART standardization and may provide further improvements in assisted reproduction outcomes.

CONCLUSION

Vitrification is an essential practice in ART clinics for ensuring optimal patient outcomes. However, vitrification is labor intensive and time consuming, and outcomes vary between operators and ART clinics. The Gavi system automates the equilibration of embryos prior to vitrification using a novel closed-system device. This allows precise control of the many vitrification variables—a very difficult feat if performed manually. Testing of Gavi system prototypes—the alpha system reported previously[13] and the pre-production and production units reported here—has shown equivalent *in vitro* outcomes to those of the gold-standard Cryotop® system. Further *in vitro* testing of production units is currently underway, and it is expected that usage in a clinical setting will begin in 2015. The Gavi system has the potential to revolutionize and standardize vitrification, and opens up the possibility of automating other complicated IVF procedures.

UPDATE

Since the submission of this chapter the Gavi verification and validation and clinical evaluations has been completed and the instrument has been CE marked.

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8 Vitrification at minimum volume: From basic science to clinical application

Amir Arav

WHAT IS VITRIFICATION?

Vitrification is the process by which a liquid is supercooled to a temperature at which the viscosity is so high that the liquid can be defined as being at an amorphous glassy solid state, having no ice crystals. The understanding of the vitrification process has deepened over the years and has been applied for cryopreservation. Currently, it is the method of choice for oocyte and embryo cryopreservation.

HISTORY OF VITRIFICATION

Basil Luyet stated in 1940: "Some of the oldest investigations on subcooling were made by Gay Lussac (1836) who observed that water can be subcooled to –12°C when it is enclosed in small tubes."[1] Supercooling is cooling under the freezing point without crystallization, and it had already been thought of at the beginning of the nineteenth century. It was done by the great French chemist and physicist Joseph Louis Gay Lussac. He is known mostly for his two laws of gases and for his work on alcohol–water mixtures. Gay Lussac found that water can be cooled to –12°C when enclosed in small tubes without freezing, finding with this discovery the basis of vitrification.[2]

In 1804, Gay Lussac ascended in a hot-air balloon and noticed that the drops in the clouds were not frozen despite the subzero temperatures. He later published the discovery of the effect of small volumes of water droplets on supercooling; indeed, the size of water drops in clouds is about 8–10 μm, which maintains them at a liquid state at the subfreezing temperature of –5°C. In 1898, Gustav Heinrich Johann Apollon Tammann pointed out that a large number of substances can be obtained as glasses and suggested that this property might be universal.[3]

In 1858, Johann Rudolf Albert Mousson sprayed droplets of water less than 0.5 mm in diameter onto a dry surface and observed that the smaller the drops, the longer they stayed subcooled.[4] However, the volume is not the only important factor for achieving supercooling; among other factors that might have an influence on inducing crystallization, as were mentioned by Luyet, are the cooling velocity and the concentration of the supercooled or supersaturated solutions. Luyet wrote: "To avoid freezing, the temperature should drop at a rate of some hundred degrees per second, within the objects themselves," and also: "The only method of vitrifying a substance is to take it in the liquid or gas state and cool it rapidly so as to skip over the zone of crystallization temperatures in less time than is necessary for the material to freeze. Therefore, one should consider the crystallization velocity. It is evident that when the crystals grow faster one must traverse the crystallization zone more rapidly if one wants to avoid crystallization."[1]

THE FIRST CELLS SURVIVING AFTER VITRIFICATION

In 1938, Basil J. Luyet and Eugene L. Hodapp published the first successful vitrification of sperm.[5] They demonstrated for the first time the successful cryopreservation of frog sperm by vitrification using a 2 M sucrose solution in small drops. Luyet started his research with colloids (i.e., gelatin, milk, or agar) and found that their water content determines the possibility or impossibility of vitrification. In general, solutions containing 50% gelatin could vitrify successfully with layers of 0.3 mm in thickness (by the method of immersion in liquid nitrogen [LN]), while solutions containing 90% water could vitrify only with smears of a few microns in thickness.[1] We recently repeated Luyet's experiment using human sperm and obtained a very high survival rate of sperm "vitrified" in liquid air (–190°C) using a solution that does not contain intracellular cryoprotectants but is based only on sugars and proteins.[6]

In 1949, Polge et al.,[7] trying to repeat Luyet's results, discovered the cryoprotective property of glycerol and so opened the field to slow freezing.

Currently, there are two methods for gamete cryopreservation: slow freezing and vitrification. Slow freezing has the advantage of using low concentrations of cryoprotectants (CPs), which are associated with chemical toxicity and osmotic shock. Vitrification is a rapid method that reduces chilling sensitivity and crystallization damage caused to cells.

For many years, slow freezing and not vitrification has been the method of choice, since vitrification was not achieved easily due to the need for high CP concentrations and relatively high-volume samples to be cryo-stored.

THE MINIMUM DROP SIZE TECHNIQUE: MY PERSONAL STORY

The first successful vitrification of mouse embryos using a relatively large-volume sample was done in 1985.[8] These

researchers vitrified mouse embryos with 6.5 M of glycerol in a large volume inside a 0.25-mL straw plunged into LN. At that time, I was a veterinary student at the University of Bologna, Italy, and I met Bill Rall, who told me about the exciting work he did on mouse embryos. Two years later, I started to work with Boris Rubinsky on the cryomicroscopy of oocytes and embryos, and in our laboratory, we used to prepare oocytes for histology evaluation by fixing them with a small drop over a microscopic slide. This gave me the idea for using the same technique for vitrification in a small drop, which I later called the "minimum drop size" (MDS) (Figure 8.1).[9–12] The volume we used for vitrification was in the range of 0.07–0.1 µL, and the concentration of the vitrification solution (VS) was about 50% lower than of the VS used for large-volume vitrification (Figures 8.1 and 8.2).[10]

We called it the MDS as this was the minimal size that allowed us to keep oocytes or embryos without damage due to desiccation. Vitrification of embryos, on the other hand, although initially attempted in the late 1980s, has not been clinically applied until recently. Vitrification is currently producing very satisfactory outcomes by means of different methodologies using a minimum volume principle both for oocytes and embryos.[13,14]

FACTORS AFFECTING VITRIFICATION

The velocity of cooling, which is a major factor in the vitrification process, depends on the thermal mass of the sample and on its surface area. To achieve rapid cooling, we should use materials that have the lowest heat mass and maximum surface-to-volume ratio. Gregory M. Fahy and William F. Rall published in 2007 the critical cooling rates needed to vitrify aqueous solutions containing different concentrations of CPs[15]; from this, it was extrapolated that for pure water, a cooling rate greater than 100 million °C/min is needed to form a glass state without crystallization (Figure 8.3). Also, it is interesting to note that for 15% (v/v) of most CPs, a cooling rate of almost 1 million °C/min is needed, which is extremely difficult to achieve. We showed that 15% (v/v) of CPs can vitrify at a relatively slow cooling rate when the volume of the drop is 0.07 µL.[10] Therefore, it is more feasible and easier to achieve vitrification by lowering the sample volume than by increasing the cooling rate.

James H. Walton and Roy C. Judd measured the velocity of ice crystal growth and found that it is in the range of 65 mm/s.[16] This means that if we wish to avoid crystallization in a drop having a diameter of 0.01 mm, we will need a velocity of 1/6500 mm/s, which is 0.0001 s. If we cool from room temperature to −180°C, this means we need to drop 200°C at a rate of 0.1 microseconds, or at 120×10^6 °C/min. This is actually very similar to the cooling rate that was estimated by Balds and Bruggeler (Figure 8.3).[1,15] However, since achieving this cooling rate is virtually impossible, the best possible way to achieve vitrification of pure water is in a small drop (diameter of 10 µm) at relatively slow cooling rates. This indicates that volume has an independent effect on the probability of vitrification.

Later attempts at vitrifying pure water have been made by a few investigators; L. Hawkes[17] published an experiment in which a drop of solid amorphous water

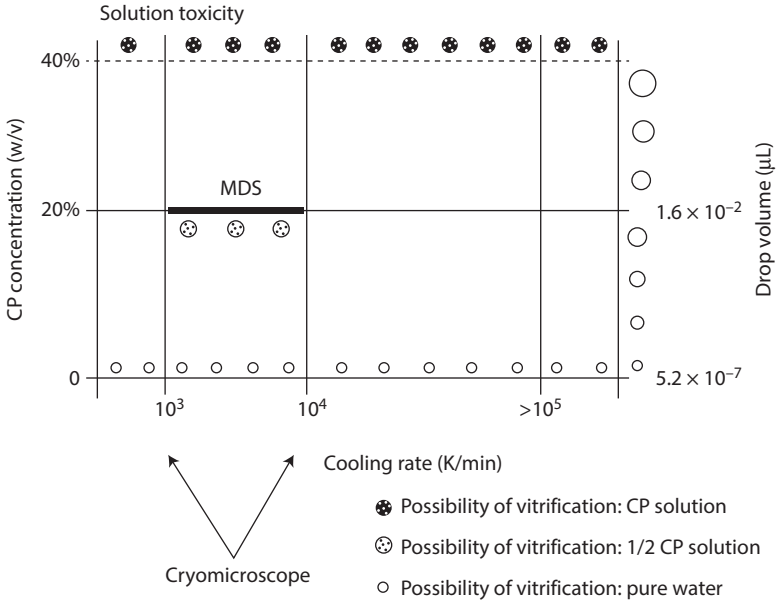

Figure 8.1 **The effect of volume, cooling rate, and CP concentration on vitrification. (CP = cryoprotectant; MDS = minimum drop size.)**

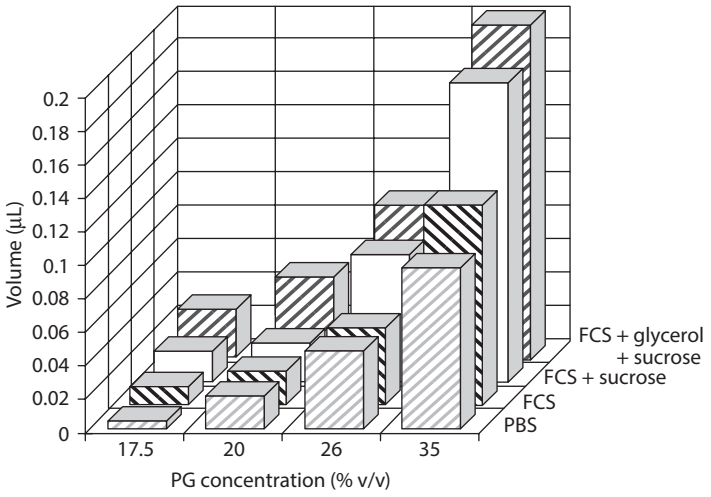

Figure 8.2 **The probability of vitrification as it depends on cooling rate, volume, and composition of the solution. As is noted in Figure 1 and 2 taken from Arav (1989),[9] the probability of vitrification increases as the volume of the sample decreases. At 0.02 µL, it is possible to achieve vitrification even with only 17.5% propylene glycol (PG) + 2.5% glycerol + 20% fetal calf serum (FCS) in phosphate-buffered saline (PBS). However, the minimum drop size was designed in the range of 0.035 µL, which requires about 30% cryoprotective agent (26% PG and 2.5% glycerol and sucrose) and is very similar to the concentration levels used currently (Figure 8.3).**

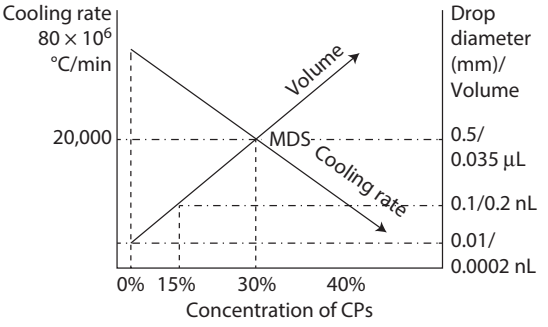

Figure 8.3 **The probability of a vitrification solution containing different concentrations of CPs (the volume is calculated for a half sphere). (CP = cryoprotectant; MDS = minimum drop size.)**

was obtained during rapid cooling. Burton and Oliver obtained, from steam, some solid water in which X-ray analysis did not reveal any crystalline structures.[18] As we can see, these achievements were mainly due to the small sample volume, and not the velocity of cooling.

IMPORTANT FACTORS THAT SHOULD BE CONSIDERED

1. *Cooling and warming rate*—high cooling rates can be achieved with LN or LN slush, and a warm water bath for warming. When using LN, the sample is plunged into LN, resulting in cooling rates of hundreds to tens of thousands of degrees Celsius per minute, depending on the container, the volume, thermal conductivity, solution composition, etc.[19] To achieve LN slush, the LN needs to be cooled close to its freezing point (−210°C). The VitMaster is a device that generates LN slush (IMT Ltd, Ness Ziona, Israel), which reduces the temperature of LN to between −205°C and −210°C by applying negative pressure. When LN slush is formed, the cooling rate is dramatically increased. The cooling rate is especially enhanced in the first stage of cooling, when cooling down from room temperature to 0°C. The cooling rate is enhanced two to six times more than plunging into LN (−196°C) with 0.25-mL straws, or any other device such as open-pulled straws (OPS) or electron microscope (EM) grids.[11] It was shown for oocytes and embryos that increasing the cooling rate improves survival rates by up to 37%.[20] In Figure 8.2, we can see that cooling at 80 million °C/min is needed to vitrify pure water, while cooling at 20,000 million °C/min is needed to vitrify 30% of CPs using a small volume in the range of 0.1 µL (the MDS technique), and only 15% of CPs is needed if we reduce the diameter of the drop to 100 µm.[21] This means that it is easier to achieve vitrification by reducing the volume rather than increasing the cooling rate. It was recently demonstrated by Seki and Mazur that the warming rate is dominant over the cooling rate for mouse oocyte vitrification[22]; however, to reduce chilling damage, which occurs during cooling and warming, we should keep the cooling rate as rapid as possible.
2. *Viscosity* of the medium in which the embryos are suspended, or the glass transition capability of the

solution at low temperatures, is defined by the concentration and behavior of various CPs and other additives during vitrification. The higher the concentration of CPs, the higher the glass transition temperature (T_G), thus lowering the chance of ice nucleation and crystallization. Different CPs and other additives have different toxicities, penetration rates, and T_Gs. A combination of different CPs is often used to increase viscosity, increase T_G, and reduce the level of toxicity. So in the cattle industry, to avoid handling of the post-warmed embryos and allow direct transfer, ethylene glycol (EG) is often used as the permeating CP because of its high penetration rate[23] and its high glass transition ability.[24]

3. *Volume*—the smaller the volume, the higher the probability of vitrification.[9–12] Smaller volumes allow better heat transfer, thus facilitating higher cooling rates. Although small volume has an independent effect on the probability of nucleation, as was discovered 200 years ago by Guy Lussac, only recently have techniques been developed to reduce sample volume, with an explosion of methods appearing in the literature during the last decade. These techniques can generally be divided into two categories: surface techniques and tubing techniques.[12] The surface techniques include the EM grid,[25] MDS,[9,11,12] Cryotop,[26] Cryoloop,[27,28] Hemi-straw,[29] solid surface,[30] nylon mesh,[31] Cryoleaf,[32] direct cover vitrification,[33] fiber plug,[34] vitrification spatula,[35] Cryo-E,[36] plastic blade,[37] and Vitri-Inga.[38] To the tubing techniques belong the plastic straw,[8] OPS,[39,40] closed-pulled straw,[41] Flexipet-denuding pipette,[42] superfine OPS,[43] CryoTip,[44] pipette tip,[45] high-security vitrification device,[46] sealed pulled straw,[47] Cryopette,[48] Rapid-i,[49] and JY Straw (RC Chian, personal communication). Each of these two groups has its specific advantages. In the surface methods, the size of the drop (0.1 μL) can be controlled, a high cooling rate is achieved because these systems are open, and high warming rates are achieved by direct exposure to the warming solution. The tubing systems have the advantage of achieving high cooling rates in closed systems, thus making them safer and easier to handle. Decreasing the vitrified volume and increasing the cooling rate allows a moderate decrease in CP concentration, so as to minimize their toxic and osmotic hazardous effects.[47] Combining these three factors can result in the following general equation for the probability of vitrification:

Arav "equation":

$$\text{Probability of vitrification} = \frac{\text{Cooling/Warming rate} \times \text{Viscosity}}{\text{Volume}}$$

OOCYTE VITRIFICATION

Oocytes are very different from sperm or embryos with respect to cryopreservation. The volume of the mammalian oocyte is in the range of three to four orders of magnitude larger than that of the spermatozoa, thus substantially decreasing the surface-to-volume ratio. However, this is not the reason why the oocytes are sensitive to low temperatures and to slow freezing; that is, mature oocytes are very sensitive to slow freezing, and even after fertilization, the volume of the oocytes remains the same and their sensitivity is reduced to a minimum. In fact, this is the best stage for freezing human oocytes (2 pro-nuclei [2PN] freezing).[50] The reason why oocytes are nonfreezable is due to their chilling sensitivity, which occurs at different cellular levels: the zona pellucida, plasma membrane, meiotic spindle, cytoskeleton, etc.

The plasma membrane of oocytes at the metaphase II (MII) stage has a low permeability coefficient, thus making the movement of CPs and water slower.[51] In addition, the freeze–thaw process causes premature cortical granule exocytosis, leading to zona pellucida hardening, thus making sperm penetration and fertilization impossible.[52–54] These later consequences can be overcome by the use of intracytoplasmic sperm injection (ICSI). Oocytes also have a high cytoplasmic lipid content that increases chilling sensitivity.[51] They have less sub-membranous actin microtubules,[55] making their membranes less robust. Cryopreservation can cause cytoskeleton disorganization and chromosome and DNA abnormalities.[56] The meiotic spindle, which has been formed at the MII stage, is very sensitive to chilling and may be compromised as well.[57] It does, however, tend to recover to some extent after thawing or warming and during *in vitro* culture; this recovery is faster following vitrification than following slow freezing.[57] Oocytes are also more susceptible to the damaging effects of reactive oxygen species.[58] Many of these parameters change after fertilization, making embryos less chilling sensitive and easier to cryopreserve.[55,59,60]

Vitrification requires the presence of high concentrations of CPs. The presence of CP in the VS decreases the probability of intracellular crystallization, which is considered to cause the most damage when very rapid cooling takes place, but the higher concentrations of the CPs are toxic and cause osmotic injury to the oocytes even without cooling. It is therefore important to minimize the damage caused to cells by the osmotic stress and/or chemical toxicity. No ideal CPs that meet the requirements of all different species and developmental embryonic stages have been found; vitrification studies should therefore be preceded by osmotic and cytotoxic studies. Different methods have been used to reduce this "solution effect": (i) short time of exposure to CPs[24,61]; (ii) use of low-toxicity CPs[62] or mixtures of them[63]; (iii) addition of nonpermeating CPs[24]; (iv) reduction of the CP concentration[62]; and (v) exposure at low temperatures.[62] Among

these methods, the use of nonpermeating CPs is very useful, either because the shrinkage of the oocyte and consequently the amount of water inside the cell that may crystallize during rapid cooling and warming is lower,[62] or because of the reduction of the amount of the CP that penetrates the cell, thus reducing the possible toxic effects.[64] In addition, the carbohydrates used as nonpermeating CPs have a stabilizing effect on membranes.[65] In a study reported by ourselves,[66] trehalose was less harmful than sucrose. Determination of the Boyle–Van't Hoff relationship for both sucrose and trehalose produced the same regression line, so it is possible that this beneficial effect could be a consequence of its interaction with the membrane polar lipid groups by the trehalose.[65] We also showed that only 10 minutes of exposure are required for equilibration in propylene glycol and dimethyl sulfoxide (DMSO) solutions or mixtures of them,[66] as the membrane is very permeable to both of them. The results of the vitrification provide evidence that propylene glycol can be used successfully. Indeed, the *in vitro* fertilization (IVF) rate of the bovine oocytes vitrified in a solution containing 40% (w/v) propylene glycol was 37%, and is not different from the results obtained using a slow-freezing protocol.[67] The viability of vitrified mouse embryos was successfully increased by reducing the concentration of the CP.[68] However, concentrated solutions of permeating CPs are required for successful cryopreservation of oocytes when rapid cooling and warming rates are used. In earlier reports on immature pig oocytes, we showed that when lower concentrations of CPs are used, despite apparent vitrification, membrane destruction was unavoidable.[69] In 1990, M. Kasai was the first to describe the use of EG for mouse embryo vitrification.[70] Today, the most frequent solutions for oocytes vitrification are based on a mixture of DMSO and EG.[13,44]

Small Volumes Can Resolve Many Problems that Occur During Vitrification

Three major problems are associated with vitrification: (1) crystallization (during cooling); (2) devitrification (crystallization during storage or during warming); and (3) fractures of the glassy solution that cause devitrification due to the release of energy by the fracture. Surprisingly, at 1 µL, fractures appeared only when the concentration of the VS was high (100% VS = 38% EG, 0.5 M trehalose and 4% bovine serum albumin in tissue culture medium [TCM]), but not at lower concentrations.[12] This means that the probability of fractures increases with the increasing of the T_G or the viscosity of the VS. At low concentration of VS (50% VS), fractures were observed only at very high cooling rates. We suggest here a simple explanation of this phenomenon, based on the following equations:

$$\text{Probability of fracturing} = \mathbf{CR} \times \mu \times \boldsymbol{V}$$

$$\textit{Since probability of vitrification is} = \mathbf{CR} \times \mu \times 1/\boldsymbol{V}$$

1. Increasing the cooling rate (CR) will increase the probability of vitrification; however, it will also increase the probability of fractures.
2. Increasing the viscosity (µ) will also increase the probability of vitrification because the T_G will increase,[19] thus increasing the probability of fractures.
3. The only parameter that will increase the probability of vitrification, and at the same time decrease the probability of fractures, is to reduce the volume (V) to the value of the MDS.

The reason for the increasing probability of fractures in high concentrations of VS is thought to be related to the T_G. We know that fractures can form only at temperatures below that at which the liquid turns into glass (the T_G) and above the LN temperature (–196°C). We also know that a solution with higher CP concentrations will have a higher T_G. Therefore, if the temperature gradient increases, as in the case of higher T_G, then the probability of fracturing will also increase. Finally, the results of the vitrification of bovine oocytes at the MII or germinal vesicle (GV) stage, with a concentration of 75% v/v of VS/phosphate-buffered saline, have been reported.[71] We achieved 72% and 38% cleavage and blastocyst formation rates, respectively, for the vitrified MII oocytes, and 27% and 14% cleavage and blastocyst formation rates, respectively, for oocytes vitrified at the GV stage. We conclude that the new vitrification procedure, which features small volumes, direct contact with supercooled LN and low concentrations of VS, reduces chilling injury and provides a high probability of vitrification in the absence of glass fractures.

WHY VITRIFICATION WITH OPEN SYSTEMS WORKS BETTER FOR OOCYTES

Open versus Closed Systems

Most of the methods that work very well use direct exposure to LN, which introduces a potential risk of contamination and cross-contamination[72]; in fact, contamination of bovine embryos by viral pathogens during storage in LN has already been reported.[72,73] Most likely, volume is the important factor for successful vitrification in open systems, as it was demonstrated that open systems give better results than closed systems, and these were exclusively for surface systems.[74–76] If we analyze the differences between the two systems, we can see that the warming rate is not the reason for these differences, as it is the same in both systems (we open the container in LN and plunge it directly into a warm dilution medium in a closed system as well). The VS is also not very much different for both systems, and the cooling rate in both systems is similar (actually, in small tubes like Cryopette, the cooling rate is very high). Therefore, the only factor that differs between the two systems is the volume of the drop; while in the open system the drop volume is reduced to a minimum (in the range of 0.1 µL or less), the volume in the

closed system is in the range of 0.5 µL or more in order to avoid rehydration and desiccation damage during the introduction to the container and heat sealing.

CLAir SYSTEM

CLAir is an automatic bench-top device for producing sterile liquid air. The device employs LN and produces clean liquid air, which is supplied into a specially designed sterile cup. Liquid air is at the same temperature range of LN (between –190°C and –196°C) and thus can be used for cryopreservation. CLAir comes in two sizes (CLAir and CLAir XL, FertileSafe, Israel), which are able to produce 70 mL and 350 mL of clean liquid air every 10 minutes, respectively. The temperature of liquid air is slightly higher than LN (–190°C) due to the presence of 20% liquid oxygen with a temperature of –180°C. However, the cooling rate is very similar in LN or clean liquid air. We have vitrified bovine GV and MII oocytes and human oocytes by direct immersion into sterile Styrofoam cups containing liquid air and assessed them for survival after warming. Control oocytes were vitrified with standard commercial LN.

In these studies, human MII oocytes were donated for research (with Institutional review board [IRB] approval) and assessed for survival after vitrification–warming by examining re-expansion and morphology. Vitrification was performed using the Cryoleaf (Origio, Denmark) for bovine oocytes and the Cryotop (Kitazato, Japan) for human oocytes. The cooling rates between liquefied air and LN were above 20,000°C/min for each volume of cryogenic liquid used (50, 25, or 10 mL). Bio-burden tests showed no contamination in liquid air as opposed to commercial LN. In bovine experiments, 20 GV and 22 MII oocytes were vitrified using clean liquid air, while 10 GV and 10 MII oocytes were controls cryopreserved in LN. The recovery rate was 90% for GV and 91% for MII. The survival rate was 88% (16/18) for GV and 95% (19/20) for the MII. A total of 88% (16/18) of GV oocytes matured, and the MII cleavage rate was 78% (15/19) (no differences with control rates). Of the seven human MII oocytes, four were vitrified with liquid air and three with LN, with experiments repeated three times. Upon warming, a 100% survival rate (12/12 for liquid air and 9/9 for LN) was obtained in both groups. We conclude that the use of clean liquid air instead of LN is a breakthrough methodology for cryopreservation and, most importantly, eliminates the potential risk of contamination from commercial LN during vitrification with open systems.

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9 Vitrification of oocytes: General considerations and the use of the Cryotec method

Masashige Kuwayama

WHY DO WE NEED TO CRYOPRESERVE HUMAN OOCYTES?

In most papers dealing with this topic, a long list of simple and obvious reasons to answer this question can be found. These answers will also be listed below. However, let this review start with a more general and rarely mentioned argument: the handicapped situation of women from the standpoint of reproduction.

Although gender-based discrimination is less and less acceptable in most human societies, nature still preserves the right to make a seemingly unfair but strong distinction between males and females in certain areas including—obviously—reproduction. In most mammalian species, females bear most of the weight of reproduction, including discomfort related to reproductive cycles and pregnancy, pain of labor, and nursing of babies. In humans, even with the best intentions, male partners cannot share most of these sacrifices, and most women accept them as inevitable parts of their full life, which are at least partially compensated by the most intimate relationship with their babies. However, there are additional, even more frustrating differences between males and females regarding the possibility of distributing and preserving genetic material. A healthy male can produce many millions of sperm cells every day, while the number of oocytes is highly restricted in many mammals, including humans, to one or two per month. Moreover, males normally preserve their reproductive ability for their whole life, while the time for women is rather limited. Theoretically, they may become pregnant and have babies before their menopause—which also means almost halving the available time compared to males—but in practice, even this period is drastically shortened by the fact that the quality of oocytes decreases sharply after the age of 35, restricting the real freedom to reproduce without concern and fear to approximately 15 years, coinciding exactly with the time that is the most critical to establishing a professional career for a lifetime.

We may be referring to the inevitable order or laws of nature, but aging and loss of teeth or hair may also be referred to as such, not to mention blindness, deafness, or other serious handicaps. We make considerable and fully justified efforts to eliminate these unfair differences created by life and nature and we institute legislative and financial help to ensure equal opportunities in many fields. We probably should focus more on this half of humankind to alleviate their handicapped situation in the field of reproduction.

Unfortunately, although not at all according to their original intentions, modern reproductive technologies in the human have made the situation even worse. With the application of intracytoplasmic sperm injection (ICSI), one gamete from both genders may be enough to produce an offspring. Compared to the enormous number of sperm available, the ten-, sometimes twenty-fold artificial increase in number of available oocytes (as the result of an expensive, painful, and sometimes risky medical intervention) does not fully compensate for this imbalance. It is even more embarrassing that with ICSI, incompletely capacitated or matured, handicapped, immotile, or dying spermatozoa can still be successfully used for fertilization, while the slightest deviation in morphology, function of oocytes (including those caused by the supportive medical intervention itself), or the smallest inaccuracy in the stage of maturation may seriously compromise their further developmental competence.

Finally, when we consider storage possibilities of the male and female gametes of mammals, the difference is even more frustrating. In many domestic species, large commercial networks are dealing with the collection, deep-freezing, and distribution of the sperm of valuable animals, by using highly standardized procedures, and with an efficiency that almost completely eliminates the need for natural mating or artificial insemination with fresh semen. The establishment of the "cryoprotectant-free vitrification" method[1] for spermatozoa also proves the extreme tolerance of male germ cells toward cryoinjuries, although the term itself may raise some concerns. In the mouse, a technology has also been developed enabling sperm transportation in a sealed envelope by ordinary mail. On the other hand, the recovery, laboratory handling, and especially cryopreservation of mammalian oocytes from live animals is still regarded as a challenge and restricted (with a few exceptions) to the experimental field. The situation is no better in humans: sperm can be easily collected, frozen, stored, and utilized in small aliquots, creating the commercial distribution of this supposedly valuable male genetic "stuff" as a prosperous business in some countries. In contrast, the storage and use of the female gamete is seriously restricted by the above-mentioned biological, technical, and psychological problems related to collection, as well as by legal measures

restricting experimental use and/or donations in many countries, and by the poor and inefficient technologies available for their cryopreservation. Until recently, due to the cumulative effect of these factors, the efficiency of the whole procedure was so low that practically every baby born after oocyte cryopreservation deserved a scientific publication.

Establishment and widespread application of an efficient and safe cryopreservation method for oocytes would not eliminate differences in reproductive flexibility between females and males, but may mean a solution to many specific problems and eventually reverse the actual trend of this unacceptable artificial widening of the gap between the two genders. Accordingly, this area deserves special attention, and should be regarded as more than just a subject of scientific ambition of a few, accidentally selected scientists, making this an area that is benignly and respectfully disregarded by the vast majority of reproductive specialists.

The need for change in the general attitude toward oocyte cryopreservation is even more justified by the recent rapid advancement in technology now outlining the perspective of a real breakthrough, and offering a definite solution right now in many important fields, including the following:

1. Malignant diseases where systemic anticancer treatment is required[2]
2. Surgical procedures resulting in loss of ovarian function[3]
3. Treatment of patients with polycystic ovarian syndrome[4,5]
4. Patients with ovary hyperstimulation syndrome[3]
5. Poor responders to ovarian stimulation[3]
6. Patients at risk of ovarian function loss through premature menopause[3]
7. In cases of male factor infertility or problems associated with difficulty of sperm collection, inadequate seminal samples, or nonviable spermatozoa at the time of oocyte retrieval[3]
8. To overcome ethical concerns and legal restrictions in several countries associated with embryo cryopreservation[6]
9. Cryobanking oocytes for young women who wish to delay motherhood for various reasons (career, lack of appropriate partner, etc.)
10. Cryobanking oocytes for egg donation programs or for research purposes[7]

WHY IS CRYOPRESERVATION OF OOCYTES DIFFICULT?

Some of the reasons for this, including the size, shape, and cell number, are quite obvious.

It is well known that oocytes are the largest cells of the human body. In cryobiology, the size, or rather the mass, is a decisive factor. Suspensions of somatic cell cultures can be cryopreserved with high efficiency and without any sophisticated approach by using simple media, a refrigerator, and a deep freezer or liquid nitrogen. For the even smaller bacteria and viruses, we meet the frustrating evidence every day: they are present in almost every liquid nitrogen tank, and preserve their viability without any protection and in spite of our best intentions (although apart from the size, some other factors—for example, their simple structure—may also play roles in this resistance). In reproductive biology, we just referred to the above-mentioned differences between cryotolerance of spermatozoa and oocytes that can be at least partially attributed to the differences in volume. Quite controversially, the cumulative mass of cells decreases exponentially during the first week of embryo development, and at the expanded blastocyst stage, it may become as low as 1/10–1/100 of that of the oocyte, with obvious similar decreases of the water content. Although the solution accumulated in the blastocoele may mean a potential source of damage either by ice crystal formation or through the accumulation and slow dilution of toxic cryoprotectants,[8–10] these mechanisms obviously cause more harm when they occur intracellularly in the oocyte.

Apart from the size, the shape of the oocyte is also most unfortunate. The almost perfect sphere slows down the formation of an equal distribution of any substance, including permeable cryoprotectants coming from outside or released from the oocyte. Accordingly, for a relatively long period of time, a continuous concentration gradient from the periphery to the center or vice versa exists, resulting in toxic damage in one part while providing less than optimal protection in the other. From this point of view, the change in shape caused by the osmotic effect at equilibration may offer some kind of benefit, but it may also contribute to the damage to the cytoskeleton (see later in this chapter).

The third major factor is the lowest possible cell number. From this point of view, the oocyte resembles a gambler who puts all of their money on the very first bet: all or nothing. Multicellular embryos can survive and compensate for as much as 50% loss of their cells (and supposedly also some level of injury in the remaining ones), as demonstrated by biopsies, bisection of embryos, or just the less-than-optimal culture conditions, quite apart from the cryopreservation experience. The oocyte has only one chance, and there is no backup to regenerate from a serious injury. We have to use an extremely careful approach to get out of the game as winners!

Unfortunately, apart from the factors listed above, there are still many other factors that contribute to the sensitivity of oocytes to cryoinjuries. Chilling injury, which occurs at relatively high temperatures and induces irreversible damage of the cytoplasmic lipid droplets, lipid-rich cell membranes, and microtubules, mostly affects the latter two structures in the human oocyte

(in contrast, for example, to pigs), as in humans, cytoplasmic lipid droplets are less abundant. On the other hand, the membranes are extremely sensitive and rapidly undergo a transition from the liquid state to the gel state, an irreversible process that is detrimental for future development. For unknown reasons, just a step ahead, after fertilization, the membranes of zygotes are much less sensitive to this type of injury.[11]

The depolymerization of microtubules, misalignment of the chromosomes, and the possible increased risks of aneuploidy are frequently emphasized and have wide experimental backgrounds,[12] although in the human, comparative examinations may not entirely confirm the seriousness of this problem,[13] and the supposed beneficial effects of some agents (cytoskeleton relaxants or stabilizers) are not fully proven. Similar to somatic cell nuclear transfer, spindle reorganization may occur surprisingly efficiently, and the number of chromosomal abnormalities in children born after oocyte vitrification does not seem to show a significant increase.

A strange and not completely understood phenomenon is the change in cryosensitivity of oocytes during the maturation process. Although there is only a minimal difference between their size and shape, immature oocytes are usually more sensitive to cryopreservation than mature (Meiosis II [MII] phase) oocytes.[11,15] The contrary might be supposed based on the known sensitivity of the meiotic spindle to chilling. More research is needed to understand the reasons for this difference, and the alteration of the sensitivity of membranes may be one of the possible explanations.

The osmotic shock at equilibration may result in shrinking and misshaping of the oocytes, supposedly damaging the cytoskeleton. However, the effect of other agents (e.g., pronase digestion of the zona pellucida) induces much more serious deformation, followed by surprisingly rapid recovery and maintenance of developmental competence. On the other hand, the osmotic shock that may occur during dilution may result in extensive swelling, rupture of the membrane, lysis, and immediate death of the oocytes.

Hardening of the zona pellucida, attributed by some authors to premature cortical granule release, may cause decreased rates of fertilization.[13]

Fracture is a common consequence of all cryopreservation procedures[16] and does not seem to occur more frequently in oocytes than in embryos. However, while the consequences for zona fracture may be similar for both, embryos may survive some level of cell membrane damage, while for the oocyte, any injury at this level is evidently fatal.

WHAT IS THE BEST APPROACH?

Based on the points listed above, the principles of a successful cryopreservation strategy can be outlined. Although infrequent in biological study, theory is mostly justified by practice, although it should be confessed that the sequence of events was (as usual) inverted: the empirically established methods were retrospectively supported by the subsequent detailed theoretical analyses of events.

Firstly, we need a method that minimizes chilling injury. So far, in mammalian embryos and oocytes, two approaches have been successfully applied for this purpose: the removal of the lipid droplets (by high-speed centrifugation and micromanipulation, although the latter step is not required with the use of some recent techniques)[17] and by radically increasing the cooling and warming rate to minimize the duration of exposure to dangerous temperatures. As human oocytes contain relatively low amounts of lipids, centrifugation does not significantly improve survival chances. On the other hand, all forms of traditional slow-rate freezing are obviously less appropriate for the purposes of oocyte cryopreservation than high-rate cooling vitrification strategies.

The large cell mass and spherical shape of the oocyte necessitates the use of highly permeable cryoprotectants with low toxicity. As in many areas of vitrification in mammalian embryology, ethylene glycol is the candidate of choice for this purpose. According to earlier investigations in rabbits,[18] the permeability of ethylene glycol is facilitated by dimethyl sulfoxide (DMSO). Further studies have also demonstrated that DMSO may have a beneficial effect on spindle polymerization, and consequently a protective effect on oocyte vitrification.[3] Although various proportions of DMSO and ethylene glycol were extensively tested for the vitrification of bovine oocytes and embryos, the best results were always achieved with a 1:1 mixture (G. Vajta, unpublished data). To facilitate dehydration, thus decreasing the chances of intracellular ice formation, the addition of nonpermeable cryoprotectants is also required. Various substances, including polymers with low toxicity, were suggested for the purpose; however, the traditionally used sugars (i.e., sucrose or trehalose) seem to be more appropriate. Curiously, although trehalose has been reported many times to be superior, in the past few years, it has gradually disappeared from the list of frequently used cryoprotectants.

In the past few years, two basically different strategies of equilibration before cooling have been applied.[19] It was proposed that dehydration may even be more important than cryoprotectant concentration for the prevention of ice crystal formation, suggesting extremely short equilibration times both for the diluted and concentrated cryoprotectant solutions.

This strategy was successfully applied subsequently by many others for domestic animal oocytes and embryos. However, more recently, another approach has received more attention and seems to be more efficient for mammalian oocytes: an extended equilibration in a rather diluted first cryoprotectant solution, followed by a short, but slightly prolonged incubation in a second, relatively concentrated vitrification solution containing, in addition, a

nonpermeable cryoprotectant.[20–22] Although the time of the exposure is significantly increased, the cumulative toxic effect (as a result of the lower concentration) may be the same or even lower, and the prolonged equilibration may ensure proper penetration of cryoprotectant, providing appropriate protection to the entire oocyte. The other way to minimize the toxic and osmotic effects of cryoprotectants is to decrease the required concentration while maintaining the ice-free solidification pattern. Currently, the only practical way to achieve this goal is with an extreme increase in cooling rates. Among the various tools applied for this purpose, electron microscopic grids, Cryoloop, and Cryotop seem to be the most appropriate choices, although recently, similar results were achieved with the open pulled straw technique.[23] Either directly by the higher rate of cooling or indirectly by the decreased toxic and osmotic effect, the Cryotop and Cryoloop vitrification approaches with a mixture of relatively low concentrations of DMSO and ethylene glycol do not seem to cause serious anomalies in the spindle structure, and may ensure relatively high developmental rates.

As mentioned, fracture damage is not specific to oocyte cryopreservation, although the consequences may be more detrimental. Fortunately, the open vitrification systems have drastically reduced the occurrence of this type of damage. Retrospectively, it may be supposed that in a closed system, the extreme pressure changes caused by rapidly cooling or warming air bubbles induce dislocations in the partially solidified solution, and with a scissor-like effect cut the zona pellucida or the cell membranes. In the open systems, such mechanical forces are almost completely avoided. The extremely small volume of solutions used also minimizes the chance of fractures. Accordingly, this type of damage is almost entirely eliminated by the application of the ultra-rapid open vitrification systems.

Finally, the problem of zona hardening and subsequent low levels of fertilization has been eliminated entirely with the discovery and subsequent widespread application of ICSI. Although not included in the original goals, the application of ICSI after cryopreservation has contributed much to increases of efficiency, and opened the gate to widespread application of oocyte cryopreservation.

THE MOST RECENTLY DEVELOPED CLINICAL VITRIFICATION PROTOCOL: THE CRYOTEC METHOD

The first consistently successful vitrification protocol of human oocytes was reported by Kuwayama et al.,[24] incorporating the Cryotop as the vitrification carrier. In his experiments at the Kato Ladies Clinic (the largest *in vitro* fertilization [IVF] center in the world), almost 95% of oocytes survived vitrification and ICSI, and cleavage rates did not differ from those of controls in our laboratory. When blastocyst transfer was applied, 45% of vitrified oocytes developed to healthy babies (Figures 9.1 and 9.2). Other groups began applying this Cryotop approach for oocyte vitrification with good success[3]: 89.2% survival rates have been reported after Cryotop vitrification of oocytes, as well as a 56.5% pregnancy rate (13 of 23 patients) with an average of 4.63 embryos transferred to patients. This Colombian group also achieved the first baby born after oocyte vitrification in South America (E. Lucena, unpublished data). From Mexico, 401/445 (90.1%) survival and 34.1% pregnancy rates were reported after Cryotop vitrification.[25] In Valencia, Spain,[26] a total of 225 MII oocytes were vitrified, of which 217 (96.5%) survived cryopreservation and 165 (76.0%) were normally

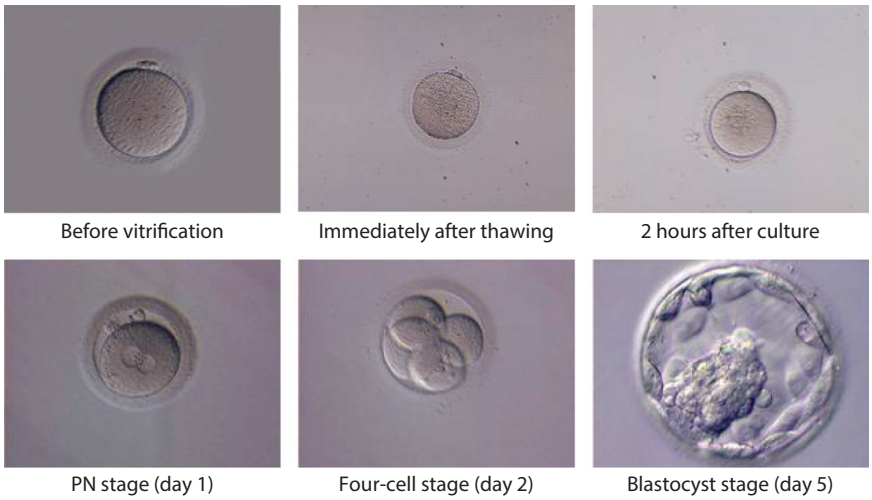

Figure 9.1 **Inverted microscopic pictures of human oocytes before and after vitrification, intracytoplasmic sperm injection and *in vitro* culture (on days 1, 2, and 5). PN = pronucleus.**

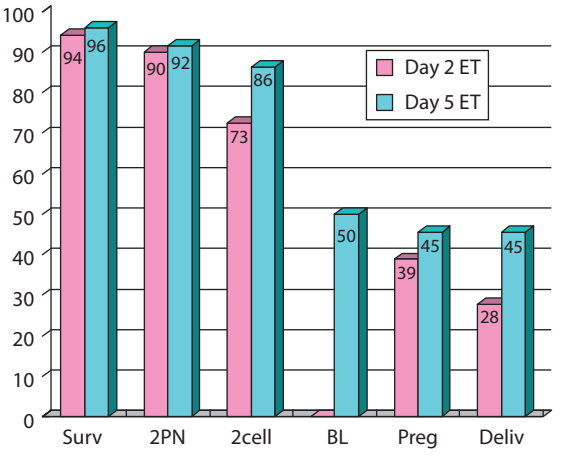

Figure 9.2 **Results of oocyte cryopreservation performed at Kato Ladies Clinic with Cryotop vitrification. Columns refer to percentages of vitrified oocytes surviving vitrification (Surv), developing to the two pronucleate embryo stage (2PN), proceeding to cleavage (2cell), developing to the blastocyst stage (BL), resulting in pregnancy (Preg), and resulting in the delivery of healthy babies (Deliv). The total numbers of oocytes used for day 2 and day 5 embryo transfer were 86 and 25, respectively. Finally, 18 and 11 embryos were transferred on days 2 and 5, respectively (2.9 and 1 embryos per recipient).**

fertilized after ICSI, and this was not different from the controls. A total of 93.9% of zygotes underwent cleavage on day 2, and the blastocyst per fertilized oocyte rate (22.4%) did not differ from the control. Twenty-one embryo transfers were performed with embryos from vitrified oocytes, resulting in 13 pregnancies (61.9% pregnancy and 37.2% implantation rates).

The Cryotop method has been in widespread use in more than 40 countries during the last decade, and a total of 70,000 healthy babies have been obtained from vitrified oocytes, and all the while the protocol has been improved several times for better survival and easier handling to reduce the potential for human error by embryologists at all stages of oocytes and embryos. This completed noninvasive clinical vitrification method has been called the Cryotec method. Now, the Cryotec method is being rapidly adopted in many countries and successfully applied, so that the Cryotec method is routinely providing almost 100% survival for both human oocytes and embryos.

Here, we provide some technical details required for successful cryopreservation of MII-phase human oocytes. The commercially available vitrification kit (Repro-Support Medical Research Centre, Tokyo, Japan) contains the Cryotec vitrification container, a filmstrip attached to a plastic handle also equipped with a cap to cover the filmstrip for safe handling and storage (Figure 9.3), and all media required for washing, equilibration, vitrification, warming, and dilution. These solutions are based on minimal essential medium (MEM) supplemented with hydroxypropyl cellulose and xanthan gum as serum replacement, and containing ethylene glycol, DMSO, and endotoxin-free trehalose as permeating and nonpermeating cryoprotectants. All media and manipulations should be performed at 25–27°C, except for warming, in which the medium should be warmed to 37°C. A pulled, fire-polished glass pipette with a 140–150-µm inner diameter is suggested for all of the manipulations for oocytes vitrification.

Oocytes can be vitrified 1–6 hours after the ovum pick-up, immediately after denudation. Oocytes are first placed on the surface of the equilibration solution that contains the permeating cryoprotectants. Oocytes immediately shrink to approximately 50% volume of their original size, and then start to recover. When oocyte volume has recovered to the original size, it is time to end this step. This normally takes 12–15 minutes for oocytes.

After the completion of the equilibration step, oocytes are then placed in the bottom of vitrification solution, which is a solution that has higher concentrations of cryoprotectants. Oocytes start to rise to the surface because of the different specific gravities between the equilibration and vitrification solutions, and the purpose of this step is to complete the exchange of the solutions from the equilibration to the vitrification solution, which is completed automatically (Figure 9.4a).

This is followed by oocyte loading onto the Cryotec (filmstrip), a carrier device set on the vitrification plate (Figure 9.4b), and then directly plunged into liquid nitrogen (Figure 9.4c). The Cryotec is then covered with the cap while still under liquid nitrogen to avoid mechanical damage during transfer to the container and storage.

At warming, the film part of the Cryotec should be submerged quickly into the 37°C warming solution in a warming plate (Figure 9.4d) to achieve the required 42,000°C/min warming rate. After 10 seconds, the oocytes are gently removed from the surface of the Cryotec by themselves, and start to float in the warming solution. After 1 minute, the dilution should be continued

Figure 9.3 **Cryotec vitrification container (a) without and (b) with cover cap.**

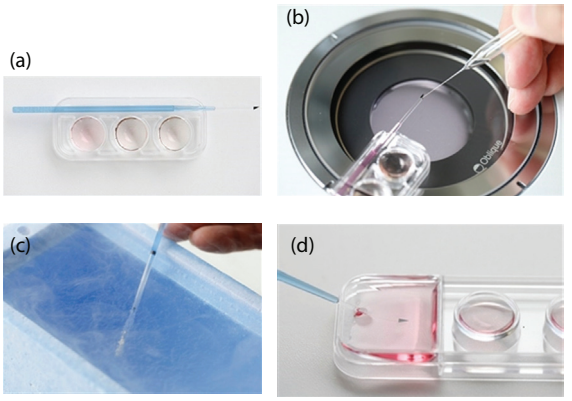

Figure 9.4 **Cryotec vitrification protocol: (a) equilibration of equilibration solution/vitrification solution/vitrification solution (ES/VS/VS), (b) loading of oocyte, (c) ultra-rapid cooling, and (d) ultra-rapid warming.**

in dilution solution, washing solutions 1 and 2 for 3, 5, and 1 minutes, respectively. Oocytes should be cultured for an additional 2 hours before the ICSI. Morphologies before and after vitrification, ICSI, and embryo culturing are shown in Figure 9.1.

INITIAL OOCYTE VITRIFICATION RESULTS ACHIEVED WITH THE CRYOTEC METHOD

Gandhi et al.[27] introduced the protocol details of the Cryotec method as one of the latest versions of Dr. Kuwayama's various novel vitrification protocols, and they introduced their clinical results in which they reported the same rates of fertilization (83.0% versus 80.9%), cleavage (96.5% versus 94.4%), pregnancy (56.3% versus 54.9%), implantation (28.0% versus 31.1%), and live births (45.0% versus 45.1%) between fresh and vitrified oocytes when using the Cryotec method.

Kagalwala et al.[28] reported a direct comparison of the Cryotop and the Cryotec methods: from 611 vitrified oocytes, survival rates, fertilization rates, and cleavage rates of the Cryotec and Cryotop methods were 97.2% and 95.1%, 90.7% and 86.2%, and 96.9% and 91.9%, respectively. After embryo transfer (ET), pregnancy rates of the Cryotec and Cryotop methods were 54.8% and 40.6%. Furthermore, when oocytes vitrified by the Cryotop method were warmed by the Cryotec method, higher survival (92.5% versus 87.8%) and higher pregnancy rates (46.5% versus 40.6%) than the original warming method were obtained. Although the results by the Cryotop method were excellent for human clinical use, superior outcomes for all steps after warming were nevertheless obtained by the Cryotec method.

The Cryotec method is now used at an increasing number of laboratories worldwide for oocyte vitrification in more than 40 countries. Almost all of these laboratories indicate survival, *in vitro* developmental, and eventual pregnancy rates that are much higher than those achieved previously with conventional vitrification and traditional freezing.

CONCLUSION

The high number of healthy babies born (more than 70,000 babies) in over 40 countries after Cryotop and Cryotec vitrification worldwide over 10 years clearly proves that oocytes cryopreservation by this successful vitrification approach is emerging as a very useful tool in the human reproduction field across many applications. All data obtained from many different laboratories (including survival, fertilization, embryo development, and pregnancy rates) suggest that oocytes vitrified with this technology are highly viable, and their developmental competence is comparable with that of fresh oocytes. The increasing evidence proves that Cryotec vitrification may offer solutions for women with various fertility problems, and may participate in the compensation for the handicap of women from the standpoint of reproduction.

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10 Safety of vitrification and cryostorage and optimization of cryopreservation protocols

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INTRODUCTION

As vitrification is a cryopreservation technique increasingly applied in clinical practice for reproductive cells and tissue, the main focus of this chapter is to analyze the risks related to this technique and to propose possible solutions. Since there may be concerns regarding the safety of vitrification procedures and cryostorage, due to the contact of cell/tissue/carrier with liquid nitrogen (LN_2), these aspects are comprehensively treated in the "Safety of vitrification and cryostorage" section.

Furthermore, since today different cryopreservation protocols with various cryoprotectant formulations can be used both for vitrification and slow freezing, the "Optimization of cryopreservation protocols" section of this chapter will describe the possibility of using a single "universal warming protocol" for any frozen cell, irrespective of the freezing protocol and cryoprotectant cocktail used at freezing.

SAFETY OF VITRIFICATION AND CRYOSTORAGE

Vitrification Carriers

Open carriers

During vitrification, cells and tissue need to be cooled and warmed at an extremely rapid rate.[1] This can be achieved by using specific "open carriers" such as the open pulled straw,[2] Cryoloop,[3] hemi-straw,[4] Cryotop,[5] Cryoleaf,[6] Cryolock,[7] Vitri-inga,[8] etc.; these "open carriers" are generally preferred for oocytes.[9–13] However, these systems cannot avoid the hypothetical risk of microorganism contamination during the vitrification procedure, if the LN_2 is accidentally contaminated.[14,15] The sterilization of LN_2 before vitrification procedures is thus recommended to ensure the safety of clinical application.

Straw-in-straw closed carriers

Another option for vitrification is the closed carrier based on the "straw-in-straw" mode (high-security vitrification), designed to insulate the inner carrier containing the cells/tissue against LN_2 during vitrification by using a sealed external straw.[16,17] This system avoids direct contact between specimens and LN_2 and also any hypothetical risk of contamination and leads to good results with zygotes, cleaved embryos, blastocyst and ovarian tissue.[17–19] However, the "straw-in-straw" system causes a reduction in the rate of cooling, and is not routinely used in the clinical cryopreservation of oocytes.

Single-straw closed carriers

As an alternative to straw-in-straw, other types of closed systems, such as CryoTip[20] or Cryopette,[21] allow faster rates of cooling. These closed carriers consist of a very thin straw specifically designed to load cells with the minimum volume of cryoprotectant solution, and to be hermetically sealed; in this way, direct contact between cells and LN_2 is avoided. Unfortunately, because of their design, these systems would not avoid the transmission of microorganisms into the culture medium during the warming procedure, arising from accidental contamination of the LN_2; this is due to the direct contact between the LN_2 and the external surface of the carrier.[22,23] In practice, the contamination of cells occurs at 37°C, when any cryopreserved microorganism found in the LN_2 reactivates after thawing in the culture medium. Even though *in vitro* fertilization (IVF) culture media are supplemented with antibiotics, some micro-organisms may resist the antibiotic and infect the culture. In these circumstances, the bacterial or viral particles released into the culture medium may attach themselves to the oocyte/embryo zona pellucida, if this is cracked.[14,24] Another procedure to decontaminate the straw is to quickly wipe the carriers with 70% ethanol for disinfection at warming.[20] However, the deactivation of all microorganisms can be obtained only by a 5-minute contact between ethanol and carrier[25]; this prolonged contact time can damage human cells, which remain inside the carrier in the warmed vitrification solution, rich in potentially toxic cryoprotectants.[26] For these reasons, sterilization of the LN_2 before the vitrification procedure is recommended for the safe clinical application of these systems.

Nitrogen vapors/supercooled air vitrification

It has been demonstrated that it is possible to vitrify reproductive cells by exposure to nitrogen vapors.[27,28] Other authors have proposed inserting the carrier containing the cells into the supercooled air of a straw for instantaneous vitrification, and then to seal the open end of the straw (post-sealing method) to avoid direct contact with LN_2.[28] It is important to point out that the supercooled air inside the straw is basically composed of nitrogen vapor owing to nitrogen's rapid evaporation and molecular weight.[29] For this reason, any microorganism accidentally present in the LN_2 can also pass to the nitrogen vapor phase,[30] and thus lead to the hypothetical

contamination of the oocyte/embryo and the inner carrier. This means that contamination issues associated with LN_2 cannot be entirely avoided by using supercooled air, and the sterilization of LN_2 before evaporation is also recommended with this method.[29]

Solid surface vitrification

With these systems, vitrification is performed on the solid surface of a chilled metal block partially submerged in LN_2 (CryoLogic Vitrification Method—http://www.cryologic.com/cvm.htm). Following vitrification, specimens are inserted into a sleeve that is then fully heat sealed. Since the air around the block is composed of nitrogen vapor,[31] these systems require LN_2 sterilization as well.

Cryostorage

Currently, human cells and tissues are mainly cryostored in LN_2 or in nitrogen vapor (NV); such cryostorage is potentially hazardous because many pathogens can survive at the low temperature of LN_2/NV,[14,15,30,32–35] and they may contaminate the frozen cells or their carriers/container surface inside the cryobanks.[14,15,24,30,31,34–36]

To date, there have been no reported cases of disease transmission by transferred cryopreserved human embryos[35,37–39]; however, we have no specific studies regarding possible negative effects of LN_2/NV infectious agent contamination on the final outcome of IVF frozen cycles, although it is generally known that some of these microorganisms negatively affect gametes and embryonic development at warming.[15,40–42] In addition, vitrification is increasingly used for human cells, and this cryo-procedure appears to be riskier than slow freezing due to the direct contact between cells/tissue and LN_2 required for "open systems." The hypothetical risk of culture contamination at warming cannot be excluded even when using some "closed vitrification systems."[22,29,31]

Some precautions may be routinely used in IVF laboratories to minimize the risk of cross-contamination during cryopreservation. For example, cryostorage in hermetically sealed containers and the use of a secondary sleeve (straw-in-straw) is recommended for human specimens for both vitrification and slow freezing (Figure 10.1).[2,31,35,43,44] Cryostorage contamination may be avoided by storing the vitrified embryos in LN_2 vapor[45,46]: Cobo et al. have demonstrated that vitrified human oocytes can also be safely cryostored in LN_2 vapor.[47]

Periodic cleaning and refilling of cryo-dewars with sterile LN_2 (SLN_2) are additional precautions for minimizing the potential risk of cross-contamination; today, certified SLN_2 can be easily obtained through ultraviolet (UV) irradiation (Figure 10.2).[43,48]

Sterilization of LN_2 for vitrification via UV radiation

LN_2 is obtained by compressing gaseous nitrogen (N_2), which has a very low boiling point (–195.82°C). In its practical applications, LN_2 evaporates when released and

Figure 10.1 **Hermetical goblet for the cryostorage of vitrification carriers. A hermetical goblet made using some disposables that are commonly marketed for human cell cryostorage: Cryoflex, a polyethylene tube specifically designed for cryostorage in liquid nitrogen (Nunc, Roskilde, Denmark); a plastic Visotube for cryostorage (Cryo Bio System, L'Aigle, France); plus a stainless steel weight. The hermetical goblet must be prepared in a sterile environment (e.g., an IVF flow-hood).**

absorbs large quantities of heat, making it very effective as a coolant. Although LN_2 should have a low microbial content (in nature, very few microorganisms are able to survive at temperatures around –195°C), nevertheless the need to guarantee the absolute sterility of nitrogen is at present still felt in view of its critical applications in cryobiology. Many methods are used for obtaining clean LN_2 at the time of production, and companies that supply LN_2 can certify the level of purity of the LN_2 supplied. Owing to the particular composition of this liquid, however, it is not possible to seal the containers used to transport it, and consequently it is not possible to guarantee or certify the sterility of the LN_2 before it is actually used. Very often, between the time of leaving the manufacturer and the time of reaching the end user, the LN_2 passes through several hands, exposing it to risks of contamination. Furthermore, incorrect sanitizing or safety procedures during the handling of potentially infected biological material in the hospital laboratory or medical center where the cryogenic container is located may also lead to LN_2 contamination. It is therefore possible for the contaminated LN_2 to infect a biological sample through

Figure 10.2 **Specifically designed device for ultraviolet liquid nitrogen sterilization (Nterilizer—www.nterilizer.com).**

direct contact with it; for example, during specific cryopreservation procedures, or in the case of samples that are cryogenically preserved, in improperly sealed or damaged devices. It has also been demonstrated that LN_2 may be a potential source of infection for cryostored gametes and embryos.[14] Defects in sealing materials may cause contact of cryopreserved cells and tissues with LN_2.[15] Oocyte and embryo contamination by LN_2 may occur, for example, if any bacterial or viral particles in the LN_2 make contact with the exposed cryoprotectant and then, after thawing, attach to a cracked zona pellucida.[14,24]

It has been hypothesized that filtration and UV radiation of LN_2 may protect against contamination during the vitrification procedure.[46] The use of UV radiation to sterilize surgical material, work surfaces, and water or other liquids is widespread. UV radiation is sub-divided into UV-A (wavelength 315–400 nm), UV-B (280–315 nm), and UV-C (40–280 nm). UV-C is the most energetic and dangerous portion of UV radiation. It can cause electronic excitation, leading to the breaking of chemical bonds and the formation of unpaired electron species, known as radicals.[49] These chemical species are highly reactive and can damage DNA and cell replication, in this way deactivating the growth of all kinds of microorganisms, from viruses to fungi.[50] With some liquids, depending on their specific physical and chemical composition, the UV radiation may be absorbed before it can reach the microorganism to deactivate it. For homonuclear molecules such as N_2, the absence of a dipole moment permits electronic absorption only in the far UV spectral region (wavelength <145 nm). Hence, N_2 is largely transparent to infrared and visible radiation. The radiations from the most common commercial UV sources, usually working at wavelengths between 185 and 366 nm, are not involved in the N_2 absorption process. Since in the case of small molecules such as N_2 no significant spectral difference transpires from the absorption spectra obtained in liquid and gas phases,[51–53] it can be hypothesized that direct UV sterilization is applicable to LN_2. However, even though UV sterilization is used for liquids similar in composition to water, this method is difficult to apply to LN_2, which evaporates rapidly. The decontamination of LN_2 via UV irradiation comprises calculating the minimum irradiation time necessary to kill the microorganisms that are resistant in LN_2. This calculation takes into account that the rate at which the microorganisms are deactivated is related to the quantity of LN_2 to be sterilized, and depends on the physical properties of the container in which the LN_2 is located; the LN_2 released in a container absorbs large quantities of heat and evaporates, and it is desirable to calculate irradiation times in such a way as to complete irradiation before the nitrogen evaporates completely. Since the rate at which the microorganisms are deactivated depends on the efficiency of the UV irradiation system, which is in turn inversely proportional to the temperature at which the irradiation system operates, it is important to check the efficiency of the system by measuring the temperature of the bulb wall of the lamp so as to calculate its relative UV output, even in the presence of the highly refrigerating effect induced by the evaporation of the LN_2. The relative UV output is required to calculate the time of irradiation (T = UV dose/I; T stands for residence time and I for UV intensity). The lamp output, at various bulb wall temperatures, is usually described by the manufacturer of the UV germicidal lamp.

In a 2010 study,[54] a method for sterilizing LN_2 was proposed based on emitting the minimum dose of UV radiation necessary to kill microorganisms that can survive at the boiling point of nitrogen (–195.82°C), and which is irradiated in a temperature-controlled regimen, within a short time interval, before the LN_2 completely evaporates. This study described an experimental microbial contamination and disinfection via UV irradiation of LN_2, with the irradiation being suspended after the administration of the minimum UV dose needed to deactivate the most resistant microorganism inoculated in the open dewar (*Aspergillus niger*: 330,000 UV dose). This study confirmed that the microorganisms used in this experiment were able to survive in LN_2, but showed that decontamination of LN_2 via UV irradiation is feasible and simple. Considering that UV radiation deactivates the growth of all kinds of microorganisms, from viruses such as hepatitis (which requires 8000 UV dose) to fungi like *Aspergillus niger* (330,000 UV dose),[50] this technique

allows UV–SLN_2 to be easily obtained for every use, and in particular for a safe vitrification procedure.[54]

Three-wash procedure in sterile-certified LN_2

A reliable procedure exists to decontaminate frozen human specimens before warming.[55] This procedure consists of washing the specimens with SLN_2, and this has been shown to efficiently decontaminate vitrification carriers in extreme experimental conditions. This procedure could be routinely performed in IVF laboratories for the safe thawing of human specimens that are cryostored in "non-hermetical" cryocontainers, particularly in the case of "open" or "single-straw closed" vitrification systems.

Regulations and quality assurance

Hypothetical cell/tissue contamination by LN_2/NV requires us to guarantee the sterility of vitrification procedures, particularly in Europe, due to the directives on tissue manipulation (European Union Tissues and Cells Directive EUTCD: 2004/23/EC, 2006/17/EC, and 2006/86/EC). These directives have been issued by the European Parliament in order to increase the safety and quality of tissues—including reproductive cells—processed for human re-implantation, through the control of equipment, devices, and environment. Similar regulations may be introduced by the Food and Drug Administration at some point in the future. Such requirements will be applicable to all assisted reproductive centers in the United States.[37] Thus, both in Europe and potentially in the United States, human reproductive cells are to be treated in the same way as other nonreproductive tissues. For this reason, even though Pomeroy et al. considered the cross-contamination of infectious agents to be a negligible risk,[37] and the majority of cryobiologists and embryologists maintain that vitrification with open systems using nonsterile LN_2 is a safe practice, international regulations and quality assurance require specific procedures in embryo/oocyte/ovarian tissue cryopreservation in order to avoid any hypothetical contamination of human cells through direct contact with accidentally contaminated LN_2.

OPTIMIZATION OF CRYOPRESERVATION

Intracellular "Glassy State"

The final goal of cell cryopreservation protocols is to convert the cell to a "glassy state" without ice crystal formation, to avoid cryoinjury.[56,57] This occurs despite the behavior of different cryopreservation media when cooled below the solidification point; in fact, the medium used for vitrification appears "glassy," whereas the slow-freezing medium is "iced" due to the presence of ice crystals. As Katkov puts it, "Slow freezing is just a method of intracellular vitrification with ice being present in the extracellular compartment."[58]

For this reason, since both vitrified and slow-frozen reproductive cells/tissues have a vitrified cytoplasm, it can be hypothesized that the same warming protocol can be potentially used regardless of the freezing protocol.[59] Furthermore, in any vitrification protocol or any "vitrification–warming ready-to-use" kit, the concentration of the nonpermeating cryoprotectant in the first warming solution is 1.0 M, irrespective of the concentration and types of cryoprotectants used.[60] This suggests that a single "universal warming protocol" via vitrification warming solutions can potentially be used to warm any reproductive cell/tissue, thereby simplifying the laboratory protocol.

"Universal warming protocol" via vitrification–warming solution

It is possible to warm slow-frozen cells by using the same warming solutions as used in vitrification protocols, which may also increase their survival rates. This has been demonstrated in a study on sibling oocytes randomized for conventional rapid thawing, or rapid warming via vitrification–warming solution.[59] The study assessed the survival at 2 hours, and also examined some of the surviving oocytes for parthenogenesis. The survival rate was significantly higher (90.2%) in rapidly warmed oocytes than in rapidly thawed oocytes (74.6%), and rapidly warmed parthenotes grew faster.

An interesting observation of this study was that, during warming, slow-frozen oocytes in the rapidly warmed group displayed behavior typical of vitrified oocytes; as soon as the oocytes were expelled from the straw, they tended to float and appear "vitreous" in 1.0 M sucrose solution. They subsequently shrank, and then progressively recovered their original shape (see Figures 10.3 and 10.4).[60] For this reason, it seems critical to expel the oocytes into the first warming solution at 37°C as soon as the external ice around the straw has melted, in order to

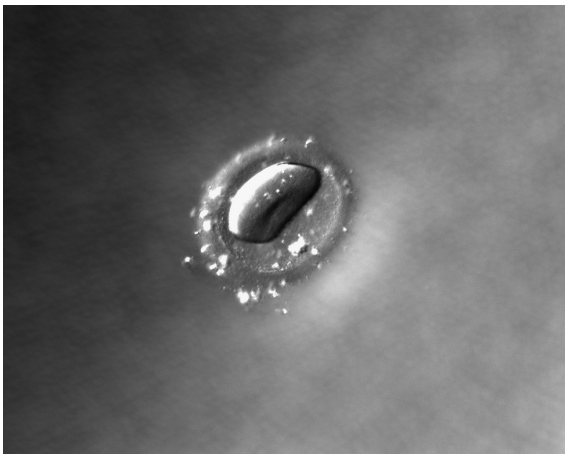

Figure 10.3 **Slow-frozen oocyte in 1 M sucrose. During the rapid warming procedure, the oocyte is expelled into 1 M sucrose at 37°C still shrunken; its rehydration occurs within 1 minute.**

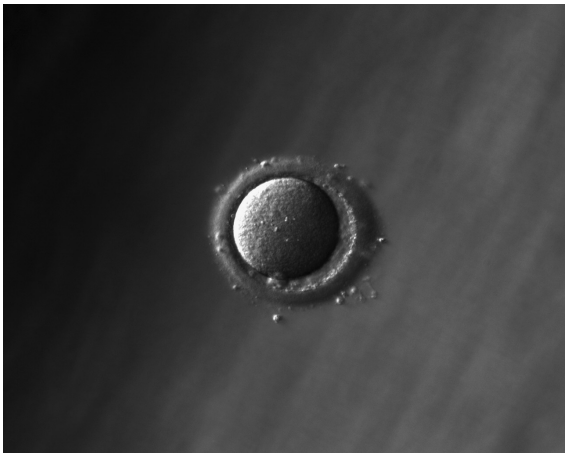

Figure 10.4 **Slow-frozen oocyte in washing solution at the end of a "universal warming procedure." After the first step in 1 M sucrose, the oocyte is moved into 0.5 M sucrose, where it shrinks again; subsequently, it is moved into a washing solution, where the oocyte completely recovers its original cell shape. The whole procedure takes 10 minutes.**

avoid or minimize fluid exchanges between oocytes and melted holding medium inside the straw.

Thus, it is possible to increase the survival of slow-frozen oocytes by using this warming protocol, and to potentially obtain results comparable to vitrification; these results were confirmed in a wider multi-center study.[61,62] Since the warming procedure is viable for oocytes, which are the most sensitive cells with the highest risk of cryoinjury, it is also potentially applicable to other less fragile reproductive cells frozen via slow freezing, such as zygotes or embryos at any stage of cleavage (from two cells to blastocysts). A further study, performed on slow-frozen embryos, confirmed the positive effect on blastocyst development of the rapid warming protocol.[63]

Furthermore, since in any protocol—or in any "ready-to-use" commercial kit—vitrification–warming solutions have the same concentration of extracellular cryoprotectants, these solutions can be used for the warming of any reproductive cell irrespective of the freezing protocols, thus streamlining laboratory activity and potentially reducing costs.

DISCUSSION

Vitrification has moved from bench to bedside, and is emerging as the preferred cryopreservation method for human reproductive cells/tissue, especially for oocytes, zygotes, cleavage-stage embryos, and blastocysts.

Regarding the risk of cell/tissue contamination through direct contact with LN_2/NV, any technique preventing hypothetical contamination must be welcome. A vitrification system that avoids any risk of contamination may be useful not only for reproductive cells/tissue but, in the future, also for other human specimens, even including whole organs. In current directives worldwide, there are no specific indications against direct contact between human specimens and LN_2/NV; for this reason, vitrification "open systems" can comply with any existing directive, as long as aseptic procedures during vitrification–cryostorage–warming are established.[31,44,45]

Despite the increase of vitrification worldwide, to date, many slow-frozen oocytes/embryos/ovarian tissue specimens have already been stored in IVF cryobanks. Existing studies of a "universal warming procedure" confirm that this increases the efficiency of slow freezing and enables survival rates comparable with vitrification, while optimizing costs and simplifying laboratory routines by using the same single "universal warming protocol."

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11 Physiological aspects of oocyte vitrification

Mark G. Larman and David K. Gardner

INTRODUCTION

It has been nearly 30 years since the birth of the first child conceived through the fertilization of a frozen human metaphase II (MII) oocyte.[1] Within the last decade, success rates and implementation of oocyte vitrification have increased dramatically. There remains, however, room for improvement. For example, why do oocytes from 10% to 20% of donors in a given donation cycle fail to tolerate the cryopreservation procedure? What negative impact does vitrification have on oocyte physiology that results in a much sharper age-related decline in clinical outcome compared to fresh oocytes?

For the most part, methods for oocyte cryopreservation have been derived by empirical approaches using survival, fertilization, and in some cases embryo development as the criteria to assess efficacy. This approach has certainly helped improve cryopreservation techniques, but provides minimal information on the physiology of the oocyte and how cryoprotectants and cryopreservation procedures affect cell function. Analyses of the effects of cryopreservation on oocyte physiology provide the opportunity to create a greater understanding about the impacts of these techniques, and to subsequently improve the results.

MEIOTIC SPINDLE

The maternal chromosomes are held suspended close to the cortex of the MII arrested oocyte by a network of microtubules, called the meiotic spindle. A correctly formed spindle and aligned chromosomes are required for the physical segregation of the chromosomes during second polar body extrusion. An intact spindle is also the site for the spatial downregulation of the M-phase promoting factor following fertilization.[2] Therefore, any disruption to the spindle and its function can also affect completion of meiosis, subsequent development, and viability, including the induction of aneuploidy.

Interestingly, the spindle is not a steady-state structure. Rather, tubulin monomers are in dynamic equilibrium, being constantly added and lost to each individual tubule. Cooling, cryoprotectants, osmotic stress, and cryopreservation itself have all been shown to induce microtubule depolymerization,[3–5] with the most likely explanation being that they affect the balance of tubulin turnover. Many studies have investigated the effects on the meiotic spindle of MII oocytes through fixation, labeling of the spindle and chromosomes with fluorescent-conjugated antibodies, and subsequent visualization through confocal microscopy. Although facilitating high-resolution images, this technique does not allow monitoring of the dynamic process within the same oocyte.

Polarized light microscopy in combination with imaging software algorithms has made it possible to image the meiotic spindle of the living and unfixed metaphase II oocyte continually, without compromising viability.[6] Rienzi and colleagues[7] used this technique to demonstrate that the spindle disappears during the thawing stages following slow freezing, but reforms in the majority of oocytes within 3 hours. This is not unsurprising considering exposures to the freeze and thaw solutions during slow freezing are carried out at room temperature. Not every study has demonstrated that the spindle is highly sensitive to temperatures lower than 37°C. Tamura et al. demonstrated that mouse oocytes maintained their spindle for up to 2 hours at room temperature, which is in contrast to many other studies.[8] Exposing the oocytes to the vitrification solutions at room temperature, however, caused depolymerization, and a loss of microtubule-organizing center proteins. Furthermore, this effect was independent of the cryoprotectants used. As the oocytes were warmed, it appears that they underwent excessive polymerization initially, but 2 hours after warming, the meiotic spindle had normalized in terms of shape and size. It seems that this recovery period is critical for subsequent survival following parthenogenetic activation, as oocytes activated before 2 hours had significantly lower survival rates.

When human oocytes were vitrified using a protocol that involves exposure to the vitrification (and warming) solutions at 37°C, the meiotic spindle remained intact (Figure 11.1).[9] This means that intracytoplasmic sperm injection (ICSI) can be carried out soon after warming, avoiding a long spindle recovery time that has been observed with slow freezing,[10] ensuring that fertilization occurs in a timely manner, and eliminating issues associated with oocyte aging. The spindle is maintained or recovers more quickly with vitrification compared to slow freezing.[9] It does appear, however, that the faster reduction in cell volume with vitrification disrupts the position of the polar body in relationship to the spindle.[11] Although it might be difficult to disrupt the spindle during microinjection with ICSI, care should be taken, as the polar body might not indicate the position of the meiotic spindle following vitrification.

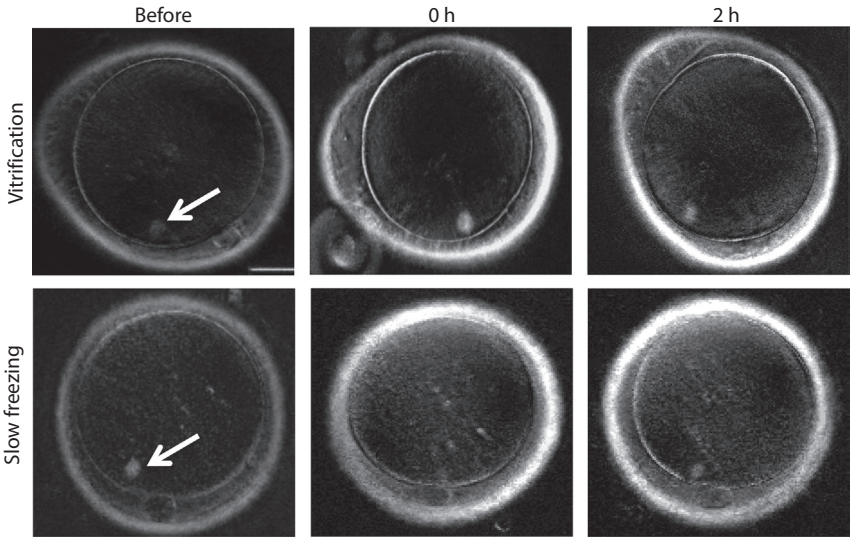

Figure 11.1 **The effect of cryopreservation on the meiotic spindle of human MII oocytes. Polarized light microscopy (Oosight™) was used to image the meiotic spindle before vitrification/freezing, immediately after warming/thawing, and after recovering for a further 2 hours. The vitrification and warming protocol was performed at 37°C, which probably explains why the spindle remained intact. Following slow freezing, it took up to 2 hours for the meiotic spindle to reform.**

It is still unclear how effectively the chromosomes maintain or reform their proper alignment following vitrification. A study of oocytes from donor patients (mean age 25.1 years) reported that vitrified oocytes had bipolar spindles, and equatorially aligned chromosomes 2–3 hours after warming,[12] whereas Coticchio and colleagues[13] reported a significantly lower rate when compared to fresh oocytes within 1 hour of warming in older age patients (mean age 38.5 years). The differences in patient age and time after warming make it difficult to draw solid conclusions, but perhaps this is one possible explanation for the decrease in results following vitrification of oocytes from older patients.[14] There are some reassuring data, however, that show that oocyte vitrification does not increase the risk of aneuploidy.[15]

INTRACELLULAR CALCIUM INCREASE AND "ZONA HARDENING" DURING OOCYTE CRYOPRESERVATION

Intracellular calcium is a ubiquitous second messenger.[16] It is not only involved in many somatic cell signaling pathways, but is pivotal in the fertilization and early embryo development of many species, including mammals.[17–21] Studies on human oocytes have been somewhat limited and, in most part, used *in vitro*-matured oocytes.[22–25] To the authors' knowledge, there is only one study measuring intracellular calcium in fresh MII human oocytes.[26] In these studies, it did not matter whether *in vitro* fertilization (IVF) or ICSI was performed, as both lead to increases in intracellular calcium. Upon fusion of the spermatozoon with the oocyte, a sperm-specific phospholipase C (PLCζ) is introduced into the cytosol of the oocyte, triggering a series of calcium oscillations.[27,28] In all mammalian oocytes, the protein triggers an initial transient intracellular calcium rise, followed by a series of shorter-duration calcium oscillations until pronuclei form. In human oocytes, the initial increase in calcium lasts approximately 3–5 minutes, with shorter-duration oscillations (2–3 minutes) occurring every 15–30 minutes.[26] It has been known for many years that the spatio-temporal profile of these calcium oscillations affects mammalian embryo development and viability.[29–34] The downstream events are still not fully elucidated, but it is clear that the calcium increases are integral in initiating and controlling a number of signaling pathways.[35–38]

The initial calcium increase causes cortical granules to fuse to the plasmalemma, release their contents into the adjacent perivitelline space, and therefore alter the zona pellucida. The proteolytic enzymes from the cortical granules target sperm binding proteins, preventing further sperm from binding, and thus blocking polyspermy. This process is referred to as zona hardening. The subsequent calcium increases are necessary for embryo development, triggering the resumption of meiosis by regulating cell cycle proteins. Embryo activation and embryo development can even be triggered without sperm by mimicking the intracellular calcium increases, demonstrating that they are necessary and sufficient to initiate embryo development. Interestingly, the calcium oscillation profile in cryopreserved human oocytes is

different compared to nonvitrified oocytes.[26] Both vitrified and slow-frozen human oocytes exhibit decreased frequency and increased duration. A change in the calcium oscillation pattern could be explained by a number of mechanisms. For example, in porcine oocytes, vitrification causes a decrease in the calcium release channel (inositol 1,4,5-trisphospahte receptor), which would negatively affect the calcium oscillations following subsequent fertilization.[39]

As with fertilization, it appears that zona hardening, which occurs during cryopreservation, is induced by an increase in calcium. It has been determined that it is the cryoprotectants, and not the physical aspects of cryopreservation, which cause the calcium increase.[40–44] Vincent and colleagues were the first to demonstrate that dimethyl sulfoxide (DMSO) causes zona hardening and consequently reduces fertilization in mouse oocytes. This was not a direct effect on the zona pellucida, as the presence of the oocyte itself was required.[40,41] Takahashi et al. demonstrated ethylene glycol (EG) causes an increase in intracellular calcium of mouse oocytes.[43] Reducing the increase in calcium with an intracellular calcium chelator decreased the cytotoxic effects of EG during long-term exposure. Larman and colleagues went on to demonstrate that EG, DMSO, and 1,2-propanediol (PrOH) all induce increases in intracellular calcium.[44,45] By removing extracellular calcium from the medium, it was possible to reduce the amount of calcium released by EG and PrOH. The response to DMSO was not affected by removing extracellular calcium from the medium, suggesting that it enters the oocyte and releases calcium most likely by disrupting the membranes of intracellular calcium-containing organelles, such as the endoplasmic reticulum and mitochondria. This direct intracellular effect of DMSO on calcium makes it stand out from other cryoprotectants, and raises concerns about its ability to alter intracellular signaling pathways.

It has been shown that the calcium increase caused by cryoprotectants during cryopreservation procedures is enough to induce zona hardening.[44,46] Using proteolytic dissolution of the zona pellucida, it was possible to measure the level of hardening. Figure 11.2a shows that the zona pellucida of vitrified mouse oocytes takes much longer to undergo dissolution by chymotrypsin than nonvitrified oocytes. If the vitrification is performed in calcium-free media, there is a significant decrease in the time it takes to dissolve the zona pellucida. The increase in intracellular calcium induced by the cryoprotectants is not only enough to cause zona hardening, but is also sufficient to significantly reduce fertilization (Figure 11.2b). Vitrified mouse oocytes have a lower fertilization rate compared to nonvitrified oocytes. Performing the vitrification in calcium-free media significantly increases the percentage of fertilized oocytes. To confirm that the reduction in fertilization of the vitrified oocytes was due to hardening of the zona pellucida, a small hole was created using a laser. By circumventing the zona pellucida, thus permitting direct access for the sperm, fertilization rates were restored.

In rat oocytes, it has been shown directly using confocal microscopy that exposure to vitrification media containing EG and DMSO caused an increase in cortical granule release, and that even more release occurred following vitrification.[47] Calcium-free vitrification media significantly decreased cortical release, and subsequently increased the fertilization rate in rat oocytes. Similarly, an increase in fertilization was observed by the same group when calcium-free vitrification media were used for mouse oocytes.[48] Although the majority of studies have been performed on other mammalian species, it appears that the human oocyte is also affected in the same way. There are a number of reports demonstrating that there is a decrease in cortical granules in cryopreserved human oocytes.[49–57] As with rodent oocytes, it appears that the release of cortical granules can also be triggered by cryoprotectants.[49,58]

ICSI provides a means to circumvent issues with zona hardening during oocyte cryopreservation. Assisted fertilization does not, however, eliminate the fact that the oocyte has seen a large increase in intracellular calcium and has initiated activation signaling pathways before the sperm has entered the oocyte. Could this explain some of the issues observed with oocyte cryopreservation? Such a study would be quite difficult to perform, but the literature certainly indicates that cryoprotectant-induced calcium increases occur in oocytes from different mammalian species.

An investigation into the concentration of calcium within the vitrification media was performed with ovine oocytes.[59] Calcium- and magnesium-free media with fetal calf serum significantly increased survival and embryo development rates when compared to a calcium-containing medium. Calcium-free media also significantly reduced parthenogenetic activation following exposure to the vitrification solutions. The negative impact of cryoprotectant-induced calcium increases are further highlighted by the fact that exposure of mouse oocytes to 1.5 mM PrOH for 4 or 10 minutes caused almost 40% and 60% of the oocytes to degenerate, respectively. In the absence of calcium, however, survival was 100%.[44]

ANALYSIS OF GENE EXPRESSION

Relatively little work has been performed on the effects of cryopreservation on the stability of mRNA and/or gene expression in oocytes. Studies comparing the efficacy of DMSO with EG have revealed that DMSO appears to have a greater detrimental effect on gene expression. For example, in mouse MII oocytes, DMSO was found to have a greater effect on the upregulation of aquaporin 7 expression than either EG or sucrose.[60] It was proposed that rather than affecting gene expression as a

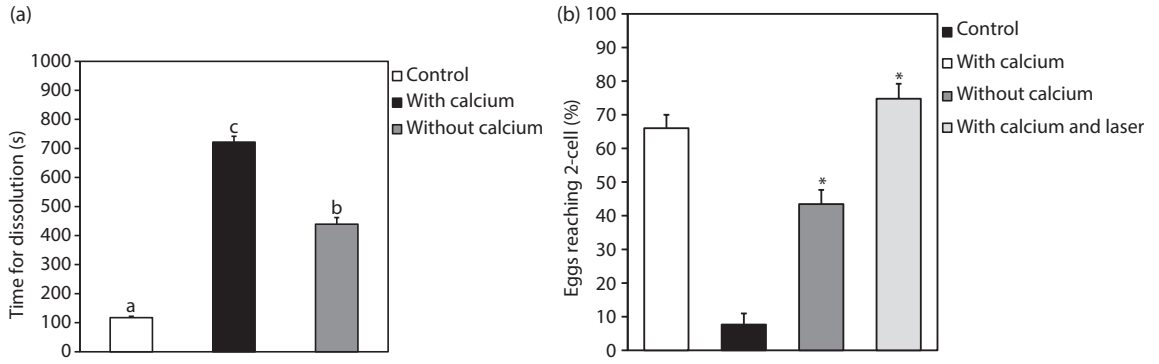

Figure 11.2 **The effect of vitrification on zona hardening and fertilization. (a) Vitrification causes hardening of the zona pellucida (ZP) of mouse MII oocytes. To assess the degree of *zona* hardening, oocytes were exposed to a 1% chymotrypsin solution to determine how long it would take to dissolve the ZP. The more cortical granule release the oocyte had experienced, the more resistant the ZP would become to enzymatic digestion. The time taken for the ZP to disappear was recorded for each treatment. Vitrification of the oocytes in calcium-containing solutions significantly increased the time taken for the ZP to disappear compared to the control (nonvitrified) oocytes. Vitrifying the oocytes in solutions that did not contain calcium significantly reduced the time for the ZP to disappear compared to vitrification with calcium-containing solutions. Different letters indicate a significant difference between treatments ($p < 0.01$). (b) The extent of zona hardening can also be examined by observing the degree of fertilization. Those oocytes with increased zona hardening will have reduced rates of fertilization. The ability of the sperm to bind, penetrate, and fertilize the oocyte was assessed by recording the number of two-cell stage embryos that were present 24 hours after insemination. Fertilization of oocytes vitrified with calcium-containing solutions was significantly reduced compared to control (nonvitrified) oocytes. A significant increase in fertilization was observed with oocytes that were vitrified with solutions that did not contain calcium ($p = 0.001$). To demonstrate that the reduction in fertilization was due to hardening, oocytes were vitrified with calcium-containing solutions and then the ZP was breached with a laser to allow direct access for the sperm.**

result of increased osmolarity, DMSO acted directly to induce changes in gene expression. Consistent with these observations, when immature bovine oocytes were vitrified with EG together with either DMSO or PrOH, it was found that the inclusion of DMSO had a significant negative impact upon the ability of oocytes to mature *in vitro*.[61] Analysis of mRNA transcript abundance in sheep oocytes revealed that when high concentrations of cryoprotectants were employed (20% EG together with 20% DMSO), there was a significant decrease in gene expression and oocyte competence.[62] The significance of this work was emphasized in a subsequent study by Habibi and colleagues on the mouse MII oocyte.[63] It was determined that the expression of *Sod1* was affected by vitrification. What is of interest is that using a total of 30% cryoprotectants (15% EG plus 15% DMSO) in the vitrification solutions had a much greater negative effect compared to the use of a total of 15% cryoprotectants. It was not determined whether there were differences between the two cryoprotectants used, but evidently, concentration of cryoprotectant influences the gene expression of the oocyte, indicating that it would be prudent to work at the lowest possible effective concentrations, and plausibly avoid DMSO. The proposed direct actions of DMSO on gene expression, similar to its action on calcium-storing organelles, reflect its high degree of solubility and penetration.

In a study by Di Pietro and colleagues, using 25 donated human MII oocytes and a total of 30% cryoprotectant, it was determined that vitrification did not affect the molecular profile, nor was it associated with the degradation of mRNA.[64] In a subsequent analysis of human MII oocytes that had failed fertilization through ICSI, Monzo and colleagues compared the impact of slow freezing or vitrification on gene expression signatures.[65] Both forms of cryopreservation were associated with altered gene expression compared with the noncryopreserved controls. While slow freezing induced a downregulation of genes connected to chromosomal structure maintenance and cell cycle regulation, vitrification induced the downregulation of genes associated with the ubiquitination pathway, comprising genes of ubiquitin-specific peptidases and subunits of the 26S proteasome. The latter was hypothesized to result in stabilization of the proteins required for oocyte function and viability, and may therefore go some way to explaining the greater efficacy of vitrification. Similarly, Chamayo and coworkers examined the impact of slow

freezing and vitrification on donated MII human oocytes, and determined that the freezing process was associated with a significant decrease in mRNA content, with only 39.4% of the mRNA being preserved. In contrast, after vitrification, 63.3% of the mRNA remained, indicating that vitrification imparts less molecular injury than slow freezing.[66]

In summary, vitrification appears to have less of an effect on gene expression in the mammalian oocyte than slow freezing, and molecular trauma is related to the concentration of cryoprotectants used.

METABOLISM AND ENZYME LEAKAGE

The metabolism of embryos is linked to their development in culture and subsequent viability post-transfer.[67–69] Stress upon the embryo usually results in compromised metabolic function, which in turn affects developmental potential.[69,70] Typically, when considering environmental stress, one thinks of pH changes, oxidative stress through the use of atmospheric oxygen in the culture system, temperature drifts, and suboptimal media formulations.[71] However, a further source of stress to oocytes is cryopreservation, where exposure to cryoprotectants, together with shifts in temperature, can impair metabolic function. Of note, all studies to date have determined that the negative effects of cryopreservation are greater for slow freezing than for vitrification.

The majority of metabolic studies have been performed on embryos, but it is likely that similar parallels can be drawn for the oocyte. The negative impact of cryopreservation on embryo metabolism was initially demonstrated in bovine embryos. The metabolic rate of blastocysts (measured by glucose consumption) following slow freezing using glycerol and sucrose was significantly decreased compared to levels before cryopreservation.[72] Subsequently, Lane et al.[73] demonstrated that slow freezing of two-cell mouse embryos induced a greater decrease in oxidative metabolism than vitrification, indicating potential damage to the mitochondria, plausibly through alterations in membrane integrity and structure. In a subsequent study on human cleavage-stage embryos, Balaban and colleagues revealed that not only was vitrification associated with higher embryo survival, but that vitrification also had significantly less of an effect on metabolic activity than slow freezing.[74] Day 3 human embryos that underwent slow freezing had a significantly lower pyruvate uptake than those embryos that had undergone vitrification, again reflecting possible disturbances to mitochondrial function.

Similarly, with regard to the oocyte, it has been shown that slow freezing of mouse oocytes resulted in a significant decrease in the uptake of the central nutrient pyruvate.[75] Although vitrification was also associated with a decrease in nutrient utilization by the oocyte compared to noncryopreserved controls, the decrease was significantly smaller than that induced by slow freezing, indicating that vitrification imparts less stress on the cell.[75] Salvetti and coworkers determined that although vitrification of rabbit oocytes resulted in a loss of intracellular ATP, if oocytes underwent slow freezing, then the loss of ATP was even more significant.[76] It is therefore feasible that using metabolic parameters will assist in identifying those treatments that have the least impact on such a central cellular function of the oocyte. As adequate energy metabolism is fundamental to numerous cell processes, it would not be prudent to select a treatment that had a negative impact on it.

One of the main negative effects of cryopreservation is damage to the plasma membrane of the cell. By quantifying the appearance of oocyte-derived proteins in the medium, it would be possible to indirectly assess membrane damage. As lactate dehydrogenase (LDH) comprises a significant amount of the total protein of the oocyte (5%),[77] quantification of LDH in the medium surrounding the oocyte makes an excellent marker of membrane integrity. It has been revealed that while slow freezing of mouse two-cell embryos results in the release of significant amounts of LDH into the medium, vitrification has almost no impact on this parameter, indicating that the damage to cell membranes is greater with slow freezing than with vitrification. Together with the data on nutrient utilization, it is tempting to speculate that slow freezing has the capacity to directly affect membranes, which are particularly sensitive to environmental perturbations. The exact aspect of the slow-freezing process that is responsible for the observed effects on oocyte and embryo metabolism has yet to be determined.

PROFILING THE OOCYTE PROTEOME USING SURFACE-ENHANCED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

Proteomic analysis of oocytes and embryos has previously been hindered by the large sample sizes required to perform standard protein analyses, such as 2D polyacrylamide gel electrophoresis. Furthermore, proteins with high or low molecular masses, or those that are either acidic or hydrophobic, can be under-represented on such gels. Recent developments in the field of mass spectrometry have facilitated the analysis of specific protein expression patterns that reflect different biological states.[78,79] Subsequently, it has been possible to use surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) to determine the proteome of small groups ($n = 5$) of oocytes and embryos.[44,80,81] Consequently, it is now possible to obtain, through the use of different protein chip types, a comprehensive profile of proteins in oocytes under different conditions, and relate this to the proteome of an *in vivo*-ovulated oocyte or *in vitro*-generated embryo.

Analysis of mouse MII oocyte protein profiles through SELDI-TOF MS following cryopreservation using either

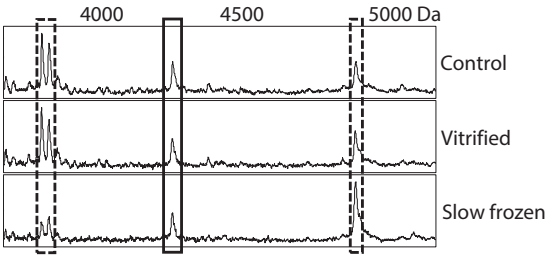

Figure 11.3 **Protein expression (3500–5500 Da) in mouse MII oocytes following cryopreservation. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry was used to investigate the effect of cryopreservation procedures on the proteome of mouse oocytes. The line plot shows that the expression level of one protein remained unchanged following cryopreservation (solid box). It was found that slow freezing affected the protein expression profile more than vitrification. In this example, slow freezing caused up- and downregulation of proteins (dashed boxes). (Modified from Gardner DK et al.** ***Theriogenology*** **2007;67:64–72.)**

slow freezing (with PrOH) or vitrification (using EG and DMSO) determined major alterations when oocytes were cryopreserved using conventional freezing techniques. Figure 11.3 shows line plots from the SELDI-TOF MS; whereas vitrified oocytes appear to be similar to noncryopreserved control oocytes, oocytes that underwent slow freezing exhibit markedly different protein profiles. A subsequent analysis of the slow-freezing procedure identified the dehydration of the oocyte, prior to seeding, as the time when alterations in the proteome are manifest. Further analysis revealed that rather than cooling from room temperature to –7°C, it is the chronic exposure to PrOH that is responsible for the aberrations in the proteome.[44]

Such observations are concordant with similar studies on embryos. A fitting example is that there certainly seems to be less cumulative damage following multiple vitrification procedures of mouse embryos.[82] Using vitrification, mouse embryos could be vitrified four times without loss of development in culture or implantation potential, though fewer fetuses developed (31.5%) compared to the noncryopreserved controls (51.7%). In contrast, mouse embryos could not survive three rounds of successive slow freezing.

CONCLUSIONS

From the literature discussed above, it appears that vitrification procedures have significantly fewer effects on oocyte physiology than those slow-freezing protocols tested, which supports its preferential use for oocyte cryopreservation. Thus, analyzing oocyte physiology assists in optimizing and/or choosing cryopreservation techniques. Such investigations will also help to determine whether there are any other variables that influence outcomes. For example, does the hormone stimulation protocol affect the ability of oocytes to withstand vitrification? Investigating the effect of cryopreservation on the meiotic spindle has demonstrated that 2 hours of recovery after warming is adequate to ensure that the spindle is intact. A recovery time might not actually be required if a protocol is used in which both vitrification and warming are performed at 37°C, which appears to maintain the spindle. Using the same analysis, it has been shown that the polar body can move during vitrification, so there could be an advantage in using polarized light microscopy to visualize the location of the spindle during ICSI, or even determine whether a meiotic spindle is present, and therefore if the oocyte is suitable for vitrification at that time. Further, it has been revealed in a number of mammalian species that performing vitrification in calcium-free media improves results. The ability to perform ICSI with human oocytes does circumvent the zona hardening, but perhaps the precocious increase in intracellular calcium caused by the cryoprotectants is detrimental to some oocytes, and should not be discounted. Many of the published studies are on donor oocytes, so there is less known about the efficacy of oocyte vitrification with older patients. The clinical outcomes following oocyte vitrification decline more rapidly with patient age than with the known age-related decline observed with fresh oocytes. Are oocytes from older patients more susceptible to the negative effects of cryoprotectant-induced calcium increases? Physiological studies are difficult to perform with human oocytes because of the paucity of material, but by conducting such analyses, it is envisaged that protocols will continue to improve and minimize the aberrations induced by cryopreservation.

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12 Vitrification of oocytes: Imprinting and disturbance in spindle formation and chromosome segregation

Tom Trapphoff

THE MEIOTIC SPINDLE

Metaphase II (MII) spindles appear to be particularly vulnerable to environmental changes. Susceptibility of microtubules to cryoprotective agents (CPAs) has been shown, as well as to thermal, oxidative, and redox stress.[1–3] Albeit transient, cryopreservation interferes without doubt with the intracellular and extracellular homeostasis and therefore poses a risk for inducing spindle abnormalities, chromatid nondisjunction, fertilization errors, and abnormal mitotic divisions during embryonic development.

The meiotic spindle is a multi-subunit complex crucial for aligning and separating chromosomes during meiosis I and II.[4] Although the spindle apparatus consists of more than 100 different proteins, it is mainly composed of microtubules of α- and β-tubulin dimers. Microtubule polymerization originates at microtubule organizing centers (MTOCs) rich in γ-tubulin and pericentriolar material located at both spindle poles to anchor the chromosomes at the equatorial plate. Specific subcellular compartments containing accumulated transcripts and proteins necessary for spindle formation, epigenetic control, and chromosome alignment are also mandatory for proper spindle functionality. Prior to and subsequent to the onset of meiosis, these factors are recruited in a stage-specific manner to ensure chromosome segregation at oocyte to embryo transition. Thus, besides direct susceptibility of microtubules to cryodamage, alterations of crucial cytoplasmic components may also be induced by cryopreservation.

Visualization Techniques

Polarization light microscopy (PLM) is based on the detection of anisotropic structures, as incident light with a defined plane of vibration becomes twisted when it passes through an anisotropic/birefringent structure. Because the meiotic spindle is birefringent while the surrounding cytoplasm exhibits isotropic characteristics, PLM provides the opportunity to study spindles noninvasively before and after vitrification without staining. Commercially available polarization light microscopes utilize liquid crystals and optimized image processing algorithms, increasing image resolution and providing the possibility to quantify microtubule density as retardance.

Epifluorescence and confocal laser scanning microscopy (CLSM) can be used to obtain detailed images of intracellularly labeled structures, including the meiotic spindle. Three-dimensional image reconstruction of distinct optical sections enables assessment of chromosome alignment at the equatorial plate, which is infeasible by noninvasive PLM. A general drawback of fluorescence microscopy is the need for fixation of the oocytes, which eliminates time-lapse assessment before and after vitrification, fertilization, and embryonic development.

Invasive techniques are powerful tools for basic research, although they cannot be adapted to assisted reproductive therapy (ART) laboratories as an analyzing tool to select oocytes that are suitable for further clinical applications. PLM is limited to assessing the spindle quantity, but it provides a useful option for verifying the presence of a meiotic spindle prior to *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) in clinical daily routine.

Vitrification of MII Oocytes

Several studies focused on spindle integrity and chromosome alignment after cryopreservation in animal models and, to a lesser extent, also in humans. However, the results were not always consistent, supposedly due to multifactorial parameters (e.g., patient age, vitrification protocol, and recovery time following vitrification). Thus, the question whether and how the meiotic spindle and chromosome alignment is subjected to cryodamage has been discussed controversially during recent years and cannot be resolved by a simple "yes" or "no."

Studies concerning spindle integrity and chromosome alignment after oocyte vitrification were initially performed in animal models.[5–7] Although the microtubular system and cytoplasmic composition in non-human models differs from that of human oocytes, early studies revealed several important findings. Using either slow-rate freezing (SRF) or vitrification methods, spindle integrity appears to be altered directly after thawing/warming due to microtubule depolymerization and disorganization, but repolymerization and reorganization occurs later dependent on time, species, and applied protocols (Figure 12.1).[5,8–10] Chen and colleagues showed that two-thirds of open-pulled straw (OPS)-vitrified mouse MII oocytes had a disrupted spindle morphology immediately after warming, while the spindle was nearly restored after 1 hour of recovery (vitrification solution [VS]: 5.5 mol/L ethylene glycol [EG] and 1.0 mol/L sucrose).[5] These findings were

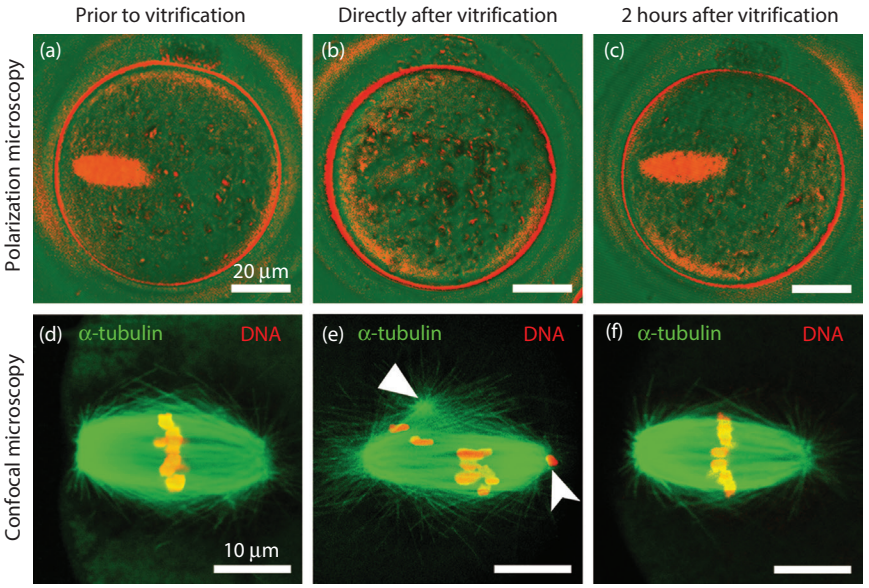

Figure 12.1 **(See color insert.) Spindle dynamics after CryoTop vitrification of murine metaphase II (MII) oocytes analyzed by noninvasive polarization light microscopy (a–c) or by confocal laser scanning microscopy (d–f) (Trapphoff T., unpublished data). Spindle characteristics prior to vitrification (a and d), directly after vitrification (b and e), and after recovery for 2 hours at 37°C (c and f). The kind of information obtained on spindle integrity differs between polarization light and fluorescence microscopy directly after vitrification and warming (b and e). Arrowhead: slightly aberrant spindle, arrow: displaced chromosome; both phenotypes occurred in about a third of *in vivo*-ovulated and vitrified murine MII oocytes directly after warming (unpublished data).**

confirmed in other model systems with different vitrification protocols and time intervals after warming.[6,10,11]

Additionally, it was demonstrated that spindle integrity depends on the applied vitrification protocol and the temperature during pre- and post-treatment. Exposure to VSs at 37°C had a profound effect on the development competence and a protective influence on the spindle structure in mice, bovines, and humans (but this increased CPA toxicity).[12–14] With respect to oocyte survival, spindle integrity, chromosome alignment, and developmental potential, minimal-volume carrier devices and equilibration in mixtures of CPAs at medium molarity, lowering the toxicity of each CPA without weakening their protective characteristics, affected the success of vitrification significantly.[15–17] For instance, grids, closed pulled straws, and OPSs preserved spindle integrity better than conventional straws due to faster cooling and warming rates (15,000–30,000°C/min versus 2500°C/min) in murines, while CryoTop vitrification appeared superior compared to OPS vitrification in bovines (VS: 20% EG, 20% dimethyl sulfoxide [DMSO], and 0.5 mol/L sucrose).[6,17] Current vitrification protocols rely commonly on mixtures of CPAs to lower their cellular toxicity, and also on small-volume vitrification methods to increase cooling and warming rates. Thus, the critical temperature window between 15°C and −15°C responsible for cryodamage is passed much faster, therefore reducing the potential damage.

Studies of human MII oocytes analyzing spindle morphology and chromosome alignment after vitrification still remain limited. Analysis of spindle retardance using noninvasive PLM after cryopreservation either by CryoLoop vitrification (VS: 16% EG, 16% propanediol [PROH], 0.65 mol/L sucrose, and 10 mg/mL Ficoll) or SRF (1.5 mol/L PROH and 0.1 mol/L sucrose) of human MII-stage oocytes demonstrated that vitrification affects the meiotic spindle only to a minor degree, while it was not preserved in slow-frozen oocytes.[12] Although in the study by Martínez-Burgos et al.[18] CryoTop vitrification, but not SRF, of human MII-stage oocytes tended to alter the angle between the meiotic spindle and the microfilament meshwork, superiority of vitrification compared to SRF regarding spindle morphology and recovery dynamics was suggested using noninvasive microscopy.[13,18]

PLM is restricted to analysis of spindle quantity, but not quality, and it was demonstrated that morphometric spindle evaluation is not consistent between polarization light and fluorescence microscopy (Figure 12.1).[19] Using CLSM, 27/43 (63%) of Cryoleaf (VS: 15% EG, 15% DMSO, and 0.5 mol/L sucrose)-vitrified human MII-stage oocytes exhibited a normal bipolar spindle with focused poles after

warming for 1 hour, compared to 22/22 (100%) from non-vitrified controls.[20] However, normal chromosome alignment at the equatorial plate and a bipolar spindle was found only in 14/43 (32%) vitrified MII oocytes, compared to 13/22 (59%) from fresh controls. This was also accompanied by an increased pole-to-pole distance.[20]

Studies using a comparable experimental setup showed that the proportion of morphologically normal spindles and well-aligned MII chromosomes was statistically not distinguishable in vitrified/warmed oocytes, compared to controls, after a recovery time of 2–3 hours.[21,22] Interestingly, the percentage of normal barrel-shaped spindles and well-aligned chromosomes was much higher in the vitrification group (51/62, 82%; VS: 15% EG, 15% PROH, and 0.5 M sucrose; Cryoleaf), compared to oocytes cryopreserved by SRF using 1.5 M PROH and 0.3 M sucrose (39/64, 60%) in the study by Cao et al.,[22] while there was no significant difference between vitrification (13/17, 76%) and SRF (19/23, 82%) in the study by Cobo.[21] Therein, chromosome alignment and the proportion of morphologically normal spindles was not distinguishable, compared to fresh controls either after vitrification (VS: 15% EG, 15% DMSO, and 0.5 mol/L sucrose; Cryotip) or after SRF using three different protocols (1.5 mol/L PROH supplemented with 0.2 or 0.3 mol/L sucrose, or with 0.3 mol/L sucrose and 137 mmol/L choline chloride). In accordance with the studies of Cobo and Cao, chromosome alignment (15/25, 60%) and spindle morphology (13/25, 52%) were not statistically different in *in vitro*-matured (IVM) and vitrified human MII-stage oocytes, compared to IVM controls ($20/31^{chrom}$, 64%; $17/31^{spind}$, 54%) in a study by Lei et al. (VS: 15% EG, 15% DMSO, and 0.5 mol/L sucrose; Cryoleaf).[23]

However, the post-warming time prior to spindle analysis and patient age (25 years,[21] 28 years,[23] and 38 years[20]) differed in the referenced studies. Oocytes from older patient groups exhibited a reduced recovery potential of the meiotic spindle compared to younger ones after cryopreservation, as demonstrated for instance after SRF.[9] As shown in animal models and after SRF of human MII-stage oocytes, spindle de- and re-polymerization exhibits dynamic characteristics, and the time point for spindle and chromosome analysis is crucial.[9,10] Although it should be noted that microtubule repolymerization is more effective in mouse oocytes exposed to cold stress than in human oocytes[3,24]; the cellular alterations that were reported after 1 hour of recovery may reflect only transient anomalies that may be restored after a prolonged recovery time, or may depend on patient cohorts that differ in age.

Overall, it is rather difficult to compare spindle quality among studies using different methodologies/parameters. Minor protocol modifications or different storage devices may have unpredictable downstream effects on spindle integrity and chromosome alignment. Therefore, it is necessary to test each cryopreservation protocol individually, and to interpret results (especially intermediate phenotypes) with caution. Nevertheless, normal spindle integrity and chromosome alignment was reported in a large subset of studies using different vitrification protocols, after an incubation period of 2–3 hours in both non-human and human oocytes. This strongly implies a negligible influence of vitrification on spindle integrity and morphology.

It is noteworthy that cryopreservation poses not only a risk for altering microtubule stability, but also interferes with proteins and mRNAs involved in cell cycle control and maintenance of the spindle. For instance, vitrification of murine MII oocytes caused a transient disappearance of the MTOC components pericentrin, NEDD1 and γ-tubulin directly after warming.[10] High-throughput analysis of gene expression profiles of vitrified human MII-stage oocytes suggested, moreover, that vitrification compromises gene expression and mRNA levels of cell cycle- and spindle-associated factors compared to fresh oocytes, although to a lesser degree than SRF.[25,26] Alterations at the molecular level may therefore induce unknown (long-lasting) downstream effects that potentially interfere with intracellular checkpoints, resulting in bypass of the spindle assembly checkpoint while not possessing an intact genome at later embryonic stages.

Chromosomal Constitution in Early Embryos and Offspring

Despite only transient changes (or intermediate phenotypes) occurring, lasting alterations during early embryonic development and in the offspring are still possible, especially when considering that early studies in mice using straw vitrification revealed an approximately threefold higher incidence of zygotes displaying aneuploidy, and an increased amount of malformed fetuses derived from vitrified oocytes.[27] In the limited number of studies on humans, no evidence for abnormal karyotypes in children born after vitrification of mature human oocytes has so far been found. Chen et al. reported one child with a normal 46,XY karyotype[28]; Song et al. reported four female and five male infants with normal karyotypes[29]; and Grifo et al., using array comparative genomic hybridization, reported one healthy, euploid male infant.[30] Similar results were reported in a follow-up study of 12 children born after SRF.[31] Additionally, animal models provide evidence for normal karyotypes in visually normal neonatal offspring after vitrification of mature oocytes.[32] Thus, results regarding chromosomal constitution in children born after vitrification of mature oocytes are reassuring, but abnormalities in early embryos leading to aborted pregnancies cannot be excluded. Pre-implantation genetic diagnosis using fluorescence *in situ* hybridization (FISH) revealed euploidy for chromosomes 13, 14, 15, 16, 18, 21, 22, and X in early cleavage-stage embryos obtained after OPS vitrification of human MII oocytes.[33] Trophectoderm biopsy using single-nucleotide polymorphism microarrays

showed no difference in the rate of embryonic aneuploidy after CryoTop[34] or Cryotip vitrification[30] of human MII-stage oocytes. Similar results were obtained after SRF of mature human oocytes.[35,36] Although the published data are reassuring, further work is needed to analyze the chromosomal status in early embryos and offspring derived from vitrified and warmed oocytes in long-term follow-up studies.

Vitrification of Immature Oocytes

Because of the sensitive nature of the MII spindles, vitrification of immature oocytes at the germinal vesicle (GV) stage followed by IVM represents an opportunity to avoid detrimental cryodamage to the spindle. To date, only a few births have been reported after cryopreservation of immature oocytes followed by IVM,[37] or vice versa.[38,39] Although the efficiency of IVM in animal models and especially in humans is still poor,[40] cryopreservation of immature oocytes represents an alternative strategy for preserving female fertility in patients for whom hormonal stimulation is not recommended, or in cases with leftover immature oocytes from stimulated cycles. Studies using SRF have shown that the spindle of IVM oocytes was compromised after cryopreservation, accompanied by delayed maturation, limited fertilization rates, and altered early embryonic development.[41–43] In terms of survival, maturation, development, and spontaneous activation rates, the use of vitrification protocols was reported to be superior compared to SRF of human oocytes.[44–46] Regarding spindle and chromosome patterns, Combelles et al. were able to show that IVM human MII oocytes vitrified at the GV stage (VS: 15% EG, 15% PROH, and 0.5 mol/L sucrose) exhibit a slightly but not significantly reduced number of bipolar spindles and properly aligned chromosomes compared to nonvitrified IVM controls.[44] Nevertheless, there were more bipolar spindles with some microtubule irregularities, and a tendency toward completely dispersed chromosomes, while morphometric parameters regarding spindle length, width, and microtubule volume were not different to controls. However, spindle irregularities and displaced chromosomes were less severe after vitrification compared to SRF (1.5 mol/L PROH, 0.3 mol/L sucrose, and 137 mmol/L choline chloride), while the general efficiency was low for both.

In animal models, normal spindle and chromosome alignment was shown after Cryoleaf (VS: 20% EG and 20% DMSO) and solid surface vitrification (VS: 17.5% EG, 17.5% propylene glycol, 0.3 M trehalose, and 50 mg/mL polyvinylpyrrolidone) of immature ovine and porcine GV oocytes followed by *in vitro* maturation,[47,48] while OPS vitrification of immature equine (VS: 20% EG, 20% DMSO, and 0.5 mol/L sucrose) and pig (VS: 40% EG) oocytes led to spindle abnormalities and misaligned chromosomes in IVM MII oocytes.[43,49] Irregularities reported in animal models may be caused by differences in CPA permeability, composition of CPA mixtures, or applied protocols.

Although some drawbacks are present and the number of studies is low, data regarding spindle morphology after vitrification of immature human oocytes followed by *in vitro* maturation are promising. Nevertheless, recent studies suggest that the overall efficiency was superior after IVM of immature GV-stage oocytes followed by vitrification when compared to the opposite strategy of vitrification followed by IVM. It was therefore concluded that immature oocytes should be vitrified after IVM at the MII stage, although the overall efficiency still remains relatively poor.[46,50] Protocol optimization and further studies are certainly needed to assess safety irrespective of the applied strategy.

Slush Nitrogen and Beneficial CPAs

Oocyte vitrification is commonly performed by plunging the carrier device directly into liquid nitrogen, with a cooling rate between 15,000°C and 30,000°C/min. The use of slush nitrogen increases the cooling rate up to 135,000°C/min.[51] Slush nitrogen avoids vaporization upon solidification ("Leidenfrost effect"), during which small gas bubbles around the specimen result in poor temperature transfer rates and cryodamage. It has been demonstrated that slush nitrogen can increase the clinical efficiency,[52] and it allows use of lower CPA concentrations to a level comparable to SRF protocols.[53] Using fast cooling by slush nitrogen, healthy meiotic spindles were present in nearly 90% of vitrified human denuded oocytes (VS: 5.5 M EG and 1 M sucrose; electron microscope grid).[51] In animal models, fewer adverse effects on the spindle morphology were also found in rabbit oocytes vitrified by slush nitrogen compared to plunging into liquid nitrogen (VS: 20% EG, 20% DMSO, 0.65 mol/L trehalose, and 10 mg/mL Ficoll).[15] Furthermore, the composition of vitrification and devitrification media also affects spindle integrity. Supplementation with hydroxyapatite nanoparticles, synthetic or organic polymers, and anti-oxidative substances can have beneficial effects on intracellular constitution.[54,55] Supplementation with anti-freeze proteins—short polypeptides that permit cellular survival in sub-freezing environments—prior to vitrification of immature murine oocytes improved spindle quality after IVM compared to nontreated controls.[55] This was accompanied by enhanced blastocyst formation and improved mRNA levels of Mad2 (a spindle checkpoint protein regulating anaphase onset), Hook1 (regulating chromosome segregation), and Eg5 (spindle maintenance). Additional cryoprotective substances or slush nitrogen therefore represent interesting options for next-generation cryopreservation techniques, but further comparative clinical studies are needed to evaluate the protective influence.

Conclusion

The question of whether and how the meiotic spindle and chromosome alignment is affected by cryodamage

has generated much controversy and a general conclusion is difficult. Obviously, vitrification of mature human MII oocytes appears to alter spindle integrity and chromosome alignment directly after vitrification and warming, while these changes disappear during recovery of cellular homeostasis. However, the biological significance of intermediate phenotypes with bipolar spindles and only minor dysfunctions is still unknown, and a potential risk cannot be excluded. Vitrification does not alter chromosome segregation either in meiosis II or in early mitotic divisions during embryonic development. No increase in birth defects and congenital malformations has been reported in neonatal offspring after vitrification compared to pregnancies from conventional IVF/ICSI and the general population.[56,57] There is evidence that fertilization, early embryonic development, implantation, and clinical pregnancy rates of vitrified and warmed human oocytes are comparable to fresh oocytes used as part of assisted reproductive techniques.[58,59] Consequently, the American Society for Reproductive Medicine has recently removed the experimental status of mature human oocyte vitrification.[60] Nevertheless, it should be considered that molecular alterations at the transcript and proteome levels may have adverse long-lasting effects, and that most of the published data were generated in a few clinics with the highest experimental experiences. Thus, results may not be generalized to the entire field of reproductive medicine. Further studies are necessary to draw a final conclusion regarding the safety and efficiency of cryopreservation, either by SRF or vitrification, to elucidate unknown downstream alterations or long-lasting effects at later stages.

GENOMIC IMPRINTING

DNA methylation and post-translational histone modifications are two major epigenetic mechanisms that regulate gene activation, or repression, without specifically modifying the DNA sequence. Genomic imprinting represents a highly specialized epigenetic mechanism for ensuring mono-allelic gene expression in the offspring. Accordingly, one parental allele becomes silenced by repressive modifications established during gametogenesis in one germ cell, while in the second germ cell, activating modifications are established to ensure gene expression in the offspring (Figure 12.2a). After fusion of both gametes, these germ cell-specific genomic modifications remain stable; contrary to this, global demethylation and reprogramming of both parental genomes occur to ensure totipotency in order to establish cell lineages and cell differentiation. DNA methylation occurs

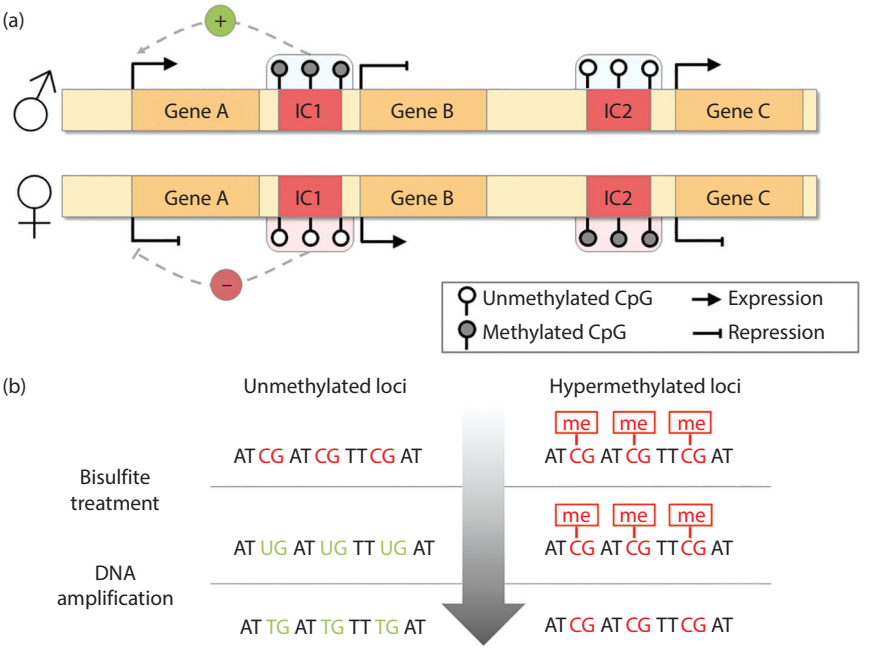

Figure 12.2 **(See color insert.) Scheme of mono-allelic gene expression in the offspring originated from parental-specific hyper- or nonmethylation of maternal and paternal imprinting centers (a). Paternal hypermethylation of IC1 represses paternal expression of gene B and activates expression of the upstream-located gene A. Hypermethylation of maternal IC2 results in paternal expression of gene C. Bisulfite treatment induced mutagenesis of hyper- and nonmethylated DNA (b). (CpG = cytosine phosphate–guanine dinucleotide.)**

Table 12.1 Examples of Imprinted Genes in Humans Used in Epigenetic Analysis

Gene	Full name	Function	Human disease
CDKN1C	Cyclin-dependent kinase inhibitor 1C	Negative regulator of cell proliferation and inhibitor of several G1 cyclin/Cdk complexes	BWS
H19	H19, imprinted maternally expressed transcript	Noncoding RNA implicated in growth suppression. Located in the *IGF2* imprinted region	BWS, Wilms' tumor
IGF2	Insulin-like growth factor II	Member of the insulin family, involved in development and growth	BWS, RSS, Wilms tumor
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily	Voltage-gated potassium channel	BWS
KCNQ10T1	KCNQ1 opposite strand/antisense transcript 1	Noncoding antisense transcript to the *KCNQ1* gene. Regulates transcription of multiple target genes	BWS
SNRPN	Small nuclear ribonucleoprotein-associated protein N	Involved in mRNA processing and tissue-specific alternative splicing events	PWS, AS
UBE3A	Ubiquitin-protein ligase E3A	Member of the ubiquitin protein degradation system	PWS, AS

Note: AS = Angelman syndrome; BWS = Beckwith–Wiedemann syndrome; PWS = Prader–Willi syndrome; RSS = Russell–Silver syndrome.

on cytosines at position C5 in cytosine phosphate–guanine dinucleotides (CpG). CpGs are located in GC-rich genomic regions known as imprinting centers (ICs) of approximately 1–4 kb in size, and they regulate the expression or repression of one or several neighboring genes (Figure 12.2a).

The establishment and maintenance of genomic imprinting is essential for mammalian development, and nearly 150 genes are known to be imprinted (Table 12.1).[61] In primordial germ cells, global DNA methylation becomes erased, including inherited parental-specific genomic imprints. *De novo* methylation of sex-specific DNA methylation patterns starts post-natal after the onset of oogenesis, and establishment of genomic imprinting is completed in mature oocytes (Figure 12.3).[62,63] DNA methyltransferase 3a (DNMT3A) and its co-factor DNMT3L are crucial for *de novo* methylation during oogenesis. So far, three maternal factors are known to prevent demethylation of imprinted genes during epigenetic reprogramming at early embryonic stages—STELLA, ZFP57, and KAP1[64–66]—while DNMT1 is essential for restoring DNA methylation patterns following DNA replication.[67] Loss of the DNA methylation imprinting pattern has crucial consequences during embryogenesis and in the offspring due to an altered gene–dose effect. STELLA-, DNMT1-, and DNMT3A-knockout mutants in mice showed, for instance, an altered/arrested embryonic development, or a precocious death a few weeks after birth.[68,69] As the use of cryopreservation either by SRF or by vitrification continues to rise worldwide, it is of upmost importance to assess the safety of both techniques with respect to the erasure, establishment, and maintenance of genomic imprinting.

Techniques

Detection of epimutations in small amounts of DNA is not simple, due to the fact that an average cell with one representative methylation profile does not exist, and imprinting diseases are quite rare.[70] Thus, one of the most challenging tasks is to identify these rare events masked within a pool of several oocytes or embryos. Most common techniques are based either on methylation-sensitive polymerase chain reaction (PCR) or enzymatic DNA restriction, or on bisulfite treatment of DNA molecules. The latter leads to a conversion of cytosine residues to uracil, while methylcytosine bases remain unaffected (Figure 12.2b). Thus, bisulfite treatment induces distinct mutations depending on the methylation pattern of individual cytosine residues. Bisulfite treatment followed by DNA amplification and sequencing (BiSeq) represents the gold standard for methylation analysis. BiSeq allows analysis of several gene loci of differentially methylated regions simultaneously. Recently published studies indicate that analysis of up to 25 genes in single cells or embryos is possible at one time, but such multiplex methylome analysis of several gene loci is time consuming.[71] Current methods typically analyze up to five genes, and therefore provide information only for a small subset of the entire methylome. Whole-genome sequencing could eliminate these restrictions, but this requires microgram quantities of DNA, while there are currently no reliable methods in the picogram range. Nevertheless, recent studies using amplification-free whole-genome bisulfite sequencing obtained methylome maps from only nanogram quantities of DNA, which, however, still would correspond to several hundred to a thousand oocytes.[72,73]

It is also noteworthy to mention that methylome analysis is prone to somatic DNA contamination, and

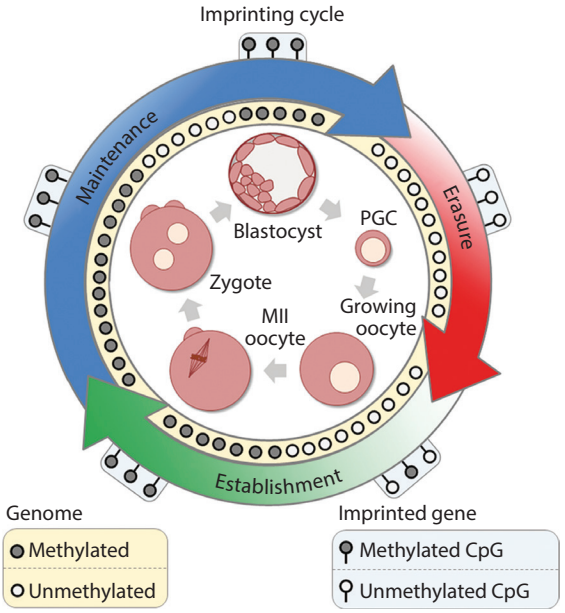

Figure 12.3 **(See color insert.) Imprinting cycle in mice. Erasure, establishment, and maintenance of imprinted genes (outer circle) during F_0 to F_1 transition. Genome-wide demethylation (including imprinted genes) occurs in primordial germ cells (PGCs) until embryonic day 13.5. Post-natal *de novo* establishment of sex-specific methylation marks occurs during oogenesis (pre-natal in males) and maintenance of genomic imprinting occurs until the formation of PGCs in the F_1 generation. Epigenetic methylation marks of nonimprinted genes (inner circle) are also erased in PGCs, followed by methylation events during oogenesis. After fertilization, active (paternal genome) and passive (maternal genome) demethylation of both parental genomes occurs, followed by *de novo* reprogramming during embryonic development. (MII = metaphase II.)**

may exhibit the possibility of stochastic amplification of just a single or only a few molecules from a starting sample of several dozen oocytes or individual blastocysts ("amplification bias"). This bias can lead to results that are not representative of the studied sample. However, preferential amplification can be excluded due to stochastic distribution of bisulfite-treated DNA molecules prior to amplification and sequencing,[71,74] while inclusion of the pluripotency-related gene *OCT4* in the multiplex assay, which is unmethylated in early embryos and oocytes and hypermethylated in somatic cells, allows the detection of DNA contamination. Therefore, although some drawbacks are present, improved BiSeq protocols represent the best opportunity to date for studying methylation patterns of several gene loci in one assay.

Imprinting Diseases and ART

Human imprinting diseases include Angelman syndrome (AS), Beckwith–Wiedemann syndrome (BWS), and Prader–Willi syndrome (PWS), and they predispose individuals to different tumor types, including ovarian cancer and Wilms' tumor.[61] The BWS critical region at chromosome 11p15.5 includes two ICs: IC1 that regulates the expression of *IGF2* and *H19*, and IC2 that controls the expression of *CDKN1C*, *KCNQ10T1*, and *KCNQ1*. BWS is associated with increased methylation at the normally nonmethylated maternal IC1, or a loss of methylation of the normally hypermethylated maternal IC2.[61] The critical regions at chromosome 15q11–q13 for AS and PWS include an IC that regulates the expression of *SNRPN* and *UBE3A*. AS and PWS children have a higher incidence of altered *SNRPN* methylation and failures to express the maternal allele of *UBE3A*, while loss of the *IGF2* imprinting profile represents the most common epimutation responsible for some cancer types.[61,75–77] ARTs, particularly cryopreservation, interfere with the critical time windows for the acquisition or maintenance of epigenetic markers, and doubtlessly pose a risk for altering DNA methylation patterns either directly or indirectly by affecting factors involved in epigenetic control. Hormonal stimulation, *ex situ* fertilization, and *in vitro* embryo culture have been reported to increase the incidence of epimutations in early pre- and post-implantation embryos, and in extra-embryonic tissue both in animal models and humans.[78–81] The general influence of ART with respect to the increased prevalence of imprinting diseases in children born after ART-treatment has been hotly debated. Certain recent studies showed no indication of an increased risk of imprinting diseases in children conceived after ART.[82,83] Conversely, some studies described a four- to nine-fold increase in the risk of BWS for children conceived *in vitro*, although the absolute risk remained very low.[84,85] Moreover, some studies suggested a link between hormonal or ICSI treatment and AS,[75,86] while a recent study did not find a significant association between the incidences of PWS or AS and IVF or ICSI treatments.[87]

Cryopreservation and Genomic Imprinting

Changes in epigenetic patterns were shown following exposure to extra- and intracellular stresses in various cell types, including oocytes and preimplantation embryos.[88–92] For instance, oxidative stress leads to chemical conversion of deoxyguanine to 8-hydroxy-2′-deoxyguanosine accompanied by repression of DNA methyltransferase activity at neighboring cytosines,[90] an altered *IGF2*/*H19* methylation pattern in the mouse prostate,[91] or a limited expression of DNMTs in human embryos that have been cryopreserved.[92] Oocyte cryopreservation therefore doubtlessly increases the probability of cellular methylation processes being altered. A large number of studies investigated the influence of IVF, ICSI, or *in vitro* culture on CpG methylation of imprinted

genes, while there is less information regarding the influence of cryopreservation either by SRF or vitrification. To date, there has been only one study of the CpG methylation pattern of two imprinted genes after the vitrification of immature human oocytes followed by *in vitro* maturation at the MII stage.[93] BiSeq analysis did not reveal significant differences for *KCNQ1OT1* and *H19* methylation, although the level of *H19* epimutations increased from 8% in IVM controls (3/34 analyzed alleles from 48 MII oocytes) to 17% after vitrification and IVM (5/29 analyzed alleles from 36 MII oocytes). For *KCNQ1OT1*, loss of methylation was found in 2/37 alleles from 20 IVM MII oocytes and in 1/28 alleles from 17 vitrified and IVM MII oocytes. It was concluded that vitrification does not cause an increased frequency of epimutations compared to IVM controls,[93] although the sample size was relatively low and the number of analyzed genes was limited.

In animal models, global DNA methylation patterns were significantly altered either after SRF or vitrification of mature bovine oocytes.[94] Thus, it seems that cryopreserved oocytes are more vulnerable to epimutations, but it should be noted that global DNA methylation was analyzed qualitatively by CLSM, without distinguishing between epigenetic DNA marks and the methylation status of imprinted genes. Using a mouse model, the genomic integrity of two imprinted genes was assessed in pups after the cryopreservation of whole ovaries by SRF followed by orthotopic ovarian re-transplantation and natural mating.[95] Methylation-sensitive DNA digestion and southern blotting revealed normal *H19* and *KCNQ1OT1* methylation patterns, suggesting that cryopreservation followed by ovarian transplantation does not disrupt the proper acquisition of genomic imprinting.

Using an alternative methodology to preserve female fertility, the genomic integrity of three imprinted genes was assessed after CryoTop vitrification of individual murine pre-antral follicles, followed by *in vitro* folliculogenesis and oogenesis.[74] Using BiSeq, normal methylation levels were reported for *SNRPN* (49/50 alleles), *IGF2R* (15/15 alleles), and *H19* (58/58 alleles) in mature GV oocytes compared to nonvitrified ($45/47^{SNRPN}$; $16/16^{IGF2R}$; $39/39^{H19}$) or *in vivo*-grown controls ($40/40^{SNRPN}$; $16/16^{IGF2R}$; $38/38^{H19}$). Thus, the published data do not provide evidence of an increased risk of epimutations after the cryopreservation of immature oocytes, and acquisition of genomic imprinting appears normal compared to controls. On the other hand, it was demonstrated using BiSeq that vitrification of murine pre-implantation embryos led to a loss of methylation of the *H19*/*IGF2* IC in early fetuses.[96] Although loss of methylation was also found in the IVF group, epimutations were more severe in the vitrification group. In another study using bovine two-cell embryos and BiSeq, the methylation level of *H19* was significantly increased in vitrified two-cell embryos and their derived blastocysts.[97] In accordance with studies that reported epigenetic perturbations during IVF, ICSI, and embryo culture, maintenance of genomic imprinting after cryopreservation seems to be more sensitive to epimutations when compared to the establishment of methylation marks after the cryopreservation of immature germ cells.

Although some epimutations were reported after the cryopreservation of germ cells and early pre-implantation embryos, data regarding imprinting diseases in children conceived after cryopreservation do not provide evidence for an increased incidence of adverse neonatal outcomes or birth defects. Such an analysis was described by Noyes et al.[57]: this study included 392 children conceived after oocyte vitrification, 532 children resulting from SRF, and 12 children from a combination of both techniques. Additionally, children born after SRF or vitrification of cleavage-stage embryos or blastocysts exhibited the same prevalence of epigenetic diseases as children born after the transfer of fresh embryos.[98–100] However, it should be noted that methylation patterns from abortions and stillbirths revealed an increased prevalence for imprinting alterations.[101] Thus, inappropriate methylation patterns of imprinted genes induced by ART treatment may contribute to spontaneous pregnancy loss.

Conclusion

The published data do not provide evidence of an increased risk of imprinting mutations after cryopreservation of oocytes *per se*. Epimutations and imprinting diseases were reported in some studies after ART treatment, including hormonal stimulation, *ex situ* fertilization, and embryo culture, but others could not replicate these findings. ART treatment is mainly a consequence of underlying subfertility or infertility of the patients. This makes it difficult to distinguish between *de novo* epimutations as a consequence of ART or whether potentially present dysfunctions in the parental generation (which cause subfertility or infertility) lead to genomic disorders in the offspring. Thus, the combination of *ex situ* treatment and the fertility history of patients undergoing ART is an important factor to keep in mind. Cryopreservation of immature oocytes does not seem to have a profound impact on the acquisition of genomic imprinting. Methylation patterns after *in vitro* follicle culture, orthotopic re-transplantation followed by endogenous *in vivo* folliculogenesis and oogenesis, as well as after IVM past cryopreservation were comparable to fresh controls. However, the influence of human MII oocyte cryopreservation has not been investigated so far, while two studies reported an elevated level of epimutations after the vitrification of bovine and murine pre-implantation embryos. It appears that the maintenance of genomic imprinting in later stages seems to be more sensitive to epimutations when compared to *ex situ* treatment prior to or during the establishment of DNA methylation. Although it is far too early to draw conclusions about the risk of imprinting mutations and birth defects after the vitrification of

oocytes, the current literature is reassuring. An increased incidence of imprinting diseases in live births after the cryopreservation of oocytes as well as pre-implantation embryos has not been identified. However, one of the most challenging tasks for future work is to verify the safety of the cryopreservation of immature and mature human oocytes or embryos with respect to the acquisition and maintenance of genomic imprinting.

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13 Metabolic profile of day 3 embryos arising from vitrified oocytes

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INTRODUCTION

The main challenge of assisted reproduction technology (ART) has always been to achieve pregnancies resulting in healthy live births for infertile couples and patients. Embryo selection methods have historically been based on microscopically evaluating morphology at different time points that have resulted in a significant improvement in pregnancy rates[1,2] and a reduced number of multiple pregnancies. Moreover, using new instruments that provide continuous images of embryos has further improved embryo selection and resulting pregnancy rates.[2,3]

The addition of new genomic, transcriptomic, proteomic, and metabolomic technologies that supersede traditional morphological methods has led to the proposal of new approaches for improving embryo selection that can provide more information and/or increase the accuracy of selection. For example, by analyzing embryo culture media, the metabolite content can be determined noninvasively, which might be used to assess embryo viability. Nutrients required for embryo development and implantation may change during *in vitro* embryo culture, and these variations might provide us with information about the metabolic activity of the embryo. Indeed, several retrospective studies have reported associations between the metabolomic profile and clinical outcomes.[4–7] The metabolism of embryos cultured for *in vitro* fertilization (IVF) often varies because embryos are adapting to the stressful conditions that they are subjected to by the variations in culture conditions.[8,9] However, other procedures, such as cryopreservation, are even more demanding on the embryo, and can adversely affect metabolism and development and thus also clinical results. Several reports describe the effects of cryopreservation on embryos or oocytes; for example, Zhao et al. report a reduction in the expression of DNA methyltransferase 1 (Dnmt1) mRNA expression in mouse oocytes.[10] At the metabolic level, studies also found effects on the metabolism of murine embryos frozen in late pre-implantation development.[11] In humans, Tachataki et al. reported that freezing embryos altered their gene expression profiles, and that day 2 frozen embryos contained less mRNA for the tuberous sclerosis gene *TSC2* than fresh day 2 embryos. However, several studies report the births of healthy children after the prior use of different vitrification approaches,[12] which provide information about the survival, embryonic development, and clinical outcomes obtained from using vitrified oocytes. Nonetheless, there is little evidence in the literature regarding the effects that these techniques may have on early human embryo metabolism.

VITRIFICATION PROCEDURES

One of the most pressing challenges in ART is the improvement of cryopreservation in order to standardize its use in clinical practice. There are different kinds of human female gamete cryostorage used for both subfertile and fertile women, including fertility preservation patients. The most common method is the use of low temperatures to completely stop all cellular metabolic and chemical reactions. Work done in the 1930s led to the discovery that sperm can survive cryostorage at –160°C,[13] paving the way for successful embryo cryopreservation and the first pregnancy after cryopreservation in 1983[14]; it has since become a cornerstone of ART.

Studies have shown that vitrification—the solidification of an aqueous solution without ice crystal formation facilitated by high-concentration cryoprotectants (CPA)—coupled with very fast cooling rates is a very efficient method for oocyte cryopreservation.[15] However, the toxicity of high-concentration CPAs is a major drawback that has led to the development of several devices and methodologies to counteract this effect.

These methods now have pregnancy rates similar to those achieved with standard fresh-oocyte procedures, sparking the creation of "egg banks" for ovum donation.[15,16] One controlled clinical trial with a large sample size failed to prove the superiority of fresh-donated oocytes over donated vitrified oocytes in infertility treatments.[15] Oocyte vitrification also has been very successfully used for infertile patients,[17] as well as in the management of low-response patients.[18] Although recent protocols and techniques report high clinical pregnancy and post-birth survival rates from cryopreserved oocytes, these rates remain low in some cases because of variation in the patient's age, oocyte quality, the type and concentration of CPA used, and the specific protocol used, among other variables.

-OMICS TECHNOLOGIES

The introduction of new genomics, transcriptomics, proteomics, and metabolomics platforms several years ago has given rise to many new ways to measure and analyze genes, mRNAs, proteins, and metabolites, which may

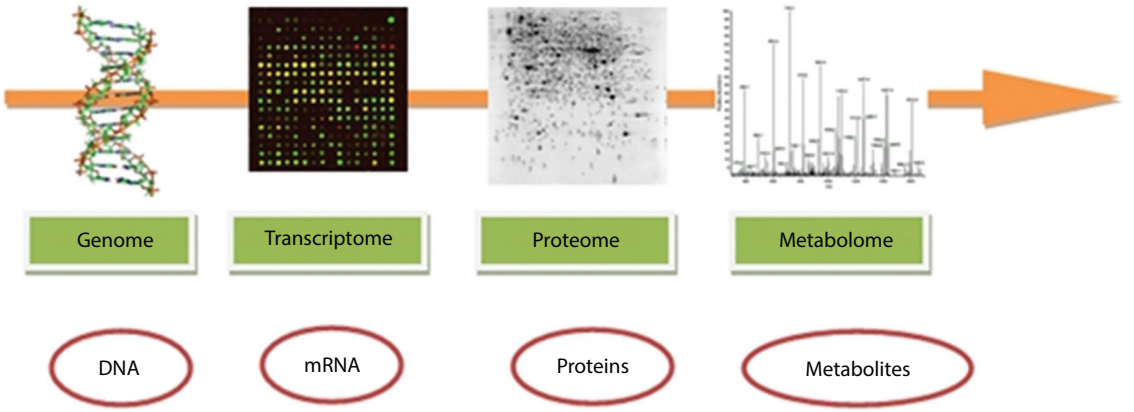

Figure 13.1 **-Omics sciences. New tools in molecular biology for multilayer global analysis, from DNA to metabolites.**

change how embryos are diagnosed and selected (Figure 13.1). These -omics technologies study the processes and interactions between cells, their metabolic byproducts, and their phenotypic changes, but necessitate powerful screening and analysis systems. These technologies have changed the classical molecular biology approach, directing it toward global analysis. Indeed, recent research using these technologies has aimed to define genetic, proteomic, and metabolomic embryonic profiles to allow better understanding of embryo viability.[5,19,20]

METABOLOMICS

Metabolomics studies the dynamic inventory of metabolites, using them as small molecular biomarkers to represent functional phenotypes in biological systems, and attempts to determine and quantify the metabolites associated with physiological and pathological states.[21] Low-molecular-weight metabolites are the final products of cell regulatory processes and can therefore reveal biological responses to several genetic, nutritional, and environmental influences.[22] The complete array of small-molecule metabolites contained in a cell or biological system constitutes its metabolome.[23] Metabolomics is a multidisciplinary science requiring the cooperation of chemists, biologists, and bioinformaticians.

The metabolome greatly varies, has a wide dynamic range, and is chemically complex and heterogeneous. In addition, automatic techniques for its analysis and measurement are still limited, and are influenced by the fact that many metabolites are still unknown, that very few extraction protocols are available, and that extraction yields are low.[21] Therefore, metabolomics is a science that is complementary to the genomic and proteomic disciplines, but with several advantages over them, as described in Box 13.1.[24] There are, however, several spectroscopy methods available for metabolite detection and analysis, and the integration of different analysis systems is enabling more complete descriptions of metabolomic profiles. These are being used to contrast normal and disease physiology, including in the early diagnosis of neurodegenerative diseases, and for screening fetal disease.

With regard to ART, evaluating cultured embryos might highlight different metabolic markers and profiles, providing quantitative parameters that we may be able to link to their implantation capacity,[7] thus significantly improving the embryo selection process. In addition, this is a noninvasive technique based on sampling the metabolites secreted into the culture medium by embryos during *in vitro* development. This makes the culture medium a useful source of information about the metabolic state and health of these embryos that additionally can be combined with other evidence (e.g., morphological, kinetic, etc.).

Different metabolomic parameters have been measured in developing embryos using noninvasive techniques.

Box 13.1 Advantages of Metabolomic Analysis

- Metabolic control theory[25] and experimental studies have shown that the concentration of a particular enzyme within a metabolomic pathway does not significantly change the biochemical reactions that take place but can significantly change metabolite concentrations.
- The metabolome is the final product of genetic expression and represents the functional level of a cell; therefore, changes in the metabolome should be much more biologically relevant than those in the proteome and transcriptome.[26]
- Research has shown that metabolic fluids are regulated both by gene expression and environmental factors, and so measuring the final metabolic products (metabolites) gives a more global picture.[27]

For example, an inverse relationship was found between pyruvate absorption and embryo viability.[28] Moreover, glucose absorption was higher in human blastocysts with better morphology.[29] A recent study also tried to correlate the presence of the amino acids glycine, leucine, and asparagine in embryo culture media with clinical pregnancy rates,[8] and another study using embryo culture media from 30 patients with known outcomes related different metabolomic profiles to implantation rates.[30]

However, despite these promising data, use of these techniques in the IVF laboratory has been limited by their high cost, the need for specialized equipment, and the long turnaround time required to obtain results, rendering it impossible to use this information in a clinical context for fresh oocytes. Although many challenges lie ahead before this technology can be used to predict fresh embryo viability, metabolomics may be beneficial in the cryopreservation field for using vitrified oocytes where result turnaround time constraints are less important. Moreover, considering the deep cytoplasmic changes and interactions that occur during the vitrification procedure, it is likely that inferences made from metabolomic profiling will be the only way to evaluate these subcellular disturbances. However, because this is a new technique, validation with different approaches using classic parameters such as morphology and cleavage rate may be necessary.

METABOLOMIC ANALYSIS PLATFORMS

Platforms for metabolome analysis should be sensitive and give a high yield in order to discriminate a high number of metabolites in a sample. Despite the relative success of current technologies, because of the wide diversity of complex metabolites contained in any one sample, it is not possible to analyze the complete metabolome with only one platform.

The most widely used technologies are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), which is usually performed after liquid chromatography (LC) separation; both technologies offer broad structural and conformational information on multiple chemical groups from only one procedural step, and each have advantages and disadvantages and offer complementary information about an ample group of metabolites without the need for preselecting which analytes should be detected.[31] The main advantage of MS is its sensitivity and the low minimum sample volume required; however, it is a destructive technique. Moreover, not all databases are complete and so, depending on the instrument and protocol used, results may not be reproducible. In contrast, NMR is a robust, nondestructive technique that is more reproducible than MS because the data obtained is universal across platforms. Unfortunately, NMR is less sensitive than MS, needs larger amounts of input sample, and has a higher metabolite detection threshold.

MULTIVARIATE STATISTICAL ANALYSIS

Multivariate statistical analysis offers a way of obtaining information from the large quantity of data generated from MS and NMR sample spectra by generating a series of multivariable profiles (one for each sample) represented in a k-dimensional space, where the position (coordinates) depend on the value of each descriptor (metabolite) describing the system (sample). Projection methods that convert multidimensional data tables into two-dimensional models are very useful for analyzing profile sets, and are the basis of metabolomic studies. These techniques make it possible to detect tendencies in the data because points that cluster represent similar multivariable profiles, whereas distant points represent different descriptors.

The specific statistical analysis used depends on the type of study being developed. Several types of methods may be applied to metabolomic data. The most used multivariable analysis methods are principal component analysis (PCA)[32] and partial least squares (PLS). PCA is a nonsupervised method used to determine the inner structure of a dataset without any prior information about it, whereas PLS is a supervised regression method whose objective is prediction within a set of data. Figure 13.2 shows an example of a PCA.

THE EFFECT OF VITRIFICATION ON HUMAN OOCYTES: METABOLOMIC PROFILE ANALYSIS OF EMBRYOS

Oocyte vitrification has been successfully applied in clinical practice, making the establishment of egg-banking programs for ovum donation possible; this technology is also proving to be an effective approach for fertility preservation purposes. An increasing body of literature shows the efficiency and consistency of the technology, along with the growing number of healthy babies born after oocyte vitrification, which has contributed greatly to the definitive validation of this approach. However, despite this clinical evidence, it has been argued that there is still a lack of basic research addressing the possible effects of vitrification on oocytes.

As we know, metabolism is intrinsic to embryo health, and so many studies have focused on identifying noninvasive metabolic markers associated with successful embryonic development. Special focus has been placed on identifying markers associated with oxidative phosphorylation, Na^+/K^+-ATPases, redox reactions, and amino acid metabolism.[8,33–36] Metabolomic profiling represents an opportunity to obtain biochemical fingerprints that may be useful for biological classification or to develop new diagnostic methods. Moreover, the compounds identified may provide insights into the biochemical events leading to a disease state or classification, and therefore provide opportunities for intervention.

In 2007, Seli et al. published the first study using a metabolomic approach for assessing the viability of human embryos.[5] They used near-infrared and Raman

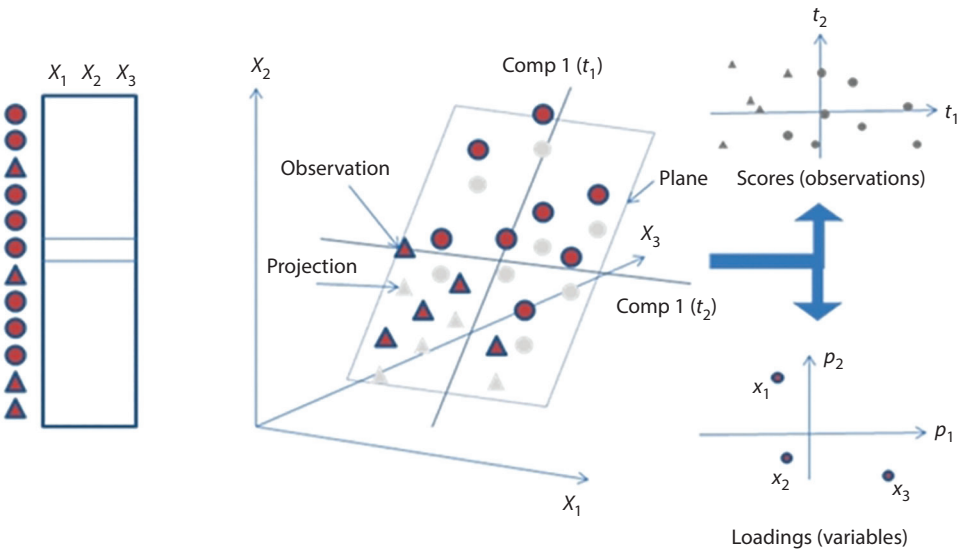

Figure 13.2 **Principal component analysis. Dots represent relationships between groups of data.**

spectroscopy to compare the metabolic profile of embryo-conditioned media from positive and negative implantations after transfer.[30] In a follow-up study, viability markers were identified using a NMR metabolic platform and statistically significant differences were found in glutamate concentrations between embryos that resulted in pregnancies and/or births and those that ended in implantation failure.[37] Other recent studies have identified genes, proteins, and metabolomic profiles that can detect which oocytes or embryos are viable, and have used these characteristics to measure their implantation capacity.[17,20,38]

A system has also been developed that aims to obtain comprehensive metabolite profiles by quantitatively and qualitatively analyzing the metabolites produced/secreted by an organism, tissue, or even a single cell.[39] Low-molecular-weight metabolites represent the final products of cellular metabolism and therefore reveal the responses of biological systems to a variety of genetic, nutritional, and environmental conditions.[22] These non-invasive, quantitative techniques are the focus of intense research to determine their value as predictors of embryo viability, pregnancy rates,[5,38] and even their ability to detect aneuploid embryos,[38,40] although a prospective randomized trial found no benefit to using these methods for pregnancy prediction.[41]

In 2013, Dominguez et al. evaluated the effects of oocyte vitrification on the global metabolomic profiles of embryos developed from vitrified oocytes compared to the profiles observed in embryos from fresh oocytes in an oocyte donor program.[42] A total of 190 spent medium samples were collected from 65 selected patients (34 fresh and 31 vitrified oocyte patients, respectively). Each 50 µL sample was collected from the spent embryo culture medium from the vitrification group (n = 59), the fresh group (n = 65), and a matched control medium group (n = 66) using corresponding media from the same batch in the same conditions, but without embryos (Figure 13.3). The samples

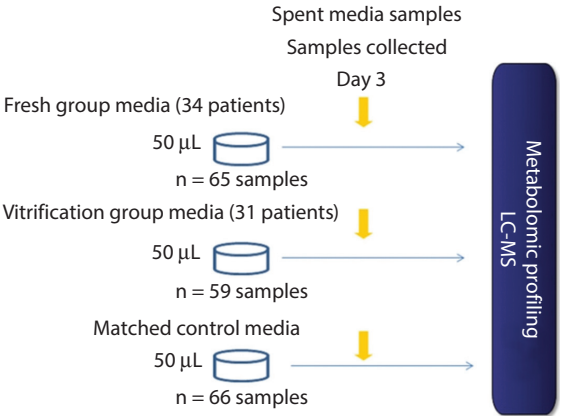

Figure 13.3 **Experimental design of the study. Microdrops were taken from 190 spent culture media from embryos that had been cultured from day 1 to day 3. Collection was performed on day 3 by collecting 50 µL of culture media. The samples were collected from the fresh group (n = 65), the vitrification group (n = 59), and the matched control media group (n = 66) in the LC-MS study. Metabolomic analysis was performed in two rounds due to the instrument capacity limitations.[42] (LC-MS = liquid chromatography mass spectrometry.) (Adapted from Dominguez F et al., *Fertil Steril* 2013;99(2):565–72.)**

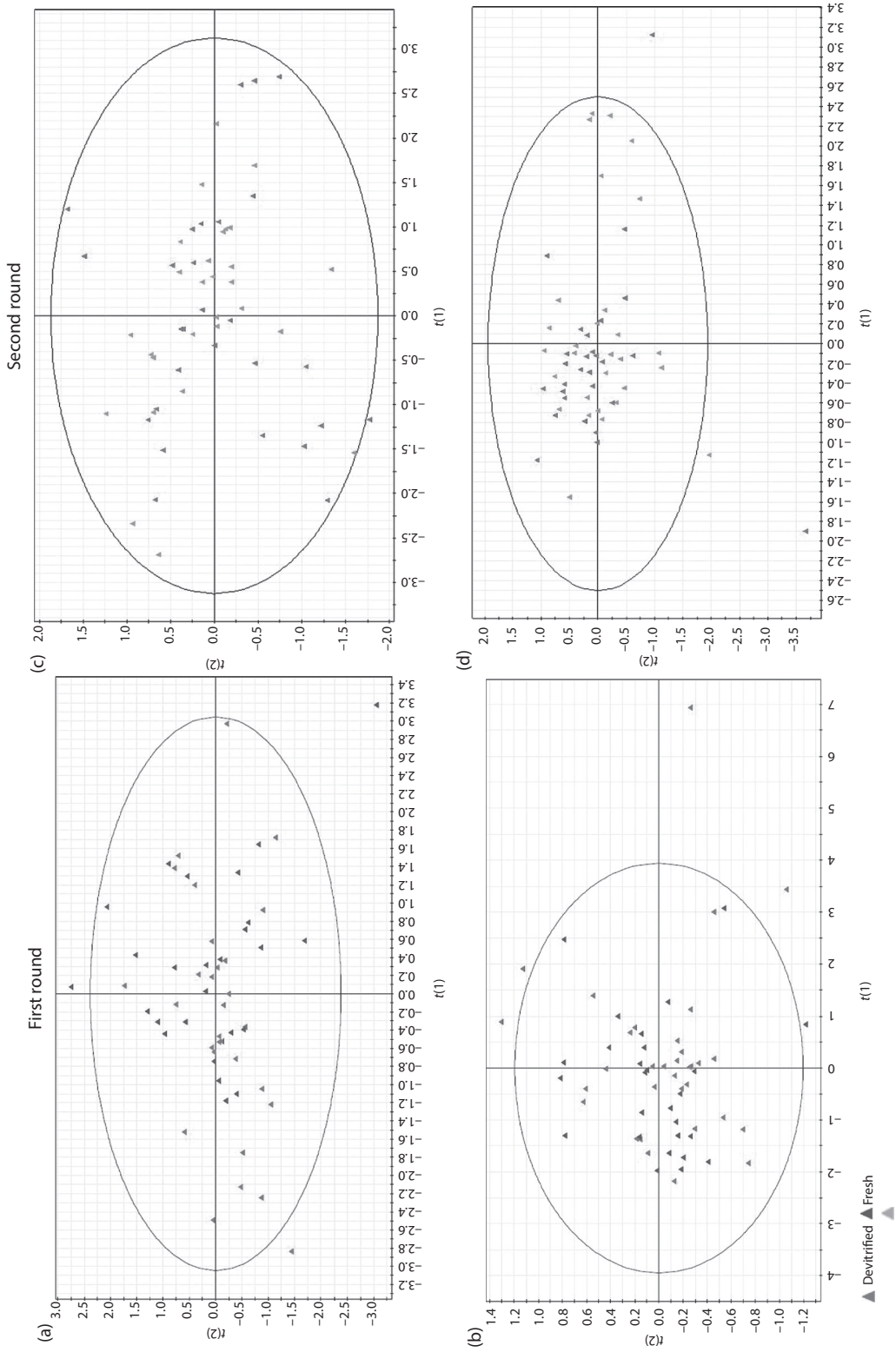

Figure 13.4 **(See color insert.)** Principal component analysis representing all of the metabolites analyzed for each sample in the two rounds. Blue/green triangles represent the fresh group, and red triangles represent the vitrification group. No separation between groups was observed using positive (a and c) or negative (b and d) sample ionization in either of the analysis rounds.

were analyzed using ultra-performance LC with MS for global metabolomic profiling, and then 10 µL sample aliquots were transferred to microtubes and derivatized for amino acid analysis and analyzed by LC-MS (Figure 13.3). The experimental design of this study involved microdrops being taken from 190 spent culture media from embryos that had been cultured from day 1 to day 3. Collection was performed on day 3 by collecting 50 µL of culture medium. The samples were collected from the fresh group (n = 59), the vitrification group (n = 65), and the matched control medium group (n = 66) in the LC-MS study. Metabolomic analysis was performed in two rounds due to the instrument capacity limitations. In the first round (consisting of 31 fresh and 31 vitrification group embryo media and 33 matched control spent media), hundreds of metabolites (including amino acids) were compared and statistically analyzed for differences between the fresh and vitrified outcome groups. After performing global metabolomic and amino acid profiling, PCA failed to find any statistically significant differences between the fresh and vitrified oocyte populations, indicating that other metabolic differences between the samples (e.g., patient-to-patient variability or analytical variation) were greater than those between the sample groups (Figure 13.4a and b). Although univariate statistical analysis revealed a series of metabolites, including tryptophan and phenylalanine, which were statistically significantly different between these groups, none remained significant after Bonferroni's correction for multiple testing. Common metabolites involved in embryo development, such as lactate and glucose, were also checked, and no statistically significant differences were found between the fresh and vitrification groups (Figure 13.5).

In the second round of experiments, 29 and 34 embryo culture media originating from fresh and vitrified oocytes, respectively, and 33 matched controls were analyzed in the same way. Again, multivariate data analysis techniques failed to find any differences between the fresh and vitrified oocyte populations (Figure 13.4b and c). However, univariate statistical analysis did reveal

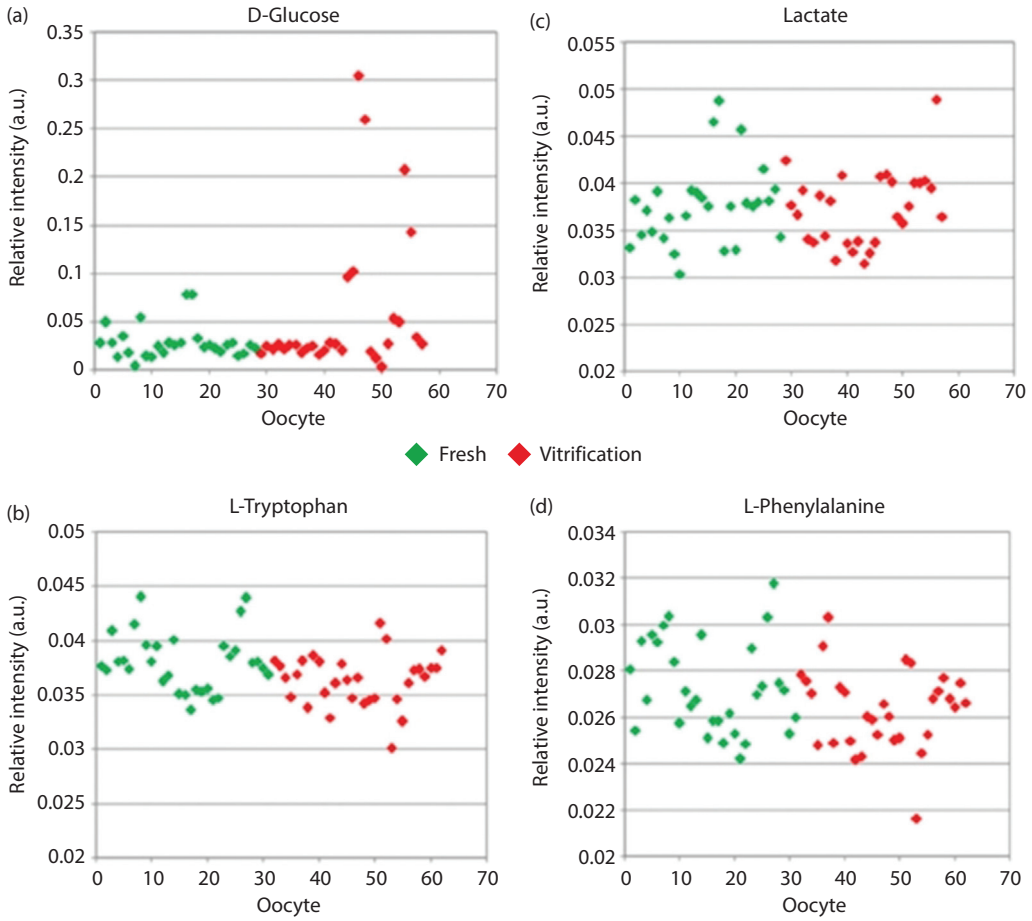

Figure 13.5 **(See color insert.) Distribution of measurements of (a) glucose, (b) tryptophan, (c) lactate, and (d) phenylalanine in both fresh (green rhombuses) and vitrification groups (red rhombuses).**

some potential markers that were statistically different between the fresh and vitrified groups, but only one of these remained statistically significant after correction for multiple testing. To identify this metabolite, mass spectra recorded in the positive and negative ion modes were analyzed to determine the most likely parent ion *m/z* value, and the compound was identified as 2,5,7,8-tetramethyl-2-(2'carboxyethil)-6-hydroychroman (alpha-CEHC). The exact mass information obtained was checked against the ChemSpider online databases using the Human Metabolome, LipidMaps, and peptides sub-databases.

This clinical outcome is consistent with other reports: perinatal data also corroborate the safety of the technology, showing no differences for the parameters analyzed in babies born after oocyte vitrification and those born after fresh oocyte donation. Although some small differences in individual metabolites are found, embryos developed from vitrified oocytes and those originating from fresh oocytes do not significantly differ in their spent medium global metabolomic profiles. This suggests that the vitrification protocol does not disturb the general metabolism of these embryos, a finding that is also supported by the clinical results.

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14 Vitrification of human oocytes for *in vitro* fertilization patients

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INTRODUCTION

Since the first successes obtained in the second half of the twentieth century,[1,2] human sperm freezing and embryo cryopreservation are today considered indispensable techniques available to all patients who utilize IVF therapy, such that they are routinely performed, and account for a large percentage of assisted pregnancies. By contrast, oocyte cryopreservation has been largely neglected, mostly due to the remarkable technical difficulties related to its special cell structure and sensitivity. Nevertheless, since the first human pregnancy reported from frozen eggs,[3] considerable efforts have been made to improve on a suboptimal protocol. In this regard, the 2004 Italian Law No. 40 that regulates assisted reproductive technology (ART) is considered a key turning point. In fact, the limit of inseminating no more than three oocytes per IVF cycle, coupled with the legal prohibition against cryopreserving supernumerary embryos for reasons other than serious, documented, and not predictable adverse effects affecting women's health, obliged embryologists to invest time and energy in developing effective cryopreservation methods.[4] Fortunately, all of these efforts have ultimately been rewarded with excellent results,[5–18] such that currently IVF clinics worldwide are able to enjoy the benefits of this amazingly useful procedure. In fact, apart from restrictive legislation and/or ethical concerns, oocyte cryopreservation is of utmost importance in many aspects of ART, offering several valid solutions to clinical, logistical, and social problems. First of all, ovum donation programs allow a more convenient, flexible, and cost-effective approach to the coordination between donor and recipient. Moreover, these programs can be precious tools for fertility preservation both for medical indications (e.g., cancer patients undergoing gonadotoxic chemotherapy/radiotherapy and eventual oophorectomy, and for women suffering from premature ovarian failure arising from certain genetic disorders) or for social reasons (e.g., for preserving fertility in post-pubertal females without a partner, and to preserve fertility for women approaching menopause). Last but not least, oocyte cryopreservation may provide useful alternatives and advantages in infertility programs. In fact, it allows oocyte cryopreservation in the following cases: (i) collection of supernumerary eggs to keep for future use, thus avoiding the hormonal treatment necessary to sustain multiple follicular growth; (ii) risk of ovarian hyperstimulation syndrome, when ethical concerns and/or legal restrictions limit embryo freezing; (iii) as an alternative option to embryo freezing to avoid potential entanglement in case of divorce and separation of couples; (iv) IVF procrastination due to no available/inadequate sperm sample; and (v) reported previous implantation failures with good-quality embryos.[4,19,20]

As already mentioned, oocyte cryopreservation protocols have evolved considerably over the years in terms of type and concentration of cryoprotectants used, such as cooling rates shifting from "slow freezing" to the more effective "vitrification" methodology. Briefly, whereas slow freezing involves the gradual cell dehydration achieved with the combination of low cryoprotectant concentrations and slow cooling rates, the principle of vitrification relies on the initial exposure to high concentrations of cryoprotectant agents, followed by single-step ultra-rapid cooling in liquid nitrogen (–196°C) in order to achieve a solid glass-like state. The introduction of vitrification has dramatically increased cryopreservation efficiency in terms of survival and, more importantly, pregnancy rates, such that it is today considered to be the method of choice to preserve both gametes and embryos, being finally freed of the restrictive label "experimental."[20]

OOCYTE VITRIFICATION IN INFERTILITY PROGRAMS: RESULTS

As already mentioned, the advent of vitrification improved many aspects of the entire oocyte cryopreservation procedure, while at the same time being reassuringly harmless.[21] Notably, recent reports revealed that vitrification does not compromise *in vitro* development and clinical outcomes, being similar to that of its fresh counterparts.[8–14] Unfortunately, many of the studies published so far refer to ovum donation programs or extremely good-prognosis patients, and are therefore strongly biased because of the high-quality oocytes involved.[8–11] The study of the standard infertile population must then be considered imperative in order to confirm and validate the clinical value of the technique.

To our knowledge, the first prospective randomized clinical trial conducted in an infertile population establishing the efficacy of the vitrification procedure for maintaining oocyte competence to develop *in vitro* was designed by our group in 2010.[12] This noninferiority trial actually aimed to compare the *in vitro* performance of fresh and vitrified–warmed sibling oocytes obtained in consecutive intracytoplasmic sperm injection (ICSI) cycles in a standard infertility program between September 2008 and March

2009 (thus before the edict of the Italian Constitutional Court that radically changed the horizon of IVF in Italy by definitively allowing embryo freezing). All of the patients involved were not older than 42 years of age and yielding more than six normal-appearing mature oocytes were undergoing ICSI treatment with an ejaculated sperm count of >500,000 motile spermatozoa/mL (in order to exclude negative paternal effect). According to Italian Law No. 40, only three oocytes per patient were injected, with the remaining been vitrified according to the protocol described by Kuwayama and colleagues.[22,23] In order to limit potentially confounding factors, extra care was taken during the oocyte manipulation to minimize extra stress, namely: (i) long exposure to 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered media with uncertain temperature control (denudation was carried out in a closed chamber with temperature plus gas control, with rapid oocyte morphology evaluation by the stereomicroscope at 40× magnification); (ii) prolonged denuded oocytes in *in vitro* culture (denudation applied immediately before treatment); and (iii) oocyte aging (ICSI was performed at 2 hours following warming). As a consequence, the only difference eventually observed between fresh and vitrified oocytes was the vitrification procedure itself.

A total of 124 patients from 124 ICSI cycles met the above inclusion criteria (33.8% of treated couples). Fifty four patients became pregnant in the fresh cycle, and therefore were not involved in the warming cycle during the study period. Of the remaining 70 patients, 40 underwent a first-round warming cycle, for a total of 124 oocytes warmed. The survival rate was 96.7% (120/124 oocytes survived), confirming the superiority of vitrification over slow freezing as already cited.[8,24] The fertilization rate per injected oocyte was not significantly different between fresh (83.3%) and vitrified–warmed sibling oocytes (79.2%); given the high survival rate obtained with the vitrification procedure, the number of degenerated eggs was negligible and did not affect the overall fertilization rate (76.6% per warmed oocytes). Even if a slightly decreased normal 2 pronuclei (2PN) morphology rate was observed in the vitrified–warmed group (which did not reach statistical significance), the cleavage rate and embryo quality were similar. In fact, day 2 embryo development and top-quality embryos were respectively 100% and 52% using fresh oocytes and 97.9% and 51.6% using their warmed oocyte counterparts. Importantly, the evaluation of the vitrification effectiveness should rely on the assessment of pregnancy and delivery rates, rather than on merely *in vitro* outcomes. In fact, the cryopreservation procedure may induce much sublethal cellular damage that is not routinely appreciated in an IVF laboratory, but affects embryo development and viability in different and at later developmental stages, thus leading to implantation failures or miscarriages. Even if the evaluation of pregnancy and implantation rates was beyond the purpose of this particular study, Rienzi and colleagues observed a promising trend in the vitrified–warmed group, with a 30% ongoing clinical pregnancy rate and 17.2% ongoing implantation rate.[12]

A subsequent prospective longitudinal cohort study from our group actually confirmed the *in vitro* outcomes and further focused on clinical results.[13] In particular, the authors stated a negative trend in the overall clinical outcomes when embryos derived from vitrified oocytes were transferred, but there was a very high cumulative effect on IVF success, comparable to those obtained with fresh and cryopreserved embryos[25] and more evident in young women. It is important to note that, in this study, the "fresh" and "vitrification" groups could not be directly compared: first of all, the second group included all of the patients who did not become pregnant in the fresh cycle, thus potentially revealing a different prognosis. Moreover, gonadotropin stimulation and superovulation rather than spontaneous hormonal cycle and surge may account for the different endometrial receptivity. As a consequence, the clinical data must be considered in a larger prospective trial, and vitrification should be proposed in order to increase the chances of cumulative success.[13]

Beyond pregnancy and implantation rates, the true outcome in IVF is undoubtedly the delivery of a healthy baby. In this regard, the overall efficiency and consistency of oocyte vitrification have been investigated in a recent observational cohort multicentric study, conducted between October 2006 and April 2010 in three different European clinics (GENERA [Gynecology, Endocrinology, Embryology and Reproductive Medicine]—Rome, Italy; Ca' Granda Ospedale Maggiore Policlinico—Milano, Italy; IVI—Valencia, Spain) and involving patients of less than 43 years of age enrolled for standard infertility programs.[15] A total of 486 warming cycles in 450 couples were performed, accounting for 2721 vitrified oocytes warmed. This large study provides three levels of informative data. First of all, the *in vitro* outcomes (i.e., survival, fertilization, and embryo development rates) and clinical data (i.e., pregnancy and implantation rates) were found to be consistent with previously published studies.[8–13] Moreover, no significant differences among the three centers were observed, except for the direct consequences of different legal restrictions (i.e., the number of oocytes injected and the blastocyst stage transfer policy). Finally, the third and probably most interesting finding consists of the evaluation of the different factors predictive of a healthy birth. In fact, the logistic regression analysis enabled the identification of three elements influencing the final IVF outcome, namely: (i) the maternal age; (ii) the number of vitrified metaphase II oocytes; and (iii) the day of transfer after fertilization. Furthermore, the recursive partitioning analysis permitted the design of an intuitive decision-making model able to predict the probability of delivery according to the above-mentioned patient characteristics, thus being extremely useful for proper counseling and patient selection. First of

all, this study established an inverse correlation between maternal age and delivery, with a decrease of 7% in delivery rate for each year of increase in maternal age. Moreover, it was calculated that the delivery rate doubles (from 22.6% to 46.4%) in cases in which more than eight oocytes were vitrified, if the other two variables were fixed. In cases of less than eight oocytes being vitrified, the outcomes are reduced for women aged more than 38 years (12.6% versus 27.5%). Finally, in cases of more than eight available vitrified oocytes, there was clearly an increased potential for blastocyst stage transfer to be performed (62.1% versus 40.7%).

The age-specific probability of live birth with cryopreserved oocytes has been more recently estimated by Pelin and colleagues[26] through an individual patient data meta-analysis. The authors propose 36 years as the upper age limit to accurately counsel patients for a reasonable chance of success, even if conception and live birth can occur as late as 44 years of age. In our opinion, these data confirm the value of egg cryopreservation, despite the established and distinct age-related decline that will occur after any estimated cutoff age. So taken together, this information, far from being dogmatic, should be regarded as a significant instrument for appropriate counseling and informed decision-making.

Notably, all of the studies described above assessed the effectiveness of the vitrification procedure in normally responding women. However, a substantial subpopulation of infertile patients is represented by low responders, namely patients with a low number of oocytes retrieved and suboptimal oocyte maturation and embryo quality, factors that invariably lead to higher cycle/transfer cancellation rates. The difficult clinical management of these patients has promoted the evaluation of oocyte accumulation through vitrification as an alternative option to disappointing stimulation regimens.[17] A recent prospective study conducted between January 2007 and December 2009 by the IVI group (Spain) enrolled 724 low-responder patients (≤5 oocytes retrieved per stimulation cycle; follicular stimulating hormone (FSH) > 11 IU/mL on cycle day 3; antral follicular count (AFC) < 6 among both ovaries; antimullerian hormone (AMH) < 5 pmol/L) divided into two groups: 242 patients (594 cycles) who decided to accumulate oocytes through vitrification for later insemination (LR [low responder]-Accu-Vit) and 482 patients (587 cycles) who preferred to undergo standard ovarian stimulation and subsequent embryo transfer (LR-fresh). Notably, there were observed no statistically significant differences between fertilization, cleavage, and top-quality embryo rates between the two groups. On the contrary, there was a fourfold lower cancellation rate in the LR-Accu-Vit group (9.1%) compared to the LR-fresh group (34%), consequently leading to a higher cumulative live birth rate (36.4% versus 23.7%), in spite of similar implantation and live birth rates per transfer. Moreover, a positive trend was observed with cryo-embryo transfers, which further increased the cumulative outcomes. In our opinion, the striking results obtained in this study are extremely important: first of all, they underline the high effectiveness of vitrification even in poor-prognosis infertile patients. Additionally, the clear benefit in terms of reduced anxiety levels, coupled with the lower cycle cancellation rate, is a major recommendation for continuing this type of approach, rather than switching too readily to ovum donation.

DISCUSSION

Oocyte cryopreservation is considered a major technique that is offered in IVF clinics worldwide, regardless of differing national legislations. In fact, its broad applicability encompasses ovum donation and infertility programs incorporating medical and social egg freezing. The advent of vitrification for oocyte cryopreservation, finally freed of the label "experimental,"[20] represents a significant improvement over previous protocols, thus presenting new opportunities for therapy in assisted reproduction. However, in spite of the recent excellent successes, its clinical application is still quite restricted, and is often applied to just a select patient population, namely young women often enrolled in ovum donation programs. Actually, it has been well established that its high effectiveness can be used even in the treatment of infertile patients with different prognoses, enabling the achievement of high cumulative pregnancy and live birth rates.[12,13,15,17] Among the various factors influencing the outcomes of oocyte vitrification are advanced maternal age, as well as the number and quality of oocytes retrieved, and these remain critical issues to be assessed for these women.[15,17,26] Nevertheless, proper patient selection coupled with an appropriate counseling and a suitable transfer often enable achievement of remarkable results for these poor-prognosis patient categories. It is therefore our firm opinion that the overall efficiency of oocyte vitrification justifies its application in routine IVF therapy.

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15 Oocyte vitrification: Donor "egg banking"

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HISTORY AND INDICATIONS FOR OOCYTE DONATION

In vitro fertilization (IVF) has been an established treatment option for couples challenged by infertility since 1978.[1] However, some couples may not be able to conceive using their own eggs. Most frequently, the reason is the advanced maternal age when no eggs or no viable eggs can be obtained. Less frequently, menopause can start early due to premature ovarian failure. Additionally, ovaries can be dysfunctional due to certain medical conditions, or as a result of treatment against cancer. In all of these situations, using donor egg is indicated. In some genetically inheritable conditions (present in the wife/female partner), the couple may also opt for donor oocytes, instead of using their own.[2]

The first pregnancy using a donor egg was reported by the Monash group in 1983.[3] Within a short period of time, egg donation was introduced in several countries and became an established part of routine IVF treatment.[4–9] It is of importance to note that there are a number of countries where gamete donation is regulated by law, and can forbid it completely (like Germany currently), or certain restrictions are imposed in many countries, as in the UK, Italy, Australia, France, and Sweden,[10–12] which are just a few examples. On the other hand, there are some countries where there is no or very minimal regulation on gamete (oocyte) donation. In these countries, the availability of egg donors typically satisfies the demand of the recipients, while in countries with different restrictions, typically the demand for egg donors is much higher than the availability of donors. As an example, in the United States, where there is no specific federal regulation on egg donation, about 10,000 treatment cycles were performed using egg donations in 2012, as reported by the Society of Assisted Reproductive Technology, corresponding to approximately 10% of all IVF cycles (https://www.sartcorsonline.com/rptCSR_PublicMultYear.aspx?ClinicPKID=0).

SIMILARITIES AND DIFFERENCES BETWEEN FRESH AND CRYO-BANK DONATIONS

Historically, oocyte donations have been performed "fresh," due to "technical challenges" with egg cryopreservation (which will be discussed later on),[6] but lately, there has been a rapid increase in the use of recently established donor egg banks as an alternative option.[13] Obviously, there are several aspects in which fresh egg donation is similar to "frozen egg" donation through donor egg banks, including: (1) donor recruitment and the type of donors; (2) donor screening and testing; (3) matching donors to recipients; (4) donor stimulation/egg retrieval procedures; (5) insemination of donor oocytes/embryo culture; and (6) preparation of the recipient for embryo transfer. However, there are also a few critically important differences between fresh and "frozen egg" donations, including: (7) price of treatment; (8) available number of donor eggs (and embryos); (9) waiting time; and (10) need for "synchronization."

1. Donors are typically recruited either by a donor agency or by the IVF clinic (donor egg banks typically act as a "combination" of a donor agency and IVF clinic—initially being part of an IVF program, then it may develop into a separate organization to recruit donors specifically for a given egg bank). Donors can be "known" (where the recipient knows the identity of the donor) or "unknown" (where the identity of the donor is not known to the recipient). Some countries require by law that even an unknown donor should provide their identity to the offspring at a certain age when conceived using their gametes, such as in the UK.[14] Depending on a country's legislation, a donor can be paid, be compassionate (not paid), or compensated only minimally. "Altruistic" donors are typically a relative or friend of a patient who requires donor eggs, or may be an IVF patient who is willing to donate some of her own eggs to a recipient (typically for little or no compensation). The amount of compensation for "paid donors" is not restricted in some countries and it is mainly dictated by the "market demand," such as in the United States, Spain, and Greece—though typically there is a "historical range," considered to be the highest in the United States, but somewhere between $3,000 and $12,000 per donation.[15]
2. Donors are typically "pre-selected" based on different physical parameters including: age (in the United States, it is typically younger than 30 years of age; in Spain, it is younger than 35 years of age); body mass index (BMI); personal history, including educational level; and medical and family history are also typically recorded, and may play a role in further selection of the donor. Psychological

evaluation, in addition to medical physical examination, is typically performed. Genetic testing for the most frequent inheritable genetic diseases (e.g., alpha-thalassemia, beta-thalassemia, cystic fibrosis, fragile X syndrome, mucolipidosis, sickle cell disease, spinal muscular atrophy, and Tay–Sachs disease, among others) is also performed. Finally, testing for different infectious diseases is also performed (such as for *Treponema pallidum* [syphilis], gonorrhea, West Nile virus, chlamydia, human immunodeficiency virus [HIV]-1, HIV-2, hepatitis C virus [HCV], hepatitis B virus [HBV] [hepatitis B core antibody (HBcAb) and Immunoglobulin G (IgG) + Immunoglobulin M (IgM)], HBV [hepatitis B surface antigen (HBsAg)] as required in the United States by the Food and Drug Administration).[16] Similar infectious testing is required in the European Union (EU) based on the EU tissue directives.[17]

3. Matching donors to recipients is an important process that usually involves both the recipient and the donor "agent" (a "donor nurse" from an IVF clinic or "donor agent" if it is a donor agency or donor egg bank). Recipients usually take into consideration the physical characteristics of the donor, preferentially resembling the patient—but educational level, medical history, and genetic information are also important factors in the decision-making. On many occasions, there is a website of the clinic or agency, or the donor egg bank, where available donors are posted and the recipient can browse the information to help them select their preferred donor (some examples: http://www.bhed.com/; http://www.thedonorsource.com/; https://myeggbank.com/; https://www.donoreggbankusa.com; http://www.theworldeggbank.com/; http://www.ivi-fertility.com/en/patients/assisted-reproduction-treatments/egg-donation/).
4. Donor ovarian stimulation is performed similarly to that which an IVF patient undergoes, including the use of medications that help to develop several follicles at the same time (called follicle-stimulating hormone [FSH]). When follicles are at the adequate size (typically, the leading follicles should be over 17–19 mm in diameter), the final maturation is triggered by an human chorionic gonadotropin (hCG) injection, and 35–37 hours later, oocytes are aspirated from the follicles surgically under sedation. A potential risk of ovarian stimulation is ovarian hyperstimulation syndrome (ovarian hyperstimulation syndrome [OHSS]; symptoms include dizziness/nausea, abdominal bloating, pain in the abdomen, sudden weight gain, decreased urination, and shortness of breath). Since OHSS can be severe, to the point that the patient needs hospitalization, it is a priority to prevent it. Using the antagonist stimulation protocol with an agonist trigger (instead of hCG), it has been demonstrated that OHSS can be avoided, while maintaining the number and quality of oocytes retrieved.[18,19] Because egg donors are most at risk for OHSS (due to their young age and tendency to respond overly well to follicle stimulating hormone [FSH]), it is now becoming standard to use only the "antagonist/agonist protocol" for all egg donors.
5. Insemination of donor oocytes and embryo culture is performed similarly to other IVF cases.[20,21] Depending on the sperm parameters of the partner of the recipient, conventional insemination may be performed, or intracytoplasmic sperm injection (ICSI) if sperm parameters are suboptimal.[22] If cryopreserved donor oocytes are used, then ICSI is the standard procedure for insemination.[23] Embryo culture is standard *in vitro* fertilization (IVF) procedure, which is typically performed in microdroplets for 3–5 days after insemination, when morphologically adequate embryo(s) are transferred and or cryopreserved.[23]
6. Preparation of the recipient for embryo transfer is a highly standardized procedure. Uterine preparation is typically carried out using a oral or transdermal estrogen or estradiol valerate 6 mg intramuscularly (IM) every 3 days. Vaginal progesterone (Crinone 8%; 90 mg twice a day) or IM progesterone (50 mg once daily) starts on day 15 of estrogen therapy. Embryo transfer is performed on day 4 (if cleavage-stage embryo[s] are transferred) or on day 6 of progesterone therapy (if blastocyst-stage embryo[s] are transferred). Pregnant recipients continue to receive the same dosages of progesterone and estrogen until an estimated gestational age of 10 weeks.[24]

   As mentioned earlier, there are some aspects in which there is a major difference between "fresh" and "frozen" donor egg treatment.
7. The price of treatment is typically lower when oocyte donation is performed through a donor egg bank, using cryopreserved oocytes, compared to "fresh donation." The reason for this is that egg donors are typically producing a larger number of usable oocytes, which are divided among several recipients in the donor egg bank setting, while during fresh donation, typically all eggs from one donor are donated to one recipient. Therefore, the cost of a donor cycle (which includes donor compensation, testing, screening, medications, IVF costs, etc.) is split between several recipients, resulting in significant savings when using a donor egg bank, compared to a fresh donation cycle. There are of course some exceptions to this situation: during a fresh donation, the eggs from one donor can also be split among more recipients, which also makes the donor egg cycle cheaper; however, it becomes more challenging to synchronize multiple recipients with one donor (see below). Alternatively, it is

also possible that a donor egg bank is doing "one-to-one" donation (i.e., all eggs cryopreserved from one donor are given to only one recipient), such as is done in some European donor egg banks (e.g., Instituto Valenciano de Infertilidad [IVI]). This latter approach can be explained (in part at least) by the fact that donor costs in Spain are significantly lower than in the United States; therefore, splitting donor eggs and donor costs would not result in significant savings in that specific setting.

8. Available numbers of donor eggs (and embryos) are typically also different in a donor egg bank setting compared to fresh (one-to-one) donation. It is typical that a recipient receives between five to eight mature, cryopreserved oocytes from a donor egg bank, which limits the number of embryos as well. During fresh donation, on the other hand, commonly all mature eggs are provided to a recipient, usually resulting in a larger number of embryos. For example, in a study by Nagy and colleagues, when the same donors were used both for fresh and for "cryo-egg" donation (in different donor stimulation cycles), the average number of eggs was 23 and the average number of embryos was 15 per recipient in the fresh donation. In the "cryo-egg" donation cycles, there were seven eggs and six embryos per recipient.[25] Importantly, in the same study, there was no difference in the results, in that both implantation and clinical pregnancy rates were similarly high in both groups ("fresh" and "frozen" donor egg).[25] The only significant difference was observed in the number of embryos cryopreserved: in the fresh donation group it was 17.3, and in the "cryo" donation group it was 1.6 per recipient.[25] While cryopreserved, supernumerary embryos provide a "safety back-up" option if a recipient does not get pregnant in the initial cycle (or if the recipient wishes to have more children), most of the time recipients usually wish to have only one child, as most recipients are typically in their forties, when an extended family is not planned. This therefore means that the number of extra embryos after "cryo-egg" donation is minimized, which ultimately means fewer ethical/moral issues for patients (and clinicians) as to the fate of those frozen embryos.
9. The waiting time for a treatment cycle is usually different as well when using a "fresh" donor compared to a donor egg bank. Even in a country where there are no restrictions on egg donation (such as the United States), it may take several months to a year to find a fresh donor that a recipient considers to be an acceptable match. However, when using a donor egg bank, typically there is a wide range of donors to choose from, whose oocytes are already retrieved and stored cryopreserved. Thus, it is not only easier to find an acceptable match, but the treatment can start within weeks, with no need to wait for the donor to start her stimulation.
10. The need for "synchronization" is a clear challenge when using fresh donation, not only for the reason of adjusting the stimulation of the donor with the protocol of the recipient, but also in terms of finding an acceptable time period when both the donor and recipient can undergo the procedures at the same time. When using donor egg banking, both donors and recipients can choose the time that best fits them, as their treatment procedures are independent from each other. Because many of the donors are students, they have more specific times of the year when they can donate more conveniently; and similarly, recipients can also choose the time period that best fits their schedules. This convenience factor makes donor egg banking so much more successful, in addition to the reduced financial burden with no wait time.

CRYOPRESERVATION AS A TECHNOLOGICAL REQUIREMENT

Cryopreservation has been one of the basic IVF procedures for several decades. Successful cryopreservation of human semen was reported in 1954.[26] Cryopreservation of embryos was introduced in humans in 1983 by establishing a pregnancy using a frozen–thawed cleavage-stage embryo.[27] Since that time, embryo cryopreservation has spread worldwide, and has now become a routine procedure. On the other hand, cryopreservation of human oocytes has remained ambiguous. Although the first success with a cryopreserved egg was reported in 1986,[28] just 3 years after embryo cryopreservation, the efficiency remained low, making it unsuitable for routine clinical use. For nearly two decades, there were only a few case reports reflecting the technical challenges with oocyte cryopreservation.[29–31] The first report of pregnancies from cryopreserved donor eggs was in 1998 from a study performed by Tucker et al.[32] Nevertheless, there were very few births reported using cryopreserved oocytes during these decades, and according to Noyes and colleagues,[33] there were fewer than a thousand babies born during the period of 1986–2008 from cryopreserved eggs.

These low success rates may be attributed to two main causes: (1) the type of cryopreservation technique applied; and (2) the sensitivity of human oocytes to cryopreservation. Based on the initial relatively high success rates with embryos using slow freezing, the same technique was applied for oocyte cryopreservation as well. However, as has been demonstrated later, slow freezing is not the optimal choice for egg cryopreservation. Secondly, human oocytes are large cells with high cytoplasmic volumes and with relatively small surfaces. Additionally, mature, metaphase II (MII) oocytes have a strongly temperature-sensitive spindle that can easily be disrupted when exposed

to lower temperatures, even if just for a few minutes.[34] Moreover, the membrane properties of a MII oocyte are substantially different from a similarly sized zygote, possibly because of the type and density of aquaporin, a protein channel that is responsible in part for water transport.[35] It has also been described in animal models that exocytosis of cortical granules may occur in response to cryopreservation,[36] possibly resulting in the hardening of the zona pellucida. This may lead to lower fertilization rates when conventional insemination is performed after thawing; and since ICSI was not developed until 1992,[37] this further added to the challenge of egg cryopreservation. For these reasons, today, ICSI is regarded as a standard procedure for inseminating oocytes that have been cryopreserved.[38,39] Despite much research and protocol modification, slow freezing remains largely inefficient for human oocyte cryopreservation.[40–44]

VITRIFICATION AS A SUPERIOR TECHNOLOGY FOR OOCYTE CRYOPRESERVATION

Not until the introduction of a technique called vitrification in human oocyte cryopreservation[45] was it possible to obtain improved results. During vitrification, the same cryoprotectants are used as during slow freezing—propanediol, dimethyl sulfoxide (DMSO), glycerol, and ethylene glycol (EG) (permeating cryoprotectants); and glucose, sucrose, and Ficoll (nonpermeating cryoprotectants)—but at different concentrations. During vitrification, a combination of cryoprotectants (most frequently EG/DMSO plus sucrose) has a much higher concentration than that which is applied in slow freezing; however, the exposure time to those solutions is much shorter.[46–48] In addition to the higher concentration of cryoprotectants, a very high speed of cooling has to be achieved in order to be able to obtain vitrification. Vitrification occurs when a solution becomes solid without crystallization; this is also called as a "glass-like" state, which is fundamentally different than slow freezing, where the extracellular solution crystallizes. To achieve the highest cooling rate, it is typical to use a "minimal volume" (such as 0.5 μL) on an "open" carrier, such as the CryoTop or CryoLock, which ensures a cooling rate of approximately 20,000°C/min; this is in stark contrast to the cooling rate during slow freezing, where, at the most critical stage, a 0.3°C/min cooling rate is provided by a programmable freezer.[48,49]

Given the higher cryoprotectant concentrations applied during vitrification, there is a concern about the potential toxic effects of cryoprotectants on the cell.[50] However, a recent study by Vanderzwalmen and colleagues elegantly demonstrated that intracellular cryoprotectant concentrations are actually higher after slow freezing compared to vitrification.[51]

When comparing outcomes of slow freezing with vitrification, it is clear that vitrification is a much more efficient approach for oocyte cryopreservation (and actually also for embryo cryopreservation).[47,52–54] Today, most professionals are using a vitrification protocol that is very similar to that which Kuwayama developed and referred to as the "Kitazato" or "Cryotech" technique.[55,56]

It has also been demonstrated that vitrification has less impact on meiotic spindle organization of the oocyte than slow freezing.[57,58] Embryos obtained from slow-frozen oocytes, however, did not exhibit higher rates of chromosomal abnormalities,[38] despite the higher rate of abnormal spindle morphology.[59] Recently, it was also shown that embryos do not have higher aneuploidy rates when oocytes have been subjected to vitrification.[60] Other studies, looking at the physiology, gene expression, or protein composition of oocytes (or derived embryos), showed that vitrification preserves better oocyte function than slow freezing.[61–65] As a result of much improved outcomes following oocyte cryopreservation (in particular using vitrification), the American Society of Reproductive Medicine has recently lifted the experimental label from the procedure (as was also recommended by some professionals[66]), which has made it possible to introduce it in the clinical routine for different purposes.[67]

DONOR OOCYTE BANKING: STRUCTURE AND FUNCTION

Having the ability to efficiently cryopreserve oocytes has made it possible to use this technology for various indications. Most professionals were expecting that the major use of egg cryopreservation would be for the purpose of fertility preservation (both for medical and social reasons); however, the interest for this currently is still relatively limited. On the other hand, many IVF patients are now using the option of freezing part of their oocytes (instead of freezing embryos), and having only a small number of oocytes inseminated (elective limited insemination), to prevent having larger number of embryos created, which later may create ethical/moral problems about what to do with them if they no longer wish to use them for reproduction purposes.[68] Additionally, egg cryopreservation may be used to rescue an IVF cycle when the husband/partner of the patient is unexpectedly not able to provide a semen sample on the day of egg retrieval. However, the major use of oocyte cryopreservation currently is for the purpose of donor egg banking. This is not really surprising, if we consider that sperm donation has been done through frozen sperm banking for nearly four decades.[69,70] Similarly, now with the emergence of donor egg banks, one can expect that oocyte donation will transition from fresh to frozen eggs. This is facilitated by many factors. Firstly, many studies that investigated the vitrification technique were using donor oocytes in humans (it is usually easier to obtain oocytes from donors for research purposes), thus there is plenty of evidence available on the efficient use of donor eggs, where results are comparable to fresh donation.[25,71–77] Additionally, there is growing evidence that cryopreservation/vitrification of oocytes does not carry increased risks for birth defects of

babies born from the procedure, which is important from the safety point of view for the technique.[33,77–80]

Donor egg banks initially were developed within the framework of an already-existing IVF clinic to supply their own recipients. Some of these clinics later expanded their donor pools, creating a larger number of donors and cryopreserved donor eggs being available, which could be offered to other clinics and their patients. Looking at the size, structure, and function of these donor egg banks, there are potentially three different types: (1) a donor egg bank that is associated with a single IVF clinic, which only supplies the recipients of the clinic; (2) a donor egg bank that is based on collaboration between several IVF clinics, and (most or all of) those IVF clinics contribute to the "donor pool" by processing donors by performing donor egg retrieval and egg cryopreservation; these cryopreserved donor eggs are then available for patients/recipients of any of those clinics that are part of this association; and (3) a donor egg bank with a single or multiple IVF center(s) that are associated in order to allow donor egg retrieval and donor egg cryopreservation; these cryopreserved oocytes are then available to virtually any IVF clinics (and to any patients) who might wish to use them. This third type of donor egg bank is probably the most similar to donor sperm banks in structure and function. One would expect that with further development of donor egg banks, they will eventually more likely function like donor sperm banks.

As donor egg banking is a very recent development, there are as yet no established procedures on reporting their results and obtaining other relevant information. For this reason, three well-known donor egg banks were contacted directly, and their outcome data were requested. It is much appreciated that all three of them were willing to provide these data. It is also important to note that, due to the differences of patient population, treatment approach, structure/function, data collection, and reporting, none of these outcome data should be compared directly. Some of the basic outcome data from Donor Egg Bank USA are presented in Table 15.1. Some of the basic outcome data from My Egg Bank North America are presented in Table 15.2. Some of the basic outcome data from IVI are presented in Table 15.3.

When looking at some of these outcome parameters, it is interesting to note that the different donor egg banks do have many similarities, even though they were developed independently from each other, as referred to in an earlier publication.[75]

Table 15.1 Outcome Data of Donor Egg Bank USA

Criteria	Donor egg bank USA
Donation cycles	2,192 egg lots, 13,636 frozen eggs
Recipient cycles	1,303
Mean age of recipients	42.2 years
Total (mean ± SD) oocytes cryopreserved per donor	13,636 (22.17 ± 0.82)
Total (mean ± SD) oocytes warmed per recipient	8,096 (6.21 ± 0.76)
Total (mean ± SD) oocytes survived per recipient	6,966 (5.45 ± 1.61)
Total (mean ± SD) oocytes for intracytoplasmic sperm insemination	6,906 (5.30 + 1.61)
Average 2PN intracytoplasmic sperm insemination fertilization rate	76.6%
% of good-quality embryos on day 3 (per inseminated oocyte)	64.7%
% of good-quality embryos on day 5 (per embryo subjected to extended culture)	60.5%
Implantation rate	33.6%
Total embryos cryopreserved	1,133
Clinical pregnancies (rate/transfer)	49.1%
Infants born	547
Embryo warming cycles with transfers (originating from cryopreserved donor egg frozen embryos)	269
Clinical pregnancies from embryo warming cycles	38.3%
Infants born from embryo warming cycles	78

Source: Courtesy of Donor Egg Bank USA.

Table 15.2 Outcome Data of My Egg Bank North America

Egg donor cycles	1,035
Vitrified oocytes	23,060
Mean oocytes/donor	22.3
Recipient cycles	3,424
Oocytes warmed	21,462
Oocytes warmed/recipient	6.3
Survival rate	88%
Fertilization rate	78%
Clinical pregnancies	1,781
Clinical pregnancy rate	52%
Babies born (to date)	1352

Source: Courtesy of My Egg Bank North America.

SUMMARY

Donor egg banking is a newly emerged service providing an alternative to fresh oocyte donation. The technological base of this service is a recently developed, highly efficient oocyte cryopreservation process. Donor egg banking provides several advantages over fresh oocyte donation, such as no need for synchronization (independent schedules for both the donor and recipient), no waiting (the

Table 15.3 Outcome Data of IVI Donor Egg Bank

Oocyte donation cycles	3,467
Oocytes cryopreserved (total)	40,741
Oocytes cryopreserved/donor	11.8
Number of embryo transfers/donation	3,050/3,382 (90.1%)
Number of day 3 transfers	1,627 (53.8%)
Number of blastocyst transfers	1,399 (46.3%)
Number of embryos replaced	5,695
Implantation rate	39.1
Ongoing pregnancy/cycle	1,398 (40.3%)
Number of babies born "fresh ET"	1,674
Number of babies born (deliveries) "cryo-transfers"	632

Source: Courtesy of IVI Donor Egg Bank.

recipient can start immediately), large donor selection (an easier match procedure), quarantine of eggs is possible, results are similar to fresh egg donation, few or no supernumerary embryos (fewer moral/ethical dilemmas), and it is economically less burdensome. It is likely that within a few years we shall experience further development and expansion of donor egg banks, which hopefully will provide additional benefits for patients requiring donor eggs.

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16 Fertility preservation for oncology patients

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INTRODUCTION

About 90,000 reproductive-age women are diagnosed with cancer annually in the United States, with the most common malignances being breast, hematologic, gynecologic, and central nervous system (CNS) cancers.[1] Approximately 25,500 of these cases are breast cancer, with the majority of women having local (61%) or regional (32%) disease.[2] Almost all of these women require chemotherapy with or without endocrine therapy for cure.[3] Thankfully, the majority of young women who undergo breast cancer treatments survive their disease (e.g., 99% of local and 84% of regional breast cancer cases), as do those with other cancer diagnoses, albeit curative treatments can lead to a delay in childbearing and/or render the patient infertile, if not sterile.[2,4] This translates to a relatively high demand for parenthood following cancer treatment. Many women express concerns regarding treatment-related infertility, allowing this risk to influence whether or not they undergo potentially life-saving chemotherapy and/or endocrine therapy.[5,6] The concerns are not unwarranted, as female cancer survivors are significantly less likely to achieve parenthood, with the probability of a first child after cancer diminished by 50% compared with the general population.[7]

Chemotherapeutic drugs, and particularly alkylating agents, cause a significant depletion in the primordial follicle pool and can result in considerable ovarian reserve compromise. One proposed mechanism for the impairment is an upregulation of the PI3K/PTEN/Akt pathway, leading to accelerated follicular recruitment and "burnout."[8] Some cancer-treatment regimens are thought to damage granulosa cells within early growing follicles.[9] Ovarian damage is evidenced by markedly lower post-treatment anti-mullerian hormone (AMH) levels, a hormone produced by granulosa cells that inhibits primordial follicle recruitment.[10,11] Serum AMH decline associated with chemotherapy is known to be dose and regimen dependent, but it is important to appreciate that even low-risk chemotherapeutic regimens can compromise fertility and result in premature ovarian failure as measured by AMH levels, antral follicle count, and/or by clinically evident amenorrhea and subfertility.

Historically, few options existed for the preservation of fertility, namely undergoing a cycle of *in vitro* fertilization (IVF) with embryo cryopreservation. However, the option of creating embryos is not acceptable to all women for a variety of reasons, most significantly when there is lack of partner or sperm source and, less often, ethical and/or religious objections to cryopreserving embryos. The ability to cryopreserve oocytes has revolutionized reproductive autonomy for women, and in fact, when offered the choice at our center between oocyte and embryo cryopreservation, the majority of cancer patients choose oocyte cryopreservation (OC) as a means to preserve their fertility.[12] After more than 10 years of research on OC, the American Society for Reproductive Medicine (ASRM) has lifted its experimental label and this technology is now considered standard of care as a fertility preservation (FP) measure.[13,14]

APPROACH TO THE FP PATIENT

Effectively, caring for the FP patient first requires timely referral from the oncologist to the reproductive endocrinologist. The American Society of Clinical Oncology and the ASRM recommend that all oncologists discuss FP with patients of reproductive age, as well as with the parents or guardians of children and adolescents if therapy has the potential to compromise fertility.[15] While FP referrals are increasing as oncologists become better informed, the proportion of patients referred for FP counseling remains relatively low; one study estimated that less than 10% of all eligible patients (<40 years of age) were referred, even when FP was completely government funded.[16–19] Once referred, patients are often faced with a limited time frame during which they can pursue FP options prior to initiating chemotherapy or radiation. In one study, women with breast cancer requiring neo-adjuvant chemotherapy on average had only 14 days (range 6–26 days) between FP consultation and initiation of chemotherapy; this abbreviated time frame further supports the urgency with which FP must be addressed.[20]

In the United States, the monetary expense of a FP cycle may be cost prohibitive for more than a quarter of patients, and cost influences FP decision-making in half of all eligible patients.[21,22] Insurance coverage for FP services remains inconsistent, and in most states coverage is nonexistent, often leaving patients responsible for the full cost of FP services. In states where third-party reimbursement exists for infertility treatment, coverage unfortunately does not include preventive FP measures including oocyte or embryo cryopreservation.[23] In countries such as the United States where costs are mostly self-incurred, finances more often impact the decision to pursue FP, and therefore these issues should be addressed

as early as possible. The immediacy with which treatment decisions must be made and cycles financed prior to initiating treatment is a significant barrier to patients lacking ample savings. In contrast, in countries such as The Netherlands, where government medical programs cover FP costs, and therefore financial considerations generally do not factor into an individual's decision to pursue FP, surveyed patients were noted to have less decisional conflict about pursuing FP.[19]

Given the tremendous investment involved, including time, emotional, physical, and financial costs, it is imperative that patients are shepherded through the FP process by a multidisciplinary team specialized in all facets of FP treatment. This multifaceted team should include, at a minimum, the patient's oncologist, reproductive endocrinologist, nursing staff, embryology laboratory staff, and patient liaisons. In many institutions, the team also includes psychologists, social workers, and researchers, as well as stress-relief programs to provide seamless care for the patient from the earliest moments of cancer diagnosis. The term "oncofertility" was coined to represent this critical multidisciplinary relationship, and the most effective approach to treating FP patients comes in the form of this patient-centered multidisciplinary care team.[24–27]

Preparing for the FP Process

Patients should be referred for a FP consultation as soon as possible following cancer diagnosis in order to expedite the process prior to chemotherapy or radiation; the reproductive endocrinologist's team should therefore be flexible and able to accommodate last-minute FP appointments as the need arises. Immediately following the initial FP visit, patients benefit from an individual educational orientation session with a dedicated FP team member. Patients are advised to bring a family member, spouse, or friend with them to this visit, as many patients report feeling overwhelmed by the large amount of information transmitted at their initial consultation, leading to an inability to recall many of the details of this visit. Patients often schedule multiple physician visits in a short period of time; however, this often leaves the patient feeling burdened by the quantity and complexity of information. Open communication between patients and providers is crucial during this often overwhelming time for the patient and her family.

During the initial FP visit, a brief review of the normal menstrual cycle and ovarian physiology may be provided, followed by an explanation of how the menstrual cycle is manipulated to pursue oocyte or embryo cryopreservation. A sample calendar is reviewed with the patient to provide a better understanding of her FP schedule. Nursing and physician staff can then review medication administration, provide hands-on injection training and discuss injection troubleshooting. It is imperative to gauge how much information the patient is absorbing, and how she is coping psychologically. It is often beneficial to refer the patient to the team psychologist. Women beginning FP treatment report higher levels of depression and anxiety compared with infertile patients pursuing similar treatments, and many may benefit from professional help.[28] Patients are often surprised by the commitment required during an FP cycle, which can result in patient attrition following the initial visit.

After the initial educational session, the patient will benefit from consultation with the center's financial department to review her financial resources and options, and to apply for medication grants or other relevant FP grants. However, it is important that patients understand that not every patient will qualify for financial assistance, and even if assistance is provided, she may be responsible for the cost of medication refills and future cryostorage expenses. Hopefully, as FP treatment becomes more mainstream, insurance carriers will begin to cover these important services, lessening the patient's financial burden.

APPROACH TO THE MINOR PATIENT

Approximately 16,000 children and adolescents under the age of 19 are diagnosed with cancer every year, with the most common cancers being leukemia, lymphoma, and CNS tumors.[29] Around 1 in 285 children will experience cancer before 20 years of age, but as survival rates approach 80%–90% in childhood leukemia and lymphoma, there are a growing number of cancer survivors for whom survivorship issues like fertility will be an important concern.[30] Ovarian stimulation and oocyte retrieval can be performed in young girls with a functioning hypothalamic–pituitary–ovarian axis. Ovarian stimulation and OC has even been successfully achieved in premenarchal girls on the crest of puberty in our center, as well as by others, further suggesting that menarche is not a prerequisite for OC in patients who have not yet completed the pubertal transition.[31]

The only FP option currently available to very young girls (i.e., remote from puberty) is ovarian tissue cryopreservation (OTC), and only through enrollment in experimental protocols. Transplantation of human cryopreserved ovarian tissue into mice suggests that a high number of follicles survive after transplantation, a large number of primordial follicles remain dormant, and the tissue is responsive to gonadotropins.[32] To date, 24 live births have been reported worldwide from human ovarian tissue transplantation in postpubertal women, including one who was 17 years of age at the time of tissue harvest who now has three children following transplantation.[33] The largest case series involving OTC in children and adolescents (<16 years) includes 58 patients with a mean age of 10.4 years, with the youngest child under 1 year of age; unfortunately, no births have yet been reported from this cohort.[33,34] Due to the young nature of this field and the limited number of ovarian tissue transplantation procedures that have been performed,

the safety and efficacy of the technology as a standard FP measure is yet to be proven.

All of the emotional, physical, medical, and financial considerations that apply to FP in adults also apply to FP in minors, but special considerations must be addressed. In young and adolescent girls considering FP, it is important to maintain an age-appropriate approach, particularly with regard to the patient's sexual history. In virginal patients, it is best to avoid vaginal ultrasonography during initial evaluation or ovarian stimulation/follicular monitoring in order to lower anxiety and avoid risk of injury. Transabdominal ultrasonography is more appropriate for visualizing the ovaries in these cases. Contemporary transabdominal ultrasound machines can afford adequate visualization, particularly once the ovaries have been stimulated to mature multiple follicles. At the time of oocyte retrieval, a smaller-shaft vaginal ultrasound probe can be utilized, preparing both the patient and parents/guardians that minimal bleeding and/or hymenal tearing may occur. The same can be applied to women of any age who remain virginal or decline the use of vaginal ultrasonography. At our center, we have routinely used this approach with success.

The care team must also be cognizant of the young patient's developmental age and ability to cope with her recent diagnosis, the demand to be present for frequent follicular monitoring and blood draws, and the possibility that she fears needles and injections.[35] Every attempt should be made to make the young patient feel more comfortable, including maintaining consistency with her nursing and physician care team, and being cognizant of her emotional maturity level regarding matters as personal as her fertility.

Ethical and Legal Issues Surrounding FP in Minors

FP in children and adolescents under the age of 18 poses unique issues due to questions of informed consent, autonomy, and ownership. While minors are not recognized as legally competent decision-makers, issues of autonomy become more complicated where reproductive rights are concerned. The constitution protects the reproductive rights of minors just as it does those of adults, and parents cannot deprive minors of future reproductive capacity. Parental consent is required for medical intervention in a minor, but the parent's right to decide on a child's treatment is not absolute, particularly when it comes to interventions deemed to be "elective": "First, if the treatment is not medically necessary for the minor, it must not be unreasonably harmful. Second, the treatment must be to the benefit of the minor, and not just to the benefit of the minor's parents or other family members."[36]

When caring for minors, providers must balance responsibilities to the patient as well as to the parents/guardians, and address sensitive issues regarding the emotional and physical consequences of invasive gynecologic procedures in a child. Also of extremely high importance is the issue of gamete disposition in the event of a minor's death.[37,38] The Tennessee Supreme Court ruled that "the existence of the right (of procreational autonomy) itself dictates that decisional authority rests *in the gamete-providers alone*," and this ruling should extend to all gamete-providers, including children and adolescents.[39] Minors undergoing OC who are, by definition, peri- or post-pubertal, and presumably emotionally mature enough to pursue FP, should thus be the only ones with authority to make decisions regarding the disposition of their own gametes. In the case of gametes that are already cryopreserved, no decision should be made as to the use or disposition of the gametes until the individual has reached the age of legal maturity. As a programmatic policy, any minor patient undergoing FP treatment should be contacted for re-consent and consideration of gamete disposition once adulthood is reached, as minor ownership of and laws surrounding property is limited at the younger age.

DISPOSITION OF GAMETES AND/OR EMBRYOS

Consent surrounding the disposition of cryopreserved gametes/tissue in a cancer patient, particularly in the setting of questionable survival, can be complex. Prior to initiating FP treatment, disposition of cryopreserved oocytes, embryos, or ovarian tissue must be addressed in the case of the patient's death. Options include discarding or destroying the gametes/tissue versus donating them/it for research. Alternatively, the tissue can be donated to a family member with the intent of having the deceased person's child, albeit posthumous reproduction remains controversial. Disposition ambiguity is most commonly addressed by the following: "When the decedent's wishes are unknown, a presumption against using gametes for posthumous reproduction should apply."[40] Adding to the dilemma is the limited property ownership capacity of a minor, again making secondary consent regarding gamete disposition a practice standard when the patient comes of age.

Importantly, if gametes are to be donated for use by another specified individual, an addendum should be included in the consent form naming that individual and confirming that the individual is aware of this designation. In these situations, additional infectious disease screening and testing should be performed on the patient ("oocyte donor") whenever possible, in order to satisfy requirements of the Food and Drug Administration (FDA) for directed-oocyte donation.

FP TREATMENT STRATEGY

Historically, embryo cryopreservation was considered the standard-of-care FP measure available to reproductive-age cancer survivors; however, advances in OC in experienced hands have now demonstrated success rates comparable to IVF.[41–43] The availability of OC has revolutionized FP, allowing its pursuit without the requirement

of sperm (directed or donor) at the time of ovarian stimulation and cryopreservation. Embryo cryopreservation may be the treatment of choice for a patient in a stable partnership or marriage, although even in this setting, it may not be acceptable if ethical objections to embryo storage exist.[44] In addition, with the high marital divorce rate occurring in the general population, and in particular among survivors of cancer, reproductive autonomy through OC is preferable in many circumstances.[45] When appropriate, embryo cryopreservation may still carry a distinct advantage in that blastocyst formation has been noted to be consistently higher when using fresh oocytes rather than following OC with warming; consequently, embryo cryopreservation may have the distinct advantage of affording greater numbers of supernumerary blastocysts to be cryopreserved when compared to cycles using frozen–thawed oocytes.[43,46]

Whether FP should be pursued at all requires clinical judgment along with informed decision-making. Factors include the extent and stage of disease, the patient's perceived desire for future parenting, as well as the patient's overall physical and mental health, including comorbidities. The patient's oncologist should be directly involved in the decision-making process; that is, it is not the role of the reproductive specialist to judge, but rather to provide information and counseling regarding the risks and benefits of FP options.

There is no recommended absolute upper or lower age limits within the reproductive span for pursuing OC, but, as with all assisted reproductive technologies, the most important clinical predictor of outcome is oocyte age at the time of cryopreservation.[47] As discussed above, a mature or near-mature hypothalamic–pituitary–ovarian axis is the threshold at which a peri-menarchal or postpubertal adolescent can pursue ovarian stimulation. Other than menopause, no such threshold or optimal upper age limit exists, although at present, success has not yet occurred using oocytes cryopreserved past the age of 42 years, and patients should be counseled accordingly.[48] The appropriate time to pursue FP is "as soon as possible" once a cancer diagnosis has been made. In an older woman, only the patient, her oncologist, and her reproductive endocrinologist can decide whether pursuing FP is worth the investment, and only after assessing the risks and benefits of FP.

CONTROLLED OVARIAN STIMULATION FOR THE FP PATIENT

Ovarian stimulation protocols are tailored to each individual patient. While controlled ovarian stimulation (COS) protocols for FP may not differ dramatically from conventional COS treatment, the following special circumstances may apply.

Random-Start Protocols

In a typical COS cycle, gonadotropin administration begins on day 2 or 3 of the patient's menstrual bleeding (i.e., the early follicular phase), and stimulation typically continues for 10–14 days. For referred FP patients who have only a brief window of opportunity during which to undergo COS, the menstrual cycle may need to be "overridden," with stimulation regimens beginning at a time other than the early follicular phase.[49] Thus, gonadotropin treatment can be randomly initiated within the cycle or, alternatively, gonadotropin-releasing hormone (GnRH) antagonist with or without oral contraceptive pill co-administration can be given for several days prior to initiating gonadotropin therapy. Studies of women undergoing "random-start" protocols are encouraging; thus, initiating gonadotropin injections during the mid-follicular or even mid-luteal phase appears to produce outcomes equivalent to a conventional early follicular-phase start.[50–52]

Aromatase Inhibitors and Selective Estrogen Receptor Modulators

Women with estrogen receptor-positive tumors, such as those that occur in the breast or endometrium, may benefit from the addition of an aromatase inhibitor (e.g., letrozole), or alternatively a selective estrogen receptor modulator (e.g., tamoxifen), to lessen estrogen stimulation during COS.[53] Despite an absence of randomized controlled trials, in addition to no proven requirement for this modification given the brief treatment interval, this protocol maintains an excellent safety profile, with serum estradiol maintained at relatively lower levels throughout the treatment cycle.[54] Lower estrogen levels may provide an added advantage by diminishing the risk of venous thromboembolism (VTE) in a patient at increased risk due to malignancy. Data derived from post-menopausal women receiving estrogen-containing hormone-replacement therapy demonstrate that those administered transdermal estradiol (having significantly reduced serum estradiol levels as compared to women receiving oral therapy) had a significantly lower risk for VTE.[55] Theoretically, one could argue that all cancer patients should be given a lower-dose estrogen protocol to lessen VTE risk. Future research is needed in this area, and VTE will be discussed again later in the chapter. Despite the aforementioned advantages of estrogen suppression during COS, these protocols remove the ability/advantage of using estrogen levels to gauge ovarian response to medication, dosing titration, and determination of the appropriate timing and administration of the final maturation trigger. These disadvantages are currently outweighed by the advantage of diminished circulating estradiol in a patient with a hormone-sensitive tumor.

GnRH Agonist Trigger

Traditional ovulation trigger with human chorionic gonadotropin (hCG) may be problematic in women who intend to begin chemotherapy treatment immediately upon completing FP. hCG retains prolonged activity and has a low clearance rate due to a high degree of glycosylation,[56]

consequently, chemotherapy may be delayed due to symptoms of ovarian hyperstimulation, as well as concerns about low-level hCG representing early pregnancy in a patient receiving chemotherapy. GnRH agonists (GnRHa) are used with great success in GnRH antagonist treatment cycles as a means of triggering the final stage of oocyte maturation. In a small retrospective study, outcomes in breast cancer patients using a GnRHa trigger were equivalent to those of women who had been prescribed traditional hCG.[57] GnRHa may be administered alongside low-dose hCG (~1000 units of intramuscular hCG, or the equivalent dose of recombinant subcutaneous hCG) as a "dual trigger," if concern exists regarding a patient's ability to respond appropriately to a GnRHa trigger with an appropriate pituitary luteinizing hormone (LH) release (i.e., peri-pubertal, hypothalamic amenorrhea).[58] Women with hypogonadotropic hypogonadism, who have demonstrated inappropriately low FSH or LH levels during the early follicular phase or prior to the administration of GnRH antagonists, should be flagged as inappropriate candidates for a single-agent GnRHa trigger.

Treatment protocols should be individualized depending on the patient's age, diagnosis, hypothalamic status, and time constraints. In our center, patients with lymphomas have sometimes demonstrated a poorer response to GnRHa triggers, and in these patients we preferentially utilize a combined GnRHa–hCG trigger for oocyte maturation whenever possible.

FP AND OC LABORATORY PROTOCOLS

The science behind cryobiology and the cryopreservation of embryos and oocytes has been well documented and thoroughly reviewed by past authors.[59–61] As noted earlier, historically, the only option available to patients seeking FP was embryo cryopreservation using controlled-rate slow cooling, originally reported by Testart et al.[62] Success rates using thawed embryos have varied, and depended on an array of factors including the following: embryonic developmental stage, embryo quality, freezing method, and post-thaw manipulation (e.g., removal of lysed cells and/or disruption of zona pellucida integrity). In recent years, there has been a shift away from slow cooling in favor of vitrification as the choice embryo cryopreservation method, and with this has come significantly improved outcomes.[63] Despite advances, embryo cryopreservation continues to fall short in meeting the needs of all patients, particularly those lacking a male partner. For this reason, it is imperative that fertility clinics become experienced and adept at performing OC and warming.

By virtue of its large size (~120 microns), high water content (80%), low surface-to-volume ratio, presence of the meiotic spindle, unique permeability to cryoprotective agents (CPAs), and spherical shape, the human oocyte presents unique challenges to cryopreservation and thawing processes. These qualities make the oocyte exquisitely sensitive to temperature modification, albeit both slow cooling and vitrification methods have been successfully applied to this gamete.[64–66] The principle behind both techniques is the avoidance of intracellular ice crystal formation, a process known to damage cell membranes and critical intracellular organelles such as the meiotic spindle. In recent years, vitrification has become the predominant methodology employed by clinics; importantly, reproducible survival, implantation, and pregnancy rates using this method are now achievable.[65–68]

OC LABORATORY PROCESSES

In preparation for OC, ovarian stimulation and oocyte retrieval are performed in a routine fashion as with traditional IVF. Immediately following follicular aspiration, oocytes are transferred to the laboratory and washed in modified media. They are then placed in bicarbonate-buffered medium supplemented with protein, and allowed to incubate at 37°C in 6% carbon dioxide (CO_2). Approximately 90 minutes later, oocytes are stripped of all cumulus and corona cells using a combination of enzymatic (Cumulase, Halozyme Therapeutics, San Diego, CA) and mechanical techniques, and oocytes are examined for maturity. Metaphase II (mature) oocytes are identified by the presence of a polar body in the peri-vitelline space and are preferentially selected for cryopreservation; however, metaphase I oocytes (those lacking both a polar body and a visible germinal vesicle [GV]), as well as GV oocytes, may also be cryopreserved, but are less likely to result in pregnancy success. Advances in *in vitro* maturation technologies may improve outcomes using immature oocytes in the future, so for this reason, in cancer and young reproductive-age patients having a low oocyte yield at retrieval, oocytes of all stages are currently cryopreserved at our center.

Slow Cooling and Subsequent Thaw

At our center, slow cooling of oocytes has been performed using a technique similar to that described by Fabbri et al.[69] Briefly, oocytes are first washed in a solution containing phosphate-buffered saline (PBS) and then transferred to an equilibration solution containing 1.5 mol/L propanediol (PROH). They are then placed in a loading solution containing 1.5 mol/L PROH plus 0.3 mol/L sucrose. All solutions are supplemented with protein (Plasmanate, Talecris Biotherapeutics, Inc., Research Triangle Park, NC; or human serum albumin [HSA], Cooper Surgical, Inc., Trumbull, CT). Oocytes are then loaded into cryopreservation straws (Conception Technologies, San Diego, CA) and transferred into a controlled-rate freezer (Planer Kryo, Planer Products Limited, Sunbury, UK), where the temperature is gradually lowered from ±20°C to –7°C at a cooling rate of –2°C/min. Ice nucleation is induced manually at –7°C. The temperature is then decreased to –30°C at a rate of –0.3°C/min and then rapidly to –150°C at a rate of –50°C/min.

Straws are then placed into canes and transferred to liquid nitrogen tanks for storage.

During subsequent thaw cycles, straws containing the cryopreserved oocytes are removed from liquid nitrogen, and briefly air-warmed until all traces of ice have disappeared. Straws are then placed into a 30°C water bath for 40 seconds. The CPAs are removed in a stepwise fashion by passing the oocytes through solutions containing decreasing concentrations of the agents as follows: 1.0 mol/L PROH plus 0.3 mol/L sucrose; 0.5 mol/L PROH plus 0.3 mol/L sucrose; 0.3 mol/L sucrose plus PBS; and finally into PBS. All solutions are supplemented with protein (Plasmanate or HSA). Surviving oocytes are then transferred to fresh pre-equilibrated culture media and incubated in a 37°C and 6% CO_2 gas atmosphere until intracytoplasmic sperm injection (ICSI) is performed.

Vitrification Method and Subsequent Warming

Vitrification differs from slow cooling in that oocytes are exposed to shorter yet significantly higher concentrations of CPA, and cooling rates are appreciably faster (i.e., ultrarapid). Quick and high exposure to CPA along with ultrarapid cooling have been described as creating a glass-like state in which the oocytes are suspended, hence "vitrified," from the Latin *vitrum* meaning "glass." Cooling rates in vitrification often exceed 15,000°C/min and are achieved by employing exquisitely small fluid volumes, often with direct exposure to liquid nitrogen. Although several commercially available oocyte vitrification kits exist, none have yet been approved for usage by the FDA.

At our center, vitrification is accomplished as follows: oocytes are first equilibrated in media containing the lowest concentration of CPA (0.30 mol/L ethylene glycol [EG] plus 0.55 mol/L dimethyl sulfoxide [DMSO]) to achieve the first level of dehydration. They are then placed in sequential equilibration solutions with incrementally increasing concentrations of CPA (EG 0.30–1.35 mol/L, and DMSO 0.55–1.10 mol/L) for a total of five dilutions over several minutes. Finally, the oocytes are placed in a vitrification solution containing 2.7 mol/L EG, 2.1 mol/L DMSO, and 0.5 mol/L sucrose for 1–1.5 minutes. All solutions are prepared using PBS and supplemented with protein (Plasmanate). Oocytes are then loaded onto Cryolocks (BioTech Inc., Alpharetta, GA) (usually two oocytes per carrier) and immediately plunged directly into liquid nitrogen, where they are capped and placed in goblets and canes for liquid nitrogen cryostorage.

When the patient is ready to undergo a warming cycle, the Cryolocks containing vitrified oocytes are uncapped under liquid nitrogen immediately before warming. The Cryolocks are then quickly moved from the liquid nitrogen, and the tips where the oocytes are located are immediately immersed in pre-warmed (37°C) thaw medium. The oocytes are then released from the device into 1 mol/L thaw medium. All subsequent handling is performed at room temperature. The oocytes are moved through sequential equilibration solutions containing incrementally decreasing concentrations of sucrose (0.5–0 mol/L) for a total of eight dilutions. Oocytes are then washed once more in sucrose-free medium, and then placed in pre-warmed and equilibrated culture medium (Global media, LifeGlobal Group, IVFonline.com) in a 37°C (6% CO_2) incubator until ICSI is performed.

UNIQUE CONCERNS IN PATIENTS WITH CANCER

Venous Thromboembolism

Cancer, as well as the chemotherapy and hormonal agents used in cancer treatment, all have procoagulant effects, promoting VTE.[70,71] The risk of VTE in cancer patients is significantly greater than in the general population, ranging from 0.6% to >18%.[72] However, a review of the available literature on the risk of thrombosis in women with cancer and in those undergoing ovarian stimulation draws the following conclusions: (1) the cohort of women with cancer seeking FP are likely to be at a lower risk of thrombosis compared to the average cancer patient; (2) avoidance of moderate-to-severe ovarian hyperstimulation syndrome (OHSS) is likely the most important way of preventing complications such as VTE; and (3) anti-thrombotic prophylaxis should be administered sparingly, and only to those patients with additional risk factors or those who develop OHSS.[73]

Thrombocytopenia

Women with hematologic malignancies may have an elevated risk of thrombocytopenia, placing these patients at an elevated risk of surgical bleeding during oocyte retrieval. As with all patients, a complete blood count should be obtained prior to initiating ovarian stimulation, and in patients with thrombocytopenia, the decision to pursue FP should be made in consultation with the oncologist, the reproductive endocrinologist, and the patient. Patients with severe thrombocytopenia may be considered for platelet transfusion immediately preceding oocyte retrieval, followed immediately by transfer to an inpatient hospital ward for close monitoring. The risks and benefits of treatment should be thoroughly discussed at length before proceeding.

Ovarian and Pelvic Masses

In women who have an ovarian tumor and are planning to undergo ovarian stimulation and OC prior to ovarian surgery, if the contralateral ovary is present and unaffected, oocytes can usually be retrieved from that ovary. Oocytes may also be carefully and deliberately retrieved from the affected ovary or ovaries; however, if attempting such a procedure, careful attention should be paid to avoiding puncture of the tumor during retrieval. If a question exists regarding the boundaries of the tumor, it may be prudent to avoid retrieval of oocytes in that area. Intraoperative tumor rupture is thought to result in peritoneal tumor

metastasis through spillage of tumor cells, leading to a higher stage and therefore a poorer prognosis, and for this reason particular caution should be taken when operating in close proximity to a suspicious tumor.[74] In addition, in patients that have relatively large tumors in the pelvic area (e.g., a bladder, cervical, or gastrointestinal malignancy), increased care must be taken when performing oocyte retrieval as local vasculature may be intensified, increasing the risk of bleeding in these patients.

FP OUTCOMES

Clinical Stimulation Parameters

While OC remains in its relative infancy, clinical outcomes are overwhelmingly reassuring. One center reported fewer oocytes retrieved from patients with hormone-dependent cancer compared with those with nonhormone-dependent cancer, suggesting that certain malignancies may affect ovarian response prior to therapy.[75] However, currently available literature supports the notion that FP patients who have not yet been exposed to chemotherapy have similar ovarian reserve parameters, responses to gonadotropin stimulation, and yields of oocytes and embryos compared with controls.[76–78]

Outcomes following oocyte thaw or warming

Data from our center suggest that pre-cryopreservation oocyte quality is equivalent regardless of the indication for OC.[64,67,79] Meiotic spindles are almost always visible prior to cryopreservation and following oocyte thaw, oocyte survival following thaw/warming routinely approaches 80%–90%, and live birth outcomes using cryopreserved oocytes are equivalent to IVF with fresh oocytes. Four randomized controlled trials have demonstrated comparable live birth rates following IVF using cryopreserved and fresh oocytes.[41,42,80,81] Notably, other data have suggested impaired blastocyst formation rates following fertilization of cryopreserved as opposed to fresh oocytes, with concomitantly fewer good-quality supernumerary embryos available following embryo transfer.[43,46] However, no increase in aneuploidy has been noted in blastocysts derived from cryopreserved oocytes.[46,82] Importantly, a review of over 900 children born following OC demonstrated no increase in congenital anomalies, and another review of 200 infants born following oocyte vitrification showed no difference in mean birth weight or incidence of congenital anomalies compared to fertile and infertile controls.[83,84] Given the relatively recent application of OC technology, no long-term data are available on outcomes of these children, but early data are reassuring.

CONCLUSION

OC is changing the landscape of FP, allowing women to maintain reproductive autonomy when faced with a life-altering diagnosis. This technology can offer hope to women confronted with potential fertility loss due to cancer or nonmalignant diseases, and is increasingly providing reproductive flexibility to women facing age-related fertility decline.[85] As the technology spreads worldwide and indications for OC expand, cryopreservation will undoubtedly continue to open reproductive doors for women.

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17 Vitrification of human ovarian tissue

Mona Sheikhi and Outi Hovatta

Cryopreservation of human ovarian tissue has been carried out for more than 18 years.[1,2] Successful studies in experimental animals with live births following transplantation of cryostored ovarian tissue in rodents and sheep had been performed earlier.[3–6]

In the beginning, slow-programmed freezing was utilized. It was also widely applied clinically. For the time being, we can estimate that more than 30 children have been born in this way. They have been summarized in several articles. The first live birth after slowly frozen human ovarian tissue was reported already in 2004,[7] followed by many others later on.[8–16] Vitrification of human ovarian tissue was the evident next step for improving this methodology.

Vitrification of rodent ovarian tissue was performed at first.[17–20] Rodents have a looser structure within their ovarian tissue, but successful vitrification of ovarian tissue was soon reported in rabbits and nonhuman primates. Ovarian tissue survival after transplantation into rat uteri,[21] showing the survival of vitrified–warmed ovarian tissue after uterine transplantation. Sheep was the obvious next species to study,[22] metaphase II were obtained after *in vitro* maturation of isolated follicles from vitrified sheep ovarian tissue. Corbiere et al.[23] were able to vitrify whole sheep ovaries by perfusing them through their vessels with vitrification solution, and bathing them in the solution. The survival of the tissue was not optimal in the sheep ovary, probably due to the large size of the ovary and the time required for the vascularization,[24] although the birth of four lambs after auto-transplantation of vitrified warmed ovarian cortical tissue into ewes was reported.

Nonhuman primates have been excellent for modelling human ovarian vitrification.[25] Vitrified ovarian tissue was obtained from five adult baboons, and the tissue was autografted after 5 months. The vitrification medium contained ethylene glycol (EG) and dimethyl sulfoxide (DMSO) as cryoprotectants. After vitrification, warming, and long-term grafting, the follicles were functional and morphologically normal as demonstrated by growth and immunostaining for Ki67, anti-mullerian hormone and growth differentiation factor-9. In addition, the stromal tissue appeared normal and vascularized.

Another group confirmed the feasibility of a closed vitrification system using macaque ovarian cortical tissue.[26] They used a combination of EG and polymers, and a combination of EG and DMSO with cooling in liquid nitrogen (LN_2) vapor and a two-step warming procedure. The samples were vitrified in high-security straws (Cryobiosystemes–IMV, Aigle, France). The viability of the tissue was confirmed by normal light microscopic morphology of the follicles and stromal tissue, the growth of the follicles in culture, and by the production of progesterone, androstenedione, and estradiol. Only the rate of antrum formation was slightly smaller in the tissue following vitrification than it was in the control tissue during culture.

VITRIFICATION VERSUS SLOW FREEZING OF HUMAN OVARIAN TISSUE

In our fertility unit at Karolinska University Hospital, Huddinge, we have offered ovarian tissue cryostorage since 1998, using at first the method that we developed at Imperial College at Hammersmith Hospital in London, UK.[1] We then improved it by using a serum-free medium.[27] This slow-programmed freezing in our unit has been proven to be effective, as determined by a live birth after orthotopic transplantation.[28]

We carried out a study in which we compared the slow controlled-rate freezing using 1,2-propanediol (PrOH) and sucrose as cryoprotectants for the vitrification of pieces of ovarian tissue from the same donors who underwent Cesarean section.[29] The outcome was measured by light and electron microscopy in fresh, cryostored, and fresh and cryostored cultured tissue. The cryoprotectants used in slow freezing were PrOH–sucrose and EG–sucrose. For vitrification, tissues were incubated for 5 or 10 minutes in three solutions containing a combination of DMSO, PrOH, EG, and polyvinylpyrrolidone (PVP). Vitrification using a combination of PrOH, EG, DMSO, and PVP was comparable to slow freezing in terms of preservation of follicles in human ovarian tissue. Ovarian stroma had significantly better morphological integrity after vitrification than after controlled-rate freezing. We did not find any light or electron microscopic signs of apoptosis in vitrified or slow-frozen human ovarian tissue.

Amorim et al.[30] compared slow freezing and two vitrification protocols for human ovarian tissue using EG and polymers as cryoprotectants. They demonstrated normal morphology of the follicles after warming and at 1 week after xenografting. Vitrification with both protocols caused less apoptosis, as shown by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, when compared to slow freezing.

Previous studies had used protocols incorporating direct contact with LN_2 for the first attempts at ovarian

tissue vitrification; however, we decided to develop a vitrification system in which no direct contact with LN_2 was needed.

Vitrification was carried out using 1.8 mL NUNC cryotubes (Nunclon, Roskilde, Denmark) with an internal thread cap to make it leak-proof. The tissue pieces were carefully transferred into the cryotube with a minimum volume of the vitrification medium. We did this because it is very difficult to sterilize LN_2. There are reports indicating that microbes are able to survive in LN_2.[31] Sterilizing LN_2 is difficult, and filters do not necessarily remove small viruses. LN_2 has a surface tension that prevents filtering without pressure, and applying pressure to this easily evaporating liquid may cause an explosion. Using a vacuum is feasible, but this is not simple, and it requires special equipment. We studied a closed and simple vitrification method by enclosing the ovarian tissue sample within a cryotube that was tightly sealed and then immersed into LN_2. According to our experiments, vitrification in cryotubes was fast enough to allow full morphologic integrity of all components of human ovarian tissue to be maintained, as revealed by light and electron microscopy.[32] Electron microscopy or caspase-3 immunostaining did not reveal any signs of apoptosis, and we did not see any necroses in the tissues either.

To avoid any performance-related risks during the laboratory procedure that might be overly complicated, we simplified the methods from the use of three permeating cryoprotectants to one, in which we used only EG with the addition of nonpermeating Ficoll 70 (w/v). This approach has resulted in as good an integrity and viability in tissue culture as the combination of the three cryoprotectants used in the previous protocol.[33] All of our procedures have been chemically defined and are free of any xeno-products (Figure 17.1a and b).

Since our first article describing human ovarian tissue vitrification,[29] several articles regarding ovarian tissue vitrification have been published. Many of them confirm our results,[34–36] although some authors have reported necroses after vitrification.[37] Importantly, it has been noted that estradiol release in culture has been similar in both control and post-vitrification tissue.[38]

The differences in these results may be explained by the fact that vitrification is not a single procedure. There are many contributing factors, such as the time of incubation and warming, and the concentrations of the various components in the vitrification solutions vary between studies. Additionally, the culture conditions also vary. This procedure is always attempting to strike a balance between proper tissue penetration by the cryoprotectants and their inherent toxicity of the components to the ovarian tissue.

CONCLUSIONS

From our experience and a number of other studies, it is clear that cryostorage of human ovarian tissue is feasible.

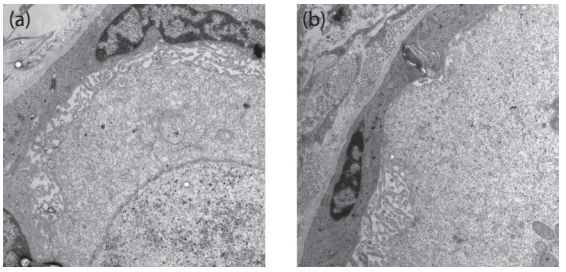

Figure 17.1 **Transmission electron micrographs of vitrified human ovarian tissue. (a) Human ovarian follicles within cortical tissue that was vitrified using a combination of dimethyl sulfoxide, 1,2-propanediol, and ethylene glycol as permeating cryoprotectants. There is a morphologically normal oocyte with well-preserved mitochondria and a granulosa cell with normal morphology. The cellular membranes look unaffected, and the extracellular matrix filaments outside the granulosa cell layer appear intact. Intact protrusions between the oocyte and the granulosa cell can be clearly seen. (b) Human ovarian tissue vitrified using only ethylene glycol as a permeable cryoprotectant. Intact structure of the membranes, the cytoplasm of the oocyte, the granulosa cells, and the extracellular matrix can be seen, as well as the finger-like protrusions between the oocyte and the granulosa cells.**

It is indeed possible to obtain normal electron microscopic morphology and viability, growth, and hormone secretion after tissue culture is performed following warming of ovarian tissue. The viability of the ovarian stroma has been shown to be somewhat better preserved, according to several more recent reports. Vitrification of human ovarian tissue has not been utilized extensively as yet, so at this point we still lack the final proof of successful vitrification for human ovarian tissue in the form of clinical pregnancies and live births; nevertheless, we believe that these will come in due course, probably paving the way for vitrification to become the standard approach to ovarian tissue cryopreservation.

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18 Vitrification of cleavage-stage embryos and blastocysts and their neonatal outcomes

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INTRODUCTION

In assisted reproductive technology (ART), cryopreservation of embryos has become important for the best use of supernumerary embryos. During the steps of the cryopreservation of embryos, there is a risk of various types of injury.[1,2] Among them, the formation of intracellular ice appears to be the most damaging. The first strategy for preventing intracellular ice from forming was to adopt a lower concentration of cryoprotectants and a long slow-cooling stage. This slow-freezing method has proven effective for the embryos of a wide range of mammalian species. Unlike embryos of laboratory animals and domestic animals, in which dimethyl sulfoxide (DMSO), glycerol, or ethylene glycol (EG) are commonly used as the cryoprotectants (cryoprotective agents [CPAs]), human embryos at early cleavage stages have most often been frozen in a solution of propanediol (PROH) supplemented with sucrose,[3] although those at the blastocyst stage have more frequently been frozen with glycerol and sucrose.[4–6] With slow freezing, however, it is difficult to eliminate injuries occurring from ice formation completely. Furthermore, the slow-freezing method requires a relatively long period to be undertaken before the embryos are finally stored in liquid nitrogen (LN_2).

In 1985, Rall and Fahy[7] applied the innovative approach of "vitrification," in which injuries related to ice crystal formation are minimized by using very high concentrations of CPA together with rapid temperature change. The definition of vitrification is the solidification of a solution at a low temperature without the formation of ice crystals, by increasing the viscosity using high cooling rates.[1,7] The rapid cooling process can minimize chilling injury and osmotic shock to the embryos. With improvements in recent decades, vitrification has become the most reliable strategy, not only because it is technically simple, but also because it can lead to high survival and implantation rates. To induce vitrification in LN_2 or supercooled air,[8] the solution must contain a high concentration of CPAs. This approach simplifies the cooling process, because embryos can be rapidly cooled directly in LN_2. Although embryos subjected to vitrification are potentially liable to being affected by the toxicity of the high concentration of CPAs, the method has been refined and proven to be effective for the cryopreservation of embryos at various stages of development in laboratory and domestic species. In 1998, it was demonstrated that vitrification using an EG-based vitrification solution (EFS40)[9] with conventional cryostraws was relatively effective for human embryos at the four- to eight-cell stage.[10] The effectiveness of vitrification was confirmed for human embryos at the 8–16-cell stage[11] and the morula stage,[12] also using EG-based solutions.[13]

Over the past decade, there have been several advances in vitrification technologies such that it can provide high clinical efficiency along with better clinical outcomes. It is proposed that vitrification will become the most suitable method for the cryopreservation of any cells and tissues in the near future. Thus, this chapter will focus on vitrification technologies for cryopreservation in human ART.

PRINCIPLES OF VITRIFICATION

The basic procedure for vitrification is simple. Embryos are suspended in a vitrification solution and then plunged in LN_2 or supercooled air. Embryos are warmed rapidly and diluted quickly with a sucrose solution. The most important stage is the exposure of embryos to the vitrification solution before rapid cooling. In order to prevent intracellular ice from forming, a longer period of exposure is desirable. However, if the exposure is too long, embryos suffer from the toxicity of the CPA solution. Therefore, the optimal exposure time for successful vitrification must be a compromise between preventing the formation of intracellular ice and preventing toxic injury. Ironically, embryos may be injured by the toxicity of the cryoprotectant before enough cryoprotectant can permeate inside the embryos. To prevent this, a two-step procedure is commonly adopted, in which embryos are first equilibrated in a more dilute (e.g., 10%) CPA solution, followed by a brief (30–60-second) exposure to a vitrification solution before embryos are cooled with LN_2. The optimal exposure time in the vitrification solution depends not only on the CPA solution, but also on the temperature, since both the permeability of embryos and the toxicity of the CPA are largely influenced by the temperature.[6,7]

In vitrification, the selection of the CPA requires extreme care because its concentration can be as high as 6 M, which can make the toxicity of these compounds a key limiting factor in cryobiology. The most appropriate characteristics of a penetrating CPA are low toxicity and high permeability. For the cryopreservation of human embryos, PROH and DMSO have been used as the dominant CPAs, although glycerol is used when embryos are

frozen at the blastocyst stage.[6] As a less toxic CPA, EG is commonly and widely used.[2] However, few comparative studies have examined the effect of the CPA on the survival of vitrified embryos.

CLEAVAGE-STAGE EMBRYO VITRIFICATION

In 1998, an investigation was conducted to find a suitable CPA and suitable conditions for exposing embryos to a vitrification solution using eight-cell mouse embryos.[10] The survival rates of eight-cell mouse embryos vitrified in various solutions after exposure to the solutions for 0.5 and 2 minutes at 20°C and 25°C were measured. The highest rates of survival were obtained with EG-based solutions, regardless of the time and temperature. Although none of the vitrified embryos were morphologically normal when the embryos were vitrified after 0.5 minutes of exposure to any mixture of 30% CPA, the survival rate was over 90% when embryos were treated for a longer time (2 minutes) at a higher temperature (25°C), or when embryos were treated with a higher concentration of EG (EFS40) at a higher temperature (25°C).

In addition, a small saccharide (e.g., sucrose) and a macromolecule (e.g., Ficoll 70, bovine serum albumin [BSA], or polyvinylpyrrolidone [PVP]) are frequently included in vitrification solutions. These nonpermeating agents are much less toxic, and are known to promote vitrification of the solution.[9] Therefore, their inclusion can reduce the toxicity of the solution by decreasing the concentration of the permeating agent required for vitrification. In addition, inclusion of a saccharide promotes shrinkage of embryos, and thus reduces the amount of intracellular cryoprotectant, which will also reduce the toxic effect of the permeating CPA.[9] At the same time, the osmotic action of saccharides plays an important role in minimizing the swelling of embryos during dilution, since a quick dilution is necessary to prevent the toxic effects of the CPA solution.

PROTOCOLS AND CLINICAL RESULTS OF CLEAVAGE-STAGE EMBRYO VITRIFICATION

There are several protocols that have been introduced for human cleavage-stage embryo vitrification. However, in these protocols, the basic concept is similar, and the differences between the protocols are related to the type and concentration of CPAs and duration of exposure to CPAs. A summary of those protocols and clinical outcome are briefly described as follows and appear in Tables 18.1 and 18.2.

Vitrification Using Conventional Cryo-Straws for Cleavage-Stage Embryos

This is a two-step protocol for vitrification with straw as a container using EG-based solutions, EFS20 and EFS40, and has been described previously.[1,10] The two solutions (EFS20 and EFS40) are used for pretreatment and vitrification, respectively, and contain EG diluted to 20% (v/v) or 40% (v/v), plus a Ficoll–sucrose solution. This method has proven suitable for human cleavage-stage embryos,[10] including in Mukaida et al. (unpublished data). In 1998, the effectiveness of this vitrification method for human cleavage-stage embryos was confirmed.[10]

Vitrification Using the Cryoloop for Human Cleavage-Stage Embryos (Tables 18.1 and 18.2)

An improvement to cleavage-stage ultrarapid vitrification came with the Cryoloop.[14–17] This method is effective for human cleavage-stage embryos, for which conventional vitrification using a straw was found to be less effective. The protocol for vitrification using the Cryoloop can be found in the following section on the vitrification of blastocysts.

In 2008, Balaban et al.[18] reported a two-step protocol using Cryoloop vitrification for human cleavage-stage embryos cryopreservation. Their protocol (Table 18.1) was originally described by Larman et al.[19] Two steps of dehydration and equilibration of CPA were applied prior to cooling. The embryos were loaded onto the Cryoloop (Hampton Research, Aliso Viejo, CA), transferring as little medium as possible, typically around 50 nL. For warming, multiple steps of rehydration with several different sucrose concentrations were performed. Clinical outcomes were as follows (Table 18.2): a total of 73 women subsequently underwent vitrified–warmed embryo transfers, where a mean number of 3.3 embryos were warmed (n = 241). The cryosurvival rate was 92.1%, and all blastomeres were intact in 72.1% of the embryos after the warming procedure. The mean number of embryos transferred was 2.3 (n = 168), and clinical pregnancy and ongoing pregnancy rates of 49.3% and 45.2% were achieved, respectively. The implantation rate was 29.7% (n = 50), resulting in a multiple pregnancy rate of 36.1% (n = 13: 1 triplet, 12 twins). At the time of reporting, 8 of the ongoing 33 pregnancies had had successful deliveries of healthy children (2 twins, 6 singletons). Moreover, in this study, it was shown that vitrification was a more effective approach for cryopreserving human embryos than conventional slow freezing.

In 2007, Desai et al.[20] reported the post-vitrification development, pregnancy outcomes, and live births for Cryoloop vitrification of human cleavage-stage embryos. Tables 18.1 and 18.2 include their protocol and results, which presented consecutive vitrification–warming cycles performed over a 2.5-year interval.

In 2005, Rama Raju et al.[21] reported a modified protocol for the vitrification of human eight-cell embryos using the Cryoloop technique. The protocol, including the type of CPA and duration of exposure, is different from the one by reported by Desai (Table 18.1).[20] Table 18.2 includes results to show the effectiveness of their protocol.

Vitrification Using Cryotops for Human Cleavage-Stage Embryos

Since the vitrification approaches of the Cryotop and Cryoloop use similar minimal-volume cooling systems,

Table 18.1 Summary of the Protocols Regarding Concentration, Time, and Properties of the Vitrification Solution for Day 2–3 Human Embryo Cryopreservation

	Mukaida et al.[10]	**Desai et al.[20]**	**Rama Raju et al.[21]**	**Kuwayama et al.[22]**
Type of container	Cryo-straw	Cryoloop (1)	Cryoloop (2)	Cryotop
Temperature	Room (25–27°C)	Warm stage (37°C)	Warm stage (37°C)	Room (25–27°C)
Equilibration	EGFS20: 20% EG	7.5% EG + 7.5% DMSO	10% EG	7.5% EG + 7.5% DMSO
Step	2 min	2 min	5 min	5–10 min*
Vitrification	EGFS40: 40% EG	15% EG + 15% DMSO + F + S	40% EG + S	15% EG + 15% DMSO + S
Step	1 min	35 s	30 s	1 min
Cooling system	Vapor-phase LN_2 (3 min), then plunged into LN_2	Plunged into LN_2 directly (ultrarapid cooling)	Plunged into LN_2 directly (ultra-rapid cooling)	Plunged into LN_2 directly (ultrarapid cooling)
Warming	One step	Two steps	Four steps	Two steps
Step	0.5 M S (5 min)	0.25 M S (2 min)	1 M S (2.5 min)	1 M S (1 min)
		0.125 M S (3 min)	0.5 M S (2.5 min)	0.5 M S (3 min)
			0.25 M S (2.5 min)	
			0.125 M S (2.5 min)	

Note: In the first row, the authors and reference number of each protocol are indicated. In the second row, the types of containers used for vitrification are indicated. In the third row, the temperature of each protocol performed is indicated. In the fourth row, the first step of the vitrification protocol that required dehydration and permeation of the cryoprotective agent (CPA) is described; the type of CPA and duration of exposure to CPA are indicated. Permeating CPAs are shown in bold. In the fifth row, the second step of the vitrification protocol prior to plunging LN_2 is described, in the same way as the fourth row. In the sixth row, the method of cooling is indicated. In the seventh row, the warming steps are described as single or multiple steps of different sucrose concentrations. (DMSO = dimethyl sulfoxide; EG = ethylene glycol; F = Ficoll; LN_2 = liquid nitrogen; S = sucrose.)

*The duration of equilibration is adjusted according to the time needed for re-expansion of the vitrified embryos. Cryoloop (1): reported by Desai et al. in 2007.[20] Cryoloop (2): reported by Rama Raju et al. in 2005.[21]

Table 18.2 Summary of the Clinical Results in Each Vitrification Approach for Day 2–3 Embryos

	Mukaida et al.[10]	**Desai et al.[20]**	**Rama Raju et al.[21]**	**Results of Nagata Clinic**
Type of container	Cryo-straw	Cryoloop (1)	Cryoloop (2)	Cryotop
Age (years)	Not available	34.1 ± 4.5	31.3 ± 4.5	35.0 ± 4.5
No. of cycles	127	77	40	604
				346 patients
Survival rate	Not available	201/236	121/127	1701/1774
		85%	95%	95.9%
Cleavage rate[a]	486/661	184/236	Not available	1289/1774
	76%	78%		72.7%
Pregnancy rate	34/127	34/77	14/40	164/604
	26.8%	44.2%	35.0%	27.2%
Implantation rate	Not available	40/201	18/121	192/1442
		19.9%	14.9%	13.3%
Delivery rate[b]	22/127	Not available	13/40	118/604
	17%		32.5%	19.5%

Note: Cryoloop (1): reported by Desai et al. in 2007.[20] Cryoloop (2): reported by Rama Raju et al. in 2005.[21]

[a] Including survival and further cleavage rate.

[b] Including on-going pregnancy.

the basic concept of these protocols are the same. The following protocol was originally introduced by Kuwayama.[22]

For vitrification using a Cryotop, the protocol is similar to blastocyst vitrification using a Cryoloop technique described in the section on blastocyst vitrification. The differences with the protocol using the Cryotop are duration of equilibration of the CPA for cooling steps and concentration of sucrose for warming steps. Embryos with 70% or more intact blastomeres are considered as survivors and kept in culture until transfer on the following day (Table 18.1).

Table 18.2 includes the results from the use of the Cryotops at Nagata Clinic to show the effectiveness of this clinical application for human cleavage-stage embryos.

BLASTOCYST VITRIFICATION

Recent advances in culture systems with sequential media have made it possible to develop human *in vitro* fertilization (IVF) embryos to the blastocyst stage more routinely. As the blastocyst is better suited to the uterine environment and blastocyst formation is one of the more useful informational criteria for selecting more viable embryos, blastocyst transfer has become a promising option for raising the overall pregnancy rate.[23,24] Accordingly, the need to cryopreserve human blastocysts is increasing. Menezo et al.[3] cryopreserved human blastocysts that were developed in a co-culture system using the slow-freezing method with glycerol and obtained reasonable clinical results (27% pregnancy rate and 17% implantation rate). However, results reported by other clinics have not been consistent.[25–27] Menezo et al.[3] speculated that the cryopreservation outcome might be influenced by the culture conditions, such as a co-culture system.

Recently, human blastocysts were successfully vitrified in straws.[28] However, our own attempts to vitrify human blastocysts using straws resulted in only 45% complete survival (39/86, unpublished data). Vanderzwalmen et al.[29] also reported a low pregnancy rate with human blastocysts vitrified in straws. This is probably because human blastocysts are much less permeable to CPA and water, since it has been observed that they shrink more slowly than mouse and bovine blastocysts in the CPA solution. This suggests that human blastocysts are more likely to be injured by intracellular ice crystal formation.

Increased rates of cooling and warming can help circumvent the problem of intracellular ice formation in less permeable embryos. Faster rates of cooling and warming can be achieved by minimizing the volume of the solution with which embryos are vitrified (i.e., by using minute tools such as electron microscopic grids,[30] open pulled straws,[31] Cryoloops,[2,32] or Cryotop[22]). We showed that the transfer of human blastocysts vitrified with Cryoloops can lead to successful births.[14] Since this original report, we have continued to use this vitrification approach for the cryopreservation of blastocysts on day 5 and day 6.

Currently, several established blastocyst vitrification protocols have been reported. As one of the examples, this chapter includes our protocol of blastocyst vitrification and a summary of the clinical outcomes for the last 10 years, which confirm the safety and the effectiveness of the Cryoloop technique for the cryopreservation of human blastocysts.

PROTOCOL FOR BLASTOCYSTS VITRIFICATION (FIGURE 18.1)

The protocol for the Cryoloop vitrification of blastocysts was adopted from the work of Lane and Gardner[17] with slight modifications.[14–16] Procedures of vitrification involve equilibration and vitrification steps carried out at 37°C in 7.5% DMSO and 7.5% EG for 2 minutes, and 15% DMSO, 15% EG, 1% Ficoll 70, and 0.65 M sucrose for 30–45 seconds in human tubal fluid (HTF)/human serum albumin (HSA). At the end of 30–45 seconds, the blastocysts are loaded on a small nylon loop (Hamilton Research, Laguna Niguel, CA), and are plunged directly into LN_2. They are warmed by placing the tip of the Cryoloop into 0.5 M sucrose in HTF/HSA, and are kept there for 2 minutes, followed by 0.25 M sucrose in HTF/HSA for 3 minutes. With the use of the Cryoloop as a container, the vitrified blastocyst almost floats in the

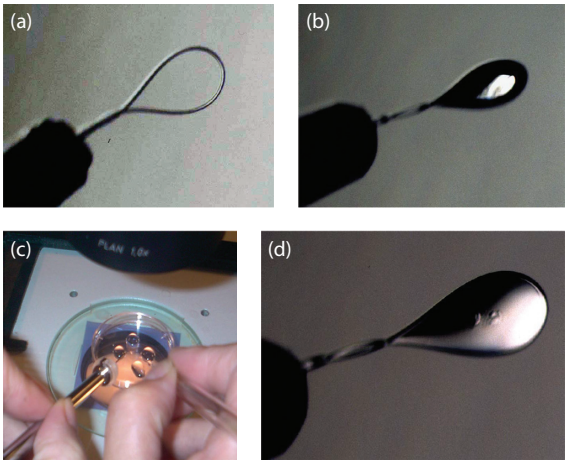

Figure 18.1 **(a) A minute nylon loop (20 μm wide, 0.5–0.7 mm in diameter) mounted on a stainless steel pipe under 100× magnification. (b) A thin layer of the vitrification solution on the nylon loop after dipping the loop into the vitrification solution. (c) Under a dissecting microscope, the capping portion of the cryovial with the Cryoloop attached by a stainless steel handling rod for manipulation (held by left hand), and a pulled Pasteur pipette with blastocysts (held by the right hand). Prior to loading, blastocysts are rinsed several times in small drops of final vitrification solution (solution II) on the lid of a culture dish. (d) Blastocysts on the loop with a thin layer of vitrification solution.**

thin filmy layer of the droplet on the nylon loop, and heat conduction to the blastocyst becomes homogenous and extremely high. With the extremely high cooling rate, full equilibration of CPA is not necessary to avoid ice crystal formation, and inside the cell is a so-called "meta-stable situation." This is why around 3 minutes of CPA exposure is enough to reach the vitrified status inside the cells. This duration of exposure is shorter than that of other vitrification approaches. Shorter exposure of CPA is more favorable due to avoiding the exposure of the potentially toxic agent to the cells.

WARMING OF BLASTOCYSTS, ASSISTED HATCHING, AND ASSESSMENT OF SURVIVAL

With the cryovial submerged in LN_2, the vial is opened with the aid of the stainless steel rod, and the loop containing blastocysts is removed from the LN_2 and placed directly and quickly into the well containing the 0.5 mol/L sucrose solution. Blastocysts immediately fall from the loop into the solution. Thus, blastocysts are warmed and are diluted instantly at around 37°C, adjusted by the stage warmer. After 2 minutes, the blastocysts are transferred to the 0.25 mol/L sucrose solution. After an additional 3 minutes, blastocysts are washed and are kept in the base medium for 5 minutes. During this 5 minutes, assisted zona hatching (AH) is always performed on warmed blastocysts with either acidic Tyrode's solution, as previously described,[33,34] or by multiple shots of a laser pulse to the zona pellucida (ZP) of the warmed blastocysts. Recently, laser equipment manufactured by Research Instrument Limited (Saturn 5 Active™ RI, Cornwall) has been able to create multiple laser pulses automatically along the ZP in the biopsy mode. This approach has allowed embryologists to perform AH very easily.

About 2–3 hours after warming, the appearance of the blastocysts is examined on an inverted microscope at 400 × magnification, and survival is assessed based on the morphological integrity of the blastomeres, inner cell mass and trophectoderm, and re-expansion of the blastocoele. The surviving blastocysts are scored as to developmental stage, and are graded according to quality as described in the section on the grading of blastocysts.

PATIENTS AND GRADING OF BLASTOCYSTS

For the following three categories of patients groups, vitrified blastocyst transfer has been performed in our clinic: group 1—patients who had their fresh embryos transferred on day 2–3, and all the remaining embryos were cultured to allow those that developed into blastocysts to be vitrified; group 2—patients who received transfers of fresh blastocysts and had all their remaining supernumerary blastocysts vitrified; group 3—patients who had no fresh embryo transfer because of ovarian hyperstimulation syndrome (OHSS) symptoms, or who were attempting vitrified blastocyst transfer intentionally along with a controlled endometrial cycle (CEC) supplemented with exogenous female hormones to overcome multiple implantation failures, because uterine receptivity under CEC has been reported to be better than during hyperstimulated cycles in donor egg-sharing program studies. After two or three failures of implantation following fresh transfer attempts, fresh blastocyst transfer is intentionally avoided with our clinical concept based on our clinical experience. One of the reasons for this is that ovarian hyperstimulation does not always create a suitable uterine receptivity nor environment for implantation, due to super-physiological levels of female hormones, when compared to controlled endometrial preparation using exogenous hormones.

On day 5 or 6 after the oocyte pick up, blastocyst development was examined. On day 5 or 6, each embryo that developed to the blastocyst stage was scored depending on the developmental stage, and graded according to quality criteria[35] with slight modifications.[15]

Briefly, blastocysts were first given a numerical score from 1 to 6 on the basis of their degree of development. Secondly, the blastocysts were graded in three ranks based on morphological appearance. For example, the inner cell mass was graded as A (many tightly packed cells), B (several loosely grouped cells), or C (few cells); and the trophectoderm was graded as A (many cells forming a cohesive epithelium), B (fewer cells forming a loose epithelium), or C (very few large cells).

When patients had their fresh embryos transferred on day 2–3, all the remaining embryos were cultured to allow those that developed into blastocysts to be vitrified. Patients who received transfers of fresh blastocysts had all their remaining supernumerary blastocysts vitrified. On day 5, if at least one supernumerary blastocyst was graded as A or B, all the blastocysts of the patient were vitrified regardless of the developmental stage and the grading. In a few cases, compacted morulae with early cavity formation were also vitrified with the blastocysts. If all the blastocysts of the patient were graded C, they were not cryopreserved. On day 6, if at least one blastocyst had a large blastocoele (i.e., scored as 3–6), and was graded as A or B, all the developed blastocysts scored as 3–6 were vitrified.

ARTIFICIAL SHRINKAGE OF EXPANDED BLASTOCYST

In 2003, it was reported that blastocyst survival rates were dependent on the developmental stage, and were negatively correlated with the expansion of the blastocoele.[15] The survival rates of early blastocysts with a smaller blastocoele cavity, scored 1 and 2 according to quality criteria,[35] were 87% (48/55) and 97% (62/64), respectively. Also, full blastocysts lacking an expanded blastocoele cavity, which were scored 3, had a survival rate of 89% (99/111). The total survival rate of blastocysts scored 1–3 together was 91% (209/230). However, the survival rate of both expanded and hatching blastocysts,

scored 4 and 5, respectively, was 85.0% (288/339), which was significantly lower than that of the group scored 1–3 ($p < 0.05$). It was therefore postulated that a large blastocoele might lessen potential to survive cryopreservation due to ice crystal formation during the rapid cooling phase of vitrification. In order to overcome this problem, shrinkage of the blastocoele was thought to be an appropriate approach. Several studies reported an increase in the survival rate of blastocysts when the volume of the blastocoele was artificially reduced with a glass micro-needle[36] (a 29-gauge needle)[37] or micropipetting with a hand-drawn Pasteur pipette.[38]

Since 2003, we have therefore added artificial shrinkage (AS) after puncturing the blastocoele with a micro-needle, or laser pulse using the Cryoloop technique prior to vitrification, to improve the survival rate and clinical outcomes of our vitrified blastocyst transfer programs. In 2006, we reported the effectiveness of AS prior to vitrification, including the confirmation of the safety of this procedure.[39] Initially, AS was carried out using a glass micro-needle to collapse the blastocoele (Figure 18.2). After complete shrinkage of the blastocyst, it was vitrified and stored in a LN_2 tank.

Since September 2004, a laser pulse generated by the laser system ZILOS-tk™ (Hamilton Thorn Bioscience Inc., Beverly, MA) has been introduced to perform the AS, instead of micro-needle puncture. The inner cell mass should be located away from the targeted point of the laser pulse. One single laser pulse (200 ms) targeted at the cellular junction of the trophectoderm cells creates a hole to induce collapsing of the blastocoelic cavity (Figure 18.3). The blastocoele of the expanded blastocyst shrinks almost immediately. With the use of this laser system, it is not necessary to hold and locate the expanded blastocyst with a holding pipette connected to a micro-manipulator. The laser technique makes the procedures simple and convenient.[40] Recently, the laser system Saturn 5 Active™ (Research Instrument Limited, RI, Cornwall) has been used either for AH or AS as an improved system of laser equipment.

CLINICAL RESULTS OF AS PROCEDURES

In order to show the effectiveness of the AS method, we summarized the results of 270 cycles in Table 18.3. Results of vitrified expanded and hatching blastocysts in our previous study reported in 2003 served as a control group (without AS). The survival rate of both expanded and hatching blastocysts, scored 4 and 5, respectively, was 85.0% (288/339). A statistical difference was noted between the study and the control groups ($p < 0.05$). When the pregnancy rate of the study group was compared with the control group, a statistically significant improvement was noticed in the AS group (60.2% versus 34.1%; $p < 0.01$).

We also performed preliminary comparisons between the results achieved by using micro-needle or laser pulse for blastocoele shrinkage, to show the difference of methodologies for AS. The survival rates achieved

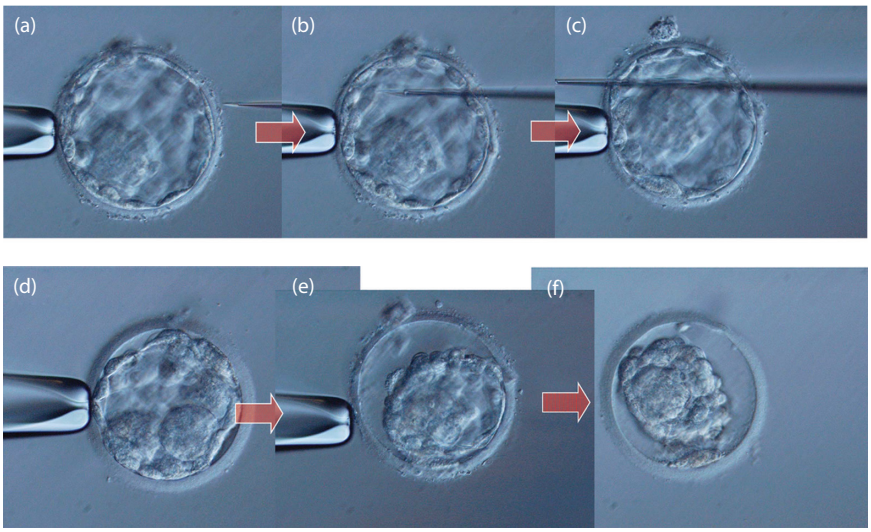

Figure 18.2 **Artificial shrinkage of expanded blastocyst with the micro-needle. (a) Holding the expanded blastocyst with a holding micropipette connected to a micro-manipulator. (b) Insertion of the micro-needle inside the blastocoele at a point away from the inner cell mass. (c) Puncture through the blastocoele and removing the micro-needle gradually. (d) Beginning of shrinkage 10 seconds after puncture. (e) Partial shrinkage 30 seconds after puncture. (f) Complete shrinkage 1 minute after puncture.**

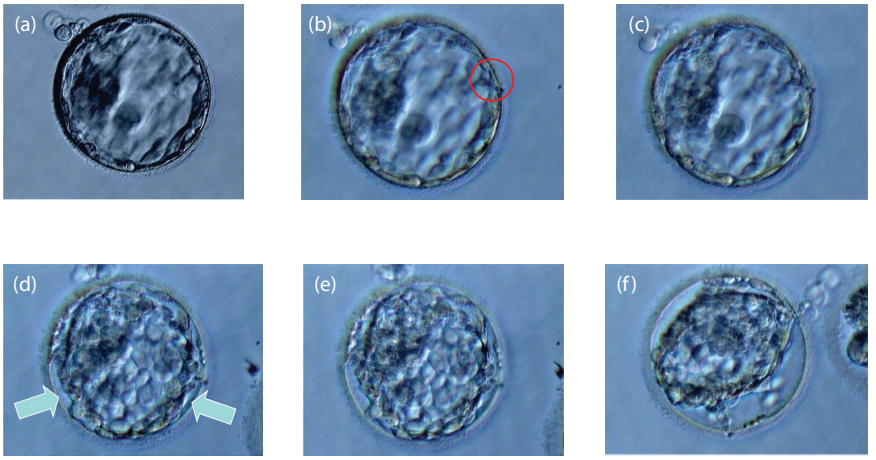

Figure 18.3 **Artificial shrinkage of an expanded blastocyst with a single laser pulse. (a) Prior to the artificial shrinkage. (b) A single laser pulse at the point of the cellular junction of the trophectoderm cell at a point away from the inner cell mass (circle indicated). (c) Beginning of shrinkage 5 seconds after laser shot. (d) Shrinkage 10 seconds after laser shot, and arrows indicating formation of the peri-vitelline space because of contraction. (e) Shrinkage 20 seconds after laser shot. (f) Almost complete shrinkage 30 seconds after laser shot.**

Table 18.3 Characteristics of Patients and Survival of Vitrified Human Blastocysts with (Study Group) or without (Control Group) Artificial Shrinkage

	Study group	Control group
No. of patients	245	76
Average age (years)	35.6	34.0
Mean no. of previous *in vitro* fertilization/intracytoplasmic sperm injection attempts	2.1	2
No. of initiated vitrified blastocyst cycles	270	—
No. of cycles with vitrified blastocyst transfer	266	85
No. of cancelled cycles due to no survival of vitrified blastocyst (%)	4 (1.5%)	—
No. of blastocysts vitrified	502	339
No. of vitrified blastocysts survived	488	288
Survival rate	97.2%	85.0%*
No. of vitrified blastocysts transferred	448	—
Mean no. of blastocysts transferred	1.7	—
Clinical pregnancies	160	29
Clinical pregnancies (%)	60.2%	34.1%**

Note: Control group refers to our previous study (Mukaida et al.[15]).
*$p < 0.05$, **$p < 0.01$.

with the two methods were similar (micro-needle: 97.2% versus laser pulse: 97.5%). The mean number of survived blastocysts transferred was also similar. Clinical pregnancy, implantation, and miscarriage rates were also similar. No statistical difference was observed in the results achieved with the two methods.[39]

CLINICAL RESULTS OF VITRIFIED BLASTOCYST TRANSFER

Table 18.4 summarizes the clinical results of our vitrified blastocyst transfer program using the Cryoloop between the years 2000 and 2013.

Clinical results and perinatal outcomes of vitrified blastocyst transfer using a Cryoloop performed since the beginning of 2000 until the end of 2013 (14 years) are summarized. A total of 12,941 blastocysts originating from 8,440 cycles were vitrified and warmed. The mean age was 36.6 years.

After warming for transfer, 12,339 (95.3%) vitrified blastocysts survived. In 93 cycles (1.1%), no blastocysts

Table 18.4 Clinical Outcomes of Vitrified Blastocyst Transfer at the Tokyo and Hiroshima Human Assisted Reproductive Technology (HART) Clinics (2000–2013)

Criteria	Data
Total no. of attempted cycles	8440
Total no. of warmed–vitrified BL	12,941
Total no. of survived BL	12,339
Survival rate	95.3%
No. of transferred cycles	8347
Mean no. of BL transferred	1.35
No. of clinical pregnancies (%/BT)	3948 (47.3%)
No. of implantations (%)	4362 (38.6%)
No. of births (babies; boy:girl)	2483 (2757; 1413:1344)
No. of miscarriages (%)	967 (24.5%)

Note: BL = blastocyst; BT = blastocyst transfer.

survived or surviving blastocysts were obtained, but embryo transfer was cancelled because the number of cells that survived and the quality of the blastocysts were not considered to be suitable for transfer. A total of 11,295 blastocysts were transferred in 8,347 cycles. The mean number of blastocysts transferred per cycle was 1.35. From 2009 to 2013, the mean number was 1.13 per cycle. Of 8,347 transfers, 3,948 cycles resulted in clinical pregnancy (confirmed by gestational sac in the uterus); the pregnancy rates were 46.8% per warming cycle and 47.3% per transfer. The implantation rate was 38.6% (4,362/11,295).

A total of 2757 babies were born from 2483 deliveries. Since 1413 babies were boys and 1344 were girls, no bias in the sex ratio was observed. Cesarean sections were performed in 1227 deliveries, and the mean gestational age was 38.1 weeks. The mean birth weight of overall births from vitrified blastocysts was 2823 g. However, in the singleton delivery outcome (2229 births), the mean gestational age was 39.4 weeks, and mean birth weight was 3071 g. This is not statistically different from the national statistics on ART conception reported in 2009 in Japan. A total of 240 births were twins (9.7%) and 14 births were triplets (0.6%). Forty three cases had either congenital birth defects or peri-natal complications (1.6%), including six chromosomal abnormalities (two trisomy 18 and six trisomy 21), nine multiple anomalies, one stillbirth due to hydrocephalus, six stillbirths of unknown causes during delivery (at 25, 29, 30, 32, 37, and 39 weeks of gestation), two anencephaly, one spina bifida, eleven congenital heart or major vessel malformations, three minor anomalies in the hands and/or feet, one congenital esophageal obstruction, one biliary duct obstruction, one Cornelia de Lange syndrome, and one Treacher Collins syndrome.

A total of 967 pregnant cycles ended in miscarriage (24.5%). A comparison of 1187 pregnancies established from fresh blastocyst transfers in our group of clinics during the same period shows that 249 (21.1%) resulted in miscarriages, and no statistical difference was observed between them. This was also similar to that which we reported previously in 2005.[40]

RESULTS WITH COMPARISON TO SLOW FREEZING

There are two main categories of embryo cryopreservation techniques: slow-cooling methods and vitrification. Slow-cooling methods involve a step-wise programmed decrease in temperature along with manual seeding at approximately a temperature of −7°C. The duration of the procedure requires about 2 hours, as well as the use of expensive programmed instruments. As described above, vitrification solidifies the oocytes/embryos into a glass-like state, thus avoiding the formation of both intra- and extra-cellular ice. Regardless of whether vitrification is clinically more effective than slow-cooling methods or not, it is necessary to perform well-established randomized control studies. Since our cryopreservation program clearly revealed better clinical outcomes obtained with vitrification when compared with that of slow cooling at the blastocyst stage, to have performed a randomized clinical trial would have been extremely difficult at our private ART center. In numerous studies investigating the different embryo cryopreservation techniques, few of them were properly constructed to truly evaluate whether one technique was superior to the other or not. In order to compare embryo cryopreservation methods, a systematic review and meta-analysis of the literature has been performed by Abdel-Hafez in 2010.[41] From the 2700 publications, they found 11 prospective and randomized studies. Five were excluded because they did not show data as required, and six were included. In four of the six studies, there was a direct comparison between vitrification and slow freezing that suggested better results were obtained with vitrification. The embryo survival rate was significantly higher with vitrification than with slow freezing, and implantation and pregnancy rates were also higher in two of the studies.[22,42] Finally, the group of Desai made the following conclusion: results of the current meta-analysis showed that embryo vitrification is superior to slow freezing based on direct comparison of embryo survival and clinical pregnancy rates.[43] Ongoing pregnancy and implantation rates were also higher with vitrification as compared with slow freezing. In oocyte cryopreservation, several studies have also indicated that vitrification appears to be superior to slow-freezing methods, leading to improved rates of oocyte survival, fertilization, and embryonic development *in vitro*.[44,45] They showed less disruption of spindle integrity and chromosome alignment in the vitrified human oocytes when compared with the slow-frozen human oocytes. Therefore, they concluded that the lower rate of embryonic development from the slow-frozen human oocytes might be related to the greater level of damage to spindle integrity and chromosome alignment.

SUMMARY

For embryo cryopreservation, the vitrification method has many advantages over the classically established conventional slow-freezing method: (1) injuries related to ice are less likely to occur; (2) survival of embryos can be maintained at a higher level if conditions for embryo treatment are optimized; and (3) embryos can be cryopreserved by a simple method in a short period without an expensive, sophisticated programmable freezer. Therefore, vitrification is suitable for human embryos in which a small number of embryos are cryopreserved frequently. Human embryos at early cleavage stages can be cryopreserved by conventional vitrification using cryostraws or by ultrarapid vitrification using Cryoloops. Human blastocysts are more efficiently cryopreserved by

the ultrarapid approach. Clinical outcomes show that the vitrification of blastocysts using the Cryoloop technique results in high survival and high pregnancy rates, which confirm the safety of this procedure, as seen in our perinatal evaluation.

At first, vitrification was introduced as an alternative approach for the cryopreservation of human gametes and embryos; however, vitrification, with recent technical improvements, has become a more reliable strategy, not only because it is very simple, but also because it can lead to high clinical efficiency, along with better clinical outcome. In particular, ultrarapid vitrification opens a new era for oocyte and blastocyst cryopreservation, as described in this chapter. Classically, adequate equilibration of CPA and dehydration is necessary to cryopreserve gametes and embryos. However, the extremely high cooling rate achieved by direct plunging into LN_2 with a minimal volume (≤0.5 μL) of final vitrification solution, including vitrified cells, can obtain high cryosurvival rates and better viability, and allow us to avoid ice crystal formation even with the lower concentrations of CPA, which could cause devitrification (i.e., ice crystal formation) if conventional cooling was to be applied. The 2012 annual report of the ART registry of Japan, organized by The Japanese Society of Obstetrics and Gynecology, showed a remarkable increase in the proportion of cryopreservation activity in clinical ART programs. Briefly, in 2012, a total of 326,426 ART cycles were performed (IVF: 82,108; intracytoplasmic sperm injection [ICSI]: 125,229; frozen embryo transfer [FET]: 119,089), and a total of 37,953 babies were born (IVF: 4740; ICSI: 5498; FET: 27,715) from these ART procedures. When the proportions of live birth outcomes are compared between ART procedures, the summary indicates that almost three-quarters of ART babies born (73.0%) were generated via cryopreservation.[46] The clinical benefits of cryopreservation, as we mentioned before, are major reasons why there is an increasing proportion of ART babies born following cryostorage, rather than following fresh embryo transfer. The reasons include: apparently higher pregnancy and implantation rates when compared with fresh transfers; the avoidance of higher risks for OHSS; and also the provision of significant convenience for the scheduling of transfer timings. This trend will inevitably be continued, and a lot of clinical programs might be ready to eliminate the transfer of fresh embryos altogether.

More recently, this ultrarapid vitrification approach has been considered as applicable for ovarian tissue and stem cell cryopreservation, and with proper preparation of ovarian tissue, such as 1 cm × 1 mm segmentation, in addition to a properly designed container, high survival and better post-warming viability may well be expected from this vitrification approach.[47] Ultimately, vitrification will become the most suitable and routinely applied method for cryopreservation of any cells and tissues.

IMPORTANT POINTS OF THIS CHAPTER

1. In human ART treatment, cryopreservation of embryos is one of the most useful approaches for utilizing supernumerary embryos after controlled ovarian hyperstimulation to increase the chance of pregnancy per IVF attempt (cumulative pregnancy rate).
2. To store human eggs/embryos under LN_2 while maintaining their viability, intracellular ice crystal formation should be avoided completely with proper equilibration of the CPA for dehydration inside the egg/embryo. Also, proper rehydration during the warming steps is necessary by reducing the osmotic stress with a sucrose solution.
3. In order to cryopreserve human embryos in LN_2, either a conventional slow-cooling method or vitrification method may be performed, but there are major differences between them in terms of cooling rate (slow or ultrarapid) and the nature of equilibrating the embryos with CPA (either high or low concentrations, and different durations of exposure).
4. The vitrification method is becoming increasingly used due to clinically favorable outcomes when compared to the conventional slow-cooling approach, because of the simplicity of the technique itself, and also its higher cryosurvival rates and clinical results.
5. The blastocyst stage seems to be the most reliable and appropriate stage for the cryopreservation of human embryos with the vitrification approach, because confirmation of embryo development to the blastocyst stage is thought to be one of the best quality-assurance criteria for potential embryo viability, and additionally, the intracellular permeability of the CPA and size/characteristics of the cells of the blastocyst are more suitable for cryopreservation. Therefore, high cryosurvival and implantation rates are now being obtained with vitrified human blastocyst programs.

DISCLOSURES

Conflicts of interest: Tetsunori Mukaida and Chikahiro Oka declare that they have no conflicts of interest.

Human rights statements and informed consent: all procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients for being included in the study.

Animal studies: all institutional and national guidelines for the care and use of laboratory animals were followed.

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19 Vitrification of human blastocysts: Clinical realities and neonatal outcomes

Juergen Liebermann

INTRODUCTION

The announcement in 1972 of the survival of mouse embryos after cryopreservation at –196°C and thawing was groundbreaking.[1] Since then, the impact of cryopreservation on the growth and improved efficiency of assisted reproduction in humans has become increasingly appreciated. The percentage of frozen human embryos has risen steadily over the years, with approximately one and a quarter million babies born following cryopreservation. Moreover, cryopreservation has also been shown to increase overall pregnancy rates, while allowing for further selection of embryos. Indeed, it is possible to achieve implantation and pregnancy rates with frozen–thawed embryos as high as those achieved with fresh embryos. Routine *in vitro* blastocyst culture and cryopreservation have been shown to increase pregnancy rates, while allowing for better selection of embryos. In the early days of human *in vitro* fertilization (IVF) history, before reliable cryopreservation protocols, there was an emphasis on transferring as many embryos as possible into patients. With more reliable cryopreservation techniques such as "vitrification," lower numbers of embryos are now being transferred, resulting in less high-order multiple pregnancies, as well as increased healthy implantations. In addition, decreased numbers of embryos are transferred, so increasing the potential for more embryos to be placed into frozen storage, thus reducing the number of fresh cycles. The fundamental objectives for successful cryostorage of cells in liquid nitrogen (LN_2) at –196°C can be summarized as follows: (1) arresting the metabolism reversibly; (2) maintaining structural and genetic integrity; (3) achieving acceptable survival rates after thawing; (4) maintenance of developmental competency post-thaw; and (5) the technique has to be reliable and repeatable.

Cryopreservation slows or totally prevents unwanted physical and chemical changes. The major disadvantage of using cryostorage is that it can lead to the crystallization of water, and thereby can create new and unwanted physical and chemical events that may injure the cells that are being preserved. Vitrification, however, avoids ice formation altogether during the cooling process by establishing a glassy or vitreous state, wherein molecular translational motions are arrested without structural reorganization of the liquid in which the reproductive cells are suspended. To achieve this glass-like solidification of living cells for cryostorage, high cooling rates in the range of 2500–30,000°C/min or greater, in combination with high concentrations of cryoprotectants, are used. During vitrification, water is transformed directly from the liquid phase into a glassy vitrified state. The physical definition of vitrification is the solidification of a solution at low temperature, not by ice crystallization, but by extreme elevation in viscosity during cooling.[2,3] A primary strategy for vitrifying cells and tissue is to increase the speed of thermal conductivity, while decreasing the concentration of the vitrificants to reduce their potential toxicity. In general, the rate of cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are inversely related. In addition, recent publications have shown the relatively greater importance of warming rates over cooling rates with regard to the survival of oocytes subjected to a vitrification procedure.[4,5]

The earliest attempts at using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985.[6] In 1993, successful vitrification of mouse embryos was demonstrated.[7] Furthermore, bovine oocytes and cleavage-stage embryos were vitrified and warmed successfully a few years later.[8] In 1999 and 2000, successful pregnancies and deliveries after vitrification and warming of human oocytes were reported.[9,10] Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in assisted reproductive technology (ART), oocytes and blastocysts appear to have received a significant boost in survival rates by avoiding ice crystallization through use of vitrification.[11] In general, vitrification solutions (VS) are aqueous cryoprotectant solutions that do not freeze when cooled at high rates to very low temperatures. Vitrification is very simple, requires no expensive programmable freezing equipment, and relies principally on the placement of the embryo in a very small volume of vitrification medium that must be cooled at extreme rates not obtainable in traditional enclosed cryostorage devices such as straws and vials.

Although initially reported in 1985 as a successful cryopreservation approach for mouse embryos,[6] vitrification took a backseat for a number of years in human assisted reproduction. One perceived "drawback" held by embryologists who are not familiar with the vitrification technique is the use of high concentrations of cryoprotectants, which does mean that VS are potentially more toxic

than their counterpart solutions used for conventional slow freezing. This is necessitated by the practical limit of the rate of cooling, and the biological limit of tolerance of the cells for the concentration of toxic cryoprotectants being used to achieve the cryopreserved state. It is important to note that recently published papers[12–15] have shown that the use of relatively high concentrations of cryoprotectants, such as 15% (v/v) ethylene glycol (EG) used in an equimolar mixture with dimethyl sulfoxide (DMSO), had no negative effect on the perinatal outcomes from blastocyst transfers following vitrification when compared with those from fresh blastocyst transfers.

Cryoprotective agents (CPAs) are essential for the cryopreservation of cells. Basically, two groups of cryoprotectants exist: (1) permeating (e.g., glycerol, EG, and DMSO) and (2) nonpermeating (e.g., disaccharides, proteins, and polymers) agents. The key component of a VS is the permeating agent. These compounds are hydrophilic nonelectrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the "freezing point" of the solution. As the permeating CPA is responsible for potential toxicity (a key limiting factor in cryobiology), different cryoprotectants have been tested for their relative toxicity, and the results indicate that EG (MW 62.02) is the least toxic, followed by glycerol. In general, the permeating CPA should be chosen firstly by their permeating property, and secondly on the basis of their potential toxicity. Additionally, these highly permeating cryoprotectants are also more likely to diffuse rapidly out of the cells, so that the cells quickly regain their original volume upon warming, thus preventing osmotic injury. The second component of a VS consists of the nonpermeating CPAs, such as disaccharides (e.g., sucrose), which do not penetrate the cell membrane, but help to draw out more water from cells by osmosis, and therefore lessen the exposure time of the cells to the toxic effects of the permeating CPAs. The nonpermeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. In addition, permeating agents are able to bind with intracellular water, and therefore water is very slowly removed from the cell. Hence, the critical intracellular salt concentration is reached at a lower temperature. Removal of the CPA during warming can present a very real problem in terms of trying to reduce toxicity to the cells. Firstly, because of the toxicity of the VS, quick dilution of them after warming is necessary; and secondly, during dilution, water permeates more rapidly into the cell than the cryoprotective additive diffuses out. As a consequence of the excess water inflow, the cells are threatened by injury from osmotic swelling. In this situation, the nonpermeating sucrose acts as an osmotic buffer to reduce the osmotic shock. During warming, using a high extracellular concentration of sucrose (e.g., 1.0 M) counterbalances the high concentration of the CPAs in the cell, as it reduces the difference in osmolarity between the intra- and extra-cellular compartments. The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling.[16–18]

However, the blastocyst as a chill-sensitive entity has a characteristic that is unique to its stage: a fluid-filled cavity called the blastocoele. A decrease in survival rate after vitrification has been noted when the volume of the blastocoele cavity is increased. This is likely due to insufficient permeation of CPAs into the blastocoele, with residual water in the cavity increasing the potential for ice crystal formation during cooling, and diluting the CPAs reaching the inner cells. Residual fluid in the blastocoele can therefore reduce post-warming survival of embryos. Vanderzwalmen et al.[19] showed that survival rates in cryopreserved, expanded blastocysts could be improved by artificial reduction of the blastocoele cavity, and others consider that blastocoele collapse is necessary pre-vitrification on whichever day the blastocyst forms.[19,20]

Although one study has suggested that expanded blastocysts show no significant differences in viability, implantation potential, or pregnancy outcome when frozen on day 5 versus day 6,[21] our "body of data"[13–15] refutes the comparable implantation rates (IRs) for blastocysts cryopreserved on days 5 or 6. While there are several possibilities as to why day 6 blastocysts perform less well than day 5 blastocysts, one explanation could be that the older blastocysts are often more expanded, with a significantly larger blastocoele impacting water loss and CPA absorption. Being mindful of previously published research on the artificial collapse (AC) of blastocysts prior to cryopreservation, we looked for an opportunity that could potentially help us improve the outcomes for day 6 blastocysts using AC of the blastocoele prior to vitrification. Further, if the concept showed any improvement for the day 6 blastocysts, it might also be applied to day 5 blastocysts that had well-expanded blastocoeles.

AC of the blastocoele can be performed using different techniques, such as micro-needles, sucrose solutions, or lasers.[20,22–26] In 2003 and 2004, two groups independently reported a beneficial effect by applying AC to blastocysts prior to vitrification. Son et al.[22] observed an improved clinical pregnancy rate (cPR) of 48% and an IR of 29% with the use of AC. Hiraoka et al.[23] collapsed day 5 and day 6 blastocysts by manually pipetting embryos until they collapsed, and achieved a cPR of 50%, with an IR of 33% after warming. Moreover, Mukaida et al.[20] found that the survival rate of vitrified blastocysts was negatively correlated with the size of the blastocoele. They speculated that a large blastocoele may disturb the efficacy of vitrification. They collapsed the blastocoele by puncturing it with a micro-needle, or by making a hole between two trophectoderm cells with a laser pulse. After applying AC, the survival improved from 86% to 97.2%. Moreover, their pregnancy rate went up from 34.1% to 60.2%, with an IR of 46.7%. Iwayama et al.[24] used a laser pulse, or

osmotic shock resulting from exposure of the whole embryo to sucrose, and the IR was significantly higher in both groups compared to the control group without AC (59.7% and 49.3% versus 34.2%). Furthermore, Hur et al.[25] looked at the effect of AC achieved using a 29-gauge needle or laser pulse on clinical outcomes in fresh transfers, and they observed a significant increase in the cPR in the study group compared to the control group (58.8% versus 39.0%).

All publications mentioned, including Liebermann and Conaghan,[26] conclude that AC has a beneficial effect both in frozen blastocyst transfers and for overall cumulative pregnancy and IRs.

MATERIALS AND METHODS

Materials

1. HSV (High Security Vitrification Kit [catalog # 022137]; CryoBioSystem)
2. Heat Sealer (CryoBioSystem)
3. Polycarbonate micropipettes, 300 µm end hole (MidAtlantic Diagnostics# KFPIP-1170-10BS)
4. Brady TLS 2000 Thermal Label Printer
5. Brady Labels (PTL-19-427)
6. 90 × 15 mm Petri dish (Nuncleon # 150362)
7. Center-well organ culture dish (Falcon 3037)
8. Styrofoam container
9. Visotubes 10 mm (IMV 5561)
10. Cryo Canes Aluminum (ThermoScientific 5015-0001)

Reagents

1. Serum substitute supplement (SSS) (Irvine)
2. Modified human tubal fluid (mHTF) (Irvine)
3. Vit-Kit—Freeze (Irvine Scientific # 90133DSOC)
4. Vit-Kit—Thaw (Irvine Scientific # 90137DSOC)

Equipment

1. Dissecting stereo microscope (Olympus SZX-12, Bausch Lomb or Leica) with warming stage
2. Laminar flow hood (Origio)
3. Inverted microscope (Olympus IX-71)
4. Infrared 1.48-µm diode laser (Hamilton Thorne—Zilos laser [Hamilton Thorne Research, Beverly, MA])

Methods

Stepwise blastocyst vitrification procedure

Vitrification of blastocysts is undertaken utilizing a "closed system" (HSV: CryoBio System, L'Aigle, France; FDA 510(k) clearance for cleavage-stage embryos in blastocysts) (see "Addendum," Notes 1 and 2) after a two-step loading with cryoprotectant agents at 24°C (see "Addendum," Notes 3 and 4). If AC is done prior to vitrification, then the blastocyst is put on an inverted microscope equipped with a laser system (Zilos-tk, Hamilton Thorne), the junction of two trophectoderm cells in each blastocyst is located, and one shot (100% power, 500 µs pulse length) is applied to the cell junction. The blastocyst is then moved back into the incubator for 5–10 minutes.[19] Briefly, blastocysts have to be placed in equilibration solution, which is the base medium (M199 with 20% serum supplement substitution [SSS] containing 7.5% [v/v] EG and 7.5% [v/v] DMSO) (see Table 19.1). After 5–7 minutes, the blastocysts need to be washed quickly in VS, which is the base medium containing 15% (v/v) DMSO, 15% (v/v) EG, and 0.5 M sucrose, for 45–60 seconds and transferred onto the HSV using a micropipette. Immediately after the loading of not more than two blastocysts in less than a 1-µL drop on the HSV, the straws are heat sealed, then plunged in LN_2, and finally stored inside 5-mL LN_2 prefilled canes (Visotube Rond, IMV, France). Each single step is described in detail below.

1. Aseptic techniques are required at all stages. For equilibration and vitrification procedures, ensure the benchwarmer is at room temperature (~24°C) (see "Addendum," Notes 3 and 4).
2. Take reagents from the refrigerator and allow them to warm to room temperature.
3. Move blastocysts to freeze into a separate well. Bring this dish to the inverted microscope and with the embryo positioned with the laser objective, use a single pulse to hit the blastocysts between two trophectoderm cells to collapse the embryo. Place the dish back into the incubator for 5–10 minutes.
4. Label a Petri dish with the patient's name under the lid as follows: HTF-HEPES, equilibration solution (ES), and VS. Prepare 2 × 50 µL of HTF-HEPES (+20% SSS–mHTF), 2 × 50 µL of ES, and 4 × 50 µL of VS (see Figure 19.1).
5. Brady labels should include the patients' last name, first name, accession number, Master Patient Index number, and date plus the number and type of embryos.
6. Before vitrification, use a stripper tip with a 200-µm end hole for loading the blastocysts on the top.
7. Fill a Styrofoam container with LN_2.

Table 19.1 Summary of Vitrification and Warming Solutions (Irvine Vit-Kit "Freeze" and "Thaw")

	Composition		
	Ethylene glycol (%)	**Dimethyl sulfoxide (%)**	**Sucrose (M)**
Equilibration solution (ES)	7.5	7.5	0
Vitrification solution (VS)	15	15	0.5
Thawing solution (TS)	0	0	1.0
Diluent solution (DS)	0	0	0.5
Wash solution (WS)	0	0	0

Note: M-19 9 + 20% SSS (Wash solution).

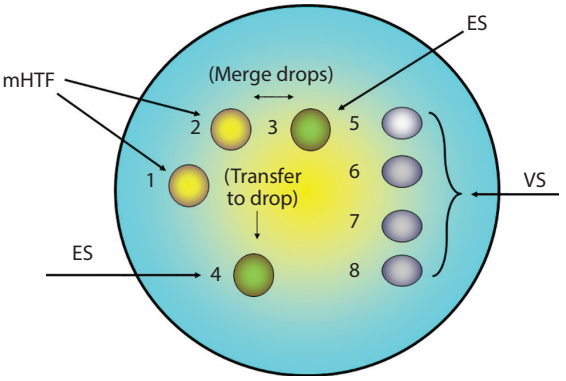

Figure 19.1 **Setup for the vitrification procedure on a plain 90-mm dish lid surface. (VS = vitrification solution; ES = equilibration solution; mHTF = modified human tubal fluid.)**

8. Each sample that is vitrified will be done in a separate hood and verified by a second embryologist before proceeding. Vitrify good expanded/hatching blastocysts on day 5/6/7.
9. Remove embryos from culture dishes using a stripper tip into the modified human tubal fluid (mHTF) (drop 1), gently aspirating to remove any residual culture medium.
10. Pipette from mHTF (drop 1) to the other drop of mHTF (drop 2) and immediately merge it with the first drop of ES (drop 3). Set the timer for 5 minutes.
11. After 5 minutes, transfer the embryos to the remaining drop of ES (drop 4). Set the timer for 3 minutes. Place the embryos on the top of the drop and let them settle to the bottom.
12. Load the blastocysts in a VS back-loaded stripper tip, and rinse through the four droplets of VS (drops 5–8); clean the tip between each droplet.
13. Placement into the VS and loading of the Cryotop should take less than 1 minute, so that the total incubation time in VS is 30 seconds. After 30 seconds, gently transfer them to the tip of the HSV by using a stripper tip to load the blastocyst(s) in as small a volume (less than 0.5 µL) as possible onto the edge of the stick (see "Addendum," Note 5).
14. Visually confirm the placement (see "Addendum," Note 6).
15. Before loading, apply the label to the open end of the empty straw. Load the HSV stick into the empty straw, placing the side with the embryos first. Use the blue handle to make sure the stick is in as far as it can go. Then, using the heat sealer, seal the open end of the stick and plunge the whole straw into the LN_2. Place the straw in a precooled aluminum cane for further storage (see "Addendum," Notes 7 and 8).
16. Store at the cane in a nitrogen tank.
17. Make sure to record the cane location on the freezing worksheet and cryo-inventory log.
18. Complete all paperwork and recheck that all vial locations are logged in the embryo inventory.

Stepwise blastocyst warming procedure

It is important to mention that, regardless of the day of cryopreservation of the embryo (whether day 5, 6, or 7), at thawing, blastocysts should be treated as if they had been cryopreserved on the fifth day of development. To remove the cryoprotectants, blastocysts need to be warmed and diluted in a three-step process. With the HSV submerged in LN_2, the inner straw should be removed, and then the carrier with the blastocysts can be removed from the LN_2 and placed directly into a prewarmed (37°C) organ culture dish containing 1 mL of 1.0 M sucrose (see "Addendum," Note 9). Blastocysts can be picked up directly from the HSV and placed in a fresh drop of 1.0 M sucrose at 24°C, and immediately connected with a drop of 0.5 M sucrose. After 5 minutes, blastocysts are transferred to a 0.5 M sucrose solution, and connected with drops of base medium for an additional 5 minutes. When switching the embryos between different concentrations of warming solutions, be sure to fill up the pipette with the next-lowest concentration of warming solution before picking up the cells for moving into the next dilution (see "Addendum," Note 10). Finally, the blastocysts are washed in the base medium for 3 minutes, then returned to culture medium (SAGE+20%SSS) (SAGE Blastocyst Medium, Trumbull, CT, USA) in the incubator until transfer. Each single step is described in detail below.

1. Take reagents from the refrigerator and allow them to warm to room temperature. All cryoprotectants are removed at 24°C.
2. Place a 200-µL drop of thawing solution (TS) on a Petri dish and place on a warming plate (see "Addendum," Note 9).
3. Label a Petri dish with the patient's name under the lid as follows: TS, diluent solution (DS), and washing solution (WS). Prepare 1×50 µL of TS, 4×50 µL of DS, and 6×50 µL of WS (see Figure 19.2).
4. Before warming, use a stripper tip with a 200-µm end hole for removing the blastocysts from the HSV tip.
5. Fill a Styrofoam container with LN_2.
6. Confirm the location and identification with a second embryologist before warming any HSV kit. Warm one kit at a time.
7. Each sample that is warmed is done in a separate hood and verified by a second embryologist before proceeding.
8. With the HSV kit under LN_2, open the kit by cutting the outer straw. Use the blue handle to remove the inner stick.

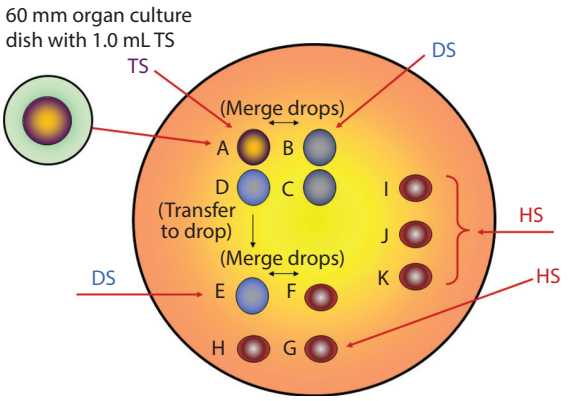

Figure 19.2 **Setup for the warming procedure on a plain 90-mm dish lid surface.**

9. Submerge the HSV kit directly in the prewarmed 37°C drop containing TS, which should be as close as possible to the LN_2 Styrofoam container (see "Addendum," Note 11). As soon as the HSV kit contents liquefy (within 1 second), try to locate the blastocyst(s) before removing it (them) with a stripper tip. After locating all of the blastocysts, remove them from the HSV tip and place them in the droplet of TS (drop A) at room temperature, then connect this immediately with the first droplet of DS (drop B). Wait for shrinkage and re-expansion.
10. When they start to wrinkle, connect them with the second droplet (drop C) and finally with the third droplet of DS (drop D).
11. When they stop reacting and start to re-shrink, transfer the blastocysts to 0.5 M sucrose (drop E) by placing them at the top of this drop so that they float to the bottom. When the reaction is complete, connect this with first of WS (drop F; wait for about 90% re-expansion).
12. After 100% expansion, connect with the second droplet (drop G), then with the third droplet (drop H) of WS. Turn on the benchwarmer, and finally dilute through a series of three wash drops of holding solution (HS) (drops I–K).
13. Place the blastocysts into a culture dish, and put the dish in the incubator for subsequent culture.
14. Record the survival and appearance of all blastocysts. Update the log with warming data, and notify the physician of the result (see "Addendum," Note 12).

RESULTS ON BLASTOCYST VITRIFICATION

Between 2004 and September 2014, the Fertility Centers of Illinois "IVF Laboratory River North" (Chicago) has vitrified 23,517 blastocysts from 5968 patients (Table 19.2). The majority of blastocysts were vitrified on day 5 (52.0%), 46.0% on day 6, with a minority on day 7 (2.0%). After nearly 11 years of vitrifying blastocysts using an open (Cryotop) as well as closed (HSV) system, and more than 4500 VET with an average number of 1.7 embryos transferred, the perinatal outcomes are as follows: the number of babies delivered until February 2013 is 1495 (766 girls and 729 boys) (Table 19.3). No abnormalities were recorded.

Table 19.2 Retrospective Data from 5968 Patients (Average Age 34.3 ± 4.9 Years) with Blastocyst Cryopreservation by Vitrification between 2004 and September 2014

	Day of development			
	Day 5	Day 6	Day 7	Total
Number of blastocysts vitrified	12,206	10,854	457	23,517
	(52.0%)	(46.0%)	(2.0%)	(100%)

Table 19.4 summarizes the data on gestational age (GA) and live birth weight (LBW) from 1363 children born. A total of 926 babies were from vitrified day 5 and day 6 transfers, whereas 437 babies were born following fresh elective single-embryo transfers (eSET). Furthermore, comparing the 437 newborns from the eSET group to the 926 newborns from frozen-vitrified group (vitrified blastocyst transfers [VBTs]), there was a significant difference in mean age of the patients ($p < 0.001$). In addition, looking at GA and LBW of newborns derived from vitrified day 5 blastocysts ($n = 561$) and day 6 blastocysts ($n = 365$), the following data were observed: t-test results indicate no significant difference in GA ($p = 0.71$) nor birth weight ($p = 0.124$) for the frozen transfers based on day of development. Obviously, GA is highly correlated with birth weight, but there is no significant difference in GA between eSET and VBT. However, Table 19.4 shows that one-way analysis of variance (ANOVA) of LBW by both groups indicates a statistically significant difference

Table 19.3 Perinatal Outcome of Vitrified Blastocysts After More than 4100 Transfers between 2004 and 2013 (Babies Delivered Until February 2013)

		Day of development	
	Day 5+ Day 6	Day 5	Day 6
Deliveries (total)	1209	748	461
Babies born (total)	1495	939	556
Female	766	488	278
Male	729	451	278
Singletons	931 (77.0)	561 (75.0)	370 (80.0)
Twins	270 (22.0)	183 (24.5)	87 (19.0)
Triplets	8 (1.0)	4 (0.5)	4 (1.0)

Note: Percentages are indicated between brackets.

Table 19.4 Live Birth Weight (LBW) and Gestational Age (GA) of Babies (n = 1363) Delivered after Fresh Elective Single-Blastocyst Transfers (eSBT; n = 437) Compared to Those Delivered from Vitrified Blastocyst Transfers (VBT; n = 926) and Related to Day of Development after VBT (Day 5 vs. Day 6)

			Day of development	
	eSBT—Day 5	VBT—Day 5 + Day 6	VBT—Day 5	VBT—Day 6
Average age (years)	31.1 ± 3.1***	34.7 ± 4.9***	34.7 ± 5.0	34.8 ± 4.8
Deliveries	437	926	561	365
Female	238*	479*	288	191
Male	199*	447*	273	174
GA (weeks)	38.1 ± 2.5*	37.9 ± 2.7*	38.0 ± 2.6*	37.9 ± 2.9*
LBW (g)	3294.9 ± 609.4**	3390.1 ± 710.6**	3419.1 ± 712.6*	3345.5 ± 705.8*

Note: T-test (Satterthwaite unequal variance): *p > 0.05: **p = 0.013; ***p < 0.001.

between the means of eSET and VBT (p = 0.013) with heavier babies in the VBT group. This observation is confirmed and published by Pinborg et al. [27]. As shown in Table 19.4, the χ^2 statistic indicates no difference in the gender distribution between fresh and vitrified transfers (p = 0.3454). Running a t-test on gender weight combining the gender from fresh with vitrified transfers indicates a significant difference in average weight by gender (p < 0.001; Table 19.5). Males were on average 157.2 g heavier than females. Table 19.5 shows the weight distribution for babies born in both groups. Overall, 110 babies weighed less than 2500 g (considered as low birth weight), 159 babies born from both groups weighed between 4000 and 4500 g (considered as large birth weight), and 33 babies weighed more than 4500 g (considered as macrosomic birth weight).

Table 19.5 Distribution (%) of Live Birth Weight (LBW) of Babies (n = 1363) Delivered after Fresh Elective Sngle Blastocyst Transfers (eSBT; n = 437) Compared to Those Delivered from Vitrified Blastocyst Transfers (VBT; n = 926) and Gender Specific Live Birth Weight (LBW) and Gestational Age (GA) between Fresh and Frozen Embryo Transfers Combined

	eSBT—Day 5 (n) [%]	VBT—Day 5 + Day 6 (n) [%]	Total (n)
LBW (g) <2500	34 [*7.8*]	76 [*8.2*]	110
LBW (g) ≥4000	38 [*8.7*]	154 [*16.6*]	192
LBW (g) ≥400 and ≤4500	32 [*7.3*]	127 [*13.7*]	159
LBW (g) >4500	6 [*1.4*]	27 [*2.9*]	33
Gender	**Girls from eSBT and VBT**	**Boys from eSBT and VBT**	
N	717	646	
GA (weeks)	37.9 ± 2.6*	38.0 ± 2.6*	
LBW (g)	3284.1 ± 663.8**	3441.3 ± 691.0**	

Note: Percentages are indicated between brackets; T-test and χ^2 test: *p > 0.05; **p < 0.001.

The outcomes with regard to day of development and age of the patient between 2004 and July 2014 are summarized in Tables 19.6 and 19.7. In good-prognosis patients under 35 years old when transferring day 5 blastocysts, ongoing pregnancy and IRs of 48.0% and 42.3%, respectively, were noted (Table 19.6). In contrast, when transferring day 6 blastocysts in patients younger than 35 years of age, ongoing pregnancy and IRs of 34.0% and 30.0%, respectively, were recorded (Table 19.7).

In October 2007, the Fertility Centers of Illinois "IVF Laboratory River North" moved forward from the use of an open carrier system (Cryotop—embryos are in direct contact with LN_2) to a closed system (embryos are sealed before contact with LN_2). Using a closed carrier (HSV) for aseptic vitrification, the following data from day 5, day 6, and day 7 blastocysts were observed and are summarized in Table 19.8: (a) cPR: 53.92% versus 41.6% versus 18.5%; (b) ongoing pregnancy rate (oPR): 45.6% versus 32.2% versus 14.8%; and (c) IR: 42.2% versus 30.2% versus 1.3% (Table 19.8). As shown in Table 19.8, oPRs, cPRs, and IRs occurring in the day 5 blastocyst group were significantly higher than when transferring day 6 or even day 7 blastocysts.

Between 2007 and July 2014, the Fertility Centers of Illinois "IVF Laboratory River North" performed 1675 VBTs without collapsing prior to vitrification, with a mean patient age of 35.4 ± 5.0 years (group A), and in 972 VBTs (group B) with a mean patient age of 35.4 ± 4.9 years, where AC was performed prior to vitrification (Table 19.9). On average, 1.7 embryos were transferred in groups A and B, which means 30% of all VBTs were single-embryo transfers. Survival in group A versus group B was not significantly different (98.7% versus 99.6%). However, there was a significant improvement in group B compared with group A for the following: (a) cPR: 58.2%

Table 19.6 Retrospective Outcome Data (2004–September 2014) at the Fertility Centers of Illinois, Chicago from Vitrified Day 5 Blastocysts with Regard to Patient Age

	Patient age (years)				
	<35	**35–37**	**38–40**	**>40**	**Donor**
Average age (years)	31.2 ± 2.4	35.9 ± 0.8	38.9 ± 0.8	42.6 ± 2.1	43.6 ± 4.7
Cycles	1285	539	351	171	252
Transfers	1284	538	349	171	252
Blastocysts survived	98.4	98.2	98.8	98.1	98.9
Blastocysts transferred (mean)	1.8	1.7	1.8	1.8	1.7
Positive pregnancy/VET (%)	62.3	59.9	56.4	52.6	59.1
Clinical pregnancy/VET (%)	55.0	51.0	46.0	41.0	52.0
Ongoing/delivered pregnancies (%)	48.0	41.0	36.0	29.0	42.0
Implantations	954	350	203	87	170
Implantation rate (%)	42.3	38.9	32.5	28.5	38.6

Note: VET = vitrified embryo transfer.

Table 19.7 Retrospective Outcome Data (2004–July 2014) at the Fertility Centers of Illinois, Chicago from Vitrified Day 6 Blastocysts with Regard to Patient Age

	Patient age (years)				
	<35	**35–37**	**38–40**	**>40**	**Donor**
Average age (years)	31.2 ± 2.3	36.0 ± 0.8	38.9 ± 0.8	42.6 ± 1.8	43.3 ± 4.8
Cycles	834	439	320	197	147
Transfers	827	434	319	195	146
Blastocysts survived	97.4	98.5	98.5	96.9	99.6
Blastocysts transferred (mean)	1.8	1.7	1.8	1.7	1.7
Positive pregnancy/VET (%)	49.1	44.7	44.5	42.1	45.9
Clinical pregnancy/VET (%)	42.0	37.0	39.0	33.0	37.0
Ongoing/delivered pregnancies (%)	34.0	29.0	30.0	22.0	27.0
Implantations	449	204	159	76	63
Implantation rate (%)	30.0	26.9	27.8	22.4	24.7

Note: VET = vitrified embryo transfer.

versus 43.8%; (b) oPR: 50.9% versus 36.0%; and (c) IR: 44.0% versus 32.7% (Table 19.9).

When the vitrified–warmed blastocysts were divided into day 5 and day 6 groups, the following data were observed (Table 19.10): in 983 VBT transferring day 5 blastocysts from group A (n = 983; mean age of 35.3 ± 5.1 years), the IRs, cPRs, and oPRs were 37.4%, 48.7%, and 40.9%, respectively, compared to 48.0%, 61.9%, and 55.1%, respectively, of day 5 blastocysts from group B (n = 637; mean age of 35.0 ± 4.9 years). As shown in Table 19.10, IRs, cPRs, and oPRs occurring from the day 5 blastocysts in group B were significantly higher than from the day 5 blastocyst in group A (χ^2; $p < 0.001$ for any comparison).

If we compare day 6 in group A (n = 692; mean age of 35.6 ± 4.9 years) with day 6 outcomes in group B (n = 335; mean age of 36.3 ± 4.9 years), the following data in terms of IR, cPR, and oPR were observed: 26.2%, 36.7%, and 29.0% versus 36.7%, 51.3%, and 43.0%, respectively (Table 19.9). As shown in Table 19.9, IRs, cPRs, and oPRs occurring in the day 6 blastocysts of group B were significantly higher than when transferring day 6 blastocysts from group A (χ^2; $p < 0.001$ for any comparison).

In Table 19.11, the results for patients under 35 years of age in group A (no assisted collapsing) and B (assisted collapsing) are summarized. Comparing day 5 from group A (n = 480; mean age of 31.2 ± 2.3 years) with day

Table 19.8 A Comparison of Retrospective Data from the Cryopreservation Program (Fertility Centers of Illinois, Chicago) of Vitrified Day 5, Day 6, and Day 7 Blastocysts Using Aseptic Vitrification Technology between October 2007 and July 2014

	Day 5 + Day 6 + Day 7	Day 5	Day 6	Day 7
Patient's age (years)	35.5 ± 5.0	35.3 ± 2.3	36.0 ± 4.8	35.7 ± 3.7
Transfers	2959	1803	1129	27
Blastocysts warmed	5186	3099	2041	45
Blastocysts survived	5138 (99.1)	3079 (99.4)	2014 (98.7)	45 (100.0)
Blastocysts transferred	5034	3017	1972	45
Blastocysts transferred (mean)	1.7	1.7	1.7	1.7
Implantations	1873 (37.2)	1272 (42.2)[a]	595 (30.2)[a]	6 (1.3)[a]
Positive pregnancy/VET	1709 (57.7)	1132 (62.8)[a]	567 (50.2)[a]	10 (37.0)[a]
Clinical pregnancy/VET	1447 (48.9)	972 (53.9)[a]	470 (41.6)[a]	5 (18.5)[a]
Ongoing/delivered pregnancies	1189 (40.2)	821 (45.6)[a]	364 (32.2)[a]	4 (14.8)[a]
Live births	905	598	303	4

Note: Percentages are indicated between parentheses. VET = vitrified embryo transfer; [a]$p < 0.001$.

Table 19.9 A Comparison of Retrospective Data from the Cryopreservation Program (Fertility Centers of Illinois, Chicago) of Vitrified Blastocysts without Artificial Collapse (AC; Group A) and with AC (Group B) Using Aseptic Vitrification Technology between 2007 and July 2014

	Technique	
	Group A (without AC)	Group B (with AC)
Patient age (years)	35.4 ± 5.0	35.4 ± 4.9
Transfers	1675	972
Blastocysts warmed	3004	1709
Blastocysts survived	2966 (98.7)	1703 (99.6)
Blastocysts transferred	2911	1680
Blastocysts transferred (mean)	1.7	1.7
Implantations	952 (32.7)[a]	739 (44.0)[a]
Positive pregnancy/VET	861 (51.4)[b]	666 (68.5)[b]
Clinical pregnancy/VET	733 (43.8)[b]	566 (58.2)[b]
Ongoing/delivered pregnancies	603 (36.0)[b]	495 (50.9)[b]

Note: Percentages are indicated between parentheses. VET = vitrified embryo transfer; [a]$p < 0.01$; [b]$p < 0.001$.

5 from group B (n = 334; mean age of 31.2 ± 2.2 years), we found the following for IR, cPR, and oPR: 41.1% versus 52.7%, 51.9% versus 66.5%, and 45.6% versus 59.9%, respectively. Looking at day 6 outcomes for group A versus group B, we observed the following for IRs, cPRs, and oPRs: 31.4% versus 41.7%, 43.0% versus 58.3%, and 36.1% versus 50.0%, respectively (see Table 19.11).

CONCLUSIONS AND FUTURE DIRECTIONS

Vitrification is a very promising cryopreservation method with many advantages and an increasingly consistent clinical track record. A standardized vitrification protocol applicable to all stages of the preimplantation embryo may not be realistic because of (a) different surface-to-volume ratios; (b) differing cooling rate requirements between oocytes, zygotes, cleavage-stage embryos, and blastocysts; and (c) variable chill sensitivity between these different developmental stages. Currently, however, the most widely used protocol applied to any embryo stage is the two-step equilibration in an equimolar combination of the cryoprotectants EG and DMSO, at a concentration of 15% each (v/v), supplemented with 0.5 mol/L sucrose. For the adoption of vitrification in ART, as with all new technologies, there has been initial resistance, but as clinical data have been accrued, this technology is becoming more commonly adopted as a standard procedure in many IVF programs worldwide. With this increased use in human assisted

Table 19.10 A Comparison of Retrospective Data from the Cryopreservation Program (Fertility Centers of Illinois, Chicago) of Vitrified Day 5 and Day 6 Blastocysts without Artificial Collapse (AC; Group A) and with AC (Group B) Using Aseptic Vitrification Technology between 2007 and July 2014

	Technique			
	Group A (without AC)		Group B (with AC)	
	Day 5	Day 6	Day 5	Day 6
Patient age (years)	35.3 ± 5.1	35.6 ± 4.9	35.0 ± 4.9	36.3 ± 4.9
Transfers	983	692	637	335
Blastocysts warmed	1736	1268	1096	613
Blastocysts survived	1721 (99.1)	1245 (98.2)	1092 (99.6)	611 (99.7)
Blastocysts transferred	1687	1224	1083	597
Blastocysts transferred (mean)	1.7	1.8	1.7	1.8
Implantations	631 (37.4)[a]	321 (26.2)[c]	520 (48.0)[a]	219 (36.7)[c]
Positive pregnancy/VET	558 (56.8)[b]	303 (43.8)[d]	455 (71.4)[b]	211 (63.0)[b]
Clinical pregnancy/VET	479 (48.7)[b]	254 (36.7)[d]	394 (61.9)[b]	172 (51.3)[d]
Ongoing/delivered pregnancies	402 (40.9)[b]	201 (29.0)[d]	351 (55.1)[b]	144 (43.0)[d]

Note: Parentheses are indicated between brackets. VET = vitrified embryo transfer. Day 5: [a]$p < 0.01$; [b]$p < 0.001$; day 6: [c]$p < 0.01$; [d]$p < 0.001$.

Table 19.11 A Comparison of Retrospective Data from the Cryopreservation Program (Fertility Centers of Illinois, Chicago) of Vitrified Day 5 and Day 6 Blastocysts without Artificial Collapse (AC; Group A) and with AC (Group B) Using Aseptic Vitrification Technology in Patients Younger than 35 Years of Age between 2007 and July 2014

	Technique			
	Group A (without AC)		Group B (with AC)	
	Day 5	Day 6	Day 5	Day 6
Patient age (years)	31.2 ± 2.3	31.5 ± 0.6	31.2 ± 2.2	31.3 ± 2.6
Transfers	480	316	334	120
Blastocysts warmed	862	591	566	219
Blastocysts survived	848 (98.4)	578 (97.8)	563 (99.5)	218 (99.5)
Blastocysts transferred	829	564	560	218
Blastocysts transferred (mean)	1.7	1.8	1.7	1.8
Implantations	341 (41.1)[a]	177 (31.4)[b]	295 (52.7)[a]	91 (41.7)[b]
Positive pregnancy/VET	279 (58.1)[a]	158 (50.0)[b]	253 (75.7)[a]	83 (69.2)[b]
Clinical pregnancy/VET	249 (51.9)[a]	136 (43.0)[b]	222 (66.5)[a]	70 (58.3)[b]
Ongoing/delivered pregnancies	219 (45.6)[a]	100 (36.1)[b]	200 (59.9)[a]	46 (50.0)[b]

Note: Values are numbers unless otherwise described; percentages are indicated between parentheses. VET = vitrified embryo transfer. Day 5: [a]$p < 0.001$; day 6: [b]$p < 0.001$.

reproduction will come evolution of the vitrification process as it is fine-tuned to clinical needs, so pushing forward its development to higher levels of clinical efficiency, utilization, and universal acceptance.

PRACTICAL IMPLICATIONS FOR VITRIFYING AT THE BLASTOCYST STAGE

Our data have shown that freezing at the blastocyst stage provides excellent survival, implantation, and cPRs. To achieve these outcomes, consider these points: (a) without a successful blastocyst vitrification storage program, extended culture should never be attempted; (b) the blastocyst is composed of more cells and is therefore better able to compensate for cryoinjury; and (c) the cells are smaller, which makes cryoprotectant penetration faster. On average, fewer embryos per patient are cryostored, but each one has a greater potential for implantation when thawed.

ADDENDUM: SPECIAL NOTES FOR THE CLINICAL EMBRYOLOGIST

1. Special care must be given to the selection of the vitrification carrier type. It is necessary to use types of carrier or vessel material with rapid heat transfer that also support the process of uniform heat exchange to achieve higher cooling rates.
2. In addition, although no reports of contamination in human IVF following cryopreservation exist, the user should be encouraged to choose a closed carrier system, which, in our experience, works for blastocysts without any problems.
3. To minimize the toxicity of the cryoprotectant, a stepwise exposure of cells to precooled concentrated solutions (approximate room temperature of 24°C) is recommended.
4. Utilizing higher concentrations of cryoprotectant allows shorter exposure times to the cryoprotectant, but be careful—the potential toxicity of the cryoprotectant increases at higher concentrations. As almost all cryoprotectants are toxic to some extent, it is important to carefully monitor the duration of exposure to the final cryoprotectant before plunging into LN_2.
5. To facilitate vitrification by higher cooling rates, it is also necessary to minimize the volume of the VS as much as is practical (preferably less than 1 μL). From this point of view, it is very important to use a small pulled pipette. Furthermore, by collecting the blastocysts in one place and loading no more than two blastocysts at the same time in the pipette, it is possible to keep the volume small. However, if the load of media is too large, it can still be reduced before plunging in LN_2 by "drawing down" the droplet to flatten the blastocysts slightly while removing all surplus VS.
6. To ensure that the blastocysts are loaded on the carrier, perform the loading process under a stereomicroscope. Always confirm the number of loaded blastocysts.
7. After sealing the carrier, submerge the carrier loaded with the blastocysts directly in LN_2 by passing rapidly through the vapor phase (nitrogen gas).
8. Store the cryo-cane in a prechilled polyvinyl chloride (PVC) cryo-sleeve sitting in the goblet in the dewar. It is essential to maintain exposure of the HSV to LN_2 at all times to eliminate the risk of warming and devitrification.
9. Before moving the carrier quickly from the LN_2 into the warming solution, pull a glass pipette or have a stripper tip (micropipette) ready. Fill the pipette with a small amount of the first warming solution (TS). When using the HSV as the vitrification carrier, rinse the open edge of the straw after placing it into the prewarmed (37°C) 1.0 M sucrose; because the droplet is so small, it warms immediately, and it is essential to pull/stir the blastocysts off the carrier surface as soon as possible to avoid any toxic effect of the VS. A stirring motion is recommended when plunging into the warm TS to agitate the cell off the carrier surface without the need to remove it actively.
10. When switching the cells between different concentrations of warming solutions, fill up the pipette with the next-lowest concentration of warming solution, before picking up the blastocysts to move into the next concentration.
11. In general, during vitrification and warming, the LN_2 Styrofoam box needs to be as close as possible to the working area to minimize any lag in cooling and warming rates.
12. Be aware of the expiration dates of the vitrification and warming media; once opened, the shelf life is 6 weeks (Irvine Scientific; according to the manufacture).

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20 Development and hatching of human blastocysts after vitrification and warming

Joe Conaghan and Sergio Vaccari

INTRODUCTION

Blastocyst vitrification is now a routine procedure that allows patients to store high-quality embryos indefinitely with a high expectation of pregnancy post-warming.[1] Patients with multiple blastocysts will routinely transfer just a single fresh embryo (elective single-embryo transfer) to avoid multiple pregnancy,[2] with the knowledge that their remaining embryos can be successfully frozen and stored until needed. At the time of warming, it will again be ideal to use just a single embryo from the freezer to maintain the efficiency and safety of the process, particularly in younger patients, those who experienced a loss or miscarriage after a previous transfer, or if the embryo is known to be euploid after chromosome analysis.[3] In attempting to use a single embryo and to give the patient the greatest possible chance of pregnancy, the embryologist is charged with recovering, warming, and culturing the embryo, and with accurately assessing the viability of the embryo before transfer. In addition, the embryologist needs to be aware of procedures that may limit or enhance the embryo's chance of implantation,[4] such as timing of warming and transfer relative to uterine receptivity,[5] culturing the embryo for an appropriate time before transfer, and assisted hatching (AH).[6]

Assessing Blastocyst Viability Post-Warming

Immediately after warming and rehydration of the blastocyst, some method for determining viability must be applied to determine whether the embryo has survived and is suitable for transfer.[7] Although vitrification is technically difficult, when it is applied correctly, the vast majority of blastocysts are expected to survive and be transferred.[8]

Embryo Culture after Warming and before Transfer

It is likely that all blastocysts will be cultured for short periods of time after warming, for anything from just a few minutes to overnight in the incubator. While it is the authors' contention that no embryo can benefit from unnecessary time in culture, it is essential to have a dish of culture medium equilibrated and ready for the warmed embryo for holding until transfer. In addition, some short period of observation may aid in embryo assessment, and some re-expansion of the blastocyst in culture may be evidence of viability after warming.[7,8]

AH: Is It Necessary after Vitrification?

The utilization of AH for fresh[9,10] and frozen[11] embryos remains controversial, and the benefits, if any, have been difficult to establish.[12,13] The technique was developed and became popular in the early 1990s using enzymatic digestion or mechanical zona pellucida (ZP) thinning or breaching.[9,10] Several Cochrane reviews have assessed the literature over the years, beginning in 2003[14] with the conclusion that AH does increase the odds of a clinical pregnancy in *in vitro* fertilization (IVF) patients, but cautioning that live birth data were lacking. A subsequent review showed an additional increase in the multiple pregnancy rate as result of AH.[15] However, the pregnancy benefit trended downward as more studies accumulated,[16,17] and as a result, this procedure has not been universally applied or accepted.[18,19] Nevertheless, guidelines for its use exist from the American Society for Reproductive Medicine,[13] although these do not address using the procedure on frozen embryos except in cases of repeat implantation failure. Discussion has occurred on the usefulness of AH specifically for frozen embryos,[10,11,20–27] but without much focus on freezing-related changes to the ZP that might specifically necessitate AH after warming.

MATERIALS AND METHODS

The embryos used in the work described here were all surplus to patient needs and were scheduled to be discarded. Patients consented to have the embryos thawed and observed prior to discard.

Blastocyst vitrification was achieved using a Vit Kit (Irvine Scientific, Santa Ana, CA), which contains an equilibration solution (ES) and a vitrification solution (VS). Blastocysts were laser collapsed as described previously,[28] before being incubated in ES for 8 minutes and VS for 60 seconds at room temperature. Embryos were loaded into Cryotips (Irvine Scientific), which were sealed before being plunged into liquid nitrogen. Embryos that had been biopsied for genetic testing had a 30-µm hole made in the ZP on day 3 of development. For warming, Cryotips were plunged into a 37°C water bath for 3 seconds before being washed through solutions of 1, 0.5, and 0 M sucrose (thaw kit; Irvine Scientific) at room temperature.

Immediately after warming, embryos were cultured in well-of-the-well (WOW) dishes[29] loaded with G2 medium (both from Vitrolife Inc, Gothenburg, Sweden)

supplemented with synthetic serum substitute (SSS: Irvine Scientific), and placed in gas-controlled incubators on the Primo Vision time-lapse imaging system (Vitrolife).[30] Embryos were imaged every 5 minutes for up to 72 hours after warming.

RESULTS

Warming of Blastocysts

Embryologists assessed embryos for survival during warming and at the time of placing the blastocysts in culture. In rare cases, embryos were clearly observed to have died during vitrification and warming, and displayed characteristics such as lysed cells and blackened areas encompassing some or all of the cell population (Figure 20.1).[31,32] Occasionally, an embryo looked perfectly normal after warming and even began to re-expand during the first few hours in culture, only to collapse and die within 6–12 hours of warming (Figure 20.2). These latter embryos were more difficult to identify as dying immediately at warming or during the first 4 hours in culture.

Re-Expansion of Blastocysts in Culture

There is no consensus on a method for measuring embryo viability after vitrification, although appearance and expansion in culture are used subjectively.[7] Many embryologists like to culture embryos for 1 or more hours after warming,[23,24] and use criteria such as the appearance of the embryo and re-expansion of the blastocoele[7] as evidence of survival. Using the Primo Vision time-lapse technology system and software, we were able to observe embryo expansion in real time and record events such as the cycles of collapse and re-expansion that occur in many embryos.[33]

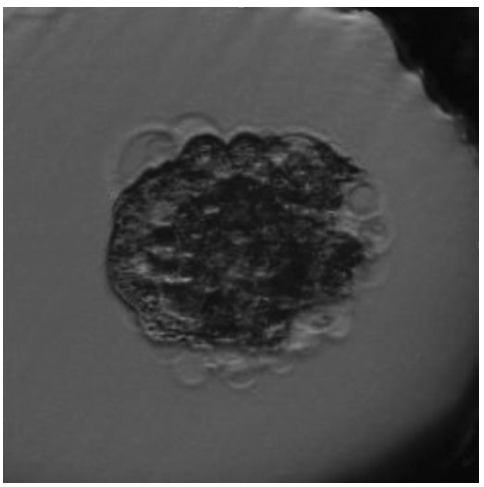

Figure 20.1 **Occasionally an embryo is clearly dead after warming. This zona-free embryo is dark and contracted and the outer cells are seen to be lysing, as evidenced by the halos around the cells on the outside of the embryo. This pattern of cell lysis is similar to that obtained when trophectoderm cells are deliberately lysed to isolate or stain only inner cell mass cells.**[31,32]

To assess re-expansion, the volume of each embryo was measured (Figure 20.3). The average diameter of the embryo was taken and the volume calculated using the formula πr^2. If the embryo was hatching, the volume of each half was calculated separately, and then the total volume calculated by adding the halves. The process was repeated after 1 hour in culture to determine whether any re-expansion had occurred.

Arbitrarily, and in the absence of published guidelines, we looked for a 20% increase in blastocyst size during the first hour in culture. In a pilot experiment, 47 aneuploid embryos that were being discarded by patients were available for study. Only 27 (57%) of the blastocysts showed any increase in volume in the first hour after warming and just 19 (40%) met the target of re-expanding by 20%.

We then repeated the process by taking measurements from photographs of embryos being transferred to patients. Embryos were quickly photographed with a digital camera, and using software attached to the Zylos laser system (Origio Inc, Måløv, Denmark), their diameters were recorded after warming and after 1 hour in culture. Fifty patients participated in the study, all of whom were having elective single-embryo transfers. Thirty-five out of 50 embryos expanded by 20% or more after 1 hour, and 22 of these led to clinical pregnancies. Significantly fewer pregnancies were seen among embryos that failed to re-expand by 20% during the first hour after warming (Table 20.1).

Time Taken to Escape from the ZP

While the re-expansion profile appeared to predict the likelihood of implantation, it was also observed that many embryos that appeared healthy and expanded normally in culture failed to escape from the confines of the ZP. In general, for embryos that were considered high quality before vitrification, such as expanded or hatching blastocysts with high cell numbers, complete hatching was achieved in the first 24 hours after warming. However, the properties of the ZP had clearly changed as a result of the vitrification procedure, and the elasticity or stretchability appeared to be severely reduced. In embryos with a small opening in the zona as a result of laser AH, each embryo had to escape through the existing hole without any stretching, rupture, or ripping of the ZP occurring (Figure 20.4). The zona appeared to have completely hardened and did not give way as the embryo tried to escape. This delayed the hatching process and caused embryos to collapse several times during hatching, requiring the embryo to begin re-expanding over and over again. Typically, embryos underwent four to six rounds of collapse and re-expansion and took 24 or more hours to hatch (Figure 20.5). Embryos that had not

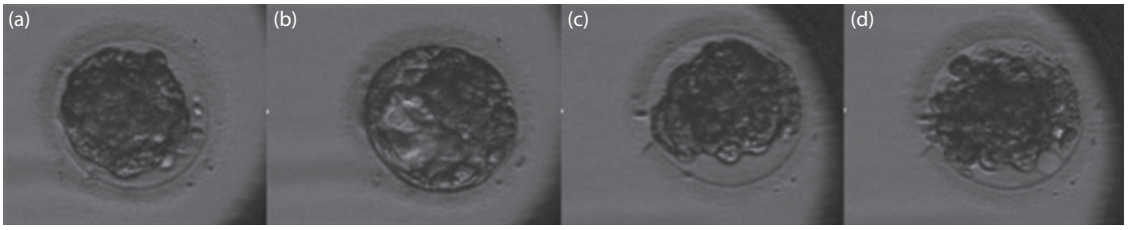

Figure 20.2 **This blastocyst was assessed as having survived warming and was in a normal collapsed state when placed in culture (a). The embryo began to expand, with fluid accumulating in the blastocoele during the first hour in culture (b). At 4 hours, the embryo had collapsed again (c) and was clearly dead and fragmenting after 6 hours (d).**

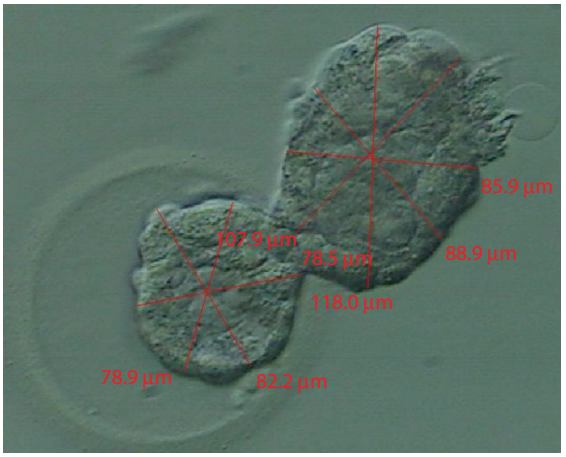

Figure 20.3 **(See color insert.) The volume of each blastocyst was calculated based on the diameter of the embryo, or the parts of the embryo if it was in a figure 8 configuration, as shown here. The volume was recorded immediately after warming and again after 1 hour in culture.**

Table 20.1 Clinical Outcomes Based on Blastocyst Expansion in the First Hour after Warming

	Patients	Positive serum hCG (%)	Clinical pregnancy (%)
All transfers	50	36 (72)	26 (52)
≥20% embryo expansion	35	28 (80)	22 (63)†
<20% embryo expansion	15	8 (53)	4 (27)†

Note: hCG: human chorionic gonadotrophin. Values with the same superscript † symbol are significantly different from each other using Fisher's exact test.

escaped from the ZP after 48 hours in culture never managed to hatch fully.

Properties of the ZP after Vitrification

As seen in Figure 20.4, the ZP appears inflexible after vitrification and, at least *in vitro*, can be a serious impediment to the hatching process. As embryos re-expanded and pushed up against the ZP, there was no expansion or thinning, as is routinely observed with fresh embryos. All the embryos studied had a small hole in the ZP as is typical for D3 AH. This hole, by virtue of its small size and in association with the hardened zona, made it difficult for embryos to completely hatch. As mentioned above, high-quality embryos mostly overcame this challenge, but the characteristics of embryos after warming affected their ability to hatch. In particular, embryos that had already partially hatched (such as the one shown in Figure 20.4) struggled to escape the entrapment of the zona, which had a firm grip around the middle of the embryo. The zona behaved like a corset, pinching the embryo and causing the two halves of the embryo to expand and collapse independently. As a result, not all embryos in this configuration were able to escape and died trying (Figure 20.6). Embryos that appeared normal and underwent good re-expansion were often so trapped that they were unable to free themselves from the constricting ZP, which also affected their ability to expand uniformly.

Making Bigger Holes in the ZP

Since embryos appear to struggle to escape from the zona after vitrification, we looked at the effect of making bigger openings through which they could hatch, as shown by other researchers.[26,34] Figure 20.7 is a schematic of a freshly warmed and collapsed blastocyst where a significant cut through the zona is being made with a laser. This procedure aims to remove approximately one-third of the zona and it is performed immediately after warming when the embryo is in its most collapsed state. The laser is set on a 450-µs pulse and fired 10–15 times. The result of applying the laser like this is that the embryo "falls" right out of the ZP as soon as it begins to expand (Figure 20.8). The time taken to hatch is reduced from an average of 24 hours with conventional AH to 4 hours with more aggressive hatching. In addition, the cycles of expansion and contraction are mostly eliminated, since the embryo is not restricted in any way.

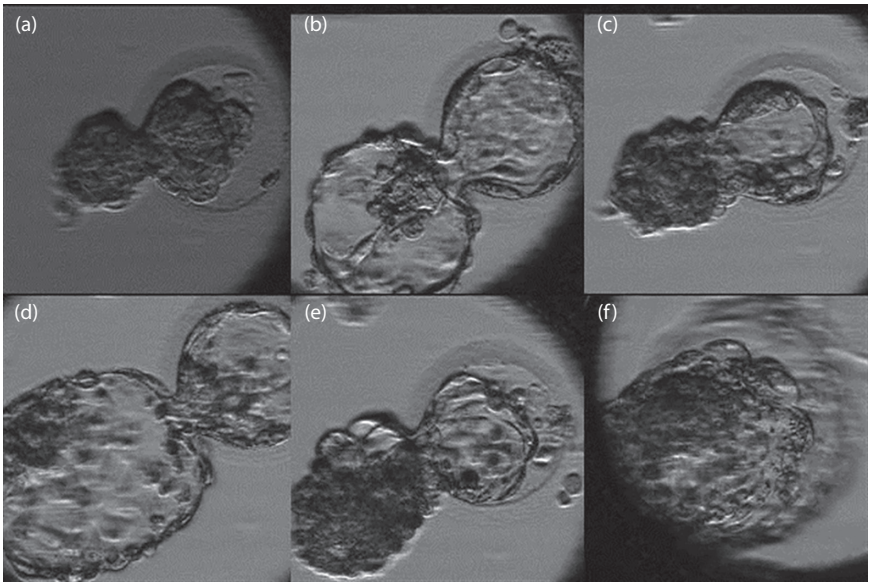

Figure 20.4 **Immediately after warming, the collapsed blastocyst (a) is placed in culture. The embryo struggles to escape through a hole in the zona pellucida that resulted from assisted hatching on day 3 of development. Several rounds of expansion (b), collapse (c), expansion (d), and collapse (e) occur, before finally the embryo hatches completely (f). This embryo collapsed four times and took 24 hours to hatch. Note that the size, thickness, and properties of the zona pellucida do not change in relation to the expansion of the embryo.**

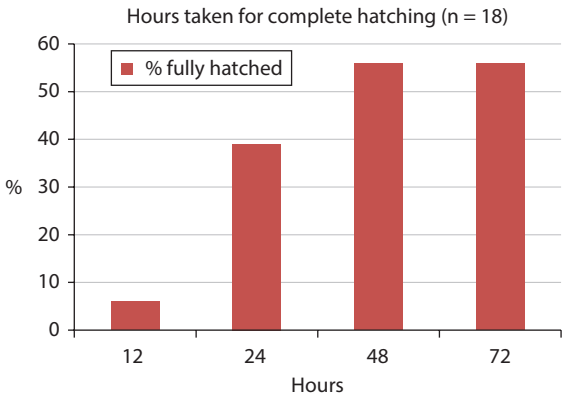

Figure 20.5 **Embryos that had been hatched on day 3 (D3) of development and subsequently were vitrified and warmed were slow to hatch completely from the zona pellucida. There was no increase in the number that hatched after 48 hours in culture.**

Hatching and Continued Expansion

Embryos that remained trapped inside the ZP died relatively quickly (Figure 20.9). Their inability to escape through a normal day 3 (D3) AH hole likely had a high energy cost since it required repeated cycles of collapse and re-expansion. Twenty percent of blastocysts trapped in this way stopped expanding in the first 12 hours in culture and 90% were dead after 48 hours.

Does Slow Freezing Affect Zona Properties?

Embryos that had been slow frozen over 10 years ago were thawed and their expansion profile in culture studied. These embryos were created at a time when AH and genetic screening of embryos was not so prevalent, and when zona hardening due to cryopreservation was not considered a risk. Upon thawing, the ZP did show what appeared to be normal expansion and thinning, but embryos had great difficulty actually breaking the zona and escaping (Figure 20.10).

Characteristics of the ZP after Oocyte Vitrification

After observing the behavior of the ZP in response to expansion in vitrified embryos, we were curious to see whether vitrified oocytes would have similar trouble with hatching when they reached the blastocyst stage. Time-lapse imaging of these blastocysts revealed a pattern that was similar to that seen with slow-frozen embryos (Figure 20.11). Embryos appeared to expand normally but were nonetheless unable to hatch.

DISCUSSION

This study has for the first time used time-lapse imaging to characterize the expansion and hatching profiles for oocytes and blastocysts that were vitrified and warmed, and for slow-frozen and thawed embryos.

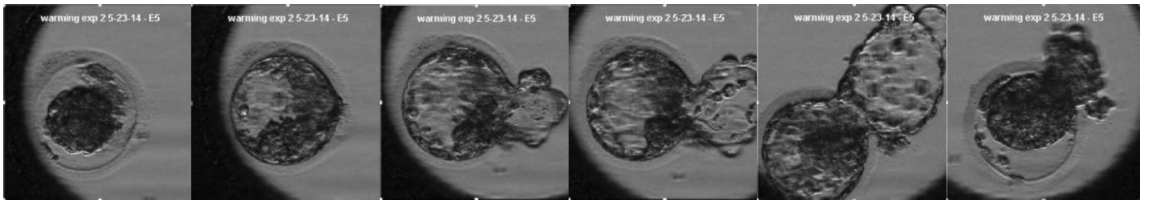

Figure 20.6 **This average-quality embryo quickly re-expanded after warming, but was unable to escape from the zona pellucida. The embryo collapsed and died after three rounds of expansion and collapse, and never had more than 50% of the cellular mass outside the zona.**

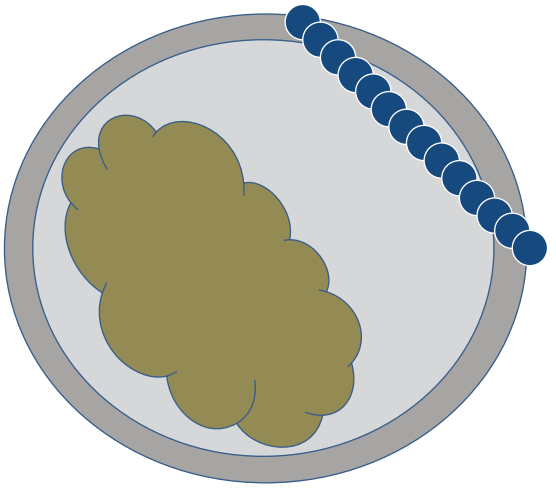

Figure 20.7 **Schematic showing the proposed hatching method for vitrified blastocysts that have not yet re-expanded. Using a 450-µs laser pulse fired 10–15 times at an area of the zona well removed from the embryo, a hole is made that is large enough to allow the embryo to fall out of the zona as it re-expands. The laser shots are depicted as a line of dark circles.**

The observations suggest that the ZP is modified or hardened in a way that could impair blastocyst hatching. This observation is consistent with that of Larman et al.,[35] who showed that the mouse ZP took five-times longer to dissolve in chymotrypsin when compared to unvitrified controls. Larman et al.[35] also showed that the effect was not due to cryoprotectant exposure alone. It should be noted, however, that our observations were made on embryos that were subjected to prolonged culture after warming, and that they may not in fact reflect the behavior of embryos in the uterus. Whether or not embryos have trouble hatching in the uterus, or even undergo repeated collapse and re-expansion cycles, remains unknown. It is also possible that factors produced in the uterus,[36] or by the embryo itself in its more natural environment, may aid in dissolving or weakening the ZP, such that hatching is not difficult. Nevertheless, any ZP that went through the vitrification process will be harder than that around a fresh embryo, and this should be taken into consideration at the time of warming and transfer. AH is a procedure that has not been widely accepted by the medical and scientific community,[13] and in detailed studies of its use with frozen embryo transfers, some authors found a benefit[23,24] and some did not.[25,37] The work presented here supports previous studies that show the positive impact of large holes in the ZP on implantation and pregnancy rates in frozen embryo transfer cycles.[22,26] It is also worth noting that since hatching occurs almost a day earlier when the ZP has a large hole, it may be necessary for physicians to examine the timing of blastocyst warming and transfer in relation to the patient's cycle and progesterone therapy.

Vitrification of blastocysts is a wonderful technology that has allowed embryologists to successfully preserve embryos with the expectation of high implantation rates after warming. However, determining exactly which embryos are viable after vitrification is subjective, and no clear method has been established that has been widely accepted and used. In some cases, embryos are transferred immediately after warming based on morphological assessment alone, but most embryos are likely to be kept in culture for some defined time period[23,24] to determine whether they are viable and continue to develop after returning to their pre-vitrification morphology. But there is no consensus on how long embryos should be kept in culture and how much expansion is considered normal, nor is there even a method for measuring expansion over time that is related to viability. Since we now perform IVF treatments in an era in which embryo freezing is routine,[38,39] and is used in some clinics for all embryos to avoid ovarian hyperstimulation,[40] to facilitate genetic testing,[41,42] to return embryos to the uterus in a natural cycle,[43] or it is simply preferred for convenience,[44] it is more important than ever to be able to assess embryo viability. Historically, viability is assessed subjectively as discussed above, leading embryologists to believe that vitrification gives extremely high or even 100% embryo survival. We have presented evidence that survival may be less than is first assumed based on the traditional methods of assessment. Time-lapse imaging clearly shows that embryos that appear perfectly normal,

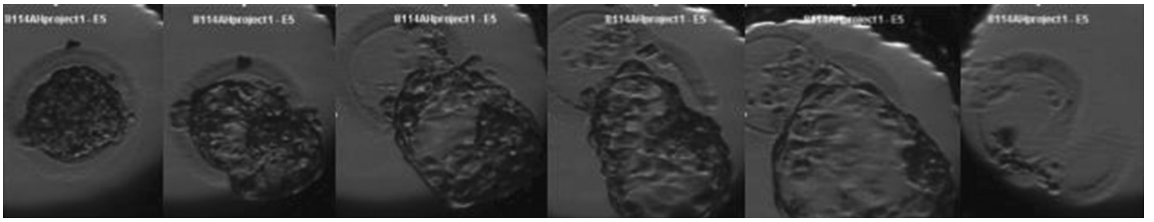

Figure 20.8 **This embryo had a large hole cut in the zona pellucida before being placed in culture. The embryo expanded without restriction, never cycled through collapse and re-expansion, and fell out of the zona 3 hours after warming. The panel on the right shows the large cut in the zona that remained after the embryo had been removed.**

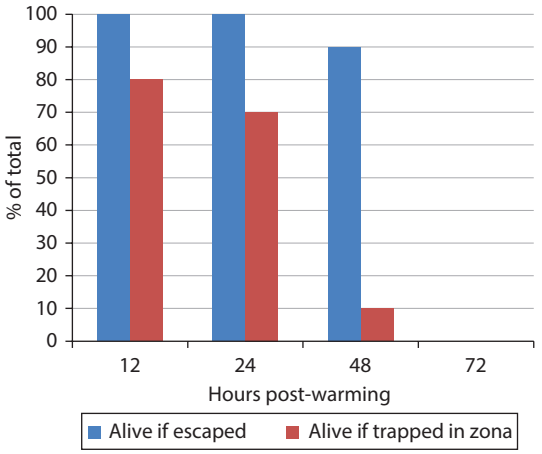

Figure 20.9 **Embryos that were able to escape from the zona pellucida continued to re-expand in culture for up to 48 hours. However, embryos that failed to escape showed a remarkably reduced ability to keep expanding, with only 80% still growing at 12 hours and 10% at 48 hours.**

and even begin to re-expand after warming, sometimes collapse and die in the first 12 hours. Since no embryo ever benefited from extended time in culture, embryologists will not always want to keep embryos in the laboratory for extended periods of time, so some test of viability would be useful. The test that we applied here—looking for a 20% increase in blastocyst size in the first hour after warming—is not perfect, but some noninvasive test like this could have value. We plan further refinements of this test, but for now, an opportunity exists for embryologists to develop new assessment methods for warmed embryos that are more reliable and less subjective than current methods. In addition, we may find that we currently overestimate embryo survival, and this could lead to further refinements in the vitrification methodology.

Pre-implantation genetic screening is rapidly becoming popular as a method for avoiding the transfer of aneuploid embryos and for the high implantation rates that result from the transfer of a single euploid embryo.[45,46] Often this technology utilizes vitrification of all blastocysts to allow embryos to be biopsied on day 5 and 6, and even day 7,[47] and thereby having all samples batched and analyzed together. Preserving the embryos also gives the genetics laboratory more time in which to perform the analysis, and allows the IVF patient to return for a frozen embryo transfer at a convenient time, and when the reproductive organs have returned to normal after hyperstimulation of the ovaries.

To perform a blastocyst biopsy, a hole must be made in the ZP so that a few cells can be recovered for testing. If this hole is too small, the embryo could be damaged during biopsy and the embryologist will struggle to collect cells for analysis. If the hole is too big, the entire embryo may be pulled out of the ZP during the biopsy attempt, making it technically more difficult to perform the biopsy and increasing the risk to the embryo. The perfect hole is large enough to allow some cells to herniate through the opening, but small enough to hold the embryo inside the ZP during biopsy. If the embryo is then frozen or vitrified, it is likely that this hole is insufficient for easy hatching, and may in fact be detrimental to the embryo. In particular, blastocysts that are not of the absolute highest quality, and those that are in a figure 8 configuration, with some of the embryo inside the ZP and some outside (see Figure 20.4 as an example), are at the highest risk for getting stuck or trapped in the ZP. It is even conceivable that part of the embryo could bud off or get pinched by the zona while inside the uterus, leading to a higher-multiple pregnancy risk.[48,49]

Further studies are needed to validate and confirm the results presented here. These data were collected over a relatively short period of time, and with only moderate numbers of embryos studied. Even fewer vitrified oocytes and slow-frozen blastocysts were observed. However, as with most studies, when we started looking more closely at these embryos, we saw things that we did not expect. The post-vitrification ZP acted like an iron fist around the blastocyst, and was unyielding to repeated hatching attempts by most embryos. Viability, as measured by continued expansion, was less than we expected, and while the small day 3 breach of the ZP facilitated embryo biopsy, it prevented many embryos from hatching after

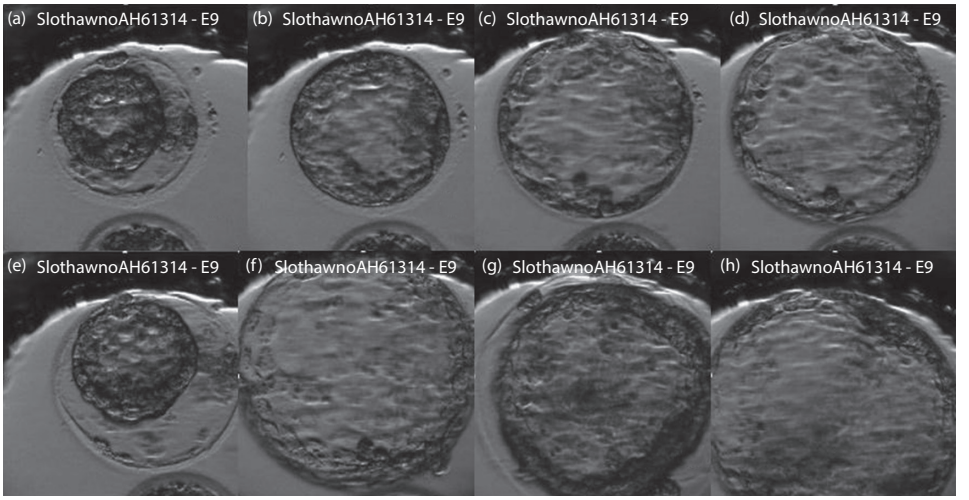

Figure 20.10 **A slow-frozen and thawed blastocyst allowed to re-expand in culture appears initially to be capable of expanding without restriction (a–d) and grows significantly in size. However, the embryo is unable to break through the zona despite 48 hours in culture, and suffers two collapsing (e and g) and re-expansion (f and h) events after which it continues to expand. Although the zona thinned considerably (visible in g) and the embryo almost outgrew the well in which it resided, it never hatched.**

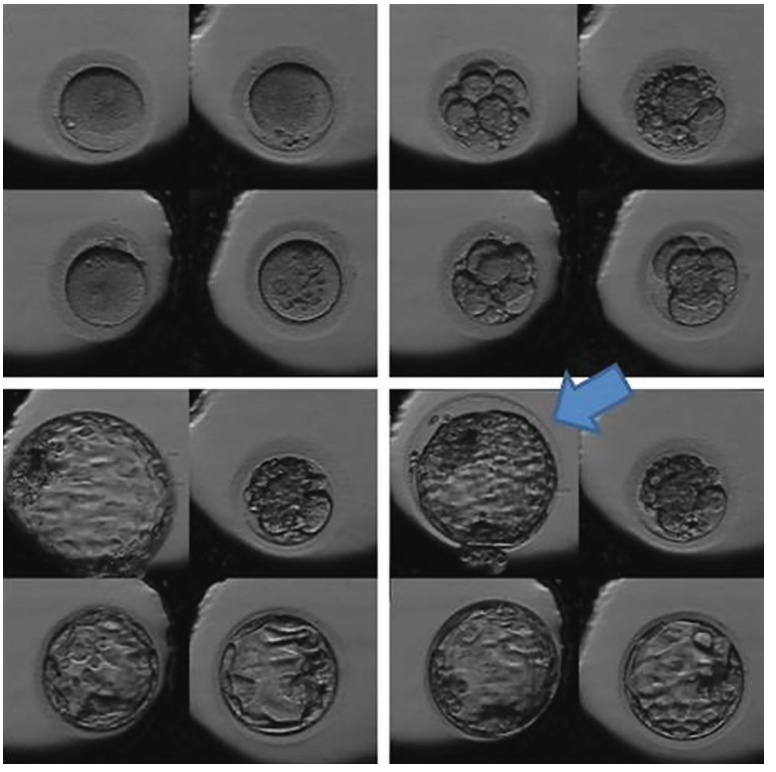

Figure 20.11 **Four vitrified oocytes warmed, fertilized, and cultured to day 6 of development. Pronuclei are visible in the upper left panel on day 1. The upper right panel shows the embryos on day 3. The lower panels show blastocyst development on day 5 (left side) and day 6 (right side). Note the largest blastocyst collapsing after thinning and stretching of the zona pellucida (arrow). None of the embryos hatched spontaneously, although the arrowed embryo did squeeze a few cells through the zona at the 6 o'clock position.**

vitrification. Not all embryos implant after vitrification, even when they are known to be euploid. Some of these failures may be iatrogenic and may be avoided. It appears that there is still room to improve success rates with vitrified embryos.

CONCLUSIONS

1. While a small number of embryos can be clearly identified as failing to survive vitrification and warming, there will be other embryos that appear to be alive and re-expanding that will die within 12 hours of warming.
2. There is no clear method for establishing blastocyst viability after vitrification and warming. A model based on percentage increase in volume over time may allow prediction of implantation rates.
3. The evidence presented here supports the idea that vitrification of blastocysts causes ZP hardening.
4. The small hole made in the ZP of a day 3 embryo is unlikely to be large enough to allow vitrified embryos to hatch easily. Hatching may be delayed, or completely impeded in some embryos. It is possible that some embryos may spend so much time and energy trying to hatch that they are unable to subsequently implant.
5. The time taken for blastocysts to re-expand and hatch is related to the size of the hole made in the ZP. Large holes facilitate immediate hatching and reduce or eliminate cycles of collapse and re-expansion.
6. Embryos that escape from their ZP stay alive for a longer time in culture.
7. Slow-frozen blastocysts and vitrified oocytes appear capable of stretching and thinning the ZP, but cannot easily break through and hatch.

ACKNOWLEDGMENTS

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21 Does storage of vitrified blastocysts have an impact on implantation potential and birth rate?

Barbara Wirleitner, Nicolas H. Zech, and Pierre Vanderzwalmen

CRYOPRESERVATION OF HUMAN GAMETES AND EMBRYOS, ITS APPLICATION, AND THE IMPORTANCE OF STABILITY DURING STORAGE

Around 8%–12% of couples of reproductive age experience difficulties conceiving, and may seek treatment using assisted reproductive technology (ART). Since the birth of the first *in vitro* fertilization (IVF) child Louise Brown in 1978, a number of new techniques have been developed. Hormone-stimulation protocols were established to obtain a number of oocytes per cycle and ovarian pick-up. Intracellular sperm injection was implemented to treatment couples diagnosed with severe male infertility. All these techniques have contributed to the success of ART, and have led toward a time in which it is now standard to obtain more than one (or two) embryos per stimulation cycle. Therefore, cryopreservation of embryos has become an essential part of IVF. Nowadays, many aspects even favor the "freeze-all" strategy, in which embryos are cryopreserved after ovarian stimulation and IVF, and then transferred in a subsequent cryo-cycle, avoiding artificial hormonal levels.[1] Embryo and oocyte freezing is applied for fertility preservation in cancer patients, and also in women planning delayed child bearing. Due to this technological progression, the storage time of cryopreserved embryos has arisen as an important issue. According to differing legislation in different countries, storage of cryopreserved gametes and embryos may be utilized for several years, or even decades.

TECHNIQUES FOR CRYOPRESERVATION OF EMBRYOS AND OOCYTES

In human IVF, cryopreservation started with use of slow freezing (SF) technology in the 1980s. With SF, embryos are exposed to cryoprotectants (CPs) such as 1,2-propanediol (PrOH) or dimethyl sulfoxide (DMSO), followed by a gradual lowering of the temperature down to the "seeding" temperature just below the freezing point (at around −6°C to −7°C), at which point extracellular ice crystal formation is artificially induced. During this process, cells are slowly dehydrated, and intracellular concentrations of CPs increase. SF has proven to be an excellent technique for zygotes and cleavage-stage embryos; however, for oocytes and blastocysts, survival rates after SF have mostly been unsatisfyingly low. Another technique had to be found for these stages, and for this, the vitrification (VIT) procedure has been established as more optimal.

In contrast to SF, VIT works (theoretically) by complete avoidance of intra- and extra-cellular ice formation. VIT is the solidification of a supercooled fluid without the formation of crystalline structures. The molecules in the fluid solidify in a disorganized pattern, and no molecular re-arrangements occur. Only when structural changes occur, as happens more commonly with SF, is there a significant risk of poor survival for cryopreserved gametes or embryos.

In order to obtain the vitrified state, viscosity and thus the concentrations of CPs in the incubation solutions are notably higher in VIT as compared to SF. Concerns arise as the majority of the CPs used may show possible harmful effects on cells and embryos, which will be discussed in more detail below. Despite the fact that several thousand IVF children worldwide have been born already after VIT, basic aspects and concerns regarding this technique remain to be elucidated. New applications such as fertility preservation have led to long storage times of vitrified gametes or embryos, but the question as to whether the state of the vitrified embryo is stable over time remains to be elucidated.

CONCERNS ABOUT SAFETY OF CRYOPRESERVED CELLS AND TISSUE

Stability of Cryopreserved Cells and Tissue during Storage in SF

The main danger for embryos cryopreserved by SF where crystallization occurs is the accumulation of radiation over time. The influence of a cumulative dose of radiation has been studied. The authors found that a radiation dosage equivalent to 2000 years of normal background radiation levels do not impair embryo survival or increase the incidence of mutations in offspring.[2–4] A few studies on SF embryos and the impact of time have been published; however, the results remain controversial. In SF mouse embryos, live births were reported after 20 years of storage,[5,6] and good embryo survival was also observed in other species, including sheep[7] and rabbits[8] after cryopreservation for 13 and 14 years, respectively. In humans, successful pregnancies have been reported after the transfer of SF cleavage-stage embryos stored for 7.5 and 8.9 years.[9,10] Similarly, a live birth was obtained after the transfer of a SF zygote stored for 8 years.[11] In a larger study, the authors found no reduction in pregnancy rates with prolonged storage time in SF.[12] In contrast,

other authors observed a reduction of 20% in the survival rate of SF human embryos when stored for 6–15 months, compared to 1 month of storage.[13] In a multiple stepwise logistic regression analysis, the clinical outcomes for SF cleavage-stage embryos stored for 1–6 months or 24–108 months were evaluated. No adverse effects on embryo survival or implantation rates were observed, but a tendency toward a lower pregnancy rate after prolonged cryopreservation was reported.[14]

Special Aspects of the Vitrified State

In addition to the aforementioned risk of accumulated radiation over time, further concerns arise with VIT. The stability of VIT cells during storage is questionable, as it represents the solidification of a supercooled viscous fluid without the formation of crystalline structures. It was shown that considerable molecular mobility persists near and just under the glass transition temperature (T_G), reducing the presumed stability of the vitrified state. VIT on the molecular level is the loss of translational and rotational degrees of freedom over a particular measurement timescale, leaving only bond vibrations within a fixed molecular structure. Residual molecular mobility below the T_G allows VIT objects to very slowly contract, release heat, and decrease entropy during relaxation toward equilibrium. Although diffusion is practically nonexistent below the T_G, small local movements of molecules related to relaxation are important and have consequences for cryobiology.[15] This is due to that fact that amorphous solids are formed when liquids are cooled very fast and molecules move too slowly to orient themselves. It is commonly admitted in the field of thermodynamic cryobiology that once the vitrified state is formed, it ages. Aged amorphous materials show decreased physical and chemical reactivity when compared to un-aged materials.

In seeds stored in liquid nitrogen, apparent time-dependent deteriorations were proven over a period of years. One possible reason for this is a degradation mechanism driven purely by vibrations of adjacent molecules.[16,17]

Concentrations of CPs

CPs are inevitable in SF as well as VIT. In contrast to SF, far higher concentrations of CPs are applied in VIT in the incubation solution, and their potentially toxic effects on cells and embryos have to be noted. However, the toxicity of CPs decreases with temperature and biological and biochemical activity. Therefore, duration and temperature during incubation with CPs are especially crucial.

Frequently applied in SF as well as VIT is DMSO, which is known to alter DNA methylation and potentially change the epigenetic profile of cells.[18] Congenital abnormalities might be induced by these epigenetic alterations. A consistently discussed phenomenon in this context is the "large offspring syndrome" in animals. In humans, the "Beckwith–Wiedemann syndrome," which is accompanied by overgrowth, enlarged organs, and overweight, has been described.[19] Recent studies report increased birth weights of children born after SF as well as VIT embryo transfer.[20,21] In VIT, predominantly ethylene glycol (EG) and DMSO are used, whereas the majority of SF protocols use PrOH, which was found to display higher genotoxicity. PrOH produces DNA damage *in vitro*, leading to chromosomal mutations.[22,23] Further, PrOH shows cytotoxic effects, as it lowers the survival rate of embryos and induces parthenogenetic activation.[24]

As stated at the beginning of this chapter, in VIT, concerns arise from the use of solutions with high concentrations of CPs. However, the incubation time in CPs before cooling in liquid nitrogen is far shorter in VIT as compared to SF. Recently, a study was conducted analyzing the intracellular concentrations of CPs in SF and VIT in order to evaluate their possible harmful effects. It was shown that only a small percentage of CPs transverse the cell membrane during VIT. Further, it was observed that the intracellular concentration of CPs in VIT zygotes is even lower than in SF.[25] Chapter 3 focuses on this topic in more detail.

Although it is known that temperature is important for evaluating the toxicity of CPs, and the duration and protocol for the incubation of cells with CPs are especially critical, the duration and temperature during storage could also play a role. Little is known yet regarding the possible impact of CPs during storage time.

Direct Contact with Liquid Nitrogen in Open-Device VIT and Long-Term Storage

Additional potential damage for VIT cells arise from the use of "open" devices. In order to increase cooling rates, gametes and embryos vitrified on open VIT carrier devices are plunged directly in liquid nitrogen. Apart from the potential risk of contamination during VIT, storage in open devices risks cross-contamination and physicochemical problems during storage. As a technologically young innovation in VIT, closed carrier devices were implemented in order to circumvent the direct contact of embryos with liquid nitrogen, with the goal of preventing infection with pathogens during VIT, as well as cross-contamination with reactive chemical compounds during storage.[26,27] Impairment of cells and embryos due to chemical reactions in liquid nitrogen during the storage time is avoided. This aspect of aseptic storage is of particular interest for long-term storage.

RESULTS FROM VITRIFIED BLASTOCYSTS AFTER LONG-TERM STORAGE

Study Design to Evaluate the Effect of Storage Time on Vitrified Blastocysts

As VIT is a young technique in ART, little is known about the stability of VIT cells over time and the health of the babies born after cryostoring embryos and oocytes for several years. In a retrospective study, the impact of

storage time on aseptically cryostored VIT blastocysts was studied by evaluating the outcomes of 603 embryo transfers of vitrified–warmed blastocysts during a period of 3 years.[28] Survival rates, implantation potentials, and birth rates, as well as the health of the children born, were analyzed.

All blastocysts were vitrified in closed devices (Vitrisafe) in EG/DMSO solutions as described previously.[26] The straws were kept in conventional liquid nitrogen storage tanks until embryo transfer, for a period of up to 6 years. At 3–6 hours prior to embryo transfer, blastocysts were warmed in sucrose solutions. Endometrial development was supported by estrogen supplementation and progesterone administration. Fourteen days after embryo transfer (ET), patient urine was tested for β-hCG; for positive patients, an ultrasound was performed at 6–8 weeks of gestation to confirm the presence of a fetal heartbeat. In a later follow-up, the birth rate and the health of the children born were evaluated.

The results were grouped according to storage time of blastocysts; no statistically significant difference in female age at the time of VIT was observed.

Survival of Vitrified Blastocysts over Time

A similar number of VIT blastocysts were warmed per ET (mean 3.2–3.6 embryos) in all groups. Survival rates as evaluated 3 hours post-warming ranged from 81.8% to 89.9% (Table 21.1). No statistically significant differences were observed between the groups, and no significant decreases in blastocyst survival over time were observed.

Clinical Outcomes after Transfer of Vitrified Blastocysts over Time

Similar to survival rates, no statistically significant differences in the pregnancy rates between the storage groups were observed. No declines between blastocysts stored for 0–3 months (48.0%) and those stored for 48–60 months (46.2%) were found (Table 21.2). A trend toward a decline in pregnancy rate in blastocysts stored for 6–12 months and for 12–24 months was seen (38.5% and 39.4%, respectively), which could be explained by patient histories. Patients in these groups were more often diagnosed with recurrent implantation failure (data not shown). In addition, when considering abortion or birth rates, no significant differences between the different storage groups were detected (Table 21.3).

Table 21.1 Survival Rates of Vitrified–Warmed Blastocysts in Relation to Storage Time

Storage time	No. of cycles	Mean female age at vitrification (years)	Survival rate (%)
0–3 months	100	34.5	83.0
3–6 months	169	35.0	89.9
6–12 months	99	35.2	84.3
12–24 months	92	34.4	82.0
24–36 months	90	34.4	81.8
36–48 months	27	33.6	87.8
48–60 months	26	32.4	83.1

Table 21.2 Clinical Outcomes after the Transfer of Vitrified–Warmed Blastocysts in Relation to Storage Time

Storage time	Pregnancy rate (%)	Ongoing pregnancy rate (%)	Abortion rate (%)	Birth rate (%)
0–3 months	48.0	40.0	7.0	33.0
3–6 months	38.5	30.2	1.8	27.8
6–12 months	39.4	33.3	5.1	28.3
12–24 months	50.0	33.7	2.2	32.6
24–36 months	53.3	47.8	8.5	38.9
36–48 months	48.2	40.7	—	40.7
48–60 months	46.2	38.5	7.4	26.9

Birth Weight and Gestational Age of Babies Born

After 38 multiple gestations and 147 singleton gestations, 226 babies were born. Only three babies were born with very low birth weights of <1500 g (1.3%), and three babies of >4500 g were reported (1.3%). Mean gestational age at birth was 39.1 weeks for singletons and 36.0 weeks for twins.

To study the birth weight of newborns in more detail, sibling children were compared. Patients who had previously given birth to a singleton in their fresh cycle, and then delivered another singleton after subsequent cryo-ET of VIT blastocysts were selected. In these 42 patients, the mean birth weights of children born after fresh ET was 3198 g, while the siblings born after cryo-ET weighed an average of 3406 g, and so no statistically significant difference was observed.

Table 21.3 Gestational Age and Birth Weight of Children Born after the Vitrification of Blastocysts

Parameter	Singleton gestation	Multiple gestation
No. of children	147	79
Mean birth weight (g)	3400 ± 562	2508 ± 406
<1500 g	2	1
<2500 g	2	38
2500–4000 g	124	40
4001–4500 g	16	—
>4500 g	3	—
Mean gestation age (weeks)	39.1	36.0
<28 weeks	1	—
<32 weeks	1	1
<37 weeks	13	19
Term birth	132	18

Congenital Malformation in Babies Born after Transfer of Vitrified Blastocysts

In the follow-up, multiple births and the health of babies were evaluated according to storage time. Two babies were born with multiple malformations: one girl after ET of a blastocyst stored for 12–24 months was diagnosed with stenosis, polysyndactyly, a palatine cleft, and a ventricular septum defect due to a deletion on chromosome 7. Another child derived after ET of a blastocyst stored for 48–60 months was diagnosed with hydronephrosis, clubfeet, and a single umbilical artery. In this case, no genetic background for the malformations was found. One minor malformation was reported for a twin girl born with a cleft palate after blastocyst storage for 36–48 months. Two children with trisomy 21 were reported, both in the 6–12-month storage group. Further, two stillbirths were reported (both singletons), both after storage for 0–6 months. In both cases, no medical reason for the stillbirth was detected. No trend toward a higher incidence of congenital malformation after a longer storage time of VIT blastocysts was found, although a larger number of children will need to be analyzed to entirely exclude any negative impact.

IS THE STORAGE OF VITRIFIED EMBRYOS (GAMETES) SAFE OVER TIME?

The aim of cryopreservation in ART is to preserve gametes or embryos. The time periods of cryostorage are increasing with new applications (e.g., fertility preservation ranging from months, possibly even to decades), meaning that consideration of cryostability over time is an increasingly important issue. Studies on SF embryos are controversial; some showed that the risk for birth defects is comparable with fresh ET,[29,30] or even lower,[31] whereas other authors found a significantly higher rate of congenital malformations after frozen embryo transfer.[32] The effect of cryostorage time on SF embryos is under debate.[12,14]

Knowledge about VIT embryos is still limited, with eight publications on VIT blastocysts—amongst them four case reports—showing no statistical differences in mean gestational age, birth weight, pre-term birth, and congenital birth defects when compared to fresh ET (summarized by Wennerholm et al.[30]). Although some case reports describe successful births of healthy babies after a prolonged period of cryostorage, it is still unknown how long an embryo can remain frozen and be successfully and safely transferred.[33]

In the data presented, no negative effects of prolonged storage for up to 6 years on the viability of VIT blastocysts has so far been observed. Further, no statistically significant differences in implantation rates, numbers of pregnancies, or birth rates were observed. Recent publications report that children derived from SF embryos are more often born large for gestational age, or even diagnosed with macrosomia, and it was hypothesized that this might be linked to the epigenetic changes induced by the freeze–thaw procedure. In the presented patient cohort with VIT blastocyst transfer, 2% of singletons were born >4500 g, which is a far lower rate than the reported 4.5% after SF, or 2.9% after fresh ET.[34]

There was an abortion rate in patients after the transfer of VIT blastocysts of 4.3% after a positive heartbeat was observed. The incidence of stillbirth was 2 in 191 births (1.0%). Severe malformations were reported in 4 out of 224 children (1.8%). These findings support other papers showing that VIT and the fresh transfer of blastocysts have similar neonatal outcomes.[35]

In summary, the data reassure use that VIT is a safe technique, and that cryostorage time does not have a negative impact on outcomes and children born. However, it has to be kept in mind that the data were observed using a "closed" aseptic device, thereby avoiding direct contact between the embryo and the liquid nitrogen during the VIT procedure, and importantly during the storage period. This hermetically sealed protection can also be obtained in SF. The ensuing results might therefore not be directly translated to the use of VIT with open devices, where this hermetically sealed protection of the embryos is not provided. In open devices, the constant contact with liquid nitrogen containing reactive chemical compounds might potentially promote embryo damage.

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22 Ovarian tissue vitrification—Clinical realities and outcomes

Sherman Silber

PREFACE

The developed world is in the midst of a widespread infertility epidemic. Economies in Japan, the United States, southern Europe, and even China are threatened by a decreasing population of young people having to support an increasing population of elderly people and retirees.[1] Infertility clinics are emerging throughout the world in huge numbers because of a worldwide decline in fertility as women age and become less fertile.[2] In her teenage years, a woman has a 0.2% chance of being infertile, and by her early twenties, it is up to 2%. By her early thirties, it is up to 20%.[2,3] Most modern women today do not think of having a baby until their mid-thirties, and by then, over 25% are infertile, simply because of aging and the decline in the number and quality of their oocytes. This is clearly demonstrated by the high pregnancy rate via using donor oocytes from young women placed into the uteruses of older women.[2–4]

Until recently, oocyte freezing had very poor to no success, and so ovary tissue slow freezing was the only preservation method we could rely upon. Of course, now we also have a favorable option of retrieving oocytes after ovarian stimulation and egg retrieval, using vitrification instead of slow freezing for cryopreservation.[5,6]

However, as we will discuss later in this chapter, many programs are not even aware of their terrible results with oocyte freezing because they are either using a brainless commercial product, or they simply are not using the best protocol perfectly. Success with oocyte freezing should be 95%–99%, but most clinics come nowhere near this. We will explain this in this chapter, but meanwhile, we expect that there will be many unhappy "fooled" women complaining about this in the next 5–10 years. Nonetheless, ovarian tissue freezing and transplantation still have great advantages over egg freezing. There does not need to be a prior delaying stimulation cycle, as ovarian tissue freezing would delay cancer treatment by only a few days. Furthermore, one cycle of ovarian stimulation and egg freezing does not ensure successful pregnancy as much as an entire ovary would, and finally, transplanting ovarian tissue back not only restores fertility, but also restores endocrine function. We hope that in this book and in this chapter, we can clarify this so that all clinics can avoid the confusion of the literatus and get the 95% results that patients expect.

OVARIAN TISSUE VITRIFICATION: CLINICAL REALITIES AND OUTCOMES

Fresh Series of Identical Twins with Premature Ovarian Failure

Let us take the clinical evolution of this technology in logical order. The first successful fresh human ovary transplantation was reported between a pair of remarkable monozygotic twins discordant for premature ovarian failure (POF) using a cortical grafting technique.[7] This key event allowed us to assess the results of fresh transplantation unclouded by the confusion that might have been caused by freezing. The transplantation technique has subsequently been refined over a larger series of nine consecutive successful fresh ovary transplants in identical twins (plus two fresh allotransplants to be treated separately), with resumption of normal hormonal cycling and menstruation in all cases, eventually leading to 14 pregnancies and 11 healthy babies born from the 9 fresh identical twin recipients.[8–11] This unusual consecutive series of fresh ovary cortical transplants helped us also to refine the techniques necessary for successful preservation of fertility for cancer patients using ovarian tissue freezing, with six additional successful pregnancies from nine frozen transplants. This unusual series also helped to establish a method for distinguishing between the egg loss from transplant ischemia versus the egg loss from cryopreservation. We now can report long-term follow-up (up to 8 years) of this original series of fresh transplants, and add to it our more recent experience with cryopreserved ovarian tissue.

Micro-hematoma formation under the graft was avoided by micro-bipolar cautery and micro-pressure stitches of 9-0 nylon. Constant pulsatile irrigation with heparinized saline prevented adhesions (Figure 22.1a–d).

Ovarian Cryopreservation

All of the frozen cases in the past that transplanted back into the patient utilized the slow-freeze approach.[12–14] However, we now use vitrification exclusively for cryopreservation in humans because of the results of *in vitro* viability analysis in humans, as well as *in vivo* transplant studies in the bovine and human.[11,15]

The goal of the *in vitro* study was to determine which method produced a higher cell survival rate: slow freeze or vitrification. The high viability (92%) of oocytes in control (fresh) specimens indicated only minimal damage to

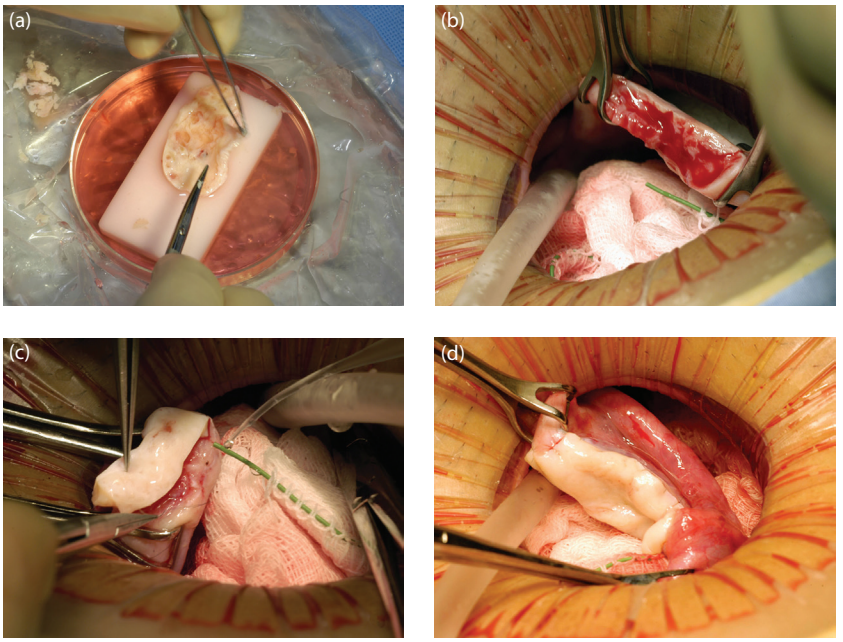

Figure 22.1 **Steps in the procedure of ovarian transplantation between monozygotic (MZ) twin sisters: (a) preparation of donor ovarian cortex by dissection in a Petri dish on ice; (b) preparation of recipient ovarian medulla; (c) attaching donor cortical tissue to recipient ovarian medulla; (d) attaching thawed donor cortical tissue for re-transplant to the recipient medulla.**

the eggs.[11] Overall, 2301 oocytes were examined from 16 specimens. Results within each of the three groups revealed no significant differences between fresh and vitrified tissue, but the viability of slow freeze-cryopreserved tissue was less than half that of vitrified tissue or controls (42%) ($p < 0.01$). Transmission electron microscopy has also been used to analyze ovarian tissue that had been either cryopreserved by slow freezing or vitrified by ultra-rapid freezing, showing vitrification to be superior.[16]

Standard H&E histology showed no difference between pre-freeze ovarian tissue and post-vitrification ovarian tissue (Figure 22.2a and b).

Finally, quantitative histologic study of primordial follicles in the bovine after vitrification and transplantation back to the cow 2 months later remarkably showed no follicle loss.

The basic science concept of vitrification, whether for eggs, embryos, or tissue, is to completely avoid any ice crystal formation by using a very high concentration of cryoprotectant and a very rapid rate (virtually "instant") of cooling. This is quite different from classic slow-freeze cooling, which relies on a partial and very gradual removal of water from the cell by encouraging ice crystal formation preferentially on the outside of the cell, drawing water out.

Using the vitrification technique, cortex tissue of each ovary is cut into slices of $1 \times 10 \times 10$ mm. Ovarian tissues are initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in handling medium (HM; HEPES-buffered TCM-199 solution supplemented with 20% serum substitution DMSO; cat. no. D2650; Sigma Aldrich, St. Louis, MO) for 25 minutes, followed by a second equilibration in 20% EG and 20% DMSO with 0.5 mol/L sucrose for 15 minutes. Ovarian tissues are then placed in a minimum volume of solution (virtually "dry") onto a thin metal strip (Cryotissue: Kitazato BioPharma, Fujinomiya, Japan), and submerged directly into sterile liquid nitrogen,[17] following which the strip is inserted into a protective container and placed into a liquid nitrogen storage tank (Figure 22.3).

For thawing, the protective cover is removed and the Cryotissue metal strip is immersed directly into 40 mL HM solution at 37°C supplemented with 1.0 mol/L sucrose for 1 minute. Then, ovary tissues are transferred into 15 mL of 0.5 mol/L sucrose HM solution for 5 minutes at room temperature, and washed twice in HM solution for 10 minutes before viability analysis or transplantation. No ice crystal formation occurs during any of these vitrification procedures.[15]

One of our twin recipients became pregnant at 39 years of age without medical assistance after her fifth menses, 8 months after transplantation. She delivered a healthy baby girl at full-term, then conceived again at 42 years of age, and delivered a healthy baby boy, again at full-term, 4 years after her transplant. Her ovary is still functioning to date after 7 years, and she conceived again at 45 years of age with another healthy boy.

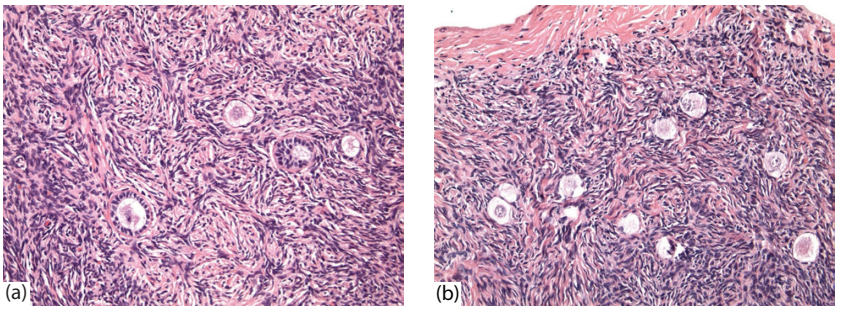

Figure 22.2 **Histology (a) pre- and (b) post-vitrification of ovarian tissue.**

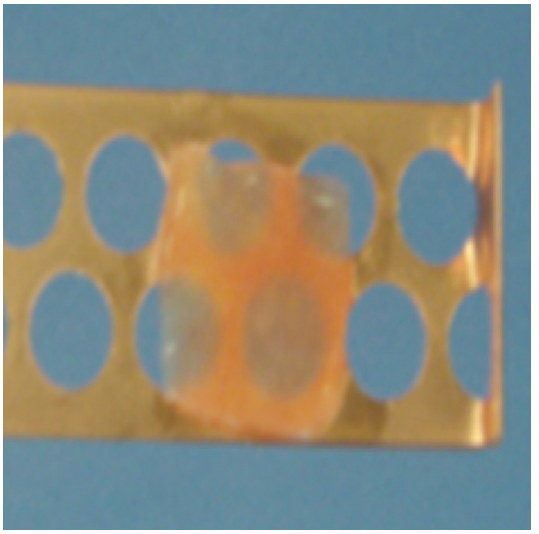

Figure 22.3 **Ovarian tissue slice.**

Table 22.1 Worldwide Frozen Ovarian Cortex Tissue Transplant Pregnancies

Case #	Diagnosis	Babies	Which Center
1	Hodgkin's	1	Donnez
2	Neuro tumor	1	Donnez
3	Non-Hodgkin's	1	Meirow
4	Hodgkin's	1	Demeestere
5	Ewing's	3	Andersen
6	Hodgkin's	1	Andersen
7	Premature ovarian failure	1	Silber
8	Hodgkin's	2	Silber
9	Polyangiitis	1	Piver
10	Breast cancer	2	Pellicer
11	Sickle cell	1	Piver
12	Hodgkin's	2	Revel
Totals: 12 patients		17 babies	8 centers
Fresh + frozen		28 babies	Silber – 14 babies

Note: The estimated outcomes presented in this table are based on a survey performed in October 2013 by Dr. Silber.

This newly favorable experience with ovarian cortex grafting is not limited just to our center.[18] Equally robust results are being experienced in Belgium, France, Spain, Denmark, and Israel. Frozen ovarian grafts (even with the slow-freeze technique) in Denmark are lasting over 5 years, and many spontaneous pregnancies have been reported with no need for *in vitro* fertilization (IVF) or other ancillary treatment. At the time of this writing, over 28 healthy babies have been born from ovarian tissue grafting fresh and frozen, and most involved no IVF and resulted from regular intercourse with no special treatment (Table 22.1).

Frozen Cortical Ovarian Transplantation

The most common benefit of ovarian transplantation is not the unusual cases of fresh grafting in identical twins, but rather the protection of the fertility and future endocrine function of young women undergoing cancer treatment.[11,15,19–26] Since 1996, we have frozen ovary tissue for 68 young women with cancer or at risk for POF, of whom 16 had spare frozen tissue subjected to detailed viability testing before cryopreservation and after thaw. Only one had ovarian metastasis, a young woman with widespread breast cancer metastasis throughout her entire body. Otherwise, none of our other 61 cases had any tumor cells in their ovaries. The reason for the remarkable absence of ovarian metastasis might possibly be due to the fibrous avascular nature of the ovarian cortex (Anderson C, personal communication). In fact, the reason why fetal ovarian tubules (which in the fetal male become seminiferous tubules) invade the fibrous cortex and become follicles is that the dense fibrous tissue of the cortex (which in the fetal and adult testis is just tunica albuginia) is needed to suppress the resting follicles from developing all at once prematurely. In addition to these 68 pathological cases, seven women have had ovarian tissue frozen simply to allow them to have the possibility of bearing children at an

older age, because they had to delay childbearing for strong personal or economic reasons.

Future Prospects for Ovarian Tissue Transplantation

After ovarian transplantation, all patients were able to attempt natural conception every month without medical assistance. In fact, the commonly held view that egg freezing is a proven technique and ovary tissue transplantation is "experimental" is belied by the fact that most of the successful pregnancies resulting from fertility preservation in cancer patients thus far have been from frozen ovary tissue, and few at the date of this writing have come from frozen oocytes.[18] Of course eventually they will have long term results to report, but not yet. However, for cancer patients, ovarian tissue does have a better record currently than egg freezing. Most of our cured cancer patients who have "young" ovarian tissue frozen feel almost grateful that they had cancer, because otherwise they would share the same fear that all modern, liberated women have about their "biological clock."

At the time of this writing, we are aware of numerous other births after implanting ovarian tissue, to a total of over 37 live births thus far.[18,26–31] Thus, despite initial skepticism, this technique is now gaining worldwide acceptance and is being enthusiastically received by young women of reproductive age with cancer.

EGG AND EMBRYO VITRIFICATION

As mentioned in the preface, many centers that perform oocyte vitrification are not doing it well, and their protocols lead to terrible egg survival rates. Among their errors are too rapid and changing osmolality with dangerously aggressive osmotic shifts. Also, there is a common failure in failing to create a rapid enough "freeze," and worse yet, not a rapid enough "thaw." The commercial kits that are designed to make this "easy" often fail in this regard, and closed freezing is worse than open freezing for rapid "freeze and thaw." Vitrification for freezing eggs or embryos was first suggested in the mid-1980s.[32,33] However, it was not until 2005 that a highly efficient method was published, which stimulated a huge wave of justified enthusiasm for this approach to egg and embryo freezing.[4,6,34,35] But the details of this successful "bridge technique" have been lost, or have given way to poor-quality commercialization (Figure 22.4a–f).

The concept behind vitrification is not just its potential simplicity (given that no freezing machine is required), but that it must completely eliminate ice crystal formation. Instead of clinical IVF programs having to weigh carefully the risks to pregnancy rate posed by embryo or egg freezing, both can now be cryopreserved without concern in virtually any case in which there would be a clinical advantage. With the new vitrification methodology,

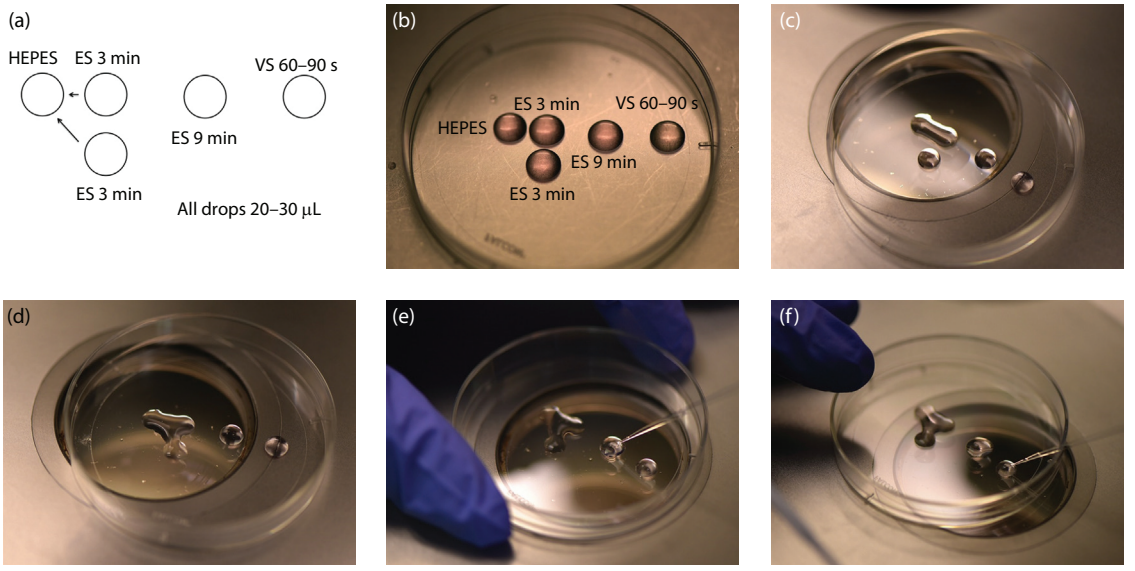

Figure 22.4 **(a) The "bridge" technique for oocyte freezing. (b) Setup for "bridge" equilibration. (c) First "bridge" between ES and isotonic HEPES media; 3 minutes. (d) Second "bridge" equilibration; 3 minutes. (e) Transfer to full concentration ES; 9 minutes. (f) Transfer from ES to VS; 60–90 seconds. High cryoprotectant concentrations are not toxic. It is only the rapid osmotic shifts that kill the egg or embryo and give the incorrect impression of toxicity. To avoid over-rapid osmotic shifts (that are more poorly tolerated by the egg than the embryo), the original "bridge" technique is best. ES solution droplets are first "bridged" over to the iso-osmotic solution the egg is in, and 3 minutes later, another droplet of ES solution is "bridged" over to the original solution very gradually and continuously raising the osmolality of the solution the oocyte is resting in.**

there seems to be no difference between fresh and cryopreserved eggs or embryos, so long as the principles perfected in 2005 are followed.

For vitrification, the cryoprotectant solution is a combination of EG and DMSO (cat. no. D2650; Sigma Aldrich, St. Louis, MO). The embryo or egg is transferred initially into gradually increasing concentrations of equilibration solution (7.5 mol/L EG and 7.5% DMSO in 20% synthetic serum substitute [SSS]) for 10–15 minutes, followed by placement for over 1 minute in vitrification solution (15 mol/L EG and 15 mol/L DMSO in 20% SSS and 0.5 mol/L sucrose).

Contrary to popular myth, leaving it in vitrification media for less than 1 minute does not allow for enough cryoprotectant permeation. Also, this is best accomplished by the "bridge technique" to allow the most gradual increase in osmolality. The embryo is not left in a droplet, as that would slow the cooling rate. All excess fluid is removed by pipette from the Cryotop platform so that there is only a thin film of fluid surrounding it, in order to allow for the most rapid temperature drop and warming rate later. The embryo is then directly immersed into liquid nitrogen. The Cryotop containing the embryo is then placed in a canister in the liquid nitrogen tank for storage.

In the warming step, embryos are placed in decreasing concentrations of sucrose solutions to remove the cryoprotectants. The Cryotops are first rapidly plunged into a 37°C dish containing warming solution (1.0 mol/L sucrose) for 1 minute. The embryos are then slowly introduced in a stepwise fashion to dilution solutions (0.75 mol/L, 0.5 mol/L, and 0.25 mol/L sucrose). A wash solution (0.0 mol/L sucrose) is slowly added to the embryos in the dilution solution, and the final rinse for the embryos is in 100% wash solution.[6] This protocol was designed to avoid too rapid osmotic shifts that could be caused by such high concentrations of cryoprotectant.

The high concentrations of cryoprotectant are actually not toxic. The appearance of toxicity comes only from too rapid an osmotic shift. The ultrarapid rate of cooling (–23,000°C/min) and the high-end concentration of cryoprotectant lowers the freezing point dramatically, and allows the ice crystallization phase to be completely avoided.

With vitrification, mature retrieved oocytes can be successfully cryopreserved with 95% success. For embryos, there really is no difference at all between fresh and frozen.[35,36]

New technology in cryopreservation via vitrification allows us to remove ovary tissue and freeze it to protect it from sterilizing cancer treatment in young women, as well as to freeze individual mature eggs. Our latest published data reveals a very high pregnancy rate with either fresh or frozen ovary tissue transplants of over 76%.[37] It also allows us to stop the aging of the ovary and eggs, which is the major cause of the current worldwide infertility epidemic. It will protect the future fertility potential of young women with cancer, and will also allow an expansion of the reproductive lifespan in any young woman who wishes to delay childbearing, or delay her age of menopause.

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23 Vitrification of human testicular tissue, spermatogonia, and spermatozoa

Christine Wyns, Gael Abou-Ghannam, and Jonathan Poels

INTRODUCTION

Improvement of cryopreservation techniques for human male germ cells is highly warranted for optimal clinical management of male infertility in the context of assisted reproduction programs, as well as fertility preservation for cancer patients.

Vitrification has been developed as an innovative strategy for gamete and gonad preservation, with a view to minimizing cell or tissue damage due to ice crystal formation and solution effects, as observed during slow freezing. Vitrification involves a transition from a liquid phase to a vitreous phase, which occurs at a low temperature following very fast cooling by directly immersing of samples in liquid nitrogen (LN_2).[1] Use of high cooling rates means water molecules have less time to escape from cells, preventing cell shrinkage and osmotic cell damage. Addition of high cryoprotectant (CPA) concentrations increases viscosity, thereby preventing movement of water molecules with solute concentrations and ice crystallization. Ease of use, speed, and absence of need for freezing equipment have contributed to the rapidly growing interest in this technique.

Application of vitrification to mature or immature male germ cell suspensions and immature gonadal tissue, as well as lessons learned from reported empirical protocols, will be reviewed in this chapter.

VITRIFICATION OF SPERMATOZOA

It is widely known that cryopreservation has detrimental effects on spermatozoa.[2,3] Spermatozoa are cryo-sensitive cells, showing a decrease in sperm motility of 50%–90% after cryopreservation by conventional slow freezing using permeable CPAs, whether programmed or not as with LN_2 vapor freezing.[4–6] Classical vitrification using high concentrations of permeable CPAs is also unsuitable for spermatozoon preservation due to lethal osmotic and cytotoxic effects.[7,8] Insights into the cryobiology of spermatozoa related to their physical features have been summarized.[9] Briefly, their intracellular milieu, containing large amounts of proteins, nucleotides, and sugars and a small quantity of water,[10] is responsible for their high intracellular viscosity that determines the possibility of vitrifying these cells without permeable CPA and at relatively low cooling rates.[11] The small size of the spermatozoon head may be an additional advantage for ensuring intracellular vitrification.

Since extrapolation of observations in animals to humans is limited by the differing sizes of sperm heads, which in turn appear to be correlated to cryostability,[11–13] this chapter will focus on human spermatozoa. Only a few teams have studied the vitrification of human spermatozoa.[14–17] Isachenko and colleagues were the first to report the successful vitrification of human spermatozoa in the absence of toxic permeable CPAs by directly plunging a sperm suspension into LN_2.[14,15] Changes in the mitochondrial membrane potential, as a marker of mitochondrial activity, were found to be dependent on the vitrification medium. The best results were achieved with a combination of 0.25 M sucrose and albumin, providing protection for about 65% of spermatozoa.[18] Furthermore, CPA-free cryopreservation by vitrification was achieved using very fast cooling rates (ranging from 150–250°C/min to 7.2×10^5°C/min) and instant warming in a warm medium, with an approximately 40% reduction in motility of spermatozoa, but unaffected DNA integrity.[19] Overall, depending on the vitrification method used and the quality and preparation of the original sperm sample, motility levels of up to 60% could be reached for normozoospermic ejaculates,[20] and 20% for oligozoospermic samples after warming.[21,22] Open systems with direct contact with LN_2 are preferentially used to achieve the highest cooling rates, but because these systems are at risk of microbiological contamination, methods allowing aseptic vitrification have been developed.[23]

Vitrification of spermatozoa has been compared to standard LN_2 vapor freezing. A significant improvement of 11.6% in post-thaw motility was achieved when vitrifying swim-up prepared spermatozoa with no CPAs,[14] and almost two-fold higher residual motility after vitrification using 0.25 M sucrose and 1% LGPS (LifeGlobal Protein supplement, IVF online, Guelph, Ontario) and fast warming at 37°C for 10 seconds was observed.[24] In addition, no statistical differences were noted for parameters such as viability, recovery rate, or percentage of morphologically normal spermatozoa with nondamaged DNA, and levels of membrane changes related to "cryocapacitation" were also similar.[21] Higher rates of plasma and acrosomal membrane integrity were attained.[21,22] An increased mitochondrial membrane potential was also reported after vitrification,[17] although the results were not always consistent, probably due to use of different vitrification protocols.[18] While lower DNA fragmentation levels were

observed after vitrification by some authors,[17] others did not find any clear benefit in terms of DNA integrity.[24,25] When compared to controlled programmed slow freezing, similar motility and DNA integrity were achieved with both techniques.[20] Solid-surface vitrification (SSV), as a variant form of vitrification, has also been compared to the slow-freezing technique.[16] Using sperm-freeze CPA (Fertipro NV, Beernem, Belgium) for both protocols, sperm motility, morphology, and vitality were not found to be different. However, a slightly significant difference was detected in favor of SSV for DNA integrity and morphological parameters, such as tail defects.

Since there is evidence that vitrification of the intracellular compartment of spermatozoa occurs even with slow cooling rates, thereby avoiding intracellular ice formation,[9] this questions the benefits of using very high cooling rates to cryopreserve spermatozoa. Indeed, ultrarapid freezing in vapor before immersion in LN_2, yielding five- to ten-times higher cooling rates than those used in conventional slow freezing, has also been investigated, both with the addition of CPAs[26–29] and without.[19,26,28] Compared to CPA-free vitrification, which allowed 60% residual motility, this method was not found to differ in terms of sperm motility, fertilization ability, or DNA integrity outcomes.[19]

Placing vitrified samples directly in a warm solution involves the use of very high warming rates, which is of paramount importance for the successful vitrification of spermatozoa. Indeed, if rewarming takes place too slowly, recrystallization of the vitrified intracellular solution occurs, with the development of large intracellular crystals.[30] Furthermore, due to its physical features, the intracellular compartment of the sperm cell will vitrify during rapid cooling, but will not devitrify until it reaches approximately –30°C during warming. This causes damage to spermatozoa as a result of an osmotic imbalance, with exposure of the outer surface of the plasmalemma to the stresses of osmotic shock due to highly concentrated extracellular fluid.[9]

The reproductive potential of vitrified sperm was finally proved by obtaining healthy live births, reported after intracytoplasmic sperm injection with vitrified spermatozoa,[31] following intrauterine insemination of vitrified semen,[32] and after rapid freezing of testicular sperm.[33]

VITRIFICATION OF IMMATURE MALE GERM CELLS OR TISSUE

Cryopreservation of spermatogonial stem cells (SSCs) or immature testicular tissue (ITT) containing SSCs is so far the only approach that can be proposed to preserve fertility in young boys whose fertility is threatened by gonadotoxic treatments, since spermatozoa are not produced before puberty. To restore fertility from cryostored tissue, auto-transplantation of testicular cells, cellular aggregates, or tissue, as well as *in vitro* maturation of SSCs, need to be considered.[34]

VITRIFICATION OF SSC SUSPENSIONS

Only one study has reported the vitrification of human diploid germ cell suspensions. In this study, cell viability was higher with vitrification using open pulled straws compared to three different slow-freezing protocols,[35] but similar recovery and viability rates were previously achieved by others with slow freezing.[36]

LESSONS LEARNED FROM VITRIFICATION OF ITT IN ANIMALS

ITT vitrification has been investigated in different species,[37–41] including nonhuman primates.[42] Protocols were empirically developed or adapted from those established for other reproductive cells or tissues. Table 23.1 summarizes studies on vitrification protocols for ITT, showing the CPAs and systems used.

Since open systems, with their advantage of higher cooling rates, run the risk of microbiological contamination by direct contact with LN_2, therefore closed systems have been used by most teams. For closed-system vitrification, different tissue carriers are available: closed plastic cryovials,[38,43] the straw-in-straw method,[44] and aluminum boats partially immersed in LN_2, known as SSV.[39,45,46] Higher cooling rates were obtained with aluminum, being a better temperature conductor, which may explain the discrepant results in studies on the vitrification of pig ITT, with the best outcomes achieved with SSV by Abrishami et al.[39]

Most vitrification protocols use a combination of two permeable CPAs, the most common being dimethyl sulfoxide (DMSO) and ethylene glycol (EG). Observations point to the importance of decreasing exposure times to high concentrations of CPAs in order to minimize their toxicity. ITT vitrification was initially studied in pigs.[38,39] After vitrification and xenotransplantation, Zeng et al.[38] observed decreased germ cell viability and similar differentiation potential to conventional slow freezing, albeit delayed compared to fresh tissue grafts.[38] When comparing different exposure times to CPAs, Abrishami et al. found better cell viability after 5 minutes of equilibration in DMSO, and complete and similar differentiation to fresh tissue when exposed for 5 or 15 minutes to glycerol, or for 5 minutes to DMSO. Furthermore, the proportion of elongated spermatids was higher when exposure times were the shortest.[39]

Promising results were also obtained in mice using an open vitrification system and comparing it with slow freezing with 0.7 M DMSO.[40] Although markers of cytotoxicity and cellular necrosis were lower after vitrification and warming, higher apoptosis levels and decreased intratubular cell density were observed after 1 day of organotypic culture of the tissue. However, this difference in cell density was not detected on day 3 of culture because of increased cell proliferation. This observation, plus the similar diameters of seminiferous tubules found in both fresh and vitrified–warmed tissue, suggest that the tissue is not—or is only slightly—affected by vitrification.

Table 23.1 Vitrification Protocols for Immature Testicular Tissue in Animals

References	Species	Vitrification cryoprotectants	Vitrification systems: open/closed
Bono-Mestre et al., 2009[37]	Zebrafish	DMSO 20%, EG 20%, FBS 20%	Closed
Zeng et al., 2009[38]	Pigs	EG, NaCl 0.9%, raffinose 0.5 M	Closed
Abrishami et al., 2010[39]	Pigs	VS1: DMSO 15%, EG 15%, FBS 20%, sucrose 0.5 M VS2: glycerol 7%, EG 15%, FBS 20%, sucrose 0.5 M	Closed
Curaba et al., 2011[40]	Mice	DMSO 20%, EG 20%, HSA 25 mg/mL	Open
Gouk et al., 2011[44]	Mice	EG 40%, sucrose 0.6 M	Closed
Poels et al., 2012[42]	*Macacca mulatta*	DMSO 15%, EG 15%, sucrose 0.5 M, HSA 25 mg/mL	Open
Baert et al., 2012[45]	Mice	DMSO 15%, EG 20%, sucrose 0.5 M, FBS 20%	Closed
Liu et al., 2012[41]	Japanese quail	DMSO 15%, EG 15%, sucrose 0.5 M, FBS 20%	Open
Gholami et al., 2013[43]	Mice	DMSO 15%, EG 15%, FBS 20%	Closed

Note: DMSO = dimethyl sulfoxide; EG = ethylene glycol; FBS = fetal bovine serum; HSA = human serum albumin; VS = vitrification solution.

Furthermore, vitrification did not appear to increase the expression of apoptosis-related genes in SSCs.[43] Higher cell viability was also reported after vitrification in a closed system compared to both slow and rapid freezing (71.5% of cells were viable after spermatogonial enrichment versus 82.9% in control tissue).[44] Recovery of complete spermatogenesis similar to fresh tissue samples was eventually achieved after vitrification in a closed system and allografting.[45]

The potential of vitrification to preserve ITT of nonhuman primates has been studied by means of xenografting in a nude mouse model. Besides the preservation of seminiferous tubule integrity and the survival and proliferation of spermatogonia, the functionality of Leydig cells was demonstrated.[42] The homogeneous distribution of seminiferous tubule integrity was achieved in fragments of up to 16 mm^3 when 1 mm-thick slices were vitrified.[42] Appropriate fragment size is a major concern for vitrification, since tissue damage increases with size due to longer penetration times. This leads to the overexposure of surface cells to CPA concentrations and slower cooling rates, which are related to greater amounts of tissue and enlarged vapor coats around fragments when immersed in LN_2.[46] Since vitrification is time consuming, establishing the maximum size of fragments yielding optimal outcomes after vitrification may be crucial for laboratories.

The importance of appropriate warming rates has also been the focus of vitrification studies. The superiority of a rapid warming protocol over a slower one has been demonstrated. Indeed, while no difference in graft vascularization was observed between fresh and vitrified–warmed tissue at 40°C, vascularization was less developed when warming at room temperature.[41] Besides achieving complete SSC differentiation after vitrification, efforts in the field were finally rewarded by the demonstration of true reproductive potential in pigs[47] and Japanese quails,[48] with normal reproductive development reported in pigs produced with sperm from vitrified xenografted ITT.[49] Such encouraging results in animals confirm the potential of vitrification in the field of fertility preservation.

VITRIFICATION OF ITT IN HUMANS

Vitrification of human ITT has been evaluated *in vitro* in an organotypic culture system[50] and *in vivo* in a nude mouse transplantation model.[51] Prior to vitrification, the tissue was pretreated with an equilibration solution consisting of 7.5% EG, 7.5% DMSO, and 0.25 M sucrose, supplemented with 25 mg/mL human serum albumin (HSA) for 10 minutes at 4°C. It was then transferred to the vitrification solution containing 15% EG, 15% DMSO, 0.5 M sucrose, and 25 mg/mL HSA. After removal of the surrounding vitrification medium, the tissue was placed in open cryostraws and plunged into ultraviolet-sterilized LN_2, according to the protocol of Parmegiani et al.[52] It was stored in sealed, precooled cryotubes. For warming, the cryotubes were removed from the LN_2 and the straws were quickly immersed in a 35°C warming solution containing sucrose (1 mol/L) supplemented with 25 mg/mL HSA, before being transferred to three consecutive baths of warming solutions with decreasing sucrose concentrations. Ten-day organotypic culture of vitrified–warmed tissue showed *in vitro* spermatogonial survival, but did not allow further functional evaluation of human SSCs after vitrification.[50]

As shown in Figure 23.1, good preservation of seminiferous tubule integrity was obtained after transplantation of the vitrified ITT, with strong cell cohesion and adhesion to the basement membrane and no sclerosis.[51] A comparative study investigating the functionality of tissue after transplantation in a nude mouse model showed that its developmental potential was similar with

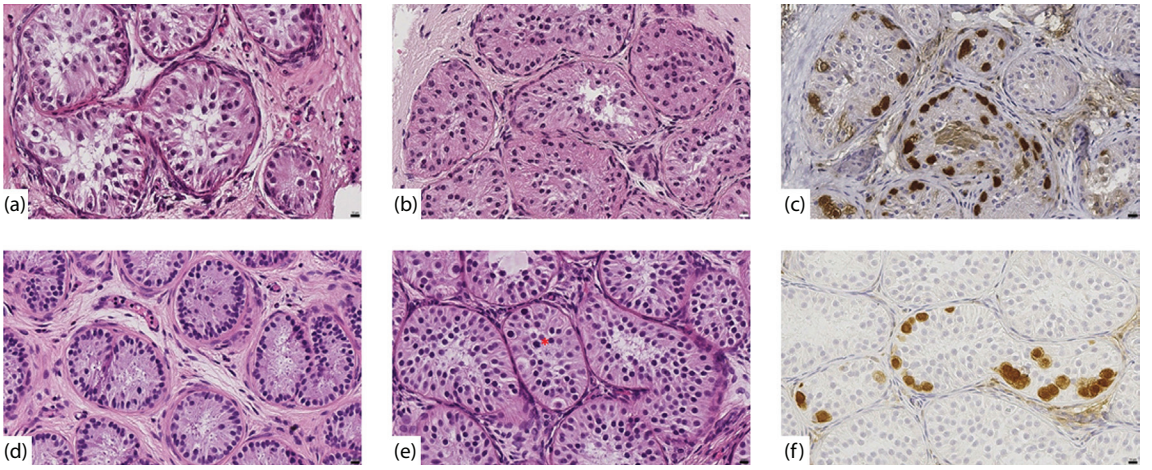

Figure 23.1 **(See color insert.) Fresh and vitrified human immature testicular tissue grafted for 6 months to castrated nude mice. Hematoxylin–eosin staining of fresh grafted (a and d) and vitrified grafted (b and e) tissue of 2- and 9-year-old donors, respectively. Spermatogonial differentiation to the pachytene stage was achieved in vitrified grafted tissue of the 9-year-old donor (e, asterisk). Immunostaining of spermatogonia with MAGE-A4 antibody can be seen in the vitrified grafted tissue of the 2-year-old (c) and 9-year-old (f) donors. Magnification 400×. Scale bar: 10 μm.**

both vitrification and conventional slow freezing[51] currently used in clinical practice.[53] Survival, proliferation capacity, and early differentiation up to the pachytene stage were demonstrated in vitrified SSCs, and functional Leydig cells were observed in vitrified tissue. Moreover, the functional potential of the tissue appeared to be similar to fresh tissue grafts, indicating that not only the cryopreservation method, but possibly also the xenotransplantation model may be implicated.[51]

Although vitrification offers a number of theoretical advantages over slow freezing (e.g., avoidance of ice crystal formation), the data are thus far insufficient to modify clinical practice and to exclude subtle cryodamage of the SSC niche.

CONCLUSION

The feasibility and efficacy of the vitrification of human spermatozoa have been demonstrated. However, besides proving the reproductive potential of vitrified spermatozoa, their genetic integrity should be further investigated on larger numbers to confirm that damage does not exceed that incurred by conventional freezing.

Vitrification of immature human male germ cells appears to be a promising strategy, yielding preservation of ITT integrity, as well as SSC survival and capacity for proliferation and early differentiation. Nevertheless, there is no proof as yet of complete functionality of the tissue in humans, and a number of unknowns remain (e.g., speed of tissue penetration of CPA, length of equilibration period to achieve appropriate CPA concentrations without increasing their toxicity due to prolonged exposure, and variations in cooling rates influenced by carrier systems [open versus closed]). So far, there is no evidence that vitrification of ITT is superior to the slow freezing already used in clinical practice in the context of clinical trials. Until the reproductive potential of frozen ITT protocols has been proved for SSC cryopreservation, methods still need to be further investigated.

One key motivation for pursuing research on gamete and gonad vitrification is to widen the diffusion of the technique to centers where there is no availability of cryopreservation equipment.

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24 Vitrification in pluripotent stem cell banking: Requirements and technical solutions for large-scale biobanks

Julia C. Neubauer, Axel F. Beier, Frank Stracke, and Heiko Zimmermann

INTRODUCTION

Pluripotent stem cells (PSCs) have been of central interest in biomedical research since the first isolation of human embryonic stem cells (hESCs) in 1998.[1] PSCs have unique potential for novel therapies of degenerative diseases,[2,3] due to their ability to differentiate into cells of all three germ layers[4,5] and their theoretical property of unlimited proliferation.[6] Studying these cells advances the understanding of human developmental processes as well as cellular differentiation processes. Furthermore, with the discovery of "induced" PSCs (iPSCs), new possibilities in the area of drug discovery and compound development have appeared: whereas hESCs can only be isolated from the inner cell mass of blastocysts during the early development of embryos usually created by *in vitro* fertilization, iPSCs are artificially generated by epigenetic reprogramming techniques from somatic cells.[7,8] This offers for the first time the possibility of producing patient- or disease-specific PSCs and integrating specific mutations (e.g., using Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein [CRISPR/Cas]-technology[9]) to generate model cells for compound screenings. New cell handling tools (e.g., surface-based hanging drop cultivation[10]) will enable single-cell diagnostic and new high-throughput/high-content screening for drug discovery. In the future, PSCs may even be used to replace damaged or nonfunctional cells and tissue, enabling personalized regenerative medicine.

However, prospective applications will need high-quality stem cells or stem cell-derived progenitors that are permanently available, reliable, and well characterized.[11] Until now, thousands of different stem cell lines have been isolated and cultivated by researchers in various scientific fields all over the world, using different protocols for the isolation, identification, and cultivation of the cells. Hence, the standardization of processes and reproducibility of protocols are limited. Additionally, long-term culture may change cell characteristics and increase the risk of contamination with viruses or mycoplasma. Therefore, cryobanking of defined and fully characterized human PSCs is in the focus of several European Union-funded projects, even coordinated by the pharmaceutical industry (www.ebisc.org), illustrating the high relevance of this cell system.

One major problem of PSCs is the biological complexity of these cells, which is significantly higher than normal cell lines. They usually grow as three-dimensional cell clusters ("colonies"), and are often cultivated on a feeder layer (a nonproliferating murine cell line) to maintain their pluripotency.[1] Additionally, PSCs need cellular contacts to each other in order to survive, which leads to serious problems during cryopreservation. Because of the colony's three-dimensional structure and size (<100 µm to >1 mm), fast and homogenous removal of the thermal energy while cooling and permeation of cryoprotectants into the inner part of cell clusters before freezing is extremely limited.[12–14] This increases the risk of large ice crystal formation within the colony, disruption of cell–cell contacts, and serious cell damage. Although the use of a selective Rho-associated kinase inhibitor (ROCKi, Y27632[15]) significantly improves the survival of cells during passaging and dissociation,[16,17] mechanisms of cell death during cryopreservation are still not completely understood, and recovery rates remain insufficient.

Slow-rate cooling and rapid thawing protocols are the current state of the art in the banking of various cell types. These allow sterile storage of samples in the vapor phase of liquid nitrogen, the cryopreservation of large quantities of PSCs, and they are comparatively easy to perform.[18] However, such protocols yield very low recovery rates, and commercial providers of hESCs guarantee a recovery of just 0.1%–1%.[19] To increase efficiency, some cryobanks, especially in the field of reproductive medicine, use rapid-freezing protocols in small-volume straws.[20,21] In this "vitrification" process, the liquid solidifies without crystallization and the growth of ice crystals. The procedure of vitrification was discovered and extensively investigated by Luyet and colleagues in 1967,[22,23] and only some years later was it proven to be a very efficient tool for the preservation of sensitive and complex cell systems (e.g., oocytes in reproduction medicine).[24] The idea behind vitrification-based storage is to prevent the specimen from biochemical degeneration by low temperatures, without the corruptive effects of phase separation and biomolecular reorganization that occurs during slow equilibrated cooling. Hence, unlike slowly cooling the sample to a stable equilibrium state, fast vitrification results in a metastable off-equilibrium state of the sample—virtually a "snap-shot" of its room temperature molecular composition. Physically, all liquids of the sample solidify to a glass-like state by an increase of the viscosity without any ice crystallization. The cell volume

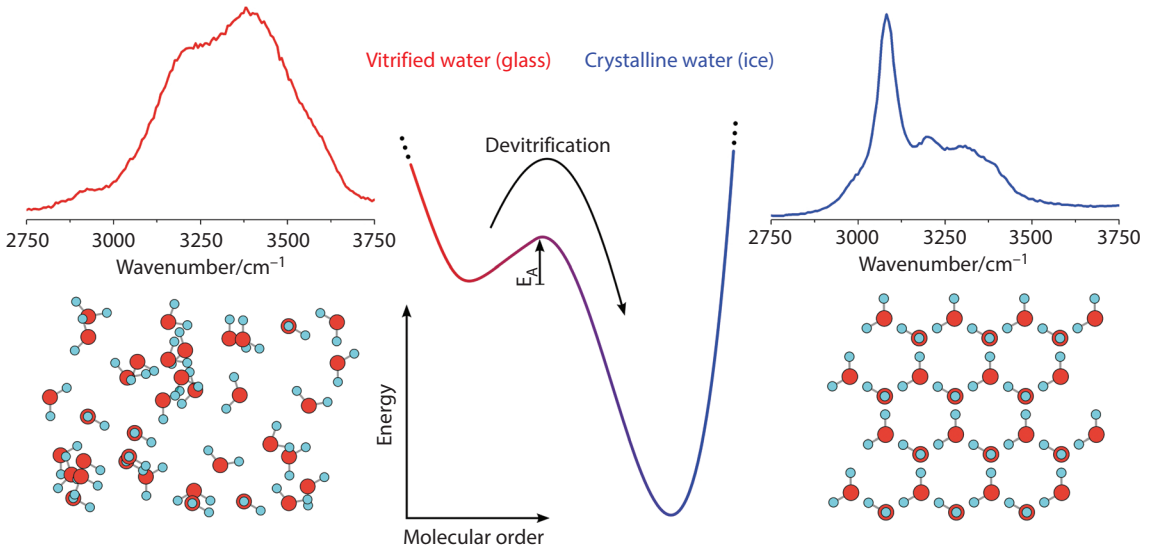

Figure 24.1 **(See color insert.) Schematic of the devitrification process in an energy diagram, the corresponding molecular arrangements, and the Raman spectra of liquid water (15°C) and ice (–115°C). Liquid water resembles the spectral shape of vitrified water quite well. It was used here as the vitrification of macroscopic amounts of pure water is practically impossible. E_A is the activation energy for devitrification.**

remains unchanged, and the biomolecules persist in their room temperature conformations. But the vitrified state is fragile, tending to change to the stable, crystallized state under the release of the latent heat. This process is called devitrification, and can be observed by Raman spectroscopy (Figure 24.1). Once the devitrification process is triggered, the entire sample will irreversibly crystallize, resulting in complete sample loss.

In order to avoid devitrification, the sample must strictly be kept below its glass transition temperature (T_G). Put simply, at temperatures above the T_G, the thermal energy of the glassy sample exceeds the activation energy (E_A) of molecular reorientation and crystallization may start. Whereas the T_G of pure water is at about –137°C,[25,26] high concentrations of vitrification additives like dimethyl sulfoxide (DMSO), ethylene glycol (EG) and various carbon hydrates can be used to raise the T_G to a certain extent,[27] but commonly used vitrification media still possess T_G values significantly below –100°C.[28]

Although vitrification procedures in straws are very efficient, they can only be applied for the storage of small sample volumes with only up to ten-cell clumps. Additionally, the workflow is difficult to perform even for trained staff, and minor changes of the incubation times can lead to a complete loss of samples. Hence, automation and upscaling of this complex procedure is not possible, so vitrification is not yet suitable for the operation of large biobanks. Finally, vitrified samples have to be stored in liquid nitrogen, risking contamination with viruses or cross-contamination with other cell types,[29,30] preventing usage in Good Manufacturing Practice (GMP)-compliant workflows.

The aim of this chapter is therefore the derivation of requirements for large-scale biobanks concerning efficient cell handling protocols and storage technologies for PSCs on the basis of demanding vitrification procedures. Furthermore, solutions using state-of-the-art technologies are shown and a reference workflow is described.

PROCEDURES AND WORKFLOWS FOR THE VITRIFICATION OF HUMAN PSCs

Human PSCs (hPSCs) are very sophisticated cell systems, as these cells grow in multicellular colonies. hPSCs can be maintained in their undifferentiated state by co-culturing with mouse or human embryonic fibroblasts, or using growth media containing special growth factors and cytokines. The applications of hPSCs range from regenerative medicine to developmental biology, as these cells are capable of differentiating into cells of all three germ layers.

There is an increasing demand for permanently available hPSCs and hPSC-derived cells, but freezing and storage at cryogenic temperatures is still the only strategy for storing viable biological material over long periods with minimal alterations, sufficient viability, and conservation of functionality.

Most cryopreservation protocols of hPSCs are currently based on freezing cells in suspension using slow-rate protocols. For adherent cell systems, like PSCs, these procedures include cell detachment from the cultivation surface using cell-damaging bio-active factors

(i.e., enzymes and divalent cation chelators) and re-adhesion after thawing. Detachment of adherent cells and cell colonies affects cell–cell and cell–matrix junctions, membrane integrity, and cell morphology. In this impaired state, cells are additionally exposed to the highly stressful mechanisms of freezing (i.e., solution effects or intracellular ice) during slow-rate protocols. Furthermore, re-establishment of full adhesion after thawing leads to a prolonged recovery time and a delayed proliferation.

These effects have been intensively investigated for hESCs. One of the first studies in this field in 2001 revealed that only 16% of the hESC clumps slowly frozen in 10% DMSO could be recovered.[31] Additionally, the hESC colonies frozen in suspension possessed a smaller colony size in comparison to the nonfrozen control after cultivation for 2 weeks, and showed significantly higher grades of differentiation. Until now, combination of different cryoprotective agents (CPAs)[32] and dissociation of cell aggregates into single cells using the addition of the ROCKi Y27632[16] improved the cryopreservation efficiency of hPSCs, achieving viability of over 85% and 80% confluency after 3–4 days with maintained pluripotency.[33]

Nevertheless, detachment of adherent cells and cell colonies, and in particular the disruption of cell–cell contacts during dissociation into single cells, causes massive cell stress and significantly affects the cell physiology.

Therefore, cryopreservation of adherent cells in their native states reduces, on the one hand, cellular stress induced by cell detachment and dissociation, and on the other hand it limits the necessary recovery time afterwards, as re-attachment of cells is not necessary at all. But cryopreservation of adherent hESCs with slow-rate protocols also resulted in a low efficiency, as cell viability decreased to approximately 30% within 90 minutes after thawing.[34,35] These data indicate that beside detachment and dissociation of cells, ice crystal formation inside and outside of cells during slow-rate freezing is the limiting factor for successful cryopreservation.

To avoid ice nucleation inside the cells, vitrification is the method of choice and has already been successfully applied to hPSCs.[31] But vitrification in straws still means detachment of cells, is only applicable for low numbers of cell clumps, and is very labor intensive and time sensitive. Therefore, combination of surface-based freezing with the vitrification technology would enable efficient cryopreservation of high cell numbers with low cell stress, even in ready-to-use multiwell formats suitable for direct integration in high-throughput screenings.

Surface-Based Vitrification

Development of an efficient surface-based vitrification approach requires a closer look at the established and well-functioning vitrification procedures of hPSCs in straws. Reubinoff et al. established the open pulled straw vitrification method for hESCs in 2001,[31] which had already been highly effective for the cryopreservation of embryos.[21] This protocol was then optimized and slightly modified by Richards et al.[36] in 2004 (Figure 24.2).

The corresponding procedure includes dissociation of undifferentiated hESC clumps by mechanical cutting into fragments consisting of 300–400 cells. For the vitrification procedure, a sterile four-well cell culture plate containing the following solutions is prepared at room temperature: (i) well 1 contains the holding medium ES–HEPES–HSA (embryonic stem–2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethane sulfonic acid (a buffer)–human serum albumin) consisting of 80% DMEM (Dulbeccos Modified Eagle Medium) (vol/vol) (high-glucose DMEM) and 20% HSA (vol/vol) buffered to 20 mM HEPES; (ii) well 2 contains the vitrification solution 1 (VS1) consisting of 80% ES–HEPES–HSA (vol/vol), 10% DMSO (vol/vol), and 10% EG (vol/vol); (iii) well 3 contains the vitrification solution 2 (VS2) consisting of 30% ES–HEPES–HSA (vol/vol), 30% ES–HEPES–HSA with 1 M sucrose (vol/vol), 20% DMSO (vol/vol), and 20% EG (vol/vol); and (iv) well 4 contains the warming solution 1 (WS1) consisting of 80% ES–HEPES–HSA (vol/vol) and 20% ES–HEPES–HSA with 1 M sucrose (vol/vol).

An embryo straw is prepared by loading it through the open end with VS2 until about 20 mm of the straw is filled using a syringe with a connector, provided with the straws (Figure 24.3). Then, about 5 mm of the straw is filled with air by aspiration.

The preparation of the cell clumps for vitrification is a multi-step procedure with a gradual increase of the medium osmolarity before cryopreservation and a gradual adjustment to physiological conditions after thawing. For vitrification, the dissected colony fragments are initially washed once in modified phosphate-buffered saline (PBS +/+). Then, five to eight colony fragments are transferred to well 1 of the four-well dish containing the holding medium ES–HEPES–HSA using a sterile glass Pasteur pipette to remove the PBS. The hESC fragments are then transferred to well 2 containing VS1 and are incubated for 1 min. For the final loading of the straw, a 20-µL drop of VS2 is placed into the middle of a 35-mm Petri dish.

When the minute-long incubation in VS1 is over, the hESC fragments are transferred to well 3 containing VS2 for 5 seconds. Then, the clumps are transferred to the 20-µL drop of VS2 placed in the middle of a 35-mm Petri dish using a sterile glass Pasteur pipette, with a minimal volume of VS2. From there, the hESC fragments are immediately loaded into the prepared embryo straw, followed by a 5-mm air gap and about 20 mm of WS1 (Figure 24.3). Both ends of the straw are directly sealed using a heat sealer, and the straw is plunged into liquid nitrogen as quickly as possible.

For thawing, again a sterile four-well cell culture plate containing the following solutions is prepared at room temperature: (i) well 1 contains WS1; (ii) well 2 contains warming solution 2 (WS2) consisting of 90% ES–HEPES–HSA (vol/vol) and 10% ES–HEPES–HSA with

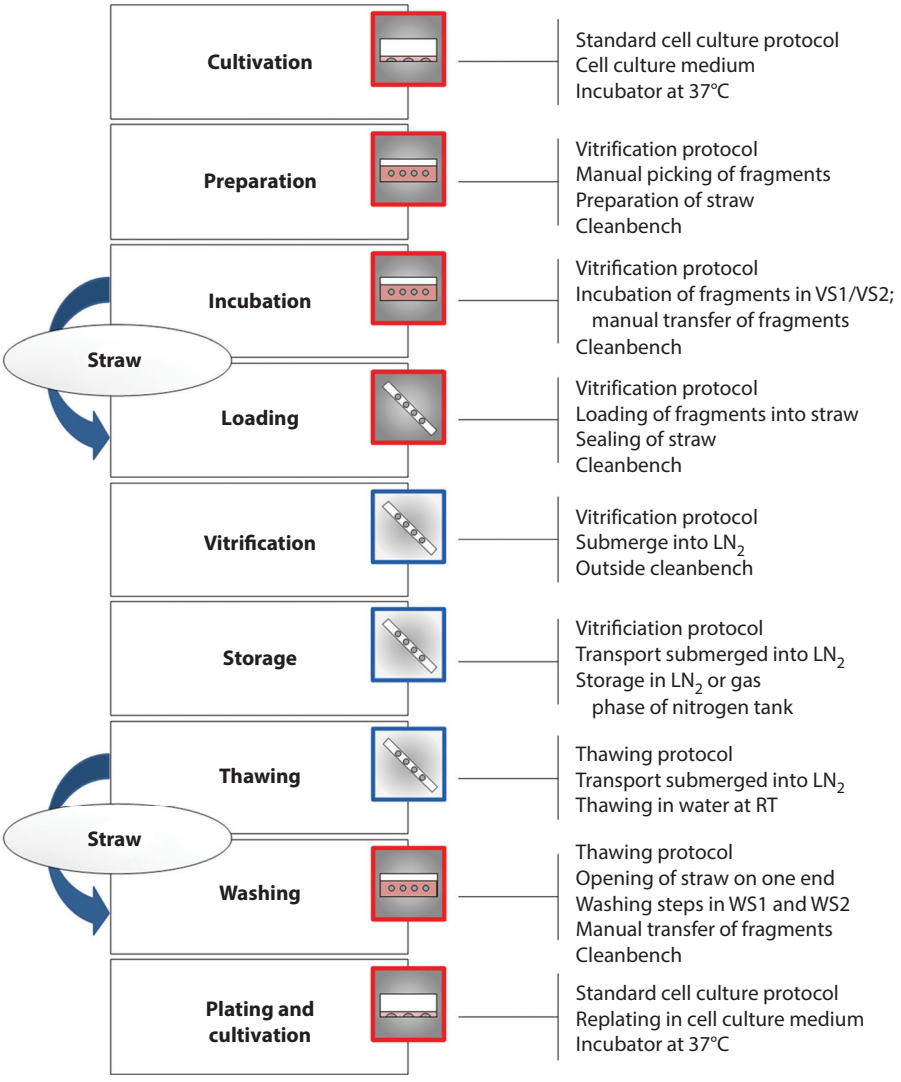

Figure 24.2 **(See color insert.) Workflow of the open pulled straw vitrification method for human embryonic stem cells by Richards et al. (LN_2 = liquid nitrogen; VS = vitrification solution; WS = warming solution.) (From Richards M et al. *Stem Cells* 2004;22:779–89. With permission.)**

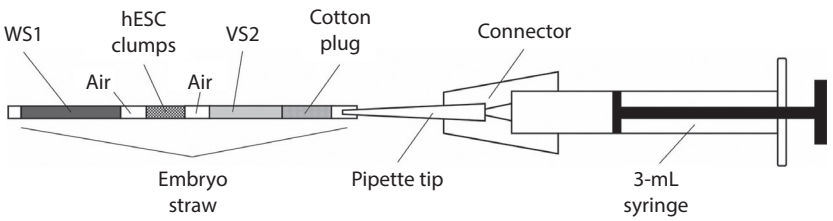

Figure 24.3 **Schematic drawing of the filled embryo straw connected to the syringe, according to Richards et al. (hESC = human embryonic stem cell; WS = warming solution.) (From Richards M et al. *Stem Cells* 2004;22:779–89. With permission.)**

1 M sucrose (vol/vol); and (iii) wells 3 and 4 contain the holding medium.

The straw is removed from the nitrogen storage and is plunged immediately into a vessel containing water at room temperature for 5 second. Then, the straw is disinfected by wiping it with 70% ethanol or isopropanol, and opened using sterile scissors cutting both ends of the straw. The entire content of the straw is placed into well 1 containing WS1, and is incubated for 1 minute. Then, the hESC clumps are transferred into well 2 containing WS2 and are incubated for 5 minutes. In the last step, the clumps are transferred into well 3 containing the holding medium for 5 minutes, then finally transferred into well 4 for washing, also containing the holding medium. Now the clumps are ready for re-plating or usage.

The vitrification protocol of Richards et al. possesses high complexity, is very labor intensive, and can be somewhat error prone. Even small deviations from the protocol regarding incubation times can result in significant cell loss due to the severe cellular stress caused by the high osmolarity of the vitrification solutions. Even the original manuscript of Reubinoff et al.[31] reported only survival of about 30% of the undifferentiated hESC fragments, whereas Richards et al.[36] reached over 75%. Additionally, only a maximum of ten hESC fragments can be vitrified at one time, requiring mechanical dissection of the colonies.

Therefore, transfer of the protocol from Richards et al.[36] to adherent cell systems would result in an easy-to-handle vitrification procedure for higher cell numbers. Incubation times can be observed more strictly, as cells are immobilized on the cultivation surface, so transfer and handling is simplified. Additionally, heat transfer of adherent, flat cells and cell clumps is higher than of detached cells and colonies with a round morphology.

Hence, the vitrification solutions and incubation times proposed by Richards et al.[36] have been applied to adherent hESCs cultivated on special cell culture-treated coverslips, so-called Thermanox©, with a diameter of 13 mm[37] (Figure 24.4). In addition, a standard slow-rate protocol has been used with adherent hESCs to investigate whether avoidance of cell stress during cell detachment and dissociation may significantly improve cryopreservation efficiency.

For this, Thermanox© coverslips are prepared by making two parallel, about 3-mm long cuts into the plastic coverslip using sterile scissors. Afterwards, the resulting part is bent up, building a handlebar for better handling of the coverslip (Figure 24.5a and b).

Additionally, some identification marks (e.g., lines) are made onto the untreated side of the coverslip using a sterile scalpel to enable recognition and distinction of the hESC colonies. Afterwards, the coverslips must be sterilized by incubation in 70% ethanol for 20 minutes, and subsequent ultraviolet radiation for 15 minutes.

Then, up to five prepared Thermanox© coverslips are placed in a 60-mm cell culture dish (Figure 24.5c), and hESCs are seeded using the standard cultivation conditions (coating with 0.1% gelatin and co-cultivation with mitotically inactivated primary mouse embryo fibroblast [PMEF] cells) onto the coverslips.

After cultivation for 5–7 days, hESCs are vitrified by taking the Thermanox© coverslips at the handlebar and incubating them for 1 minute in cell culture medium containing 10% DMSO and 10% EG. Then, coverslips are incubated for 5 seconds in culture medium containing 20% DMSO, 20% EG, and 300 mM sucrose. Finally, the coverslips are directly plunged into liquid nitrogen and stored in the vapor phase of a nitrogen storage tank.

For thawing, the Thermanox© coverslips are removed from the nitrogen storage tank and are directly incubated for 1 minute in culture medium containing 200 mM sucrose. Afterwards, cells are incubated for 5 minutes in culture medium containing 100 mM sucrose, and are then transferred to normal cell culture medium for further cultivation or application.

For comparison with slow-rate freezing of adherent hESC colonies, hESCs are seeded using the standard cultivation conditions (coating with 0.1% gelatin and co-cultivation with mitotically inactivated PMEFs) into a 35-mm cell culture dish with identification marks on the underside. After cultivation for 4–6 days, the culture medium is removed and 1 mL of the standard cryopreservation medium recommended by WiCell containing 60% culture medium, 30% FCS (fetal calf serum), and 10% DMSO is added and incubated for 30 minutes at 4°C. Then, cells are frozen at −1°C/min from 4°C to −80°C using a computer-controlled freezer, and are stored in the vapor phase of a nitrogen storage tank.

For thawing, the cell culture dish is removed from the nitrogen storage tank and incubated in a water bath without submerging the lid. Afterwards, 2 mL cell culture medium is added drop-wise for dilution of the cryopreservation medium. Finally, the medium is changed with cell culture medium for further cultivation or application.

Viability of hESC colonies after adherent vitrification and slow-rate freezing was determined using live/dead staining directly after thawing, after 24 hours, and after 48 hours. Microscopic images showed that, with slow-rate protocols, most hESC colonies were still adherent directly after thawing, but with large dead areas, mainly in the center of the colonies (Figure 24.6a). Twenty-four hours after thawing, the colonies possessed a disintegrated morphology, as most of the dead cells detached from the surface (Figure 24.6c). Proliferation of the surviving cells for 48 hours induced recovery of colony morphology (Figure 24.6e).

In comparison, hardly any cell or colony loss was detected using adherent vitrification directly after thawing (Figure 24.6b), after 24 hours (Figure 24.6d), or after 48 hours (Figure 24.6f). Most cells were viable and colony morphology was compact at every time point.

Based on the microscopic fluorescence images, the viable area of the same colony was determined before and

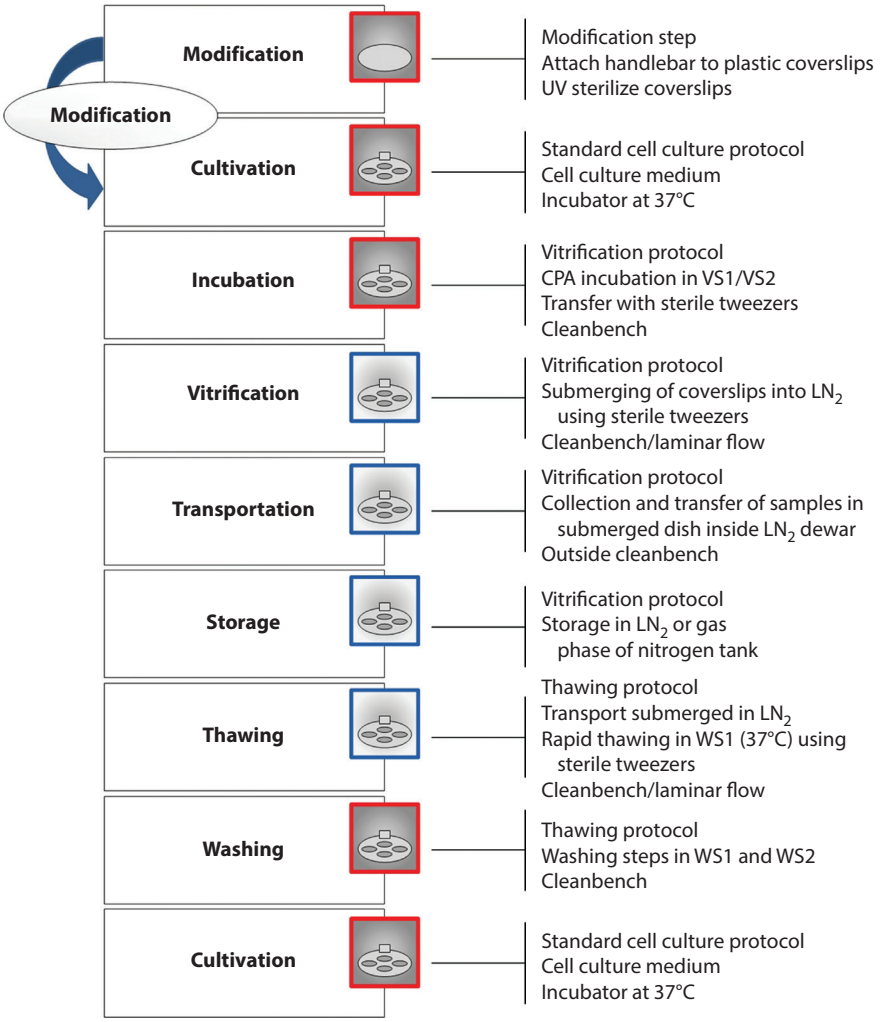

Figure 24.4 **(See color insert.) Workflow of the surface-based vitrification method for human embryonic stem cells by Beier et al. (CPA = cryoprotective agent; LN_2 = liquid nitrogen; UV = ultraviolet; VS = vitrification solution; WS = warming solution.) (From Beier AF et al. *Cryobiology* 2011;63:175–85. With permission.)**

after cryopreservation. For this, pictures of identical colonies have been taken before and after cryopreservation on the basis of the identification marks on the underside of the 35-mm cell culture dish or Thermanox© coverslips. Based on these images, direct comparison of the colony survival is possible.[37] After slow-rate freezing of adherent hESC colonies, 51% ± 22% of the colony area remained adherent and viable directly after thawing (Figure 24.7). Twenty-four hours later, the viable colony area slightly decreased to 44% ± 25%. At only 48 hours after thawing, slowly frozen hESC colonies recovered, started to proliferate again, and reached an adherent and viable colony area of 73% ± 36%.

In contrast, hardly any cell loss was detected using surface-based vitrification, as 89% ± 10% of the hESC colonies remained viable and adherent directly after thawing (Figure 24.7). At 24 and 48 hours later, considerable cell proliferation was determined, with colony areas of 167% ± 38% and 254% ± 81%, respectively.

In addition to colony recovery and survival, maintained expression of stemness markers is absolutely crucial for efficient cryopreservation of hESCs. Therefore, the expression of the transcription factor Oct-4 and cell surface marker Tra-1-81 was measured using flow cytometric analysis (Figure 24.8). Results showed that stemness markers were maintained after surface-based vitrification (Figure 24.8a and c) and were higher than in the non-frozen control colonies (Figure 24.8b and d).

The data revealed that vitrification can be applied to adherent cells and results in highly efficient recovery rates of large cell numbers with maintained stemness. Additionally, comparison with slow-rate freezing of adherent hESC colonies showed that high cooling rates

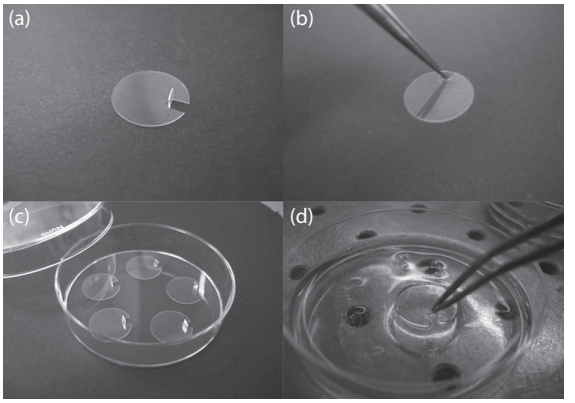

Figure 24.5 **Preparation and handling of the Thermanox© coverslips for vitrification. Addition of a handlebar for better treatment (a and b), arrangement of coverslips in a 60-mL cell culture dish for cell seeding (c) and handling of coverslips during cell incubation in different cryo-media (d). (From Beier AF et al. *Cryobiology* 2011;63:175–85. With permission.)**

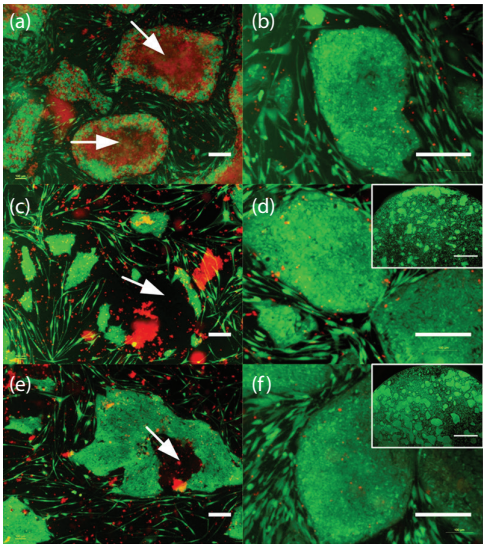

Figure 24.6 **(See color insert.) Microscopic fluorescence images of human embryonic stem cells at different time points after slow-rate freezing (a, c, and e) and adherent vitrification (b, d, and f). Cells have been stained with live/dead staining (fluorescein diacetate/ethidium bromide [FDA/EB]) directly after thawing (a and b), after 24 hours (c and d) and after 48 hours (e and f). Scale bars indicate 200 μm (a–f) and 2 mm (inserts). (From Beier AF et al. *Cryobiology* 2011;63:175–85. With permission.)**

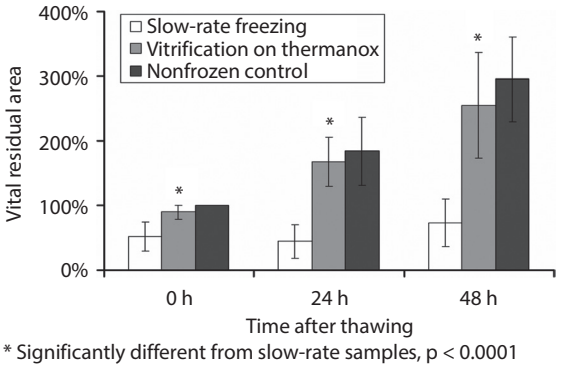

Figure 24.7 **Viable and adherent colony areas at different time points after adherent slow-rate freezing and vitrification. Colony areas of the same colony were compared before and after adherent slow-rate freezing and vitrification. Additionally, the colony areas of nonfrozen human embryonic stem cell colonies were determined. (From Beier AF et al. *Cryobiology* 2011;63:175–85. With permission.)**

are necessary to enable cell survival, as slow-rate freezing led to significant loss of cell numbers and viability. Due to their three-dimensional colony structure and high numbers of tight and gap junctions, optimal cell dehydration is impeded and intracellular ice crystals have the possibility to propagate throughout the whole colony, resulting in complete colony loss.[38] Decrease of viable and adherent hESC colonies 24 hours after thawing is a sign of apoptosis, probably induced by alterations in cytoplasm caused by intracellular ice formation during slow-rate freezing.[39] Hence, intracellular ice crystal formation seems to be the critical factor during the cryopreservation of hESCs, and surface-based vitrification is the optimal method for the efficient cryopreservation of ready-to-use cells.

The method of surface-based vitrification has already been successfully applied to several other sensitive cell types that are difficult to cryopreserve using standard slow-rate protocols; for example, human iPSCs and iPSC-derived hepatocyte-like cells.

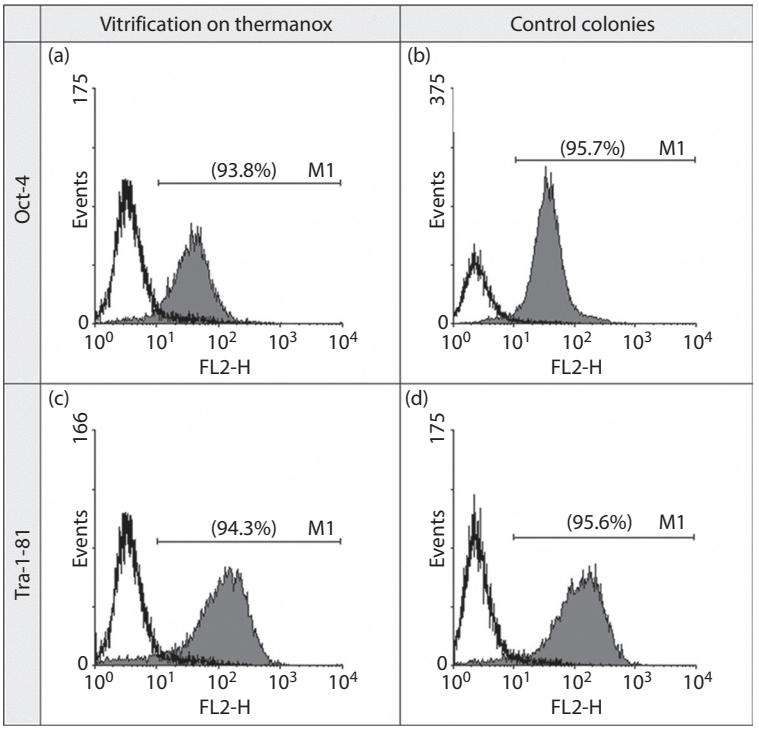

Figure 24.8 **Flow cytometric analysis of stemness markers after surface-based vitrification. Expression levels of transcription factor Oct-4 (a and b) and cell surface marker Tra-1-81 (c and d) of human embryonic stem cells passaged once after surface-based vitrification (a and c) and of nonfrozen control colonies (b and d) were determined. (From Beier AF et al. *Cryobiology* 2011;63:175–85. With permission.)**

But surface-based vitrification of cells on Thermanox© coverslips also possesses several limitations. The polymer of the coverslips is not fully resistant to the high thermal requirements of cell cultivation and vitrification (temperature range from +37°C to −196°C). Hence, thermal cracks occur in about 5%–10% of the coverslips, resulting in disruption and damage of the affected hESC colonies.[37] Additionally, the cells on the coverslips have direct contact with liquid nitrogen in order to reach the necessary high cooling rates for vitrification. Usually, however, liquid nitrogen is not sterile and cross-contamination between samples is possible, as various bacteria and viruses are able to survive at these temperatures.[29] Although the production of sterile liquid nitrogen is possible, costs would significantly increase for sterile banking of cells under GxP regulations using the surface-based vitrification technique on Thermanox© coverslips. Therefore, adaptation of this highly efficient method has been done to make this a GMP-compliant vitrification device.[40]

GMP-Compliant Surface-Based Vitrification

To guarantee GMP compliance of the surface-based vitrification method, direct contact of cells with liquid nitrogen has to be avoided without significantly decelerating the cooling rate of the procedure. Therefore, a self-assembled disposable, the so called TWIST substrate, which strictly separates cells and liquid nitrogen using a two chamber system was developed (Figure 24.9). This TWIST substrate is based on a cell culture-treated imaging dish with a thin foil as the cultivation surface.[40] The foil possesses good optical quality and high thermal conductivity.

The TWIST substrate consists of a sterile, closable cultivation chamber and an open liquid nitrogen chamber. The concept of the TWIST substrate is based on using it in two different positions: for cultivation, the substrate is used in an "upright position" (Figure 24.9a), with the cultivation chamber on top. In this position, cells can be seeded, expanded, monitored, and prepared for cryopreservation sealed with a lid guaranteeing a closed and sterile environment. Additionally, the "upright position" is used for washing steps, cell recovery, and cultivation after thawing. Then, there is an "upside-down position" with the liquid nitrogen chamber on top (Figure 24.9b). In this position, the vitrification itself is executed by filling liquid nitrogen into this chamber, onto the cultivation foil. On the other side of the foil, the cells are now covered with only a very thin liquid layer. In this position, the cells are also stored in liquid nitrogen storage tanks. For thawing, 37°C warm water is filled in the liquid nitrogen chamber, enabling a very fast thawing of the adherent cells.

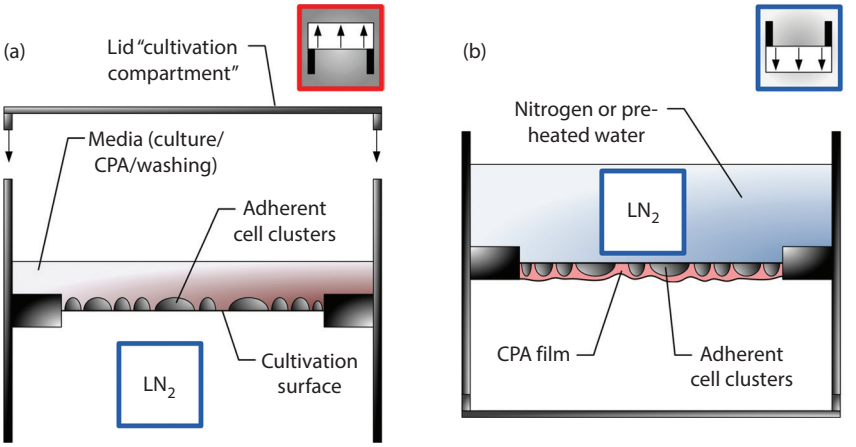

Figure 24.9 **(See color insert.) Outline and application of the TWIST substrate. The TWIST substrate is a two-chamber system with a closable, sterile cultivation chamber and a LN_2 chamber. In the cultivation chamber, cells are seeded, expanded, monitored, and prepared for cryopreservation by incubation in CPAs (a); for surface-based vitrification, the cryo-medium is removed, the substrate is turned over ("twisted"), and LN_2 is filled into the LN_2 chamber (b). (CPA = cryoprotective agent; LN_2 = liquid nitrogen.) (From Liu BL, McGrath J. *Acta Biochim Biophys Sin (Shanghai)* 2005;37:814–8. With permission.)**

Figure 24.10 illustrates every step of the GMP-compliant surface-based vitrification workflow and shows the corresponding position of the TWIST substrate. Cells remain adherent for the complete procedure, and no direct contact with potentially contaminated liquid nitrogen is necessary.

For evaluation of the GMP-compliant surface-based vitrification procedure, hESC colonies were cultivated and vitrified in the TWIST substrate using the workflow and the solutions described above. Preparation of media used and analysis of the viable and adherent hESC colony area was performed as described in Beier et al.[37]

Pictures of the hESC colonies have been taken before and after vitrification, with cells stained using a live/dead dye at different time points after thawing (Figure 24.11). Hardly any cell loss was detected directly after thawing (Figure 24.11e) or 24 hours later (Figure 24.11f). Additionally, vitrified colonies showed a compact and tight morphology without any signs of differentiation after passing and further cultivation (Figure 24.11i and j). Nevertheless, slight localized cell loss was determined in some areas at the border of the cultivation surface (Figure 24.11f, asterisk).

Quantitative analysis of the microscopic images showed that 99% ± 1% viable and adherent hESC colonies were detected directly after thawing (data not shown). After 24 hours, the colony area increased to 155% ± 24% in comparison to 161% ± 32% of the nonfrozen control.

In addition, maintenance of stemness markers after GMP-compliant surface-based vitrification was determined using flow cytometric analysis. The results showed that expression of the transcription factor Oct-4 and the cell surface marker Tra-1-81 after vitrification using the TWIST substrate was as high as for the nonfrozen control colonies (Figure 24.12).

These data revealed that efficient surface-based vitrification is adaptable to GxP regulations by avoiding direct contact of cells with liquid nitrogen and by using a closed cultivation chamber to guarantee sterility. Based on the TWIST substrate, vitrification of ready-to-use cells in different formats is possible, including patient-derived primary cell material or disease-relevant, induced pluripotent stem (iPS) cell-derived model systems in 96- or 384-multiwell formats for high-throughput screenings in the field of drug discovery or target finding. Furthermore, large-scale cryopreservation and storage of highly sensitive cells for therapeutic approaches, in which large cell numbers are necessary, is possible by development of an adapted TWIST substrate with a large cultivation surface (e.g., T175 cm^2 vitrification flask).

Nevertheless, the GMP-compliant surface-based vitrification also has some drawbacks: although the number of thermal cracks was reduced using a cell culture-treated foil for cell cultivation and vitrification to <5%, further optimization of the polymer used is necessary for complete avoidance of cracks. Additionally, some cell loss was detected at the border of the cultivation area, as here the cryo-medium could not be removed completely, resulting in a thicker liquid layer at the edge. Therefore, the thermal capacity is increased in these areas, and the applied cooling rate is lower than in the middle part of the TWIST substrate, preventing successful vitrification of the hESC colonies. In conclusion, formation of a liquid meniscus has to be avoided in order to guarantee a homogenous

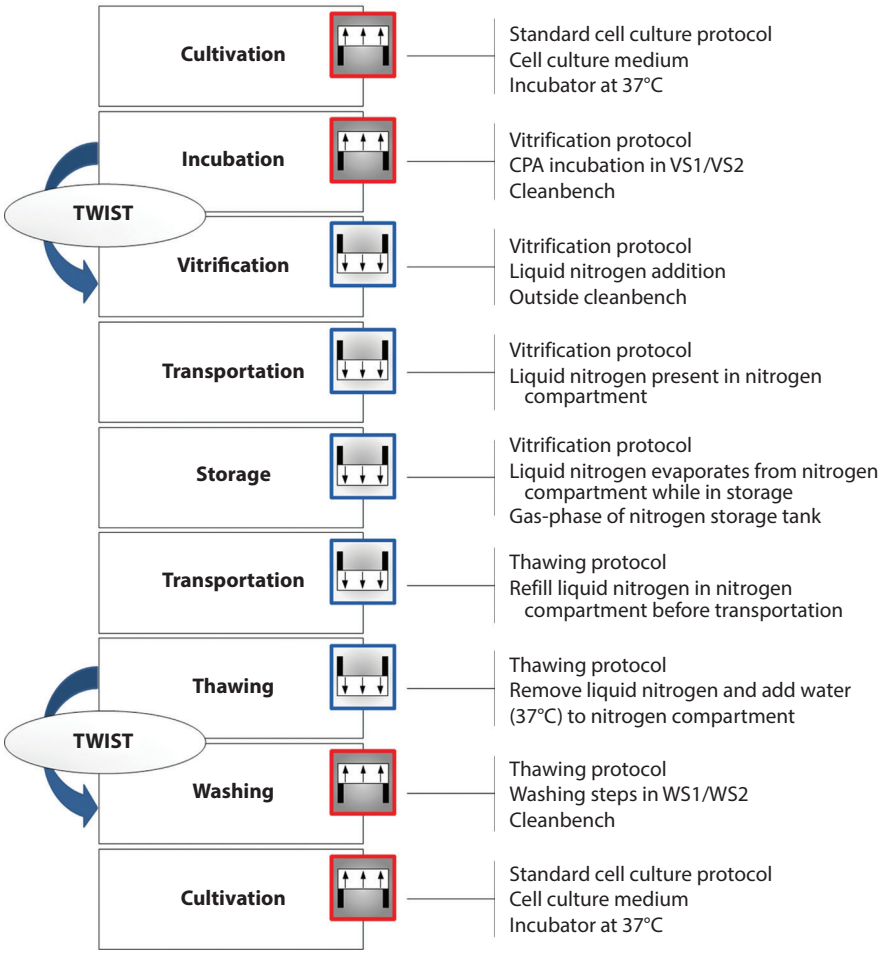

Figure 24.10 **(See color insert.) Workflow of the GMP-compliant surface-based vitrification procedure. Every step of the workflow and the corresponding positions of the TWIST substrate are summarized here. (CPA = cryoprotective agent; VS = vitrification solution; WS = warming solution.) (From Beier AF et al. *Cryobiology* 2013;66:8–16. With permission.)**

low thermal capacity and ultra-fast cooling rates using the surface-based vitrification method.

Another drawback of vitrification protocols in general is the use of very high concentrations of toxic CPAs.[41,42] These high concentrations, usually in the range of 40%, are required to achieve successful vitrification of the samples without ice crystallization.[43] Due to the toxicity and high osmolarity of the applied media, incubation steps within the vitrification protocols are generally very short in order to keep the detrimental effects on the cells at a minimum. However, due to the nature of some vitrification workflows, the handling of the samples can be awkward and cumbersome, leading to a high dependency of protocol effectiveness on the manual skills of the operator. Therefore, the development of less toxic CPA media compositions and the precise definition of CPA incubation times could lead to an improved applicability of vitrification techniques.

In addition to DMSO and EG, there are a variety of potential CPAs reported to be feasible for vitrification.[44–47] In particular, lower-strength glass formers are reported to show reduced toxicity when compared to strong glass formers like DMSO.[43] Strong glass formers, on the other hand, need lower cooling rates to vitrify. Figure 24.13 shows a comparison of several potential CPAs according to their effectiveness in surface-based vitrification of hESC colonies. The efficiency has been determined by viability assessment directly after thawing and after a 24-hour recovery period. In addition to DMSO and EG, the substances formamide, 1,2-propanediol (PD), and 2,3-butanediol were tested. Also included in the study were various mixtures of the tested components, since it is common to mix good glass-forming CPAs with poorer glass formers in order to eliminate ice formation, while at the same time incurring a reduced toxicity penalty (Figure 24.13, variations 1–4).[45]

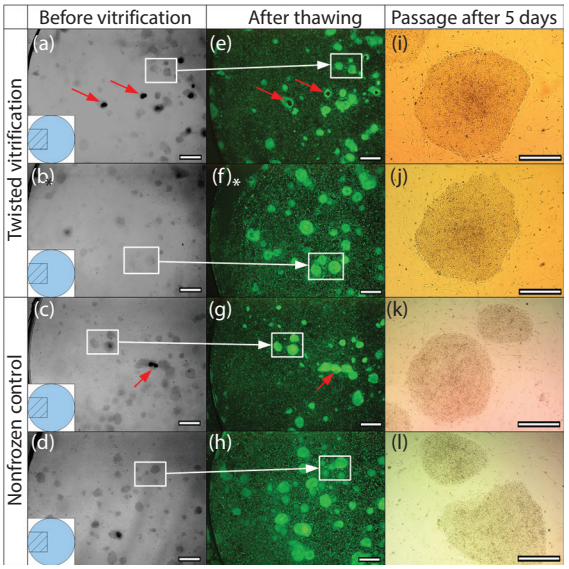

Figure 24.11 **(See color insert.) Microscopic images of human embryonic stem cell (hESC) colonies before and after vitrification using the GMP-compliant surface-based vitrification method. Pictures of the same hESC colonies have been taken before (a–d) and after vitrification (e and f), in comparison to a nonfrozen control (g and h). For determination of the viable and adherent colony area, hESC colonies have been stained with a live/dead dye (FDA/EB) directly after thawing (b) or 24 hours after thawing (f). The nonfrozen hESC colonies have been stained on the day of vitrification (g) or 24 hours later (h). Vitrified (i and j) and nonfrozen hESC colonies (k and l) have been passaged and cultivated further to determine signs of differentiation. Red arrows indicate thicker, probably differentiated areas of the hESC colonies that are lost after vitrification (e) in comparison to the nonfrozen control (g). Scale bars indicate 1 mm (a–h) and 500 μm (i–l). (From Beier AF et al.** ***Cryobiology*** **2013;66:8–16. With permission.)**

DMSO, EG, and PD all showed high post-thawing survival rates, while formamide and 2,3-butanediol led to an almost complete cell loss immediately after thawing. While most of the tested substances and mixtures resulted in very high cell survival and good recovery rates, the least spontaneous post-thawing differentiation rate was achieved with 20% PD and 20% EG, suggesting a reduced toxicity of this medium composition when compared to other mixtures (Figure 24.13, variation 3; data on differentiation rate not shown). The concentration of the PD/EG medium used could be further reduced to 20% PD and 15% EG without a reduction in post-thaw viability (Figure 24.13, variation 4). This indicates that the potentially less toxic PD/EG combination might be a valuable alternative for the commonly used DMSO/EG medium in the surface-based vitrification of hESC and iPSC colonies. However, extensive long-term analysis on the toxicity of this medium composition is needed to ensure its effectiveness in hESC and human iPSC banking.

Another working point for the improvement of vitrification techniques for human ESCs and iPSCs is the clear definition of CPA concentrations and incubation times in the applied workflows. The optimal CPA concentration should allow sufficient avoidance of ice crystallization while keeping toxic detrimental effects of the media at a minimum. The same goes for the incubation times in the respective media. These should be chosen to be long enough to enable sufficient penetration of the hESC and iPSC colonies to allow for an efficient avoidance of ice crystallization in the whole sample, while keeping negative toxicity effects as low as possible.

To emphasize this, the effects of different concentrations and incubation times of the commonly used CPA medium, containing equal volumes of DMSO and EG, on adherent hESC colonies have been evaluated (Figure 24.14).

The concentration range was chosen from 20% up to 70% total CPA concentration, and evaluated according to the vital residual areas of the colonies before and after vitrification. The results showed an optimal concentration of CPAs at around 40% (Figure 24.14a and b). Lower concentrations led to an insufficient ability of the cryoprotective media to avoid ice crystallization, leading to the loss of complete colonies (data not shown). Higher concentrations (50%–70%) led to cell death, which was probably caused by the high toxicity of the applied media.

Incubation time dependency was evaluated by variation of the surface-based vitrification protocol described above in regard to incubation times (Figure 24.14c–e and g). The standard protocol implies a 60-second incubation

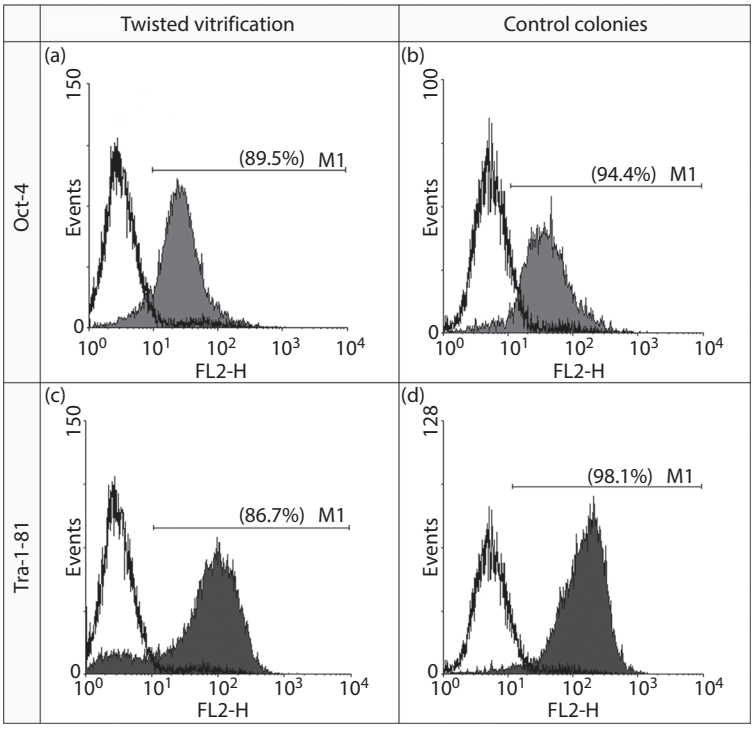

Figure 24.12 **Flow cytometric analysis of stemness markers after GMP-compliant surface-based vitrification. Expression levels of the transcription factor Oct-4 (a and b) and cell surface marker Tra-1-81 (c and d) of human embryonic stem cells passaged once after surface-based vitrification in the TWIST substrate (a and c), and of nonfrozen control colonies (b and d) were determined. (From Beier AF et al. *Cryobiology* 2013;66:8–16. With permission.)**

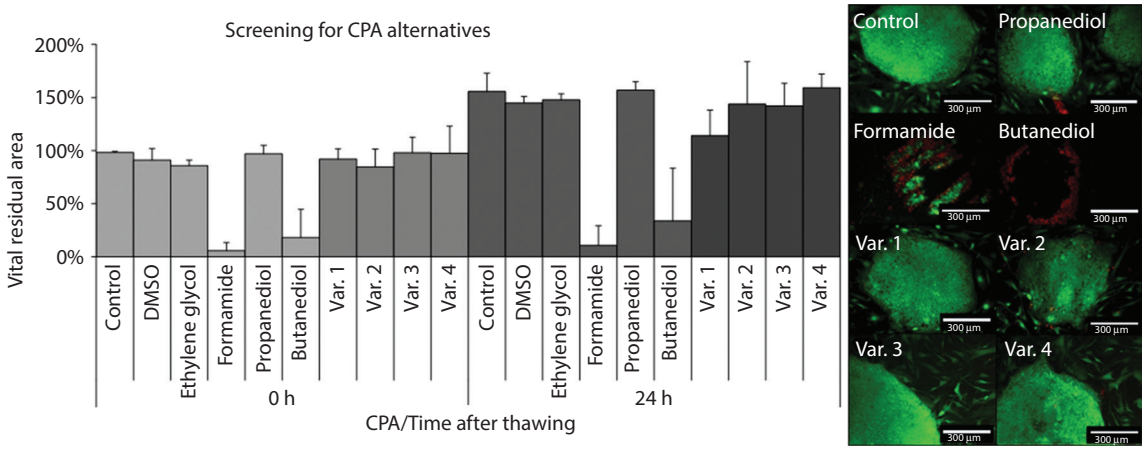

Figure 24.13 **(See color insert.) Comparison of potential vitrification CPAs according to their effectiveness in surface-based vitrification. Concentrations of CPAs have been 40% except for in Var. 4, which shows a less-concentrated version of Var. 3. Images have been stained with EB and FDA and show the corresponding colonies directly after thawing. Var. 1–4 show different mixtures of the CPAs used. Var. 1: 10% propanediol, 10% formamide, 10% ethylene glycol, and 10% butanediol. Var. 2: 13% propanediol, 13% ethylene glycol, and 13% butanediol. Var. 3: 20% propanediol and 20% ethylene glycol. Var. 4: 20% propanediol and 15% ethylene glycol. (CPA = cryoprotective agent; DMSO = dimethyl sulfoxide; Var. = variation.)**

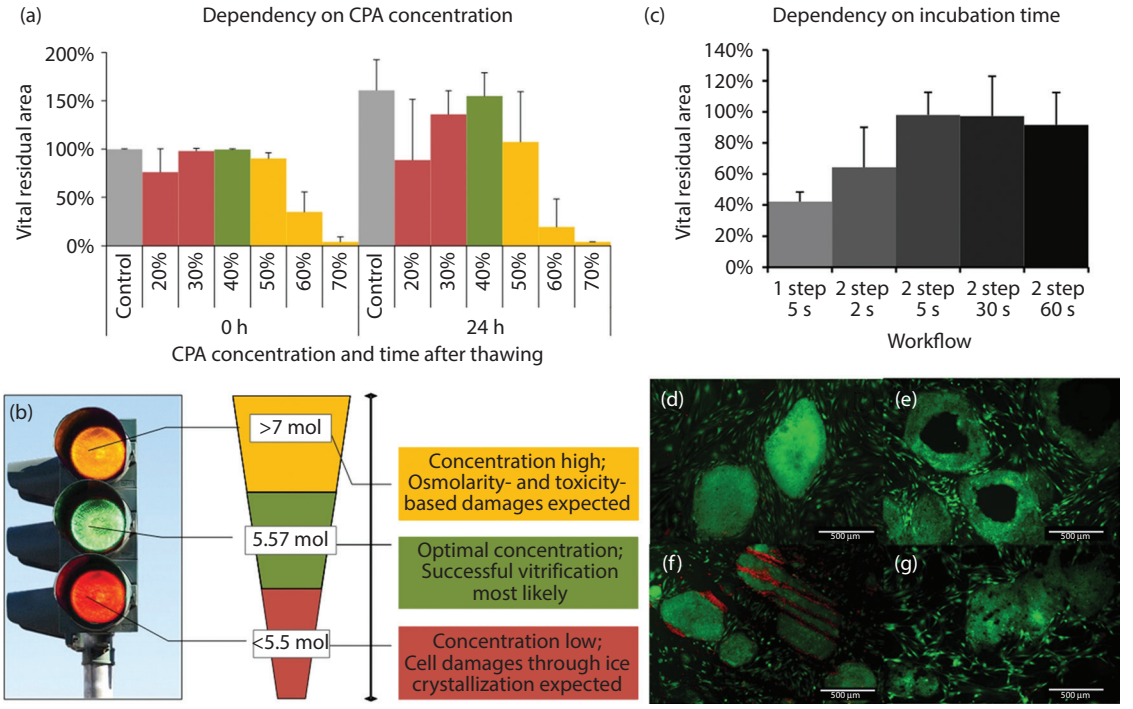

Figure 24.14 **(See color insert.) Evaluation of optimal CPA concentration range (a and b) and incubation time (c) in surface-based vitrification. CPA concentration range experiments were done with media containing equal amounts of dimethyl sulfoxide and ethylene glycol in comparison to a nonfrozen control sample (d). The effects of too short an incubation time (e), too long an incubation time (g), and damage by inadequate surface material are shown. Images have been stained with FDA/EB. (CPA = cryoprotective agent.)**

step in media containing 10% DMSO and 10% EG, followed by a 5-second incubation step in media comprising 20% DMSO and 20% EG. This second high-concentration incubation step was varied in incubation time. Analysis of post-thaw viability showed an optimal incubation time of between 5 and 30 seconds. Shorter incubation times led to incomplete CPA penetration of the thicker inner parts of the colonies, resulting in major cell death in these areas (Figure 24.14e). Longer incubation times (>30 seconds) led to high survival rates, but resulted in a more "frisky" colony morphology after thawing (Figure 24.14g), probably due to toxicity based damage to cell–cell contacts and cell membranes. Also, spontaneous differentiation rate appears to be higher (data not shown).

Integration in Automated Workflows

Due to their capability to differentiate into cells of all three germ layers, PSCs are also an important model system for toxicology screens. In particular, human iPSCs that can be derived from patients with specific genetic disorders offer new possibilities for the development of effective drug treatments. Until now, approval of novel drugs has mainly been based on successful animal testing. In the context of the Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals, it is expected that 68,000 chemicals have to be tested in about 54 million animal trials.[48] Today, there are great expectations that hPSCs can replace and reduce the necessary amount of animal testing. For the performance of large high-throughput screenings of several thousand compounds and chemicals, cells have to be available in miniaturized small-volume compartments, like 96- or 384-multiwell plates, to reduce costs of compounds and media, as well as the necessary cell number. Additionally, standard cultivation of cells in two dimensions as monolayers affects cell characteristics[49] and is not comparable to the three-dimensional environment within the human body. Therefore, results from candidate screens for drug discovery and development that are performed on cells cultivated as monolayers are often not transferrable, and fail in human clinical trials. To maintain cell–cell interactions and the regulatory networks necessary for expressive cell reactions after exposure to chemicals or drug candidates, cultivation of cells as 3D micro-tissues is necessary. Recent advances in micro-tissue production have highlighted the potential of scaffold-free cell aggregates in maintaining tissue-specific functionality.[50] One common procedure for the formation of micro-tissue is

the hanging drop method. Here, small microliter drops of cell suspension are placed on the inner side of the lid of a bacterial cell culture dish, over several microliters of water or PBS acting as the evaporation buffer. Within the drop, all cells are concentrated in the lower part of the drop due to gravity, forming one homogeneous cell aggregate. The hanging drop cultivation induces differentiation of PSCs (e.g., into cardiomyocytes or neurospheres) by preventing cell–matrix contacts, and is therefore also an important tool for the generation of disease-specific cell models. To avoid differentiation and maintain the expression of stemness markers, provision of adhesion surfaces in the drop by the addition of micro-carriers is possible.[10] For enabling permanent availability of micro-tissues for high-throughput screenings, cryopreservation strategies have to be developed, preferably able to be directly integrated into the hanging drop workflow. One promising strategy for integrated cryopreservation of micro-tissues is the adaptation of pellet-freezing procedures for the hanging drop technology. Using this method, human iPSC aggregates in 20-µL drops are dropped into liquid nitrogen, using the same vitrification and warming solutions as mentioned above (Figure 24.15a).

After vitrification and thawing, human iPSC aggregates have been re-plated and cultivated further (Figure 24.15d) in comparison to a nonfrozen control (Figure 24.15b) and human iPSC aggregates frozen in standard cryovials using slow-rate protocols (Figure 24.15c). Analysis of the adhesion rate after 24 hours normalized to the nonfrozen control resulted in a higher adhesion rate for the vitrified than for the slow-frozen samples, with values between 16% ± 3% and 24% ± 6%, depending on the different basal media and additives used. Additionally, expression of a stemness marker was investigated at 7 days post-thaw (Figure 24.15e–g), showing low signs of differentiation in all samples.

Furthermore, the pellet freezing of cell aggregates in small sample volumes can also be used for cells cultivated on micro-carriers (Figure 24.16) (e.g., for preventing differentiation of human iPSCs in the hanging drop). For this, human iPSCs adherent on micro-carrier form a three-dimensional cell–micro-carrier agglomerate (Figure 24.16d) in 20-µL drops that were dropped into liquid nitrogen, using the same vitrification and thawing solutions as mentioned above (Figure 24.16a).

The viability of vitrified human iPSCs adherent on micro-carriers has been investigated using live/dead staining (FDA/EB) directly after thawing and 24 hours later, normalized to the nonfrozen control (Figure 24.16b), and was analyzed as described elsewhere.[14] Directly after thawing, human iPSCs showed high viability values. But 24 hours later, a significant decrease was detectable, probably due to apoptosis processes within the first 8 hours. Assessment of stemness was also performed on vitrified human iPSCs adherent on micro-carriers using anti-HESCA-2 antibody with Alexa-Fluor-488 directly after thawing (Figure 24.16e) and 24 hours later (Figure 24.16f) in comparison to a nonfrozen control (Figure 24.16c and d). At all of the time points after thawing, human iPSCs showed expression of the stemness marker that was comparable to the control.

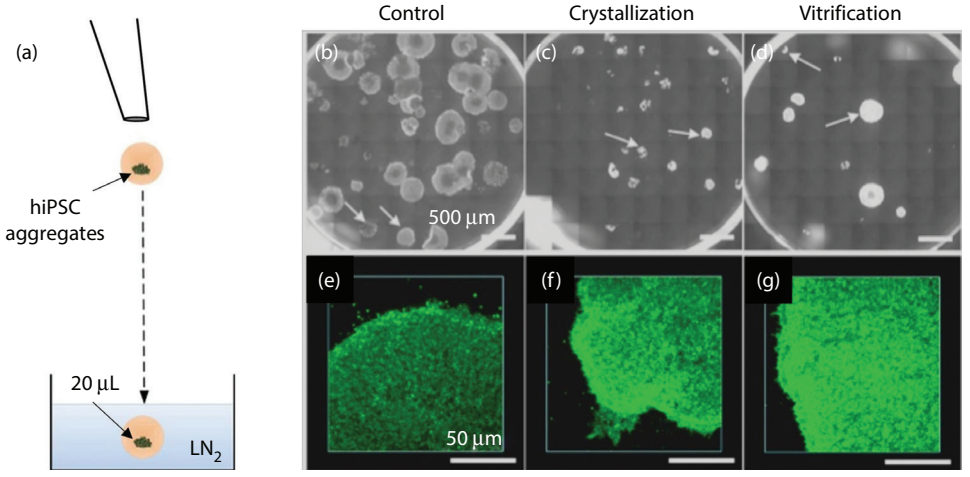

Figure 24.15 **(See color insert.) Cryopreservation of hiPSC aggregates using slow-rate protocols and pellet vitrification. For pellet vitrification, 20-µL drops of vitrification medium containing one hiPSC aggregate each were dropped into LN_2 (a). Adhesion of aggregates was monitored after slow-rate freezing (c) and pellet vitrification (d), in comparison to a nonfrozen control (b). Stemness was analyzed with immunofluorescence staining using anti-HESCA-2 antibody with Alexa-Fluor-488 after cultivation for 7 days (e–g). (hiPSC = human induced pluripotent stem cell; LN_2 = liquid nitrogen.)**

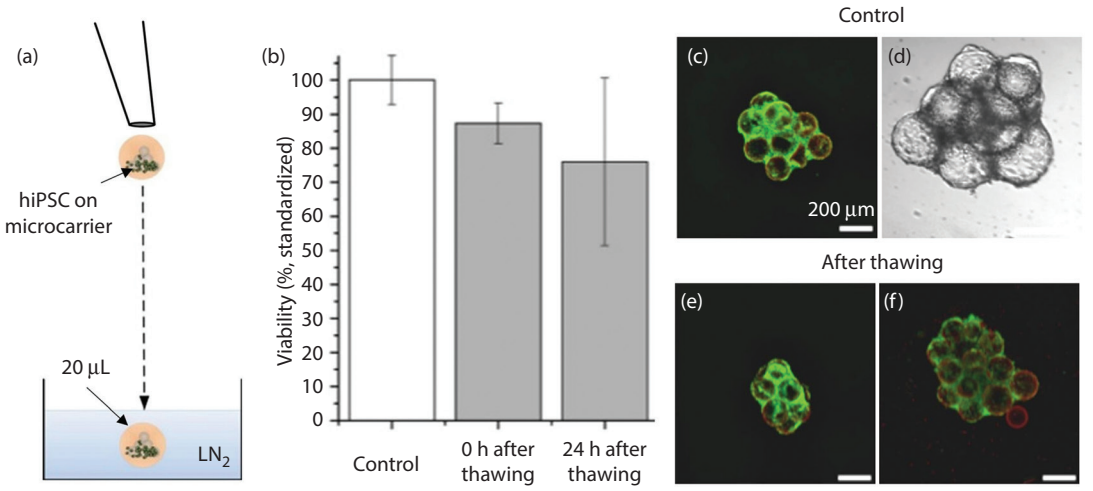

Figure 24.16 **(See color insert.) Cryopreservation of hiPSC on micro-carriers using pellet vitrification. For pellet vitrification, 20-µL drops of vitrification medium containing hiPSCs adherent on micro-carriers were dropped into LN_2 (a). Viability of vitrified cells was analyzed using live/dead staining (FDA/EB) directly after thawing and 24 hours later (b). Stemness of a nonfrozen control (c and d) directly after thawing (e) and 24 hours later (f) was determined with immunofluorescence staining using anti-HESCA-2 antibody with Alexa-Fluor-488. (hiPSC = human induced pluripotent stem cell; LN_2 = liquid nitrogen.)**

Hence, a first workflow could be established for creating the preconditions to integrate the vitrification procedure into automation workflows for high-throughput screenings or cell differentiation. Full integration can be realized by using commercially available hanging-drop plates on pipette or liquid-handling robots (Figure 24.17). These hanging-drop plates possess a perforated surface, so that pipette robots have access from above to generate droplets, perform medium exchange, or add compounds. With this setup, complete automation of the pellet vitrification workflow described above is possible by adding the different vitrification solutions after automated micro-tissue formation, and by dripping the drops containing the micro-tissues into liquid nitrogen placed beyond the perforated hanging-drop plates.

Integration of the micro-tissue formation into robotic platforms also offers the possibility of performing miniaturized compound screens on these systems. The drop access from above enables liquid-handling units to perform medium changes during cultivation and add CPAs for cryopreservation, and also add compounds for drug discovery and development. One possible application of this is the embryonic stem cell test for analyzing the embryotoxicity of compounds on mouse embryonic stem

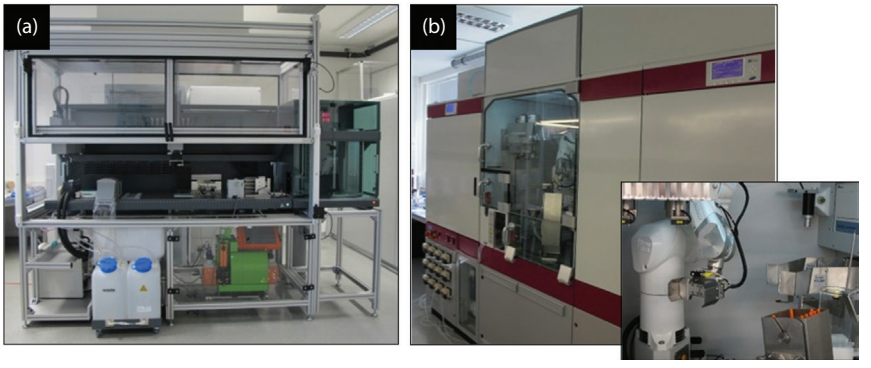

Figure 24.17 **Robotic platforms for the integration of stem cell workflows. The Freedom EVO 200 platform (TECAN Group AG, Männedorf, Switzerland) enables very dynamic and flexible adaptation of different stem cell workflows (a), whereas the CompacT SelecT platform (TAP Biosystems, Royston, UK/Sartorius Stedim Biotech, Aubagne, France) is an established system used *inter alia* by pharmaceutical industries (b).**

cells during differentiation into cardiomyocytes in the hanging drop.

Furthermore, cell culture platforms are already used for the automated production of biologicals (e.g., of HIV pseudoviruses for the development of HIV vaccines).[51]

STORAGE, MANIPULATION, AND CRYOPHYSICAL VALIDATION OF VITRIFIED SAMPLES

Another major concern in the use of surface-based vitrification techniques is the dependence of sample viability on storage temperature. Maintenance of the correct temperature during banking, and especially during transportation to and from the laboratory to the storage tank, has great impact on cell survivability, recovery, and preservation of pluripotency. Temperatures of vitrified samples cannot exceed the T_G (around –130°C), or devitrification can cause a complete loss of viability by rapid ice crystallization throughout the complete sample.[43] These shortcomings hamper the more widespread use of vitrification techniques, since regular banking systems often show fluctuations in temperature through opening and closing of the tanks, and therefore cannot guarantee a constant sample temperature below –130°C. This makes the development of specialized banking systems for vitrified samples a major criterion when aiming for widespread use of vitrification techniques.

Prevention of Devitrification during Long-Term Storage

The importance of correct storage temperature is shown in Figure 24.18. Vitrified hESC samples were stored at –80°C in a commercially available freezer and at –170°C in the gas phase of a liquid nitrogen storage tank. Samples were thawed after 1 day, 1 week, and 1 month, and their viability was evaluated after a 24-hour recovery period. The results show a clear dependence of cell survivability on storage temperature. After only 24 hours of storage at –80°C, the viability of the vitrified samples went down by more than 90%, as compared to samples stored below the T_G. This implies that reliable liquid nitrogen banking systems are a basic and definitive prerequisite for the efficient application of vitrification workflows in the field of hESC and iPSC research and clinical application.

Current biobanking technologies are based on liquid nitrogen storage tanks for the storage of samples in the vapor phase of liquid nitrogen. Within these systems, a temperature of about –160°C is guaranteed, so storage of vitrified samples is possible. But during sample input and output, the closed cold chain cannot be maintained, as the sample racks of the storage tanks have to be pulled out to room temperature. Handling of high sample numbers can take several minutes, leading to a significant warming of the complete sample rack up to above –80°C during sample input and output (Figure 24.19).

This can lead to an adverse effect on slow-frozen samples in standard cryovials due to recrystallization processes, but may also result in complete cell loss in vitrified samples caused by devitrification. Additionally, the humidity in the ambient air causes the formation of ice and frost on the stored samples during the storage process, increasing the risk of contamination and significantly reducing the readability of labels and barcodes. Finally, manual removal and insertion of the storage rack is accompanied by uncontrolled collisions and vibrations, which result in sufficient E_A to induce ice crystallization in metastable, vitrified samples.

Hence, standard cryostorage technologies used in state-of-the-art biobanks are not applicable for the efficient storage of vitrified samples, and can also lead to the reduced sample quality of slow-frozen samples. In order to address these drawbacks, the hermetic storage concept was developed in collaboration with ASKION GmbH (Gera, Germany), combined with internal and optional external automation of the storage process (Figure 24.20). For this, a vacuum-isolated access tower is mounted on conventional cryotanks that can be cooled down to about –130°C with dry nitrogen gas, preventing condensation of ice from ambient air (Figure 24.20a). The frozen samples are transported in liquid nitrogen-cooled transport vessels that are connected to the cooled access tower. The samples are then transferred from the transport vessel to the final storage position by an integrated gripper. Hence, a closed cold chain and an ice-free environment are guaranteed during sample input and output. In addition, each rack is mechanically connected to a special lift system, enabling smooth movement of the storage racks, significantly reducing the collisions and vibration of the samples. Especially for vitrified samples, this feature prevents devitrification and increases sample quality. Several cryotanks can be connected by a rail system for automated transport and connection of the cooled transport vessel containing the samples with the access tower (Figure 24.20b). The sample management software in combination with integrated barcode readers enable permanent sample tracking and storage of sample history and information. Additionally, temperature sensors at different positions inside the storage system enable the preparation of a temperature profile for each sample during the whole storage duration.

Comparison of storage systems with the protective hood concept and conventional storage systems showed that, for slow-frozen peripheral blood mononuclear cells, temperature fluctuations during sample storage using the conventional liquid nitrogen tank resulted in reduced cell viability, recovery, and T-cell functionality in contrast to the storage system with an access tower.[52]

In summary, new storage and biobanking systems are available that enable efficient and vibration-free storage of vitrified samples, preventing warming of samples above the T_G and hence devitrification.

Safe Handling of Vitrified Samples

Beside the storage process, handling of frozen samples bears the risk of exceeding the T_G, thus causing ice

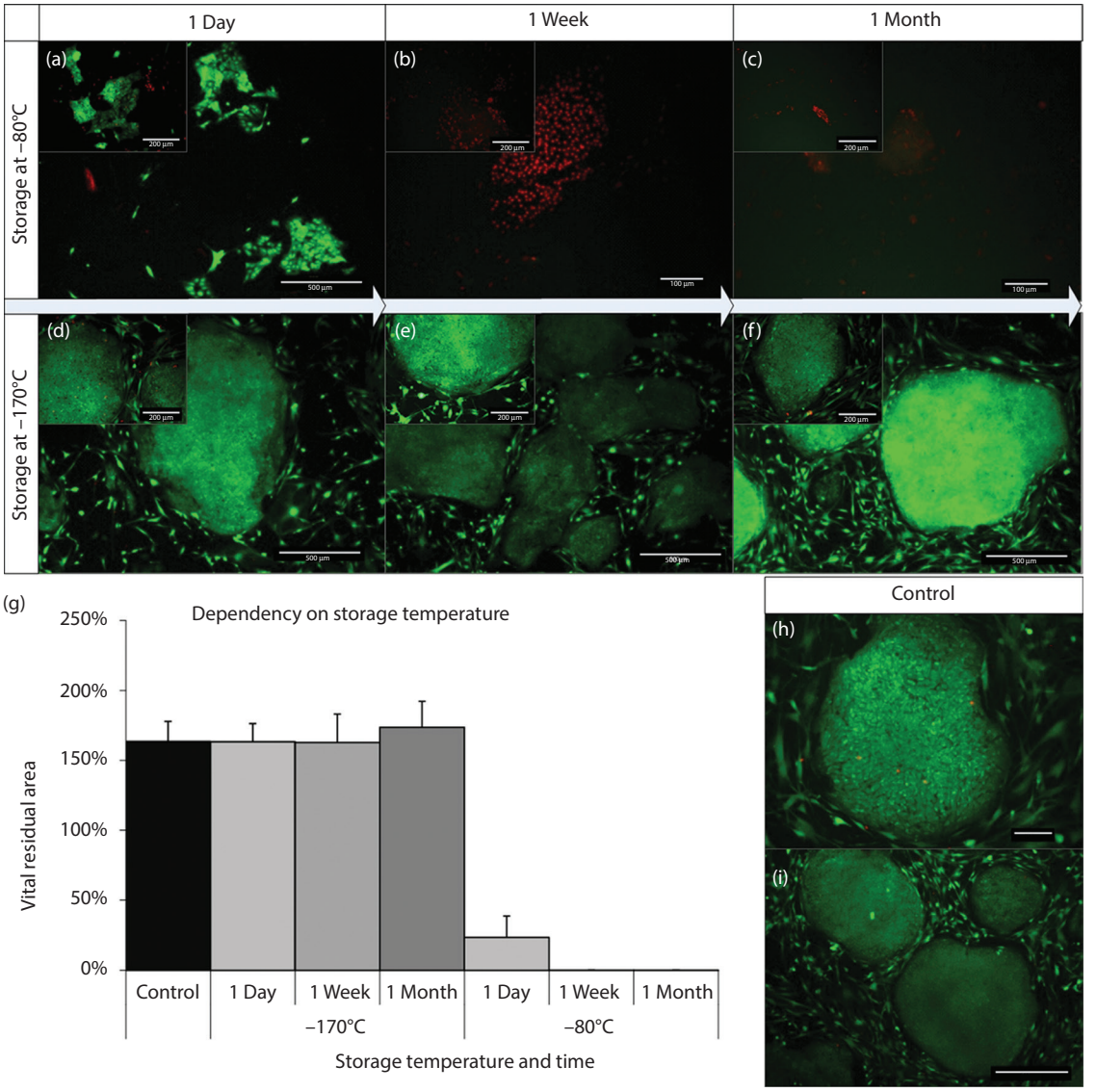

Figure 24.18 **(See color insert.) Comparison of different storage temperatures for vitrified human embryonic stem cell colonies. Cells have been vitrified via surface-based vitrification and stored at –80°C (a–c) and –170°C (d–f). Samples were thawed after 1 day (a and d), 1 week (b and e), and 1 month (c and f). Evaluation was done after a 24-hour recovery period via staining with FDA/EB and comparison of the vital residual areas (g). A nonfrozen control sample has been included (h and i).**

crystallization in vitrified samples. Especially in large biobanks, re-sorting of frozen samples is often necessary (e.g., during preparation of sample shipment or for optimizing the storage capacity).

Therefore, a working space that can be cooled down to –130°C for the handling of frozen, especially vitrified samples, is necessary to guarantee sample quality. The cryo-workbench developed in collaboration with ASKION GmbH (Figure 24.21a) is cooled down by pumping liquid nitrogen into the base of the handling room, creating a temperature gradient from –170°C to about 8°C (Figure 24.21b). Frozen samples can be relabeled or re-sorted inside the cryo-workbench without interrupting the cold chain. Additionally, up to three independently operating controlled-rate freezers are integrated in the cryo-workbench (Figure 24.21c). These freezers are based on an elevator principle, making use of the temperature gradient inside the working space. The freezer consists of a platform for taking up the samples with two temperature sensors for measuring the ambient temperature

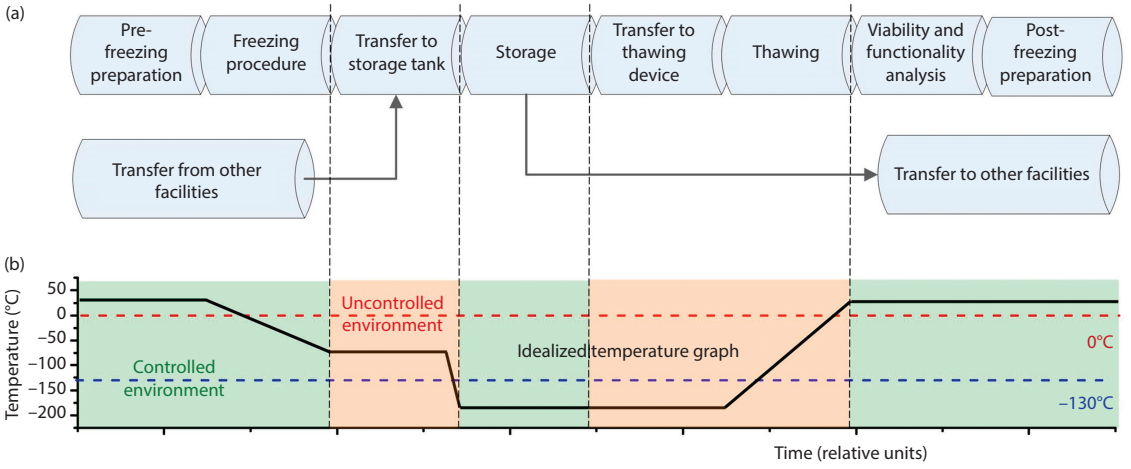

Figure 24.19 **Overview of the workflow and temperature profile during sample storage. General workflow of sample processing, cryopreservation, and storage in biobanks today (a) and related temperature profiles for the cryopreservation and storage of samples (b).**

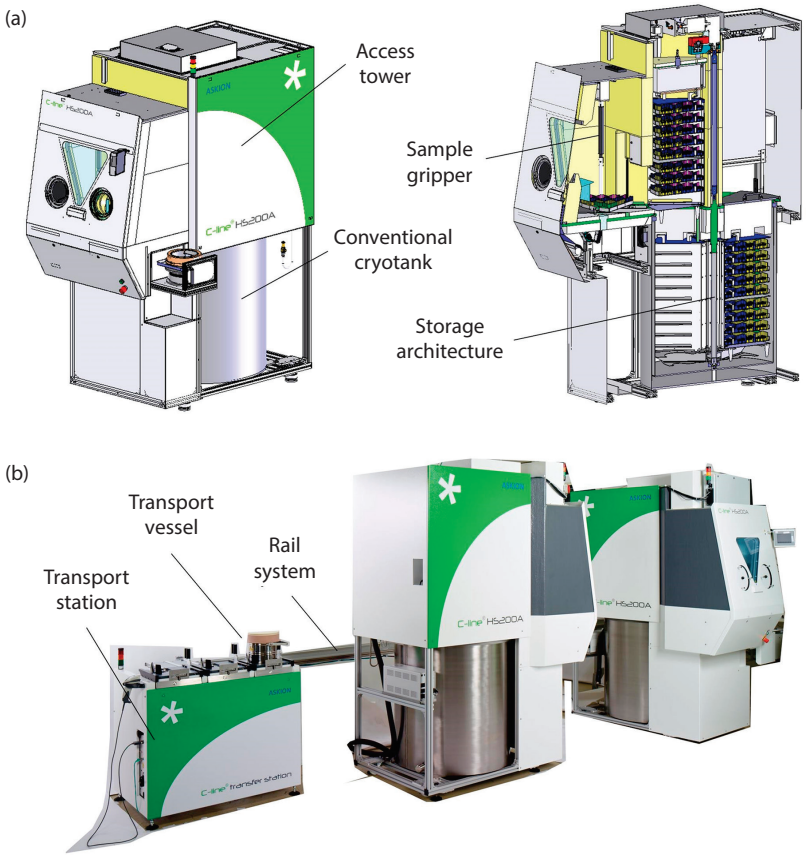

Figure 24.20 **Automated biobank with vacuum-isolated access tower mounted on a conventional liquid nitrogen cryotank (ASKION GmbH, Gera, Germany). Plan and cross-section of the storage tank with access tower (a) and image of the automated biobank with transport station and rail system (b). The access tower can be cooled down to about –130°C with dry nitrogen gas, guaranteeing a closed cold chain and preventing condensation of ice from ambient air.**

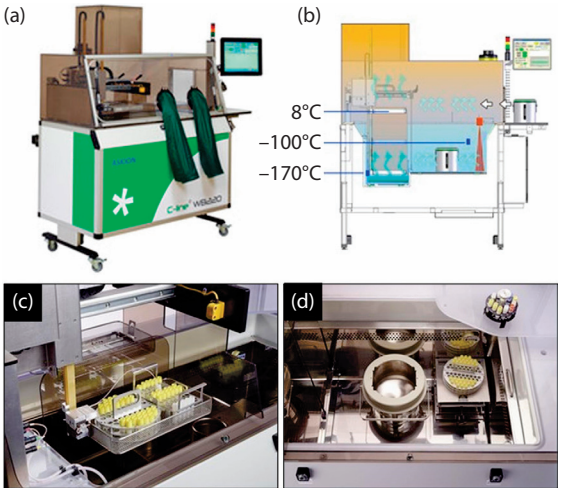

Figure 24.21 **Cryo-workbench with integrated controlled-rate freezer. Image of the cryo-workbench (a) and plan of the system with the applied temperature gradient (b) with integrated controlled-rate freezer (c). This handling system enables the manipulation of frozen samples during re-sorting, relabeling, or for taking inventory with an uninterrupted cool chain below –130°C (d).**

and the temperature in a reference sample. The platform moves downwards inside the handling room, resulting in a sensor-controlled temperature decrease according to the temperature profile that is programmed. Finally, the frozen samples can be transferred to the cooled transport vessels and are transferred to the storage tank (Figure 24.21d). Additionally, sample tracking and taking inventory of samples is implemented by a barcode-based sample management system.

In summary, today, innovative biobanking systems are available guaranteeing a closed cold chain and sample temperature below –130°C, while also making the safe handling and storage of vitrified samples in biobanks possible.

Noninvasive Validation of Vitrified Samples

The T_G can be easily exceeded during retrieval of samples, transport, or due to technical failures. If a sample or even an entire sample collection has experienced such an interrupted cool chain, risk of crystallization—and hence destruction—of the biological content is high. Unfortunately, the event of ice crystallization is invisible to visual inspection. An interim reanimation of the sample for inspection purposes is not possible since it is costly, time-intensive, and significantly harms the sample. To overcome this drawback, Raman spectroscopy can be used to discriminate unscathed, vitrified samples from crystalized ones without rewarming. Using a smart confocal probe design even allows for assessment of the sample state in a closed container. The drastic thermal gradient between the storage space and the ambient air can be passed through by fiber optics, which are comparably insensitive to temperature influences, so the optical probe in the cold can be connected to the light source and the Raman detection instrument at ambient temperature.

Raman spectroscopy detects molecular vibrations like infrared spectroscopy, but is based on inelastic scattering[53,54] and may thus be acquired by emission spectroscopy setups akin to fluorescence. No exogenous markers are needed since the molecular vibrations of water can be used directly. Due to the different intermolecular forces and arrangements between liquid/vitrified water and crystalline ice, their molecular vibration spectra can be clearly discriminated (Figures 24.1 and 24.22).

Utilizing a confocal probe geometry assessment of a sample becomes possible. By this means, a suspicious sample may be inspected for ice formation at storage temperature. Because the spectral behaviors of liquid and glassy water as well as ice are known, the sample status can be directly determined without any need for comparison. It is even possible to observe ice formation from the vitrified state in real time. Figure 24.22 shows a sequence of Raman spectra of an initially vitrified aqueous solution of DMSO (20% by volume), EG (20%), and 300 mM sucrose, which was rewarmed at 1 K/min, beginning at –135°C. At about –120°C, ice formation starts, giving rise to the striking ice band at 3100 cm^{-1}. After a few minutes, the entire sample volume is crystalized (an analogous study is shown in Doerr et al.[28]).

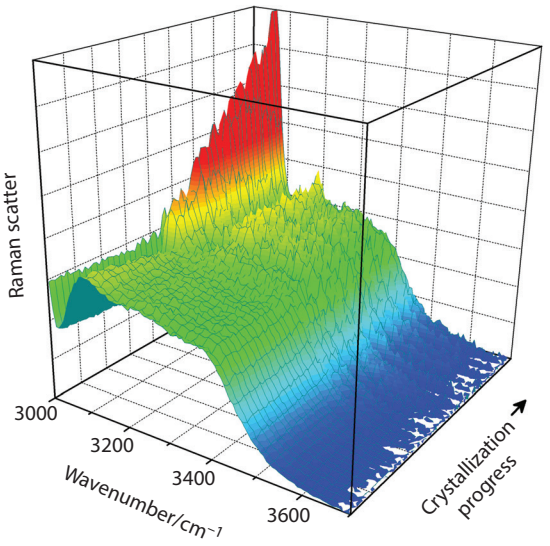

Figure 24.22 **(See color insert.) Waterfall plot of a Raman spectral sequence showing ice formation from a vitrified medium. The medium composition was 300 mM sucrose solution with 20% dimethyl sulfoxide and 20% ethylene glycol. The medium was vitrified at a temperature of –135°C, then heated to –95°C at a rate of 1 K/min.**

The benefit of the technique is, however, limited if fluorescent species are present, which cover the much weaker Raman emission. While we found cellular auto-fluorescence to be a minor problem, fluorescent media additives like phenol red may considerably hamper any Raman measurement.

Raman spectroscopy reveals information about any (main) constituent of a sample, as long as it is not a noble gas or a metallic compound, so it has the potential to tell us about the status of slowly frozen samples, as well.[55–57] But here the analysis and interpretation of the data are much more complicated, since we can no longer make use of the predominant constituent—water. In future, this technique could offer new possibilities for the non-invasive validation of vitrified samples in large-scale biobanks integrated in a wide quality control and management system.

SUMMARY AND OUTLOOK

This chapter describes innovative cryopreservation strategies and banking technologies for the efficient storage of vitrified samples in response to the open demands of large-scale biobanks. Vitrification has shown itself to be highly suitable for the cryopreservation of complex and sensitive cell systems like PSCs, with good maintenance of cell viability and functionality if protocols are strictly adhered to in terms of vitrification media and incubation times, and if a closed cold chain is guaranteed in order to prevent harmful devitrification. Adaptation of the established vitrification technique in open pulled straws to the surface-based vitrification technique enables exact implementation of the complex protocols, storage of large cell numbers, and further optimization to a GxP-compliant vitrification procedure (see section "Procedures and workflows for the vitrification of human PSCs"). Additionally, innovative banking technologies using the hermetic storage concept with integrated quality control can prevent warming of samples over the T_G during sample input and output, as well as during sample re-sorting or labelling, enabling the efficient storage of vitrified samples (see section "Storage, manipulation, and cryo-physical validation of vitrified samples").

Finally, the procedures described here offer further possibilities for future applications. The combination of the surface-based vitrification technique in cell culture plates or on micro-carriers with the evolving technology of thermos-responsive or generally switchable surfaces enables modulation of cell adhesion during the cryopreservation process. This can result in controlled cell detachment processes of all cells or only defined cell populations (e.g., differentiated cells) during the thawing step. Additionally, further implementation of the complete workflow consisting of iPS generation, expansion, differentiation, and cryopreservation in automated systems will increase throughput, reproducibility, and standardization, and will increase benefits for the community, especially the possibility of automating the cryopreservation of cells in a ready-to-use format upon thawing in high-throughput applications, as this will overcome the drawback of there being a clear lack of surface-based sterile freezing that could be integrated in industry-standard plate- or flask-based workflows ready for automation processes in high-throughput applications. Surface-based freezing has the double advantage of: (i) cell dissociation/detachment not being necessary, therefore retaining normal cell–cell and cell–matrix interactions to reduce cell stress; and (ii) cell re-attachment on thawing not being necessary, thereby promoting recovery, thus reducing cell death and manufacturing timelines.

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25 Scrying the future: The ongoing transformation of reproductive medicine through vitrification

Kevin S. Richter, James R. Graham, and Michael J. Tucker

Reproductive medicine is in the midst of an exhilarating revolution. Few innovations have had as transformative an impact on the field of assisted reproduction as vitrification. This impact will only increase as development and refinement of vitrification technology and methodology continues. The benefits associated with vitrification of cells and tissues have profound consequences for nearly every aspect of assisted reproductive practices. Damage-free storage of gametes and embryos, made possible for the first time through vitrification, dramatically improves the efficacy and practicality of a variety of current procedures and unlocks an entirely new realm of possibilities for fertilization and embryo transfer strategies, oocyte donation, and elective fertility preservation.

EMBRYO CRYOPRESERVATION

The earliest reported pregnancies and successful live births following transfers of previously cryopreserved embryos were published in 1983 and 1984.[1,2] Since then, embryo cryopreservation has been increasingly incorporated as a standard adjunct to fresh *in vitro* fertilization (IVF) treatment cycles. From 2003 to 2013, the number of cryopreserved embryo transfer procedures performed in the United States nearly tripled, growing from 17,494 in 2003 to 40,643 in 2013 (Figure 25.1). During that time span, the proportion of all embryo transfer procedures that involved cryopreserved rather than fresh embryos also more than doubled, from 18.7% in 2003 to 38.4% in 2013 (Figure 25.1). The rates of increase over time in both the number and percentage of cryopreserved embryo transfers remained relatively constant from 2003 to 2009, but since then, both of these rates have been progressively accelerating.

As a consequence of the widespread and increasing use of embryo cryopreservation, hundreds of thousands of children have been born worldwide. However, despite this substantial success, it is well established that, historically, the implantation potential of cryopreserved embryos has been significantly lower than that of embryos transferred while fresh. The viability deficit of cryopreserved embryos relative to fresh embryos is clearly evident in a comparison of outcomes of fresh versus cryopreserved embryo transfers to donor oocyte recipients. Unlike autologous transfers, recipients of donor oocytes are not subject to the potential disruption of endometrial receptivity caused by stimulation medications, and protocols for endometrial preparation are identical regardless of whether embryos are transferred while fresh or following cryopreservation. Thus, any outcome differences are more accurately reflective of the relative viability of the embryos transferred. National results for the United States, reported by the Society for Assisted Reproductive Technology (SART), reveal that, in transfers of embryos derived from donor oocytes, live birth rates are consistently much higher for fresh transfers compared to transfers of cryopreserved embryos. From 2003 to 2013, live birth rates for cryopreserved embryo transfers were 28% to 41% lower compared to fresh transfers (Figure 25.2), despite the fact that similar numbers of embryos were transferred per cycle. An essentially equal viability deficit has been reported for embryo transfers to gestational carriers, in which the live birth rate for fresh transfers was 51% compared to 34% for cryopreserved embryo transfers to gestational carriers not having a fresh transfer first.[3]

VITRIFICATION ENHANCES CRYOPRESERVED EMBRYO VIABILITY

Until recently, embryo cryopreservation has been performed almost exclusively via "slow-freezing" protocols. Mounting evidence increasingly demonstrates that embryo vitrification, as an alternative to traditional slow freezing, can substantially reduce if not virtually eliminate this historical "cryopreservation deficit." A systematic review and meta-analysis of post-cryopreservation survival rates has reported that cryosurvival is significantly higher with vitrification versus slow freezing of both cleavage-stage and blastocyst-stage embryos.[4] Nearly all (97.5%) of the vitrified cleavage-stage embryos survived compared to 84.1% of the slow-frozen cleavage-stage embryos, while 89.9% of the vitrified blastocysts survived compared to 75.4% of the slow-frozen blastocysts. A randomized trial published soon afterwards added to the evidence that cleavage-stage vitrification, compared to slow-freezing, resulted in significantly higher cryosurvival (94.8% versus 88.7%).[5] In addition, pyruvate uptake (an indicator of metabolic rate), embryos with 100% blastomere survival (77.9% versus 51.4%), and subsequent blastocyst formation (60.3% versus 49.5%) were all found to be significantly higher with vitrification. A more recent systematic review and meta-analysis evaluating pregnancy outcomes found that both clinical pregnancy rates (38.7% versus 33.1%) and ongoing pregnancy rates (34.7% versus 27.1%), as well as implantation rates (29.3% versus 24.2%), were significantly higher with embryo vitrification versus slow freezing.[6] In a population-based cohort study of all (more than 30,000)

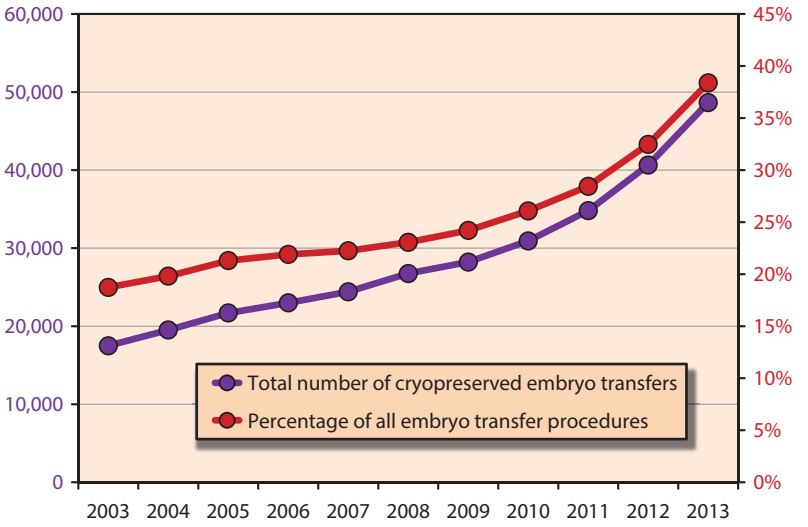

Figure 25.1 **Total annual numbers of cryopreserved embryo transfer procedures (nondonor and donor oocytes combined) and the percentage of cryopreserved embryo transfers among all embryo transfers (fresh and cryopreserved) performed in the United States from 2003 to 2013. (Adapted from data reported by the Society for Assisted Reproductive Technology [www.sart.org].)**

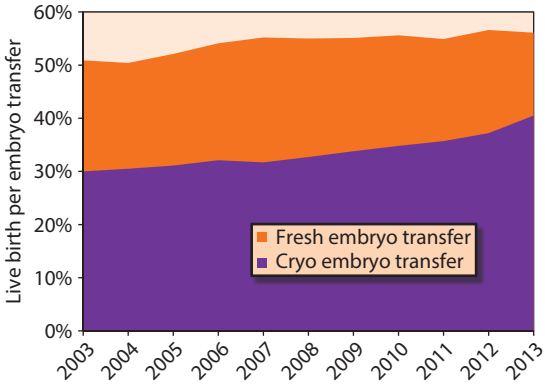

Figure 25.2 **Annual live birth rates per embryo transfer procedure for *in vitro* fertilization cycles in which donor oocytes were used, comparing outcomes of fresh embryo transfers to transfers of embryos that had been cryopreserved, among all reported cycles performed in the United States from 2003 to 2013. (Adapted from data reported by the Society for Assisted Reproductive Technology [www.sart.org].)**

autologous cryopreserved blastocyst transfer cycles performed in Australia and New Zealand over a 3-year period, vitrification compared to slow freezing resulted in more transfers per warming/thawing attempt (95.6% versus 90.6%), more transferred embryos per warmed/thawed blastocyst (84.5% versus 71.4%), and higher pregnancy and birth rates per transfer (32.7% versus 23.8% and 24.8% versus 17.7%).[7]

Our Own Experience of Transitioning from Slow Freezing to the Vitrification of Blastocysts

Shady Grove Fertility Reproductive Science Center, encouraged by the increasing published evidence supporting embryo vitrification, made the transition from slow-freeze protocols[8] used through December 2008 to vitrification[9] for all cryopreserved embryos beginning in January 2009. Embryos were cryopreserved at the blastocyst stage on day 5, 6, or 7 after oocyte retrieval and fertilization, according to the day on which they developed to the expanded blastocyst stage, with a clearly visible inner cell mass (ICM) and trophectoderm. Only good-quality blastocysts were cryopreserved (minimum ICM/trophectoderm score of BB according to Gardner and Schoolcraft's grading system[10]). The morphological criteria for supernumerary blastocyst cryopreservation were the same regardless of the day on which they were cryopreserved, and remained the same before and after the switch to vitrification. Between January 2003 and April 2012, we performed 4597 transfers of 7598 autologous cryopreserved blastocysts, consisting of 2842 transfers using slow freezing and 1755 transfers using vitrification protocols (Table 25.1), providing what is to date perhaps the largest reported single-center clinical experience comparing slow freezing to vitrification of human embryos.

Blastocysts cryopreserved by vitrification exhibited substantially reduced cryodamage compared to those cryopreserved using slow-freeze protocols. As has been reported by others,[11–15] vitrified blastocysts were more likely to survive warming (96% versus 92%). In addition, surviving blastocysts had a much higher percentage of

Table 25.1 Treatment Outcomes Compared between Slow Freezing and Vitrification among Autologous Cryopreserved Blastocyst Transfer Cycles

	Slow freezing	Vitrification	p-value
Cycles	2842	1755	—
Age at cryopreservation (years)	33.5	33.7	0.078
Embryo survival per cycle	91.9%	95.6%	<0.0001
Intact cells per surviving embryo	88.7%	95.3%	<0.0001
Embryos per transfer	1.71	1.56	<0.0001
Total embryos transferred	4862	2736	—
Positive hCG per transfer	1304 (45.9%)	1204 (68.6%)	<0.0001
Biochemical pregnancy per positive hCG	312 (23.9%)	190 (15.8%)	<0.0001
Clinical pregnancy per transfer	992 (34.9%)	1014 (57.8%)	<0.0001
Implantation per embryo transferred	1212 (24.9%)	1285 (47.0%)	<0.0001
Live birth per transfer	717 (25.2%)	780 (44.4%)	<0.0001
Live-born children per transferred embryo	829 (17.0%)	939 (34.3%)	<0.0001
Clinical pregnancy loss	275 (27.8%)	234 (23.1%)	0.017

intact cells (95% versus 89%). A comparison of the frequency distributions of observed post-warming cryodamage between slow freeze and vitrification illustrates substantial differences (Figure 25.3). Most vitrified embryos (73%) had little or no visible cryodamage (≤5% cell loss), while only 34% of the slow-frozen embryos fared as well. Conversely, only 8% of the vitrified embryos suffered greater than 10% cell loss, while 40% of the slow-frozen embryos lost more than 10% of their cells. Under 2% of vitrified embryos were less than 80% intact, compared to 12% of the slow-frozen embryos.

Despite the transfer of significantly fewer blastocysts per cycle, pregnancy and birth outcomes were much better following vitrification (Table 25.1). Compared to slow freezing, vitrification resulted in significantly more positive serum human chorionic gonadotropin (hCG) tests (relative increase of 49%). Similar to a few previous reports,[14–16] we observed significantly improved implantation, pregnancy, and birth rates. Our data indicated relative increases of 89% for implantation, 66% for clinical pregnancy, and 76% for live births with vitrification compared to slow freezing. Vitrified blastocysts were twice as likely to result in live-born children compared to slow-frozen blastocysts. Conversely, early pregnancy loss occurring after confirmation of positive serum hCG but before ultrasound confirmation of clinical pregnancy (i.e. biochemical pregnancies) had a statistically significant relative reduction of 34%, and pregnancy loss following ultrasound confirmation of clinical pregnancy was significantly lower as well, with a relative reduction of 17%.

Predictably, clinical pregnancy rates and live-birth rates resulting from single-blastocyst transfers declined significantly with increasing patient age at the time of the fresh oocyte retrieval and embryo cryopreservation, whether embryos were cryopreserved using slow-freeze or vitrification protocols (Figure 25.4; $p < 0.0001$ for all). Within each age group, clinical pregnancy rates were higher with vitrification than with slow freezing, although the difference was not statistically significant for the 41–42-year age group (<35 years: 54% versus 30%, $p < 0.0001$; 35–37 years: 53% versus 21%, $p < 0.0001$; 38–40 years: 31% versus 19%, $p = 0.03$; 41–42 years: 32% versus 17%, $p = 0.32$). Live-birth rates were also higher for vitrification versus slow freezing within each age group, but statistically significantly so only for patients under 38 years (<35 years: 42% versus 22%, $p < 0.0001$; 35–37 years: 39% versus 17%, $p < 0.0001$; 38–40 years: 19% versus 12%, $p = 0.16$; 41–42 years: 24% versus 4%, $p = 0.1$). Statistical power was very limited in the 41–42-year age group, with sample sizes of only 23 and 25 transfers for slow freezing and vitrification, respectively.

Whether using slow freezing or vitrification, success rates among single-blastocyst transfer cycles declined as the time it took for embryos to develop to the expanded blastocyst stage, indicated by the day of cryopreservation, increased (Figure 25.5). Among slow-frozen embryos, clinical pregnancy rates differed significantly between day 5 and day 6 cryopreservation (42% versus 27%, $p < 0.0001$) and between day 6 and day 7 cryopreservation (27% versus 10%, $p < 0.0001$). Live birth rates also declined significantly with each progressive day in the slow-freeze group (day 5 versus 6: 34% versus 19%, $p < 0.0001$; day 6 versus 7: 19% versus 6%, $p < 0.0001$). Among transfers of single vitrified blastocysts, the modest declines in pregnancy and birth rates from day 5 to day 6 cryopreservation were not statistically significant (pregnancy: 54% versus 50%, $p = 0.34$; birth: 42% versus 37%, $p = 0.1$). However, day 7 vitrification was associated with significantly lower outcomes for both pregnancy (15%) and birth (10%) compared to either day 5 or day 6 vitrification ($p < 0.0001$ for all).

Within each cryopreservation day, vitrification compared to slow freezing was consistently associated with higher clinical pregnancy (day 5: 54% versus 42%, $p = 0.008$; day 6: 50% versus 27%, $p < 0.0001$; day 7: 15% versus 10%, $p = 0.4$) and live-birth rates (day 5: 42% versus 34%, $p = 0.05$; day 6: 37% versus 19%, $p < 0.0001$; day 7: 10% versus 6%, $p = 0.4$), although the statistical significance of these trends could not be confirmed within the day 7 cryopreservation groups. The small sample size of blastocysts vitrified on day 7 ($n = 41$) limited the power of comparisons within this day.

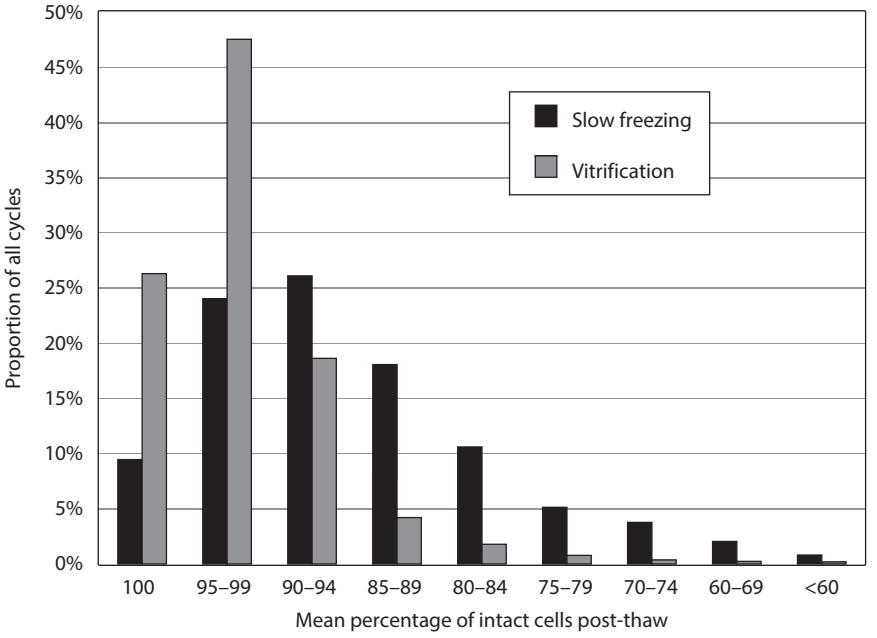

Figure 25.3 **Frequency distribution of observed post-warming cryodamage, quantified as the percentage of intact cells among all blastocysts surviving the cryopreservation and warming process, compared between slow freezing (black) and vitrification (grey).**

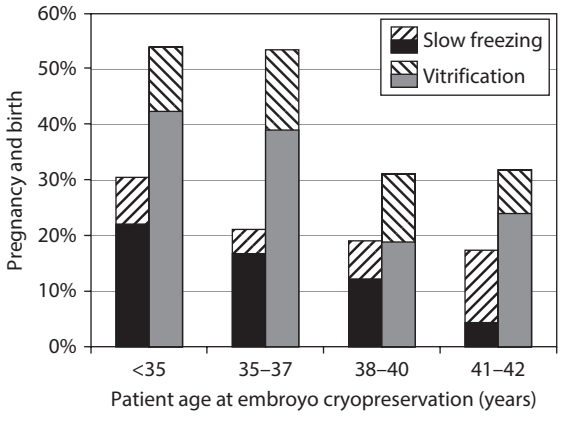

Figure 25.4 **Clinical pregnancy and live-birth rates among autologous single cryopreserved blastocyst transfers, according to patient age at the time of the fresh *in vitro* fertilization cycle from which embryos were cryopreserved. Compared between slow freezing (pregnancy in downward pattern and birth in black) and vitrification (pregnancy in upward pattern and birth in grey).**

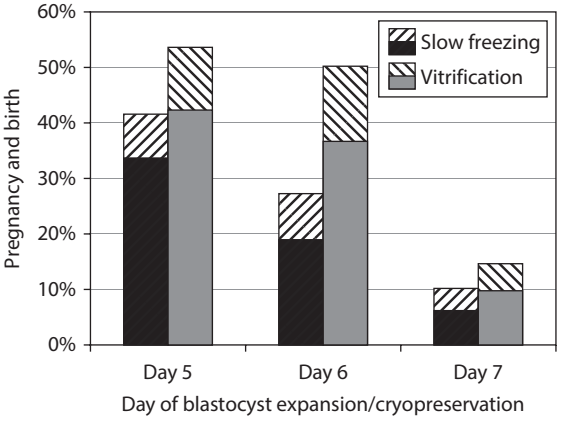

Figure 25.5 **Clinical pregnancy and live-birth rates among autologous single cryopreserved blastocyst transfers, according to the day after oocyte retrieval on which embryos developed to the expanded blastocyst stage and were cryopreserved. Compared between slow freezing (pregnancy in downward pattern and birth in black) and vitrification (pregnancy in upward pattern and birth in grey).**

Clinical pregnancy and live-birth rates among single-blastocyst transfer cycles both declined steadily and rapidly as the percentage of intact cells in transferred embryos declined, regardless of the method of cryopreservation (Figure 25.6; $p < 0.0001$ for all,). Only one of 99 slow-frozen embryo transfers and none of the six vitrified embryo transfers with fewer than 75% intact cells resulted in live births. Even among embryos with relatively little cryodamage (≤10% cell loss), the negative

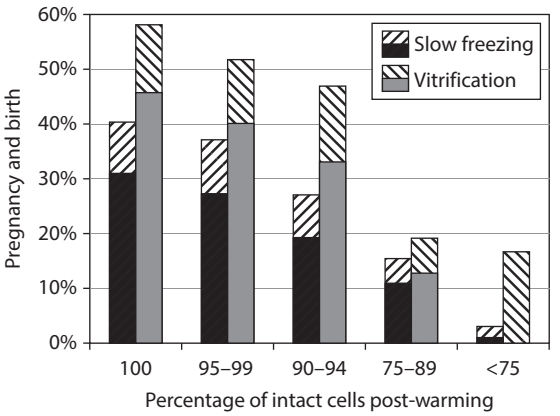

Figure 25.6 **Clinical pregnancy and live-birth rates among autologous single cryopreserved blastocyst transfers, according to the percentage of intact cells following cryopreservation and warming. Compared between slow freezing (pregnancy in downward pattern and birth in black) and vitrification (pregnancy in upward pattern and birth in grey).**

effect of cryodamage was evident. When the analyses were limited to blastocysts that were at least 90% intact, there were still statistically significant declines in both pregnancy and birth rates among both slow-frozen and vitrified blastocysts ($p = 0.0009$ and $p = 0.0017$ for slow freezing; $p = 0.0066$ and $p = 0.0052$ for vitrification).

Within each of the top three categories of cell survival, vitrification was associated with significantly higher pregnancy rates than slow freezing (100%: 58% versus 40%, $p = 0.0004$; 95%–99%: 52% versus 37%, $p = 0.0003$; 90%–94%: 47% versus 27%, $p = 0.0002$). Live-birth rates were also significantly higher for vitrification compared to slow freezing in each of the top three cell survival categories (100%: 46% versus 31%, $p = 0.0026$; 95%–99%: 40% versus 27%, $p = 0.0008$; 90%–94%: 33% versus 19%, $p = 0.004$). The significantly higher (by 13%–20%) pregnancy and birth rates for vitrification versus slow freezing, even among blastocysts in which observable cryodamage appeared to be the same, is particularly interesting. This result suggests that while differences in visible cryodamage partially explain differences in pregnancy and birth rates between vitrification and slow freezing, slow freezing relative to vitrification causes additional damage to embryos that is not apparent upon microscopic examination and yet adversely affects embryo viability.

In light of this accumulating evidence of greater viability retention associated with embryo vitrification, it may not be a coincidence that among donor oocyte transfer cycles the relative difference in birth rates between cryopreserved and fresh transfers has been attenuating noticeably in recent years as vitrification has begun to replace slow freezing (Figure 25.2). Live-birth rates from cryopreserved embryo transfers consistently remained at approximately 41% lower relative to fresh transfers from 2003 to 2008 (when nearly all cryopreservation was by conventional slow-freezing protocols), while thereafter the relative deficit in birth rates for cryopreserved relative to fresh transfers has declined annually (39% in 2009; 37% in 2010; 35% in 2011; and 34% in 2012; 28% in 2013) as embryo vitrification has been increasingly incorporated into clinical practices.

SHIFTING PARADIGMS IN EMBRYO TRANSFER STRATEGIES

The greatly enhanced survivability and viability associated with embryo vitrification suggests the need for a paradigm shift in the approach to assisted reproduction. The conventional approach to IVF is to transfer the highest-quality embryos while fresh, usually more than one per transfer, under the assumption that this will maximize their chances for implantation by avoiding the damaging effects of cryopreservation. As refinements in vitrification protocols lead to continued reductions in cryodamage, further limiting the adverse effects of cryopreservation on embryo viability, the rationale for multiple embryo transfer, or even fresh embryo transfer at all, wanes. With little compromise to embryo viability, clinical outcomes with cryopreserved embryos can approach those achieved with fresh transfers. In fact, live-birth rates have been reported to be equivalent for vitrified compared to freshly transferred blastocysts of equal morphological quality.[17]

Outcomes of vitrified embryo transfers may even surpass those of fresh transfers given that cryopreserved embryo transfer circumvents the known adverse effects of ovarian hyperstimulation on endometrial receptivity and endometrium–embryo developmental synchrony.[18] Dramatic hyperstimulation-induced alterations in endometrial histology (regardless of the stimulation protocol used or the fertility status of the subjects) and a clear negative association between the severity of these alterations and the likelihood of pregnancy have recently been documented,[19] providing strong evidence for compromised endometrial quality in stimulated cycles. Indeed, a meta-analysis of 633 cycles randomized to either fresh or cryopreserved embryo transfer reported relative differences exceeding 30% in favor of cryopreserved embryo transfer for both clinical pregnancy (50% versus 38%) and ongoing pregnancy (47% versus 36%).[20] One of the trials included in this meta-analysis, in addition to significant improvements in clinical and ongoing pregnancy rates, also reported a dramatic improvement in implantation rates with cryopreservation (71% versus 39%).[21] A retrospective study of single fresh or cryopreserved blastocyst transfers that were well matched for age, day of blastocyst expansion, blastocyst and ICM diameter, and trophectoderm cell count demonstrated significantly higher clinical pregnancy with cryopreservation (56% versus 27%).[22] Among our own patients undergoing transfers of

thousands of vitrified blastocysts at Shady Grove Fertility RSC, we observed an implantation rate of 47% among all autologous transfers regardless of age and an implantation rate of 54% among patients under 35 years old at the time of blastocyst vitrification, success rates that are well above the most recently reported (www.sart.org, 2013) implantation rates for autologous fresh transfers of those under 35 years of age for either our center specifically (42%) or for the nation as a whole (39%).

In addition to equivalent if not better pregnancy and live-birth rates with vitrified rather than freshly transferred embryos, there is a growing body of evidence demonstrating that cryopreserved embryo transfers result in substantially better perinatal outcomes than fresh embryo transfers. A systematic review and meta-analysis of singleton IVF pregnancies concluded that, compared to fresh embryo transfers, cryopreserved embryo transfers were significantly less likely to be associated with perinatal mortality (RR = 0.68), pre-term birth (RR = 0.84), low birth weight (RR = 0.69), small for gestational age (RR = 0.45), and antepartum hemorrhage (RR = 0.67).[23] More recent studies have found that pregnancies resulting from cryopreserved rather than fresh embryo transfers were less likely to experience third-trimester bleeding or placenta previa,[24] and provided further confirmation that the resulting children were born later and heavier,[17,24–26] and thus were more comparable to children from natural conceptions. It has also been reported that blastogenesis birth defects are significantly more common with fresh embryo transfers, but not with cryopreserved embryo transfers, compared to natural conceptions.[27]

Based on such evidence of higher success rates and improved perinatal outcomes associated with cryopreserved embryos, there is increasing advocacy for abandoning fresh embryo transfers altogether in favor of transferring cryopreserved embryos into a more hospitable and receptive endometrial environment undisrupted by ovarian hyperstimulation necessary to produce a supraphysiological cohort of mature oocytes.[28–31] With the minimization of cryodamage possible through vitrification, it now appears likely that vitrification of all embryos for transfer in a later unstimulated cycle may provide the highest potential for success, and may soon become the norm for IVF treatments.

Under these conditions, the imperative to transfer multiple embryos while still fresh, and in fact the clinical benefits of multiple embryo transfer at all (except perhaps, but not necessarily, in cases of poor embryo quality), virtually disappear. With the minimization of cryodamage resulting in equivalent or better chances of achieving pregnancy following vitrification rather than fresh transfer, transfer of multiple embryos (whether fresh or cryopreserved) will not increase the chances of eventual success from an oocyte retrieval cycle. In fact, transfer of embryos one at a time will maximize the potential for success and the number of children that can result from a single oocyte retrieval procedure, as well as maximizing the perinatal health of any resulting children. Firstly, a succession of single-embryo transfers eliminates the drawbacks of "putting one's eggs all in one basket." Assuming that cycle-to-cycle variability in endometrial receptivity exists (which is practically a given), a succession of single-embryo transfers would minimize the potential for all viable embryos to be transferred to a nonreceptive uterus. It would have the additional advantage of avoiding any possible competition among viable embryos for implantation sites, growing space, nutrient supply, and other maternal resources, thus maximizing the potential for the optimal growth of each embryo.

We have actually documented this effect among cryopreserved embryo transfers in our own data from Shady Grove Fertility RSC. In an analysis of vitrified blastocyst transfers,[32] we found a relative increase of 13.6% in the number of live-born children per transferred embryo among cryopreserved blastocysts transferred singly rather than in pairs (adjusted means = 38.08% versus 33.52%). This 13.6% relative difference suggests that, given the same number of vitrified blastocysts, 13.6% more children could be born if they were transferred one at a time rather than two at a time. Predictably, the children resulting from single vitrified blastocyst transfers were also healthier at birth, being born on average 1 week later (and only one-sixth as likely to be very or extremely pre-term) and 581 g (20%) heavier (and only one-tenth as likely to be very low birth weight) compared to children born from transfers of two vitrified blastocysts.

EMBRYO VITRIFICATION WHEN FRESH TRANSFER IS CONTRAINDICATED

In addition to the more general benefits of embryo vitrification already described, there are a variety of special case situations in which fresh embryo transfer may be particularly contraindicated. One common contraindication to fresh embryo transfer is either the presence of significant symptoms of ovarian hyperstimulation syndrome (OHSS) prior to scheduled transfer and/or conditions suggesting a high risk for developing moderate-to-severe OHSS (e.g. polycystic ovary syndrome [PCOS], previous history of OHSS, high or rapidly increasing serum estradiol before hCG trigger, large number of developing follicles during ovarian stimulation, or large number of retrieved oocytes[33,34]). OHSS is a potentially serious—in rare cases even life threatening—complication that generally only occurs in the context of ovarian stimulation in conjunction with IVF treatment.[35] It is the leading cause of IVF-related mortality among nonpregnant women (approximately three deaths per 100,000 stimulated cycles).[36] It is induced and exacerbated by exogenous hCG from the trigger injection and/or by the endogenous hCG produced by implanted embryo(s).[34] In the presence or with high risk of OHSS, postponing transfer will therefore

either lessen the severity of OHSS or prevent moderate-to-severe OHSS requiring intervention from occurring at all.

Fresh embryo transfer may also be contraindicated when there are indications of unusually poor endometrial receptivity during the fresh cycle. In addition to the already discussed general disruption to normal endometrial development caused by ovarian hyperstimulation for IVF, some specific conditions are known to be predictive of unusually low chances of success with fresh embryo transfer. For example, a thin endometrial lining at the time of trigger administration is known to be associated with significantly lower pregnancy rates.[37–40] Elevated serum progesterone levels, defined variously by a threshold between 0.8 and 3.0 ng/mL, at the time of trigger administration have also been linked to significantly poorer success rates with autologous fresh embryo transfers.[41] There are also situations in which unanticipated health issues, personal issues, emergencies and the like prevent undergoing an embryo transfer procedure as planned.

In all such occasions and in similar circumstances, in the absence of a reliable and effective embryo cryopreservation program, forgoing fresh embryo transfer could necessitate discarding the entire embryo cohort and wastage of the entire treatment cycle. A suboptimal cryopreservation program would reduce embryo viability and decrease the chances of success once a suitable time for embryo transfer arrives. With the high viability maintained through the use of current vitrification protocols, however, embryos can be stored for later use with high chances of success in all of these situations.

PRE-IMPLANTATION GENETIC SCREENING AND EMBRYO VITRIFICATION

The ability to minimize cryodamage through the use of vitrification also has important implications for the practicality of incorporating pre-implantation genetic screening (PGS) as a clinically advantageous adjunct to IVF treatment. The first iteration of PGS, using fluorescence *in situ* hybridization analysis of a minority subset of the chromosome complement derived from a cleavage-stage biopsy, has proven to be detrimental.[42] This failure of early PGS attempts appears to be largely due to dramatic reductions in embryonic developmental potential (39% relative reduction in implantation with progression to a live-born infant) when a cleavage-stage biopsy is performed.[43] More recently, alternative methods incorporating comprehensive chromosome screening (CCS)[44,45] and trophectoderm biopsy at the blastocyst stage, while as-yet not conclusively proven, appear to hold more promise of benefit.[45–49] Blastocyst-stage biopsy appears to preserve developmental potential much more so than cleavage-stage biopsy,[43] but leaves little time for evaluation before embryos must be transferred if transferred while fresh. CCS enables analysis of all 23 pairs of chromosomes, and as the technology matures, the emergence and enhancement of whole-genomic sequencing[50] will make available a growing abundance of increasingly detailed information upon which assessments of embryo viability and health can be made. Vitrification of biopsied embryos would provide ample time for conducting and interpreting increasingly complex genetic analyses, offer appropriate genetic counseling to patients, and allow patients and clinicians to consider and make informed clinical decisions based on this information.

OOCYTE VITRIFICATION

Compared to the historically widespread practice of embryo cryopreservation, oocyte cryopreservation has been, until recently, no more than a minor footnote in the field of assisted reproduction. However, with the rise of vitrification, oocyte cryopreservation is rapidly becoming an integral and major component of assisted reproductive technology (ART). Clinical application of oocyte cryopreservation had been impeded by disappointingly low success rates with the slow-freezing protocols used for nearly all attempts until 2003. Through 2002, fewer than 80 births from cryopreserved oocytes had been reported worldwide.[51] By the end of 2008, the total number of births from cryopreserved oocytes, while still very low relative to overall ART births, had grown to over 900, with 532 through slow freezing and 392 through vitrification.[51]

Oocyte survival is significantly higher with vitrification,[52,53] and multiple groups have published reports of post-vitrification oocyte survival rates greater than 95%.[54–57] Fertilization rates, embryo cleavage rates, and top-quality embryo rates are also all significantly higher with vitrification compared to slow freezing.[52] A review of all published series reports from 1998 to 2008 demonstrated that the number of children born per thawed/warmed oocyte was more than twice as high with vitrification than with slow freezing (2.4% versus 5.2%), and by 2008, the majority of newborns were the result of oocyte vitrification rather than slow freezing.[51]

Fertilization, embryonic development, embryo quality, implantation, pregnancy and birth rates, and obstetric and perinatal complications following oocyte vitrification have been shown in numerous studies to be comparable to those of fresh oocytes.[52,54,56–60] Electron microscopy has revealed that vitrification of mature human oocytes maintains good ultrastructural preservation,[61] and metabolic profiling has failed to disclose any significant differences between embryos derived from vitrified versus fresh oocytes.[62] Such studies demonstrate that oocytes can be cryopreserved with little or no damage using vitrification. The consistency and repeatability[63] of such positive outcomes with this method of oocyte cryopreservation has spurred the increasingly rapid adoption of oocyte vitrification into assisted reproduction programs. In 2013, the American Society for Reproductive Medicine (ASRM) and the SART issued

a joint committee opinion,[64] endorsed by the American College of Obstetrics and Gynecology,[65] concluding that oocyte vitrification and warming should no longer be considered experimental based on good evidence that fertilization and pregnancy rates are similar for vitrified–warmed oocytes as with fresh oocytes when used for IVF/intracytoplasmic sperm injection treatment, with no apparent increase in chromosomal abnormalities, birth defects, or developmental deficits in resulting offspring, further opening the door to the use of oocyte vitrification in standard clinical practice.

DONOR VITRIFIED OOCYTE BANKING

Oocyte donation by healthy young women plays a substantial role in assisted reproduction, providing reproductive options for women whose oocyte quality is poor or whose ovarian reserves are depleted. In the United States, more than 17,000 transfers of embryos derived from donor oocytes were performed in 2013, accounting for 13.8% of all embryo transfers performed. The ability to successfully cryopreserve oocytes enables substantial changes in the ways in which donor oocytes can be provided to patients in need.

Traditionally, oocyte donation could only be performed fresh. With fresh transfer, careful synchronization of donor and recipient cycles is required to ensure that recipients are maximally receptive when embryos are ready to implant. Cycle synchronization is even more complicated and challenging when fresh donor cohorts are shared among multiple recipients, a practice that makes more efficient and cost-effective use of the large oocyte cohorts often produced by donors,[66–69] but which is only practical in relatively large programs with numerous concurrently cycling recipients. In most cases, donor cohorts used fresh are unshared, often resulting in more viable oocytes and embryos than patients require for their family-building goals. An ever-present risk in fresh oocyte donation cycles is that cycles may be cancelled due to an unexpectedly poor response by the donor, resulting in the cancellation of retrieval or, in the case of shared cohorts, retrieval of too few oocytes to share among all intended recipients. Cycles may also be cancelled due to inadequate endometrial development in the intended recipient, in which case, without the option of oocyte cryopreservation, the donated oocytes would go unused and wasted. In either case, the intended recipient incurs considerable time and expense for cycle cancellation.

Advancements in oocyte cryopreservation, particularly vitrification, have led to the establishment of numerous donor egg banks. By early 2012 (while oocyte cryopreservation was still labeled as "experimental" by societal guidelines), there were already at least seven commercial egg banks operating in the United States (all but one of them cryopreserving by vitrification), maintaining more than 3000 oocytes obtained from nearly 300 individual donors.[70] As of April 2012, more than 600 clinical pregnancies had been established using oocytes dispensed by these banks. In 2013, the first year for which donor oocyte banking data was included in the annual summary report of SART member clinics, cryopreserved banked donor eggs accounted for more than 20% of all donor egg embryo transfers performed in the United States, and these banked donor egg cycles resulted in 960 live births.

Such egg banks provide a multitude of benefits to recipient patients. No treatment delay or synchronization is necessary; donor oocytes have already been retrieved and can be thawed/warmed whenever the recipient is ready to use them. Cycles are never cancelled due to inadequate donor response. Eggs are not thawed/warmed until the intended recipient demonstrates adequate endometrial development, so poor development in a given cycle is relatively inconsequential. Sharing large cohorts of oocytes is simple; each recipient from a donor egg bank typically receives five to seven mature eggs, so a single donor retrieval can usually provide enough eggs to supply two or three (and sometimes more) recipients. The simplicity of cohort sharing allows egg banks to distribute donor oocytes to a much larger number of recipients, and at a significantly reduced cost per patient. Cryopreserved oocytes can be safely shipped between distant treatment facilities, so they can provide recipients with a much larger and more diverse donor pool from which to select compared to a fresh donor program that must depend on the potential donors available at the time in the local geographic area. In addition, oocyte banking, in contrast to fresh oocyte donation, allows for quarantining and thorough evaluation for infectious agents prior to clinical use, as is standard practice for donor sperm. Given these many and substantial advantages, as well as the clinical outcomes for vitrified oocytes that are comparable to fresh oocytes, it seems inevitable that egg donation—likely in the near future—is destined to follow the model of sperm donation, with fresh donations being entirely replaced with cryopreserved (mainly vitrified) donor egg banking.

MEDICALLY INDICATED FERTILITY PRESERVATION THROUGH OOCYTE VITRIFICATION

Fertility preservation through oocyte cryopreservation for medical indications has been in use for some time on a very limited scale, and with only limited success in the past due to the substantially reduced viability associated with earlier oocyte cryopreservation protocols. Despite limited success, these attempts continued, since they often provided the best—and sometimes the only—chances for these women to bear children in the future. The current potential of oocyte vitrification greatly enhances the applicability and success of medically indicated fertility preservation.

One of the primary medical indications for fertility preservation is anticipated gonadotoxic medical treatments

among women of childbearing years.[64] Chemotherapy or radiation therapy, which often reduce fertility, are frequently administered as treatments for a variety of oncological conditions, as well as some autoimmune and hematological diseases. Oocyte vitrification has been used with good success among women diagnosed with cancer who used their vitrified oocytes following successful cancer treatments.[71] Additionally, oophorectomy may be required by some patients for either malignant or benign conditions, or undergone prophylactically in women with BRCA mutations or other genetic conditions associated with a high risk for ovarian cancer. It is estimated that one in every 46 women in the United States will develop invasive cancers by 39 years of age,[72] demonstrating the wide applicability of a truly effective method like oocyte vitrification to preserved fertility options for these patients. In the past, embryo cryopreservation was the most reliable option available to young women facing such treatments, but many women in this position are without a partner with whom they intend to build a family. Given the proven success of oocyte vitrification, this option should be, and likely will become, a standard component of pretreatment counseling for younger women planning gonadotoxic therapies.

Additional medical indications for oocyte vitrification include a variety of genetic conditions associated with premature ovarian failure, such as Turner syndrome, fragile X syndrome, and deletions of the X chromosome. If these conditions are diagnosed at an early age, oocyte vitrification is likely to provide such women with the best chances of conceiving with their own eggs.

Other potential but as-yet unproven alternatives to mature oocyte retrieval and vitrification for medically indicated fertility preservation are currently being investigated. Collection of immature oocytes followed by *in vitro* maturation and vitrification holds potential as an option for fertility preservation without the delay required for ovarian stimulation and retrieval of mature oocytes.[73–75] This approach could be particularly advantageous when a delay in gonadotoxic therapy is contraindicated. Cryopreservation of ovarian cortical tissue or whole ovaries for later transplantation has also met with some initial success, and may eventually become a viable treatment option both for women and prepubertal girls. Preliminary experience with ovary and ovarian tissue cryopreservation suggests that viability retention may be much better with vitrification rather than slow freezing, with better morphological integrity of stroma, higher rates of oocyte survival, reduced follicular DNA damage, and increased post-transplantation activation of the resting follicle pool.[76–79]

ELECTIVE OOCYTE VITRIFICATION

The success of oocyte vitrification has led to the emergence of elective fertility preservation in anticipation of the normal age-related decline in fertility potential as an entirely new industry.[80,81] Only a short time ago, elective oocyte cryopreservation was essentially unheard of, as it was considered too expensive and unreliable to be recommended without a compelling medical reason, given the low success rates associated with the oocyte cryopreservation techniques available at the time. However, spurred by the emergence of compelling evidence demonstrating success rates with vitrified oocytes rivaling those of fresh eggs, as well as the repeal of the ASRM/SART "experimental" label, the floodgates have opened to an explosive expansion of this fledgling industry. There are well over 100 independent medical centers in the United States alone that now routinely perform elective oocyte cryopreservation as a standard clinical practice for women seeking to store a number of their eggs for the future.

The trends of delaying reproduction until a later age, particularly in more industrialized societies, and the increasingly rapid decline in female fertility potential beginning at approximately 30 years of age are well understood. These trends are largely responsible for the growing demand for and use of ART in general. However, delaying treatment until a time when reproduction is desired (e.g., when the woman is in or past her mid-30s) is likely to substantially reduce her chances of success. Therefore, with oocyte vitrification becoming a clinically viable option, this opportunity is now being offered and marketed to women who are not yet ready to start a family, but who anticipate wanting to do so in the future. For such women, oocyte vitrification enables them to collect and store what are expected to be relatively viable eggs while they are still young, for availability when they are ready to start building a family at a later age when their fertility potential is diminishing. While far from a guarantee, banking of their own eggs while young provides these women with some degree of insurance, if and when they eventually decide they are ready to have children. A survey of reproductive-aged women (21–40 years) conducted in 2010, when there was little awareness of the efficacy and possibilities of oocyte vitrification, indicated that nearly a third of the respondents might consider elective oocyte cryopreservation for themselves.[82] Since then, public awareness and acceptance of fertility preservation possibilities available through oocyte vitrification have increased considerably. Very recently, major companies including Facebook and Apple announced that they will cover the costs of elective egg cryopreservation for fertility preservation as a benefit to their employees, signaling a high acceptance level for this procedure. Given that elective oocyte cryopreservation could be much more broadly applicable to any young women contemplating delayed but future reproduction, and not just to patients currently experiencing reproductive difficulties, demand for fertility preservation procedures could conceivably eventually far surpass demand for traditional assisted reproductive practices.

OTHER APPLICATIONS OF OOCYTE VITRIFICATION

In addition to donor egg banking and fertility preservation, the ability to achieve clinical results with oocyte vitrification that are comparable to using fresh oocytes has several other clinically important applications.

Oocyte vitrification can be an invaluable option in autologous IVF cycles in which there is an unanticipated unavailability of sperm on the day of oocyte retrieval. While infrequent, there are occasions in which the male partner is unable to be present on the day of retrieval or otherwise unable to produce a usable sample. In such cases, without a viable oocyte cryopreservation program, the retrieved eggs would simply be discarded and the entire cycle wasted—a potentially devastating consequence for couples investing considerable effort and expense to overcome their infertility. With the option of oocyte vitrification, the retrieved eggs can be reliably stored for later use with little clinical consequence. Similarly, for couples in which the male suffers from severe male factor infertility, this ability to store oocytes can allow for repeated sperm recovery attempts to accumulate enough usable sperm before the eggs are warmed for use.

The success of oocyte vitrification also provides an important alternative to the insemination of entire cohorts of retrieved oocytes. Many patients have religious, moral, and/or ethical reservations (or legal restrictions, as has been the case in Italy) against creating more embryos than they will necessarily use in their infertility treatments. For these patients, oocyte vitrification may provide a more acceptable alternative while still maximizing the clinical utility of the eggs that are retrieved. Small numbers of eggs can be inseminated at a time, referred to as elective limited insemination, reducing or preventing the creation of surplus untransferred embryos that must be either cryopreserved or discarded. The remaining oocytes can be kept in storage until later use, if desired, without significant loss in viability.

VITRIFICATION OF SPERM

Compared to its considerable impact on oocyte and embryo cryopreservation, vitrification has relatively little applicability to sperm cryopreservation. Conventional slow freezing of sperm, a standard component of clinical practice for decades, has for the most part been adequate. However, sperm vitrification does present some potential benefits over conventional sperm cryopreservation protocols. Vitrification has recently been reported to be a simple and cost-effective way of storing sperm without the use of conventional cryoprotectants, while improving post-warming quality, as indicated by increased motility, higher mitochondrial membrane potential, and reduced DNA fragmentation compared to conventionally cryopreserved samples.[83,84] It has also been demonstrated that vitrification is effective at cryopreserving small numbers of sperm, which is not practical with conventional cryopreservation techniques,[85–88] an advantage that has significant clinical value for men with severe oligozoospermia and azoospermic men requiring surgical sperm retrieval.

CONCLUSIONS

The transformative impact of vitrification on the field of assisted reproduction is only just beginning to be realized. Embryo cryopreservation will continue to play an increasingly dominant role in IVF treatment cycles, eventually supplanting fresh embryo transfers entirely, due to the higher birth rates per transferred embryo that can be achieved through embryo vitrification, coupled with the healthier perinatal outcomes of those resulting children. In a continuing effort to maximize the number of children who are born per oocyte retrieved and also to maximize the health of those children, single-embryo transfer, aided by oocyte and embryo vitrification, will become the norm rather than the exception. The alluring but elusive promise of PGS, coupled with vitrification to provide ample time for the analysis and interpretation of increasingly vast quantities of accessible genetic information, may finally be realized. Vitrified donor oocyte banking will replace fresh oocyte donation, simplifying scheduling and treatment, increasing availability to more patients in need, expanding the pool of donors from which each recipient may choose, enabling more effective screening of infectious agents, and reducing treatment costs. Oocyte vitrification is also providing the first truly effective treatment for medically indicated fertility preservation, and has led to the birth of an entirely new industry of elective fertility preservation that may eventually eclipse traditional assisted reproduction in patient volume.

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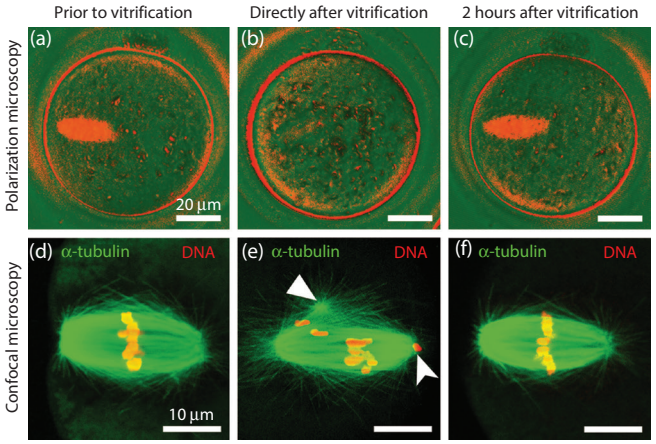

Figure 12.1 **Spindle dynamics after CryoTop vitrification of murine metaphase II (MII) oocytes analyzed by noninvasive polarization light microscopy (a–c) or by confocal laser scanning microscopy (d–f) (Trapphoff T., unpublished data). Spindle characteristics prior to vitrification (a and d), directly after vitrification (b and e), and after recovery for 2 hours at 37°C (c and f). The kind of information obtained on spindle integrity differs between polarization light and fluorescence microscopy directly after vitrification and warming (b and e). Arrowhead: slightly aberrant spindle, arrow: displaced chromosome; both phenotypes occurred in about a third of *in vivo*-ovulated and vitrified murine MII oocytes directly after warming (unpublished data).**

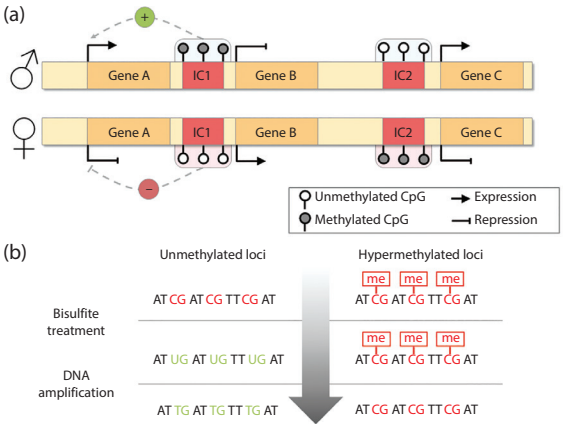

Figure 12.2 **Scheme of mono-allelic gene expression in the offspring originated from parental-specific hyper- or nonmethylation of maternal and paternal imprinting centers (a). Paternal hypermethylation of IC1 represses paternal expression of gene B and activates expression of the upstream-located gene A. Hypermethylation of maternal IC2 results in paternal expression of gene C. Bisulfite treatment induced mutagenesis of hyper- and nonmethylated DNA (b). (CpG = cytosine phosphate–guanine dinucleotide.)**

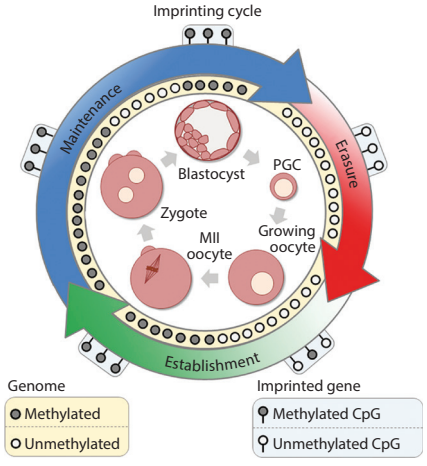

Figure 12.3 **Imprinting cycle in mice. Erasure, establishment, and maintenance of imprinted genes (outer circle) during F_0 to F_1 transition. Genome-wide demethylation (including imprinted genes) occurs in primordial germ cells (PGCs) until embryonic day 13.5. Post-natal *de novo* establishment of sex-specific methylation marks occurs during oogenesis (pre-natal in males) and maintenance of genomic imprinting occurs until the formation of PGCs in the F_1 generation. Epigenetic methylation marks of nonimprinted genes (inner circle) are also erased in PGCs, followed by methylation events during oogenesis. After fertilization, active (paternal genome) and passive (maternal genome) demethylation of both parental genomes occurs, followed by *de novo* reprogramming during embryonic development. (MII = metaphase II.)**

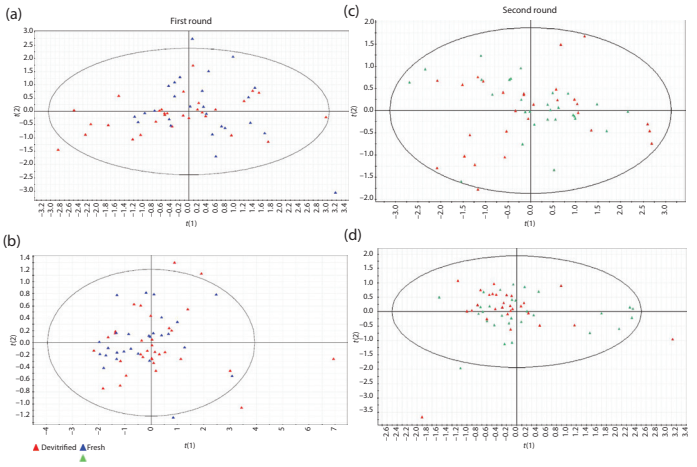

Figure 13.4 **Principal component analysis representing all of the metabolites analyzed for each sample in the two rounds. Blue/green triangles represent the fresh group, and red triangles represent the vitrification group. No separation between groups was observed using positive (a and c) or negative (b and d) sample ionization in either of the analysis rounds.**

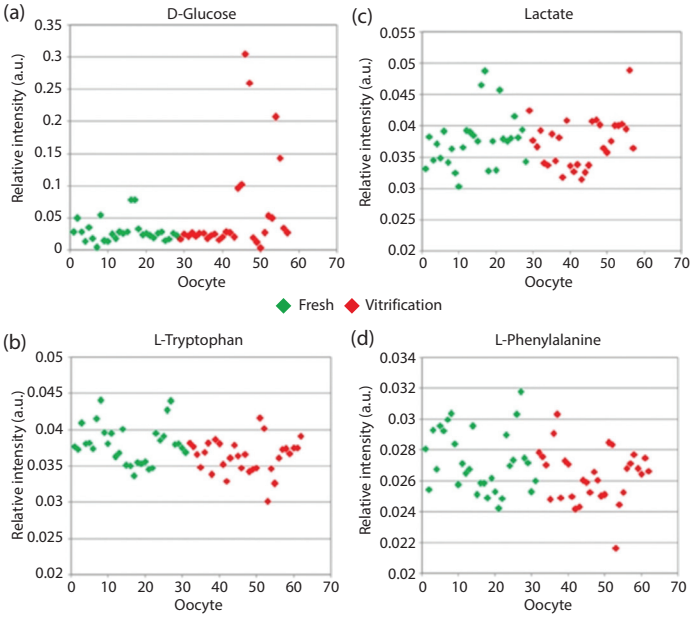

Figure 13.5 **Distribution of measurements of (a) glucose, (b) tryptophan, (c) lactate, and (d) phenylalanine in both fresh (green rhombuses) and vitrification groups (red rhombuses).**

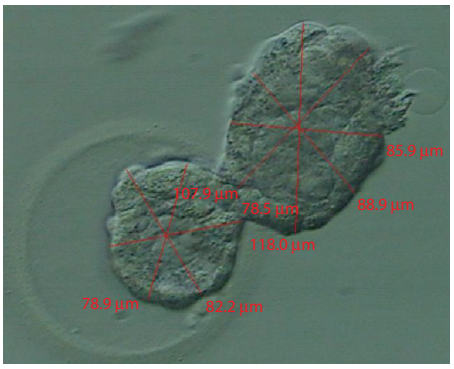

Figure 20.3 **The volume of each blastocyst was calculated based on the diameter of the embryo, or the parts of the embryo if it was in a figure 8 configuration, as shown here. The volume was recorded immediately after warming and again after 1 hour in culture.**

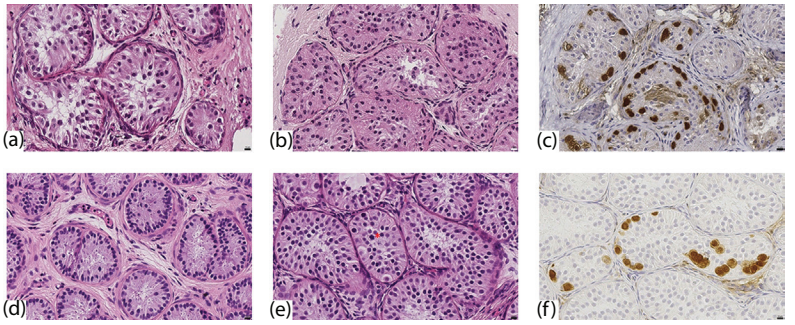

Figure 23.1 **Fresh and vitrified human immature testicular tissue grafted for 6 months to castrated nude mice. Hematoxylin–eosin staining of fresh grafted (a and d) and vitrified grafted (b and e) tissue of 2- and 9-year-old donors, respectively. Spermatogonial differentiation to the pachytene stage was achieved in vitrified grafted tissue of the 9-year-old donor (e, asterisk). Immunostaining of spermatogonia with MAGE-A4 antibody can be seen in the vitrified grafted tissue of the 2-year-old (c) and 9-year-old (f) donors. Magnification 400×. Scale bar: 10 µm.**

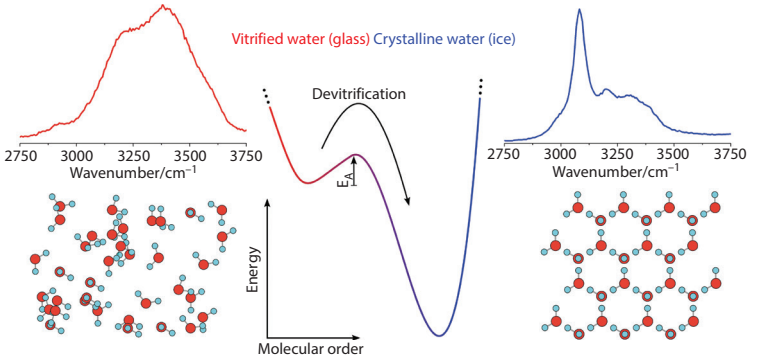

Figure 24.1 **Schematic of the devitrification process in an energy diagram, the corresponding molecular arrangements, and the Raman spectra of liquid water (15°C) and ice (–115°C). Liquid water resembles the spectral shape of vitrified water quite well. It was used here as the vitrification of macroscopic amounts of pure water is practically impossible. E_A is the activation energy for devitrification.**

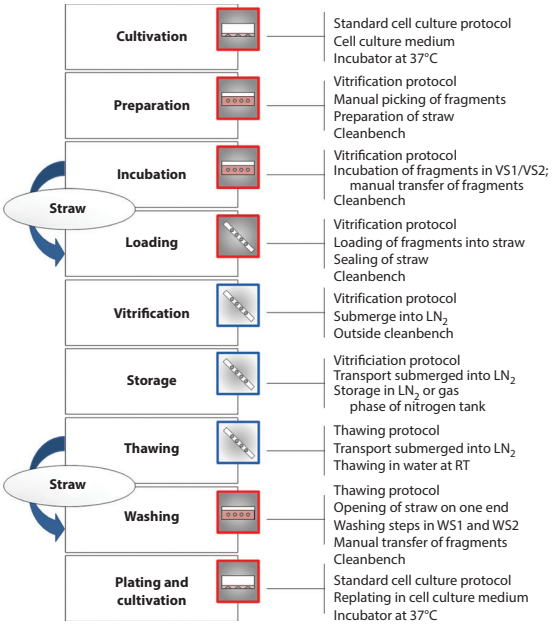

Figure 24.2 **Workflow of the open pulled straw vitrification method for human embryonic stem cells by Richards et al. (LN_2 = liquid nitrogen; VS = vitrification solution; WS = warming solution.) (From Richards M et al. *Stem Cells* 2004;22:779–89. With permission.)**

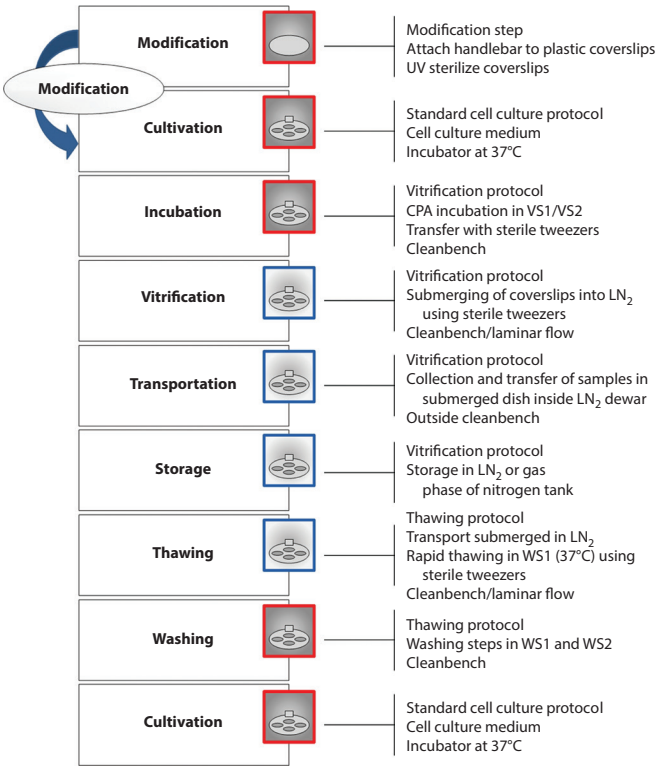

Figure 24.4 Workflow of the surface-based vitrification method for human embryonic stem cells by Beier et al. (CPA = cryoprotective agent; LN_2 = liquid nitrogen; UV = ultraviolet; VS = vitrification solution; WS = warming solution.) (From Beier AF et al. *Cryobiology* 2011;63:175–85. With permission.)

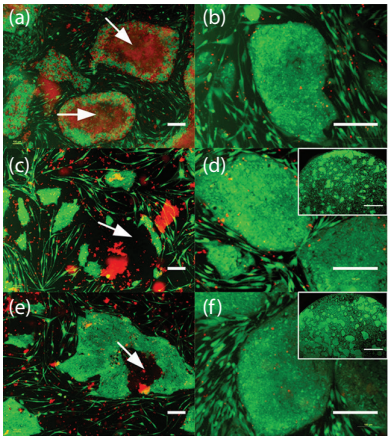

Figure 24.6 Microscopic fluorescence images of human embryonic stem cells at different time points after slow-rate freezing (a, c, and e) and adherent vitrification (b, d, and f). Cells have been stained with live/dead staining (fluorescein diacetate/ethidium bromide [FDA/EB]) directly after thawing (a and b), after 24 hours (c and d) and after 48 hours (e and f). Scale bars indicate 200 μm (a–f) and 2 mm (inserts). (From Beier AF et al. *Cryobiology* 2011;63:175–85. With permission.)

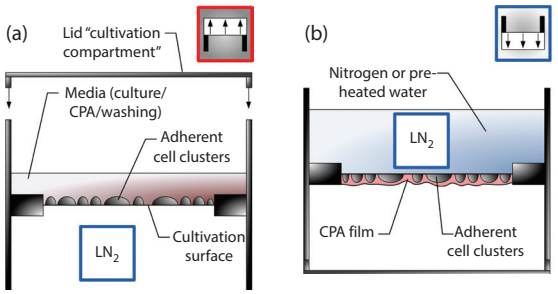

Figure 24.9 Outline and application of the TWIST substrate. The TWIST substrate is a two-chamber system with a closable, sterile cultivation chamber and a LN_2 chamber. In the cultivation chamber, cells are seeded, expanded, monitored, and prepared for cryopreservation by incubation in CPAs (a); for surface-based vitrification, the cryo-medium is removed, the substrate is turned over ("twisted"), and LN_2 is filled into the LN_2 chamber (b). (CPA = cryoprotective agent; LN_2 = liquid nitrogen.) (From Liu BL, McGrath J. *Acta Biochim Biophys Sin (Shanghai)* 2005;37:814–8. With permission.)

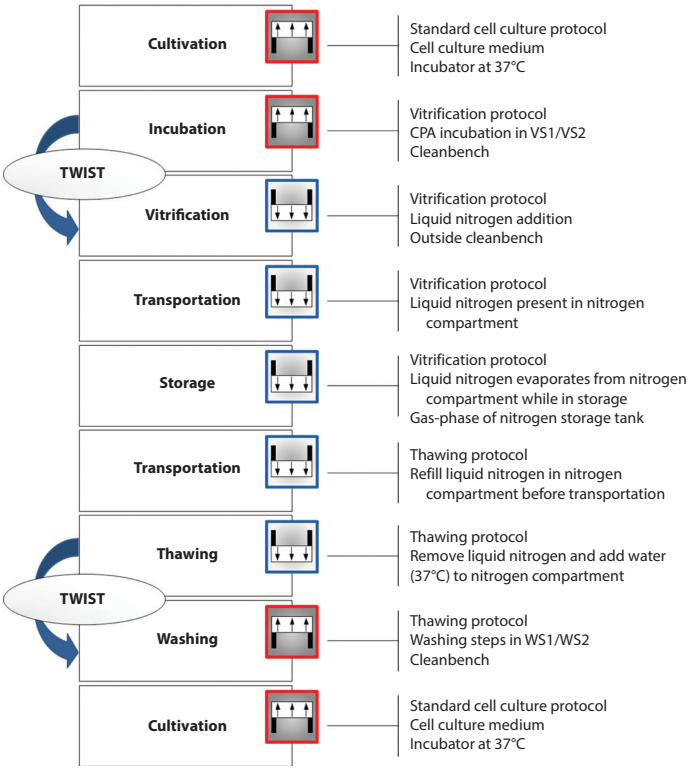

Figure 24.10 Workflow of the GMP-compliant surface-based vitrification procedure. Every step of the workflow and the corresponding positions of the TWIST substrate are summarized here. (CPA = cryoprotective agent; VS = vitrification solution; WS = warming solution.) (From Beier AF et al. *Cryobiology* 2013;66:8–16. With permission.)

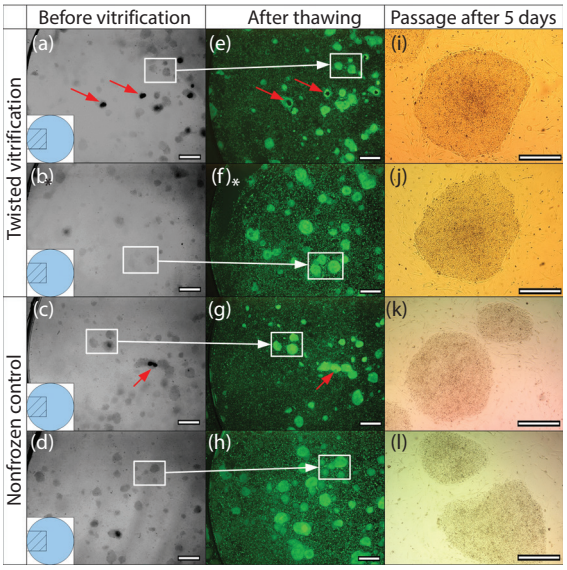

Figure 24.11 Microscopic images of human embryonic stem cell (hESC) colonies before and after vitrification using the GMP-compliant surface-based vitrification method. Pictures of the same hESC colonies have been taken before (a–d) and after vitrification (e and f), in comparison to a nonfrozen control (g and h). For determination of the viable and adherent colony area, hESC colonies have been stained with a live/dead dye (FDA/EB) directly after thawing (b) or 24 hour after thawing (f). The nonfrozen hESC colonies have been stained on the day of vitrification (g) or 24 hours later (h). Vitrified (i and j) and nonfrozen hESC colonies (k and l) have been passaged and cultivated further to determine signs of differentiation. Red arrows indicate thicker, probably differentiated areas of the hESC colonies that are lost after vitrification (e) in comparison to the nonfrozen control (g). Scale bars indicate 1 mm (a–h) and 500 µm (i–l). (From Beier AF et al. *Cryobiology* 2013;66:8–16. With permission.)

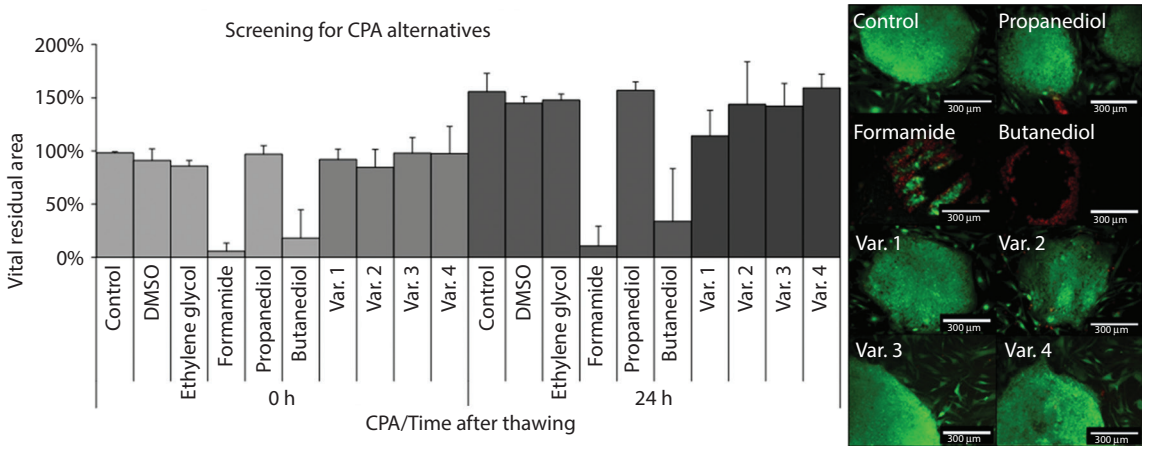

Figure 24.13 **Comparison of potential vitrification CPAs according to their effectiveness in surface-based vitrification. Concentrations of CPAs have been 40% except for in Var. 4, which shows a less-concentrated version of Var. 3. Images have been stained with EB and FDA and show the corresponding colonies directly after thawing. Var. 1–4 show different mixtures of the CPAs used. Var. 1: 10% propanediol, 10% formamide, 10% ethylene glycol, and 10% butanediol. Var. 2: 13% propanediol, 13% ethylene glycol, and 13% butanediol. Var. 3: 20% propanediol and 20% ethylene glycol. Var. 4: 20% propanediol and 15% ethylene glycol. (CPA = cryoprotective agent; DMSO = dimethyl sulfoxide; Var. = variation.)**

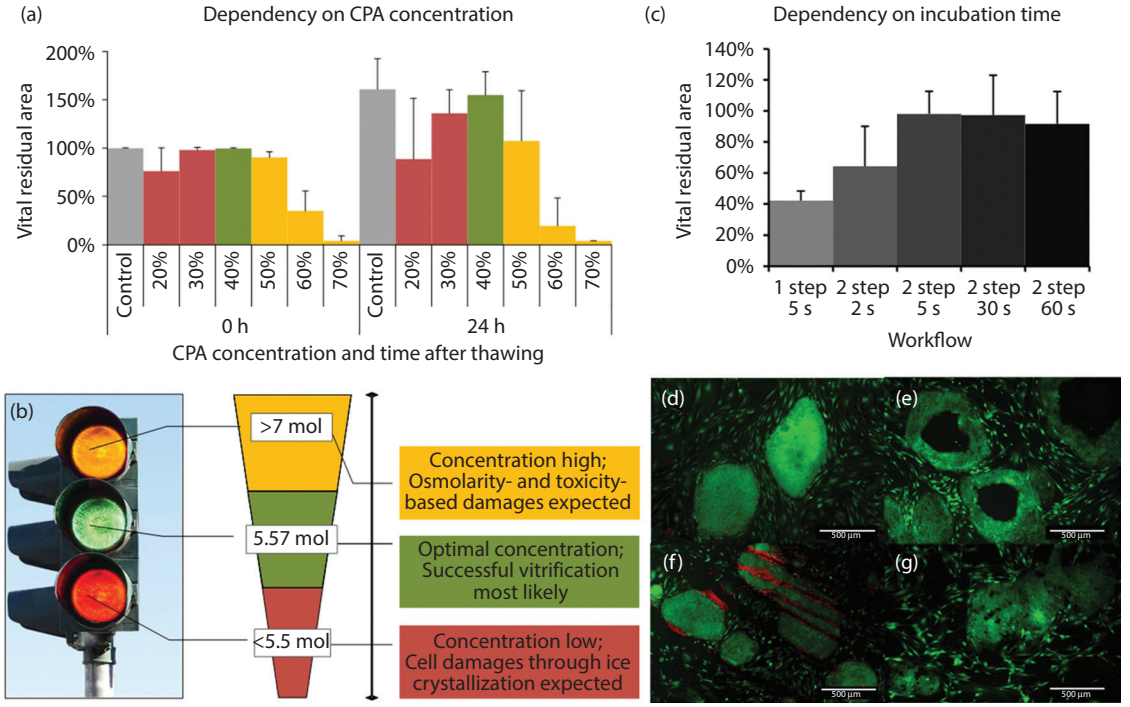

Figure 24.14 **Evaluation of optimal CPA concentration range (a and b) and incubation time (c) in surface-based vitrification. CPA concentration range experiments were done with media containing equal amounts of dimethyl sulfoxide and ethylene glycol in comparison to a nonfrozen control sample (d). The effects of too short an incubation time (e), too long an incubation time (g), and damage by inadequate surface material are shown. Images have been stained with FDA/EB. (CPA = cryoprotective agent.)**

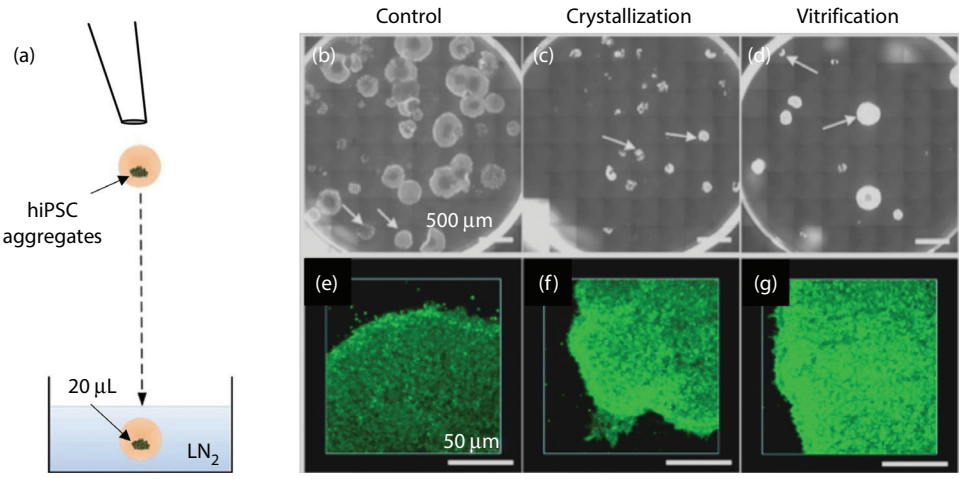

Figure 24.15 **Cryopreservation of hiPSC aggregates using slow-rate protocols and pellet vitrification. For pellet vitrification, 20-µL drops of vitrification medium containing one hiPSC aggregate each were dropped into LN_2 (a). Adhesion of aggregates was monitored after slow-rate freezing (c) and pellet vitrification (d), in comparison to a nonfrozen control (b). Stemness was analyzed with immunofluorescence staining using anti-HESCA-2 antibody with Alexa-Fluor-488 after cultivation for 7 days (e–g). (hiPSC = human induced pluripotent stem cell; LN_2 = liquid nitrogen.)**

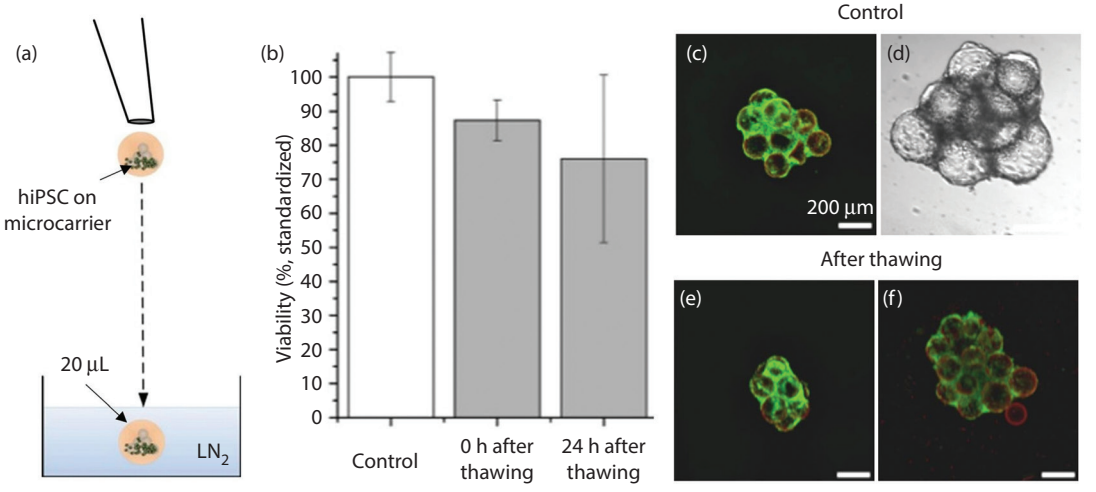

Figure 24.16 **Cryopreservation of hiPSC on micro-carriers using pellet vitrification. For pellet vitrification, 20-µL drops of vitrification medium containing hiPSCs adherent on micro-carriers were dropped into LN_2 (a). Viability of vitrified cells was analyzed using live/dead staining (FDA/EB) directly after thawing and 24 hours later (b). Stemness of a nonfrozen control (c and d) directly after thawing (e) and 24 hours later (f) was determined with immunofluorescence staining using anti-HESCA-2 antibody with Alexa-Fluor-488. (hiPSC = human induced pluripotent stem cell; LN_2 = liquid nitrogen.)**

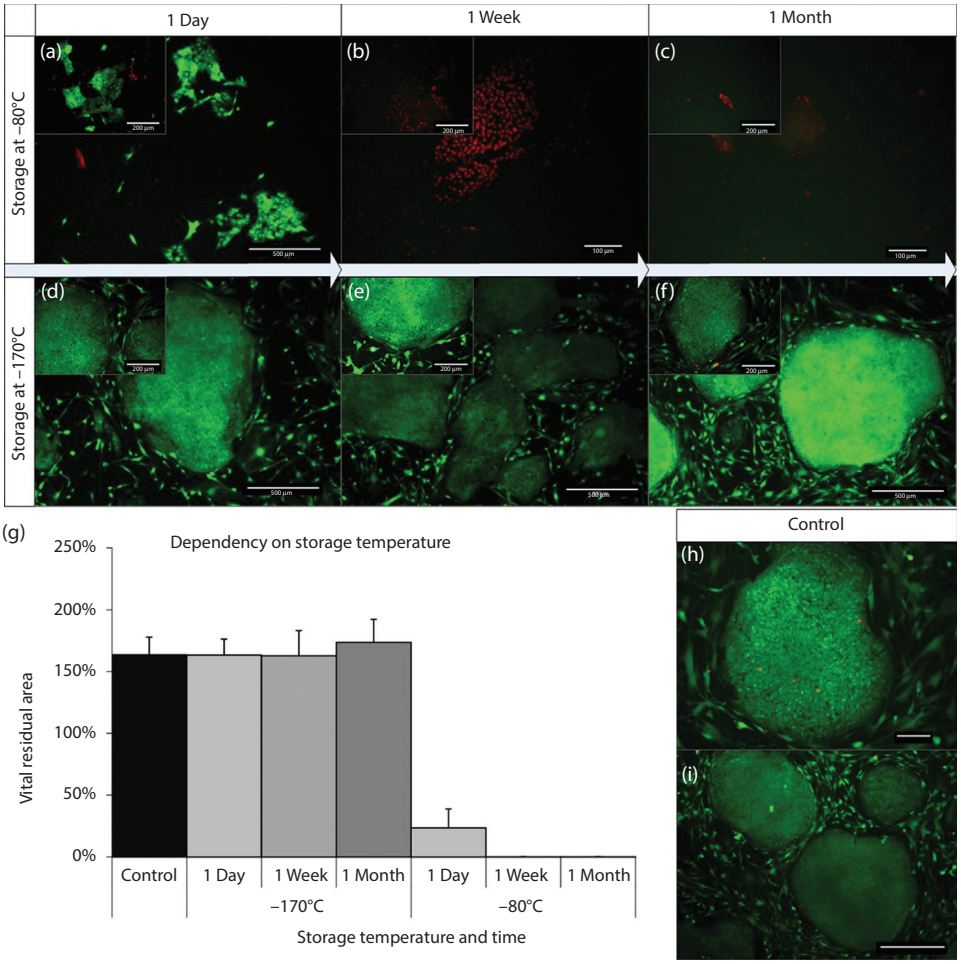

Figure 24.18 **Comparison of different storage temperatures for vitrified human embryonic stem cell colonies. Cells have been vitrified via surface-based vitrification and stored at –80°C (a–c) and –170°C (d–f). Samples were thawed after 1 day (a and d), 1 week (b and e), and 1 month (c and f). Evaluation was done after a 24-hour recovery period via staining with FDA/EB and comparison of the vital residual areas (g). A nonfrozen control sample has been included (h and i).**

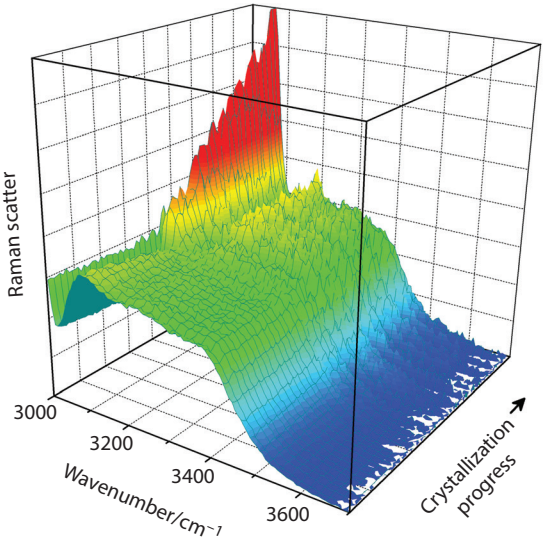

Figure 24.22 **Waterfall plot of a Raman spectral sequence showing ice formation from a vitrified medium. The medium composition was 300 mM sucrose solution with 20% dimethyl sulfoxide and 20% ethylene glycol. The medium was vitrified at a temperature of –135°C, then heated to –95°C at a rate of 1 K/min.**

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