10. Human embryo cryopreservation and its effects on embryo morphology

James J Stachecki and Klaus Wiemer

CRYOPRESERVATION AND ITS EFFECTS ON EMBRYO MORPHOLOGY

Cryopreservation may have a variety of effects on embryo morphology, ranging from subtle damage on intracellular organelles, cytoplasm, or processes that can negatively affect normal cell development, to overtly blatant effects such as the lysis of one or more blastomeres. Many underlying factors can influence the outcome of cryopreservation, e.g. the quality of the oocyte or embryo itself will often determine the effects of the process on subsequent morphology. The type of freezing protocol used (slow-cooling or vitrification) and the concentration and type of cryoprotectant(s) will also have an effect on subsequent morphology. The goal of this chapter is to offer an insight into the question: how does cryopreservation affect subsequent embryo morphology? We address this question in three major sections: background, types of cryopreservation damage, and causes of damage.

BACKGROUND

Cells have been successfully cryopreserved for over 100 years. Glycerol was discovered to have protective effects on sperm cells in 1949, and since that time both gametes and embryos have been successfully frozen from a number of mammalian species including mice, sheep, cows, pigs, horses, hamsters, rats, cats, and humans. The foundation of modern cryobiology was established throughout the 1940s, 1950s, and 1960s by Lovelock, Meryman, Polge, Smith, Levitt, Luyet, Mazur, and others.[1–4] Further advances in animal oocyte and embryo cryopreservation were achieved during the 1970s by Whittingham, Willadsen, Leibo, Mazur, and Wilmut,[5–7] and these prepared the way for human oocyte and embryo storage in the 1980s. Protocols for human embryo storage were refined during the subsequent years up to 2000, so that they have now become a routine procedure in IVF clinics throughout the world. Cryopreservation exposes embryos and oocytes to numerous types of stress, and the fact that these cells can survive and go on to form a viable fetus after freezing and thawing is truly remarkable. However, freeze–thawing protocols are not perfect, and many cells do not survive and/or go on to develop normally.

CONSEQUENCES OF CRYOPRESERVATION-INDUCED DAMAGE

Damage can occur at any time throughout the cryopreservation process, and may be manifested in different ways and at different times. Extreme types of damage such as intracellular ice formation (IIF) and cell fracture will lead to immediate cell lysis and death, and these are easily documented through routine microscopic observation of morphology. Damage can also occur on a cellular structural/functional level, involving intracellular organelles, and this is more difficult to diagnose.[8] There is very little ultrastructural data documenting morphological damage in cryopreserved oocytes and embryos. However, differences in appearance between fresh and cryopreserved embryos are sometimes mentioned in the discussion section of manuscripts that focus on various freezing techniques. From these studies we can gather at least some information about the effect of cryopreservation on embryo morphology. However, reported differences in morphology postcryopreservation may not be universal, and are likely to be due to the specific protocol and overall set of circumstances used in the study. Cryopreservation procedures may not inevitably result in specific observable morphological aberrations, but the embryo might still be

affected. In other words, the fact that one investigator using propanediol finds 80% embryo mortality, does not necessarily mean that propanediol is a toxic or inferior cryoprotectant. Likewise, if the same investigator shows 95% embryo survival with dimethylsulfoxide (DMSO), this does not mean that DMSO is the best or the safest cryoprotectant to use. Ultimately, regardless of morphological appearance, the most important feature is that an embryo should remain viable after cryopreservation.

CRYOPRESERVATION THEORY

A basic understanding of the cryopreservation procedure is important in order to better comprehend common morphological characteristics observed following cryopreservation. The first theoretical basis for cryopreservation of cells was proposed by Mazur[3] and later applied to mouse embryos[5,9,10] and other species including cows and sheep.[7,11]
Conventional cryopreservation methods consist of several steps:

(1) Pre-equilibration: embryos are exposed to a simple salt solution containing a permeable cryoprotectant (1,2-propanediol, DMSO, glycerol, ethylene glycol, etc.) and usually a low concentration of non-permeable cryoprotectant (sucrose).
(2) Cooling: after a brief time of exposure to allow uptake of cryoprotectant and initial dehydration, the cells are cooled rapidly to a temperature slightly below the melting point of the solution (usually around −7°C).
(3) Seeding: at this point the container with the cells is super-cooled in a process known as 'seeding' so that ice forms in the extracellular solution.
(4) Slow cooling: upon ice formation and further cooling at a slow rate (usually <1°C/min to below −30°C), the osmolarity of the extracellular solution increases as water freezes to ice, causing the cells to dehydrate with the increasing tonicity.
(5) Plunging/vitrification: dehydration continues during slow-cooling until the cells are plunged into liquid nitrogen, usually at a temperature below −30°C. At this point the intracellular cryoprotectant concentration is high enough so that the remaining intracellular water will vitrify, preventing IIF.
(6) Thawing and rehydration: during thawing, the dehydrated cells are exposed to hypotonic conditions and rehydrate as the cryoprotectant is removed.[12]

TYPES OF DAMAGE

INTRACELLULAR COMPONENTS

The effects of cryopreservation are not always evident; embryos may be adversely affected by cryopreservation at the intracellular level, with the potential of altering the function of the intracellular organelles and cytoplasm. Protein structure and function, as well as metabolism can also be affected. It is likely that embryos require a period of 'recovery' following cryopreservation, before they are able to continue normal intracellular function. Therefore, those embryos that are able to compensate for lost or decreased function are the ones most likely subsequently implant to and develop further. We have noted that human embryos following thawing often begin to develop initially slightly slower than their fresh in vitro counterparts. Following extended culture, frozen embryos may resume normal rates of development prior to transfer.

EMBRYO ORIGIN

The origin of the embryo itself may have a profound impact on the survival of embryos following cryopreservation. In cattle, we have noted that in vivo oocytes have a large perivitelline space; cleaved embryos have uniform blastomeres, and compaction at the morula stage is not irregular. In most cases, expanded blastocysts contain distinct inner cell masses that are not dark in appearance. In contrast, in vitro 1-cell embryos have a very small perivitelline space, blastomeres are irregular in early cleavage-stage embryos, and morulae tend to exhibit irregular patterns of compaction. Resulting blastocysts retain a dark appearance and a slightly more irregular shape.

Evidence suggests that in vitro developed bovine embryos may be more sensitive (to cryopreservation) than their in vivo counter parts.[13,14] A reduced implantation rate for in vitro produced blastocysts following cryopreservation was noted. Reduced survival and pregnancy rates after transfer of in vitro produced bovine embryos when compared with in vivo embryos has also been reported by others.[15,16] The reason for this is unclear, other than the fact that in vivo produced embryos are inherently different to in vitro embryos. Some of the inherent differences may be due to suboptimal maturation and culture systems, inability to adapt cryopreservation methodologies to suit the kinetics of in vitro embryos, and inabilities to manipulate energy substrates or biochemical pathways following thawing.

INTRACELLULAR LIPIDS

It is well known that embryos from certain species including goat, pig, and cow are substantially more sensitive to injury from a reduction in temperature than are mouse or human embryos. This chill sensitivity or direct chilling injury is defined as an irreversible damage from exposure to low temperatures. Martino et al[17] describe the effect of embryo stage, cooling temperature, and cooling duration on subsequent fertilization and development in vitro of bovine oocytes. Leibo and Loskutoff[18] noted that in vitro and in vivo cattle embryos have different buoyant densities. In vivo embryos will sink in 2.35 M sucrose solutions, while their in vitro counterparts will float in solutions containing more than 1.6 M sucrose. This may be due to altered ratios of lipids to proteins found in cell membranes. In vitro produced bovine embryos have a higher lipid content, making them less buoyant, and this lower buoyancy renders the embryo more sensitive to chilling and freezing when compared with its in vivo counter part. Horvath and Seidel[19] pointed out that membranes with higher cholesterol concentrations are more fluid at lower temperatures, and are thus more chill-resistant. They demonstrated this effect by loading cumulus–oocyte complexes with cholesterol-loaded methyl-beta-cyclodextrin; these cholesterol-loaded oocytes showed a higher survival after vitrification than did the non-loaded controls. However, no data on further development and birth were given. Arav et al[20] also showed that there is a difference in lipid phase transition, and the temperature at which this phase transition took place was directly related to the effect of temperature on the plasma membrane. Phase transition is simply the progression from one phase or physical state to another. For example from liquid to solid or from liquid to gas. During cooling, 'liquid' water will progress to 'solid' ice at a warmer temperature than would oil or lipids, or the cryoprotectants propanediol and DMSO. Differences in lipid phase transition were observed between in vivo and in vitro matured oocytes. This suggests that alteration of membrane composition affects chill sensitivity, and subsequently survival following freezing. Nagashima et al.,[21] showed that chilling sensitivity of porcine embryos was directly related to their lipid content. Embryos that were partially or fully delipidated survived cooling better than control non-delipidated embryos, and delipidated non-cooled embryos did not differ in their development compared with control embryos. In a more recent manuscript, Beeb et al.,[22] noticed that early stage porcine blastocysts could survive vitrification and produce piglets following centrifugation of lipids within the embryos prior to cooling. This was the first report of piglets from frozen early-stage blastocysts with lipid removal. It may be possible that the variation found in in vitro human embryos is in part due to the amount and distribution of lipids found in the cell membranes. In accordance with this hypothesis, Ghetler et al[23] showed that human zygotes had a higher resistance to chilling injury compared with oocytes of different stages of maturation. Since many species that have been studied to date exhibit variable, and more specifically, higher lipid content in the cell membranes of in vitro derived embryos compared to their in vivo counterparts, it is reasonable to suppose that human in vitro derived embryos may also exhibit this variation.

INTRA- AND INTERSPECIES DIFFERENCES

The effects of cryopreservation on intracellular organelles may not be limited to in vitro produced embryos.

A recent study suggests that high quality cattle embryos from different breeds appeared morphologically similar to their non-frozen counterparts.[24] The type of freezing protocol used did have a slight effect on cellular appearance within the embryo itself. At the intracellular level, embryos from two types of cattle exhibited minor cytoplasmic injuries as well as vacuolization of the nucleus. These types of findings are similar to those found in cells that have been subjected to hyperosmotic stress. The authors also noted that embryos from Bos tarus had higher amounts of intracellular lipids than embryos from Bos indicus, and notably embryos from Bos tarus had a slightly better morphological appearance than their Bos indicus counterparts.

Likewise, Dinnyes et al[25] showed that embryos from different strains of mice treated with the same cryopreservation protocol responded differently, with variable survival and development rates. The in vivo development of cryopreserved embryos was also influenced by genotype and cryopreservation method (slow-cooling vs vitrification). Although genotype did not affect the ability of embryos to develop (all the embryos could develop), the differences in overall embryo development after cryopreservation were related to the genotype and not the sensitivity to cryo injury. In other words, differences in the developmental ability of embryos of different genotypes became apparent only after cryopreservation. Furthermore, variability often noted within human embryos from different patients may be due to the genetic background of the patient population. The 'freezablity' of human embryos from different genetic backgrounds has never been evaluated.

CORTICAL GRANULES

Cortical granule release occurs naturally during egg activation and/or fertilization. The release of cortical granules leads to zona hardening, as a natural block to polyspermy. Zona hardening due to both cortical granule release and culture in vitro, may impair the embryo's ability to hatch during the time of implantation. Cryopreservation may also contribute to zona hardening over and above what would occur naturally, and this may affect blastocyst hatching.[26] Likewise, oocyte freezing may cause premature hardening of the zona, an effect possibly related to premature cortical granule release as observed in several species.[27–32] Vincent et al[27] showed that exposure to DMSO caused a reduction in cortical granules in mouse oocytes, and Ghetler et al[32] similarly found that exposure to propanediol causes cortical granule release from human oocytes, observing a significant reduction of cortical granules in electron micrographs of frozen–thawed oocytes. However, Wood et al[29] discovered that under certain conditions, cortical granule release could be avoided during cryopreservation, and that other alterations in the zona pellucida were to blame for reduced fertilization rates of cryopreserved mouse oocytes. Whatever changes occur to modify the zona following cryopreservation, this can be overcome by choosing either artificial zona opening or ICSI as the method of choice for insemination of cryopreserved oocytes.[33–37]

MEIOTIC SPINDLE

Detailed morphological analysis of the nucleus and/or spindle (oocytes) requires the use of a PolScope, immunohistochemistry, FISH, or other specialized technique. Spindle re-formation and function play an important role in the further development of the oocyte following cryopreservation, and numerous studies on the effect of freezing on spindle morphology have been reported (for reviews see references 38 and 39). Improper chromosome segregation could lead to aneuploidy and genetic errors, which may result in embryonic and fetal abnormalities. With the use of numerous different cryopreservation protocols, a wide variety of outcomes have been reported regarding spindle morphology, as well as actin and microfilament disruption postcryopreservation. These range from almost 0% to nearly 100% re-formation and normal spindle morphology.[8,40] This range of results makes it difficult to interpret the true situation with regards to potential spindle damage after cryopreservation. In a recent report by Stachecki et al[38] a more global view of spindle disassembly and reassembly was presented by investigating the effects of cryopreservation on spindle morphology in three evolutionarily distinct mammalian species: mouse,

bovine, and human. Their observations suggest that other results may be misleading, due to the fact that the majority of the investigators report only 35–65% oocyte survival after thawing. Therefore, if the freezing technique were sufficiently stressful to kill half of the eggs, it is likely that the remaining oocytes might not have normal function, and this may be manifested in poor or improper spindle re-formation. Stachecki et al[38] further demonstrated that their cryopreservation technique yields significantly higher survival rates with oocytes from all three species after freeze–thawing, in the range of 80–100%. When returned to a physiological temperature (37–39°C) for 30–90 minutes, nearly all of the oocytes were able to reassemble their spindle, and the majority (≥70%) had a normal barrel-shaped spindle with chromosomes aligned. Results for frozen–thawed oocytes were statistically similar to non-frozen control oocytes. It is important to bear in mind that the appearance of the spindle does not necessarily correlate with its function: a spindle that does not have a perfect appearance may nonetheless have normal function, and vice versa. Therefore, spindle function needs to be further investigated by analyzing second polar bodies and/or the genetic makeup of embryos that result from frozen eggs. Interestingly, there has been very little attention to spindle function associated with embryo freezing. Although most blastomeres are in interphase and a spindle is not present during cryopreservation, a spindle still has to form in order for the cell to progress through mitosis, and freeze–thawing could have detrimental effects on this process.

EMBRYO ULTRASTRUCTURE

Cryopreservation can have effects on the zona pellucida and cell membrane, in addition to intracellular components. For example, the zona can be damaged by cracking, splitting, elongation, and distortion, caused by different stresses during freezing and/or thawing. However, the zona is not always damaged during cryopreservation, or it may be damaged to differing degrees, some of which may not be microscopically apparent.

Anyone who has dropped an ice cube into a glass of warm water, soda, or similar solution has seen and heard it crack. This is caused by the rapid thermal expansion that occurs within the ice cube when it is removed from the freezer, around −20°C, and placed into a substantially warmer environment, such as a 23°C liquid, a temperature difference of only 43°C. This amount of heat exchange is enough to fracture an ice cube, and yet when a straw or vial is removed from liquid nitrogen at −196°C and placed in room temperature air (or water) the temperature difference is over 200°C. This dramatic change can and does, at least in some cases, cause fracture damage to the contents of the straw or vial, most often reported as zona cracking. Rall and Meyer[41] specifically studied the mechanism behind such injury. They compared thawing rates and surmized that their observations were consistent with the view that zona damage is directly associated with thermal-induced fracturing of the cryoprotectant suspension during rapid changes of temperature that occur during thawing. Stachecki et al[42,43] specifically studied thawing rates of mouse oocytes and observed similar evidence of zona cracking from thawing too rapidly. They also reported significant changes in survival and development with different thawing regimens.

Moreira da Silva and Metelo[44] recently described the effects of cryopreservation methods on the physical properties of the zonae pellucidae of in vitro produced bovine embryos, in order to explain the loss of embryo developmental capacity following freezing and thawing. These authors noted that pore size in the zona was correlated with viability following cryopreservation. When bovine embryos were frozen either by slow methods or by vitrification, pore size and subsequent viability was most affected by vitrification. Pore size was smallest (0.27 μm) after vitrification, compared with 0.34 μm for slow cooled and 0.48 μm for the control embryos, respectively. Of interest, the survival rate of embryos following vitrification was lower; indicating that pore size within the zona could be related to viability. Results indicate alterations in the zona might be caused by the steps of cryopreservation process itself which may be responsible for irreversible damage on subsequent development of bovine embryos.

In many cases, the effects of cryopreservation are not overtly evident. The embryo may appear to

be microscopically normal following the thawing process, when in fact minute changes have occurred. It is possible that freeze/thawing can induce changes that negatively affect cell membranes and organelles. In the case of frozen–thawed blastocysts, distinctive cell types within the embryos themselves may be affected differently by this process. Detailed effects of these processes on embryos can be assessed by ultrastructure studies, and these have noted that in vitro produced bovine embryos have a higher proportion of degraded trophectoderm and dead inner cell mass (ICM) cells following thawing, when slow freezing methods were used.[45] The methodologies used to freeze these embryos greatly affected the proportion of embryos with dead cells within the ICM. Embryos frozen after introducing the cryoprotectant in one step had a higher proportion of necrotic cells within the ICM than did blastocysts frozen after introducing the cryoprotectant in three steps. These authors also noted that the basic salt solutions used for cryopreservation influenced the proportion of necrotic cells within the ICM, and that necrotic cells were extruded from the trophectoderm junction when the embryos were cultured prior to fixation. Morulae that were frozen, thawed, and cultured prior to fixation often produced blastocysts that were delayed in development, with reduced cell numbers within the trophectoderm as well as the ICM.

The ultrastructural effects of cryopreservation on equine embryos have also been studied.[46] Blastocysts were non-surgically recovered from mares, and some were frozen using a medium containing 10% glycerol with a slow freezing protocol. Embryos were thawed and fixed for subsequent evaluation. Wilson et al[46] observed that embryos that were exposed to glycerol only (without freezing) had changes in lipid droplets within the ICM and changes in the appearance of mitochondria. Embryos that were cryopreserved prior to fixation also had structural changes that were associated with the cryoprotectant used, as well as the freezing procedure itself. Specifically, the mitochondria associated with the ICM and trophoblast were affected, and the greatest damage was associated with the cells within the ICM. These data suggest that glycerol did not sufficiently permeate the embryo prior to freezing. In other words, proper dehydration may have not occurred, allowing ice crystals to form. This would be particularly true for cells within the ICM, since the reduced permeation of glycerol through the embryo would be most evident within the ICM. This might be due to the presence of junctional processes between the trophoblast cells that could reduce the permeation of cryoprotectants into the ICM. For the cryoprotectant to come in contact with the ICM, it must first pass through the trophoblast cells that might limit permeation because of the junctional processes or some other physical (cell or membrane) barrier.

Ultrastructural analyses of in vitro cultured human blastocysts also revealed structural damage.[47] This study revealed that tight junctions could not be detected following cryopreservation and thawing of expanded blastocysts, although cells remained in close apposition; however, tight junctions were detected in the cryopreserved specimens. The collapse of the cavity during the freeze–thaw process may have caused the disruption of the apically located zona occludentes. More recently, Escriba et al[48] detected no ultrastructural changes following cryopreservation of human blastocysts. These authors noted that epithelial junctions formed by apical tight junctions and basal desmosomes remained intact and polarized. Cells within the ICM retained their shape and intercellular junctions. These data differ from those previously reported by Wiemer et al[47] using a different freezing protocol. Escriba et al[48] used vitrification techniques, whereas the embryos frozen by Wiemer et al[47] were frozen using a slow freezing technique. It seems that vitrification may be the best method for freezing human embryos. More recently Wiemer et al (unpublished data) used an optimized vitrification method known as S^3-vitrification, with an improvement in results. With donated or spare material, survival rates of blastocysts have been in excess of 90%. In addition, we have achieved a pregnancy rate in excess of 50% in a small group of patients.

Nottola et al (personal communication) analyzed fresh and frozen thawed human oocytes using light microscopy and transmission electron microscopy (TEM). Oocytes were frozen with a conventional slow cooling protocol using 0.1 mol/l or 0.3 mol/l

sucrose, and oocytes had fairly good preservation and normal organization of the cytoplasm following freezing. There was a reduced amount and density of cortical granules in some of the frozen cells. They also found slight to moderate vacuolization, specifically in the group with 0.3 mol/l sucrose. The spindles in the oocytes were morphologically normal after thawing, and a normal complement of mitochondrial aggregates was found in both fresh and frozen oocytes. It is difficult to determine all of the effects of freezing, as only the oocytes that survived were analyzed; however, the authors suggest that the use of high sucrose concentrations in freezing protocols should be approached with care until more information is known about its effects on embryo development.

EMBRYO QUALITY

The initial quality of embryos prior to cryopreservation is also a determining factor in their resistance to the freezing process. In a recent study, cattle embryos of differing quality were cryopreserved, thawed, and evaluated at the ultrastructural level.[49] Apoptosis was assessed using terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) for embryos of varying quality prior to cryopreservation, and both electron microscopy and TUNEL analysis showed that the number of lyzed cells and apoptotic cells increased following cryopreservation. The degree of apoptosis was directly related to the initial morphology of the embryo prior to freezing: embryos of good morphology had significantly lower degrees of apoptosis than embryos of poor morphology. In higher quality blastocysts, apoptotic cells were more prevalent within the ICM, whereas in blastocysts of lesser quality, apoptotic cells were randomly distributed. The data suggest that embryos of higher quality (based on their appearance) have better resistance to the damage that is often associated with cryopreservation. Of interest, seasonal effects were also noted in this study. Embryos collected during the seasonally stressful time, when natural forage is less abundant, had a higher proportion of lyzed cells following cryopreservation than embryos produced during more ideal conditions.

As previously mentioned, embryos that are derived from IVF might be more susceptible to effects induced by cryopreservation. Similar to data found with cattle embryos, the quality of embryos prior to cryopreservation has a significant effect on the morphology following thawing. Both cattle and human embryos are affected by the osmotic effects associated with the introduction and removal of cryoprotectants, and tight and gap junctions were disrupted in human embryos.[47] In cattle, there was evidence of vacuolization of the nucleus,[24] apoptotic cells were present within the inner cell mass of high quality blastocysts, or throughout the cells of lower quality cattle embryos.[49] In some cases, IVF centers prefer to freeze cleavage stage embryos at the 8-cell stage, with a rationale that their extended culture conditions may not be optimal for blastocyst culture, or their experience with blastocyst cryopreservation is less than optimal. Freezing embryos at the 8-cell stage (day 3) allows high quality embryos to be frozen immediately following the embryo transfer procedure. The advantage of this practice is that no further culture is required. This practice works well in order to reduce the work load in the IVF laboratory as well as laboratories that have poor blastocyst development rates. Freezing 8-cell embryos often maximizes the chance of pregnancy from a single stimulated cycle, after frozen embryo transfer. In general, only embryos of the highest quality are frozen, due to the stresses involved with cryopreservation, which can further reduce an embryo's chance of full development. Additionally, embryos of suboptimal morphological quality sometimes, but not always, have poorer survival rates and a significantly higher proportion of lyzed blastomeres. The transfer of embryos with lyzed/degenerate blastomeres is associated with lower pregnancy and implantation rates.[50] The same authors noted that the birth rate was three times higher after the transfer of fully intact embryos, when compared with the transfer of damaged embryos. No attempt to remove any damaged cells was carried out in this study.

The reason for blastomere lysis in high quality embryos undergoing cryopreservation is unclear. It is possible that these cells lyze due to the presence of intracellular ice caused by incomplete dehydration

of the cell, reduced permeability, membrane weakness, or inappropriate thawing conditions. The potentially deleterious effect of contraction and re-expansion of the cells during dehydration and rehydration processes may also be a possible cause. Furthermore, the mechanism whereby embryos become compromised by the presence of lyzed cells is not clear, but it is possible that the lyzed and/or damaged cell might create a locally 'toxic' condition that could affect subsequent development.

Cohen et al[51,52] developed the assisted hatching technique in order to bypass potential implantation failure associated with impaired embryo hatching, and this also allows access to necrotic cells or fragments, which can be removed via the breach created in the zona pellucida. Assisted hatching has been most advantageous for patients that have a history of producing suboptimal embryos or those with thickened zonae,[53–55] and it has also been applied to thawed cleavage stage embryos. Following assisted hatching and removal of necrotic cells, pregnancy rates increased from 17% to 45.7% compared with embryos that did not have necrotic cells removed.[56] Other studies have also noted that the removal of lyzed cells following assisted hatching improved implantation and pregnancy rates.[57,58]

Based upon personal experience as well as the current literature, it seems that removal of lyzed blastomeres in freeze–thawed embryos after assisted hatching may be advantageous, both in alleviating impaired hatching due to potential zona hardening, and in order to eliminate potentially toxic effects of necrotic products.

CELLULAR FUNCTION

The effects of cryopreservation on cellular metabolism are difficult to assess morphologically, but this important aspect must also be considered. Disruption of mitochondrial membranes leads to loss of protons and reduces the oxidative potential for ATP production. Rieger et al[59,60] noted that cryopreservation of horse and cattle embryos caused an increase in glutamine production, possibly due to a disruption in mitochondrial ATP production and therefore a flux in the Krebs cycle. Gardner et al[61] also noted that the process of freezing and thawing on IVF-produced bovine blastocysts had a significant effect on nutrient uptake and utilization. The freeze–thaw process had a negative impact on the rate of glucose and pyruvate uptake as well as lactate production, and the viable embryos did not recover the metabolic activity that was recorded prior to freezing. Gardner et al[61] noted that damage to the mitochondria may have resulted in changes in oxidative phosphorylation, thus increasing oxygen consumption.

The review of intracellular effects of cryopreservation by Smith and Silva[8] mentions that cryopreservation may alter the nuclear envelope, causing downstream disruption in replication and/or transcription. Van Blerkom[62] showed that although germinal vesicle (GV)-stage mouse oocytes survived vitrification and were capable of resuming meiosis and undergoing normal chromosomal and cytoplasmic maturation to metaphase II, profound alterations in the structure and organization of the cytoplasm, nucleus, nucleolus, and chromatin occurred during the dehydration stage. The majority of cytoplasmic and nuclear perturbations returned to normal post-thawing, but the potential for adverse development after fertilization remains. Enzymatic regulation and protein structure/function before and after cryopreservation have not yet been assessed.

CAUSES OF DAMAGE

It is not always easy to determine the exact causes of damage that can occur throughout the cryopreservation process, since the dysmorphism(s) induced can have several origins. The problems that can occur during cryopreservation differ in association with slow cooling and with vitrification. Problems associated with slow cooling that have been described include IIF and osmotic effects, whereas chemical toxicity is a major obstacle with current vitrification techniques. However, this interpretation may not be entirely correct, because it does not encompass sodium-loading, other ion effects, and the possibility of problems as yet undiscovered.

Damage can occur at any step in the cryopreservation process, and the variety of aberrations that

can occur is dependent upon the protocol used for cryopreservation. Some protocols and media result in less damage, and close examination of each step in the procedure can give an indication of the common types of damage that can occur.

Two types of cryoprotectants are commonly used to preserve human oocytes and embryos: permeable and impermeable. Permeable cryoprotectants include propanediol, glycerol, DMSO, and ethylene glycol. Sucrose and trehalose are impermeable. All of these cryoprotectants cause the oocyte/embryo to undergo changes in osmolarity. The subsequent contraction and expansion known collectively as solution effects might be partially responsible for some of the changes commonly found in embryos following cryopreservation. During pre-equilibration, the cell is exposed to a hyperosmotic environment that allows some dehydration, along with uptake of permeable cryoprotectant(s). If the dehydration is too severe, membrane damage can occur, sometimes resulting in blebbing of the membrane. The cryoprotectant can be toxic to the cell if the concentration is too high. The toxicity of cryoprotectants is also related to the temperature at which they are used: the higher the temperature, the greater the toxicity.[63] Cryoprotectant toxicity may result in immediate cell lysis, or lysis after thawing. The pre-equilibration process is usually performed in a series of steps, in order to reduce the stress of dehydration and facilitate cryoprotectant uptake.

During initial cooling, temperature shock may occur and damage the cell, resulting in lysis or degeneration following thawing. Temperature shock is most likely to occur in species that are 'chill-sensitive', including bovine and porcine,[17,21] whereas mouse and human embryos seem better able to tolerate cooling from incubator temperatures to zero or below.

The process of seeding can also cause cell damage. Seeding is usually done by touching the side of a cryovial or straw with liquid nitrogen-cooled forceps, and if the cooled area is too close to the cells themselves, they may freeze and then die upon thawing. Seeding is usually carried out at a position away from the location of the cells, but damage can still occur if the seeding temperature is not appropriate.[64,65] For example, if seeding is carried out at a temperature of less than −10°C, the ice crystal may form and grow too rapidly, causing heterogeneous ice formation intracellularly. The cell may also become deformed in supercooled areas of liquid between growing ice crystals. If the seeding temperature is too high, above −4.5°C, the ice crystal may melt and never progress, with very rapid extracellular ice formation when the temperature falls below −15°C, resulting in possible cell death upon thawing.

During slow cooling, the remaining solutes become more concentrated as water freezes, and this exerts hypertonic pressure on the cell, resulting in its further dehydration and osmotic stress. At this time, the concentration of cryoprotectant increases to potentially lethal levels. If the cell is not sufficiently dehydrated during the slow cooling process, and the intracellular concentration of cryoprotectant is not sufficient to intercalate with the residual water inside the cell, intracellular ice may form and kill the cell. If the cell is excessively dehydrated, it may be incapable of rehydrating sufficiently to resume normal function after thawing.[66]

Numerous types of injury can occur during thawing. The process of taking a cell from a resting temperature of −196°C and warming it to 0°C or higher over the period of a minute or less, is an extreme temperature change. For example, a straw is usually held in room temperature air for a period of time before further warming in a water bath (usually 30°C). Thawing that is too rapid can result in large and/or small fractures in the zona pellucida or the cell itself, as described previously. These fractures are caused by a non-uniform change in the volume of the medium during rapid phase changes that occur during thawing. [41,67] If the cell membrane fractures it will lyze immediately, but even if the cell membrane does not fracture, intracellular components may be fractured or damaged leading to the eventual demise of the cell. During re-warming, ice can form once again if the vitrified solution warms at a rate that permits the process of devitrification. This occurs when the temperature reaches a point where the molecular mobility of water increases so that water molecules can move and rearrange themselves from a disorderly amorphous vitrified position to an orderly

crystalline position. This happens well below the melting point, and is therefore a potentially lethal problem.[68,69] IIF at this stage can lead to cell lysis and/or damage to organelles or other intracellular components. Damage of this type may not be apparent from simple observation of morphology.

During the final step of thawing, the previously frozen cell will be very dehydrated and must undergo rehydration and removal of cryoprotectant(s) in order to continue development. Because water permeates more rapidly than does cryoprotectant, the cell may swell and lyze in the process of trying to remove the cryoprotectant. Sucrose is usually used in the step-out process in order to reduce the osmotic effects.[70,71] Damage at this stage or during the next several hours of culture may be manifested as cell expansion and rupture, lysis, or a darkening of the cytoplasm and cell death. Sometimes the cell appears to have survived thawing, but after culture fails to develop, lyzes, or degenerates.

Kasai et al[71] discussed embryo dysmorphism following cryopreservation; they tried to mimic common types of cellular damage by subjecting mouse blastocysts to a range of treatments, including the use of excess cryoprotectant to induce toxic injury, inducing IIF, exposure to hyper- and hypotonic solutions, and rapid thawing to induce zona/cytoplasm fractures. By specifically trying to induce damage they were able to relate the morphological appearance of the cell to the type of cryodamage, and clearly demonstrated a relationship between different types of damage and the method of cryopreservation. Stachecki et al[43] showed that it is relatively easy to freeze mouse eggs using a standard slow cooling protocol with a high break-point (the temperature at which the cells are plunged into liquid nitrogen or solidified) of −20°C, compared with the more conventional −30°C break-point. It is often that we learn more by experiments that supposedly 'fail' than when experiments 'work' and all of the cells survive, and through this experiment that attempted to kill oocytes under stressful experimental conditions, it was found that mouse eggs could be exposed to stresses previously thought impossible, and still form viable pups.

CONCLUSIONS

Although embryo cryopreservation has been used for over 25 years, there are still many aspects that we do not understand about the process. The majority of studies report only morphological observations of survival and subsequent development rates. These reports have allowed modifications to protocols that have improved the success of cryopreservation, but there is still room for improvement. A few investigations have delved into the subcellular alterations that can occur during freezing and thawing, and these more detailed studies have given us a more complete understanding of the effects of freezing on embryos and oocytes. Although a vast array of cellular and subcellular components and processes can be affected by cryopreservation, the resilience of the embryo in resisting and/or adapting to these changes is remarkable. As we have alluded to above, the nucleus, cytoplasm, internal and external membranes, etc. are all subject to alteration during the process of freezing. Some of these alterations, such as lyzed blastomeres, are manifested upon thawing, and others, including genetic abnormalities, are apparent only following subsequent development over time. Therefore, early as well as late effects must be considered when optimizing a cryopreservation protocol.

As mentioned throughout this chapter, there are many similarities between human embryos and cattle embryos. The ability to study cattle embryos or those of other species may allow us to understand more fully the effects that cryopreservation has on human embryos. Indeed, most of the current vitrification protocols have been used for years with cattle embryos and have only recently been tested on human embryos. Bovine and mouse embryos are readily available, and they therefore provide good models for testing cryopreservation protocols. The data obtained from these models can serve as a general baseline of what to expect when trying to adapt protocols to humans or other mammalian species. However, more in depth analyses, specifically with human embryos and eggs, is necessary in order to optimize freezing and thawing protocols.

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