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Cryopreservation of Mammalian Embryos: The State of the Art[1]

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Abstract

Moore K, Bonilla AQ. Cryopreservation of Mammalian Embryos: the State of the Art. ARBS Annu Rev Biomed Sci 2006;8:19-32. Embryo cryopreservation has been a useful tool for embryology since the early 1970s. It has become an essential part of assisted reproductive technologies, allowing long term storage of valuable embryos from lab animals, livestock, endangered species and humans. Initially, work was done with conventional slow cooling technologies, using cryoprotectants, such as glycerol or ethylene glycol and sucrose. More recently, however, greater advances have been made with vitrification, a procedure that bypasses ice crystal formation and places the embryo in a glass-like state. This technology is receiving greater attention as it is simpler, faster and less expensive, and provides the embryo with greater protection from cryoinjury. Successful cryopreservation requires a good understanding of the proper use of cryoprotectants, sugars, and macromolecules in order to allow the highest survival rates and ultimately pregnancy and live offspring following embryo transfer. Although this technology has been in existence for over three decades, it still yields variable results. It is clear now that success is dependent upon many variables, including embryo stage and quality, embryo species and derivation, cooling and warming rates and culture conditions, to name a few. Efforts to optimize these conditions will further enhance survival and future developmental potential of the embryo. This review will briefly summarize the current understanding of embryo cryopreservation technologies, including key areas of concern and strategies to achieve the greatest successes, as well as possible future directions for this field.

Keywords: embryo, cryoprotectant, slow freeze, vitrification, thaw, viability, embryo transfer

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1. Introduction

The goal of embryo cryopreservation is long term storage and reproducible high survival rates of embryos following warming, leading to the successful establishment of pregnancy and live offspring following embryo transfer. It has become an essential component of the artificial reproductive technologies, allowing for storage of valuable germplasm produced by in vitro embryo production, intracytoplasmic sperm injection - ICSI, and cloning. Embryo cryopreservation also provides an option when superovulation yields are greater than recipient availability. Additionally, cryopreservation provides a method to preserve embryos from exotic or endangered species, and allows for import and export between countries. Furthermore, cryopreservation of embryos provides a means for reducing animal space requirements, and protects valuable animal lines from potential loss due to environmental disasters, genetic drift and infectious diseases (Landel, 2005). With this said, there is still a lot of variability and inconsistencies in survival of cryopreserved embryos. This review will summarize the latest information known about conventional freezing, but will focus primarily on the recent advances in vitrification and possible future directions for the field of embryo cryopreservation.

There are six steps to successful embryo cryopreservation: exposure to cryoprotectant, cooling to subzero temperatures, storage, thawing or warming, removal of cryoprotectant and return to a physiological environment (Liebermann *et al.*, 2003). Two important parameters determine the success of all cryopreservation protocols: 1) rate of cells regaining equilibrium in response to cooling, and 2) speed of freezing (Liebermann *et al.*, 2003). Cryoinjury can occur from formation of intracellular or extracellular ice crystals, chemical toxicity, osmotic injury, and/or fracture damage (Kasai *et al.*, 2002), and is dependent upon such things as size and shape of cells, membrane permeability, embryo quality and stage, species, and embryo origin (in vivo derived or in vitro produced) (Vajta & Kuwayama, 2006). There are two main methods for cryopreserving embryos, conventional slow cooling/freezing and vitrification. Each of these will be addressed below followed by conditions that should be considered in order to get optimal results for embryo survival.

2. Conventional Slow Cooling

Conventional slow cooling is based on the principle of dehydration, where cooling rates are optimized to remove water from the embryo, preventing cryoinjury from ice crystal formation while minimizing chemical toxicity, and osmotic stress from exposure to high concentrations of salts (Campos-Chillon *et al.*, 2006). Slow cooling methods allow solution exchange between the intracellular and extracellular compartments without inducing serious osmotic effects (Vajta & Kuwayama, 2006). The chilling sensitivity of the embryo is dependent on the stage of development, and on the culture conditions under which it developed (Pollard & Leibo, 1994). Moreover, the success of slow cooling depends on achieving the optimal equilibrium between the rate at which water can leave the cells and the rate at which it is converted into ice (Visintin *et al.*, 2002). Equilibrium is achieved at low cryoprotectant concentrations and slow cooling rate, allowing dehydration to occur during cooling (Nawroth *et al.*, 2005).

Using the slow freezing method, the dehydration of the embryo is usually achieved by placing the embryo in a solution containing 10 to 11% of a penetrating cryoprotectant. The temperature is then lowered, and ice crystal growth is initiated by "seeding" (Visintin *et al.*, 2002), which is done by touching a column of solution with a supercooled instrument. As the ice crystals grow, the water in the solution is converted to a solid state, increasing the concentration of extracellular solutes, which draw water out of the cells (Visintin *et al.*, 2002). The most common conventional cryopreservation procedure for embryos consists of equilibration in cryoprotectant for 5-10 min at 20-25ºC, seeding at -5 to -9ºC, and cooling at 0.3 to 0.6ºC/min down to -33 to -40ºC, followed by plunging into liquid nitrogen (Fair *et al.*, 2001; Nedambale *et al.*, 2004; Visintin *et al.*, 2002; Campos-Chillon *et al.*, 2006; Mucci *et al.*, 2006).

Embryos were first frozen from the mouse using conventional slow cooling in 1972 (Whittingham *et al.*, 1972). Using this technology, Wilmut & Rowson (1973) successfully froze the first bovine in vivo derived embryos (d 10-13), producing a live calf following thawing and embryo transfer. The first human born from a cryopreserved embryo was not reported until 1983 (Trounson & Mohr, 1983). Interestingly, in vitro produced (IVP) embryos were shown to be more sensitive to cryoinjury (Leibo, 1986; Hasler *et al.*, 1995). This delayed the success of the first cryopreserved IVP embryos until 1987, when calves were produced from cryopreserved d8 embryos (Fukuda *et al.*, 1990).

Today, conventional methods are considered fairly routine, yet still produce variable results. Rall (1992) reported 14% pregnancy following transfer of slow frozen/warmed embryos. However, Van Wagtendonk-de Leeuw *et al.* (1997) recorded overall pregnancy rates of 45.1% for slowly frozen embryos. Agca *et al.* (1998) also observed a lower pregnancy rate for conventionally frozen embryos. Voelkel & Hu (1992b) recorded pregnancy rates at 60 days of gestation for embryos frozen in ethylene glycol or glycerol were 62 and 39%, respectively. Dochi *et al.* (1995) reported pregnancy rates of 69%, 52% and 60% for ethylene glycol (EG), ethylene glycol+sucrose (EG + SUC) and control medium glycerol+sucrose (GLY + SUC), respectively. Using a freezing method combining 1.36 M glycerol and 0.25 M sucrose in PBS, Massip *et al.* (1987) obtained a pregnancy rate of 51.8% from direct transfer. Upon direct nonsurgical transfer of day 7 expanded blastocysts frozen in 3.6 M EG, a pregnancy rate of 43% was achieved (Sommerfeld & Niemann, 1999). Pregnancy rates of bovine embryos frozen with glycerol 5-10%, glycerol 10% or ethylene glycol 1.5M were 40.4, 39.1 and 45.4%, and calving rates were 40.4, 36.9 and 44.3%, respectively (Martinez *et al.*, 2002). These data confirm that conventional methods are still variable and not optimal for protecting embryos from cryoinjury.

3. Vitrification

Vitrification is a newer method that eliminates both intracellular and extracellular ice formation, producing instead a glass-like state. At sufficiently low temperatures, solutions become very viscous and solidification occurs without ice crystal formation. It does this through dehydration and the extreme elevation in viscosity caused by ultra rapid cooling rates, from 15,000 to 30,000ºC/min (Liebermann *et al.* 2003). The glass transition state is ~ -130ºC (Kasai & Mukaida, 2004), but varies depending upon components in the vitrification solution. Vitrification of solutions has been known since 1948 (Kauzmann, 1948), but was first used for preserving embryos from the mouse in 1985 (Rall & Fahy, 1985). A year later the first bovine embryos successfully vitrified were reported (Massip *et al.*, 1986). Considerable efforts have been made since the mid 1980's developing simpler protocols, and more stable and less toxic solutions for vitrification.

Improvements have been made by using less toxic and more permeable chemicals, by using a combination of cryoprotectants to reduce toxicity, using a stepwise approach to equilibration and increasing cooling and warming rates (Vajta & Kuwayama, 2006). Factors that determine whether a vitrification solution will remain uncrystalized are the total solute concentration (> 40%), the capacity of the cryoprotectant to form glass, and the rate of cooling/warming (Shaw *et al.*, 1997). Increasing the cooling/warming rate can also decrease the amount of solute required to form a stable glass.

Vitrification is carried out in a viscous vitrification solution containing several of the following components: basal medium buffered with phosphate or HEPES, cryoprotectant, macromolecules, saccharides, and proteins. These same components are also utilized in conventional freezing protocols, but at different concentrations and under different conditions. Each of these components will be addressed below, but more emphasis will be given here to vitrification, but points of interest and comparisons will be mentioned for slow cooling.

4. Cryoprotectants

The primary components in successful freezing and vitrification solutions are cryoprotectants. Permeating cryoprotectants are essential for dehydrating intracellular water. Additionally, they lower the freezing point, thus giving more time for dehydration. Careful selection of cryoprotectants must be made however, first for toxicity and second for permeability. Cryoprotectants are toxic at high concentrations, but toxicity is minimized at low temperatures, and with short periods of exposure (Agca *et al.*, 1998).

In the beginning, embryos were conventionally cryopreserved with glycerol (1-1.5M), resulting in acceptable pregnancy rates, but its use required sequential dilution of the cryoprotectant upon thawing (Hasler *et al.*, 1995). Martinez *et al.* (2002) observed that glycerol (5-10%) was more effective for freezing morula than blastocysts (59 and 20% pregnancy rates, respectively). Dochi *et al.* (1998) compared different cryoprotectants, and obtained higher pregnancy rates for glycerol than for ethylene glycol or propylene glycol (48.6%, 44.7% and 36%, respectively). As this field of research progressed, other cryoprotectants began to be utilized in an attempt to reduce variability and toxicity.

Several common cryoprotectants used in embryo cryopreservation are listed here in order of embryo toxicity, from least to most toxic: ethylene glycol (EG), propanediol, glycerol, dimethyl sulfoxide (DMSO), and acetamide. However, this is somewhat controversial, due to variable results related to species, embryo stage and culture conditions. Survival was shown to be better with propanediol than EG for mouse embryos, indicating possible species differences (Emiliani *et al.*, 2000). Valdez *et al.* (1992) showed on the contrary that EG was the least toxic to mouse embryos and successfully used a combination of 20% EG, 20% DMSO and 10% 1, 3-butanediol. Additionally, EG is known to be the most permeable cryoprotectant (Szell *et al.*, 1989), which is preferable, leading to shorter incubation prior to cooling and more rapid removal during warming (Kasai & Mukaida, 2004). Permeability, however, varies by embryo stage and species. Interestingly, EG is a very weak glass forming molecule, whereas DMSO is a better glass forming molecule, so often times the two are combined in vitrification solutions (Valdez *et al.*, 1992).

Menezo (2004) suggested that EG may be of some concern for freezing human embryos, as it had negative effects on rat organogenesis. Klug *et al.* (2001) had reported that EG (100-200 mM) and its metabolites (0.3-6 mM) result in harmful effects on embryonic development and organogenesis in the d9.5-11.5 rat embryo. However, this was after prolonged exposure to EG (48h) at elevated temperatures. Embryos are cryopreserved in greater concentrations of EG, but are done so at ultralow temperatures and for only a matter of seconds. Furthermore, on warming the EG is quickly removed, due to its high permeability, so embryos do not stay at this elevated concentration for long. It is therefore unlikely that this is of concern to later embryonic development of vitrified or slow cooled embryos.

The goal in vitrification is to maximize cooling rate and minimize cryoprotectant concentrations in order to avoid toxicity issues (Liebermann *et al.*, 2003). As cooling rate increases, concentration of cryoprotectants can be decreased, making solutions less toxic (Vajta & Kuwayama, 2006). Additionally, a two step equilibration is recommended, first in a lower concentration of cryoprotectant, followed by a brief exposure to high concentration and plunging into liquid nitrogen. Valdez *et al.* (1992) showed that both one step and two step methods were effective for vitrifying mouse embryos, but obtained more live offspring with the two step method (45% *versus* 54.2%, respectively). Furthermore, the two step approach was preferable for vitrifying bovine embryos, increasing survival and hatching post-warming (Mahmoudzadeh *et al.*, 1995). Today, most vitrification protocols utilize this two step approach in order to yield optimal results.

5. Sugars

Non-permeating saccharides are another key ingredient of most embryo cryopreservation protocols. Sugars help in the dehydration of cells by raising osmolality, as well as preserving structural integrity. Preequilibration in sugars can help draw out more water, and lessen the time of exposure to the more toxic permeating cryoprotectants. Furthermore, they can reduce the amount of cryoprotectant required to produce a state of vitrification. Sugars also serve as an osmotic buffer, reducing osmotic shock at warming, by reducing the speed and magnitude of cell swelling (Liebermann *et al.*, 2003).

The sugars used in cryopreservation are categorized as monosaccharides (glucose, fructose, sorbitol, mannitol), disaccharides (sucrose, trehalose), and polysaccharides (raffinose) (Kuleshova *et al.*, 1999; Fair *et al.*, 2001; Dattena *et al.*, 2004; Mavrides & Morroll, 2005; Campos-Chillon *et al.*, 2006). They are virtually non-toxic at cooler temperatures, but can be harmful at elevated temperatures (Kasai & Mukaida, 2004). Kuleshova *et al.* (1999) evaluated the role of the sugars listed above at 0, 0.5 and 1M in an EG based vitrification system. Interestingly, monosaccharides were effective in promoting vitrification on a wt/wt base equivalent to EG, but raised the glass transition state. They also found disaccharides and polysaccharides to be more problematic, due to difficulty with dissolving and precipitation from solution at room temperature or 4ºC. Furthermore, higher concentrations of disaccharides were needed to form a stable glass (Kuleshova *et al.*, 1999). This study suggested that monosaccharides may be better than disaccharides currently used in vitrification protocols, due to their low embryo toxicity, and ability to form glass at lower total solute concentrations.

6. Macromolecules

Use of macromolecules in vitrification has become more popular recently, as it increases viscosity during supercooling and helps avoid extracellular ice crystal formation. Often times, non-permeating macromolecules are less toxic than cryoprotectants and can be used to partially replace them, thus making the solution less toxic (Liebermann *et al.*, 2003). Moreover, polymers are known to protect against zona cracking. Doumoulin *et al.* (1994) showed that 10% Dextran 10,000 MW, 70,000 MW and 300,000 MW helped protect against zona damage, but development to blastocyst was greatest with Dextran 70,000 MW and 300,000 MW. However, the glass transition state and melting points can be markedly altered by the various polymers and should be considered when formulating vitrification solutions (Shaw *et al.*, 1997).

Several macromolecules commonly used in embryo cryopreservation are polyethylene glycol 8000 (PEG), polyvinylpyrrolidone (PVP) 360,000, Ficoll 70,000 or 400,000, polyvinyl alcohol (PVA) and Dextran. Kuleshova *et al.* (2001) showed that Ficoll and Dextran were not embryo toxic. Although addition of Ficoll to vitrification solutions was not beneficial for improving survival of bovine embryos (Laowtammathron *et al.*, 2005). Yet, Ficoll was successfully used for cryopreserving human embryos (Zech, 2005), hamster embryos (Lane *et al.*, 1999) and bovine embryos (Mahmoudzadeh *et al.*, 1995; Lane *et al.*, 1999). PVP at 30% was toxic unless first dialyzed (Kuleshova *et al.*, 2001). These contradictions confirm the ideas suggested above by Shaw *et al.* (1997), that macromolecules can alter the vitrification properties of solutions leading to variability, and should be considered when designing vitrification conditions.

Other possibilities to enhance cryopreservation solutions include the use of biological components and proteins. These include the use of bovine serum albumin, serum and egg yolk in cryopreservation solutions to reduce cryoinjury. However, each of these are possible sources of infectious agents, can result in change of freezing characteristics (Shaw *et al.*, 1997), and can exhibit problems due to batch variation. Most research now is working to develop more defined systems, in order to remove such sources of variability.

7. Storage

During cryopreservation, embryos are taken from physiological to subzero temperatures and finally stored in liquid nitrogen (LN_2). Conventional freezing and vitrification protocols achieve this in much different ways (see Fig. 1). Problems can arise due to chilling, low permeability or toxicity of cryoprotectants. Chilling injury occurs within a critical temperature zone from 15 to -5ºC, and can be avoided by rapidly passing through this zone (Liebermann *et al.*, 2002a). To enhance this, storage volume is minimized to reduce insulation from LN_2 vapor, which forms when embryos are plunged into LN_2, thus increasing cooling rates. Vitrification is typically done in very small volumes, from 0.6 to 2 µl (Kasai & Mukaida, 2004). Another strategy to reduce cryoinjury is to vitrify under vacuum, effectively increasing the cooling rate in a LN_2 slush, as seen with the VitMaster (IMT, Israel) (Arav *et al.*, 2000). Increased cooling rates also reduce toxicity issues of cryoprotectants, as discussed above.

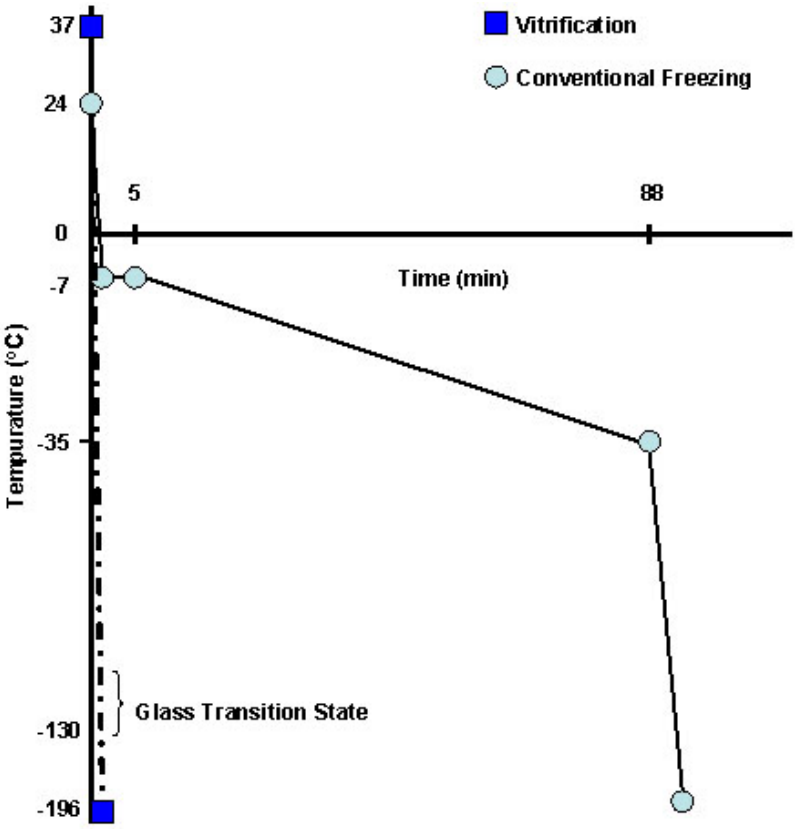

Figure 1. Comparison of conventional slow cooling method with vitrification method.

There are many storage devices now available that allow storage of embryos in small volumes. Embryos frozen using conventional methods typically use 0.5 or 0.25 cc sealed straws for storage. Vitrification methods have been much more creative in reducing storage volumes by using one of many options: open pulled straws (OPS) (Vajta *et al.*, 1998; Lazar *et al.*, 2000), mini straws (Vanderzwalmen *et al.*, 2002), flexipet-denuding pipets (Liebermann *et al.*, 2002b), electron microscopy copper grids (Son *et al.*, 2003), hemi straws (Liebermann *et al.*, 2002b; Vanderzwalmen *et al.*, 2003), cryoloops (Lane *et al.*, 1999), gel loading pipet tips (Tominaga & Hamada, 2001; Niasari-Naslaji *et al.*, 2006), and microdrops (Landa & Tepla, 1990). Vitrification of mouse pronuclear stage embryos was improved when frozen on a metal surface over that of conventional French straws (Bagis *et al.*, 2005). The cryoloop was taken from X-ray crystallography and utilized for successful vitrification of both hamster and bovine embryos (Lane *et al.*, 1999). Vajta *et al.* (1998) showed that the OPS method circumvents chilling injury, reduces cryoprotectant toxicity and osmotic damage due to its ultrarapid cooling (> 20,000°C/min) and warming in small volumes. This method has been used successfully for oocytes and all preimplantation stage embryos from many species, and is still one of the most commonly used methods to date. More recently, gel loading tips were shown to be a more cost effective alternative to OPS, yielding similar results (Tominaga & Hamada, 2001).

One concern for storing vitrified embryos is liquid nitrogen, as a possible source of viral contamination, since vitrified embryos come into direct contact with LN_2. Bielanski *et al.* (2000) showed that 21% of embryos were contaminated with virus when stored in contaminated LN_2. Very few reports indicate such problems, yet this may be due to lack of screening. Three possible remedies for this situation are to use only filter sterilized LN_2, store embryos in LN_2 vapor or store embryos in newer closed

container systems, such as the cryoloop, the cryotip, vitset or sealing a straw within a straw (see Vajta & Kuwayama, 2006 for review on this topic).

8. Additional Factors Contributing to Embryonic Survival

Warming cryopreserved embryos is an area that can have dramatic effects on embryo survival and future development. Hochi *et al.* (1996) found that the survival of in vitro produced bovine morula and blastocysts was improved by very slow cooling/ freezing, but slow warming appeared to cause injuries. Slow warming of blastocysts has been associated with lower survival at all cooling rates (Van Wagtendonk-de Leeuw *et al.*, 1997). Warming frozen straws can be done by exposure to the air or 20-28ºC for 10s, followed by 10-30s in a 22 to 35ºC water bath (Fair *et al.*, 2001; Martinez *et al.*, 2002; Nedambale *et al.*, 2004; Moreira da Silva & Metelo, 2005). Warming in a water bath from -196 to 37ºC translates to > 4460ºC/min (Liebermann *et al.*, 2002a), whereas warming in the air at room temperature results in < 196ºC/min, often resulting in zona damage (Shaw *et al.*, 1997). In general, rapid warming is also more beneficial for improving survival of vitrified embryos (Liebermann *et al.*, 2002a). However, one report showed that vitrification in straws flanked by 0.5M sucrose and thawed on ice (4ºC, 10 min) gave the best viability and hatching of bovine IVP embryos, which is promising for use in field conditions (Gutierrez-Adan *et al.*, 1999).

Culture conditions have a major influence on the developmental outcome of in vitro produced embryos and their survival following cryopreservation. Sensitivity to chilling can be the result of embryo stage and the culture conditions embryos were produced in (Leibo & Loskutoff, 1993). Blastocyst stage embryos are frozen more successfully than early cleavage or pronuclear stage embryos, possibly since they have already surpassed embryonic genome activation (Menezo, 2004). Earlier stage embryos are more prone to cryoinjury (Liebermann *et al.*, 2003), which may also be due to their having fewer cells, which when damaged are less likely to survive. Another possible explanation is that embryos at different developmental stages utilize different metabolic pathways, which could make them more prone to cryoinjury. Furthermore, in vitro produced embryos are known to be more sensitive to cryoinjury than in vivo derived embryos (Leibo, 1986; Hasler *et al.*, 1995). Several ideas will be presented below that should be considered in the culture of embryos, either prior to cryopreservation or following warming in order to achieve the greatest successes.

Supplementing culture systems with serum can prove problematic for embryos that are to be cryopreserved. Bovine embryos cultured in CR1aa without serum prior to vitrification had better survival post-warming (Tominaga *et al.*, 2000; Mucci *et al.*, 2006). Mucci *et al.* (2006) found no difference in blastocyst production for cultures supplemented with amino acids and estrous cow serum and/or BSA, but post-thaw survival rates were higher for medium containing amino acids and BSA (51.9% *versus* 25%). Similarly, Rizos *et al.* (2003) found that serum in culture with SOF reduced cryotolerance, as well as altered the relative abundance of developmentally important mRNAs in vitrified bovine embryos.

Interestingly, serum containing medium has been shown to increase lipid droplet size and number (2-6 μ and > 6 μ) in bovine IVP embryos, while those produced in serum-free systems had lipid droplets of smaller size (2 μ) (Abe *et al.*, 2002). When compared, in vivo derived embryos were found to have a buoyant density of > 1.3, whereas in vitro produced embryos were < 1.2 (Leibo & Loskutoff, 1993). This could be due to a higher lipid to protein ratio in the IVP embryos, making them float in medium. This lipid accumulation may also reduce cryotolerance, thus reducing survival post-warming. Furthermore, culture with serum resulted in a greater increase in immature mitochondria over serum-free culture, possibly effecting metabolic rates (Abe *et al.*, 2002). Contrary to this finding, Men *et al.* (2005) showed that culture of porcine embryos in serum improved the ability of the embryos to survive vitrification. Use of serum in culture will therefore depend on species of interest, but most efforts continue towards producing culture protocols free of serum in hopes of giving more reliable defined systems.

Altering the lipid content of embryos, whether through removal of serum or other routes, is aimed to improve embryo cryotolerance. Addition of linoleic acid-BSA to culture improved survival of slow cooled (Hochi *et al.*, 1999) and vitrified bovine embryos (Laowtammathron *et al.*, 2005). Soybean lecithin was tested to minimize membrane damage, however no significant improvement was found for frozen-thawed blastocyts after 48h in culture (Guyader-Joly *et al.*, 1999). They speculated that the efficacy of lecithin in embryo cryopreservation may be reduced by the negative effects of spontaneous lipid peroxidation, which occurs during aerobic incubation. Seidel (2006) reported that the use of phenazine

ethosulfate (PES) in culture lowers lipid content in bovine embryos and improves cryosurvival. Delipidation of cleavage stage bovine and porcine embryos, through centrifugation and removal through micromanipulation also seems to improve their cryosurvival (Ushijima *et al.*, 1999; Tominaga *et al.*, 2000). Otoi *et al*. (1997) suggested that cellular damage from cryopreservation may be due to the large amounts of lipids found in pig and cattle embryos, since its removal or polarization reduced cryoinjury. However, Liebermann *et al.* (2002a) warned that delipidation may remove possible building blocks required for development of cell membranes in the developing embryo.

Oxygen free radicals formed during cryopreservation can cause cellular damage and loss of embryo viability. Addition of EDTA (0.1mM) and/or glutathione (GSH; 1 mM) to culture prior to vitrification improved development of mouse embryos from the morula to blastocyst stage (Aksoy *et al.*, 1999). Possible explanations are extracellular GSH oxidation in the presence of reactive oxygen species reduces oxidative reactions of unsaturated cell membrane lipids, promoting cell integrity. EDTA also protects against free radical damage and autoperoxidation of lipids. Furthermore, Voelkel & Hu (1992a) determined that an atmosphere of low oxygen (5%) gave superior survival to high oxygen (20%) for bovine embryos that were later cryopreserved. As another way to regulate oxygen free radicals, addition of β-mercaptoethanol (100 mM) to culture post-warming was also found to substantially improve survival of vitrified bovine embryos, even in low oxygen environments (Nedambale *et al.*, 2006). Clearly, efforts to reduce the effects of oxygen free radicals in culture should be standard practice for improving survival of cryopreserved embryos.

Co-culture is an older culture technique that is known to improve development in vitro, as well as improve cryotolerance. Granulosa cell co-culture was superior to epithelial cell co-culture for improving cryosurvival (see Leibo & Loskutoff, 1993). Hasler *et al.* (1995) studied two different co-culture systems (TCM 199 or Buffalo Rat Liver (BRL) cells in Menezo's B2 medium), and recorded that bovine embryos resulting from BRL cell co-culture developed faster than those cultured with TCM 199, however there was no difference in pregnancy rates after freeze-thaw. Vero cell co-culture has also been shown to improve cryosurvival of mouse embryos (Desai *et al.*, 2000). Although helpful for promoting cryotolerance, co-culture is another variable that is undefined and leads to variability across batches. For this reason, most protocols are moving away from using this approach for producing embryos to be cryopreserved.

Culture media and supplements are additional areas of concern when producing IVP embryos to be cryopreserved. Nedambale *et al.* (2004) compared four culture media: CR1aa, KSOM, SOF and sequential culture in KSOM and SOF and determined that bovine embryos cultured sequentially in KSOM and SOF had higher survival following vitrification and warming. Growth factors are also a possibility for improving viability post-warming. IGF-1 and -2, and EGF have been shown to be of benefit on post-warming viability (Desai *et al.*, 2000). Much work is still needed in determining the optimal culture conditions both for the production of IVP embryos to be cryopreserved, as well as optimizing post-warming culture conditions in order to achieve increased survival and development post-warming.

Survival and hatching post-warming are generally acceptable indicators of viability of cryopreserved embryos, especially when dealing with livestock, as costs and time to produce live offspring can be prohibitive. Sommerfeld & Niemann (1999) using a protocol with 3.6 M ethylene glycol obtained day 7 blastocysts with a hatching rate of 81%, which was similar to that of the non-frozen controls (76%). Other indicators of survival, such as live-dead stains, cell counts and glucose utilization may also prove useful. Further research is warranted to find reliable markers of cryotolerance and embryo survival. However, results for viability are best demonstrated by production of live offspring. Frozen bovine embryos can be thawed, and transferred nonsurgically into recipients under conditions quite similar to those used for artificial insemination (Leibo, 1984). The use of ethylene glycol as a cryoprotectant has allowed direct transfer after thawing without preceding dilution, because of its high penetration rate and low toxicity (Voelkel & Hu, 1992b; Dochi *et al.*, 1995; Martinez *et al.*, 2002). Martinez *et al.* (2002) recorded better pregnancy rates from direct transfer using ethylene glycol (1.5M) with sucrose (0.1M), as compared with sucrose (0.3M). Direct transfer of vitrified embryos is also possible, but variable. Saha *et al.* (1996) were successful in producing three normal calves from the direct transfer of five vitrified/warmed d7 in vivo derived embryos. Greater efforts are needed to develop reliable protocols for direct transfer of vitrified embryos, which result in consistently high pregnancy rates and production of live offspring.

9. Vitrification versus Conventional Freezing

Vitrification has a great advantage over conventional freezing due to its simplicity, speed and does not require additional equipment, making it more user friendly especially for on-farm procedures (Table 1). Slow cooling has been proven effective, but requires more time, and use of expensive equipment. Mahmoudzadeh *et al.* (1994) recorded lower survival rates for IVP blastocyst stage embryos for slow freezing than for vitrification (34.9% and 81.9%, respectively), but the overall hatching rate following the two cryopreservation methods was not significantly different. Agca *et al.* (1998) reported that pregnancy rate of conventionally frozen embryos was lower than that of fresh or vitrified embryos. Mucci *et al.* (2006) showed that survival of vitrified bovine embryos substantially out performed conventional slow freezing (43% vs. 12%, respectively). These results indicate vitrification has an advantage over conventional freezing for improving embryo survival.

Table 1. Comparison of embryo vitrification with conventional slow cooling.

Attribute	Vitrification	Slow Cooling
Direct Contact with LN2	Yes	No
Ice Crystalization	No	Yes
Cryoprotectant Concentration	High (40-60%)	Low (8-10%)
Sample Volumes	1-2 µl	100-250 µl
Cooling Rates	15,000-30,000 °C/min	0.3-0.6 °C/min
Time	Rapid (s)	Slower (min)
Expense	inexpensive	expensive
Technical Expertise	simple	simple
Embryo Stages	all	all
Direct Embryo Transfer	yes, variable	yes

The type and degree of cryoinjuries may vary depending on the method of cryopreservation. Cryoinjury in bovine embryos may be selective for one cell type within an embryo, and its extent and nature are dependent on its developmental stage. Damage to day 7 blastocysts after freezing included loss of integrity of trophoblast cell plasma membranes, leading to collapse of the blastocoele (Mohr & Trounson, 1981). Moreover, these authors showed that for day 13 embryos after freezing and thawing, the ICM cells were structurally intact while the trophectoderm had substantial damage to all cell components (Mohr & Trounson, 1981). Kaidi *et al.* (2001) observed a greater decrease in cell number for conventionally frozen embryos than for vitrified ones. The decrease in trophectoderm cells was greater in frozen blasts as compared to controls (38% *versus* 20%), but no difference was observed for the number of inner cell mass cells (24 and 27%, respectively). Vitrification could eventually replace conventional slow freezing methods, due to its relative ease and superior results for preventing cryoinjury.

10. Recent Advances an Future Directions

Reduction in temperature can result in cold shock injury, and varies with species. It is associated with changes in membrane permeability and cellular microfilament structure (Mavrides & Morroll, 2005). Visintin *et al.* (2002) recorded as one of the most common degenerative features, the swelling of cells with cytoplasmic injuries and nuclear vacuolization, cellular lyses, and cells with discontinuous plasma membranes, which were similar to characteristics observed during the processes of cellular death at necrosis. Vitrification of porcine morula and blastocysts display microfilament disruptions and reduced survival post-warming (Dobrinsky *et al.*, 2000). By stabilizing the cytoskeleton with cytochalasin B prior to vitrification, these authors improved survival and development of vitrified porcine blastocysts post-warming, and produced live offspring following embryo transfer. However, Chen *et al.* (2005) found that pretreatment of mouse blastocysts with cytochalasin B provided no benefit for improving survival following

vitrification and warming. Pretreatment of equine embryos with cytochalasin B reduced cell death post vitrification and warming, but depolymerization of actin filaments was not reversed within 6h of culture, possibly compromising survival (Tharasanit *et al.*, 2005). Therefore, artificial cytoskeletal stabilization may only be useful in some species, but still needs to be determined.

Dehydration of blastocyst- and expanded blastocyst-stage embryos at vitrification may be impaired due to difficultly of cryoprotectants to permeate the blastocoelic cavity, increasing the susceptibility to ice crystal formation. By artificial reduction of blastocoelic fluid, osmotic shock, permeability issues and ice crystal formation can all be reduced. Viability, implantation and pregnancy rates have all been improved in human embryos that have been artificially collapsed by microsuction prior to vitrification (Vanderzwalmen *et al.*, 2002; Son *et al.*, 2003). Microsuction of blastocoelic fluid prior to vitrification of mouse blastocysts improved survival post-warming to rates comparable to nonvitrified controls, increasing live young (Chen *et al.*, 2005). Unique attempts to improve dehydration of embryos, such as this may further enhance cryosurvival of embryos from many more species, allowing survival similar to non-cryopreserved controls.

More recently, a study showed the effects of freeze/thaw on the zona pellucida. Moreira da Silva & Metelo (2005) evaluated the zonae of bovine morula and blastocysts cryopreserved by slow cooling or vitrification, as compared with controls. They found a significant reduction in zona pore size and number for vitrified embryos over controls. For slow cooled embryos there was a 46% decrease in total pore size *versus* 73% reduction for the vitrification group (Moreira da Silva & Metelo, 2005). The authors indicated that this may inhibit nutrient exchange in culture, as well as hatchability. Furthermore, Vincent *et al.* (1991) showed that 0.7M sucrose can cause zona hardening in mouse oocytes. Further advances in culture conditions, as well as optimizing vitrification solution reagents may help to rectify these problems, thus improving survival and development after warming.

Vitrification has become quite useful for storage of micromanipulated embryos. Nguyen *et al.* (2000) demonstrated that cloned embryos were more sensitive to cryopreservation and successfully vitrified them using a lower concentration of EG (39%). This may be due to greater access of the cryoprotectant, caused by the rent in the zona pellucida of micromanipulated embryos. Cloned embryos produced by hand-made cloning were also effectively vitrified and warmed, producing a live calf (Tecirlioglu *et al.*, 2003). As assisted reproductive technologies become more refined and successful, efforts to improve cryopreservation protocols to enhance survival of these manipulated embryos will be imperative.

Several social implications have also developed over time with the increased successes of assisted reproductive technologies and human embryo cryopreservation. As of 2002, there were more than 400,000 cryopreserved human embryos stored in the United States alone (Hoffman *et al.*, 2003). Of these 88% were slated for use by patients, whereas less than 3% were planned for research purposes. Ethical issues will need to be addressed as to the long term storage or disposal of this growing resource. Further complications arise in the cases of death, divorce or spousal disagreement. Currently, it has not been determined whether the embryo has rights, and whether embryos should be donated or adopted (Bankowski *et al.*, 2005). To date, the use of spare embryos for research and production of embryonic stem cells is highly controversial and politically, hotly debated. These issues will need to be addressed in the near future in order to deal with difficulties arising from the development of large quantities of abandoned embryos.

In conclusion, the last decade has seen greater overall successes with embryo survival following vitrification for many species. Further research is warranted however, to ensure a safe reliable means for storing viable embryos. Optimizing culture conditions of IVP embryos, both prior to cryopreservation and post warming, are as essential as developing reliable vitrification protocols that allow for direct embryo transfer, improving pregnancy rates and ultimately the production of viable offspring.

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