Review

Current trends, biological foundations and future prospects of oocyte and embryo cryopreservation

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Abstract

For a long time, the cryopreservation of gametes and embryos remained a major hurdle for the clinicians and scientists in terms of success. However, recent technical advancement in the field of cryobiology has opened up various options for freezing gametes and embryos at different developmental stages. The tendency of the IVF world to switch over to natural-cycle IVF and to elective single-embryo transfer has put cryotechnology in the forefront of research. Still, the intricacies of the cold-induced changes in human gametes and embryos that could affect the intracellular and developmental processes need to be known. The transcriptomics, proteomics and metabolomic platforms hold promise for elucidating these complex processes during cryopreservation processes.

Keywords: cryopreservation, embryo, infertility, IVF, oocytes, ovarian tissue

Introduction

The first pregnancy using cryopreserved mature oocytes was reported by Chen (1986). Since then, cryopreservation protocols for gametes, embryos and ovarian tissues have evolved substantially. Researchers have studied many variables that can affect the success of the process including temperature (during cryoprotectant addition, freezing and thawing), type of cryoprotectant, additives, vessels for holding the gametes/embryos, type of machinery, osmotic properties of solutions and several mathematical models for developing an effective protocol. However, except for some very recent (unpublished) achievements, the pregnancy rate with frozen–thawed gametes/embryos is still below that of fresh cycles.

At the same time, worldwide rates of infertility continue to rise. Indeed, infertility now affects every sixth couple in western countries. Moreover, in some countries, up to 3% of all registered births result from assisted conception. Thus, there is a stronger-then-ever need for freeze–thaw protocols that safely and successfully cryopreserve gametes, embryos and ovarian tissues for use in assisted reproductive technologies. This review will discuss recent developments in cryopreservation regimens, their advantages/disadvantages and biological backgrounds and avenues for future prospects.

Cryopreservation of oocytes

There are several advantages of freezing gametes and embryos for later use in assisted reproduction. For example, the process reduces concentrations of gonadotrophins for ovarian stimulation and protects oocyte competence and reduces the overall cost for multiple IVF attempts. In an interesting study done by the French national register on in-vitro fertilization (FIVNAT, 1994), 465 pregnancies from frozen embryos were compared with 8757 pregnancies from fresh embryo transfers. There were fewer premature infants and small-for-gestational
age (SGA) infant rate was lower in the group of pregnancies originating from frozen embryos. In singleton pregnancies, the SGA rate of newborns originating from frozen–thawed embryos was half that of the pregnancies coming from fresh embryo transfers. It is remarkable to note that the main difference between the two groups of patients was that ovarian stimulation was mild or even absent in the frozen-embryo replacement cycles (Olivennes et al., 2002).

Oocyte cryopreservation has wider clinical implications than embryo freezing. Women who have no spouse, who wish to delay their motherhood or stand to lose ovarian function due to surgery or radio/chemotherapy can maintain their fertility via oocyte cryopreservation. It also avoids ethical, religious and legal issues surrounding embryo cryopreservation. Moreover, oocyte cryopreservation remains the only option in countries where embryo freezing is forbidden by local regulations such as Italy, where strict laws have been in place since 2004 (Benagiano and Gianaroli, 2004).

**Experimental evidence and biological factors that determine oocyte cryosurvival**

It has been well documented that oocytes experience both reversible and irreversible damage during cryopreservation. This includes hardening of the zona pellucida, premature release of cortical granules, denaturation of the microtubules and misalignment of the chromosomes. Immunofluorescent studies on fixed cells, and very recently PolScope-based live imaging of the spindle apparatus, indicate that spindles from both animal and human oocytes are susceptible to the freeze–thaw process. The spindle is a dynamic structure composed of heterodimeric units of α- and β-tubulin. They are assembled into 13 protofilaments arranged side by side, which are assembled continuously at one end and removed at the other in a treadmill fashion. The polymerized tubulin, the major component of the spindle microtubules, is in equilibrium with the free tubulin pool within the ooplasm (Vincent and Johnson, 1992). For an oocyte spindle to be considered normal, the chromosome must be located at the metaphase plate and have intact bonds between the microtubules and kinetochores. Stray chromosomes may result from perturbations inflicted upon the spindle polymers from the freeze–thaw process. The major challenge in cryopreservation stems from the limited ability of human oocytes to repair their spindle upon return to physiological temperatures, which is in contrast to the mouse oocyte (Mandelbaum et al., 2004). The latter is more resistant to freeze damage (Stachecki et al., 2004), which casts doubts on its suitability as a model for optimization of human oocyte cryopreservation regimens.

Although the chromosomes reassemble themselves and align along the spindle equator at cell rewarming, there is a risk of chromosomal loss and aneuploidy during the first maturation division (Pickering et al., 1998). Human oocytes can withstand a wide range (~600–3000 mOsm) of aniosmotic conditions, but in terms of membrane integrity and enzymatic functions, the tolerance level of meiotic spindles to non-physiological conditions is considerably narrow (Mullen et al., 2004). A recent investigation reported significant variations in osmotic response and tolerance limits to changes in osmolarities and in oolemma permeability of human oocytes to commonly used cryoprotective agents (Van den Abbeel et al., 2007). That study also indicated that there may be a considerable difference in the tolerance level to cryoprotective agents between oocytes of in-vivo or in-vitro origin other than the genetic influence for the expression of important cryobiological characteristics of mammalian oocytes. Several lines of evidence also indicate that mammalian oocytes express water channels (aquaporins), the expression pattern of which may affect how oocytes at various stages of maturity respond to cryopreservation (Ford et al., 2000; Edashige et al., 2003).

When using the same freezing and thawing procedures, mature human oocytes had survival rates that were lower than that of embryos (Tucker et al., 1998a). The cytoplasmic membranes of oocytes, which have fewer submembranous actin microfilaments, are more fragile to cryopreservation (Gook et al., 1993) than cleavage-stage embryos. The volume-to-surface ratio of oocytes is greater, making the dehydration process more difficult and more likely to lead to meiotic spindle disturbances, chromosomal dispersion, failure of normal fertilization and failure to develop (Eroglu et al., 1998a).

In one study, PolScope analysis revealed a limited amount of meiotic spindles in living human oocytes after cooling–rewarming (Wang et al., 2001). It has been suggested that a 3 h post-thaw incubation could help restore meiotic spindles after slow freeze and thawing cycles, thus facilitating normal fertilization, preferably by intracytoplasmic sperm injection (ICSI) (Chen et al., 2005). Considerable variations in the spindle recovery rate have been documented based on the type of cryopreservation protocols that are used. One method, ultra-rapid cooling (vitrification), seems to help maintain the spindle apparatus (Gardner et al., 2007). However, well-designed slow-freezing protocols that use a higher sucrose concentration have also been shown to maintain spindle integrity (Nottola et al., 2006; Coticchio et al., 2007).

In an attempt to improve the survival of denuded or cumulus enclosed metaphase II (MII)-stage oocytes, Fabbri et al. (2001) increased the sucrose concentration in cryopreservation media to 0.3 mol/l and the exposure time to 15 min in the slow freeze–rapid thaw method that would dehydrate the oocytes/cumulus-enclosed oocytes more adequately. They achieved a survival rate of 82%, which was significantly higher than the rates associated with the 0.1 mol/l (34%) and 0.2 mol/l (60%) sucrose concentrations. A higher sucrose concentration during the rehydration step of the thawing procedure may keep osmotic stress within tolerable limits and could also be a contributor to increased survival (Fabbri et al., 2001). It is important to completely remove the cryoprotectant and establish the original water content and thereby a normal physiological state, and currently practiced thawing procedures and their effects on volume excursions in oocytes during rehydration need refinement to avoid any osmotic stress-induced vulnerability (Coticchio et al., 2007).

Coticchio et al. (2006) provided evidence that during slow cooling, spindle and chromosome configuration are safely preserved provided that a higher sucrose (0.3 mol/l) concentration in the freezing solution is used. It is interesting to observe that cryopreservation with either the 0.1 or 0.3 mol/l sucrose protocols has given rise to different spontaneous...
abortion rates (20.0% and 14.2%, respectively) (Borini et al., 2006b; Bianchi et al., 2007). Studies using PolScope show low and high spindle birefringence, an indicator of differences in spindle organization (or tubulin fibre density), during freeze-thaw cycles (Bianchi et al., 2005). It has been suggested that the reduced ability for spindle reformation during the post-thaw of human oocytes may be linked to the fact that they contain fewer foci of pericentriolar material – the site containing microtubule organizing centres (MTOC) – than do murine oocytes (Paynter, 2000). Further insights from analysis of microtubule regulatory proteins (like protease activated receptors PAR1 and PAR3) might reveal damage inflicted on the spindle apparatus during oocyte freeze-thaw process (Sun and Schatten, 2006). Assessment of tubulin organization by confocal laser scanning microscopy in the same thawed oocyte that has been analysed for its birefringence pattern may extend the knowledge on fine cytoarchitecture of microtubular structures and optimal fertilization time required after oocyte thawing.

A low sodium/sodium-depleted, choline-based medium can significantly improve oocyte storage outcome in terms of survival after thawing, fertilization rates and in-vitro and in-vivo developmental competence (Stachecki et al., 1998, 2002). The replacement of sodium with choline in the base solution may prevent salt-induced cryopreservation injury (solution effect), resulting in improved survival rates and embryonic development in vitro. It is argued that equilibration in commonly used freezing media causes an excess of sodium to be pumped into the ooplasm. Since the functions of Na-K pumps may be impaired during the freeze-thaw process, Na could accumulate intracellularly, which is detrimental to cell viability. Unlike the sodium ion, the choline ion is not thought to cross the cell membrane and it may have a stabilizing effect on the membrane by protecting against freezing damage. Another protective mechanism of choline-based medium is believed to relate to an increase in viscosity at low temperatures. A total of 11 human live births have been reported in the literature using the choline-based freezing method (Quintans et al., 2002; Azambuja et al., 2005).

Boldt et al. (2003) compared two slow-freezing protocols: sodium-depleted (choline-substituted) freezing medium containing 1.5 mol/l 1,2 propanediol and 0.2 mol/l sucrose and seeding at −6°C versus a sodium-based freezing medium containing 1.5 mol/l propanediol and 0.1 mol/l sucrose with a seeding at −3°C. Among the two groups, the choline group showed a significantly higher survival rate than sodium-based group (74.4% versus 12.3%) after thawing. In the sodium-depleted group, the fertilization rate was 59%, and of 11 embryo transfers, four pregnancies and five live births were reported (Boldt et al., 2003). It is difficult to say whether the better outcome in the choline-group was attributable only to sodium-depletion in the freezing medium. The authors have used a higher sucrose concentration along with a higher seeding temperature, both of which have been reported to increase oocyte survival after thawing (trad et al., 1999; Fabbri et al., 2001). Despite the suggestions put forward that choline-based media could prevent the solution effect, the better survival and pregnancy outcome reported with sodium-rich basal media by several groups invite further well-controlled studies comparing sodium-containing and sodium-depleted cryopreservation media on human oocytes (preferably based on a good number of oocytes) or in non-human primate models on freeze-thaw outcome.

In spite of the pitfalls arising from the technique of oocyte cryopreservation, other biological factors such as the quality of the gametes, age of the patient and the in-vivo and in-vitro microenvironment of growing oocytes has to be considered (Mandellaum et al., 2004; Edgar and Gook, 2007). Among the various indicators of developmental potential of embryos derived from cryopreserved oocytes, day-3 blastomere number and an early cleavage at 25-h after insemination can be useful markers of selection (Edgar et al., 2000; Bianchi et al., 2005). One major obstacle is the extent to which the clinics can use various biological indicators of oocyte competence and embryo viability on cryopreserved oocytes, based on the strict legal restriction imposed in some European countries. Disturbances in the maintenance of Ca²⁺ signalling due to freezing and thawing-induced defects in mitochondrial polarity have also been reported (Jones et al., 2004). Using fluorescent Ayim reporter JC-1, it has been documented that cryopreservation can diminish the ability of pericortical mitochondria to retain high polarity and to form J-aggregates. The observation that some oocytes of infertile patients are J-aggregate negative and, therefore, developmentally compromised (Van Blerkom et al., 2002) need to be considered during oocyte cryopreservation experiments.

**Oocyte vitrification: replacing the traditional slow cooling strategies?**

Vitrification is a cryopreservation method during which solutions become solid, forming a glass-like state, which avoids crystallization (Rall and Fahy, 1985; Shaw and Jones, 2003). By preventing the formation of ice, vitrification also prevents the progressive concentration of salt and other solutes during cooling but at the expense of potential damage from a much higher initial concentration of cryoprotectant (Pegg, 2005). By increasing the cooling and warming rates to 20,000–200,000°C/min, however, this cryoprotectant concentration can be diminished to a less harmful level.

The vitrification technique is simpler, more convenient and more effective than the slow-cooling method (Vajta and Nagy, 2006). In 2001, Kuwayama et al. modified the vitrification technique by increasing the high rates of cooling and warming to reduce the concentration of cryoprotectants to 15% dimethyl sulphoxide (DMSO), 15% ethylene glycol (EG) and 0.5 mol/l sucrose in an a minimum volume cooling plate (Kuleshova et al., 1999; Katayama et al., 2003). This technique minimizes the damage associated with cryopreservation such as intracellular and extracellular ice crystal formation, chilling injuries and osmotic shock (Lucena et al., 2006). Over the years, the use of various types of vessels has been considerably refined to minimize the volume of solution needed to quickly submerge the carrier in liquid nitrogen. These vessels include cryoloop (Lane et al., 1999), open pulled straws (Vajta et al., 1998), cryotop (Kuwayama and Kato, 2000), cryotip (Kuwayama et al., 2005a), cryolafe (Chian et al., 2005), flexipet denuding pipette (Liebermann et al., 2002), electron microscope grids (Steponkus et al., 1990), direct cover vitrification (Chen et al., 2006). The cooling and warming rates for cryostraw (25 µl) are 2500 and 1300°C/min, respectively (Rall and Fahy, 1985), whereas these values for open pulled straws (1 µl), cryotop (0.1 µl) and cryotip (1 µl) are 16,700 and 13,900°C/min (Vajta et al., 1998), 23,000
and 42,100°C/min (Kuwajama et al., 2005b) and 12,000 and 24,000°C/min (Kuwajama et al., 2005c), respectively. In another study, Liebermann and Tucker (2002) found the hemistraw system to be a superior carrier than cryoloop and obtained a significantly higher (37.7% versus 29.4%) embryo compaction rate.

It is said that for a given cell, the maximum initial concentration that can be used without impairment of viability depends on the temperature, the rate at which the cryoprotectant is added and removed and the total time of exposure (Pegg, 2005). Although in the last few years, the use of slow-freezing protocols have increased the survival rate to 90% (Stachecki et al., 2006), the pregnancy rate per thawed oocyte remains poor (Boldt et al., 2006: 4.2% 15/361; Borini et al., 2006a: 2.3% 16/705), appearing to be much lower in comparison with vitrification (Kuwajama et al., 2005a: 11.2%, 12/107; Lucena et al., 2006: 8.2%, 13/159; Antinori et al., 2007: 11.8%, 39/330). Antinori et al. (2007) have also reported an implantation rate of 13.2% using cryotop vitrification of human oocytes (Figure 1). Very recently, Cobo et al. (2008) described an oocyte donation programme where the results achieved with vitrified versus fresh oocytes did not differ from those of unvitrified controls, with pregnancy rates over 60% in both groups after blastocyst transfer. Moreover, according to a recent investigation, 200 infants conceived following oocyte vitrification were not associated with an increased risk of adverse obstetric and perinatal outcomes (Chian et al., 2008).

Huang et al. (2007) compared vitrification with slow freezing using mouse oocytes and choline-based media. Survival, fertilization and early embryonic development rates were higher in the vitrification group. Analysis of spindle integrity and chromosome alignment indicated that there were fewer abnormalities in the vitrified oocytes than in the slow-frozen oocytes. In spite of that, the incidence of aneuploidy was similar between the two cryopreservation groups. Using a mouse model, Bos-Mikich et al. (1995) also found no increase in the incidence of aneuploidy following oocyte vitrification. Compared with vitrification, oocytes cryopreserved using slow freezing produced blastocysts with significantly fewer cells (including a lower inner cell mass:trophectoderm ratio) and reduced viability following embryo transfer (Lane and Gardner, 2001).

The results from the study by Huang et al. (2007) also indicate that most vitrified oocytes regain normal meiotic spindles and chromosome configuration after 1-h post-thaw incubation. In contrast, a significantly higher percentage of oocytes in the slow-freezing group exhibited abnormal morphology of spindle organization and chromosome alignment than the vitrified oocytes.

A number of studies have speculated that commonly used cryoprotectants and the type of cryopreservation process play a role in the deregulation of cell cycle machinery such as calcium oscillation and calcium signalling pathways (Takahashi et al., 2004; Larman et al., 2006; Larman et al., 2007). Larman et al. (2006) demonstrated that two cryoprotectants commonly used in vitrification protocols (DMSO and ethylene glycol) cause transient increases in intracellular calcium in mouse MII oocytes. The increase in calcium is sufficient to cause zona hardening, presumably through triggering cortical granule exocytosis, which is a calcium-dependent event (Kline and Kline, 1992). By removing extracellular calcium, it was demonstrated that the source of the calcium increase in response to ethylene glycol was mainly extracellular in contrast to intracellular for DMSO. In a continuation of their study, Larman et al.

![Figure 1](image.png)

Figure 1. Human metaphase II oocytes prior to, during and after various steps of vitrification using the Cryotop™ method. (a) Before vitrification; (b) in equilibration solution; (c) in vitrification solution; (d) in dilution solution; (e) in washing solution; (f) in the culture media 5 min after the final washing step.
(2007) verified that propanediol also causes a significant rise in intracellular calcium. Since calcium is a ubiquitous cellular signalling messenger, strict homeostatic regulation of the calcium concentration is of utmost importance for cellular functions. Moreover, integral cell cycle proteins such as M-phase-promoting factor and mitogen-activated protein kinase (MAPK) have been shown to be regulated by calcium (Marangos and Carroll, 2004). Osmotic pressure changes to the oocyte could induce oocyte activation (Whittingham, 1980) through Ca\(^{2+}\) release and thereby a high rate of cortical granule exocytosis and pronuclear formation. Amiloride, a pharmacological agent and an inhibitor of Na\(^+\)/H\(^+\) exchange, has been shown to inhibit cortical granule exocytosis and protein phosphorylation caused by osmotic pressure changes associated with cryopreservation (Inagaki et al., 1996). The treatment has also been reported to limit the chromosomal dispersion to some extent.

Although ICSI can circumvent zona hardening due to oocyte cryopreservation (Kazem et al., 1995; Porcu et al., 2000), most thawed oocytes that have undergone a cortical granule reaction have already undergone an activation process prior to actual fertilization that leads to deregulation of cell cycle control proteins. The deregulation of cortical granule exocytosis by the freeze–thaw process and possible upstream perturbations in Ca\(^{2+}\) signalling pathways and downstream fetal development anomalies should be addressed in future studies based on the report by Ozil and Huneau (2001). These authors reported the consequences of experimentally induced Ca\(^{2+}\) signalling on the morphology of fetuses derived from parthenogenetically activated rabbit oocytes. The observation that the use of calcium-free media during the freeze–thaw process reduces the abnormal increase in intracellular calcium and associated detrimental physiological effects justifies its use with common cryoprotectants (Larman et al., 2007). It should be noted, however, that excellent results can be achieved with common, calcium-containing media in human oocyte vitrification, as mentioned earlier.

Overall, babies born through the use of cryopreserved human oocytes do not have an increased incidence of abnormal karyotypes or congenital malformations (Porcu et al., 2000). However, the case number is still relatively small (around 600 to 1000 worldwide) and more data are needed to verify the results. Even though an upsurge of experimental evidence in the current literature has substantiated the beneficial effect of well-designed vitrification protocols for mature oocytes, it is possible (although not very likely) that better results may be achieved using classical controlled-rate freezing of oocytes, provided further improvements in each step of the procedure are made, especially in the rehydration step after thawing, a less well addressed area of research.

Sugars, antifreeze proteins and antioxidants: key players for a prospective outcome?

In nature, a variety of organisms such as arctic frogs, tardigrades, salamanders, some nematodes, rotifers, insects, brine shrimp, bacteria, yeasts, fungi and their spores and various plant seeds survive extreme dehydration conditions (Crowe et al., 1992; Potts, 1994). The inherent ability to survive such extreme conditions correlates with a spectrum of physiological adaptations that include the accumulation of large amounts of intracellular sugars. Among the sugars, trehalose, sucrose and glucose have been found to be particularly effective in the protection of macromolecules and subcellular structures from the harmful effects of excessive dehydration (Crowe et al., 1992). The addition of sugars to cryoprotectants has multiple benefits: sugars can cause an osmotic gradient across the cell membrane, which augments dehydration of the cell before freezing of extracellular water, and sugars are capable of preserving the structural and functional integrity of cell membrane at low water activities (Hotamisligil et al., 1996). Adding sugars to the vitrification solution could enhance viscosity, whereby incubation of cells in this solution before vitrification will help withdraw more water from the cells and reduce their exposure to the toxic effects of the cryoprotectants (Orief et al., 2005). Furthermore, it has been reported that a vitrification solution that contains added sugars could significantly improve the survival rate of vitrified bovine blastocysts (Saito et al., 1994). Toth et al., (1994) showed that the maturation rate of frozen human immature oocytes improved when sugars were added. An appropriate osmotic stress in the vitrification solution is an important factor for improving the maturation rate of vitrified–thawed immature oocytes, which could be regulated by sugars. Fabbri et al. (2001) demonstrated that increasing the sucrose concentration from 0.1 mol/l to 0.3 mol/l in a freezing medium improved the survival rate of human oocytes after a slow-freezing rapid-thawing programme and prevented the formation of intracellular ice. On the other hand, Mullen et al. (2004) verified that an increased sucrose concentration imposed a greater osmotic stress and increased the likelihood of damage to the spindle. Ultrastructural studies using different concentrations of sucrose in the freezing media indicate that a lower concentration of sucrose (0.1 mol/l) causes a more conspicuous loss of cortical granules than a higher concentration (0.3 mol/l) (Nottola et al., 2006), which might partly explain the better results in cryopreservation of MII oocytes employing a higher sugar concentration (Fabbri et al., 2001).

In a study by Eroglu et al. (2002), the use of a combination of small amounts (0.15 mol/l) of intracellular trehalose (a sugar being utilized by some organisms to cope with extreme freezing and desiccation) with its moderate extracellular (0.5 mol/l) concentration effectively protected human oocytes against cryopreservation-associated stresses in the absence of any other cryoprotectant. In a subsequent study, they showed that the microinjected trehalose was progressively eliminated during mouse embryonic development (Eroglu et al., 2005). However, such invasive procedures need to be further assessed with extensive molecular and developmental biological studies before they can be applied to human oocytes. An interesting observation is that bovine cumulus–oocyte complexes (COC) that were matured in vitro in the presence of trehalose had better membrane stability (assessed by the propidium iodide/Hoechst differential staining) after vitrification–thawing procedures than the controls (Berlinguer et al., 2007). However, there were no differences in the fertilization or cleavage rates between the test and control groups. Trehalose has been considered to be beneficial either in counteracting the osmotic effect or specifically interacting with membrane phospholipids (Crowe and Crowe, 1984). Kim et al. (1986) reported an improvement in the viability of mouse morula frozen ultra-rapidly in the presence of trehalose and found that trehalose is superior to sucrose during freezing. However, Abe et al. (2005) obtained similar results with either trehalose or sucrose...
during bovine germinal vesicle (GV)-stage oocyte vitrification using a stepwise protocol.

Another candidate that holds promise for cryopreservation is antifreeze proteins. Many species of ectothermic animals, plants and microbes inhabiting cold environments produce antifreeze proteins/polypeptides to protect themselves from freezing damage (Fletcher et al., 2001; Marshall et al., 2004). Since their discovery 35 years ago (DeVries and Wohlschlager, 1969), there has been a growing interest in the various families of antifreeze proteins (AFP), antifreeze glycoproteins and their mutants. AFP can lower the freezing temperature of a solution noncolligatively without affecting the melting temperature (thermal hysteresis) and thus can prevent freezing of body fluids (Liu et al., 2007). AFP can also inhibit ice recrystallization, of which the large ice crystals grow at the expense of smaller ones, thus preventing cell damage during freeze–thaw cycles. It is generally accepted that AFP function through adsorption of their flat ice-binding surfaces onto particular planes of ice crystals and prevent or inhibit further ice growth (Fletcher et al., 2001). Cryomicroscopic observations indicate that antifreeze glycoproteins cause phenomena that are typical of vitrification (absence of visible crystallization, transparency of medium) (Karanova et al., 1995), which is distinctly different to the cryoprotectant DMSO. Microinjection of AFP into teleost embryos has been shown to increase the survival rate significantly (Robles et al., 2006, 2007). AFP have unique properties: they can protect cell membranes from cold-induced damage (Rubinsky et al., 1991) and inhibit ice recrystallization (Knight and Duman, 1986). It should be noted, however, that the initial enthusiasm regarding the use of AFP has diminished in the new millennium, partially because of the achievements made using new vitrification techniques, where such supplementation did not result in a considerable improvement.

In the early 1990s, considerable work was published on AFP in cryopreservation. Recently, interest in AFP has given rise to new research amongst cryobiologists. Currently, a number of experimental approaches are being pursued to understand how antifreeze proteins interact with ice and thereby decipher the underlying molecular mechanism (Sander and Tkachenko, 2004; Liu et al., 2007; Wierzbicki et al., 2007). Theoretical modelling and molecular dynamic computer simulations of fish type-I kinetic ice inhibitor protein at the ice–water interface have recently been studied by some groups. This has provided new insight into the unique molecular properties that determine the ability of AFP to inhibit ice growth at the ice–water interface (Jorov et al., 2004; Yang and Sharp, 2005; Wierzbicki et al., 2007).

Adding antifreeze glycoproteins at 1 mg/ml has been shown to improve the fertility rates of mouse oocytes cryopreserved in 6 mol/l DMSO (O’Neill et al., 1998). The same authors have also reported that antifreeze glycoproteins could also be bound to the surface of growing ice-crystals, inhibiting the addition of any further water molecules. Positive results were also obtained with porcine embryos, which are highly susceptible to chilling injury because of their high lipid content (Chen et al., 1995; Fei et al., 1995). Hays et al. (1996) reported that antifreeze glycoproteins found in the blood of polar fish could inhibit leakage of intracellular contents across membranes during thermotropic phase transitions. AFP also holds promise in the preservation of ultrastructural functions of organs during the freeze–thaw process. Electron microscopy has shown that subzero cryopreservation of mammalian hearts for transplantation using type-I or type-II AFP (15–20 mg/ml) maintains myocardite structure and mitochondrial integrity (Amir et al., 2003). It is interesting to note that type-III AFP from ocean pout (Zoarcus americanus, a demersal eel-like species found in the northwest Atlantic from Labrador to Delaware), when expressed in transgenic mouse oocytes, was able to protect the fecundity of whole ovaries after vitrification (Bagis et al., 2008).

Since oocyte plasma membranes contain significant amounts of polyunsaturated fatty acids, they are particularly vulnerable to oxidative attack. It has been reported that lipid peroxidation of membrane phospholipids is adversely involved in embryo development. Some reports have indicated that cryopreservation can damage the antioxidant enzymes protecting against lipid peroxidation and that freeze–thaw stress can be modified by incubating the embryo in the presence of inhibitors of membrane lipid peroxidation (Tarin and Trounson, 1993). These results suggest the possibility that the process of cryopreservation induces the production of reactive oxygen species (ROS) or alters the antioxidant enzyme potential of oocytes, leading to lipid peroxidation of their plasma membranes and resulting in reduced survival potential by a perturbation of membrane structure and permeability. Loss of cell membrane function via lipid peroxidation might interfere with transport systems such as pH regulatory systems on the cell membrane, and disruption of organelle membranes could affect transport systems such as mitochondrial transport systems essential for oxidative phosphorylation, the major energy-generating pathway of the early embryo (Lane et al., 2002).

In addition, it is possible that loss of survival and fertilization competence following oocyte cryopreservation is also mediated by the mechanism of the cytotoxic action of peroxynitrite and NO2. Supplementation of 50 IU/ml of superoxide dismutase (SOD) or the concomitant addition of SOD and haemoglobin to the freezing and thawing media has been shown to improve survival and fertilization of mouse oocytes (Dinara et al., 2001). It has been suggested that peroxynitrite, formed by the interaction of O2 − and NO3, may exert a cytotoxic effect on mouse metaphase II oocytes during cryopreservation. In addition, inclusion of Lycium barbarum polysaccharide in the vitrification solution was shown to reduce the production of ROS, thereby preventing plasma membranes from lipid peroxidation and stabilizing membrane structure and permeability of porcine oocytes (Huang et al., 2008).

The work by Lane et al. (2002) demonstrates that including 0.1 mmol/l ascorbate when cryopreserving mouse cleavage-stage and blastocyst-stage embryos is beneficial to subsequent embryo development and maintenance of normal cell function. Ascorbate assists embryo development by stimulating development of the inner cell mass following cryopreservation. The beneficial effects of ascorbate were most evident in mouse embryos that were slow frozen compared with those that were vitrified, which substantiates the fact that the damage from oxygen radicals is greater after slow freezing. Ascorbate also reduced hydrogen peroxide generation significantly among vitrified embryos but failed to do so in the slow-frozen ones. These results indicate that the slow-freezing procedure augments the production of hydrogen peroxide considerably more than the ultra-rapid vitrification procedure. Future studies on antioxidative enzymes and NO scavengers may lead to a better understanding of the biochemical processes that occur during oocyte/embryo cryopreservation. Estimating the
total antioxidant capacity and measuring ROS concentrations in the follicular fluid of retrieved oocytes will help select patients whose gametes require antioxidant supplementation during cryopreservation and subsequent in-vitro cultures. The implication that ROS-mediated damage to oocyte developmental competence and embryo viability in infertile patients have raised concerns and need further research to identify the pathology behind this condition (Agarwal et al., 2005).

The expression of various biomarkers of oxidative stress such as SOD, Cu-Zn SOD, Mn SOD, glutathione peroxidase, glutamyl synthetase and lipid peroxides has been demonstrated in normally cycling human ovaries (Agarwal et al., 2005). A delicate balance exists between ROS and antioxidant enzymes in ovarian tissue. Because hypoxic conditions following retransplantation of ovarian tissues induce ROS generation and the freeze–thaw cycle depletes the tissue’s antioxidant capacity, antioxidant treatment might arguably be warranted. Studies of bovine ovarian tissue transplantation showed that ascorbic acid reduced apoptosis (Kim, 2003).

**Cryopreservation of immature oocytes: imminent challenge for cryobiologists**

Establishing technology that can vitrify oocytes at the prophase-I (GV) stage and other stages of maturation may also be important for clinical and experimental use. Because cumulus cells must be connected to oocytes through gap junctions for immature oocytes to mature, vitrification protocols may prove beneficial. Developing techniques that can successfully cryopreserve GV-stage oocytes will also aid GV transplantation, which requires a large number of donor GV oocytes. Removing GV oocytes from older women and transplanting them into younger enucleated oocytes has been proposed as a novel option for reducing aneuploidies (Takeuchi et al., 2001). This will also lead to an in-depth elucidation of the differences between nuclear and cytoplasmic maturation processes.

In GV oocytes, chromatin remains in a decondensed state. In addition, microtubule-organizing centres are not formed, and the microtubular system is not yet organized. The survival rate and developmental potential of these oocytes decrease after freezing. Cryopreservation of immature oocytes has been reported with varying degrees of success in several mammalian species such as mice (Eroglu et al., 1998b; Moffa et al., 2002), cattle (Suzuki et al., 1996), buffalo (Wani et al., 2004), pigs (Fujihira et al., 2004) and humans (Tucker et al., 1998b; Isachenko et al., 2006). Birth after slow freezing of immature human oocytes (Tucker et al., 1998b) and biochemical pregnancy after vitrification of immature human oocytes (Wu et al., 2001) has been reported. However, at least one study reported irreversible damage to the cytoskeleton of porcine GV and MII-oocytes after vitrification, which could be an important factor affecting developmental competence (Rojas et al., 2004). Protocols for the collection of fully grown GV-stage human oocytes have been devised by modifying mature oocyte collection techniques (Trounson et al., 1994) and by reducing hormonal stimulation. Several lines of evidence indicate that immature oocytes collected from stimulated ovaries are more resistant to cryopreservation and have lower incidences of chromosomal and spindle abnormalities than immature oocytes from unstimulated cycles (Baka et al., 1995; Park et al., 1997).

The freeze–thaw process can rupture the zona pellucida, cause cumulus cells to detach, and interrupt gap junctions between the cumulus cells and oocyte, further influencing subsequent oocyte development and maturation (Wu et al., 2006). Such structural changes might be attributed to severe dehydration resulting from osmotic effects of the cryoprotectants. To avoid formation of intracellular ice crystal during vitrification, it is essential for the oocytes to dehydrate (Fabbrì et al., 2000). On the other hand, dehydration more or less affects cellular structures. Therefore, damage to microvilli resulting from vitrification could reduce the viability of oocytes. Vitrification of immature porcine oocytes lead to a reduction of their mitochondrial matrix density, but the extent to which these changes affect oocyte development is unclear (Wu et al., 2006).

A porcine GV oocyte vitrification study by Wu et al. (2006) employed an open pulled straw vitrification method using 20% EG (equilibration) and 40% EG with 0.6 mol/l sucrose (vitrification solution). However, successful production of blastocysts was reported by Aono et al. (2003) following ultra-rapid vitrification with stepwise equilibration of GV-stage mouse oocytes. It is interesting to note that, in the above study, the blastocyst hatching rates were 8.8% for single-step equilibration, 15.6% for two-step equilibration and 22.8% for 10-step equilibration. This suggests that the process of gradual equilibrium conversion of the cryoprotectant agent seems to adjust the permeability of the plasma membrane, which may contribute to maintaining the connection between the oocyte and cumulus cells and/or a decrease in rapid changes in osmotic pressure. The three-dimensional COC is likely to be particularly prone to physical disruption caused by ice crystal formation. Even in the absence of ice crystal formation during vitrification, the vast difference in size between the oocyte and its associated cumulus cells means that they are likely to react differently to the stresses applied during cryopreservation. The same group has reported a modified protocol for stepwise pre-equilibration called SWEID, which ultrarapidly vitrifies mouse GV oocytes. After cryopreservation, the GV oocytes were fertilized in vitro and cultured. Some of the 2-cell-stage embryos were then transferred, resulting in live birth (Aono et al., 2005).

Abe et al. (2005) reported the favourable effect of stepwise pre-equilibration prior to vitrification of bovine immature oocytes. They showed that after stepwise exposure to cryoprotectants, the in-vitro maturation rate and the normality of cytoplasmic organelles in the vitrified GV oocytes were most similar to those in the control, resulting in production of viable blastocysts. In the single-step exposure, most oocytes showed a highly vacuolated cytoplasm with many ruptured mitochondria (Abe et al., 2005). Since vitrification is a non-equilibrium cryopreservation method that needs relatively high concentrations of cryoprotectants, a stepwise addition of cryoprotectants may reduce the toxic effect of cryoprotectants and minimize damage due to extreme cell-volume expansion (Rall, 1987).

Chilling injury, a major cause of irreversible changes to the cytoplasmic membranes, occurs at subphysiological temperatures during the transition of lipids from the liquid crystalline phase to the gel phase (lipid phase transition). The phospholipid:cholesterol ratio and nature of the fatty acid composition of oocyte cytoplasmic membranes – which show varying seasonal as well as inter- and intra-species variation – could also account for the complexity of oocyte survival during the freeze–thaw process. Experimental manipulation of the lipid-phase transition temperature by...
polysaturated fatty acids has been shown to reduce chilling injuries to oocytes and embryos (Hochi et al., 1999; Zeron et al., 2002). It is believed that, in addition to being an integral part of the cell membrane, cholesterol is also present in the lipid-enriched microdomains (lipid rafts), which can influence receptor functions (Westover et al., 2003). Since the maturation process of frozen–thawed immature oocytes requires several growth factors and proper interaction between the oocytes and receptors, the integrity of the cell membrane, with its functional ligand-receptor system, needs to be preserved during cryopreservation.

Embryo development status improved when bovine oocytes were treated with cholesterol-loaded methyl-β-cyclodextrin (CLC) prior to vitrification (Horvath and Seidel, 2006). Moreover, when oocytes were exposed to cyclodextrin containing 4-nitrobenzo-2-oxa-1,3-diazol-labelled cholesterol for about 1 h, fluorescence was observed in both the COC and oocytes, indicating that oocytes as well as cumulus cells incorporated the cholesterol from CLC through diffusion. However, the results also showed that CLC may be effective in incorporating the cholesterol into cell membranes only when it is added to chemically defined media. Otherwise, the cholesterol may be sequestered in the presence of additives such as fetal calf serum.

Isachenko and Nayudu (1999) have reported a high survival rate and meiotic normality together with good preservation of attached cumulus cells using a simple new vitrification procedure. They showed that 37°C pre- and post-freeze exposure could significantly improve both survival and normal spindle configuration of mouse GV oocytes after in-vitro maturation. Egg yolk (a natural complex mixture of phospholipids and antioxidants) was found to produce further beneficial effects on both the oocyte and cumulus cell integrity, with the best effects being obtained at 37°C with the inclusion of egg yolk both before and after the freezing. With this protocol, the normal survival on post-thaw was greater than 80% with an intact and attached cumulus complex. In addition, there was an 84% maturation rate and a 45% normal metaphase configuration. However, concerns regarding possible disease transmission hampered widespread use of egg yolk, and a similar effect could also be achieved with other protein supplements. It is suggested that, based on the interrelationships of the cytoskeleton and the cellular membrane (Younis et al., 1996), additives that protect the integrity of the cellular membrane during each step of cryopreservation might also be expected to produce an improvement in the survival and normality of the oocytes after exposure to cryoprotectants and cooling (Isachenko and Nayudu, 1999).

It is well documented that the oocyte cytoskeleton is damaged by the cryopreservation process, which could lead to significant changes in the organization and trafficking of molecules and organelles (Vincent and Johnson, 1992). The completion of several dynamic events, including homologous chromosome separation, spindle anchorage, spindle rotation, vesicle and organelle transport and pronuclear apposition (mouse) requires interaction between microfilaments and microtubules in oocytes (Sun and Schatten, 2006). Another study also emphasizes that the dynamic equilibrium between polymerized and free tubulins in mammalian oocytes is extremely sensitive to temperature change (Zenses et al., 2001). Younis et al. (1996) studied the deleterious effect of glycerol (not a common cryoprotectant for female gametes) at different equilibration temperatures on F-actin organization of rhesus monkey oocytes (GV stage). They found a lower destabilizing effect of glycerol when the addition was combined with low temperature.

The use of DMSO has produced several beneficial effects on oocyte microfilaments similar to glycerol (Vincent et al., 1990). In addition to the changes in F-actin organization, an irregular shrinkage pattern was observed when non-human primate GV oocytes were exposed to hypertonic solutions. Interestingly, MII oocytes under similar conditions showed a uniform shrinkage pattern. The irregular shrinkage of GV oocytes is said to be caused by differences in the microfilament organization in oocytes at various maturational stages, particularly in reference to the transzonal projections, which anchor the plasma membrane of GV and MI oocytes to the zona pellucida and corona cells (Younis et al., 1996). GV oocytes treated with ethylene glycol tetracetate acid – a calcium chelator that disengages junctional contacts – showed a uniform shrinkage pattern after exposure to hypertonic conditions. Ultrastructural study has revealed that the disruption of the actin filament system caused by DMSO was associated with changes in the lengths and distribution of oolemma microvilli.

The freeze–thaw process could cause irreversible changes in nuclear and cytoplasmic organization in immature oocytes in spite of an apparent normal progression to the MI stage shown by polar body extrusion (Boise et al., 2002). Since microtubules play a major role in mediating cytoplasmic maturation during the early stages of meiosis by facilitating mitochondrial and mRNA distribution and cortical positioning of the MI spindle, disruption of the microtubule assembling or the depolymerization/polymerization dynamics during cryopreservation could have a major negative impact on development. The MI block and erratic MI spindle usually observed following the post-thaw maturation stage are suggested to be due to detrimental influences on MAPK activities that are initiated after GV breakdown and remain at a high level throughout the transition from MI to MII (Lu et al., 2002; Comizzoli et al., 2004). Comizzoli et al. (2004) recently showed that exposure of cat GV-stage oocytes to 1.5 mol/l propanediol at 25°C had little effect on cell cycle regulatory mechanisms as observed by a normal developmental pattern and chromosome integrity, which was similar to that of the controls. However, EG appeared to be more harmful to the cat oocyte than propanediol and was suggested to have a possible detrimental effect on M-phase promoting factor (MPF) and MAPK regulators (c-mos, cyclin B) or other factors influencing meiotic progression and cytokskeletal organization (Wu et al., 1999; Kim et al., 2000) during the MI–MII transition (Comizzoli et al., 2004). Dobrinsky et al. (2000) reported increased survival of porcine blastocysts following treatment with cytochalasin B before vitrification. Verlhac et al. (2000) showed that the Mos-MAPK pathway controls the activity of the actin microfilament network, perhaps through myosin IIα in mouse oocytes, and proper positioning and active movement of organelles are essential for oocyte growth, maturation and fertilization.

The literature documents contrasting results regarding human oocyte survival rate after cryopreservation with or without the cumulus oophorus. In one study, the presence of the cumulus mass or the partial or total removal of the cumulus cells did not significantly modify the oocyte survival rate (Mandelbaum et al., 1988). Similar results were obtained by Fabri et al. (2001) who used a higher sucrose concentration. However, Gook et al. (1993) observed reduced survival of oocytes that were cryopreserved along with the cumulus corona mass. In contrast, Imoedemhe and
Sigue (1992) reported a better survival rate in oocytes that were frozen intact with cumulus cells (54%) than in denuded oocytes (27%) where they used 0.25 mol/l sucrrose in the cryoprotectant agent. It seems that the cryopreservation regimen dictates the survival rate of cumulus-enclosed oocytes. It is suggested that the cumulus mass may offer some protection against sudden osmotic changes and stresses that could occur by rapid influx/or efflux of the cryoprotectant agent during freezing and thawing procedures (Fabri et al., 2001). One explanation for the greater osmotic sensitivity in the GV oocytes is that their hydraulic conductivity is two times lower than that of in-vitro matured MII oocytes (Agca et al., 2000). Since oocyte–somatic cell interactions are vital for the cytoplasmic and nuclear maturation of immature oocytes (Albertini et al., 2001), it is important to design freeze–thaw protocols for an intact COC that are beneficial in view of recent developments in in-vitro maturation systems. Lack of proper meiotic competence, fertilization and embryo development of frozen–thawed and in-vitro cultured GV-stage oocytes, even in the presence of fresh cumulus cells, indicate that intercellular connections (gap junctions) mediated transfer of paracrine factors are mandatory for in-vitro maturation of cryopreserved cumulus-enclosed immature oocytes (Ruppert-Lingham et al., 2003).

The study by Ruppert-Lingham et al. (2003) on the effect of DMSO exposure and cryopreservation of GV COC indicates that cumulus–oocyte cross-connections remain intact when exposed to the cryoprotectant, but the disruption is initiated due to the freeze–thaw procedure. Two other studies have also reported a loss of cumulus cells from COC following the freeze–thaw process using slow-cooling programmes (Cooper et al., 1998; Goud et al., 2000). Low-sodium (choline-based) media were used by Goud et al. (2000) on immature human oocytes, and they found no differences in the maturation rates when cumulus-enclosed GV oocytes were compared with the controls. They did, however, report a considerable loss of cumulus cells after thawing. Although ICSI has been advocated for the fertilization of cryopreserved and thawed oocytes, classical IVF must be promoted along with the need for a cryopreserved and intact COC (Li et al., 2005; Coticchio et al., 2007). Cellular and genetic perturbations emanating from the stressful process of cryopreservation combined with the effects of microinjection cannot be ruled out as having an effect on the molecular structure of oocytes and causing long-term post-natal consequences. One recent study found untoward effects of the ICSI procedure on the wellbeing of children born by this procedure and the deregulation at the epigenetic level (Varghese et al., 2007a).

**Zygote and cleavage-stage embryos: are slow-cool freezing methods giving way to vitrification?**

Since the first pregnancy after replacement of frozen–thawed human embryos was reported (Trounson and Mohr, 1983), embryo cryopreservation has become a routine technique in assisted conception programmes. The use of frozen embryos together with refinements in embryo quality evaluation have facilitated the current shift of embryo transfer policy towards single-embryo transfer with the consequent reduction of the risk of unwanted multiple pregnancies. However, overall, cryopreservation leads to a 30–40% reduction in the implantation potential (Edgar et al., 2000; El-Toukhy et al., 2003). Cytoplasmic dysmorphism of the oocyte has been shown to affect the developmental capacity of the derived embryos in fresh IVF cycles. A similar adverse effect is reflected in freeze–thaw cycles with the developmental capacity of frozen–thawed embryos related to the morphology of the oocyte from which they originate (Balaban et al., 2006). It has been suggested that the success rate of embryo cryopreservation can be improved by selecting embryos for transfer based on their capacity for sustained cleavage during a 24-h culture period in vitro. In this context, freezing embryos at the zygote stage may be an interesting option based on recent reports showing high survival and pregnancy rates using zygote-stage vitrification and post-thaw culture for 24 h before embryo transfer (Al-Hasani et al., 2007): in this study of 849 vitrified pronuclear zygotes, 339 pronuclear zygotes were thawed, resulting in an 89% survival rate and a pregnancy rate of 36.9%, both of which are higher than those obtained with the slow-freezing method (10.2%). The post-thaw embryo cleavage rate in vitro is also a good predictor of the embryo implantation rate (Van der Elst et al., 1997). Based on the observation that freeze–thaw cycles may have some deleterious (hardening) effects on the zona pellucida, partial zona dissection or laser-assisted hatching may be beneficial in improving the implantation rate following transfer of frozen–thawed embryos. Even though partially damaged embryos following cryopreservation can give rise to term pregnancies, all recent studies involving high numbers of frozen-embryo transfer cycles agree that the developmental potential of partially damaged frozen–thawed embryos is inferior to that of embryos that remain fully intact after thawing. It is postulated that damaged blastomeres exert a toxic effect on the growth potential of intact ones. In a recent randomized prospective study, there was no difference in pregnancy/implantation rates when lysed cell removal was performed on thawed embryos (Prados et al., 2006).

Another line of investigation has suggested that the type of ovarian stimulation regimen affects oocyte and embryo quality and the success of frozen-embryo transfer cycles. The type of gonadotrophin preparation used for ovarian stimulation may influence embryo quality and its potential to implant in fresh cycles. A recent study showed that a higher proportion of embryos derived from IVF patients stimulated with highly purified human menopausal gonadotrophin survived cryopreservation than those stimulated with recombinant FSH and appeared to have a higher potential for development and implantation (Ziebe et al., 2006). These studies confirm that it is not only the refinements in cryopreservation protocols that will improve results of frozen-embryo transfer cycles but also derivation of good-quality oocytes and thereby embryos with optimal genetic integrity and physiological status via proper clinical management (such as ideal gonadotrophin treatment).

Although embryos subjected to vitrification are likely to be injured by the toxicity of the high concentration of cryoprotectant, many vitrification methods have been refined in recent years and proved effective for the cryopreservation of embryos at various stages of development in laboratory and domestic species. In 1998, it was shown that vitrification using an EG-based vitrification solution (EFS40) (Kasai et al., 1990) with conventional cryostraws was effective for human embryos at the 4–8-cell stage (Mukaida et al., 1998). The effectiveness of vitrification was confirmed for human embryos at the 8–16-cell stage (Saito et al., 2000) and the morula stage (Yokota et al., 2001) also using EG-based vitrification solutions. Human blastocysts are much less permeable to cryoprotectant and water and have been observed to shrink more rapidly than it takes conventional cryopreservation.
slowly than mouse and bovine blastocysts when placed in the cryoprotectant solution. This suggests that they are more likely to be injured by intracellular ice. One major reason for this has been attributed to the blastocelic fluid. Artificial reduction of the blastocoele cavity prior to the vitrification procedure has yielded favourable results. Among a total of 725 vitrified blastocysts, high rates of survival (80%) and pregnancy (37%) were reported by Mukaida et al. (2003) using cryoloop-based vitrification.

A recent study demonstrates that the average characteristics of pregnancies and labour as well as perinatal health exhibited by infants (n = 34) born as a result of embryo transfer after vitrification are not different from that of the overall population average (Potapov et al., 2006). To vitrify the embryos, this group used a solution containing EG, trehalose, Ficoll, hyaluronan and human serum albumin and 0.25 ml cryostraw as the vessel; the total exposure time was 5 min. The embryos were then plunged into liquid nitrogen. As mentioned earlier, babies born after vitrification of oocytes did not exhibit increased rates of developmental abnormalities (Chian et al., 2008).

Genomic and post-genomic approaches in cryotechnology

Global embryonic gene expression in humans occurs at the 4–8 cell stage (Braude et al., 1988). The maternal proteins and mRNA that were synthesized during oogenesis are responsible for the initial cleavages of mammalian embryos. Alterations in gene expression patterns detailed by reverse transcription polymerase chain reaction or microarray analysis can be of help to elucidate the effects of ovartin stimulation, culture conditions and any in vivo or ex-vivo manipulations such as cryopreservation.

Tachataki et al. (2003) has shown that there is a gradual decrease in the expression pattern of the TCS2 gene throughout human preimplantation development; the level of expression is high during the unfertilized state and falls to a low level when global activation of the embryonic genome occurs. This gradation of the transcriptome expression pattern was lost in unfertilized aged oocytes. There was a considerable drop in TCS2 mRNA when D-2 embryos were rapidly thawed. This drop was not significant when D-3 embryos were subjected to such protocols. The study revealed that human embryos frozen on day 2 appeared to be more susceptible to temperature change than embryos frozen on day 3, which may be a consequence of the activation of the embryonic genome.

The transcriptome analysis of cells and tissues by microarray techniques and further elucidation of the molecular and functional status of cells under particular conditions by proteomic and metabolomic approaches hold great promise in assisted reproductive technologies to improve the success rate (Katz-Jaffe and Gardner, 2007; Singh and Sinclair, 2007; Varghese et al., 2007b). Temperature reductions are known to negatively affect the stability of RNA secondary structures, which have been suggested to lead a rate-limiting step of translation initiation in prokaryotes (Zangrossi et al., 2000). The gene expression profile as assessed by microarray techniques showed that vitrification of embryos results in a broad spectrum of consequences: cell metabolism and regulatory- and stress-related genes seem to be most effected (Mamo et al., 2006). Several of these enhanced biological processes as revealed by overrepresentation of transcriptomes are said to compensate for cold-related reduction in enzyme activities and to synthesize stress-protective molecules. Interestingly, of all the genes studied for cold response, a higher number of differentially expressed genes (22/180, 12.2%) were located in chromosome 7 in mice. This study identified several new candidate marker genes for cryosurvival. In cryopreserved mouse embryos and ovarian tissues, cold response gene expression patterns have indicated that embryo growth is arrested and that DNA damage-inducible genes are present (Liu et al., 2003; Mamo et al., 2006). The results of these studies may help further improve cryopreservation protocols.

In addition, recent analysis of the oocyte proteome using time-of-flight mass spectrometry following cryopreservation revealed that slow freezing significantly affects protein expression (Larman et al., 2007). When proteomics was used to compare slow freezing and vitrification methods, the results showed that vitrified MII oocytes closely resembled the non-frozen controls whereas the MII oocytes that underwent slow freezing exhibited markedly different protein profiles with either up- or down-regulation of 19 positively charged and 21 negatively charged proteins (P < 0.05). Further analysis of the oocyte’s proteome revealed that it was during the dehydration stage prior to seeding in the slow-freezing protocol when these alterations occurred (Gardner et al., 2007). The observation that specific proteins were significantly up-regulated after slow freezing is said to be associated with the length of time the oocytes are exposed to the toxic cryoprotectant. This exposure may induce stress-related responses and the up-regulation of stress proteins and/or apoptosis. In contrast, vitrification has a minimal impact, indicating that it has a fundamental advantage in the cryopreservation of oocytes.

Kim et al. (2006) studied the proteome of cryopreserved bovine ovarian tissue. They found alterations in protein profiling and expression in ovarian grafts after transplantation. Most of the significant proteins identified after transplantation were proteins related to tissue survival and metabolism such as actin, laminin and antioxidant (glutathione S-transferase). Glutathione-S-transferase is an enzyme that is usually up-regulated in oxidative stress, and mRNA for this enzyme has also been shown to be up-regulated in mouse embryos under cold response (Mamo et al., 2006). Among the slow-freeze and vitrification groups, changes in the protein profile of the vitrified and transplanted ovarian tissue were more consistent in the fresh controls (Kim et al., 2006).

Low-molecular-weight metabolites signify the end-products of cell regulatory processes and therefore reveal the response of biological systems to a variety of environmental or genetic influences. As physiological changes to a cell resulting from gene overexpression or deletion/silencing are amplified through the hierarchy of the transcriptome and proteome, these changes are said to be more readily measured through the metabolome (Singh and Sinclair, 2007). Moreover, the metabolome is downstream of gene function and therefore considered to be a superior measure of cellular activities at the physiological level compared with transcriptomic and proteomic approaches. The metabolic profiling of frozen–thawed embryos has been reported as a novel marker of embryo viability and growth potential irrespective of the embryo grade (Stokes et al., 2007). In spite of reassuring data on the post-natal safety of human embryo cryopreservation, animal studies indicate that frozen–thawed embryos could affect metabolism in the late preimplantation development (Emiliani et al., 2000) and have
more subtle long-term, as well as post-natal, effects that are manifested late in development (Dulioist et al., 1995). It is suggested that cryopreservation-induced stress may alter homeostasis, metabolism, cell integrity and developmental potential (Stokes et al., 2007). Cryopreserved embryos with the capacity to develop to the blastocyst stage had a lower rate of amino acid depletion, appearance and turnover than arresting embryos. Metabolic parameters are therefore imperative when identifying treatment protocols that least interfere with key cellular functions of the oocytes (Gardner et al., 2007). Furthermore, when monitoring metabolism through pyruvate uptake is considered, mouse oocytes and developing embryos following slow freezing were shown to be more metabolically impaired than those that were vitrified (Lane and Gardner, 2001; Lane et al., 2002). Analysis of membrane integrity through the retention of the enzyme lactic dehydrogenase also indicated that slow freezing induces significantly more plasma membrane damage than vitrification (Lane et al., 2002). Non-invasive footprinting of glycolytic activity (Lane and Gardner, 1996) and knowledge of fatty acid metabolism and uptake of embryos (Haggarty et al., 2006) may also be used one day to help select cryopreserved/vitrified embryos. Research so far indicates that vitrification procedures have less of an impact on oocyte/embryo proteome and energy metabolism than slow freezing.

Conclusions

‘A tendency to adopt techniques widely when they show promise often occurs at the expense of further refinement.’ Gook DA and Edgar DH (1999).

Although animal oocytes and germinal tissues have provided extensive information on the biological effects of the freeze–thaw process, caution must be exercised when extrapolating results from the animal studies to human applications because of the proven variability in the chill sensitivity of oocytes (Stachecki et al., 2004) and tissues among different species. In this regard, non-human primate models (Songsasen et al., 2002) may offer a useful experimental system because of the close relationship between Homo sapiens and other primates based on an evolutionary and genetic point of view. Moreover, there is much to learn from studying cold adaptation in some organisms – which is the result of millions of years of evolution – and the organic molecules that protect their protoplasm from extreme desiccation. The use of sugars and antifreeze proteins in the cryopreservation of gametes and embryos need to be studied further. A complex and as yet poorly understood growth and differentiation process that comprises several months of co-ordinated intra-ovarian development yields healthy and viable oocytes in human beings. Consequently, mimicking normal oogenesis and folliculogenesis in transplants at heterotrophic sites of the body or in vitro are major challenges (Smirtz, 2004). Although the use of preimplantation genetic diagnosis has been advocated in clinical oocyte-freezing programmes (Boiso et al., 2002), the invasive nature of the procedure on the wellbeing of the resultant embryos is still being debated. Non-invasive methods such as proteomic and metabolomic profiling of the embryo-spent media and EmbryoScope™-based measurements of conceptus respiratory and blastomere activity have already been reported (Lopes et al., 2006). This may soon offer a suitable selection method for cryopreserved gametes and embryos.

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