Symposium: Cryopreservation and assisted human conception

An overview of oocyte cryopreservation

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Abstract

The ability to cryopreserve human oocytes and store them indefinitely would be beneficial for cancer patients at risk of becoming sterile after therapy, allow women to delay reproduction, and alleviate religious concerns associated with embryo storage. In 1986, Chen was the first to report a pregnancy originating from a frozen–thawed human oocyte. Although over 100 babies have been born from oocyte storage since then, pregnancy rates remain unacceptably low. Adapting embryo cryopreservation techniques to oocyte storage has had limited success and new reproducible methods are needed. Problem areas other than intracellular ice formation and osmotic effects need to be identified. A broad approach of critical analysis should be conducted regarding the entire cryopreservation process from pre-equilibration and cooling, to thawing and step-out. All established facets deserve reanalysis in order to assess which aspects can be optimized or changed so that cellular demise can be avoided and cellular viability enhanced. New methods, including the use of choline-based media and vitrification have proven useful in increasing survival and pregnancy rates in some clinics. Other methods yet untested, such as injection of complex carbohydrates into the oocyte, deserve further studies. Vitrification research has led to the formulation of new ideas and has demonstrated the flexibility of cells to survive cryopreservation. Although successful, vitrification protocols are potentially harmful and technically challenging, due to elevated cryoprotectant concentrations and rapid cooling rates. Bovine embryo vitrification methods have been used to store human oocytes and embryos, particularly blastocysts with some success. Vitrification solutions containing high molecular weight polymers have also proved beneficial by reducing solution toxicity. In general, further advances are needed to improve human oocyte storage before widespread routine clinical use.

Keywords: *choline chloride, cryopreservation, human, mouse, oocytes*

Introduction

Cryopreservation of gametes and embryos is highly desirable for numerous reasons, primarily to store excess genetic material and to control the timing and precision of embryo transfer. Oocyte storage is especially appealing because it: (i) allows women to delay reproduction until later in life, for instance after establishing a career, thereby providing more reproductive choices; (ii) can help alleviate religious or other ethical concerns of embryo storage; (iii) makes 'egg banks' possible, eliminating donor–recipient synchronization problems and standardization of the number of eggs for the recipient; and (iv) permits women at risk of becoming sterile to cryopreserve oocytes prior to radio- or chemotherapy or ovariectomy.

Since the discovery of glycerol's protective effects on sperm cells in 1949, both gametes and embryos have been successfully frozen from a number of mammalian species including mice, sheep, cows, pigs, horses, hamsters, rats, cats and humans. Throughout the 1950s and 1960s, the foundation of modern cryobiology was laid by Lovelock, Meryman, Polge, Smith, Levitt, Luyet, Mazur, and others. During the 1970s, further discoveries by Whittingham, Willadsen, Liebo,

Mazur and Wilmut prepared the way for human oocyte and embryo storage in the 1980s. The last 20 odd years up to 2000 have seen refinements of protocols for human embryo and sperm storage, such that they have become a routine procedure in IVF clinics throughout the world. Nevertheless, the techniques remain inefficient, especially considering oocyte cryopreservation.

The purpose of this paper is to review the existing practices for human oocyte cryopreservation, to discuss the potential biological and technical hurdles, and to propose avenues for improvement.

Background

In 1986, Chen was the first to report a pregnancy originating from a frozen–thawed human oocyte. This work came under scrutiny when it was suggested that cryopreservation showed higher levels of chromosomal anomalies when compared with fresh eggs (Johnson and Pickering, 1987; Pickering and Johnson, 1987; Sathananthan *et al*., 1988). This seriously slowed work in human oocyte cryopreservation, such that during the 9 years following the first pregnancy, only a few (including Chen himself) achieved pregnancy originating from a frozen–thawed human oocyte; a total of only five births up to 1995 (Al-Hasani *et al*., 1987; Van Uem *et al*., 1987; Chen, 1988). Meanwhile, embryo cryopreservation had not only become routine, but increasingly more successful. Oocyte freezing remained a low priority for clinicians, not only because of the unacceptably low success rate, but also because continuing murine work did not at first allay fears of possible chromosomal abnormalities.

Another potential problem was that changes induced by freezing and thawing significantly inhibited fertilization in mouse oocytes through a process referred to as 'zona hardening' (Vincent *et al*., 1990; Wood *et al*., 1992; George and Johnson, 1993). Cortical granule release that occurs naturally during sperm–oocyte fusion acts to block polyspermy through alteration of the zona proteins ZP2 and ZP3; however, Wood *et al*. (1992) found no evidence to support the hypothesis that cortical granules were prematurely released during cryopreservation. In a later study by Carroll *et al*. (1993), it was discovered that addition of fetal calf serum to the freezing and thawing medium significantly improved fertilization rates after murine oocyte cryopreservation.

Further research on mouse and human oocytes started to show that cryopreservation was not as detrimental as prior studies suggested (Gook *et al*., 1993, 1994). Also, intracytoplasmic sperm injection (ICSI) was being used to ensure fertilization and prevent polyspermia of frozen–thawed oocytes (Gook *et al*., 1995; Kazem *et al*., 1995). After 1995, Tucker and Massey in the United States, Fabbri and Porcu in Italy, and other laboratories around the world had produced babies from frozen–thawed oocytes (Tucker *et al*., 1996, 1998; Porcu *et al*., 1997, 1998, 1999a,b; Antinori *et al*., 1998; Borini *et al*., 1998; Nawroth and Kissing, 1998; Polak de Fried *et al*., 1998; Vidali *et al*., 1998; Yang *et al*., 1998, 1999; Young *et al*., 1998; Porcu, 1999). After some years, the number of normal children born from frozen oocytes was over 20.

By the year 2000, there was renewed interest in egg freezing (Cha *et al*., 2000; Porcu *et al*., 2000, 2002; Fabbri *et al*., 2001; Porcu, 2001; Yang *et al*., 2002; Boldt *et al*., 2003; Fosas *et al*., 2003; Marina and Marina, 2003). Some cryopreservation protocols employed conventional slow cooling; some used variations of rapid freezing and vitrification; and investigations in this area became numerous (Pensis *et al*., 1989; Hunter *et al*., 1995; Hong *et al*., 1999; Kuleshova *et al*., 1999a; Chen *et al*., 2000; Chung *et al*., 2000; Ali, 2001; Wu *et al*., 2001; Kuleshova and Lopata, 2002; Liebermann and Tucker, 2002; Wininger and Kort, 2002; Liebermann *et al*., 2003; Yoon *et al*., 2003).

To date (2004) there have been around 100 children born from oocyte freezing, but the number of offspring produced per number of oocytes frozen was seldom greater than 1–5%. Marina and Marina (2003) reported four births from 99 oocytes frozen (4%). Porcu *et al*. (1999a) have done the majority of work in clinical human oocyte freezing, and reported 16 pregnancies from 1502 thawed eggs (1%). Yang *et al*. (1999) reported seven pregnancies using 120 oocytes (5.8%). Ahuja *et al*. (1998) reported a 2% average number of pregnancies in UK IVF centres. All this shows that the use of human embryo cryopreservation protocols for freezing human oocytes is very inefficient.

Even with modification of freezing protocols and an improvement in survival rates, it is difficult to obtain a high percentage of fertilized and normal cleaving embryos after oocyte cryopreservation. Initial survival rates for frozen–thawed human oocytes are around 50–70% of all oocytes frozen, but that number can be significantly reduced after pronuclear formation (fertilization), and cleavage beyond the 2-cell stage. As a notable exception, Marina and Marina (2003) reported similar pregnancy rates for oocytes and embryos after cryopreservation; but to the authors' knowledge, this work has not been reproduced elsewhere.

Genetic state of oocytes

The reasons why oocytes are more difficult to cryopreserve than embryos are uncertain, so investigators looked into differences between the two cell types inasmuch as they affect freezing and thawing. The most obvious is their genetic state, as the DNA of mature, unfertilized oocytes is compacted into chromosomes that are aligned on a metaphase plate, while the majority of DNA in embryos exists as decondensed chromatin at interphase. Cryobiologists realized early on the importance of the cell cycle for understanding cellular viability. It can therefore assumed that the physical state of the DNA may be altered by cryopreservation, and the chromatin and spindle structure and cryoprotectant exposure have been analysed before and after cooling. It is known that the spindle becomes disorganized and the DNA could therefore be altered; so the genetic integrity of resulting embryos is suspect. However, it is unlikely that the physical state of the DNA would effect oocyte survival (membrane intactness) upon thawing.

Physical conditions, including temperature, and chemicals such as taxol, colcemid, nocodazole, as well as cryoprotectants like dimethylsulphoxide (DMSO) and 1,2-propanediol (PrOH), can affect microtubule polymerization (for review, see Vincent and Johnson, 1992). Alterations to the second meiotic spindle caused by cryoprotectant exposure and/or temperature change have been well documented. Specific alterations, including the depolymerization of microtubules during the

cryopreservation of metaphase II oocytes, pose a potential problem for normal spindle function after storage. Spindle depolymerization during the cryopreservation process may be irrelevant to future survival and development. Whether the spindle can re-polymerize and segregate chromosomes correctly after cryopreservation is the more important question.

Indeed, it may not matter if the spindle depolymerizes, so long as it can regain functionality. Some reports say the spindle apparatus of cryopreserved MII oocytes depolymerizes and can only rarely reform properly. Other studies show that it usually does repolymerize correctly (human: Sathananthan *et al*., 1988; Pickering *et al*., 1990; Gook *et al*., 1993; Almeida and Bolton, 1995; Baka *et al*., 1995; Zenzes *et al*., 2001; Boiso *et al*., 2002; murine: Johnson and Pickering, 1987; Pickering and Johnson, 1987; Sathananthan *et al*., 1988, 1992; Van der Elst *et al*., 1988; Aigner *et al*., 1992; Gook *et al*., 1993; Eroglu *et al*., 1998; bovine: Aman and Parks, 1994; Martino *et al*., 1996b; Saunders and Parks, 1999; Wu *et al*., 1999; rabbit: Vincent *et al*., 1989). One recent study reported the minute-to-minute changes in polymerization state during cooling of human oocytes (Zenzes *et al*., 2001). In general, the differences between these reports lie in the details of how the cryopreservation procedure occurred. There is general agreement that the microtubules of mammalian oocytes will depolymerize during cryopreservation. However, cryopreservation is not necessarily lethal if the spindle does not repolymerize following thaw. Atypical protocols rather than the generic process may lead to spindle failure in oocytes.

Indeed, most studies lack follow-up data correlating spindle de- and repolymerization with function. Some studies have scrutinized fertilization and development and analysed the state of the chromosomes after cooling or cryopreservation (murine: Glenister *et al*., 1987; Bongso *et al*., 1988; Kola *et al*., 1988; Aigner *et al*., 1992; Bouquet *et al*., 1992; Eroglu *et al*., 1998; Stachecki and Willadsen, 2000; Stachecki *et al*., 2002; bovine: Fuku *et al*., 1992; Otoi *et al*., 1995; Martino *et al*., 1996a; Suzuki *et al*., 1996; Azambuja *et al*., 1998; Saunders and Parks, 1999; human: Gook *et al*., 1994). Although there have been studies on chromosome dispersal and the risk of aneuploidy, the majority of these report fertilization rates that are only somewhat lower than with non-frozen oocytes. Furthermore, several authors report normal in-vitro and in-vivo development. Clearly, more work needs to be done in this area.

Besides the microtubules that make up the spindle apparatus, other cytoskeletal elements, particularly those associated with the cell membrane, have been shown to play a role in oocyte survival following cryopreservation. Compounds including the cytochalasins, EGTA and taxol, which affect the cytoskeleton, have been used as stabilizers prior to or during cryopreservation and have had, in some cases, beneficial effects (Younis *et al*., 1996; Park *et al*., 2001; Rho *et al*., 2002; Vieira *et al*., 2002). However, these and similar compounds have not made their way into standard clinical freezing protocols.

Many papers analysing the effect of cryopreservation on spindle reformation report relatively low oocyte survival rates of 50–70% (mouse and human). It is not surprising that spindles are affected and look abnormal when the stress of their cryopreservation procedure kills nearly half the cells. It is reasonable to assume that spindle reformation and functionality would benefit from a protocol where all or most oocytes survived. Recently published data on mouse, bovine, and human oocytes also show that if the oocytes are frozen and thawed properly, the spindle can and will reform (Stachecki and Willadsen, 2004). It should be noted that in fresh, non-cryopreserved oocytes the percentage of normal spindles is only around 90%, so 100% success cannot be expected even if it was possible to determine accurately the presence of a spindle prior to cryopreservation. It appears that if the oocytes are treated properly there should be few problems with spindle reformation.

Functionality after reformation is a different matter. Of the numerous births resulting from frozen–thawed oocytes, no abnormalities to date have been reported (Fosas *et al*., 2003; Marina and Marina, 2003) despite concerns that cryopreservation may disrupt the chromosomal apparatus (meiotic spindle) in mature oocytes and increase chromosome abnormalities in the resulting embryos. However, long-term follow-up studies have not been reported.

Some other studies on zona hardening, digyny, cooling sensitivity, and osmotic tolerance have added more questions rather than answers (Pickering and Johnson, 1987; Carroll *et al*., 1989; George and Johnson, 1993). Since 1970, only minor modifications to clinical cryopreservation practice have occurred, with no significant breakthroughs, save some of the vitrification techniques. It has therefore become more than obvious that oocytes need a different approach to cryopreservation than embryos, although the fundamentals of that approach, including dehydration, addition of cryoprotectant to bind the remaining water and cooling to storage temperatures, would be similar.

Cryopreservation theory

The first theoretical basis for cryopreservation of cells was proposed by Mazur (1965) and later applied to mouse oocytes (Mazur *et al*., 1984). For example, human embryos are exposed to a simple isotonic salt solution containing a permeable cryoprotectant (PrOH) and usually, a low concentration of non-permeable cryoprotectant (sucrose). After a brief exposure time to allow uptake of cryoprotectant, the cells are cooled rapidly to a temperature slightly below the melting point of the solution (usually around –7°C). At this point, the container with the cells is 'seeded' so that ice forms in the extracellular solution. Upon ice formation and further cooling (slow cooling to below –30°C), the osmolarity of the extracellular solution increases as water freezes out as ice. With the increasing tonicity, the cells dehydrate. Dehydration continues during slow cooling until the cells are plunged into liquid nitrogen, usually at a temperature below –30°C. At this point, the intracellular cryoprotectant concentration is high enough that the remaining intracellular water will vitrify, preventing intracellular ice formation (IIF). During rewarming, ice can form once again if the vitrified solution warms at a rate permitting the process of devitrification. This happens when the temperature is high enough that the molecular mobility of water has increased to a point where the water molecules can move and rearrange themselves from a

disorderly amorphous vitrified position to an orderly crystalline position. This occurs well below the melting point, and is therefore a potentially lethal problem (Mazur and Schmidt, 1968; Luyet, 1970). During thawing, the dehydrated cells are exposed to hypotonic conditions and rehydrate along with cryoprotectant removal (Mazur, 1977).

Intracellular ice formation and osmotic effects

Slow cooling protocols derived and modified from mouse and bovine embryo freezing work in the early 1970s do not work well for the storage of unfertilized oocytes, but have been able to produce a low percentage of offspring. Oocytes can therefore be frozen effectively, but only if the stresses imposed on them are reduced or removed. New information is needed by using very different approaches within the same context of dehydrating the cell, binding the remaining water with a cryoprotectant, and cooling to specific temperature(s) before storage in liquid nitrogen. For success, it is necessary to identify problem areas and then modify the approach to work around them.

However, there are a number of unknowns. When a cell lyses after thawing, the typical logic is that IIF and/or osmotic effects were responsible. However, commonly this analysis is not based on experimentation, but on the seemingly logical, potential problems of IIF and osmotic effects. Hence, the freezing protocol is either modified, or abandoned because 'it does not work'. In 1990 and 1991, Toner *et al*. specifically investigated IIF in mouse oocytes cooled in phosphate-buffered saline (PBS) without cryoprotectants and confirmed two distinct mechanisms of IIF: the first catalysed by extracellular ice and the second heterogeneously by intracellular structures (Toner *et al*., 1990, 1991). They discuss these mechanisms in the presence of cryoprotectants and conclude that both seeding (mechanism I) and heterogeneous nucleation (mechanism II) are rendered ineffective by the addition of cryoprotectants. Leibo, McGrath, and Cravalho in 1975, and again in 1977 and 1978 (Leibo, 1977; Leibo *et al*., 1975, 1978) show that mouse oocyte survival is inversely related to IIF. Their graph shows that the curve denoting IIF crosses the x-axis and becomes zero (0% IIF) at and below a cooling rate of 1°C/min. This is substantially faster than the current protocol standard rate of 0.3°C/min used for freezing mammalian embryos and oocytes, and accordingly IIF would not occur when a slower rate is used. In another study, Ashwood-Smith *et al*. (1988) looked at IIF in mouse oocytes using a cryomicroscope, reconfirming previous literature concluding that if IIF was present (occurred at –14°C using a solution of PBS, 10% MeOH and 10% dextran), the cells would lyse upon thaw due to a number of mechanical forces, including internal ice expansion rupturing membranes. They also say that cells could have been pierced by extracellular ice crystals, but earlier Luyet and Gehenio (1940) denied the existence of this mechanistic failure, stating 'experience has shown that cells/tissues can tolerate the presence of extracellular ice and despite morphological appearances exerts no mechanical pressure on the cells and certainly does not puncture them'. Ashwood-Smith *et al*. (1988) argued that mammalian eggs are much larger than the red blood cells used in Luyet's paper, and physical forces could still play a significantly damaging role. However, if these forces were lethal, it would not be possible to obtain near 100% survival of mouse embryos using a variety of cryoprotectants and protocols currently employed.

Therefore, it can be suggested that other stresses cause cell death, although mechanical forces would still contribute to the overall stress upon the cell, especially re-expansion or sheering forces upon rewarming (causing zona cracking, for instance). The aforementioned studies collectively indicate that IIF is not necessarily a serious problem in modern protocols where penetrating cryoprotectants, slow cooling, and appropriate rewarming strategies are used.

In one of the more interesting articles, Trad *et al*. (1999) studied seeding and IIF in human and mouse oocytes, and found there was a relationship between IIF in human oocytes and seeding temperature. They reported IIF rates of 79% at –8°C, 100% at –6.5°C, 33% at –6.0°C, 17% at –5.5°C, and 0% at –4.5°C. Although 1.5 mol/l PrOH was used, a cooling rate of 120°C/min was employed; however, these environmental conditions do not exactly match conditions that occur within a cryo-straw. Although it was difficult, if not impossible to maintain a seed in a standard 0.25 ml straw loaded with 1.5 mol/l PrOH, and held at –4.5°C in a BioCool freezer, the fact remains that the higher seeding temperature in Trad's paper proved superior. They did not use sucrose in their freezing medium, which, in and of itself, may account for their poor survival results when using a seeding temperature of –6.0°C or lower. Their results are interesting, even though difficult to explain or reproduce.

The other potential problem of 'solution effects' involves the change in a solution or cytoplasm that result from dehydration, increased solute concentration, pH changes, and precipitation of solutes (Mazur, 1965; Mazur *et al*., 1984). Half a century ago, Lovelock (1953) showed that the increasing extracellular solute concentration that occurs during cooling when water freezes out as ice, was responsible for red blood cell lysis (>0.8 mol/l NaCl) and that the addition of a cryoprotectant (glycerol) reduced the amount of ice formed at any temperature, thereby effectively reducing the concentration of electrolytes produced. It has indeed been known for a long time that 'solution effects' must be reduced to such a degree that cells can withstand cryopreservation. However, several more recent studies have reported higher tolerance levels to osmotic stress. Agca *et al*. (2000) exposed bovine oocytes to PBS with increasing concentrations of sodium chloride to increase the osmolarity to supra-physiological concentrations (up to 4800 mOsm). They found that oocytes exposed to 2400 mOsm or lower developed to the blastocyst stage, albeit at slightly reduced rates as compared with untreated controls. Van Os and Zeilmaker (1986) exposed mouse zygotes to solutions up to 3100 mOsm (added NaCl) apparently without detriment to blastocyst formation.

Others have exposed human or mouse oocytes and embryos to varying concentrations of cryoprotectants or sugars without cooling and found that cells have a considerable tolerance to heightened osmotic conditions (Oda *et al*., 1992; McWilliams *et al*., 1995; Hotamisligil *et al*., 1996). When Toner *et al*. (1993) cooled mouse zygotes to –40°C in moderate to high concentrations of NaCl (300–2400 mOsm) without cryoprotectants, all the embryos lysed after rewarming.

However, if the osmolarity of the PBS solution was increased (up to 2400 mOsm) using another salt (choline chloride), the majority of the cells remained intact despite the absence of cryoprotectants. Furthermore, these zygotes were plunged directly into the highly concentrated solutions and not slowly dehydrated over a range of increasing osmotic concentrations, yet they survived.

This study showed that cellular demise from osmotic stress could be due to the type of stressor and not necessarily the ability of the cell to handle osmotic shock. Indeed, during the normal course of cryopreservation, cells (mouse embryos for example) are exposed to very high osmolarities yet >90% survive. If osmotic stress were as lethal as originally suggested, the majority of the cells would die; yet this does not happen. Even when human oocyte freezing is considered, around half of the cells are intact after thawing and around 70% of the blastomeres in human embryos survive freezing.

Therefore, lethal IIF and osmotic effects may play a smaller part in cellular demise during cryopreservation than generally thought, even though they can be lethal if the cells are not treated properly. There are probably other problems that may have been concealed by IIF and osmotic effects.

Optimizing cryopreservation

The fact that oocytes and embryos can survive freezing to subzero temperatures, storage for extended time periods, and rewarming to physiological temperatures is remarkable. The difficulties in learning about the process are vastly increased by the fact that during cooling and storage, the cells are inside containers that do not allow for visualization, let alone other types of analysis. This means that for the most part, only visual observations of gross morphology are made prior to and after the entire cryopreservation process. In order to significantly improve survival rates and development to healthy offspring, it is necessary to understand the entire cryopreservation process. This includes understanding the stresses that cells may tolerate. Oocytes and embryos are incredibly complex, highly organized living systems that do not react well to environmental changes, and although they can show resilience under certain conditions, every step in the cryopreservation process is critically important when trying to obtain a high survival and viability rate. For a new approach to cryopreservation of oocytes, it is necessary to keep an open mind and take nothing for granted unless specifically tested with appropriate controls.

It is known that the type and concentration of cryoprotectants affect oocyte survival and during the pre-freeze, cells are exposed to permeable and impermeable cryoprotectants in a saline solution. Although osmotic stress can kill or injure oocytes, they can in fact withstand a considerable amount of osmotic stress and still survive. The initial insult to the oocyte when placed into the freezing medium is small compared with the stress induced after seeding. When slow cooling is used, initial cryoprotectant concentrations are around 2 mol/l. Direct immersion into such a solution should not seriously decrease viability, if at all. Blebbing of the cytoplasm can occur if the osmolarity is too great, depending on the species, but this can be minimized or prevented by stepwise exposure to cryoprotectants. This is usually done in two steps: (i) freezing medium with 1.5 mol/l PrOH followed by (ii) freezing medium with 1.5 mol/l PrOH and 0.2 mol/l sucrose. However, is blebbing of the cytoplasm detrimental? Mouse oocytes have been shown to survive blebbing and go on to fertilize normally. It has been found that after stepping the cells out of cryoprotectant, they reverted to their normal appearance (unpublished data). Additionally, the cryoprotectant concentration used in most slow-cooling protocols is low enough not to be toxic to human oocytes.

Cryoprotectant toxicity is associated with the exposure concentration, temperature, and type of cryoprotectant (Rall, 1987; Wood *et al*., 1993; Hotamisligil *et al*., 1996). It continues to be an area of controversy, especially in vitrification protocols that use highly concentrated cryoprotectant solutions. In order to determine if a solution is toxic, the experiment must be carefully designed in order to generate results that can be correctly interpreted.

Mouse oocytes have been frozen in 0.75 mol/l as well as 3 mol/l PrOH (half and twice the normal cryoprotectant concentration) without observing significant differences in survival, which suggests that 1.5 mol/l PrOH is a relatively safe concentration. The time the oocytes are exposed to the cryoprotectant is also important. Standard incubation times range from 10 min to over 30 min, and can involve numerous solutions leading up to the final 1.5 mol/l PrOH + 0.2 mol/l sucrose solution typically used. When incubating oocytes (mouse or human) in cryoprotectant, they start by shrinking but after around 10 min swell to just under their original volume, due to the intake of cryoprotectant and water. Mouse oocytes were frozen by directly placing them into the final 1.5 mol/l PrOH + 0.2 mol/l sucrose solution and immediately loading them into straws, and initiating cooling without any equilibration time. Survival and fertilization rates were found to be similar to controls that were equilibrated for 10–15 min. Therefore, multiple solutions and extended incubation times may in fact not be necessary, at least for mouse oocytes.

Among the myriad protocol items, the temperature during pre-equilibration is also important. Earlier studies using mouse oocytes showed that exposure to DMSO was detrimental at temperatures above 20°C and had to be done at 5°C (Wood *et al*., 1992; Carroll, 1993; George and Johnson, 1993; Van der Elst *et al*., 1993; George *et al*., 1994). It was found that exposure to cryoprotectant at room temperature was not detrimental, depending on the freezing solution used (Stachecki *et al*., 1998a,b).

Other aspects of the cryopreservation procedure, including initial cooling and seeding, cooling, break point, thawing and step-out, need critical analysis and rethinking to obtain near perfect survival and development rates. In-depth analysis of these areas is beyond the scope of this review.

Mouse experiments

During initial studies with mouse oocytes, it was postulated that once intracellular ice formation and osmotic stress were properly addressed, they should become minor or negligible problems. Attention was focussed on the medium in which the oocytes were frozen, and it was found that replacing all sodium ions in the medium with choline ions allowed mouse

oocytes to be frozen effectively. The effects of plunge temperature (degree of dehydration) and thawing regimes on mouse oocyte survival (Stachecki and Willadsen, 2000) were investigated. All this supported previous findings by showing that the composition of the freezing solution can have dramatic effects on oocyte survival.

With further experimentation, it was found that minor modifications had little effect, and only when mouse oocytes were destroyed by radically altering methods that some beneficial observations were made. For instance, mouse oocytes displayed a much higher capacity to survive plunging into liquid nitrogen at relatively higher temperatures (–20°C) than had been expected (Van den Abbeel *et al*., 1994). To demonstrate the effectiveness of the protocol and freezing medium (Stachecki *et al*., 2002), numerous pups were obtained for the first time ever, from oocytes plunged into liquid nitrogen at –20°C, and without the use of a programmable freezer.

Proper thaw rates were absolutely critical for obtaining high survival rates, and demonstrated that reproductively competent offspring developed from frozen–thawed oocytes after being transferred at the 2-cell or blastocyst stage to pseudopregnant mice. The thawing rate was found to be just as important as the freezing rate, a point overlooked in many existing protocols. Indeed, it was found that the majority of mouse oocytes plunged at –20°C would have lysed if a standard thaw rate had been used for oocytes plunged at –33°C (Stachecki and Willadsen, 2000). Instead, by using a slight modification in the thaw rate, an interesting contradiction was found. A near 100% survival of oocytes plunged at –20°C was obtained; a survival rate that was never observed when oocytes were plunged at –33°C, which is a temperature where the cells would be more dehydrated and less likely to form intracellular ice. This further proved the hypothesis that IIF was not the principal cause of oocyte demise.

In continuing work (unpublished studies), it became apparent that the cryopreservation medium composition, method, and extent of cellular dehydration, equilibration time with cryoprotectant, plunge temperature, and thawing regime are key factors that need to be carefully orchestrated in order to maximize oocyte survival. After several modifications of the protocol and medium, mouse oocytes could be cryopreserved with an initial survival rate after thawing of over 95% and with a blastocyst formation rate of 75% per all oocytes frozen. This is a significant improvement over previously published data.

By focusing on medium composition (ionic and osmolar), length of cryoprotectant exposure, dehydration, effects of cooling, plunge temperature, thawing regime, and cryoprotectant removal instead of the potential problems of IIF and osmotic effects, it was possible to modify protocols and successfully freeze unfertilized mouse oocytes in a simple and reproducible manner. This approach was then applied to cryopreserving oocytes from other species, including humans.

Human oocyte experiments

In initial studies using discarded human oocytes that failed to fertilize, a higher concentration of sucrose (0.2 versus 0.1 mol/l) and longer equilibration time (20 min versus 10 min) were found to be beneficial to oocyte survival, similar to observations made later by Fabbri *et al*. (1998). As an extension of this observation, Fabbri *et al*. (2001) used 0.3 mol/l sucrose and obtained even greater survival, after which Fosas *et al*. (2003) tested this out clinically, resulting in the delivery of five babies. The exact reasons why these methods worked as well as they did have yet to be fully understood. At first glance, it would seem apparent that increasing the sucrose concentration would dehydrate the oocyte to a greater extent, having an outcome of greater initial survival after thawing. However, the exact effect of additional sucrose can only be speculated on, and may or may not have anything to do with reducing IIF, simply because lethal IIF may not be occurring. It could be that the extra sucrose protects the membrane in some manner.

Experience with freshly collected, donated and research-consented oocytes has indicated that elevating the concentration of sucrose (0.1–0.2 mol/l) was beneficial, yet even with this, initial survival rates were poor at best (around 50%, unpublished data), although those oocytes that had survived, fertilized and developed at regular control rates until the experiments were terminated on day 3. The data showed that the ability of fresh oocytes to remain intact after thawing was reduced compared with aged oocytes (oocytes that were immature, germinal vesicle [GV] or meiosis I [MI] stage, when collected and matured overnight) and that modifications to the initial protocol were necessary. This contradicts the popular idea that freshly collected human oocytes will withstand cryopreservation better than aged oocytes or oocytes matured overnight, and could be caused by changes in the membrane composition as an oocyte matures.

Human oocytes that were intact upon thawing were fixed and stained after culture at 37°C for 1 h, to assess spindle reformation. It was found that although the spindle will disassemble during cryopreservation, the spindle can reform in frozen–thawed human oocytes when the eggs are incubated at 37°C after thawing (27/35, 77.1%, oocytes with barrel-shaped spindle and chromosomes aligned on the metaphase plate; Stachecki and Willadsen, 2004). In much the same way, it was also found that spindles will reform with mouse and bovine oocytes (Stachecki and Willadsen, 2004).

Although other investigators have reported consistent human oocyte survival rates around 70% using conventional embryo freezing methodologies, considerable loss occurs after insemination and culture, resulting in relatively few embryos suitable for transfer. Tucker and others have reported that survival rates were the major obstacle to oocyte cryopreservation. Fertilization rates are similar to fresh oocytes, but ICSI can lead to cell death more frequently in frozen–thawed oocytes than fresh. Embryo development of these frozen–thawed oocytes is an additional problem.

The future

There has been little progress in the approach to oocyte freezing, as only minor modifications of existing protocols have been made since Chen produced the first offspring from cryopreserved human oocytes. However, a few articles have

incorporated new ideas in their methods of storing oocytes.

One recent article suggested injecting trehalose into oocytes (Eroglu *et al*., 2002). The results showed that intracellular and extracellular trehalose alone could aid in human oocyte cryopreservation. The oocytes were not plunged in liquid nitrogen, but cooled to only –60°C. Lethal IIF did not form in injected cells; but because trehalose is an impermeable sugar, there may be problems with cellular removal after thaw, which could affect fertilization and subsequent development. Additionally, one would have to inject the oocyte twice, once to get the trehalose in and then again to inject a spermatozoon. Nonetheless, this paper demonstrates a novel application and adds to the growing body of information on cryopreservation.

The substitution of choline chloride for sodium chloride is a major alteration to the standard cryopreservation protocol, and there are good reasons. The most common ingredient besides water is sodium in the form of sodium chloride in all cryopreservation media, including mammalian cell culture media, and it is 10–100 times more abundant than any other single ion in the medium. To remove this ion and substitute it with a totally different ion (choline) is a radical alteration to the cellular environment, and it seemed to us improbable that cells would survive in a medium totally devoid of the most common ion, sodium for an extended period of time. Nevertheless, no overt sign of toxicity has been observed (no cells died) when mouse and human oocytes or embryos were cultured in a sodium-free medium for 24 h.

Like other organic osmolytes including betaines, choline has been known to have osmoprotective and cryoprotective effects on liposomes, erythrocytes, plants, and mouse embryos (Lloyd *et al*., 1992, 1994; Toner *et al*., 1993). The osmolyte glycinebetaine has been found in animals, bacteria, fungi, algae, and many drought- and salt-tolerant plants (Rhodes and Hanson, 1993). Some of these compounds protect enzymes and membranes from cold (Lynch *et al*., 1979; Krall *et al*., 1989; Van Winkle *et al*., 1990), salt (Jolivet *et al*., 1983; Hanson *et al*., 1994) and freezing damage (Zhao *et al*., 1992; Huang *et al*., 2000). It is possible that choline has similar effects on the membrane integrity of oocytes and embryos. In support of this hypothesis, Toner *et al*. (1993) has shown that during cooling, zygote cell membranes are far more tolerant to hyperosmotic stress from a mixture of sodium and choline ions than from sodium ions alone.

Choline's ability to help mouse oocytes survive cryopreservation was tested by removing all of the sodium and replacing it with an equal osmolar amount of choline (Stachecki *et al*., 1998b). Nothing else was altered in the protocol except the freezing medium. At that time, it was generally thought that slow freezing of mouse oocytes in PrOH following a standard 10–15 min equilibration at room temperature in sodium chloride solutions would lead to poor survival and in-vitro development. Most of the publications during the early 1990s only used DMSO, even though PrOH was popular for embryo freezing (Schroeder *et al*., 1990; Wood *et al*., 1992; Carroll *et al*., 1993; George and Johnson, 1993; Van der Elst *et al*., 1993; George *et al*., 1994; Karlsson *et al*., 1996; Stachecki *et al*., 1998b). In one study (Stachecki *et al*., 1998b), it was shown that most of the oocytes died in the normal sodium-based medium but when sodium was removed and choline was used, >80% of the oocytes survived freezing and thawing. Further work has confirmed the beneficial effects of choline (Stachecki *et al*., 1998a, 2002; Stachecki and Willadsen, 2000). The exact mechanism by which choline works is unknown (so is that of sodium, for that matter), but if sodium loading occurs during freezing, this would be eliminated using a choline-based medium. It is likely that choline is responsible for the improved survival and development rates observed.

Simply switching the medium from a sodium base to a choline base may improve oocyte survival, but the entire cryopreservation process must be optimized to maximize results. Since the first report using choline in 1998 (Stachecki *et al*., 1998b), several laboratories have used a choline-based freezing medium to freeze human oocytes and to date more than 10 babies have been born (Azambuja *et al*., 2002; Quintans *et al*., 2002; Boldt *et al*., 2003), Not all laboratories have duplicated the initially high survival rates; this is most likely because cryopreservation protocols have many variables (Lane and Gardner, 2001; Quintans *et al*., 2002; Boldt *et al*., 2003).

Vitrification

The idea of vitrification, or achieving a glass-like state, was first described in 1860, and then again in 1937 by Luyet (1937). It was not until nearly 50 years later in 1985 that Rall and Fahy described vitrification as a potential alternative to slow cooling (Rall and Fahy, 1985). Although it is relatively successful for embryo storage, the process has not so far been reproducible for oocytes of any species; however, this seems to be contradicted by the report of Bos-Mikich (1993). Nevertheless, there have been several recent publications using vitrification to store human oocytes (Pensis *et al*., 1989; Hunter *et al*., 1995; Hong *et al*., 1999; Kuleshova *et al*., 1999a; Chen *et al*., 2000; Chung *et al*., 2000; Ali, 2001; Wu *et al*., 2001; Kuleshova and Lopata, 2002; Liebermann and Tucker, 2002; Wininger and Kort, 2002; Liebermann *et al*., 2003; Yoon *et al*., 2003).

Vitrification involves exposure of the cell to high concentrations of cryoprotectant(s) for brief periods of time, usually at or near room temperature, followed by rapid cooling in liquid nitrogen. The high osmolarity of the vitrification solution rapidly dehydrates the cell and submersion into liquid nitrogen quickly solidifies the cell, so that the remaining intracellular water does not have time to form damaging ice crystals. A similar situation occurs during slow cooling; the cells are dehydrated over a longer period of time and then plunged into liquid nitrogen at much lower temperatures. Vitrification tries to mimic the extra- and intracellular conditions that exist and allow for survival when cells are plunged (around –30°C) during slow cooling.

In the past decade, vitrification studies have been among the most interesting because so little is known about how to vitrify oocytes, and as it is different from slow cooling, investigators have tried many novel approaches leading to several findings. Many types and combinations of permeable and impermeable cryoprotectants have been used from propanediol and glycerol, to Ficoll and trehalose. A lot of the methods were borrowed from murine and bovine vitrification studies (Shaw *et al*.,

1992; Martino *et al*., 1996b; Vajta *et al*., 1998). Vitrification seems to be an acceptable alternative to slow cooling in species that exhibit sensitivity to a reduction in temperature, including cows and pigs, in which there have been numerous investigations. Along with different cryoprotectants, various storage devices have been used including open-pulled straws, cryoloops, electron microscopy grids, as well as regular cryostraws (for review, see Liebermann *et al*., 2002).

Compared with slow freezing, however, vitrification might pose a threat to survival because of the toxicity of the highly concentrated cryoprotectants and the temperatures at which they are used (Hotamisligil *et al*., 1996; Mukaida *et al*., 1998). The higher risk involved with vitrification limited the number of attempts to use this technique for human oocyte storage until Kuleshova *et al*. (1999) documented the birth of a healthy girl from vitrifying oocytes in open-pulled straws, a method adopted from a successful bovine vitrification study (Vajta *et al*., 1998).

Since then two main concepts have become associated with vitrification; first, that high concentrations of cryoprotectants are toxic and exposure (to the final and highest concentrations) should be reduced to 30 s or less (Shaw *et al*., 1992; Martino *et al*., 1996b), and second, that faster cooling increases survival. The first idea is that toxicity is more of a concern with vitrification because of the high cryoprotectant concentration (4–6 mol/l). The toxic effects of highly concentrated vitrification solutions have been well established. Most protocols include a pre-equilibration period using a reduced cryoprotectant concentration, prior to a very short (<30 s) incubation in the final vitrification solution (Shaw *et al*., 1992; Hunter *et al*., 1995; Hong *et al*., 1999; Chung *et al*., 2000; Wu *et al*., 2001; Yoon *et al*., 2003) all based, in part, upon the work of Martino *et al*. (1996b). However, this is not the only method that can work. By contrast, Chen *et al*. (2000) vitrified human oocytes in straws, and found that human oocytes could be exposed to the vitrification solution for either 60 s or 90 s prior to plunging in liquid nitrogen and still survive.

Hotamisligil *et al*. (1996) reported no significant differences in development of mouse oocytes incubated in 6 mol/l ethylene glycol for 5 min, as compared with controls. By contrast, exposure of mouse oocytes to 8 mol/l ethylene glycol proved lethal. Recently, Isachenko *et al*. (2003), showed that human pronuclear oocytes sustained greater damage to intracellular organelles when cooled without cryoprotectants, which therefore afford protection, although possibly toxic at high concentrations. Using a modified open-pulled straw technique, 71% of the zygotes survived and several pregnancies were reported.

The second concept proposes that if the cell is dehydrated and then cooled fast enough, everything will 'freeze' in place and damage will not have time to occur as a vitrified amorphous, glass-like solid will form instead of crystals. Similar thinking applies to the thawing of a vitrified solution, which must take place at a relatively fast rate to prevent crystal organization upon rewarming. In order to freeze faster, several new methods have been used. The first to come along was the open-pulled straw (Vajta *et al*., 1998), which reduced the diameter of a conventional 0.25 ml straw, reducing the amount of liquid that needed to be loaded, and increasing the vitrification rate.

Subsequently, other methods, including electron microscope grids and nylon loops that allowed direct contact with liquid nitrogen, and the use of minute volumes, were able to increase the vitrification speed considerably (Martino *et al*., 1996b; Hong *et al*., 1999; Lane and Gardner, 2001; Wu *et al*., 2001; Liebermann *et al*., 2003). However, care must be taken to interpret results correctly, making no assumptions about the cause of cell death. If cells die during vitrification, it may or may not be because the cryoprotectant concentration was toxic, or ice did in fact form, or the cooling rate was too slow.

With continued success and pregnancies being reported, vitrification is well on its way to being used clinically, but a few obstacles need to be overcome (Kuleshova and Lopata, 2002; Rall, 2003). First, several reports of viral contamination in liquid nitrogen have appeared in the literature and are cause for concern when not vitrifying in a sealed container (Bielanski *et al*., 2000, 2003; Kuleshova and Shaw, 2000). Second, the common technique of placing cells into a highly concentrated vitrification solution, loading them onto a grid, loop, or into a straw, and plunging, all in less than 30 s remains technically challenging; and more importantly, leaves little or no room for error, despite what some investigators say. Third, the consistency of results with vitrification protocols is often poor. An average survival rate of 70% may be considered good only if there are enough results to give a convincing average (Liebermann *et al*., 2002). Some reports possibly avoid these problems, among them mouse oocytes (Wood *et al*., 1993) and bovine blastocysts (Kaidi *et al*., 1998, 1999, 2000) have been successfully vitrified in sealed straws that have been placed for a period of several minutes in liquid nitrogen vapours prior to plunging. They show that a longer time period (enough to load the cells into a straw and seal the straw, >1 min) and a reduced rate of cooling (liquid nitrogen vapours) can be used while still obtaining very good results. If slower rates of cooling and longer equilibration times are in fact possible, as these studies demonstrate, perhaps viral contamination and speed of technique difficulties could be avoided (Yokota *et al*., 2000, 2001). It is disconcerting that investigators using liquid nitrogen vapours for cooling commonly do not report the temperature of the vapours, as they can undergo extreme fluctuations (–20 to –170°C) across a few cm: a source of significant variance in cooling rate and possibly experimental outcome.

There are many methods that could work to vitrify cells successfully. Martino *et al*., (1996b), suggested that cell dehydration is more important than having a large amount of cryoprotectant inside the cell. The short equilibration times employed in many vitrification protocols seem to confirm this idea. Kuleshova *et al*. added high molecular weight polymers (Ficoll or dextran) to the vitrification solution, and thereby reduced the amount of penetrating cryoprotectants necessary to vitrify oocytes and/or embryos (Kuleshova *et al*., 1999b, 2001). This novel approach worked well and provides an option for vitrification that reduces the toxicity of the final solution, which in turn may allow for longer equilibration times, a necessary consideration to eliminate errors when trying to vitrify cells in under 30 s as specified in many of today's protocols.

Conclusion

In conclusion, both slow cooling and vitrification seem promising for storing a variety of cell types, including human oocytes. If the problems outlined above can be overcome and survival rates become consistently high (>90%), then this technology has a real chance to benefit patients. More information regarding the reasons for cellular demise during and/or after freezing needs to be established. Survival after rewarming is but the first hurdle to overcome, to be followed by orderly fertilization and subsequent development *in vitro* and *in vivo* and long-term health. Determining levels of cellular tolerance during each step of the cryopreservation process will help generate effective protocols. Experimentation and application of new methods are sure to supply a clearer picture.

References

Agca Y, Liu J, Rutledge JJ *et al*. 2000 Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. *Molecular Reproduction and Development* **55**, 212–219.

Ahuja KK, Simons EG, Mostyn BJ *et al*. 1998 An assessment of the motives and morals of egg share donors: policy of 'payments' to egg donors requires a fair review. *Human Reproduction* **13**, 2671–2678.

Aigner S, Van der Elst J, Siebzehnrubl E *et al*. 1992 The influence of slow and ultra-rapid freezing on the organization of the meiotic spindle of the mouse oocyte. *Human Reproduction* **7**, 857–864.

Al-Hasani S, Diedrich K, van der Ven H *et al*. 1987 Cryopreservation of human oocytes. *Human Reproduction* **2**, 695–700.

Ali J 2001 Vitrification of embryos and oocytes with 5.5 mol/l ethylene glycol and 1.0 mol/l sucrose. *Human Reproduction* **16**, 1777–1779.

Almeida PA, Bolton VN 1995 The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote* **3**, 357–365.

Aman RR, Parks JE 1994 Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biology of Reproduction* **50**, 103–110.

Antinori S, Dani G, Selman H *et al*. 1998 Pregnancy after sperm injection into cryopreserved human oocytes. *Human Reproduction* **13**, 157–158.

Ashwood-Smith MJ, Morris GW, Fowler R *et al*. 1988 Physical factors are involved in the destruction of embryos and oocytes during freezing and thawing procedures. *Human Reproduction* **3**, 795–802.

Azambuja RM, Kraemer DC, Westhusin ME 1998 Effect of low temperatures on in-vitro matured bovine oocytes. *Theriogenology* **49**, 1155–1164.

Azambuja R, Stachecki J, Badalotti M *et al*. 2002 Nascimento de feto a termo apos de oocitos. *Journal Brasileiro De Reproducao Assistida* **6**, 119–121.

Baka SG, Toth TL, Veeck LL *et al*. 1995 Evaluation of the spindle apparatus of in-vitro matured human oocytes following cryopreservation. *Human Reproduction* **10**, 1816–1820.

Bielanski A, Nadin-Davis S, Sapp T *et al*. 2000 Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology* **40**, 110–116.

Bielanski A, Bergeron H, Lau PC *et al*. 2003 Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology* **46**, 146–152.

Boiso I, Marti M, Santalo J *et al*. 2002 A confocal microscopy analysis of the spindle and chromosome configurations of human oocytes cryopreserved at the germinal vesicle and metaphase II stage. *Human Reproduction* **17**, 1885–1891.

Boldt J, Cline D, McLaughlin D 2003 Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. *Human Reproduction* **18**, 1250–1255.

Bongso A, Chye NS, Sathananthan H *et al*. 1988 Chromosome analysis of two-cell mouse embryos frozen by slow and ultrarapid methods using two different cryoprotectants. *Fertility and Sterility* **49**, 908–912.

Borini A, Baffaro M, Bonu M *et al*. 1998 Pregnancies after freezing and thawing human oocytes. Preliminary data. *Human Reproduction* **13**, 124–125.

Bos-Mikich A, Wood MJ, Candy J, Whittingham DG 1993 Cytogenetic analysis and developmental potential of vitrified mouse embryos. *Biology of Reproduction* **53**, 780–785.

Bouquet M, Selva J, Auroux M 1992 The incidence of chromosomal abnormalities in frozen–thawed mouse oocytes after in-vitro fertilization. *Human Reproduction* **7**, 76–80.

Carroll J, Warnes GM, Matthews CD 1989 Increase in digyny explains polyploidy after in-vitro fertilization of frozen–thawed mouse oocytes. *Journal of Reproduction and Fertility* **85**, 489–494.

Carroll J, Wood MJ, Whittingham DG 1993 Normal fertilization and development of frozen–thawed mouse oocytes: protective action of certain macromolecules. *Biology of Reproduction* **48**, 606–612.

Cha KY, Chung HM, Lim JM *et al*. 2000 Freezing immature oocytes. *Molecular Cellular Endocrinology* **169**, 43–47.

Chen C 1986 Pregnancy after human oocyte cryopreservation. *Lancet* **1**, 884–886.

Chen C 1988 Pregnancies after human oocyte cryopreservation. *Annals of the New York Academy of Sciences* **541**, 541–549.

Chen SU, Lien YR, Chao K *et al*. 2000 Cryopreservation of mature human oocytes by vitrification with ethylene glycol in straws. *Fertility and Sterility* **74**, 804–808.

Chung HM, Hong SW, Lim JM *et al*. 2000 In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. *Fertility and Sterility* **73**, 545–551.

Eroglu A, Toth TL, Toner M 1998 Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. *Fertility and Sterility* **69**, 944–957.

Eroglu A, Toner M, Toth TL 2002 Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. *Fertility and Sterility* **77**, 152–158.

Fabbri R, Porcu E, Marsella T *et al*. 1998 Oocyte cryopreservation: birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Human Reproduction* **13** (suppl. 4), 98–108.

Fabbri R, Porcu E, Marsella T *et al*. 2001 Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Human Reproduction* **16**, 411–416.

Fosas N, Marina F, Torres PJ *et al*. 2003 The births of five Spanish babies from cryopreserved donated oocytes: comments on oocyte cryopreservation. *Human Reproduction* **18**, 1417–1421.

Fuku E, Kojima T, Shioya Y *et al*. 1992 In vitro fertilization and development of frozen-thawed bovine oocytes. *Cryobiology* **29**, 485–492.

George MA, Johnson MH 1993 Use of fetal bovine serum substitutes for the protection of the mouse zona pellucida against hardening during cryoprotectant addition. *Human Reproduction* **8**, 1898–1900.

George MA, Johnson MH, Howlett SK 1994 Assessment of the developmental potential of frozen-thawed mouse oocytes. *Human Reproduction* **9**, 130–136.

Glenister PH, Wood MJ, Kirby C *et al*. 1987 Incidence of chromosome anomalies in first-cleavage mouse embryos obtained from frozen–thawed oocytes fertilized in vitro. *Gamete Research* **16**, 205–216.

Gook DA, Osborn SM, Johnston WI 1993 Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. *Human Reproduction* **8**, 1101–1109.

Gook DA, Osborn SM, Bourne H *et al*. 1994 Fertilization of human oocytes following cryopreservation; normal karyotypes and

absence of stray chromosomes. *Human Reproduction* **9**, 684–691.
Gook DA, Schiewe MC, Osborn SM *et al*. 1995 Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. *Human Reproduction* **10**, 2637–2641.
Hanson AD, Rathinasabapthi B, Rivoal J *et al*. 1994 Osmoprotective compounds in the Plumbaginaceae: a natural experiment in metabolic engineering of stress tolerance. *Proceedings of the National Academy of Sciences of the USA* **91**, 306–310.
Hong SW, Chung HM, Lim JM *et al*. 1999 Improved human oocyte development after vitrification: a comparison of thawing methods. *Fertility and Sterility* **72**, 142–146.
Hotamisligil S, Toner M, Powers RD 1996 Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol. *Biology of Reproduction* **55**, 161–168.
Huang J, Hirji R, Adam L *et al*. 2000 Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiology* **122**, 747–756.
Hunter JE, Fuller BJ, Bernard A *et al*. 1995 Vitrification of human oocytes following minimal exposure to cryoprotectants; initial studies on fertilization and embryonic development. *Human Reproduction* **10**, 1184–1188.
Isachenko V, Selman H, Isachenko E *et al*. 2003 Modified vitrification of human pronuclear oocytes: efficacy and effect on ultrastructure. *Reproductive BioMedicine Online* **7**, 211–216.
Johnson MH, Pickering SJ 1987 The effect of dimethylsulphoxide on the microtubular system of the mouse oocyte. *Development* **100**, 313–324.
Jolivet Y, Hamelin J, Larher F 1983 Osmoregulation in halophytic higher plants: the protective effects of glycinebetaine and other related solutes against the oxalate destabilization of membranes in beet root cells. *Zeitschrift für Pflanzenphysiologie* **109S**, 171–180.
Kaidi S, Donnay I, Van Langendonckt A *et al*. 1998 Comparison of two co-culture systems to assess the survival of in vitro produced bovine blastocysts after vitrification. *Animal Reproduction Science* **52**, 39–50.
Kaidi S, Van Langendonckt A, Massip A *et al*. 1999 Cellular alteration after dilution of cryoprotective solutions used for the vitrification of in vitro-produced bovine embryos. *Theriogenology* **52**, 515–525.
Kaidi S, Donnay I, Lambert P *et al*. 2000 Osmotic behavior of in vitro produced bovine blastocysts in cryoprotectant solutions as a potential predictive test of survival. *Cryobiology* **41**, 106–115.
Karlsson JO, Eroglu A, Toth TL *et al*. 1996 Fertilization and development of mouse oocytes cryopreserved using a theoretically optimized protocol. *Human Reproduction* **11**, 1296–1305.
Kazem R, Thompson LA, Srikantharajah A *et al*. 1995 Cryopreservation of human oocytes and fertilization by two techniques: in-vitro fertilization and intracytoplasmic sperm injection. *Human Reproduction* **10**, 2650–2654.
Kola I, Kirby C, Shaw J *et al*. 1988 Vitrification of mouse oocytes results in aneuploid zygotes and malformed fetuses. *Teratology* **38**, 467–474.
Krall JP, Edwards GE, Andreo CS 1989 Protection of pyruvate, Pi dikinase from maize against cold lability by compatible solutes. *Plant Physiology* **89**, 280–285.
Kuleshova LL, Lopata A 2002 Vitrification can be more favorable than slow cooling. *Fertility and Sterility* **78**, 449–454.
Kuleshova LL, Shaw JM 2000 A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen. *Human Reproduction* **15**, 2604–2609.
Kuleshova L, Gianaroli L, Magli C *et al*. 1999a Birth following vitrification of a small number of human oocytes: case report. *Human Reproduction* **14**, 3077–3079.
Kuleshova LL, MacFarlane DR, Trounson AO *et al*. 1999b Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. *Cryobiology* **38**, 119–130.
Kuleshova LL, Shaw JM, Trounson AO 2001 Studies on replacing most of the penetrating cryoprotectant by polymers for embryo cryopreservation. *Cryobiology* **43**, 21–31.
Lane M, Gardner DK 2001 Vitrification of mouse oocytes using a nylon loop. *Molecular Reproduction and Development* **58**, 342–347.
Leibo SP 1977 Fundamental cryobiology of mouse ova and embryos. In: *The Freezing of Mammalian Embryos*. CIBA Foundation Symposium 52. Elsevier, Amsterdam, pp. 69–96.
Leibo SP, McGrath JJ, Cravalho EG 1975 Microscopic observation of intracellular ice formation in mouse ova as a function of cooling rate. *Cryobiology* **12**, 579.
Leibo SP, McGrath JJ, Cravalho EG 1978 Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. *Cryobiology* **15**, 257–271.
Liebermann J, Tucker MJ 2002 Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. *Reproduction* **124**, 483–489.
Liebermann J, Nawroth F, Isachenko V *et al*. 2002 Potential importance of vitrification in reproductive medicine. *Biology of Reproduction* **67**, 1671–1680.
Liebermann J, Tucker MJ, Sills ES 2003 Cryoloop vitrification in assisted reproduction: analysis of survival rates in >1000 human oocytes after ultra-rapid cooling with polymer augmented cryoprotectants. *Clinical and Experimental Obstetrics and Gynecology* **30**, 125–129.
Lloyd AW, Baker JA, Olliff CJ *et al*. 1992 The evaluation of N-modified betaines as erythrocyte cryoprotectants. *Cryo-Letters* **13**, 337–348.
Lloyd AW, Oliff CJ and Rutt KJ 1994 A study of modified betaines as cryoprotective additives. *Journal of Pharmacy and Pharmacology* **46**, 704–707.
Lovelock J 1953 The haemolysis of human red blood cells by freezing and thawing. *Biochimica et Biophysica Acta* **10**, 414–426.
Luyet B 1937 The vitrification of organic colloids and protoplasm. *Biodynamica* **1**, 1–14.
Luyet B 1970 Physical changes occurring in frozen solutions during rewarming and melting. In: Wolstenholme G *et al*. (eds) *The Frozen Cell*. Churchill, London, pp. 27–50.
Luyet B, Gehenio P 1940 Life and death at low temperatures. *Biodynamica*, Normandy, MO, p. 261.
Lynch RD, Schneeberger EE, Geyer RP 1979 Alterations in L fibroblasts lipid metabolism and morphology during choline deprivation. *Experimental Cell Research* **122**, 103–113.
Marina F, Marina S 2003 Comments on oocyte cryopreservation. *Reproductive BioMedicine Online* **6**, 401–402.
Martino A, Pollard JW, Leibo SP 1996a Effect of chilling bovine oocytes on their developmental competence. *Molecular Reproduction and Devevelopment* **45**, 503–512.
Martino A, Songsasen N, Leibo SP 1996b Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biology of Reproduction* **54**, 1059–1069.
Mazur P 1965 Causes of injury in frozen and thawed cells. *Federation Proceedings* **24**, 175–182.
Mazur P 1977 Slow-freezing injury in mammalian cells. *Ciba Foundation Symposium*, pp. 19–48.
Mazur P, Schmidt JJ 1968 Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast. *Cryobiology* **5**, 1–17.
Mazur P, Rall WF, Leibo SP 1984 Kinetics of water loss and the likelihood of intracellular freezing in mouse ova. Influence of the method of calculating the temperature dependence of water permeability. *Cell Biophysics* **6**, 197–213.
McWilliams RB, Gibbons WE, Leibo SP 1995 Osmotic and physiological responses of mouse zygotes and human oocytes to mono- and disaccharides. *Human Reproduction* **10**, 1163–1171.
Mukaida T, Wada S, Takahashi K *et al*. 1998 Vitrification of human embryos based on the assessment of suitable conditions for 8-cell

mouse embryos. *Human Reproduction* **13**, 2874–2879.
Nawroth F, Kissing K 1998 Pregnancy after intracytoplasmatic sperm injection (ICSI) of cryopreserved human oocytes. *Acta Obstetricia et Gynecologica Scandinavica* **77**, 462–463.
Oda K, Gibbons WE, Leibo SP 1992 Osmotic shock of fertilized mouse ova. *Journal Reproduction and Fertility* **95**, 737–747.
Otoi T, Yamamoto K, Koyama N *et al*. 1995 In vitro fertilization and development of immature and mature bovine oocytes cryopreserved by ethylene glycol with sucrose. *Cryobiology* **32**, 455–460.
Park SE, Chung HM, Cha KY *et al*. 2001 Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using taxol, a cytoskeleton stabilizer. *Fertility and Sterility* **75**, 1177–1184.
Pensis M, Loumaye E, Psalti I 1989 Screening of conditions for rapid freezing of human oocytes: preliminary study toward their cryopreservation. *Fertility and Sterility* **52**, 787–794.
Pickering SJ, Johnson MH 1987 The influence of cooling on the organization of the meiotic spindle of the mouse oocyte. *Human Reproduction* **2**, 207–216.
Pickering SJ, Braude PR, Johnson MH *et al*. 1990 Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertility and Sterility* **54**, 102–108.
Polak de Fried E, Notrica J, Rubinstein M *et al*. 1998 Pregnancy after human donor oocyte cryopreservation and thawing in association with intracytoplasmic sperm injection in a patient with ovarian failure. *Fertility and Sterility* **69**, 555–557.
Porcu E 1999 Freezing of oocytes. *Current Opinion in Obstetrics and Gynecology* **11**, 297–300.
Porcu E 2001 Oocyte freezing. *Seminars in Reproductive Medicine* **19**, 221–230.
Porcu E, Fabbri R, Seracchioli R *et al*. 1997 Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertility and Sterility* **68**, 724–726.
Porcu E, Fabbri R, Seracchiolo R *et al*. 1998 Birth of six healthy children after intracytoplasmic sperm injection of cryopreserved human oocytes. *Human Reproduction* **13**, 124.
Porcu E, Fabbri R, Ciotti P *et al*. 1999a Cycles of human oocyte cryopreservation and intracytoplasmic sperm injection: results of 112 cycles. *Fertility and Sterility* **72**, S2.
Porcu E, Fabbri R, Ciotti PM *et al*. 1999b Ongoing pregnancy after intracytoplasmic sperm injection of epididymal spermatozoa into cryopreserved human oocytes. *Journal of Assisted Reproduction and Genetics* **16**, 283–285.
Porcu E, Fabbri R, Damiano G *et al*. 2000 Clinical experience and applications of oocyte cryopreservation. *Molecular and Cellular Endocrinology* **169**, 33–37.
Porcu E, Fabbri R, Ciotti P *et al*. 2002 Oocytes or embryo storage? *Fertility and Sterility* **169**, S15.
Quintans CJ, Donaldson MJ, Bertolino MV *et al*. 2002 Birth of two babies using oocytes that were cryopreserved in a choline-based freezing medium. *Human Reproduction* **17**, 3149–3152.
Rall WF 1987 Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology* **24**, 387–402.
Rall WF, Fahy GM 1985 Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. *Nature* **313**, 573–575.
Rho GJ, Kim S, Yoo JG *et al*. 2002 Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. *Molecular Reproduction and Development* **63**, 464–470.
Rhodes D, Hanson AD 1993 Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 357–384.
Sathananthan AH, Ng SC, Trounson AO *et al*. 1988 The effects of ultrarapid freezing on meiotic and mitotic spindles of mouse oocytes and embryos. *Gamete Research* **21**, 385–401.
Sathananthan AH, Kirby C, Trounson A *et al*. 1992 The effects of cooling mouse oocytes. *Journal of Assisted Reproduction and Genetics* **9**, 139–148.
Saunders KM, Parks JE 1999 Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro-matured bovine oocytes. *Biology of Reproduction* **61**, 178–187.
Schroeder AC, Champlin AK, Mobraaten LE *et al*. 1990 Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro. *Journal of Reproduction and Fertility* **89**, 43–50.
Shaw PW, Bernard AG, Fuller BJ *et al*. 1992 Vitrification of mouse oocytes using short cryoprotectant exposure: effects of varying exposure times on survival. *Molecular Reproduction and Development* **33**, 210–214.
Stachecki JJ, Willadsen SM 2000 Cryopreservation of mouse oocytes using a medium with low sodium content: effect of plunge temperature. *Cryobiology* **40**, 4–12.
Stachecki JJ, Willadsen SM 2004 Spindle organization after cryopreservation of mouse, human, and bovine oocytes. *Reproductive BioMedicine Online* **8**, 664–672.
Stachecki JJ, Cohen J, Willadsen SM 1998a Cryopreservation of unfertilized mouse oocytes: The effect of replacing sodium with choline in the freezing medium. *Cryobiology* **37**, 346–354.
Stachecki JJ, Cohen J, Willadsen SM 1998b Detrimental effects of sodium during oocyte cryopreservation. *Biology of Reproduction* **59**, 395–400.
Stachecki JJ, Cohen J, Schimmel T *et al*. 2002 Fetal development of mouse oocytes and zygotes cryopreserved in a nonconventional freezing medium. *Cryobiology* **44**, 5–13.
Suzuki T, Boediono A, Takagi M *et al*. 1996 Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by a one-step dilution method in vitro. *Cryobiology* **33**, 515–524.
Toner M, Cravalho EG, Armant DR 1990 Water transport and estimated transmembrane potential during freezing of mouse oocytes. *Journal of Membrane Biology* **115**, 261–272.
Toner M, Cravalho EG, Karel M *et al*. 1991 Cryomicroscopic analysis of intracellular ice formation during freezing of mouse oocytes without cryoadditives. *Cryobiology* **28**, 55–71.
Toner M, Cravalho EG, Stachecki J *et al*. 1993 Nonequilibrium freezing of one-cell mouse embryos. Membrane integrity and developmental potential. *Biophysical Journal* **64**, 1908–1921.
Trad FS, Toner M, Biggers JD 1999 Effects of cryoprotectants and ice-seeding temperature on intracellular freezing and survival of human oocytes. *Human Reproduction* **14**, 1569–1577.
Tucker M, Wright G, Morton P *et al*. 1996 Preliminary experience with human oocyte cryopreservation using 1,2-propanediol and sucrose. *Human Reproduction* **11**, 1513–1515.
Tucker MJ, Wright G, Morton PC *et al*. 1998 Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. *Fertility and Sterility* **70**, 578–579.
Vajta G, Holm P, Kuwayama M *et al*. 1998 Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development* **51**, 53–58.
Van den Abbeel E, Van der Elst J, Van Steirteghem AC 1994 The effect of temperature at which slow cooling is terminated and of thawing rate on the survival of one-cell mouse embryos frozen in dimethyl sulfoxide or 1,2-propanediol solutions. *Cryobiology* **31**, 423–433.
Van der Elst J, Van den Abbeel E, Jacobs R *et al*. 1988 Effect of 1,2-propanediol and dimethylsulphoxide on the meiotic spindle of the mouse oocyte. *Human Reproduction* **3**, 960–967.
Van der Elst JC, Nerinckx SS, Van Steirteghem AC 1993 Slow and ultrarapid freezing of fully grown germinal vesicle-stage mouse oocytes: optimization of survival rate outweighed by defective blastocyst formation. *Journal of Assisted Reproduction and Genetics* **10**, 202–212.
Van Os HC, Zeilmaker GH 1986 Volumetric behaviour and survival of mouse zygotes and embryos in hyperosmotic media. *Human Reproduction* **1**, 95–98.
Van Uem JF, Siebzehnrubl ER, Schuh B *et al*. 1987 Birth after cryopreservation of unfertilized oocytes [letter]. *Lancet* **1**, 752–753.
Van Winkle LJ, Haghighat N, Campoine al. 1990 Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *Journal of Experimental Zoology* **253**, 215–219.

Vidali D, Dani G, Antinori M *et al*. 1998 Oocyte cryopreservation is a viable alternative option for patients who refuse embryo freezing. *Fertility and Sterility* **70**, S138.

Vieira AD, Mezzalira A, Barbieri DP *et al*. 2002 Calves born after open pulled straw vitrification of immature bovine oocytes. *Cryobiology* **45**, 91–94.

Vincent C, Johnson MH 1992 Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. *Oxford Reviews of Reproductive Biology* **14**, 73–100.

Vincent C, Garnier V, Heyman Y *et al*. 1989 Solvent effects on cytoskeletal organization and in-vivo survival after freezing of rabbit oocytes. *Journal of Reproduction and Fertility* **87**, 809–820.

Vincent C, Pickering SJ, Johnson MH 1990 The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present. *Journal of Reproduction and Fertility* **89**, 253–259.

Wininger JD, Kort HI 2002 Cryopreservation of immature and mature human oocytes. *Seminars in Reproductive Medicine* **20**, 45–49.

Wood MJ, Whittingham DG, Lee SH 1992 Fertilization failure of frozen mouse oocytes is not due to premature cortical granule release. *Biology of Reproduction* **46**, 1187–1195.

Wood MJ, Barros C, Candy CJ *et al*. 1993 High rates of survival and fertilization of mouse and hamster oocytes after vitrification in dimethylsulphoxide. *Biology of Reproduction* **49**, 489–495.

Wu B, Tong J, Leibo SP 1999 Effects of cooling germinal vesicle-stage bovine oocytes on meiotic spindle formation following in vitro maturation. *Molecular Reproduction and Development* **54**, 388–395.

Wu J, Zhang L, Wang X 2001 In vitro maturation, fertilization and embryo development after ultrarapid freezing of immature human oocytes. *Reproduction* **121**, 389–393.

Yang D, Blohm P, Winslow L *et al*. 1998 A twin pregnancy after microinjection of human cryopreserved oocyte with a specially developed oocyte cryopreservation regime. *Fertility and Sterility* **70**, S239.

Yang D, Blohm P, Cramer L *et al*. 1999 A successful human oocyte cryopreservation regime: survival, implantation and pregnancy rates are comparable to that of cryopreserved embryos generated from sibling oocytes. *Fertility and Sterility* **72**, S86.

Yang D, Winslow L, Blohm P *et al*. 2002 Oocyte donation using cryopreserved donor oocytes. *Fertility and Sterility* **78**, S14.

Yokota Y, Sato S, Yokota M *et al*. 2000 Successful pregnancy following blastocyst vitrification: case report. *Human Reproduction* **15**, 1802–1803.

Yokota Y, Sato S, Yokota M *et al*. 2001 Birth of a healthy baby following vitrification of human blastocysts. *Fertility and Sterility* **75**, 1027–1029.

Yoon TK, Kim TJ, Park SE *et al*. 2003 Live births after vitrification of oocytes in a stimulated in vitro fertilization–embryo transfer program: freezing immature oocytes. *Fertility and Sterility* **79**, 1323–1326.

Young E, Kenny A, Puigdomenech E *et al*. 1998 Triplet pregnancy after intracytoplasmic sperm injection of cryopreserved oocytes: case report. *Fertility and Sterility* **70**, 360–361.

Younis AI, Toner M, Albertini DF *et al*. 1996 Cryobiology of non-human primate oocytes. *Human Reproduction* **11**, 156–165.

Zenzes MT, Bielecki R, Casper RF *et al*. 2001 Effects of chilling to 0 degrees C on the morphology of meiotic spindles in human metaphase II oocytes. *Fertility and Sterility* **75**, 769–777.

Zhao Y, Aspinall D, Paleg L 1992 Protection of membrane integrity in *Medicago sativa* l. by glycinebetaine against the effects of freezing. *Journal of Plant Physiology* **140**, 541–543.

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