Reproductive BioMedicine Online (2011) **23**, 298–306

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SYMPOSIUM: OOCYTE CRYOPRESERVATION
REVIEW

Theoretical and experimental basis of oocyte vitrification

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Abstract In the last decades significant advances have been made in successful cryopreservation of mammalian oocytes. Human oocyte cryopreservation has practical application in preserving fertility for individuals at risk of compromised egg quality due to cancer treatments or advanced maternal age. While oocyte cryopreservation success has increased over time, there is still room for improvement. Oocytes are susceptible to cryodamage; which collectively entails cellular damage caused by mechanical, chemical or thermal forces during the vitrification and warming process. This review will delineate many of the oocyte intracellular and extracellular structures that are/may be stressed and/or compromised during cryopreservation. This will be followed by a discussion of the theoretical basis of oocyte vitrification and warming, and a non-exhaustive review of current experimental data and clinical expectations of oocyte vitrification will be presented. Finally, a forward-thinking vision of a potential means of modifying and improving vitrification and warming procedures and success will be proposed.

KEYWORDS: oocyte, vitrification

Introduction

The Scottish anatomist and surgeon, John Hunter, pioneered the process of cryopreservation with visions of prolonged storage of tissues that could be revitalized following suspended animation in cryostorage. Dr Hunter speculated that life could be rekindled following freezing of the entire human body (for review, see Leibo, 2004). In

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2011.05.003

the 1940s it was discovered that addition of glycerol to protect against cryodamage greatly enhanced survival of cryopreserved living cells (Polge et al., 1949; Smith, 1961). This gave rise to the investigational concept of cryoprotectants. In hindsight, the use of cryoprotectants is logical considering that in insects biological systems exist whereby sugars and sugar-alcohols are used as cryoprotectants to withstand severe winter temperatures (Pegg, 1987). Experience in cryopreservation of various cell types led to the appreciation that as cell size increases, difficulty in cryopreservation also increases (Mazur et al., 1972). This concept is of particular importance in mammalian oocyte and embryo cryopreservation. Cryobiology advances over the last 200 years have supported significant advances in cryopreservation of living cells, including spermatozoa, preimplantation embryos and more recently oocytes. While cryopreservation of spermatozoa and embryos has had significant advances in the last three decades, oocyte cryopreservation has only recently seen consistent success. Due to their specific characteristics such as cell size, membrane permeability and intracellular functions oocytes are very susceptible to cryodamage; which involve cellular damage caused by mechanical, chemical or thermal forces during the cryopreserving process.

Promise of oocyte cryopreservation

The first report of oocyte cryopreservation, reanimation and use to generate embryos and a viable pregnancy (Chen, 1986) originated from slow-rate freezing–thawing and was followed by many years of limited success. More recently, resurgent interests and efforts have focused on oocyte cryopreservation by both slow-rate freezing (Bianchi et al., 2007; Boldt et al., 2003; Borini et al., 2004; Porcu et al., 1997; Tucker et al., 1998; Winslow et al., 2001) and vitrification (Katayama et al., 2003; Kuleshova et al., 1999a; Kuwayama et al., 2005; Yoon et al., 2003). Oocyte cryopreservation holds clinical and practical promise for many patient populations; including women at risk of losing fertility due to chronic disease and/or treatment (i.e. cancer therapies using radio- or chemotherapies; for review, see Huang et al., 2007) and those with genetic predisposition to infertility (i.e. Turner syndrome; Kavoussi et al., 2008). Oocyte cryopreservation allows flexibility for assisted reproductive programmes if initial treatment cycles must be halted for unforeseen reasons (Gera et al., 2010). Oocyte cryopreservation significantly reduces the medical management and financial burden within an oocyte donation treatment cycle, allows quarantine of oocytes for infectious disease testing of donors and enhances protection of recipients (Hammarberg et al., 2008). In the future, patients who choose or are required to postpone childbirth until advanced age, when oocytes normally have a decreased developmental competence, could cryopreserve oocytes at a young age thus increasing the chances of establishing an isogenetic pregnancy at a more advanced age. Finally, oocyte cryopreservation provides another option to patients concerned with ethical and legal issues of embryo cryopreservation.

Oocyte cryopreservation-induced stress

During cryopreservation, cells are exposed to numerous stressors including mechanical, thermal and chemical (Mazur et al., 1972; Meryman, 1971), which can lead to compromised cell function and cell death. Cellular stress is defined as altered cellular and/or subcellular processes involved with significant disturbance from a homeostatic state. In relation to the oocyte cryopreservation, external stressors can cause oocyte stress or cryodamage that may be transient disturbances from the homeostatic state and sublethal or be permanent and lethal. In general it has been demonstrated that oocytes are more sensitive to cryodamage than later embryonic stages (Friedler et al., 1988). It is important to recognize that not all stresses cause actual damage. In consideration of cellular structures and events that can be stressed and/or damaged by oocyte cryopreservation one must consider specific intracellular organelles within the nucleus and cytoplasm, their normal developmental and homeostatic functions, their ability to respond to stresses and the potential consequences of compromising their intracellular spatial orientation and functions with cryodamage (for review, see Smith and Silva E Silva, 2004). Most oocyte cryopreservation today is performed with mature metaphase II (MII) oocytes and thus the nuclear structure and cryodamage will not be considered here. The ultrastructural configuration of the cytoplasm is continually changing dependent upon the stage of the meiotic and mitotic cell cycle. Thus potential effects of cryo-induced stress on cytoplasmic function will inherently be related to stage of the cell cycle.

Many studies in the last two decades have focused on oocyte cryopreservation by slow-rate freezing and vitrification, stress, microtubule polymerization, spindle structure and potential 'damage' of the meiotic spindle (Bromfield et al., 2009; Coticchio et al., 2010b; Gomes et al., 2008; Pickering and Johnson, 1987). Proper organization and function of the oocyte cytoskeleton are essential for normal segregation of chromosomes, spindle rotation, cytokineses and pronuclei/nuclei formation (Maro et al., 1986; Schatten et al., 1985). In oocytes the major microtubular structure is the spindle, which is responsible for spatial organization and subsequent migration of chromosomes during meiotic divisions. Disruption of the microtubular network within the oocyte can result in scattering and/or displacement of the spindle and thus changes in the chromosomal complement within the cell. Research has demonstrated that exposure of oocytes to cooling (Aman and Parks, 1994; Pickering and Johnson, 1987; Pickering et al., 1990), cryoprotectants (Johnson and Pickering, 1987; Vincent et al., 1989) or the freeze–thaw process (Aigner et al., 1992) can cause depolymerization and disorganization of spindle microtubules. However, numerous reports have demonstrated that the metaphase spindle microtubules that depolymerize during oocyte cryopreservation by both slow-rate freezing and vitrification can repolymerize and form normal structural and functional spindles post-cryopreservation when given time and the proper temperature environment (**Figure 1**; (Bromfield et al., 2009; Coticchio et al., 2010b; Gomes et al., 2008)). A current area of interest is in determining whether differences exist between meiotic stages when

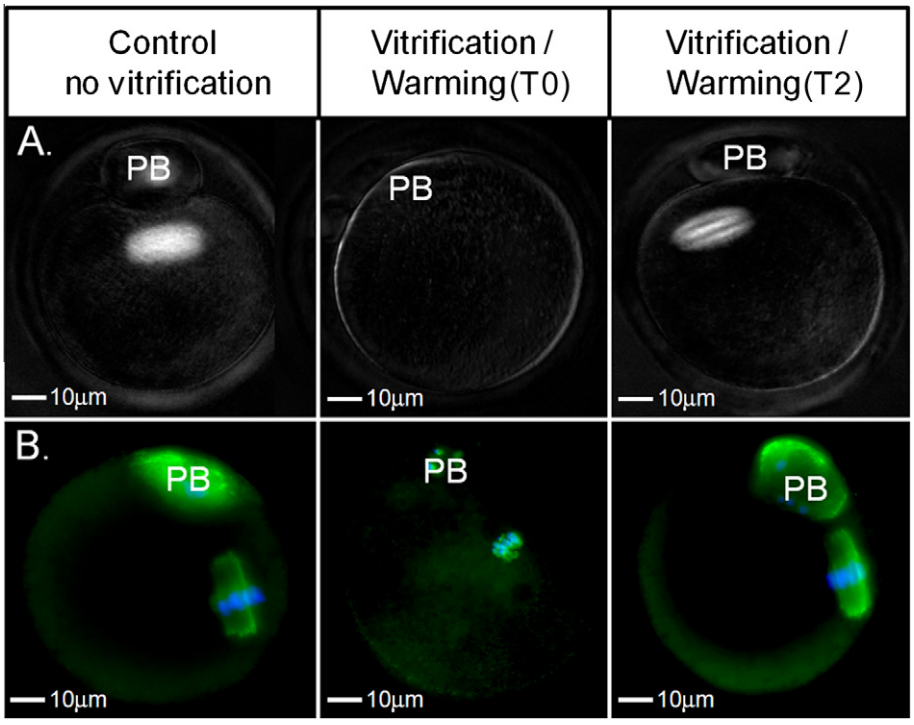

Figure 1 Analysis of mouse metaphase II oocyte spindles by (**A**) polymerized filter microscopy and (**B**) and immunocytochemistry without vitrification and warming, initially after vitrification and warming (T0) and after 2 h incubation (T2) at 37°C. These data demonstrate that while spindles can depolymerize during vitrification and cooling, they can repolymerize if given the proper environmental conditions. Blue = Hoechst; green = anti-β-tubulin/fluorescein isothiocyanate; PB = polar body. Bars = 10 μm.

spindles are present (i.e. metaphase I, anaphase I, telophase I and metaphase II) and ability of microtubules to depolymerize and repolymerize with fidelity. If there are differences in the ability of spindles to depolymerize and repolymerize, how does this impact subsequent meiotic progression, fertilization, embryo development, pregnancy and health of the resulting offspring?

Other primary cytoplasmic structures to consider in oocyte cryopreservation, cryo-induced stress and potential damage are microfilaments composed of polymerized actin and intracellular spatial distribution of intracellular organelles, such as ribosomes, Golgi apparati and mitochondria. In human oocytes, microfilaments have been found organized in a uniform layer enveloping the cortex (Pickering et al., 1988) and are necessary for regulating spindle rotation, polar body extrusion, pronuclear migration, intracellular trafficking of molecules and organelles and cytokinesis (Le Guen et al., 1989; Schatten et al., 1986; Vincent and Johnson, 1992; Wang et al., 2000). Cooling of oocytes, exposure to permeating cryoprotectants and the slow-rate freezing and vitrification processes have not been demonstrated to alter microfilament polymerization (De Santis et al., 2007; Rojas et al., 2004; for review, see Smith and Villa-Diaz, 2007). Additionally, oocytes contain a pool of non-replicating yet functional mitochondria from which all cells of the resultant embryos directly inherit. Mitochondria play a vital role in the metabolism of energy-containing compounds in the oocyte cytoplasm to provide ATP for fertilization and preimplantation embryo development. One current view of mitochondrial function is that reduced meiotic competence, fertilizability of oocytes and developmental failure in the preimplantation embryo could result from pre-existing oocyte mitochondrial DNA (mtDNA) defects (Perez et al., 2000), from age-related accumulation of mtDNA mutations (Keefe et al., 1995) and/or abnormal distributions of mitochondria in the oolemma (Nagai et al., 2006; Van Blerkom et al., 2000). It is interesting to consider that abnormal distribution of oocyte mitochondria could result from compromised microfilament function after oocyte cryopreservation by either slow-rate freezing or vitrification. Mitochondrial swelling has been observed after oocyte cryopreservation (Hochi et al., 1999; Valojerdi and Salehnia, 2005). Whether mitochondria can recover from swelling, or what will be the developmental consequences of it, is unknown. Recently, Thouas et al. (2006) demonstrated that sublethal mitochondrial injury in mouse oocytes is heritable by resultant embryos and results in post-implantation pathologies similar to those reported for clinically subfertile women, including recurrent implantation failure or miscarriage and decreased live birthweight. Some elegant studies looking at the ultrastructure of human oocytes have recently demonstrated a high percentage of atypical, small and slender mitochondria—smooth endoplasmic reticulum aggregates following vitrification (Nottola et al., 2009) and normal-appearing mitochondria—smooth endoplasmic reticulum after slow-rate freezing (Coticchio et al., 2010a). Methods to circumvent or minimize oocyte cryopreservation-induced stress should be goals for the future in making oocyte cryopreservation more efficient and safe.

Theoretical basis of oocyte vitrification

Currently, there are two methods used to cryopreserve mammalian oocytes: slow-rate freezing and vitrification (for review, see Bernard and Fuller, 1996). Independent of the methodology used for cryopreservation, effects on oocyte cellular functions can compromise the ability to develop normally following the cryopreservation–re-animation process.

In general, slow-rate freezing attempts to control biophysical properties of freezing, like cooling and warming rates, in conjunction with cryoprotectants to minimize adverse cellular events. This method allows cells to be cooled to very low temperatures while minimizing intracellular ice crystal formation and at the same time attempting to minimize the detrimental influences of increased solute concentrations and osmotic stress (Friedler et al., 1988). Thus one can appreciate that with slow-rate freezing extracellular ice formation drives cellular dehydration through an equilibrium process. On the other hand, vitrification, a form of rapid cooling, utilizes very high concentrations of cryoprotectant that becomes an extremely viscous supercooled liquid without forming ice crystals, which are a major cause of intracellular cryodamage. Vitrification can be considered a non-equilibrium approach to cryopreservation (Rall and Fahy, 1985). The vitrified solids therefore contain the normal molecular and ionic distributions of the original liquid state and can be considered an extremely viscous, supercooled liquid (Rall, 1987). In this technique oocytes are dehydrated by brief exposure to a concentrated solution of cryoprotectant before plunging the samples directly into liquid nitrogen. Application of vitrification for both oocytes and embryos is an area of current focus for many clinical, rodent and domestic animal production laboratories. An excellent review of the history of vitrification and potential advantages is available (Kuleshova et al., 1999b). It is important to recognize that vitrification is not a new technique and was not recently 'developed' by any single individual, and in fact there are numerous solutions, cryocontainers and methodologies that are available and work well. It is important to recognize that the majority of recent reports relating to human oocyte cryopreservation, especially vitrification, are empirical observations and not controlled studies testing one parameter versus another. For example, does inclusion of dimethylsulfoxide (DMSO) help or hinder vitrification? Should oocytes be cryopreserved with or without cumulus cells? Does exposure of oocytes to equilibration and vitrification solution need to be at 22, 25 or 37°C? Does the volume of vitrification solution surrounding the oocyte influence post-warming survival and function? Finally, does scientific evidence exist that one cooling rate is beneficial over another once one gets beyond a set rate? These theoretical issues, which are many times discussed as dogma, will be addressed below.

Experimental basis of oocyte vitrification

Prior to discussion of experimental evidence supporting or refuting theoretical queries stated above, the laboratory methods need to be briefly explained. This protocol is discussed in more detail elsewhere (Smith and Fioravanti, 2006). In addition, this protocol has been used in the only prospective randomized trial to date, as far as is known comparing slow-rate freezing and vitrification of human oocytes (discussed below). Denuded MII oocytes were placed into a 20 μl drop of human tubal fluid medium with Hepes (HTF-H) and 12 mg/ml serum synthetic substitute (SSS) for 1 min prior to merging with an adjacent 20 μl drop of equilibration solution (7.5% v/v ethylene glycol, 7.5% v/v DMSO, 12 mg/ml SSS in HTF-H). After 2 min, a second 20 μl drop of equilibration solution was merged with drops containing oocytes. Two minutes later oocytes were removed and pipetted into a fresh 20 μl drop of equilibration solution for 3 min. Oocytes were subsequently pipetted into separate 20 μl drops of vitrification solution (15% v/v ethylene glycol, 15% v/v DMSO, 0.5 mol/l sucrose, 12 mg/ml SSS in HTF-H) for 5 s, 5 s, 10 s and 90 s. All solution exposures were performed at room temperature or approximately 22°C. During the final 90 s in vitrification solution, oocytes were loaded into vitrification containers, sealed and submerged in liquid nitrogen. For warming, oocytes in closed containers were rapidly transferred from liquid nitrogen into a 37°C water bath for 3 s. After containers were opened and the contents expelled, straws were rinsed with ~1 μl of initial warming solution (1.0 mol/l sucrose, 12 mg/ml SSS in HTF-H), expelled as a drop and merged with the straw content drop for 1 min. Oocytes were then transferred into a 20 μl drop of initial warming solution for an additional minute followed by movement through 2 × 20 μl drops of dilution solution (0.5 mol/l sucrose, 12 mg/ml SSS in HTF-H) for 2 min each and finally through three 20 μl drops of wash solution (12 mg/ml SSS in HTF-H) for 2 min per drop. Oocytes were then moved into pre-equilibrated 37°C bicarbonate-buffered media supplemented with SSS to a total protein content of 12 mg/ml and placed into a humidified 37°C environment with 7.0% CO_2 and air until intracytoplasmic sperm injection is performed.

Numerous reports have demonstrated that human oocytes (Katayama et al., 2003; Kuleshova et al., 1999a,b; Kuwayama et al., 2005) can be successfully vitrified. Additionally, many programmes have excellent experience with slow-rate freezing of oocytes (Bianchi et al., 2007; Boldt et al., 2003; Borini et al., 2004; Chen, 1986; Tucker et al., 1998; Winslow et al., 2001). While a meta-analysis has been performed comparing reported outcomes of human oocyte slow-rate freezing and vitrification (Oktay et al., 2006), no investigational comparative study of human oocyte cryopreservation technologies existed, until recently (Smith et al., 2010). Here will be presented some of the study centres' experimental findings on oocyte vitrification, with a clear appreciation that numerous programmes have reported similar or better pregnancy success rates following oocyte vitrification, albeit many times with very different patient populations.

From January 2005 to April 2009, 230 patients with infertility performed oocyte cryopreservation as a means of eliminating and/or reducing numbers of embryos cryopreserved following infertility treatment with IVF. Those patients who did not become pregnant within their ovarian stimulation (non-cryopreserved) IVF cycle, or who requested an oocyte thawing/warming to obtain an additional pregnancy, returned to the clinic to re-animate

oocytes with subsequent laboratory interventions in an attempt to initiate a pregnancy. This equated to 30 cases of oocyte thaw from slow-rate freezing and 48 cases of warming from vitrification. Once an oocyte thawing/warming cycle was initiated, a semen sample was collected from male partners, analysed and used to prepare spermatozoa for insemination. Initial survival rate was significantly different between frozen (67%) and vitrified (81%; $P < 0.001$) cryopreserved oocytes. Survival of oocytes at 4 h after thawing/warming was still significantly higher in the vitrification group ($P < 0.01$). The percentage of inseminated cryopreserved and surviving oocytes that fertilized normally was significantly lower following freezing (67%) compared with vitrification (77%; $P < 0.03$). The percentage of fertilized zygotes that cleaved was significantly different between cryopreservation techniques ($P < 0.01$). On day 3 (day 0 = day of oocyte thawing/warming), average embryo development was significantly compromised following oocyte thaw compared with warming. Highest quality embryos resulting from oocyte cryopreservation were transferred on day 3 and numbers of embryos transferred were not significantly different between frozen (3.2 ± 0.3) and vitrified (3.1 ± 0.1) oocyte groups.

Because the objective of this trial was to compare oocyte cryopreservation technologies, clinical pregnancy rates were compared in relation to number of oocyte thawing or warming cycles performed and not with respect to number of embryo transfers performed (discussed further below). A significant difference ($P < 0.02$) was observed in clinical pregnancy rates (gestational sac and heartbeat) at 13% (4/30) per frozen–thawed cycles compared with 38% (18/48) per vitrification–warmed cycles.

A few important points from these data require further discussion. The initial survival following vitrification–warming was 81%, and while this is lower than some reports of survival following human oocyte vitrification of 95% or more (Cobo et al., 2008; Katayama et al., 2003; Kuwayama et al., 2005), it is representative of a survival rate (81%) calculated when one averages reported human oocyte survival following vitrification–warming (Chian et al., 2008; Katayama et al., 2003; Kuleshova et al., 1999b; Kuwayama et al., 2005; Kyono et al., 2005; Lucena et al., 2006; Wu et al., 2001; Yoon et al., 2003). Similarly, in this study initial survival of slow-rate frozen–thawed oocytes was 67%, again comparable to an average of 64% calculated from literature reports (Boldt et al., 2003; Borini et al., 2007; Chen, 1986; Kyono et al., 2005; Porcu et al., 1997, 1998; Quintans et al., 2002; Winslow et al., 2001). Higher cleavage rates and embryo morphological scores during early development following fertilization of vitrified–warmed oocytes in comparison to frozen–thawed oocytes emphasizes the impact of mechanical, chemical and thermal (Mazur et al., 1972; Meryman, 1971) stressors on oocytes; and how these influences can be recognized at a later stage of embryo development. Finally, this study emphasized the important issues of step-wise efficiencies, loss of attrition, cumulative impact of oocyte perturbations and subsequent embryo development and influence on final measures of success. Even though similar average numbers of oocytes were thawed or warmed per cycle, the reduced efficiency in survival, fertilization and cleavage following slow-rate freezing–thawing equated to 11 patients who did not have embryos for transfer, whereas only one patient in the oocyte vitrification–warming arm did not have an embryo transfer.

These data, from the prospective randomized trial comparing human oocyte slow-rate freezing and vitrification, are presented primarily to compare and contrast results in an experiment actually designed for that reason. It is tempting to compare study A with study B to draw conclusions about one protocol versus another, or one theoretical parameter of vitrification versus another; however, such comparisons have significant short-comings and are ill-advised. In addressing the theoretical issues of vitrification stated above, human data will be used when possible, yet many times this study will need to revert to animal model data from designed comparative studies.

There has been long-standing debate as to which, how much or what combination of permeating cryoprotectants one should use in vitrification. Recently, a novel approach in the mouse model has been proposed with low concentrations of 1,2-propanediol (Lee et al., 2010). While there may be benefits of using low concentrations of 1,2-propanediol it was recently demonstrated that at high concentrations both 1,2-propanediol and ethylene glycol induced genotoxic effects on Chinese hamster ovary cells, whereas DMSO did not (Aye et al., 2010). 1,2-Propanediol alone as the permeating cryoprotectant can be used to vitrify but does cause increased intracellular calcium and physiological effects that can be alleviated by using calcium-free media (Larman et al., 2007). These reports demonstrate the lack of consensus on use of permeating cryoprotectants for vitrification.

Should MII oocytes be vitrified with or without cumulus cells? The majority of experience in human MII oocyte vitrification has been with denuded oocytes because of the physical complexity of vitrifying cumulus-enclosed MII oocytes. In the bovine model system it was recently reported that survival, cleavage and blastocyst development rate was not significantly different following vitrification and warming with or without cumulus cells (Zhou et al., 2010).

Should exposure of oocytes to equilibration and vitrification solutions be performed at 20, 25, 30 or 37°C? Searching PubMed has yet to find a publication that describes the direct testing and comparison of these oocyte exposure temperatures in a systematic and scientific manner. Empirically, many researchers have worked at room temperature or approximately 22°C during vitrification and warming solution exposures and outcomes are represented above.

Cooling rate during the actual vitrification process has been suggested to be important. While there are cooling rates essential for vitrification, the question is: do higher cooling rates result in improved vitrification–warming outcomes? In a recent review (Saragusty and Arav, 2011), comparing vitrification of oocytes and embryos using liquid nitrogen or liquid nitrogen slush (2–6 times higher cooling rate compared with liquid nitrogen) the reports are extremely variable and inconclusive. In some species (bovine) and experiments when cryosurvival is suboptimal (Arav and Zeron, 1997; Santos et al., 2006), liquid nitrogen slush was beneficial. However, other reports in rodent and rabbit models where cryosurvival was greater than 80% with liquid nitrogen alone, the slush was not significantly better (Cai et al., 2005; Seki and Mazur, 2009). Again, a firm conclusion is wanting.

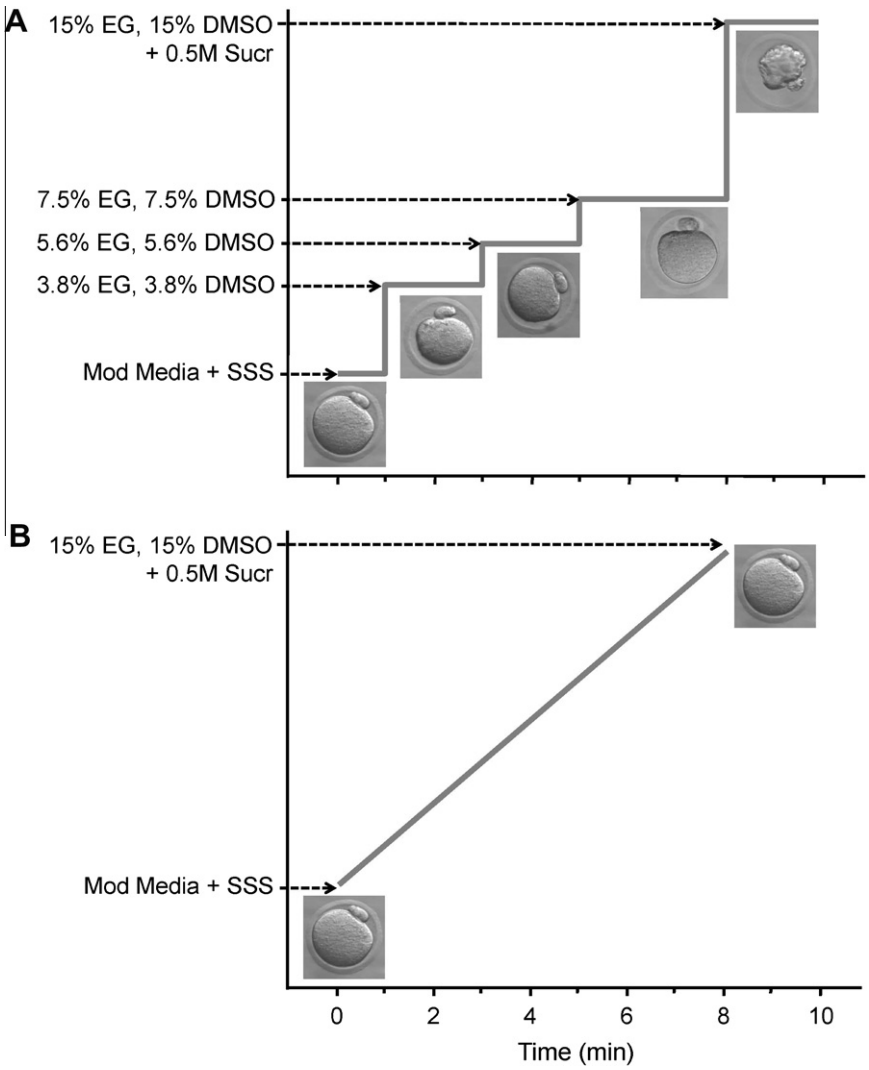

Figure 2 (**A**) Schematic representation of altered mouse oocyte size and shape during stepwise exposure to equilibration and vitrification solutions in preparation for vitrification. (**B**) Theoretical representation of solution exposures in an automated means with gradual changes in solution exposure and the potential to replace intracellular water with permeating cryoprotectants without significant alterations in cell size and shape. DMSO = dimethylsulfoxide; EG = ethylene glycol; Mod Media = modified media; Sucr = sucrose; SSS = serum synthetic substitute.

What about the volume of cryosolution surrounding the oocyte on, or in, one of the plethora of non-commercially and commercially available vitrification containers currently available; (Saragusty and Arav, 2011). It has been stated that the smaller the volume, the higher the rate of heat transfer, the higher the cooling rate and the higher the probability of vitrification (Yavin and Arav, 2007). However, once a threshold cooling rate is obtained, and vitrification occurs, is there an added benefit of faster cooling rates? One must query if the experiment has been performed to demonstrate that vitrification in 0.1, 0.5, 1.0, 5.0 or 100 μl of vitrification are comparable or different? Considering that evaporation, surface area and initial volume can impact osmolality of solutions (Heo et al., 2007), maybe we should be concerned about using too little solution? Most importantly, experimental data should drive conclusive statements regarding these and other practical procedural steps.

Future thoughts

Currently there are extensive discussions and debates as to which vitrification container is best and which combination of permeating and non-permeating cryoprotectants are most efficient in supporting oocyte cryopreservation and long-term safety. All vitrification containers work and the efficiencies are highly technician-dependent. Sound advice is to select a vitrification container that meets needs and regulatory demands and to practise extensively with that container, and efficiency will be achieved. Thinking beyond containers and solutions, maybe researchers should be con-

sidering mechanisms to precisely control the mixing and exposure of oocytes to various vitrification and warming solutions. As oocytes are exposed to increasing concentrations of permeating and non-permeating cryoprotectants the cell itself physically shrinks and re-expands. The same is true as one replaces intra-oocyte permeating cryoprotectants with water during the warming process. Considering the importance of spatial orientation of the cytoskeleton and developmental-stage-specific distribution of intracytoplasmic organelles, like mitochondria, there are the questions: are these structures and organelles displaced by these shrinking—re-expansion events, and does this impact subsequent developmental competence as embryos? Might there be a benefit from removing these step-wise additions of solutions, and the resulting cell-shape perturbation (**Figure 2A**), by designing systems for vitrification that are experimentally developed and tested to transit the oocyte from no cryoprotectant to vitrification solution permeated in a gradual progressive manner (**Figure 2B**)? One could envision that a microfluidics device where solutions are moved over cells, instead of cells through solutions, could provide an automated method of delivering solutions to cells in a gradient fashion. Importantly, experiments would need to be performed to demonstrate the utility of such a device and the theoretical benefit of gradual exposure and/or removal of cryosolutions. Might the same approach of gradual replacement of permeating cryoprotectant with water in the warming process be superior? Data exist to suggest that added steps during the warming process, and reducing the differences between steps, can be beneficial for cell survival and function (Aono et al., 2005; Kuwayama et al., 1994). Whether such systems of automating the vitrification—warming procedure and moving and mixing solutions in a systematic fashion, instead of manually moving cells through drops of different cryosolutions, will be advantageous remains to be tested and reported.

Clinical and laboratory practitioners of assisted reproductive technologies are indebted to the numerous cryobiologists, whose basic and translational research have contributed significantly to current success of oocyte cryopreservation. Knowledge continues to accumulate regarding mechanisms important in oocyte and early embryo development, such as the importance of protein structural/functional relationships to normal gene expression, protein translation, intracellular trafficking, epigenetic modifications and cell development. Independent of whether this information is gained through studies on cell-lines, somatic cells, gametes or embryos, ultimately researchers will assess normalcy of these cell functions in relation to cryopreservation. Such an approach will provide data that will lead to optimization of technical procedures and will likely be the avenue by which future oocyte and embryo cryopreservation success is improved and refined.

Acknowledgements

The authors would like to express their appreciation to Dr Carrie Cosola-Smith for critical review of this manuscript. They would like to thank Drs. Nagy and Noyes for the invitation to participate in this collection of reviews. They apologize to those whose work has not been cited owing to space limitation. Finally, they thank the reviewers for valuable input.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 25 August 2010; refereed 4 May 2011; accepted 5 May 2011.