Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes

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Abstract

Despite the success of embryo cryopreservation, routine oocyte freezing has proved elusive with only around 200 children born since the first reported birth in 1986. The reason for the poor efficiency is unclear, but evidence of zona pellucida hardening following oocyte freezing indicates that current protocols affect oocyte physiology. Here we report that two cryoprotectants commonly used in vitrification procedures, dimethyl sulfoxide (DMSO) and ethylene glycol, cause a large transient increase in intracellular calcium concentration in mouse metaphase II (MII) oocytes comparable to the initial increase triggered at fertilization. Removal of extracellular calcium from the medium failed to affect the response exacted by DMSO challenge, but significantly reduced the ethylene glycol-induced calcium increase. These results suggest that the source of the DMSO-induced calcium increase is solely from the internal calcium pool, as opposed to ethylene glycol that causes an influx of calcium across the plasma membrane from the external medium. By carrying out vitrification in calcium-free media, it was found that zona hardening is significantly reduced and subsequent fertilization and development to the two-cell stage significantly increased. Furthermore, such calcium-free treatment appears not to affect the embryo adversely, as shown by development rates to the blastocyst stage and cell number/allocation. Since zona hardening is one of the early activation events normally triggered by the sperm-induced calcium increases observed at fertilization, it is possible that other processes are negatively affected by the calcium rise caused by cryoprotectants used during oocyte freezing, which might explain the current poor efficiency of this technique.

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Introduction

The ability to routinely cryopreserve human oocytes will have a significant impact on assisted reproductive technology (ART). Changes in government guidelines in certain countries now restrict the number of oocytes that can be inseminated. This means there is now an increased need for gamete cryopreservation. Furthermore, not only will oocyte cryopreservation circumvent the ethical and legal problems associated with embryo freezing, but it will also benefit numerous patient groups including women at risk of ovarian function loss through premature menopause, surgical treatments or radio/chemotherapy (for review see Falcone *et al.* 2004). An increasing number of women are deciding to delay starting a family and although currently controversial, one could envisage cryostorage of oocytes from younger women to alleviate the problems associated with maternal ageing and the sharp drop in egg quality that occurs between 30 and 40 years of age.

Currently, there are two techniques employed for cryopreservation - slow-freezing and vitrification. The principal difference between these two procedures, when using the same freezing straws, is the rate of cooling (approximately 1 vs >1000 °C/min respectively). For both protocols, first the cells must be treated with a combination of cell-permeating and non-permeating agents to minimize ice formation that can severely damage the cell. Previously, high concentrations of cryoprotectant were required for vitrification, but novel containers and sub-microliter volumes result in super-fast cooling and subsequent warming rates (>20 000 °C/min; Steponkus *et al.* 1990, Mukaida *et al.* 2001) so that concentrations closer to those used in slow-freezing can now be used (Mukaida *et al.* 2002, Kasai & Mukaida 2004).

Embryo freezing has been implemented very successfully in ART and now allows supernumerary embryos generated through *in vitro* fertilization (IVF) to be routinely

frozen and stored (Liebermann 2003, Veeck 2003). Blastocysts thawed and subsequently transferred yield an acceptable pregnancy rate, which demonstrates that cryopreserved human blastocysts do not have a dramatic loss of viability (Gardner *et al.* 2003, Leibo 2004, Veeck *et al.* 2004). However, the oocyte has proved to be far more troublesome (Coticchio *et al.* 2004). Chen (1986) reported the first pregnancy following IVF of a cryopreserved human oocyte. Despite this, to date only around 200 children have resulted from oocyte cryopreservation techniques (Leibo 2004). Together with the fact that the number of births per number of oocytes frozen is typically no greater than 3%, it is clear that oocyte cryopreservation is an inefficient process at present.

The stark difference between the success of oocyte and embryo cryopreservation has yet to be strictly explained, but differences in cell size and membrane permeability have been proposed (Leibo 2004, Stachecki & Cohen 2004). The oocyte is unique in that the maternal DNA is held suspended in the cytoplasm on the meiotic spindle and not within the protective confines of the nuclear membrane, as it is for the majority of the time during subsequent preimplantation development. Damage to the DNA and/or microtubules could explain the limited success of oocyte cryopreservation. However, a further consideration is that the oocyte is arrested in a state primed for activation and changes in its environment can cause parthenogenetic activation.

A current potential problem with oocyte freezing is the induction of a primary activation event, zona hardening, which significantly inhibits IVF (Matson *et al.* 1997) and may affect subsequent implantation. Zona hardening is brought about by fusion of cortical granules to the plasma membrane and the release of their contents into the zona pellucida layers (for review see Sun 2003). The membrane fusion event is calcium-dependent and is normally triggered by the increase in intracellular calcium initiated by sperm-egg fusion (Kline & Kline 1992). Upon fusion, the sperm triggers a series of calcium oscillations. The initial calcium increase lasts for around 5 min and causes the cortical granules to fuse with the plasma membrane and release their contents into the zona pellucida layers (Tahara *et al.* 1996). Proteolytic enzymes target sperm binding proteins to prevent further sperm from fertilizing the egg. Zona hardening can be overcome by intracytoplasmic sperm injection (ICSI) (Porcu *et al.* 2000), but the fact that the oocyte has undergone the cortical granule reaction indicates that the oocyte has been artificially activated prior to actual fertilization. This could, therefore, explain the poor efficiency of oocyte freezing, for even though a sperm is injected via ICSI, the oocyte has already begun its activation events such as cortical granule exocytosis and possible down-regulation of cell cycle control proteins.

It has previously been reported that the cryoprotectant dimethyl sulfoxide (DMSO) causes zona hardening and a reduced rate of fertilization (Vincent *et al.* 1991). The effect of DMSO is not directly on the zona, but

requires the presence of the oocyte (Vincent *et al.* 1990). DMSO reduces the number of cortical granules (Vincent *et al.* 1991), suggesting that the DMSO-induced zona hardening is caused by cortical granule exocytosis. Since DMSO has been shown to cause a transient intracellular calcium rise in various cell lines (Morley & Whitfield 1993) and fusion of cortical granules to the oocyte plasma membrane is calcium-dependent (Kline & Kline 1992, Tahara *et al.* 1996), it is tempting to suggest that DMSO triggers cortical granule release by increasing intracellular calcium. DMSO appears not to be the only cryoprotectant that increases calcium since high concentrations of ethylene glycol (EG, 10–40%) have been shown recently to increase intracellular calcium in mouse oocytes (Takahashi *et al.* 2004).

Therefore, we have investigated whether exposure to cryoprotectants causes a calcium increase that leads to zona hardening. The data presented here show that the two cryoprotectants used in vitrification protocols, DMSO and EG, cause large transient increases in intracellular calcium in mouse oocytes. The increase in calcium is sufficient to cause zona hardening, which significantly reduces fertilization. By making the vitrification medium nominally calcium-free we have determined that zona hardening is significantly reduced, which allows IVF without the use of ICSI or laser-assisted zona removal. Furthermore, calcium-free vitrification does not seem to affect embryo developmental rates and cell number/allocation.

Materials and Methods

Unless otherwise stated all Materials were obtained from Sigma Chemical Co. (St Louis, MO, USA). This work received prior approval from the Institutional Animal Care and Users Committee. Sperm was collected from 12- to 20-week-old F1 (C57BL/6 × CBA) male mice and oocytes were collected from 4- to 5-week-old F1 (C57BL/6 × CBA/Ca) female mice (Jackson Laboratory, Bar Harbor, ME, USA). Female mice were administered 5 iu pregnant mare's serum gonadotropin and 48–52 h later 5 iu human chorionic gonadotropin (hCG) were administered. Oocytes were collected 12.5–13.5 h post-hCG and denuded by incubation in G-MOPS (Lane & Gardner 2004) (supplemented with 5 mg/ml human serum albumin (HSA)) with 0.5 mg/ml hyaluronidase. Oocytes were washed three times and held in droplets of G-MOPS under Ovoil (Vitrolife, Gothenburg, Sweden) at 37°C.

Intracellular calcium changes were monitored by loading the oocytes with 10 µmol l⁻¹ Indo-1 AM (Molecular Probes, Eugene, OR, USA) for 30 min. The loading media included 250 µmol l⁻¹ sulfinpyrazone to reduce dye compartmentalization and extrusion (Lawrence *et al.* 1997). Single oocytes were held in G-MOPS with or without calcium (with a holding pipette) in a heated chamber on a Nikon TE300 microscope equipped with an SFX-2 microfluorimeter (Solamere Technology Group, Salt Lake City, UT, USA). Oocytes were incubated in calcium-free

G-MOPS for 30 min before cryoprotectant exposure. Solutions without calcium were termed 'nominally-free' since calcium was simply omitted from the stock solution. To ensure rapid exposure that would best mimic the vitrification procedure, oocytes were held in a small droplet (approx. 20 μ l) of G-MOPS before 980 μ l of the cryoprotectant solution (made up in G-MOPS with 12 mg/ml HSA) was added to the heated chamber (37 °C).

Vitrification and warming were carried out at 37 °C in a base solution of G-MOPS supplemented with 12 mg/ml HSA. Vitrification was carried out using a two-step method and cryoloop, as previously described (Lane & Gardner 2001). Briefly, oocytes were placed in the initial cryoprotectant solution (8% DMSO, 8% EG v/v; equates to 1.13 mol l⁻¹ DMSO, 1.43 mol l⁻¹ EG) for 1 min. Oocytes were then moved to the second solution (16% DMSO, 16% EG v/v, 0.65 mol l⁻¹ sucrose; equates to 2.25 mol l⁻¹ DMSO, 2.86 mol l⁻¹ EG) for less than 30 s before being pipetted onto a cryoloop, which had been preloaded with this solution. The cryoloop was then plunged into a cryovial filled with liquid nitrogen to facilitate vitrification. For warming, oocytes were moved through 1 ml serial dilutions of sucrose (0.25 mol l⁻¹ for 1 min; 0.125 mol l⁻¹ for 2 min; 0 mol l⁻¹ for 5 min) to reduce osmotic stress (Lane & Gardner 2001). Oocytes were allowed to recover for 1 h. At this stage oocytes were either fertilized or the degree of zona hardening was determined.

To load the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM), oocytes were incubated with 10 μ mol l⁻¹ BAPTA-AM (Molecular Probes) for 30 min. For zona hardening assessment, oocytes were transferred to a 1% w/v solution of chymotrypsin (in G-MOPS) on a heated stage (37 °C) and the integrity of the zona pellucida was monitored over time, the end point being when the zona was no longer clearly visible (Matson *et al.* 1997). For fertilization, oocytes were transferred to G-FERT (Vitrolife). Twenty oocytes were placed in 20 μ l droplets of G-FERT. One microliter sperm suspension that had been allowed to capacitate for 2 h in 1 ml G-FERT was added to each 20 μ l droplet. When required, laser-assisted fertilization was carried out using a Fertilase 670 nm wavelength laser system (MTM Medical Technologies, Montreux, Switzerland) to create a 5–10 μ m hole in the zona. Oocytes were then maintained at 37 °C in 6% CO₂, 5% O₂ and 89% N₂. Twenty four hours after the sperm were added, oocytes were scored for development to the two-cell stage. Those embryos that had cleaved were transferred to G1 medium (Gardner & Lane 2004) and cultured for a further 24 h before being transferred to G2 medium (Gardner & Lane 2004) for an additional 48 h. On day 4 and day 5, embryos were scored for developmental stages. Cell number and allocation to the trophectoderm and inner cell mass were assessed using differential cell staining (Hardy *et al.* 1989). Statistical analysis was performed using either Students unpaired *t*-test or ANOVA followed by Bonferonni comparison.

Results

Current vitrification protocols typically employ both DMSO and EG. To investigate the effect of these two cryoprotectants on intracellular calcium concentration, mouse oocytes were loaded with the ratiometric fluorescent indicator, Indo-1 and the cryoprotectant solution rapidly applied. Both 1.5 mol l⁻¹ DMSO and EG caused transient increases in intracellular calcium that increased the fluorescence ratio 2–3 times and lasted for approximately 5 min (Fig. 1a,b). The ratio increase caused by both cryoprotectants was similar to the initial increase caused by sperm entry, although slightly longer in duration (Fig. 1c). It appears that the kinetics of calcium release are different as the mean base-to-peak ratio for DMSO (3.20 ± 0.16) was elevated compared with EG (2.66 ± 0.18 ; $P = 0.06$).

In an attempt to reduce the amount of calcium being released, oocytes were incubated in calcium-free media prior to application of the cryoprotectants (again in calcium-free media). The effect of calcium-free treatment was different for DMSO compared with EG (Fig. 1d,e). The DMSO-induced increase in calcium was unaffected by the absence of extracellular calcium. The mean base-to-peak ratio increase was not significantly different for DMSO challenge in the presence or absence of calcium (3.20 ± 0.16 and 3.11 ± 0.15 respectively; $P = 0.68$; Fig. 2a). In contrast, the EG response was significantly decreased by removal of calcium (2.66 ± 0.18 and 1.58 ± 0.1 respectively; $P = 0.0001$; Fig. 2b).

It has previously been reported that removing calcium from the cryoprotectant medium reduces the cytotoxicity of high levels of EG (Takahashi *et al.* 2004). Therefore, reducing the increase in intracellular calcium in response to cryoprotectant exposure offers an advantage in improving cell survival. Since an increase in calcium normally triggers cortical granule release, which leads to zona hardening, it was determined whether removing extracellular calcium also reduced zona hardening following cryoprotectant exposure during vitrification. Oocytes were either vitrified in the presence or absence of extracellular calcium. Zona hardening was then assessed by monitoring the time taken for zona dissolution in 1% chymotrypsin (Matson *et al.* 1997). Figure 3a shows that vitrified oocytes in the presence of calcium have a zona pellucida that takes 6 times longer to dissolve than non-vitrified control oocytes. However, removing extracellular calcium significantly reduces (by 40%) the zona hardening induced during vitrification.

To demonstrate that it is indeed a rise in intracellular calcium that triggers zona hardening during the vitrification procedure and not a direct physical effect, oocytes were loaded with the calcium chelator, BAPTA-AM, which has been used to block calcium increases at fertilization and mitosis entry in mouse embryos (Kono *et al.* 1996, Tombes *et al.* 1992). By directly preventing an increase in intracellular calcium with BAPTA, zona

Figure 1 The effect of DMSO and EG on intracellular calcium in metaphase II (MII) mouse oocytes. Mouse oocytes loaded with Indo-1 were held in a heated chamber (37 °C) and changes in calcium were monitored with a PMT (photomultiplier tube)-based system. Oocytes were treated with 1.5 mol l⁻¹ DMSO (a, d) or EG (b, e) in the presence or absence of calcium (*n* = greater than 12 for each treatment, with at least 6 replicates). (c) The initial increase in calcium triggered by sperm at fertilization.

hardening was further reduced to levels not significantly different from non-vitrified oocytes (Fig. 3a). A previous report has suggested that it is part of the freeze/thaw process and not cryoprotectant exposure

that physically alters the zona pellucida (Wood *et al.* 1992). To examine this, oocytes were treated with the same vitrification regime (i.e. vitrification and warming solutions) without exposure to liquid nitrogen and

Figure 2 Mean base-to-peak ratio increase following DMSO and EG exposure in the presence and absence of extracellular calcium. The increase in calcium was measured by the fold-increase in ratio from base to peak (*n* = greater than 12 for each treatment, with at least 6 replicates). (a) The difference between DMSO challenge in the presence or absence of calcium was insignificant (*P* = 0.68), whereas (b) EG showed a significant reduction in calcium release in the absence of extracellular calcium. ***P* = 0.0001.

Figure 3 The effect of removing extracellular calcium from the vitrification medium on zona hardening and subsequent fertilization. Oocytes were collected from F1 hybrid (C57BL/6 × CBA) female mice at 12.5 h post-hCG. (a) Oocytes were divided into 3 treatment groups and a non-vitrified control. Following vitrification in the presence or absence of calcium or treatment with BAPTA-AM, the oocytes were incubated in 1% chymotrypsin to assess the level of zona hardening. The time taken for the zona to completely disappear was recorded for each treatment (n = greater than 60 eggs for each treatment, with at least 3 replicates). Calcium-free vitrification and BAPTA-AM treatment significantly reduced zona hardening, when compared to vitrification with calcium (*; P = 0.001). Zona hardening following vitrification with or without calcium was statistically different from control (a; P = 0.001). (b) Following vitrification (>95% survival rate) oocytes were fertilized 15 h post-hCG in G-FERT. Laser-assisted fertilization (Laser) was included as an additional treatment group. The ability of the sperm to penetrate the zona and fertilize the oocyte was assessed by monitoring the number of two-cell stage embryos 24 h after addition of sperm (n = greater than 250 eggs for each treatment). Calcium-free vitrification and laser-assistance significantly increased fertilization and development to the two-cell stage, when compared to vitrification with calcium (*; P = 0.001). Development to the two-cell stage, following vitrification with or without calcium was statistically different from control (a; P = 0.001, b; P = 0.01).

subsequent vitrification. The time for zona dissolution following this treatment was 780.2 ± 26.42 s, indicating that the effect on the zona is caused by the cryoprotectant solution and not by the freeze/thaw process.

The outcome of zona hardening following vitrification is severely reduced fertilization rates with IVF. This is due

not only to the zona hardening, but also to a specific change in a sperm binding protein. The zona pellucida glycoprotein (ZP2) becomes converted to the fertilized form, which does not support sperm binding. This has resulted in the use of ICSI or partial zona removal following freezing to permit sperm access to the plasma membrane and fertilization (Carroll *et al.* 1990, Kazem *et al.* 1995, Porcu *et al.* 2000). Since zona hardening is significantly reduced in the absence of extracellular calcium, we wanted to determine if this increased the fertilization rate and subsequent development to the two-cell stage. Oocytes were vitrified in the presence or absence of extracellular calcium and then fertilized *in vitro*. Development to the two-cell stage was assessed at 24 h. Oocytes vitrified in the presence of calcium had very poor fertilization rates and poor development to the two-cell stage (less than 10%; Fig. 3b). Removing the extracellular calcium significantly increased the number of oocytes reaching the two-cell stage to a rate that approached the non-vitrified control (Fig. 3b). To show that it was zona hardening that reduced the level of fertilization and development, oocytes that had been vitrified in the presence of calcium had a 5–10 μ m hole made in the zona with a laser. When sperm access to the oocyte was permitted with this laser-assistance procedure, fertilization and two-cell development were comparable to non-vitrified oocytes (Fig. 3b).

Removing extracellular calcium appears to significantly reduce zona hardening and therefore dramatically improve fertilization rates. To ensure that the removal of calcium during the vitrification procedure does not compromise embryo development, fertilized oocytes were subsequently cultured and monitored for developmental rate to the blastocyst stage and cell number/allocation through differential staining. Oocytes that had been fertilized and developed to two-cell embryos were cultured in sequential media for a total of 96 h. Embryos were scored on day 4 and day 5 for embryo development. The vitrification protocol we used has previously been reported not to affect embryo development or cell number/allocation (Lane & Gardner 2001). It was found that carrying out calcium-free vitrification did not affect embryo development (Fig. 4a). On day four 39.1% of control (non-vitrified) embryos had reached the blastocyst stage, which was comparable to the number of embryos following calcium-free vitrification (37.2%). On day five the percentage of embryos from the control and calcium-free vitrification treatments that had reached the hatching blastocyst stage was also similar (94.2 and 92.2% respectively).

Using differential staining (Hardy *et al.* 1989) cell number and allocation between the trophectoderm (TE) and inner cell mass (ICM) were determined for non-vitrified control embryos and those that were vitrified in calcium-free media. Figure 4b shows that as with the regular vitrification protocol the calcium-free treatment had no detrimental effect on TE or ICM numbers.

Figure 4 The effect of calcium-free vitrification on embryo development. (a) Two-cell embryos derived from either non-vitrified (control) or calcium-free vitrified oocytes were cultured for 5 days. Embryo development was scored on day 4 and day 5 for morula, blastocyst and hatching blastocyst formation (n = greater than 140 embryos for each treatment). (b) Following the day 5 score, cell numbers composing the trophectoderm (TE) and inner cell mass (ICM) were determined using differential cell staining (n = greater than 60 for each treatment). No statistical differences were observed.

Discussion

Despite the high success rate of embryo cryopreservation and subsequent clinical pregnancies, efficient human oocyte cryopreservation remains elusive. Pronucleate stage embryos have been frozen using numerous techniques and different mammalian species, which suggests that the surface area to volume ratio, *per se*, does not account for the problematic nature of oocyte freezing (Coticchio *et al.* 2004). The permeability of the oocyte to cryoprotectants and their ability to replace intracellular water is another well documented consideration (Pfaff *et al.* 1998, Pedro *et al.* 2005), but rarely is the cell cycle stage contemplated. The oocyte is primed for activation and completion of meiosis, which is triggered by the sperm. The sperm delivers the signal to resume development by introducing a sperm-specific protein upon sperm-egg fusion (Saunders *et al.* 2002). The protein (phospholipase C zeta) triggers calcium oscillations that last several hours and control a number of activation events such as cortical granule exocytosis (block to polyspermy) and maturation promoting factor (MPF)

down-regulation (Lorca *et al.* 1991, Kline & Kline 1992, Nixon *et al.* 2002, Marangos & Carroll 2004) which allows completion of meiosis. Precocious initiation of cortical granule exocytosis has already been demonstrated during cryopreservation (Carroll *et al.* 1990). Together with the observation that the cryoprotectant agent DMSO causes zona hardening through cortical granule release, which is a calcium-dependent event, the aim of this study was to investigate if vitrification affects intracellular calcium.

Previous studies have reported that 1.5 mol l^{-1} 1,2-propanediol increased intracellular calcium in aged mouse oocytes (Litkouhi *et al.* 1999) and high levels of EG caused sustained calcium increases (Takahashi *et al.* 2004). However, to our knowledge no study has investigated the effect of cryoprotectants (DMSO and EG) at levels commonly used for vitrification on young oocytes (14–17 h post-hCG). By loading mouse oocytes with a fluorescent calcium indicator and exposing them to DMSO and EG, we have found that both of these agents cause a dramatic transient increase in intracellular calcium. The amplitude of the calcium increase caused by the cryoprotectants is comparable to the initial increase triggered at fertilization, but lasts approximately 50% longer in duration (Saunders *et al.* 2002). DMSO appeared to induce a larger and more sustained increase in calcium compared with EG, which may be attributable to the higher permeability of the oocyte to DMSO (Paynter 2005). The exact mechanism for the cryoprotectant-induced calcium increase is unknown. The lipophilic properties of DMSO and EG would be expected to have a non-specific effect on the plasma membrane and other internal membranes such as the endoplasmic reticulum, which would lead to either calcium influx and/or calcium release from internal stores. However, the osmotic contraction induced by cryoprotectants might also contribute to the increase in intracellular calcium.

Removing external calcium failed to affect the intracellular calcium increase caused by DMSO, which suggests that internal stores, such as the endoplasmic reticulum, are the source of the rise. This is in agreement with observations with several different cell lines (Morley & Whitfield 1993). In contrast, observations presented here show that with EG, removal of external calcium significantly reduces the intracellular calcium increase, which suggests the majority of the EG-induced calcium increase is via influx across the plasma membrane from the medium.

Earlier studies using a fluorescent lectin binding assay to assess cortical granule exudate reported no premature release following freezing of mouse or human oocytes (Wood *et al.* 1992, Gook *et al.* 1993). However, as discussed by Wood and colleagues (1992) it is possible that the assay may not be sensitive enough to detect a lower threshold of release or the population of cortical granules released may not contain the appropriate binding substrate. Since the amount of calcium released is comparable to that at fertilization, it would be capable of causing cortical granule exocytosis, which would explain zona hardening. This conclusion is also in agreement with the findings of

Vincent and colleagues (1990), who demonstrated that the oocyte must be present for zona hardening to occur following exposure to DMSO. The data presented here show that cryoprotectant exposure alone causes zona hardening. Since introduction of the calcium chelating agent, BAPTA, prevents zona hardening, it appears that the cryoprotectants used increase intracellular calcium to levels sufficient to induce cortical granule release and subsequent zona hardening. Removing calcium during vitrification significantly reduces the degree of zona hardening, which dramatically improves fertilization and development to the two-cell stage.

Although zona hardening and its negative effect on fertilization rates can be circumvented through ICSI or partial zona removal, cortical granule release is but one of the initial activation events. A large increase in calcium might also be detrimental for further embryo development by affecting cell cycle proteins such as MPF whose down-regulation is normally triggered by the calcium oscillations at fertilization (Lorca *et al.* 1991, Marangos & Carroll 2004). Calcium is a ubiquitous signaling agent that is involved in a number of biological processes, so strict homeostatic regulation of release is critical (for review see Berridge *et al.* 1998). A sustained level of micromolar calcium is sufficient to cause the inappropriate activation of calcium-dependent enzymes such as proteases and phospholipases, which can have severe cellular consequences (Orrenius *et al.* 2003). Furthermore, oocytes activated with high frequency calcium oscillations can undergo apoptosis (Gordo *et al.* 2000, 2002). A large increase in intracellular calcium caused by cryoprotectants may therefore decrease viability and explain the low efficiency of oocyte cryopreservation. Further support for this idea is provided by removing extracellular calcium and introducing BAPTA to reduce the intracellular calcium rise during cryoprotectant exposure. Takahashi and colleagues (2004) demonstrated that reducing the calcium increase in mouse oocytes with such an approach significantly reduced the calcium rise caused by 40% EG (for 3 min), which, in turn, increased the number of intact oocytes from 0% to 100%.

The results presented here demonstrate that both DMSO and EG cause a transient increase in intracellular calcium. This increase in calcium is responsible for zona hardening and explains the requirement for zona removal or ICSI to permit fertilization. Zona hardening, caused by exposure to the cryoprotectant, can be overcome by inclusion of bovine serum albumin (BSA) in the medium (George *et al.* 1992). This, together with the employment of lower temperatures during cryoprotectant exposure, would account for the apparent lack of zona hardening and high fertilization rates reported in some previous studies (Carroll *et al.* 1990, Hotamisligil *et al.* 1996, dela Pena *et al.* 2001). It is thought that the active component in BSA is fetuin (George & Johnson 1993) which presumably acts as either a competitive substrate for proteolytic enzymes released from the cortical granules or as a direct inhibitor.

However, in human IVF a more defined system is required and human serum albumin has been shown not to afford the same protection as BSA (George & Johnson 1993). Lowering the temperature during cryoprotectant exposure prevents zona hardening, most likely through the decrease in temperature-sensitive enzymatic activity and cellular function, but by carrying out the procedure at 37 °C it alleviates potential cooling injury to the cytoskeleton (Pickering & Johnson 1987, Pickering *et al.* 1990).

The data presented here demonstrate that the cryoprotectants, EG and DMSO, increase intracellular calcium, which leads to the induction of zona hardening. Subsequent removal of calcium from the vitrification medium facilitated IVF and development to the two-cell stage at a rate approaching that of control (non-vitrified) oocytes. The calcium-free treatment did not affect embryo development rates or cell number/allocation in the blastocyst, which suggests that the removal of calcium during cryopreservation is not detrimental. In this present study we wished to examine the effect of calcium-free vitrification on a common protocol that used EG and DMSO. However, the fact that the response to DMSO challenge was unaffected by removing extracellular calcium suggests that DMSO should either be omitted from the cryoprotectant solutions or reduced to a concentration that does not dramatically increase intracellular calcium. The kind of analysis presented here provides the opportunity for further experiments, which include using the measurement of calcium during cryoprotectant challenge to empirically determine the optimum concentration and duration of exposure for vitrification protocols that minimize the increase in oocyte intracellular calcium. By carrying out such studies it is anticipated that not only will zona hardening be significantly reduced, but also that normal cell function will be facilitated allowing IVF and thereby increasing the efficiency of oocyte cryopreservation.

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