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Tolerance of lamb and mouse oocytes to cryoprotectants during vitrification

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Research Article

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Summary

Mouse and lamb oocytes were vitrified with, or exposed to, different cryoprotectants and evaluated for their effects on their survival and developmental competence after *in vitro* fertilization (IVF) and activation treatments. Control oocytes remained untreated, whilst the remainder were exposed to three different combinations of vitrification solutions [dimethyl sulfoxide (DMSO) + ethylene glycol (EG), EG only, or propanediol (PROH) + EG] and either vitrified or left unfrozen (exposed groups). Oocytes in the control and vitrified groups underwent IVF and developmental competence was assessed to the blastocyst stage. In lambs, survival rate in vitrified oocytes was significantly lower than for oocytes in the exposed groups ($P < 0.05$). Blastocyst development was low in vitrified oocytes compared with controls (<6% vs 38.9%, $P < 0.01$). Parthenogenetic activation was more prevalent in vitrified lamb oocytes compared with controls ($P < 0.05$). No evidence of zona pellucida hardening or cortical granule exocytosis could account for reduced fertilization rates in vitrified lamb oocytes. Mouse oocytes demonstrated a completely different response to lamb oocytes, with survival and parthenogenetic activation rates unaffected by the vitrification process. Treatment of mouse oocytes with DMSO + EG yielded significantly higher survival and cleavage rates than treatment with PROH + EG (87.8% and 51.7% vs 32.7% and 16.7% respectively, $P < 0.01$), however cleavage rate for vitrified oocytes remained lower than for the controls (51.7% vs 91.7%, $P < 0.01$) as did mean blastocyst cell number (33 ± 3.1 vs 42 ± 1.5, $P < 0.05$). From this study, it is clear that lamb and mouse show different tolerances to cryoprotectants commonly used in vitrification procedures, and careful selection and testing of species-compatible cryoprotectants is required when vitrifying oocytes to optimize survival and embryo development.

Introduction

Embryo cryopreservation is now considered to be a routine procedure for humans and most domestic animals. This refined technique results in high post-thaw survival rates and acceptable implantation and birth rates. Oocyte cryopreservation was developed to improve domestic animal breeding and maintain the biodiversity of endangered animals (Vajta, 2000; Pukazhenthi and Wildt, 2004), and remains a much more challenging procedure. In humans, oocyte cryopreservation enables the reproductive capacity to be retained in cancer patients who may suffer infertility either from disease or treatment (Ali and Sirard, 2002). It offers women the option to delay reproduction during career development, and avoids the ethical and moral issues associated with embryo cryopreservation. Oocyte freezing also provides the opportunity to create egg banks that are used in some countries to synchronize and optimize donor – recipient timing and standardize the number of eggs being transferred to recipients (Stachecki and Cohen, 2004).

Slow freezing has been widely used and is a well established method to preserve embryos (Veeck *et al*., 1993; Veeck *et al*., 2004), but recently vitrification is becoming the method of choice for preserving either cleaved or blastocyst stage embryo due to a higher survival rate (Loutradi *et al*., 2008). Slow cooling requires low concentrations of cryoprotectants, with controlled stepwise cooling rates of between −0.3 and −2°C/min to prevent formation of intracellular ice crystals (Shaw and Jones, 2003). Vitrification is a faster, simple procedure and often cheaper than slow cooling and has been reported to reduce chilling injury and osmotic shock, resulting in improved survival rates and embryo development (Lane and Gardner, 2001; Kuwayama *et al*., 2005; Gautam *et al*., 2008; Cobo *et al*., 2013a, 2013b). In oocyte cryopreservation, more recently better results have been achieved using the vitrification procedure when compared with slow cooling (Lane and Gardner, 2001; Kuwayama *et al*., 2005; Gautam *et al*., 2008; Cao *et al*., 2009; Cobo *et al*., 2013b; Levi Setti *et al*., 2014).

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However, when compared with slow cooling, vitrification often requires twice the concentration of permeating cryoprotectants in the cryo-medium and can be lethal for oocytes if the exposure time is too long, the volume is too large or the rates of cooling are too slow. Ideally, exposure of oocytes to the vitrifying solution should be less than a minute to minimize the toxic effects and the cooling rate should be around −50,000 to −200,000°C/min to prevent the formation of intra- and extracellular ice crystals (Shaw and Jones, 2003; Kuwayama *et al*., 2005).

The permeating capacity and toxicity of intracellular cryoprotectants vary among the different agents. For example, 1,2 propanediol and 2,3 butanediol were less cytotoxic than dimethyl sulfoxide (DMSO) and ethanediol when umbilical vein endothelial cells were exposed to these agents (Wusteman *et al*., 2002). Despite this, DMSO achieves a better solid glass state upon vitrification when compared with two other commonly used cryoprotectants, acetamide or ethylene glycol (EG) (Fahy *et al*., 2004). To date, EG or a combination of DMSO and EG or PROH and EG are the most common cryoprotectants either commercially or in house production to be used in mouse, cattle, goat, lamb, fox, cat and human oocyte and embryo cryopreservation (Kuwayama *et al*., 2005; Succu *et al*., 2007a; Gautam *et al*., 2008; Berthelot-Ricou *et al*., 2013; Larman and Gardner, 2014; Fernandez-Gonzalez and Jewgenow, 2016; Cao *et al*., 2017).

Embryos derived from vitrified oocytes differ in their development between species. High survival, and high blastocyst and pregnancy rates have been reported in the mouse (Lane and Gardner, 2001), but in other animals such as lamb (Succu *et al*., 2007a, 2007b, 2008), buffalo (Gautam *et al*., 2008; Liang *et al*., 2012), pig (Somfai *et al*., 2007), goat (Begin *et al*., 2003), fox (Cao *et al*., 2017), and cat (Fernandez-Gonzalez and Jewgenow, 2016) embryo development rates still remain low, probably due to a variety of factors including disruption of meiotic spindles and chromosomes (Albarracin *et al*., 2005; Succu *et al*., 2007a, 2007b; Berthelot-Ricou *et al*., 2013; Cao *et al*., 2017), parthenogenetic activation (Tian *et al*., 2007), premature cortical granule rupture and zona hardening, which decreased sperm penetration at fertilization (Fuku *et al*., 1995; Tian *et al*., 2007), and decreased glutathione production (Cao *et al*., 2017). The common method for insemination of vitrification-warmed human oocytes is intracytoplasmic sperm injection (ICSI), which avoids zona hardening issues (Kuwayama *et al*., 2005; Chang *et al*., 2008; Cao *et al*., 2009; Cobo *et al*., 2013a). However, in animal studies, in which large numbers of oocytes are often processed, *in vitro* fertilization (IVF) remains a popular method for insemination of vitrification-warmed oocytes (Succu *et al*., 2007b, 2008; Gautam *et al*., 2008). A few large studies in cattle and humans have reported high fertilization rates, blastocyst rates and pregnancies from post-vitrified oocytes by either IVF or ICSI (Vajta *et al*., 1998; Papis *et al*., 2000; Tong *et al*., 2012; Kuwayama *et al*., 2005) but most results have been variable and no universal method has yet been established.

The objectives of this study were to evaluate the survival rate of mouse and lamb oocytes after exposure to different combinations of commonly used cryoprotectants and vitrification itself, fertilizing ability, developmental competence of fertilized or activated lamb and mouse vitrified-warmed oocytes and the incidence of cortical granule exocytosis and zona hardening in lamb oocytes.

Materials and methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Oocyte collection, selection and in vitro maturation (IVM): lamb

As our laboratory did not have the facilities to perform *in vivo* maturation in lamb, those oocytes were *in vitro* matured. Ovaries were obtained from lambs (3–9 months) within 2 h of slaughter. Ovaries were washed three times in pre-warmed saline supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (GIBCO, New Zealand). Oocytes were aspirated from follicles of diameter 2–6 mm into tissue culture medium (TCM)-199 handling medium (MP Biomedicals, Solon, OH, USA), supplemented with 2.5 mM sodium hydrogen carbonate, 1 mM glutamine, 2 mM sodium pyruvate, 22.5 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2% (v/v) lamb serum (GIBCO) and supplemented with 5000 IU/ml heparin (Pfizer, WA, USA). Oocytes with at least two layers of cumulus cells and homogenous cytoplasm were washed in TCM handling medium and cultured under mineral oil in 500 µl IVM medium (M-199 with 25 mM Na bicarbonate, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml follicle-stimulating hormone (FSH) (Folltropin V, Bioniche, Canada), 5 µg/ml luteinising hormone (LH) and 20% (v/v) lamb serum) in four-well Petri dishes (Nunclon, Nalge Nunc International, Denmark) for 24–26 h in a Mini-incubator (MINC, COOK Medical, Brisbane, Australia) using a humidified atmosphere of 6% CO_2, 5% O_2 in air at 39°C.

Mature oocyte collection: Mouse

F1 C57B/J × CBA mice aged 4–5 weeks were superovulated by intraperitoneal (IP) administration of 7.5 IU of pregnant mare serum gonadotrophin (PMSG; Folligon; Intervet, Australia) followed 48–60 h later with intraperitoneal (i.p.) 7.5 IU human chorionic gonadotrophin (hCG, Chorulon, Intervet). Mice were killed by cervical dislocation 13–14 h post hCG, and mature cumulus–oocyte complexes were collected from the oviduct into HEPES–KSOM (kalium simplex optimized medium) supplemented with 0.5% (v/v) non-essential amino acid (GIBCO), 1% (v/v) essential amino acid (GIBCO) and 0.3% (w/v) BSA (GIBCO), modified from Summers *et al.* (1995). Cumulus cells were denuded by treatment with 60 IU hyaluronidase in HEPES–KSOM for 30–60 s before transfer into modified Tyrode's (mT6) medium, adapted from Fraser (1984).

Oocyte vitrification and warming

Vitrification and warming solutions were prepared in HEPES–KSOM medium for mouse and HEPES–synthetic oviductal fluid (SOF) for lambs. Three vitrification solutions (VS) were tested: 2.3 M dimethylsulfoxide (DMSO) + 3 M EG; 6 M EG only; 2.3 M 1,2 propanediol (PROH) + 3 M EG. All VS were supplemented with 0.75 M sucrose. Vitrification was performed at 37°C. Corresponding equilibration solutions (ES) contained half the stated concentration of cryoprotectants and lacked sucrose. Denuded oocytes were placed in ES, equilibrated for 3 min, transferred to VS, then with minimal solution (less than 3 µl) placed on a fibreplug, touched to a pre-cooled steel block in liquid nitrogen and sealed in a pre-cooled straw (CVM kit, Cryologic, Victoria,

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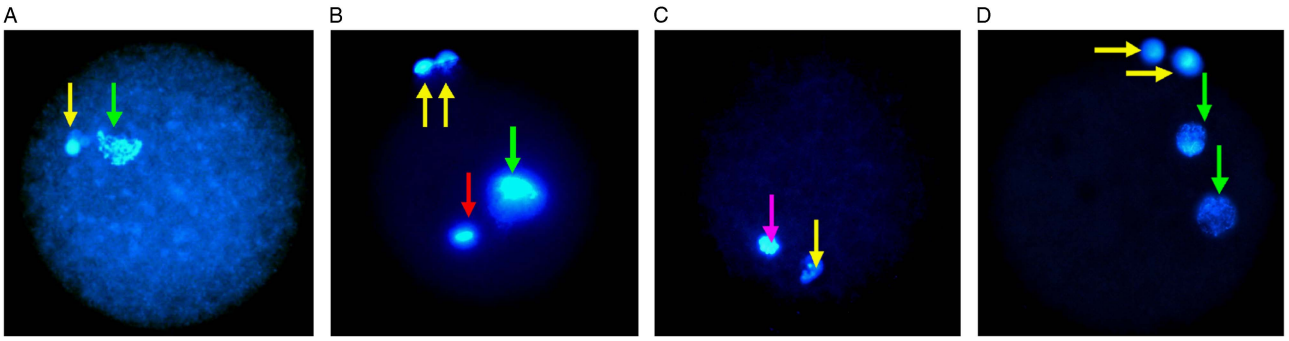

Figure 1. Fluorescence microscopy of vitrified-warmed lamb oocytes after insemination. The positions of pronuclei (PN) are marked with a green arrow and the polar body (PB) with a yellow arrow. (*A*) Parthenogenetic activation demonstrated by the presence of 1 PN and 1 PB. (*B*) Abnormal fertilization, with 1 PN, 2 PB and a sperm head is indicated by a red arrow. (*C*) Mature MII unfertilized oocyte with 1 PB and meiotic spindle is indicated by a pink arrow. (*D*) Normal fertilization indicated by 2 PN and 2 PB.

Australia) for storage in liquid nitrogen. The time taken from leaving ES to vitrification on the block was between 30 to 40 s. Vitrified oocytes were warmed at 37°C serially through three solutions (0.3 M, 0.25 M, 0.15 M sucrose in HEPES–SOF medium for lamb and HEPES–KSOM for mouse) for 5 min each, then washed in fresh HEPES handling medium. All VS (PROH and EG) were from the same batch, except for DMSO in which a fresh ampoule was opened every 3 weeks. Control oocytes were left fresh without exposure to cryoprotectants and vitrified. Exposed groups of cryoprotectants consisted of oocytes that were exposed to the cryoprotectants, but not vitrified to observe the toxicity of cryoprotectants on oocyte survival only. Vitrified-warmed oocytes were left for 2 h before fertilization.

In vitro fertilization (IVF) and in vitro culture (IVC)

Lambs

In total, 20–30 matured oocytes were transferred into 500 µl modified SOF, adapted from Tervit *et al.* (1972), supplemented with 2% (v/v) lamb serum. Frozen ram sperm pellets were thawed in a 5-ml glass tube whilst shaking at 37°C water bath for 10 s then layered on a discontinuous 45%/90% Percoll gradient in 12-ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 600 *g* for 15 min. The supernatant was removed and the pellet was diluted with HEPES–SOF medium and centrifuged at 300 *g* for 6 min. Oocytes were inseminated with 2×10^6 motile spermatozoa/ml for 18 h in an incubator with an humidified atmosphere of 6% CO_2, 5% O_2 in air at 39°C. Oocytes were cleaned free of cumulus and attached spermatozoa were removed by manual pipetting. Some presumptive zygotes were fixed and stained to observe normal, abnormal and parthenogenetic activation and the rest of oocytes were transferred into Sydney IVF cleavage medium (COOK® Medical, cat. no. K-SICM 50, Brisbane, Australia) to observe oocyte developmental competence. On day 3, cleaved oocytes with four or more blastomeres were transferred into Sydney IVF blastocyst medium (COOK® Medical, cat. no. K-SIBM 50). Cleavage and blastocyst rates were assessed at day 2 and day 8 after insemination.

Mouse

Fresh spermatozoa were collected from the cauda epididymis of C57B/J × CBA males, placed into 1 ml mT6 in 12 ml Falcon tubes and incubated at 37°C, in 5% CO_2 in air for 30–60 min. Oocytes were inseminated with 2.5×10^5 motile spermatozoa/ml in 50 µl mT6 droplets in 35 mm Falcon dishes, covered with mineral oil for 4 h in an incubator under an atmosphere of 5% CO_2 in air, at 37°C, and then transferred into Sydney IVF cleavage medium for 2 days and into Sydney IVF blastocyst medium on day 3. Cleavage and blastocyst rates were assessed at day 1 and day 4 after insemination. Blastocysts derived from control and vitrified oocytes were assessed for cell count on day 4. Those blastocysts were incubated with Hoechst 33342 stain (5 µg/ml in HEPES–KSOM) for 30 min at 37°C and assessed immediately under UV fluorescence.

Staining for lamb pronuclei (for evidence of normal, abnormal and parthenogenetic activation)

At 18 h post-fertilization, lamb presumptive zygotes were stained with Hoechst 33342 dye, 5 µg/ml in HEPES–SOF medium for 30 min at 39°C, then assessed by UV fluorescence microscopy (Leica DMR Upright Fluorescence Microscope, Germany). Oocytes were identified as normally and abnormally fertilized and parthenogenetic activation as previously described (Han and Gao, 2013; Liang *et al.*, 2012; Tian *et al.*, 2007; Gomez *et al.*, 1997). Normal fertilization was identified by the appearance of two polar bodies (2 PB) and two pronuclei (2 PN). The oocytes were scored as parthenogenetic when there was no sperm observed but a single pronucleus or 2 PN in the cytoplasm with only 1 PB or no PB. Oocytes with penetrated sperm or a decondensed sperm head in the cytoplasm or with 2 PB and more than 2 PN were assessed as having abnormal fertilization. Oocytes with a metaphase spindle and 1 PB but no evidence of sperm penetration were classified as unfertilized oocytes (Fig. 1C). Germinal vesicle and metaphase I stage oocytes were identified and excluded.

Parthogenetic activation on mouse oocytes

To observe parthenogenetic activation in the mouse, oocytes were cultured for 1 day without insemination and recorded as parthenogenetic embryos if cleavage occurred within 24 h. As a positive control, mouse oocytes were activated with parthenogenetic medium (10 mM $SrCl_2$, 5 µg/ml cytochalasin B and 10 mM EGTA in HEPES–KSOM) as described by Kishigami and Wakayama (2007). Briefly, denuded mature oocytes were transferred into parthenogenetic medium and cultured for 6 h then washed in HEPES–KSOM medium before being transferred to Sydney IVF cleavage and blastocyst medium.

Staining for cortical granules

In vitro matured lamb oocytes, either unfrozen (control group) or 1–4 h post-warming, were removed of their zona pellucida using

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Table 1. Effect of different cryoprotectants and vitrification on survival and degeneration of lamb oocytes

Status of oocytes	No. of oocytes	Post-warming survival rate (4 h post-warming) per oocytes*	Degeneration rate (18 h post-insemination) per warming oocytes
Control	190	N/A	N/A
Vitrified			
DMSO + EG	171	136 (79.5%)^{a}	2 (1.5%)
EG	156	106 (67.9%)^{b}	2 (1.9%)
PROH + EG	183	142 (77.6%)a	7 (4.9%)
Exposed			
DMSO + EG	34	33 (97.1%)^{c}	0 (0%)
EG	30	27 (90%)^{c}	0 (0%)
PROH + EG	36	35 (97.2%)^{c}	0 (0%)

*From the total number of inseminated oocytes from vitrified groups, some were cultured to observe embryo development and the rest were fixed and stained to assess normal and abnormal fertilization status and parthogenetic activation. Control-112 cultured, 78 fixed and stained; DMSO + EG-85 cultured, 51 fixed and stained; EG-61 cultured, 45 fixed and stained; PROH + EG-85 cultured, 57 fixed and stained.
^{a,b,c}Different superscripts within columns denotes statistical difference ($P < 0.05$).
N/A: not applicable.

0.5% (w/v) pronase in HEPES–SOF medium at 39°C. Oocytes were continuously observed for zona dissolution using a stereomicroscopy and the time taken for complete dissolution was recorded. The zona-free oocytes were washed in 0.01% (v/v) Triton-X in HEPES–SOF medium then fixed in 4% (w/v) paraformaldehyde. After washing three times (5 min each) in blocking solution containing 10 mM glycine in HEPES–SOF medium, oocytes were permeabilized with 0.1% (v/v) Triton-X 100, then incubated for 30 min at 39°C in 1 µmol/l fluorescein isothiocyanate conjugated to *Lens culinaris* agglutinin (FITC–LCA) and washed four times for 5 min each in blocking solution and were mounted in glycine solution in twin cavity slides for observation using confocal laser scanning microscopy (CLSM, Leica TCS NT upright equipped by CLSM, Germany). Next, 5 µm sections were imaged from the surface to a depth of 20 µm. The grey level intensity of FITC–LCA was measured at 20 µm using Image J (National Institutes of Health, USA) and calculated relative to surface area.

Statistical analysis

Data for survival, normal and abnormal fertilization, parthenogenetic activation and embryo development were analysed using Fisher's exact test. Blastocyst total cell number and zona pellucida dissolving time were assessed by paired *t*-test. A non-parametric repeated measures analysis of variance (ANOVA) was used to compare measured grey level intensities for FITC–LCA staining using Statgraphics software (Statpoint Technologies Inc., Virginia, USA). Experiments were replicated at least three times in each treatment group. All graphed data were presented as mean ± standard error of the mean (SEM).

Results

Effect of different cryoprotectants and vitrification on mouse and lamb oocyte survival rates

Survival rates of lamb oocytes after passing through the warming solutions for all exposed groups were >90%. Post-warming survival rates (vitrified groups) for lamb oocytes vitrified in DMSO + EG (79.5%), EG (67.9%), and PROH + EG (77.6%) were significantly lower ($P < 0.05$) than for exposed oocytes (97.1, 90 and 97.2%, respectively). DMSO + EG and PROH + EG resulted in better post-warm survival rates than EG (79.5 and 77.6% compared with 67.9% respectively, $P < 0.05$; Table 1).

In contrast with lamb oocytes, mouse oocytes vitrified in PROH + EG had a lower survival rate than those in DMSO + EG (32.7% vs 87.8%, $P < 0.01$) and 10 times greater degeneration rate following culture (15.2% vs 1.6%, $P < 0.01$). A similar result was obtained for oocytes exposed to PROH + EG and DMSO + EG (survival 25.8% vs 93%, $P < 0.01$; degeneration rate following culture 37.5% vs 2.5%, $P < 0.01$, respectively; Table 2). Vitrification using EG alone was only tested in lambs and was not tested for embryo development in the mouse, due to poor survival rates (5/34, 14.7%) in preliminary experiments.

Effect of different cryoprotectants and vitrification on normal and abnormal fertilization, and parthenogenetic activation in mouse and lamb oocytes

In lambs, normal fertilization rates in DMSO + EG (17.6%), EG (22.2%), PROH + EG (10.5%) were significantly lower than in the controls (47.4%, $P < 0.01$; Table 3; Fig. 1D). The incidence of parthenogenetic activation was significantly higher in DMSO + EG (35.3%, $P < 0.01$), 24.4% in EG ($P < 0.05$), 26.3% in PROH + EG ($P < 0.01$) compared with controls (9%; Table 3; Fig. 1A). There was no significant difference in abnormal fertilization rate including polyspermia in treatment groups compared with controls (Table 3; Fig. 1B). In contrast with lambs, only one of 35 mouse oocytes (2.9%) in DMSO + EG group developed into two cells without insemination, but did not develop to the blastocyst stage; In contrast, in the positive control of parthenogenetic activation, 100% (10/10) of oocytes grew into two cells and 70% (7/10) developed to blastocysts (Table 4).

Effect of different cryoprotectants and vitrification on lamb and mouse oocyte developmental competence

Post IVF, cleavage rate of all vitrified-warmed lamb oocytes was similar and statistically lower than control oocytes (DMSO + EG

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Table 2. Effect of different cryoprotectants and vitrification on survival and degeneration of mouse oocytes

Status of oocytes	No. of oocytes	Post-warming survival rate (4 h post-warming) per oocytes*	Degeneration rate (18 h post-insemination) per warming oocytes
Control	46	N/A	N/A
Vitrified			
DMSO + EG	139	122 (87.8%)[a]	2 (1.6%)[a]
PROH + EG	101	33 (32.7%)[b]	5 (15.2%)[b]
Exposed			
DMSO + EG	43	40 (93%)[a]	1 (2.5%)[a]
PROH + EG	31	8 (25.8%)[b]	3 (37.5%)[b]

*From 46 fresh control oocytes, 36 were fertilized to observe embryo development and 10 oocytes were used as negative controls to observe parthenogenetic activation in mouse oocytes. In the DMSO + EG vitrified group, 87 oocytes were inseminated and cultured and 35 oocytes were not inseminated to assess parthenogenetic activation on day 1.
[a,b]Different superscripts within columns denote statistical difference ($P < 0.01$).

Table 3. Effect of different cryoprotectants and vitrification on normal and abnormal fertilization, and parthenogenetic activation of lamb oocytes

Status of oocytes	No. of oocytes	Normal fertilization per oocytes (%)	Abnormal fertilization per oocytes (%)	Parthenogenetic activation per oocytes (%)
Control	78	37 (47.4%)[a]	7 (9%)	7 (9%)[c]
Vitrified				
DMSO + EG	51	9 (17.6%)[b]	4 (7.8%)	18 (35.3%)[d]
EG	45	10 (22.2%)[b]	1 (2.2%)	11 (24.4%)[d]
PROH + EG	57	6 (10.5%)[b]	2 (3.5%)	15 (26.3%)[d]

Different superscripts within columns denote statistical difference: *a* vs *b*: $P < 0.01$, *c* vs *d*: $P < 0.05$.

Table 4. Effect of different cryoprotectants, vitrification and induced chemical activation in the absence of sperm on mouse oocyte development

Status of oocytes	No. of oocytes	Cleavage rate per oocytes (%)	Blastocysts rate per cleaved (%)
Fresh	10	0	0
Fresh activated	10	10 (100%)	7 (70%)
Vitrified			
DMSO + EG	35	1 (2.9%)	0
Exposed			
DMSO + EG	14	0	0

32.9%, EG 32.8% and PROH + EG 21.2% vs control 84.8%, $P < 0.01$), and blastocyst development from cleaved embryos was lower for vitrified groups (PROH + EG and EG) < 6% compared with the control 38.9% ($P < 0.01$; Table 5). In contrast with lambs, cleavage rate of vitrified-warmed oocytes with PROH + EG had a 30% lower cleavage compared with DMSO + EG ($P < 0.01$, Table 6). A trend for reduced blastocyst development in the PROH + EG group was also evident, however this was not significant as only small sample numbers could be analyzed due to extensive degeneration and poor cleavage rates for these oocytes. Compared with control oocytes, the proportions of embryos from PROH + EG and DMSO + EG groups that cleaved and formed blastocysts was significantly lower ($P < 0.01$ and $P < 0.05$ respectively); with significantly fewer cell number of blastocysts in the DMSO + EG treatment group (33 ± 3.1) compared with the control group (42 ± 1.5, $P < 0.05$; Table 6).

Cortical granule exocytosis and zona dissolution times in post-warmed vitrified lamb oocytes

Two treatment groups; DMSO + EG and PROH + EG were observed for cortical granules exocytosis. No differences were found in the timing of the zona pellucida dissolution between fresh and vitrified lamb oocytes (Fig. 4). The relative grey level intensity for FITC–LCA in oocyte cross-sections, each measured at a depth of 20 μm, was around three times greater in vitrified lamb oocytes compared with the controls (Fig. 2; $P < 0.05$). Overall, the higher fluorescent intensity in vitrified oocytes represented diffuse staining throughout the ooplasm (Fig. 3). Cytoplasmic staining was not apparent in control oocytes, which demonstrated only cortical fluorescence, around 2 μm beneath the oolemma (Fig. 3*A*).

Discussion

Mouse oocytes were extremely compromised in VS containing EG alone, and due to very poor survival rates in preliminary experiments, vitrification in EG was not tested further. Mouse oocyte survival using DMSO + EG during vitrification was significantly higher than those vitrified with PROH + EG. Combined with the observation that similar survival rates were obtained using the same cryoprotectant treatments without vitrification, this suggests that PROH, not the vitrification process itself, was detrimental in the mouse model. In general, successful

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Table 5. Effect of different cryoprotectants and vitrification on lamb oocyte developmental competence

Status of oocytes	No. of oocytes	Cleavage rate per oocytes (%)	Morulae rate per cleaved (%)	Blastocyst rate per cleaved (%)
Control	112	95 (84.8%)^{a}	40 (42.1%)^{a}	37 (38.9%)^{a}
Vitrified				
DMSO + EG	85	28 (32.9%)^{b}	4 (14.3%)^{b}	0 (0%)
EG	61	20 (32.8%)^{b}	5 (25%)^{a,b}	1 (5%)^{b}
PROH + EG	85	18 (21.2%)^{b}	2 (11. 1%)^{b}	1 (5.6%)^{b}

Different superscripts within columns denote statistical difference ($P < 0.01$).

Table 6. Effect of different cryoprotectants and vitrification on mouse oocyte developmental competence

Status of oocytes	No. of oocytes	Cleavage rate per oocytes (%)	Blastocyst rate per cleaved (%)	Total blastocyst cell number (± standard error of the mean (SEM))
Control	36	33 (91.7%)^{a}	27 (81.8%)^{d}	42 ± 1.5^{f}
Vitrified				
DMSO + EG	87	45 (51.7%)^{b}	25 (55.6%)^{e}	33 ± 3.1^{g}
PROH + EG	24	4 (16.7%)^{c}	1 (25%)^{e}	N/A

Different superscripts within columns denote statistical difference: *a* vs *b*, c: $P < 0.01$, *b* vs *c*: $P < 0.01$, *d* vs *e*: $P < 0.05$, *f* vs *g*: $P < 0.05$.

vitrification requires permeating cryoprotectant concentrations to be around 4.5 M (Shaw and Jones, 2003). Using 2.3 M PROH satisfied this requirement when combined with 3 M EG. Nevertheless, 2.5 M PROH has been reported to cause degeneration of > 80% of mouse oocytes (Mullen, 2007), and only 32% oocytes survived after exposure to 1.5 M PROH with 38% cleavage rate (Gook *et al*., 1993). Of oocytes that were subsequently slow cooled, only 4% survived and none cleaved following insemination. However, Huang *et al*. (2008) reported that 91.8% of oocytes survived after vitrified with 2 M PrOH + 2.6 M EG, but only 30.5% developed into blastocysts (Huang *et al*., 2008). The mechanism behind toxic injury from PROH in the mouse is not fully understood. Propanediol is known to decrease the viability of oocytes by altering the oocyte proteome due to a prolonged elevation of intracellular calcium levels in mouse oocytes (Larman *et al*., 2007). The cytoskeletal actin molecule in mouse oocytes was also found to be sensitive to PROH, causing an alteration of the actin molecule and inducing fragility (Maro *et al*., 1984). Moreover, it has been shown that VS containing PROH induced more DNA damage on mouse oocytes compared with DMSO (Berthelot-Ricou *et al*., 2013). As vitrified mouse oocyte in the DMSO + EG group has higher embryo development compared with the PROH + EG group, it was demonstrated that the former combination is more suitable for preserving mouse oocytes.

Unlike mice, oocytes collected from abattoir-sourced lamb ovaries did not demonstrate a specific toxicity to DMSO, PROH or EG. Survival rates after exposure to VS without vitrification were similar using either PROH + EG or DMSO + EG, and both demonstrated better post-warm survival than EG alone. It has been proposed that a combination of cryoprotectants was more successful than using a single agent (Van der Elst *et al*., 1992), therefore reducing the required concentration for individual cryoprotectants, and possibly reducing overall toxicity. Nevertheless the rate of lamb oocyte lysis following the vitrification process was high for all groups. Lamb oocytes are remarkably different from murine oocytes, they are larger, have a higher intracellular lipid content and have poor post-warming recovery of meiotic spindles (Succu *et al*., 2007a, 2007b). The sensitivity of lamb oocytes to cooling injury may result from destruction of intracellular lipids through a mechanism of lipid phase transition (LPT) in which membranes, as they cool and reheat, will change their form. For example, unsaturated fatty acids in non-lamellar forming lipids assume a hexagonal II phase structure that alters membrane function and leads to ion leakage and cell death (Arav and Zvi, 2008; Quinn, 1985).

Parthenogenetic activation was investigated in mouse oocytes vitrified using DMSO + EG and no evidence for a greater incidence of parthenogenetic activation was found. In contrast, higher parthenogenetic activation rates have been reported in mouse oocytes vitrified using PROH compared with DMSO as a cryoprotectant (Shaw and Trounson, 1989). Chemicals containing hydroxyl (–OH) groups such as PROH are reported to stimulate parthenogenetic activation of mouse oocytes (Shaw and Trounson, 1989; Van der Elst *et al*., 1992). In this experiment, the low numbers of oocytes surviving exposure to, or vitrification with, PROH precluded examination of parthenogenetic activation. By contrast, lamb parthenogenetic activation rate was significantly increased in all treatment groups. Moreover, there was an increased trend of parthenogenetic activation for DMSO + EG than for EG alone. This is supported by previous reports of a higher incidence of parthenogenetic activation in lamb oocytes using DMSO + EG (63.9%) compared with untreated oocytes (8.2%) (Tian *et al*., 2007).

In both species, the number of embryos that reached the two-cell stage and developed into blastocysts after vitrification and insemination was significantly higher for unfrozen oocytes than for all vitrified groups. The only available source of ovine oocytes was from pre-pubertal lamb ovaries, therefore this may have been the reason for the relatively low blastocyst development. It has been demonstrated that there was lower blastocyst development in younger lambs compared with adult ewes (Succu *et al*., 2007b). The blastocyst rate post-vitrified-warmed adult sheep oocytes was reported to be around 0 – 17% (Marco-Jimenez *et al*., 2012; Succu *et al*., 2007a, 2008) and 0% from young lamb oocytes (Succu *et al*.,

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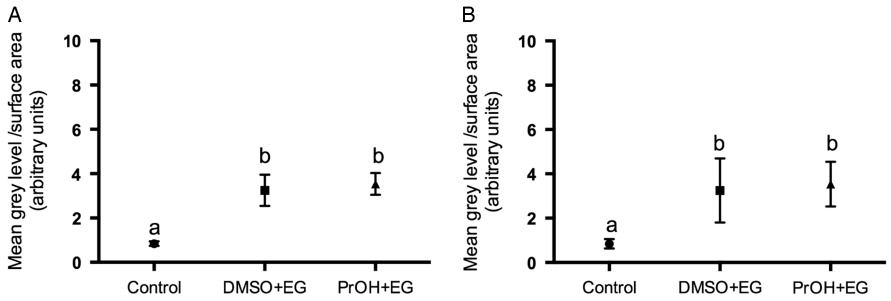

Figure 2. Grey level intensity of fresh control and vitrified-warmed oocytes (DMSO + EG and PROH + EG) stained with fluorescein isothiocyanate conjugated to *Lens culinaris* agglutinin (FITC–LCA) to demonstrate the presence of α-D-mannose and α-D-glucose in cortical granules. Different superscripts denote significant differences ($P < 0.05$).

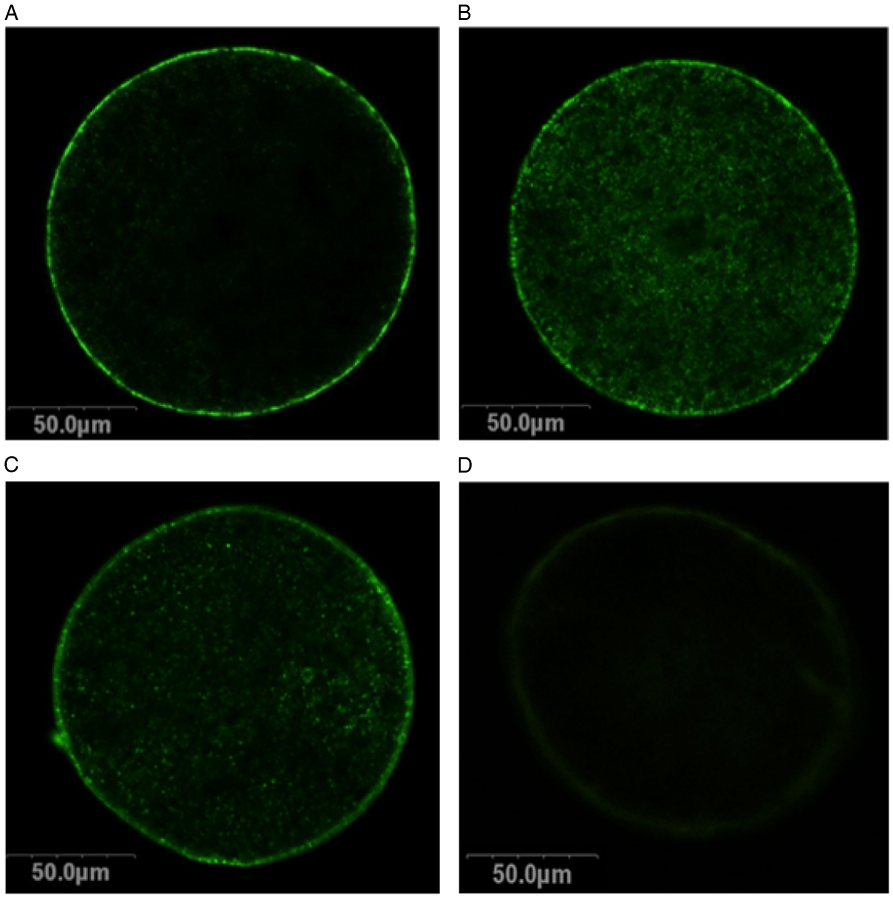

Figure 3. Cortical granules in lamb oocytes. Cross-sections of lamb oocytes labelled with FITC–LCA to stain cortical granules and imaged at a depth of 20 µm from the plasma membrane. Cortical granules in control oocytes (*A*) were identified at the cortical edge of control oocytes, about 2 µm beneath the oolemma with very little staining within the cytoplasm. Oocytes vitrified using DMSO + EG (*B*) and PROH + EG (*C*) exhibit diffuse cortical granule staining throughout the cytoplasm. Negative controls (*D*) exhibited no autofluorescence.

2007b). From this present study, normal fertilization rate of vitrified-warmed oocytes was low compared with fresh controls. In human studies, most reports using vitrified oocytes utilize ICSI (Chang *et al*., 2008; Cobo *et al*., 2013a; Kuwayama *et al*., 2005; Cao *et al*., 2009) and few have attempted IVF (Tong *et al*., 2012, 2013), giving an indication that vitrification is yet to be perfected to avoid compromising the membranes. Although, some reports have shown that increased fertilization rates can be achieved with ICSI as it can bypass the membrane barriers (Palermo *et al*., 1992, 2009). It has also been reported that this technique increases the incidence of aneuploidy (Bernardini *et al*., 1997) and impairs embryonic development (Griffiths *et al*., 2000) in fresh oocytes. It has been demonstrated that blastocyst development from vitrified-warmed buffalo oocytes fertilized by either IVF or ICSI showed similar embryo development (Liang *et al*., 2012; Attanasio *et al*., 2010). Conversely, Lane and Gardner (2001) reported that there was no significant difference in blastocyst development from vitrified-warmed oocytes when compared with unfrozen oocytes (96.3% vs 95.4% respectively). In this latter study, however, a different insemination method (zona drilling) was used for post-warmed oocytes, which possibly negating the zona hardening issue.

The reduced fertilization rate of vitrified oocytes and its relationship with cortical granule exocytosis has been widely debated. Cortical granule release following initial sperm penetration is

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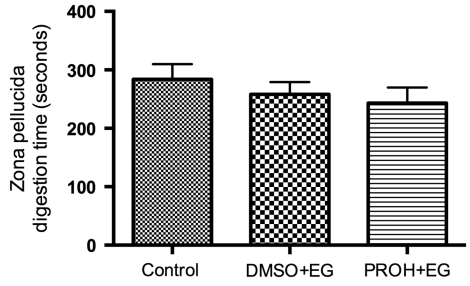

Figure 4. Zona pellucida digestion time (s) for lamb oocytes. Zona pellucida dissolving time (mean ± standard error of the mean (SEM)) in fresh control and vitrified-warmed lamb oocytes (DMSO + EG and PROH + EG) are not significantly different between groups.

known to prevent further sperm entry, and is related to 'hardening' of the zona pellucida due to the cortical granule reaction. Reports on cortical granule exocytosis following oocyte vitrification are conflicting: some have shown a reduction in the number of cortical granules in post-warmed oocytes (George and Johnson, 1993; Schalkoff *et al*., 1989; Tian *et al*., 2007) whilst others report normal cortical granule density and distribution (Gook *et al*., 1993; Jones *et al*., 2004; Manna *et al*., 2001; Wood *et al*., 1992; Tong *et al*., 2012). Here, we compared fresh lamb oocytes with those that had been vitrified using two combinations of cryoprotectants, DMSO + EG and PROH + EG and warmed, to determine if zona hardening or cortical granule exocytosis may have contributed to the poor fertilization rate. Cortical granules were retained within the cytoplasm of vitrified oocytes, and also showed no evidence of zona hardening, as determined by normal zona dissolution times. Some papers reported that there is no difference in zona pellucida dissolution times for unfrozen and vitrified oocytes if *in vitro* matured (Tian *et al*., 2007; Tong *et al*., 2012). In this study, there was no evidence of cortical granule exocytosis in lamb oocytes. In fact, vitrified oocytes showed a brighter overall staining for FITC-conjugated *Lens culinaris* agglutinin (LCA) throughout the cytoplasm. Ghetler *et al*. (2006) reported a higher intensity of fluorescence in post-warmed oocytes stained with LCA, but they interpreted this as evidence for cortical granule exocytosis (Ghetler *et al*., 2006). Miyara *et al*. (2003) reported that almost 80% of oocytes displaying cytoplasmic staining for LCA showed disorganized meiotic spindles and metaphase plates (Miyara *et al*., 2003). Interestingly, they also found that the pattern of cortical granule abnormalities in vitrified oocytes was similar to those in immature oocytes.

FITC-conjugated *Lectin culinaris* agglutinin (LCA) was used for its ability to bind to α-D-mannose and α-D-glucose moieties that are abundant in cortical granules (Ducibella *et al*., 1988; Lee *et al*., 1988). One explanation for the increase in cytoplasmic LCA staining is that the cortical granules from vitrified oocytes may have become disorganized or degenerated after vitrification. Vacuoles containing degenerated cortical granules have been reported in post-warmed oocytes (Ghetler *et al*., 2006; Hyttel *et al*., 2000). Hyttel *et al*. (2000) reported an abundance of small membrane-bound vesicles containing cortical granules in the cytoplasm 0–4 h after warming, whilst 22 h later the vacuoles containing degenerate cortical granules suggesting alteration of cell biology of post-warming oocytes (Hyttel *et al*., 2000). The brighter LCA fluorescence in the cytoplasm may be caused by greater non-specific binding of LCA to mucopolysaccharide moieties on cytoplasmic components other than cortical granules that are exposed after freezing. Whilst cortical granule exocytosis and zona hardening were not apparent, it is possible that the lower fertilization rate in post-warmed oocytes could be due to intrinsic modifications to the zona pellucida that are independent of cortical granule exocytosis as a consequence of cryoprotectant treatment and/or vitrification (Wood *et al*., 1992). Partial release of cortical granules that cannot be detected by LCA staining might also have occurred (Manna *et al*., 2001).

This paper provides evidence that the effects of different cryoprotectants for oocyte vitrification in different species are not uniform. Limited success was obtained using EG alone or PROH + EG for mouse oocyte vitrification compared with DMSO + EG. Lamb oocytes demonstrated very poor fertilization and embryo development post-vitrification, and high parthenogenetic activation rates after treatment with all cryoprotectants, highlighting both the sensitivity of lamb oocytes to chilling injury and cryoprotectant toxicity during the vitrification process. There was no evidence to suggest that cortical granule exocytosis or zona hardening contributed to the poor fertilization rate of lamb oocytes. This study demonstrated a clear difference in the susceptibility of mouse and lamb oocytes to the cytotoxic effects of cryoprotectants used in the vitrification procedure. Cryoprotectant combinations therefore need to be extensively tested and carefully selected for the species being cryopreserved to optimize success rates.

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Ethics. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. All experiments using mice were approved by the Monash University Animal Ethics Committee.

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