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ORIGINAL ARTICLE *Embryology*

Antioxidants increase blastocyst cryosurvival and viability post-vitrification

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STUDY QUESTION: What is the effect of antioxidants acetyl-*L*-carnitine, *N*-acetyl-*L*-cysteine and α-lipoic acid (A3) in vitrification and warming solutions on mouse blastocyst development and viability?

SUMMARY ANSWER: The combination of three antioxidants in vitrification solutions resulted in mouse blastocysts with higher developmental potential *in vitro* and increased viability as assessed by both an outgrowth model *in vitro* and fetal development following uterine transfer.

WHAT IS KNOWN ALREADY: The antioxidant combination of acetyl-*L*-carnitine, *N*-acetyl-*L*-cysteine and α-lipoic acid present in IVF handling and embryo culture media has significant beneficial effects on mouse embryo and fetal development, especially under oxidative stress.

STUDY DESIGN, SIZE, DURATION: The study was a laboratory-based analysis of an animal model. Rapid cooling through vitrification was conducted on F1 mouse blastocysts, with antioxidants (A3) supplemented in vitrification and/or warming solutions, followed by culture and embryo transfer.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Pronucleate oocytes were collected and cultured in groups to Day 4 blastocysts. Expanded blastocysts were vitrified and warmed in solutions with and without the A3 antioxidants and cultured for a further 24 h. Blastocyst cell number and allocation, apoptosis and histone acetylation levels were all quantified, and viability through outgrowths and transfers assessed.

MAIN RESULTS AND THE ROLE OF CHANCE: Mouse blastocysts vitrified with no antioxidants had significantly lower cell numbers ($P < 0.001$) and higher apoptotic cells ($P < 0.05$) compared to non-vitrified embryos. Addition of combined A3 antioxidants to the vitrification and warming solutions resulted in a significant increase in inner cell mass cell (ICM) number ($P < 0.001$) and total cell number ($P < 0.01$), and an increase in outgrowth area ($P < 0.05$), which correlated with the increased fetal weight ($P < 0.05$), crown rump length ($P < 0.05$) and limb development ($P < 0.05$) determined following transfer compared to embryos with no antioxidants. Furthermore, while blastocyst vitrification significantly reduced acetylation levels ($P < 0.05$) compared to non-vitrified embryos, the inclusion of A3 antioxidants helped to ameliorate this.

LIMITATIONS, REASONS FOR CAUTION: Embryo development was only examined in the mouse.

WIDER IMPLICATIONS OF THE FINDINGS: Results in this study demonstrate that vitrification and warming of blastocysts have significant detrimental effects on embryo histone acetylation and subsequent viability. The presence of antioxidants in the vitrification solutions helps to alleviate the negative effects of cryopreservation. Our data indicate that antioxidants need to be present in the medium at the time of exposure to increased oxidative stress associated with vitrification and that prior exposure (i.e. during culture or IVF alone) is insufficient to protect cells against cryo-induced injury. Hence, A3 antioxidants may assist in maintaining the viability of vitrified human embryos in ART through the reduction of oxidative stress.

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Key words: acetylation / acetyl-*L*-carnitine / acetyl-*L*-cysteine / α-lipoic acid / blastocyst / cryopreservation / development / embryo transfer / reactive oxygen / stress

Introduction

Cryopreservation of human gametes and embryos is an essential and routine procedure in human assisted reproduction. For the first 20 years of IVF, cryopreservation was achieved with slow freezing protocols (Trounson and Mohr, 1983; Cohen *et al*., 1985), which over the past two decades have largely been superseded by rapid cooling through vitrification (Lane *et al*., 1999; Stehlik *et al*., 2005; Sparks, 2015). The use of cryopreservation in human IVF is increasing as more clinics move away from fresh transfers to the transfer of vitrified embryos (Shapiro *et al*., 2014) into a natural cycle, and as more clinics adopt preimplantation genetic testing, requiring the cryopreservation of the embryo while genetic analyses are performed (Schoolcraft and Katz-Jaffe, 2013). Further, an increasing use of oocyte cryopreservation for oocyte donation, as well as for social and oncofertility reasons, demands that vitrification procedures are optimized (Kuwayama *et al*., 2005; Cobo *et al*., 2011).

There exist some reassurances that cryopreservation of gametes and embryos is a safe and reliable procedure (Ozgur *et al*., 2015; Sekhon *et al*., 2018). In women with good prognosis, frozen embryo transfer (FET) has been reported to have a higher singleton live birth rate than fresh embryo transfer (Li *et al*., 2019; Wei *et al*., 2019). However, follow-up studies on resultant children have reported conflicting outcomes. Large-scale cohort analyses have determined that while FET is associated with a reduction in preterm birth, small for gestational age and placental previa and abruption (Wennerholm *et al*., 2013; Ishihara *et al*., 2014), others have reported that cryopreservation is associated with higher singleton birth weight and being large for gestational age compared to fresh embryo transfer and naturally conceived singletons (Pinborg *et al*., 2014; Beyer and Griesinger, 2016; Spijkers *et al*., 2017; Anav *et al*., 2019; Maris *et al*., 2019). These latter data infer that there could be some cryo-induced trauma to the embryo, which is subsequently manifested downstream (Barsky *et al*., 2016; Anav *et al*., 2019). However, the influence of endometrial programming during a cryopreserved cycle cannot be dismissed (Ginstrom Ernstad *et al*., 2019; von Versen-Hoynck *et al*., 2019).

Cryopreservation requires the exposure of cells to high levels of cryoprotectants and increased osmolalities, as well as to extreme changes in temperature, all of which can have dramatic effects on gamete and embryo physiology (Fuller and Paynter, 2004; Gardner *et al*., 2007; Larman *et al*., 2007; Dalcin *et al*., 2013; Somoskoi *et al*., 2015) and gene expression (Tachataki *et al*., 2003; Larman *et al*., 2011; Stinshoff *et al*., 2011; Monzo *et al*., 2012; Sahraei *et al*., 2018). The physico-chemical processes involved in cryopreservation induces osmotic stress in reproductive cells (Fuller and Paynter, 2004). Osmotic stress in turn can activate the generation of superoxide anions through the activation of NADPH oxidase (Tatone *et al*., 2010). Several groups have reported that cryopreservation, either through slow freezing or vitrification, is associated with an increase in reactive oxygen species (ROS). Lane *et al*. (2002) reported significant production of hydrogen peroxide in cryopreserved two-cell mouse embryos, with slow freezing having a greater negative effect than vitrification. Similarly both Somfai *et al*. (2007) and Gupta *et al*. (2010) reported an increase in hydrogen peroxide levels in vitrified pig oocytes, while Somfai *et al*. (2007) also quantified a concomitant decrease in glutathione (GSH) following cryopreservation. This documented increase in oxidative stress associated with cryopreservation is subsequently manifest in perturbations in metabolic function and gene expression patterns (Boonkusol *et al*., 2006; Mamo *et al*., 2006; Balaban *et al*., 2008; Succu *et al*., 2008; Larman *et al*., 2011; Bartolac *et al*., 2018). Further, vitrification-induced modifications of histone methylation and/or acetylation in oocytes and embryos have been reported, thereby linking cryopreservation to alterations in epigenetic state (Wang *et al*., 2010; Zhao *et al*., 2013; Chen *et al*., 2016; Jahangiri *et al*., 2018; Sahraei *et al*., 2018). Additionally, the repair of cryo-induced damage to cell structure and function requires the generation of energy which induces an increase in ROS production (Tatone *et al*., 2010).

Individual antioxidants have been shown to be effective in ameliorating oxidative stress in mammalian embryos during cryopreservation. Ascorbate was first shown to improve mouse embryo development and subsequent blastocyst viability following freezing (Tarin and Trounson, 1993; Lane *et al*., 2002), or vitrification (Lane *et al*., 2002). Furthermore, the inclusion of ascorbate significantly reduced intracellular hydrogen peroxide levels (Lane *et al*., 2002). The beneficial effect of exogenous ascorbic acid during vitrification was subsequently confirmed in the porcine model (Castillo-Martin *et al*., 2014; Castillo-Martin *et al*., 2015). The combination of three antioxidants [10 μM acetyl-*L*-carnitine (LC)/10 μM *N*-acetyl-*L*-cysteine (NAC)/5 μM α-lipoic acid (ALA)] has been shown to confer significant benefits to mouse IVF and culture, and subsequent embryo and fetal development (Truong *et al*., 2016; Truong and Gardner, 2017). Of interest, all these antioxidants have been shown individually to have beneficial effects during the cryopreservation process. NAC supplemented to vitrification and warming solutions has been demonstrated to improve mitochondrial distribution patterns and successfully eliminate ROS, resulting in increased development of vitrified oocytes (Yue *et al*., 2016). Cysteine is also the rate limiting factor in GSH synthesis (Anderson and Meister, 1983; de Matos *et al*., 1995; de Matos and Furnus, 2000), and hence it can impact on embryo development and quality by increasing antioxidant capacity through its antioxidant properties and supply of GSH. GSH supplemented during vitrification and warming of mouse germinal vesicle (GV) stage-oocyte has likewise been shown to increase blastocyst development (Moawad *et al*., 2017) indicating a protective role in oxidative damage and enhanced cryotolerance.

LC supplemented during vitrification and IVM of GV-stage mouse oocytes has been shown to improve subsequent blastocyst development (Moawad *et al*., 2013). In addition, spindle assembly, chromosomal alignment and mitochondrial distribution were also improved (Moawad *et al*., 2014). The increase in mitochondrial oxidative activity resulted in significantly improved metaphase II (MII) oocyte quality and blastocyst development (Moawad *et al*., 2013; Moawad *et al*., 2014).

ALA has been shown to protect mouse embryos against oxidative stress (Linck *et al*., 2007) and has been found to reduce DNA damage (Selvakumar *et al*., 2006; Ibrahim *et al*., 2008). When used in sperm cryopreservation there was an increase in sperm survival with improvements in motility, membrane integrity and acrosome integrity in buffalo sperm, and increased motility in boar sperm resulting in a higher pregnancy rate and litter size (Dominguez-Rebolledo *et al*., 2010; Shen *et al*., 2016; Fayyaz *et al*., 2017). Cryopreservation of pre-antral follicles with ALA has also shown higher rates of survival, antrum formation

and MII oocytes (Hatami *et al*., 2014). Thus, the multi-factorial effects of ALA on embryos, gametes and reproductive cells may contribute to the reduction of oxidative stress during cryopreservation in numerous ways.

The aim of this study was therefore to determine, in mice, the effects of LC, NAC and ALA on embryo development, histone acetylation and viability following vitrification and warming.

Materials and Methods

Culture of blastocysts

Pregnant mare's serum gonadotrophin (PMSG, 5 IU; Folligon, Intervet, Buckinghamshire, UK) was administered i.p. to 3–4 week old F1 virgin hybrid female mice (C57BL/6 × CBA). HCG (5 IU; Chorulon, Intervet) was administered 48 h later and female mice mated with F1 male mice. Mice were housed under a 12 h light–12 h dark photoperiod with food and water available *ad libitum*. A vaginal plug the following morning was used to indicate successful mating. At 22 h post-hCG, pronucleate oocytes (2PN) were collected in prewarmed GMOPS-PLUS (GMOPS-PLUS, Vitrolife AB, Gortborg, Sweden) handling medium. Cumulus cells were denuded with GMOPS-PLUS containing 300 IU/ml hyaluronidase (bovine testes type IV, Sigma Aldrich, NSW, Australia). Embryos from five mice were pooled, washed twice in GMOPS-PLUS medium and once in G1 medium containing 5 mg/ml human serum albumin (HSA; Gardner and Truong, 2019). After 48 h, embryos were transferred to G2 medium containing 5 mg/ml HSA (Gardner and Truong, 2019) for a further 24 h of culture. All cultures were performed in 35 mm petri dishes (Falcon, BD Biosciences, NJ, USA) under paraffin oil (OVOIL, Vitrolife) at 6% CO_2 and in either 20% or 5% oxygen in a humidified multi-gas incubator at 37°C (MCO-5M; Sanyo Electric, Osaka, Japan), with or without antioxidants in the culture media. *In vivo* developed blastocysts were flushed from the uteri of superovulated and mated females on Day 4. All mice experimentation was approved by The University of Melbourne, Animal Ethics Committee.

Vitrification of blastocysts

Day 4 expanded blastocysts were vitrified using the RapidVit Blast kit (Vitrolife) and the cryo-loop as the cryodevice (Lane *et al*., 1999). Blastocyst vitrification required three solutions (Vitri 1 Blast, Vitri 2 Blast and Vitri 3 Blast) which were prewarmed in four-well Nunc dishes at 37°C for 20 min. All manipulations were carried out on a 37°C heated stage. Ten blastocysts at a time were placed in 1 ml Vitri 1 Blast solution for at least 5 min and a maximum of 20 min. Embryos were moved to 1 ml Vitri 2 Blast solution for 2 min. From the 1 ml of prewarmed Vitri 3 Blast solution, a 20 µl drop was made on the lid of a dish and embryos transferred to the drop for 45 sec and mixed by pipetting up and down. With 10 s remaining, embryos were collected in a minimal volume and placed onto the Cryo-loop and immediately plunged into a Cryo-vial secured in a cryo-straw submerged in liquid nitrogen. Treatment groups had antioxidants supplemented into each of the vitrification solutions. Control groups had no antioxidants added to the vitrification solutions.

Warming of vitrified blastocysts

Warming of vitrified blastocysts was carried out using the RapidWarm Blast Kit (Vitrolife). Blastocyst warming required three solutions (Warm 1 Blast, Warm 2 Blast and Warm 3 Blast) which were prewarmed in four-well Nunc dishes at 37°C for 20 min. All manipulations were carried out on a 37°C heated stage. Immediately after vitrification, the Cryo-loop was removed from liquid nitrogen and dipped into 1 ml Warm 1 Blast solution to allow embryos to fall from the device. Embryos were left for 2 min and then transferred to 1 ml Warm 2 Blast solution for 3 min. Embryos were transferred to 1 ml Warm 3 Blast solution for 5–10 min before rinsing twice in medium G2, and embryo culture was continued in groups of 10 in medium G2 with no antioxidants for a further 24 h. Treatment groups had antioxidants supplemented into each of the blastocyst warming solutions. Control groups had no antioxidants added to the warming solutions.

Treatments

Dose response of antioxidants for cryopreservation

2PN oocytes were cultured from Day 1 to Day 4 in G1/G2 media without antioxidants. Day 4 expanded embryos were vitrified in cryopreservation solutions in the presence or absence of varying concentrations of antioxidants (10 µM to 2 mM LC/10 µM to 2 mM NAC/5 µM to 1 mM ALA). Embryos were immediately warmed in warming solutions in the presence or absence of the respective concentrations of antioxidants as in the vitrification solutions. Surviving embryos were cultured for a further 24 h in G2 media with no antioxidants (Fig. 1A). Embryo development was analysed and followed by differential nuclear staining and apoptosis staining as outlined below.

Antioxidants in culture media and cryopreservation solutions

2PN oocytes were cultured from Day 1 to Day 4 in media with antioxidants (10 µM LC/10 µM NAC/5 µM ALA) or without antioxidants. Day 4 expanded embryos were vitrified and immediately warmed in cryopreservation solutions in the presence or absence of antioxidants, creating four groups (Fig. 1B). Surviving embryos were cultured for a further 24 h in G2 media with no antioxidants. Embryo development was analysed and followed by differential nuclear staining as outlined below.

Antioxidants in vitrification and/or warming solutions

2PN oocytes were cultured from Day 1 to Day 4 in media without antioxidants. Day 4 expanded embryos were vitrified in vitrification solutions in the presence (10 µM LC/10 µM NAC/5 µM ALA) or absence of antioxidants. Embryos were immediately warmed in warming solutions in the presence or absence of antioxidants, creating four groups (Fig. 1C). Surviving embryos were cultured for a further 24 h in G2 medium with no antioxidants. Embryo development was analysed and followed by differential nuclear staining as outlined below.

Determination of apoptotic cells by TUNEL staining

A FITC-conjugated *in situ* cell death detection kit (DeadEnd Fluorometric TUNEL, Promega, Sydney, Australia) was used according to the manufacturer's instructions. Embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 1 h at room temperature and

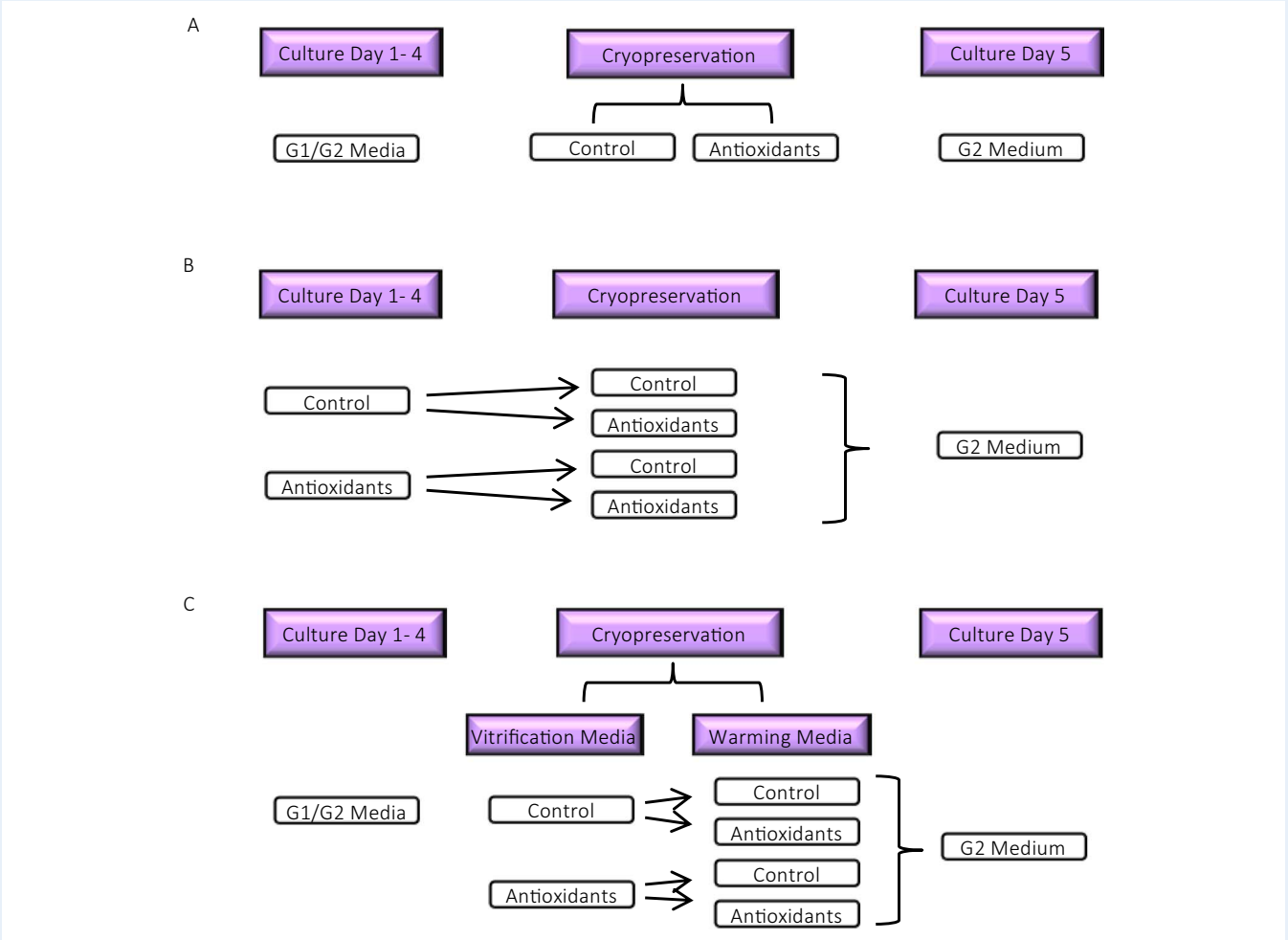

Figure 1 Experimental design for the analysis of antioxidants. (**A**) Design for the analysis of antioxidants in a dose response in cryopreservation solutions. Mouse embryos were cultured in media with no antioxidants for 4 days. Day 4 blastocysts were cryopreserved and warmed in solutions with and without antioxidants at varying concentrations. Surviving blastocysts were cultured for a further 24 h in media without antioxidants. (**B**) Design for the analysis of antioxidants in culture and/or cryopreservation solutions. Mouse embryos were cultured in the presence or absence of antioxidants [10 μM acetyl-*L*-carnitine (LC)/10 μM *N*-acetyl-*L*-cysteine (NAC)/5 μM α-lipoic acid (ALA)] for 4 days. Day 4 blastocysts were cryopreserved and warmed in solutions with and without antioxidants, thereby creating four groups. Surviving blastocysts were cultured for a further 24 h in media with no antioxidants. (**C**) Design for the analysis of antioxidants in vitrification and/or warming solutions. Mouse embryos were cultured in media with no antioxidants for 4 days. Day 4 blastocysts were vitrified in solutions with and without antioxidants and warmed in solutions with and without antioxidants, thereby creating four groups. Surviving blastocysts were cultured for a further 24 h in media with no antioxidants.

washed three times in GMOPS-PLUS. Embryos were then permeabilized with 0.125% Triton in 0.1% sodium citrate for 30 min at room temperature then washed three times with GMOPS-PLUS. Positive and negative controls were treated with DNase I (50 IU/ml; Promega) for 1 h at room temperature. After washing three times in GMOPS-PLUS, positive controls and treatment groups were incubated in TUNEL mixture containing deoxynucleotidyl transferase and fluorescein-conjugated dUTP for 1 h at 37°C in a humidified dark chamber. Negative controls were incubated with the fluorescent label in the absence of terminal transferase. After washing three times in GMOPS-PLUS embryos were incubated with 10 μg/ml bisbenzimide in GMOPS (Hoechst 33342; Sigma Aldrich) for 15 min at room temperature in the dark. Embryos were washed three times in GMOPS-PLUS and immediately glycerol mounted on glass slides and examined under a fluorescence microscope (Nikon Eclipse TS100, Osaka, Japan).

Inner cell mass and trophectoderm allocation

Blastocysts underwent a modified differential nuclear stain to determine both inner cell mass (ICM) and trophectoderm (TE) development (Hardy *et al.*, 1989). Following pronase treatment to remove the zona and following complement-mediated lysis, propidium iodide was used to label TE nuclei, leaving the ICM nuclei unlabelled. All nuclei were subsequently stained with bisbenzimide and blastocysts mounted in

glycerol. Differentially stained blastocysts were imaged under a fluorescence microscope (Nikon Eclipse TS100) and cells counted using ImageJ software (ImageJ, MD, USA).

Acetylation status of histones H3K9 and H3K27

Vitrified Day 4 expanded blastocysts were warmed and cultured for a further 24 h, before removal from culture drops, and fixed in 4% PFA (Sigma Aldrich) for 15 min. Following permeabilization in 0.25% Triton X-100 (Sigma Aldrich) in PBS for 15 min, blocking for non-specific binding was performed for 1 h at room temperature using blocking base solution consisting of 0.5% bovine serum albumin (BSA, Sigma Aldrich) and 1% Tween 20 (Sigma Aldrich) in PBS and supplemented with 5% donkey serum (Invitrogen, CA, USA). Primary antibody (Rabbit αH3K9ac or Rabbit αH3K27ac 1:250; Abcam Cambridge, UK) was applied overnight at 4°C in a humidified chamber. Embryos were washed three times (for 15 min per wash) with blocking solution before applying secondary antibody (Donkey α Rabbit Alexa Fluor 488 1:2000; Invitrogen) for 50 min in a humidified chamber at room temperature in the dark. Embryos were washed three times (20 min per wash) with blocking solution and all nuclei subsequently stained with bisbenzimide. Embryos were washed in blocking solution before being mounted in 100% glycerol. Controls were embryos treated with donkey serum and blocking solution with no antibodies, or with Rabbit IgG at equivalent concentrations (H3K9ac Rabbit IgG 1:200 and H3K27ac Rabbit IgG 1:100; Santa Cruz Biotechnology, Dallas, Texas, USA). Embryos were imaged under a fluorescence microscope (Nikon Eclipse TS100) at matching magnification and cells analysed using ImageJ software.

Blastocyst outgrowth

Day 4 expanded blastocysts were vitrified and warmed as described above either with (10 μM LC/10 μM NAC/5 μM ALA) or without antioxidants supplemented to the cryosolutions prior to use. Immediately after warming embryos were cultured in G2 medium with no antioxidants, in groups of 10 at 20% oxygen for 4 h. Blastocysts that re-expanded after 4 h were used for outgrowth assays (Binder *et al.*, 2014). Flat-bottomed 96-well tissue culture dishes were rinsed with sterile PBS and coated with a solution of 10 μg/ml fibronectin (BD Biosciences). Fibronectin was reconstituted in sterile distilled water to a stock concentration of 1 mg/ml and applied overnight at 4°C. Coated wells were rinsed with sterile PBS and incubated with a 4 mg/ml BSA in PBS solution for 2 h. Wells were subsequently rinsed with PBS, followed by G2 medium and then filled with 150 μl of G2 medium, supplemented with 5% fetal calf serum and equilibrated at 37°C under paraffin oil (OVOIL) under 20% oxygen for 4 h before the addition of blastocysts. Expanded and hatching blastocysts were placed individually into the coated wells (one embryo per well) and incubated for 96 h. Outgrowth was examined, and images were taken at 48, 72, 90 and 96 h after transfer to outgrowth plates with an inverted microscope (Nikon Eclipse Ti) equipped with a heated stage at 37°C and an XYZ camera. Images were taken using NIS Elements BR 3.00, SP7 Laboratory Imaging software (Nikon) and the extent of outgrowth for each treatment was determined using ImageJ. All images were analysed at matching magnification.

Embryo transfers

Synchronous embryo-uterine transfers were performed with Day 4 vitrified blastocysts treated either with (10 μM LC/10 μM NAC/5 μM ALA) or without antioxidants supplemented to the cryosolutions prior to use and surgically transferred to the uteri of a Day 4 pseudo-pregnant female mice. After anesthetizing with isoflurane gas, five embryos were transferred with a polished glass pipette into the lumen of each uterine horn of recipient female mice, with each mouse receiving embryos from control and antioxidant groups. Alternate groups were transferred to both the right and left horns per recipient to avoid any preferential implantation bias. Ten days later, pregnant females were sacrificed, and fetal development and/or resorptions recorded. Crown-rump length, and fetal and placental weights were measured, and morphological grades of fetal ear, eye and limb development were determined (Lane and Gardner, 1994).

Statistical analysis

Cell number data analysis for all treatments was compared to the non-vitrified group or control with no antioxidants and data were subjected to a one-way ANOVA followed by Bonferroni multiple comparisons test or Student's *t*-test. Proportion data were compared using a 3×2 contingency table. Prior to analysis all groups were tested for normality using Bartlett's test. Biological significance was considered at a *P*-value of 0.05. GraphPad Prism (San Diego, CA, USA) was used to perform all analyses.

Results

Dose response of antioxidants in blastocyst cryopreservation solutions

A dose response for the A3 antioxidant combination (10 μM LC/10 μM NAC/5 μM ALA) in blastocyst cryopreservation solutions at 10 (×10), 25 (×25), 50 (×50), 100 (×100) and 200 times (×200) the concentrations was assessed by vitrifying Day 4 blastocysts and analysing the resultant percentage of blastocyst survival, total cell numbers and apoptotic rates (Table I). All vitrified groups had similar blastocyst survival rates. Embryos in controls with no antioxidants and in groups treated with A3 ×10, A3 ×25, A3 ×50, A3 ×100 and A3 ×200 antioxidant concentrations resulted in significantly lower total cell numbers ($P < 0.001$) compared to non-vitrified embryos. Embryos in group A3 (10 μM LC/10 μM NAC/5 μM ALA) resulted in the highest total cell numbers that were similar to non-vitrified embryos and were significantly higher than control (untreated) embryos.

The number of apoptotic cells in control embryos with no antioxidants and A3 ×200 were significantly higher, while all other groups showed similar numbers of apoptotic cells compared to non-vitrified embryos. In addition, all groups, other than A3 ×200, resulted in a similar percentage of apoptotic cells compared to non-vitrified embryos. Interestingly, when compared to the control group with no antioxidants, embryos that had antioxidants at dose A3 ×25, A3 ×50 and A3 ×100 had a significantly lower number of apoptotic cells. In *in vivo* flushed embryos, a baseline of apoptosis frequency in preimplantation embryos was established with the mean number ± SEM of apoptotic cells and percentage apoptotic cells 2.22 ± 0.55 and 4%, respectively.

Table I Effect of antioxidants on blastocysts cultured at 20% oxygen and vitrified on day 4.

	Control	A3	A3 ×10	A3 ×25	A3 ×50	A3 ×100	A3 ×200	Non-Vitrified
No. 2PN embryos cultured	33	30	28	40	37	38	33	40
No. vitrified day 4 blastocysts	33	29	28	40	37	36	33	N/A
No. blastocysts (post vitrification)	30	27	26	36	35	34	31	N/A
% blastocyst survival	90.9%	93.1%	92.9%	90.0%	94.6%	94.4%	93.9%	N/A
No. total cells 24 h post vitrification	77.39 ± 4.57***	95.40 ± 5.6#	69.36 ± 3.7***	80.21 ± 3.9***	75.30 ± 4.04***	82.12 ± 4.63***	74.57 ± 6.01***	105.88 ± 4.4
No. apoptotic cells 24 h post vitrification	6.13 ± 0.75*	5.88 ± 0.89	3.96 ± 0.55	2.96 ± 0.54##	3.03 ± 0.50##	3.83 ± 0.62##	14.40 ± 2.86**	3.04 ± 1.36
% apoptotic cells	9%	5%	6%	4%	4%	4%	11%	4%

2PN: pronucleate oocytes. Combination of antioxidants (A3) comprises 10 μM LC/10 μM NAC/5 μM ALA. Antioxidants were in cryopreservation solutions. Controls were embryos that had no antioxidants in cryopreservation solutions. Three biological replicates.
Asterisks denote significant differences from non-vitrified embryos. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
#Denotes significant differences from control embryos that had no antioxidants cryopreservation solutions. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$
Data analysis for all treatments was compared to the non-vitrified group or control embryos with no antioxidants in cryopreservation solutions and data were subjected to a one-way ANOVA followed by Bonferroni multiple comparisons test.

Effect of antioxidants on blastocyst development when present in embryo culture and/or cryopresevation solutions

Embryos cultured at 20% oxygen and cryopreserved with no antioxidants resulted in significantly lower total and TE cell numbers compared to non-vitrified embryos (Table II). Similarly, embryos that were cultured with antioxidants but cryopreserved without antioxidants had lower total and TE cell numbers compared to non-vitrified embryos. In contrast, embryos that had antioxidants in both culture and cryopreservation solutions or solely in cryopreservation solutions had a similar cell number to non-vitrified embryos. These two groups also had higher total cell numbers when compared to embryos cultured and cryopreserved with no antioxidants. Similarly, embryos that were cultured at 5% oxygen and had antioxidants solely in cryopreservation solutions also exhibited increased total cell (109.6 ± 3.7 versus 98.02 ± 4.4; $P < 0.05$) and ICM number (24.90 ± 1.7 versus 18.15 ± 1.2; $P < 0.01$) compared to embryos with no antioxidants.

Embryos in which antioxidants were added solely to vitrification solutions and to both vitrification and warming solutions had significantly increased total, TE and ICM numbers compared to embryos that were not exposed to antioxidants (Table III). In addition, these two groups also had significantly increased total and ICM cells when compared to embryos that had antioxidants solely in the warming solutions.

Acetylation of blastocysts derived from *in vitro* culture and vitrified blastocysts

Embryos vitrified at the blastocyst stage had significantly lower levels of H3K9ac and H3K27ac compared to non vitrified embryos ($P < 0.001$; Fig. 2). This was irrespective of whether antioxidants were present or absent in the vitrification solutions. However, blastocysts that were cryopreserved in the presence of antioxidants had higher levels of H3K9ac and H3K27ac compared to vitrified embryos with no antioxidants, although the presence of antioxidants did not eliminate the negative effects of cryopreservation on acetylation levels.

Viability of vitrified blastocysts determined by outgrowths *in vitro*

Vitrified blastocysts (with no exposure to antioxidants either in culture or during vitrification and warming) and antioxidant groups (embryos not exposed to antioxidants in culture but exposed during vitrification and warming) attached to fibronectin in culture at the same rate (83%). However, embryos vitrified with antioxidants exhibited a significant increase in outgrowth area and perimeter compared to blastocysts vitrified with no antioxidants (Fig. 3).

Viability of vitrified blastocysts determined by synchronous embryo transfer

Synchronous transfer of blastocysts derived from culture under 20% oxygen and vitrified and warmed in the presence of antioxidants, resulted in increased fetal weight (190.19 ± 4.61 mg versus 174.29 ± 5.52 mg; $P < 0.05$), crown rump length (11.09 ± 0.10 versus 10.76 ± 0.11; $P < 0.05$) and limb development (14.89 ± 0.07 versus 14.56 ± 0.11; $P < 0.05$) and an overall increase in estimated fetal age (14.89 ± 0.05 versus 14.64 ± 0.09; $P < 0.05$) when compared to embryos not exposed to antioxidants during vitrification and warming (Table IV). There were similar rates of implantation, fetal development and sex ratios. Similarly, embryos cultured at 5% oxygen to the blastocyst stage and vitrified and warmed with antioxidants showed increases in both crown rump length (11.29 ± 0.08 versus 10.74 ± 0.12; $P < 0.001$) and ear development (14.90 ± 0.05 versus 14.63 ± 0.11; $P < 0.05$), culminating in an increase in estimated fetal age (14.94 ± 0.02 versus 14.75 ± 0.08; $P < 0.05$) (Table V). Although there were trends to higher fetal and placental weights in the antioxidant group when

Table II Effect of antioxidants in culture or cryopreservation media on blastocysts cultured at 20% oxygen.

Culture Cryopreservation	Control Control	Control A3	A3 Control	A3 A3	Non-Vitrified
No. 2PN embryos cultured	80	80	80	80	80
No. vitrified day 4 blastocysts	80	80	80	80	N/A
No. blastocysts (post vitrification)	78	77	77	76	N/A
% blastocyst survival	97.5%	96.3%	96.3%	95.0%	N/A
No. total cells 24 h post vitrification	76.38 ± 2.95***	93.00 ± 2.58###	80.25 ± 3.06***	93.08 ± 2.46###	104.13 ± 2.37
No. ICM cells	20.59 ± 1.06	25.08 ± 1.02##	22.83 ± 1.11	23.50 ± 0.96	21.40 ± 1.13
No. TE cells	60.45 ± 2.76***	67.91 ± 2.23	61.98 ± 2.62**	68.72 ± 2.46	77.93 ± 3.18

Combination of antioxidants (A3) comprises 10 μM LC/10 μM NAC/5 μM ALA. Antioxidants were in culture and/or cryopreservation solutions. Controls were embryos that had no antioxidants in culture and cryopreservation solutions. Four biological replicates.
Asterisks denote significant differences from non-vitrified embryos. $^{**}P < 0.01$, $^{***}P < 0.001$.
#Denotes significant differences from control group that had no antioxidants in both culture and cryopreservation solutions. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.
Data analysis for all treatments was compared to the non-vitrified group or control group with no antioxidants in both culture and cryopreservation solutions and data were subjected to a one-way ANOVA followed by Bonferroni multiple comparisons test.

Table III Effect of antioxidants in vitrification or warming media on blastocysts cultured at 20% oxygen.

Vitrification Warming	Control Control	Control A3	A3 Control	A3 A3
No. 2PN embryos cultured	60	60	60	60
No. vitrified day 4 blastocysts	60	60	60	60
No. blastocysts (post vitrification)	56	57	54	56
% blastocyst survival	93.3%	95.0%	90.0%	93.3%
No. total cells 24 h post vitrification	73.03 ± 3.87	82.56 ± 3.34	102.10 ± 4.31***###	101.30 ± 3.92***###
No. ICM cells	17.00 ± 1.05	19.52 ± 1.12	22.77 ± 1.23**###	22.84 ± 1.30**###
No. TE cells	62.12 ± 3.41	63.04 ± 3.07	79.33 ± 3.71**	78.46 ± 3.56**

Combination of antioxidants (A3) comprises 10 μM LC/10 μM NAC/5 μM ALA. Antioxidants were in vitrification and/or warming solutions. Three biological replicates. Controls were embryos that had no antioxidants in vitrification and warming solutions.
Asterisks denote significant differences from control group vitrified and warmed with no antioxidants. $^{**}P < 0.01$, $^{***}P < 0.001$.
#Denotes significant differences from embryos warmed with antioxidants. $^{\#\#}P < 0.01$.
Data analysis for all treatments was compared to the control group with no antioxidants in both vitrification and warming solutions and data were subjected to a one-way ANOVA followed by Bonferroni multiple comparisons test.

embryos were cultured under 5% oxygen, these were not statistically significant.

Discussion

Consistent with the benefits of the combined antioxidants seen in mouse IVF and embryo culture (Truong and Gardner, 2017; Truong *et al*., 2016), here we report that antioxidants provide significant protection against the damaging effects of ROS during vitrification, culminating in increased embryo development and fetal outcome post transfer. The beneficial effects were seen when antioxidants were added to the cryopreservation solutions, irrespective of whether embryos had been cultured in the presence of antioxidants, resulting in total cell numbers comparable to non-vitrified embryos. Unlike previous studies, which showed that the greatest benefit of antioxidants occurred when they were present in both sperm and oocyte IVF media (Truong and Gardner, 2017), antioxidants solely in culture media were not able to prevent the reduced cell number following cryopreservation. As such, these data indicate that antioxidants need to be present at the time of exposure to increased oxidative stress associated with vitrification and that prior exposure is insufficient to protect cells against cryo-induced injury. This confirms the necessity of antioxidants to be present throughout all stages of *in vitro* manipulations (Truong and Gardner, 2017), especially during times of high oxidative stress such as cryopreservation (Lane *et al*., 2002).

Data further indicate that the oxidative damage acquired during cryopreservation is likely to occur predominantly during the vitrification process rather than the warming phase, as antioxidants added solely to warming solutions, conferred no benefit to blastocyst development. In contrast, when antioxidants were added to both vitrification and warming solutions, or solely in vitrification solutions, there was a significant increase in blastocyst cell number following culture. When compared to the non-vitrified embryos, there was a reduction in cell number, although this decrease was somewhat ameliorated by the presence of antioxidants during the vitrification step. This lowering of blastocyst cell number, even in the presence of antioxidants during

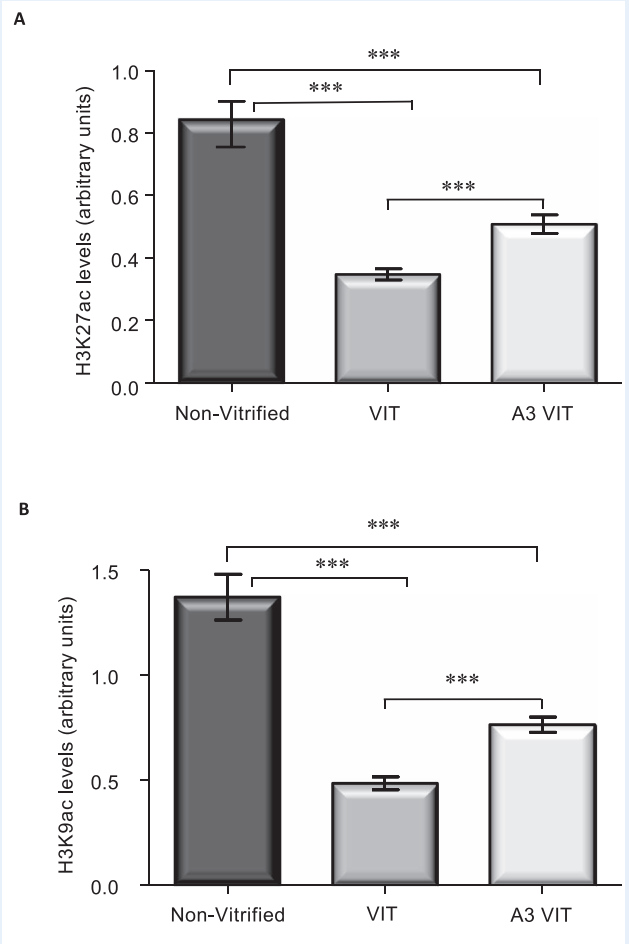

Figure 2 Effect of antioxidants present during vitrification and warming on histone acetylation. (**A**) Histone H3K27ac levels in cultured mouse blastocysts and blastocysts vitrified (VIT) with or without antioxidants. (**B**) Histone H3K9ac levels in cultured mouse blastocysts and blastocysts vitrified with or without antioxidants. Antioxidant combination (A3) comprises 10 μM LC/10 μM NAC/5 μM ALA. Controls were *in vitro* cultured, non-vitrified blastocysts. Data are expressed as mean ± SEM. $n = 30$ embryos per treatment, from three independent biological replicates. Data were subjected to a one-way ANOVA followed by Bonferroni multiple comparisons test. Asterisks denote significant differences. $^{***}P < 0.001$.

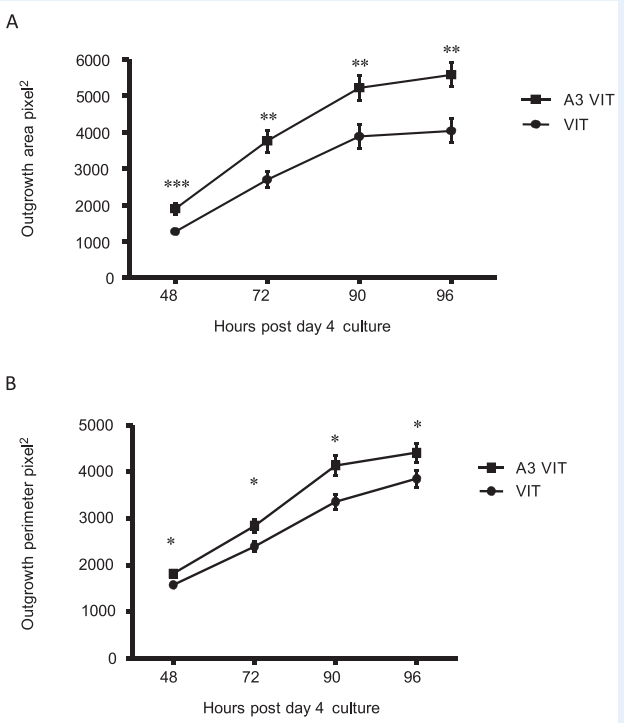

Figure 3 Effect of antioxidants present during vitrification and warming on blastocyst outgrowth. Blastocyst outgrowth was significantly increased in mouse embryos cultured at 20% oxygen and vitrified and warmed in the presence of antioxidants. (**A**) Area of embryos outgrown after vitrification. (**B**) Perimeter of outgrowths after vitrification. Antioxidant combination (A3) comprises 10 μM LC/10 μM NAC/5 μM ALA. Data are expressed as mean ± SEM. $n = 35$ embryos outgrown per treatment, from three independent biological replicates. Data were subjected to Student's *t*-test. Asterisks denote significant differences from vitrified embryos with no antioxidants. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cryopreservation, may be a result of reduced metabolic activity associated with cryopreservation (Gardner *et al*., 1996; Balaban *et al*., 2008), which would lead to a delay in cleavage times.

Analysis of histone acetylation revealed a decrease in both H3K9ac and H3K27ac levels of cryopreserved blastocysts compared to non-vitrified embryos. Although the inclusion of the three antioxidants could not totally prevent the cryo-induced increase in deacetylation, the negative impact was significantly alleviated. H3K9ac is an epigenetic marker which represents transcriptionally active chromatin and is targeted for deacetylation by sirtuin 1 (SIRT1) and SIRT6 (Morris, 2013; Tatone *et al*., 2015). SIRT6 is known to maintain telomere integrity and modulate DNA repair and genome integrity (Wu and Morris, 1998; Finkel *et al*., 2009). Likewise, the functions of SIRT1 contribute to chromatin organization, DNA repair and genome stability (Finkel *et al*., 2009; Furukawa *et al*., 2007). Thus, aberrant changes to histone acetylation state could lead to changes in gene expression and genetic stability. Further work is therefore required on regulation of the epigenetic state, specifically histone acetylation and methylation levels, by exogenous antioxidants. Although in the present study combined LC, NAC and ALA were quite effective, it is highly plausible that other antioxidants may have additional protective benefits.

Implantation potential of vitrified blastocysts was evaluated through both an outgrowth assay and embryo transfers. Blastocysts vitrified with antioxidants displayed an increased ability to outgrow as demonstrated by an increase in outgrowth area and perimeter, indicating greater implantation potential. In addition, the increase in ICM number of embryos cryopreserved with antioxidants also suggests a correlation with increased outgrowth capacity and viability (Lane and Gardner, 1997). Conversely, the reduced outgrowth and ICM number of blastocysts vitrified without antioxidants is indicative of compromised implantation potential, plausibly due to the cryopreservation process. Post-implantation developmental capacity of vitrified blastocysts, assessed through embryo transfers, revealed reduced fetal development following blastocyst cryopreservation with no antioxidants relative to those cryopreserved with antioxidants, providing further evidence of the

Table IV Fetal development following Day 4 synchronous embryo transfer of vitrified blastocysts cultured from 2PN at 20% oxygen.

Parameter	VIT	A3 VIT
Implantation per transfer	61.43%	62.86%
Fetal development per transfer	35.71% (25/70)	35.71% (25/70)
Fetal development per implantation	58.14% (25/43)	56.82% (25/44)
Resorption per transfer	25.71% (18/70)	27.14% (19/70)
Fetal weight (mg)	174.29 ± 5.52	190.19 ± 4.61*
Placental weight (mg)	89.26 ± 5.39	95.08 ± 5.07
Crown-rump length (mm)	10.76 ± 0.11	11.09 ± 0.10*
Limb morphological grade	14.56 ± 0.11	14.89 ± 0.07*
Eye morphological grade	14.60 ± 0.12	14.91 ± 0.06
Ear morphological grade	14.76 ± 0.11	14.86 ± 0.08
Estimated fetal age	14.64 ± 0.09	14.89 ± 0.05*

Data are expressed as % mean ± SEM. $n = 70$ blastocysts transferred per group.
*$P < 0.05$.
Combination of antioxidants (A3) comprises 10 μM LC/10 μM NAC/5 μM ALA. Antioxidants were in vitrification and warming solutions. Control (VIT) were embryos that had no antioxidants in vitrification and warming solutions.
Data were subjected to a Student's *t*-test. Proportion data were compared using a 2 × 2 contingency table.

Table V Fetal development following Day 4 synchronous embryo transfer of vitrified blastocysts cultured from 2PN at 5% oxygen.

Parameter	VIT	A3 VIT
Implantation per transfer	49.00%	49.00%
Fetal development per transfer	31% (31/100)	30% (30/100)
Fetal development per implantation	63.27% (31/49)	61.22% (30/49)
Resorption per transfer	18% (18/100)	19% (19/100)
Fetal weight (mg)	184.50 ± 6.07	196.32 ± 4.75
Placental weight (mg)	98.94 ± 4.41	109.54 ± 4.50
Crown-rump length (mm)	10.74 ± 0.12	11.29 ± 0.08***
Limb morphological grade	14.82 ± 0.07	14.95 ± 0.04
Eye morphological grade	14.81 ± 0.09	14.97 ± 0.02
Ear morphological grade	14.63 ± 0.11	14.90 ± 0.05*
Estimated fetal age	14.75 ± 0.08	14.94 ± 0.02*

Data are expressed as % mean ± SEM. $n = 100$ blastocysts transferred per group.
*$P < 0.05$, ***$P < 0.001$.
Combination of antioxidants (A3) comprises 10 μM LC/10 μM NAC/5 μM ALA. Antioxidants were in vitrification and warming solutions. Control (VIT) were embryos that had no antioxidants in vitrification and warming solutions.
Data were subjected to a Student's *t*-test. Proportion data were compared using a 2 × 2 contingency table.

detrimental effects of this procedure and a benefit of antioxidants. Together these data demonstrate a role of antioxidants in maintaining the viability potential of cryopreserved embryos for implantation and fetal development. Of note, the effects of antioxidants were more pronounced in embryos cultured at 20% oxygen compared to 5%, reflecting the greater stress placed on embryo physiology in atmospheric oxygen (Wale and Gardner, 2013). Furthermore, the fetal and placental weights of embryos cultured at 20% were lower than embryos cultured at 5% with and without antioxidants. This provides further evidence of the high stress and damage incurred during cryopreservation and is also consistent with other studies showing the detrimental effects of cumulative stress (Wale and Gardner, 2013; Kelley and Gardner, 2016; Truong *et al*., 2016). Therefore, accumulation of *in vitro* stresses, such as culture at atmospheric oxygen and cryopreservation, can have crucial consequences on embryo development and viability. However, direct statistical comparisons could not be conducted between fetal development of transfers from embryos cultured at 20% and 5% due to them being conducted at separate times. Nevertheless, fetal development rate and fetal and placental weight were lower and resorption rate higher when embryos were cultured at 20% than in 5% oxygen. Addition of antioxidants, while beneficial to fetal development compared to controls in 5% oxygen, did not have the same impact as seen at 20% oxygen. Importantly, the increase in fetal weights associated with vitrification in the presence of antioxidants did not result in fetal overgrowth compared to *in vivo* controls (Truong *et al*., 2016), but rather the antioxidants alleviated the downstream detrimental effects of vitrification.

In this study, we did not determine whether it was the exposure to the cryoprotectants or the process of vitrification which imparted the oxidative stress in the blastocysts. Bartolac *et al*. (2018), using the porcine model, determined that vitrification, and not exposure to cryoprotectants, induced changes in the expression of the imprinted genes insulin growth factor IGF2 and IGF2 receptor. However, previous work on the mouse oocyte proteome revealed that exposure to cryoprotectants alone was sufficient to induce changes (Katz-Jaffe *et al*., 2008). Given the documented genotoxicity of cryoprotectants used in vitrification (Aye *et al*., 2010; Berthelot-Ricou *et al*., 2011), further studies are required to assess the protective actions of antioxidants against cryoprotectants.

In conclusion, the presence of antioxidants during cryopreservation conveys significant beneficial effects on embryo development, plausibly through the reduction of oxidative stress, resulting in increases in cell numbers, reduced apoptotic cells, increased acetylation levels and subsequent improved fetal development. The addition of antioxidants to cryopreservation solutions could therefore represent a valuable strategy to improve embryo development and viability. These data hold promise for the improvement of FET transfer outcome in clinical IVF, and studies are currently underway to evaluate this.

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Authors' roles

DKG conceived the project, developed the research plan and contributed to the writing of the manuscript. TT assisted with the research plan, undertook the laboratory work and contributed to the writing of the manuscript.

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Conflict of interest

None declared.

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