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ORIGINAL ARTICLE *Embryology*

Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions

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STUDY QUESTION: What is the intracellular concentration of cryoprotectant (ICCP) in mouse zygotes during vitrification (VIT) and slow-freezing (SLF) cryopreservation procedures?

SUMMARY ANSWER: Contrary to common beliefs, it was observed that the ICCP in vitrified zygotes is lower than after SLF, although the solutions used in VIT contain higher concentrations of cryoprotectants (CPs).

WHAT IS KNOWN ALREADY: To reduce the likelihood of intracellular ice crystal formation, which has detrimental effects on cell organelles and membranes, VIT was introduced as an alternative to SLF to cryopreserve embryos and gametes. Combined with high cooling and warming rates, the use of high concentrations of CPs favours an intracellular environment that supports and maintains the transition from a liquid to a solid glass-like state devoid of crystals. Although the up-to-date publications are reassuring in terms of obstetric and perinatal outcomes after VIT, a fear about exposing gametes and embryos to high amounts of CPs that exceed 3–4-fold those found in SLF was central to a debate initiated by advocates of SLF procedures.

STUDY DESIGN, SIZE, DURATION: Two experimental set-ups were applied. The objective of a first study was to determine the ICCP at the end of the exposure steps to the CP solutions with our VIT protocol ($n = 31$). The goal of the second investigation was to compare the ICCP between VIT ($n = 30$) and SLF ($n = 30$). All experiments were performed in triplicates using mouse zygotes. The study took place at the GIGA-Research Institute of the University of Liège.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Cell volume is modified by changes in extracellular osmolarity. Hence, we estimated the final ICCP after the incubation steps in the VIT solutions by exposing the cells to sucrose (SUC) solutions with defined molarities. The ICCP was calculated from the SUC concentration that produced no change in cell volume, i.e. when intra- and extracellular osmolarities were equivalent. Cell volume was monitored by microscopic cinematography. ICCP was compared between SLF and VIT based on the principle that a high ICCP lowers the probability of (re)crystallization during warming but increases the probability of over-swelling of the cell due to fast inflow of water. The survival rates of mouse zygotes after SLF or VIT were compared using either (i) various warming rates or (ii) various concentrations of SUC in the warming dilution medium.

MAIN RESULTS AND THE ROLE OF CHANCE: The ICCP in mouse zygotes during the VIT procedure prior to plunging them in liquid nitrogen was ~2.14 M, i.e. one-third of the concentration in the VIT solution. After SLF, the warming rate did not affect the zygote survival rate. In contrast, only 3/30 vitrified zygotes survived when warmed slowly but as many as 30/30 zygotes survived when warming was fast (>20 000°C/min). Vitrified zygotes showed significantly higher survival rates than slow-frozen zygotes when they were placed directly in

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the culture medium or in solutions containing low concentrations of SUC ($P < 0.01$). These two experiments demonstrate a lower ICCP after VIT than after SLF.

LIMITATIONS, REASONS FOR CAUTION: The results should not be directly extrapolated to other stages of development or to other species due to possible differences in membrane permeability to water and CPs.

WIDER IMPLICATIONS OF THE FINDINGS: The low ICCP we observed after VIT removes the concern about high ICCP after VIT, at least in murine zygotes and helps to explain the observed efficiency and lack of toxicity of VIT.

STUDY FUNDING / COMPETING INTEREST(S): The study was funded by the FNRS (National Funds for Scientific Research). The authors declare that they have no competing interests.

Key words: vitrification / slow freezing / intracellular concentration and osmolarity / cryoprotectant / toxicity

Introduction

Water is the major component of the cell and its solidification is the main factor that has to be controlled during the process of cryopreservation. Intracellular formation of ice crystals must be avoided during both cooling and warming phases, in order to avoid its detrimental effect on cellular organelles and membranes (Luyet, 1937; Leibo *et al.*, 1978; Mazur *et al.*, 2005).

In order to reduce the likelihood of intracellular ice crystal formation, vitrification (VIT) was introduced as an alternative to the slow freezing (SLF) cryopreservation procedure (Rall and Fahy, 1985; Massip *et al.*, 1986). VIT abolishes the intracellular ice crystal formation found during SLF (Quinn, 2010). VIT is the conversion of a super-viscous, supercooled liquid into a glassy state when it is cooled below its glass transition temperature (T_g; Fahy *et al.*, 1987).

In the assisted reproduction technology (ART) field, it is now recognized that VIT is a very effective way to cryopreserve MII oocytes (Kuwayama *et al.*, 2005; Cobo *et al.*, 2010, 2012a; Rienzi *et al.*, 2012), zygotes (Al-Hasani *et al.*, 2007; Vanderzwalmen *et al.*, 2012) and embryos at different stages of development (Isachenko *et al.*, 2007; Balaban *et al.*, 2008; Vanderzwalmen *et al.*, 2009; Van Landuyt *et al.*, 2011; Cobo *et al.*, 2012b). However, since the first announcement of successful VIT in a mammalian model in 1985 (Rall and Fahy, 1985) and in human embryos in 1997 (Vanderzwalmen *et al.*, 1997), more than 10 years elapsed before the emergence of raising interest and implementation of this technique in the ART disciplines. Although, reliable and promising results with blastocysts have been published, the VIT technique gained interest only when it was shown that it was considerably superior to SLF for the cryopreservation of unfertilized human oocytes (Smith *et al.*, 2010; Martínez-Burgos *et al.*, 2011).

How can this reluctance to move to VIT be explained? One part of the answer concerns the nature of the cryoprotectant solution (CPsol) used in this technique. By comparison to SLF, higher concentrations and different types of CPs are used. Exposure of the biological material to high concentrations of CPsol is one of the conditions that support the emergence of a glassy state. Usually, oocytes or embryos are first exposed to two non-vitrifying solutions (nVS1 and nVS2) containing cell-penetrating CPs (2.3–3.2 M; Kuwayama *et al.*, 2005; Vanderzwalmen *et al.*, 2009). Next, they are exposed for a short time (45–60 s) to a vitrifying solution (VS) containing high concentrations of penetrating (4.8–6.4 M; Kuwayama *et al.*, 2005; Vanderzwalmen *et al.*, 2009) and non-penetrating (0.5–0.75 M) CPs before being plunged into liquid nitrogen (LN2).

Our protocol (Vanderzwalmen *et al.*, 2009, 2012) allows the CPs to penetrate the zygote during their exposure to nVS1 and nVS2. The exposure is short and the initial volume of the zygote is not recovered during this process since there is insufficient time for intra- and extracellular CP concentrations to equilibrate. In the last step, embryos are exposed to the VS (6.4 M) where sucrose (SUC) and Ficoll, as non-penetrating CPs, induce dehydration. Thereby various intrinsic macromolecules like (glyco)proteins, salts and also penetrating CPs are concentrated, generating an intracellular environment that enables a vitreous state to be reached and maintained during the cooling and warming processes.

An extracellular vitrified state is allowed by the high concentrations of CPs in the VS that encapsulates the embryo in a vitrifying sheath. Exposure to these high concentrations of CPs rises concerns that high intracellular concentrations of CPs (ICCPs) might reached during VIT and be harmful or even toxic for the cell. For a long time this concern was sufficient to make many practitioners favour SLF because it uses lower concentrations of penetrating CPsol (1.5 M) even though very good survival and implantation rates had been reported with VIT. A serious debate on the safety of exposure of oocytes or embryos to CP concentrations that exceed 3–4-fold those found in SLF posed questions about their potential cytotoxicity.

In that context, the aim of this research is to discover how high the ICCP is when VIT and SLF cryopreservation procedures are performed.

Two investigations were undertaken with mouse zygotes as models. The objective of the first study was to determine the ICCP at the end of the exposure steps to the CPsol used in our VIT protocol. The CP molarity observed inside the cells was then compared with the one in the VS. The ICCP was estimated using cell volume measurements. For that purpose, mouse zygotes were exposed to nVS1, nVS2 and VS before immersing them in SUC solutions with various molarities. No variation in the cell volume indicated equivalent osmotic pressures on both sides of the membrane. Using a cinematographic recording system, we have determined the SUC concentration with which no modification of the cell volume was observed, allowing inference of the ICCP. We compared it to VS.

The goal of the second study was to evaluate which of the cryopreservation techniques (VIT versus SLF) produced the higher final ICCP. A high ICCP decreases the probability of (re)crystallization during the warming step but increases the probability of over-swelling of the cell due to fast inflow of water. Based on this assumption, two protocols were designed allowing comparison of the survival rates of

mouse zygotes after SLF and VIT with (i) various warming rates and (ii) various concentrations of SUC in the warming dilution medium.

Materials and Methods

Stimulation, retrieval and culture of mouse zygotes

Only FVB/N inbred mice (Janvier, France) were used in this study. Five to 7-week-old FVB/N females were stimulated with 3.5 IU of pregnant mare serum gonadotrophin (PMSG; Folligon, Intervet, The Netherlands) injected i.p. followed by an i.p. injection of 5 IU of human chorionic gonadotrophin (hCG; Chorulon, Intervet) 46 h later. Soon after, each female was mated with a male of the same strain. The mice with a vaginal plug were sacrificed by cervical dislocation 12 h *post-coitum*; the ampulla was opened and zygotes were collected and placed in culture in M16 medium at 37°C under 5% CO_2.

Cryopreservation protocols

VIT protocol

Exposure steps to the CPsol. All steps were performed at room temperature (RT ~25°C). CPsol were prepared in home-made M2 medium (Quinn *et al*., 1982). The zygotes were first exposed to nVS1: 5% v/v dimethyl sulfoxide (DMSO, Sigma, Germany)—5% v/v ethylene glycol (EG, Sigma) (nVS1 = 1.6 M) for 3 min and then to nVS2: 10% v/v DMSO—10% v/v EG (nVS2 = 3.2 M) for 3 min. Afterwards they were transferred to VS containing 20% v/v EG—20% v/v DMSO, 25 μM (10 mg/ml) Ficoll (70 000 MW, Sigma) and 0.5 M SUC (Sigma) (VS = 6.4 M for the penetrating CPs) for 45–60 s. As additional control, two alternative solutions of our VS were used: solution 10/10 (sol 10/10) containing 10% v/v EG—10% v/v DMSO, 25 μM Ficoll and 0.5 M SUC and solution 25/25 (sol 25/25) containing 25% v/v EG—25% v/v DMSO, 25 μM Ficoll and 0.5 M SUC.

Loading on the carrier and cooling step. Zygotes were placed onto the Vitrisafe gutter (VitriMed, Austria) in a small quantity of VIT solution (<1 μl). The gutter was inserted in a 0.3 ml CBS straw (CryoBioSystem, France) and this latter was immediately sealed at both ends with a thermosealer (SYMS2, CryoBioSystem) before being plunged into LN2. These processes took no more than 60 s.

The Vitrisafe plug, designed for aseptic VIT ensures the hermetic isolation of the zygotes from the LN2 and thereby recapitulates the aseptic storage of cells cryopreserved in closed SF devices (Vanderzwalmen *et al*., 2009).

SLF procedures

Exposure to the CPsol and freezing. The SLF protocol described by Renard and Babinet (1984) was used here with minor modifications. Zygotes were exposed for 25 min at RT to a 1.5 M solution of 1–2 propanediol (PROH, Sigma) diluted in M2 medium, before they were loaded in a straw (0.25 ml French straw, CryoBioSystem). Fifteen zygotes were loaded in each straw. Straws were then placed in a precooled (0°C) freezing machine (Embryo-Freeze, Biotronics, UK). Temperature was decreased at 2°C/min until −7°C. Five minutes later, the seeding was performed on one end of each straw. After 7.5 min, the temperature was decreased at a rate of 0.3°C/min until −35°C. At this temperature, the straws were plunged directly into LN2.

Determination of the ICCP and its comparison between VIT and SLF

First study

Assessment of the ICCP at the end of the exposure steps to nVS1, nVS2 and VS by cellular volume monitoring. Cells react to changes in extracellular osmolarity by volume alterations (Fig. 1a). The measure of the osmotic pressure gradient between two solutions separated by a semipermeable membrane is called tonicity. Cells exposed to hypotonic or hypertonic solutions initially react either by swelling (hypotonic solutions) or by shrinkage (hypertonic solutions) due to flows of water but later recover as permeant solutes equilibrate across the cell membrane.

We used this mechanism to estimate the final ICCP after the exposure steps to the nVS1, nVS2 and VS, by transferring the zygotes into solutions containing defined SUC concentrations. We inferred the ICCP from the molarity of the SUC solution that did not induce any direct change in the cell volume after exposure, by converting the known osmolarity of the SUC solution into corresponding molarity.

Assessment of volume variations by microscopic cinematography. Our method was adapted from the procedure of Paynter *et al*. (2001). Observations of the behaviour of mouse zygotes in the various CPsol were performed on the lid of a 10 cm petri dish. Fifty microliters drops of successive CPsol were aligned one after the other on a horizontal line, and were covered with mineral oil (Fertipro, Belgium; Fig. 2).

The determination by cell volume variations of ICCP during the various steps was done on one zygote at a time. To estimate the original volume, the zygote was first kept in M2 culture medium. At the end of the consecutive exposures to nVS1 and 2 and VS, the zygote was immersed in an SUC solution with a defined molarity and the variation of its volume was recorded. The molarity of the SUC solutions was gradually increased from 0 to 2.14 M until almost no modification of the volume was observed.

A minimum of three fully interpretable assays was performed for each SUC concentration. Only zygotes keeping a homogeneous spherical shape during all the sequential exposure steps were considered for measurements.

The observations were performed under an inverted microscope (IX51; Olympus) equipped with a camera (Olympus DP50) and software that records the pictures (AnalySIS version 3.2; Olympus).

In order to maintain the zygote on the same focal plane during the transit from one CPsol to the next, a holding pipette fixed on a micromanipulator (TransferMan NK2, Eppendorf) was installed. The mineral oil coverage strongly limited the volume of medium that was transferred from one droplet to the other, thereby limiting media mixing (Fig. 3B).

The areas of the zygotes were measured manually with the image analysis software (AnalySIS). The volume of the corresponding zygotes could then be calculated from their deduced radius. The results were plotted on a graph representing the volume variations in relation to the exposure time in the different solutions.

Second study

Comparison of the ICCP after SLF or VIT. The same procedure of volume follow-up cannot be performed during SLF due to the process itself (Fig. 1b). After equilibration with 1.5 M PROH, the dehydration of the cells continues during the slow cooling equilibrium process in reaction to the extracellular ice crystals formation. During this period the zygote lies inside a straw fixed in a programmable cooling machine and thereby is not accessible for any volume variation follow-up. For this reason, two indirect strategies were adopted to compare the survival rates between various warming conditions after SLF and VIT procedures, and therefore, to compare corresponding ICCP levels.

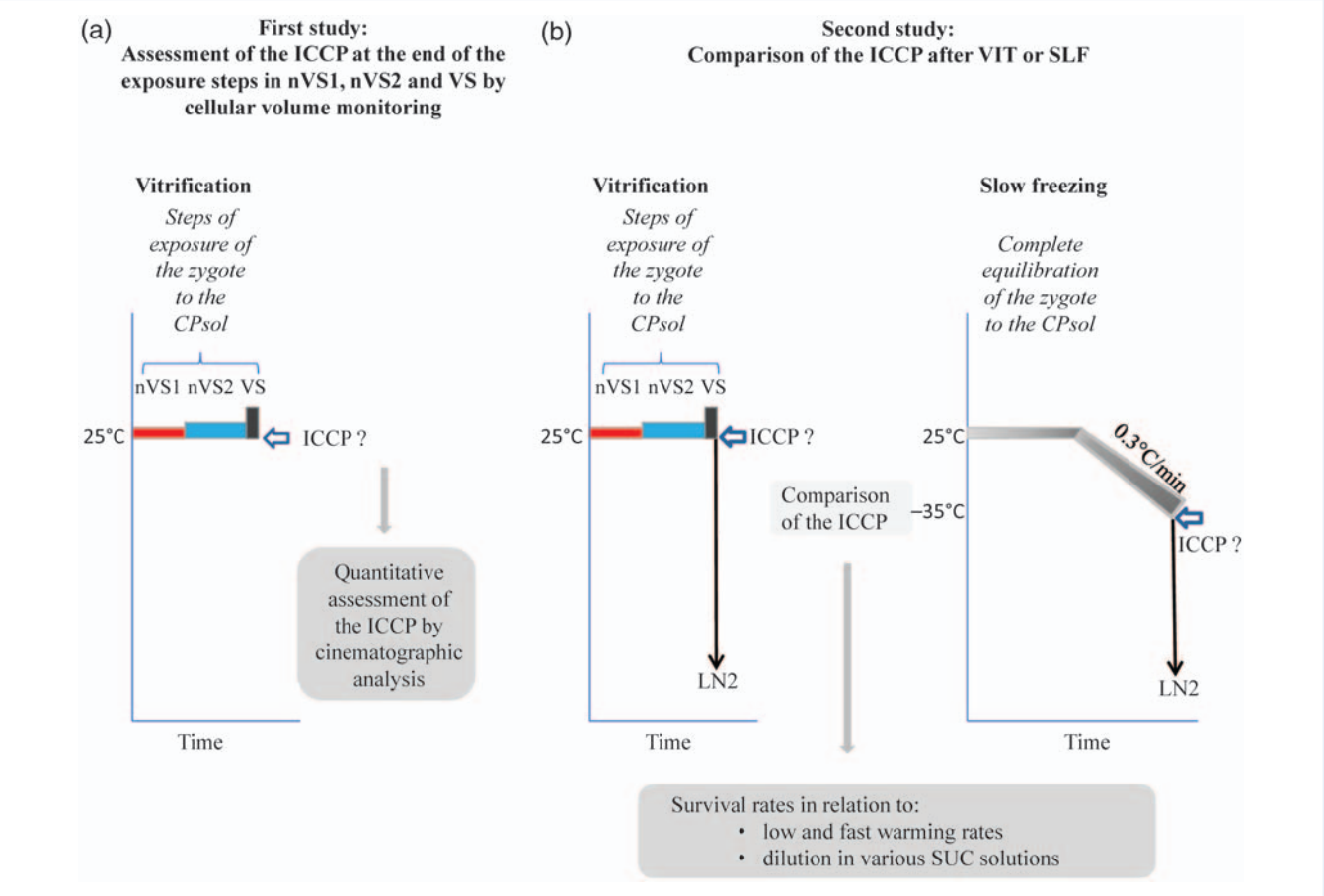

Figure 1 (**a**) Experimental design for the assessment of the ICCP when VIT cryopreservation procedure was performed. ⇦ Time point where the ICCP was evaluated. (**b**) Experimental design for the comparison of the ICCP between VIT and SLF cryopreservation procedures. ⇦ Time point where the ICCPs were compared. nVS1/2, non vitrifying solution 1/2; VS, vitrifying solution; CPsol, cryoprotectant solution.

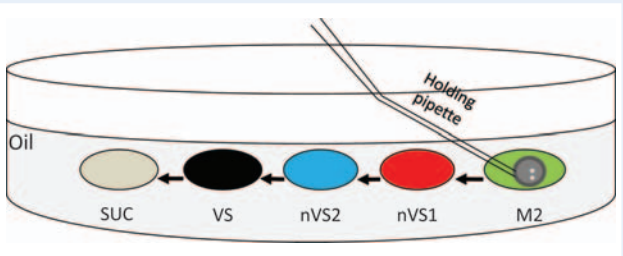

Figure 2 Schematic representation of the method used to monitor the change in volume of zygote during exposure to M2, nVS1, nVS2, VS and various molarities of SUC. Transfer of one zygote from one 50 µl droplet of CPsol to the other one with the help of a holding pipette in order to maintain it on the same focal plane. nVS1/2, non vitrifying solution 1/2; VS, vitrifying solution; SUC, sucrose.

Strategy number one: comparison of survival rates after VIT or SLF in relation to the warming rate. Three assays on 10 zygotes each were performed for both SLF and VIT groups. It is well known that increasing the ICCP decreases the probability of recrystallization and cell lysis when low warming rates are applied. According to this assumption, one experiment was conducted comparing the rates of zygote survival after rapid (37°C) and slow warming (25°C) following either SLF or VIT. The warming rate was estimated for each condition using two digital thermometers (GMH 3230; Greisinger Electronic and 2110T; Research Instruments).

For thawing after SLF, one set of the straws was plunged in a water bath at 37°C until the ice has disappeared (warming rate ~2280°C/min), whereas the second set was taken out of the LN2 and kept in the air until complete melting (warming rate ~680°C/min). After thawing, the straws were emptied in a 30 mm Petri dish containing 3 ml of 1 M SUC solution. After 5 min, the zygotes were washed twice in M2 medium before being transferred to 50 µl droplet of M16 medium under oil and cultured at 38°C in an atmosphere containing 5% CO_2.

For warming after VIT, the upper end of the protective straw was cut while still held in LN2, and the Vitrisafe gutter holding the zygotes was removed. The gutter was then either warmed in the air (warming rate ~1140°C/min) before being immersed in a 1 M SUC solution, or was directly warmed in the 1 M SUC solution (warming rate ~21 000°C/min). In both cases, 0.8 ml of 1 M SUC in a 4-well plate (Nunc, Denmark) at RT was employed. After 1 min in the 1 M SUC solution, the zygotes were transferred to 0.75, 0.5, 0.25 and 0.125 M SUC solutions for 1, 1, 2 and 2 min, respectively. Subsequently, the zygotes were directly immersed in 3 ml of M2 medium for 15 min and rinsed twice before further culture in

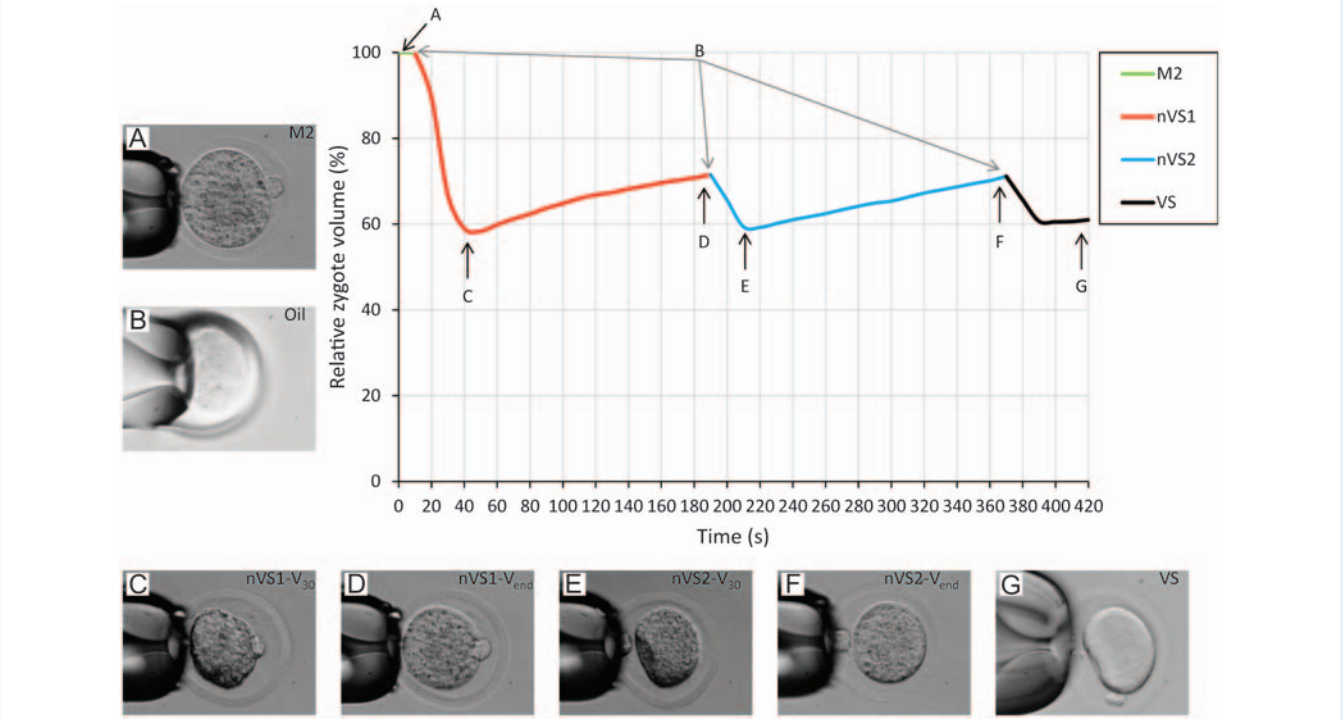

Figure 3 Cinematographic study: evolution of the relative volume of one zygote through the various CPsol. Letters on the graph refer to corresponding pictures. A: $t = 0$ in M2 medium; B: passage from one drop to another drop through the oil; C: $t = 30$ s in nVS1; D: $t = 180$ s: end of the nVS1 step; E: $t =$ after 30 s in nVS2; F: $t =$ after 180 s in nVS2. G: end of the exposure to VS. V_{30}, volume after 30 s of exposure to a specific CPsol; V_{end}, volume at the end of a period of exposure to a specific CPsol. nVS1/2, non vitrifying solution 1/2; VS, vitrifying solution.

50 μl droplet of M16 medium under oil at 38°C in an atmosphere containing 5% CO_2.

Strategy number two: comparison of survival rates after VIT or SLF in relation to dilution in various SUC concentrations. Higher ICCP requires transfer to a warming medium containing a higher SUC concentration to minimize overswelling of the cell due to fast inflow of water through the cell membrane. In this experiment, the percentages of lysed zygotes were evaluated in SUC-containing warming solutions of increasing concentration, either after VIT or SLF.

Slow-frozen zygotes were first thawed in a 37°C water bath and then transferred to the SUC solution. Vitrified zygotes were directly immersed in the SUC solution. Various molarities of SUC in M2 medium were used. The survival rates of the zygotes were determined 3 h after warming.

Statistical analysis

All experiments were repeated at least three times. The results were pooled. Analyses were performed by Student's *t*-test or by χ^2 test. A *P*-value of <0.05 was considered as statistically significant.

Ethical approval

The animal housing and all experimental methods were performed with the approval of the Animal Ethics Commission of University of Liège, Belgium (file nr 297). All procedures were conducted in accordance with the Federation of European Laboratory Animal Science Associations guidelines. All the staff working with animals, including researchers followed the required legal trainings.

Results

First study: assessment of the ICCP at the end of the exposure steps to nVS1, nVS2 and VS by cellular volume monitoring

First, the variations of the cell volume during the exposure steps to the CPsol: nVS1, nVS2 and VS were determined (Fig. 3). The zygotes ($n = 31$) reacted to exposure to the CPsol by a reduction (shrinkage) followed by a partial restoration (swelling) of the initial volume. In the nVS1, the cell first decreased to 58.3 ± 5.1% and subsequently recovered to 71.5 ± 3.9% of its initial volume. Hence in nVS2, shrinkage to 59.2 ± 5.0% followed by a swelling to again 71.5 ± 4.8% was observed. In the VS, only a decrease in cell volume occurred to finally reach a mean of 60.5 ± 5.2% of the initial (Fig. 3G).

Shrinkage corresponds to the initial water outflow from the zygote exposed to a hypertonic solution, whereas swelling is due to the subsequent inflow of penetrating CPs followed by water. The absence of swelling after cell shrinkage in VS solution is a consequence of the presence of the non-penetrating CPs SUC and Ficoll in the solution. In nVS1 and nVS2, incomplete equilibration of CPs does not permit to return to the initial volume (final mean volume = 71.5%). Dehydration occurring immediately after exposure to a more concentrated CPsol increases the intracellular concentration of previously penetrated CPs.

After the VS step, zygotes were exposed to one of the following SUC solutions: 0 M ($n = 3$), 0.30 M ($n = 4$), 0.45 M ($n = 3$), 0.82 M

($n = 4$), 1.40 M ($n = 6$), 1.82 M ($n = 6$) and 2.14 M ($n = 5$) (Fig. 4a). The cells swelled rapidly when exposed to 0–1.82 M SUC solutions; the degree of swelling varied from more than doubling of the volume in 0 M SUC to an increase of ~10% in 1.82 M SUC. When incubated in 2.14 M SUC solution only minimal changes in cell volume occurred. This reduced volume modification indicates that an osmotic quasi-equilibrium exists between intracellular and extracellular compartments. Therefore during VIT, the intracellular osmolarity approaches 2.14 Osm/L before the zygotes are plunged in LN2.

To confirm that exposing zygotes to nVS1, nVS2 and the routinely used VS, results in an approximate iso-osmoticity between ICCP and the 2.14 M SUC solution, we analysed volume variations of mouse zygotes after exposure to two alternative VS solutions: sol 10/10 or sol 25/25.

In both supplementary groups, cinematographic analysis was performed on four zygotes per group ($n = 9$ for sol 10/10; $n = 15$ for sol 25/25; Fig. 4b). After 60 s exposure to sol 10/10, we observed an immediate and dramatic reduction in cell volume upon immersion in the 2.14 M SUC solution. In contrast, after exposure to sol 25/25, an increase in volume was observed. We have shown above that after exposure to VS, only faint variation in the volume was observed, reflecting an iso-osmotic situation. Exposures to the two alternative solutions reflect hypo-osmotic (sol 10/10) or hyper-osmotic (sol 25/25) situations.

Second study: comparison of the ICCP after SLF or VIT

Figure 5 shows the survival rates after slow or fast warming of 60 vitrified and 60 slow-frozen zygotes. After SLF, no significant difference in the survival rates was noticed either if slow warming (80%; 24/30) or rapid warming (77%; 23/30) was applied. After VIT however, only 10% (3/30) of the zygotes survived when the warming process took place in air (slow warming). When the tip of the Vitrisafe was directly plunged in a SUC bath to reach a warming rate of $>20\,000^{\circ}$C/min, 100% (30/30) of the zygotes survived the warming procedure (rapid warming). The difference in terms of survival rates was statistically significant ($P < 0.001$).

The survival rates after the transfer of 90 vitrified and 90 slow-cooled zygotes to various concentrations of SUC solutions after thawing is presented in Fig. 6. Slow-frozen zygotes that were directly diluted in the culture medium (0 M SUC) showed a significantly lower ($P < 0.001$) survival rate (7%; 2/30) than the vitrified zygotes (37%; 11/30). In the 0.25 M and 0.5 M SUC solutions, survival rates in the SLF group were 43% (13/30) and 70% (21/30), respectively. Again, vitrified zygotes showed significantly better survival rates with 73% (22/30) in 0.25 M ($P < 0.01$) and 97% (29/30) in 0.5 M SUC solutions ($P < 0.001$).

Discussion

During the VIT process, and before rapidly cooling the biological material in LN2, cells are exposed to at least two steps of gradual increase of the concentrations of CPs in the nVS1, nVS2 and VS. The aim is to create an intracellular environment that supports the transition from a liquid to a solid glass-like state for defined cooling and warming rates. This means that before plunging the biological material in LN2, the intracellular compartment has to be conditioned to allow the emergence and maintenance of a vitreous state (Fahy et al., 1987).

The fear of exposing gametes and embryos to such high amounts of CPs was the central part of a debate initiated by advocates of the SLF procedure, using low concentration of penetrating CP. Indeed, when compared with SLF where embryos are equilibrated with CPsol of 1.5 M, the final concentration of penetrating CPs in the VS is considerably higher, ranging from 4.8 M (Kuwayama et al., 2005) to 6.4 M (Vanderzwalmen et al., 2009; Quinn, 2010).

To reassure those who are concerned by the high concentration of CPs in VIT and to provide them with an objective answer, we estimated in this study the ICCP of a vitrified mouse zygote. In a second approach, we specified which of both techniques, SLF or VIT, results in the highest ICCP.

To our knowledge, this is the first manuscript reporting an estimation of the true ICCP immediately before plunging the zygote in LN2.

It is well accepted that all CPs are potentially toxic. The impact of the CPs on the safety of VIT has always been a major scientific concern. Although the up-to-date publications are reassuring in terms of obstetric and perinatal outcomes (Chian et al., 2008; Liebermann, 2009; Nagy et al., 2009; Noyes et al., 2009; Forman et al., 2012; Mukaida et al., 2012), further studies were necessary to solve the apparent contradiction between the observed efficiency/safety of VIT and the anticipated higher ICCP of vitrified cells. In a recent study, Chatzimeletiou et al. (2012) analysed blastocysts that were aseptically vitrified. They concluded that although there was a significantly higher incidence of abnormal spindles in the vitrified group than in the fresh control, the high survival rates after thawing and the large proportion of normal spindle/chromosome configurations suggested that VIT did not adversely affect the development of human blastocysts and the ability of spindles to form and continue normal cell divisions. Additionally, Wirleitner et al. (2012) showed that the length of the storage period of vitrified blastocysts in closed devices had no negative impact. Neither survival nor developmental potential were impaired after 6 years in the LN2 tank, nor was any influence on the health of born children detected.

If the concentration of CPs had been very high in the cells, would it have been possible to obtain life birth rates almost comparable with fresh embryo transfers (Rienzi et al., 2010)? Bearing this in mind, is it justified to favour SLF because of concern about the use of high CPs in VIT?

The reason to use high concentration of CPs in VIT is to embed the zygote in a vitrifying extracellular sheath to avoid ice crystal formation that may have detrimental effects. The use of macromolecules such as Ficoll, that remain outside the cells, increases the medium viscosity and protects the outer part of the embryo against crystallization. In general, the VS contains the minimal concentration of CPs that allows a liquid to reach a vitrified state under defined cooling and warming conditions.

The cinematographic analysis allowed us also to estimate the degree of penetration of CPs inside the cells. The concentration of CPs inside the cell after the two first steps of exposure to the first two CPsols (nVS1 and nVS2) was too low to allow a vitrified state to be achieved and maintained. The zygotes in our VIT protocol did not reach complete equilibration after short periods of exposure to nVS1 and nVS2. Hence, zygotes were exposed for a brief period to VS containing SUC, to dehydrate them so that an intracellular vitrifying state could be established. This increased the intracellular osmolarity following from

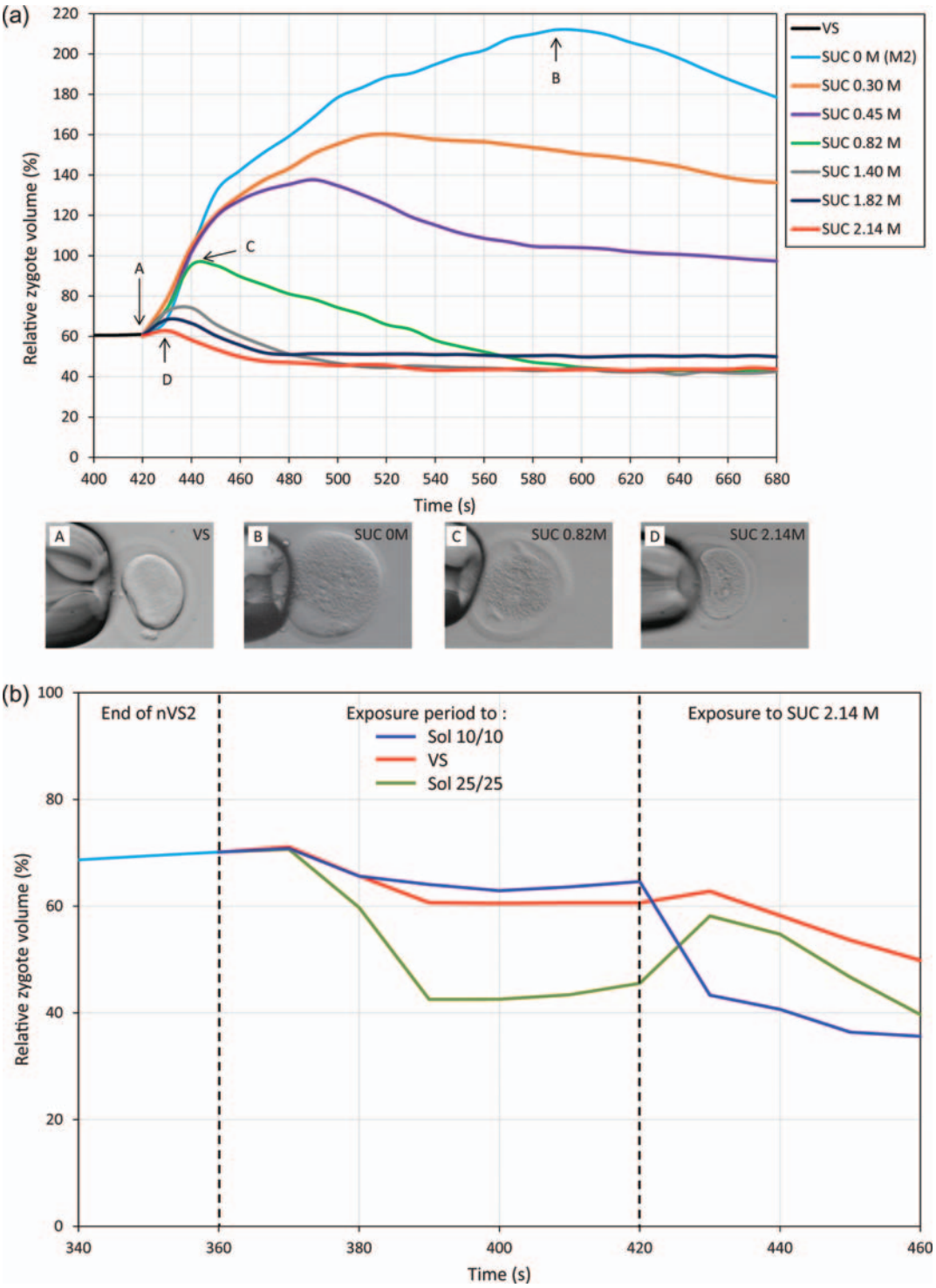

Figure 4 (**a**) Evolution of the relative zygote volume when immersed in solutions with various concentrations of SUC following the exposure steps to nVS1, nVS2 and VS (Fig. 3). Letters on the graph refer to corresponding pictures. A: end of the exposure to VS (4A is the same time point as Fig. 3G). B–D: pictures of zygotes when they have reached their maximal volume in SUC solutions of 0, 0.82 and 2.14 M, respectively. (**b**) Evolution of the relative zygote volume when immersed in 2.14 M SUC solution following the exposure steps to nVS1, nVS2 and VS or alternative solutions (sol 10/10 or sol 25/25). nVS1/2, non vitrifying solution 1/2; VS, vitrifying solution; SUC, sucrose.

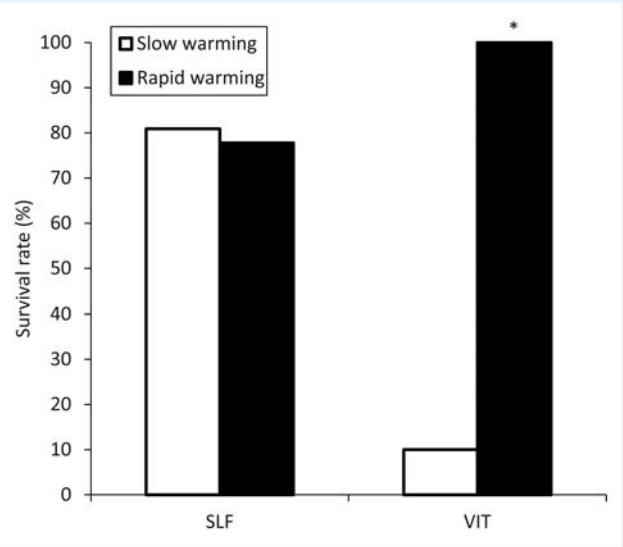

Figure 5 Comparison of survival rates after VIT or SLF in relation to the warming rate. *$P < 0.001$; 3 replicates of 10 zygotes in each group.

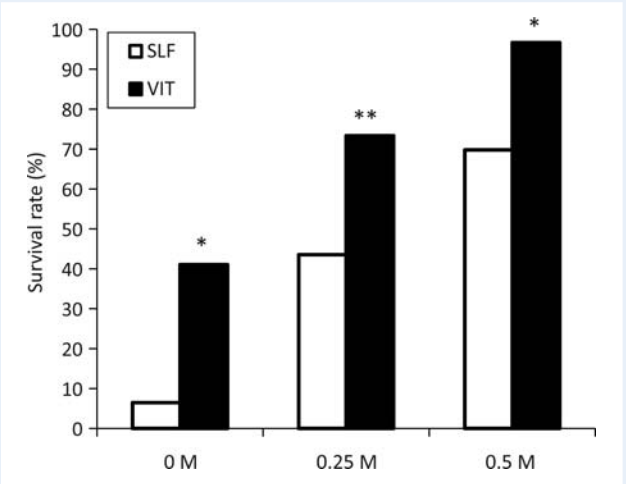

Figure 6 Percentages of intact zygotes after VIT or SLF in relation to various SUC concentrations. *$P < 0.01$, **$P < 0.02$; 3 replicates of 10 zygotes in each group.

increases in the concentrations of the previously entered CPs as well as intracellular salts and (glyco)proteins. The cytoplasm contains various intrinsic macromolecules and salts that were concentrated during the final dehydration step favouring the amorphous state. This in turn generates an intracellular environment that promotes maintenance of a vitreous state when cells are rapidly plunged into LN2 or warmed. The CPs are only part of the intracellular solutes that contribute to the intracellular osmolarity, so the true ICCP is even lower than 2.14 M. This low ICCP helps to explain why VIT gives better results than SLF: although high extracellular concentration of CPs are needed to allow VIT, only a fraction of them enter the cell, thereby reducing their toxicity.

The next step aimed at comparing the ICCPs after SLF and VIT. Two experiments have shown that the ICCP was lower in our VIT protocol than in a conventional SLF protocol.

We observed a significantly higher rate of lysed zygotes after SLF than after VIT whatever the SUC concentration in the medium they were transferred to after thawing. Immediately after warming, SLF zygotes presented obvious signs of over-swelling, which finally induced the lysis of the zygotes. This can be explained by a rapid and finally lethal inflow of water into the cells. This is the signature of a higher intracellular osmolarity in SLF when compared with the one present in vitrified cells. The higher the ICCP, the higher the SUC concentration in the warming medium must be to minimize an over-swelling of the cells.

It has been shown that warming speeds play an even more important role for cell survival after VIT than the cooling rates (Seki and Mazur, 2008, 2009). A high warming speed after VIT prevents cell lysis. This is because in a VIT process, the ICCP is too low to prevent recrystallization during slow warming when the temperature exceeds T_g (approximately $-120^{\circ}C$). If the warming speed is not fast enough, the supercooled liquid is rapidly transformed into ice crystals in the interval between T_g and the melting temperature (T_m).

The contrasting observation that survival rates after SLF are poorly affected by the warming rate, suggests that the ICCP is higher after SLF and can prevent intracellular crystallization.

As critical warming rates are higher than the critical cooling rates, the minimal concentration of CPs to prevent re-crystallization during warming must be higher than during cooling. This means that it might be easier to maintain a vitrified state during cooling than during the warming process, given the same concentration of CPs. If the warming rate is reduced by using devices that insulate the drop containing the zygotes, thereby increasing thermal inertia, higher ICCPs are needed in order to reduce the likelihood of re-crystallization. However, these higher concentrations of CPs might be toxic for the cells.

Out of two studies using mouse zygotes in conjunction with a time lapse analysis, we can conclude that the ICCP during VIT is a lot lower than the CP concentrations present in the VS solution. We have clearly shown that the ICCPs in the vitrified zygotes are, in contrast to the common beliefs, even lower than the ones observed after a SLF procedure.

During SLF, when cooling is applied at a rate of $0.3^{\circ}C$/min, cells are equilibrated with a low concentration of a penetrating CP (PROH) whose concentration increases during all the cooling process as a consequence of the increasing formation of extracellular ice crystals (solution effect). Knowing that a cell survives a cryopreservation procedure only if the development of intracellular ice crystals is avoided, survival after SLF reflects the presence of an intracellular vitrified state. SLF has been the standard cryopreservation method for >25 years without being aware of the presence of a vitrified intracellular state obtained with a very high ICCP. So, people anxious of toxic consequences of high ICCP during cryopreservation should shift from SLF methods to the VIT protocols achieving consistently lower ICCP. Moreover, this shift could also be justified by the much shorter exposure to CPs during a VIT process than during SLF, and ultimately by the higher efficiency of VIT.

From all these experiments, we can conclude that the ICCP reached after VIT is not as high as it was previously thought. As a

consequence, we can demystify the problem of high level of CPs inside the cell during VIT, thereby solving the apparent contradiction with the higher VIT efficiency. However, this study was performed on mouse zygotes. A similar experiment should be re-conducted on human oocytes and embryos to confirm our observations. Nevertheless, we are quite confident that our conclusions could be extrapolated to other species since they reflect the efficiency and safety of the VIT procedure reported in the literature.

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Authors' roles

P.V. and F.J.E. were responsible for the core of concept design, for the experimental work described herein and the writing of the manuscript. D.C. was involved in several aspects of experimental work, data analysis and finalizing of the manuscript. B.R. and S.V. participated in mouse preparation, zygotes collection and cryopreservation. B.W. and N.Z. played a role in manuscript review and input. All authors were involved in manuscript preparation and P.V., F.J.E., D.C. and L.G. approved the final submitted version.

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Conflict of interest

The authors declare that they have no competing interests.

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