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Optimising vitrification of human oocytes using multiple cryoprotectants and morphological and functional assessment

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Abstract. Oocyte vitrification is a clinical practice that allows preservation of fertility potential in women. Vitrification involves quick cooling using high concentrations of cryoprotectants to minimise freezing injuries. However, high concentrations of cryoprotectants have detrimental effects on oocyte quality and eventually the offspring. In addition, current assessment of oocyte quality after vitrification is commonly based only on the morphological appearance of the oocyte, raising concerns regarding its efficiency. Using both morphological and functional assessments, the present study investigated whether combinations of cryoprotectants at lower individual concentrations result in better cryosurvival rates than single cryoprotectants at higher concentrations. Surplus oocytes from IVF patients were vitrified within 24 h after retrieval using the Cryotop method with several cryoprotectants, either individually or in combination. The morphological and functional quality of the vitrified oocytes was investigated using light microscopy and computer-based quantification of mitochondrial integrity, respectively. Oocyte quality was significantly higher using a combination of cryoprotectants than vitrification with individual cryoprotectants. In addition, the quality of vitrified oocyte varied depending on the cryoprotectants and type of combination used. The results of the present study indicate that observations based purely on the morphological appearance of the oocyte to assess the cryosurvival rate are insufficient and sometimes misleading. The outcome will have a significant implication in the area of human oocyte cryopreservation as an important approach for fertility preservation.

Additional keywords: Cryotop, human oocyte, immunofluorescence, mitochondria, vitrification.

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Introduction

In recent years the importance of oocyte cryopreservation as a clinical tool of assisted reproductive technologies has increased for many reasons (Pegg 2005; Vajta and Kuwayama 2006; Galeati *et al.* 2011), including, but not limited to, allowing fertility to be improved by preserving oocytes before the loss of ovarian function due to aging, disease, surgery, chemotherapy or radiotherapy (Chen *et al.* 2003; Isachenko *et al.* 2006; Batuhan and Safaa 2010; Trapphoff *et al.* 2010). Oocyte cryopreservation is used not only to preserve fertility in women and thus avoid logistical issues required to produce an embryo through IVF, but also to avoid some of the ethical issues involved with the storage and disposal of surplus embryos (Oktay *et al.* 2006). Through cryobanking, oocyte cryopreservation can extend fertility, giving young women the opportunity to establish their professional careers before building a family and also benefit women in need of oocyte donation due to ovarian failure by allowing easier and immediate access to oocytes (Isachenko *et al.* 2006; Cobo *et al.* 2008).

Vitrification is a relatively new technique for oocyte cryopreservation achieved by fast cooling rates in a minimum volume of liquid containing high concentrations of cryoprotectant agents (CPAs). This technique entails the solidification of a solution into a glassy state at low temperatures without the formation of ice crystals, minimising injury to oocytes (Ambrosini *et al.* 2006; Martínez-Burgos *et al.* 2011). Although this reduces freezing injury caused by ice crystal formation, the safety of these CPAs in relation to the health of the offspring has not been fully verified. In addition, there is an increased probability of toxic, osmotic and other forms of injury causing differential shrinking and morphological changes in the cell during equilibration in CPAs or media that can result in cell death (Vajta 2000; Trapphoff *et al.* 2010). Although human oocytes have been successfully vitrified, the degree and exact

effect of CPA toxicity is poorly understood. Although the pregnancy rates involved with oocyte vitrification are comparable to those with fresh oocytes (Cobo *et al.* 2008; Nagy *et al.* 2009; Rienzi *et al.* 2010), the fragile ultrastructure of the mammalian oocyte, along with the toxic effects of CPAs, may result in adverse effects on offspring (Cobo *et al.* 2008).

Vitrification requires high concentrations of CPAs, such as dimethyl sulfoxide (DMSO), propanediol (PROH) and ethylene glycol (EG; Trapphoff *et al.* 2010). Specifically, extensive investigations into CPAs for vitrification have shown that very high concentrations of EG can be used with minimal toxicity (Gook and Edgar 2007). Innovations such as the Cryotop method have been introduced to minimise exposures to CPAs and have been applied successfully to cryopreserve human oocytes and embryos and are now commonly used in many IVF clinics (Ruvalcaba *et al.* 2005; Antinori *et al.* 2007; Cobo *et al.* 2008). Subsequent reports have shown that mature human oocytes vitrified using the Cryotop method at a slightly lower concentration of EG resulted in high rates of survival, fertilisation and development to the blastocyst stage (Kuwayama *et al.* 2005; Lucena *et al.* 2006). Therefore, we hypothesised that lower concentrations of a combination of CPAs, used with the Cryotop method, may result in less toxicity and higher rates of oocyte cryosurvival and eventually pregnancy and births.

The quality of a vitrified oocyte plays a critical role in female fertility because it not only affects the developmental potential of the oocyte, but also plays a crucial role in fertilisation and the subsequent development of the embryo (Rienzi *et al.* 2010). Some reports have shown that compromised oocyte quality can lead to developmental defects, reduced fertility and epigenetic defects that affect the long-term health of the offspring (Mtango *et al.* 2008). Although previous studies have been conducted to determine the negative effects of high concentrations of individual CPAs on oocytes, the observations have been based purely on the gross morphology and appearance of the oocytes (Cao *et al.* 2009; Bonetti *et al.* 2011). The integrity of the oocyte organelles and ultrastructures, such as mitochondria, chromosomal DNA and microtubules, cannot be determined on the basis of gross morphology alone under light microscopy; therefore, more detailed biochemical, functional and/or ultrastructural studies are required.

Mitochondria are the most abundant cell organelles in oocytes and their activity is essential because they provide ATP for energy-demanding cellular activities and specifically for normal spindle formation and chromosome segregation (Nagai *et al.* 2004; Eichenlaub-Ritter *et al.* 2011). The spindle, which is composed of α-tubulin, is extremely sensitive to temperature and disruption of the meiotic spindle can result in chromatid non-disjunction, aneuploidy and poor embryonic development (Park *et al.* 1997; Noyes *et al.* 2010). Therefore, the amount and distribution of these organelles may reflect the quality of oocytes. Nonetheless, most of the studies conducted to determine the integrity of subcellular oocyte structures have focused on mitochondrial or microtubule distribution or integrity alone (Chatzimeletiou *et al.* 2012), all while using only one type of CPA at a high concentration (Bonetti *et al.* 2011). This raises the necessity for performing more comprehensive and non-biased quantitative studies.

The present paper reports the results of a study using both morphological and functional parameters to determine whether a combination of multiple CPAs at lower concentrations will have less toxicity and result in better oocyte cryosurvival than the use of single CPAs at higher concentrations. The study also aimed to determine which CPA or combination of CPAs results in the least damage and the most viable oocytes.

Materials and methods

Human oocytes

Oocytes were collected from women aged between 23 and 44 years undergoing infertility treatments at a local IVF clinic (PIVET Medical Centre, Perth, WA, Australia) and following standard clinical protocols for cycle stimulation and IVF. Approximately 40% of women were between 23 and 35 years of age, 30% were between 35 and 39 years of age and other 30% were 40 years or older. The causes of infertility were mixed: either male or female infertility, a combination of both or unexplained. All patients were stimulated using antagonist plus recombinant FSH (MSD, North Ryde, NSW, Australia) at customised dosages described by the Clinic algorithm (Yovich *et al.* 2012) to avoid hyperstimulation. Ovulation was induced by 10 000 IU human chorionic gonadotrophin (hCG; MSD), with oocyte recovery 35–36 h later (Yovich and Stanger 2010). Denuded immature oocytes (40 h after hCG) were not used for clinical purposes and were donated for the present study by consenting patients. The study was approved by the Curtin University Human Research Ethics Committee. Oocytes were cultured overnight in fertilisation medium (Sage Inc., a Cooper Surgical Company, Bedminster, NJ, USA) at 37°C with 5% CO_2, during which time some oocytes matured *in vitro*, as evidenced by the expulsion of the first polar body; these oocytes were classified as mature oocytes. All oocytes were graded 1–4 according to morphological viability using light microscopy (Xia 1997; Fig. 1). Oocytes graded 1 and 2 were considered viable, whereas Grade 3 and 4 oocytes were not. Only Grade 1 oocytes were included in the present study. Oocytes were either subjected to vitrification before further assessment or were assessed without vitrification ('fresh' oocytes). To have a comparable distribution of oocytes derived from different patients among the eight different groups evaluated (fresh and seven CPA combinations), randomisation was performed throughout sample collection and use. For example, if we obtained eight Grade 1 oocytes from a given patient, one oocyte would go to the 'fresh' group and one would go to each of the CPA combination groups for vitrification. However, because this number was not always the case in reality, oocytes were allocated to the eight treatment groups in a continuous and sequential manner.

Vitrification and warming

Seven different combinations of cryoprotective solutions were tested during vitrification based on the Cryotop method of vitrification and warming (Kuwayama *et al.* 2005), with slight modification, using Cryotops (Kitazato BioPharma, Shizuoka, Japan). Modifications included the use of different CPAs (Sigma-Aldrich, St Louis, MO, USA) and human serum albumin (HSA; Sage Inc.). Details of the CPAs and the combinations

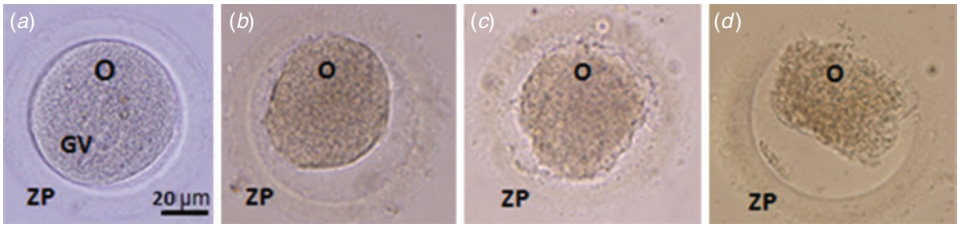

Fig. 1. Oocyte morphology and grading. This study was performed twice, before and after vitrification. Oocyte grading was as follows: (*a*) Grade 1, homogeneous cytoplasm, intact membrane; (*b*) Grade 2, homogeneous but contracted cytoplasm (large sublaminal space), intact membrane; (*c*) Grade 3, heterogeneous cytoplasm, degenerative, intact membrane; (*d*) Grade 4, lysed oocyte, broken membrane. GV, germinal vesicle; O, ooplasm; ZP, zona pellucida.

Table 1. Composition and concentrations of different combinations of cryoprotectant agents

Different concentrations of cryoprotectant agents (CPAs) in equilibration and vitrification solutions were prepared either individually or in combination in HEPES buffer. EG, ethylene glycol; PROH, propanediol; DMSO, dimethyl sulfoxide

Cryoprotectant combination	Equilibration solution	Vitrification solution
EG	15% EG	30% EG
PROH	15% PROH	30% PROH
DMSO	15% DMSO	30% DMSO
EG+PROH	7.5% e.g. + 7.5% PROH	15% e.g. + 15% PROH
EG+DMSO	7.5% e.g. + 7.5% DMSO	15% e.g. + 15% DMSO
PROH+DMSO	7.5% PROH + 7.5% DMSO	15% PROH + 15% DMSO
PROH+DMSO+EG	5% PROH + 5% DMSO +5% EG	10% PROH + 10% DMSO + 10% EG

used, together with the composition of the equilibration and vitrification solutions, are given in Table 1. Oocytes were warmed to 37°C for 50 s, then placed in diluent solution for 3 min and washed twice for 5 min. All solutions were supplemented with 20% HSA before use. Warmed oocytes were assessed for their morphological appearance and graded accordingly to determine the effect of vitrification. All these oocytes, regardless of their morphological grading, were then used for further investigation, as shown in Fig. 2, which describes the overall experimental design.

Labelling of viable mitochondria

Vitrified and fresh oocytes were labelled using MitoTracker Red CMX (Molecular Probes, Eugene, OR, USA) with a wavelength 594 nm. This fluorescent dye stains functioning mitochondria in live cells, regardless of membrane potential, and is important for evaluating the viability and distribution of mitochondria. Oocytes were incubated with 100 nM MitoTracker Red in phosphate-buffered saline (PBS) at 37°C, 5% CO_2, for 30 min (Stojkovic *et al.* 2001). Oocytes were then washed three times in the same buffer for 10 min before being processed for further studies.

Immunofluorescent labelling and microscopy

All oocytes, including those prelabelled with MitoTracker Red, were fixed for 30 min in 4% paraformaldehyde in PBS and permeabilised using 0.02% Triton X-100 in PBS for 40 min at room temperature. An indirect two-step immunofluorescent method was used, as described previously (Almahbobi *et al.* 1992). After incubation in 4 µg mL^{-1} mouse anti-cytochrome *c* monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C followed by a 10 min wash, oocytes were incubated in buffered 4 µg mL^{-1} goat anti-mouse IgG secondary antibody conjugated to Alexa 488 (Molecular Probes) for 1 h at room temperature. The same procedure was applied to other oocytes using 4 µg mL^{-1} rabbit anti-α-tubulin (Santa Cruz Biotechnology) and 2 µg mL^{-1} donkey anti-rabbit antibody conjugated to Alexa 594 (Molecular Probes). Negative controls for immunofluorescent labelling were performed using preimmune serum (1 : 10) instead of the primary antibodies. Oocytes were mounted on glass slides using anti-Fade aqueous mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Molecular Probes) to avoid photobleaching and to counterstain chromosomes.

For the distribution and quantification of labelled mitochondria and α-tubulin, oocytes were examined using an inverted semiconfocal microscope (200M Axiovert; Carl Zeiss, Sydney, NSW, Australia) equipped with a ×20 objective and 594 and 488 nm filters to detect both MitoTracker Red CMX and α-tubulin in addition to cytochrome *c*, respectively. The intensity of the fluorescent signal was quantified using computer-based software connected to the semiconfocal microscope (Carl Zeiss). To this end, the fluorescence emission from each oocyte was quantified in 50 × 1 µm equatorial serial sections through the centre of oocyte, where the DNA and polar bodies are located (Fig. 3).

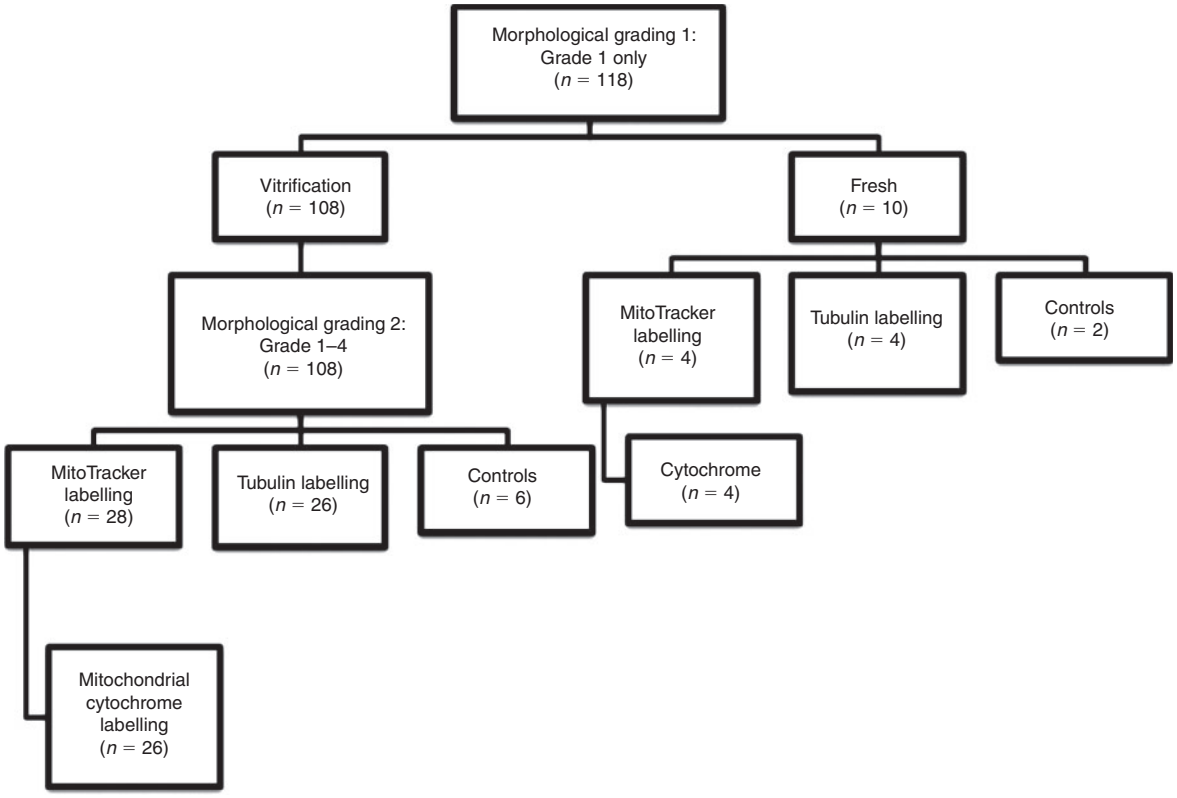

Fig. 2. Flow chart of the experimental design and the number of oocytes in each category of assessment. MitoTracker Red staining and cytochrome *c* oxidase labelling were performed on the same oocytes. *n*, number of oocytes.

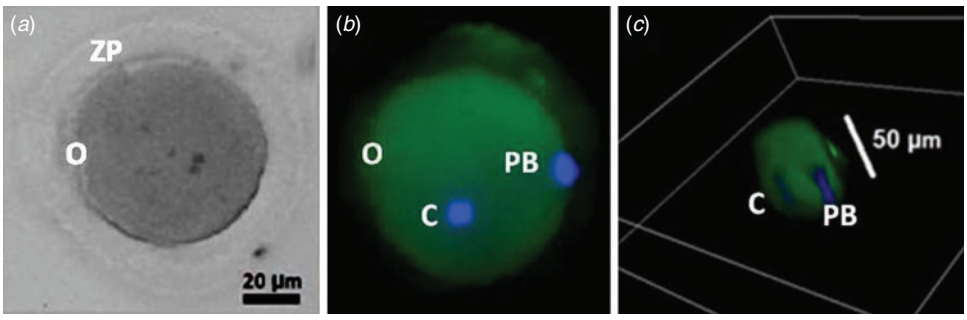

Fig. 3. Quantification of mitochondria and tubulin in whole-mount oocytes. (*a*) Phase contrast image of a mature oocyte. (*b*) Immunofluorescence labelling of cytochrome *c* oxidase of the same oocyte in (*a*). (*c*) A 50-µm fraction of the same oocyte in (*a*) and (*b*) selected for the quantification of cytochrome *c* oxidase. This fraction includes the chromatin (C) and polar body (PB). Note that the serial visual sections in (*c*) are slightly distant of each other, giving a stretchy appearance. O, ooplasm; ZP, zona pellucida.

Statistical analysis

Results were analysed using Prism version 5 (Graph Pad Software, La Jolla, CA, USA), one-way ANOVA and Student's *t*-tests, with statistical significances set at $P < 0.05$. One-way ANOVA was used to compare the amount of mitochondria and α-tubulin in oocytes vitrified using each of the CPA solutions with that in fresh oocytes. To analyse individual groups, *t*-tests were performed. To be considered viable, oocytes were graded according to cut-off values relative to quantified results for fresh oocytes: oocytes that exhibited 75%–100% similarity to results for fresh oocyte were considered viable and competent, those with 50%–74% similarity were considered viable but not competent and those exhibiting 0%–50% similarity were considered non-viable.

Results

Assessment of oocyte morphological appearance

Results of oocyte cryosurvival rates using different individual and combinations of CPAs are summarised in Fig. 4. The highest percentage of viable oocytes was recovered in EG+PROH and,

to a lesser degree, with DMSO, EG+DMSO and PROH+DMSO+EG compared with fresh oocytes. This was significantly higher ($P < 0.0001$) when compared with any of the most harmful CPAs (EG, PROH, and PROH+DMSO).

Assessment of mitochondrial integrity and distribution

In living vitrified oocytes, viable mitochondria were distributed evenly throughout the cytoplasm based on MitoTracker Red labelling (Fig. 5*a*), similar to fresh oocytes (data not shown). After fixation and permeabilisation of vitrified and fresh oocytes, mitochondrial membrane recovery was demonstrated in a similar pattern, based on cytochrome *c* oxidase immunolabelling (Fig. 5*b*). As a result, dual visualisation using superimposed images revealed perfect colocalisation (Fig. 5*c*). The same oocyte was photographed using normal phase contrast optics (Fig. 5*d*). Negative controls showed no detectable labelling (data not shown).

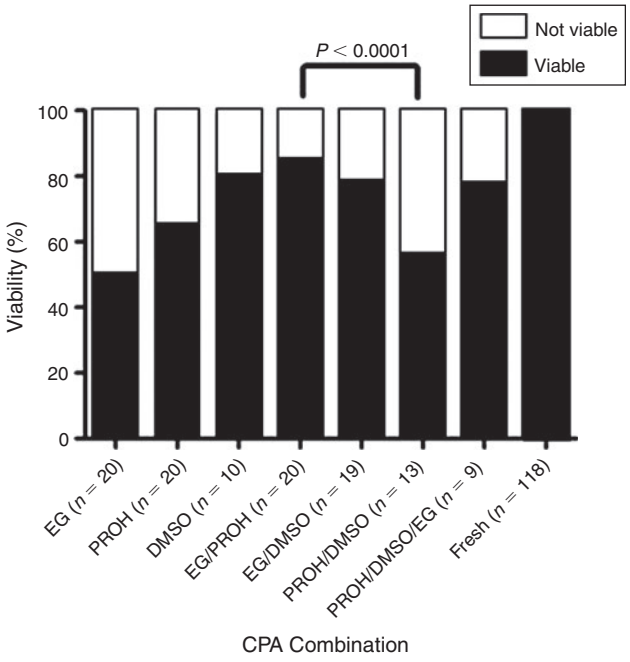

Fig. 4. Rate of cryosurvival in different cryoprotectant solutions. The percentage of viable (solid bars; Grade 1 and 2) and non-viable (open bars; Grade 3 and 4) oocytes was calculated per the total number of oocytes used in each individual group of cryoprotectant agents (CPAs). Significant ($P < 0.0001$) differences were found between the cryosurvival rates in different solutions. *n*, number of oocytes; EG, ethylene glycol; PROH, propanediol; DMSO, dimethyl sulfoxide.

Quantitative analysis of mitochondrial retrieval in living oocytes after vitrification and warming using MitoTracker Red showed considerable variations compared with fresh oocytes (Fig. 6*a*). Significantly higher ($P < 0.01$) amounts of viable mitochondria were recovered when using certain CPAs, such as EG+PROH, DMSO and EG+DMSO, with the least recovered following the use of PROH+DMSO+EG. Similarly, when the oocytes were fixed and permeabilised, the pattern of mitochondrial recovery, based on cytochrome *c* oxidase immunolabelling, was similar to that in viable oocytes, with a significantly higher ($P < 0.04$) recovery of total mitochondria observed following the use of EG+PROH and EG+DMSO compared with seen with the most detrimental CPAs, such as PROH+DMSO+EG (Fig. 6*b*).

Assessment of α-tubulin and distribution

After fixation and permeabilisation of oocytes, α-tubulin labelling showed homogeneous distribution throughout the ooplasm, regardless of oocyte maturity (Fig. 7*a*). The negative control for immunolabelling is shown in Fig. 7*b*, with the phase contrast image of the same oocyte shown in Fig. 7*c*.

Quantification of microtubules based on α-tubulin immunolabelling after vitrification varied, with significantly higher ($P < 0.008$) amounts of α-tubulin recovered following the use of EG+DMSO and EG+PROH as well as EG and PROH (Fig. 8). Interestingly, the amount of α-tubulin recovered in immature oocytes was significantly higher ($P < 0.0005$) than in mature oocytes (Fig. 8).

The overall results of the different assessments and grading of CPAs are given in Table 2. Of the entire series of seven individual and combinations of CPAs, EG+PROH and EG+DMSO showed the most successful outcomes in terms of morphological and functional parameters and the least detrimental effects. Conversely, the most detrimental effects were shown for EG alone and the combination of PROH+DMSO (Table 2).

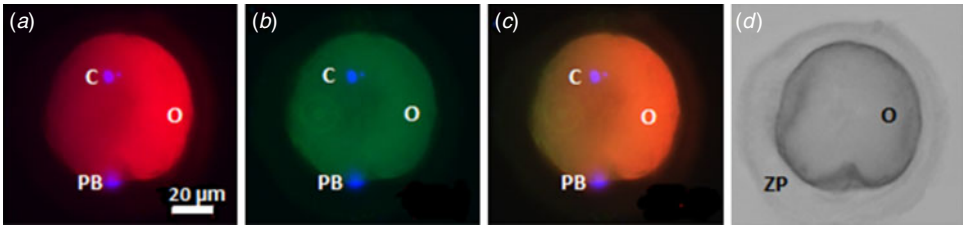

Fig. 5. Fluorescent and immunofluorescent labelling of mitochondria and their distribution. (*a*) Live mitochondrial labelling by MitoTracker Red, (*b*) mitochondrial cytochrome *c* oxidase immunofluorescent labelling, (*c*) double-labelling image of MitoTracker Red and cytochrome *c* oxidase demonstrating perfect colocalisation and (*d*) phase contrast image of the same mature oocyte shown in (*a–c*). C, chromosomes; O, ooplasm; PB, polar body; ZP, zona pellucida.

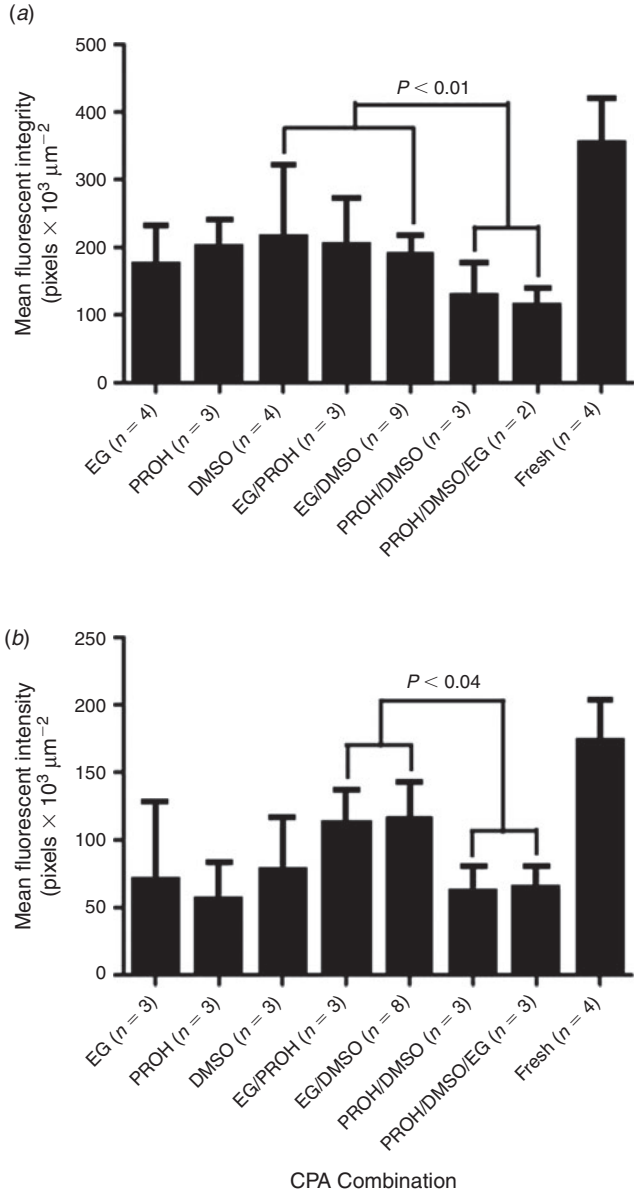

Fig. 6. Quantification of mitochondrial recovery after vitrification, compared with fresh oocytes in different solutions of cryoprotectant agents (CPAs). (*a*) Recovery of viable mitochondria based on MitoTracker Red labelling. (*b*) Recovery of total mitochondria based on cytochrome *c* oxidase immunolabelling. Mitochondrial recovery was calculated in a 50-µm central area of the entire oocyte vitrified by different CPA combinations. Data are the mean ± s.d. Significant differences were found, as indicated, using *t*-tests. *n*, number of oocytes; EG, ethylene glycol; PROH, propanediol; DMSO, dimethyl sulfoxide.

Discussion

The present study reports several interesting outcomes that are important for the development of an optimal method for human oocyte vitrification. First, we report for the first time that the use of a combination of CPAs at lower concentrations yields the most successful results for human oocyte vitrification compared with using a single CPA at a high concentration. The toxicity of DMSO on mouse oocytes is reduced by combining it with PROH or EG (Amorim *et al.* 2011; Ali and Shelton 2007). It should be noted that some single CPAs showed good outcomes comparable to those seen for CPA combinations, but only in certain individual assessments, such as morphology in the case of DMSO, mitochondrial viability in the case of PROH and tubulin recovery in the case of EG. However, no one single CPA was efficient in terms of the overall morphological and functional integrity of the oocytes. In contrast, the combination of PROH+DMSO showed the least satisfactory results, which requires further investigation. It is not clear why the use of some combinations of CPAs, in which the total concentration is equal to that of an individual CPA within that combination, results in better cryosurvival rates than CPAs used individually.

Oocytes were vitrified after an extended period of culture following oocyte retrieval, which would have detrimental effects on oocyte performance after freezing (Hunter *et al.* 1992) and embryo development (Parmegiani *et al.* 2008). In the present study, applying the Cryotop method to human oocytes resulted in an up to 85% survival rate, which is towards the higher end of the range of outcomes reported (68.6%–94%) using the same method of vitrification (Katayama *et al.* 2003; Yoon *et al.* 2003; Kuwayama *et al.* 2005; Lucena *et al.* 2006; Nagy *et al.* 2009; Smith *et al.* 2010; Cobo *et al.* 2011). Whether these oocytes exhibit some other adverse effects in terms of developmental competence is beyond the scope of the present study. In addition, the highest concentrations of CPAs used in the present study was 30%, which is within the currently used range of 20%–40% CPA (Cobo *et al.* 2011).

Assessments based on both gross morphology and quantitative analysis of subcellular structural integrity, such as that of the mitochondria and microtubules, are required to determine the quality of the oocyte. Although certain combinations of CPAs, such as EG+DMSO+PROH, resulted in the best morphology, they were the most detrimental in terms of total mitochondrial and tubulin retrieval after vitrification, indicating that the oocyte's morphological appearance alone is not an efficient measure of the quality of vitrified oocytes and can be misleading.

Fluorescent and immunofluorescent labelling of mitochondria in the present study revealed homogeneous staining throughout the ooplasm. This is probably due to large numbers of mitochondria in oocytes and whole-mount imaging at low magnifications. It has been reported that even immature oocytes contain approximately 100 000 mitochondria and each is metabolically active (Eichenlaub-Ritter *et al.* 2011). In contrast, an inadequate pattern of mitochondrial redistribution throughout the ooplasm is a marker of cytoplasmic immaturity and has been strongly linked to reduced quality and developmental potential in aged oocytes and embryos (Wang and Sun 2007; Eichenlaub-Ritter *et al.* 2011).

Most interestingly, the selection of the optimal CPA combination in the present study was based specifically on the highest rate of retrieval of functional mitochondria after vitrification. This is the first time mitochondrial distribution and retrieval rates have been used as parameters for assessing human oocyte quality after vitrification. Labelling with MitoTracker Red

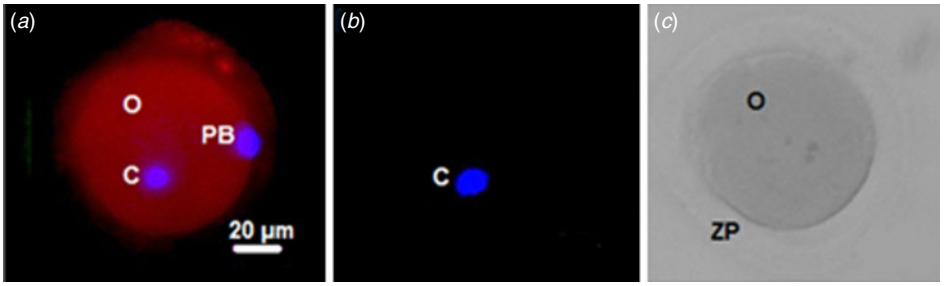

Fig. 7. Immunofluorescent labelling of tubulin and its distribution. (*a*) Immunofluorescent labelling of α-tubulin in a mature oocyte. (*b*) Negative control using preimmune serum in an immature oocyte. Note the absence of a 4′,6′-diamidino-2-phenylindole-labelled polar body. (*c*) Phase contrast image of the mature oocyte in (*a*). C, chromosomes; O, ooplasm; PB, polar body; ZP, zona pellucida.

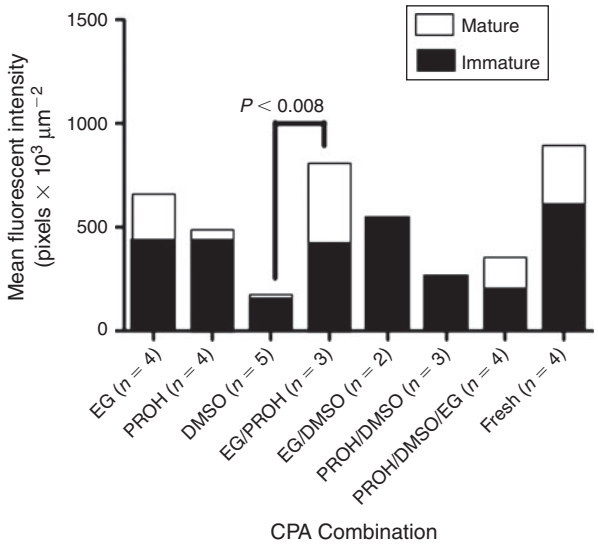

Fig. 8. Quantification of α-tubulin retrieval after vitrification compared with that in fresh oocytes. Tubulin recovery after vitrification was based on α-tubulin immunofluorescent labelling. Tubulin recovery was calculated in a 50-μm central area of the entire oocyte vitrified using different combinations of cryoprotectants. More tubulin was recovered in immature (solid bars) than in mature (open bars) oocytes. Data are the mean ± s.d. Significant differences were found, as indicated, using *t*-tests. *n*, number of oocytes; EG, ethylene glycol; PROH, propanediol; DMSO, dimethyl sulfoxide.

Table 2. Overall grading of the efficiency of cryoprotectant agents
The efficiency of cryoprotectant agents (CPAs) was evaluated on the basis of the quality of the oocytes after vitrification. Grading was estimated from data shown in Figs 3, 5, 7 compared with the quality of fresh oocytes. ✓✓✓, 90%–100%; ✓✓, 70%–90%; ✓, 50%–70%; –, <50% oocyte quality. EG, ethylene glycol; PROH, propanediol; DMSO, dimethyl sulfoxide

	Morphology	Viable mitochondria	Cytochrome *c* oxidase	α-Tubulin
EG	✓	✓	✓	✓✓
PROH	✓	✓✓	–	–
DMSO	✓✓	✓✓	✓	–
EG+PROH	✓✓	✓✓	✓✓✓	✓✓✓
EG+DMSO	✓✓	✓✓	✓✓✓	✓✓✓
PROH+DMSO	✓	✓	✓	–
PROH+DMSO+EG	✓✓	✓✓	✓	✓
Fresh	✓✓✓	✓✓✓	✓✓✓	✓✓✓

reveals the presence of functional mitochondria through oxidation by actively respiring cells, with the dye becoming sequestered in the mitochondria (Reyes *et al.* 2011). However, irrespective of the functionality of the mitochondria, cytochrome *c* oxidase immunolabelling reveals the total amount of mitochondrial membrane retained after vitrification, permeabilisation and washing. Therefore, the more damaged the mitochondria are due to vitrification and permeabilisation, the more fragmented membrane extraction occurs in washing, resulting in lower fluorescence intensity. Thereby, the present study allowed comparison of functioning mitochondria between fresh and vitrified oocytes, as well as comparison of total mitochondrial content between fresh and vitrified oocytes.

The present report does not show the presence of the meiotic spindle in mature oocytes, but instead shows depolymerised microtubules labelled with anti-α tubulin in both mature and immature oocytes. Previous studies in different species reported that oocyte spindles are extremely sensitive to low temperatures (Park *et al.* 1997; Wang *et al.* 2001; Keefe *et al.* 2003; Noyes *et al.* 2010; Chang *et al.* 2011) and failure to visualise the meiotic spindle in human *in vitro*-matured oocytes has been associated with low mitochondrial content (Reyes *et al.* 2011). More recently, it has been reported that the incidence of abnormal spindles is higher in vitrified blastocysts compared with fresh embryos (Chatzimeletiou *et al.* 2012). We believe that the changes in temperature and/or extended culture after oocyte retrieval may contribute to the process of microtubule depolymerisation. Nevertheless, the correlation between the presence or absence of the meiotic spindle and early embryo development has been controversial, with some studies reporting no observed correlation (Cohen *et al.* 2004).

In the present study, the levels of recovered tubulin were significantly higher in immature than mature oocytes and varied depending on the type of CPA used, justifying the validity of our results regardless of the present form of tubulin. Similar to the

case of mitochondria, the more depolymerised the microtubules are, due to vitrification and permeabilisation, the greater the extraction of depolymerised microtubules during washing.

From the above assessments, the overall outcome indicates that the use of EG+PROH or EG+DMSO at a concentration of 15% for each CPA will be the most optimal and least detrimental alternative method for human oocyte vitrification. This conclusion is based on a comprehensive study using a wide range of CPA combinations and assessments of both morphological and functional criteria. In conclusion, vitrification using a combination of CPAs is recommended, because this causes the least detrimental effects. In addition, analysis based on the morphological and functional integrity of the oocyte is required to determine which oocytes have the best quality and hence the highest developmental competence. The outcome of the present study has clinical significance because it optimises vitrification for IVF application and offers a guide to the safest and least toxic method of vitrification to increase and maintain oocyte viability for offspring. However, further investigation to assess functional integrity in a clinical setting using non-invasive methods may be necessary.

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