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Impact of vitrification on the mitochondrial activity and redox homeostasis of human oocyte

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STUDY QUESTION: Do the extreme conditions of vitrification affect mitochondrial health and reactive oxygen species (ROS) levels of human oocytes?

SUMMARY ANSWER: Vitrification of discarded human oocytes shifts the intracellular redox potential towards oxidation but does not alter the mitochondrial potential or intracellular ROS levels.

WHAT IS KNOWN ALREADY: Recent studies have reflected increased ROS levels in warmed young oocytes and have highlighted the temporal dynamic loss of mitochondrial potential that could, therefore, lead to a decrease in ATP production, impairing embryo development. Mitochondrial function can also be evaluated *in vivo* by the FAD/NAD(P)H autofluorescence ratio, which reflects the respiratory chain activity and is considered as a marker of the intracellular redox state.

STUDY DESIGN, SIZE, DURATION: A total of 629 discarded Metaphase II (MII) oocytes collected from June 2013 to April 2014 were included in this control (fresh oocytes, $n = 270$) versus treatment (vitrified oocytes, $n = 359$) study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Discarded MII oocytes were donated to research by young (<27 years old) and reproductively aged (>36 years old) women who underwent ovarian stimulation for IVF at a university-affiliated private fertility clinic. Redox state was assessed by measuring the FAD/NAD(P)H autofluorescence ratio, while ROS and mitochondrial activity were reported by *in vivo* labelling with carboxy-H2DCFDA and JC-1, respectively.

MAIN RESULTS AND THE ROLE OF CHANCE: Young and aged oocytes showed high and similar survival rates (81.8 versus 83.1%, not significant). Confocal microscopy revealed that the FAD/NAD(P)H ratio was significantly higher in vitrified oocytes than in fresh oocytes, suggesting a significant shift towards the oxidized state in oocytes after vitrification, regardless of the maternal age. Mitochondrial distribution was not affected by vitrification. Furthermore, it was not possible to resolve any difference in mitochondrial potential using JC-1 potentiometric dye or in reactive oxygen species (ROS) production (assessed with H2-DCFDA staining) between fresh and vitrified oocytes. Therefore, measurement of intracellular redox potential by autofluorescence imaging may be a more sensitive method to assess oxidative stress or mitochondrial demise in human oocytes because it showed a higher resolving power than JC-1 staining and displayed less variability than H2-DCFDA staining.

LIMITATIONS, REASONS FOR CAUTION: Owing to sample availability, MII discarded oocytes (*in vitro* matured oocytes and unfertilized oocytes 20 h after ICSI) were included in the study. These discarded oocytes do not necessarily reflect the physiological condition of the MII human oocyte.

WIDER IMPLICATIONS OF THE FINDINGS: Although vitrified oocytes yield comparable clinical outcomes compared with fresh oocytes, lower cleavage and blastocyst rates can be observed during *in vitro* culture. Data here obtained suggest that the redox state of human oocytes could be affected by vitrification. Therefore, the importance of adding protective antioxidant molecules to the vitrification solution and to the post-warming culture medium to improve embryo cleavage deserves some research.

STUDY FUNDING/COMPETING INTEREST(S): This research project was supported by the Valencian Government (Val+i+D program, M.N.-C.), INCLIVA Foundation for health research (G.S.-A.) and by the University of L'Aquila and Regione Abruzzo ('Reti per l'Alta Formazione' – P.O.F.S.E. Abruzzo 2007–2013 G.D.E.). No conflicts of interest were declared.

Key words: oocyte vitrification / mitochondrial activity / oxidative stress / redox metabolism / maternal age

Introduction

The ability to achieve a highly efficient method of oocyte cryopreservation has represented a great achievement for clinical practice. Nowadays, this valuable tool has become a routine laboratory practice in clinical assisted reproductive technologies and is no longer considered experimental by scientific societies (ESHRE Task Force on Ethics and Law *et al.*, 2012; Practice Committees of American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2013). Applications of oocyte preservation include fertility preservation for social (Stoop *et al.*, 2011) or medical reasons (Noyes *et al.*, 2011; Cobo *et al.*, 2013), oocyte banking for donation programmes (Nagy *et al.*, 2009a; Cobo *et al.*, 2010), oocyte accumulation in low-responder patients (Milan *et al.*, 2010; Cobo *et al.*, 2012), prevention of hyperstimulation syndrome (Herrero *et al.*, 2011b) and oocyte storage when sperm collection is not possible. In addition, it represents a valid ethical alternative to embryo cryopreservation (Nagy *et al.*, 2009b).

In the past few years, previous cryopreservation techniques such as slow freezing have shown both low clinical results and low reproducibility. In contrast, oocyte vitrification has shown better outcomes (Smith *et al.*, 2010; Cobo and Diaz, 2011, Herrero *et al.*, 2011a, Cil *et al.*, 2013). In recent years, it has allowed the development of cryopreservation programmes with comparable success rates to those achieved with fresh oocytes in terms of ongoing pregnancy, implantation, blastocyst formation, embryo cleavage and fertilization (Cobo *et al.*, 2008; Rienzi *et al.*, 2010; Garcia *et al.*, 2011; Parmegiani *et al.*, 2011; Trokoudes *et al.*, 2011; Goldman *et al.*, 2013). According to this evidence, it seems that vitrification does not impair the potential of vitrified oocytes to develop into competent embryos. However, at Day 3 of development, a reduced developmental potential showing lower cleavage and usable blastocyst rates after oocyte vitrification has also been reported, suggesting that cryopreservation procedures may compromise oocytes physiology (Forman *et al.*, 2012; Siano *et al.*, 2013).

A number of studies in human and other mammalian species have hypothesized that extreme conditions imposed by vitrification, such as osmotic stress and cryoprotectant toxicity could disturb several oocyte structures, such as the zona pellucida (Khalili *et al.*, 2012), actin filaments (Gook and Edgar, 2007; Bogliolo *et al.*, 2014), meiotic spindle (Coticchio *et al.*, 2009; Boiso *et al.*, 2002) and mitochondria (Jones *et al.*, 2004) as well as calcium homeostasis (Gualtieri *et al.*, 2011), and the induction and the release of cortical granules (Nottola *et al.*, 2009). Furthermore, both slow freezing and vitrification procedures have negatively affected the expression of genes involved in the regulation of DNA structure, cell cycle progression, mitochondrial homeostasis and energy production (Chamayou *et al.*, 2011; Monzo *et al.*, 2012).

Such changes could be a further challenge for women of advanced reproductive age due to their reduced oocyte quality (Wilding *et al.*, 2005) and lower ability to respond to stress (Tatone, 2008). Furthermore, a higher rates of meiotic aberrations (Wilding *et al.*, 2005) and aneuploidies with increasing maternal age have been well documented (Eichenlaub-Ritter, 2012; Kurahashi *et al.*, 2012). In addition, an altered mitochondrial activity, that could lead to an abnormal formation of the meiotic apparatus (Wilding *et al.*, 2003), and a reduced ability to counteract ROS (Eichenlaub-Ritter *et al.*, 2004) along with (Wilding *et al.*, 2005) a higher expression of apoptosis markers and a lower expression of molecules related to energy production and stress response (Steuerwald *et al.*, 2007) have also been related to aging.

Mitochondria are essential organelles involved in a variety of cellular activities (Duchen, 2000). Mitotracker Green and JC-1 staining has shown that vitrification induces an altered mitochondrial distribution and a decreased mitochondrial potential in mouse oocytes, respectively (Lei *et al.*, 2014b), while in failed fertilization (Chen *et al.*, 2012) or *in vitro* matured (Lei *et al.*, 2014a) human Metaphase II (MII) oocytes, vitrification causes a temporal reduction of the mitochondrial membrane potential, the driving force for mitochondrial ATP production in cells and a sensitive indicator of cell damage (Chen *et al.*, 2012). For warmed mammalian oocytes, this alteration of the normal mitochondrial function, in addition to the induced damage to the endogenous antioxidant systems, could be responsible of the increment in the levels of ROS observed in vitrified oocytes (Somfai *et al.*, 2007; Gupta *et al.*, 2010). In addition, it has been reported that cryopreservation significantly increases ROS production in young mouse oocytes but not in oocytes from reproductively older mouse (Tatone *et al.*, 2011) and decreases the abundance of transcripts coding for proteins involved in redox homeostasis, such as the thioredoxin reductase 1 in discarded MII human oocytes (Monzo *et al.*, 2012). In the present work, it was hypothesized that vitrification and maternal age could be causing intracellular oxidative stress, negatively impacting the mitochondrial health of human oocytes.

Materials and Methods

Human oocytes

This study was approved by the local ethics committee; all patients signed an informed consent form and all of the data were anonymous. From June 2013 to April 2014, a total of 629 discarded MII oocytes were included in this study: 308 oocytes from the group of 18- to 26-year-old women (young women; YW) and 321 oocytes from the group of 37- to 46-year-old women (reproductively aged women; AW), all of whom were undergoing ovarian stimulation for IVF at IVI Valencia. Oocytes were analysed separately depending on maternal age, to study the possible effect of aging.

Approximately half of the MII oocytes were *in vitro* matured oocytes: germinal vesicle oocytes that had undergone 22 h of *in vitro* maturation (IVM) in 25 ml of CCM, Vitrolife, 37°C 5% CO_2 (Escrich *et al.*, 2012), whereas the other half of the MII oocytes came from microinjected oocytes that had failed to exhibit signs of fertilization 20 h after retrieval. All patients had overall fertilization rates of >70% and severe male factor ART patients were excluded.

Oocytes were collected every day and assigned into the fresh or the vitrified oocytes groups.

Vitrification—warming protocol

Oocytes were vitrified and warmed following the standard method routinely used at IVI. Briefly, oocytes were gradually equilibrated in 300 μl of 7.5% (v/v) ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in TCM199 medium at room temperature for 12 min. Then, oocytes were transferred

into the vitrification solution (15% EG, 15% DMSO and 0.5 trehalose) for 1 min, loaded on the Cryotop strip and immediately plunged into sterile liquid nitrogen. Vitrified oocytes were kept in a liquid nitrogen tank between 8 and 10 days. For warming, the Cryotop was quickly immersed into the warming solution (1 M trehalose) for 1 min at 37°C. Oocytes were then transferred into 0.5 M trehalose in TCM199 medium at room temperature for 3 min. Finally, oocytes were washed for 6 min in TCM199 before being incubated in culture medium (Fertilization Medium, Cook Medical, Sydney, Australia) covered with oil at 37°C for 2 h before staining. All materials required for vitrification were obtained from Kitazato (Tokyo, Japan).

After warming, oocytes with rounded shape, homogenous cytoplasm and intact oolemma were classified as surviving oocytes.

Fluorescence labelling and confocal microscopy

Evaluation of mitochondrial distribution and activity

The mitochondrial membrane potential-sensitive fluorescent dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Molecular Probes, USA) was used for both determining the mitochondrial location and activity. JC-1 accumulates within the mitochondria and has a dual emission (Wilding *et al.*, 2001; Jones *et al.*, 2004; Eichenlaub-Ritter, 2012). Mitochondria with high membrane potential difference ($\Delta\Psi_m$) are considered active and functional and show red fluorescence. Mitochondria with low $\Delta\Psi_m$ are considered inactive or inoperative, and correspond to green fluorescence. The red/green ratio gives information about the global mitochondrial activity. This ratio was calculated by dividing pixel by pixel the red fluorescence by the green fluorescence.

A stock concentration of 3.35 mM of JC-1 in DMSO was prepared. Both fresh and vitrified oocytes were stained with 1.5 μM JC-1 diluted in culture medium (Fertilization Medium, Cook Medical, Sydney, Australia) for 30 min at 37°C (Jones *et al.*, 2004). Fluorescence was observed under a confocal microscope (Olympus FV1000). Oocytes were excited at 488 nm, and emission wavelengths were filtered through a 515–530 nm bandpass filter (green emission) or a 585 nm longpass filter (red emission). Laser conditions were kept constant for all the experiments.

The mitochondrial distribution pattern was determined by eye by three different operators (M.N.-C., G.S.-A. and G.D.E.) to avoid operator bias. The grading patterns were based on previous studies (Wilding *et al.*, 2001).

Intracellular oxidative stress estimation by measuring ROS levels

Intracellular ROS levels produced by individual oocytes were assessed by using the molecular probe 6-carboxy-2′, 7′-dichlorodihydrofluorescein (DCF) diacetate (6-carboxy-H2DCFDA, Molecular Probes), as previously described (Tatone *et al.*, 2011). This non-fluorescent molecule diffuses through the cell membrane. Once inside the cell, acetate groups are removed by intracellular esterases and oxidized by ROS to the highly fluorescent molecule DCF (Karlsson *et al.*, 2010). The brightness of this fluorescence reflects the intracellular extent of ROS but no information as to the kind of ROS generated in the cell can be inferred.

A stock solution of 10 mM H_2DCF-DA in DMSO was prepared. Both fresh and vitrified oocytes were incubated for 1 h at 37°C in culture medium (Fertilization medium, Cook) supplemented with 10 μM carboxy-H_2DCFDA. After washing with culture medium to remove the traces of dye, oocyte fluorescence was observed under a confocal microscope (Olympus FV1000, excitation wavelength 488 nm and emission 520 nm bandpass). Simultaneously, oocytes DNA was stained with Hoechst 33342 (Molecular Probes, 5 μg/ml).

Measuring the oocyte FAD and NADH autofluorescence as biomarkers of intracellular redox state

Intracellular redox state was measured by imaging NAD(P)H and FAD autofluorescence according to Dumollard *et al.* (2007b). Blue autofluorescence emitted by the pyridine nucleotides NADH and NADPH in their reduced form was excited with UV light (360 or 351 nm) on confocal laser scanning microscopy and emission was collected using a 435–485 nm bandpass filter. NAD(P)H autofluorescence is located both in the mitochondria and in the cytoplasm. The fluorescence of oxidized flavoproteins (FAD), that is located only inside the mitochondria, was excited at 488 nm and emitted fluorescence was collected through 505–550 bandpass filter. The FAD/NAD(P)H ratio, described as the 'redox ratio', was calculated by dividing pixel by pixel the fluorescence intensity of FAD by the fluorescence intensity of NAD(P)H. Since such endogenous fluorescence was rather weak, the pinhole was opened wide and the camera's gain was increased. To allow comparison between experiments, these settings were maintained in all the experiments.

To determine whether fluorescence was due to NAD(P)H and FAD emission, the mitochondrial uncoupler FCCP (1 μM, Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) as well as the inhibitor of the respiratory chain complex IV, CN− (1 mM, Carbonyl cyanide *m*-chlorophenylhydrazone, Calbiochem, UK), were added to the chamber as 10× solutions. The mitochondrial chain inhibitor CN− caused the reduction of NAD+ and FAD (see Results). In another pool of oocytes, the addition of 1 μM FCCP, an uncoupler that eliminates the proton gradient across the mitochondrial membranes, provoked the oxidation of NAD(P)H and $FADH_2$, and as a consequence the FAD/NAD(P)H ratio increased.

Image acquisition and analysis

A digital camera (Nikon) attached to the microscope acquired the images and mean grey values. As with previous studies (Wilding *et al.*, 2003), laser power settings were kept constant for all experiments. Oocytes were positioned with the polar body in the plane of focus. A single scan through the centre of the oocyte was used for the analysis. Oocytes were imaged in groups of three or four in each replicate, separately enough to avoid photo bleaching.

Fluorescence intensity was measured using ImageJ software (NIH). Background substraction was performed before analysing.

Statistical analysis

Quantitative data obtained from fresh and vitrified oocytes from YW and reproductively AW were compared.

First of all, an exploratory data analysis was performed using the Stem and Leaf plot. For the intracellular oxidative stress estimation, 10 values were eliminated as they were considered as outliers (see Results section). Secondly, a *t*-student was applied in order to evaluate whether the oocyte origin (IVM or fertilization failure) was a confounding effect. Finally, the data were analysed using a univariate linear regression (as the experiments had two independent variables: age and treatment, and just one dependent variable).

All data were plotted as mean ± standard error of the mean unless stated otherwise. All plots and statistical analysis were performed using Sigma Plot and Sigma Stat software packages (Statistics Package for Social Sciences, Erkrath, Germany). *P*-Values <0.05 were considered statistically significant.

Results

Oocytes source

Owing to sample availability, two oocyte sources (IVM or fertilization failure) were analysed in order to increase the sample size. Although there should be physiological differences between *in vivo* and *in vitro* matured oocytes, the statistical analysis (*t*-student) revealed that, for each set of experiments, there were no differences according to the oocyte's origin. Therefore, the oocytes origin was not a confounding variable.

Oocyte survival rate

After warming, degenerated or darkened oocytes or oocytes that had lost their original shape were evaluated as non-viable oocytes. A total of 359 MII oocytes underwent vitrification and warming; 82.5% (296/359) of them survived. Only surviving oocytes were stained. Oocytes from YW and reproductively AW presented high and comparable survival rates (over 80%) when observed at 2 h after warming, with no differences depending on maternal age (81.8 versus 83.1%, NS).

Evaluation of mitochondrial distribution and activity

A total of 146 oocytes were used in this set of experiments ($n_{YW\ fresh} = 36$; $n_{YW\ vitrified} = 40$; $n_{AW\ fresh} = 30$; $n_{AW\ vitrified} = 40$).

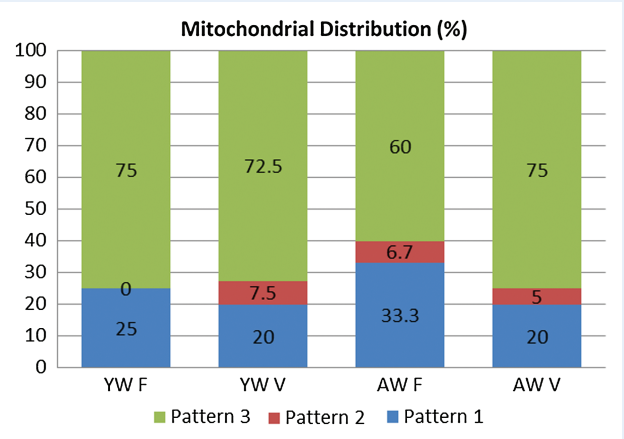

Figure 1 Mitochondrial distribution patterns in fresh (F) and vitrified (V) oocytes of young women (YW) and reproductively aged women (AW). All comparisons were not significant (*P*-value > 0.05). Pattern 1: granular and clumped aggregation; Pattern 2: smooth and homogenous distribution and Pattern 3: mixed pattern (Patterns 1 and 2 combined).

The mitochondrial distribution pattern was determined by observing intracellular localization of JC-1 green signal (Wilding *et al.*, 2001). Different oocytes showed different distribution patterns (Fig. 1): a granular and clumped aggregation (Pattern 1), a smooth and homogenous distribution (Pattern 2) or a combination of Patterns 1 and 2 (Pattern 3, Fig. 2). As shown in Fig. 1, the percentages of oocytes showing the different patterns were comparable in all the groups. Therefore, neither age nor vitrification affected oocyte mitochondrial distribution.

Interestingly, mitochondrial patterns based on JC-1 green staining were correlated with the oocyte morphological appearance under phase contrast microscope (Van Blerkom and Henry, 1992), i.e. oocytes displaying a granular pattern of mitochondria observed with JC-1 also showed a granular cytoplasm when observed in phase contrast (Fig. 2).

Although three different patterns were observed, the mixed distribution pattern (Pattern 3) was the most common pattern found in oocytes from all the groups, with the 'granular pattern' most commonly found under the plasma membrane, while the 'smooth pattern' was observed towards the centre of the oocyte (Fig. 2).

JC-1 was used to estimate the mitochondrial membrane potential ($\Delta\Psi_m$) by measuring the red/green fluorescence ratios. It was observed that fresh and warmed oocytes from both YW and reproductively AW showed similar JC-1 ratios (Fig. 3). Since JC-1 staining did not reflect any ratio changes, it can be assumed that vitrification did not cause an alteration of the mitochondrial activity, regardless of age and that age itself did not decrease the mitochondrial activity either.

Intracellular oxidative stress estimation by measuring ROS levels

DCF fluorescence intensity of each age group (YW and AW) was evaluated in fresh and vitrified oocytes (Fig. 4). Fluorescence data from a total of 10 oocytes (two oocytes from the YW fresh group, oocytes from the YW vitrified group, two oocytes from the AW fresh group and four oocytes from the AW vitrified group) were considered as outlier

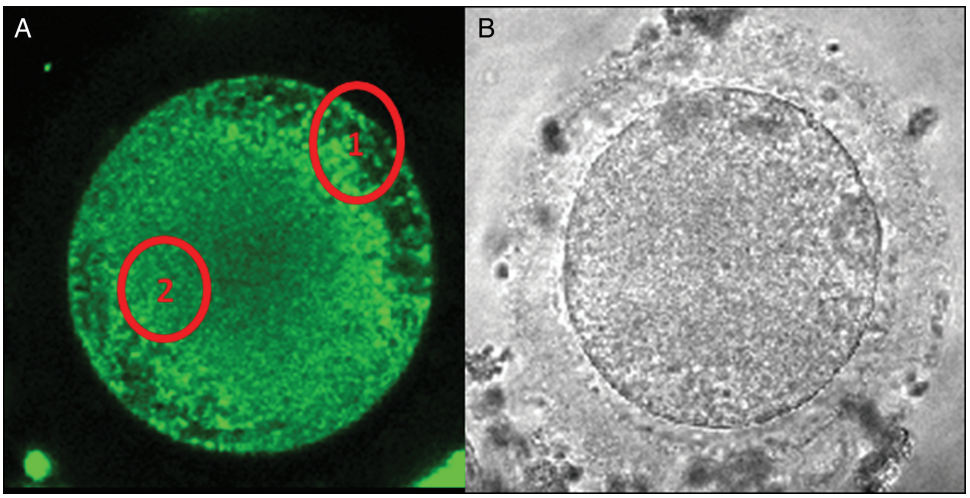

Figure 2 The mitochondrial distribution pattern was related with the oocyte appearance under the microscope. Fluorescence (**A**) and optical (**B**) images of an oocyte with mitochondrial a mixed distribution pattern; the 'granular pattern' (1) most commonly found under the plasma membrane, while the 'smooth pattern' (2) was observed towards the centre of the oocyte.

values after the exploratory analysis of the data and were not considered for the analysis. Therefore, 220 oocytes were employed in this set of experiments ($n_{YW\ fresh} = 54$; $n_{YW\ vitrified} = 56$; $n_{AW\ fresh} = 54$; $n_{AW\ vitrified} = 56$).

Theoretically, low DCF fluorescence should reflect a low level of intracellular ROS species, whereas higher DCF fluorescence intensity should indicate higher levels of ROS (Fig. 4). In the present study, it was observed that fresh oocytes from both YW and reproductively AW showed similar pixel intensity (around 300 A.U.). DCF fluorescence seemed to be increased by vitrification in oocytes from both YW and AW but no statistical difference was found (P-value >0.05) because of the high variability in DCF fluorescence among vitrified oocytes (Fig. 5). Therefore, H2DCF staining could not determine an increase in oxidative stress induced by vitrification.

Co-enzymes NADH and FAD as biomarkers for mitochondrial function

FAD/NADH fluorescence intensity ratio, described as 'redox ratio' (Dumollard *et al.*, 2007b), was used to determine relative changes in the cell oxidation–reduction state, which reflect changes in the metabolic state of the cell. In this case, 190 oocytes were analysed ($n_{YW\ fresh} = 40$; $n_{YW\ vitrified} = 46$; $n_{AW\ fresh} = 52$; $n_{AW\ vitrified} = 52$).

Figure 6 shows that fresh oocytes from both YW and reproductively AW had a similar redox state (P-value >0.05), indicating that age did not alter the intracellular redox state. On the contrary, a higher ratio FAD/NADH was observed in vitrified oocytes (P-value <0.005) from both groups, indicating that vitrification caused an oxidation of the intracellular redox potential, regardless of age.

When mitochondrial chain inhibitor CN− was added to a pool of oocytes, it caused the reduction of NAD+ and FAD and decreased the FAD/NAD(P)H ratio to 0.389 ± 0.025. In another pool of oocytes, the addition of 1 μM FCCP, an uncoupler that eliminates the proton gradient across the mitochondrial membranes, provoked the oxidation of NAD(P)H and $FADH_2$, and as a consequence the FAD/NAD(P)H ratio increased to 7.113 ± 0.918.

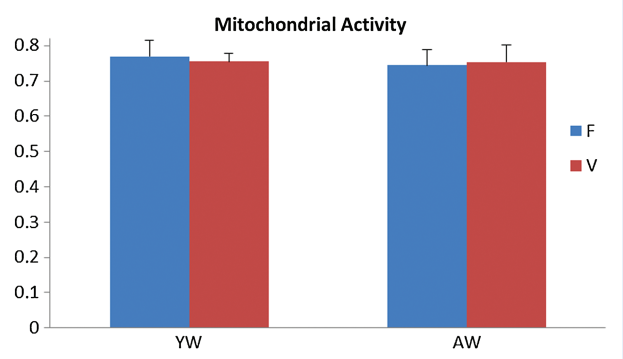

Figure 3 Histogram showing the JC-1 red/green fluorescence ratio, expressed as mean pixel intensity from fresh (F) and vitrified (V) oocytes of YW and reproductively AW. Sample size and pixel intensity ± SD: **YW F** $n = 36$, 0.769 ± 0.047; **YW V** $n = 40$, 0.755 ± 0.044; **AW F** $n = 30$, 0.744 ± 0.023; **AW V** $n = 40$, 0.754 ± 0.049.

Discussion

The present work shows that vitrification oxidizes the intracellular redox potential measured by autofluorescence imaging without affecting the mitochondrial electrical potential. This oxidation of the intracellular redox potential could not be characterized by H2DCF staining due to the variability of the staining obtained with this ROS-sensitive dye. Interestingly, oxidation of the intracellular redox potential by vitrification was independent of age.

Recent advances in cryobiology have made it possible to optimize vitrification protocols. This highly efficient technique has notably improved the efficacy of oocyte cryopreservation, in terms of oocyte survival and IVF outcomes (Cobo and Diaz, 2011). However, whether vitrification affects oocyte physiology remains to be determined. It is well known that mitochondria play a pivotal role in oocyte competence: they are essential for the production of the energy required to perform all of the cellular processes coordinated, such as programmed cell death or spindle formation (Dumollard *et al.*, 2007a). ATP is produced by oxidation of metabolites in the cytosol (glycolysis, proteolysis and lipolysis) or in the mitochondria via oxidative phosphorylation. In the mitochondria, energy is stored as an electrical membrane potential ($\Delta\Psi_m$) established by the operation of the respiratory chain enzymes which drives the

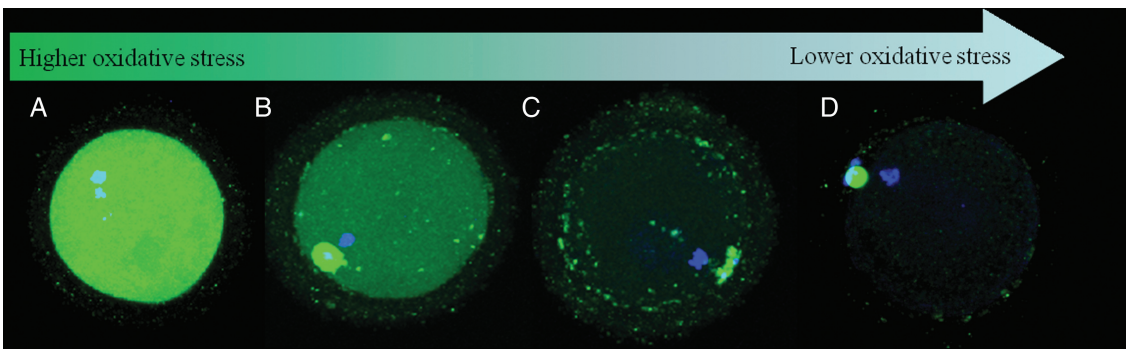

Figure 4 H2DCF stained oocytes (600×). Green fluorescence is due to DCF-induced fluorescence. Differences in DCF fluorescence intensity reflect different ROS levels. Simultaneously, oocyte DNA was stained with Hoechst 33342 (Molecular Probes, blue signal, 5 μg/ml).

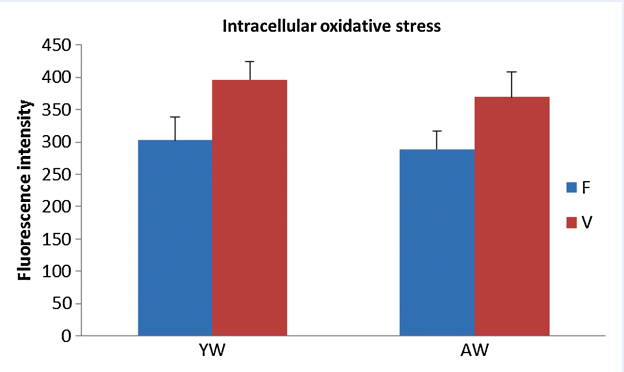

Figure 5 Histogram showing DCF-induced fluorescence from fresh (F) and vitrified (V) oocytes of YW and reproductively AW. Sample size and pixel intensity ± SD: **YW F** n= 54, 302.85 ± 36.18; **YW V** n = 56, 396.14 ± 28.32; **AW F** n = 54, 288.79 ± 27.48; **AW V** n = 56, 369.39 ± 38.92.

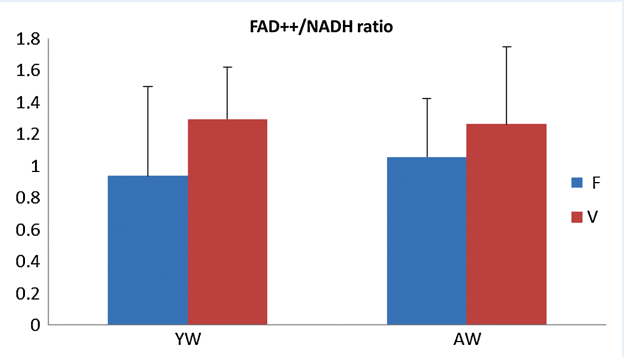

Figure 6 Histogram showing FAD/NADH autofluorescence ratio from fresh (F) and vitrified (V) oocytes of YW and reproductively AW. Sample size and pixel intensity ± SD: **YW F** n = 40, 0.938 ± 0.563; **YW V** n = 46, 1.295 ± 0.326; **AW F** n = 52, 1.057 ± 0.366; **AW V** n = 52, 1.263 ± 0.488.

conversion of ADP to ATP (Chen *et al.*, 2012). Mitochondrial dysfunction in oocytes has been correlated with embryo arrest *in vitro* (Thouas *et al.*, 2004). A number of studies have assessed the $\Delta\Psi_m$ using JC-1 (Szabadkai and Duchen, 2008). It has been found that slow-frozen discarded human MII oocytes have a loss of high potential (Jones *et al.*, 2004). Vitrification has also been reported to induce an abnormal mitochondrial distribution and a decreased mitochondrial activity in mouse oocytes (Lei *et al.*, 2014b). In bovine oocytes, ATP content decreased due to vitrification (Zhao *et al.*, 2011). However, in human MII oocytes that failed to fertilize only a temporary reduction of mitochondrial high potential was shown in vitrified/warmed oocytes, followed by a spontaneous recovery to levels seen in fresh oocytes after 4 h-culture (Chen *et al.*, 2012). Similarly, ATP levels in vitrified oocytes after a 3 h-culture were comparable to those measured in human fresh oocytes 3 h after incubation in culture conditions (Manipalviratn *et al.*, 2011). Nevertheless, it has been suggested that for both and within cohorts of oocytes, ATP content can vary significantly (Van Blerkom *et al.*, 1995). The findings obtained here argue in favour of a less harmful effect of vitrification on mitochondrial membrane potential and mitochondrial distribution 2 h after warming. This is consistent with our clinical results (Cobo and Diaz, 2011), fertilization rates and previous reports that suggest that the vitrification protocol here employed does not disturb the development of embryos generated from warmed oocytes (Cobo *et al.*, 2010). Another possible explanation is that JC-1 staining has poor resolving power, being unable to show small differences in the mitochondrial potential.

In addition, these results on the mitochondrial activity and their distribution in fresh (discarded) oocytes showed no relation with maternal age, although this could be due to the nature of the oocyte. In contrast, another study showed a lower mitochondrial membrane potential as maternal age increased (Wilding *et al.*, 2001, 2003), although this has been shown to be unrelated to early apoptotic events (Gualtieri *et al.*, 2009). In fact, a decrease in oxidative phosphorylation efficiency is among the current theories for explaining the reduced reproductive efficiency with maternal aging (Wilding *et al.*, 2005; Eichenlaub-Ritter, 2012). Although mitochondrial potential does not seem to be affected by the vitrification/warming procedures employed here, mitochondrial metabolism is affected in vitrified oocytes as shown in the present work by the clear oxidation of FAD after vitrification. Such oxidation of mitochondrial redox potential could be reflected in lower cytosolic ATP levels.

The data presented herein show that the mitochondrial distribution pattern correspond closely to the oocyte appearance under the microscope (Van Blerkom and Henry, 1992). A mixed distribution was the most common pattern found in oocytes in all the groups, with a granular pattern most commonly found under the plasma membrane and a smooth distribution observed towards the centre of the oocyte. Interestingly, the mitochondrial aggregation pattern observed is not related with the mitochondrial activity, as it can be observed that both granular and smooth distribution exhibited areas of high and low mitochondrial activity. Both oocytes from reproductively aged and YW showed similar mitochondrial distribution patterns, and the osmotic dehydration and cell shrink caused by the cryoprotectors did not affect the mitochondrial distribution after oocytes warming. Although a previous work observed alterations in mouse oocytes vitrification (Lei *et al.*, 2014b), this could be species specific (Li *et al.*, 2006; Yin *et al.*, 2006).

Mitochondria are a source of ROS and free radicals in mammalian oocytes, even under physiological conditions (Takahashi *et al.*, 2013). Despite the fact that ROS are essential participants in a wide range of cellular processes (Bergamini *et al.*, 2004), the inability of the antioxidant defense to quench an increased production of ROS creates a biochemical imbalance known as oxidative stress. Such oxidative stress is capable of disrupting many cellular components and activities, possibly resulting in developmental blockage (Velez-Pardo *et al.*, 2007; Agarwal *et al.*, 2012) and cell death (Karlsson *et al.*, 2010).

A previous study showed increased ROS levels in fresh mouse oocytes from reproductively aged females compared with oocytes from young mice, most probably due to their diminished ability to challenge stress conditions (Tatone *et al.*, 2011). Similar findings were observed by others in porcine studies (Gupta *et al.*, 2010) and lower antioxidant systems were found in bovine oocytes (Zhao *et al.*, 2011). In contrast, similar ROS levels in oocytes from YW and AW were observed both in fresh and vitrified oocytes. In this study, interestingly, although no statistical differences were observed in vitrified oocytes, a trend towards increased oxidation can be suggested from the data. As the data show, the DCF signal is very variable and such variability could be masking

differences between the samples. The high variability in the DCF measurements could arise from the H2DCF staining protocol used for 1 h. As H2DCF can be photo-oxidized, any small change in the environment could have brought about such artefactual oxidation of DCF (thereby generating variability). Therefore, the DCF data could suggest that there might be difference in ROS levels but the variability would mask it.

Here, a different approach was tried to assess alteration in mitochondrial metabolism as neither JC-1 nor H2DCF staining revealed any deleterious impact of vitrification on oocytes. In mice, vitrification has been associated with a decrease in nutrient consumption when compared with non-cryopreserved oocytes (Lane and Gardner, 2001). Such impaired nutrient consumption should be reflected in the intracellular redox potential even if it is not large enough to impact on $\Delta\Psi_m$ or oxidative stress. The intracellular redox potential can be assessed by estimating the amount of redox couples participating in oxidation and reduction reactions such as NADH/NAD+, NADPH/NADP+ or FADH2/FAD (in the mitochondria). The endogenous autofluorescence of NAD(P)H and FAD was, therefore, imaged to estimate the intracellular redox potential (Dumollard *et al.*, 2007b; Heikal, 2010; Buschke *et al.*, 2012). Although traditional biochemical techniques, such as enzymatic methods, cycling assays or chromatography analyses, have been used to estimate cellular levels of NADH and FAD, taking advantage of their autofluorescence does not require the destruction of the oocytes (Heikal, 2010).

The data showed that fresh oocytes from YW and reproductively AW have a similar intracellular redox state, suggesting a similar cytosolic or mitochondrial oxidative metabolism. In addition, the data suggest that the FAD/NADH ratio is significantly increased after vitrification. Surprisingly, this alteration was independent of maternal age. As NAD(P)H and FADH2 participate ubiquitously in numerous redox reactions, it is not straight forward to infer which metabolic pathway is affected. Nevertheless, an increased oxidation of FADH2 shows that the mitochondrial redox potential is more oxidized after vitrification. Higher oxidation in the mitochondria could be due to either uncoupling of mitochondria (which could be correlated with higher oxygen consumption in future experiments) or to a shortage of Kreb's cycle intermediates (leading to decreased ATP levels and decreased oxygen consumption) or a more deleterious and general oxidation of the mitochondria. However, the latter is unlikely because it was not possible to measure a lower mitochondrial potential in vitrified oocytes. There is also evidence that oxidative stress will result in an oxidation of NADH, therefore decreasing intracellular NADH levels, prior to the onset of apoptosis (Liang *et al.*, 2007; Heikal, 2010). In addition changes in the FAD/NADH ratio would precede an increase in ROS production and cellular oxidative stress (Yang *et al.*, 2008). Decreased cytosolic NADH levels reflect increased oxidation in the cytosol that could be due to diminished GSH, thereby decreasing the antioxidant defense and resulting in higher ROS. Further work is necessary to ascertain whether increased ROS production can be observed after vitrification/warming using this protocol.

Most probably, the additive effects of reduced antioxidant defense mechanisms (Monzo *et al.*, 2012), elevated ROS and higher oxidative stress (although no significant) has triggered significant changes in the redox balance. For this reason, future works could be focused on determining the possible benefits of antioxidants supplementation to vitrification media to recover the redox balance and minimize oocyte damage. It is interesting that oocytes from both YW and reproductively AW had a significantly altered redox balance, suggesting that age was not affecting the oocyte susceptibility to vitrification.

Since this study was performed on *in vitro* matured and unfertilized oocytes, future work is needed to determine whether these effects occur on fully competent MII oocytes. Results based on our large experience with oocyte vitrification show that, although there are some cases of a small penalty in terms of oocyte survival and embryo quality, the on-going pregnancy rate is comparable with that obtained in fresh cycles (Cobo *et al.*, 2008; Cobo and Diaz, 2011). The alteration of the redox potential (measured by the FAD/NAD(P)H ratio) could be indicative of the higher oxidative stage in oocytes after vitrification procedures. That could eventually impair blastocyst development. New assays to test the effect of antioxidant supplementation during vitrification procedures are currently under investigation.

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Authors' roles

M.J.D.S.M., C.T., M.N.-C. and R.D. developed the study design. M.N.-C., G.S.A. and G.D.E. collected and analysed the samples and performed the data analysis. All the authors participated in the interpretation of data and manuscript drafting, edition and critical discussion.

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Conflict of interest

None declared.

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