—Original Article—

Ultrastructural characteristics of human oocytes vitrified before and after *in vitro* maturation

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Abstract. The development of an effective program that combines *in vitro* maturation (IVM) and cryopreservation for immature oocytes would represent a novel advance for *in vitro* fertilization (IVF), especially as a means to preserve the fertility of women in unique situations. The aim of this study was to analyze the ultrastructural characteristics of human oocytes, obtained after controlled ovarian stimulation, to determine whether IVM is best performed before or after vitrification. To this end, we analyzed the following features in a total of 22 MII oocytes: size, zona pellucida and perivitelline space, mitochondria number, M-SER (mitochondria-smooth endoplasmic reticulum) aggregates and M-V (mitochondria-vesicle) complexes, the number of cortical granules and microvilli, and the presence of vacuolization using transmission electron microscopy (TEM). Each oocyte presented a rounded shape, with an intact oolemma, and was surrounded by a continuous zona pellucida and perivitelline space. Statistical analysis comparing oocytes vitrified before or after IVM indicated that there were no significant differences between examined characteristics.
Key words: Cryopreservation, Electron microscopy, *In vitro* maturation

Approximately 80% of oocytes obtained after ovarian cycle stimulation (OS) are in metaphase II (MII). The remaining oocytes, in metaphase I (MI) and prophase I (PI), are usually discarded due to their low capacity for embryonic development [1]. Moreover, there is evidence for an increase in the incidence of aneuploidy in embryos obtained through OS and *in vitro* maturation (IVM) [2], with a clear decrease in successful embryo implantation [3]. If a small number of MII oocytes are obtained, IVM with oocytes in MI or PI can help increase the number of fertilized oocytes and hence the number of embryos that can be transferred to the patient [4]. Additionally, hormonal cycles are unnecessary in order to obtain the immature oocytes, avoiding ovarian hyperstimulation syndrome (OHS) and allowing women with a low response to gonadotropin stimulation to increase their fertility [5]. Moreover, women undergoing oncological treatment can also benefit, as it allows treatment to begin immediately [6]. Consequently, it is important to develop and optimize a protocol that allows PI oocytes to be successfully vitrified and matured via IVM, greatly improving the opportunity to preserve fertility [7]. Therefore, it is crucial to identify any similarities or differences in oocytes matured *in vitro* to establish objective criteria for assessing

oocyte quality and promote research aimed at improving maturation techniques. In this regard, Coticchio *et al*. 2016 [8] have established that most, but not all, oocyte ultrastructural features can develop normally *in vitro*.

On the other hand, mature MII oocytes can be difficult to cryo-preserve using current techniques [9] due to certain specific features, such as a relatively large volume. This leads to a low surface-to-volume ratio, high water content, a high degree of cytoplasmic specialization (including cytoskeletal characteristics), and precise chromosomal arrangement [10]. In fact, ultrastructural damage is one of the main adverse effects associated with cryopreservation due to the toxic effects of cryoprotectants, the formation of ice crystals, and osmotic stress [11]. The meiotic spindle is especially sensitive to the cryopreservation process. Cryopreservation of immature PI oocytes could avoid some of these issues, especially those related to spindle and chromosome cryodamage, as they are protected by the nuclear membrane. However, PI oocytes still need to be matured *in vitro* [12] but current protocols for oocyte cryopreservation and maturation are suboptimal and clinical success has only been obtained in a limited number of cases [13]. It is therefore essential to define some objective criteria to establish how oocyte quality may be affected by cryopreservation, with a view to supporting or ruling out the applicability of different protocols and assessing the possible health risks for children born from cryopreserved oocytes [14].

During oocyte maturation, we can distinguish between two distinct processes, nuclear maturation and cytoplasmic maturation. In the nuclear maturation phase, meiotic processes restart, going from PI to MII. Cytoplasmic maturation includes changes to the ooplasm that are

necessary for the oocyte's future development [15]. Synchronization of these two processes ensures normal fertilization and successful embryonic development [16]. Transmission electron microscopy (TEM) is a valuable research tool that can be used to determine an oocyte's cytoplasmic maturation status. Within oocytes, the most abundant organelles are mitochondria. These often associate with the membrane of the smooth endoplasmic reticulum (SER) or small vesicles and these associations play an important role in the production of useful substances during fertilization and in membrane neoformation during early embryogenesis [17]. They may also act to regulate free calcium levels and ATP production, and have a role in several cellular activities at fertilization, including calcium signal mediation [18]. Mitochondria-smooth endoplasmic reticulum (M-SER) aggregates are very abundant in MII oocytes and are considered a marker for normal cytoplasmic maturation. In contrast, high numbers of mitochondria-vesicle (M-V) complexes are an indicator of cellular aging due to exceeding the IVM time. This can adversely affect fertilization and the early stages of embryonic development [19]. It is also necessary to analyze the arrangement of cortical granules and the degree of vacuolization [18].

Assessing oocyte quality is based on many morphometric criteria. In order to evaluate both structural and ultrastructural oocyte characteristics, several parameters should be assessed using both light microscopy (LM) and TEM [20]. These include oocyte shape and dimension, zona pellucida (ZP) texture, perivitelline space (PVS) appearance, oolemma integrity and density, mitochondria, M-SER aggregate and M-V complex number, quantity of cortical granules (CG) and their arrangement [10], and the presence of ooplasmic vacuolization [21].

The aim of the present study was to evaluate ultrastructure oocyte characteristics in order to determine whether IVM should be performed before or after vitrification, as little is known about the efficiency or consequences of cryopreservation in immature and *in vitro* matured oocytes.

Materials and Methods

This study was reviewed and accepted by the Ethical and Scientific Committee of the La Fe Hospital, Valencia, Spain. Signed informed consent was obtained from all participants.

Oocyte collection

Controlled ovarian stimulation of patients was performed with a short antagonist protocol using (150–300 IU/day) rec-FSH (Gonal F 1050; Merck and Co, Madrid, Spain) and GnRH (Orgalutran®; MSD and Co, Hoddesdon, UK) for pituitary suppression. Triggering was performed through the administration of 250 mcg of rec-hCG (Ovitrelle, Merck, London, UK) when there were at least three follicles > 16 mm present. Oocyte retrieval was performed via vaginal puncture guided by ultrasound 36 h later. Cumulus-oocyte complexes were removed using hyaluronidase (SynVitro® Hyadase; Origio®, Màlov, Denmark) solution for a maximum of 30 sec with a denuding pipette (Flexipet® Denuding Pipette, Cook® Medical, Bloomington, IN, USA). In total, 22 MII immature oocytes were identified through the presence of a germinal vesicle (PI stage) and included in this study. Of these, 10 were vitrified before IVM (group

1) and 12 were vitrified after IVM (group 2).

In vitro maturation

Healthy oocytes were placed in an IVM medium consisting of blastocyst medium (CCM™, Vitrolife®, Göteborg, Sweden) supplemented with human menopausal gonadotropin (hMG, Menopur® 75 U.I., Ferring®, Madrid, Spain) and serum substitute (SSS, IrvineScientific®, Santa Ana, CA, USA) under paraffin oil at 37ºC in a 6% CO_2 humidified atmosphere. After 24 and 48 h of culture, mature oocytes were identified by the presence of the first polar body using an inverted microscope (Olympus, IX70, Tokyo, Japan). The general distribution of cytoplasmic organelles during oocyte maturation is shown in Fig. 1.

Oocyte vitrification

Oocytes (PI and MII) were vitrified in Kitazato® (KITAZATO Vitrification/Thawing media, Biopharma, Shizuoka, Japan) medium using the Cryotop® system (KITAZATO Vitrification/Thawing media), according to a modified drop protocol proposed by Wang *et al.* 2013 [22]. Oocytes were equilibrated in a 20 µl drop of basic solution (BS) for 1 min before the drop was merged with a 20 µl drop of equilibration solution (ES) for 3 min. Next, a second 20 µl drop of ES was merged for a further 3 min. The oocytes were then placed in a new 20 µl drop of ES for 6 min. After equilibration, the oocytes were transferred to four drops of vitrification solution (VS) and loaded into the Cryotop® to be stored in liquid nitrogen. The total time for the final process was 60 sec. All procedures were performed at room temperature (22–25ºC).

Oocyte warming was performed based on procedures specific to the Kitazato® Kit. Each Cryotop® was removed from liquid nitrogen and quickly submerged in 1 ml of thawing solution (TS) at 37ºC for 1 min. The oocytes were then transferred to 300 µl of dilution solution (DS) for 3 min and then transferred to 300 µl of washing solution (WS). After 5 min, the oocytes were washed in new WS medium and placed in IVM medium. These procedures were performed at room temperature (22–25ºC).

The survival rate after thawing was evaluated microscopically 2 to 3 h after culture and was based on observations of the morphology and integrity of the oocyte membrane.

Electron microscopy

The oocytes were fixed and processed for TEM analysis using methods previously described by Nottola *et al.*, 2007 [23]. Oocyte fixation was performed using 2% glutaraldehyde (SIC, Rome, Italy) in phosphate buffered saline (PBS). After fixation for at least 2 days at 4ºC, samples were rinsed in PBS, postfixed with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS and rinsed again in PBS. The oocytes were then embedded in small blocks of 2% agar (Sigma-Aldrich, St. Louis, MO, USA) approximately 5 × 5 × 1 mm in size. These were then dehydrated in an ascending series of ethanol concentrations, immersed in propylene oxide (Electron Microscopy Sciences, Hatfield, PA, USA) for solvent substitution, and finally embedded in epoxy resin EPON-812 (Electron Microscopy Sciences, Hatfield, PA, USA). Semithin sections of 1.0 µm thickness were cut serially with a glass knife on a Leica LKB-III ultramicrotome and then mounted on gelatinized slides, stained
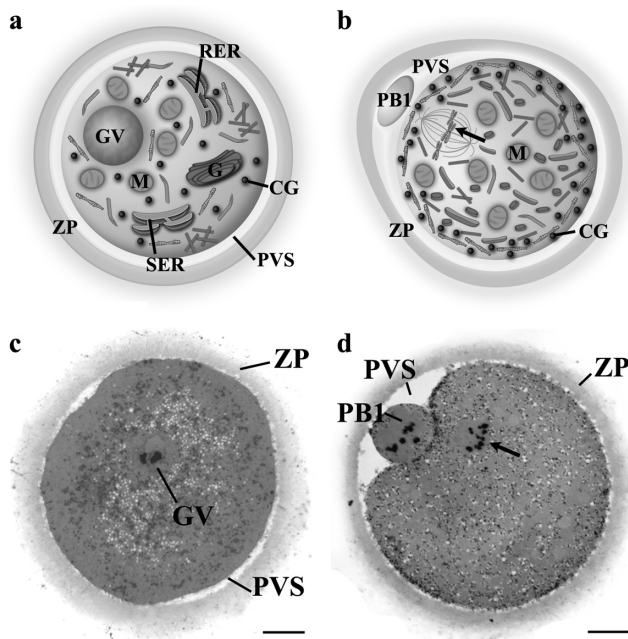
Fig. 1. General distribution of cytoplasmic organelles during human oocyte maturation. Diagram of PI oocytes (a) Diagram of metaphase-II oocytes, (b) Micrographs of PI, (c) and metaphase-II (d) shown by LM. GV = germinal vesicle, ZP = zona pellucida; PVS = perivitelline space, PB1 = 1ˢᵗ polar body; M = mitochondria; G = Golgi apparatus; CG = cortical granules; RER = rough endoplasmic reticulum; SER = smooth endoplasmic reticulum. Note the metaphase II oocyte with chromosomes at the equator (arrow), cortical granules disposed beneath the oolemma, mitochondria uniformly distributed throughout the cytoplasm, fragmented Golgi apparatus, and reorganization of the cytoskeleton (modified from *Mao et al.,* 2014 [32]). Bar: 20 μm (c, d).

Fig. 2. Light and electron micrographs of human oocytes vitrified before (group 1) and after (group 2) *in vitro* maturation. Group 1 (a, b) and group 2 (c, d). Continuous zona pellucida (ZP) and perivitelline space (PVS) can been observed (a–d). Note the regular presence of microvilli (mv) (b, d). A row of electron-dense cortical granules (CG) is seen under the oolemma (O) (b, d). M = Mitochondria, V = vesicle, PB1 = 1ˢᵗ polar body. Bar: 10 μm (a, c); 2 μm (b, d).

with 0.5% toluidine blue, and examined under a Leica DMRB light microscope. Photomicrographs were taken with a Lumenera Infinity microscope camera (Microsercon, SLU, Madrid, Spain). Ultrathin sections were cut with a diamond knife and double-contrasted with uranyl acetate 5% and lead citrate 2.5%. These were examined under a JEOL JEM-1400 Plus transmission electron microscope equipped with a Gatan Orius digital camera (Gatan, Pleasanton, CA, USA) for image capture.

Morphometric analysis

In this study, only oocytes that were visually ascertained to be of good quality using LM were selected for ultrastructural analysis. Features used to evaluate quality included the presence of a regular and rounded shape, a clear and moderately granular cytoplasm, a narrow PVS with the first polar body, and an intact and colorless ZP.

The evaluation of organelle density was performed through the collection of TEM micrographs of whole surface profiles at 6300 × magnification on three equatorial ultra-thin sections per oocyte (distance between the sections was 3–4 μm). These images were digitally enlarged to aid identification of organelles.
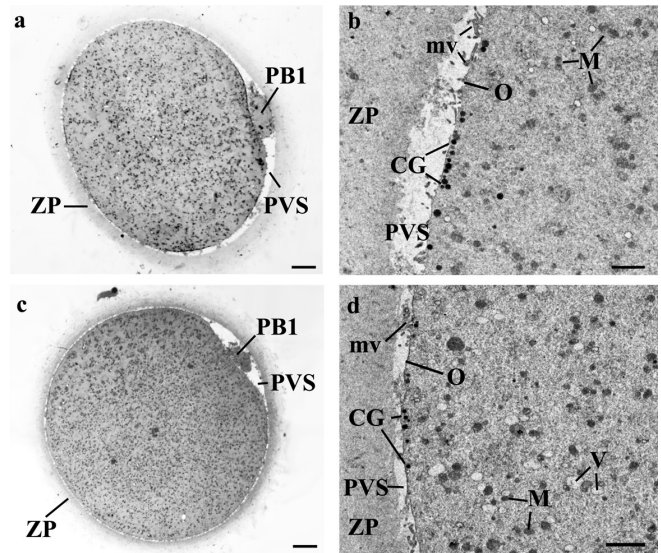
ImageJ software [24] was used to measure the dimensions of mitochondria, CG, microvilli, and vesicles. For each experimental group, at least six oocytes were selected for statistical analysis.

Statistical analysis

All data were expressed as a mean ± standard deviation and compared using unpaired t-tests (R software v3.3.1, https://www.r-project.org/). Differences in values were considered significant if $P < 0.05$. Mitochondria and vesicle values were expressed as the number of each per 100 μm^2, while CG and microvilli values were expressed as the number per 10 μm of the linear surface profile.

Results

General features

Through LM examination of semithin sections, both groups (oocytes vitrified before and after IVM) possessed good quality oocytes, as evidenced by their regular rounded shape, a maximum diameter of 90–105 μm, the cytoplasm showing a uniform and fine granular texture, and were surrounded by a continuous ZP and PVS (Figs. 2a and 2c). TEM analysis at low magnification (Figs. 2b and 2d) revealed that organelles in both groups were abundant and uniformly dispersed in the homogeneous ooplasm, with slight microvacuolization of the cytoplasm. However, some Golgi apparatus were occasionally observed in oocytes vitrified before IVM (Fig. 3a).

Mitochondria

Ultrastructural analysis using TEM revealed that the most numerous and commonly identified organelles were mitochondria. These often associated with the SER to form large M-SER aggregates or, less
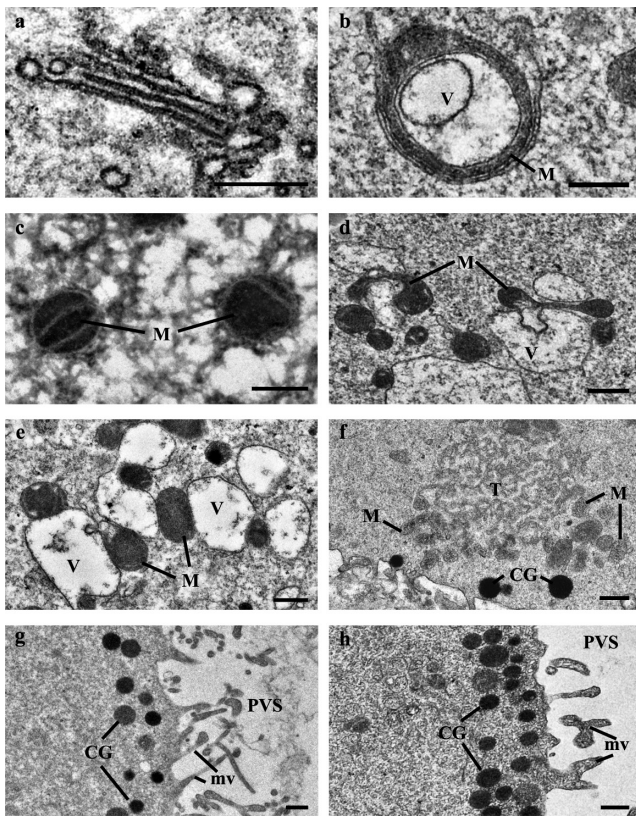
Fig. 3. Microphotograph of different organelles found in human oocytes vitrified before (group 1) (a, c, e, g) and after *in vitro* maturation (group 2) (b, d, f, h). a) Golgi apparatus. b) Some SER small vesicles (V) are encircled by flattened, crescent-shaped mitochondria (M). c) Rounded mitochondria endowed with few transversal cristae. d–e) M-V complexes. Note elongated forms with a central constriction. f) M-SER aggregates. g, h) Cortical granules (CG) disposed just beneath the oolemma. SER = smooth endoplasmic reticulum; T = aggregates of tubules; mv = microvilli; PVS = perivitelline space. Bar: 250 nm (a–c); 500 nm (d–h).

frequently, associated with small vesicles (V) to form M-V complexes (Figs. 3b–3e). Mitochondria were localized homogenously across the entire cytoplasm and were rounded or oval in profile, with few transversal cristae and a matrix showing moderate electrodensity (Fig. 3c). We also found elongated mitochondrial forms confined to the center of the ooplasm (Fig. 3d). Large M-SER aggregates were located in the cortical areas of the ooplasm (Fig. 3f). The number of mitochondria was slightly higher in group 2. The mean number $\pm$ SD of mitochondria per 100 μm^2 was 45.6 $\pm$ 7.5 and 54.33 $\pm$ 8.6 in oocytes vitrified before and after IVM, respectively. However, there was no statistically significant difference in the number of mitochondria among groups (P = 0.119) (Fig. 4a).

Vesicles

In all observed oocytes, typical numbers of SER vesicles and complexed M-V were present. The mean number $\pm$ SD of vesicles per 100 μm^2 was found to be 6.69 $\pm$ 3.1 and 7.25 $\pm$ 1.8 in oocytes

vitrified before and after IVM, respectively. There was no significant difference among groups (P = 0.930) (Fig. 4b).

Cortical granules

Using electron microscopy, we were able to identify up to three rows of spherical CG immediately below the oolemma through electron-dense matrix differences (Figs. 3g and 3h). However, a few isolated CG were occasionally detected in the inner ooplasm of some oocytes in group 1 (oocytes vitrified before IVM). Morphometric analysis revealed that the mean number $\pm$ SD of cortical granules per 10 μm of the linear surface profile was 5.68 $\pm$ 2.5 and 8.99 $\pm$ 3.1 in oocytes vitrified before and after IVM respectively, and there was no significant difference (P = 0.3535) (Fig. 4c).

Microvilli

Similar ultrastructural features were found in both groups. Oocytes were surrounded by a regular oolemma, with numerous microvilli arranged in a typical pattern projecting into a PVS (Figs. 3g and 3h). There was no significant difference in the number of microvilli per 10 μm of the linear surface profile between the two groups (mean number $\pm$ SD: 15.16 $\pm$ 2.2 and 16.21 $\pm$ 2.9 in oocytes vitrified before and after IVM, respectively, P = 0.8361) (Fig. 4d).

Discussion

In recent years, the recovery of immature oocytes and subsequent IVM has been found to be an attractive alternative to *in vitro* fertilization (IVF) [25]. Although IVM methods are not yet fully optimized for humans [26], there are recent reports of healthy infants being born following IVM [27, 28]. However, little research has been carried out into whether IVM should be performed before or after vitrification. Zhang *et al.* 2011 [29] have suggested that vitrification of immature oocytes may be a better way to preserve microtubule organization and reduce cytoskeletal spindle damage. On the other hand, other studies [30, 31] report that the IVM procedure is more efficient when it is performed before oocyte vitrification. In the present work, we have investigated the ultrastructural changes that occur in the cytoplasmic organelles of oocytes in both cases.

Cytoplasmic maturation is a complex process, but electron microscopy allows oocyte quality to be assessed based on morphometric criteria. The criteria used for the identification of cytoplasm immaturity are the presence of numerous CG dispersed in the oocyte cortex instead of being positioned beneath the oolemma, the presence of Golgi complexes still forming cortical vesicles, the absence of mitochondria in the oocyte cortex, the absence of SER tubule aggregates, and the presence of SER large vesicles without associated mitochondria. Our analysis confirmed that all oocytes in our study could be considered good quality when assessed by LM and TEM. In both groups, oocytes were round in shape and the oolemma, ZP, and PVS appeared continuous. In fact, CG were found in similar numbers beneath the oolemma, with only a few isolated CG located in the subcortex of oocytes in group 1 (oocytes vitrified before IVM). Moreover, CG showed variation in electrodensity, which represents an important parameter to evaluate cytoplasm maturation. Sparsely electrodense CG can be interpreted as immature organelles or as an early morphological sign of exocytosis [14, 23]. However, low
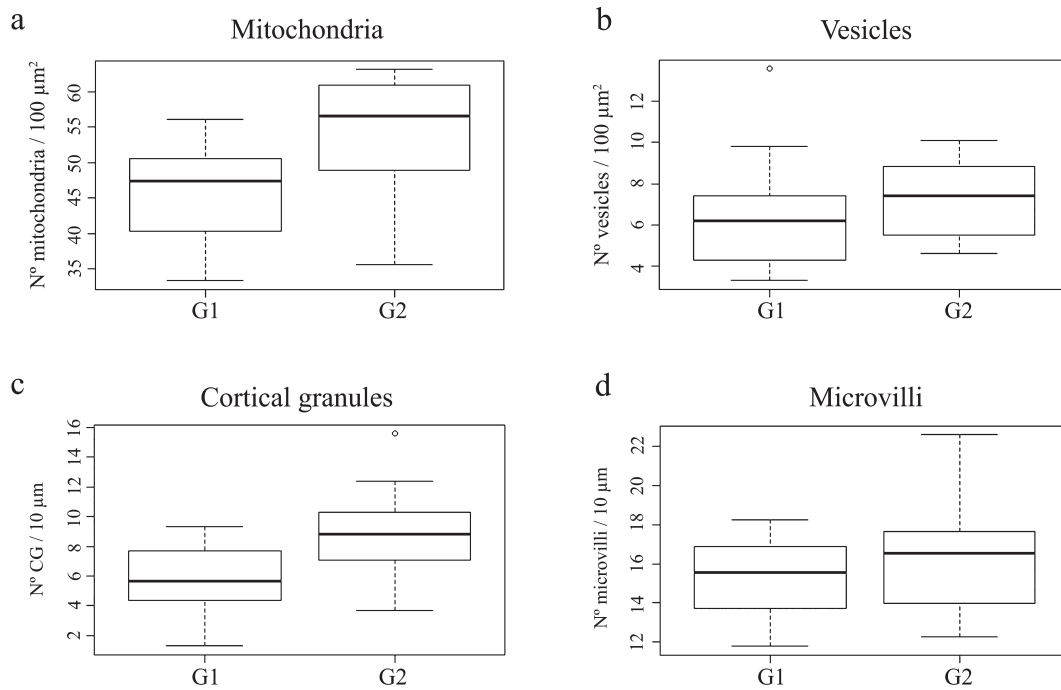
a

Mitochondria

b

Vesicles

c

Cortical granules

d

Microvilli

Fig. 4. G1: Oocytes vitrified before IVM. G2: Oocytes vitrified after IVM. The graphs represent the number of cytoplasmic organelles, number of mitochondria per 100 μm^2 (a), number of vesicles per 100 μm^2, (b) number of cortical granules per 10 μm linear surface profile, (c) and number of microvilli per 10 μm linear surface profile (d). Values for each group are expressed as mean ± SD. No statistical differences were found between the two groups.

levels of vacuolization have also been observed, which could be considered a marker of high quality oocytes [32].

In a small number of oocytes vitrified before IVM, some Golgi complexes were observed. Previous studies [33] have reported that Golgi apparatus are rarely found in MII oocytes. This may suggest that oocytes vitrified before IVM are of a lower quality then those vitrified afterwards. However, this could also be interpreted as an increase in protein production in the ooplasm.

The number and status of mitochondria were also similar between both groups, with a comparable number, shape, internal architecture, and texture. The mitochondria showed no signs of apoptosis, as reported in other studies that have examined IVM of oocytes [29]. Mitochondria, M-SER aggregates, and M-V complexes play a role in the production of materials useful for maturation and fertilization [34]. Mitochondrial abnormalities may also be associated with embryo incompetence [35, 36]. Our results have shown that the mitochondria in both groups did not have a disorganized matrix or the presence of dark vesicles.

With regards to vesicles, SER elements may dynamically acquire different shapes (tubules or vesicles), although they belong to the same system of interconnected membranes [21]. In this respect, during the maturation of the oocyte, the aggregation of SER tubules appears to be the earliest observable event. Subsequently, these tubules become surrounded by mitochondria, forming mitochondria-SER aggregates. Moreover, small M-V complexes are found in both immature and mature oocytes [12, 37].

Finally, the ultrastructure of the microvilli showed a normal pattern

in both groups. This characteristic is very important as improper microvilli distribution contributes to fertilization failure through ineffective spermatozoon-oocyte fusion [38].

In conclusion, human oocytes can be vitrified before or after IVM, but oocytes cryopreserved after IVM show slight improvements in terms of the ultrastructural characteristics of cytoplasm maturation. However, further studies are needed to confirm these results owing to the limited number of samples used.

Acknowledgments

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