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The effect of vitrification on ultrastructure of human in vitro matured germinal vesicle oocytes

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ABSTRACT

Objective: To describe the possible effects of cryotop vitrification on maturation rate and ultrastructural morphology of human in vitro matured germinal vesicle (GV) oocytes.
Study design: A total of 301surplus immature GV oocytes obtained from infertile patients were allocated into two groups: (i) GV oocytes (n = 150) matured in vitro (fIVM), and (ii) GV oocytes (n = 151) that were first vitrified, then matured in vitro (vIVM). Supernumerary fresh in vivo matured oocytes (n = 10) were used as controls. The maturation media was Ham's F10 supplemented with FSH + LH and human follicular fluid. After 36 h of incubation, the oocytes were investigated for nuclear maturation and ultrastructural changes using transmission electron microscopy (TEM).
Results: Oocyte maturation rates were reduced ($P < 0.001$) in vIVM (45.92%) in comparison with fIVM oocytes (75.33%). The rate of degeneration was also significantly higher in vIVM than in the fIVM group (44.4% vs. 6.0%). Large and numerous mitochondria and minute vesicles of smooth endoplasmic reticulum (SER) complexes (MV complexes) were observed in both fIVM and vIVM groups. In addition, TEM revealed a drastic reduction in amount of cortical granules (CGs) at the cortex of vitrified-warmed GV oocytes, as well as appearance of vacuoles and small mitochondria-SER aggregates in the ooplasm.
Conclusion: The vitrification procedure is associated with ultrastructural alterations in specific oocyte microdomains, presumably related to the reduced competence of cryopreserved oocytes for maturation. This information emphasizes the need for further work on advancing the cryotechnology of human oocytes.

1. Introduction

Oocyte cryopreservation has wider clinical implications than embryo freezing [1]. It is now possible for women who have no partner or are about to lose their ovarian function because of surgery or chemo/radiotherapy to store their oocytes for future use [2] It also provides an alternative to embryo preservation to avoid ethical issues and legal restrictions. For IVF patients, freezing the excess oocytes could avert repeated oocyte retrieval from the patients themselves or be a source for oocyte donation [3]. Recently, progress in improving human oocyte cryopreservation has been made, evident by numerous reports describing pregnancies and live births [4]. Recent progress involved application of vitrification that markedly improved the survival rate of oocytes, indicating that it is a promising cryopreservation technique [5].

One of the major problems associated with cryopreservation of mature oocytes is the sensitivity of the meiotic spindle to low temperature and cryoprotectants. This could be avoided by cryopreserving at the germinal vesicle (GV) stage when the chromosomes are within the nuclear membrane [6]. The disadvantage of cryopreservation of immature oocytes, however, is the fact that in vitro maturation (IVM) is required after thawing. It should be noted that IVM is not easily performed with human oocytes, and only a few successful pregnancies from cryopreserved immature oocytes have been reported so far [7]. Chian et al. reported pregnancies and live births as a result of natural IVF cycles with oocytes that were subjected to IVM [8]. This technology may therefore provide a benefit for patients, oocyte donation, and the study of cryopreservation of immature oocytes. Later, Cao et al. showed that there were no differences in survival rates between

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oocytes vitrified at the GV or at the mature stage, but the potential of oocyte maturation is diminished by GV vitrification [9].

Evaluation of the oocytes by phase-contrast microscopy (PCM) is an important predictive marker of oocyte quality, currently utilized to evaluate the success of ART programs [10]. Low-resolution morphological assessment, however, is not always a sufficient measure of oocyte fertilization potential and developmental competence [11]. Therefore transmission electron microscopy (TEM) assessment, integrated with other investigational approaches, seems effective in estimating how cooling rates and cryoprotectants affect oocyte structural integrity during freeze-thawing. It seems that vitrification affects the ultrastructural morphology of the human mature oocyte [12]. This research aimed to investigate, by application of TEM, the possible effect of cryotop vitrification on maturation and ultrastructural features of human GV oocytes retrieved from ICSI cycles that were matured in vitro.

2. Materials and methods

2.1. Source of oocytes

A total of 301 GV and 10 MII oocytes were collected with informed consent from 137 women aged 20–40 years who underwent ICSI cycles. This study was approved by the ethics committee of our institute in Yazd, Iran. The patients underwent ovarian stimulation using a long stimulation protocol [13]. Human chorionic gonadotrophin (hCG; 10,000 IU) was administered 36 h before oocyte collection. The collected oocytes were cultured in IVF media (Vitrolife Co., Switzerland) and placed in an incubator (6% CO_2; 37 °C) for 1–2 h. Cumulus cells were removed enzymatically with 80 IU/ml hyaluronidase (Sigma Co, USA) and mechanically by glass pipettes. Denuded oocytes were observed under a stereo microscope (Olympus Co, Japan) to assess maturity. The oocytes that extruded the first polar body (1PB) were considered mature and used for ICSI. GV oocytes were determined by the absence of 1PB and presence of a GV nucleus. Only supernumerary MII oocytes from canceled cycles due to azoospermia were used as controls. Oocytes were divided into three groups of fresh IVM (fIVM; $n = 150$), vitrified IVM (vIVM; $n = 151$) and controls ($n = 10$).

2.2. Follicular fluid preparation

Human follicular fluid (HFF) was prepared as described previously [14]. HFF was obtained from patients who underwent follicular puncture. After centrifugation at 3500 RPM for 10 min, the blood and granulosa cells were settled, and pure HFF was inactivated in a water bath at 56 °C for 30 min. Finally, HFF was filtered with 0.22 μm filters.

2.3. In vitro maturation

Fresh (fIVM group) and vitrified (vIVM group) immature oocytes were transferred into maturation medium (2–3 oocytes per 50 μl droplet under mineral oil) for 36 h at 37 °C in a humidified atmosphere of 6% CO2 in air. The maturation medium was Ham's F10 (Biochrom Co, Germany) supplemented with 0.75 IU LH + 0.75 IU FSH (Ferring Co, Germany) with 40% HFF. To assess maturation, the oocytes were observed under an inverted microscope (Nikon Co, Japan) which was determined by the presence of the 1PB.

2.4. Vitrification

Immature oocytes were frozen utilizing a modified vitrification method [15]. Initially, the oocytes were equilibrated in a solution containing 7.5% ethylene glycol (EG, Merck Co, Germany), 7.5% dimethyl sulphoxide (DMSO, Merck Co, Germany) in Ham's F10 medium supplemented with 20% human serum albumin (HSA, Plasbumin Co, USA) for 10 min at room temperature (RT). They were then transferred to vitrification solution containing 15% EG, 15% DMSO and 0.5 M sucrose (Sigma Co, USA) in Ham's F10 medium supplemented with 20% HSA for 1 min at RT. Then, the oocytes were loaded on a cryotop in a volume of <1 μl, and immediately submerged into liquid nitrogen for storage.

For thawing, the cryotops were directly plunged into pre-incubated Ham's F10, 20% HSA and 1 M sucrose solution for 1 min. Subsequently, thawed oocytes were rehydrated in Ham's F10 and 20% HSA (v/v) containing 0.5 and 0.25 M sucrose at RT for 3–5 min in each, respectively. Finally, the oocytes were rinsed in Ham's F10 and 20% HSA for 3–5 times. After this stage, the oocytes were transferred into IVM medium for 36 h in incubator and checked after 1 h for survival [16]. Post-warming survival rate was assessed using morphological criteria, indicated by the absence of overt cell degeneration, elongated shape, thick or distorted zona, expanded perivitelline space (PVS) and dark cytoplasm.

2.5. Electron microscopy

Ten oocytes from each group were fixed and processed for TEM as described by Nottola et al. [12]. Oocytes were fixed in 1.5% glutaraldehyde (Sigma) for 2–5 days at 4 °C, embedded in 1% gelose (Sigma), and exposed to 1% osmium tetroxide (SIC, Rome, Italy). Then, the samples were dehydrated in increasing concentrations of ethanol, immersed in propylene oxide for solvent substitution and individually embedded in Epon 812 (SIC). The oocytes were then sectioned for both light and electron microscopy. For light microscopy, the oocytes were sectioned at a thickness of 0.5–1 μm and stained with toluidine blue. Ultrathin sections (60–80 nm) were cut and stained with uranyl acetate and lead citrate. These sections were observed and photographed with a TEM (Zeiss Co., Germany).

2.6. Statistical analysis

Differences in oocyte maturational stages between the groups were calculated and compared using Student's *t*-test and Chi-squared test. A *P*-value of <0.05 was considered statistically significant. Calculations were performed by SPSS software (version 16, USA).

3. Results

There were no significant differences in characteristics of age, etiology of infertility, and total number of retrieved oocytes between the fIVM and vIVM groups (Table 1). The majority of the patients had male factor infertility. The rates of oocyte survival and their maturation are shown in Table 2. The survival rate was as high as 89.4% post-thaw in the vIVM group. The maturation rate of the GV oocytes was 45.92% (62/135) when the oocytes were vitrified and then underwent IVM. This was lower than the fIVM

Table 1
Characteristics of patients in fresh IVM and vitrified IVM groups.

Variable	Fresh-IVM ($n = 150$)	Vitrified-IVM ($n = 151$)	*P*-value
Age (years) (mean ± SD)	29.3 ± 5.9	32.2 ± 5.5	NS
Female factor infertility	64 (50.6)	63 (49.4)	NS
Male factor infertility	69 (50.0)	69 (50.0)	NS
Both (male and female factors infertility)	17 (45.5)	19 (54.5)	NS

†NS, not significant; values inside parentheses represents (%)

Table 2
Comparisons of maturation rates of human oocytes in two groups of IVM.

Variables	Fresh IVM ($n=150$)	Vitrified-IVM ($n=135$)	*P*-value
MII oocyte (matured)	113 (75.33)	62 (45.92)	<0.001*
Oocyte arrest	23 (15.33)	11 (8.14)	–
Degeneration	9 (6)	60 (44.4)	<0.001*
Parthenogenesis	5 (3.33)	2 (1.48)	–

Values inside parentheses represents (%)
* Significant difference between fresh IVM and vitrified-IVM

group ($P < 0.001$). Also, the rate of degeneration was significantly higher in the vIVM than the fIVM group (44.4% vs. 6.0%).

3.1. Ultrastructure of MII oocytes

The zona pellucida (ZP) of control MII oocytes consisted of closely packed electron dense fibrillar material. The perivitelline space (PVS) was uniform with scarce debris. Numerous long thin microvilli were uniformly distributed on the continuous oolemma, except in the zone of PB extrusion. Round cortical granules had an electron dense appearance, located just beneath the oolemma (Figs. 1a and 2a). The majority of the oocyte organelles consisted of aggregates of smooth endoplasmic reticulum (SER) surrounded by mitochondria (M-SER aggregates). Small mitochondria-vesicle (MV) complexes were another finding (Fig. 3a). The mitochondria were usually spherical or elliptical shaped. Their arc-like or transverse cristae were irregularly placed on the periphery and parallel to the outer mitochondrial membrane (Fig. 3a).

3.2. Ultrastructure of GV oocytes after IVM

The ultrastructural characteristics of IVM oocytes had some similarity to the control group, but the most notable feature of in vitro matured oocytes was the presence of numerous and large MV complexes within the cytoplasm (Fig. 3b). The mitochondria,

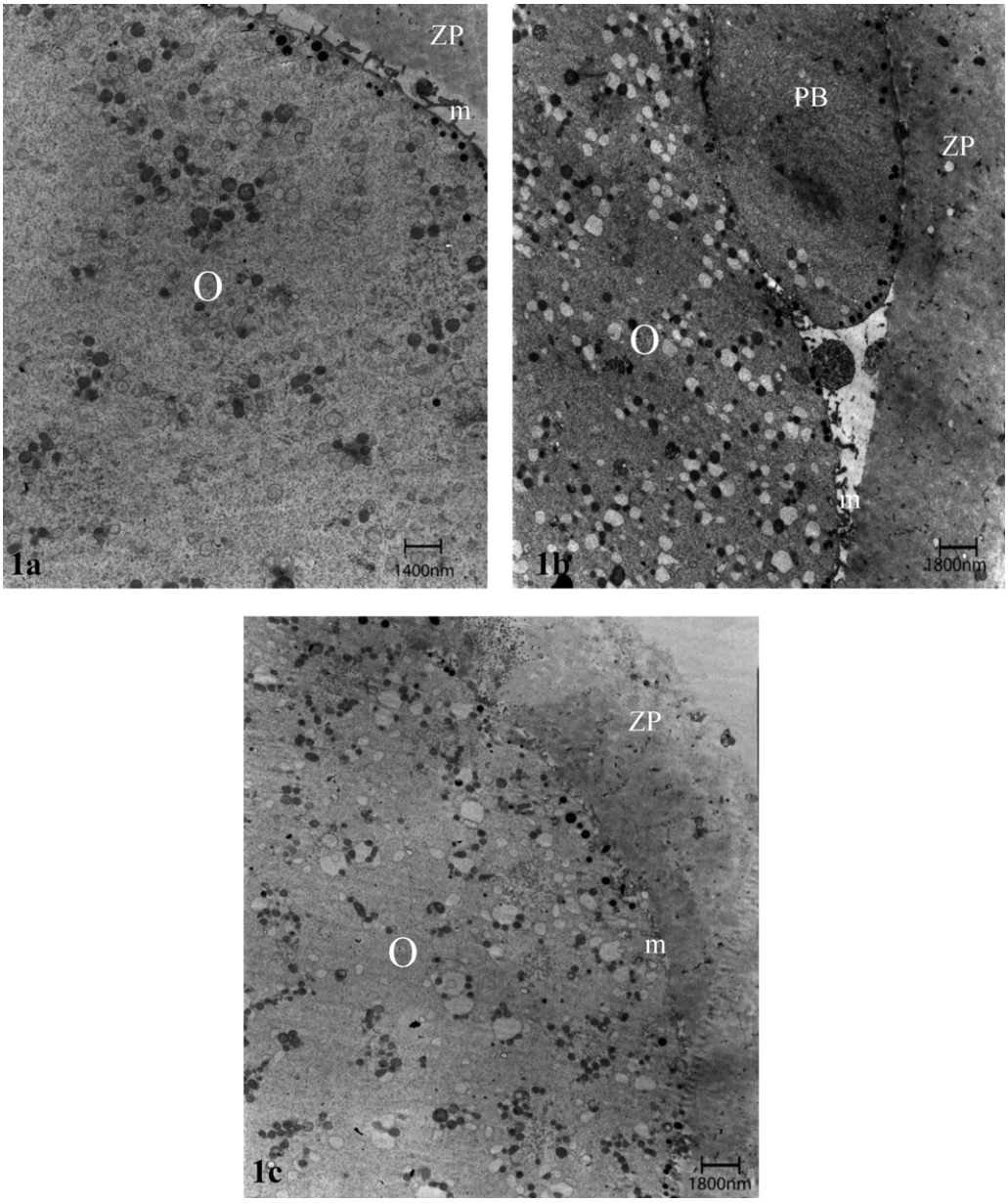

Fig. 1. Control oocyte (a), GV stage oocyte after IVM (b), Vitrified-Thawed GV stage oocyte after IVM (c). The general morphology and organelle microtopography are shown by TEM. O = oocyte; ZP = zona pellucida; M = microvilli; PB = polar body.

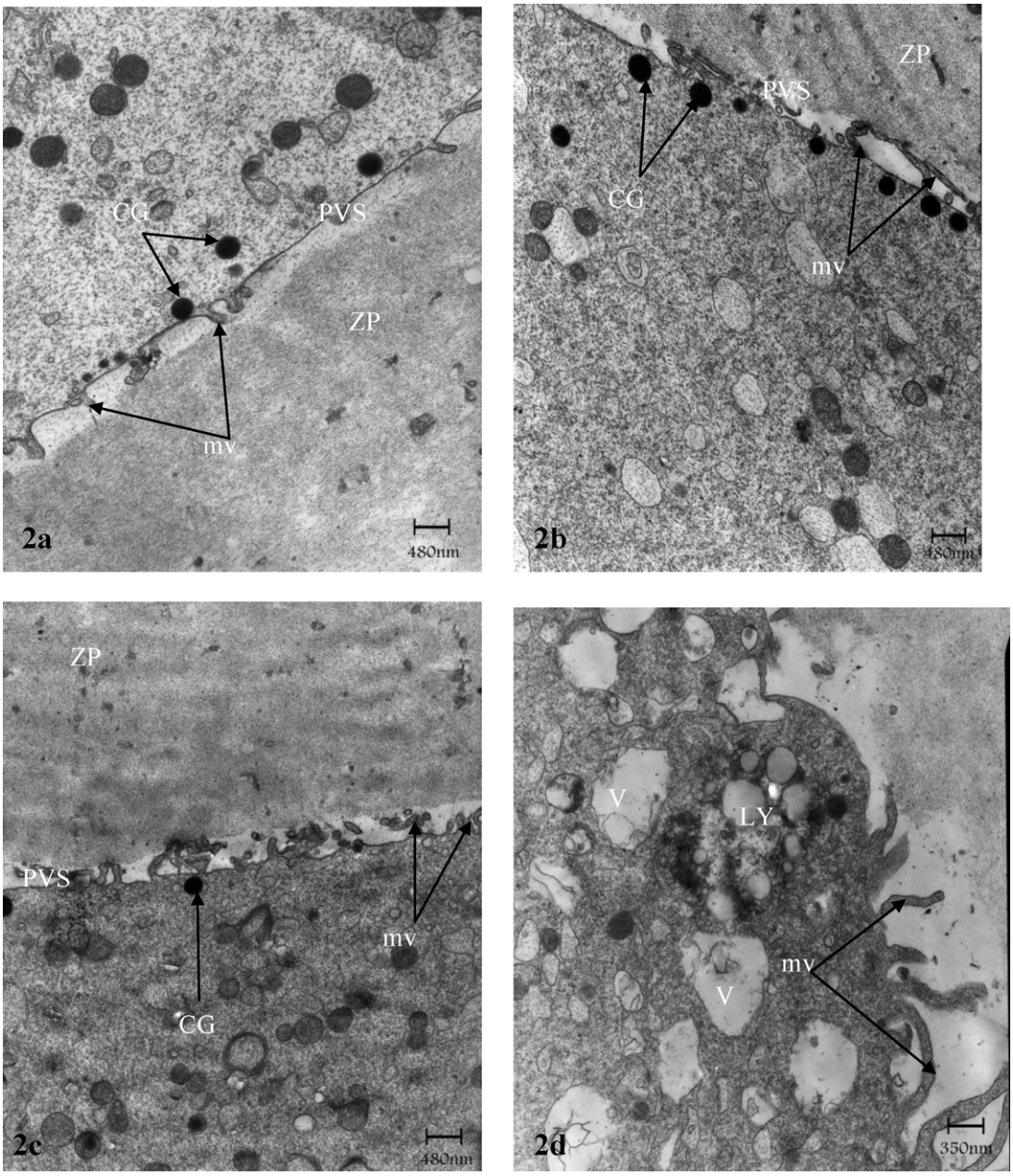

Fig. 2. Ultrastructure of control oocyte (a), GV stage oocyte after IVM (b), Vitrified-Thawed GV stage oocyte after IVM (c and d). A rim of electron-dense cortical granules (arrows) is seen just beneath the oolemma of the fresh oocytes (a and b). Instead, cortical granules are sparse and form a discontineus layer in vitrifie-warmed oocytes (c and d). Note the increased compaction of the inner aspect of the ZP in (c) and (d) in comparison with the looser texture in (a) and (b). Microvilli are numerous and long on the oolemma of the fresh and vitrified-warmed oocytes (a–d). Vacuoles are seen in vitrified-warmed oocyte (d), sometimes associated with lysosomes. ZP = zona pellucida; mv = microvilli; CG = cortical granules; PVS = perivitelline space; V = vacoule; LY = lysosome.

cortical granules, microvilli, PVS and ZP were similar to those of the controls (Figs. 1b, 2b and 3b–c).

3.3. Ultrastructure of vitrified-thawed GV oocytes after IVM

The peripheral cortical granules were prematurely released into the subzonal space, and reduced in number (Fig. 2c). Reduction or absence of cortical granules was sometimes associated with an increased compaction of the inner aspect of the ZP (Figs. 1c and 2c–d). Small M-SER aggregates were found randomly distributed in the ooplasm (Fig. 3d–e). Large MV complexes were also seen (Fig. 3d–e). Vacuoles were empty and surrounded by a membrane often interrupted (Fig. 2d). Sometimes, secondary lysosomes were found associated with them (Fig. 2d). Mitochondria, oolemma, microvilli and PVS were similar to both control and fIVM groups (Figs.2c–d and 3e).

4. Comment

The aim was to evaluate the effect of cryotop vitrification on maturation and ultrastructural features of human GV oocytes retrieved from ICSI cycles. Although there are at least 900 live births resulting from slow-frozen and vitrified MII oocyte methods [17], GV cryopreservation has distinct advantages compared to MII oocytes. GV oocytes may circumvent the problem of cryo-spindle injury that may occur in mature oocytes, since the chromosomes remain inside the nuclear membrane [6]. Moreover, freezing of immature oocytes allows cancer patients to preserve their fertility

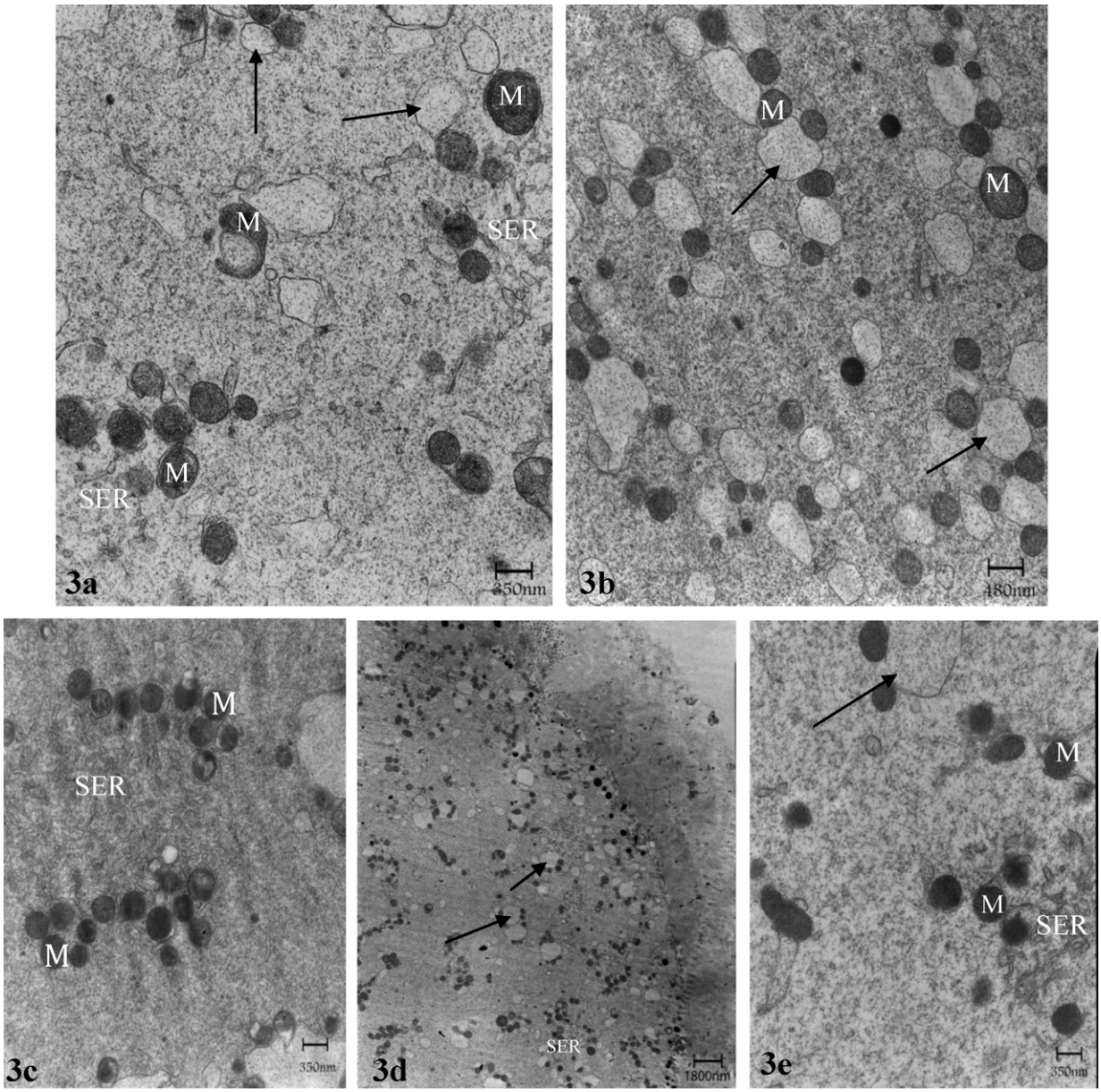

Fig. 3. Control oocyte (a), GV stage oocyte after IVM (b), Vitrified-Thawed GV oocyte after IVM (c). Mitochondria are generally rounded and provided with few peripheral or transverse cristae. Dumbbell shaped, possibly dividing mitochondria can be occasionally find in the ooplasm (a). Note the presence of complexes between mitochondria and vesicles of SER in a, b, d, e (arrows). Voluminous aggregates between mitochondria and elements of SER are seen (a, c, e). SER = smooth endoplasmic reticulum; M = mitochondria.

before receiving chemo/radiotherapy without delaying treatment or experiencing risks related to ovulation induction [18]. In the present study, almost 90% of the oocytes survived and the maturation rate was over 45%. In addition, a significant reduction in maturation rate was seen in vitrified GV oocytes. This is probably due to the negative effect of vitrification on the outcome of oocyte maturation [19]. Maturation and developmental retardations of cryopreserved oocytes are related to critical disturbances of various cell components, such as the chromosome segregation apparatus, the intracellular Ca^{+} signaling system, and the cytoskeleton [20]. Wu et al. indicated that 59% of oocytes retrieved from unstimulated ovaries survived freezing, and 64% of these oocytes subsequently matured in vitro [7]. Their findings are different to ours, which is probably due to differences in the source of oocytes and method of freezing. Also, Cao et al. reported that the survival and maturation rates of their GV oocytes after thawing were 85.4% and 50.8%, respectively, which are in agreement with ours, but our rates of maturity following IVM without vitrification were higher when compared with those of Cao et al. [9].

Differing reports of maturation rates after IVM are probably due to several factors, including media composition, hormonal or growth factor supplementions, the source of oocytes (stimulated versus unstimulated cycles) and whether or not cumulus cells were retained with the oocytes [21]. It is physiologically more appropriate to leave GVs enclosed in their cumulus during cryopreservation, because it can improve the process of IVM after thawing [22], together with survival and blastocyst formation rates [23]. Minasi et al., however, mentioned that presence of cumulus cells has no beneficial effect on the MII oocyte competency in cryopreservation program [24].

Recently, Asimakopoulos et al. insisted upon the efficiency of cryopreservation of immature human oocytes using vitrification before application of IVM [25]. Also, Zhang et al. demonstrated that vitrification is superior to the slow freezing method in terms of

survival and developmental competence of human failed-matured oocytes [3]. Based on clinical studies, it seems that the oocyte biological functions affected by the two cryopreservation techniques are different, with a more deleterious impact of the slow freezing procedure [26]. It has been demonstrated that slow frozen/thawed and vitrified MII oocytes are characterized by differential down-regulation of specific transcripts, suggesting that both cryopreservation procedures lead to loss of the mRNA content [27]. Oocyte developmental competence depends on the accumulation of maternal proteins and mRNAs during oogenesis, and reduced developmental competence may be one of the main reasons for IVF failure.

The ultrastructural study demonstrated that vitrification affects the pericortical distribution of cortical granules and SER in oocytes. Cortical granules were reduced in density and were damaged in vitrified-warmed oocytes, similar to other findings [28]. Ultrastructural evidence of premature exocytosis of cortical granules from human oocytes exposed to either PROH or DMSO was demonstrated by Schalkolf et al. [29]. Premature exocytosis of cortical granules into the PVS led to ZP hardening, which may block sperm penetration at fertilization level [30]. We indicated that cryopreservation resulted in the appearance of vacuoles within the ooplasm, which might demonstrate structural damage, which is similar to the study of Ghetler et al. [31]. The appearance of vacuoles is a non-specific response to cryodamage or osmotic stress, which are usually regarded as a degenerative process [32]. This concept is supported by the finding of associations among vacuoles, multivesicular bodies and lysosomes in the ooplasm after slow cooling [33]. Previous studies reported that multivesicular bodies and lysosomes must be regarded as signs of regressive when associated with vacuoles in mature oocytes [34]. Therefore, the presence of vacuoles in human oocytes might finally lead to a reduced competence to fertilization and/or impaired embryo development [35]. Ultrastructural alterations to M-SER aggregates with preservation of mitochondrial integrity were in agreement with a previous study [33]. Nottola et al. [12] and Khalili et al. [32] reported that the alterations of M-SER aggregates in vitrified-warmed oocytes is more probably due to the utilizing of EG in vitrification solutions than to the cryoinjury itself. In addition, they stated that the fine alterations in the arrangement of M-SER aggregates might lead to disturbance in calcium homeostasis and as a consequence, to the fertilization reduction. It is believed that M-SER aggregates are precursor of MV complexes [36]. Large and numerous MV complexes observed in both fresh and vitrified IVM oocytes are probably due to prolonged culture – a sign of aging. Ultrastructural changes to MV complexes in the current study were similar to a previous report [37]. Also, mitochondria and accompanied membranes have a role in the production of materials useful at maturation and fertilization. In addition, these organelles may regulate local concentrations of free calcium and ATP production. It has been suggested that the shape and distribution of mitochondria were related to the level of cell metabolism, proliferation and differentiation [38]. Dumollard et al. suggested that modulation of calcium signaling is carried out by oocyte M-SER [39].

In conclusion, this work demonstrated that the maturation potential is reduced by cryotop vitrification of the GV oocytes. Vitrification may generate ultrastructural alterations in specific oocyte microdomains, presumably responsible for the reduced potential of oocytes for maturation. This emphasizes the need for further studies on modification of this cryopreservation technique.

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