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FREEZE/THAW STRESS INDUCES ORGANELLE REMODELING AND MEMBRANE RECYCLING IN CRYOPRESERVED HUMAN MATURE OOCYTES

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appear influenced by freeze-thawing. However, except for CGs, membrane alterations seem to undergo a partial or, more rarely, an almost complete recovery at the end of the rehydration. This study also shows that such a membrane remodeling is mainly represented by a dynamic process of transition between M-SER aggregates and MV complexes, both able of transforming into each other. Vacuoles and CG membranes may take part in the membrane recycling mechanism.

FREEZE/THAW STRESS INDUCES ORGANELLE REMODELING AND MEMBRANE RECYCLING IN CRYOPRESERVED HUMAN MATURE OOCYTES.

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ABSTRACT

Purpose: Cryopreservation may affect oocyte morphology and competence. Little is known about influence of thawing and rehydration on oocyte integrity. Our aims were to evaluate the ultrastructure of human metaphase II oocytes subject to slow freezing and fixed after thawing, at different intervals during post-thaw rehydration. **Methods:** Samples were studied by light and transmission electron microscopy. **Results:** We found that vacuolization was present in all cryopreserved oocytes, reaching a maximum in the intermediate stage of rehydration. Mitochondria-smooth endoplasmic reticulum (M-SER) aggregates decreased following thawing, particularly in the first and intermediate stages of rehydration, whereas mitochondria-vesicle (MV) complexes augmented in the same stages. At the end of rehydration vacuoles and MV complexes both diminished and M-SER aggregates increased again. Cortical granules (CGs) were scarce in all cryopreserved oocytes, gradually diminishing as rehydration progressed. **Conclusions:** In conclusion, a) vacuoles may form during freezing and/or at thawing but increase during rehydration; b) significant changes of opposite trend in the number of M-SER aggregates and MV complexes occur during freeze-thawing; c) CG exocytosis proceeds during the whole freeze-thawing procedure. Thus, all ooplasmic membranes appear influenced by freeze-thawing. However, except for CGs, membrane alterations seem to undergo a partial or, more rarely, an almost complete recovery at the end of the rehydration. This study also shows that such a membrane remodeling is mainly represented by a dynamic process of transition between M-SER aggregates and MV complexes, both able of transforming into each other. Vacuoles and CG membranes may take part

in the membrane recycling mechanism.

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Key words: oocyte, vacuoles, organelles, cryopreservation, human, ultrastructure

INTRODUCTION

Oocyte cryopreservation currently represents a valuable procedure among assisted reproductive technologies (ART) that bypasses some ethical, moral and religious dilemmas associated with the storage of embryos. It is in fact a valid solution for women who have to repeat in vitro fertilization (IVF) treatments avoiding the risk of ovarian hyperstimulation syndrome, and for women who may lose their ovarian function due to surgery, cancer treatments or premature menopause [1]. Oocyte cryostorage may also represent a possibility to counteract future infertility for healthy women who decided to postpone childbearing due to educational or socio-economic pressures (social freezing) [2]. Despite such a paramount impact of oocyte cryopreservation, the mature, metaphase II (MII) human oocyte is more difficult to cryopreserve than the cleavage-stage embryo [3]. This is due to oocyte peculiar features, such as large size (low surface –to- volume ratio), high water content, elevated degree of cytoplasmic specialization and sensitivity of the chromosome segregation machinery [4-9]. Indeed some structural domains of the mammalian MII oocytes, such as zona pellucida (ZP), cortical granules (CGs) and other organelles, cytoskeletal components and, particularly, meiotic spindle are sensitive to the process of cryopreservation, due to the negative effects exerted by low temperatures, formation of intracellular ice crystals, osmotic stress and toxicity of the substances used as cryoprotectants (for references, see: [10-17]). Thus, light and transmission electron microscopy (LM and TEM) are powerful tools of investigation and evaluation of the impact that the above factors may have on human oocyte microstructure during freeze-thawing.

Vacuolization from a slight to a moderate extent is an important dysmorphism that has been frequently detected by both LM and TEM in the ooplasm of human mature oocytes subjected to cryopreservation, mainly when slow freezing is applied [12,17,18]. Vacuoles are also present in ageing or degenerating oocytes, whereas in fresh, healthy MII oocytes they are very scarce or

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virtually absent [19-23]. Thus, the occurrence of vacuolization in frozen-thawed oocytes may be considered a form of structural damage explainable as a non-specific response of the oocyte to cryoinjury and/or osmotic stress. In addition, since oocytes subjected to different protocols of slow freezing may show different degrees of vacuolization [12-14,17,24,25], it should not be ruled out that oocyte vacuolization may be dependent, at least in part, upon the type and/or concentration of the cryoprotectants. Oocyte dysmorphisms may be related to poor clinical outcomes [26]. Although the effects of oocyte vacuolar dysmorphism on embryo development may remain controversial [27], it is a common finding that vacuolated MII oocytes show poor fertilization rates [28]. If fertilized, vacuolated oocytes cleave with difficulty [22] or present a failure of the preimplantation development [29,30]. However, while oocyte vacuolization seems to be associated with IVF failure, the genesis of vacuoles and the morphodynamics of vacuole formation have not yet been fully understood.

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Well-defined composite associations between mitochondria and cytoplasmic membranes are characteristically found in the ooplasm of fully grown human oocytes, named mitochondria-smooth endoplasmic reticulum (M-SER) aggregates and mitochondria-vesicle (MV) complexes [19,22,31,32]. Mitochondria and associated cytoplasmic membranes may play a role in production of substances useful at fertilization and/or in rapid neoformation of membranes during early embryogenesis [20,33,34]. Mitochondria-SER aggregates may also regulate local levels of free calcium and ATP production, thus acting on different cellular activities including the mediation of an “explosive” calcium signal at fertilization [23,35-38]. Thus, disturbances in morphology and function of these organelle associations may lead to a reduced oocyte competence for fertilization. In this regard, the presence of very large M-SER aggregates, sometimes related to gonadotropin hyperstimulation [21] has been generally associated with compromised embryo development and implantation [39,40], even though different opinions have been recently expressed [41-43]. On the contrary, underdeveloped M-SER aggregates have been found in a percentage of human mature

1 oocytes subjected to vitrification [15] or to a slow freezing protocol based on the use of ethylene
2 glycol as cryoprotectant agent [14], whereas other studies on slow-frozen oocytes treated with
3
4 propanediol (PrOH) did not evidenced qualitatively detectable ultrastructural alterations in M-SER
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6 aggregates [12,13,17]. However, a quantitative morphometric analysis on mitochondria and
7
8 associated membranes has not been carried out up to now in human cryopreserved oocytes.
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11 Several researchers, using TEM, have identified an abnormal reduction of the amount of
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13 CGs in mature oocytes of some mammals, including humans, after the application of different
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15 cryopreservation protocols [12-15,17,24,44-49]. Ultrastructural evidence of premature CG release
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17 has been also found after the simple contact of the oocyte with some cryoprotectants, as described
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19 by Schalkoff et al. [50] in human oocytes exposed to either 1,2-PrOH or dimethylsulfoxide at room
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21 temperature (RT). Contrasting data have been reported by Jones et al. [51], who found an
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23 abundance of CGs in the ooplasm of human PrOH-cryopreserved oocytes, although these
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25 observations do not preclude the possibility that a partial CG exocytosis in some other areas would
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27 not be detected. Thus, keeping under constant observation the presence and amount of CGs in
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29 human oocytes after the freeze-thawing procedure is extremely important. In fact, precocious
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31 oocyte activation - with a consequent decrement of oocyte developmental competence - is a
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33 phenomenon that may eventually be demonstrated with the appearance of premature CG exocytosis
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44 With the aim to give a contribution in solving some questions related to the quality, timing
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46 and entity of organelle alterations occurring during human oocyte cryopreservation, the present
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48 report was intended to evaluate presence and amount of: a) ooplasmic vacuolization, b) organelle-
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50 specific associations such as M-SER aggregates and MV complexes, and c) CGs in human MII
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52 oocytes subject to slow freezing and examined after thawing, at different intervals during post-thaw
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54 rehydration. Morphological data have been collected and evaluated though an integrated LM, TEM
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56 and morphometric approach.
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MATERIALS AND METHODS

Source of oocytes

This study was approved by Institutional Review Board of the participating Clinics. Oocytes were obtained from patients undergoing ART treatment with their informed consent and according to the current Italian laws. Only oocytes donated from women (N = 32) younger than 33 years (mean \pm standard deviation, SD: 31.36 ± 1), whose infertility was due to male or disovulatory factors, were used. Controlled ovarian hyperstimulation was induced with long protocols using GnRH agonist and rFSH, according to the standard clinical procedures routinely employed by the participating Clinics [52]. Ten thousand IU of hCG were administered 36 h prior to oocyte collection. After retrieval, oocytes were cultured in IVF media (Cook IVF, Brisbane, Australia, or Sage IVF Inc, Trumbull, CT, USA). Complete removal of cumulus mass and corona cells was performed enzymatically using hyaluronidase (80 IU/ml), and mechanically by using fine bore glass pipettes. Only oocytes devoid of any sort of dysmorphism at phase contrast microscopy (PCM) examination, showing an extruded first polar body (PBI), thus presumably at the MII stage, were assigned to the control or experimental groups. According to their assignment, oocytes were either frozen or fixed after a period of 3-4 h following retrieval.

Freezing procedure

Freezing was performed according to the two-step PrOH dehydration. In detail, the oocytes were equilibrated sequentially in solutions containing respectively 0.75 mol/l PrOH + 20% plasma protein supplement (PPS) and 1.5 mol/l PrOH + 20% PPS in Dulbecco's phosphate-buffered saline (PBS) (7.5 min for each step). Further, oocytes were transferred for 5 min into the loading solution

1 (1.5 mol/l PrOH + 0.2 mol/l sucrose + 20% PPS in PBS). Oocytes were finally loaded in plastic
2 straws (Paillettes Crystal 133 mm; Cryo Bio System, France), individually or in small groups
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4 (maximum three oocytes per straw). Straw temperature was lowered through an automated Kryo 10
5 series III biological freezer (Planer Kryo 10/1,7 GB) from 20°C to -8°C at a rate of -2°C/min).
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7 Manual seeding was performed at -8°C. This temperature was maintained in a hold interval of 10
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9 min in order to allow uniform ice propagation. Temperature was then decreased to -30°C at a rate
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11 of -0.3°C/min and finally rapidly to -150°C at a rate of -50°C/min. Finally, straws were directly
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13 plunged into liquid nitrogen and stored for later use.
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22 Thawing procedure

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24 Thawing was carried out at RT. Straws were removed from liquid nitrogen, warmed in air
25 for 30 sec and then plunged in a water bath at 37°C for 40 sec. The thawing solutions contained a
26 gradually decreasing concentration of PrOH and a constant 0.3 mol/l sucrose concentration.
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29 Thawed oocytes were firstly released in 1.0 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS (solution 1)
30 and incubated for 5 min. Afterwards they were transferred in 0.5 mol/l PrOH + 0.3 mol/l sucrose +
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32 20% PPS (solution 2) for additional 5 min. Finally, oocytes were placed in 0.3 mol/l sucrose + 20%
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34 PPS (solution 3) for 10 min before final dilution in PBS + 20% PPS (solution 4) for 20 min (10 min
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36 at RT and 10 min at 37°C). All freezing and thawing solutions were manufactured by Cook IVF,
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49 Electron microscopy

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52 Only oocytes with highest morphological scores at PCM examination [53] were selected for
53 electron microscopy analysis. A total of 60 MII oocytes were included in this study. Fifteen of them
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55 were fixed after 3-4 h following retrieval and assigned to the control group. The other 45 oocytes,
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1 after being cultured for 4 h, were subjected to freeze-thawing as described above and fixed in
2 glutaraldehyde after thawing, at different intervals during post-thaw rehydration, as follows: Group
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4 A, oocytes fixed after the passage in thawing solution 1 (N = 15); Group B, oocytes fixed after the
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6 passage in thawing solution 2 (N = 15); Group C, oocytes fixed after the passage in thawing
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8 solution 3 (N = 15).
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11 Oocytes were processed for LM and TEM as previously described [12-15,54]. Oocyte
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13 fixation was performed in 1.5% glutaraldehyde (SIC, Rome, Italy) in PBS solution. After fixation
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15 for 2–5 days at 4°C, the samples were rinsed in PBS, post-fixed with 1% osmium tetroxide (Agar
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17 Scientific, Stansted, UK) in PBS, and rinsed again in PBS. Oocytes were then embedded in small
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19 blocks of 1% agar of about 5×5×1 mm in size, dehydrated in ascending series of ethanol (Carlo
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21 Erba Reagenti, Milan, Italy), immersed in propylene oxide (BDH Italia, Milan, Italy) for solvent
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23 substitution, embedded in Epoxy resin (Agar Scientific, Stansted, UK) and sectioned by a Reichert-
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25 Jung Ultracut E ultramicrotome. Semithin sections (1 µm thick) were stained with toluidine blue,
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27 examined by LM (Zeiss Axioskop) and photographed using a digital camera (Leica DFC230).
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29 Ultrathin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids and
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31 contrasted with saturated uranyl acetate followed by lead citrate (SIC, Rome, Italy). The ultrathin
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33 sections were examined and photographed using a Zeiss EM 10 and a Philips TEM CM100
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35 Electron Microscopes operating at 80KV. Images were acquired using a GATAN charge-coupled
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37 device camera.
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48 Ultrastructural parameters

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50 The following parameters were evaluated by LM and TEM and taken into consideration for
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52 the qualitative morphological assessment of the structural and ultrastructural preservation of
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54 oocytes: general features (including shape and dimensions), presence and extent of ooplasmic
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1 vacuolization, microtopography, type and quality of the organelles, integrity of the oolemma, ZP
2 texture and appearance of the perivitelline space (PVS) [15,55].
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4 PBI and MII spindle features were not systematically assessed by LM and TEM, due to their
5 detection only in sections lying on appropriate planes.
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8 9 10 11 Statistical analysis

12 The presence of vacuoles, M-SER aggregates and MV complexes was evaluated at the LM
13 level on at least 3 equatorial semithin sections per oocyte (distance between the sections: 3-4 μm).
14 For each section, counting was performed on a single panoramic image of each oocyte, obtained
15 combining together several pictures of different oocyte portions taken at x100 magnification by
16 using the function Photoshop's Photomerge (PS Adobe Photoshop CS3). The amount of vacuoles,
17 M-SER aggregates and MV complexes was expressed in number of vacuoles or organelle
18 associations per 100 μm^2 of the oocyte area. Only structures (vacuoles, SER networks, vesicles)
19 greater than or equal to 0.5 μm in diameter were counted.
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34 The evaluation of CG density was performed through collection of TEM micrographs of
35 whole surface profiles at x6300 magnification on 3 equatorial ultrathin sections per oocyte. The
36 images were further magnified on the PC screen to easily recognize and count CGs. Values were
37 expressed as the number of CGs for 10 μm of the oocyte linear surface profile [12,15,25].
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44 All data were expressed as mean \pm SD and compared by one-way analysis of variance
45 (ANOVA) and Tukey's test as post-hoc test (GraphPad InStat). Differences in values were
46 considered significant if $P < 0.05$.
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57 **RESULTS**

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Control oocytes

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2 A total of 15 fresh, control oocytes were observed. When analyzed by LM, these oocytes
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4 appeared rounded in shape, 90-100 μm in diameter (ZP excluded), provided with a homogeneously
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6 textured ooplasm in which vacuoles were rarely seen (Fig.1a). Morphometric analysis revealed that
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8 the mean number \pm SD of vacuoles per 100 μm^2 was 1.06 ± 0.18 in the control group (Table 1;
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10 Graph 1). By LM and low magnification TEM the organelles, including numerous, large M-SER
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12 aggregates and small MV complexes, appeared scattered in the ooplasm (Fig. 1a,b). By
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14 morphometric analysis the mean number \pm SD of M-SER found in 100 μm^2 was 0.96 ± 0.01 while
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16 the mean number \pm SD of MV in 100 μm^2 was 0.60 ± 0.29 (Table 1; Graphs 2,3). A continuous,
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18 intact ZP, approximately 10-12 μm thick, completely surrounded the oocyte, which was separated
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20 from the inner zona aspect by a narrow PVS (Fig.1a,b). By TEM, mitochondria (0.5 - 1 μm in
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22 diameter), rounded or oval - in relation to the orientation of the cutting section - and provided with
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24 arched *cristae*, were numerous and characteristically associated with networked SER tubules with a
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26 diameter varying from 1 to 5 μm , forming the M-SER aggregates (Fig.1b,c). MV complexes
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28 appeared as small vesicles with a diameter of about 0.5 μm , filled with flocculent, slightly
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30 electrondense material and surrounded by mitochondria (Fig.1b,c). Rounded, electrondense CGs,
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32 varying in diameter from 300 to 400 nm, were abundant and stratified in one/two layers in
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34 subolemmal areas (mean number \pm SD of CGs per 10 $\mu\text{m} = 9.07 \pm 0.45$) (Fig 1d.; Table 1; Graph
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47 Numerous microvilli of variable length projected from the oolemma into the PVS (Fig. 1d).
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49 In sections lying on appropriate planes, the PBI was detected in the PVS and the MII spindle was
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51 found in the ooplasm.
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Cryopreserved oocytes

1 In total, 45 mature cryopreserved oocytes, 15 for each experimental group (A, B, C), were
2 analyzed. A preliminary evaluation was performed by LM (Fig.2a-c). All the oocytes were rounded,
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4 with a diameter ranging from 90 to 100 μm , provided with a homogeneous ooplasm and surrounded
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6 by a regular, uninterrupted ZP. No overt differences in oocyte shape/dimensions were detected
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8 between cryopreserved and control oocytes and among cryopreserved oocytes belonging to
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10 different experimental groups.
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14 By LM, circular areas of different sizes and shapes in which staining and matter consistency
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16 were reduced, identified as vacuoles, were numerous in the ooplasm of the cryopreserved oocytes
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18 belonging to all the experimental groups (Fig. 2). With regard to their distribution, vacuoles
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20 populated both inner and outer oocyte areas, but appeared more concentrated in the deeper ooplasm.
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22 Morphometric analysis revealed that the mean number \pm SD of vacuoles per 100 μm^2 was $7.20 \pm$
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24 1.50 (group A), 17.05 ± 5.50 (group B), 9.50 ± 5.20 (group C). Thus, vacuoles were numerous in
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26 group A (difference between control group and group A was statistically significant, $P < 0.001$). In
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28 addition, vacuoles further increased with the progression of rehydration, reaching a maximum
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30 amount in group B (difference between groups A and B was statistically significant, $P < 0.05$) and
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32 diminishing again at the end of the rehydration process (difference between groups A and C was not
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34 statistically significant, $P = 0.3$) (Table 1; Graph 1).
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41 By LM and low magnification TEM, the organelles appeared evenly dispersed in the
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43 ooplasm of all frozen-thawed oocytes, as in the control samples, irrespective of the experimental
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45 group (A,B,C) (Fig.2). However, M-SER aggregates significantly diminished following thawing,
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47 and such a decrease in number was particularly evident in the oocytes belonging to groups A and B.
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49 In fact, the mean number \pm SD of M-SER was 0.20 ± 0.03 in group A (Control vs group A,
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51 $P < 0.001$) and 0.04 ± 0.03 in group B (Control vs group B, $P < 0.001$) (Table 1; Graph 2). On the
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53 contrary MV complexes, small and scarce in control oocytes, augmented in number after thawing,
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55 being especially abundant in the oocytes belonging to group B. Specifically, the mean number \pm SD
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1 of MV in 100 μm^2 was 1.12 ± 0.40 in group A (Control vs group A, $P < 0.05$) and 2.36 ± 0.42 in
2 group B (Control vs group B, $P < 0.001$). (Table 1; Graph 3). At the end of the rehydration process
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4 organelle associations showed an opposite trend: in fact, M-SER aggregates increased again in
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6 number - though never reaching the abundance shown in control oocytes - whereas MV complexes
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8 diminished in the oocytes belonging to group C, being respectively 0.87 ± 0.05 (Control vs group C,
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10 $P < 0.001$) and 0.67 ± 0.29 (Control vs group C, $P = 0.6$). (Table 1; Graphs 2,3).
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14 By TEM, vacuoles, ranging in size from 0.5 to 4 μm , appeared delimited by membranes that
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16 were at times interrupted and characterized, in some parts, by densely organized indentations or
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18 niches. The inside of these compartments was scarcely electron-dense in comparison to the
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20 surrounding cytoplasm and occasionally contained cell debris (Fig. 3). Sometimes, secondary
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22 lysosomes were found in the proximity of the vacuoles (Fig. 3a,d). In the group B the more
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24 pronounced vacuolization was often associated with an increased density of the cytoplasmic matrix
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26 (Fig.3b).
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31 A normal pattern of organelles was usually detected by TEM in ooplasm of the
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33 cryopreserved oocytes belonging to all experimental groups, including the ooplasmic areas adjacent
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35 to vacuoles (Figs. 3a,c; 4a). With this regard mitochondria, M-SER aggregates and MV complexes
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37 did not show overt qualitative ultrastructural changes if compared to those organelles and organelle
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39 associations found in control oocytes (Fig. 4b,c). However, a percentage of small M-SER
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41 aggregates was found (with a diameter of SER networks of 1-2 μm), particularly in the oocytes
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43 belonging to groups A and B, whereas unusually large MV complexes (up to 2.5 μm in vesicular
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45 diameter) were sometimes found in the oocytes belonging to group B.
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51 TEM analysis also revealed that CGs were scanty, arranged in a discontinuous layer, and
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53 sometimes scarcely electron-dense in the cryopreserved oocytes belonging to all experimental
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55 groups in respect to those found in the control group (Fig. 5). In addition, when a morphometric
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57 evaluation was performed, the mean number \pm SD of CGs per 10 μm was 6.28 ± 1.12 (group A),
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3.17 ± 0.28 (group B), 2.33 ± 0.50 (group C), suggesting that CGs underwent an initial reduction at the beginning of rehydration (difference between control group and group A was statistically significant, P<0.001) and further decreased in number as rehydration progressed (differences between groups A and B and between groups B and C were statistically significant, P<0.001 and P<0.05 respectively) (Table 1; Graph 4).

Numerous microvilli were also seen bordering the oolemma and projecting into the PVS (Figs. 2d-f; 5a-c). In sections lying on appropriate planes, the PBI was detected in the PVS and the MII spindle was found in the ooplasm.

DISCUSSION

In humans, numerous studies suggest that post-thaw survival rates of oocytes that have undergone slow freezing are inferior to those of oocytes subjected to vitrification procedures [56]. In addition, oocyte vitrification, compared to slow freezing, probably increases implantation and pregnancy rates [57,58]. The results, however, as reported in the Italian ART registry, are not homogeneous among clinics and protocols [59] since there is a wide variation in pregnancy rates among different centers [58]. Further, as it results from a general survey of the literature, the total number of women and pregnancies in the included trials were low and the evidence was limited by imprecision [57]. Moreover, ultrastructural dysmorphisms have been identified in both vitrified-warmed and frozen-thawed human MII oocytes, although at a different extent [17].

In this regard, while vitrification seems to have a clear role in ART, continued research to establish optimal slow freezing methods for human MII oocytes seems required, which may assist in alleviating concerns over safety issues related to vitrification, such as storage, transport and the

1 use of very high cryoprotectant concentrations [60]. Slow freezing of oocytes can thus be a still
2 valid tool in IVF practice when performed with a suitable protocol [61,62].
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4 The purpose of this study was firstly to investigate the phenomenon of vacuolization in
5 human MII oocytes subjected to slow freezing, since presence and extent of this ultrastructural
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The purpose of this study was firstly to investigate the phenomenon of vacuolization in human MII oocytes subjected to slow freezing, since presence and extent of this ultrastructural dysmorphism can be an important indicator of oocyte quality after cryopreservation. Secondly, we aimed to assess in the same oocytes the morphodynamics of typical oocyte organelles and organelle associations (M-SER aggregates, MV complexes, CGs) during freeze-thawing. In particular, we paid special emphasis to the structural and ultrastructural modifications occurring just after thawing, during the rehydration steps. In fact, although several factors have been successfully optimized in the protocols used to cryopreserve human oocytes, up to now post-thaw rehydration conditions received a limited attention [5,63,64]. Different rehydration conditions seem also to influence the survival of vitrified-warmed human oocytes [65]. Rehydration could, indeed, sensitize the oocyte and make it particularly vulnerable, since removal of the intracellular cryoprotectant and the re-establishment of the original water content occurring during this procedure are both sources of osmotic stress for the cell. In this view, it seems essential to know in detail in which step/steps of the rehydration procedure cryoinjuries may occur, in order to optimize rehydration conditions, too.

In this study we performed oocyte freezing according to the two-step PrOH dehydration protocol which, in our previous studies, appeared more affected by vacuolization than one-step PrOH protocol [12]. In this way we could deeply investigate the phenomenon of vacuolization. The oocytes were fixed after thawing, at different intervals during post-thaw rehydration and, precisely, after the passage in thawing solution 1 (group A), thawing solution 2 (Group B) and thawing solution 3 (Group C).

General features

1 All the oocytes showed similar shape, dimensions and overall appearance, irrespective of
2 their classification (control or experimental groups A, B, and C). Thus, neither freezing nor
3 thawing, including the different steps of post-thaw rehydration, seemed to associate with any
4 significant variation in volume and/or general appearance of the oocytes. This feature well
5 correlates with previous observations on human mature oocytes subjected to different protocols of
6 slow freezing [12-14,17] or vitrification [15,17,55,66], further emphasizing that current
7 cryopreservation protocols do not significantly impair oocyte general architecture.
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17 By LM and TEM the organelles appeared uniformly dispersed in the ooplasm of all the
18 oocytes. However, organelle-specific differences were found between control and frozen-thawed
19 oocytes as: extent of vacuolization and differences in the number of M-SER aggregates, MV
20 complexes and CGs.
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28 Vacuoles

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31 We found a slight to moderate vacuolization in the cryopreserved oocytes belonging to all
32 experimental groups. Vacuoles were instead only occasionally present in the ooplasm of fresh
33 oocytes. In particular, vacuoles were already found in the group A, after oocyte exposure to the
34 thawing solution 1, suggesting that they may form during freezing and/or at thawing. Further,
35 vacuoles increased in number as post-thaw rehydration proceeded. They reached a maximum
36 amount in group B, after oocyte exposure to the thawing solution 2, which contains the lowest
37 concentration of PrOH during PrOH step-wise dilution. Finally, vacuoles decreased again in
38 number at the end of the rehydration process (group C), after oocyte exposure to the thawing
39 solution 3, which is PrOH-free. A measurable number of vacuoles, however, remain in the ooplasm
40 of frozen-thawed oocytes, indicating that their recovery is largely incomplete.
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56 An extensive, typical vacuolization is commonly observable in the mature oocytes of some
57 mammals, like cattle and sheep [67,68]. Vacuoles are also physiologically found in a small amount
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1 in human immature, GV-stage oocytes [22,66]. True vacuoles are instead a quite uncommon feature
2 in the ooplasm of human MII oocytes. Thus, their abundance in frozen-thawed oocytes can be
3 interpreted as a manifestation of oocyte stress during cryopreservation. As introduced above, the
4 degree of oocyte vacuolization significantly increases by applying slow freezing [12-14,17,69],
5 whereas data on the presence of vacuoles in vitrified oocytes appear still undefined and
6 controversial, possibly depending on the vitrification system adopted (open or closed) and/or on the
7 device used as oocyte support [15,17,66,69,70]. Taken together, however, all these data indicate
8 that vacuoles are less represented in vitrified than in slow-frozen oocytes.
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19 In our cryopreserved samples, vacuoles were thoroughly distributed in the ooplasm, with a
20 slight tendency to accumulate in the deeper ooplasm. Vacuoles, in general, may derive from
21 swelling and coalescence of Golgi and/or SER membranes [21,71], possibly associated to
22 cytoskeletal defects [14,22]. In the mature oocyte SER membranes, when transforming into
23 vacuoles, become interrupted and lose their close association with mitochondria, typical for M-SER
24 aggregates and MV complexes, acquiring degenerative features [38]. Disruption of such a
25 “molecular hug” between mitochondria and SER [72] may contribute to the occurrence of altered
26 calcium transients in cryopreserved oocyte. Peripheral vacuoles may also originate from oolemmal
27 invaginations and/or clusters of endocytotic vesicles forming in the oocyte cortex, as it occurs in
28 oocytes exposed to cryoprotectants only [12,50]. At this regard, however, we should make a
29 distinction between vacuoles and mere “oolemmal pouches”. From an ultrastructural point of view,
30 in fact, the former appear delimited by membranes sometimes interrupted or indented. The latter are
31 instead more composite in nature, being bordered by an intact oolemma provided with microvilli
32 and surrounded by a thin rim of CGs [66]. However, we cannot exclude that part of the vacuoles
33 may represent an involution of primitive pouch-like oolemmal invaginations. In cryopreserved
34 oocytes vacuoles could also derive from altered, swollen mitochondria [46,47] or from the fusion of
35 degenerating CGs, associated to an extensive loss of the electron-dense granule content [48].
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1 In this study, we sometimes identified secondary lysosomes adjacent to vacuoles. Vacuoles
2 and lysosomes may fuse, forming structures with a mixed content involved in the degradation of
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4 ooplasmic material via autophagy [73]. Some authors recently hypothesize that autophagic
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6 activation in cryopreserved oocytes could be a natural, adaptative response to “cold” stress [74,75].
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9 In addition, we found that the more marked vacuolization of the oocytes belonging to group
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11 B was often associated with an increased density of the ooplasm. All these features are similar to
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13 those found in post-mature, atretic oocytes unfertilized after *in vitro* insemination [20]. This is a
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15 further prove that regressive changes in cryopreserved oocytes are consequent to altered
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17 cytoplasmic dynamics that are particularly expressed when the cryoprotectant PrOH reaches its
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19 lowest concentration in the second step of rehydration.
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26 M-SER aggregates and MV complexes

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28 In this study we also found that significant variations, of opposite trend, occurred during
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30 freeze-thawing in size and number of M-SER aggregates and MV complexes.
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33 In particular, M-SER aggregates, large and abundant in the ooplasm of fresh controls,
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35 decreased in size and number after thawing, particularly in the oocytes belonging to groups A and B
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37 (first and intermediate stages of post-thaw rehydration), indicating a special sensitivity of these
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39 aggregates to cryoprotectant exposure and, particularly, to PrOH withdrawal. On the contrary, M-
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41 SER aggregates significantly increased in group C, where PrOH was completely removed from the
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43 thawing solution and a recovery of metabolic activities occurred. An opposite trend was observed
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45 for MV complexes. These structures were, in fact, typically small and scarce in fresh controls but
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47 showed an increase in size and number after thawing, being especially numerous and large in the
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49 oocytes belonging to groups A and B, to decrease again in group C, at the end of rehydration. In this
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51 regard, it is worth noting that the changes described for MV complexes closely resemble those
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53 previously reported for vacuoles.
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On the basis of these findings, we suggest that ooplasmic membranes, whose dynamic structure may be regulated by cytoskeletal activity, as occurs in other cells [76], become capable of transforming into each other under an appropriate stimulus. According to this view, SER elements could dynamically acquire different shapes (tubules or vesicles) depending on the metabolic/structural needs of the cell, actually belonging to the same system of interconnected membranes. In fact, transitional figures with intermediate characteristics between tubules and vesicles have been observed by TEM [20]. In particular, we speculate that M-SER aggregates and small MV complexes, commonly found in the ooplasm of MII oocytes before freezing, can both give rise to numerous, large MV complexes after thawing and during rehydration, through a generous SER membrane reassembly. This is confirmed by previous studies in which the authors suggested that aging and/or prolonged culture can elicit a similar transition into the oocyte. In fact, numerous and large MV complexes were found in MII oocytes sampled from reproductively aged patients (oocytes aged in vivo) [38] or kept in vitro for 9 up to 48 hours (oocytes aged in vitro) [17,20,38]. Large MV complexes are also present in GV oocytes that have reached MII stage after 24 hour-culture (in vitro matured oocytes) [77,78]. Therefore, M-SER to MV transition do not seem related only to aging or culture period but can be also induced in cryopreserved oocytes by step-wise dilution of PrOH during post-thaw rehydration.

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In this study we also originally reported through a morphological approach that a reversal of this phenomenon of membrane “recycling” occurs at the end of freeze-thawing, when the rehydration process is completed and culture conditions regain a more physiological state. As a consequence of this, the large vesicular component of MV complexes could shrink again to form small vesicles and tortuous, anastomosing SER tubules of M-SER aggregates. This well correlates with previous reports on human oocytes subjected to slow freezing and treated with PrOH, which did not evidence at the end of the procedure of slow freezing any qualitative ultrastructural change of these aggregates in respect to fresh controls [12,13,17]. However, from a quantitative

1 morphometric analysis, we hereby reported a complete recovery only for MV complexes, whereas
2 M-SER aggregates do not reach the number found in fresh controls, thus undergoing a reliable but
3 partial recovery at the end of the rehydration process.
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7 Cryopreservation has been reported to affect calcium oscillation in the human oocytes [79].
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9 In this vein, since, as reported above, calcium levels in the oocyte are regulated by a correct cross-
10 talk between mitochondria and associated ooplasmic membranes, membrane reassembling during
11 rehydration may produce altered, although temporary, calcium transients, possibly interfering with
12 oocyte competence to fertilization.
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19 It seems also worth noting that, irrespective of the above described diffuse recycling of
20 ooplasmic membranes, associated mitochondria appear concerned neither by freezing nor by
21 thawing and its sequential post-thaw rehydration steps, maintaining unaltered their ultrastructure.
22 This finding further reinforces the concept that both slow freezing and vitrification procedures do
23 not significantly affect mitochondrial structure in human MII oocytes [12-15,17].
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31 Finally, on the basis of what discussed above, we cannot rule out that vacuoles and their
32 membranes can play an active role in the membrane recycling that occurs during freeze-thawing. In
33 fact, the membranes of M-SER aggregates and MV complexes could sometimes derail during their
34 reassembling, becoming oriented toward vacuole transformation.
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43 Cortical granules

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45 In this study we revealed that CGs were scarce in the oocytes belonging to all experimental
46 groups in respect to those found in the fresh control group. CGs, that are trapped by a network of
47 actin filaments in the subplasmalemmal area of fresh MII oocytes until fertilization occurs [21],
48 gradually diminished as post-thaw rehydration progressed, reaching their lowest concentration in
49 group C, and thus revealing the occurrence of a gradual but progressive loss during freeze-thawing.
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58 This feature well correlates with previous reports in which an ubiquitary reduction in number of
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1 CGs was found at the end of the cryopreservation procedure, irrespective of the protocol applied
2 (slow freezing, vitrification with closed or open devices) [12-15,17]. As previously noted, a reduced
3 amount and electron-density of CGs in cryopreserved oocytes may be due to the occurrence of a
4 premature exocytosis of the CG content into the PVS with the consequent hardening of the inner
5 aspect of the ZP, a filamentous extracellular matrix that covers the oocyte/embryo until
6 implantation [80]. Such hardening, caused by the increase in density and compactness of the ZP
7 filaments, leads to a predictable low fertilizability of the oocyte through the block of sperm
8 penetration, only overcome by ICSI procedures [12].
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19 The novelty of our observations on CG morphodynamics during cryopreservation is related
20 to the following considerations. Firstly, the whole freeze-thawing procedure may induce CG loss
21 during cryopreservation. The reduction of CGs was in fact already evident in oocytes from group A.
22 This means that the CG release could begin during freezing and/or at thawing. Secondly, the further
23 reduction of CG observed in groups B and C, indicates that the CG exocytosis does not stop after
24 thawing, but continues throughout the following phases of rehydration. Such CG loss is thus the
25 only phenomenon, among those described in this study, apparently not subjected to any kind of
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39 Several studies have shown that the mere exposure of mature oocytes to cryoprotectants
40 leads to a reduction in the number and electron-density of CGs [24,50]. Therefore, also on the basis
41 of these reports, we can further emphasize that the progressive CG loss reported in this study may
42 be related not only to low temperatures, but also to the processes of cryoprotectant addition
43 (dehydration step) and removal (rehydration step). Cryoprotectant addition, in particular, has been
44 reported to have a role in inducing a precocious oocyte activation by increasing calcium intake. In
45 fact, exposure to PrOH in calcium-containing media is likely to induce a premature calcium
46 increase sufficient to trigger CG exocytosis in human oocytes and consequent zona hardening
47 [25,81]. More recently, Gualtieri et al., by using the calcium ionophore A23187, observed a
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1 significant delay of the recovery of intracellular calcium to basal levels in frozen-thawed oocytes
2 [69]. According to the results obtained in our work, it can be assumed that the concentration of
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4 cytosolic calcium, once altered during dehydration, may further increase during the rehydration and
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6 consequent PrOH withdrawal, thus resulting in the described continuous, progressive CG release.
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9 In addition, the gradual loss of CGs during freeze-thawing leads us to hypothesize that the
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11 membranes of the exocytosed granules may not only be reintegrated in the oolemma but may also,
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13 at least in part, contribute to the above described ooplasmic membrane recycling.
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19 Conclusions and Future Perspectives

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21 In this report we have revealed that oocyte ultrastructural dysmorphisms related to
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23 cryopreservation and possibly responsible of low oocyte fertilizability not only occur during
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25 freezing and thawing, in a strict sense, but also during post-thaw rehydration. These cellular
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27 alterations, induced by low temperatures and by osmotic and chemical forces produced during
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29 cycles of dehydration-rehydration as well, may alter the distribution and activity of oocyte cellular
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31 components.
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36 In particular, although slow freezing appears to ensure a good overall preservation of the
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38 oocyte, nevertheless vacuolization and CG release remain crucial limits. It seems also worth noting
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40 that all systems of ooplasmic membranes appear significantly affected by freeze-thawing but,
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42 except for CGs, their alterations seem to undergo a partial or, more rarely, an almost complete
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44 recovery after thawing, at the end of the rehydration process. In addition, the observed variations in
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46 the number of M-SER aggregates and MV complexes, occurring during freeze-thawing, suggest
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48 that a dynamic process of transition between these two forms of organelle associations may occur.
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51 At this regard, it should not be excluded that vacuole and CG membranes, and oolemma as well,
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53 may take part in the recycling mechanism. Such shuttle of membranes, starting during freezing
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55 and/or at thawing but mainly occurring during rehydration, may be related to alterations of the
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1 cytoskeletal stiffness [82] presumably due to PrOH administration and/or withdrawal [83,84]. We
2 cannot exclude, of course, that the described membrane restructuring is also related to calcium
3 disturbances. From a merely morphological point of view, this recycling reveals a sort of
4 morphogenetic multipotence of the oocyte cytomembranes, possibly eliciting membrane turnover
5 and delivery or clearance of substances (CG content, cryoprotectants, calcium, other solutes?), as
6 postulated for other cells [85].
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14 Finally, a similar ultrastructural approach could be applied in the future to the study of the
15 rehydration process in slow-frozen oocytes undergoing rapid warming [62] and in vitrified-warmed
16 oocytes belonging to both conventional and low-cryoprotectant vitrification protocols [86].
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FIGURE CAPTIONS

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5 **Fig. 1** Fresh human MII oocytes. The general features and organelle microtopography are shown by
6 LM (Fig.1a) and TEM (Fig. 1b). Note the rounded shape of the oocyte (O), the narrow PVS, the
7 intact ZP and the uniform distribution of organelles in the ooplasm. Among the organelles,
8 numerous mitochondria (M), M-SER aggregates and MV complexes can be found. By TEM, details
9 of a M-SER aggregate and of several, small MV complexes are seen in Fig.1c. A rim of CG is also
10 seen just beneath the oolemma in Fig.1d. mv: microvilli. Bar is: 45 μm (Fig.1a); 5 μm (Fig.1b); 1
11 μm (Fig.1c); 2 μm (Fig.1d)
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26 **Fig. 2** Cryopreserved human MII oocytes. By LM (Fig.2a-c) and TEM (Fig. 2d-f) no overt
27 difference in shape, dimensions and organelle distribution is seen among the oocytes (O) belonging
28 to group A (Fig. 2a,d), B (Fig. 2b,e) and C (Fig. 2c,f) and between fresh (see Fig. 1) and
29 cryopreserved oocytes (Fig. 2). Note the intact ZP (Fig. 2a-c) and the presence of microvilli (mv) on
30 the oolemma (Fig. 2d-f). Numerous vacuoles (Va) are seen in all cryopreserved oocytes,
31 particularly abundant in group B (Fig. 2b,e). The apparent reduced dimensions, enlargement of the
32 PVS and increased ZP thickness of the oocyte shown in Fig. 2c is an effect of the section plane (not
33 equatorial). arrowheads: MII spindle with chromosomes; star: large vacuole possibly due to a gas
34 bubble; M: mitochondria; SER: smooth endoplasmic reticulum; MV: MV complexes; asterisk:
35 remnants of the PBI. Bar is: 45 μm (Fig. 2a); 40 μm (Fig. 2b); 35 μm (Fig. 2c); 2 μm (Fig. 2d-f)
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54 **Fig. 3** Cryopreserved human MII oocytes. By TEM, vacuoles (Va) are present in the ooplasm of the
55 cryopreserved oocytes belonging to group A (Fig. 3a,d), B (Fig. 3b) and C (Fig. 3c). Vacuoles
56 frequently appear empty (Fig. 3a-d) and may be delimited by a discontinuous membrane (Fig. 3d).
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1 A close association between vacuoles and lysosomes (Ly) is seen in Fig. 3a,d. Mitochondria (M),
2 SER networks and MV complexes are seen in the areas among vacuoles (Fig. 3a,c). Note the
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4 increased density of the cytoplasmic matrix in group B (Fig. 3b) in comparison with fresh controls
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7 (Fig. 1b-d) and groups A and C (Fig. 3a,c,d). Bar is: 1 μm (Fig. 3a-c); 0.5 μm (Fig. 3d)
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15 **Fig. 4** Cryopreserved human MII oocytes. Oocytes belonging to group A, representative pictures of
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17 mitochondria (M), M-SER aggregates and MV complexes, as seen by TEM. Note the presence of
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19 well preserved mitochondria (Fig. 4a-c) and of typical M-SER aggregates (Fig. 4a,c) and MV
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21 complexes (Fig. 4a,b) of various sizes. Differently from vacuoles, the vesicles belonging to MV
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23 complexes are filled with a slightly electrondense material, are surrounded by an intact membrane
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25 and are closely associated to mitochondria. Va: vacuoles. Bar is: 1 μm (Fig. 4a-c)
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34 **Fig. 5** Cryopreserved human MII oocytes. In cryopreserved oocytes belonging to group A (Fig. 5a),
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36 B (Fig. 5b) and C (Fig. 5c) CG appear by TEM sparse or isolated, forming a discontinuous layer.
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38 mv: microvilli. Bar is: 1 μm (Fig. 5a-c)
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Figure 1

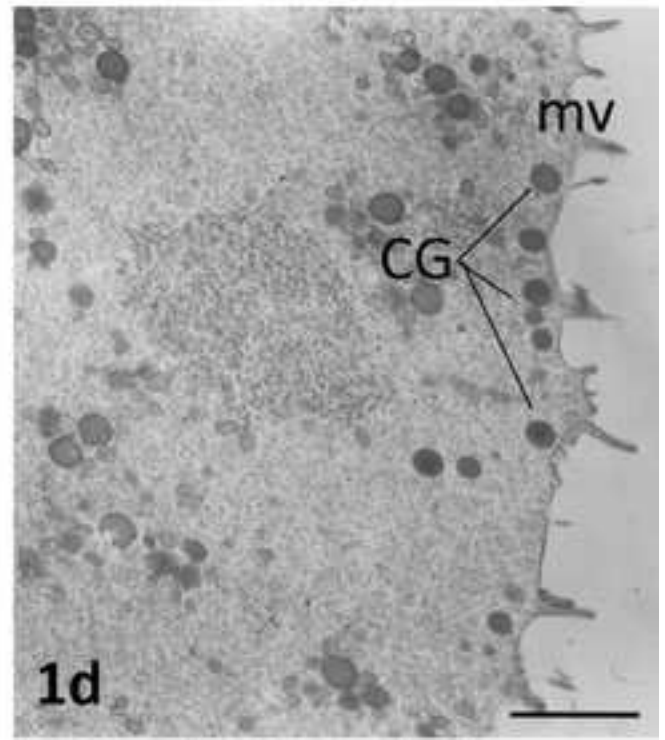
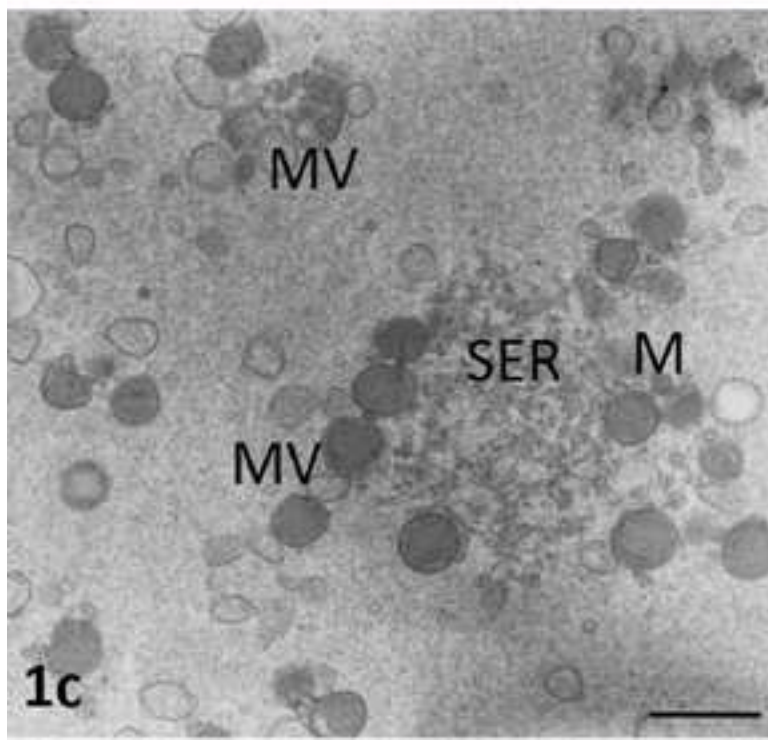
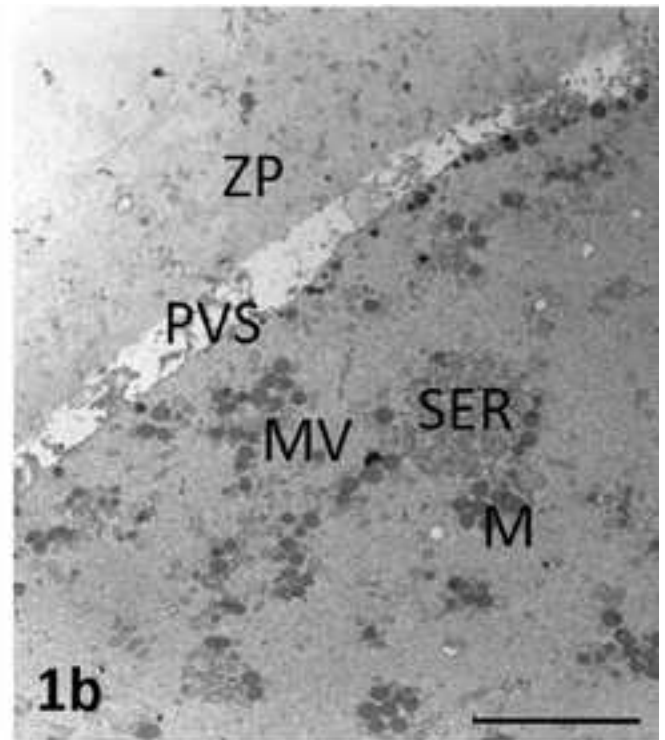
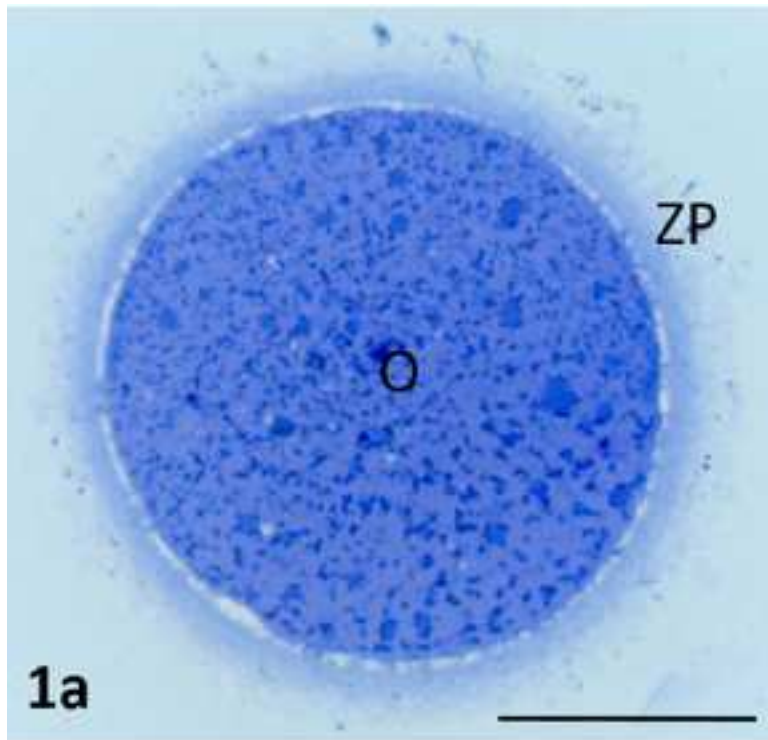


Figure 2

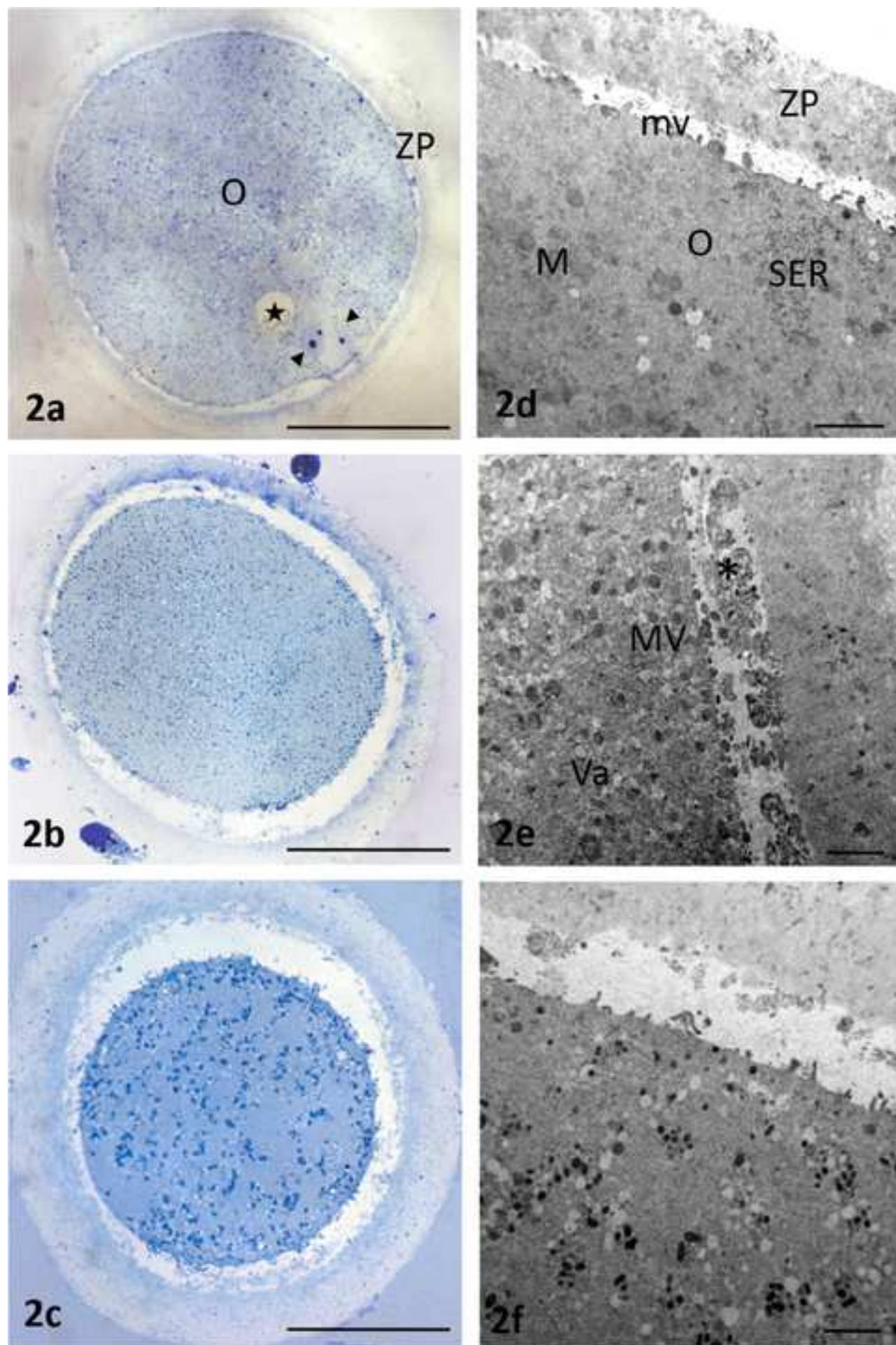


Figure 3

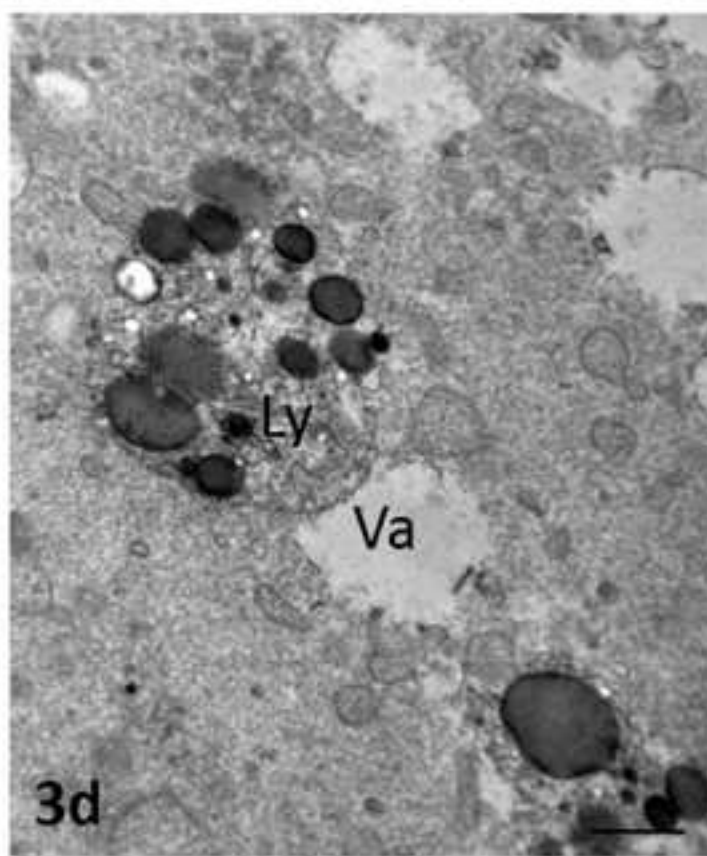
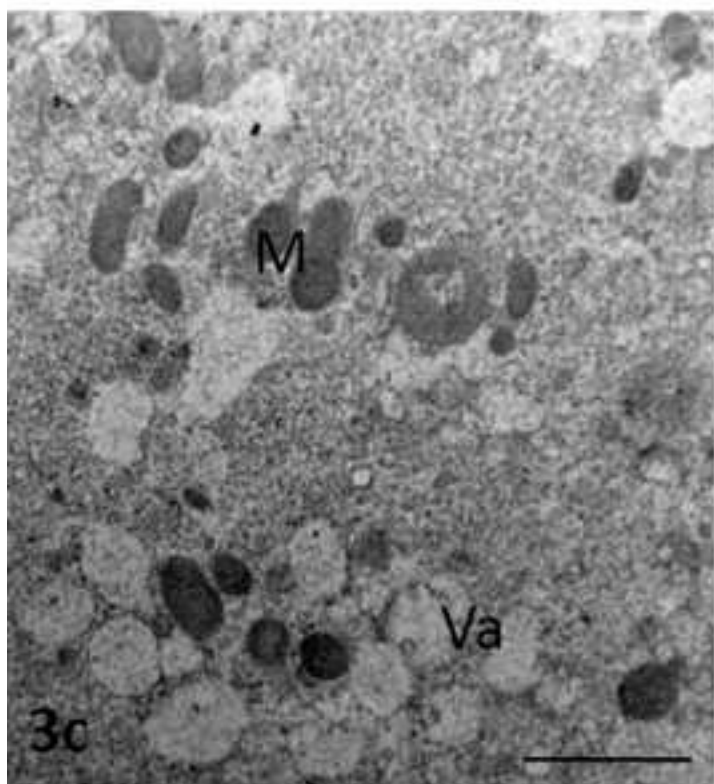
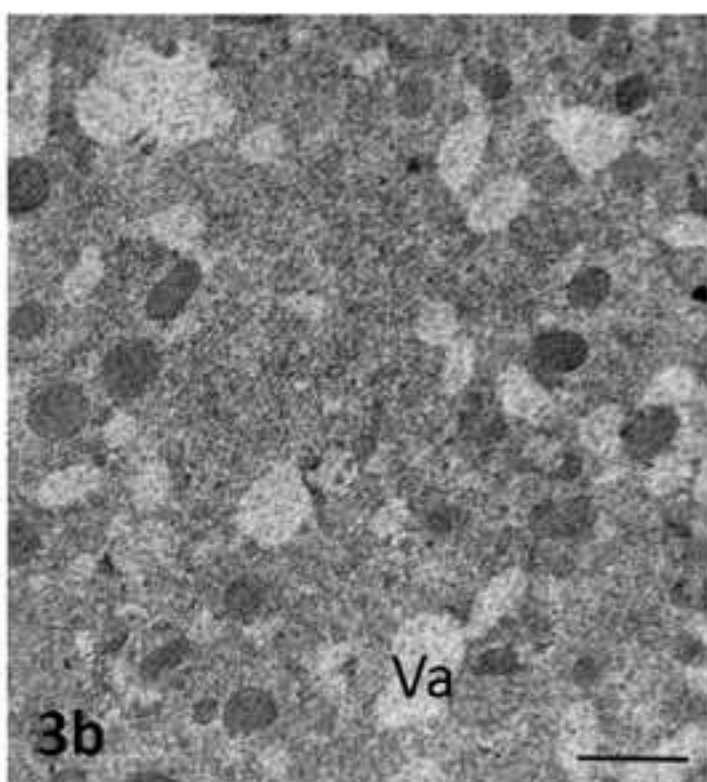
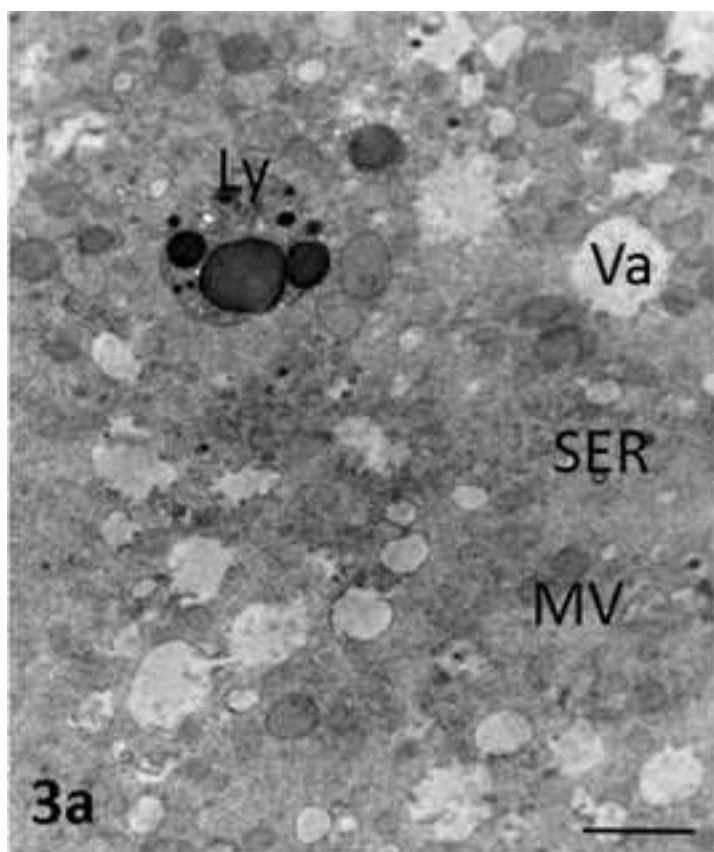


Figure 4

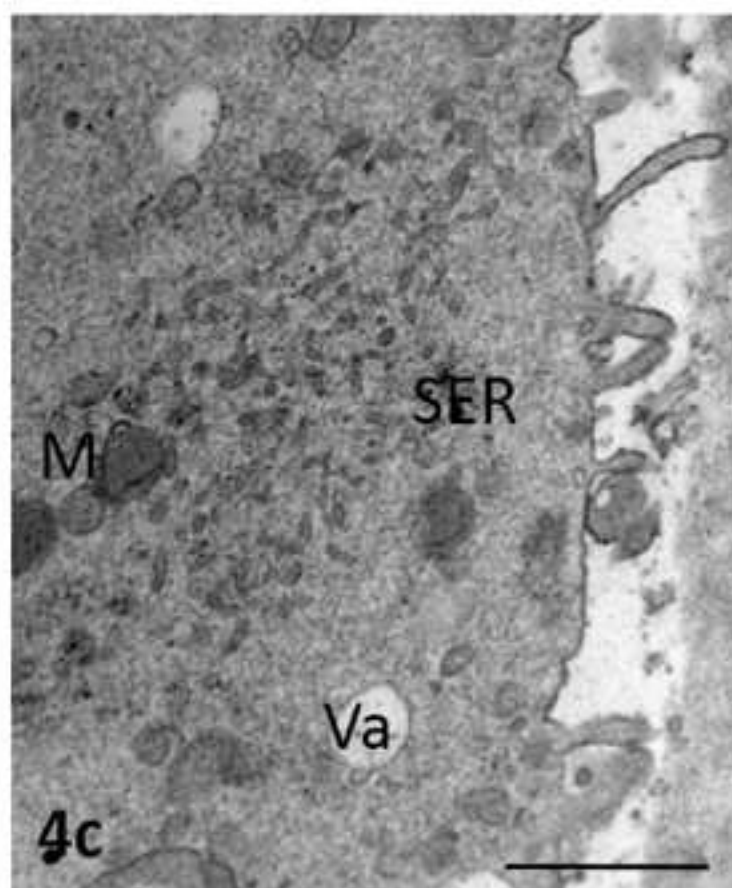
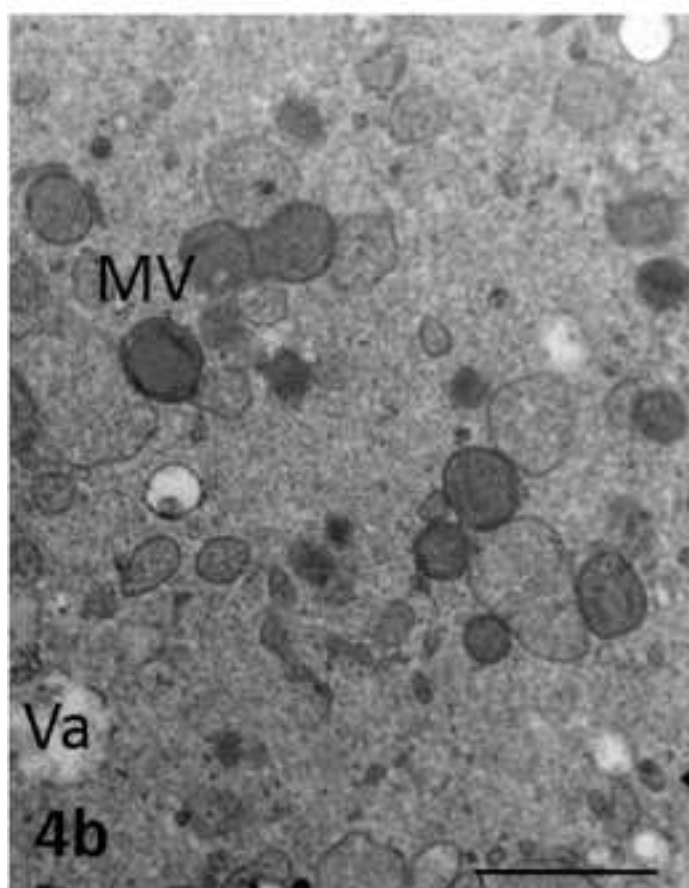
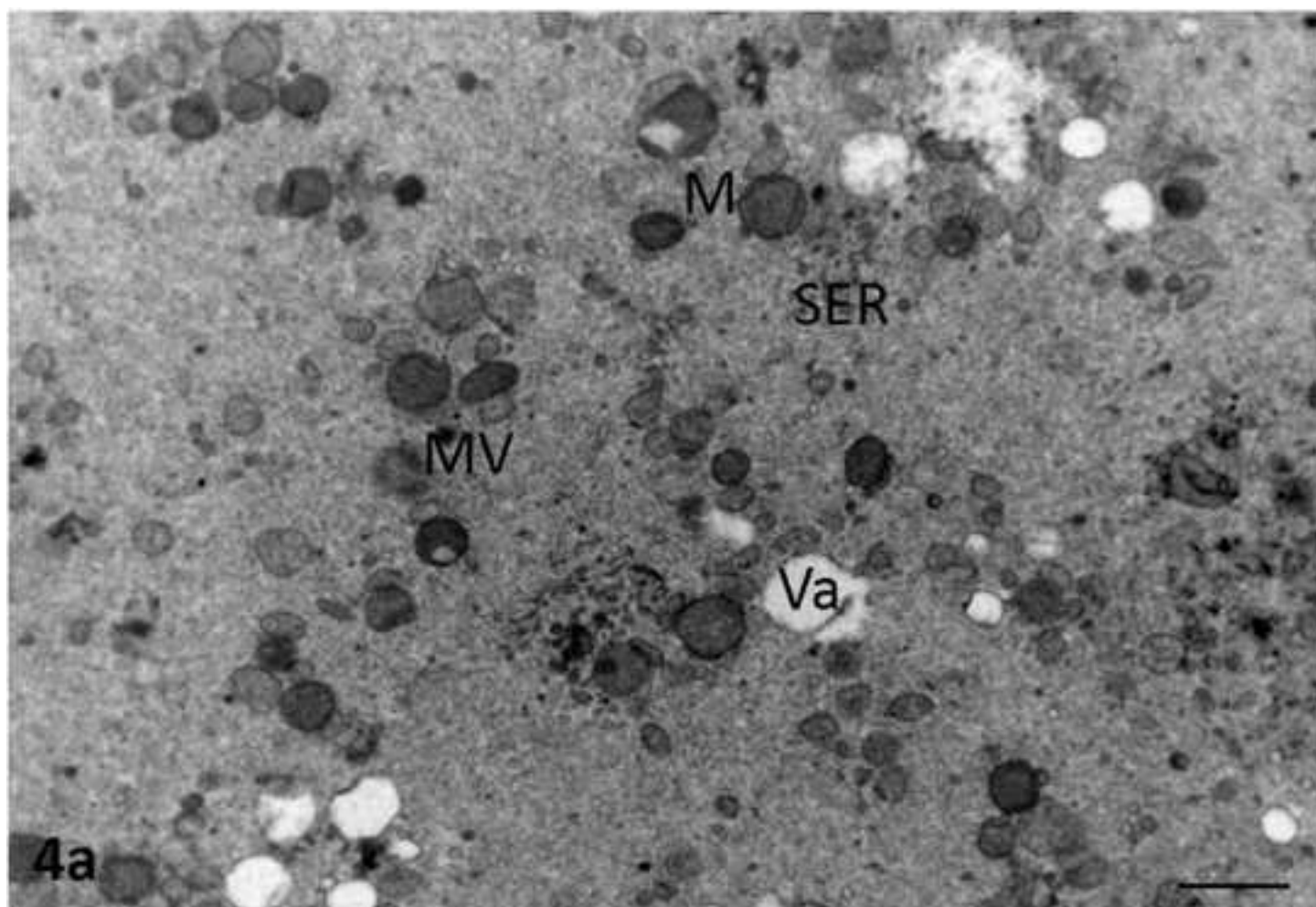
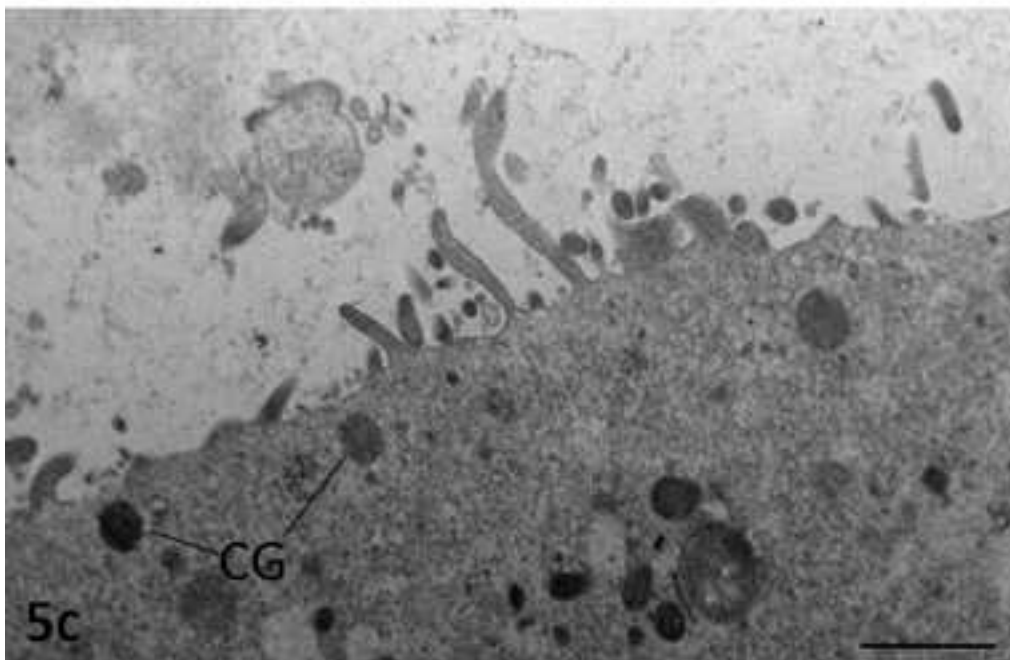
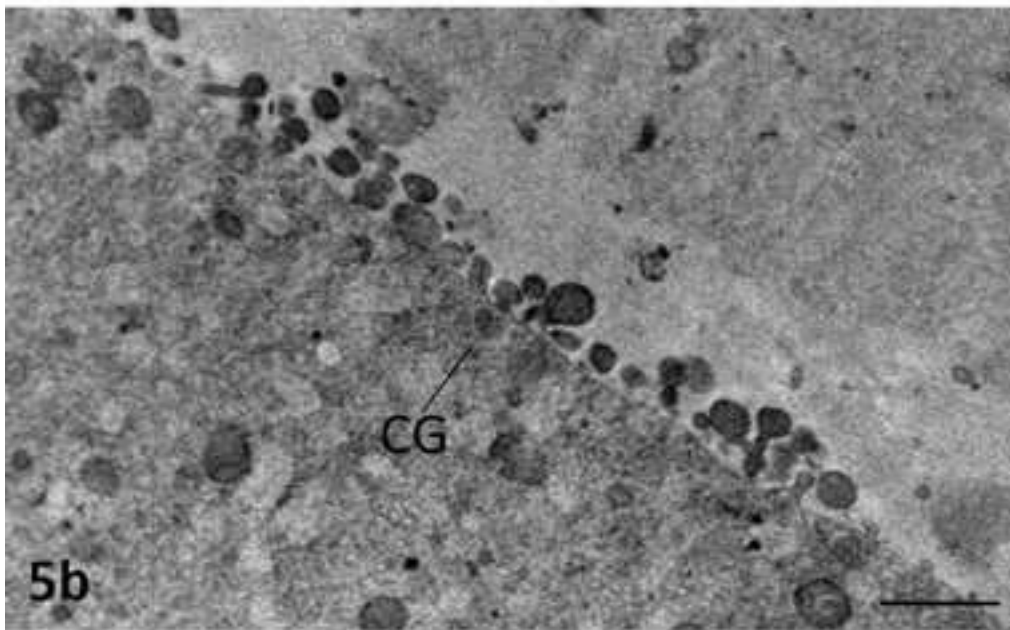
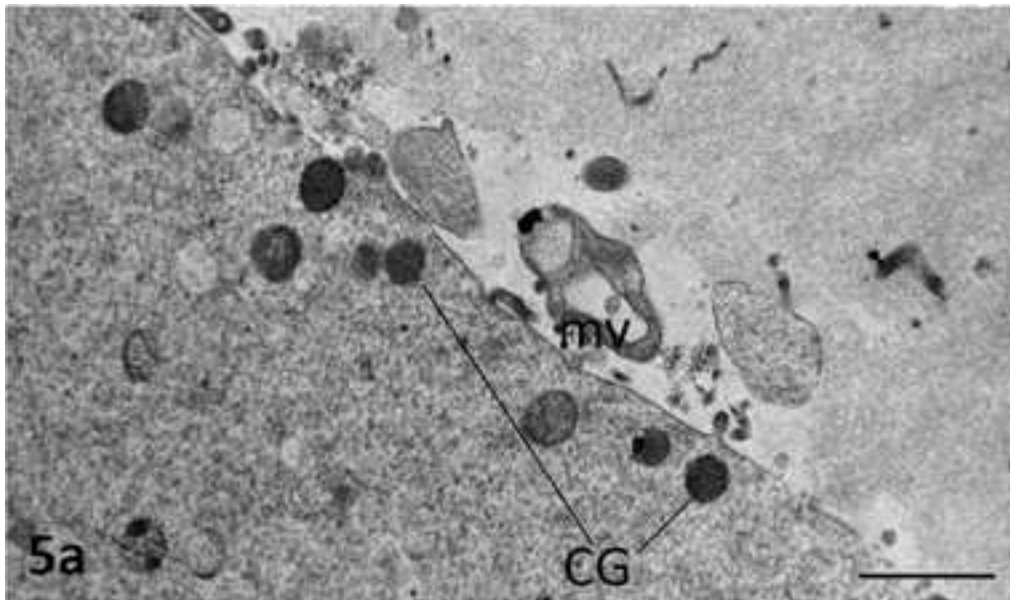
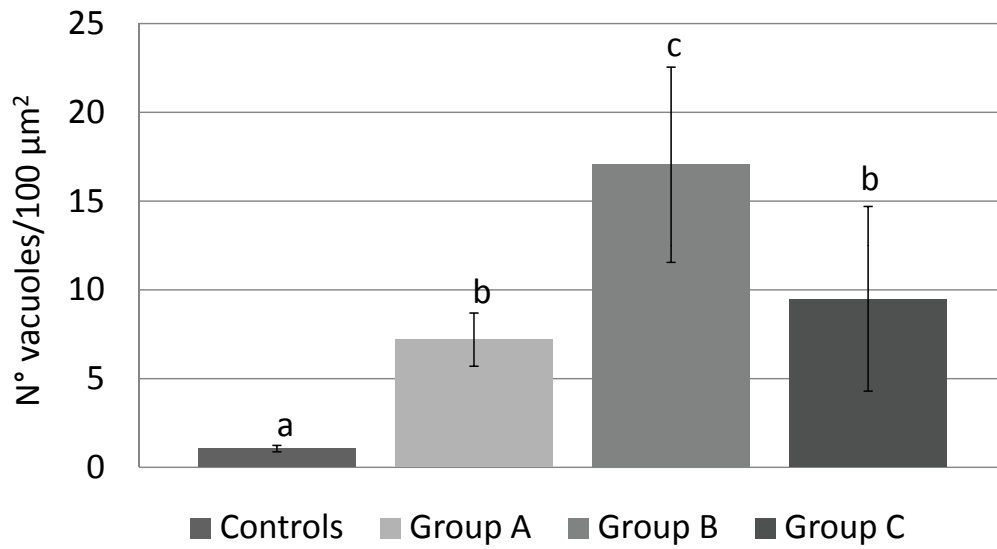


Figure 5

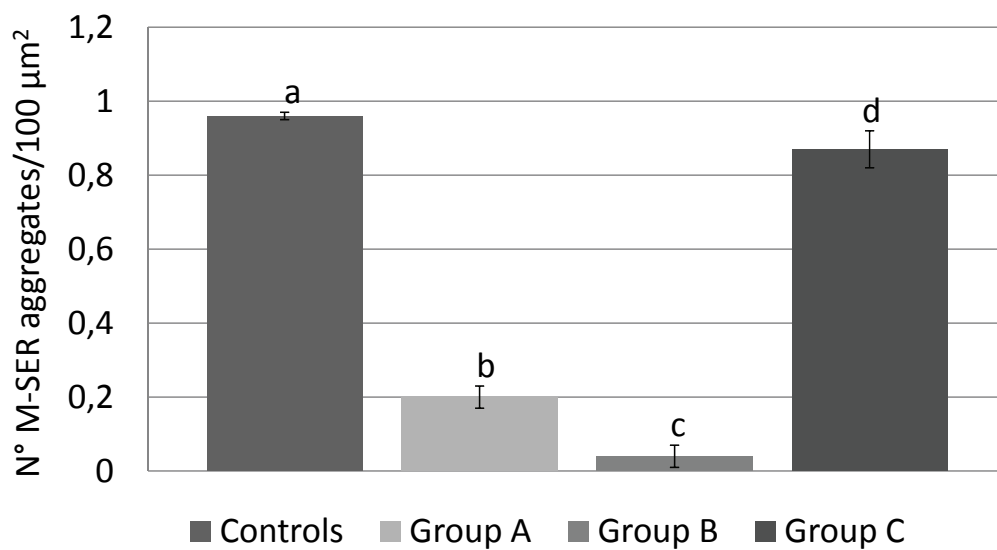


Graph 1



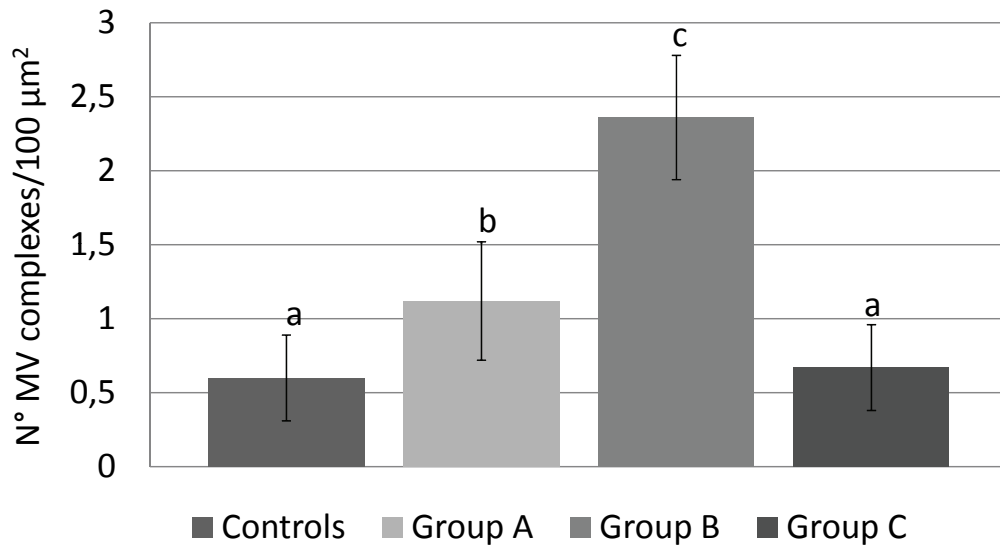
Number of vacuoles (vacuole diameter $\geq 0.5 \mu\text{m}$) per $100 \mu\text{m}^2$ of oocyte area. Values for each group are expressed as mean \pm SD. Different letters indicate significant differences ($P < 0.05$).

Graph 2



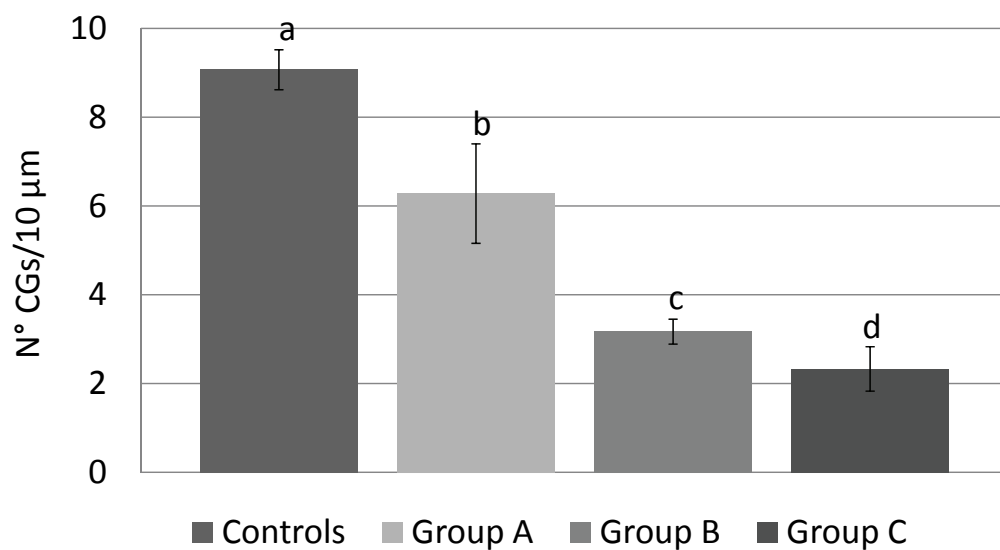
Number of M-SER aggregates (SER network diameter $\geq 0.5 \mu\text{m}$) per $100 \mu\text{m}^2$ of oocyte area. Values for each group are expressed as mean \pm SD. Different letters indicate significant differences ($P < 0.05$).

Graph 3



Number of MV complexes (vesicle diameter $\geq 0.5 \mu\text{m}$) per $100 \mu\text{m}^2$ of oocyte area. Values for each group are expressed as mean \pm SD. Different letters indicate significant differences ($P < 0.05$).

Graph 4



Number of CGs per $10 \mu\text{m}$ of oocyte linear surface profile. Values for each group are expressed as mean \pm SD. Different letters indicate significant differences ($P < 0.05$).

Table 1. Morphometric evaluation of vacuolization pattern and organelle morphodynamics in human MII oocytes subjected to slow freezing and fixed after thawing, at different intervals during post-thaw rehydration.

	Controls (Fresh)	Cryopreserved		
		Group A	Group B	Group C
N° of vacuoles/100 μm^2 (vacuole diameter $\geq 0.5 \mu\text{m}$)	1.06 ± 0.18^a	7.20 ± 1.50^b	17.05 ± 5.50^c	9.50 ± 5.20^b
N° of M-SER aggregates/100 μm^2 (SER network diameter $\geq 0.5 \mu\text{m}$)	0.96 ± 0.01^a	0.20 ± 0.03^b	0.04 ± 0.03^c	0.87 ± 0.05^d
N° of MV complexes/100 μm^2 (vesicle diameter $\geq 0.5 \mu\text{m}$)	0.60 ± 0.29^a	1.12 ± 0.40^b	2.36 ± 0.42^c	0.67 ± 0.29^a
N° of CGs/10 μm	9.07 ± 0.45^a	6.28 ± 1.12^b	3.17 ± 0.28^c	2.33 ± 0.50^d

Values are expressed as mean \pm SD. Statistical analysis is calculated between columns. Different letters indicate significant differences ($P < 0.05$).