Conditioned Mediums and Human Oocytes in Vitro Maturation

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ABSTRACT

Optimizing in oocyte culture media and in vitro maturation (IVM) system have been the controversial subjects. The aim of present study was to determine the effect of human umbilical cord mesenchymal stem cells (hUCM) conditioned medium, on ultrastructure of immature human oocytes after IVM.247 germinal vesicle (GV) oocytes were obtained from 117 patients who underwent an ICSI cycle. GV oocytes classified into: vitrified IVM (vIVM) and fresh IVM (fIVM);Two IVM mediums: 1- Alpha Minimum Essential Medium (α-MEM) and 2- conditioned medium (CM) supernatants derived from human umbilical cord Mesenchymal stem cells (hUCM).After 36h, the maturation rate and morphological feature of matured oocytes in four groups were evaluated; 40 matured oocytes (MII) were randomly obtained for ultrastructural electron microscopy study, which were later compared with 10 fresh in vivo matured oocytes, cancelled from ICSI. The highest maturation was evident in the hUCM fIVM, and lowest, in α- MEM vIVM (85.18% vs 71.42%). The mitochondria–vesicle (MV) complexes increased in IVM oocytes. Among IVM groups, the highest decrease in cortical granule distribution was in α- MEM vIVM, but the greatest presences of mitochondria–SER (M-SER) aggregates were in hUCM fIVM. In vIVM oocytes, oolemma was irregular with little microvillous organization.

The hUCM media have shown optimal efficacy for improving oocyte maturation and fine ultrastructural conservation; also there is a correlation between the oocyte ultrastructural feature and culture medium.

Key Words: Vitrification, Ultrastructure, IVM, Mesenchyme Stem Cells (Mscs), Oocyte

eIJPPR 2018; 8(2):64-71

HOW TO CITE THIS ARTICLE: Hakimeh Akbari, Seyed Hassan Eftekhar Vaghefi, Abbas Shahedi,Victoria Habibzadeh, Tooraj Reza Mirshekari, Majid Asadi Shekari, Reza Nejatbakhsh. (2018). "Conditioned mediums and human oocytes in vitro maturation" , international journal of pharmaceutical and phytopharmacological research, 8(2), pp.64-71.

INTRODUCTION

In the ovarian stimulation during assisted reproductive technology (ART), approximately 20% of human oocytes are immature, thus, cryopreserving immature oocytes in germinal vesicle stage (GV), and then, in vitro maturation (IVM) can suggest compensations. Also ethical issues can lead to avoiding embryo cryopreservation; consequently the use of immature oocytes for cryopreservation may circumvent some of the limitations associated with the vitrification/warming of mature oocytes, specifically relating to the functional integrity of the meiotic spindle and policy of resulting embryos. [1-4]. However, low survival rate of immature oocyte cryopreservation was reported, which is a serious problem [5]. Nevertheless, in vitrified MII oocytes, poor development was evident [6]. Not only nuclear maturation and cytoplasmic development of GV oocytes are not affected by vitrification, but also vitrified GV oocytes are protected from cryo-damage during IVM [7-9]. Some recovery mechanisms may occur during culturing in the IVM medium; however, one of the disadvantages of GV oocyte cryopreservation is that the vitrified GV oocyte must be warming before IVM [7-9]. Some factors such as the oocytes' meiotic stages, warming and cooling rates of

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest .
Received: 20 November 2017; **Revised:**12 March 2018; **Accepted:** 25 March 2018

vitrified oocytes influence the capacity of the cell to survive cryopreservation [10-12]. Depending on the type of cryopreservation protocols, type of maturation medium, and the developmental stage of the cell, effects of cryopreservation can be damaging to the cellular organization, depolimerization of microtubules and microfilaments, altered plasma membrane bilayer structure, functional and morphological cell impairment [12-14].Conditioned medium derived stem cell contains various cytokines, tissue regenerative agents, bioactive factors and growth factors, which were secreted from the stem cells, could recover oocyte meiotic in vitro maturation and subsequent embryo development. Furthermore different culture condition and the numerous conditioned media have been tested in several kinds of diseases show improvement in treatment Process and healing[15, 16]. In vitro studies have shown that stem cells (MSCs) actively prevent the function of several immune cells through secreted cytokines, growth factors and enzymes; Similarly, conditioned medium supernatants, derived from human umbilical cord Mesenchymal stem cells(hUCM), might provide useful factors for the culture medium, counting cytokines, amino acids, and vitamins, which affect serum component[16, 17]; might be useful for nuclear and cytoplasmic oocyte maturation and development, following in vitro fertilization (IVF). Either serum components in in vitro maturation medium, furthermore, could avoid zonapellucida hardening which could have a harmful effect on reproduction [17, 18].Oocyte quality is the most important factor enabling oocytes to be mature and normally fertilized [19]. Several studies showed an association between the rate of fertilization and morphological quality of IVM oocytes, yet phase contrast microscopy couldn't actually reveal analytical signs of oocyte quality, oocyte cytoplasmic maturation, and developmental competence, while electron microscopy can act as an effective device to evaluate ultrastructure features of IVM oocytes [13, 20, 21].While paracrine effects of (MSCs) have been proposed previously, human oocyte protection by hUCM secretions has never been established; since it is known that growth factors and cytokines stimulate meiotic progress, also meiotic improvement related with IVM processes; the aim of this study was to compare, the efficacy of hUCM conditioned medium, by means of Transmission electron microscopy (TEM), on maturation rate and ultrastructural features of human GV oocytes obtained from ICSI cycles after vitrification.

METHODS

Study period and patients

This experimental study included 247 germinal vesicle (GV) oocytes were obtained from 117 patients (30.5±5.6 years old) who practiced an ICSI cycle. All the patients underwent evaluation by the KermanMedical University's Ethical Committee (n: 93/692); and admitted toAfzalipour Infertility Center in Kerman (Kerman, Iran). Written informed consent was gotten from all participants. Criterion for female inclusion was the age lower than 35 years old; and the exclusion criteria were endometriosis, low ovarian response, abnormal chromosome, chronic anovulation, fewer than five oocytes on retrievaland poly cystic ovarian syndrome (PCOs).

Ovarian stimulation

Ovarian stimulation was attained by long protocol via administration of a combination of (GnRH) gonadotropin-releasing hormone and FSH (follicular stimulating hormone)(Serono, Geneva, Switzerland). Then, following follicular growth via transvaginal ultrasound, when adequate matured follicle was reached, injection of human chorionic gonadotropin (hCG) (IBSA Co, Switzerland) 10,000 IU was started; 36 hours later, the oocyte collection was carried out through, ultrasound-guided laparoscopy by using a single-lumen aspiration needle (Smiths Medical International, UK)[22].

Oocyte preparation

Next, cumulus oocyte complexes (COCs) were picked up and transferred in G-IVF culture medium (Vitrolife, Sweden) under mineral oil (Vitrolife, Sweden) in incubator for 2–3 hours. The COCs were denuded using 80 IU hyaluronidase (Sigma Co, USA) and underwent mechanical dissection by pipetting the COCs. Nuclear maturity of the denuded oocytes, presence of first Polar Body (PB), was evaluated under the dissecting microscope. Oocytes were classified as immature (GV or MI) or mature (MII). Then, MII oocytes were used for IVF or ICSI procedures, but GV oocytes were washed in 3-4 drops of washing media (Sage IVF),then vitrified or fresh GV oocytes, were incubated at 37°C and 5 % CO2 in two maturation media; after 48h the oocytes were evaluated for the first polar body presence by microscope(Olympus Co, Japan)[23].

IVM methodology

Following denudation, germinal vesicle oocytes were studied in four groups.

Group1: fresh GV oocytes were matured in α-MEM (Sigma Co, USA). Group 2: vitrified GV oocytes, following warming were matured in α-MEM. Group 3: fresh GV oocytes were matured in MSCs conditioned medium. Group 4: vitrified GV oocytes, following warming were matured in MSCs conditioned medium.

Two types of IVM culture mediums were used in this study:

Medium I: Alpha Minimum Essential Medium (α-MEM) as the basic control medium [24].

Medium II: conditioned medium (CM) supernatants derived from hUCM[25].

Vitrification

Vitrified immature oocytes according to Al-Hasani's (2007) vitrification method: At first, germinal vesicle oocytes were washed in Ham's F10 medium added with 20% (HSA) human serum albumin (Plasbumin Co, USA) solution. Then, they were submerged in an equilibration solution (ES) containing 7.5% dimethyl sulfphoxide (DMSO)(Merck Co, Germany) + 7.5% ethylene glycol (EG)(Merck Co, Germany), for 5 to 15 minutes, at room temperature, then, the oocytes were transferred to the vitrification solution (VS) containing 15% DMSO + 15% EG + 0.5 mol/l sucrose at room temperature for 50-60 seconds. Next, immediately, the oocytes were located inside cryotops (Vitrolife, Sweden) and instantly plunged into fluid nitrogen, and caps were fixed on the cryotop; finally, these oocytes were reserved for several months in the fluid nitrogen storage tank [26].

Warming

Next, the warming of the oocytes was carried out in the warming solution [Ham's F10 medium supplemented with 20% (HSA) as the basic medium] through four stages: 1) 1.0 mol/l sucrose (50-60 Seconds), 2) 0.5 mol/l sucrose (3 min), 3) 0.25 mol/l sucrose (3 min), and finally, 4) Ham's F10 medium added with 20% (HSA) (3-5 min), then the oocytes were placed randomly in one of the IVM culture media which was used for this study in incubator for 48 hours, but the oocytes viability was evaluated 2-3 hours after IVM by stereomicroscope [27].

MSC isolation and culture

The hUCM cells were provided from Kerman Medical University (Kerman-Iran); these cells were positive in mesenchymal stromal cell markers; CD44, CD73, CD90 andCD105. Also, the hUCM were induced to differentiate into adipocyte, osteocyte and neural cells [28].The α-MEM media was used as the basic control medium. These cells were cultured according to the Ling's method [29]. At first these cells were washed, with PBS (phosphate buffered saline) containing 100 mg/mL streptomycin (Gibco), 100 U/mL penicillin (Gibco) and collagenase I (Sigma), then these cells cultured in α-mem medium (Gibco) supplemented with 10% FBS (Gibco), 100 mg/mL streptomycin (Gibco) and 100 U/mL penicillin (Gibco), in the incubator. The medium was changed and after attaining complete cell confluence, they were trypsinized. After changing the medium and gathering of supernatant, it was filtered with 0.2µm membrane for immediate use.

Electron Microscopy

Randomly, 10 matured oocytes (MII) from each IVM-medium were obtained and as control, 10 (MII) oocytes matured in-vivo from patients that were cancelled because of azoospermia on the puncture day of oocyte retrieval, were prepared for the transmission electron microscopy (TEM) study. The oocytes were prepared for TEM according to Nottola et al method [30]. At first, the oocytes were fixed in glutaraldehyde 1.5% (Sigma, USA) in 0.1 M phosphate-buffered saline (PBS) solution at 4 °C for 2-5 days, then they were washed in the PBS buffer for 10 min, and subsequently, the oocytes were fixed in osmium tetroxide 1% (Agar, UK) in PBS buffer far away from light, and washed again in PBS buffer. To facilitate oocyte removal, they were put in small thin blocks of agar 1% (Sigma, USA). The oocytes were dehydrated in arising ethanol concentrations, immersed in propylene oxide for solvent replacement and exclusively embedded in Epon 812 resin (Agar, UK). Semi-thin sections (thickness from 0.5 to 1 µm) were then stained with toluidine blue, for light microscopy evaluation (Zeiss, Germany).Five ultrathin sections (thickness from 60 to 80 nm) for each sample, were prepared and stained with uranyl acetate (7 minutes) and lead citrate (13 minutes). Finally, these sections were photographed at 80KV by a TEM (Zeiss, Germany) [31]. The subsequent factors such as overall structures (as well as shape and dimensions), integrity of the oolemma, type and quality of the cytoplasmic organelles, zonapellucida (ZP) quality, and presence of the perivitelline space (PVS) and random existence of the first polar body have been assessed by light microscopy and TEM [7, 32].

Statistical analysis

Differences of the maturation parameters in IVM oocytes between the groups were calculated and compared by using Chi-square test and for non-parametric data Kruskal-Wallis test, using SPSS software (version 21, USA). Statistically, a P-value of <0.05 was considered significant.

RESULTS

There were no significant differences in maternal age ($P < 0.756$), etiology of infertility ($P < 0.432$) and infertility duration ($P < 0.227$).

The viability of vitrified oocytes was evaluated following warming. Consequently, the survival rate was 88.54%; so, 116alive GV oocytes, out of the original 131, were IVM. In medium I (α-MEM) oocyte maturation rates were reduced in vIVM (71.42%) compared to fIVM (72.58%).While in medium II (hUCM-CM)) oocyte maturation rates of vIVM (79.24%) in comparison to fIVM (85.18%). And maturation rate in hUCM-CM were significantly higher than those of α-MEM medium

(82.21% vs. 72%) (P value: 0.000). Also oocyte arrest in MI was significantly different (P value: 0.036) (Table1). Within the columns, the oocyte Maturation rate and MI arrest did differ significantly ($p<0.05$) according to Chi-square test.

Ultrastructure of MII Oocyte as control group

The oolemma of control MII oocytes (in vivo) which were continuous and contained several long thin microvilli, were regularly dispersed on the oolemma, except in the region of polar body extrusion. The zona pellucida (ZP) was composed of a thoroughly packed electron dense fibrilar substantial (Figure 1). The perivitelline space (PVS) was constant with occasional debris (Figure 1). Round cortical granules with an electron dense appearance was located directly underneath the oolemma. The widespread oocyte organelles involved aggregates of smooth endoplasmic reticulum (SER) enclosed by round or oval shaped mitochondria (M-SER aggregates) (Figure 1).

Ultrastructure of fresh and vitrified in vitro matured Oocytes, in both media

Assessment of semi-thin sections under the light microscope (LM) often exposes structural impairments unnoticeable by LM. Light microscopic analysis of IVM oocytes showed only negligible differences between fresh and vitrified oocytes, such as larger perivitelline space and irregular shape. No major differences in size, shape and total organelle distribution were found between fresh and vitrified oocytes. The zonapellucida thickness of the vitrified oocytes was increased (Figure 1).

In both media, oolemma of IVM oocytes was unbroken and continuous. Some long microvilli were dispersed on the oolemma of fresh IVM oocytes, while in the vitrified IVM oocytes, oolemma was determined to have irregular and little microvillous arrangements (Figure2).Within in vivo-matured oocytes the cortical granule and M-SER aggregates were increased (Fig2a).This study indicated that the cortical granules distribution was reduced in vIVM oocytes compared to fIVM, as the highest decrease in cortical granule distribution was in α-MEM vIVM (Figure 2).

Mitochondria-smooth endoplasmic reticulum (M-SER aggregates), varied in size and shape in vitrified IVM oocytes as M-SER aggregates accomplished oval or slender in shape and then smaller in size compared to fresh oocytes in both mediums (Figure 2) in (2e) and (Figure 3) [(3f) (fIVMhUCM)]. Within in vitro-matured oocytes, the highest presence of M-SER aggregates was in fIVMhUCM culture medium (Figure 2).Commonly were found in oocytes, several complexes of small mitochondria-vesicle (MV)(Fig 3a). The MV complexes were increased in IVM oocytes (Fig 3 b, c, d, e).

DISCUSSION

The low effectiveness of existing commercial IVM mediums and its costs; on the other hand, the Potential mechanisms of paracrine MSC-conditioned medium (MSC-CM) therapeutic adequacy could be the idea of using MSC-CM In this study; so, we investigate whether secretions of human umbilical cord MSC, can be employed to ameliorate maturation of human oocytes during IVM process. The IVM conditions and culture medium component, impact on nuclear and cytoplasmic oocyte maturation; through cellular and molecular processes, the proteomic profile and mRNA content[33].In this study, the maturation rate of oocytes was reduced in the vIVM compared to the fIVM group (75.33% vs 78.88%), with the highest maturation in MSCs fIVM and the lowest in α-MEM vIVM (85.18% vs 71.42%). Accordingly, maturation rate in MSCs-CM was higher than α-MEM medium in fresh and vitrified groups. Ling et al. indicated that MSCs-CM produced a higher oocyte maturation rate (91.2%) than α-MEM (63.5%) in mice [29]. Their outcomes are different from our oocyte maturation rate, which may be due to differences in the source of MSC, type of oocytes and method of cell culture. Also, Shahedi et al. reported that the maturation rates of their human GV oocytes were higher in fIVM (75.33%) rather than in vIVM (45.92%) [34], which are in agreement with our findings, but our maturity rates following IVM were higher, which is probably due to differences in the IVM mediums. Deficiency in the oocyte quality and in vitro culture medium caused reduced energy metabolism and developmental potential; then for the optimal oocyte meiotic maturation, must cytoplasmic organelles changes which attend the oocyte growth. TEM is the only method for assessment of Intracellular damage and morphological differences during IVM [35-37]. The hUCM medium improved the rate of in vitro oocyte maturation and oocyte viability after warming, rather than α-MEM. On the other hand, Parekkadan et al. indicated that approximately 30% of MSC-CM consisted of an enormous range of molecules involved in immunomodulation and chemotactic cytokines and growth factors as potential mediators of the therapeutic effect of MSC-CM [25]. Our findings determined that, MSC-CM medium, probably protect oocyte maturation and increase oocyte survival after vitrification, through growth factors and cytokines; also, Eppig et al. suggested that, supernatants derived from hUCM cells, might provide useful factors for the culture medium, as well as cytokine and vitamins that may have specific effects on serum component, oocyte maturation and developmental competence [18]. In the present study, the vitrified IVM oocytes seemed regular in shape, extents and consistency

of the ooplasm under light microscopy examination, as in the fresh IVM oocytes in both groups. According to some studies, in agreement with ours, good conservation of vitrified oocytes of several types of mammals and conservation protocols does not significantly damage oocyte features [38-40]. On the other hand, Boonkusol et al. detected that the vitrification process influences ultra-structural conditions of the mature oocytes [41].Their outcomes are different from ours, which may be due to differences in the source of oocyte, and oocyte meiosis stages which were vitrified. The micro tubular spindle damage during cryopreservation in human GV oocytes is fewer compared to mature oocyte [7-9], which could indicate the reason of high survival and maturation of vitrified GV oocytes in this study. Absence or decrease of cortical granules in vIVM oocytes was occasionally related with an improved compaction of the inner feature of the ZP, because the occurrence of a premature exocytosis of the cortical granule content into the PVS with the resulting hardening of the inner feature of the ZP, also, ZP hardening and cortical granule exocytosis, may cause the penetration of sperm and no fertilization [42-44].Several studies support our results for ZP hardening and decrease in the number of cortical granules after vitrification [13, 34, 38, 41, 45]. This study detected that both fresh and vitrified IVM oocytes were enclosed by continuous oolemma. This finding was in agreement with Notolla et al, Familiari et al; and Khalili et al [31, 39, 40, 42]. On the other hand, microfilaments are involved in fertilization development and cleavage which can be altered by cryoprotectants and cooling [46]. Rojas et al. detected the variation in microtubules of the spindle and absent microfilaments [12]. Vincent et al. noticed reorganization subsequent to deletion of the cryoprotectants[47] which was in agreement with our findings. In this study, some long microvilli were dispersed on the oolemma of fIVM oocytes rather than vIVM oocytes, while in the vIVM oocytes, oolemma was determined to be irregular and with little microvillous arrangement; this was in agreement with Vincent et al. [47], and was presented to reduce the length of actin filaments which is regarded as an advantage during cryopreservation, as cell's volume decrease in the dehydration course. Microtubules and microfilaments are fragile webs, in response to cryopreservation in vitrified oocytes. Oocytes must be able to recover the cytoskeleton structure after cryopreservation, as cytoskeleton damage might affect cell division and survival [8, 48]. Swain et al. showed that the cause of a fewer rate of fertilization and developmental potency of cryopreserved oocytes might be inappropriate microvilli distribution [21].Valojerdi et al. described structural impairment of microvilli in cryopreserved oocytes [45]. mitochondria–SER (M-SER) aggregates with conservation of mitochondrial reliability were in agreement with Nottola et al and shahedi et al, describing that the differences of M-SER aggregates in vitrified IVM oocytes is possibly due to the use of ethylene glycol (EG) in vitrification not the cryopreservation injury during vitrification [30,34] .The organization of mitochondria–vesicle(MV) complexes and the good structure of mitochondria were alike in both fresh and vitrified IVM samples, which were in agreement with Nottola et al [40].The M-SER aggregates are sign of MV complexes, also the existence of large MV complexes in IVM oocytes, are might because of aging and culture period during IVM; moreover sensitivity of M-SER aggregates to cryoprotectant exposure leads to reduction of M-SER aggregates in size and number, but the recovery mechanisms of metabolic activities that occur at the end of the warming process to shrink the large MV complexes and formed small MV complexes; similarly the M-SER aggregates reformed again [32, 38]. The variations in the organization of M-SER aggregates may lead to disorders of calcium homeostasis and, consequently influence reproduction outcomes [30, 31]. Mitochondria and associated cytoplasmic membranes, during early embryogenesis period, may play a key role in reproduction and reorganization of membranes by controlling the effects of calcium concentrations and ATP production; therefore, they act on fertilization incidents [49]. On the other hand, Jones et al.[43]noticed that within cryopreserved human oocytes, mitochondria couldn't form normal aggregates, in which the oocyte may have no effects on ATP production but may have a reduced capacity to control the levels of intracellular free calcium. Also proper mitochondrial structural preservation may suggest that human oocytes could be more tolerant to vitrification; similarly, Valojerdi et al showed that in mouse oocytes, mitochondria maintain good tolerance against vitrification [45]. On the other hand, differences in size and shape of M-SER aggregates showed marks of injury in mitochondria and SER tubules, which could have an adverse effect on fertilization and developmental competence [38, 50].

CONCLUSION

The present study indicated that, the CM derived of hUCM have shown optimal efficacy and a favorable microenvironment for improving oocyte maturation and fine ultrastructural conservation; through the secretion of growth factors and cytokines; also there is a relationship between the oocyte ultrastructural feature and IVM medium condition.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Afzalipour Infertility Center who helped with study and the data collection. Moreover this work was supported by the grant number 93-548 provided by Deputy of Research of Kerman University of Medical Sciences, Kerman, Iran.

Conflict of interest: The authors confirm that there are no conflicts of interest.

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Table 1. The maturation rate and nuclear maturation stage of oocytes in groups

Groups of IVM	Number of IVM GV oocyte	Arrest in GV stage (n)	Arrest in MI stage(n)	Number of MII oocyte	Oocyte Maturation rate
fIVM α-MEM	62	7	10	45	72.58%
vIVM α-MEM	63	15	3	45	71.42%
fIVM hUCM	54	7	1	46	85.18%
vIVM hUCM	53	4	7	42	79.24%
P value	0.439	0.534	0.036	0.091	0.000

GV arrest: Oocyte arrested at the germinal vesicle stage
MI arrest: Oocyte arrested at metaphase of the first meiotic division

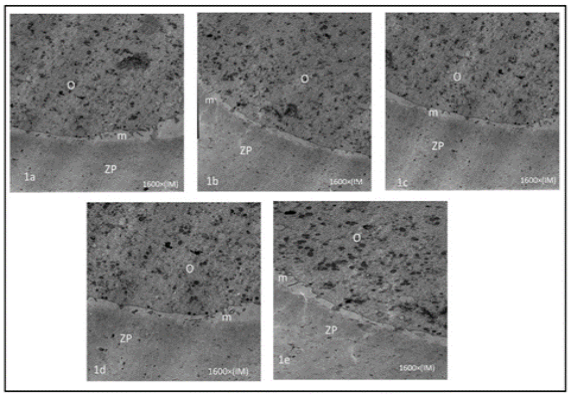

Fig. 1. General Fine Structure and Organelle Microtopography are shown by Transmission Electron Microscopy.Control oocyte (a), fIVM α-MEM (b), vIVM α-MEM (c), fIVMhUCM (d), vIVMhUCM (e). The general morphology and organelle microtopography are shown by Transmission Electron Microscopy (TEM). O = oocyte; ZP = zonapellucida; m = microvilli

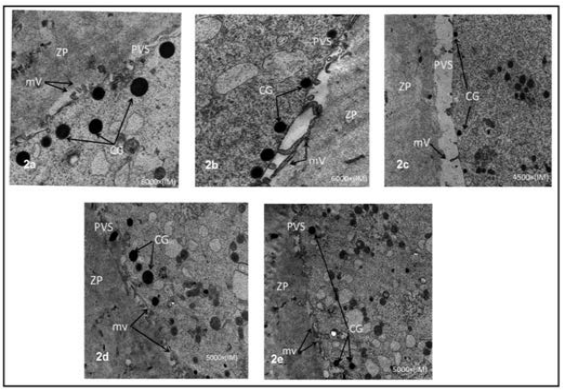

Fig. 2. Ultrastructure of Control oocyte (a), fIVM α-MEM (b), vIVM α-MEM (c), fIVMhUCM (d), vIVMhUCM(e). Round cortical granules with an electron dense arrival that was located just underneath the oolemma (a,b,c,d,e). The number of cortical granules was reduced in vIVM oocytes (c,e) rather than f IVM(b,d). Note the increased compaction of the inner aspect of the ZP in (c) and (e) in comparison with the looser texture in (a, b and d). Voluminous aggregates between mitochondria and elements of SER (M-SER) are seen (e). Several long Microvilli are seen in the control oocytes (a). Also long microvilli were dispersed on the oolemma of fresh IVM oocytes (b, d) rather than in vitrified IVM oocytes (c, e), while in the vitrified IVM oocytes (c, e), oolemma was determined to have irregular and little microvillous arrangements.ZP = zonapellucida; mv = microvilli; CG = cortical granules; PVS = perivitelline space

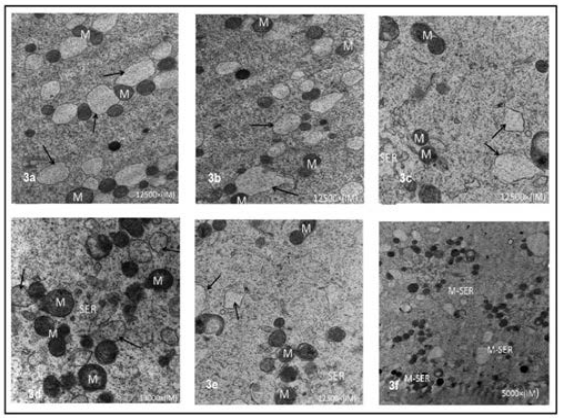

Fig. 3. Control oocyte (a), fIVM α-MEM (b), vIVM α-MEM (c), fIVMhUCM (d), vIVMhUCM (e). Mitochondria are generally rounded and provided with few peripheral or transverse cristae (a and d). Dumbbell shaped, possibly dividing mitochondria can be occasionally found in the ooplasm (d and e). Note the presence of complexes between mitochondria and vesicles of SER in a, b, c, d, e (arrows). The MV complexes was increased in IVM oocytes; Mitochondria-smooth endoplasmic reticulum (M-SER aggregates), varied in size and shape [(3f) (fIVMhUCM)]. SER = smooth endoplasmic reticulum; M=mitochondria. M-SER=Mitochondria- smooth endoplasmic reticulum aggregates