Original Article

Ultrastructural Characteristics of Mouse Preantral Follicles Isolated from Vitrified and Non Vitrified Ovaries after *In Vitro* Maturation

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Abstract

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Objective: The purpose of the present study was to verify the ultrastructural characteristics of cultured mouse preantral follicles from fresh and vitrified –warmed ovarian tissues.

Materials and Methods: The ovaries of 14-day-old mice (n = 5) were vitrified using a mixture of ethylene glycol, ficoll 70 and sucrose. The preantral follicles were isolated and cultured in α-minimum essential medium supplemented with 5% fetal bovine serum, 100 mIU/ml recombinant follicle stimulating hormone, 1% insulin, transferrin and selenium, 20ng/ml murine recombinant epidermal growth factor for four days. The follicles were fixed in 2.5% glutaraldehyde and post fixed with 1% osmium tetroxide and processed for transmission electron microscopic studies.

Results: Cultured preantral follicles of the control group on day 4 consisted of an oocyte surrounded by several layers of polyhedral granulosa and flattened theca cells. The granulosa cells and oocytes were in close connection. Several granulosa cell projections could be seen in the outer layer of zona pellucida. Polyribosomes and rough endoplasmic reticulum cisternae were observed in the most samples in association with mitochondria. The ultrastructure of vitrified follicles was similar to the control. The homogenous zona pellucida surrounded the oocytes but the granulosa cell projections did not enter it. The subzonal space was wider than that of the control group.

Conclusion: Ovarian vitrification and subsequent *in vitro* growth, *in vitro* maturation and *in vitro* fertilization with minimal ultrastructural changes in oocyte could be an alternative to preserve fertility of infertile women.

Keywords: *In Vitro* Maturation, Ultrastructure, Mouse Preantral Follicle, Vitrification

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Introduction

In vitro maturation (IVM) of follicles derived from fresh and cryopreserved ovarian tissues provide a possible strategy for fertility preservation in several mammalian species (1).

Vitrification is a new strategy which has been applied recently for cryopreservation of ovarian tissue and some modifications were performed to enhance its efficiency (2-7).

Chen et al. have developed an innovative vitrification method using less concentrated cryoprotectants and direct application of liquid nitrogen to the ovarian tissue which indicated as direct cover vitrification to improve this technique (5).

Different results have been published on the effects of cryopreservation on the ultrastructure of ovarian follicles after warming (3, 8, 9). Nagia et al. showed that the reduced developmental competence of oocytes with diffuse and fragmented mitochondria was caused by vitrification and thawing (8). Whereas, Hasegawa et al. reported the cortical region of ovaries

maintained good morphologic structure after vitrification and warming (9).

Also in some investigations, the first follicular isolation have been performed then, vitrification and in vitro maturation were assessed, e.g. Dela Pena et al. (10) isolated the preantral follicles mechanically from the mice ovaries. Then, they vitrified the follicles in a solution containing ethylene glycol. They have shown that the proportion of viable oocytes granulosa cells complex from the vitrified follicles was 10% lower than that of the fresh preantral follicles after culture. The developmental competence of the oocytes derived from both vitrified and fresh preantral follicles grown in vitro was lower than that of the oocytes grown in vivo (10).

Recently Choi et al. (11) demonstrated that the developmental rate of primordial follicles was significantly lower in slow-frozen and vitrified ovaries than the fresh controls after five days of culture.

However, according to the ultrastructure investigations, it was shown that the cryopreservation could affect the fine structure of ovarian tissue. It might be possible that some of these changes be reversible during IVM or be critical and affect the subsequent follicular development. There was not any report about the ultrastructural changes of follicles derived from vitrified ovaries after IVM.

Thus, the aim of this study was to evaluate the ultrastructure of cultured isolated preantral follicles from vitrified-warmed mouse ovarian tissue and to compare them with those of the control group.

Materials and Methods

Animal

Five 14-day-old female NMRI mice were cared and used according to the Tarbiat Modares University guide for the care and use of laboratory animals and they were killed by cervical dislocation. Their ovaries were removed and stored in α- minimal essential medium (α- MEM; Gibco, U.S.A) supplemented with 10% fetal bovine serum (FBS; Gibco, U.K). Then, they were considered randomly in fresh (non- vitrified) and vitrified groups. 14-day-old mice ovaries contain a homogenous population of preantral follicles.

Vitrification and Warming of Ovaries

All reagents were prepared from Sigma (Germany) except those mentioned. The vitrification procedure was based on the Salehnia et al. method (3). Briefly, the ovaries were immersed in vitrification medium containing 40% (V/V) ethylene glycol, 30% (W/V) ficoll 70, 1 mol sucrose for 5 min in room temperature. Then, the ovaries were placed in the straw with a minimum volume of the vitrification medium at room temperature, transferred to nitrogen vapor for 30 sec and then, immersed and stored in liquid nitrogen for one week.

For warming, the straws were warmed at room temperature for 30 seconds .Then, placed in 25°C water bath for 20 sec and finally the content transferred into 1, 0.5 and 0.25 mol sucrose for 5 min at room temperature, respectively. The recovered ovaries were transferred to α- MEM supplemented with 5% FBS for washing and equilibrating.

Preantral Follicle Isolation and Culture

The preantral follicles from fresh and vitrified-warmed ovaries with a diameter of 140-170 µm were mechanically isolated using 29-guage needle under stereomicroscope. Isolated follicles containing several layers of granulosa cells with a centrally located healthy oocyte and a thin layer of theca cells were randomly selected. The isolated follicles were cultured individually in 20 µl droplets of α- MEM (Gibco; UK) supplemented with 5% FBS, 100 mIU/ml recombinant follicle stimulating hormone (rFSH or Gonal-f; Serono, Switzerland), 1% insulin, transferrin, and selenium (ITS; Gibco, UK), 20ng/ml murine recombinant epidermal growth factor (mrEGF; Sigma, Germany), 100 µg/ml penicillin and 50 µg/ml streptomycin under mineral oil in a humidified atmosphere of 5% CO_2 in air at 37 °C for 4 days (12). Four samples for each vitrified and control groups were collected randomly for ultrastructural study.

Electron Microscopy

The cultured preantral follicles derived from vitrified and non-vitrified ovaries (n = 4 for each group) were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 2 hours, and post fixed with 1% osmium tetroxide in the same buffer for 2 hours. After dehydration in an ascending series of ethanol, specimens were placed in propylene oxide and embedded in Epon 812 (TAAB, UK). Semithin sections (0.5 µm) were stained with toluidine blue for observation with light microscopy. Ultrathin sections (60-80 nm) were stained with uranyl acetate and lead citrate and examined under transmission electron microscopy (TEM) (Zeiss EM 900, Germany).

Results

Ultrastructure of cultured isolated preantral follicles in non vitrified group

Cultured preantral follicles on day 4 consisted of an oocytes surrounded by several layers of polyhedral granulosa and flattened theca cells. The granulosa cells and oocytes were in close connection (Fig 1).

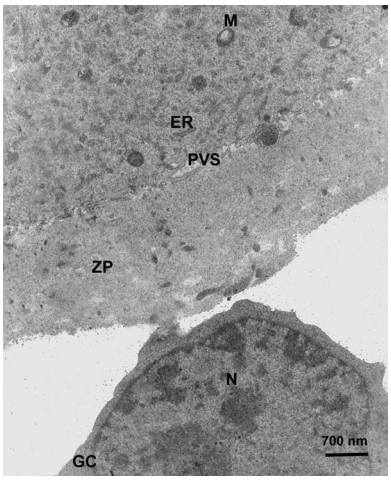

Fig 1: Electron micrograph of the cultured preantral follicles in control group showing the close contact between oocyte and granulosa cells (A); GC, granulosa cell; N, nucleus; ZP, zona pellucida; ER, endoplasmic reticulum; M, mitochondria; PVS, perivitteline space.

The zona pellucida was observed as a thick layer around the oocytes. Several granulosa cell projections could be seen in the thickness of zona pellucida especially in its outer part.

The large and torturous oocyte microvillus was extended into narrow perivitteline space. Oocyte was ovoid with a very homogeneous cytoplasm. The most abundant organelles were the round or ovoid shaped mitochondria, which presented few peripheral cristae and electron-dense granules. Polyribosomes and rough endoplasmic reticulum cisternae were observed in most samples in association with mitochondria, however some were seen isolated within the cytoplasm. Small cisternae of Golgi complex were sometimes observed. A lot of longitudinal and cross section of cytokeratin intermediate filament with striated appearance were observed among of the oocyte cytoplasm.

The granulosa cells had normal morphology and organelles distribution (Fig 2). Their nuclei were ovoid or round with euchromatin in the inner part and small peripheral aggregates of heterochromatin. Round or cylindrical mitochondria and rough endoplasmic reticulum tubules were the most evident organelles.

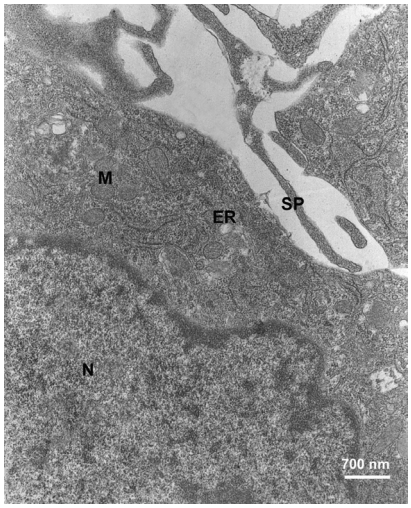

Fig 2: Electron micrograph of the cultured preantral follicles in control group showing the granulosa cell; M, mitochondria; ER, endoplasmic reticulum; N, nucleus; SP, space between granulosa cells.

Ultrastructure of cultured isolated preantral follicles in vitrified groups

The ultrastructure of follicles was similar to the control. The homogenous zona pellucida surrounded the oocytes but the granulosa cell projections did not enter it. The subzonal space was wider than that of the control group (Fig 3).

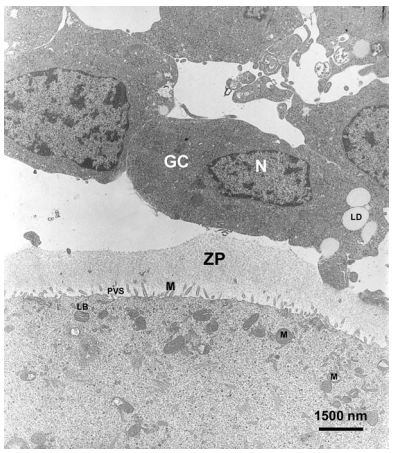

Fig 3: The ultrastructure of cultured isolated preantral follicles from vitrified group (D); GC, granulosa cell; N, nucleus; ZP, zona pellucida; PVS, perivitteline space; M, mitochondria and microvilli; LB, lamellar body.

The large and cylindrical microvillus of oocytes was extended from the outer surface of oocyte to the perivitteline space.

In cortical region of ooplasm, there were mitochondrial condensation and multivesicular body aggregation which contained different types of vacuoles. Some of them had electron dense appearances and some were clearer. Also the lamellar bodies were observed in the periphery of the cytoplasm. The intermediate filaments of cytokeratin were abundant throughout the cytoplasm (Fig 4).

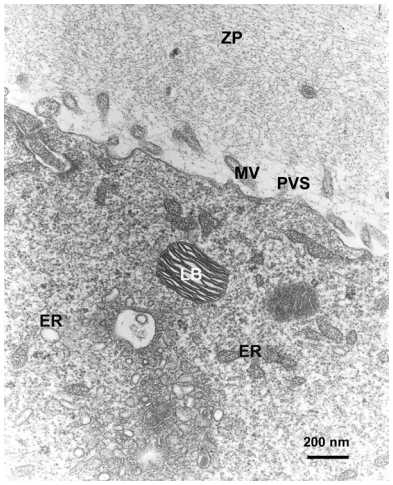

Fig 4: The ultrastructure of cultured isolated preantral follicles from vitrified group; ZP, zona pellucida; MV, microvilli; PVS, perivitteline space; ER, endoplasmic reticulum; LB, lamellar body.

There were distinct dilated rough endoplasmic reticulum, mitochondria and some fat droplets in the granulosa and theca cells.

Discussion

In vitro maturation is an alternative technique to evaluate the efficiency of cryopreservation (13-15). Our previous study showed that the vitrified-warmed mouse ovarian tissue could preserve its developmental capacity. During culturing period, the follicular size was increased and developmental potential in both vitrified and non-vitrified groups was the same (12). As reported earlier, the mean diameters of the fresh and vitrified follicles on the second day were 229.42 ± 30.40 and 222.55 ± 33.4 and on the fourth day were 340.45 ± 61.05 and 299.50 ± 65.55 respectively.

TEM images of this study showed that the distribution of the most organelles in oocyte and follicular cells were normal in both vitrified and non vitrified groups.

However, several studies demonstrated that cryopreservation of mouse ovarian tissues by vitrification had some effects on the oocyte such as mitochondrial changes (3,8,16,17). Nagia et al. (8) showed that the reduced developmental competence of oocytes with diffuse and fragmented mitochondria were caused by vitrification and thawing. They suggested that abnormal mitochondrial distribution in MII oocytes is a cause of developmental retardation and therefore normal mitochondrial distribution could be used as a criterion for selection of good oocytes.

Ultrastructural observations in this study revealed that four days after culture of the isolated preantral follicles, derived from vitrified ovary, the mitochondria had normal morphology similar to that of the control. But in our previous study, some oocyte s mitochondria were swelled in vitrified ovaries just after warming (3).Our explanation is that this minor ultrastructural change could be improved during culturing period.

Our findings provide more evidence for the study conducted by Santos et al. (17), in that, they showed the possibility of preservation of preantral follicles for a long period and that their TEM examination demonstrated that cryopreserved preantral

follicles had normal ultrastructure (17). Also Mazoochi et al. (18) recently showed by TEM study that, vitrified ovaries showed a well-preserved ultrastructure and no sign of apoptosis was observed morphologically or by TUNEL technique in neither fresh nor vitrified–warmed mouse immature ovaries.
The electron micrograph of cultured preantral isolated follicles of the control groups showed a lot of granulosa cell projections which penetrated into outer part of zona pellucida thickness, whereas, in vitrified groups the number of these projections were decreased. The subzonal space was wider than that of the control group. These changes might be due to shrinkage of the cells during equilibration procedure. Although these changes could not influence the developmental capacity of vitrified samples compared to control group (12).

Conclusion

Thus, ovarian cryopreservation by vitrification and subsequent in vitro growth, in vitro maturation and in vitro fertilization with minimal ultrastructural changes of oocyte could be an alternative method for restoring young cancer patients' fertility. The practical information presented here could be applicable to human ovarian tissues.

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