Supplementation of culture media with vitamin E improves mouse antral follicle maturation and embryo development from vitrified ovarian tissue

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Abstract

Aim: The aim of this study was to evaluate the effects of preventive vitamin E (α-tocopherol) on antral follicle development and embryogenesis of oocytes obtained after vitrification of mouse ovarian tissue.

Methods: Female Balb/c mice were killed by cervical dislocation after the injection of pregnant mare's serum gonadotrophin (10 IU) and their ovaries were randomly divided into three groups: control or non-vitrified ($n = 10$), vitrification 1 (5, 10% ethylene glycol + 5, 10% dimethylsulfoxide) ($n = 15$), and vitrification 2 (10, 15% ethylene glycol + 10, 15% dimethylsulfoxide) ($n = 15$) with ascending concentration of cryoprotectants. After toxicity tests and vitrification–warming, mechanically isolated antral follicles were cultured in α-minimum essential medium, which was supplemented with or without α-tocopherol (100 µM). The follicular maturation rates and embryo development were collected and assessed. Also, the viability, morphology and ultrastructure of derived antral follicles from vitrified ovaries were analyzed.

Results: The morphology and ultrastructure of follicles were well preserved in the vitrified groups and α-tocopherol supplementation of culture media significantly increased the proportion of oocytes that reached metaphase II blastocyst rates compared to non-α-tocopherol supplemented media ($P < 0.01$).

Conclusion: Vitamin E improves *in vitro* maturation rates and blastocyst rates of oocytes that are isolated from vitrified ovarian tissue.

Key words: antral follicles, *in vitro* culture, vitrification, α-tocopherol.

Introduction

Although the vitrification method is still widely applied for cryopreservation of ovarian tissue, it has been shown that vitrification is often accompanied by ultrastructural damage to the granulosa cells and oocyte, such as accumulation of vesicles and loss of mitochondrial cristae.[1–4] Moreover, the developmental rates of ovarian tissue and embryo quality are still low. Vitrification protocols have depended on several factors, such as suitable cryopreservation techniques, number of equilibrations, type and concentration of cryoprotectant, warming steps and cryopreservation devices.[5–7]

Several studies have reported that low molecular weight cryoprotectant agents, such as ethylene glycol (EG) and dimethylsulfoxide (DMSO), could be useful solutions for preservation of ovarian tissue.[8–12] Vitrification causes changes in the structure of the membrane and follicles' mitochondria.[13,14] Thus, in this study, the ultrastructure of vitrified ovaries was analyzed for assessment of cryoprotectant solutions. We concluded in our previous investigation that the morphological

integrity and ultrastructure of follicles were preserved after ovarian tissue vitrification with ascending concentration of EG and DMSO.[14] In this regard, step-by-step vitrification in ascending concentration of EG and DMSO was used for ovarian tissue cryopreservation. Two methods, including graft of ovarian tissue and *in vitro* maturation of follicles, were applied for obtaining a mature oocyte from frozen–thawed ovaries[15,16] wherever possible as a useful method for vitrification solutions assessment.

The previous study showed that reactive oxygen species (ROS) levels were increased in the absence of antioxidants during follicle culture and maximum levels of ROS occurred in 48 h of culture in vitrified samples.[17] Overproduction of ROS in cultured follicles can have a deleterious effect on oocyte quality, fertilization rate and embryogenesis. Therefore, ROS must be decreased during culture of ovarian follicles and where the follicular fluid is rich in antioxidants. Moreover, to protect cultured follicles from the deleterious effect of ROS, the addition of an antioxidant is essential.

Alpha-tocopherol (vitamin E) is a predominant lipid-soluble antioxidant that has been considered as a primary free radical scavenger in biological membranes.[17–19] Alpha-tocopherol scavenges peroxyl radicals from polyunsaturated fatty acid in membrane phospholipids or lipoproteins that do not spread the radical chain, thereby protecting against lipid peroxidation.[19] Some researches have reported that α-tocopherol improves folliculogenesis, oocyte quality, fertilization rates and embryo development.[20–22] Lisboa *et al.* reported that supplementation of α-tocopherol maintains the survival of cattle preantral follicles and promotes activation of primordial follicles after 6 days of *in vitro* culture.[23]

The antioxidant activity of α-tocopherol is dose-dependent and higher concentrations may have a toxic effect on follicular development. In order to determine the optimal concentration, Jeong *et al.* tested 0, 50, 100 and 200 μM of α-tocopherol for embryo culture.[24] A higher frequency of blastocyst formation was obtained in the 100-μM concentration.[24] Considering the above, 100 μM α-tocopherol is a suitable level for follicular development and might be chosen for the *in vitro* culture system. We aimed to investigate the preventive effects of 100 μM α-tocopherol on the development of antral follicle, rate of fertilization and embryogenesis of oocytes obtained from vitrified ovarian tissue in mice. In this way, we aimed to achieve the optimal culture conditions for follicular development and to improve oocyte maturation and development in *in vitro* fertilization clinics.

Methods

Chemicals

All media and chemicals were bought from Sigma-Aldrich unless otherwise indicated.

Animals and ovarian tissue preparation

Female Balb/c mice ($n = 40$), aged 5–6 weeks old, were lodged and used in accordance with the International Animal Care and Use Committee of Tabriz University of Medical Sciences. The mice were housed in an environmentally controlled room on a 12-h light/12-h dark photoperiod at 22–24 °C. Food and water were supplied without limitation. The mice were injected intraperitoneally with 10 IU of pregnant mare's serum gonadotrophin (PMSG) to synchronize the estrus cycle. Forty-eight hours after the injection of PMSG by cervical dislocation, the mice were killed and their ovaries (~2 mm^3) were dissected free of fat and mesentery and straight away transferred to dissection medium in 100-μL drops, consisting of α-minimum essential medium (α-MEM) added with 20% fetal bovine serum (FBS).

Preparation of the equilibration and vitrification solution

After ovarian tissue collection, the mice were randomly divided into three groups: control or non-vitrified, vitrification 1 (5, 10% EG + 5, 10% DMSO) and vitrification 2 (10, 15% EG + 10, 15% DMSO). The cryoprotectant and warming solutions were prepared in Dulbecco's phosphate-buffered saline (DPBS).

Three concentrations of cryoprotectants in the equilibration and vitrification solutions (VS) in this research consist of: 5% EG and 5% DMSO in DPBS with 20% FBS and 0.5 M sucrose (VS1), 10% EG and 10% DMSO in DPBS with 20% FBS and 0.5 M sucrose (VS2), and 15% EG and 15% DMSO in DPBS with 20% FBS and 0.5 M sucrose (VS3).

Vitrification and warming

First, ovarian tissue in the vitrification 1 group was equilibrated in the VS1 for 8 min, and then placed in VS2 at room temperature for 2 min. In the vitrification 2 group, ovarian tissue was also equilibrated in the VS2 for 8 min, then placed in VS3 at room temperature for 2 min; then the ovarian tissue was put in 1.8-mL plastic cryotubes with a minimum volume of the VS, placed on nitrogen vapor for 20 s and finally plunged into liquid nitrogen and kept for 1 week.

For warming, vitrified ovaries were placed on nitrogen vapor for 20 s, warmed at room temperature for 20 s and

then placed in a 25 °C water bath for 20 s. Then, the contents of each cryotube were expelled into 100 μL of descending concentrations of sucrose (1, 0.5 and 0.25 M) and DPBS at room temperature for 5 min.

Before follicle isolation, warmed ovaries were equilibrated in α-MEM medium with 20% FBS for 30 min in an incubator.

Follicle morphology

For histological assessment, vitrified and fresh ovaries were fixed in Bouin's solution, dehydrated in ethanol, clarified with xylene, embedded in paraffin wax, serially sectioned at 5 μm, stained with hematoxylin–eosin and analyzed under a light microscope (magnification ×400).

For this study, antral follicles were those with two or more layers of cuboidal granulosa cells and those that had an antrum. Follicular quality was evaluated and follicles were classified as normal or degenerate. The follicles were classified as normal by intact oocyte and complete layer of granulosa cells or degenerated by vacuolization, pyknotic and shrinkage of oocyte and granulosa cells.

Ultrastructure of follicle

All chemicals were obtained from TAAB Laboratories Ltd. Vitrified and non-vitrified ovaries were randomly collected ($n = 3$ from each group) after equilibration in medium for 30 min, fixed in 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) for 2 h, and post fixed with 1% osmium tetroxide in the same buffer for 2 h. After dehydration in an ascending series of ethanol, they were placed in propylene oxide and embedded in Epon 812. The ovarian tissue was cut into 0.5-μm sections (semithin sections), stained with toluidine blue and observed in light microscopy.

Ultrathin sections (60–80 nm) were contrasted with uranyl acetate and lead citrate and examined by electron microscopy (Zeiss). The granulosa cells and oocyte were analyzed by cytoplasmic organelles, vacuolization, mitochondrial formation and basement membrane of cells.

Follicle isolation

Antral follicles from ovaries were isolated by mechanical dissection under a stereomicroscope and were selected according to the following criteria: (i) intact follicle with several layers of granulosa cells and some adhering theca cells; (ii) antrum present; (iii) visible, round and central oocyte; and (iv) follicle diameter between 220 and 370 μm. Then, isolated follicles were transferred to main culture medium.

Survival of antral follicles

The survival rates of antral follicles from non-vitrified and vitrified ovaries were assessed using trypan blue staining.[25] The isolated follicles from the ovaries were transferred to new microdroplets of medium (20 μL) and were stained by 0.4% trypan blue and examined under an inverted microscope. For each group, only follicles containing layers of membrane-enclosed granulosa cells with a centrally located oocyte were examined. The follicles were scored as viable or degenerate: viable ones were not stained and the oocyte and the surrounding granulose cells were clear and degenerated follicles stained blue.

In vitro maturation of antral follicles

Isolated antral follicles were cultured individually in a culture dish (35-mm Petri dishes) containing 50-μL droplets of culture medium under decontaminated mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 in air at 37 °C for 4 days.

The culture medium consisted of α-MEM (pH = 7.2) supplemented with 20% FBS, 19/0 mM sodium pyruvate, 75 μg/mL recombinant follicle stimulating hormone (rFSH or Gonal-f), luteinizing hormone (0/5 μg/mL), 50 μg/mL penicillin G, 50 μg/mL streptomycin, and 100 μM vitamin E (α-tocopherol). The α-MEM culture media supplemented with FBS and without vitamin E (α-tocopherol) was considered as the control group. Every 48 h of culturing, culture medium from each drop was replaced by renewed medium. The survival rate of the follicles was checked by assessment of follicle morphology under inverted microscope.

In vitro ovulation induction

On the 4th day of culture, final oocyte maturation and ovulation were induced by addition of 1.5 IU/mL recombinant human chorionic gonadotrophin (rhCG; Organon) to the media. Released oocytes were recorded as: germinal vesicle (GV); when the GV was absent, as germinal vesicle breakdown (GVBD); when absence of both a germinal vesicle and a first polar body, as metaphase I (MI); and when the first polar body was extruded, as metaphase II (MII). The proportions of GV, MI and MII were evaluated in all groups of study 48 h after hCG addition.

Fertilization rates and embryo culture

Spermatozoa were extracted from the cauda epididymis of 7–8-week-old male Balb/c mice and capacitated for 1.5 h into a 500-μL drop of Tyrods medium (T6)

supplemented with 5 mg/mL of bovine serum albumin in the incubator. The collected MII oocytes from all experimental and control groups were transferred to T6 medium containing capacitated spermatozoa, and after fertilization, they were cultured for 120 h.

Statistical analysis

The survival, degeneration and developmental rates of follicles and oocytes were evaluated by Tukey, analysis of variance (ANOVA) and follicular diameter analyzed by Means, ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Survival rates of isolated antral follicles after vitrification–warming

The survival rates of antral follicles derived from the fresh and the vitrification 1 and 2 groups were 84.9%, 66.2% and 67.2%, respectively (Table 1). There were significant differences in the survival of antral follicles between the fresh and vitrified groups ($P < 0.05$).

Table 1 Number and percentage of intact and degenerated follicles of various stages isolated from vitrified and non-vitrified ovarian tissue after Trypan blue staining

Group	Total number of follicles	Number of antral follicles (%)	
		Int	Deg
Control	186	158 (84.9)	28 (15.1)
Vit1	275	182 (66.2)*	93 (33.8)*
Vit2	290	195 (67.2)*	95 (32.8)*

*Percentage significantly different ($P < 0.05$) from the control and supplemented groups. Deg, degenerated; Int, intact; Vit, vitrification.

Morphology of antral follicles after vitrification–warming

The morphology of follicles was well preserved in the vitrified groups and was similar with the non-vitrified or fresh group (Fig. 1a). The vitrification 1 group showed more signs of degeneration and cryoinjury, such as shrinkage and picnosis of the oocyte, cytoplasmic retraction, numerous cytoplasmic vacuoles and detachment of innermost granulosa layer and oocyte (Fig. 1b). However, in the vitrification 2 group, slight cryodamage was observed, including the disruption of intercellular contacts among granulosa cells and the oocyte of antral follicles (Fig. 1c).

Antral follicles ultrastructure after vitrification–warming

The ultrastructural analysis showed that the integrity of cell organelles was well preserved in the vitrification 2 concentration and this was very similar to the non-vitrified groups. The granulosa cells exhibited a well-developed Golgi complex and both smooth and rough endoplasmic reticulum and highly variable numbers of vesicles spread throughout the ooplasm. Most mitochondria had continuous membranes and normal cristae but irregularly shaped or swollen mitochondria were rarely observed in the cytoplasm of granulosa cells. The oocytes were surrounded by several layers of cuboidal granulosa cells with a continuous basement membrane in antral follicles (Fig. 2a–c).

Although the ultrastructure of antral follicles that vitrified in the vitrification 1 cryoprotectant was similar to fresh control follicles, in some cases, granulosa cells, oocytes and techa cells had numerous vacuoles and elongated mitochondria with a few cristae (Fig. 2b).
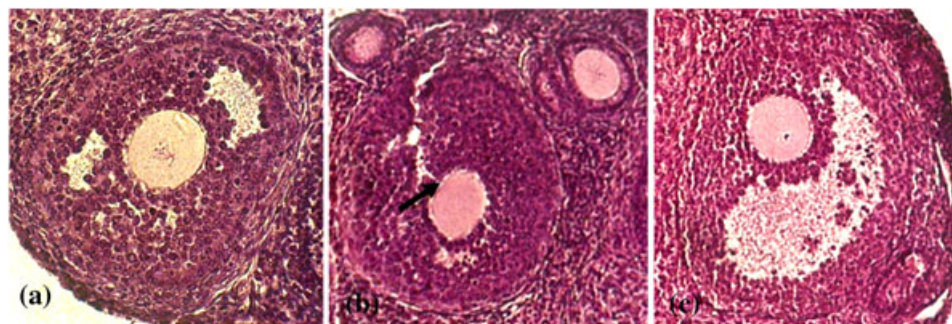

Figure 1 Morphological images of the mouse antral follicles. (a) Non-frozen group. (b) Vitrification 1 group. (c) Vitrification 2 group. Black arrow shows slight disruption of contact between innermost granulosa cells and oocyte in vitrification 1 group. Scale bar = 50 μm.
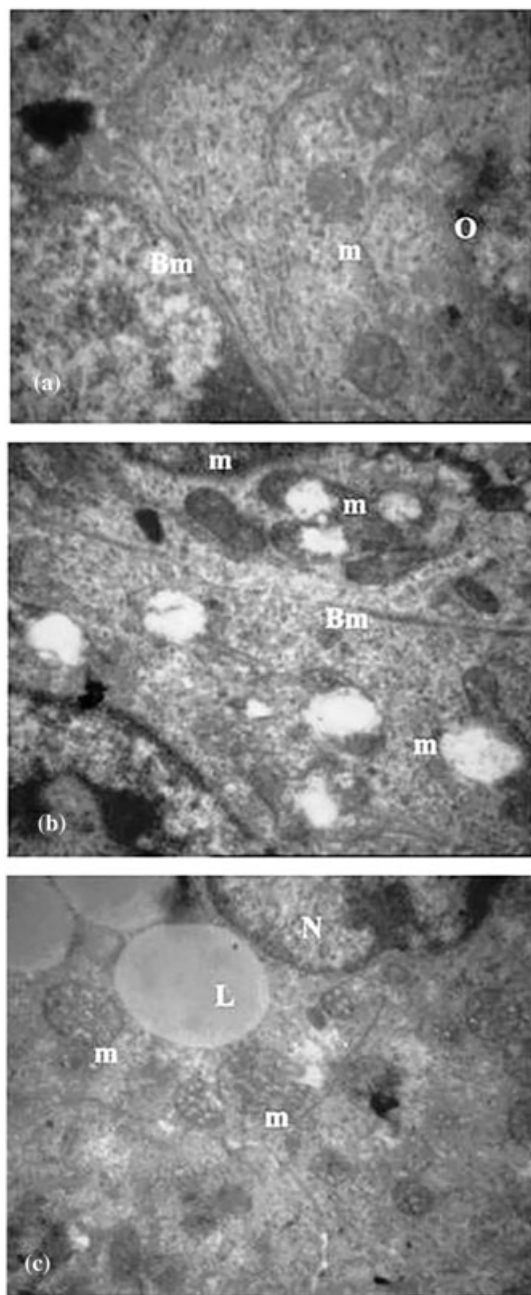
Figure 2 The electron micrograph of granulosa cells of antral follicles showing arrangement of mito chondria (m) and basal membrane (Bm). (a) Numerous mitochondria with typical or regular shape and normal cristae, continuous Bm in control group. (b) Numerous elongated mitochondria with tubular–vesicular cristae and ruptured Bm in vitrification 1 group. (c) Some round mito chondria with atypical cristae, nucleus (N), and numerous lipid droplets (L) in vitrification 2 group ×7750.

Although vacuolization and deformity of mitochondria were observed in the ooplasm and the cytoplasm of granulosa cells, other organelles did not change after vitrification of ovarian tissue, compared with fresh ovaries.

In vitro follicular viability

Altogether, approximately 800 good-quality antral follicles were cultured with or without α-tocopherol in the present study.

By day 4 of culture, the survival rates of isolated follicles from the control, vitrification 1 and 2 groups that were cultured in the absence of α-tocopherol in medium were 74%, 59% and 59%, respectively. That of follicles cultured in vitamin-E-supplemented media were 89%, 77% and 80%, respectively (Table 2).

The control group had a significantly higher proportion of antral follicles that survived to the end of the culture as compared to the vitrified group ($P < 0.001$). The addition of α-tocopherol to the media significantly increased the viability rate of antral follicles ($P < 0.001$). Therefore, follicular viability was affected by α-tocopherol supplementation.

In vitro follicular growth

Follicular diameters and changes during culture on days 0, 2 and 4 are presented in Table 3. Follicular diameter did not differ among the treatment groups at the beginning of culture. However, follicles cultured in α-tocopherol had a larger diameter than those in the unsupplemented groups, but there was no difference in follicular diameter on different days of culture ($P > 0.05$).

In vitro follicular ovulation rate

After 4 days' culture of antral follicles, hCG (1.5 IU/mL) was supplemented to induce ovulation. The numbers of cumulus-oocyte complexes (COC) that were derived (after 24 h) from the antral follicles were counted, in order to evaluate the ovulation rate. The addition of 100 μM α-tocopherol in culture medium increased ovulation rates of follicles (60% to 80%, $P < 0.05$) in the vitrified groups but the incidence of ovulation was not significantly different among the treatment and control groups (Table 2).

In vitro follicular development

The rates of maturation to the MII oocyte in the control, vitrification 1 and 2 groups that cultured in non-α-tocopherol-supplemented media were 41%, 19% and 24%, respectively. Those of follicles cultured in

Table 2 Development of cultured antral follicles in supplemented media with or without vitamin E (α-tocopherol) 100 μM after 4 days

Group	Total follicles (%)	No. survived follicles (%)	No. degenerated follicles (%)	No. ovulated follicles (%)	Developmental GV (%)	Stage of MI (%)	Oocytes MII (%)
Control	180	133 (73.9)	47 (26.1)	110 (82.7)	34 (30.9)	31 (28.2)	45 (40.9)
Vit1	143	85 (59.4)*	58 (40.6)*	47 (55.3)*	20 (42.6)	18 (38.3)	9 (19.1)*
Vit2	140	82 (58.6)*	58 (41.4)*	54 (65.8)*	22 (40.7)	19 (35.2)	13 (24.1)*
Control + VE	110	98 (89.1)	12 (10.9)	86 (87.8)	11 (12.8)	21 (24.4)	54 (62.8)**
Vit1 + VE	133	103 (77.4)	30 (22.6)	75 (72.8)	16 (21.3)	25 (33.3)	34 (45.4)
Vit2 + VE	117	94 (80.3)	23 (19.7)	78 (83.0)	15 (19.2)	24 (30.8)	39 (50.0)

*Percentage significantly different ($P < 0.001$) from the control and supplemented groups. **Percentage significantly different with all groups without Vit2 + VE. ($P < 0.05$). GV, germinal vesicle; MI, metaphase I; MII, metaphase II; VE, vitamin E; Vit, vitrification.

Table 3 Diameter of isolated antral follicles (μm) that cultured. The follicular diameter (μm) of cultured antral follicles in α-tocopherol-free and α-tocopherol supplemented media (100 μM for 4 days)

Group	Day 0	Day 2	Day 4
Control	267.5 ± 29.6	308.6 ± 27.7	350.6 ± 17.5
Vit1	284.1 ± 18.7	307.2 ± 18.7	338.8 ± 17.7
Vit2	282.7 ± 26.4	304.8 ± 23.8	339.7 ± 16.5
Control + VE	267.0 ± 33.3	292.9 ± 27.3	329.4 ± 16.6
Vit1 + VE	271.2 ± 21.6	293.5 ± 24.4	329.7 ± 16.3
Vit2 + VE	277.5 ± 20.6	303.3 ± 16.1	342.9 ± 17.7

[1]There was no significant difference between groups ($P > 0.05$). The first day of culturing was considered as day 0. VE, vitamin E; Vit, vitrification.

medium containing α-tocopherol were 63%, 45% and 50%, respectively (Table 2).

The proportion of oocytes in the MII stage at day 4 of maturation was significantly higher in the control group compared with the verified group ($P < 0.001$). Addition of α-tocopherol to the maturation medium revealed that a significantly higher ($P < 0.001$) proportion of oocytes reached MII compared to those in non-α-tocopherol-supplemented media. There was no significant difference between the control and vitrified groups in the presence of α-tocopherol. Therefore, follicular maturation was affected by α-tocopherol supplementation (Fig. 3a,b).

Fertilization rate and embryo culture

After 24 h of sperm oocyte co-incubation, the proportions of oocytes that were fertilized were 69% (control), 30% (vitrification 1), and 38% (vitrification 2) in the absence of α-tocopherol and 81% (control); and 79% (vitrification 1) and 82% (vitrification 2) in the presence of α-tocopherol (Fig. 3c,d; Table 4). The number of matured oocytes that fertilized and reached blastocyst stage was higher in the control group compared to both the vitrified groups ($P < 0.001$) and 5 days after insemination, a significantly higher proportion of oocytes reached the blastocyst stages in the α-tocopherol-supplemented

medium compared with the unsupplemented groups ($P < 0.001$).

The blastocyst formation was significantly lower in follicles from the vitrification 1 group that cultured in α-tocopherol media compared with treatment control (29% vs 41%). Moreover, the embryonic development was increased significantly in the control + vitamin E supplementation group compared to all other groups ($P < 0.05$).

Discussion

These studies were designed to determine whether the antioxidant vitamin E would protect *in vitro* maturation and development of isolated follicles from the deleterious effects of cryoprotectants. More mature oocytes developed into embryos during the blastocyst stages when the culture medium was supplemented with 100 μM vitamin E when compared with the non-treatment groups.

Because the vitrification solution is normally supercooled at a low temperature in liquid nitrogen, it can be crystallized during warming, which leads to the cryodamage of ovarian tissue. In order to prevent cryodamage during freezing–warming, the presence of
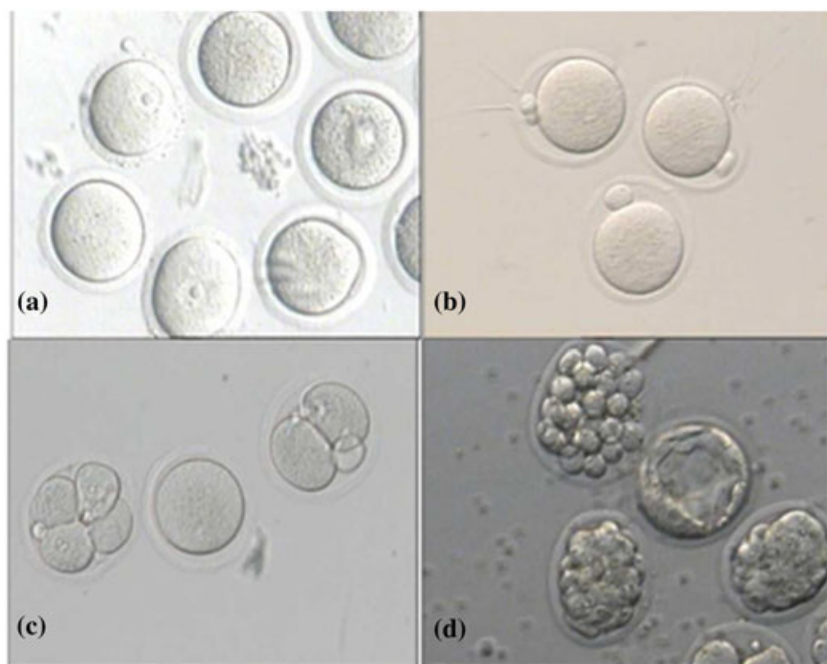
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Figure 3 *In vitro* maturation (released oocytes) and *in vitro* development (embryo formation) of isolated antral follicles. (a) Oocyte in germinal vesicle (GV) and metaphase I (MI) stages. (b) Oocytes in MII stages. (c) Embryo in two and four cell stages. (d) Embryo in morula and blastocyst stages.

high concentrations of permeable cryoprotective agents is essential. The first successful method for vitrifying mouse ovarian tissue is addition of EG as permeable agents. Our previous study confirmed that EG in combination with DMSO had the highest protective effects and least ultrastructural damage on the ovarian follicle.[14] Thus, in the present study we used mixed cryoprotectants (EG and DMSO) for ovarian tissue vitrification. The cryopreservation of ovarian tissue leading to disruption of the meiotic spindles thereby decreases fertilization rate and embryo quality. Therefore the use of DMSO might have a protective effect by spindle polymerization.[26]

The second successful method is the addition of a macromolecule, such as sucrose, in equilibration and vitrification solutions. The addition of sucrose confirmed

previous reports that this is more efficient for follicular viability and ultrastructure.[4,14,15] Sucrose has high solubility and low viscosity, which could facilitate the exit of water from the cell and decrease the formation of ice crystals; thus, it could protect the cells during the freezing–warming process.

In agreement with previous findings, this study indicated that optimum survival can be attained with 10-min equilibration in 10% EG + DMSO with 20% sucrose (VS2) and vitrification in 15% EG + DMSO with 20% sucrose (VS3) for ovarian tissue. This allows sufficient time for the cryoprotectant to permeate into the granulosa cells and results in a high proportion of oocytes of normal morphology, fertilization and development to two-cell embryos and blastocysts after thawing and dilution.[14] However, in our study, no

Table 4 Fertilization and developmental rates of oocytes derived from cultured follicles in vitrified and non-vitrified ovaries in the presence of vitamin E

Group	No. oocytes that reached MII	No. fertilized oocytes (%)	No. two cells (%)	No. four cells (%)	No. blastocysts (%)
Control	45	31 (68.9)	18 (40.0)	12 (26.7)	9 (20.0)
Vit1	9	3 (30.3)*	2 (22.2)*	0 (0.0)*	0 (0.0)*
Vit2	13	5 (38.5)*	3 (23.1)*	1 (7.7)*	0 (0.0)*
Control + VE	54	44 (81.5)	33 (61.1)	26 (48.1)	22 (40.7)**
Vit1+ VE	34	27 (79.4)	17 (50.0)	12 (35.3)	10 (29.4)
Vit2+ VE	39	32 (82.1)	22 (56.4)	17 (43.6)	15 (38.5)

*Percentage significantly different ($P < 0.001$) from the control and supplemented groups. **Percentage significantly different with all groups without Vit2 + VE ($P < 0.05$). MΠ, metaphase II; VE, vitamin E; Vit, vitrification.

significant difference was observed in the survival, fertilization and developmental rate of frozen–thawed ovarian tissue.

The *in vitro* culture of antral follicles is an important method to evaluate the effects of cryopreservation on follicular quality. This finding indicates that a high proportion of frozen–thawed ovarian tissue that undergoes normal fertilization can develop into two-cell embryos and subsequently into expanded blastocysts.

Ultrastructural analysis of the present study was consistent with previous observations where the fertilization and development rates of vitrified oocytes were significantly lower than those of the controls, indicating that some damage occurred in the organelle of granulosa cells and oocytes, such as membrane cells and mitochondria.

Intracellular ice formation may have occurred during freezing–thawing and the hardness of the zona pellucida could have affected the subsequent fertilization and development of the oocyte.[27,28] These results suggest that the mitochondria mediated an apoptosis and oxidative stress process.[29]

The discrepancy between viability, morphology and ultrastructural changes suggested that vitrification may affect different intra/extracellular components that are necessary for cellular functions.

The mitochondrial organization and cytoskeletal network are essential for cryopreservation assessment.[30] The main functions of mitochondria are the energy-producing, metabolic activation and regulating cell viability.[31] Mitochondria seem to be the most sensitive to the cryopreservation process; these procedures cause changes in mitochondrial function that may affect ovarian tissue quality. Moreover, cryopreservation causes a change in the cell membrane fluidity and induces mitochondrial malfunction, leading to ROS formation.[32] Thus, we hypothesize that an increase in ROS production in the frozen–thawed ovarian tissue may be related to mitochondrial malfunction. Supplementation of culture media with α-tocopherol could help to minimize the production of ROS by the abnormal mitochondria, and thus reduce the risk of oxidative stress.

Some researchers have suggested that the low growth rate of the frozen–thawed follicles might have resulted from the delayed proliferation, lower concentrations of growth factors or initial cell death of the granulosa cells in response to the freezing–thawing process.[33,34]

Under normal conditions, cells can deal with ROS with scavenging mechanisms, such as Superoxide Dismutase (SOD) and catalase, but in the vitrification there is a possibility that these protective defense systems might be lost.

It is well known that the overproduction of ROS levels and lipid peroxidation of granulosa cells and oocyte membrane can affect follicular function after cryopreservation.[17,35,36] Various antioxidants have been used to supplement *in vitro* culture media, especially α-tocopherol. Alpha-tocopherol (vitamin E) is well known as an ROS scavenger in *in vivo* and *in vitro* conditions[18,19,37] and is the most important antioxidant present in ovarian tissue and follicular fluid. The antioxidant activity of α-tocopherol in preventing free-radical-induced tissue damage is accepted by most investigators and is believed to be the primary free radical scavenger and to inhibit lipid peroxidation in the mammalian cell membrane.[18,19,21,37,38]

There was an increase in the number of viable and intact ovarian follicles after supplementation of α-tocopherol in culture media. Lisboa *et al.* reported that α-tocopherol maintains the survival of cattle preantral follicles and promotes activation of primordial follicles after 6 days of *in vitro* culture.[23] Successful maturation of oocyte, fertilization and blastocyst rates depends on the coordination between growth and development of the ovarian follicles. Perhaps the supplementation of vitamin E may be enhanced by the effectiveness of hormones and growth factors in culture medium. In the present study, the follicle diameters, which were measured in order to evaluate growth, showed an increase of follicular diameter during culturing of isolated follicles from all fresh and vitrified ovaries. The main biological function of vitamin E is likely to protect polyunsaturated fatty acids in membranes and maintain iron and other metals in a reduced state.[39] Peroxidation of cell membrane lipids can lead to structural damages, affecting function and permeability of membranes, eventually resulting in irreversible cell death. As a fat-soluble vitamin, α-tocopherol may be more efficient on the cell membrane than other antioxidants to protect against lipid peroxidation.

The results of the present study have shown that vitamin E supplementation of the culture media significantly increased the percentage of oocytes that reached MII stage and increased blastocyst formation. After α-tocopherol supplementation in the vitrified groups, the follicle viability, morphology and structure were significantly higher than that of the unsupplemented groups. Indeed, the increase of ROS was administrated in the absence of antioxidants during 48 h of follicular culture in vitrified samples.[17] Therefore, in our sample, the frozen addition of α-tocopherol confirms the necessity of antioxidant supplementation during *in vitro* maturation of follicles.

These studies suggest that supplementation of culture media with α-tocopherol at a concentration of 100 μM allowed the maintenance of the equilibrium between antioxidants and pro-oxidants in the follicular culture, reducing the O2 concentration, and minimizing ROS production. Furthermore, this procedure inhibits establishment of oxidative stress conditions and thereby improves follicular maturation and embryo development. Kitagawa *et al.* showed that increased ROS levels and decreased antioxidant concentration in the culture media of oocyte maturation were responsible for embryonic fragmentation.[35]

In conclusion, our results showed that culture of antral follicles from vitrified ovaries with 100 μM α-tocopherol improves the developmental rate of mouse follicles *in vitro* and the blastocyst formation. These results introduce a potential improvement of vitamin E to preserve the fertility potential by minimizing the level of free radicals that might occur during vitrification of ovarian tissue and this could be useful during assisted reproductive techniques.

Acknowledgments

Disclosure

There is no conflict of interest.

References

1. Shaw J, Jones G. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Hum Reprod Update* 2003; **9**: 583–605.
2. Choi WJ, Yeo HJ, Shin JK, Lee S, Lee JH, Paik WY. Effect of vitrification method on survivability, follicular growth and ovulation of preantral follicles in mice. *J Obstet Gynaecol Res* 2007; **33**: 128–133.
3. Boonkusol D, Faisaikarm T, Dinnyes A, Kitiyanant Y. Effects of vitrification procedures on subsequent development and ultrastructure of in vitro-matured swamp buffalo (Bubalus bubalis) oocytes. *Reprod Fertil Dev* 2007; **19**: 383–391.
4. Abedelahi A, Salehnia M, Abdolamir A, Hajizadeh E. Comparison of ultrastructure and morphology of mouse ovarian follicles after conventional and direct cover vitrification using different concentrations of ethylene glycol. *Iran J Reprod Med* 2009; **7**: 45–52.
5. Vajta G, Holm P, Kuwayama M *et al.* Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev* 1998; **51**: 53–58.
6. Wani N, Maurya S, Misra A, Saxena V, Lakhchaura B. Effect of cryoprotectants and their concentration on in vitro development of vitrified-warmed immature oocytes in buffalo (Bubalus bubalis). *Theriogenology* 2004; **61**: 831–842.
7. Schiewe M, Rall W, Stuart L, Wildt D. Analysis of cryoprotectant, cooling rate and in situ dilution using conventional freezing or vitrification for cryopreserving sheep embryos. *Theriogenology* 1991; **36**: 279–293.
8. Dhali A, Manik R, Das S, Singla S, Palta P. Post-vitrification survival and in vitro maturation rate of buffalo (Bubalus bubalis) oocytes: Effect of ethylene glycol concentration and exposure time. *Anim Reprod Sci* 2000; **63**: 159–165.
9. Kasai M, Komi J, Takakamo A, Tsudera H, Sakurai T, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J Reprod Fertil* 1990; **89**: 91–97.
10. Chen SU, Chien CL, Wu MY, *et al.* Novel direct cover vitrification for cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in mice. *Hum Reprod* 2006; **21**: 2794–2800.
11. Rahimi G, Isachenko E, Sauer H *et al.* Effect of different vitrification protocols for human ovarian tissue on reactive oxygen species and apoptosis. *Reprod Fertil Dev* 2003; **15**: 343–349.
12. Wiweko B, Maidarti M, Mansyur E *et al.* Ovarian tissue vitrification as a method for fertility preservation: A study of follicle number and morphology after vitrification. *IVF Lite* 2014; **1**: 148–152.
13. Valojerdi MR, Salehnia M. Developmental potential and ultrastructural injuries of metaphase II (MII) mouse oocytes after slow freezing or vitrification. *J Assist Reprod Genet* 2005; **22**: 119–127.
14. Nasrabadi HT, Gavami M, Akbarzadeh A, Beheshti R, Mohammadnejad D, Abedelahi A. Preservation of mouse ovarian tissue follicle morphology and ultra-structure after vitrifying in biotechnological protocols. *J Ovarian Res* 2015; **8**: 7.
15. Abedelahi A, Rezaei-Tavirani M, Mohammadnejad D. Fertility preservation among the cancer patients by ovarian tissue cryopreservation, transplantation, and follicular development. *Iran J Cancer Prev* 2013; **6**: 123.
16. Desai N, AbdelHafez F, Ali MY *et al.* Mouse ovarian follicle cryopreservation using vitrification or slow programmed cooling: Assessment of in vitro development, maturation, ultra-structure and meiotic spindle organization. *J Obstet Gynaecol Res* 2011; **37**: 1–12.
17. Abedelahi A, Salehnia M, Allameh A, Davoodi D. Sodium selenite improves the in vitro follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxide activity. *Hum Reprod* 2010; **25**: 977–985.
18. Olson S, Seidel G. Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. *Biol Reprod* 2000; **62**: 248–252.
19. Liebler DC. The role of metabolism in the antioxidant function of vitamin E. *Crit Rev Toxicol* 1993; **23**: 147–169.
20. Barzegari Firouzabadi F, Javid A, Rezaei ZS. An in vitro study of follicle stimulating hormone (FSH) and α-tocopherol effects on the maturation of preantral follicle-enclosed oocytes from immature mice. *RJMS* 2010; **17**: 7–15.
21. Tareq K, Akter QS, Khandoker MA, Tsujii H. Selenium and vitamin E improve the in vitro maturation, fertilization and culture to blastocyst of porcine oocytes. *J Reprod Dev* 2012; **58**: 621–628.

22. Soleimani Mehranjani M, Noorafshan A, Hamta A *et al*. Effects of vitamin E on ovarian tissue of rats following treatment with p-nonylphenol: A stereological study. *Iran J Reprod Med* 2010; **8**: 1–9.

23. Lisboa LA, Andrade ER, Hertel MF *et al*. Viability and growth of cattle preantral follicles after in vitro culture of ovarian fragments in α-tocopherol. *Reprod Fertil Dev* 2009; **22**: 318–319.

24. Jeong YW, Park SW, Hossein MS *et al*. Antiapoptotic and embryotrophic effects of alpha-tocopherol and L-ascorbic acid on porcine embryos derived from in vitro fertilization and somatic cell nuclear transfer. *Theriogenology* 2006; **66**: 2104–2112.

25. Nagano M, Atabay EP, Atabay EC, Hishinuma M, Katagiri S, Takahashi Y. Effects of isolation method and pre-treatment with ethylene glycol or raffinose before vitrification on in vitro viability of mouse preantral follicles. *Biomed Res* 2007; **28**: 153–160.

26. Eroglu A, Toth T, Toner M. Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. *Fertil Steril* 1998; **69**: 944–957.

27. Mandelbaum J, Anastasiou O, Levy R, Guérin J, De Larouziere V, Antoine J. Effects of cryopreservation on the meiotic spindle of human oocytes. *Eur J Obstet Gynecol Reprod Biol* 2004; **113**: S17–S23.

28. Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Eur J Obstet Gynecol Reprod Biol* 2005; **20**: 3385–3389.

29. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309.

30. Rho GJ, Kim S, Yoo JG, Balasubramanian S, Lee HJ, Choe SY. Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. *Mol Reprod Dev* 2002; **63**: 464–470.

31. Van Blerkom J. Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. *Proc Natl Acad Sci U S A* 1991; **88**: 5031–5035.

32. Ahn HJ, Sohn IP, Kwon HC, Jo DH, Park YD, Min CK. Characteristics of the cell membrane fluidity, actin fibers, and mitochondrial dysfunctions of frozen-thawed two-cell mouse embryos. *Mol Reprod Dev* 2002; **61**: 466–476.

33. Newton H, Illingworth P. In-vitro growth of murine pre-antral follicles after isolation from cryopreserved ovarian tissue. *Hum Reprod* 2001; **16**: 423–429.

34. Cortvrindt R, Smitz J, Van Steirteghem A. Ovary and ovulation: A morphological and functional study of the effect of slow freezing followed by complete in-vitro maturation of primary mouse ovarian follicles. *Hum Reprod* 1996; **11**: 2648–2655.

35. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. *Theriogenology* 2004; **62**: 1186–1197.

36. Hatami S, Zavareh S, Salehnia M, Lashkarbolouki T, Ghorbanian MT, Karimi I. Total oxidative status of mouse vitrified pre-antral follicles with pre-treatment of alpha lipoic acid. *Iran Biomed J* 2014; **18**: 181.

37. Sies H, Stahl W. Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. *Am J Clin Nutr* 1995; **62**: 1315S–1321S.

38. Chow CK. Vitamin E and oxidative stress. *Free Radic Biol Med* 1991; **11**: 215–232.

39. Yamamoto K, Niki E. Interaction of α-tocopherol with iron: Antioxidant and prooxidant effects of α-tocopherol in the oxidation of lipids in aqueous dispersions in the presence of iron. *Biochim Biophys Acta* 1988; **958**: 19–23.