Ultrastructural and Morphalogical Changes of Mouse Ovarian Tissues Following Direct Cover Vitrification with Different Cryoprotectants

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Abstract

Background: Cryopreservation of mammalian ovaries has been reported with different levels of success. Cryopreservation of ovarian tissue may be a potential alternative for treatment of infertility and many attempts have been done to improve the efficiency of ovarian cryopreservation. The objective of the present study was to compare the direct cover vitrification (DCV) with ethylene glycol (EG), dimethyl sulfoxide (DMSO) and EG plus DMSO.

Methods: Eighty five mice were sacrificed by cervical dislocation and their ovaries were cryopreserved in the presence of 5% EG or DMSO alone or as mixture, 10% EG or DMSO alone or as mixture and a group with ascending concentrations of cryoprotectants. After toxicity testing and vitrification warming, the ovaries were fixed for histological and ultrastructural studies. In addition, the viability of mechanically isolated follicles was studied by trypan blue staining. All data were compared by ANOVA ($p<0.05$).

Results: Ovarian tissues frozen in EG plus DMSO in ascending concentrations retained a higher percentage of morphologically normal and or viable follicles than tissues frozen in 10 M EG plus DMSO or in either concentration of EG and DMSO alone ($p<0.001$). Ultrastructural analysis of ovarian tissues frozen in ascending concentrations of EG plus DMSO showed that these follicles were well preserved and it was very similar to the control group.

Conclusion: Cryopreservation of ovarian tissue in EG plus DMSO is the most effective method for preserving the structural integrity of follicles within the ovary.

Introduction

O varian tissue cryopreservation is a successful technique that enables the storage of the large numbers of follicles and preserves the structural integrity of somatic and reproductive cells. Therefore, many attempts have been made to improve cryopreservation conditions by using simple and efficient vitrification procedures (1-3). Vitrification of ovarian tissue is a useful method for preservation of female mouse germ cells and live pups following transplantation of vitrified-warmed ovarian tissue (4-6).

However, it has been shown that vitrification is often associated with ultrastructural damage of follicular cells and oocytes (7-11).

Vitrification protocols depended on several factors such as appropriate cryopreservation techniques, type and concentration of cryoprotectant, number of equilibration, warming steps and cryopreservation devices (12-14). Toxicity of cryoprotectant is the hot spot of cryobiology studies; therefore, the main challenge is to find the optimal concentration of cryoprotectant solution.

Cryoprotectant toxicity is probably reduced by lowering the temperature of exposure. Therefore, the recent advances in the cryopreservation of ovarian tissue were designed based on the development of ultra-rapid vitrification procedures, including several modifications, such as direct cover vitrification (DCV). Recently developed DCV method is suitable for freezing ovarian tissue with a high follicular viability (15, 16). In DCV as an ultrarapid cooling technique, vitrification (cryoprotectant) solutions have direct contact with the liquid nitrogen (N2).

Chen et al. showed that DCV could facilitate the maximum cooling rate of vitrification and prevent ice crystal injury (16). The isolated follicles from cryopreserved ovarian tissues by DCV can develop in *in vitro* culture (17), however ovarian tissue cryopreservation by supercool of EG and DMSO affect maturation and development of follicles (18). The data on the success of this method for ovarian tissue cryopreservation remain limited and conflicting and therefore further investigation is required.

However, it was previously reported that EG is an efficient and optimal cryoprotectant for cryopreservation of mouse ovarian tissue with a little change in the structure of ovarian follicles because of low toxicity and rapid permeation from cell membrane (19, 20). However, optimal cryoprotectant is one of the important factors in ovarian tissue vitrification, but still many of them are toxic to cells.

It has been revealed that combination of EG+DMSO with sucrose could improve follicular preservation (21, 22), whereas others have reported no added benefit on combination of cryoprotectants (EG+DMSO) (23). In contrast, Nagano et al. showed that exposure of secondary follicles to EG (2 *M*) causes morphological alterations in follicles (24).

Although the cryodamage may be decreased following ovarian tissue vitrification, the optimal cryoprotectant with low toxicity is important for ovarian tissue vitrification.

Despite many reports on improvement of vitrification procedure, the quality is still lower in vitrified than non-vitrified cells and tissues. There are no reports about the characteristics of follicular structure after DCV.

The aim of this study was modified direct cover vitrification of mouse ovarian tissue using different concentrations of EG and/or DMSO and its mixture. Also, histological examination, viability and ultrastructural analyses were evaluated.

Methods

Chemicals: All chemicals and media used in the study were purchased from Sigma-Aldrich Co. (Hamburg-Germany) unless otherwise indicated.

Animals and Ovarian Tissue Preparation: Five to six week-old Balb/c female mice (n=85) were housed in light and temperature controlled condition (12 *hr* light/12 *hr* dark, 22-24°C and 55% humidity). Food and water were freely available at all times. All the experiments using animals were in accordance with the International Animal Care and ethical Committee of Tabriz University of Medical Sciences.

Animal were sacrificed by cervical dislocation and their ovaries were dissected free of fat and mesentery. One ovary of each mouse was considered for vitrification procedure and the other as the intact control.

The equilibration and vitrification solution: The collected ovaries were randomly allocated to three groups: control or non-vitrified (n=8), toxicity test (n=18) and DCV (n=59). The ovaries were vitrified based on the Chen method (16). The cryoprotectant and warming solutions were prepared in Dulbecco's phosphate-buffered saline (DPBS). In this experiment, six concentrations of cryoprotectants in the equilibration and vitrification solutions were used: 5% EG in DPBS with 0.5 *M* sucrose and 20% FBS (group DCV1), 10% EG in DPBS with 0.5 *M* sucrose and 20% FBS (group DCV2), 5% DMSO in DPBS with 0.5 *M* sucrose and 20% FBS (group DCV3), 10% DMSO in DPBS with 0.5 *M* sucrose and 20% FBS (group DCV4), 5% EG+5% DMSO in DPBS with 0.5 *M* sucrose and 20% FBS (group DCV5), 10% EG+10% DMSO in DPBS with 0.5 *M* sucrose and 20% FBS (group DCV6). In DCV7 group, ovaries were equilibrated in DCV5 and DCV6 solution respectively, and then vitrified in DCV6 solution.

Vitrification and warming: Ovarian tissue was equilibrated in the DCV1-6 solution at room temperature for 8 *min*, and then placed in vitrification solution at room temperature for 2 *min*. In DCV7 group, ovarian tissue was equilibrated in DCV5 (4 *min*) and then DCV6 (4 *min*) solutions at room temperature and placed in the vitrification solution of DCV6 for 2 *min*.

The cryotubes were partially filled with a minimum volume of the vitrification solution. The liq-

uid nitrogen was directly applied onto the vitrification solution (DCV), then the cap of the cryotubes was closed and they were placed into liquid nitrogen and stored for one week.

For warming, vitrified ovaries were warmed in room temperature for 10 *s* and then placed in 25 *°C* water bath for 10 *s*. The content of each cryotube was expelled into 1 *ml* of descending concentrations of sucrose (1, 0.5 and 0.25 *M*) and DPBS at room temperature for 10 *min*.

The warmed ovaries were equilibrated for 30 *min* in α-MEM medium supplemented with 20% fetal bovine serum (FBS) before preparing them for viability evaluation. For toxicity test, the ovaries were exposed to the cryoprotectant solution and passed through all stages of vitrification and warming procedure except plunging in liquid nitrogen.

Ovarian follicular viability: Fresh and vitrified-warmed ovaries were placed in 100 *μl* micro-droplets of medium as described earlier. Ovarian follicles in different stages were mechanically isolated from the ovaries using 29-gauge needles under a stereomicroscope (SZ-STS, Olympus, Tokyo, Japan) and were transferred to new micro-droplets (20 *μl*) of medium and covered with mineral oil.

For each group, only follicles containing layers of membrane-enclosed granulosa cells with a centrally located oocyte were examined. It was stained using 0.4% trypan blue and examined under an inverted microscope. The follicles were classified as viable or non-viable; in non-viable follicles, oocyte or the surrounding granulosa cells had blue coloration and viable ones were not stained and the oocyte and the surrounding granulosa cells were clear (Figure 1) (7).

Histological evaluation: The follicle morphology was examined by histological staining and by using light microscope (magnification ×400) after vitrification and warming. Vitrified, non-vitrified and toxicity tested ovaries were embedded in paraffin wax and serially sectioned at 5 *μm*-thickness. Then every 10th section of each ovary was mounted on glass slides, and stained with hematoxylin and eosin.

For this study, primordial follicles were defined as those containing single layer of flattened granulosa cells surrounding the oocyte (30-50 *μm*), primary follicles as those with single layer of cuboidal granulosa cells (50-90 *μm*), and secondary follicles as those with two or more layers of cu-

boidal granulosa cells and no antrum (100-150 *μm*). For each group, only follicles with a visible nucleus in the oocyte were considered for counting to avoid duplicate counts of a follicle.

The follicles were histologically classified as normal, when they contained an intact oocyte and complete layer of granulosa cells (Figure 2A). The follicle was regarded as influenced if it had an intact oocyte with partial detachment of the oocyte from surrounding granulose cells (Figure 2B). The follicle was regarded as degenerated if it contained a cytoplasmic microvacuolation, pyknotic nucleus, shrunken ooplasm, and/or disrupted granulosa cells (Figure 2C).
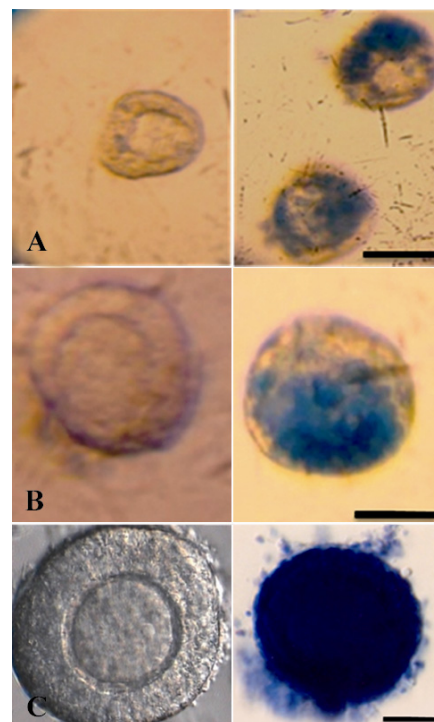

Figure 1. Trypan Blue staining for viability of follicles from vitrified ovarian tissues in different stages. A: Primordial follicles. B: Primary follicle; C: Secondary follicle. Scale bar=30 *μm*. In non-viable follicles, oocyte or the surrounding granulosa cells had blue coloration and viable ones were not stained
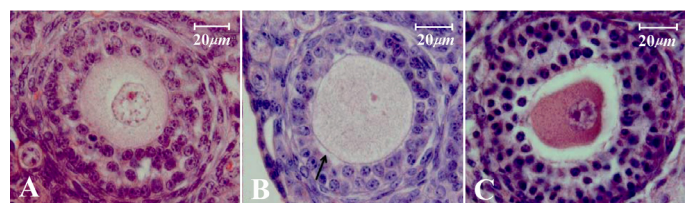

Figure 2. Morphology of the secondary follicles after direct cover vitrification; A: normal or intact follicle; B: influenced follicle whit slightly disruption of contact between innermost granulosa cells and oocyte (black arrow); C: degenerated follicle

Electron microscopy: All chemicals were obtained from TAAB Laboratories Ltd. (Berkshire, UK). Vitrified and non-vitrified ovaries were randomly collected (n=3 from each groups) after equilibration in medium for 30 *min* and fixed in 2.5% glutaraldehyde in PBS (pH=7.4) for 2 *hr*, and post fixed with 1% osmium tetroxide in the same buffer for 2 *hr*. Following dehydration in an ascending series of ethanol, specimens 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 for evaluation by electron microscopy (Zeiss-Gottingen-Germany).

The granulosa cells and oocyte were studied to find the integrity of the cytoplasmic membrane, the mitochondrial cristae, nuclear membrane, density of cytoplasm, cytoplasmic organelles, the numbers and size of vesicles in the cytoplasm and attachment of the cytoplasm to granulosa cells.

Statistical analysis: Quantitative variables were expressed as mean±SD. Categorical variables were presented as values or percentages. One-way analysis of variance (ANOVA) and Tukey's test were used to compare the proportion of normal morphology or variable number of follicles for non-vitrified and vitrified ovaries. Statistical analysis was done with SPSS software 16.0. A $p<0.05$ was considered statistically significant.

Results

The ovarian follicles were mechanically isolated from fresh and vitrified ovaries including 638 primordial follicles (approximately 80 follicles in each group), 721 primary follicles (approximately 90 follicles in each group) and 640 secondary fol-licles (approximately 80 follicles in each group) were examined for their viability (Table 1). There were no significant differences in viability of primordial and primary follicles in all verified concentrations ($p>0.05$).

Although there were no significant differences in viability of secondary follicles of control and combination of cryoprotectants (DCV5-7 groups), in combination of cryoprotectant (EG+DMSO), a higher percentage of viable secondary follicles was observed than single cryoprotectant after cryopreservation ($p<0.05$).

Follicle morphology: The normality of various developmental stages of follicles in the non-vitrified and vitrified groups are presented in table 2. The percentages of normal follicles in toxicity tested groups were the same as those of vitrification tested groups and no significant differences could be found ($p>0.05$) (Table 3).

The number of normal primordial follicles in DCV1 and DCV3 were significantly lower than other groups ($p<0.05$). Primordial follicles with an oocyte surrounded by one layer of flattened or flattened cuboidal granulosa cells (Figure 3.) and granulosa cells had a high nuclear/cytoplasmic ratio and laid on a continuous basement membrane.

In total, 119 to 138 primary follicles were examined for morphological analysis in fresh and DCV groups.

The percentage of normal primary follicles observed in ovarian tissue vitrified in combination of cryoprotectants (DCV5-7) was not significant compared with control, while the percentage of normal primary follicles in single cryoprotectant groups (DCV1-4) was lower than control group ($p<0.05$).

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

Group	Total number of follicles	Primordial follicles (%)			Primary follicles (%)			Secondary follicles (%)		
		Total	Int	Deg	Total	Int	Deg	Total	Int	Deg
Control	247	84	82(98)	2(2)	82	80(97)	2(3)	81	78(97)	3(3)
DCV1	248	77	70(89)	7(11)	91	83(91)	8(9)	80	68(85)	12(15) [a,b]
DCV2	276	86	80(92)	6(8)	97	89(92)	8(8)	93	81(86)	12(14) [a]
DCV3	268	88	80(90)	8(10)	95	87(91)	8(9)	85	73(85)	12(15) [a,b]
DCV4	238	74	67(92)	7(8)	91	83(92)	8(8)	73	61(84)	12(16) [a,b]
DCV5	224	80	75(94)	5(6)	88	83(95)	5(5)	56	50(88)	6(12)
DCV6	237	74	70(94)	4(6)	87	83(95)	4(5)	76	69(90)	7(10)
DCV7	261	75	72(96)	3(4)	90	87(97)	3(3)	96	92(96)	4(4)

a: $p<0.05$ versus non-vitrified control group; b: $p<0.05$ versus vitrified tissue in DCV7 group
DCV= Direct Cover Vitrification

Table 2. Number and percentage of intact (Int), influenced (Inf) and degenerated (Deg) follicles of various stages evaluated in vitrified and non-vitrified ovarian tissue after Hematoxylin- eosin staining

Group	Total number of follicles	Primordial follicles (%)				Primary follicles (%)				Secondary follicles (%)			
		Total	Int	Inf	Deg	Total	Int	Inf	Deg	Total	Int	Inf	Deg
Control	532	255	253(99)	1(0.5)	1(0.5)	133	129(97)	2(2)	2(1)	144	136(94)	5(4)	3(2)
DCV1	574	280	265(95) [a, b]	8(3)	7(2)	124	108(86) [a, b]	8(7)	8(7)	170	53(36) [a, b]	81(44)	36(20)
DCV2	541	235	227(96)	4(2)	4(2)	152	134(88) [a, b]	10(7)	8(5	154	60(39) [a, b]	43(28)	51(33)
DCV3	494	262	245(94) [a, b]	9(3)	8(3)	119	104(87) [a, b]	10(9)	5(4)	113	27(23) [a, b]	53(48)	33(29)
DCV4	542	255	246(96)	6(2)	3(2)	126	190(86) [a, b]	10(8)	7(6)	161	53(38) [a, b]	80(46)	28(16)
DCV5	519	302	290(96)	9(3)	3(1)	101	92(91)	4(4)	5(5)	116	49(42) [a, b]	48(42)	19(16)
DCV6	632	354	343(97)	8(2)	3(1)	138	128(93)	6(4)	4(3)	140	62(45) [a, b]	46(33)	32(22)
DCV7	758	470	460(98)	7(1)	3(1)	122	115(94)	4(4)	3(2)	166	144(86)	14(9)	8(5)

a: $p<0.05$ versus non-vitrified control group; b: $p<0.05$ versus vitrified tissue in DCV7 group
DCV= Direct Cover Vitrification

Table 3. The percentage of intact (Int), influenced (Inf) and degenerated (Deg) follicles at different developmental stages evaluated in toxicity tested ovaries

Group	Total number of follicles	Primordial follicles (%)				Primary follicles (%)				Preantral follicles (%)			
		Total	Int	Inf	Deg	Total	Int	Inf	Deg	Total	Int	Inf	Deg
DCV1	357	200	192(96)	6(3)	2(1)	88	82(94)	3(3)	3(3)	69	65(95)	3(4)	1(2)
DCV2	356	190	182(96)	4(2)	4(2)	93	86(93)	4(4)	3(3)	73	70(96)	2(3)	1(1)
DCV3	385	205	194(95)	6(3)	4(2)	100	95(95)	4(4)	1(1)	80	75(94)	2(2)	3(4)
DCV4	355	186	178(96)	5(3)	3(1)	78	73(94)	4(5)	1(1)	91	86(95)	1(1)	4(4)
DCV5	364	173	166(96)	5(3)	2(1)	103	98(95)	3(3)	2(2)	88	84(96)	2(2)	2(2)
DCV6	375	220	213(97)	4(2)	1(1)	90	87(97)	2(2)	1(1)	65	63(97)	1(2)	1(1)
DCV7	385	219	210(98)	7(3)	2(1)	95	91(96)	4(4)	0(0)	75	72(97)	2(2)	1(1)

DCV= Direct Cover Vitrification

In primary follicles, the oocyte was surrounded by one layer of cuboidal granulosa cells (Figure 3A). The morphology of primary follicles was well preserved in the combination of cryoprotectants groups and the oocytes cytoplasm was clear and normal. Also, the granulosa cells were intact and firmly attached to the related basement membranes (Figures 3 G-H).

In total, 1164 secondary follicles were histologically examined in non-vitrified and vitrified groups. A very low rate of degeneration was observed in non-vitrified ovarian tissue and DCV7 groups (1.90% *vs*. 4.97%). The ovarian tissue exposed to DCV7 solution had significantly higher normal follicles in comparison to those exposed to DCV1-6 solutions.

Secondary follicles consisted of an oocyte surrounded by two or more layers of granulosa cells though the number of layers was very irregular (Figures 2A-C).

In this study, an attempt was made to evaluate morphologically normal follicles that they were either round or oval oocytes by presenting a well-
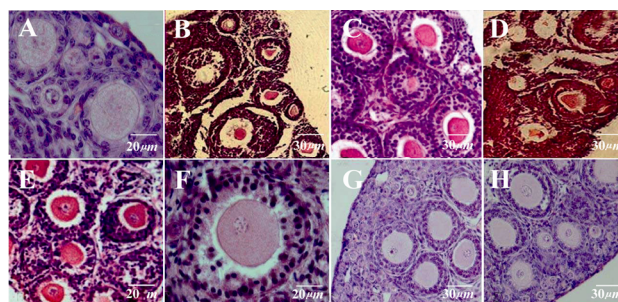

Figure 3. Light microscopic images of mouse ovarian tissue; A: non-frozen; B: DCV1 group that vitrified alone in 5% EG, C: DCV2 group that vitrified in 10% EG; D: DCV3 group that vitrified alone in 5% DMSO, E: DCV4 group that vitrified in 10% DMSO, F; DCV5 group that vitrified in 5% EG + 5% DMSO, G: DCV6 group that vitrified in 10% EG + 10% DMSO and H: DCV7 group that equilibrated in DCV5 and then DCV6 and finally vitrified in DCV6 concentration

delimited nucleus with uncondensed chromatin, surrounded by healthy granulosa cells juxtaposed to oocyte (Figure 3A). The morphology of follicles was well preserved in the DCV7 and the cytoplasm of the secondary follicles and oocytes was normal and also the granulose layers and the-

ca interna and externa were intact and firmly attached to the related basement membranes. The stromal cells were normal with distinct boundaries and prominent nucleus (Figure 3H).

Two types of degeneration could be distinguished. The most predominant degeneration type was characterized by the disruption of intercellular contacts among innermost granulosa cells and the oocyte.

This type of degeneration and cryoinjury was observed most often in secondary follicles in vitrified ovarian tissue in DCV1-6 groups (Figures 3B-G). However, this kind of degeneration was also observed in low level in the control and DCV7 group (Figure 3H).

The other type of degeneration was characterized by shrinkage and pyknosis of the oocyte and granulosa cells, detachment of the follicle from the surrounding stroma, cytoplasmic retraction and numerous cytoplasmic vacuoles. This kind of degeneration was observed with great frequency in secondary follicles of ovarian tissue vitrified in DCV1-6 groups (Figures 3B-G).

In all cases, there was no difference between the percentages of normal primordial, primary and secondary follicles of toxicity tests and vitrification groups (p>0.05).

Therefore, ovarian tissue frozen in combination of cryoprotectants had significantly higher percentages of normal follicles than single cryoprotectants.

Ultrastructural ovarian tissue: The ultrastructure of the ovarian tissue was well preserved in DCV7 concentration and it was very similar to the non-vitrified group. In addition, there was no evidence of subcellular alterations in ovaries vitrified in DCV7 solution. All ovarian tissues vitrified in single cryoprotectant showed a poor ultrastructure. Indeed, compared to oocyte, follicular granulosa cells showed a good ultrastructure.

Primordial follicle ultrastructure: In primordial follicles, oocytes were surrounded by 4-8 flattened granulosa cells and it had shown a homogenous cytoplasm with a large number of vesicle and centrally located nucleus.

In mixed cryoprotectants, the mitochondria were predominantly round with normal cristae and continuous membranes without electron-dense granules. The rough endoplasm reticulum cisternae and organelle was very well developed and the cellular membranes of oocyte and granulosa cells were in close connection.
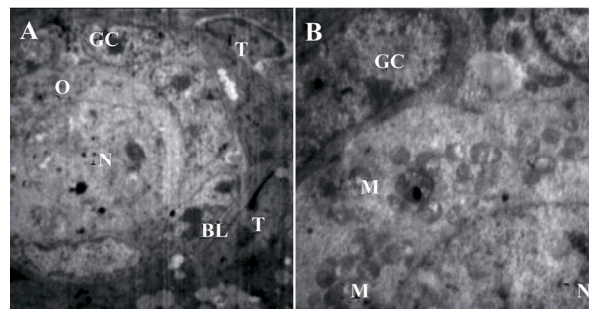

Figure 4. Photomicrograph of early primary follicle (intermediary follicle) in the DCV7 group showing the oocyte (O) encircled by one layer of both flattened and cuboidal granulosa cells (GC). Note the slightly centric nucleus (N), a homogeneous basal lamina (BL) and the theca cells (T) showing fibroblasts with elongated nuclei. The nucleus and the cytoplasmic organelles are well preserved in all combination cryoprotectants (EG +DMSO) groups. TEM: Ax1293, Bx 4646
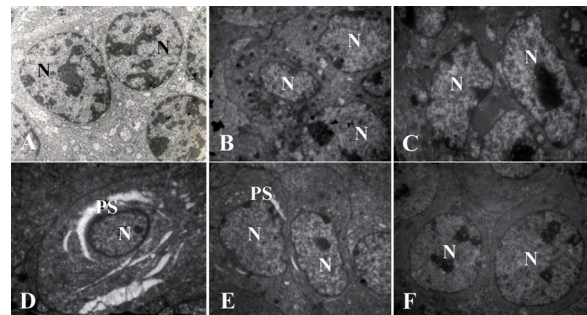

Figure 5. The electron micrograph of granulosa cells of secondary follicles from control or non-frozen (Ax4795), DCV1 (Bx2784) and DCV2 (Cx3597) DCV5 (Dx3597), DCV6 (Ex2156) and DCV7 (Fx2784). The granulosa cells in single cryoprotectant appeared to have cytoplasmic retraction with nuclear shrinkage (A, B). In DCV5 and DCV6 groups were some perinuclear space (D, E). Granulosa cells were well preserved in non-frozen and DCV 7 groups (A, F). N: nucleus, PS: perinuclear space

In single cryoprotectants, elongated mitochondria were observed in some cases and their cristae disappeared.

Primary follicle ultrastructure: A single layer of 8-20 granulosa cells surrounded the oocyte of primary follicles. In the primary follicles, ooplasm remained full of vesicles and the large amount of organelle was very similar to the one observed in the primordial follicles, but elongated mitochondria were observed in some cases and gap junctions were observed between oocyte and granulosa cell membranes in primary follicles of ovarian tissue vitrified in single cryoprotectants (Figures 4A, B).

Secondary follicles ultrastructure: The oocytes were surrounded by two or three layers of cuboidal granulosa cells in secondary follicles.
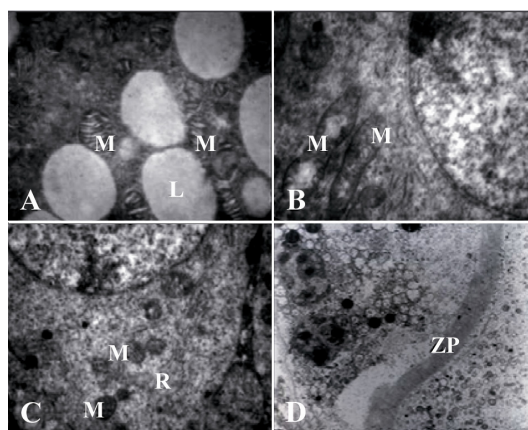
Figure 6. The electron micrograph of mithochondria (M) arrangement of secondary follicles. A: Numerous lipid droplets (L) are found in close association with atypical or irregularly shaped mithochondria with longitudinally oriented cristae in DCV 1-4 groups, TEM:x10000. B: Numerous elongated mithochondria with tubular-vesicular cristae in DCV 5-6 groups, TEM:x10000. C: Note the presence of numerous round mithochondria with continuous membranes and normal cristae and numerous parallel stacks of RER in DCV7 group, TEM:x7750, D: The zona pellucid (ZP) was fully developed and forming a thick layer around the oocyte of secondary follicle combination cryoprotectants. TEM:x10000

The integrity of cell organelles in the secondary follicles in controls (Figure 5A) and DCV7 group (Figure 5F) was well preserved. Round mitochondria with continuous membranes and normal cristae were still more abundant, and elongated mitochondria were rarely observed (Figure 6C). Patches of ZP material were observed in all structures and were usually associated with erect microvilli (Figure 6D).

Our ultrastructural studies in single cryoprotectants groups showed that after warming of ovaries, noticeable changes occurred in the organelles of oocytes and follicular cells (Figures 5B, C and Figure 6A). In addition, extremely vacuolated oocytes with nuclear shrinkage and elongated mitochondria with a few cristae were observed as well (Figure 6A). In some cases, granulose cells had disappeared and were swollen. Moreover, they exhibited irregularly-shaped nuclei, irregular distribution of cytoplasmic organelles and vacuolation (Figures 5D, 5F and Figure 6B).

Discussion

Our data showed that direct cover vitrification of ovarian tissue using combination of cryoprotectants (stepwise concentration of EG with DMSO) had no harmful effect on the morphology and ultrastructure of ovarian tissue, where the cell structure is complex.

Many studies consider vitrification to be the best optimal cryopreservation technique for whole organ with heterogeneous and complex structure (19-25). Thus, ovarian tissue cryopreservation involves a compromise between effects on the oocyte, follicular cells and stroma. This makes it difficult to select an appropriate cryoprotectant concentration and cooling rate for ovarian tissue.

An important factor in the selection of a vitrification solution will be its toxicity and permeability into cells. EG and DMSO are the most commonly used permeable cryoprotectants in vitrification; EG has highest permeability due to lowest molecular weight compared with DMSO (62.07 *g/mol vs.* 78.13 *g/mol*) (26).

In this study, EG+DMSO was better than EG or DMSO for ovarian tissue vitrification; when ovaries equilibrated and vitrified in combination of cryoprotectants have enough penetration into cells and avoid the intracellular ice formation, then they could improve the efficiency of cryoprotectants.

A disadvantage of the vitrification procedure is toxicity of the cryoprotectant agents, which can cause cell damage. To overcome cryoprotectant toxicity, in this study, the cooling rate was decreased by direct plunging of ovarian tissue to nitrogen and exposing ovarian tissue to combination of cryoprotectants (5% to 10%). This could probably be due to sufficient permeation of the cryoprotectants into the ovarian tissue and reduction of osmotic stress; therefore, maybe it improves the success of cryopreserving follicles in different stages.

In our study, when single cryoprotectants were applied, the morphologically normal follicles reduced from 94% to 34%, which was consistent with previous reports in which mouse ovarian tissues were vitrified either in DMSO alone (3) or in a mixture of DMSO, acetamide, propylene glycol (PG) and polyethylene glycol (15, 18, 25, 27) or when ovarian tissues were vitrified in DMSO, acetamide and PG (28-30).

In this study, the importance of the presence of both non-permeating (sucrose) and permeating (EG and DMSO) cryoprotectants in a vitrification solution was shown for successful follicular preservation.

Non-permeating cryoprotectants such as sugars could reduce ice crystal formation during cryopreservation, stabilize lipid membrane and facilitate the dehydration and rehydration of cells prior to cooling (31). The previous studies approved these results. *i.e.*, the protective effect of vitrifica-

tion on the ultrastructure of follicles obtained after vitrifying/warming with EG and sucrose (21, 32).

In agreement with Choi et al., more resistance of primordial follicle to cryodamage were observed in vitrified ovarian tissues compared to other stages. Primordial follicles have small size, low number of granulose cells around the small oocyte, and there is the absence of the zona pellucida and peripheral cortical granules in them (33). In addition, the primordial follicles have more potential to repair sublethal damage to organelles during their growth phase (34). In our study, more than 90% of primordial follicles survived in all vitrification groups. Moreover, the primordial follicles were free of ultrastructural sign of damages.

The follicular changes after vitrifying-warming suggested the sensitivity of the secondary follicles to cryoprotectant solutions relative to that of the primordial and primary follicles. This can be explained by the increased complexity of the growing follicle and gap junctional communication, which makes them more sensitive to cryodamage (35). This hypothesis was in agreement with results of Luz et al that demonstrated the secondary follicle has lower viability and normal morphology than other follicular development stages (36).

Our ultrastructural studies showed that after vitrifying-warming of ovaries in DCV7 group, no noticeable changes occurred in the organelles of oocytes and follicular cells and the integrity of cell organelles was well preserved. This method may be an efficient alternative for cryopreservation of mammalian ovarian tissue and can be used to improve fertility among cancer patients.

The mitochondrial organization and morphology and cytoskeletal network is a necessary key factor for cryopreservation assessment (37, 38). The primary function of mitochondria is to generate ATP, necessary for cell survival and cell function. The movement of mitochondria within different areas of the cell is mediated by cytoskeletal network of microtubules (41). Our results suggested that the mitochondrial damages during ovarian tissue vitrification might be involved in the reduced viability of ovarian follicles. Moreover, mitochondria played an important role in Ca^{2+} signaling that mediated oocyte activation and development, and also apoptosis (39). As was mentioned before, the ultrastructural alternation in mitochondria in mixed cryoprotectants group, especially DCV7 group was lower than the single cryoprotectants groups. These results suggested that the follicles from combination of cryoprotectants group have developmen-

tal potential in comparison to single cryoprotectants groups (Figures 5A-D).

Conclusion

In conclusion, following exposure of ovarian tissue to sucrose, DMSO, EG in alone or a mixture of these cryoprotectants by using DCV, a combination of EG and DMSO and sucrose appeared to be the best cryoprotectant for the preservation of follicular morphology and viability.

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Conflict of Interest

The authors declare no conflict of interest.

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