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Comparison of ultrastructure and morphology of mouse ovarian follicles after conventional and direct cover vitrification using different concentrations of ethylene glycol

Ali Abedelahi[1]M.Sc., Mojdeh Salehnia[1]Ph.D., Allameh Abdolamir[2]Ph.D., Ebrahim Hajizadeh[3] Ph.D.

1 Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
2 Department of Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
3 Department of Biostatistics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

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Abstract

Background: Many attempts have done to improve cryopreservation of mammalian ovaries using simple, economical and efficient technique "vitrification".

Objective: The aim of the present study was to compare the mouse ovaries cryopreservation by direct cover vitrification (DCV) using different concentrations of ethylene glycol (EG) with conventional vitrification methods (CV).

Materials and Methods: Ninety NMRI mice were sacrificed by cervical dislocation; their ovaries were divided into three main experimental groups: control or non-vitrified group, CV group and DCV groups with 4, 6 and 8M EG as cryoprotectant. After vitrification-warming, the viability of mechanically isolated follicles and the morphology of ovarian follicles by light and electron microscopes were studied.

Results: The normality of primary and preantral follicles in non-vitrified and CV groups were higher than those achieved by DCV groups ($p<0.001$). The survival rates of isolated follicles in non-vitrified, CV and DCV groups with 4M, 6M and 8M ethylene glycol were 98.32, 96.26, 84.10, 85.46 and 84.56 %, respectively and in DCV groups it was lower than other groups ($p<0.001$). The ultrastructure of ovarian follicles was well preserved in CV technique. The follicles in DCV groups appeared to have vacuolated oocyte with nuclear shrinkage and irregular distribution of cytoplasmic organelles. Their mitochondria were located mainly in the sub cortical part of the oocyte and the granulosa cells demonstrated some signs of degeneration.

Conclusion: DCV of mouse ovarian tissue using only EG has induced some alteration on the fine structure of follicles. The integrity of mouse ovarian tissue was affected by DCV technique more than CV.

Key words: *Cryopreservation, Direct cover vitrification, Ethylene glycol, Preantral follicle, Ultrastructure.*

Introduction

Cryopreservation of ovarian tissue is an alternative technique to preserve the fertility of mammalian species. Many attempts have been made to improve cryopreservation conditions using simple, economical and efficient technique "vitrification" (1-5). Vitrification of ovarian tissue has been reported with different levels of success in mouse and live pups were produced from transplanting vitrified-warmed ovarian tissue to mice (6-11). However it have been shown that vitrification is often associated with ultrastructural damage to the follicular cells and oocyte (5, 12, 13). Ethylene glycol (EG) has been widely used in cryopreservation of embryo and oocyte with low

Corresponding Author:
Mojdeh Salehnia, Department of Anatomy, Tarbiat Modares University, P. O. BOX: 14115-111, Tehran, Iran.
E-mail: mogdeh@dr.com, Salehnim@modares.ac.ir

toxicity effect (14-17). However, controversial reports were published regarding to the effects of EG on the structure, function and developments of follicles derived from vitrified samples (5, 13, 18-21). Salehnia and colleagues showed that 40% EG in combination with sucrose and Ficol (EGFS40) is an efficient cryoprotectant in vitrification of mouse ovaries with little changes in the fine structure of follicles (5). The vitrification technique did not induce apoptosis in mouse and human ovarian tissue after warming (13, 21). Recently Santos *et al* reported that the vitrification of goat ovarian tissue using EG did not significantly alter the percentages of normal follicles (22).

In contrast Nagano *et al* showed exposure of preantral follicles to 2M EG induced morphological alterations in follicles (20).

Some investigations are undertaken to improve the vitrification condition with alteration in the cooling rate (18, 23). Chen *et al* showed that direct covering of vitrification solution by liquid nitrogen may facilitate the maximum cooling rate of vitrification and prevent ice crystal injury (18).

As mentioned before, in several methods the high concentrations of EG were employed in combination with sucrose and other macromolecules and there were controversial reports in this regards (5, 13, 21). However there is a limited attention to use a single cryoprotectant agent such as EG in vitrification procedure and our hypothesis is that the usage of simple solution may has less toxic effects than complex solution on the ovarian tissue.

Thus, the aim of this study was to evaluate the vitrification of mouse ovarian tissue by direct cover vitrification (DCV) method using different concentrations of EG as a single cryoprotectant agent and compare this technique with CV method.

Materials and methods

Chemicals

All chemicals and media were purchased from Sigma-Aldrich (Hamburg, Germany) unless otherwise indicated.

Animals and ovarian tissue preparation

Ninety 12-14 day-old female NMRI mice were cared for and used according to the guide for the care and use of laboratory animals of Tarbiat Modares University and housed under a 12-h light/12-h dark regime at 22–24°C. Food and water were freely available at all times.

The mice were sacrificed by cervical dislocation and their ovaries (~2mm^3) were dissected free of fat and mesentery. One ovary of each mouse was reserved for vitrification procedure and the other for the intact control.

Experimental design

After ovarian tissue collection, they were divided into three main experimental groups: control or non-vitrified, experiment I: conventional vitrification and experiment II: direct cover vitrification with different concentrations of EG (4, 6 and 8M) as cryoprotectant. Also in each experiment the toxicity tested groups were considered.

Experiment I

Conventional vitrification procedure: The ovaries were vitrified based on the Salehnia CV method (5). Briefly, the ovaries were equilibrated in vitrification medium containing 40% ethylene glycol (v/v), 30% Ficoll 70 (w/v), and 1M sucrose supplemented with 4mg/ml bovine serum albumin (EGFS40; Ethylene glycol, Ficol, Sucrose) for 5 minutes in room temperature. Then, they were put in 1.5 ml plastic cryotubs with a minimum volume of the vitrification medium, placed on nitrogen vapor for 10 sec and then plunged into liquid nitrogen and kept for one week. For warming, vitrified ovaries were warmed in room temperature for 10 sec and then placed in a 25°C water bath for 10 sec. The contents of each cryotubs were expelled into 1ml of descending concentrations of sucrose (1, 0.5 and 0.25M) and PBI (phosphate buffer I) at room temperature for 5 minutes.

Warmed ovaries were equilibrated for 30 minutes in HEPES-Modified Tissue Culture Medium 199 (TCM199) supplemented with 5% fetal bovine serum (FBS) before preparing them for morphological evaluation.

Toxicity test I: The ovaries were exposed to the cryoprotectant solution EGFS40 and passed through all stages of vitrification and warming procedure except plunging in liquid nitrogen.

Experiment II

Vitrification solutions: In this experiment the equilibration solutions were composed of 2, 4, 6 and 8M ethylene glycol in PBI. Then each solution evaluated for vitrifying or glass formation during plunging in liquid nitrogen. Only the 2M EG solution did not opaque during this test, therefore, the concentrations of 4, 6 and 8 M were considered as final vitrification solutions.

Direct cover vitrification procedure

In this group the ovaries were dehydrated (equilibrated) in three stepwise manners as follows:

A: 2 min in 2M EG then 3 min in 4M EG.

B: 1 min in 2M EG, 2 min in 4M EG then 2 min in 6M EG.

C: 1 min in 2M EG, 1 min in 4M EG, 1 min in 6M EG then 2 min in 6M EG

After dehydration of samples they were put in a 1.5-ml plastic cryotubs. The liquid nitrogen was directly applied onto the vitrification solution then the cap of the cryotubs was closed and they were placed into liquid nitrogen and stored for one week (18). For warming, the cryotubs were warmed in room temperature and then placed in 25°C water bath for 10 sec in each steps. The contents of each cryotubs were expelled into Petri dishes and the ovaries were serially transferred into 1ml of 1, 0.5 and 0.25M sucrose and PBI medium for 5 minutes.

Toxicity Test II

In these experiments the ovaries were exposed to the cryoprotectant solutions as mentioned in group A, B and C (equilibration periods) and passed through all stages of vitrification procedures except plunging in liquid nitrogen. After dehydration, the cryoprotectants were immediately removed from the tissue by transferring the ovaries into 1, 0.5 and 0.25M sucrose solutions for 5 minutes at room temperature.

Evaluation of the ovarian follicular viability using trypan blue

The survival rates of the isolated preantral follicles from non-vitrified and vitrified ovaries were determined using trypan blue staining (24). Preantral follicles with 120-150 μm in diameter were mechanically isolated from the ovaries using 29-gauge needles under a stereomicroscope and transferred to new microdroplets (20 μl) of medium and covered with oil.

The follicles containing layers of membrane-enclosed granulosa cells with a centrally located oocyte were examined. They were stained using 0.4% trypan blue and examined under an inverted microscope. The follicles were scored as surviving or degenerated: degenerated follicles stained blue, and surviving ones were not stained (13).

Histological evaluation

To assess the integrity of follicles after freezing and thawing, the follicular morphology was examined by histological staining. Vitrified, non-vitrified and toxicity tested ovaries were fixed in Bouin's solution and were embedded in paraffin wax and serially sectioned at 6-μm-thickness, every 10th section of each ovary was mounted on glass slides, and stained with haematoxylin and eosin. All sections were examined using light microscope at a magnification of ×400. For this study, primordial follicles were defined as those containing of flattened granulosa cells surrounding the oocyte, primary follicles as those with one layer of cuboidal granulosa cells, and preantral follicles as those with two or more layers of cuboidal granulosa cells and no antrum. 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 histological classified as normal, when they contained an intact oocyte and intact granulosa cells. They were classified as degenerated, when they contained a piknotic oocyte nuclei, shrunken ooplasm, and/or disorganized granulosa cells.

Electron microscopy

All chemicals were obtained from TAAB Laboratories Ltd. (Berkshire, UK). Vitrified and non-vitrified ovaries were randomly collected (n = 4 from each groups) after equilibration in medium for 30 minutes and fixed in 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) for 2h, and post fixed with 1% osmium tetroxide in the same buffer for 2h. After dehydration in an ascending series of ethanol, specimens were placed in propylene oxide and embedded in Epon 812. Semi thin sections (0.5 μm) were stained with toluidine blue for light microscopy. Ultrathin sections (60-80 nm) were contrasted with uranyl acetate and lead citrate and examined by electron microscopy (Zeiss, Gottingen, Germany).

Statistical analysis

Statistical analysis was done with SPSS 13.0 software. The survival rates of follicles in vitrified, non-vitrified and toxicity tested groups were compared by one-way analysis of variance (ANOVA) and Tukey's test. $p<0.05$ was considered to be statistically significant.

Results

The viability of isolated preantral follicles

Percentages of viable preantral follicles derived from vitrified, non-vitrified and toxicity tested ovaries after trypan blue staining is presented in Table I. The survival rates of isolated preantral follicles were significantly lower in DCV groups

than CV and non-vitrified groups ($p<0.001$). However, there was not significant difference between three groups of DCV (4, 6 and 8M EG) in this regard ($p>0.05$). There is not any significant differences between all toxicity tested groups with non vitrified group ($p>0.001$).

The percentage of normal follicles after toxicity test and vitrification

The normality of various developmental stages of follicle in the vitrified, controls and non-vitrified groups are presented in table II.

No significant differences were observed between the normality of primordial follicles in all groups of study ($p>0.001$). The percentages of normal primary follicles in DCV using 4, 6 and 8M EG were significantly lower with non-vitrified and CV groups ($p<0.05$).

The normality of preantral follicles in three DCV groups were significantly lower than non-vitrified and CV groups ($p< 0.001$). Statistical analysis of the percentages of normal follicles in toxicity tested groups with those of vitrification tested groups demonstrated no significant differences ($p>0.05$).

Table I. The follicular viability of preantral follicles isolated from fresh, vitrified and toxicity tested ovaries.

	Non-vitrified	Conventional	Direct cover			Conventional	Direct cover		
			4M	6M	8M		4M	6M	8M
Total number	92	85	111	102	106	85	85	90	90
No. of survived	90	82	93	88	90	82	82	87	87
Percentage of survived	97.82	96.47	83.78[a]	86.27[a]	84.90[a]	96.47	96.47	96.66	96.66

a: Significant differences with non-vitrified controls, conventional vitrification and toxicity tested groups ($p<0.001$). EGFS40: the medium containing Ethylene Glycol, Ficol and Sucrose.

Table II. Number of survived follicles (%) at different developmental stages in vitrified, non-vitrified and toxicity tested ovaries.

Stage of follicles	Non-Vitrified	Vitrification				Toxicity test			
		Conventional	Direct Cover			Conventional	Direct Cover		
			4 M	6 M	8 M		4 M	6 M	8 M
Primordial	1061/1076 (98.61)	961/978 (98.26)	1140/1164 (97.94)	1068/1090 (97.99)	1146/1170 (97.95)	1058/1075 (98.41)	1061/1078 (98.43)	1064/1082 (98.33)	1054/1072 (98.32)
Primary	112/115 (97.39)	89/94 (94.68)	85/92 (92.39)[a]	71/77 (92.20)[a]	79/86 (91.86)[a]	77/80 (96.25)	76/80 (95)	82/85 (96.47)	88/91 (96.70)
Preantral	412/423 (97.40)	380/394 (96.44)	302/367 (82.8)[b]	301/378 (79.62)[b]	367/423 (86.76)[b]	381/395 (96.45)	414/427 (96.95)	501/516 (97.01)	455/471 (96.59)

a: Significant differences with non-vitrified controls and conventional vitrification groups ($p<0.05$) b: Significant differences with non-vitrified controls and conventional vitrification groups ($p< 0.001$). EGFS40: the medium containing Ethylene Glycol, Ficol and Sucrose.

Follicular morphology of preantral follicles after vitrification and warming

The morphology of ovarian tissue in non-vitrified and CV groups and its related toxicity tested group was similar and normal (Figure 1A and B). However in CV group, some cryoinjury was observed including the disruption of intercellular contacts among granulosa cells and the oocyte of preantral follicles (Figure 1B). But DCV groups showed more signs of degeneration and cryoinjury in preantral follicles (Figure 1D-F).

The disruption of intercellular contacts among innermost granulosa layer and oocyte, nuclear piknosis and cytoplasmic retraction were prominent. These follicles also exhibited numerous cytoplasmic vacuoles which were better shown in their semithin sections (Figure 2A-C). The morphology of follicles were well preserved in the DCV related toxicity tested groups (Figure 1G-I).

Ultrastructure of oocytes

The ultrastructure of oocyte was well preserved in conventional vitrification technique and it was very similar to the control group. The oocyte had shown uniform cytoplasm with usual organelles. A typically round germinal vesicle was seen on the center of the oocyte (Figure 3A, B) and its ultrastructure in CV group was the same as non-vitrified control. The perinuclear envelope also was shown normal integrity in CV group (Figure 3B). Mitochondria smooth endoplasmic reticulum, Golgi complex and cytokeratin intermediate filaments were the most prominent organelles which distributed among of the ooplasm (Figure 3C). Mitochondria were typically rounded and they had normal cristae and continuous mitochondrial membranes. Smooth endoplasmic reticulum (SER) was observed in close association with mitochondria (Figure 3C).

The preantral follicles in DCV group appeared to have vacuolated oocyte with nuclear shrinkage.

Irregular distribution of cytoplasmic organelles was prominent and their mitochondria were located mainly in the sub cortical part of the oocyte (sub zonal area; Figure 3D) and they were disappeared around the germinal vesicle (Figure 3E). Also the high power electron micrograph showed the disappearance of cristae in some of the mitochondria. Large vacuoles also were formed among of the ooplasm (Figure 3E, F). The intermediate filaments of cytokeratin were disappeared in the oocyte cytoplasm in DCV groups (Figure 3D-F).

Ultrastructure of granulosa cells

The oocytes were surrounded by two or three layers of cuboidal granulosa cells in preantral follicles. At the periphery, all the follicles from fresh and frozen-thawed tissue were surrounded by a continuous basement membrane and this structure were preserved during both vitrification procedures. In non-vitrified (Figure 4A) and conventional vitrified (Figure 4B) ovaries, the ultrastructure of granulosa cell organelles was normal. The mitochondria with a low-density matrix and normal tubular cristae, fat droplets, elements of endoplasmic reticulum and a well-developed Golgi complex were present in the follicular cell but in some conventional vitrified samples, mitochondria showed a reduction of mitochondrial cristae (Figure 4B). The granulosa cells in DCV groups demonstrated some signs of degeneration (Figure 4C-F). The follicular cells showed irregularly-shaped nuclei and accumulation of membrane - bound vesicles and loss of mitochondrial cristae (Figure 4D, F).

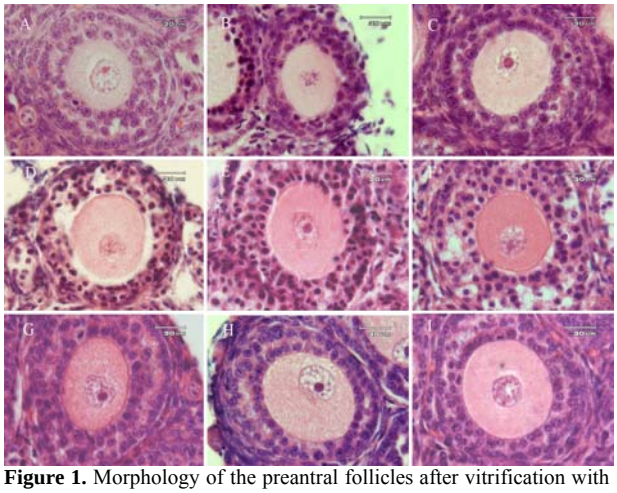

Figure 1. Morphology of the preantral follicles after vitrification with conventional and direct cover techniques and their related toxicity tested groups using hematoxylin and eosin staining. The normal morphology of preantral follicles in non-vitrified (**A**), conventional vitrified (**B**) and its related toxicity tested groups (**C**) were seen. Some morphological changes were seen in preantral follicles in direct cover vitrification groups using 4M EG (**D**), 6M EG (**E**) and 8M EG (**F**). The normal structure of preantral follicles in toxicity tested groups related to direct cover vitrification methods using 4M EG (**G**), 6M EG (**H**) and 8M EG (**I**).

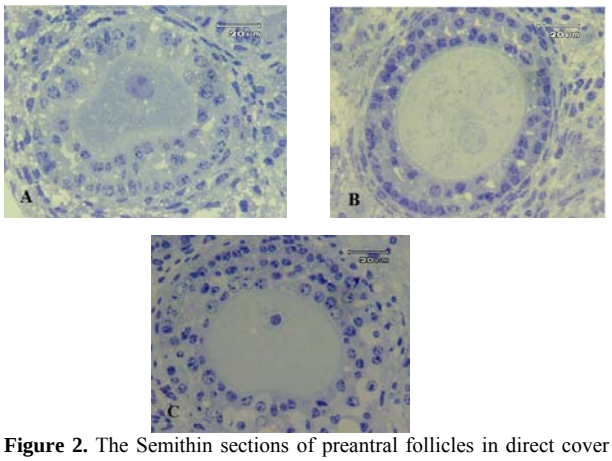

Figure 2. The Semithin sections of preantral follicles in direct cover vitrification groups using 4M EG (**A**), 6M EG (**B**) and 8M EG (**C**). The oocyte shrinkage was prominent in 4M EG group and the intercellular space between granulosa cells were observed in 4 and 6M EG. Some vacuoles were seen in granulosa cells in 8M EG group.

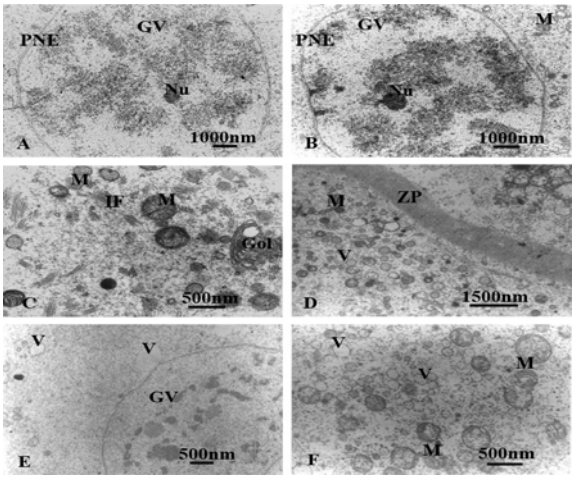

Figure 3. The electron micrograph of oocytes at germinal stage of preantral follicles from control (**A**), conventional vitrification (**B,C**) and direct cover vitrification groups (**D-F**) using 6M EG (**D**) and 8 M EG (**E, F**). GV: germinal vesicle, Nu: nucleolus, PNE: perinuclear envelope, M; mitochondria, IF: intermediate filaments, Gol: Golgi complex, ZP: zona pellucida, V: vesicle.

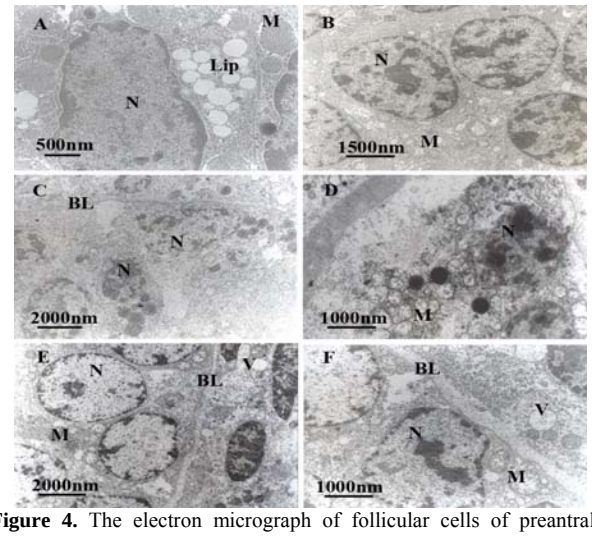

Figure 4. The electron micrograph of follicular cells of preantral follicles from control (**A**), conventional vitrification (**B**) and direct cover vitrification groups (**C-F**) using 4M EG (**C**), 6M EG (**D**) and 8M EG (**E, F**). N: nucleus, M; mitochondria, Lip: lipid droplet, BL: basal lamina, V: vesicle.

Discussion

Toxicity tests in our experiments revealed that cryoprotectants in all concentrations had no toxic effect on the follicles. The percentages of morphologically normal or viable primary and preantral follicles had reduced in DCV groups using 4, 6 and 8M EG rather than non-vitrified and CV groups ($p<0.05$). Also our TEM observations revealed that the organelles of granulosa cells and oocyte were well preserved in CV but the ovaries cryopreserved in DCV had shown some signs of ultrastructural abnormalities. These alterations may be induced by osmotic damage due to cryoprotectant slower permeation. The similar ultrastructural observations were shown in bovine oocytes vitrified by the open pulled straw method (25).

Also the polar accumulation of lipid droplets and vesicles in the subcortical part of oocytes were reported by Boonkusol *et al* and they suggested that this is associated with the dehydration and shrinkage of the oocytes during vitrification (12).

On the other words, our results demonstrated that vitrification of mouse ovarian tissue using a mixture of EG, sucrose and Ficol achieving better results than using only EG. It is suggested that the usage of macromolecules such as disaccharides and Ficol during cryopreservation procedure could prevent the ice crystal formation (15, 20, 26). During the equilibrium phase, the cells initially shrink as water flows out, and then swell as water and cryoprotectant agent enter. The addition of macromolecules such as sucrose in equilibration and vitrification solution could facilitate the exit of water from the cell and decrease the ice crystal formation, thus it could protect the cells during cooling and warming process (22). In agreement with this suggestion the previous studies reported the improvement of the survival of follicles and beneficial effects of EG in combination with macromolecules and sucrose during vitrification procedure on the morphology of mouse and human ovarian follicles (5, 22, 27).

In contrast to our observation Chen *et al* reported that the proportion of viable follicles was lower in CV than those achieved from DCV ($p<0.01$) and the ultrastructure of primordial follicles derived from DCV was more preserved than that achieved from CV group (18). These different results may be due to differences between techniques, including cryoprotectant composition and concentration, exposure time, and container. Different types of container were used by investigators such as cryovial and straw and theses may affect the results. Also the effects of cryoprotectant on the follicular viability may be depended on the method and steps which have been used during cooling and warming. More study is needed in these regards.

Moreover, a remarkable damage to mitochondria was observed in electron micrograph in DCV groups and there are some reports regarding to the mitochondrial damaged by cryopreservation (5, 28, 29). The mitochondria is an important regulators of apoptosis (30), and plays a critical role during fertilization and embryo development (31), their damage may affect on the developmental capacity of the oocyte and embryos. As we mentioned the ultrastructural alteration in mitochondria in CV groups was lower than the DCV groups. This data suggested that the follicles in CV groups have more developmental potential than DCV and our previously reports showed the in vitro maturation rates of isolated follicles derived from CV methods was similar to the non-vitrified and the CV method did not induce apoptosis just after warming (13,19).

Our data obtained from histological study and viability tests showed that the percentage of normal primordial follicle in two vitrification methods was higher than primary and preantral follicles ($p<0.001$). It means that the primordial follicles are more resistant to the cryoinjury than other stages of follicles.

It may be due to their special characteristics including: their small size, a low number of granulosa cells around the small oocyte and an absence of the zona pellucida. These results are in agreement with findings of previous studies (3, 6, 7, 32).

Also the similar results were obtained by Hovatta *et al* after cryopreservation of ovarian tissue using slow freezing technique and they showed a great number of primordial follicles (70%–90%) were well preserved and they were more resistant to cryoinjury and ischemic damage than other follicular stages (33). In addition, Kim *et al* have been reported primordial follicles to have more potential to repair their organelles damage during their prolonged growth phase (34).

In conclusion, our ultrastructural observation in correlation with histological and viability analysis showed the DCV of mouse ovarian tissue using only EG as cryoprotectant has induced some alteration on the fine structure of follicles than CV

method. Also the survival rate of follicles in DCV groups was lower than CV, thus the integrity of mouse ovarian tissue is more affected by DCV technique than CV.

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