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Reactive oxygen species level, mitochondrial transcription factor A gene expression and succinate dehydrogenase activity in metaphase II oocytes derived from *in vitro* cultured vitrified mouse ovaries

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Abstract

The aim of this study was to evaluate the effects of ovarian tissue vitrification and two-step *in vitro* culture on the metaphase II (MII) oocyte reactive oxygen species (ROS) level, mitochondrial transcription factor A *(*TFAM) expression and succinate dehydrogenase (SDH) activity. After collection of neonatal mouse ovaries, 45 ovaries were vitrified and the others (n = 45) were considered as control. All ovaries were cultured for seven days, and their isolated preantral follicles were cultured in three-dimensional culture system. After 12 days, the follicular development and oocyte maturation were evaluated and compared in vitrified and non-vitrified ovaries. The collected MII oocytes were inseminated with capacitated spermatozoa. Then, the fertilization, embryonic development, ROS level, TFAM gene expression and SDH activity of oocytes were assessed and compared. There was no significant difference between morphology and percentage of normal follicles between vitrified and non-vitrified ovaries at the beginning of culture. The follicular development and hormone level in the vitrified group was significantly lower than non-vitrified group and the ROS concentration in the vitrified group was significantly higher than non-vitrified group after one-week culture. After follicular culture, there was no significant difference in follicular development, oocyte maturation, fertilization rate, TFAM gene expression, ROS level and mitochondrial SDH activity between the groups. This study showed that ovarian tissue vitrification influences the follicular development through increase in ROS level during culture but these harmful effects may be recovered during the follicular culture period. Thus, vitrification and ovarian culture method should be improved.

میزان رادیکال‌های آزاد اکسیژن، بیان ژن فاکتور رونویسی میتوکندریایی نوع A و فعالیت آنزیم سوکسینات دهیدروژناز تخمک متافاز دو حاصل از تخمدان‌های انجمادی کشت شده موش

چکیده

هدف مطالعه حاضر بررسی اثرات انجماد بافت تخمدان و کشت دو مرحله‌ای آن بر میزان رادیکال‌های آزاد اکسیژن (ROS) و میزان بیان ژن فاکتور رونویسی میتوکندریایی نوع A (TFAM) و نیز فعالیت آنزیم سوکسینات دهیدروژناز (SDH) تخمک متافاز دو(MII) بود. پس از تهیه تخمدان‌های موش نابالغ ۴۵ عدد ازتخمدانها انجماد شیشه ای شدند و ۴۵ عدد به عنوان کنترل در نظر گرفته شد. تمام تخمدان‌ها به مدت یک هفته کشت شده و فولیکول‌های پره انترال آنها جداسازی شده و در سیستم کشت سه بعدی کشت شدند. پس از ۱۲ روز، تکوین فولیکولی و بلوغ تخمکی در تخمدان های منجمد شده و غیر منجمد شده مورد ارزیابی و مقایسه قرار گرفت. تعدادی از تخمک‌های MII در حضور اسپرم‌های ظرفیت‌گیری شده، تلقیح شدند. سپس، باروری، تکوین جنین‌ها، میزان ROS، بروز ژن TFAM و فعالیت آنزیم SDH تخمکها ارزیابی و مقایسه شد. تفاوت معنی‌داری بین گروه‌های انجمادی و غیر انجمادی از نظر مرفولوژی و درصد فولیکول‌های نرمال در ابتدای کشت مشاهده نشد. پس از یک هفته کشت، گروه انجمادی تکوین فولیکول و سطح هورمون کمتری و میزان ROS بیشتری را در مقایسه با گروه غیر انجمادی داشت. پس از کشت فولیکولی، تفاوت معنی‌داری بین دو گروه از نظر تکوین فولیکولی، بلوغ تخمکی، درصد لقاح و میزان ROS ، بروز ژن TFAM و فعالیت آنزیم SDH دیده نشد. این مطالعه نشان داد که انجماد شیشه‌ای بافت تخمدان با افزایش میزان ROS در طی کشت، تکوین فولیکولی را متأثر می سازد اما این اثرات مخرب در طی دوره کشت فولیکولی بهبود می یابند. لذا اصلاح روش انجماد و کشت تخمدان ضروری است.

واژه های کلیدی: انجماد شیشه‌ای، فاکتور رونویسی میتوکندریایی نوع A، تخمک متافاز دو، آنزیم سوکسینات دهیدروژناز

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Introduction

Ovarian tissue vitrification is a suitable technique for maintaining fertility in the patients who have malignancy that needs chemotherapy or radiotherapy.[1] Several studies reported the successful results, regarding to vitrification of ovarian tissue in various species.[2] After cryopreservation of ovarian tissue, *in vitro* culture of tissue is an alternative method for follicular development.[3] Recently, attempts have been focused on *in vitro* culture of ovarian tissue as a whole organ or tissue fragments.[4,5] However, effects of vitrification and *in vitro* culture of ovarian tissue on the development of follicles are under investigation and there are controversial reports.[4-7] Many improvements have been achieved in vitrification and *in vitro* culture of ovarian tissue techniques, but there are different results about the impact of these methods on the development of follicles and the normal morphology of ovarian tissue.[8,9] Organ culture provides three-dimensional micro-environment similar to *in vivo* condition to support physical characteristics of the follicles for their growth and development.[10-13] Moreover a number of investigations showed that the *in vitro* development of the follicles in vitrified mouse ovaries was significantly lower than non-vitrified once.[14,15] The ovarian tissues were successfully cultured in two-steps protocol but embryonic development of oocytes derived from the cryopreserved cultured ovaries is still low.[4,16] These negative effects of vitrification and subsequently *in vitro* culture on follicular development may be due to the high level of reactive oxygen species (ROS).[17-19] Moreover, low level of ROS is vital for normal physiology of cells but its high level causes oxidative stress that may influence follicular development.[20,21] The enzymatic and non-enzymatic antioxidants lead to balance in ROS level in ovarian tissues.[22,23] In addition during *in vitro* culture, different exogenous factors such as oxygen tension, visible light, handling of specimen may increase the ROS level.[19,22-24]

The mitochondria, as a critical organelle for oocyte maturation and development, may damage during process of cryopreservation and *in vitro* maturation.[4,25,26] Oxidative phosphorylation (OXPHOS) system of mitochondria is involved in cellular energy metabolism. It consists five multimeric complexes that are located in the inner membrane of mitochondria. Unlike other complexes, complex II or succinate dehydrogenase (SDH) does not participate in proton transfer. SDH has regulatory role in energy metabolism because this enzyme not only transfers electron in respiratory chain but it is also involved in Krebs cycle.[27] Therefore, SDH plays an important role in connection of two pathway of cellular metabolism and all of its subunits are encoded by nuclear genome.[28] Therefore, nuclear-mitochondrial communication is very important for mitochondrial function.[29] Mitochondrial transcription factor A (TFAM) is a regulatory key for the maintenance and stability of mitochondrial genome and its gene is encoded by genomic DNA.[30] Transcription and replication of mitochondrial DNA (mtDNA) is essential for mitochondrial biogenesis during development.[31] However, vitrification and *in vitro* culture may affect follicular development and oocyte quality. Therefore, this study designed to evaluate the effect of vitrification and two step *in vitro* culture of ovarian tissue on oocyte fertilization rate, embryonic development, ROS level, TFAM gene expression and mitochondrial SDH activity.

Materials and Methods

Chemicals. All reagents were purchased from Sigma Aldrich (Hamburg, Germany) except otherwise indicated.

Animal and ovarian tissue collection. In this study, National Medical Research Institute (NMRI) 7 days old neonate female (n = 45) and 7-8 weeks old adult male mice (n = 10) were used. All animals were housed under conditions with 14: 10 (hours) light: dark cycles, at 20 to 24 ˚C and 40 to 50% humidity. Approval for this study was achieved from the Animal Research Ethical Committee of the Tarbiat Modares University (Ref No: 52/1637). Female mice were sacrificed by cervical dislocation[32-34] then, their dissected ovaries were placed into α-minimal essential medium (α-MEM; Gibco™, Paisley, Scotland) supplemented with 5% fetal bovine serum (FBS; Gibco™) before any assessment.

Experimental design. The ovaries of neonate female mice were randomly divided into vitrified (n = 45) and non-vitrified (n = 45) groups, then the ovaries in both groups were cultured for 7 days on insert. After that their preantral follicles were isolated and cultured in three-dimensional culture system for 12 days. During this culture period the developmental rates of follicles were evaluated. After collecting the MII oocytes, the fertilization rate, embryonic development, TFAM gene expression and succinate dehydrogenase activity of released MII oocyte in both groups were assessed. Moreover, the levels of ROS and 17-β estradiol (E2) were analyzed during ovarian tissue culture and *in vitro* follicular culture.

Vitrification and warming. The ovaries (n = 45) were vitrified as previously described.[35] Briefly, the ovaries were transferred into vitrification solution (EFS40) containing 40% ethylene glycol (v/v), 30% Ficoll 70 (w/v), and 1 mol sucrose supplemented with 10% bovine serum albumin (BSA) for five min at room temperature. Then, they were loaded on Cryolock™ and were plunged on liquid nitrogen for one week. Afterward, the Cryolock™ was immersed in 1 mol sucrose solution containing 10% BSA in α-MEM medium at 37 ˚C and sequentially was placed in 0.5, 0.25 mol sucrose and medium solution for five min at room temperature. After warming, the ovaries were incubated for 1 hr in α-MEM medium supplemented with 5% FBS under mineral oil at 37 ˚C in a humidified 5%

CO_2 in air. Then some of vitrified warmed and fresh (non-vitrified) ovaries were fixed for histological examination (n = 5 in each group).

Ovarian culture. The ovaries in both groups (n=40 in each group) were cultured in α-MEM medium supplemented with 5% FBS, 1% insulin, transferrin, and selenium (ITS; Gibco), 100 mIU mL^{-1}recombinant follicle stimulating hormone (rFSH or Gonal-f; Serono, Aubonne, Switzerland) after transferring on inserts (Millicell-CM, 0.4-µm pore size; Millipore Corp, Billerica, Germany) in 24-well plates at 37 ˚C, 5% CO_2 for one week. Every other day, half of the medium in each well was changed with fresh medium.[14]

Histological evaluation. The ovaries at the beginning of the culture (n=5 in each group) and after seven days *in vitro* organ culture period (n=5 in each group) were fixed in Bouin's solution, processed and embedded in paraffin wax. The collected serially sections with five intervals were stained by hematoxylin and eosin (H & E) and were mounted on a glass slide. The tissue sections were observed under light microscope. Classification of follicles was described previously and follicles with an intact oocyte and organized granulosa cells were considered as normal follicles. Degenerated follicles had piknotic oocyte nuclei, shrunken ooplasm, and/or disorganized granulosa cells.[4]

Encapsulation and three-dimensional culture of isolated preantral follicles. The isolated preantral follicles (n=500 in each group) from cultured ovaries in both groups were encapsulated in sodium alginate as described previously.[11] Briefly, sodium alginate was dissolved in deionized water at a concentration of 1% (w/v) then activated charcoal was added. The provided sodium alginate solution was filtered and diluted with sterile phosphate buffer saline (PBS) at a concentration of 0.5% (v/v) at room temperature. The follicles were individually placed inside droplets of sodium alginate (5 µL), then they were slowly throwing into a cross-linking solution (50 mM $CaCl_2$ and 140 mM NaCl) for 2 min, then alginate beads were removed and rinsed in a-MEM media. Encapsulated follicles were cultured in α-MEM medium supplemented with 5% FBS, 100 mIU mL^{-1} recombinant follicle stimulating hormone (rFSH or Gonal-f; Serono), 1% insulin, transferrin, and selenium (ITS; Gibco), 100 mg/ml penicillin, and 50 ng mL^{-1} streptomycin under mineral oil at 37 ˚C with 5% CO_2 for 12 days. During the culture period the half of culture media was removed and changed with fresh media every other day. The collected media were stored at – 20 ˚C for hormonal analysis.

***In vitro* ovulation induction.** Ovulation induction was performed by adding 1.5 IU mL^{-1} human chorionic gonadotropin hormone (HCG; Sereno) to the culture media after 12 days of *in vitro* culture of follicles. The collected MII oocytes from *in vitro* condition were randomly selected for insemination and assessment of the embryo development, analysis of TFAM gene expression, level of ROS and evaluation of mitochondrial succinate dehydrogenase activity.

***In vitro* fertilization and embryo culture.** A number of MII collected oocytes were inseminated with capacitated spermatozoa derived from cauda epididymis of 7-8 weeks old male NMRI mice (n = 10) in human tubal fluid (HTF, Irvine Scientific; Santa Ana, USA) medium supplemented with 15 mg mL^{-1} BSA for 4–6 hours. Then the oocytes were removed and placed into 20 µL drops of global medium (Life Global; Guilford, USA) with five mg/ml BSA under mineral oil at 37 ˚C and 5% CO_2 in air. Fertilization and developmental rate of blastocyst embryos were evaluated.

ROS assay in cultured ovarian tissues and collected oocytes. Concentration of ROS was measured in two steps. First, in the vitrified and non-vitrified ovaries after 7 days culture (n = 5 in each group) and secondly in collected MII oocytes derived from two groups (n = 60 for each group in 3 repeats). The samples were incubated in 40 mmol L^{-1} of tris–HCl buffer (pH = 7.0) containing 5 mmol L^{-1} 2′, 7′ dichlorodihydro-fluorescein diacetate (Merck, Hamburg, Germany) at 37 ˚C for 30 min. They were washed with PBS, lysed by lysis buffer, sonicated at 50W for 2 min, centrifuged at 4 ˚C and 10,000 *g* for 20 min, and the supernatant monitored using a spectrofluorometer at 488 nm excitation and at 525 nm emissions.

Hormonal assay in cultured ovarian tissues and collected oocytes. The level of E2 was measured in collected media in two steps by a Microplate Enzyme Immunoassay kit (Monobind, Lake Forest, USA) with sensitivity = 6.50 pg mL^{-1}). Media collected from vitrified and non-vitrified cultured ovaries on day 3 and 7 of culture period (n = 15 for each group) and on day 2 and 12 of follicular *in vitro* culture period in both groups.

Oocyte RNA extraction and cDNA synthesis. Total RNA was extracted from collected MII oocytes in both groups (n = 30 oocytes in each group; 10 oocytes for each replicate of experiments) by RNeasy Mini Kit (Roche Molecular Biochemicals, Mannheim, Germany) and stored at – 80 ˚C. The cDNA was synthesized by the cDNA kit (Thermo Scientific, EU) according to the manufacturer's instructions at 42 ˚C for 60 min then, stored at – 20 ˚C.

Real-time RT-PCR. Primer pairs for amplifying TFAM gene were designed using GenBank at NCBI. The primers sequences are shown in Table 1. β-actin was used as house-keeping gene. Real time thermal cycler (Applied Biosystems, Foster City, USA) was used for analyzing gene expression. QuantiTect SYBR Green RT-PCR kit (Applied Biosystems) was employed for amplifying the targeted genes. Amplification of reference and target genes was performed in the same run, for each sample. Thermal protocol of real time RT-PCR was programmed as: the holding step at 95 ˚C for 5 min, cycling step at 95 ˚C for 15 sec, 58 ˚C for 30 sec, and 72 ˚C for 15 sec, which was followed by a melt curve step at 95 ˚C for 15 sec, 60 ˚C for 1 min, and 95 ˚C for 15 sec. Determining of relative

Table 1. Designed primer sequences used for real-time PCR.

Gene	Primer pair sequence (5´-3´)	Accession number	PCR product size (bp)
β-actin	F: TGTGACGTTGACATCCGTAA R: GCTAGGAGCCAGAGCAGTAA	NM-007393	64
TFAM	F: AAGGGAATGGGAAAGGTAGA R: AACAGGACATGGAAAGCAGAT	NM-011045	76

quantitation for target genes was performed using Pfaffl method. All experiments of real time RT-PCR were done three times.

Mitochondrial succinate dehydrogenase activity. The distribution and activities of SDH in MII oocytes were detected using the method described by Vivarelli *et al.*[36] Briefly, samples were washed in PBS supplemented with bovine serum albumin (BSA) per mL, then they placed in one drop (0.8 mL^{-1}) of a solution [12-5 mM-tris-HCl (pH 7-4); 1-25 mg BSA per mL; 1-25 mM-CaCl2; 1 mM-NaCN (pH 7-4); 62-5 mM-disodium succinate, pH 7-4]. After transferring MII oocytes on Cryotop® with small volume of the same solution, the Cryotop® were frozen and thawed at 37 ˚C then 0.1 ml of 10 mg nitro blue tetrazolium per 5 mg phenazine methosulphate were added, and the MII oocytes were incubated at 37 ˚C for 15 min. For controls, MII oocytes were incubated in the same solution consisted of 90 mM-NaCl instead of substrate. Stained MII oocytes were placed in the center of glass slide. These slides were observed under a light microscope. The intensity of the enzyme activity was determined by the amount of deposited reaction products. The light microscopic images of individual oocytes (n=5 in each groups) were taken at 400 magnification and entered into Digimizer software system (version 5.3.3; MedCalc Software, Mariakerke, Belgium). The intensity of the enzyme activity in each oocyte was quantitative (pixel value) and measured using Image J (version 1.50; National Institute of Mental Health, Bethesda, USA).

Statistical analysis. The SPSS (version 21.0; SPSS Inc., Chicago, USA) was used for data analysis. Values are given as mean ± SE. The data were compared with independent t-test. A p value less than 0.05 was considered as statistically significant.

Results

Light microscopy. The morphology of vitrified and non-vitrified ovaries before and after culture period was demonstrated in Figure 1. Growing follicles were obvious at day 7 of culture in non-vitrified and vitrified ovaries whereas, degenerated follicles were shown in central area of both groups. However, it was more prominent in vitrified group.

Percentage of normal follicles. The percent of normal follicles in non-vitrified and vitrified groups at the beginning of culture and one week after culture were summarized in Table 2. After 7 days *in vitro* culture the percentage of normal follicles at primordial stage was lower while the percent of follicles at primary and preantral stages were higher in non-vitrified group than vitrified group.

Developmental rate of follicles and embryo. Developmental rate of follicles in two studied groups was presented in Table 3. The survival and antrum formation in non-vitrified group were 75.14% and 61.81% and these rates in vitrified group were 73.08% and 58.60%, respectively. The percentage of matured MII oocytes in non-vitrified and vitrified groups were 30.75% and 30.65%, respectively and the fertilization and blastocyst rates were 78.86% and 46.66% in non-vitrified and were 74.58% and 42.50% in vitrified groups respectively (Table 3). There was no significant difference between vitrified and non-vitrified groups in these regards ($p > 0.05$).

Hormonal assay in cultured ovaries and follicles. The concentration of E2 during the ovarian organ culture and follicular culture was compared in both vitrified and non-vitrified groups (Fig. 2). The levels of E2 during ovarian culture were increased in two groups. The level of

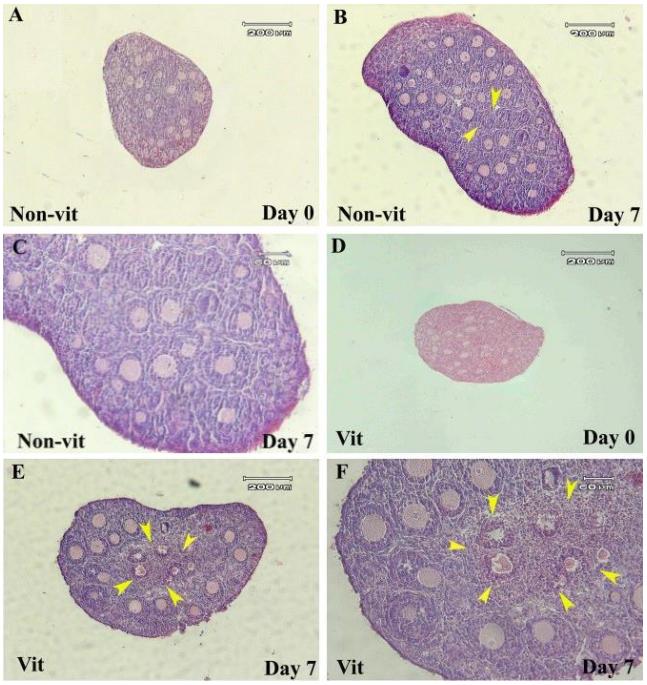

Fig. 1. Photomicrographs of vitrified and non-vitrified whole mouse ovarian sections using hematoxylin and eosin staining during *in vitro* culture. The morphology of mouse non-vitrified (A) and vitrified (D) ovaries at the beginning of culture (on-cultured), cultured non-vitrified ovaries with low magnification (B) and with higher magnification (C), the vitrified cultured ovary with low magnification (E) and with higher magnification (F) was shown. Arrowheads: Degenerated follicles in central area.

Table 2. The number and percentage of follicles at different developmental stages in all examined groups.

Group	Total number	Normal follicles (%)	Degenerated follicles (%)	Primordial follicle (mean ± SE)	Primary follicle (mean ± SE)	Preantral follicle (mean ± SE)
Non vitrified *	2638	2535 (96.09)	103 (3.90)	2349 (92.70 ± 1.79)	134 (5.23 ± 1.45)	52 (2.05 ± 0.40)
Vitrified *	2369	2254 (95.14)	115 (4.85)	2089 (92.69 ± 1.78)	117 (5.17 ± 1.49)	48 (2.12 ± 0.36)
Cultured non- vitrified	1485	1359 (73.87)	388 (26.12)	716 (65.30 ± 1.16)[a]	108 (9.78 ± 1.16)[a]	273 (24.90 ± 2.57)[a]
Cultured vitrified	1247	920 (73.77)	327 (26.22)	647 (70.32 ± 0.67)[b]	104 (11.29 ± 1.40)[b]	169 (18.37 ± 0.84)[bc]

* These two groups are the ovaries at the beginning of culture (Non-cultured). The percentage of follicles was calculated based on the normal follicles. [a] indicates significant difference with the non-vitrified group at the beginning of culture ($p < 0.001$). [b] indicates significant difference with vitrified ovaries at the beginning of culture ($p < 0.001$). [c] indicates significant difference with cultured non-vitrified ovaries ($p < 0.05$).

E2 on day 7 of organ culture in vitrified group was significantly lower than non-vitrified group ($p < 0.05$), but there was no significant difference between these groups on day 12 of follicular culture.

ROS level in cultured ovaries and follicles. The level of ROS in cultured ovaries and isolated follicles were indicated as mM H_2O_2, and were shown and compared in Figure 3. After one week *in vitro* culture of ovaries the level of ROS in vitrified group were significantly higher than non-vitrified group (Fig. 3A; $p < 0.05$). But at the end of follicular culture there was no significant difference between the ROS concentration in these two groups (Fig. 3B).

Oocyte succinate dehydrogenase activity. The succinate dehydrogenase reaction was seen as purple formazan deposit in cytoplasm of MII oocytes and there was no reaction in negative control samples (Fig. 4A-4C). The phase contrast micrograph of these oocytes was shown in Fig. 4D-4F. The intensities of enzyme activity in MII oocytes derived from non- vitrified and vitrified ovaries were 121.60 ± 6.65, 108.20 ± 1.80 respectively (Fig. 5). There was no significant difference between two groups.

Oocyte TFAM Gene expression. The ratio of expression of TFAM to housekeeping genes in MII oocytes derived from non-vitrified and vitrified ovaries were 0.10± 0.02 and 0.07 ± 0.02, respectively. There was no significant difference between two groups.

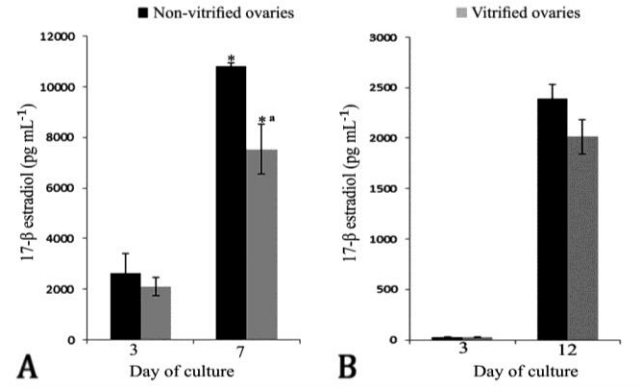

Fig. 2. The comparison of E2 levels during the ovarian organ culture and follicular culture in both vitrified and non-vitrified groups. Concentration of E2 during ovarian culture period (A) and during follicular culture (B). * Asterisk indicates significant difference with day 3 in each group ($p < 0.05$). [a] indicates significant differences with non-vitrified group ($p < 0.05$).

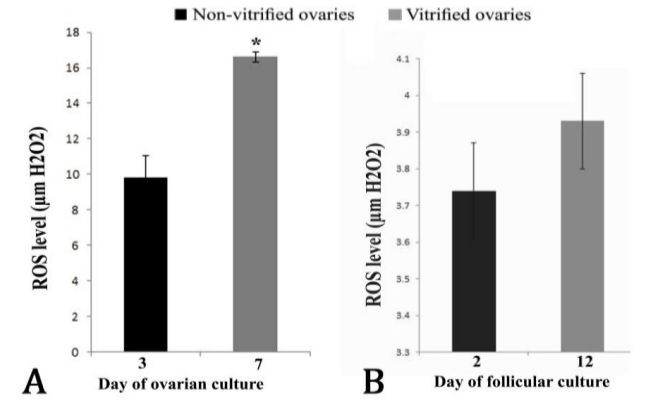

Fig. 3. The concentration of ROS was indicated as mM H_2O_2 in vitrified and non-vitrified ovaries after 7 days culture (A) and MII oocytes derived from isolated follicles (B). * Asterisk indicates significant difference with non-vitrified group ($p < 0.05$).

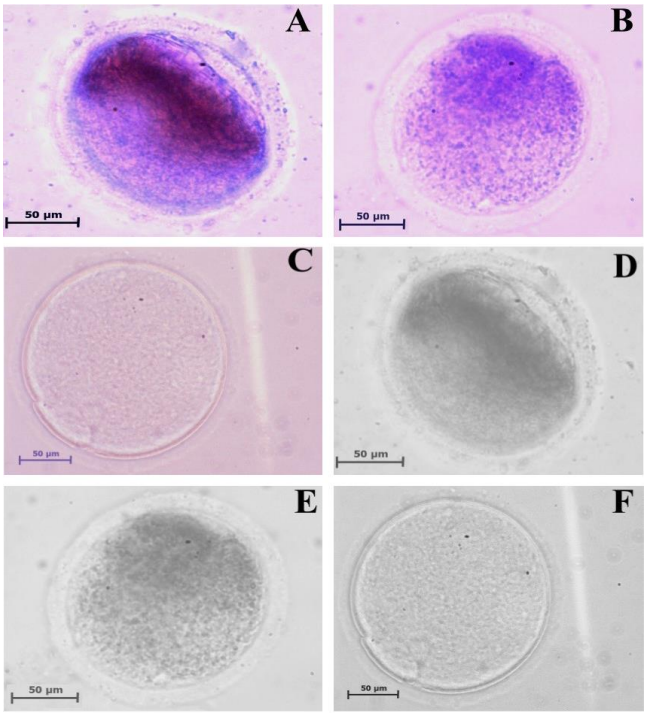

Fig. 4. Photomicrographs of succinate dehydrogenase cyto-enzymology in MII oocytes collected from studied groups. Succinate dehydrogenase activity was demonstrated as purple formazan deposit in cytoplasm of MII oocytes in cultured non-vitrified (A) and vitrified ovaries (B) and there was no reaction in negative control samples (C). The phase contrast micrograph of previous oocytes was shown in D-F respectively.

Table 3. The comparison of maturation, fertilization and developmental rates of MII oocytes derived from cultured isolated follicles in studied groups. The percentage was calculated based on the survived follicles.

Group	No. of follicles	Survived (%)	Antrum formation (%)	MII (%)	No. of Inseminated MII	Fertilization (%)	Blastocyst (%)
Non-vitrified	565	423 (75.14)	265 (61.81)	125 (30.75)	24	19 (78.86)	9 (46.66)
Vitrified	541	394 (73.08)	238 (58.60)	122 (30.65)	20	15 (74.58)	6 (42.50)

MII: metaphase II. There was no significant difference between vitrified and non-vitrified groups ($p > 0.05$).

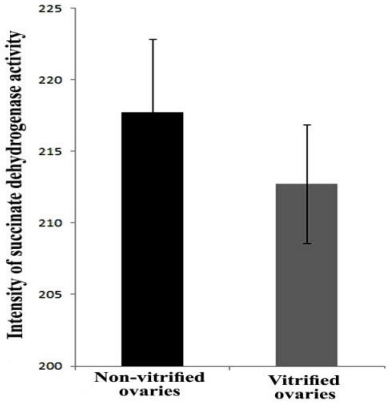

Fig. 5. The intensity of succinate dehydrogenase activity of MII oocytes was compared (G) and there was no significant difference between vitrified and non-vitrified groups ($p > 0.05$).

Discussion

In spite of similar morphology in vitrified and non-vitrified groups at the beginning of the culture, the proportion of preantral follicles, were decreased in vitrified group in comparison with non-vitrified group 7 days after culture. It seems that the harmful effects of vitrification on the integrity and structure of ovarian tissue were better demonstrated during one week *in vitro* culture and these effects did not appear in short term (after warming). The similar observations were reported previously.[14,15] The light microscopy observation of cultured ovaries demonstrated that the degeneration of follicles was prominent in the vitrified ovaries. Also, the low level of E2 production and the high level of ROS in vitrified samples after one-week organ culture were confirmed the reason of degeneration of follicles in vitrified group. It seems that the vitrification of ovarian tissue might lead to hypoxic condition and the increase of ROS concentration and these can affect the follicular development after one-week culture of the vitrified ovaries. Similar reports also showed that an increase in ROS level after cryopreservation of ovaries may influence the follicular development.[14,37,38]

In the other point of view, *in vitro* condition also could induce an increase in ROS level.[18,21,39] Low level of E2 in vitrified group in the present study may be due to decrease in the proportion of normal preantral follicles. However, the levels of this hormone in both groups were enhanced after culturing in order to follicular development. An increase in estrogen production after culture period may be due to increase in proliferation of granulosa and theca cells.[40]

In the present study for evaluation of the effects of ovarian vitrification on the quality of oocytes, several assessments were performed and our results showed the level of ROS, TFAM gene expression, SDH activity and secretion of hormone in MII oocytes were not significantly different in vitrified and non-vitrified groups. It seems that the damaged follicles by the vitrification process, were ignored after isolation at the second steps of culture and the only intact and healthy follicles were selected and cultured thus, the effect of vitrification was not shown properly. It can be also supposed that the harmful effect of vitrification method may be stopped during the follicular culture period. Previous studies showed the similar results.[4,41] Abdi *et al.* reported that ROS levels of oocytes derived from vitrified and non-vitrified ovaries after two steps culture were not significantly different.[5] Results of this study showed that vitrification did not affect *TFAM* gene expression. TFAM molecules are vital for maintenance and biogenesis of mitochondrial DNA (mtDNA).[42-43] The human TFAM is a 25 kD protein which has a nuclear-encoded high-mobility group (HMG) box protein[45] and plays an important role in regulation of mtDNA transcription and replication.[44-47] The functions of the *TFAM* gene consist stabilizing and repairing mtDNA molecules and packaging mtDNA into a nucleoid-like complex.[48,49] Results of the present study indicated that vitrification did not change activity of SDH as one of the mitochondrial respiratory chain enzymes, which plays important role in energy metabolism.[50]

In conclusion this study showed that mouse ovarian tissue vitrification had effects on the follicular development through increase in ROS level during organ culture but these harmful effects of vitrification method may recover during the follicular culture period. Thus, vitrification and ovarian organ culture method should be improved.

Conflict of Interest

The authors declare that thre is no conflict of interest.

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