# Function of vitrified mouse ovaries tissue under static magnetic field after autotransplantation

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| Article Info | Abstract |
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| **Article history:**<br><br>Received: 16 July 2016<br>Accepted: 06 December 2016<br>Available online: 15 September 2017<br><br>**Key words:**<br><br>Apoptosis<br>Autotransplantation<br>Magnetic field<br>Mouse<br>Ovarian vitrification | This study was designed to investigate the effects of applying 1 mT static magnetic field (SMF) during the vitrification process, on the viability of ovarian follicles after vitrification-warming and autotransplantation. The study was conducted in two phases. In the first phase, ovaries of female NMRI mice (6 to 8 weeks old) were randomly divided into three groups: 1- Freshly isolated ovaries fixed in Bouin solution (control group), 2- Ovaries vitrified-warmed without exposure to magnetic field (V1 group) and 3- Ovaries exposed to magnetic field during equilibration step of the vitrification process (V2 group). In the second phase, the vitrified (V1 and V2 groups) and fresh ovarian tissues were autografted into the back muscles of the mice from which the ovaries were extracted. In both phases, morphological aspects and molecular characteristics of active-apoptotic caspase-3 antibody were evaluated. Results indicated the lower percentages of morphologically intact primordial, primary and antral follicles in the V1 group (67.6, 49.5 and 17.6%, respectively) than those of control (97.3, 85.4 and 42.1%, respectively) and V2 (94.1, 78.8 and 40.9%, respectively) groups. In addition, the mean percentages of morphologically intact follicles in the V1 group were statistically lower than those in other groups, after transplantation. The rate of apoptosis in preantral follicles of the V1 group was significantly higher than that in the other groups. It was concluded that exposure of mice ovaries to SMF during vitrification resulted in greater resistance to injuries.<br><br> |

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**عملکرد بافت تخمدان شیشه‌ای شده موش تحت میدان مغناطیسی ثابت پس از پیوند خودی**

**چکیده**

مطالعه حاضر به منظور بررسی اثر میدان مغناطیسی ثابت با شدت یک میلی‌تسلا در فرایند انجماد شیشه‌ای بافت تخمدان موش بالغ و نیز بررسی تغییرات مورفولوژیکی فولیکول‌ها پس از ذوب و پیوند انجام شد. مطالعه در دو مرحله انجام شد. در مرحله اول، تخمدان موش‌های ماده (۶ الی ۸ هفته‌ای) نژاد NMRI به طور تصادفی در سه گروه قرار گرفتند: ۱)گروه کنترل: تخمدان‌های تازه که بلافاصله پس از جدا شدن مورد بررسی بافت‌شناسی قرار گرفتند، ۲) گروه V1: تخمدان‌ها بدون قرارگیری در معرض میدان مغناطیسی منجمد و ذوب شدند و ۳) گروه V2: تخمدان‌ها در مرحله تعادل در معرض میدان مغناطیسی قرار گرفته سپس منجمد و ذوب شدند. در مرحله دوم، تخمدان‌های منجمد-ذوب شده و تازه به عضلات پشت همان موش دهنده پیوند زده شدند. در هر دو مرحله بررسی‌های مورفولوژیکی و ارزیابی میزان مرگ سلولی توسط نشانگر کاسپاز-۳ انجام شد. نتایج نشان داد که پس از انجماد و ذوب، میانگین درصد فولیکول‌های آغازین، اولیه و آنترال سالم در گروه V1 (به ترتیب ۶۷/۶، ۴۹/۵ و ۱۷/۶ درصد) از نظر آماری پائین تر از گروه‌های کنترل (به ترتیب ۹۷/۳، ۸۵/۴ و ۴۲/۱ درصد) و V2 (به ترتیب ۹۴/۱، ۷۸/۸ و ۴۰/۹ درصد) بودند. همچنین میانگین درصد فولیکول‌های سالم پس از پیوند در گروه V1 اختلاف معنی‌داری را نسبت به گروه‌های دیگر نشان داد. نرخ آپوپتوز در فولیکول‌های پیش آنترال، در گروه V1 به طور قابل توجهی بالاتر از گروه‌های دیگر بود. بنابراین می‌توان نتیجه گیری نمود که قرارگیری تخمدان‌ها در معرض میدان مغناطیسی ثابت در طی انجماد منجر به حفظ بهتر فولیکول‌ها در برابر صدمات می‌گردد.

**واژه های کلیدی:** آپوپتوز، انجماد شیشه‌ای تخمدان، پیوند خودی، موش، میدان مغناطیسی

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## Introduction

Preservation of fertility is an extensively researched topic that is driven by the need to perpetuate life and preserve rare species of animals. Cryopreservation is one of the most common and frequently used methods for the preservation of fertility. Among the many methods of cryopreservation studied, the vitrification method has assumed much importance in recent years.[1] The advantage of vitrification is that the formation of intra and extracellular ice crystals is suppressed by the high rates of cooling employed. However, the downside to vitrification is the toxicity caused by the high concentrations of cryoprotectants used during the process.[2] Therefore, it is important to optimize the vitrification process to minimize injuries, while retaining its advantages.

Many methods have been studied to minimize toxicity effects of cryopreservation. Fast water exclusion from cells could potentially reduce the toxicity effects of cryoprotectants and there have been a number of studies exploring the processes that can accelerate water exclusion from cells during vitrification.[4] Recently, magnetic freezing[3] has received much attention in this area because magnetic fields (MF) can potentially accelerate the release of water from cells/tissues,[4] and thus prevent toxicity. Lee and coworkers have suggested that magnetic cryopreservation is an effective method for storage of dental pulp tissue and requires only small amounts of cryoprotectant.[5] Furthermore, published data suggest that freezing blastocysts when subjected to a magnetic field, leads to inhibition of apoptosis after thawing.[6] Thus, it seems that MF can be useful in ovarian vitrification and warming procedures.

Most earlier studies on magnetic freezing have been performed with time-varying MFs, but there have been a few studies on the effect of static magnetic fields (SMFs) on cryopreservation of biological specimens.[7,5] The SMFs are time-independent fields that are generally classified on the basis of intensity as weak (< 1 mT), moderate (1 mT to 1 T), strong (1 to 5 T), and ultra-strong (> 5 T).[8] The SMFs with moderate intensity have been shown to be effective on biological systems and can interact directly with moving charges (ions, proteins, etc.) and magnetic materials found in tissues through several physical mechanisms.[9]

This study aimed at establishing the effects of the static magnetic field on whole ovarian tissue vitrification and the follicular morphology of ovarian tissue after warming and autotransplantation.

## Materials and Methods

**Preparation of mouse ovary and experimental design.** Forty-five 6- to 8-week-old female NMRI mice weighing 20 to 30 g were obtained from the animal house of Royan Institute (Tehran, Iran). They were kept at appropriate conditions (18 to 22 ℃, 12/12 hr light/dark cycle) with free access to food and water. The study was conducted in two phases. In the first phase, both ovaries were removed from female mice, the right ovaries were put in tissue culture medium 199 (Sigma, Munich, Germany) containing 10% human serum albumin. Then ovaries were randomly divided into three groups: 1) freshly isolated ovaries were fixed in Bouin's solution and served as the control group, 2) ovaries were vitrified-warmed without exposure to a magnetic field (V1 group) and 3) ovaries were exposed to 1 mT SMF during the equilibration step of the vitrification process (V2 group). In the second phase, the vitrified (V1 and V2 groups) and fresh ovarian tissues were autografted into the back muscle of the mice.

**Static magnetic field production.** In the present study, an electromagnetic device (designed and manufactured by the engineering group of Royan institute) capable of producing a constant and uniform magnetic field of around 1 mT was used. The magnetic field was generated using two poles of a ferrite core, wrapped with 2,000 turns of a copper wire. The input power of the device was 220 v alternating current (50 Hz) which was converted to 4 A direct current for the wire coil to generate a uniform magnetic flux between the poles.

**Vitrification and warming processes.** The samples (whole ovaries) in experimental groups V1 and V2 were vitrified using the Behbahanian *et al.* vitrification method with minor modifications.[10] Briefly, the ovaries were transferred into an equilibration medium for 15 min at room temperature. The ovaries were then immediately immersed in the vitrification medium, for 30 min at 4 ℃. After dehydration, the ovarian tissues were subjected to a cryopin,[11] a needle of an insulin syringe, plunged into liquid nitrogen. A simple warming procedure was then applied in just one step for 10 min at room temperature. The ovaries were transferred into N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid (HEPES) tissue culture medium (HTCM; Sigma) containing 1 M sucrose supplemented with 20% human serum albumin (HAS; Sigma). To recover the ovaries after rehydration, warmed ovaries were incubated at 37 ℃ for 30 min in an incubator with 5% $CO_2$. They were then fixed in Bouin's solution (Sigma) for subsequent evaluation (n = 5 in each group) or thawed ovaries were orthotopically transplanted into the back muscle of the experimental mice (n = 10 in each group).

**Transplantation.** Thawed and fresh ovaries were immediately transplanted into the back muscle of the same female mouse from which the left ovary was removed. Briefly, after the mouse was anesthetized, a gap with the length of approximately 1 cm was created in the skin over the backbone, and the muscle beneath the skin was then exposed. The ovary was inserted within the muscle and after transplantation, the muscle and skin were closed by suture. The mice were allowed to live for three weeks.

**Morphological evaluations and immunohisto-chemistry study.** After casting in paraffin, the ovaries were serially sectioned into 6 μm thick samples and stained with Hematoxylin and Eosin (H & E). Stained samples were evaluated using light microscopy (400×) and the numbers of intact and dead follicles were counted as described by Liu.[12] To prevent mis-calculations or repetitive counting of the follicles, only those with observable nuclei of oocytes were counted.

Immunohistochemical study was performed with an anti-active caspase-3 antibody. Before incubation with the primary antibody (ab4051; Abcam, Cambridge, UK), tissue sections were deparaffinized and rehydrated in xylene, followed immediately by rehydration in ethanol with serially decreasing concentration. Antigen retrieval was performed using sodium citrate buffer (pH = 6.0) for about 20 min at 98 °C. The tissue sections were then pretreated with 3% hydrogen peroxidase for 30 min to inhibit internal peroxidase activity. In this step, the tissue samples were incubated with the primary antibody (dilution 1: 100, overnight at 4 °C). The tissues were washed using PBS (three times, once every 5 min), and then treated using a secondary antibody diluted 1: 200 (ab97051), for 1 hour at 37 °C. Subsequently, the slides were exposed to a solution containing a chromogen (Diaminobenzidine: DAB) for 2 min at room temperature. The sections were then counterstained with hematoxylin for 10 sec. Finally, after washing and dehydration of the tissues with ethanol solutions of increasing concentration and finally with xylene, immunohistochemistry stained sections were sealed and evaluated using light microscopy.

In this experiment, the follicles that contained caspase-3 positive oocyte or more than 30% granulosa cells were considered to be apoptotic follicles. In order to quantify, ten sections of each ovary were subjected to immunohistochemistry studies.

**Statistical Analysis.** SPSS software (version 18.8; SPSS Inc., Chicago, USA) was used for statistical analysis. The numbers of morphologically intact, dead and apoptotic follicles in all experimental groups were compared by one-way analysis of variance and Duncan's test. A $p$ value less than 0.05 was considered to be statistically significant.

## Results

**Histological assessment.** The mean percentages of morphologically intact primordial, primary and antral follicles in the V1 group (67.6, 49.5 and 17.6%, respectively) were statistically lower than those of the control (97.3, 85.4 and 42.1%, respectively) and V2 (94.1, 78.8 and 40.9%, respectively) groups (Table 1; $p < 0.05$). There were no significant differences in intact antral follicles among all groups. There were significant differences in morphologically intact follicles between the V1 group and other groups. In tissue sections, good quality follicles at different growth stages were observed in the control and V2 groups (Fig. 1). The space between neighboring granulosa cells was seen in the V1 group.

**Prevalence of programmed cell death.** Expression of the caspase-3 protein was not observed in the cells of surface epithelium and primordial and primary follicles, but several apoptotic caspase-3 positive cells were detected in degenerating corpus luteum (Fig. 2). The percentage of apoptotic preantral and antral follicles, differed significantly in V1 (6.6 ± 1.2 and 7.0 ± 0.5) and control (2.6 ± 0.3 and 1.3 ± 0.3), and V2 (3.1 ± 1.2 and 2.6 ± 0.6) groups (Table 2).

**Histological assessment after transplantation.** The mean percentages of morphologically intact primordial, primary, preantral and antral follicles in the V1 (35.4, 23.7, 16.6 and 7.6%, respectively) group were statistically lower than in other experimental groups (Table 3; $p < 0.05$).

**Table 2.** Mean percentages of apoptotic follicles (%) at different developmental stages in vitrified and non-vitrified ovaries. Data are presented as mean ± SEM.

| Groups* | Preantral follicles | Antral follicles |
|---|---|---|
| **Control** | 2.6 ± 0.3 [b] | 1.3 ± 0.3 [b] |
| **V1** | 6.6 ± 1.2 [a] | 7.0 ± 0.5 [a] |
| **V2** | 3.1 ± 1.2 [b] | 2.6 ± 0.6 [b] |
| *p*-values | 0.01 | 0.01 |

* Control: Fresh ovaries were immediately subjected to immuno-histochemical evaluation, V1: Ovaries were vitrified-warmed without exposure to the magnetic field and V2: Ovaries were exposed to the magnetic field in equilibrium step of vitrification process. Values within a column with similar superscripts are not significantly different ($p < 0.05$).

**Table 1.** Number (mean percentages) of morphologically intact follicles at different developmental stages in vitrified and non-vitrified ovaries.

| Groups* | Primordial follicles | | Primary follicles | | Preantral follicles | | Antral follicles | |
|---|---|---|---|---|---|---|---|---|
| | Total | Number of follicles | Total | Number of follicles | Total | Number of follicles | Total | Number of follicles |
| **Control** | 261 | 254 (97.3)[a] | 131 | 112 (85.4)[a] | 68 | 38 (55.8)[a] | 19 | 8 (42.1)[a] |
| **V1** | 232 | 157 (67.6)[b] | 119 | 59 (49.5)[b] | 43 | 17 (39.5)[ab] | 17 | 3 (17.6)[b] |
| **V2** | 256 | 241 (94.1)[a] | 142 | 112 (78.8)[a] | 59 | 28 (47.4)[a] | 22 | 9 (40.9)[a] |
| *p*-values | 0.01 | | 0.03 | | 0.14 | | 0.04 | |

* Control: Fresh ovaries were immediately subjected to histological evaluation, V1: Ovaries were vitrified-warmed without exposure to the magnetic field and V2: Ovaries were exposed to magnetic field in equilibrium step of vitrification process. Values within a column with similar superscripts are not significantly different ($p < 0.05$).
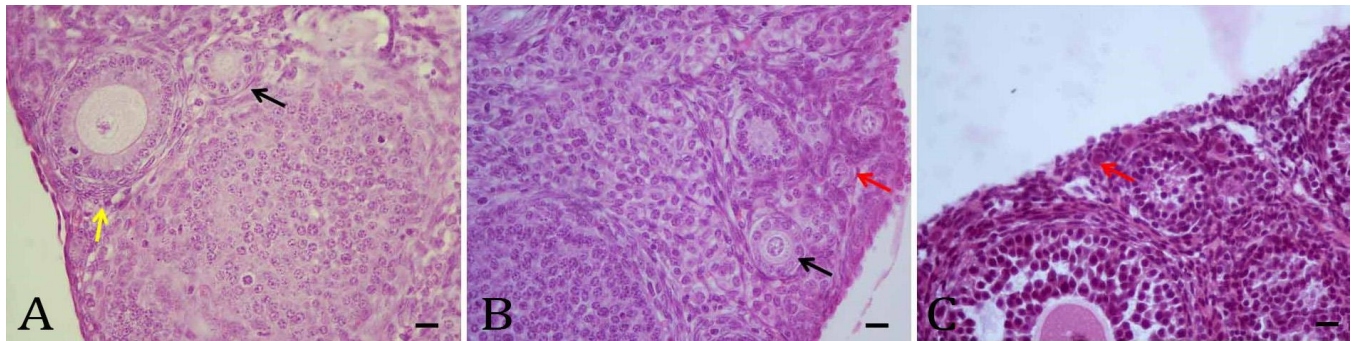
**Fig. 1.** Morphology of mouse ovarian cortical histological sections. A: Fresh ovaries were immediately subjected to histological evaluation (control), B: Ovaries were vitrified-warmed without exposure to the magnetic field (V1) and C: Ovaries were exposed to the magnetic field in equilibrium step of vitrification process (V2). The red arrow indicates primordial follicles. The black arrow indicates primary follicles. The yellow arrow indicates preantral follicles (H & E, Bar = 10 μm).

There were no statistically significant differences in intact follicles at different developmental stages between the control and V2 groups. A weak sign of inflammation was observed in all the experimental groups (Fig. 3).

**Prevalence of programmed cell death after transplantation.** Evaluation of apoptotic incidence after transplantation of mouse ovaries did not show any caspase-3 positive follicles in the primordial and primary stages. The mean percentage of preantral apoptotic follicles in the V1 group (11.3% ± 2.1) was significantly higher than that in the other groups. The mean percentages of apoptotic antral follicles were not statistically significant different among the groups (Table 4 and Fig. 4).

**Table 4.** Mean percentages of apoptotic follicles (%) at different developmental stages in transplanted mouse ovaries into back muscle. Data are presented as mean percent ± SEM.

| Groups* | Preantral follicles | Antral follicles |
|---|---|---|
| Control | 4.8 ± 1.2 [b] | 4.1 ± 1.2 [a] |
| V1 | 11.3 ± 2.1 [a] | 6.1 ± 1.6 [a] |
| V2 | 5.4 ± 1.1 [b] | 4.5 ± 1.3 [a] |
| *p*-values | 0.04 | 0.24 |

* Control: Fresh ovaries were immediately transplanted to back muscle, V1: Ovaries were vitrified-warmed without exposure to the magnetic field then transplanted and V2: Ovaries were exposed to the magnetic field just in equilibrium step then vitrified and warmed, after that transplanted to back muscle. Values within a column with similar superscripts are not significantly different (*p* < 0.05).
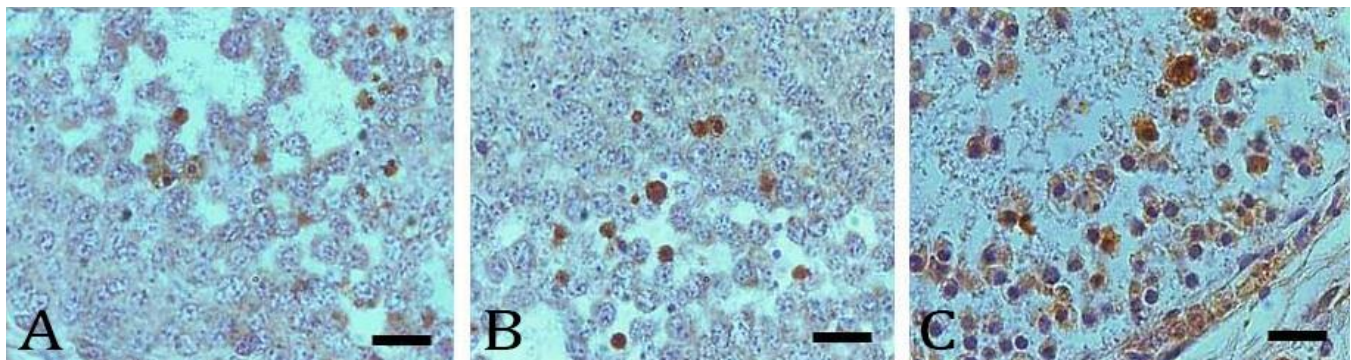


**Fig. 2.** Caspase 3 immunohistochemistry test. A: Fresh ovaries were immediately subjected to immunohistochemical evaluation (control), B: Ovaries were vitrified-warmed without exposure to the magnetic field (V1) and C: Ovaries were exposed to the magnetic field in equilibrium step of vitrification process (V2). Positive staining is shown as brown coloration of the cytoplasm of the cells (Bar = 10 μm).

**Table 3.** Number (mean percentages) of morphologically intact follicles at different developmental stages 21 days after transplantation.

| Groups* | Primordial follicles | | Primary follicles | | Preantral follicles | | Antral follicles | |
|---|---|---|---|---|---|---|---|---|
| | Total | Number of follicles | Total | Number of follicles | Total | Number of follicles | Total | Number of follicles |
| Control | 103 | 75 (72.8)[a] | 74 | 36 (48.6)[a] | 26 | 7 (28.9)[a] | 12 | 2 (16.6)[a] |
| V1 | 93 | 33 (35.4)[b] | 59 | 14 (23.7)[b] | 18 | 3 (16.6)[b] | 13 | 1 (7.6)[b] |
| V2 | 122 | 90 (73.7)[a] | 81 | 39 (48.1)[a] | 29 | 9 (31.0)[a] | 15 | 2 (13.3)[a] |
| *p*-values | 0.02 | | 0.01 | | 0.03 | | 0.03 | |

* Control: Fresh ovaries were immediately transplanted to back muscle, V1: Ovaries were vitrified-warmed without exposure to the magnetic field then transplanted and V2: Ovaries were exposed to the magnetic field just in equilibrium step then vitrified and warmed, after that transplanted to back muscle. [ab] Values within a column with similar superscripts are not significantly different (*p* < 0.05).
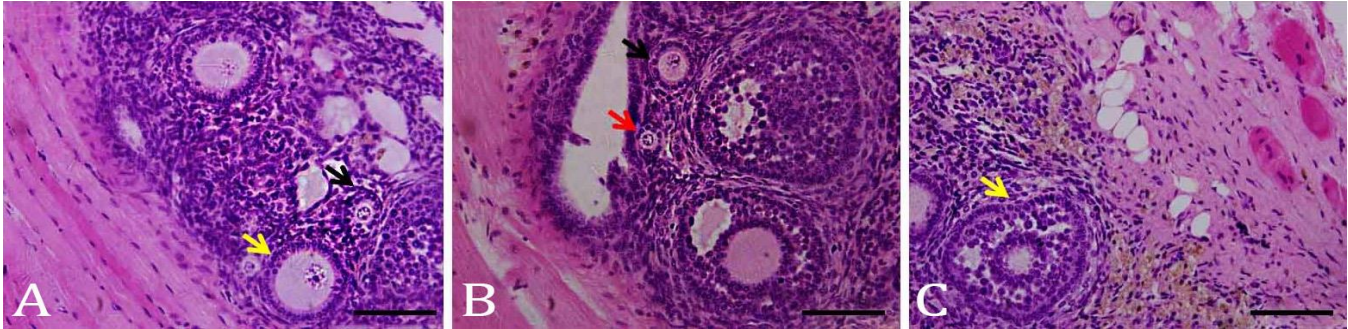
**Fig. 3.** Morphology of mouse ovarian cortical histological sections after transplantation. A: Fresh ovaries were immediately transplanted to back muscle (control), B: Ovaries were vitrified-warmed without exposure to the magnetic field then transplanted (V1) and C: Ovaries were exposed to the magnetic field just in equilibrium step then vitrified and warmed, after that transplanted (V2). The red arrow indicates primordial follicles. The black arrow indicates primary follicles. The yellow arrow indicates preantral follicles. (H & E, Bar = 20 μm).
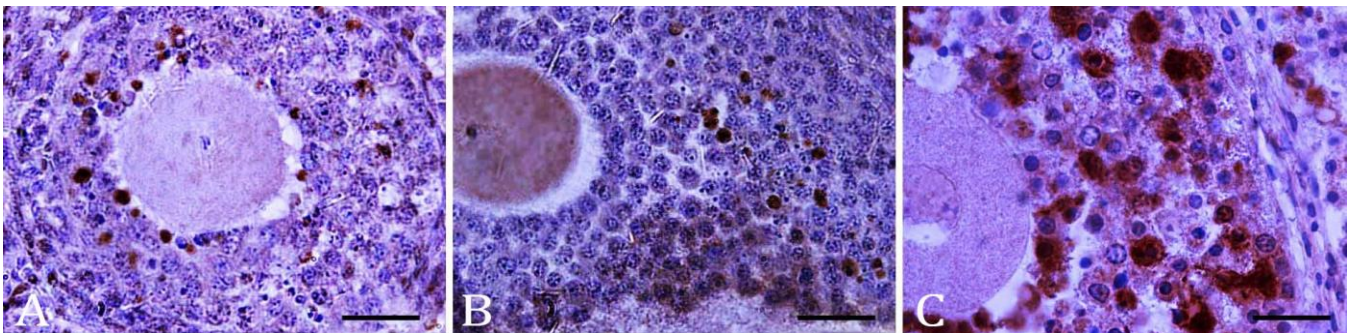


**Fig. 4.** Caspase 3 immunohistochemistry test. A: Fresh ovaries were immediately transplanted into the back muscle (control), B: Ovaries were vitrified-warmed without exposure to the magnetic field then transplanted (V1) and C: Ovaries were exposed to the magnetic field just in equilibrium step then vitrified and warmed, after that transplanted into the back muscle (V2). Positive staining is shown as brown coloration of the cytoplasm of the cells (Bar = 10 μm).

## Discussion

Ovarian tissue cryopreservation has been found to be a useful process to preserve primordial follicles. Vitrification is considered as a promising cryopreservation technique for such ovarian tissue preservation. One of the disadvantages of vitrification method is the use of high concentrations of toxic cryoprotectants that can be harmful to the cells and tissues.[2] Therefore, increasing the rate of replacement of water with cryoprotectants is necessary, especially for the prevention of damage due to osmotic pressure.[11] In the current study, SMF was used during ovarian tissue vitrification.

Studies on the interaction of SMFs with biological specimens have been gaining increasing attention in recent years.[8] Kawata and colleques have studied the effect of applying MF during cryopreservation of periodontal ligament teeth banking.[13] Lee and coworkers suggested that MF applied during cryopreservation of biomaterials, may improve the penetration of cryo-protectants.[5] However, there have been few reports on the application of the magnetic field for cryopreservation of ovarian tissue.[14,15]

The results of H & E staining showed that vitrified ovaries maintained their natural appearance. Follicles contained oocytes at the stage of the germinal vesicle with uniform cytoplasm and the central nucleus. In some vitrified ovaries, injuries resulting from vitrification were observed, and such injuries included disorganization between oocytes and inner granulosa cells, pyknotic nucleus, shrinkage of the cytoplasm and necrosis in a number of follicles. Nuclear staining was slightly high in some of the vitrified groups. These changes in the V1 group were more specific. We found that the V2 group best retained the morphological integrity of the follicles. In general, the maximum and minimum injuries to primordial follicles were seen in the V1 and V2 groups, respectively.

Previous studies have reported that moderate MF is sufficient to change the plasma membrane permeability of cells and alter ion movement across the membrane. These studies also reported that an increase in calcium ion uptake is occurred due to channel activation by MF.[16,17] Teodori and coworkers showed that SMF increases the cell survival againsr damaging agents via increased $Ca^{2+}$ influx in U937 cells.[18] In the present study, the better preservation of follicles in the V2 group might be attributed to the increased membrane permeability induced by SMF and as a result caused an increase in intracellular calcium ions.

In the present study, we used caspase-3 as an apoptosis marker, which, in its active form is used as a marker for apoptotic death in the early stages of apoptosis. The incidence of programmed cell death in the preantral follicles in the control and V2 groups were significantly less than the vitrified group without exposure to SMF. Results pointed to improved follicular survival when ovarian tissues were exposed to SMF during the equilibrium step of the vitrification process. Indeed, in an earlier study it has been suggested that SMFs of moderate intensities decrease cell death by apoptosis induced by several agents in various human cell types.[19]

To understand ovarian function after vitrification and warming, we transplanted the ovaries of groups of the control, V1 and V2 groups into the back muscle of mice from which the ovaries were extracted. Healthier primordial follicles were seen in the group of transplanted vitrified-warmed ovaries that were exposed to SMF during the first step of vitrification process than in the group that was not subjected to SMF during vitrification process.

The mechanisms by which SMFs influence the vitrification process are not completely clear. One explanation that has previously been proposed by Rosen is that the application of SMFs may induce magnetic re-orientation of membrane phospholipids (specifically the acyl chains of the molecules) via diamagnetic anisotropy effects,[19,20] which affects membrane permeability. It has also been reported that magnetic fields could accelerate the release of water from cells/tissues, and thus prevent the toxicity effects of cryoprotectants.[4] Our results have also confirmed that the SMF-exposed follicles of the V2 group exhibited greater resistance to vitrification and warming. Thus, it is reasonable to suggest that the 1 mT SMF exposure decreases damage to ovarian tissues during vitrification process and is helpful in ovarian cryo-preservation. However, the exact mechanism of the effect of SMF on ovarian vitrification is not clear.

In conclusion, SMF was shown to be an effective method for ovarian tissue banking. SMF coupled with the vitrification procedure increased survival rates of vitrified-warmed ovarian follicles. This study showed that the effects of SMF cryoprotective action arose from its influence on water removal from the cell during the vitrification procedure. Further studies are necessary to determine the exact mechanism of SMF effects on ovarian vitrification.

## Acknowledgments

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