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Survival rate of preantral follicles derived from vitrified neonate mouse ovarian tissue by cryotop and conventional methods

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Abstract. The aim of this study was to investigate the growth and survival rate of preantral follicles isolated from vitrified ovarian tissue by Cryotop and conventional methods. The ovaries of 14-day-old mice were separated and divided into four groups as following: Cryotop group, vitrified by Cryotop; CV (Conventional; CV) group, vitrified by conventional straw; toxicity test group and control group. After warming the vitrified ovaries, isolated preantral follicles from four groups were cultured for 4 days to compare survival rate and follicular growth between above-mentioned groups. Survival rate (97.3%) in toxicity test group alike the control group (98.7%) were significantly higher ($P < 0.05$) than the Cryotop (92.7%) and CV (47.7%) groups. Increase in follicle diameters after 4 days in Cryotop and CV groups was significantly lower ($P < 0.05$) than the control and toxicity test groups, but growth and survival rate of follicles in Cryotop group was significantly higher ($P < 0.05$) than the CV group. These results demonstrated that ovarian tissue vitrification by Cryotop highly preserves the viability rate of preantral follicles.

Keywords: Vitrification, ovarian tissue, preantral follicles, cryotop

1. Introduction

Since ovaries are large stores of oocytes, cryopreservation is a feasible method to preserve female reproductive potential, especially in young patients with cancer or in women at risk of premature ovarian failure [14,24,25]. In assisted reproductive technology, cryopreservation methods for embryos have been developed to a considerable level but are still insufficient for ovarian tissue [8]. In comparison with an oocyte or embryo which is a single unit, the ovary is a complex structure composed of several

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different types of cells such as stroma cells and follicles formed by the oocyte, granulosa and theca cells, cryopreservation of ovarian tissue is more difficult because different cell types have different requirements for optimal survival [10,32]. There are two protocols for ovarian tissue cryopreservation. One is slow freezing and the other is a rapid cooling method called vitrification [8].

In general practice, ovarian cryopreservation is often carried out by the slow freezing which is an expensive method due to the employment of sophisticated freeze-control machines. In addition, this procedure can cause damage by intracellular ice crystal formation.

However, cryopreservation by vitrification involves very rapid cooling, in which solutions go directly from the aqueous phase to glass phase (amorphous solid) without exposure to the crystalline state where the damage can occur. Thus cooling ($-2/500\,^{\circ}$C/min) in the presence of high concentration of cryoprotectants avoids intracellular ice crystal formation [27,31]. Cryopreservation by vitrification has been successfully applied in ovarian tissue from mouse [30] and human [11] with minimal change in morphology. Successful pup births after *in vitro* culture of oocyte-granulosa cell complex (OGC) from preantral follicles of vitrified ovaries have been reported in mice [8].

To achieve the rapid cooling and warming of tissue in vitrification, very small volumes of vitrification solution are necessary, which together with the tissue that has to be vitrified, are cooled by immersion in liquid nitrogen [31]. So far different strategies have been used in order to reduce the volume of vitrification solution were used, including the use of grids for electron microscopy [20], glass capillaries [9], open-pulled plastic straw [35], solid-surface vitrification [6] and cryoloop [18,21,22,37]. The key to success in these methods was a reduction in the amount of vitrification medium surrounding the samples which allowed them to rapidly pass through a critical temperature zone in the presence of a cryoprotective agent [1,36]. A new freezing technique that was used for vitrification of oocytes or/and embryos by Kuwayama et al. for the first called Cryotop [15] that recently applied for ovarian tissue [8,12]. The Cryotop vitrification method is probably the latest minimum volume vitrification approach that has resulted in the highest number of babies born after vitrification of human embryos and after cryopreservation of human oocytes worldwide [17]. The aim of this study has been to develop a vitrification procedure that optimally preserves the morphology, viability and *in vitro* development capacity of mouse preantral follicles. Ovaries have been vitrified according two different procedures: conventional vitrification (CV) and Cryotop by using sucrose and mixture of dimethylsulfoxide (DMSO) and ethylene glycol (EG). Ovaries frozen using these protocols have also been subsequently thawed, and their preantral follicles were cultured for 4 days.

2. Materials and methods

2.1. Source of ovarian tissue

Female NMRI (Naval Medical Research Institute) mice (Pasteur Institute; Tehran, Iran) were housed and bred at Royan institute resource and were kept at the temperature of 20–25 °C and 50% humidity in light-controlled condition (12 hours light to 12 hours dark) and provided with sterile food and water ad libitum. Mouse ovaries were taken from 14-day-old mice that were killed by cervical dislocation.

The enveloping tissue was dissected from the collected ovaries and divided into four groups: vitrification by Cryotop (Cryotop group), vitrification by conventional straw (CV group), toxicity test group and control.

2.2. Vitrification methods

2.2.1. Cryotop method

Ovaries were immersed in an equilibration solution composed of 7.5% DMSO (Sigma, Germany) and 7.5% EG (Sigma, Germany) in HEPES-buffering TCM 199(Gibco, USA) (PH 7.4) with 20% HSA (Octapharma, Switzerland) for 15 minutes at room temperature, and then transferred into the vitrification solution was composed of 15% EG, 15% DMSO, and 0.5 M sucrose (Sigma, Germany) in HEPES-buffering TCM 199(PH 7.4) with 20% HSA for 30 minutes. Each pair of ovaries was put on the polyester sheet of Cryotop (Kitazato CO. Ltd., Fujinomiya, Japan) with a minimum volume of the vitrification solution. The sheets were plunged immediately into liquid nitrogen and capped, then transferred to a larger storage container and stored for 3-weeks.

For warming after pulling up from liquid nitrogen, the sheets were placed directly in a warming solution composed of 1M sucrose in HEPES-buffering TCM199 (PH 7.4) with 20% HSA for 10 minutes at room temperature. The ovaries detached from the sheet were transferred and incubated in α-MEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS: Fraction V; Sigma, Germany) and an antibiotic solution that was composed of 100IU of penicillin G (Sigma, Germany), 100 μg of streptomycin (Sigma, Germany) and 0.25 μg of amphotericin B (Gibco, USA) for 30 minutes.

2.2.2. CV method

As for Cryotop method ovaries were treated with equilibration vitrification media and afterwards loaded into conventional straws. The CV method was based on a protocol for vitrification of mouse oocytes [3] as follows: the 0.25-ml straw (IMV, L Aigel, France) was filled with 1 cm of vitrification medium, 0.5 cm of air, 2 cm of vitrification media containing ovary, 0.5 cm of air, and 3.5 cm of vitrification medium. It was plunged immediately into liquid nitrogen and stored for 3-weeks. For warming, the straw was taken out and held in the air in room temperature for 30 s. Then it was cut with scissors and the contents containing the ovary were expelled into warming solutions as above.

2.3. Toxicity test

The ovaries were treated with equilibration-vitrification media and afterwards the cryoprotectants were removed as described above without immersion in liquid nitrogen.

During all the steps of vitrification and warming process in all three groups, ovaries were continuously mixed using a shaker

2.4. Histological examination

Ovaries in all groups were fixed in bouin's fixative overnight and then processed and embedded in paraffin blocks. The ovaries were sectioned at 5 μm thickness. They were mounted and stained with hematoxylin and eosin (H&E).

2.5. Mechanical isolation and selection of preantral follicles for in vitro culture

Each ovary in of all four groups were placed in 200 μl of α-MEM droplets that were supplemented with 10% v/v FBS and an antibiotic solution (composed of 100 IU penicillin, 100 μg streptomycin and 0.25 μg amphotericin B) and follicles were released from the ovary using mechanical dissection and selected for culture by the following criteria: i) intact round follicular structure with two or three layers of granulosa cells and those diameter was 95–130 μm. ii) the oocytes had to be visible, round and centrally located within the follicles.

Table 1
Comparison of survival rate of preantral follicles isolated from ovaries in four groups after 4 days culture

Follicles	No. of follicles		
	Cultured	Survived	Degenerate
Control group	466	460 (98.7%)[a]	6 (1.3%)
Toxicity test group	148	144 (97.3%)[b]	4 (2.7%)
Cryotop group	355	329 (92.7%)[c]	26 (7.3%)
CV group	153	73 (47.7%)[d]	80 (52.3%)

[a,b] Not significantly different a vs. b ($P > 0.05$).
[b,c] Significantly different b vs. c ($P < 0.05$).
Significantly different a vs. c, a vs. d, b vs. d, c vs. d ($P < 0.0001$).

2.6. In vitro culture of preantral follicles

Isolated preantral follicles were individually cultured in α-MEM supplemented with 100 mu/ml human follicle stimulating hormone (hFSH; Serono, Switzerland), ITS (insulin, 10 μg/ml; transferrin, 5.5 μg/ml; selenium, 6.7 ng/ml; Gibco, USA) and 5% v/v FBS [5]. Twenty follicles were individually cultured in a culture dish (60 mm tissue culture dish; Falcon) containing 10 vl droplets covered with 5 ml mineral oil (Sigma, Germany) at 37°C in a humidified atmosphere of 5% CO_2. On the second day of culture, 10 vl medium was added to each droplet. On the 1st and 4th days of culture, diameter of the follicles excluding the theca stroma was estimated by measuring two perpendicular diameters (length and width) with an ocular micrometer under the invert microscope (Nikon).

Follicle survival in culture was considered positive when follicles retained a normal structure and oocyte remained surrounded by granulosa cells attached to the culture dish.

2.7. Statistical analysis

The percentage of survived preantral follicles after 4 days of culture was compared between all of the groups. Differences were assessed by Z test. $P < 0.05$ was considered statistically significant.

Comparison of survived preantral follicles diameters between all different groups with Fresh ovary/control group was performed using Dunnet test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Histological examination of ovaries

Morphological evaluation was performed to compare the structure of growing and primordial follicles in four groups.

Growing and primordial follicles in sections of ovaries in Cryotop group and toxicity test group showed a similar morphology to the control ovarian section. No major differences were observed in these sections (Fig. 1A, B, C).

The majority of preantral follicles in the sections from Cryotop group ovaries showed oocytes with smooth cytoplasm and central germinal vesicles surrounded by granulosa cells, as shown in toxicity test group and control ovarian sections (Fig. 2A, B, C).

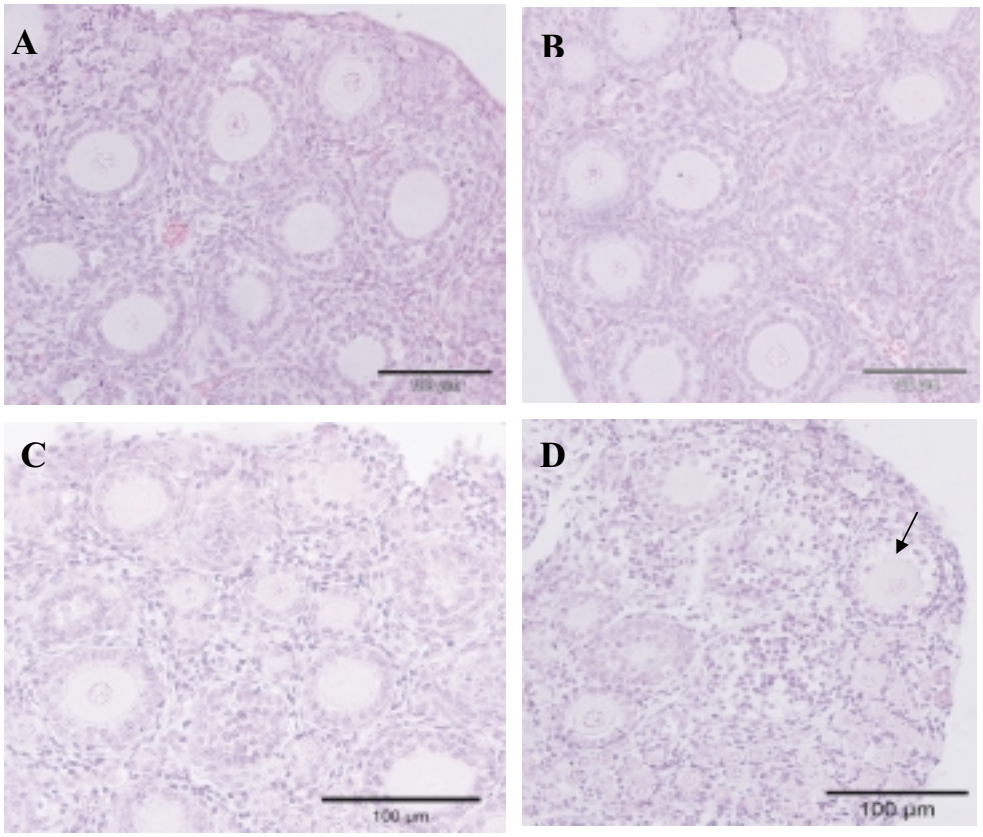

Fig. 1. Influence of cryoprotectant toxicity and vitrification-warming on mouse ovaries in histological examination. (A) Parafian section from control mouse ovary. (B) Section of ovary after toxicity test. (C) Section of ovary that vitrified by Cryotop. (D) Section of ovary that vitrified by conventional straw. A, B and C sections are shown to be of a comparatively similar morphological appearance, but in section D detached area between the granulosa cells and granulosa cell-oocyte were observed (arrows). (H&E staining).

The Cryotop group ovarian sections were quite similar to the control ovarian section in which the granulosa cells surrounding the oocyte had a well organized cell to cell contact and had also maintained the granulosa cell-oocyte contact (Fig. 2A, C), but in the CV group ovarian sections, cell to cell contact in granulosa cells surrounding oocyte were not seen, also detached area between the granulosa cells and granulose cell-oocyte were observed (Fig. 2D).

3.2. Survival rate of preantral follicles

In the present study, follicles were considered to be survived if they retained a normal structure, with close contact between the oocytes and the surrounding granulosa cells (Fig. 3).

Follicles that failed to survive were defined as those that had lost their three-dimensional spherical structure, and oocyte was not remained surrounded by granulosa cells in culture. Table 1 depicts the follicles survival rate for *in vitro* culture that was evaluated after 4 days. Survival rate (97.3%) in toxicity test group, alike the control group (98.7%) was significantly higher ($P < 0.05$) than the Cryotop (92.7%) and CV (47.7%) groups, and survival rate in Cryotop group was significantly higher ($P < 0.05$) than the CV group.

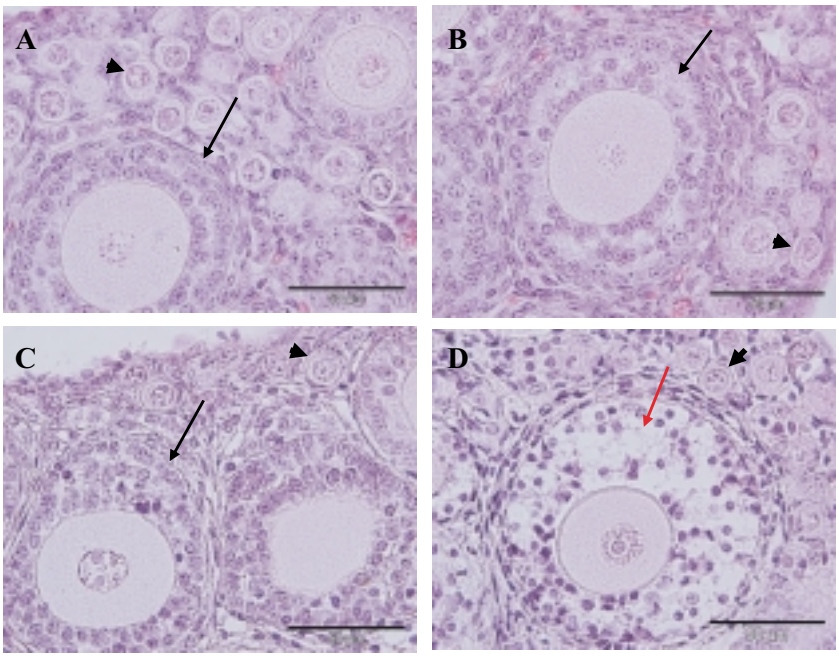

Fig. 2. Histological analysis of mouse ovarian sections (H&E staining) for comparison of primordial and growing follicles. (A) Control ovarian section. (B) Section of ovary after toxicity test. (C) Section of ovary that vitrified by Cryotop. (D) Section of ovary that vitrified by conventional straw. No significant differences were observed in primordial and preantral follicles between A, B and C. In section D, primordial follicles were not deference with the other sections but in the preantral follicles, granulosa cells surrounding the oocyte were missed cell to cell contact (thin arrow). (Primordial follicles: arrowhead; preantral follicles: black arrow).

3.3. Follicular growth rate

Mean of diameters of follicles in Cryotop, CV, toxicity test and control groups, on the day of commencement were: 111.1 $\pm$ 9.09, 102.1 $\pm$ 8.9, 113 $\pm$ 12.01 and 120.2 $\pm$ 9.62 micron, and day 4 were: 127.8 $\pm$ 19.59, 110.9 $\pm$ 14.94, 145.9 $\pm$ 26.48 and 156.3 $\pm$ 30.04 micron, respectively; which demonstrated that follicular growth rates in toxicity test and control groups were significantly higher than the Cryotop and CV groups. Also, growth rate in Cryotop group was significantly higher than the CV group, as shown in Table 2.

4. Discussion

Cryopreservation of ovarian tissue should be seriously considered for patients who need treatments due to fertility impairment in the future; such as pelvic, extra pelvic and/or systemic malignant diseases, as well as non-malignant diseases [7]. Vitrification has important advantages in comparison to the conventional cryopreservation (slow freezing) of tissue.

With this method, the formation of amorphous crystal-like structures rather than that of actual crystals is induced [10], probably because of the high osmolarity of the vitrification solution. Furthermore, during vitrification, cooling is so rapid that structural damage attributable to crystal formation does not occur, and a glass-like solid structure forms instead of crystals [34]. For a slow freezing procedure, the concentrations of cryoprotectants are limited to 1 or 1.5 M, and the toxicity of cryoprotectants is

Table 2
Comparison of follicular growth after 4 days between different groups with control. Dunnet test: Comparisons significant at the 0.05 level are indicated by ***

Group comparison	Difference between means of diameter growth	Simultaneous 95% confidence limit
Toxicity Test vs. control	−3.135	−12.625 6.354
Cryotop group vs. control	−19.456	−25.651 −13.261 ***
CV group vs. control	−27.329	−34.999 −19.660 ***

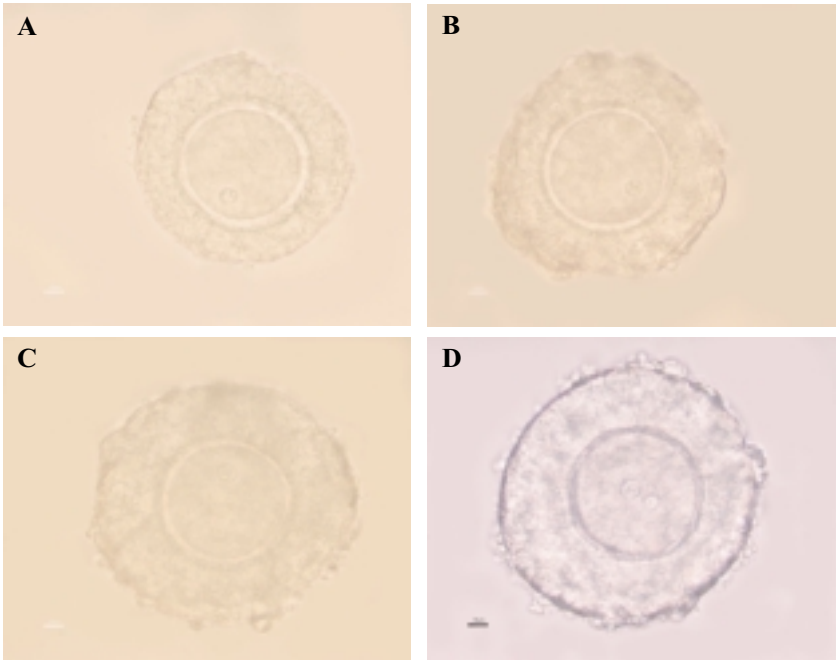

Fig. 3. Isolated preantral follicles from: (A) control group, (B) toxicity test group, (C) Cryotop group and (D) CV group. Bar = 10 μm.

relatively low. However, in vitrification the concentrations of cryoprotectants used are much higher and sometimes can be as high as 8 M [4,13]. Therefore, there is a strong correlation between concentration of cryoprotectant and cooling-warming rates during the vitrification process [4] that can increase the risk of toxic and osmotic damage [16]. Therefore, it seems more important to select cryoprotectants with low toxicity for the vitrification procedure. DMSO and EG are the most commonly used permeable cryoprotectants [33]. Rodrigues et al. showed that the DMSO and EG are the best cryoprotectants [28, 29]. Referring to Hasegawa et al. [8], in the present study we also used EG, DMSO and sucrose as cryoprotectant and toxicity of cryoprotectants. After *in vitro* culture of preantral follicles and histological examination, no significant differences was observed between control and cryoprotectant treated groups. In treated groups, cryoprotectants used in this procedure had no adverse effects on morphology, growth and survival rate of preantral follicles.

The *in vitro* culture of preantral follicles is an important tool to evaluate the success of follicular cryopreservation. Once preantral follicles are submitted to the freezing/thawing procedure, dehydration and rehydration during cryoprotectant removal can cause changes in the morphology and viability of the follicles. To reacquire their normal morphology and to observe the effects of cryopreservation,

equilibration in a warm medium is necessary; this can be achieved by short-term *in vitro* culture. The process of re-warming the cryopreserved tissue in a nutrient-rich environment allows the follicular cells to re-establish metabolic activity, normal cell volume, and cell-cell contacts [26].

As mentioned above, to evaluate the success of vitrification-warming process and to observe that the follicles of vitrified ovaries will be able to survive and continue their growth after warming, these follicles were transferred to culture medium for *in vitro* culture. After warming, the morphology of Preantral follicles which have been obtained from vitrified ovaries by Cryotop were quite similar to the control group. The survival rate of these follicles following 4 days of culture was also high (92.7%) and were able to reinitiate their growth in the culture condition. Therefore, these follicles could highly reacquire their normal activity. Although, immediately after warming, the quality of most of the follicles that had been obtained from vitrified ovaries by CV was low, these follicles were unable to survive and reacquire their normal morphology and volume in culture condition. Therefore, their survival rate was low (47.7%).

In this study, the growth rates of preantral follicles derived from both vitrified groups were lower in comparison to control group.

Newton et al. have suggested that the low growth rate of the frozen-thawed follicles might have been the result of the delayed proliferation or initial cell death of the granulosa cells in response to the freezing and thawing process [23]. The results of this study showed the superiority of the Cryotop procedure over CV, due to the following reasons:

In the Cryotop group, ovarian tissue with only a minimal volume of vitrification solution on its surface was loaded on the polyester sheet of cryotop and then submerged directly into liquid nitrogen. This allowed an extremely high cooling rate. This increase in cooling rate was due to the reduction of vitrification solution volume and the direct contact of samples with liquid nitrogen. Kuwayama et al. reported that the average values of the cooling and warming rates with the Cryotop method is 23,000 °C/min and 42,000°C/min [16]. In contrast, in conventional straws, cooling rate is 2500°C/min [27]. This lower cooling and warming rate may be a result of indirect contact of the sample with liquid nitrogen due to the high volume of vitrification solution surrounding the sample and the preventive effects of the walls of the straw. Also, an obstacle to increase the cooling rates in the CV method is that by plunging a warmer straw into liquid nitrogen an isolating layer of N_2 boiling, gas phase is created around it for several seconds. The Cryotop method has solved these problems [19].

Moreover, in the Cryotop method ovaries in a small amount of vitrification solution can be directly warmed and immediately diluted into the dilution solution. This reduces exposure to unsuitable temperatures and concentrated cryoprotectants. In contrast, the conventional straw is warmed in the air and then cut with scissors. The ovaries in a larger vitrification volume are expelled into the dilution solution and then placed into another dilution solution. This allows more time to pass through the inappropriate conditions [2].

In conclusion, these results suggest that in spite of significant difference in survival rate between control and Cryotop groups, ovarian tissue vitrification by Cryotop highly preserves the viability rate of the preantral follicles and can be used successfully for vitrification of ovarian tissue.

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