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*Original Article*

# Vitrification of mouse oocyte versus slow freezing: evaluation of ultrastructure and developmental potential

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**Abstract:** Development of oocyte cryopreservation techniques have lead to major breakthroughs in human assisted reproductive technology, but the effect of cryopreserving oocytes is not ideal. This study compared the ultrastructure and developmental potential between the two approaches applied to mouse oocytes. In the control group, oocytes were cultured immediately, while in the vitrification and slow freezing groups, the oocytes were cultured after vitrification-warming and slow freezing-thawing procedures. The morphology and angle of spindle deviation with the first polar body were estimated by Polscope. Moreover, the measurement of the thickness and density of zona pellucida was also carried out by Polscope. The image of the morphology of oocyte was obtained by SEM, while the morphological characteristics of mitochondria and perivitelline space were observed by TEM. The slow freezing approach reduced the number of ICSI embryos that developed to the 2-cell or blastocyst stage as compared to the vitrification-thawing group. A higher quality of oocytes was observed by the vitrification-thawing protocol as compared to the slow freezing-thawing protocol. The angle of spindle deviation concerning the first polar body was in a better grade of vitrification-thawing group as compared to the slow freezing-thawing group. The developmental potential was superior in the vitrification-thawing group that was evaluated by the percentage of 2-cell and blastocyst after ICSI. These preliminary results indicated that cryopreservation significantly affected the conservation of oocytes. Oocyte vitrification by the cryotop method seems to be better than slow freezing based on the spindle deviation angles, oocyte morphology, and developmental potential.

**Keywords:** Vitrification, slow freezing, mouse oocyte, ultrastructure, developmental potential

## Introduction

Development of oocyte freezing technology has become a modern birth insurance. In humans, oocyte cryopreservation presents an attractive option for the treatment of infertility [1, 2], especially for two groups: young female cancer patients with a desire of preserving their fertility before chemoradiotherapy [3] and those requiring to preserve their fertility for late pregnancy [4]. The extreme sensitivity of spindle and mitochondria to temperature and renders easy hardening of zona pellucida during freezing and thawing, thus selecting the optimal cryogenic method is crucial.

Currently, most pregnancies from cryopreserved oocytes are achieved by the slow programmable freezing method [5]. Slow-freezing allows for cryopreservation to occur at a sufficiently slow rate to permit adequate cellular dehydration while minimizing the intracellular ice formation. The first successful protocol applied in 1972 for mammalian embryo cryopreservation required a cooling rate of -1°C/min to -70°C [6]. Tissue and gamete cooling performed using this method is referred to as equilibrium freezing [7]. Subsequently, slow-rate cooling was only applied to approximately -30°C [8]. As a result, the intracellular water content was converted into small intracellular ice crystals or glass. To avoid extensive crystallization, rapid warming was required.

For vitrification, although the first pregnancies with human cleavage-stage embryos were

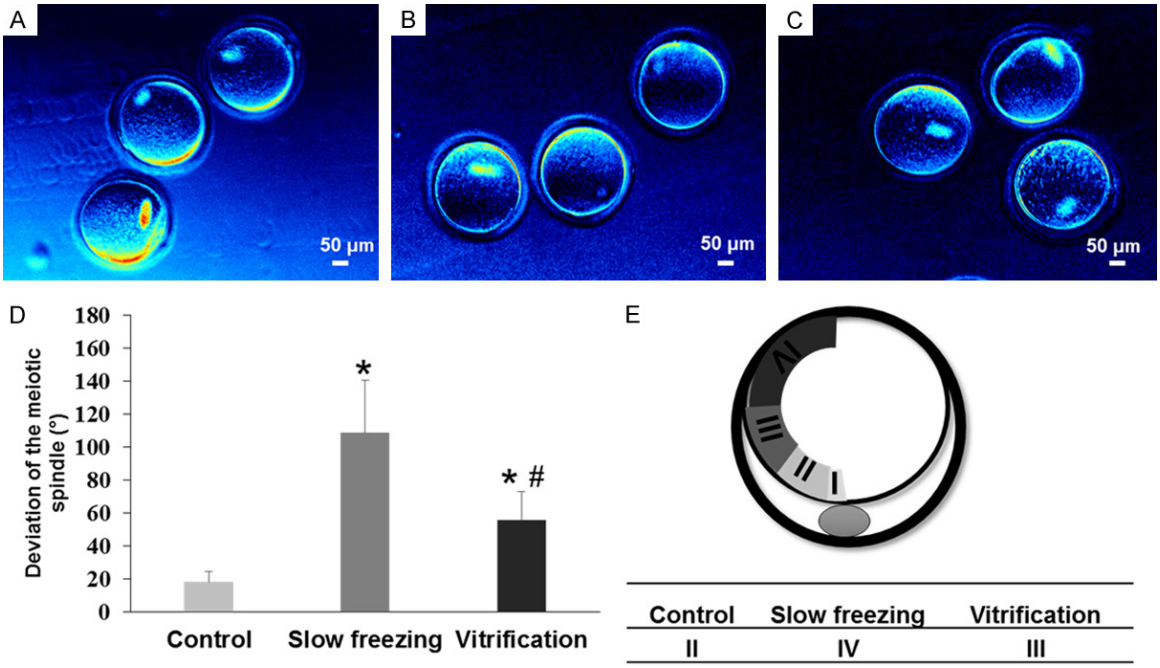


**Figure 1.** Morphological features of the spindle in metaphase II oocytes were investigated by Polscope. (A-C) Morphological observation of the spindle, scalebar =50 μm. (A) Control group; (B) Slow freezing group; (C) Vitrification group. (D) The angle of spindle deviation with regard to the first polar body measured by Polscope. (E) Schematic illustration (upper) represent the four oocyte groups (spindle deviation angles of 0-5° is panel I; 6-45° is panel II; 46-90° is panel III; 91-180° is panel IV, respectively) and classification (lower) according to the results in (D). Visualized using the Polscope system with regard to the location of the first polar body. These angles, measured between the line connecting the oocyte center with the meiotic spindle and that connecting the oocyte center with the first polar body, are 18° (classified as II) in the control group, 109° (classified as IV) in the slow freezing group, 56° (classified as III) in the vitrification group.

obtained using dimethylsulfoxide (DMSO) as the cryoprotectant, the approach was replaced rapidly [9]. For nearly 20 years, the protocol combining 1.5 M propylene glycol (PROH) with 0.1 M sucrose as the permeable and non-permeable cryoprotectants, respectively, has been the used widely [10]. The freezing curve adopted the use of a programmable freezing machine designed to provide accurate and consistent cooling parameters. Briefly, the sample was exposed to a relatively rapid cooling rate of 2°C/min to around -7°C, followed by a manual seeding to induce ice crystal formation in the solution. Then, a consistent slow cooling rate of 0.3-1.0°C/min is applied before the freezing device is plunged into liquid nitrogen after reaching temperatures around -40 to -70°C utilizing the Trounson and Mohr approach [11], respectively. The modifications to the concentration of sucrose and the use of other cryoprotectants such as glycerol have been investigated for the cryopreservation of oocytes [2]. A detailed analysis of biological principles and development of various slow-freezing procedures have been described previously [12].

Cryopreservation of oocytes is advantageous as it allows long-term storage of supernumerary oocytes produced by IVF, and it can also be used for sperm function testing, oocyte donation programs, and in women receiving radio- or chemotherapy. A few reports have described the success of vitrification of oocytes. However, the best oocyte cryopreservation protocol has yet to be determined [13, 14] and only a few term pregnancies have resulted from cryopreserved human oocytes [15, 16], which might be associated with the susceptibility of the human metaphase II oocytes to freezing-thawing damage that leads to poor survival, fertilization, and development of cryopreserved human oocytes [17, 18]. Herein, we studied the quality of oocytes after vitrification-thawing or slow freezing-thawing approaches by detecting the ultrastructure and developmental potential.

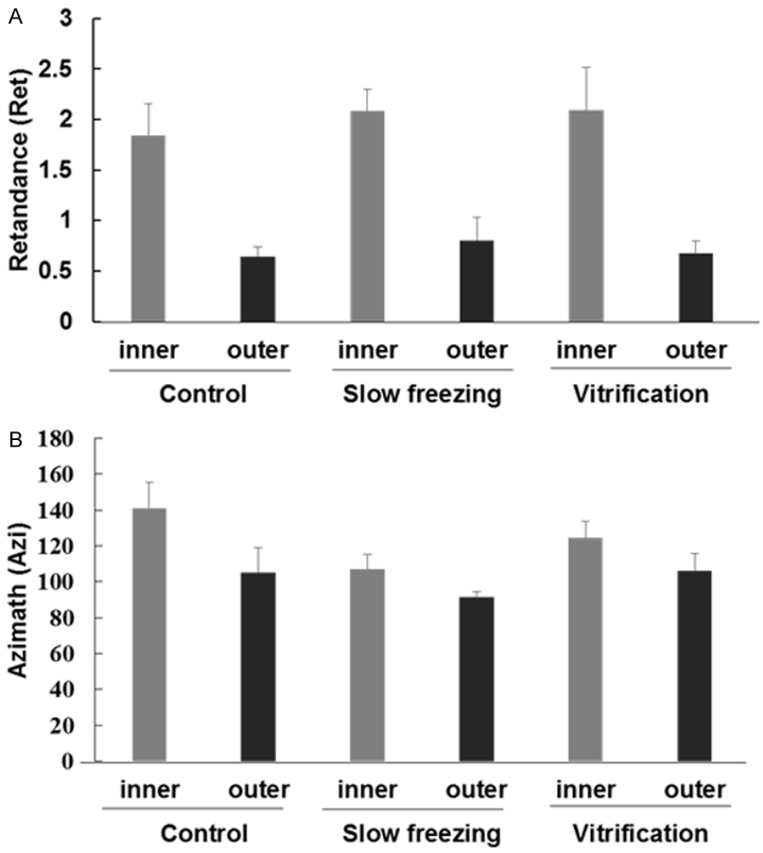


**Figure 2.** Thickness (Retandance) and density (Azimuth) of the zona pellucida in mouse oocytes were measured in three groups by Polscope. A. The inner and outer retandance of zona pellucida; B. The inner and outer azimuth of zona pellucida.

## Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All animals were maintained and handled in accordance with the requirements of the Institutional Animal Care and Use Committee of the Xiamen University.

*Preparation of animals*

Six-week-old animals are purchased from the Animal Institute of Xiamen University. F1 hybrid mice (C57BL/6 female × DBA/2 male) were maintained at a cycle of 14 hours light (light on at 06:00 a.m.) and 10 hours dark, 40-60% humidity, and 22-25°C. Food and water were provided *ad libitum*. After two weeks of acclimatization, mice were intraperitoneally injected with pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) with a 48-hour interval in order to induce superovulation. Cumulus-oocyte complexes were collected from oviducts 12 hours after hCG treatment and recovered in G-IVF plus medium (Vitrolife, Sweden). The cumulus cells were dispersed with 300 IU/mL hyaluronidase (Vitrolife, Sweden), and then, isolated oocytes were washed in G-IVF plus medium three times for experimental usage. Mouse sperms were collected from the cauda epididymis of euthanized male C57BL/6 mice and released into G-IVF plus media. In some cases, the sperms were frozen in the same media (without cryopreservation) in a -80°C freezer. For heat treatment, the sperms were incubated for a specified period in a water bath at 56°C, and then, sonicated for 10 seconds before being added to drops containing the oocytes.

*Vitrification freezing and thawing procedure*

The cryotop method for oocyte vitrification was used as described previously by Chian et al. [19]. The mouse oocytes were equilibrated in equilibrium solution (ES) consisting of 7.5% (v/v) ethylene glycol (EG), 7.5% DMSO in the base medium (G-MOPS with 10% HSA Vitrolife) at room temperature for 5 minutes. Subsequently, these oocytes were placed in the vitrification solution (VS) comprising of 15% EG, 15% DMSO, and 0.5 mol/L sucrose (Merck, Munich, Germany) in the base medium for 1 minute, followed by loading them on the Cryotop (Kitazato, Fuji, Shizuoka, Japan) tip (3-5 oocytes/Cryotop), immediately immersed in liquid nitrogen, and stored for 1 day. For warming, Cryotops were instantly placed in 1.0 mol/L sucrose solution at 37°C for 1 or 3 minutes in 0.5 mol/L, and 0.25 mol/L at room temperature. Finally, the oocytes were washed in the base medium and incubated in G-IVF plus medium for 2 hours.

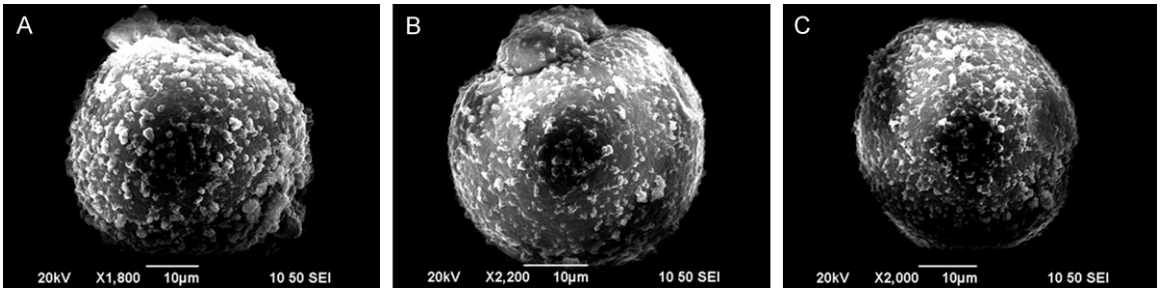


**Figure 3.** Morphological images of mouse oocyte after thawing procedure by SEM. A. Control group; B. Slow freezing group; C. Vitrification group. Scalebar = 10 μm.

*Slow freezing and thawing*

The slow freezing method was described previously [20]. The preantral oocytes were exposed to 1.5 mol/L 1,2-propanediol (PROH) in G-MOPS supplemented with 20% HSA and 0.1 mol/L sucrose for 20 minutes, loaded into the plastic straws (Cryostraw), and transferred into an automated cryologic 10 series III freezer (Melbourne, Australia). The start temperature was 22°C, which decreased gradually to -7°C (2°C/min); at this point, the seeding of the straws was induced. After holding for 10 minutes, the straws were cooled to -30°C at the rate of 0.3°C/min and held at this temperature for 10 minutes. Then, the straws were rapidly cooled to -196°C and stored in liquid nitrogen for 1 day. For thawing, the straws were transferred to room temperature for 30 seconds, followed by 30 seconds shaking in a 37°C water bath. The cryoprotectants were removed by passing the oocytes through decreasing concentrations of PROH (1, 0.5, 0.25, and 0 mol/L) in 0.2 mol/L sucrose in G-MOPS supplemented with 20% HSA (5 min for each step). Finally, the oocytes were washed two times in G-MOPS plus and incubated in G-IVF plus medium for 2 hours.

*Observation of spindles using the light microscope (Polscope)*

The morphological features of the spindle were imaged by placing each oocyte in a 5 μL drop of G-MOPS plus medium covered with prewarmed paraffin oil (Vitrolife, Sweden) at 37°C in a thermostatic system (δTC3 Culture Dish System; Bioptech, Butler, PA, USA). The oocytes were examined under an inverted microscope (Zeiss Axiovert 200; Zeiss, Oberkochen, Germany) equipped with a Cohu analog video camera, a Polscope containing liquid crystals, an electro-optical controller, as well as, a computerized image analysis system (Metamorph software; Universal Imaging, West Chester, PA, USA). The time of observation for each oocyte was within 20 seconds.

*Evaluation of oocytes by transmission electron microscopy (TEM) and scanning electron microscope (SEM)*

The oocytes were fixed (2.5% glutaraldehyde) for 4 hours on the cover glass and washed 3 times with 0.1 M pH 7.4 phosphate-buffered saline (PBS). The samples were maintained at 4°C before SEM and TEM analysis. Data were analyzed and collected by SEM (JEOL JSM-7500, Japan) and TEM (JEOL JSM-1000, Japan).

*Statistical analysis*

The experiments were performed in three replicates. Statistical analysis was performed using SPSS software version 17 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SEM. After testing the data for normal distribution and equal variance, differences between the two groups were analyzed using Student's unpaired t-test. $P<0.05$ was considered as statistically significant.

## Results

*Morphological features of the spindle*

The morphological features of the spindle in a metaphase II oocyte used Polscope (**Figure 1A-C**). The normal spindle appeared at a high rate in the control and vitrification groups as compared to the slow freezing group. The angle between the spindle and first polar body was calculated (**Figure 1D**) and classified according

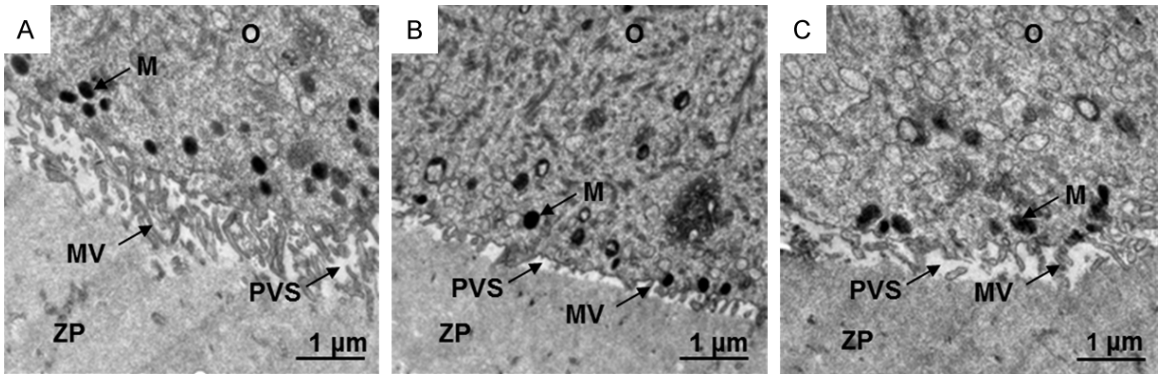


**Figure 4.** Perivitelline space and microvilli in the three groups were evaluated by TEM. A. Control group; B. Slow freezing group; C. Vitrification group. M = mitochondria (arrows); ZP = zona pellucida; MV = microvilli (arrows); PVS = perivitelline space (arrows); O = oocyte. Scalebar = 1 μm.

to that described previously by Rienzi et al. [21]. Briefly, the oocytes in which the meiotic spindle was detected at the time of ICSI were divided into four groups according to the angle of meiotic spindle deviation from the polar body position and measured in the equatorial plane of the oocyte where both the meiotic spindle and the polar body were focused. The angle of deviation was 0±5°, 6±45°, 46±90°, and >90° for groups I, II, III, and IV, respectively (**Figure 1E**). As a result, the quality of oocytes in the three groups showed a descending order as control group (II), vitrification group (III), slow freezing group (IV).

*Measurement of thickness and density of zona pellucida*

The thickness and density of zona pellucida of mouse oocytes were measured by Polscope. The results showed that the thickness (Retandance) and density (Azimuth) of the zona pellucida did not differ significantly among the three groups (**Figure 2A**, **2B**).

*Morphological images of mouse oocytes*

These oocytes were analyzed by SEM. (**Figure 3A-C**) shows more protrusions on the oocyte surface in the control group as compared to the other two groups. In addition, the shape of the oocyte in the slow-freezing group was slightly abnormal in morphology.

*Observation of perivitelline space and mitochondria*

The images of perivitelline space and mitochondria were collected by TEM. **Figure 4** shows no difference in the density of the filamentous texture of the inner aspect of the zona pellucida (ZP) in the three groups. The oocytes of the three groups were surrounded by an integrated and regularly structured plasma membrane provided with numerous microvilli stretching into a perivitelline space (PVS). However, the density of microvilli and the width of PVS was decreased in the slow freezing group. An abnormal structure of the mitochondria was observed in the slow freezing and vitrification groups (**Figure 5A**, **5C**).

*Percentage of embryo (ICSI) development into the blastocyst stage*

**Figure 6** demonstrates that embryonic development into 2-cell and blastocysts was reduced in embryos resulting from ICSI in the slow freezing group as compared to the control and vitrification groups. Thus, the slow freezing approach may result in a decrease in the number of ICSI embryos developed into the 2-cell and blastocyst stages.

## Discussion

Mouse oocyte cryopreservation is vital as it provides preliminary data that may be indirectly applicable to human oocyte cryopreservation. The cryopreserved-thawed mouse oocytes can be used for other experiments since mice are frequently used at several IVF centers for quality control. Thus, studies on mouse oocyte cryopreservation provide basic cryobiological data [22].

The first success with oocyte freezing was achieved in the mouse in 1977 [23]. Live off-

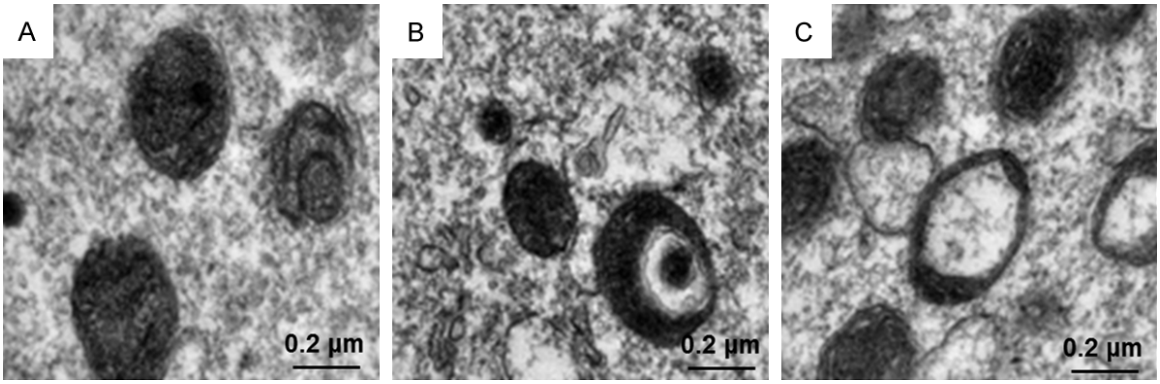


**Figure 5.** Morphological characteristics of the mitochondria in mouse oocyte were observed by TEM. A. Control group; B. Slow freezing group; C. Vitrification group.

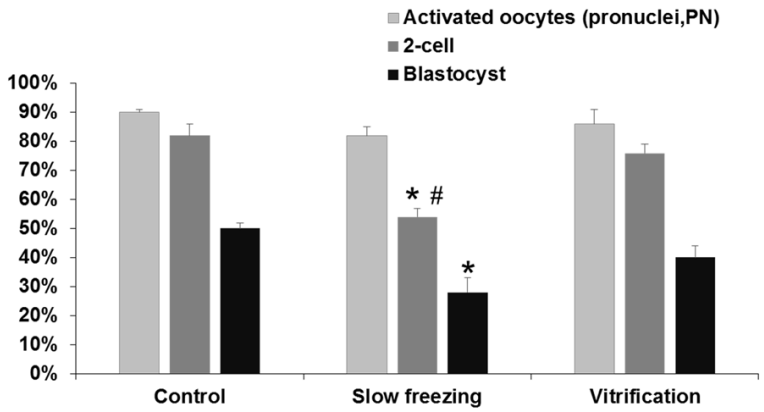


**Figure 6.** Percentage of embryo developmental to the blastocyst stage after ICSI compared to slow freezing and vitrification. **P*<0.05 compared to the control group; #*P*<0.05 compared to the vitrification group.

spring were obtained after IVF of mouse oocytes previously frozen with DMSO and stored in liquid nitrogen at -196°C. However, several issues with oocyte freezing were discovered. Freeze-thaw induced hardening of the zona pellucida, and fertilization rates decreased in the frozen-thawed mouse oocytes [24, 25]. The cytoplasmic disturbance in the post-thaw immature oocyte results in defective blastocyst development, which might be attributed to the loss of association between oocyte and cumulus cells induced by cryopreservation [26]. The current study explored the ultrastructure and developmental potential that may be caused by different protocols.

In contrast to slow-freezing, vitrification is a cryopreservation method that allows solidification of the cell(s) and the extracellular milieu into a glass-like state without the formation of ice. The most widely used vitrification method for mammalian embryos requires the use of high initial concentrations of the cryoprotectants, low volumes, and ultra-rapid cooling-warming rates [27]. To date, the most commonly used protocol for both oocyte and embryo vitrification involves the combination of 15% DMSO, 15% EG, and 0.5 M sucrose in a minimum volume (≤1 μL) [28]. Recently introduced, vitrification is now considered a promising technical innovation [29, 30]. Also, in this study vitrification had a superior efficiency for the cryopreservation of mature mouse oocytes. Furthermore, in this study, cryotop vitrification and slow freezing procedures were used for cryopreservation of mouse oocytes. The results of spindle deviation angles were found to be distinctly smaller in the vitrification group (56°) as compared to that in the slow freezing group (109°). According to some reports, the small spindle deviation angles indicate a better quality of oocyte [21]. ICSI data showed a low percentage of ICSI embryos that developed into the 2-cell and blastocyst stage in the slow freezing group as compared to the vitrification group. The high percentage of survival and maturation rates proved the success of the vitrification procedure. These findings are consistent with the report of Valojerdi et al. [31] and Lin et al. [32].

In conclusion, oocyte vitrification by the cryotop method is better than the slow freezing as observed the parameters of spindle deviation

angles, oocyte morphology, and developmental potential. Thus, vitrification is currently the widely used method for oocyte cryopreservation due to better results than slow freezing, although standardized utilization is yet to be applied.

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## Disclosure of conflict of interest

None.

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