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Original Article

Achieving oocyte survival and stable spindles after vitrification using closed pulled straws regardless of zona status

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ABSTRACT

Objectives: Embryo cryopreservation is a well-established technique at *in vitro* fertilization centers but the best protocol for vitrification of oocytes and zona manipulation remains inconclusive. This study aimed to determine if the closed pulled straws (CPS) method and zona drilling for vitrification provide a higher survival rate for thawed oocytes.

Materials and Methods: Female MF1 mice were superovulated by injection of gonadotropins. Cumulus-oocyte complexes were derived from excised fallopian tubes and the oocytes were divided into the following three groups: (1) one-hole zona drilling by laser, ($n = 40$); (2) intact zona ($n = 48$); and (3) zona digestion by pronase ($n = 43$). The control group consisted of 40 nonvitrified oocytes. After thawing, surviving oocytes were stained for spindles and chromosomes after 1-hour and 3-hour incubations, and compared to controls.

Results: There were no significant differences in the survival rates among Groups 1 (34/40, 85%), 2 (34/48, 71%), and 3 (30/43, 70%), but there were significant differences compared with the control oocytes (100%). After 1-hour and 3-hour incubations, vitrified oocytes in the three groups did not have significantly fewer normal spindles than the controls (1 hour: Group 1, 70.5%; Group 2, 64.7%; and Group 3, 66.6% vs. control 90%). There was also no significant difference in the percentage of oocytes with a normal spindle shape between the 1-hour and 3-hour recovery times (3 hours: Group 1, 88.2%; Group 2, 88.2%; and Group 3, 80%; control, 95%).

Conclusions: The *zona pellucidum* with CPS method has no effect on spindle injury and oocyte survival. Sufficient culture time for the recovery of the meiotic spindle is imperative for fertilization of vitrified oocytes. A CPS vitrified oocyte has the advantages of high survival and preserved good spindles.
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1. Introduction

Improvements in slow freezing and vitrification techniques have opened the possibility of efficiently storing human oocytes and using them for *in vitro* fertilization treatment after thawing [1–4]. Cryopreservation of an oocyte can reduce the need for ovarian freezing in women undergoing chemotherapy [5], and the need to repeat ovarian hyperstimulation [6] and restrict embryo freezing [7]. Unfortunately, the results so far have not matched the level of interest. By the end of 2008, only about 900 babies worldwide had come from cryopreserved oocytes as opposed to tens of thousands from frozen embryos [8]. The procedure is still considered experimental.

Trace amounts of ice formation within a cell during cryopreservation is lethal [9]. Vitrification is used to avoid intracellular ice formation by suspending the cells in a very high concentration of solutes. The water in the system is converted from liquid to glass with no ice formation. With a high warming rate, the system does not convert from glass to ice during warming.

There are two firmly held premises in the vitrification approach. One is that avoiding ice formation in cells and obtaining high survival demands the highest cooling rates. Consequently, a series of devices has been developed in the last decade to achieve cooling rates of ≥10,000 °C/min by permitting the manipulation of very small volumes of oocyte suspensions. The closed-pulled straw

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(CPS) method is one of them [10]. The second premise is that the vitrification solution in which the cells are suspended must have a very high concentration of a mixture of nonelectrolytic solutes. High concentrations of ethylene glycol (EG) and sucrose have been used to protect oocytes from cryopreservation injury [11]. This composition is relatively typical of most vitrification solutions in containing mixtures of permeating and nonpermeating solutes.

The *zona pellucida* (ZP) is a thick extracellular coat that surrounds all mammalian eggs and preimplantation embryos. The ZP could be a barrier to the effects of osmotic equilibration during the freezing process. In previous study, cryoprotectant agents could permeate easily to both the trophectoderm and inner cell mass of embryos. The absence of the ZP could enhance this phenomenon [12]. Indeed, effects on osmotic equilibration of the ZP during and after addition of the vitrification solution are avoided if the ZP is first removed, with no negative effects on embryo survival [13].

Our previous study compared vitrification with a slow freezing method [11]. Propanediol was used for slow control-rate freezing and EG for vitrification. There were more normal spindles and better fertility in the vitrification group than in the slow control-rate freezing group.

The present study aimed to further evaluate the zona effect in vitrification with CPS, and to compare postvitrification morphology and survival of intact zona, zona-drilled and zona-free mouse oocytes.

2. Materials and methods

2.1. Mice

MF1 hybrid mice were used. The mice were kept under constant environmental conditions with a 12-hour light and dark cycle and a constant temperature of 25 °C. The Tzu Chi University committee for animal experimentation approved the experimental procedures.

2.2. Media

All media were prepared from analytical grade chemicals (Sigma-Aldrich Co., St Louis, MO, USA). The oocytes were collected and cultured in human tubal fluid medium (HTF; #90125; Irvine Scientific, Santa Ana, CA, USA).

2.3. Oocyte collection

Six-week-old female mice were superovulated via intraperitoneal injection of 5 IU equine chorionic gonadotropin (Folligon; Intervet International, Boxmeer, The Netherlands), followed by 5 IU human chorionic gonadotropin (hCG) (Chorulon, Intervet International) in 0.2 mL saline after 48 hours. At 13–14 hours after hCG injection, oocyte-cumulus complexes were collected from the oviducts into HTF containing 60 IU/mL hyaluronidase (type IV-S; Sigma) for 5 minutes. The adherent cumulus cells were removed by gentle pipetting and washed in HTF. The oocytes were then placed into 20 μL drops of HTF previously equilibrated under mineral oil in 5% CO_2 in air at 37 °C for at least 30 minutes before vitrification.

The oocytes were divided into the following three groups: (1) one-hole zona drilling by laser, ($n = 40$); (2) intact zona ($n = 48$); and (3) zona digestion by pronase ($n = 43$). The control group consisted of 40 nonvitrified oocytes.

2.4. Laser drilled hole in the ZP

Denuded oocytes were used in zona drilling with laser equipment (IVF Workstation and Zona Laser Treatment system; Hamilton Thorne Instruments, Beverley, MA, USA). The IVF Workstation used a compact diode laser attached to an Olympus IX-70 inverted microscope (Olympus America, New York, NY, USA) below the objective turret. The Laser-Assisted Hatching software (Hamilton) was designed for easy positioning, focusing, and measurement of oocytes, and simple alignment of the laser. The laser had three preset energy intensities [low (35 mW), medium (45 mW), and high (55 mW)] that could be delivered in a single 25 millisecond pulse with a single click of the mouse controller. Low power was used for perforating very thin (<10 μm) zona or to minimize exposure, medium power for drilling the zona of most oocytes (10–15 μm), and high power for perforating thick (>15 μm) or hard ZP. After zona drilling, the oocytes were washed with HTF three times and cryopreserved by vitrification.

2.5. Dissolution of the whole ZP

The zona was removed using a 20 IU/mL solution of pronase (5.6 IU/mg; 100 mg; Sigma P-8811). The oocytes were transferred to the pronase solution in phosphate-buffered saline (PBS). After dissolution of the zona, zona-free oocytes were then gently rinsed several times to wash off excess pronase and were returned to the standard culture media until vitrification. The procedure was performed under an inverted microscope (Olympus America) fitted with a stage warmer set at 37 °C. The dissolution time for the ZP was recorded as completed when its border was no longer clearly defined under 200× magnification after pronase treatment.

2.6. Manufacture of the pulled straws

The 0.25 mL plastic straws (IVM, l'Aigle, France) were heat-softened over a hot plate and pulled manually. The pulled straws were cut at the tapered end with a blade. The inner diameter of the tip was 0.8 mm, with a wall thickness of ~0.07 mm [10].

2.7. Oocyte vitrification in CPS and warming

Exposure of oocytes (4–6 at a time) to cryoprotectants was done on the microscope stage set at 37 °C. Cohorts of oocytes, as indicated, were placed into 20 μL drops of HTF containing 1.5M EG for 1 minute at 37 °C. Oocytes were then transferred to HTF containing 5.5M EG for 1 minutes at 37 °C. Using a syringe, the tip of the pulled straw was loaded with 2 mm of vitrification medium, 2 mm air, 2 mm vitrification medium containing oocytes, 2 mm of air, and 2 mm of vitrification medium [10]. The CPS was then plunged into liquid nitrogen for cooling and storage.

After 5 days of storage, the CPS was removed from the liquid nitrogen for warming. The opposite end of the pulled straw was sealed using an index finger. The contents were then expelled into a drop of 0.5M sucrose (400 μL) using the increase in air pressure in the tube caused by the thermal change. The oocytes were then transferred into 0.5M, 0.25M, and 0.125M sucrose solutions in a four-well dish, and immersed for 2.5 minutes in each solution. The oocytes were then washed, transferred into the culture medium, and incubated.

2.8. Definition of morphological survival

Oocytes were defined as having morphologically survived if the cells had an intact ZP (in the intact zona group) and plasma

membrane and refractive cytoplasm. They were counted and recorded.

2.9. Fluorescent staining of meiotic spindles and chromosomes

The survival of the oocytes was determined by observing the oocyte morphology with presentation of a regular oocyte shape and diameter, the presence of an intact ZP and oolemma, a clear perivitelline space, normal oocyte size, and no evidence of ooplasmic degeneration. The morphologically surviving oocytes from the three treatment groups were examined for spindles and chromosome after 1-hour and 3-hour incubations. The control oocytes were also tested for comparison. The oocytes were preserved in 2% formaldehyde (Merck, Darmstadt, Germany) with 0.02% Triton X-100 (Merck) at 37 °C for 30 minutes. They were incubated with anti-α-tubulin monoclonal antibody (Sigma) in Dulbecco's PBS (DPBS) with 0.5% bovine serum albumin (BSA) for 45 minutes and washed in 0.01% Tween-20 (Merck) for 15 minutes. Tubulin was stained by fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin G (Sigma) for 45 minutes, while propidium iodide (5 mg/mL, Sigma) was used to stain the chromatin for 15 minutes. Excess antibody and dye were washed out in 0.01% Tween-20 for 15 minutes. The oocytes were transferred into DPBS with 0.5% BSA for 60 minutes and then wet mounted.

2.10. Observation of spindles and chromosomes

Fluorescence was observed using a laser-scanning confocal microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany) with an argon–krypton laser. The images were recorded on a host computer. The localization of tubulin and chromatin were revealed by FITC and propidium fluorescence.

2.11. Statistical analysis

The statistical package for social sciences (SPSS, Version 17.0; SPSS Inc., Chicago, IL, USA) software was used in all of statistical assessments of the experimental results. Data pertaining to oocyte survival and normal spindle incidence were analyzed using Pearson's Chi-square test to determine whether there were significant differences between the control and experimental groups. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Survival of oocytes

The morphologic survival rate of mature oocytes was 85% in the one-hole zona, 71% in the intact zona, and 70% in the no zona groups (Table 1). The survival rate of post-thawed mature oocytes was not significantly different among the three zona condition groups with vitrification cryopreservation ($p > 0.05$).

Table 1
Morphologic survival rate of different freeze–thaw conditions.

Condition	Oocytes	Survived oocytes	Percentage (%)
One-hole zona	40	34	85%
Intact zona	48	34	71%
No zona	43	30	70%
Control	40	40	100%

The overall p-value was <0.05, by contingency table analysis; $p < 0.05$ was noted in vitrified oocytes compared to controls; $p > 0.05$ was noted in comparison to the three groups of vitrified oocytes.

3.2. Spindle morphology analysis

After a 1-hour incubation postdilution, the oocytes from the one-hole zona, intact zona, and no zona groups had significantly lower percentages of normal spindles than the control group (Table 2; $p < 0.05$). However, differences among the three groups were not apparent. The majority of abnormal chromosomal patterns consisted of reduced spindles (Fig. 1).

After a 3-hour postdilution incubation, more vitrified oocytes in the three groups recovered normal spindles than those with the 1-hour incubation (Table 3). However, there was no statistically significant difference in spindle morphology between the 3-hour and 1-hour incubations. The percentages of oocytes with normal spindles in the three groups were lower than that of the controls but the differences were not significant. The one-hole and intact zona groups had higher percentages of normal chromosomes, similar to that of the controls, but higher than that of the no zona group. However, the differences were not statistically significant.

4. Discussion

In this study, mouse oocytes vitrified using CPS had a similar chance of morphologic survival regardless of zona status. Damage to oocytes during vitrification has been demonstrated to be dependent on the interaction of all the steps, including the rate of increase of exposure of the cells to cryoprotectants by mixing them repetitively, the temperature and time of exposure, and methods of dilution [14].

Traditionally, a programmed slow freezing method is widely used for embryo freezing with good survival. However, unlike embryos, unfertilized oocytes have specific characteristics, such as a very large cell volume and low cell-membrane permeability, compared with preimplantation embryos and other somatic cells [15]. Because of these characteristics, unfertilized oocytes are damaged easily by changes in osmotic pressure and physical stress. As demonstrated by our previous study, it is difficult to cryopreserve unfertilized oocytes by the conventional slow-freezing method, which involves the addition of cryoprotectants into the ooplasm and subsequent dehydration of the ooplasm by slow cooling [11].

Vitrification has been used to resolve the problems of slow cooling for oocyte cryopreservation. During vitrification, intracellular ice formation can be avoided by suspending the cells in very high concentrations of solutes, including ones that permeate the cell, and cooling them at high rates to temperatures below −100 °C. As a result, the water in the system is converted from a liquid to a glass with no ice formation [16]. This approach also requires high warming rates to ensure that the system does not convert from glass to ice during warming.

There are many vitrification methods including those using electron microscope grids [17], nylon mesh [18], open-pulled straws [19], CPS [10], cryoloops [20], microdrop methods [21], and cryotops [22]. The CPS has the advantages of achieving high survival and preserving good spindles [10]. In this study, CPS was

Table 2
Spindle morphology of mouse oocytes 1 hour after thawing.

Condition	Oocytes	Spindle morphology, n (%)	
		Normal	Abnormal
One-hole zona	17	12 (70.5)	5 (29.3)
Intact zona	17	11 (64.7)	6 (35.3)
No zona	15	10 (66.6)	5 (33.2)
Control	20	18 (90)	2 (10)

The overall p was >0.05 for normal spindle, by Chi-square test.

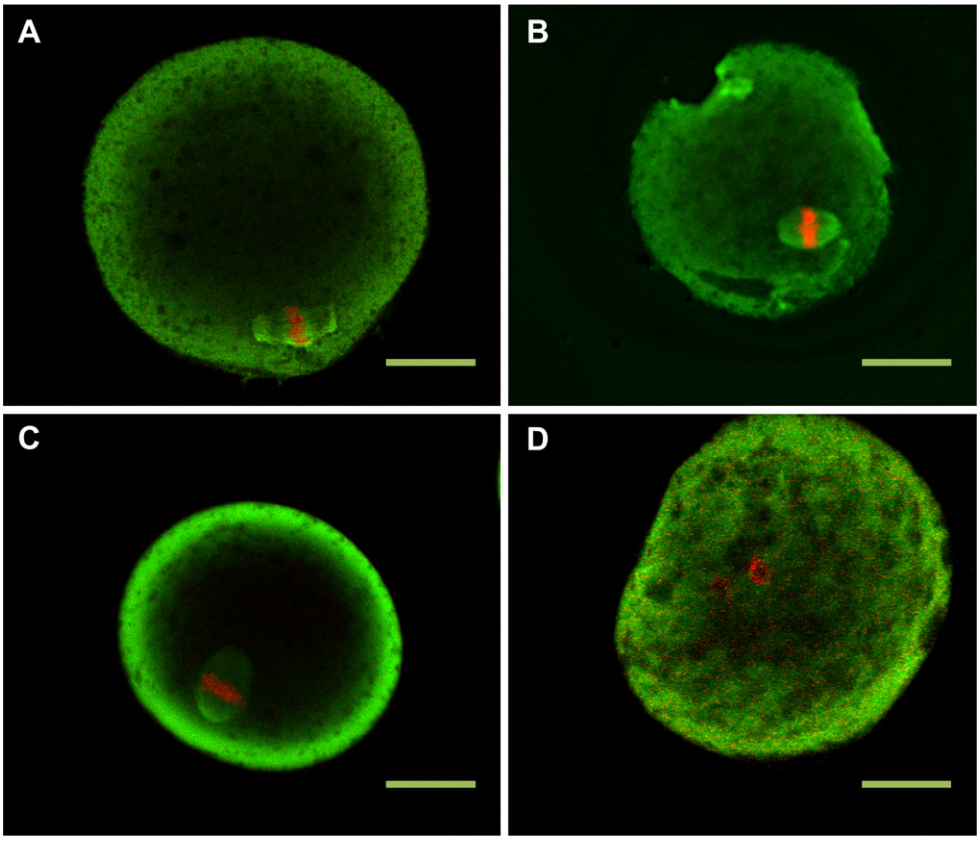

Fig. 1. Spindles and chromosomes of control oocytes and those in the three vitrified oocyte groups. (A) A control oocyte containing a normal barrel-shaped spindle in the ooplasm, with the chromosomes aligned regularly on the metaphase plate. (B) After a 1-hour incubation postdilution, an oocyte vitrified in a closed pulled straw (CPS) has a normal spindle and normal chromosomes. (C) After 3 hours' incubation, an oocyte vitrified in a CPS has recovered its normal spindle organization with compact chromosomes. (D) After 3 hours' incubation, an oocyte vitrified in a CPS shows disruption of the spindle with dispersion of chromosomes. Scale bar = 20 μm.

chosen for the vitrification method. However, there were no significant differences in survival and spindle morphology among the three treated oocyte groups.

The visual changes in spindles after vitrification were linked with function effects of oocytes on fertilization and development [23]. A sufficient restoration of the cytoskeleton after incubation is critical for fertilization events [24]. In this study, vitrified oocytes had lower percentages of good spindle morphology after a 1-hour incubation. After 3 hours' incubation, the spindle morphology improved. However, there were no significant differences in spindle morphology between 1 hour and 3 hours regardless of zona status. The most appropriate time for recovery of spindles after dilution deserves further study.

Anzai et al reported high recovery, survival, and fertility rates in zona-drilled mouse oocytes using vitrification cryopreservation (97%, 94%, and 60%, respectively) [25]. In the zona-intact group, the recovery, survival and fertility rates were 90%, 98%, and 11%. There was no difference between recovery and survival, although there was difference in the fertilization rate. In our study, we evaluated the effects of vitrified zona-drilled, zona-intact and zona-free oocytes. There was also no difference between survival and spindle morphology. However, we did not evaluate the fertilization rate among these three groups.

Table 3
Spindle morphology of mice oocytes 3 hours after thawing.

Condition	Oocytes	Spindle morphology, *n* (%)	
		Normal	Abnormal
One-hole zona	17	15 (88.2)	2 (11.8)
Intact zona	17	15 (88.2)	2 (11.8)
No zona	15	12 (80)	4 (26.6)
Control	20	19 (95)	1 (5)

The overall p was >0.05 for normal spindle, by Chi-square test.

4.1. Limitations

The numbers of oocytes were not large enough to reach statistical significance. Fertilization ability, such as 2-cell embryos and the derived offspring, was not evaluated in the postvitrification oocytes. Anti-infectivity ability in zona-drilled oocytes was also not evaluated *in vitro*.

4.2. Conclusions

Vitrification is effective not only for cryopreservation of early embryos [26], but also for mouse and rat unfertilized oocytes [27]. The vitrification procedure is very effective for cryopreservation of unfertilized oocytes from mice regardless of the zona condition. The ZP with the CPS method has no effect on spindle injury and oocyte survival. Sufficient culture time for recovery of the meiotic spindle is needed for fertilization of vitrified oocytes. A CPS vitrified oocyte has the advantages of high survival and preserved good spindles.

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