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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.

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