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The Crucial Role of Zona Pellucida in Cryopreservation of Oocytes by Vitrification

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Abstract

Mammalian oocytes have a proteinaceous hydrogel-like outer shell known as the zona pellucida (ZP) that semi-encloses their plasma membrane and cytoplasm. In this study, we cryopreserved mouse oocytes either with or without ZP by vitrification. Our results show that the presence of an intact ZP could significantly improve the post-vitrification survival of oocytes to 92.1% from 13.3% for oocytes without ZP. Moreover, there was no significant difference in embryonic development between fresh and cryopreserved oocytes with ZP after *in vitro* fertilization (IVF). Further atomic force microscopy (AFM) analysis showed that the intact oocytes with ZP have an elastic modulus that is more than 85 times higher than that of oocytes without ZP. This may partially explain the important role of ZP in protecting the oocytes by resisting the mechanical stress due to possible ice formation during cryopreservation by vitrification. Collectively, this study reveals a new biophysical role of ZP during vitrification of oocytes and suggests microencapsulation of the many mammalian cells without a ZP in ZP-like hydrogel is an effective strategy to improve their survival post cryopreservation by vitrification.

Keywords

zona pellucida; oocyte; cryopreservation; vitrification; AFM

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1. Introduction

The mammalian oocytes consist of a cytoplasm inside a plasma membrane that is surrounded by a transparent outer shell known as the zona pellucida (ZP) (Fig. 1). The ZP is made of hydrogel of glycoproteins and has been found to perform important biological roles in oogenesis and fertilization (i.e., interaction between sperm and oocytes), development of fertilized oocytes (i.e., embryos) before the embryos hatch out of the ZP, and protection of oocytes and pre-hatching embryos from infection by bacterial or fungal agents in the reproductive tract before implanting into the uterus wall [11,19,30–32]. By mimicking the ZP with a semipermeable shell of alginate hydrogel, our recent studies show that the miniaturized biomimetic 3D microenvironment semi-enclosed in the ZP-like alginate hydrogel shell could maintain the stemness of pluripotent stem cells significantly better than the conventional 2D culture in Petri dish or 3D culture in homogeneous bulk hydrogel [1,2,35]. This biomimetic 3D microenvironment was further found to greatly facilitate the enrichment of cancer stem-like cells compared to the conventional 3D bulk suspension culture [23]. These observations are understandable because the microenvironment semi-enclosed in the ZP is the native home of stem cells from the totipotent-pluripotent (i.e., zygote-blastocyst) stage [2,4,18,21,24,26,35]. Furthermore, our recent studies show that alginate hydrogel microencapsulation could significantly improve the survival of mesenchymal and embryonic stem cells after cryopreservation by vitrification (i.e., cooling to deep cryogenic temperature without intentionally seeding extracellular ice to dehydrate cells at a high subzero temperature, usually $> -10^{\circ}\text{C}$) using a low concentration ($\sim 2\text{ M}$) of cell-membrane permeable cryoprotectant (CPA) [13,34]. This is attributed to the exceptional capability of alginate hydrogel in damping ice formation during cooling and inhibiting ice recrystallization during the warming stage of a typical vitrification procedure. Therefore, we hypothesize that the ZP (hydrogel of glycoproteins) can protect oocytes similarly to the alginate hydrogel in protecting mesenchymal and embryonic stem cells during vitrification. To test this hypothesis, we conducted vitrification of deer mouse oocytes both with and without ZP in this study.

2. Materials and Methods

2.1. Animals

Peromyscus maniculatus bairdii (BW stock) deer mice were purchased from the Peromyscus Genetic Stock Center at the University of South Carolina, Columbia, SC. They were bred and maintained on a 16:8 h light-dark cycle. All animal use procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University (IACUC #:2011A00000084), and all efforts were made to minimize animal suffering.

2.2. Chemicals

L-15 Leibovitz-glutamax, leukemia inhibitory factor (LIF), and fetal bovine serum (FBS) were purchased from Invitrogen, Chemicon, and Hyclone, respectively. Unless specifically noted otherwise, all other chemicals were purchased from Sigma (USA).

2.3. Isolation of oocytes and removal of zona pellucida from oocytes

To isolate deer mouse oocytes, female deer mice (12 to 14 weeks of age) were injected with 5 IU of pregnant mare serum gonadotropin (PMSG) [5]. The mice were euthanized at 56 h later and their ovaries collected and placed in L-15 Leibovitz-glutamax medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). Cumulus-oocytes complexes (COCs) were then isolated manually by carefully and gently puncturing antral follicles in the collected ovaries using a pair of 30-gauge needles attached to disposable syringes under a stereoscope. The COCs in the antral follicles from both the outer (ovarian surface) and inner (deep ovarian tissue) layer of ovarian cortex were retrieved. *In vitro* maturation (IVM) of the immature oocytes contained in the isolated COCs was then conducted by culturing the COCs for 19 h in minimum essential medium- α containing Earle's salts (Invitrogen) supplemented with 10 mg/ml streptomycin sulfate, 75 mg/ml penicillin G, 5% (v/v) heat-inactivated FBS, 5 ng/ml epidermal growth factor (EGF), and 1000 IU/ml leukemia inhibitory factor (LIF) [5]. The medium was covered with 250 μ l of mineral oil in a 4-well plate at 37 °C in 5% CO₂ air. After IVM, COCs were collected and incubated in M2 medium (Millipore, Billerica, MA, USA) containing 200 IU/ml hyaluronidase at 37 °C for up to 3 min to remove cumulus cells. The samples were further washed twice in fresh M2 medium to obtain clean oocytes. Mature oocytes at the metaphase II (MII) stage were determined by the appearance of the first polar body (Fig. 1) and only MII oocytes were used for further experiments.

To remove zona pellucida (ZP) from oocytes, they were incubated in M2 medium containing 0.5% pronase for 5 min at 37 °C and then washed by gentle pipetting in M2 medium. This process is similar to detach cells in Petri dish using trypsin and has been shown not to cause any significant damage or stress to oocytes [20,22,25]. This is understandable since pronase is an enzyme that specifically digests the extracellular matrix and zona pellucida is part of the extracellular matrix of oocytes.

2.4. Vitrification of oocytes

For vitrification, the oocytes (with or without zona pellucida) were first suspended for 15 min in a droplet of 30 μ l of pre-equilibrium solution composed of 15% 1,2-propanediol (PrOH) in FHM medium (Sigma, St. Louis, MO, USA) supplemented with 20% FBS (FHM20). The pre-equilibrium solution was HEPES-buffered to maintain its pH at 7.2. The oocytes were then transferred into a droplet of 30 μ l of vitrification solution consisting of 15% PrOH and 1.3 M trehalose in FHM20 for 1 min before loading into a quartz microcapillary (QMC) (Charles Supper Company, Natick, MA, USA) for vitrification. The oocyte-laden QMC was then plunged into liquid nitrogen and held in there for at least 3 minutes. Afterward, the QMC was transferred from liquid nitrogen into a solution consisting of 0.2 M trehalose in 1 $\times$ phosphate-buffered saline (PBS) at 37 °C to warm up the sample. After warming, the oocytes were pushed out of the QMC by injecting FHM20 from the funnel head into the QMC and washed three times by transferring them consecutively in three droplets of 30 μ l of FHM20. Lastly, they were either used for *in vitro* fertilization study or transferred and cultured at 37 °C for 10 minutes in M2 medium with 5 μ M calcein AM and 5 μ M ethidium homodimer (Invitrogen) for live (green) and dead (red) staining, respectively.

2.5. *In vitro* fertilization (IVF) and embryo culture

For IVF, 5 MII oocytes (fresh or after vitrification) were incubated with 2×10^4 sperm for 4.5 h in a 200 μ l droplet of TYH medium [10]. To obtain sperm for IVF, 12 to 14-week old male deer mice were euthanized by cervical dislocation and epididymides were collected by dissection. They were then placed in the central well of an IVF dish with 1 ml TYH medium, a modified Krebs-Ringer bicarbonate solution containing 5.56 mM glucose, 1.0 mM sodium pyruvate, 4 mg/ml bovine serum albumin (BSA), and antibiotics. After making 5–7 longitudinal cuts using a syringe needle on each epididymis, they were incubated for 20 min at 37 °C in 5% CO₂ air to allow for sperm dispersion. The sperm suspensions were then further incubated for 2 h at 37 °C in 5% CO₂ air for capacitation.

Fertilized oocytes were subsequently cultured in a drop of 50 μ l of Hoppe and Pitts embryo culture medium [16] modified by removing sodium lactate and increasing the sodium chloride concentration to 5.97 g/ml at 37°C in 5% CO₂ air [4]. Development of the parthenogenetically activated and *in vitro* fertilized oocytes was monitored under a phase contrast light microscope (Nikon 80i) for the formation of two-pronuclei and two-cell embryos. The two-cell embryos were further cultured on MEF feeder (cell density: 2×10^4) layer in either ESC or embryo medium in a 96-well plate. The MEF feeder layer was made by inactivating the MEFs at passage 3 with 10 μ g/ml mitomycin. The embryonic stem cell (ESC) medium was made of knockout DMEM supplemented with 15% knockout serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol (β ME, Gibco), 2mM L-glutamine, antibiotics (1% penicillin/streptomycin), and 1000 U ml⁻¹ leukemia inhibition factor (LIF, Millipore).

2.6. Measurement of the elastic modulus of oocytes

We measured the overall elastic modulus of MII oocytes with and without ZP using an asylum MFP-3D-BIO atomic force microscopy (AFM) together with a NanoWorld PNP-TR AFM cantilever at the same time (i.e., immediately after removing the zona pellucida with pronase). For the AFM measurement, oocytes were transferred into a Petri dish filled with M2 medium to prevent dehydration of the cells. After the oocytes sunk down on the bottom surface of the Petri dish as a result of gravity, the AFM cantilever was manually brought to be in contact with the oocytes and positioned at their center to prevent possible side movement of the oocytes during measurement. Single force nano-indentation was performed by the AFM system and the obtained force curves were further analyzed using the Hertz's theory [33] to calculate the stiffness, which is built in the software of the AFM instrument and detailed in its user's manual. The total compressive strain applied was ~0.1 and the compressive strain rate was 0.05 s⁻¹ for the AFM study.

2.7. Statistical analysis

A generalized linear model (PROC-GLM) and one-way ANOVA in a Statistical Analysis System (SAS) program was used for statistical analysis to determine the *p* value between various conditions. The differences were taken as significant when the *p* value was less than 0.05.

3. Results and Discussion

The QMC used in this study for oocyte vitrification has an outer and inner diameter of 300 μm and 280 μm , respectively. A typical picture of the QMC loaded with oocytes is shown in Fig. 2a. Due to the miniaturized diameter, ultrafast cooling rate ($\sim 1.5 \times 10^5$ $^{\circ}\text{C}/\text{min}$) can be achieved by plunging the QMC into liquid nitrogen [12]. As a result, no apparent ice formation in the vitrification solution consisting of 15% PrOH and 1.3 M trehalose in FHM20 was observable (i.e., the solution appeared transparent) after plunging it into liquid nitrogen (Fig. 2b). In contrast, apparent ice formation with a whitish or opaque appearance was evident in the FHM20 solution without any CPA after plunged into liquid nitrogen in the same way (Fig. 2b). We held the oocyte-laden QMCs in liquid nitrogen for at least 3 minutes before transferring the sample from the liquid nitrogen into a warming solution consisting of 0.2 M trehalose in $1\times$ phosphate-buffered saline (PBS) at 37 $^{\circ}\text{C}$ to warm up the sample. This holding time was used because it has been shown to be more than sufficient to cool the sample in the QMC to the temperature of liquid nitrogen and all kinetics including that of cell injury is suspended at the deep cryogenic temperature [6,12].

Typical phase and fluorescence micrographs showing the morphology of viable and dead oocytes either with or without ZP after vitrification and the quantitative viability data are given in Fig. 3 and Table 1, respectively. The viability of oocytes without ZP was only 13% after the aforementioned vitrification procedure although it was 100% ($n=12$) before vitrification. By contrast, the viability was significantly ($p < 0.05$) improved to 92% for oocytes with an intact ZP after vitrification conducted in the same way. Furthermore, with a different set of experiments, we showed that there was no significant difference in terms of embryonic development after *in vitro* fertilization between the oocytes with ZP after vitrification and fresh oocytes with ZP (Fig. 4 and Table 2). These results indicate long-term functional survival of oocytes with an intact ZP after the vitrification procedure. Due to the dismal survival of oocytes without ZP after vitrification, we focused our IVF and embryonic development studies on the fresh and vitrified oocytes with an intact zona pellucida. Of note, the study on the embryonic development of deer mice is still in its infancy and few *in vitro* fertilized oocytes could be developed to beyond the 4-cell stage [4,5,29]. Although they are beyond the scope of this work, further studies are warranted to further confirm our observation using oocytes from species with well-established protocols of IVF allowing for embryonic development to obtain blastocysts for further implementation to produce viable and normal animals.

As shown in Fig. 3, for the dead oocytes with ZP after vitrification, the overall size of the oocytes within ZP did not change much at all although the cytoplasm appears damaged (homogenized), suggesting the strong mechanical property of the ZP in resisting possible mechanical damage due to ice formation during the cryopreservation procedure. This is particularly important during the warming step of cell cryopreservation by vitrification, when ice formation/recrystallization often occurs although ice formation can usually be minimized during the cooling step of a typical vitrification protocol [3,7–9,13–15,17,27,28]. Therefore, we further measured the elastic modulus of the oocytes with and without ZP using AFM. For the AFM measurement, the AFM cantilever was positioned at the center of oocytes to prevent possible side movement of the oocytes during measurement (Fig. 5a).

Indeed, the elastic modulus of oocytes with ZP is nearly two orders of magnitude higher than that of oocytes without ZP (2598 ± 778 Pa *versus* 30 ± 16 Pa, $p < 0.05$) (Fig. 5b). This mechanical strength of ZP may contribute to its protection of oocytes during vitrification, in addition to the possible capability of damping ice formation during cooling and inhibiting ice recrystallization during warming by the glycoprotein hydrogel of ZP [13,34]. Further studies are needed to confirm this hypothesis by encapsulating the oocytes without zona pellucida in hydrogel microcapsules of different degrees of elasticity and identifying a correlation between oocyte survival and the degree of elasticity. However, it is beyond the scope of this work as it is challenging to microencapsulate the stress-sensitive oocytes without a zona pellucida in hydrogel microcapsules with high survival for further vitrification at this time.

In summary, this study reveals a new important biophysical role of ZP in protecting oocytes during vitrification in addition to its biological functions reported previously in the literature. This is partially due to its strong mechanical properties compared to the plasma membrane of oocytes. These data further supports that microencapsulation of the many mammalian cells without a ZP in ZP-like hydrogel is an effective strategy to improve their survival post cryopreservation by vitrification.

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Fig. 1.

A typical micrograph together with schematic illustrations showing the anatomic structure of mammalian oocyte. The cell consists of a cytoplasm inside a plasma membrane that is surrounded by a transparent outer shell known as the zona pellucida (ZP). The ZP is made of hydrogel of glycoproteins. Scale bar: 40 μm

Fig. 2.

Quartz microcapillary (QMC) for vitrification of oocytes. (a) A typical picture of the quartz microcapillary (QMC) used in this study for vitrification together with a zoom-in view showing four oocytes loaded in the QMC. (b) a typical imaging showing apparent vitrification (transparent or clear appearance) *versus* apparent ice formation (whitish or opaque appearance) of the vitrification *versus* FHM20 solutions. The FHM20 solution is the FHM medium supplemented with 20% fetal bovine serum. The vitrification solution is the FHM20 solution with cryoprotectants (CPAs). Scale bar: 10 mm.

Fig. 3. Typical phase and fluorescence micrographs showing the morphology of viable and dead oocytes after vitrification. Scale bar: 40 μ m.

Control (fresh)

2-Pronuclei

2-cell

4-cell

Vitrified

Fig. 4.

Typical micrographs of 2-pronuclei, 2-cell, and 4-cell embryos during embryonic development of fresh and vitrified MII oocytes from deer mice after *in vitro* fertilization (IVF): The two arrows indicate the two pronuclei. The cells in the background of the 4-cell stage embryos are mouse embryonic fibroblasts (MEFs) as the feeder layer for culturing 2-cell embryos to develop them to the 4-cell stage. Scale bar: 40 μ m.

Fig. 5.

Mechanical property of oocytes with and without zona pellucida (ZP) determined by atomic force microscopy (AFM). (a) Typical micrographs showing the setup for measuring the mechanical properties of oocytes with and without ZP using AFM. (b) Quantitative data of the elastic modulus of oocytes with and without ZP determined by AFM. A total of 6 oocytes were studied for each group. Scale bar: 70 μm . The asterisk indicates $p < 0.05$.

Table 1

Viability of MII oocytes with and without zona pellucida (ZP) after vitrification with 15% PrOH and 1.3 M trehalose using quartz microcapillary (QMC): A total of six animals were used to isolate the MII oocytes. The survival of oocytes with an intact zona pellucida after vitrification is significantly higher than that of oocytes with zona pellucida ($p < 0.05$).

Type	Number of MII oocytes	Number (%) of	
		Live MII oocytes	Dead MII oocytes
With ZP	38	35 (92)	3 (8)
Without ZP	30	4 (13)	26 (87)

Table 2

Embryonic development after *in vitro* fertilization (IVF) of MII oocytes either without (control) or with vitrification using quartz microcapillary (QMC): A total of six animals were used to isolate the MII oocytes.

Group	No. of MII oocytes	% of 2-pronuclei embryos	No. (%) of 2-cell embryos	No. (%) of 4-cell embryos
Control	42	86 ^a	9 (21)	2 (22)
Vitrification	40	80 ^b	7 (18)	1 (14)

^a A total of 22 fertilized oocytes was monitored for the formation of two pronuclei.

^b A total of 20 fertilized oocytes was monitored for the formation of two pronuclei.

^c Percentage of MII oocytes.

^d Percentage of total 2-cell embryos.