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Developmental competence and gene expression of immature oocytes following liquid helium vitrification in bovine

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ABSTRACT

The objective of this study was to develop an effective ultra-rapid vitrification method and evaluate its effect on maturation, developmental competence and development-related gene expression in bovine immature oocytes. Bovine cumulus oocyte complexes were randomly allocated into three groups: (1) controls, (2) liquid nitrogen vitrification, and (3) liquid helium vitrification. Oocytes were vitrified and then warmed, the percentage of morphologically normal oocytes in liquid helium group (89.0%) was significantly higher ($P < 0.05$) than that of the liquid nitrogen group (81.1%). When the vitrified–thawed oocytes were matured in vitro for 24 h, the maturation rate in liquid helium group (50.6%) was higher ($P < 0.05$) than liquid nitrogen group (42.6%). Oocytes of liquid helium vitrification had higher cleavage and blastocyst rates (41.1% and 10.0%) than that of liquid nitrogen vitrification (33.0% and 4.5%; $P < 0.05$) after in vitro fertilization. Moreover, the expression of GDF9 (growth/differentiation factor-9), BAX (apoptosis factor) and ZAR1 (zygote arrest 1) was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) when the vitrified–thawed oocytes were matured 24 h. The expression of these genes was altered after vitrification. Expression of GDF9 and BAX in the liquid helium vitrification group was not significantly different from that of the control, however there were significant differences between the liquid nitrogen vitrification group and control. In conclusion, it was feasible to use liquid helium for vitrifying bovine immature oocytes. There existed an association between the compromised developmental competence and the altered expression levels of these genes for the vitrified oocytes.

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Introduction

The fast development of assisted reproductive technologies has led to an increase in the need of mammalian oocytes [2]. The easiest way of accessing oocytes at any time is to preserve them at very low temperatures. Since the 1980's, the oocyte cryopreservation studies have been increased gradually, most of which are at metaphase II (MII). However, the cryopreservation of immature oocytes have been rarely reported [27]. Until recently, the vitrification of immature oocytes has been taken more and more attention since there is no meiotic spindle present and the genetic material is confined within the nucleus [16]. Currently, liquid nitrogen vitrification is a fast-freezing and successful technique in several species [8,11,12,18,23] that preserves oocytes by preventing the formation of damaging ice crystals and other injuries [5,7,21]. However, the vitrification has not been widely used in bovine production because of the relatively poor embryos development from frozen oocytes. The reason may be due to the less-than-ideal cooling rates [28] during the vitrification process. Furthermore, vitrification in large volume of solution not only affects oocyte developmental competence [31,36], but also damages DNA in cumulus oocyte complexes (COCs) [17] and loses cytoplasmic mRNA [26]. Therefore, difference of gene expression levels in oocytes cryopreservation has become an effective method for oocytes cryoinjury research. At present, it is of great interest to develop an approach to achieve vitrification of mammalian oocytes, which could combine the advantages of the existing vitrification methods while avoiding their shortcomings.

A new technology called the liquid helium vitrification has recently been developed in our laboratory. We are trying to use the liquid helium (−269 °C, a liquid can be applied at the lowest temperature now) instead of the liquid nitrogen (−196 °C). Theoretically, this can be done by ultra-fast freezing of bovine immature oocytes to a vitrified state at cryogenic temperatures. Moreover,

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we used the OPS vitrification method based on its ease of use and the superior efficiencies in mammals [13,32]. Immature oocytes of bovine were used as the experimental material during the whole experiments. The vitrification effects of two different cryogens (liquid nitrogen and liquid helium) on oocytes were compared, then the developmental competence of oocytes and the expression level of the genes related development of oocytes which include GDF9 (growth/differentiation factor-9), BAX (apoptosis factor) and ZAR1 (zygote arrest 1) were examined. The objective of the current study was to use the liquid helium to cryopreserve the bovine immature oocytes and explore the relationship between the late-developmental competence and expression of these genes for the vitrified oocytes.

Materials and methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. All the media used in the present study were supplemented with penicillin (100 IU/mL) and streptomycin (0.1 mg/mL) and filter (0.22 μm) sterilized prior to use.

Collection and selection of COCs

Bovine ovaries (Chinese yellow cattle, Bos taurus) were transported from the local abattoir to the laboratory in physiological saline solution at 35 °C within 4 h after slaughter. Oocytes were recovered by aspiration of 2–8 mm follicles using a 12-gauge needle attached to a 10 mL syringe. Oocytes with at least 3 layers of intact, compact cumulus cells were selected for in vitro maturation (IVM) and vitrification [4,25].

Preparation of open pulled straw (OPS)

French mini straws were slightly melted over a flame and pulled by hands until the inside diameter decreased from 1.7 mm to approximately 0.8 mm and the wall thickness of the central part decreased from approximately 0.15 mm to 0.07 mm. The straws were then cut at the narrowest point of the pulled portion [26,35].

Vitrification and warming of COCs

Liquid nitrogen vitrification

Briefly, COCs were handled in a basic vitrification medium (BV) consisting of TCM 199-Hepes + 20% (v/v) FBS. Then all oocytes were moved into vitrification solution-1 (VS1) for 3 min. VS1 contained BV with 10% Dimethyl sulfoxide (Me_2SO) and 10% Ethylene glycol (EG). Next, oocytes were exposed to BV with 20% Me_2SO, 20% EG and 0.5 M sucrose (vitrification solution-2; VS2). A single droplet, approximately 1.5 μL and containing 5–7 oocytes, after a quick passage in VS2, was loaded into a straw by the capillary effect and then directly submerged into liquid nitrogen [4,20,35]. The time interval between the contact with VS2 and cooling did not exceed 30 s.

For warming [13], the OPS containing vitrified COCs were taken out from liquid nitrogen and immersed in pre-warmed (at approximately 38 °C) BV with 0.25 M sucrose (Warming solution 1; W1). The oocytes were transferred to W1 by gently blowing on one end of the OPS. Five minutes later, the COCs were transferred to BV with 0.15 M sucrose (Warming solution 2; W2) for 5 min, and then washed twice in the BV for 5 min each. The warmed oocytes were transferred to maturation media and cultured as described in "*In vitro maturation of COCs*".

Liquid helium vitrification

The method of liquid helium vitrification was similar with that of liquid nitrogen vitrification. Oocytes were also exposed to BV with 20% Me_2SO, 20% EG and 0.5 M sucrose. When OPS with COCs was prepared, it was immediately plunged into liquid helium 10 s, then pulled it up and preserved into liquid nitrogen. The time interval between the contact with VS2 and the immersion into liquid helium did not exceed 30 s.

For warming, the tip of OPS was placed into 0.25 M sucrose solution (38 °C). Then, the warming method was same as that of liquid nitrogen vitrification.

Evaluation of oocyte morphology

Vitrified–thawed oocytes were washed twice with handling medium and observed by invert microscope (NIKON2000-U, Japan). Oocytes were considered normal with the following characteristics: oocytes with spherical and symmetrical shape with no sign of membrane damage, swelling, vacuolization, leakage of the cellular content, ruptured zona pellucida or vitelline membrane, and fragmented cytoplasm [22]. Morphologically normal oocytes were used for IVM and other subsequent experiments.

In vitro maturation of COCs

COCs were washed three times in HEPES-buffered TCM-199 (GIBCO BRL, Grand Island, NY, USA) and then washed three times in IVM medium ($NaHCO_3$-buffered TCM-199) on a heating stage at 37 °C in the sterile room. The maturation medium consisted of TCM-199 with 25 mM HEPES, 2 mM $NaHCO_3$, 10% FBS, 10 ng/mL epidermal growth factor, 0.5 mg/mL follicle-stimulating hormone (FSH), 10 mg/mL luteinizing hormone (LH), and 1.0 mg 17β-estradiol. Approximately 25–30 COCs were cultured in 50 μL microdrops overlaid with mineral oil for 24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air [35]. The criteria used for assessing maturation of oocytes were as follows: the oocytes were denuded by treating them with hyaluronidase (100 IU/mL) and then examined for extrusion of the 1st polar body (PB I).

In vitro fertilization (IVF)

Sperm separation was carried out using a swim-up procedure as previously described [4,24]. First, cryopreserved semen (0.25 mL; Simmental cattle sire) was taken out from liquid nitrogen and thawed in a water bath for 15 s, and then added to a centrifuge tube containing 2 mL IVF medium (consisting of 6 mg/mL bovine serum albumin, 10 IU/mL heparin, and 5 mM caffeine). After 40 min of incubation at 38.5 °C, the upper layer of supernatant containing the motile sperm was removed. The sperm were centrifuged for 5 min at 500g and the supernatant aspirated to leave a pellet of approximately 50 μL in volume for fertilization (final concentration, 1×10^6 sperm/mL). COCs were washed three times with IVF medium and transferred to 50 μL drops of sperm suspension for fertilization. Incubation of oocytes and sperm cells together was carried out for 16 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air [35].

In vitro culture (IVC)

Presumptive zygotes were washed three times in IVC medium, which consisted of TCM-199 with 25 mM HEPES and 10% (v/v) FBS and 2.5 mM $NaHCO_3$. The zygotes were transferred to 50 μL drops of IVC medium with granulosa cell monolayers [6] and then cultured at 38.5 °C. Cleavage rates were recorded on Day 2 (Day 0 = day of insemination), and the rate of embryo development (early blastocysts) was recorded on Day 7 [35].

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