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Effect of liquid helium vitrification on the ultrastructure and related gene expression of mature bovine oocytes after vitrifying at immature stage

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ABSTRACT

This study aimed to investigate the developmental potential of and the ultrastructural changes and gene expression differences resulting from liquid helium (LHe; −269 °C) vitrification in immature bovine oocytes. Immature oocytes were randomly divided into three groups: fresh oocytes (control, negative control), oocytes vitrified in liquid nitrogen (LN group, positive control), and oocytes vitrified in LHe (LHe group). In experiment 1, the rates of normal morphology, maturation, cleavage, and blastocyst in the LHe group were higher than those in the LN group (87.1% vs. 80.5%, 51% vs. 48%, 41.7% vs. 36.8%, and 13% vs. 8.5%, respectively; $P < 0.05$), and the rates of development in the control group (100%, 73.2%, 62%, and 39.8%) were higher than those in the treated groups ($P < 0.05$). In experiment 2, oocytes displayed various degrees of injury at the ultrastructural level after vitrification, but more severe degeneration was observed in the LN group, such as formation of several lipid droplets, swelling of mitochondria, and absence of cortical granules. Compared with the LN group, fewer lipid droplets, relatively intact mitochondria, and clustered cortical granules were distributed in the cytoplasm of oocytes in the LHe group. In experiment 3, the mRNA expression levels of *p53*, *CDC20*, *Eg5*, and *Npm2* were investigated by real-time quantitative polymerase chain reaction. Expression levels of the kinesin *Eg5* and the apoptotic gene *p53* expression levels were higher in the LN group compared with the control and LHe groups ($P < 0.05$). CDC20 and Npm2 expression did not differ significantly between the LN and LHe groups ($P > 0.05$), the CDC20 expression in the LN and LHe groups were lower than control group ($P < 0.05$), the Npm2 expression in LHe group was lower than control group ($P < 0.05$), but there was no significant difference between the LN and control groups ($P > 0.05$). In conclusion, LHe vitrification decreased the negative effect of cryoinjury on the ultrastructure of some organelles and the expression of some related genes, thereby improving the viability of immature bovine oocytes compared to LN vitrification.

1. Introduction

Successful cryopreservation of oocytes is important for conservation of female genetics in domestic animals and wild animals, as well as assisted reproduction in humans. Several methods of oocyte cryopreservation have been employed in several mammalian species. However, the

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efficiency is still very low because of the unique structure of oocytes and their sensitivity to cooling [1].

To date, significant attention has focused on the cryopreservation of mature oocytes. However, mature oocytes are sensitive to cooling, which can result in disruption of their chromosomes and meiotic spindle injuries [2–4]. Compared with mature oocytes, immature oocytes have an advantage in terms of their intracellular structure because their chromatin is in a decondensed state. Because no microtubular system is formed in these cells, cryopreservation at this stage may yield better results [5]. In addition, cryopreservation of immature oocytes expands the choice of timing and location of artificial embryo production. However, the efficiency of immature oocyte cryopreservation is still very low despite some reports of success [6]. Therefore, the optimal method for cryopreservation of immature oocytes is yet to be developed.

Different methods for cryopreservation of oocytes have been tested for a long time. Classic freezing protocols have been gradually replaced by vitrification methods. Vitrification is a procedure that improves the cooling rate and shortens the time of exposure to cryoprotectant solutions without the formation of ice crystals [7,8]. Several successful vitrifications of bovine oocyte were reported with cryodevices, such as open pulled straws (OPSs), solid surface vitrification, and cryotops [9–11] and using liquid nitrogen (LN) as the cryogen. Although vitrification avoids ice crystal formation inside oocytes, it requires higher concentrations of cryoprotectants, which are toxic and may cause osmotic injury in oocytes [12,13]. A rapid cooling rate during vitrification is the key to successful vitrification to avoid cryoinjury in sensitive cells. Yavin et al. [14] reported that LN slush (−210 °C) could be used as an innovative vitrification method instead of LN (−196 °C) vitrification to achieve a high cooling rate, thereby simultaneously enabling reduction in cryoprotectant concentration. To achieve LN slush, the LN needs to be cooled close to its freezing point (−210 °C) by applying negative pressure. When LN slush is formed, the cooling rate is dramatically increased [15]. To achieve a higher cooling rate for vitrification, our laboratory attempted to use liquid helium (LHe; −269 °C) as a new cryogen instead of LN and LN slush. Liquid helium is the least active element and has stable refrigeration effect. Although it is generally applied in the field of superconducting technology, it has not been used in the field of cryobiology. In our previous work, the effect of LHe on developmental competence and expression of some development-related genes (*GDF9*, *BAX*, and *ZAR1*) in bovine immature oocytes were evaluated. The results showed that LHe vitrification was a feasible method for vitrifying bovine immature oocytes [16]. Then, the effectiveness of the LHe vitrification method for bovine immature oocytes with the OPS system was validated, and a mixture of 17.5% (vol/vol) DMSO and 17.5% (vol/vol) ethylene glycol (EG) was determined as the optimal cryoprotectant for this purpose [17]. Based on these findings, ultrastructure of some organelles and expression of other related genes were investigated in this study to analyze the effects of LHe vitrification on immature bovine oocytes (Chinese yellow cattle, *Bos taurus*).

Ultrastructural alteration and molecular damage of oocytes occur mainly because of cold injury during vitrification. Fuku et al. [18] and Diez et al. [19] reported that oocytes lost the integrity of their cell structure and some organelles, with observations such as swelling of mitochondria, disappearance of all microvilli, and reduced and irregular cortical granules following vitrification. Decrease in levels of mRNA is thought to be associated with degradation or consumption of some mRNA molecules [20]. Determining changes in expression of some key genes has become an effective method for detecting oocyte cryoinjury on cryopreservation. Such genes include the cell apoptosis–related gene *p53*, the cell-cycle regulatory gene *CDC20*, the cell division–related gene *Eg5*, and the maternal effect gene *Npm2*. The *p53* gene is related to the apoptotic pathway and can be a good candidate for detection of certain types of cell degradation [21]. *CDC20* is required for spindle assembly, chromosomal segregation, and PB1 extrusion during bovine oocyte maturation [22]. As a member of the kinesin-related motor protein, the kinesin-5 motor protein encoded by the *Eg5* gene is highly expressed in proliferating cells. During interphase, *Eg5* is associated with ribosomes and is required for optimal synthesis of nascent polypeptides [23]. *Npm2* is a maternal effect gene and is critical for nuclear and nucleolar organization during the initial stages of embryonic development, before activation of the embryonic genome. *Npm2* knockout females have fertility defects because of failed preimplantation embryo development [24]. *Npm2* may function as a histone chaperone to help remodel chromatin in oocytes and early embryos [25]. The alteration of expression of these key genes might cause abnormal embryo compaction and poor embryo development.

Therefore, the objectives of this study were (1) to evaluate the developmental competence of bovine oocytes following LHe vitrification; (2) to test ultrastructural changes in immature bovine oocytes after freezing by LHe; and (3) to investigate the levels of mRNA of some related genes, such as *p53*, *Eg5*, *CDC20*, and *Npm2*, in oocytes vitrified by LHe.

2. Materials and methods

2.1. Reagents and supplies

Unless otherwise specified, all reagents were purchased from Sigma (St. Louis, Mo, USA). Medium 199 (M-199, Gibco BRL, Grand Island, NY, USA), fetal bovine serum (FBS, Zhe Jiang Tian Hang Biotechnology Co., Ltd., China), and LHe (Chinese Academy of Sciences, China) were also used.

2.2. Collection of cumulus oocyte complexes (COCs)

Ovaries were collected from a local abattoir and transported to our laboratory in saline (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 g/mL) at 37 °C within 4 hours of slaughter. Cumulus oocyte complexes were aspirated from 2- to 8-mm follicles using a 10 mL syringe with a 12-gauge needle at room temperature. COCs with more than two layers of compact cumulus cells and uniform