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Successful vitrification of bovine immature oocyte using liquid helium instead of liquid nitrogen as cryogenic liquid

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ABSTRACT

The objectives of this study were to compare the effectiveness of liquid helium (LHe) and liquid nitrogen (LN_2) as cryogenic liquid for vitrification of bovine immature oocytes with open-pulled straw (OPS) system and determine the optimal cryoprotectant concentration of LHe vitrification. Cumulus oocyte complexes were divided into three groups, namely, untreated group (control), LN_2 vitrified with OPS group, and LHe vitrified with OPS group. Oocyte survival was assessed by morphology, nuclear maturation, and developmental capability. Results indicated that the rates of normal morphology, maturation, cleavage, and blastocyst (89.3%, 52.8%, 42.7%, and 10.1%, respectively) in the LHe-vitrified group were all higher than those (79.3%, 43.4%, 34.1%, and 4.7%) in the LN_2-vitrified group ($P < 0.05$) although the corresponding rates in both treated groups decreased compared with the control group (100%, 75.0%, 64.9%, and 40.8%; $P < 0.05$). Normal calves were obtained after the transfer of blastocysts derived from LHe- and LN_2-vitrified oocytes. The effects of the different vitrification solutions (EDS30, EDS35, EDS40, EDS45, and EDS50) in LHe vitrification for bovine immature oocytes vitrification were examined. No difference was found in the rates of morphologically normal oocytes among the EDS30 (87.9%), EDS35 (90.1%), EDS40 (89.4%), and EDS45 (87.2%) groups ($P > 0.05$). The maturation rate of the EDS35 group (65.0%) was higher than those of the EDS30 (51.3%), EDS40 (50.1%), EDS45 (52.1%), and EDS50 groups (36.9%; $P < 0.05$). No significant differences were observed in the cleavage and blastocyst rates between the EDS35 (49.0% and 12.1%) and EDS40 (41.7% and 10.2%) groups. However, the cleavage and blastocyst rates in the EDS35 group were higher ($P < 0.05$) than those of the EDS30 (36.2% and 6.8%), EDS45 (35.9% and 5.8%), and EDS50 (16.6% and 2.2%) groups. In conclusion, LHe can be used as a cryogenic liquid for vitrification of bovine immature oocytes, and it is more efficient than LN_2-vitrified oocytes in terms of blastocyst production. EDS35 was the optimal cryoprotectant agent combination for LHe vitrification in this study.

1. Introduction

Highly efficient oocyte cryopreservation is important for assisted reproduction technology in humans and for preserving genetic material in domestic or endangered species. However, mammalian oocyte cryopreservation remains challenging. Contemporary techniques for oocyte cryopreservation in mammals are often marred by low survival and developmental rates. Furthermore, most of the present investigations are still focused on mature oocytes rather than on immature oocytes because of the difficulties encountered in the cryopreservation for immature oocytes [1,2]. The main damage in vitrified mature oocytes is meiotic spindle disorganization followed

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by microtubule depolymerization [3,4]. Immature oocytes have no meiotic spindle, and the genetic material is confined within the nucleus, which is a theoretically ideal characteristic for cryopreservation. However, immature oocytes are extremely sensitive to cryopreservation because of the damage at the cytoplasmic membrane [5] and significant damage to gap junction integrity, disrupting communication between cumulus cells and oocytes [6]. Immature oocytes are more readily accessible in significant numbers in ovaries without the need for *in vivo*/*vitro* maturation, increasing the availability of gamete for research applications or innovative assisted reproduction techniques.

Controlled freezing and vitrification are performed in oocyte cryopreservation. Compared with controlled freezing, vitrification reduces the required time and equipment cost for cryopreservation [7]. Thus, vitrification has become a competitive alternative to controlled freezing [8]. Vitrification is a simple cryopreservation method of directly submerging a cell in liquid nitrogen (LN_2) after brief exposure to a cryoprotectant agent (CPA). This process has substantially low harmful effect on cells, such as oocytes and embryos, that have high chilling sensitivities. The success rate of vitrification depends on three key factors, namely, cooling rate, concentration of CPAs added to the vitrification solution (VS), and sample volume [9]. Rapid cooling rate theoretically reaches the glass transition temperature more effectively, but vitrifying a single drop (1 μL) of pure water requires a cooling rate of up to 10^8 °C/min, which is virtually impossible to achieve [10]. Therefore, vitrification usually uses a VS with high CPA concentration that lowers the ice formation temperature and increases the glass transition temperature [11]. These changes result in successful vitrification under achievable cooling rate. However, high concentration of CPAs induces vitrification and simultaneously induces detrimental toxicity and permeability damage to oocytes [8]. Smaller sample volumes allow better heat transfer, thus facilitating greater cooling rate. Therefore, the smaller the volume, the higher the probability of vitrification [12]. Consequently, reducing the CPA concentration by enhancing the cooling rate and decreasing the sample volumes is important in vitrification. The typical methods for enhancing cooling rates, such as minimum drop size [12], open-pulled straw (OPS) [13], Cryotop [14], CryoLoop [15], and electron microscopy grid methods [16], rely on techniques that reduce sample volumes. The OPS method is more advantageous than the other rapid cooling methods. Aside from having high cooling and warming rates, this method is simple, rapid, and inexpensive and facilitates the handling of vitrified cells [13].

Alterations in the cryogenic liquid can optimize the sample container and increase the desired cooling rates [16,17]. The use of propane for plunging has been previously explored with some reported drawbacks because of the possible permeability of cells to this molecule [18]. A more desirable approach is the utilization of slush nitrogen, in which liquid and solid nitrogen coexist. Slush nitrogen as a cryogenic liquid has a temperature of approximately −205 °C to −210 °C, and its main benefit is the reduction of vaporization when subjected to relatively high temperatures [19,20]. The cooling rate, when LN_2 slush is used, is especially enhanced in the first stage of cooling (from 20 °C to −10 °C) when the slush is two to six times higher than LN_2 (−196 °C) with 0.25-mL straw or any other device, such as OPS or electron microscope grids [12]. Increasing the cooling rate improves the survival rates of oocytes and embryos by up to 37% [21]. Very few studies on liquid helium (LHe), which has the lowest boiling point (−269 °C), have been reported. This compound has been used in our laboratory as a cryogenic liquid. In an earlier article, we reported that normal morphology and post-thaw developmental competence of immature bovine oocytes in LHe vitrification were better than those in LN_2 vitrification [22].

Vitrification can only be induced in exceptional situations, that is, with dangerously high CPA concentrations and/or with extreme increase in cooling and warming rates. In vitrification, as the cooling rate increases, the CPA concentration can be lowered and *vice versa* [8]. We speculated that the temperature (−269 °C) of LHe will increase the cooling rate to reduce the concentration of CPAs needed for vitrification. In the present study, we used LHe to vitrify bovine immature oocytes with OPS method in different CPA concentrations. The normal morphology and post-thaw developmental competence in the vitrified-thawed oocytes were assessed and compared with those of the fresh control oocytes. We determined the effectiveness of LHe as a cryogenic liquid using the OPS method described by Vajta et al. [13] and explored the optimal concentration of CPAs for LHe vitrification.

2. Material and methods

2.1. Chemicals and supplies

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Plastic dishes and tubes were obtained from Nunc (Roskilde, Denmark). The OPSs were made in our laboratory from 0.25-mL French straws (IMV, L'Aigle, France).

2.2. Oocyte recovery

Ovaries from bovine (Chinese yellow cattle, *Bos taurus*) were transported from a local abattoir to the laboratory in physiological saline at 38.5 °C within 1 to 2 hours. Cumulus oocyte complexes (COCs) from follicles with 2 to 8 mm in diameter were aspirated using an 18-gauge needle attached to a 10-mL syringe. Only COCs with three or more layers of cumulus cells and homogeneous cytoplasm were selected for further processing.

2.3. Preparation of OPSs

After removing the cotton plug, the 0.25-mL French straws were softened by heating them over the flame of a special alcohol lamp. The straws were then pulled manually 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 to 0.07 mm. The straws were cooled in air and then cut with a razor blade at the narrowest point.