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Effect of antifreeze glycoprotein 8 supplementation during vitrification on the developmental competence of bovine oocytes

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ABSTRACT

The purpose of this study was to investigate the effect of antifreeze glycoprotein 8 (AFGP8) supplementation during vitrification on the survival, fertilization, and embryonic development of bovine oocytes and the underlying molecular mechanism(s). Survival, fertilization, early embryonic development, apoptosis, DNA double-strand breaks, reactive oxygen species levels, meiotic cytoskeleton assembly, chromosome alignment, and energy status of mitochondria were measured in the present experiments. Compared with that in the nonsupplemented group; survival, monospermy, blastocyst formation rates, and blastomere counts were significantly higher in the AFGP8-supplemented animals. Oocytes of the latter group also presented fewer double-strand breaks and lower cathepsin B and caspase activities. Rates of normal spindle organization and chromosome alignment, actin filament impairment, and mitochondrial distribution were significantly higher in the AFGP8-supplemented group. In addition, intracellular reactive oxygen species levels significantly decreased in the AFGP8-supplemented groups, maintaining a higher $\Delta\Psi m$ than that in the nonsupplemented group. Taken together, these results indicated that supplementation with AFGP8 during vitrification has a protective effect on bovine oocytes against chilling injury.

1. Introduction

Cryopreservation of oocytes is a powerful technique for preserving fertility, especially in adult women/pubertal females with premature loss of ovarian function due to surgery, chemotherapy, or radiotherapy. In addition, an effective oocyte cryopreservation program also serves as a means of assisted reproductive technology by storing the donated oocyte. For instance, oocytes can be frozen in advance in the case of failed sperm collection. Cryopreservation of oocytes through vitrification is widely conducted owing to its uncomplicated procedure, high survival rates of the oocytes and embryos, and subsequently, high pregnancy rates [1]. Although vitrification can effectively prevent intercellular ice-crystal formation and decrease chilling injury [2], the sensitivity of oocyte membranes to ice-crystal formation, and that of the cytoskeleton fibers to low temperatures and cryoprotectants, is frequently associated with substantially reduced viability of frozen-thawed oocytes [3]. The permeability of water and cryoprotectants to the plasma membrane is very important for cryopreservation of oocytes and embryos during freeze-thawing [4,5]. Various types of cryoprotectants, such as dimethyl sulfoxide (DMSO), 1,2- propanediol, and/or 1,2-ethandiol (also called ethylene glycol, [EG]) have been used in different combinations for vitrification of

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mammalian oocytes and embryos [6]. These permeable cryoprotectants reduce cell dehydration and shrinkage, thereby, limiting osmotic and mechanical injury to membranes and other cellular structures. High concentrations of membrane-permeable cryoprotectants are necessary to protect mammalian cells from osmotic stress injury during freezing; however, studies report that some cryoprotectants are chemically toxic [7,8].

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs), collectively referred to as AF(G)Ps, are found in various Arctic and Antarctic fish, as well as in amphibians, plants and insects, thus playing an important role in their survival in subzero environments [9–11]. These proteins and peptides have ice-binding affinities and can kinetically suppress the temperature for ice-crystal formation, thus preventing mechanical cell damage caused by ice regrowth [12,13]. It is believed that these agents depress the freezing point to a level 200 to 300 fold lower than ordinary cryoprotectants (such as sugars and polyols) and salts [14,15]. Recently, improved survival rates have been reported in animal models using modified vitrification procedures, particularly those involving the use of AFPs-supplemented freezing and thawing solutions [16,17]. Antifreeze proteins supplements in the freezing and thawing solution protect cell membranes against cold-induced injury by inhibiting ice recrystallization, leading to improved survival rates and embryonic development potential *in vitro*. In contrast to AFPs, AFGPs possess very different primary, secondary, and tertiary structures, composed of repeated tripeptide units (alanyl-alanyl-threonyl) with minor sequence variations [18,19]. This unique property of AFGPs has attracted significant interest because of their potential applications in a variety of fields including medicine and assisted reproductive technology. Several reports have reported the benefits of AFGPs supplementation during cryopreservation, organ and cell transplant, and cryosurgery [20–22].

AFGPs are categorized into eight subclasses, each with a unique molecular weight and amino acid sequence. Among the several types of AFGPs, AFGP8 has the lowest molecular weight fraction (2.6 kDa). It consists of alanyl-alanyl-threonyl $(AAT^*)_{n\,=\,4}$ AA, in which T^* is threonine with O-linked α-D-N-acetylgalactosamine. Furthermore, it is reported that AFGP8 can depress the freezing temperature by forming hexagonal bipyramidal ice crystals [19,23].

In the present study, AFGP8 was applied during the vitrification of in vitro-matured bovine oocytes. Its protective feature against cryo-induced damage was investigated by evaluating the survival, fertilization, and early embryonic development of frozen-thawed bovine oocytes after AFGP8 supplementation during the vitrification process. Furthermore, various functional features, such as cell apoptosis, DNA double-strand breaks (DSBs), meiotic cytoskeleton assembly, chromosome alignment, cortical granules (CGs) distribution, mitochondrial distributions, reactive oxygen species (ROS) levels and energy status of mitochondria, were evaluated and compared after AFGP8 supplementation.

2. Materials and methods

Animal experiments were performed according to the Guiding Principles for the Care and Use of Animals of Chungbuk National, Republic of Korea. Antifreeze glycoprotein 8 was chemically synthesized by following previously reported procedures [19,23]. All other chemicals used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.1. Collection and IVM of oocytes

Bovine ovaries were collected from a local slaughterhouse, and transported to the laboratory at 37 °C in saline supplemented with penicillin G (75 mg/mL) and streptomycin sulfate (50 mg/mL). Follicles that were 3 to 8 mm in diameter were aspirated. Cumulus-oocyte complexes (COCs) surrounded by a minimum of three cumulus cells were selected for subsequent culture. The COCs were washed thrice in Tyrode Lactate Hepes (TL-HEPES) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA) and gentamycin (0.05 g/L). Next, the COCs were washed thrice in, and then transferred to, the maturation medium [tissue cultured medium-199 supplemented with 0.1 g/L sodium pyruvate, 0.6-mM L-cysteine, 10 μg/mL FSH, 10 μg/mL estradiol, 10% (v/v) fetal bovine serum [Gibco BRL (Burlington, Ontario, Canada), 10099–141], and 1% penicillin-streptomycin]. Maturation was performed by culturing approximately 50 COCs in 4-well dishes containing 500 μL of maturation medium. The medium was covered with mineral oil, and the plates were incubated at 39 °C in a humidified atmosphere containing 5% CO_2 for 22 hours. After IVM, the cumulus cells were removed from the oocyte by gentle pipetting in TL-HEPES supplemented with 1 mg/mL hyaluronidase and 0.1% PVA. Only the oocytes that displayed normal morphologies and polar bodies were selected for further studies.

2.2. Vitrification and warming of oocytes

Oocytes were vitrified in vitrification solution with or without AFGP8 supplementation. Antifreeze glycoprotein 8 was dissolved in PBS to yield a stock of 100 mM, aliquoted, and stored in the dark at −20 °C. The optimal concentration of AFGP8 was predetermined by a dose-response study, at which the highest survival was noted (Supplementary Table 1). The final concentration of AFGP8 was 1 mM. Oocyte vitrification was performed as previously described [24], with slight modifications. The holding medium, which was used to handle oocytes during vitrification and warming, was composed of HEPES-buffered tissue cultured medium-199 (Gibco BRL) supplemented with 20% fetal calf serum. For vitrification, metaphase II-stage oocytes were placed in equilibrium solution (holding medium, containing 7.5% EG and 7.5% DMSO) for 10 minutes. The oocytes were then transferred to a vitrification solution (holding medium, containing 15% EG, 15% DMSO, and 0.5 M sucrose) for 1 minute. Next, the oocytes were loaded onto a cut standard straw (the middle part of a 0.25-mL straw was heated, pulled to an inner diameter of 0.1 mm, and then cut at an angle of 45°) in sets of 3 to 5 under a stereomicroscope (SMZ745, Nikon, Tokyo, Japan), and covered with the remaining part of straw. Before vitrification, most of the vitrification solution transferred with the oocytes was removed from the straw and only a thin layer (<0.1 μL) was retained to cover the oocytes. Subsequently, the straw containing the oocytes was