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Effect of the exposure to methyl-β-cyclodextrin prior to chilling or vitrification on the viability of bovine immature oocytes ☆

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ABSTRACT

The present study aimed to evaluate the effect of methyl-β-cyclodextrin (MβCD) as a cholesterol loader to change oocyte plasma membrane and increase its tolerance toward cryopreservation. The first and second experiments were conducted to investigate if MβCD could improve nuclear and cytoplasmic maturation after oocyte exposure to cold stress for 10 or 30 min, respectively. No differences ($P > 0.05$) in either experiment in the metaphase II (MII) rate of oocytes exposed to MβCD and cold stress; but these oocytes presented lower maturation rates than control groups. In the second experiment, a lower percentage of oocytes showed degenerated chromatin ($P < 0.05$) after exposure to 2 mg/mL of MβCD compared to the group exposed to 0 mg/mL. However, no differences among treatments were observed in cytoplasmic maturation. Groups exposed to cold stress demonstrated a lower ($P < 0.05$) capacity for embryonic development compared to the control groups. In the third experiment immature oocytes were exposed to MβCD and then, vitrified (cryotop). After warming, we observed that the ability to reach MII and chromatin degeneration were altered ($P < 0.05$) by MβCD. The blastocysts rate ($P < 0.05$) on D7 was higher in the 2 mg/mL MβCD group, but an identical finding was not observed on D8 ($P > 0.05$). Chromatin degeneration was higher in the vitrification groups. We conclude that MβCD improved nuclear maturation by reducing oocyte degeneration after cold stress or vitrification; however, more studies are required to clarify the usefulness of MβCD use in oocyte cryopreservation.

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Introduction

The ability to preserve the female gamete is becoming an integral part of assisted reproductive techniques (ARTs) as it increases the availability of oocytes for use in such techniques. Successful cryopreservation of the oocyte would allow for the preservation of genetic resources of farm and wild animals as well as the preservation of gametes of women with premature loss of ovarian function. However, because of their large size and marked sensitivity to cooling, the cryopreservation of oocytes is very difficult in most mammals.

Up to now, the standard method used to cryopreserve mammalian oocytes has been slow freezing, which includes slow cooling rates and low concentrations of cryoprotectants agents. Vitrification, which uses rapid cooling rates and a very high concentration of cryoprotectants to prevent the formation of ice crystals, usually replaces slow freezing. This method has been utilized in several species of domestic animals, such as sheep [7], horses [34], cats [16] and cattle [21,33]. However, the overall success of the oocytes in developing to the blastocyst stage is still very low.

Multiple attempts have been made for improving the efficiency of oocyte vitrification by maximizing the cooling rate and minimizing the cryoprotectant concentration. One approach for achieving a rapid cooling rate is to reduce the volume of the vitrification solution. In this regard, various methods have been proposed, initially MDS was developed by Arav in 1992 [28], and then many other devices were developed such as Open Pulled Straw (OPS) [35], cryoloop [13], hemi-straw [37] and cryotop [12]. Among these methods, cryotop uses a very small amount of vitrification solution and is reportedly more practical and efficient for cryopreserving bovine oocytes [21,22]. Even with the advantages of the cryotop method compared to others, the results obtained with vitrification of bovine oocytes remain unsatisfactory [5,19,21,22,42].

The cell damage that occurs during cryopreservation is caused by several factors, such as osmotic stress, toxicity of the

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cryoprotectants used, formation of ice crystals with consequent damage to cellular organelles [29] and direct chilling injury (DCI). Chilling injuries trigger the destabilization of cell membranes during the thermotropic phase transition from the fluid phase to the gel phase during the cooling process, which is considered one of the major obstacles to the success of cryopreservation of oocytes [3,27].

Irreversible damage to membrane integrity caused by chilling during the lipid phase transition is directly related to the quantity of lipids present [3]. Cholesterol is a major structural lipid constituent of the membrane and regulates its function. Therefore, the cholesterol/phospholipid ratio is a vital determinant of plasma membrane fluidity and stability during cryopreservation [10]. Membranes with high concentrations of cholesterol are more fluid at low temperatures and consequently more resistant to damage during cooling [40,41]. To increase membrane fluidity and permeability at low temperatures, cholesterol can be added to the plasma membrane, thereby providing an alternative method for increasing oocyte tolerance for cryopreservation.

Cyclodextrins can act as carrier molecules for the incorporation of cholesterol into plasma membranes [1,10,25]. Cyclodextrins are water-soluble cyclic oligosaccharides consisting of glucose units (α-D-glucopyranoside) joined by connections typeα-1,4 that contain a hydrophobic center capable of integrating lipids. Due to its structure, free cyclodextrin can selectively deplete cholesterol from isolated or intact membranes from a variety of cells, including spermatozoa and oocytes [23], whereas cyclodextrins preloaded with cholesterol deliver cholesterol to the plasma membrane. Therefore, this simple approach can be used prior to cryopreservation to change the membrane composition and minimize membrane damage.

Methyl-β-cyclodextrin (MβCD) is the most potent cyclodextrin family member with respect to its affinity for cholesterol binding. Moreover, it was showed that cholesterol improve bovine [1,25] and equine [20] sperm viability after cryopreservation [23].

One study demonstrated that cholesterol carried by cyclodextrin entered cumulus cells and oocytes, which improved the survival of vitrified mature bovine oocytes [10]. No further studies have investigated this simple approach to reduce oocyte cold sensitivity.

In the present study, we used MβCD to load cholesterol from fetal calf serum (FCS) and deliver it to the oocyte plasma membrane. The purpose of this study was to investigate the effect of MβCD exposure on the in vitro maturation rates and developmental ability of cold-stressed as well as vitrified immature bovine oocytes.

Materials and methods

Chemicals and supplies

Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO, USA). Cryotop devices were purchased from Ingámed (Maringá, PR, Brazil).

Oocyte recovery

Ovaries from crossbred cows (*Bos indicus* × *Bos taurus*) were collected immediately after slaughter and transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 g/mL) at 35 °C. Cumulus oocyte complexes (COCs) were aspirated from 3- to 8-mm diameter follicles with an 18-gauge needle and pooled in a 15-mL conical tube. After 10 min, COCs were recovered and selected in holding medium consisted by HEPES-buffered TCM-199 (GIBCO® BRL) supplemented with 10% FCS. Only COCs with homogenous cytoplasm and at least three layers of cumulus cells were used in the experiments.

Cholesterol-loaded methyl-β-cyclodextrin (MβCD) preparation

In a glass tube, a stock solution (SS) with 1 g of methyl-β-cyclodextrin was dissolved in 2 mL of methanol and stored at −20 °C [10]. To load cholesterol from FCS, the SS was diluted with different concentrations (1, 2 or 3 mg) of MβCD in 1 mL of HEPES-buffered TCM-199 (GIBCO® BRL) supplemented with 20% FCS. The solution was incubated overnight at 38.5 °C.

Vitrification and warming

Oocyte vitrification was performed as previously described [12] with slight modifications. The holding medium (HM), which was used to handle oocytes during vitrification and warming, was composed of HEPES-buffered TCM-199 (GIBCO® BRL) supplemented with 20% FCS. For vitrification, groups were first washed three times in an equilibrium solution composed of 7.5% ethylene glycol and 7.5% dimethylsulfoxide (Me_2SO) dissolved in HM for a total of 9 min. Oocytes were transferred to a vitrification solution of 15% ethylene glycol, 15% Me_2SO and 0.5 M of sucrose in HM where they were incubated for 45–60 s. Next, the oocytes were placed into the cryotop device in sets of 3–5 under a stereomicroscope. Before vitrification, most of the solution that was transferred with the oocytes was removed from the device, and only a thin layer (<0.1 μl) remained to cover the oocytes. Subsequently, the cryotop device was immediately submerged into liquid nitrogen. Warming was performed immediately after vitrification by immersing the cryotop end into a drop of HM supplemented with 1 M of sucrose for 1 min pre-warmed at 37 °C. The oocytes were transferred to HM medium supplemented with 0.5 M of sucrose for 3 min, respectively, and finally to the original holding medium. Afterwards, the oocytes were placed in the culture dishes to mature or were fixed for maturational stage evaluation.

Oocyte maturation and assessment of meiotic progression

After warming, COCs were washed and transferred (groups of 25–30) to a 200 μL drop of maturation medium under silicone oil and incubated for 22 h at 39 °C in 5% CO_2 in air. The maturation medium was TCM-199 supplemented with 10% FCS (v/v), 10 mg/mL of FSH and antibiotics (100 IU/mL of penicillin and 50 mg/mL of streptomycin). CCOs were distributed into 4 groups, each group represented one maturation period. The first one was fixed immediately after selection, before IVM; the second group was fixed with 8 h of IVM; the third was fixed 22 h of IVM and the fourth group completed IVM period and was fixed with 24 h of IVM.

For meiotic progression evaluation, oocytes were denuded and fixed for at least 48 h with acetic alcohol (1:3). On the day of the evaluation, these oocytes were placed on a slide, covered with a coverslip and were stained with 1% lacmoid in 45% glacial acetic acid. The maturational stage of each oocyte was determined using phase contrast microscopy. Oocytes were classified as follows: immature – did not reach metaphase II; mature – showed metaphase II plate; abnormal – any chromosomal aberrations (diploid, abnormal metaphase II, multidirectional spindle, chromosomal dispersion); degenerate – diffuse or undefined chromatin.

In vitro fertilization (IVF) and embryo culture (IVC)

Following maturation, COCs (groups of 25–30) were transferred to a 200-μL drop of fertilization medium. For fertilization, frozen semen from a Nelore bull previously tested in the lab for IVF was used. Motile spermatozoa were obtained by the Percoll method

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