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Bovine oocyte membrane permeability and cryosurvival: Effects of different cryoprotectants and calcium in the vitrification media

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ABSTRACT

The cryopreservation process must be improved to enhance oocyte cryosurvival and functionality. Two protocols with different cryoprotectants (CPAs), containing either ethylene glycol (EG), dimethyl sulfoxide (DMSO) and sucrose (EGDMSO) or 1,2-propanediol and sucrose (PrOH) were evaluated. In both protocols, calcium (Ca^{2+}) free or -containing base media were tested. Oocytes were subjected to vitrification or only exposed to CPAs without immersion in liquid nitrogen. Oocyte's viability, cortical granules location and competence for development after fertilization were assessed. Finally, fatty acid composition and membrane permeability of oocytes exposed to CPAs were analyzed. Independently of Ca^{2+} concentration in the vitrification media, the development rates were higher in oocytes vitrified with EGDMSO protocols ($p = 0.0005$). After warming, higher cleavage rates were obtained in EGDMSO + Ca^{2+} compared to the PrOH without Ca^{2+} protocol ($p = 0.02$). Oocytes exposed to PrOH without Ca^{2+} presented lower cleavage rates compared to control ($p = 0.04$). An enhanced premature zona hardening in vitrified oocytes as well as lower concentrations of the fatty acids c11:18:1 and 20:4n-6 in cumulus oocyte complexes exposed to PrOH protocols were identified. The oocytes minimum volume and permeability were affected by the exposure to PrOH and Ca^{2+} ($p \leq 0.007$). In conclusion, the most effective protocol for bovine oocytes cryopreservation combines EG and DMSO, independently of Ca^{2+} concentration in the media. A higher toxicity and an incomplete depletion of water during PrOH loading may hamper oocyte viability. The type of CPAs and Ca^{2+} interfered differentially on oocyte pathways to functionality, and this should be considered when choosing a cryopreservation protocol.

1. Introduction

Cryopreservation of the female gamete can cause damage, such as oocyte cytoskeleton disorganization, spindle and chromosome abnormalities, alteration in the distribution of cortical granules, high rates of polyspermy and altered gene expression [39]. To minimize cryodamage, vitrification appears to be the best alternative for preserving oocytes [2]. Vitrification is a multistep method that involves exposing oocytes to increasing concentrations of permeable cryoprotectants (CPAs), often associated with other non-permeable CPAs to prevent intracellular and extracellular ice crystallization, before subjecting them to ultrarapid cooling in liquid nitrogen. Oocyte warming, conversely, subjects the gamete to decreasing concentrations of non-permeable CPAs to control hypoosmotic shock during rehydration [2,29]. Factors that affect oocyte viability and competence after cryopreservation such as the presence of different CPAs and/or absence of calcium (Ca^{2+}) in the vitrification media were reported [7,15,39]. Nonetheless, the extent of the oocyte cryoinjuries is highly variable, depending not only on CPA concentrations, chemical nature and permeability but also on the sample volume and cooling rate or even on the donor species or the oocyte lipid content [2,21,29]. Statements from many authors highlight the essential need for technical modifications of current oocyte vitrification protocols. Mature oocytes are broadly accepted as easier to cryopreserve, since immature oocytes are less

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permeable to CPAs [5,6]. Cytoplasmic insufficiencies underlie compromised fertilization and low developmental competence of vitrified oocytes [10]. This is incompatible with the crucial role of mammalian ooplasm to support fertilization and early embryonic development [35]. Cryoprotectants commonly used in vitrification protocols, such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG), can cause a premature increase in intracellular Ca^{2+} in mature oocytes from mouse [19] and ovine [38]. Following the fusion of the sperm with oocyte membranes, the sperm triggers a prolonged series of low-frequency oscillations in oocyte cytoplasmic Ca^{2+} concentration through the release of sperm factor phospholipase C zeta and consequently of diacylglycerol and inositol 1,4,5-trisphosphate (IP3), which activates the release of Ca^{2+} from the endoplasmic reticulum [42]. This cascade of events seems to be impaired in vitrified/warmed oocytes. It has been reported that after exposure to CPAs, an increase of intracellular Ca^{2+} reduced the male pronucleus formation in ovine [40] and pig oocytes [37]. This premature rise of Ca^{2+} may also induce the parthenogenic activation of oocytes and cortical granule exocytosis causing zona pellucida hardening [38]. Moreover, removal of Ca^{2+} from the vitrification medium improved the embryo development to the two-cell stage in murine oocytes [19]. Ca^{2+}-free or -reduced media were also effective for improving the outcomes of oocyte vitrification protocols in rat and sheep [7,38]. Interestingly, it was also evidenced that the type of CPA may also influence the source of Ca^{2+} rise as well as the magnitude and duration of this intracellular transient [20]. Additionally, the type of CPA may have impact on the oocyte plasma membrane permeability and osmotic swelling. Moreover, different CPAs imply different level of toxicity and protection to cryoinjuries, affecting the survival of oocytes after vitrification [4,11,39].

This prompted us to investigate the effect of Ca^{2+} in the vitrification solutions using different cocktails and concentrations of CPAs, such as EG plus DMSO and sucrose (EGDMSO), or 1,2-propanediol and sucrose (PrOH), on the developmental competence of vitrified/warmed bovine oocytes. In this study we performed CPAs toxicity experiments to better understand how the two main steps of the vitrification process, CPAs exposure/removal or chilling, might affect the oocyte survival. In addition, the permeability and composition of oocyte plasma membranes as well as cortical granules exocytosis and consequent zona pellucida hardening were also determined.

2. Methods

All chemicals used were purchased from Sigma Aldrich Chemical Co. (St. Louis, USA) unless specified otherwise.

2.1. Oocyte collection and in vitro maturation

Oocyte collection and maturation was performed as previously described [18]. Briefly, cross-bred 6–12 months old heifer oocytes aspirated from slaughterhouse ovaries with at least three layers of compact cumulus cells and an evenly granulated cytoplasm were selected for maturation. Cumulus oocyte complexes (COC) were matured in TCM 199 with 10% serum, 10 μgmL^{-1} FSH, 100 μM glutathione and antibiotics, in an incubator at 38.8 °C with humidified air and 5% CO2 during 22 h.

2.2. Oocyte cryopreservation

Mature COC were gently pipetted to remove the excessive cumulus cells. Then two distinct protocols for oocyte vitrification were tested. The first was currently used in our Lab adapted from Matos et al. [21]. For vitrification, mature oocytes with at least one layer of cumulus cells were immersed for 5 min in holding medium [HM: synthetic oviductal fluid (SOF) medium [27] with or without Ca^{2+} plus 20% fetal calf serum (FCS)], supplemented with 7.5% EG and 7.5% DMSO (1.34M and 1.06M, respectively). Oocytes were then transferred to the vitrification solution (HM plus 15% EG, 15% DMSO and 0.5 M sucrose). After 15s, COC were loaded in a cryotop. In total, oocytes were exposed to vitrification solution for 30–45 s prior to immersion in liquid nitrogen. For warming, the oocytes were immersed during 1 min in a warming solution (0.5 M sucrose in HM) at 38.5 °C and transferred to a serial of diluted solutions (0.25, 0.1 and 0 M sucrose in HM) at a 5 min interval. Oocytes were then maintained in HM.

The second protocol was based on Criado et al. [3] using a low concentration of CPA and always performed with Ca^{2+} free or –containing base medium. For vitrification, the mature oocytes were washed in Ca^{2+} free or -containing HM, then transferred to a 2.0 M PrOH in Ca^{2+} free or -containing HM for 10 min and finally to the vitrification solution (2.0 M PrOH and 0.5 M sucrose). COC were loaded into a cryotop prior to immersion in liquid nitrogen (LN_2). This last step should be performed in 60 s. For warming, ultravitrified oocytes were immersed in a 2.0M PrOH and 0.5M sucrose solution at 38.5 °C for 1 min. Subsequently, they were transferred to a 1.0 M PrOH and 0.5 M sucrose in HM solution for 7 min and then to a 0.3 M sucrose in HM solution for 9 min. Finally, COC were transferred to HM for 2 min.

In both protocols, all media were kept at room temperature (*25* °C) prior to vitrification and at 38.5 °C for warming. Solutions without Ca^{2+} were obtained by using Ca^{2+}-free base medium solutions. After warming, only intact oocytes showing no signs of degeneration and with at least one layer of cumulus cells were considered viable (experiment 1: viable n = 346 and non-viable n = 89) and hence selected for in vitro fertilization (IVF). In experiment 2, mature oocytes were exposed to CPAs in Ca^{2+} free or -containing solutions according to the same process as above but without loading the oocytes in cryotops and subsequent immersion in LN_2.

2.3. Oocyte fertilization and embryo culture

Oocyte fertilization was performed with frozen–thawed semen from the same Holstein bull following swim-up procedures [27]. In vitro fertilization medium consisted of modified Tyrode's medium supplemented with 5.4 USP mL^{-1} heparin, 10 mM penicillamine, 20 mM hypotaurine and 0.25 mM epinephrine. Sperm concentration was adjusted to 2×10^6 spermatozoa mL^{-1}. Sperm and oocytes were co-incubated for 22 h (IVF = day 0). Then, presumptive zygotes were placed into droplets (25 μL, 25 each) of SOF supplemented with BME and MEM amino acids, glutamine, glutathione and BSA, layered with mineral oil, and cultured at 38.8 °C in a humidified atmosphere with 5% O2, 5% CO2 and 90% N2. After assessing cleavage at 48 h, embryo development proceeded in SOF plus 10% FCS. Cleavage (2–4 cell embryos) and day 7/8 embryo (morula and blastocysts, being most embryos at the blastocyst stage) development rates were calculated as a proportion of cultured oocytes.

2.4. Cortical granules labeling

Vitrified-warmed or CPA exposed oocytes were washed several times in PBS without calcium chloride ($ClCa^{2+}$) to eliminate cumulus cells and fixed with freshly prepared 4% (v/v) paraformaldehyde for 1 h at room temperature. Fixed oocytes were then washed twice in a solution of PBS-PVP (0.1 M PBS, pH 7.4, with 0.3% (w/v) PVP) and permeated with PBSS (0.1 M PBS, pH 7.4, with 0.1% saponin) for 10 min. Samples were finally incubated at room temperature in the dark with 5 μgmL^{-1} fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) in PBS for 30 min [41]. After staining, oocytes were washed and 5 to 7 oocytes transferred to 2 μL mowiol in a clean slide. Samples were kept in the dark at 4 °C until assessment within 48 h. Oocytes were then observed under a fluorescence microscope (Olympus BX40, BP 470–490 filter, UPlanFI 20 × /0.50 objective lens). After labeling, and according to the relocalization of the cortical granules, oocytes were classified into three categories A) peripheral cortical granules location (line), where they were all visible near the oolemma