Cryobiology 73 (2016) 324–328

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

The use of antifreeze protein type III for vitrification of *in vitro* matured bovine oocytes

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ARTICLE INFO

Article history:
Received 11 May 2016
Received in revised form
30 August 2016
Accepted 7 October 2016
Available online 8 October 2016

Keywords:
Oocyte
Cryopreservation
AFP III
Meiotic spindle
Embryo production

ABSTRACT

The aim of this study was to evaluate the use of antifreeze protein type III (AFP III) into vitrification medium on meiotic spindle morphology of *in vitro* matured bovine oocytes as well as the fertilization and blastocyst rates. Mature *cumulus*-oocyte complexes (COC) were distributed in four groups: control (untreated), vitrified without supplementation (AFP_0) or supplemented with 500 (AFP_{500}) or 1000 ng/mL (AFP_{1000}) into vitrification solutions. Samples from each group were used to analyze the organization of meiotic spindle by confocal microscopy and the remaining COC were submitted to *in vitro* fertilization and culture for eight days. Control group exhibited only 15% of abnormal spindle. However, the spindle morphology was affected in all vitrified groups regardless to AFP concentration: 75.8%, 76.1% and 69.2% ($P > 0.05$) for AFP_0, AFP_{500} and AFP_{1000}, respectively. Similar cleavage rate was obtained among the vitrified groups ($AFP_0 = 17.9\%$, $AFP_{500} = 16.9\%$ and $AFP_{1000} = 17.8\%$), but lower ($P < 0.05$) compared with control group (68.7%). At Day 5 of culture, embryo production rate of AFP_{500} (30.8%) and AFP_{1000} (25.0%) were similar to control group (49.4%). However, at Day 8 of culture, AFP_0, AFP_{500} and AFP_{1000} groups exhibited lower ($P < 0.05$) blastocyst rates (10.0%, 3.8% and 9.4%, respectively) when compared to control (41.1%). In conclusion, AFP III did not preserve meiotic spindle organization against the cryoinjuries. However, the use of AFP III improved embryo development at Day 5 of culture, although this effect was not maintained up to the blastocyst formation.

1. Introduction

Cryopreservation is an important tool for assisted reproductive biotechnologies since it has several potential applications, such as biodiversity maintenance and development of breeding programs. However, the cryopreservation of oocytes is still a challenging task because of sensitive nature of these cells to chilling [1,2] and thus it remains inefficient for bovine oocytes [8].

Several studies have been developed in attempt to improve the efficiency of oocyte cryopreservation, such as applying the use of different cryoprotectants [32], cytoskeleton stabilizer [23] and cellular lipid metabolism modifiers [4]. In this perspective, the search for new alternatives to improve oocyte cryopreservation remains a central area of investigation.

In recent decades, studies identified a class of polypeptides important to the survival of fishes in frozen Arctic waters. These proteins were denominated as antifreeze proteins (AFP) and classified as: i) AFP type I, that comprises 60% alanine with helical structures with sizes from 3.3 to 4.3 kDa; ii) AFP type II, which is rich in cysteine with 14 kDa of size; iii) AFP type III (AFP III), that contains 66 amino acids with size of 7 kDa and rarely alanine or cysteine residues; and iv) glycosylated protein or antifreeze glycoprotein that consists of the repetition of alanine-alanine-threonine peptides in each disaccharide threonine and shows molecular weight ranging from 2.6 to 33.7 kDa [6,20,33].

AFP acts in solutions decreasing kinetically the temperature of formation of ice by thermal hysteresis activity (disparity between freezing and melting temperature), which reduce the freezing point without affecting melting point and osmotic pressure of a solution

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http://dx.doi.org/10.1016/j.cryobiol.2016.10.003
0011-2240/

[15,19,27]. Moreover, AFP retard recrystallization and depress the freezing temperature below the melting point [29]. The AFP mechanism at the molecular level is still an active research area, however some theories suggest that AFP act by adsorption to ice surface, and therefore restrict ice growth regions between molecules where the protein is adsorbed. For this feature, the growth of crystal ice is curved [5]. Overall, this process protects cell membranes against cold-induced injury. Furthermore, the protection abilities of AFP identify them as potential natural ice modulators in the conservation of frozen products [29]. In this perspective, recent studies have shown that supplementation with AFP can be used in the cryopreservation medium of sperm [25], oocytes [12] and embryos [20].

AFP III supplementation (500 ng/mL) into vitrification media exerted positive effects during vitrification of mature mouse oocytes [12]. In this study, the AFP III improved cryosurvival, fertilization, and subsequent embryonic development, and, suggested a cryoprotective effect by stabling meiotic spindle and preserving membrane integrity and intracellular ATP contents.

We hypothesized that AFP III could also improve the efficiency of bovine oocytes vitrification. Therefore, the aim of this study was to evaluate the use of AFP III into vitrification medium on meiotic spindle integrity of mature bovine *cumulus*-oocyte complexes (COC) and their further development after *in vitro* fertilization and embryo culture.

2. Material and methods

Except otherwise indicated, chemicals were purchased from Sigma Chemical Co. (Saint Louis, MO, USA) and plastics from Nunc (Nalgen Nunc International, Roskilde, Denmark).

2.1. Oocyte collection and in vitro maturation (IVM)

Ovaries obtained from the local slaughterhouse were transported to the laboratory in 0.9% (w/v) NaCl solution containing antibiotics (Pentabiótico, Fort Dodge, Campinas, Brazil). Ovaries were washed in pre-warmed fresh saline and oocytes were aspirated from all visible follicles between 2 and 8 mm in diameter with an 18-gauge needle attached to a disposable syringe.

COC were selected under a stereomicroscope (SMZ 800, Nikon, Tokyo, Japan) and only oocytes enclosed in a compact *cumulus* with an evenly granulated cytoplasm were selected for experiments. COC were rinsed in medium consisting of HEPES-buffered TCM199, 10% fetal bovine serum (FBS), 0.2 mM of sodium pyruvate and 1% of antibiotic and antimycotic solution.

For IVM, COC were washed twice and distributed into groups of 45–50 in four-well dishes containing 500 μL of maturation medium which consisted in TCM199 supplemented with 10 ng/mL Epidermal Growth Factor, 1 μg/mL estradiol, 20 μg/mL FSH/LH (Pluset, Calier, Barcelona, Spain), 200 mM L-glutamine, 100 mM cysteamine, 5% FBS, 0.2 mM of sodium pyruvate and 1% of antibiotic and antimycotic solution. COC were incubated for 22 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Vitrification and warming

AFP III was acquired from A/F Protein Inc. (Waltham, MA, USA). Thus, after IVM, COC in four replicates were randomly allocated in each group: control (untreated), vitrified without supplementation (AFP_0) and supplemented with 500 ng/mL (AFP_{500}) or 1000 ng/mL (AFP_{1000}) AFP III into vitrification solution 1 (VS1) and 2 (VS2). The concentrations of AFP were determined by studies [12,13] and previous experiments performed in our laboratory. Open pulled straw (OPS) method was used as previously described by Vajta et al. [28] with few modifications and all procedures were carried out at room temperature (20–25 °C).

The base medium for preparation of all cryoprotectant solutions was holding medium (HM), which consisted in HEPES-buffered TCM199 supplemented with 20% (v/v) FBS. COC were first washed in HM for 3 min and processed in VS1 (7.5% DMSO and 7.5% ethylene glycol) in HM for 15 min approximately. Then, COC were transferred into two successive drops of VS2 (16% DMSO, 16% ethylene glycol and 1 M sucrose) in HM for 20 s each. Finally, the COC were loaded in OPS (Minitub, Tiefenbach, Germany) and plunged into liquid nitrogen.

For warming, the tip of the straw was plunged in warming solution 1, which consisted in HM plus 1 M sucrose pre-warmed at 37 °C, incubated for 1 min, transferred into warming solution 2, HM containing 0.5 M sucrose, incubated for 3 min and washed twice in HM for 5 min. Therefore, the COC were kept in HM for approximately 2 h before being used for further experiments. Samples of COC vitrified/warmed from each group were used to analyze the organization of meiotic spindle by confocal microscopy. The remaining COC were submitted to *in vitro* fertilization and culture for eight days.

2.3. Evaluation of meiotic spindle

For immunostaining, COC were kept in HM after warming and thereafter, denuded, fixed and permeabilized in 4% (w/v) paraformaldehyde, 0.5% (v/v) Triton X-100 in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) for 20 min at room temperature. Samples were washed three times in PBS-BSA, and transferred into blocking solution (PBS-BSA supplemented with 5% of goat pre immune inactivated serum) and kept in this solution for 2 h at room temperature. Coincubation with primary antibodies in PBS supplemented with 1% of BSA, monoclonal anti α-tubulin (1:1000) were carried out for 1 h at 37 °C, and 2 h at room temperature, respectively. Oocytes were washed three times in PBS-BSA for 30 min and incubated with secondary antibody, Daylight 594 (Thermo Fisher Scientific, Waltham, MA USA) (1:500) in PBS-BSA for 2 h in a dark at room temperature. Then, oocytes were washed three times in PBS-BSA for 30 min and incubated with Hoechst 33,342 (1/1000) for 10 min in dark at room temperature, washed one time in PBS-BSA for 10 min and mounted on microscope slide and covered with a coverslip. The slides were stored at 4 °C and protected from light until observation. The laser-scanning C2 Nikon confocal microscope (Nikon, Tokyo, Japan) was used to examine tubulin (TRITC; excitation 488 nm) and chromatin (DAPI; excitation 405 nm).

Spindle morphology and chromosomes were classified, as previously described [3], into two categories: normal (barrel-shaped with chromosomes clustered as a discrete bundle at the metaphase plate and microtubules crossing the length of the spindle from pole to pole) or abnormal (microtubules not organized as typical spindles or some microtubules disassembled with chromosomes showing some degree of disarrangement or displacement from the metaphase plate).

2.4. In vitro fertilization (IVF)

Vitrified/warmed and untreated COC were washed two times in IVF-TALP medium [28], and then transferred in groups of 50 into four-well dishes containing 450 μL of IVF-TALP per well supplemented with 30 μg/mL heparin (Calbiochem, Merck, Darmstadt, Germany), 30 μg/mL penicillinamine, 15 μM hypotaurine, 1 μM epinephrine.

Two straws of semen from the same bath were used per replicate throughout experiments. Motile spermatozoa were obtained

by centrifugation of frozen-thawed semen on a discontinuous Percoll density gradient (55%/90%) at 700 *g* for 25 min at room temperature. The pellet was washed twice in 2 mL Sperm-TLP [24], with few modifications, and centrifuged at 500 and 400 *g* for 10 min each one. After the final wash, the pellet was resuspended in IVF-TALP. Fifty microliters of the sperm suspension were finally added to the 450 μL of IVF-TALP, producing a final sperm concentration of 2×10^6 cells/mL. Spermatozoa and COC were coincubated for 18 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.5. In vitro culture (IVC)

Presumptive zygotes were vortexed and washed three times in HEPES-buffered TCM199, 10% FBS, 0.2 mM of sodium pyruvate and 1% of antibiotic and antimycotic solution, to remove *cumulus* cells and attached spermatozoa. Zygotes were washed two times in SOF medium [10] supplemented with BME amino acids (50×), MEM amino acids (100×), 5% FBS and transferred by groups of 50 into four-well petri dishes containing 500 μL drops of culture medium covered with 300 μL of mineral oil. The presumptive zygotes were cultured for eight days at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. Embryo development was recorded on Day 2 (cleavage), 5 (morula), 7 and 8 (blastocysts) post-IVF.

2.6. Statistical analysis

Data were statistically analyzed using the GraphPad Prism 5.0a software. The chi-square test was used to compare data among the experimental groups. Rates of days 5 (morula), 7 and 8 (blastocysts) were calculated from the total of embryos cleaved. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Meiotic spindle

Fig. 1 shows representative images of normal and abnormal meiotic spindle structures analyzed for each group. The spindle integrity was significantly affected in the vitrified groups with or without AFP III supplementation, when compared to the control group ($P < 0.05$). The observed abnormalities were disorganization of microtubules (as non-typical spindles) or unidentifiable spindle (as aberration of chromatin arrangement, clumping or dispersal from the spindle center).

In control group, it was observed only 15% (6/41) of COC with abnormal spindle. This occurrence was significantly different when compared to the others experimental groups ($P < 0.05$). Nonetheless, no significant differences ($P > 0.05$) were observed among the vitrified groups, which exhibit abnormalities in 75.8% (25/33), 76.1% (35/46) and 69.2% (27/39) of oocytes treated with AFP_0, AFP_{500} and AFP_{1000}, respectively (Fig. 2).

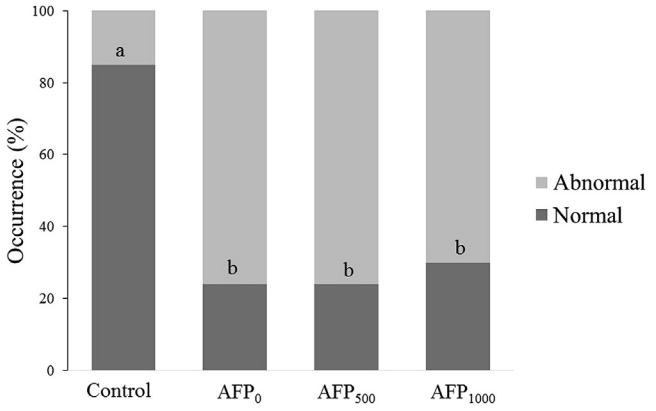

Fig. 2. Proportion of normal and abnormal meiotic spindle organization from vitrified-warmed matured COC vitrified with or without AFP III supplementation in vitrification media. a,b: $P < 0.05$.

3.2. Embryo development

The effect of AFP III supplementation on embryo development from *in vitro* matured COC are presented in Table 1. In control group, the cleavage rate was higher ($P < 0.05$) when compared to the vitrified groups. However, similar cleavage rate was obtained among the vitrified groups ($P > 0.05$). At Day 5, the embryo production in AFP_{500} (30.8%) and AFP_{1000} (25.0%) groups showed no difference ($P < 0.05$) when compared to control group (49.4%). However, the AFP_0, AFP_{500} and AFP_{1000} groups exhibited lower blastocyst rates at Day 8 (10.0%, 3.8% and 9.4%, respectively) when compared to control (41.1%; $P < 0.05$).

4. Discussion

The main objective of the current study was to assess the effects of supplementation with AFP III in vitrification media during vitrification of *in vitro* matured bovine COCs. This study was the first one to use the AFP III in vitrification media of bovine oocytes. Recent studies concerning vitrification of mouse oocytes showed

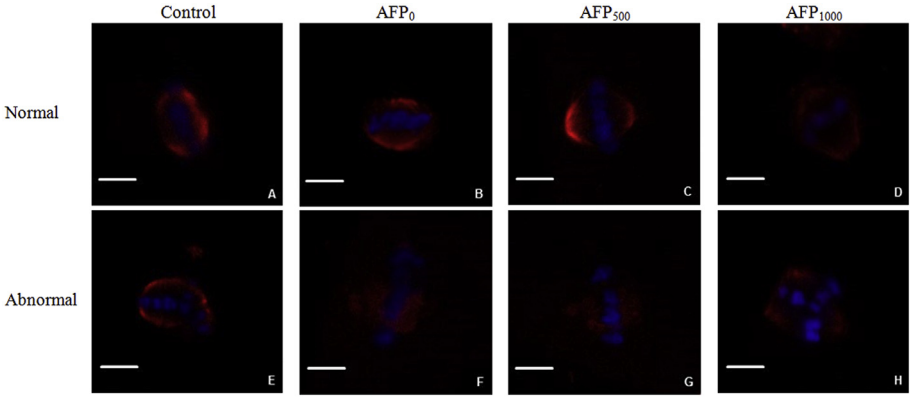

Fig. 1. Representative images of and meiotic spindle organization from *in vitro* matured bovine oocytes vitrified with different concentrations of AFP III. Tubulin (red), chromosomes (blue). Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Development of vitrified-warmed matured COC with antifreeze protein type III supplementation in vitrification media.

Groups	N	Day 2	Day 5	Day 7	Day 8	Hatched/total
		Cleaved (%)	Morula (%)	Blastocysts (%)		blastocysts (%)
Control	262	180 (68.7)[a]	89 (49.4)[a]	60 (33.3)[a]	74 (41.1)[a]	31 (17.2)[a]
AFP_0	223	40 (17.9)[b]	6 (15.0)[b]	2 (5.0)[b]	4 (10.0)[b]	0 (0)[b]
AFP_{500}	154	26 (16.9)[b]	8 (30.8)[ab]	0 (0.0)[b]	1 (3.8)[b]	0 (0)[b]
AFP_{1000}	180	32 (17.8)[b]	8 (25.0)[ab]	2 (6.3)[b]	3 (9.4)[b]	0 (0)[b]

* Rates of Days 5, 7 and 8 were calculated from the total of embryos cleaved; Values with different letters in superscripts within same column are significantly different ($P < 0.05$).

that the concentration of 500 ng/mL of AFP III improved the blastocyst rates in mature [12] and immature [13] oocytes. Therefore, the concentration of 500 ng/mL was tested to verify if it was possible to get similar results and a higher concentration (1000 ng/mL) trying to optimize AFP III cryoprotection effect.

In concentrations of 500 and 1000 ng/mL, the addition of AFP III in vitrification media did not improve cleavage rates and embryo development after vitrification-warming. However, at Day 5, the rate of development in control group was similar to morulas from AFP III supplemented groups, suggesting some positive effect provided by the addition of this protein in the vitrification media.

The embryo development was analyzed to evaluate if this protein confers positive effect on vitrification of bovine oocytes as previous demonstrated in mouse oocytes [12,13,31]. However, the cryopreservation of an oocyte is a challenging task because of its sensitive nature to chilling due to several factors, such as size, shape, permeability, species of origin, lipid content, that affect its survival and developmental competence after cryopreservation [2]. In contrast to that observed in our study, the addition of AFP III in the vitrification of immature mouse oocytes at a concentration of 500 ng/mL improved cleavage rate (91.5% vs. 69.8% with and without AFP, respectively) and blastocyst development (56.1% vs. 33.6% with and without AFP, respectively) [13]. Similarly, Jo et al. [12] observed that in same concentration, the supplementation of AFP III in vitrification of mature mouse oocytes improved the survival rate after warming (95% vs. 85% with and without AFP, respectively). Furthermore, in the same study, the cleavage rate (94.6%) and the development of blastocysts (89.1%) were better than vitrified group without AFP III (84.5% for cleavage rate and 68.9% for blastocyst development).

AFP III supplementation was also evaluated in vitrification of mature mouse oocytes at concentration of 2.5 mg/mL [31]. In this study, the fertilization rate in the fresh control group was higher than the vitrification group and vitrification with addition of AFP III group. Meanwhile, the fertilization rate in the vitrification with addition of AFP III group (46.7%) was higher than that in the group vitrified without AFP III (31.7%), however, blastocyst formation rate of these groups showed no statistical difference.

In our study, we have chosen the OPS method because it is simple, less expensive, reduces the volume to be vitrified, narrowing the insulating layer between the cooling agent and the vitrification medium [28]. However, studies demonstrated that vitrification by OPS of mature bovine oocytes resulted in very low blastocyst rates, as 0% [22], 1.6% [7] and 2.3% [23]. We hypothesized that AFP III could improve OPS rates, however our results showed similar embryo development in accordance with other authors.

In all previously mentioned studies, AFP III was used with Cryotop vitrification method. This method, developed by Kuwayama et al. [17], allows the cryopreservation of oocytes in a minimum of vitrification solution (~0.1 μL) resulting in an extremely high cooling rate in liquid nitrogen (>23,000 °C). Among the vitrification techniques commonly used, Cryotop have demonstrated superiority for vitrification of mature oocytes [21], including when compared to OPS method used in this study. It appears that Cryotop can better preserve the cellular structure and therefore the ability of these oocytes to fertilization. In recent study, the oocyte vitrification with Cryotop method resulted in 20.2% of blastocyst rate from bovine mature oocytes [4]. However, the application of this technique is more expensive than OPS.

Another problem was the influence of large amounts of cytoplasmic lipid commonly found in oocytes of farm animals. Most of the lipid droplets are in close proximity to the plasma membrane (primary site of cryoinjuries) and other organelles such as mitochondria and endoplasmic reticulum [14,16]. In contrary, mouse oocytes have less proportion of lipids content [9], and consequently can tolerate better the cold stress by vitrification. We hypothesized that this factor could have contributed to the absence of effect of AFP III over bovine embryo production after warming observed in this study.

The meiotic spindle plays an essential role in normal chromosome segregation, for normal fertilization and subsequent embryonic development, the chromosome alignment and meiotic spindle assembly are basic requirements [10,30]. Previous studies have shown that, high concentration of cryoprotectants required to achieve vitreous state may exert a highly detrimental effect on spindle configuration [11]. In our study were used EG and DMSO, cryoprotectants common in many vitrification protocols. According Ledda et al. [18] they are considered to be toxic due to their cell permeating nature and high concentrations needed to induce vitrification. Moreover, many cryoprotectants are known to induce changes in microtubule organization [26].

Jo et al. [12] found that AFP treatment could be beneficial to preserve spindle and chromosome stability during mouse oocyte vitrification. These authors suggest that AFP is able to protect oocyte membrane through decreasing freezing temperature of solution, indicating that AFP supplementation is beneficial to preserve membrane integrity during vitrification and warming of mouse oocytes. However, in bovine oocytes, our results indicated that there are no differences by the addiction of AFP III among the vitrified groups. The proportion of oocytes with abnormal spindle and chromosomal morphology in vitrification with AFP III group was higher than that in the vitrification without supplementation group. Jo et al. [12] also suggested the specific interactions between AFP and cellular membranes during vitrification should be better understood. Thus, we consider that AFP III did not improve the bovine oocyte development probably due to its inefficiency to protect the chromosomes and meiotic spindle during the cryopreservation treatment.

Jo et al. [13] found that the use of AFP III in vitrification of immature bovine oocytes significantly reduced morphological changes in chromosome structure, which helped to preserve the dynamics of the meiotic spindle/chromosomes after *in vitro* maturation. In the evaluation of related genes of meiotic spindle integrity, it was demonstrated that increasing in expression of *Mad2* and *Hoo1*, and reduction in expression of *Eg5* could have contributed to preservative effect of AFP III over spindle integrity

during vitrification. Wen et al. [31] suggested that AFP III may exert its cryoprotective effect via mechanisms that inhibit the extracellular ice crystals formation and recrystallization during thawing, thus stabilizing the spindle morphology.

In summary, AFP III did not preserve meiotic spindle organization against the cryoinjuries caused by cryopreservation method. Although the use of AFP III has improved embryo development at Day 5 of culture, this effect was not maintained up to the end of culture (Day 8). Finally, to improve the efficiency of this technique, further investigations need to be carried out with more AFP concentrations and different vitrification tools.

Acknowledgments

The authors would like to thank the Medical Mycology Center/Federal University of Ceará (Fortaleza, Brazil) for analysis in confocal microscopy platform and Mr. Maykon Costa (Molecular Biology Institute of Paraná, Brazil) for the kind donation of the AFP III. This study was financed by CAPES (Brasília, Brazil) according to the grant number 728/11.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.cryobiol.2016.10.003

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