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VITRIFICATION OF THAI NATIVE CATTLE OOCYTES: EFFECTS OF ETHYLENE GLYCOL CONCENTRATIONS AND EXPOSURE TIME, LINOLEIC ACID ALBUMIN AND CHOLESTEROL-LOADED METHYL-B-CYCLODEXTRIN

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Abstract

The present study aimed to improve the oocyte vitrification procedure for preservation of Thai native cattle genetic resources. In Experiment I, oocytes were exposed to various doses (2%, 4% and 6%) of ethylene glycol (EG) in vitrification solution I (VS-I) for different equilibration times (10 or 20 min) before being exposed to VS-II and then subjected to vitrification. Experiment II was divided into two parts: (a) oocytes were matured in medium supplemented with linoleic acid albumin (LAA) (1% or 2%) and then vitrified; (b) matured oocytes were preincubated with cholesterol-loaded methyl-β-cyclodextrin (CLC) (1% or 2%) and then vitrified. Equilibration of oocytes by exposure to 6% EG in VS-I for 10 min (Experiment I), and *in vitro* maturation of immature oocytes in medium supplementation with 2% LAA (Experiment II) were the most effective methods; vitrified/thawed oocytes showed higher rates of survival and subsequent embryonic development compared with the other experimental groups.

Keywords: cholesterol; cryoprotectant; linoleic acid albumin; oocyte cryopreservation; tropical cattle

INTRODUCTION

Tropical-breed cattle, including Thai native cattle (TNC) (*B. indicus*), have a high ability to adapt to a hot climate. Their genetic resources are necessary for breeding heat-tolerant cattle to improve beef cattle production as temperatures in the tropics continue to increase due to global warming (1). However, the genetic diversity of these native cattle worldwide has been rapidly declining over the last few decades, and some breeds are at risk of extinction (2). Oocyte cryopreservation, particularly vitrification (rapid freezing), has recently provided methods for genetic preservation of endangered animal species (3, 4). However, these techniques have been largely unsuccessful because of the low viability and rates of fertilization of frozen/thawed bovine oocytes, as well as the unsatisfactory developmental competence of blastocysts (5). Therefore, improved vitrification protocols are needed.

High concentrations of cryoprotectant agents (CPAs) are necessary for the vitrification method. Ethylene glycol (EG)

has been used as the base of the CPAs in the vitrification solution (VS) (6, 7, 8). However, it has been reported that EG at high concentrations has a toxic effect on bovine oocytes (9). Recently, equilibration of bovine oocytes in a low concentration of EG before exposure to a high concentration was shown to be an effective method of reducing toxic effects and osmotic stress during vitrification. However, there is a paucity of data on the most effective EG concentration and time of exposure for TNC oocyte cryopreservation.

In addition, supplementation of linoleic acid albumin (LAA) in the maturation medium or preincubation of matured oocytes with cholesterol-loaded methyl-β-cyclodextrin (CLC) is other candidate methods for increasing cryotolerance during vitrification (10, 11, 12). However, there have been only a few reports to compare the effectiveness of the two methods on the bovine oocyte cryopreservation (13). Furthermore, there is limited information available on cryopreservation of oocytes from TNC and other indigenous cattle in Southeast Asia.

The aim of this study was to develop a simple, efficacious vitrification procedure for freezing TNC oocytes. The present study was designed to evaluate the effects of (a) various EG concentrations in the equilibration solution and different times of exposure, and (b) supplementation of LAA in the maturation medium or preincubation of mature oocytes with CLC on the survival rate after vitrification and viability of frozen/thawed oocytes development after IVF.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tissue culture media 199 (TCM-199) (HEPES and bicarbonate) and fetal calf serum (FCS) were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Collection of Oocytes

TNC ovaries were obtained from a village abattoir located in the vicinity of Khon Kaen, Thailand, and transported to the laboratory in 0.9% normal saline solution at ambient temperature. The cumulus-oocyte complexes (COCs) were recovered by aspiration from 2 to 6 mm follicles using an 18-gauge needle connected to a 10 mL syringe containing modified Dulbecco's phosphate buffered saline (mDPBS) supplemented with 10% FCS. The ovarian follicular fluid was pooled in 15 mL conical tubes and allowed to settle to the bottom of the tubes over a 10-15 min period. COCs were selected under a stereomicroscope and washed five times with mDPBS supplemented with 10% FCS; those with more >3 layers or 2 layers of cumulus cells and a uniform cytoplasm (grades A and B) were subjected to *in vitro* maturation (IVM) (14).

In vitro maturation

COCs were matured using the procedure previous described by Ratto et al. (15), the COCs were washed three times with mDPBS supplemented with 10% FCS and twice with basic medium (BM; TCM-199 with 20% FCS). Groups of 20 COCs were cultured in 100 µL droplets of maturation medium (10% FCS, 10 µg/mL luteinizing hormone (LH), 1 µg/mL estradiol (E2), 0.5 µg/mL follicle stimulating hormone (FSH), 50 IU/mL penicillin G sodium and 50 mg/mL streptomycin in TCM-199). Droplets containing oocytes were covered with mineral oil; the Petri dishes were then placed in an incubator at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 24 h.

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

CLC was prepared as previously described by Purdy and Graham (16), 200 mg cholesterol (Sigma) was dissolved in 1 mL chloroform. In a separate glass tube, 1 g methyl-β-cyclodextrin was dissolved in 2 mL methanol. A 0.45 mL aliquot of the cholesterol solution was added to the cyclodextrin solution and stirred until the combined solution was clear, after which the

mixture was poured into a glass petri dish; solvents were then removed using a stream of nitrogen gas. The resulting crystals were allowed to dry for an additional 24 h, at which time they were removed from the dish and stored in a glass container at 22°C. A working solution of the CLC was prepared by adding 50 mg CLC to 1 mL TCM-199 with 1 mg/mL polyvinylpyrrolidone (PVP) at 38.5°C and mixing the solution briefly using a vortex mixer.

Vitrification and warming procedures

After 22 h of IVM, oocytes were partially denuded of cumulus cells by brief exposure to 0.1% hyaluronidase followed by gentle pipetting in TCM-199 supplemented with 1 mg/mL PVP. Those with approximately 2-4 layers of cumulus cells on the surface were subjected to vitrification. Oocyte vitrification was performed using 0.25 mL cryo-plastic straws (CPS) (Cryo Bio System, L'Aigle, France), as described by Prentice et al. (14).

In experiment I oocytes were exposed to 2%, 4% or 6% EG in TCM-199 with 20% FCS (vitrification solution I; VS-I); in experiment II, oocytes were exposed to the most effective VS-I obtained from experiment I at 38.5°C. In both experiments, after oocytes were exposed to VS-I they were transferred through three 20 µL droplets of VS-II (35% EG, 20% FCS and 1 M sucrose in TCM-199) and exposed at 38.5°C for <30 s. Groups of five oocytes were then loaded into 0.25 mL CPS and directly plunged into liquid nitrogen (LN_2). For warming, the straws were individually immersed in a 50 mL tube containing 38.5°C warming solution (20% FCS and 1 M sucrose in TCM-199) in a dry block heater for 30 s. Then, the straw contents were emptied in 30 mm petri dish containing warming solution. After warming, COCs were washed three times with BM. Groups of 20 retrieved oocytes were transferred immediately into 100 µL/droplets of BM and incubated for an additional 2 h at 38.5 C under a humidified atmosphere of 5% CO_2 in air before evaluation of oocyte viability. All groups were treated for a total of 24 h maturation time.

Evaluation of oocyte viability

After 2 h of warming, oocyte viability was evaluated by fluorescein diacetate (FDA) staining, according to the method previously described by Mohr and Trounson (17), frozen/thawed oocytes were treated with 2.5 µg/mL FDA in PBS supplemented with 5 mg/mL bovine serum albumin (BSA) at 38.5°C for 2 min in darkness and then washed three times in PBS supplemented with 5 mg/mL BSA. Oocytes were evaluated under UV irradiation using a fluorescence microscope (IX71; Olympus, Tokyo, Japan) equipped with a U-MWIB3 filter with an excitation wavelength of 460 to 495 nm and an emission wavelength of 510 nm. Oocytes expressing bright green fluorescence were regarded as living and were used in subsequent experiments.

In vitro fertilization (IVF)

Sperm used for IVF were prepared using the swim-up method according to Parrish et al. (18) with minor modifications. The frozen Thai native semen was thawed at 37 °C in a water bath for 30 s. The swim-up method was carried out by placing 200 µL of the semen into the bottom of a 5 mL tube containing 2 mL tyrode's albumin lactate pyruvate-HEPES (TALP-HEPES) medium supplemented with 0.3% fatty acid free BSA and was incubated at 38.5°C, 5% CO_2 under humidified atmosphere for 1 h to allow live spermatozoa to swim- up. After that, 1 mL of the upper phase of the medium was transferred to a 15 mL tube and centrifuged at 800xg at 38°C for 5 min. Then supernatant was removed and sperm pellets were re-suspended in TALP-HEPES medium and washed twice at 485xg for 5 min. Thereafter, supernatant was removed again and sperm pellets were re-suspended in 1.5 mL TALP-IVF medium (TALP-IVF) consisting of TALP supplemented with 0.3% fatty acid free BSA, 3.0 µg/mL heparin and 3.0 µg/mL penicillamine. The solution was diluted to a final sperm concentration of 3×10^6 sperms/mL before

fertilization (3×10^5 sperms in 100 µL IVF droplet). In the IVF procedure, after a 24-h oocyte culture period, oocytes were washed three times with TALP-IVF medium and a group of 20 COCs was placed in 100 µL/droplets of TALP-IVF medium in culture dish covered with mineral oil and incubated with sperms at 38.5°C under humidified atmosphere of 5% CO_2 in air for 18 h (19).

In vitro Culture (IVC)

The zygotes were cultured in modified synthetic oviductal fluid with amino acid (mSOFaa) (20). Briefly, approximately 18-20 h after insemination, the zygotes was washed four times in mSOFaa and once in G-mSOFaa (mSOFaa with 0.5 µg/mL glutamine and 3 mg/mL BSA). The zygotes were immediately transferred into 100 µL droplets of G-mSOFaa, covered with mineral oil and cultured at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 for 2 days (Day 0 = day of insemination). The cleavage rates were determined at Day 2 post-insemination under stereo zoom microscopy. The embryos, two-cell stage or beyond, were washed twice in C-mSOFaa (mSOFaa with of 0.5 mg/mL glucose, 1 µg/mL citrate and 3 mg/mL BSA). The embryos were then transferred immediately into 100 µL droplets of C-mSOFaa with bovine oviductal epithelial cells, covered with mineral oil and cultured at 38.5°C under a humidified atmosphere of 5% CO_2 in air for an additional 5 days (8 days in total). The culture medium was replaced with fresh medium every other day. The cleavage and day 8 blastocysts rates were recorded. The expanding or fully expanded blastocyst with a distinct trophectoderm and eccentrically located inner cell mass were acceptable according as a standards previous described by Rizos et al. (21).

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA). Comparison of means among treatments was performed by Tukey's multiple comparison tests using Statistical Analysis System software, ver. 9.2 (SAS Institute, Cary, NC, USA). For all analyses, a probability of $P<0.05$ was defined as statistically significant.

Experimental Designs

Experiment I: This experiment studied the effects of exposure to various doses of EG in VS-I and different exposure times on the survival rate and viability of frozen/thawed oocytes. The experimental design was set up according to a factorial procedure (3 × 2) based on three EG concentrations and two equilibration times for a total of six treatment groups plus one control group: group 1 (fresh control) - COCs were IVM for 24 h; groups 2, 3 and 4 - after 22 h IVM, oocytes were exposed to 2%, 4% and 6% EG, respectively, in VS-I for 10 min before being exposed to VS-II, followed by vitrification; groups 5, 6 and 7- the procedure was performed as for groups 2, 3 and 4 except the equilibration time in VS-I was changed to 20 min.

Experiment II: This experiment evaluated the effects of LAA supplementation in IVM medium or preincubation of mature oocytes with CLC on the survival rate and viability of frozen/thawed oocytes. The most effective VS-I obtained from experiment I was used. COCs were assigned to five treatment groups plus one control group: group 1 (fresh control) - COCs were IVM for 24 h; group 2 - after 22 h of IVM, oocytes were vitrified (vitrification control); groups 3 and 4 -COCs were matured in IVM medium supplemented with 1% and 2% LAA, respectively, for 22 h and then vitrified; groups 5 and 6 - after maturation in IVM medium for 22 h, oocytes were preincubated with 1 and 2 mg/mL (respectively) CLC in TCM-199 with 1 mg/mL PVP for 30 min, followed by vitrification.

RESULTS

Experiment-I Among the vitrification groups, oocytes exposed to 6% EG in VS-I for 10 min (group 2) had the highest survival rate and developmental competence ($P<0.05$). However, oocytes exposed to 6% EG in VS-I for 20 min (group 7) showed the

Table 1. Effect of EG concentrations and times of exposure in VS-I on frozen-thawed TNC oocyte survival and subsequent embryonic development

*Groups	Time of exposed (min)	Survival (%)	IVF	Cleaved (%)	No.% of Day 8 blastocyst
Fresh control[1]	-	207/207 (100.0%)[a]	207	188 (90.8±1.1)[a]	80 (38.6±0.9)[a]
2% EG[2]	10	130/207 (62.8±0.6)[e]	126	50 (39.7±1.1)[e]	1 (0.8±0.4)[e]
4% EG[3]	10	158/207 (76.3±0.9)[c]	158	90 (57.0±1.9)[c]	16 (10.1±0.4)[c]
6% EG[4]	10	181/207 (87.6±1.0)[b]	181	117 (64.7±1.4)[b]	25 (13.8±0.7)[b]
2% EG[5]	20	123/207 (59.5±1.5)[f]	119	52 (43.7±2.0)[e]	2 (1.7±0.6)[e]
4% EG[6]	20	145/207 (70.3±0.3)[d]	145	73 (50.3±2.1)[d]	12 (8.3±0.9)[d]
6% EG[7]	20	105/207 (50.7±1.0)[g]	105	32 (30.5±2.1)[f]	0

*Group 1, after 24 h IVM COCs; groups 2-7, after 22 h IVM, oocytes were exposed to VS-I containing EG, then exposed to VS-II. Six replications were performed. Data within the same columns with different superscript letters are statistically different ($P \leq 0.05$). Percentage data are presented as mean±SEM.

lowest post-thaw survival rates and a complete absence of day 8 blastocyst formation. Rates of survival, cleavage and day 8 blastocyst formations significantly decreased after vitrification in all groups compared with the control group, as shown in Table 1.

Experiment-II

An increasing concentration of LAA and CLC in medium was shown a positive effect for vitrification. The survival rates of frozen-thawed oocytes in the group of IVM oocyte in maturation medium supplemented with 2% LAA in following vitrification (group 4) and preincubated of oocytes with 2 mg/mL CLC in BM medium for 1 h following vitrification (group 6) was higher when compared with others vitrification groups ($P<0.05$). The cleavage rates and day 8 blastocyst rate, however, was shown to be higher in group 4 than in other vitrification groups ($P<0.05$). The interesting about the results from this current study is that CLC enhanced blastocysts rate of vitrified oocytes. However, cleavage rates and day 8 blastocyst rate in all vitrification groups was lower than the fresh control group ($P<0.05$) as shown in Table 2.

DISCUSSION

The present study, equilibration of TNC oocytes by exposed to 6% EG in VS-I for 10 min following exposure to VS-II together with vitrification within < 30 s by using the CPS vitrification method was shown a higher frozen-thawed oocyte survival rate, cleavage and Day 8 blastocyst formation rate (64.7±1.4; 87.6±1.0 and 21.5±1.2, respectively) than equilibration by exposed to 2% and 4% EG in VS-I before exposure to VS-II and subjected to vitrification. Papis et al. (22), however, reported that equilibration of bovine oocytes by exposed to 3% or 4% EG in VS-I for 15 min following by exposure in VS-II (31%

Table 2. Effect of oocyte IVM in LAA maturation medium or preincubated with CLC on frozen-thawed TNC oocyte survival and subsequent embryonic development

*Groups	Survival (%)	IVF	Cleaved (%)	No.% of Day 8 blastocyst
Fresh control[1]	232/232 (100)[a]	232	207 (89.2±2.5)[a]	93 (40.1±1.0)[a]
Vitri-control[2]	197/232 (85.0±2.1)[d]	197	124 (63.0±2.1)[d]	29 (14.7±1.1)[d]
1% LAA[3]	199/232 (85.8±2.0)[cd]	199	144 (72.4±2.1)[c]	42 (21.1±0.9)[c]
2% LAA[4]	208/232 (89.7±0.7)[bc]	208	160 (77.0±2.9)[b]	52 (25.0±1.4)[b]
1 mg/mL CLC[5]	230/265 (87.6±1.4)[bcd]	230	126 (54.8±1.7)[e]	31 (13.5±0.9)[d]
2 mg/mL CLC[6]	210/232 (90.5±0.7)[b]	210	149 (71.0±1.8)[c]	45 (21.4±1.1)[c]

*Group 1, after 24 h IVM of COCs; group 2, after 22 h IVM, oocytes were vitrified; groups 3 and 4, COCs were IVM in 1% or 2% LAA maturation medium for 22 h and then vitrified; groups 5 and 6, after 22 h IVM, oocytes were preincubated with 1 or 2 mg/mL CLC in basic medium for 30 min and then vitrified. Six replications were performed. Data within the same columns with different superscript letters are statistically different ($P \leq 0.05$). Percentage data are presented as mean±SEM.