Toxicity Test of Sucrose and Trehalose Prior to Cryopreservation in Immature Bovine Oocytes

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ABSTRACT

The purpose of this study was to determine toxic effect of sucrose and trehalose prior to cryopreservation on nuclear maturation and embryonic development in immature bovine oocytes. All cryoprotectant was prepared in tissue culture medium 199-HEPES (TCM 199-HEPES) with 10% fetal bovine serum (FBS). Immature oocytes were exposed to 1.2 M ethylene glycol (EG) and 0.1 M sucrose or 1.2 M EG and 0.1 M trehalose for 3 min and then were exposed to 3.2 M EG and 0.25 M sucrose or 3.2 M EG and 0.25 M trehalose for 1 min. Oocytes treated with cryoprotectants were exposed to 0.25 M sucrose or 0.25 M trehalose for 5 min and then 0.1 M sucrose or 0.1 M trehalose for 5 min. Depending on type of sugar added to cryopreservation solution, oocytes were allocated to sucrose group and trehalose group, respectively. Oocytes exposed to TCM 199-HEPES with 10% FBS were considered as control. Oocytes were cultured in TCM 199 supplemented with 10% FBS, 5 ng/ml epidermal growth factor, 0.01 IU/ml luteinizing hormone, and 1 μg/ml estradiol for 24 h in 39℃, 5% CO_2. Nuclear maturation was assessed by staining oocytes with 1% aceto-orcein. Oocytes were fertilized *in vitro* and were cultured in TCM 199 supplemented with 10% FBS, 5 mM sodium pyruvate, and antibiotics in 39℃, 5% CO_2. The rates of cleavage and blastocyst, and cell number in blastocyst were assessed. Metaphase Ⅱ rates were not different among experimental groups regardless of type of sugar. The cleavage rate of trehalose group (73.3%) was significantly higher ($p<0.05$) than those of sucrose group (62.8%) and control group (60.8%). The blastocyst rate was significantly higher in trehalose group ($p<0.05$). Mean cell number in blastocyst were not different among experimental groups, although cell number of blastocyst in trehalose group was significantly higher on day 7 ($p<0.05$). In conclusion, sucrose and trehalose were not toxic to immature bovine oocytes prior to cryopreservation. In particular, trehalose was more effective on embryonic development.

(Key words : immature bovine oocytes, sucrose, trehalose, nuclear maturation, embryonic development)

INTRODUCTION

Cryopreservation of immature oocytes has been increased since reproductive technologies, such as *in vitro* maturation, *in vitro* fertilization and *in vitro* embryo production has developed (Huang *et al.*, 2008). Cryopreservation of immature oocytes would be a significant advance for basic and commercial applications (Candy *et al.*, 1994; Kim *et al.*, 2007; Schroeder *et al.*, 1990). Immature oocytes do not have an organized meiotic spindle, and so cryopreservation may be an alternative approach instead of metaphase Ⅱ (Albarracin *et al.*, 2005; Vieira *et al.*, 2002).

Recently, vitrification has been used as a useful method to cryopreserve mammalian oocytes and embryos (Albarracin *et al.*, 2005; Cetin and Bastan *et al.*, 2006; Kim *et al.*, 2007). Vitrification solutions contain permeating cryoprotectant, macromolecules, and sugars. Ethylene glycol (EG), due to its high permeability and low toxicity, has been found to be convenient for vitrification of immature oocytes (Cetin and Bastan, 2006; Cha *et al.*, 2000; Hurt *et al.*, 2000). Thus, EG was used as a permeating cryoprotectant in this study.

The protective action of sugars is very complex, attributable to a number of their special properties (Fabbri *et al.*, 2000). The addition of sugars to penetrating cryoprotectant aids in the dehydration of oocytes and embryos (Hotamisligil *et al.*, 1996; Takahashi and Kanagawa, 1985). Sugars lowered the toxicity of permeating cryoprotectants (Ahammad *et al.*, 2002; Palasz *et al.*, 2000). Sutton (1992) reported that the critical cooling rate required to avoid ice crystallization in solutions with permeating cryoprotectants was altered following the addition of sugars. Kuleshova *et al.* (1999) found that the overall contribution made by the sugar to the vitrification solution depended on the type of sugar that was used. Effect of sugars on oocyte cryopreservation has been focused on studies on sucrose con-

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centration (Coticchio *et al.*, 2006; De Santis *et al.*, 2007; Nottola *et al.*, 2007). However, trehalose as well as sucrose has been effectively used for oocyte cryopreservation (Atabay *et al.*, 2004; Gasparrini *et al.*, 2007). However, it is difficult to determine the toxic effect of sugars before cryopreservation since the effects of sugars might be changed during cryopreservation in above studies. In the present study, toxic effect of both sucrose and trehalose on *in vitro* maturation and embryonic development was determined by exposing oocytes to cryopreservation solution prior to cryopreservation.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Co. (St. Louis, Mo, USA). Tissue culture media 199 (TCM 199) and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen Corporation, Grand Island, USA).

1. Collection of Oocyte

Cattle ovaries were obtained from abattoir. Collected ovaries were transported in 0.9% saline supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) and maintained at 25~27℃. The ovaries were washed three times in 0.9% sterilized saline. Cumulus oocytes complexes (COCs) were collected by aspiration of follicles using an 18-gauge needle connected to 10 ml syringe, and were recovered in a petri dish. Oocytes surrounded by a compact cumulus mass with evenly granulated cytoplasm were selected.

2. Toxicity Test

Immature bovine oocytes were exposed to cryoprotectant to determine toxic effect of both sucrose and trehalose prior to cryopreservation.

All cryoprotectant was prepared in TCM 199-HEPES with 10% FBS. Immature oocytes were exposed to 1.2 M EG and 0.1 M sucrose or 1.2 M EG and 0.1 M trehalose for 3 min and then were exposed to 3.2 M EG and 0.25 M sucrose or 3.2 M EG and 0.25 M trehalose for 1 min. Depending on type of sugar added to cryopreservation solution, oocytes were allocated to sucrose group and trehalose group, respectively. Oocytes exposed to TCM 199-HEPES with 10% FBS were considered as control. Oocytes treated with cryoprotectants were exposed to 0.25 M sucrose or 0.25 M trehalose for 5 min and then 0.1 M sucrose or 0.1 M trehalose for 5 min. All treatment was conducted on slide warmer at 38℃. Other macromolecules was not added to cryopreservation solution to prevent additional their interaction with ethylene glycol and sugars.

3. *In Vitro* Maturation (IVM)

COCs were washed 3~4 times in TCM 199-HEPES containing 5 mM sodium pyruvate, and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, 0.025 μg/ml amphotericin B) and were washed three times with maturation medium: TCM 199 supplemented with 10% FBS, 5 mM sodium pyruvate, 0.05 ng/ml epidermal growth factor, 0.01 IU/ml luteinizing hormone, 1 μg/ml estradiol, and antibiotics. A group of 20~25 oocytes was placed in 100 μl drop of maturation medium under sterile mineral oil at 39℃ in an atmosphere of 5% CO_2 in air for 24 h.

4. *In Vitro* Fertilization

Frozen semen was thawed in a water bath at 38℃ for 30 sec. Live spermatozoa were recovered using Percoll density gradient (Yu *et al.*, 2002). Spermatozoa were washed twice with Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975) containing 10 μg/ml heparin and 5 mM caffeine-sodium benzoate by centrifugation at 500 g for 5 min. Sperm pellet was mixed with BO medium containing 1% bovine serum albumin, 10 μg/ml heparin and 2.5 mM caffeine-sodium benzoate. Final concentration of spermatozoa was adjusted to 6×10^6 sperm/ml. Oocytes and spermatozoa were co-cultured under mineral oil at 39℃ in an atmosphere of 5% CO_2 in air for 6 h.

5. *In Vitro* Culture

The presumptive zygotes were cultured in TCM 199 supplemented with 10% FBS, 5 mM sodium pyruvate, and antibiotics.

6. Assessment of Meiotic Stage

Oocytes denuded by vigorous shaking in 3% sodium citrate solution were fixed in a 4-well culture dish (Nunc, Rochester, NY, USA) containing 500 μl of ethanol : acetic acid (3 : 1 v/v) for 48 h. They were then dispensed onto a slide to which a cover slip was added. Each sample was stained with aceto-orcein [1% (w/v) orcein in 45% (v/v) acetic acid] and destained with glycerol : acetic acid : distilled water (1 : 1 : 3, v/v). Oocytes were evaluated under a biological microscope at 400 × magnification. Meiotic stages were classified as previously described (Romar and Funahashi, 2006) as being at germinal vesicle, germinal vesicle breakdown, metaphase Ⅰ, anaphase Ⅰ, telophase Ⅰ, or metaphase Ⅱ.

7. Rates of Cleavage and Blastocyst, and Cell Number in Blastocyst

The rate of cleavage was examined on day 2 and blastocyst rate was examined on day 7, 8, 9, and 10, respectively. Blastocysts were stained with Hoechst 33342 (5 μg/ml) to count cell number under fluorescent microscope at magnification of 400 ×.

8. Statistical Analysis

Each experiment was replicated 5 times. All percentage data was subjected to arcsine transformation. All data and data sets are presented as the mean ± SE. Maturation of oocytes and development of embryos following IVF were analyzed by Duncan's multiple range test using the Statistical Analysis System ver. 8× (SAS, Cary, NC, USA). $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

1. Nuclear Maturation

The toxicity test of sucrose and trehalose as cryoprotectant was conducted prior to cryopreservation in this study. The effect of sucrose and trehalose on nuclear maturation was described in Table 1. Nuclear maturation rates were not different among experimental groups.

The toxicity of components is of great importance in any cryopreservation protocol (Kuleshova *et al.*, 1999; Magnusson *et al.*, 2008; Wani *et al.*, 2004). Particularly, sugar aids the dehydration of oocytes and embryos and lowered the toxicity of permeating cryoprotectants (Ahammad *et al.*, 2002; Hotamisligil *et al.*, 1996; Palasz *et al.*, 2000; Takahashi and Kanagawa, 1985). In the present study, sucrose and trehalose did not decrease maturation rate of oocytes compared to control. We demonstrated that sucrose and trehalose were not toxic to oocytes prior to cryopreservation.

2. Cleavage and Blastocyst Rates

The rates of cleavage and blastocyst were described in Fig. 1. Cleavage rate and blastocyst rate in trehalose group were significantly higher than those in sucrose group and control ($p<0.05$). However, there was no significant difference between sucrose group and control group.

Kuleshova *et al.* (1999) found that the overall contribution made by the sugar to the vitrification solution depended on the type of sugar that was used. Trehalose has been found to have a protective action related to an osmotic effect and to a specific interaction with membrane phospholipids (Kim *et al.*, 1986). We infer that trehalose might be more protective to oocyte membrane and might affect subsequently embryonic development.

3. Cell Number in Blastocyst

Cell number in blastocyst was compared according to developmental day of blastocyst (Table 2). In control group, cell number in blastocyst on day 7 was significantly higher ($p<0.05$). The similar tendency was shown in trehalose group. Cell number in blastocyst on day 7 was significantly higher ($p<0.05$). However, there was no difference among developmental days in sucrose group. In comparison among experimental groups, cell number of blastocyst in control and trehalose groups was significantly higher than that in sucrose group on day 7 ($p<0.05$). However, mean cell number in blastocyst was not different among experimental groups.

Table 1. *In vitro* maturation of immature bovine oocytes exposed to ethylene glycol supplemented with sucrose or trehalose to test their toxicity

Nuclear stage	Nuclear maturation (%)		
	Control	Sucrose	Trehalose
Metaphase Ⅱ	86.1±11.0	88.8±8.0	87.9±7.6

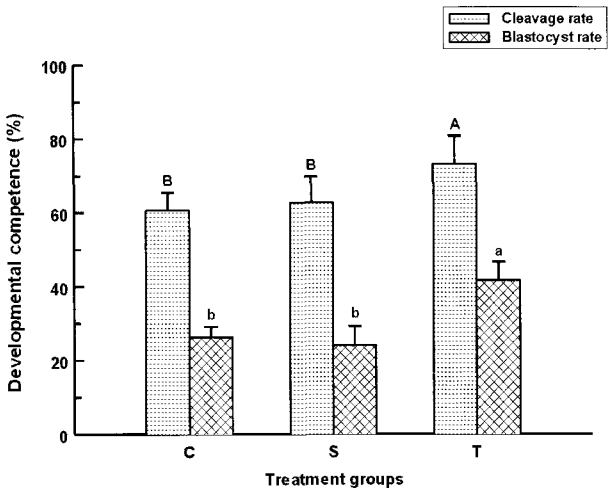

Fig. 1. *In vitro* fertilization following exposure of immature bovine oocytes to ethylene glycol supplemented with sucrose or trehalose to test their toxicity. C: Control, S: Oocytes exposed to EG with sucrose, T: Oocytes exposed to EG with trehalose. The data represent mean±SE.
^{A,B,a,b} Values with different superscripts are significantly different ($p<0.05$).

On day 7, cell number of blastocyst in trehalose group was higher than that in sucrose group. Difference of osmometric behavior might be one of explanation. The osmometric behavior of mouse oocytes in the presence of different extracellular sugars is an important variable factor when considering optimization of cryopreservation protocols using sugars (Bhowmick *et al.*, 2002; Kuleshova *et al.*, 1999). The difference between osmometric behaviors of different extracellular sugars may affect cytoplasmic maturation and subsequent developmental ability (Abe *et al.*, 2005). These differences might affect mitosis in blastocyst development. However, we could not determine sucrose and trehalose as a non-permeating cryoprotectant to protect oocytes during cryopreservation. Thus, effect of sucrose and trehalose after cryopreservation should be demonstrated in future experiment.

In conclusion, trehalose as well as sucrose were not toxic to immature bovine oocytes prior to cryopreservation. Trehalose was more effective on embryonic development. This result could be a basic data to select appropriate type of sugar for cryopreservation of immature bovine oocytes.

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Table 2. Cell number in blastocyst produced following exposure of immature bovine oocytes to ethylene glycol supplemented with sucrose or trehalose to test their toxicity

Groups	Cell number in blastocyst (n) on developmental day				
	Day 7	Day 8	Day 9	Day 10	Mean
Control	120.3±3.0^{a,A}	85.5±10.1^{A,B}	52.5±5.9^{B}	82.0±0.0^{A,B}	85.1±4.8
Sucrose	81.3±7.0^{b}	87.6± 8.0	87.0±0.0	73.5±8.8	82.4±6.0
Trehalose	107.8±9.7^{a,b,A}	93.3± 9.9^{A,B}	88.3±8.1^{A,B}	60.0±4.4^{B}	87.4±8.0

The data represent mean ± SE.

^{a,b} Values in the same column with different superscripts differ significantly ($p<0.05$).

^{A,B} Values in the same raw with different superscripts differ significantly ($p<0.05$).

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