Effect of Taxol Pre-treatment to *In Vitro* Matured Bovine Oocytes on Spindle Morphology and Embryonic Development Following Vitrification

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ABSTRACT

The purpose of this study was to determine the effects of Taxol pre-treatment to *in vitro* matured bovine oocytes, and sucrose and trehalose added to vitrification solution on spindle morphology and embryonic development following cryopreservation. Bovine oocytes were collected from ovaries and matured in tissue culture medium 199 (TCM 199) supplemented with 10% Fetal Bovine Serum (FBS), 0.05 ng/ml epidermal growth factor, 0.01 IU/ml luteinizing hormone and 1 μg/ml estradiol for 22 h in 39℃, 5% CO_2, TCM 199-HEPES containing 20% FBS was used as basic medium (BM) to prepare vitrification solution. Oocytes were pre-treated with 1 μM Taxol in maturation medium for 15 min prior to vitrification. Oocytes were exposed to 1.6 M ethylene glycol (EG) and 1.3 M dimethyl sulfoxide (DMSO) in BM and then were exposed to 3.2 M EG, 2.6 M DMSO and 0.5 M sucrose in BM or 3.2 M EG, 2.6 M DMSO and 0.5 M trehalose in BM. Oocytes with cumulus cells and oocytes without cumulus cells were considered as control 1 and control 2, respectively and held in TCM 199-HEPES at 39℃. Oocytes were frozen using modified solid surface vitrification and were stored in cryotubes in liquid nitrogen for more than 1 week. Frozen oocytes were thawed in TCM 199-HEPES containing 0.5 M, 0.25 M and 0.1 M sucrose in BM for 2 min, respectively or 0.5 M, 0.25 M and 0.1 M trehalose in BM for 2 min, respectively. Immunoflurorescence staining of oocytes was performed to assess spindle morphology and chromosome configuration of oocytes. The rates of cleavage and blastocyst were examined following *in vitro* fertilization. Normal spindle morphology rate of oocytes pre-treated with Taxol prior to vitrification was not higher than that of other vitrified groups. Taxol pre-treatment did not increase cleavage and blastocyst formation rates, although control groups showed significantly higher rates ($p<0.05$). Percentages of normal spindle and embryonic development were not significantly different among vitrified groups regardless of type of sugar. In conclusion, Taxol pre-treatment of oocytes before cryopreservation did not reduce the damage induced by vitrification and subsequently did not improve embryonic development following vitrification. Trehalose may be used as an alternative non-permeating cryoprotectant in vitrification solution.

(Key words : bovine oocytes matured *in vitro*, vitrification, Taxol, sucrose, trehalose)

INTRODUCTION

Cryopreservation of oocytes has important roles in the preservation and management of genetic resources, low-cost international movement of selected genetics, and rapid dissemination of germ plasm via *in vitro* embryo production, genetic engineering and nuclear transfer procedures (Gupta *et al.*, 2007). Recently, vitrification method has been used for cryopreservation of mammalian oocytes (Vajta, 2000). Rall and Fahy (1985) reported that intracellular ice formation might be overcome by vitrification. The vitrification methods that have been reported are open pulled straw, nylon loop system, solid surface vitrification (SSV), and cryo-loop. Modified method of SSV has been successfully used for oocyte cryopreservation (Gupta *et al.*, 2007; Zhang *et al.*, 2008).

It has been reported that meiotic spindle of oocytes is very sensitive to low temperature. Abnormal spindle and chromosomes were observed after cryopreservation (Aman and Parks, 1994; Morato *et al.*, 2008; Pickering and Johnson, 1987; Pickering *et al.*, 1990). The main damage observed during vitrification was an abnormal spindle configuration mainly due to the disorganization or disassembly of meiotic microtubules (Albarracin *et al.*, 2005).

One possible way to reduce sensitivity of oocytes to cryopreservation may be use of cytoskeleton stabilizer. The addition of Taxol, a cytoskeleton stabilizer, to vitrification solution, improved the post-warming development of mature oocytes in mice (Park *et al.*, 2001). Taxol added to the vitrification solution stabilized meiotic spindle and chromosomes during freezing and improved the post-warming development of immature

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human oocytes and mature murine, porcine, bovine oocytes (Fuchinoue *et al.*, 2004; Fujihira *et al.*, 2005; Shi *et al.*, 2006).

In vitrification of the oocytes, the factors that influence on the survival are the toxicity of the cryoprotectant, the composition of the vitrification solution and the freezing and thawing speed (Cuello *et al.*, 2004). Cryoprotectant used for vitrification is composed of permeating cryoprotectant, glycerol, ethylene glycol (EG), propylene glycol, and dimethyl sulfoxide (DMSO) and non-permeating cryoprotectant, sugars, and macromolecules. Each cryoprotectant has different toxicities and permeabilities which are their weak point. Thus, EG has been used for cryopreservation of matured bovine oocytes because of the toxicity in DMSO and glycerol (Martino *et al.*, 1996). Glucose and sucrose have been commonly used as non-permeating cryoprotectant (Kim *et al.*, 1996; Rayos *et al.*, 1994). Oocyte cryopreservation has been performed to compare different way, such as, cryoprotectant, cryoprotectant concentration, and exposure time to cryoprotectant (Bagis *et al.*, 2004; Cetin and Bastan, 2006; Yamada *et al.*, 2007).

In the present study, the purpose was to determine the effects of Taxol pre-treatment to *in vitro* matured bovine oocytes prior to vitrification and sucrose and trehalose added to vitrification solution on spindle morphology and embryonic development following vitrification.

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 and *In Vitro* Maturation

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.

COCs were washed 3~4 times in TCM 199-HEPES containing 5 mM sodium pyruvate, antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, 0.025 μg/ml amphotericin B) and washed three times with maturation medium. Maturation medium was composed of 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.

2. Cryopreservation

The oocytes were vitrified by modified solid surface vitrification (SSV) method as described by Dinnyes *et al.* (2000). Oocytes were partially or completely denuded of cumulus cells. Then oocytes were handled in a basic medium (BM), consisting of TCM 199-HEPES with 20% FBS. All procedures were performed on a heated surface at 39℃. Groups of 5~10 oocytes were exposed to 1.6 M EG and 1.3 M DMSO in BM for 30 sec, and then exposed to 3.2 M EG, 2.6 M DMSO, and 0.5 M sucrose in BM or 3.2 M EG, 2.6 M DMSO, and 0.5 M trehalose in BM. Oocytes were aspirated into a pipette with 1~2 μl vitrification solution and dropped onto a metal surface precooled to −150 to −180℃, and instantly vitrified, then moved with a forceps cooled in liquid nitrogen (LN_2) into a 1 ml cryotube and stored in LN_2 for more than 1 week.

Taxol (paclitaxel) was prepared as 1 mM stock solution in DMSO and stored in aliquots at −20℃ until use. Oocytes were exposed to maturation medium with 1 μM Taxol for 15 min at 39℃ and vitrified by SSV method.

Oocytes were allocated to six experimental groups: oocytes with cumulus cells (control 1), oocytes without cumulus cells (control 2), oocytes pre-treated with 1 μM Taxol and vitrified in vitrification solution with trehalose, oocytes pre-treated with Taxol and vitrified in vitrification solution with sucrose, oocytes vitrified in vitrification solution with trehalose, and oocytes vitrified in vitrification solution with sucrose. Control 1 and control 2 held in TCM 199-HEPES at 39℃. Control 2 was prepared by pipetting of COCs in 0.6 mg/ml (w/v) hyaluronidase in TCM 199-HEPES.

3. Thawing

Oocytes vitrified in vitrification solution supplemented with sucrose were dropped into 0.5 M sucrose in BM for 2 min, and then transferred into 0.25 M, and 0.1 M sucrose in BM for 2 min, respectively. Oocytes vitrified in vitrification solution supplemented with trehalose were thawed in 0.5 M trehalose in BM, and then transferred into 0.25 M, and 0.1 M trehalose for 2 min, respectively. After being washed for 2~3 times with BM and maturation medium, the oocytes were

incubated in maturation medium for 1 h.

4. Spindle and Chromosome Immunostaining

Immunoflurorescence staining of oocytes was performed by the method described by Shi *et al.* (2007). Cumulus cells of oocytes were removed with 3% sodium citrate and were fixed in 4% formaldehyde containing 0.3% Triton x-100 in phosphate buffered saline (PBS) for 40 min. They were then washed in PBS containing 0.1% polyvinyl alcohol, 1% bovine serum albumin (BSA) and 1% sodium azide (PPB) three times at room temperature. The fixed oocytes were exposed to PPB containing 10% goat serum for 10 min at room temperature, and then were stored in PPB containing mouse anti-α-tubulin monoclonal antibody (1:100) at 4℃ overnight. After three washes in washing buffer, the oocytes were incubated in PPB containing fluorescein isothiocyanate-conjugated anti-mouse IgG (1:40) for 1 h in the dark at room temperature. The oocytes were washed with PPB for three times and incubated with in PPB containing 10 μg/ml propidium iodide for 15~20 min. The oocytes were washed with PPB for three times. Finally, the oocytes were mounted on glass slide and spindle morphology and chromosome organization were examined under fluorescence inverted microscope at 400× magnification. Spindle morphology and chromosomes were classified by the analysis described by Shi *et al.* (2007).

5. *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) medium 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% BSA and 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.

6. *In Vitro* Culture

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

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 4 times. All percentage data was subjected to arcsine transformation. All data and data sets are presented as the mean ± SE except spindle morphologies and chromosome alignments. Spindle morphologies and chromosome alignments, and development of embryos following *in vitro* fertilization 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

Table 1 shows the effect of Taxol pre-treatment on spindle morphology and chromosome alignment following vitrification.

Table 1. Spindle morphologies and chromosome alignments observed in matured bovine oocytes after vitrification

Groups	No. of MⅡ oocytes	Spindle configuration, *n* (%)			Chromosome alignment, *n* (%)		
		Normal	Abnormal	Absent	Dispersed	Decondensed	Absent
C1	74	52 (70.2)	21 (28.3)	1 (1.4)	14 (18.9)	6 (8.1)	2 (2.7)
C2	48	25 (52.1)	18 (37.5)	5 (10.4)	12 (25.0)	9 (18.8)	2 (4.2)
TT	28	11 (39.3)	9 (32.1)	8 (28.6)	8 (28.6)	9 (32.1)	0 (0)
TS	29	10 (34.5)	14 (48.3)	5 (17.2)	10 (34.5)	9 (31.0)	0 (0)
T	41	22 (53.7)	13 (31.7)	6(14.6)	10 (24.4)	8(19.5)	1 (2.4)
S	37	18 (48.6)	10 (27.0)	9 (24.3)	8 (21.6)	11 (29.7)	0 (0)

Control 1 (C1): Oocytes with cumulus cells were not vitrified, Control 2 (C2): Oocytes without cumulus cells were not vitrified, Taxol-Trehalose (TT): Oocytes were vitrified in vitrification solution with trehalose after pre-treatment with 1 μM Taxol, Taxol-Sucrose (TS): Oocytes were vitrified in vitrification solution with sucrose after pre-treatment with 1 μM Taxol, Trehalose (T): Oocytes were vitrified in vitrification solution with trehalose, Sucrose (S): Oocytes were vitrified in vitrification solution with sucrose.

Vitrified oocytes showed lower percentage of normal spindle configurations. However, there was no significant difference among experimental groups regardless of vitrification. Rates of abnormality and absent spindle were not significantly different, although there were numeral differences among groups. When chromosomes alignment was compared, there was no significant difference among groups irrespective of classification of chromosomal abnormalities. Pre-treatment of Taxol in oocytes before vitrification did not improve spindle configuration and chromosome alignment following vitrification. Percentage of normal spindle was not significantly different among vitrified groups regardless of type of sugar added to vitrification solution. Fig. 1 shows the morphology of spindle and chromosomes observed in experimental groups.

The effect of Taxol pre-treatment on cleavage rate and blastocyst rate following vitrification is shown in Fig. 2. The rates of cleavage and blastocyst in control 1 and control 2 were significantly higher than those in vitrified oocytes. However, there was no significant difference among vitrification groups regardless of Taxol pre-treatment. When blastocyst formation rate among vitrified groups were compared according to sugars added to vitrification solution, oocytes vitrified in vitrification solution containing sucrose showed higher blastocyst formation rate without significant difference.

DISCUSSION

In the present study, effect of Taxol pre-treatment to *in vitro* matured bovine oocytes prior to vitrification on spindle morphology and chromosomes alignment, and embryonic development was determined following vitrification. In addition, effect of sucrose and trehalose was compared as non-permeating cryoprotectant.

Oocyte cryopreservation has been required as a useful method to develop reproductive biotechnologies. SSV is one of useful and simple methods to vitrify oocytes and is modified to improve its efficiency (Dinnyes *et al.*, 2000; Gupta *et al.*, 2007). In this study, we used a stainless round bowl with flat bottom as a modified SSV to prevent oocyte from dropping oocytes out of stainless material.

One of main problems to cryopreserve matured oocytes is meiotic spindle and chromosomes damage. Cytoskeleton stabilizing agents have been used to protect oocytes spindle from freezing. Taxol (paclitaxel) has been known to stabilize the kinetics of spindle microtubles (Caplow and Zeeberg, 1982; Jor-

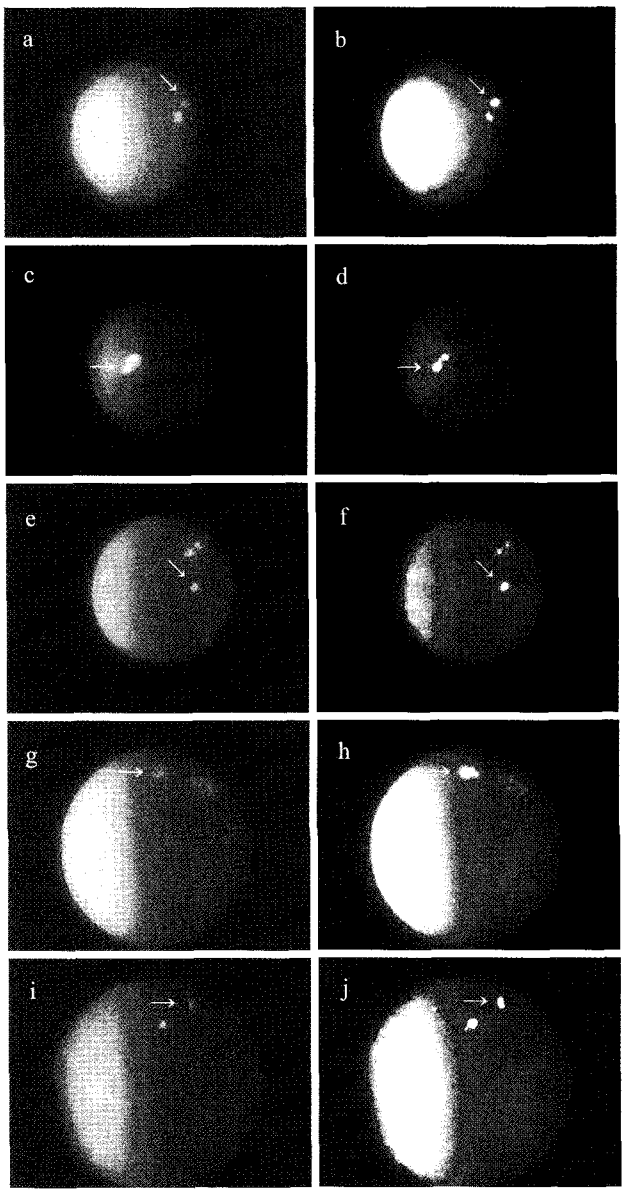

Fig. 1. Fluorescence images of *in vitro* matured bovine oocytes after vitrification with or without Taxol pre-treatment. Oocytes were immunocytochemically stained using a mouse anti-α-tubulin monoclonal antibody and to visualize the microtubules (green) and counterstaining with propidium iodide (PI) to visualize the chromosome (red). Control 1 (a, b): Normal barrel-shaped MII spindle with microtubules forming a clear meiotic spindle with chromosomes aligned (200x). Taxol-Trehalose (c, d): Abnormal spindle structure associated with partly disorganized chromosome (200x). Taxol-Sucrose (e, f): Abnormal spindle structure associated with disorganized chromosome (200x). Trehalose (g, h): Abnormal associated spindle structures and dispersed chromosome (400x). Sucrose (i, j): The absence of microtubules and chromosome with an aberrant less condensed appearance (400x). First polar body (arrow).

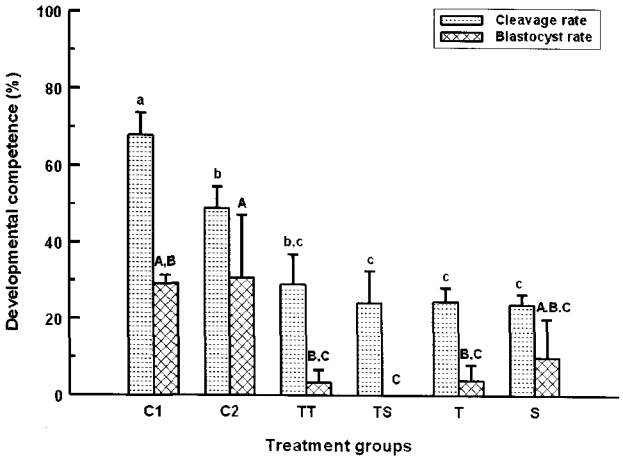

Fig. 2. Cleavage rates and blastocyst rates observed bovine fertilized oocytes after vitrification with or without Taxol pre-treatment. The data represent mean ± SE. Control 1 (C1): Oocytes with cumulus cells were not vitrified, Control 2 (C2): Oocytes without cumulus cells were not vitrified, Taxol-Trehalose (TT): Oocytes were vitrified in vitrification solution with trehalose after pre-treatment with 1 μM Taxol, Taxol-Sucrose (TS): Oocytes were vitrified in vitrification solution with sucrose after pre-treatment with 1 μM Taxol, Trehalose (T): Oocytes were vitrified in vitrification solution with trehalose, Sucrose (S): Oocytes were vitrified in vitrification solution with sucrose. Values with different superscript are significantly different ($p<0.05$).

dan *et al.*, 1993). However, Taxol did not stabilize meiotic spindle of oocytes during cryopreservation and did not improve embryonic development after freezing-thawing in the present study. This result agrees with that of Fujihira *et al.* (2005). They described that Taxol did not affect on the developmental competence of porcine matured oocyte following vitrification, although different species was used. Zhang *et al.* (2008) also reported that survival of oocytes, and rates of cleavage and blastocyst were not different regardless of Taxol treatment. On the contrary, Morato *et al.* (2008) reported that pre-treatment of oocytes with Taxol before vitrification reduced the damage induced by vitrification and potentially improved the development of vitrified oocytes. This was in contrast with our findings. On the other hand, Park *et al.* (2001) and Shi *et al.* (2006) reported that Taxol added to vitrification solution improved post-warming development of mature murine, porcine, and bovine oocytes. Thus, we may consider vitrifying oocytes in vitrification solution containing Taxol in future study.

In this study, cleavage rate of cumulus-enclosed oocytes was significantly higher than that of cumulus-free oocytes ($p<0.05$) and blastocyst rate was not different between them. Park *et al.* (2001) found that preimplantation development of cumulus-enclosed oocytes were higher than that of cumulus-free oocytes after vitrification. On the other hand, Zhang *et al.* (2008) reported that rates of survival and embryonic development were not significantly different between cumulus-enclosed and cumulus-free ovine oocytes. Cumulus cells of oocytes were generally removed to vitrify small volume. Considering our result and other results described above, cumulus cells may not be a main factor to vitrify oocytes successfully.

In pilot study, oocytes exposed to cryopreservation solution with trehalose prior to cryopresevation showed higher cleavage and blastocyst formation rates. Thus, we expected trehalose added to vitrification solution might improve survival of oocytes after freezing-thawing. However, the difference of spindle and chromosome morphology and embryonic development between two sugars was not observed.

In the present study, 1 μM Taxol pre-treatment before vitrification did not stabilize meiotic spindle and subsequent did not improve embryonic development. Further studies are required to demonstrate the effect of Taxol with different concentrations as a cytoskeleton stabilizer. In addition, different kinds of cytoskeleton stabilizers should be compared with Taxol.

In conclusion, Taxol pre-treatment of oocytes before cryopreservation did not reduce the damage induced by vitrification and subsequently did not improve embryonic development following vitrification. Trehalose may be used as an alternative non-permeating cryoprotectant in vitrification solution.

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