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Pretreatment of *in vitro* matured bovine oocytes with docetaxel before vitrification: Effects on cytoskeleton integrity and developmental ability after warming

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ABSTRACT

The stabilization of spindle fibersis important for successful vitrification of bovine oocytes because microtubules and other cytoskeleton fibers (CSF) can be damaged during vitrification, resulting in failure of fertilization after thawing. Docetaxel, a stabilizing agent, could potentially reduce CSF damage of bovine oocytes induced during vitrification. However, there have been no reports on the effects of docetaxel on their vitrification. Experiment 1 was conducted to investigate the effects of various doses of docetaxel (0.0, 0.05, 0.5, 5.0 and 50 μM) in preincubation medium of *in vitro* matured (IVM) bovine oocytes on their developmental ability after *in vitro* fertilization (IVF). The results show that 0.05 μM docetaxel had no adverse effect on embryo development, while docetaxel at a concentration of ⩾0.5 μM inhibited development. Experiments 2 and 3 were conducted to investigate the effects of preincubation of IVM bovine oocytes with 0.05 μM docetaxel for 30 min prior to vitrification-warming on CSF integrity (Experiment 2), and on oocyte survival and viability after IVF (Experiment 3). When preincubated with 0.05 μM docetaxel for 30 min before vitrification, post-thawed oocytes had less CSF damage and higher survival rates compared with those untreated with docetaxel before vitrification. Surviving oocytes also had higher rates of cleavage and development to the blastocyst stage after IVF. In conclusion, preincubation of IVM bovine oocytes with 0.05 μM docetaxel for 30 min before vitrification was effective at preventing CSF damage during vitrification, and improving oocyte viability after warming and subsequent cleavage and blastocyst formation after IVF.

1. Introduction

Vitrification of oocytes is a powerful method for preservation of genetic diversity in endangered animal species and farm animals through establishment of oocyte banks [42]. However, damage of the cytoskeleton fibers (CSF) of oocytes during vitrification is the main cause of abnormal spindle configuration and reduced viability of frozen–thawed oocytes [29]. Recently, it was reported that the stabilization of CSF before vitrification could be beneficial for reducing CSF damage in oocytes [24]. In fact, several cytoskeleton stabilizing agents, such as cytochalasin B and D (CB, CD) [40,49] and paclitaxel (Taxol®) [38], have been used to protect oocytes from CSF damage during vitrification. Furthermore, it is well documented that preincubation of matured oocytes with paclitaxel can reduce CSF damage after vitrification-warming and improve embryo development in humans [19], mice [31], pigs [38] and cattle [36].

Docetaxel is a recently identified member of a class of anti-cancer drugs, taxane diterpenoids, which also includes paclitaxel [5]. However, the modes of action of these two drugs are different. Docetaxel has been shown to promote both the rate and extent of tubulin polymerization into stable microtubules (MT) and inhibit MT depolymerization when exposed to ultra-low temperature; the rate and extent of tubulin polymerization promoted by docetaxel were twice those of paclitaxel [41]. Furthermore, the effective affinity of docetaxel for the MT binding site is 1.9-fold greater than that of paclitaxel [20]. In addition, docetaxel induces a twofold greater decrease of the critical concentration of GTP-tubulin required for tubulin polymerization when compared with paclitaxel [20,9].

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Taken together, docetaxel may be more efficient than paclitaxel at stabilizing CSF of oocytes during vitrification and improving their viability after vitrification-warming. However, the toxicity of docetaxel on bovine oocytes has never been investigated. Furthermore, the effects of pretreatment of *in vitro* matured (IVM) bovine oocytes with docetaxel before vitrification on CSF integrity and oocyte survival after vitrification-warming, and rates of their subsequent embryo development, have not been reported. Therefore, the present study was conducted to investigate: (1) the effects of various doses of docetaxel in the preincubation medium on IVF of bovine oocytes; (2) the effects of preincubation with docetaxel before vitrification on CSF integrity of oocytes; and (3) the effects of preincubation with docetaxel before vitrification on oocyte survival after warming, and their rates of cleavage and subsequent development to the blastocyst stage after IVF.

2. Materials and methods

Unless otherwise stated, all chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tissue culture medium (TCM-199; HEPES buffer with Earle's salts and sodium bicarbonate) and fetal calf serum (FCS) were obtained from Life Technologies (Carlsbad, CA, USA).Stock solutions of docetaxel (01885; Sigma–Aldrich) were prepared at 100 mM concentration in dimethylsulfoxide (DMSO).

2.1. Oocyte collection

Bovine (*Bos indicus*) ovaries were obtained from a local abattoir and transported to the laboratory in normal saline solution (0.9% NaCl with 0.1 g/L streptomycin) at ambient temperature. The cumulus-oocyte complexes (COCs) were recovered by aspiration from antral follicles (2–6 mm) using an 18-gauge needle connected to a 10 mL syringe containing modified Dulbecco's Phosphate Buffered Saline (mDPBS) with 10% FCS. The ovarian follicular fluid was pooled in 50 mL conical tubes and allowed to settle to the bottom of the tube during a 5 min interval. The COCs were selected under a stereomicroscope and washed five times with mDPBS with 10% FCS; those with more than two or three layers of cumulus cells and uniform cytoplasm (grades A and B) were subjected to *in vitro* maturation (IVM) [33].

2.2. IVM of oocytes

COCs were cultured using the procedure previously described [34]. Briefly, the COCs were washed three times with mDPBS supplemented with 10% FCS and three times with TCM-199 supplemented with 20% FCS. Groups of 20 COCs were then cultured for 24 h in 100 μL droplets of IVM medium (TCM-199 supplemented with 20% 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) at 38.5 °C under a humidified atmosphere of 5% CO_2 in air.

2.3. Vitrification and warming of IVM oocytes

Vitrification and warming of IVM oocytes were performed according to the method previously described by Dinnyés et al. [14]. Briefly, cumulus cells of IVM oocytes were partially removed by gentle pipetting with a pulled pipette using 0.1% hyaluronidase in TCM-199. Subsequently, oocytes were washed three times with basic medium (BM) consisting of TCM-199 supplemented with 20% FCS; groups of five oocytes were then placed in an equilibration medium consisting of 4% ethylene glycol (EG) [29] in BM supplemented with 20% FCS at 39 °C for 12–15 min. Thereafter, oocytes were rinsed three times in 20 μL droplets of vitrification solution consisting of 35% EG, 5% polyvinylpyrrolidone (PVP) and 0.4 M trehalose in BM for 25–30 s. They were then dropped as 1–2 μL droplets directly onto the surface of a steel cube that had been covered with aluminum foil and cooled to around −150 to −180 °C by partial immersion in liquid nitrogen (LN_2). The droplets were instantaneously vitrified. Using nitrogen-cooled forceps, the vitrified droplets were moved into 1.5 mL LN_2-filled cryovials and stored in a LN_2 tank for 3 weeks. Thereafter, the vitrified droplets containing oocytes in cryovials were moved immediately by using nitrogen-cooled forceps into a 35 mm petri dish containing with BM supplemented with 0.3 M trehalose at 39 °C for 2 min, followed by treatment with BM supplemented with 0.15, 0.075 and 0.0375 M trehalose for 1 min each. Then, the retrieved oocyte were washed and transferred to BM until used in the following procedures.

2.4. Evaluation of oocyte survival after vitrification-warming

At 2 h after warming, survival of vitrified oocytes was evaluated by fluorescein diacetate (FDA) staining, according to the method described by Mohr and Trounson [28]. Briefly, oocytes were treated with 2.5 μg/mL FDA in mDPBS supplemented with 5 mg/mL bovine serum albumin (BSA) at 38.5 °C for 2 min in darkness and then washed three times with mDPBS supplemented with 5 mg/mL BSA. Oocytes were evaluated under a fluorescence microscope (IX-71; Olympus, Tokyo, Japan) with UV irradiation using a U-MWIB3 filter with an excitation wavelength of 460–495 nm and emission at 510 nm. Oocytes expressing bright green fluorescence were regarded as living and were used in subsequent experiments.

2.5. Microtubule (MT) and chromosome (CM) staining

Microtubule (MT) and chromosome (CM) staining were performed according to the method previously described by Adona et al. [3]. Briefly, at 2 h after warming, oocytes freed from cumulus cells were fixed in 3% paraformaldehyde supplemented with 0.6% Triton X-100 in mDPBS with 0.1% polyvinyl alcohol (PP) [37] for 30 min. Subsequently, they were washed three times in PP and blocked in 3% goat serum in PP for 45 min. Then, oocytes were stained with FITC-conjugated anti-α-tubulin antibody (1:100 in PP) for 1 h. After staining, oocytes were washed three times with PP and stained with 10 μg/mL propidium iodide (PI) for 15 min, then washed in PP and mounted between a slide and a coverslip in glycerol. MT and CM morphology was observed under fluorescence microscopy.

MT morphology was classified into two categories – (1) normal: barrel-shaped, with chromosomes clustered as a discrete bundle at the metaphase plate and MT crossing the length of the spindle from pole to pole, or extending from the spindle poles to the chromosomes. MT within the polar body had no discernible organization and instead appeared as an amorphous mass intertwined with chromatin occupying the perivitelline space. (2) Abnormal: MT were not organized into typical spindles, or some were disassembled. Details of MT patterns found are provided in Fig. 1. CM organization was classified into two categories – (1) dispersed: CM were scattered throughout the cytoplasm or dispersed in a few zones of the cytoplasm; (2) condensed: CM with an aberrant, less condensed appearance. Details of CM patterns found are provided in Fig. 1.

2.6. Staining of cortical granules (CG) and mitochondria (MC)

Cortical granules (CG) were detected following a protocol described previously [8], with modifications. Briefly,

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