Effects of pretreating in vitro matured of native Thai cattle oocyte with docetaxel before vitrification on their viability

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Abstract. The present study was to investigate the efficacy of docetaxel (DT) at reducing microtubule damage during vitrification and maintaining the developmental competence of vitrified-warmed oocytes in native Thai cattle. Native Thai cattle cow oocyte from ultrasound-guided transvaginal follicular aspiration (UTFA) were subjected to in vitro maturation (IVM). Then, the IVM oocytes were pretreating with DT in various concentration (0.0, 1.0, 2.0 and 3.0 μM) and subjected to cryopreservation by Cryotop vitrification method. The survival rate of vitrified-warmed oocyte, cleavage and blastocyst formation rate after in vitro fertilization (IVF), and blastocyst nucleic cell numbers were evaluated in this study. Pretreatment of IVM native Thai cattle oocytes with 0.05 μM DT before vitrification resulted in significantly higher (P < 0.05) rates of oocyte survival and cleavage after IVF, and subsequent blastocyst formation on Days 7-8, hatching and hatched on Days 8-9, compared with oocytes pretreated with 0.5 and 1.0 μM DT before vitrification or those vitrified and the control group. Pretreatment of IVM native Thai cattle oocytes with 0.05, 0.5 and 1.0 μM DT before vitrification without side effects on blastocyst nucleic cell numbers. In summary, pretreatment of IVM native Thai cattle oocytes with 0.05 μM DT before vitrification improved survival of vitrified-warmed oocytes, fertilization and developmental competence.

1. Introduction
At the present, several native cattle species in world were dramatically decreased because the culture and way of life of farmers were changed. Moreover, the livestock production system was changed from smallholding farmers to a commercial farming system and needed higher production. This change was responsible for a loss of genetic diversity and extinction of native cattle species [1, 2]. Therefore, worldwide countries needed to conserve native cattle breeds and try to maintain their populations, in order to provide an important genetic resource for cattle breeding in the improvement of commercial cattle breeds in various countries [3]. In fact, native cattle were resistant to insect and parasite diseases, heat tolerance and environment adaptation from climate change and global warming. The conservation of native cattle in natural environments was difficult because there are not incentives for low economic return. Therefore, there are great risks to extinction in the future [4]. In the present, the cryopreservation of animal gametes like oocytes or spermatozoa was essential biotechnology for ex situ conservation of endangered animal species. This is genomic banking for animal breeding or restoration of endangered or extinct animal species in the future [5].
A previous study in cryopreservation of bovine oocytes was successful in an improvement of oocyte quality for freezing. This included development of new cryopreservation methods like slow freezing and vitrification, development of freezing media and containers. The percentage of in vitro fertilization of frozen oocytes and their development to the blastocysts stage was limited after warming [6]. Several studies have been reported that the oocyte cytoskeleton fiber (CSF) was broken during cryopreservation [7,8,9]. This cryoinjury problem was limited to frozen-thawed oocyte development after warming. In fact, the cytoskeleton stabilization of oocytes by pretreating the oocytes with a cytoskeleton stabilizer agent like Taxol before freezing has been investigated and improved frozen-thawed oocyte development showed a decrease in oocyte cytoskeleton damage [10,11,12,13]. DT is another cytoskeletal stabilizer agent investigated to protected the bovine oocyte cytoskeleton from damage during cryopreservation and showed was the more effective in protecting the bovine oocyte cytoskeleton damage than Taxol [9].

Therefore, the aim of the present study was to investigate the efficacy of DT at reducing microtubule (MT) damage during vitrification and maintaining the developmental competence of vitrified-warmed oocytes native Thai cattle.

2. Materials and Methods
The research protocol was approved by the Animal Ethical Research Committee of Princess of Naradhiwas University, Thailand.

2.1. Experimental design
IVM native Thai cattle oocytes were collected from donor native Thai cows in the same batch of a UTFA session were separated into five treatment groups consisting of Group 1: IVM oocytes were subjected to IVF/IVC (fresh control group), Group 2: IVM oocytes were subjected to vitrification (Vitri-control group), Group 3: IVM oocytes were pretreated with 0.05 μM for 30 min before vitrification, Group 4: IVM oocytes were pretreated with 0.5 μM DT before vitrification and Group 5: IVM oocytes were pretreated with 1.0 μM DT before vitrification. Then, the vitrified-warmed oocytes in all treatment groups were subjected to IVF and IVC. The IVF rate, blastocyst formation rate on Day 7-8, blastocyst hatching rate on Day 8-9 and Day 7 blastocyst cell numbers were compared among treatment and control groups. The experiment was replicated eight times.

2.2. Oocyte collection in native Thai beef cattle
Ten native Thai cows (4.13 ± 0.14 years old and 218.52 ± 24.82 kg; mean ± SEM) were synchronized of estrus according to the method previously described [14]. Briefly, the cow was estrus synchronized by injecting 500 μg prostaglandin F2α (PGF2α) (Estrumate, Coopers, Berkhamsted, England) twice at an interval of 12 d and the cow estrous behaviors was observe every 12 h after last PGF2α injection. All dominance follicles (DF) (≥4 mm in diameter) were removed by ultrasound-guided follicular aspiration at 48-h after estrus and was setting for 0 h of superstimulation follicular growth program. Cow were received 100 mg of follicle stimulating hormone (FSH: Folltropin-V) dissolved in polyvinylpyrrolidone (PVP) at 36 h by a single dose administration after removed DF; the oocyte collection was performed by using ultrasound-guided transvaginal follicular aspiration (UTFA) method at 72 h after follicular aspiration. Then, at 36-h after the preceding UTFA was the time set for stimulation of follicular growth by single dose injecting 100 mg of FSH for the subsequent UTFA session. Each cow was performed in 8 UTFA session.

The UTFA were performed according to the method previously described [14]. Briefly, the cow was handling in a cattle chute and was then prepared for UTFA by intramuscular injection of 0.02 mg/kg BW Xylazine (L.B.S. Laboratory Ltd., Bangkok, Thailand) and the epidural anesthesia with 3-4 mL of 2.0% lignocaine (Union drug laboratories Ltd., Bangkok, Thailand). The UTFA was performed by using a sector intravaginal 7.5-MHz transducer (Honda Electronics Co., Ltd, Japan) with a 17G×490 mm cow ova vacuum needle (Misawa Medical, Tokyo, Japan) connecting with aspiration pump (Vakuum pumpe; Minitüb GmbH, Tiefenbach, Germany) with a vacuum of 120 mmHg and 22
mL/min aspiration rate through a disposable aspiration needle. The aspirated follicular fluid was kept in modified Dulbecco’s phosphate buffered saline (mDPBS), supplemented with 1.0% fetal calf serum and 125 IU/mL heparin in 50 mL tubes. The oocyte collection were finding under stereomicroscopic control inside a petri dish and recovered oocyte >1 layers of cumulus cells were subjected to the in vitro maturation (IVM) in this study.

2.3. IVM of native Thai cattle oocytes
IVM of native Thai cattle oocytes are retrieved from the UTFA method were performed by the method previous described [9] by minorly modified. Briefly, the IVM medium was TCM-199 (Gibco™ Medium 199; Thermo Fisher Scientific, Waltham, MA, USA) added with 5% (v/v) newborn calf serum (NBCS) (N4637, Sigma-Aldrich), 0.5 μg/mL FSH (F2293, Sigma-Aldrich), 50 IU/mL penicillin (P3032, Sigma-Aldrich) and 50 mg/mL streptomycin (S9137, Sigma-Aldrich). The oocyte was subjected to IVM by washing twice in IVM medium. After washing twice with IVM medium, the groups of 15-20 oocyte were cultured in 100 μL droplets of IVM medium with liquid paraffin covered (18512, Sigma-Aldrich) in tissue culture dishes for 22 h at 38.5 °C in 5% CO2 in air with a high humidity.

2.4. Pre-treating of IVM native Thai cattle oocytes with DT before vitrification
DT (01885, Sigma-Aldrich) stock solutions of were prepared by dissolving it in dimethyl sulfoxide (DMSO) (D454, Sigma-Aldrich) at concentrations of 0.1mM and keep them at -30 °C in 10 μL aliquots. The working solutions of 0.05, 0.5 and 1 μM DT were performed by dissolving stock solutions in 10,000, 1,000 and 100 times with TCM medium and supplemented with 3 mg/mL PVP (P0930, Sigma-Aldrich). After IVM, matured oocyte was partially removed expanded cumulus cells by treatment with 0.5 mg/mL hyaluronidase (H4272, Sigma-Aldrich) for 1 min, leaving for two or three layers of cumulus cells on the surface. The oocytes were then incubated in DT working solution for 30 min at 38.5 °C in 5% CO2 in air with high humidity. They were then washed three times in TCM-199 medium and maintain in TCM-199 medium at 38.5 °C until further use.

2.5. Vitrification and warming of native Thai cattle oocytes
IVM native Thai cattle oocytes were kept in a base medium (BM) until vitrification. The BM was TCM-199 added with 20% (v/v) NBCS. Cryotop® (Kitazato, Tokyo, Japan) vitrification of oocytes was performed as described previously [8] with minorly modifications. The vitrification medium was preparation, consisting of VS-1: BM with 2.0% (v/v) ethylene glycol (EG) (E9129, Sigma-Aldrich) and 2.0% (v/v) DMSO and VS-2:BM with 17.5 % (v/v) EG, 17.5 % (v/v) DMSO, 50 mg/mL PVP and 0.3 M trehalose (T0167, Sigma-Aldrich). Vitrification was performed by incubating groups of 5-10 matured oocyte for 12 to 14 min in VS-1 at 38.5 °C. The matured oocytes were then washed 3 times in 20 μL droplet of VS-2 at 37 °C for 30s. Thereafter, they were immediately placed on a Cryotop sheet with a minimum volume of VS-2 medium and immediately immerse in liquid nitrogen. The vitrified oocyte in all treatment group were kept in a liquid nitrogen tank for up to two weeks. Thawing of vitrified oocytes was performed by immersing the Cryotop sheets in 0.3 M trehalose in BM for 2 min, followed by 0.15 M, 0.075 M and 0.0375 M trehalose in BM for 1 min each. The live vitrified oocytes were evaluated for an intact plasma membrane after warming. Then retrieved live oocytes were washed and kept in BM medium until further use. All procedures were performed at 37 °C on a warm plate.

2.6. In vitro fertilization (IVF) of IVM oocytes and in vitro culture (IVC) of embryos
For IVF, frozen semen from a native Thai cattle bull was thawed in warming water at a 37 °C for 1 min and then moved to 3 mL of a 90% Percoll® (P4937, Sigma-Aldrich) solution in a 15 mL tube and centrifuged at 740×g for 10 min. The spermatozoa sedimet was then resuspended with 6 mL Tyrode’s albumin lactate pyruvate-HEPES (TALP-HEPES) medium and centrifuged at 540×g for 5 min at 37 °C. The final concentration of spermatozoa was adjusted to 3 × 10^6 sperm/mL. The oocytes were
washed twice in TALP-IVF medium at 38.5 °C. Then, groups of 20 oocytes were IVF with spermatozoa in 100 μL droplets of TALP-IVF medium. The oocytes and spermatozoa were co-incubated for 6 h at 38.5 °C in 5% CO2 in air with a high humidity. At the end of IVF, IVF oocyte were removed of cumulus cells by gentle pipetting in IVC medium. Thereafter, groups of 15 to 20 were cultured in IVC medium in separate culture drops and covered with paraffin oil. IVC was performed in 100 μL drops of Charles Rosenkrans (CR1) [15] medium with amino acids (CR1aa) [16] added with 5% NBCS and 0.25 mg/mL of linoleic acid albumin (L8384, Sigma-Aldrich). Embryos were cultured for 9 days at 38.5 °C in 5% CO2, 5% O2 and 90% N2 in air with a high humidity.

2.7. Evaluation of trophectoderm (TE) and inner cell mass (ICM) cell numbers in blastocysts
Day 7 blastocysts (Day 0 = day of IVF) were subjected to TE and ICM cell number evaluation by a differential staining protocol [17], with modifications. Briefly, blastocysts were washed three time in phosphate buffered saline (PBS) and PBS added with 3 mg/mL PVP. Then, the TE cell were staining was performed by incubation them in 0.1 mg/mL propidium iodide (PI) (P4170, Sigma-Aldrich) and 0.2% (v/v) Triton X-100 in PBS for 1 min. The ICM cells staining was performed by incubation them in 25 μg/mL Hoechst 33342 (B2261, Sigma-Aldrich) dissolved in 99.5% ethanol for 5 min. The stained embryos were washed in glycerol and mounted them on individual glass microscope slides flattened in glycerol by cover slips to a level where all nuclei appeared at the same focal plane, and visualized using a fluorescent microscope (Olympus) under UV light with excitation at 330-385 nm and emission at 420 nm. A visualized image of each embryo under fluorescent microscope was captured, and the numbers of TE (pink) and ICM (blue) cell were counted by using ImageJ 1.40 software [18].

2.8. Statistical analysis
All data were analyzed by one-way ANOVA at a \( P < 0.05 \) significance level followed by Tukey’s multiple comparison test using SAS v. 9.2 software (SAS Institute, Cary, NC, USA). Percentage data were arcsine transformed before analysis.

### Table 1. Survival of frozen-thawed IVM native Thai cattle oocytes pretreated with different concentration of DT before vitrification and their subsequent development after IVF.

| Pretreatment with DT | Vitrification | Total n | Live † (%) | Cleaved, n (% live). | Developed to blastocyst, n (% live). |
|----------------------|--------------|---------|-------------|----------------------|------------------------------------|
| -                    | -            | 125     | 119 (95.2±3.1) | 103 (86.6±3.5) a | 41 (34.5±3.6) b | 66 (55.5±2.7) c |
| -                    | +            | 130     | 105 (80.8±3.3) c | 69 (65.7±4.3) c | 25 (23.8±2.3) c | 33 (31.4±3.5) c |
| DT (0.05 μM)         | +            | 120     | 108 (90.0±3.1) b | 83 (76.9±3.1) b | 39 (36.1±2.1) b | 43 (39.8±2.3) b |
| DT (0.50 μM)         | +            | 132     | 110 (83.3±2.8) c | 75 (68.1±3.2) c | 28 (25.5±3.5) c | 37 (33.6±3.3) c |
| DT (1.00 μM)         | +            | 125     | 93 (74.4±2.6) d | 49 (52.7±3.1) d | 16 (17.2±3.5) d | 24 (25.8±3.8) d |

Eight replications were performed. Data are presented as mean ± SEM. †Oocyte survival is expressed as the rate of oocytes with intact plasma membrane after warming. *Day 0 = the day of IVF.

Values with different superscripts in the same column are significantly different at \( P < 0.05 \) (one-way ANOVA).

3. Results
As shown in Table 1, pretreated of IVM native Thai cattle oocytes with 0.05 μM DT before vitrification resulted in significantly higher (\( P < 0.05 \)) oocyte survival rates after warming, the
cleavage rate, and the blastocyst formation rate on Day 7 and Day 8 were compared with pretreatment with 0.5 and 1.0 μM DT before vitrification and those vitrified without any pretreatment. Nevertheless, the oocyte survival rates after warming, subsequent cleavage, and blastocyst formation rate on Day 7 and Day 8 after IVF in all vitrified groups were significantly lower (P < 0.05) than the control group.

The hatching and hatched blastocyst formation rates on Day 8 and Day 9 in the group pretreated with 0.05 μM DT before vitrification and the control group were similar. Vice versa, the hatching and hatched blastocyst formation rates on Day 8 and Day 9 in both pretreated with 0.05 μM DT before vitrification and the control group were significant higher (P < 0.05) than those of pretreated with 0.5 μM and 1.0 μM DT before vitrification and vitrification control group, as shown in Table 2.

Table 2. Blastocyst hatching during IVC of embryos derived from IVF of vitrified-warmed IVM native Thai cattle oocytes pretreated with different concentration of DT before vitrification.

| Pretreatment with DT | Vitrification | Total blastocysts | Day 8*, n (% total) | Day 9*, n (% total) |
|----------------------|---------------|-------------------|---------------------|--------------------|
|                      |               |                   | Hatching | Hatched | Hatching | Hatched |
| -                    | -             | 40                | 10       | (25.0±1.5)a | 23       | (57.5±1.6)a |
|                      | +             | 21                | 5        | (23.8±1.6)b | 10       | (47.6±1.6)b |
| DT (0.05 μM) +       | 23            | 6                 | (26.1±1.8)c | 13       | (56.5±1.8)c |
| DT (0.50 μM) +       | 22            | 5                 | (22.7±1.2)c | 10       | (45.5±1.5)c |
| DT (1.00 μM) +       | 20            | 4                 | (20.0±1.4)c | 7        | (35.0±2.1)c |

Eight replications were performed. Data are presented as mean ± SEM. *Day 0 = the day of IVF. Values with different superscripts in the same column are significantly different at P < 0.05 (one-way ANOVA).

In addition, the investigated number of nuclei in Day 7 blastocysts was found that an insignificant difference among groups in the total number of nuclei (cells), number of TE and ICM cells and the ratio of ICM cells, as shown in Table 3.

Table 3. Number and proportion of TE and ICM cells in blastocysts* derived from IVF of vitrified-warmed native Thai cattle oocytes pretreated with different concentration of DT before vitrification.

| Pretreatment with DT | Vitrification | Blastocysts evaluated, n | No. of nuclei TE | No. of nuclei ICM | Total cells | ICM ratio (%) |
|----------------------|---------------|--------------------------|------------------|-------------------|-------------|---------------|
| -                    | -             | 26                       | 46.6±3.559.3±2.105.3±6.4 | 32.0±2.1 |
|                      | +             | 15                       | 45.6±4.258.8±2.104.2±5.3 | 30.4±1.9 |
| DT (0.05 μM) +       | 20            | 49.9±3.864.3±3.111.6±5.7 | 29.8±2.0 |
| DT (0.50 μM) +       | 17            | 45.4±4.158.1±2.105.5±4.6 | 29.3±2.3 |
| DT (1.00 μM) +       | 16            | 45.9±3.257.6±3.100.5±6.2 | 30.1±1.8 |

Eight replications were performed. TE = trophoderm; ICM = inner cell mass. *Day 7 blastocysts (Day 0 = the day of IVF). Data are presented as mean ± SEM.

4. Discussion

DT is a microtubule (MT) stabilizing agent like Taxol [19]. DT have MT as their target organs. In fact, when it bonds with MT it acts to link the α-β tubulin dimer in MT. Moreover, DT improved the MT-associated proteins (MAPs) and α-β tubulin dimmers binding; it affects to improve the stabilizer of MT [20]. Therefore, MT were not damage or broken in the ultralow temperature when pretreat with DT before freezing [21]. Furthermore, it has been reported that DT can decrease the concentration of
tubulin-critical concentration to induce the depolymerization of tubulin for polymerization of MT formation [22]. Then, the increase of MT in the cell were assisted in completing the mitochondria (MC) distribution. Moreover, DT stimulated MT organizing centers (MTOC) to produce the MT from tubulin free in the cell, resulting in MT and tubulin that were in equilibrium in the cell [23]. In addition, DT stimulated the MT and tubulin equilibrium with the depolymerization mechanism and stimulation of cytoskeleton fibers (CSF) damage repair in the cell [24]. In fact, the post warming frozen cell showed normal growth and development after warming with stabilized MT with DT before freezing [21].

The present study investigated of the efficiency of various concentration of DT to protected native Thai cattle oocyte from MT degenerated during freezing by vitrification methods. The results in this study indicated the high rate of survival, IVF, blastocyst formation, hatching and hatched blastocyst formation on Day 8 and Day 9 of vitrified-warmed native Thai cattle oocytes in the group pretreated with 0.05 μM DT before vitrification. In fact, previous studies have investigated the efficiency of pretreatment of the IVM bovine oocyte with DT before vitrification and also found that vitrified-warmed bovine oocytes in the groups of pretreated with 0.05 μM of DT before vitrification showed higher MT integrity, survival rate, IVF and blastocyst formation rate in comparison with untreated with DT before vitrification [21, 9]. Chasombat et al. [21] and Pitchayapipatkul et al. [9] reported that 0.05 μM of DT is an effective dose for reducing MT disassembly and maintaining the developmental competence of M-II bovine oocytes during vitrification compared to 1.0 μM taxol or untreated oocytes with a MT stabilizer agent before vitrification. Moreover, they were reported that preincubated bovine oocytes with 0.05 μM DT before vitrification and vitrified-warmed oocytes had less CSF degenerated and higher survival rates compared with those untreated with DT before vitrification. These results indicated that DT could stabilize MT by causing tighter links between a- and b-tubulin dimers and by enhancing microtubular cross-linking following changes in the conformation and binding of high-molecular-weight MAPs [25]. It was able to prevent MT degenerate or depolymerization during vitrification of the bovine oocytes.

Chasombat et al. [21] investigated the toxicity of DT on IVM bovine oocytes by pretreatment them with 0.0, 0.05, 0.5, 5.0 and 50 μM DT before IVF and found that 0.05 μM DT had no adverse effect on IVM, IVF and IVC, while DT at a concentration of ≥ 0.5 μM inhibited their development of the oocyte to metaphase II stage (MII). In fact, the present study that investigated the efficiency of pretreated IVM native Thai cattle oocytes with 0.05, 0.5 and 1.0 μM before vitrification found that vitrified-warmed native Thai cattle oocyte in the group of pretreated with 0.5 and 1.0 μM of DT before vitrification had lower rates of survival, IVF and blastocyst formation, hatching and hatched blastocyst formation on Day 8 and Day 9 in compared with the group of pretreated 0.05 μM DT before vitrification. Moreover, when evaluation of TE and ICM cell numbers in this study showed a steady decrease of TE, ICM and total cells of Day 7 blastocysts with the increased concentrations of DT (0.05, 0.5 and 1.0 μM) in treatment groups, but it showed insignificant difference among treatment groups. This result confirmed the previous study that the 0.5 and 1.0 μM of DT have a toxic effect on vitrified-warmed native Thai cattle oocytes. Furthermore, Chasombat et al. [21] and Pitchayapipatkul et al. [9] also found that pretreated bovine oocytes with 0.05 μM DT before vitrification had an adverse effect on blastocyst quality in terms of the number of TE and ICM cells in D7 blastocysts in comparison with the control group or groups untreated with the DT before vitrification. In fact, a previous study reported that high concentrations of DT an blockade cell development by inhibiting nuclear and cytoplasmic maturation, thus restrain the cell development rate [26]. Moreover, Schrevel et al. [27] reported that DT at concentrations ≥ 0.5 μM inhibited cell development due to its binding with tubulin of MT, which effected on the stabilization of the MT polymerization. This results in the loss of balance among MT and tubulin, thus causing disturbance with the function of key enzymes such as cyclin, which is responsible for stimulating cyclin-dependent kinases (CDKs) and the maturation-promoting factor (MPF). Therefore, when cyclin, CDKs and MPF function abnormally in the cell cycle, then cell was discontinuing development to the next stage. Moreover, it has been
reported that DT at high concentrations induces the pro-apoptotic proteins Bax and Bak while it inhibits the anti-apoptotic proteins Bcl-2 and Bcl-x-long, thus induced cell apoptosis [28].

In conclusion, pretreatment of IVM native Thai bovine oocytes with 0.05 μM DT before vitrification showed improved survival in vitrified-warmed oocytes, fertilization and developmental competence. Further research will be needed to evaluate the developmental competence to term of the resultant blastocysts transfer to native Thai cow recipients.

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