

Ultrasound-guided Transvaginal Follicular Aspiration and Development of Vitrified-thawed Thai Indigenous Beef Cattle (*Bos indicus*) Oocytes after *in vitro* Fertilization

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Abstract

This study was done to evaluate the efficiency of ultrasound-guided transvaginal follicular aspiration without hormonal pre-stimulation for collecting the oocytes of indigenous Thai beef cattle (*Bos indicus*), and to assess the vitrification method for their preservation. All animals between 2.5-12 years were categorized according to age (i.e. heifer, young cow, and old cow). A medium-sized follicle (ϕ 3-8 mm.) had the greatest distribution among the various size classes ($p \leq 0.05$), and the mean of oocytes collected from each group was not significantly different. Post-thawed survival was 60.78, 53.70 and 57.50%, with a cleavage rate of 38.71, 37.93 and 39.13%, and a blastocyst embryo of 6.45, 3.45 and 4.35% among heifers, young cows and old cows, respectively, which was significantly less than the fresh control group and solution control group ($p \leq 0.05$). The present study suggests that OPU in Thai indigenous beef cattle without hormonal pre-stimulation can be routinely done for an extended period of time. The age of donor does not affect the number and quality of oocytes aspirated, post-thawed viability and the number of embryos developing after IVF.

Keywords: cryopreservation, GMP, IVF, OPU

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บทคัดย่อ

การเจาะเก็บโอโอไซต์ด้วยเครื่องคลื่นความถี่สูงในโคพื้นเมืองไทย (*Bos indicus*)

และการพัฒนาของโอโอไซต์ที่ผ่านการแช่แข็งแบบวิทริฟิเคชันหลังการปฏิสนธิภายนอกร่างกาย

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การศึกษานี้มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพการเจาะเก็บโอโอไซต์ผ่านทางช่องคลอดด้วยเครื่องคลื่นความถี่สูงในโคเนื้อพันธุ์พื้นเมืองไทยโดยไม่ใช้ฮอร์โมนกระตุ้นรังไข่ก่อนการเจาะเก็บและการเก็บรักษาโอโอไซต์ที่เจาะเก็บได้ในรูปแบบแช่แข็งโดยวิธีวิทริฟิเคชันจัดแบ่งโคอายุระหว่าง 2-12 ปี ออกเป็น 3 กลุ่ม ตามช่วงอายุ (โคสาว แม่โคอายุน้อย แม่โคอายุมาก) ผลการศึกษาพบว่า พอลลิเคิลที่พบบนรังไข่ส่วนใหญ่เป็นพอลลิเคิลขนาดกลาง (ϕ 3-8 มม.) ($p \leq 0.05$) และจำนวนโอโอไซต์ที่เจาะเก็บได้เฉลี่ยในโคแต่ละกลุ่มไม่แตกต่างกัน อัตราการรอดชีวิตของโอโอไซต์หลังการละลายมีค่าเท่ากับร้อยละ 60.78, 53.70 และ 57.50 อัตราการแบ่งตัวหลังปฏิสนธิภายนอกร่างกายมีค่าเท่ากับร้อยละ 38.71, 37.93 และ 39.13 และการพัฒนาของเอ็มบริโอเข้าสู่ระยะบลาสโตซิสมีค่าเท่ากับร้อยละ 6.45, 3.45 และ 4.35 ในโคสาว แม่โคอายุน้อย และแม่โคอายุมาก ตามลำดับ โดยผลที่ได้มีค่าต่ำกว่ากลุ่มควบคุมโอโอไซต์และกลุ่มควบคุมสารละลายวิทริฟิเคชัน ($p < 0.05$) การเจาะเก็บโอโอไซต์ผ่านทางช่องคลอดด้วยเครื่องคลื่นความถี่สูงในโคเนื้อพันธุ์พื้นเมืองไทยโดยไม่ใช้ฮอร์โมนกระตุ้นรังไข่ก่อนการเจาะเก็บ สามารถเจาะเก็บต่อเนื่องเป็นงานประจำได้ โดยที่อายุของโคตัวให้ไม่มีผลต่อจำนวนและคุณภาพของโอโอไซต์ที่เจาะเก็บได้ อัตราการรอดชีวิตของโอโอไซต์หลังการทำละลาย และการพัฒนาของเอ็มบริโอหลังการปฏิสนธิภายนอกร่างกาย

คำสำคัญ: การแช่แข็ง ปิเปตแก้วขนาดเล็ก ปฏิสนธิภายนอกร่างกาย การเจาะเก็บโอโอไซต์

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Introduction

Thai indigenous beef cattle (*Bos indicus*) play a prominent role in rural livestock production in Thailand, where they serve as a food source, for draught power, and as a part of the cultural structure of the society. Due to their tolerance of intense sunshine and humidity and better resistance to ticks and tropical diseases, they are often crossed with exotic breeds to form meat herds (Wannapat, 2004; Boonyanuwat et al., 2009).

Agricultural modernization over the last 25 years has dramatically reduced the numbers, and threatened loss of the genetic resource of a pure breed of Thai indigenous beef cattle (Boonyanuwat et al., 2005). The downward trend in the local purebred market and slaughterhouse suggests that the Thai indigenous beef cattle breed will be lost if no conservation efforts are made. Drucker (2010) reported that 16% of livestock breeds have been lost

over the last 100 years and over 20% of the remaining breeds are at risk as production systems pose the greatest threat to livestock diversity throughout the world. To address the various aspects of rescuing genetic material, *in situ* conservation of live populations is the most realistic way to conserve breeds of livestock regarded as threatened, and is logistically and financially unfeasible (Taberlet et al., 2008). Assisted reproductive technologies (ART) are of interest in the field of preservation of endangered animal species, as these emerging technologies have the potential to increase reproductive efficiency, speed up genetic progress, expand the number of animals that can be bred from superior parents and improve other biotechnologies that use oocytes (Ledda et al., 2001; Pilling et al., 2007).

Oocyte cryopreservation represents an alternative method of preserving female genetic resources (Massip, 2003). The main sources of bovine oocytes have been from slaughterhouse ovaries, rarely are pure breed ovaries recovered, making it difficult

to apply the technology to these breeds. Thus, transvaginal ultrasound-guided ovum pick-up (OPU) with a non-invasive technique for retrieving oocytes from a live donor is possibly a better source for oocytes. This technique, together with cryopreservation of oocytes and *in vitro* embryo production (IVP), could be the way to conserve the genetics of Thai indigenous beef cattle.

The success rate of OPU in cattle is affected by number and quality of follicles present at the time of collection. The follicular population is related to the state of estrous cycle (Garcia and Salaheddine, 1998). Sakhong et al. (2011) reported that in Thai native cattle greater numbers of follicles emerged in heifers than in cows. Furthermore, Rizos et al. (2005) observed more follicles aspirated and COCs collected from heifers compared to lactating cows. Lerner et al. (1986) discriminated decreased numbers of oocytes per embryos accompanied by lowered fertilization rates and lower embryo quality with advanced age of the donors. In addition, Su et al. (2009) observed the highest cleavage and blastocysts rates in young cows followed by middle aged and older cows. Thereby, age of oocyte donors may influence the number and quality of oocytes collected and their development.

The present study was therefore undertaken among Thai indigenous beef cattle to (a) determine the efficiency of transvaginal ultrasound-guided aspiration of ovarian follicles without hormone pre-stimulation for collecting oocytes in differences donor age, and (b) explore the potential of developmentally-competent of post-thawed vitrified oocytes derived OPU for their preservation.

Materials and Methods

This experiment was conducted in the northeastern region of Thailand, between February and May, 2010. A total of 15 Thai indigenous beef cattle (5 heifers, 2.80 ± 0.15 years of age on average, 5 young cows, 5.60 ± 0.51 years of age on average, and 5 old cows, 9.40 ± 0.75 years of age on average) with a 3.0-3.5 body condition score (BCS; 1-5 scoring), raised at the Animal Farm, Khon Kaen University were used. Body weight of the cows ranged between 183 and 274 kg and were 2.5 and 12 years. Manipulations were performed once a week for 10 weeks. The protocol and management of the animals were reviewed and approved by the Animal Ethics Committee of Khon Kaen University, based on the Animal Ethics Experimentation Guideline of the National Research Council of Thailand. All chemicals and media used for the oocytes and embryos were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated. Lymphoprep™ was obtained from AXIS-SHIELD PoC AS (Also, Norway) and penicillin-streptomycin from Gibco (Grand Island, NY, USA).

Experimental design: The estrous cycles of our Thai indigenous beef cattle were synchronized before the experimental period *as per* Sakhong et al. (2011). Animals were categorized according to their age, but assigned to their respective treatment group by a

completely randomized design. The efficacy of ultrasound-guided follicle aspiration in Thai indigenous beef cattle for vitrification was then investigated; according to the different reproductive status of donors, number of visible follicles recovered and quality of the cumulus oocyte complexes (COCs). The utilization of vitrification procedure for cryopreservation of the oocytes, cleavage rate and development of embryos were also determined. Non-vitrified oocytes from slaughter house were used as the fresh controls and oocytes that were performed according to the vitrification method, but were not dipped into liquid nitrogen, were used as the control solution. The cleavage and subsequent development to the blastocyst stage were determined at days 2 and 8 after fertilization respectively.

Oocyte aspiration from Thai indigenous beef cattle: During oocyte collection via ultrasound-guided aspiration, the animals were restrained in a chute, and epidural anesthesia (1 ml/100 Kgbw of 2% (v/v) lidocaine HCl) was administered before manipulation. Follicular aspiration was performed using an ultrasound machine (Honda HS-2000) with a 7.5 MHz transvaginal transducer with a needle guide, a single lumen 17-gauge 490 mm long sterile needle (COVA-needle; Misawa Medical, Tokyo, Japan) and a vacuum pressure of 120 mmHg (Minitub N86 KN.18, Tiefenbath, Germany). The flushing medium was Dulbecco's phosphate buffered saline (DPBS) supplemented with 2% (v/v) penicillin-streptomycin, 50 units/ml heparin, and 2% fetal bovine serum (FBS).

The number and size of follicles in each ovary were determined and characterized on the basis of diameter as small (≤ 3 mm), medium (3-8 mm) and large (> 8 mm) size. The probe carrier was deeply inserted into the vagina and the needle penetrated the vaginal wall into the follicle while the ovary was fixed rectally. Ultrasound imagery allowed the ovary to be positioned so that the needle could be guided to aspirate the medium size of COCs. After collapsing the follicular wall, the needle was withdrawn and the next follicle approached. Upon completing each cow, the fluid was searched for COCs within 30 min of aspiration.

In vitro maturation of oocytes: The recovered COCs were graded according to oocyte cytoplasm aspect and number and morphology of cumulus cell layers *as per* Viana et al. (2004), as follows: Grade A: compact COCs, more than three layers of cumulus cells and oocyte with homogeneous cytoplasm, Grade B: compact COCs, with three or two layers of cumulus cells and oocyte with homogeneous cytoplasm, Grade C: compact COCs with one layers of cumulus cells, or oocyte with slightly heterogeneous cytoplasm, Grade D: oocytes showing complete removal of cumulus cells from less than 1/3 of zona pellucida (ZP) surface or showing expansion of cumulus cell and Grade E: oocytes with expanded cumulus cells

They were transferred to the holding medium comprising TCM-199 HEPES + 10% fetal bovine serum (FBS). The COCs were washed three times with the *in vitro* maturation (IVM) medium consisting of TCM-199 supplemented with 10% FBS, 5

µg/ml follicle stimulating hormone, 1 µg/ml estradiol-17β, 5 µg/ml Luteinizing hormone and 0.2 mM sodium pyruvate. The 10-12 recovered COCs were matured in 50 µl droplets of the IVM medium, covered with paraffin oil, in a 35 mm petri-dish for 22 hrs in an incubator at 38.5°C with a moist atmosphere containing 5% CO₂ in air (Wani et al., 2004).

Vitrification and warming: Oocytes handling was performed at ambient temperature while all media were maintained at 37°C on a hot plate. The oocytes grades A, B and C with an expanded multilayer cumulus and homogenous ooplasm were selected and washed three times in TCM-199 HEPES medium. The expanded cumulus-enclosed oocytes were exposed to 100 µl of equilibration solution (1.5 M EG + 1.5 M DMSO + 20% FBS) for 2 min before exposing them to the final vitrification solution (3.0 M EG + 3.0 M DMSO + 0.5 M Sucrose + 35% (v/v) Lymphoprep™ + 10% FBS), which were adapted from Checurea and Seidel Jr. (2007). The expanded oocytes were loaded into the glass micropipette (GMP) vessel with the latest vitrification solution, and dipped horizontally in liquid nitrogen. All the procedures from exposure of the oocytes to final vitrification solution to dipping of the loaded GMP in liquid nitrogen were done within 45 sec. After the GMP was stored in liquid nitrogen for a minimum of 1 month, they were thawed in a stepwise manner in 1,000 µl of 0.50, 0.25, and 0.00 M sucrose solutions in TCM-199 HEPES supplement with 20% FBS at 38.5°C for 5, 5 and 10 min, respectively. All of the oocytes were washed three times in a maturation solution then cultured in IVM medium for 3 hours.

Assessment of thawed oocytes: After thawing, all of the oocytes were assessed for viability as previously described (Sripunya et al., 2010). Briefly, after 3 hours of maturation, all of the oocytes were washed three times in DPBS + 4 mg/ml BSA (mDPBS) then incubated in the dark at 37°C in mDPBS + 0.1 mg/ml fluorescence diacetate (FDA) for 2 min. The oocytes were washed five times in mDPBS, then a droplet was placed into the same solution in a petridish covered with mineral oil and viewed using reverse fluorescence stereomicroscopy (Nikon, Japan). The surviving oocytes with viable cytoplasm fluoresced green whereas damaged ones did not. Only oocytes with a viability and morphologically normal appearance were counted, and used for *in vitro* fertilization (IVF) for 18 hours.

In vitro fertilization of oocytes: The spermatozoa were prepared for insemination by modification of a method described by Wani et al. (2004). Briefly, the contents of two 0.25 ml straws of frozen Thai indigenous beef cattle semen were thawed in water at 37°C for 1 min. The semen was washed twice with TALP medium, containing 10 µg/ml heparin by centrifugation at 1800 rpm for 5 min each. The spermatozoa were suspended in TALP medium containing 0.5% fatty acid free BSA, 10 µg/ml heparin and 5 mM caffeine, and 50 µl droplets of these were incubated for 1 hr at 38.5°C in a CO₂ incubator before insemination. After 22 hours of *in vitro* maturation, 10-12 oocytes were washed three times in TALP

medium (containing fatty acid-free BSA, heparin and caffeine) and introduced into 50 µl droplets of processed spermatozoa (10-12 × 10⁶ spermatozoa/ml) and left for 18 hours in a CO₂ incubator at 38.5°C. At the end of the sperm-oocyte incubation, the oocytes were separated from sperm droplets and washed with Synthetic oviductal fluid (SOF) and cultured for 8 days.

In vitro culture: At approximately 18 hours post-insemination, presumptive zygotes were denuded by vortexing and washed three times in SOF before being transferred in groups of 15 to 50 µl culture droplets of SOF under mineral oil. The dishes were incubated with a moist atmosphere of 5% CO₂ at 38.5°C in a CO₂ incubator. The cleavage rates were recorded at day 2 after insemination as was the proportion of oocytes developing to the blastocyst stage accessed at day 8 after insemination.

Statistical Analysis: Statistical comparisons between and within treatments were carried out by a General linear model technique (SAS Institute Inc., 2004). Statistical significance was established at $p \leq 0.05$. The number of aspirated follicles and the recovery rate during various sessions for different donor ages were compared using the least square means. The numbers of normal oocytes recovered after thawing from the vitrified groups, the number of oocytes cleaved, and the total number of morulas and blastocysts developed from the oocytes vitrified in the difference classes of donors were compared using the chi-square test.

Results

Efficacy of ultrasound-guided follicle aspiration in Thai indigenous beef cattle: Data on follicle populations of various size categories by age of donors is presented in Table 1. No difference was found in the number of various sizes of follicles according to the age of donors. By contrast, a medium-sized follicle was the most common follicle size class ($p \leq 0.05$), and no difference in the numbers of follicles between the left and right ovaries was found. The numbers of oocytes of various grades recovered from donors are presented in Table 2. A total of 272 COCs were recovered by aspiration from 1,473 visible follicles, for an overall rate of 18.47% (ranging from 13.97-23.06%) while 2.27 COCs were harvested per donor. The mean number of total follicles and oocytes recovered did not differ significantly among groups of donors. The mean numbers of medium size follicles aspiration and oocytes recovered per donor in various sessions were different ($p \leq 0.05$) and the oocyte recovered from first to subsequent sessions were affected (Table 3).

Utilization of vitrified oocytes in Thai indigenous beef cattle: The M-II oocytes from three different age groups were loaded into the glass micropipette (GMP) with the vitrification solution and stored in liquid nitrogen for at least 1 month. Post-thawed oocytes and survival of oocytes are presented in Table 4. After thawing, 145 (66.82%) from the 217 vitrified oocytes were recovered, only 83 (57.24%) of these were judged viable.

Table 1 Means of various sizes of follicles (small, ≤ 3 mm; medium, 3-8 mm; large, >8 mm) in Thai indigenous beef cattle detected by ultrasonography using transvaginal probe (7.5 MHz) during OPU sessions

Donor class	No. of OPU session	No. of visible follicles		
		Small	Medium	Large
Heifers	40	2.74 \pm 0.79 ^b	12.05 \pm 0.54 ^a	1.00 \pm 1.15 ^b
Young cows	40	2.65 \pm 0.83 ^b	12.58 \pm 0.54 ^a	1.00 \pm 1.09 ^b
Old cows	40	2.50 \pm 0.81 ^b	10.93 \pm 0.56 ^a	1.13 \pm 1.22 ^b

Values (lsmeans \pm SEM) in same row with different superscripts differ significantly ($p \leq 0.05$).

Table 2 Successful ultrasound-guided follicle aspiration and recovery rate among Thai indigenous beef cattle of different age groups

Donor class	No. of aspired follicles	No. of recovered oocytes (%)	Recovered oocytes/donor ²	No. of cumulus oocyte complexes quality (%) ¹			
				Grade A	Grade B	Grade C	Grade D
Heifers	490	113 (23.06)	2.75 \pm 0.22 ^a	14 (12.38) ^b	25 (22.12) ^b	53 (46.90) ^a	21 (18.58) ^b
Young cows	525	95 (18.10)	2.38 \pm 0.22 ^a	12 (12.63) ^b	10 (10.53) ^b	49 (51.58) ^a	24 (25.26) ^{ab}
Old cows	458	64 (13.97)	1.55 \pm 0.22 ^b	7 (10.94) ^b	20 (31.25) ^{ab}	24 (37.50) ^a	13 (20.31) ^b

¹Values in same row with different superscripts differ significantly ($p \leq 0.05$).

²Values (lsmeans \pm SEM) in same column with different superscripts differ significantly ($p \leq 0.05$).

Table 3 Means of medium size follicles aspiration and oocytes recovered per donor among Thai indigenous beef cattle of different session

Session	No. of donor	No. of medium size follicles/donor	Oocytes recovered/donor
First	15	9.87 \pm 0.97 ^c	2.60 \pm 0.36 ^b
Second	15	13.40 \pm 0.97 ^a	3.73 \pm 0.36 ^a
Third	15	11.80 \pm 0.97 ^{ab}	2.53 \pm 0.36 ^b
Fourth	15	13.00 \pm 0.97 ^a	1.67 \pm 0.36 ^{cb}
Fifth	15	13.73 \pm 0.97 ^a	2.67 \pm 0.36 ^{cb}
Sixth	15	11.00 \pm 0.97 ^{ab}	1.93 \pm 0.36 ^{cb}
Seventh	15	11.73 \pm 0.97 ^{ab}	1.87 \pm 0.36 ^{cb}
Eighth	15	13.67 \pm 0.97 ^a	1.20 \pm 0.36 ^c

Values (lsmeans \pm SEM) in same column with different superscripts differ significantly ($p \leq 0.05$).

Table 4 Recovery viability of post-thawed oocytes and development of embryos from oocytes vitrified among Thai indigenous beef cattle of different age groups

Treatment	No. of oocytes (%) examination				No. of blastocyst (%)
	Post-thawed recovery	Viability	Insemination	Cleavage	
Fresh control	-	-	22	17 (77.27) ^a	6 (27.27) ^a
Solution control	-	-	24	17 (70.83) ^a	6 (25.00) ^a
Heifers	51 (62.20)	31 (60.78)	31	12 (38.71) ^b	2 (6.45) ^b
Young cows	54 (68.35)	29 (53.70)	29	11 (37.93) ^b	1 (3.45) ^b
Old cows	40 (71.43)	23 (57.50)	23	9 (39.13) ^b	1 (4.35) ^b

Values in same column with different superscripts differ significantly ($p \leq 0.05$)

Discussion

The results of our investigation demonstrate that: (1) recovery of oocytes from Thai indigenous beef cattle (taken via transvaginal ultrasound-guide aspiration) following vitrification and IVF can

The most common morphological damages to oocytes were degenerated cytoplasm (4.15%), shrinkage of cytoplasm (1.84%) or rupture of the zona pellucida with loss of cytoplasm (0.92%). Based on our results, there was no difference detected among the post-thaw recovery and viability of the oocytes among the groups of donors. The proportion of embryos developing to cleavage (38.55%) and blastocyst (4.82%) was lower than those of the fresh control and solution control groups ($p \leq 0.05$). The number of vitrified oocytes recovered and the development of embryos from oocytes vitrified among the different classes of donors of Thai indigenous beef cattle are shown in Table 4.

develop to the blastocyst stage; (2) different age classes of donors did not affect the percentage of oocyte recovery in terms of number, quality or blastocyst yield; and (3) weekly repeated OPU procedures without hormonal stimulation over a long period had no effects on ovarian function. These results suggest that we may collect oocytes from Thai

indigenous beef cattle using the OPU method throughout their lifespan after puberty and that the use of OPU together with cryopreservation of the oocytes can preserve oocytes of Thai indigenous beef cattle in an oocyte bank for subspecies conservation.

A total of 1,473 follicles were aspirated from 120 sessions, giving a mean of 12.28 aspirated follicles per cow, and a total number of 2.27 oocytes harvested per donor. These data are less than in earlier reports without FSH treatment: 3.45 in an Indian breed (Manik et al., 2003) and with FSH stimulation: 4.00-5.25 in southern Thai native cattle (Thongsodsang et al., 2004), 4.40-7.00 in a Gyr breed (Viana et al., 2004), 2.46 in northern Thai native cattle (Apimeteetumrong et al., 2009), and 2.22 in pregnant Angus breed treated with eCG (Aller et al., 2012). These results may be affected by a number of factors such as age of oocytes donors, stimulation protocol, time interval and gonadotropin dosage used (Bungartz et al. 1995; Presicce et al. 1997). It has been reported previously that a number of follicle emergence per wave were 4.00-7.71 follicles and the use of PMSG in Thai native cattle increased the numbers and sizes of follicles in heifers more than in cows (Sakhong et al., 2011; 12). Moreover, twice a week of follicle aspiration gave a higher number of harvestable follicles and more oocytes recovered than once a week (Garcia and Salaheddine, 1998).

We found that approximately 46.32% of COCs were classified as grade 3, although previous studies reported that 41.04% were grade 2 among northern Thai native cattle (Apimeteetumrong et al., 2009) and 32% were grades 1 and 2 among Holstein cows (De Roover et al., 2005). The various sizes of follicular populations may explain the resultant oocyte yields observed in our study. The low mean number of oocytes recovered per donor obtained in this study could have been caused by the characteristic of the ovary in this breed. Thai indigenous beef cattle have a small ovary size (1.51x2.29 cm²; Sakhong, 2011) which is located distally to the small coiled horn of the uterus, and tightens the ligament that supports the female reproductive organs. It is, therefore, difficult to control the position of the ovaries when the needle penetrates during OPU. Sakhong et al. (2012) observed that the use of PMSG, which had comparable responses between heifers and cows in Thai native cattle, can induce follicular growth and hyperplasia of ovary. Thus, additional gonadotropin treatment may be beneficial to handle ovaries and higher oocyte yields per cow per OPU collection. However, a more frequent collection schedule (every 3-4 days) will prevent a dominant follicle from impacting negatively on smaller follicles and the cow will not cycle, will not form a corpus luteum and will not produce high levels of estrogen as she would normally (Wangtendonk-de Leeuw, 2006). Furthermore, the needle tip bevel and the aspiration procedure affect the morphology of COCs and the developmental capacity of the oocytes after IVF (Bols et al., 1996), as increasing the aspiration vacuum results in a decrease in the number of compact COCs.

In this study we used the vitrification method and GMP as the cryo-device, which enabled their subsequent *in vitro* development after IVF. The narrower diameter of the glass capillary of the GMP vessel ought to yield a higher cooling rate (Vajta et al., 1998), which should increase the viability of vitrified oocytes after thawing, capability to IVF and subsequent development to the blastocyst stage. The viability of oocytes immediately post-cryopreserved was difficult to assess in this study due to the presence of cumulus cells; notwithstanding, the fluorescein diacetate (FDA) staining was used to assess viability of the post-thawed oocytes. The viability of post-thawed oocytes (53.70-60.78%), cleavage (37.93-39.13%) and development to the blastocyst stage (3.45-6.45%) was similar to vitrified oocytes collected from slaughterhouses (i.e. 72.90% and 4.17% for cleavage and blastocyst yields, respectively) (Magnusson et al., 2008), and those preserved using Cryotop vitrification (53.2% and 8.7% cleavage rate and blastocyst yields on day 8, respectively) (Sripunya et al., 2010). One possible explanation for the low developmental rates could be that cold storage oocytes causes meiotic spindle depolymerization which can lead to loss of chromosomes and subsequent aneuploidy (Wood et al., 1992). Notwithstanding, we observed similar rates of cleavage rate subsequent blastocyst formation on day 8 of vitrified oocytes from heifers, young cows and old cows group. These results indicate that the solution, the GMP method and warming regimen used in the present study allowed their normal fertilization and were effective for cryopreservation of M-II Thai indigenous beef cattle oocytes.

A stress to cumulus cells during the freezing procedure might be reflected in an adverse effect on the oocyte (Ambrosini et al., 2006), and treating the oocyte with a high concentration of CPA and cooling rate of M-II oocytes may trigger biochemical reactions which can result in hardening of the zona pellucida of oocyte (Shaw et al., 2000; Ambrosini et al., 2006), that prevents the penetration of spermatozoa into the oocyte. The granulosa cells of the cumulus and the zona pellucida play important roles in the development of competence of immature oocytes (Ambrosini et al., 2006). In addition, the role of the cumulus cells during vitrification will protect M-II oocytes against vitrification-induced damage. Kuwaya et al. (2005) reported higher survival and blastocyst rate for cumulus-enclosed than cumulus-free human M-II oocytes after vitrification. Tharasanit et al. (2009) found that cumulus-enclosed equine M-II oocytes preserved their meiotic spindle and chromatin quality better during vitrification than denuded oocytes.

In summary, the use of OPU together with vitrification of the oocytes using a glass micropipette facilitated preservation efforts among Thai indigenous beef cattle. Importantly, repeated aspirations were possible, the aspirated COCs were viable and vitrified oocytes were able to develop to blastocyst stages upon IVF. Further studies are needed to investigate the efficiency of embryo transfer using the OPU vitrified oocytes in Thai indigenous beef cattle.

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