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Output ที่ได้จากโครงการ

1. ตีพิมพ์ในวารสารวิชาการนานาชาติที่มี impact factor 2 เรื่อง

1.1. Liang Y.Y., Ye, D.N., Laowtammathron, C., Phermthai, T., Nagai, T., and Parnpai, R. **2010.** Effects of chemical activation treatment on development of swamp buffalo (*Bubalus bubalis*) oocytes matured *in vitro* and fertilized by intracytoplasmic sperm injection. ***Reprod. Domestic Anim.*** doi: 10.1111/j.1439-0531.2010.01636.x (IF = 1.606) **เอกสารแนบหมายเลข 1**

1.2. Srirattana, K., Lorthongpanich, C., Laowtammathron, C., Imsoonthornruksa S., Ketudat-Cairns, M., Phermthai, T., Nagai, T. and Parnpai, R. 2010. Effect of donor cell types on developmental potential of cattle (*Bos taurus*) and swamp buffalo (*Bubalus bubalis*) cloned embryos. ***J. Reprod. Dev.*** 56: 49-54. (IF = 1.697) **เอกสารแนบหมายเลข 2**

1.3. Liang, Y., Phermthai, T., Nagai, T., Somfai, T. and Parnpai, R. 2010. *In vitro* development of vitrified buffalo oocytes following parthenogenetic activation and intracytoplasmic sperm injection. ***Theriogenology*** Submitted 21 September 2010; Revised and re-submitted 28 December 2010; Accepted 31 December 2010 (IF = 2.073) **เอกสารแนบหมายเลข 3**

2. Manuscript กำลังเขียนเพื่อส่งไปตีพิมพ์

2.1. Liang, Y., Phermthai, T., Nagai, T., Somfai, T. and Parnpai, R. 2011. Survival rates of matured buffalo oocytes after vitrification by Microdrop and Cryotop and subsequent embryos development after intracytoplasmic sperm injection. จะส่งไปตีพิมพ์ในวารสาร ***Anim. Reprod. Sci.*** (IF = 1.563) **เอกสารแนบหมายเลข 4**

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3. ส่งผลงานไปประชุมวิชาการในการประชุมระดับนานาชาติ 3 เรื่อง

3.1. Liang, Y., Phermthai, T., Nagai, T., Somfai, T. and **Parnpai, R.** 2010. Developmental rates of vitrified buffalo oocytes following parthenogenetic activation and intracytoplasmic sperm injection. Proceeding of The 9th World Buffalo Congress, 25-28 April, 2010, Buenos Aires, Argentina, p.850-857. **เอกสารแนบหมายเลข 5**

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4. ส่งผลงานไปประชุมวิชาการในการประชุมระดับชาติ 3 เรื่อง

4.1. หยวนหยวน เหลียง และ รังสรรค์ พาลพ่าย. 2553. อัตราการอยู่รอดของไข่สุกกระบือปลักที่ผ่านการ vitrification ด้วยวิธี Microdrop และ Cryotop และการเจริญเติบโตของตัวอ่อนภายหลังการฉีดตัวอสุจิเข้าในไซโตพลาสซึม. ***เรื่องเต็มการประชุมทางวิชาการมหาวิทยาลัยเกษตรศาสตร์ครั้งที่ 48 เล่มที่ 3 สาขาสัตวแพทยศาสตร์.*** กรุงเทพฯ, หน้า 95-102. **เอกสารแนบหมายเลข 8**

4.2. ขวัญฤดี แก้วมุงคุณ กาญจนา ปัญญาไว ศิวัช สังข์ศรีทวงษ์ และ **รังสรรค์ พาลพ่าย.** 2553. ผลของโกรทแฟ็กเตอร์ต่อการเจริญเติบโตของฟอลลิเคิลขนาดเล็กภายนอกร่างกายในกระบือปลัก: การศึกษาเบื้องต้น. ***การประชุมวิชาการเกษตร ครั้งที่ 11 ประจำปี 2553 คณะเกษตรศาสตร์ มหาวิทยาลัยขอนแก่น,*** หน้า 309-313. **เอกสารแนบหมายเลข 9**

4.3. หยวนหยวน เหลียง อนวัช แสงมาลี สุเมธ อิ่มสุนทรรักษา จันทร์เจ้า ล้อทองพานิชย์ กนกวรรณ ศรีรัตนานุชจรินทร์ ศรีปัญญา วันวิสาข์ ผิวสร้อย ขวัญฤดี แก้วมุงคุณ ชุติ เหล่าธรรมธร ดานา เย มารินา เกตุทัต-คาร์นส์ และ รังสรรค์ พาลพ่าย. 2552. ผลของการใช้สารเคมีกระตุ้นการแบ่งตัวต่อการพัฒนาของตัวอ่อนกระบือภายหลังการ ICSI. ***เรื่องเต็มการประชุมทางวิชาการของมหาวิทยาลัยเกษตรศาสตร์ครั้งที่ 47 เล่มที่ 3 สาขาสัตวแพทยศาสตร์.*** กรุงเทพฯ, หน้า 78-86. **เอกสารแนบหมายเลข 10**

5. รางวัลที่ได้รับซึ่งเป็นผลมาจากการทำวิจัยเรื่องนี้

5.1. รางวัลแทนคุณแผ่นดิน สาขาวิทยาศาสตร์ ประจำปี 2550 จากเครือเดอะเนชั่น

6. การดำรงตำแหน่งในองค์กรด้านกระบือนานาชาติ

เมื่อเดือนตุลาคม 2552 ในการประชุม The 6th Asian Buffalo Congress (ABC) ณ ประเทศปากีสถาน หัวหน้าโครงการวิจัยได้รับการคัดเลือกให้ดำรงตำแหน่ง President of Asian Buffalo Association และเมื่อเดือนเมษายน 2553 ในการประชุม The 9th World Buffalo Congress ณ ประเทศอาร์เจนตินา หัวหน้าโครงการวิจัยได้รับการคัดเลือกให้ดำรงตำแหน่ง President of International Buffalo Federation (IBF) และในฐานะที่เป็น Standing Committee ของ IBF หัวหน้าโครงการวิจัยได้จัดทำแผนการเสนอตัวให้ประเทศไทยเป็นเจ้าภาพจัดการประชุม The 10th World Buffalo Congress และ The 7th Asian Buffalo Congress ณ ภูเก็ต ในเดือนพฤษภาคม

2556 ซึ่งได้รับการลงคะแนนจากสมาชิกให้เป็นเจ้าภาพจัดการประชุมดังกล่าวได้ ดังโปสเตอร์ประชาสัมพันธ์การประชุมที่แนบมาด้วย

ภาคผนวก

เอกสารแนบหมายเลข 1

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Effects of Chemical Activation Treatment on Development of Swamp Buffalo (*Bubalus bubalis*) Oocytes Matured *In Vitro* and Fertilized by Intracytoplasmic Sperm Injection

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Contents

The objective of this study was to optimize the activation protocol for buffalo oocytes after intracytoplasmic sperm injection (ICSI). The release of the second polar body (PB) at 3, 6 and 9 h after ICSI of *in-vitro* matured oocytes activated either with 5 μM ionomycin (Io) or with 7% ethanol (EtOH) was preliminary examined. The highest rate of second PB extrusion occurred at 3 h of activation, and the second PB extrusion in EtOH group was significantly higher than that in Io group. Oocytes that extruded the second PB were selected and cultured either with 1.9 mM 6-dimethylaminopurine (6-DMAP) for 3 h or with 10 μg/ml cycloheximide (CHX) for 5 h. Significantly higher rate of oocytes formed 2 pronuclei in EtOH combined with CHX (EtOH + CHX) (62%) group compared to those of Io + CHX (42%) and EtOH + 6-DMAP (48%) groups ($p < 0.01$) whereas Io + 6-DMAP group showed intermediate value (58%). Significantly higher blastocyst formation rates were obtained in Io + 6-DMAP (29%) and EtOH + CHX (24%) groups than in Io + CHX (6%) and EtOH + 6-DMAP (17%) groups. Our results indicate that buffalo ICSI oocytes are effectively activated by combination treatment of Io with 6-DMAP and EtOH with CHX resulting in the highest cleavage and blastocyst formation rates.

Introduction

The importance of swamp buffalo (*Bubalus bubalis*) in many area of the world is equal to cattle in terms of meat and milk production, and labour, especially because of its resistance to hot climate, stress and diseases. Although traditional-assisted reproduction technologies are currently utilized in this species, the efficiency of reproduction is somewhat less than that of cattle because of inherent reproductive problems such as silent oestrous signs (Esposito et al. 1992; Zicarelli et al. 1997), long calving interval, delayed age of puberty and low number of primordial follicles (Van Ty et-al. 1994). As an assisted reproduction technology, artificial insemination using frozen spermatozoa has been established and applied for the genetic improvement of swamp buffalo (Muer et al. 1988; Shukla and Misra 2007). However, frozen buffalo spermatozoa sometimes show immobility after thawing (Muer et al. 1988) which may cause reduced fertility. To overcome this problem intracytoplasmic sperm injection (ICSI), the injection of a single spermatozoon directly into cytoplasm bypassing the zona pellucida and oolemma is the optimum procedure. As the egg will theoretically be fertilized using only a single sperm, this method is considered a standard way to produce normal diploid embryos; therefore, it may be the preferable way to produce the next generation in swamp buffalo. This technique has successfully been applied for buffaloes (Lu et al. 2006). Although ICSI alone seems to be sufficient to trigger the events of oocyte activation in some species such as hamsters, mice and humans (Perreault et al. 1988; Kimura and Yanagimachi 1995; Tesarik and Sousa 1995; Kuretake et al. 1996), certain species such as cattle (Rho et al. 1998) and pigs (Lee et al. 2003) require additional activation of the oocytes after ICSI to assure their development.

The events of oocyte activation including the release of cortical granules, accomplishment of meiosis, second polar body (PB) extrusion and pronucleus formation require the inactivation of protein kinases such as maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) (Liu and Yang 1999; Sun and Nagai 2003). During fertilization, the activity of these kinases collapses by repetitive elevation (oscillations) of intracellular calcium triggered by the sperm (Ducibella and Fissore 2008). Such effect can be imitated artificially by certain drugs; In cattle, for instance, treatment of oocytes with calcium ionophore alone decreased cyclin B (a subunit of MPF) level within 1 h and cyclin B level rose again 4–15 h after the treatment (Liu and Yang 1999). Ethanol (EtOH) was reported, for the first time, to be effective for the activation of *in vitro*-matured bovine oocytes (Nagai 1987) and has been used to activate buffalo oocytes (Parnpai and Tasripoo 2003) by promoting the formation of inositol 1,4,5-triphosphate (IP3) at the plasma membrane and inducing the extracellular calcium influx which would lead to a high intracellular calcium concentration (Ilyin and Parker 1992). Another chemical that elevates Ca^{2+} in oocytes is Ionomycin (Io) that has been successfully used in buffalo nuclear transfer, to induce repeated transient intracellular calcium rising (Saikhun et al. 2004). As full activation of oocytes leading to pronuclear formation requires the proper inactivation of both MPF and MAPK activities, a combination of the calcium rise and the inhibition of MPF and MAPK activities either by protein synthesis inhibitors [such as cycloheximide (CHX)] or by protein phosphorylation inhibitors [such as 6-dimethylaminopurine (6-DMAP)] is considered a reasonable approach to obtain optimal activation of oocytes.

From another perspective, activation treatment following ICSI increases the possibility of parthenogenetic embryo development. Activation procedures aimed at making buffalo oocytes suitable for ICSI differ from

those aimed at producing parthenogenesis; haploidy is required for the former whereas diploid is required for the latter. Treatment with CHX or 6-DMAP following the calcium rise reagents after ICSI would immediately inhibit the extrusion of the second PB resulting in triploid or parthenogenetic diploid embryos (Liu et al. 1998). Therefore, the selection of buffalo oocytes by the presence of the second PB, together with the additional activation treatment with CHX or 6-DMAP, may decrease the triploidy and parthenogenesis.

With the goal of producing diploid activated oocytes for ICSI, the objective of this study was to optimize the activation procedure. We compared the effects of four combined activation treatments using EtOH, Io, CHX and 6-DMAP on second PB extrusion, pronuclear formation, cleavage and subsequent development of buffalo ICSI oocytes. Preliminarily, the efficiency of EtOH and Io was examined by the release of second PB following ICSI.

Materials and Methods

Except where otherwise indicated, all reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Oocyte collection and *in vitro* maturation

Abattoir-derived buffalo ovaries were transported to the laboratory within 4 h and kept in physiological saline (0.9% NaCl). Cumulus–oocyte complexes (COCs) were collected from the follicles 2–6 mm in diameter using a 21-gauge needle attached to a 10-ml syringe as described earlier (Muenthaisong et al. 2007) for buffalo ovaries. Each of 20 COCs was washed five times with PBS supplemented with 0.1% polyvinylpyrrolidone (PVP), and three times with IVM medium, then cultured in 100-μl droplets of IVM medium covered with mineral oil in a humidified atmosphere of 5% CO_2 in air at 37°C for 21 h.

The *in vitro*-matured COCs were mechanically denuded by repeated pipetting with a fine-tip pipette in 0.2% hyarulonidase and washed five times in the Emcare holding medium (ICP-Bio, Auckland, New Zealand). Oocytes with a visible first PB were defined to be MII and selected for the ICSI.

Sperm preparation for ICSI

A straw of frozen buffalo semen from the same bull was thawed at 37°C for 30 s. Thawed spermatozoa were washed twice by centrifugation at 500 × *g* for 7 min in Brackett and Oliphant medium (Brackett and Oliphant 1975) supplemented with 10 mM caffeine (caffeine-BO) as described previously with some modification (Suteevun et al. 2006). The sperm pellet was resuspended in caffeine-BO at a concentration of 8×10^6 sperm/ml.

ICSI procedures

Sperm injection was performed under an inverted microscope (IX71; Olympus, Tokyo, Japan) with a micromanipulator (M0188NE; Narishige Tokyo, Japan). The inner diameter of the sperm injection needle was 8–10 μm, and the inner diameter of the holding pipette was 20 μm. Three droplets of solution were prepared on the lid of 60-mm culture dish and covered with paraffin oil. The first droplet was PVP solution (ICSI®, Vitrolife, Sweden) for washing the pipette, the second droplet was the sperm suspension with PVP solution (1 : 5) and the third droplet was Emcare medium for the ICSI procedure. Approximately 10 MII oocytes were placed in the droplet of Emcare medium. A single, motile buffalo spermatozoon was immobilized against the bottom of the second droplet. The tail of the sperm was loaded first with a minimum volume of the medium into the injection pipette, and then the injection pipette was moved to the droplet containing the oocytes. An oocyte was aspirated by the holding pipette at the 9 o'clock position, with the first PB being at either 12 or 6 o'clock position. The immobilized sperm was injected into the ooplasm at 3 o'clock position. Sham injection of oocytes was conducted by using the same procedure as sperm injection, except that no sperm was loaded in the injection pipette.

Activation of ICSI oocytes

Within 1 h of the injection, the injected oocytes (both ICSI and Sham groups) were activated by exposure either to 7% EtOH in Emcare medium for 5 min or to 5 μM Io in Emcare medium for 5 min, and then subsequently cultured in TCM199 + 10% FCS to allow extrusion of the second PB. The completion of the second meiosis was determined by the extrusion of the second PB observed at 3, 6 and 9 h after activation, and the optimal starting time for the further chemical activation treatments was decided. Injected oocytes without activation treatment were treated as the control group.

The injected and activated oocytes which extruded the second PB at 3 h of activation were selected and transferred to mSOF medium supplemented with either 1.9 mM 6-DMAP or 10 μg/ml CHX and cultured for 3 and 5 h, respectively, at 38.5°C under humidified atmosphere of 5% CO_2 in air. The oocytes treated with combined activations were grouped as Io + 6-DMAP, Io + CHX, EtOH + 6-DMAP and EtOH + CHX groups.

Nuclear staining

The pronuclear formation status in oocytes was evaluated after 18 h of ICSI. Total 193 ICSI oocytes were mounted on glass slides, immersed in ethanol: acetic acid (3 : 1, w:v) for 24 h and stained with 1% (w/v) aceto-orcein for observation. As all the evaluated oocytes presented a second PB, the absence of an intact spermatozoon or male pronucleus was considered as ICSI failure. Male and female pronuclei were identified by their position relative to the remaining sperm tail or mid-piece and to the second PB of the oocytes.

In vitro embryo culture

After the combined activation treatment, presumptive zygotes were further cultured in mSOF medium (Gardner

et al. 1994) supplemented with 3 mg/ml fatty acid–free BSA at 38.5°C under a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 2 days. The day of ICSI was considered Day 0. After that, 8-cell-stage embryos were selected and co-cultured with buffalo cumulus cells in mSOF medium at 38.5°C under humidified atmosphere of 5% CO_2 in air for additional 5 days. The medium was changed daily and the development of embryos was recorded at the same time for medium changing.

Evaluation of blastocyst cell number

The Sham-injected and ICSI blastocysts harvested from each treatment group at 7 days of IVC were counter-stained to distinguish total cell numbers. The blastocysts were fixed in 4% paraformaldehyde for 15 min, and then washed twice in PBS at room temperature. They were transferred to PBS containing 5 μg/ml Hoechst 33342 for 5 min. After being washed in PBS, they were transferred on a clean glass slide and covered with a cover slide. The total cell numbers were counted under a fluorescent microscope (IX71; Olympus, Tokyo, Japan) at 400 × magnification.

Experimental design

Experiment 1: The timing and frequency of second PB extrusion were determined after stimulation by EtOH or Io. Oocytes after ICSI or sham injection were treated with either 7% EtOH or 5 μM Io or without activation (Control) and cultured for 9 h. The extrusion of second PB was recorded at 3, 6 and 9 h after activation treatment.

Experiment 2: Pronuclear formation and second PB extrusion in ICSI oocytes was assessed after combined activation with Io or EtOH and CHX or 6-DMAP.

Experiment 3: In vitro embryo development and cell numbers in blastocysts were investigated after combined activation with Io or EtOH and CHX or 6-DMAP of ICSI and sham-injected oocytes.

Statistical analysis

Data were analysed by ANOVA using the statistical analysis systems. The differences between groups were considered to be statistically significant at a probability value of 0.05 or less.

Results

Induction of the second PB release by EtOH or Io

As shown in Table 1, a total of 104 injected buffalo oocytes including sham injection group without activation (control group) did not extrude the second PB after culture. The highest second PB extrusion rate (68%) was obtained in the EtOH group after 3 h of ICSI among all the groups ($p < 0.05$). When compared within the same treatment, most of the second PB extrusion occurred at 3 h after ICSI in both EtOH and Io groups. The number (%) of oocytes extruding the second PB at 3 h of activation out of total number of oocytes with the second PB within 9 h of activation was 73/87 (84) and 50/66 (76), respectively. There was no difference in the second PB extrusion rates between EtOH and Io groups in sham-injected oocytes and in both groups the most of the second PB extrusion occurred at 3 h of injection as well. These results show that, based on the percentage of second PB extrusion, EtOH was more effective for the activation of buffalo oocytes following ICSI than Io.

Table 1. The effect of exposure of intracytoplasmic sperm injection and sham-injected oocytes to 7% EtOH or 5 μM Io on the second PB extrusion in swamp buffalo

Chemical treatments	Sperm injection	Number of oocytes examined	Number (%) of oocytes extruded the second PB			
			Times after activation			
			3 h	6 h	9 h	Total
5 μM Io	+	108	50 (46)^{b}	15 (14)^{a}	1 (1)	66 (61)^{b}
	−	102	44 (43)^{b}	6 (6)^{ab}	0 (0)	50 (49)^{b}
7% EtOH	+	107	73 (68)^{a}	13 (12)^{a}	1 (1)	87 (81)^{a}
	−	101	54 (53)^{b}	5 (5)^{ab}	0 (0)	59 (58)^{b}
Control*	+	52	0 (0)^{c}	0 (0)^{c}	0 (0)^{c}	0 (0)^{c}
	−	52	0 (0)^{c}	0 (0)^{c}	0 (0)^{c}	0 (0)^{c}

EtOH, ethanol; Io, ionomycin; PB, polar body.
*Control means no activation.
^{a,b,c}Means within columns with different superscripts significantly differ ($p < 0.05$ ANOVA).

Table 2. Pronuclear formation after intracytoplasmic sperm injection of buffalo oocytes followed by various activation protocols

Activation treatments	Number oocytes examined	Number (%) oocytes exhibiting			
		2PB + 2PN[1]	2PB + 1PN[2]	2PB + Sperm[3]	2PB[4]
Io + 6-DMAP	45	26 (58)^{ab}	16 (36)^{a}	1 (2)	2 (4)^{b}
Io + CHX	50	21 (42)^{b}	21 (42)^{a}	2 (4)	6 (12)^{a}
EtOH + 6-DMAP	48	23 (48)^{b}	18 (38)^{a}	1 (2)	6 (13)^{a}
EtOH + CHX	50	31 (62)^{a}	15 (30)^{a}	1 (2)	3 (6)^{b}

EtOH, ethanol; Io, ionomycin; 6-DMAP, 6-dimethylaminopurine; CHX, cycloheximide; PB, polar body; PN, pronucleus/pronuclei.
^{a,b}Means within columns with different superscripts differ ($p < 0.05$ ANOVA).
[1]Oocytes extruded a second PB and had 2 pronuclei.
[2]Oocytes extruded a second PB and had 1 pronucleus.
[3]Oocytes extruded a second PB and had an intact sperm head.
[4]Oocytes extruded a second PB and had no pronucleus.

Pronuclear formation in ICSI oocytes

As shown in Table 2, a significantly higher proportion of ICSI oocytes extruded a second PB and had 2 pronuclei (2PB + 2PN) in EtOH + CHX group (62%) than those in Io + CHX (42%) and EtOH + 6-DMAP (48%) groups; however, there was no difference from the Io + 6-DMAP group (58%). The proportion of activated oocytes having a single pronucleus showed no difference among oocytes of the four activation treatments.

Effect of combined activation treatment after ICSI on embryonic development *in vitro*

As shown in Table 3, the cleavage rates of ICSI oocytes in Io + 6-DMAP, EtOH + 6-DMAP and EtOH + CHX groups (76%, 69% and 78%, respectively) were significantly higher than those in Io + CHX group

Table 3. *In vitro* development of buffalo embryos derived from intracytoplasmic sperm injection followed by different activation treatments

Activation treatments	Sperm injection	No. (%) of oocytes: Injected	Extruded second polar body	Cultured	No. (%) cleavage	No. (%) of embryos developed to: 8-cell stage	Morulae	Blastocysts
Io + 6-DMAP	+	187	84 (45)^{b}	84	64 (76)^{a}	47 (56)^{a}	28 (33)^{a}	24 (29)^{a}
	−	102	44 (43)^{b}	44	27 (61)^{bc}	19 (43)^{bc}	8 (18)^{d}	6 (14)^{b}
Io + CHX	+	170	84 (49)^{b}	84	44 (52)^{c}	19 (23)^{d}	11 (13)^{c}	5 (6)^{c}
	−	102	46 (45)^{b}	46	23 (50)^{c}	10 (22)^{d}	2 (4)^{d}	2 (4)^{c}
EtOH + 6-DMAP	+	132	85 (64)^{a}	85	59 (69)^{ab}	35 (41)^{bc}	21 (25)^{b}	14 (17)^{b}
	−	101	54 (53)^{b}	54	28 (52)^{c}	18 (33)^{cd}	8 (15)^{d}	4 (7)^{bc}
EtOH + CHX	+	125	83 (66)^{a}	83	65 (78)^{a}	40 (48)^{ab}	27 (33)^{ab}	20 (24)^{a}
	−	97	49 (51)^{b}	49	29 (59)^{bc}	19 (39)^{bc}	8 (16)^{d}	6 (12)^{b}

EtOH, ethanol; Io, ionomycin; 6-DMAP, 6-dimethylaminopurine; CHX, cycloheximide.
^{a,b,c,d}Means within columns with different superscripts differ ($p < 0.05$ ANOVA).

(52%) and sham-injected control group in all activation treatments. Significantly higher blastocyst formation rates were obtained in Io + 6-DMAP and EtOH + CHX groups (29% and 24% respectively) than those in Io + CHX and EtOH + 6-DMAP groups (6% and 17%, respectively) and sham-injected groups. The Io + CHX group showed the lowest blastocyst formation rate among all the groups. There was no difference in cleavage and blastocyst formation rates between ICSI and sham-injected oocytes in Io + CHX oocytes. Within the sham-injected group, the cleavage rate of these four treatments was not different; however, the blastocysts formation rate in the Io + CHX group (4%) was significantly lower than that in Io + 6-DMAP (14%) and EtOH + CHX (12%) groups. There was no difference in blastocyst rates among sham-injected oocytes in Iσ + 6-DMAP, EtOH + 6-DMAP and EtOH + CHX groups.

Cell numbers in day 7 blastocysts derived from injected oocytes

As shown in Table 4, the cell number of blastocysts obtained from ICSI oocytes in EtOH + CHX group (88.9 ± 23.0) was significantly higher than that in Io + CHX (53.8 ± 14.9); however, it did not differ from those in Io + 6-DMAP (79.3 ± 21.7) and EtOH + 6-DMAP (74.1 ± 13.9) groups. Within each treatment group, the cell number of blastocysts in ICSI and sham-injected oocytes was not different.

Table 4. Cell numbers of blastocysts derived from injected oocytes followed by various activation treatments

Activation treatments	Sperm injection	No. of embryos examined	Cell numbers (Mean ± SD)
Io + 6-DMAP	+	9	79.3 ± 21.7^{ab}
	−	6	64.3 ± 22.7^{bc}
Io + CHX	+	4	53.8 ± 14.9^{bc}
	−	2	38.5 ± 9.2^{c}
EtOH + 6-DMAP	+	7	74.1 ± 13.9^{ab}
	−	4	70.8 ± 9.9^{b}
EtOH + CHX	+	10	88.9 ± 23.0^{a}
	−	6	72.0 ± 14.6^{ab}

EtOH, ethanol; Io, ionomycin; 6-DMAP, 6-dimethylaminopurine; CHX, cycloheximide.
^{a,b,c}Means within columns with different superscripts differ ($p < 0.05$ ANOVA).

Discussion

In this study, we investigated the second PB extrusion, pronuclear formation, cleavage and development to the blastocyst stage of buffalo ICSI oocytes to determine the optimal activation protocol. To our knowledge, this is the first study to report high percentages of buffalo ICSI oocytes developing to the blastocyst stage by the combination of chemical activation treatments such as Io + 6-DMAP or EtOH + CHX. These two combinations supported the highest second PB extrusion and resulted in the highest blastocyst rates and cell numbers per embryo. The activation protocol combining Io with 6-DMAP has also been proven to get the best results in sheep (Shirazi et al. 2008), goats (Ongeri et al. 2001) and cattle (Rho et al. 1998). Combining EtOH with CHX treatment was also reported to achieve high activation rates for both young and ageing bovine oocytes (Presicce and Yang 1994a,b).

In this study, without additional chemical activations, none of the oocytes extruded the second PB and developed, indicating that mere sperm injection was not sufficient to activate buffalo oocytes. In other words, an additional activation treatment of buffalo oocytes following ICSI was necessary for the oocytes to complete the meiosis, subsequent pronuclear formation and embryo development. The same necessity of additional activation treatments for the oocytes after ICSI to assure their development was reported for other species such as cattle (Rho et al. 1998) and pigs (Lee et al. 2003). In contrast, in hamsters (Uehara and Yanagimachi 1976), rabbits (Keefer 1989) and humans (Tesarik et al. 1994), the mechanical processes during ICSI, penetration of an ICSI needle through the zona pellucida and cytoplasmic suction, were reported to be enough to activate oocytes. The reason why only mechanical activation and the existence of spermatozoon in the cytoplasm could not induce extrusion of the second PB of oocytes to complete the meiosis processes in cattle, pigs and swamp buffalo is not clear, which maybe related to the packaging of chromatin in spermatozoa that was reported to be more stable in cattle than in other species like human, mouse and hamster (Perreault et al. 1988).

A series of multiple intracellular calcium ion transient increases were exhibited in fertilized mammalian oocytes (Miyazaki et al. 1986, 1993; Fissore et al. 1992; Fissore and Robl 1994). After sperm–oocyte fusion, these early

calcium ion responses have important roles in the exocytosis of cortical granules and the resumption of meiotic arrest in mouse oocytes (Kline and Kline 1992). The completion of oocyte meiosis was monitored by the rate of oocytes extruding the second PB after activation in this study, and it was found that EtOH treatment was more effective to activate buffalo ICSI oocytes than Io treatment (Table 1). This could be attributed to the different ways of calcium mobilization induced by EtOH and Io. EtOH activates oocytes by promoting the rapid potentiation of inositol 1,4,5-trisphosphate (IP3)-mediated Ca^{2+} release through stimulation of IP3 formation at the plasma membrane, and then it induces a single calcium rise coming from both extracellular entry and intracellular stores (Shilina et al. 1993). On the other hand, Io induces a single Ca^{2+} rise in mammalian oocytes through the release of Ca^{2+} only from internal stores (Vincent et al. 1992). The current result was consistent with that for cattle in which EtOH was considered to be a more effective calcium rise reagent to induce the second PB extrusion from oocytes than Ca^{2+} ionophore IA23187 (Nakada and Mizuno 1998). In cattle, EtOH can induce a greater rise of Ca^{2+} and longer Ca^{2+} releasing duration than those observed at fertilization (Nakada and Mizuno 1998).

Oocytes exposed to a single chemical (e.g., calcium ionophore; EtOH) or electrical stimulus would induce a transient rise in intracellular calcium; however, this is inadequate for full activation of oocytes, resulting in the incomplete cortical granule formation (CG exocytosis) (Wang et al. 1998) and failures of pronuclear formation, mRNA recruitment and DNA synthesis (Susko-Parrish et al. 1994; Schultz and Kopf 1995; Soloy et al. 1997). Studies have demonstrated that the activation protocols with the best results were those that combined Ca^{2+} rise treatment and inhibition of MPF activity on ICSI and somatic cell nuclear transfer in bovine embryos (Rho et al. 1998; Bhak et al. 2006). Thus, combination of a stimulation for Ca^{2+} rise and an inhibition of either protein synthesis with CHX or protein phosphorylation with 6-DMAP can be a reasonable approach for the establishment of the optimal activation method for buffalo oocytes. Inhibition of protein synthesis can enhance oocyte activation by inducing temporal changes in MPF and MAPK. Cycloheximide not only inhibits the cyclin B synthesis and maintains low activity of MPF, but also inhibits the phosphorylation of MAPK after pronucleus formation in oocytes (Liu and Yang 1999). Also, dephosphorylation of MAPK can be induced by 6-DMAP leading to earlier pronuclear development (Susko-Parrish et al. 1994; Soloy et al. 1997; Leal and Liu 1998). Recently, Abdalla et al. (2009) demonstrated that combined activation treatment of bovine ICSI oocytes with Io and EtOH yield higher blastocyst rate than Io combined with CHX or 6-DMAP.

After receiving the first intracellular calcium rise, the MII oocytes continue to resume meiosis until the extrusion of the second PB. In this study, most of the second PB extrusions from the buffalo oocytes were detected after 3 h of calcium rise treatment. The reason why others did not extrude the second PB in these circumstances at that time is not clear. However, it might be attributed to disintegration of their meiotic spindle. When those showing delayed extrusion of the second PB after 6 and 9 h of EtOH or Io treatment were cultured in IVC medium with 6-DMAP or CHX, they had a significantly lower ability to develop to the blastocyst stage (Liang YY, Parnpai R, unpublished data) compared with those of oocytes which extruded the second PB after 3 h of treatment. It is possible that the delayed extrusion of the second PB might be relevant to the ability of oocytes to resume meiosis. The oocytes that needed a longer time to finish meiosis might have started the cell cycle later than those which finished meiosis within 3 h of the treatment. This might be the one of the reasons why the oocytes with delayed extrusion of the second PB had lower development ability compared to those which extruded the second PB within 3 h of the treatment. In cattle it was reported that 93% of oocytes extruded the second PB within 3 h of Io treatment following ICSI (Fulka et al. 1991; Susko-Parrish et al. 1994; Rho et al. 1998). The results in this study indicated that 3 h is the optimal time for the selection of oocytes which extruded the second PB after EtOH or Io treatment following ICSI in buffalo.

In this study, the cleavage and blastocyst formation rates for ICSI oocytes were significantly higher than those for sham-injected oocytes in EtOH + CHX and Io + 6-DMAP groups, suggesting that the spermatozoon could enhance oocyte activation and subsequent development in those groups. It has been reported that the sperm cell is a natural activator of oocytes (Bootman and Berridge 1995). In mice (Vitullo and Ozil 1992; Jones and Whittingham 1996; Lawrence et al. 1998), pigs (Sun et al. 1992) and cattle (Nakada and Mizuno 1998; Tosti et al. 2002), sperm penetration induces periodical and transient increases in the free intracellular calcium concentration for several hours. It also has been suggested that after fusion of sperm and oocyte, a sperm CSF is released into the oocyte, which generates the cytoplasmic calcium increase (Fissore et al. 1998; Kimura et al. 1998). However, our study has demonstrated that a mere sperm injection or sham injection into IVM buffalo oocytes without any additional activation procedures resulted in no activation and that in EtOH + CHX and Io + 6-DMAP groups sperm injection combined with activation treatment resulted in higher activation and development than activation treatment alone. This suggests that the synergetic effect on oocyte activation of sperm and activation treatment is necessary to guarantee high rates of embryo development by ICSI in buffaloes. It is of interest that oocytes activated by the sham injection extruded the second PB and developed into blastocysts, however, with a lower rates and embryonic cell numbers compared to ICSI oocytes. Embryos generated from sham-injected oocytes are considered haploid, and the developmental potential of haploid embryos was reported to be much lower than that of diploids in mice (Tarkowski and Rossant 1976). In this study, only the ICSI oocytes had the possibility to develop as diploids. The reduced cell number of blastocysts and lower rate of *in vitro* embryo development in sham-injected oocytes suggest that such haploid embryos might have insufficient DNA to support themselves to develop into early stage embryos compared with diploids.

In conclusion, our study demonstrated that activation of the ICSI swamp buffalo oocytes with EtOH gave the highest 2 pronuclei formation, and that combination treatments with Io + 6-DMAP and EtOH + CHX gave the highest cleavage and blastocyst formation rates.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Authors contributions

We thank these people for the contribution of the completion of this research and its publication. YY Liang did the experiment and drafted the article. DN Ye,C Laowtammathron and T Phermthai analysed the data. T Nagai and T Somfai consulted the results and corrected the final version of the draft. R Parnpai designed the experiment, discussed and commented on the article.

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—Full Paper—

Effect of Donor Cell Types on Developmental Potential of Cattle (*Bos taurus*) and Swamp Buffalo (*Bubalus bubalis*) Cloned Embryos

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Abstract. This study investigated the effect of donor cell types on the developmental potential and quality of cloned swamp buffalo embryos in comparison with cloned cattle embryos. Fetal fibroblasts (FFs), ear fibroblasts (EFs), granulosa cells (GCs) and cumulus cells (CCs) were used as the donor cells in both buffalo and cattle. The cloned cattle or buffalo embryos were produced by fusion of the individual donor cells with enucleated cattle or buffalo oocytes, respectively. The reconstructed (cloned) embryos and *in vitro* matured oocytes without enucleation were parthenogenetically activated (PA) and cultured for 7 days. Their developmental ability to the blastocyst stage was evaluated. The total number of trophectoderm (TE) and inner cell mass (ICM) cells and the ICM ratio in each blastocyst was determined by differential staining as an indicator of embryo quality. The fusion rate of CCs with enucleated oocytes was significantly lower than for those of other donor cell types both in cattle and buffalo. The rates of cleavage and development to the 8-cell, morula and blastocyst stages of cloned embryos derived from all donor cell types did not significantly differ within the same species. However, the cleavage rate of cloned cattle embryos derived from FFs was significantly higher than those of cattle PA and cloned buffalo embryos. The blastocyst rates of cloned cattle embryos, except for the ones derived from CCs, were significantly higher than those of cloned buffalo embryos. In buffalo, only cloned embryos derived from CCs showed a significantly higher blastocyst rate than that of PA embryos. In contrast, all the cloned cattle embryos showed significantly higher blastocyst rates than that of PA embryos. There was no difference in ICM ratio among any of the blastocysts derived from any of the donor cell types and PA embryos in both species. FFs, EFs, GCs and CCs had similar potentials to support development of cloned cattle and buffalo embryos to the blastocyst stage with the same quality.

Key words: Cattle, Differential staining, Embryo development, Nuclear transfer, Swamp buffalo

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Cattle (*Bos taurus* and *Bos indicus*) and swamp buffalo (*Bubalus bubalis*) are important domestic animals, providing high quality milk, meat and work power. However, buffalo populations in many countries have been decreasing due to their low fertility and less demand from humans. In the near future, the buffalo will become an endangered species. Somatic cell nuclear transfer (SCNT) is one of the strategies for propagation of exotic buffalos. The first successful buffalo SCNT was reported in 1999 [1]. Thereafter, Saikhun *et al.* [2] transferred cloned swamp buffalo embryos derived from fetal fibroblasts to recipients, and pregnancy was maintained for more than 90 days. However, none of the embryos supported development to term. Simon *et al.* [3] transferred 16 cloned water buffalo embryos derived from fetal fibroblasts to recipients, but no pregnancy was reported. Shi *et al.* [4] transferred 42 cloned buffalo blastocysts derived from fetal fibroblasts and granulosa cells to 21 recipients, and four recipients became pregnant (19%). Three of these recipients developed to term, but two of the newborns died after birth, and only one cloned buffalo derived from fetal fibroblasts survived. The success rate of producing cloned buffalo offspring is still low, and there are multiple factors influencing the viability of clones. One of the key factors is the donor cell type, which has a significant effect on the efficiency of SCNT and the development of cloned rabbit embryos [5]. There are many kinds of somatic cells, such as mammary epithelial cells, ovarian cumulus cells, fibroblasts from skin and internal organs, oviduct epithelial cells, granulosa cells, muscle cells, uterine epithelial cells, Sertoli cells, macrophages, blood leukocytes, lymphocytes, natural killer T cells, mature B and T cells, olfactory cells, neural stem cells and myoblasts, that can be used as donor cells for SCNT (reviewed by Campbell *et al.* [6]). Embryonic and fetal cells have been reported to be more successful candidates for SCNT when compared with adult cells or terminally differentiated cells in cattle [7, 8]. This might be related to the fact that embryonic and fetal cells have rapid growth and potential for many cell divisions prior to senescence in culture [9–11]. On the other hand, among adult cell types for SCNT, cumulus cells appear to be the best choice according to their cloning efficiency and the smallest proportion of abnormalities in resultant cloned animals [12]. Also, highly efficient production of cloned calves using

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cumulus cells as donor cells has been reported [13–15]. However, cumulus cells have variously been reported as better [16] or worse [17] nuclear donor cells as compared to fibroblasts in rabbits. Comparison of the reported cloning efficiency for each donor cell type is difficult due to variations in the nuclear-transfer procedures, source and quality of recipient oocytes, age and genotype of the donor cells and embryo culture systems used in the studies [18]. Furthermore, there have been few reports about the effect of donor cell types on the developmental potential of cloned buffalo embryos [4, 19]. Thus, in the present study, we investigated the effect of donor cell types, fetal fibroblasts, ear fibroblasts, granulosa cells and cumulus cells, on the developmental potential and blastocyst quality of cloned swamp buffalo embryos in comparison with cloned cattle embryos under the same SCNT system.

Materials and Methods

Chemicals and media

All reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise specified.

Donor cell preparation

Ear fibroblasts (EFs): EFs were prepared according to the method of Suteevun *et al.* [20]. In brief, ear skin tissues were biopsied from a heifer cattle or a female buffalo and then transported to the laboratory at 4 C in modified Dulbecco's phosphate buffered saline (mDPBS). Skin tissues were removed from cartilage and cut into small pieces (about 1 mm^2) before being placed in a 60-mm culture dish (Nunc, Roskilde, Denmark) and covered with a glass slide. Five milliliters of αMEM supplemented with 10% fetal bovine serum (FBS) were added in the dish and then cultured under a humidified atmosphere of 5% CO_2 in air at 37 C for 8–10 days. At subconfluence, outgrowths of the EFs were harvested using 0.25% trypsin/EDTA in Ca^{2+}- and Mg^{2+}-free PBS and seeded in 5 ml αMEM supplemented with 10% FBS in a 25-cm^2 culture flask (Nunc). EFs were frozen at the third passage of cell culture in αMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO) and kept in liquid nitrogen.

Fetal fibroblasts (FFs): The skin tissues of female cattle and buffalo fetuses at 80–90 days of gestation were enzymatically digested with 0.25% trypsin/EDTA and agitated for 20 min at room temperature. The disaggregated cells were washed in αMEM supplemented with 10% FBS by centrifugation at 600 × *g* for 5 min and then cultured in 25-cm^2 culture flasks under a humidified atmosphere of 5% CO_2 in air at 37 C. At subconfluence, the FFs were harvested, cultured and frozen as EFs.

Granulosa cells (GCs): The GCs were isolated from follicles 2–8 mm in diameter of cattle and buffalo ovaries. GCs were disaggregated with 0.25% trypin/EDTA for 3 min and then washed in αMEM supplemented with 10% FBS by centrifugation at 600 × *g* for 5 min. GCs were cultured in 25-cm^2 culture flasks under a humidified atmosphere of 5% CO_2 in air at 37 C. At subconfluence, the GCs were harvested, cultured and frozen as EFs.

Frozen-thawed donor cells were cultured in 35-mm culture dishes (Nunc) for 2–3 days before use as donor cells and were used for up to eight passages. A few minutes before injection, the proliferating donor cells were harvested by standard trypsinization and resuspended in TCM199-Hepes.

Cumulus cells (CCs): Fresh CCs were mechanically removed from *in vitro* maturated cattle and buffalo oocytes by repeated pipetting using a fine-tip pipette in mDPBS supplemented with 0.2% hyalurodinase and immediately used as donor cells.

Oocyte preparation

Collection and *in vitro* maturation (IVM) of cattle and buffalo follicular oocytes were performed according to the methods of Parnpai *et al.* [1]. Cattle and buffalo ovaries were collected at a local abattoir and transported to the laboratory in 0.9% NaCl at room temperature. Cumulus-oocyte complexes (COCs) were collected by aspiration of follicles 2–8 mm in diameter using a 21-gauge needle attached to a 10-ml syringe. COCs were washed 5 times with mDPBS supplemented with 0.1% polyvinyl pyrolidone (PVP) and then washed 3 times in IVM medium. Twenty COCs were cultured in 100 μl droplets of IVM medium overlaid with mineral oil under a humidified atmosphere of 5% CO_2 in air at 38.5 C for 22 h. After IVM, the cumulus cells were mechanically removed from the oocytes by repeated pipetting using a fine-tip pipette in mDPBS supplemented with 0.2% hyaluronidase and subsequently washed 5 times in TCM199-Hepes before enucleation.

Somatic cell nuclear transfer

IVM cattle and buffalo oocytes were placed in TCM199-Hepes supplemented with 5 μg/ml cytochalasin B for 15 min. The zona pellucida above the first polar body was cut with a glass needle. A small volume (about 5–10%) of the cytoplasm beneath the first polar body was squeezed out. Complete enucleation was confirmed by staining the squeezed out cytoplasm with 5 μg/ml Hoechst 33342 and visualizing the stained nucleus in the cytoplasm under a fluorescence microscope (IX71, Olympus, Tokyo, Japan). An individual donor cell (14–16 μm in diameter) was inserted into the perivitelline space of each enucleated oocyte. The donor cell-cytoplast couplet was sandwiched between a pair of electrodes in Zimmermann fusion medium [21], and two direct current pulses (24 V, 15 μsec for cattle and 26 V, 17 μsec for buffalo) were generated by a fusion machine (SUT F-1, Suranaree University of Technology) for cell-cytoplast fusion. The reconstructed couplets (embryos) were activated by 7% ethanol in TCM199-Hepes for 5 min at room temperature and cultured in a modified oviduct synthetic fluid with amino acids (mSOFaa) medium [22] supplemented with 1.25 μg/ml cytochalasin D (CD) and 10 μg/ml cycloheximide (CHX) at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 5 h [23]. Each donor cell and oocyte type was treated with the same procedures.

Parthenogenetic activation (PA)

At 26 h after IVM, matured cattle and buffalo oocytes were activated in 7% ethanol in TCM199-Hepes for 5 min at room temperature and then cultured in mSOFaa medium supplemented with 1.25 μg/ml CD and 10 μg/ml CHX at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 5 h.

Table 1. Effect of donor cell type on developmental potential of cloned cattle and buffalo embryos*

Species	Donor cell type	No. of couplets fused (%)	No. of embryos cultured	No. of embryos cleaved (%)	No. (%) embryos developed to		
					8-cell	Morula	Blastocyst
Cattle	FF	113/132 (85.6)^{b}	110	108 (98.2)^{a}	88 (80.0)^{a}	60 (54.5)^{a}	45 (40.9)^{a}
	EF	111/121 (91.7)^{a}	111	100 (90.1)^{ab}	78 (70.3)^{abc}	60 (54.1)^{a}	43 (38.7)^{a}
	GC	111/124 (89.5)^{ab}	111	101 (91.0)^{ab}	76 (68.5)^{ab}	51 (45.9)^{ab}	46 (41.4)^{a}
	CC	118/145 (81.4)^{c}	108	100 (92.6)^{ab}	65 (60.2)^{abc}	46 (42.6)^{ab}	40 (37.0)^{ab}
	PA	–	105	86 (81.9)^{bc}	48 (45.7)^{c}	43 (41.0)^{ab}	27 (25.7)^{cd}
Buffalo	FF	120/136 (88.2)^{ab}	119	100 (84.0)^{bc}	76 (63.9)^{abc}	38 (31.9)^{b}	26 (21.8)^{cd}
	EF	112/130 (86.2)^{b}	112	96 (85.7)^{bc}	71 (63.4)^{abc}	37 (33.0)^{b}	30 (26.8)^{cd}
	GC	108/122 (88.5)^{ab}	102	88 (86.3)^{bc}	69 (67.6)^{ab}	35 (34.3)^{b}	25 (24.5)^{cd}
	CC	117/143 (81.8)^{c}	104	86 (82.7)^{bc}	63 (60.6)^{abc}	35 (33.7)^{b}	29 (27.9)^{bc}
	PA	–	104	82 (78.8)^{c}	58 (55.8)^{bc}	36 (34.6)^{b}	20 (19.2)^{d}

*Five replicates were performed. Different superscripts within a column indicate significant differences (P<0.05). FF=fetal fibroblasts, EF=ear fibroblasts, GC=granulosa cells, CC=cumulus cells, PA=parthenogenetic activation.

In vitro culture (IVC) of embryos

The cloned and PA embryos were cultured in a mSOFaa medium (20 embryos/ 100 μl) at 38.5 C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 2 days. Embryos at the 8-cell stage were selected and co-cultured with cattle oviductal epithelial cells in a mSOFaa medium (10 embryos/ 100 μl) at 38.5 C under a humidified atmosphere of 5% CO_2 in air for an additional 5 days (7 days in total). A half volume of the IVC medium was replaced daily, and the development of the embryos was recorded [1].

Differential staining of blastocysts

The cloned and PA blastocysts at day 7 of culture were counterstained to distinguish cells of the inner cell mass (ICM) and trophectoderm (TE) according to the method of a previous report [20] with some modifications. Briefly, the zonae pellucidae of the blastocysts were removed by exposure to 0.5% protease in mDPBS supplemented with 0.1% Polyvinyl pyrolidone, and then the zona-free blastocysts were washed in the medium. The zona-free cattle or buffalo blastocysts were incubated for 45 min in 10% rabbit anti-cattle spleenocyte antibodies for cattle embryos or 10% rabbit anti-buffalo spleenocyte antibodies for buffalo embryos, respectively. Both antibodies were generated in our laboratory according to Iwasaki *et al.* [24]. After incubation, the blastocysts were transferred into a mixture of 10% guinea pig complement, 10 μg/ml propidium iodide and 10 μg/ml Hoechst 33258 and further incubated for 30 min. The blastocysts were mounted with glycerol on glass slides and covered with cover slips. The ICM cells (blue) and TE cells (red) were counted under an ultraviolet light fluorescence microscope.

Experimental design

The effect of donor cell type on the developmental potential of cattle and buffalo cloned embryos was investigated. Four donor cell types were compared. Cattle and buffalo FFs, EFs, GCs and CCs were fused with enucleated oocytes of each species. The rates of fusion, cleavage, development to the 8-cell, morula and blastocyst stages as well as the ICM and TE cell numbers of cloned and PA embryos were compared.

Statistical analysis

Statistical analysis of data was evaluated by completely randomized design (CRD) with Statistical Analysis System (SAS Institute, Cary, NC, USA). Analysis of Variance (ANOVA) and comparison of means by Duncan's Multiple Range Test (DMRT) were performed.

Results

Fusion rates of reconstructed cattle and buffalo oocytes

As shown in Table 1, the fusion rate of EFs (91.7%) with enucleated oocytes in cattle was significantly higher than that of FFs (85.6%) but no different from that of GCs (89.5%), and the CCs (81.4%) showed the lowest fusion rate among all donor cell types (P<0.05). In buffalo, no significant difference was found among the fusion rates for FFs (88.2%), EFs (86.2%) and GCs (88.5%). However, the CCs (81.8%) showed the lowest fusion rate among all donor cell types (P<0.05). When the fusion rates of each donor cell type were compared between cattle and buffalo, the fusion rates of FFs, GCs and CCs were not significantly different between them. However, the fusion rate for cattle EFs with cattle oocytes was significantly higher than that for buffalo EFs with buffalo oocytes.

Effect of donor cell types on the development of cattle and buffalo cloned embryos

Among cattle and buffalo, the rates of cleavage and development to the 8-cell, morula and blastocyst stages of cloned embryos derived from all donor cell types were not significantly different (Table 1). However, in cattle, the cloned embryos derived from FFs (98.2%) showed a significantly higher cleavage rate than that for PA embryos (81.9%), while the embryos derived from FFs (80.0%) and GCs (68.5%) showed significantly higher developmental rates to the 8-cell stage than that of the PA embryos (45.7%); the embryos derived from all the donor cell types showed significantly higher blastocyst formation rates (37.0–41.4%) than

that of the PA embryos (25.7%). In buffalo, only the blastocyst formation rate of cloned embryos derived from CCs (27.9%) was significantly higher than that of the PA embryos (19.2%), and other developmental parameters did not differ among the donor cell types and PA embryos.

When the development of embryos derived from each donor cell type and PA embryos was compared between cattle and buffalo, the cleavage rate of cloned cattle embryos derived from FFs was significantly higher than those of cloned buffalo and cattle PA embryos. The developmental rate to the 8-cell stage of cloned embryos was the same among the cattle and buffalo embryos. In contrast, the developmental rates to the morula stage of cloned cattle embryos derived from FFs (54.5%) and EFs (54.1%) were significantly higher than those of buffalo cloned and PA embryos (31.9–34.6%). The blastocyst formation rates of cloned cattle embryos derived from FFs, EFs and GCs were significantly higher than those of cloned buffalo and PA embryos. No significant difference was observed in the percentages of blastocysts derived from CCs in between cloned cattle and buffalo embryos.

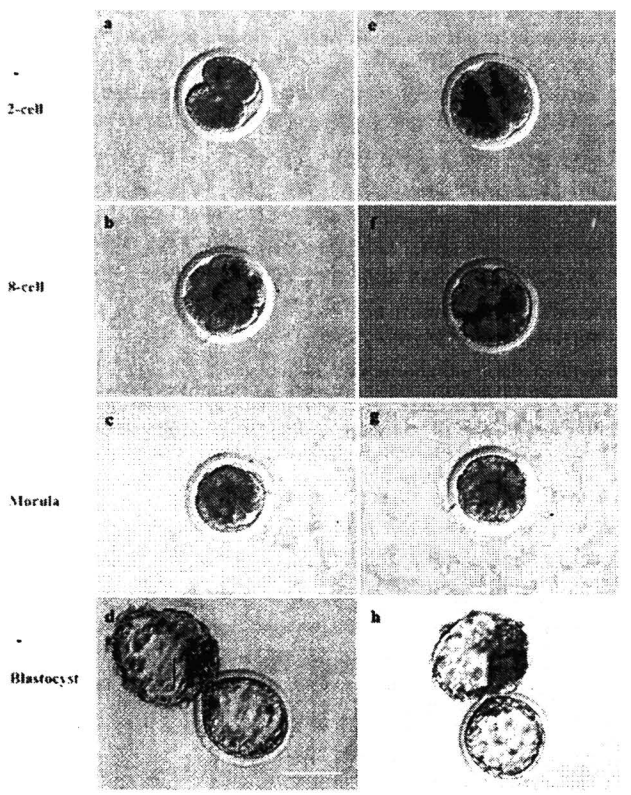

Fig. 1. Cattle (a–d) and buffalo (e–h) SCNT embryos at various stages after *in vitro* culture.

Embryonic cell number

As shown in Table 2, in both cattle and buffalo, similar numbers of cells were found in the TE and ICM with both cloned and PA blastocysts (Table 2). The cattle and buffalo blastocysts derived from cloned and PA embryos showed no significant difference in the ICM ratio irrespective of donor cell type (2.98–3.09).

Discussion

Our results demonstrate the developmental potential to the blastocyst stage of cloned cattle and swamp buffalo embryos derived from four different cell types, FFs, EFs, GCs and CCs. To our knowledge, this is the first comparison study about the effects of FFs, EFs, GCs and CCs on developmental potential in cattle and buffalo cloned embryos using the same system. Our results suggest that FFs, EFs, GCs and CCs can be reprogrammed via SCNT and result in the same potential, within the same species, to support development of the resultant embryos to the blastocyst stage in both cattle and buffalo. However, the blastocyst formation rates for cloned cattle embryos derived from all the donor cell types, except for CCs, were higher than those for cloned buffalo embryos obtained by the same SCNT system. This may have been caused by the embryo culture system because the system used in this study was established for *in vitro* production of cattle embryos [1]. Furthermore, buffalo embryos may have lower developmental potential to the blastocyst stage than cattle embryos. Nevertheless,

Table 2. Number of trophectoderm (TE) and inner cell mass (ICM) cells in cattle and buffalo blastocysts

Species	Donor cell types	No. blastocysts examined	Mean (± S.E.M.) number of cells in blastocyst*		
			TE	ICM	ICM ratio (%)
Cattle	FF	5	104.4 ± 0.83	33.6 ± 0.42	24.5 ± 0.21
	EF	5	91.6 ± 0.77	29.8 ± 0.43	24.6 ± 0.14
	GC	5	100.2 ± 1.01	33.0 ± 0.57	24.9 ± 0.22
	CC	5	105.6 ± 1.01	34.8 ± 0.57	24.8 ± 0.15
	PA	5	102.2 ± 0.79	33.4 ± 0.46	24.6 ± 0.17
Buffalo	FF	5	94.4 ± 1.16	31.4 ± 0.65	25.2 ± 0.23
	EF	5	98.0 ± 0.88	31.8 ± 0.49	24.6 ± 0.21
	GC	5	103.8 ± 0.59	34.6 ± 0.25	25.1 ± 0.21
	CC	5	115.4 ± 0.65	38.6 ± 0.35	25.1 ± 0.13
	PA	5	91.2 ± 0.87	30.4 ± 0.47	25.1 ± 0.16

*No statistical difference was obtained ($P>0.05$). FF=fetal fibroblasts, EF=ear fibroblasts, GC=granulosa cells, CC=cumulus cells, PA=parthenogenetic activation.

the ICM ratio in the cloned and PA blastocysts was not significantly different between the cattle and buffalo. Thus, the quality of cloned embryos seems to be similar irrespective of the donor cell types and species. Although many studies have examined cloned buffalo, the success rates for production of offspring have remained low [2, 3]. Only one cloned swamp buffalo has survived after birth, in 2007 [4], and that study reported that FFs and GCs have the same potential to support development to the blastocyst stage of cloned buffalo embryos and that no significant difference was found in the pregnancy rates after transfer to recipient animals. However, two of the newborn calves derived from FFs and GCs died after birth, while another calf from the FFs grew up and is still alive today [4]. Although cloned embryos derived from FFs, EFs, GCs and CCs showed the same developmental rates to the blastocyst stage in buffalo, only the cloned buffalo embryos derived from CCs showed a significantly higher blastocyst formation rate than that of the buffalo PA embryos in this study. Considering that the PA embryos were treated in the same manner, except for enucleation of oocytes and somatic cell fusion, as cloned embryos, it is possible that CCs could enhance the developmental ability of *in vitro* matured buffalo oocytes after SCNT. To investigate whether CCs can support production of cloned buffalos (full-term development), further experiments on transfer of clone embryos derived from CCs into recipients are needed in the future.

When the effect of each donor cell type on the rate of fusion with recipient oocytes was investigated in each species, all of the donor cell types showed higher fusion rates, even compared with the rates previously reported [13, 14, 23, 25–29]. The fusion rate of EFs with cattle oocytes was significantly higher than that of buffalo oocytes with EFs, but the reason for this difference in fusion rate is not clear. On the other hand, CCs showed the lowest fusion rate among all the donor cell types reconstructed with either cattle or buffalo oocytes. The reason for the low fusion rate for CCs is not clear, but it may be due to the fact that the size of CCs is smaller than those of other fibroblasts; normal fibroblasts are two-times the size of CCs.

When compared within the same species, there was no significant difference among FFs, EFs, GCs and CCs in ability to support cleavage and embryo development to the 8-cell, morula and blastocyst stages. Our results are similar to previous reports in which cloned cattle embryos derived from fetal and adult fibroblasts showed no difference in blastocyst formation rates [30, 31]. Moreover, Shi *et al.* [4] reported that buffalo embryos derived from either FFs or GCs have the same ability to develop to the blastocyst stage and that there is no significant difference in pregnancy rate after transfer to recipients between them. In other species, Ogura *et al.* [32] found no significant difference among CCs, fibroblasts and Sertoli cells in ability to support full-term development of cloned mice. Yang *et al.* [5] found that CCs and FFs have the same potential to support development of cloned rabbit embryos to the blastocyst stage. In contrast, many reports on cattle have suggested that CCs appear to be the best choice because of the highest cloning efficiency among the cells examined [12, 15, 18, 25, 33–35]. Also, in buffalos, Shah *et al.* [19] produced cloned embryos by a hand-made cloning (HMC) technique and found that the blastocyst formation rate of embryos derived from CCs was better than those of embryos derived from FFs or adult fibroblasts. Similarly, Wakayama *et al.* [36] reported that the live birth rate of embryos derived from CCs was higher than those of embryos derived from neuronal and Sertoli cells in mice. However, Saikhun *et al.* [37] showed that cloned buffalo embryos derived from FFs have a higher blastocyst formation rate than those of embryos derived from CCs and oviduct cells. Thus, it can be suggested that there are no clear relationships in the blastocyst formation rates among embryos derived from fetal and adult fibroblasts or somatic cell types. These conflicting results may be due to the different cell culture systems, cloning protocols, embryo culture systems used and skills of lab personnel.

In conclusion, the donor cell types, FFs, EFs, GCs and CCs, had the same ability to support cloned embryos to develop to the blastocyst stage within the same species. The quality of cloned embryos derived from all four donor cells was similar in both cattle and buffalo. However, the pregnancy rate and development to term of cloned embryos derived from the different donor cell types need further investigation.

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IN VITRO DEVELOPMENT OF VITRIFIED BUFFALO OOCYTES FOLLOWING PARTHENOGENETIC ACTIVATION AND INTRACYTOPLASMIC SPERM INJECTION

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Abstract

The objective of this study was to investigate the potential of swamp buffalo oocytes vitrified-warmed at the metaphase of the second meiotic cell division (M-II) stage to develop to the blastocyst stage after parthenogenetic activation (PA) or intracytoplasmic sperm injection (ICSI). In Experiment 1, we examined the effects of exposure time of oocytes to cryoprotectants (CPA) on their *in vitro* development after PA. *In vitro* matured (IVM) oocytes were placed in 10% dimethylsulfoxide (DMSO) + 10% ethylene glycol (EG) for 1 min and then exposed to 20% DMSO + 20% EG + 0.5 M sucrose for 30 s, 45 s or 60 s (1min+30s, 1min+45s and 1min+60s groups, respectively). The oocytes were then exposed to warming solution (TCM199 HEPES + 20% FBS and 0.5M sucrose) for 5 min and then washed in TCM199 HEPES + 20% FBS for 5 min. IVM oocytes without CPA treatments served as a control group. The viability assessed by fluorescein diacetate (FDA) staining was 100% in all groups. The developmental rates after PA to the blastocyst stage between 1min+30s (16%) and control (26%) groups did not differ significantly, but they were significantly higher than those in 1min+45s (10%) and 1min+60s (2%) groups. In Experiment 2, we examined the effect of two CPA exposure times, 1min+30s and 1min+45s on the *in vitro* development after PA of oocytes vitrified by the microdrop method. The viabilities in vitrified 1min+30s, 1min+45s and the control (without CPA treatments) groups were not different (97%, 95% and 100%, respectively). The development of surviving oocytes to the blastocyst stage in the vitrified 1min+30s group (8%) was significantly higher than that in the vitrified 1min+45s group (4%) and significantly lower than those in control group (26%). In Experiment 3, we examined the effect of two CPA exposure times, 1min+30s and 1min+45s on *in vitro* development after ICSI of vitrified oocytes. Viabilities in vitrified oocytes among 1min+30s, 1min+45s and control groups were not different (96%, 91% and 100%, respectively). After ICSI, vitrified-warmed oocytes were

activated and oocytes with the second polar body were cultured for 7 days. The development of ICSI oocytes to the blastocyst stage in the vitrified 1min+30s group (11%) was significantly higher than that in the vitrified 1min+45s (7%) group and significantly lower than those in control group (23%). In conclusion, our study demonstrated that the 1min+30s CPA treatment regimen could yield the highest blastocyst formation rates after PA and ICSI for oocytes vitrified by the microdrop method.

Keywords: buffalo oocytes; microdrop; vitrification; ICSI.

1. Introduction:

Nowadays, the buffalo is the major milk and meat producing farm animal in many developing countries. Buffalo oocytes obtained from slaughterhouse ovaries and matured *in vitro* are useful sources for reproductive procedures such as IVF and intracytoplasmic sperm injection (ICSI), in which mainly cryopreserved spermatozoa are used. Cryopreservation of oocytes is also very important in preserving female gametes for future use. Efficient oocyte cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in the buffalo species.

Cryopreservation of mammalian oocytes has become more successful using vitrification as an alternative to cryopreservation compared with slow cooling methods, in recent years [1,2].The vitrification process induces a glass-like solidification of living cells at low temperatures. The unique advantage of the vitrification process is the elimination of ice crystal formation, the most severe cause of cryoinjury. Insufficient cooling rates of oocytes were considered one of the principal

obstacles in vitrification technology [3]. Since the first report on buffalo oocyte vitrification by using French straw [4], some reports have been published regarding the cryopreservation of buffalo oocytes [5-11]. In 2004, the first successful production of a buffalo blastocyst derived from *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of vitrified-warmed oocytes was reported [5]. In order to overcome the problem of insufficient cooling rates several methods have been proposed using very small amounts of solution. Some improved vitrification methods have been successfully used for oocyte cryopreservation, including cryotop [12], cryoloop [13], open pulled straw (OPS) [14], glass micropipette [15], microdrop [16], electron microscope grids [17] and solid surface vitrification [18].

Among the various methods of vitrification, the microdrop method is considered easy and inexpensive, as it excludes the use of any specialized devices to introduce oocytes into liquid nitrogen. The microdrop method is the simplest way of vitrification by dropping oocyte - containing solutions directly into liquid nitrogen. This method was first proposed for mouse embryos [19], and then successfully applied to bovine embryos, zygotes and oocytes [20,21,22,8]. After cryopreservation or treatment with cryoprotectant (CPA), structural changes in the zona pellucida (ZP) have been shown to reduce fertilization rates [23,24]. Although the mechanism of ZP hardening of the cryopreserved oocytes is unclear, it seems to be caused by the premature release of cortical granules that induce the zona reaction [24] resulting in lower incidences of sperm penetration into oocytes. This consequence of ZP hardening could be overcome by micromanipulation techniques such as intracytoplasmic sperm injection (ICSI) [23, 25-28].

Permeation of a certain amount of CPA into the oocytes is essential for cryosurvival of oocytes. The amount of CPAs within the cell increase with the duration of exposure [29]; however, the toxicity of the CPA must be considered, with an optimum exposure time being favorable for this

purpose. Nevertheless the optimal CPA treatment regimen for the vitrification of IVM buffalo oocytes has not been determined. The aim of this study was to investigate the effect of exposure time in vitrification solution on the post-thaw viability and the developmental competence of IVM vitrified-warmed swamp buffalo oocytes after parthenogenetic activation (PA) or ICSI. Differential cell staining was applied to assess the qualitative aspects of blastocysts that were derived from fresh or vitrified-warmed oocytes.

2. Materials and methods:

2.1 Experimental design

Experiment 1 was performed to test the toxicity of vitrification solution. After treatment with the equilibration solution for 1 min, *in vitro* matured (IVM) oocytes at the metaphase of the second meiotic cell division stage (M-II) were exposed to vitrification solution for 30, 45 or 60 s (1min+30s, 1min+45s and 1min+60s groups, respectively) and then transferred to warming solution. Surviving oocytes selected by the fluorescein diacetate (FDA) test were then subjected to PA and then *in vitro* cultured. The development of oocytes exposed to the vitrification solution was compared with CPA-untreated but FDA stained oocytes (FDA-exposed). To test the possible side effects of FDA staining, the oocytes without CPA and FDA treatments were also activated and cultured (control).

Experiment 2 was performed to assess the developmental ability of vitrified-thawed oocytes induced by parthenogenetic activation (PA). On the basis of the results from Experiment 1, only the 1 min+30 s and 1 min+45 s CPA treatment regimens were used to vitrify M-II oocytes (1min+30s

and 1min+45s groups, respectively). Vitrified oocytes were stored in liquid nitrogen containers for 7 to 14 days. After warming, all surviving (FDA positive) oocytes were subjected to PA and their *in vitro* developments were compared to those of activated FDA-exposed and control oocytes.

Experiment 3 was performed to assess the development of intracytoplasmic sperm injection (ICSI) embryos generated from vitrified-thawed M-II oocytes. Oocytes vitrified by the 1 min+30 s and 1 min+45 s treatment regimens (1min+30s and 1min+45s groups, respectively) were warmed and all of the surviving (FDA positive) oocytes were subjected to ICSI. Their *in vitro* developments were compared to those of FDA-exposed and control oocytes.

2.2 Chemicals and media

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. The medium used for IVM was TCM199 supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 0.02 AU/mL FSH (Antrin, Denka Pharmaceutical, Tokyo, Japan), 50 iu/mL hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1 μg/mL estradiol-17β. The Emcare holding medium (EHM, ICP Bio, Auckland, New Zealand) was used as the basal medium throughout the process of ICSI and PA. The medium for embryo culture was modified synthetic oviduct fluid supplemented with amino acids and 0.3% fatty acid-free BSA (mSOF) [30].

2.3 Oocyte collection and in vitro maturation

Buffalo ovaries were obtained from slaughterhouses and kept in 0.9% NaCl during transport

139 to the laboratory within 4 h at room temperature. Cumulus-oocyte complexes (COCs) were
140 collected from follicles 2 to 8 mm in diameter using a 21-gauge needle attached to a 10 mL syringe.
141 Each of 20 COCs was cultured in 100 μL droplets of IVM medium covered with mineral oil in a
142 humidified atmosphere of 5% CO_2 in air at 38.5 °C for 21 h. After IVM culture, cumulus cells were
143 removed from oocytes by gentle pipetting with a fine glass pipette in EHM supplemented with 0.1%
144 hyarulonidase, and the oocytes were subsequently washed 3 times in EHM. Oocytes with a visible
145 first polar body (PB) were defined to be M-II oocytes and selected for the subsequent experiments.
146
147 *2.4 Vitrification and warming*
148
149 The M-II oocytes were vitrified-warmed by using the microdrop method. Groups of five
150 oocytes were washed in TCM199-Hepes + 20% FBS before being placed in TCM199-Hepes + 20%
151 FBS containing 10% dimethylsulfoxide (DMSO) and 10% ethylene glycol (EG) for 1 min, and then
152 exposed in TCM199-Hepes + 20% FBS containing 20% DMSO, 20% EG and 0.5 M sucrose for 30,
153 45 or 60 s (1min+30s, 1min+45s or 60 s 1min+60s groups, respectively) at 22 to 24 °C. The oocytes
154 were then directly dropped with about 2 μL vitrification solution into liquid nitrogen. For storage,
155 vitrified microdrops were placed in a pre-cooled cryovial filled with liquid nitrogen using a
156 pre-cooled forceps and kept for one to two weeks. The vitrified microdrops were warmed by
157 immersing directly into 3 mL of 0.5 M sucrose in TCM199-Hepes + 20% FBS at 38.5 °C for 5 min,
158 transferred to TCM199-Hepes + 20% FBS for 5 min, and then kept in the TCM199-Hepes + 20%
159 FBS under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h. Some oocytes were treated
160 with CPA and washing solution without the cooling and warming process ("CPA-exposed" group).
161

162 *2.5 Evaluation of oocyte viability*

163

164 Oocyte viability was evaluated by FDA staining according to the method previously
165 described by Mohr and Trounson [31]. Briefly, oocytes were treated with 2.5 μg/mL FDA in PBS
166 supplemented with 5 mg/mL BSA at 38.5 °C for 2 min in a dark room and then they were washed
167 three times in PBS supplemented with 5 mg/mL BSA and evaluated under a fluorescent microscope
168 (IX71, Olympus, Tokyo, Japan) with UV irradiation using a U-MWIB3 filter with an excitation
169 wavelength of 460 to 495 nm and emission at 510 nm. Oocytes expressing a bright green
170 fluorescence were regarded as living ones and were used subsequently.

171

172 *2.6 PA of oocytes*

173

174 The M-II oocytes were subjected to the PA treatment as previously described [32]. Briefly,
175 the oocytes were first treated with 7% ethanol in the EHM for 5 min at room temperature, and then
176 incubated with 10 μg/mL cycloheximide (CHX) and 1.25 μg/mL cytochalasin D (CD) in mSOF
177 medium + 10 % FBS under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 5 h.

178

179 *2.7 ICSI and oocytes activation*

180

181 Frozen spermatozoa were thawed at 37°C, and semen was placed in the bottom of snap –cap
182 centrifuge tube containing 1 mL Brackett and Oliphant (BO) medium [33] supplemented with 10
183 mM caffeine (caffeine-BO) for sperm swim up for 30 min. The top of 700 μL of medium was then
184 collected and placed in a conical centrifuge tube. ICSI was performed according to the method of

185 Liang et al. [34]. Briefly, 1 μL sperm suspension was transferred into oil-covered 5 μL 10%
186 polyvinylpyrrolidone (PVP) solution and a single, motile buffalo spermatozoon was immobilized
187 against the bottom of the dish, loaded tail first with a minimum volume of medium into the
188 injection pipette and then injected into the cytoplasm of a buffalo oocyte. Within 1 h of the injection,
189 the injected oocytes were activated by exposure to 7% ethanol in EHM for 5 min, and then
190 subsequently cultured in TCM199 + 10% FBS for 3 h to allow extrusion of the second PB. Injected
191 and activated oocytes which extruded the second PB were selected and transferred to mSOF
192 medium supplemented with 10 μg/mL CHX and cultured for 5 h at 38.5 °C under humidified
193 atmosphere of 5% CO_2 in air.
194
195 *2.8 In vitro culture*
196
197 The PA and ICSI oocytes were further cultured in mSOF medium (20 embryos/100μL)
198 under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C for 2 days. Thereafter,
199 embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelium cells in
200 mSOF medium under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 5 days, as
201 previously reported [35]. Half of the medium was replaced with fresh medium every day and the
202 embryo development was recorded at the same time as medium changing. The cleavage rates were
203 recorded on Day 2 (the day of PA or ICSI was considered as Day 0), and the development of
204 embryos to blastocyst stage was recorded on Day 7. In ICSI experiment, the rates of cleavage and
205 blastocyst formation were calculated out of fertilized oocytes that extruded the second PB after
206 ICSI.
207

208 *2.9 Differential cell staining*

209

210 The blastocysts harvested on Day 7 were stained to distinguish cells of the inner cell mass
211 (ICM) and trophectoderm (TE), as previously reported [36]. Briefly, the zona pellucida was
212 removed from each blastocyst by exposure to 0.5% protease. After washing with mDPBS
213 supplemented with 0.1% PVP, the zona-free blastocysts were incubated in 100 μL of 10% rabbit
214 anti-buffalo spleenocyte antibodies for 45 min, and then transferred into a 100 μL mixture of 10%
215 guinea pig complement, 10 μg/mL propidium iodide and 10 μg/mL Hoechst 33258 for 45 min. The
216 blatocysts were mounted on glass slides with glycerol and covered with a cover slide. The ICM
217 (blue) and TE cells (red) were counted under a fluorescent microscope at 330 to 380 nm, allowing
218 determination of the total number of cells for blastocysts and the percentage of ICM cells based on
219 the total number of cells.

220

221 *2.10 Statistical analysis*

222

223 The experiments were replicated at least three times in each treatment group. The data for
224 blastocysts cell numbers were expressed as mean±SD. As regards the comparison of oocytes
225 survival rate and embryo development among control groups and vitrified groups, one-way ANOVA
226 and CRD using the Statistical Analysis Systems software (SAS Inst. INC.,Cary, N.C.,USA) were
227 analyzed in order to determine whether difference between these test groups existed at a statistically
228 significant level. The differences between groups were considered to be statistically significant at a
229 probability value of 0.05 or less.

230

231 **3. Results**

232

233 *3.1 Experiment 1. Parthenogenetic development of CPA-treated oocytes.*

234

235 Survival and *in vitro* development of buffalo M-II oocytes following CPA-exposure and PA
236 treatment are shown in Table 1. The viability of oocytes determined by the FDA test in all groups of
237 CPA treatment and the control were 100%. The cleavage rate and embryo development in the 1
238 min+30 s and 1min+45 s groups were significantly higher than in the 1 min+60 s group, but lower
239 than in the FDA-exposed and control groups. The blastocyst formation rate did not significantly
240 differ among the 1 min+30 s (16%), FDA-exposed (22%) and control groups (26%), but they were
241 significantly higher than those of the 1 min+45 s (10%) and 1 min+60 s (2%) groups. The number
242 of total and ICM cells were similar among the 1 min+30 s, 1 min+45 s, FDA-exposed and control
243 groups and were higher than those in the 1 min+60 s group.

244

245 *3.2 Experiment 2. Parthenogenetic development of vitrified oocytes.*

246

247 *In vitro* development of oocytes following vitrification and PA treatment is shown in Table 2.
248 The oocyte viability rates among the vitrified 1 min+30 s (97%), 1 min+45 s (95%) and the control
249 groups (100%) did not differ. The cleavage and blastocyst rates were influenced when the oocytes
250 were subjected to vitrification. The development of PA oocytes to the blastocyst stage in the
251 vitrified 1 min+30 s group (8%) was significantly higher than that in the vitrified 1 min+45 s group
252 (4%) but significantly lower than those in the FDA-exposed (24%) and control groups (26%). The
253 total cell numbers of blastocysts in the 1 min+30 s (71.6±18.3) and 1 min+45 s (69.9±19.6) groups

were not significantly different from that in the FDA-exposed (78.5±24.6) and control (80.0±23.1) groups. There was no difference in the ICM cell numbers among the four groups.

3.3 Experiment 3. In vitro development following vitrification and ICSI.

In vitro development of buffalo M-II oocytes following vitrification and ICSI treatment is shown in Table 3. The oocytes viability rates among the vitrified 1 min+30 s (96%), 1 min+45 s (91%) and control (100%) groups did not differ. After activation of ICSI oocytes by ethanol, the second PB extrusion rate in the 1 min+30 s (43%) group was significantly higher than that in the 1 min+45 s group (35%) but significantly lower than those in the FDA-exposed (56%) and control groups (59%). The cleavage and blastocyst formation rates in the 1 min+30 s group (67 and 11%, respectively) were also significantly higher than those in the vitrified 1 min+45 s group (50 and 7%, respectively) and significantly lower than those in the FDA-exposed (86% and 21%, respectively) and the control groups (86% and 23%, respectively). There was no significant difference in total cell numbers among the four groups. However, the TE cell numbers of both control groups were significantly higher than those of vitrified groups.

4. Discussion

The major finding of this study is that buffalo M-II oocytes could be successfully cryopreserved by vitrification with microdrop method which was verified by their development to the blastocyst stage after PA and ICSI. Vitrification is a simple, rapid and cost-effective method of cryopreserving mammalian cells. Using this method, cryopreserved cells are less likely to

277 experience solution effects and intracellular ice formation [37]. However, a negative consequence of
278 this method is believed to be the increased probability of different kinds of stresses other than ice
279 crystal formation such as osmotic injury, toxic effect of cryoprotectants, concentrated intracellular
280 electrolytes or zona hardening [38]. To achieve vitrification of solutions, radical increases of
281 cooling rates are required.

282 The microdrop method involves dropping the oocyte-containing solution directly into liquid
283 nitrogen. The success of this method is due to elimination of the insulation effect of the container
284 wall. Warming of the oocytes is equally rapid when vitrified samples are directly dropped into a
285 warm solution [16]. In the present study more than 90% of the vitrified-warmed oocytes following
286 microdrop method had a normal esterase enzyme activity as determined by FDA staining, proving
287 that microdrop method was effective for buffalo M-II oocytes cryopreservation. One essential factor
288 in cryosurvival is the permeation of a certain amount of CPA into the oocyte, increasing with the
289 duration of exposure. However, the toxicity of the CPA must be considered, with an optimum
290 exposure time being favorable for this purpose.

291 In the present study, we investigated the effects of different exposure time to
292 equilibration/vitrification solutions. The survival and developmental rates in the 1min+30 s group
293 were higher than those in 1 min+45 s or 1 min+60 s groups. Toxicity testing of different exposure
294 time to equilibration/vitrification solutions showed that the embryo developmental rate decreased
295 with increasing exposure time. Parthenogenetic development of buffalo M-II oocytes after CPA
296 treatment in the 1 min+30 s group did not differ significantly from control groups, suggesting that
297 exposure of oocytes to equilibration solution for 1 min and vitrification solution for 30 s without
298 cooling and warming processes did not affect the embryo development. However, increasing the
299 exposure time to vitrification solution to 60 s resulted in a reduction in the embryo developmental

300 rate, probably due to excessive permeation of the CPA into the oocyte causing a higher toxicity
301 level in the 1min+60s treatment. In mouse, bovine and rabbit oocytes, exposure to CPA can result in
302 microtubule disorganization [39-41]. The very short exposure time to a high concentration of EG
303 before cooling may reduce its toxic effects.

304 This study has demonstrated that vitrification applied on buffalo oocytes decreased second
305 PB formation, cleavage and blastocyst rates when compared with fresh oocytes. For successful ICSI
306 in bovine and buffalo species, applying activation to oocytes is considered to be a key factor [42].
307 The second PB formation is often taken to indicate the ability of oocytes to accomplish meiosis.
308 Our study revealed that the vitrification and warming procedure could reduce the ability of oocytes
309 to accomplish the second meiosis division, which might be caused by the altered spindle formation.
310 A possible reason may be damage to the meiotic spindle, as has been frequently observed in
311 cryopreserved oocytes [43]. Freezing injury would lead to spindle disorganization, microtubules
312 loss or clumping and premature cortical granules which can cause zona hardening [23, 44]. The
313 ICSI into frozen-thawed bovine oocytes enhanced the pronucleus formation, cleavage and
314 blastocyst development compared with IVF [45], suggesting that the release of cortical granules
315 from vitrified oocytes led to zona-hardening and low fertilization rates [46]. In our study, cleavage
316 and blastocyst rates in the ICSI control groups were significantly higher than those of vitrified
317 groups. The rate of development to the blastocyst stage in the 1min+30s group was significantly
318 higher than that in the 1min+45s group and significantly lower than those in control groups, but did
319 not differ between the FDA-exposed and control groups. Similar observations were made for the
320 total cell numbers of Day 7 blastocysts. This indicated that the vitrification procedure resulted in
321 retarded embryonic development and that 30 s exposure to the vitrification solution was the most
322 effective regimen for buffalo M-II oocyte vitrification. For bovine oocytes, 25-35 s for the final

equilibration seem to be a general agreement about the rather optimal protocol [14,16-17] which probably derived from some early experiements [47]. Our research is also similar to recent studies of buffalo oocytes vitrification on 30 s of final equilibration for the same cryoprotectants and OPS vitrification method [48].

In our study, oocyte viability was determined by FDA which selectively stains cells with normal esterase enzyme activity and oolemma integrity. We compared the control and FDA-exposed oocytes treated with FDA to determine the toxicity of the FDA staining procedure. Our results showed no difference in the second PB extrusion rates, embryo development and blastocyst cell numbers between FDA treated and untreated control oocytes. This indicates that FDA staining does not have any negative effects upon embryo development *in vitro* and thus can be safely used for viability testing of oocytes.

To the best of our knowledge, the present study is the first report of ICSI-derived blastocysts from buffalo oocytes vitrified at the M-II stage by microdrop method. In conclusion, microdrop, a simple, economical and rapid procedure, allowed M-II buffalo oocytes to be vitrified, fertilized by ICSI and to develop to the blastocysts stage. The results of this study demonstrated that buffalo oocytes vitrified by the microdrop method with the 1 min+30 s regimen could yield reasonable blastocyst formation rates after ICSI. Furthermore, the FDA staining for viability checking had no detrimental effect on the embryo development *in vitro*.

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474
475
476

77 Table 1. *In vitro* development of buffalo M-II* oocytes after exposure to CPA and the quality of blastocysts following PA

Treatment groups	No. oocytes	FDA viability (%)	Cleavage (%)	No. (%) of oocytes developed to				No. of BL examined	Mean (±S.D.) blastocyst cell No.		
				8-C	16-C	Mor	BL		Total cell	TE cell	ICM cell
1min+30s	111	111(100)	97(87)^{b}	64(58)^{ab}	36(32)^{b}	25(23)^{b}	18(16)^{ab}	8	68.2±21.4^{ab}	48.1±6.3	19.6±8.4^{ab}
1min+45s	111	111(100)	92(83)^{b}	59(53)^{b}	31(28)^{b}	20(18)^{b}	11(10)^{bc}	5	70.7±17.5^{ab}	49.5±7.1	19.1±7.3^{ab}
1min+60s	111	111(100)	76(68)^{c}	42(38)^{c}	17(15)^{c}	8(7)^{c}	2(2)^{c}	2	62.0±9.0^{b}	48.5±7.5	13.5±1.5^{b}
FDA-exposed	96	96(100)	85(89)^{ab}	64(67)^{ab}	43(45)^{a}	33(34)^{a}	21(22)^{a}	8	76.6±19.5^{a}	55.2±5.1	20.1±7.5^{a}
Control	96	-	90(94)^{a}	66(69)^{a}	46(48)^{a}	35(36)^{a}	25(26)^{a}	8	77.1±17.8^{a}	56.0±6.4	20.9±6.7^{a}

78 * Metaphase of the second meiotic cell division.

79 ^{a,b,c} Means within columns with different superscripts differ (P<0.01 ANOVA).

80 Treatment groups: 1min+30s = 1 min equilibration followed by 30 sec of exposure to vitrification solution;

81 1min+45s = 1 min equilibration followed by 45 sec of exposure to vitrification solution;

82 1min+60s = 1 min equilibration followed by 60 sec of exposure to vitrification solution.

83 Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

Table 2. *In vitro* development of buffalo M-II* oocytes and quality of blastocysts following vitrification and PA.

Treatment groups	No. oocytes	FDA viability (%)	Cleavage (%)	No. (%) of oocytes developed to				No. of BL examined	Mean (±S.D.) blastocyst cell No.		
				8-C	16-C	Mor	BL		Total cell	TE cell	ICM cell
1min+30s	130	126(97)^{ab}	84(67)^{b}	42(33)^{b}	24(19)^{b}	15(12)^{b}	10(8)^{b}	9	71.6±18.3	49.1±4.7	21.4±9.5
1min+45s	132	126(95)^{b}	58(46)^{c}	23(18)^{c}	10(8)^{c}	7(6)^{c}	5(4)^{c}	5	69.9±19.6	48.2±4.3	20.2±5.8
FDA-exposed	118	118(100)^{a}	105(89)^{a}	81(69)^{a}	53(45)^{a}	41(35)^{a}	28(24)^{a}	10	78.5±24.6	59.0±6.1	22.7±4.2
Control	117	-	110(94)^{a}	82(70)^{a}	56(48)^{a}	43(37)^{a}	31(26)^{a}	10	80.0±23.1	59.2±5.3	23.8±6.0

* Metaphase of the second meiotic cell division.

^{a,b,c} Means within columns with different superscripts differ (P<0.01 ANOVA).

Treatment groups: 1min+30s = 1 min equilibration followed by 30 sec of exposure to vitrification solution;

1min+45s = 1 min equilibration followed by 45 sec of exposure to vitrification solution;

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

92 Table 3. *In vitro* development of buffalo M-II* oocytes and quality of blastocysts following vitrification and ICSI.

93

Treatment groups	No. oocytes	FDA viability (%)	ICSI success (%)	2nd P B (%)	Cleavage (%)	No. (%) of oocytes developed to				No. of BL examined	Mean (±S.D.) blastocyst cell No.		
						8-C	16-C	Mor	BL		Total cell	TE cell	ICM cell
1min+30s	147	141(96)^{ab}	141(100)	61(43)^{b}	41(67)^{b}	24(39)^{b}	17(28)^{b}	9(15)^{b}	7(11)^{b}	5	99.1±11.2	61.0±9.1^{b}	31.2±6.7
1min+45s	146	133(91)^{b}	132(99)	46(35)^{c}	23(50)^{c}	14(30)^{b}	8(17)^{c}	5(11)^{b}	3(7)^{c}	3	94.8±14.7	60.2±5.8^{b}	30.5±9.6
FDA-exposed	126	126(100)^{a}	126(100)	70(56)^{a}	60(86)^{a}	47(67)^{a}	31(44)^{a}	23(33)^{a}	15(21)^{a}	9	101.7±12.3	70.0±6.8^{a}	32.2±4.5
Control	130		130(100)	77(59)^{a}	66(86)^{a}	50(65)^{a}	34(44)^{a}	24(31)^{a}	18(23)^{a}	9	104.2±10.6	69.2±8.6^{a}	35.5±3.1

94 * Metaphase of the second meiotic cell division.

95 ^{a,b,c} Means within columns with different superscripts differ (P<0.01 ANOVA).

96 Treatment groups: 1min+30s = 1 min equilibration followed by 30 sec of exposure to vitrification solution;

97 1min+45s = 1 min equilibration followed by 45 sec of exposure to vitrification solution;

98 Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

99 Rates of cleavage, 8-C, 16-C, Mor and BL were calculated out of fertilized (2nd P B) oocytes.

เอกสารแนบหมายเลข 4

SURVIVAL RATES OF MATURED BUFFALO OOCYTES AFTER VITRIFICATION BY MICRODROP AND CRYOTOP AND SUBSEQUENT EMBRYOS DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION

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ABSTRACT

In the present study, effects of two types of vitrification solution, and of two types of vitrification technique on the survival rate of vitrified-warmed buffalo MII oocytes were examined. Furthermore, the developmental capacity of vitrified-warmed buffalo MII oocytes following intracytoplasmic sperm injection (ICSI) was investigated. *In vitro* matured (IVM) oocytes were randomly separated into 6 groups and cryopreserved by using 1) Cryotop method combined with VA solution (10% DMSO+10% EG for 1 min and then 20% DMSO+20%EG+0.5M sucrose for 30 sec), (Cryotop+VA) 2) Cryotop method combined with VB solution (4% EG for 12-15 min and then 35% EG+5% polyvinylpyrrolidone and 0.4 M trehalose for 30 sec), (Cryotop+VB) 3) Microdrop method combined with VA solution, (Microdrop+VA) 4) Microdrop method combined with VB solution, (Microdrop+VB) 5) Control oocytes stained with fluorescein diacetate (FDA), 6) Fresh control oocytes without staining by FDA. The survival rate of oocytes was examined by FDA staining and surviving oocytes were subjected to ICSI. The recovery rates were similar among groups. The oocytes viability of VA solution (Microdrop+VA: 93% and Cryotop+VA: 97%) were significantly higher than in VB solution (Microdrop+VB: 79%, Cryotop+VB: 81%), but significantly lower than control groups (100%). The second polar body extrusion rate and the capacity of embryo development to the blastocyst stage in control group and fresh control groups were significantly higher than vitrified groups, but no difference among vitrified groups. There was no difference between control and fresh control group in second polar body extrusion and embryo development. In conclusion, VA solution could yield higher survival rate of vitrified oocytes, Cryotop and Microdrop are equally suitable techniques for buffalo oocytes vitrification.

INTRODUCTION

According to the buffalo is the major milk and meat producing farm animal in many developing countries, the *in vitro* embryo production of buffalo become popular nowadays. Cryopreservation of oocytes has great importance in buffalo for the efficient oocytes cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in the buffalo species.

Oocytes cryopreservation is still an open challenge in most mammalian species, due to the extreme sensitivity of gametes to chilling injuries. Vitrification is the process that induces a glass-like solidification of living cells during freezing. There are many species successfully cryopreserved by vitrification, such as bovine (Vieira et al., 2002), mouse (Wood et al., 1993), equine (Maclellan et al., 2002) and human oocytes (Katayama et al., 2003).The unique advantage of the vitrification process is elimination of ice crystal formation, the most dangerous cause of cryoinjury. Many factors affected the efficacy of vitrification, such as cryoprotectant concentration, exposure time, container and temperature. There are some improved vitrification methods succeed in mammalian oocytes cryopreservation such as Cryotop (Kuwayama and Kato, 2000), Cryoloop (Oberstein et al., 2001), OPS (open pulled straw) (Vajta et al., 1998), GMP (glass micropipette) (Kong et al., 2000), Microdrop (Papis et al., 2000), and EMG (electron microscope grids) (Martino et al., 1996). Among the various methods of vitrification, Microdrop vitrification was easy and convenient, as it excluded the use of any specialized device using minimum volumes of vitrification to introduce oocytes into liquid nitrogen. One of the most successful ultra-rapid vitrification technique is the Cryotop vitrification that has resulted in excellent survival and developmental rates with

human and bovine MII oocytes (Kuwayama et al., 2005) when cryopreserved oocytes were fertilized *in vitro*. Cryoprotectants influence the ability of buffalo oocytes to survive cryopreservation (Wani et al., 2004). Several studies demonstrated that EG would be the ideal cryoprotectant (Shaw et al. 1997; Cetin and Bastan 2006), because it penetrates membranes faster than glycerol (Cha et al. 2000) and is less toxic than other permeable cryoprotectants (Martino et al. 1996; Cha et al. 2000; Dinnyes et al. 2000). Moreover, cryoprotectant mixtures may have some advantages over solutions containing only one permeable cryoprotectant (Vajta et al. 1998; Chian et al. 2004).

In addition, change in the zona pellucida (ZP) has shown to induce lower fertility rates (Carroll et al., 1990; Vincent et al., 1990). These structural changes causing lower fertility could be overcome by micromanipulation technique such as ICSI (Carroll et al., 1990; Kazem et al., 1995; Karlsson et al., 1996; Porcu et al., 1997).

The aims of this study were to investigate the effects of two types of vitrification technique Microdrop and Cryotop and two types of cryoprotectant solution on the post-warm viability, embryos developmental competence of vitrified buffalo MII oocytes subjected to ICSI.

METERIALS AND METHODS

Unless stated otherwise, all reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA)

Oocyte collection and *in vitro* maturation

Buffalo ovaries were obtained from a slaughterhouse and transported to the laboratory within 4 h in physiological saline (0.9% NaCl). Cumulus-oocyte complexes (COCs) were collected from the follicles 2–8 mm in diameter and were matured *in vitro* for 22 h. The medium for IVM was TCM 199 supplemented with 10% fetal bovine serum, 0.02 AU/mL FSH(Antrin, Denka Pharmaceutical, Tokyo, Japan), 50 iu/mL hCG(Chorulon, Intervet, Boxmeer, Netherlands) and 1μg/mL estradiol-17ß. After maturation, cumulus cells were gently removed by pipetting, and the oocytes with a visible first polar body were selected for the following experiments. The Emcare holding medium (EHM, ICP Bio, Auckland, New Zealand) was used as the basal medium throughout the process of ICSI. The medium for embryo culture was modified synthetic oviduct fluid supplemented with amino acids and 0.3% fatty acid-free BSA (mSOFaa) (Gardner et al., 1994).

Vitrification and warming

The M-II oocytes were vitrified-warmed by either the Cryotop minimum volume cooling procedure, originally reported by Kuwayama and Kato (Kuwayama and Kato, 2000) or Microdrop vitrification method (Papis et al., 2000).

Two different cryoptotectant solutions were used to vitrify buffalo MII oocytes. Oocytes vitrified by VA method was described as following. *In vitro* matured oocytes were placed in TCM199-Hepes + 20% FBS (basic medium; BM) + 10% dimethylsulfoxide (DMSO) + 10% ethylene glycol (EG) for 1 min and then exposed in BM + 20% DMSO + 20% EG + 0.5 M sucrose for 30 sec. Finally, a group of 5 oocytes were directly dropped with about 2 μL vitrification solution into liquid nitrogen or placed on a sheet of each Cryotop in a small

112 volume of the vitrification solution (<1μL). For storage, vitrified oocytes were kept in liquid
113 nitrogen for 1-2 weeks. The vitrified Microdrops or Cryotops were warmed by directly
114 immersing into 3 ml of 0.5 M sucrose in BM for 5 min, and then transferred to the BM for 5
115 min. Then they were kept in the BM under a humidified atmosphere of 5% CO_2 in air at
116 38.5 °C for 1 h.

117 VB method was originally described by Dinnyes (Dinnyés et al., 2000). Briefly, the
118 oocytes were treated with an equilibration medium which consisted of 4% (v/v) EG in BM for
119 12 to 15 min at 38.5 °C followed by 35% (v/v) EG, 50 mg/ml polyvinyl pyrrolidone (PVP)
120 and 0.4 M trehalose for 30 s. Vitrified oocytes were warmed by transferring
121 Microdrops/Cryotops into a warming solution (0.3 M trehalose in BM) at 38.5°C. One to 2
122 min later, the oocytes were consecutively transferred for 1-min into each 500-μl droplets of
123 BM supplemented with 0.15 M, 0.075 M and 0.0375 M trehalose, respectively. And then they
124 were cultured under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h.

125

126 **Evaluation of oocyte viability**

127 Oocyte viability was evaluated by fluorescein diacetate (FDA) staining according to
128 the method previously described by Mohr and Trounson (Mohr and Trounson, 1980). Briefly,
129 oocytes were treated with 2.5 μg/mL FDA in PBS supplemented with 5 mg/mL BSA at 38.5
130 °C for 2 min in a dark room and then they were washed three times in PBS supplemented
131 with 5 mg/mL BSA and evaluated under a fluorescent microscope (IX71, Olympus, Tokyo,
132 Japan) with UV irradiation using a U-MWIB3 filter with excitation wavelength of

460–495 nm and emission at 510 nm. Oocytes expressing a bright green fluorescence were regarded as living ones and were used subsequently.

ICSI

Straw of frozen spermatozoa were thawed at 37°C for 30 sec. Thawed spermatozoa were gently placed to the bottom of the 1mL Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975), supplemented with 10 mM caffeine (caffeine-BO) in a centrifuge tube for sperm swim up for 30 min and then the supernatant was collected and centrifuged at 500×g for 5 min. The sperm pellet was washed twice with 1mL of caffeine-BO by centrifugation at 500×g for 5 min. The sperm pellet was resuspended in the caffeine-BO at concentration of 8×10^6 sperm/mL. ICSI was performed as previously described by Liang et al [Liang et al., 2010]. Briefly, three droplets of media covered with mineral oil were prepared on the lid of 60 mm culture dish for the ICSI procedure; the first droplet was polyvinylpyrolidone (PVP) solution (ICSI®, Vitrolife, Gothenburg, Sweden) for washing the pipette, the second droplet was the sperm suspension diluted with PVP solution (1:5), and the third droplet was EHM for the ICSI procedure. Approximately 10 MII oocytes were placed in the droplet of EHM. A single, motile buffalo spermatozoon was immobilized against the bottom of the PVP droplet, loaded tail first with a minimum volume of medium into the injection pipette and then injected into the cytoplasm of a buffalo oocyte. Within 1 h of the injection, the injected oocytes were activated by exposure to 7% ethanol in EHM for 5 min, and then subsequently cultured in TCM199 + 10% FBS for 3 h to allow extrusion of the second PB. With the purpose of producing haploid activated oocytes for ICSI, the injected

and activated oocytes which extruded the second PB were selected and transferred to mSOF medium supplemented with 10 μg/mL CHX and cultured for 5 h at 38.5 °C under humidified atmosphere of 5% CO_2 in air.

***In vitro* culture**

The presumptive zygotes were cultured in the mSOFaa medium under a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C for 2 days. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelium cells (BOEC) under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 5 days (Parnpai et al., 1999). Half of the medium was replaced with fresh medium every day and the embryo development was recorded at the same time of medium changing. The cleavage rates were recorded on Day 2 (the day of ICSI was considered as Day 0), the development of embryos to blastocyst stage was recorded on Day 7.

Differential cell staining

The blastocysts harvested on Day 7 were stained to distinguish cells of the inner cell mass (ICM) and trophectoderm (TE), as previously reported (Suteevun et al., 2006). Briefly, the zona pellucidae of blastocysts were removed by exposure to 0.5% protease. After washing with mDPBS supplemented with 0.1% PVP, the zona-free blastocysts were incubated in 100 μL of 10% rabbit anti-buffalo spleenocyte antibodies for 45 min, and then transferred into a 100 μL mixture of 10% guinea pig complement, 10 μg/mL propidium iodide and 10 μg/mL Hoechst 33258 for 45 min. The blatocysts were mounted on glass slides with glycerol and

177 covered with cover slide. The ICM (blue) and TE cells (red) were counted under a fluorescent
178 microscope at 330–380 nm, allowing determination of the total number of cells for blastocysts
179 and the percentage of ICM cells based on the total number of blastocysts.
180
181
182 **Statistical analysis**
183 Experiments were replicated at least three times in each treatment group. Data were
184 analyzed by ANOVA using the statistical analysis systems. The differences between groups
185 were considered to be statistically significant when $P < 0.05$.
186
187 **RESULTS**
188
189 **Effect of vitrification techniques on recovery and viability of oocytes**
190 Oocytes were vitrified either by Microdrop or Cryotop with VA or VB solution (Fig.1).
191 After warming, the recovery rate between Microdrop and Cryotop in VA solution (96 and
192 100%, respectively) was no difference, which was also found in VB solution for Microdrop
193 and Cryotop (98 and 100%, respectively). Among the groups, there was no difference in the
194 oocytes recovery rate when compared with control groups.
195 The viability of oocytes examined by FDA staining was illustrated in Table 2. The
196 proportion of live oocytes vitrified with VA solution was significantly higher than VB
197 solution but lower than fresh control group (93-97%, 79-81% and 100% respectively). And
198 the results of oocytes survival rate were nearly indentical for both Microdrop and Cryotop in

199 same vitrification solution.

200

201 **Effect of vitrification methods on ICSI results**

202 After ICSI, there was no difference in the 2nd polar body formation rate between
203 fresh control (+) FDA (56%) and (-) FDA (57-59%) but they were significantly higher than
204 those of Microdrop (39% in VA, 37% in VB, repectively) and Cryotop (43% in VA; 40% in
205 VB, respectively). The 2nd polar body formation between Microdrop and Cryotop were no
206 significant difference in VA or VB. The cleavage rate of fresh control (+) FDA (79-82%) and
207 (-) FDA (81-83%) was no significant difference, but they were significantly higher than those
208 of Microdrop (65% in VA, 66% in VB, respectively) and Cryotop (67% in VA, 72% in VB,
209 respectively). The blastocyst rate of fresh control (+) FDA (21%) and (-) FDA (19-22%) was
210 no significant difference, but they were significantly higher than those of Microdrop (8% in
211 VA, 5% in VB, respectively) and Cryotop (10% in VA, 11% in VB, respectively). The
212 blastocyst formation rate of Microdrop (8%) and Cryotop (10%) was no significant difference
213 in VA, and VB (5%, 11%, respectively).

214

215 **Cell number in day 7 ICSI-derived blastocyst from vitrified oocytes**

216 The cell number of ICSI-derived blastocyst from vitrified oocytes was showed in Table
217 2. According to the blastocyst quality, there was no significantly difference among the
218 vitrified groups and control groups, except that of Microdrop method in VB solution, which
219 was significantly lower than that of control groups in the total cell number and ICM cell
220 number.

221

222 **DISCUSSION**

223 The major finding of this study is that buffalo MII oocytes could be cryopresered by
224 vitrification with Microdrop and Cryotop methods and the oocytes were suitable for ICSI
225 produce.

226 The recovery rate is the number of remaining oocytes after expulsion of solution from
227 the container during warming. Its analysis is important because cells must tolerate a sequence
228 of volumetric contractions and expansions because of different concentrations of solutions,
229 and they can be lost or rupture during this process. Moreover, the recovery rate can be
230 influenced by the container and vitrification solution as the oocytes might be lost during the
231 last step of vitrification by sticking on the transfer pipette. After vitrification and warming of
232 buffalo MII oocytes, 96-100% recovery rate was taken from both VA and VB solution, which
233 demonstrated that this two vitrification solution and two containers did not affect the oocytes
234 recovery rate. The viability of vitrified oocytes after warming in VA solution was
235 significantly higher than in VB solution, but the same vitrification solution Microdrop and
236 Cryotop arrive the indentical survival rate. Our study revealed that VA method has the higher
237 cryopreservation efficacy than VB system.

238 Cryotop is a minimum volume procedures where the carrier tool, a thin film attached to
239 a plastic holder is equipped with a protective plastic tube (Kuwayama et al., 2005). Oocytes
240 are loaded on the film, the solution is almost entirely removed by aspiration, and the sample
241 is immersed into liquid nitrogen. Subsequently, for safe storage, the plastic tube is pulled over
242 the film. The method is easy to learn and perform, the cooling and warming rates are higher

than those achievable with OPS, and the simple manipulation decreases the risk of inconsistency.

Microdrop method involves dropping an oocytes-containing solution directly into liquid nitrogen, the success of this method is due to elimination of the insulation effect of the container wall. Warming of the oocytes is equally rapid when vitrified sample are directly dropped into a warm solution (Paris et al., 2000). One essential factor in cryosurvival is permeation of a certain amount of cryoprotectant into the oocyte, which increase with the duration of exposure. However, the toxicity of the cryoprotectant must be avoided, with an optimum exposure time being favorable for this purpose.

In the present study, we investigated the effect of VA, VB methods and Microdrop, Cryotop technique on the oocyte viability after vitrified-warmed and embryo development following ICSI. This study has demonstrated that ICSI into frozen-thawed oocytes decreased the rates of 2^{nd} polar body formation, cleavage and blastocyst development when compared with the fresh oocytes. The 2^{nd} polar body formation was judged as the ability of oocytes accomplishing meiosis. Our study revealed that frozen-thawed procedure could reduce the oocytes ability to accomplish the second meiosis.

Regarding the effect of vitrification technique, the recovery rates, viability of oocytes, embryo development rates and blastocyst quality that derived from Cryotop were higher than Microdrop method.

Cryotop method that containing the most less vitrification solution, which can greatly increase the cooling speed had been applied to pronuclear-stage rabbit zygotes (Hochi et al., 2004), pre-hatching stage porcine embryos (Esaki et al., 2004), germinal vesicle-stage whale

265 COCs (Iwayama et al., 2004), denuded *M-II* bovine oocytes (Chian et al., 2004),
266 SCNTderived bovine and swamp buffalo blastocysts (Laowtammathron et al., 2005) and
267 denuded M-II oocytes and IVF-derived blastocysts in humans (Kuwayama et al., 2000;
268 Katayama et al., 2003), was successfully extended to both denuded M-II and enucleated
269 swamp buffalo oocytes in the present study. On the other way, the success of Microdrop
270 method is due to elimination of the insulation effect of the container wall. Warming of the
271 oocytes is equally rapid when vitrified samples are directly dropped into a warm solution
272 (Papis et al., 2000). Our results supported the view that Cryotop was regarded to be the
273 effective tool for oocytes or embryos vitrification (Kuwayama et al., 2000) according to the
274 embryo development.

275 The higher survival rate were obtained from VA solution which using the CPA
276 combination of EG+DMSO. Albarracyn et al., (2005) reported that oocytes exposed only to
277 20% EG and 20% DMSO showed a similar appearance to the control. The comparison of two
278 vitrification medium (VA, VB) with similar results showed flexibily of oocytes to tolerate
279 cryopreservation. DMSO was used in combination with EG in VA, whereas EG was alone
280 used for equilibration and in comination with PVP for vitrification in VB. In bovine, oocytes
281 have higher permeability to DMSO than to EG (Agca et al., 1998), this may account for VA
282 working better than VB according to the oocyte viability. The reduction of the survival and
283 embryo development could be resulting from possible multifactorial causes, including toxic
284 effects of the CPA, ultrastructural damage to the oocytes, and deleterious effects on the
285 chromosomes and other cytoplasmic structure (Johnson and Pickering 1987)

286 In conclusion, our study indicated that Cryotop method could yield high survival rate

of *in vitro* matured swamp buffalo oocytes which could develop to blastocyst stage after ICSI. The vitrification method of VA could yield high survival rate of frozen-thawed *in vitro* matured buffalo oocytes. But the development competence of frozen-thawed *in vitro* matured buffalo oocytes was significantly lower than fresh control oocytes.

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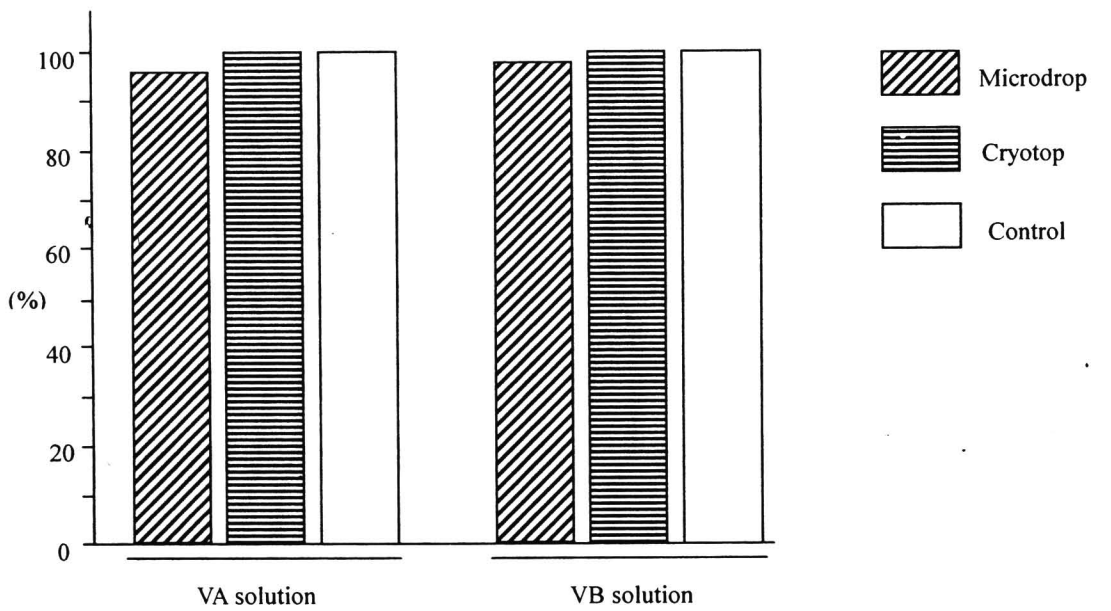
Figure 1. Recovery rate of vitrified-warmed buffalo MII oocyte



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406 Figure 2. Survival rates of vitrified-warmed buffalo MII oocytes stained by FDA

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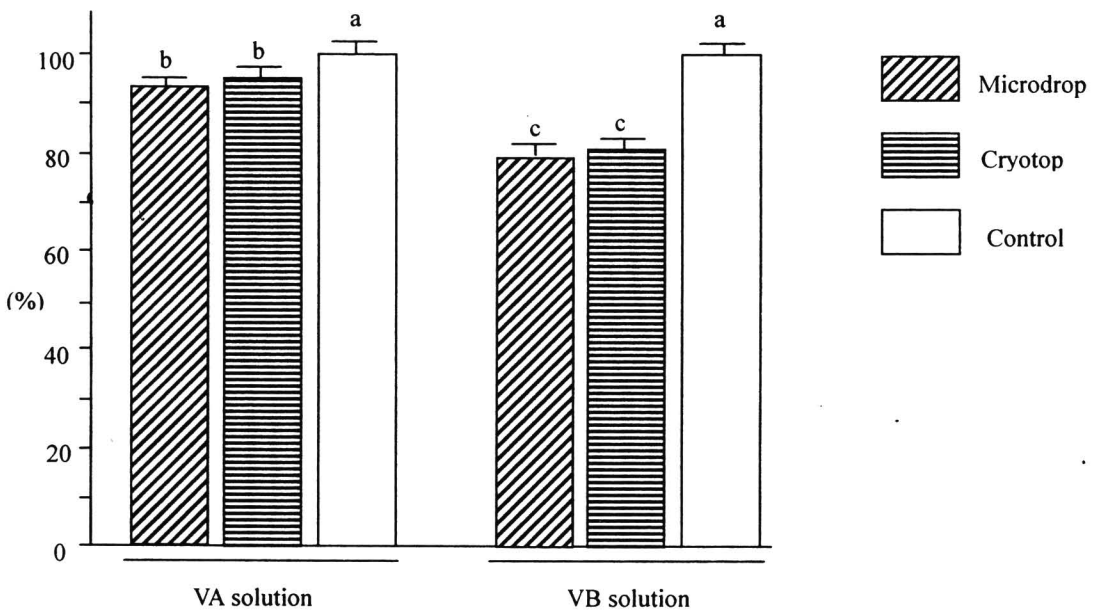

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410 [a,b,c] Means within columns with different superscripts differ (P<0.01 ANOVA).

411 Table1. Development of ICSI derived embryos from vitrified-warmed buffalo MII oocytes.

Vitrification solutions	Methods	No. of ICSI	ICSI success	2nd polar body	Cleavage rate	No. (%) of oocytes developed to			
						8-C	16-C	Mor	BL
VA	Microdrop	127	124(98)	48(39)^{b}	31(65)^{b}	16(33)^{b}	11(23)^{b}	5(10)^{b}	4(8)^{b}
	Cryotop	140	139(99)	60(43)^{b}	40(67)^{b}	23(38)^{b}	15(25)^{b}	9(15)^{b}	6(10)^{b}
	Control*	124	124(100)	70(56)^{a}	55(79)^{a}	40(57)^{ab}	27(39)^{a}	21(30)^{a}	15(21)^{a}
	Fresh Control	124	124(100)	73(59)^{a}	59(81)^{a}	42(58)^{ab}	27(37)^{a}	17(23)^{ab}	14(19)^{a}
VB	Microdrop	111	110(99)	41(37)^{b}	27(66)^{b}	15(37)^{b}	9(22)^{b}	3(7)^{b}	2(5)^{bc}
	Cryotop	117	116(99)	46(40)^{b}	33(72)^{ab}	20(43)^{b}	11(24)^{b}	7(15)^{b}	5(11)^{b}
	Control*	111	111(100)	62(56)^{a}	51(82)^{a}	39(63)^{a}	25(40)^{a}	19(31)^{a}	13(21)^{a}
	Fresh Control	115	115(100)	65(57)^{a}	54(83)^{a}	41(63)^{a}	25(38)^{a}	18(28)^{a}	14(22)^{a}

412 ^{a,b} Means within columns with different superscripts differ (P<0.01 ANOVA).

413 Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst

414

415 Table2. Quality analysis of the blastocysts derived from Day-7 (Buffalo oocytes vitrified-ICSI)

Vitrification solution	Methods	No. of embryos examined	Blastocyst cell numbers (Mean ± S.D)		
			Total cell	TE cell	ICM cell
VA	Microdrop	4	90.5 ± 12.0 [ab]	58.6 ± 7.1	30.7 ± 5.2 [a]
	Cryotop	6	92.6 ± 9.8 [ab]	56.0 ± 10.2	32.6 ± 8.4 [a]
	Control*	8	99.4 ± 10.7 [a]	67.5 ± 9.3	31.8 ± 9.7 [a]
	Fresh Control	8	97.1 ± 11.3 [a]	62.2 ± 6.5	32.1 ± 7.3 [a]
VB	Microdrop	2	72.0 ± 13.0 [b]	47.5 ± 8.5	24.5 ± 4.5 [b]
	Cryotop	5	87.5 ± 11.2 [ab]	53.2 ± 7.3	28.6 ± 7.1 [ab]
	Control*	8	96.8 ± 14.3 [a]	62.5 ± 6.7	30.1 ± 8.5 [a]
	Fresh Control	8	99.7 ± 9.1 [a]	63.0± 8.2	30.9 ± 6.4 [a]

416 [a,b] Means within columns with different superscripts differ (P<0.01 ANOVA).

417 Abbreviations: TE = trophectoderm; ICM = inner cell mass.

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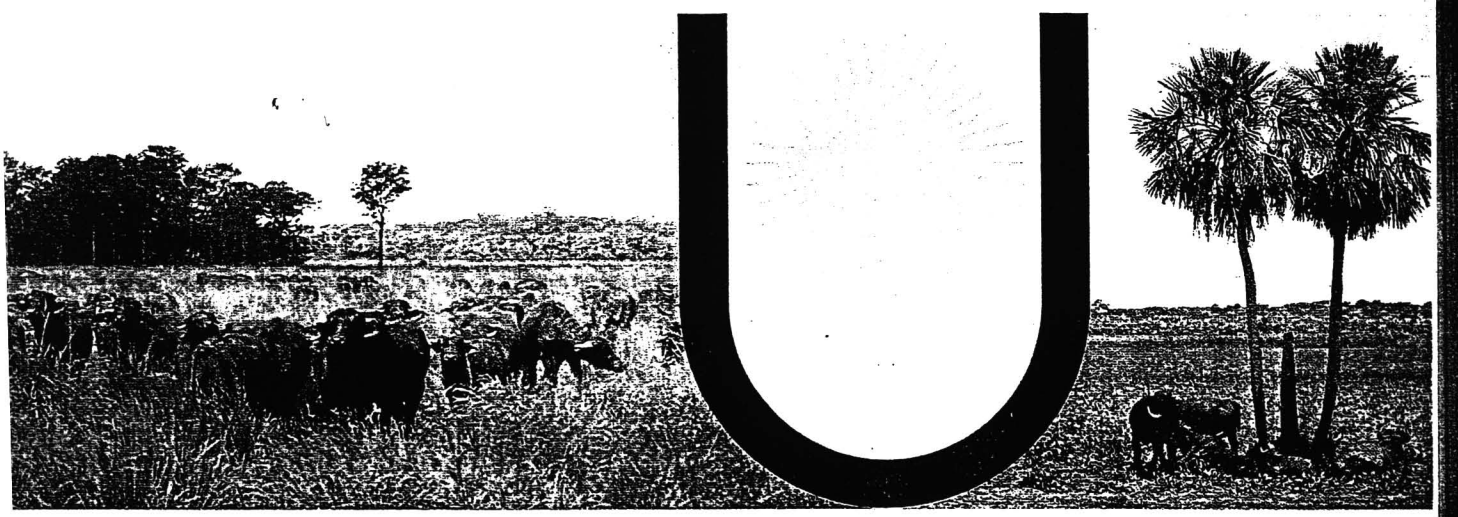

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Developmental Rates of Vitrified Buffalo Oocytes Following Parthenogenetic Activation and Intracytoplasmic Sperm Injection

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ABSTRACT

The objective of this study was to investigate the potential of swamp buffalo oocytes vitrified-warmed at the MII stage to develop to the blastocyst stage after parthenogenetic activation (PA) and intracytoplasmic sperm injection (ICSI). In the first experiment, we examined the toxic effects of cryoprotectants on *in vitro* development. *In vitro* matured oocytes were placed in 10% dimethylsulfoxide (DMSO) + 10% ethylene glycol (EG) for 1 min and then exposed to 20% DMSO + 20% EG + 0.5 M sucrose for 30 sec (1+30), 45 sec (1+45) or 60 sec (1+60). The oocytes were exposed to warming solution (0.5M sucrose) for 5 min and then washed in TCM199 HEPES + 20%FBS for 5 min. Oocyte viability was assessed by fluorescein diacetate (FDA) staining. Surviving oocytes were parthenogenetically activated and cultured for 7 days. The viability in all groups of CPA treatment and the control were 100%. The development rates to the blastocyst stage among CPA exposed 1+30 (17%), control (23%) and fresh control (control without FDA assay) (27%) did not differ significantly, but they were significantly higher than those in CPA exposed 1+45 (9%) and 1+60 (1%) groups. In the second experiment, we examined the effect of two CPA exposure times, 1+30 and 1+45 on the *in vitro* development for 7 days after PA of oocytes vitrified by the microdrop method. The viability in vitrified 1+30, 1+45 and the control groups was not different (97%, 95% and 100%, respectively). The development of surviving oocytes to blastocyst stage in the vitrified 1+30 group (8%) was significantly higher than that in the vitrified 1+45 group (4%) and significantly lower than those in control and fresh control groups (24% and 26%, respectively). In the third experiment, we examined the effect of two CPA exposure times, 1+30 and 1+45 on *in vitro* development after ICSI of vitrified oocytes. The FDA viability in vitrified 1+30, 1+45 and control groups (100%) was not different (96%, 91% and 100%, respectively). After ICSI vitrified-warmed oocytes were activated and oocytes with the 2nd polar body were cultured for 7 days. The development of ICSI oocytes to the blastocyst stage in the vitrified 1+30 group (11%) was significantly higher than that in vitrified 1+45 (7%) and significantly lower than those in the control and fresh control (21% and 23%, respectively). In conclusion, our study demonstrated that the 1+30 CPA treatment regimen could yield the highest blastocyst rates for oocytes vitrified by the microdrop method and that the FDA viability test had no effect on the embryo development.

Keywords: buffalo oocytes, microdrop, vitrification, ICSI.

INTRODUCTION

Nowadays, buffalo is the major milk and meat producing farm animal in many developing countries. Buffalo oocytes obtained from slaughterhouse ovaries and matured *in vitro* are useful sources for reproductive procedures such as *in vitro* fertilization (IVF) and ICSI, in which cryopreserved spermatozoa are used. Cryopreservation of oocytes also has great importance to preserve female gamete for future use. Efficient oocyte cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in the buffalo species.

Cryopreservation of mammalian oocytes has become more successful using vitrification as an alternative to cryopreservation compared with slow cooling methods in recent years (Chian et al., 2004; Vajta and Nagy, 2006). Vitrification is the process that induces a glass-like solidification of living cells at low temperatures. The unique advantage of the vitrification process is the elimination of ice crystal formation, the most dangerous cause of cryoinjury. Insufficient cooling rate of oocytes was considered as one of the obstacles in vitrification technology (Vajta, 1997). In order to overcome this problem, several methods have been proposed that use very small amounts of solution. There are some improved vitrification methods which has been successfully used for oocyte cryopreservation such as cryotop (Kuwayama and Kato, 2000), cryoloop (Lane et al., 1999), open pulled straw (OPS; Vajta et al., 1998), glass micropipette (GMP; Hochi et al., 1994), microdrop (Papis et al., 2000), electron microscope grids (EMG; Martino et al., 1996) and solid surface vitrification (SSV, Dinnyés et al., 2000).

Since the first report on buffalo oocytes vitrification by using French Straw (Dhali et al., 1999), there are some reports regarding the cryopreservation of buffalo oocytes (Wani et al., 2004; Gasparrini et al., 2007; Muenthaisong et al., 2007; Boonkusol et al., 2007; Sharma and Loganathasamy, 2007; Gautam et al., 2008; Mahmoud et al., 2008). The first successful production of buffalo blastocyst derived from *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of vitrified-warmed oocytes (Wani et al., 2004).

Among the various methods of vitrification, the microdrop method is considered easy and cheap, as it excludes the use of any specialized device to introduce oocytes into liquid nitrogen. The microdrop method is a simplest way of vitrification by dropping oocyte containing solutions directly into liquid nitrogen. This method was first proposed for mouse embryos (Landa and Tepla, 1990), and then successfully applied for in bovine embryos, zygotes and oocytes (Riha et al.,1991; Yang and Leibo,1999; Papis et al., 2000). During cryopreservation or treatment with cryoprotectants structural changes in the zona pellucida (ZP) has been shown to reduce fertilization rates (Carroll et al., 1990; Vincent et al., 1990). Although the mechanism of ZP hardening of the cryopreserved oocytes is unclear, it seems to be caused by the premature release of cortical granules (Vincent et al., 1990) resulting in lower incidences of sperm entry into oocytes. This consequence of zona hardening could be overcome by micromanipulation techniques such as ICSI (Carroll et al., 1990; Kazem et al., 1995; Karlsson et al., 1996; Porcu et al., 1997).

One essential factor in cryosurvival is permeation of a centain amount of cryoptotectant into the oocytes, which increase with the duration of exposure; however, the toxicity of the cryoprotectant must be avoided, with an optimum exposure time being favorable for this purpose. The aim of this study was to investigate the exposure time in vitrification solution on the post-thaw viability and the developmental competence of vitrified-warmed swamp buffalo oocytes after parthenogenetic activation (PA) or ICSI. Differential cell staining was applied to assess the qualitative aspects of blastocysts that were derived from fresh or vitrified-warmed oocytes.

MATERIALS AND METHODS

Chemicals and media

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. The medium used for IVM was TCM199 supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 0.02 AU/mL FSH (Antrin, Denka Pharmaceutical, Tokyo, Japan), 50 iu/mL hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1 µg/mL estradiol-17ß. The Emcare holding medium (ICP Bio, Auckland, New Zealand) was used as the basal medium throughout the process of ICSI and parthenogenetic activation. The medium for embryo culture (IVC medium) was modified synthetic oviduct fluid supplemented with amino acids (mSOFaa) (Gardner et al., 1994) and 0.3% fatty acid-free BSA.

Oocyte collection and *in vitro* maturation

Buffalo ovaries were obtained from a slaughterhouse and transported to the laboratory within 4 h at room temperatur The ovaries were placed in 0.9% NaCL during transport to laboratory. Cumulus-oocyte complexes (COCs) were collecte from the 2-8 mm follicles using a 21-gauge needle and were *in vitro* cultured in IVM medium for 22 h. After maturation cumulus cells were removed by gentle pipetting with a fine glass pipette, and the oocytes were subsequently washed : times in Emcare medium. Oocytes with a visible first polar body were selected for the following experiments.

Vitrification and warming

The M-II oocytes were vitrified-warmed by the Microdrop method originally reported by Papis et al. (2000). Groups of 5 oocytes were washed in TCM199-Hepes + 20% FBS before placed in TCM199-Hepes + 20% FBS + 10% DMSO + 10% ethylene glycol (EG) for 1 min, and then exposed in TCM199-Hepes + 20% FBS + 20% DMSO +20% EG + 0.5 M sucrose for 30 (1+30), 45 (1+45) or 60 sec (1+60) at 22-24 °C. The oocytes were then directly dropped with about 2 µL vitrification solution into liquid nitrogen. For storage, vitrified microdrops were placed in a pre-cooled cryovial filled with liquid nitrogen using a pre-cooled forceps and kept for 1-2 weeks. The vitrified microdrops were warmed by directly immersing into 3 mL of 0.5 M sucrose in TCM199-Hepes + 20% FBS at 38.5 °C for 5 min, and then transferred to the TCM199-Hepes+ 20% FBS for 5 min. Then oocytes were kept in the TCM199-Hepes + 20% FBS under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h. Some oocytes were treated with cryoprotectants and warming solution without the cooling and warming process ("CPA-exposed" group).

Evaluation of oocyte viability

Oocyte viability was evaluated by FDA staining according to the method previously described by Mohr and Trounson (1980). Briefly, oocytes were treated with 2.5 µg/mL FDA in PBS supplemented with 5 mg/mL BSA at 38.5 °C for 2 min in a dark room and then washed three times in PBS supplemented with 5 mg/mL BSA and evaluated under an epifluorescence microscope with UV irradiation using a U-MWIB3 filter with excitation wavelength of 460-495 nm and emission at 510 nm. Oocytes expressing a bright green fluorescence were regarded as living ones and were used subsequently.

Parthenogenetic activation (PA)

The MII oocytes were subjected to the PA treatment as described previously (Laowtammathron et al., 2005). Briefly, the oocytes were first treated with 7% ethanol in the Emcare holding medium for 5 min, and then incubated with 10 µg/mL cycloheximide (CHX) and 1.25 µg/mL cytochalasin D (CD) in mSOF medium + 10 % FBS for 5 h at 38.5 °C under humidified atmosphere of 5% CO_2 in air.

ICSI

Straws of frozen spermatozoa were thawed in a 37°C water bath for 30 sec. Thawed spermatozoa were gently placed to the bottom of the 1mL BO medium supplemented with 1 mM caffeine for sperm swim up for 30 min and then the supernatant was collected and centrifuged at 500×g for 5 min. The sperm pellet was washed twice with 1mL of BO-medium by centrifugation at 500×g for 5 min. The sperm pellet was resuspended in the BO-medium and diluted approximately 1:5 with polyvinyl pyrrolidone (PVP). Sperm injection was performed with inverted microscope (IX71, Olympus). The inner diameter of the sperm injection needle was 8-10 µm, and the inner diameter of the holding pipette was 20 µm. Three droplets of media covered with mineral oil were prepared on 60 mm petri dish cover for the ICSI procedure; the first droplet was 10% PVP medium for washing pipette, the second droplet was the sperm suspension diluted with 10% PVP medium (1:5), and the third droplet was Emcare holding medium for the ICSI procedure. A single, motile buffalo spermatozoon was immobilized against the bottom of the PVP droplet, loaded tail first with a minimum volume of medium into the injection pipette and then injected into the cytoplasm of a buffalo oocyte. Within 1 h of the injection, the injected oocytes were activated by exposure to 7% EtOH in Emcare medium for 5 min, and then subsequently cultured in TCM199 + 10% FCS for 3 h to allow extrusion of the second polar body. With the purpose of producing haploid activated oocytes for ICSI, the injected and activated oocytes which extruded the second polar body were selected and transferred to mSOF medium supplemented with 10 µg/mL CHX and cultured for 5 h at 38.5 °C under humidified atmosphere of 5% CO_2 in air.

In vitro culture and differential cell staining

The PA and ICSI oocytes were cultured in 100 µL microdrops (20 oocytes per microdrop) of the IVC medium covered by paraffin oil under a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C for 2 days. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelium cells (BOEC) under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 5 days, as reported previously (Parnpai et al., 1999). Half of the medium was removed every day from each drop and replaced with fresh medium. The cleavage rates were recorded on Day 2, the development of embryos to the blastocyst stage was recorded on Day 7 (the day of PA or ICSI was considered Day 0).

The blastocysts harvested on Day 7 were stained to distinguish cells of the inner cell mass (ICM) and trophectoderm (TE), as reported previously (Suteevun et al., 2006). Briefly, zona pellucidae of blastocysts on Day 7 were removed by 0.5% protease. After washing with mSOFaa medium, the zona-free blastocysts were incubated in 100 µL of 10% rabbit anti-buffalo spleenocyte antibodies for 45 min, and then transferred into a 100 µL mixture of 10% guinea pig complement, 75 mg/mL propidium iodide (PI) and 100 µg/mL Hoechst 33258 for 45 min. The ICM (blue) and trophectoderm cells (red) were counted under a fluorescent microscope at 330–380 nm, allowing determination of the total number of cells for blastocysts and the percentage of ICM cells based on the total number of blastocyst cells.

Experimental design

Experiment 1 was performed to test the toxicity of Vitrification solution. After treatment with the equilibration solution for 1 min oocytes were exposed to vitrification solution for 30 (1+30), 45 (1+45) or 60 sec (1+60) and then to warming solution. Surviving oocytes selected by the FDA test were then subjected to PA and then *in vitro* cultured. The development of oocytes exposed to the vitrification solution for were compared with that of untreated oocytes (Control). To test the possible effects of FDA staining some oocytes without CPA and FDA treatment were also activated and cultured (Fresh control).

Experiment 2 was performed to assess the developmental ability of vitrified-thawed oocytes induced by PA. On the basis of the results from Experiment 1, only the 1+30 and 1+45 CPA treatment regimens were used to vitrify M-II oocytes. Vitrified oocytes were stored in liquid nitrogen containers for several days or weeks. After warming all surviving (FDA positive) oocytes were subjected to PA and their *in vitro* development was compared to those of activated Control and Fresh control oocytes.

Experiment 3 was performed to assess the development of ICSI embryos generated from vitrified-thawed oocytes. Oocytes vitrified by the 1+30 and 1+45 CPA treatment regimens were warmed and all of the surviving (FDA positive) oocytes were subjected to ICSI. Their *in vitro* development was compared to those of Control and Fresh control oocytes following ICSI.

Statistical analysis

The experiments were replicated at least three times in each treatment group. Data were analyzed by one-way ANOVA using the statistical analysis systems (SAS). The differences between groups were considered to be statistically significant when $P < 0.05$.

RESULTS

Experiment 1. Parthenogenetic development of CPA-treated oocytes.

Survival and *in vitro* development of buffalo MII oocytes following CPA-exposed and PA treatment is summarized in Table 1. The viability of oocytes determined by the FDA test in all groups of CPA treatment and the Control were 100%. The cleavage rate and embryo development in the 1+30 and 1+45 groups were significantly higher than in the 1+60 group, but lower than in the Control and Fresh control groups. The development of oocytes after PA to the blastocyst stage did not differ significantly among the 1+30 (17%), Control (23%) and Fresh control groups (27%) but were significantly higher than those in 1+45 (9%) and 1+60 (1%) groups. The number of total cells and ICM cells were similar among the 1+30, 1+45, Control and Fresh control groups and were higher than those in 1+60 group (Table 1).

Experiment 2. Parthenogenetic development of vitrified oocytes.

In vitro development of oocytes following vitrification and PA treatment is summarized in Table 2. The rates of li oocytes among the vitrified 1+30 (97%), 1+45 (95%) and the Control groups (100%) were not different. The cleava and blastocyst rates were influenced when the oocytes were subjected to vitrification. The development of PA oocytes t the blastocyst stage in the vitrified 1+30 group (8%) was significantly higher than that in the vitrified 1+45 group (4% and significantly lower than those in the Control (24%) and Fresh control (26%). The total cell numbers of blastocysts i the 1+30 (71.6±18.3) and 1+45 (69.9±19.6) groups were lower than that in the Control (78.5±24.6) and Fresh contr (80.0±23.1) groups. There was no difference in the ICM cell numbers among the four groups.

Experiment 3. In vitro development following vitrification and ICSI.

In vitro development of buffalo MII oocytes following vitrification and ICSI treatment is summarized in Table 3. The rate of live oocytes among the vitrified 1+30 (96%), 1+45 (91%) and Control (100%) groups were not different. Afte activation of ICSI oocytes by ethanol the 2nd polar body extrusion rate in the 1+30 (43%) group was significantly highe than that in the 1+45 group (35%) and significantly lower than those in the Control (56%) and Fresh control group (59%).The cleavage and blastocyst rates in the 1+30 group (67%, 11%) were also significantly higher than that in the vitrified 1+45 group (50%, 7%) and significantly lower than those in the Control and the Fresh control groups (86%, 21% and 86%, 23%, respectively). There was no significant difference in total cell numbers among the four groups. However, the ICM number of Fresh control embryos was higher than those of the other three groups (Table 3).

DISCUSSION

The major finding of this study is that buffalo MII oocytes could be cryopreserved by vitrification with microdrop method and the oocytes were suitable for ICSI produce. Vitrification is a simple, rapid and cost-effective method of cryopreservation mammalian cells. Using this method cryopreserved cells are less likely to experience solution effects and intracellular ice formation (Fahy et al., 1984). However, a negative consequence of this strategy is the increased probability of nearly all forms of injury except for those caused by ice crystal formation. To achieve vitrification of solutions, a radical increase of both the cooling rates and the concentration of cryoprotectants are required.

Microdrop method involves dropping an oocyte-containing solution directly into liquid nitrogen, the success of this method is due to elimination of the insulation effect of the container wall. Warming of the oocytes is equally rapid when vitrified samples are directly dropped into a warm solution (Papis et al., 2000). One essential factor in cryosurvival is the permeation of a certain amount of cryoprotectant into the oocyte, which increases with the duration of exposure. However, the toxicity of the cryoprotectant must be avoided, with an optimum exposure time being favorable for this purpose.

In the present study, we investigated the effect of different vitrification solution exposure time. The 1+30 group showed higher survival rate and embryos developmental rate than 1+45 or 1+60. A toxicity test of the different vitrification solution exposure time showed that the embryo development rate decreased with the exposure time extended. The very short exposure to a high concentration of EG before cooling and after thawing reduced its toxic effects; moreover, the toxic effects during thawing were minimized by the direct dilution method. We attempted to handle oocytes in solutions at or close to body temperature of buffalo, followed by quick cooling.

This study has demonstrated that ICSI into vitrified-warmed oocytes decreased the rates of 2nd polar body formation, cleavage and blastocyst development when compared with the fresh oocytes. The 2nd polar body formation was judged as the ability of oocytes accomplishing meiosis. Our study revealed that the vitrification and warming procedure could reduce the oocytes ability to accomplish the second meiosis. A possible reason may be the damage of meiotic spindle which has been frequently observed in cryopreserved oocytes (Chen et al., 2003).

Developmental rate into blastocyst in the 1+30 group was significantly higher than that of the 1+45 group and significantly lower than control groups, but did not differ between the Control and Fresh control groups. Similar observation were made in total cell number of day 7 blastocysts. This indicated that the vitrification procedure resulted in retarded embryonic development and that 30 sec exposure to the vitrification solution was the most effective regimen for buffalo MII oocyte vitrification.

In our study, oocyte viability was determined by FDA staining which in stained cells indicates normal esterase enzyme activity and oolemma integrity. We compared the Fresh control and Control oocytes treated with FDA to determine the toxicity of FDA staining procedure. Our results showed no difference in the the 2nd polar body extrusion rate, the embryo development and the blastocyst cell number between Fresh control and Control oocytes. This indicates that FDA staining does not exert any negative effect upon embryo development and thus it can be safely used for viability testing of oocytes.

In conclusion, our study demonstrated that buffalo oocytes vitrified by the microdrop method with the 1+30 CPA regimen could yield high blastocyst rates after ICSI and that the FDA viability checking had no effect on embryo development.

Table 1. *In vitro* development of CPA-exposed buffalo MII oocytes and quality of blastocysts following parthenogenetic activation.

Treatment	No. oocytes	FDA viability (%)	Cleavage (%)	No. (%) of oocytes developed to				Mean (±S.D.) blastocyst cell No.		
				8-C	16-C	Mor	BL	Total cell	TE cell	ICM cell
1+30	111	111(100)	97(87)^{b}	64(58)^{ab}	36(32)^{b}	25(23)^{b}	18(16)^{ab}	68.2±21.4^{ab}	48.1±6.3^{ab}	19.6±8.4^{ab}
1+45	111	111(100)	92(83)^{b}	59(53)^{b}	31(28)^{b}	20(18)^{b}	11(10)^{bc}	70.7±17.5^{ab}	49.5±7.1^{ab}	19.1±7.3^{ab}
1+60	111	111(100)	76(68)^{c}	42(38)^{c}	17(15)^{c}	8(7)^{c}	2(2)^{c}	62.0±9.0^{b}	48.5±7.5^{ab}	13.5±1.5^{b}
Control	44	44(100)	40(91)^{ab}	30(68)^{ab}	22(50)^{a}	16(36)^{a}	10(23)^{a}	76.6±19.5^{a}	55.2±5.1^{a}	20.1±7.5^{a}
Fresh control [(-) FDA]	44	-	42(95)^{a}	31(70)^{a}	23(52)^{a}	17(39)^{a}	12(27)^{a}	77.1±17.8^{a}	56.0±6.4^{a}	20.9±6.7^{a}

^{a,b,c} Means within columns with different superscripts differ (P<0.01 ANOVA).

Treatment groups: 1+30 = 1 min equilibration followed by 30 sec of exposure to vitrification solution;

1+45 = 1 min equilibration followed by 45 sec of exposure to vitrification solution;

1+60 = 1 min equilibration followed by 60 sec of exposure to vitrification solution.

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

Table 2. *In vitro* development of buffalo MII oocytes and quality of blastocysts following vitrification and parthenogenetic activation.

Treatment	No. oocytes	FDA viability (%)	Cleavage (%)	No. (%) of oocytes developed to				Mean (±S.D.) blastocyst cell No.		
				8-C	16-C	Mor	BL	Total cell	TE cell	ICM cell
1+30	130	126(97)^{ab}	84(67)^{b}	42(33)^{b}	24(19)^{b}	15(12)^{b}	10(8)^{b}	71.6±18.3^{ab}	49.1±4.7^{ab}	21.4±9.5
1+45	132	126(95)^{b}	58(46)^{c}	23(18)^{c}	10(8)^{c}	7(6)^{c}	5(4)^{c}	69.9±19.6^{ab}	48.2±4.3^{ab}	20.2±5.8
Control	118	118(100)^{a}	105(89)^{a}	81(69)^{a}	53(45)^{a}	41(35)^{a}	28(24)^{a}	78.5±24.6^{a}	59.0±6.1^{a}	22.7±4.2
Fresh control [(-) FDA]	117	-	110(94)^{a}	82(70)^{a}	56(48)^{a}	43(37)^{a}	31(26)^{a}	80.0±23.1^{a}	59.2±5.3^{a}	23.8±6.0

^{a,b,c} Means within columns with different superscripts differ (P<0.01 ANOVA).

Treatment groups: 1+30 = 1 min equilibration followed by 30 sec of exposure to vitrification solution;
1+45 = 1 min equilibration followed by 45 sec of exposure to vitrification solution;
1+60 = 1 min equilibration followed by 60 sec of exposure to vitrification solution.

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

Table 3. *In vitro* development of buffalo MII oocytes and quality of blastocysts following vitrification and ICSI.

Treatment	No. oocytes	FDA viability (%)	ICSI success (%)	2nd PB (%)	Cleavage (%)	No. (%) of oocytes developed to				Mean (±S.D.) blastocyst cell No.		
						8-C	16-C	Mor	BL	Total cell	TE cell	ICM cell
1+30	147	141(96)^{ab}	141(100)	61(43)^{b}	41(67)^{b}	24(39)^{b}	17(28)^{b}	9(15)^{b}	7(11)^{b}	99.1±11.2^{a}	61.0±9.1^{b}	31.2±6.7^{ab}
1+45	146	133(91)^{b}	132(99)	46(35)^{c}	23(50)^{c}	14(30)^{b}	8(17)^{c}	5(11)^{b}	3(7)^{c}	94.8±14.7^{ab}	60.2±5.8^{b}	30.5±9.6^{ab}
Control	126	126(100)^{a}	126(100)	70(56)^{a}	60(86)^{a}	47(67)^{a}	31(44)^{a}	23(33)^{a}	15(21)^{a}	101.7±12.3^{a}	70.0±6.8^{a}	32.2±4.5^{ab}
Fresh control [(-) FDA]	130	-	130(100)	77(59)^{a}	66(86)^{a}	50(65)^{a}	34(44)^{a}	24(31)^{a}	18(23)^{a}	104.2±10.6^{a}	69.2±8.6^{a}	35.5±3.1^{a}

^{a,b,c} Means within columns with different superscripts differ (P<0.01 ANOVA).

Treatment groups: 1+30 = 1 min equilibration followed by 30 sec of exposure to vitrification solution;
1+45 = 1 min equilibration followed by 45 sec of exposure to vitrification solution;
1+60 = 1 min equilibration followed by 60 sec of exposure to vitrification solution.

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

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$P < 0.05$. In Experiment 1, the effects of IGF-I and EGF in DMM (TCM-199 supplemented with FSH, LH, 17β-estradiol, Na pyruvate, gentamycin sulfate, and polyvinyl alcohol) were evaluated in 4 treatment groups (DMM as a control, DMM + EGF, DMM + IGF-I, and DMM + EGF + IGF-I). Cleavage, morula, and blastocyst production rates were higher in the DMM + EGF + IGF-I (83.1 ± 0.5, 47.7 ± 0.5, and 30.8 ± 1.2) than in the DMM (72.9 ± 1.3, 37.0 ± 1.1, and 20.2 ± 1.2), DMM + EGF (77.2 ± 1.0, 43.2 ± 2.1, and 26.1 ± 1.8), or DMM + IGF-I (79.9 ± 0.6, 43.3 ± 1.2, and 25.7 ± 1.3) groups ($P < 0.05$). In Experiment 2, the effects of Cys in DMM were evaluated. The addition of Cys to DMM + EGF + IGF-I resulted in a mean blastocyst rate of 35.0 ± 0.9% compared with 31.5 ± 1.3% in DMM + EGF + IGF-I alone ($P < 0.05$); however, mean cleavage rates (84.5 ± 1.3 *v.* 82.8 ± 1.0) did not differ ($P = 0.32$). In Experiment 3, the effects of DMM + EGF + IGF-I + Cys, SDMM (TCM-199 supplemented with FSH, LH, 17β-estradiol, Na pyruvate, gentamycin sulfate, and BSA) + EGF + IGF-I + Cys, and UDMM (TCM-199 supplemented with FSH, LH, 17β-estradiol Na pyruvate, gentamycin sulfate, and FBS) + EGF + IGF-I + Cys on cleavage and embryo developmental were compared. The UDMM supplemented with EGF, IGF-I, and Cys resulted in higher proportions of cleavage, morula yields, and blastocyst yields ($P < 0.05$) than the DMM or SDMM supplemented with EGF + IGF-I + Cys. There was no significant difference between DMM and SDMM in the proportions of oocytes reaching the morula and blastocyst stages. In conclusion, an efficient system for *in vitro* production of sheep blastocysts was developed by using a defined oocyte maturation system combined with growth factors, hormones, and an antioxidant, but the UDMM resulted in higher cleavage, morula yields, and blastocyst yields than the DMM or SDMM.

210 CLEAVAGE ASSESSMENT AT DAY 2 TO PREDICT BLASTOCYST DEVELOPMENTAL POTENTIAL IN PORCINE *IN VITRO*-FERTILIZED EMBRYOS

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Porcine embryos could be a valuable tool to study preimplantation development, implantation, and pregnancy, but to do this it is necessary to establish an efficient *in vitro* embryo production system. Because the cause of high mortality in embryos during preimplantation development is not clear, a noninvasive method of determining the developmental potential of cleavage-stage embryos is needed. The objective was to evaluate the developmental potential of Day 2 embryos in a porcine *in vitro* fertilization (IVF) system. Specifically, this study was conducted to examine the relationship between embryo morphology 48 h after IVF on rates of blastocyst formation 5 days later. To prepare *in vitro* maturation (IVM) of porcine oocytes, cumulus–oocyte complexes were obtained from slaughterhouse-derived ovaries and matured in M-199 medium supplemented with 10% pig follicular fluid and 0.57 mM cysteine for 44 h and then freed from cumulus cells. After IVM, cumulus-free oocytes were coincubated with frozen–thawed sperm (2×10^6 cells mL^{-1}) and 2 mM caffeine for 6 h. Inseminated embryos were cultured in NCSU-23 medium that was supplemented with 0.5 mM pyruvate and 0.5 mM lactate. Data were analyzed by ANOVA and Duncan's test ($P < 0.05$). Morphology data on a total of 919 embryos were analyzed retrospectively. Forty-eight hours after insemination, embryos were classified into the following 5 groups based on the cleavage state: 1 cell, 2 cells, 4 cells, 5 to 8 cells, and fragmentation. These groups were cultured another 120 h and then evaluated for blastocyst formation. Blastocyst formation rates were significantly higher in the 4-cell (38.07%) and 5- to 8-cell (40.65%) cleaving groups than in the other groups ($P < 0.05$). In contrast, the 2-cell and fragmentation groups produced 7.5 and 2.9% blastocysts, respectively. Data suggest that embryos reaching 4 cells and 5 to 8 cells by 48 h after insemination have high developmental competence, and this parameter may be useful to predict the development of preimplantation embryos and their ability to establish pregnancy.

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211 *IN VITRO* PRODUCTION OF SWAMP BUFFALO EMBRYOS BY INTRACYTOPLASMIC SPERM INJECTION: EFFECT OF CHEMICAL ACTIVATION TREATMENTS

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Intracytoplasmic spern injection (ICSI) in the buffalo has not yet been well examined. Several factors involved affect the success rates of this technique, particularly the postinjection activation procedure. The objective of this study was to evaluate the effects of chemical activation treatments on *in vitro* development of oocytes after ICSI. A single spermatozoa was injected into the cytoplasm of an *in vitro*-matured oocyte using a micromanipulator under an inverted microscope. The ICSI oocytes were assigned to the following chemical activation treatments: (1) exposed to 5 μM ionomycin (Io) in Emcare medium for 5 min and placed in Emcare medium for 3 h, or (2) exposed to 7% ethanol (EtOH) in Emcare medium for 5 min and placed in Emcare medium for 3 h. The treated oocytes that extruded a second polar body were then selected and cultured either in (A) 1.9 mM 6-dimethylaminopurine (6-DMAP) in mSOF medium for 3 h, or (B) 10 μg mL^{-1} of cychloheximide (CHX) for 5 h. The treated oocytes were further cultured in mSOF medium supplemented with 3 mg mL^{-1} of fatty acid-free BSA at 38.5°C under a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 2 d. Thereafter, 8-cell-stage embryos were selected and co-cultured with buffalo cumulus cells in mSOF medium at 38.5°C under a humidified atmosphere of 5% CO_2 in air for another 5 d. The medium was changed daily and the development of embryos was recorded at the same time the medium was changed. The sham-injected oocytes were treated and cultured along with ICSI oocytes. With 8 replications for each activation treatment, 336 oocytes were used for ICSI. With 6 replications for each activation treatment, 211 oocytes were used for sham injection. The cleavage of ICSI oocytes treated with Io + 6-DMAP, EtOH + 6-DMAP, and EtOH + CHX was 76.2, 69.4, and 78.3%, respectively, which was significant higher ($P < 0.01$) than ICSI oocytes treated with Io + CHX (52.4%) and also significant higher ($P < 0.01$) than sham-injected oocytes in all treatments. The highest blastocyst rate was observed in ICSI oocytes treated with Io + 6-DMAP (28.6%), which was not significantly different from ICSI oocytes

treated with EtOH + CHX (24.4%). The blastocyst rates of ICSI oocytes treated with Io + 6-DMAP and EtOH + CHX were significantly higher than ICSI oocytes treated with Io + CHX (5.9%) and EtOH + 6-DMAP (16.5%) and also were significantly higher than sham-injected oocytes in all treatments. In conclusion, our study demonstrated that activated ICSI of swamp buffalo oocytes with Io + 6-DMAP or EtOH + CHX gave the highest cleavage and blastocyst rates.

This work was supported by the Thailand Research Fund and Suranaree University of Technology.

212 EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR ON THE EARLY DEVELOPMENT AND POLYSPERMY RATE OF OVINE EMBRYOS PRODUCED *IN VITRO*

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To investigate the effect of vascular endothelial growth factor (VEGF) on the early development and polyspermy rate of ovine embryos *in vitro*, 2 experiments were conducted with human recombinant $VEGF_{165}$ supplemented to the media during maturation, fertilization, and culture *in vitro*, respectively. Ovaries were collected from ewes at a local slaughterhouse. All oocytes surrounded by a multilayer of cumulus cells were collected and rinsed 3 times in maturation medium (control medium and treatment medium, respectively). A total of 100 oocytes in each group were cultured in 4-well plates (Nunc) containing 800 μL of maturation medium at 38.5°C in an atmosphere of 5% CO_2 with saturated humidity. Four replicates of each experiment were conducted. Statistical analyses were conducted by ANOVA with SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as means, and $P < 0.05$ was considered significant. In Experiment 1, to investigate the effect of VEGF on the early development of ovine embryos *in vitro*, VEGF was used at 5 ng mL^{-1} (treatment group A) and 10 ng mL^{-1} (treatment group B) in maturation medium (TCM-199 + BSA), HSOF fertilization medium, and SOF culture medium. The results showed that the maturation rate was increased significantly ($P < 0.01$), from 75.76% in the control treatment to 83.98 and 80.23% in treatment group A and treatment group B, respectively. The cleavage rate was increased from 75.85% in the control group to 79.39% in treatment group A ($P > 0.05$). The development rates of morulae (45.03%) and blastocysts (23.54%) in treatment group A were significantly higher ($P < 0.01$) than those in the control group (38.94 and 18.09%, respectively). In addition, the development rates of blastocysts in treatment group B (21.05%) were lower than those in treatment group A ($P > 0.05$) and higher than those in the control group ($P > 0.05$). In Experiment 2, to investigate the effect of VEGF on the polyspermy rate of ovine embryos *in vitro*, 5 ng mL^{-1} of VEGF was used in TCM-199 + BSA maturation medium in this experiment. The results showed that the fertilization rate after 18 h of IVF was increased significantly ($P < 0.01$), from 75.75% in the control group to 83.86% in the treatment group, and that the polyspermy rate was decreased significantly ($P < 0.01$), from 12.64% in the control group to 7.68% in the treatment group. These results indicate that VEGF significantly improved the maturation and fertilization rates of ovine oocytes and, consequently, the rate of embryo development *in vitro*, especially when the medium was supplemented with 5 ng mL^{-1} of VEGF. The VEGF obviously decreased the polyspermy rate and bated the phenomenon of polyspermy in the process of ovine oocyte IVF.

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213 POLARIZED LIGHT MICROSCOPY REVEALS AN ASSOCIATION BETWEEN ZONA BIREFRINGENCE INTENSITY AND EMBRYONIC DEVELOPMENT IN BOVINE OOCYTES AND ZYGOTES

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To increase the efficiency of human IVP, noninvasive parameters to predict the developmental competence of oocytes and zygotes would be useful for selecting the right embryo to transfer. However, human oocytes and zygotes for experimental studies are rare. Therefore, using a bovine model with a precisely large sample size was the aim of the present study to investigate whether zona birefringence intensity (ZBI), measured by polarization light microscopy of metaphase II (MII) oocytes and zygote-stage embryos, was associated with subsequent development. In this study, ZBI was measured through 2 different parameters, the birefringence peak (PV, average signal strength of polarized light) and the birefringence peak combined with the surface of the signal (CV, average signal strength of polarized light multiplied by the surface of the signal), by using polarized light microscopy and OCTAX polarAIDE-software. Statistical analysis was done by the Tukey-Kramer test using SAS version 9.1. Cumulus–oocyte complexes (COC) were recovered from slaughterhouse ovaries by aspiration. After *in vitro* maturation, MII oocytes were denuded and activated parthenogenetically (4-min exposure to 5 μM Ionomycin, followed by 3.5 h of incubation in 2 mM 6-DMAP) or fertilized *in vitro* before denudation. Subsequently, ZBI of MII oocytes (directly after activation) and zygotes (24 h after the beginning of IVF) were measured. To allow tracking of subsequent development, embryos were cultured individually in a well-of-well (WoW) culture system until Day 7 in CR1aa medium (5% CO_2, 38.7°C, humidified air). When parthenogenetically activated embryos were cultured ($n = 365$), 287 (78.6%) cleaved and 115 (31.5%) reached blastocyst stage. The ZBI of

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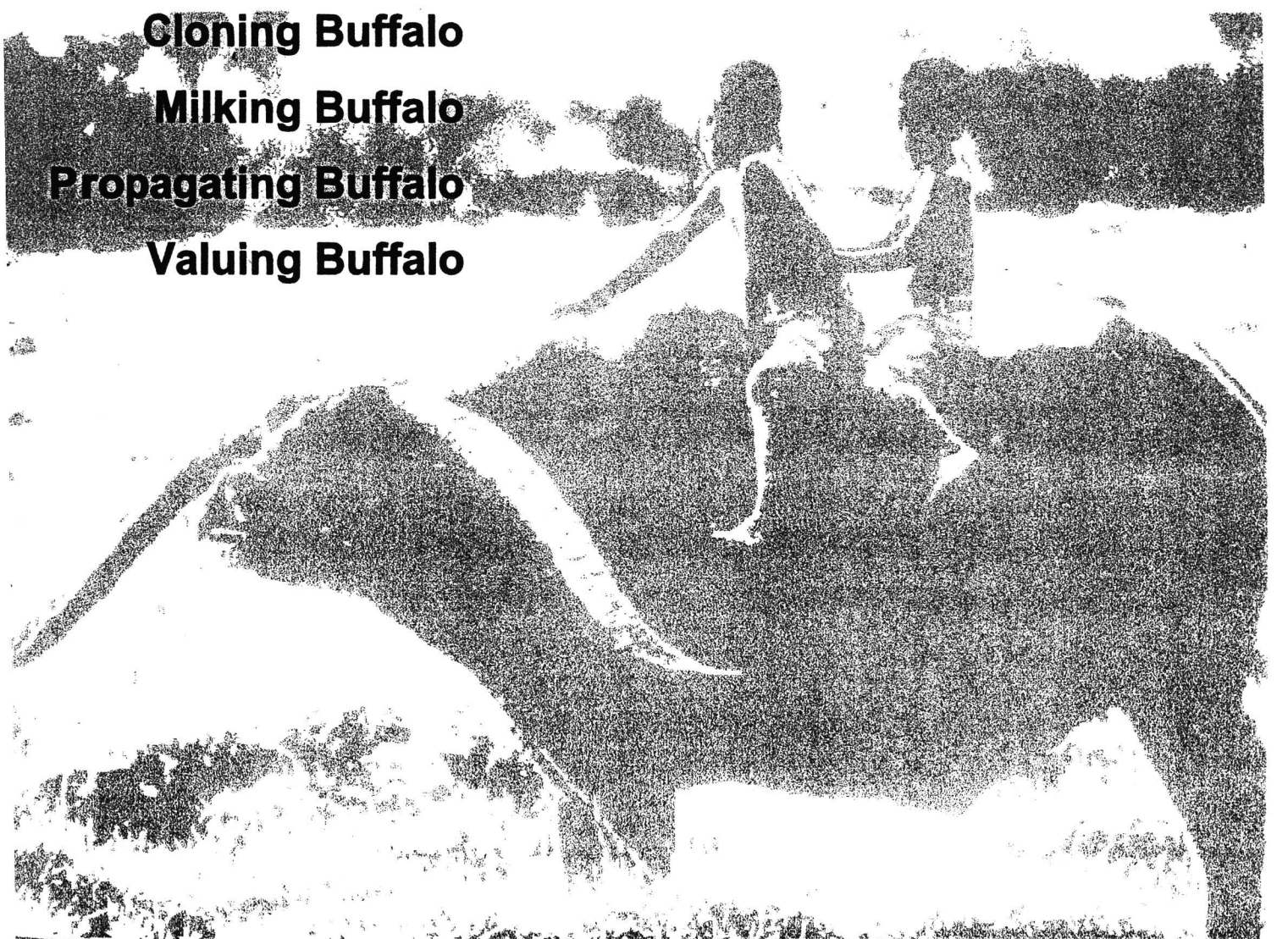

Somatic cell nuclear transfer in swamp buffalo

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Swamp buffaloes are an important livestock species that provides milk, meat and draft power, and thus significantly contributes to the economy in Southeast Asia. The population of swamp buffalo in Thailand has been dramatically decreased due to their low fertility and less demands from people. Several assisted reproductive techniques such as artificial insemination, embryo transfer, *in vitro* fertilization (IVF), genome resource banking (semen, oocyte, embryo, somatic cell banks) and somatic cell nuclear transfer (SCNT) have been implemented in buffalo production. Although the SCNT buffaloes were successfully produced, the success rate is still extremely low. Most recently, our studies have revealed that donor cells from fetal, ear skin, granulosa and cumulus cells had the similar potential to support the development of SCNT buffalo embryos. However, the blastocyst formation rate of SCNT buffalo embryos was lower than that of bovine SCNT embryos using the same source of somatic cells from cattle.

เอกสารแนบหมายเลข 8


เรื่องเต็มการประชุมทางวิชาการ ครั้งที่ ๔๘ มหาวิทยาลัยเกษตรศาสตร์
เล่มที่ 3 สาขาสัตวแพทยศาสตร์
(Subject: Veterinary Medicine)
The Proceeding of 48th Kasetsart University Annual Conference
พื้นเศรษฐกิจชาติ เกษตรศาสตร์กู้วิกฤต พิชิตโลกร้อน
The Roles of Agriculture Science in Fueling Economic Revival, Resolving the Crisis and Battling Global Warming

อัตราการอยู่รอดของไข่สุกกระบือปลักที่ผ่านการ vitrification ด้วยวิธี Microdrop และ Cryotop และการเจริญเติบโตของตัวอ่อนภายหลังการฉีดตัวอสุจิเข้าในไซโตพลาสซึม

Survival rates of matured buffalo oocytes after vitrification by Microdrop and Cryotop and subsequent embryos development after intracytoplasmic sperm injection (ICSI)

หยวนหยวน เหลียง[1] และ รังสรรค์ พาลพ่าย[1]

Yuanyuan Liang[1] and Rangsun Parnpai[1]

บทคัดย่อ

การทดลองเปรียบเทียบวิธีการการแช่แข็ง 2 ชนิดคือ MicrodropและCryotop ด้วยน้ำยาแช่แข็ง 2 ชนิดคือ VA (10% EG + 10% DMSO นาน 1 นาที ตามด้วย 20% EG + 20% DMSO + 0.5M sucrose นาน 30 วินาที) และVB (4% EG นาน 12-15 นาที ตามด้วย 35% EG + 5% PVP + 0.4M trehalose นาน 30 วินาที) และใช้ไข่สดเป็นกลุ่มควบคุมตรวจสอบอัตราการอยู่ของไข่ที่ผ่านการแช่แข็งโดยย้อมด้วยfluoresceindiacetate (FDA) นำไข่ที่มีชีวิตรอดมาทำICSIและกระตุ้นด้วยสารเคมี จากการทดลองไม่พบความแตกต่างทางสถิติระหว่างอัตราการอยู่รอดของไข่ในกลุ่มควบคุม (100%) และไข่แช่แข็งด้วยวิธีMicrodrop (93%, VA; 79%, VB) และCryotop (97%,VA;81%.VB)นอกจากนี้พบว่าน้ำยาVAให้อัตราอยู่รอดของไข่สูงกว่าน้ำยาVBการแช่แข็งด้วยวิธีCryotopให้ตัวอ่อนระยะบลาสโตซีสสูงกว่าวิธีMicrodrop การทดลองนี้แสดงให้เห็นว่าไข่สุกกระบือที่แช่แข็งด้วยวิธีMicrodrop และCryotopสามารถเจริญเติบโตถึงระยะบลาสโตซีสหลังจากทำ ICSI.

ABSTRACT

This study compared Microdrop and Cryotop vitrification techniques, with two protocols: VA (10% EG + 10% DMSO for 1 min, followed by 20% EG + 20% DMSO + 0.5M sucrose for 30 s) versus VB (4% EG for 12-15 min, followed by 35% EG + 5% PVP + 0.4M trehalose for 30 s). The frozen-thawed oocytes viability was evaluated by fluorescein diacetate (FDA) staining. The surviving oocytes were performed ICSI then were chemically activated. The viability of oocytes in fresh control (100%) was higher than that of Microdrop (93%, VA; 79%, VB) and Cryotop (97%, VA; 81%, VB) vitrified oocytes. VA showed higher oocytes survival rate than VB. The Cryotop technique showed higher blastocysts than Microdrop technique. The results indicated that vitrified-thawed buffalo oocytes by Microdrop and Cryotop techniques could develop to blastocyst stage after ICSI.

Nowadays, buffalo is the major milk and meat producing farm animal in many developing countries. Cryopreservation of oocytes has great importance in buffalo. Efficient oocytes

Key Words: swamp buffalo, MIIoocyte,vitrification, cryotop, microdrop

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INTRODUCTION

cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in the buffalo species.

Vitrification is the process that induces a glass-like solidification of living cells during freezing. The unique advantage of the vitrification process is elimination of ice crystal formation, the most dangerous cause of cryoinjury. There are some improved vitrification methods succeed in mammalian oocytes cryopreservation such as Cryotop (Kuwayama and Kato, 2000), Cryoloop (Oberstein et al., 2001), OPS (open pulled straw) (Vajta et al., 1998), GMP (glass micropipette) (Kong et al., 2000), Microdrop (Papis et al., 2000), and EMG (electron microscope grids) (Martino et al., 1996). Among the various methods of vitrification, Microdrop vitrification was easy and convenient, as it excluded the use of any specialized device to introduce oocytes into liquid nitrogen. One of the most successful ultra-rapid vitrification technique is the Cryotop vitrification that has resulted in excellent survival and developmental rates with human and bovine MII oocytes (Kuwayama et al., 2005) when cryopreserved oocytes were fertilized *in vitro*. In addition, change in the zona pellucida (ZP) has shown to induce lower fertility rates (Carroll et al., 1990; Vincent et al., 1990). These structural changes causing lower fertility could be overcome by micromanipulation technique such as ICSI (Carroll et al., 1990; Kazem et al., 1995; Karlsson et al., 1996; Porcu et al., 1997).

The aim of this study was to investigate the effects of two vitrification procedures Microdrop and Cryotop and two cryoprotectant procedures on the post-thaw morphology, embryos developmental competence of vitrified buffalo MII oocytes subjected to ICSI.

METERIALS AND METHODS

Unless stated otherwise, all reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA)

Oocyte collection and *in vitro* maturation

Buffalo ovaries were obtained from a slaughterhouse and transported to the laboratory within 4 h in physiological saline (0.9% NaCL). Cumulus-oocyte complexes (COCs) were collected from the follicles 2–8 mm in diameter and were matured *in vitro* for 22 h. The medium for IVM was TCM 199 supplemented with 25 mM HEPES, 10% fetal bovine serum, 0.02 AU/mL FSH, 50 iu/mL hCG and 1μg/mL estradiol-17ß. After maturation, cumulus cells were gently removed by pipetting, and the oocytes with a visible first polar body were selected for the following experiments.

Vitrification and warming

The M-II oocytes were vitrified-warmed by either the Cryotop minimum volume cooling procedure, originally reported by Kuwayama and Kato (Kuwayama and Kato, 2000) or Microdrop vitrification method (Papis et al., 2000).

Two different cryoptotectant systems were used to vitrify buffalo MII oocytes. Oocytes vitrified by VA method was described as following. *In vitro* matured oocytes were placed in TCM199-Hepes + 20% FBS (basic medium; BM) + 10% dimethylsulfoxide (DMSO) + 10% ethylene glycol (EG) for 1 min and then exposed in BM + 20% DMSO + 20% EG + 0.5 M sucrose for 30 sec. Finally, a group of 5 oocytes were directly dropped with about 2 µl vitrification solution into liquid nitrogen or placed on a sheet of each Cryotop in a small volume of the vitrification solution (<1µL). For storage, vitrified oocytes were kept in liquid nitrogen for 1-2 weeks. The vitrified Microdrops or Cryotops were warmed by directly immersing into 3 ml of 0.5 M sucrose in BM for 5 min, and then transferred to the BM for 5 min. Then they were kept in the BM under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h.

VB method was originally described by Dinnyes (Dinnyés et al., 2000). Briefly, the oocytes were treated with an equilibration medium which consisted of 4% (v/v) EG in BM for 12 to 15 min at 38.5 °C followed by 35% (v/v) EG, 50 mg/ml polyvinyl pyrrolidone (PVP) and 0.4 M trehalose for 30 s. Vitrified oocytes were warmed by transferring Microdrops/Cryotops into a warming solution (0.3 M trehalose in BM) at 38.5°C. One to 2 min later, the oocytes were consecutively transferred for 1-min into each 500-µl droplets of BM supplemented with 0.15 M, 0.075 M and 0.0375 M trehalose, respectively. And then they were cultured under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h.

Evaluation of oocyte viability

Oocyte viability was evaluated by fluorescein diacetate (FDA) staining according to the method previously described by Mohr and Trounson (Mohr and Trounson, 1980). Oocytes expressing a bright green fluorescence were regarded as living ones and were used subsequently.

ICSI

Straws of frozen spermatozoa were thawed in a 37°C water bath for 30 sec. After 30 min swimming up, the supernatant medium was centrifuged at 500×g for 5 min. Sperm pellet was washed twice with BO-medium by centrifugation at 500×g for 5 min. Sperm suspensions were diluted approximately 1:5 with PVP. A single, motile buffalo spermatozoon was immobilized against the bottom of the PVP droplet, loaded tail first with a minimum volume of medium into the injection pipette and then injected into the cytoplasm of a buffalo oocyte. The fresh MII oocytes with or without FDA staining conducted by ICSI were treated as control groups.

Activation and *in vitro* culture

The injected oocytes were activated in 7% EtOH in Emcare medium for 5 min, and then subsequently cultured in TCM199 + 10% FCS for 3 h to allow extrusion of the second polar body. The oocytes extruded the second polar body were selected and transferred to mSOF medium supplemented with 10µg/mL CHX for 5 h. The zygotes were cultured in the mSOF medium under a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C for 2 days. Thereafter, embryos at the 8-

cell stage were selected and co-cultured with bovine oviductal epithelium cells (BOEC) under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 5 days (Parnpai et al., 1999).

Statistical analysis

Experiments were replicated at least three times in each treatment group. Data were analyzed by ANOVA using the statistical analysis systems (SAS). The differences between groups were considered to be statistically significant when $P < 0.05$.

RESULTS

Effect of vitrification methods on viability of oocytes

The viability of oocytes was examined by FDA staining. The viability of oocytes vitrified by Cryotop (97%) was higher than Microdrop (93%) but lower than fresh control (100%) in VA medium. The viability of oocytes by Cryotop (81%) and by Microdrop (79%) was no significant difference, which lower than the fresh control oocytes (100%) in VB method.

Effect of vitrification methods on ICSI results

After ICSI, the 2nd polar body formation of fresh control (+) FDA (56%) and (-) FDA (57-59%) was no significant difference, but they were significantly higher than those of Microdrop (39%, VA; 37%, VB) and Cryotop (43%, VA; 40%, VB). The 2nd polar body formation between Microdrop and Cryotop were no significant difference in VA or VB. The cleavage rate of fresh control (+) FDA (79-82%) and (-) FDA (81-83%) was no significant difference, but they were significantly higher than those of Microdrop (65%, VA; 66%, VB) and Cryotop (67%, VA; 72%, VB). The blastocyst rate of fresh control (+) FDA (21%) and (-) FDA (19-22%) was no significant difference, but they were significantly higher than those of Microdrop (8%, VA; 5%, VB) and Cryotop (10%, VA; 11%, VB). The blastocyst formation rate of Microdrop (8%) and Cryotop (10%) was no significant difference in VA, but Cryotop (11%) was higher than Microdrop (5%) in VB.

DISCUSSION

The major finding of this study is that buffalo MII oocytes could be cryopresered by vitrification with Microdrop and Cryotop methods and the oocytes were suitable for ICSI produce.

Cryotop is the minimum volume procedures where the carrier tool, a thin film attached to a plastic holder is equipped with a protective plastic tube (Kuwayama et al., 2005). Oocytes are loaded on the film, the solution is almost entirely removed by aspiration, and the sample is immersed into liquid nitrogen. Subsequently, for safe storage, the plastic tube is pulled over the film. The method is easy to learn and perform, the cooling and warming rates are higher than those achievable with OPS, and the simple manipulation decreases the risk of inconsistency.

Microdrop method involves dropping an oocytes-containing solution directly into liquid nitrogen, the success of this method is due to elimination of the insulation effect of the container wall. Warming of the oocytes is equally rapid when vitrified sample are directly dropped into a warm solution (Paris et al.,

2000). One essential factor in cryosurvival is permeation of a certain amount of cryoprotectant into the oocyte, which increase with the duration of exposure. However, the toxicity of the cryoprotectant must be avoided, with an optimum exposure time being favorable for this purpose.

In the present study, we investigated the effect of VA, VB methods and Microdrop, Cryotop technique on the oocyte viability after vitrified-warmed and embryo development following ICSI. This study has demonstrated that ICSI into frozen-thawed oocytes decreased the rates of 2nd polar body formation, cleavage and blastocyst development when compared with the fresh oocytes. The 2nd polar body formation was judged as the ability of oocytes accomplishing meiosis. Our study revealed that frozen-thawed procedure could reduce the oocytes ability to accomplish the second meiosis.

Regarding the effect of vitrification technique, the recovery rates, viability of oocytes, embryo development rates and blastocyst quality that derived from Cryotop were higher than Microdrop method. Maybe because of the volume of vitrification medium contained with Cryotop was much more less than Microdrop method, which can greatly increase the cooling speed. Cryotop vitrification which had been applied to pronuclear-stage rabbit zygotes (Hochi et al., 2004), pre-hatching stage porcine embryos (Esaki et al., 2004), germinal vesicle-stage whale COCs (Iwayama et al., 2004), denuded M-II bovine oocytes (Chian et al., 2004), SCNTderived bovine and swamp buffalo blastocysts (Lanctammathron et al., 2005) and denuded M-II oocytes and IVF-derived blastocysts in humans (Kuwayama et al., 2000; Katayama et al., 2003), was successfully extended to both denuded M-II and enucleated swamp buffalo oocytes in the present study. Our results supported the view that Cryotop was regarded to be the effective tool for oocytes or embryos vitrification (Kuwayama et al., 2000).

The comparison of two vitrification medium (VA, VB) with similar results showed flexibiliy of oocytes to tolerate cryopreservation. DMSO was used in combination with EG in VA, whereas EG was alone used for equilibration and in comination with PVP for vitrification in VB. In bovine, oocytes have higher permeability to DMSO than to EG (Agca et al., 1998), this may account for VA working better than VB according to the oocyte viability.

In conclusion, our study indicated that Cryotop method could yield high recovery and survival rate of *in vitro* matured swamp buffalo oocytes which could develop to blastocyst stage after ICSI. The vitrification method of VA could yield high survival rate of frozen-thawed *in vitro* matured buffalo oocytes. But the development competence of frozen-thawed *in vitro* matured buffalo oocytes was significantly lower than fresh control oocytes.

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Table1. Embryos development of ICSI derived from vitrified buffalo MII oocytes (Microdrop vs. Cryotop) by Vitrification solution A.

Treatments	No. oocytes	No. (%) recovery	FDA viability	No. ICSI	ICSI success	2nd PB rate	Cleavage rate	No.(%) oocytes developed to			
								8-C	16-C	Mor	BL
Microdrop	143	137(96)	127(93)[a]	127	124(98)	48(39)[a]	31(65)[a]	16(33)[a]	11(23)[a]	5(10)[a]	4(8)[a]
Cryotop	145	145(100)	140(97)[a]	140	139(99)	60(43)[a]	40(67)[a]	23(38)[a]	15(25)[a]	9(15)[b]	6(10)[b]
Fresh control +FDA	124	124(100)	124(100)[b]	124	124(100)	70(56)[b]	55(79)[b]	40(57)[b]	27(39)[b]	21(30)[b]	15(21)[b]
Fresh control	124			124	124(100)	73(59)[b]	59(81)[b]	42(58)[b]	27(37)[b]	17(23)[b]	14(19)[b]

[a,b] Means within the same columns with different superscripts differ (P<0.01)

Table2. Embryos development of ICSI derived from vitrified buffalo MII oocytes (Microdrop vs. Cryotop) by Vitrification solution B.

Treatments	No. oocytes	No. (%) recovery	FDA viability	No. ICSI	ICSI success	2nd PB rate	Cleavage rate	No. (%) of oocytes developed to			
								8-C	16-C	Mor	BL
Microdrop	143	140(98)	111(79)[a]	111	110(99)	41(37)[a]	27(66)[a]	15(37)[b]	9(22)[b]	3(7)[b]	2(5)[b]
Cryotop	144	144(100)	117(81)[a]	117	116(99)	46(40)[a]	33(72)[a]	20(43)[b]	11(24)[b]	7(15)[ab]	5(11)[ab]
Fresh control+FDA	111	111(100)	111(100)[b]	111	111(100)	62(56)[b]	51(82)[b]	39(63)[b]	25(40)[b]	19(31)[b]	13(21)[b]
Fresh control	115			115	115(100)	65(57)[b]	54(83)[b]	41(63)[b]	25(38)[b]	18(28)[b]	14(22)[b]

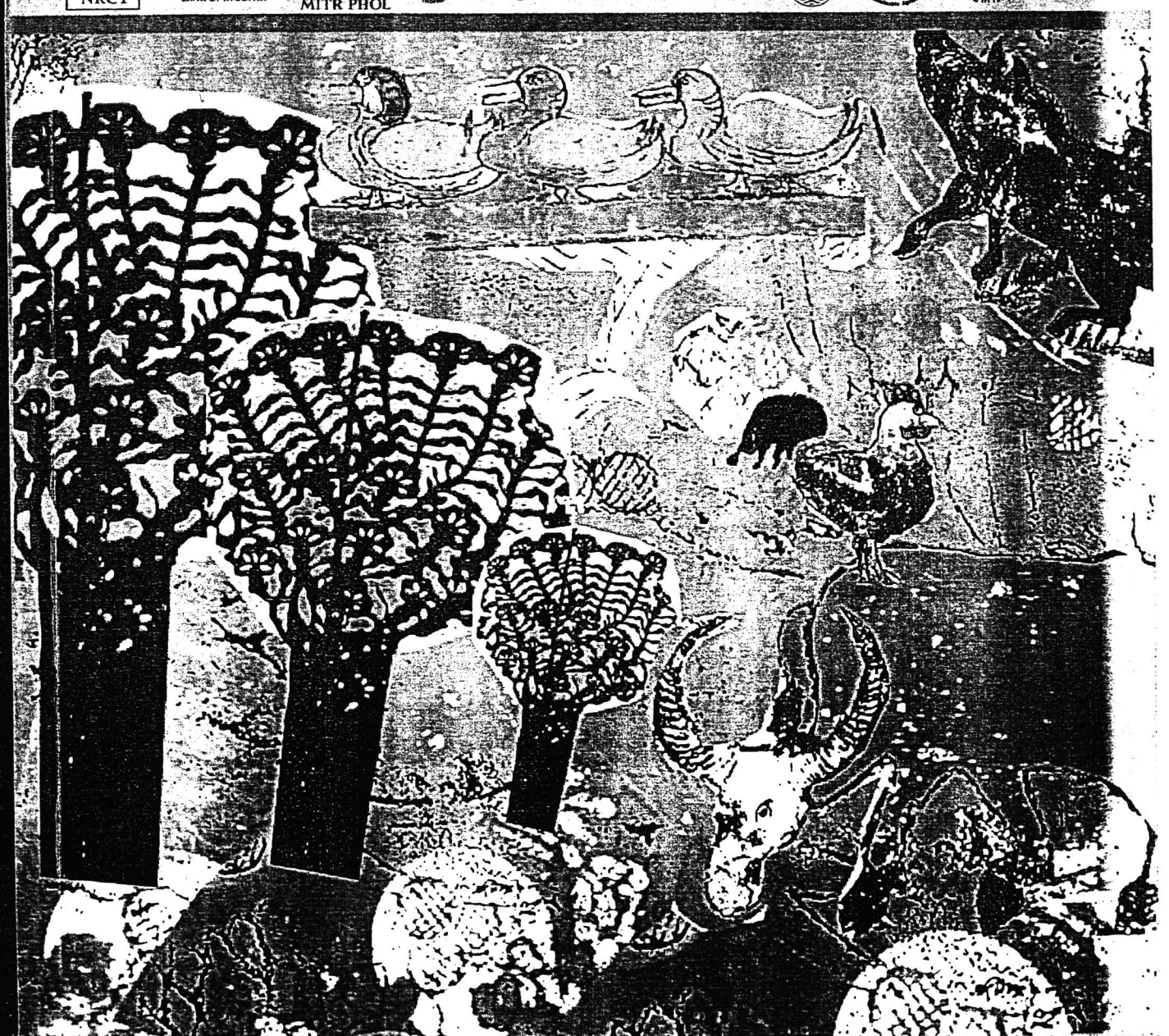
[a,b] Means within the same columns with different superscripts differ (P<0.01)

เอกสารแนบหมายเลข 9

ประชุมวิชาการเกษตร ครั้งที่ 11

ประจำปี 2553

คณะเกษตรศาสตร์ มหาวิทยาลัยขอนแก่น



ผลของโกรทแฟ็กเตอร์ต่อการเจริญเติบโตของฟอลลิเคิลขนาดเล็กภายนอกร่างกายในกระบือปลัก: การศึกษาเบื้องต้น

Effects of various growth factors on *in vitro* development of swamp buffalo early antral follicles: A preliminary study

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บทคัดย่อ

การศึกษาผลของโกรทแฟ็กเตอร์ที่มีต่อการเจริญเติบโตของฟอลลิเคิลขนาดเล็กภายนอกร่างกายในกระบือปลัก แบ่งฟอลลิเคิลออกเป็น 3 กลุ่ม ตามขนาดเส้นผ่าศูนย์กลาง กลุ่มที่ 1 ขนาด 200-399 ไมโครเมตร กลุ่มที่ 2 ขนาด 400-599 ไมโครเมตร และ กลุ่มที่ 3 ขนาด 600-799 ไมโครเมตร การทดลองแบ่งออกเป็น 4 กลุ่ม คือ ทรีทเม้นท์ 1 ไม่มีการเติมโกรทแฟ็กเตอร์ (กลุ่มควบคุม), ทรีทเม้นท์ 2 เติม 50 นาโนกรัม/มิลลิลิตร basic fibroblast growth factor (bFGF), ทรีทเม้นท์ 3 เติม 100 นาโนกรัม/มิลลิลิตร insulin-like growth factor-I (IGF-I) และ ทรีทเม้นท์ 4 เติม 50 นาโนกรัม/มิลลิลิตร epidermal growth factor (EGF) การวัดการเจริญเติบโตของฟอลลิเคิลจะวัดขนาดเส้นผ่าศูนย์กลางของฟอลลิเคิลในวันที่ 7 และ วันที่ 14 การเลี้ยงฟอลลิเคิลกระบือปลักในกลุ่มที่ 1 และ กลุ่มที่ 2 พบว่ามีเพียงน้ำยาที่เติม bFGF เท่านั้นที่สามารถทำให้ฟอลลิเคิลมีการเจริญเติบโตแต่อัตราการเพิ่มขึ้นของขนาดฟอลลิเคิลจากวันที่ 0 ถึง 7 นั้นไม่มีนัยสำคัยทางสถิติ ($P>0.05$) ส่วนในวันที่ 7 ถึง 14 พบการเพิ่มขนาดของฟอลลิเคิลเป็นไปอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ในกลุ่มที่ 3 ฟอลลิเคิลที่เลี้ยงในน้ำยาที่เติม bFGF และ กลุ่มควบคุม วันที่ 0 ถึง 7 มีการเจริญเติบโตของฟอลลิเคิลมากกว่า ฟอลลิเคิลที่เลี้ยงในน้ำยาที่เติมโกรทแฟ็กเตอร์ EGF และ IGF-I อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) แต่ในวันที่ 7 ถึง 14 พบว่าการเจริญเติบโตของฟอลลิเคิลเป็นไปอย่างไม่มีนัยสำคัญทางสถิติ ($P>0.05$)

คำสำคัญ: กระบือปลัก ฟอลลิเคิล โกรทแฟ็กเตอร์

Abstract

This study was carried out to examine the effect of growth factors on swamp buffalo early antral follicle growth. The follicles were divided into 3 groups, depended on their diameters, group I; 200-399 µm, group II; 400-599 µm and group III; 600-799 µm. These follicles were embedded in collagen gel and cultured in *in vitro* growth (IVG) medium supplemented with growth factors by 4 treatments: Treatment 1, no supplementation of growth factors as a control; Treatment 2, 50 ng/ml basic fibroblast growth factor (bFGF); Treatment 3, 100 ng/ml insulin-like growth factor-I (IGF-I) and Treatment 4, 50 ng/ml epidermal growth factor (EGF) growth factor (EGF). The follicles were cultured in 4-well dish for 14 days. The diameter of follicle was measured at day 7 and 14. The results showed that the diameter of follicle at day 0-7 in group I and group II that had been cultured in IVG medium+bFGF were able to increased follicle diameter but no significantly difference ($P>0.05$), whereas at day 7-14 follicles in group 1 and 2 were significant increased follicle diameter ($P<0.05$). On the other hand, follicles in group III that had been cultured in IVG medium+bFGF and control group had higher increase follicle diameter ($P<0.05$) than IVG medium+EGF and IVG medium+IGF-I. However, at day 7-14, follicle that had been cultured in IVG medium+bFGF and control group were able to increased follicle diameter but no significant difference ($P>0.05$).

Keywords: Swamp buffalo, Early antral follicles, Growth factors

บทนำ

ภายในรังไข่ของสัตว์เลี้ยงลูกด้วยนมจะประกอบด้วยฟอลลิเคิลระยะต่างๆ จำนวนมาก (Slomianka, 2006) ซึ่งฟอลลิเคิลเหล่านี้เมื่อได้รับการกระตุ้นจากฮอร์โมนจะทำให้เกิดการเจริญขึ้นมา เช่น เกิดช่องว่างภายใน (antrum), มีการเพิ่มขึ้นของเซลล์แกรนูโลซา (granulosa cell) และขนาดของไข่ (Alm และคณะ 2006) ในระหว่างการเจริญเติบโตมีฟอลลิเคิลเพียงบางส่วนเท่านั้นที่เติบโตเป็นกราเฟียน ฟอลลิเคิล (graafian follicle) และมีการตกไข่ แต่มีฟอลลิเคิลจำนวนมากภายในรังไข่ที่ไม่ได้เติบโตจนเป็นกราเฟียน ฟอลลิเคิล ซึ่งฟอลลิเคิลเหล่านั้นจะฝ่อไปในระหว่างที่มีการเจริญเติบโตเรียกว่าการเกิด atresia (Leigh และ คณะ, 2004) เนื่องจากการพัฒนาของฟอลลิเคิลบนรังไข่ ในช่วงที่ฟอลลิเคิลยังไม่มีช่องว่างภายในเรียกว่าพรีแอนทรัลฟอลลิเคิล (preantral follicle) จะยังไม่ถูกควบคุมด้วยฮอร์โมนโกนาโดโทรปินหรือฮอร์โมนมีผลน้อยมาก การเจริญหรือการฝ่อสลายไปของฟอลลิเคิล จะถูกควบคุมด้วยกลไกภายในรังไข่เอง โดยฟอลลิเคิลใบใหญ่กว่าจะคอยยับยั้งฟอลลิเคิลใบเล็กกว่าไม่ให้เจริญหรือให้ฝ่อสลายไป (ศูนย์วิจัยการผสมเทียมและเทคโนโลยีชีวภาพการผลิตปศุสัตว์, 2007) จากเหตุผลดังกล่าวจึงได้มีการพัฒนาเทคนิคในการเลี้ยงฟอลลิเคิลขนาดเล็กเพื่อให้หรีแอนทรัลฟอลลิเคิลสามารถเจริญเป็นกราเฟียน ฟอลลิเคิลได้มากขึ้นก่อนที่จะฝ่อ และการเลี้ยงฟอลลิเคิลยังเป็นอีกทางเลือกหนึ่งที่จะทำให้ได้ไข่เพิ่มขึ้นและสามารถนำมาใช้ในการปฏิสนธิหรือนำมาใช้ในการทดลองอื่นๆ เช่น การปฏิสนธิในหลอดแก้ว (IVF), การฉีดตัวอสุจิเข้าในไข่ (ICSI), การทำโคลนนิ่ง นอกจากนี้การเลี้ยงฟอลลิเคิลยังทำให้เข้าใจกลไกการเจริญเติบโตของฟอลลิเคิลและไข่ภายในฟอลลิเคิลได้อีกด้วย Yamamoto และคณะ (1999) รายงานการนำไข่โคที่ได้จากการเลี้ยงฟอลลิเคิลมาใช้ในการทำปฏิสนธิในหลอดแก้วจนได้ลูกโคเกิดขึ้น โดยใช้ฟอลลิเคิลขนาด 0.5-0.7 มิลลิเมตร เลี้ยงในสภาวะที่ตรึงด้วยคอลลาเจนเจลในน้ำยา TCM 199 เป็นเวลา 14 วัน Gupta และคณะ (2002) และ Santos และคณะ (2006) ศึกษาโกรทแฟ็กเตอร์ที่เหมาะสมกับการเลี้ยงฟอลลิเคิลขนาดเล็กของกระบือพบว่า ฟอลลิเคิล สติมูเลติ้ง ฮอร์โมน (FSH) และ อินซูลินทรานสเฟอร์รินเซเลเนียม (ITS) เหมาะสมในการเลี้ยงฟอลลิเคิลขนาดเล็กของกระบือโดยใช้ระยะเวลาในการเลี้ยง 7 และ 15 วัน การเลี้ยงฟอลลิเคิลในกระบือนั้นยังไม่ประสบผลสำเร็จ จากรายงานการศึกษาก่อนหน้านี้พบว่าโกรทแฟ็กเตอร์สามารถช่วยให้ฟอลลิเคิลเจริญเติบโตได้แต่ก็มีความแตกต่างกันไปในสัตว์แต่ละชนิด ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของโกรทแฟ็กเตอร์ชนิดต่างๆที่มีต่อการเจริญเติบโตของฟอลลิเคิลขนาดเล็กในกระบือปลัก

อุปกรณ์และวิธีการ

การแยกฟอลลิเคิลออกจากรังไข่

ทำการเก็บรังไข่กระบือปลักจากโรงฆ่าสัตว์ไว้ในน้ำเกลือ (0.9% NaCl) ล้างรังไข่ด้วย 70% เอททานอล นาน 1 นาที จากนั้นล้างรังไข่ 3 ครั้ง ในน้ำเกลือ แล้วใช้กรรไกรตัดไขมันและเนื้อเยื่อเกี่ยวพันที่ติดมากับรังไข่ออกให้หมด ใช้ใบมีดเฉือนเอาเฉพาะชั้นคอร์เทคออกเป็นชิ้นเล็กๆ ให้มีขนาดหนาประมาณ 1-2 มิลลิเมตร นำชิ้นของรังไข่ที่ได้ ล้าง 3 ครั้ง ในน้ำเกลือ จากนั้นใส่ในจานเลี้ยงเซลล์ที่มี 0.1% collagenase (17104-019, Gibco) และ 40 units/ml DNase (10 104 159 001, Roche) จากนั้นนำไปไว้ในตู้อบที่อุณหภูมิ 37°C ภายใต้บรรยากาศที่มี 5% CO_2 นาน 1 ชั่วโมง จากนั้นใช้พลาสเจอร์ปิเปตดูดเอนไซม์ทิ้ง แล้วเติม HEPES (H-4034, Sigma) - buffered TCM 199 (M-5017, Sigma) ลงไปเพื่อล้างเอนไซม์ ล้างด้วย HEPES-buffered TCM 199 จำนวน 2 ครั้ง จากนั้นเติม HEPES-buffered TCM 199 ลงในจานเลี้ยงเซลล์ และนำเนื้อเยื่อรังไข่ที่ผ่านการย่อยด้วยเอนไซม์ไปคัดแยกเอาเฉพาะฟอลลิเคิลโดยใช้ปากคีบปลายแหลมช่วยในการแยก หลังจากแยกเสร็จแล้ววัดขนาดของฟอลลิเคิลโดยทำภายใต้กล้อง inverted microscope ซึ่งต่อกับกล้อง CCD แบ่งฟอลลิเคิลออกเป็น 3 กลุ่ม ตามขนาดเส้นผ่าศูนย์กลาง โดยกลุ่มที่ 1 ขนาด 200-399 ไมโครเมตร กลุ่มที่ 2 ขนาด 400-599 ไมโครเมตร และ กลุ่มที่ 3 ขนาด 600-799 ไมโครเมตร

การเลี้ยงฟอลลิเคิลขนาดเล็กในสภาวะที่ตรึงด้วยคอลลาเจนเจล

เตรียมสารละลายคอลลาเจนเจลโดยใช้ collagen gel type I (Cellmatrix Type I, Nitta Gelatine Inc., Osaka, Japan) นำสารละลาย 3.0% acid collagen ผสมกับ 10 เท่าของน้ำยา TCM 199 และ 0.05 N sodium hydroxide solution ซึ่งมี 22 mg/ml $NaHCO_3$ และ 47.7 mg/ml HEPES ในอัตราส่วน 8:1:1 (v:v:v) ระหว่างการเตรียมสารละลายคอลลาเจนต้องทำในน้ำแข็งเนื่องจากสารละลายคอลลาเจนสามารถแข็งตัวได้ที่อุณหภูมิห้อง แบ่งสารละลายคอลลาเจนออกเป็นสองส่วนสำหรับทำชั้น base layer และ top layer จากนั้นนำสารละลายที่แบ่งไว้สำหรับทำชั้น base layer เติมลงใน 4 well dish (Nunc) โดยใส่ well ละ 200 ไมโครลิตร แล้วนำไปไว้ในตู้อบที่อุณหภูมิ 37°C ภายใต้บรรยากาศที่มี 5% CO_2 นาน 5 นาที เพื่อให้เจลแข็งตัว จากนั้นนำฟอลลิเคิลที่แบ่งกลุ่มไว้วางบนชั้น base layer โดยวางฟอลลิเคิล 4 ใบต่อ well จากนั้นทำชั้น top layer โดยนำสารละลายคอลลาเจนที่ใช้ทำชั้น top layer แช่ในน้ำอุณหภูมิ 25° C นาน 5 นาที จากนั้นเติมสารละลายคอลลาเจนทับลงไปบนชั้น base layer ซึ่งมีฟอลลิเคิลวางอยู่ โดยใส่ well ละ 200 ไมโครลิตร แล้วนำไปไว้ในตู้อบที่อุณหภูมิ 37°C ภายใต้บรรยากาศที่มี 5% CO_2 นาน 10 นาที จากนั้นเติมน้ำยาเลี้ยงฟอลลิเคิลซึ่งประกอบด้วย TCM 199 เติมด้วย 10 μg/ml FSH, 2 mM glutamate, 0.23 mM sodium pyruvate, 2 mM hypoxanthine, 1% ITS, 0.1 mg/ml streptomycin และ 100 IU/ml penicillin ลงไป 500 ไมโครลิตร ทำการเปลี่ยนน้ำยาทุกๆ 2 วัน โดยดูดน้ำยาเก่าออก 250 ไมโครลิตร แล้วเติมน้ำยาใหม่ลง 250 ไมโครลิตร โดยเลี้ยงฟอลลิเคิลนาน 14 วัน จะวัดอัตราการเจริญเติบโตของฟอลลิเคิลที่วันที่ 7 และ 14 ภายใต้กล้อง inverted microscope ซึ่งต่อกับกล้อง CCD น้ำยาเลี้ยงฟอลลิเคิลจะมีการเติมโกรทแฟ็กเตอร์ตามแต่ละการทดลอง โดยแบ่งการทดลองออกเป็น 4 กลุ่ม คือ ทรีทเม้นท์1 ไม่มีการเติมโกรทแฟ็กเตอร์ (กลุ่มควบคุม), ทรีทเม้นท์ 2 เติม 50 นาโนกรัม/มิลลิลิตร bFGF, ทรีทเม้นท์ 3 เติม 100 นาโนกรัม/มิลลิลิตร IGF-I และ ทรีทเม้นท์ 4 เติม 50 นาโนกรัม/มิลลิลิตร EGF โดยแต่ละการทดลองใช้ฟอลลิเคิลครั้งละ 12 ใบ ทำซ้ำทั้งหมด 5 ครั้งในแต่ละทรีทเม้นท์ รวมทรีทเม้นท์ละ 60 ใบ

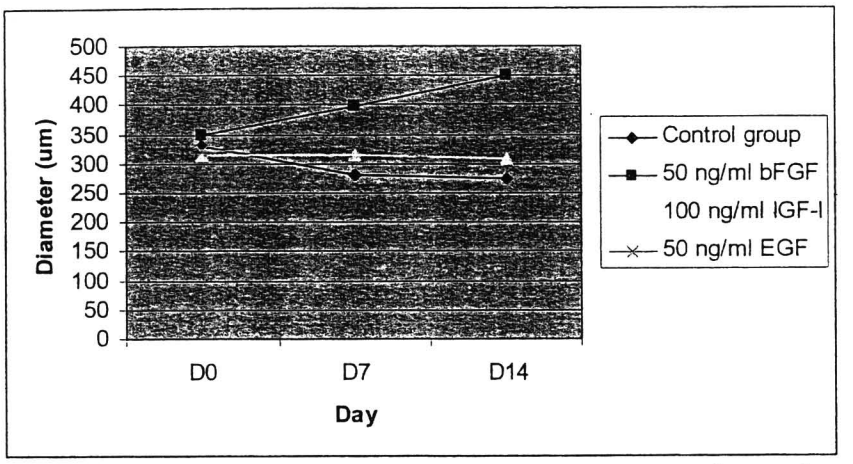
วิเคราะห์ผลการทดลอง วางแผนการทดลองแบบ CRD โดยแบ่งการทดลองออกเป็น 4 กลุ่ม คือ ทรีทเม้นท์1 ไม่มีการเติมโกรทแฟ็กเตอร์ (กลุ่มควบคุม), ทรีทเม้นท์ 2 เติม bFGF, ทรีทเม้นท์3 เติม IGF-I และ ทรีทเม้นท์ 4 เติม EGF ทำทรีทเม้นท์ละ 5 ซ้ำใช้โปรแกรม SAS ในการวิเคราะห์ผลทางสถิติ

ผลการทดลองและวิจารณ์

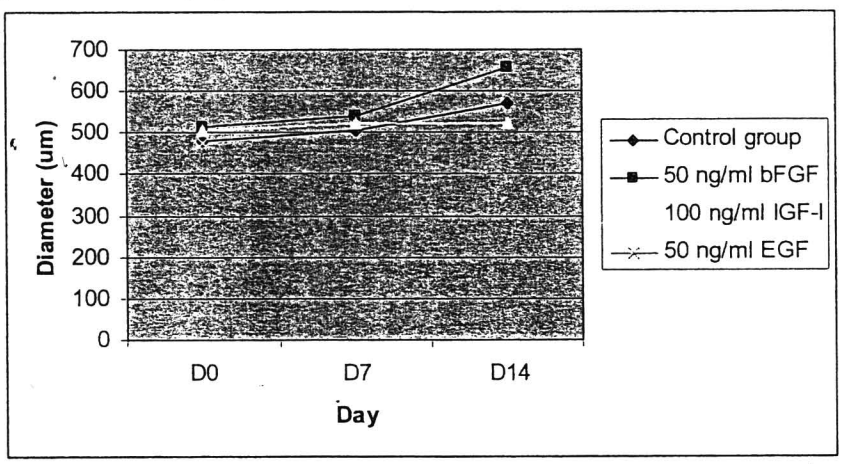
การเลี้ยงฟอลลิเคิลกระบือปลักในกลุ่มที่ 1 (Ø 200-399 ไมโครเมตร) และ กลุ่มที่ 2 (Ø 400-599 ไมโครเมตร) พบว่ามีเพียงน้ำยาที่เติม bFGF เท่านั้นที่สามารถทำให้ฟอลลิเคิลมีการเจริญเติบโตแต่อัตราการเพิ่มขึ้นของขนาดฟอลลิเคิลจากวันที่ 0 ถึง 7 นั้นไม่มีนัยสำคัยทางสถิติ ส่วนในวันที่ 7 ถึง 14 พบการเพิ่มขนาดของฟอลลิเคิลเป็นไปอย่างมีนัยสำคัญทางสถิติ ในกลุ่มที่ 3 (Ø 600-799 ไมโครเมตร) ฟอลลิเคิลที่เลี้ยงในน้ำยาที่เติม bFGF และ กลุ่มควบคุม มีการเจริญเติบโตของฟอลลิเคิลโดยการเพิ่มขนาดของฟอลลิเคิลจากวันที่ 0 ถึง 7 เป็นไปอย่างมีนัยสำคัญทางสถิติ แต่ในวันที่ 7 ถึง 14 พบการเพิ่มขนาดของฟอลลิเคิลเป็นไปอย่างไม่มีนัยสำคัญทางสถิติ การทำให้ฟอลลิเคิลกลุ่มที่ 1 เจริญเติบโตภายในห้องทดลองทำได้ยากมากเนื่องจากฟอลลิเคิลมีขนาดเล็กและไม่สามารถที่จะสร้างแอนทรัม (antrum formation) ได้เอง ส่วนฟอลลิเคิลในกลุ่มที่ 2 และ 3 นั้นมีแอนทรัมขนาดเล็กอยู่แล้วตั้งแต่เริ่มเข้าเลี้ยง ดังนั้นฟอลลิเคิลบางใบจึงสามารถเจริญเติบโตและขยายแอนทรัมเพื่อเพิ่มขนาดฟอลลิเคิลได้ดีกว่ากลุ่มที่ 1 ฟอลลิเคิลบางใบจึงสามารถเจริญเติบโตได้โดยที่ไม่ต้องใช้โกรทแฟ็กเตอร์ อย่างไรก็ตามจากผลการทดลองพบว่าฟอลลิเคิลที่เลี้ยงในน้ำยาที่เติมด้วย bFGF สามารถเจริญเติบโตและเพิ่มขนาดได้ดีกว่าฟอลลิเคิลที่เลี้ยงในน้ำยาที่ไม่เติมโกรทแฟ็กเตอร์

สรุปและข้อเสนอแนะ

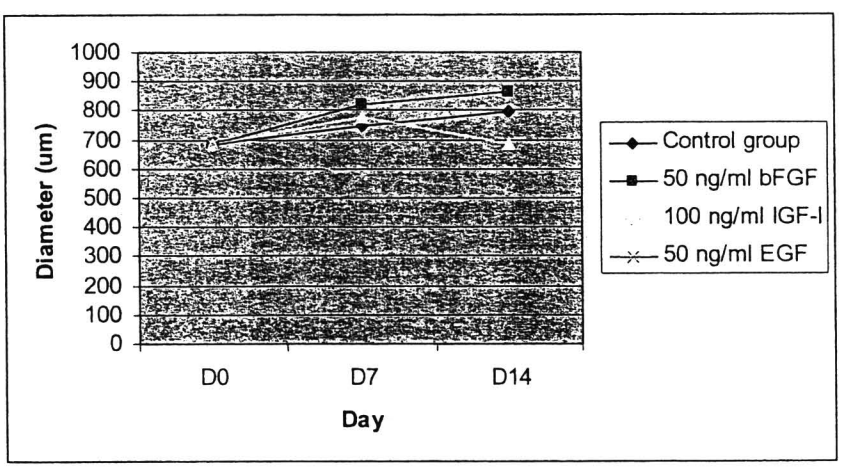
จากการทดลองพบว่าน้ำยาเลี้ยงฟอลลิเคิลที่เติม bFGF สามารถเพิ่มขนาดของฟอลลิเคิลได้ดีกว่าน้ำยาที่เติม IGF-I และ EGF อย่างไรก็ตามการทดลองนี้ยังไม่เสร็จสมบูรณ์เนื่องจากควรจะเลี้ยงฟอลลิเคิลโดยใช้น้ำยาสองตัวรวมกัน (EGF+IGF-I, EGF+bFGF, IGF-I+bFGF) และเลี้ยงโดยใช้โกรทแฟ็กเตอร์ทั้งสามตัวรวมกัน (EGF+IGF-I+bFGF) ซึ่งผลที่ได้อาจจะแตกต่างจากการการเลี้ยงโดยใช้โกรทแฟ็กเตอร์เพียงตัวเดียว



รูปที่ 1 แสดงการเจริญเติบโตของฟอลลิเคิลในกลุ่มที่ 1 (Ø 200-399 ไมโครเมตร) ตั้งแต่เริ่มเลี้ยงจนถึงวันที่ 14



รูปที่ 2. แสดงการเจริญเติบโตของฟอลลิเคิลในกลุ่มที่ 2 (Ø 400-599 ไมโครเมตร) ตั้งแต่เริ่มเลี้ยงจนถึงวันที่ 14



รูปที่ 3. แสดงการเจริญเติบโตของฟอลลิเคิลในกลุ่มที่ 3 (Ø 600-799 ไมโครเมตร) ตั้งแต่เริ่มเลี้ยงจนถึงวันที่ 14

กิตติกรรมประกาศ

การทดลองนี้ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย มหาวิทยาลัยเทคโนโลยี สุรนารี และ JSPS-NRCT scientific cooperation program

เอกสารอ้างอิง

ศูนย์วิจัยการผสมเทียมและเทคโนโลยีชีวภาพการผลิตปศุสัตว์. 2007. สรีรวิทยาระบบสืบพันธุ์โคเพศเมีย.บทความเชิงวิชาการด้านเทคโนโลยีชีวภาพ. แหล่งที่มา: http://www.dld.go.th/biotech/Source%20Docs/ From%20 Dr%20 Chirut/AI_Article/C-2_Cow_Repro_System.pdf. September 10, 2007.

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เอกสารแนบหมายเลข 10

เรื่องเต็มการประชุมทางวิชาการ ครั้งที่ ๔๗ มหาวิทยาลัยเกษตรศาสตร์

The Proceeding of 47th Kasetsart University Annual Conference

เล่มที่ 3 สาขาสัตวแพทยศาสตร์

(Subject: Veterinary Medicine)

เกษตรนำไทย : อาหารและพลังงานทดแทนสู่สมดุลอย่างยั่งยืน

Agricultural Science Leading Thailand : Food and Alternative Energy for Sustainable Balance

๔๗

ผลของการใช้สารเคมีกระตุ้นการแบ่งตัวต่อการพัฒนาของตัวอ่อนกระบือภายหลังการICSI

Effect of chemical activation treatments on the development of swamp buffalo (*Bubalus bubalis*) follicular oocytes following intracytoplasmic sperm injection

หยวน หยวน เหลียง อนวัช แสงมาลี สุเมธ อิ่มสุนทรรักษา จันทร์เจ้า ล้อทองพานิชย์
กนกวรรณ ศรีรัตนา นุชจรินทร์ ศรีปัญญา วันวิสาข์ ผิวสร้อย ขวัญฤดี แก้วมุงคุณ
ชุติ เหล่าธรรมธร ดานนา เย มาริน่า เกตุทัต-คาร์นส์ และ รังสรรค์ พาลพ่าย

Yuan yuan Liang Anawat Sangmalee Sumeth Imsoonthornruksa Chanchao Lorthongpanich
Kanokwan Srirattana Nucharin Sripunya Wanwisa Phewsoi Kwanrudee Keawmungkun
Chuti Laowtammathron Danna Ye Mariena Ketudat-Cairns and Rangsun Parnpai

บทคัดย่อ

ในการทดลองนี้ ได้ทำการศึกษาถึงความจำเป็น และผลของการใช้สารกระตุ้นการแบ่งตัว ต่อการพัฒนาของไข่กระบือภายหลังการ ICSI ผลการศึกษาพบว่าอัตราการแบ่งตัวของตัวอ่อนในกลุ่มที่ได้รับการกระตุ้นด้วย EtOH+CHX มีค่าสูงกว่ากลุ่ม Io+6-DMAP และสูงกว่ากลุ่ม EtOH+6-DMAP และ Io+CHX อย่างมีนัยสำคัญ อัตราการเจริญเข้าสู่ระยะบลาสโตซีส สูงที่สุดในกลุ่ม Io+6-DMAP (30%) และกลุ่ม EtOH+CHX (25%) ซึ่งมีค่าสูงกว่ากลุ่ม Io+CHX (6%) และกลุ่ม EtOH+6-DMAP (18%) อย่างมีนัยสำคัญ สรุปว่า การกระตุ้นให้เกิดการแบ่งตัวของไข่กระบือภายหลังการ ICSI ด้วย Io+6-DMAP หรือ EtOH+CHX ให้ผลดีที่สุด ทั้งในส่วนของอัตราการแบ่งตัว และการเจริญเข้าสู่ระยะบลาสโตซีสต์

ABSTRACT

Intracytoplasmic sperm injection (ICSI) in buffalo has not yet well examined. This study assessed the necessity for activation and the effects of chemical activation treatments on in vitro development of oocytes after ICSI. The single spermatozoa was injected into the cytoplasm of an in vitro matured oocyte using micromanipulator under inverted microscope. The ICSI oocytes were assigned to chemical activation treatments: 1) exposed to 5 µM ionomycin (Io) in Emcare medium for 5 min and placed in Emcare medium for 3 h 2) exposed to 7% ethanol (EtOH) in Emcare medium for 5 min and placed in Emcare medium for 3 h. Then the oocytes which treated with Io and EtOH that extruded 2nd polar body were selected and cultured either in A) 1.9 mM 6-dimethylaminopurine (6-DMAP) in mSOF medium for 3 h or B) 10 µg/mL cychloheximide (CHX) for 5 h. The cleavage of ICSI oocytes treated with EtOH+CHX was higher than that treated with Io+6-DMAP and was significantly higher than EtOH+6-DMAP and Io+CHX groups. The highest blastocyst rate was observed in ICSI oocytes treated with Io+6-DMAP (30%) which did not significant different with ICSI oocytes treated with EtOH+CHX (25%). The blastocyst rates of ICSI oocytes treated with Io+6-DMAP and EtOH+CHX showed significantly higher than that treated with Io+CHX (6%) and EtOH+6-DMAP (18%) and also

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[1]ศูนย์วิจัยเทคโนโลยีตัวอ่อนและเซลล์ต้นกำเนิด, สาขาวิชาเทคโนโลยีชีวภาพ, สำนักวิชาเทคโนโลยีการเกษตร, มหาวิทยาลัยเทคโนโลยีสุรนารี
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significant higher than sham injected oocytes in all treatments. In conclusion, our study demonstrated that activation of the ICSI swamp buffalo oocytes with Io+6-DMAP or EtOH+CHX gave the highest cleavage and blastocyst rates.

Key Words: ICSI, chemical activation, embryos development, swam buffalo

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INTRODUCTION

The importance of swamp buffaloes (*Bubalus bubalis*) in many parts of the world is equal to cattle for its meat, milk and labor, especially its resistance to climate, stress and diseases. However, inherent reproductive problems like silent oestrous signs, long calving interval, delayed age of puberty, low number of primordial follicles limit the productivity of buffalo. Assisted reproductive technologies applied on buffalo embryos *in vitro* production have received increasing interest in the recent time.

In natural fertilization, whether the oocytes can further develop depends on the activation of oocytes by sperm, which undergoes the acrosome reaction and penetrates the zonal pellucid and fuse with the oolemma. Intracytoplasmic sperm injection (ICSI) is a kind of assistant reproduction technologies, which sends the sperm directly into cytoplasm by passing the zona pellucida and oolemma, and fertilizes the egg just need a single sperm in theory. Since the first ICSI applied in mammals was reported on hamster (Uehara and Yanagimachi, 1976), several kinds of animal species such as mice (Kimura and Yanagimachi, 1995), sheep (Catt et al., 1996), rabbits (Hosoi et al., 1998), horses (Cochran et al., 1998), cattle (Hamano et al., 1999), domestic cats (Gomez et al., 2000), and pigs (Martin, 2000; Kolbe and Holtz, 2000; Lai et al., 2001) have been produced by the application of this technique. However, up to now there is still no report on the ICSI applied in buffalo.

The activation of oocytes is necessary for the success of fertilization and further development of oocytes to embryos whether it is natural fertilization or *in vitro* fertilization. Some species like hamsters, mice and human (Perreault et al., 1988; Kimura and Yanagimachi, 1995; Tesarik and Sousa, 1995; Kuretake et al., 1996) only ICSI alone seem to be sufficient to trigger the events of oocyte activation. In contrast, other species such as cattle (Rho et al., 1998) and pig (Martin, 2000) required additional activation of the oocyte after sperm injection. Up until recently, no information has been done on buffalo oocyte activation following ICSI treatment. In previous research, we found that ICSI alone is insufficient to activate the buffalo oocytes.

The mechanism of artificial stimuli activating oocytes is inducing the increase of intracellular Ca^{2+} level which is similar to what is done by sperm. Ethanol (EtOH) has been used to activate buffalo oocytes (Parnpai and Tasripoo, 2003) by promoting the formation of inositol 1,4,5-triphosphate (IP3) at plasma membrane and induce the extracellular calcium influx which would form a large

intracellular calcium concentration (Ilyin and Parker, 1992). In buffalo nuclear transfer, Ionomycin (Io) was used to induce repeated transient intracellular calcium rising (Saikun et al., 2004). Exposure to single chemical cannot induce late events of mRNA recruitment, pronuclear formation, DNA synthesis and cleavage (Susko-Parrish et al., 1994; Schultz et al., 1995; Soloy et al., 1997). Thus, combination of a Ca^{2+} rise and an inhibition of either protein synthesis with cycloheximide (CHX) or protein phosphorylation with 6-dimethyl amino purine (6-DMAP) can be an approach for activation of oocytes.

The objective of the present study was to evaluate the effects of combined activation treatments of buffalo oocytes following ICSI on *in vitro* embryo development.

MATERIALS AND METHODS

Oocyte collection and *in vitro* maturation

Buffalo ovaries were obtained from a slaughterhouse and transported to the laboratory within 4 h. The ovaries were placed in physiological saline (0.9% NaCl) in thermal containers. Cumulus-oocyte complexes (COCs) were collected from the follicles 2–8 mm in diameter using a 21-gauge needle and were matured *in vitro* for 22 h. After maturation, cumulus cells were gently removed by pipetting, and the oocytes were subsequently washed 3 times in Emcare medium (ICP-Bio, Auckland, New Zealand). Oocytes with a visible first polar body were selected for the ICSI.

Sperm preparation for ICSI

Straws of frozen spermatozoa were thawed in a 37°C water bath for 30 sec. Thawed spermatozoa were gently injected to the bottom of the BO-medium supplemented with 1 mM caffeine for sperm swimming up, after 30 min and then the supernatant medium were centrifuged at 500×g for 5 min. Deposit was washed twice with BO-medium by centrifugation at 500×g for 5 min. The sperm pellet was resuspended in the BO-medium. Sperm suspensions were diluted approximately 1:5 with 10% polyvinylpyrrolidone (PVP).

ICSI procedures

Sperm injection was performed using an Olympus inverted microscope with a Narishige micromanipulator. The inner diameter of the sperm injection needle was 8-10 µm, and the inner diameter of the holding pipette was 20 µm. The first droplet was 10% PVP medium for washing pipette, the second droplet was the sperm suspension with 10% PVP medium (1:5), and the third droplet was Emcare medium for the ICSI procedure. These droplets were covered with mineral oil. A single, motile buffalo spermatozoon was immobilized against the bottom of the PVP droplet, loaded tail first with a minimum volume of medium into the injection pipette and then injected into the cytoplasm of a buffalo oocyte. Sham-injected buffalo oocytes were manipulated similarly without injection of the sperm cell.

Chemical activation treatment

Injected oocytes were exposed to 5 μM Io in Emcare medium for 5 min and placed in TCM199 medium for 3 h or exposed to 7% EtOH in Emcare medium for 5 min and placed in TCM199 medium for 3 h to permit extrusion of their second polar body. The oocytes with a visible second polar body were transferred to a drop of 1.9 mM 6-DMAP in TCM199 for 3 h or 10 μg/ml CHX in TCM199 for 5 h.

In vitro culture

After activation treatment, survival oocytes were co-cultured with granulosa cell monolayer in TCM199 supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), as described previously with some modification (Lu et al., 2007)). Embryos were cultured under a humidified atmosphere of 5% CO2 in air at 38.5°C for 7 d. Half of the medium was changed every 48 h, and recorded development of embryos from Day 2 to Day 7.

Statistical analysis

Data were analyzed by ANOVA using the statistical analysis systems (SAS). The differences between groups were considered to be statistically significant when $P < 0.05$.

RESULTS

In vitro embryo development of buffalo oocytes following ICSI and activation treatment is showed in Table1. Total of 104 Metaphase-II buffalo oocytes were injected with and without sperm cell,in control treatment (no activation treatment), no oocyte extruded the second polar body and develop to 2-cell stage. The cleavages of ICSI oocytes treated with Io + 6-DMAP, EtOH + 6-DMAP and EtOH + CHX were 75%, 68% and 76% respectively, which were significantly higher ($p<0.01$) than that oocytes treated with Io + CHX (51%) and also significantly higher ($p<0.01$) than sham injected oocytes in all treatments. The high cleavage and blastocyst rates were obtained after the activation treatment of the injected oocytes with Io + 6-DMAP and EtOH + CHX. The lowest cleavage and blastocyst rates were obtained from the activation treatment of Io + CHX. The cleavage and blastocyst rates of EtOH+ 6-DMAP treatment were significantly higher ($p<0.01$) than that of Io + CHX treatment but significantly lower than that of Io + 6-DMAP and EtOH + CHX treatment.

In the sham-injected group, the cleavage of oocytes within these four treatments has no significantly difference. The blastocyst rates of oocytes with treatment Io + 6-DMAP, EtOH + 6-DMAP and EtOH + CHX were 13%, 9%, and 11% respectively, which were significantly higher ($p<0.01$) than that treated with Io + CHX (2%). There was no significant difference among Io + 6-DMAP and EtOH + 6-DMAP or EtOH + CHX treatments on the blastocyst rate.

Table 1. *In vitro* development of buffalo oocytes after ICSI .

Activation treatment	Sperm Injection	No. of oocytes	Cleaved (%)	No (%) of embryos developed to 8-C	16-C	Mor	BL
Io+6-DMAP	+	64	48(75)^{a}	36(56)^{a}	30(47)^{a}	23(36)^{a}	19(30)^{a}
Io+6-DMAP	-	55	32(58)^{b}	24(44)^{bc}	18(33)^{bc}	11(20)^{b}	7(13)^{bc}
Io + CHX	+	70	36(51)^{b}	15(21)^{d}	10(14)^{d}	10(14)^{bc}	4(6)^{de}
Io + CHX	-	49	24(49)^{b}	13(27)^{d}	8(16)^{d}	4(8)^{cd}	1(2)^{e}
EtOH+6-DMAP	+	68	46(68)^{a}	26(38)^{c}	18(26)^{c}	16(24)^{b}	12(18)^{b}
EtOH+6-DMAP	-	54	31(57)^{b}	27(50)^{ab}	21(39)^{b}	10(19)^{b}	5(9)^{cd}
EtOH+CHX	+	72	55(76)^{a}	33(46)^{bc}	26(36)^{b}	24(32)^{a}	18(25)^{a}
EtOH+CHX	-	53	31(59)^{b}	23(43)^{bc}	19(36)^{b}	10(19)^{b}	6(11)^{c}
Control*	+	52	0^{c}	0^{e}	0^{e}	0^{d}	0^{e}
Control*	-	52	0^{c}	0^{e}	0^{e}	0^{d}	0^{e}

^{a,b,c,d,e} Means within columns with different superscripts differ (P<0.01)

* Control means no activation treatment after sperm injection or sham-injection

DISCUSSION

The result of this study showed that the additional activation treatment of buffalo oocytes following ICSI was very necessary for embryo development. This finding had been confirmed by a result of control experiment, which there was no oocyte can reach two-cell stage. Higher percentage of embryo development were obtain with ICSI plus chemical stimulation. Unlike in the hamster (Uehara and Yanagimachi, 1976), rabbit (Keefer, 1989) and human (Tesarik et al., 1994), which mechanical processes during performing ICSI penetration of an ICSI needle through zona pellucida and cytoplasmic suction were strongly enough to activate oocytes, but only mechanical activation could not activate the buffalo oocytes.

Oocytes are naturally activated by sperm. Oocyte activation and parthenogenesis can be induced by artificial, Ca^{2+} oscillation and inactivation of both MPF and MAPK be used to evaluate the artificial activation. Oocytes exposed to a single chemical (e.g., calcium ionophore; EtOH) or electrical stimulus would induce a transient rise in intracellular calcium, but it is inadequate to full term activation as the incomplete CG exocytosis, failure of pronuclear formation, mRNA recruitment and DNA synthesis (Soloy et al.,1997). Thus, combination of a Ca^{2+} rise and an inhibition of either protein synthesis with CHX or protein phosphorylation with 6-DMAP can be an approach for avtivation of oocytes (Susko-Parrish et al., 1994). Inhibition of protein synthesis can enhance oocyte activation by induces temporal change in MPF and MAPK. CHX not only inhibit the cyclin B synthesis and maintain low activity of MPF, but also inhibit the phosphorylation of MAPK after oocytes form pronuclear (Soloy et al., 1997).

In the current study, the cleavage and blastocyst rates in sham-injected groups were significantly lower than sperm-injected groups in all activation treatments. These results indicated that

the injected spermatozoa contributed to the activation process. Previous studies (Kimura et al., 1998) suggest that spermatozoa carry a sperm-borne oocytes-activating factor (SOAF) into oocytes.

In this study, the comparison of 4 kinds of activation treatment combination has been done to find the effective way for the buffalo oocyte activation following ICSI. The result demonstrated that the high cleavage and blastocyst rates were obtained after the ICSI produced buffalo embryos were activated with Io + 6-DMAP and EtOH + CHX. There was no significant difference between this two treatments ($P>0.05$) on the cleavage and blastocyst rate.

Artificial activation of oocytes aims to mimic the action of sperm calls during fertilization (Nakada and Mizuno, 1998). The cell-signaling process may be helpful in understanding this. MII oocytes are characterized by high leves of MPF and MAPK activity (Alberts et al., 2004) corresponding to high levels of cyclin B. The fertilization triggers a transient increase intracellular calcium, which would inactivate the CSF and MPF by degrade the cyclin B or dephosphorylate cdc2 kinase. The events of oocytes activation include the release of cortical granules, accomplishment of miosis, second polar body extrusion and pronucleus formation. In buffalo oocytes, Io is an popular activating agent currently used in buffalo nuclear transfer protocol (Saikhun et al.,2004) which induce induces repetitive transient rises of Ca2+ lasting for several hours. EtOH has been used to activate buffalo oocytes (Parnpai et al., 2003) by promoting the formation of inositol 1,4,5-triphosphate (IP3) at plasma membrane and induce the extracellular calcium influx which would form a large intracellular calcium concentration (Ilyin and Parker, 1992). A single stimulus followed by 6-DMAP, an inhibitor of phosphorylation, or CHX, an inhibitor of protein synthesis, can prevent the rise in histone kinase and permit pronuclear formation. Different from CHX or IVF induced activation, 6-DMAP induced dephosphorylation of MAPK, and therefore earlier pronuclear development. Combined treatments of these agents were more effective in inducing activation and development of young bovine ooocytes than any single treatment alone (Liu et al., 1998).

Higher cleavage and blastocyst rates in Io + 6-DMAP and EtOH + CHX and similar embryo development to 2-cell, morula and blastocyst suggest that both treatments may have followed similar signaling events.

In summary, the present data indicated that effective way for activation of the buffalo oocytes following ICSI is Io + 6-DMAP or EtOH + CHX. Further studies, particularly the oocyte activation, need to focus on the increase of normal diploid that will regularly result in chromosomally normal embryos, fetus, and calf.

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