Effect of Dibutyryl cAMP Together with FSH and EGF during In Vitro Maturation on Sperm Aster Formation and Blastocyst Development after Intracytoplasmic Sperm Injection

Chikako Kani[1, 2], Akiko Kuwahata[2], Masanori Ochi[2] and Toshitaka Horiuchi[1]*

[1]*Graduate School of Comprehensive Scientific Research, Prefectural University of Hiroshima, Hiroshima 727-0023, Japan*
[2]*Ochi Yume Clinic Nagoya, Aichi 460-0002, Japan*

Abstract: *Bovine intracytoplasmic sperm injection (ICSI) is a useful technique in the production of calves. Generally, oocytes matured in vitro are used for bovine ICSI, but the developmental competence is lower than that of oocytes matured in vivo. The objective of this study was to evaluate the effect of dibutyryl cAMP (dbcAMP) during in vitro maturation (IVM) on meiotic maturation, sperm aster formation, and blastocyst development after ICSI. Cumulus-oocyte complexes were cultured in TCM199 supplemented with 100 μM dbcAMP in the presence or absence of follicle-stimulating hormone (FSH) and epidermal growth factor (EGF) in the following treatment four groups: an untreated control, dbcAMP, FSH/EGF, and FSH/EGF/dbcAMP. The percentage of MII oocytes at 21 h did not differ among the four groups (66.7–73.9%). The percentages of sperm aster formation in the FSH/EGF and FSH/EGF/dbcAMP groups (87.1% and 85.7%, respectively) and the diameter of sperm asters in the FSH/EGF/dbcAMP group (81.6 $\pm$ 2.7 μm) were significantly greater than those in the other groups. The blastocyst rate in the FSH/EGF/ dbcAMP group (42.7%) was significantly greater those that in the other groups. Thus, adding dbcAMP together with FSH and EGF to IVM medium stimulated the sperm aster formation in bovine oocytes and increased the blastocyst rate after ICSI.*
Key words: *In vitro maturation, Oocytes, Dibutyryl cAMP, Intracytoplasmic sperm injection, Bovine*

Introduction

Bovine intracytoplasmic sperm injection (ICSI) is useful for the production of calves when the supply of gametes is limited [1]. However, the development rate of ICSI embryos remains insufficient for practical use. In humans and mice, oocytes matured *in vivo* have usually been used for ICSI [2], but in cattle, oocytes matured *in vitro* are mainly used [3]. The developmental competence of oocytes matured *in vitro* is lower than that of in oocytes matured *in vivo* [4]. One of the causes of low development rate of ICSI embryos is thought to be the incomplete maturation of bovine oocytes *in vitro.* In a preliminary study, we showed that the blastocyst rate after ICSI of bovine oocytes matured *in vivo* was higher than that of in oocytes matured *in vitro* [5].

Furthermore, the sperm aster formation rate of oocytes matured *in vivo* after ICSI was higher than that of in oocytes matured *in vitro* [4]. Bull spermatozoa contain centrosomes, the cell's microtubule-organizing center [6]. Once incorporated into an oocyte, the bovine sperm centrosome organizes the microtubules of the sperm aster [7–9]. The microtubules are cytoskeletal elements, which are essential for pronuclear migration and apposition and the union of the male and female genomes during fertilization. The organization of the sperm aster during fertilization is related to the developmental competence of an oocyte [9]. The evaluation of sperm aster formation is thus an important criterion in the assessment of the developmental competence of bovine oocytes matured *in vitro*.

The addition of serum, gonadotropins, and growth

factors to *in vitro* maturation (IVM) medium stimulates meiotic maturation and the developmental competence of oocytes matured *in vitro*, and they act via the adenylate cyclase pathway to produce the intracellular messenger, cAMP [10, 11]. Cyclic AMP plays a significant role in the regulation of mammalian oocyte maturation [12], which can be increased in oocytes by adding the following agents to IVM medium: cAMP analogues, such as dibutyryl cAMP (dbcAMP); activators of adenylate cyclase, such as follicle-stimulating hormone (FSH) or invasive adenylate cyclase (iAC); and phosphodiesterase (PDE) inhibitors such as milrinone or rolipram [13].

In bovine, adding iAC [10], and milrinone or together with FSH [14] to IVM medium improved the subsequent oocyte developmental potential. In porcine, adding dbcAMP combined with equine gonadotrophin (eCG) and human chorionic gonadotropin (hCG), or epidermal growth factor (EGF) to the IVM medium improved the blastocyst rate [15–17]. However, in bovine, the effect of dbcAMP, or its combination with FSH and EGF on the developmental competence of oocytes matured *in vitro* has not been reported, even though the combination of FSH and EGF during IVM improved the subsequent blastocyst development [18].

Therefore, the purpose of this study was to evaluate the effect of adding dbcAMP in the presence or absence of FSH and EGF to IVM medium on meiotic maturation, microtubule organization, and the developmental competence of bovine oocytes matured *in vitro* after ICSI.

Materials and Methods

Chemicals and media

Unless otherwise stated, all chemicals used in this study were purchased from Nacalai Tesque (Kyoto, Japan). Sp-TLP medium [19] supplemented with 10 mM pentoxifylline (Sigma-Aldrich Chemicals, St. Louis, MO, USA) was used for sperm washing and preparation [3]. HEPES-buffered TCM-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA) supplemented with 0.2 mM sodium pyruvate and 50 μg/ml gentamycin sulfate (Sigma-Aldrich) was supplemented with 1 mg/ml recombinant human albumin (rHA; Vitrolife AB, Kungsbacka, Sweden), and with or without 1 IU/ml recombinant human FSH (Gonal-F; Serono, Geneva, Switzerland) and 50 ng/ml recombinant human EGF (Upstate, Lake Placid, NY, USA), and/or 100 μM dibutyryl cAMP (Sigma-Aldrich) for the IVM medium. M2 medium [20] supplemented with 5% fetal bovine

serum (FBS; Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) was used for handling oocytes and ICSI. Modified synthetic oviduct fluid (mSOF) [21, 22], supplemented with 20 μl/ml essential amino acids solution (x50, Gibco BRL), 10 μl/ml non-essential amino acids solution (x100, Gibco BRL), 1 mM glycine, 2 mM taurine, ITS supplement (final concentration of 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium; Sigma-Aldrich), and 6 mg/ml fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich), was used as the culture medium for ICSI oocytes.

Collection and in vitro maturation of bovine oocytes

Bovine ovaries were obtained at a slaughterhouse and transported to our laboratory in 0.9% saline at 35–36ºC. After confirming negative results for bovine spongiform encephalopathy, cumulus-oocyte complexes (COCs) were aspirated from follicles with a diameter of 2 to 8 mm, and oocytes surrounded by at least two layers of compact cumulus cells and with an evenly granulated cytoplasm were selected for IVM. A group of 20 to 30 COCs was cultured for 21 h in 500 μl of TCM199 supplemented with or without 1 IU/ml FSH + 50 ng/ml EGF, and/or 100 μM dbcAMP at 38.5ºC under 5% CO_2 in air. After maturation, the oocytes were freed from the cumulus cells by a brief incubation with 2 mg/ml hyaluronidase (Sigma-Aldrich) in M2 medium, followed by gentle pipettings for 1 min. Oocytes with an extruded first body were defined as matured. They were maintained in 50 μl of TCM199 supplemented with 10%FBS under paraffin oil at 38.5ºC under 5% CO_2 in air until used for ICSI.

Intracytoplasmic sperm injection (ICSI)

A straw of frozen spermatozoa from a Japanese Black bull was thawed in a 37ºC water bath for 30 s. Thawed spermatozoa were washed with BSA-free SP-TALP medium [19] supplemented with 10 mM pentoxifylline by centrifugation at 500 × *g* for 5 min. Pelleted spermatozoa were resuspended in the same medium. To prepare for ICSI, 5 μl of sperm suspension were mixed with 10 μl of M2 medium containing 12% PVP K90 (MP Biochemical, OH, USA).

ICSI was performed with a piezo-driven micromanipulator (PMM-150FU; Prime Tech, Ibaraki, Japan) according to the method reported previously [3, 23, 24]. Briefly, immediately before sperm injection, a motile spermatozoon was immobilized by breakage of its tail with the tip of the injection needle against the bottom of the dish. The immobilized spermatozoon was sucked tail first into a blunt-ended injection needle with an outer

diameter of 7–8 μm. An oocyte was held by the holding pipette while the polar body was positioned at the 12 o'clock position. The immobilized spermatozoon was then sucked tail first into the injection pipette. The zona pellucida was drilled by applying several piezo pulses. The spermatozoon was pushed forward until its head approached the tip of the pipette. The injection pipette was advanced mechanically into the ooplasm until its tip reached the center of the oocyte. The oolemma was punctured by a single piezo pulse and the spermatozoon was injected into the ooplasma. The injected oocytes were transferred into 50 μl of TCM199 supplemented with 10%FBS under paraffin oil and kept at 38.5ºC under 5% CO_2 in air.

Ethanol treatment of oocytes after ICSI

Sperm-injected oocytes were cultured in TCM199 supplemented with 10% FBS for 4 h. The oocytes were then treated for 5 min with 7% ethanol in TCM 199 containing 1 mg/ml PVP-40 (Sigma-Aldrich) as previously reported [3, 23, 24]. The ethanol-treated oocytes were washed several times with TCM199 supplemented with 10% FBS and transferred to mSOF medium [21, 22]. Most of the ethanol-treated oocytes were used for embryo culture. Some of the oocytes were fixed and stained for observation of sperm aster formation and chromatin at 6 h after ICSI.

Embryo culture

The ethanol-treated oocytes were cultured in groups of 10–15 in 50-μl drops of mSOF medium supplemented with 6 mg/ml fatty-acids-free BSA at 38.5ºC under 5% CO_2, 7% O_2, and 88% N_2. Cleavage and blastocyst rates were assessed at 54 h and 196 h after ICSI, respectively.

Immunocytochemical detection of microtubules and DNA in bovine oocytes after ICSI

Zonae pellucidae were removed from the oocytes by brief treatments with 0.75% pronase (Kaken Chemicals, Tokyo, Japan) prepared in protein-free M2. After 30 min of recovery at 38.5ºC, zona-free oocytes were attached to polylysine-coated coverslips and extracted for 15 min in buffer M (25% (v/v) glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 50 mM imidazole, and 1 mM 2-mercaptoethanol at pH 6.8) containing 0.2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), 1% (v/v) Triton X-100 detergent, and 5% methanol, followed by fixation in cold methanol for 10 min according to the methods of Simerly and Schatten [25]. Fixed oocytes were then permeabilized overnight in 0.1 mM phosphate-buffered saline containing 0.1% (v/v) Triton X-100. Microtubules were labeled with a mixture of monoclonal antibody against α- and β-tubulin (clone B-5-1-2 and clone 2-28-33; diluted 1:200; Sigma-Aldrich) and acetylated α-tubulin (clone 6–11B–1; diluted 1:200; Sigma-Aldrich). The primary antibodies were detected by fluorescein–conjugated goat anti–mouse immunoglobulin G (diluted 1:40; Zymed, San Francisco, CA, USA). DNA was detected after labeling with 5 μg/ml DAPI.

Coverslips were mounted in an anti-fade medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and the slides were examined using conventional epifluorescence microscopy (Optiphot-2; Nikon, Japan). The images were acquired using a Hamamatsu C-4742 Digital Camera (Hamamatsu Photonics K.K., Hamamatsu, Japan) controlled by Fluro-Pro 3.0 software (Media Cybernetics, Bethesda, MD, USA), and were recorded digitally. The size of sperm asters was calculated by measuring the diameter of the sperm aster at the largest point according to Navara *et al.* (1996) [9] using Image-Pro Plus 4.0 software (Media Cybernetics, Bethesda, MD, USA).

Experimental design

In Experiment 1, the effects of dbcAMP on the timing of germinal vesicle breakdown (GVBD) and meiotic maturation of bovine oocytes were investigated. COCs were cultured in TCM199 supplemented with or without dbcAMP in the presence or absence of FSH + EGF in the following four treatment groups: none (the untreated control), dbcAMP, FSH/EGF, and FSH/EGF/dbcAMP. To evaluate GVBD, the oocytes were fixed for 48 h with aceto-ethanol (acetic acid:ethanol, 1:3, v:v), stained with 1% acetoorcein, and examined under a phase-contrast microscope. In Experiment 2, sperm aster formation at 6 h after ICSI was visualized by immunofluorescent staining in the four groups. The appearance of each sperm aster and the diameter of each sperm aster were compared among the four groups. In Experiment 3, the effect of dbcAMP during IVM on the *in vitro* development of ICSI embryos was investigated.

Statistical analysis

The percentage data of GVBD were compared using the chi-square test. The percentage data of nuclear maturation and embryonic development in each replicate were arcsine -transformed before being subjected to one-way analysis of variance (ANOVA). The diameters of sperm asters were also analyzed by one-way ANOVA. All of the experiments were

replicated at least three times. Differences among the means of different groups were compared by Bonferroni's multiple comparison test using GraphPad Prism 5 (GraphPad Software; La Jolla, CA, USA). The percentage data of sperm aster formation were analyzed using the chi-square test. Differences were considered significant at values of $P < 0.05$.

Results

Figure 1 shows the effects of dbcAMP in the presence or absence of FSH and EGF on the timing of GVBD of bovine oocytes. Bovine oocytes were matured *in vitro* without any supplements (none: the untreated control), or supplemented with dbcAMP, FSH/EGF, or FSH/EGF/ dbcAMP. In the untreated control, the percentage of GVBD at 3 h after the onset of IVM was significantly (P <0.05) higher than in the other groups (35.5% vs. 3.1%, 0% and 0%, respectively). At 6 h post-IVM, the percentage of GVBD in the dbcAMP group was significantly (P <0.05) lower than in the other groups (38.1% vs. 76.9%, 80.8% and 81.8%). At 9 h post-IVM, there were no significant differences among the four groups.

Table 1 shows the results of the effects of dbcAMP on nuclear maturation of bovine oocytes. The percentages of MII oocytes at 21 h in the four groups were respectively 66.7 ± 5.4%, 73.9 ± 1.8%, 69.5 ± 1.5%, and 72.7 ±1.9%, without significant difference (P > 0.05). The addition of dbcAMP did not affect meiotic maturation of bovine oocytes.

Figure 2 shows the microtubule organization and chromatin configurations in bovine oocytes at 6 h after ICSI. The sperm aster, a radical microtubule array extending from the sperm centrosome, was organized in the untreated group (Fig. 2A). In the dbcAMP group, the male pronucleus was surrounded by a microtubule
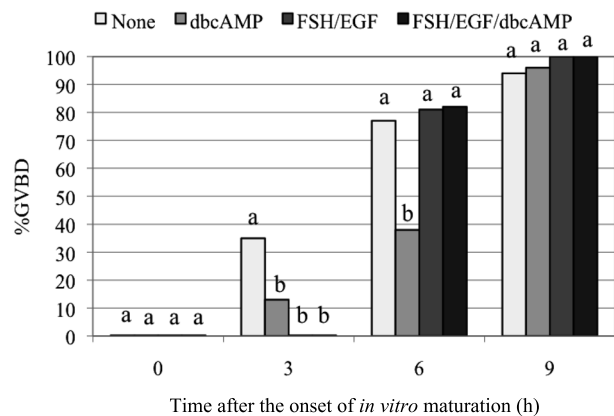

Fig. 1. Effect of dibutyryl cAMP in the presence or absence of FSH and EGF on timing of germinal vesicle breakdown (GVBD) of bovine oocytes. Each bar represents 30~35 oocytes. Bars with different letters (a, b) at the same times indicate significant differences (P <0.05).

array, and the microtubules had contact with the female pronucleus (Fig. 2B). In the FSH/EGF group, a large sperm aster was organized around the male pronucleus, but no microtubules were organized around the female pronucleus (Fig. 2C). In the FSH/EGF/dbcAMP group, both male and female pronuclei were surrounded by a microtubule array, and the microtubules were fully elongated in the ooplasma (Fig. 2D).

Figure 3 shows the results of the effects of dbcAMP during IVM on sperm aster formation and size in bovine oocytes at 6 h after ICSI. The percentage of sperm aster formation in the dbcAMP group (54.8%) was significantly lower than in the untreated group (17.2%). In the FSH/ EGF and FSH/EGF/dbcAMP groups (87.1% and 85.7%, respectively), the percentage of sperm aster formation was significantly higher than in the other groups. The diameter of sperm aster in the untreated group (50.1 ±

Table 1. Effect of dibutyryl cAMP on nuclear maturation of bovine oocytes

Supplement	No. of COCs cultured (no. of replicates)	No. (% ± SE) of MII oocytes at 21 h*
None	147 (3)	97 (66.7 ± 5.4)
dbcAMP	137 (3)	101 (73.9 ± 1.8)
FSH/EGF	154 (3)	107 (69.5 ± 1.5)
FSH/EGF/dbcAMP	143 (3)	104 (72.7 ± 1.9)

COCs: Cumulus-oocyte complexes; dbcAMP: 100 μM dibutyryl cyclic adenosine monophosphate; FSH: 1 IU recombinant human follicle-stimulating hormone, EGF: 50 ng/ml recombinant human epidermal growth factor.
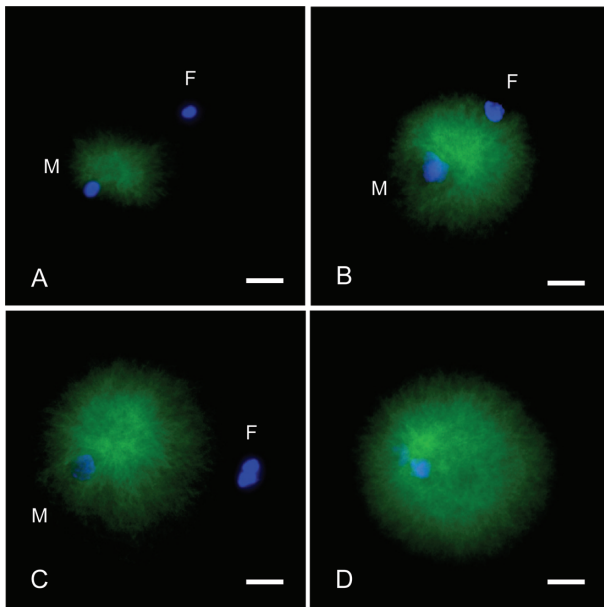*There were no significant differences among the groups (P > 0.05).

Fig. 2. Microtubule (green) organization and chromatin (blue) configurations in bovine oocytes at 6 h after intracytoplasmic sperm injection. Bovine oocytes were matured *in vitro* in TCM199 without any supplements (A), supplemented with dibutyryl cAMP (dbcAMP) (B), FSH and EGF (C), or FSH, EGF and dbcAMP (D). (A) A small sperm aster was organized around the sperm centrosome, but no microtubules were organized around the female nucleus. (B) The male pronucleus was surrounded by a microtubule array, and the microtubules had contact with the female pronucleus. The microtubules were larger than that in the untreated group (A). (C) Microtubules elongated, forming a radically arrayed sperm aster, and there were no microtubules organized around the female nucleus. Microtubules were a little larger than in the dbcAMP group (B). (D) Microtubules were extensively organized around the male and female pronuclei. M: male pronucleus, and F: female pronucleus. Scale bar = 30 μm.

Fig. 3. Effect of dibutyryl cAMP (dbcAMP) during *in vitro* maturation on the appearance of sperm aster formation (A) and the diameter of sperm aster formation (B) in bovine oocytes fertilized by ICSI. The bovine oocytes were matured *in vitro* in TCM199 without any supplements (the untreated group, none: n = 29), supplemented with dibutyryl cAMP (dbcAMP: n = 31), with FSH and EGF (FSH/EGF: n = 31), and with FSH, EGF, and dbcAMP (FSH/EGF/dbcAMP: n = 28). Bars with different letters (a-c) are significantly different ($P < 0.05$).

8.6 μm) was significantly smaller than in the other the groups. In the dbcAMP and FSH/EGF groups, the diameters of sperm asters (69.8 ± 3.3 μm and 70.8 ± 1.9 μm, respectively) were significantly larger than in the untreated group. The diameter of sperm asters in the FSH/EGF/dbcAMP group (81.6 ± 2.7 μm) was larger than in the others groups. Supplementing the IVM medium with dbcAMP stimulated sperm aster formation and size in bovine oocytes at 6 h after ICSI.

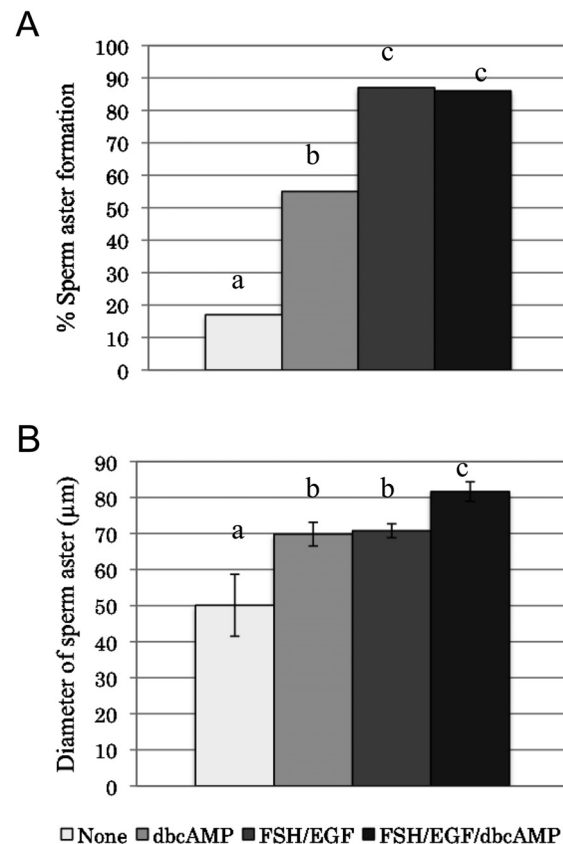Table 2 shows *in vitro* development of bovine oocytes matured in the presence of dbcAMP and fertilized by ICSI. The survival rate after ICSI was not significantly different among the groups. The cleavage rate in the FSH/EGF/dbcAMP group was higher than in the untreated group (93.8% vs. 76.3%) and was similar to that in the dbcAMP or FSH/EGF group (82.7% and 89.7% respectively). The blastocyst rate in the FSH/EGF/dbcAMP group (42.7%) was higher than in the other groups. In the dbcAMP or FSH/EGF group, the blastocyst rate was significantly higher than in the untreated group (respectively 23.8% and 23.6% vs. 12.9%). Supplementing the IVM medium with dbcAMP together with FSH and EGF significantly improved the proportion of ICSI embryos developing into blastocysts.

Table 2. Effect of dibutyryl cAMP during *in vitro* maturation on subsequent embryo development after ICSI

| Supplement | No. of oocytes | | No. (% ± SEM) of embryos developed to stage* | | | |
	sperm-injected (no. of replicates)	survived (%)	≥2-cell [54 h]	8-cell [72 h]	Morulae [148 h]	Blastocysts [196 h]
None	97 (3)	85 (88)	65 (76.3 ± 3.6)[a]	39 (45.2 ± 10.8)[a]	23 (26.8 ± 5.0)[a]	13 (12.9 ± 1.0)[a]
dbcAMP	101 (3)	92 (91)	76 (82.7 ± 3.7)[ab]	55 (59.8 ± 5.8)[a]	36 (39.2 ± 5.4)[ab]	22 (23.8 ± 3.6)[b]
FSH/EGF	107 (3)	97 (91)	87 (89.7 ± 1.5)[ab]	42 (43.3 ± 9.6)[a]	31 (32.6 ± 7.1)[ab]	23 (23.6 ± 0.3)[b]
FSH/EGF/dbcAMP	104 (3)	96 (92)	90 (93.8 ± 1.6)[b]	64 (66.4 ± 4.7)[a]	54 (55.7 ± 5.5)[b]	41 (42.7 ± 1.5)[c]

dbcAMP: 100 μM dibutyryl cyclic adenosine monophosphate; FSH: 1 IU recombinant human follicle-stimulating hormone; EGF: 50 ng/ml recombinant human epidermal growth factor.
*The percentage of embryos developed to each stage is shown as the number of surviving oocytes.
[a–c]Values with different superscripts within the same column are significantly different (P <0.05).

Discussion

In the present study, we demonstrated that adding 100 μM dbcAMP to IVM medium did not affect the proportion of metaphase II (MII) oocytes at 21 h of culture, but did increase the percentage of sperm aster formation in bovine oocytes at 6 h after ICSI. Furthermore, treating bovine COCs with dbcAMP in the presence of FSH and EGF increased the size (diameter) of sperm asters in the bovine oocytes matured *in vitro* after ICSI, and improved the blastocyst rate of ICSI embryos.

In the present study, treating bovine COCs *in vitro* with a low concentration of 100 μM dbcAMP in the presence or absence of FSH and EGF, did not affect meiotic maturation. Treating bovine COCs *in vitro* with a high concentration of 2.5–10 mM dbcAMP only decreased the frequency of germinal vesicle breakdown (GVBD) at 6 h in bovine oocytes, but meiotic resumption *in vitro* was transiently inhibited [26]. High concentrations (1–5 μg/ml) of iAC in IVM medium inhibited the resumption of meiosis, while low concentrations (0.01–0.1 μg/ml) resulted in high rates of maturation to the MII stage [10]. In the presence of a low concentration of dbcAMP, most oocytes matured *in vitro* progressed to the metaphase II stage, and meiotic resumption of bovine oocytes *in vitro* was not inhibited by dbcAMP.

In the present study, treating bovine COCs *in vitro* with dbcAMP together with FSH and EGF had a positive effect on oocyte cytoplasmic maturation, and improved oocyte developmental potential. Supplementation of IVM medium with only dbcAMP resulted in an increased blastocyst rate compared with that in the absence of treatment. The results show that during IVM, cAMP played a role in the regulation of oocyte cytoplasmic maturation, and it may be a key molecule in the acquisition of developmental competence by oocytes matured *in vitro*.

Thomas *et al.* (2004) demonstrated that, treating bovine COCs with the type 3- or 4-specific PDE inhibitor, milrinone or rolipram during IVM, together with FSH advanced embryo development to the blastocyst stage after IVM-IVF [14]. Similarly, Luciano *et al.* (2004) showed that treating bovine COCs with low concentrations of iAC during IVM resulted in increased rates of development to the blastocyst stage [10]. Regarding dbcAMP in porcine, Funahashi *et al.* (1997) [15] and Kim *et al.* (2008) [17] reported that treating porcine COCs with 1 mM dbcAMP combined with eCG and hCG, or with the addition of EGF for the first 20–22 h within a total of 44 h of IVM increased the development rate to the blastocyst stage after IVF. Likewise, in this study, treating bovine COCs with a low concentration of dbcAMP combined with FSH and EGF during IVM increased the blastocyst rate compared to the treatment of FSH and EGF without dbcAMP.

In the present study, the percentage of sperm aster formation in bovine oocytes matured *in vitro* was increased by the addition of dbcAMP to the IVM medium without FSH and EGF. The higher the microtubules formation rate in the bovine oocytes matured *in vitro*, the better the blastocyst rate. The cytoplasmic quality of bovine oocytes matured *in vitro* treated with dbcAMP affected microtubule formation from the sperm centrosome after ICSI. The microtubules lengthened to form a sperm aster as the sperm DNA decondensed, and elongated, eventually filling the entire cytoplasm.

Commonly, microtubules are organized by maternal α- and β-tubulin in oocytes. Bovine spermatozoa retain γ-tubulin. Paternal γ-tubulin is largely inaccessible in the mature spermatozoa until the disulfide bond has been reduced. The coiled-coil infrastructure of the centrosome regulates the exposure to, and binding sites for, γ-tubulin. γ-Tubulin is created by a combination of some paternal but mostly maternal protein in the

ooplasma during fertilization [6, 8, 27]. Recruitment of maternal γ-tubulin to the sperm centrosome occurs after sperm incorporation. After a sperm is exposed to cytoplasmic components of the oocyte, centrosomal constituents, for example MPM-2, induce the phosphorylation of the centrosomal protein, and the centrosomes become competent at nucleating microtubule growth into sperm asters [8]. Thus, γ-tubulin nucleates the microtubules for the sperm aster. These complex mechanisms support not only the sperm components but also the cytoplasmic components in oocytes.

The mechanisms by which bovine oocyte cytoplasmic maturation is improved by the addition of dbcAMP together with FSH and EGF during IVM are unclear. In this study, we observed that treating bovine oocytes with 100 μM dbcAMP, or with FSH and EGF in the presence or absence of dbcAMP induced delayed GVBD as compared with spontaneous maturation in the untreated control. However, treating bovine oocytes with FSH and EGF in the presence or absence of dbcAMP induced GVBD a similar time course. Thomas *et al.* (2004) reported that in the presence of FSH, type 3- and 4-specific PDE inhibitors delayed GVBD and only slightly extended cumulus cells-oocyte gap junctional communication over the first 9 h, but that they completely blocked meiotic resumption during this period [14]. This inhibition would be expected to facilitate prolonged exchange of positive regulatory molecules and metabolites from the cumulus cells to the oocyte, improving oocyte cytoplasmic quality.

The delay of meiotic resumption induced by dbcAMP in this study may have affected oocyte cytoplasmic maturation via the same mechanism as PDE inhibitors. FSH activates adenylate cyclase, leading to cAMP accumulation in the cumulus cells [12]. High levels of cAMP in cumulus cells would rapidly diffuse into the oocytes via gap junctions [11]. Although cumulus cells generate increased ooplasma cAMP content, the oocyte PDE degrades cAMP rapidly. Thus, the ooplasma cAMP level would increase with combined treatment of COCs with a PDE inhibitor, or dbcAMP together with an adenylate cyclase stimulator, such as FSH. A high level of cAMP not only prevents immediate meiotic resumption *in vitro* but also controls progression beyond the metaphase-I stage [28]. In addition, a high level of cAMP in oocytes activates cAMP-protein kinase A (PKA), and also protein kinase C (PKC) also plays an important role in the signal transduction of FSH. Both the PKA and PKC pathways may modulate the developmental capacity of bovine IVM oocytes.

Furthermore, EGF together with dbcAMP has a critical role in the resumption of meiosis and supports nuclear and cytoplasmic maturation of porcine oocytes. Akaki *et al.* (2009) reported that supplementation with EGF family members and dbcAMP during the first 20 h of IVM supported the meiotic progress and developmental competence of porcine oocytes [29]. In our present study, a combination of FSH, EGF, and dbcAMP was added to IVM medium, but the interactions among these factors were not clear. It remains to be demonstrated what their interactions are in enhancing the developmental competence of bovine oocytes.

In conclusion, we demonstrated that adding a low concentration (100 μM) of dbcAMP to IVM medium in the presence or absence of FSH and EGF did not affect nuclear maturation at 21 h of bovine IVM. However, treating the COCs with dbcAMP together with FSH and EGF during IVM increased the rate of sperm aster formation and the size of the sperm asters at 6 h after ICSI. Thus, treating bovine oocytes with dbcAMP together with FSH and EGF during IVM improved the rate of early embryonic development to the blastocyst stage after ICSI. This outcome indicates that supplementation of IVM medium with FSH, EGF and dbcAMP, affects the cytoplasmic maturation of bovine oocytes and increases the developmental competence of bovine oocytes which have been matured *in vitro*.

References

1) Horiuchi, T. and Numabe, T. (1999): Intracytoplasmic sperm injection (ICSI) in cattle and other domestic animals: problems and improvement in practical use. J. Mamm. Ova Res., 16, 1–9.

2) Yanagimachi, R. (2005): Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in human and animals. Reprod. Biomed. Online, 10, 247–288.

3) Horiuchi, T., Emuta, C., Yamauchi, Y., Oikawa, T., Numabe, T. and Yanagimachi, R. (2002): Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: a methodological approach. Theriogenology, 57, 1013–1024.

4) Rizos, D., Ward, F., Duffy, P. and Boland, M.P. (2002): Consequences of bovine oocyte maturation, fertilization, or early embryo development in vitro versus in vitro: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev., 61, 234–248.

5) Horiuchi, T., Takenaka, M., Kani, C., Emuta, C., Ogata, Y. and Matushige, T. (2007): Sperm aster formation and blastocyst development of in vitro-matured bovine oocytes fertilized by intracytoplasmic sperm injection. Reprod. Fertil. Dev., 301–302, abstract.

6) Schatten, G. (1994): The centrosome and its mode of inheritance: The reduction of the centrosome during the gametogenesis and its restoration during fertilization. Dev. Biol., 165, 299–335.

7) Sutovsky, P. and Schatten, G. (1997): Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. Biol. Reprod., 56, 1503–1512.

8) Payne, C., Rawe, V., Ramalho-Santos, J., Simerly, C. and Schatten, G. (2003): Preferentially localized dynein and perinuclear dynactin associate with nuclear pore complex proteins to mediate genomic union during mammalian fertilization. J. Cell Sci., 116, 4727–4738.

9) Navara, C.S., First, N.L. and Schatten, G. (1996): Phenotypic variations among paternal centrosomes expressed with the zygote as disparate microtubule lengths and sperm aster organization: Correlations between centrosome activity and developmental success. Proc. Natl. Acad. Sci. USA, 93, 5384–5388.

10) Luciano, A.M., Pocar, P., Milanesi, E., Modina, S., Rieger, D. and Gandolfi, F. (1999): Effect of different levels of intracellular cAMP on the in vitro maturation of cattle oocytes and their subsequent development following in vitro fertilization. Mol. Reprod. Dev., 54, 86–91.

11) Eppig, J.J. (1989): The participation of cyclic adenosine monophosphate (cAMP) in the regulation of meiotic maturation of oocytes in the laboratory mouse. J. Reprod. Fertil. Suppl., 38, 3–8.

12) Luciano, A.M., Modina, S., Vassena, R., Milanesi, E., Lauria, A. and Gandolfi, F. (2004): Role of intracellular cyclic adenosine 3', 5'-monophosphate concentration and oocyte-cumulus communications on the acquisition of the developmental competence during in vitro maturation of bovine oocyte. Biol. Reprod., 70, 465–472.

13) Gilchrist, R.B. and Thompson, J.G. (2006): Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. Theriogenology, 67, 6–15.

14) Thomas, R.E., Thompson, J.G., Armstrong, D.T. and Gilchrist, R.B. (2004): Effect of specific phosphodiesterase isoenzyme inhibitors during in vitro maturation of bovine oocytes on meiotic and developmental capacity. Biol. Reprod., 71, 1142–1149.

15) Funahashi, H., Cantley, T.C. and Day, B.N. (1997): Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine in vitro fertilization. Biol. Reprod., 57, 49–53.

16) Bag, M.A., Nottle, M.B., Grupen, C.G. and Armstrong, D.T. (2006): Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental porcine oocytes. Mol. Reprod. Dev., 73, 1326–1332.

17) Kim, J.S., Cho, Y.S., Song, B.S., Wee, G., Park, J.S., Choo, Y.K., Yu, K., Lee, K.K., Han, Y.M. and Koo, D.B. (2008): Exogenous dibutyryl cAMP affects meiotic maturation via protein kinase A activation; it stimulates further embryonic development including blastocyst quality in pigs.

18) Harper, K.H. and Brackett, B.G. (1993): Bovine blastocyst development after in vitro maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. Biol. Reprod., 48, 409–416.

19) Parrish, J.J., Suko-Parrish, J. Winer, M.A. and First, N.L. (1988): Capacitation of bovine sperm by heparin. Biol. Reprod., 38, 1171–1180.

20) Quinn, P., Barros, C. and Whittingham, D.C. (1982): Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J. Reprod. Fertil., 66, 161–168.

21) Takahashi, Y. and First, N.L. (1992): In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology, 37, 963–978.

22) Holm, P., Booth, P.J., Schmidt, M.H., Greve, T. and Callesen, H. (1999): High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology, 52, 683–700.

23) Emuta, C. and Horiuchi, T. (2001): Effects of timing of activation and aging of bovine oocytes fertilized by intracytoplasmic sperm injection (ICSI) on the cleavage and subsequent embryonic development in-vitro. J. Reprod. Dev., 47, 399–405.

24) Oikawa, T., Takada, N., Kikuchi, T., Numabe, T. and Horiuchi, T. (2005): Evaluation of activation treatments for blastocysts production and birth of viable calves following bovine intracytoplasmic sperm injection. Anim. Reprod. Sci., 86, 187–194.

25) Simerly, C. and Schatten, G. (1993): Techniques for localization of specific molecules on oocytes and embryos. Methods Enzymol., 225, 516–553.

26) Sirard, M.A. and First, N.L. (1988): In vitro inhibition of oocyte nuclear maturation in the bovine. Biol. Reprod., 39, 229–234.

27) Simerly, C., Zoran, S.S., Payne, C., Dominko, T., Sutovsky, P., Navara, C.S., Salisbury, J.L. and Schatten, G. (1999): Biparental inheritance of γ-tubulin during human fertilization: Molecular reconstitution of functional zygotic centrosomes in inseminated human oocytes and cell-free extracts nucleated by human sperm. Mol. Biol. Cell, 10, 2955–2969.

28) Nogueira, D., Albano, C., Adriaenessens, T., Cortvrindt, R., Bourgain, C., Devroey, P. and Smitz, J. (2003): Human oocytes reversibly arrested in prophase I by phosphodiesterase type 3 inhibitor in vitro. Biol. Reprod., 69, 1042–1052.

29) Akaki, Y., Yoshioka, K., Noguchi, M., Hoshi, H. and Funahashi, H. (2009): Successful piglet production in a chemically defined system for in-vitro production of porcine embryos: Dibutyryl cyclic AMP and epidermal growth factor-family peptides support in-vitro maturation of oocytes in the absence of gonadotropins. J. Reprod. Dev., 55, 446–453, 2009.