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Artificial control maturation of porcine oocyte by dibutyryl cyclicAMP

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In this study, we investigated the effects of various durations of dibutyryl cyclic AMP (dbcAMP) treatment on the in vitro maturation (IVM) and subsequent development of parthenogenetically activated embryos. Immature porcine oocytes were cultured with or without 1 mM dbcAMP during the first 20, 28, or 36 h of culture, and then incubated for an additional 24 h without dbcAMP. The expression of *Wee1B*, *Myt*, and *Cdc25B* and the level of maturation promoting factor (MPF) in metaphase II oocytes were analyzed by real-time PCR (qRT-PCR) and enzyme linked immunosorbent assay (ELISA), respectively. The distribution of actin microfilaments in oocytes was also assessed. Subsequently, apoptotic cells in blastocysts from each group were visualized by transferase-mediated dUTP nick-end labeling staining. Results showed that oocytes extruded the first polar body between 12 and 18 h after being released from dbcAMP. MPF activity in oocytes at 28 +24 h and 36+24 h after dbcAMP treatment was higher than that in the control group. Significantly more blastocysts were present among embryos in 28+24 h (54.28% vs. 39.11%, $P < 0.05$) and 36 + 24 h (47.24% vs. 32.94%, $P < 0.05$) groups than among embryos cultured in the absence of dbcAMP. However, the number of total and apoptotic cells was not significantly different between groups. The distribution of actin microfilaments was abnormal in oocytes cultured for 60 h without dbcAMP. In addition, the expression of *Wee1B*, *Myt*, and *Cdc25B* was higher in the control group at 44 h than in the dbcAMP group, but there were no differences in expression at the other time points. In conclusion, dbcAMP treatment delays oocyte maturation and maintains oocyte quality.

Keywords: dbcAMP; oocyte maturation; maturation control

Introduction

The successful application of artificial reproductive technologies (ART) requires oocytes of high quality. After ovulation or in vitro maturation (IVM), mammalian oocytes arrest at metaphase II (MII) until they are activated by penetrating spermatozoa or artificial stimuli. In mammals, the optimal window for fertilization or artificial activation is only a few hours, and oocytes undergo aging if cultured for a prolonged period without fertilization or activation. This results in a decrease in the rate of embryo development, a failure of fertilization, and a decrease in the number of cloned offsprings. In actual production process, maximum number of healthy embryos that could be produced everyday was limited by operation durations in the lab. To obtain more embryos, it must consume myriad time which is directly caused aging and results in decrease of embryos quality. A high percentage of aged porcine oocytes exhibit abnormal morphology or fragmentation and spontaneously parthenogenesis (Kim et al. 1996; Gable & Woods 2001), or disruption of the cytoskeleton (Suzuki et al. 2002). However, the mechanisms involved in oocyte aging are still not clear.

Oocyte aging is thought to be caused by changes in the activity of important factors such as maturation promoting factor (MPF) and mitogen-activated protein kinase. MPF is the most important index for the maturation of oocytes. Other factors such as calcium (Yoon & Fissore 2007) and reactive oxygen species (Goud et al. 2008), which directly affect MPF activity, may also contribute to oocyte aging. Recently, many compounds have been reported to prevent abnormalities in oocytes by preventing the decrease in MPF activity. For example, caffeine-induced dephosphorylation of the catalytic subunit of MPF, p34cdc2, elevates MPF activity and improves the development of somatic cell nuclear transfer embryos. MG132 also maintains MPF activity by inhibiting its degradation, which prevents oocyte aging and enhances embryo development.

During meiotic resumption, MPF acts as a lamin kinase, phosphorylating lamins on phosphorylation sites and causing nuclear lamina disassembly (Peter et al. 1990). The disassembly of the nuclear lamina, in turn, is a prerequisite for nuclear envelope breakdown (Gerace & Burke 1988). After meiotic resumption, gradually accumulated cyclin levels are converted into the sharp activation of MPF. Greatwall kinase requires activation by MPF. Once it is turned on, it phosphorylates Ensa, which switches off PP2A-B55δ. This is essential to protect MPF substrates from dephosphorylation. It simultaneously influences the auto-regulatory loop enhancing MPF activity by protectin Cdc25 and Wee1/Myt1 from dephosphorylation. At the end of maturation, MPF activity peaks and then decreases gradually. Because oocyte aging only occurs after maturation, the control of MPF activity, which is

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essential for germinal vesicle breakdown (GVBD), may help to control oocyte maturation and provide a source of healthy oocytes for subsequent experiments.

Many compounds inhibit oocytes at the germinal vesicle (GV) stage reversibly (Schultz et al. 1983; Kubelka et al. 2000; Ma et al. 2003). For example, dbcAMP, an analog of cyclicAMP (cAMP), is an important regulator of meiosis in oocytes. Previously, treatment of prepubertal porcine oocytes for 20–22 h with dbcAMP has been shown to increase the synchrony of meiotic progression and the incidence of blastocyst formation following in vitro fertilization (IVF) (Funahashi et al. 1997). In another study, exposure of prepubertal porcine oocytes to dbcAMP for the first 22 h of IVM was also found to increase the incidence of monospermic fertilization (Mattioli et al. 1994a). It is speculated that treatment of prepubertal oocytes with dbcAMP during IVM increases the cAMP level (Bagg et al. 2006), which correlates with a slower meiotic progression during IVM and an improvement in cytoplasm maturation and developmental competence. Although it has been demonstrated that exposure of oocytes to dbcAMP for 20–22 h increases development, it is not known whether dbcAMP controls maturation and MPF activity after maturation.

In this study, we investigated whether dbcAMP controls maturation by quantifying MPF activity. We also assessed oocyte quality after maturation by examining the distribution of β-actin, developmental competence, and apoptosis after parthenogenetic activation.

Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

Collection of porcine oocytes, IVM, and embryo culture

Ovaries from prepubertal gilts were collected from a local slaughterhouse. Follicles that were 3–6 mm in diameter were aspirated, and cumulus oocyte complexes (COCs) that were surrounded by a minimum of three cumulus cells were selected for analysis. COCs were washed three times in Tyrode's albumin lactate pyruvat-HEPES (TL-HEPES) supplemented with 0.1% polyvinyl alcohol (PVA) (v/v). Before being transferred to IVM medium, COCs were washed three times in TCM-199 supplemented with 0.1 g/L sodium pyruvate, 0.6 mM L-cysteine, 10 ng/mL epidermal growth factor, 10% porcine follical fluid (v/v), 10 IU/mL luteinizing hormone (LH), and 10 IU/mL follicle-stimulating hormone (FSH). Groups of 25 COCs were transferred to 100 μL droplets of IVM medium with or without 1 mM dbcAMP and incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 (v/v) in air for different durations based on the experimental design. Following treatment, COCs were cultured in fresh medium for an additional 24 h and then cumulus cells were removed by pipetting in the presence of 0.1% hyaluronidase (w/v) for 2–3 min. The cumulus-free, MII-arrested oocytes were activated for parthenogenesis with 5 μM Ca^{2+} ionophore for 5 min. After 3 h of culture in porcine zygote medium-5 (PZM-5) containing 7.5 μg/mL cytochalasin B, porcine embryos were washed three times in PZM-5 containing 0.4% bovine serum albumin (BSA) (w/v) and then transferred into fresh medium under mineral oil for 7 days at 38.5 °C in a humidified atmosphere of 5% CO_2 (v/v) in air. During culture, the percentage of 2- and 4-cell embryos and blastocysts was determined.

Evaluation of nuclear dynamics in oocytes

To investigate the inhibition of GVBD, COCs were treated with dbcAMP, denuded with 0.1% hyaluronidase, stained with 10 μg/mL Hoechst,33,342, and mounted underneath a coverslip. Oocytes were examined by fluorescence microscopy at 400× magnification and meiotic stages were classified based on previously described criteria (Motlik & Fulka 1976). GVX to GVIV were classified as GV.

Transferase-mediated dUTP nick-end labeling (TUNEL) assay

Approximately ten blastocysts were washed three times in Dulbecco's phosphate-buffered saline (dPBS) (pH 7.4) containing 1 mg/mL PVA and fixed in 3.7% paraformaldehyde (w/v) in dPBS/PVA for 1 h at room temperature. After fixation, the embryos were washed with dPBS/PVA and permeabilized with 0.5% Triton X-100 (v/v) for 1 h at room temperature. The embryos were then washed twice with dPBS/PVA and incubated in fluorescein-conjugated Deoxyuridine Triphosphate (dUTP) and terminal deoxynucleotidyl transferase enzyme (in situ cell death detection kit, Roche, Mannheim, Germany) in the dark for 1 h at 37 °C. The embryos were counterstained with 50 μg/mL RNase A in 40 μg/mL propidium iodide for 1 h at 37 °C to label nuclei, washed in PBS/PVA, mounted underneath a coverslip, and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

In vitro p34cdc2 kinase activity assay

p34cdc2 kinase activity was quantified with the Mesacup cdc2 kinase assay (Cat. no. 5234; MBL, Nagoya, Japan) according to the method described by Shoujo et al. (2000). With this method, the correlation coefficient between p34cdc2 kinase activity (as determined by the cdc2 kinase assay) and histone H1 kinase activity (as measured by radioactive detection) can be as high as 0.9961.

Briefly, 5 μL of oocyte extract (containing 20 oocytes) was mixed with 45 μL of kinase assay buffer (25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) [pH 7.5] containing 10 mM $MgCl_2$, 10% MV peptide solution [w/v; SLYSSPGGAYC, MBL], and 0.1 mM adenosine triphosphate [ATP]). The mixture was incubated for 30 min at 30 °C. The reaction was terminated with 200 μL phosphate-buffered saline (PBS; pH 7.5) containing 50 mM ethylene glycol tetraacetic acid (EGTA). Phosphorylation of MV peptides was detected by an enzyme linked immunosorbent assay (ELISA). Values were expressed as optical densities (OD). This experiment was repeated three times.

Immunofluorescence and scanning laser confocal microscopy

Oocytes were denuded of cumulus cells and fixed in 4% paraformaldehyde (w/v) for 30 min at room temperature. Oocytes were blocked and permeabilized in PBS (pH 7.5) containing 1% Trixon-100 (v/v) and 3% BSA (w/v) at 37 °C for at least 1 h. Oocytes were incubated with a β-actin-Rhodamine antibody (1:100 dilution) for 1 h at 37 °C, followed by three washes in PVA–PBS. Oocytes were stained with 5 μM Hoechst,33,342 for 5 min, washed three times in PVA–PBS, and mounted underneath a coverslip. Microscopy was performed using a laser-scanning confocal microscope with a 63× oil immersion objective (Zeiss LSM 710 META, Germany). Images were processed with Zen software (Carl Zeiss).

Real-time PCR (qRT-PCR) assay

Approximately 50 MII oocytes were washed in Ca^{2+}- and Mg^{2+}-free PBS (pH 7.5), followed by mRNA extraction using a Dynabeads mRNA direct kit (Dynal Biotech Asa, Oslo, Norway) according to the manufacturer's instructions. The synthesis of cDNAs was performed by reverse transcription of RNAs using an oligo $(dT)_{12–18}$ primer and SuperScript reverse transcriptase. The expression of *Wee1B* (forward: 5′-GGGTGACAATGGTGACAACAAA-3′ and reverse: 5′-AAGACGGGAAGTAGGAGGACGA-3′), *Myt* (forward: 5′-TGGGATGACGACAGC ATAGGG-3′ and reverse: 5′-AGGTTCCGAGGCTCAAAGG-3′), and *Cdc25B* (forward: 5′-ACTACCCTCACCCAGACCA-3′ and reverse: 5′-GCGTCGGATAGCAAACTG-3′) was then detected by real-time PCR with specific primer pairs and a Dynamo SYBR qPCR kit. The PCR reaction was performed according to the manufacturer's instructions. The threshold cycle value represented the cycle number at which the fluorescence level of the sample was significantly above background levels. The cycling conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of amplification and quantification at 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s. *Gapdh* was used as an internal standard.

Statistical analysis

All data were analyzed using SPSS (version 11.0, USA) Differences in the percentages of oocytes developing to a particular stage were analyzed by analysis of variance (ANOVA) and differences between treatment groups were

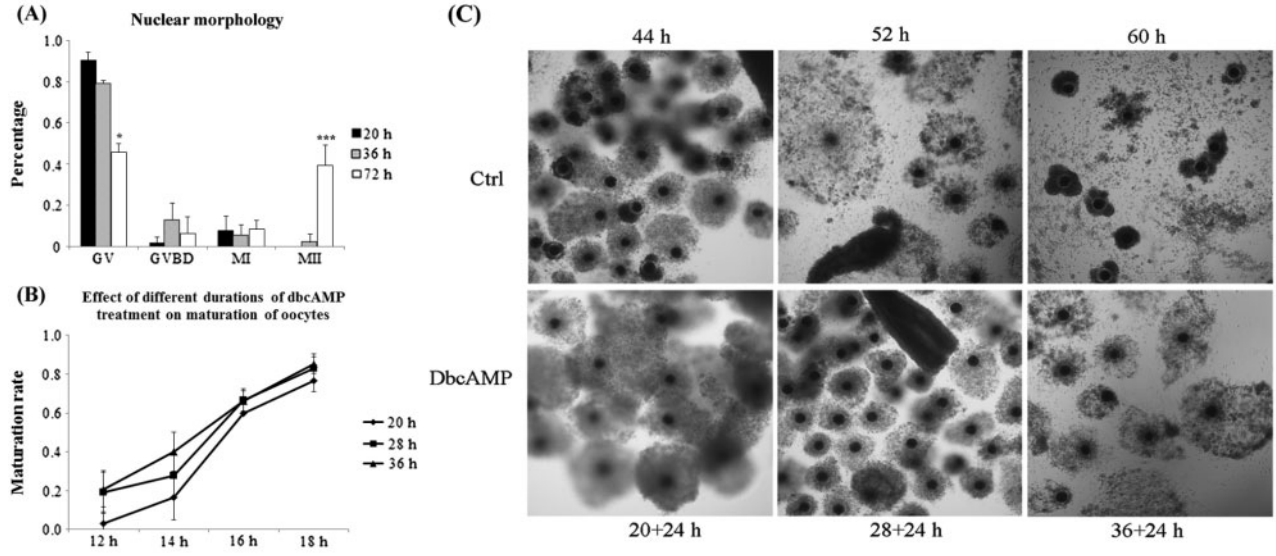

Figure 1. Maturation of porcine COCs after dbcAMP treatment. (A) Ability of dbcAMP to inhibit GVBD. Oocytes were treated with dbcAMP for 20, 36, or 72 h. Nuclear dynamics were assessed microscopically, and nuclei were classified as GV, GVBD, MI, and MII. (B) Maturation profile of oocytes treated with dbcAMP for different periods of time. Oocytes were treated with dbcAMP for 20, 28, or 36 h, followed by 12, 14, 16, or 18 h of culture in the absence of dbcAMP. (C) Images of expanded cumulus cells in cultured COCs at different maturation times in the presence or absence of dbcAMP. COCs were treated with dbcAMP for 20, 28, or 36 h, followed by incubation for an additional 24 h. In the control group, COCs were cultured for 44, 52, or 60 h. Ctrl, untreated oocytes at different times; dbcAMP, dbcAMP-treated oocytes.

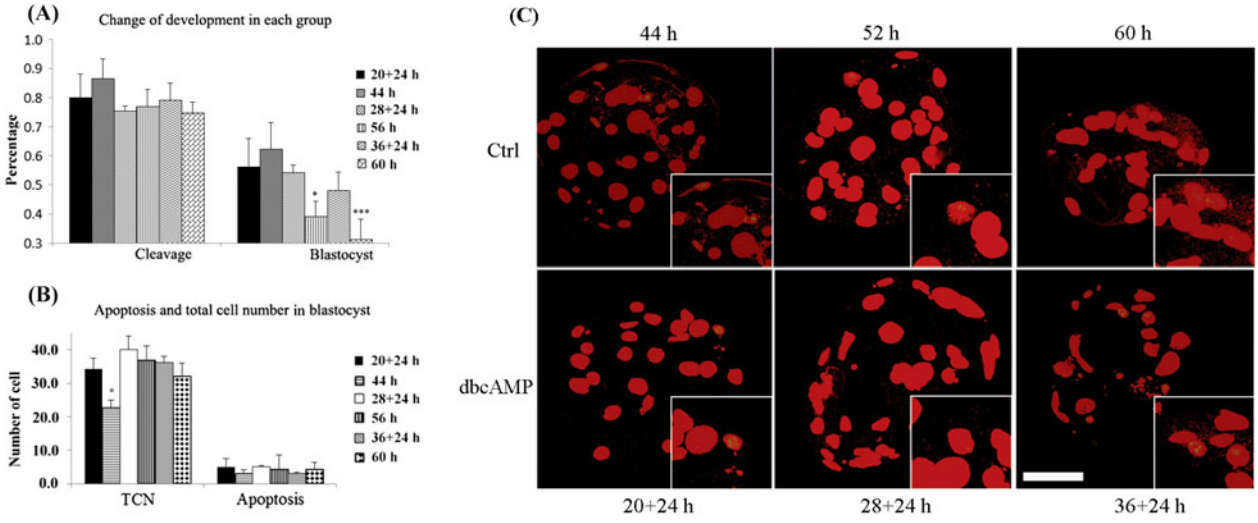

Figure 2. Embryo development after dbcAMP treatment. (A) Development of dbcAMP-treated oocytes after IVM for different periods of time (20+24 h, 28+24 h, and 36+24 h). Oocytes were parthenogenetically activated, and development rate was assessed. "*", significantly different compared with 44 h group ($P < 0.05$), "***", significantly different compared with 44 h group ($P < 0.001$). (B) and (C) The number of total and apoptotic cells in porcine blastocysts developed in vitro. Chromatin content was determined by propidium iodide (red) staining; fragmented DNA was labeled by the TUNEL reaction (green). The colocalization of red and green signals yielded a yellow signal. Scale bar = 50 μm.

evaluated with Duncan's multiple comparison test. A paired Student's *t*-test was used to compare relative gene expression levels. $P < 0.05$ was considered significant.

Experimental design

Experiment 1:The effect of dbcAMP on the maturation of porcine oocytes. To investigate the inhibition of GVBD by dbcAMP, immature oocytes were treated with 1 mM dbcAMP for 0, 20, 36, and 72 h. Nuclear dynamics were assessed after treatment.

As dbcAMP maintained oocytes at the GV stage for at least 36 h, oocytes were treated with 1 mM dbcAMP for 0, 20, 28, and 36 h, and then cultured in the absence of dbcAMP for an additional 24 h. The rate of maturation was assessed every 2 h onwards of 12 h.

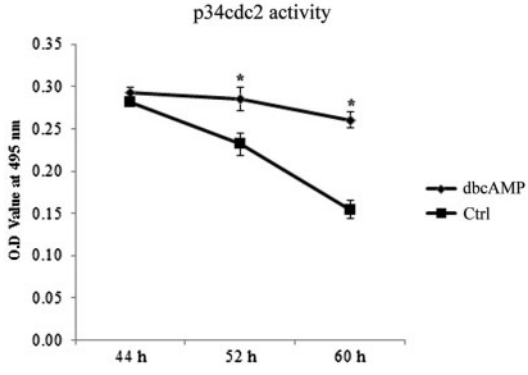

Figure 3. MPF activity in MII oocytes. MPF was isolated from oocytes and used to phosphorylate the MV peptide, a specific MPF substrate. Phosphorylated MV peptide was quantified by ELISA, which reflected MPF activity. Data are expressed as the percentage ± SEM of three independent replicates of three experiments. *, significantly different among dbcAMP treatment and nontreatment group at same time point ($P < 0.05$).

Experiment 2: The effect of dbcAMP on the development of parthenogenetic embryos cultured in vitro. As dbcAMP delayed the maturation of oocytes, developmental rate and blastocyst quality were assessed after parthenogenetic activation.

Experiment 3: The effect of dbcAMP on MPF content in MII oocytes. Oocyte maturation was delayed by dbcAMP. Thus, MPF content, an indicator of blastocyst quality, was quantified after maturation.

Experiment 4: The effect of the duration of dbcAMP treatment on the expression of Wee1B, Myt, *and* Cdc25B *in oocytes*. To determine why MPF content was high in oocytes after dbcAMP treatment, the expression of *Wee1B*, *Myt*, and *Cdc25B* was quantified by qRT-PCR.

Experiment 5: The effect of dbcAMP treatment on the distribution of actin microfilaments in MII oocytes. To determine whether dbcAMP treatment could improve actin morphology, the distribution of actin microfilaments was assessed by immunofluorescence staining.

Results

Effect of dbcAMP on porcine oocyte maturation

COCs were cultured with dbcAMP for 20, 36, and 72 h. By 20–36 h, most oocytes cultured in the presence of dbcAMP were arrested at the GV stage, but only 41.67% of oocytes were arrested at the GV stage after 72 h. Fifty percent of oocytes enucleated the first polar body at 72 h of culture (Figure 1A).

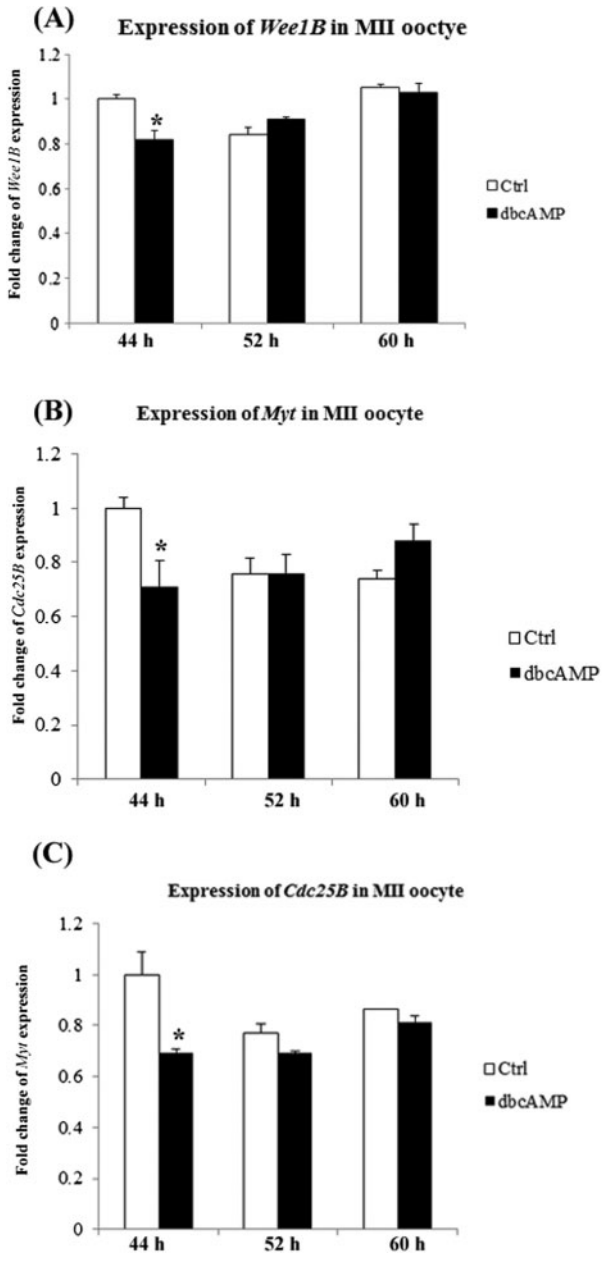

Figure 4. Expression of (A) *Wee1B*, (B) *Myt*, and (C) *Cdc25B* in MII oocytes after treatment with dbcAMP as determined by real-time PCR. Expression was detected in 20+24 h, 28+24 h, and 36+24 h groups and in corresponding control groups (44, 52, and 60 h). *Wee1B*, *Myt*, and *Cdc25B* expression was normalized to the expression of *Gapdh*. Results are from three independent experiments. *, significantly different between dbcAMP treatment and nontreatment group ($P < 0.05$).

Oocytes were cultured in IVM medium with or without 1 mM dbcAMP for 20, 28, or 36 h and then incubated in the absence of dbcAMP for an additional 24 h. After treatment, oocytes in each group displayed a similar maturation profile (Figure 1B). Most of oocytes reached MII stage between 12 and 18 h after dbcAMP treatment. There was also no significant difference in the MII rate at 18 h among each group. The morphology of COCs and oocytes after maturation are shown in Figure 1(C). After 60 h of maturation, the cumulus cells in the control group were darker and displayed an apoptosis manner than those in the dbcAMP group.

Effect of dbcAMP on the development of parthenogenetic embryos cultured in vitro

The development of parthenogenetic embryos was evaluated by examining cleavage and blastocyst formation (Figure 2A). With increasing maturation time, embryo development in the control group decreased significantly (62.32% at 44 h vs. 39.18% at 52 h, $P < 0.05$ and 32.94% at 60 h, $P < 0.001$, respectively). However, there was an increase in blastocyst formation in dbcAMP-treated 28+24 h and 36+24 h groups compared with the control group at corresponding time points (54.28% vs. 39.11% at 52 h and 47.24% vs. 32.94% at 60 h, respectively; $P < 0.05$). The number of total and apoptotic cells in blastocysts in each group is shown in Figure 2(B) and 2(C). There were no significant differences in the number of total and apoptotic cells among groups.

Effect of dbcAMP treatment on MPF content in MII oocytes

We measured the MPF content of 20 oocytes in each group with an ELISA. The results are shown in Figure 3 (values are expressed as OD). While there was no significant difference in MPF content between 20+24 h and 44 h groups (Figure 4), there was a significant increase in 28+24 h and 36+24 h groups compared with the control group (0.2770 vs. 0.2267 at 52 h and 0.2536 vs. 0.1478 at 60 h, respectively; $P < 0.05$). However, there was no significant difference in MPF content among the 20, 28, and 36+24 h groups. Within the control group, MPF content decreased significantly as the length of the culture period increased (28.11 at 44 h vs. 22.67 at 52 h vs. 14.78 at 60 h; $P < 0.05$).

Effect of the duration of dbcAMP treatment on the expression of Wee1B, Myt, *and* Cdc25B

The expression of *Wee1B*, *Myt*, and *Cdc25B* was measured in oocytes after maturation (Figure 4). The pattern of expression was similar across the three genes. At 44 h, the expression of the three genes was higher in the control group than in the dbcAMP group. There were no significant differences in expression at the other time points.

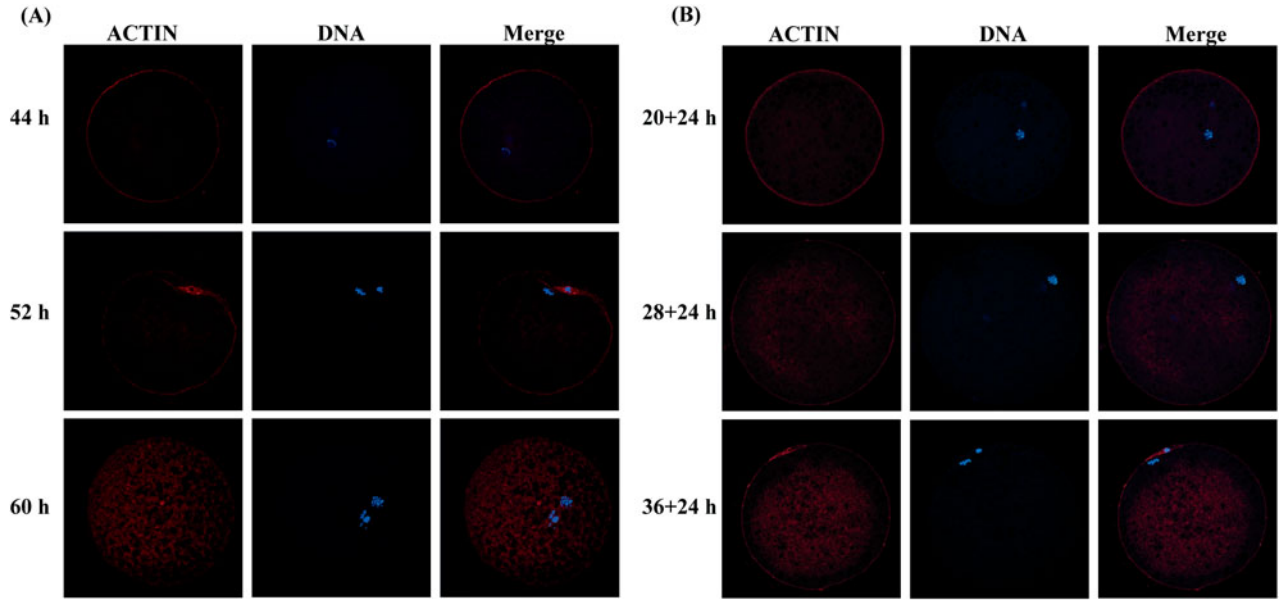

Figure 5. Distribution of actin microfilaments in oocytes in control (A) and dbcAMP (B) groups. Oocytes were stained with a rhodamine-conjugated β-actin antibody (red) and counterstained with Hoechst,33,342 (blue). A signal was detected in 20+24 h, 28+24 h, and 36+24 h groups and in corresponding control groups (44, 52, and 60 h).

Effect of dbcAMP treatment on actin microfilament distribution in MII oocytes

The distribution of actin microfilaments in MII oocytes was assessed after maturation. Actin microfilaments were present at the plasma membrane and formed a shell like structure in oocytes at 44 and 52 h after dbcAMP treatment (Figure 5A). However, actin microfilaments were present in the whole cytoplasm at 60 h. Contrast, at 36+24 h group, strongly and obviously microfilament signal located on he membrane, the mophology as same as that in 44 h. All three dbcAMP groups showed a similar microfilament localization and signal intensity (Figure 5B).

Discussion

The present study is the first report to investigate the effect of dbcAMP on the maturation of porcine oocytes. We report that dbcAMP did not affect oocyte development and MPF activity.

In this study, approximately half of oocytes were arrested at the GV stage, even after exposure to dbcAMP for 72 h. These results illustrate that dbcAMP is a strong inhibitor of GVBD. dbcAMP, an analog of cAMP, is a key regulator of the meiotic cell cycle in oocytes. In the mammalian ovary, oocytes arrest at the initial stage of meiotic division until the release of pre-ovulatory gonadotrophins that stimulate meiotic resumption (Richard & Sirard 1996). In vivo gonadotropins trigger a transient increase in oocyte cAMP content, which subsequently decreases to trigger GVBD (Mattioli et al. 1994b; Petr et al. 1996). A high intracellular level of cAMP maintains oocyte meiotic arrest by activating cAMP-dependent protein kinase (PKA), which in turn maintains MPF, another key regulator of the meiotic cell cycle, in its inactive form (pre-MPF) (Bornslaeger et al. 1986). We show that inhibition of MPF by dbcAMP blocked meiotic resumption, even after exposure for 72 h.

In this study, most oocytes extruded the first polar body between 12 and 18 h after dbcAMP treatment, which indicates that the effects of dbcAMP were nontoxic and reversible. The results were confirmed by parthenogenetic activation, higher blastocyst formation were obtained in dbcAMP group while decreasing blastocyst rate were obtained in nontreatment group. Furthermore, a greater percentage of aged oocytes reached the 2- to 4-cell stage, which may be due to the formation of two pronuclei after activation (Kim et al. 1996). However, few embryos in the age group developed into blastocysts. To understand these results better, MPF activity was measured after maturation. While MPF activity in the control group decreased with time in culture, MPF activity remained high in the dbcAMP group. After release from dbcAMP, PKA is inactivated, followed by the dephosphorylation of pre-MPF by CDC25. Activated MPF turns off PP2A-B55δ and active Cdc25 and promotes pre-MPF activation. The positive feedback loop contributes to the high level of MPF after dbcAMP release. We also demonstrate that the rate of blastocyst development is correlated strongly with MPF activation. Low MPF activity in aging oocytes increases the tendency for spontaneous oocyte activation

(Nagai 1987), subsequent fragmentation, and abnormal cleavage (i.e., unequal blastomeres) (Kikuchi et al. 1995).

WEE1B, MYT, and CDC25B directly regulate the activity of MPF (Oh et al. 2010, 2011). In this study, all three genes were highly expressed in the control group at 44 h compared with the dbcAMP treatment group. There were no significant differences in expression at the other time points. The reason results in the difference are still unclear. Although the expression of these genes was variable at 44 h, corresponding protein levels may be different. Stability of expression showed that there might be a persistent translation to supply sufficient protein for MPF regulator. Alternatively, WEE1B, MYT, and CDC25B may also be controlled by PKA. Additional studies are needed to understand the mechanism of action better.

The distribution of actin microfilaments after dbcAMP treatment in this study was similar to results reported by Kim et al. (1996). After 60 h of maturation, actin microfilaments were present throughout the cytoplasm and not at the plasma membrane. While after dbcAMP treatment, normal actin morphology still be observed even if in 36+24 h group. One of the roles of actin microfilaments is to retain the chromatin at the proper position in the oocyte cortex (Kim et al. 1996). Aging disrupts actin microfilaments such that atypical development results after parthenogenetic activation (Kim et al. 1996). It may also result in a low rate of development as reported in this study.

In conclusion, dbcAMP can regulate porcine oocyte maturation. There were no changes in oocyte development, MPF activity, and the actin cytoskeleton after dbcAMP treatment. The present study provides a new means to prevent oocyte aging and to improve the production of healthy embryos.

Acknowledgments

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