

Removal of cumulus cells around 20 h after the start of *in vitro* maturation improves the meiotic competence of porcine oocytes via reduction in cAMP and cGMP levels

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Abstract. We examined the effect of the timing of removing cumulus cells surrounding porcine oocytes from small follicles (SFs, < 3 mm in diameter) and medium follicles (MFs; 3–6 mm in diameter) on the meiotic and developmental competence of the oocytes. Cumulus-oocyte complexes (COCs) were collected from SFs and MFs, and the oocytes were denuded at 0, 20, and 44 h after the start of *in vitro* maturation (IVM), and the meiotic progression of the oocytes was assessed at the end of the IVM period. The incidence of mature oocytes was significantly affected by both the origin of the COCs and the time when the oocytes were denuded. Although the percentage of mature oocytes was always higher when the COCs were collected from MFs than that when the COCs were collected from SFs, the maturation rate was significantly higher when the oocytes were denuded at 20 h than when they were denuded at 44 h after the start of IVM. When the mature oocytes were activated electrically, the developmental competence of the oocytes denuded at 20 and 44 h to reach the blastocyst stage did not differ, whereas the competence of the MF-derived oocytes was significantly higher than that of SF-derived oocytes. When the intracellular cAMP and cGMP levels in SF-derived oocytes were examined at 24 h of IVM, the levels of both were significantly decreased only in the oocytes denuded at 20 h. In conclusion, denuding oocytes at 20 h of IVM caused a significant reduction in ooplasmic cAMP and cGMP levels and increased the meiotic competence of the oocytes without any reduction in blastocyst formation, even in the case of SF-derived oocytes.

Key words: cAMP, cGMP, Cumulus cells, *In vitro* maturation, Pig

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The flow of the second messenger molecules, guanosine 3',5'-cyclic monophosphate (cGMP) and adenosine 3',5'-cyclic monophosphate (cAMP) from cumulus/granulosa cells via gap junctions is well-known to play important roles in maintaining meiotic arrest and in resuming meiosis in mammalian oocytes [1, 2]. Although high levels of cGMP increase the levels of cAMP by inhibiting phosphodiesterase 3A activity and consequently maintaining oocyte meiotic arrest, the LH surge appears to reduce cGMP levels allowing for the resumption of meiosis [2]. In fact, the presence of dibutyryl cAMP, as well as gonadotropins, during the first 20 h and their absence for 24 h during the following *in vitro* maturation (IVM) period has been shown to induce not only synchronous meiotic progression but also cytoplasmic maturation of porcine oocytes [3]. However, our recent study using the same biphasic IVM protocol demonstrated that intracellular cAMP in the cumulus cell mass was significantly increased by exposing COCs to dibutyryl cAMP and gonadotropins

at the start of IVM and reduced after removing these agents, but that ooplasmic cAMP levels did not drastically change before and just after removing both supplements [4]. Although intracellular cGMP levels in cumulus cells also gradually dropped after the start of the IVM culture, the levels did not change in oocytes [4].

Since the functional gap junctions between cumulus cells and the oocytes may be maintained by a high level of cAMP in cumulus cells, disconnection of this functional communication will reduce intracellular cGMP and cAMP levels in oocytes and induce the resumption of meiosis. Interestingly, it has been reported that although the functional coupling between cumulus cells and the oocyte substantially decreases through the IVM period, the presence of a specific inhibitor of phosphodiesterase 3, cilostamide, maintained this functionality for up to the first 24 h during the IVM period [5]. Several studies have also demonstrated that an interruption of the communication between cumulus cells and the oocyte using gap-junctional inhibitors [6] or by directly denuding oocytes during the IVM period [7] will induce the resumption of meiosis and cause maturation. Recently, we have also demonstrated that removing the cumulus cells from cumulus-oocyte complexes (COCs) which had been exposed to gonadotropins and dibutyryl cAMP for 20 h significantly improved the meiotic competence of the denuded oocytes even when the COCs were collected from small follicles (< 3 mm in diameter; SF) [8], suggesting that intracellular cAMP levels may not drop suddenly

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even after dibutyryl cAMP was removed 20 h after the start of IVM.

For the *in vitro* production of porcine embryos, COCs have usually been collected from medium-sized follicles (3 to 6 mm in diameter; MF), since it is well known that MF-derived oocytes have higher meiotic and developmental competences than those derived from SFs, which are the most abundant in ovaries [9–11]. Although we could improve the meiotic competence of SF-derived oocytes by denuding 20 h after the start of IVM [8], we still do not understand the developmental competence of the denuded oocytes and the changes in cAMP and cGMP levels that occur in oocytes before and after denuding 20 h after the start of IVM.

Thus, the objective of this study was to evaluate the effect of denuding SF-derived oocytes at different times during IVM on their meiotic and developmental competences, compared with the effects of MF-derived oocytes as a positive control. In addition, to ensure that denuding 20 h after the start of IVM affects intracellular cGMP and cAMP levels, we also analyzed the levels of these important second messengers in SF-derived oocytes.

Materials and Methods

Chemicals and media

Sodium chloride, KCl, HCl, NaOH, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, KH_2PO_4 , gentamicin-sulfate and paraffin liquid were obtained from Nacalai Tesque Inc (Kyoto, Japan), whereas $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and mannitol were purchased from Ishizu Pharmaceutical (Osaka, Japan), eCG (Serotropin) and hCG (Gonotropin) were purchased from ASKA Pharmaceutical (Tokyo, Japan). Unless specified, all the other chemicals were purchased from Millipore Sigma (St. Louis, MO, USA).

The medium used for collecting and washing COCs was a modified TL-HEPES-PVA medium [3]. The basic IVM medium used in the current research was a BSA-free chemically defined medium, Porcine Oocyte Medium (POM, Research Institute for the Functional Peptides, Yamagata, Japan) modified by supplementation with 50 μM β -mercaptoethanol (mPOM) [12]; which has been shown to support the successful development to the blastocyst stage of oocytes following IVF [13] and successful piglet production [12].

The media used for the parthenogenesis protocols were an electroporation solution consisting of 0.25 M mannitol, 0.5 mM HEPES, 100 μM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 100 μM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.01% (w/v) polyvinylalcohol (PVA) at a pH of 7.2 [14] and a basic medium, Medium-199 with Earle's salts (Gibco, NY, USA) modified by the addition of 3.05 mM glucose, 2.92 mM hemi-calcium lactate, 0.91 mM sodium pyruvate, 12 mM sorbitol, 75 $\mu\text{g}/\text{ml}$ potassium penicillin G, and 25 $\mu\text{g}/\text{ml}$ gentamicin (mM199) [15].

The medium used for early embryo culture was Porcine Zygote Medium (PZM) [16] which consisted of 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4 , 0.4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25 mM NaHCO_3 , 0.2 mM sodium pyruvate, 2 mM L(+)-lactic acid, 2 mM L-glutamine, 5 mM hypotaurine, 10 $\mu\text{g}/\text{ml}$ gentamicin, 2% (v/v) BME amino acids solution, 1% (v/v) MEM non-essential amino acids solution, and 3% (w/v) PVA.

All the culture media (except for the modified TL-HEPES-PVA and the electroporation solution) were equilibrated under paraffin oil at 39°C in an atmosphere of 5% CO_2 in air overnight prior to

use. TL-HEPES-PVA medium was always used at room temperature and the electroporation solution was pre-warmed overnight at 39°C.

Preparation of COCs

Oocytes were obtained from prepubertal gilts at a local slaughterhouse and brought into the laboratory into a 0.9% (w/v) saline solution supplemented with 75 mg/l potassium penicillin G and 50 mg/l streptomycin-sulfate within 2 h at 25°C [17]. Only ovaries without any corpora lutea on the surface were used for experiments. The cumulus-oocyte complexes were aspirated from the SFs or MFs located on the ovarian surface using an 18-gauge needle attached to a 10 ml disposable syringe and collected separately into a 50 ml centrifuge tubes. After washing three times with modified TL-HEPES-PVA medium at room temperature, only the SF- or MF-derived COCs with uniform ooplasm, and with at least three layers of clear and compact cumulus cells, were selected for the experiments.

Experiment 1: Effect of removing cumulus cells at different times of IVM on the meiotic and developmental competences of the SF- or MF-derived oocytes

COCs derived from SFs or MFs were randomly distributed into groups (30–35 COCs per group) and cultured separately in a 300 μl drop of mPOM supplemented with 1 mM dibutyryl cyclic AMP (dbcAMP), 10 IU/ml eCG and 10 IU/ml hCG under paraffin oil for 20 h at 39°C in an atmosphere of 5% CO_2 in air. Then, the COCs were washed three times in mPOM without dbcAMP and gonadotropins and then cultured in droplets with 300 μl of the same medium for another 24 h period under the same conditions. At 0, 20, and 44 h of IVM, the oocytes were denuded by pipetting with 0.1% (w/v) hyaluronidase. After washing three times in equilibrated mPOM solution, the denuded oocytes continued to be cultured until a total IVM time of 44 h. At the end of the IVM period, the denuded oocytes were observed under a stereomicroscope for the presence of the first polar body (PB) extruding into the perivitelline space, and only mature oocytes with PBs were selected for the parthenogenesis protocol.

Mature oocytes with PBs were washed three times with the electroporation solution (pH 7.2) and then transferred to a drop of the same medium filled between the electrodes of an activation chamber. A single electrical pulse (DC: 1.2 kV/cm, 30 μsec) was given to the oocytes to induce parthenogenetic activation using a BTX Electro-Cell Manipulator 2001M (BTX, San Diego, CA, USA). The stimulated oocytes were then washed three times in mM199 solution supplemented with 0.4% (w/v) BSA and 5 $\mu\text{mol}/\text{l}$ cytochalasin B and were cultured at 39°C in an atmosphere of 5% CO_2 in air for 4 h. After this, the oocytes were washed three times in PZM and cultured in a drop of the same medium covered with paraffin oil under the same conditions for 5 days. Two days after the start of the culture, the cleavage rate was observed and non-cleaved oocytes were removed from the culture media. Five days after the start of the culture, the blastocyst formation rate was observed and the blastocysts were fixed for at least 24 h in 4% PFA at 4°C, washed with PBS solution supplemented with 0.1% (w/v) PVA (PBS-PVA), stained in a Vectashield™ mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope to count the cell number in each blastocyst.

Table 1. Effect of removing cumulus cells during IVM on meiotic and developmental competence of porcine SF- and MF-derived oocytes

	Time (h) when oocytes were denuded	No. of oocytes examined	No. (% ± SEM) # of oocytes matured	No. (% ± SEM)* of oocytes		Mean no. of cells in a blastocyst	Total efficiency (% ± SEM) # to form blastocyst
				cleaved	developed to the blastocyst stage		
MF	0	180	92 (50.8 ± 4.6) ^{Ac}	82 (88.8 ± 2.2) ^A	27 (27.3 ± 5.8) ^{Ab}	32.2 ± 8.4	14.7 ± 3.8 ^{Ab}
	20	183	151 (82.5 ± 4.2) ^{Aa}	139 (91.8 ± 2.4) ^A	69 (44.9 ± 5.6) ^{Aa}	32.8 ± 3.9	37.7 ± 6.2 ^{Aa}
	44	188	143 (76.0 ± 3.4) ^{Ab}	130 (90.9 ± 1.2) ^A	69 (48.5 ± 4.5) ^{Aa}	34.0 ± 2.6	36.7 ± 3.1 ^{Aa}
SF	0	186	70 (37.6 ± 1.8) ^{Bc}	57 (81.7 ± 4.3) ^B	2 (2.7 ± 1.7) ^{Bb}	25.0 ± 1.4	1.1 ± 0.6 ^{Bb}
	20	181	116 (64.1 ± 2.6) ^{Ba}	100 (85.9 ± 4.8) ^B	27 (23.3 ± 4.9) ^{Ba}	31.9 ± 1.7	15.1 ± 3.5 ^{Ba}
	44	182	93 (51.2 ± 1.9) ^{Bb}	74 (79.1 ± 7.0) ^B	24 (25.8 ± 8.0) ^{Ba}	32.0 ± 2.5	13.0 ± 3.9 ^{Ba}
Result of 2-way ANOVA							
Origin of COCs			< 0.001	< 0.05	< 0.001	0.077	< 0.001
Time when oocytes were denuded			< 0.001	0.410	< 0.001	0.169	< 0.001
Interaction			0.225	0.881	0.275	0.392	0.905

^{AB} Different superscript letters indicate significant differences between SF- and MF-derived oocytes independently of the time the oocytes were denuded. ^{abc} Different superscript letters indicate significant differences among SF- or MF-derived oocytes denuded at different times. # Percentage of oocytes examined. * Percentage of oocytes matured. SF, small follicles; MF, medium follicles; COCs, cumulus-oocyte complexes.

Experiment 2: Analyses of intracellular cAMP and cGMP levels of oocytes around the time of cumulus cell removal

Groups of approximately 35 COCs derived from SFs and MFs were cultured separately in a drop of 350 µl of mPOM for a total of 24 h, as described in experiment 1. At 20 h of IVM culture, some of the SF-derived oocytes were denuded (DSF) and then cultured for a further 4 h. At 16 and 24 h after the start of IVM, the intracellular cAMP and cGMP levels in the oocytes were analyzed. Briefly, all the oocytes were denuded and the zona pellucida was removed with 0.1% (w/v) protease in PBS-PVA solution. After washing three times with PBS-PVA, the zona-free oocytes in each group were stored in microtubes with 10 µl of PBS-PVA at -80°C until their later analysis. To measure the levels of cAMP and cGMP, a direct immunoassay kit for cAMP or cGMP (Arbor Assays, Ann Arbor, MI, USA) and an iMark microplate reader (Bio-Rad, Hercules, CA, USA) were used according to the manufacturer's instructions. Briefly, the zona-free oocytes were lysed with 250 µl of sample diluent, and vortexed for 10 min at room temperature. The lysed oocyte samples were centrifuged at 2,200 × g for 10 min at 4°C and 200 µl of the supernatant was treated with 10 µl of acetylated reagent (one part of acetic anhydride with 2 parts of triethylamine) and then used for the assay. After adding 50 µl of a special plate primer to all the wells of a microtiter plate, 50 µl of standard or sample were added to each well. Following this, 25 µl of cAMP or cGMP peroxidase conjugate was added to all the wells and the binding reaction was started by adding 25 µl of the anti-cAMP or anti-cGMP antibodies. After 2 h, the plate wells were washed four times and 100 µl of substrate were added to all the wells and incubated for 30 min at room temperature, followed by the addition of 50 µl of stop solution. Color intensities were measured using a microplate reader at 450 nm. The concentration of cAMP or cGMP per oocyte was calculated using the Microplate Manager 6 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data obtained from at least four replicate experiments were

processed using a two-way (Experiments 1; with data from five replicates) or a one-way ANOVA (Experiment 2; with data from 4–7 replicates) using the IBM SPSS Statistics 21 software for Windows. Findings were considered to be statistically significant if the P value was less than 0.05, and when there was a significant effect, the values were compared with a Tukey's multiple comparisons test. Before performing the statistical analysis, all the percentage data in our experiments were subjected to arc-sine transformation if there were values > 90% or < 10%, and all the data from the immunoassays were square root transformed. All the data were posteriorly transformed back into percentages or means for the Table and Figures and are expressed as means ± SEM.

Results

Experiment 1

As shown in Table 1, the incidence of MF- and SF-derived mature oocytes with PBs were significantly higher when these were denuded at 20 h after the start of IVM (82.5 ± 4.2% and 64.1 ± 2.6%, respectively), as compared with the positive controls which were cultured as COCs through the whole culture period (76.0 ± 3.4% and 51.2 ± 1.9%, respectively). On the other hand, much less MF- and SF-derived oocytes matured when these were denuded before the start of IVM (50.8 ± 4.6% and 37.6 ± 1.8%, respectively). Overall, the maturation rate of SF-derived oocytes was significantly lower than that of MF-derived oocytes.

Two days after parthenogenetic activation, the incidence of oocyte cleavage did not differ between the times when the oocytes were denuded, whereas the percentage of cleaved SF-derived oocytes was significantly lower than that of MF-derived oocytes. Five days after activation, the percentage of MF- and SF-derived oocytes that developed to the blastocyst stage did not differ between when they were denuded at 20 h after the start of IVM (44.9 ± 5.6% and 23.3 ± 4.9%, respectively) and when they were denuded at the end of IVM (48.5 ± 4.5% and 25.8 ± 8.0%, respectively). When the

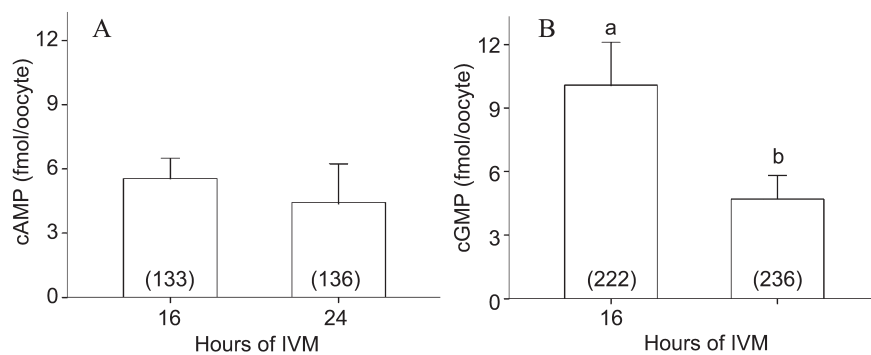


Fig. 1. Intracellular cAMP (A) and cGMP levels (B) in MF-derived oocytes at 16 h and 24 h of IVM. The numbers in parentheses indicate the total number of oocytes assayed in each group. Data are shown as means \pm SEM derived from four (A) and seven (B) replicates. The bars represent cAMP or cGMP levels in oocytes which were cultured as COCs. Bars with different letters indicate a significant difference.

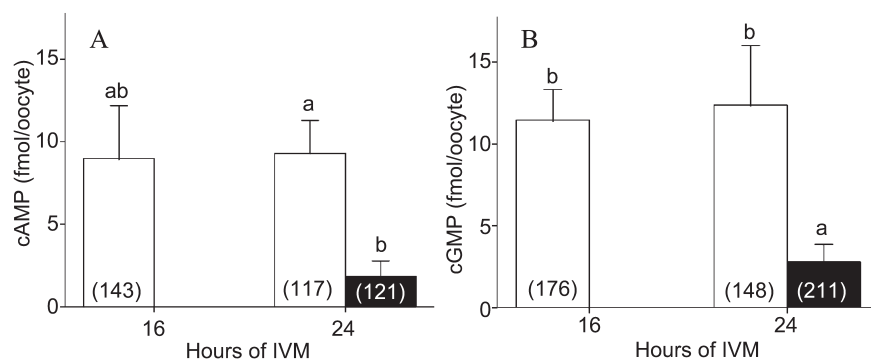


Fig. 2. Intracellular cAMP (A) and cGMP levels (B) of SF-derived oocytes at 16 h and 24 h of IVM. The numbers in parentheses indicate the total number of oocytes assayed in each group. Data are shown as means \pm SEM derived from 4–5 (A) and 6–7 (B) replicates. The white bars represent cAMP or cGMP levels in oocytes which were cultured as COCs. The black bars show the cAMP or cGMP levels in SF-derived oocytes which were denuded 20 h after the start of IVM. Bars with different letters indicate a significant difference.

oocytes were denuded before the start of IVM, the incidences of MF- and SF-derived oocytes that developed to the blastocyst stage ($27.3 \pm 5.8\%$ and $2.7 \pm 1.7\%$, respectively) were significantly lower than those in the other groups. However, there were no significant differences in the number of cells in a blastocyst among any of the different groups. The capacity of SF-derived oocytes to cleave and develop to the blastocyst stage was significantly lower than that of MF-derived oocytes.

Experiment 2

The intracellular cAMP and cGMP levels in MF-derived oocytes at 16 and 24 h of IVM are shown in Fig. 1. There were no significant differences in cAMP levels in MF-derived oocytes between 16 h (when the COCs were exposed to dibutyryl cAMP) and 24 h (4 h after the COCs were transferred to fresh dibutyryl cAMP-free media) after the start of IVM (5.6 ± 1.0 fmol/oocyte and 4.4 ± 1.8 fmol/oocyte, respectively; $P = 0.47$, Fig. 1A), whereas the intracellular cGMP levels in MF-derived oocytes were drastically decreased ($P < 0.05$) after transfer to fresh dibutyryl cAMP-free media (10.1 ± 2.0 fmol/oocyte at 16 h to 4.7 ± 1.1 fmol/oocyte at 24 h after the

start of IVM, Fig. 1B).

The intracellular cAMP and cGMP levels in SF-derived oocytes at 16 and 24 h of IVM are shown in Fig. 2. When the COCs were cultured in the presence of dibutyryl cAMP and gonadotropins for 20 h, and then in the absence of these agents for 4 h, the intracellular cAMP levels of the SF-derived oocytes did not change significantly ($P = 0.946$) between 16 h and 24 h after the start of culture (9.0 ± 3.2 fmol/oocyte and 9.3 ± 2.0 fmol/oocyte, respectively; Fig. 2A). Under the same culture conditions, however, when the SF-derived oocytes were denuded at 20 h, the cAMP levels of the oocytes (1.8 ± 0.9 fmol/oocyte) at 24 h after the start of culture were significantly lower ($P < 0.05$) than the controls cultured as COCs (9.3 ± 2.0 fmol/oocyte; Fig. 2B). As with the cAMP levels, when the COCs were cultured in the presence of dibutyryl cAMP and gonadotropins for 20 h, and then in the absence of these agents for 4 h, the intracellular cGMP levels in the SF-derived oocytes did not change significantly ($P = 0.998$) between 16 and 24 h after the start of culture (11.5 ± 1.9 fmol/oocyte and 12.4 ± 3.7 fmol/oocyte, respectively; Fig. 2B). However, when the SF-derived oocytes were denuded 20 h after the start of culture, the intracellular cGMP levels of the oocytes at 24 h

after the start of the culture (2.8 ± 1.1 fmol/oocyte) were significantly lower ($P < 0.05$) than those in the controls (12.4 ± 3.7 fmol/oocyte) which were cultured as COCs for 24 h (Fig. 2B).

Discussion

In the present study, we demonstrated differences in intracellular cGMP levels in oocytes derived from either MFs or SFs immediately after removing dibutyryl cAMP and gonadotropins from the culture medium. We also found that denuding the oocytes following the culture of COCs in the presence of dibutyryl cAMP for 20 h induced a significant reduction in oocyte intracellular cAMP and cGMP levels, and consequently improved their meiotic competence to reach the metaphase-II stage. In addition, we also showed that the developmental competence of the oocytes was not decreased by this treatment.

Our study confirms previous results [9, 10, 18, 19] that show that MF-derived oocytes have higher meiotic and developmental competences *in vitro* than SF-derived oocytes. The developmental competence of oocytes appears to be acquired during the final process of follicular development through an interaction with their companion somatic cells [20]. We have shown that the number of cumulus cells surrounding an SF-derived oocyte was significantly lower than a MF-derived oocytes and that supplementation of the IVM media with a cumulus cell mass significantly improved both the ooplasmic diameter and the meiotic competence of the oocytes to reach the metaphase-II stage following IVM [21]. We have also suggested that differences in the levels of vascular endothelial growth factor secreted from cumulus cells reflects the meiotic and developmental competences of SF-derived porcine oocytes and supplementation of IVM medium with vascular endothelial growth factor improves their competence [19]. Since it is not well known if the interaction between cumulus cells affects intracellular cAMP and cGMP levels, which are well known to regulate meiotic resumption in oocytes [22], or if the interaction is similar between SF- and MF-derived oocytes during IVM, we examined the effect of denuding oocytes 20 h after the start of IVM in our standard culture system [3].

It is common knowledge that the surrounding cumulus cells supply both cGMP [23] and cAMP [24] to the oocyte via gap junctions, and that cGMP, which inhibits the activity of cyclic nucleotide phosphodiesterase 3A, plays a role in maintaining oocyte meiotic arrest by impeding the degradation of ooplasmic cAMP [25]. In the present study, when the COCs were cultured in the presence of gonadotropins and dibutyryl cAMP for 20 h, and cultured further in the absence of these agents, we found that the intracellular cAMP levels in MF-derived oocytes were not affected between 16 and 24 h after the start of culture, but that the cGMP levels were significantly decreased after removal of the gonadotropins and dibutyryl cAMP. On the other hand, under the same culture conditions, we also observed that both intracellular cAMP and cGMP levels in SF-derived oocytes did not change between 16 and 24 h after the start of culture. These results appear to be consistent with previous studies [4, 26]. It has been reported that gap-junctional communication between surrounding cumulus cells and the oocyte is maintained in porcine COCs until the first 24–32 h after the start of IVM, even after the cumulus cell mass has started to expand around 16 h of IVM [27]. Furthermore, our

current results show that denuding SF-derived oocytes 20 h after the start of culture might cause a significant decrease in both intracellular cAMP and cGMP levels in these oocytes. These results suggest that the interaction between oocyte and the surrounding cumulus cells might still play an active role to keep both the intracellular cAMP and cGMP levels of SF-derived oocytes even around 16 h and 24 h after the start of culture. Furthermore, denuding oocytes at 20 h could be effective at reducing the levels of both cAMP and cGMP in order to accelerate the resumption of meiosis.

Our current results also demonstrate that a reduction in the intracellular cGMP and cAMP levels in SF-derived oocytes by denuding the oocytes significantly improves the maturation rate after IVM culture. This result is consistent with our [7] and other's previous reports using carbenoxolone, a gap-junction inhibitor [25]. The presence of a cumulus cell mass surrounding the oocyte during the latter half of the IVM culture may not be required for meiotic progression to the metaphase-II stage [7]. Therefore, interrupting the communication between the oocyte and the surrounding cumulus cells at the appropriate time, seems to be effective at promoting the resumption of meiosis in oocytes allowing them to reach the metaphase-II stage, probably by allowing a reduction in cAMP levels [28] and consequently activating mitogen-activated protein kinase [29]. Furthermore, our data did not show a significant improvement in the developmental competence of SF-derived oocytes to reach the blastocyst stage by denuding the cumulus cells 20 h after the start of IVM. It has been widely reported that the surrounding cumulus cells provide the oocyte with nutrients and other molecules such as ions or transcripts necessary for achieving good cytoplasmic maturation [30, 31]. If the communication between the oocyte and the surrounding cumulus cells is disconnected at a relatively early period of IVM, the oocytes probably fail to grow and mature due to a lack of these factors [31, 32]. However, our current results clearly demonstrate that interrupting the communication between the oocyte and the surrounding cumulus cells around the mid-stage of IVM did not reduce the developmental competence of SF-derived oocytes. The presence of cumulus cells during the 20 h after the start of IVM may be sufficient for the oocytes to receive a sufficient amount of enabling molecules from the surrounding cumulus cells.

In conclusion, denuding oocytes 20 h after the start of IVM causes a significant reduction in ooplasmic cAMP and cGMP levels and increases the meiotic competence to reach the metaphase-II stage, without any reduction in the developmental competence of the SF-derived oocytes.

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