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Meiotic arrest maintained by cAMP during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes

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Summary

We investigated effects of invasive adenylate cyclase (iAC), 3-isobutyl-1–methylxanthine (IBMX) and dibutyryl cyclic AMP (dbcAMP) on porcine oocyte *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and subsequent embryonic development. Porcine oocytes were collected in Hepes-buffered NCSU-37 supplemented with or without 0.1 μg/ml iAC and 0.5 mM IBMX. IVM was performed in a modified NCSU-37 supplemented with or without 1 mM dbcAMP for 22 h and then without dbcAMP for an additional 24 h. After IVF, oocytes were cultured *in vitro* for 6 days. After 12 h of IVM, no difference in nuclear status was observed irrespective of supplementation with these chemicals during collection and IVM. At 22 h, most (95%) of the oocytes cultured with dbcAMP remained at the germinal vesicle (GV) stage, whereas 44.3% of the oocytes cultured without dbcAMP underwent GV breakdown. At 36 h, oocytes cultured with dbcAMP had progressed to prometaphase I or metaphase I (MI) (32.6% and 49.3%, respectively), whereas non-treated oocytes had progressed further to anaphase I, telophase I or metaphase II (MII) (13.6%, 14.3% and 38.0%, respectively). At 46 h, the rate of matured oocytes at MII was higher in oocytes cultured with dbcAMP (81%) than without dbcAMP (57%), while the proportion of oocytes arrested at MI was lower when cultured with dbcAMP (15%) than without dbcAMP (31%). The rate of monospermic fertilisation was higher when oocytes were cultured with dbcAMP (21%) than without dbcAMP (9%), with no difference in total penetration rates (58% and 52%, respectively). The blastocyst rate was higher in oocytes cultured with dbcAMP (32%) than without dbcAMP (19%). These results suggest that a change in intracellular level of cAMP during oocyte collection does not affect maturational and developmental competence of porcine oocytes and that synchronisation of meiotic maturation using dbcAMP enhances the meiotic potential of oocytes by promoting the MI to MII transition and results in high developmental competence by monospermic fertilisation.

Keywords: cAMP, *In vitro* maturation, Oocyte, Pig

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Introduction

The success of *in vitro* maturation and fertilisation (IVM/IVF) in pigs has been improved in many respects in the last decade, but is still not as good as that in cattle. Besides the problem of polyspermy, insufficient cytoplasmic and nuclear maturation can cause low fertilisation and blastocyst rates. The success of nuclear and cytoplasmic maturation of oocytes is a crucial point of efficiency in IVM/IVF systems.

During oocyte collection and *in vitro* culture, spontaneous maturation can start since germinal vesicle (GV) oocytes resume meiosis spontaneously when removed from the follicle (Pincus & Enzmann, 1935). A large variation in nuclear morphology of GV-stage pig oocytes was found just after collection (Funahashi *et al*., 1997*a*; Nagai *et al*., 1997) and after a certain time of culture (Funahashi *et al*., 1997*a*). It has been shown that there are differences in GV configuration and polypeptide synthesis between oocytes obtained from different-sized antral follicles, causing heterogeneity in overall developmental competence within oocytes collected for IVM/IVF (Mcgaughey *et al*., 1979). Since the source of oocytes may differ (from different individuals and different-sized follicles), the developmental competence of GV-stage pig oocytes used for IVM/IVF can also vary within the population used in one experiment, and therefore some oocytes can start meiosis earlier than others causing heterogeneity in maturation status among the oocytes after IVM. To overcome this phenomenon, synchronisation of nuclear maturation is necessary.

A high level of intercellular cAMP is responsible for activating cAMP-dependent protein kinase (PKA), which controls meiotic arrest of oocytes at the GV stage (Bornslaeger *et al*., 1986; Cameron, 1987). For elevating the level of cAMP within mammalian oocytes, gonadotropins such as FSH and LH acting through follicular cells are responsible (Bornslaeger & Schultz, 1985; Mattioli *et al*., 1994; Shimada *et al*., in press). Resumption of meiosis and germinal vesicle breakdown (GVBD) are associated with an irreversible cascade starting with the reduction in intra-oocyte cAMP that is followed by PKA inactivation and the activation of mitogen-activated protein kinase (Schultz *et al*., 1983; Bornslaeger *et al*., 1986; Sun *et al*., 1999). Spontaneous maturation is supposed to occur by the interruption of metabolism between the follicle components (granulosa cells and/or follicular fluid) and the oocyte in which cAMP is maintained at a high level. Addition of dibutyryl cyclic AMP (dbcAMP), a membrane-permeable cAMP analogue, to IVM medium during the first 20 h of maturation inhibits GVBD and has a uniform effect on the nuclear stage of pig oocytes (Funahashi *et al*., 1997*b*). The use of a combination of invasive adenylate cyclase (iAC) and 3-isobutyl-1-methylxanthine (IBMX) during oocyte collection increased the meiotic and subsequent embryonic developmental competence of IVM/IVF bovine oocytes (Luciano *et al*., 1999), suggesting that changes in the level of intracellular cAMP during collection might affect further meiotic or developmental competence of oocytes.

The objective of the present study was to examine the effect of intracellular cAMP during oocyte collection and *in vitro* culture on nuclear maturation, fertilisation and subsequent embryonic development of porcine oocytes. Maturation media supplemented with or without IBMX and iAC were used for oocyte collection, and the following oocyte maturation culture was performed in the presence or absence of dbcAMP.

Materials and methods

Oocyte collection and *in vitro* maturation

Prepuberal porcine ovaries from cross-bred gilts (Landrace × Large White) were obtained from the local abattoir and transported to the laboratory in Dulbecco's phosphate-buffered saline (PBS) within 2 h at 35 °C. Dissection of follicles 3–6 mm diameter and collection of cumulus–oocyte complexes (COCs) were performed in a collection medium supplemented with or without iAC and IBMX: The basic collection medium (BCM) (used as control) was NCSU37 (Petters & Wells, 1993) supplemented with 50 μM β-mercaptoethanol (M-7522; Sigma Chemical Co., St Louis, MO), 25 mM HEPES, 1 mg/ml polyvinyl alcohol (PVA) (P-8136; Sigma), 100 U/ml penicillin G potassium (Sigma) and 0.1 mg/ml streptomicin sulfate (Sigma). The osmolarity was adjusted to 0.285 osmol/kg, the pH was regulated to 7.3. Complete collection medium (CCM) was BCM supplemented with 0.5 mM IBMX (I-7018; Sigma) and 0.1 μg/ml iAC (635–088;, adenylate cyclase toxin; Alexis Biochemicals, Lausanne, Switzerland). COCs were cultured in a maturation medium, which was modified NCSU-37 containing 10% (v/v) pig follicular fluid (PFF), 50 μM β-mercaptoethanol, 0.6 mM cysteine, 10 IU/ml PMSG (PMS 1000 IU; Nihon Zenyaku Kogyo, Koriyama, Japan), 10 IU/ml hCG (Puberogen 500 unit; Sankyo, Tokyo, Japan) and 1 mM dbcAMP (D-0627; Sigma). Some COCs matured without dbcAMP were used as control. After the first 22 h of maturation, the COCs were transferred into 500 μl maturation medium without any hormonal or dbcAMP supplement and cultured for an additional 24 h. The COCs were cultured in batches of 20–30 in 500 μl of maturation medium in four-well dishes at 39 °C under 5% O_2 (adjusting CO_2 and N_2 to 5% and 90%, respectively).

In vitro fertilisation (IVF) and *in vitro* culture (IVC)

IVF and IVC were carried out as described previously (Kikuchi *et al*., 2002). After 46 h of maturation culture, COCs were transferred into 100 μl droplets of fertilisation medium, which was Pig-FM (Suzuki *et al*., 2002) modified with 2 mM caffeine and 5 mg/ml bovine serum albumin (BSA, Fraction V; Sigma), covered by mineral oil. About 25 oocytes per 100 μl medium were fertilised by epididymal spermatozoa from a Landrace

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boar that had been frozen-thawed (Kikuchi *et al*., 1998) and preincubated (for 15 min; Nagai *et al*., 1988), the final concentration being 1×10^5/ml.

After co-incubation of gametes for 3 h, cumulus cells and attached spermatozoa were removed from the oocytes by pipetting through a fine glass pipette. They were transferred into IVC medium. Two types of IVC medium were prepared (Kikuchi *et al*., 2002). The basic IVC medium was NCSU-37 modified with the addition of 0.4% (w/v) BSA and 50 μM β-mercaptoethanol. IVC-PyrLac (basic IVC medium plus 0.17 mM sodium pyruvate and 2.73 mM sodium lactate) was used from day 0 (the day of IVF was defined as day 0) to day 2, and IVC-Glu (basic medium plus 5.55 mM glucose) was used from day 2 to day 6. IVM-IVF oocytes were cultured at 38.5 °C under 5% O_2.

Statistical analysis

Each treatment of each experiment was replicated at least three times. Statistical analyses of IVM data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.01$) using GLM procedures of the Statistical Analysis System (SAS Institute, Cary, NC).

Data of the IVF and IVC results were analysed by chi-square test ($p < 0.05$). Data are expressed as mean ± SEM.

Experimental design

Experiment 1
To evaluate the effects of IBMX and iAC in collection medium and dbcAMP in maturation medium, respectively, on nuclear progression and oocyte maturation, COCs were collected using BCM and CCM collection media, and cultured *in vitro* with or without 1 mM dbcAMP for 22 h, then cultured in the medium without hormones and dbcAMP supplement. Nuclear progression during IVM was evaluated after fixation at 12, 22, 36 and 46 h of culture. Chromatin condensation (GV stage) in the oocytes was classified according to Motlik & Fulka (1976).

Experiment 2
To study the effect of IBMX and iAC in collection medium and dbcAMP in maturation medium on fertilisation parameters, COCs were matured in the presence or absence of 1 mM dbcAMP, and then fertilised *in vitro*. The inseminated oocytes were fixed at 10 h after the insemination. Only the oocytes with a male pronucleus(ei) and/or decondensed sperm head(s) with corresponding sperm tails were judged as penetrated. Zygotes with one female and one male pronucleus (or decondensed sperm head) and with two polar bodies were classified as normally (monospermic) fertilised oocytes.

Experiment 3
Effect of IBMX and iAC in collection medium and dbcAMP in maturation medium for oocytes on their subsequent embryonic development after IVF. On day 6, all the IVM/IVF embryos were fixed and evaluated for the rate of blastocyst formation and the cell number in each blastocyst.

Results

Experiment 1

No difference in chromatin condensation between oocytes collected and/or matured in the presence or absence of cAMP was observed at 12 h of culture (Fig. 1). In both in the dbcAMP– and dbcAMP+ groups almost all the oocytes remained at the GV stage, where GVII (48.6 ± 5.8% and 47.6 ± 8.0%, respectively) and GVIII (39.3 ± 4.6 and 38.6 ± 5.2%, respectively) were dominant. Only a very few remained at GVI or reached more condensed stages of chromatin (GVIV). The rate of degenerated oocytes was the same between the groups.

Significant differences in nuclear progression of oocytes matured with or without dbcAMP were detected at 22 h of culture (Fig. 2). By this time, GVBD occurred at a high rate (44.3 ± 8.1%) in oocytes that were matured in the absence of dbcAMP; the rest remained at GV stage and 9.6 ± 5.2% of oocytes had already reached metaphase I (MI). The nuclear status of oocytes that were cultured with dbcAMP was GV stage, which is similar to that at 12 h of culture, with an unremarkable rate (1.0 ± 1.0%) of oocytes that

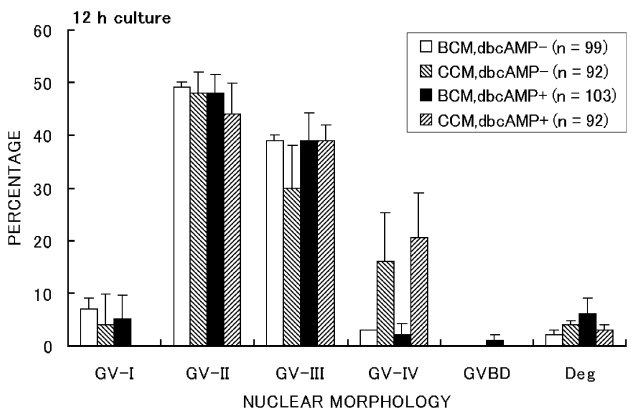

Figure 1 Nuclear morphology (mean ± SEM) of oocytes after 12 h of culture in four different treatments. BCM, basic collection medium; CCM, complete collection medium; dbcAMP–, cumulus–oocyte complexes (COCs) cultured in the absence of 1 mM dbcAMP; dbcAMP+, COCs cultured in the presence of 1 mM dbcAMP. Numbers of oocytes examined in different treatment groups are given in parentheses.

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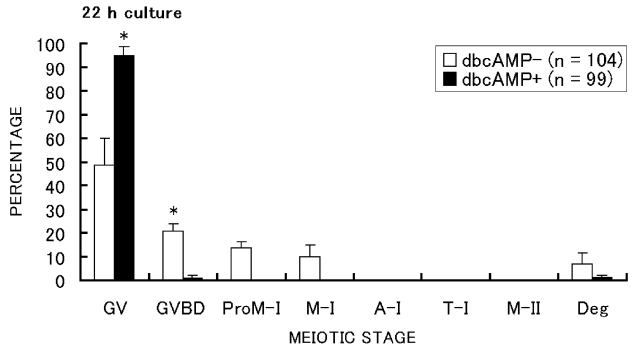

Figure 2 Distribution (mean ± SEM) of meiotic stage of porcine oocytes after 22 h culture with or without 1 mM dbcAMP. An asterisk above the bar indicates a significant difference ($p < 0.01$). Numbers of oocytes examined in different treatment groups are given in parentheses.

underwent GVBD. The rate of degenerated oocytes in this period of culture was the same in the treatment groups. No difference in nuclear stage between oocytes collected with different levels of cAMP was observed at this period (data not shown).

By 36 h of culture, most oocytes had undergone GVBD in both the dbcAMP– and dbcAMP+ groups (89.3 ± 2.4% and 93.6 ± 3.4%, respectively). A considerable proportion (38.0 ± 6.4%) of the oocytes matured in the absence of dbcAMP had reached metaphase II (MII) by this time and a significant proportion were at MI (16.6 ± 5.3%) (Fig. 3). The remaining oocytes that underwent GVBD were at prometaphase I (proMI) (5.0±1%), telophase I (TI) (14.3 ± 4.6%) or anaphase I (AI) (13.6 ± 2.6%). In contrast, in the dbcAMP+ group a significantly higher proportion of oocytes were at MI (49.3±7.3%) or proMI (32.6±2.3) stage but none of the oocytes showed an MII phase nucleus (Fig. 3). No difference in nuclear stage between oocytes collected with different levels of cAMP was observed at this period of culture (data not shown).

By the end of the maturation period (at 46 h of culture), the majority (56.6 ± 7.8%) of the oocytes that were matured without dbcAMP reached MII phase, while a large proportion (30.6 ± 8.4%) of the oocytes remained arrested at MI (Fig. 4). A higher proportion (81.0 ± 6.5%) of oocytes treated with dbcAMP during the first 22 h of culture reached MII phase and a significantly lower proportion (15.0 ± 4.5%) remained at MI phase by the end of culture (Fig. 4). No significant difference in the rate of degenerated oocytes was observed between the dbcAMP– and dbcAMP+ groups by the end of the culture period (10.6 ± 1.6% and 3.0 ± 1.7%, respectively). No difference in nuclear morphology was observed between the oocytes that were collected with different levels of cAMP (data not shown).

Experiment 2

The penetration rate in the control (BCM, dbcAMP–) group was 45.6 ± 7.7% and no difference in penetration rate was observed between the treatment groups (Fig. 5). The rate of monospermic fertilisation was 9.3 ± 1.4% when no iAC in the collection medium and/or dbcAMP in IVM medium was used. A significant increase (20.6 ± 3.0%) in the normal monospermic fertilisation rate was observed when dbcAMP was used during IVM. There was no significant difference in monospermic fertilisation rates between the treatment groups with different concentrations of cAMP in collection medium when dbcAMP was absent (9.3 ± 1.4% and 15.2 ± 1.6%) or present (20.6 ± 3.0% and 11.5 ± 3.6%) in maturation medium. The number of penetrating spermatozoa per oocyte was 2.11 in the control (BCM, dbcAMP–) group, and this value did not change significantly when iAC or dbcAMP was used (2.02 and 2.06, respectively).

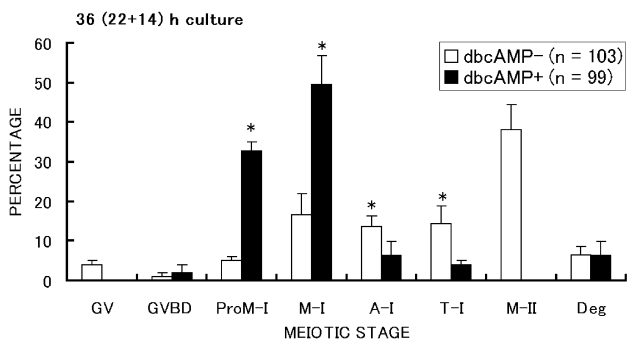

Figure 3 Distribution (mean ± SEM) of meiotic stage of porcine oocytes after an additional 14 h cultivation following 22 h of culture (36 h in total) with or without 1 mM dbcAMP. An asterisk above the bar indicates a significant difference ($p < 0.01$). Numbers of oocytes examined in different treatment groups are given in parentheses.

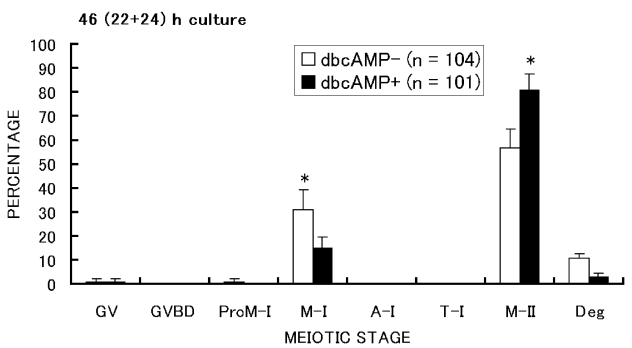

Figure 4 Distribution (mean ± SEM) of meiotic stage of porcine oocytes after an additional 24 h cultivation following 22 h of culture (46 h in total) with or without 1 mM dbcAMP. An asterisk above the bar indicates a significant difference ($p < 0.01$). Numbers of oocytes examined in different treatment groups are given in parentheses.

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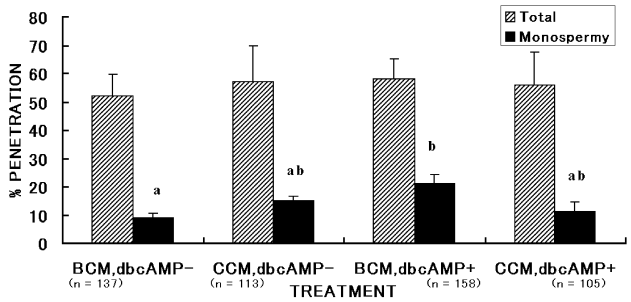

Figure 5 Fertilisation results 10 h after IVF of oocytes obtained in different collection media and cultured in the absence or presence of 1 mM dbcAMP for the first 22 h of the total 46 h maturation period. BCM, basic collection medium; CCM, complete collection medium; dbcAMP–, cumulus–oocyte complexes (COCs) cultured in the absence of 1 mM dbcAMP; dbcAMP+, COCs cultured in the presence of 1 mM dbcAMP. Data are presented as mean ± SEM. Different letters above the bars represent significant differences ($p < 0.05$). Numbers of oocytes examined in different treatment groups are given in parentheses.

Experiment 3

The blastocyst rate of the oocytes collected with BCM and matured in the presence of dbcAMP was significantly higher than that of oocytes collected with BCM and matured without dbcAMP (32.1 ± 5.7% and 20.6 ± 2.9%, respectively) (Fig. 6). The supplementation of collection medium with iAC and IBMX resulted in no difference in the rate of blastocysts (25.7 ± 8.4%). The combination of iAC in collection medium and dbcAMP in maturation medium did not cause a significant change in the blastocyst rate (26.7 ± 6.8%). There was no significant difference in blastocyst quality relative to the number of cells in blastocysts obtained by the different oocyte collection and maturation methods (Fig. 7).

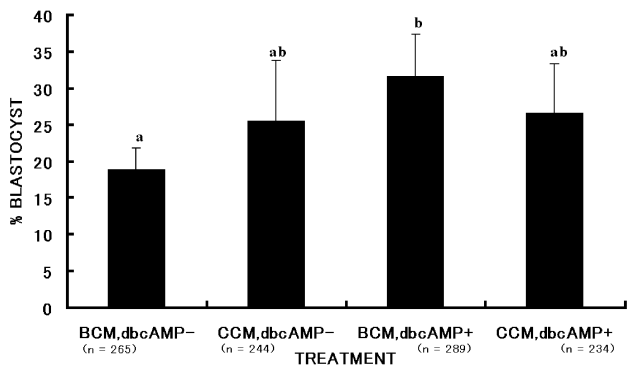

Figure 6 *In vitro* developmental rates to blastocyst stage of porcine oocytes obtained in different collection media and cultured in the absence or presence of 1 mM dbcAMP for the first 22 h of the total 46 h maturation period. BCM, basic collection medium; CCM, complete collection medium; dbcAMP–, COCs cultured in the absence of 1 mM dbcAMP; dbcAMP+, COCs cultured in the presence of 1 mM dbcAMP. Data are presented as mean ± SEM. Different letters above the bars represent significant differences ($p < 0.05$). Numbers of oocytes examined in different treatment groups are given in parentheses.

Discussion

The present results confirm, also in the swine, that intercellular cAMP can cause meiotic arrest of mammalian oocytes at GV stage. To elevate the intercellular cAMP level artificially, we used the phosphodiesterase inhibitors IBMX and iAC. Recently, the use of 0.5 mM IBMX was reported to arrest GVBD in porcine oocytes without any negative effect on the formation of LH receptors (Shimada *et al.*, in press). iAC is an is an enzyme purified from the bacterium *Bordetella pertussis* (Wolff *et al.*, 1980). It enters and elevates the intercellular cAMP content of mammalian cells effectively (Confer *et al.*, 1984). The successful use of iAC dialysed urea extract to elevate the cAMP level in rat oocytes was reported (Aberdam *et al.*, 1987). It has also been demonstrated that iAC can inhibit meiosis of both cumulus-enclosed and cumulus-free bovine oocytes in a dose-dependent manner through accumulating intercellular cAMP and without decreasing the developmental competence (Aktas *et al.*, 1995). The successful use of a combination of iAC and IBMX to enhance developmental competence of bovine oocytes was reported when these chemicals were added to oocyte collection medium (Luciano *et al.*, 1999), suggesting that the intracellular level of cAMP during collection might also affect the further developmental competence of oocytes. In the present study in swine,

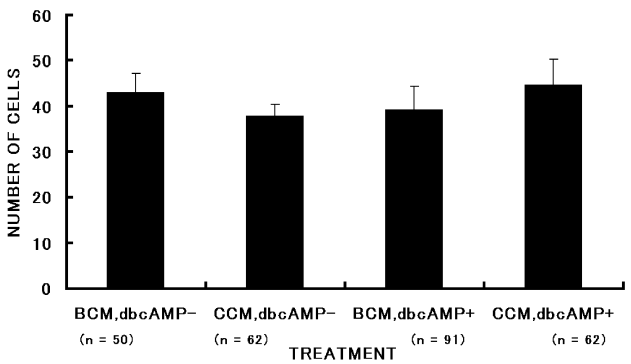

Figure 7 Number of cells in blastocysts after *in vitro* culture of IVF oocytes obtained by the different oocyte collection and maturation methods. BCM, basic collection medium; CCM, complete collection medium; dbcAMP–, COCs cultured in the absence of 1 mM dbcAMP; dbcAMP+, COCs cultured in the presence of 1 mM dbcAMP. Data are presented as mean ± SEM. Numbers of oocytes examined in different treatment groups are given in parentheses.

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however, the chromatin condensation, nuclear maturation and further developmental competence of oocytes to the blastocyst stage did not differ when collection media supplemented with or without IBMX and iAC were used regardless of the usage of dbcAMP during IVM (Fig. 1). This result indicates that a decreased concentration of cAMP in the medium during oocyte collection did not affect maturation of porcine oocytes and subsequent development after IVF; consequently, the initiation of spontaneous maturation might occur during the incubation of the oocytes in our IVM system. Otherwise a difference in nuclear status between the oocytes collected with or without cAMP supplement would have been detected after 12 h of incubation.

During the initial stage of maturation *in vivo*, LH elevates intercellular cAMP in porcine oocytes (Mattioli *et al*., 1994) and similar phenomenon occurs *in vitro* if COCs are exposed to FSH prior to LH (Shimada *et al*., in press). Since FSH and LH act positively upon IVM of porcine oocytes (Mattioli *et al*., 1991), a transient meiotic arrest maintained by cAMP seems to be beneficial for normal maturation of oocytes. The fact that LH enhances IVM in pigs more effectively when oocytes are exposed to this hormone only during the first 20 h of maturation culture (Funahashi *et al*., 1994) suggests that the timing of meiotic arrest must be crucial and should be maintained during the first half of maturation. Moreover, dbcAMP-maintained meiotic arrest during the first 20 h of IVM of porcine oocytes successfully synchronised nuclear maturation and resulted in an increased rate of blastocyst formation after IVF but without affecting the final rate of matured oocytes and the rate of monospermic fertilisation (Funahashi *et al*., 1997*b*). However, dbcAMP and forskolin are known to increase the GVBD rate of *in vitro* matured mouse oocytes through stimulating cumulus cells to secrete a diffusible meiosis-inducing substrate (Guoliang *et al*., 1994) and similar results were reported when porcine oocytes were exposed to FSH or forskolin (Xia *et al*., 2000). When we used 1 mM dbcAMP to synchronise nuclear maturation of oocytes, no difference was observed in the nuclear morphology of oocytes after culture for 12 h irrespective of dbcAMP supplementation. This result suggests a certain synchronisation of nuclear maturation of oocytes that were cultured in the absence of dbcAMP. A possible reason of this phenomenon might be that the gonadotropic hormones PMSG and hCG were present in the maturation medium of both the dbcAMP-treated and control oocytes. Gonadotropic hormones are known to elevate the intracellular cAMP level of mammalian oocytes (Bornslaeger & Schultz, 1985; Mattioli *et al*., 1994; Shimada *et al*., in press).

The present results suggest clearly that synchronisation of meiosis with dbcAMP during the first 22 h of maturation results in a higher maturation rate and an increased proportion of monospermic zygotes (in other words, reduced polyspermy) after IVF. In parallel with the result of Funahashi *et al*. (1997*b*), we also found a more synchronised GVBD with the use of dbcAMP while the nuclear status of oocytes cultured without dbcAMP showed heterogeneity during IVM, and no difference in GVBD rate was observed between the control and the dbcAMP-treated groups. We suggest that dbcAMP seems to manifest its beneficial effect on meiotic competence during the MI to MII transition, since a higher rate of MII oocytes was observed when dbcAMP was added during the first 22 h of IVM and without this drug, more oocytes remained at MI phase by the end of culture (Fig. 4). This outcome is in accordance with the results of Shimada & Terada (2002) who found that cAMP plays an important role in the regulation of meiotic progression beyond the MI stage in porcine oocytes. On the other hand, we had a higher rate of monospermic fertilisation when the meiotic process was synchronised by an elevated level of cAMP (Fig. 5). The reason for this outcome is unclear at present; however, there could be possibilities as follows: There seems to be a difference in the diversity of nuclear status and cytoplasmic maturity between oocytes treated with or without dbcAMP. Distribution of cortical granules (CGs) in porcine oocytes during IVM is in tune with nuclear maturation (Wang *et al*., 1997), suggesting a relationship between nuclear and cytoplasmic maturation. Moreover it was shown that cytoplasmic maturation changes (e.g. CG distribution) occur in accordance with meiotic resumption (Sun *et al*., 2001). According to this phenomenon, a higher rate of fully mature oocytes might manifest in a higher percentage of normal fertilisation, while without synchronisation of meiosis a more heterogeneous population might be obtained including immature and aged oocytes, thus resulting in a higher rate of abnormal fertilisation such as polyspermy. Asynchronous meiotic maturation of porcine oocytes cultured *in vitro* is known to occur, resulting in a considerable population of aged oocytes that are susceptible to polyspermic fertilisation (Grupen *et al*., 1997). After maturation for 36 h, in the present study, a high incidence of nuclear maturation was observed among the oocytes that were cultured without dbcAMP, a remarkable proportion (38 ± 6.42%) having already finished the meiotic process. These oocytes might have been aged after culture for 46 h at the time of IVF, resulting in heterogeneity in cytoplasmic maturation among oocytes at MII phase. Contrary to this, oocytes after a certain period of meiosis synchronisation showed more homogeneity in terms of nuclear maturation and presumably cytoplasmic maturation as well, which could result in a lower rate of polyspermy.

Other possible ways that dbcAMP could affect monospermic fertilisation should also be considered. Increasing the intercellular cAMP content in COCs

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might affect oocyte cytoplasmic maturation directly as well, by enhancing metabolism between the cumulus cells and the oocytes. Flagg-Newton *et al.* (1981) reported that mammalian cells exposed to cAMP and dbcAMP show increased junctional permeability. FSH and dbcAMP are known to elongate the period of coupling and metabolic cooperation between cumulus cells and oocyte during *in vitro* culture in mice (Salustri & Siracusa, 1983). It has also been suggested that a certain level of intercellular cAMP might affect developmental competence of bovine oocytes through enhancing communication between the oocyte and cumulus cells (Modina *et al.*, 2001). Increased permeability of gap junctions between cumulus cells and oocyte might promote normal fertilisation since cumulus cells are known to support the oocyte with an unknown factor(s) that is (are) necessary for normal cytoplasmic maturation, fertilisation and further embryonic development. Moreover, a direct effect of cAMP on the block to polyspermy is also conceivable. dbcAMP was found to stimulate the activity of tissue-type plasminogen activator (tPA) in porcine COCs during *in vitro* culture (Kim & Menino, 1995). tPA is synthesised in mouse and rat oocytes during meiosis (Haurte *et al.*, 1985) and released during activation of rat oocytes, suggesting a possible role in the zona reaction (Zhang *et al.*, 1992), which may affect the block to polyspermy in pigs. Taking these factors into consideration, we suggest that the elevated rate of blastocyst formation might have been caused by the higher rate of monospermic fertilisation after meiotic synchronisation of oocytes with dbcAMP. Funahashi *et al.* (1997), however, found a higher rate of blastocyst formation following IVF of dbcAMP-treated oocytes but without any effect on monospermic fertilisation. In parallel with those results, an increased level of intercellular cAMP in porcine COCs by LH did not affect the IVF rate and monospermic fertilisation but resulted in an improved developmental competence of oocytes to the blastocyst stage after IVF (Shimada *et al.*, in press). These results are discrepant and further experiments are needed to clarify the relationship between monospermy caused by cAMP treatment and subsequent development to the blastocyst stage.

In the present study we inseminated COCs instead of denuded oocytes in order to achieve better fertilisation results. There might have been a certain proportion of oocytes arrested at MI stage among the fertilised oocytes according to the nuclear status of each treatment group, and this fact might have affected the blastocyst rates. On the basis of the MII rates at the end of IVM, and presuming that MI-arrested oocytes cannot a form blastocyst, we re-calculated the blastocyst rates. It was found that the blastocyst rate of dbcAMP-treated oocytes is still significantly higher than that of the control group (43.4% and 33.0%, respectively). However, porcine oocytes arrested at MI stage can be activated (Kikuchi *et al.*, 1999) and seem to be able to develop to the blastocyst stage following IVF (Somfai *et al.*, unpublished observation).

An increased meiotic potential and developmental competence of dbcAMP-treated oocytes is reported in this study. Porcine oocytes with a lower meiotic and/or developmental competence can upgrade their meiotic and developmental ability due to the elongated period of metabolism between the cumulus cells and the oocyte through the maintenace of meiotic arrest at GV stage by dbcAMP treatment.

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