Effect of Treatment of *In Vitro* Matured Pig Oocytes with Calcium Ionophore on Monospermic Penetration *In Vitro*

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ABSTRACT : The present study examined whether treatment of *in vitro* matured pig oocytes with calcium ionophore (A23187) could prevent polyspermic penetration *in vitro*. When oocytes cultured for maturation for 33, 36 or 44 h were subsequently treated with 50 μM A23187 in medium with fetal calf serum (FCS) for 1, 2 and 3 h and then cultured for 12 h without spermatozoa, virtually no activation occurred. In the absence of FCS, however, 31-42, 45-49 and 56-64% of oocytes were activated, respectively. When oocytes treated with 50 μM A23187 in medium with FCS for 3 h were inseminated in vitro, the penetration rates (14-57%) were lower ($p<0.01$) with a higher ($p<0.01$) incidence (35-67%) of monospermy compared with untreated oocytes (69-80% penetration and 15-17% monospermy). However, sperm penetration was completely blocked in all oocytes treated with A23187 in the absence of FCS. When oocytes matured for 33 h were treated with different concentrations of A23187 for 3 h and inseminated *in vitro*, the penetration rate did not change but there was an increased incidence ($p<0.05$) of monospermy at 10-20 μM and 2.5-5 μM A23187 in the presence and absence of FCS, respectively, compared with at 0 μM A23187. With these lower concentrations of A23187, treatment of oocytes for at least 60 and 30 min in the presence and absence of FCS, respectively, was required to increase the incidence of monospermy without reducing penetration rate. These results indicate that a high concentration (50 μM) of A23187 in medium without FCS, but not in medium with FCS, stimulated *in vitro* matured pig oocytes to induce parthenogenetic activation and a complete block to sperm penetration *in vitro*. However, treatment of oocytes with lower concentrations of A23187 (10-20 μM and 2.5-5 μM) both in the presence and absence of FCS maintained sperm penetration *in vitro* and increased the incidence of monospermy. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 2 : 172-178)***

Key Words : Pig, Oocyte, *In Vitro* Fertilization, Monospermy, Calcium Ionophore

INTRODUCTION

In mammalian oocytes, cortical granules (CGs) play an important role(s) in blocking polyspermic penetration. When sperm penetration and activation are initiated, the intracellular free calcium concentration increases in the oocytes of several animals (Jaffe, 1985; Miyazaki et al., 1986; Kline and Kline, 1992; Fissore et al., 1992) including pigs (Sun et al., 1992; Machaty et al., 1997). Most of the events associated with oocyte activation, such as CG exocytosis, resumption of meiosis and cell division, are the results of the increased intracytoplasmic calcium concentration. Experimental evidence indicates that all of these events are important for normal fertilization and early development (Yanagimachi, 1994). For example, the contents of the CGs disperses into the perivitelline space by exocytosis in response to sperm penetration (Dandekar and Talbot, 1992; Hoodbhoy and Talbot, 1994) and modifies the zona pellucida (Yanagimachi, 1994). Thus oocytes establish the functional block to polyspermic penetration.

Polyspermic penetration has been one of the unresolved problems concerning pig oocytes matured and inseminated *in vitro* (Niwa, 1993). Cran and Cheng (1986) and Yoshida et al. (1993) reported that although pig oocytes matured both *in vivo* and *in vitro* released CGs' contents upon sperm penetration, oocytes penetrated *in vivo* released CGs' contents more quickly than those penetrated *in vitro*, and the contents of CGs dispersed in the perivitelline space *in vivo* but not *in vitro*, after exocytosis. Wang et al. (1997b) have also observed delayed and partial CG exocytosis in pig oocytes matured and inseminated *in vitro*. Thus polyspermy may be the result of slow or partial CG exocytosis under *in vitro* conditions.

The calcium ionophore (A23187) is an artificial stimulator widely used in the activation of mammalian oocytes. In the pig, oocytes matured *in vitro* for 46-48 h were activated by A23187 in medium without fetal calf serum (FCS) (Hagen et al., 1991; Saito et al., 1993; Grocholova et al., 1997; Wang et al., 1998), but not in medium with FCS (Wang et al., 1997b). The purpose of the present study was to examine the penetration *in vitro* of *in vitro* matured pig oocytes treated with A23187 with or without FCS and to determine the potential role of A23187 in producing monospermic oocytes.

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MATERIALS AND METHODS

Media

The basic medium used for manipulation of oocytes and for the *in vitro* fertilization was TCM-199B (Wang et al., 1991), which was tissue culture medium (TCM) 199 (with Earle's salts; Gibco Lab., Grand Island, NY, USA) supplemented with 3.05 mM D-glucose, 2.92 mM hemicalcium lactate, 0.91 mM sodium pyruvate, 75 μg/mL potassium penicillin G, 50 μg/mL streptomycin sulfate and 10% (v/v) heat-inactivated FCS (Gibco). The maturation medium used was TCM-199B (pH 7.4) supplemented with 10 IU/mL eCG (Serotropin; Teikoku-Zoki Co., Tokyo, Japan) and 10 IU/mL hCG (Puberogen; Sankyo Co., Tokyo, Japan). Fertilization medium was TCM-199B (pH 7.8) supplemented with 10 mM caffeine sodium benzoate (Sigman Chemical Co., St. Louis, MO, USA).

Collection and maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution containing 75 μg/mL potassium penicillin G and 50 μg/ml streptomycin sulfate at 35-37°C. Oocytes were aspirated from antral follicles of 2-5 mm in diameter with an 18-gauge needle attached to a 10-mL disposable syringe (Wang and Niwa, 1997). Oocytes surrounded by a compact cumulus and with an evenly granulated cytoplasm were selected and washed 3 times with maturation medium. Ten to fifteen oocytes were transferred to a 100 μL drop of the same medium, which had previously been covered with warm paraffin oil in a polystyrene culture dish (35×10 mm; Falcon No. 1008, Becton Dickinson Labware, Lincoln Park, NJ, USA) and equilibrated in an atmosphere of 5% CO_2 in air for more than 3 h, and cultured for 33-44 h depending on the experiment at 39°C under the same atmospheric conditions. After culture, oocytes were transferred to newly prepared maturation medium (100 μL) with or without 10% (v/v) FCS.

Calcium ionophore treatment

Calcium ionophore A23187 (Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma) at a concentration of 2 mM and stored at -20°C until use. The A23187/DMSO solution was then diluted to experimental concentrations with maturation medium with or without 10% (v/v) FCS and 100 μL of diluted A23187/DMSO was added to the medium (100 μL) containing oocytes. The oocytes were then incubated in a CO_2 incubator for 1-3 h depending on the experiment. After treatment, oocytes were washed 4-5 times in fertilization medium and cultured with or without spermatozoa for 12 h for examination of activation or fertilization.

In vitro fertilization of oocytes

After treatment with A23187, oocytes were washed 5 times with previously equilibrated fertilization medium, and then 10-15 oocytes were placed into a 50 μL drop of the same medium in a polystyrene culture dish (35×10 mm; Falcon) and kept in a CO_2 incubator (5% CO_2 in air at 39°C) for about 30 min until spermatozoa were added (Wang et al., 1994).

Frozen ejaculated spermatozoa (3 pellets each with a 100 μL volume) were thawed in 2 mL of previously equilibrated caffeine-free fertilization medium at 39°C for 1 min (Wang et al., 1991). After thawing, 6 mL of the same medium were added and spermatozoa were washed 3 times by centrifugation at 550 g for 5 min each. The final sperm pellet was resuspended in the same medium to give a sperm concentration of 10^7 spermatozoa/ml. A 50 μL aliquot of sperm suspension was added to the 50 μL drop of fertilization medium containing oocytes and the mixture was cultured at 39°C in 5% CO_2 in air.

Examination of oocytes

To assess nuclear activation and sperm penetration, oocytes treated with A23187 and cultured for 12 h without or with spermatozoa were mounted on slides, fixed for about 72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope at a magnification of ×200 or ×400. Oocytes that proceeded to anaphase II or female pronuclear formation were considered parthenogenetically activated. Oocytes were considered penetrated when they had one or more decondensing sperm nucleus and/or male pronucleus with corresponding sperm tail(s).

Statistical analysis

All proportional data were subjected to arc-sin transformation and the transformed values were analyzed using one-way ANOVA. When ANOVA revealed a significant treatment effect, each treatment was compared by Fisher's protected LSD test.

RESULTS

Activation of oocytes following treatment with 50 μM A23187

When oocytes matured *in vitro* for 33, 36 and 44 h were treated with 50 μM A23187 in medium with FCS for 1, 2 and 3 h and cultured for 12 h in fertilization medium without A23187, virtually no parthenogenetic activation occurred; only a very small percentage (7 and 9%, respectively) of the oocytes matured for 44 h and treated for 2 or 3 h were activated (data not shown). However, when

treated in medium without FCS for 1, 2 and 3 h. 31-42%, 45-49% and 56-64% of the oocytes matured for 33, 36 and 44 h were parthenogenetically activated, respectively (figure 1). The proportion of activated oocytes did not increase significantly as the time of A23187 treatment increased, but more oocytes were activated after 44 h of culture than after 33 h ($p<0.05$).

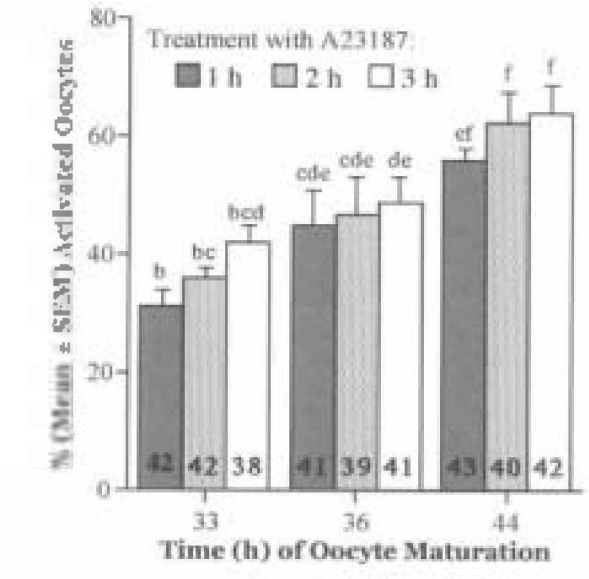

Figure 1. Activation of pig oocytes treated with 50 μM A23187 in medium without fetal calf serum for 1, 2 and 3 h at various times of maturation. Oocytes were examined 12 h after treatment. Experiments were repeated 3 times. Numbers of oocytes examined in each treatment group are shown within bars. Bars with different letters are significantly different ($p<0.05$).

Penetration *in vitro* of oocytes following treatment with 50 μM A23187

When oocytes matured for 33, 36 and 44 h were inseminated and cultured for 12 h after treatment with 50 μM A23187 in medium with FCS for 3 h, a lower ($p<0.01$) penetration rate (14-57%) was observed compared with untreated oocytes (69-80%; table 1). However, the proportion of monospermic oocytes was significantly higher in A23187 treated (35-67%) than untreated (15-17%) oocytes and the number of spermatozoa in penetrated oocytes was significantly ($p<0.01$) lower in treated (1.2-2.2 per oocyte) than untreated (3-3.5 per oocyte) oocytes. In treated oocytes, the penetration rate decreased ($p<0.01$) but the proportion of monospermy increased ($p<0.01$) when maturation time increased from 33 h to 44 h.

No sperm penetration was observed in oocytes treated with A23187 in medium without FCS (data not shown).

Penetration *in vitro* of oocytes following treatment with different concentrations of A23187 for different times

When oocytes matured for 33 h were treated with different concentrations of A23187 in medium with FCS for 3 h and inseminated *in vitro*, the penetration rate (74-83%) was not different among oocytes treated with 0-20 μM A23187, but was significantly ($p<0.05$) reduced (51%) after treatment with 40 μM A23187 (table 2). However, significantly ($p<0.05$) higher rates (36-49%) of monospermy and fewer number (1.4-2.0) of spermatozoa per oocyte were observed in oocytes treated with 10-40 μM than in controls (21% and 3.1 per oocyte, respectively) treated with 0 μM A23187. In contrast, in oocytes treated in medium without FCS (table 3), 10 μM virtually and 20 μM A23187 completely blocked sperm penetration, but similar penetration rates (88-93%) and significantly ($p<0.01$) increased incidence (22-31%) of monospermy were observed at 2.5 and 5 μM A23187 compared with 0 μM

Table 1. Penetration *in vitro* of oocytes after treatment with 50 μM A23187 in medium with FCS

Oocyte maturation time (h)	A23187 treatment	No. of oocytes examined	No. of oocytes Penetrated (%; mean±SEM)	No. of monospermic oocytes (%;mean±SEM)*	No. (mean±SEM) of spermatozoa in Penetrated oocytes
33	-	59	47 (79.7±2.7)^{a}	7 (14.9±2.0)^{a}	3.5±0.2^{a}
	+	60	34 (56.7±4.3)^{b}	12 (34.9±2.5)^{b}	2.2±0.2^{b}
36	-	58	45 (77.6±1.5)^{a}	7 (15.6±2.2)^{a}	3.2±0.1^{a}
	+	60	23 (38.3±3.2)^{c}	13 (58.2±8.1)^{c}	1.8±0.2^{b}
44	-	58	40 (68.9±2.1)^{d}	7 (17.3±2.1)^{a}	3.0±0.2^{a}
	+	57	8 (13.8±2.5)^{e}	5 (66.7±11.8)^{c}	1.2±0.1^{c}

Oocytes were treated with (+) or without (-; with 2.5% DMSO) A23187 for 3 h before insemination; experiments were repeated four times.

* Percentage of the total number of oocytes penetrated.

^{a,b,c,d,e} Values with different superscripts within each column differ significantly, $p<0.01$.

Table 2. Penetration *in vitro* of oocytes after treatment with different concentrations of A23187 in medium with FCS

Concentration of A23187 (μM)	No. of oocytes examined	No. of oocytes penetrated (%; mean±SEM)	No. of monospermic oocytes (%; mean±SEM)*	No. (mean±SEM) of spermatozoa in penetrated oocytes
0	56	46 (83.4±4.3)^{a}	9 (20.6±3.2)^{a}	3.1±0.3^{a}
5	60	49 (81.7±7.4)^{a}	13 (27.1±6.2)^{ab}	2.5±0.3^{ab}
10	57	44 (77.1±1.7)^{a}	16 (36.2±2.0)^{bc}	2.0±0.2^{b}
20	58	43 (74.2±2.9)^{a}	19 (44.3±2.2)^{cd}	1.6±0.1^{c}
40	58	29 (50.7±10.1)^{b}	14 (48.6±4.3)^{d}	1.4±0.0^{c}

Oocytes were cultured for 33 h and then treated with A23187 for 3 h before insemination; experiments were repeated four times.
* percentage of the total number of oocytes penetrated.
^{a,b,c,d} Values with different superscripts within each column differ significantly, $p<0.05$.

Table 3. Penetration *in vitro* of oocytes after treatment with different concentrations of A23187 in medium without FCS

Concentration of A23187 (μM)	No. of oocytes examined	No. of oocytes penetrated (%; mean±SEM)	No. of monospermic oocytes (%; mean±SEM)*	No. (mean±SEM) of spermatozoa in penetrated oocytes
0	46	42 (92.3±4.5)^{a}	4 (9.8±1.0)^{a}	3.3±0.2^{a}
2.5	49	45 (92.9±7.2)^{a}	10 (22.4±2.8)^{b}	3.0±0.4^{ab}
5	55	48 (87.7±9.5)^{a}	14 (30.5±4.2)^{b}	2.7±0.6^{b}
10	51	10 (19.5±1.0)^{b}	6 (56.3±6.3)^{c}	1.6±0.2^{c}
20	40	0	0	0

Oocytes were cultured for 33 h and then treated with A23187 for 3 h before insemination; experiments were repeated four times.
* percentage of the total number of oocytes penetrated.
^{a,b,c} Values with different superscripts within each column differ significantly, $p<0.01$.

controls (92% penetration and 10% monospermy).

When oocytes were treated with 20 μM A23187 in medium with FCS for 0, 15, 30, 60, 120 and 240 min, no difference in the penetration rate (89-98%) was observed among oocytes treated for 0-120 min but the percentage of monospermy was significantly ($p<0.01$) higher in oocytes treated for 60-120 min (37%) than in those treated for 0-30 min (18-20%) (table 4). In oocytes treated for 240 min, penetration rate (55%) was significantly ($p<0.01$) reduced but monospermic penetration (45%) was not different from oocytes treated for 60-120 min. Similar results (table 5) were obtained when oocytes were treated with 5 μM A23187 in medium without FCS except that the incidence of monospermy was significantly ($p<0.01$) increased starting from 30 min of treatment.

DISCUSSION

A23187 is one of the most effective artificial stimulators that has been widely used in the activation of oocytes in animals such as mouse (Tawia and Lopata, 1992; Ducibella et al., 1990), hamster (Hoshi et al., 1992), rat (Zhang et al., 1992), bovine (Soloy et al., 1997; Wang et al., 1997a) and human (Winston et al., 1991; Balakier and Casper, 1993), although the effectiveness varies among species. In the pig, it has been reported that A23187 induced oocyte activation (Hagen et al., 1991; Saito et al., 1993; Wang et al., 1997b, 1998; Grocholova et al., 1997), but different protocols yielded very variable results. For example, in oocytes matured for 44-48 h *in vitro* and treated with 50 μM A23187 in medium without FCS for 5 min, nearly 50% (Hagen et al., 1991; Saito et al., 1993; Wang et al., 1998) or 67% (Grocholova et al., 1997) were activated. However, Wang et al. (1997b) reported that when oocytes matured for 46 h *in vitro* were treated with 30 μM A23187 in medium with FCS for 1 h, they were not induced to undergo nuclear activation although released CGs' contents. In the present study, treatment with an increased concentration (50 μM) of A23187 in medium with FCS for 3 h did not consistently induce nuclear activation in oocytes matured for 33-44 h. However, when the same treatment was applied in the absence of FCS, the proportion of parthenogenetic oocytes increased as time of maturation increased, suggesting that the effectiveness of A23187 to induce oocyte activation is largely affected by FCS and the stage of maturation.

Sperm penetration *in vitro* was prevented in activated oocytes (Wang et al., 1998). In the present study, when oocytes were inseminated after treatment with 50 μM A23187 for 3 h in medium contaning FCS, sperm penetration occurred, although the penetration rate reduced and the incidence of monospermy increased, as the time for oocyte maturation increased from 33 to 44 h. However,

Table 4. Penetration *in vitro* of oocytes after treatment with 20 μM A23187 in medium with FCS for differ times

Treatment time (min)	No. of oocytes examined	No. of oocytes penetrated (%; mean±SEM)	No. of monospermic oocytes (%; mean±SEM)*	No. (mean±SEM) of spermatozoa in penetrated oocytes
0	51	50 (97.9±2.1)[a]	10 (20.2±2.7)[a]	5.1±0.6[a]
15	52	49 (94.7±1.8)[a]	9 (17.9±3.2)[a]	4.2±0.3[a]
30	53	52 (98.3±1.7)[a]	11 (21.1±1.5)[a]	4.2±0.5[a]
60	55	51 (88.8±2.6)[a]	18 (36.5±1.3)[b]	1.9±0.2[b]
120	56	50 (89.4±4.7)[a]	18 (36.8±6.5)[b]	2.0±0.2[b]
240	60	33 (54.8±5.9)[b]	15 (44.9±4.4)[b]	2.0±0.1[b]

The oocyte maturation time plus the pretreatment time of A23187 was 36 h in each treatment group before insemination; experiments were repeated four times.

* Percentage of the total number of oocytes penetrated.

[a,b] Values with different superscripts within each column differ significantly, $p<0.01$.

Table 5. Penetration *in vitro* of oocytes after treatment with 5 μM A23187 in medium without FCS for differ times

Treatment time (min)	No. of oocytes examined	No. of oocytes penetrated (%; mean±SEM)	No. of monospermic oocytes (%; mean±SEM)*	No. (mean±SEM) of spermatozoa in penetrated oocytes
0	42	37 (88.1±2.5)[a]	6 (16.3±0.9)[a]	3.4±0.3[a]
15	39	32 (82.0±2.6)[a]	6 (18.9±1.1)[a]	3.2±0.2[a]
30	41	33 (80.4±2.7)[a]	9 (27.4±1.5)[b]	2.4±0.2[b]
60	40	33 (82.5±2.1)[a]	10 (33.5±3.7)[b]	2.2±0.1[bc]
120	39	33 (84.8±4.3)[a]	10 (30.2±1.7)[b]	2.2±0.1[bc]
240	41	24 (59.0±4.0)[b]	10 (41.7±4.1)[c]	1.8±0.1[c]

The oocyte maturation time plus the pretreatment time of A23187 was 36 h in each treatment group before insemination; experiments were repeated three times.

* Percentage of the total number of oocytes penetrated.

[a,b,c] Values with different superscripts within each column differ significantly, $p<0.01$.

sperm penetration was completely blocked in oocytes treated in medium without FCS at all maturation times. It has been reported that FCS does not prevent premature CG exocytosis but inhibits zona reactions in the mouse (Ducibella et al., 1990; De Felici and Siracusa, 1985). A remarkable increase in polyspermic penetration *in vitro* in bovine oocytes inseminated in medium with FCS (Tajik et al., 1993) also suggests that FCS inhibits CG exocytosis and/or zona reactions. Wang et al. (1998) have described as their unpublished data that, when *in vitro* matured pig oocytes were treated with A23187 both in TCM-199 and modified Tris-buffered medium plus 10% FCS and then inseminated in the same medium, sperm penetration was not prevented even though CG exocytosis had occurred. These results indicated that pretreatment with A23187 in medium containing FCS induced partial CG exocytosis, leading to reduction of polyspermic penetration in pig oocytes.

To investigate the ability of A23187 to regulate polyspermic penetration *in vitro*, we examined the effect of concentrations and treatment time of A23187 on sperm penetration *in vitro*. For this purpose, we used oocytes *in vitro* matured for 33 h, because in these oocytes the incidence of parthenogenetic activation was lower and the penetration rate *in vitro* was higher after treatment with 50 μM A23187 in the absence and presence of FCS, respectively, compared with oocytes cultured for 44 h. Furthermore, in the same culture conditions as employed in the present study, 75% of oocytes reached metaphase-I (M-I) to metaphase-II (M-II) 30 h after the start of culture and there were no differences in the penetration rate and in the proportion of penetrated oocytes with male and female pronuclei after IVF between oocytes cultured for 30 and 36 h although at the latter time 73% of oocytes had reached M-II (Wang et al., 1994). In addition, CGs migrated to the cortex and formed a monolayer just beneath the oolemma when pig oocytes reached M-I to M-II *in vitro* and no further increase in the number of CGs was observed from M-I to M-II (Wang et al., 1997c). The results obtained in the present study provide evidence that treatment of oocytes cultured for 33 h with lower concentrations of A23187 for at least 30-60 min increased the incidence of monospermy without reducing the penetration rate. Based on evidence that the vast majority of CGs in pig oocytes are released after sperm penetration *in vitro* but the CGs contents remain immediately above the plasma membrane, it was suggested

that, following IVF in the pig, vital constituents of CGs remain unavailable for interaction with the zona pellucida and therefore, continued penetration of the oocyte by spermatozoa is possible (Cran and Cheng, 1986). If this is the case, it seems likely that pretreatment with the appropriate concentrations of A23187 would not only induce CG exocytosis but also promote subsequent reactions of the contents with the zona pellucida and thus regulate continued penetration of spermatozoa into the treated oocytes *in vitro*.

It was shown in the present study that a higher concentration (10-20 μM) of A23187 and a longer time (60 min) for treatment were required for treatment in the presence than in the absence of FCS to increase the incidence of monospermy without reducing penetration rate. It has been suggested that, if FCS was present in medium containing A23187, it would bind to the zona pellucida and reduce the available level of calcium, which is involved in CG exocytosis, and as a result the cortical reaction could not be induced; however, when the calcium level in the medium was raised from 1.8 mM to 7.64 mM, CG exocytosis occurred and the contents dispersed (Cran and Cheng, 1986). In the present study, the treatment medium (maturation medium) contained a comparatively low concentration (2.92 mM) of calcium. Therefore, in the present experimental conditions, a higher concentration of A23187 and a longer time of treatment may be required in the presence than in the absence of FCS to induce the zona reaction, which regulates continuous sperm penetration into oocytes.

In conclusion, the results of present study indicate that a high concentration (50 μM) of A23187 in medium without FCS, but not in medium with FCS, stimulates *in vitro* matured pig oocytes to induce parthenogenetic activation and a complete block to sperm penetration *in vitro*. However, treatment of oocytes with lower concentrations (10-20 μM and 2.5-5 μM in the presence and absence of FCS, respectively) of A23187 for shorter periods (at least 60 min and 30 min in the presence and absence of FCS, respectively) increases the incidence of monospermy without reducing penetration rate. Therefore, the treatment may have a practical use in pig IVF to produce large numbers of normal embryos.

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