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Activation and Penetration *In Vitro* of Pig Oocytes Treated with Calcium Ionophore

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Abstract. The present study examined the effects of pre-treatment of pig oocytes with different concentrations (0–50 μ M) of calcium ionophore A23187 (CaA) on their activation, development and penetration *in vitro*. Although untreated oocytes were not activated and did not cleave in culture, high proportions of treated oocytes did so and 20% of oocytes developed to the blastocyst stage when treated with 6.25 μ M CaA for 2 min. However, these proportions were reduced in a concentration-dependent manner. When inseminated *in vitro* with 1×10^6 spermatozoa/ml, the penetration rate of oocytes treated with 6.25 μ M CaA was similar to that of untreated oocytes. However, fewer oocytes treated with 12.5 and 50 μ M CaA were penetrated than untreated oocytes. On the other hand, the proportion of monospermy of oocytes treated with 6.25 μ M CaA was higher than the values in oocytes not treated or treated with 50 μ M CaA. The time required for zona dissolution of oocytes treated with 6.25 and 12.5 μ M CaA was not different from that in untreated oocytes, but oocytes treated with 50 μ M CaA required a longer time than untreated oocytes, indicating that zona solubility by protease does not reflect penetrability of oocytes *in vitro*. When oocytes were inseminated with different concentrations (1 – 10×10^6 cells/ml) of spermatozoa, the highest penetration rate was observed at 1×10^6 cells/ml in untreated oocytes and a similar result was obtained in oocytes treated with 6.25 μ M CaA. There was no difference in the rate of monospermy in untreated oocytes among different concentrations of spermatozoa, but in treated oocytes, higher proportions of monospermy were observed at 0.5 – 5×10^6 than 10×10^6 cells/ml. At 1×10^6 cells/ml, the proportion of monospermy was higher in treated than untreated oocytes. These results suggest that pre-treatment of pig oocytes with 6.25 μ M CaA, an appropriate concentration, inhibits polyspermic penetration *in vitro* when insemination occurs with spermatozoa at a concentration of 1×10^6 cells/ml.

Key words: Calcium ionophore A23187, *In vitro* fertilization (IVF), Monospermy, Pig, Polyspermy
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A series of repetitious and transient increases in the concentration of the intracellular free calcium ion ($[Ca^{2+}]_i$) is induced in activated oocytes after fusion with spermatozoa in many mammals [1–4], including pigs [5, 6]. One of the important roles of the $[Ca^{2+}]_i$ increase in penetrated oocytes is to induce cortical granule (CG) exocytosis, which is responsible for the zona reaction that participates in the block to polyspermy in most mammals [7–9].

The zona reaction and the block to polyspermic penetration are easily induced in pig oocytes under normal physiological conditions [10], but a high incidence of polyspermy in pig oocytes matured and penetrated *in vitro* has been reported by many researchers [11–13]. Although pig oocytes matured both *in vivo* and *in vitro* release CG contents upon sperm penetration, oocytes penetrated *in vivo* release CG contents more quickly than those penetrated *in vitro* [14]. Furthermore, CG contents disperse in the perivitelline space of *in vivo*

penetrated oocytes, but not *in vitro* penetrated oocytes, after CG exocytosis [14–16]. Delayed and partial CG exocytosis has also been reported in pig oocytes matured and inseminated *in vitro* [17]. On the other hand, a high concentration of spermatozoa is generally used for *in vitro* fertilization (IVF) and this condition may be one of the reasons for the polyspermic penetration that occurs even in fertilization *in vivo* in pigs [10]. Under such conditions, spermatozoa may continue to penetrate oocytes before the oocytes establish a complete zona reaction [18]. Although it is not known how this phenomenon can be prevented *in vitro*, oviductal secretions [16, 19, 20], epithelial cells [21, 22], exogenous hyaluronan [23], and fertilization promoting peptide or adenosine [24] have all been suggested to participate in the prevention of polyspermy in pig oocytes.

Calcium ionophore A23187 (CaA) is one of the most effective chemical stimulators for the activation of oocytes and has been widely used in many animals. In oocytes activated by CaA, $[Ca^{2+}]_i$ increase is induced and becomes a trigger for CG exocytosis [5, 9]. Although CG exocytosis is responsible for a zona reaction, only a partial block of sperm penetration is observed in mouse [25] and rat [26] oocytes treated with CaA. This may be due to incomplete CG exocytosis in CaA-treated oocytes as observed in pig oocytes [14, 17, 27]. The CaA-induced activation and CG exocytosis of oocytes may be dose-dependent [27–29]. A dose-dependent activation has been observed in modified Tris-buffered medium (mTBM) [27], but not in phosphate buffered saline (PBS) [30]. Furthermore, the activation rate of oocytes treated with a fixed dose of CaA did not increase with increased treatment time [31, 32]. On the other hand, sperm penetration *in vitro* of pig oocytes was dose-dependently inhibited by pre-treatment with CaA, with reduced rates of polyspermy [27]. It has been shown that this inhibition did not occur but the rate of monospermy increased when maturing pig oocytes (matured for 33 h in culture) were treated with lower concentrations of CaA in medium with or without fetal bovine serum (FBS) [32]. These results indicate that if pig oocytes fully matured for 44 h in culture could be activated with lower concentrations of CaA synchronously with sperm penetration, it might be possible to increase the incidence of monospermy without reducing the penetration rate *in vitro*. The present study was

designed to investigate this possibility using pig oocytes matured for 44 h in culture examining: 1) the rates of activation, early development and penetration *in vitro*, and zona dissolution of oocytes treated with different concentrations of CaA, and 2) the effects of sperm concentrations on monospermic penetration *in vitro* of oocytes treated with a fixed concentration of CaA.

Materials and Methods

Media

The basic medium used for the manipulation of oocytes and spermatozoa was tissue culture medium 199 (TCM-199; with Earle's salts; Gibco, Grand Island, NY, USA) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 μ g/ml potassium penicillin G, and 50 μ g/ml streptomycin sulfate. The medium was designated basic TCM-199 (bTCM-199). The medium used for *in vitro* maturation (IVM) of oocytes was bTCM-199 (pH 7.4) supplemented with 10% (v/v) pig follicular fluid (pFF), 0.57 mM L-cysteine (Sigma Aldrich Chemical Co., St. Louis, MO, USA), 10 IU/ml equine chorionic gonadotropin, and 10 IU/ml human chorionic gonadotropin. The pFF was withdrawn into a 10-ml disposable syringe through an 18-gauge needle from superficial follicles (2–5 mm in diameter) on maturing pig ovaries. The pFF was centrifuged at $1,500 \times g$ for 15 min at room temperature (24–25 C) to remove any cells or debris, and the supernatant was stored at –20 C until used [33]. The medium used for IVF of oocytes was bTCM-199 (pH 7.8) supplemented with 2.92 mM hemi-calcium lactate, 2.5 mM caffeine-sodium benzoate (Sigma), and 2 mg/ml BSA (A-7888; Fraction V, Sigma). The IVF medium was essentially the same as TCM-199B used by Wang *et al.* [34], except that 2 mg/ml BSA was substituted for FBS.

Preparation of oocytes

Ovaries isolated from maturing gilts at a local slaughterhouse were transported to the laboratory within 1–1.5 h of slaughter in 0.9% (w/v) NaCl solution containing 75 μ g/ml potassium penicillin G and 50 μ g/ml streptomycin sulphate at 37–39 C. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (2–5 mm in diameter) through an 18-gauge needle into a 10-ml disposable syringe

and washed three times with modified HEPES-buffered Tyrode's medium containing 0.1% (w/v) polyvinylalcohol (HEPES-TL-PVA). The complexes with uniform ooplasm and a compact cumulus cell mass were washed again three times with IVM medium. Fifty COCs were transferred to 500 μ l of the same medium, which had previously been covered with warm paraffin oil (Nacalai Tesque Inc., Kyoto, Japan) in a four-well culture plate (Nunc, Roskilde, Denmark) and equilibrated in an atmosphere of 5% CO₂ in air for a minimum of 3 h, and were cultured for 44 h at 39 C under the same atmospheric conditions. After culture, oocytes were freed from cumulus cells by repeated passage through a fine pipette in IVM medium containing 0.1% hyaluronidase from bovine testes (Sigma). The denuded oocytes were washed three times with caffeine-free IVF medium before treatment with Ca-ionophore A23187 (CaA; C-7522, Sigma).

Treatment of oocytes with CaA

The CaA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM, and the stock solution was kept at -32 C until used. The stock solution (5 μ l) without dilution and diluted two, four, and eight times with caffeine-free IVF medium was added to 495 μ l of the same medium covered with paraffin oil in a four-well culture plate (Nunc) to give 50, 25, 12.5 and 6.25 μ M CaA, respectively. For the control (0 μ M CaA), no stock solution was added. The maximal concentration of DMSO introduced into the medium was 1.0% (v/v) in the presence of 50 μ M CaA. The four-well culture plate was kept on a warm plate at 39 C and 20–30 denuded oocytes were introduced into each drop of medium for treatment with different concentrations of CaA for 2 min.

Assessment of activation and development of CaA treated oocytes

Oocytes treated or not treated with CaA were washed six times with IVF medium and transferred into 50 μ l of the same medium covered with paraffin oil in a culture dish ($\varnothing$ 35 $\times$ 10 mm, Becton and Dickinson, Franklin Lakes, NJ, USA). At 12 h after the start of culture at 39 C in 5% CO₂ in air, oocytes were fixed with 25% (v/v) acetic acid in ethanol, stained with 1% (w/v) orcein, and examined under a phase-contrast microscope at magnifications of $\times$ 200 and $\times$ 400 for activation.

Oocytes with one female pronucleus and second polar body or with two pronuclei were considered as activated. For the development of oocytes, they were first cultured for 6 h in IVF medium containing 2.5 μ g/ml cytochalasin B (CB; C-6762, Sigma) to induce activation producing two female pronuclei and then for 6 h in the medium without CB. After this two-step culture, oocytes were washed three times with NCSU-23 medium [35] and were further cultured in the same medium in 5% CO₂ in air at 39 C. Oocytes were examined for cleavage and development to blastocysts at 36 and 156 h after the start of the final culture, respectively, under a stereoscopic microscope at $\times$ 100.

Assessment of solubility of zona pellucida (ZP) of CaA treated oocytes

Solubility of the ZP was assessed as described previously [16]. Briefly, oocytes treated with CaA were cultured for 4.5 h in IVF medium and then washed six times with HEPES-TL-PVA. Eight or ten oocytes were transferred into 500 μ l of HEPES-TL-PVA containing 0.1% (w/v) protease (P-6911; Sigma) and were continuously observed for dissolution of the ZP at room temperature under a stereoscopic microscope at $\times$ 100. The time required for dissolution of the ZP in 50% of the oocytes was designated as the time for dissolution.

Assessment of sperm penetration in vitro

Frozen ejaculated spermatozoa (three pellets having a 200- μ l volume and containing about 4×10^6 spermatozoa/pellet) were thawed in 2 ml of PBS containing 0.1% (w/v) BSA (A-8022; Fraction V, Sigma), 100 μ g/ml potassium penicillin G and 75 μ g/ml streptomycin sulphate at 37 C. After thawing, 8–10 ml of the same medium was added and the spermatozoa were then washed twice by centrifugation, each time at 550 $\times$ g for 4 min. After washing, the sperm pellet was resuspended to give various concentrations (1 – 10×10^6 cells/ml) of spermatozoa according to the different experiments with IVF medium. The sperm suspension (50 μ l) was introduced into 50 μ l of IVF medium containing 10–15 denuded oocytes and covered with paraffin oil in a polystyrene culture dish (35 $\times$ 10 mm). At 12 h after the start of culture at 39 C in 5% CO₂ in air, oocytes were fixed, stained and examined for sperm penetration under a phase-contrast microscope at magnifications of $\times$ 200 and $\times$ 400. Oocytes were considered penetrated when

they had one or more morphologically changed sperm nucleus and corresponding sperm tail(s).

Statistical analysis

All data obtained from four to six replicates were analyzed using one-way ANOVA. Proportional data were subjected to arc-sin transformation and the transformed values were used for the analyses. When ANOVA revealed a significant treatment effect, multiple comparisons and comparisons between two treatments were made by Fisher's protected least significant difference and Student's *t*-test, respectively, using the STATVIEW IV program (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Activation and early development of CaA treated oocytes

As shown in Fig. 1, no oocytes were activated when they were not treated with CaA. However, when treated with 6.25 μM CaA, a high proportion ($87.6 \pm 2.3\%$) of oocytes was activated. Although a similar activation rate was obtained in oocytes treated with 12.5 μM CaA ($82.7 \pm 4.0\%$), the rates were significantly ($P < 0.05$) reduced when the concentration of CaA was increased to 25 ($68.6 \pm 7.6\%$) and 50 μM ($41.7 \pm 7.6\%$). As shown in Fig. 2, when oocytes were cultured after treatment with CaA, the proportion of oocytes cleaved at 48 h of culture was significantly ($P < 0.05$) reduced in a concentration-dependent manner. At 168 h of culture, the proportion of blastocyst formation was not different between oocytes treated with 6.25 ($19.8 \pm 6.6\%$) and 12.5 ($18.5 \pm 6.9\%$) μM CaA, but it was significantly ($P < 0.05$) reduced in oocytes treated with 25 ($2.8 \pm 2.0\%$) and 50 ($3.0 \pm 2.1\%$) μM CaA.

Penetration *in vitro* and solubility of the ZP of CaA treated oocytes

A high proportion ($70.4 \pm 3.7\%$) of oocytes that were not treated with CaA were penetrated *in vitro* when they were inseminated with a fixed concentration (1×10^6 cells/ml) of spermatozoa (Fig. 3A). This value was similar to that ($62.7 \pm 6.2\%$) in oocytes treated with 6.25 μM CaA. However, penetration rates in oocytes treated with 12.5 ($49.8 \pm 6.0\%$) and 50 ($24.6 \pm 5.2\%$) μM CaA were

Fig. 1. Activation of pig oocytes matured *in vitro*, treated with various concentrations of Ca-ionophore A23187 (CaA) for 2 min in IVF medium, and cultured for 12 h in the same medium. Values obtained from four replicates are expressed as mean $\pm$ SEM. The numbers of oocytes examined are indicated in parentheses. Values with different letters differ significantly ($P < 0.05$).

Fig. 2. Early development of pig oocytes matured *in vitro* and treated with various concentrations of Ca-ionophore A23187 (CaA) for 2 min in IVF medium. The treated oocytes were cultured for 6 h in IVF medium supplemented with 2.5 $\mu\text{g}/\text{ml}$ cytochalasin B (CB) and for a further 6 h in the medium without CB. Then the oocytes were transferred into NCSU 23 medium and cultured for their development. The oocytes cleaved to the 2- to 4-cell stages and developed to blastocysts were observed 48 and 168 h after the start of culture, respectively. Values obtained from four replicates are expressed as mean $\pm$ SEM. The numbers of oocytes examined are indicated in parentheses. Values with different letters within each category of the development differ significantly ($P < 0.05$).

Fig. 3. Penetration *in vitro* and solubility of the zona pellucida (ZP) of pig oocytes treated with various concentrations of Ca-ionophore A23817 (CaA) for 2 min. Oocytes inseminated with frozen-thawed boar spermatozoa at a concentration of 1×10^6 cells/ml were examined 12 h after insemination for penetration (A) and monospermy (B). Values obtained from six replicates are expressed as percentage (mean $\pm$ SEM) of inseminated oocytes. The numbers of oocytes inseminated are indicated in parentheses. For assessment of solubility of the ZP, oocytes treated with CaA were cultured for 4.5 h in IVF medium and then 8 or 10 oocytes were introduced into modified HEPES-buffered Tyrode's medium containing 0.1% (w/v) polyvinylalcohol and 0.1% (w/v) protease. The time required for dissolution of the ZP in half the number of oocytes was designated as the time for dissolution (C). Values obtained from four replicates are expressed as mean $\pm$ SEM. Values with different letters in each measurement differ significantly ($P < 0.05$).

Fig. 4. Total penetration (A) and monospermic penetration (B) *in vitro* of pig oocytes treated (6.25 μM) or not treated (0 μM) with Ca-ionophore A23817 (CaA) and inseminated with various concentrations of spermatozoa. Values obtained from five replicates are expressed as % (mean $\pm$ SEM) of inseminated oocytes. The numbers of oocytes inseminated are indicated in parentheses. Values with different letters within each concentration of CaA differ significantly ($P < 0.05$). Values with an asterisk differ significantly from that in untreated oocytes within the same sperm concentration ($P < 0.05$).

significantly ($P < 0.05$) lower than untreated oocytes. On the other hand, the proportion of monospermic penetration in oocytes treated with 6.25 μM CaA ($41.5 \pm 4.5\%$) was not significantly different to that ($30.1 \pm 4.6\%$) in oocytes treated with 12.5 μM CaA, but it was significantly ($P < 0.05$) higher than the values in oocytes not treated ($23.8 \pm 3.4\%$) or treated with 50 μM CaA ($14.9 \pm 2.0\%$) (Fig. 3B). When oocytes were treated with 0.1% protease after treatment with CaA, the time required for dissolution of the ZP was not different among oocytes treated with 6.25–50 μM CaA (175.0 ± 18.7 – 215.5 ± 21.6 sec) (Fig. 3C). However, oocytes treated with 50 μM CaA required a significantly ($P < 0.05$) longer time (215.5 ± 21.6 sec) than that (136.3 ± 13.3 sec) in untreated oocytes for dissolution of

the ZP.

Penetration in vitro of oocytes treated with 6.25 μ M CaA and inseminated with various concentrations of spermatozoa

The penetration rate ($80.5 \pm 6.5\%$) of untreated oocytes was significantly ($P < 0.05$) higher when they were inseminated with 1.0×10^6 spermatozoa/ml than 0.5, 5.0 or 10.0×10^6 spermatozoa/ml ($62.2 \pm 2.0 - 65.8 \pm 4.0\%$) (Fig. 4A). A similar result was obtained in oocytes treated with 6.25 μ M CaA, but there was no significant difference in penetration rates between oocytes inseminated with 1.0 ($70.2 \pm 4.3\%$) and 5.0 ($62.4 \pm 2.4\%$) $\times 10^6$ spermatozoa/ml. Although a significantly ($P < 0.05$) higher proportion of untreated oocytes was penetrated than treated oocytes when inseminated with 0.5×10^6 spermatozoa/ml, there was no difference in penetration rates with the other sperm concentrations. There was no significant difference in the proportions of monospermic penetration when different concentrations of spermatozoa were used for insemination in untreated oocytes ($29.5 \pm 3.9 - 37.8 \pm 4.9\%$) (Fig. 4B). However, in treated oocytes, significantly ($P < 0.05$) higher proportions of monospermic penetration were obtained using 0.5 – 5.0 ($40.6 \pm 2.5 - 54.6 \pm 8.8\%$) than 10.0 ($21.6 \pm 5.4\%$) $\times 10^6$ spermatozoa/ml. At a sperm concentration of 1.0×10^6 cells/ml, a significantly ($P < 0.05$) higher proportion of monospermy was observed in treated ($54.6 \pm 8.8\%$) than in untreated ($31.9 \pm 3.4\%$) oocytes.

Discussion

It has been reported that activation of pig oocytes is not induced properly by treatment with 30–50 μ M CaA for 30–60 min in medium with FBS [32, 36]. However, in medium without FBS and with a higher concentration (7.5 mM) of calcium, treatment with CaA for only 5 min activated oocytes in a concentration-dependent manner [27]. At a fixed concentration (50 μ M) of CaA, more oocytes were activated when treated for 2 min than 0.5 and 1 min in the same medium, although there was no difference in activation rates between oocytes treated for 2 and 5 min [31]. In the present study, when oocytes were treated with CaA for 2 min in medium with a lower concentration (3.26 mM) of calcium, the proportions of oocytes

activated were reduced as the concentration of CaA increased from 6.25 to 50 μ M. This contrasts with the observation of Wang *et al.* [27] and may be due to the different concentrations of calcium in the medium used. In the study of Wang *et al.* [31], many of the pig oocytes treated with 50 μ M CaA for 5 min in medium without calcium formed condensed chromatin and no microtubules were observed in these oocytes. These have been reported to reduce activation rates [31] and therefore a detrimental effect of CaA may occur in a concentration-dependent manner, such as when the oocytes are treated with CaA in medium containing a lower concentration of calcium.

It has been reported in mice that diploid embryos possess higher developmental ability than haploid embryos [37]. In pigs, electrically activated oocytes showed a higher incidence of diploid formation than CaA-activated oocytes and the former showed higher rates of blastocyst formation than the latter [38]. Therefore, in the present study, to study developmental competence of oocytes treated with CaA, the treated oocytes were first cultured for 6 h in IVF medium containing CB and further for 6 h in the same medium without CB to induce diploid formation. When oocytes were cultured in NCSU-23 medium for 36 h after the two-step culture, the cleavage rate was reduced as the concentration of CaA increased from 6.25 to 50 μ M. After a longer period (156 h) of culture, the proportion of oocytes that developed to blastocysts was also lower in those treated with 25 and 50 μ M than 6.25 and 12.5 μ M CaA. This may be due to the lower activation rate in oocytes treated with higher concentrations of CaA under the present experimental conditions, in which we examined activation rate of oocytes treated only with CaA, but not with CaA + CB. It has been reported that the amplitude of the intracellular calcium transients in pig oocytes treated with CaA increased in a concentration-dependent manner [27]. Although an increase of intracellular calcium is important for initiation of activation and development of oocytes, the present results show that treatment with increased concentrations of CaA does not necessarily stimulate activation and development of oocytes. Since activation rate and developmental ability are higher in pig oocytes treated with two electric pulses inducing two calcium transients than CaA [27], this suggests that the number of calcium transients may be more important than the

intracellular calcium increase for inducing activation and development of pig oocytes.

The $[Ca^{2+}]_i$ increase in pig oocytes treated with CaA is responsible for a zona reaction in which zona hardening occurs [27]. In the present study, the time requirement for dissolution of ZP of pig oocytes treated with different concentrations of CaA was examined to assess the degree of zona hardening. The results obtained showed that the dissolution time was significantly increased in oocytes treated with 50 μM CaA, but not in oocytes treated with 6.25 and 12.5 μM CaA, compared with untreated oocytes, suggesting that zona hardening was not promoted by treatment with the lower concentrations of CaA to a level that could be detected in this study. Nevertheless, when inseminated with a fixed concentration (1×10^6 cells/ml) of spermatozoa, similar penetration rates were obtained between untreated oocytes and those treated with 6.25 μM CaA. However, the penetration rates of oocytes treated with 12.5 to 50 μM CaA were significantly reduced compared with untreated oocytes. These results suggest that zona solubility by protease does not necessarily reflect the penetrability of oocytes *in vitro*. The lack of a close correlation between zona solubility and penetrability of pig oocytes *in vitro* has also been suggested by Wang *et al.* [27].

One of the most interesting observations in the present study was that treatment of oocytes with 6.25 μM CaA for 2 min followed by insemination with 1.0×10^6 spermatozoa/ml increased the incidence of monospermy without reducing the penetration rate compared with untreated oocytes. To our knowledge, our previous investigation [32] and the present study are the first to have found that polyspermic penetration *in vitro* can be regulated by treatment of pig oocytes with CaA. In our previous study [32], treatment of pig oocytes with 5 μM CaA for longer periods (30–120 min) in medium without FBS also increased the incidence of monospermy without reducing the penetration rate. In that study, cumulus-enclosed oocytes matured only for 33 h in culture were treated with CaA in IVM medium without BSA, while in the present study, oocytes freed from cumulus cells after full maturation for 44 h in culture were treated with CaA in IVF medium with BSA. Therefore, it is difficult to compare the results obtained in the present study with those obtained in the previous

study. However, since oocytes that have reached metaphase II develop full ability to induce CG exocytosis in all animals examined [39], including pigs [17], oocytes matured for 33 h may require a longer period of treatment with CaA to induce CG exocytosis and zona reaction than those matured fully for 44 h. The mechanism regulating polyspermic penetration in pig oocytes treated with CaA was unclear in the previous [17, 32] and the present studies. A possible explanation is that a lower and incomplete CG exocytosis in pig oocytes penetrated *in vitro* [14] may be modified by the treatment of oocytes with CaA before insemination. It has been reported that most CGs are released equally from both oocytes matured *in vivo* and *in vitro*, providing evidence that both CG exocytosis itself and the events after exocytosis must play important role(s) in establishing a functional block to polyspermy [40]. Therefore, it seems likely that moderately induced CG exocytosis in oocytes treated with appropriate concentrations of CaA is compensated for by sperm penetration and the subsequent reaction of the CG contents with the ZP is enhanced to regulate continuous sperm penetration *in vitro*.

In the present study, penetration rates of oocytes both not treated and treated with 6.25 μM CaA, were significantly increased when inseminated with 1.0 rather than 0.5×10^6 spermatozoa/ml, but these rates gradually reduced as the sperm concentration increased up to 10.0×10^6 cells/ml. Although there were no differences in the proportions of monospermy between untreated and treated oocytes inseminated with 0.5, 5.0 and 10.0×10^6 spermatozoa/ml, a significantly higher proportion of monospermy was observed in treated than untreated oocytes inseminated with 1.0×10^6 spermatozoa/ml. These results indicate that the sperm concentration used for insemination *in vitro* is also an important factor in the increased proportion of monospermy in CaA treated oocytes.

In conclusion, the results of the present study indicate that the treatment of pig oocytes *in vitro* with 6.25 μM CaA for 2 min is effective for inducing activation, cleavage and blastocyst formation. This treatment also increases the incidence of monospermy without reducing penetration rates, when oocytes are inseminated with a concentration of 1.0×10^6 spermatozoa/ml.

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