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A Comparison of Degree of Cortical Granule Exocytosis in Zona-Free Pig Oocytes Induced by Different Artificial Stimulators

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ABSTRACT: Cortical granule (CG) exocytosis occurs when the first sperm fertilizes an oocyte to modify the extracellular environment and zona pellucida of the oocyte thereby preventing polyspermy. Apart from sperm penetration, many artificial stimulators have been reported to induce CG exocytosis in mammalian oocytes, including pig oocytes. However, little detailed classification of the degree of exocytosis after the stimulation have been done, especially in zona-free oocytes. This study is, therefore, aimed to investigate the degree of exocytosis in zona-free pig oocytes after the stimulation with artificial stimulators. Oocytes were matured *in vitro* for 48 hr. Zona-free matured oocytes were then stimulated with each stimulator, including sperm insemination as a positive control group. The degree of exocytosis was monitored at 5 min, 6, 12 and 24 hr after stimulation. The results showed that calcium ionophore A23187 induced 100% of the oocytes to release the granules, and induced the maximum percentage of oocytes exhibiting type I CG exocytosis (67.8%) at 24 hr after stimulation. Apart from oocytes cultured alone, the highest percentage of oocytes that did not release their cortical granules was seen in the oocytes treated with puromycin at 6 hr after treatment (31.2%). Of the stimulators calcium ionophore A23187 seems to be the most effective at inducing cortical release, while puromycin and sulphadiazine have very little effect on CG exocytosis. The potentials of artificial stimulators on the release of cortical granules may lead to their application in preventing polyspermy. However, further studies are needed to investigate whether or not the release of cortical granules in zona-free pig oocytes induced by artificial stimulators, such as calcium ionophore A23187, can prevent polyspermy *in vitro*.

KEYWORDS: cortical granule exocytosis, pig oocyte, oocyte maturation, zona pellucida, polyspermy.

INTRODUCTION

Cortical granule (CG) exocytosis normally occurs when the first sperm penetrates an egg. The release of cortical granules modifies the extracellular environment and zona pellucida to block additional sperm from penetrating the fertilized egg [1]. Moreover, it has been reported that altered zona morphology may cause polyspermic penetration in pigs[2]. Many artificial stimulators have been previously reported to induce CG exocytosis in the matured pig oocytes. Calcium ionophore A23187[3,4] and an electrical pulse[5] can produce CG exocytosis. Ethanol has also been reported to elicit CG exocytosis in oocytes of mouse eggs, similar to that which occurs during fertilization[6]. In pigs, treatment of matured oocytes with 7% ethanol induced an increase in intracellular pH, accompanied by the parthenogenetic activation of the oocytes[4], but there were no reports in its effect on CG exocytosis. The use of antibiotic reagents such as cycloheximide has been reported to result in oocyte activation in many species[7,8], yet again there were no reports in its effect on CG exocytosis.

In addition, polyspermy is one of the unresolved problems that exist regarding pig oocytes that are matured and inseminated *in vitro*[1]. The polyspermy rate was significantly higher in *in vitro*-matured pig oocytes (65%) than those in ovulated oocytes (28%). CGs of ovulated oocytes appeared more aggregated than those of *in vitro*-matured oocytes [9].

Cortical granule (CG) exocytosis of matured zona-intact pig oocytes has been classified into three categories after *in vitro* fertilization: complete CG exocytosis and even distribution of exudates in the entire perivitelline space (type I); complete exocytosis and partial distribution of exudate (type II) and incomplete CG exocytosis (type III) [10]. The incidence of oocytes with type I exocytosis was higher in oocytes matured *in vivo* than in those matured *in vitro* [10].

From the published literature, there appeared to be no report on the degree of CG exocytosis in zona-free oocytes. The present study is, therefore, designed

to examine the effects of artificial stimulators: calcium ionophore A23187, 7% (v/v) ethanol and antibiotic reagents (puromycin and sulphadiazine), in inducing the release of cortical granules in zona-free matured pig oocytes. Puromycin was used in the present study because it has been reported to normally be used for oocyte activation *in vitro*[11,12]. Sulphadiazine was also chosen as a stimulator in the present study because it has been widely used as a feed additive to prevent infection in pigs[13]. Therefore, both reagents were used as antibiotics representatives. The results from this study would provide new information to allow better understanding of the release of CG exocytosis in zona-free pig oocytes and its role in polyspermy prevention.

Materials and Methods

Oocyte Collection and Maturation

Pig ovaries were removed from the animals at a slaughterhouse. The ovaries were immediately transferred to the laboratory in normal saline (0.9% (w/v) NaCl) at 18-24°C. The oocytes were aspirated from 2-8 mm ovarian follicles using 10 cc sterile syringes and 18-G sterile, disposable needles. The oocytes with a compact cumulus mass (more than 3 layers of tight cumulus cells) and evenly granulated cytoplasm were selected for use.

The maturation process was performed in oocyte maturation medium containing HEPES-buffered TCM-199 (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% (v/v) heat-treated fetal calf serum (56°C for 30 min.) and 10 IU/ml of PMSG, 10 IU/ml of hCG and 1 mg/ml of 17-b estradiol (Sigma Chemical Co.). The pH was adjusted to 7.3-7.4 using 1 M NaOH. Selected oocytes were washed 3 times in maturation medium and placed into 50 ml maturation medium droplets (10-15 oocytes per droplet) in 60 mm plastic Petri dishes under 10 ml sterile paraffin oil. The oocytes were left to undergo maturation for 48 hr in a high humidity CO_2 incubator (5% CO_2 in air at 39°C). After the maturation period, cumulus cells were removed from the oocytes by vortexing in 0.3 M mannitol solution containing 0.3 mg/ml of hyaluronidase. The denuded, matured oocytes were finally washed 3 times in maturation medium and were then ready for use in the experiment.

Preparation of Zona-Free Oocytes

The denuded matured oocytes with zona intact were washed three times with the modified Medium 199 supplemented with 10 mmol/l caffeine sodium benzoate and 4 mg/ml BSA (Sigma Chemical Co.) at pH 7.4. Then, the zona pellucida was removed using 0.25% pronase (Pronase E, Sigma Chemical Co.) and the oocytes were washed in modified medium (TCM199) several times before use.

Sperm Collection

Semen was freshly collected from mature white boars, whose average age was 2 years old. A sperm-rich fraction (100-250 ml) was collected from each animal by the gloved-hand method[14]. Then, the fraction was filtered through 2 layers of gauze fixed to pre-warmed (37°C) thermos flasks.

Determining the Effects of Artificial Stimulators on CG Exocytosis in Zona-Free Pig Oocytes, in Comparison with Sperm Insemination (Positive Control) and Zona-Free Oocytes Cultured Alone (Negative Control)

Sperm Insemination (Positive Control)

The semen was washed three times by centrifugation (1,000 g for 3 min) with 0.9% (w/v) NaCl supplemented with 1 mg/ml of BSA (Fraction V; Sigma Chemical Co.). At the end of washing, the pellets containing spermatozoa were resuspended at a concentration of 2×10^8 cells/ml in modified Medium 199 at pH 7.8. Then, the sperm suspension was incubated for 90 min at 39°C in an atmosphere of 5% CO_2 in air. Ten zona-free matured oocytes were washed three times with modified Medium 199 supplemented with 10 mmol/L caffeine sodium benzoate and 4 mg/ml BSA (Sigma Chemical Co.) at pH 7.4. After being washed, ten oocytes were placed into a 50 ml droplet of the modified medium (TCM199) under paraffin oil. Fifty millilitres of diluted preincubated spermatozoa were added to 50 ml of medium containing the oocytes giving a final concentration of spermatozoa at 1×10^6 cells/ml. The co-culture was kept at 39°C in an atmosphere of 5% CO_2 in air for 6 hr.

After the incubation, the oocytes were washed 3 times with the modified medium and transferred to 500 ml of fresh medium containing 100 mg/ml peanut agglutinin labelled with fluorescein isothiocyanate (FITC-PNA; Sigma Chemical Co.) which has been used to label cortical granules[10]. The degree of CG exocytosis was observed under an inverted fluorescent microscope using the classifications modified from the study of Kim et al. (1996)[10] at 5 min, 6, 12 and 24 hr post-insemination.

Calcium Ionophore A23187

Ten zona-free oocytes were exposed to 100 mM calcium ionophore A23187 (Sigma Chemical Co.) for 5 min. Then, they were stained with FITC-PNA and monitored the degree of exocytosis under the fluorescent microscope at 5 min after stimulation, and at 6, 12 and 24 hr of incubation.

7% Ethanol

Ten zona-free oocytes were exposed to 7% (v/v) ethanol for 5 min [4]. Then, they were examined for CG exocytosis at 5 min after stimulation, and at 6, 12 and 24 hr of incubation, as mentioned earlier.

Puromycin and Salphadiazine

Sets of ten zona-free oocytes each were exposed to either 10 mg/ml puromycin or 10 mg/ml sulphadiazine (Sigma Chemical Co.) for 5 min. Then, they were stained with FITC-PNA to monitor the degree of exocytosis under the fluorescence microscope at 5 min, 6, 12 and 24 hr of incubation.

Zona-Free Oocyte Cultured Alone (Negative Control)

Ten zona-free matured oocytes were left alone in the medium without stimulation. Degrees of CG exocytosis were monitored at 5 min, 6, 12 and 24 hr of incubation.

Classifications of CG Exocytosis

The classifications of CG exocytosis used in the present study were modified from the classifications described by Kim et al. (1996)[10]. Therefore, the classifications of CG exocytosis in the present study were classified into three categories (Fig 1): complete CG exocytosis around the oolemma (type I); incomplete CG exocytosis (type II) and no CG exocytosis (type III)

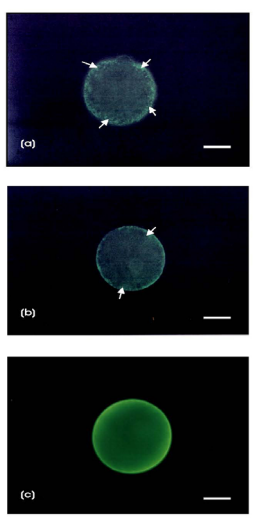

Fig 1. Zona-free pig oocytes from this study demonstrating different types of CG exocytosis. Type I CG exocytosis (a) showing a complete distribution of cortical granules around the oolemma (arrows); type II CG exocytosis (b) showing partial distribution of cortical granules around the oolemma (arrows) and type III CG exocytosis (c) showing no release of cortical granules. Scale bar = 0.2 mm.

Statistical Analysis

The numbers of eggs exhibiting the CG exocytosis in each category were converted into percentages and an average degree of CG exocytosis was calculated in each group from three replications. One way analysis of variance with Bonferroni and Dunnett's test was performed to estimate the effect of each stimulator on the release of cortical granules. A p-value of <0.05 was considered to be statistically significant.

Results

The percentages of zona-free matured oocytes exhibiting type I CG exocytosis are presented in figure 2. There were no oocytes exhibiting type I CG exocytosis in oocytes cultured alone in every incubation period examined. The percentage of oocytes exhibiting type I CG exocytosis after stimulation with 7% ethanol (52.4±6.3) was significantly higher ($P<0.01$) than oocytes stimulated by sperm insemination (positive control group) (26.7±5.3) at 5 min after stimulation. The percentages of oocytes exhibiting type I CG exocytosis were greatly increased after stimulated with calcium ionophore for 6 hr (63.2±8.2) compared to 5 min, and the percentage was significantly higher ($P<0.01$) than other stimulators. The highest percentage of oocytes exhibiting type I CG exocytosis were also seen at 24 hr of incubation in the calcium ionophore stimulated group (67.8±8.4). However, this was not significantly different when compared with the positive control group (58.9±11.9). The percentages of oocytes exhibiting type I CG exocytosis stimulated by puromycin were slightly increased from 5 min to 24 hr of incubation, but it failed to reach significant levels.

The percentages of zona-free matured oocytes exhibiting type II CG exocytosis are presented in figure 3. There were no oocytes exhibiting type II CG exocytosis in oocytes cultured alone at 5 min of incubation period, but these were seen at 6, 12 and 24

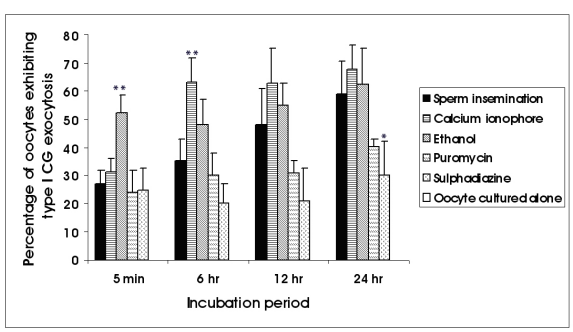

Fig 2. A comparision of the percentages of zona-free oocytes exhibiting type I CG exocytosis after stimulation by different stimulators at various incubation periods.
** $P<0.01$ vs Sperm insemination (ANOVA Post hoc Dunnett Test)
* $P<0.05$ vs Sperm insemination (ANOVA Post hoc Dunnett Test)

hr of incubation period with the highest percentage (27.8±6.9) was seen at 12 hr of incubation. At 5 min of incubation, the percentage of oocytes exhibiting type II CG exocytosis was highest in the sperm insemination group (61.9±2.9). This was significantly higher than oocytes stimulated by 7% ethanol (39.4±2.4) and sulphadiazine (47.5±2.6). At 6 hr of incubation, the percentages of oocytes exhibiting type II CG exocytosis was still highest in the sperm insemination group (56.7±5.6). This was significantly higher ($P<0.05$) than oocytes stimulated by calcium ionophore (32.9±11.2). At 12 and 24 hr of incubation, the percentages of oocytes exhibiting type II CG exocytosis were not significantly different among the groups examined, with the highest percentage seen in oocytes stimulated by sulphadiazine.

The percentages of zona-free matured oocytes exhibiting no CG exocytosis (type III) are presented in fig 4. Oocytes cultured alone showed the highest percentage of type III CG exocytosis at every incubation period, especially at 5 min of incubation (100%). These were significantly different with other stimulators at every incubation period. However, the percentages of oocytes exhibiting no CG exocytosis were significantly higher in oocytes stimulated by sulphadiazine and puromycin when compared with sperm insemination group at 6, 12 and 24 hr of incubation.

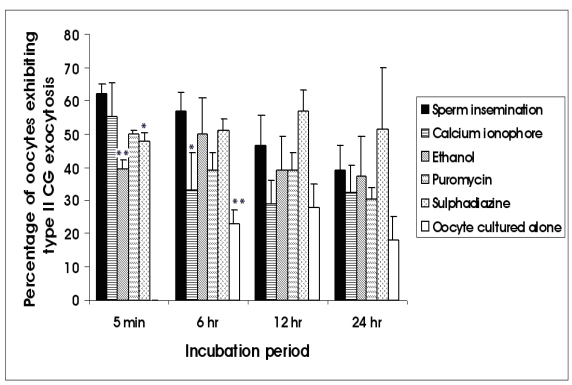

Fig 3. A comparision of the percentages of zona-free oocytes exhibiting type II CG exocytosis after stimulation by different stimulators at various incubation periods.
** P<0.01 vs Sperm insemination (ANOVA Post hoc Dunnett Test)
* P<0.05 vs Sperm insemination (ANOVA Post hoc Dunnett Test)

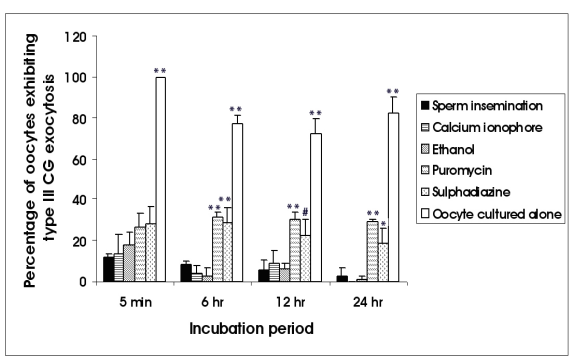

Fig 4. A comparision of the percentages of zona-free oocytes exhibiting type III CG exocytosis after stimulation by different stimulators at various incubation periods.
** P<0.01 vs Sperm insemination (ANOVA Post hoc Dunnett Test)
* P<0.05 vs Sperm insemination (ANOVA Post hoc Dunnett Test)
# P=0.05 vs Sperm insemination (ANOVA Post hoc Dunnett Test)

Discussion

The release of cortical granules is normally induced by the elevated $[Ca^{2+}]_i$ caused by the sperm penetration[15]. Fertilization triggers a wave of CG exocytosis in the egg that is a consequence of an increase in intracellular free calcium concentration. It has been reported that cortical granules contain a high concentration of total calcium. They represent a major cortical storage site of calcium in the egg, and exchange part of their accumulated calcium by an ATP dependent mechanism[16]. One of the role of $[Ca^{2+}]_i$ influx in the fertilized oocytes is to cause CG exocytosis, which in turn induces a zona reaction that participates in the block to polyspermy in most mammals[17,18].

In the present study, zona-free oocytes activated by sperm penetration showed a gradual increase in the release of CG exocytosis. Oocytes released the maximum percentage of type I CG exocytosis (59%) 24 hr after sperm insemination and only 2% of oocytes could not produce CG exocytosis at 24 hr after stimulation. These results were similar to those found in zona-intact pig oocytes which showed 86.3% of CG exocytosis after stimulation with sperm penetration[5]. Wang et al. (1997)[1] reported that all oocytes penetrated by spermatozoa were activated and released cortical granules from ooplasm at 18 hr after insemination. Complete CG exocytosis was observed in 45% of zona-intact oocytes, and none was observed in nuclear-inactivated oocytes. In the present study, 59% of zona-free oocytes showed complete CG exocytosis 24 hr after sperm insemination. This finding could reflect different responses to sperm penetration between zona-intact and zona-free pig oocytes on the release of cortical granules.

Calcium ionophore A23187 seemed to be the most effective artificial stimulator at inducing CG exocytosis in zona-free pig oocytes in the present study. Most of the oocytes activated by A23187 produced CG exocytosis at 24 hr after stimulation. A23187 could induce the maximum percentage of zona-free oocytes exhibiting type I CG exocytosis (68%) at 24 hr after stimulation. These results were slightly different from previous studies in zona-intact pig oocytes which

reported that A23187 induced 75.7% of cortical granules to be released from the oocytes, and only 10% of the oocytes showed complete CG exocytosis[3,5]. These results could suggest that calcium ionophore A23187 can be used as an effective artificial stimulator to induce the release of cortical granules *in vitro*. In addition, calcium ionophore A23187 has been reported to be one of the most effective artificial stimulators that have been widely used in oocyte activation in animals[7]. It is generally thought that A23187 directly induces the influx of extracellular calcium and can also activate oocytes[19,20]. It has been reported that the effect of A23187 is the direct induction of the influx of extracellular calcium influx and intracellular H^+ efflux, thus resulting in increases in the intracellular calcium concentration and intracellular pH[21]. Wang et al. (1998)[22] reported that cumulus-free pig oocytes exposed to 0-100 mM A23187 showed an increase in the amplitude of the intracellular calcium transients, percentage of pronuclear formation and percentage of CG exocytosis in a concentration-dependent manner.

The present study showed that ethanol was another artificial stimulator that can induce CG exocytosis very quickly. Zona-free oocytes stimulated with 7% (v/v) ethanol produced the highest percentage of type I CG exocytosis (52%) at 5 min after stimulation when compared with the positive control group (sperm penetration) and other stimulators. The ethanol in induction of CG exocytosis in zona-free oocytes was consistently high up to 24 hr after stimulation, as only 0.83% of oocytes examined did not release their granules. Ethanol has also been reported to elicit the cortical exocytosis in the oocytes of some species, similar to that occurs during fertilization[6]. Treatment of bovine oocytes with 7% (v/v) ethanol for 5 min resulted in 71.7% of the oocytes being activated as shown by the resumption of meiosis and the formation of female pronuclei[23]. It has been suggested that ethanol induces a single transient rise in $[Ca^{2+}]_i$ in oocytes, and the duration of the rise in $[Ca^{2+}]_i$ was significantly longer than that caused by spermatozoa at fertilization[24]. In pigs, treatment of matured oocytes with 7% (v/v) ethanol induced an increase in the intracellular pH accompanied by parthenogenetic activation of the oocytes[4]. In addition, Shiina et al. (1993)[25] reported that the release of calcium from intracellular stores and the influx of extracellular calcium contribute to the increase in $[Ca^{2+}]_i$ can be induced by ethanol.

Sulphadiazine and puromycin have very little effects to CG exocytosis when compared with other stimulators. About 24% and 25% of zona-free oocytes released type I CG exocytosis at 5 min after sulphadiazine and puromycin stimulations, respectively. In addition, stimulations of oocytes with sulphadiazine and puromycin showed significant higher percentages of oocytes that did not release CG when compared with the positive control (sperm insemination) at 24 hr of incubation. These results could suggest that sulphadiazine and puromycin should not be used as artificial stimulators on CG exocytosis, even though puromycin has been reported to be widely used for oocyte activation[11,12,26]. Possibly, puromycin needs a longer period of stimulation (> 5 min) to induce the release of CG exocytosis, especially in pigs. These results could also suggest that the use of sulphadiazine during infection treatment does not affect the fertilization process in pigs.

This study appeared to be the first to demonstrate the degree of CG exocytosis in zona-free matured pig oocytes induced by artificial stimulators, including antibiotic reagents. The results indicate that some artificial stimulators such as calcium ionophore A23187 and 7% (v/v) ethanol can effectively induce CG exocytosis in zona-free pig oocytes matured *in vitro*. In addition, oocytes stimulated with both stimulators produced a quicker release of cortical granules than sperm penetration and other artificial stimulators examined. Calcium ionophore A23187, in particular, induced all oocytes examined to release the granules after 24 hr of stimulation. Similarly, a high percentage of type I CG exocytosis was induced by calcium ionophore. In contrast, oocytes stimulated with antibiotic agents, sulphadiazine and puromycin, showed only slight increases in CG exocytosis. The release of cortical granules induced by each artificial stimulator may be modulated by an increase in intracellular calcium concentration in the oocyte. The differences in the degree of CG exocytosis induced by different artificial stimulators may reflect their potentials in modulating intracellular calcium concentration in the oocyte. The potentials of artificial stimulators in inducing CG exocytosis, such as calcium ionophore A23187, may be used to assess their application for preventing polyspermy in pigs. It has been reported that the time for dissolution of the zona pellucida was increased in the oocytes treated with 25-100 mM A23187, and only 3-4% of oocytes were penetrated by spermatozoa after treatment with 50-100 mM A23187[22]. However, further studies are needed to investigate whether or not the release of cortical granules without inducing a zona reaction can prevent polyspermy in zona-free pig oocytes *in vitro*.

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