Degree of Cortical Granule Exocytosis in *in vitro*-matured Porcine Oocytes Induced by Different Artificial Stimulators

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Abstract

**Background:** Cortical granule exocytosis occurs when the first sperm fertilizes oocyte. Apart from sperm penetration, many artificial stimulators have been reported to induce cortical granule exocytosis in mammalian oocytes, including pigs. However, little detailed classifications of the degree of exocytosis after the stimulation have been done.
Aim: This study is designed to investigate the degree of exocytosis in porcine oocytes matured in vitro after the stimulation with artificial stimulators.

Materials and Methods: Denuded matured oocytes were stimulated with each stimulator, including sperm insemination as a control group. Degrees of exocytosis were monitored at 5 min, 6, 12 and 24 hr after stimulations.

Results: The results showed that calcium ionophore induced 100% of oocytes to release the granules at 6 hr after treatment while ethanol induced the maximum percentage of oocytes exhibiting complete (type I) cortical granule exocytosis (34.93%) at 12 hr after stimulation. In contrast, stimulations with sulphadiazine and puromycin showed very little effect on the release of cortical granules. About 29% and 12% of oocytes did not release their granules (type IV) at 24 hr after sulphadiazine and puromycin stimulation, respectively.

Discussion and Conclusion: These results suggest that calcium ionophore A23187 and 7% ethanol can induce cortical granule exocytosis in a similar manner with sperm penetration. The release of cortical granules after stimulation with each stimulator may be caused by the increase in intracellular calcium concentration in the oocytes. However, further studies are needed to investigate the potential of each stimulator in the modifications of cortical granules to prevent polyspermy for in vitro fertilization.

Keywords: Cortical granule; cortical granule exocytosis; oocyte activation; in vitro fertilization; polyspermy

Introduction

Cortical granule (CG) exocytosis normally occurs when the first sperm penetrates an egg to modify the extracellular environment and blocks additional sperm to penetrate the fertilized egg [1]. Many artificial stimulators have been previously reported to induce the cortical granule exocytosis in the matured porcine oocytes. Calcium ionophore A23187 [1-6] and electrical pulse can produce cortical granule exocytosis [7]. Ethanol has also been reported to elicit the cortical granule exocytosis in oocytes of some species, similarly to that occurs during fertilization [8]. In porcines, treatment of matured oocytes with 7% ethanol induced an increase in intracellular pH, accompanied by the parthenogenetic activation of the oocytes [6], but there were no reports in its effect on cortical granule exocytosis. The use of antibiotic reagents such as cycloheximide has been reported to result in oocyte activation in many species [9,10], yet again there were no reports in its effect on cortical granule exocytosis. In addition, polyspermy is one of the unresolved problems that exist regarding pig oocytes matured and inseminated in vitro [2]. Polyspermy rate was significantly higher in in vitro-matured pig oocytes (65%) than those in ovulated oocytes (28%).

From the published literature, the precise effects of artificial stimulators on CG exocytosis in porcine oocytes are still controversial. The present study is,
therefore, designed to examine the ability of different artificial stimulators: calcium ionophore A23187, 7% (v/v) ethanol and antibiotic agents (puromycin/sulphadiazine), in inducing the release of cortical granules in \textit{in vitro} matured porcine oocytes. The results from this study would provide the potential stimulator for inducing CG exocytosis in porcine oocytes, and this could be useful in developing the techniques to prevent polyspermy in \textit{in vitro} fertilization especially in porcine. The use of antibiotics to induce cortical granule exocytosis could also provide us new information whether or not the consumption of the antibiotics has an effect in the difficulties in fertilization in both commercially-reared porcine and human.

\textbf{Materials and Methods}

\textbf{Oocyte collection and maturation}

Porcine 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) supplemented with 10% (v/v) heat-treated fetal calf serum (56°C for 30 min.) and 10 iu PMSG ml\(^{-1}\), 10 iu hCG ml\(^{-1}\) and 1 \(\mu\)g 17-\(\beta\) estradiol ml\(^{-1}\) (Sigma Chemical CO, St Louis, MO). The pH was adjusted to 7.3-7.4 using 1M NaOH. Selected oocytes were washed 3 times in maturation medium and placed into 50 \(\mu\)l maturation medium droplets (10-15 oocytes per droplet) in 60 mm plastic Petri dish 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 maturation period, cumulus cells were removed from the oocytes by vortexing in 0.3 M mannitol solution containing 0.3 mg hyaluronidase ml\(^{-1}\). The denuded, matured oocytes were finally washed 3 times in maturation medium and ready for use in the experiment.

\textbf{Sperm collection}

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

\textbf{Determining of CG exocytosis in \textit{in vitro}-matured porcine oocytes after stimulation}

\textbf{Sperm insemination}

The semen was washed three times by centrifugation (1000 g for 3 min.) with 0.9 (w/v) NaCl supplemented with 1 mg BSA ml\(^{-1}\) (Fraction V; Sigma Chemical CO, St Louis, MO). At the end of washing, the pellets containing
Spermatozoa were resuspended at a concentration of 2 x 10^8 cells ml^-1 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% CO₂ in air. Ten matured oocytes were washed three times with modified Medium 199 supplemented with 10 mmol caffeine sodium benzoate l^-1 and 4 mg BSA ml^-1 (Sigma Chemical CO, St Louis, MO) at pH 7.4. After being washed, ten oocytes were placed into a 50 μl droplet of the modified Medium 199 under paraffin oil. Fifty millilitres of diluted preincubated spermatozoa were added to 50 μl of medium containing the oocytes giving a final concentration of spermatozoa at 1 x 10^6 cells ml^-1. The co-culture was kept at 39°C in an atmosphere of 5% CO₂ in air for 6 hr.

After the incubation, the oocytes were washed 3 times with the modified medium and transferred to 500 μl of fresh medium containing 100 μg peanut agglutinin labelled with fluorescein isothiocyanate (FITC-PNA; Sigma Chemical CO, St Louis, MO) ml^-1 which has been used to labelled calcium release during CG exocytosis [11]. The degree of CG exocytosis was observed under the inverted fluorescent microscope using the classifications modified from the study of Kim et al. (1996) [11] at 5 min, 6, 12 and 24 hr post-insemination.

**Calcium ionophore A23187**

Ten matured oocytes were exposed to 100 μM Calcium ionophore A23187 (Sigma Chemical CO, St Louis, MO) 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 matured oocytes were exposed to 7% (v/v) ethanol for 5 min [6]. Then, they were examined for CG exocytosis at 5 min after stimulation, and at 6, 12 and 24 hr of incubation as mentioned earlier.

**Antibiotic agents (puromycin and sulphadiazine)**

Ten matured oocytes were exposed to 10 μg puromycin ml^-1 (Sigma Chemical CO, St Louis, MO) 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. Another ten matured oocytes were exposed to 10 μg sulphadiazine ml^-1 (Sigma Chemical CO, St Louis, MO) for 5 min. Then, they were observed for the degree of CG exocytosis as described above.

**Classifications of CG exocytosis**

The classifications of CG exocytosis used in the present study were therefore modified from the classifications described by Kim et al. (1996) [11]. CG exocytosis was classified into four categories (Figure 1.): complete CG exocytosis with the even distribution of exudate in the entire perivitelline space (Type I); complete CG exocytosis with or without partial distribution of exudate in the perivitelline space (Type II); incomplete CG exocytosis (Type III) and no CG exocytosis (Type IV).
Figure 1: Denuded, matured porcine oocytes exhibiting four types of cortical granule (CG) exocytosis. (a) complete CG exocytosis with an even distribution of exudate in the entire perivitelline space (PVS) (Type I); (b) complete CG exocytosis with partial distribution of exudate in the perivitelline space (Type II); (c) incomplete CG exocytosis (Type III), and (d) no CG exocytosis (Type IV). Cortical granules (arrow). Bars represent 0.01 mm.

Statistical analysis

In each treatment, the experiment was repeated three times with an average number of eggs being examined of 86.05. The numbers of eggs exhibiting the CG exocytosis in each category were converted into percentages and an average of the degree of CG exocytosis was calculated in each group. One way analysis of variance (ANOVA) 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 matured oocytes exhibiting type I CG exocytosis were presented in table 1. High percentages of matured oocytes exhibiting type I CG exocytosis were induced quickly by ethanol and calcium ionophore at 5 min after stimulation, while puromycin cannot induce type I CG exocytosis at this interval. However, the data just failed to reach significant level (P = 0.07). At 6 hr after the treatment, the numbers of oocytes exhibiting type I CG exocytosis were significantly higher (P<0.05) in ethanol, calcium ionophore and sperm insemination groups with the percentages of 20.55, 15.88 and 11.23 respectively, while there were still no oocytes exhibiting type I CG exocytosis in the sulphadiazine and puromycin treated groups. The pattern of oocytes exhibiting type I CG exocytosis at 12 hr of incubation was similar to those seen at 6 hr interval. The percentage of oocytes presenting type I CG exocytosis was highest in sperm penetration group at 24 hr of incubation, while sulphadiazine induced no oocytes exhibiting type I CG exocytosis.

Table 1: Percentage of matured oocytes exhibiting type I cortical granule exocytosis after stimulated by each stimulator

<table>
<thead>
<tr>
<th>Incubation time after stimulation</th>
<th>Percentage of oocytes exhibiting type I cortical granule exocytosis</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sperm insemination</td>
<td>Ethanol</td>
</tr>
<tr>
<td>5 min</td>
<td>1.39±2.41</td>
<td>11.30±2.68</td>
</tr>
<tr>
<td>6 h</td>
<td>11.23±4.71</td>
<td>20.55±1.26</td>
</tr>
<tr>
<td>12 h</td>
<td>15.00±0.00</td>
<td>34.93±0.73</td>
</tr>
<tr>
<td>24 h</td>
<td>31.09±6.26</td>
<td>25.18±1.87</td>
</tr>
</tbody>
</table>

Data are means and standard deviations
Level of significance is tested using one way ANOVA

The percentages of oocytes exhibiting type II CG exocytosis after being stimulated with each stimulator in each interval were presented in table 2. At 5 min after stimulation, the percentages of type II CG exocytosis were higher (P<0.01) in the oocytes treated with calcium ionophore (64.41%), ethanol (51.60%) and puromycin (50.44%) when compared with those of sperm penetration (31.27%) and sulphadiazine (34.23%). At 6 hr, the percentage of type II CG exocytosis was highest (P<0.01) in the oocytes stimulated with calcium ionophore (55.20%) and smallest in the oocytes stimulated with sulphadiazine (20.70%). There were no significant differences in the oocytes examined at 12 and 24 hr intervals.
Table 2: Percentage of matured oocytes exhibiting type II cortical granule exocytosis after stimulated by each stimulator

<table>
<thead>
<tr>
<th>Incubation time after stimulation</th>
<th>Sperm insemination</th>
<th>Ethanol</th>
<th>Sulphadiazine</th>
<th>Calcium Ionophore</th>
<th>Puromycin</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>31.27±3.24</td>
<td>51.60±3.58</td>
<td>34.23±7.82</td>
<td>64.41±10.33</td>
<td>50.44±13.32</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>6 h</td>
<td>43.66±6.25</td>
<td>43.67±2.39</td>
<td>20.70±3.25</td>
<td>55.20±13.56</td>
<td>48.83±12.21</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>12 h</td>
<td>41.67±2.89</td>
<td>48.30±4.24</td>
<td>43.09±2.68</td>
<td>51.89±15.43</td>
<td>55.00±18.03</td>
<td>ns</td>
</tr>
<tr>
<td>24 h</td>
<td>47.22±4.81</td>
<td>51.61±2.79</td>
<td>38.32±3.99</td>
<td>63.78±26.52</td>
<td>57.84±13.98</td>
<td>ns</td>
</tr>
</tbody>
</table>

Data are means and standard deviations
Level of significance is tested using one way ANOVA

The percentages of oocytes exhibiting type III CG exocytosis were presented in table 3. Sperm penetration produced the maximum percentage of oocytes presenting type III CG exocytosis (P = 0.001) at 5 min interval (42.04%), while calcium ionophore produced the minimum (21.18%). On the other hand, sperm penetration (21.14%) induced the smallest percentage of type III CG exocytosis at 6 hr, whereas sulphadiazine induced the highest (43.96%). Similarly sulphadiazine also induced the maximum percentages of type III CG exocytosis at 12 and 24 hr with the significant levels of P<0.01 and P<0.05 respectively.

Table 3: Percentage of matured oocytes exhibiting type III cortical granule exocytosis after stimulated by each stimulator

<table>
<thead>
<tr>
<th>Incubation time after stimulation</th>
<th>Sperm insemination</th>
<th>Ethanol</th>
<th>Sulphadiazine</th>
<th>Calcium Ionophore</th>
<th>Puromycin</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>42.04±2.25</td>
<td>24.28±1.84</td>
<td>31.77±2.99</td>
<td>21.18±5.98</td>
<td>28.67±5.60</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>6 h</td>
<td>21.14±0.52</td>
<td>28.15±2.39</td>
<td>43.96±3.46</td>
<td>28.92±3.70</td>
<td>32.37±2.56</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>12 h</td>
<td>21.67±2.89</td>
<td>9.09±2.17</td>
<td>34.61±1.34</td>
<td>20.61±13.94</td>
<td>27.41±5.59</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>24 h</td>
<td>14.61±2.77</td>
<td>16.27±3.18</td>
<td>32.66±1.33</td>
<td>10.97±12.02</td>
<td>25.69±10.61</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Data are means and standard deviations
Level of significance is tested using one way ANOVA

The percentages of oocytes presenting no CG exocytosis (type IV) were shown in table 4. Calcium ionophore showed the smallest percentages of type IV CG exocytosis in every interval examined, especially there were no oocytes presenting type IV CG exocytosis at 6, 12 and 24 hr intervals. In contrast, oocytes stimulated with sulphadiazine showed the maximum percentages of oocytes releasing no cortical granules. The percentages of type IV CG exocytosis were gradually decreased in the oocytes treated with sperm penetration, ethanol and puromycin from 5 min to 24 hr interval.
Table 4: Percentage of matured oocytes exhibiting type IV cortical granule exocytosis after stimulated by each stimulator

<table>
<thead>
<tr>
<th>Incubation time after stimulation</th>
<th>Percentage of oocytes exhibiting type IV cortical granule exocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sperm insemination</td>
</tr>
<tr>
<td>5 min</td>
<td>25.3±1.53</td>
</tr>
<tr>
<td>6 h</td>
<td>23.9±2.77</td>
</tr>
<tr>
<td>12 h</td>
<td>21.7±2.89</td>
</tr>
<tr>
<td>24 h</td>
<td>7.1±2.66</td>
</tr>
</tbody>
</table>

Data are means and standard deviations
Level of significance is tested using one way ANOVA

Discussion

In the present study, 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 (31%) 24 hr after sperm insemination and only 7% of oocytes could not produce CG exocytosis at 24 hr after stimulation. These results are in agreement with the recent study which reported that sperm penetration induced 86.3% of cortical granules to be released from the porcine oocytes [12] and all oocytes penetrated by spermatozoa were activated and released CGs from ooplasm at 18 hr after insemination [7].

Calcium ionophore A23187 seemed to be the most effective artificial stimulator that can induce CG exocytosis in in vitro-matured porcine oocytes in the present study. Most of the oocytes activated by A23187 produced CG exocytosis. A23187 can induce CG exocytosis quicker than other stimulators examined with the most effective interval that oocytes exhibited type I CG exocytosis was 12 hr after stimulation. The results were similar to our previous report that calcium ionophore A23187 induced 100% of CG exocytosis in zona-free porcine oocyte and 67.8% exhibiting type I CG exocytosis 24 hr after stimulation [13]. In a similar manner, A23187 has been reported to induce 75.7% of cortical granules to be released from the porcine oocytes [12]. Calcium ionophore A23187 has been reported as one of the most effective artificial stimulators widely used in oocyte activation in animals [9]. It is generally thought that A23187 directly induces the influx of extracellular calcium and can also activate oocytes [14,15]. 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 intracellular calcium concentration and intracellular pH [16]. It has been reported that cumulus-free pig oocytes exposed to 0-100 μM A23187 showed increase in the amplitude of the intracellular calcium transients, percentage of pronuclear formation and percentage of CG exocytosis in a concentration-dependent manner [5].
7% ethanol was another artificial stimulator that can induce CG exocytosis very quickly. Oocytes stimulated with 7% ethanol produced the highest percentage of type I CG exocytosis (35%) at 12 hr when compared with the control group (sperm insemination) and other stimulators. Ethanol has also been reported to elicit the cortical exocytosis in the oocytes of some species, similarly to that occurs during fertilization [8]. Bovine oocytes treated with 7% ethanol for 5 min resulted in 71.7% of the entire oocytes being activated as shown by the resumption of meiosis and the formation of female pronuclei [17]. It has been suggested that ethanol induces a single transient rise in $[\text{Ca}^{2+}]_i$ in oocytes, and the duration of the rise in $[\text{Ca}^{2+}]_i$ was significantly longer than that by spermatozoa at fertilization [18]. In porcine, treatment of matured oocytes with 7% ethanol induced an increase in intracellular pH accompanied by parthenogenetic activation of the oocytes [6]. In addition, it has been reported that the release of calcium from intracellular stores and the influx of extracellular calcium contribute to the increase in $[\text{Ca}^{2+}]_i$ can be induced by ethanol [19].

Sulphadiazine and puromycin have very little effects to CG exocytosis when compared with other stimulators. Only 4% of oocytes released type I CG exocytosis at 5 min after sulphadiazine stimulation. On the other hand, there were no oocyte release type I CG exocytosis at 24 hr after stimulation while over 29% showed no CG release. Similarly, oocytes stimulated with puromycin showed no type I CG exocytosis at 5 min and 6 hr intervals. Only 5% of oocytes exhibited their type I at 24 hr interval. It has been reported that human oocytes exposed to puromycin resulted in oocyte activation [20-22]. Treatment of aged human oocytes with 10 $\mu$g puromycin ml$^{-1}$ for 6-8 hr and further cultured for 12-15 hr induced 90.5% of oocyte activation [23]. Even though, puromycin has been reported to induce oocyte activation, it may need a longer period of stimulation (> 5 min) to induce the release of CG exocytosis.

**Conclusion**

This study appeared to be the first to demonstrate the degree of CG exocytosis induced by artificial stimulators, including antibiotic agent (sulphadiazine) which is normally used in pigs. The results indicate some potential artificial stimulators such as calcium ionophore A23187 and 7% ethanol can induce CG exocytosis in porcine oocytes matured in vitro in a similar manner with sperm penetration. 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 6 h of stimulation. High percentage of type I CG exocytosis was induced by ethanol. In contrast, oocytes stimulated with antibiotic agents, sulphadiazine and puromycin, showed only slight effect in inducing CG exocytosis. The release of cortical granules induced by each artificial stimulator may be modulated by in 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 preventing polyspermy. However, further studies are needed to investigate the effect of each stimulator in the modifications of cortical granules to prevent polyspermy in porcine oocytes fertilized in vitro.

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