Effect of the Duration of Preincubation on the Ability of Pig Spermatozoa to Penetrate Oocytes in vitro

Keita SUZUKI, Tadashi MORI and Hiroshi SHIMIZU
Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo-shi 060

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Abstract Pig spermatozoa are generally preincubated prior to in vitro fertilization (IVF) to accomplish penetration into oocytes. This study was carried out to establish the optimum condition of preincubation for boar spermatozoa which have low ability of penetration. To investigate whether a long preincubation period and the presence of bovine serum albumin (BSA) in the preincubation medium improve the ability of these spermatozoa to penetrate oocytes in vitro, spermatozoa were preincubated in media with and without BSA, and for various periods. The rate of oocyte penetration by spermatozoa preincubated for 8h was significantly higher than that by spermatozoa preincubated for 1.5h (control). On the other hand, there was no difference in the rates of oocytes penetration by spermatozoa preincubated in a medium containing BSA and in a medium with no BSA.

Key words: pig spermatozoa, preincubation of spermatozoa, in vitro fertilization

In our previous paper, we reported different abilities of spermatozoa collected from different boars and/or different ejaculates even from the same boars to penetrate oocytes in vitro. These differences in the ability of spermatozoa to penetrate oocytes makes it difficult to establish a widely usable system of pig IVF in which we can obtain a high rate of penetrated oocytes using any semen sample. Sperm-preincubation is an important process in IVF for spermatozoa to achieve penetrating ability. However, little is known about the effect of the duration of preincubation and the presence of BSA in the preincubation medium on the penetrating ability of spermatozoa which have low abilities of penetration. Therefore, in this study we investigated these two points.

Materials and Methods

The methods used in this study for oocyte maturation, sperm preparation and co-incubation of oocytes and spermatozoa were the same as those described in our previous report. In vitro fertilization:

All the semen samples used in this study were collected from three Landrace boars (Boar A, B and C). Semen from these boars was used for artificial insemination, and had produced normal piglets. In a preliminary examination of in vitro fertilization (IVF), semen samples from Boar A and B showed a lower penetration rate of oocytes than the semen samples from
Condition of pig sperm preincubation

Boar C. After the spermatozoa (sperm-rich fraction from ejaculates) were washed, spermatozoa were preincubated in a medium containing 0.1% polyvinylalcohol (Sigma) (Pm-PVA, mTyrode's solution\(^6\)) without bovine serum albumin (BSA) at 37°C under an atmosphere of 5% CO\(_2\) in air. The concentration of spermatozoa was 1 × 10\(^8\) sperm/ml during preincubation.

To examine the effect of the duration of preincubation and the presence of BSA in the Pm on the ability of spermatozoa to penetrate oocytes in vitro, the spermatozoa from Boar A were incubated for 0, 1.5, 4, 8 and 12 h in Pm-PVA and for 1.5 h in Pm containing 0.1% BSA (Fraction V, Sigma) (Pm-BSA), and the spermatozoa from Boar B were incubated for 0, 1.5, 4 and 8 h in Pm-PVA and Pm-BSA. The spermatozoa from Boar C were preincubated for 1.5 h in Pm-PVA to compare the penetrating ability with the spermatozoa from Boar A and B.

After in vitro maturation in modified Waymouth's medium\(^8\) for 44-45 h, oocytes were washed and introduced into droplets of fertilization medium (Fm, mTyrode's solution with 2 mM caffeine and 0.1% PVA, without BSA\(^8\)). Preincubated spermatozoa were washed, diluted in Fm and introduced into Fm droplets. The final sperm concentration was adjusted to 1 × 10\(^6\) sperm/ml. Co-incubation of spermatozoa and oocytes was performed at 39°C under 5% CO\(_2\) in air atmosphere for 8 h. After co-incubation, the oocytes were fixed with 25% acetic alcohol for a minimum of 24 h, and stained with 1% aceto-orcein. The number of spermatozoa which penetrated oocytes were assessed under phase contrast

<table>
<thead>
<tr>
<th>Boar</th>
<th>No. of trials</th>
<th>Preincubation period (h)</th>
<th>Pm(^2) with (+) or without (−) BSA</th>
<th>No. of oocytes Penetrated/GVBD(^3)</th>
<th>Monospermy (%)(^4)</th>
<th>No. of penetrated spermatozoa /a oocyte(^5)</th>
<th>Sperm motility (% range) Just after transfer to Fm</th>
<th>After 8 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>0</td>
<td>1/46 (2.1)</td>
<td>1 (100.0)</td>
<td>1.0</td>
<td>60–70</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>9/71 (12.7)</td>
<td>8 (88.9)</td>
<td>1.1</td>
<td>80–90</td>
<td>90</td>
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<td></td>
<td></td>
<td>3</td>
<td>2/61 (2.6)</td>
<td>2 (100.0)</td>
<td>1.0</td>
<td>70–80</td>
<td>90</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>4</td>
<td>16/46 (33.3)</td>
<td>13 (81.3)</td>
<td>1.3</td>
<td>80–90</td>
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<td></td>
<td></td>
<td>8</td>
<td>30/52 (57.7)</td>
<td>20 (66.7)</td>
<td>1.5</td>
<td>70–80</td>
<td>80</td>
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<tr>
<td></td>
<td></td>
<td>12</td>
<td>12/45 (24.5)</td>
<td>8 (66.7)</td>
<td>1.3</td>
<td>50–60</td>
<td>70–80</td>
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<tr>
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<td>1.0</td>
<td>70–80</td>
<td>40–60</td>
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<td>4</td>
<td>10/68 (14.7)</td>
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<td>37 (46.3)</td>
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<td>10–30</td>
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<td>8</td>
<td>73/113 (64.6)</td>
<td>30 (41.1)</td>
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<td>10–20</td>
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<tr>
<td>C</td>
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<td>95/74 (74.3)</td>
<td>22 (40.0)</td>
<td>2.0</td>
<td>60–70</td>
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</table>

\(^1\) All preincubated spermatozoa were inseminated with oocytes in the same Fertilization medium (Fm : protein-free mTyrode's solution).

\(^2\) Preincubation medium

\(^3\) Oocytes underwent germinal vesicle break down

\(^4\) Percentage of oocytes penetrated

\(^5\) Penetrated oocyte
microscopy. Oocytes having decondensed sperm heads or male pronuclei with corresponding sperm tails were judged to be penetrated.

**Sperm motility**: For measuring sperm motility, the preincubated and washed spermatozoa were also introduced into the Fm droplets containing no oocyte. Sperm motilities were measured under phase contrast microscopy just after transfer to the Fm droplets and after 8h of incubation under the same conditions as sperm-oocyte co-incubation. Before examination, the spermatozoa in the Fm droplets were mixed gently by pipetting to avoid head-to-head agglutination.

**Statistical analysis**: The statistical significance of the results was analyzed by $\chi^2$ tests.

**Results and Discussion**

**Effect of the duration of preincubation**: The duration of preincubation greatly affected the ability of spermatozoa to penetrate oocytes *in vitro*. The spermatozoa from Boar A preincubated for 8h penetrated more oocytes (57.7%) than spermatozoa preincubated for 1.5h (12.7%). When the spermatozoa from Boar B were preincubated for 8h, the rate of oocyte penetration by spermatozoa was higher (69.6%) than that in any other period of preincubation (1.8, 2.0 and 9.5% by spermatozoa preincubated for 0, 1.5 and 4h, respectively). Post reports have also suggested that the period of sperm preincubation is an important factor in pig spermatozoa penetration of oocytes *in vitro*. The optimal length of preincubation varied according to environmental conditions and individual boars. In this study, the results showed that the optimal length of sperm preincubation was 8h for Boar A and B. It was thought that 8h-preincubation of spermatozoa altered the composition of sperm cell membranes, mainly the composition of the lipid bilayer that determined the fluidity of cell membranes. Thus, it was thought that an 8h-preincubation period induced the cell membranes of spermatozoa to become fluid, thus, increasing the rate of oocyte penetration by spermatozoa. On the other hand, the results showed that 12h of preincubation decreased the rate of sperm penetration. The cause of this decrease may be excess fluidity of sperm cell membranes.

**Effect of BSA**: BSA in the Pm could not increase the penetrating ability of spermatozoa (Boar A: 2.5% and 12.7%; Boar B: 3.7% and 2.0%, by spermatozoa preincubated for 1.5h in Pm–BSA and Pm–PVA, respectively). No significant difference was observed in the rates of oocyte penetration by spermatozoa from Boar B preincubated between Pm–BSA and Pm–PVA for each preincubation period. It was also observed that albumin modulated the composition of sperm plasma membranes. Thus, it was thought that spermatozoa could easily capacitate under the presence of BSA. In this study, the presence of BSA in the preincubation medium did not increase the pig sperm ability to penetrate oocytes *in vitro*. This agreed with the results in our previous study.

**Sperm motility**: The penetration rate of high motile spermatozoa was not always higher than that of low motile samples. As there was no correlation between the rate of oocyte penetration and sperm motility, the motility of sperm samples could not be used as an indicator for the ability to penetrate oocytes.

Further study is required to clarify the mechanisms behind the different abilities of spermatozoa to penetrate oocytes. In this study, the low rate of oocyte penetration by spermatozoa was increased by a long sperm preincubation period (8h). This method may be useful for improving the penetrating ability of pig spermatozoa with a low ability of penetration *in vitro*. 
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References


