Beneficial effect of serum on the fertilizability of mouse oocytes matured in vitro

T. S. Choi, M. Mori*, K. Kohmoto and Y. Shoda

Department of Animal Breeding, Faculty of Agriculture, University of Tokyo, 1–1, Yayoi-1-chome, Bunkyo-ku, Tokyo 113, and *Department of Animal Science, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422, Japan

Summary. Mouse oocytes matured in vitro in chemically defined medium were not penetrated by spermatozoa. The time required for dissolution of the zona pellucida of such oocytes by α-chymotrypsin was much longer than that for ovulated oocytes. Addition of fetal calf serum to the medium for maturation of oocytes improved the incidence of sperm penetration and shortened the time of enzymic dissolution of the zona pellucida. These results suggest that the low rate of fertilization of oocytes matured in vitro is mainly due to qualitative changes of the zona pellucida, which could be overcome by a factor or factors in fetal calf serum.

Introduction

Follicular oocytes spontaneously resume meiotic division when they are liberated from the follicle and cultured in vitro in a chemically defined medium (Tsafriri, 1978). These oocytes are arrested at the metaphase of the second meiotic division, which is the equivalent stage as for ovulated oocytes. However, oocytes matured in vitro in chemically defined medium have very low rates of sperm penetration and pronucleus formation (Cross & Brinster, 1970; Mukherjee, 1972; Cross, 1973; Minato & Toyoda, 1980). It is also reported that the presence of serum in culture medium during maturation in vitro can improve the incidence of sperm penetration (Minato & Toyoda, 1982) and further development of fertilized eggs (Schroeder & Eppig, 1984). Since the first barrier of sperm penetration is the zona pellucida, we investigated qualitative differences of the zona pellucida of oocytes matured in medium with various concentrations of serum by assessing resistance to dissolution by α-chymotrypsin.

Materials and Methods

The experimental animals used in this study were jcl-ICR mice. They were either raised in our own colony or obtained from CLEA JAPAN (Tokyo, Japan). Animals were kept in an air-conditioned room at 21–23°C under a controlled light/dark cycle (lights on 05:00–19:00 h) and supplied with laboratory animal chow (NMF, Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum.

Medium used for the maturation of oocytes and for in-vitro fertilization was modified Krebs–Ringer–bicarbonate (Toyoda et al., 1971), which consists of 119.37 mM-NaCl, 4.78 mM-KCl, 1.17 mM-CaCl₂, 1.19 mM-KH₂PO₄, 1.19 mM-MgSO₄, 25.07 mM-NaHCO₃, 1.00 mM-sodium pyruvate, 5.56 mM-glucose, 75 mg penicillin/l, 50 mg streptomycin/l, and 4 g bovine serum albumin/l (fraction V; Boehringer, Mannheim, West Germany). The medium was sterilized by Millipore filter (pore size: 0.45 μm) and a 0.4 ml portion was placed in the centre of a plastic culture dish (35 × 10 mm) covered with 5–6 ml paraffin oil. In some experiments, the medium was supplemented with fetal calf serum (HyClone, Lot No. 1111523, Logan, UT, U.S.A.), which was inactivated by heating at 56°C for 30 min.

Female mice were given intraperitoneally 5 i.u. PMSG at 21–23 days of age and killed 46–48 h later by cervical dislocation. The ovaries were, cleaned, cleaned of adhering blood by blotting on a sheet of sterile filter paper, and placed in Heps medium, which was the same as the culture medium except for the addition of 20 mM-Heps and 8.00 mM-NaOH and the reduction of NaHCO₃ concentration to 5.08 mM. Oocytes were obtained from large follicles by puncturing with a sharp needle under a dissecting microscope. Only oocytes with complete cumulus cells were
selected. The oocytes were washed twice in the medium and cultured for 14–16 h at 37°C in an atmosphere of 5% CO₂ in air. To obtain the ovulated oocytes, coeval female mice were injected with 5 i.u. PMSG followed by injection of 5 i.u. hCG 46–48 h later. The oviducts were removed 14–16 h after the hCG injection. The diluted ampullae were immediately submersed in paraffin oil in a culture dish. A clump of eggs was obtained by tearing the diluted ampulla with a 27-gauge needle and was gently moved to a drop of the culture medium which was located in the centre of the dish.

For in-vitro fertilization, spermatozoa were collected from the cauda epididymidis of mature mice and were incubated for 1–2 h at 37°C before insemination to allow capacitation. The final concentration of spermatozoa for fertilization was adjusted to approximately 10⁵ spermatozoa/ml. All oocytes were examined 6 h after insemination. These were stained with lacmoid and observed under a phase-contrast microscope (× 400).

For the test of resistance of the zona pellucida to enzymic dissolution, cumulus-free oocytes were obtained by vigorous pipetting of oocytes matured in vitro or by brief incubation of ovulated oocytes with 0-1% hyaluronidase dissolved in the culture medium. About 10 cumulus-free oocytes were transferred to 100 μl of a solution of 0.3% α-chymotrypsin (bovine pancreas, Sigma, St Louis, MO, U.S.A.) dissolved in the Heps medium without BSA. During incubation at 37°C, oocytes were examined at 5-min intervals under a dissecting microscope, and the presence of the zona pellucida was recorded. The complete disappearance of the zona pellucida was regarded as the completion of dissolution.

Results and Discussion

After 14 h of culture of oocytes with adherent cumulus cells in media supplemented with various concentrations of fetal calf serum, oocytes extruding one polar body were selected, and fertilized in vitro. Table 1 shows that the incidence of sperm penetration gradually increased with the increase in the concentration of fetal calf serum. Without the supplement of fetal calf serum, the zona and/or vitellus was penetrated in only 12% of the oocytes, while the addition of 10% fetal calf serum increased this rate to 79%.

To demonstrate the qualitative difference of the zona pellucida of these oocytes, their resistance to 0.3% α-chymotrypsin was examined. The results are shown in Fig 1. The time for the dissolution of zona pellucida with α-chymotrypsin was dependent on the concentration of fetal calf serum in the medium during maturation in vitro. Increased resistance of the zona pellucida to proteolytic dissolution has been considered as a post-fertilization event and referred to as zona pellucida hardening. De Felici & Siracusa (1982) reported that it also occurs during the maturation of oocytes in a chemically defined medium, and they referred to it as spontaneous zona pellucida hardening. Our results indicate that the presence of fetal calf serum in the medium influences spontaneous zona pellucida hardening as well as the incidence of sperm penetration. De Felici et al. (1985) have reported that follicular fluid contains factors that prevent spontaneous zona pellucida hardening during in-vitro maturation of mouse oocytes. Such factors could possibly be derived from serum.

<table>
<thead>
<tr>
<th>Conc. of fetal calf serum* (%)</th>
<th>No. of oocytes examined†</th>
<th>No. of penetrated oocytes</th>
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<tr>
<td></td>
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<td>Total (%)</td>
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<tr>
<td>0</td>
<td>50</td>
<td>6 (12)</td>
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<tr>
<td>0.1</td>
<td>44</td>
<td>9 (20)</td>
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<tr>
<td>1</td>
<td>51</td>
<td>30 (59)</td>
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<tr>
<td>10</td>
<td>47</td>
<td>37 (79)</td>
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PVS = perivitelline space; VS = vitelline space; 1PN = one pronucleus; 2PN = two or more pronuclei.

*Oocytes with adherent cumulus cells were cultured for 14 h in the media supplemented with various concentrations of fetal calf serum.
†Cumulative no. of oocytes of two independent experiments, in which 8 and 10 animals were used.
Fig. 1. Effects of fetal calf serum during the maturation of oocytes in vitro on the time required for dissolution of the zona pellucida with α-chymotrypsin. Oocytes obtained from follicles were cultured in medium supplemented with 0% (△), 0.1% (▲), 1% (□), 10% (■) or 30% (○) fetal calf serum. After 14 h, oocytes which had one polar body were selected, freed from cumulus cells, washed twice with the culture medium and incubated in 0.3% α-chymotrypsin. The disappearance of the zona pellucida was examined at 5-min intervals under a dissecting microscope. The results for ovulated oocytes (●) are also shown. Values are the average of 3 independent experiments.

Fig. 2. Dissolution of the zona pellucida with α-chymotrypsin. Oocytes obtained from follicles (■) or zonae pellucidae isolated from them (□) were cultured for 14 h and the time of dissolution of zona pellucida with α-chymotrypsin was assessed. Ovulated oocytes (●) or the zonae pellucidae isolated from them (○) were also examined for their dissolution time. Values are the average of 4 independent experiments.

It is generally accepted that zona pellucida hardening is caused by the secretory products of cortical granules released from the oocyte during the cortical reaction of the oocytes (Schmell & Gulyas, 1980). To clarify the role of oocytes on spontaneous zona pellucida hardening, zonae pellucidae isolated from follicular oocytes by vigorous pipetting were cultured in vitro for 14 h. The resistance to dissolution with α-chymotrypsin was tested and compared with that of zonae
pelliculidae isolated from ovulated oocytes or intact oocytes. Figure 2 shows that spontaneous zona pellucida hardening of the oocytes matured in vitro differed from that of ovulated oocytes, and was not influenced by the presence of oocytes within it.

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References


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