IN VITRO FERTILIZATION OF BOVINE OOCYTES
BY SPERMATOZOA CAPACITATED IN VITRO

Kenneth R. Bondioli and Raymond W. Wright, Jr.
Washington State University, Pullman 99164

Summary

In vitro fertilization of ovulated bovine oocytes was attempted with fresh or frozen semen after capacitation in vitro. Sperm incubations and oocyte cultures were performed in a Krebs Ringer bicarbonate medium containing Na pyruvate, glucose and 3% bovine serum albumin. High ionic strength (HIS) medium was prepared by adding NaCl to provide an osmolality of 370 to 380 mOsmol/kg. Donor cows were treated with prostaglandin F2α and a series of follicle-stimulating hormone injections and ovulated oocytes were recovered 72 h after prostaglandin treatment. Fresh or frozen semen was: 1) placed directly into a microdrop of standard medium (SM) under oil; 2) washed by centrifugation with SM and placed in a microdrop of SM or 3) pretreated with HIS for 10 min, washed and placed into a microdrop of SM. In all cases, spermatozoa were preincubated for 3 h at a concentration of ~10^6 cells/ml before addition of oocytes. Oocytes were incubated with spermatozoa for 6 h, transferred to fresh medium and cultured for 24 h. When spermatozoa were placed directly into a microdrop, three of 34 (9%) oocytes were penetrated, but none divided. Spermatozoa washed with SM penetrated 20 of 45 (44%) oocytes and three (7%) divided. Spermatozoa incubated in HIS penetrated 14 of 47 (30%) oocytes and five (11%) divided. The washing of spermatozoa with standard medium was equally as effective as incubation with high ionic strength medium in inducing in vitro capacitation of bovine spermatozoa.

Introduction

Since the initial reports of Austin (1951) and Chang (1951) of a need for spermatozoa to reside in the female reproductive tract for some hours before gaining the ability to penetrate an oocyte, many efforts have been directed towards eliminating the requirement of the female reproductive tract in the process. Capacitation of mammalian spermatozoa in vitro has been reported following a variety of treatments, including exposure to bovine serum albumin (BSA), serum, follicular fluid, cumulus cells, adrenal extracts and Sendai virus (Rogers, 1978; Wright and Bondioli, 1981).

Bovine spermatozoa have been capacitated in vitro by incubation in high ionic strength (HIS) medium (Brackett et al., 1980). Recently (Brackett et al., 1982) a 40% fertilization rate has been achieved with spermatozoa capacitated in this manner in addition to the birth of a live calf following in vitro fertilization. In these experiments, spermatozoa were treated in a manner previously reported for in vitro capacitation of rabbit spermatozoa (Brackett and Oliphant, 1975; Brackett et al., 1978). Treatment of mouse spermatozoa with HIS medium has been shown to elute protein from the spermatozoa (Oliphant and Brackett, 1973). Hosoi et al. (1981) also reported that rabbit epididymal spermatozoa washed with isotonic medium penetrated oocytes as readily as those exposed to HIS medium. It would also be expected that such treatment would extract peripheral proteins from the plasma membrane (Singer, 1974). Rabbit spermatozoa were as effectively capacitated by washing with isotonic medium as by incubation in HIS medium (Brackett and Oliphant, 1975). Hosoi et al. (1981) also reported that rabbit epididymal spermatozoa washed with isotonic medium penetrated oocytes as readily as those exposed to HIS medium. These studies of capacitation of rabbit spermatozoa pose some questions as to the importance of exposure to...
HIS medium in capacitation of bovine spermatozoa. The objective of this study was to investigate the importance of HIS medium in capacitation of bovine spermatozoa.

Materials and Methods

Medium. The standard medium used for capacitation of spermatozoa and in vitro fertilization was essentially the same as that used by Brackett and Oliphant (1975) except a commercially available antibiotic-antimycotic mixture was used instead of sodium penicillin. It was composed of 112 mM NaCl, 4.02 mM KCl, 2.25 mM CaCl₂, .83 mM NaH₂PO₄, .52 mM MgCl₂, 37 mM NaHCO₃, 13.9 mM glucose, 1.25 mM sodium pyruvate, 3 mg bovine serum albumin/ml⁴ and 10 ml/liter of 100X antibiotic-antimycotic solution⁵. High ionic strength medium was prepared by the addition of NaCl to provide an osmolarity of 370 to 380 mOsmol/kg as measured by a vapor pressure osmometer⁶. All media were filtered with a .2 μm Millipore filter.

Oocyte Collection. Mature cows of mixed breeding were used as oocyte donors. Estrous cycles were synchronized by two injections of prostaglandin F₂α (PGF₂α), of 25 or 50 mg each, with the higher dose used in large dairy cows. Superovulation was accomplished by administration of follicle-stimulating hormone (FSH) at 12-h intervals over a 5-d period beginning on the 7th day after the first PGF₂α injection. Each cow received a declining dose of 5, 5, 4, 4, 3, 3, 2, 2, 1, 1 mg FSH injection. Ovulated oocytes were collected at slaughter 72 h after the second PGF₂α injection by cannulation of the oviduct through the ostium and retrograde flushing with Hams F10 medium.

Treatment of Spermatozoa. Both freshly ejaculated and frozen semen stored in straws were used in different replicates of the experiment. All fresh ejaculated semen was collected from a single bull and all frozen semen was from a single ejaculate from a different bull.

Figure 1. Bovine oocyte fertilized in vitro by spermatozoa washed with isotonic medium. Two pronuclei (arrows) are visible. The oocyte was fixed with 10% neutral formalin and stained with hematoxylin. Magnification 1,000X.
Fresh semen was collected with an artificial vagina and concentration of spermatozoa determined by turbimetric means (Willett and Buckner, 1951). Frozen semen was thawed in a 35°C water bath for 1 min and concentration of spermatozoa determined by counting using a hemacytometer.

An aliquot of semen was used to inseminate 100-μl drops of standard medium under paraffin oil immediately after collection or thawing and the remainder diluted with 2 ml of standard medium. Spermatozoa were washed by centrifugation (4 min at 600 x g) an the supernatant removed by aspiration. Resulting packed cells were resuspended in 2 ml of standard or HIS medium and incubated for 10 min. At the end of the incubation period, spermatozoa were recentrifuged and resuspended in 2 ml of standard medium and used to inseminate microdrops of standard medium under paraffin oil. In all cases, the final concentration of spermatozoa in the microdrops was ~10^6 cells/ml.

In Vitro Fertilization. Spermatozoa treated as previously described were preincubated for 3 h before five to 10 ovulated oocytes were added per drop. Spermatozoa and oocytes were incubated together for 6 h on a platform rocker in a humidified incubator under a 5% CO₂ in air atmosphere. After 6 h, oocytes were transferred to fresh drops of standard medium and cultured for an additional 24 h. At the end of culture oocytes were fixed in 10% neutral formalin and stained with hematoxylin. Stained oocytes were observed for the presence of spermatozoa within the vitellus, formation of male and female pronuclei and multiple nuclei in apparently cleaved oocytes (figures 1 and 2). The proportion of oocytes penetrated or undergoing division for the different treatments was analyzed by chi-square procedures (Steel and Torrie, 1960).

**Experimental Design.** In vitro fertilization was attempted on 126 recently ovulated oocytes in eight replicates of the experiment. All three spermatozoal treatments described above were represented in each replicate. All oocytes collected for each replicate were pooled and assigned randomly to the treatments. In five replicates (51 oocytes) fresh semen was used and in three replicates (75 oocytes) frozen semen was used.

**Results and Discussion**

There was no difference (P>.10) in fertiliza-
TABLE 1. IN VITRO FERTILIZATION OF BOVINE OOCYTES BY SPERMATOZOA CAPACITATED IN VITRO

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. penetrateda/ no. inseminated</th>
<th>% penetrated</th>
<th>No. dividing</th>
<th>% dividing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation in standard medium</td>
<td>3/34</td>
<td>9b</td>
<td>0</td>
<td>0d</td>
</tr>
<tr>
<td>Washed with standard medium and preincubation in standard medium</td>
<td>20/45</td>
<td>44c</td>
<td>3</td>
<td>7de</td>
</tr>
<tr>
<td>Treatment with HIS medium and preincubation in standard medium</td>
<td>14/47</td>
<td>30c</td>
<td>5</td>
<td>11e</td>
</tr>
</tbody>
</table>

aOocytes dividing are included in the number penetrated.
b,c,d,ePercentages in the same column with different superscripts differ (P<.05).

fertilization rates observed when fresh semen was used compared with frozen semen, thus these results were pooled for comparison of fertilization rates between different spermatozoal treatments.

Results of in vitro fertilization trials are summarized in table 1. Bovine spermatozoa simply preincubated in standard medium were not effectively capacitated because only three of 34 (9%) oocytes were penetrated in this treatment. One oocyte had a spermatozoan within the vitellus, another had a swollen sperm head and the third had two pronuclei. Spermatozoa pretreated for 10 min with either standard or HIS medium followed by a second wash penetrated oocytes more readily (44% and 30%, respectively). When spermatozoa were pretreated with standard medium three of the 20 penetrated oocytes had a swollen sperm head and the remainder had two pronuclei or formed two-cell embryos with a nucleus in each blastomere. When spermatozoa were pretreated with HIS medium all 14 penetrated oocytes had two pronuclei or formed two-cell embryos with a nucleus in each blastomere. These results are comparable to those observed with rabbit spermatozoa. Ejaculated (Brackett and Oliphant, 1975) and epididymal (Brackett et al., 1978; Hosoi et al. 1981) rabbit spermatozoa washed once penetrated a low percentage of oocytes, while spermatozoa treated with standard or HIS medium penetrated oocytes readily.

There was no difference (P>.10) between penetration rates of oocytes after insemination with spermatozoa pretreated with standard (44%) or HIS medium (30%). Rabbit spermatozoa treated with standard or HIS medium also penetrated oocytes at equal rates (Brackett and Oliphant, 1975; Brackett et al., 1978; Hosoi et al., 1981). No attempt was made in this study to monitor removal of seminal plasma components during exposure to standard or HIS medium. However, when the removal of seminal plasma antigens from rabbit spermatozoa was monitored (Brackett and Oliphant, 1975) the same standard medium used in this study removed only a small fraction of such antigens during a 20-min exposure. The results of this study and those conducted with rabbit spermatozoa suggest that whatever change occurs during capacitation in this system, it is not related to any change induced by HIS medium. The fertilization rate observed in this study with spermatozoa pretreated with standard medium are comparable to those reported by Brackett et al. (1982) with spermatozoa treated with HIS medium. In that study a 43% fertilization rate was observed with 15% dividing in culture and one oocyte resulting in a live calf following transfer to the oviduct of a recipient cow.

In these experiments, penetration of oocytes was observed with frozen semen as well as with fresh semen. Brackett et al. (1980) observed no penetration in a limited number of bovine oocytes when frozen semen was used, while penetration was observed with fresh semen. In the present study, all penetration by spermatozoa simply preincubated in standard medium were in experiments employing frozen semen. When spermatozoa were pretreated with standard or HIS medium, fertilization rates were similar for frozen and fresh semen.
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Literature Cited


