EFFECT OF ORVUS ES PASTE ON ACROSOME MORPHOLOGY, MOTILITY AND FERTILIZING CAPACITY OF FROZEN-THAWED BOAR SPERM

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SUMMARY

In four experiments the surfactant, Orvus ES Paste (OEP) was incorporated in semen extender to determine its effects on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. In experiment 1, the effects of 0, .5, 1, 1.5 and 2% OEP in the Beltsville F5 (BF5) extender containing 20% egg yolk were compared. Maximum post-thaw results for percentage of acrosomes with a normal apical ridge (NAR) were obtained with 1 to 1.5% OEP. In experiment 2, incubation of boar sperm for 1 hr with as little as .1% OEP in an extender devoid of egg yolk had a marked deleterious effect on acrosome morphology and sperm motility. In experiment 3, the beneficial effect of OEP on acrosomes of boar sperm was more pronounced after the 2-hr cooling rate than after the 6- or 12-hr cooling rates. In contrast, the beneficial effect of OEP on sperm motility was independent of cooling rate. In experiment 4, the competitive fertilizing capacity of boar sperm frozen in BF5 containing 0 or .5% OEP was compared. Number of ova fertilized by sperm frozen with 0 or .5% OEP (43 vs 81) differed from a 50:50 ratio (P<.01). These results indicate that OEP was a beneficial component of the extender and significantly enhanced cryopreservation of boar sperm under conditions employed.

(Key Words: Competitive Fertilization, Acrosomes, Motility, Swine Sperm, Frozen Semen.)

INTRODUCTION

Even though sperm were the first mammalian cells to be successfully frozen and thawed (Polge et al., 1949), freeze preservation of sperm from most species of animals has been achieved only in recent years. The primary deterrent to development of procedures to freeze mammalian sperm has been the paradoxical situation whereby glycerol acts as a cryo-protective agent toward sperm motility of most species, but reduces the fertilizing capacity of swine (Polge, 1956; Graham et al., 1971), fowl (Polge, 1951; Neville et al., 1970) and horse sperm (Demick et al., 1976).

Boar sperm are particularly sensitive to glycerol during storage at 20 C and during freezing and thawing (Wilmot and Polge, 1974). When boar semen was frozen in 4 to 8% glycerol, the level reported for maximum post-thaw motility, then no fertility was obtained (Polge, 1956; Settergren, 1958; King and Macpherson, 1967). However, successful pregnancies with intracervical insemination of frozen boar semen were obtained with 0 to 2% glycerol (Crabo and Einarsson, 1971; Graham et al., 1971; Pursel and Johnson, 1971).

Research directed toward finding more suitable cryoprotective compounds have met with limited success (Graham and Crabo, 1972; Wilmot and Polge, 1977). Graham et al. (1971) reported that addition of Orvus ES Paste (OEP), a synthetic detergent and wetting agent, to a semen extender decreased freeze-thaw damage to boar sperm. Subsequently, OEP has been incorporated into several other boar semen extenders (Romeny et al., 1974; Pursel and Johnson, 1975).

Experiments reported here were conducted to obtain additional information about the effects of OEP on acrosome morphology, motility and fertilizing capacity of frozen boar sperm.

MATERIALS AND METHODS

Boar semen was collected by the gloved-hand technique into a 250-ml insulated vacuum bottle; the opening was covered with a milk filter disc to...
separate the gel particles. Semen was fractionated visually into sperm-rich and sperm-poor portions at collection with only the sperm-rich portion being used. Each ejaculate served as a replicate in experiments 1, 2 and 3.

Experiment 1. This experiment was conducted to (1) determine whether inclusion of OEP in the Beltsville F5 extender (BF5) would affect post-thaw acrosome morphology and motility of boar spermatozoa and (2) determine the optimum concentration of OEP in the BF5 extender.

Semen from two ejaculates from each of eight boars was processed by the Beltsville procedure (Pursel and Johnson, 1975), which included a 2-hr holding time after collection, removal of seminal plasma by centrifugation, resuspension in BF5 extender, cooling from 22 to 24 C to 5 C over a 2-hr period, dilution with BF5 containing 2% glycerol, and freezing in .2 ml pellets on dry ice. The BF5 extender was prepared using 0, .5, 1, 1.5 or 2% OEP and 20% egg yolk (Pursel and Johnson, 1975). The insoluble fraction of egg yolk was removed by centrifugation at 12,000 g for 10 minutes. Each sample contained 5 ml of pelletated semen and a total of $3 \times 10^9$ sperm. After being stored 1 to 3 days in liquid nitrogen, pellets were removed, held in a Styrofoam container for 3 min and then thawed in 12.5 ml of Beltsville thawing solution (BTS) that was prewarmed to 50 C (Pursel and Johnson, 1975).

Experiment 2. This experiment was conducted to determine whether OEP would have a direct effect on sperm when egg yolk was not present in the extender. Semen from one ejaculate from each of five boars was used. Aliquots containing $3 \times 10^9$ sperm were centrifuged at 300 g for 10 min, and seminal plasma was removed by aspiration. Sperm were resuspended with 2.5 ml of BF5 extender containing 20% egg yolk and .5% OEP or 2.5 ml of BF5 extender containing 0% egg yolk and 0, .1, .3 or .5% OEP. Samples were not frozen but were held at 22 to 24 C for 1 hr, and then motility and acrosome morphology were assessed.

Experiment 3. This experiment was conducted to determine whether there was a relationship between OEP and cooling rate. Semen was used from one or two ejaculates from each of 10 boars. Semen was processed as described in Experiment 1 using BF5 extender containing 0 or .5% OEP and 20% egg yolk. The extended semen was cooled from 22 to 24 C to 5 C over a period of 2, 6 or 12 hr in waterbaths with differing insulation properties and differing volumes of water before being frozen. Six pellets per sample were thawed in 2.5 ml of BTS that was preheated to 50 C.

Experiment 4. This experiment was designed to compare the competitive fertilizing capacity of boar sperm frozen in BF5 extender containing 0 or .5% OEP. Semen from two or three boars was pooled and redivided into aliquots containing $3 \times 10^9$ sperm. Sperm in one-half of the aliquots were marked (T) with 2 mg of TEPA (Tris(l-aziridinyl)phosphine oxide) per $10^9$ sperm for 10 min, using the procedure described by Pursel et al. (1975), and those in the other half of the aliquots were unmarked (C). The marked and unmarked sperm were frozen in BF5 extender containing 0 or .5% OEP and 20% egg yolk. Ova fertilized by TEPA-marked sperm have retarded cleavage and can be distinguished from ova fertilized by unmarked sperm.

Each sample of $3 \times 10^9$ sperm was thawed in 20 ml of BTS that had been preheated to 50 C. The two treatment samples of $3 \times 10^9$ sperm each were combined just before insemination, i.e., T with 0% OEP and C with .5% OEP or T with .5% OEP and C with 0% OEP. Twenty-one gilts were inseminated with the mixed semen. Gilts were inseminated twice per estrous period with $6 \times 10^6$ sperm per insemination using a spiral-tip catheter.

Ova were recovered about 162 hr after onset of estrus, fixed in acetic alcohol for at least 24 hr, stained with aceto orcein, and examined as whole mounts for cleavage and nuclear development. Ova that contained pronuclei to four nuclei were considered to have been fertilized by a TEPA-marked sperm. Ova that had developed into morula or blastocysts were considered to have been fertilized by an unmarked sperm (Pursel et al., 1975).

Sperm Evaluation. Immediately after the pellets were thawed two 1-ml sub-samples were removed. One sub-sample was held at 37 C for 30 min after which the percentage of motile sperm was estimated at 37 C by phase-contrast microscopy at 250X. The other sub-sample was used for acrosome examination by phase-contrast microscopy as described by Pursel et al. (1972). Two slides were examined for each sample with 100 acrosomes assessed per slide and the percentage of sperm with a normal apical ridge (NAR) was determined. All samples were coded before evaluation so that sample identity was not known to the evaluator.

Statistical Analysis. The percentages of NAR acrosomes and motility were subjected to arcsin transformation before analysis of variance. In experiments 1 and 2, orthogonal comparisons were performed. In experiment 4, the proportions of ova fertilized were analyzed by chi-square.
TABLE 1. EFFECT OF CONCENTRATION OF OEP ON POST-THAW ACROSOME MORPHOLOGY AND MOTILITY OF BOAR SPERM

<table>
<thead>
<tr>
<th>OEP (%)</th>
<th>NAR acrosomes %</th>
<th>Motility %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>56 ± 2.4a</td>
<td>36 ± 2.4</td>
</tr>
<tr>
<td>.5</td>
<td>63 ± 2.3</td>
<td>42 ± 2.5</td>
</tr>
<tr>
<td>1</td>
<td>67 ± 1.2</td>
<td>41 ± 2.4</td>
</tr>
<tr>
<td>1.5</td>
<td>68 ± 2.1</td>
<td>36 ± 1.8</td>
</tr>
<tr>
<td>2</td>
<td>61 ± 2.0</td>
<td>27 ± 2.3</td>
</tr>
</tbody>
</table>

aMean (± SE) for 16 replications.

RESULTS AND DISCUSSION

Experiment 1. The post-thaw percentages of NAR acrosomes and motile sperm differed among treatments (P<.005 for each comparison, table 1). These percentages changed linearly and quadratically as the level of OEP increased (P<.005 for each comparison). The quadratic response resulted from increasing percentages for .5 to 1.5% OEP concentrations and from decreasing percentages for the 2% OEP concentration.

These results confirm the original observations of Graham et al. (1971) regarding OEP's providing a beneficial effect during the freezing of boar sperm. However, the present results indicated that 1 to 1.5% OEP was the optimum concentration for the sperm acrosomes and .5 to 1% OEP resulted in the highest post-thaw motility. Graham and Crabo (1972) reported that .25% OEP was the optimum concentration for post-thaw motility under their conditions.

Experiment 2. Boar sperm incubated for 1 hr at 25 C with as little as .1% OEP in the extender devoid of egg yolk had a marked deleterious effect on acrosome morphology and sperm motility (table 2). In contrast, the same extender containing 20% egg yolk and .5% OEP had no detrimental effect on either acrosome morphology or sperm motility as compared to the extender containing neither egg yolk nor OEP. The percentage of NAR acrosomes and motile sperm decreased linearly as the concentration of OEP in the extender (without egg yolk) increased (P<.005 for each comparison).

These results lend support to the hypothesis of Graham et al. (1971) that OEP exerts its beneficial action through alteration of egg yolk constituents rather than the hypothesis of a direct effect of OEP on the cellular membranes (Graham et al., 1971).

Experiment 3. The post-thaw percentages of NAR acrosomes and motile sperm were higher for those samples frozen with OEP in the extender than those frozen without OEP (P<.005 for each comparison, table 3). The percentage of NAR acrosomes for boar semen processed without OEP increased linearly as the cooling time increased. In contrast, the percentage of NAR acrosomes for boar semen processed with .5% OEP was little affected by cooling time. This difference resulted in a cooling time x OEP interaction (P<.005). Post-thaw motility did not show a similar interaction.

These results indicate that the beneficial effect of OEP on the acrosomes of boar semen was more

TABLE 2. EFFECTS OF OEP AND ABSENCE OR PRESENCE OF EGG YOLK ON ACROSOME MORPHOLOGY AND MOTILITY OF UNFROZEN BOAR SPERM AFTER 1 HR OF INCUBATION AT 25 C

<table>
<thead>
<tr>
<th>Extender Composition</th>
<th>% egg yolk</th>
<th>NAR acrosomes %</th>
<th>Motility %</th>
</tr>
</thead>
<tbody>
<tr>
<td>% OEP</td>
<td>20</td>
<td>92 ± 2.1a</td>
<td>86 ± 2.4</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>93 ± 1.4</td>
<td>52 ± 10.7</td>
</tr>
<tr>
<td>.5</td>
<td>0</td>
<td>71 ± 8.8</td>
<td>38 ± 10.7</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>32 ± 14.8</td>
<td>22 ± 12.7</td>
</tr>
<tr>
<td>.5</td>
<td>0</td>
<td>21 ± 14.2</td>
<td>18 ± 13.5</td>
</tr>
</tbody>
</table>

aMean (± SE) for five replications.

TABLE 3. EFFECTS OF COOLING TIME AND ABSENCE OR PRESENCE OF OEP ON POST-THAW ACROSOME MORPHOLOGY AND MOTILITY OF BOAR SPERM

<table>
<thead>
<tr>
<th>Cooling time (hr)</th>
<th>OEP (%)</th>
<th>NAR acrosomes %</th>
<th>Motility %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>35 ± 3.0a</td>
<td>15 ± 2.4</td>
</tr>
<tr>
<td>6</td>
<td>.5</td>
<td>52 ± 3.8</td>
<td>28 ± 4.1</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>48 ± 3.1</td>
<td>14 ± 2.3</td>
</tr>
</tbody>
</table>

aMean (± SE) for 16 replications.
TABLE 4.  Fertilizing Capacity of Boar Sperm Frozen with or Without .5% OEP

<table>
<thead>
<tr>
<th>Sperm mixture&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of gilts</th>
<th>Insem.</th>
<th>With fert. ova</th>
<th>.5%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/.5% + C/0%</td>
<td>11</td>
<td>6</td>
<td>37 (T)</td>
<td>15 (C)</td>
<td></td>
</tr>
<tr>
<td>C/.5% + T/0%</td>
<td>10</td>
<td>6</td>
<td>44 (C)</td>
<td>28 (T)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>12</td>
<td>81 (65.3)</td>
<td>43 (34.7)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>T = TEPA, C = control, and 0 and .5% = OEP.

pronounced after the 2-hr cooling rate than after the 6- or 12-hr cooling rates. In contrast, the beneficial effect of OEP on sperm motility was independent of the cooling rate.

**Experiment 4.** Fertilized ova were recovered from 12 of the 21 gilts inseminated with the sperm mixtures (table 4). A total of 150 ova were recovered from the 12 gilts; 59 ova had cleaved normally (16 or more nuclei), 65 ova had delayed cleavage caused by fertilization with a TEPA-treated sperm (four nuclei or less), 25 ova were unfertilized, and one ova was not classifiable (nine nuclei). The number of ova fertilized by sperm frozen with 0 or .5% OEP, 43 and 81 ova, respectively, differed significantly from a 50:50 ratio (P<.01, table 4). The post-thaw percentages of NAR acrosomes (67.3 vs 50.6) and motile sperm (43.3 vs 22.5%) were higher (P<.005) for sperm in .5% OEP than for those in 0% OEP.

TEPA-marked sperm fertilized 52% of the fertilized ova that were recovered. These results confirm our earlier observation that TEPA-marked sperm compete equally well with unmarked sperm (Pursel et al., 1975).

These results indicate that the use of .5% OEP in the BF5 extender significantly enhanced the preservation of fertilizing capacity concomitant with an increase in post-thaw percentages of NAR acrosomes and motile sperm. These results are based on fertilized ova from only 12 gilts, however, if the present competitive fertilization trial was as sensitive to fertility differences in swine as was reported for cattle (Beatty et al., 1969) the results would be equivalent to an insemination trial involving over 2000 gilts. Beatty et al. (1969) found that a competitive fertility test using a genetic marker increased the sensitivity of estimating relative fertility by more than 170 times that of the usual insemination trial in cattle. Martin and Dziuk (1977) also reported greater sensitivity of the competitive fertility test as compared to the usual insemination trials in chickens and swine.

**LITERATURE CITED**


