Effect of rapid warming of boar semen on sperm morphology and physiology

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Summary. The effect of rapid dilution (1:8 with BTS or 1:6.5 with KRP) and temperature change on sperm morphology and physiology were studied using boar spermatozoa pre-diluted in BF5 diluent. Rapid dilution of cold semen (5°C) with a warm solution (37°C) caused marked acrosomal changes which were most prominent in the anterior region. The acrosomal damage appeared to be caused mainly by rapid warming. In contrast to rapid cooling, rapid warming had little effect upon motility, glutamic-oxaloacetic transaminase release and respiration.

Introduction

It is well known that the rapid cooling of semen causes irreversible changes in the morphology and physiology of spermatozoa, although it has been considered that rapid warming of cold semen has no such effect (Watson, 1981). However, damage due to warming after thawing occurs in bovine semen (Senger, Becker & Hillers, 1976; De Abreu, Berndtson, Smith & Pickett, 1979).

It is generally accepted that boar spermatozoa require fast rates of freezing and thawing (Polge, 1976) and if rapid warming does have deleterious effects these would most likely occur if frozen pellets were rapidly warmed by being placed in a hot thawing solution. In the present study, therefore, the effect of rapid warming and dilution on the morphology and some physiological aspects of boar spermatozoa have been examined.

Materials and Methods

Preparation of spermatozoa

The sperm-rich fraction of semen from mature boars was cooled to 15°C over a 2-h period and then diluted 1:2 with Hülsenberg VIII diluent (Richter, Romeny, Weitze & Zimmermann, 1975; 5.75 g glucose, 0.25 g lactose, 0.45 g trisodium citrate dihydrate, 0.35 g disodium EDTA, 0.12 g NaHCO₃ and 0.04 g KCl per 100 ml). Immediately after dilution, the suspension was centrifuged at 350 g for 15 min, the supernatant was discarded and the spermatozoa were resuspended in BF5 diluent (Pursel & Johnson, 1975; 1.2 g N-Tris-(hydroxymethyl)methyl-2-aminomethane sulphonic acid, 0.2 g Tris(hydroxymethyl)aminomethane, 3.2 g glucose, 0.5 ml Orvus ES Paste (Proctor and Gamble, Cincinnati, Ohio) and 20 ml egg yolk per 100 ml) to half the volume of the original sperm-rich fraction. The diluted sperm suspension was next cooled to 5°C over 1.5 h and diluted further 1:1 with BF5 diluent containing 2% (v/v) glycerol. It was stored at 5°C for up to 1 h until subjected to experimental treatments. Semen samples at 5°C were divided into two: one was kept at 5°C and the other slowly warmed to 37°C by transferring 6 ml in a test tube (16 × 120 mm) to a water bath at 37°C. The following treatments were made:
The semen was diluted by rapidly pipetting 0.2 ml semen into 1.6 ml BTS (Pursel & Johnson, 1975; 3.7 g glucose, 0.6 g trisodium citrate dihydrate, 0.125 g NaHCO₃, 0.125 g disodium EDTA and 0.075 g KCl per 100 ml). Both samples at 5°C were allowed to equilibrate for 1 min and then warmed to 37°C by transferring the test tube to the water bath.

**Experiment 1: influences on motility, acrosomal morphology and glutamic-oxaloacetic transaminase release**

Motility was estimated after 0, 1, 3 and 5 h incubation at 37°C by placing 10 µl semen between a coverslip (18 × 18 mm) and a slide coated with anhydrous caffeine (Bamba & Kojima, 1978) and examining on a warm stage at 37°C. Motility was expressed as the percentage of progressively motile spermatozoa.

For the evaluation of acrosomal morphology, semen was diluted 1:1 with formal citrate after 0, 1, 3 and 5 h incubation. Part of the sperm suspension (10 µl) was placed between a coverslip and a slide and examined using differential interference-contrast microscopy at a magnification of 750; 200 spermatozoa were examined from each sample and the proportion possessing a smooth crescent-shaped apical ridge (normal acrosome) was estimated.

Duplicate samples were centrifuged at room temperature (1000 g, 10 min) immediately after the rapid dilution treatments, and the supernatants were analysed for glutamic-oxaloacetic transaminase (EC 2.6.1.1) (Okuda, 1973). Glutamic-oxaloacetic transaminase activity was expressed as Karmen units (one unit is the amount of transaminase in a 1 ml sample which decreases the absorbance of NADH at 340 nm by 0.001 in 1 min at 37°C). The results were expressed as units/ml of sample containing 10⁸ cells. Replicate tests were made on 6 ejaculates, one from each of 6 boars. Data were subjected to analyses of variance and the differences between treatment means were tested by Tukey’s procedure (Steel & Torrie, 1960).

**Experiment 2: influences on respiration**

Samples for the measurement of respiration were treated as in Exp. 1 except that 1 ml semen was rapidly diluted with 6.5 ml Ca²⁺-free Krebs–Ringer–phosphate buffer (Umbreit, Burris & Stauffer, 1957). A 3-ml sample of the diluted sample was immediately transferred to a 20-ml Warburg flask containing 0.3 ml 20% (w/v) KOH solution in the centre well. The flask was shaken at 120 strokes per min. After 10 min equilibration at 37°C, oxygen uptake was measured for 1 h. Motility and acrosomal evaluations were made on samples before measurement of respiration. Replicate tests were made on 4 ejaculates, one from each of 4 boars.

**Experiment 3: influences on ultrastructure**

Samples for transmission electron microscopy were processed as described by Cran, Dott & Wilmington (1982). For scanning electron microscopy, samples were fixed in BTS containing 1% (w/v) formaldehyde and 1% (w/v) glutaraldehyde, mounted on polylysin-coated coverslips and passed through graded alcohols to amyl acetate. They were critical-point dried and coated with gold.
Rapid warming of boar semen

Results

Rapid dilution + warming caused an immediate decrease in the proportion of normal acrosomes (Table 1) which was comparable to that of rapid dilution + cooling treatment. Rapid dilution at 37°C resulted in a lower proportion of normal acrosomes compared to rapid dilution at 5°C and, further, the percentage of normal acrosomes present after 5 h incubation as a proportion of those at 0 h was less at 37°C than at 5°C. However, the decrease was very much less than the effect of rapid dilution + warming. Rapid dilution + cooling caused an immediate decrease in motility, whereas loss of motility occurred only with a time lag of some 3 h with rapid dilution + warming. However, the effect of rapid dilution + warming on motility and rapid dilution at 37°C on acrosomal morphology differed considerably between ejaculates. The amount of glutamic-oxaloacetic transaminase released was not affected by rapid dilution + warming.

Table 1. Effect of rapid dilution and temperature change of boar semen on acrosomal morphology, sperm motility and release of glutamic-oxaloacetic transaminase (GOT)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 h</th>
<th>1 h</th>
<th>3 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal acrosomes (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapid dilution at 5°C</td>
<td>89.5±4.4</td>
<td>77.1±10.0</td>
<td>67.5±9.4</td>
<td>58.5±19.9</td>
</tr>
<tr>
<td>Rapid dilution + warming</td>
<td>12.0±8.5**</td>
<td>5.8±5.7**</td>
<td>4.0±3.6**</td>
<td>2.5±2.3**</td>
</tr>
<tr>
<td>Rapid dilution at 37°C</td>
<td>66.8±19.1*</td>
<td>54.5±25.1*</td>
<td>44.5±20.9*</td>
<td>35.6±21.8*</td>
</tr>
<tr>
<td>Rapid dilution + cooling</td>
<td>9.1±3.6**</td>
<td>1.6±1.5**</td>
<td>2.1±3.1**</td>
<td>1.5±3.6**</td>
</tr>
<tr>
<td>Motility (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapid dilution at 5°C</td>
<td>85.0±0</td>
<td>81.6±6.0</td>
<td>74.1±3.7</td>
<td>70.8±10.2</td>
</tr>
<tr>
<td>Rapid dilution + warming</td>
<td>84.1±2.0</td>
<td>80.8±3.7</td>
<td>49.1±18.8**</td>
<td>30.3±21.3**</td>
</tr>
<tr>
<td>Rapid dilution at 37°C</td>
<td>85.0±0</td>
<td>83.3±2.5</td>
<td>76.6±2.5</td>
<td>75.0±3.1</td>
</tr>
<tr>
<td>Rapid dilution + cooling</td>
<td>8.5±5.7**</td>
<td>5.5±5.8**</td>
<td>3.3±5.8**</td>
<td>0.3±0.8**</td>
</tr>
<tr>
<td>GOT (units)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapid dilution at 5°C</td>
<td>79.5±31.2</td>
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<td></td>
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<tr>
<td>Rapid dilution + warming</td>
<td>72.8±20.7</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rapid dilution at 37°C</td>
<td>72.4±20.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapid dilution + cooling</td>
<td>102.2±27.4*</td>
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</tbody>
</table>

Values are mean ± s.d. for 6 observations.
Means within the same column statistically different from the value for rapid dilution at 5°C: *P<0.05, **P<0.01.

Table 2. Effect of rapid dilution and temperature change on respiration of boar semen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O₂ uptake (µl/10⁸ cells for 1 h at 37°C)</th>
<th>Normal acrosomes (%)</th>
<th>Motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid dilution at 5°C</td>
<td>9.6±2.5</td>
<td>83.0±4.5</td>
<td>72.5±6.4</td>
</tr>
<tr>
<td>Rapid dilution + warming</td>
<td>9.5±2.3</td>
<td>1.7±1.7**</td>
<td>68.7±8.5</td>
</tr>
<tr>
<td>Rapid dilution at 37°C</td>
<td>10.1±3.1</td>
<td>69.0±9.0</td>
<td>68.7±6.2</td>
</tr>
<tr>
<td>Rapid dilution + cooling</td>
<td>7.5±3.8</td>
<td>10.0±6.1**</td>
<td>5.0±10.0**</td>
</tr>
</tbody>
</table>

Values are mean ± s.d. for 4 observations.
**Statistically significant from value for rapid dilution at 5°C (P<0.01).

The rate of respiration was not affected by rapid dilution + warming or by rapid dilution at 37°C (Table 2). The lowest respiration rate was observed in samples subjected to rapid dilution + cooling. Similar changes in acrosomal morphology and motility to those described above were recorded.
As shown in Plate 1, there were marked differences between normal spermatozoa (Pl. 1, Figs 1, 2 & 8), those subjected to rapid dilution + cooling (Pl. 1, Figs 3, 4 & 9) and rapid dilution + warming (Pl. 1, Figs 5, 6, 7, 10 & 11). With phase-contrast microscopy the surface of sperm heads after rapid dilution + cooling was seen to be very rough (Pl. 1, Fig. 3) and the heads had a fuzzy appearance (Pl. 1, Fig. 4). On the other hand, after rapid dilution + warming spermatozoa had a relatively smooth surface, but loss of the apical ridge (Pl. 1, Figs 5 & 6), swelling (Pl. 1, Fig. 5) and a dark area in the proximal part of the acrosome (Pl. 1, Fig. 6) were observed. In eosin–nigrosin smears, damaged acrosomes were observed in spermatozoa after rapid dilution + warming, but they were not stained with eosin (Pl. 1, Fig. 7).

Scanning electron micrographs demonstrated that after rapid dilution + cooling most spermatozoa had an irregular protrusion along the apex of the head (Pl. 1, Fig. 9). In such spermatozoa the equatorial segment was often visible (Pl. 1, Fig. 9). The general appearance of spermatozoa after rapid dilution + warming was similar to that in Pl. 1, Fig. 5. However, most exhibited a rough surface on the proximal part of the acrosomes (Pl. 1, Figs 10 & 11). In a few cases, a distinct wrinkling across the middle part of the acroome was observed (Pl. 1, Fig. 11). When no temperature changes were involved during rapid dilution no discernible changes in the ultrastructure of the spermatozoa could be detected by transmission electron microscopy (Pl. 2, Fig. 12). Rapid dilution + warming, however, induced marked changes which were most prominent in the anterior region of the acrosomes (Pl. 2, Figs 13 & 14). These involved an invagination of the outer acrosomal membrane into the acrosomal matrix, frequently producing extensive sheets of membrane. Concomitantly a tubular network was found within the acrosome which, as far as could be determined, was not physically connected with the vesiculation of the outer acrosomal membrane. Although both membrane forms were occasionally found in the equatorial segment, they were most common in the proximal region of the acroome.

Rapid dilution + cooling (Pl. 2, Fig. 15) also induced changes similar to those found after rapid dilution + warming. In addition, the plasma membrane frequently lifted off the surface of the spermatozoon. During rapid dilution + warming, the process of vesiculation was very rapid, the first indication being clearly evident within 15 sec after dilution (Pl. 2, Fig. 16). Prolonged

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**PLATE 1**

**Figs 1 and 2.** Spermatozoa with a normal acrosome. They have a smooth crescent-shaped apical ridge. Fig. 1, Nomarski; Fig. 2, phase contrast. × 1250.

**Figs 3 and 4.** Spermatozoa after rapid dilution + cooling. The surface of the sperm head is very rough (Fig. 3, Nomarski) and the head appearance is fuzzy (Fig. 4, phase contrast). × 1250.

**Figs 5 and 6.** Spermatozoa after rapid dilution + warming. Note loss of the apical ridge (Figs 5 & 6), swelling (Fig. 5, arrow; Nomarski) and a dark area in the proximal region of the acrosome (Fig. 6, arrow; phase contrast). × 1250.

**Fig. 7.** Spermatozoa stained with eosin and nigrosin after rapid dilution + warming. They have damaged acrosomes but are not stained with eosin. × 1250.

**Fig. 8.** Spermatozoa with normal acrosomes. SEM, × 7000.

**Fig. 9.** Spermatozoa after rapid dilution + cooling. Most of the spermatozoa have an irregular protrusion along the apex of the head (arrow). The equatorial segment (arrowhead) is visible in one spermatozoon. SEM, × 7000.

**Figs 10 and 11.** Spermatozoa after rapid dilution + warming. The surface of the head is relatively smooth, except in the proximal part. In some cases, a distinct wrinkling traverses the mid-part of the acrosome and demarcates a smooth and rough area of the head (Fig. 11, arrow). SEM; Fig. 10, × 7000; Fig. 11, × 15 000.
incubation at 37°C resulted in changes similar to those observed after rapid dilution + cooling, with detachment of the plasma membrane (Pl. 2, Fig. 17).

**Discussion**

The results of the present study showed clearly that rapid dilution of cold semen with a warm solution exerts a detrimental effect on the acrosome. The decrease in the proportion of normal acrosomes was significantly greater in samples subjected to rapid dilution + warming than to rapid dilution at 37°C. This observation suggests that the acrosomal damage caused by rapid dilution + warming is mainly due to the rapid warming component and that dilution itself results in a greater temperature change being detected by individual spermatozoa than otherwise would have been the case. Preliminary experiments showed that such acrosomal damage occurred regardless of type of diluents (including seminal plasma) for both pre-dilution and rapid dilution + warming; therefore the acrosomal change appears to be a common reaction to rapid warming.

According to Senger *et al.* (1976), post-thaw warming of bovine spermatozoa damages the acrosomes. However, the decrease in the proportion of normal acrosomes in boar spermatozoa was much greater than that observed in bovine spermatozoa, and may be due to the difference in the warming rates and/or to the susceptibility of boar and bovine spermatozoa to stress. In another preliminary study, the change of semen temperature was recorded during rapid dilution + warming treatment (Yokogawa-Hokushin ER 182 recorder with iron-constantan thermocouple, Tokyo). In boar semen the temperature rose from 5 to about 33°C in 1 sec and then to 37°C in about 20 sec. On the other hand, the warming rate of bovine semen employed by Senger *et al.* (1976) was slower (about 15 sec from 0 to 30°C). In addition, the susceptibility of spermatozoa to rapid warming appears to vary with species; bovine spermatozoa are not as sensitive as those of boar and rabbit (K. Bamba, C. E. Adams & D. G. Cran, unpublished observation).

In contrast to the effect of rapid cooling, the effect of rapid warming was specific for acrosomes. Motility, glutamic-oxaloacetic transaminase release and respiration were little affected. In addition, spermatozoa remained unstained with eosin after rapid warming, suggesting that the plasma membranes were also little affected. These characteristics of rapid warming appear to be similar to

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**PLATE 2**

**Fig. 12.** Spermatozoa after rapid dilution at 37°C. There is no evidence of structural alteration. × 15 000.

**Fig. 13.** Section through the tip of a spermatozoon after rapid dilution + warming. Prominent invaginations of the outer acrosomal membrane forming extensive lacunae within the acrosome are present (arrows) together with some tubules (arrowheads). × 40 000.

**Fig. 14.** Apical region of a spermatozoon treated as in Fig. 2. The acrosome at the apical ridge is filled with a tubular network. × 40 000.

**Fig. 15.** Spermatozoon after rapid dilution + cooling. The plasma membrane (arrow) has lifted from the surface of the cell and there has been a loss of acrosomal content in the apical region of the acrosome (arrowhead). × 26 500.

**Fig. 16.** Spermatozoon after rapid dilution + warming and incubated at 37°C for 15 sec. There are marked undulations of the outer acrosomal membrane together with prominent invaginations. × 30 000.

**Fig. 17.** Spermatozoon treated as in Fig. 16 but incubated for 3 h at 37°C. There has been marked swelling of the acrosome and loss of its content. Note the retention of a region of density at the original site of the apical ridge (arrow). × 30 000.
those of glycerol in several respects; acrosomal vesiculation occurred in the presence of glycerol at 5°C and its occurrence did not influence the motility and metabolism of boar spermatozoa (Jones, 1973; Murdoch & Jones, 1978). As far as could be determined by differential interference-contrast microscopy, the head of rapidly warmed spermatozoa was comparatively smooth. However, scanning electron micrographs revealed ‘roughness’ and wrinkling in the proximal region of the acrosome. Ultrastructural examination by transmission electron microscopy showed a tubular network in the acrosomal matrix induced by rapid warming. The change is similar to that of ageing (Jones, 1973) and of deep-frozen boar spermatozoa (Larsson, Einasarsson & Nicander, 1976). The existence of these ultrastructural changes in rapidly warmed spermatozoa suggests that they would become more critical after prolonged incubation in vitro and in vivo.

Frozen pellets of pig semen are thawed in a warm thawing solution to obtain rapid thawing rates. Under these conditions, rapid dilution and warming should be taken into account as a stress experience in addition to freezing and thawing. So far as the thawing method described by Pursel & Johnson (1975) is concerned, the possibility of rapid warming which may cause acrosomal damage appears to be unlikely; when pellets (10 ml) were dropped into 25 ml BTS at 50°C, they thawed in about 20 sec and the terminal temperature of the thawed semen was about 20°C. However, it may become critical when more thawing solution and/or a higher thawing temperature are employed.

Further work is necessary to elucidate the mechanism of warm shock in relation to that of cold shock. The fact that the acrosomes of boar spermatozoa are very sensitive to rapid warming indicates a need for further experiments of the method of thawing frozen semen from this species.

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References


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