Further studies on rapid dilution and warming of boar semen

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Summary. Studies have been carried out to investigate factors related to the induction of warm shock in boar spermatozoa. Rapid dilution *per se* caused visible damage to acrosomes when the sample contained 7.5% or more glycerol. This dilution effect was greater at lower temperatures. Acrosomal damage was greatly reduced by raising the dilution temperature from 15 to 25°C, suggesting that a change in the physico-chemical characteristics of the acrosomal membrane occurred between these temperatures. During rapid dilution with warming, the dilution rate, the magnitude of the temperature change and the terminal temperature had a significant influence on acrosomal integrity; a terminal temperature of 35°C was much more detrimental than one of 25°C. The first sign of acrosomal damage was observed 15 sec after rapid dilution + warming and the damage was nearly maximal by 60 sec. An antioxidant, butylated hydroxytoluene (BHT), was effective against both rapid cooling and warming, while glycerol, dimethylsulphoxide and propylene glycol were ineffective in preventing warm shock.

Keywords: boar spermatozoa; warming and dilution; acrosome; structure

Introduction

Boar spermatozoa are severely damaged by warm shock (Bamba & Cran, 1985). It is likely that this is a very important factor during the thawing of frozen semen. In view of this, it is clear that further studies are required to elucidate the mechanism(s) by which it acts and to find effective conditions for its prevention. The present communication describes a series of experiments designed to examine the effects of temperature, dilution rate, and cryoprotectants on the prevention or initiation of acrosomal damage associated with warm shock.

Materials and Methods

Semen. Boar semen was collected manually from mature boars and the sperm rich fraction was used. Epididymal spermatozoa were collected by flushing the lower part of each cauda epididymidis with Hülseberg VIII diluent (Richter *et al.*, 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). The testes were obtained from freshly slaughtered boars.

Preparation of spermatozoa. Ejaculated semen was processed in basically the same manner as described in the previous report (Bamba & Cran, 1985). Semen was cooled over 2 h to room temperature (20–25°C) and diluted with Hülseberg VIII diluent. Immediately after dilution the sperm suspension was centrifuged at 350 g for 15 min. Epididymal and ejaculated spermatozoa were then processed in a similar manner. The supernatant was discarded and the spermatozoa were resuspended in BF5 diluent (Pursel & Johnson, 1975; for details of this and BTS, see report by Bamba & Cran, 1985). The partly diluted spermatozoa were cooled to 5°C over 2 h and diluted 1:1 (v/v) with BF5 diluent containing cryoprotective agent; the dilution was made by adding two equal volumes at 10-min intervals. The diluted semen samples were subjected to experimental treatments immediately.

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Rapid dilution was performed by pipetting 0.2 ml of semen rapidly into a test tube containing 1.6 ml Medium BTS (Pursel & Johnson, 1975). Unless otherwise stated, the test tube was kept in a water bath at the given temperature. After rapid dilution, the sample was equilibrated to the given temperature for 2 min and then warmed to 35 or 37°C. Samples were equilibrated for at least 5 min before evaluation of sperm quality.

Motility was estimated as described by Bamba & Cran (1985) and was expressed as the percentage of progressively motile spermatozoa. Samples for acrosomal evaluation were diluted 1:1 (v/v) with Medium BTS containing 0.4% formaldehyde and examined by phase-contrast or differential interference-contrast microscopy at $\times 750$. At least 200 spermatozoa were examined for each sample. Samples before dilution (controls) were diluted 1:1 with formol-BTS at the same temperature. Photographs were taken of samples fixed in 1% glutaraldehyde and 0.4% formaldehyde.

Samples for electron microscopy were processed as described by Cran *et al.* (1982).

Replicate tests were made on ejaculates from different boars. Data were subjected to analyses of variance and the differences between treatment means were tested by Tukey's procedure (Steel & Torrie, 1960).

Results

Experiment 1: influence of glycerol concentration

Semen samples containing 0.0, 1.0, 2.5, 5.0, 7.5 and 10.0% (v/v) glycerol were rapidly diluted 1:8 (v/v) with Medium BTS as follows: (1) rapid dilution at 5°C, (2) rapid dilution + warming from 5 to 37°C and (3) rapid dilution at 37°C after gradual warming. Replicate tests were made on 5 ejaculates.

For all treatments the effect of glycerol was relatively small when at concentrations <5%. However, significantly lower percentages of normal acrosomes were present after dilution at 5°C with 7.5 or 10% glycerol (Table 1). The appearance of the damaged spermatozoa was similar to that following warm shock (Fig. 3). Rapid dilution at 37°C also resulted in acrosomal damage but this was not as severe as that following dilution at 5°C and it was further clear that the inclusion of glycerol did not prevent warm shock following rapid temperature elevation. The proportion of normal acrosomes was also lower following gradual warming regardless of glycerol concentration. As shown in Figs 1 and 2 the inclusion of 1 or 10% glycerol at 5°C did not result in vesiculation of the acrosomes. However, the apical ridges appeared to be slightly flattened and there were slight signs of invagination of the outer acrosomal membrane at a concentration of 10% glycerol. Acrosomal vesiculation took place in response to warm shock regardless of glycerol concentration.

Table 1. Effects of glycerol concentration and temperature on acrosomal morphology in rapid dilution treatments

Glycerol (%)	Temperature of semen:	5°C			37°C	
	Temperature of Medium BTS:	Control	5°C	37°C	Control	37°C
0		93.4	89.6	10.0	91.0	84.0
1.0		94.6	89.2	10.2	90.0	85.6
2.5		93.4	88.0	8.4	88.6	87.4
5.0		92.4	81.8	8.4	87.2	84.6
7.5		91.8	52.6**	6.2	85.8	77.6
10.0		88.4	6.8**	3.2	84.4	58.6**

Values are mean % of normal acrosomes for 5 observations.

**Significantly different from 0% glycerol value ($P < 0.01$).

Experiment 2: influence of the temperature of rapid dilution and glycerol concentration

Semen samples containing 1 or 10% glycerol were warmed gradually in a stepwise fashion from 5 to 15 to 25 to 37°C with 5 min at each temperature. At each temperature, the samples were rapidly diluted 1:8 (v/v) with Medium BTS at the same temperature as the samples. Replicate tests were made on 5 ejaculates.

Table 2. Effects of rapid dilution at various temperatures on acrosomal morphology

Temperature of dilution	Glycerol concentration	
	1%	10%
Before dilution (5°C)	90.2	84.8
Dilution at: 5°C	88.4	4.4**
15°C	86.6	9.2**
25°C	84.0	46.2**
37°C	78.8*	57.8**
After gradual warming (37°C)	82.8	81.0

Values are mean % of normal acrosomes for 5 observations.

Statistically significant from the value for before dilution at 5°C (* $P < 0.05$, ** $P < 0.01$).

Table 3. Time course of acrosomal change after rapid dilution + warming treatment

Incubation time after dilution	% normal acrosomes
Control (before dilution)	94.5
0 sec	92.7
15	74.7**
60	22.0**
300	10.5**

Values are means for 4 observations.

**Significantly different from control value ($P < 0.01$).

In the samples containing 1% glycerol the proportion of normal acrosomes decreased with a rise in the dilution temperature (Table 2). However, in those samples containing 10% glycerol there was a marked effect on acrosomal integrity which was ameliorated with temperature elevation. Rapid dilution at 5, 15 and 25°C (Fig. 4) induced vesiculation in semen containing 10% glycerol.

Experiment 3: time course of acrosomal change after rapid warming

Semen samples containing 1% (v/v) glycerol were rapidly diluted 1:8 (v/v) with Medium BTS at 37°C. After 0, 15, 60 and 300 sec from dilution they were fixed 1:1 with the primary fixative used for electron microscopy (2% glutaraldehyde, 1% formaldehyde, 0.2 M-sucrose, 1.5 mM-CaCl₂ in 0.1 M-cacodylate buffer, pH 7.3). Replicate tests were made on 4 ejaculates.

At 15 sec following treatment there was a significant decrease in the number of normal acrosomes which reached maximal values by 300 sec (Table 3). At the level of the electron microscope it was evident that the first signs of invagination of the outer acrosomal membrane were present by 15 sec (Fig. 5). At 60 sec the spermatozoa had the full complement of structural features associated with warm shock.

Experiment 4: influence of dilution rate

Semen samples (0.2 ml) containing 1 and 10% glycerol at 5°C were quickly pipetted into thin-walled test tubes containing 0–1.6 ml Medium BTS at 37°C. Replicate tests were made on 4 ejaculates.

While in such an experiment it is difficult to distinguish between the effect of temperature and the degree of dilution, nevertheless it was clear that as the degree of dilution increased there was a higher percentage of acrosomal damage (Table 4). As in previous experiments a detrimental effect of a high glycerol concentration was observed.

Experiment 5: influence of the magnitude of temperature change and temperature zone

Semen samples containing 1% glycerol were warmed in a stepwise fashion from 5 to 15 to 25 to 35°C. At each temperature, semen samples were rapidly diluted 1:8 (v/v) with Medium BTS at 5, 15, 25 and 35°C. In this manner temperature differentials ranging from +30 to -30°C were achieved. Replicate tests were made on 6 ejaculated and 6 epididymal sperm samples.

The acrosomes of ejaculated and epididymal spermatozoa showed basically similar responses to the rapid temperature changes together with dilution (Table 5). However, epididymal spermatozoa were rather more sensitive to these stresses than were those in ejaculates. While the percentage of normal acrosomes decreased slightly during gradual warming from 5 to 35°C this did not approach significance. In treatments in which the spermatozoa were subjected to an increase or decrease in temperature the degree of integrity of the acrosomes was influenced by the magnitude of the temperature differential and the temperature zone. When treatments with the same temperature change were compared, acrosomal damage tended to occur at a higher temperature zone during warming and at a lower zone during cooling. Rapid warming from 5 to 35°C resulted in the greatest degree of acrosomal damage even more than that found after cooling through the same temperature differential. The mean percentage of motility was unaffected by rapid warming but decreased significantly after rapid cooling from 35 to 5°C (ejaculated and epididymal spermatozoa, $P < 0.01$) and from 25 to 5°C (ejaculated spermatozoa, $P < 0.05$).

Experiment 6: immunocytochemical detection of acrosomal damage after temperature shocks

Semen samples containing 1% glycerol at 5°C and those warmed gradually to 37°C were rapidly diluted 1:8 (v/v) with Medium BTS at 5 and 37°C. After rapid dilution, the samples were incubated at 37°C for 2 h. Smears were made at the beginning and at the end of the incubation, and detection of acrosomal damage was carried out as described by Harrison & Fléchon (1980). At least 200 spermatozoa were examined and categorized into those which were fluorescent (acrosin positive) and those which were not (acrosin negative). Those cells categorized as being acrosin-positive ranged from having weak to strong fluorescence. Replicate tests were made on 4 ejaculates.

Fig. 1. Spermatozoon with a normal apical ridge. TEM, $\times 26\,500$.

Fig. 2. Spermatozoa diluted in BF5 diluent containing 10% glycerol at 5°C. Note flattened apical ridge and a slight sign of invagination. TEM, $\times 29\,250$.

Fig. 3. Spermatozoa after rapid dilution at 5°C (the spermatozoa in BF5 containing 10% glycerol were diluted rapidly with BTS medium at 5°C). Note the loss of apical ridge and a dark area in anterior part of head. Phase contrast, $\times 1250$.

Fig. 4. Spermatozoon after rapid dilution at 5°C (the spermatozoon was treated as in Fig. 3). Note prominent invagination and ruffling of outer acrosomal membrane. TEM, $\times 34\,000$.

Fig. 5. Spermatozoon after 15 sec of rapid dilution + warming. The appearance is similar to that of spermatozoa in Fig. 2. Small invaginations are visible. TEM, $\times 45\,000$.

Fig. 6. Warm-shocked spermatozoa with filament-like (arrow heads show both ends) or droplet-like (arrow) particle. Nomarski, $\times 1250$.

Table 4. Effects of dilution rates on acrosomal morphology in rapid dilution + warming treatments

Treatment	Concentration of glycerol	
	1%	10%
Before dilution (5°C)	91.5	89.5
Slow warming to 37°C	87.2	82.7
Dilution rate (semen:BTS)		
1:0	81.7	71.1
1:1	70.5	42.7
1:2	39.2**	20.5**
1:4	17.5**	5.0**
1:8	5.0**	1.5**

Values are means of % normal acrosomes for 4 observations.

**Significantly different from values before dilution ($P < 0.01$).

Table 5. Effects of temperature of semen and Medium BTS on acrosomal morphology in rapid dilution treatments

Temperature (°C)			% normal acrosomes	
Semen	BTS	Difference	Ejaculated sperm.	Epididymal sperm.
5	—	Control	94.0	93.6
15	—	(before dilution)	92.0	91.6
25	—		92.8	91.5
35	—		90.8	87.8
5	5	0	91.3	87.5*
15	15		90.0	82.8**
25	25		87.6	81.8
35	35		86.5	80.1
5	15	+10	90.6	83.8**
15	25		89.6	81.0**
25	35		76.3**	65.8**
15	5	-10	85.6	82.3**
25	15		88.8	83.0
35	25		84.1	71.6*
5	25	+20	86.1**	74.0**
15	35		18.6**	4.1**
25	5	-20	64.0**	59.8**
35	15		80.5	68.6*
5	35	+30	14.8**	4.0**
35	5	-30	24.5**	25.6**

Values are means for 6 observations.

* $P < 0.05$, ** $P < 0.01$ compared with control value for that semen temperature.

Table 6. Effects of rapid dilution treatments on acrosin release

Incubation	Treatment	Acrosin positive	Normal acrosomes	Motility
0 h	Rapid dilution at 5°C	93.7	95.1	90.0
	Rapid dilution + warming	92.5	3.7**	90.0
	Rapid dilution at 37°C	93.0	90.6	90.0
	Rapid dilution + cooling	60.1*	14.1**	18.7**
2 h	Rapid dilution at 5°C	91.0	84.7	87.5
	Rapid dilution + warming	68.3	1.0**	85.0
	Rapid dilution at 37°C	90.1	77.3	87.5
	Rapid dilution + cooling	20.7**	2.8**	12.5**

Each value is the mean % of 4 observations.

* $P < 0.05$, ** $P < 0.01$ compared with values for rapid dilution at 5°C.

Table 7. Effects of cryoprotective agents (G, glycerol; D, DMSO; P, propylene glycol) on motility and acrosomal morphology in rapid dilution treatments

Treatment	Motility			Normal acrosomes		
	G	D	P	G	D	P
Rapid dilution at 5°C	89.0	66.0**	89.0	87.4	84.4	85.4
Rapid dilution + warming	89.0	89.0	89.0	3.0	3.2	3.6
Rapid dilution at 37°C	89.0	89.0	89.0	79.0	79.4	81.0
Rapid dilution + cooling	15.0	10.0	12.0	6.2	3.6	5.0

Each value is the mean % of 5 observations.

** $P < 0.01$ compared with value for G.

Rapid dilution + cooling resulted in an immediate reduction of acrosin-positive spermatozoa with a further decrease after 2 h incubation (Table 6). There was no change in the percentage of acrosin-positive spermatozoa immediately following rapid dilution + warming. However, after incubation for 2 h the number fell to some 75% of the control level.

Experiment 7: influence of cryoprotective agents

Semen samples containing 1% (v/v) glycerol, dimethyl sulphoxide (DMSO), or propylene glycol at 5°C were rapidly diluted 1:8 (v/v) with Medium BTS at 5 and 37°C. Rapid dilution was also made on samples gradually warmed to 37°C. Replicate tests were made on 5 ejaculates.

None of the cryoprotectants significantly reduced the incidence of the damage resulting from warm shock (Table 7). In addition, only DMSO had a significant effect ($P < 0.01$) on motility following rapid dilution at 5°C.

Experiment 8: influence of butylated hydroxytoluene (BHT)

Ejaculated semen was diluted 1:1 (v/v) with Medium BTS immediately after collection. The partly diluted semen was further diluted 1:1 (v/v) with (1) Medium BTS, (2) Medium BTS containing 2% (v/v) DMSO, and (3) Medium BTS containing 2% DMSO and 4 mM-butylated hydroxytoluene (BHT) at 37°C; they were added in two equal portions 10 min apart. The diluted semen samples were further incubated at 37°C for 20 min, and then rapidly diluted 1:8 (v/v) with Medium BTS at 5

Table 8. Effects of BHT and DMSO on motility and acrosomal morphology in rapid dilution treatments

Treatment	Motility			Normal acrosomes		
	None	DMSO	DMSO + BHT†	None	DMSO	DMSO + BHT
Control at 37°C	90.0	87.5	0 (10.1)	97.8	97.5	96.7
Rapid dilution + cooling	0	0.2	0 (0)	0	0	73.0**
Control at 5°C	77.5	78.7	0 (11.2)	91.0	89.5	90.7
Rapid dilution + warming	71.2	78.7	0 (8.7)	13.8	16.3	60.6**

Each value is the mean % of 4 observations.

** $P < 0.01$ compared with value for no treatment.

†Although progressive motility was not observed in the presence of BHT, some spermatozoa were slightly motile (values in the parentheses are the means of % motile spermatozoa).

and 37°C. Rapid dilution was also made on the samples cooled down to 5°C gradually. Replicate tests were made on 4 ejaculates.

The addition of BHT to the medium had a profound effect on the motility of spermatozoa (Table 8). While some cells maintained a slight degree of motility, progressive motility was abolished. On the other hand, there was a clear protective influence against acrosomal damage sustained during both warm and cold shock.

Discussion

It has been established that boar spermatozoa are adversely affected by the presence of high concentrations of glycerol (Polge, 1978). In the present study, the effects of rapid dilution and warming were first examined in relation to glycerol concentration. Although the decrease in the proportion of normal acrosomes due to the inclusion of 10% glycerol was comparatively small, there were slight alterations in the apical ridges at this concentration as seen by transmission electron microscopy. It is possible that such altered acrosomes would have an increased sensitivity to osmotic or temperature shock. On the other hand, pronounced acrosomal damage was observed when samples containing 7.5 or 10% glycerol were rapidly diluted at 5 and 15°C. This damage was reduced as the temperature of dilution was raised, particularly from 15 to 25°C, indicating a change in the permeability of the outer acrosomal membrane to cryoprotectants presumably through the phase transition of the membrane lipids at these temperatures. However, visible acrosomal damage was still observed in samples containing 10% glycerol after rapid dilution at 37°C.

Similar temperature-dependent effects of rapid dilution have been noted in human red blood cells (Woolgar & Morris, 1973), mouse lymph node cells (Thorpe *et al.*, 1976) and mouse embryos (Whittingham *et al.*, 1979). In the case of sperm cells, the removal of high concentrations of glycerol should be done gradually at temperatures above 25°C to minimize osmotic shock. When glycerol levels were lower than 2.5%, the effect of rapid dilution was relatively small regardless of the dilution temperature, suggesting that acrosomal damage caused by rapid dilution + warming could largely be attributed to the warming component at low glycerol concentrations. In contrast, both warming and dilution components are important factors affecting acrosomal integrity in samples containing high glycerol concentrations.

The dilution rate was an important factor during rapid dilution and warming. The proportion of normal acrosomes decreased significantly at a dilution rate of 1:2 and acrosomal damage increased with increasing rates of dilution, which is likely to be due to the increased rates of warming as far as samples containing low concentrations of glycerol were concerned. In addition, it was shown that a

terminal temperature of 35°C was much more detrimental to acrosomes than one of 25°C. On thawing pellets in solution, attention should be given to its volume and initial temperature such that the terminal temperature of the thawed semen does not exceed 25°C.

During slow warming of semen from 5 to 35 or 37°C, the proportion of normal acrosomes decreased by some 5% in samples containing 1% glycerol (Table 1, 2, 4 and 5). In addition, pipetting 0.2 ml of semen at 5°C into a test tube at 37°C caused a reduction in the percentage of normal acrosomes. Such an injurious effect of warming would be inevitable when pellets are thawed on a hot plate or straws thawed in hot water. Pursel & Park (1985) reported that the highest percentage of motility and normal acrosomes were observed when a 5-ml straw was thawed by immersion in a water bath at 52°C for 40 sec (terminal temperature; 15.2°C). It is likely that the terminal temperature is an important factor when thawing semen without a thawing solution.

To prevent warm shock it is preferable to include a protective agent in the diluent. It has been shown that BHT, an antioxidant, protected bull (Hammerstedt *et al.*, 1978), ram (Hammerstedt *et al.*, 1978; Watson & Anderson, 1983) and boar (Pursel, 1979) spermatozoa from cold shock. In the present study this proved to be effective in preventing acrosomal damage resulting from both cold and warm shock. The use of BHT might, therefore, be one approach for the prevention of warm shock during rapid thawing of frozen semen. However, since BHT adversely affects sperm motility, further studies on its use are required. On the other hand, permeable cryoprotective agents such as glycerol, propylene glycol and DMSO were ineffective against warm shock.

Epididymal spermatozoa were more sensitive to warming stress than those in the ejaculate. With regard to susceptibility to cold shock in the boar a complex interaction exists between the spermatozoa and the seminal fluid (Watson, 1981). Susceptibility to cold shock occurs in the distal corpus epididymidis (Watson & Plummer, 1985). Since the epididymal spermatozoa used in this study were collected from the cauda epididymidis, the inherent characteristics of these cells may be similar to those in the ejaculate. Therefore, seminal plasma may be involved in the differences in susceptibility to warm shock.

After rapid dilution + warming, acrosomal vesiculation commenced within 15 sec and was nearly maximal by 60 sec. However, the membranes of such warm shocked spermatozoa did appear to be altered since subsequent incubation for 2 h at 37°C resulted in release of acrosin. In addition, cells with a filament and/or droplet-like structure were frequently observed after storage of warm shocked cells in the fixative at 4°C overnight. It is suggested that matrix materials within the acrosome were released through small punctate ruptures in the membranes. In cold-shocked spermatozoa such structures were not observed, possibly since the membrane damage was so great that release of acrosomal contents occurred abruptly.

Although a full understanding of warm shock may require a biophysical approach, the present study has indicated some avenues which may provide improvements in current techniques of freezing and thawing boar semen.

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