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Morphological Changes as Observed by Light Microscopy of the Acrosome of Boar Spermatozoa Subjected to Deep Freezing

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Abstract

The effects of chilling (5°C) and deep freezing in liquid nitrogen (−196°C) on the acrosomal morphology of diluted boar spermatozoa were evaluated by light microscopy using Giemsa stain. A large reduction in numbers of spermatozoa with normal acrosomes was recorded as the temperature fell from 30 to 5°C but the greatest damage occurred during the freeze–thaw cycle.

Introduction

When spermatozoa are subjected to cold shock they suffer increased acrosome damage (Healey 1969; Pursel *et al.* 1972, 1974; Watson and Martin 1972; Visser and Salamon 1974), irreversible loss of motility (Blackshaw 1954; Boender 1968) and increased membrane permeability to stains (Hancock 1951; Dott and Foster 1972).

Boender (1968) has demonstrated that the extent of acrosomal damage was determined by the severity of cold shock. Boar spermatozoa acquired resistance to cold shock when incubated *in vitro* at 30°C (Pursel *et al.* 1973) but susceptibility to cold shock increased as the dilution rate increased from 1:2 to 1:10. Healey (1969) showed that freezing damaged the ultrastructure of the spermatozoa of some domestic species (bull, ram, boar, stallion and chinchilla). The purpose of this investigation was to classify the alterations to acrosome morphology of boar spermatozoa that occur during freezing.

Materials and Methods

Semen from one Landrace and five Large White boars was collected by the gloved-hand method into a 500-ml insulated vacuum bottle, the opening of which was covered with gauze. At collection the gel fraction was separated by the gauze filter and only the sperm-rich portion was used. Two collections from each boar were made 3 days apart.

The semen was incubated at 30°C for 2 h and then aliquots of the sperm-rich fraction of each ejaculate were diluted 1:4 (initial volume:final volume) with BF5 diluent (Pursel and Johnson 1972). The diluted semen samples were gradually cooled to 5°C in 2 h at which stage glycerol was added to give a final concentration of 2% (v/v). Diluted semen was frozen in 0·5-ml Cassou-type straws (Cassou 1964) and stored in liquid nitrogen (24–72 h) until thawed for microscopic examination.

Frozen semen was thawed in a water-bath at 30°C and the percentage of motile spermatozoa was assessed at ×400 under a coverslip on a warm stage (37°C). Smears were prepared (Hancock 1952) from samples of diluted semen at 30°C, after equilibration with glycerol at 5°C and after freezing and thawing. Acrosomal morphology was evaluated at ×1250 with a Zeiss Neofluar 100/1·30 phase-contrast objective using the following scoring system: 0, normal acrosome; 1, slight

to moderate damage; 2, severe damage; and 3, missing acrosome. Two slides were made for each treatment and the first 100 spermatozoa observed in a traverse of the semen smear were evaluated. The percentage of spermatozoa in each acrosomal score category and the mean acrosomal score were then calculated. Slides and straws were coded and presented in a random manner for examination so that the observer did not know their identity.

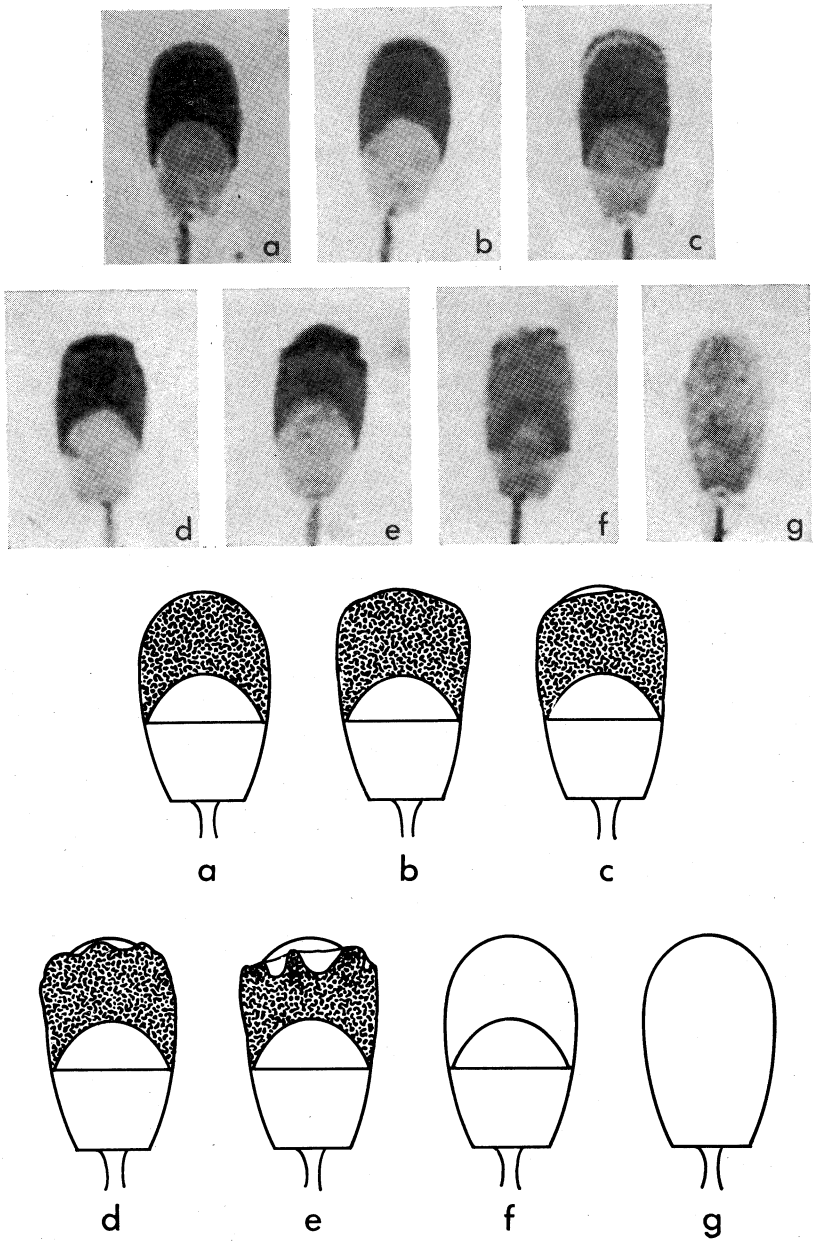

Fig. 1. Normal spermatozoon (*a*) and spermatozoa showing slight (*b*, score 1) moderate (*c*, score 1) and severe (*d*, *e*, both score 2) damage and spermatozoa with missing acrosomal cap (*f*, *g*, both score 3) with retention (*f*) and loss (*g*) of the equatorial segment. A schematic representation of these morphological changes is also given in the corresponding line drawings.

Normal acrosomes were classified as those with a distinct apical ridge that formed a smooth crescent at the apical border of the head and with the acrosomal cap smoothly adherent to the nucleus (Pursel *et al.* 1972). Damaged acrosomes were those which had undergone any change in morphology (Boender 1968; Saacke *et al.* 1968; Watson and Martin 1972; Visser and Salamon 1974).

Results

The extent of acrosomal damage or loss in spermatozoa for each of the acrosomal score categories is shown in Figs 1*a*–1*g*. The most frequent form of acrosome damage observed was vacuolation and lifting of the acrosome. This occurred either as a regular pattern in which the acrosome separated from the head leaving a crescent-shaped space between the two or as an irregular form where the loose acrosome remained attached to the head at one or more points and frequently folded back onto itself. The latter was observed in 58% of post-freeze spermatozoa in acrosomal score category 2. Swelling or vacuolation in the apical area was observed in a number of otherwise apparently normal spermatozoa, which had regular outlines with reduced density immediately posterior to the apical ridge. In spermatozoa of score category 3 the acrosome was either absent with retention of the equatorial segment or both acrosome and equatorial segment were absent.

The effects of cooling and deep freezing on the percentage of motile spermatozoa, mean acrosomal scores and the percentage of spermatozoa falling into each acrosomal score category are given in Table 1. A large reduction in the proportion of spermatozoa with normal acrosomes (score 0) occurred as the temperature dropped from 30°C to 5°C, but the greatest damage occurred during the freeze–thaw cycle (Table 1). The percentage of motile spermatozoa declined sharply during cooling (from 87 to 57%) and after the freeze–thaw cycle only 15% of spermatozoa remained motile.

Table 1. Effect of chilling and freezing of boar semen on the percentage of spermatozoa in each acrosomal score category, acrosomal score and percentage of motile spermatozoa

Treatment	Spermatozoa (%) in acrosomal score category				Mean acrosomal score	Motile spermatozoa (%)
	0	1	2	3		
Dilution at 30°C	87·2	9·1	3·1	0·6	0·17	87
Glycerol equilibration at 5°C	38·3	49·7	11·1	0·9	0·75	57
Freezing and thawing	8·5	26·7	59·0	5·8	1·62	15

Discussion

The three main categories described by Watson and Martin (1972) of damaged acrosomes of bull and ram spermatozoa following deep freezing were (1) bubbling and swelling of the acrosome, (2) separation of acrosome from spermatozoal head and (3) loss of the acrosome. They showed that the acrosomes of ram spermatozoa were more severely damaged by deep freezing than those of bull, with categories (1) and (2) predominating in both species. The present study showing mean acrosomal score as a composite of both the degree of deterioration of individual spermatozoa and the proportion of spermatozoa showing damage agrees with those of Watson and Martin (1972) and Boender (1968) but neither Watson and Martin or Boender described the damaged acrosome folding back on itself.

Boender (1968) recorded loss of the apical ridge as the major morphological change of boar spermatozoa aged *in vitro*, and vacuolation and complete loss of the acrosome and then loss of the equatorial segment as further evidence of deterioration of the sperm head. Areas with reduced density of stain immediately posterior to the apical ridge appear to represent early stages of vacuolation and lifting of the acrosome in cold-shocked spermatozoa. However, although the influence of cold shock on the acrosome was instantaneous and Boender (1968) could not decide whether vacuolation preceded or followed death of the spermatozoa, Pursel *et al.* (1972) showed that boar spermatozoa with acrosomal damage were motile and observed a high proportion of loose acrosomal caps (which represented the final stage of damage) and missing apical ridges following cold shock. Pursel *et al.* (1973) also showed that when incubation time preceding cold shock was increased from 1 to 5 h, the proportion of acrosomes with normal apical ridges and spermatozoal motility were significantly increased. Samples diluted before incubation were more susceptible to cold-shock damage than those diluted after incubation and they concluded that dilution during incubation interfered with the development of resistance to cold shock. Although seminal plasma may have interacted with the spermatozoa during incubation to produce this increased resistance, results of other trials indicated that the ability of spermatozoa to develop cold-shock resistance during incubation was inherent within the cells.

Healey (1969) noted that the acrosome of frozen bull spermatozoa was normal, but freezing severely damaged the acrosome of the boar and ram. Damaged frozen–thawed chinchilla spermatozoa exhibited progressive motility, supporting the claim that in these species damage to the acrosome preceded death of the spermatozoa (Healey 1969). Acrosomal injury, observed with frozen boar spermatozoa, has been associated with infertility by Graham *et al.* (1971) and Pursel and Johnson (1971).

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