The effects of cooling to 5°C and freezing and thawing on the ultrastructure of bull spermatozoa

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Summary. Semen from 6 bulls was examined under the transmission electron microscope immediately after collection, after dilution and cooling to 5°C and after freezing and thawing. Conception rates were determined following artificial insemination of the frozen and thawed semen. Dilution and cooling to 5°C caused acrosomal swelling in about 50% of the spermatozoa. Subsequent freezing and thawing caused considerable ultrastructural changes to the acrosomes (disruption of the plasma and outer acrosomal membranes and dispersion of the acrosomal contents) and middle pieces (breakage of the plasma membrane and a reduction in the electron density of the mitochondrial matrix) of a high proportion of spermatozoa. The average non-return rate following insemination of semen from 5 of the bulls was 61.6% and higher ($P < 0.001$) than for the sixth bull (15%). Although this difference in semen viability was also demonstrated in the structural studies (acrosome, $P < 0.05$; middle piece, $P < 0.001$), more work is required to assess the relationship between structure and function of spermatozoa.

Introduction

For many species progress has been slow in developing methods for the deep-frozen storage of semen (Jones, 1971). A major problem has been that spermatozoa lose viability during processing so that following artificial insemination they can no longer ascend and survive in the female reproductive tract in order to fertilize an ovum (Mattner, Entwistle & Martin, 1969; Polge, Salamon & Wilmut, 1970). As this loss in viability has been correlated with structural changes (Healey, 1969; Quinn, White & Cleland, 1969; Pedersen & Lebech, 1971; Nath, 1972) which cannot all be detected with the light microscope (Tasseron, Amir & Schindler, 1977), it is necessary to examine further the ultrastructure of spermatozoa to assess the value of such an approach and to provide a basis for comparative studies. Earlier work on this project was carried out to quantitate and improve methods of preparing spermatozoa for transmission electron microscopy and to assess the effects of the preparative procedures on sperm structure (Jones, 1973a, b, c, 1975). These methods were used to determine the effects on sperm structure of diluting and cooling semen to 5°C and of freezing and thawing. Since, at this stage of the project, it is important to relate the structural changes to sperm viability the experiment was carried out using sub-samples of semen which were tested for fertilizing capacity (Linford, Glover, Bishop & Stewart, 1976).

Materials and Methods

The 3 Friesian and 3 Hereford bulls used in this study had, between them, previously produced a wide range of conception rates following artificial insemination. Semen was collected into an artificial vagina and processed by the procedures used routinely at the Cattle Breeding Centre,

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Reading. One ejaculate was collected from each bull and diluted at 30°C with an unglycerolated diluent before cooling to 5°C over 30 min. An equal volume of diluent containing 14% (v/v) glycerol was added in two stages 15 min apart to give a final diluent composition of 83% (v/v) ultra heat-treated skim milk, 10% (v/v) egg yolk, 7% v/v glycerol, 12.5 mg fructose/ml, 500 i.u. penicillin/ml and 500 µg streptomycin sulphate/ml. Before use the diluent was cleared of particulate matter by centrifugation at 5000 g for 30 min (5°C) and only the supernatant was collected for use. The semen was equilibrated at 5°C for 6 h and frozen in 0.5 ml polyvinyl-chloride straws suspended 4 cm above liquid nitrogen. Each straw contained 20×10^6 spermatozoa and was stored at -196°C for at least 28 days before thawing in air for use. Semen was used only for first inseminations and conception rates were determined as non-returns to oestrus within 16 weeks of insemination.

Sub-samples of semen were taken for microscopic examination before the initial dilution at 30°C, just before freezing, and after freezing and thawing. For electron microscopy samples were concentrated into about 0.3 ml by centrifugation at 700 g for 10 min, removal of the supernatant and mixing with a Pasteur pipette. Spermatozoa were fixed in PFG-cacodylate (Jones, 1973a) and prepared for examination according to the methods described by Jones (1973a). These included coding and randomization of samples and taking low power (initial magnification, $\times 5000$) electron micrographs of different areas of the Araldite sections prepared from each treatment. Prints (photographic enlargement, $\times 2$) were examined and all the sections of spermatozoa through the appropriate plane were scored according to the type and degree of structural change occurring in the head and middle piece. Usually 100 sections of heads and 100 of middle pieces were scored per treatment and the frequencies of each type are expressed as mean percentages in Tables 1-4. Heads of spermatozoa were scored as corresponding to one of the types shown in Pl. 1, Figs 1 to 9 of Jones & Martin (1973) and described in the headings of Tables 1 and 3. Sections of middle pieces were classified into the types shown in Pl. 1, Figs 10 to 14 of Jones & Martin (1973) and described in the headings of Tables 2 and 4. The χ^2 analyses described by Claringbold (1961) were carried out on the frequency data and the statistical significance of treatment effects was assessed with an F-test using the error mean square calculated in the analysis.

Table 1. Assessment of the structure of the heads of freshly collected bull spermatozoa (means of 6 ejaculates from different bulls) and the effects on this structure of cooling to 5°C and freezing and thawing

	Scored as type shown in Plate 1 of Jones & Martin (1973)								
	Fig. 1	Fig. 2	Fig. 3	Fig. 4	Fig. 5	Fig. 6	Fig. 7	Fig. 8	Fig. 9
Plasma membrane:									
intact	+	+	+	+	-	-	-	-	-
broken or lost	-	-	-	-	+	+	+	+	+
Acrosome:									
swollen	-	+	+	+	-	-	+	+	-
vesiculated	-	-	+	+	+	-	-	-	-
vacuolated	-	-	-	-	-	-	-	+	-
loss of outer membrane	-	-	-	+	-	-	-	-	+
loss of contents	-	-	-	-	+	-	-	-	+
Semen treatment									
(1) Undiluted	69	7	2	1	1	1	2	4	13
(2) Cooled to 5°C	14	51	3	3	1	0	7	3	18
(3) Frozen and thawed	10	18	2	2	1	5	7	10	45
1 versus 2 + 3	$P < 0.001$								
2 versus 3	$P < 0.001$								

Results

Tables 1 and 2 show the effect of processing on the structure of heads and middle pieces of spermatozoa. Cooling semen to 5°C caused acrosomal swelling in a high proportion of spermatozoa and freezing and thawing caused rupture of the outer acrosomal membrane and the plasma membrane covering the acrosome, and loss of the acrosomal contents (Table 1). However, there was a large error term in the χ^2 analysis (χ^2_{50} for the interaction of treatments, replicates and sperm type = 261.97, $P < 0.001$). This was partly because the initial semen quality

Table 2. Assessment of the structure of the middle pieces of freshly collected bull spermatozoa (means of 6 ejaculates from different bulls) and the effects on this structure of cooling to 5°C and freezing and thawing

	Scored as type shown in Plate 1 of Jones & Martin (1973)					
	Plasma membrane intact			Plasma membrane broken		
	Mitochondria normal (Fig. 10)	Mitochondria condensed (Fig. 11)	Mitochondria pale (Fig. 12)	Mitochondria normal (Fig. 13)	Mitochondria condensed* (Fig. 13)	Mitochondria pale (Fig. 14)
Semen treatment						
(1) Undiluted	51	8	6	1	0	34
(2) Cooled to 5°C	56	7	13	1	0	24
(3) Frozen and thawed	25	2	37	1	0	36
1 versus 2 + 3	$P < 0.001$					
2 versus 3	$P < 0.001$					

* Scored as similar to type shown in Pl. 1, Fig. 11 (Jones & Martin, 1973), but with a broken plasma membrane.

Table 3. Characteristics of deep-frozen and thawed bull semen: structure of heads, percentage of unstained spermatozoa in eosin-nigrosin smears and 16-week non-return rates after artificial insemination

	Scored as type shown in Plate 1 of Jones & Martin (1973)										Conception rate	
	Fig. 1	Fig. 2	Fig. 3	Fig. 4	Fig. 5	Fig. 6	Fig. 7	Fig. 8	Fig. 9	% unstained	No. of cows	% non-return
Plasma membrane:												
intact	+	+	+	+	—	—	—	—	—			
broken or lost	—	—	—	—	+	+	+	+	+			
Acrosome:												
swollen	—	+	+	+	—	—	+	+	—			
vesiculated	—	—	+	+	+	—	—	—	—			
vacuolated	—	—	—	—	—	—	—	+	—			
loss of outer membrane	—	—	—	+	—	—	—	—	+			
loss of contents	—	—	—	—	+	—	—	—	+			
Bull No.												
1	9	31	3	2	0	1	12	17	25	62	99	60
2	6	19	1	2	1	13	6	12	40	41	79	63
3	9	15	1	0	0	9	12	11	43	42	123	59
4	15	14	1	6	1	4	4	9	46	43	88	64
5	20	30	7	0	0	4	8	6	25	45	93	63
6	0	0	1	1	2	0	2	6	88	20	60	15
Mean 1–5 versus 6	$P < 0.05$					$P < 0.05$					$P < 0.001$	

for Bull 6 was poor (18 and 50% of the spermatozoa in columns 1 and 8 respectively in Table 1), but also because there was considerable variation between the other bulls in their response to semen processing.

Cooling to 5°C had little effect on the structure of the middle pieces, but freezing and thawing caused breakage of the plasma membrane and loss of some of the mitochondrial matrix of a considerable proportion of spermatozoa (Table 2). As with counts of acrosomal damage there was a large error term in the χ^2 analysis ($\chi^2_{30} = 212.20$, $P < 0.001$) due to the initial poor semen quality of Bull 6 and the variability between the other animals in the response to semen processing.

Tables 3 and 4 show the semen characteristics and conception rates after artificial insemination of the 6 ejaculates. The statistical analysis showed that the average conception rate obtained for Bull 6 was lower than that for the other bulls (mean of 61.6%) and semen from Bull 6 also had a higher proportion of spermatozoa showing acrosomal and middle piece degeneration, a lower count of percentage unstained spermatozoa and a lower mean score of percentage motile spermatozoa than did semen from the other bulls.

Table 4. Characteristics of deep-frozen and thawed bull semen: structure of the middle piece, scores of percentage motile and counts of percentage tail-less heads

Scored as type shown in Plate 1 of Jones & Martin (1973)								
Plasma membrane intact			Plasma membrane broken			% motile	% tailless heads	
Bull	Mitochondria normal (Fig. 10)	Mitochondria condensed (Fig. 11)	Mitochondria pale (Fig. 12)	Mitochondria normal (Fig. 13)	Mitochondria condensed*	Mitochondria pale (Fig. 14)		
1	60	4	21	4	1	10	60	0
2	23	3	52	1	0	21	50	4
3	17	1	52	1	1	28	20	2
4	16	0	43	0	0	41	40	32
5	29	3	42	1	0	25	50	17
6	2	0	13	0	0	85	5	33
Mean 1-5 versus 6			$P < 0.001$			$P < 0.05$ $P < 0.05$		

* Scored as similar to type shown in Pl. 1, Fig. 11 (Jones & Martin, 1973), but with a broken plasma membrane.

Discussion

These studies show that freezing and thawing caused structural changes to a considerable proportion of bull spermatozoa. The changes involved damage to the plasma membrane over the acrosome and the outer acrosomal membrane, causing disruption of the acrosomal contents, and damage to the mitochondria and plasma membrane of the middle piece. These findings are in agreement with the earlier qualitative studies of ram (Quinn *et al.*, 1969; Nath, 1972) and human (Pedersen & Lebech, 1971) spermatozoa. Healey (1969) also showed that freezing and thawing caused qualitatively similar acrosomal damage in boar, chinchilla and ram spermatozoa. However, he reported that the ultrastructure of bull spermatozoa was much the same before and after freezing. As the bulls used in the present study were not selected as producers of high quality semen (see 'Materials and Methods') it is suggested that the difference in response to freezing between this study and that reported by Healey (1969) may be due to variation between bulls as well as possibly differences in processing techniques.

Cooling bull spermatozoa to 5°C mainly caused swelling of the acrosome whereas freezing and thawing caused disruption of the acrosome and damage to the middle piece of a high

proportion of spermatozoa. Consequently, it seems most likely that any loss in viability attributed to the processing of semen occurs during the freezing and thawing process.

Bulls were selected for use in this study because some had previously given semen samples of quite different motility, but the same fertility, whilst others had previously given semen of similar motility, but quite different fertility. For reasons which cannot be explained these differences were (except for Bull 6) not recorded during this study. The finding that Bulls 1–5 were more fertile than Bull 6 indicates that structural studies may be useful for assessing sperm function, but this will only be resolved in studies involving more sensitive tests of fertility.

The quantitative approach used in these studies to assess the structure of spermatozoa is necessary since semen contains a heterogeneous population of spermatozoa and it is considered that male fertility affects conception rates according to the number of viable spermatozoa in an inseminate and their life-span in the female reproductive tract (Jones, 1975). However, this work indicates that more information is required to interpret the relationship between sperm structure and function. Table 3 shows that the average conception rate for Bulls 1 to 5 was 61.6% although an average of only 11.8% of the spermatozoa were scored as 'normal' (Table 3, column 1). This suggests that other spermatozoa may be capable of fertilizing an ovum. Since acrosomal swelling is a preliminary stage of the acrosome reaction (Yanagimachi & Noda, 1970) this process should not affect the ability of the spermatozoa to fertilize an ovum. Consequently, if the spermatozoa represented in columns 1 and 2, Table 3, were considered structurally capable of fertilizing an ovum they make a total of 33.6% viable spermatozoa in the sample. This corresponds to an inseminate of 6.72×10^6 viable spermatozoa, a number which is considered to be sufficient to achieve satisfactory conception rates (Olds, Beath, Carpenter & Lucas, 1953; Jondet, 1968; Stewart & Bennett, 1968).

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