Fertility of boar spermatozoa after freezing in the absence of seminal vesicular proteins

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Because exposure to seminal plasma enhances the detrimental effects of cold shock on boar spermatozoa (Lasley & Bogart, 1944; Pursel, Johnson & Rampacek, 1972), many procedures for preserving semen by deep freezing employ sperm-rich ejaculate fractions (Pursel & Johnson, 1975; Larrson & Einarsson, 1975), thereby excluding much of the seminal plasma. Our previous studies showed that a basic protein fraction of seminal vesicle origin binds irreversibly to boar spermatozoa at ejaculation (Moore & Hibbitt, 1976) and during cooling promotes sperm membrane disruption (Moore, Hall & Hibbitt, 1976). We therefore tested the possibility that boar spermatozoa could be frozen more effectively in the absence of seminal vesicular fluid.

Spermatozoa from intact boars and from boars without seminal vesicles (Davies, Hall, Hibbitt & Moore, 1975) were frozen in various diluents and comparisons were made of the sperm quality after thawing and the conception rates of gilts.

Whole ejaculates were collected from three intact boars and four boars without seminal vesicles (Large White breed), and spermatozoa were frozen to -196°C by a method based on that of Pursel & Johnson (1975). Previous tests with fresh semen inseminations had indicated that the fertility of all the boars was good. Semen samples exhibiting good progressive motility of spermatozoa at 37°C were transferred in a polythene bag to a 28°C water bath. Antibiotics (Streptopen: Glaxo Ltd; 1000 i.u./ml) were added and the semen was cooled to 22°C over 2 h. After centrifugation at 700 g for 15 min, the spermatozoa were resuspended at a concentration of 1.2×10^9 cells/ml in a buffer solution, pH 7.5 (40 mM-Tes, 17 mM-tris and 180 mM-glucose) containing one of the following: (a) 20% (v/v) egg yolk; (b) 2% (w/v) casein; or (c) 2% (w/v) casein, 2.5 mM-phosphatidyl serine and 10 mM-cholesterol. Aliquots (5 ml) were cooled to 5°C at $6^{\circ}\text{C}/\text{h}$ and then diluted with an equal volume of the same diluent to which 2% (v/v) glycerol had been added. These spermatozoa were immediately pellet frozen (in 0.2 ml volumes) on solid CO_2 (Nagase & Niwa, 1964) and transferred to liquid nitrogen for storage (1–3 weeks).

Fifty semen pellets (10 ml) were thawed by first placing them in a styrofoam box (6×20 cm) for 3 min and they were then transferred to 50 ml of IVT diluent (du Mesnil du Buisson & Jondet, 1961) at 50°C . Each sample was assessed for post-thaw motility at 37°C . The percentage of eosinophilic (live/dead) spermatozoa was estimated from nigrosin-eosin smears examined the same day. Random samples were centrifuged and the supernatants assayed for L-aspartate 2-oxoglutarate aminotransferase (GOT) activity as described by Moore *et al.* (1976). Enzyme release was measured as a percentage of the total soluble enzyme activity of the cell. The latter was determined by homogenizing spermatozoa in IVT diluent, centrifuging at 10,000 g for 30 min and then assaying enzyme activity in the supernatant.

Gilts in oestrus were cervically inseminated with semen thawed within the previous 30 min. Each gilt was inseminated 24 and 32 h after first standing to a boar and the reproductive tracts were examined 6 weeks later. Fetuses and corpora lutea were counted and the fetuses were examined superficially for abnormalities. The ovaries of non-pregnant gilts were inspected for cyclic activity.

Results and discussion

As shown in Table 1, for each diluent, spermatozoa from boars without seminal vesicles gave slightly higher conception rates than those from intact animals although these differences were not statistically significant. Overall conception rates were 35% for spermatozoa from boars without

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Table 1. Details of pregnancies in gilts after insemination with frozen-thawed semen

Diluent	Boars	No. of gilts pregnant/no. inseminated	Average fetal litter size	Average no. of corpora lutea
(a) 20% egg yolk	Intact	4/12	6.5	11.1
	Vesiculectomized	5/12 (36%)	6.8	11.6
(b) 2% casein	Intact	1/10	8	12.0
	Vesiculectomized	2/10 (15%)*	8.0	11.7
(c) 2% casein + cholesterol + phosphatidyl serine	Intact	4/10	8.8	12.1
	Vesiculectomized	4/9 (42%)*	8.3	12.7

* Significantly different ($P \leq 0.05$) from the other diluents.

Values in parentheses indicate overall rate with that diluent.

vesicles and 29% for spermatozoa from intact boars. The diluents with 20% egg yolk (a) and 2% casein with cholesterol and phosphatidyl serine (c) provided significantly greater ($P \leq 0.05$) cryoprotection than did that with 2% casein (b). However, the features used to evaluate sperm viability varied only slightly for the spermatozoa in the three diluents (Table 2). The least fertile samples of spermatozoa (from intact boars and frozen in 2% casein) released the highest levels of GOT and although they gave high post-thaw motility (35–40%) they displayed the greatest proportion of eosinophilic ('dead') spermatozoa. In general, levels of enzyme release from the spermatozoa after thawing were three times those before freezing, indicating that substantial membrane damage had occurred in at least some spermatozoa during freezing and thawing, irrespective of the diluent composition. The conception rates with the egg yolk diluents were lower than those reported by Larsson & Einarsson (1976) and Pursel & Johnson (1975), perhaps because of breed differences or variations in the cooling, freezing and thawing procedures.

At ejaculation, seminal vesicular proteins bind to spermatozoa which subsequently become more susceptible to cold shock (Moore *et al.*, 1976). It is not clear, however, what effect these proteins have on the spermatozoa during freezing and thawing. Crabo & Hunter (1975) have suggested that sperm-coating proteins may enhance the fertility of frozen-thawed boar spermatozoa by preventing their early elimination from the female tract. Larsson & Einarsson (1976) concluded that an important function of the thawing diluents (including seminal plasma and protein-free seminal plasma) was to preserve this 'protective' protein coating. Our results indicate that the presence or absence of seminal vesicular fluid at ejaculation has only a small effect on the fertilizing ability of spermatozoa frozen and thawed in suitable diluents. The contributions made by the prostatic secretions cannot be assessed, but it can be concluded that proteins from the seminal vesicles are not important for maintaining sperm fertility after freezing and thawing. From the results of previous experiments (Moore *et al.*, 1976), it was hoped that the proportion of surviving spermatozoa from vesiculectomized boars would be greater at 5°C and that a greater number of cells capable of fertilization would therefore persist after freezing and thawing. These hopes were not realized: spermatozoa from boars without seminal vesicles gave conception rates which were not significantly different from those obtained using spermatozoa from intact boars, and the viability of the spermatozoa before and after freezing (as measured by the features shown in Table 2) was apparently the same for all the animals. The reason for this is unclear. The cryoprotective agents used in this study may have reduced the deleterious effects of the seminal vesicular proteins on spermatozoa from intact animals without enhancing the viability of spermatozoa from boars without seminal vesicles. The high levels of enzyme release and eosinophilic cells after thawing, although not an accurate indication of sperm fertilizing capacity (Linford, Glover, Bishop & Stewart, 1976), suggest that for all treatments most spermatozoa were damaged during freezing and thawing, although the percentage motility was similar before and after freezing.

Table 2. Enzyme release (GOT), motility, and eosinophilic cells of boar spermatozoa before freezing (5°C) and after thawing

Diluent	Boar	Enzyme release*		Motility (%)		Eosinophilic spermatozoa (%)	
		5°C	After thawing	5°C	After thawing	5°C	After thawing
(a) 20% egg yolk	Intact	26 (22–29)	82 (73–94)	36 (32–38)	30 (25–32)	36 (34–41)	71 (64–81)
	Vesicul-ectomized	25 (23–28)	77 (70–79)	30 (28–31)	37 (35–40)	35 (29–42)	67 (58–71)
(b) 2% casein	Intact	33 (28–37)	86 (79–97)	31 (24–33)	37 (35–40)	38 (36–39)	83 (76–96)
	Vesicul-ectomized	23 (20–26)	73 (68–83)	30 (28–37)	26 (25–29)	32 (26–33)	72 (66–75)
(c) 2% casein + cholesterol + phosphatidyl serine	Intact	26 (23–30)	84 (81–89)	38 (35–42)	27 (25–30)	32 (29–35)	79 (76–81)
	Vesicul-ectomized	25 (19–27)	84 (79–84)	30 (24–34)	28 (25–30)	31 (29–34)	70 (62–76)

Values are given as the mean of 4 samples and the range (in parentheses).

* As % of the total amount of sperm soluble enzyme (3.62 units/10⁹ spermatozoa).

As expected, the diluent composition at freezing had a marked effect on sperm fertility. The addition of cholesterol and phosphatidyl serine greatly enhanced the cryoprotective effect of casein diluent. Butler & Roberts (1975) have demonstrated that phosphatidyl serine protects boar spermatozoa specifically against the effects of rapid cooling, and preliminary experiments with various phospholipids and cholesterol in this laboratory (Moore, 1975) showed that a combination of phosphatidyl serine and cholesterol preserved the viability of boar spermatozoa cooled to 5°C. The present work indicates that this cryoprotection continues during freezing and thawing to a level at least equal to that obtained with egg yolk. It is noteworthy that Pace & Graham (1974) implicated a lipoprotein fraction of egg yolk as the active factor in protecting bovine spermatozoa during cooling.

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