EFFECTS OF SOME PHOSPHATIDYL COMPOUNDS ON BOAR SPERMATOZOA FOLLOWING COLD SHOCK OR SLOW COOLING

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Rapid cooling of the spermatozoa of most mammalian species leads to irreversible decrease in motility and metabolism (Milovanov, 1934; Chang & Walton, 1940), leakage of intracellular proteins and enzymes (Mann & Lutwak-Mann, 1955), increase in the proportion of differentially stained cells (Easley, Mayer & Bogart, 1942) and changes in cellular cation concentrations (Blackshaw & Salisbury, 1957). These effects could all result from changes in permeability which suggests that the cell membrane is the principal target of injury. The visible disruption of membranes, particularly in the acrosomal region (Hancock, 1952), supports this hypothesis. Phospholipids are of major importance in membrane structure and ram, bull and boar spermatozoa are known to release phospholipid into the surrounding medium on cold shock and freezing (Darin-Bennett, Poulos & White, 1973).

Various compounds, most notably milk and egg yolk, have been added to semen diluents in attempts to overcome the effects of cold-shock, with some success (Lardy & Phillips, 1939; Thacker & Almquist, 1953). These compounds are rich sources of phospholipids and it has been shown that lecithin is probably the active component in egg yolk which protects bull spermatozoa (Kampschmidt, Mayer & Herman, 1953).

Whole ejaculate boar spermatozoa (without protective additives) are sensitive to cooling *per se* and contrast with those of other species in that increasing numbers are killed as the temperature is reduced below 15°C. Boar epididymal spermatozoa are much more resistant than ejaculated spermatozoa to cooling and, in common with those of other species, are also more resistant to cold shock (Lasley & Bogart, 1944a; Lasley & Mayer, 1944). As spermatozoa pass through the epididymis and ductus deferens and are ejaculated, their resistance to cold shock is gradually reduced (Lasley & Bogart, 1944b) and is accompanied by a decrease in cellular phospholipid (Grogan, Mayer & Sikes, 1966; Quinn & White, 1967). There is some evidence that the seminal plasma may be involved in increasing the sensitivity of ejaculated spermatozoa to temperature reduction. Spermatozoa from the sperm-rich fraction, which contains only a low proportion of seminal plasma, are less sensitive to cooling than those from the whole ejaculate (Pursel, Johnson & Rampacek, 1972).

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Certain protein components of the seminal plasma have been shown to precipitate progressively as the temperature is reduced from 14°C to 4°C (Roberts, Bournsnel & Brown, 1974). Roberts & Bournsnel (1974) have shown that addition of phosphatidyl compounds to boar seminal plasma markedly increased this precipitation. It is possible that, during cooling of whole semen, the plasma proteins compete with the spermatozoa for the available phospholipid. Phosphatidyl compounds were therefore added to whole semen before cooling in an attempt to increase sperm survival.

Phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine were each suspended at 10 mM concentration in a diluent containing, per 100 ml, lactose (4 g), tris (2 g), citric acid (1.15 g) and fructose (0.5 g). Aliquots (300 µl) of whole ejaculate were added to 100 µl diluent containing phospholipid in thin-walled glass test-tubes (1.0 cm i.d.) at room temperature. The final concentration of phospholipid in the diluted semen was 2.5 mM. Semen added to diluent without phospholipid was included as a control and all treatments were carried out in triplicate. After various periods of equilibration at room temperature, the tubes were placed in a water-bath at 30°C for 10 min and were then plunged directly to 5°C and held there for 15 min. The tubes were subsequently rewarmed to 30°C and the % motile spermatozoa was estimated. Assessment of acrosome morphology was also carried out using spermatozoa from the same samples that had been fixed in formol-saline (100 spermatozoa/sample were assessed using phase-contrast microscopy and the percentage with

Table 1. The effect of various phosphatidyl compounds and equilibration time before cold shock on % motile spermatozoa and the % spermatozoa with normal acrosomes following exposure

<i>Sperm suspending medium</i>	<i>Equilibration time before cold shock (hr)</i>				
	0	$\frac{1}{2}$	1.0	2.0	4.0
Diluent alone	10.6 (1.3)	10.6 (1.7)	16.1 (1.0)	22.2 (5.3)	21.7 (5.0)
Diluent + phosphatidylserine	13.3 (0.7)	36.6 (4.3)	76.1* (6.7)	80.0* (22.0*)	77.8* (9.3)
Diluent + phosphatidylcholine	6.1 (0.3)	7.2 (1.3)	11.1 (6.0)	14.4* (4.3)	7.8* (4.3)
Diluent + phosphatidylethanolamine	10.6 (0.3)	11.1 (2.0)	14.5 (2.7)	21.7 (5.7)	25.0 (3.0)

Figures in parentheses are the % of spermatozoa with normal acrosomes.

* Within each equilibration period, values are significantly ($P < 0.001$) different from those of spermatozoa suspended in diluent alone.

normal acrosomes was determined). The results are shown in Table 1. Similar results were obtained when the diluted semen was cooled slowly to 5°C over a period of 3 to 4 hr.

Among the compounds examined, phosphatidylserine alone protected the spermatozoa against the damaging effects of exposure to cold. Equilibration at room temperature for approximately 1 hr was required to obtain the maximum protective effect. The semen in diluent without phospholipid also gradually

increased in resistance to cold shock as the time before treatment lengthened. A similar effect was noted in whole semen in the absence of diluting media (Pursel *et al.*, 1972). In an attempt to distinguish between these effects, semen samples were held at room temperature for various periods before cold shock; phosphatidylserine was either present throughout the equilibration or it was added immediately before cold shock and undiluted controls (without phosphatidylserine) were also included. The cold-shock procedure has been described above and the results are shown in Table 2.

Table 2. The effect of time of addition of phosphatidylserine and equilibration before cold shock on % motile spermatozoa following exposure

Time of addition of phosphatidylserine	Equilibration time before cold shock (hr)						
	0	$\frac{1}{2}$	1.0	1.5	2.0	3.0	4.0
None added (undiluted semen)	15	20 ^a	23.9 ^a	27.2 ^a	26.7 ^a	22.8 ^a	25.6 ^a
Before equilibration	18.3	53.4 ^b	58.6 ^b	64.5 ^b	63.3 ^b	65.0 ^b	68.6 ^b
After equilibration (before cold shock)	18.3	35.5 ^c	47.2 ^c	61.7 ^b	65.0 ^b	62.2 ^b	63.8 ^b

Within each equilibration period, different letters indicate significant differences ($P < 0.001$).

The beneficial effect of equilibration may be associated with changes in the sperm membrane which increase its resistance to cold shock. The resistance is much enhanced by the presence of phosphatidylserine which accelerates the attainment of the maximum resistance if present throughout the equilibration, or acts immediately if added after equilibration.

The diluent used for suspending the phospholipids did confer some resistance to cold shock on the spermatozoa compared to those in undiluted semen. To ensure that the action of the phosphatidylserine was not dependent on the presence of this diluent, sperm Ringer (Mann, 1964), 300 mM-sucrose, 2% sodium citrate and tris-buffered saline were also used as media. In each case, the beneficial effect of the presence of phosphatidylserine was apparent.

In order to determine more precisely the concentration of phosphatidylserine required to protect the spermatozoa, it was added at 0.125-, 0.25-, 0.5-, 1.0- or 2.0-mM final concentration to semen from two boars and cold-shock treatment was carried out approximately $1\frac{1}{2}$ hr after collection of the semen. More than 60% motile spermatozoa survived cold shock with a final concentration of 0.5 mM-phosphatidylserine in the diluted semen. This was significantly ($P < 0.001$) higher than with a concentration of 0.25 mM, or below. Increasing the concentration of phosphatidylserine above 0.5 mM did not significantly increase the motility. The pattern of response in terms of % spermatozoa with normal acrosomes was similar but, in each case, was not as high as the % motile spermatozoa. No significant differences were observed between the two boars.

These comparisons were carried out using a single batch of phosphatidylserine (B.D.H. Chemicals, Poole, Dorset). When comparisons between various concentrations of two different batches were carried out, using semen from one boar, highly significant differences ($P < 0.001$) between the batches in their effectiveness in protecting against cold shock were observed. One batch gave

maximum protection in terms of motility at a final concentration of 0.5 mM while similar protection with the other batch was not achieved until a concentration of 1.0 mM was used. At concentrations above 1.0 mM in the diluted semen, both batches conferred equally high resistance on the spermatozoa. It is possible that this apparent difference in concentration requirements is merely a reflection of biological variation between the sources of the phospholipid or variation in extraction procedure.

Since phosphatidylserine is an unstable compound and may undergo changes during preparation, its effectiveness in protecting against cold shock was compared directly with that of the related compounds, phosphatidic acid, L-serine and DL-O-phosphoserine. Each compound was suspended in the original diluent at 10 mM concentration giving a final concentration of 2.5 mM in the diluted semen. The semen in which phosphatidylserine was present during cold shock retained 60% motile spermatozoa. This was significantly ($P < 0.001$) higher than the 30% or less of motile spermatozoa in semen containing the other compounds, though their presence did increase ($P < 0.05$) the motility after cold shock compared with samples in diluent alone (21% motile) and undiluted semen (13% motile). The semen with phosphatidylserine present also showed a non-significantly higher percentage of spermatozoa with normal acrosomes, 22% compared with 12% or less for the other compounds and the controls.

The action of phosphatidylserine in protecting boar spermatozoa against cold shock appears to be a specific function of this substance since other phosphatidyl and related compounds were ineffective. Boar seminal haemagglutinin inhibition tests (Roberts & Bournsnel, 1974) have also shown considerable variations related both to the phosphatidyl compound and the red cell species employed. These observations strengthen the view that the precise conditions necessary for protection of the spermatozoa probably reflect particular structural features of the sperm membrane. Such structural features may be unique to each species; preliminary investigations with bull and ram spermatozoa indicate that the protective effects of phosphatidyl compounds against cold shock in spermatozoa are markedly species-specific.

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