Fertility of dairy cattle after artificial insemination with semen frozen in a lipoprotein diluent

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Summary. A lipoprotein separated from egg yolk by centrifugation and column chromatography was used to prepare a diluent containing 2.9% trisodium citrate. $2\text{H}_2\text{O}$ (pH 7.0) and 7% glycerol. Ejaculates from Friesian bulls were split between this and two control diluents containing egg yolk, glycerol and citrate or lactose and used to inseminate once 1782 unselected Friesian cows. By 16 weeks after insemination with semen frozen in the egg yolk-lactose, egg yolk-citrate or lipoprotein-citrate diluents, 66.4, 67.1 and 66.1% of cows, respectively, had not returned to service.

Egg yolk was originally found to protect bovine spermatozoa during cooling to low temperatures above freezing (Phillips, 1939) and is now widely regarded as an essential ingredient of diluents employed for the freezing of spermatozoa. Variation between batches of eggs and the potential for transmission of pathogens have, however, prompted a search for alternatives to egg yolk.

Attempts to identify the active egg-yolk component have concentrated on phospholipids (Kampschmidt, Mayer & Herman, 1953), phosphatidyl choline (Martin, 1963; Lanz, Pickett & Komarek, 1965; Masuda & Nishikawa, 1972), various lipid extracts and lipoprotein fractions (Bialy, Ludwick, Hess & Ely, 1957; Gebauer, Pickett, Komarek & Gauryan, 1970; Pace & Graham, 1974; Watson, 1976) and specific lipoproteins (Masuda & Nishikawa, 1972). Laboratory assessment of semen has indicated that many of these preparations are capable of reducing cell damage and maintaining motility of spermatozoa after cooling or freezing and thawing.

In this laboratory, lipoprotein fractions have been prepared from egg yolk by centrifugation and column chromatography (Foulkes, 1977) and the non-destructive techniques employed gave preparations of high solubility. The fractions were effective in protecting spermatozoa during freezing and thawing, as measured by the maintenance of cellular integrity and retention of motility. The protective capacity of the most effective and abundant of the egg-yolk lipoproteins was investigated by artificial insemination after the freezing of bovine semen in a diluent prepared from fraction 3.

Methods

Egg yolk was separated from egg white and traces of albumin were removed by blotting on filter paper. The lipoprotein 3 fraction, prepared as described previously (Foulkes, 1977), eluted at a position just before bovine serum albumin (BSA). Successive preparations were combined and their volume reduced with a Bio-Fibre 80 ultrafiltration device (Bio-RaD Laboratories Ltd, Bromley, Kent). Contaminants were removed by repeated washing with 2.9% trisodium citrate. $2\text{H}_2\text{O}$ pH 7.0) and reduction of the volume to dryness, and the diluent buffer was added.

Three diluents, all including 500 µg streptomycin sulphate/ml, were prepared as follows. (1) The lipoprotein-citrate diluent was prepared in two parts, without glycerol (A) and containing 14% glycerol (B). The protein content of the lipoprotein fraction was determined (Lowry, Rosebrough, Farr & Randall, 1951) and each part was adjusted to contain 10 mg protein/ml in 2.9% trisodium citrate. $2\text{H}_2\text{O}$ (pH 7.0). (2) The egg yolk-citrate control diluent was also prepared in two parts; Part A contained egg yolk in 2.9% trisodium citrate. $2\text{H}_2\text{O}$, pH 7.0 (20:80 v/v) and Part B contained egg yolk, 2.9% trisodium citrate. $2\text{H}_2\text{O}$, pH 7.0, and glycerol (20:66:14 by vol.). (3) The egg yolk-lactose control diluent consisted of egg yolk, 11% lactose and glycerol (20:75:3.4:7 by vol.).

Two collections were obtained from each of 3 fertile Friesian bulls by using an artificial vagina. The semen was maintained at 30°C and each ejaculate was split between the three diluents. Lipoprotein-citrate and egg yolk-citrate diluents were added in two stages. Initial dilution was performed with the unglycerolated Part A and the diluted semen was cooled to 5°C over 30 min. An equal volume of the appropriate glycerolated part (Part B) was added in two steps, 15 min apart, to give a final glycerol concentration of 7%. The lactose diluent was added in its entirety at 30°C to provide a glycerol concentration of 4.7% and the diluted semen was cooled to 5°C over 30 min. Diluted semen from each collection and treatment was equilibrated for 6 h at 5°C before 0.25 ml polyvinylchloride straws were filled and sealed to provide 20×10^6 spermatozoa/straw. Semen was frozen on horizontal racks 4 cm above the surface of liquid nitrogen in a Union Carbide LR320 wide-necked container. A temperature of -120°C was attained in 7 min and the straws were then transferred to a goblet containing liquid nitrogen. The motility of spermatozoa in the ejaculates and in the diluted semen after freezing and thawing was recorded blind by an independent observer (Table 1).

After storage for 28 days, equal numbers of coded straws from each collection and treatment were thawed and used for the insemination of 1782 unselected Friesian cows of known identity. Each cow was inseminated once only and the number of cows not returning to service in the 16 weeks after insemination was recorded to provide a measure of fertility.

Table 1. Motility of bovine spermatozoa in ejaculated semen (assessed on a scale from 0 to 10) and in diluted semen after freezing and thawing (as % of motile cells)

Bull	Sample	Ejaculated spermatozoa	Frozen-thawed spermatozoa		
			Egg yolk-lactose	Egg yolk-citrate	Lipoprotein-citrate
1	(a)	5.0	38	33	55
	(b)	5.5	30	28	45
2	(a)	5.0	33	55	43
	(b)	5.5	30	45	45
3	(a)	4.5	30	28	55
	(b)	6.5	33	45	50
Mean		5.4	35	39	49

Table 2. Non-return rates of cows (99/group) 16 weeks after first insemination with semen stored in different diluents

Bull	Sample	Diluent		
		Egg yolk-lactose	Egg yolk-citrate	Lipoprotein-citrate
1	(a)	57.0	68.3	67.7
	(b)	69.7	64.0	69.0
2	(a)	62.9	69.5	66.7
	(b)	63.6	62.6	62.6
3	(a)	73.2	62.2	66.7
	(b)	72.0	75.8	63.6
Mean		66.4	67.1	66.1

Results and discussion

Observations of motility (Table 1) indicated that spermatozoa had apparently survived freezing and thawing well in all three diluents. The motilities recorded for the lipoprotein diluent tended to be slightly higher than for the other diluents, but this may have been the result of the ease with

which individual spermatozoa could be observed in this clear diluent. The 16-week non-return rates recorded for each diluent (Table 2) showed that use of the lipoprotein diluent had resulted in levels of fertility indistinguishable from those obtained with the control diluents.

This field trial has shown that a lipoprotein prepared from egg yolk is capable of protecting spermatozoa during the freezing and thawing procedures used in artificial insemination at a diluent level equivalent to that in whole egg yolk, thus confirming previous in-vitro findings (Foulkes, 1977). The protective action is unlikely to be exclusive to egg-yolk lipoproteins because diluents containing milk have been used for many years (Thacker & Almquist, 1951). The lipoprotein preparation we used had chromatographic characteristics similar to those of pig serum lipoproteins (Kalab & Martin, 1968). Hillyard, White & Pangburn (1972) have equated some egg-yolk lipoproteins with chicken serum lipoproteins, and Holdsworth, Michell & Finean (1974) have demonstrated the transfer of lipoproteins from plasma to egg yolk. Lipoproteins from these and other sources may be similarly effective in protecting spermatozoa.

Foulkes (1977) was unable to demonstrate an association between particular lipids and spermatozoa following the use of diluents prepared with radioactive egg yolk, but there was some evidence of the binding of labelled lipoproteins to the cells. Information is now required on the nature of the interaction between lipoproteins and spermatozoa, particularly the means by which the sperm membrane is apparently reinforced to survive the rigours of freezing and thawing. Knowledge of this mechanism would accelerate the formulation of defined diluents from sources free from disease risks and could facilitate the freezing of spermatozoa from other species.

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