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Effects of Egg Yolk, Glycerol and the Freezing Rate on the Viability and Acrosomal Structures of Frozen Ram Spermatozoa

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Abstract

The influence of egg yolk, glycerol and the freezing rate on the survival of ram spermatozoa and on the structure of their acrosomes after freezing was investigated. Egg yolk was shown to be beneficial not only during chilling but also during freezing; of the levels examined, 1·5% gave the greatest protection. Although the presence of glycerol in the diluent improved the survival of spermatozoa, increasing concentrations produced significant deterioration of the acrosomes. With closely controlled linear cooling rates, no overall difference was detected in the survival of spermatozoa frozen at rates between 6 and 24°C per min. However, a significant interaction between freezing rate and the inclusion of glycerol in the diluent showed that glycerol was less important at the highest freezing rate. A sudden cooling phase near to the freezing point following the release of the latent heat of fusion was not detrimental to spermatozoa.

Introduction

Since its protective role was first demonstrated by Phillips (1939), egg yolk has been a common constituent of diluents used for the chilling and deep freezing of spermatozoa. It is well known that egg yolk is protective to spermatozoa during chilling and storage above 0°C but its protective effect during freezing has not been clearly demonstrated. Most evidence suggests that it is beneficial to ram spermatozoa during freezing but a minority of reports have claimed that it was of no benefit (Platov and Sevcova 1967; Salamon 1970).

The survival of spermatozoa during freezing is dependent on a number of interrelated factors amongst which are the diluent composition and the freezing rate (Jones 1971). For example, the optimum level of glycerol was found to be about 7% for ram spermatozoa frozen in ampoules (First *et al.* 1961) whereas 2–4% was satisfactory for spermatozoa frozen by the 'pellet' method, where a smaller volume and a faster freezing rate were used (Lightfoot and Salamon 1969; Salamon 1970). No comparable studies of the glycerol requirement have been made with semen frozen in straws, although for the commercial production of frozen bull semen, 7% is commonly used with success (Milk Marketing Board 1967).

Reports of the survival of ram spermatozoa at different freezing rates are rare. In general, slower freezing rates have given better results than faster rates when semen was frozen in ampoules (Hill *et al.* 1959; Entwistle and Martin 1972), but Salamon (1970) obtained good revival with pelleted semen frozen at quite high rates. In the experiments reported here, variations in diluent composition have been studied in relation to the freezing rate for diluted ram semen frozen in straws.

Materials and Methods

The composition of the diluent used in these studies was 247 mM glucose, 17 mM fructose, 49 mM NaCl, 5 mM KCl, 10 mM each of Na_2HPO_4 and NaH_2PO_4. Penicillin G (500 i.u./ml) and dihydrostreptomycin (500 i.u./ml) were included. Egg yolk was added at the levels indicated in the experiments and the larger yolk globules were removed by centrifugation at 1000 *g* for 10 min. The level of egg yolk is given as that before centrifugation and no correction is made for the portion removed by this process.

Semen was collected by electro-ejaculation (Blackshaw 1954) of Merino rams. After initial dilution at 30°C with diluent containing no glycerol, the semen was cooled to 5°C at a constant rate of 0·21°C per minute. A second volume of diluent containing 15% (v/v) glycerol was added at 5°C, to bring the final dilution of the semen to 1 : 20 and the glycerol level to 7·5%. In experiment 4, the glycerol level was varied as an experimental factor.

The glycerolated semen was held at 5°C, and during this period plastic straws (1·7 mm diameter, 133 mm long; Cassou, L'Aigle, France) were filled and their polyvinyl alcohol seals set by holding them in a water-bath at 5°C. Freezing was commenced not less than 3·75 h (expt 1) or 5 h (expts 2–4) after the initial dilution. The straws were frozen in a cabinet whose temperature was controlled from +5 to −55°C. The freezing rate could be varied between 0·25 and 24°C fall per minute and could be interrupted by a pause at any stage during which the temperature was held constant (expts 1 and 2). Up to 100 straws, held in a horizontal position, could be frozen in a single batch. The frozen samples at −55°C were plunged into liquid nitrogen (−196°C) and were held for at least 48 h before thawing for the assessment of the motility and the preparation of smears for staining with Giemsa. The straws were thawed by immersion in a water-bath at 37°C.

All samples were examined under the microscope within 5 min of thawing, on a warm stage at 37°C. Motility was estimated on a 0–4 scale in which half-grades were scored (Emmens 1947) and the percentage motile estimated to the nearest 10% (Martin 1963). The Giemsa staining and scoring technique has been described elsewhere (Watson and Martin 1972). Briefly, the degree of damage to the acrosomes of spermatozoa was estimated on a 0–3 scale, 0 indicating undamaged spermatozoa and 3 loss of the acrosome.

Only a single freezing treatment could be conducted at any one time. Where more than one such treatment was involved (expts 1, 2 and 4) the sequence of freezing treatments in successive replications of the experiment was determined by a latin square design. By this means, the main effect of freezing treatments was separated from the possible influence of differences in equilibration time. The 'between-day' effect (replications) and the equilibration time trend were also obtained as main effects. Some confounding did, however, result from this design in the interactions of freezing treatment and replication factors, and these interactions were not dissected but were pooled as a residual error term in the analyses of variance.

Experimental designs and methods relevant to each experiment were as follows.

Experiment 1

This was a 2^2 factorial design in which two replicates (ejaculates) were frozen on each of four days using a 4 by 4 latin square to determine the sequence of freezing treatments as outlined above.

Two freezing rates, 1 and 12°C per minute, were tested with or without a 3-min pause at the freezing point of the samples. Matching samples were also frozen in ampoules following method B of Entwistle and Martin (1972). Egg yolk was included in the diluent at 6% (v/v).

Experiment 2

In this case the freezing process was halted for 0, 3, 6 or 12 min at the freezing point and egg yolk was incorporated in the diluent at 3, 6 or 12% (v/v). Thus the design was a 3 by 4 factorial and replication was the same as experiment 1. The sequence of applying the 'pause' treatments followed a 4 by 4 latin square and a total of eight ejaculates were used as replicates.

Experiment 3

Four methods of preparation of semen before freezing were tested and six replicates of the experiment were completed. The preparations were:

(1) No egg yolk added to the diluent during either chilling or freezing;

(2) No egg yolk in diluent during chilling and 3% egg yolk present at freezing;
(3) 3% egg yolk in diluent during chilling but removed before freezing;
(4) 3% egg yolk present during both chilling and freezing.

Samples of semen were diluted at 30°C in the appropriate diluents for chilling and cooled to 5°C. All samples were then centrifuged at 1000 *g* for 10 min, the supernatant was removed and the spermatozoa were resuspended in the appropriate diluents for freezing. This second set of diluents all contained glycerol at a level of 7·5%. A freezing rate of 12°C per minute was used.

Experiment 4

The experiment was a 3^3 factorial design replicated with six ejaculates, a pair per day, in a 3 by 3 latin square for allocation of the sequence of freezing rates applied. Levels of the factors were: egg yolk 0, 1·5 or 3% (v/v); glycerol 0, 2·5 or 7·5% (v/v); freezing rate 6, 12 or 24°C per min.

Table 1. Effects of a pause at the freezing point and of concentration of egg yolk on ram spermatozoa deep-frozen to −196°C

Values are mean scores from eight replicates with ejaculates from different rams. For details of experiments, see text

Expt No.	Treatment	Motility	% motile	Acrosome changes
1	Freezing at 1°C per minute	1·45	19·00	—
	Freezing at 12°C per minute	1·67	21·25	—
	No pause at freezing point	1·59	20·64	—
	3-min pause at freezing point	1·53	19·61	—
	Sperm frozen in ampoules	1·72	20·31	—
2	Pause of 0 min	1·88	29·38	0·90
	Pause of 3 min	1·82	25·74	0·91
	Pause of 6 min	1·91	26·25	0·97
	Pause of 12 min	1·91	28·33	0·91
	3% egg yolk	1·82	26·67	0·91
	6% egg yolk	1·96	28·91	0·94
	12% egg yolk	1·85	26·72	0·92

Results

The survival of spermatozoa after freezing and thawing in experiment 1 is presented in Table 1. No significant differences were recorded between freezing rates of 1 and 12°C per minute, and the introduction of a pause in freezing was without effect. The overall survival of spermatozoa frozen in straws was comparable with that of those frozen in ampoules. A significant between-day contrast for motility ($P < 0·05$) was recorded and there were significant differences between ejaculates within days ($P < 0·05$).

The results of experiment 2, motility scores and acrosome damage after freezing and thawing, are shown in Table 1. There were no significant differences in survival or in acrosome damage between freezing treatments, and varying the egg yolk level between 3 and 12% had no effect. Significant contrasts between days and between ejaculates within days were observed for all scores.

The four methods of preparation of semen before freezing in experiment 3 showed that the presence of egg yolk during chilling gave a marked improvement in the

survival of spermatozoa after freezing ($P < 0{\cdot}001$) and reduced acrosome damage ($P < 0{\cdot}05$) (Table 2). Moreover, a smaller improvement in survival was seen when egg yolk was included in the freezing diluent [$P < 0{\cdot}01$ (motility score); $P < 0{\cdot}001$ (percentage motile score)] although no effect was detected on the acrosomes. The presence of egg yolk was more effective during chilling than during freezing in increasing the percentage of spermatozoa surviving after freezing ($P < 0{\cdot}05$) and in reducing the degree of acrosome damage resulting from freezing ($P < 0{\cdot}001$). There were differences in survival ($P < 0{\cdot}001$) and acrosome damage ($P < 0{\cdot}05$) between ejaculates and some significant differences between semen samples from individual rams in response to the various treatments.

Table 2. Experiment 3: effect of egg yolk on mean survival and acrosome scores of ram spermatozoa chilled and frozen to −196°C

Treatment	Egg yolk treatment		Motility	% motile	Acrosome score
	Chilling	Freezing			
1	−	−	1·33	17·21	1·33
2	−	+	1·79	25·42	0·99
3	+	−	2·02	28·75	0·95
4	+	+	2·13	32·08	1·08

Summary of analyses of variance

Source of variation	D.F.	Variance ratios Motility	% motile[A]	Acrosome score
Diluent treatments	3			
1 and 2 *v.* 3 and 4	1	25·00***	42·92***	4·80*
1 and 3 *v.* 2 and 4	1	7·60**	21·25***	2·50
Interaction	1	3·00	5·31*	13·10***
Ejaculates	5	13·88***	38·06***	2·50*
Treatment × ejaculates	15	2·04*	2·98**	0·80
Sampling (error) variance	72	0·25	25·48	0·10

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

[A] Data transformed to angles for analysis.

Again, in experiment 4 (Table 3) the presence of egg yolk improved both the motility of spermatozoa and the percentage motile after freezing ($P < 0{\cdot}001$). The lower level, 1·5%, was superior to 3% in motility score ($P < 0{\cdot}05$), but there were no significant effects of egg yolk levels on the percentage of motile spermatozoa. Damage to acrosomes was less severe in the presence of egg yolk ($P < 0{\cdot}01$), and 1·5% egg yolk was more effective than 3% ($P < 0{\cdot}05$). The survival of spermatozoa was increased by the presence of glycerol ($P < 0{\cdot}001$), but no advantage was seen with 7·5% compared with 2·5% for the two scores of spermatozoal activity. Acrosome damage was significantly greater in the presence of glycerol ($P < 0{\cdot}001$) and the degree of acrosome disruption was greater at the higher level ($P < 0{\cdot}001$). The main effect of freezing rates was not significant for any of the scores.

Significant interactions indicate that the motility of spermatozoa frozen with 7·5% glycerol was greater than that of spermatozoa frozen with 2·5% glycerol in the absence of egg yolk, but in its presence there was little difference between the two levels of

glycerol (presence of egg yolk by level of glycerol, $F_{1,88} = 8{\cdot}82$; $P < 0{\cdot}01$). The percentage of spermatozoa surviving after freezing at 24°C per minute was greater than that after freezing at 6°C per minute when no glycerol was present, but, even at the fastest freezing rate, revival was lower than where glycerol was included in the diluent (level of glycerol by linear component of freezing rate, $F_{1,88} = 13{\cdot}73$; $P < 0{\cdot}001$). Only minor differences in survival were observed between the cooling rates employed when glycerol was included in the diluent.

Table 3. Experiment 4: effects of egg yolk, glycerol and freezing rate on the survival and acrosome scores of ram spermatozoa deep-frozen to −196°C

Treatment	Motility	% motile	Acrosome score
A. Egg yolk level (%)			
0	1·16	11·37	1·22
1·5	2·05	27·06	1·00
3·0	1·87	24·63	1·13
B. Glycerol level (%)			
0	1·23	10·81	0·96
2·5	1·85	24·46	1·00
7·5	1·99	27·78	1·35
C. Freezing rate (°C/min)			
6	1·60	20·74	1·10
12	1·71	21·07	1·12
24	1·76	21·24	1·13

Summary of analyses of variance

Source of variation	D.F.	Variance ratios		
		Motility	% motile[A]	Acrosome score
A. Egg yolk	2			
Egg yolk *v.* none	1	135·82***	160·65***	10·13*
1·5% *v.* 3·0%	1	4·94*	1·59	5·50*
B. Glycerol	2			
Glycerol *v.* none	1	100·76***	167·45***	14·63***
2·5% *v.* 7·5%	1	3·06	3·57	42·25***
C. Freezing rates	2	2·06	0·98	0·13
C′. Equilibration time trend	2	1·24	1·64	0·50
D. Ejaculates/days	5	12·12***	10·85***	4·63***
A×B	4	2·76*	2·42	0·50
A×C	4	0·24	0·18	1·25
B×C	4	2·29	5·30*	1·00
A×B×C	8	0·53	1·25	1·25
A×D	10	2·94*	3·42**	2·38*
B×D	10	1·94	2·22	0·88
A×B×D	20	1·82*	1·27	0·75
Residual (error) variance	88[B]	0·17	31·97	0·08

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

[A] Data transformed to angles for analysis.

[B] 90–2 (for C′) = 88 degrees of freedom for 'error'.

Discussion

In previous studies in this laboratory, semen has been frozen in 1-ml aliquots sealed in glass ampoules. However, uncontrolled variations in the freezing rate and

pattern of crystallization occur within the sample because of its volume and dimensions, and this has hindered more critical studies of the freezing conditions. In addition, the freezing curve recorded by a thermocouple within the sample has a characteristic deviation from linearity (Entwistle and Martin 1972).

The use of the plastic 'mini-straw' offers several advantages to the study of spermatozoa during freezing, in that the sample volume is 0·25 ml and no point is more than 1 mm from the surface. When crystallization is initiated it spreads rapidly from the nucleation point in both directions along the straw. Because of the large surface area in relation to the volume of the straw, the latent heat of fusion can be removed rapidly, and a freezing rate approaching linearity can be achieved. It is therefore possible to produce specified freezing rates more exactly within the sample. The initial experiment in this series clearly demonstrated that the survival of spermatozoa frozen in straws was comparable to that of spermatozoa frozen in ampoules.

The introduction of a pause in freezing at the temperature of crystallization reduced the temperature differential between the straws and the cabinet while a substantial part of the latent heat of fusion of the water was evolved and extracted. No advantage was gained by this modification in freezing (expts 1 and 2) and it may therefore be concluded that a temperature fluctuation coinciding with the formation of ice crystals was not detrimental to the spermatozoa.

Entwistle and Martin (1972) found that less than 6% egg yolk in the diluent used for freezing ram spermatozoa in ampoules resulted in reduced survival. In experiment 2, however, no difference was observed between the survivals of spermatozoa in diluents with egg yolk levels ranging from 3 to 12%, and the degree of acrosome disruption was similar in all diluents. If egg yolk is at all beneficial during the freezing of spermatozoa in straws, the critical level for optimum survival must therefore lie below 3%. Experiment 3 demonstrated that egg yolk was beneficial during both chilling and freezing but the suggestion that its presence during chilling was of greater importance may be an oversimplification of the situation. In the treatment in which the diluent containing yolk was removed before freezing (treatment 3, Table 2), traces of egg yolk would have remained. These might have been sufficient to give some protection during freezing, particularly if the protective substances in yolk are bound to the cell surface. The acrosomes of spermatozoa both chilled and frozen in the presence of egg yolk were more damaged than those of spermatozoa with egg yolk present only during chilling or freezing. Nevertheless the differences were slight, and the major conclusion is clearly that egg yolk was advantageous.

It was evident from the results of experiment 4 that a very low concentration of egg yolk is required, since there were only small differences between diluents containing 1·5 and 3% egg yolk. The interaction of egg yolk and glycerol in the motility scores indicated that the level of glycerol could be reduced providing egg yolk were present. Contrary to the findings of Saroff and Mixner (1955) with frozen bull spermatozoa, there was no evidence that egg yolk interfered with the protective action of glycerol.

A glycerol level of 2·5% in the diluent gave a survival comparable with that of 7·5%, while a small but consistent proportion of spermatozoa survived freezing in the absence of glycerol. The degree of acrosome damage was directly related to the level of glycerol, a finding similar to that of Wilmut and Polge (1972) for boar spermatozoa. This result suggests that a lower level of glycerol than the 7% commonly used for bull spermatozoa (Milk Marketing Board 1967) may be advantageous.

Since egg yolk apparently did not have this deleterious effect, and since its presence reduced the requirement for glycerol, the levels of these substances need further investigation in order to determine concentrations giving maximal survival with minimal structural alterations. The freezing rate was also shown to be relevant to those considerations, since lower levels of glycerol could be used when faster freezing rates were employed. For the pellet-freezing of ram spermatozoa, where very high freezing rates are employed, it has been shown that only 2–4% glycerol is required (Lightfoot and Salamon 1969; Salamon 1970). It therefore appears that the colligative, or water-binding, properties of glycerol, in reducing the crystallization of ice and concentration of solute, are of less importance when the sample is cooled rapidly through the critical regions of the freezing curve.

An explanation is required for the complex relationships between glycerol and egg yolk, and between glycerol and the freezing rate. High-molecular-weight components of egg yolk (Watson and Martin 1974) may stabilize cell membranes, making the cells more resistant to damage. Then the action of glycerol in diminishing the proposed causes of cryo-injury, i.e. solution effects (Mazur 1970) or dehydration (Litvan 1972), may be less important. The use of faster freezing rates might simultaneously reduce the requirement for a cryoprotective such as glycerol by limiting the time during which these effects can occur. Without an adequate theory of cryo-injury these conclusions remain speculative, but they do furnish hypotheses on which to base future experiments.

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