EFFECTS OF COMPOSITION OF DILUENT, METHOD OF ADDITION OF GLYCEROL, FREEZING RATE, AND STORAGE TEMPERATURE ON THE REVIVAL OF RAM SPERMATOZOA AFTER DEEP-FREEZING

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Abstract

The addition of 6% (v/v) egg yolk to a synthetic diluent [247 mM glucose, 49 mM NaCl, 5 mM KCl, 5 mM phosphate buffer, and 7·5% (v/v) glycerol] improved the survival of ram spermatozoa after deep-freezing. Judging by the activity of spermatozoa after thawing, a single addition of glycerol to the diluted semen at 5°C was as effective as multiple additions giving a graded increase of glycerol concentration over a period of 20 min. Reciprocal replacement of the sodium chloride of the diluent by an increase in the concentration of the phosphate buffer showed that motility of the spermatozoa after thawing was depressed when the level of phosphate exceeded 10 mM.

A rapid rate of freezing of 1-ml aliquots of semen in glass ampoules in the neck of a liquid nitrogen container gave lower survival of spermatozoa than a slower rate in dry ice (average rates to −40°C of 1 and 2 degrees Celsius per minute respectively).

Spermatozoa survived a period of 2 weeks of storage in liquid nitrogen better than when stored in dry ice. There was a significant decline in the revival rate of samples prepared and frozen from four successive ejaculates collected by electro-ejaculation at intervals of 3 days.

I. Introduction

Emmens and Blackshaw (1950, 1955), Blackshaw and Emmens (1953), and Hill, Godley, and Hurst (1959) have reported revival of ram spermatozoa deep-frozen in diluents based on mixtures of egg yolk and sodium citrate. However, Jones and Martin (1965) have indicated that, particularly after 20- or 40-fold dilution of the semen, survival was poorer in an egg yolk–citrate diluent than in diluents based on milk or synthetic diluents containing a high proportion of lactose.

Various preparations of cow milk have been used in diluents for deep-freezing ram spermatozoa and satisfactory revival on thawing has been recorded (Blackshaw 1960*a*, 1960*b*; First *et al.* 1961; Martin 1961; Jones and Martin 1965). More recently, synthetic diluents containing sugars and electrolytes, together with small proportions of preparations from milk or egg yolk, have been developed which give a degree of protection to ram spermatozoa during freezing and thawing which is comparable to, or in some cases exceeds, that given by diluents based on heated milk or fresh egg yolk (Jones 1965; Jones and Martin 1965).

Apart from the studies of Hill, Godley, and Hurst (1959), and Jones (1965), using 1-ml aliquots of diluted semen in glass ampoules, and Salamon (1970), using a drop of diluted semen of up to 0·3 ml volume, i.e. "pellet freezing" (Nagase and Niwa 1963), the effects of freezing rate and temperature of storage on the survival of deep-frozen ram spermatozoa have not been examined in any detail.

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II. Materials and Methods

(a) *General Procedure*

Ram semen was collected by electro-ejaculation (Blackshaw 1954), diluted at 30°C within 30 min of collection, and cooled to 5°C in 2 hr. Glycerol was added to the diluted semen by a further dilution after the sample had been held at 5°C for 1·5 hr and freezing was started after another 1·5 hr at 0–5°C. A final semen dilution rate of 40-fold was used in all experiments.

In all experiments, suitable volumes of egg yolk were dispersed in a mixture composed of 247 mM glucose, 49 mM NaCl, 5 mM KCl, 4 mM sodium phosphate buffer (pH 7), and 17 mM fructose together with 500 i.u. each of penicillin and streptomycin per millilitre. The second-stage diluent containing glycerol had the same content of these yolk-containing substances together with 15% (v/v) glycerol. Each mixture was centrifuged at 700 *g* for 10 min and the upper fatty layer removed. The remainder of the supernatant was used as the diluent and the waxy plug at the bottom of the centrifuge tube was discarded. Thus, the actual content of yolk is approximately 15–20% lower than that stated. However, the procedure was necessary as in earlier preparations, in which egg yolk was simply shaken with the synthetic diluent, the presence of undispersed fat globules in the diluted semen made accurate observation of the spermatozoa impossible.

All samples were frozen in 1 ml borosilicate glass ampoules in which the aliquot formed a cylinder of diameter 8 mm and height 20 mm.

All frozen samples were thawed in a water-bath at 37°C and specimens were examined microscopically whilst held on a warm stage also at 37°C. Intensity of motility was scored on a scale 0–4 (Emmens 1947) and the percentage of motile spermatozoa was estimated to the nearest 10%.

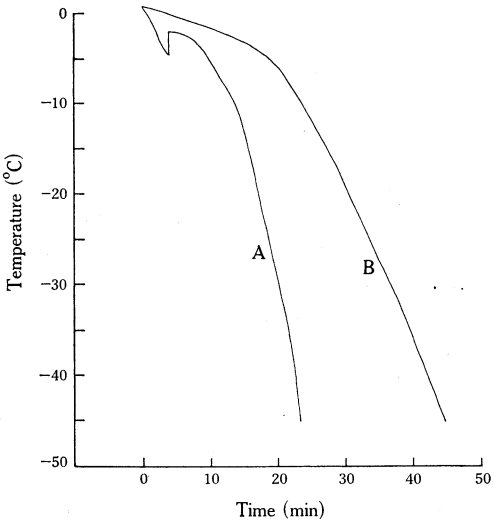

Fig. 1.—Freezing rates used in experiment 3. Rate A was obtained by suspending the ampoules in the neck of a liquid nitrogen flask (Linde, LR35–9) and the slower rate B was given by a container patterned on that described by Polge and Lovelock (1952).

All specimens were examined after coding their identity and randomization of the order in which they were seen (Martin 1963). In each experiment either two or three samples from each ejaculate were prepared for each combination of levels of factors and in the analyses of variance for the data from experiments 1 and 2 the "between-samples" variance term was used as the estimate of error variance. The form of the analyses of variance for experiment 3 is shown in Table 3. Here the variance has been partitioned three ways, i.e. "between-rams", "within-rams", and "sample variance per treatment within ejaculates". Both percentages of motile and of unstained spermatozoa were transformed to angles for the analyses of variance (Claringbold, Biggers, and Emmens 1953).

(b) Procedures Specific for Individual Experiments

(i) *Experiment 1*

The glycerol-containing solution was mixed with an equal volume of diluted semen in one addition or as three additions, 10 min apart, of 0·2, 0·4, and 0·4 units of the volume of the sample of chilled diluted semen.

(ii) *Experiment 2*

An increasing proportion of the sodium chloride of the diluent was replaced by isosmolar quantities of sodium phosphate buffer (pH 7) to give the levels shown in Table 2. Glycerol was added to all samples by a single addition of the second-stage diluent.

In both experiments samples were frozen at rate B (Fig. 1) and stored at −79°C for 48 hr before thawing.

TABLE 1

EXPERIMENT 1: EFFECT OF EGG YOLK CONTENT OF DILUENT AND METHOD OF ADDITION OF GLYCEROL TO DILUTED RAM SEMEN ON THE SURVIVAL AFTER DEEP-FREEZING

All values are means

Main effects	*n*	Motility (0–4)	% Motile
A. Egg yolk levels (%, v/v):	24		
1. 0		2·42	23·8
2. 6·5		2·81	37·9
3. 13·0		2·72	34·8
Level of significance:			
0 *v.* 6·5 and 13·0%		$P < 0·001$	$P < 0·001$
6·5 *v.* 13·0%		n.s.	n.s.
B. Method of introducing glycerol to diluent	36		
Single addition		2·65	31·4
Three additions		2·65	32·6
Level of significance:		n.s.	n.s.
C. Ejaculates	18		
1		2·86	40·7
2		2·33	22·5
3		2·88	36·3
4		2·58	19·4
Level of significance:		$P < 0·001$	$P < 0·001$
Significance of interactions:			
A×B		n.s.	n.s.
A×C		$P < 0·01$	$P < 0·01$
B×C		n.s.	n.s.
A×B×C		n.s.	n.s.
Error variance	(48)	*0·38*	*25·62*

(iii) *Experiment 3*

Ejaculates were collected from each of four rams at intervals of 3 days. Two rates of freezing were used and are shown in Figure 1. The notch in the early part of curve A indicates that the sample supercooled before crystallization of ice occurred. After freezing, ampoules were stored either in dry ice (−79°C) or liquid nitrogen (−196°C) for 2 weeks before thawing.

Spermatozoa in smears made from the diluted semen and stained with congo red–fast green FCF (Entwistle, unpublished data) were classified as stained or unstained. One hundred spermatozoa were examined in each smear and the count expressed as a percentage of unstained spermatozoa.

III. Results

The results of experiment 1 are summarized in Table 1. There was no significant effect of an increase in level of egg yolk in the diluent from 6·5 to 13% (v/v) but the revival rate in the absence of egg yolk was significantly lower than in diluents

Table 2

Experiment 2: effect of electrolyte composition of diluent on activity of deep frozen ram spermatozoa after thawing

All values are means

Main effects			*n*	Motility (0–4)	% Motile
A. Electrolyte composition*			16		
	NaCl (mM)	Phosphate buffer (mM)			
1.	57	0		2·87	38·3
2.	50	5		2·90	42·8
3.	43	10		2·84	37·7
4.	28·5	20		2·87	35·2
5.	0	40		2·84	33·9
Level of significance:					
Linear				n.s.	$P < 0·01$
Remainder				n.s.	n.s.
B. Egg yolk levels			40		
3·0% (v/v)				2·53	35·3
6·0% (v/v)				2·90	39·9
Level of significance:				$P < 0·05$	$P < 0·01$
C. Ejaculates			20		
1				3·00	44·8
2				2·97	41·7
3				2·50	26·0
4				3·00	38·6
Level of significance:				$P < 0·001$	$P < 0·001$
Significance of interactions					
A × B				n.s.	n.s.
A × C				n.s.	$P < 0·05$
B × C				n.s.	n.s.
A × B × C				n.s.	n.s.
Error variance			(40)	*0·06*	*17·10*

* 5 mM KCl present in all diluents.

containing egg yolk. The method of addition of the glycerol-containing portions of the diluent had no effect on the survival of spermatozoa during freezing. The significant interaction of yolk level × ejaculate indicated the differences in degree of response of ejaculates to the presence or absence of egg yolk in the diluent.

TABLE 3

EXPERIMENT 3: EFFECT OF RATE OF FREEZING AND TEMPERATURE OF STORAGE ON REVIVAL OF DEEP FROZEN RAM SPERMATOZOA PREPARED FROM SUCCESSIVE EJACULATES

Treatment	Motility (0–4)	% Motile	% Unstained
A. Freezing rate (Fig. 1)			
1. In liquid nitrogen vapour ("rapid")	2·99	33·0	22·5
2. In dry ice ("slow")	3·26	42·0	28·4
B. Storage temperature			
1. −196°C	3·16	39·8	25·6
2. −79°C	3·08	35·1	25·3
C. Rams			
1	3·20	40·2	31·1
2	3·18	39·0	28·0
3	2·84	28·4	15·6
4	3·26	42·5	28·2
D. Ejaculates			
First	3·26	41·2	28·6
Second	3·12	37·5	25·5
Third	3·10	37·7	24·3
Fourth	3·00	33·5	23·3

Summary of Analyses of Variance:

Source of variation	D.F.	Variance ratios: Motility	% Motile	% Unstained
A. Freezing rates	1	16·12*	104·43**	28·31
B. Storage temperature	1	1·39	28·28*	0·11
C. Rams	3	8·28	52·52**	43·61**
Interactions				
A×B	1	0	1·88	0·01
A×C	3	0·30	2·04	0·32
B×C	3	0·46	1·51	1·14
A×B×C (error I)	3	2·15	0·46	1·77
D. Collection intervals	3	5·84	5·97**	7·65***
Interactions				
A×D	3	4·50*	2·84	4·53**
B×D	3	0·34	0·07	0·48
C×D	9	2·96	5·80***	19·17***
Pooled higher-order interactions (error II)	30	1·04	0·87	0·51
Sample variance per treatment within ejaculates (error III)	64	*0·25*	*21·70*	*18·82*

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

Reciprocal replacement of sodium chloride by phosphate in the electrolyte portion of the diluent caused a significant reduction in the percentage of motile spermatozoa in experiment 2 (Table 2). Significantly poorer survival of spermatozoa was observed after a change in the level of egg yolk in the diluent from 6·0 to 3·0% (v/v). The interaction of electrolyte composition of diluent × ejaculates indicated that the degree of response to the change in phosphate level (or simultaneously, a fall in chloride content) differed from ejaculate to ejaculate.

Results and summary analyses of variance for experiment 3 are presented in Table 3. Revival was significantly lower in those samples in which the rapid freezing rate was used. Judging from the scores of percentage motile only, storage at —196°C gave superior preservation of deep-frozen spermatozoa. There was a significant decline in revival rate after freezing of the ejaculates collected at intervals of 3 days. This reduction was more marked in those samples frozen in liquid nitrogen vapour (significant interaction of freezing rate × ejaculate in both motility score and percentage of unstained spermatozoa). The revival rate in the third ejaculate used from rams 2 and 3 was better than for the earlier ejaculates and this is the reason for the significant ram × ejaculate interaction.

IV. Discussion

In these experiments, the revival rate was best when the diluent contained 6 or 6·5% (v/v) egg yolk. Levels of this order are considerably lower than those used by Blackshaw (1955*a*, 1955*b*), Jones and Martin (1965), Salamon and Lightfoot (1969) and Salamon (1970) who all employed diluents which contained sodium citrate. However, First *et al.* (1961) noted a tendency toward decreased survival of ram spermatozoa frozen in diluents based on milk as the level of egg yolk was increased from 3 to 24% (v/v). Evidently the nature of the response to change in content of egg yolk in the diluent is, in part, related to the composition of the remainder of this diluent.

Although Feredean and Brăgăru (1964) and Lightfoot and Salamon (1969*a*) have investigated some effects of the method of addition of glycerol to the diluted ram semen before freezing, there appear to be no reports of slow addition of glycerol at 5°C. Our findings indicate that at this temperature there is no advantage in a three-step increment in glycerol level over addition of all the glycerol at once, to give a final content of 7·5% (v/v), and are in contrast to those reported on the preparation of deep-frozen bull semen (Miller and VanDemark 1954; O'Dell and Almquist 1957; Choong and Wales 1964). However, with the "pellet-freezing" technique, one-step dilution with the glycerol containing diluent at 30°C appears to be as satisfactory as dilution with the glycerolated diluent after cooling to 5°C (Lightfoot and Salamon 1969*a*).

To define an optimal preparative method will evidently involve the nature of diluents, rate of glycerol addition, and the temperature (or temperatures) at which glycerol is introduced to the mixtures. Choice of cooling rate, first to the point of freezing and then to the storage temperature of —79°C or lower, has been substantially an empirical process with some workers reporting success with slow rates of cooling where relatively high levels of glycerol have been used (Hill, Godley, and Hurst 1959;

Jones and Martin 1965) and others, using lower glycerol levels in diluents having a high content of di- or trisaccharide, have reported success using a "pelleting" technique of freezing which involves freezing at rates of the order of 100°C/min (Salamon 1968, 1970; Salamon and Lightfoot 1969; Lightfoot and Salamon 1969*a*, 1969*b*). Mazur (1968, 1970) has published data on the effects of cooling rate and survival of various cells (yeasts, human erythrocytes, mouse marrow stem cells, and hamster lung cells from tissue culture) and has shown in these experiments that cooling rate, nature and level of freeze protective agent (e.g. glycerol, dimethyl sulphoxide, polyvinylpyrrolidone) and other components of the suspending medium interact strongly. From his work it is clear that an exact definition of a freezing rate which causes little or no death of ram spermatozoa can only be made after more is known of such factors as the permeability of the cell membrane to water, the resistance of the spermatozoon to osmotic stress at sub-zero temperatures, and the nature of the crystallization pattern of both water and the solutes present in the diluent.

There is no obvious explanation for the change in resistance to freezing of spermatozoa collected from the four rams in a period of 10 days. There was no large variation in the characteristics of semen obtained over the collection period, and no changes in diet, environment, or management were evident. However, as significant interactions of ejaculate sequence with freezing rate and with rams were detected in these experiments, and as similar trends have been noted by other workers (Salamon and Lightfoot 1967), further experiments are necessary to elucidate the relationship of frequency of ejaculation to the resistance of ram spermatozoa to deep-freezing.

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VI. References

Blackshaw, A. W. (1954).—A bipolar rectal electrode for the electrical production of ejaculation in sheep. *Aust. vet. J.* **30**, 249.

Blackshaw, A. W. (1955*a*).—The effect of equilibration and the addition of various sugars on the revival of spermatozoa from −79°C. *Aust. vet. J.* **31**, 124.

Blackshaw, A. W. (1955*b*).—Factors affecting the revival of bull and ram spermatozoa after freezing to −79°C. *Aust. vet. J.* **31**, 238.

Blackshaw, A. W. (1960*a*).—The effects of pH, tonicity and the temperature of glycerolisation on the revival of ram and bull spermatozoa after freezing to −79°C. *Aust. vet. J.* **36**, 376.

Blackshaw, A. W. (1960*b*).—The effects of milk diluents on the viability of ram spermatozoa and their revival after freezing. *Aust. vet. J.* **36**, 432.

Blackshaw, A. W., and Emmens, C. W. (1953).—Survival of deep frozen mammalian spermatozoa. *Vet. Rec.* **65**, 872.

Choong, C. H., and Wales, R. G. (1964).—The effects of glycerol addition and equilibration on the revival of bull spermatozoa frozen in reconstituted skim milk. *Res. Vet. Sci.* **5**, 228.

Claringbold, P. J., Biggers, J. D., and Emmens, C. W. (1953).—The angular transformation in quantal analysis. *Biometrics.* **9**, 467.

Emmens, C. W. (1947).—The motility and viability of rabbit spermatozoa at different hydrogen-ion concentrations. *J. Physiol.* **106**, 471.

Emmens, C. W., and Blackshaw, A. W. (1950).—The low temperature storage of ram, bull and rabbit spermatozoa. *Aust. vet. J.* **26**, 226.

EMMENS, C. W., and BLACKSHAW, A. W. (1955).—The fertility of frozen ram and bull semen. *Aust. vet. J.* **31**, 76.

FEREDEAN, T., and BRĂGĂRU, F. (1964).—Contribution to the study on diluents for preserving ram semen. *Revta. Zootek. Med. vet. Buc.* **14**, 53.

FIRST, N. L., HENNEMAN, H. A., MAGEE, W. T., and WILLIAMS, J. A. (1961).—The storage of ram semen. *J. Anim. Sci.* **20**, 74.

HILL, J. R., GODLEY, W. C., and HURST, V. (1959).—Effect of glycerol equilibration time, glycerol level, and rate of temperature descent on the freezing of ram spermatozoa. *J. Anim. Sci.* **18**, 614.

JONES, R. C. (1965).—The use of dimethyl sulphoxide, glycerol, and reconstituted skim milk for the preservation of ram spermatozoa. II. The influence of diluent composition and processing time during freezing to −79°C with dimethylsulphoxide or glycerol or both compounds. *Aust. J. biol. Sci.* **18**, 887.

JONES, R. C., and MARTIN, I. C. A. (1965).—Deep-freezing ram spermatozoa: the effects of milk, yolk citrate and synthetic diluents containing sugar. *J. Reprod. Fert.* **10**, 413.

LIGHTFOOT, R. J., and SALAMON, S. (1969*a*).—Freezing ram spermatozoa by the pellet method. II. The effects of method of dilution, dilution rate, glycerol concentration, and duration of storage at −5°C prior to freezing on survival of spermatozoa. *Aust. J. biol. Sci.* **22**, 1547.

LIGHTFOOT, R. J., and SALAMON, S. (1969*b*).—Freezing ram spermatozoa by the pellet method. III. The effects of pellet volume, composition of the thawing solution, and reconcentration of the thawed semen on survival of spermatozoa. *Aust. J. biol. Sci.* **22**, 1561.

MARTIN, I. C. A. (1961).—The viability of diluted ram spermatozoa at 37°C, 5°C, and −79°C. Proc. Conf. Artif. Breed. Sheep in Australia, Univ. N.S.W. (Ed. E. M. Roberts.) p. 143.

MARTIN, I. C. A. (1963).—The influence of diluents and processing time after ejaculation on the revival of deep-frozen bull spermatozoa. *J. agric. Sci., Camb.* **61**, 55.

MAZUR, P. (1968).—Physical and chemical changes during freezing and thawing of cells, with special reference to blood cells. Proc. XIth Int. Congr. Int. Soc. Blood Trans., Sydney 1966. [Bibliotheca Haematologica No. 19. Pt. 3. pp. 764–77. (Ed. L. P. Hollander.) (Karger: Basel and New York.)]

MAZUR, P. (1970).—Cryobiology—the freezing of biological systems. *Science, N.Y.* **168**, 939.

MILLER, W. J., and VANDEMARK, N. L. (1954).—The influence of glycerol level, various temperature aspects and certain other factors on the survival of bull spermatozoa at sub-zero temperatures. *J. Dairy Sci.* **37**, 45.

NAGASE, H., and NIWA, T. (1963).—Studies on deep-freezing technique for bull semen. III. Deep-freezing of bull semen in tablet form. *Jap. J. Anim. Reprod.* **9**, 93.

O'DELL, W. T., and ALMQUIST, J. O. (1957).—Freezing bovine semen. I. Techniques for freezing bovine spermatozoa in milk diluents. *J. Dairy Sci.* **40**, 1534.

POLGE, C., and LOVELOCK, J. E. (1952).—Preservation of bull semen at −79°C. *Vet. Rec.* **64**, 396.

SALAMON, S. (1968).—Deep-freezing of ram semen: recovery of spermatozoa after pelleting and comparison with other methods of freezing *Aust. J. biol. Sci.* **21**, 351.

SALAMON, S. (1970).—Survival of ram spermatozoa following pellet freezing below −79°C. *Aust. J. biol. Sci.* **23**, 459.

SALAMON, S., and LIGHTFOOT, R. J. (1967).—Effect of cold shock, liquid storage and pellet freezing on successive ram ejaculates. *Aust. J. agric. Res.* **18**, 959.

SALAMON, S., and LIGHTFOOT, R. J. (1969).—Freezing ram spermatozoa by the pellet method. I. The effect of diluent composition on survival of spermatozoa. *Aust. J. biol. Sci.* **22**, 1527.