THE USE OF DIMETHYL SULPHOXIDE, GLYCEROL, AND RECONSTITUTED SKIM MILK FOR THE PRESERVATION OF RAM SPERMATOZOA

I. THE TONICITY AND TOXICITY OF DIMETHYL SULPHOXIDE AND RECONSTITUTED SKIM MILK AT 30 AND 5°C

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[*Manuscript received December* 29, 1964]

Summary

The viability of ram spermatozoa in milk and buffered saline diluents containing dimethyl sulphoxide and in milk diluents alone was investigated during incubation studies at 30 and 5°C. Dimethyl sulphoxide did not contribute to the tonicity of a diluent. Dimethyl sulphoxide (5% v/v) or glycerol in a diluent depressed spermatozoal activity during incubation at 30 or 5°C. Dimethyl sulphoxide retarded motility more than glycerol, especially with incubation at 5°C, whilst glycerol rendered a greater proportion of cells immotile than did dimethyl sulphoxide, this effect being significant only after incubation at 30°C.

Reconstituted skim milk was better at 9% (w/v) than at 7 or 11% (w/v) at both incubation temperatures. Spermatozoa do not survive as well in a reconstituted skim milk diluent prepared for only 4 hr as in a diluent prepared and stored at 5°C for 1 or 2 days before use. Heating to 85°C for 5 min removed the toxic factor in pasteurized milk but had no effect on the reconstituted skim milk diluent.

I. INTRODUCTION

A review by Emmens and Robinson (1962) describes diluents used for storage of ram spermatozoa at 30, 5, and −79°C. Trials by Salamon (1956) and Salamon and Robinson (1962*a*, 1962*b*) showed semen diluted in heated cows milk to be at least equal in fertility to undiluted semen or semen diluted in yolk–citrate–glucose. Dauzier (1956) demonstrated successful chilled storage for 12–24 hr in a milk diluent. Martin (1961) found that reconstituted skim milk (11% w/v) did not maintain the survival of spermatozoa during storage at 5°C as well as a yolk–citrate diluent and a number of synthetic diluents containing either egg yolk or casein, but milk was best for protecting spermatozoa during deep-freezing.

Of a number of sugars and alcohols tested, as well as inositol and urea (Emmens and Blackshaw 1950), none protected ram spermatozoa during freezing and thawing as well as glycerol, although the addition of a sugar to a glycerol-containing diluent improved revival. No other compounds have been used to prepare deep-frozen ram spermatozoa. Lovelock and Bishop (1959) reported that dimethyl sulphoxide was

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superior to glycerol in protecting erythrocytes, but inferior when used for freezing bull spermatozoa. Ashwood-Smith (1961*a*, 1961*b*) and Persidsky and Richards (1963) respectively found dimethyl sulphoxide equal to and better than glycerol for freezing bone marrow cells. Other workers have used dimethyl sulphoxide to freeze several strains of human cervical carcinoma (HeLa), chicken embryo cells, human and monkey embryonic kidney cells, lung cells (Dougherty 1962; Porterfield and Ashwood-Smith 1962; Greaves, Nagington, and Kellaway 1963), and trypanosomes (Walker and Ashwood-Smith 1961).

As dimethyl sulphoxide has proved so successful for deep-freezing other mammalian tissues and has been used for the preservation of bull spermatozoa, and since poor fertility (5–10% of ewes lambing) has followed insemination of ewes with ram semen deep-frozen in diluents containing glycerol (Emmens and Blackshaw 1955;

TABLE 1

FACTORIAL COEFFICIENTS USED FOR CONTRASTS IN EXPERIMENT 4

Source of Variation	Glycerol (% v/v)				Dimethyl Sulphoxide (% v/v)			
	0	5	10	15	0	5	10	15
Protective agents (*B*)								
(1) Controls contrast	−1	0	0	0	+1	0	0	0
(2) Absence *v.* presence of protective agents	−3	+1	+1	+1	−3	+1	+1	+1
(3) Dimethyl sulphoxide *v.* glycerol	0	−1	−1	−1	0	+1	+1	+1
(4) 5% *v.* 15% glycerol	0	−1	0	+1	0	0	0	0
(5) (5%+15%) *v.* 10% glycerol	0	+1	−2	+1	0	0	0	0
(6) 5% *v.* 15% dimethyl sulphoxide	0	0	0	0	0	−1	0	+1
(7) (5%+15%) *v.* 10% dimethyl sulphoxide	0	0	0	0	0	+1	−2	+1

First, Sevinge, and Henneman 1961), the author has examined the value of dimethyl sulphoxide for the preparation of deep-frozen ram spermatozoa. This paper is a report of preliminary experiments in which the effect of dimethyl sulphoxide on ram spermatozoa incubated at 30 and 5°C was studied before testing it as a protective agent during deep-freezing. In addition, various preparations of reconstituted milk powder were examined for their suitability as diluents in which to preserve ram semen.

II. Materials and Methods

Semen was collected by electrical stimulation (Blackshaw 1954) and diluted within 10–40 min. Only samples containing a high proportion of spermatozoa with good activity were used.

(*a*) *Diluents and Processing*

All diluents contained 0·3% (w/v) fructose. Diluents in experiments 1–3 contained 0·01M sodium dihydrogen phosphate, 0·01M dibasic sodium phosphate, and 0·154M sodium chloride. The commercial preparation, "Hunter Valley Powdered Milk" (non-fat), was used in the reconstituted skim milk diluents described in experiments 4 and 5. In the studies at 5°C the diluted semen was chilled from 30°C over 2 hr.

(*b*) *Scoring and Analyses*

The system of coding and randomization of treatments described by Martin (1963*a*) was used in all experiments. Semen was examined microscopically as a thin film between a slide and coverslip on a warm stage at 38°C. In experiments 1–3 single motility scores were made on a scale 0–4 (Emmens 1947). This score was doubled to remove fractions, and in this form it was the motility index used as unit observation

TABLE 2

MEAN MOTILITY SCORES FROM EJACULATES OF FOUR RAMS AFTER SPERMATOZOA WERE INCUBATED IN SOLUTIONS CONTAINING 20mM PHOSPHATE BUFFER AND VARYING CONCENTRATIONS OF SODIUM CHLORIDE AND DIMETHYL SULPHOXIDE

Experiment No.	Sodium Chloride		Dimethyl Sulphoxide		Mean Motility Score
	Molarity (mM)	Relative Tonicity (%)	Molarity (mM)	Concn. as (% v/v)	
1 (semen incubated for 1·5 hr at 30°C)	0	0	308	2·19	0
	38	25	231	1·64	1·25
	77	50	154	1·09	2·88
	116	75	77	0·55	3·75
	154	100	0	0	3·75
2 (semen incubated for 1·5 hr at 30°C)	0	0	154	1·09	0·15
	0	0	308	2·19	0·15
	0	0	462	3·28	0·09
	0	0	616	4·37	0·17
	123	80	154	1·09	3·07
	123	80	308	2·19	2·79
	123	80	462	3·28	2·56
	123	80	616	4·37	2·34
3 (semen incubated for 6·5 hr at 5°C)	92	60	0	0	1·81
	154	100	0	0	1·88
	216	140	0	0	1·06
	92	60	462	3·28	1·81
	154	100	462	3·28	1·81
	216	140	462	3·28	1·56

in the analyses. In all other experiments an estimate of the proportion of motile cells was made as well as the motility score. These were transformed to angles for analysis. The analysis of response was carried out on the automatic digital computer SILLIAC (Claringbold 1957) and Table 1 shows the orthogonal coefficients used to partition the contrasts in experiment 4. Description of experimental contrasts as "linear" (*L*) and "quadratic" (*Q*) follows that of Cochran and Cox (1957).

III. Results

(a) *Tonicity of Dimethyl Sulphoxide*

The osmotic activity of dimethyl sulphoxide was tested in three experiments. Semen was incubated at 30 or 5°C for 1·5 or 6·5 hr, respectively, in diluents to which were added various concentrations of dimethyl sulphoxide. Two experiments were similar in design to those described by Martin (1963*b*). Table 2 illustrates the 100, 75, 50, 25, and 0% (v/v) replacement of buffered isotonic sodium chloride (0·154M) by isosmotic dimethyl sulphoxide (0·308M, 2·18% v/v). Dimethyl sulphoxide alone was not a suitable diluent, but increasing concentrations of saline to 0·116M produced

Table 3

Experiment 4: effect of levels of milk solids, dimethyl sulphoxide, and glycerol on the survival of ram spermatozoa stored at 30 and 5°C

Treatment	Spermatozoa Incubated for 4 hr at 30°C		Spermatozoa Incubated for 24 hr at 5°C	
	Mean Motility Score	Percentage Motile	Mean Motility Score	Percentage Motile
Milk solids (*A*)				
7% (w/v)	2·47	41·9	2·22	35·9
9% (w/v)	2·44	41·3	2·39	46·6
11% (w/v)	2·00	33·3	1·97	37·7
Protective agents (*B*)				
Nil	3·08	53·9	2·85	53·9
5% (v/v) glycerol	2·71	45·4	2·58	41·7
10% (v/v) glycerol	2·25	26·3	2·25	30·4
15% (v/v) glycerol	1·38	16·3	1·88	28·3
5% (v/v) dimethyl sulphoxide	2·50	39·2	2·58	48·8
10% (v/v) dimethyl sulphoxide	2·25	45·8	1·96	33·8
15% (v/v) dimethyl sulphoxide	1·17	29·6	0·58	29·6
Ejaculates (*C*)				
1	2·29	45·0	2·42	53·1
2	2·46	45·6	2·54	60·2
3	2·31	34·2	1·92	22·1
4	2·15	30·4	1·90	24·8

a significant linear increase ($P<0·001$) in spermatozoal survival. Buffered solutions of dimethyl suphoxide varying from half the molarity of isosmotic dimethyl sulphoxide to twice its molarity were tested in the presence and absence of isotonic saline (Table 2). The addition of saline significantly improved the revival for all levels of dimethyl sulphoxide. In a 2×3 experimental design replicated four times (Table 2) duplicate motility observations were made of semen incubated at 5°C for 6·5 hr in 0·092M, 0·154M, and 0·216M sodium chloride in the presence and absence of 0·462M dimethyl sulphoxide. Although increasing the concentration of saline produced a significant linear decrease ($P<0·01$) in mean motility there was no significant difference between the effect of the presence and absence of dimethyl sulphoxide.

(*b*) *Toxicity of Dimethyl Sulphoxide*

The pH of a 10% (v/v) dimethyl sulphoxide milk diluent was 6·75; that of a similar solution containing no dimethyl sulphoxide was 6·60. This could not explain any toxicity of dimethyl sulphoxide.

Experiments 1–3 indicated that the toxicity of dimethyl sulphoxide could be tested in diluents in which its tonicity was assumed to be zero. Table 3 describes two experiments having the same design (expt. 4, $2\times3\times4^2$ factorials) in which the toxicity of dimethyl sulphoxide was tested in diluted semen stored at 30 or 5°C.

TABLE 4

SUMMARY OF ANALYSES OF VARIANCE ON DATA IN EXPERIMENT 4

Source of Variation	Degrees of Freedom	Variance Ratios			
		Incubation at 30°C		Incubation at 5°C	
		Motility Score	Percentage Motile	Motility Score	Percentage Motile
Milk solids (*A*)	2				
7% *v.* 11% (*L*)	1	16·60***	7·23*	7·01*	0·28
9% *v.* mean of 11 and 7% (*Q*)	1	4·16*	1·93	13·26***	11·83**
Protective agents (*B*)	7				
(1) Controls contrast	1	0·20	0·01	0·66	0·08
(2) Absence *v.* presence of protective agents	1	92·24***	46·73***	98·25***	36·13***
(3) Dimethyl sulphoxide *v.* glycerol	1	1·64	11·15**	35·19***	1·13
(4) 5% *v.* 15% glycerol (*L*)	1	50·20***	36·52***	21·13***	6·21*
(5) 10% *v.* mean of 5 and 15% glycerol (*Q*)	1	1·64	0·91	0·02	0·94
(6) 5% *v.* 15% dimethyl sulphoxide (*L*)	1	50·20***	3·75	168·42***	14·88***
(7) 10% *v.* mean of 5 and 15% dimethyl sulphoxide (*Q*)	1	6·54*	6·50*	7·89*	0·76
Ejaculates (*C*)	3	1·85	8·73***	18·88***	51·22***
Interactions					
$A\times B$	14	1·55	1·42	2·69	1·55
$A\times C$	6	0·47	3·28**	1·91	0·91
$B\times C$	21	1·03	1·44	1·51	1·29
Residual mean square	42	0·85	62·48	0·57	72·58

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

The factors were: (1) reconstituted skim milk levels of 7, 9, and 11% (w/v); (2) protective agents (i.e. glycerol and dimethyl sulphoxide); (3) levels of protective agent of 0, 5, 10, and 15% (v/v); (4) replications with ejaculates from four rams. Table 4 presents the analyses of variance for experiment 4. On the mean motility

scores after storage at 30°C, 7 and 9% of milk solids maintained best survival, 7% being slightly better than 9%. However, after storage at 5°C, 9% milk solids was best and 7% maintained motility significantly better than 11% milk solids.

The significant interaction between levels of milk solids and ejaculates in the analysis of scores of the percentage motile spermatozoa after incubation at 30°C

TABLE 5

EXPERIMENT 5: EFFECT OF HEAT TREATMENT AND STORAGE TIME AT 5°C ON THE SURVIVAL OF RAM SPERMATOZOA IN RECONSTITUTED AND PASTEURIZED SKIM MILK DILUENTS INCUBATED AT 30°C FOR 7 HR

Treatment	Mean Motility Score	Percentage Motile
Pasteurized milk		
Milk not heated, stored 4 hr, 1 day, 2 days	0	0
Milk heated, stored 4 hr	2·00	46·2
Milk heated, stored 1 day	2·38	43·7
Milk heated, stored 2 days	2·12	36·2
Reconstituted milk		
Heat treatment (*A*)		
Milk not heated	2·60	44·2
Milk heated	2·67	47·0
Storage time (*B*)		
Stored 4 hr	1·96	32·7
Stored 1 day	2·94	52·0
Stored 2 days	3·00	52·0
Milk solids (*C*)		
7% (w/v)	2·60	46·8
9% (w/v)	2·89	52·1
11% (w/v)	2·39	37·9
Ejaculates (*D*)		
1	2·78	51·6
2	3·48	51·9
3	2·16	30·8
4	3·56	71·2

occurred because increasing levels of milk solids were toxic to the spermatozoa in ejaculates 3 and 4, but beneficial to ejaculate 2, while best activity was observed in 9% milk solids (with ejaculate 1) and 7% was better than 11%. When this interaction was used as the estimate of error to test the main effect of milk levels, there was no significant difference between levels.

The presence of either dimethyl sulphoxide or glycerol decreased spermatozoal survival during storage at either 30 or 5°C. At 30°C scores of progressive motility were not significantly different for spermatozoa incubated in either dimethyl sulphoxide or glycerol, but a smaller proportion were motile after incubation in glycerol than after incubation in dimethyl sulphoxide. However, after incubation at 5°C,

an approximately equal proportion of spermatozoa was motile in the diluents containing dimethyl sulphoxide and glycerol. Motility scores for surviving spermatozoa were much higher in glycerol than in dimethyl sulphoxide.

The factors in experiment 5 (Table 5), a $2\times3\times4^2$ factorial, were: (1) the diluent preparation was either not heated or was held at 85°C for 5 min; (2) the diluent was stored at 5°C before use for 4 hr, 1 day, and 2 days; (3) reconstituted skim milk levels of 7, 9, and 11%, and skim pasteurized milk; (4) replications with ejaculates from four rams. As pasteurized milk was obviously toxic when not heated,

TABLE 6

SUMMARY OF ANALYSES OF VARIANCE OF DATA† IN EXPERIMENT 5

Source of Variation	Degrees of Freedom	Variance Ratios	
		Motility Score	Percentage Motile
Heat treatment (*A*)	1	0·39	0·91
Storage time (*B*)	2		
1 day *v.* 2 days (*L*)	1	0·21	0·01
0 day *v.* mean of 1 and 2 days (*Q*)	1	65·34***	61·44***
Milk solids (*C*)	2		
7% *v.* 11% (*L*)	1	2·33	5·63*
9% *v.* mean of 7 and 11% (*Q*)	1	11·21**	13·18***
Ejaculates (*D*)	3	26·81***	36·08***
Interactions			
Pooled treatment × treatment interactions	8	0·94	0·66
$A\times D$	3	2·38	1·03
$B\times D$	6	6·78***	9·00***
$C\times D$	6	1·64	3·03
Residual mean square	40	0·89	48·05

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

† Analysis of data of spermatozoal survival in pasteurized milk is not included in this table (see text).

and was a poorer diluent than reconstituted milk after the heat treatment, the portion of the experiment relating to spermatozoal survival in pasteurized milk was not included in the analyses of variance described in Table 6. Although, on visual observation, the milk solids appeared to dissolve completely within 4 hr of preparing the solution, the use of this diluent did not maintain spermatozoal motility as well as diluents prepared 1 or 2 days previously and stored at 5°C until used. The interaction $B\times D$ (Table 6) indicates that this effect was only observed for two ejaculates (ejaculates 1 and 3); the other two survived equally well in diluents stored for 0, 1, or 2 days before use. However, the main effect, storage time of diluent (*B*), was still statistically significant ($P<0·05$) when the $B\times D$ interaction was used as the estimate of error. The best response was obtained with 9% (w/v) milk solids, both 7 and 9% being better than 11%.

IV. Discussion

Previous reports (Ashwood-Smith 1961*a*; Persidsky and Richards 1963) indicate that 10–15% (v/v) dimethyl sulphoxide is needed in a freezing diluent. On a molecular basis this could constitute more than a four-fold increase in tonicity, and unless dimethyl sulphoxide freely permeated spermatozoa, they would not survive in such a hypertonic diluent. Experiments 1, 2, and 3 in this paper confirm Lovelock's (1954) claim that dimethyl sulphoxide penetrates spermatozoa quickly.

Differences in the toxicity of dimethyl sulphoxide relative to glycerol when observed at 30 and 5°C are similar but vary in statistical significance. Thus, for motility scores, glycerol is slightly less toxic than dimethyl sulphoxide at 30°C. This difference is much larger and is statistically significant after incubation at 5°C. The situation is reversed for scores of percentage active cells. The apparently different expression of toxicity may be due to a different mode of action of the two compounds, or perhaps, dimethyl sulphoxide takes longer than glycerol to arrest completely the motility of spermatozoa, although its effect is more quickly registered as a decrease in activity. The toxicity of dimethyl sulphoxide cannot be attributed to a change in the pH of the diluent.

Experiment 5 was designed to investigate further the properties of reconstituted milk and the interaction described between milk levels and ejaculates in experiment 4. Since a previous batch of the reconstituted milk preparation had proved to be toxic the comparison between the heated and non-heated diluent was made. Contrary to Blackshaw's (1960) finding, pasteurized milk held at 85°C for 5 min before storage was inferior to reconstituted milk as a diluent. Ejaculates differed in their capacity to survive in various concentrations of reconstituted milk; generally, 9% (w/v) is best for storage at 30 and 5°C. A period of storage, possibly no longer than 12 hr, is necessary to prepare a milk protein diluent to ensure protection of spermatozoa during dilution and storage. Perhaps hydration of the complex conjugated proteins in milk may be associated with a relatively slow reorientation of the tertiary structure of these proteins.

V. Acknowledgments

The author thanks Dr. I. C. A. Martin and Professor C. W. Emmens for advice and criticism. The work was carried out with the aid of grants from the Australian Wool Board.

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