REVIVAL OF BULL SPERMATOZOA AFTER DEEP-FREEZING TO -79°C FOLLOWING EQUILIBRATION IN DILUENTS OF VARIOUS TONICITIES

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Summary. With 1% lecithin and 1.0 M-glycerol, the activity of deep-frozen bull spermatozoa after thawing was better if they were frozen in an isotonic diluent than if frozen in a hypertonic ($1.2\times$) solution (the solution referred to as isotonic was: 50 mM-sodium citrate, 62 mM-fructose and 20 mM-sodium phosphate buffer). There was no significant difference between the mean effects of addition of the glycerol either immediately the diluted semen had been cooled to 5°C (equilibration) or just prior to freezing after a period of storage at 5°C equivalent to the equilibration period (ageing). A period of 4 hr of storage at 5°C prior to freezing gave the best mean motility scores and percentage motile, but the percentage of spermatozoa unstained by congo-red-nigrosin solutions continued to rise over a period of 18 hr storage.

Yolk-containing diluents, with the tonicity range of 0.90, 1.05 and $1.20\times$, were tested and hypotonic diluents were superior to hypertonic. Equilibration for up to 18 hr had no significant effect on the scores of activity of spermatozoa after thawing, but, again, there was a highly significant rise in the percentage of unstained spermatozoa.

INTRODUCTION

Lovelock & Polge (1954) have shown the importance of the concentration of electrolyte of the diluent in which spermatozoa are deep-frozen. Blackshaw (1960) found that best revival rates of deep-frozen bull spermatozoa were given when the diluent was close in tonicity to that of 0.1 M-sodium citrate. It has been found that tonicity, sugar content of the diluent and the period of storage of the diluted semen at 5°C before deep-freezing interact and affect revival rates after freezing (Martin, 1963), and that the fructose content of the diluent and the period of equilibration at 5°C before freezing influence both the revival rate and fertility of deep-frozen bull semen (Martin & Emmens, 1961). The experiments reported in this paper were undertaken to define the effects of these factors more clearly and to find what effect changes in tonicity during the period of dilution and cooling at 5°C had on the equilibration effect and on overall revival rates.

MATERIALS AND METHODS

In both experiments bull semen was diluted 20-fold in the lecithin or yolk-containing diluents shown in Table 1. Lecithin was prepared by precipitating an alcohol and ether soluble extract of egg yolk in acetone (Blackshaw, 1954) and this extract, a white, waxy solid, was stored under acetone at -40°C until required. After drying *in vacuo*, it readily formed suspensions in the diluents used. Tonicity is stated relative to the 60 mM-sodium citrate, 62 mM-fructose and

TABLE 1
COMPOSITION OF DILUENTS USED IN THE EXPERIMENTS

	Lecithin (% w/v)	Egg-yolk (% v/v)	Sodium citrate (mM)	Fructose (mM)	Phosphate buffer (pH 7.0) (mM)	Glycerol (M)
EXPERIMENT 1						
<i>First-stage diluents:</i>						
Tonicity = 1.0	1	—	60	62	20	—
= 1.2	1	—	72	74	24	—
<i>Glycerol-containing diluents:</i>						
Tonicity = 0.8	1	—	48	50	16	2
= 1.0	1	—	60	62	20	2
= 1.2	1	—	72	74	24	2
= 1.4	1	—	84	87	28	2
EXPERIMENT 2						
<i>First-stage diluents:</i>						
No fructose						
Tonicity = 0.90	—	50	32	—	8	—
= 1.05	—	50	44	—	11	—
= 1.20	—	50	56	—	14	—
Containing fructose						
Tonicity = 0.90	—	50	24	25	8	—
= 1.05	—	50	33	37	11	—
= 1.20	—	50	42	43	14	—
<i>Glycerol-containing diluents:</i>						
No fructose						
Tonicity = 0.90	—	—	72	—	18	2
= 1.05	—	—	84	—	21	2
= 1.20	—	—	96	—	24	2
Containing fructose						
Tonicity = 0.90	—	—	54	56	18	2
= 1.05	—	—	63	65	21	2
= 1.20	—	—	72	74	24	2

20 mM-phosphate buffer diluent so that the tonicity coefficient 1.2 indicates that the concentration of each component of the diluent was raised 1.2 times. In designing diluent for Experiment 2, egg yolk was assumed to have a tonicity of 1.0; 1 % w/v lecithin was considered to have a negligible effect on the osmotic pressure of the diluents.

The initial dilution was made at 30°C within 30 min of the collection of the semen and subsequent cooling of the diluted semen to 5°C took 2 hr. The terms 'aged' and 'equilibrated' are used in the same sense as in a previous paper

(Martin, 1963), i.e. the glycerol-containing solution was added to the diluted semen as soon as it had cooled to 5° C for 'equilibration' and, for 'ageing', the glycerol-containing diluent was added immediately prior to the commencement of freezing. In all treatments glycerol was added at 5° C in one addition of an equal volume of the 2.0 M-glycerol solution to the diluted semen, giving a final strength of glycerol of 1.0 M.

Samples from each treatment were frozen in 1 ml ampoules using a freezing container patterned on that of Polge & Lovelock (1952) and the deep-frozen semen was stored at -79° C for at least 2 days and thawed immediately prior to examination in a water bath at 40° C. Scores of the survival of spermatozoa after deep-freezing were made by three criteria, motility score, per cent motile and per cent unstained by congo-red-nigrosin (Martin, 1963).

TABLE 2

EXPERIMENT 1: MEAN EFFECTS OF EQUILIBRATION, STORAGE TIME AT 5° C AND TONICITY ON THE REVIVAL RATES OF DEEP-FROZEN BULL SPERMATOZOA (FACTORIAL DESIGN $2^3 \times 3^2$)

Treatment		Motility score	Motile spermatozoa (%)	Unstained spermatozoa (%)
Final tonicity	1.0	3.04	30.0	40.7
	1.2	2.72	21.0	43.5
Tonality of first diluent	1.0	2.92	27.5	44.1
	1.2	2.85	23.5	40.2
'Ageing' equilibration		2.92	25.6	41.9
		2.85	25.4	42.4
Time of storage at 5° C	0 hr	2.79	26.4	32.3
	4 hr	3.06	29.2	44.7
	18 hr	2.79	20.8	49.4
Ejaculates (replicates)	1	3.25	32.9	52.4
	2	2.46	16.5	43.0
	3	2.94	27.1	31.0

The experiments were factorial designs, $2^3 \times 3^2$ and $2 \times 3^2 \times 4$, and the SILLIAC electronic computer was used to prepare the analyses of variance (Claringbold, 1957). The method of describing experimental contrasts as 'linear' and 'quadratic' and the corresponding partition of interaction variance using orthogonal polynomials follows that of Cochran & Cox (1957).

RESULTS

The survival rates of bull spermatozoa deep-frozen in diluents of final tonicity 1.0 and 1.2 are shown in Table 2. In addition to this factor of final tonicity, the initial dilution, made at 30° C, was into either a diluent of 1.0 or 1.2 tonicity, so that the effects of change of tonicity from 1.0 to 1.2, or vice versa, at the time of glycerol addition were also tested. Ageing and equilibration in the Period 0,

D*

4 and 18 hr were tested in all combinations with the four tonicity treatments. From Tables 2 and 3, an isotonic diluent (at the time of freezing) was superior to the hypertonic and there was no difference in the overall means between samples equilibrated or aged. Best motility results were scored in those treatments stored for 4 hr at 5° C prior to deep-freezing and this period gave the highest percentage of motile spermatozoa. However, storage for 18 hr showed a continued and significant rise in the number of unstained spermatozoa.

Examination of the interaction of tonicity of the diluent at the time of freezing and period of storage prior to freezing revealed that the motility

TABLE 3
SUMMARY OF ANALYSES OF VARIANCE OF DATA FOR EXPERIMENT I

Source of variation	d.f.	Variance ratios		
		Motility score	Motile spermatozoa(%)	Unstained spermatozoa (%)
A. Final tonicity	1	14.24***	19.91***	4.02
B. First-stage tonicity	1	0.67	4.51*	6.87*
C. Equilibration	1	0.67	0.09	0.12
D. Storage time				
Linear 0 versus 18 hr	1	0.00	5.45*	85.65***
Quadratic 4 versus mean of 0 and 18 hr	1	9.10**	7.77**	5.65*
E. Ejaculates (replications)	2	29.58***	28.19***	68.44***
Interactions				
A × B	1	0.02	2.30	1.03
A × C	1	0.67	0.54	0.00
A × D				
Linear × Linear	1	7.91**	3.29	1.14
Linear × Quadratic	1	0.86	0.16	0.17
B × C	1	0.67	5.68*	3.09
B × D	2	0.84	0.60	1.77
C × D	2	0.19	0.68	2.88
A × E	2	0.84	0.50	0.94
B × E	2	6.00**	5.58**	3.01
C × E	2	4.06*	2.79	0.44
D × E	4	1.40	0.55	4.98**
Residual	45	0.129†	29.6†	14.6†

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

†Residual mean square.

scores, after thawing, of spermatozoa frozen after 0 hr storage at 5° C was significantly higher in the isotonic than in the hypertonic diluent (mean motility scores of 3.12 and 2.46, respectively whereas, after 18 hr storage, the corresponding scores were 2.83 and 2.75).

Another interaction, which just reached significance ($P < 0.05$) on analysis of the data on percentage of motile spermatozoa was the response to initial tonicity and the subsequent processing method of equilibration or ageing. The mean percentage of motile spermatozoa after deep-freezing was 21.4 % if initially diluted with the hypertonic diluent and subsequently equilibrated, 25.6 % for both dilution into hyper- or isotonic diluents and aged before freezing, and

TABLE 4

EXPERIMENT 2: MEAN EFFECTS OF EQUILIBRATION, FRUCTOSE AND TONICITY CHANGES ON SPERMATOZOA DEEP-FROZEN IN EGG-YOLK-CONTAINING DILUENTS (FACTORIAL DESIGN $2 \times 3^2 \times 4$)

Treatment		Motility score	Motile spermatozoa (%)	Unstained spermatozoa (%)
Equilibration time				
	0 hr	2.67	24.2	23.3
	1 hr	2.73	27.7	32.3
	18 hr	2.67	29.6	50.7
Tonicity				
	0.90	2.85	30.4	36.4
	1.05	2.85	27.1	35.3
	1.20	2.31	23.9	34.7
Fructose absent in diluent		2.69	25.4	35.0
Fructose present in diluent		2.65	28.9	35.8
Ejaculates (replicates)				
	1	2.89	30.0	37.2
	2	2.69	27.2	40.9
	3	2.58	26.4	35.2
	4	2.58	25.0	28.6

TABLE 5

SUMMARY OF ANALYSES OF VARIANCE FOR DATA OF EXPERIMENT 2

Source of variation	d.f.	Variance ratios		
		Motility score	Motile spermatozoa (%)	Unstained spermatozoa (%)
A. Equilibration time				
Linear 0 versus 18 hr	1	0.00	2.68	180.98***
Quadratic 1 versus mean of 0 and 18 hr	1	0.04	0.09	4.50
B. Tonicity				
Linear 0.90 versus 1.20	1	22.43***	7.42**	0.97
Quadratic 1.05 versus mean of 0.90 and 1.20	1	7.48**	0.01	0.10
C. Fructose	1	0.20	1.74	0.38
D. Ejaculates	3	2.91*	0.85	10.81***
Interactions				
A × B	4	1.15	0.26	1.51
A × C	2	0.20	0.15	0.29
B × C	2	0.00	0.70	0.09
A × D	6	0.61	0.66	4.53**
B × D	6	4.22**	8.43***	0.73
C × D	3	0.73	0.62	0.85
Residual	40	0.157†	42.48†	19.35†

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

†Residual mean square.

29.4% if equilibration followed the use of an isotonic first-stage diluent. Interactions of ejaculates with treatment were detected and indicate a variation in the degree of response to a treatment. In no case was there a reversal of response in contradiction of the mean values for the treatment.

Egg yolk was used in the diluent for the second experiment (Tables 4 and 5) in which the effects of a range of tonicities were tested in the presence and absence of fructose, using a period of up to 18 hr equilibration at 5° C, the contrast of storing the semen for a period before the addition of glycerol being omitted. There were small and non-significant changes in the motility and percentage of motile spermatozoa after deep-freezing, but, again, there was a highly significant improvement in the percentage of unstained spermatozoa in 18 hr. The activity of spermatozoa in 0.90 tonicity was significantly higher than in diluents of 1.20 tonicity. The incorporation of 62 mM-fructose in the diluent had no apparent effect. Apart from the interactions of ejaculates and tonicity and equilibration period, which again indicated variations in degree of response from ejaculate to ejaculate, there were no other significant effects.

DISCUSSION

It has been customary to prepare the glycerol-containing diluents with glycerol added v/v to a citrate or phosphate buffer (Smith & Polge, 1950) with the result that spermatozoa were frozen in a slightly hypotonic diluent. When the sugar-containing diluents proposed by Emmens & Blackshaw (1950) were used, the addition of the sugar and glycerol containing diluent raised the tonicity of the semen diluent and altered the ratio of electrolytes to non-electrolytes (sugars). The diluents used in this and previous experiments (Martin, 1963) were prepared assuming that glycerol had no effect on their tonicity relative to bull seminal plasma.

As far as possible, the composition of the first and second (glycerol-containing) diluents were kept constant in the ratio of fructose to citrate and phosphate buffer, and where no change in tonicity at the time of glycerol addition was required, the lecithin-containing diluents of Experiment 1 only differed by the content of glycerol in the latter.

The use of yolk in diluents for tonicity studies posed problems. For, unless yolk was included in both the initial and glycerol-containing solutions, marked changes in the composition of the semen diluent would occur at the time of glycerol addition apart from the very rapid rise in glycerol content. However, the diluents used in Experiment 2 were chosen because, in respect of the other details of processing, the fructose-free, 0.90 tonicity combination resembled the diluents used by Polge & Rowson (1952) and the diluent containing 58 mM-fructose and at a tonicity of 1.20, was similar to those used by Emmens & Blackshaw (1950) and Blackshaw (1955). Conflicting opinions on the need for a period of equilibration were held by these two groups of workers. For similar reasons, the equilibration period of 1 hr was included in the design of the second experiment as, in two papers on experiments on the fertility of deep-frozen semen (Emmens & Martin, 1957; Martin & Emmens, 1958) using the arabinose- or fructose-containing diluents of Emmens & Blackshaw (1950) and

Blackshaw (1955), equilibration for the short period of 1 hr gave regularly poorer results in the counts of spermatozoa unstained by congo-red-nigrosin than 18 hr (overnight) equilibration and fertility results were similarly lowered.

Isotonic diluents were more satisfactory than hypertonic diluents and, in Experiment 2, hypotonic diluents were superior to hypertonic, judging on scores of activity of spermatozoa, whereas in this range of 0.90 and 1.20 relative tonicity, the staining reaction was not significantly altered. It would seem that the equilibration effect is not dependent on the presence of glycerol, or to any great degree on the composition, or changes in composition during processing, of the diluent. Although change from a hypertonic diluent to an isotonic for equilibration, or the maintenance of hypertonicity throughout equilibration gave poorer percentages of motile spermatozoa than samples held in isotonic diluents throughout dilution and equilibration, these changes were not sufficient to alter the permeability of the spermatozoa to congo-red.

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