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DEEP-FREEZING RAM SPERMATOZOA: THE EFFECTS OF MILK, YOLK-CITRATE AND SYNTHETIC DILUENTS CONTAINING SUGAR

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Summary. In a factorial experiment skim milk, egg-yolk–citrate and synthetic diluents composed of fructose or lactose, sodium chloride and phosphate buffer containing 3·0% w/v of a lyophilized preparation of non-dialysable solids from (cow) milk were used as diluents for deep-freezing ram spermatozoa and for incubating spermatozoa at 37° C after thawing. All samples of semen were diluted forty-fold before freezing. Egg-yolk–citrate was inferior to skim milk as a diluent for freezing spermatozoa and for incubating spermatozoa after thawing. Of the two synthetic diluents, lactose synthetic was better for freezing spermatozoa whilst fructose synthetic was better for incubating spermatozoa after thawing. Only spermatozoa frozen in the lactose synthetic diluent and resuspended after thawing in the fructose synthetic survived incubation at 37° C for 2 hr as well as spermatozoa frozen and incubated in skim milk diluents.

Two factorial experiments compared egg-yolk–citrate and skim milk as diluents for freezing semen diluted from ten- to forty-fold. At ten-fold dilution, revival was much the same after freezing in milk or egg-yolk–citrate. A twenty- or forty-fold dilution was better than a ten-fold dilution in milk, but revival was depressed at these higher dilution rates after freezing in egg-yolk–citrate. When semen was frozen at a ten-fold dilution it was advantageous to resuspend spermatozoa in a diluent free of glycerol after thawing. A diluent based on Krebs–Henseleit–Ringer solution containing 0·5% w/v of non-dialysable milk solids was better for incubating spermatozoa after thawing than egg-yolk–citrate or milk.

A period of 5 hr equilibration at 5° C before freezing was better than 30 min equilibration.

INTRODUCTION

Emmens & Robinson (1962) conclude that cow milk preparations compare favourably with conventional yolk–citrate media as diluents for ram spermatozoa although this is not consistent for all reports. Salamon & Robinson (1962) found no difference between yolk–glucose–citrate and heated skim cow

milk when semen was used 'fresh' or after storage at 10 to 15° C, but after storage from 0 to 72 hr at 5° C highest fertility followed the insemination of semen diluted in yolk–glucose–citrate. In-vitro studies by Martin (1961) showed that yolk–citrate was better than 11% reconstituted skim milk for storage at 5° C but that milk was the better diluent for deep-freezing spermatozoa. However, Blackshaw found that spermatozoa survived deep-freezing best in a yolk–citrate diluent in one experiment (Blackshaw, 1955), but scored approximately equal revival for spermatozoa frozen in the two diluents in another experiment (Blackshaw, 1960b). There are no comparisons of the fertility of spermatozoa frozen in milk or yolk–citrate. However, only approximately 5% of ewes lambed after insemination with semen deep-frozen in yolk–citrate (Emmens & Blackshaw, 1955), whilst when heated homogenized milk was used (First, Sevinge & Henneman, 1961), 23% of 135 ewes lambed after insemination with frozen semen in an experiment, but in another experiment no lambs were born after ninety-four ewes were inseminated (i.e. an overall lambing rate of 14%).

Further studies of diluents (reconstituted skim milk, egg-yolk–citrate and two synthetic diluents) for the deep-freezing of ram spermatozoa and the effects of dilution rate are described in this paper.

MATERIALS AND METHODS

Semen was collected from Merino rams by electro-ejaculation (Blackshaw, 1954). Within 10 to 45 min of collection samples showing wave motion, with a score of 3·5 to 4·0 (Emmens, 1947), were diluted at 30° C to half the volume required for freezing in the diluents shown in Table 1. After chilling to 5° C in 2 hr, equal volumes of diluted semen and glycerol-containing diluent were mixed as three additions over 30 min in Experiment 1 and one addition in Experiments 2 and 3. The final rates of semen dilution for freezing are shown in Tables 2, 4 and 7.

After the addition of glycerol spermatozoa were equilibrated at 5° C for 4 hr in Experiment 1, 30 min in Experiment 2 and 30 min or 5 hr in Experiment 3, then 1 ml samples were frozen in a device patterned on that described by Polge & Lovelock (1952). This gave a freezing rate of 0·5 to 1° C min to −10 to −15 °C after which the rate increased to about 3° C min.

All frozen semen was stored at least 24 hr at −79° C and thawed to approximately 5° C in a water bath at 37° C. The thawed spermatozoa were scored for revival and then centrifuged at 700 ***g*** for 10 min. The supernatant was removed (except for one treatment as required in the design of Experiment 2) and the spermatozoa resuspended for incubation at 37° C in diluents shown for each experiment (Tables 2, 4 and 7). In Experiment 1 these diluents were the same as the first-stage (30° C) diluents except in the case of egg-yolk–citrate which was 25% yolk, 60 mM-sodium citrate and 15 mM-phosphate buffer. A solution based on Krebs'–Henseleit–Ringer was used as shown in Tables 4 and 6 in Experiment 2 and for all treatments in Experiment 3. This diluent was a mixture of 4 parts by volume of Krebs'–Henseleit–Ringer (Mann, 1954) with 1 part 0·1 M-sodium phosphate buffer and contained 17 mM-fructose and 0·5% w/v of a lyophilized

preparation of non-dialysed solids from heated (92° C for 10 min) skim cow milk (Martin, unpublished).

Scoring and analyses of results

The system of coding and randomization of treatments described by Martin (1963) was used in all experiments. On thawing, semen was examined as a thin film between a slide and coverslip on a microscope warm stage at 38° C. Scores of progressive motility (range 0 to 4; Emmens, 1947) and percentage of motile cells were made. Congo-red–nigrosin smears were prepared (Blackshaw, 1955) for each treatment before centrifugation in Experiments 1 and 2, but in

TABLE 1

COMPOSITION OF THE DILUENTS USED IN THE EXPERIMENTS

Constituent	*Yolk–citrate*				*Reconstituted skim milk*		*Lactose synthetic*		*Fructose synthetic*	
	54 mM-*sodium citrate*		60 mM-*sodium citrate*							
	30°C	5°C	30°C	5°C	30°C	5°C	30°C	5°C	30°C	5°C
Egg yolk (% v/v)	50	–	50	–	–	–	–	–	–	–
Skim milk powder (%w/v)	–	–	–	–	9	9	–	–	–	–
Lyophilized non-dialysable heated skim cow milk (% w/v)	–	–	–	–	–	–	3	3	3	3
Sodium citrate (mM)	40	69	40	80	–	–	–	–	–	–
Sodium chloride (mM)	–	–	–	–	–	–	31	31	31	31
Dibasic sodium phosphate (mM)	5	9	5	10	–	–	10	10	10	10
Sodium dihydrogen phosphate (mM)	5	9	5	10	–	–	5	5	5	5
Potassium dihydrogen phosphate (mM)	–	–	–	–	–	–	5	5	5	5
Lactose (mM)	–	–	–	–	–	–	185	185	–	–
Fructose (mM)	17	140	17	140	17	140	17	140	202	325
Glycerol (M):										
Experiments 1 and 2	–	1·90	–	–	–	1·90	–	1·90	–	1·90
Experiment 3	–	1·77	–	1·77	–	1·77	–	–	–	–

Experiment 3 spermatozoa were resuspended in Krebs'–Henseleit–Ringer before staining. One hundred spermatozoa were counted per smear, classified as stained or unstained and expressed as percentage unstained. These counts, as well as scores of percentage motile spermatozoa, were transformed to angles for the analyses of variance which were performed by the SILLIAC (Claringbold, 1957) in Experiments 2 and 3. The set of orthogonal coefficients used to partition contrasts in Experiment 1 are shown in Table 2. In the analyses of variance of scores made upon thawing in Experiments 1 and 2, the variance between ampoules within treatments was used as the estimate of error to test the significance of treatment effects. A residual variance, composed of higher than first-order interactions, was used as the estimate of experimental error in all other analyses.

RESULTS

Experiment 1, a 4^3 factorial design, tested skim milk, egg-yolk–citrate and fructose and lactose synthetics as diluents for freezing spermatozoa and for subsequent incubation at 37° C after thawing (Table 2). Summaries of the analyses of variance are shown in Table 3. Where ejaculate × diluent interactions were significant (scores of motility upon thawing and percentage

TABLE 2

EXPERIMENT 1: CHARACTERISTICS OF DEEP-FROZEN RAM SPERMATOZOA IMMEDIATELY AFTER THAWING AND THEIR SUBSEQUENT VIABILITY AFTER INCUBATION AT 37° C FOR 2 HR (SEMEN DILUTED FORTY-FOLD BEFORE FREEZING, MEANS FROM FOUR EJACULATES)

Treatment		(i) *Mean score on thawing*			*Coefficients used for the contrasts made in subsequent analyses*					
		Motility	*% Motile*	*% Unstained*						
Diluent for freezing					*A*	*B*	*C*			
Skim milk		3·12	55·6	41·3	−1	0	−1			
Yolk–citrate		3·09	47·5	36·4	+1	0	−1			
Fructose synthetic		3·12	42·5	47·5	0	−1	+1			
Lactose synthetic		3·37	51·9	36·1	0	+1	+1			
Diluent for freezing	*Diluent for incubation*	(ii) *Mean scores after 2 hr at 37° C*								
		Motility	*% Motile*	*% Unstained*	*A*	*B*	*C*	*D*	*E*	*F*
Skim milk	Skim milk	2·75	50·0	36·2	−1	0	−1	−1	0	−1
	Yolk–citrate	2·50	30·0	30·5	−1	0	−1	+1	0	−1
	Fructose synthetic	2·62	37·5	35·0	−1	0	−1	0	−1	+1
	Lactose synthetic	2·25	25·0	22·7	−1	0	−1	0	+1	+1
	Mean	2·53	35·6	31·1						
Yolk–citrate	Skim milk	2·12	12·5	22·5	+1	0	−1	−1	0	−1
	Yolk–citrate	1·12	9·0	28·2	+1	0	−1	+1	0	−1
	Fructose synthetic	1·50	8·7	27·0	+1	0	−1	0	−1	+1
	Lactose synthetic	1·00	7·5	19·5	+1	0	−1	0	+1	+1
	Mean	1·44	9·4	24·3						
Fructose synthetic	Skim milk	1·62	16·2	17·2	0	−1	+1	−1	0	−1
	Yolk–citrate	1·37	20·2	15·7	0	−1	+1	+1	0	−1
	Fructose synthetic	1·50	10·2	23·5	0	−1	+1	0	−1	+1
	Lactose synthetic	1·37	8·7	17·5	0	−1	+1	0	+1	+1
	Mean	1·47	13·9	18·5						
Lactose synthetic	Skim milk	2·87	37·5	27·7	0	+1	+1	−1	0	−1
	Yolk–citrate	2·25	30·0	24·2	0	+1	+1	+1	0	−1
	Fructose synthetic	3·00	45·0	33·7	0	+1	+1	0	−1	+1
	Lactose synthetic	2·87	32·5	26·2	0	+1	+1	0	+1	+1
	Mean	2·75	36·2	28·0						

motile and percentage unstained spermatozoa after incubation) the variance of the interaction was then used as the estimate of experimental error for testing the significance of responses to the pertinent treatment. After applying this criterion in the relevant cases, the significant treatment effects were:

Diluent for freezing spermatozoa

(1) The scores of percentage of motile of unstained spermatozoa on thawing and those of motility and percentage of motile spermatozoa after incubation showed milk to be better than yolk–citrate.

(2) The percentage of motile spermatozoa on thawing was higher in the lactose than the fructose synthetic diluent, but there were better percentages of unstained spermatozoa in the fructose diluent. All scores made after incubation showed lactose to be better than fructose.

(3) Taken together, milk and yolk diluents were better than the synthetic diluents for measures of percentage of motile spermatozoa, but the ranking was reversed for scores of percentage of unstained spermatozoa upon thawing.

TABLE 3

EXPERIMENT 1: SUMMARIES OF THE ANALYSES OF VARIANCE

Source of variation	*Degrees of freedom*	*Variance ratios*		
		Motility score	*% Motile*	*% Unstained*
1. Scores made immediately after thawing				
Diluents for freezing				
(A) Milk versus yolk–citrate	1	0·14	13·53***	5·33*
(B) Fructose versus lactose synthetic	1	10·00**	20·50***	25·18***
(C) Milk and yolk–citrate versus synthetic diluents	1	6·32*	9·01**	4·06*
Ejaculates (replicates)	3	2·74	27·35***	74·77***
Diluents × replicates	9	3·29*	0·86	1·24
Between samples (ampoules)	48	*0·050*†	*14·06*†	*15·29*†
2. Scores made after incubation at 37° C for 2 hr				
Diluents for freezing				
(A) Milk versus yolk–citrate	1	23·69***	55·08***	5·19*
(B) Fructose versus lactose synthetic	1	32·50***	44·55***	13·20***
(C) Milk and yolk–citrate versus synthetic diluents	1	0·62	0·54	5·13*
Diluents for incubation				
(D) Milk versus yolk–citrate	1	5·59*	4·70*	0·13
(E) Fructose versus lactose synthetic	1	1·56	2·61	9·23**
(F) Milk and yolk–citrate versus synthetic diluents	1	0·15	1·55	0·01
Ejaculates (replicates)	3	0·84	5·21**	35·19***
Freezing × incubation diluents	9	0·59	1·13	0·92
Freezing diluents × ejaculates	9	0·86	1·67	2·39*
Incubation diluent × ejaculates	9	1·00	2·51*	1·83
Residual (error)	27	*0·416*†	*37·49*†	*19·82*†

† Variance.

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

Diluent for incubating spermatozoa

(1) On scores of motility, milk was better than yolk–citrate.

(2) The percentage of unstained spermatozoa showed fructose synthetic to be better than lactose synthetic.

(3) Coefficients other than the orthogonal set described in Table 2 were used to show that the proportion of motile spermatozoa frozen then incubated after thawing in lactose synthetic was significantly smaller ($F_{1,27} = 6·4, P<0·05$) than the proportion of spermatozoa which survived freezing in the lactose synthetic then incubation in the fructose synthetic diluent. Further, the proportion of spermatozoa which survived freezing and incubation in milk

was not significantly different ($F_{1,27} = 0{\cdot}97$) from the proportion of spermatozoa which survived freezing in lactose synthetic then incubation in fructose synthetic. The importance of these findings is stated in the Discussion.

The treatments and results of Experiment 2, a $2^2 \times 3 \times 5$ factorial design, are shown in Table 4. Table 5 summarizes the analyses of variance. The significant treatment effects were:

(1) All measures of response showed that milk was a better diluent than yolk–citrate for freezing spermatozoa. An interaction of freezing diluent and ejaculates (A × C) for scores of percentage of motile cells on thawing showed that the difference in revival of spermatozoa frozen in milk and yolk–citrate varied in magnitude from ejaculate to ejaculate; however, the difference was still significant when tested with the A × C interaction.

TABLE 4

EXPERIMENT 2: EFFECTS OF DILUTION RATE, THE DILUENTS USED FOR FREEZING AND FOR INCUBATION AFTER THAWING ON THE REVIVAL OF DEEP-FROZEN RAM SPERMATOZOA AND ITS SUBSEQUENT SURVIVAL DURING INCUBATION AT 37° C FOR 1 HR (MEANS FROM FIVE EJACULATES)

Treatment	(1) *Mean scores on thawing*			(2) *Mean scores after 2 hr at 37° C*	
	Motility	% *Motile*	% *Unstained*	*Motility*	% *Motile*
Freezing diluent (A) and dilution rate (B)					
Skim milk, diluted ten-fold	2·87	36·7	30·3	2·47	24·7
Skim milk, diluted forty-fold	3·30	53·3	43·4	2·90	39·3
Yolk–citrate, diluted ten-fold	2·87	33·3	19·6	2·03	22·7
Yolk–citrate, diluted forty-fold	2·30	26·7	15·9	1·37	16·0
Diluent for incubation (C)					
(1) Type used for freezing, but containing 17 mM-fructose and no glycerol	–	–	–	2·18	26·0
(2) Krebs'–Henseleit–Ringer	–	–	–	2·58	29·5
(3) Resuspended in diluent used for freezing	–	–	–	1·83	21·5

(2) All measures of activity of spermatozoa showed an interaction of diluent used for freezing and dilution rate (Table 4). Thus, spermatozoa in samples diluted ten-fold survived freezing, thawing and incubation at 37° C equally well in either diluent; however, a forty-fold dilution in milk increased survival rates, whereas survival decreased at the same dilution in yolk–citrate.

(3) For scores of motility, Krebs'–Henseleit–Ringer was the best diluent for incubating spermatozoa after thawing.

(4) If spermatozoa frozen in yolk–citrate were resuspended after thawing in the Krebs'–Henseleit–Ringer diluent they survived incubation nearly as well as spermatozoa frozen in milk (Table 6 and A × C interaction, Table 5).

(5) Replacement of the diluent used for freezing by a similar diluent without glycerol and with a reduced fructose content for incubation was beneficial when semen was diluted ten-fold (score of 2·45 versus 1·65), but not forty-fold (score of 1·90 versus 2·00) before freezing (interaction B × C, Table 5).

TABLE 5

EXPERIMENT 2: SUMMARIES OF ANALYSES OF VARIANCE

Source of variation	*Degrees of freedom*	*Variance ratios*		
		Motility scores	*% Motile*	*% Unstained*
1. Scores made immediately after thawing				
(A) Diluent	1	26·47***	63·76***	18·52***
(B) Dilution rate	1	0·47	3·91	0·66
(C) Ejaculates	4	17·79***	34·38***	2·12
Interactions				
A × B	1	26·47***	35·16***	3·72
A × C	4	2·79	4·83**	0·22
B × C	4	1·06	1·02	0·11
A × B × C	4	2·33	3·27	0·09
Between samples (ampoules)	40	*0·57*†	*24·00*†	*153·53*†
2. Scores made after incubation at 37° C for 1 hr				
(A) Diluent for freezing	1	38·97***	38·25***	–
(B) Dilution rate	1	0·55	0·44	–
(C) Diluent for incubation				
(i) 1 versus 3	1	3·29	1·38	–
(ii) 2 versus mean 1 and 3	1	11·84**	7·85**	–
(D) Ejaculates (replicates)	4	4·33**	19·33***	–
Interactions				
A × B	1	12·19**	26·05***	–
A × C				
(i) Milk versus yolk–citrate after incubation in 1 or 3	1	2·42	0·54	–
(ii) Milk versus yolk–citrate after incubation in 1, 2 and 3	1	5·73*	10·41**	–
B × C				
(i) ten versus forty-fold dilution after incubation in 1 or 3	1	5·44*	0·54	–
(ii) ten versus forty-fold dilution after incubation in 1, 2 and 3	1	0·02	0·12	–
A × D	4	1·16	2·08	–
B × D	4	1·25	0·50	
C × D	8	1·31	3·72**	–
Residual	30	1·49†	41·60†	–

† Residual variance.
*** $P<0·001$; ** $P<0·01$; * $P<0·05$.

TABLE 6

INTERACTION OF DILUENT USED FOR FREEZING AND DILUENT USED FOR INCUBATION IN EXPERIMENT 2 (MEANS FROM TEN OBSERVATIONS)

Semen incubated after thawing in:	*Mean score after incubation*			
	Motility		*% Motile*	
	Semen frozen in		*Semen frozen in*	
	Skim milk	*Yolk–citrate*	*Skim milk*	*Yolk–citrate*
(1) Diluent used for freezing but containing 17 mM-fructose and no glycerol	2·65	1·70	33·0	19·0
(2) Krebs'–Henseleit–Ringer	2·80	2·35	31·0	28·0
(3) Resuspended in diluent used for freezing	2·60	1·05	32·0	11·0

Experiment 3 (a $2 \times 3^2 \times 4$ factorial; Tables 7 and 8) was designed to compare two levels of citrate in egg-yolk–citrate with skim milk as freezing diluents when all spermatozoa were resuspended in Krebs'–Henseleit–Ringer for incubation after thawing. The significant effects were:

(1) For scores of motility on thawing a yolk–citrate diluent containing 54 mM citrate was better than one containing 60 mM-citrate.

(2) From all scores of activity of spermatozoa both 54 mM and 60 mM-citrate with yolk were inferior to milk as a diluent.

TABLE 7

EXPERIMENT 3: EFFECTS OF DILUENTS USED FOR FREEZING, DILUTION RATE AND EQUILIBRATION ON THE REVIVAL RATES OF DEEP-FROZEN RAM SPERMATOZOA AND THEIR SUBSEQUENT SURVIVAL DURING INCUBATION AT 37° C FOR 1 HR IN A KREBS'–HENSELEIT–RINGER DILUENT (MEANS FROM FOUR EJACULATES)

Treatment	*Mean motility score*		*Mean % motile*		*Mean % unstained by congo-red after thawing and washing*
	Thawed	*Incubated*	*Thawed*	*Incubated*	
Freezing diluent (A) and dilution rate (B)					
Skim milk, diluted ten-fold	2·56	2·44	21·9	18·1	16·5
Skim milk, diluted twenty-fold	2·69	2·63	35·0	23·8	28·9
Skim milk, diluted forty-fold	2·75	2·38	31·3	23·1	24·3
Egg yolk in 60 mM-sodium citrate					
diluted ten-fold	2·50	2·31	31·9	22·5	27·6
diluted twenty-fold	2·13	1·88	24·4	12·5	23·6
diluted forty-fold	1·13	1·25	6·3	8·1	16·8
Egg yolk in 54 mM-sodium citrate					
diluted ten-fold	2·56	2·56	30·0	26·9	28·4
diluted twenty-fold	2·25	2·06	20·6	13·8	17·0
diluted forty-fold	1·88	1·75	19·3	11·3	17·1
Equilibration (C)					
0·5 hr	2·13	1·93	21·3	13·2	13·6
5·0 hr	2·42	2·35	27·8	22·4	30·8

(3) The (ii) × (i) component of the interaction of diluent for freezing and dilution rate shows that for all responses measured, citrate was slightly better than milk as a diluent for freezing at ten-fold dilution, but at a forty-fold rate milk was clearly superior (Table 7). For the (i) × (ii) component of the interaction, scores of percentage motile spermatozoa on thawing showed that the detrimental effect of dilution in yolk–citrate was greater when the higher concentration of citrate (60 mM) was used in the diluent. In the (ii) × (ii) component of the interaction, for counts of percentage unstained spermatozoa, a twenty-fold was better than either a ten- or forty-fold dilution in milk and again the effect of dilution in yolk–citrate was greatest when the higher concentration of citrate (60 mM) was used in the diluent.

(5) In all responses measured, a period of 5 hr equilibration was better than 30 min.

(6) Equilibration interacted with dilution rate in the analysis of scores of motility after thawing. At ten-fold dilution the mean motility scores were

2·22 and 2·88 after 30 min and 5 hr equilibration, respectively, but at forty-fold dilution the equivalent scores were 1·94 and 1·96.

(7) The interaction of equilibration and ejaculates in the scores of percentage of unstained spermatozoa occurred because equilibration gave a greater improvement in scores for some ejaculates than others.

TABLE 8

EXPERIMENT 3: SUMMARIES OF ANALYSES OF VARIANCE

Source of variation	*Degrees of freedom*	*Variance ratios*				
		Motility score		*% Motile*		*% Unstained*
		Thawed	*Incubated*	*Thawed*	*Incubated*	*Thawed*
A. Diluent						
(i) Egg-yolk; 60 mM versus54mM-citrate	1	4·35*	2·35	0·71	1·21	1·35
(ii) Egg-yolk–citrate versus milk	1	20·95***	8·37*	8·29**	6·54*	0·62
B. Dilution rate						
(i) ten-fold versus forty-fold	1	17·41***	10·05**	9·90**	7·06*	2·46
(ii) twenty-fold versus mean ten- and forty-fold	1	0·93	0·17	1·65	0·02	0·87
C. Equilibration	1	5·69*	6·27*	6·60*	13·18***	50·51***
D. Ejaculates (replicates)	3	8·82***	3·21*	9·56***	5·29**	30·87***
Interactions						
A × B						
(i) × (i)	1	3·51	0·25	4·88*	0·21	0·01
(ii) × (i)	1	14·71***	4·10*	18·84***	9·26**	10·15**
(i) × (ii)	1	0·78	0·19	3·01	0·26	2·04
(ii) × (ii)	1	0·26	0·34	0·86	0·82	4·16*
A × C	2	0·74	1·02	0·05	0·11	0·22
B × C						
(i) 0·5 versus 5·0 hr equilibration when semen diluted ten- or forty-fold	1	4·95*	2·35	2·85	0·25	1·26
(ii) 0·5 versus 5·0 hr equilibration when semen diluted ten-, twenty- and forty-fold	1	0·23	0·28	0·00	0·67	0·31
C × D	3	0·87	0·75	2·46	1·05	4·05*
Pooled remaining treatment × ejaculate interactions	12	0·81	0·99	1·08	1·16	0·35
Residual	40	*1·08*†	*1·99*†	*56·61*†	*66·41*†	*50·13*†

† Residual variance.

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

DISCUSSION

Experiment 1 in this paper shows that frozen spermatozoa survive incubation after thawing equally well in a synthetic or a milk diluent providing the main sugars in the synthetic diluents are lactose during freezing and fructose during incubation. It appears that diluents containing citrate used either for freezing or incubation after thawing decrease the viability of ram spermatozoa. This

effect could explain part of the failure to achieve high conception rates with deep-frozen ram semen and certainly gives reason for the popular acceptance of milk as a diluent for artificial insemination work (Emmens & Robinson, 1962). Experiments 1 and 2 showed that there is some variability in the effect of citrate from ejaculate to ejaculate; but even if spermatozoa frozen in yolk–citrate do not show damage on thawing, this effect is highly likely to appear if the spermatozoa are either further diluted (washed) or incubated in citrate.

The effects observed in these studies of dilution rate on the revival of spermatozoa frozen in milk or egg-yolk–citrate, are consistent with previously published results (Blackshaw, 1960b; Martin, 1961).

In these studies the concentration of milk solids was the same (9% w/v) in the diluent used for the initial semen dilution and in the diluent containing glycerol. If we accept 9% milk as an isosmotic solution for ram spermatozoa (Jones, 1965) the final tonicity of the diluent, including added fructose, is 123%. Blackshaw (1960a) used 100 mM-sodium citrate as an isosmotic solution, and thus the tonicity of the egg-yolk–citrate diluent described by Blackshaw, Emmens, Martin & Heyting (1957) is 116% when 7% glycerol is used, since glycerol replaces part of the sodium citrate. Blackshaw (1960a) varied the tonicity of this diluent by modifying the sodium citrate concentration and froze semen in 40, 54 and 66 mM-sodium citrate (relative tonicities of 101, 115 and 127% respectively) using 7·5% glycerol. Best survival followed the use of 54 mM-sodium citrate. Experiment 3 in these studies showed that increasing the sodium citrate concentration (54 to 60 mM) in an egg-yolk–citrate diluent, to adjust its tonicity up to that of the milk diluent, decreased the survival of spermatozoa.

A high error variance was obtained in the analysis of percentage of unstained spermatozoa in Experiment 2. It was frequently difficult to see spermatozoa in a stained preparation from a diluent containing egg-yolk due to the aggregations of egg-yolk in the dried smear and this probably affected the observer's ability to make an objective score. However, elimination of this technical difficulty in Experiment 3, by resuspending thawed spermatozoa before preparing Congo red stains, showed milk to be a better diluent than yolk–citrate when twenty- and forty-fold dilution rates were used.

Reasons cannot be offered for the better revival following freezing of semen diluted twenty- or forty-fold rather than ten-fold in milk, but possible factors are that proximity of spermatozoa in ten-fold diluted semen may be physically undesirable during freezing, a critical concentration of some toxic metabolic by-product is not reached at higher dilutions and dilution may be in effect just a greater degree of replacement of seminal plasma by a more suitable diluent in which to freeze spermatozoa.

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