DILUENTS FOR THE PRESERVATION OF RAM SPERMATOZOA

I. DILUENTS USED AT 37°C AND 5°C CONTAINING CASEIN

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[*Manuscript received December* 6, 1965]

Summary

Ram semen was diluted 40- to 50-fold in various synthetic diluents and the motility of the spermatozoa scored after incubation at 37°C and after slow cooling to, and storage at, 5°C.

Levels of potassium above 10 m-equiv. were deleterious at both temperatures, the addition of 5 m-equiv. calcium depressed motility at 37°C but improved survival rates of spermatozoa stored at 5°C. The inclusion of 6 m-equiv. magnesium in diluents had no detectable effect on motility scores.

Replacement of the sodium chloride component (123 mM) of a diluent buffered with 20 mM phosphate buffer with an isosmotic lactose solution showed that diluents with a content of this sugar ranging from 61 to 246 mM were superior at both 37 and 5°C to that containing 123 mM sodium chloride and no lactose. Diluents containing 184 mM fructose, glucose, lactose, or sucrose and 31 mM sodium chloride were all better for the storage of ram spermatozoa at 5°C than the diluent containing 123 mM sodium chloride.

Motility scores of spermatozoa incubated at 37°C were slightly, but significantly, lower in diluents containing 2·0% casein than in those with 0·5% casein. Scores at 5°C showed that 2·0% casein gave much better survival than 0·5% casein.

I. INTRODUCTION

In experiments to study the effects of changes in osmotic pressure, electrolytes, and pH of diluents used at room temperatures, Blackshaw and Emmens (1951) found that ram spermatozoa have maximal motility at about pH 7·0, irrespective of the proportion of sodium chloride or glucose present, and hypotonic diluents were more deleterious than hypertonic diluents under all conditions studied. Blackshaw (1953*a*) and White (1953*a*, 1953*b*, 1953*c*) studied the effects of high dilution on ram spermatozoa. Of the substances tested for their protective value against the deleterious effects of high dilution, the inclusion of 4 mM potassium chloride in the diluent was most important. Blackshaw (1953*a*) also demonstrated that some protection was given by the inclusion of ram seminal plasma, accessory secretions from a vasectomized ram, or by starch, glycogen, egg albumin, plasma albumin, and plasma gamma-globulin, all substances of high molecular weight. Ram spermatozoa are particularly sensitive to temperature shock (i.e. a rapid reduction in temperature to 0°C causes an irreversible depression in motility), but Blackshaw (1954) found that lecithin extracted from egg yolk gave considerable protection. Thus, the physiological

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requirements of pH, tonicity, electrolytes, and protection from the effects of high dilution rates and sudden temperature changes given must be met by any diluent suitable for the preservation of ram spermatozoa at reduced temperatures.

The use of milk as a diluent for ram semen has been reviewed by Emmens and Robinson (1962) who concluded that, in insemination trials, milk diluents of one type or another compared favourably with conventional diluents (e.g. yolk–citrate, yolk–citrate–glucose, and yolk–phosphate). In most of these experiments the dilution rates were low (up to tenfold dilution) and the semen was used immediately without attempting storage at 5°C. Salamon and Robinson (1962*a*, 1962*b*) reported that, at between three- and fivefold dilution, heated cows milk was superior to ewes milk and that egg yolk–citrate–glucose (Ozin 1956) was better than heated cows milk. However, fertility results in these insemination trials were poor after the first day of storage of the diluted semen at 5°C and added to the general experience that any period of greater than 24 hr of storage at 5°C was likely to give unsatisfactory conception rates.

The use of a biological fluid, such as milk, with a variable composition adds difficulty to research on the chemical and physical factors required for a satisfactory semen diluent. Choong and Wales (1962) and Wales and Choong (1963) used synthetic diluents containing casein and lactose in studies of the effects of cold shock and ultraviolet radiation on ram and bull spermatozoa. These two substances were studied further in experiments in this paper for their value in preserving spermatozoa at 37 and 5°C and from these results, research into the effects of other proteins and sugars was developed and will be the subject of later papers. All these studies were *in vitro*, but the variation in degree of success in insemination trials using diluted, preserved ram semen shows that fundamental studies of ram spermatozoa must be undertaken before an improvement can be expected in the fertility of diluted semen prepared for insemination.

II. Materials and Methods

Semen was collected from Merino rams by electro-ejaculation. Ejaculates were used in experiments if they had high initial motility and a concentration of spermatozoa exceeding $1 \cdot 5 \times 10^9$/ml. Typically, the semen used in these experiments had a spermatozoal count of 3×10^9/ml.

Diluents were prepared from A.R. grade chemicals assuming 154 mM NaCl, 100 mM phosphate buffer (pH = 7), and 308 mM sugar to be isotonic with ram spermatozoa (Blackshaw and Emmens 1951). As factorial experimental designs were frequently used, details of the combinations of diluents used in each experiment are given together with the results in the next section. Antibiotics, 500 i.u./ml of each of sodium penicillin G and dihydrostreptomycin sulphate were added to all diluents and, regardless of other sugars present in the diluent, all diluents contained $11 \cdot 4$ mM (2 mg/ml) fructose.

Casein (British Drug Houses) and bovine plasma globulin (Commonwealth Serum Laboratories) were obtained commercially in dry powder form. Both proteins dissolved readily at the concentrations used.

In all experiments semen was initially diluted at 30°C no more than 40 min after collection and the final dilution rate in all experiments lay between 40- and 50-fold. Diluted semen was cooled from 30 to 5°C in 2 hr.

Open tubes were used for incubation at 37°C and storage at 5°C.

The activity of spermatozoa was scored from samples in thin films under glass coverslips using a microscope warm stage at 37°C to heat the samples. A motility score (scale: 0–4, Emmens 1947) was used to describe the velocity of progression of the spermatozoa and a visual estimation of the percentage of motile spermatozoa was made.

In experiments 1, 2, and 3, a motility index was prepared by summing the motility scores made during the incubation period for each sample and these added scores were used as unit observation in the analyses of variance.

Analyses of variance were made on the sets of data for each experiment. Wherever possible the SILLIAC electronic computer was used for these analyses (Claringbold 1957) but, where the computer programmes were unsuited to the experimental design, a desk calculator was used and, in these cases, the orthogonal coefficients used to partition the responses to treatments are tabulated later in the paper near the table of results. The terms "linear" (L) and "quadratic" (Q) and the interaction coefficients derived from them are the standard orthogonal polynomials used to describe dose response lines and have been fully described by Fisher and Yates (1953) and Cochran and Cox (1957). Such analyses are presented in summary form in the tables, with the residual variance at the base of each column of variance ratios.

Each ejaculate in these experiments was used for a complete replication of the experimental design, so that, in the analyses, the term "ejaculates" always represents replicates and no individual ram gave more than one ejaculate for each experiment.

III. Results

Lactose content, the ratio of potassium to sodium, and the levels of magnesium and calcium were studied in a $2^2 \times 3^3$ factorial experiment design. The basic diluent contained 21 mM KCl, 63 mM NaCl, 70 mM lactose (i.e. 2·5%), 20 mM phosphate buffer, 2·0% casein, 0·5% globulin, and 2 mg/ml fructose. This was varied as required by the levels of factors in the design and the addition of the higher levels of lactose also meant that the tonicity of the diluent was increased. Table 1 summarizes the responses and their significance in this experiment. The lowest level of lactose (i.e. the isotonic diluent) gave best results both on incubation at 37°C for 3 hr and storage at 5°C for 5 days. An increasing proportion of potassium depressed survival rates at both temperatures, 5 m-equiv. calcium depressed the motility index at 37°C, but increased scores at 5°C.

Experiment 2 was another factorial design (3^4) in which lower levels of potassium and casein than those in experiment 1 were tested and the content of magnesium and calcium was varied simultaneously. The diluents were based on 70 mM lactose, 85 mM NaCl, 20 mM phosphate buffer, and 2 mg/ml fructose. The

TABLE 1

EXPERIMENT 1: MEAN MOTILITY INDEXES FOR RAM SPERMATOZOA INCUBATED AT 37°C FOR 3 HR AND AT 5°C FOR 5 DAYS, SHOWING THE EFFECTS OF LACTOSE, POTASSIUM, SODIUM, CALCIUM, AND MAGNESIUM ON THE ACTIVITY OF SPERMATOZOA

The experiment was a $2^2 \times 3^2$ factorial replicated using three ejaculates from different rams; the number of observations from which the mean for each level of a factor was calculated is given

Constituents of Diluent and Levels Used	No. of Observations	Tonicity of Diluent	Mean Motility Index	
			37°C (maximum score = 16)	5°C (maximum score = 12)
Lactose				
70 mM (2·5% w/v)	36	1·0	8·78	4·21
105 mM (3·75% w/v)		1·12	7·59	3·45
140 mM (5·00% w/v)		1·25	5·59	2·69
Potassium/sodium ratio				
1 : 3 (21 m-equiv. : 63 m-equiv.)	36		9·11	3·91
1 : 1 (42 m-equiv. : 42 m-equiv.)			7·43	3·42
3 : 1 (63 m-equiv. : 21 m-equiv.)			5·43	3·04
Magnesium level				
0 m-equiv.	54		7·34	3·28
6 m-equiv.			7·30	3·63
Calcium level				
0 m-equiv.	54		8·17	2·57
5 m-equiv.			6·48	4·33

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		37°C	5°C
Lactose (A)			
2·5% *v.* 5·0% (L)	1	236·89***	37·75***
Remainder (Q)	1	5·11	0·00
Potassium/sodium ratio (B)			
21 m-equiv. potassium *v.* 63 m-equiv. potassium (L)	1	315·10***	12·40***
Remainder (Q)	1	0·75	0·50
Magnesium (C)	1	0·57	3·02
Calcium (D)	1	99·23***	76·34***
Ejaculates (E)	2	364·18***	18·45***
Interactions			
First-order treatment (pooled)	13	0·91	0·94
$B \times E$	4	4·26**	0·33
$D \times E$	2	0·95	4·45*
Pooled remaining treatment × ejaculate	6	2·29	0·71
Residual variance	74	19·3	109·7

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

motility indexes for 3·5 hr of incubation at 37°C showed that the absence of potassium, calcium, magnesium, and 0·5% casein gave significantly better results (Table 2).

TABLE 2

EXPERIMENT 2: EFFECTS OF POTASSIUM, CALCIUM, MAGNESIUM, AND CASEIN ON THE MOTILITY OF RAM SPERMATOZOA INCUBATED AT 37°C FOR 3·5 HR OR 5°C FOR 5 DAYS

A 3^3 factorial design, replicated with three ejaculates, was used; the number of observations from which the mean for each level of each factor was calculated is given

Constituents of Diluent and Levels Used	No. of Observations	Mean Motility Index (maximum score = 16 for both temperatures)	
		37°C	5°C
Potassium level (m-equiv.)			
0	27	12·36	6·29
10		11·88	6·88
20		11·35	6·56
Magnesium/calcium levels (m-equiv.)			
0/0	27	12·07	4·71
3/2·5		11·90	6·66
6/5		11·64	8·35
Casein (%, w/v)			
0·5	27	12·03	5·61
1·0		11·83	6·60
2·0		11·74	7·51

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		37°C	5°C
Potassium (*A*)			
Presence *v.* absence	1	37·27***	4·19*
10 m-equiv. *v.* 20 m-equiv.	1	13·94***	2·18
Magnesium/calcium (*B*)			
Presence *v.* absence	1	6·05*	212·89***
3/2·5 levels *v.* 6/5 levels	1	3·39	59·82***
Casein (*C*)			
0·5% *v.* 2·0% (*L*)	1	4·21*	73·37***
1·0% *v.* (0·5 and 2·0%) (*Q*)	1	0·21	0·02
Ejaculates (*D*)	2	3·40*	9·39***
Interactions			
First-order treatment	12	1·28	2·14
First-order ejaculate × treatment	12	2·06	4·00***
Residual variance	48	6·7	64·4

* $P < 0·05$. *** $P < 0·001$.

However, at 5°C, 10 m-equiv. potassium and up to 6 m-equiv. magnesium and 5 m-equiv. calcium improved survival. Casein (2%) was more protective than 0·5 or 1·0% casein.

Table 3 summarizes the behaviour of ram spermatozoa at 37 and 5°C in diluents prepared by mixing the basic diluents of (*A*) 246 mM lactose, 10 mM KH_2PO_4, 10 mM Na_2HPO_4, and (*B*) 123 mM NaCl with the same content of phosphate buffer in the proportions 0 : 4, 3 : 1, 1 : 1, 1 : 3, and 4 : 0. All diluents contained 2·0% casein, 2 mg/ml fructose, and 5 m-equiv. calcium. At both 37 and 5°C, spermatozoal survival was improved by an increasing proportion of lactose.

TABLE 3

EXPERIMENT 3: MEAN MOTILITY INDEXES FOR RAM SPERMATOZOA STORED IN DILUENTS CONTAINING MIXTURES OF LACTOSE AND SODIUM CHLORIDE FOR 3·5 HR AT 37°C AND 5 DAYS AT 5°C

Results are means from three ejaculates

Sodium Chloride/Lactose Levels (mM) in Diluent			Mean Motility Index		Coefficients Used for Contrasts			
			37°C	5°C	*a*	*b*	*c*	*d*
Sodium Chloride		Lactose						
123	:	0	6·0	2·9	−1	0	0	+3
93	:	61	8·3	3·5	0	−1	−1	−2
62	:	123	11·3	7·5	0	0	+2	−2
31	:	185	12·0	9·3	0	+1	−1	−2
0	:	246	11·4	8·7	+1	0	0	+3

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		37°C	5°C
Replacement of sodium chloride by lactose			
Coefficient *a*	1	6·81*	12·92**
Coefficient *b*	1	3·10	13·22**
Remainder	2	1·10	0·76
Ejaculates	2	3·20	3·11
Residual variance	8	6·50	3·86

* $P < 0·05$. ** $P < 0·01$.

Table 4 presents a summary of the survival of spermatozoa chilled for 4 days in sugar-containing diluents. All diluents contained 20 mM phosphate buffer (containing 10 m-equiv. potassium), 3% casein, and 5 m-equiv. calcium. Reconstituted skim milk (10% w/v preparation of commercially prepared skim milk powder) with 2 mg/ml fructose was compared with the synthetic diluents and three scores of activity were made during the period at 5°C. In the analysis of variance, variance ratios were calculated for the diluent contrasts using the ejaculate × diluent interaction as error term and the residual variance of high-order interactions was used for the ratios testing factors involving time (Table 4). A significantly higher percentage of spermatozoa survived in milk than in the synthetic diluents, but the greatest differences were shown between the diluent containing 123 mM NaCl (*A*) and those containing sugars, either mono- or disaccharides (*B*, *C*, *D*, and *E*). Motility scores fell off rapidly during

storage in the high-salt diluent (*A*) which shows as a significant interaction of diluent and time. Ejaculates 1 and 2 did not survive chilling as well as 3 and 4 and this accounts for the significant ejaculate × time interaction.

TABLE 4

EXPERIMENT 4: MEAN MOTILITY SCORES AND PERCENTAGE OF MOTILE SPERMATOZOA OBSERVED DURING A PERIOD OF STORAGE FOR 4 DAYS AT 5°C IN VARIOUS SUGAR-CONTAINING DILUENTS

Results are means from four ejaculates; maximum motility score = 4

Constituents of Diluents and Levels Used	Mean Motility Scores	No. of Motile Spermatozoa (%)	Coefficients Used to Partition Variance				
			a	*b*	*c*	*d*	*e*
Sodium chloride (123 mM) (*A*)	2·08	27·9	+4	0	0	0	−1
Sodium chloride (31 mM)							
+fructose (185 mM) (*B*)	3·00	38·3	−1	−1	−1	0	−1
+glucose (185 mM) (*C*)	3·00	40·0	−1	−1	+1	0	−1
+lactose (185 mM) (*D*)	2·79	37·5	−1	+1	0	−1	−1
+sucrose (185 mM) (*E*)	3·04	39·6	−1	+1	0	+1	−1
Reconstituted skim milk (10% w/v powdered milk)	2·92	46·3	0	0	0	0	+5

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		Motility Scores	% Motile
Diluents			
Coefficient *a*	1	40·83***	13·79**
Coefficient *b*	1	0·44	0·04
Coefficient *c*	1	0·00	0·12
Coefficient *d*	1	2·06	0·33
Coefficient *e*	1	1·00	7·02*
Ejaculates	3	15·11***	14·68**
Diluents × ejaculates	15	0·18†	53†
Time at 5°C (three scores made over 4 days)	2	70·54***	83·05***
Diluents × time	10	2·77*	1·83
Ejaculates × time	6	0·54	3·94**
Residual variance	30	0·13‡	18‡

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

† Interaction variance used to compute variance ratios for diluent contrasts.

‡ Residual variance used to compute variance ratios for contrasts with storage time as a factor.

IV. DISCUSSION

The results of experiments 1 and 3 show a satisfactory working hypothesis for the design of diluents to be that a 308 mM solution of sugar is isotonic, as well as theoretically isosmotic, with 154 mM sodium chloride. In addition, the use of a high proportion of lactose replacing sodium chloride in the diluent prolongs the survival of spermatozoa at 5°C. As far as these experiments show, glucose, fructose, and sucrose, when tested at 185 mM content in the diluent, are as valuable as lactose.

This effect is not simply due to the provision of sugar as a metabolic substrate as all diluents, including those with 123 mM sodium chloride, also contained 11·4 mM fructose. In experiment 3 where lactose (which cannot be utilized by spermatozoa) was used, there was a progressive increase in survival as the sugar content was raised from 61 to 185 mM. Different aspects of the value of sugar in a diluent for the preservation of spermatozoa by deep-freezing have been presented by Emmens and Blackshaw (1950), Blackshaw (1954), Martin and Emmens (1961), Choong and Wales (1963), and Martin (1963, 1965*a*, 1965*b*), and a more detailed examination of various sugars arising from the studies reported in this paper is presented by Lapwood and Martin (1966). It appears that the inclusion of a sugar in the diluents in which spermatozoa are chilled or frozen alters the physical properties of the diluent and in some way protects the function of the cell.

Whenever potassium was used at higher levels than 10 m-equiv. the viability of the spermatozoa was lowered. O'Shea and Wales (1964) have described a similar effect and Choong and Wales (1963), in experiments on deep-freezing bull semen, also observed that levels of potassium in synthetic diluents should not be as high as are found in milk and they suggested that a substantial part of the potassium in milk is bound and is not present as an ionized electrolyte. However, the original observation made by White (1953*a*, 1953*b*) on the need for 4–5 m-equiv. potassium in a diluent remains unchallenged and the presence of a small amount of potassium is a necessary part of a diluent for the survival of highly diluted ram spermatozoa at both body temperature and for chilled storage.

Blackshaw (1953*b*) demonstrated that the presence of calcium ions in a glucose–saline diluent depressed the motility of ram spermatozoa incubated at 37°C for 5 hr and this is confirmed in the first experiment in this paper. However, the response was reversed for spermatozoa stored at 5°C for 5 days and survival was enhanced by the inclusion of 5 m-equiv. calcium in the diluent. A reversal of response was also observed when the content of casein in the diluent was varied. Higher levels depressed motility scores at 37°C but survival at 5°C was significantly improved when the casein level was increased from 0·5 to 2·0%. The samples were cooled slowly from 30 to 5°C to avoid temperature shock so that the protective effect of casein at 5°C is probably in addition to that described by Choong and Wales (1962) in their studies of cold shock.

In experiment 4, the presence of sugar improved survival of spermatozoa in the synthetic diluents, but none of these was as good as that prepared from skim milk. Numerous attempts have been made to refine egg-yolk buffer or milk diluents or to substitute synthetics for them and these are reviewed adequately in Mann (1964), but none of these has been widely used in regular artificial insemination practice. The results presented in this paper show that the temperature intended for storage will influence the choice of substances and levels of these substances used, and more intensive studies of the protein, sugar, and electrolyte content of the diluent are warranted. Apart from any practical value for use for insemination, the development of such synthetic diluents could have considerable fundamental value in determining factors affecting the structure, function, and senescence of the living cell.

V. Acknowledgments

I wish to thank Professor C. W. Emmens for constructive comment on this paper. This work was aided by grants from the Australian Wool Board.

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