EFFECT OF OSMOLALITY AND PHOSPHATE, 'TRIS', 'TES', 'MES', AND 'HEPES' HYDROGEN ION BUFFERS* ON THE MOTILITY OF BULL SPERMATOZOA STORED AT 37 OR 5°C

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Abstract

Bull spermatozoa survived incubation, in unstoppered tubes at 5 and 37°C, better in solutions of 250 m-osmoles/kg than in solutions of higher or lower osmolalities regardless of the concentrations of sodium chloride or citrate in the solutions relative to the concentrations of phosphate, Tris, TES, MES, or HEPES. When buffer solutions contributed 120 m-osmoles/kg to a total diluent strength of 250 m-osmoles/kg, the ranking from best to worst on the basis of survival of spermatozoa was: MES, HEPES, TES, Tris, and phosphate. Comparisons when 50 mM concentrations of the buffers were used showed the same ranking except that HEPES was as good as MES.

I. Introduction

The hydrogen ion buffers used for storing spermatozoa or for studies of their metabolism have not been completely suitable. Phosphate, citrate, veronal, and Tris are satisfactory as buffers for semen diluents (Phillips 1939; Salisbury, Fuller, and Willet 1941; Blackshaw 1953; Wales and White 1958; Davis, Bratton, and Foote 1963; Steinbach and Foote 1967; Foote 1969); however, none are entirely inert with respect to cellular function. Phosphate tends to precipitate most polyvalent cations and depresses spermatozoal respiration in some species (Salisbury and Lodge 1962; Lodge *et al.* 1963; Wales and Wallace 1964; Murdoch and White 1966). Citrate is a weak buffer and may be toxic to spermatozoa (Jones and Martin 1965). Tris, below pH 7·5, also has poor buffering capacity, and due to its primary aliphatic amine may be "inhibitory" (Good *et al.* 1966) to some cells. These latter authors recognized the inadequacies of the available hydrogen ion buffers for use in biological research. They designed and synthesized a number of organic compounds and then tested them for side-effects and buffering capacity within a suitable pH range. Ten were zwitterionic and had low affinities for binding metals. They were better than phosphate or Tris, as measured by the Hill reaction and phosphorylation-coupled oxidation of succinate by bean mitochondria. Of these, TES and HEPES gave the most active and stable mitochondrial preparations. MES also showed promise.

* Abbreviations are as follows: Tris, tris(hydroxymethyl)aminomethane; TES, *N*-tris(hydroxymethyl)methyl-2-aminomethanesulphonic acid; MES, 2-(*N*-morpholino)ethanesulphonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

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Since buffers with these properties would be highly suitable for metabolic studies of spermatozoa and possibly for storing spermatozoa, TES, HEPES, and MES were tested for their effects on the survival of bull spermatozoa at 37 and 5°C.

II. Materials and Methods

Samples of bull semen containing a high proportion of spermatozoa with good activity were diluted within 40 min of collection for use in the experiments. TES, MES, and HEPES were purchased from Calbiochem (Los Angeles, California); other chemicals were analytical grade from various sources. The phosphate buffer was prepared from the sodium salts. All other solutions were adjusted to pH 7·0 with either hydrochloric acid or sodium hydroxide. On completion of the incubation studies the pH of each solution was remeasured and was found unchanged.

The osmolality of the diluents was measured with an osmometer (Advanced Instruments Inc., Newton Highlands, Massachussetts). As the observed osmolalities of the diluents were slightly higher than expected from the sum of osmolalities of the component solutions, only the mean observed osmolality of the diluents is stated in the results. Individual solutions did not vary more than 7 m-osmoles/kg from these means, which have been rounded to ± 2 m-osmoles/kg to simplify presentation.

All solutions contained 4 m-equiv. of potassium and 10 mmoles (expts. 1 and 2) or 6 mmoles (expts. 3 and 4) of fructose, and 500 i.u. of penicillin and 500 μg of streptomycin per millilitre, 1% (v/v) egg yolk in experiments 1–3 and 5% (v/v) egg yolk in experiment 4. The yolk was used only at levels sufficient to give the spermatozoa some protection during cooling to 5°C in order to minimize interference with tests of the effect of diluent composition.

Semen was diluted 75-fold. The diluted samples were incubated in 5-ml capacity open polyethylene tubes in a water-bath and the tubes were coded and randomized. At hourly or 2-hourly intervals during incubation at 37°C and once a day for incubation at 5°C, subsamples were prepared as a thin film between a slide and coverslip on a microscope warm stage at 37°C. The samples were examined, using a magnification of $\times 320$, the rate of progressive motility was scored from 0 to 4 (Emmens 1947) and the percentage of motile spermatozoa was estimated. Each score of motility was doubled to remove fractions and used as a unit observation in the analyses of variance. Scores of percentage motile were transformed to angles (Claringbold, Biggers, and Emmens 1953) for analysis. The orthogonal polynomial coefficients tabulated by Fisher and Yates (1957) were used to partition levels of a factor. However, to test the hypotheses examined as factor 1 in experiments 1, 3, and 4 a Helmert matrix of coefficients (Irwin 1949) was used to rank compounds. The analyses of variance for experiment 4 (Table 5) is shown; for the other experiments the error variances, with degrees of freedom and the statistical significance of treatment main effects, are shown in the relevant tables. For brevity only main effects and first-order interactions that are statistically significant are described in the text.

III. Results

Preliminary experiments were carried out to determine what concentrations of sodium chloride and sodium citrate were optimal for spermatozoa under the conditions of these experiments. Solutions of either of the salts, varying in osmolality from 180 to 340 m-osmoles/kg were used separately to dilute semen samples for incubation at 37 and 5°C. Best survival was observed in solutions of about 240 m-osmoles/kg regardless of which salt was used. This corresponds to 130 mM sodium chloride and 85 mM sodium citrate solutions. Although this osmolality is lower than normally accepted as optimal for bull spermatozoa it was chosen because it gave the best results under the conditions tested. Consequently, solutions of 240 m-osmoles/kg were tested further in experiment 1, a 5×3 factorial design replicated with ejaculates from four bulls (Table 1). Phosphate, Tris, TES, MES, and HEPES were used in

concentrations to contribute 115 m-osmoles/kg, and the final diluents were adjusted to 205, 240, and 275 m-osmoles/kg by varying the concentration of sodium chloride.

TABLE 1

EXPERIMENT 1: MEAN SCORES OF SPERMATOZOA WHICH SURVIVED AFTER INCUBATION IN SOLUTIONS OF VARIOUS BUFFERS FOR 11 HR AT 37°C OR 2 DAYS AT 5°C

Buffer concentrations such that osmolality of each was 115 m-osmoles/kg

Factor and level	11 hr at 37°C		2 days at 5°C	
	Motility scores	Percentage motile	Motility scores	Percentage motile
Buffer				
(1) 50 mM phosphate	0·83	14·2	0·71	12·5
(2) 65 mM Tris	1·46	29·2	0·75	22·9
(3) 95 mM TES	1·79	43·3	1·42	40·8
(4) 70 mM MES	1·63	35·8	1·25	39·2
(5) 95 mM HEPES	1·75	37·1	1·46	43·3
P: (1) *v.* (2)	**	*	n.s.	n.s.
Mean of (1) and (2) *v.* mean of (3), (4), and (5)	***	***	***	***
Molarity of sodium chloride and osmolality of final solutions				
(1) 40 mM NaCl, 205 m-osmoles/kg	1·48	25·5	1·25	30·5
(2) 59 mM NaCl, 240 m-osmoles/kg	1·65	37·5	1·10	34·8
(3) 77 mM NaCl, 275 m-moles/kg	1·35	32·8	1·00	30·0
P: Linear	n.s.	*	*	n.s.
Quadratic	*	*	n.s.	n.s.
Error variance (D.F. = 24)	*0·69*	*60·5*	*0·53*	*94·5*

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

Spermatozoa survived incubation at 37°C best in solutions of 240 m-osmoles/kg, and scores of percentage motile spermatozoa were greater for the highest osmolality than for the lowest. An interaction of buffer and osmolality ($P < 0·05$) showed that the mean motility scores of samples stored in solutions adjusted to 205 and 275 m-osmoles/kg differed depending upon the buffer used. Thus for TES the mean scores were respectively 2·13 and 1·13, for MES they were 1·38 and 1·68, and for HEPES they were 1·75 and 1·75. A similar interaction involving only TES and HEPES was recorded for scores of percentage motile spermatozoa. The respective mean scores for TES were 45·0 and 30·0, and for HEPES they were 25·0 and 41·3. The mean motility scores for samples stored at 5°C increased as the osmolality of the diluent decreased.

Comparisons between buffers showed that for the 37°C incubation Tris was better than phosphate and for 37 and 5°C incubation Tris and phosphate were not as good as TES, MES, and HEPES. Considering that phosphate was used at a lower concentration than Tris and the concentrations of both phosphate and Tris were lower than TES, MES, and HEPES, the conclusions should be the same for solutions of the buffers used at equal concentrations.

As the effect of varying osmolality was small in experiment 1 this factor was reconsidered in an experiment using phosphate and Tris buffers over a wider range of

osmolalities (expt. 2, Table 2). Analyses of variances showed that Tris was better than phosphate as judged by all measures of response ($P < 0{\cdot}01$ for scores of percentage of motile spermatozoa after incubation at 5°C, and $P < 0{\cdot}05$ for other responses). For storage at 37°C it was best to adjust the osmolality of phosphate diluents to 220–266 m-osmoles/kg; however, there was little effect of varying the osmolality of Tris-containing diluents (quadratic component of percentage motile scores at 37°C, $P < 0{\cdot}05$; interaction, $P < 0{\cdot}05$).

TABLE 2

EXPERIMENT 2: EFFECT OF VARYING THE CONCENTRATION OF SODIUM CHLORIDE ON THE SURVIVAL OF SPERMATOZOA IN PHOSPHATE AND TRIS SOLUTIONS AFTER INCUBATION FOR 13 HR AT 37°C OR 2 DAYS AT 5°C

Values given are means from four ejaculates

Buffer	Sodium chloride concn. (mM)	Osmolality of diluent (m-osmoles/kg)	13 hr at 37°C		2 days at 5°C	
			Motility scores	Percentage motile	Motility scores	Percentage motile
50 mM phosphate	25	174	0·67	15·0	1·50	40·0
	50	220	1·33	40·0	1·33	46·7
	75	266	1·00	40·0	0·83	26·7
	100	312	1·00	23·3	0·50	16·7
Mean			1·00	29·6	1·04	32·5
65 mM Tris	25	174	2·00	43·3	2·17	60·0
	50	220	1·83	46·7	1·67	50·0
	75	266	1·33	40·0	1·00	53·3
	100	312	1·33	43·3	1·17	40·0
Mean			1·63	43·3	1·50	50·8

For samples stored at 5°C scores of motility ($P < 0{\cdot}001$) and percentage motile spermatozoa ($P < 0{\cdot}05$) increased as osmolality decreased. The interaction of buffer and osmolality observed for the 37°C study was also significant for motility scores in the 5°C study, showing again that the effect of varying osmolality was less for Tris-containing diluents than phosphate-containing diluents.

The diluents in experiments 1 and 2 were slightly opaque when egg yolk was added and survival of spermatozoa seemed lower than normal. Therefore, citrate ion instead of chloride was used in experiment 3 (Table 3). Further, since the concentration of sodium chloride was confounded with osmolality of the diluent in the previous experiments, the importance of the confounding was tested by varying either buffer or sodium citrate concentration. A constant concentration of 50 mM phosphate, 98 mM TES, 73 mM MES, or 100 mM HEPES (i.e. 120 m-osmoles/kg) was used with varying sodium citrate concentrations to obtain the appropriate osmolality. Alternately, a constant 43 mM of sodium citrate (also 120 m-osmoles/kg) was used with varying concentrations of the buffers. The experiment was replicated with ejaculates from six bulls.

Poor survival was observed in the phosphate-containing diluents, so these results were excluded from the analyses of variance. However, for comparison the

TABLE 3

EXPERIMENT 3: MEAN SCORES OF SPERMATOZOA WHICH SURVIVED AFTER INCUBATION FOR 6 HR AT 37°C OR 1 DAY AT 5°C

Factor and level	6 hr at 37°C		1 day at 5°C	
	Motility scores	Percentage motile	Motility scores	Percentage motile
I. Buffer				
Sodium phosphate†	0·44†	5·5†	0·98†	12·5†
(1) TES	2·42	47·6	2·24	37·5
(2) MES	2·14	42·6	2·21	43·1
(3) HEPES	2·10	39·9	2·31	39·4
P: TES *v.* mean of MES and HEPES	n.s.	***	n.s.	**
MES *v.* HEPES	n.s.	n.s.	n.s.	*
II. Osmolality of diluent				
(1) 190 m-osmoles/kg	2·32	34·3	2·47	41·4
(2) 250 m-osmoles/kg	2·54	54·9	2·63	52·1
(3) 310 m-osmoles/kg	1·79	41·0	1·65	26·5
P: Linear	**	*	***	***
Quadratic	**	***	**	***
III. Osmolality adjusted to value determined by factor II by:				
(1) varying buffer concn.; sodium citrate concn. constant (120 m-osmoles/kg)	2·20	42·8	2·22	38·6
(2) varying sodium citrate concn.; buffer concn. constant (120 m-osmoles/kg)	2·23	44·0	2·28	41·4
Error variance (D.F. = 64)	*1·29*	*58·8*	*1·10*	*35·0*

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

† Not used in calculation of other mean effects.

mean responses for samples stored in phosphate are included in Table 3. The following effects were statistically significant:

Incubation at 36°C:

(1) Both scores of survival were higher for samples stored in solutions of 250 m-osmoles/kg than 190 and 310 m-osmoles/kg. For scores of motility, solutions of 190 m-osmoles/kg were better than solutions of 310 m-osmoles/kg. However, for scores of percentage motile spermatozoa this ranking was reversed.

(2) For scores of percentage motile spermatozoa TES was better than MES and HEPES. However, there was an interaction of buffer and osmolality ($P < 0·05$). Thus for final strengths of 190, 250, and 310 m-osmoles/kg respectively, mean scores for TES-containing diluents were 36·7, 55·4, and 50·8; for MES-containing diluents they were 30·4, 60·0, and 37·5; and for HEPES-containing diluents they were 35·8, 49·2, and 34·7.

(3) An interaction of factors II and III for scores of percentage motile sperm shows that in the diluents of lowest osmolality spermatozoa survived best when TES, MES, or HEPES were used at higher osmolalities than sodium citrate. Comparing samples stored in 120 m-osmoles/kg of sodium citrate with the mean for samples stored in 120 m-osmoles/kg of TES, MES, or HEPES: the mean scores for solutions with final osmolalities of 190 m-osmoles/kg differed considerably (25·8 *v.* 42·8); for solutions of 250 m-osmoles/kg (58·6 *v.* 51·1) and 310 m-osmoles/kg (43·9 *v.* 38·1) the differences were small.

Incubation at 5°C:

(1) Best survival occurred in solutions of 250 m-osmoles/kg while solutions of 190 m-osmoles/kg gave better survival than solutions of 310 m-osmoles/kg.

(2) The main effect of buffer was not significant for the scores of percentage motile when the main effect was tested with the significant ($P < 0{\cdot}05$) interaction of buffer and ejaculates. An interaction of buffer and osmolality was observed for scores of motility ($P < 0{\cdot}05$) and percentage motile ($P < 0{\cdot}01$). Considering scores of percentage motile: for final osmolalities of 190, 250, and 310 m-osmoles/kg the means for TES-containing diluents were respectively, 40·8, 47·9, and 23·8; for MES-containing diluents they were 38·3, 55·0, and 35·8; and for HEPES-containing diluents they were 45·0, 53·3, and 20·0.

(3) For samples stored in solutions of 120 m-osmoles/kg of the buffers and varying concentrations of citrate, mean scores of percentage motile sperm were much the same; 41·1, 41·7, and 41·4 respectively for TES, MES, and HEPES. However, the means differed and were respectively 33·9, 44·4, and 37·5 in solutions containing 120 m-osmoles/kg of sodium citrate and varying concentrations of the buffer (interactions of factors I and III for scores of percentage motile, $P < 0{\cdot}05$).

As equal osmolalities of Tris, TES, MES, and HEPES were used in the previous experiments, the effect of type and concentration of buffer was confounded. Consequently, in experiment 4 (a 4×2^2 factorial experiment replicated with ejaculates from four bulls; Table 4) the buffers were compared at equal concentrations and equal osmolalities. The solutions were adjusted to 250 m-osmoles/kg with either sodium chloride or sodium citrate. For comparison semen samples were also stored in an egg yolk–citrate diluent (Blackshaw *et al.* 1957); clearly spermatozoa did not survive as well in this as in the solutions of test buffers (Table 4).

The analyses of variance showed a number of first-order interactions between factors. For brevity these are not explained in detail as they describe variations in the amount of response (and not changes in rank order) due to treatments which, presumably, are present because spermatozoa in the poorer buffers were the most sensitive to treatment during the incubation periods. When there were interactions of treatment main effects with ejaculates in the analyses of variance the main effect was tested using the interaction variance as the denominator in the F-test; where

appropriate only the effects that were statistically significant after this test are shown in Table 4.

TABLE 4

EXPERIMENT 4: MEAN SCORES OF SPERMATOZOA WHICH SURVIVED AFTER INCUBATION FOR 14 HR AT 37°C OR 7 DAYS AT 5°C IN VARIOUS BUFFER SOLUTIONS

Final osmolalities of buffer solutions 250 m-osmoles/kg. Solutions adjusted with sodium chloride or sodium citrate

Factor and level	14 hr at 37°C		7 days at 5°C	
	Motility scores	Percentage motile	Motility scores	Percentage motile
I. Buffer (concn.)				
(1) Tris (50 and 68 mM)	1·13	24·1	0·94	21·0
(2) TES (50 and 98 mM)	1·25	29·7	1·78	36·6
(3) MES (50 and 73 mM)	2·13	51·9	2·53	54·7
(4) HEPES (50 and 100 mM)	1·72	43·4	2·41	49·1
P: Tris *v.* mean of TES, MES, and HEPES	***	***	***	***
TES *v.* mean of MES and HEPES	***	***	***	***
MES *v.* HEPES	*	n.s.	n.s.	n.s.
II. Buffer concn. and osmolality adjustments				
(1) buffer concn. constant (50 mM); osmolality varied	1·83	44·8	1·66	41·9
(2) buffer concn. varied; osmolality constant (120 m-osmoles/kg)	1·28	29·7	2·17	38·8
P:	***	***	***	n.s.
III. Buffer adjusted to 250 m-osmoles/kg with:				
(1) sodium chloride	1·66	42·2	1·66	37·8
(2) sodium citrate	1·50	32·3	2·17	42·8
P:	n.s.	n.s.	***	n.s.
Error variance (D.F. = 33)	*0·89*	*38·8*	*1·08*	*48·2*
Control: egg yolk–citrate	0·38	20·0	0·25	2·8

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

Incubation at 37°C:

(1) HEPES and MES were better than TES and these were all better than Tris. MES was only superior to HEPES when used at a lower concentration than HEPES (i.e. comparison at equal osmolality). For scores of percentage motile the magnitude of these effects varied from ejaculate to ejaculate.

(2) The second factor (II) showed that spermatozoa survived best if the greater proportion of the diluent's osmolality was made up with either sodium chloride or sodium citrate rather than the test buffers, The magnitude of this effect also varied from ejaculate to ejaculate.

(3) An interaction of the third factor (III) with ejaculates (for scores of percentage motile) showed that for two ejaculates it was better to adjust the diluents to 250 m-osmoles/kg with sodium chloride than sodium citrate. An interaction of factors I and III showed that the advantage with sodium chloride was only when it was used in Tris- and TES-buffered diluents.

Incubation at 5°C:

(1) The ranking of buffers was the same as for the 37°C incubations in that HEPES and MES were better than TES and these were all better than Tris; an interaction (of factors I and II) showed that TES was only inferior to MES and HEPES when compared at unequal concentration (i.e. equal osmolality). The magnitude of the differences between buffers varied from ejaculate to ejaculate.

(2) The ranking of levels of factor II, according to mean motility scores (and for scores of percentage motile for three of the four ejaculates), was the reverse of that observed for the 37°C incubation. Thus, spermatozoa survived better in solutions containing higher concentrations of the test buffers (120 m-osmoles/kg and above 68mM) than in 50 mM solutions of sodium chloride or citrate.

(3) It was better to use sodium citrate than sodium chloride in the Tris- and TES-containing diluents but approximately the same results were obtained when either was used in the other buffers.

IV. Discussion

In general spermatozoa survived best in solutions of 250 m-osmoles/kg regardless of the substance used to adjust the diluents osmolality. However, compared to solutions commonly accepted as optimal for bull spermatozoa, these are hyposmotic to 154 mM sodium chloride or 100 mM sodium citrate (both approximately 280 m-osmoles/kg), but isomotic to 100 mM sodium phosphate buffer. Nevertheless, these results are in agreement with the graphs published by Blackshaw and Emmens (1951). Further, considering that the effect of diluent osmolality on survival of spermatozoa is decreased by increasing the protein component of the diluent (Jones and Foote 1972), it is possible that in the presence of sufficient milk protein or egg yolk spermatozoa may tolerate a range of osmolalities from 240 to 280 m-osmoles/kg.

Under the conditions studied it may be concluded that TES, MES, and HEPES are better than Tris or phosphate for storing bull spermatozoa. In experiments 1 and 3 in which the incubation periods were short, differences between TES, MES, and HEPES were small and less important than the effects of osmolality. When compared in experiment 4, using longer periods of incubation in isotonic diluents, MES and HEPES were superior to TES, and for one measure of response MES was better than HEPES. However, both MES and HEPES are worthy of further consideration for metabolic and storage studies for spermatozoa. Indeed, both may be useful under different conditions, as at 20°C the pK_a of MES is 6·15 and for HEPES it is 7·55.

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