EFFECTS OF POTASSIUM ON RAM SPERMATOZOA DURING CHILLING TO AND STORAGE AT 5° C

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Summary. Potassium (16 to 64 mM) adversely affected ram spermatozoa during cooling from 30 to 5° C, while magnesium was beneficial. The effect of potassium was independent of the rate of chilling or the dilution of semen between 1 in 10 and 1 in 20, but it was decreased by increasing the sodium content of the diluent and was increased by the presence of phosphate. The addition of potassium after chilling did not affect subsequent motility.

High potassium levels during chilling were detrimental to washed ram spermatozoa. Low levels, on the other hand, helped to maintain the motility of washed cells during the period of storage. The addition of seminal plasma did not modify the effect of potassium on washed cells.

INTRODUCTION

A number of workers have confirmed that potassium (4 to 5 mM) stimulates the motility of washed ram and bull spermatozoa incubated *in vitro* at 37° C (Lardy & Phillips, 1943; White, 1953a, b, c; Blackshaw, 1953a, b). More recently potassium has been shown to increase the viability of washed and unwashed dog and fowl spermatozoa (Wales & White, 1958a, b).

Despite the evidence of the importance of potassium for in-vitro survival at 37° C, there is little mention of its effects on spermatozoa at low temperatures. Choong & Wales (1963) found that the addition of 10 mM potassium to synthetic diluents did not affect the survival of bull spermatozoa deep frozen to –79° C after 2 or 18 hr equilibration. In other experiments, these workers found that 36 to 85 mM potassium at a Na : K ratio of 2 : 1 depressed the revival of deep frozen bull spermatozoa (C. H. Choong & R. G. Wales, unpublished data, 1963). Kampschmidt, Meyer & Herman (1953), however, were not able to demonstrate any difference between potassium carbonate and sodium carbonate buffers with respect to the viability of bull spermatozoa stored at 5° C for up to 240 hr after cooling. Apart from these observations on bull spermatozoa, the effect of this cation during storage, either at 5° C or at sub-zero temperatures, has not received attention. In the present paper, the effect of potassium ions on ram spermatozoa has been studied during cooling to 5° C and storage at this temperature.

MATERIALS AND METHODS

Semen was collected from rams after electrical stimulation with the bipolar rectal electrode (Blackshaw, 1954) and only apparently normal ejaculates with good initial motility were used. During collection and transport to the laboratory, ejaculates were protected from sudden temperature changes. Experiments were started as soon after collection as practicable and, in general, samples had begun to chill within 30 min of collection.

Unless otherwise indicated in the paper, the isotonic basic diluent (pH 7.0) contained 20 mM mono- and di-sodium phosphate buffer, 76 mM sodium chloride, 88 mM lactose, 3% casein and 1.1 mM fructose as substrate for the spermatozoa. Preliminary experiments showed that levels of fructose between 1 and 6 mM in the diluent caused no change in the potassium effect during chilling. When required, potassium and magnesium were added to the diluents as the chloride salts and sodium chloride content adjusted to keep the diluent isotonic. In some cases, where indicated, phosphate buffer was replaced by 20 mM veronal-HCl buffer (pH 7.0) or 20 mM trishydroxymethylaminomethane (tris) buffer (pH 7.0).

For some studies, the spermatozoa were lightly washed. This was done by diluting the semen 1 : 2 with casein-free basic diluent, and centrifuging it at 2000 rev/min (about 400 g) for 10 min. The supernatant was replaced with an equal volume of diluent and the spermatozoa dispersed and recentrifuged. The second supernatant was removed and the volume was adjusted to that of the original semen.

In general, 0.2 ml aliquots of each ejaculate were mixed with 1.8 ml of diluent in a test tube at 30° C and the semen suspensions were cooled to 5° C by placing in a beaker containing water at 30° C and standing in a refrigerated cabinet. Where required, chilling rates could be modified, but cooling from 30 to 5° C normally occupied 3 hr.

After reaching 5° C the samples were stored in the refrigerated cabinet for 42 hr, surrounded by a water jacket to avoid sudden fluctuations in temperature. Immediately after reaching 5° C, and 18 and 42 hr later, aliquots (approx. 0.5 ml) were removed from each chilled sample with a cooled pasteur pipette and incubated at 37° C for 10 min before the evaluation of motility. Samples were randomized and readings were made on a warm stage under the low power microscope at $\frac{1}{2}$ hr intervals for 1 $\frac{1}{2}$ hr. The activity of the spermatozoa was given a score between 4 and 0 at each reading (Emmens, 1947). Since quarter grades were frequently used, all scores were multiplied by four and the indices of motility were calculated by summing the observations ($\times 4$) over the 1 $\frac{1}{2}$ hr period (Emmens, 1948).

All experiments were of a factorial design and the significance of the results has been assessed by analysis of variance. All main effects and their first-order interactions were isolated and tested for significance, but, in the summary form for the tables, only the first-order interactions which were significant have been presented separately, and non-significant first-order interactions have been combined.

In order to estimate the effects of treatments during the period at 5° C, differences in motility at 0, 18 and 42 hr have been isolated and tested for signi-

ficance. However, since the aliquots assessed at these times were all drawn from the same chilled sample, the effects of time trends and their interactions have been treated as within-sample variates and tested against the separate within-sample error. In the tables, the within-sample and the between-sample sources of variation are presented separately together with their separate error mean squares.

TABLE 1

MEAN MOTILITY INDICES OF RAM SPERMATOOZOA COOLED AT VARYING RATES IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF POTASSIUM

Potassium conc. (mM)	Time of cooling											
	15 min				1 hr				3 hr			
	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total
0	23	20	16	59	29	24	23	76	36	32	25	93
4	22	21	15	58	27	25	20	72	36	30	27	93
16	22	18	17	57	29	25	23	77	33	30	28	91
64	20	16	10	46	26	20	18	64	32	26	22	80
Total	87	75	58	220	111	94	84	289	137	118	103	358

Summary of the analysis of variance

Source of variation	Degrees of freedom	Variance ratios
<i>Between chilled samples</i>		
A Effect of potassium	3	32.95**
B Effect of chilling rate	2	344.90**
C Ejaculate differences	3	59.00**
First order interactions		
B × C	6	52.97**
Other interactions	15	1.24
Between-sample error	18	4.1
<i>Within chilled samples</i>		
D Effect of sampling time	2	135.49**
First-order interactions		
D × C	6	9.17
Other interactions	10	1.38
Within-sample error	78	4.7

Each sample was evaluated at 0, 18 and 42 hr after reaching 5° C and values are the means for four ejaculates.

** $P < 0.01$.

RESULTS

The results of the first experiment, together with the summary of the analysis of variance, are shown in Table 1. Four ejaculates of ram spermatozoa were cooled to 5° C at different rates in a potassium-free diluent and in diluents containing 4, 16 and 64 mM potassium chloride. Rates of cooling were so arranged that

samples cooled from 30 to 5° C in 3 hr could be compared with those cooled in 1 hr or in 15 min. Samples that were cooled to 5° C over 3 hr in a potassium-free diluent survived best. Survival began to decline with a potassium concentration of 16 mM and motility was very depressed when the diluent in which the

TABLE 2
RESPONSE OF CHILLED RAM SPERMATOZOA TO THE ADDITION OF
POTASSIUM BEFORE AND AFTER CHILLING

Potassium conc. (mM)		Ratio semen : diluent during chilling							
Pre-chilling	Post-chilling	1:20				1:10			
		0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total
0	0	25	19	18	62	28	23	24	75
16	8	23	20	19	62	27	23	24	74
64	32	22	15	12	49	26	20	18	64
0	0	25	21	19	65	29	24	25	78
0	8	25	21	21	67	26	23	23	72
0	32	25	19	19	63	26	22	24	72
Total		145	115	108	368	162	135	138	435

Summary of the analysis of variance

Source of variation	Degrees of freedom	Variance ratio
<i>Between chilled samples</i>		
A Effect of potassium	2	19.05**
B Time of potassium addition	1	16.68**
C Effect of semen dilution	1	96.17**
D Ejaculate differences	3	87.44**
First-order interactions		
A × B	2	6.74**
C × D	3	5.60**
Other interactions	12	0.63
Between-sample error	23	5.7
<i>Within chilled samples</i>		
E Effect of sampling time	2	87.76**
First-order interactions		
E × C	2	3.74*
E × D	6	10.22**
Other interactions	6	2.20
Within-sample error	80	4.5

Values are the mean motility indices for four ejaculates evaluated at 0, 18 and 42 hr after chilling.

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

spermatozoa were cooled contained 64 mM potassium. Rapid cooling was detrimental and to ensure good revival in all subsequent experiments, semen samples were chilled over a 3-hr period. Following cooling, however, the motility of the surviving cells declined at a similar rate during the next 42 hr irrespective of the treatment during cooling. This indicated that potassium

probably had a detrimental effect only during chilling. Therefore the next experiment was designed to determine whether potassium had an effect during the 42 hr at 5° C.

Aliquots of ram semen were mixed with four and nine volumes of a potassium-free diluent and with similar quantities of diluents containing 16 and 64 mM potassium. After cooling to 5° C, the samples were mixed with equal volumes of

TABLE 3

EFFECT OF VARYING CONCENTRATIONS OF SODIUM ON THE DETRIMENTAL EFFECTS OF POTASSIUM TO RAM SPERMATOOA CHILLED TO 5° C

Final sodium conc. (mM)	Potassium concentration (mM)											
	0				12				36			
	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 h	Total	0 hr	18 hr	42 hr	Total
40	17	16	15	48	14	13	16	43	10	9	10	29
60	15	15	16	46	15	13	14	42	12	11	12	35
100	15	16	17	48	16	15	15	46	14	10	12	36
Total	47	47	48	142	45	41	45	131	36	30	34	100

Summary of the analysis of variance

Source of variation	Degrees of freedom	Variance ratio
<i>Between chilled samples</i>		
A Effect of potassium	2	60.93**
B Effect of sodium	2	1.32
C Ejaculate differences	2	128.30**
First-order interactions		
A × B		
Linear × linear	1	6.21*
Other	3	1.05
Other first-order interactions	8	1.97
Between-sample error	8	2.8
<i>Within chilled samples</i>		
D Effect of sampling time	2	5.26**
First-order interactions		
D × C	4	21.67**
Other interactions	8	0.61
Within-sample error	40	3.5

Samples were examined at 0, 18 and 42 hr after reaching 5° C and mean motility indices for three ejaculates are given.

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

precooled potassium-free diluent to give a final potassium concentration of 8 or 32 mM. The motility of these samples was then compared with others similarly diluted with the potassium-free diluent, chilled to 5° C and then mixed with equal volumes of precooled potassium-containing diluents to give final concentrations of 8 and 32 mM potassium. Thus, samples cooled in the presence of potassium could be compared with samples in which potassium was added after

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reaching 5° C. The results of four ejaculates, together with the summary of the analysis of variance, are shown in Table 2. Even at the high concentrations of semen used, the effects of dilution were evident. The more concentrated samples survived chilling better and showed a slower decline in motility during the 42 hr storage at 5° C. Motility was depressed in samples where large amounts of

TABLE 4

EFFECTS OF VARYING CONCENTRATIONS OF PHOSPHATE BUFFER (pH 7.0) ON THE DEPRESSION CAUSED BY POTASSIUM DURING CHILLING RAM SPERMATOZOA

Phosphate conc. (mM)	Potassium concentration (mM)											
	0				16				64			
	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total
10	25	25	22	72	25	22	22	69	22	18	21	61
20	27	23	24	74	25	19	21	65	21	15	16	52
40	28	25	23	76	27	21	20	68	17	13	9	39
Total	80	73	69	222	77	62	63	202	60	46	46	152

Summary of the analysis of variance

Source of variation	Degrees of freedom	Variance ratio
<i>Between chilled samples</i>		
A Effect of potassium	2	92.93**
B Effect of phosphate	2	8.00**
C Ejaculate differences	3	202.18**
First-order interactions		
A × B		
Linear × linear	1	33.06**
Other	3	2.80
A × C	6	14.54**
B × C	6	1.25
Between-sample error	12	6.3
<i>Within chilled samples</i>		
D Effect of sampling time	2	50.61**
First-order interactions		
D × A	4	1.53
D × B	4	4.39**
D × C	6	6.47**
Within-sample error	56	4.4

Mean motility indices for four ejaculates evaluated at 0, 18 and 42 hr after cooling are given in the table.

** $P < 0.01$.

potassium were present during chilling, but was not affected by its addition after reaching 5° C.

In subsequent experiments, other components of diluents were scanned for their possible interactions with potassium during cooling to 5° C. The effect of sodium concentration on the depression caused by potassium during chilling is shown in Table 3. Sodium chloride (18, 36 and 72 mM) was included in

potassium-free diluents and in diluents containing 12 and 36 mM potassium chloride. The diluents were buffered with mono- and di-sodium phosphates and isotonicity was maintained by varying the lactose content. The total sodium content of these diluents was approximately 40, 60 and 100 mM respectively. There was a significant interaction between potassium and sodium in the analysis of

TABLE 5

MEAN MOTILITY INDICES OF FOUR RAM EJACULATES AFTER COOLING TO 5° C IN THE PRESENCE OF VARYING CONCENTRATIONS OF POTASSIUM AND TRIS BUFFER (pH 7.0)

Potassium conc. (mM)	Concentration of tris buffer (mM)											
	0				10				40			
	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total
0	35	31	34	100	31	28	26	85	26	25	25	76
16	40	29	25	94	28	24	25	77	25	24	23	72
64	27	26	21	74	24	24	21	69	26	24	23	73
Total	102	86	80	268	83	76	72	231	77	73	71	221

Summary of the analysis of variance

Source of variation	Degrees of freedom	Variance ratio
<i>Between chilled samples</i>		
A Effect of potassium	2	17.14**
B Effect of tris	2	21.23**
C Ejaculate differences	3	6.95**
First-order interactions		
A × B	4	3.86*
A × C	6	2.23
B × C	6	8.84**
Between-sample error	12	11.1
<i>Within chilled samples</i>		
D Effect of sampling time	2	10.25**
First-order interactions		
D × C	6	4.03**
Other interactions	8	1.27
Within-sample error	56	20.7

All diluents contained 20 mM phosphate buffer (pH 7.0) and motility was estimated at 0, 18 and 42 hr after chilling.

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

variance. As the sodium content and the Na : K ratio of the diluent increased, the depression caused by high potassium decreased.

Phosphate at 10, 20 and 40 mM and potassium at 0, 16 and 64 mM were studied in all combinations and the results, together with the summary of the analysis of variance, are shown in Table 4. Increase in phosphate concentration when potassium was absent improved motility slightly. There was, however, an interaction between potassium and phosphate and the detrimental effect of potassium during cooling was greatest in the presence of 40 mM phosphate. In

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addition, during the period of storage, motility was not maintained as well when both high potassium and high phosphate were included in the diluent.

In further experiments there was no significant effect of 4, 16 or 64 mM potassium during chilling when phosphate buffer in the diluent was replaced by tris buffer. On the other hand, the effect of potassium occurred when veronal

TABLE 6
EFFECTS OF POTASSIUM AND MAGNESIUM ON THE RESPONSE OF WASHED AND UNWASHED RAM SPERMATOOZOA TO CHILLING

Treatment	Potassium conc. (mM)	Magnesium concentration (mM)							
		0				11			
		0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total
Unwashed	0	17	17	16	50	18	16	18	52
	16	16	14	14	44	17	18	17	52
Washed	0	12	9	6	27	14	12	7	33
	16	11	9	11	31	13	12	12	37

Summary of analysis of variance

Source of variation	Degrees of freedom	Variance ratio
<i>Between chilled samples</i>		
A Effect of potassium	1	0.23
B Effect of magnesium	1	25.50**
C Washing effect	1	267.42**
D Ejaculate differences	3	113.55**
First-order interactions		
A × C	1	15.75**
C × D	3	22.89**
Other interactions	8	0.59
Between-sample error	13	2.9
<i>Within chilled samples</i>		
E Effect of sampling time	2	11.79**
First- and second-order interactions		
E × A	2	4.56*
E × C	2	5.06*
Other first-order interactions	8	2.16
E × A × C	2	7.53**
Other second-order interactions	22	0.45
Within-sample error	26	3.5

Mean motility indices for four ejaculates examined at 0, 18 and 42 hr after reaching 5° C are given.

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

buffer was used to replace phosphate. An experiment was designed to see if tris would change the effect of potassium when phosphate was present in the diluent. Tris (pH 7.0) was added to a potassium-free diluent containing 20 mM phosphate and the effect of these diluents during chilling was compared with those of similar diluents containing 16 or 64 mM potassium. The results for four ejaculates, together with the summary of the analysis of variance, are shown in

Table 5. Both tris and potassium were found to depress motility of chilled samples, but the effect of the tris varied from ejaculate to ejaculate. At high potassium levels the effects of tris and potassium were less than would be expected from the sum of their separate effects and high potassium had a similar effect in the absence of tris as in its presence.

The effects of washing on the response of ram spermatozoa to chilling in the presence of low levels of potassium and magnesium are shown in Table 6.

TABLE 7

MOTILITY OF WASHED RAM SPERMATOZOA AFTER THE ADDITION OF 10% SEMINAL PLASMA OR 10% DIALYSED SEMINAL PLASMA TO POTASSIUM-FREE AND POTASSIUM-CONTAINING DILUENTS

Potassium conc. (mM)	Additives after washing											
	Nil (control)				Seminal plasma				Dialysed seminal plasma			
	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total	0 hr	18 hr	42 hr	Total
0	23	21	24	68	29	24	27	80	26	26	27	79
64	16	16	18	50	24	17	22	63	21	18	22	61

Summary of the analysis of variance

Source of variation	Degrees of freedom	Variance ratio
<i>Between chilled samples</i>		
A Effect of potassium	1	208.15**
B Effect of additives		
(i) Control versus plasma	1	43.31**
(ii) Effect of dialysis	1	0.66
C Ejaculate differences	3	14.60**
First-order interactions		
A × C	3	11.52**
Other interactions	8	2.77
Between-sample error	6	4.8
<i>Within chilled samples</i>		
D Effect of sampling time	2	16.65**
First-order interactions	12	1.74
Within-sample error	34	5.3

Mean values for four ejaculates examined at 0, 18 and 42 hr after chilling are given.

** $P < 0.01$.

Washed spermatozoa were more severely depressed by chilling than were unwashed cells. Magnesium (11 mM) was beneficial both to washed and unwashed spermatozoa. Potassium, on the other hand, had a differential effect and, although it slightly depressed the motility of unwashed spermatozoa, it stimulated the motility of washed cells. The beneficial effect of potassium to washed cells was due to the maintenance of motility during the period of storage rather than to initial stimulation. The motility of washed cells immediately after chilling in the presence of potassium was lower than that of washed cells chilled

in a potassium-free diluent. The reverse was true, however, after 42 hr, and the motility of cells was much lower in potassium-free than in potassium-containing diluents.

The effect of chilling on washed ram spermatozoa in the presence of 64 mM potassium is shown in Table 7. Seminal plasma (10% v/v) and dialysed seminal plasma (10% v/v) were added to the diluents before chilling and both were found to have beneficial effects on the chilled cells. The presence of 64 mM potassium in the diluent during chilling severely depressed motility. The high potassium level did not have a beneficial effect during the period at 5° C and the decline in motility of spermatozoa in potassium-containing diluents was similar to that in potassium-free diluents.

DISCUSSION

The results clearly show that potassium ions are detrimental to ram spermatozoa during chilling from 30 to 5° C. In no experiment was there interaction between potassium concentration and the time at which unwashed spermatozoa were examined after reaching 5° C. Thus it would seem that the adverse effect of potassium is confined to the chilling period and that after the spermatozoa reach 5° C, potassium no longer exerts a detrimental effect. Thus potassium had no effect on motility when added after the spermatozoa reached 5° C. In fact, with washed spermatozoa the presence of 16 mM potassium during chilling and storage helped to maintain motility during storage even though its presence during chilling decreased motility.

High levels of potassium have been reported to be slightly toxic to mammalian spermatozoa (White, 1953c; Wales & White, 1958a, b). However, Salisbury & Lodge (1962) maintain that the inhibition caused by high levels of potassium is due to changes in the Na : K ratio rather than in potassium content. In the present studies, the effect of high potassium (36 mM) during chilling was modified by the presence of sodium and was greatest when the Na : K ratio was low (1 : 1), but was still evident with Na : K ratios as high as 3 : 1. It would not seem, therefore, that Na : K ratio is the only or most important factor modifying the effects of potassium during chilling. For example, the presence of phosphate increased the detrimental effects of 64 mM potassium even when the Na : K ratio remained unchanged.

Tris buffer has been shown to penetrate human red-cell membranes (Omachi, Macey & Waldeck, 1961). Amines also competitively interfere with the uptake of potassium by bacteria (Macleod & Onofrey, 1954), while in other organisms it is implied that tris might interfere with organic metabolites. In the experiments reported here, potassium in the presence of 20 mM tris buffer did not significantly affect ram spermatozoa during chilling. When 20 mM phosphate was added, however, both tris and potassium were harmful during chilling, but their effects were not additive. From these experiments with tris it might be argued that high intracellular potassium levels during chilling are responsible for the adverse effects caused by the inclusion of potassium in the diluent. As in bacteria, tris possibly decreases potassium uptake by spermatozoa and so decreases the adverse effect of this cation during chilling. The situation is further

complicated by the addition of phosphate which itself increases the effects of potassium. Even under these circumstances tris and potassium in combination caused changes less than one might expect from the sum of their separate effects.

Cell membrane changes probably account for the irreversible damage caused by sudden cooling of spermatozoa to 0° C known as 'cold shock'. The phenomenon is accompanied by loss of ions from the cell (Blackshaw & Salisbury, 1957) and there is disruption of the cell surface (Walton, 1957). It seems unlikely, however, that the effect of potassium during chilling at moderate rates is associated with permeability changes, since increasing the rate of chilling (which would be expected to increase cell membrane changes) did not change the reaction of the cell to the presence of potassium during chilling.

Since dialysed seminal plasma was as effective as non-dialysed, the beneficial effect of seminal plasma on washed ram spermatozoa during storage at 5° C was due to high molecular weight substances such as proteins. Miscellaneous high molecular weight substances protect spermatozoa against the detrimental effects of excessive dilution and washing (Emmens & Swyer, 1948; Blackshaw, 1953b; Wales & White, 1961, 1963) and casein helps to protect ram and bull spermatozoa against cold shock (Choong & Wales, 1962). These substances probably act by the physical action of preventing loss of intracellular materials such as potassium and magnesium. The protection afforded by seminal proteins during chilling may occur by a similar mechanism. The finding that low levels of potassium maintained the motility of washed and chilled ram spermatozoa during storage at 5° C would suggest that leaching of this cation during washing was one of the important factors in producing the depression during storage caused by washing.

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