THE USE OF VARIOUS DILUENTS FOR DEEP-FREEZING BULL SPERMATOZOA

By C. H. Choong*† and R. G. Wales*

[*Manuscript received May* 6, 1963]

Summary

Non-dialysable milk solids added to synthetic diluents improved the revival of deep-frozen bull spermatozoa. The use of casein led to a similar revival.

In the dialysable portion of milk, lactose was an important constituent protecting spermatozoa against the detrimental effect of freezing, but in synthetic diluents it was inferior to fructose.

The addition of low levels of potassium, magnesium, and calcium chlorides or sodium citrate to the diluent did not significantly improve the revival of deep-frozen semen. Levels of potassium similar to those in milk were detrimental.

The addition of fructose after chilling improved revival even when 144 mM fructose was already present in the diluent.

I. Introduction

In the past, numbers of diluents have been used in the preservation of bull spermatozoa at −79°C. Some of the most successful of these have been various milk preparations including whole milk, skim and canned milk, and reconstituted powdered milk. Although information is now available on the use of these preparations as semen diluents (O'Dell and Almquist 1954, 1957; O'Dell and Hurst 1956; Jakobsen 1956; Jones, Perkins, and Seath 1956; Blackshaw 1960), little is known about the individual compounds or combinations of compounds in milk that make it so useful.

Experiments with such a complex biological fluid as milk suffer from the disadvantage that its composition cannot be submitted to adequate experimental control. A diluent with all the beneficial qualities of milk but of known composition would be of value in investigating the effects of deep-freezing on spermatozoa. In this paper, diluents with a composition broadly based on that of milk have been prepared and used to investigate these effects.

II. Materials and Methods

(*a*) *Techniques*

An artificial vagina was used for the collection of bull semen (Walton 1933) and only apparently normal ejaculates with high initial motility were used. Aliquots of each ejaculate were diluted at 30°C with nine volumes of each diluent used in the experiment and the semen–diluent mixtures were chilled slowly to 5°C over 2 hr. An equal volume of the corresponding precooled glycerol–diluent mixture

* Department of Veterinary Physiology, University of Sydney.

† Present address: Central Animal Husbandry Station, Kluang, Johore, Malaysia.

[containing 15% (v/v) glycerol and 85% (v/v) diluent used for initial dilution] was added to the chilled suspensions to give a final glycerol concentration of 7·5%. In all but the final experiment, the glycerol–diluent mixture added to the chilled suspensions contained no additional sugar.

Unless otherwise stated in the results, the spermatozoa were allowed to equilibrate for 18 hr with glycerol before being sealed in ampoules and frozen. In the experiments where different periods of equilibration were studied, it was necessary to freeze the treatment groups at different times. In order to minimize confounding of treatment differences with differences between times of freezing, duplicate samples of each treatment group were sealed in separate ampoules. One set of ampoules was placed in one freezing unit and the duplicate set placed in another. Each freezing unit was then cooled to —79°C separately. In all experiments, the cooling rate recommended by Polge and Lovelock (1952) was used and samples were stored at —79°C for at least 24 hr before thawing.

After thawing, the spermatozoa were incubated at 30°C for 20 min before estimating motility and percentage of motile spermatozoa. Readings were made on a warm stage under the low-power microscope at ½-hr intervals for 2 hr. Motility was scored by the system of Emmens (1947) and the percentage of motile spermatozoa was estimated to the nearest 5%. The indices of motility and percentage motile were calculated by summing observations over the 2-hr period for each sample. Thus they give an index of survival over the observation period rather than initial revival after thawing.

In one experiment, bull spermatozoa were chilled for 24 hr in various diluents. During this short period of storage, no antibacterials were added to the diluents.

(*b*) *Diluents*

All diluents were isotonic, of pH 7·0, and were made from A.R. chemicals and distilled water. For the first experiment, the diluents had the following composition:

(1) Sodium chloride diluent: 0·154M NaCl.
(2) Fructose–sodium chloride diluent: 0·077M NaCl, 0·154M fructose.
(3) Calcium-free Krebs–Ringer ("Ringer") phosphate–fructose diluent (Umbreit, Burris, and Stauffer 1949): 0·01M Na_2HPO_4, 0·005M KCl, 0·001M KH_2PO_4, 0·001M $MgCl_2$, 0·132M NaCl, 0·0056M fructose.

The composition of diluents used in other experiments are given in the tables showing the results of these experiments. All were buffered with 20 mM NaH_2PO_4–Na_2HPO_4 and isotonicity was maintained by varying the sodium chloride content. These diluents are broadly based on data for the composition of cow's milk (Altman 1961).

(*c*) *Preparation of Dialysed Milk Extracts*

A 10-ml sample of reconstituted skim milk powder was dialysed against 100 ml distilled water for 24 hr, the dialysate replaced with 100 ml distilled water, and dialysis continued for another 24 hr. "Cellophane" was used as semi-permeable membrane. The dialysable fraction was evaporated to dryness *in vacuo* at 40°C and made up to the original volume with distilled water. The non-dialysable portion was lyophilized and made up to the original volume with the appropriate isotonic diluent.

(*d*) *Statistical Analysis*

For the analyses of variance of the results, all main effects and their first-order interactions were isolated and tested for significance. In the summary form for the tables, however, only the first-order interactions which were significant have been presented as separate variance ratios. All non-significant interactions have been combined and their pooled variance used to calculate the variance ratio.

III. Results

Samples of reconstituted skim milk (10%w/v) were dialysed and the dialysed milk extracts added to diluents used in the deep-freezing experiments. Non-dialysable milk solids (3%w/v) were dissolved in isotonic sodium chloride, isotonic sodium chloride–fructose, and in Ringer diluent and compared with casein (3%w/v) in the same diluents. As preliminary tests showed that the reconstituted dialysable portion of milk depressed motility slightly, it was diluted with an equal volume of Ringer solution for the test. The results for four ejaculates and a summary of the analyses of variance are given in Table 1.

The spermatozoa survived freezing best in reconstituted skim milk. Although solutions containing 3% casein and non-dialysable solids gave similar results, the effect of these additives varied with the diluent in which they were dissolved. A balanced salt solution was slightly better than sodium chloride but the addition of fructose greatly enhanced revival. Dialysable milk solids were not effective in sustaining viability during deep freezing.

The effects of albumin, globulin, and casein (all 3%w/v) on the survival of chilled spermatozoa were compared by adding these proteins to diluents buffered with mono- and disodium phosphates and containing 22 mM fructose as substrate for the spermatozoa. Other constituents were varied to test the effects of 3%(w/v) lactose and a sodium/potassium ratio similar to milk. Casein was the only protein to increase viability of four bull ejaculates chilled to 5°C for 24 hr and globulin was slightly toxic. In view of the general poor viability of the samples containing albumin and globulin after 24 hr at 5°C, only the casein-containing solutions were used as diluents for deep-freezing bull spermatozoa. Even then, revival was unsatisfactory for the four ejaculates used and only in the potassium-free lactose-containing diluent was there a measurable revival. Revival in lactose-free diluents and in those containing potassium was negligible.

The effects of low concentrations of potassium, magnesium, and calcium, alone and in combination, were tested in diluents containing either lactose or an equivalent amount of fructose. These diluents were buffered with mono- and disodium phosphates and contained 3%(w/v) casein. Aliquots of three bull ejaculates were diluted in each of the diluents and deep frozen after 2 or 18 hr equilibration. The results and the summary of the analyses of variance are shown in Table 2. Fructose was better than lactose, and calcium improved the motility of surviving cells. Potassium and magnesium had no effect. Although 18 hr equilibration was overall better than 2 hr the effect varied from ejaculate to ejaculate and the effect of equilibration was not significant when tested against the significant ejaculate × equilibration interaction.

The diluents of composition shown in Table 3 were used to compare the effects of casein and non-dialysable milk solids (all 3%w/v). Within the diluents, the effect

TABLE 1

COMPARISON OF THE EFFECT OF CASEIN AND NON-DIALYSABLE MILK SOLIDS ON THE REVIVAL OF BULL SPERMATOZOA FROZEN TO −79°C

Revival of four ejaculates in casein and non-dialysable milk solids is compared with revival in reconstituted skim milk and dialysable milk solids

Diluent No.	Additive	Diluent Composition	Mean Motility Index	Mean Percentage Motile Index
A	Casein	NaCl	6	15
B	Casein	NaCl–fructose	19	40
C	Casein	Ringer	9	18
D	Non-dialysable milk solids	NaCl	5	11
E	Non-dialysable milk solids	NaCl–fructose	12	29
F	Non-dialysable milk solids	Ringer	10	21
G	Nil	Reconstituted skim milk	22	54
H	Dialysable milk solids	50% Ringer	3	8

Summary of the Analyses of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		Motility	Percentage Motile
Ejaculate differences	3	2·4	4·3*
Effect of diluents:			
Between diluents A–F			
Difference between additives	1	2·4	2·4
Effect of diluents			
Ringer *v.* NaCl	1	4·6*	4·3*
NaCl+fructose *v.* rest	1	24·4**	31·4**
Interaction: additives × diluents	2	2·5	1·6
Milk *v.* diluents A–F	1	32·2**	65·1**
Dialysable milk solids *v.* diluents A–G	1	19·3**	26·0**
Residual	21	14	52

* $P<0·05$. ** $P<0·01$.

of adding 7 mM citrate, the amount present in milk, was tested and lactose was again compared with an equivalent amount of fructose. Fructose was also included in the

TABLE 2

REVIVAL OF BULL SPERMATOZOA AFTER FREEZING TO −79°C IN VARIOUS DILUENTS

All diluents were buffered with 20 mM mono- and disodium phosphates and contained 3%(w/v) casein. Values are means for three ejaculates

Sugar (144 mM)	K (10 mM)	Mg (2 mM)	Ca (0·5 mM)	Mean Motility Index			Mean Percentage Motile Index		
				Equilibration		Total	Equilibration		Total
				2 Hr	18 Hr		2 Hr	18 Hr	
Fructose	—	—	—	16	19	35	34	48	82
	—	—	+	19	19	38	38	45	83
	—	+	—	19	20	39	45	52	97
	—	+	+	13	21	34	25	63	88
	+	—	—	17	15	32	38	40	78
	+	—	+	19	21	40	43	49	92
	+	+	—	17	18	35	33	45	78
	+	+	+	18	20	38	35	48	83
Total				138	153	291	291	390	681
Lactose	—	—	—	12	15	27	24	35	59
	—	—	+	14	19	33	32	52	84
	—	+	—	12	11	23	26	30	56
	—	+	+	12	13	25	26	32	58
	+	—	—	9	12	21	21	28	49
	+	—	+	11	18	29	28	44	72
	+	+	—	10	12	22	19	28	47
	+	+	+	15	16	31	34	38	72
Total				95	116	211	210	287	497

Summary of the Analyses of Variance

Source of Variation	Degrees of Freedom	Variance Ratios		Source of Variation	Degrees of Freedom	Variance Ratios	
		Motility	Percentage Motile			Motility	Percentage Motile
Fructose *v.* lactose	1	46·3**	15·7**	First-order interactions:			
Effect of potassium	1	0·5	0·6	Equilibration × ejaculate	2	5·3*	5·2*
Effect of magnesium	1	0·2	0·1	Other first-order interactions	18	0·7	0·3
Effect of calcium	1	9·2**	2·5	Higher-order interactions	68	1·0	475·7†
Effect of equilibration	1	8·9**	13·3**				
Ejaculate differences	2	161·3**	57·0**	Duplicate variance	96	26·3	145·2

* $P<0·05$. ** $P<0·01$.

† Higher-order interactions variance was used as error as it was significantly larger than the duplicate variance.

corresponding glycerol–diluent mixtures added at 5°C to give concentrations of 0·625 and 1·25%(w/v) and these samples compared with others diluted with glycerol–diluent mixtures which did not contain fructose. The semen suspensions were frozen after 2 or 18 hr equilibration and the results for four ejaculates and a summary of the analyses of variance are given in Table 4.

The substitution of fructose for lactose in the original diluents was beneficial and non-dialysable milk solids were better than casein. However, there was an interaction between these factors and although the motility of surviving cells in the casein–lactose diluent was low, the substitution of fructose for lactose was so beneficial that motility in the fructose–casein diluent almost equalled that in lactose– or fructose–milk solid diluent. In addition, the beneficial effect of substitution of fructose for lactose was slightly less after 18 hr equilibration.

TABLE 3

COMPOSITION OF DILUENTS USED TO COMPARE THE EFFECTS OF CASEIN AND NON-DIALYSABLE MILK SOLIDS ON THE REVIVAL OF FROZEN BULL SPERMATOZOA

Diluent No.	Phosphate Buffer (mM)	Sodium Citrate (mM)	Fructose (mM)	Lactose (mM)	Sodium Chloride (mM)
1	20	7	0	144	45
2	20	7	144	0	45
3	20	0	0	144	72
4	20	0	144	0	72

The addition of fructose to the glycerol-containing diluent improved revival both when lactose and fructose were present in the original diluent. Citrate slightly improved revival but its effect varied from ejaculate to ejaculate and the overall effect was not significant when tested against the citrate × ejaculate interaction.

IV. Discussion

The non-dialysable fraction of milk is an important part of milk diluents used for semen preservation. Its effect, however, varies with the solution in which it is dissolved and there is probably a significant contribution of both non-dialysable and dialysable portions to make milk such a good diluent. The use of 3% casein leads to similar revival to that in non-dialysable milk solids but other proteins such as albumin and globulin cannot act as substitutes. In well-prepared non-dialysable fractions, the presence of residual lipids or combinations of proteins probably explains any superiority to casein.

TABLE 4

COMPARISON OF CASEIN AND NON-DIALYSABLE MILK SOLIDS AS ADDITIVES FOR DEEP-FREEZING BULL SPERMATOZOA

Composition of diluents used given in Table 3. Mean values for four ejaculates given

Additive	Diluent No.	Mean Motility Index: Fructose Added after Chilling (%) 0	0·625	1·25	Total	Mean Percentage Motile Index: Fructose Added after Chilling (%) 0	0·625	1·25	Total
2 Hr Equilibration									
Non-dialysable milk (3%w/v)	1	28	29	30	87	73	79	88	240
	2	32	36	36	104	88	110	128	326
	3	28	30	27	85	71	81	74	226
	4	32	34	35	101	90	108	104	302
Total		120	129	128	377	322	378	394	1094
Casein (3%w/v)	1	22	24	21	67	55	57	53	165
	2	31	32	34	97	79	83	101	184
	3	19	20	20	59	48	51	53	152
	4	28	31	30	89	71	83	76	230
Total		100	107	105	312	253	274	283	731
18 Hr Equilibration									
Non-dialysable milk (3%w/v)	1	30	31	30	91	86	84	83	253
	2	31	34	33	98	88	109	113	310
	3	27	29	32	88	76	76	93	245
	4	31	31	33	95	88	86	103	277
Total		119	125	128	372	338	355	392	1085
Casein (3%w/v)	1	21	23	23	67	55	58	58	171
	2	29	33	31	93	77	91	86	254
	3	21	22	20	63	54	55	51	160
	4	24	28	28	80	62	74	71	207
Total		95	106	102	303	248	278	266	792
Grand total		434	467	463	1364	1161	1285	1335	3702

Summary of the Analyses of Variance

Source of Variation	Degrees of Freedom	Variance Ratios: Motility	Percentage Motile	Source of Variation	Degrees of Freedom	Variance Ratios: Motility	Percentage Motile
Lactose *v.* fructose in diluent (*A*)	1	225·4**	183·1**	Interactions: First-order			
Casein *v.* non-dialysable milk solids (*B*)	1	187·3**	193·3**	*A* × *B*	1	28·2**	2·7
				A × *C*	1	1·3	6·6*
Effect of citrate (*C*)	1	19·7**	23·2**	*A* × *D*	2	1·7	4·2*
Effect of fructose (*D*)				*A* × *E*	1	12·8**	10·4**
Linear	1	15·5**	22·5**	*A* × *F*	3	1·0	8·1**
Quadratic	1	5·0*	2·5	*B* × *F*	3	16·0**	34·8**
Effect of equilibration (*E*)	1	1·8	1·0	*C* × *F*	3	4·0**	7·7**
				D × *F*	6	1·6	3·4**
Ejaculate differences (*F*)	3	284·8**	185·6**	*E* × *F*	3	25·1**	22·5**
				Other	9	0·6	0·8
				Higher-order	150	1·0	1·2
				Duplicate variance	192	16·0	274·7

* $P<0·05$. ** $P<0·01$.

Both casein and non-dialysable milk solids protect spermatozoa against cold shock (Choong and Wales 1962) and it is tempting to suggest that milk proteins may have a similar role during rapid cooling at sub-zero temperatures. Proteins and other large molecules have been found to protect spermatozoa against the effects of dilution (Blackshaw 1953; Wales and White 1961, 1963) and it has been suggested that their beneficial effect is due to maintenance of the integrity of the cell membrane. During preservation at sub-zero temperatures, the cell membrane of spermatozoa is probably affected by freezing and thawing and milk proteins may well protect against undue increases in permeability.

In the milk dialysate, lactose is important as a constituent of semen diluents. In synthetic diluents, however, fructose was superior to lactose. Moreover, it is surprising to find that the addition of even more fructose after chilling improved the revival of spermatozoa in a diluent already containing 144 mM fructose. This additional fructose may help to offset any adverse effects of the glycerol added at the same time, or the higher concentration of the hexose *per se* may be beneficial.

As the use of diluents with sodium/potassium ratios and concentrations similar to that of milk decreased revival, the potassium of milk is probably bound in such a way that it is not available to the spermatozoa as an ionized electrolyte. In short-term incubations of spermatozoa, potassium is beneficial and very high levels have to be reached before motility is depressed (White 1953*a*, 1953*b*; Wales and White 1958*a*, 1958*b*). During ice formation potassium levels in the unfrozen fraction of the diluent possibly rise high enough to become detrimental.

In the experiments reported here, there was little evidence of any interaction between various constituents of diluents and, so far, it would seem that each constituent has an independent effect which is added to that of others.

V. Acknowledgments

The authors are indebted to Professor C. W. Emmens for his interest and advice. One of the authors (C. H. C.) is grateful for a scholarship awarded by the Colombo Plan Organization.

VI. References

Altman, L. P. (1961).—"Blood and Other Body Fluids." (Federation of American Societies for Experimental Biology: Washington.)

Blackshaw, A. W. (1953).—*J. Gen. Physiol.* **36**: 449.

Blackshaw, A. W. (1960).—*Aust. Vet. J.* **30**: 249.

Choong, C. H., and Wales, R. G. (1962).—*Aust. J. Biol. Sci.* **15**: 543.

Emmens, C. W. (1947).—*J. Physiol.* **106**: 471.

Jakobsen, F. (1956).—Proc. 3rd Int. Congr. Anim. Reprod., Cambridge. p. 30.

Jones, W. M., Perkins, J. R., and Seath, D. M. (1956).—*J. Dairy Sci.* **11**: 1574.

O'Dell, W. T., and Almquist, J. O. (1954).—*J. Dairy Sci.* **37**: 652.

O'Dell, W. T., and Almquist, J. O. (1957).—*J. Dairy Sci.* **40**: 1534.

O'Dell, G. D. and Hurst, V. (1956).—*J. Dairy Sci.* **39**: 1156.

Polge, C., and Lovelock, J. E. (1952).—*Vet. Rec.* **64**: 396.

UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. (1949).—"Manometric Techniques." (Burgess Publ. Co: Minneapolis.)

WALES, R. G., and WHITE, I. G. (1958*a*).—*J. Physiol.* **142**: 494.

WALES, R. G., and WHITE, I. G. (1958*b*).—*Aust. J. Biol. Sci.* **11**: 589.

WALES, R. G., and WHITE, I. G. (1961).—*Aust. J. Biol. Sci.* **14**: 637.

WALES, R. G., and WHITE, I. G. (1963).—*J. Reprod. Fertil.* **5**:67.

WALTON, A. (1933).—"The Technique of Artificial Insemination." (Imperial Bureau of Animal Breeding and Genetics: Edinburgh.)

WHITE, I. G. (1953*a*).—*Aust. J. Exp. Biol. Med. Sci.* **31**: 193.

WHITE, I. G. (1953*b*).—*Aust. J. Biol. Sci.* **6**: 716.