EFFECT OF COMPOSITION OF THE THAWING SOLUTION ON SURVIVAL OF RAM SPERMATOZOA FROZEN BY THE PELLET METHOD

By S. SALAMON* and M. R. BRANDON*

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Abstract

Six factorial experiments were conducted to examine the effects of thawing solutions consisting of non-electrolyte or of a combination of non-electrolyte and sodium citrate on the survival of ram spermatozoa pellet frozen with a raffinose–citrate diluent.

When only non-electrolyte was present in the thawing solution best performance with fructose was achieved at higher tonicity than with inositol, glucose, lactose, or raffinose. With thawing solutions consisting of two components cell survival improved with a decrease in sodium citrate and simultaneous increase in non-electrolyte concentration. Inositol and glucose required higher concentration than lactose and fructose.

The best and similar survival rates following 1 : 4 pre-freezing and 1 : 3 (pellets : thawing solution, v/v) thawing dilution were obtained with 388·5 mM fructose and with inositol or glucose both at 210 mM concentration and each combined with 40 mM sodium citrate.

I. INTRODUCTION

Several investigators (reviewed by Lightfoot and Salamon 1969*a*, 1969*b*) have reported that relatively poor revival of spermatozoa is obtained when pellet-frozen bull, ram, jackass, or stallion semen is thawed without using a thawing solution. Few studies are available on the effect of composition of thawing solutions, and for pellet-frozen ram semen appear to be limited to those of Salamon (1968) and Lightfoot and Salamon (1969*b*). The information obtained by the latter authors indicated that further studies were needed to find thawing solution(s) that would improve initial revival on thawing and survival of spermatozoa during incubation.

Consequently a series of experiments were designed and the results obtained are presented in the present paper.

II. MATERIALS AND METHODS

Semen was collected by artificial vagina from Merino rams, which were included as a factor in all experiments. Ejaculates of good initial motility were diluted (1 : 4) at 30°C with a diluent consisting of 166·5 mM raffinose, 68 mM sodium citrate, 15% (v/v) egg yolk, and 5% (v/v) glycerol. The diluted semen was cooled to 5°C over 1 hr and held at that temperature for a further 1 hr period before pelleting (0·13–0·15 ml) on dry ice (Nagase and Niwa 1963, 1964). Using a cooled Pasteur pipette, four drops of semen were placed in rapid succession into cavities on the surface

* Department of Animal Husbandry, University of Sydney, Sydney, N.S.W. 2006.

of a block of dry ice. The semen was kept on dry ice for 3–4 min, then the frozen pellets were stored in liquid nitrogen for at least 48 hr before thawing for examination. The pellets were thawed in test tubes containing the thawing solution and held in a water-bath at 37°C. The dilution rate at thawing in all experiments was 1 : 3 (pellets : thawing solution, v/v).

The percentage of motile spermatozoa was assessed under a coverslip on a warm stage (37°C) immediately after thawing and at 2-hr intervals during subsequent 6-hr incubation at 37°C.

Freezing point depression (Δ, deg C) of the thawing solutions was determined by a Fiske cryoscope.

Data were examined by analyses of variance, following angular transformation and, where appropriate, by regression analyses as described previously (Salamon and Lightfoot 1969).

III. Experimental and Results

(a) *Experiment 1*

The experiment was of $5 \times 4 \times 4$ factorial design and examined the following factors:

(1) Type of solution: inositol *v.* fructose *v.* glucose *v.* lactose *v.* raffinose.

(2) Concentration of solution: 166·5 *v.* 222·0 *v.* 277·5 *v.* 333·0 mM.

(3) Rams: three pooled ejaculates from each of four rams.

All three factors—type and concentration of solution and rams—had significant effects ($P < 0{\cdot}001$) on the percentage of motile spermatozoa assessed during incubation.

Table 1

Experiment 1: effects of type and concentration of thawing solution on the percentage of motile spermatozoa during post-thawing incubation

Concentration of Thawing Solution (mM)	Motile Spermatozoa (%) for following Thawing Solutions:					Means
	Inositol	Fructose	Glucose	Lactose	Raffinose	
166·5	17·2	14·7	12·8	21·2	15·0	16·1
222·0	20·2	14·0	18·4	22·4	21·0	19·4
277·5	28·9	23·5	31·2	24·4	21·1	25·7
333·0	31·8	29·2	31·2	18·0	16·3	24·9
Means	24·3	20·0	23·2	21·5	18·2	

There was an interaction ($P < 0{\cdot}001$) between type and concentration of solution (Table 1). Survival rates improved with increasing concentration of inositol, fructose, and glucose, indicating that higher levels could have given better results, whereas the optimum levels for both lactose and raffinose were within the range examined.

Further significant first-order interactions occurred between type of solution and incubation time, and concentration of solution and incubation time ($P < 0{\cdot}001$). The viability of spermatozoa in the first case was best maintained during the 6-hr incubation time with inositol and glucose and in the second case with concentrations of 277·5 and 333·0 mM.

(*b*) *Experiment 2*

In this experiment ($4 \times 7 \times 3$ factorial) a wider concentration range was examined and the following factors were included:

(1) Type of solution: inositol *v.* glucose *v.* fructose *v.* lactose.

(2) Concentration of solution: 222·0 *v.* 277·5 *v.* 333·0 *v.* 388·5 *v.* 444·0 *v.* 499·5 *v.* 555·0 mM.

(3) Rams: four pooled ejaculates from each of three rams.

The analysis of variance revealed that both type and concentration of solution had significant effects on the survival of spermatozoa ($P < 0{\cdot}001$). There was, however, an interaction between these factors (Table 2). Best results for fructose

TABLE 2

EXPERIMENT 2: INTERACTION BETWEEN TYPE AND CONCENTRATION OF THAWING SOLUTION ON THE MEAN PERCENTAGE MOTILE SPERMATOZOA DURING 6-HR POST-THAWING INCUBATION

Concentration of Thawing Solution (mM)	Motile Spermatozoa (%) for following Thawing Solutions:				Means
	Inositol	Glucose	Fructose	Lactose	
222·0	26·9	28·5	12·2	25·9	23·0
277·5	39·6	40·9	29·5	29·9	34·9
333·0	38·8	40·0	34·0	16·3	31·0
388·5	34·1	40·0	43·0	10·3	30·8
444·0	31·1	30·1	42·6	8·7	27·0
499·5	30·1	32·5	42·6	8·7	27·3
555·0	23·8	29·0	37·1	5·4	22·3
Means	31·4	34·3	33·8	14·0	

solution were obtained at relatively high concentration, whereas lower levels were required for inositol, glucose, and especially for lactose.

There was a second-order interaction ($P < 0{\cdot}001$) involving type and concentration of solution and incubation time.

Table 3 and Figure 1 present the regression of mean percentage of motile spermatozoa during the 6-hr post-thawing incubation on thawing solution freezing point depression. The regressions for fructose and lactose showed significant deviation and those for inositol and glucose non-significant deviation from linearity. Quadratic regressions of the four types of thawing solutions differed markedly as also did their position ($P < 0{\cdot}001$).

(*c*) *Experiment 3*

The experiment was of a $5 \times 3 \times 3 \times 3$ factorial design involving the following factors:

(1) Type of non-electrolyte: inositol *v.* glucose *v.* fructose *v.* lactose *v.* raffinose.

(2) Concentration of non-electrolyte: 34·4 *v.* 44·4 *v.* 54·4 mM.

(3) Concentration of sodium citrate: 65·0 *v.* 72·8 *v.* 80·6 mM.

(4) Rams: four pooled ejaculates from each of three rams.

TABLE 3

EXPERIMENT 2: REGRESSION OF THE MEAN PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION ON THE FREEZING POINT DEPRESSION OF THE THAWING SOLUTION (Δ)

Thawing Solution	Quadratic Regression*	Significance of Quadratic Deviation from Linearity
Inositol	$Y = 12{\cdot}0 + 74{\cdot}2\Delta - 55{\cdot}7\Delta^2$ (3·3) (0·2) (0·3)	$0{\cdot}05 < P < 0{\cdot}10$
Glucose	$Y = 14{\cdot}2 + 65{\cdot}4\Delta - 43{\cdot}9\Delta^2$ (3·4) (0·3) (0·4)	$0{\cdot}10 < P < 0{\cdot}20$
Fructose	$Y = -38{\cdot}0 + 178{\cdot}5\Delta - 99{\cdot}5\Delta^2$ (7·4) (0·2) (0·4)	$P < 0{\cdot}01$
Lactose	$Y = 51{\cdot}3 + 47{\cdot}4\Delta - 13{\cdot}2\Delta^2$ (7·4) (0·3) (0·5)	$P < 0{\cdot}05$

* Values in parenthesis are standard errors.

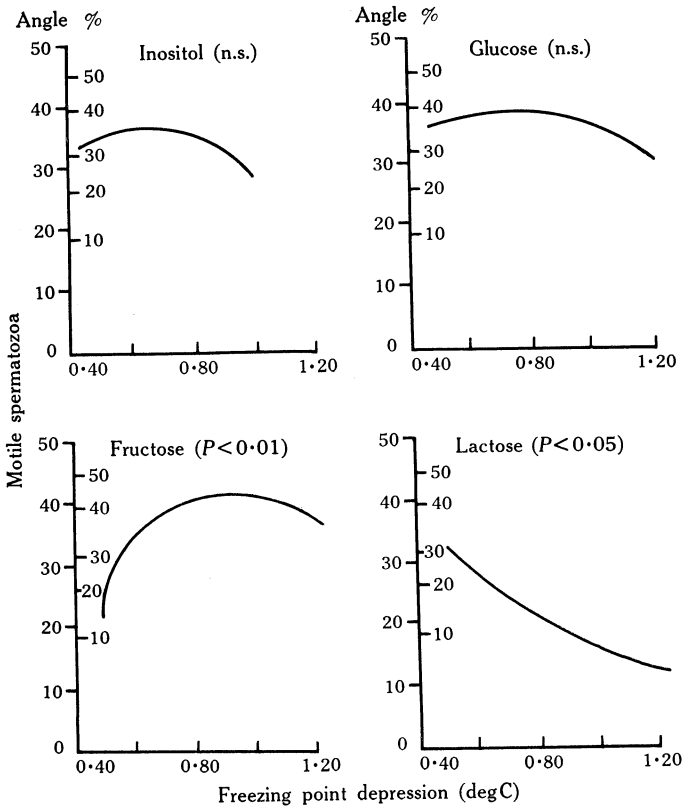

Fig. 1.—Quadratic regressions of mean percentage of motile spermatozoa assessed during post-thawing incubation on the freezing point depression of the thawing solutions.

The mean percentage of motile spermatozoa assessed during the 6-hr post-thawing incubation was significantly affected by the type and concentration of non-electrolyte ($P < 0{\cdot}001$) and by level of sodium citrate in the thawing solution ($P < 0{\cdot}01$).

Table 4 shows that there was little difference between the non-electrolyte components of the solution when assessed immediately after thawing, but during subsequent incubation spermatozoa thawed in glucose were more viable than when thawed in lactose and inositol, and especially in raffinose and fructose solutions ($P < 0{\cdot}001$). The mean percentages of motile spermatozoa for non-electrolyte levels of 34·4, 44·4, and 54·4 mM concentrations were 30·0, 31·2, and 33·4% respectively.

TABLE 4

EXPERIMENT 3: EFFECT OF COMPOSITION OF THE THAWING SOLUTION ON THE PERCENTAGE OF MOTILE SPERMATOZOA ASSESSED DURING POST-THAWING INCUBATION

Incubation Time (hr)	Motile Spermatozoa (%) for following Types of Non-electrolyte:					Motile Spermatozoa (%) for Sodium Citrate Levels (mM) of:			Means
	Glucose	Lactose	Inositol	Raffinose	Fructose	65·0	72·8	80·6	
0	40·8	41·1	41·5	39·4	40·8	36·8	41·7	43·8	40·7
2	38·5	39·4	33·5	30·6	32·6	33·8	37·9	33·0	34·9
4	35·3	33·6	29·7	25·0	26·1	32·2	31·3	26·3	29·9
6	30·8	24·9	23·6	18·4	14·5	25·2	22·4	17·5	21·6
Means	35·5	34·6	31·9	28·0	28·0	31·9	33·1	29·8	

Mean results for 80·6 mM sodium citrate were poorer than for 72·8 mM ($P < 0{\cdot}01$) and 65·0 mM levels ($P < 0{\cdot}05$), but there was an interaction between sodium citrate concentration and incubation period ($P < 0{\cdot}001$) (Table 4). There was no effect for ram spermatozoa.

None of the regressions of mean percentage motile spermatozoa during 6-hr post-thawing incubation on the thawing solution freezing point depression were significant, but their position varied markedly ($P < 0{\cdot}001$).

(*d*) *Experiment 4*

The experiment was of $4 \times 5 \times 2 \times 3$ factorial design and included the following factors:

(1) Type of non-electrolyte: inositol *v.* glucose *v.* fructose *v.* lactose.

(2) Concentration of non-electrolyte: 34·4 *v.* 54·4 *v.* 74·4 *v.* 94·4 *v.* 114·4 mM.

(3) Concentration of sodium citrate: 72·8 *v.* 80·6 mM.

(4) Rams: four pooled ejaculates from each of three rams.

All factors, except the level of sodium citrate, had significant effects ($P < 0{\cdot}001$) on the mean percentage of motile spermatozoa during incubation. There was a second-order interaction ($P < 0{\cdot}01$) involving type and concentration of non-electrolyte and

level of sodium citrate (Table 5). Increasing concentrations of glucose and lactose improved the results when combined with 72·8 mM sodium citrate but the high concentration of these sugars (114·4 mM) with 80·6 mM sodium citrate showed a depressing effect. The situation was reversed when increasing concentrations of inositol were combined with the two sodium citrate levels [(glucose and lactose *v.* inositol) × (sugar level, quadratic) × (72·8 *v.* 80·6 mM citrate level) $P < 0{\cdot}01$]. There was also an improvement in cell survival, although of smaller magnitude, when increasing concentrations of fructose were combined with 72·8 mM sodium citrate, but little or no effect was observed with the 80·6 mM sodium citrate level [(fructose *v.* rest) × (sugar level, linear) × (72·8 *v.* 80·6 mM citrate level) $P < 0{\cdot}05$].

TABLE 5

EXPERIMENT 4: EFFECTS OF COMPONENTS AND THEIR CONCENTRATION IN THE THAWING SOLUTION ON THE MEAN PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Type of Non-electrolyte	Concn. (mM)	Motile Spermatozoa (%) for Sodium Citrate Levels (mM) of: 72·8	80·6	Means
Inositol	34·4	24·1	20·9	
	54·4	27·2	20·9	
	74·4	33·2	25·8	27·6
	94·4	37·5	27·6	
	114·4	28·4	32·1	
Glucose	34·4	23·3	18·9	
	54·4	18·6	23·8	
	74·4	24·5	34·0	28·0
	94·4	34·1	36·5	
	114·4	37·5	31·3	
Fructose	34·4	16·9	22·5	
	54·4	18·3	22·3	
	74·4	25·5	22·1	22·7
	94·4	25·6	24·3	
	114·4	27·4	22·9	
Lactose	34·4	21·2	18·4	
	54·4	26·1	28·6	
	74·4	32·9	30·6	28·7
	94·4	34·1	33·2	
	114·4	35·3	27·7	
Means		27·4	26·1	

The effect of post-thawing incubation was influenced by most factors in the experiment, and the existence of a complex situation was reflected by the fact that besides first-order interactions ($P < 0{\cdot}001$) there were detected also second-order interactions ($P < 0{\cdot}05$, $P < 0{\cdot}01$), involving incubation time and one or two of the factors examined.

(e) *Experiment 5*

In this experiment ($4 \times 4 \times 3 \times 3$ factorial) inositol, glucose, fructose, and lactose were used, but each at a wider concentration range (70, 140, 210, 280 mM) and combined with 40, 60, and 80 mM levels of sodium citrate. Four pooled ejaculates from each of three rams were used.

TABLE 6

EXPERIMENT 5: RELATIONSHIP BETWEEN COMPONENTS AND THEIR CONCENTRATION IN THE THAWING SOLUTION ON THE MEAN PERCENTAGE OF MOTILE SPERMATOZOA DURING POST-THAWING INCUBATION

Type of Non-electrolyte	Concn. (mM)	Motile Spermatozoa (%) for Sodium Citrate Levels (mM) of: 40	60	80	Means
Inositol	70	13·1	19·0	17·6	21·1
	140	30·1	31·8	11·3	
	210	40·5	32·9	8·5	
	280	36·2	15·1	9·6	
Glucose	70	25·4	27·4	13·6	27·9
	140	33·7	35·3	21·3	
	210	40·5	39·0	18·6	
	280	40·5	39·0	9·0	
Fructose	70	11·0	12·9	7·0	4·6
	140	23·3	4·2	9·8	
	210	5·1	0·3	0·1	
	280	6·5	0·1	0·0	
Lactose	70	7·1	22·2	15·4	9·4
	140	34·6	28·8	5·8	
	210	23·4	7·3	1·0	
	280	4·2	0·2	0·1	
Means		21·8	16·2	7·3	

The results are summarized in Table 6. Type and concentration of non-electrolyte solution and level of sodium citrate interacted ($P < 0{\cdot}001$). With different levels of glucose and of sodium citrate a higher number of successful combinations were achieved than with inositol–citrate, lactose–citrate, and especially with fructose–citrate levels. Best results for glucose were observed when used at 210 and 280 mM concentrations and combined with either 40 or 60 mM sodium citrate levels. Similar concentrations of inositol preferred 40 mM level of sodium citrate. There were fewer successful combinations in the case of lactose and fructose which performed best at 140 mM concentration combined with 40 mM sodium citrate level.

Viability of spermatozoa during post-thawing incubation was influenced by each type and concentration of non-electrolyte, level of sodium citrate ($P < 0{\cdot}001$), and, as in experiment 4, these factors were also involved in second-order interactions ($P < 0{\cdot}01, P < 0{\cdot}001$).

(*f*) *Experiment 6*

In this experiment thawing solutions which showed the best performance in experiments 2, 4, and 5 and some additional media were compared using four pooled ejaculates from each of three rams (Table 7).

TABLE 7

EXPERIMENT 6: SURVIVAL OF SPERMATOZOA FOLLOWING THAWING IN VARIOUS SOLUTIONS

Bracketed values do not differ significantly

Thawing Solution	$n = 12$		$n = 48$		$n = 36$	
	Freezing Point Depression (deg C)	Motile Spermatozoa (%)	Ram No.	Motile Spermatozoa (%)	Incubation Period (hr)	Motile Spermatozoa (%)
Inositol 210 mM—sodium citrate 40 mM	0·62	46·6	1	38·5	0	43·8
Fructose 388·5 mM	0·79	45·8	2	31·6	2	40·7
Glucose 210 mM—sodium citrate 40 mM	0·65	44·6	3	43·6	4; 6	36·7; 30·4
Glucose 277·5 mM	0·56	40·4				
Calcium-free Krebs–Ringer phosphate	0·64	39·9				
Inositol 277·5 mM	0·52	38·7				
Long-life milk (U.H.T.)	0·54	37·0				
Glucose 144·4 mM—sodium citrate 72·8 mM	0·59	36·9				
Inositol 94·4 mM—sodium citrate 72·8 mM	0·53	36·4				
Lactose 114·4 mM—sodium citrate 72·8 mM	0·59	36·0				
Skim milk* (9 g/100 ml)	0·54	28·4				
Glucose 44·4 mM—sodium citrate 80·6 mM (control)	0·50	24·7				
		$P < 0·001$		$P < 0·001$		$P < 0·001$† n.s.‡

* Bonlac non-fat milk. † Linear. ‡ Quadratic, cubic.

Survival of spermatozoa following thawing in various solutions differed markedly ($P < 0·001$). The best and relatively high cell survival rates were obtained with inositol 210 mM–sodium citrate 40 mM, fructose 388·5 mM, and with glucose 210 mM–sodium citrate 40 mM. The results for glucose at 277·5 mM level were somewhat lower, but indistinguishable from the former solutions. Glucose 44·4 mM–sodium citrate 80·6 mM, which was included as a control treatment, showed the poorest performance.

The semen from the three rams differed significantly in their behaviour on thawing and there was a ram × incubation interaction ($P < 0·05$). Thawing solutions which gave the best mean results were also better in maintaining the viability of spermatozoa during the 6-hr post-thawing incubation (thawing solution × incubation, $P < 0·01$).

IV. DISCUSSION

There is a complex situation in the freeze–thaw procedure, as injury to the spermatozoa occurs at both freezing and thawing stages. Due to the difficulties encountered in detecting and measuring the degree of injury at either of the two

stages, evaluation of solutions used for thawing pellet-frozen spermatozoa is also complicated.

While it seems unlikely that the thawing solution would have any importance when spermatozoa were lethally damaged during the freezing process, it could, however, have either a deleterious or a protective effect to the cells which have maintained their integrity during freezing and remained in a "dormant" state until thawing. Besides the protective effect, some kind of repairing action provided by the thawing solution to the sperm cells not lethally injured during freezing may also be possible. The concept of "freeze-thaw-induced latent injury" proposed by Sherman (1967) should similarly be considered, according to which the deleterious effect of freezing and thawing will not be manifested at or shortly after thawing, but with time after thawing. Further, it has been demonstrated that thawing temperature is important (Salamon 1968), and that the solution acting beneficially on the recovery of cells will not necessarily be an optimal milieu for maintenance of viability of spermatozoa as the time elapses from thawing (Lightfoot and Salamon 1969*b*).

When designing the present experiments we were aware of the fact that a relationship exists between composition of pre-freezing diluent and of thawing solution (Lightfoot and Salamon 1969*b*), nevertheless the freezing environment as a factor has been omitted and only one freezing diluent was included throughout all tests. This was done because the primary intention in this study was to find solution(s) which would give maximal recovery on thawing and maintain viability of spermatozoa at a high level during post-thawing incubation when they are pellet-frozen with a raffinose–citrate diluent already examined in laboratory and fertility tests (Salamon and Lightfoot 1969, 1970).

The experiments showed that ram spermatozoa pellet-frozen in the raffinose–citrate diluent and subsequently thawed in solutions consisting of either one component (non-electrolyte) or two components (non-electrolyte and sodium citrate) could yield satisfactory survival rates. The effect of solution, however, depended largely on the type and concentration of the components, and the situation was further complicated by many interactions which occurred during post-thawing incubation.

When only one component was present in the thawing solution, the best performance with fructose was achieved at relatively higher tonicity than with inositol, glucose, or lactose. Raffinose solution generally proved to be a poorer thawing medium.

When thawing solutions consisting of two components were used the recovery of spermatozoa on thawing and their viability during subsequent incubation was influenced by the levels of both non-electrolyte and electrolyte in the solution. Cell survival generally improved with a decrease in sodium citrate level and simultaneous increase in the concentration of non-electrolyte component. The rate of increase in concentration, however, depended on the type of non-electrolyte. Inositol and glucose preferred higher concentration than either lactose or fructose. There was no apparent relationship between freezing point depression of the two-component solution and percentage of motile spermatozoa following thawing, which indicated that the non-electrolyte–sodium citrate ratio in the medium rather than tonicity *per se* was important.

In view of the finding that the concentration of non-electrolyte component should be increased on account of the sodium citrate level, it is not surprising that in experiment 6 the control solution (glucose 44·4 mM–sodium citrate 80·6 mM) showed the poorest performance. This solution was used in previous studies and it is evident now that more efficient thawing media are available.

The final and comparative test (expt. 6) showed that several media can be used for thawing, but the best survival rates may be expected with 388·5 mM fructose and with inositol or glucose both at 210 mM concentration and each combined with 40 mM citrate level. Measurements of freezing point depression revealed that while the fructose solution was hypertonic, both inositol–citrate and glucose–citrate were isotonic in respect to ram semen. All others, except the calcium-free Krebs–Ringer solution, were hypotonic thawing media. The long-life milk which showed a relatively satisfactory performance merits attention, as it could be of practical use in particular circumstances.

Finally two points should be mentioned. First, that 1 : 4 prefreezing and 1 : 3 (pellets : thawing solution, v/v) thawing dilutions were used here and variation in dilution rates at either stage of processing semen could probably yield different performance (Lightfoot and Salamon 1969*a*). Secondly, ejaculates pellet-frozen from individual rams could be expected to behave differently when thawed in these solutions.

V. Acknowledgments

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