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Effect of Composition of Tris-based Diluent on Survival of Boar Spermatozoa following Deep Freezing

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Abstract

Six factorial experiments were conducted to examine the effects of concentrations of 2-amino-2-hydroxymethylpropane-1,3-diol (tris), sugars, erythritol, catalase, ethylenediaminetetra-acetic acid (EDTA), glycerol and egg yolk in the freezing diluent on the survival of boar spermatozoa after freeze–thawing.

When different tris and fructose concentrations were combined in glycerol–egg yolk media, the spermatozoa tolerated a relatively wide range in diluent tonicity. With glucose and lactose, however, the choice of concentrations of tris and sugar was limited.

Erythritol in the tris–fructose–egg yolk diluent provided considerable protection to spermatozoa in the absence of glycerol. The presence of catalase or EDTA in the tris-based diluent did not improve recovery of spermatozoa upon thawing, but both agents had a beneficial effect on the post-thawing survival.

Based on the viability of spermatozoa during *in vitro* incubation the tris (250 mM)–fructose (111 mM)–EDTA (15 mM)–citric acid (79·5 mM) diluent containing 3% (v/v) glycerol and 15% (v/v) egg yolk was selected. The proportion of spermatozoa with undamaged acrosomes following the freeze–thaw–incubation procedures in this diluent, however, was relatively low.

Introduction

Freezing of boar semen in diluents containing 2-amino-2-hydroxymethylpropane-1,3-diol (tris) has been reported by several investigators (Bader 1964; Rohloff 1967; Grove *et al.* 1968; Leidl 1968; Allmeling 1970; Rohloff and Allmeling 1970, 1971; Pursel and Johnson 1971; Salamon 1973). There appears, however, to be no published information on various concentrations of tris in the diluent as main component and on its combination with other agents.

The experiments reported in the present study were conducted to examine the effects of tris concentration, type and concentration of sugar, and concentration of catalase, ethylenediaminetetra-acetic acid (EDTA) and erythritol in the freezing diluent on the survival of boar spermatozoa following freeze–thawing.

Materials and Methods

Semen was collected from one Berkshire and two Large White boars by the manual method, and only the sperm-rich fraction of the ejaculates was used. After being filtered through several layers of sterile gauze the semen was diluted (1 : 2) at 30°C. The components of tris-based diluents used in all experiments and of glucose diluting medium included in addition in experiment 6 are described in the Results. The pH of tris-based diluents and of thawing solutions was adjusted to 7·0 with an appropriate amount of citric acid. The concentration of egg yolk in the diluent, when not included as a factor, was 22·5% (v/v). The glycerol concentration in the diluting media was 7·5% (v/v) in experiment 1, 6% (v/v) in experiment 2, 3% (v/v) in experiment 6, and varied in experiments 3, 4 and 5.

The diluted semen was cooled to 5°C in 2 h, then pelleted (0·1 ml) on dry ice (Nagase and Niwa 1964). The semen was kept on dry ice for 3 min, after which the frozen pellets were transferred into liquid nitrogen and stored for 24–72 h before thawing for examination. The pellets were thawed in dry test tubes, or in test tubes containing thawing solution (experiment 2), and held in a water-bath at 37°C. When thawing in a solution, the dilution ratio at thawing was 1 : 2 (pellets : thawing solution, v/v). Immediately after thawing of the pellets the test tubes were transferred into a water-bath at 30°C and the percentage of motile spermatozoa was assessed under a coverslip on a warm stage (37°C). Further assessments of the proportion of motile spermatozoa were made at 2-h intervals during incubation at 30°C for 4 h (experiments 1 and 3) or for 6 h (experiments 2, 4, 5 and 6).

In experiment 6 the spermatozoa were examined also for acrosome damage, immediately after thawing and after 6 h incubation, on smears stained and cleared according to Dott and Foster (1972). Three smears were prepared for each treatment and on each smear 100 cells were examined under a phase-contrast microscope using the yellowish-green part of a continuous interference filter (Leitz Wetzlar). The osmolality of the diluents in experiments 1 and 6 was measured with a Fiske Osmometer (Uxbridge, Mass., U.S.A.).

The vials containing the semen samples for incubation and the smears were coded and presented for examination in random order, so that the observer did not know their identity.

Motility data for each experiment, following angular transformation, were examined by analysis of variance for a split-plot experiment, post-thawing incubation being the sub-plot. In the experiments in which boars were included as a factor and significant first-order interaction was revealed between boars and other factors, the interaction mean square was used to test the relevant main effect. In experiment 6 the data for acrosome damage were examined by the standard analysis of variance.

Experimental Details and Results

Experiment 1

This experiment was of $4 \times 3 \times 4$ factorial design, using pooled semen from three boars, and incorporated the following factors:

1. Concentration of tris in diluent	150 *v.* 200 *v.* 250 *v.* 300 mM
2. Sugar in diluent	fructose *v.* glucose *v.* lactose
3. Concentration of sugar in diluent	55·5 *v.* 111·0 *v.* 166·5 *v.* 222·0 mM

Survival of spermatozoa varied depending on the concentration of tris and type of sugar in the diluent ($P<0·05$; Table 1). When fructose was included in the freezing diluent, tris at 200 and 250 mM was better than at 150 and 300 mM concentrations. Survival of spermatozoa declined with increasing tris concentration when glucose and particularly when lactose was incorporated in the diluent.

There was an interaction between type of sugar and sugar concentration in the diluent ($P<0·001$; Table 1). While fructose performed equally well at all concentrations examined, glucose proved best at 55·5 mM, with lower but similar results at 111–222 mM concentrations. Survival of spermatozoa declined sharply with increasing concentrations of lactose.

The linear regressions of the mean percentage of motile spermatozoa (y) during the 4-h post-thawing incubation on the osmolality (x; expressed as milliosmoles) of the tris–sugar diluents were as follows:

tris–fructose $y = 27·66+0·005(\pm 0·007)x$; D.F. 1, 14; $F<1$, n.s.
tris–glucose $y = 45·31-0·043(\pm 0·012)x$; D.F. 1, 14; $F = 12·07$, $P<0·05$.
tris–lactose $y = 56·18-0·091(\pm 0·008)x$; D.F. 1, 14; $F = 143·99$, $P<0·001$.

Quadratic regressions for the three tris–sugar diluents were not significant. The relationship between motility of spermatozoa and osmolality of diluent, within the range examined, was linear rather than curvilinear for tris–glucose and tris–lactose, whereas there was no apparent relationship for tris–fructose.

Survival of spermatozoa during post-thawing incubation was influenced by concentration of tris ($P<0{\cdot}01$) and type of sugar in diluent ($P<0{\cdot}05$), and these factors were also involved in a second-order interaction (tris concentration × sugar type × incubation period; $P<0{\cdot}05$). The best recovery and survival of spermatozoa were obtained when tris at 200 or 250 mM was combined with fructose.

Table 1. Effect of composition of pre-freezing diluent on the mean percentage of motile spermatozoa during 4 h of post-thawing incubation (experiment 1)

Values in parentheses are osmolality (m-osmole/kg) of diluent used for calculation of regression. Values for glucose were the same as those for fructose

Sugar in diluent	Concn of sugar (mM)	Concentration of tris in diluent (mM): 150	200	250	300	Means[A]
Fructose	55·5	26·0 (262)	31·3 (312)	31·8 (366)	26·5 (422)	28·9
	111·0	27·6 (326)	29·1 (383)	33·4 (438)	31·8 (490)	30·6
	166·5	28·1 (401)	32·4 (454)	33·4 (509)	28·6 (553)	30·5
	222·0	28·6 (469)	34·5 (525)	30·7 (590)	26·5 (632)	30·1
	Means[B]	27·6	31·8	32·4	28·3	30·0
Glucose	55·5	33·4	31·8	31·8	24·5	30·3
	111·0	27·6	34·0	27·6	9·5	23·9
	166·5	32·4	27·0	22·5	17·4	24·6
	222·0	29·1	24·5	22·0	21·6	24·2
	Means[B]	30·6	29·3	25·9	17·9	25·9
Lactose	55·5	29·7 (266)	30·7 (318)	24·5 (373)	16·5 (417)	25·1
	111·0	27·6 (340)	23·0 (387)	16·1 (444)	9·5 (498)	18·5
	166·5	16·1 (412)	14·4 (469)	2·8 (526)	1·8 (579)	7·4
	222·0	11·7 (502)	0·6 (552)	5·1 (612)	0·3 (661)	3·1
	Means[B]	20·7	14·3	10·6	5·3	12·1
Overall means		26·2	24·7	22·2	15·9	

[A] Interaction: type of sugar × concentration of sugar.
[B] Interaction: type of sugar × concentration of tris.

Experiment 2

This was of 4 × 5 × 3 factorial design and examined the following factors:

1. Concentration of catalase (from bovine liver; BDH) in tris (250 mM)–fructose (111 mM)–glycerol–egg yolk diluent — 0 *v.* 100 *v.* 300 *v.* 500 enzyme units (e.u.)/ml
2. Method of thawing — In dry test tubes *v.* solution A with no catalase *v.* solution A with 100 e.u./ml catalase *v.* solution A with 300 e.u./ml catalase *v.* solution A with 500 e.u./ml catalase (solution A = 250 mM tris–111 mM fructose)
3. Boars — Sperm-rich fraction of ejaculates from each of three boars

Inclusion of catalase in either the freezing diluent or in the thawing solution had no effect on the mean percentages of motile spermatozoa. There was, however, an

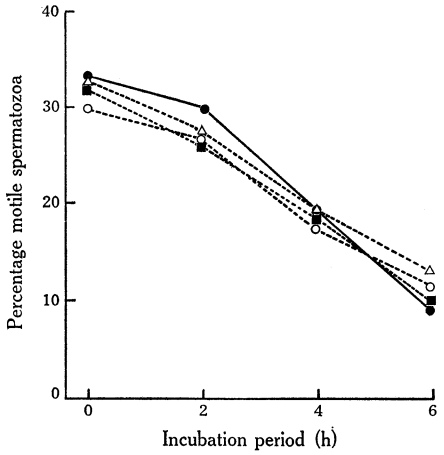


Fig. 1. Effect of concentration of catalase in diluent on the survival of spermatozoa during post-thawing incubation (experiment 2).
● 0 catalase
△ 100 e.u./ml catalase
■ 300 e.u./ml catalase
○ 500 e.u./ml catalase

interaction between catalase in the freezing diluent and survival of spermatozoa during post-thawing incubation (Fig. 1) which was due to a steeper decline in survival

Table 2. Effect of glycerol and erythritol concentrations in the diluent on the percentage of motile spermatozoa during post-thawing incubation (experiment 3)

Erythritol concentration (M)	Incubation period (h)	Glycerol concentration (% v/v): 0	3·75	7·50	Means
0	0	16·1	29·1	31·8	25·3
	2	14·8	26·5	27·9	22·8
	4	2·5	15·7	17·1	10·5
	Means	10·0	23·5	25·3	19·0
0·5	0	24·3	31·8	28·6	28·2
	2	21·4	29·1	24·3	24·9
	4	10·5	18·9	15·5	14·8
	Means	18·3	26·4	22·6	22·3
1·0	0	30·2	29·1	25·0	28·1
	2	28·1	24·3	20·8	24·3
	4	17·0	16·8	11·9	15·2
	Means	24·8	23·2	18·9	22·3
1·5	0	30·2	18·9	9·5	18·8
	2	23·8	12·8	7·4	14·0
	4	16·4	7·1	2·6	7·8
	Means	23·2	12·5	6·1	13·2
Overall means		18·7	21·1	17·4	

of spermatozoa frozen in the diluent containing no catalase [(0 catalase *v.* 100, 300, 500 e.u./ml catalase) × incubation period; linear $P<0{\cdot}01$, quadratic $P<0{\cdot}05$].

Experiment 3

The factors included in this experiment ($3 \times 4 \times 3 \times 3$ factorial) were:

1. Tris concentration in diluent — 200 *v.* 250 *v.* 300 mM†
2. Concentration of *meso*-erythritol (biochemical) in diluent — 0 *v.* 0·5 *v.* 1·0 *v.* 1·5M
3. Glycerol concentration in diluent — 0 *v.* 3·75 *v.* 7·5% (v/v)
4. Boars — Sperm-rich fraction of ejaculates from each of three boars

Table 3. Summary of analysis of variance of results of experiment 3 (see Table 2)

Source of variation	D.F.	M.S.	*F*
Boars (A)	2		21·86***
Glycerol concentration (B)	2		11·16***
Tris concentration (C)	2		<1·0
Erythritol concentration (D)	3		4·31
Interactions			
B×D	6		49·59***
A×D[A]	6	198·18	10·94***
A×C[B]	4	72·54	4·01*
Remainder first-order interactions	14		1·99
Pooled second-order interactions	44		<1·0
A×B×C×D (error 1)	24	18·11	
Incubation period (E)	2		789·68***
Interactions			
D×E	6		5·60***
C×E	4		2·60*
B×E	4		4·82**
A×E	4		39·47***
B×D×E	12		2·73**
A×D×E	12		11·68***
A×B×E	8		8·28***
Remainder second-order interactions	28		1·63
A×C×D×E	24		2·24*
Remainder third-order interactions	64		1·36
A×B×C×D×E (error 2)	48	3·58	

[A] Mean square used to test the effect of erythritol concentration (D).
[B] Mean square used to test the effect of tris concentration (C).
*$P<0·05$. **$P<0·01$. ***$P<0·001$.

The results are summarized in Table 2 and the analysis of variance is presented in Table 3. There was an interaction between concentration of glycerol and of erythritol ($P<0·001$) and these factors were also involved in a second-order interaction (glycerol concentration × erythritol concentration × incubation period; $P<0·01$). Few spermatozoa survived the freeze–thaw and subsequent incubation procedures when both glycerol and erythritol were absent, or when these agents were combined at high concentrations (7·5% glycerol and 1·5M erythritol). Erythritol at 1·0M concentration yielded similar results to 3·75 and 7·50% glycerol.

† Each concentration combined with 111 mM fructose.

The mean percentages of motile spermatozoa for 200, 250 and 300 mM tris concentrations were similar (17·9, 19·7 and 19·6% respectively), but the decrease in viability of cells during the 4-h post-thawing incubation was steeper for 200 mM than for 250 and 300 mM tris concentrations (tris concentration × incubation period interaction, $P<0·05$).

Experiment 4

In this 3 × 4 : 3 factorial experiment, using pooled semen from two boars, the following factors were included:

1. Concentration of glycerol in diluent* 0 *v.* 3 *v.* 6% (v/v)
2. Concentration of EDTA (disodium salt) in diluent 0 *v.* 5 *v.* 10 *v.* 15 mM
3. Three replicates at thawing (: 3)

Survival of spermatozoa during post-thawing incubation was influenced by both glycerol and EDTA concentrations in the diluent. Recovery and survival of spermatozoa was superior in diluent containing 3% rather than no or 6% glycerol (glycerol concentration × incubation period interaction, $P<0·05$; Table 4). Survival of

Table 4. Effect of glycerol concentration in the diluent on the percentage of motile spermatozoa during post-thawing incubation (experiment 4)

Incubation period (h)	Glycerol concentration (% v/v): 0	3	6	Means
0	26·9	36·6	34·1	32·5
2	24·2	35·4	32·5	30·6
4	21·7	31·7	26·5	26·5
6	13·5	26·5	19·3	19·5
Means	21·3	32·5	27·9	

spermatozoa during post-thawing incubation was also better when EDTA was included in the diluent (0 *v.* 5, 10, 15 mM EDTA × incubation period interaction, $P<0·001$; Fig. 2). Although there was no significant interaction between glycerol and EDTA concentration, EDTA in the diluent gave some protection to spermatozoa frozen in the absence of glycerol. Best results were obtained with the diluent containing 3% glycerol and 5 or 10 mM EDTA.

Experiment 5

This experiment was of 3 × 3 × 4 : 3 factorial design using pooled semen from two boars, and examined the following factors in the tris (250 mM)–fructose (111 mM) diluent:

1. Glycerol concentration in diluent 0 *v.* 3 *v.* 6% (v/v)
2. Erythritol concentration in diluent 0 *v.* 0·5 *v.* 1·0M
3. EDTA concentration in diluent 0 *v.* 5 *v.* 10 *v.* 15 mM
4. Three replicates at thawing (: 3)

* Pre-freezing diluent: tris (250 mM)–fructose (111 mM).

Concentrations of glycerol, erythritol and EDTA in the basic diluent all had significant effects ($P<0\cdot001$) on the mean percentage of motile spermatozoa during post-thawing incubation. Increasing the concentration of erythritol improved

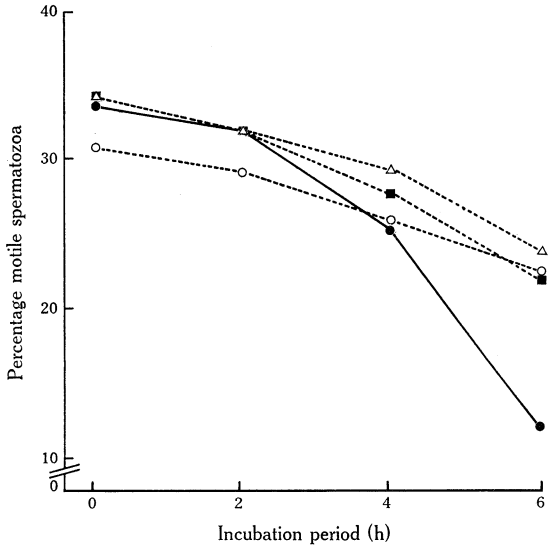


Fig. 2. Effect of concentration of EDTA in diluent on the survival of spermatozoa during post-thawing incubation (experiment 4).
● 0 EDTA
△ 5 mM EDTA
■ 10 mM EDTA
○ 15 mM EDTA

the sperm survival in the absence of glycerol, but had a depressing effect with concomitant increase of concentration of glycerol (glycerol concentration × erythritol concentration interaction, $P<0\cdot001$; Fig. 3). This pattern, however, was influenced

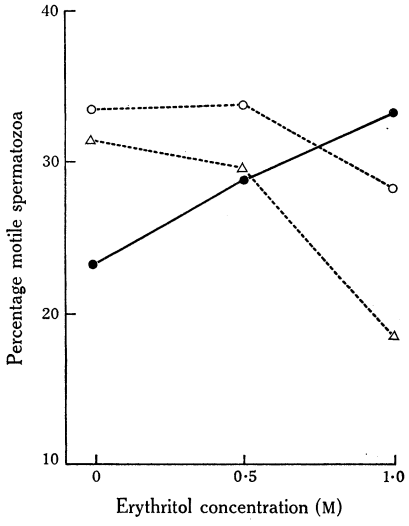


Fig. 3. Effect of concentration of glycerol and erythritol in the diluent on the mean percentage of motile spermatozoa during 6 h post-thawing incubation (experiment 5).
● 0 glycerol
○ 3% v/v glycerol
△ 6% v/v glycerol

also by the EDTA in the diluent (glycerol concentration × erythritol concentration × 0 *v.* 5, 10, 15 mM EDTA, $P<0\cdot001$; Table 5). EDTA improved the survival of spermatozoa when erythritol and glycerol were not included, or when these components were combined at high concentrations (1·0M erythritol and 6% glycerol) in the diluent.

The beneficial effect of EDTA on the viability of spermatozoa was evident during the post-thawing incubation (0 *v.* 5, 10, 15 mM EDTA × incubation period interaction, linear $P<0{\cdot}001$). Table 6 shows that although motility upon thawing was slightly better in the absence of EDTA, its inclusion at 5, 10 or 15 mM concentrations in the diluent resulted in substantially better survival rates during the subsequent 6-h incubation.

Table 5. Effects of concentration of glycerol, erythritol and EDTA in the diluent on the percentage of motile spermatozoa during 6 h post-thawing incubation (experiment 5)

EDTA concentration (mM)	Erythritol concentration (M)	Glycerol concentration (% v/v): 0	3	6	Means
0	0	18·3	31·3	27·7	25·5
	0·5	24·0	31·7	25·8	27·1
	1·0	32·1	25·4	14·0	23·4
	Means	24·6	29·4	22·2	25·3
5	0	26·6	32·9	35·0	31·4
	0·5	31·3	34·2	30·9	32·1
	1·0	35·0	29·0	17·2	26·7
	Means	30·9	32·0	27·3	30·0
10	0	22·7	34·2	32·9	29·8
	0·5	28·9	36·2	31·7	32·2
	1·0	34·1	29·7	20·4	27·9
	Means	28·4	33·3	28·1	29·9
15	0	25·0	36·2	30·5	28·9
	0·5	31·7	33·7	30·9	32·1
	1·0	31·7	29·7	22·9	28·0
	Means	29·4	33·2	28·0	30·2
Overall means		28·3	31·9	26·4	

Experiment 6

This experiment (3 × 4 : 3) examined three diluents each with four concentrations of egg yolk (Table 7), and using three replicates at dilution from pooled semen of two boars (: 3). The concentration of glycerol in each diluent was 3% (v/v).

The mean percentage of motile spermatozoa was higher for the two tris diluents than for the glucose one ($P<0{\cdot}01$), and also when egg yolk was included in the extending medium ($P<0{\cdot}001$). Increasing the concentration of egg yolk improved the initial motility of spermatozoa upon thawing; however, the decline of their viability during subsequent incubation became steeper as the egg yolk level increased (egg yolk concentration × incubation period interaction, $P<0{\cdot}01$), and this was more pronounced for tris–fructose than for tris–fructose–EDTA and glucose diluents (type of diluent × egg yolk concentration × incubation period interaction, $P<0{\cdot}05$).

The results for acrosome examination are presented in Table 8. The mean percentages of spermatozoa with normal appearing acrosomes (type I) and of those

with slight damage (type II) after the freeze–thaw–incubation procedures were higher for the two tris diluents than for the glucose one. Damage to the acrosomes during post-thawing incubation depended on the diluent used (type of diluent × incubation

Table 6. Effect of concentration of EDTA in the diluent on the percentage of motile spermatozoa (experiment 5)

Incubation period (h)	EDTA concentration (mM): 0	5	10	15	Means
0	36·4	35·8	34·2	34·5	35·2
2	30·5	33·8	32·0	32·9	32·3
4	23·6	30·2	30·4	30·0	28·5
6	13·1	21·0	23·5	23·6	20·1
Means	25·3	30·0	29·9	30·2	

period interactions; Table 8). Percentage of spermatozoa with normal acrosomes (type I) declined more steeply during incubation for glucose than for tris–fructose and tris–fructose–EDTA media. Although only a few spermatozoa were severely damaged

Table 7. Effect of type of diluent and egg yolk concentration on the percentage of motile spermatozoa (experiment 6)

Freezing diluent[A]	Incubation period (h)	Egg yolk concentration (% v/v): 0	7·5	15·0	22·5	Means
Tris (250 mM)–fructose (111 mM) (367–421)[B]	0	25·0	29·7	34·5	32·9	30·5
	2	20·1	25·0	31·3	31·3	26·8
	4	14·9	23·5	22·0	25·0	21·2
	6	11·3	20·1	17·0	18·8	16·7
	Means	17·5	24·5	25·9	26·8	23·6
Tris (250 mM)–fructose (111 mM)–EDTA (15 mM) (409–467)[B]	0	25·0	28·1	28·1	31·3	28·1
	2	23·5	28·1	28·1	26·5	26·5
	4	20·6	23·5	22·0	25·0	22·8
	6	15·3	22·0	22·0	20·1	19·8
	Means	21·0	25·4	25·0	25·6	24·2
Glucose (315 mM) (344–444)[B]	0	20·6	25·0	25·0	28·1	24·6
	2	20·6	23·5	25·0	25·0	23·5
	4	15·3	23·5	23·5	22·0	21·0
	6	15·3	20·6	18·8	17·0	17·9
	Means	17·9	23·1	23·0	22·9	21·7
Overall means		18·8	24·3	24·6	25·1	

[A] Glycerol concentration in the diluents was 3% (v/v).
[B] Range in osmolality (m-osmole/kg) for different egg yolk concentrations.

(type IV) immediately after thawing when using the two tris diluents, the proportion of spermatozoa with such damage was markedly increased during 6 h post-thawing incubation. Inclusion of egg yolk in the diluents contributed only slightly to the

maintenance of integrity of the acrosome. However, the proportion of spermatozoa with severely damaged acrosomes was significantly higher when using media without egg yolk ($P<0\cdot01$).

Table 8. Percentage of spermatozoa with normal and damaged acrosomes after freeze–thawing and incubation procedures (experiment 6)

Freezing diluent[A]	Incubation period (h)	Acrosome classification:[B] I	II	III	IV	V
Tris–fructose	0	21·9	24·0	41·7	8·1	2·1
	6	10·5	18·8	32·1	30·6	6·3
	Means	15·8	21·3	36·8	18·0	3·9
Tris–fructose–EDTA	0	19·9	29·9	42·2	5·3	1·0
	6	8·7	22·6	36·8	24·5	6·1
	Means	13·8	26·2	39·5	13·4	3·0
Glucose	0	18·0	21·0	25·4	31·3	1·6
	6	4·5	12·1	37·9	35·7	8·1
	Means	10·2	16·3	31·5	33·4	4·2
Interaction: Diluent × incubation period		$P<0\cdot05$	n.s.	$P<0\cdot01$	$P<0\cdot01$	n.s.

[A] See Table 7 for full composition.
[B] For description of different types see Figs 4–8.

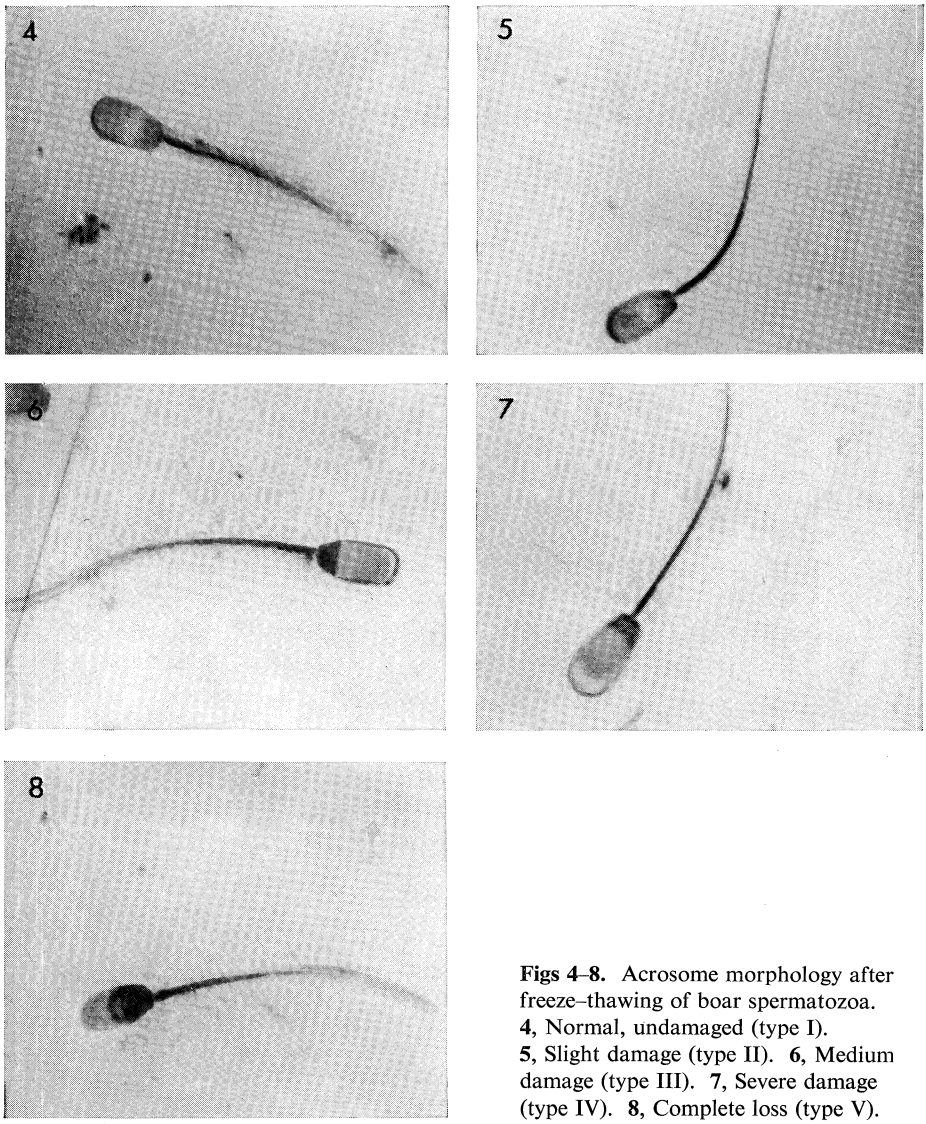
Discussion

The optimum concentration of tris in the diluent is generally reported to be 200 and 250 mM for bull semen (reviewed by Foote 1972) and 300 mM for ram semen (Salamon and Visser 1972). Igboeli (1970) found that tris concentrations of 200 and 250 mM were more suitable than 150 mM for liquid storage of boar semen, whereas Pursel *et al.* (1972) did not detect a difference in the resistance of spermatozoa to cold shock when using tris concentrations varying from 82 to 246 mM. In the majority of reports on the freezing of boar semen that were mentioned in the Introduction tris was used at concentrations of 200 mM in the diluent, except in the Beltsville F_3 extender of Pursel and Johnson (1971).

The results of this study showed that boar spermatozoa can tolerate a relatively wide range in tris concentrations, but the most acceptable rates of survival of spermatozoa after the freeze–thawing procedure can be expected with tris concentrations from 200 to 250 mM in the freezing diluent.

The type and concentration of sugar included in the tris medium also had an effect on the survival of spermatozoa. Fructose proved to be a more suitable diluent component than glucose or lactose. The tonicity of diluents in which different concentrations of fructose and of tris were combined varied from 262 to 632 m-osmoles; nevertheless, this wide tonicity range was well tolerated by the spermatozoa. In contrast, the diluents containing glucose and lactose gave best results in the osmolality ranges of 262–469 and 266–373 m-osmoles respectively (Table 1). Thus, the type of sugar included in the tris medium was more important than the tonicity of the diluent.

Based on the survival of spermatozoa during *in vitro* incubation, the medium containing 250 mM tris, 111 mM fructose and 79·5 mM citric acid was selected as most suitable. The osmolality of this medium was 438 m-osmoles and it was hypertonic to boar semen (osmolality of semen, 308 m-osmoles). The type of sugar in the tris-based diluent was found to have an effect also on the resistance of boar spermatozoa to cold shock (Pursel *et al.* 1972) and on the freezability of ram semen (Salamon and Visser 1972).



Figs 4–8. Acrosome morphology after freeze–thawing of boar spermatozoa. **4**, Normal, undamaged (type I). **5**, Slight damage (type II). **6**, Medium damage (type III). **7**, Severe damage (type IV). **8**, Complete loss (type V).

Diluents containing catalase or EDTA were reported to improve the survival of spermatozoa during storage of liquid semen of the bull (e.g. Foote 1967; Popov 1968; Macmillan 1970; Bredderman and Foote 1971), ram (Honmode 1965) and boar (Pliško 1965; Sadovnikova 1966; Senegačnik 1969; Igboeli 1970; Podaný and

Muzikant 1970). Both of these substances allegedly protect the spermatozoa against the toxic effects of hydrogen peroxide formed by oxidation of aromatic amino acids present in the extender, and their beneficial effect on survival of spermatozoa is more pronounced during storage at 37°C (Macmillan *et al.* 1972; Shannon and Curson 1972). In this study both catalase and EDTA in the tris–fructose diluent improved the survival of boar spermatozoa during the post-thawing incubation at 30°C. In addition, EDTA also provided some protection during the freeze–thawing procedure (Table 5). It should be mentioned however that, contrary to these results, in a series of additional tests in which combination of different concentrations of glucose and EDTA were used in the pre-freezing diluents (with appropriate adjustment of pH), the post-thawing survival of spermatozoa was repeatedly very poor. Thus, the effectiveness of EDTA may vary depending on the composition of the basic diluent used for freezing.

Erythritol in the tris–fructose diluent provided considerable protection to spermatozoa, and at 1·0 and 1·5M concentrations its protective effect was similar to that of glycerol (Table 2). Furthermore, when erythritol (1·0M) and EDTA (5 or 10 mM) were combined in the tris extender, survival of spermatozoa was even better than in the presence of glycerol (Table 5). The low molecular weight polyols were examined also by Salamon *et al.* (1973), who found that their protective effect depended on the composition of the basic diluent, and that in the inositol–yolk extender glycerol was superior to xylitol. It may well be presumed that the protective effect of a low molecular weight polyol, like that of EDTA, is more pronounced in tris than in other diluting media.

In the final and comparative test (experiment 6) tris–fructose–yolk, with or without EDTA, proved to be a more suitable extender than glucose–yolk, as judged by motility and integrity of spermatozoa after the freeze–thaw–incubation procedures. Nevertheless, the proportion of spermatozoa with undamaged acrosomes for the former medium was still relatively low. Further investigation is necessary to prevent the considerable freeze–thaw damage to spermatozoa with concomitant improvement of their viability.

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