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Examination of Some Processing Methods for Freezing Boar Semen

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Abstract

Five experiments were conducted to examine the effect of processing methods and diluents on survival and morphology of boar spermatozoa after freezing.

Post-thawing survival of spermatozoa was better for Beltsville-F_3 (BF_3) than for tris–fructose–EDTA freezing diluent when the seminal plasma and glycerol were removed prior to freezing (method A). Both freezing diluents yielded similar viability results when the spermatozoa were frozen in the presence of seminal plasma and glycerol (method B). Viability of spermatozoa after thawing was better when glycerol concentration in the prefreezing diluent (method A) or in the freezing medium (method B) was 2·5 and 5·0 rather than 7·5%. Cooling of diluted semen to 5°C beyond 4 h decreased the post-thawing survival of spermatozoa. The proportion of spermatozoa with undamaged acrosomes after processing and thawing by different methods was indistinguishable and relatively low.

When the semen was frozen at cell concentrations ranging from 0·25 to $2{\cdot}0 \times 10^9$/ml, the viability of spermatozoa declined with increasing concentration following freezing in BF_3 and S-1 diluents. Viability results were very similar for all cell concentrations examined when tris–fructose–EDTA diluent was used, indicating the possibility of freezing boar semen in a concentrated state.

Introduction

Boar semen has been processed for deep-freezing by various methods (reviewed by Einarsson 1973; Paquignon and du Mesnil du Buisson 1973; Schmidt *et al.* 1974; Cheng and Wung 1975). The methods differ in essential details, such as the type of diluent used for processing, the method of dilution and glycerolization, time of cooling to 5°C, and sperm concentration of semen at freezing.

The experiments reported here were undertaken to examine the effect of some processing methods for freezing on the viability and morphology of boar spermatozoa.

Materials and Methods

Semen was collected from Berkshire, Landrace, Large White and Saddleback boars by the manual method, and only the sperm-rich fraction of the ejaculates was used. Each sample was filtered through gauze and the concentration of spermatozoa determined by a colorimeter calibrated against haemocytometer counts. Dilution of semen, or resuspension of spermatozoa following centrifugation and removal of the seminal plasma, was carried out at 30°C.

The diluents used in all five experiments were Beltsville-F_3 (BF_3; Pursel and Johnson 1971) and tris–fructose–EDTA (Visser and Salamon 1974). The pH of the latter diluent was adjusted to 7·0 by including 75 mM citric acid in the medium. The diluents included in addition to the former media were TES–glucose–yolk (Crabo *et al.* 1972; expt 2), S-1 (expts 3, 4) and S-2 (expt 5). The S-1 diluent, elaborated by us, consisted of 250 mM tris, 75 mM citric acid, 15 mM EDTA, and 1% (w/v) casein. The S-2 extender was a modification of S-1 by inclusion also of 55·5 mM glucose.

In experiment 1 the processing method of Visser and Salamon (1974) was slightly modified by diluting the semen to a sperm concentration of $0{\cdot}25 \times 10^9$ per ml, instead of using a dilution ratio of 1 : 2. When the semen was processed according to the method of Pursel and Johnson (1971),

the initial diluent was removed after cooling to 5°C by centrifugation and the spermatozoa were resuspended with diluent containing no glycerol to a cell concentration of $0{\cdot}25 \times 10^9$ per ml.

In experiments 3 and 4 the semen was centrifuged at 600 *g* for 10 min at room temperature. After removal of the seminal plasma, the spermatozoa were resuspended with the diluent containing 5% glycerol to a concentration of $0{\cdot}25 \times 10^9$ per ml in experiment 3 and $0{\cdot}5 \times 10^9$/ml in experiment 4. In both experiments the resuspended samples were cooled to 5°C in 3 h and then the initial diluent was removed by centrifugation at 1000 *g* for 10 min. For freezing, the spermatozoa were resuspended with cooled diluent (5°C) containing no glycerol to concentrations indicated under Experimental Details and Results.

In Experiment 5, the fresh semen was diluted at a ratio of 1 : 2 (semen : diluent, v/v), then cooled to 5°C in 4 h and centrifuged at 1000 *g* for 10 min. After removal of the diluent–seminal plasma mixture, the spermatozoa were resuspended with the initial diluent (5°C) to a cell concentration of $1{\cdot}0 \times 10^9$ per ml and frozen. The diluents used for initial dilution and resuspension of spermatozoa contained 2% or no glycerol. When glycerol was present at initial dilution it was absent at resuspension, and when glycerol was absent at dilution it was present at 2% concentration in the suspending medium.

In all experiments the semen was frozen on dry ice by the pellet method (Nagase and Niwa 1964), and the frozen pellets (0·2 ml) were stored in liquid nitrogen for at least 48 h before thawing for examination. The pellets were thawed at 37°C in dry test tubes, except in experiment 2 where reconstituted skim milk ('Bonlac', 10% w/v, heated to 95°C for 10 min) and the freezing diluent (containing no glycerol, egg yolk, or casein) were used as thawing solutions. In the latter case, the dilution ratio at thawing was 1 : 1 (pellets : thawing solution, v/v). Immediately after thawing of the pellets (at 37°C) the test tubes were transferred into a water-bath at 30°C. When different sperm concentrations at freezing were involved (expts 3 and 4) the thawed semen was extended to a cell concentration of $0{\cdot}25 \times 10^9$ per ml, or to a ratio of 1 : 2 (v/v; expt 5) with the respective freezing diluent containing no cryoprotective agent.

The percentage of motile spermatozoa was assessed under a coverslip on a warm stage (37°C) shortly after transferring the thawed semen to 30°C, or after post-thawing extension at this temperature. Further assessment of the proportion of motile spermatozoa was made at 2-h intervals during incubation at 30°C for 6 h (expts 1, 3, 4 and 5). The acrosome morphology of spermatozoa following freezing and thawing, and after 6 h incubation (expt 2) was examined on smears stained and cleared according to Dott and Foster (1972). The smears were examined with a phase-contrast microscope (1500× magnification) using a continuous interference filter (Leitz Wetzlar). One hundred spermatozoa were counted on each smear, classified as having normal or damaged acrosomes and expressed as percentage normal acrosomes.

The data, 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 significant first-order interaction was found between boars and other factors, the interaction mean square was used to test the relevant main effect.

Experimental Details and Results

Experiment 1

The factors included in this experiment ($2 \times 2 \times 3 \times 4 \times 3$ factorial) were the following:

(1)	Type of diluent	Tris–fructose–EDTA *v.* BF_3
(2)	Processing method	Method of Pursel and Johnson (1971) *v.* method of Visser and Salamon (1974)
(3)	Cooling time to 5°C	2 *v.* 4 *v.* 6 h
(4)	Glycerol concentration in diluent	0 *v.* 2·5 *v.* 5·0 *v.* 7·5% (v/v)
(5)	Boars	Sperm-rich fraction of ejaculates from each of three boars (Berkshire, Large White, Saddleback)

There was an interaction between type of diluent, processing method and post-thawing incubation period ($P = 0{\cdot}05$; Table 1), which was due to better survival of spermatozoa during the 6-h incubation for the semen processed by the method of Pursel and Johnson (1971) and frozen in the BF_3 diluent.

Table 1. Effects of type of diluent, processing method, and cooling time to 5°C on the percentage of motile spermatozoa during post-thawing incubation (experiment 1)

Type of diluent	Incubation period (h)	Processing method[A]		Cooling time to 5°C (h)			
		A	B	2	4	6	Means
Tris–fructose–EDTA	0	23·3	23·6	23·8	24·4	22·2	23·5
	2	24·1	21·8	24·9	23·6	20·3	22·9
	4	18·2	17·2	19·0	18·9	15·3	17·7
	6	11·1	11·1	12·6	11·7	9·1	11·1
	Means	18·8	18·2	19·9	19·4	16·4	18·5
BF_3	0	22·1	27·9	26·0	26·8	22·1	24·9
	2	24·0	21·5	22·7	22·8	22·8	22·8
	4	18·4	19·9	20·4	19·0	18·1	19·2
	6	12·1	15·0	13·9	14·3	12·4	13·5
	Means	18·9	20·9	20·6	20·6	18·6	19·9

[A] Method A according to Visser and Salamon (1974), except that the semen was diluted to a sperm concentration of $0{\cdot}25 \times 10^9$/ml, instead of 1 : 2 dilution ratio. Method B according to Pursel and Johnson (1971).

Table 2. Effects of processing method, cooling time to 5°C, and glycerol concentration in the diluent on the percentage of motile spermatozoa during post-thawing incubation (experiment 1)

Processing method[A]	Incubation period (h)	Cooling time to 5°C (h)			Glycerol concn in diluent (%)[B]				
		2	4	6	0	2·5	5·0	7·5	Means
A Visser and Salamon (1974)	0	24·0	24·6	19·6	15·5	22·0	26·4	27·6	22·7
	2	24·2	25·4	22·6	16·6	25·2	26·5	28·6	24·1
	4	18·9	19·3	16·7	15·3	19·8	19·0	19·2	18·3
	6	11·2	13·0	10·6	9·5	15·5	12·1	9·5	11·6
	Means	19·3	20·3	17·1	14·1	20·5	20·7	20·6	18·9
B Pursel and Johnson (1971)	0	25·8	26·6	24·7	23·3	27·0	26·1	26·4	25·7
	2	23·4	21·1	20·5	19·0	23·1	22·9	21·6	21·6
	4	20·6	18·6	16·6	16·1	19·7	19·8	18·6	18·6
	6	15·5	12·9	10·8	11·3	14·1	13·9	12·8	13·0
	Means	21·2	19·6	17·8	17·2	20·8	20·5	19·7	19·5
Overall means		20·2	20·0	17·5	15·6	20·6	20·6	20·1	

[A] The concentration of spermatozoa at freezing for both methods was $0{\cdot}25 \times 10^9$/ml.
[B] When processing by the method of Pursel and Johnson (1971) the diluents containing 0, 2·5, 5·0 and 7·5% glycerol were removed, after cooling to 5°C, by centrifugation and the spermatozoa re-suspended with diluent containing no glycerol.

Cooling the diluted semen to 5°C in 2 or 4 h before freezing yielded similar results, but prolongation of the cooling period to 6 h decreased the survival of spermatozoa ($P < 0{\cdot}01$). There were interactions between cooling time, type of diluent and post-

thawing incubation period ($P < 0{\cdot}05$; Table 1), and between cooling time, processing method and post-thawing incubation period ($P < 0{\cdot}01$; Table 2). Nevertheless, the survival of spermatozoa during incubation for both diluents and for the two processing methods was lower when the semen was cooled to 5°C in 6 h than in 2 or 4 h (Tables 1 and 2).

The mean percentage of motile spermatozoa was lower in the absence of glycerol than in the presence of 2·5, 5·0 or 7·5% glycerol in the diluent ($P < 0{\cdot}005$), the results of the three glycerol concentrations being similar. There was a second-order interaction between glycerol concentration, processing method and post-thawing incubation period ($P < 0{\cdot}005$; Table 2) which was due to a steeper decline in viability of spermatozoa during incubation when the semen was processed by the method of Visser and Salamon (1974) and frozen in diluents containing 5·0 and 7·5% glycerol.

Table 3. Percentage of spermatozoa with normal acrosomes after processing and thawing by different methods

Incubation period (h)	Processing method			Thawing method			Means
	Pursel and Johnson (1971)	Crabo *et al.* (1972)	Visser and Salamon (1974)	In dry test tubes	In skim milk	In diluent buffer	
0	19·4	15·7	18·3	17·6	17·1	18·6	17·8
6	9·9	8·4	9·8	8·8	8·5	10·8	9·4
Means	14·3	11·8	13·8	12·8	12·5	14·5	

Experiment 2

In this experiment (3 × 3 × 2 × 3 factorial) the acrosome morphology of spermatozoa was examined using pooled semen of two boars (Berkshire and Large White). The factors included were the following:

(1) Processing method (the diluent used for each method is in square brackets) — Method of Pursel and Johnson (1971) [BF_3] *v.* method of Crabo *et al.* (1972) [TES–glucose–yolk] *v.* method of Visser and Salamon (1974) [tris–fructose–EDTA]

(2) Thawing method — In dry test tubes *v.* skim milk *v.* freezing diluent (containing no egg yolk, glycerol or casein)

(3) Post-thawing incubation period — 0 *v.* 6 h

(4) Three replicates at dilution

The results are presented in Table 3. There was no detectable difference between the processing or thawing methods with regards to the mean percentage of spermatozoa with normal acrosomes. The proportion of undamaged spermatozoa was significantly reduced after 6 h post-thawing incubation ($P < 0{\cdot}005$).

Experiment 3

The factors examined in this experiment (3 × 3 × 3 factorial) were the following:

(1) Type of diluent — Tris–fructose–EDTA *v.* BF_3 *v.* S-1

(2) Concentration of spermatozoa during freezing — $0{\cdot}25$ *v.* $0{\cdot}50$ *v.* $0{\cdot}75 \times 10^9$ per ml

(3) Boars — Sperm-rich fraction of ejaculates from each of three boars (Berkshire, Large White, Saddleback)

The results are summarized in Table 4. There was no difference between the mean percentage of motile spermatozoa for the sperm concentrations examined and for the boars. The three diluents differed significantly ($P < 0{\cdot}005$), the best results being obtained with the tris–fructose–EDTA extender. There was an interaction between type of diluent and post-thawing incubation ($P < 0{\cdot}05$). Survival rate during the post-thawing incubation was best when the spermatozoa were frozen in tris–fructose–EDTA diluent at a concentration of $0{\cdot}75 \times 10^9$ per ml.

Table 4. Effects of type of diluent and sperm concentration on the percentage of motile spermatozoa during post-thawing incubation (experiment 3)

Type of diluent	Incubation period (h)[A]	Sperm concn during freezing ($\times 10^9$/ml)[B]			
		0·25	0·50	0·75	Means
Tris–fructose–EDTA	0	31·3	31·3	32·9	31·8
	6	20·1	20·1	22·0	20·8
	Means	25·6	26·0	28·5	26·7
BF_3	0	28·1	31·3	28·1	29·1
	6	18·8	18·8	18·8	18·8
	Means	22·3	23·0	23·8	23·0
S-1	0	20·6	23·5	25·0	23·0
	6	9·5	17·0	15·3	13·8
	Means	17·1	19·8	20·3	19·0
Overall means		21·6	22·9	24·1	

[A] Data for 2- and 4-h incubation periods not presented.
[B] For details in obtaining the indicated sperm concentrations see Materials and Methods.

Experiment 4

In this experiment ($3 \times 4 \times 3$ factorial) a wider range of concentration of spermatozoa in the semen during freezing was examined and the following factors were included:

(1) Type of diluent — Tris–fructose–EDTA *v.* BF_3 *v.* S-1

(2) Concentration of spermatozoa during freezing — $0{\cdot}5$ *v.* $1{\cdot}0$ *v.* $1{\cdot}5$ *v.* $2{\cdot}0 \times 10^9$ per ml

(3) Boars — Sperm-rich fraction of ejaculates from each of three boars (Berkshire, Large White, Saddleback)

The results are summarized in Table 5. While the viability of spermatozoa for the sperm concentrations examined was very similar when the semen was frozen in tris–fructose–EDTA diluent, it declined with increasing cell concentration following freezing in BF_3 and S-1 diluents, and this was more pronounced for the BF_3 freezing

medium (tris–fructose–EDTA *v.* BF_3 × sperm concentration, linear; $P < 0{\cdot}05$). The mean percentage of motile spermatozoa during the 6 h post-thawing incubation was higher for tris–fructose–EDTA than for the other diluents ($P < 0{\cdot}005$).

Experiment 5

The experiment was of $3 \times 4 \times 3$ factorial design using pooled semen from two boars (Landrace and Large White) and examined the following factors:

(1) Type of diluent — Tris–fructose–EDTA *v.* BF_3 *v.* S-2

(2) Glycerol treatment — A, No glycerol in diluent during cooling to 5°C or during freezing; *v.* B, 2% glycerol in diluent during cooling to 5°C and during freezing; *v.* C, 2% glycerol in diluent during cooling to 5°C, but removed before freezing; *v.* D, glycerol absent in diluent during cooling to 5°C, but 2% present during freezing.

(3) Three replicates at dilution

The semen was frozen at a sperm concentration of $1{\cdot}0 \times 10^9$ per ml.

Table 5. Effects of type of diluent and sperm concentration on the percentage of motile spermatozoa during post-thawing incubation (experiment 4)

Type of diluent	Incubation period (h)[A]	Sperm concn during freezing ($\times 10^9$/ml)[B]				
		0·5	1·0	1·5	2·0	Means
Tris–fructose–EDTA	0	32·9	31·3	32·9	31·3	32·1
	6	22·0	21·6	25·0	22·0	22·6
	Means	27·7	27·6	28·5	26·1	27·5
BF_3	0	34·5	32·9	31·3	26·5	31·3
	6	23·0	22·0	17·0	13·2	18·6
	Means	26·7	27·3	23·1	19·4	24·1
S-1	0	23·5	25·0	23·5	23·5	23·9
	6	17·0	11·3	9·5	9·5	11·7
	Means	20·3	18·0	17·3	15·9	17·8
Overall means		24·8	24·1	22·8	20·3	

[A] Data for 2- and 4-h incubation periods not presented.
[B] For details in obtaining the indicated sperm concentrations see Materials and Methods.

The results are summarized in Table 6. The mean percentage of motile spermatozoa was higher for the tris–fructose–EDTA than for BF_3 and S-2 diluents ($P < 0{\cdot}005$). There was a significant difference between the glycerol treatments ($P < 0{\cdot}005$). The presence of glycerol at any stage during processing of semen for freezing improved the survival of spermatozoa (A *v.* B, C, and D; $P < 0{\cdot}005$). The percentage of motile spermatozoa during the post-thawing incubation was higher when glycerol was present in the diluent during freezing compared to when it was removed before freezing (C *v.* D; $P < 0{\cdot}005$). When the glycerol was present during freezing, it made no difference whether it was added before of after cooling the semen to 5°C (B *v.* D).

The only significant interaction detected by the analysis was between type of diluent and post-thawing incubation period ($P < 0{\cdot}005$) which was due to the steeper decline in viability of spermatozoa during the 6-h incubation for BF_3 and S-2 than for the tris–fructose–EDTA diluent.

Table 6. Effects of type of diluent and glycerol treatment on the percentage of motile spermatozoa during post-thawing incubation (experiment 5)

Type of diluent	Incubation period (h)[A]	Glycerol treatment[B]				
		A	B	C	D	Means
Tris–fructose–EDTA	0	29·7	36·2	32·9	36·2	33·7
	6	22·0	32·9	28·1	31·3	28·5
	Means	26·9	35·0	30·9	34·1	31·7
BF_3	0	23·5	34·5	25·0	32·9	28·9
	6	15·3	17·0	18·8	18·8	17·4
	Means	20·1	27·0	21·9	25·6	23·6
S-2	0	20·6	31·3	23·5	32·9	26·9
	6	14·9	22·0	14·9	20·6	18·0
	Means	19·0	26·1	19·3	26·1	22·6
Overall means		21·9	29·3	23·9	28·6	

[A] Data for 2- and 4-h incubation periods not presented.
[B] Glycerol treatment: A, no glycerol in diluent during cooling to 5°C or during freezing; B, 2% glycerol in diluent during cooling to 5°C and during freezing; C, 2% glycerol in diluent during cooling to 5°C, but removed before freezing; D, glycerol absent in diluent during cooling to 5°C, but 2% present during freezing.

Discussion

The two semen processing methods examined in experiment 1 differed in the following aspects. The method of Pursel and Johnson (1971) involved removal of the seminal plasma, resuspension of spermatozoa with glycerolated diluent, followed by cooling to 5°C and centrifugation at this temperature to remove the glycerol-containing diluent, and finally resuspension of spermatozoa with the freezing medium containing no glycerol. The processing according to Visser and Salamon (1974) was simpler, and consisted of dilution of the freshly collected semen with glycerol-containing diluent, cooling to 5°C and freezing. Thus, by using the latter method the spermatozoa were frozen in the presence of seminal plasma and glycerol, but both these substances were removed when the freezing was carried out by the method of Pursel and Johnson (1971).

The viability of spermatozoa after freeze–thawing was better for the method of Pursel and Johnson (1971) when BF_3 rather than tris–fructose–EDTA diluent was used. Both diluents yielded similar viability results when the semen was processed according to the method of Visser and Salamon (1974) (see Table 1). A prolonged period (6 h) of cooling of the diluted semen to 5°C and high glycerol concentration (7·5%) decreased the post-thawing survival of spermatozoa.

Although processing of semen for freezing had an effect on the viability of spermatozoa, the proportion of undamaged sperm cells for the methods examined was indistinguishable and relatively low (Table 3). This indicates that, apart from estimation

of viability, attention should also be paid to the morphological picture of the frozen–thawed spermatozoa when assessing by laboratory means any processing method for freezing, or the parameters linked with the method.

The fact that a substantial proportion of boar spermatozoa is damaged during the freeze–thawing procedure raises the problem of introducing a sufficient number of undamaged spermatozoa into the female reproductive tract during insemination (via the cervix). This can be achieved by inseminating a large number of total spermatozoa, for which, however, semen frozen in a concentrated state is required. Such an attempt was undertaken by Graham and Crabo (1972) who found that freezing of boar semen in TEST–yolk buffer at a cell concentration above $0{\cdot}25 \times 10^9$ per ml depressed the recovery of spermatozoa. Pursel and Johnson (1975), however, were successful in freezing boar semen in BF_5 diluent at a sperm concentration of $0{\cdot}6 \times 10^9$ per ml. The results of post-thawing viability of spermatozoa in experiments 3 and 4 support the finding of the latter workers, and furthermore indicate that boar semen can be frozen at higher sperm concentration. Among the diluents examined here, the tris–fructose–EDTA medium proved suitable for freezing of semen at a sperm concentration as high as $2{\cdot}0 \times 10^9$ per ml.

It is pertinent to mention that, apart from the diluents used, there was also a difference in the processing method for freezing concentrated semen in the present study and in the work of Pursel and Johnson (1975). While these workers added the glycerol to the cooled semen immediately before freezing, glycerolization in experiments 3 and 4 of this study occurred during the 3-h cooling period, and the glycerol was removed before freezing. Examination of different glycerol treatments in experiment 5 revealed no difference between addition of glycerol before cooling and after cooling, but the presence of glycerol during freezing was beneficial for maintenance of viability of spermatozoa (Table 6).

It should be noted that in the experiments reported here on freezing of boar semen in a concentrated state only the viability of spermatozoa was used for assessment, and this remains to be supplemented by morphological examination of spermatozoa and fertility tests.

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