DEEP FREEZING OF BOAR SEMEN

II.* EFFECTS OF METHOD OF DILUTION, GLYCEROL CONCENTRATION, AND TIME OF SEMEN–GLYCEROL CONTACT ON SURVIVAL OF SPERMATOZOA

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Abstract

Five factorial experiments were conducted to examine the effects of glycerol concentration and processing procedures prior to freezing on the revival of boar spermatozoa upon thawing.

The results after addition of glycerol to the semen at 30°C (one-step dilution) or after cooling to 5°C (two-step dilution) depended on glycerol concentration and the time of exposure of spermatozoa to glycerol during cooling to 5°C or during storage at this temperature or both. One-step dilution with cooling for 0·5 hr required higher glycerol concentration than 1·0- and 2·0-hr cooling periods. Storage of semen at 5°C after both one-step and two-step dilutions decreased the revival rates. After two-step dilution, the contact of semen with glycerol for 5 s to 10 min was sufficient for protection of spermatozoa during freezing. Glycerol concentrations providing maximum protection during 5–20 s, 30 s, and 5–10 min contact periods were 8, 6, and 4% respectively.

I. INTRODUCTION

Preparative procedures of boar semen prior to freezing by the conventional slow method have been examined by several workers (reviewed by King and Macpherson 1966), but information relevant to the pellet-freezing technique is limited. Bamba *et al.* (1968) found that optimum protection to sperm cells during pellet freezing was given by 1·5–3·0% final glycerol concentration. Graham *et al.* (1971) claimed that holding the sperm-rich fraction of boar ejaculate at 22°C for 2 hr before dilution improved the recovery of spermatozoa on thawing, and reduced the loss of glutamic oxalacetic transaminase enzyme from the cells. In other reports on pellet freezing of ejaculated semen (Rohloff 1967; Leidl 1968; Dalrymple and Macpherson 1969; Rohloff and Allmeling 1970, 1971) or epididymal boar spermatozoa (Grove *et al.* 1968) preparative methods were either not compared or insufficient information is provided.

The experiments reported here examined the effects of glycerol concentration and processing procedures prior to freezing on the revival of boar spermatozoa upon thawing.

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II. Materials and Methods

Semen was collected from Large White, Landrace, and Large Black boars by the manual method and only the sperm-rich portion of the ejaculates showing good initial motility was used.

The diluent was egg yolk–inositol 180 mM in all experiments and in experiment 2 egg yolk–glucose 315 mM was also used. The concentration of egg yolk in the diluted semen was 15% (v/v), but glycerol concentration was always included as a factor. The final dilution ratio was 1 : 2 (semen : diluent) except in experiment 5 in which it was 1 : 3.

The semen was diluted at 30°C by one addition of the glycerol-containing diluent, or with the non-glycerolated portion when the two-step method of dilution was employed. In the latter case, the glycerolated diluent fraction was added either 30 min after the first dilution (expt. 2) or following cooling to 5°C. When using the two-step dilution technique and 1 : 2 final dilution, half of the total amount of diluent needed was non-glycerolated and the other half glycerolated, except in experiment 5, where the final dilution was 1 : 3 [see Section III(*e*)].

The semen was pelleted (0·09 ml, expts. 1–4; 0·12 ml, expt. 5) on dry ice following cooling to 5°C, or after different periods of storage at this temperature (expt. 2) and subsequently stored in liquid nitrogen at −196°C for 24–48 hr before thawing for examination.

The pellets were thawed in dry test tubes shaken in a water-bath at 37°C. The percentage of motile spermatozoa following thawing was estimated as described previously (Salamon *et al.* 1973).

The experiments were of factorial design using three samples from pooled ejaculates of two boars for the prefreezing dilution (: 3) and three samples at thawing (: 3) for each treatment.

Data for all experiments, following angular transformation, were examined by analysis of variance using the samples at dilution and at thawing (: 3 : 3 in design), or in experiment 2 the samples at dilution (: 3) and the means of the three thawing samples. The mean square for within samples at thawing was used as error term in all, except experiment 2.

III. Experimental and Results

(*a*) *Experiment 1*

This experiment (3×3 : 3 : 3 factorial) examined the effect of time of cooling to 5°C (0·5, 1·0, 2·0 hr) following one-step dilution at 30°C and glycerol concentration (2·5, 5·0, 7·5%, v/v in 1 : 2 diluted semen). Additional semen samples were also extended with "control" diluents (containing no glycerol), cooled at the indicated rates, and subsequently pelleted.

There was no difference between the percentages of motile spermatozoa prefreezing for the three glycerol concentrations when cooled at different rates, all of which were similar to those observed for the controls. There were no surviving spermatozoa in the control samples following pelleting and subsequent thawing.

The analysis of variance on the post-thawing data revealed a cooling time × glycerol concentration interaction ($P < 0{\cdot}01$; Table 1). The recovery rates improved with increasing concentration of glycerol when the semen was cooled in 0·5 hr, but 5% glycerol gave better results than 2·5 and 7·5% concentrations if the cooling was extended to 1·0- or 2·0-hr periods.

(*b*) *Experiment 2*

The experiment was of 2×2×3×3 : 3 factorial design as shown below:

(1) Type of diluent: egg yolk–glucose 315 mM *v.* egg yolk–inositol 180 mM.

(2) Glycerol concentation: 2·5 *v.* 5% (v/v in 1 : 2 diluted semen).

(3) Method of dilution: (i) one addition of glycerolated diluent at 30°C;
(ii) addition of non-glycerolated and of glycerolated portions at 30°C with 30-min intervals;
(iii) addition of non-glycerolated diluent at 30°C and of glycerolated portion at 5°C.

(4) Time of storage at 5°C prior to freezing ("aging"): 0 *v.* 2 *v.* 4 hr.

The percentage of motile spermatozoa prefreezing was not affected by aging or by variation of glycerol concentration.

TABLE 1

EXPERIMENT 1: INTERACTION BETWEEN TIME OF COOLING TO 5°C AND GLYCEROL CONCENTRATION ON THE PERCENTAGE OF MOTILE SPERMATOZOA FOLLOWING THAWING

Cooling time to 5°C (hr)*	Glycerol concn. (% v/v)†			Means
	2·5	5·0	7·5	
0·5	24·5	25·5	28·6	26·2
1·0	22·0	28·1	24·0	24·7
2·0	27·0	29·1	26·5	27·6
Means	24·5	27·6	26·4	26·1

* After one-step dilution at 30°C with the glycerol-containing diluent. Pelleting on dry ice was done immediately after cooling to 5°C.

† In 1 : 2 diluted semen.

The analysis of variance on the post-thawing data was carried out by using the three samples at dilution and the means of three samples at thawing. The results are presented in Table 2. The mean recovery rate for egg yolk–glucose was higher

TABLE 2

EXPERIMENT 2: SURVIVAL OF SPERMATOZOA IN RELATION TO TYPE OF DILUENT, METHOD OF DILUTION, GLYCEROL CONCENTRATION, AND TIME OF STORAGE AT 5°C PRIOR TO FREEZING

$n = 54$		$n = 36$		$n = 54$		$n = 36$	
Diluent type	Motile sperm (%)	Method of dilution*	Motile sperm (%)	Glycerol concn. (% v/v)†	Motile sperm (%)	Time of storage at 5°C (hr)	Motile sperm (%)
Egg yolk–glucose	25·0	(i) One-step	24·7	2·5	22·5	0	28·2
Egg yolk–inositol	23·5	(ii) Two-step	23·7	5·0	27·2	2	24·4
		(iii) Two-step	26·0			4	22·0
P	<0·01		<0·001		<0·001	Linear	<0·001
						Quadratic	n.s.

* See Section III(*b*). † In 1 : 2 diluted semen.

than for egg yolk–inositol diluent ($P < 0{\cdot}01$). The most efficient method of dilution was the two-step method, by adding the non-glycerolated diluent at 30°C and the

glycerolated portion after cooling to 5°C. Glycerol at 5% concentration in the diluted semen gave better protection to spermatozoa than 2·5% glycerol ($P < 0·001$). Storage of semen at 5°C following cooling was not beneficial and the recovery rates declined as the time of storage increased (linear, $P < 0·001$). There was a diluent type × glycerol concentration interaction ($P < 0·05$), nevertheless the best recovery with both glucose and inositol diluents was obtained when 5% rather than 2·5% glycerol was used. There were no other interactions.

(*c*) *Experiment 3*

The experiment (3 × 3 : 3 : 3 factorial) included the following factors:

(1) Glycerol concentration: 2 *v.* 4 *v.* 8% (v/v in 1 : 2 diluted semen).

(2) Method of dilution:
- (i) one addition of glycerolated diluent at 30°C;
- (ii) addition of non-glycerolated diluent at 30°C and of glycerolated portion at 5°C. Interval between second dilution and freezing 8–10 min;
- (iii) as in method (ii), but the glycerolated diluent was mixed, using the pelleting pipette, in vials just before pelleting. Interval between second dilution and pelleting 5–20 s.

There was an interaction between method of dilution and glycerol concentration ($P < 0·01$; Table 3). While increasing concentration of glycerol had a depressing effect when the one-step dilution at 30°C was employed [method (i)], the recovery rates improved with increasing glycerol concentration when adopting the two-step method of dilution with contact of spermatozoa with the protective agent for 5–20 s [method (iii)]. In the case of two-step dilution with sperm–glycerol contact for 8–10 min [method (ii)] 4% glycerol was the optimum concentration.

TABLE 3

EXPERIMENT 3: INTERACTION BETWEEN METHOD OF DILUTION AND GLYCEROL CONCENTRATION ON THE PERCENTAGE OF MOTILE SPERMATOZOA FOLLOWING THAWING

Method of dilution*	Glycerol concn. (% v/v)†						Means
	2·0		4·0		8·0		
(i) One-step	(67)‡	25·5	(67)	24·0	(62)	21·9	23·8
(ii) Two-step	(58)	25·0	(62)	29·1	(58)	26·5	26·9
(iii) Two-step	(63)	23·0	(62)	25·0	(52)	26·5	24·8
Means		24·5		26·0		24·9	25·2

* See Section III(*c*). † In 1 : 2 diluted semen.

‡ Values in parentheses are percentages of motile spermatozoa pre-freezing.

(*d*) *Experiment 4*

In this experiment (3 × 5 : 3 : 3 factorial) the time of storage of semen at 5°C following two-step dilution (30 s, 5 min, 50 min) and glycerol concentration (4, 6, 8, 10, 12% v/v in 1 : 2 diluted semen) were examined.

The results are presented in Table 4. The mean cell recovery rate for the 30 s storage of semen at 5°C prior to freezing was higher than for 5 and 50 min storage periods ($P < 0{\cdot}01$). There was a decrease in recovery rates with increasing concentration of glycerol in the diluted semen (linear, $P < 0{\cdot}001$) and the effect was more pronounced when the glycerol was in contact with spermatozoa for 5 and 50 min (glycerol concentration × time of storage; $P < 0{\cdot}05$). Recovery was very similar with all three storage periods when the glycerol concentration was 4%.

TABLE 4

EXPERIMENT 4: EFFECT OF TIME OF STORAGE OF DILUTED SEMEN AT 5°C PRIOR TO FREEZING AND OF GLYCEROL CONCENTRATION ON THE PERCENTAGE OF MOTILE SPERMATOZOA FOLLOWING THAWING

Glycerol concn. (% v/v)*	Time of storage at 5°C†			Means
	30 s	5 min	50 min	
4	29·7	30·2	29·7	29·8
6	30·2	27·6	28·1	28·6
8	26·0	21·9	20·8	22·9
10	20·5	17·0	17·6	18·3
12	17·0	12·6	16·7	15·4
Means	24·5	21·5	22·3	22·7

* In 1 : 2 diluted semen.

† After addition of glycerolated diluent portion at 5°C; cooling of partially (1 : 1) diluted semen from 30 to 5°C in 2·5 hr.

(e) *Experiment 5*

The factors examined in this experiment (3 × 5 : 3 : 3 factorial) were glycerol concentration (2, 4, 8% v/v in 1 : 3 diluted semen) and method of dilution. The latter factor varied in the following manner:

(1) One addition of glycerol containing diluent at 30°C (control).

(2) Addition of non-glycerolated diluent at 30°C (1 : 1) and of glycerolated portion at 5°C just before pelleting (1 : 1, diluted semen : diluent). Time of contact of spermatozoa with glycerol 15–20 s.

(3) First addition as in method (2), second addition at 5°C by pipetting on dry ice alternatively diluted semen from first-step dilution and glycerolated diluent. Order of droplets in quick succession was: one drop of diluted semen + one drop of diluent + one drop of diluted semen + one drop of diluent, total of four drops.

(4) As in method (3), except that the order of droplets was: two drops of semen diluted with non-glycerolated diluent + two drops of glycerolated diluent.

(5) As in method (3), except that the order of droplets was: two drops of glycerolated diluent + two drops of semen diluted with non-glycerolated diluent.

The semen samples diluted at 30°C to final rate (method 1) or with the non-glycerolated diluent (methods 2–5) were cooled to 5°C in 2 hr.

There was an interaction between method of dilution and glycerol concentration. Table 5 shows that while the recovery rates improved with increasing glycerol concentration from 2 to 8% when spermatozoa were in contact with the protective agent for a very short time (methods 2, 3, 4, 5), the optimum glycerol concentration in the control treatment (1) was 4% [(method 1 *v.* methods 2, 3, 4, 5) × glycerol concentration, linear and quadratic, $P < 0{\cdot}001$]. The revival rates of spermatozoa in method (2) with 8% glycerol and for the control treatment (1) with 4% glycerol were similar. Dilution methods (3), (4), and (5), in which the partially diluted semen came in contact with the glycerolated diluent on the dry ice, yielded markedly lower results with all glycerol concentrations than methods (1) and (2).

TABLE 5

EXPERIMENT 5: EFFECT OF METHOD OF DILUTION AND OF GLYCEROL CONCENTRATION ON THE PERCENTAGE OF MOTILE SPERMATOZOA FOLLOWING THAWING

Method of dilution*	Glycerol concn. (% v/v)†			Means
	2·0	4·0	8·0	
1—One-step (control)	19·8	29·1	20·8	23·1
2 Two-step	17·4	24·5	29·1	23·5
3 Two-step	9·0	11·8	15·7	12·0
4 Two-step	7·9	8·9	13·1	9·9
5 Two-step	5·9	7·9	11·3	8·3
Means	11·5	15·5	17·6	14·8

* See Section III(*e*). † In 1 : 3 diluted semen.

IV. DISCUSSION

A strict recommendation whether to add the glycerol to the semen at 30°C (one-step dilution), or after cooling to 5°C (two-step dilution) cannot be made, as the cell recovery rates depended upon the glycerol concentration and on the time of exposure of spermatozoa to the protective agent during cooling to 5°C or during storage (aging) at this temperature or both. The one-step dilution method followed by 0·5 hr cooling to 5°C required higher glycerol concentration than 1·0- and 2·0-hr cooling periods (Table 1). On the other hand, prolonged exposure of semen to glycerol at 5°C (aging) after either the one-step or the two-step method of dilution decreased the revival of spermatozoa on thawing (Table 2). When the glycerol was added to the semen after cooling, contact of spermatozoa with the glycerol for 5–20 s was sufficient for protection of cells during freezing. The results, however, with short contact periods depended on the concentration of the protective agent. Thus, glycerol concentrations providing maximum protection during 5–20 s, 30 s, and 5–10 min contact periods were 8, 6, and 4% respectively. The shortest sperm–glycerol contact achieved by pipetting semen and glycerol-containing diluent on dry ice also required 8% glycerol, the highest concentration examined in this method, although revival was much lower than by adding the glycerol to the semen in vials just before pelleting

(Table 5). When the latter method of glycerol addition was adopted, glycerol concentrations exceeding 6% and in contact with spermatozoa for 30 s decreased the recovery of spermatozoa (Table 4).

Post-thawing motility of bull spermatozoa is increased by reducing to 10 s the length of exposure of spermatozoa to glycerol (Berndtson and Foote 1969, 1972), and maximum revival was obtained by using higher glycerol concentrations than were necessary after exposure for 6 hr. The authors surmize that the glycerol had not fully penetrated the spermatozoa within 10 s and that as the cells were dehydrated at the time of pelleting they were better able to survive freezing and thawing.

Measurement of the rate of penetration of glycerol into spermatozoa which are suspended in physiological media is difficult. The time taken for lysis of cells suspended in aqueous solutions of penetrating compounds has been used as an index of relative permeability (Drevius 1971, 1972). When bull and boar spermatozoa were suspended in glycerol solution, membrane rupture occurred in less than 8 s with bull spermatozoa (Drevius 1971), and in less than 2 s with boar spermatozoa which had been cooled to 5°C (Wilmut 1971). Thus there would seem to be a species difference in the rate of penetration of glycerol into the spermatozoa.

V. References

Bamba, K., Kojima, Y., and Iida, I. (1968).—Studies on deep-freezing of boar semen. V. The effect of honey-yolk diluent on the livability of frozen boar spermatozoa. *Jap. J. Zootech. Sci.* **39**, 415.

Berndtson, W. E., and Foote, R. H. (1969).—The survival of frozen bovine spermatozoa following minimum exposure to glycerol. *Cryobiology* **5**, 398.

Berndtson, W. E., and Foote, R. H. (1972).—The freezability of spermatozoa after minimal pre-freezing exposure to glycerol or lactose. *Cryobiology* **9**, 57.

Dalrymple, J. R., and Macpherson, J. W. (1969).—Low temperature preservation of boar spermatozoa. *Can. J. Anim. Sci.* **49**, 45.

Drevius, L.-O. (1971).—Permeability coefficients of bull spermatozoa for water and polyhydric alcohols. *Exp. Cell Res.* **69**, 212.

Drevius, L.-O. (1972).—The permeability of bull spermatozoa to water, polyhydric alcohols and univalent anions and the effects of the anions upon the kinetic activity of spermatozoa and sperm models. *J. Reprod. Fert.* **28**, 41.

Graham, E. F., et al. (1971).—Preliminary report on procedure and rationale for freezing boar semen. *A. I. Digest* **19**(1), 12.

Grove, D., Bollwahn, W., and Mahler, R. (1968).—Versuche zur Gewinnung und Tiefgefrierkonservierung von Nebenhodenschwanzsperma beim Eber. *Dt. tierärztl. Wschr.* **75**, 35.

King, G. J., and Macpherson, J. W. (1966).—Boar semen studies. I. Laboratory evaluation of processing phases. *Can. J. Comp. Med.* **30**, 332.

Leidl, W. (1968).—Neue Verfahren in der Künstlichen Besamung der Wirbeltiere. Proc. 6th Int. Congr. Anim. Reprod., Paris. Vol. 2. p. 951.

Rohloff, D. (1967).—Tiefgefrierung von Ebersperma in Pelletform bei −196°C. *Zuchthygiene* **2**, 75.

Rohloff, D., and Allmeling, G. (1970).—Beitrag zur Eberspermatiefgefrierung. *Zuchthygiene* **5**, 88.

Rohloff, D., and Allmeling, G. (1971).—Zur Tiefgefrierung von Ebersamen. *Berl. Münch. tierärztl. Wschr.* **84**, 330.

Salamon, S., Wilmut, I., and Polge, C. (1973).—Deep freezing of boar semen. I. Effects of diluent composition, protective agents, and method of thawing on survival of spermatozoa. *Aust. J. biol. Sci.* **26**, 219.

Wilmut, I. (1971).—The preservation of boar semen. Ph.D. Thesis, University of Cambridge.