

EFFECT OF INITIAL FREEZING TEMPERATURE, ADDITION OF GLYCEROL AND DILUTION ON THE SURVIVAL AND FERTILIZING ABILITY OF DEEP-FROZEN RAM SEMEN

G. COLAS

*I.N.R.A.-Station de Physiologie de la Reproduction,
Nouzilly, 37380 Monnaie, France*

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Summary. The influence of temperature, addition of glycerol, initial freezing temperature, method of dilution, level of glycerol in the diluted semen, equilibration time and type of diluent on the survival and fertilizing capacity of deep-frozen ram spermatozoa was studied. The fertilizing capacity of semen frozen according to the best conditions was compared with that of 'fresh' semen.

The addition of glycerol at +30°C resulted in a highly significant decrease in the mean proportion of motile spermatozoa immediately after thawing compared with the effect of addition at +4°C. The immersion of the straws at -55°C significantly reduced the revival of the spermatozoa compared with initial freezing at lower temperatures. The exposure time to glycerol had no significant effect on the survival of spermatozoa after thawing and incubation, but fertility was significantly higher with 4% than with 2% glycerol. The I.N.R.A. diluent provided better sperm survival and a significantly higher conception rate than did lactose-egg yolk extender. The semen frozen according to the best conditions (about 50% of the samples) had a fertilizing ability similar to that of 'fresh' semen when the proportion of motile spermatozoa before, and after 1 or 3 hr of incubation was equal to or above 45, 40 and 30% respectively.

INTRODUCTION

The setting up of new intensive methods for sheep breeding has recently aroused particular interest in the storage of ram semen at very low temperature. Several studies have been conducted concerning this problem but, in spite of some encouraging results, it seems that it has been impossible up to the present time to freeze ram spermatozoa without reducing their fertilizing ability (Salomon, 1971, 1972). Most of the manipulations which are necessary for the preparation of the spermatozoa, before freezing, are generally assessed only after tests *in vitro*, which are not always related to the fertility of the semen. For example, in the pig, a slight variation in the level of glycerol can considerably

modify the fertilizing rate and this cannot be predicted from tests *in vitro* (Graham, Rajamannan, Schmehl, Maki-Laurila & Bower, 1971; Wilmut & Polge, 1972).

Important progress in the freezing of boar semen has recently been achieved by Wilmut & Polge (1972), Graham & Crabo (1972), Richter & Leidicke (1972) and Paquignon & du Mesnil du Buisson (1973) who have used various media, but similar detailed studies have not been carried out for sheep.

This paper presents the results of a series of experiments in which certain stages in the deep-freezing of ram semen have been subjected to analysis *in vitro* and *in vivo*.

MATERIALS AND METHODS

Experimental design

The following factors were studied in five experiments: Exp. 1, the temperature at which glycerol was added and the subsequent cooling rate; Exp. 2, the method of dilution of the semen—constant rate (v/v) versus constant sperm concentration; Exp. 3, the final concentration of glycerol in the diluted semen and the equilibration time; Exp. 4, the comparison of Diluent 1 versus Diluent 2; Exp. 5, the fertilizing ability of semen frozen according to the best combination of the above factors as compared with semen stored at +15°C (referred to as 'fresh semen' since the duration of storage was ≤ 12 hr).

Collection and evaluation of semen

Semen was collected by means of an artificial vagina from Île-de-France and Lacaune rams during the non-breeding season for tests *in vitro* and during the breeding season for artificial insemination.

The semen volume, motility (scored on a scale of 0 to 5) and concentration (estimated by a photocolormeter) were recorded for each ejaculate immediately after collection. Only ejaculates with good initial motility ($> 4/5$) were used for fertility tests. The experiments *in vitro* were all performed on split ejaculates.

Treatment of semen

The semen was diluted to a constant rate (1:4, semen:diluent) or to a constant sperm concentration of 900×10^6 /ml after addition of glycerol. Dilution was performed either in one step at 30°C by a single addition of a diluent containing 321 mM-lactose and 20% fresh egg yolk (v/v) and 5% glycerol (Nagase & Graham, 1964), or in two steps at 30°C and 4°C. The diluent added at 30°C for the two-step dilution contained no glycerol but was otherwise similar to that used for the one-step dilution. The diluent added for the two-step dilution at 4°C contained 5% or 10% glycerol.

Two types of glycerolated extenders were used: Diluent 1 was a solution of 80% (v/v) lactose (343 mM) and egg yolk (20%, v/v) and Diluent 2 was the patented I.N.R.A. diluent*. The non-glycerolated fraction of this diluent was prepared from reconstituted skim-milk as used for the storage of the fresh

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semen (Colas, Dauzier, Courot, Ortavant & Signoret, 1968). The milk suspension was added to powdered skim-milk (4 g powder/100 ml reconstituted skim-milk), and the pH was adjusted to 6.60 to 6.65 with a concentrated solution of 800 mM-trisodium citrate.

In practice, the two-step dilution was carried out in the following manner. When diluting to a constant rate (1:4), one part of semen was added to two parts of lactose-egg yolk solution at 30°C and, after cooling to 4°C, a further two parts of diluent containing glycerol were added. When diluting to a constant sperm concentration, the final volume of diluted semen to give a sperm concentration of 900×10^6 /ml was calculated. Sufficient non-glycerolated diluent was then added to the semen at 30°C to give a volume equal to three-fifths of the final volume required. After cooling to 4°C, a volume of diluent containing glycerol equal to two-fifths of the final volume was added. Both methods of dilution provided a final concentration of glycerol in the diluted semen (before freezing) of either 2% or 4%, only when diluting to a constant sperm concentration (900×10^6 spermatozoa/ml). The number of molecules of glycerol per sperm cell in the final concentration of 4% glycerol was exactly double that in the semen containing 2% glycerol. When diluting to a constant rate (1:4), the final concentration of glycerol was always 4%. Semen was chilled from 30°C to 4°C ($\pm 1^\circ\text{C}$) over approximately 2 hr and immediately extended at this temperature with glycerolated diluent which was added on two occasions 20 min apart.

After 20 or 150 min, the extended semen (0.45 ml) was put into plastic straws which were suspended for 8 min at different temperatures (-55°C , -75°C , -90°C , -105°C , -125°C) in the vapour above liquid nitrogen before being plunged into the liquid for storage. In the present study, these temperatures are designated the 'initial temperature of freezing'.

For fertility trials, the duration of storage did not exceed 2 months.

Examination of the frozen semen

A system of coding of treatments was used in all experiments *in vitro* to avoid subjective bias.

Semen was thawed at 37 to 38°C for 30 sec. It was then resuspended in 2 ml trisodium citrate solution (87 mM, pH 8.2). The percentage of motile spermatozoa was estimated microscopically at a magnification of $\times 200$ at 37 to 38°C immediately after thawing (Exps 1 and 2) or after incubation for 0, 1 and 3 hr at 37 to 38°C (Exps 3, 4 and 5).

The ejaculates prepared for fertility trials (one straw/ejaculate) were examined by the same methods. In Exps 1 and 2, only ejaculates were used in which the revival rate immediately after thawing was equal to or above 45%. In Exps 3, 4 and 5, the percentages of motile spermatozoa after incubation for 0, 1 and 3 hr had to be 45, 40 and 30%, respectively, or better.

Thus, in all experiments, the minimum number of motile spermatozoa used for artificial insemination for each dose, as estimated immediately after thawing, was 180×10^6 .

Treatment of the ewes and insemination techniques

A total of 647 ewes was inseminated. Ewes were from the I.N.R.A. laboratory

flock (Île-de-France; Exp. 1) or from commercial flocks (Lacaune, Suffolk, Île-de-France and Romanov × Charmoise; Exps 2 to 5). Oestrus was synchronized by inserting vaginal sponges impregnated with 40 mg fluorogestone acetate for 14 days. At the time of withdrawal of the sponges, the ewes were injected with 400 i.u. PMSG (Colas & Brice, 1970).

Before insemination, straws were plunged into a water bath at 37 to 38°C for 30 sec. Ewes were then inseminated with one straw of thawed semen according to the routine method of the laboratory: either during the first oestrus induced by treatment without preliminary inspection for heat at 50 and 60 hr after the withdrawal of the vaginal sponges, or during the second oestrus following treatment. Two inseminations were made 12 hr apart. Some ewes from the same flocks acted as controls (Exp. 5) and were inseminated twice at natural oestrus (500×10^6 spermatozoa/ewe) with fresh semen diluted to 1×10^9 spermatozoa/ml in skim milk and stored at 15°C for no more than 12 hr (Colas *et al.*, 1968).

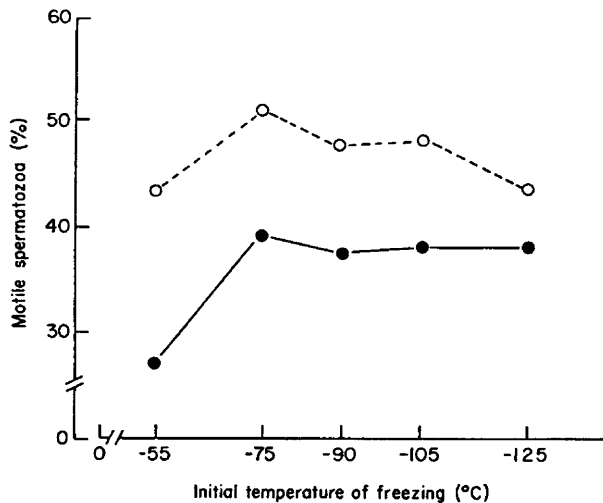
Analysis of data

The proportions of motile cells were transformed to angles and examined by analyses of variance. Fertility results were expressed as the percentage of ewes in which pregnancy was confirmed by laparotomy 40 days after the first insemination (Exp. 1) or by lambing rates in the other experiments, and were analysed by χ^2 tests.

RESULTS

Experiment 1

The results of the comparison of the temperature (30°C and 4°C) at which glycerol was added and the freezing rates are presented in Text-fig. 1. The



TEXT-FIG. 1. The effect of the temperature at which glycerol was added (●, +30°C; ○, +4°C) and the initial temperature of freezing on the revival of ram spermatozoa after thawing (N = 22).

addition of glycerol at 30°C resulted in a highly significant ($P < 0.01$) decrease in the mean proportion of motile spermatozoa immediately after thawing (36.1% versus 47.0%) and a lower percentage of pregnant ewes (35.0% versus 48.0%; twenty-three ewes (N) in each treatment group). There was no apparent correlation between the temperature at which glycerol was added and the initial temperature of freezing on the motility of the thawed spermatozoa.

The initial temperature of freezing had a significant ($P < 0.01$) influence on revival only when it was -55°C . There was no significant difference in the proportion of motile spermatozoa after initial freezing at any of the other temperatures. The best revival of spermatozoa was obtained when the straws were cooled at a temperature of -75°C , before storage in liquid nitrogen, regardless of whether glycerol was added at $+30^{\circ}\text{C}$ or $+4^{\circ}\text{C}$.

Experiment 2

The ejaculates used were selected so that each inseminate contained approximately the same number of motile spermatozoa. The percentage of ewes which lambed was higher when semen was diluted to a constant concentration of 900×10^6 spermatozoa/ml (34.0%; N = 91) than at a constant rate of 1:4 (25.0%; N = 101) but the difference was not significant ($P > 0.05$).

Table 1. Effects of the level of glycerol in diluted semen and the equilibration time on the survival during incubation after thawing and the fertilizing ability of deep-frozen ram spermatozoa

Glycerol in diluted semen (% v/v)	Survival (% motile spermatozoa)						Fertility
	Equilibration time (min)		Time of incubation (hr)				Conception rate (%)
	20	150	0	1	3	Mean	
2	33.4	40.4	46.5	41.1	23.0	36.9	38.5 (91) $\chi^2 = 4.38$
4	39.0	38.0	48.6	42.6	24.3	38.5	55.7 (79) $P < 0.05$
Mean	36.2	39.2	47.6	41.9	23.7		

For each treatment *in vitro*, values are the means of twelve replications on split ejaculates. The ewes (number given in parentheses) were inseminated after treatment with 40 mg fluorogestone acetate and 400 i.u. PMSG.

Experiment 3

The effects of various concentrations of glycerol and the time allowed for equilibration are shown in Table 1. Increasing the exposure time of semen to glycerol before freezing had no significant effect on the viability of spermatozoa after thawing and incubation, and there was no statistical difference between the 2% and 4% glycerol concentrations. Fertility, however, was significantly higher ($P < 0.05$) with diluted semen containing 4% glycerol (55.7%) than with that containing 2% glycerol (38.5%).

Experiment 4

The survival of spermatozoa after freezing in 4% glycerol was slightly better when the semen had been extended in Diluent 2 than in Diluent 1 (37.0%

versus 33.9% respectively) but the difference was not significant (Table 2). The decline in the proportion of motile spermatozoa after incubation for 1 and 3 hr was different, however, being 21% and 60% in Diluent 1 and 0 and 41% in Diluent 2, respectively. The type of diluent had a significant effect on the fertility of the frozen semen (42.2% versus 73.5% for Diluents 1 and 2, respectively).

Table 2. The effect of type of diluent on the survival and fertilizing ability of deep-frozen ram spermatozoa during incubation after thawing

Diluent (containing 4% glycerol)	Survival (% motile spermatozoa)*				Fertility
	Time of incubation (hr)			Mean	Conception rate (%)
0	1	3			
No. 1	46.7	36.8	18.3	33.9	42.2 (45) $\chi^2 = 6.51$
No. 2	42.7	43.5	24.8	37.0	73.5 (34) $0.01 < P < 0.02$

Figures in parentheses represent the number of ewes inseminated.

* Means are values of fifteen replications on split ejaculates.

Experiment 5

For ewes in natural oestrus, insemination with semen frozen according to the best conditions found in Exps 1 to 4 (9×10^6 spermatozoa/ml, 4% glycerol and Diluent 2) gave a fertility rate of 68.3% (N = 60) compared with that of 71.3% (N = 80) for fresh semen. When insemination was carried out at oestrus induced by treatment with progestagen and PMSG, 75.0% (N = 94) of ewes lambed. None of these values was significantly different ($P > 0.05$).

DISCUSSION

Cryoprotective penetrating agents, particularly glycerol, protect cells against injury during the crystallization phase (Morris & Farrant, 1972). Since crystallization occurred between -6°C and -10°C in our experiments it did not seem logical to include it at $+30^\circ\text{C}$. Most authors, however, except Lunca (1968), have diluted ram semen immediately after collection with a glycerolated medium.

From the results obtained in the first experiment, it is concluded that the recovery rate of spermatozoa after thawing depends on the temperature at which glycerol is added. This factor has been studied by Lightfoot & Salamon (1969) whose conclusions differ from ours, but it is difficult to compare the two experiments since their freezing method was different. The differences caused by addition of glycerol at $+30^\circ\text{C}$ and $+4^\circ\text{C}$ in our experiments cannot be attributed to an insufficient penetration of the cryoprotective agent into the cell at $+30^\circ\text{C}$ since penetration is probably more complete at $+30^\circ\text{C}$ than at $+4^\circ\text{C}$ (Sherman, 1963). It is possible that glycerol is slightly toxic to ram spermatozoa, even at a concentration of 4% and that its harmful effect is less when it is added at a temperature near 0°C .

It is clear that, in the range between -75 and -125°C , the influence of

initial freezing temperature on cell recovery was not very important. This is in agreement with Salamon (1970) who found that freezing at -79°C and -120°C yielded similar results. By contrast, recovery after thawing was worse when the initial temperature of freezing was higher than -75°C . Semi-rapid freezing rates are more harmful to spermatozoa therefore than the more rapid ones.

Blackshaw (1960) found a better recovery rate when spermatozoa were equilibrated for more than 6 hr but First, Henneman & Magee (1959) and Hill, Godley & Hurst (1959) observed no differences after equilibration for $\frac{1}{2}$, 1 or 18 hr. In the present experiment, the slightly better results after equilibration for $2\frac{1}{2}$ hr suggest that glycerol penetration into the ram cell is not as rapid as has been shown in the bull (Berndtson & Foote, 1972).

The optimum level of glycerol in the diluted semen appears to be about 4% in the ram. This is in agreement with the observations of Fraser (1968) and Jones (1969), but does not confirm the results of First, Henneman, Magee & Williams (1961) who found that a final concentration of glycerol of 6 to 8% was the most satisfactory.

Better results were obtained when the semen was diluted to a constant concentration of 900×10^6 spermatozoa/ml, i.e. the quantity of glycerol/cell remaining constant, than at a constant ratio (1:4) when the quantity of glycerol/cell would be variable. This suggests that the optimal level of glycerol in the diluted semen is perhaps related to its final concentration relative to the spermatozoa. This relationship does not appear to have been considered by other authors and may be one reason for the diversity of the published findings on the freezing of ram semen.

The use of the I.N.R.A. extender containing glycerol (Diluent 2) for dilution at $+4^{\circ}\text{C}$ improves both survival of semen after incubation and its fertilizing ability. This result has three consequences. First, it shows that the nature of the diluent is very important for the protection of the biological properties of the sperm cell during the freezing process. Secondly, there is no relationship between the recovery rate immediately after thawing and the fertilizing capacity of the semen (Table 2: 46.7% motile spermatozoa for Diluent 1 and 42.7% for Diluent 2). Thirdly, the viability of spermatozoa during incubation after thawing, a test which Salamon often uses in his trials *in vitro*, gives a fairly precise idea of the quality of the semen. It remains necessary, however, to determine the practicality of reducing the examination of the semen to one measure (1 or 3 hr after incubation). We also must investigate whether the lower limits we have chosen for sperm survival can be reduced. At the present time, the rejection rate of ejaculates after freezing and thawing with the best treatment is about 50%.

We have used large numbers of spermatozoa for each insemination to achieve the results presented here. The work of Salamon (1971), Kareta, Pilch & Wierzbowski (1972) and Anderson, Aamdal & Fougner (1973) suggests that this number could be reduced without a deleterious effect on fertility, especially for insemination of ewes in natural oestrus. The fact that ewes in which oestrus was induced by fluorogestone acetate and PMSG had a slightly higher fertility than ewes in natural oestrus suggests that the hormonal treatment does not

create an environment unfavourable for deep-frozen spermatozoa, even though sperm survival may be sharply reduced (Loginova & Zheltobrukh, 1968, 1972).

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