Effect of physical parameters on ram spermatozoal motility

G.C. UPRETI, J. OLIVER, R. MUNDAY AND J. F. SMITH

AgResearch, Ruakura Agricultural Centre, Private Bag 3123,
Hamilton, New Zealand.

ABSTRACT

Spermatozoal motility is known to be influenced both by the diluent used and by the physical parameters involved in the processing of semen. We have evaluated the effect of cooling and dilution on ram spermatozoal motility in a defined semen diluent. The results indicate that ram semen is best stored at 15°C, this temperature being reached by cooling at a rate not greater than 3°C per minute. Time (up to 60 minutes from semen collection) and extent of dilution (500 or 800 x 10^6 spermatozoa per ml) were found to have no effect upon maintenance of spermatozoal motility.

Keywords Ram, spermatozoa, motility, cooling, dilution.

INTRODUCTION

Artificial insemination (AI) has contributed greatly to the development of the dairy cattle industry. Such success is attributable to the development of effective diluents for bovine spermatozoa, which permit prolonged storage at ambient temperature with excellent maintenance of viability (Foote and Dunn, 1962; Foote, 1970; Shannon, 1978).

In contrast, satisfactory diluents for ovine spermatozoa are not avilable and AI in sheep is practiced only to a limited extent. The diluents employed for bovine spermatozoa are not suitable for ram semen (Evans and Maxwell, 1987). We have, however, developed a defined ram semen diluent (RSD-1) which supports spermatozoal motility for prolonged periods at 38°C (Upreti *et al.*, 1991). We are using this diluent to investigate the effects of semen processing upon maintenance of viability, with a view to further extending storage life.

Among the physical parameters involved in semen handling, cooling and dilution have been recognised to influence spermatozoal motility (Watson, 1979; Robertson and Watson, 1986). In this communication, we describe the effects of cooling and of dilution upon ram spermatozoal motility in RSD-1.

MATERIALS AND METHODS

Animals

Semen was collected from trained Poll Dorset rams (<5 years old) which were maintained on ryegrass/clover pastures with supplementary feeding with high-protein nuts.

Semen Collection and Assessment

Semen was collected using an artificial vagina, the collection tube being maintained at 38°C. An aliquot of semen was used for assessment of spermatozoal concentration and motility, while the remainder was transferred to a water bath at 35°C. Spermatozoal concentration was determined by measuring the absorbance (580 nm) of a diluted sample (20 µl semen in 20 ml of 2% saline solution containing 0.1% formalin) the concentration being determined by reference to a standard curve. The motility of the semen was estimated microscopically at 38°C at 100 X magnification. Motility was scored on a scale of 0 to 5, 0 denoting the absence of motion, and 5 indicating rapid swirling motion. Semen samples with a spermatozoal concentration ≥1500 x 10^6 per ml and a motility score of ≥4.0 were used in this study.

Semen Dilution and Transport

Unless otherwise indicated, semen was diluted with RSD-1 (Upreti *et al.*, 1991), and equilibrated at 35°C within 10 minutes of collection. The spermatozoal concentration was adjusted to 800 x 10^6 per ml (unless specified) and the diluted semen was transported to the biochemistry laboratory in a well-insulated vacuum flask.

Trial 1 - Rate of Cooling

Test tubes (~10 ml) containing 1 ml of diluted semen at 35°C were plunged into a 15°C waterbath with or without a water-jacket containing 0, 20, 50 or 100 ml of water at 35°C and an equal volume of glass beads (~4 mm diameter) in a 250 ml beaker. The temperature change in the diluted semen was recorded at 30 sec intervals using a thermometer. The semen was held at 15°C for 24 h then incubated at 38°C and motility estimates made 6 and 24 h later.

Trial 2 - Extent of Cooling

Diluted samples of semen at 35°C in test tubes with a water-jacket containing 100 ml of water were cooled to 25, 20, 15, 10 or 4°C and held at these temperatures for 24 h. They were then incubated at 38°C, and motility estimates were made 6 and 24 h later.

Trial 3 - Time of Dilution

Semen samples were equilibrated at 35°C after collection and held for 5, 10, 20, 30 or 60 min before dilution. Upon dilution the semen samples were cooled to 15°C, held at that temperature for 4 h and then incubated at 38°C. Motility measurements were made at each time of dilution and after 0.5 and 24 h of incubation.

Trial 4 - Extent of Dilution

Samples of semen were diluted to either 800 or 500 x 10^6 spermatozoa per ml, cooled to 15°C and held at that temperature for 24 h. The samples were then incubated at 38°C for 4 h before

further dilution to 100 x 10^6 spermatozoa per ml. They were then incubated at 38°C, and motility estimates were made 8 and 24 h later.

Spermatozoal Motility Determinations

The percentage of motile spermatozoa and their progressive motility was determined by the method previously described for bovine spermatozoa (Upreti *et al.*, 1988), with minor modifications. Briefly, the cooled semen sample was diluted to a spermatozoal concentration of 100 x 10^6 per ml, using RSD-1 equilibrated at the same temperature. The diluted semen samples were incubated at 38°C under an air atmosphere. Motility parameters (percentage of motile spermatozoa and motility score on a scale of 0 to 5) were estimated microscopically (100 X magnification).

Statistical Analysis

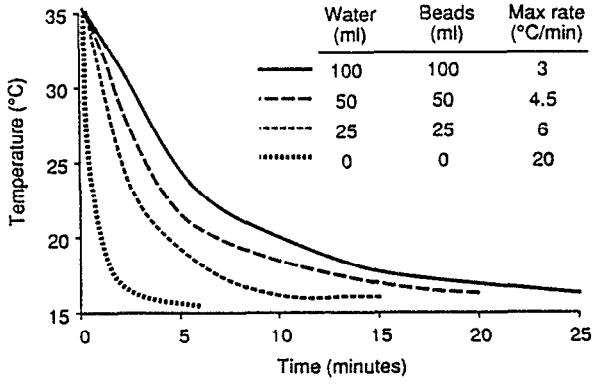
Data were analysed using the ANOVA procedure in the GENSTAT statistical package, fitting split plot models as appropriate.

RESULTS

1. Effect of Rate of Cooling

When test tubes containing 1 ml of diluted semen at 35°C were plunged into a 15°C waterbath, the rate of cooling was ~20°C per minute. The cooling followed an exponential curve and the maximum rate of cooling was calculated from the initial slope. The rate of cooling was decreased by introducing a water-jacket around the test tube. The rate varied with the amount of water in the jacket (Fig. 1).

FIGURE 1 Relationship between cooling rates and water-jacket volume. Samples were cooled from 35 to 15 °C.



With 0, 25, 50 and 100 ml of water (35°C) in the water-jacket, the cooling rates were 20, 6, 4.5 and 3°C per minute when samples were cooled to 15°C (Fig. 1). The influence of cooling on the maintenance of spermatozoal motility at 38°C showed that decreasing the rate of cooling had beneficial effects on both percent motile ($P<0.05$) and motility score. Although a cooling rate of 3°C/min was better than either the 4.5°C or 6°C/min, the differences were not statistically significant (Table 1).

TABLE 1 Effect of cooling rate on motility score and percent motile (in parentheses)

Time of incubation (h)	Cooling rate (°C/min) 20	6.0	4.5	3.0
6	2.88 (53.3)	3.17 (58.3)	3.21(60.0)	3.29 (63.3)
24	2.66 (39.3)	2.75 (45.0)	2.75(46.7)	2.83 (52.5)

Semen diluted to 800 x 10^6 spermatozoa per ml, cooled to 15 °C for 24 h. Incubated at 38 °C (100 x 10^6 spermatozoa per ml) for motility observations at 6 and 24 h.

Cooling rates of 20, 6, 4.5 and 3 °C/min were obtained by using 0, 25, 50 and 100 ml of water in the water-jacket.

Results shown are the mean of separate determinations, from six rams. For $P<0.05$ LSD = 0.24 and 4.84 within columns for score and percent motile respectively

LSD = 0.24 and 5.54 for any pair

2. Effect of Extent of Cooling

The cooling curves of diluted semen samples varied with the degree of cooling. The rates for samples cooled to 25, 20, 15, 10 and 4°C were 1.8, 2.5, 3.0, 4.0 and 4.0°C per minute respectively.

A biphasic response for both motility parameters was observed with an apparent optimum at 15°C. In general, the samples which were not subjected to cold shock and incubated at 38°C for up to 24 h maintained their motility better than the cold shocked semen samples. The differences were significant ($P<0.05$) for samples cooled to 25, 20, 10 and 4°C but not for those at 15°C (Table 2).

3. Effect of Time of Dilution

The motility of raw semen held at 35°C progressively deteriorated, with >90% reduction in motility by 60 minutes (Table 3). The loss of motility, however, was reversible by dilution. There was no difference in the maintenance of motility of the semen samples diluted up to 60 minutes from collection (Table 4).

TABLE 2 Effect of extent of cooling on motility score and percent motile (in parentheses)

Time of incubation (h)	Temperature (°C) NS	38	25	20	15	10	4
6	4.25 (75.0)	3.3 (55.0)	3.05 (49.0)	2.96 (49.0)	3.75 (61.0)	3.12 (52.0)	2.53 (32.3)
24	3.62 (67.0)	0.6 (9.0)	1.60 (25.4)	1.70 (26.5)	3.17 (48.0)	2.16 (26.5)	1.94 (22.2)

Semen diluted to 100 x 10^6 spermatozoa per ml and stored at stated temperature for 24 h (except NS) and incubated at 38°C for motility observations at 6 and 24 h.

NS: semen sample not stored but placed into incubation immediately upon dilution.

Results shown are the mean of separate determinations, from five rams.

For $P<0.05$ LSD = 0.80 and 12.98 within columns for score and percent motile respectively

LSD = 1.18 and 19.73 any other pair

TABLE 3 Motility score of undiluted semen at 35°C

Time (minutes)	Score Range	Mean ± SEM
0	5	5.0
10	5	5.0
20	1-5	4.12 ± 0.34
30	0-4	1.58 ± 0.42
40	0-3	0.63 ± 0.27
50	0-2	0.21 ± 0.17
60	0-2	0.21 ± 0.17

Motility determinations were made on 12 ejaculates from 4 rams.

4. Effect of Extent of Dilution

Diluted semen containing either 500 x 10^6 or 800 x 10^6 spermatozoa per ml stored (at 15°C for 24 h) and then incubated (at 38°C for 4 h), maintained their motility, when incubated at a spermatozoal concentration of 100 x 10^6 per ml, at 38°C for up to 24 h. The motility parameters ie. score and percent motile (within parenthesis) at 8 and 24 h were 3.29 (53.3) and 1.83 (36.7) for both 500 and 800 million spermatozoal dilution (for $P<.05$ the least significant difference for score and percent motile were 0.26 and 4.4 respectively).

DISCUSSION

The results of the present investigation show that spermatozoa not subjected to cold shock or storage maintained their motility at 38°C better than samples which were stored at various (4 to 38°C) temperatures (Table 2). However, storage of sperm is necessary for transport etc., and cooling of semen will be essential for practical use in AI.

Responses to cold shock vary with the species and with the nature of the diluent (Shannon, 1978; Watson, 1981). It is therefore necessary to evaluate the influence of cooling and dilution for each diluent. Our results have shown that cooling of ram semen in RSD-1 to 15°C is better than cooling to either 20°C or above, or 10°C or below. The greater loss of motility at 5°C compared with ambient temperature has previously been reported (Shannon, 1978; Franceschini *et al.*, 1984; Simpson and White, 1986; Weber, 1989). Such observations are also in accord with the reported phase changes in spermatozoal membranes at ~15°C (Watson, 1981; Leeuw *et al.*, 1990). A number of other investigators, however, have recommended a storage temperature of 5°C for a number of species (Parrish and Foote, 1986; Evans and Maxwell, 1987; Varner *et al.*, 1988). These discrepancies possibly reflect species differences or an effect of diluent.

The differences in the maintenance of motility for semen stored at 15°C compared with 20 and 25°C are difficult to explain. With bovine spermatozoa, storage in the range of 15 to 27°C gave satisfactory conception rates (Shannon, 1978). It is possible that in ram spermatozoa the metabolic rates at 20°C or above are sufficiently high to influence the maintenance of motility after storage.

It is generally agreed that slow cooling is better than rapid cooling (Varner *et al.*, 1988; Bouchard *et al.*, 1990; Simpson and White, 1986) and our observations on ram semen in RSD-1 support this. It must be noted, however, that both the cooling rate and the temperature to which the semen is cooled influence spermatozoal motility. We found only non-significant differences between cooling rates of 3, 4.5 and 6°C/min, in contrast to Varner *et al.*, (1988), who reported significant differences in equine spermatozoal motility in samples cooled at different rates to 4°C.

We observed a significant decrease in spermatozoal motility when undiluted semen was incubated at 35°C for up to 60 minutes. Similar observations were made by Nass-Arden and Breitbart (1990), who concluded that motility was decreased due to the acidification of semen. We also found that the loss in motility was reversible by dilution in RSD-1. Incubation of raw semen up to 60 minutes, however, did not influence subsequent maintenance of spermatozoal motility after dilution. We routinely dilute semen within 10 minutes of collection, as suggested by Evans and Maxwell (1987). It is reported that 160-200 x 10^6 spermatozoa are required for successful cervical insemination in the ewe (Evans and Maxwell, 1987). In view of the fact that the capacity of the cervical os is only ~0.25 ml, a concentration of 800 x 10^6 spermatozoa per ml is required. Dilution to 500 x 10^6 per ml did not improve the maintenance of motility. We thus conclude that for the maintenance of spermatozoal motility both the time and extent of dilution (at least up to 500 x 10^6 spermatozoa per ml) do not make any difference.

CONCLUSIONS

We suggest that for preservation of ram semen for use in cervical AI, the semen should be diluted in RSD-1 to 800 x 10^6 spermatozoa per ml and cooled to 15°C with a cooling rate of 3°C/min. Motility estimates should be made by diluting the spermatozoal suspension either immediately or after storage at 15°C, to 100 x 10^6 spermatozoa per ml and incubating the diluted suspension at 38°C.

TABLE 4 Effect of time from collection to initial dilution at 35°C on motility score and percent motile (in parentheses)

Time of incubation (h)	Time of dilution (mins) 5	10	20	30	60
0.5	4.19 (65.0)	4.25 (65.0)	4.25(65.0)	4.25 (62.5)	4.25 (62.5)
24	2.19 (40.0)	2.38 (42.5)	2.63(45.0)	2.56 (45.0)	2.56 (42.5)

Semen diluted to 800 x 10^6 spermatozoa per ml and stored at 15 °C for 4 h. Incubated at 38 °C (100 x 10^6 spermatozoa per ml) for observation at 0.5 and 24 h.

Results shown are the mean of separate determinations, from four rams. For $P<0.05$ LSD = 0.76 (12.47) for any pair.

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