FERTILITY OF RAM SPERMATOZOA FROZEN IN A TRIS-BASED DILUENT*

By D. VISSER† and S. SALAMON†

Abstract

Results of two fertility tests are presented. In the first test the mean lambing rates for semen frozen in Tris–glucose–yolk and raffinose–citrate–yolk and for fresh-diluted semen were 40·0% (28/70), 44·8% (30/67), and 65·7% (46/70) respectively. Double insemination significantly increased the fertility for both frozen and fresh semen.

In the second test semen frozen in Tris–glucose–yolk diluent was used in reconcentrated and unconcentrated state after thawing, and the mean lambing results for the two treatments were 37·5% (36/96) and 41·3% (52/126). Double insemination had no effect.

Introduction

Fertility of ram semen frozen in a raffinose–citrate–yolk diluent, thawed in the appropriate thawing solution, and subsequently reconcentrated before insemination has been examined in several studies (Lightfoot and Salamon 1970*b*; Salamon and Lightfoot 1970; Salamon 1971, 1972). Recently Salamon and Visser (1972) reported better viability of ram spermatozoa after thawing when the semen was frozen in Tris–glucose–yolk rather than in raffinose–citrate–yolk diluent. Consequently, a study was undertaken to obtain information on the fertility of semen pellet frozen in Tris-based diluent, and this communication presents the results of two experiments.

Methods

Semen was collected from five mature Merino rams by artificial vagina and ejaculates of good initial motility were pooled. In experiment 1 half of the pooled semen was extended with Tris (300 mM)–glucose (27·75 mM)–yolk and the other half with raffinose (166·5 mM)–sodium citrate (68 mM)–yolk diluent. In experiment 2 the pooled semen was diluted with Tris–glucose–yolk diluent. The diluting media contained 15% (v/v) egg yolk and 5% (v/v) glycerol. The diluted semen (1 : 4 at 30°C) was cooled to 5°C in 1 hr and held at that temperature for an additional 1 hr before pelleting (0·25 ml) on dry ice (Nagase and Niwa 1964). The frozen pellets were stored in liquid nitrogen for 1–2 weeks before use.

The pellets frozen in raffinose–citrate–yolk diluent were thawed in inositol (210 mM)–sodium citrate (40 mM) and those frozen in Tris–glucose–yolk were thawed in Tris (300 mM)–fructose (55·5 mM) thawing solution, as in previous studies the respective thawing media yielded the optimal recovery and viability of spermatozoa (Salamon and Brandon 1971; Salamon and Visser 1972).

Four pellets were dropped in each of a series of test tubes containing 2 ml of thawing solution and held in a water-bath at 37°C (thawing dilution ratio 1 : 2, pellet : thawing solution, v/v). The

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† Department of Animal Husbandry, University of Sydney, Sydney, N.S.W. 2006.

thawed semen was collected into centrifuge tubes (at 37°C), centrifuged at 1000 *g* for 10 min, and the supernatant discarded to obtain a concentration of $1 \cdot 7$–$1 \cdot 9 \times 10^9$ motile spermatozoa per millilitre. When in experiment 2 the semen frozen in Tris–glucose–yolk diluent was thawed in dry test tubes (at 37°C, four pellets per tube) it was used without centrifugation and reconcentration.

Fresh semen (expt. 1) was collected from the same rams from which ejaculates were obtained for freezing. Ejaculates from individual rams were pooled and diluted with glucose (44·4 mM)–sodium citrate (80·6 mM)–yolk (15%, v/v) diluent to a cell concentration of $1 \cdot 7$–$1 \cdot 9 \times 10^9$ per millilitre. The diluted semen was centrifuged at 1000 *g* for 10 min and then resuspended.

The volume of inseminate of both thawed reconcentrated and fresh-diluted semen was 0·1 ml in experiment 1. In experiment 2 inseminate volumes of 0·05 and 0·20 ml were used for reconcentrated and unconcentrated semen respectively, and the number of motile spermatozoa was 85–95 $\times 10^6$ per dose in both cases.

Mature Merino ewes were inseminated at the second oestrus after synchronization with intravaginal sponges (Robinson 1965). Oestrous ewes were detected by vasectomized rams previously tested by electro-ejaculation. The ewes were drafted at 0800 and 1800 hr and after each drafting randomly allocated into treatment groups. Semen from every treatment was used on each day of insemination and both single and double inseminations were carried out. The ewes drafted in the evening were inseminated at 0900–1100 hr the following day with the morning's draft ewes and the second insemination was performed at 1700–1800 hr.

Lambing was determined by udder examination 160 days after the last date of insemination. Differences between the proportion of ewes lambing in treatment groups were tested by χ^2.

TABLE 1

EXPERIMENT 1: LAMBING RESULTS FOLLOWING INSEMINATION WITH FROZEN–THAWED AND FRESH-DILUTED SEMEN

No. of inseminations	No. of ewes inseminated	No. lambing	% lambing
Frozen–thawed semen: frozen in Tris–glucose–yolk diluent			
1	35 } 70	8 } 28	22·9 } Mean
2	35	20	57·1 } 40·0
Frozen–thawed semen: frozen in raffinose–citrate–yolk diluent			
1	34 } 67	12 } 30	35·3 } Mean
2	33	18	54·5 } 44·8
Overall total and mean	137	58	42·3
Fresh-diluted semen: extended with glucose–citrate–yolk diluent			
1	35 } 70	19 } 46	54·3 } Mean
2	35	27	77·1 } 65·7

Results and Discussion

Results for the first experiment are presented in Table 1. Fertility of frozen–thawed and fresh semen differed markedly ($\chi^2_{(1)} = 10 \cdot 13$; $P < 0 \cdot 005$). There was no significant difference between the lambing rates obtained with semen frozen in Tris–glucose–yolk or in raffinose–citrate–yolk diluents ($\chi^2_{(1)} = 0 \cdot 31$; $P > 0 \cdot 50$). Double insemination resulted in a significant increase in lambing ($\chi^2_{(1)} = 13 \cdot 57$; $P < 0 \cdot 001$). There was no significant interaction.

In the second experiment the pellets frozen in Tris–glucose–yolk diluent were either thawed in a solution and reconcentrated by centrifugation or thawed in dry

test tubes without further treatment before insemination. Semen frozen in raffinose–citrate–yolk diluent was not included, as on subsequent thawing of the pellets without using a thawing solution (in this and in previous laboratory tests) the viability of spermatozoa was lower than of those frozen in Tris–glucose–yolk medium. Spermatozoa pellet frozen in the latter diluent, however, showed similar or negligible difference in survival when thawed with or without using a thawing solution. Lambing after insemination with reconcentrated and unconcentrated semen was indistinguishable (Table 2). There was also no difference in fertility following single and double inseminations, unlike in experiment 1 of the present study and in previous studies (Salamon and Lightfoot 1970; Salamon 1971). No explanation can be offered for the low response to double insemination.

TABLE 2

EXPERIMENT 2: LAMBING RESULTS FOLLOWING INSEMINATION WITH RECONCENTRATED AND UNCONCENTRATED FROZEN–THAWED SEMEN

No. of inseminations	No. of ewes inseminated	No. lambing	% lambing
	Reconcentrated semen*		
1	46 } 96	17 } 36	37·0 } Mean
2	50	19	38·0 } 37·5
	Unconcentrated semen†		
1	58 } 126	22 } 52	37·9 } Mean
2	68	30	44·1 } 41·3
Overall total and mean	222	88	39·6

* The frozen pellets were thawed in Tris–fructose solution and reconcentrated by centrifugation before insemination; dose of inseminate 0·05 ml.

† The frozen pellets were thawed without solution, no centrifugation and reconcentration before insemination; dose of inseminate 0·20 ml.

In the present study ram semen pellet frozen in Tris–glucose–yolk or in raffinose–citrate–yolk diluent and reconcentrated after thawing yielded equal fertility. Results of the second experiment merit attention, as it indicated that acceptable fertility can also be obtained when the semen pellets frozen in Tris–glucose–yolk diluent are thawed without a thawing solution and subsequently used for insemination in unconcentrated state. The number of motile cells in the inseminate (85–95 $\times$ 10^6) most probably were sufficient for the establishment of a cervical population of spermatozoa, the importance of which was shown earlier (Mattner *et al.* 1969; Lightfoot and Salamon 1970*a*; Salamon and Lightfoot 1970). Further, the careful handling of ewes during insemination to reduce stress to a minimum could have also been a contributing factor to the retention of most or all of the inseminate volume and thereby to the establishment of the cervical sperm population.

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