Effect of diluent and storage time on pregnancy rate in ewes after intra-uterine insemination

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ABSTRACT

Four trials involving the intra-uterine insemination of a total of 840 Coopworth ewes were conducted to assess the fertilization capacity of ram semen stored at 15°C. Semen from Polled Dorset rams was collected by artificial vagina, diluted with either a standard milk diluent or a synthetic diluent (RSD-1) to a concentration of $200x10^6$ sperm/ml, cooled to 15°C, placed in 0.25 ml straws and held at 15°C for various periods [Day 0 (4 h), Day 1 (28 h), Day 2 (52 h), Day 3 (76 h) and Day 4 (100 h)] before insemination. Ewes were synchronised, with CIDR devices inserted for 14 days and 400 i.u. PMSG was given at time of device removal. Laparoscopic assisted intra-uterine inseminations were performed between 52 h and 56 h after device withdrawal. A total of six inseminators were used throughout the trial series with between 3 to 5 inseminators per trial. Conception rates were determined by plasma progesterone levels at day 19 and pregnancy confirmed by real time ultrasonic scanning at day 50 post mating.

There were significant differences between trials in the proportion of ewes treated that exhibited oestrus prior to insemination with lower percentages in the non-breeding season. There was considerable discrepancy between the two methods of pregnancy detection. A high proportion of the non-tupped ewes deemed pregnant by progesterone were not pregnant at scanning. There were no differences between trials in the proportion of ewes pregnant after insemination with Day 0 semen. There were no significant differences between the diluents at any particular time of storage however, the overall pregnancy rate was higher for the milk diluent (50.1% v 45.0%). There was a significant effect of storage time, with the mean overall values for storage periods of 0, 1, 2, 3 and 4 days being 49.4, 46.5, 40.3, 29.6 and 20.0% respectively. Significant differences between inseminations were recorded ranging from 52.6% to 35.0%.

The similarity in conception rates between diluents to different storage times is in marked contrast to the estimations of percentage motile sperm obtained with these treatments. This indicates that further modification of the diluent is necessary and that for this to be achieved more critical and quantitative measurements of sperm motility and function are required.

Keywords: Ram semen, diluents, storage time, intra-uterine AI, fertility.

INTRODUCTION

One of the limiting factors to increased usage of artificial insemination in the New Zealand sheep industry is the relatively short period of fertilisable life of ram semen when stored at ambient temperatures. (Evans and Maxwell, 1987). Recent research into diluents for the storage of "fresh" ram semen has resulted in the development of a diluent (RSD-1) that maintains sperm motility both when stored at 15°C and when incubated at 39°C (ewe body temperature) for much longer periods than does the commonly used "milk" base diluent (Upreti *et al.*, 1991).

However, assessment of sperm motility in the laboratory is not necessarily a good indication of the fertility obtained following cervical insemination. Preliminary investigations were conducted to assess the fertilization capacity of ram semen stored for various periods of time in these diluents. The intra-uterine insemination technique was employed as this eliminates the problem associated with sperm movement through the cervix which exerts a major influence on conception rates.

MATERIALS AND METHODS

A series of four trials were conducted between August 1991 and June 1992. Details of treatments, ewe numbers etc. are provided in Table 1.

Semen processing

Semen from Polled Dorset rams was collected using an artificial vagina and diluted with either a) the standard milk diluent or, b) with the new synthetic diluent RSD-1; to a concentration of $200x10^6$ sperm/ml. The diluted semen was then cooled to 15°C (Upreti *et al.*, 1992a), placed into 0.25 ml "cassou" straws and held at 15°C for periods ranging from 6 h (day 0) to 102 h (day 4) depending on the trial (see Table 1).

Oestrous synchronisation and insemination

Coopworth ewes were synchronised using CIDR® type G devices (Carter Holt Harvey, Plastics Products division, Hamilton, NZ) for a period of 14 days and the i.m. injection of 400iu pregnant mare's serum gonadotrophin (PMSG; Pregnecol®, Pastoral Consultants (NZ) Ltd Otane, NZ) at time of device removal. Ewes were run with harnessed

vasectomised rams and tupping marks were recorded at 32 and 50 h after device removal.

Intrauterine insemination via laparoscopy (Killeen and Caffrey, 1982) was performed on all ewes 53-56 h after CIDR® removal. Each ewe received 0.125 ml of semen into each uterine horn (total of $50x10^6$ sperm). A total of six inseminators were used throughout the series of experiments (table 1).

TABLE 1: Outline of treatment comparisons conducted in each of four trials, number of animals and date of trial.

		Trial			
		A	B	C	D
Date		Aug .91	Dec. 91	April 92	June 92
Ewes (n=)		(50)	(50)	(50)	(30)
Diluent	Milk	√	√	√	√
	RSD-1	√	√	√	√
Semen	0 (6)	√	√	√	√
Storage time	1 (30)	-	√	√	-
days	2 (54)	-	-	√	√
(hr)	3 (78)	-	-	-	√
	4 (102)	-	-	-	√
Inseminator	1	√	√	√	
	2	√	√	√	√
	3	√	√	√	√
	4	-	-	√	√
	5	-	-	√	-
	6	√	-	-	-

Pregnancy

Pregnancy rates were determined by two methods, firstly using plasma progesterone levels measured by RIA on blood samples taken 21 days after CIDR® removal (day 19 of the cycle). Animals with values <1.00 ng/ml were designated non-pregnant (PROG). Secondly, real time ultrasonic scanning (linear array) was performed approximately 50 days after CIDR® removal and the number of foeti present recorded (SCAN).

Statistical analysis

Chi squared analysis of the proportion of ewes treated detected in oestrous and the proportion of ewes inseminated detected as pregnant were performed.

RESULT

Proportion of ewes detected in oestrus prior to insemination (tupped ewes)

There were significant (P<0.001) differences between trials in the proportion of ewes tupped prior to AI (59.8, 57.0, 88.9 and 78.1% for trials A to D respectively). Subsequent analysis was therefore performed on two data sets, one for tupped ewes only and an overall set for all ewes inseminated.

Technique for pregnancy diagnosis

The overall percentage of ewes diagnosed as pregnant was significantly (P<0.001) lower for the SCAN (43.8%) method than for the PROG method (55.0%). Of the ewes diagnosed as pregnant based on the PROG method 79.6% were confirmed pregnant by scanning. This indicates a possible level of embryonic loss in 11.2% of the total ewes inseminated.

However, there were significant (P<0.001) differences between tupped and non-tupped ewes in the proportion of the ewes diagnosed pregnant with the PROG technique that were confirmed by the SCAN technique (87.2% v 51.1%). This was reflected in the greater differences in pregnancy rate between tupped and non-tupped ewes using the SCAN method (51.8% v 21.8% P<0.001) compared to that using the PROG system (59.5% v 42.7% P<0.05). There were no indications of interactions with other treatments and subsequent analysis has been restricted to SCAN data only.

Semen treatment and inseminator effects

The overall pregnancy rates based on SCAN data are presented in table 2 and for tupped ewes only in table 3. There were no significant differences between trials for pregnancy rate of ewes inseminated with semen used on the day of collection (the common storage time for all trials) in both data sets. Thus data for the four trials were pooled.

There were no significant differences (in either data set) between the diluents at any of the storage times. However, the overall pregnancy rate was significantly (P<0.05) higher with the milk diluent than with the RSD-1 (50.1% v 45.0% and 55.8% v 47.6%) for the overall and tupped ewe data sets respectively.

There was a significant (P<0.001) effect of time of storage on pregnancy rate (in both data sets). The mean values for storage periods of 0, 1, 2, 3, and 4 days were 49.4, 46.5, 40.3, 29.6 and 20.0% (overall) and 58.5, 57.7, 45.4,

TABLE 2: Percentage of ewes pregnant after intra-uterine AI with semen stored at 15°C in either milk or RSD-1 for varying periods (All ewes).

Diluent		Milk					RSD-1				
Storage time at 15°C (days)		0	1	2	3	4	0	1	2	3	4
Trial	No. Ewes per group										
A	50	39.6	-	-	-	-	46.9	-	-	-	-
B	50	50.0	51.0	-	-	-	54.0	50.0	-	-	-
C	50	62.0	57.0	35.4	-	-	46.0	42.9	40.8	-	-
D	30	50.0	-	56.7	39.2	33.3	53.3	-	39.3	29.6	20.0
Total		50.6	54.1	43.6	39.2	33.3	49.4	46.5	40.3	29.6	20.0

TABLE 3: Percentage of ewes pregnant after intra-uterine AI with semen stored at 15°C in either milk or RSD-1 for varying periods (Restricted to ewes detected in oestrus prior to insemination - tupped ewes).

Diluent	Milk					RSD-1				
Storage time at 15°C (days)	0	1	2	3	4	0	1	2	3	4
Trial										
A	46.7	-	-	-	-	53.6	-	-	-	-
B	74.1	79.2	-	-	-	65.4	56.0	-	-	-
C	68.9	56.3	40.4	-	-	50.0	47.5	44.4	-	-
D	50.0	-	60.9	40.7	40.0	57.7	-	40.0	30.0	20.0
Total	61.3	63.9	47.7	40.7	40.0	55.6	50.8	43.1	30.0	20.0

36.2, and 30% (tupped ewes). There was no indication of interaction between storage time and diluent.

Significant ($P<0.01$) differences were seen between inseminators in both data sets (Table 4). While none of the interactions between inseminator, diluent and storage time were significant there was an indication that the poorer inseminators performed worst with the RSD-1 diluent.

TABLE 4: Effect of inseminator's on the percentage of ewes pregnant following intra-uterine insemination.

Inseminator	Tupped ewes only		All ewes	
	Number	% pregnant	Number	% pregnant
1	192	53.1	286	47.8
2	134	56.0	192	46.4
3	106	64.2	152	52.6
4	84	40.5	110	35.5
5	36	39.9	40	35.0
6	36	52.8	56	46.4

Motility measurements

Table 5 presents mean data from a separate experiment (Upreti *et al.*, unpublished) for the percentage of motile sperm (visual assessment) for semen stored in these diluents at 15°C and then incubated at 39°C, these are presented for comparative purposes and indicate the significantly better *in vitro* survival of semen diluted with RSD-1.

TABLE 5: Effect of diluent type, storage time at 15°C and length of incubation (39°C) in motility (percentage motile) of ram semen. Values are means of samples from 8 rams and the least significant difference between means is 12% (from Upreti *et al* unpublished).

Diluent	Milk			RSD-1		
Duration of incubation at 39°C (hr)	1	7	24	1	7	24
Storage time at 15°C (days)						
0	75	65	0	80	75	65
1	60	35	0	70	65	40
2	55	5	0	65	55	45
3	45	15	0	60	55	35
8	10	0	0	45	40	25

DISCUSSION

The variation between trials in the proportion of total ewes tupped prior to insemination was greater than that expected given the system of synchronisation used. The response followed a pattern in line with the breeding season and could indicate a possible failure of the synchronisation system, although the general pattern was consistent with previously reported data (Smith *et al.*, 1991a and b).

Similarly the PROG technique used for detection of pregnancy is dependant on an effective synchronisation system. While the differences between PROG and SCAN methods for the tupped ewes (7.7%) most probably reflects a true level of embryonic loss, the much greater discrepancy in the non-tupped ewes (20.9%) is more likely to be due to these ewes being asynchronous in relation to cycle stage at the time of blood sampling.

While some decline in pregnancy rate with semen storage was to be expected the overall poorer performance of the RSD-1 diluent is in complete contrast to that which was expected following the motility results after storage and incubation in the laboratory (Table 5). This means that the laboratory assessment of sperm viability (maintenance of sperm motility under incubation at 39°C) has little correlation with that semen's ability to achieve fertilisation following intra-uterine insemination in the ewe. Thus for the continued development of improved diluents more critical and quantitative measurements of the different aspects of sperm motility are obviously necessary as are the development of a robust *in vitro* method for estimation of fertilisability (Upreti *et al.*, 1992b and c).

The data indicate that using the intrauterine technique acceptable levels of pregnancy can be achieved with semen used either on the day of collection or after storage at 15°C for upto 30 hr after collection. This has been confirmed in an independent field study (Smith *et al.*, unpublished) where SCAN pregnancy rates of 81 and 85% and 77 and 71% were obtained for intrauterine insemination of Milk and RSD-1 diluted semen on days 0 and 1 respectively. These higher levels may have been due to the use of a higher concentration of semen (total dose of 150×10^6) in that trial or to the possibility of zearalenone induced subfertility in the present experimental flock (Smith *et al.*, 1992).

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