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FERTILITY OF RAM SPERMATOZOA FROZEN BY THE PELLET METHOD

II. THE EFFECTS OF METHOD OF INSEMINATION ON FERTILIZATION AND EMBRYONIC MORTALITY

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Summary. A factorial experiment was conducted to examine the effects in Merino ewes of method of insemination and semen type on embryonic loss (fertilization rate versus lambing rate).

The results showed that:

(i) frozen semen (pellet method) was of lower fertility than fresh semen (49% versus 70% respectively).

(ii) there was little embryonic mortality following either the cervical or cervical traction methods of insemination (13% and 6% respectively), but substantial loss occurred following uterine insemination (47%). Results for both fresh and frozen semen were similar in this respect.

Normal cervical insemination (two inseminations at a 12-hr interval within one oestrus) with frozen semen of high concentration ($1{\cdot}6 \times 10^9$ motile spermatozoa/ml, 0·1 ml dose) resulted in ewe fertilization and lambing rates of 58% and 50%, respectively.

INTRODUCTION

Lightfoot & Salamon (1970) have shown that the infertility following insemination with pellet-frozen ram semen was associated with an impaired pattern of transport of spermatozoa in the ewe's genital tract. This was due to failure to establish and maintain an adequate cervical population of spermatozoa, a problem which was partly overcome by using inseminates with a high concentration of motile spermatozoa. The results suggested that concentration of the thawed semen before insemination should result in improved fertility.

In an earlier study, Salamon & Lightfoot (1967) obtained high fertilization rates (88% and 93%) when the cervix was by-passed, by depositing frozen semen directly into the uterus, but subsequent survival of the zygotes was poor. Of sixty-eight ewes inseminated and retained for lambing, only twenty-three (34%) had not returned to service 22 days later, and only seventeen (25%) subsequently lambed.

In view of this evidence, the experiment reported here was designed to examine ewe fertility:

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(i) when the cervical population of spermatozoa was increased by using inseminates containing a high concentration of motile spermatozoa;

(ii) when the cervical barrier to sperm transport was by-passed by surgical insemination into the uterus.

Both fertilization and lambing were studied to determine whether, following insemination with frozen semen, excessive embryonic mortality further contributed to reduced ewe fertility, in addition to failure of fertilization.

MATERIALS AND METHODS

Experimental design

The experiment was of factorial design, $3 \times 2 \times 2$, n = 22 to 40, N = 362, as shown below.

(1) Method of insemination—cervical versus cervical traction versus uterine
(2) Type of semen—fresh versus frozen
(3) Stage of pregnancy—fertilization versus lambing

Sheep and management

Mature Merino ewes were allocated at random to treatments on a within draft basis. The methods adopted for testing vasectomized rams before joining and identifying ewes in oestrus were as described previously (Lightfoot & Salamon, 1970). Oestrous ewes were drafted from the flock at 07.00 hours daily. Ewes allocated to the cervical and cervical traction methods of insemination were inseminated twice, first within 3 hr of drafting (approximately 1 to 27 hr after onset of oestrus), and again 12 hr later. Ewes inseminated by the uterine method received one insemination 9 to 39 hr after the onset of oestrus. All inseminations were performed with 0·1 ml of semen containing 160×10^6 motile spermatozoa. Cervical (external os) and uterine inseminations were as described earlier (Lightfoot & Salamon, 1970). For cervical traction inseminations, a cervical papilla was grasped with a long pair of forceps and the cervical os withdrawn to a position just cranial of the vaginal entrance.

Semen

For freezing, semen was collected by artificial vagina and diluted (1 : 3, semen : diluent, v/v) at 30° C with a diluent consisting of 166·5 mM-raffinose, 102 mM-sodium citrate, 15% (v/v) egg yolk to which was added 5% (v/v) glycerol. The diluted semen was cooled over 1½ hr to 5° C, held at that temperature for 3 hr, then frozen as pellets (0·035 ml) on dry ice and stored in liquid nitrogen for 2 to 12 weeks before use. The pellets were thawed (1 : 3, pellets : thawing solution, v/v) in 44·4 mM-glucose–80·6 mM-sodium citrate at 37° C. The thawed semen was centrifuged at 2500 rev/min for 15 min and the supernatant discarded to achieve a concentration of $1{\cdot}6 \times 10^9$ motile spermatozoa/ml for insemination.

The fresh semen was collected from two rams and the concentration of spermatozoa was determined by haemocytometer after pooling the ejaculates. The semen was then diluted two- to three-fold with egg yolk–glucose–citrate diluent (15% by vol, 44·4 mM and 80·6 mM respectively) to a concentration of $1{\cdot}6 \times 10^9$ motile spermatozoa/ml for insemination.

Fertilization

Eggs were recovered 48 to 60 hr after insemination following mid-ventral laparotomy under local anaesthesia. The eggs were examined for cell cleavage, presence of polar bodies and number of spermatozoa on the zona pellucida.

Lambing

Vasectomized rams were joined with ewes in the lambing treatments to obtain individual non-return records. The ewes were side-numbered 2 weeks before the commencement of lambing and individual ewe records obtained by drift-lambing (Tribe & Coles, 1966) with twice daily inspections.

Statistical analyses

The statistical significance of all treatment comparisons was determined by analysis of χ^2 (Claringbold, 1961).

RESULTS

Data for ovulation, egg recovery and egg fertilization are presented in Table 1. A slightly lower proportion of eggs was recovered following uterine insemination than after insemination by the cervical and cervical traction methods (73·0%, 85·0% and 82·3%, respectively; surgical versus non-surgical insemination, $\chi^2_1 = 2{\cdot}91$; $0{\cdot}05 < P < 0{\cdot}1$).

TABLE 1

OVULATION, EGG RECOVERY AND EGG FERTILIZATION AFTER CERVICAL, CERVICAL TRACTION AND UTERINE INSEMINATION WITH FRESH AND FROZEN RAM SPERMATOZOA

Treatment		*No. of ewes inseminated for estimates of fertilization*	*No. of eggs*			*% of eggs*	
Method of insemination	*Type of semen*		*Ovulated*	*Recovered*	*Fertilized*	*Recovered*	*Fertilized*
Cervical	Fresh	23	31	27	19	87·1	70·4
	Frozen	24	29	24	11	82·8	45·8
Cervical traction	Fresh	30	33	25	17	75·8	68·0
	Frozen	22	29	26	11	89·7	42·3
Uterine	Fresh	26	32	23	20	71·9	87·0
	Frozen	28	31	23	21	74·2	91·3
Overall		153	185	148	99	80·0	66·9

The use of frozen semen, as compared with fresh, resulted in a lower proportion of eggs fertilized following both cervical (45·8% versus 70·4%) and cervical traction (42·3% versus 68·0%) insemination. Frozen and fresh semen were of equal fertility, (91·3% versus 87·0%), however, when inseminated into the uterus (type of semen × method of insemination, non-surgical versus surgical: $\chi^2_1 = 3{\cdot}49$; $0{\cdot}05 < P < 0{\cdot}1$).

The effects of method of insemination, type of semen and stage of pregnancy on ewe fertility are shown in Table 2 and the relevant analysis of χ^2 in Table 3. Two main points emerge from these comparisons.

TABLE 2

THE EFFECTS OF METHOD OF INSEMINATION AND TYPE OF SEMEN ON FERTILIZATION, LAMBING AND ESTIMATED EMBRYONIC MORTALITY

Treatment		*Stage of pregnancy*						
		Fertilization			*Lambing*			
		No. of ewes		%	*No. of ewes*			
Method of insemination	*Type of semen*	*Yielding eggs*	*Yielding fertilized eggs*	*of ewes yielding fertilized eggs*	*Inseminated*	*Lambing*	% *of ewes lambing*	*Estimated embryonic mortality* (%)*
Cervical	Fresh	21	16	76·2	35	24	68·6	10·0
	Frozen	19	11	57·9	40	20	50·0	13·6
Cervical traction	Fresh	24	16	66·7	36	27	75·0	—
	Frozen	21	9	42·9	37	11	29·7	30·8
Uterine	Fresh	19	17	89·5	31	17	54·8	38·8
	Frozen	21	19	90·5	30	12	40·0	55·8
Main effects								
Cervical		40	27	67·5	75	44	58·7	13·0
Cervical traction		45	25	55·6	73	38	52·1	6·3
Uterine		40	36	90·0	61	29	47·5	47·2
	Fresh	64	49	76·6	102	68	66·7	12·9
	Frozen	61	39	63·9	107	43	40·2	37·1
Overall		125	88	70·4	209	111	53·1	24·6

* 100 (% Fertilization minus % Lambing/% Fertilization) %.

(i) Frozen semen was of lower fertility than fresh semen (63·9% fertilization, 40·2% lambing, mean = 48·8% versus 76·6%, 66·7%, mean = 70·5%; $P < 0·001$).

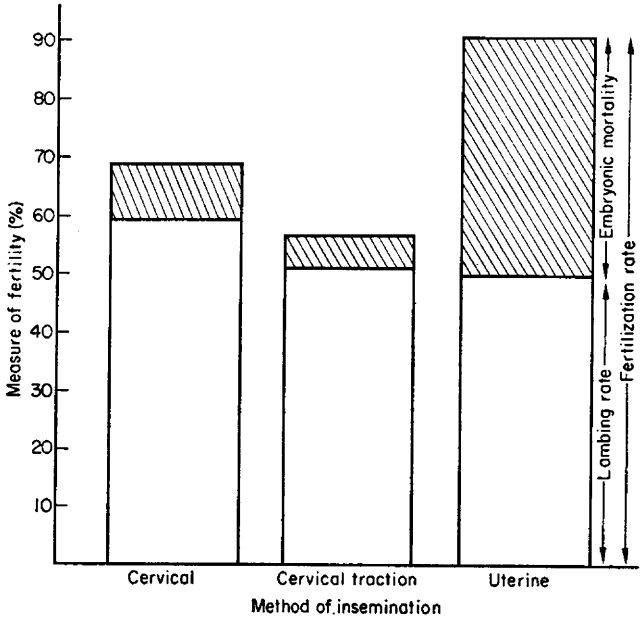
(ii) There was a significant interaction between stage of pregnancy and

TABLE 3

ANALYSIS OF χ^2 OF DATA IN TABLE 2

Effect		*d.f.*	χ^2
Method of insemination	(M)		
Within non-surgical†		1	1·69
Non-surgical versus surgical‡		1	1·37
Semen type	(S)	1	16·29***
Stage of pregnancy	(P)		
Fertilization versus lambing		1	9·71**
M × S			
Within non-surgical × S		1	1·98
Non-surgical versus surgical × S		1	3·16
M × P			
Within non-surgical × P		1	0·14
Non-surgical versus surgical × P		1	9·90**
S × P		1	1·46
M × S × P		2	0·61

** $P < 0·01$; *** $P < 0·001$.
† Cervical versus cervical traction.
‡ Cervical and cervical traction versus uterine.



TEXT-FIG. 1. The effect of method of insemination on the incidence of embryonic mortality. (Method of insemination, non-surgical versus surgical × stage of pregnancy, $P < 0·01$). Data are the means of fresh and frozen semen treatments.

method of insemination ($P<0{\cdot}01$). Thus, following insemination by the cervical and cervical traction (non-surgical) methods, lambing rates were only slightly lower than fertilization rates (13·0% and 6·3% estimated embryonic mortality respectively), but following uterine (surgical) insemination, lambing rates were much lower than fertilization rates (47·2% estimated embryonic mortality). This relationship is shown in Text-fig. 1. Results for both fresh and frozen semen treatments were similar in this respect.

The overall estimates of embryonic mortality following insemination with fresh and frozen semen were 12·9% and 37·1% respectively. This difference, examined statistically by the interaction between type of semen and stage of pregnancy, however, was not significant ($\chi_1^2 = 1{\cdot}46$; $0{\cdot}2<P<0{\cdot}3$).

Fertilized eggs were classified according to the number of spermatozoa counted on the zona pellucida immediately after recovery (Table 4). Insemination with fresh, as compared with frozen, semen resulted in a greater proportion of eggs to which numerous spermatozoa were attached ($P<0{\cdot}01$). Both

TABLE 4

CLASSIFICATION OF FERTILIZED EGGS ACCORDING TO THE NUMBER OF SPERMATOZOA ON THE ZONA PELLUCIDA

Treatment	*No. of eggs with spermatozoa on the zona pellucida*				*Mean no. of spermatozoa on zona pellucida*
	0 *sperm.*	1 *to* 5 *sperm.*	6 *to* 20 *sperm.*	>20 *sperm.*	
Type of semen					
Fresh	7	18	13	18	19·4
Frozen	5	24	12	2	6·0
P			$<0{\cdot}01$		
Method of insemination					
Cervical	8	12	7	3	8·1
Cervical traction	2	13	5	8	17·0
Uterine	2	17	13	9	15·3
P			$0{\cdot}05<P<0{\cdot}1$		

the uterine and cervical traction insemination procedures tended to yield a higher proportion of fertilized eggs with large numbers of spermatozoa on the zona pellucida ($0{\cdot}05<P<0{\cdot}10$). When both these methods were tested collectively against cervical insemination, the difference was statistically significant ($P<0{\cdot}05$).

Of the ewes that failed to lamb, approximately 80% returned to service (vasectomized rams) within 24 days of insemination and this proportion was similar in all treatments.

There were no differences between treatments in the number of blastomeres in fertilized eggs.

Of the fifty-four ewes subjected to laparotomy and uterine insemination, and in which fertilization was examined, twenty-four were inseminated before and thirty after ovulation. Of the forty-six eggs subsequently recovered, only four were unfertilized (two fresh and two frozen semen), all from ewes inseminated after ovulation. Table 5 shows no effect on lambing performance, following

TABLE 5

LAMBING DATA IN RELATION TO WHETHER UTERINE INSEMINATION WAS PERFORMED BEFORE OR AFTER OVULATION

Main effect	*No. of ewes*								
	Time of uterine insemination								
	Before ovulation			*After ovulation*			*Overall*		
	Inseminated	*Lambed*	%	*Inseminated*	*Lambed*	%	*Inseminated*	*Lambed*	%
Type of semen									
Fresh	16	8	50·0	15	9	60·0	31	17	54·8
Frozen	20	8	40·0	10	4	40·0	30	12	40·0
Overall	36	16	44·4	25	13	52·0	61	29	47·5

the use of either fresh or frozen semen, attributable to time of uterine insemination relative to ovulation.

DISCUSSION

The relatively low fertilization rates that normally accompany insemination with deep-frozen ram semen can be markedly increased by depositing the spermatozoa directly into the uterus. Eggs fertilized following insemination by this technique are characterized by numerous spermatozoa on the zona pellucida, indicating that uterine insemination improves fertilization by increasing the number of spermatozoa that reach the Fallopian tubes.

Despite the achievement of high fertilization rates with frozen spermatozoa by uterine insemination, lambing results were poor, due presumably to the occurrence of excessive embryonic mortality. This phenomenon occurred following uterine inseminations with either fresh or frozen spermatozoa in contrast to the occurrence of normal levels of embryonic mortality (Quinlivan, Martin, Taylor & Cairney, 1966a, b; Mattner & Braden, 1967) following normal cervical insemination. High embryonic losses following uterine insemination with both fresh and frozen spermatozoa is suggested by the data of Mattner Entwistle & Martin (1969) but few animals were involved and there were no normal insemination controls.

The physiological mechanisms by which surgical insemination in the sheep precipitates excessive embryonic losses are not clear. The uteri showed no response on macroscopic examination 2 days after insemination but most ewes returned to service at the normal time. From the results of Moor & Rowson (1966), this indicates that the zygotes died before Day 12. It is possible that the surgical interference brought about a response of the genital tract leading to reduced survival of otherwise normal zygotes, or to a high incidence of abnormal zygotes due to anomalous fertilization.

There are several Soviet reports concerned with excessive embryonic mortality in the ewe after surgical insemination with fresh semen (Lopyrin & Loginova, 1957; Lopyrin, Loginova & Zeltobrjuk, 1965; Lopyrin & Rak, 1965; Lopyrin & Manujlov, 1966, 1967). It has been claimed that both the semen diluent and dilution rate may affect the extent of losses found, but there were too few animals involved in most treatment comparisons to permit the drawing of valid conclusions.

The cervical traction method of insemination offered no advantage over normal cervical insemination, in contrast to earlier promising results (Salamon & Lightfoot, 1967) and the findings of Ten En Bon (1965).

There have been few reports concerned with the comparative levels of embryonic mortality following non-surgical inseminations with fresh and frozen spermatozoa. Shaffner (1942) reported that twelve of forty-eight eggs produced by hens after insemination with frozen spermatozoa were fertile, but embryonic development did not proceed for more than 10 to 15 hr. More recently, Wales & O'Shea (1968) and O'Shea & Wales (1969) have achieved limited fertility with deep-frozen spermatozoa in the rabbit, but they found no evidence that embryonic mortality was increased. Excessive embryonic mortality is unlikely

to occur following insemination with pellet-frozen bull spermatozoa, as fertility is usually similar to that obtained with chilled semen (e.g. Leipnitz, 1965; Milk Marketing Board, 1965/66; Meding, 1966). There appear to be no studies on early embryonic mortality, but comparisons of early versus late non-return rates for cows inseminated with bull spermatozoa frozen in ampoules (Salisbury, 1963, 1967) have indicated pre-natal losses of an order similar to that reported for semen after short-term liquid storage (Salisbury & Flerchinger, 1967).

In the present study, embryonic mortality in ewes inseminated with frozen spermatozoa was not significantly higher than that observed with fresh semen. This is in agreement with a recent report by Volkov (1968) who found fertilization and lambing rates of 11·9% and 15·8% respectively, following inseminations with ram semen frozen by the pellet method in a lactose–yolk diluent. Normal cervical inseminations with concentrated frozen spermatozoa in the present experiment resulted in fertilization and lambing rates of 57·9% and 50·0% respectively. Thus, losses due to embryonic mortality were of lesser importance than failure of fertilization due to impaired transport of spermatozoa.

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