PASSAGE, SURVIVAL, AND FERTILITY OF DEEP-FROZEN RAM SEMEN IN THE GENITAL TRACT OF THE EWE

By P. E. Mattner,* K. W. Entwistle,† and I. C. A. Martin†

[*Manuscript received June* 6, 1968]

Summary

The distribution of spermatozoa in the genital tract was determined in ewes killed 4 hr or 24 hr after cervical insemination with 100 million live spermatozoa from either freshly ejaculated or deep-frozen ram semen. At 4 hr, greater numbers of spermatozoa were present in the cervices, uteri, and fallopian tubes in ewes inseminated with fresh semen than in ewes inseminated with frozen semen. At 24 hr, the numbers of spermatozoa in the uteri and fallopian tubes of ewes inseminated with fresh semen had increased relative to the numbers at 4 hr but no spermatozoa were present in the uterus and fallopian tubes in ewes inseminated with frozen semen.

The proportion of eggs fertilized after intra-uterine insemination of ewes was high (six out of seven and five out of six) when 3 million freshly ejaculated live spermatozoa or 30 million live, frozen spermatozoa were used respectively, but was low (one out of seven) when a dose of 3 million live, frozen spermatozoa was used. A high incidence of fertilization occurred in ewes following laparotomy and intra-uterine insemination with large doses of fresh or frozen semen but the embryonic loss was high in both groups of ewes.

It was concluded that a primary cause of poor fertility found in ewes inseminated with deep-frozen ram semen is reduced longevity in a high proportion of the spermatozoa in the semen.

I. Introduction

The use of deep-frozen ram semen for artificial insemination has generally resulted in few lambs born (Blackshaw and Emmens 1953; Emmens and Blackshaw 1955; Salamon 1968). However, estimated conception rates, based on either lambings or on non-return to service have varied from 18 to 56% (Kuznecov and Kuprijanova 1959; First, Sevinge, and Henneman 1961; Kalev and Venkov 1961; Vlachos and Sakalof 1965).

Lopyrin and Loginova (1958) found that, at 6–7 hr after insemination of ewes with fresh or frozen semen, fewer spermatozoa were present in the cranial portion of the cervix in the ewes treated with frozen semen. They attributed this to poor longevity of the frozen spermatozoa. Salamon and Lightfoot (1967) found only a few spermatozoa in the fallopian tubes at 3, 6, 12, or 24 hr after cervical insemination of ewes with frozen semen and suggested that primary failure of sperm transport

* Division of Animal Physiology, Ian Clunies Ross Animal Research Laboratory, CSIRO, Prospect, N.S.W. 2149.

† Department of Veterinary Physiology, University of Sydney, N.S.W. 2006.

probably occurred in the cervix. Fertilization has been obtained, however, following laparotomy and the introduction of frozen semen into either the fallopian tubes or the uterus of ewes (Loginova 1962; Salamon and Lightfoot 1967; Mattner and Martin, unpublished data). Loginova (1962) and Salamon and Lightfoot (1967) obtained results indicative of a high embryonic loss following fertilization with deep-frozen spermatozoa. However, it is possible that the anaesthesia, laparotomy, and puncture of the genital tract required for intra-tubal or intra-uterine inseminations may have contributed to the losses, as control ewes similarly treated but inseminated with fresh semen were not examined.

The present experiments were undertaken to examine more fully the survival and passage of deep-frozen spermatozoa in the genital tract of the ewe, their fertilizing capacity, and the viability of the zygotes produced.

II. Materials and Methods

(*a*) *Animals and Treatments*

The numbers of animals per treatment group and the experimental factors examined are summarized in Table 1.

Merino ewes, aged 2½–5 years, were allocated at random to treatment groups. In experiment 1, oestrus was synchronized by the use of intravaginal sponges* (Robinson 1965) and the ewes were inseminated at a later natural oestrus. Onset of oestrus was detected using vasectomized rams fitted with marking harness and crayons (Radford, Watson, and Wood 1960).

(*b*) *Semen*

Semen was collected from four Merino rams by electro-ejaculation (Blackshaw 1954) and diluted 20-fold in a diluent containing 6% (v/v) egg yolk, 7·5% (v/v) glycerol, 247 mM glucose, 49 mM NaCl, 5 mM Na_2HPO_4–NaH_2PO_4 buffer, 17 mM fructose, and added antibiotics (500 i.u. each of penicillin and streptomycin/ml). The diluted semen was cooled to 5°C over 2 hr, then deep-frozen in an apparatus similar to that described by Polge and Lovelock (1952) which gave a freezing rate of 0·5–1°C/min to −10 or −15°C after which the rate was increased to about 3°C/min. Ampoules were transferred to and stored in liquid nitrogen.

For experiment 1 only, frozen semen was concentrated by centrifugation for 10 min at 700 *g* after thawing, and part of the supernatant was removed to adjust the concentration of spermatozoa to that required. Semen from the same four rams was used for inseminations with fresh semen in experiment 1. Fresh semen for experiments 2 and 3 was collected from different rams and in experiment 2 was diluted 1:5 to 1:7 with saline immediately before use. The motility and percentage of live cells were assessed and the concentration of spermatozoa was determined using a haemocytometer.

(*c*) *Insemination*

Cervical inseminations were performed by depositing the semen within the first cervical fold or on the external os of the cervix. Intra-uterine inseminations were performed following laparotomy of the ewes under Thiopentone Sodium B.P. anaesthesia. Using a microsyringe and a blunt-ended 26-gauge hypodermic needle, the semen was injected into the lumen of the uterine horn adjacent to the ovary containing a pre-ovulatory follicle.

In experiments 1 and 3, portions of each ejaculate, either fresh or frozen, were used to inseminate two ewes. One of the these was killed at the shorter and the other at the longer interval indicated in Table 1. For experiment 2, each ampoule of frozen semen was used to inseminate two ewes. One of these received the low, the other the high sperm dose shown in Table 1.

* Synchromate, Searle.

(*d*) *Examination of Reproductive Tracts*

The ewes were killed and spermatozoa and eggs were flushed from the genital tracts by the methods of Mattner and Braden (1963). Eggs were examined for spermatozoa on the zona pellucida and for segmentation and were then fixed and stained (Mattner 1963*a*) for further examination. Ewes killed 60 days after insemination were examined for the presence of a viable foetus.

(*e*) *Histological Examination of Cervices*

After being flushed with saline, each cervix was fixed with 10% formol saline and divided transversely into four blocks of equal length. The blocks were numbered sequentially from the caudal end. Four transverse sections (5 μ) were cut from each block at approximately equidistant points along its length. These were stained with haematoxylin and eosin, and counts were made of spermatozoa in each section as described by Mattner (1966).

TABLE 1

SUMMARY OF THE DESIGN AND EXPERIMENTAL FACTORS EXAMINED

Experimental Factors	Experiment 1	Experiment 2	Experiment 3
No. of animals per treatment group	8	7	9
Interval from onset of oestrus to insemination	12–16 hr	5–9 hr	5–9 hr
Route of insemination	Cervical	Intra-uterine	Intra-uterine
Fresh semen			
No. live sperm inseminated	100 million	3 million	80–120 million
Insemination volume	20–60 μl	20 μl	50 μl
Frozen semen			
No. live sperm inseminated	100 million	3 or 30 million	90–150 million
Insemination volume	100 μl	20 or 200 μl	500 μl
Interval from insemination to slaughter	4 or 24 hr	2 days	2 or 60 days

(*f*) *Statistical Analysis*

Analyses of data were carried out using a programme developed by Dr. P. J. Claringbold, Division of Animal Genetics, CSIRO, for use on the C.D.C. 3200 computer. For the analysis of variance of data from the cervical flushings and cervical section counts, the data were transformed [$\log_{10}(1+\text{count})$]. The analysis of variance of data from the cervical section counts was in the form described by Kempthorne (1952) for a hierarchical classification.

III. RESULTS

(*a*) *Experiment 1*

The mean numbers of spermatozoa flushed from the cervices, uteri, and fallopian tubes are shown in Table 2. At 4 hr after insemination, the number of spermatozoa flushed from the cervix was approximately four times greater in the ewes inseminated with fresh semen than in those inseminated with frozen semen. Spermatozoa were also present in the uteri and fallopian tubes at 4 hr after insemination in some ewes in each group but greater numbers were present in the ewes inseminated with fresh semen.

At 24 hr after insemination, the number of spermatozoa flushed from the cervices was still greater in the ewes inseminated with fresh semen. Whilst the number

of spermatozoa in the uterus and fallopian tubes of ewes inseminated with fresh semen was far greater at 24 hr than at 4 hr after insemination, no spermatozoa were found at these sites in ewes inseminated 24 hr previously with frozen semen.

TABLE 2

MEAN NUMBERS OF SPERMATOZOA FLUSHED FROM THE CERVIX, UTERUS, AND FALLOPIAN TUBES OF EWES KILLED AT 4 HR AND 24 HR AFTER INSEMINATION

Values in parentheses are the number per group in which no spermatozoa were counted in flushings. Group size = 8

Interval from Insemination to Slaughter	Division of Tract	Mean No. of Spermatozoa (±S.E.)	
		Fresh Semen	Frozen Semen
4 hr	Cervix	2,267,000±760,000	591,000±55,000
		(0)	(1)
4 hr	Uterus	10,700±3,500	1,100±740
		(1)	(5)
4 hr	Fallopian tubes	170±90	20±10
		(1)	(4)
24 hr	Cervix	233,000±68,000	1,700±900
		(0)	(3)
24 hr	Uterus	30,500±9,600	—
		(0)	(8)
24 hr	Fallopian tubes	12,000±6,900	—
		(0)	(8)

Summary of Analysis of Variance of Numbers of Spermatozoa (transformed values) in Cervical Flushings

Source of Variation	D.F.	Variance Ratios
4 hr *v.* 24 hr slaughter (*A*)	1	11·53**
Fresh *v.* frozen semen (*B*)	1	41·64***
Interaction $A \times B$	1	1·01
Error variance	28	292·9

** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

Examination of sections from the flushed cervices showed that spermatozoa were present in the cervical crypts and glands in every ewe and that greater numbers were present in the cervices of ewes that had been inseminated with fresh semen (see Table 3). There was a tendency, in each group of ewes, for the number of spermatozoa remaining in the flushed cervices to decrease from the caudal end, but the trend was not significant. The cervices had been flushed from the cranial end. Thus, the presence of spermatozoa in the cranial region of the cervix in all of the ewes killed 4 hr after insemination indicated that frozen as well as fresh spermatozoa had spread along the entire length of the cervix by this time.

(*b*) *Experiment 2*

The incidence of fertilization in ewes after intra-uterine insemination with either 3 million live fresh spermatozoa or 30 million live frozen spermatozoa was

high and did not differ significantly between groups. However, the fertilization rate in ewes inseminated with 3 million live frozen spermatozoa was low and differed significantly from that in ewes inseminated with 3 million live fresh spermatozoa, as shown in the following tabulation:

State of semen	Frozen	Frozen	Fresh
No. of live spermatozoa	3×10^6	30×10^6	3×10^6
No. of ewes from which eggs recovered	7	6	7
No. of ewes with fertilized eggs	1*	5	6*

* $\chi_1^2 = 4{\cdot}57$, $P<0{\cdot}05$.

TABLE 3

MEAN COUNTS (±S.E.) OF SPERMATOZOA IN CERVICAL SECTIONS

Treatment Group	Block 1	Block 2	Block 3	Block 4	Group Mean
4 hr, fresh	237·0±42·6	251·1±59·9	124·6±23·5	125·0±47·2	184·4±23·0
24 hr, fresh	40·6±7·4	39·9±6·2	94·2±23·3	15.1±1·3	47·4±6·7
4 hr, frozen	26·5±3·6	23·3±2·0	15·7±1·3	16·5±1·4	20·5±1·2
24 hr, frozen	20·3±2·2	18·4±1·5	18·8±1·6	12·1±1·3	17·4±0·8

Summary of Analysis of Variance of Counts of Spermatozoa (transformed values) in Cervical Sections

Source of Variation	D.F.	Mean Square	Variance Ratios
Treatments			
4 hr *v.* 24 hr slaughter	1	62,601·2	3·75
Fresh *v.* frozen semen	1	207,419·5	12·43**
Time × semen	1	35,292·8	2·11
Ewes within treatments†	28	16,686·3	4·99***
Blocks	3	2,805·0	0·83
Blocks within ewes	93	3,341·4	7·75***
Samples within blocks	384	431·5	1·12

** $P<0{\cdot}01$. *** $P<0{\cdot}001$. † Error term for treatments.

(*c*) *Experiment 3*

A high fertilization rate followed intra-uterine insemination of ewes with large numbers of fresh or frozen spermatozoa, as the following tabulation shows:

Insemination	Fresh semen	Frozen semen
Ewes with fertilized eggs/ewes from which eggs recovered	8/9	7/8
Ewes with live embryo(s) at 60 days/ ewes inseminated	2/9	3/9

All of the fertilized eggs recovered 2 days after insemination had cleaved (2–8 cells present) and appeared normal in all respects. However, the proportion of ewes with live

embryos 60 days after insemination was much lower and there was no significant difference between the two groups of ewes in the estimated embryonic loss.

IV. Discussion

The present results support those of Loginova (1962) and Salamon and Lightfoot (1967) in indicating that deep freezing and thawing of ram semen does not seriously impair the ability of the spermatozoa to penetrate and fertilize eggs. Consequently, the poor fertility found after cervical insemination of ewes with deep-frozen ram semen could be due to failure of sperm transport, poor survival of spermatozoa in the genital tract, or embryonic loss.

By 4 hr after cervical insemination of ewes with frozen semen, spermatozoa were distributed along the entire length of the cervix and some had passed into the uterus and fallopian tubes. Fewer spermatozoa were present at this time in the cervices in ewes inseminated with frozen semen than in ewes inseminated with fresh semen. However, this difference may have been due in part to the somewhat larger volume used for insemination with frozen semen (100 μl cf. 20–60 μl) and the drainage of a greater proportion of the dose back into the vagina immediately after insemination.

Although spermatozoa were present in the cervix in all ewes killed 24 hr after cervical insemination with frozen semen, none were found in the uterus and tubes in these animals. Freshly ejaculated spermatozoa can survive for more than 40 hr in the ovine cervix (Quinlan, Maré, and Roux 1933; Dauzier and Wintenberger 1952) and while live spermatozoa are present in the cervix, migration of some spermatozoa to the uterus and tubes continues as a result of their own motility (Mattner 1963*b*). Although a proportion of ram spermatozoa will survive deep freezing and show reasonable motility immediately after thawing, their longevity on incubation *in vitro* is reduced (White, Blackshaw, and Emmens 1954; O'Shea, personal communication). It is likely, therefore, that the absence of spermatozoa from the uteri and tubes of the ewes killed 24 hr after insemination with frozen semen was due to reduced longevity of the frozen spermatozoa established in the cervix rather than to the failure of any transport mechanisms external to the spermatozoa.

Nevertheless, it was clear that at least a small proportion of the spermatozoa in frozen ram semen could survive for reasonably long periods in the genital tract of the ewe and still be fertile. In experiment 2, the proportion of eggs fertilized was high when a large number of frozen spermatozoa, relative to the number of fresh spermatozoa required to effect fertilization, was introduced into the uterus in ewes approximately 10–15 hr prior to ovulation.

Loginova (1962) and Salamon and Lightfoot (1967) found that a high incidence of embryonic loss followed laparotomy and deposition of deep-frozen semen into the uterus in ewes. However, neither study included a number of ewes similarly treated with fresh semen as a control. In the present study, embryonic loss was high and of a similar level following intra-uterine insemination of ewes with either frozen or fresh ram semen. Thus, it appears that the methods employed for intra-uterine insemination, rather than the deep freezing of ram semen, may be responsible for the high level of embryonic loss observed for intra-uterine insemination of ewes with deep-frozen ram semen.

V. Acknowledgments

The technical assistance of R. Bretton, K. E. Turnbull, and S. Lane is gratefully acknowledged. The authors are in debt to Professor C. W. Emmens for helpful advice and criticism of this paper. The work was aided by a grant to one of them (K.W.E.) from the Australian Wool Board.

VI. References

Blackshaw, A. W. (1954).—*Aust. vet. J.* **30**, 249.

Blackshaw, A. W., and Emmens, C. W. (1953).—*Vet. Rec.* **65**, 872.

Dauzier, L., and Wintenberger, S. (1952).—*C.r. Séanc. Soc. Biol.* **146**, 660.

Emmens, C. W., and Blackshaw, A. W. (1955).—*Aust. vet. J.* **31**, 76.

First, N. L., Sevinge, A., and Henneman, H. A. (1961).—*J. Anim. Sci.* **20**, 79.

Kalev, G., and Venkov, T. (1961).—Proc. 4th Int. Congr. Anim. Reprod., The Hague. Vol. 4, p. 972.

Kempthorne, O. (1952).—"The Design and Analysis of Experiments." (John Wiley & Sons: New York.)

Kuznecov, M. P., and Kuprijanova, L. P. (1959).—*Trudy vses. nauchno-issled. Inst. Zivotn.* **23**, 171. [*Anim. Breed. Abstr.* **29**, 1518.]

Loginova, N. V. (1962).—*Ovcevodstvo* **8**(8), 20. [*Anim. Breed. Abstr.* **31**, 81.]

Lopyrin, A. I., and Loginova, N. V. (1958).—*Ovcevodstvo* **4**(8), 31. [*Anim. Breed. Abstr.* **27**, 72.]

Mattner, P. E. (1963*a*).—*Nature, Lond.* **199**, 772.

Mattner, P. E. (1963*b*).—*Aust. J. biol. Sci.* **16**, 877.

Mattner, P. E. (1966).—*Nature, Lond.* **212**, 1479.

Mattner, P. E., and Braden, A. W. H. (1963).—*Aust. J. biol. Sci.* **16**, 473.

Polge, C., and Lovelock, J. E. (1952).—*Vet. Rec.* **64**, 396.

Quinlan, J., Maré, G. S., and Roux, L. L. (1933).—*Ondestepoort J. vet. Sci.* **1**, 135.

Radford, H. M., Watson, R. H., and Wood, G. F. (1960).—*Aust. vet. J.* **36**, 57.

Robinson, T. J. (1965).—*Nature, Lond.* **206**, 39.

Salamon, S. (1968).—*Aust. J. exp. Agric. Anim. Husb.* **7**, 559.

Salamon, S., and Lightfoot, R. J. (1967).—*Nature, Lond.* **216**, 194.

Vlachos, C., and Sakalof, P. (1965).—*Bull. Physiol. Path. Reprod. Artif. Ins.* **1**, 71.

White, I. G., Blackshaw, A. W., and Emmens, C. W. (1954).—*Aust. vet. J.* **30**, 85.