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Pattern of Loss of Spermatozoa from the Vagina of the Ewe

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Abstract

In a series of experiments spermatozoa were inseminated blindly into the vaginae of ewes and then recovered at varying times after insemination. Most of the spermatozoa inseminated were lost by drainage through the vulva. The rate of loss was not affected by the motility of spermatozoa or oestrous state of the ewe. Initially after insemination the loss was not rapid with 82% of the inseminate still remaining in the vagina after 3 h. After this time losses were very rapid and by 9 h after insemination 18% of spermatozoa remained and by 12 h 10% remained. Spermatozoa were removed from the vagina during withdrawal of the penis after intromission and the extent of this loss varied between rams and with the volume of semen already in the vagina. Up to half the inseminate was lost in this way when there was 0·5 ml of semen in the vagina but only 11% was lost when the volume of inseminate was 0·1 ml. The unavoidable loss of spermatozoa may influence the quantity available for fertilizing ova.

Introduction

Few of the spermatozoa deposited in the vagina of the ewe reach the site of fertilization and most are voided to the exterior. The loss of large numbers of spermatozoa from the vagina by drainage was demonstrated by Conley and Hawk (1969) and Hawk and Conley (1971) who ligated the vulvo-vaginal junction in ewes after insemination. After 24 h 62% of the deposited spermatozoa were recovered from ligated ewes but less than 1% of the spermatozoa inseminated were recovered from control ewes. It has also been implied that the loss of spermatozoa from the vagina is rapid. Quinlivan and Robinson (1969) reported that spermatozoa disappeared rapidly from the vagina during the first 12 h after insemination and it was suggested that the spermatozoa are either destroyed and resorbed *in situ* or are voided to the exterior. Only 15% of spermatozoa inseminated were recovered from the reproductive tract of ewes by Lightfoot and Restall (1971) after 2 h and they attributed this to expulsion and/or drainage of mucus and spermatozoa from the vagina. In addition, Allison (1972*a*) found that natural mating had no significant effect on the numbers of spermatozoa within the vagina of ewes that had been artificially inseminated up to 2 h previously and concluded that a high proportion of the inseminate was lost within 2 h of mating. Although it is evident that many spermatozoa are lost from the vagina the pattern of loss has never been investigated in detail.

If losses of spermatozoa from the vagina are large and rapid then few spermatozoa will remain in the vagina in the first hours after insemination. In the first three experiments we examined the loss of spermatozoa from the vagina of the ewe in detail. The effects of the motility of spermatozoa, the oestrous state of the ewe and

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the volume and concentration of the inseminate on losses were considered, and the pattern of loss during 24 h was determined. There are no reports in the literature of the loss of spermatozoa from the vagina at intromission. But it might be expected that the withdrawal of the penis after intromission would contribute to the loss of spermatozoa. The fourth experiment tested this hypothesis.

Materials and Methods

Experimental Design

Experiment 1: Loss of live and dead spermatozoa from the vagina of oestrous and non-oestrous ewes

This part of the experiment had a 2 × 2 × 3 factorial design and the treatments were:

(i) Oestrous or non-oestrous ewes.
(ii) Live or dead spermatozoa inseminated into the vagina.
(iii) Time of recovery of spermatozoa.

Spermatozoa were recovered from the vaginae of the ewes either immediately or 6 or 12 h after insemination. The experiment was replicated (N = 72). In the first replicate 0·4 ml of semen was inseminated and in the second 0·3 ml was inseminated. Analysis of variance was used to analyse the data after determination of normal distribution.

Experiment 2: Effect of volume and concentration of semen on the loss of spermatozoa from the vagina

Ewes were inseminated with semen of different volumes and concentrations of spermatozoa. There were five treatments in a randomized block design each with three replicates (n = 27, N = 135). The treatments were:

(i) 0·1 ml of pooled undiluted semen.
(ii) 0·5 ml of pooled undiluted semen.
(iii) 1·0 ml of pooled undiluted semen.
(iv) 0·1 ml of pooled semen made up to a volume of 0·5 ml with physiological saline (0·9% w/v sodium chloride).
(v) 0·1 ml of pooled semen made up to a volume of 1·0 ml with physiological saline.

Spermatozoa were recovered from the vaginae of ewes either immediately or 6 or at 12 h after insemination.

Ewes were treated with progesterone to suppress oestrus. The data were converted to proportions of the spermatozoa recovered immediately after insemination and expressed as a percentage so that direct comparisons between treatments could be made after logarithmic transformation. The data were then analysed by analysis of variance.

Experiment 3: Pattern of loss of spermatozoa from the vagina over time

Twenty-one ewes treated with progesterone were randomly divided into seven groups of three. Each ewe was inseminated with 0·4 ml of dead spermatozoa. Spermatozoa were recovered from the vaginae of ewes in each group either immediately or 3, 6, 9, 12, 18 or 24 h after insemination. Each experimental group was replicated 1 week later (N = 42). After microscopic examination six samples were discarded (N = 36).

An analysis was undertaken to fit a curve to the data that described the loss of spermatozoa from the vagina of the ewe over time. A scatter plot of the data suggested a sigmoidal decay curve with a short upper tail. The Maximum Likelihood Program (Ross 1980) was used to fit a large number of families of curves to the data. Due to the large differences between the estimated variances of observations grouped over time, the observations were weighted. Weighting was done by the inverse square roots of the estimated variances and estimation was performed by weighted least squares.

Experiment 4: Effect of withdrawal after intromission on the loss of spermatozoa from the vagina

The experiment had a latin square design with five mating treatments and five ewes in each treatment. Control ewes were inseminated and then the semen was recovered immediately. In the other treatments, ewes were inseminated, a vasectomized ram was permitted to ejaculate into each ewe once and then the semen remaining in the vagina was recovered. Four different vasectomized rams were used. The experiment

was replicated three times with the same animals but each time the volume of the inseminate was either 0·1, 0·5 or 1·0 ml.

Animals

Adult Merino ewes were used in both experiments. Ewes were chosen randomly from a group of 55 entire ewes in experiments 1, 2 and 3 and from a group of 25 ovariectomized ewes in experiment 4. Both groups were run as separate flocks and were fed daily hay *ad libitum* and lupins at 200 g per head. To synchronize oestrus in experiment 1 ewes were given intramuscular injections of 20 mg of progesterone every second day for 12 days. On the 14th day 10 mg of progesterone was given and 200 i.u. of pregnant mare serum (PMS) was injected 24 h later. The PMS was obtained from mares between 60 and 70 days of pregnancy and was not purified prior to use. The gonadotrophic activity of PMS was measured in a bioassay and compared with a commercial pregnant mare serum gonadotrophin (Folligon, Intervet International). Non-oestrous ewes in experiments 1, 2 and 3 were injected with 20 mg of progesterone every second day throughout the experimental period. Oestrus was induced in the ewes in experiment 4 by administration of 20 mg of progesterone every second day for 14 days followed by a single injection of 60 μg of oestradiol benzoate (β-estradiol-3-benzoate, Sigma Chemical Company) 48 h after the last injection of progesterone (Robinson *et al.* 1956). Ewes were detected in oestrus by vasectomized rams fitted with harnesses and marking crayons (Radford *et al.* 1960).

Eleven Merino rams that had been trained to ejaculate into an artificial vagina (Salamon and Lindsay 1961) were used to supply semen in these experiments. Six vasectomized Merino rams were used to detect oestrous ewes and four of these were also used in experiment 2. All rams were fed hay *ad libitum* and a daily ration of 300 g lupins.

Treatment of Semen and Insemination of Ewes

The semen from all rams was pooled prior to insemination. Volumes and concentrations of suspensions of spermatozoa were obtained by diluting the pooled semen with physiological saline (0·9% sodium chloride). Where dead spermatozoa were required the semen was collected the day before it was needed and was refrigerated at 4°C overnight. For live spermatozoa, semen was collected just prior to insemination.

In both experiments the vagina of each ewe was flushed with 20 ml of physiological saline just before insemination to ensure that there was no cellular debris, bacteria or spermatozoa from a previous insemination. Ewes were inseminated using plastic disposable inseminating pipettes with a 2·5 mm internal and 4·5 mm external diameter. The pipettes were calibrated to the desired dose and were connected to either a 1- or 2-ml syringe. Semen was deposited blindly into the vagina. Each ewe was restrained but remained standing throughout insemination.

Recovery of Spermatozoa from the Vagina of the Ewe

Spermatozoa were recovered from the vagina of the ewe with a plastic inseminating pipette connected to a 20-ml syringe. Each ewe was elevated over a rail and a speculum used to penetrate and open the vulva. Light was shone into the vagina from a cable connected to a fibre optic light source. Physiological saline (20 ml) was flushed into the vagina with the pipette which was rotated to ensure that the vaginal walls were flushed. The contents of the vagina were withdrawn and were placed in a 50-ml volumetric flask. This procedure was repeated with another 20 ml of saline.

The contents in each flask were made up to 50 ml. Before semen samples were diluted for counting, a drop from each sample was examined under a microscope (×100 magnification). Samples which appeared to contain sources of DNA other than from spermatozoa were excluded from the experiment. Semen samples were diluted further in physiological saline.

Estimation of Numbers of Spermatozoa by Analysis for Quantity of DNA

The number of spermatozoa in each sample were counted by fluorometric analysis for DNA (Kissane and Robins 1958; Hinegardner 1971). The standards were prepared from known concentrations of spermatozoa counted in an haemocytometer.

Semen was collected from rams, pooled and the concentration determined by haemocytometer. Fifteen separate counts were made on the pooled undiluted semen sample. The first set of standards made were eventually depleted so it was necessary to collect and count a second pool of semen. There was little variation between the counts for each pooled semen sample. The first pool of undiluted semen contained 4890×10^6 spermatozoa/ml and the second pool contained 5020×10^6 spermatozoa/ml.

Standards were obtained by serial dilution of the semen pool with phosphate-buffered saline (PBS) containing per litre 0·01 mol PO_4^{3-}, 0·14 mol NaCl (pH 7·0) containing 84 mmol $CaCl_2$ per litre, 49 mmol $MgCl_2$, 2·7 mmol KCl and with 2% (v/v) formaldehyde as a preservative. Three sets of standards were made from the first pool of undiluted semen and two sets from the second. The volume of each standard used in the assays was 0·05 ml. The standards contained a known number of spermatozoa which ranged from 25×10^6 to $0{\cdot}2 \times 10^6$ and were stored at 4°C.

Due to the sensitivity of the assay it was necessary to dilute semen samples before analysis. The diluent used was PBS or, in the case of samples that had been recovered from the vagina of the ewe, physiological saline. A number of dilutions were made and compared against each standard curve until one of the curves could be used to estimate the number of spermatozoa in the sample.

The between-assay coefficient of variation using 19 sets of standards was 11·7% and the within-assay coefficient of variation ranged between 0·02 and 0·06%.

Artifact errors due to the emission intensity of seminal plasma were found to have a mean of 3·1%. Cervical mucus from ewes in oestrus and at mid-cycle did not influence the assay.

Results

Recovery of Spermatozoa from the Vagina

The recovery of spermatozoa from the vagina immediately after insemination was very high. When 0·4 or 0·5 ml of undiluted semen was inseminated the mean (±s.e.) recovery from experiments 1, 2 and 3 was 95·06 ± 3·03%. In experiment 2 the mean recovery of 1·0 ml of undiluted semen from the vagina immediately after insemination was calculated as 103·54 ± 11·60%. For 0·1 ml of undiluted semen in the same experiment the recovery was 138 ± 31·12%. The variability in this case was due to sampling errors. The spermatozoa lost or remaining in the vagina were expressed as proportions (%) of those recovered immediately after insemination.

Table 1. Mean (±s.e.) number of dead or live spermatozoa (in millions) recovered from the vaginae of oestrous or progesterone-treated ewes at 0, 6 or 12 h after insemination

The loss (%) of spermatozoa from the vagina is indicated in parentheses

Time after insemination (h)	Oestrous ewes		Non-oestrous ewes		Mean loss from all ewes (%)
	Dead sperm	Live sperm	Dead sperm	Live sperm	
0	0·7±0·06	1·01±0·10	0·78±0·07	0·76±0·13	0
6	0·14±0·08 (80)	0·13±0·07 (87)	0·26±0·04 (67)	0·39±0·15 (49)	70
12	0·13±0·07 (82)	0·10±0·05 (90)	0·27±0·10 (65)	0·15±0·05 (80)	80
Mean	0·34±0·08 (49)	0·34±0·11 (34)	0·43±0·08 (55)	0·43±0·09 (57)	51

Experiment 1

There were large and significant ($P < 0{\cdot}01$) losses of spermatozoa from the vagina with time after insemination for all treatments. Only 30% of the spermatozoa inseminated remained in the vagina after 6 h and 20% after 12 h (Table 1). Insemination of oestrous or non-oestrous ewes with live or dead spermatozoa did not affect the rate of loss from the vagina (Table 1).

Experiment 2

Many of the spermatozoa inseminated were progressively lost from the vagina. After 6 h 53% of the spermatozoa had been lost ($P < 0{\cdot}01$) and after 12 h 73% had been lost ($P < 0{\cdot}01$). There were no significant differences in the rate of loss of the different volumes and concentrations of spermatozoa from the vagina (Table 2).

Table 2. Mean (±s.e.) number of spermatozoa (in millions) recovered from the vagina at 0, 6 and 12 h after insemination for each of the five treatments

The percentage loss of spermatozoa from the vagina is given in parentheses

Time after insemination (h)	Concentration and volumes of spermatozoa: 0·1 ml semen, undil.	0·5 ml semen, undil.	1·0 ml semen, undil.	0·1 ml semen, 0·4 ml saline	0·1 ml semen, 0·9 ml saline	Mean loss from all ewes (%)
0	0·40±0·07	1·64±0·15	4·18±0·71	0·44±0·06	0·37±0·03	0
6	0·18±0·08 (55)	0·94±0·23 (43)	1·36±0·43 (67)	0·27±0·04 (39)	0·10±0·02 (73)	53
12	0·17±0·09 (57)	0·29±0·09 (82)	0·94±0·37 (77)	0·10±0·04 (77)	0·11±0·01 (70)	73
Mean	0·25±0·05 (63)	0·96±0·14 (59)	2·16±0·40 (52)	0·28±0·04 (64)	0·21±0·03 (58)	41

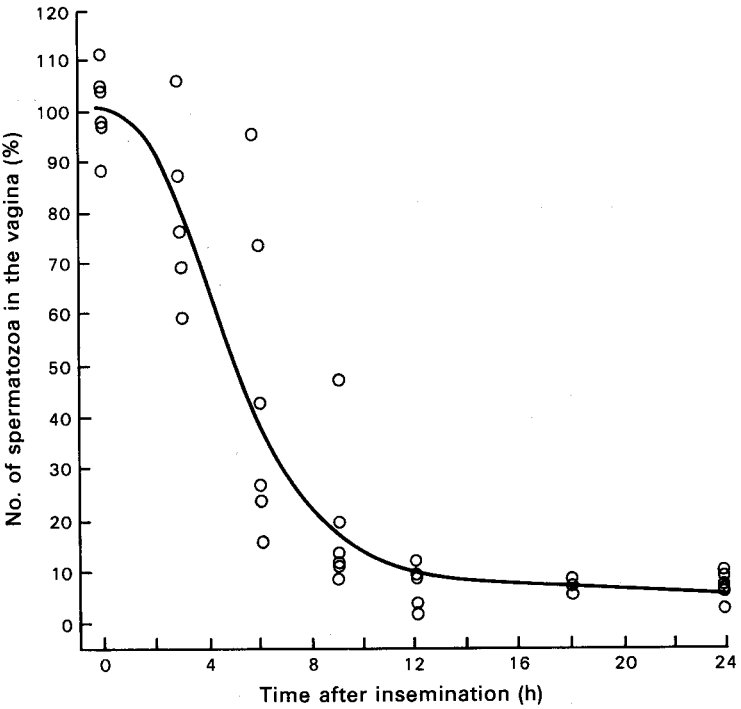

Fig. 1. A Gompertz curve describing the losses of spermatozoa over time after insemination of dead spermatozoa into the anterior vagina of ewes.

Experiment 3

The relationship between the spermatozoa remaining in the vagina and the time from insemination showed that during the first 3 h the loss of spermatozoa was slow with 82% of the spermatozoa inseminated still remaining in the vagina (Fig. 1). During the next 6 h the loss of suspensions of spermatozoa from the vagina was rapid with 41% of the spermatozoa inseminated remaining after 6 h and 18% remaining after 9 h. Only 10% of the spermatozoa inseminated were recovered after 12 h. Little further loss was then observed with 8% of the spermatozoa remaining in the vagina 18 and 24 h after insemination.

Of the number of curves fitted to these data, the Gompertz curve fitted best overall describing the pattern of loss of spermatozoa from the vagina with time (Fig. 1). The accuracy of this curve was tested by comparing predicted values of the proportion of spermatozoa remaining in the vagina over time with actual values taken from eight separate sets of data for the losses of spermatozoa. The data were highly variable and only 69% of individual values were within two standard errors of the curve. Nevertheless, a *t*-test showed the actual and predicted values to be not significantly different. When the mean numbers of spermatozoa from the eight sets of data were compared to the curve, 7 out of 8 were within two standard errors 6 h after insemination and all were within two standard errors of the curve 12 and 24 h after insemination. On the basis of this, and because the rate and magnitude of loss did not vary with the volume and concentration of the inseminate (Table 2), the curve in Fig. 1 was considered to be a realistic representation of the pattern of loss of spermatozoa from the vagina.

Table 3. Loss of insemination spermatozoa from the vagina of ewes after intromission by vasectomized rams

Volume of inseminate (ml)	10^{-6} × Mean (±s.e.) No. of spermatozoa recovered		Loss after intromission (%)
	Without intromission	With intromission	
0·1	164 ± 12·4	146 ± 8·5	11
0·5	677 ± 105·4	330 ± 55·6	51
1·0	1233 ± 376·0	825 ± 162·8	33
Mean	691 ± 162·2	412 ± 63·93	31·7

Experiment 4

Over all three volumes of inseminate, withdrawal of the ram's penis after intromission caused a significant loss of 31·7% of spermatozoa ($P < 0·01$) from the vagina (Table 3). The extent of this loss varied between the rams and between the volumes of inseminate. When 0·1 ml of semen had been inseminated control ewes had not lost significantly more spermatozoa than those ewes that had been mated (11%) but with an inseminate of 0·5 ml about half the spermatozoa (51%) inseminated had been lost and this was significant ($P < 0·05$). Even though one-third of the spermatozoa were lost due to withdrawal of the ram's penis after intromission when the inseminate was 1·0 ml, this loss was not statistically significant.

Discussion

There were large losses of spermatozoa from the vagina 6 and 12 h after insemination in both experiments 1 and 2 but none of the treatments influenced the rate of loss. Since there was no significant difference in the magnitude of losses of spermatozoa, live or dead, from the vaginae of ewes in oestrus or not in oestrus (Table 1) insemination of non-oestrous ewes with dead spermatozoa was adopted as a model in experiments 2, 3 and 4. Both the condition of the cervical mucus (Mattner 1963; Raynaud 1973; Robinson 1973) and the motility of the spermatozoa (Mattner 1966; Mattner and Braden 1969; Lightfoot and Restall 1971) are important for the entry and passage through the cervix and for the formation and retention of cervical reservoirs. Thus any deficit in the number of spermatozoa inseminated into the vagina could be attributed to loss by drainage through the vulva because when dead spermatozoa were inseminated into non-oestrous ewes very few, if any, would have migrated up the reproductive tract. It was expected that the rate of loss of spermatozoa would vary with the volume and viscosity of semen inseminated but, surprisingly, in experiment 2 the proportion of spermatozoa lost from the vagina over 12 h did not vary with either the volume or concentration of the inseminate (Table 2). Therefore the results from experiments 1 and 2 suggested that the loss of spermatozoa from the vagina was not linear but followed a much more complex pattern.

Irrespective of the pattern of loss, most spermatozoa had disappeared from the vagina within 12 h of insemination with only 10% of the number inseminated remaining (Fig. 1). Conley and Hawk (1969) and Hawk and Conley (1971) found that most of the spermatozoa deposited in the tract of the ewe had been lost from the vagina after 24 h but they did not investigate the pattern of loss within the 24-h period as was done in experiment 3. This experiment, which illustrates the quantitative pattern of losses, showed that initially after insemination the loss was not great and 82% of the inseminate remained in the vagina after 3 h. This refutes an hypothesis of a rapid loss of spermatozoa from the vagina soon after mating and is contrary to the suggestion of Allison (1972*a*) that a high proportion of the inseminate is lost within 2 h of mating. The results also disagree with other studies where rapid loss has been implied because very few of the spermatozoa inseminated have been recovered soon after insemination (e.g. Quinlivan and Robinson 1969 after 1 h; Lightfoot and Restall 1971 after 2 h). The reason for this difference may lie in the different techniques used. In our experiment spermatozoa were recovered directly from the vaginae of live ewes. In all the other experiments spermatozoa were recovered by removal and flushing of the reproductive tracts of ewes after they had been slaughtered. The accuracy of recovery in our experiments using direct flushing was close to 100% but no values were given for recovery rates in the other experiments and presumably none were estimated. By contrast, spermatozoa were lost rapidly from the vagina between 3 and 9 h after insemination and this is in agreement with Quinlivan and Robinson (1969) who reported a large reduction in the numbers of vaginal and cervical spermatozoa between 1 and 12 h after insemination. It can be concluded that the rate of loss of spermatozoa from the vagina of the ewe to the exterior is not constant but follows a pattern of gradual decline followed by a longer period of rapid loss where most of the spermatozoa disappear. After this period the small numbers of spermatozoa left disappear slowly.

Withdrawal of the ram's penis after intromission caused a loss of up to half the spermatozoa in the vagina but these losses varied between rams and with the volume of the inseminate. The variation between rams in their influence on the losses might be explained on the basis of differences in the size and shape of the penis' of rams and of the vaginae of ewes or differences in the style of mating of individual rams. When there was 0·5 ml of semen in the vagina rams removed a significant amount ($P < 0·05$) therefore in field mating it seems likely that entire rams would contribute to losses at the same time as depositing spermatozoa. If this is so, spermatozoa would not accumulate in the vagina at a rate equal to the total number in successive ejaculates, as was suggested by Synnott *et al.* (1981). Frequent ejaculation leads to a decrease in the volume and density of the ejaculate and in the total number of spermatozoa in the ejaculate (Bielanski and Wierzbowski 1961; Salamon 1962, 1964; Salamon and Lightfoot 1967; Allison 1972*b*; Jennings and Crowley 1972; Jennings and McWeeney 1976; Tomkins and Bryant 1976; Simpson and Edey 1979; Synnott *et al.* 1981). It may be expected that in the field where rams are mating continually the amount of semen in the vagina and therefore losses due to withdrawal after intromission may be less than we have measured with 0·5 ml of semen. On the other hand, for the smaller proportion of ewes that are mated many times (Tilbrook 1984), and therefore would have a larger volume of semen in their vaginae, up to half the spermatozoa already present in the vagina could be lost due to withdrawal after intromission. At the beginning of the mating season or when rams have not been working continually and have large ejaculates, withdrawal after intromission may also have a large influence on eventual numbers of spermatozoa remaining.

In conclusion, these experiments have shown that there is a pattern of loss of spermatozoa from the vagina and withdrawal of the ram's penis after intromission can contribute to this loss depending on the volume of semen in the vagina. The unavoidable loss of spermatozoa from the vagina may influence the quantity of spermatozoa available for fertilizing ova and the implications of this for fertility were investigated in a subsequent study (Tilbrook and Pearce 1986).

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