THE RECOVERY OF SPERMATOZOA FROM THE REPRODUCTIVE TRACT OF THE SPAYED EWE TREATED WITH PROGESTERONE AND OESTROGEN

A. J. ALLISON* AND T. J. ROBINSON

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

(Received 29th September 1971, accepted 13th December 1971)

Summary. Eighty spayed ewes were used in a 4×4 factorial experiment ($n = 5$) in which four doses of oestradiol benzoate (0, 12.5, 25 and 50 μg) were administered 48 hr after four progesterone regimens (0, 5, 10 and 20 mg/day for 12 days). Each ewe was inseminated 24 hr later with 0.15 ml undiluted semen ($\geq 500 \times 10^6$ spermatozoa). After a further 24 hr, the ewes were slaughtered and the vagina, cervix, uterus and Fallopian tubes were flushed for spermatozoa. The population of spermatozoa in the cervix was highly dependent upon oestrogen ($P < 0.001$) and appeared to be related to dose ($P = 0.1$). There was no significant mean effect of progesterone but there was a significant interaction with oestrogen ($P < 0.05$). High numbers of spermatozoa were associated with high doses of oestrogen and of progesterone. The numbers of spermatozoa in the vagina were not related to oestrogen and there was no relationship with the numbers in the cervix ($r = -0.011$). Penetration and maintenance of a cervical population of spermatozoa was related to the level of oestrogen and full expression required adequate progesterone influence preceding that of oestrogen.

INTRODUCTION

It is becoming increasingly evident that the inability of the reproductive tract of the ewe to maintain an adequate population of spermatozoa from the time of mating to that of fertilization is a common cause of reproductive failure. It has been implicated in 'clover infertility' (Lightfoot, Croker & Neil, 1967), in the relative infertility following synchronization of oestrus with exogenous progestagen, and in a proportion of ewes experiencing normal oestrous cycles (Quinlivan & Robinson, 1967, 1969).

The pattern of transport and survival of spermatozoa within the female reproductive tract is related to the endocrine status of the animal. Early reports indicated that spermatozoa could be transported through the reproductive tract of the ewe under widely differing endocrine states (e.g. Green & Winters, 1935; Phillips & Andrews, 1937; Warbritton, McKenzie, Berliner &

* Present address: Invermay Agricultural Research Centre, Private Bag, Mosgiel, New Zealand.

Andrews, 1937; Schott & Phillips, 1941), but these observations were based on few animals and, with the exception of those of Warbritton *et al.* (1937), were not quantitative. The probability of fertilization following such artificial practices as forced mating at an individual ovulation in anoestrus is low (see Robinson, 1951), despite reports of some spermatozoa reaching the Fallopian tubes under such conditions.

Definitive evidence concerning the quantitative effects of hormones, notably progestagen and oestrogen, on the survival and fertilizing capacity of spermatozoa in the female tract is lacking, but some interesting qualitative effects are becoming evident. Thus, Conley & Hawk (1970) report that loss of spermatozoa from the uterine lumen of ewes in the luteal phase is less than that from oestrous ewes. Further, inadequate progestagen before insemination impairs sperm transport and survival and, consequently, the chances of fertilization (Allison & Robinson, 1970). Mattner & Braden (1963) made a preliminary study on the effect of two dose levels of progesterone and of stilboestrol dipropionate on the pattern of distribution of spermatozoa throughout the reproductive tract of ovariectomized ewes $3\frac{1}{2}$ to 4 hr after mating. More spermatozoa were present in the Fallopian tubes of the fifteen oestrogen-treated ewes than of three untreated controls, but there was no demonstrable effect of the dose of either oestrogen or progesterone on the distribution of spermatozoa within any segment of the reproductive tract.

This paper presents the results of an experiment designed to examine the quantitative effects of oestrogen and progesterone on the pattern of distribution of spermatozoa in the reproductive tract of the ewe 24 hr after insemination, that is at the approximate time of fertilization.

MATERIALS AND METHODS

Experimental design

Eighty medium wool Merino ewes were ovariectomized approximately 7 weeks before commencement of the experiment. Extreme care was taken to avoid unnecessary handling of the tract, and in particular the fimbriae, in order to minimize adhesions and occlusion of the Fallopian tubes.

Ewes randomized into a factorial experiment which incorporated various endocrine treatments preceding artificial insemination:

1. dose of progesterone during a priming period of 12 days—0, 5, 10, 20 mg/day
2. dose of oestradiol benzoate (ODB) following progesterone—0, 12.5, 25, 50 μ g

Factorial 4×4 ; $n = 5$; $N = 80$.

Three animals were lost from the experiment between the times of ovariectomy and the final injection of ODB.

Administration of hormones

Artificial cycles. Immediately after ovariectomy, and for 11 days subsequently, each ewe was injected with 10 mg progesterone (intramuscularly in 1 ml peanut oil) followed 2 days later by a single injection of 25 μ g ODB. This 'artificially

induced cycle' was repeated once (commencing 3 days after the injection of ODB) before initiation of the 'experimental cycle'.

Experimental cycle. The appropriate dose of progesterone (intramuscularly in 1 ml peanut oil) was given daily at 09.00 hours for 12 days commencing 3 days after the previous injection of 25 µg ODB of the second 'artificial cycle'. The appropriate dose of oestrogen was given 48 hr later.

Insemination

Ewes were examined twice daily at 08.00 and 17.00 hours for evidence of oestrus, detected by vasectomized rams fitted with siresine harnesses and crayons. All ewes were inseminated 24 hr after the injection of ODB (or at the corresponding time in those which received no ODB), regardless of whether or not they exhibited oestrus.

Semen was collected by artificial vagina from three rams and samples of high quality were pooled, following visual assessment of density and motility. Each ewe was inseminated with 0.15 ml undiluted semen estimated to contain not less than 500×10^6 spermatozoa.

Following insemination, ewes remained undisturbed in a small pen until just before slaughter.

Recovery of spermatozoa

Twenty-four hours ($\pm \frac{1}{2}$ hr) after insemination, each ewe was slaughtered and the whole reproductive tract was removed and ligated at the base of the Fallopian tubes (0.6 mm proximal to the uterotubal junction) and at either end of the cervix, as described by Mattner & Braden (1963). Each tract was placed in a small plastic bag before removal to the laboratory for dissection. The four sections of each tract were flushed with 0.9% saline in the following manner:

Fallopian tubes: from the uterine end, each with 2 ml, into a single labelled 5-ml glass vial;

uterus: placed in a filter funnel and flushed from the cranial end using approximately 10 ml for each horn;

cervix: laid open in a filter funnel, after cutting down one side, and washed with 10 ml saline from the vaginal end; then inverted and washed with another 10 ml from the uterine end;

vagina: laid open in a filter funnel and washed with 20 ml of saline.

Clean instruments were used for each section of the tract and the hands of the operator were thoroughly washed after each flushing. All uterine, cervical and vaginal flushings were collected in 40-ml polythene bottles and, with those from the Fallopian tubes, were frozen at -20°C for subsequent thawing, staining and counting.

Estimation of numbers of spermatozoa

After thawing, all samples were stained using Eosin B-Fast Green F.C.F. (Hackett & McPherson, 1965). Individual samples were thoroughly mixed and a 1-ml aliquot of each vaginal, cervical and uterine sample was distributed evenly onto four slides and carefully covered by a 20 × 40-mm coverslip. The

spermatozoa were allowed to settle and the numbers present were estimated as described by Allison & Robinson (1970). The volume of each flushing was measured and the total number of spermatozoa estimated. A similar procedure was adopted for the tubal flushings, except that the whole volume distributed on six slides was used.

Values were calculated for the three missing animals and analyses of variance and of covariance were conducted on the estimated numbers of spermatozoa recovered, using the following transformations on y :

Vagina (no zero values) $\log_{10}(1.0x + 0.0)$

Cervix (twelve zero values) $\log_{10}(1.0x + 0.5)$

where x = number of spermatozoa in thousands.

RESULTS

Ewes in oestrus

The three ewes lost from the experiment were randomly distributed among treatments.

Thirty ewes were in oestrus at the time of insemination, and there was a clear relationship with dose of ODB: 0 μg , 0/20; 12.5 μg , 2/18; 25 μg , 10/19; 50 μg , 18/20.

TABLE 1
NUMBER OF EWES FROM WHICH SPERMATOZOA WERE RECOVERED
FROM VARIOUS DIVISIONS OF THE REPRODUCTIVE TRACT

Treatment	No. of ewes	Spermatozoa recovered from:				
		Vagina	Cervix	Uterus	Fallopian tubes	Either uterus or tubes
Oestrogen (μg)						
0	20	20	16	2	2	4
12.5	18	18	15	2	2	4
25	19	19	16	4	5	7
50	20	20	18	5	4	8
Progesterone (mg/day)						
0	18	18	16	3	2	5
5	20	20	14	4	2	6
10	19	19	17	2	2	4
20	20	20	18	4	7	8
Total for each treatment	77	77	65	13	13	23

N.B. Only three ewes yielded spermatozoa from both uterus and tubes.

Number of spermatozoa recovered

Of the seventy-seven ewes for which data are available, eleven had occlusion of one Fallopian tube. Spermatozoa were recovered from the vaginae of all seventy-seven ewes and from the cervixes of sixty-five, and these data were suitable for formal analysis. On the other hand, only twenty-three ewes yielded spermatozoa from either the uterus or the Fallopian tubes and, of these, only three yielded spermatozoa from both sources (Table 1).

These data were not suitable for formal analysis and there were no clear effects of treatment, although there was a trend towards higher numbers with increasing doses of both steroids.

The data for the vagina and cervix were characterized by enormous variability. Table 2 presents the arithmetic means together with the corrected

TABLE 2
NUMBERS OF SPERMATOZOA RECOVERED FROM THE VAGINA AND CERVIX OF EWES
TREATED WITH VARYING DOSES OF PROGESTERONE AND OESTROGEN

Dose of progesterone (mg/day)	Vagina					Cervix				
	0	Dose of oestrogen (μ g)				0	Dose of oestrogen (μ g)			
		12.5	25	50	Mean		12.5	25	50	Mean
A. Arithmetic means										
0	666.8	138.3	139.6	89.7	258.6	1.4	7.9	12.8	36.7	14.7
5	9.0	12.5	21.8	43.1	21.6	0.4	59.3	34.0	14.7	27.1
10	93.6	75.4	80.6	26.4	69.0	3.0	7.4	72.0	108.8	47.8
20	89.4	29.4	172.9	54.7	86.6	3.2	5.4	46.0	132.6	46.8
Mean	214.7	63.9	103.7	53.5	109.0	2.0	20.0	41.2	73.2	34.1
B. Corrected log means										
0	134.6	54.9	78.5	77.4	81.9	1.3	7.9	4.9	6.3	4.4
5	6.7	10.8	10.7	26.6	12.0	0.2	28.5	12.5	4.1	5.4
10	58.7	45.1	78.4	21.3	45.9	2.4	3.1	21.1	22.0	7.9
20	25.8	13.4	53.1	36.9	28.7	2.6	1.7	20.9	67.9	9.6
Mean	34.2	24.4	43.3	35.6	33.7	1.3	6.2	13.0	14.3	6.5

Each individual value is the mean for five ewes, except for three missing values. All data are expressed in thousands.

TABLE 3
ANALYSES OF VARIANCE OF DATA IN TABLE 1

Source of variation	Degrees of freedom	A. Vagina			B. Cervix		
		Mean square	F	P	Mean square	F	P
Dose of oestrogen	(3)	0.213	0.53				
0 versus Σ 12.5, 25, 40 μ g	1				9.066	19.19	<0.001
12.5, 25, 50 μ g linear	1				1.198	2.54	$\approx$ 0.1
quadratic	1				0.234		
Dose of progesterone	(3)				0.403	0.85	
0 versus Σ 5, 10, 20 mg	1	3.964	9.81	<0.01			
5, 10, 20 mg linear	1	1.439	3.56	<0.1			
quadratic	1	2.065	5.11	<0.05			
Interaction	9	0.283	0.70		1.019	2.16	<0.05
Within (Error)	61	0.404			0.473		
Total	76						

mean values obtained following reversion of the $\log_{10}$ means. Table 3 presents the analyses of variance.

There was no relationship between the numbers of spermatozoa recovered from the vagina and from the cervix (correlation coefficients; gross, $r = +0.021$; residual, $r = -0.011$).

The mean numbers recovered from the vagina were influenced by the pre-treatment with progesterone. Significantly fewer were recovered from those ewes treated with progesterone ($P < 0.01$) and, within the progesterone treatments, there was an effect of dose which had a linear ($0.05 < P < 0.1$) and quadratic ($P < 0.05$) component. Oestrogen had no effect and was not involved in any interaction.

The mean numbers recovered from the cervix were markedly influenced by the dose level of oestrogen. Significantly more were recovered from those

TEXT-FIG. 1. The distribution of numbers of spermatozoa recovered from the cervix of ewes treated with oestradiol benzoate-ODB (data pooled for dose of progesterone) 0 versus Σ 12.5, 25, 50; $P < 0.01$.

ewes treated with oestrogen ($P < 0.001$) and, within the oestrogen-treated ewes, there was an apparent effect of dose which approached significance ($P \approx 0.1$). The nature of the effect of oestradiol is shown in Text-fig. 1. The proportion of ewes which yielded large numbers of spermatozoa ($> 20,000$) increased with increasing doses of oestrogen ($P < 0.01$). Progesterone had no average effect on mean numbers of spermatozoa but was involved in an interaction with oestrogen ($P < 0.05$), the nature of which is illustrated in Text-fig. 2. Pretreatment with the higher doses of progesterone appeared necessary for maximum expression of the effect of oestrogen.

TEXT-FIG. 2. The nature of the interaction between dose of progesterone (●, 0; ▲, 5; ■, 10; △, 20 mg/day) and dose of oestrogen on the number of spermatozoa recovered from the cervix of ewes.

DISCUSSION

Two factors operating within the female genital tract have been implicated in the transport of spermatozoa, namely uterine motility and the characteristics of the cervical mucus. Both are influenced by the ovarian hormones and, in view of observations such as those of Noyes, Adams & Walton (1959) and Mattner & Braden (1969), it is not surprising that ewes which received no oestrogen yielded so few spermatozoa from the cervixes 24 hr after insemination. Noyes *et al.* (1959) found that the proportion of ova fertilized following transfer to artificially inseminated spayed rabbit does was clearly related to increasing dose of oestrogen. By-passing the cervix by inseminating directly into the uterus decreased the minimum effective dose of oestrogen. Mattner & Braden (1969) found many fewer spermatozoa in the cervixes 4 and 24 hr after insemination of ewes late in oestrus—by which time ovarian oestrogen production has fallen (Moore, Barrett, Brown, Schindler, Smith & Smyth, 1969)—as compared with insemination early. Previously, Mattner & Braden (1963) had reported no effect of steroid hormones on the number and distribution of spermatozoa within the cervix and uterus of ovariectomized ewes 3½ to 4 hr after mating. The apparent discrepancy between their earlier (1963) results

and ours can be explained on the basis either of the few animals used, coupled with the enormous variation between individuals, or of the difference in time after insemination or mating when recoveries of spermatozoa were effected.

The action of oestrogen on the production and physical characteristics of cervical secretions is well documented (Vickery & Bennett, 1968), and Bishop (1961) concluded that oestrogen-induced hypersecretion of cervical mucus increased the viability of spermatozoa as well as their capacity to penetrate. More recently, Lindsay & Francis (1968) have demonstrated increasing production of cervical mucus in spayed ewes injected with increasing doses of stilboestrol. In spayed and postmenopausal women, cervical mucus is scanty and impenetrable, but can be transformed into the characteristic mucus of ovulation by giving oestrogen (Abarbanel, 1946; Pommerenke & Viergiver, 1946).

The effect of ovarian hormones on the motility of the reproductive tract is also well documented (Reynolds, 1965), and contractions of the uterus (Hartman, 1957) and of the vagina (Krehbiel & Carstens, 1939; Akester & Inkster, 1961) have both been implicated in the entry of spermatozoa into the cervix. Motility of the cervix and uterus is minimal when the tract is under the influence of progesterone (Reynolds, 1937; Brinsfield, 1968) and increases during the follicular phase.

The relative importance of these two effects of the ovarian hormones on the initial establishment and consequent maintenance of a cervical population of spermatozoa adequate for fertilization in the ewe remains a matter for debate. The anatomical structure of the cervix of the ewe and the increasing evidence of a relationship between the nature of cervical mucus and fertility (Smith, 1971) suggest, however, that the characteristics of the mucus may be more important than uterine motility.

The numbers of spermatozoa recovered were much lower than those reported in entire ewes (Mattner, 1963; Mattner & Braden, 1963, 1967; Quinlivan & Robinson, 1969). Since recovery was achieved only 24 hr after insemination, it can be argued that the observed pattern of response to the endocrine treatments is not necessarily a reflection of the pattern of initial penetration and establishment of a cervical population. Indeed, data of Quinlivan & Robinson (1967) concerning the pattern of transport and survival of spermatozoa in the tract of ewes at a normal oestrus, as compared with one controlled by exogenous progestagen, show that the initial pattern of penetration and establishment may show little relationship to subsequent survival. Our data, therefore, simply show that oestrogen is directly involved in the initial establishment of a cervical population and in its maintenance for 24 hr, without differentiating between establishment and maintenance nor, for that matter, between whether the spermatozoa are alive or dead at 24 hr.

The absence of any demonstrable relationship between the numbers of spermatozoa recovered from the vagina on the one hand and the cervix on the other indicates a complete dissociation between the two populations 24 hr after insemination. The endocrine environment which favours the establishment and survival of a cervical population appears to differ markedly from that needed for survival in the vagina. The effect of pretreatment with progesterone in depressing the survival of the latter population probably has biological signi-

ificance in view of the high order of statistical significance ($P < 0.01$) but the marginally significant effect of dose is less convincing in view of the variability characteristic of the data.

The significant interaction between dose of oestrogen and the preceding progesterone regimen on the cervical population implies that a balance of endocrine effects is involved. Imbalance, besides affecting the quantitative and qualitative characteristics of cervical secretions and the motility of the tract, may affect the capacity of the spermatozoa to survive. Noyes (1959) concluded that normal transport of spermatozoa and ova does not occur when oestrogen alone is acting on the female genital tract, while Lindsay & Francis (1968) found that although progesterone is inhibitory to the production of cervical mucus, it is a necessary prerequisite for a normal pattern of production in spayed ewes ingesting oestrogenic forage. Further, the observations of Conley & Hawk (1970) suggest that progesterone could be involved in the capacity of spermatozoa to survive in the female reproductive tract. Our data imply an interaction between progesterone and oestrogen in this context.

It appears that adequate progesterone priming of the ewe is a necessary prerequisite for a pattern of production of cervical mucus which favours the establishment and subsequent survival of a population of spermatozoa in the cervix. Earlier observations on the inadequacy of treatment with exogenous progestagen to provide an environment suitable for the maintenance of populations of spermatozoa in the female tract sufficient for fertilization in a high proportion of ewes (Quinlivan & Robinson, 1967, 1969) and the relationship between numbers of spermatozoa recovered and dose of progestagen used for the control of the time of ovulation and oestrus (Allison & Robinson, 1970) support this concept.

ACKNOWLEDGMENTS

Financial support was provided by G. D. Searle (Aust.) Ltd and the Australian Research Grants Committee. One of the authors (A.J.A.) was the recipient of an Australian Commonwealth Scholarship. We wish to thank Mr J. Ellsmore for technical assistance and Mr L. N. Balaam for advice concerning the statistical analyses of the data.

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