FERTILIZATION AND SURVIVAL OF FERTILIZED EGGS IN THE EWE FOLLOWING SURGICAL INSEMINATION AT VARIOUS TIMES AFTER THE ONSET OF OESTRUS

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Summary

Two experiments are described in which fertilization and development of fertilized eggs to lambs were studied following cervical and surgical (tubal and uterine) insemination of ewes at various times after the onset of oestrus. The experiments also provided information on the time of ovulation and on the fertilizable life of the ovine egg.

The majority of ewes (76%) ovulated between 12 and 24 hr after the onset of oestrus and eggs remained highly fertilizable for 12–18 hr after ovulation.

Almost complete failure of fertilization and lambing followed cervical insemination carried out later than 24 hr after the onset of oestrus, whereas surgical insemination at 54 hours gave some fertilization (3 of 16 ewes fertile). There was no marked difference between fertilization and lambing performance of cervically inseminated ewes (20 of 96 ewes fertile; 11 of 96 lambed), whereas there was a marked difference following surgical insemination (37 of 96 ewes fertile; 6 of 96 lambed).

Surgical insemination at all times gave a high incidence of fertilized eggs containing small anucleate particles. Late surgical insemination resulted in a number of grossly abnormal fertilized eggs. Nine of 23 eggs fertilized by surgical insemination at 36 and 48 hr were either polyspermic, or contained multinucleate blastomeres. The viability of the types of abnormal eggs and their contribution to poor lambing performance following surgical insemination are discussed.

I. INTRODUCTION

Estimates of the fertilizable life of the ovine egg vary from 10 to 24 hr (Green and Winters 1935; Dauzier and Wintenberger 1952; Thibault 1967). The estimates have been obtained by mating at specific times after the onset of oestrus and the accuracy of the estimates must be dependent upon two assumptions. First, that ovulation occurs at a uniform time after the onset of oestrus, and second, that rate of transport of spermatozoa to the site of fertilization is uniformly rapid throughout oestrus. However, neither assumption appears to be strictly valid. Killeen and Moore (1970*a*) and Mattner and Braden (1969) have shown that the efficiency of transport of spermatozoa diminishes in late oestrus and individual variations in time elapsing between the onset of oestrus and ovulation have been well documented (Killeen 1969).

Capacitation of ram spermatozoa and the time taken for the process of capacitation to be completed could further affect estimates of the fertilizable life of the egg. However, Mattner (1963*a*) suggested that capacitation can be completed in the reproductive tract of the ewe in 1·5 hr. If capacitation is completed at a uniformly

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rapid rate throughout oestrus then there would be little effect upon estimates of fertilizable life.

The present study was designed to define the fertilizable life of the ovine egg and to investigate the effect of age of egg at the time of fertilization on its subsequent development.

II. Materials and Methods

A flock of 350 mature, cyclic, Merino ewes, 10 vasectomized rams, and 6 entire rams of proven fertility were used in two experiments (see following tabulations). The ewes were run with vasectomized rams and were inspected for oestrus at 3-hourly (experiment 1) or 6-hourly (experiment 2) intervals. The mid-point between successive inspections was taken as the mean time of onset of oestrus and subsequent time intervals were related to this estimated mean.

(*a*) *Experiment 1*

Ewes were inseminated either with pooled freshly ejaculated semen which had been collected by artificial vagina from at least three rams, or similar semen after it had been incubated for 6 hr in ligated fallopian tubes of oestrous ewes. Animals used for incubation had been first detected in oestrus no more than 12 hr previous to incubation. A ligature was placed around the tube adjacent to the utero-tubal junction and 0·2–0·3 ml fresh semen was injected through the fimbria, and another ligature was secured around the tube adjacent to the fimbria. Semen was recovered by removing the fimbria ligature, a polythene cannula was inserted into the tube, and the semen was exhausted through the cannula by flushing with 1 ml normal saline injected into the tube near the utero-tubal junction. Only those samples which contained progressively motile spermatozoa were used for insemination.

Inseminations were carried out under general anaesthesia (Nembutal) and semen was deposited into the ampulla of the tubes or tip of the uterine horns corresponding to ovaries containing a recent ovulation or finite follicle. Irrespective of type of semen used similar volumes were placed in each tube (0·01 ml) or uterine horn (0·05 ml). All ewes were laparotomized 27 hr after the time of insemination and the fallopian tubes and portion of the uterine horns were flushed with cold normal saline. Eggs recovered from the flushings were examined as unstained preparations.

A summary of the design of experiment 1 is set out in the following tabulation:

(1) Time of insemination (hr after onset of oestrus $\pm 1 \cdot 5$ hr)—30 *v.* 36 *v.* 42 *v.* 48 *v.* 54;

(2) Treatment of semen—nil *v.* incubated;

(3) Site of insemination—uterus *v.* fallopian tube.

Factorial: $5 \times 2 \times 2$; $n = 4$ ewes; total ewes = 80.

(*b*) *Experiment 2*

Inseminations using pooled freshly ejaculated semen were made into the cervix (0·20 ml) or uterine horns (0·05 ml) in ewes 12–48 hr after they were first detected in oestrus. Uterine inseminations were carried out as in experiment 1, whilst standard non-surgical techniques were used for cervical inseminations.

Following insemination the ewes were either allowed to go to term or they were laparotomized for egg recovery at 30 hr after insemination (ewes inseminated at 24, 36, and 48 hr after the onset of oestrus) or at 42 hr after insemination (ewes inseminated at 12 hr). These times were adopted to ensure that the majority of fertilized ova would have cleaved at least once when recovered. The eggs were examined as unstained preparations and again after staining with 1% orcein. The design of experiment 2 may be summarized as follows:

(1) Time of insemination (hr after onset of oestrus ± 3 hr)—12 *v.* 24 *v.* 36 *v.* 48;

(2) Site of insemination—cervix *v.* uterus;

(3) Fate of ewes—egg recovery *v.* lambing.

Factorial: $4 \times 2 \times 2$; $n = 12$ ewes; total ewes = 192.

(c) *General*

In both experiments ewes from which no eggs were recovered were replaced by animals in which egg recovery was successful. In experiment 1 in which eggs were examined as unstained preparations, the only available criterion of fertilization was that of apparently normal cleavage, whereas in experiment 2 examination of eggs after staining with orcein enabled a critical assessment to be made of the cytological state of cleaved and uncleaved eggs.

(d) *Statistical Procedures*

Where group sizes were equal, standard analyses of variance were applied to the raw or appropriately transformed data. With unequal sized groups, analyses of χ^2 were applied to the raw data. The analyses were carried out using a computer program (CHIPARTIN; control data 3600, Canberra) which enabled the partitioning for main effects and interactions into comparisons based on individual degrees of freedom, using orthogonal polynomial coefficients weighted according to the group size for each factor level (Claringbold 1961).

III. Results

(a) *Experiment 1*

A total of 123 ewes were inseminated and all but two had ovulated at the time of insemination. The two ewes were inseminated at 30 hr and when laparotomized for

TABLE 1

EXPERIMENT 1: NUMBER OF EWES WHICH YIELDED EGGS FOLLOWING UTERINE AND TUBAL INSEMINATION

Main effects. There were no significant interactions

Main Effect	No. of Ewes Inseminated	No. which Yielded Eggs	No. which Yielded Cleaved Eggs*	No. which Yielded Uncleaved Eggs		Total No. of Eggs Recovered
				With Spermatozoa Attached	Without Spermatozoa Attached	
1. Time of insemination (hr)†						
30	20	16	9 (2)	3	4	18
36	24	16‡	10	0	5	16
42	24	15	10	1	4	15
48	24	16	7	8	1	16
54	31	16	3	7	6	16
P (linear)		n.s.	< 0·05	< 0·01	n.s.	
P (remainder)		n.s.	n.s.	n.s.	n.s.	
2. Treatment of semen						
Nil	65	40‡	24 (1)	9	6	41
Incubated	58	39	15 (1)	10	14	40
P		n.s.	< 0·05	n.s.	< 0·05	
3. Site of insemination						
Uterus	51	39‡	20 (1)	8	10	40
Tubes	72	40	19 (1)	11	10	41
P		< 0·05	n.s.	n.s.	n.s.	
Total	123	79†	39 (2)	19	20	81

* Number of ewes which yielded two eggs shown in parenthesis.

† Hours after onset of oestrus.

‡ One fractured egg recovered from one ewe—no cleavage data available.

egg recovery 27 hr later, both had ovulated. Eggs were recovered from 79 of the 123 ewes and there was an effect of site of insemination on egg recovery (Table 1). Six

ewes had two ovulations, the remainder were monovular. Two eggs were recovered from two of the six ewes and all four eggs had cleaved.

The low incidence of cleaved eggs in ewes inseminated at 48 and 54 hr was associated with an increase in the number of uncleaved eggs with spermatazoa attached to their zonae pellucidae. Incubation of semen decreased the number of ewes with cleaved eggs and increased the incidence of uncleaved eggs without spermatozoa attached to their zonae pellucidae.

Of the 41 cleaved eggs that were recovered 31 were of two cells and the remainder were of three or four cells, but there was no effect of any treatment on cell stage. Three eggs showed unequal cleavage. They had one or more small cells associated with apparently normal-sized blastomeres (Fig. 1).

(*b*) *Experiment 2*

(i) *Egg Recovery*

Information on the time of ovulation was provided by the four groups of ewes which were surgically inseminated 12, 24, 36, and 48 hr after the onset of oestrus. Ovulation was first recorded at 24 hr and by 36 hr all ewes had ovulated, as shown in the following tabulation:

Time of insemination (hr):	12	24	36	48
No. of ewes inseminated:	26	29	26	31
No. of ewes which ovulated:	0	22	26	31

At 24 hr all ewes that had not yet ovulated showed well-defined follicles and ovulation appeared to be imminent. In order to obtain 96 successful egg recoveries 118 ewes had

TABLE 2

EXPERIMENT 2: NUMBER OF EWES WHICH YIELDED EGGS FOLLOWING CERVICAL AND UTERINE INSEMINATION

Main effects

Main Effect	No. of Ewes Inseminated	No. which Yielded Eggs	No. which Yielded Fertilized Eggs		Total No. of Eggs Recovered
			Normal*	Abnormal	
Time of insemination (hr)†					
12	27	24	24 (2)	0	26
24	31	24	20 (1)	0	25
36	27	24	9	4	27
48	33	24	4 (1)	6	26
P (linear)		n.s.	< 0·001	< 0·05	
P (remainder)		n.s.	n.s.	n.s.	
Site of insemination					
Cervix	54	48	20 (1)	1	51
Uterus	64	48	37 (3)	9	53
P		n.s.	< 0·001	< 0·05	
Total	118	96	57 (4)	10	104

* No. of ewes which yielded two fertilized eggs are shown in parenthesis.

† Hours after onset of oestrus.

to be inseminated (Table 2). Two eggs were recovered from 8 of 10 ewes which shed two eggs. The remaining ewes were monovular. Of the 104 eggs that were recovered,

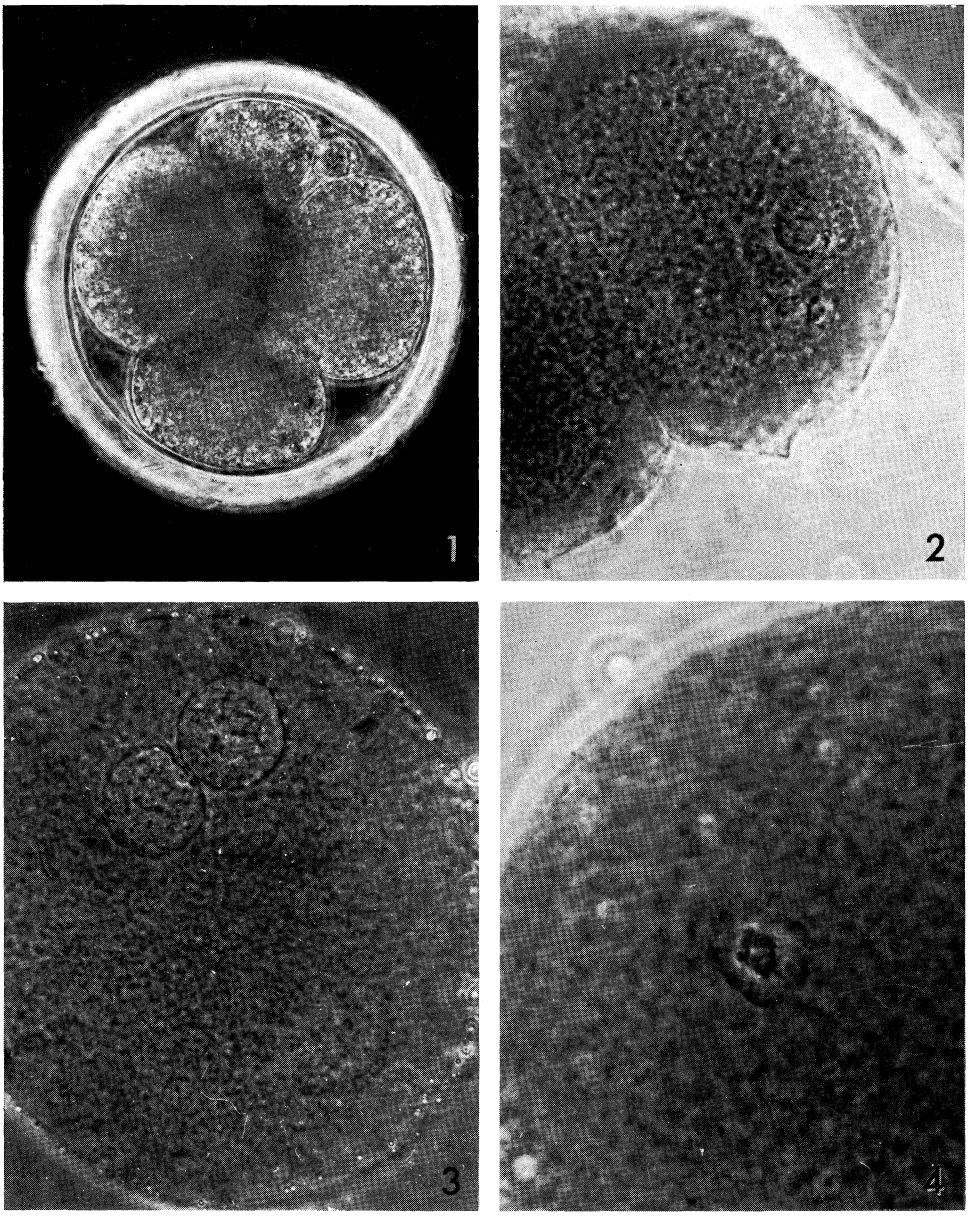

Fig. 1.—An atypical (type 1) four-cell egg with three small "cells" associated with four apparently normal blastomeres. Fresh preparation. ×280.

Fig. 2.—An abnormal (type 2) two-cell egg showing a large nucleus and several subnuclei in one blastomere. Orcein-stained. ×280.

Fig. 3.—An abnormal (type 3) one-cell egg which contained three pronuclei (two only in focal plane). Orcein-stained. ×280.

Fig. 4.—An aged, unfertilized egg recovered 66 hr after the onset of oestrus. Note compact appearance of the female nucleus. Orcein-stained. ×600.

33 showed no evidence of penetration or activation by spermatozoa. The chromatin of unfertilized eggs was characteristically compact (Fig. 4) and, except for one egg recovered following uterine insemination at 48 hr, no spermatozoa were found attached to the zonae pellucidae of unfertilized eggs. The remaining 71 eggs were fertilized; 62 had cleaved at least once while nine were of one cell and contained at least two well-formed pronuclei. Where two eggs were recovered from individual ewes both were either fertilized or unfertilized.

Three types of eggs of unusual appearance were recorded:

Type 1: Eggs with anucleate particles generally smaller than normal blastomeres (Fig. 1)—15 eggs.

Type 2: Individual blastomeres with more than one nucleus (Fig. 2)—7 eggs.

Type 3: Eggs of one cell with more than two pronuclei (Fig. 3)—3 eggs.

The occurrence of all three types of eggs was more common following uterine insemination. Of the 25 eggs involved, all but two were recovered after uterine insemination. Type 1 eggs were "atypical" rather than abnormal and for the purposes of statistical analyses they have been considered to be normal fertilized eggs. Types 2 and 3 showed gross nuclear aberrations and have been considered to be abnormal fertilized eggs.

There were marked effects of site of insemination and of time of insemination on the proportion of ewes which yielded normal fertilized eggs (Table 2) and a large portion of these effects was due to the complete absence of normal fertilized eggs in ewes cervically inseminated at 36 and 48 hr after the onset of oestrus (Table 3).

TABLE 3

EXPERIMENT 2: NUMBER OF EWES WITH NORMAL FERTILIZED EGGS OR LAMBS FOLLOWING EITHER CERVICAL OR UTERINE INSEMINATION

$n = 12$

Time of Insemination (hr)*	Cervical Insemination			Uterine Insemination			Totals	
	No. with Fertilized Eggs	No. with Lambs	Total	No. with Fertilized Eggs	No. with Lambs	Total	With Fertilized Eggs	With Lambs
12	12	8	20	12	4	16	24	12
24	8	3	11	12	1	13	20	4
36	0	0	0	9	1	10	9	1
48	0	0	0	4	0	4	4	0
Total	20	11	31	37	6	43	57	17

Significant main effects	Significant interactions
Time of insemination (linear): $P < 0{\cdot}001$	Site of insemination × fate of ewes: $P < 0{\cdot}001$
Site of insemination (cervix *v.* uterus): $P < 0{\cdot}05$	Time of insemination × fate: $P < 0{\cdot}05$
Fate of ewes (fertile *v.* lambed): $P < 0{\cdot}001$	Time × site: $P < 0{\cdot}05$

* Hours after onset of oestrus.

(ii) *Lambing Performance*

Only 17 of 96 ewes allowed to go to term lambed and when fertilization and lambing data were compared major discrepancies were observed (Table 3). The

difference between fertilization and lambing performance was most marked following uterine insemination. Of 48 ewes cervically inseminated 20 gave fertilized eggs and 11 of 48 lambed, whereas following uterine insemination 37 ewes had fertilized eggs but only 6 lambed. The effect of time of insemination on fertilization was somewhat different from its effect on lambing performance and the effect of time of insemination was further modified by site of insemination. There was complete failure of normal fertilization and lambing in ewes cervically inseminated at 36 and 48 hr after the onset of oestrus, whereas with uterine insemination at 36 and 48 hr some ewes had normal fertilized eggs (nine and four), but only one ewe lambed.

IV. Discussion

Both experiments provided information on the time of ovulation and on the fertilizable life of the ovine egg. In experiment 2 the majority of ewes had ovulated by 24 hr after the onset of oestrus and by 30 hr in experiment 1 ovulation was almost complete. Assuming that the majority of ewes ovulated about 24 hr after the onset of oestrus then experiment 1 showed that eggs remained highly fertile for 12–18 hr. No decrease in the number of ewes with cleaved eggs occurred until 48 hr had elapsed between the onset of oestrus and insemination. It is possible that the fertilization process in the older eggs was somewhat retarded and a number of uncleaved eggs recovered after insemination at 48 and 54 hr may have been fertilized. However, in experiment 2 in which eggs of a similar age were subjected to more critical examination only 9 of 42 uncleaved eggs had been fertilized.

Experiment 1 did not provide any positive evidence on the need for capacitation of ram spermatozoa. If capacitation is necessary and can be achieved in 1·5 hr (Mattner 1963*a*) then it is unlikely that the cell stage of fertilized eggs is sufficiently critical to detect differences in the rapidity of penetration and activation of eggs by incubated and non-incubated spermatozoa. Tubal incubation, as in the rabbit, may not efficiently capacitate spermatozoa (Adams and Chang 1962). The fallopian tubes do not provide a favourable environment for the survival of ram spermatozoa (Quinlan, Maré, and Roux 1933; Mattner 1963*b*). The lowered incidence of cleaved eggs and the relatively high incidence of cleaved eggs without spermatozoa attached to their zonae pellucidae which were associated with the use of incubated spermatozoa would indicate a loss of viability during incubation in the tubes.

Surgical insemination either into the fallopian tubes or the uterus as late as 54 hr after the onset of oestrus gave some fertilization, but there was almost complete failure of fertilization with cervical insemination carried out later than 24 hr after the onset of oestrus. Similarly, no ewe inseminated cervically at 36 and 48 hr subsequently lambed. It seems likely as suggested by Mattner and Braden (1969) and shown by Killeen and Moore (1970*a*) that faulty transport of spermatozoa from the cervix to the tubes in late oestrus was responsible for failure of fertilization in late cervically inseminated ewes.

Surgical insemination resulted in a number of eggs of unusual appearance. In experiment 1 the only "unusual" eggs that could be detected were those which had small particles associated with normal-sized blastomeres. The staining procedures used in experiment 2 showed that these particles were devoid of nuclei. Further and probably more serious nuclear abnormalities were detected in experiment 2 (types 2

and 3). Type 2 and type 3 eggs were almost invariably associated with late uterine insemination whilst those of type 1 were distributed at random throughout the various times of insemination. Although the present experiments provide no direct evidence on the viability of these eggs, it has been shown that eggs of type 1 are capable of full development (Hancock and Hovell 1961; and Killeen and Moore 1970*b*) and should therefore be classed as "atypical" rather than abnormal. The severe nuclear aberrations of type 2 and type 3 eggs probably preclude full development and as in other species (see Hunter 1967) they resulted from the exposure of "aged" eggs to spermatozoa. Faulty transport of spermatozoa in ewes inseminated late in oestrus would not allow fertilization of aged eggs. Thus under normal conditions of natural service and artificial insemination it is doubtful if these types of severe nuclear abnormalities play any significant part in reproductive wastage.

Loss of abnormal eggs of types 2 and 3 could be responsible for some, but certainly not all, of the difference between fertilization and lambing performance following uterine insemination. Similar discrepancies have been observed by Salamon and Lightfoot (1967) and it now appears that loss of fertilized eggs following uterine (or tubal) insemination is due to excessively rapid transport of eggs within the reproductive tract (Killeen and Moore 1970*b*).

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