THE ROLE OF PROGESTERONE IN IMPLANTATION IN THE SHEEP

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Summary

Merino ewes were ovariectomized on days 4, 8, or 12 of pregnancy and injected daily from that time with 1, 4, or 16 mg progesterone in oil. The ewes were killed on day 20 and examined for embryos. There was a clear effect of dose of progesterone on the number and viability of embryos. Of the ewes receiving 1, 4, and 16 mg progesterone there were 0 out of 12, 2 out of 11, and 11 out of 14 with viable embryos on day 20. The effect of increasing progesterone was also seen in significantly increased glandular and luminal epithelial cell heights in ewes receiving 16 mg per day.

Plasma progesterone was measured daily during days 1–5 and twice daily during days 6–15 of pregnancy and of the oestrous cycle. The numbers of corpora lutea and embryos in these animals were verified at laparotomy on days 15–18. The difference between pregnant and non-pregnant ewes with regard to plasma progesterone was not significant until days 16–17. Both pregnant and non-pregnant ewes had highest levels of progesterone during the period day 10 to day 14. Different ewes had peak values at different times so that the mean progesterone values took the form of a variable plateau, lasting several days, of 2–3 ng/ml.

Peak levels of progesterone appeared in the plasma at times consistent with the idea that this hormone is responsible for the rapid preimplantation growth phase of the embryo during days 11–15.

I. Introduction

While in the mouse and rat it has been clearly established that both oestrogen and progesterone are required for implantation, the situation in the ewe has not been closely examined. It is known that when ovariectomy is performed on day 20, pregnancy can be maintained in the ewe by injections of progesterone until the placenta assumes this role around day 50 (Neher and Zarrow 1954; Moore and Rowson 1959; Alexander and Williams 1966). There are two reports where ovariectomy was performed during the first few days of pregnancy and the ewes treated from this time with mixtures of progesterone and oestradiol (Foote *et al.* 1957; Moore 1963). It appeared from these that progesterone alone was sufficient for implantation and normal embryo development.

The purpose of the present study was to examine the progesterone requirements for implantation in the ewe especially with regard to the time of involvement in relation to the preimplantation phenomena described in an earlier paper (Bindon 1971). This was approached first by performing ovariectomy at various times and studying the dosage requirements of progesterone for embryo survival. Secondly, direct measurements of plasma progesterone were made in some detail during the period day 7 to day 14 of pregnancy during which time rapid preimplantation growth of the embryo takes place.

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II. Materials and Methods

(a) *Sheep*

There were 43 Merino ewes for experiment 1 and 23 Merino ewes for experiment 2. The ewes were aged 3–5 years, weighed 45–50 kg, and included both parous and non-parous animals. They were maintained indoors at Sydney University where they experienced the natural fluctuations of light and temperature but were protected from rainfall and wind. The ewes were group-fed on a diet of equal parts *Medicago sativa* and *Triticum spp.* chaff in sufficient quantity to allow slow growth.

(b) *Detection of Oestrus*

Vasectomized rams equipped with marking crayons were placed with the ewes for a preliminary period to record the regularity of oestrous cycles. Ewes intended for pregnancy studies were allowed repeated services by rams of known fertility on the day of oestrus (i.e. day 1). All ewes were run with vasectomized rams after service to check returns to service.

(c) *Laparotomy and Ovariectomy*

Mid-ventral laparotomy was performed after infusion of the site with local anaesthetic [3% lignocaine with adrenaline (1:50,000)]. Ovariectomy was performed after further infusion of the ovarian vascular plexus. In experiment 2 the uteri of pregnant ewes were flushed *in vivo*. This was achieved by injecting 15 ml of 0·9% NaCl into the uterine horn after clamping off the uterotubal junctions. The flushing media were collected into a funnel positioned near a small incision into the uterine lumen. All ewes received an intramuscular injection of 1 million units of penicillin (mixture of benzathine, procaine, and potassium penicillin G) on the day of surgery and again 24 hr later. The animals were fasted from food and water for 12 hr before surgery.

(d) *Examination of Reproductive Tracts*

The ewes in experiment 1 were killed on day 20 of pregnancy. The uteri were removed and flushed with 10 ml of 0·9% NaCl. When large embryos were present these were measured (see Table 1) after examination under a binocular microscope. Foetal heartbeat was conveniently established by squeezing the heart region with fine forceps. Smaller embryos and unfertilized ova were located by examining 2-ml aliquots of the flushing medium at a magnification of $\times 45$.

After flushing, a 1-cm segment of the uterine horn was removed and fixed in Bouin's fluid. This sample was taken from the side corresponding to the ovary containing the corpus luteum prior to ovariectomy. The samples were coded and later dehydrated, embedded in Paraplast, and sectioned in duplicate at a thickness of 8 μm. The sections were stained with haematoxylin and eosin. Histological measurements were made as previously described (Bindon 1971).

(e) *Progesterone Injections and Determinations*

In experiment 1 the appropriate dose levels were prepared by adding progesterone dissolved in ethanol to refined peanut oil and evaporating the ethanol by heating. The solutions were prepared so as to yield 1, 4, and 16 mg progesterone per millilitre of oil. Daily injections were given intramuscularly into alternate legs in the gluteal region.

Plasma progesterone determinations in experiment 2 were performed on duplicate 2-ml aliquots of plasma by the competitive protein-binding method described by Basset and Hinks (1969) and Thorburn, Bassett, and Smith (1969). This test, based on the competitive protein-binding principle, responds to compounds other than progesterone, including 17α-hydroxy-4-pregnen-3,20-dione and 20α-hydroxy-4-pregnen-3-one. The results are more correctly "total plasma progestagen" levels although progesterone has been indicated as the dominant compound measured in plasma of non-pregnant sheep (Thorburn, Bassett, and Smith 1969). For convenience the results are referred to as plasma progesterone. The values reported here have not been corrected for procedural losses. Studies using tritiated progesterone in plasma revealed that the extraction method (double extraction with five volumes of light petroleum) yielded a recovery of 76–85% of added steroid. All samples were processed in duplicate. When duplicates differed by more

than 15% the sample was re-assayed, again in duplicate. Blood samples (10–15 ml) were drawn from the jugular veins into heparinized syringes, centrifuged at 4°C for 20 min at 2000 r.p.m., and the plasma stored at −20°C.

III. Experimental Procedure and Results

(a) *Experiment 1: Progesterone Requirements for Implantation after Ovariectomy on Days 4, 8, or 12*

Ewes were randomized on day 1 of pregnancy into treatment groups within the factorial experimental design:

Day of ovariectomy	4, 8, or 12
Daily dose progesterone from day of ovariectomy	1, 4, or 16 mg

The first injection of progesterone was given 1 hr before ovariectomy to ensure that some progesterone was in the circulation at all times. There were nine treatment groups, with five ewes per group. These were reduced in some cases as a result of the failure of the ewes to ovulate. All ewes were killed on day 20 and the uteri flushed for embryos which were measured as described in Table 1. All the data are presented for completeness but for purposes of discussion the data for the three times of ovariectomy may be pooled since this factor was without significant effect. The most significant feature of the results is the clear dose effect of progesterone. Of those ewes receiving 16 mg/day all had embryos on day 20 and 11 out of 14 had heartbeat when the foetal heart was stimulated in the flushing medium after recovery. A brief summary of the pooled results is shown below:

Dose of Progesterone (mg)	Proportion of Ewes with Embryos	Proportion of Embryos with Heartbeat	Proportion of Embryos Degenerating
1	7 out of 14	0 out of 7	5 out of 7
4	9 out of 12	2 out of 9	6 out of 9
16	14 out of 14	11 out of 14	2 out of 14

There is thus a daily requirement of 4–16 mg progesterone for normal implantation and development. The embryos from ewes receiving 16 mg were of normal size since three ewes killed at this time in normal pregnancy had embryos with a mean crown–rump length of 6·6 mm.

The embryos without heartbeat were represented by either fragmented segments of trophoblast or by embryos that were considered normal but retarded in development. There was one of the latter type in each of the groups ovariectomized on days 4 and 8 and given 1 mg progesterone per day. These were equivalent in development to days 9 and 10 of normal pregnancy. It is possible that here the conditions for inducing delayed implantation in the ewe may have been approached, although the small numbers involved allow no firm conclusions to be reached.

The histological data on these ewes is presented in Table 2, again after pooling the ewes from the three times of ovariectomy. Both the uterine and glandular epithelial cell heights show significant linear increases with increasing dose of progesterone. It is significant that, with 16 mg progesterone, both these parameters reach the magnitude of that in ewes in normal pregnancy (Bindon 1971). The

TABLE 1

DIMENSIONS OF SHEEP EMBRYOS RECOVERED ON DAY 20 AFTER OVARIECTOMY AND PROGESTERONE TREATMENT DURING EARLY PREGNANCY

Day of Ovariectomy	Daily Dose of Progesterone (mg)	No. of Ewes	No. with Embryos on Day 20	No. of Degenerating Embryos	Embryo Dimensions*					Embryos with Positive Heartbeat
					C–R or E.D. (mm)	A.L. (mm)	A.W. (mm)	T.L. (mm)	T.W. (mm)	
4	1	6	3	2	0·05‡	—	—	0·45	0·21	0
	4	4	2	1	0·30‡	—	—	7·8	3·6	0
	16	5	5	1	6·83±0·9	46·6±16·4	13·3±6·2	72	—	3
8	1	4	2	1	0·21 by 0·17	—	—	0·90	0·90	0
	4	5	4†	4	0·14 by 0·66	—	—	20	2·0	1
	16	4	4	1	5·87±1·3	36·2±29·6	5·9±4·1	200	—	3
12	1	4	2	2	—	—	—	—	—	0
	4	3	3	1	5·63±0·7	32·0±12·2	14·1±3·8	200	—	1
	16	5	5	0	5·65±0·8	29·3± 7·1	8·1±2·8	200	—	5

* C–R, crown–rump length; E.D., diameter of embryonic disc; A.L., allantoic length; A.W., allantoic width; T.L., trophoblast length; T.W., trophoblast maximum width.

† One ewe had twin embryos.

‡ Embryo spherical.

association of maximum epithelial cell height with a high proportion of normal embryos is obvious from Table 2. The number of glandular coils was not influenced by dose of progesterone.

TABLE 2

HISTOLOGICAL MEASUREMENT IN UTERI OF EWES KILLED ON DAY 20 AFTER OVARIECTOMY AND PROGESTERONE TREATMENT DURING EARLY PREGNANCY

Results are expressed as means ± S.E.

No. of Ewes	Daily Dose of Progesterone (mg)	(a) No. of Glandular Coils	(b) Height of Uterine Epithelium (μm)	(c) Height of Glandular Epithelium (μm)	No. of Ewes with Normal Embryos on Day 20
12	1	6·08±0·81	16·38±1·02	9·78±0·54	0
11	4	4·73±0·43	23·46±1·98	10·19±0·74	2
14	6	5·93±0·61	29·81±1·66	13·66±0·92	11

Bartlett's test for homogeneity of variances:
(a) $\chi^2_{(2)} = 4·45$ (n.s.); (b) $\chi^2_{(2)} = 4·44$ (n.s.); (c) $\chi^2_{(2)} = 4·15$ (n.s.)

Summary of overall analyses of variance

Source of Variation	D.F.	Mean Squares (a)	Mean Squares (b)	Mean Squares (c)	*F* Ratios (a)	*F* Ratios (b)	*F* Ratios (c)
Between groups	2	6·34	5836·1	597·6	1·24	18·59***	8·04**
Between ewes	34	5·12	313·9	74·4			

0·001 < *P* < 0·01. * *P* < 0·001.

(b) Experiment 2: Detailed Measurement of Plasma Progesterone during the First 15 Days of the Oestrous Cycle and Early Pregnancy

Merino ewes that had experienced regular oestrous cycles were randomized into groups that were subsequently served by either entire or vasectomized rams. Blood samples were taken between 0900 and 1000 hr on the morning of days 1–5 and between 0900–1000 hr and 2100–2200 hr on days 6–15. Most ewes were sampled also at 0900–1000 hr on days 15–18. All ewes were laparotomized after blood sampling on days 15–18. No blood samples were collected after laparotomy. The number of corpora lutea were recorded and the uteri of the pregnant ewes flushed with 0·9% NaCl. The numbers of ewes in the various classifications are shown in Table 3. Fortunately, there were eight or nine ewes in the classifications of most interest—i.e. a single ovulation represented by a single embryo and a single ovulation in the case of the non-pregnant ewes.

The plasma samples (about 650) were stored at −20°C then analysed for progesterone during a period of about 6 weeks. To avoid any chance of bias or error due to gradual improvement or deterioration in the technique, the samples were mixed in

the deep-freeze and then numbered 1 to n by an independent observer. The samples were analysed in random order and kept in code until they were all completed. This step is considered important since day to day fluctuations in accuracy of measurement would be distributed throughout all animals, rather than producing spuriously low or high values in all samples of a particular ewe.

TABLE 3

CLASSIFICATION OF MERINO EWES USED IN BLOOD PROGESTERONE STUDY

Classification at Laparotomy on Days 15–18	Ewes Served by:	
	Entire Ram	Vasectomized Ram
1 ovulation; 1 embryo	9*	0
1 ovulation; no embryo	0	9
2 ovulations; 2 embryos	1	0
2 ovulations; 1 embryo	1	0
2 ovulations; no embryo	1	2

* One ewe with 1 ovulation and 1 degenerating embryo.

The results have been expressed in two ways. Firstly, as shown in Table 4, the period of study has been divided into days 1–5, 6–7, 8–9, 10–11, 12–13, 14–15, 16–17. A comparison between pregnancy and the oestrous cycle has been made for each time period using an overall comparison of variance within and between groups.

TABLE 4

COMPARISON OF PLASMA PROGESTERONE LEVELS IN PREGNANT AND NON-PREGNANT EWES DURING DAYS 1–17

Single ovulations only

Days being Compared	Pregnant Ewes		Non-pregnant Ewes		Significance of Difference*
	No. of Samples	Progesterone Level (ng/ml)	No. of Samples	Progesterone Level (ng/ml)	
1– 5	30	0·27±0·06	38	0·32±0·05	n.s.
6– 7	24	0·68±0·12	28	0·91±0·17	n.s.
8– 9	29	0·95±0·10	32	1·04±0·17	n.s.
10–11	32	1·92±0·67	36	1·02±0·22	n.s.
12–13	35	1·61±0·43	36	1·20±0·39	n.s.
14–15	25	1·54±0·63	29	0·95±0·19	n.s.
16–17	18	0·97±0·20	18	0·44±0·18	$P<0·05$

* Assessed from the overall analyses of variance within and between groups for each comparison. Data transformed to logarithms for analysis.

Variances were generally heterogeneous and the data were therefore transformed to logarithms before analysis. The complete analyses are not presented but the significance of differences between pregnant and non-pregnant ewes is shown in Table 4.

Secondly, the mean values for all times of examination for both groups of ewes are shown in Figure 1. The pattern of mean values tends to obscure the fact that

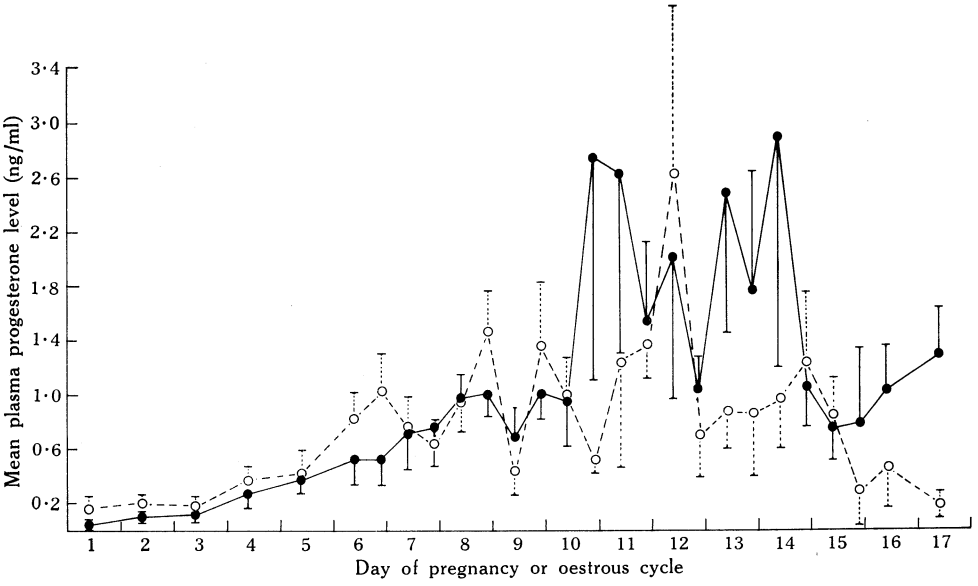

Fig. 1.—Mean plasma progesterone levels in eight pregnant (●) and nine non-pregnant (○) ewes (single ovulations only in each case) up to day 17 after service. Blood samples were taken at 1000 hr on days 1–5 and 16–17 and at 1000 hr and 2200 hr on days 6–15. Vertical bars represent standard errors.

TABLE 5

MAGNITUDE AND TIME OF OCCURRENCE OF MAXIMUM PLASMA PROGESTERONE VALUES RECORDED DURING THE OESTROUS CYCLE AND EARLY PREGNANCY

Single ovulations only

Pregnant Ewes				Non-pregnant Ewes			
Tag No.	Maximum Progesterone Level (ng/ml)	Occurrence Day	Occurrence Time	Tag No.	Maximum Progesterone Level (ng/ml)	Occurrence Day	Occurrence Time
866	1·83	12	10 p.m.	877	7·2	12	10 a.m.
860	15·8	14	10 a.m.	868	1·85	14	10 a.m.
859	2·6	13	10 p.m.	889	1·95	10	10 a.m.
879	8·0	13	10 p.m.	882	3·0	16	10 a.m.
865	15·2	10	10 p.m.	884	8·0	11	10 a.m.
875	11·0	13	10 a.m.	862	3·0	14	10 p.m.
873	8·8	12	10 a.m.	867	2·5	11	10 p.m.
890	1·95	14	10 p.m.	874	12·4	12	10 a.m.
				871	3·9	9	10 p.m.

there were quite large peaks for some ewes around the middle of the luteal phase as shown in Table 5. Since the peak values did not occur at the same time in all ewes,

they caused large variation about the various mean values shown in Figure 1. This problem may be accounted for in part by the 24-hr error in estimating the time of commencement of oestrus when ewes are inspected for oestrus once daily.

IV. Discussion

The results of experiment 1 confirm that progesterone is capable of initiating implantation and normal embryo growth in ewes ovariectomized during days 4–12. This does not preclude the possibility that other steroids may be involved since the adrenal gland was not ablated in these animals. Only hypophysectomy, followed by progesterone replacement, would resolve this question. At the time of writing there are no reports of direct measurement of plasma oestrogen levels during early pregnancy that might indicate whether this steroid is involved.

Ovariectomy was performed as early as day 4 to test the hypothesis that a continued low level of circulating progesterone (e.g. as provided by 1 mg/day) begun prior to shedding of the zona pellucida from the zygote might lead to delayed implantation as exists in the rodent. The results do not provide sufficient evidence to support the hypothesis, but two apparently delayed spherical zygotes were recovered (see Table 1). These were equivalent in size to those seen on days 9–10 of normal pregnancy. The hypothesis deserves further investigation, since a technique to delay implantation would provide, as in the rodent, a convenient means of studying the mechanisms of implantation.

The main reason for measuring plasma progesterone was to establish when pregnant and non-pregnant ewes first differ with regard to this parameter. In the Rhesus monkey, for example, the pregnant animal shows a peak of progesterone on days 9–11 that is not present in the non-pregnant animal (Neill, Johansson, and Knobil 1969), suggesting an early influence of the embryo on luteal function. To investigate this possibility in the ewe it was thought essential to know both the number of corpora lutea and (in the case of the pregnant animal) the number of embryos in the animals being compared. Both these factors could conceivably influence the level of plasma progesterone. It was also essential to measure progesterone more frequently than had been done in the past if short-term fluctuations were to be detected. Plasma progesterone in early pregnancy has not previously been studied on a daily basis, while data on the oestrous cycle are based on measurements made once daily or less (Edgar and Ronaldson 1958; Thorburn, Bassett, and Smith 1969; Smith and Robinson 1969; Stabenfeldt, Holt and Ewing 1969). Time of day, however, may not be a source of variation in plasma progesterone at least in the oestrous cycle (Thorburn, Bassett, and Smith 1969).

In a recent study by Obst and Seamark (1970) pregnant ewes had higher progesterone values from as early as day 12. The results in Table 4 show that although the mean progesterone values for pregnant ewes were higher than for non-pregnant ewes from days 10–11 onward, this difference was not statistically significant until days 16–17. The reason for this lies in the large variability within groups of animals in progesterone values during the peak luteal phase (days 10–14). Similar variability has been described by Thorburn, Bassett, and Smith (1969) and Stabenfeldt, Holt, and Ewing (1969). In the latter study the variability was due to the existence of

transient peaks of progesterone in some ewes during days 10–13. In the present study similar peaks were observed (see Table 5) but these were of greater magnitude than those of Stabenfeldt, Holt, and Ewing (1969), due possibly to the fact that measurements were made here at 10 a.m. and 10 p.m. each day during the peak luteal phase.

From Table 5 it can be seen that the eight pregnant ewes with a single corpus luteum and a single embryo all had their peak progesterone levels in the period 10 p.m. day 10 to 10 p.m. day 14. With the acknowledged 24-hr error in the timing of the start of oestrus, six of these ewes may have had peaks on day 13—the day of significance for embryo transfer (Moor and Rowson 1966). Day 13 also coincides with the onset of rapid preimplantation embryo growth. It is possible that progesterone is directly responsible for such embryo growth, especially since Wintenberger-Torrès (1967) has shown that progesterone injections increase the rate of cell division in the trophoblast between days 8 and 12.

The peak progesterone values for non-pregnant ewes (Table 5) were generally lower than during pregnancy but due to variation in the time of occurrence of the peaks the mean difference between the two groups of ewes is not significant. To define the point where the embryo first causes elevation of plasma progesterone it will be necessary to make more frequent measurements during the luteal phase of pregnant and non-pregnant ewes. Until this is done it will not be possible to fully interpret the transient, large peaks of progesterone described for some ewes in the present paper.

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