Reproductive function in prepubertal lambs: ovulation, embryo development and ovarian steroidogenesis

A. O. Trounson*, S. M. Willadsen and R. M. Moor

A.R.C. Unit of Reproductive Physiology and Biochemistry, Cambridge, U.K.

Summary. When 23 10–16-week-old Welsh Mountain lambs were treated with PMSG 19 ovulated, the number of eggs ovulated being directly correlated with the duration of progesterone pretreatment (0.5 ± 0.29 (S.E.M.) after 3 days; 7.8 ± 3.47 after 18 days). Injection of HCG at the time of the induced oestrus had no effect on ovulation. The eggs shed from immature ovaries became fertilized and developed normally when tested in the ligated rabbit oviduct for development to the morula stage and by transfer to adult ewes (1 live lamb). Luteal function in lambs with a single CL was similar to that in nonpregnant ewes; progesterone levels in entire lambs with multiple CL and in hysterectomized lambs remained elevated for at least 60 days.

The capacity of ovarian follicles from PMSG-primed lambs to secrete oestrogen, testosterone and progesterone *in vitro* was similar to that of follicles from adult ewes. However, oestrogen production by lamb follicles immediately after explantation was higher than that of adult follicles and the administration of progesterone to lambs before PMSG treatment decreased subsequent follicular testosterone production.

Introduction

The ovaries of prepubertal lambs contain large numbers of growing and vesicular follicles which by histological examination appear normal (Kennedy, Worthington & Cole, 1974). Furthermore, ovulation may be induced in lambs at 8 weeks of age and older by treatment with progesterone and gonadotrophins (Mansour, 1959) and cleavage of such ovulated eggs may be obtained after intra-uterine insemination (Land & McGovern, 1968). However, there has been no detailed examination of the normality of fertilization, embryo development, luteal function and ovarian follicular steroidogenesis in prepubertal lambs. The experiments reported in the present paper were designed to examine these factors.

Materials and Methods

Welsh Mountain lambs, 10–16 weeks of age, were used in the experiments and weighed 7.3–19.5 kg at the time of ovulation.

Experiment 1

Twenty-three lambs randomized for body weight were given daily i.m. injections of 10 mg progesterone dissolved in arachis oil for 3, 11, 13, 15 or 18 days. At the time of the penultimate progesterone injection the lambs were given a s.c. injection of 750–1000 i.u. PMSG (Folligon: Intervet, U.K.). The dose of PMSG depended on the bodyweight of the lamb, those < 12 kg receiving 750–800 i.u. and those > 12 kg 900–1000 i.u. After the last progesterone injection the lambs were run with a vasectomized ram and observed for the onset of oestrus. Most lambs showed oestrus $2\frac{1}{2}$ –3 days after

* Present address: University of Monash, Department of Obstetrics & Gynaecology, Queen Victoria Memorial Hospital, Melbourne, Victoria, Australia 3000.

the last progesterone injection and were inseminated surgically at this time with 0.01–0.02 ml freshly ejaculated ram semen placed directly into each uterine horn. At the time of insemination (Day 0) half the lambs in each progesterone treatment group served as controls while the rest were given an i.v. injection of 500 i.u. HCG (Gonadotraphon L.H.: Paines & Byrne).

Ovulated eggs were recovered on Days 2–3 by flushing the uterine horns and oviducts of lambs with 5 ml modified Dulbecco's phosphate buffer (PBI: Whittingham & Wales, 1969). The numbers of corpora lutea (CL) were recorded and the eggs obtained were examined for cleavage. The potential for further development of fertilized eggs was examined by transferring 6 2-cell eggs to the ligated oviduct of a follicular-phase rabbit for a period of 4 days and by the transfer of 3 eggs to the oviducts of 3 adult recipient ewes that were in oestrus at the same time as the donor lambs. The remaining eggs were fixed in aceto-ethanol, stained with 1% lacmoid and examined by phase-contrast microscopy.

Experiment 2

Following the induction of ovulation as described in the previous experiment, 13 lambs were used to examine luteal function. Four lambs were hysterectomized on Day 2 and jugular vein blood samples were taken from these lambs and from 9 entire lambs at 2–3 day intervals to Day 16 and thereafter at weekly intervals to Day 60. The plasma obtained after centrifugation was stored at -20°C and the progesterone concentrations in plasma samples, after extraction with n-hexane, were determined by the radioimmunoassay procedure described previously by Moor, Hay, McIntosh & Caldwell (1973). Corpora lutea persisting in the ovaries of lambs at Day 80 were dissected free of other ovarian tissue and weighed.

Experiment 3

Ovarian follicles were obtained from 13 adult ewes and 4 lambs 24 h after the injection of 1000 i.u. PMSG. Adult ewes were given PMSG 12 days after oestrus (T1), 2 lambs were given PMSG with no progesterone treatment (T2) and 2 lambs were given daily injections of 10 mg progesterone in oil for 13 days and PMSG at the time of the final injection of progesterone (T3). Ovaries were removed at laparotomy and follicles >3 mm diameter were dissected from the ovarian stroma and cultured individually as described by Moor *et al.* (1973). Culture medium was replaced every 24 h for 5 days and the medium obtained after culture for 1, 3 and 5 days was assessed for oestrogen, progesterone and testosterone by radioimmunoassay. The oestrogen and progesterone assays have been described previously (Moor *et al.*, 1973). The testosterone assay is described below. Steroid production for each follicle was expressed as ng steroid/mg wet weight of follicle tissue/24 h. For statistical analysis the steroid production of 20 randomly chosen follicles >3 mm diameter in each treatment group (T1, T2, T3) was examined by analysis of variance and *t* tests.

Testosterone assay. The procedures followed for the radioimmunoassay of testosterone were the same as those for the progesterone assay (O'Grady, Caldwell, Auletta & Speroff, 1972). Antiserum to testosterone (550/3 kindly donated by Dr B. Furr, I.C.I., Macclesfield, Cheshire, U.K.) was used at a dilution of 1:10,000 and the standard curve calculated from triplicate samples of the blank and duplicate samples of culture medium containing 0.1, 0.2, 0.5, 1.0 and 2.0 ng testosterone. The coefficient of variation of individual points ranged from 2.5–8.0% (mean = 4.7%). The limit to assay sensitivity was 0.015 ng (lower fiducial limit of counts bound at zero testosterone). The serial dilution of a known high testosterone pool of culture medium gave a regression equation of $\log x = 2.48 - 2.14 (\log y)$ and was parallel to the standard curve. The within-assay coefficient of variation (quadruplet samples) of a pool of culture medium containing 45.5 ± 0.69 ng testosterone/ml was 4.9% and the between-assay coefficient of variation (duplicate means of 20 assays) of the same pool was 6.8%. The cross-reactivity, estimated at 50% displacement of labelled testosterone, was $< 0.01\%$ for C_{21} and C_{18} steroids. The cross-reactivity of C_{19} steroids was: androstenedione $< 0.01\%$; 5α -dihydrotestosterone 100%; androstenediol 9%; 5α -androstane- $3\beta,17\beta$ -diol 41%; 5α -androstane- $3\alpha,17\beta$ -diol 43%.

Assay of 5 µl culture medium without extraction gave concentrations for samples not significantly different from those after extraction of 100 µl culture medium. Duplicate 5 µl aliquots were then taken from medium used to culture follicles to determine the production *in vitro* of testosterone and other cross-reacting androgens. It has, however, been shown previously (Seamark, Moor & McIntosh, 1974) that testosterone accounts for 80–90% of the steroids that cross-react in this assay. The term testosterone fraction will therefore be used to cover these steroids.

Results

Experiment 1

Ovulation occurred in 19 of 23 lambs treated with PMSG; injection of HCG at the onset of oestrus neither influenced the proportion of lambs that ovulated (HCG, 9/11; no HCG, 10/12 ovulated), nor had a significant effect on mean ovulation rate (HCG, 4.3; no HCG, 2.5). Mean ovulation rate increased from 0.5 ± 0.29 after 3 days of progesterone priming to 7.8 ± 3.47 after 18 days of progesterone priming. The correlation between ovulation rate (Y) and length of progesterone treatment (X) was highly significant ($r = 0.56$; $P < 0.01$); the linear regression function was $Y = 0.45X - 2.02$. Excluding lambs treated for 3 days with progesterone, the relationship was still found to be significant ($r = 0.57$; $P < 0.05$; $Y = 0.88X - 8.31$). Neither body weight nor age of lamb affected mean ovulation rate.

Most of the ovulated eggs became fertilized (77%) as judged by the presence of the tail of the penetrated spermatozoon, together with male and female pronuclei in 1-cell eggs and apparently normal nuclei in cleaved eggs of 2- to 8-cells. Four of the 6 eggs transferred to the ligated rabbit oviduct developed to morulae with 28–36 normal nuclei, and a normal lamb was born after transfer of 3 eggs to 3 recipient ewes.

Experiment 2

In lambs with a single CL, progesterone levels in peripheral plasma followed the usual pattern for the normal adult oestrous cycle (Text-fig. 1a), falling in all lambs with one CL to <0.4 ng/ml by Day 15.

The hysterectomized lambs had either 1, 3 or 4 CL and progesterone levels (Text-fig. 1b) showed a peak of variable size at Day 7. From Days 10–60 progesterone levels remained reasonably constant and the mean ($\pm$ S.E.M.) wet weight of CL at Day 80 was 222 ± 33.3 mg.

In entire lambs with 3, 5 or 40 CL, peak progesterone levels were in proportion to the number of CL and occurred on Days 7–10. In lambs with multiple ovulations progesterone levels remained elevated (> 5 ng/ml) on Day 15 and were still elevated in 2 of 3 animals at Day 60. Corpora lutea were found in the ovaries of one of these lambs at Day 80 but luteal weight was considerably less than in hysterectomized lambs (42.0 ± 6.41 mg).

Experiment 3

The mean production rates of progesterone, oestrogen and total testosterone by adult and lamb follicles are shown in Table 1. Steroid production of individual animals within groups was not significantly different. Oestrogen production declined during culture but differences due to groups were not significant. However, oestrogen production of follicles of adult ewes during the first day of culture was lower than that from follicles of lambs ($t_{58} = 6.79$; $P < 0.001$).

Production rate of the testosterone fraction was highest at Day 3 and mean production rates in follicles from T1 and T2 animals were similar. Follicles from T3 lambs produced less testosterone than those from T1 ewes (Day 1: $t_{38} = -2.15$, $P < 0.05$; Day 3: $t_{38} = -3.33$, $P < 0.01$; Day 5: $t_{38} = -2.31$, $P < 0.05$) and T2 lambs during the first 3 days of culture (Day 1: $t_{38} = -2.81$, $P < 0.01$; Day 3: $t_{38} = -2.19$, $P < 0.05$).

Text-fig. 1. Mean concentration of peripheral plasma progesterone in ewe lambs after the induction of ovulation. (a) Lambs with a single corpus luteum. (b) Hysterectomized lambs with 1-4 corpora lutea.

Progesterone production increased during culture but the differences between groups were not significant. As observed previously (Seamark *et al.*, 1974) follicles that produced small amounts of androgens produced higher progesterone levels; follicles from T3 lambs produced more progesterone on Day 5 than those from T2 lambs ($t_{38} = 2.29$, $P < 0.05$).

Discussion

The present results confirm previous reports (Mansour, 1959; Land & McGovern, 1968) that ovulation, fertilization and cleavage of embryos can be obtained in prepubertal lambs at 10-16 weeks of age; embryos developed normally in the rabbit oviduct and to term in adult recipient ewes. Normality of a proportion of embryos obtained from immature animals of other species has been demonstrated similarly by the birth of live young after transfer to adult animals (rabbits: Adams, 1953; mouse: Gates, 1956; pig: Baker & Dziuk, 1970). However, in the calf only limited development of embryos has been obtained (Onuma & Foote, 1969; Seidel, Larson & Foote, 1971; Seidel, Larson, Spilman, Hahn & Foote, 1971). Maintenance of pregnancy to term in immature animals requires the supplementation of exogenous steroids (rabbit: Abbot, 1973; pig: Ellicott, Dziuk & Polge, 1973).

Liefer, Foster & Dziuk (1972) found that pituitary LH content of 9-week-old lambs approximates that of adult ewes, indicating adequate pituitary LH for ovulation and Land, Thimonier & Pelletier (1970) reported that oestradiol induced pituitary LH release in 10-week-old lambs. The present experiments show that ovulation can occur spontaneously in the absence of injected HCG. Furthermore, LH and FSH have been detected in peripheral blood plasma of 10-16-week-old lambs (Liefer *et al.*, 1972; Tassell, Chamley & Kennedy, 1976) at levels similar to those of adult ewes during the luteal phase of the oestrous cycle. The question remains to be answered as to whether follicles of the lamb ovary can respond to endogenous gonadotrophins. It is possible that gonadotrophin binding

Table 1. Mean $\pm$ S.E.M. steroid production *in vitro* of adult ewe and lamb ovarian follicles (20 follicles >3 mm diam.) explanted 24 h after PMSG injection

Steroid production (ng/mg follicle tissue/24 h)											
Group	Progesterone treatment	No. of donors	Oestrogen			Testosterone fraction			Progesterone		
			Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
Adult (T1)	None	13	60 ± 9.1	46 ± 5.0	30 ± 5.2	10 ± 1.9	27 ± 5.5	13 ± 2.1	2.2 ± 0.60	6.0 ± 1.72	8.0 ± 2.39
Lamb (T2)	None	2	95 ± 12.8	41 ± 4.4	23 ± 3.2	12 ± 1.7	21 ± 4.1	10 ± 1.1	2.5 ± 0.60	5.9 ± 1.64	5.3 ± 1.27
Lamb (T3)	13 days	2	80 ± 12.8	33 ± 4.5	23 ± 5.3	6 ± 0.6	9 ± 1.1	8 ± 0.7	2.0 ± 0.29	9.4 ± 2.18	12.0 ± 2.39

Analyses of variance				
Steroid	Source of deviation	Degrees of freedom	F value	
Oestrogen	Group	2	0.49	
	Day of culture	2	66.92***	
	Group × Day of culture	4	4.41**	
Testosterone	Group	2	7.84**	
	Day of culture	2	14.12***	
	Group × Day of culture	4	3.24*	
Progesterone	Group	2	1.81	
	Day of culture	2	19.76***	
	Group × Day of culture	4	2.12	

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

sites on the follicle may be inadequate for stimulation, leading to ovarian insensitivity. It is clear that lamb follicles can be stimulated by PMSG, indicating that they are capable of responding to gonadotrophins, but this may be peculiar to the exogenous hormone used or to an overwhelming of the natural inhibition to follicular stimulation.

The process by which exogenous progesterone increases ovulation rate in lambs is not known and has not been reported previously, although Land & McGovern (1968) suggested that progesterone may increase follicular development when large doses of PMSG are used. In the present experiment, ovulation rate increased as the duration of progesterone treatment increased. Progesterone may act directly on the ovary by altering the follicle population or indirectly by influencing pituitary hormone secretion. Progesterone treatment of lambs had some minor effects on follicle secretion *in vitro* although it is difficult to equate these differences with ovulation rate. In fact, steroid secretion of follicles from lambs given no progesterone was more like secretion of follicles from adult ewes than secretion of follicles from lambs given progesterone. There was no evidence that induction of ovulation with PMSG promotes any further ovulatory activity in prepubertal lambs.

Steroidogenesis in the follicles of lambs stimulated with PMSG was similar to that in follicles from adult ewes, although oestrogen production by the lamb follicles was elevated immediately after implantation. Testosterone is known to be secreted by the theca interna and it has been suggested that androgens facilitate the action of LH or cyclic AMP in promoting steroidogenesis in the membrana granulosa (Moor, Hay & Seamark, 1975). Testosterone and progesterone tend to be negatively correlated (Seamark *et al.*, 1974) and it is probable that exogenous progesterone acts directly on follicles to reduce testosterone production. These results *in vitro* and the fact that normal oocytes are ovulated *in vivo* suggest that follicular function in prepubertal lambs is normal.

The CL formed after ovulation were fully functional for at least 60 days and in the intact lamb with a single CL luteolysis occurred at the time generally observed in the non-pregnant ewe. Failure of complete luteolysis was found in entire lambs with three or more CL. In this laboratory there is no evidence of an extended luteal phase in superovulated adult ewes and failure of luteolysis in lambs may be due to the relatively small uterus so that synthesis or release of prostaglandin F-2 α is inadequate to cause complete regression of more than a single CL. In the prepubertal pig, premature regression of CL terminates pregnancy and Polge (1972) suggests that this is due to insufficient endogenous gonadotrophins. In the lamb there appears to be adequate gonadotrophin support of induced CL.

Peripheral plasma progesterone levels on Days 7–10 were elevated in lambs with multiple CL and peak values were in proportion to the number of CL. Lamond & Gaddy (1972) found a similar relationship in peripheral plasma of superovulated cattle but not in spontaneously ovulating sheep. However, peripheral plasma progesterone levels in sheep are much lower than in cattle, and if plasma samples are taken from the ovarian vein progesterone levels are directly related to the number of CL in both superovulated and spontaneously ovulating ewes (Trounson & Moore, 1974).

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