Steroid production and hCG binding by ram-induced ovarian follicles in seasonally anoestrous ewes

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Summary. The introduction of rams to a group of previously isolated anoestrous ewes has been shown to stimulate ovarian follicular development and ovulation. The present experiment was carried out to determine the ability of follicles arising from this ram stimulus to produce steroids and bind hCG. Seasonally anoestrous Southdown ewes were exposed to rams for 24 h, 40 h, 3 days, 10 days or 20 days before ovariectomy. Steroid production and the concentration of hCG binding sites in follicles dissected from the ovaries were measured in vitro. The presence of a ram caused ovulation and enhanced oestradiol production by follicles, but had little effect on total androgen production or the number of hCG binding sites present in the follicles when compared to follicles from anoestrous ewes. The oestradiol concentrations in large follicles were not as high as in preovulatory follicles from cyclic ewes reported in other studies. Follicles continued to develop through the ram contact period and when incubated after 40 h and 10 days of ram contact produced high levels of progesterone, indicating partial luteinization, although the corpora lutea (CL) resulting from the induced ovulations regressed prematurely. We suggest that the lack of hCG binding sites in ram-induced follicles may be the cause of poor luteinization and suboptimal development of luteal tissue after induced ovulation in ewes during seasonal anoestrus.

Introduction

The hormonal milieu in an ovarian follicle is thought to be a reliable indicator of its potential for further development. Increased oestradiol concentration in antral fluid accompanies the growth of an ovariolytic follicle in cyclic ewes (McNatty, 1981; Carson, Findlay, Clarke & Burger, 1981). Thecal androgens are the necessary precursors to oestradiol formation and the oestrogen:androgen ratios indicate how active the aromatase enzyme system is (Carson, Findlay, Burger & Trounson, 1979) and, therefore, the potential of the follicle for growth.

The ram has been shown to stimulate ovarian follicular development in seasonally anoestrous ewes when concentrations of circulating gonadotrophins are low (Atkinson & Williamson, 1985). Ram-induced ovulations have also been reported but the resultant corpora lutea are often non-functional and regress prematurely (Oldham & Martin, 1979). The ability of ram-induced follicles and corpora lutea to produce steroid hormones or bind hCG has not been measured. The purpose of the present study was to measure the ability of ram-induced ovarian follicles and luteal tissue to produce steroids, and to determine the binding potential for 125I-labelled hCG of granulosa cells from preovulatory follicles resulting from the stimulus of ram introduction. The follicles used in

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this study are the same follicles whose development and size distribution were reported in an earlier study (Atkinson & Williamson, 1985).

**Materials and Methods**

Fifty-six (56) Southdown ewes in mid-seasonal anoestrous (August–November) were run on irrigated kikuyu (*Pennisetum clandestinum*) pasture and kept isolated from the sight and smell of rams for 3 months before the start of the experiment. Control ewes (N = 11) were held in continued isolation from rams throughout the experiment. A separate group of 6 ewes was bled daily for 20 days following ram introduction, for measurement of plasma progesterone concentrations. Rams harnessed with marker crayons were run with the remaining ewes from the beginning of the experimental period. These 'ram contact' ewes were randomly divided into groups which were exposed to rams for the following periods: 24 h, 40 h, 3 days, 10 days or 20 days. The ewes exposed for 3 days (limited exposure) were then removed from the sight or smell of rams for a further 17 days. This group (designated as 3/20) served to demonstrate whether any effects seen in ewes 20 days after ram introduction were due to the initial stimulus of ram introduction, or to the continued presence of the ram. At the completion of the specified period of ram contact, each group of ewes was bled for 6–9 h, at which time all ewes were ovarioctomized, using thiopentone anaesthesia (Intraval: May and Baker, Australia). Gonadotrophin concentrations obtained from the blood samples and the development and size distribution of the follicles have been previously reported (Atkinson & Williamson, 1985).

Upon ovarioctomy, the ovaries were placed in Minimum Essential Medium with Earle's salts and Hepes buffer (MEME: Flow Laboratories, Australia) at 4°C for transport and dissection. All follicles > 1 mm in diameter and any CL were dissected from the ovaries, measured and recorded (Atkinson & Williamson, 1985). Follicles were classified according to size as follows: > 2 mm, small follicle; 2–4 mm, medium follicle; > 4 mm, large follicle. Follicles that were opaque and not well vascularized were considered atretic and were not included in the data presentation. Follicles dissected from the ovaries of ewes (N) from the control (N = 6), 40 h (N = 6), 10 day (N = 6) and 20 day (N = 6) ram contact groups were incubated in vitro for steroid production. Because CL were observed in the 24 h ram exposure group, hCG binding was assessed on follicles 24 h after ram introduction to see whether hCG binding was increased in ovaries containing ram-induced pre-ovulatory follicles. Follicles dissected from the ovaries of ewes (N) from the 24 h (N = 5) exposure group and the remainder of the control (N = 5), 10 day (N = 5) and 20 day (N = 5) ram contact groups were used for hCG binding site measurement.

**Steroid production by follicles and luteal tissue in vitro.** Follicles were gently torn open, drained of antral fluid and rinsed in medium before being placed in the tissue culture wells. Bisected CL (all 4–8 mm in diameter) and follicles were incubated in 1 ml of the MEME incubation medium at 37°C for 24 h, which was predetermined as the optimal incubation period. The medium was then decanted and stored at −20°C before determination of steroid hormone concentrations. The incubation medium consisted of MEME supplemented with a 1% solution of essential amino acids, fungizone, glutamate, penicillin–streptomycin and 10% fetal calf serum (Flow Laboratories, Australia) as used by Henderson & Moon (1979). Oestradiol was measured in the incubation medium decanted from all follicles, while total androgens and progesterone were measured in incubation media from all large follicles and randomly selected medium and small follicles. Progesterone was measured in the incubation medium from all CL.

**Binding sites for $^{125}$I-labelled hCG on granulosa cells.** Binding sites were measured in granulosa cells harvested from follicles collected from ewes exposed to rams for 24 h, 10 days or 20 days (see Moor, 1977; Webb & England, 1982a, b, for technique). Granulosa cell suspensions were homogenized and centrifuged (27 000 g) for 30 min to obtain a crude membrane pellet. Incubation time and
temperature were pretested; optimum binding occurred after incubation for 3 h at 25°C using a minimum of 50,000 cells. ¹²⁵I-labelled hCG with a specific activity of 34 µCi/µg and an active fraction of 70% was the label used. Non-specific binding was assessed by adding an excess (100 i.u./100 µl) of unlabelled hCG (Pregnyl: Organon, Australia) to a set of tubes. Specific binding of the radiolabel to granulosa cell receptors was expressed as fmol bound per mg protein. Protein content of the washed pellet was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

**Oestradiol assay.** The incubation medium from the follicle incubations was assayed unextracted in a single antibody radioimmunoassay to obtain oestradiol-17β concentrations (modified from McNatty et al., 1976). The assay buffer (0·02 M-PBS) was modified with 0·1% BSA and 0·5% normal rabbit serum (Commonwealth Serum Laboratories, Australia) and by the exclusion of gelatin, stilboestrol and sucrose. The binding agent was serum from rabbits immunized to oestradiol-17β-6-carboxymethylxime-BSA conjugate (McNatty et al., 1976). The antiserum titre used was 1:15,000 and cross-reactivity or specificity was <3% with other steroids (McNatty et al., 1976). [2,4,6, 7-³H(N)]Oestradiol (sp. act. 92–104 Ci/mmol) was the labelled ligand (New England Nuclear, Boston, MA, U.S.A.). Oestradiol USP XIV (Schering A.G., W. Germany) was diluted for standards of the following concentrations: 10, 25, 50, 100, 200 and 500 pg/100 µl. Two blanks for incubation medium and non-specific binding were run with each assay. Non-specific binding was 6·55 ± 0·23% and the sensitivity was 25 pg/ml (incubation medium blank). Intra-assay coefficient of variation was 1·7%. Two pools of incubation medium were included in each assay for the calculation of inter-assay coefficients of variation which were 5·45% and 3·13%. The addition of 50, 100 and 200 µl in triplicate of a known concentration of incubation medium gave 595·5, 601·1 and 604·0 pg oestradiol/ml, respectively. The addition of 100 pg oestradiol/ml to a pool of incubation medium gave an assayed value of 93·62 ± 9·78 pg/ml.

**Total androgen assay.** Total androgens were measured using a single antibody technique (M. S. F. Wong & R. I. Cox, personal communication). The binding agent was serum from a sheep (457) immunized against testosterone-3-carboxymethylxime-BSA (M. S. F. Wong & R. I. Cox, personal communication), which was used at a working dilution of 1:40,000. Cross-reactivity with dihydrotestosterone (DHT), 4-androstene-3β,17β-diol and androstenedione was 98%, 47% and 4·7% respectively. All oestrogen and progesterone compounds had <1% cross-reactivity. Mean non-specific binding was 3·3% and the sensitivity of the assay was 14·0 pg/ml (incubation medium blank). [1,2,6, 7-³H]Testosterone (sp. act. 107 Ci/mmol) was the labelled ligand (Amersham, U.K.). Standards of the following concentrations were diluted from crystalline testosterone (Steraloids, Wilton, N.H., U.S.A.): 12·5, 25, 50, 100, 250 and 500 pg/100 µl. A pool of incubation medium of 199·7 ± 7·6 pg/ml was included in each assay to determine the interassay coefficient of variation, which was 6·6%. Intra-assay coefficient of variation was 2·2%.

**Progesterone assays.** An enzyme immunoassay (EIA) was used to measure progesterone from the incubation medium of randomly selected follicles and all CL. The EIA was based on techniques established by Munro & Stabenfeldt (1984), and all assay constituents came from their laboratory. Incubation medium was assayed unextracted and the sensitivity of the assay was 30 pg/ml. Cross-reactivity was <1% for all other steroids measured (Munro & Stabenfeldt, 1984) and non-specific binding was consistently <1%. Intra-plate coefficient of variation was 5·2%, and inter-plate coefficient of variation was 7·1%. Two pools of incubation medium of 3·47 ± 0·2 and 68·23 ± 0·3 ng/ml were assayed to determine inter-assay coefficients of variation, which were 9·8 and 10·6%, respectively. The standards were made up in incubation medium so no adjustments or displacement factors needed to be accounted for.

Plasma progesterone concentrations were determined by a single antibody radioimmunoassay. The antiserum was raised in an ovariectomized ewe immunized against progesterone-11α-hemisuccinate conjugated to BSA (McNatty et al., 1981). The antiserum was used at a titre of 1:6500.
diluted in 100 µl PBS. Cross-reactivity was <1% for all non-progesterone steroids tested (McNatty, Gibb, Dobson, Thurley & Findlay, 1981). Mean non-specific binding was 3·0% and the sensitivity of the assay was 0·04 ng/ml. [1,2,6,7-3H]Progesterone with a specific activity of 115·0 Ci/mmol was used (Amersham, U.K.). Standards of crystalline 4-pregnen-3,20-dione were diluted to the following concentrations in PBS assay buffer: 10, 20, 40, 80, 120 and 160 pg/100 µl. Plasma was extracted with diethyl ether (100 µl plasma in 1·5 ml diethyl ether). The ether extract was reconstituted in assay buffer. Extraction recovery was 85·6%. Two plasma pools were included in each assay for the determination of inter-assay coefficients of variation, which were 11·6 and 13·8%, respectively. Intra-assay coefficient of variation was 4·8%.

Statistical analyses. Log-logit transformations were applied to the standard curves of all steroid assays (Rodbard, 1974). The natural logarithm was taken from the in-vitro oestradiol and total androgen concentrations to reduce heterogeneity of variance. These values were analysed using an ANOVA (Nie, Hull, Jenkins, Steinbrenner & Brent, 1975), although tables of results present untransformed data.

Results

The mean plasma progesterone concentrations measured in daily blood samples taken after the introduction of the ram were consistently low (<1 ng/ml) with no major fluctuations over the 20-day sampling period in all ewes.

Six corpora lutea from the 40 h ram contact group were incubated in vitro. Each ewe that had ovulated (4 of 6) had also been marked by the ram. Progesterone production by these CL, which were 4–8 mm in diameter, was extremely variable; three CL produced <10 ng progesterone/ml incubation medium over the 24 h, another produced 270 ng/ml and the remaining two produced >2000 ng/ml. No other CL, functional or regressed, were observed in the ovaries of any other treatment group. This observation is in accord with the results of serial plasma progesterone concentrations, which indicated that no functional CL formed following the ram-induced ovulations. Oestrus detection was only recorded for the 40 h ram exposure group.

In-vitro steroid production by incubated follicles

Oestradiol production increased with increasing follicle diameter in all groups (Table 1). There was significantly higher (P < 0·01) oestradiol production by small follicles (<2 mm) collected from all ewe groups exposed continuously to the ram than by small follicles from the control (isolated) ewes or ewes subjected to limited ram exposure (3/20 days). In contrast, medium follicles (2–4 mm) from control ewes produced significantly more (P < 0·01) oestradiol than did medium follicles from any other group, with the exception of those ewes exposed to the ram for 10 days. There were no differences in oestradiol production by large follicles (>4 mm) from any ram contact group. No large follicles were present in the ovaries of the control ewes. The ewes subjected to limited ram exposure (3/20 days) had the lowest oestradiol production by all sizes of follicles, but this reached statistical significance only with the small follicles, and then it was not different from the control group.

Total androgen production did not increase with increasing follicle diameter in any ewe group, nor were there any differences in classes of follicles from any group with different continuous periods of ram contact (Table 2). There was, however, significantly lower (P < 0·01) total androgen production by the small follicles of the ewes subjected to limited ram exposure (3/20 days). The medium-sized follicles of this group also produced significantly less (P < 0·01) total androgen than did those of all other groups with the exception of the ewes exposed to the ram for 40 h. There were no significant differences in total androgen production in the large follicles from different ram contact groups.
**Table 1. Oestradiol production**\(^*\) by follicles incubated *in vitro* after dissection from the ovaries of seasonally anoestrous ewes exposed to rams for various lengths of time

<table>
<thead>
<tr>
<th>Period of ram contact</th>
<th>No. of ewes</th>
<th>Small (&lt;2 mm)</th>
<th>Medium (2–4 mm)</th>
<th>Large (&gt;4 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>6</td>
<td>149·3 ± 26·6(^a) (58)</td>
<td>2549·8 ± 131·8(^a) (39)</td>
<td>—</td>
</tr>
<tr>
<td>40 h</td>
<td>6</td>
<td>196·5 ± 24·9(^b) (124)</td>
<td>451·7 ± 61·6(^b) (23)</td>
<td>23021·5 ± 11693·4(^a) (12)</td>
</tr>
<tr>
<td>10 days</td>
<td>6</td>
<td>262·1 ± 54·0(^b) (134)</td>
<td>1329·0 ± 527·7(^b) (20)</td>
<td>26229·2 ± 8984·1(^a) (19)</td>
</tr>
<tr>
<td>20 days</td>
<td>6</td>
<td>254·1 ± 29·0(^b) (180)</td>
<td>751·8 ± 129·6(^b) (58)</td>
<td>7996·1 ± 1128·3(^a) (25)</td>
</tr>
<tr>
<td>3/20 days</td>
<td>6</td>
<td>126·6 ± 25·5(^a) (68)</td>
<td>404·8 ± 171·4(^a) (10)</td>
<td>8534·9 ± 2612·1(^a) (13)</td>
</tr>
</tbody>
</table>

\(^*\) Concentrations expressed as mean ± s.e.m. in pg/ml for the no. of follicles indicated in parentheses. Values in columns bearing different superscripts are significantly different (*P* < 0·01).

**Table 2. Total androgen production**\(^*\) by follicles incubated *in vitro* after dissection from the ovaries of seasonally anoestrous ewes exposed to rams for various lengths of time

<table>
<thead>
<tr>
<th>Period of ram contact</th>
<th>No. of ewes</th>
<th>Small (&lt;2 mm)</th>
<th>Medium (2–4 mm)</th>
<th>Large (&gt;4 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>6</td>
<td>4470·8 ± 1491·6(^a) (13)</td>
<td>22835·6 ± 15355·6(^a) (23)</td>
<td>—</td>
</tr>
<tr>
<td>40 h</td>
<td>6</td>
<td>1625·9 ± 537·1(^a) (15)</td>
<td>1653·4 ± 773·9(^bc) (13)</td>
<td>2273·7 ± 1287·4(^a) (12)</td>
</tr>
<tr>
<td>10 days</td>
<td>6</td>
<td>3270·4 ± 1429·2(^a) (15)</td>
<td>3471·1 ± 1089·2(^ab) (19)</td>
<td>2285·9 ± 639·9(^a) (18)</td>
</tr>
<tr>
<td>20 days</td>
<td>6</td>
<td>1493·2 ± 193·2(^a) (10)</td>
<td>4278·2 ± 1385·5(^ab) (16)</td>
<td>1326·4 ± 342·6(^a) (17)</td>
</tr>
<tr>
<td>3/20 days</td>
<td>6</td>
<td>251·7 ± 108·2(^b) (10)</td>
<td>368·4 ± 215·0(^c) (6)</td>
<td>1159·6 ± 833·1(^a) (12)</td>
</tr>
</tbody>
</table>

\(^*\) Concentrations expressed as mean ± s.e.m. in pg/ml for the no. of follicles indicated in parentheses. Values in columns bearing different superscripts are significantly different (*P* < 0·01).

As with the oestradiol concentrations, the oestradiol:androgen ratios increased with increasing follicle diameter in all groups (Table 3). The ratios were significantly higher (*P* < 0·05) at 40 h of ram contact in the small and medium follicles compared with the ratios of the control group or the 10 days ram contact group (Table 3). The group subjected to limited ram exposure had oestradiol:androgen ratios that were significantly higher (*P* < 0·05) in all classes of follicles than any other group (Table 3).

Progesterone production increased significantly (*P* < 0·05) in small follicles from all ewes exposed to the ram (Table 4) and was highest from small follicles collected after 40 h and 10 days of ram contact. In-vitro progesterone production by small follicles collected at 20 days from both ram contact groups, although lower than the 40 h and 10 day groups, was statistically higher (*P* < 0·05) than that by small follicles from the control ewes. A similar pattern occurred in the medium follicles.
Table 3. Oestradiol: androgen ratios* calculated as the group ratio from mean oestrogen and total androgen production from the group

<table>
<thead>
<tr>
<th>Period of ram contact</th>
<th>No. of ewes</th>
<th>Small (&lt;2 mm)</th>
<th>Medium (2-4 mm)</th>
<th>Large (&gt;4 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>6</td>
<td>0.66 ± 0.06a</td>
<td>0.82 ± 0.05ae</td>
<td>-</td>
</tr>
<tr>
<td>40 h</td>
<td>6</td>
<td>0.76 ± 0.03b</td>
<td>0.91 ± 0.05b</td>
<td>1.32 ± 0.30a</td>
</tr>
<tr>
<td>10 days</td>
<td>6</td>
<td>0.67 ± 0.05a</td>
<td>0.85 ± 0.06a</td>
<td>1.30 ± 0.06a</td>
</tr>
<tr>
<td>20 days</td>
<td>6</td>
<td>0.75 ± 0.02b</td>
<td>0.79 ± 0.03c</td>
<td>1.27 ± 0.04a</td>
</tr>
<tr>
<td>3/20 days</td>
<td>6</td>
<td>0.87 ± 0.08e</td>
<td>1.09 ± 0.12d</td>
<td>1.46 ± 0.13b</td>
</tr>
</tbody>
</table>

* Values expressed as mean ± s.e.m. Values in columns bearing different superscripts are significantly different (P < 0.05).

Table 4. In-vitro follicular progesterone production* from seasonally anoestrous ewes exposed to rams for various lengths of time

<table>
<thead>
<tr>
<th>Period of ram contact</th>
<th>No. of ewes</th>
<th>Small (&lt;2 mm)</th>
<th>Medium (2-4 mm)</th>
<th>Large (&gt;4 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>6</td>
<td>0.13 ± 0.02a</td>
<td>0.75 ± 0.2a</td>
<td>-</td>
</tr>
<tr>
<td>(13)</td>
<td></td>
<td>(23)</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>40 h</td>
<td>6</td>
<td>3.35 ± 1.5b</td>
<td>8.68 ± 2.3b</td>
<td>24.35 ± 3.95a</td>
</tr>
<tr>
<td>(15)</td>
<td></td>
<td>(13)</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td>10 days</td>
<td>6</td>
<td>6.43 ± 2.6b</td>
<td>4.10 ± 1.3b</td>
<td>66.36 ± 28.8a</td>
</tr>
<tr>
<td>(15)</td>
<td></td>
<td>(19)</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>20 days</td>
<td>6</td>
<td>0.48 ± 0.1c</td>
<td>0.59 ± 0.2a</td>
<td>23.25 ± 8.5a</td>
</tr>
<tr>
<td>(10)</td>
<td></td>
<td>(16)</td>
<td>(17)</td>
<td></td>
</tr>
<tr>
<td>3/20 days</td>
<td>6</td>
<td>0.28 ± 0.06e</td>
<td>1.32 ± 0.3a</td>
<td>15.78 ± 2.5a</td>
</tr>
<tr>
<td>(10)</td>
<td></td>
<td>(6)</td>
<td>(12)</td>
<td></td>
</tr>
</tbody>
</table>

* Values expressed as means ± s.e.m. (ng/ml) for the number of follicles indicated in parentheses.
Values within columns bearing different superscripts are different (P < 0.05).
† This group clearly contained two classes of follicles; 7 follicles produced high levels of progesterone (>1.1 µg/ml), while the remaining 5 follicles produced lower levels. The group mean was calculated from the latter class of follicles.

in which there was significantly higher (P < 0.05) progesterone production by follicles from the 40 h and 10 days ram contact groups, but the 20 day and limited exposure groups had progesterone production values similar to those of medium follicles from the control ewes. The large follicles from the 40 h ram contact group fell into two groups; 7 of the 12 follicles produced extremely high levels of progesterone (>1.1 µg/ml, see Table 4). The remaining 5 follicles in this group produced levels of progesterone equivalent to the large follicles in the other ram contact groups. There were no large follicles in the control group ewe to enable comparison.

Binding of 125I-labelled hCG to granulosa cells

There were no significant differences in the binding capacity of follicles from any ewe group
Table 5. Binding capacity of hCG to the granulosa cells of the 3 largest follicles in each ovary of seasonally anoestrous ewes exposed to rams for various lengths of time

<table>
<thead>
<tr>
<th>Period of ram contact</th>
<th>No. of ewes</th>
<th>No. of follicles</th>
<th>Binding capacity (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (controls)</td>
<td>6</td>
<td>30</td>
<td>1.24 ± 0.2a</td>
</tr>
<tr>
<td>24 h</td>
<td>6</td>
<td>30</td>
<td>1.61 ± 0.2a</td>
</tr>
<tr>
<td>10 days</td>
<td>6</td>
<td>39</td>
<td>1.59 ± 0.3a</td>
</tr>
<tr>
<td>20 days</td>
<td>6</td>
<td>32</td>
<td>1.50 ± 0.3a</td>
</tr>
</tbody>
</table>

Values bearing the same superscripts are not significantly different (P < 0.05).

(Table 5). Only 4 of the 40 ovaries removed from ewes exposed to the ram had no follicles with detectable binding sites and approximately half (42–56%) of the follicles in each ovary had detectable binding sites. The correlation between binding capacity and follicle diameter decreased as the period of continuous ram contact increased, (from isolated anoestrous ewes (r = 0.47, P < 0.01) to continuous ram contact (24 h, r = 0.42, P < 0.05; 10 days, r = 0.19, NS; 20 days, r = 0.14, NS).

Discussion

This study confirms that seasonally anoestrous ewes show increased follicular development and ovulation following the introduction of rams (Martin, 1979; Oldham, Martin & Knight, 1979). The success of ovulation induction in the present study using seasonally anoestrous Southdown ewes was similar to that reported using seasonally anoestrous Merino ewes (Oldham & Martin, 1979), although the percentage of ewes with premature regression of the resultant CL was much greater in the present study than in previous studies (Oldham & Martin, 1979).

Exposure of seasonally anoestrous ewes to rams for as little as 40 h was associated with increased oestrogen production by small follicles and the development of large oestrogenic follicles. In addition, our previous study showed increased follicle recruitment (antrum formation to <2 mm) and the development of large follicles (>4 mm), which were absent in the isolated control ewes (Atkinson & Williamson, 1985). The medium follicles of the control group ewes produced more oestradiol than did the ram contact group at 40 h, which may be an indication that in the ram contact groups the more mature medium follicles (those with greater aromatase activity) had developed to the large follicle stage under the stimulus of ram introduction, leaving behind the follicles with less aromatase activity (possibly regressing). This is a probable explanation as significantly fewer medium-sized follicles were present in the ovaries of ewes exposed to the ram for 40 h than in the control ewes (Atkinson & Williamson, 1985). Although oestradiol production was enhanced in small and large follicles from all groups continuously exposed to the ram, it was well below the level judged to indicate growth and ovulatory potential in cyclic ewes (McNatty, Smith, Makris, Osathanondh & Ryan, 1979; McNatty, Lun, Fanin, McDiarmid & Heath, 1983).

Follicular androgen production was not affected by the continuous presence of a ram. The group subjected to limited ram exposure had the lowest levels of total androgen production and circulating gonadotrophins (Atkinson & Williamson, 1985), adding evidence to the theory that LH pulses stimulate androgen production by the thecal cells (Ryan, 1979). Further, the variation in oestradiol production despite relatively constant total androgen production must be accounted for by varying aromatase activity in the different groups.
All classes of incubated follicles for which oestradiol:androgen ratios were calculated had high ratios when compared with the results of Carson et al. (1981), indicating that the majority of the follicles collected were non-atretic and had the potential to become preovulatory. Although androstenedione was not measured separately in the present study, its effect on the calculation of total androgens in the study of Carson et al. (1981) was minimal and the levels they measured were uniform across all groups. The highest oestradiol:androgen ratios calculated were produced by follicles from the ewe group that was subjected to limited ram exposure (3/20 days). The destiny of all the follicles in these ewes is most certainly atresia, despite the high oestradiol:androgen ratios. This adds evidence that, as total androgen production is constant, the active follicular aromatase system has artificially boosted the oestradiol:androgen ratios of follicles that are unlikely to become preovulatory.

The occurrence of oestrous behaviour and ovulation in 4 of the 6 animals exposed to rams for 40 h suggests that the large oestrogenic follicles that developed after ram introduction were capable of ovulation, and that the initial hypothalamic and pituitary response to ram introduction was sufficient to precipitate an ovulatory surge of LH. Ram-induced ovulations in anoestrous Merino ewes are not often accompanied by behavioural oestrus (Schinkel, 1954; Oldham & Martin, 1979) although behavioural oestrus has been observed in isolated ewes of other British breeds exposed to rams (Chesworth & Tait, 1974). In the present study no ovulations occurred after 40 h despite continued exposure to rams and the presence of large oestrogenic follicles. This indicates that, as reported in our previous study, no further ovulatory gonadotrophin surges occurred (Atkinson & Williamson, 1985).

Follicles from the ovaries of the ewes exposed to the ram for 40 h and 10 days produced levels of progesterone that were greater than or equivalent to levels produced by follicles from ewes in a luteal phase (S. Atkinson, unpublished data). Some of the large follicles collected at 40 h after ram introduction produced extremely high amounts of progesterone ( > 1.1 µg/ml, see Table 4). This indicates that these follicles detected an LH surge and responded with an increase in progesterone production (partial luteinization?) by the follicles even though the CL present in the ovaries had regressed or were in the process of regression (based on progesterone production) and the circulating concentrations of progesterone had fallen. Small and medium follicles from the ovaries of ewes that were run with the ram also produced significantly more progesterone than did the small and medium follicles from the ovaries of the isolated control ewes (Table 4). Progesterone secretion was high at 10 days after ram introduction but was depressed by 20 days, as would be expected in a luteal phase during the breeding season. These high-progesterone producing follicles were probably partly luteinized by the ram-induced LH surge and would have become atretic.

The in-vitro progesterone production by 4 of the 6 CL was less than that produced by the small and medium follicles from the same ewes. Although these 4 CL appeared morphologically functional, their progesterone production was low compared to progesterone production from newly formed CL (McNatty et al., 1981). The group of anoestrous ewes introduced to the ram and bled daily showed no increase in plasma progesterone concentrations, providing further evidence that no functional CL were formed from the ram-induced ovulation. The 2 CL that appeared morphologically functional and produced high levels of progesterone during in-vitro incubation would probably therefore have been short lived as there were no CL present in the ovaries of ewes after 10 days of ram contact and a subsequent cycle was not evident at 20 days of ram contact.

The formation of hCG receptors is normally associated with increased concentrations of FSH and oestradiol (Zelevnik, Midgley & Reichert, 1974; Webb & England, 1982a; Hunter, Southee, McLeod & Haresign, 1986). In the present study the oestradiol produced by incubated follicles increased significantly after ram introduction, but FSH levels decreased precipitously (Atkinson & Williamson, 1985). The net result is that there was no difference in the mean number of hCG binding sites in follicles > 3 mm diameter in any treatment group. This included large follicles collected from ewes 24 h after ram introduction, which presumably were destined to ovulate. Ovaries from the 2 ewes with morphologically functional CL at 40 h after ram introduction also
had follicles with hCG binding capacities similar to those of follicles from the control group ovaries. Hunter et al. (1986) found that follicles removed at the beginning of the LH surge had significantly more hCG receptors if the ewes had been pretreated with progesterone than if they were untreated. The present study was carried out during mid-seasonal anoestrus, when the natural progesterone priming (from the previous luteal phase) was absent and hCG binding was low. In addition, hCG receptors have been shown to increase with increased follicle diameter (Carson, Findlay & Burger, 1979), yet the correlation between hCG binding capacity and follicle diameter was low in the present study and it decreased as the period of ram contact increased. This indicates that a basic difference may exist between the developing follicles in a preovulatory, cyclic ewe and follicles induced in an anoestrous ewe, i.e. the failure of hCG binding sites to develop normally. We therefore suggest that the lack of hCG binding sites in the ram-induced follicles may contribute to the poor development of luteal tissue through an inadequate concentration of binding sites which facilitate lutetinization.

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