J. Reprod. Fert. (1982) **65**, 111–123 *Printed in Great Britain*

Preovulatory follicular development in sheep treated with PMSG and/or prostaglandin

K. P. McNatty, M. Gibb, C. Dobson, K. Ball, J. Coster, D. Heath and D. C. Thurley

Wallaceville Animal Research Centre, Research Division, Ministry of Agriculture and Fisheries, Private Bag, Upper Hutt, New Zealand

Summary. The patterns of growth and atresia of antral follicles including that of the presumptive preovulatory follicle were examined in sheep ovaries for a 24–48-h period after the induction of luteolysis with a prostaglandin analogue, cloprostenol or cloprostenol + PMSG. Ewes were ovariectomized at various times after the initiation of the treatments. All follicles ⩾1 mm in diameter were dissected from the excised ovaries and the antral fluid and granulosa cells recovered. Individual follicles were classified as healthy or atretic on the basis of the number of granulosa cells recovered and then subclassified as to whether they contained intrafollicular levels of oestradiol that were ⩾ or < than 100 ng/ml.

In another series of similarly treated ewes, the ovarian secretion rates of oestradiol and the intrafollicular concentrations of oestradiol in all large antral follicles (⩾5 mm diameter) as well as the levels of progesterone in peripheral plasma were measured at different times after induction of luteolysis.

The results showed that a large 'oestrogenic' follicle (⩾5 mm diameter and secreting ⩾1 ng oestradiol/min) appears around 10 h after the cloprostenol injection and that this presumptive preovulatory follicle emerges before the corpus luteum has ceased to function. Moreover, the presumptive preovulatory ('oestrogenic') follicle appears to develop from the pool of small 'oestrogenic' follicles (1–3 mm diameter) after the onset of luteolysis. The emergence of a large 'oestrogenic' follicle is accompanied by a widespread increase in atresia (>80%) in all other classes of antral follicles (⩾1 mm in diameter).

During the first 10 h of cloprostenol-induced luteolysis, PMSG (a) prevented the normal occurrence of atresia in the large follicle population; (b) enhanced oestrogen secretion in a greater proportion of large antral follicles compared to that in control animals; (c) temporarily 'rescued' and/or prevented small antral follicles (1–4 mm diameter) from undergoing atresia; but (d) had little, or no, effect on the overall population of antral follicles (⩾1 mm diameter). After 24 h, the atresia-preventing effects of PMSG were no longer discernible and the only obvious difference noted, compared to the controls, was the number of large oestrogen-secreting follicles.

Introduction

In the sheep ovary, the time taken for a primordial follicle to develop into a preovulatory structure (>5 mm in diameter) is estimated to be about 6 months (Turnbull, Braden & Mattner, 1977; Cahill & Mauléon, 1980), with most of this growing time spent in the preantral stages. Once a follicle has formed an antrum it requires only another 15 days to reach preovulatory size

0022-4251/82/030111-13$02.00/0

(Turnbull *et al.*, 1977). Most of the evidence suggests that the preovulatory follicle emerges from the pool of small antral follicles (<4 mm diameter) some time after the onset of luteal regression (Smeaton & Robertson, 1971; Land, 1973; Brand & de Jong, 1973; McNatty, Gibb, Dobson, Thurley & Findlay, 1981a). This implies that some of the antral follicles of <4 mm diameter can be mobilized for rapid development when stimulated appropriately with gonadotrophins. Moor, Hay, McIntosh & Caldwell (1973) have reported that there is a 20–30% increase in the number of antral follicles ⩾3 mm in diameter around 6 h after the injection of pregnant mares' serum gonadotrophin (PMSG). Moreover, McNatty, Gibb, Dobson & Thurley (1981b) have demonstrated that follicles <4 mm in diameter in the sheep ovary during anoestrus can be stimulated to ovulate and become normal corpora lutea within 72 h of initiating exogenous LH treatment.

After the onset of luteolysis, changes in the pattern of gonadotrophin secretion can be detected within 10 h after the induction of luteolysis with prostaglandin (PG) F-2α analogues (Baird, 1978; McNatty *et al.*, 1981b). These changes in gonadotrophin secretion are associated with the promotion of 1 or 2 follicles to ovulation despite there being 4–20 antral follicles (⩾1 mm diameter) in each ovary (Brand & de Jong, 1973; McNatty *et al.*, 1981a).

Although there is a relative abundance of data on follicle numbers and the proportions of atretic follicles throughout the oestrous cycle, there is a paucity of quantitative information pertaining to the development of a preovulatory follicle during the period from luteolysis to ovulation (Brand & de Jong, 1973; Turnbull *et al.*, 1977; Dott, Hay, Cran & Moor, 1979; Cahill & Mauléon, 1980; McNatty *et al.*, 1981a). The present experiments were undertaken to determine the patterns of antral follicle development, including that of the presumptive preovulatory follicle for a 48-h period after ewes were treated with PMSG and/or cloprostenol (I.C.I. 80,996), a PG analogue.

Materials and Methods

Animals and experimental design

All animals were parous Romney ewes aged 2½ years, 40–60 kg in body weight and at Days 9–10 of the oestrous cycle (Day 0 = day of oestrus) at the start of the studies. Ewes of this breed and aged 2½ years at the Wallaceville Research Centre, in 1979 or 1980, had a 92% probability of ovulating a single follicle between March and June when the present studies were undertaken.

Experiment I. Ewes were ovariectomized (3 at each time) at 0, 1, 3, 6, 10, 24 and 48 h after an intramuscular (i.m.) injection of 125 μg cloprostenol. A previous study at Wallaceville had shown that the mean ovulation rate in animals treated with cloprostenol (1·08) was not significantly different ($P > 0{\cdot}05$) from an age- and liveweight-matched control flock (1·05, N = 200): 4 of the treated and 3 of the control ewes failed to ovulate.

Experiment II. Ewes were ovariectomized (3 at each time) at 0, 1, 3, 6, 10 and 24 h after an i.m. injection of a mixture of cloprostenol and 500 i.u. PMSG (Folligon; Intervet, Artarmon, New South Wales, Australia).

In the above 2 experiments, all antral follicles ⩾1·0 mm diameter were dissected from the excised ovaries (389 in Exp. I, 402 in Exp. II) within 4 h of ovariectomy. From some of the follicles (181 in Exp. I, 121 in Exp. II), sufficient antral fluid was recovered for the individual measurements of androstenedione, testosterone and oestradiol. The granulosa cells were mechanically dislodged from the walls of all dissected follicles and counted as described by McNatty, Moore-Smith, Makris, Osathanondh & Ryan (1979a). On the basis of results from both human and sheep follicles (McNatty *et al.*, 1979a; McNatty, 1982) sheep follicles were classified as healthy if they contained ⩾50% of their maximum number of granulosa cells for a given follicle size and atretic if they contained <50% of this number. The follicles classified as atretic were similar to those in Stages III to V atresia by the macroscopic criteria of Moor, Hay, Dott &

Cran (1978) and Carson, Findlay, Clarke & Burger (1981). More than 80% of the ovine follicles (⩾2 mm diameter) defined as healthy contained a granulosa-cell population with a detectable level of basal and/or FSH stimulable oestrogen-synthetase activity *in vitro*. In addition, the probability of recovering a healthy looking (×40 magnification), germinal vesicle-stage oocyte from such a follicle was 68·9% (103 healthy follicles examined). By contrast 75% of those defined as atretic contained granulosa cells with low basal and/or FSH-stimulable oestrogen synthetase activity *in vitro* with only 34·6% (205 atretic follicles examined) of the oocytes appearing healthy (McNatty, 1982).

Experiments III and IV. The ovarian secretion rates of oestradiol and peripheral concentrations of progesterone were determined just before ovariectomy at 0, 1, 3, 6, 10, 24 and 48 h after the induction of luteolysis with cloprostenol (Exp. III) or with cloprostenol + PMSG (Exp. IV). For each experiment, 3 animals were examined at each time except at 48 h in Exp. IV when no animals were investigated. All large antral follicles ⩾5 mm in diameter were dissected from the excised ovaries (61 in Exp. III, 58 in Exp. IV) and the antral fluid was recovered for the measurement of oestradiol. The granulosa cells and oocytes were not recovered in these experiments.

Recovery of blood samples, ovarian tissues and antral fluid

All ewes were blood sampled and/or ovariectomized while under the influence of thiopentone sodium anaesthesia (Intravel; May and Baker, New Zealand: Mattner, Stacy & Brown, 1976). For the animals from which ovarian venous blood samples were to be collected, 25 000 i.u. heparin were administered via a jugular venous cannula. Subsequently, the largest ovarian vein draining each ovary was cannulated using the largest vinyl tube (range 2·5–4·0 mm o.d.) capable of being accommodated by the vein. After all subsidiary veins had been clamped ovarian venous blood was collected over a 1–5-min period as described by McNatty *et al.* (1981a). Jugular venous blood (40 ml) was obtained at the same time. All blood samples were centrifuged (4000 ***g*** for 15 min at 6°C) immediately after collection and aliquots of the plasmas were frozen to −20°C for subsequent hormone determinations.

After ovariectomy, the ovaries were weighed, their gross morphology recorded and then the individual antral follicles were dissected free of all extraneous tissue under a stereomicroscope. The follicle wall was split open and antral fluid was aspirated through a finely drawn-out Pasteur pipette. The granulosa cells were recovered as previously described (McNatty *et al.*, 1979a). In addition, the thecal tissue and oocytes were recovered but the results of studies on these two cell-types are not reported in this paper.

Calculation of ovarian secretion rates of oestradiol

These were determined from a knowledge of the time taken to collect a certain volume of blood (blood flow), the concentrations of oestradiol in ovarian venous blood and haematocrit value (McNatty *et al.*, 1981a).

Hormone assays

Follicular fluid. Samples of antral fluid were diluted 10- to 100-fold in 0·1 M-phosphate-buffered saline (pH 7·2) and together with internal recovery standards, were extracted twice with 5 volumes of ether and subjected to celite chromatography (Abraham, Hopper, Tulchinsky, Swerdloff & Odell, 1971). The radioimmunoassay techniques were exactly as described by McNatty, Makris, De Grazia, Osathanondh & Ryan (1979b). Column chromatography was to ensure that testosterone and androstenedione were separated from dihydrotestosterone, which was present in ovarian follicles, sometimes in appreciable amounts (K. P. McNatty, unpublished data).

The androstenedione antiserum (WA-965) was used at an initial dilution of 1:3000. Major cross-reacting steroids with this antiserum are 4-androsten-3,11,17-trione (40%), 11β-hydroxyandrostenedione (31%) and testosterone (0·4%). The detection limit of the assay for antral fluid was 2 ng/ml.

The testosterone antiserum (WA-36) was used at an initial dilution of 1:800. Major cross-reacting steroids with this antiserum are 5α-dihydrotestosterone (75%), 5β-dihydrotestosterone (75%), 5β-androstan-3α,17β-diol (37·5%), 5α-androstan-3α,17β-diol androstenedione (0·1%). The detection limit of the assay for antral fluid was 1 ng/ml.

The oestradiol antiserum (WA-27) was used at an initial dilution of 1:16 000. Major cross-reacting steroids with this antiserum are oestrone (7·3%), oestriol (1·4%), oestradiol-17α (1·4%); androstenedione and testosterone were both <0·02%. The detection limit of the assay for antral fluid was 1 ng/ml.

Plasma. For oestradiol measurements, ether extracts of the plasma (Exps III and IV), were subjected to Sephadex LH-20 column chromatography as previously described (McNatty *et al.*, 1981a). Chromatography was necessary to remove serum materials (identity unknown) which gave high blank values in the oestradiol radioimmunoassay. The assay procedure was then identical to that described by McNatty *et al.* (1981a). In Exps III and IV oestradiol measurements were made on unextracted, diluted (10- to 1000-fold) aliquots. Validation for this procedure has been reported elsewhere (McNatty *et al.*, 1981a). The detection limit of the assay for serum extracts was 5 pg/ml.

Progesterone in peripheral plasma was measured using a radioimmunoassay procedure similar to that described by Thorneycroft & Stone (1972). Details of potential cross-reacting steroids with the progesterone antiserum (WA-26) are reported elsewhere (McNatty *et al.*, 1981a).

The inter- and intra-assay coefficients of variation for all the above steroid assays were <12%.

Statistical analysis

When data at various times after induction of luteolysis were to be compared with those at time 0, an analysis of variance was performed in conjunction with the Neuman and Keuls test (De Jonge, 1963). Statistical significance was tested at both the 5% and 1% levels.

When the levels of oestradiol were to be compared with those of androgen in the same follicles or when the number of healthy follicles between different studies were to be compared, a Student's t test was performed. The effects of the treatments on the follicle populations were compared by χ^2 tests.

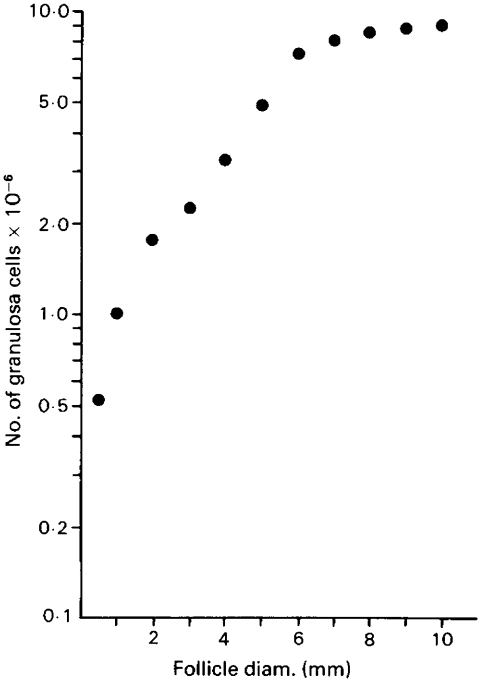
Results

Follicle diameter and the maximum number of recoverable granulosa cells

The relationship between the diameter of an antral follicle and the maximum number of recoverable granulosa cells at each diameter after the induction of luteolysis with cloprostenol (Exp. I) is shown in Text-fig. 1. This relationship can be expressed by the equation $y = 0{\cdot}23 + 0{\cdot}94x + 0{\cdot}004x^2$, where x = follicle diameter in mm and y = number of granulosa cells $\times 10^{-6}$ ($r = 0{\cdot}9893$; $P < 0{\cdot}0001$). A similar study of follicles from ewes in Exp. II revealed no significant deviation of this relationship.

Steroid levels in follicular fluid

The concentrations of aromatizable androgen (androstenedione + testosterone) and oestradiol in the follicles recovered in Exps I and II were grouped according to follicle diameter (small, 1–2 mm; medium, 3–4 mm; large, ⩾5 mm) and whether the follicles were healthy or atretic

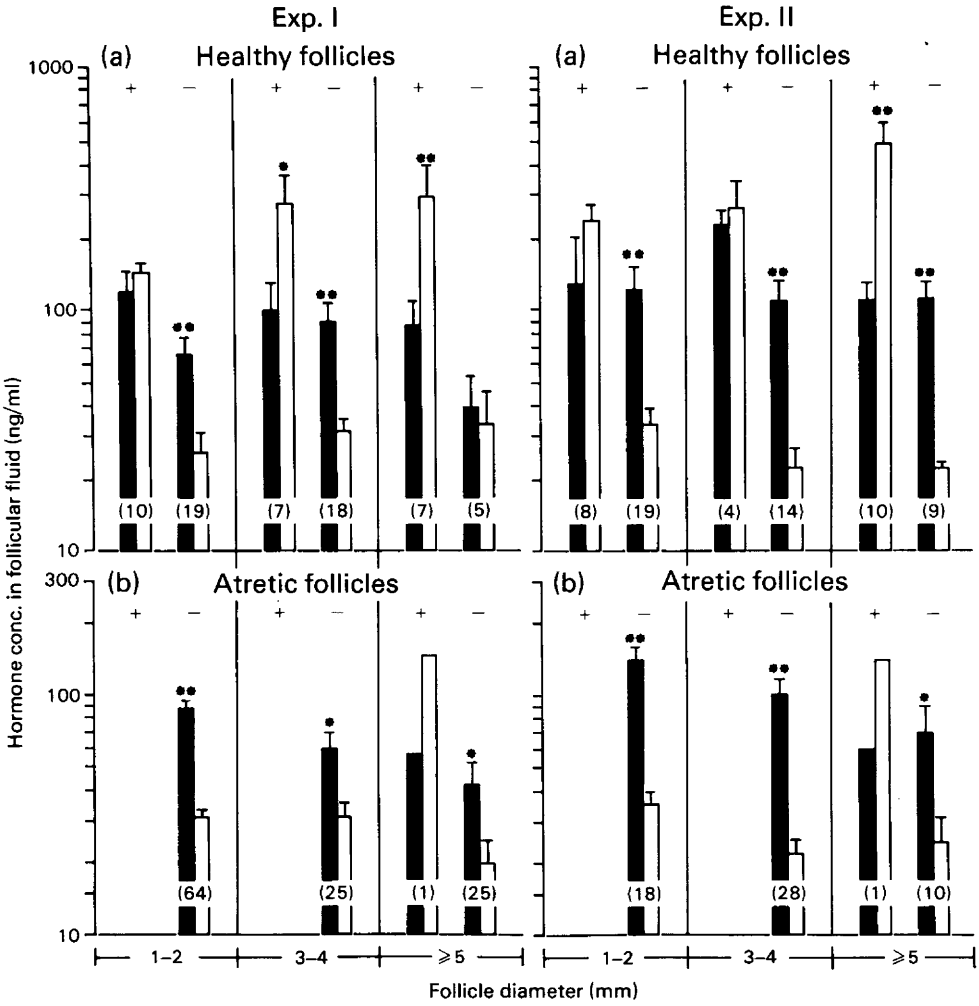


Text-fig. 1. Relationship between the maximum number of recoverable granulosa cells and follicle diameter in isolated ovarian follicles of sheep treated with cloprostenol on Days 9–10 of the oestrous cycle (N = 389, >0·5 mm diameter).

according to their number of recoverable granulosa cells (see 'Materials and Methods'), rather than in relation to different times after luteolysis. In both experiments, >95% of the follicles had oestradiol values which ranged between 10 and 70 ng/ml or 100 and 600 ng/ml, regardless of follicle size, or time after luteolysis. The follicles were therefore further subclassified according to whether they contained ⩾ or <100 ng oestradiol/ml. The hormone concentrations for the various types of follicles are shown in Text-fig. 2.

Experiment I. Only 1 out of the 115 follicles classified as atretic contained ⩾100 ng oestradiol/ml: this single follicle was 7 mm in diameter and contained 45% of the maximum number of recoverable granulosa cells for a follicle of this size. For all other atretic follicles, irrespective of whether they were small, medium or large, the concentrations of aromatizable androgen (androstenedione + testosterone) were significantly higher than those of oestradiol (small, $P < 0{\cdot}01$; medium, $P < 0{\cdot}05$; large, $P < 0{\cdot}05$; paired t test). For the healthy follicles, 34·4, 28·0 and 58·8% of those which were small, medium and large respectively contained ⩾100 ng oestradiol/ml. For the medium and large 'oestrogenic' healthy follicles, the levels of aromatizable androgen were significantly lower than those of oestradiol ($P < 0{\cdot}05$; $P < 0{\cdot}01$; paired t test).

Experiment II. Only 1 out of the 57 atretic follicles (1·8%) contained ⩾100 ng oestradiol/ml; this single follicle was 5·5 mm in diameter and contained 42% of the maximum number of recoverable granulosa cells for a follicle of this size. For all other atretic follicles, the concentrations of aromatizable androgen were significantly higher than those of oestradiol (small, $P < 0{\cdot}01$; medium, $P < 0{\cdot}01$; large, $P < 0{\cdot}05$). For the follicles classified as healthy 29·6, 22·2 and 52·6% of those which were small, medium and large respectively contained ⩾100 ng oestradiol/ml. In the large, but not small or medium-sized 'oestrogenic' (i.e. ⩾100 ng oestradiol/ml) follicles, the levels of aromatizable androgen were significantly lower than those of



Text-fig. 2. Concentrations of aromatizable androgen (androstenedione + testosterone; ■) and oestradiol-17β (□) in follicles of different sizes from the ovaries of sheep in Exp. I (cloprostenol) and Exp. II (cloprostenol + PMSG). +, follicles containing ⩾100 ng oestradiol/ml antral fluid; −, follicles containing <100 ng oestradiol/ml antral fluid. Healthy and atretic follicles are those with ⩾50% and <50% respectively of their maximum number of recoverable granulosa cells for a given follicle diameter (see Text-fig. 1). Values are means ± s.e.m. for the number of follicles indicated in parentheses. Statistically significant differences between androgen and oestradiol concentrations are indicated. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

oestradiol ($P < 0{\cdot}01$; paired t test). For the healthy 'non-oestrogenic' (i.e. <100 ng oestradiol/ml) follicles, the aromatizable androgen concentrations were significantly greater than those of oestradiol irrespective of follicle size ($P < 0{\cdot}01$, for the small, medium and large follicles; paired t test).

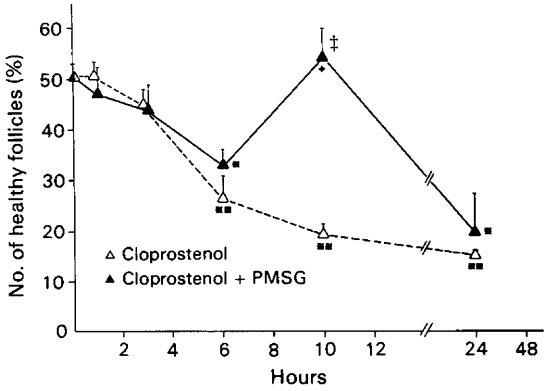
When testosterone concentrations in individual follicles were compared to those of androstenedione with respect to the health of the follicles, no significant differences in the ratios were noted in Exps I or II. Overall the mean testosterone:androstenedione ratio was $3{\cdot}4 \pm 0{\cdot}3$ ($n = 181$) in Exp. I, with 72·6% of all the follicles containing more testosterone than

androstenedione and 3·2 ± 0·4 (n = 121) in Exp. II with 62·6% of the follicles containing more testosterone.

Changes in the follicle population and proportion of healthy follicles after the onset of luteolysis

No significant differences in the total numbers of antral follicles (⩾1 or 2 mm diameter) per ewe were noted at different times after luteolysis compared to the numbers at time 0 or between Exps I and II. The mean total number of antral follicles (⩾1 mm diameter) per ewe was 26·3 (range 10–41) in Exp. I and 22·9 (range 11–36) in Exp. II. The respective mean (± s.e.m.) numbers of antral follicles >2 mm diameter (per ewe) at 0, 1, 3, 6, 10 and 24 h were 4·7 ± 0·6, 4·6 ± 1·0, 4·7 ± 1·2, 7·0 ± 1·1, 6·7 ± 1·5 and 6·3 ± 0·9 in Exp. I and 4·7 ± 0·9, 4·7 ± 0·3, 5·3 ± 0·3, 5·7 ± 0·7, 6·3 ± 2·0 and 6·7 ± 1·8 in Exp. II.

The changes in the proportion of antral follicles (⩾1 mm diameter), adjudged to be healthy on the basis of their granulosa cell populations, after the induction of luteolysis in Exps I and II are shown in Text-fig. 3. Even when all follicles ⩾5 mm in diameter were excluded each mean value differed by <1% and the statistical findings remained unchanged. At 10 h after the injection of cloprostenol, the proportion of healthy follicles in Exp. II ovaries was significantly greater than in Exp. I ($P < 0·01$).

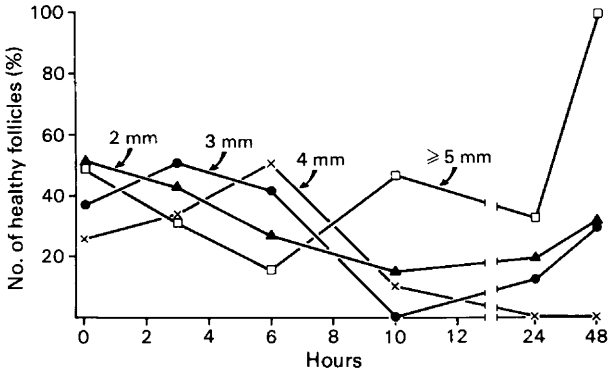


Text-fig. 3. Mean (+ s.e.m.) numbers of healthy follicles at different times after injection of cloprostenol (Exp. I) or cloprostenol + PMSG (Exp. II). Each point represents 30–91 follicles (⩾1 mm diameter) from 3 different sheep. *$P < 0·05$, **$P < 0·01$ compared to values at time 0; ‡$P < 0·01$ compared to values for Exp. I at 10 h; †$P < 0·05$ compared to values for Exp. II at 6 h (Neuman–Keuls test).

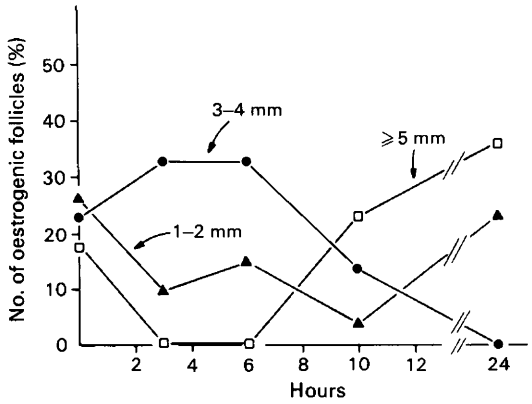
The mean percentages of healthy antral follicles ⩾5 mm diameter at 1, 3, 6, 10 and 24 h were respectively 33, 33, 17, 48 and 33% in Exp. I and 62, 77, 62, 80 and 44% in Exp. II. Significantly fewer large follicles were healthy in Exp. I (43·2%) than in Exp. II (71·4%) (Exp. I, 16/37 follicles, Exp. II, 25/35 follicles; $P < 0·025$, $\chi^2 = 5·83$).

The changing proportions of healthy follicles of various sizes in Exp. I at different times after cloprostenol treatment are shown in Text-fig. 4. Follicles 1 mm in diameter are omitted for clarity but their changing proportions were similar to those of the 2 mm group. At 48 h, all the large follicles ⩾5 mm diameter were healthy.

The proportions of 'oestrogenic' follicles of different diameters at different times after cloprostenol injection in Exp. I are shown in Text-fig. 5. At 10 and 24 h the ⩾5 mm class was predominant.



Text-fig. 4. Mean percentages of healthy follicles at different times after injection of cloprostenol (Exp. I). Each point represents 3–40 antral follicles (>1 mm diameter) from 3 different sheep.



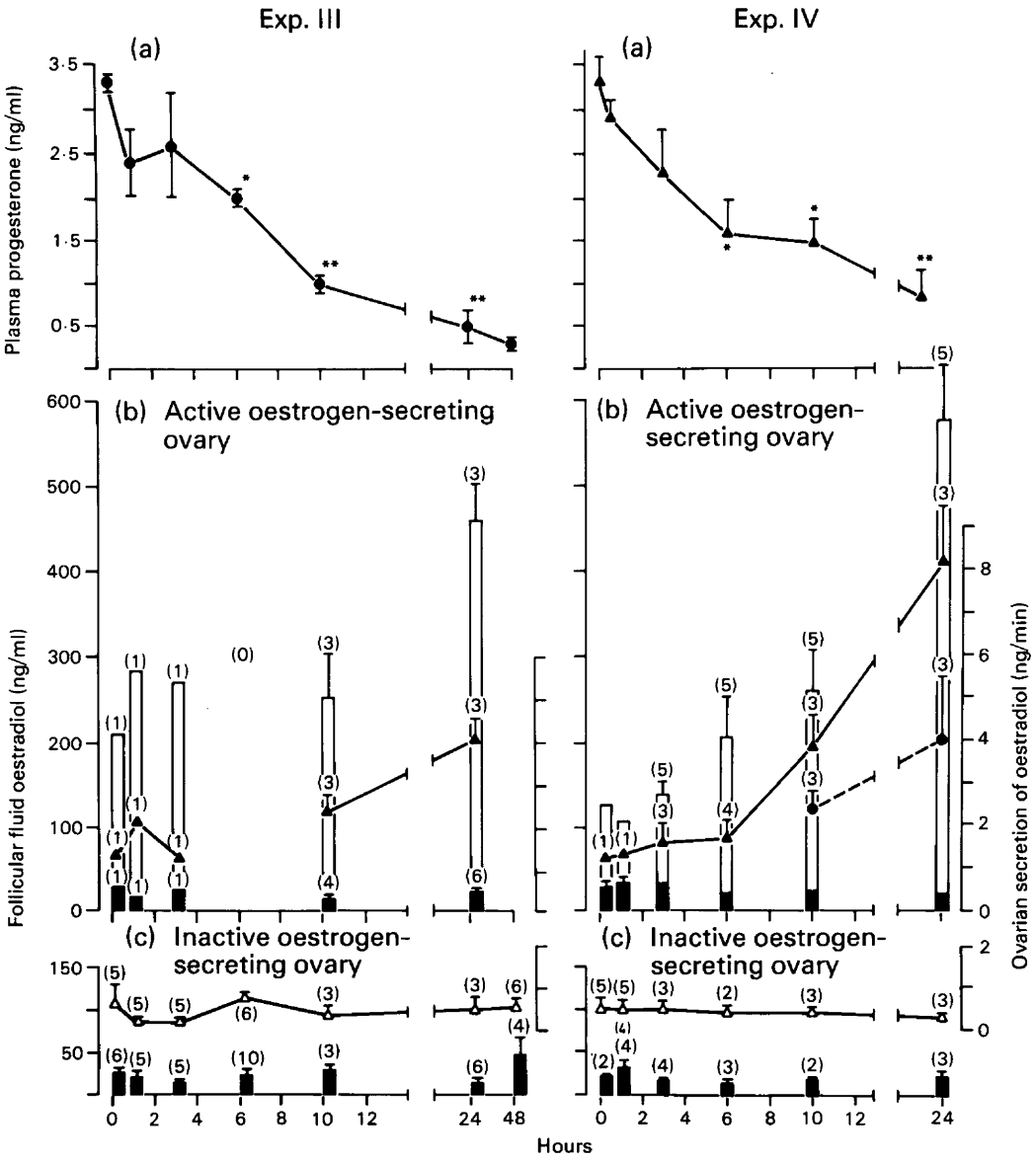
Text-fig. 5. Mean numbers of 'oestrogenic' follicles at different times after injection of cloprostenol (Exp. I). Each value represents 3–27 antral follicles (≥1 mm diameter). Since the levels of oestradiol return to <100 ng/ml 48 h after PG injection, data at this time are not included (see McNatty *et al.*, 1981a).

Ovarian secretion-rates of oestradiol and the appearance of large 'oestrogenic' follicles after induction of luteolysis

In Exps III and IV, the plasma concentrations of progesterone had fallen by 40–50% after 6 h (both $P < 0{\cdot}05$), 60–70% after 10 h (Exp. III, $P < 0{\cdot}01$; Exp. IV, $P < 0{\cdot}05$), and >80% after 24 h (both $P < 0{\cdot}01$) (Text-fig. 6).

Ovaries secreting oestradiol at a rate ≥1 ng/ml were classified as the active (oestrogen-secreting) ovaries while those secreting <1 ng/min were considered to be inactive ovaries with respect to oestrogen secretion because it has previously been shown that ovaries devoid of large antral follicles (≥5 mm diameter) secrete <1 ng oestradiol/min around the time of oestrus and after gonadotrophin stimulation on Day 10 of the oestrous cycle (McNatty *et al.*, 1981a; McNatty, Dobson, Gibb, Kieboom & Thurley, 1982).

In Exp. III, only 1/3 sheep at 0, 1 and 3 h had an active oestrogen-secreting ovary, and, each of these ovaries contained only one large 'oestrogenic' follicle. Other large follicles were also found in these active ovaries but these contained <100 ng oestradiol/ml. At 6 h, none of the animals contained an active oestrogen-secreting ovary. At 10 and 24 h, one ovary from each of



Text-fig. 6. The results of Exps III and IV showing (a) concentrations of progesterone (mean ± s.e.m.) and ovarian secretion rates of oestradiol (△, ▲) from ovaries secreting >1 ng oestradiol/min (b) (active); and those secreting <1 ng/min (c) (inactive). The concentrations of oestradiol (□ ⩾100 ng/ml; ■ <100 ng/ml) in the antral fluid of large antral follicles (⩾5 mm diameter) in the active and inactive ovaries are also shown. There were 3 sheep at each point; numbers in parentheses refer to the number of follicles (histograms) and number of ovaries (points). $^{*}P < 0.05$; $^{**}P < 0.01$ compared to levels of progesterone at time 0 (Neuman–Keuls test).

the animals studied was secreting oestradiol at ⩾1 ng/min (Text-fig. 6), and each of these particular ovaries had one large 'oestrogenic' follicle as well as several 'non-oestrogenic' large follicles. No large 'oestrogenic' follicles were found in the inactive ovaries at any time although several large follicles were present.

In Exp. IV, only 1/3 sheep at both 0 and 1 h had an active oestrogen-secreting ovary, each of these ovaries contained one large 'oestrogenic' follicle. All other large follicles in these as well as the inactive ovaries contained <100 ng oestradiol/ml. At 3, 6, 10 and 24 h, all the ewes (3 at each time) contained at least one ovary secreting oestradiol at ⩾1 ng/min and at least one 'oestrogenic' follicle (at each time interval: 1 ewe, 1 oestrogenic follicle; 2 ewes, 2 oestrogenic follicles/animal) was located in each of the active oestrogen-secreting ovaries. Other large follicles were also found in these active ovaries but these contained <100 ng oestradiol/ml. No large 'oestrogenic' follicles were ever found in any of the inactive oestrogen-secreting ovaries. All ovaries in Exps III and IV which were devoid of large follicles secreted <1 ng oestradiol/min.

The mean secretion rate of oestradiol from the active oestrogen-secreting ovaries in Exp. IV was ~2-fold higher than in Exp. III at 24 h, but the difference was not statistically significant.

Discussion

These findings have demonstrated that a large 'oestrogenic' follicle (⩾5 mm diameter) appears in the sheep ovary within 10 h after the induction of luteolysis with a PG analogue (Text-fig. 6) and that this presumptive preovulatory follicle (McNatty *et al.*, 1981a) emerges before the corpus luteum has ceased to function (Text-fig. 6). As there were no active oestrogen-secreting ovaries or large 'oestrogenic' follicles in any of the ewes at 6 h after the induction of luteolysis as well as an absence of large 'oestrogenic' follicles in at least two-thirds of the animals at 0, 1 and 3 h (Text-figs 5 and 6), the present data are consistent with the notion that the presumptive preovulatory follicle is recruited from the pool of small antral follicles after the onset of luteolysis (Smeaton & Robertson, 1971; Land, 1973; Bherer, Matton & Dufour, 1977). This concept is further supported by the findings of a progressive advancement in diameter (i.e. from 1–2 mm) in the proportion of healthy follicles with high concentrations of oestradiol during the first 10 h after induction of luteolysis (Text-figs 4 and 5). This would suggest that the population of granulosa cells in the 'chosen' follicle is capable of doubling in 6–10 h (Text-figs 1 and 4). The possibility that the patterns of follicular development following induction of luteolysis with a synthetic prostaglandin were influenced to some extent by this analogue cannot be ruled out. However, the endocrine changes in plasma following prostaglandin-induced luteolysis have been carefully documented (Chamley *et al.*, 1972) and have been found to be similar to those occurring at the end of a normal cycle (Barcikowski, Carlson, Wilson & McCracken, 1974; Baird, Land, Scaramuzzi & Wheeler, 1976). It is possible that the promotion of a small 'oestrogenic' follicle into a preovulatory structure (Text-figs 4 and 5) and the rapidly changing patterns of follicular development during the initial phases of luteolysis (Text-fig. 3) are due in part to the changing patterns of LH secretion which results from the progressive decline in progesterone output (Baird, Baker, McNatty & Neal, 1975; Baird, 1978; Legan & Karsch, 1979; McNatty *et al.*, 1981b). It is equally possible that a decline in FSH secretion during the periovulatory period (Salamonsen *et al.*, 1973; Pant, Hopkinson & Fitzpatrick, 1977) may also be partly responsible for the significant and widespread increase in antral follicle atresia particularly after the large 'oestrogenic' follicle has emerged (Text-figs 3 and 6). The injection of PMSG simultaneously with PG analogue temporarily prevented atresia of antral follicles during the first 10 h after initiating luteolysis (Text-fig. 3).

The present studies suggest that an antral follicle must accumulate (or retain) at least one half of the maximum number of granulosa cells for a given follicle diameter to have a capacity to generate an 'oestrogenic' intrafollicular environment (i.e. ⩾100 ng/ml) (Text-fig. 2). Regardless of follicle size, or exposure to PMSG, virtually all (>98%) antral follicles deficient in granulosa cells (i.e. <50% of their maximum number for a given follicle diameter) contained high levels of aromatizable androgen but only low levels of oestrogen (i.e. <100 ng/ml). Follicles at this stage of atresia secrete relatively large amounts of androgen (androstenedione + testosterone) but only

trace amounts of oestrogen *in vitro* (Moor *et al.*, 1978; Hay, Moor, Cran & Dott, 1979; K. P McNatty, unpublished data). Since such follicles contain high levels of aromatizable substrate (Text-fig. 2) and as the granulosa cell populations in such follicles are incapable of being stimulated to produce oestrogen by PMSG *in vivo* (Text-fig. 2) or by FSH or FSH plus androstenedione *in vitro* (McNatty, 1982), they could be considered to be undergoing irreversible atresia (Hay *et al.*, 1979; McNatty *et al.*, 1979a). With regard to the follicles classified as healthy, most contained granulosa cells with an FSH-stimulable oestrogen synthetase system *in vitro* (McNatty, 1982), but it is not possible to conclude whether the healthy 'androgenic' follicles in the present study were undergoing early atresia or were merely healthy growing follicles in transit through an 'androgenic' phase before becoming 'oestrogenic' (Carson *et al.*, 1981; McNatty, 1982).

PMSG is used widely to increase the ovulation rate although its mechanisms of action are still obscure. From examination of sheep ovaries 24 h after PMSG treatment, Dott *et al.* (1979) suggested that PMSG might act to prevent the normal occurrence of atresia and/or the speed at which small follicles (<2 mm diameter) develop into larger structures, but ruled out the possibility that it could act to rescue follicles from atresia. In the present studies in which ovaries were examined at shorter time intervals, PMSG was found to have (a) prevented the normal occurrence of atresia in the large follicle population during the first 10 h of PG analogue-induced luteolysis; (b) enhanced oestrogen secretion in a greater proportion of large antral follicles compared to control animals; (c) temporarily 'rescued' and/or prevented small antral follicles (1–4 mm diameter) from undergoing atresia with respect to their granulosa cell population (Text-figs 3 and 6); but (d) had little or no effect on the overall population of antral follicles (⩾1 mm diameter). The present data are therefore consistent with earlier findings suggesting that PMSG may be able to rescue follicles from atresia in some circumstances (Peters, Byskov, Himelstein-Braw & Faber, 1975; Hay *et al.*, 1979). However, with the treatment regimen employed, the potential 'rescuing' effects of PMSG were short-lived because at 24 h the proportion of healthy follicles had declined to values similar to those in the control ovaries (Text-fig. 3).

The cloprostenol + PMSG regimen used in the present study has previously been shown to increase significantly the ovulation rate compared to that in ewes treated with cloprostenol alone (Gibb, Thurley & McNatty, 1981), and the increased ovulation rate with PMSG was found to be due, almost entirely, to an increase in the number of twin ovulations. In the present study with cloprostenol + PMSG-treated animals, 2/3 animals at 3, 6, 10 and 24 h after treatment had 2 large 'oestrogenic' follicles each (Text-fig. 6). However, it is not obvious as to which follicles were recruited by PMSG for preovulatory follicular maturation. One possibility is that the temporary prevention of atresia in small follicles (1–4 mm in diameter) leads to more than one of these follicles being recruited. Another, perhaps more likely, possibility is that PMSG promoted the development of the preovulatory follicle(s) from the extant large follicle pool within the first 3 h after injection of the PG analogue + PMSG combination. Perhaps the variable ovulation rates caused by higher doses of PMSG are due in part to the number of large non-atretic follicles at the time of injection (Peters & McNatty, 1980) and in part to the differential actions of the drug on both the large and small follicle pools as discussed above.

In the present studies ovaries secreting <1 ng oestradiol/min sometimes contained small or medium-sized 'oestrogenic' follicles (data not shown). This suggests that the fate of such follicles, in ovaries with a low secretion-rate of oestradiol, is probably dictated entirely by the existing patterns of gonadotrophin secretion, with the follicles themselves, via their output of steroids, being unable to exert any major influence on the hypothalamic–pituitary axis (Baird *et al.*, 1975).

In conclusion, the present studies support the notion that the presumptive preovulatory follicle in the sheep develops from the pool of small antral follicles after the onset of luteolysis. They demonstrate that the emergence of the presumptive preovulatory follicle is accompanied by a widespread increase in atresia to all other classes of antral follicles (>1 mm diameter).

Moreover they demonstrate that the incidence of atresia during luteolysis can be modulated with PMSG.

We thank Linda Kieboom and Jean Fannin for assistance with surgery and with blood sampling, Dr S. Hillier, Dr P. F. A. Van Look, and Dr H. Peters for advice during the preparation of the manuscript; and Miss J. Pikart for the typing.

References

Abraham, C.E., Hopper, K., Tulshinsky, D., Swerdloff, R.S. & Odell, W.D. (1971) Simultaneous measurement of plasma progesterone, 17α-hydroxyprogesterone and estradiol-17β by radioimmunoassay. *Analytical Letters* **4**, 325–335.

Baird, D.T. (1978) Pulsatile secretion of LH and ovarian estradiol during the follicular phase of the sheep estrous cycle. *Biol. Reprod.* **18**, 359–364.

Baird, D.T., Baker, T.G., McNatty, K.P. & Neal, P. (1975) Relationship between the secretion of the corpus luteum to the length of the follicular phase in the ovarian cycle. *J. Reprod. Fert.* **45**, 611–619.

Baird, D.T., Land, R.B., Scaramuzzi, R.J. & Wheeler, A.G. (1976) Endocrine changes associated with luteal regression in the ewe: the secretion of ovarian oestradiol, progesterone and androstenedione and uterine prostaglandin $F_{2\alpha}$ throughout the oestrous cycle. *J. Endocr.* **69**, 274–286.

Barcikowski, B., Carlson, J.C., Wilson, L. & McCracken, J.A. (1974) The effect of endogenous and exogenous estradiol-17β on the release of prostaglandin $F_{2\alpha}$ from the ovine uterus. *Endocrinology* **95**, 1340–1342.

Bherer, J., Matton, P. & Dufour, J.J. (1977) Fate of the two largest follicles in the ewe after injection of gonadotrophins at two stages of the estrus cycle. *Proc. Soc. exp. Biol. Med.* **154**, 412–414.

Brand, A. & de Jong, W.H.R. (1973) Qualitative and quantitative micromorphological investigations of the tertiary follicle population during the oestrous cycle in sheep. *J. Reprod. Fert.* **33**, 431–439.

Cahill, L.P. & Mauléon, P. (1980) Influences of season, cycle and breed on follicular growth rates in sheep. *J. Reprod. Fert.* **58**, 321–328.

Carson, R.S., Findlay, J.K., Clarke, I.J. & Burger, H.G. (1981) Estradiol, testosterone and androstenedione in ovine follicular fluid during growth and atresia of ovarian follicles. *Biol. Reprod.* **24**, 105–113.

Chamley, W.A., Buckmaster, J.M., Cain, M.D., Cerini, J., Cerini, M.E., Cumming, I.A. & Goding, J.R. (1972) The effect of prostaglandin $F_{2\alpha}$ on progesterone, oestradiol and luteinizing hormone secretion in sheep with ovarian transplants. *J. Endocr.* **55**, 253–263.

De Jonge, H. (1963) Technieken betreffende de gemiddelde van populaties: variantie-analyse. In *In leiding tot de Medische Statistiek,* deel II, pp. 493–539. Nederlands Instituut voor Praeventieve Geneeskunde te Leiden, Leiden.

Dott, H.M., Hay, M.F., Cran, D.G. & Moor, R.M. (1979) Effect of exogenous gonadotrophin (PMSG) on the antral follicle population in the sheep. *J. Reprod. Fert.* **56**, 683–689.

Gibb, M., Thurley, D.C. & McNatty, K.P. (1981) Active immunization of Romney ewes with an androstenedione-protein conjugate. I. Effect on oestrus and ovulation rate. *N.Z. Jl agric. Res.* **24**, 5–9.

Hay, M.F., Moor, R.M., Cran, D.G. & Dott, H.M. (1979) Regeneration of atretic ovarian follicles *in vitro*. *J. Reprod. Fert.* **55**, 195–207.

Land, R.B. (1973) Ovulation rate in Finn-Dorset sheep following unilateral ovariectomy or chlorpromazine treatment at different stages of the oestrous cycle. *J. Reprod. Fert.* **33**, 99–105.

Legan, S.J. & Karsch, F.J. (1979) Neuroendocrine regulation of the estrous cycle and seasonal breeding in the ewe. *Biol. Reprod.* **20**, 74–85.

Mattner, P.E., Stacy, B.D. & Brown, B.W. (1976) Changes in total ovarian blood flow during anaesthesia and cannulation of utero-ovarian veins. *J. Reprod. Fert.* **46**, 517, Abstr.

McNatty, K.P. (1982) Ovarian follicular development from the onset of luteal regression in humans and sheep. In *IVth Reinier De Graaf Symposium on Follicular Maturation and Ovulation*, in press. Excerpta Medica, Amsterdam.

McNatty, K.P., Moore-Smith, D.M., Makris, A., Osathanondh, R. & Ryan, K.J. (1979a) The microenvironment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the population of granulosa cells and the status of the oocyte *in vivo* and *in vitro*. *J. clin. Endocr. Metab.* **49**, 851–860.

McNatty, K.P., Makris, A., De Grazia, C., Osathanondh, R. & Ryan, K.J. (1979b) The production of progesterone, androgens and estrogens by granulosa cells, thecal tissue and stromal tissue from human ovaries *in vitro*. *J. clin. Endocr. Metab.* **49**, 687–699.

McNatty, K.P., Gibb, M., Dobson, C., Thurley, D.C. & Findlay, J.K. (1981a) Changes in the concentrations of gonadotrophic and steroidal hormones in the antral fluid of ovarian follicles throughout the oestrous cycle of the sheep. *Aust. J. biol. Sci.* **34**, 67–80.

McNatty, K.P., Gibb, M., Dobson, C. & Thurley, D.C. (1981b) Evidence that changes in luteinizing hormone secretion regulate the growth of the preovulatory follicle in the ewe. *J. Endocr.* **90**, 375–389.

McNatty, K.P., Dobson, C., Gibb, M., Kieboom, L. & Thurley, D.C. (1982) Accumulation of luteinizing hormone, oestradiol and androstenedione by sheep ovarian follicles *in vivo*. *J. Endocr.* **91**, 99–109.

Moor, R.M., Hay, M.F., McIntosh, J.E.A. & Caldwell, B.V. (1973) Effect of gonadotrophins on the production of steroids by sheep ovarian follicles cultured *in vivo*. *J. Endocr.* **58**, 599–611.

Moor, R.M., Hay, M.F., Dott, H.M. & Cran, D.G. (1978) Macroscopic identification and steroidogenic function of atretic follicles in sheep. *J. Endocr.* **77**, 309–318.

Pant, H.C., Hopkinson, C.R.N. & Fitzpatrick, R.J. (1977) Concentration of oestradiol, progesterone, luteinizing hormone and follicle-stimulating hormone in the jugular venous plasma of ewes during the oestrous cycle. *J. Endocr.* **73**, 247–255.

Peters, H. & McNatty, K.P. (1980) Ovulation. In *The Ovary*, pp. 78–79. Eds H. Peters & K. P. McNatty. Granada Publishing, London.

Peters, H., Byskov, A.G., Himelstein-Braw, R. & Faber, M. (1975) Follicle growth: the basic event in the mouse and human ovary. *J. Reprod. Fert.* **45**, 559–566.

Salamonsen, L., Jonas, H.A., Burger, H.C., Buckmaster, J.M., Chamley, W.A., Cumming, I.A., Findlay, J.K. & Goding, J.R. (1973) A heterologous radioimmunoassay for follicle-stimulating hormone: application to measurement of FSH in the ovine estrous cycle and in several other species including man. *Endocrinology* **93**, 610–618.

Smeaton, T.C. & Robertson, H.A. (1971) Studies on the growth and atresia of Graafian follicles in the ovary of the sheep. *J. Reprod. Fert.* **25**, 243–252.

Thorneycroft, I.H. & Stone, S.C. (1972) Radioimmunoassay of serum progesterone in women receiving oral contraceptives. *Contraception* **5**, 129–146.

Turnbull, K.E., Braden, A.W.H. & Mattner, P.E. (1977) The pattern of follicular growth and atresia in the ovine ovary. *Aust. J. biol. Sci.* **30**, 229–241.

Received 16 June 1981