Ovarian morphology and the concentration of steroids during the oestrous cycle of sheep actively immunized against androstenedione

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Summary. Ewes were immunized against androstenedione-11 α -hemisuccinyl-bovine serum albumin (androstenedione-11-BSA) or BSA alone (controls). The ovaries were examined macroscopically and ovarian venous blood was collected at laparotomy by in-situ cannulation of the ovarian vein on Day 10 of the oestrous cycle, or by needle puncture of the ovarian vein 36 h after prostaglandin treatment. Follicular fluid was also collected from follicles >5 mm diameter in some ewes. Ovaries and adrenal glands were collected and examined histologically. Oestrone, oestradiol-17 β , androstenedione, testosterone and progesterone were measured by radioimmunoassays. Immunization against androstenedione-11-BSA was followed by an increase in the number of surface follicles >3 mm in diameter and in ovulation rate. These changes were accompanied by an increased production of androgens (especially androstenedione) and of oestrogens during the preovulatory period. There were no observed changes in the adrenal cortex or pituitary glands, but the ovaries were heavier in immunized ewes and luteinized follicles were present in the ovaries of 1 of 4 immunized ewes.

These results show that immunization against androstenedione-11-BSA leads to increased ovulation rate and suggest two possible mechanisms: (1) interference with the negative feedback effects of oestradiol-17 β and/or (2) a reduction in the incidence of follicular atresia.

Introduction

The ovaries of the ewe secrete at least 9 steroids (Baird, 1978), including androgens such as androstenedione and testosterone. Little is known of the physiological functions of these circulating androgens. Sheep immunized against androstenedione show increases in ovulation rate (Scaramuzzi, Davidson & Van Look, 1977; Van Look, Clarke, Davidson & Scaramuzzi, 1978) and in the levels of LH and progesterone in jugular venous plasma and a decrease in the levels of FSH (Martensz & Scaramuzzi, 1979). These studies show that immunization against androstenedione produces large changes in the hypothalamic-pituitary-ovarian feedback and imply that

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androstenedione is an active regulator of ovarian feedback at the hypothalamus and pituitary gland.

One of the principal functions of feedback between the hypothalamus, pituitary gland and ovaries is to ensure the regular occurrence of ovulation. Changes in these feedback systems (no matter how large) are of little biological consequence if ovulation is unaffected. Immunization against androgens (Martensz, 1977; Scaramuzzi *et al.*, 1977; Martensz & Scaramuzzi, 1979) can interfere with ovarian function and alter the ovulation rate of sheep. The present study investigates changes, in sheep actively immunized against androstenedione, in ovarian morphology and the concentration of steroids in ovarian venous blood and follicular fluid, particularly in relation to the resulting alterations in ovulation rate.

Materials and Methods

Immunization procedures

During October 1972 5 Welsh Mountain ewes were immunized with 2.1 mg androstenedione-11 α -hemisuccinyl-bovine serum albumin (androstenedione-11-BSA) in Freund's complete adjuvant (Scaramuzzi, Corker, Young & Baird, 1975). The animals were given booster immunizations in March 1973 (2.0 mg), June 1974 (2.0 mg), November 1974 (2.0 mg) and July 1975 (0.6 mg). A control group of 5 ewes was immunized against bovine serum albumin (BSA) at the same times.

A second group of 25 Welsh Mountain ewes was immunized against androstenedione-11-BSA (18 ewes) or BSA (7 ewes) in August 1975 (1.3 mg) and given booster immunizations in November 1975 (1.0 mg) and October 1976 (1.0 mg).

Group 1: cannulation of ovarian vein and collection of tissues

During September 1975 oestrus and ovulation were synchronized in the first group of ewes (because of deaths now reduced to 4 immunized and 4 control ewes) using intravaginal progestagen pessaries (Synchromate: G.D. Searle & Co., High Wycombe, Bucks). Following removal of the pessaries, all ewes commenced regular oestrous cycles of 16–19 days duration (oestrus = Day 0). Cannulation of both ovarian veins was carried out on Day 10 (range 8–11) of the third oestrous cycle following synchronization of oestrus. Anaesthesia was induced with sodium thiopentone and maintained by halothane. The ovaries were exposed through a mid-ventral incision and the surface morphology was recorded. The ovarian veins were successfully cannulated (Baird & Scaramuzzi, 1976) in 7/8 ewes and a 25 ml sample of ovarian venous blood was collected. A sample of jugular venous blood was collected by needle puncture midway through the collection of the ovarian venous blood sample. The ewe was then killed and the ovaries and adrenals removed, and processed for histology. The pituitary gland was also removed, dissected free of connective tissue and weighed.

Group 2: collection of ovarian venous blood

Oestrus and ovulation were synchronized in the second group of ewes (because of deaths now reduced to 16 immunized and 7 control ewes) in September 1976, using intravaginal pessaries as described above. Regular oestrous cycles occurred following removal of the pessaries. Premature luteal regression was induced by intramuscular injection of 100 μ g of a prostaglandin F analogue (Cloprostenol: ICI Ltd, Macclesfield, Cheshire) between Days 8 and 12 of the second oestrous cycle after synchronization. Laparotomies, as described above, were then performed 36 h (range 33–44 h) after injection of Cloprostenol, and a 10 ml sample of ovarian venous blood was collected by aspiration in 22 of the ewes (Scaramuzzi & Land, 1978). A 20 ml

sample of jugular venous blood was collected at the same time. Following collection of blood samples follicular fluid was aspirated into a 1 ml syringe, using a 23-gauge needle, from all visible follicles of >5 mm diameter.

Histology

Corpora lutea were dissected free from the ovaries. The corpora lutea and the remainder of the ovaries were weighed separately and placed in aqueous Bouin's fixative. The adrenal glands were trimmed, weighed and placed in Helly's fixative. After 24–36 h fixation the tissues were removed from the fixatives, the adrenals washed overnight in running water, and stored in 70% v/v ethanol. The tissues were embedded in paraffin wax, and sectioned at 15 μ m. Representative sections were stained with haematoxylin and eosin and examined under the light microscope.

Assay procedures

The blood samples from both experiments were collected in heparinized tubes and the plasma was removed after centrifugation at 4°C and stored at –20°C until assay. Follicular fluid was also stored at –20°C. Following acidification with 1 N-hydrochloric acid, the concentrations of steroids in plasma were measured using radioimmunoassays described in detail in other publications from this laboratory: oestrone, oestradiol-17 β and androstenedione (Baird, Burger, Heaven-Jones & Scaramuzzi, 1974; Baird, Swanston & Scaramuzzi, 1976); testosterone (Corker & Davidson, 1978); and progesterone (Scaramuzzi *et al.*, 1975). For the assay of these steroids in follicular fluid the same antisera, as indicated above, were used but the methods of extraction and separation were as described from this laboratory for follicular fluid (McNatty *et al.*, 1976) and luteal tissue (Swanston, McNatty & Baird, 1977). The characteristics of these assays used in the present analyses are listed in Table 1.

Table 1. Details of the assays used

	Antiserum	Inter-assay precision (%)	Limit of sensitivity (pg/ml)
Oestrone	029-14	12	10
Oestradiol-17 β	—*	6.1–9.5	10
Androstenedione	Shopman	15	150
Testosterone	E 01	11.5	10
Progesterone	91920/9	13	200

* The antiserum used for the assay of the oestradiol fraction was a specific one raised against 17 β -oestradiol-6(O-carboxymethyl)-oxine-BSA (Dean, Exley & Johnson, 1971; Van Look, Hunter, Corker & Baird, 1977).

The binding of exogenous tritiated steroids by jugular venous plasma from the control and immunized ewes was determined by dextran–charcoal radioassay (Scaramuzzi *et al.*, 1975) and by equilibrium dialysis against physiological saline (9 g NaCl/l) (Rivarola, Forest & Migeon, 1968).

Statistical procedures

The concentrations of steroids in ovarian venous plasma and follicular fluid were analysed using a split-plot analysis of variance and a 2-way non-orthogonal analysis of variance respectively. Before the analyses all steroid concentrations were transformed to logarithms to overcome heterogeneity of variance. Data on ovulation rates were analysed by Fisher's exact

test, follicle numbers by Wilcoxon's Rank sum test and diameter of follicles by a one-way analysis of variance. All other data were analysed using the *t* test.

Results

Group 1

The data in Table 2 show that the ovaries of ewes immunized against androstenedione-11-BSA were heavier than those from control ewes. The increase in ovarian weight resulted from both an increased number of corpora lutea and an increase in the weight of non-luteal ovarian tissue. The numbers of corpora lutea and of visible surface follicles >3 mm in diameter were higher in the immunized ewes than in the controls. The weights of the corpora lutea, adrenal and pituitary glands were not significantly affected (Table 2).

Table 2. The ovulation rate, number of surface follicles > 3 mm, and the weight of ovaries, corpora lutea, adrenal glands and pituitary glands on Day 10 of the oestrous cycle of sheep actively immunized against androstenedione-11-BSA (immunized ewes) or BSA (control ewes)

	Immunized ewes (N = 4)	Control ewes (N = 4)
Wt of ovaries including CL (g)	2.26 ± 0.24**	1.19 ± 0.18
No. of CL (ovulation rate)	2.00 ± 0.00	1.50 ± 0.25
Wt of CL (g)	0.61 ± 0.04	0.50 ± 0.05
No. of visible surface follicles per ewe (>3 mm diam.)	3.0 ± 0.3*	1.0 ± 0.3
Wt of adrenal glands (g)	1.93 ± 0.07	1.71 ± 0.09
Wt of pituitary glands (mg)	515 ± 28	667 ± 75

Values are mean ± s.e.m.

Significantly different from control values: *P < 0.05, **P < 0.01.

Steroid concentrations in jugular venous and ovarian venous plasma are presented in Table 3. The concentrations of androstenedione and testosterone were significantly elevated in the immunized ewes, but those of progesterone and oestrone were significantly elevated only in jugular venous plasma. Oestradiol concentrations were unchanged.

Histological examination of the adrenal glands showed no obvious hypertrophic or hyperplastic changes of the steroid-secreting cells of the adrenal cortex. Likewise, examination of the ovaries did not show any obvious changes in 3 of the ewes immunized against androstenedione-11-BSA (Plate 1). The other similarly immunized ewe showed luteinization of the membrana granulosa in two large follicles but exhibited regular oestrous cycles up to the end of the observations.

Group 2

The results are shown in Tables 4 and 5 and in general they are consistent with the results of Group 1. The ovulation rate and the number of visible surface follicles >3 mm in diameter were significantly increased in immunized ewes. The size distribution of these follicles was also altered so that there were relatively more larger follicles in immunized ewes. The numbers of small visible

PLATE 1

Figs 1–4. Graafian follicle (Figs 1 and 2) and corpus luteum (Figs 3 and 4) of a control ewe (immunized against BSA). Figs 1 and 3, ×132; Figs 2 and 4, ×336.

Figs 5–8. Graafian follicle (Figs 5 and 6) and corpus luteum (Figs 7 and 8) of normal appearance in a ewe immunized against androstenedione-11-BSA. Compare with Figs 1–4. Figs 5 and 7, ×132; Figs 6 and 8, ×336.

PLATE I

(Facing p. 30)

Table 3. The concentration of steroids in ovarian venous and jugular venous plasma on Day 10 of the oestrous cycle in sheep actively immunized against androstenedione-11-BSA (immunized ewes) or BSA (control ewes)

Steroid	Vein	Immunized ewes (N = 4)		Control ewes (N = 3)	
Oestrone (pg/ml)	Ovarian	109 ±	11	96 ±	18
	Jugular	31 ±	4*	19 ±	3
Oestradiol-17β (pg/ml)	Ovarian	62 ±	22	52 ±	13
	Jugular	29 ±	3	33 ±	4
Androstenedione (pg/ml)	Ovarian	12 400 ±	1520***	315 ±	62
	Jugular	5025 ±	1211***	125 ±	72
Testosterone (pg/ml)	Ovarian	385 ±	55*	188 ±	54
	Jugular	128 ±	54	44 ±	15
Progesterone (ng/ml)	Ovarian†	765 ±	73	582 ±	138
	Ovarian‡	116,	29	3.9,	8.1
	Jugular	26.3 ±	3.3***	3.1 ±	0.2

Values are mean ± s.e.m.

Significantly different from control values: * $P < 0.05$, *** $P < 0.001$ (probabilities apply to log transformed data).

† Vein draining ovaries containing corpora lutea.

‡ Vein draining ovaries not containing corpora lutea, individual values.

surface follicles < 3 mm in diameter, however, were not increased. The concentrations of oestradiol-17β and androstenedione in ovarian venous plasma were increased in the immunized ewes but testosterone concentrations were unchanged (Table 4).

Table 4. The effect of active immunization of ewes against androstenedione-11-BSA (immunized ewes) or BSA (control ewes) on ovulation rate, follicular development, and steroid levels in ovarian venous plasma during the periovulatory period

	Immunized ewes (N = 15)	Control ewes (N = 7)
No. of CL (ovulation rate)	2.3 ± 0.2***	1.1 ± 0.2
No. of visible surface follicles per ewe (> 3 mm diam.)	5.0 ± 0.5*	3.1 ± 0.7
No. of visible surface follicles per ewe (< 3 mm diam.)	11.3 ± 2.5	11.6 ± 1.4
Time from PG injection to laparotomy (h)	39.55 ± 1.02	38.38 ± 1.36
Oestradiol-17β conc. (pg/ml)	830 ± 169*	590 ± 148
Androstenedione conc. (pg/ml)	12 105 ± 1113***	1120 ± 364
Testosterone conc. (pg/ml)	310 ± 20	336 ± 54

Values are mean ± s.e.m.

Significantly different from control values: * $P < 0.05$, *** $P < 0.001$ (probabilities for the steroid concentrations apply to log transformed data).

The concentration of steroids in follicular fluid were extremely variable (Table 5). Androstenedione was the only steroid of those measured whose concentration was increased by immunization against androstenedione-11-BSA. Testosterone, although initially very low, was decreased even further by immunization. Follicles were classified as active or inactive depending on whether they contained > or < 50 ng oestradiol-17β/ml follicular fluid. Progesterone was increased in active follicles of control ewes and in all follicles from the immunized ewes. The concentration of oestradiol-17β in the ovarian vein draining an ovary containing at least one active follicle was significantly greater than that in the ovarian vein draining an ovary containing only inactive follicles (Table 5). The concentration of oestradiol-17β in the ovarian vein was significantly correlated with the concentration of oestradiol-17β in follicular fluid ($r = 0.45$; $n = 34$; $P < 0.01$).

Table 5. The concentrations of steroids in follicular fluid from active or inactive follicles (see text for definitions) and of oestradiol-17 β in ovarian venous plasma during the periovulatory period from sheep immunized against androstenedione-11-BSA (immunized ewes) or BSA (control ewes)

	Immunized ewes		Control ewes	
	Active follicles	Inactive follicles	Active follicles	Inactive follicles
Diameter of follicle (mm)	9.2 $\pm$ 0.4 (17) ^a	7.6 $\pm$ 0.3 (18) ^b	7.6 $\pm$ 0.4 (5) ^b	8.7 $\pm$ 0.7 (3) ^{ab}
Oestradiol-17 β (ng/ml)				
Follicular fluid	136.7 $\pm$ 14.3 (17) ^a	24.9 $\pm$ 4.0 (18) ^b	117.1 $\pm$ 17.9 (5) ^a	8.4 $\pm$ 5.6 (3) ^b
Ovarian vein	1.38 $\pm$ 0.27 (15) ^a	0.29 $\pm$ 0.05 (11) ^c	0.91 $\pm$ 0.18 (5) ^b	0.58 $\pm$ 0.31 (3) ^c
Androstenedione (ng/ml)	89.3 $\pm$ 27.2 (16) ^a	106.8 $\pm$ 29.7 (18) ^a	13.0 $\pm$ 2.6 (4) ^b	9.5, 3.7 (2)
Testosterone (ng/ml)	4.0 $\pm$ 0.4 (16) ^a	4.1 $\pm$ 0.8 (17) ^a	8.0 $\pm$ 2.7 (5) ^b	8.7 (1)
Progesterone (ng/ml)	114 $\pm$ 18 (17) ^a	125 $\pm$ 27 (17) ^a	78 $\pm$ 13 (5) ^b	15 $\pm$ 2 (3) ^c

Values are mean $\pm$ s.e.m. for the no. of follicles or samples indicated in parentheses. Active follicles contained >50 ng oestradiol/ml follicular fluid.

Within rows, values with different superscripts are significantly different at $P < 0.05$.

Plasma steroid binding

All the ewes immunized against androstenedione-11-BSA produced detectable antiserum titres against androstenedione. The mean androstenedione antibody titres at the time of surgery were 1/110 and 1/410 for ewes in Groups 1 and 2 respectively. The specificity of the immune response was examined in the 4 animals from Group 1 and indicated significant binding of testosterone in 2/4 ewes and of oestrone in 1/4 ewes (Martensz, 1977). No elevation in the binding of oestradiol-17 β was detected (Martensz, 1977).

As shown in Table 6, the binding of androstenedione, oestrone and testosterone by plasma was significantly elevated in the immunized ewes; the binding of oestradiol was unchanged.

Table 6. The percentage binding of tritiated steroids (15–20 pg) by a 1:5 dilution of jugular venous plasma from ewes immunized against androstenedione-11-BSA (immunized ewes) or BSA (control ewes)

	Immunized ewes (N = 4)	Control ewes (N = 4)
Oestrone	91.3 $\pm$ 2.0***	79.3 $\pm$ 0.8
Oestradiol-17 β	87.1 $\pm$ 0.5	89.2 $\pm$ 1.8
Androstenedione	99.2 $\pm$ 0.1***	56.2 $\pm$ 2.1
Testosterone	98.3 $\pm$ 0.5***	85.6 $\pm$ 1.4

Values are mean $\pm$ s.e.m.

Significantly different from control values, *** $P < 0.001$.

Discussion

All of the ewes immunized against androstenedione-11-BSA produced antisera with an increased capacity to bind tritiated androstenedione when tested in a dextran-coated charcoal radioassay or by equilibrium dialysis. The resultant decrease in metabolic clearance rate (Wickings, Becher & Nieschlag, 1976) produced greatly elevated concentrations of biologically inactive andro-

stenedione in the jugular venous plasma (Table 3). Plasma from some of the ewes immunized against androstenedione-11-BSA also contained antibodies capable of binding tritiated oestrone and testosterone, resulting in small increases in the peripheral concentrations of these steroids. No increase in the binding capacity of jugular venous plasma to tritiated oestradiol was seen in immunized ewes.

Ovulation rate and the number of visible surface follicles >3 mm in diameter were increased in the immunized ewes although FSH concentration was lower than in control animals (Martensz & Scaramuzzi, 1979). In the ewe, follicular development is thought to be a continuous process (Turnbull, Braden & Mattner, 1977a) under the control of gonadotrophins. The elevated basal levels of LH in ewes immunized against androstenedione-11-BSA (Martensz & Scaramuzzi, 1979) are associated with increased ovulation rate. Elevated LH levels have an antimitotic effect on the granulosa cells (McNatty & Sawers, 1975) and might allow follicles capable of ovulating to remain viable for a longer period than normal (Turnbull, Land & Scaramuzzi, 1977b). It has also been suggested that ovarian androgens increase the rate of follicular atresia (Louvet, Harman, Schreiber & Ross, 1975). It is possible therefore that the binding of androgens by antibodies in follicular fluid and the vascular compartment of the androstenedione-immunized ewes reduced the rate of atresia in follicles >3 mm diameter and so increased ovulation rate. The absence of any change in the number of visible follicles <3 mm in diameter, despite the presence of altered FSH and LH levels, in the androstenedione-immunized sheep suggests that these earlier stages of follicular growth are proceeding normally and are less dependent on gonadotrophins.

Steroid secretion from the ovaries varied widely from animal to animal in the immunized ewes, and in general was characterized by increased net secretion (ovarian venous minus jugular venous concentrations) of androstenedione and of oestradiol during the preovulatory period. Since LH stimulates the secretion of both androstenedione and oestradiol (McCracken, Uno, Goding, Ichikawa & Baird, 1969; Baird *et al.*, 1976), and androstenedione is a major precursor of oestradiol in the sheep ovary (Rado, McCracken & Baird, 1970), the increased concentrations of LH (Martensz & Scaramuzzi, 1979), preovulatory oestradiol and androstenedione are in agreement with each other. These data also imply that circulating oestradiol has a reduced effectiveness on the negative feedback control of LH.

The presence of elevated levels of progesterone in jugular venous plasma indicates that some alteration in the metabolism of progesterone occurred in the ewes immunized against androstenedione-11-BSA. Significant antibody titres to progesterone were also present in these animals (Martensz & Scaramuzzi, 1979).

The concentrations of oestradiol-17 β in follicular fluids from follicles >5 mm diameter were apparently bimodal in distribution and the follicles were classified accordingly as inactive (oestradiol-17 β , range 3.0–48.8 ng/ml; $n = 21$) and active (oestradiol-17 β , range 65.2–292.5 ng/ml; $n = 22$). Elevated concentrations of oestradiol in follicular fluid are characteristic of preovulatory follicles (Sanyal, Berger, Thompson, Taymor & Horne, 1974; McNatty, Hunter, McNeilly & Sawers, 1975; Fowler, Chan, Walters, Edwards & Steptoe, 1977) and it is possible that the follicles we have classified as active were indeed those follicles about to ovulate. It has been proposed that oestradiol in follicular fluid arises from androgens of thecal origin entering the granulosa cell where aromatization to oestrogens can occur under the influence of FSH (Dorrington, Moon & Armstrong, 1975), but it is unclear which androgen (androstenedione or testosterone) serves as major precursor for aromatization. It seems likely that antibodies to androstenedione enter the follicle (Edwards, 1974) leading to increased concentrations of biologically inactive androstenedione in follicular fluid (Table 5) and hence reducing the availability of the steroid for conversion to oestrogens by granulosa cells. Since the concentration of oestradiol-17 β was not reduced by immunization against androstenedione-11-BSA but follicular fluid levels of testosterone were lower in immunized ewes it is probable that most of the oestradiol-17 β in follicular fluid of immunized ewes was derived from conversion of testosterone

rather than androstenedione. Normally only follicles undergoing the final stages of preovulatory maturation would also have high concentrations of progesterone in their follicular fluid (McNatty *et al.*, 1975; Fowler *et al.*, 1977) and detectable LH receptors on their granulosa cells (R. S. Carson, J. K. Findlay, H. G. Burger & A. O. Trounson, personal communication). Both active and inactive follicles in the ovaries of immunized ewes had high concentrations of progesterone in follicular fluid, presumably premature luteinization in response to the increased basal concentration of LH (Scaramuzzi *et al.*, 1977).

We propose that immunization against androstenedione reduces the availability of biologically active androstenedione which effectively decreases the sensitivity of the hypothalamic-pituitary unit to the feedback effect of oestradiol and results in increased LH release. The elevated LH levels then somehow act to increase the number of follicles available to ovulate. Alternatively, the binding of androgens to antibodies may reduce androgen-induced atresia, thereby increasing the availability of preovulatory follicles. Similar mechanisms may exist in breeds of sheep with high ovulation rates (e.g. Finnish Landrace) and the androstenedione to oestradiol ratio might be a correlate of the ovulation rate. However, at least two other factors can also modify ovulation rate, the rate of uptake of follicles into their rapid growth phase (Turnbull, Mattner, George & Scaramuzzi, 1978) and the class (i.e. size) at which follicles mature and become capable of undergoing preovulatory changes and ovulation in response to LH (Turnbull *et al.*, 1977a).

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