Effect of long-term hypophysectomy on ovarian follicle populations and gonadotrophin-induced adenosine cyclic 3',5'-monophosphate output by follicles from Booroola ewes with or without the F gene

K. P. McNatty, D. A. Heath, N. Hudson and I. J. Clarke*

MAFTech, Wallaceville Animal Research Centre, Ministry of Agriculture and Fisheries, P.O. Box 40063, Upper Hutt, New Zealand; and Prince Henry's Institute of Medical Research, P.O. Box 118, South Melbourne, Victoria 3005, Australia

Summary. Long-term (i.e. ~70 days) hypophysectomy led to a significant ($P < 0.05$) reduction in ovarian weight but no reduction in the total number of antral follicles (>0.1 mm in diameter). In hypophysectomized ++ Booroola ewes ($N = 8$) follicles were always ≤ 3 mm and in hypophysectomized FF Booroola ewes ($N = 6$) follicles were always ≤ 2 mm in diameter; in ewes of both genotypes follicles reached diameters which were ~40% of their predicted final size at ovulation. Under in-vitro conditions, follicles from the FF and ++ hypophysectomized ewes produced significant increases in cAMP within 1 h of exposure to gonadotrophins ($P < 0.05$) although no genotypic differences in cAMP production were noted.

We conclude that ovarian follicles in FF and ++ ewes have absolute requirements for pituitary hormone on reaching diameters of 2 mm and 3 mm respectively and that appreciable numbers of antral follicles in ewes of both genotypes remain responsive to pituitary gonadotrophins despite prolonged deprivation of these hormones.

Keywords: hypophysectomy; ovarian follicles; gonadotrophins; cAMP output; Booroola ewes; F gene

Introduction

High fecundity Booroola ewes possess a major gene (F) which influences their ovulation rate (see Bindon, 1984, for review). Homozygotes (FF), heterozygotes (F+) and non-carriers (++) of the putative gene(s) are segregated on the basis of ovulation rate recordings of ≥ 5 , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982). In FF Booroola ewes ovarian follicles reach preovulatory maturity and ovulate at diameters between 2 and 4.5 mm. In contrast, follicles in ++ ewes ovulate at diameters between 5 and 7 mm (McNatty & Henderson, 1987). It has been established that the final phases of preovulatory follicular maturation have a critical requirement for pituitary luteinizing hormone (LH) (Cahill, 1979; McNatty, 1981; Baird & McNeilly, 1981) and that corpus luteum (CL) formation can be induced in hypophysectomized ewes by the sequential administration of pregnant mares' serum gonadotrophin [PMSG; a follicle-stimulating hormone (FSH) enriched material] and human chorionic gonadotrophin (Fry *et al.*, 1988). In long-term hypophysectomized (~70 days) ewes antral follicles rarely develop beyond 3 mm in diameter (Cahill, 1979), but the effect of hypophysectomy on follicle development in ewes of the Booroola genotypes is unknown. The purpose of the present study was to examine the consequences of long-term hypophysectomy on antral follicle development in Booroola FF and ++ ewes and also to test whether the residual antral follicles (if any) retained a capacity to synthesize adenosine cyclic 3',5'-monophosphate (cAMP) in response to exogenous LH and FSH *in vitro*.

Materials and Methods

Animals

The parous Booroola Merino ewes were between 5 and 6 years of age and all FF (N = 12) and ++ (N = 16) animals were assigned their genotype by pedigree analysis. Moreover, the annual ovulation rates in these animals were confirmed by laparoscopy to be ≥ 5 (FF genotype) and < 3 (++) genotype).

Six FF and 8 ++ ewes were hypophysectomized in November/December 1988 (i.e. during anoestrus) as described by Fry *et al.* (1987). All animals were housed indoors under natural lighting in a warm environment (i.e. $\sim 16^\circ\text{C}$) and fed and watered *ad libitum*. In addition all hypophysectomized animals received glucocorticoid replacement therapy (i.e. 3 mg Dexamet, s.c. once every 6 days; Chemavet Distributors Ltd, Auckland, NZ) and were bled once weekly to check for any residual FSH and LH in peripheral plasma while the untreated control ewes were bled once before ovariectomy. The LH and FSH assays were identical to those described by McNatty *et al.* (1989). For FSH and LH, the volume of plasma which was assayed was 0.1 ml with each sample being assayed for each hormone in duplicate. The standard curve standards (NIAMDD-oFSH-RP-1, biopotency $75 \times \text{NIH-FSH-3}$; NIAMDD-oLH-S23, biopotency $2.3 \times \text{NIH-LH-S1}$) and reference samples were prepared in hypophysectomized ewe plasma which contained undetectable concentrations of FSH or LH. The minimal detectable amounts for FSH and LH were both 0.02 ng/tube and the intra- and inter-assay coefficients of variation were < 4 and $< 5\%$ respectively for FSH and < 5 and $< 8\%$ respectively for LH.

Follicle dissection and culture

The ovaries of all control and hypophysectomized ewes were recovered at ovariectomy between January and April 1989. The reproductive status (i.e. anoestrus, day of oestrous cycle) of the control animals was not known before ovariectomy. The ovaries were placed in chilled ($4-6^\circ\text{C}$) medium [i.e. Minimum Essential Medium + Earle's salts + L-glutamine + 0.1% (w/v) bovine serum albumin (BSA) + 0.2 mmol/l, 1-methyl-3-isobutylxanthine (MEM + MIX)] which was also used as the dissection medium. All ovarian antral follicles > 0.1 mm in diameter and corpora lutea (CL) were dissected free of extraneous tissue using a stereodissecting microscope (SV8; C. Zeiss, Oberkochen, West Germany) at $\times 8-64$ magnification. Individual follicular diameters were measured in 0.1 mm increments using 10×25 focussing eye-pieces each fitted with a net graticule (Type 474068, C. Zeiss). The weights of the ovaries and CL were recorded.

For cAMP measurements each follicle was finely minced in 0.5 ml Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY, USA) containing 0.1% (w/v) BSA and 0.2 μmol MIX/ml (i.e. DPBS + BSA + MIX). The total minced suspension of each follicle was then transferred to a 10×75 mm plastic test-tube containing 0.5 ml DPBS + BSA + MIX or 0.5 ml DPBS + BSA + MIX + LH (2 μg NIAMDD-oLH-S24/ml) and FSH (2 μg NIAMDD-oFSH-S17/ml) capped and either incubated in a 37°C shaking water bath (200 cycles/min) for 1 h before being transferred to an 80°C water bath for 15 min or transferred directly to the 80°C water bath for 15 min. For this study all follicles > 0.1 mm in diameter were used with $\sim 50\%$ of each size class for each ewe being studied for 1 h while the others were kept as 0 h controls. All procedures before the incubation or heating steps were performed with the tissues at 4°C . Thereafter, each sample was stored at -70°C until assayed for cAMP by radioimmunoassay. Preliminary studies showed that the cAMP values accumulated by follicular tissues in DPBS + BSA + MIX + LH + FSH at 0 h or by tissues in DPBS + BSA + MIX after 1 h incubation at 37°C were not different from those by tissues in DPBS + BSA + MIX at 0 h.

cAMP

The minced follicular sample in DPBS + BSA + MIX was centrifuged at 2000 g for 15 min and 20 μl of the supernatant were then added to 80 μl assay buffer [sodium acetate buffer (0.05 M, pH 6.5) containing 0.1% (w/v) BSA]. These samples together with the cAMP standards in 100 μl of assay buffer were acetylated by the method of Harper & Brooker (1975) or assayed directly.

The cAMP antiserum (ARL) was kindly supplied by Dr A. R. La Barbera (North Western University, Chicago, IL, USA) and used at a final dilution of 1:24 000 for the acetylated assay and 1:15 000 for the non-acetylated assay. The cross-reactions of AMP, ADP, ATP and cGMP were all $< 0.00004\%$ while that for dibutyl cAMP was 0.0049%.

Iodinated cAMP derivative ($\sim 10\,000$ c.p.m.) in assay buffer containing 3% (w/v) BSA was added to each assay tube in 100 μl . The radioactive label was prepared by iodinating 2',0-monosuccinyl adenosine 3',5'-cyclic monophosphate tyrosyl methyl ester (Sigma Chemicals, St Louis, MO, USA) using the chloramine T method to a specific activity of 120–150 $\mu\text{Ci}/\mu\text{g}$.

The assay tubes were incubated overnight at 4°C and the free and bound fractions were separated using 0.2% (w/v) Norit A charcoal in 0.5 M-sodium acetate buffer (pH 6.5) with 0.25% BSA (w/v). The cAMP results were expressed as pmol cAMP/follicle. The intra- and interassay coefficients of variation were 8 and 14% respectively. The limit of detection was ≤ 0.03 pmol/follicle.

Statistical procedures

Statistically significant differences in the follicle number or cAMP data between sheep within each genotype were noted (analysis of variance, ANOVA). Thus, the data for each sheep were averaged over each 0.5 mm diameter range so that statistical comparisons with respect to incubation time or genotype were based on the number of sheep. All data with regard to follicle numbers or cAMP output were transformed by $\log_e x + 1$ to equalize the variances before statistical analysis. The log-transformed data were examined by analysis of variance. Most results are presented as geometric means together with 95% confidence limits. The results relating to the ovarian weights are presented as arithmetic means $\pm$ s.e.m.

Results

The mean ($\pm$ s.e.m.) time from hypophysectomy until ovariectomy was 69 (± 1) days for the ++ ewes ($N = 8$) and 68 (± 1) for the FF ewes ($N = 6$). Two control ++ and 2 control FF ewes were still in anoestrus as assessed by the absence of corpora lutea at ovariectomy. The mean ($\pm$ s.e.m.) numbers of corpora lutea in the remaining 6 ++ and 4 FF ewes were 1.2 (± 0.2) and 5.0 (± 1.2) respectively.

The plasma concentrations of FSH and LH in the hypophysectomized ewes were undetectable (i.e. < 0.2 ng/ml for both hormones) throughout the sampling period. At the time of ovariectomy the mean ($\pm$ s.e.m.) FSH/LH concentrations in the FF and ++ ewes were 1.8 (± 0.1)/0.4 (± 0.1) and 1.4 (± 0.1)/0.4 (± 0.1) ng/ml respectively; these hormone values were not significantly different between genotypes.

The mean ($\pm$ s.e.m.) non-luteal ovarian weights in the control and hypophysectomized FF and ++ ewes are summarized in Table 1. Although no genotype differences existed, ovaries of hypophysectomized ewes were significantly lighter ($P < 0.05$) than those of control ewes (2-way ANOVA); no interaction effect was noted between genotype and hypophysectomy.

Table 1. Non-luteal ovarian weights in control and hypophysectomized FF and ++ Booroola ewes† (means $\pm$ s.e.m.)

Treatment	Genotype	No. of ewes	Ovarian weight (g)
Control	FF	6	1.05 $\pm$ 0.09
	++	8	1.27 $\pm$ 0.11
Hypophysectomy	FF	6	0.79 $\pm$ 0.13
	++	8	0.92 $\pm$ 0.13

†The weights for all ewes refer to the mean of both ovaries after first removing luteal tissue which was sometimes present in control ewes.

* $P < 0.05$ compared to the mean of the ovaries from hypophysectomized ewes (from 2-way ANOVA).

The number of follicles > 0.1 mm in diameter in the ovaries of Booroola FF and ++ control and hypophysectomized ewes are given in Table 2. Irrespective of treatment or genotype, no differences in the overall mean numbers of follicles were observed (2-way ANOVA). Examination of follicle numbers with respect to follicular diameter, genotype and treatment by multi-factor ANOVA revealed significant main effects of genotype ($P < 0.01$), treatment ($P < 0.01$) and diameter ($P < 0.01$). However, the importance of these main effects cannot be evaluated independently since there were significant interactive effects between genotype and diameter ($P < 0.01$), treatment and diameter ($P < 0.01$) but not treatment and genotype. In FF control ewes no follicles > 4.5 mm in diameter were observed whereas in ++ control ewes there was generally at least 1 follicle/ewe > 5 mm diameter. In hypophysectomized FF ewes no follicles were observed > 2 mm in

Table 2. Ovarian follicle numbers in control and hypophysectomized FF and ++ Booroola ewes with respect to follicular diameter (mm)

Treatment (no. of ewes)	Genotype	Follicular diameter (mm)											Total
		>0.1 -0.5	>0.5 -1.0	>1.0 -1.5	>1.5 -2.0	>2.0 -2.5	>2.5 -3.0	>3.0 -3.5	>3.5 -4.0	>4.0 -4.5	>4.5 -5.0	>5.0	
Control (6)	FF	15 (9, 27)	32 (18, 56)	10 (6, 19)	16 (8, 33)	4 (1, 10)	1 (0, 3)	2 (1, 4)	1 (0, 3)	0 (0, 1)	—	—	87 (53, 140)
	++	12 (6, 21)	24 (13, 45)	9 (5, 14)	13 (7, 24)	8 (5, 13)	4 (2, 8)	3 (1, 5)	1 (0, 3)	1 (0, 1)	0 (0, 1)	1 (0, 2)	84 (57, 124)
Hypophysectomy (6)	FF	11 (5, 23)	27 (16, 44)	16 (10, 27)	9 (4, 17)	—	—	—	—	—	—	—	68 (49, 94)
	++	13 (8, 19)	22 (19, 25)	7 (4, 11)	15 (11, 22)	8 (5, 12)	4 (1, 10)	—	—	—	—	—	74 (59, 92)

Values are geometric means (and 95% confidence limits).

diameter whereas in hypophysectomized ++ ewes follicles between 2.5 and 3 mm in diameter were common; follicles of larger diameters were never observed.

The production of cAMP in follicles of different diameter, incubation times and genotype are shown in Table 3. When each treatment and genotype (++ or FF control; ++ or FF hypophysectomized) was examined separately by 2-way ANOVA (incubation time vs follicular diameter), significant main effects of time ($P < 0.01$) and diameter ($P < 0.01$) but no interactive effects for any of the groups were noted. The amounts of cAMP present at both 0 and 1 h increased overall with respect to follicular diameter. The amount of cAMP present at 0 h within each range of follicular diameter was similar for each genotype or treatment (hypophysectomy or control). In control ++ and FF ewes significant ($P < 0.01$) accumulation of cAMP was evident after 1 h in some follicles between > 1.5 and 3.0 mm in diameter. In control ewes, the proportions of follicles between > 0.1 – 1.0 mm, > 1.0 – 3.0 mm and > 3.0 mm in diameter which accumulated more cAMP after 1 h relative to 0 h were 72/178 (40.5%), 60/120 (50.5%) and 17/32 (53.1%) respectively for the ++ genotype and 75/154 (48.7%), 42/83 (50.6%) and 10/13 (76.9%) respectively for the FF genotype. In hypophysectomized ++ and FF ewes significant ($P < 0.05$) accumulation of mean cAMP was evident after 1 h in follicles between > 1.0 and 2.5 mm in diameter. In the hypophysectomized animals, the proportion of follicles between > 0.1 – 1.0 mm and > 1.0 – 2.5 mm in diameter which accumulated more cAMP after 1 h relative to 0 h was 73/150 (48.7%) and 110/138 (79.7%) respectively for the ++ genotype and 75/134 (56.0%) and 61/80 (76.2%) respectively for the FF genotype. In 1 – 1.5 mm-diameter follicles but not in the larger-diameter follicles, the hourly cAMP outputs in hypophysectomized FF and ++ ewes were each significantly higher than in the respective genotype controls ($P < 0.01$).

Discussion

In previous studies on long-term (i.e. 70 days) hypophysectomized ewes, Cahill (1979) reported an absence of atresia in preantral follicle although in antral follicles he noted an increase in atresia and a decrease in mitotic activity relative to antral follicles from control ewes. In addition, some non-atretic antral follicles < 3 mm in diameter in the hypophysectomized ewes were seen. The present studies show that long-term (i.e. ~ 70 days) hypophysectomy leads to a modest reduction in the non-luteal component of ovarian weight ($P < 0.05$; see Table 1) but no reduction in the total number of antral follicles. These studies confirm those of Cahill (1979) in demonstrating that the most dramatic effect of hypophysectomy is the absence of large antral follicles (i.e. > 3 mm in diameter).

In the present study LH and FSH were not detectable in plasma from 7 days after hypophysectomy until the end of the study. The question therefore arises as to whether some of the small antral follicles (≤ 3 mm in diameter) developed after hypophysectomy or were present at the time of hypophysectomy and simply persisted after gonadotrophin withdrawal. The evidence from immature hypophysectomized rats indicates that antral follicles cannot develop without exogenous gonadotrophins (see Goldenberg *et al.*, 1972; Richards, 1978). However, it is uncertain as to whether the findings for the immature rat are applicable to mature sheep and the results of the present studies do not resolve this issue.

In FF ewes preovulatory-sized follicles do not normally exceed 4.5 mm in diameter whereas in ++ ewes such follicles rarely exceed 7 mm (McNatty *et al.*, 1986; Table 2). After hypophysectomy, follicles in FF and ++ ewes did not exceed 2 and 3 mm respectively (Table 2). These findings indicate that follicles > 2 mm and > 3 mm for FF and ++ ewes respectively have an absolute requirement for pituitary hormones. Moreover, it appears that the genotype differences in the largest follicles in control ewes were not altered in any disproportionate way 70 days after hypophysectomy; in ewes of both genotypes after hypophysectomy the largest follicles were $\sim 40\%$ of their predicted final size at ovulation.

Some small antral follicles (1–3 mm in diameter) in both Booroola genotypes were responsive to exogenous LH + FSH with respect to cAMP synthesis *in vitro* even after hypophysectomy (Table

Table 3. Gonadotrophin-induced follicular cAMP amounts with respect to follicular diameter, incubation time and Booroola genotype

Genotype (no. of ewes)	Treatment	Incubation time (h)	Follicular diameter (mm)						
			>0.1 — 0.5	>0.5 — 1.0	>1.0 — 1.5	>1.5 — 2.0	>2.0 — 2.5	>2.5 — 3.0	>3.0
++ (8)	Control	1	0.6 (0.4, 0.9)	1.3 (1.0, 1.6)	2.6 (2.0, 3.2)	7.3* (4.8, 11.0)	11.6 (6.7, 19.7)	16.0* (9.9, 25.5)	24.0 (17.6, 32.4)
		0	0.7 (0.4, 1.1)	1.1 (0.8, 1.5)	2.4 (1.7, 3.2)	3.7 (2.4, 5.4)	7.1 (4.6, 10.8)	8.1 (5.6, 11.5)	21.2 (14.4, 31.2)
FF (6)	Control	1	0.9 (0.5, 1.5)	1.7 (0.8, 2.9)	3.1 (1.7, 5.4)	8.1* (3.9, 16.0)	5.4 (2.3, 11.8)	10.8 (4.8, 23.0)	17.6 (12.8, 28.2)
		0	0.6 (0.2, 1.1)	1.0 (0.5, 1.5)	2.0 (1.3, 2.9)	3.5 (2.4, 4.9)	5.8 (4.8, 8.6)	9.6† (5.9, 21.6)	11.5 (5.9, 21.6)
++ (8)	Hypophysectomized	1	1.0 (0.5, 1.8)	1.5 (1.0, 2.2)	5.5** (3.4, 8.7)	12.6** (8.2, 19.3)	11.8** (8.0, 17.2)	12.6 (7.9, 19.9)	—
		0	0.5 (0.3, 0.8)	1.0 (0.7, 1.3)	2.0 (1.4, 2.7)	3.9 (2.9, 5.0)	6.0 (4.9, 7.2)	7.9 (3.5, 16.8)	—
FF (6)	Hypophysectomized	1	0.8 (0.4, 1.3)	2.3 (1.0, 4.4)	10.3** (5.9, 20.9)	8.5** (4.1, 13.2)	—	—	—
		0	0.6 (0.2, 1.1)	1.2 (0.5, 2.3)	2.4 (1.9, 3.0)	3.7 (2.4, 5.5)	—	—	—

Values are geometric means (and 95% confidence limits) in pmol/follicle.

* $P < 0.05$; ** $P < 0.01$ with respect to values at 0 h (ANOVA).

†Follicles at this time were only studied from 1 ewe.

3). The proportions of follicles > 1.0 mm diameter which responded to LH + FSH (i.e. 50–80%) were similar to those of non-atretic follicles found in control ++ and FF ewes (McNatty *et al.*, 1986). Fry *et al.* (1988) showed that progesterone-primed hypophysectomized Booroola ++ and F+ ewes could be induced to ovulate with PMSG and hCG 3–4 days later. In sheep, cauterization of surface-visible follicles ≥ 2 mm in diameter did not delay ovulation compared to sham-operated controls after prostaglandin treatment during the oestrous cycle (Tsonis *et al.*, 1984); in both groups ovulation occurred 3–4 days later. Presumably, even 70 days after hypophysectomy, a proportion of gonadotrophin-responsive small antral follicles is those which can be induced to ovulate some 72–96 h later.

The present study reports no gene-specific differences in cAMP responses to gonadotrophin stimulation in contrast to an earlier report using a similar methodology (McNatty *et al.*, 1985). This discrepancy may be attributable to one or more important differences between the two studies. In the earlier study all ewes were examined at Day 10 of the oestrous cycle and at defined times after PGF-2 α treatment on Day 10. In the present study some ewes were in anoestrus whilst others were at unknown stages of the oestrous cycle. Secondly, in the present but not earlier study, a phosphodiesterase inhibitor MIX was used during ovarian collection, dissection and during gonadotrophin stimulation. Thirdly, in the present but not former study, considerable variation between sheep with each genotype meant that analysis of genotype differences was based on numbers of sheep rather than number of follicles; this variability between sheep is probably due to sheep being sampled at different reproductive phases. Despite the absence of gene differences in cAMP in follicles at recovery, follicles > 1 mm in diameter from hypophysectomized ewes were responsive to LH + FSH and were capable of synthesizing larger amounts of cAMP earlier in development than those in the control ewes. The reasons for these differences are unknown although the difference may be due to the fact that follicles in control ewes were exposed to endogenous gonadotrophins before incubation.

Although these results demonstrated the retention of a gene-specific difference with respect to the size of the largest antral follicles after long-term hypophysectomy, they do not offer further insights as to whether the principal sites of gene expression are in the ovary or elsewhere (McNatty & Henderson, 1987; Fry *et al.*, 1988; McNatty *et al.*, 1989). There are several reasons for this. Firstly, the time taken in intact sheep for a primordial follicle to develop into a preantral structure exceeds 70 days and, after hypophysectomy, the evidence from studies on mitotic rates suggests that follicular growth is slowed down even further (Cahill, 1979). Secondly, FF ewes more frequently have higher plasma FSH concentrations than do ++ ewes and the long-term consequences of this on follicle development after hypophysectomy are unknown.

In conclusion, this study shows that gonadotrophin-sensitive small antral follicles can be found in long-term hypophysectomized ewes and that ovarian follicles in FF and ++ ewes have an absolute requirement for pituitary hormone upon reaching diameters of 2 and 3 mm respectively.

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