Ultrasonography and hormone profiles of adrenocorticotropic hormone (ACTH)-induced persistent ovarian follicles (cysts) in cattle

H. Dobson, A. Y. Ribadu, K. M. Noble, J. E. Tebble and W. R. Ward

Department of Veterinary Clinical Science and Animal Husbandry, University of Liverpool, Leahurst, Neston CH64 7TE, UK

The objective of this study was to develop a model for the study of abnormal ovarian follicles in cattle by treating heifers with adrenocorticotropic hormone (ACTH) (100 iu at 12 h intervals for 7 days, beginning on day 15 of the oestrous cycle). Cortisol concentrations increased ($P < 0.05$) within 24 h after beginning ACTH treatment and cortisol and progesterone concentrations remained elevated after cessation of ACTH treatment for 8 and 4 days, respectively. The pulses and surges of LH decreased during ACTH treatment, but FSH profiles were similar to those in controls and persistent or prolonged follicles were eventually observed in all heifers. In five heifers, prolonged dominant follicles ovulated after 10 days, whereas in six heifers, persistent follicular structures were present for 20 days, but ceased to secrete oestradiol after approximately 12 days. In the heifers with persistent follicular structures, new follicles emerged when the persistent follicle became non-oestrogenic. During the last 2 days of normal follicular growth, the concentration of oestradiol was greater than it was during prolonged or persistent follicle development ($P < 0.05$). There were no differences in the growth rates or maximum diameters of abnormal follicles that had different outcomes, but oestradiol concentrations were greater in prolonged follicles that ovulated compared with those follicles that persisted ($P = 0.06$). In conclusion, stimulation with ACTH resulted in a marked deviance from normal follicular activity. The aberrations were probably caused by the interruption of pulsatile secretion of LH (but not FSH) leading to decreased but prolonged oestradiol secretion.

Introduction

There are several limitations to studies of clinical cases of spontaneous abnormal persistent ovarian follicles (cysts) in cattle. At diagnosis, persistent follicles are already established, thus it is impossible to determine how long follicular structures have been present. In addition, the circumstances affecting ovarian function at the time of diagnosis may be different from the prevailing conditions at the time of formation. It is also difficult to follow clinical cases (once diagnosed) for a long period of time without treatment, as clients request treatment as early as possible. Therefore, it may be helpful to develop a model to monitor the development of persistent ovarian follicles closely and to rationalize clinical observations. It is not known whether the morphological and endocrine differences observed in clinically diagnosed persistent ovarian follicles represent stages of a single or multiple pathophysiological mechanism (Refsal et al., 1987).

Savio et al. (1990) observed the spontaneous formation of persistent follicles in cows. Two distinct phases of persistent follicle development can be determined from their data. An oestrogenic phase that lasts up to 20 days, during which there was high oestradiol secretion; and a phase of similar duration during which the follicular structure remained detectable by ultrasonography but oestradiol secretion was decreased.

Stress has been implicated in the aetiology of spontaneously occurring persistent follicles in cattle and sheep. The preovulatory LH surge is sensitive to the inhibitory effects of exogenous adrenocorticotropic hormone (ACTH) and such inhibition results in the development of persistent follicles (Doney et al., 1976; Liptrap and McNally, 1976; Stoebel and Moberg, 1982; Dobson et al., 1988). However, the reproductive hormonal control of the consequent development of either the persistent follicle or subsequent follicles was not examined in these studies.

The objective of the present study was to: (i) treat heifers with ACTH to induce abnormal ovarian follicular development; and (ii) monitor subsequent activity of the pituitary gland and ovary by ovarian ultrasonography and plasma endocrine profiles. The results will be related to clinical findings to explain why only 50% of cows that showed spontaneously occurring persistent follicles had an
LH surge in response to exogenous oestradiol (Dobson and Nanda, 1992).

Materials and Methods

All procedures were carried out under UK Home Office regulations for experiments on living animals.

ACTH treatment, controls and blood sampling

Beginning on day 15 of a synchronized oestrous cycle, thirteen heifers received s.c. injections of 100 iu ACTH (Tetracosactrin acetate (Corticotrophin); Synacthen depot, CIBA, Horsham) every 12 h for 7 days. Seven control animals received saline (2 ml) instead of ACTH. In all animals, ultrasonography and blood sampling were carried out each day from day 14 either until ovulation and corpus luteum development, or during the formation and regression of abnormal follicles.

The frequency of blood sampling was increased to every 4 h between days 19 and 22 in seven control animals and in seven ACTH-treated heifers to detect the occurrence of an LH surge. Blood samples were taken from six control and from six ACTH-treated animals every 10 min for 6 h on days 19 and 20 for control heifers, and on days 19, 20, 24, 27, 31 and 35 for ACTH-treated animals to examine LH pulse profiles. During daily monitoring periods, blood samples were taken by tail venepuncture. Indwelling jugular catheters were inserted under local anaesthesia the day before more frequent sampling periods (that is, every 4 h or every 10 min).

Blood samples were centrifuged at 1000 g for 30 min and plasma was stored at –20°C until hormonal analysis.

Animals and ultrasonography

Nulliparous Holstein/Friesian or Hereford-cross heifers, 12–18 months of age and weighing 313–411 kg, were used. An initial reference oestrus was synchronized with two injections of cloprostenol (2 ml Estrumate, Schering-Plough, Uxbridge) 11 days apart. Heifers were observed for behavioural signs of oestrus (standing to be mounted) for at least 20 min each day. The frequency of observation was increased to every 6 h during the treatment period for both the controls and ACTH-treated animals.

Ovarian ultrasonography was carried out each day using an ultrasound scanner equipped with a 7.5 MHz rectal transducer (SSD 210 DXII, Aloka, BCF Technology, Livingstone). After freezing the image on the screen, all corpora lutea and follicles > 5 mm in internal diameter were measured using in-built callipers.

A dominant follicle was defined as the largest follicle on the ovary with > 10 mm internal diameter in the absence of other growing follicles. A dominant follicle and cohorts were defined as a follicular wave. The emergence of follicles was defined as the first day a follicle reached > 5 mm in diameter. If a dominant follicle was present for more than 3 days and subsequently could not be detected, ovulation was assumed. A prolonged follicle was defined as a dominant follicle present for more than 5 days but that eventually ovulated. Persistent follicles were defined as follicular structures of > 10 mm in diameter that were present for more than 5 days, but that did not ovulate.

Hormone measurement

Previously characterized radioimmunoassays were used to measure plasma concentrations of cortisol and progesterone (Dobson et al., 1999a), oestradiol (Beard et al., 1994), FSH (Dobson, 1978) and LH (Alam and Dobson, 1986). The FSH assay was modified to use antiserum AFP C5288113 (1:16 000) and 10 000 c.p.m. labelled FSH (AFP 5679C RP-1). The standard used for the FSH assay was AFP 5679C RP-1, and NIDDK bLH B8 was used for the LH assay. For samples in the range of those in the current study, intra- and interassay coefficients of variation for all assays were < 13%. Minimum detectable values for each assay were 0.4 ng cortisol ml⁻¹, 0.025 ng progesterone ml⁻¹, 0.9 pg oestradiol ml⁻¹, 0.1 ng FSH ml⁻¹ and 0.15 ng LH ml⁻¹. For each hormone, all samples from one animal were measured in the same assay.

An increase in LH concentration above 10 ng ml⁻¹ in two consecutive samples taken every 4 h was considered as an LH surge. The start and end of the LH surge were defined by LH values > 10 ng ml⁻¹.

Statistical analysis

Rates of growth of dominant ovulatory follicles and persistent follicles were calculated by regression analysis. The linear regression lines were analysed for parallelism using Student’s t test. The size of the follicles and hormone concentrations were log₁₀ transformed and analysed using repeated measures ANOVA, and data were partitioned on the basis of time and follicle status (control, prolonged or persistent). Similarly, durations of follicle presence, oestradiol production, and hormone concentrations on specific days were compared using Student’s t test after log₁₀ transformation. P < 0.05 was regarded as significant.

LH pulses were identified using the Munro alogarithm (Taylor, 1987) as described by Dobson et al. (1999a). The windows of analysis, which defined the number of samples used to calculate mean values for comparison, were set for the whole of each 6 h sampling period. Values were transformed before statistical analysis (log₁₀ for hormone concentration and pulse amplitude, and square root for pulse frequency). Mean values were compared between control and ACTH-treated animals on days 19 and 20 using Student’s one-tailed unpaired t test.

Results

Oestrous behaviour was observed at the time of expected oestrus in all control heifers, but was absent in ACTH-treated
heifers. None of the heifers that developed abnormal follicles subsequent to ACTH treatment were mounted more than five times within an oestrous period; oestrous periods did not exceed 24 h and there were no interoestrous periods of more than 18 days.

**Ultrasonography**

In terms of ovarian responses, the thirteen ACTH-treated animals formed three groups. In five heifers (Fig. 1), follicles emerged between days 15 and 19 but did not ovulate until 9.8 ± 1.6 days (range from 7 to 11 days) after dominance was achieved, hereafter referred to as ‘prolonged' follicles. In six heifers (Fig. 2), the dominant follicle that emerged during ACTH treatment did not ovulate. The follicular structure was maintained for an extended period and regression occurred after 19.5 ± 2.1 days (range from 17 to 23 days; P < 0.05), hereafter referred to as a ‘persistent' follicle. In the presence of persistent follicles as detected by ultrasonography, a normal dominant follicle emerged 2 days after cessation of oestradiol secretion and had a normal lifespan of 3–4 days. In the other two animals, the initial persistent follicle regressed eventually and was not detected by ultrasonography after 14 and 20 days. Within the next 2 days, other follicles emerged for durations of 6 and 5 days, respectively.

The growth rates and maximum internal diameters of control, prolonged and persistent follicles were similar up until day 23, when the control follicles ovulated. Between day 24 and day 29, both types of abnormal follicle were larger than control follicles (Fig 3; P < 0.05).

**Hormone profiles**

Cortisol concentrations during the control oestrous cycles ranged from 4.2 to 11.2 ng ml⁻¹. In ACTH-treated animals, there was an increase in cortisol concentration (P < 0.05) within 24 h after starting treatment. Cortisol concentrations remained elevated (> 20 ng ml⁻¹) after ACTH treatment, but were < 5 ng ml⁻¹ at 8 days after the last injection (Fig. 4).

The concentration of progesterone during control oestrous cycles ranged from 6.5 ± 0.6 ng ml⁻¹ in the mid-luteal phase to 0.4 ± 0.1 ng ml⁻¹ in the late follicular phase (P < 0.05). During ACTH treatment, progesterone concentrations decreased at a slower rate than in the controls, and values remained approximately 2 ng ml⁻¹ greater than in the controls (P < 0.05) for an additional 4 days (Fig. 4). There was no difference in progesterone profiles for animals that produced either type of abnormal follicle. Progesterone concentrations decreased (< 0.9 ng ml⁻¹) in the presence of abnormal dominant follicles until regression and subsequent corpus luteum formation (Figs 1 and 2).

FSH concentrations increased before the emergence of each wave of growing follicles, even during ACTH treatment (Fig. 3). Concentrations of FSH were greater on day 19 and day 20 in those animals that eventually produced persistent follicles compared with animals that produced prolonged or normal follicles, but the difference was not significant (P = 0.06). When all data up to day 34 were included, animals that produced persistent follicles showed similar FSH concentrations to those that produced prolonged follicles. For all animals, there were no increases in FSH concentration when normal or abnormal dominant follicles were secreting oestradiol.

Basal oestradiol concentrations increased from 4.9 ± 1.7 to 13.2 ± 1.4 pg ml⁻¹ (P < 0.05) during the late follicular phase of the control oestrous cycles. Between day 20 and day 27, basal oestradiol concentrations in heifers that had prolonged follicles tended to be greater (P = 0.06) than in those heifers that had persistent follicles (Fig. 3). However, oestradiol concentrations were lower in ACTH-treated than in control follicular phases (P < 0.05). Oestradiol concentrations were > 5 pg ml⁻¹ for 3.5 ± 0.9 days in controls, which was a shorter period than the 8.4 ± 1.9 and 11.8 ± 3.2 days in prolonged and persistent follicles, respectively (P < 0.05).

A preovulatory LH surge was observed in control heifers between day 19 and day 22 of the oestrous cycle, whereas in seven ACTH-treated animals sampled every 4 h, LH surges were not detected. Preovulatory LH surges were also observed in two control animals that were sampled every 10 min on day 20, hence data for these two animals on this day were not included in the analysis. Mean LH concentrations and pulsatility were reduced during ACTH treatment.
Discussion

Treatment with ACTH resulted in a decrease in LH secretion but the dominant follicle that emerged during treatment was maintained. Five heifers had prolonged follicles that maintained lower than normal oestradiol concentrations, but these follicles ovulated after approximately 10 days. By contrast, in six animals, the persistent follicle remained detectable by ultrasonography for 19.5 days, and lower amounts of oestradiol were produced and secretion ceased after approximately 12 days. There was no difference in the growth rates or maximum diameters of these abnormal follicles that had different outcomes, although they were clearly divergent from normal follicles as they were detectable by ultrasonography and secreted oestradiol for longer. Emergence of other follicles was inhibited during oestradiol secretion by prolonged or persistent follicles.

ACTH-induced reduction in pulsatile LH secretion and the obliteration of the LH surge both concur with previous observations in sheep that ACTH had suppressive effects at both the hypothalamus and pituitary gland (Phogat et al., 1997, 1999). However, the present study demonstrated that secretion of FSH and its ability to stimulate follicular emergence was not influenced by either ACTH treatment or the consequent increased cortisol and progesterone concentrations. This finding is in agreement with the study of Hamilton et al. (1995) in which differences in FSH concentrations were not detected in forming persistent follicles that had been treated with high doses of progesterone and oestradiol. For at least 8 days after ACTH treatment, FSH concentrations remained basal in the presence of abnormal follicles, and new follicles did not emerge until approximately day 30. This was the time when ACTH concentrations presumably returned to normal, as reflected by decreased cortisol concentrations. However, oestradiol concentrations were also increased until approximately day

Fig. 2. Peripheral plasma concentrations of progesterone ( ○) and oestradiol (○) (a), FSH (○) (b) and follicle internal diameters (mm) (c) in Heifer 8 that formed a persistent follicle after treatment with 100 iu ACTH every 12 h for 7 days from day 15 of the oestrous cycle.

Fig. 3. Mean (± SEM) peripheral plasma concentrations of oestradiol (a), FSH (b) and follicle internal diameters (c) for (○) six control heifers, (△) five heifers that formed a prolonged follicle and (□) six heifers that formed a persistent follicle after treatment with 100 iu ACTH every 12 h for 7 days from day 15 of the oestrous cycle. After day 34, each animal had different follicular patterns. Asterisks indicate control oestradiol values significantly different from prolonged and persistent follicle oestradiol values (P < 0.05).
for at least 10 days, which is 7–8 days longer than normal control heifers (Baird et al., 1991). However, it remains unclear why some follicles ovulated (presumably in response to an LH surge), whereas other follicles continued to secrete oestradiol at lower concentrations for another 3 days with the follicular structure remaining detectable by ultrasonography for up to 19.5 days. The observation that persistent follicles secreted oestradiol for the first half of their lifespan but reduced oestradiol secretion for the second half, would explain why half the clinical cases that had persistent follicles challenged with exogenous oestradiol did not have an LH surge (Dobson and Nanda, 1992). Clinical cases not responding with an LH surge after oestradiol administration were probably within the oestrogenic phase, whereas those that did have an LH surge were in the second non-oestrogenic half of the lifespan.

The periods during which ACTH-induced persistent follicles secreted oestradiol and were detectable by ultrasonography were both approximately half the lifespan reported by Savio et al. (1990) for spontaneously occurring structures. This difference may be accounted for by the continued extenuating circumstances in the spontaneous situation compared with the defined 7 day treatment with ACTH. In sheep, persistent follicles induced by using a GnRH antagonist and exogenous LH were also oestrogenic for approximately half their lifespan (Dobson et al., 1997). These latter follicles required pulses of exogenous LH to produce androstenedione and subsequently oestradiol. Nevertheless, even with continued exogenous LH pulses, androstenedione secretion eventually ceased. Hence, it remains to be determined how and why persistent follicles (caused by whatever means) cease androstenedione and, thus, oestradiol synthesis.

During ACTH treatment and for an additional 4 days, the concentration of progesterone was > 0.5 ng ml⁻¹. The dose of ACTH that was administered was high and the effects of lower doses are worthy of examination since stressful stimuli in ruminants do increase progesterone secretion above baseline (Dobson et al., 1999a; Phogat et al., 1999). From the present study, it was not possible to determine the source of progesterone. There is evidence that progesterone may have come from the adrenal glands or via interference with luteolysis (Watson and Munro, 1984; Cooke and Benhaj, 1989). Whatever the source, progesterone alters LH pulsatility and inhibits surge release (Hansel and Convey, 1983; Kasa-Vubu et al., 1992). These effects will lead to prolonged oestradiol secretion that, in turn, inhibits secretion of an LH surge (Ozturk et al., 1998). However, preliminary evidence from the use of RU 486, the corticoid and progesterone receptor antagonist, demonstrated that the delay in the LH surge during insulin-induced stress was not due to increased cortisol or progesterone concentrations in sheep (H. Dobson, unpublished). This finding indicates that, in the present study, ACTH or prolonged exposure to oestradiol were responsible for the inhibitory influences on the LH surge, not progesterone or cortisol (Phogat et al., 1997, 1999; Ozturk et al., 1998).

The formation of a new persistent follicle as the first follicle regressed in two heifers confirmed previous reports.

30 and it is probable that oestradiol, along with inhibin (but not ACTH, cortisol or progesterone) exerts a strong negative feedback on FSH secretion (Baird et al., 1991).

Both prolonged and persistent follicles secreted oestradiol for at least 10 days, which is 7–8 days longer than normal follicles. Exposure to increased oestradiol concentrations for more than 4 days inhibits the release of the LH surge (Brawer et al., 1993; Ozturk et al., 1998). However, it remains unclear why some follicles ovulated (presumably in response to an LH surge), whereas other follicles continued to secrete oestradiol at lower concentrations for another 3 days with the follicular structure remaining detectable by ultrasonography for up to 19.5 days. The observation that persistent follicles secreted oestradiol for the first half of their lifespan but reduced oestradiol secretion for the second half, would explain why half the clinical cases that had persistent follicles challenged with exogenous oestradiol did not have an LH surge (Dobson and Nanda, 1992). Clinical cases not responding with an LH surge after oestradiol administration were probably within the oestrogenic phase, whereas those that did have an LH surge were in the second non-oestrogenic half of the lifespan.

The periods during which ACTH-induced persistent follicles secreted oestradiol and were detectable by ultrasonography were both approximately half the lifespan reported by Savio et al. (1990) for spontaneously occurring structures. This difference may be accounted for by the continued extenuating circumstances in the spontaneous situation compared with the defined 7 day treatment with ACTH. In sheep, persistent follicles induced by using a GnRH antagonist and exogenous LH were also oestrogenic for approximately half their lifespan (Dobson et al., 1997). These latter follicles required pulses of exogenous LH to produce androstenedione and subsequently oestradiol. Nevertheless, even with continued exogenous LH pulses, androstenedione secretion eventually ceased. Hence, it remains to be determined how and why persistent follicles (caused by whatever means) cease androstenedione and, thus, oestradiol synthesis.

During ACTH treatment and for an additional 4 days, the concentration of progesterone was > 0.5 ng ml⁻¹. The dose of ACTH that was administered was high and the effects of lower doses are worthy of examination since stressful stimuli in ruminants do increase progesterone secretion above baseline (Dobson et al., 1999a; Phogat et al., 1999). From the present study, it was not possible to determine the source of progesterone. There is evidence that progesterone may have come from the adrenal glands or via interference with luteolysis (Watson and Munro, 1984; Cooke and Benhaj, 1989). Whatever the source, progesterone alters LH pulsatility and inhibits surge release (Hansel and Convey, 1983; Kasa-Vubu et al., 1992). These effects will lead to prolonged oestradiol secretion that, in turn, inhibits secretion of an LH surge (Ozturk et al., 1998). However, preliminary evidence from the use of RU 486, the corticoid and progesterone receptor antagonist, demonstrated that the delay in the LH surge during insulin-induced stress was not due to increased cortisol or progesterone concentrations in sheep (H. Dobson, unpublished). This finding indicates that, in the present study, ACTH or prolonged exposure to oestradiol were responsible for the inhibitory influences on the LH surge, not progesterone or cortisol (Phogat et al., 1997, 1999; Ozturk et al., 1998).

The formation of a new persistent follicle as the first follicle regressed in two heifers confirmed previous reports.

**Fig. 4.** Peripheral plasma concentrations of progesterone in six control heifers (○), and progesterone (○) and cortisol (○) in six heifers during and after treatment with 100 iu ACTH every 12 h for 7 days from day 15 of the oestrous cycle.

**Fig. 5.** Peripheral plasma concentrations of LH in three control heifers on day 19 of the oestrous cycle (a) and in three heifers on day 19 during treatment with 100 iu ACTH every 12 h for 7 days from day 15 of the oestrous cycle (b).
that abnormal ovarian follicles are dynamic structures (Cook et al., 1990), a frequent clinical observation. In both of these animals that had repeated persistent follicles, there was a dominant follicle of 10 mm in diameter present at the beginning of ACTH treatment and the reduction of LH pulse frequency may have been so great that it totally inhibited oestradiol secretion (Dobson et al., 1999b). However, FSH secretion was not inhibited and thus new prolonged follicles were formed that did not ovulate for a further 9–11 days.

In conclusion, treatment with ACTH to stimulate the stress endocrine axis resulted in marked changes in ovarian follicular activity, which were probably caused by the interruption of normal pulsatile and surge LH secretion leading to reduced but prolonged oestradiol secretion. Although there may be only one pathophysiological mechanism involved, it leads to both prolonged and persistent follicles. The changes revealed by daily ultrasonography and endocrinology in this experimental model help our understanding of observations in clinical cases that cannot be examined as frequently.

The authors are grateful to the Association of Commonwealth Universities for a Fellowship to A. Y. Ribadu; to H. Purcell and D. Harvey for expert technical assistance; to N. Jones and his animal staff for diligent care of the animals; and to NIDDK, Bethesda and NHPP, California for gonadotrophin assay reagents.

References

Alam MGS and Dobson H (1986) Effect of various veterinary procedures on plasma concentrations of cortisol, luteinizing hormone and proglandin F metabolite in the cow Veterinary Record 118:7–10
Doney JM, Gunn RG and Smith WF (1976) Effects of pre-mating environmental stress, ACTH, corticosterone acetate or metyrapone on oestrus and ovulation in sheep Journal of Agricultural Science, Cambridge 87:27–132