Histological and steroidogenic changes in dominant ovarian follicles during oestradiol-induced atresia in heifers

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Abstract

Histological and steroidogenic changes within dominant ovarian follicles (DFs) undergoing atresia following systemic administration of oestradiol benzoate (ODB) were characterized in beef heifers. At 5.6 ± 0.1 days after the onset of oestrus, heifers received 1 mg ODB i.m./500 kg body weight (ODB; $n = 15$) or served as controls ($n = 15$). Timing of treatment initiation was designated as hour (h) 0 on day (d) 0, and coincided with the presence of the DF of the first follicular wave (DF1). Within treatments, the DF1 was collected following ovariectomy in four animals at h 12, h 36 or after ultrasonic detection of a new wave (NW) of ovarian follicular development. In heifers of the NW groups ($n = 7$ per treatment), blood samples were collected at intervals of 20 min for 12 h beginning at h -12 , 0, 24 and 48 to characterize circulating LH patterns. Administration of ODB suppressed ($P < 0.01$) mean concentrations of LH at h 24 and h 48 by preventing ($P < 0.05$) the increase in LH pulse amplitude observed in controls, but had no effect on FSH. Follicular fluid (FF) concentrations of androgens and oestradiol were reduced at h 36 in the ODB-treated group. The diameter of the DF1 and the number of granulosa cell layers were also reduced in ODB-treated as compared with control heifers. Treatment differences were not observed in the proportion of apoptotic granulosa cells as assessed using the TUNEL assay method, and timing of a new wave of follicular development ($d 4.6 \pm 0.2$) was similar ($P > 0.1$) among treatments. A prominent characteristic of oestradiol-induced atresia of the DF1 of the oestrous cycle in heifers was a loss in oestrogenic function associated with reduced LH support. However, the timing of new follicular development may be influenced by a factor(s) other than the status of the DF undergoing oestradiol-induced atresia.

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Introduction

Strategic regulation of ovarian follicular development is an important component in the application of cattle reproductive technologies such as oestrous synchronization and embryo transfer (Bo *et al.* 2002). One approach to control follicular development in a group of cattle is to synchronously induce atresia of the dominant follicles (DFs). Removal of the functional DF causes an increase in follicle-stimulating hormone (FSH) that drives recruitment of a new cohort of follicles (Adams *et al.* 1992, Bergfelt *et al.* 1994). Oestradiol-17 β (Bo *et al.* 1995) or conjugated forms of oestradiol (Thundathil *et al.* 1998, Day & Burke 2002) have been used effectively for this purpose. The current limitations to this approach include variability in responses associated with the stage of follicular development when oestradiol is administered, and also the lack of precision in the timing of new follicular wave development (Diskin *et al.* 2002, Burke *et al.* 2003).

Induction of atresia in the DF is a prerequisite when using oestradiol to synchronize a new wave of follicular development. The developmental events occurring in DFs

throughout their growth cycle have been reviewed in Fortune (1994) and Bao and Garverick (1998). In the early stages of atresia of DFs, a reduction in aromatase activity and oestradiol synthesis is observed (Price *et al.* 1995). Loss of oestrogenicity may (Badinga *et al.* 1992, Lucy *et al.* 1992) or may not (McNatty *et al.* 1984, Xu *et al.* 1995) be associated with a reduction in androgen precursors. In atretic DFs, the granulosa cell compartment is eroded, and there is a high incidence of pyknotic nuclei. Follicular fluid (FF) oestrogen content is low, while FF concentrations of progesterone are increased in atretic DFs (Price *et al.* 1995).

Follicular atresia in mammalian ovaries is facilitated by a 'programmed-cell death' process called apoptosis (Kaipia & Hsueh 1997). During apoptosis, the nuclear DNA is fragmented by $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases (Wyllie 1980). Cells containing fragmented DNA can be detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) procedure (Gavrieli *et al.* 1992). The ability of exogenous oestradiol to induce a new wave of follicular development

in a synchronous fashion is documented in cattle (Bo *et al.* 2002, Burke *et al.* 2003). However, the underlying basis for the existing DF to lose dominance following administration of oestradiol in cattle has not previously been characterized.

The objectives of the present study were to characterize steroidogenic and cellular changes within the DF during loss of dominance and the associated changes in circulating gonadotrophins in response to administration of oestradiol benzoate (ODB). The model of oestradiol-induced atresia permits an evaluation of the temporal changes within the DF as dominance is relinquished to allow new follicular growth. An increased understanding of this process may lead to improved methods in manipulating ovarian follicular development during oestrous synchronization treatment.

Materials and Methods

Animal procedures used in these experiments were approved by The Ohio State University Agricultural Animal Care and Use Committee.

Oestrus was synchronized in 30 reproductively mature Simmental $\times$ Angus heifers weighing 462 ± 38 kg (mean $\pm$ S.D.) following one or two i.m. injections of prostaglandin $F_{2\alpha}$ (PGF; Lutalyse, Pfizer, Kalamazoo, MI, USA). Behavioural oestrus was monitored using an electronic surveillance system (HeatWatch, DDx, Denver, CO, USA). Animals were allocated at 5.6 ± 0.1 days after the onset of oestrus to receive 1 mg ODB/500 kg body weight (BW) by i.m. injection (ODB; CIDIROL, InterAg, Hamilton, New Zealand; $n = 15$) or to serve as untreated controls (C; $n = 15$). Treatments were given at this stage of the oestrous cycle as all animals were expected to have a clearly identifiable DF of the first follicular wave (DF1) after oestrus, and a functional corpus luteum. The timing of the ODB injection was designated as hour (h) 0 of day (d) 0. Diameter and location of corpora lutea and ovarian follicles ≥ 3 mm in diameter were monitored daily by transrectal ultrasonography using a 7.5 MHz transducer probe (Aloka Co. Ltd, Tokyo, Japan) beginning within 24 h of oestrus being detected. Eight animals from each treatment were bilaterally ovariectomized at either 12 h (12H) or 36 h (36H; $n = 4$ per time point). The remaining seven heifers per treatment remained intact until emergence of a new wave of follicular development was detected (new wave; NW). The frequency of ultrasonography examinations increased to every 12 h from h 60 in heifers of the NW group until emergence of a new follicular wave was detected. Emergence of a new follicular wave was defined as the day of appearance of a cohort of emerging follicles on the ovary of which at least one was ≥ 5 mm in diameter. Four heifers per treatment in the NW group were ovariectomized within 24 h (14.7 ± 3.5 h) of detection of a new wave of follicular development. The endpoint for the remaining three NW animals in each treatment was the same, but these were not ovariectomized. The

experiment was conducted over two periods (period 1 and 2) 14 days apart. The number of heifer allocated to treatments was balanced within period 1 ($n = 14$) and period 2 ($n = 16$).

Blood sampling

Blood samples were collected daily from a jugular vein of all animals after detection of oestrus and then at 6 h intervals between h -12 and ovariectomy, or until the emergence of a new follicular wave was confirmed. Blood was collected into glass tubes containing an anticoagulant (EDTA) and centrifuged at $1500g$ for 12 min. Plasma was harvested and stored at -20°C until determination of FSH, progesterone and oestradiol. Serial blood collections from a jugular vein were performed in all heifers assigned to the NW treatment groups for determination of luteinising hormone (LH). A 4 ml blood sample was taken at intervals of 20 min for periods of 12 h beginning at h -12 , 0, 24 and 48. Serial sampling was facilitated by placement of a 14-gauge indwelling catheter (Angiocath, Becton-Dickinson Infusion Therapy Systems, Inc., Sandy, UT, USA) into a jugular vein. A 2100 mm length of plastic tubing (Tygon, Norton Performance Plastics Co., Akron, OH, USA) with an internal diameter of 1.2 mm and overall capacity of 3 ml was attached to the catheter using a modified 16-gauge needle inserted through an injection adaptor (Medex, Hilliard, OH, USA). Between the removal of each blood sample, the catheter line was flushed with sterile 3.5% sodium citrate–0.9% sodium chloride solution containing an antibiotic (2 mg ml^{-1} oxytetracycline). The initial 7 ml fluid withdrawn from the catheter were discarded while the following 4 ml were retained as sample. Blood was collected into plastic tubes without anticoagulant and allowed to stand at 4°C for about 42 h before centrifugation at $4750g$ for 30 min. Sera were harvested and stored at -20°C .

Histology of the DF

Following ovariectomy, the ovary bearing the DF1 was immediately placed into ice-cold sterile PBS for 3 min. A sample of FF (approx. $150\mu\text{l}$) was collected and stored at -20°C . Within 10 min of ovariectomy, the DF1 was dissected out of the ovary and fixed in freshly prepared 4% paraformaldehyde-PBS (pH 7.2) for about 24 h and embedded in Paraplast (Oxford Labware, Missouri, MO, USA) using routine procedures. The small volume of FF removed from the DF did not impede gross dissection of the DF from the ovary. Embedded DF1s were sliced into $8\text{-}\mu\text{m}$ sections and mounted in groups of three to five consecutive sections on slides coated with aminoalkylsilane (Silane-Prep Slides, Sigma Diagnostics, St. Louis, MO, USA). The proportion of apoptotic cells in the granulosa cell compartment was determined by the TUNEL procedure using the ApoAlert DNA Fragmentation kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) in accordance with the manufacturer's instructions. An anti-photo

bleaching and nuclear counter-stain agent (DAPI, Vectashield, Vector Laboratories, Inc., Burlingame, CA, USA) was added as a final step to visualize granulosa cell nuclei. Each slide included a positive control section (DNase I-treated), the TUNEL section and a negative control section (TUNEL reaction buffer lacking TdT). Five fields within each follicle were evaluated by fluorescent microscopy at $\times 400$ magnification. Fields were chosen where the granulosa cell compartment was best visualized and was representative of the whole layer, and locations were spaced as evenly as possible around the follicle. The number of FITC (510 nm emission wavelength)-labelled granulosa cells (apoptotic) was counted in each field and divided by the total number of nucleated granulosa cells (DAPI-stained; 430 nm emission wavelength). The average total number of cells counted per field in each DF of the six treatment groups are depicted in Table 1. An average value for percent apoptotic cells was calculated for each follicle. Most cells ($>90\%$) were FITC-labelled in positive controls and no labelling was observed in negative controls. The number of layers of granulosa cells was estimated visually in all fields by detection of DAPI-stained nuclei.

Hormone RIAs

Concentrations of FSH in plasma were determined in duplicate using a double-antibody RIA (Wolfe *et al.* 1992, Burke *et al.* 2003). The average intra-assay coefficient of variation (CV) was 2.4% and the average inter-assay CV (four assays) was 11.9% for standard pools that contained 1.6 and 2.2 ng ml⁻¹ FSH. The average sensitivity was 0.2 ng ml⁻¹. Concentrations of LH in sera were determined in duplicate using a double-antibody RIA (Dyer *et al.* 1990). The average intra-assay CV was 2.9% and the average inter-assay CV (five assays) was 7.7% for standard pools containing 1.6 and 4.4 ng ml⁻¹ LH. The average sensitivity was 0.13 ng ml⁻¹.

Concentrations of progesterone in plasma were determined in duplicate using a double-antibody RIA (Anderson *et al.* 1996). The intra-assay CV was 3.4% and the inter-assay CV (three assays) was 14.9% for standard pools containing 1.4 and 6.9 ng ml⁻¹ progesterone. The average sensitivity was 0.24 ng ml⁻¹. Concentrations of progesterone in FF were determined using a kit (Progesterone Coat-a-Count, DPC, Los Angeles, CA, USA) as previously described (Burke *et al.* 2003). Validation for use in FF was performed. Parallelism was confirmed by comparing the slopes from serially diluted FF (-0.72) with the standard curve (-0.76) after natural log-logit transformation. Average recovery of progesterone across concentrations ranging from 2.2 to 7 ng ml⁻¹ was 102%. Follicular fluid was diluted 1:10 or 1:100 in Progesterone Zero Calibrator (DPC) before addition to the reaction tube. The intra-assay CV was 2.4% and the inter-assay CV (five assays) was 4.0% for standard pools containing 1.5, 2.6 and 10.1 ng ml⁻¹ progesterone. The average sensitivity was 0.05 ng ml⁻¹.

Concentrations of oestradiol in plasma were determined in duplicate using a double-extraction single-antibody RIA (Anderson *et al.* 1996). The antibody used (Dr N R Mason, Lilly Research Laboratories, Indianapolis, IN, USA) has a 71% cross-reactivity with ODB (Kesler *et al.* 1977). The same assay without ether extraction was used to determine oestradiol content in FF where samples were diluted 10^2 , 10^3 , 10^4 and 10^5 in assay buffer before addition to the reaction tube. For plasma analyses, the average intra-assay CV was 2.7% and the inter-assay CV (11 assays) was 12.2% for standard pools containing 13.7 and 20.4 pg ml⁻¹ oestradiol. For follicular fluid oestradiol, the inter-assay CV among five samples of FF with known concentrations ranging between 36.6 and 316 ng ml⁻¹ was 15.4%. Average sensitivity of the assays was 1.0 pg ml⁻¹.

Concentrations of testosterone in FF were determined in duplicate using a kit (Total Testosterone Coat-a-Count,

Table 1 Concentrations of steroid hormones in follicular fluid and histological characteristics of the granulosa cell compartment in the first dominant follicle collected at h 12 (H12), h 36 (H36) or after the emergence of a new follicular wave (NW) in heifers treated with 1 mg oestradiol benzoate (ODB) i.m./500 kg BW on day 5.6 ± 0.1 of the oestrous cycle (designated as hour 0), as compared with contemporary untreated controls.

Variable ^a	Control			ODB treated		
	H12	H36	NW	H12	H36	NW
Observations (n)	1	3	3	4	4	4
Concentrations (ng ml ⁻¹) in follicular fluid						
Oestradiol	366	258 $\pm$ 27 ^b	28 $\pm$ 18 ^c	545 $\pm$ 182	49 $\pm$ 4 ^c	1.3 $\pm$ 1 ^d
Progesterone	38	35 $\pm$ 2 ^b	28 $\pm$ 5 ^b	30 $\pm$ 6	23 $\pm$ 4 ^b	118 $\pm$ 31 ^c
Testosterone	5.2	2.7 $\pm$ 1.3 ^b	1.3 $\pm$ 0.6 ^{b,c}	15.2 $\pm$ 4.2	0.2 $\pm$ 0.1 ^c	1.5 $\pm$ 0.4 ^b
Androstenedione	2.9	1.2 $\pm$ 0.6 ^{b,d}	2.0 $\pm$ 0.6 ^{d,e}	18.8 $\pm$ 8.7	0.2 $\pm$ 0.1 ^b	3.5 $\pm$ 1.2 ^e
Histological characteristics of granulosa cells						
No. of layers ^f	8.8	11.1 $\pm$ 1.9	4.7 $\pm$ 0.4	11.0 $\pm$ 1.7	8.3 $\pm$ 0.6	2.1 $\pm$ 0.6
Total cells per field	100	65 $\pm$ 11	47 $\pm$ 6	90 $\pm$ 36	68 $\pm$ 7.3	28 $\pm$ 12
% cells apoptotic ^g	4.6	3.8 $\pm$ 3.2	25.0 $\pm$ 6.2	0.3 $\pm$ 0.3	1.9 $\pm$ 1.8	25.7 $\pm$ 6.4

^aMean comparisons confined to treatment differences among the H36 and NW groups.

^{b,c,d,e}Means within rows with no common superscripts are different ($P < 0.05$).

^fTreatment ($P < 0.05$) and time ($P < 0.01$); see text.

^gTime ($P < 0.01$); see text

DPC). Parallelism was confirmed by comparing the slopes from serially diluted FF (-0.79) with the standard curve (-0.71) after natural log-logit transformation. Average recovery of testosterone across concentrations ranging from 0.4 to 6.8 ng ml^{-1} was 105% . Follicular fluid samples were diluted $1:4$ in Testosterone Zero Calibrator (DPC). The average intra-assay CV was 2.5% and the inter-assay CV (six assays) was 11.1% for standard pools containing 1.4 , 3.5 and 9.7 ng ml^{-1} of testosterone. The average sensitivity was 0.03 ng ml^{-1} . Concentrations of androstenedione in FF were determined in duplicate using a kit (Direct Androstenedione Coat-a-Count, DPC). The slope of serially diluted FF (-0.89) was parallel with the standard curve (-0.82), and average recovery of androstenedione across concentrations ranging from 1 to 3 ng ml^{-1} was 98% . Samples of FF were diluted $1:4$ in Androstenedione Zero Calibrator (DPC). The intra-assay CV (single assay) was 3.9% with a sensitivity of 0.1 ng ml^{-1} .

Statistical analyses

Three of four DF1s in the 12H C group were accidentally ruptured during ovariectomy. Thus, initial analyses of follicular data was confined to the H36 and NW groups in a 2×2 arrangement, and secondly to the 12H ODB, 36H ODB and NW ODB treatment groups to test if the 12H ODB treatment differed from either the 36H ODB or NW ODB treatments. The interval from oestradiol treatment, or the equivalent time in controls, to maximal FSH concentrations was assessed in individual animals and these data are reported as the time of the FSH peak within treatment. The number of LH pulses, LH pulse amplitude and mean LH concentrations were determined as described by Goodman and Karsch (1980).

Data were analyzed by ANOVA using the MIXED procedure in SAS (SAS Institute Inc. 1996). A repeated measures model was used on any data collected from the same animal over consecutive time points. The repeated measures model was $Y_{ijk} = \mu + T_i + c_{j:i} + H_k + (TH)_{ik} + e_{ijk}$ where Y_{ijk} is the observation of the j^{th} heifer in the i^{th} treatment at the k^{th} time, μ is the overall mean, T_i is the fixed effect of the i^{th} treatment, $c_{j:i}$ is the random effect of the j^{th} cow within the i^{th} treatment ($c_{j:i} \sim N[0, \sigma_c^2]$), H_k is the fixed effect of the k^{th} time, $(TH)_{ik}$ is the treatment by time interaction term, and e_{ijk} is the random residual error effect ($e_{ijk} \sim N[0, \Sigma]$), where Σ is the variance-covariance structure of the residual errors with a first order autoregressive structure for repeated measurements within cows. The effect of experimental replication (period) was tested for all dependent variables and replication was included in the model if it was a significant ($P < 0.05$) source of variation. For analyses of FSH data, the pretreatment concentration of FSH (value at h 0) within animals was included as a covariate in the model to compensate for variable initial concentrations among heifers. The least square mean values of FSH are reported in the results. Data not involving repeated measures were analyzed

using the MIXED procedure in SAS v 8.1 ($Y_{ij} = \mu + T_i + e_{ij}$, with notations as defined previously). Likelihood Ratio tests using the ratios of the -2 log restricted maximum likelihoods (REML) statistic of full and reduced models were performed to test the homogeneity of the variances across treatments (Littell *et al.* 1996). The full model allowing variances to differ among treatments was used if heterogeneity was indicated. Square root transformations were performed prior to ANOVA in many instances involving steroid concentrations in FF in which treatment variances were heterogeneous or proportional to mean values. Unless stated otherwise, data are expressed as the actual mean $\pm$ S.E.M.

Results

Follicular dynamics and circulating steroid hormone concentrations

Treatments were initiated 5.6 ± 0.1 days after detection of oestrus when the DF1 was $10.8 \pm 0.3 \text{ mm}$ in diameter (at h 12) and had emerged 4.1 ± 0.2 days previously. Concentrations of oestradiol in plasma (Fig. 1a) were elevated in the ODB NW group at h 12, and although declining, remained greater ($P < 0.01$) than the C NW group up to h 96. The increase in plasma oestradiol in the ODB NW group was associated with an immediate cessation in growth of the DF1 (Fig. 1b) and a smaller ($P < 0.01$) diameter during the plateau phase as compared with the DF1 in the C NW group. Time of emergence of a new wave of follicular development after treatment initiation for the ODB NW ($d 4.8 \pm 0.3$) and the C NW ($d 4.4 \pm 0.1$) groups was not different, but was more ($P < 0.05$) variable for the ODB NW treatment. Concentration of progesterone in plasma among all heifers was $1.2 \pm 0.1 \text{ ng ml}^{-1}$ on d 0. The subsequent rise in circulating progesterone over the next five days was evident in both treatments, but was reduced ($P < 0.01$) in the ODB NW compared with the C NW group (Fig. 1c).

Gonadotrophin responses

Concentrations of FSH (Fig. 1d) were not affected by treatment, but were greater ($P < 0.05$) between h 66 and h 102 than around the time (h -12 to 18) that treatment was initiated. The average maximum values were observed at h 90 and h 96, and the first decline ($P < 0.05$) from this peak was observed at h 108. The time of peak FSH concentration was also assessed within individual animals. The average time of the FSH peak within individuals of the ODB NW ($h 79.0 \pm 8.4$) and C NW ($h 89.1 \pm 3.6$) groups was similar. However, the interval from the FSH peak to emergence of a new wave of follicular development was increased ($P < 0.01$) in the ODB NW (35.0 ± 2.9 hours) compared with the C NW group (17.1 ± 2.4 h).

Characteristics of LH secretion were compared at h -12 , 0 , 24 and 48 (Fig. 2). The frequency of LH pulses

Figure 1 (a) Plasma concentrations of oestradiol, (b) diameter of the first dominant follicle (DF1), (c) plasma concentrations of progesterone and (d) least square mean (LSM) plasma concentrations of FSH in heifers that received 1 mg ODB (oestradiol benzoate) i.m./500 kg BW (ODBNW; $n = 7$) on day 5.6 ± 0.1 of the oestrous cycle (hour (h) 0) as compared with untreated controls (CNW; $n = 7$). NW refers to the groups of heifers that were monitored through to confirmation of emergence of a new wave of follicular development. Means plotted beyond h 114 are data from six heifers per treatment group as one heifer from each treatment had been ovariectomized before these times.

(Fig. 2a) declined ($P < 0.01$) over time but did not differ among treatments. The amplitude of the LH pulses was greater ($P < 0.05$) at h 24 and h 48 than at h -12 and h 0 for the C NW group (Fig. 2b), whereas pulse amplitude remained constant ($P > 0.1$) over all periods for the ODB NW group. Mean concentrations of LH (Fig. 2c) remained constant at about 0.8 ng ml^{-1} in the C NW group. Mean values in the ODB NW were similar ($P > 0.1$) to the C NW group at h -12 and h 0, but declined ($P < 0.05$) to less than 0.6 ng ml^{-1} at h 24 to h 48, and these concentrations were less ($P < 0.05$) than in controls for the equivalent time periods.

Steroids in follicular fluid (FF)

Concentrations of oestradiol in FF were reduced fivefold ($P < 0.01$) at h 36 in the ODB as compared with the C group, and was equivalent to the C group after a new follicular wave had emerged (Table 1). A substantial decline ($P < 0.01$) in FF oestradiol concentrations occurred between h 12 and h 36 in the ODB treatment group. This decline was associated with reduced ($P < 0.05$) concentrations of testosterone and androstenedione at h 36 in the ODB treatment group as compared with control animals (Table 1). Concentrations of FF testosterone and FF androstenedione were positively correlated ($r^2 = 0.85$; $P < 0.01$). A fivefold increase ($P < 0.01$) in FF progesterone concentration was observed in the ODB treatment group between h 36 and after a new follicular wave had emerged. In contrast, FF progesterone concentrations in DF were not increased ($P > 0.1$) after new wave emergence in control animals (Table 1).

The number of granulosa cell layers and incidence of apoptosis

The overall average number of granulosa cell layers was reduced (treatment; $P < 0.05$) in the DF1 of ODB (5.2 ± 1.2) as compared with C treatments (8.3 ± 1.6). Across treatments, the number of granulosa cell layers declined from 9.7 ± 1.0 at h 36 to 3.2 ± 0.6 after emergence of a new follicular wave (time; $P < 0.01$). A time-by-treatment interaction was not detected. The number of granulosa cell layers in ODB 12H group (Table 1) was greater ($P < 0.01$) than for the ODB NW group, and tended ($P = 0.08$) to be greater than the ODB 36H group. The proportion of apoptotic granulosa cells increased (time; $P < 0.01$) from $2.9 \pm 1.7\%$ at h 36 to $25.4 \pm 4.2\%$ after emergence of a new follicular wave in a similar ($P > 0.1$) manner for ODB and C treatments (Table 1). Photographs representing the erosion of the granulosa cell membrane and the increasing extent of apoptosis across time within the ODB-treated groups are presented in Fig. 3.

Discussion

A prerequisite for synchronizing the wavelike pattern of follicular development that occurs on the ovaries of cattle using an exogenous treatment is that the existing DF ceases to be dominant. Induction of atresia can be achieved with exogenous progesterone alone, provided that the DF has developed for an extended period under a low progestagen environment (Day & Burke 2002). The mechanisms of progesterone-induced atresia of 'aged' DF have been reported (Manikkam & Rajamahendran 1997). This previous study found that an acute reduction in intrafollicular aromatase activity, oestradiol content and insulin-like growth factor

Figure 2 Frequency of LH pulses/12 h (a), amplitude of LH pulses (b) and mean concentrations of LH (c) during periods of 12 h beginning at h -12, 0, 24 and 48 in heifers that received 1 mg ODB i.m./500 kg BW (ODBNW; $n = 7$) on day 5.6 ± 0.1 of the oestrous cycle (hour (h) 0) as compared with untreated controls (CNW; $n = 7$).

availability were key features of induced atresia. The present study is the first to provide a detailed characterization of systemic hormone changes and intrafollicular events that occur during loss of dominance due to oestradiol-induced atresia. The key findings were that the oestrogenic function of the DF was effectively removed at h 36 and this was associated with reduced testosterone in FF and support from LH in the circulation. The patterns of change in circulating concentrations of FSH and the timing of the emergence of a new follicular wave of development were not different between treated and control heifers. This suggests that a factor(s) other than the oestrogenic status of the DF is critically influencing the timing of new follicular development on the ovaries of cattle following oestradiol-induced atresia of the DF.

Gonadotrophins are the major survival signals for mature antral follicles (Markström *et al.* 2002), and reduced circulating concentrations of LH (Burke *et al.* 1996) and FSH (O'Rourke *et al.* 2000) are believed to be the primary mechanism through which exogenous oestradiol perturbs follicular development. The experimental design of the present study aimed to characterize the temporal events occurring within the DF during oestradiol-induced atresia, and the temporal relationships between gonadotrophins and follicular function. It is well established that in order for a follicle to become a mature DF, a switch from being FSH-dependent to LH dependency is required (Lucy *et al.* 1992, Findlay *et al.* 1996, Gong *et al.* 1996, Crowe *et al.* 2001). A critical dependency on LH in mature DFs is further demonstrated in studies where experimentally increased LH support extended the duration of dominance well beyond normal (Sirois & Fortune 1990, Taft *et al.* 1996). Maximal expression of mRNA for LH receptor in theca and granulosa cells is observed on about day 4 after emergence (see Bao & Garverick 1998) and LH receptor density is greatest at this time in non-ovulatory DFs (Ireland & Roche 1983). In the present study, treatments were initiated when DFs were at this LH-dependent stage of maturity. On the strength of this well-established relationship, we speculate that oestradiol-induced suppression of LH was the primary driver for the observed responses in the DFs after oestradiol treatment.

It is evident from the present study that the oestrogenic capacity in the DF was rapidly decreased (i.e. within 36 h) following ODB treatment. The loss of oestrogenic function is considered unfavourable for continued survival of the follicle. Oestradiol promotes growth of the follicle in multiple ways (Rosenfeld *et al.* 2001, Schams & Berisha 2002) and is considered an anti-apoptotic factor (Knecht *et al.* 1984, Billig *et al.* 1993, Kaipia & Hsueh 1997, Lund *et al.* 1999). A direct effect of exogenous oestradiol on the DF is possible although unlikely. First, intrafollicular concentrations of oestradiol in healthy DFs are several 1000-fold greater than systemic concentrations (Ginther *et al.* 1997), even when considering the elevation in circulating concentrations due to treatment with ODB. Secondly, direct

Figure 3 Representative photomicrographs of the follicular wall of first dominant follicles (DF1) in the ODB-treated groups; 12H ODB (a to d), 36H ODB (e to h) and ODBNW (i to l). Each row represents the same DF1. Haematoxylin and eosin (e and i), DAPI (a and j), positive control (DNase treated) TUNEL (b and f), TUNEL sample (c, g and k), negative control (TdT omitted) TUNEL (d, h and l). RBCs, red blood cells; GCs, granulosa cells; AGC, apoptic granulosa cell. Magnification ($\times 400$) is similar for all photomicrographs (note the 25 μm magnification bar in i).

placement of oestradiol into ovarian stroma adjacent to the DF did not cause atresia (Bo *et al.* 2000).

Concentrations of testosterone and oestradiol in FF at h 36 in ODB-treated heifers were markedly reduced compared with controls. The observed reduction in androstenedione was not significant at the 95% confidence level. In agreement with other studies in cattle (Badinga *et al.* 1992, Lucy *et al.* 1992), the decrease in FF oestradiol

could be explained by reduced availability of androgen substrate for aromatization. The present study provides strong evidence that the oestrogenic function of the DF1 was suppressed during ODB treatment via reduced LH support for thecal tissue synthesis of oestrogenic precursor steroids. However, studies in sheep (Tsonis *et al.* 1984), and in cattle during progesterone-induced atresia of 'persistent' DF (Manikkam & Rajamahendran 1997), indicated

that the decrease in FF oestradiol during atresia was primarily related to reduced aromatase activity. Further studies are required to determine how steroidogenic enzymes, such as aromatase and 17 α -hydroxylase, respond during oestradiol-induced atresia of DFs.

Follicular fluid progesterone concentrations after new follicular wave emergence were markedly greater in DF1s of ODB-treated than control heifers. The greater concentration of progesterone in atretic follicles after new wave emergence in the ODB-treated animals suggests a more advanced state of atresia compared with that in untreated controls. The latter finding is consistent with several reports showing that FF progesterone increases as follicles become atretic (Uilenbroek *et al.* 1980, Bodensteiner *et al.* 1996). The reason for an accumulation in FF progesterone during atresia could be because theca cells remain viable and continue to produce progesterone even during apoptotic destruction of the granulosa cell compartment (Fortune & Quirk 1988, Xu *et al.* 1995). Thus the increase in progesterone may simply reflect an accumulation of substrate that is not converted to downstream steroids (Hubbard & Greenwald 1981). Additionally, a recent study has suggested that the increase in FF progesterone is an important aspect of cell death and is achieved through an FSH-stimulated increase in progesterone synthesis in granulosa cells (Gross *et al.* 2001).

The advanced state of atresia in follicles of ODB-treated animals was further supported by reduced numbers of granulosa cell layers. The maximum number of granulosa cells is attained in bovine follicles at about 9 mm in diameter (Lussier *et al.* 1987, Fortune 1994), a smaller diameter than the DF1s in the present study. Thus, the reduction in the number of granulosa cell layers in the DF1s of ODB-treated animals indicates greater degradation of the granulosa cell compartment. The TUNEL assay procedure was able to detect the increased rate of apoptosis in the DF1s after a new wave of follicular development. The rate of apoptosis (25%) was consistent with that in DFs undergoing progesterone-induced atresia (Manikkam & Rajamahendran 1997). However, the TUNEL assay used in the present study failed to detect greater rates of apoptosis at any time point in ODB-treated as compared with control heifers. It is possible that the rate of apoptosis was greater in the DF1s of the ODB-treated group at time points not measured in the present study. In agreement with previous studies (Jolly *et al.* 1994, Manikkam & Rajamahendran 1997, Yang & Rajamahendran 2000), some apoptotic granulosa cells were detected even in apparently 'healthy' DFs in the present study. The appearance of an inherent basal rate of apoptosis throughout follicular development increases the likelihood that increased degeneration of the granulosa cell compartment following ODB treatment occurred without the sampling or assay procedure being sufficiently sensitive to detect such changes. Additionally, the TUNEL assay does not detect granulosa cells that are completely degraded or, in the present study, granulosa cells that are sloughed from the granulosa cell

compartment into the antrum of the follicle. These alternative endpoints of cellular death were not assessed in the present study. Further studies are required to determine if apoptotic trigger signals, such as Fas ligand and Fas antigen (Porter *et al.* 2001), are up-regulated during the process of oestradiol-induced atresia to better establish the relative role of apoptosis during this event.

Timing of the pre-emergence increase in FSH and subsequent new follicular wave development was not different between the ODB-treated and control heifers. The second wave of follicular development normally begins on about day 10 of the oestrous cycle in heifers (Sirois & Fortune 1988), and this timing was observed in the control group of the present study as expected. Timing of a new wave of follicular development following administration of oestradiol is typically observed three to five days later (Bo *et al.* 2002, Diskin *et al.* 2002, Day & Burke 2002). The interval to new emergence in ODB-treated heifers of the present study was within these reported times, and consistent with our previous results following administration of 1 mg ODB at various stages of the oestrous cycle in beef heifers (Day & Burke 2002). As ODB treatment did not result in earlier new wave emergence, one possibility is that the DF1 continued to dominate despite a loss in estrogenic function in ODB-treated animals. This would be possible if the DF continued to secrete inhibin (Kaneko *et al.* 2002), which was not measured in the present study. Alternatively, ODB treatment could have exerted a direct effect on FSH to regulate emergence of the next follicular wave. We tested this possibility in another experiment by aspirating the DF1 and administering increasing doses of ODB from 0 to 4 mg (Burke *et al.* 2003). This latter study clearly showed that in the absence of the DF1, ODB delays the increase in FSH and the timing of new wave emergence in a dose-dependent manner. It also demonstrated that variation in timing of the pre-emergence increase in FSH and new follicle wave emergence is dependent on clearance of oestradiol from the circulation. This may have accounted for the fact that ODB-treated heifers of the present study had a more variable timing in new emergence relative to controls. In the present study, ODB may have postponed emergence by inhibiting the pre-emergence increase in FSH, even though the DF1 had entered an atretic state and had lost dominance. It is speculated that the timing of emergence of the second wave was influenced by peripheral oestradiol concentrations resulting from the ODB in this treatment group, whereas in the control animals, the time of emergence was a function of time of spontaneous atresia of the DF1.

In conclusion, administration of an atretogenic dose of ODB reduced concentrations of LH in the circulation and promoted a rapid and sustained loss in the estrogenic capacity of DFs. Erosion of the granulosa cell compartment was increased in the DF1 of ODB-treated animals, but the rate of apoptosis in granulosa cells was not increased by ODB. The extent to which ODB-induced atresia approximates the process of spontaneous atresia

requires further clarification. This approach could serve as a useful model to better understand the mechanisms that initiate atresia and the progressive nature of this process in bovine ovarian follicles.

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References

- Adams GP, Matteri RL, Kastelic JP, Ko JCH & Ginther OJ 1992 Association between surges of follicle-stimulating hormone and the emergence of follicle waves in heifers. *Journal of Reproduction and Fertility* **94** 177–188.
- Anderson LH, McDowell CM & Day ML 1996 Progesterone-induced puberty and secretion of luteinizing hormone in heifers. *Biology of Reproduction* **54** 1025–1031.
- Badinga L, Driancourt MA, Savio JD, Wolfenson D, Drost M, de la Sota RL & Thatcher WW 1992 Endocrine and ovarian responses associated with the first-wave dominant follicle in cattle. *Biology of Reproduction* **47** 871–883.
- Bao B & Garverick HA 1998 Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *Journal of Animal Science* **76** 1903–1921.
- Bergfelt DR, Lightfoot KC & Adams GP 1994 Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. *Theriogenology* **42** 895–907.
- Billig H, Furuta I & Hsueh AJ 1993 Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology* **133** 2204–2212.
- Bo GA, Adams GP, Caccia M, Martinez M, Pierson RA & Mapletoft RJ 1995 Ovarian follicular wave emergence after treatment with progestogen and estradiol in cattle. *Animal Reproduction Science* **39** 193–204.
- Bo GA, Bergfelt DR, Brogliatti GM, Pierson RA, Adams GP & Mapletoft RJ 2000 Local versus systemic effects of exogenous estradiol-17 β on ovarian follicular dynamics in heifers with progestogen implants. *Animal Reproduction Science* **59** 141–157.
- Bo GA, Baruselli PS, Moreno D, Cutaia L, Caccia M, Tribulo R, Tribulo H & Mapletoft RJ 2002 The control of follicular wave development for self-appointed embryo transfer programs in cattle. *Theriogenology* **57** 53–72.
- Bodensteiner KJ, Wiltbank MC, Bergfelt DR & Ginther OJ 1996 Alterations in follicular estradiol and gonadotropin receptors during development of bovine antral follicles. *Theriogenology* **45** 499–512.
- Burke CR, Macmillan KL & Boland MP 1996 Oestradiol potentiates a prolonged progesterone-induced suppression of LH release in ovariectomised cows. *Animal Reproduction Science* **45** 13–28.
- Burke CR, Mussard ML, Gasser CL, Grum DE & Day ML 2003 Estradiol benzoate delays new follicular wave emergence in a dose-dependent manner after ablation of the dominant ovarian follicle in cattle. *Theriogenology* **60** 647–658.
- Crowe MA, Kelly P, Driancourt MA, Boland MP, & Roche JF 2001 Effects of follicle-stimulating hormone with and without luteinizing hormone on serum hormone concentrations, follicle growth, and intrafollicular estradiol and aromatase activity in gonadotropin-releasing hormone-immunized heifers. *Biology of Reproduction* **64** 368–374.
- Day ML & Burke CR 2002 Management of follicular growth with progesterone and estradiol within progestin-based estrous synchrony systems. In *Factors Affecting Calf Crop*, pp 101–117. Eds MJ Fields, RS Sand & JV Yelich. Boca Raton, Florida: CRC Press.
- Diskin MG, Austin EJ & Roche JF 2002 Exogenous hormonal manipulation of ovarian activity in cattle. *Domestic Animal Endocrinology* **23** 211–228.
- Dyer RM, Bishop MD & Day ML 1990 Exogenous estradiol reduces inhibition of luteinizing hormone by estradiol in prepubertal heifers. *Biology of Reproduction* **42** 755–761.
- Findlay JK, Drummond AE & Fry RC 1996 Intraovarian regulation of follicular development and ovulation. *Animal Reproduction Science* **42** 321–331.
- Fortune JE 1994 Ovarian follicular growth and development in mammals. *Biology of Reproduction* **50** 225–232.
- Fortune JE & Quirk SM 1988 Regulation of steroidogenesis in bovine preovulatory follicles. *Journal of Animal Science* **66** (Supp 2) 1–8.
- Gavrieli Y, Sherman Y & Bensasson AA 1992 Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *Journal of Cell Biology* **119** 493–501.
- Ginther OJ, Kot K, Kulick LJ & Wiltbank MC 1997 Sampling follicular fluid without altering follicular status in cattle: oestradiol concentrations early in the follicular wave. *Journal of Reproduction and Fertility* **109** 181–186.
- Gong JG, Campbell TA, Bramley TA, Gutierrez CG, Peters AR & Webb R 1996 Suppression in the secretion of follicle-stimulating hormone and luteinizing hormone, and ovarian follicular development in heifers continuously infused with a gonadotropin-releasing hormone agonist. *Biology of Reproduction* **55** 68–74.
- Goodman RL & Karsch FJ 1980 Pulsatile secretion of luteinizing hormone: differential suppression by ovarian steroids. *Endocrinology* **107** 1286–1290.
- Gross SA, Newton JM & Hughes FM 2001 Decreased intracellular potassium levels underlie increased progesterone synthesis during ovarian follicular atresia. *Biology of Reproduction* **64** 1755–1760.
- Hubbard CJ & Greenwald GS 1981 Changes in DNA, cyclic nucleotides and steroids during induced follicular atresia in the hamster. *Journal of Reproduction and Fertility* **63** 455–461.
- Ireland JJ & Roche JF 1983 Development of nonovulatory antral follicles in heifers: Changes in steroids in follicular fluid and receptors for gonadotropins. *Endocrinology* **112** 150–156.
- Jolly PD, Tisdall DJ, Heath DA, Lun S & McNatty KP 1994 Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3',5'-monophosphate response to follicle stimulating hormone and luteinizing hormone, and follicular atresia. *Biology of Reproduction* **51** 934–944.
- Kaipia A & Hsueh AJW 1997 Regulation of ovarian follicle atresia. *Annual Reviews of Physiology* **59** 349–363.
- Kaneko H, Noguchi J, Kikuchi K, Todoroki J & Hasegawa Y 2002 Alterations in peripheral concentrations of inhibin A in cattle studied using a time-resolved immunofluorometric assay: Relationship with estradiol and follicle-stimulating hormone in various reproductive conditions. *Biology of Reproduction* **67** 38–45.
- Kesler DJ, Garverick HA, Youngquist RS, Elmore RG & Bierschwal CJ 1977 Effect of days postpartum and endogenous reproductive hormones on GnRH-induced LH release in dairy cows. *Journal of Animal Science* **46** 797–803.
- Knecht M, Darbon JM, Ranta JM, Baukal AJ & Catt KJ 1984 Estrogens enhance the adenosine 3',5'-monophosphate-mediated induction of follicle-stimulating hormone and luteinizing hormone receptors in rat granulosa cells. *Endocrinology* **115** 41–49.

- Littell RC, Milliken GA, Stroup WW & Wolfinger RD** 1996 In *SAS System for Mixed Models*. Cary, NC: SAS Institute Inc.
- Lucy MC, Savio JD, Badinga L, De La Sota RL & Thatcher WW** 1992 Factors that affect ovarian follicular dynamics in cattle. *Journal of Animal Science* **70** 3615–3626.
- Lund SA, Murdoch J, Van Kirk EA & Murdoch WJ** 1999 Mitogenic and antioxidant mechanisms of estradiol action in preovulatory ovine follicles: relevance to luteal function. *Biology of Reproduction* **61** 388–392.
- Lussier JG, Matton P & Dufour JJ** 1987 Growth rates of follicles in the ovary of the cow. *Journal of Reproduction and Fertility* **81** 301–307.
- McNatty KP, Heath DA, Henderson KM, Lun S, Hurst PR, Ellis LM, Montgomery GW, Morrison L & Thurley DC** 1984 Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary. *Journal of Reproduction and Fertility* **72** 39–53.
- Manikkam M & Rajamahendran R** 1997 Progesterone-induced atresia of the proestrous dominant follicle in the bovine ovary: changes in diameter, insulin-like growth factor system, aromatase activity, steroid hormones, and apoptotic index. *Biology of Reproduction* **57** 580–587.
- Markström E, Svensson EC, Shao R, Svanberg B & Billig H** 2002 Survival factors regulating ovarian apoptosis – dependence on follicle differentiation. *Reproduction* **123** 23–30.
- O'Rourke M, Diskin M, Greenan JM & Roche JR** 2000 The effect of dose and route of oestradiol benzoate administration on plasma concentrations of oestradiol and FSH in long-term ovariectomized heifers. *Animal Reproduction Science* **59** 1–12.
- Porter DA, Harman RM, Cowan RG & Quirk SM** 2001 Relationship of Fas ligand expression and atresia during bovine follicle development. *Biology of Reproduction* **121** 561–566.
- Price CA, Carrière PD, Bhatia B & Groome NP** 1995 A comparison of hormonal and histological changes during follicular growth, as measured by ultrasonography, in cattle. *Journal of Reproduction and Fertility* **103** 63–68.
- Rosenfeld CS, Wagner JS, Roberts RM & Lubahn DB** 2001 Intraovarian actions of oestrogen. *Reproduction* **122** 215–226.
- SAS Institute Inc.** 1996 In *SAS/STAT User's Guide*. Cary, NC: SAS Institute Inc.
- Schams D & Berisha B** 2002 Steroids as local regulators of ovarian activity in domestic animals. *Domestic Animal Endocrinology* **23** 53–65.
- Sirois J & Fortune JE** 1990 Lengthening the bovine estrous cycle with low levels of exogenous progesterone: A model for studying ovarian follicular dominance. *Endocrinology* **127** 916–925.
- Sirois J & Fortune JE** 1988 Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biology of Reproduction* **39** 308–317.
- Taft R, Ahmad N & Inskeep EK** 1996 Exogenous pulses of luteinizing hormone cause persistence of the largest bovine ovarian follicle. *Journal of Animal Science* **74** 2985–2991.
- Thundathil J, Kastelic JP & Mapletoft RJ** 1998 The effect of estradiol cypionate (ECP) on ovarian follicular development and ovulation in dairy cattle. *Canadian Journal of Veterinary Research* **62** 314–316.
- Tsonis CG, Carson RS & Findlay JK** 1984 Relationships between aromatase activity, follicular fluid oestradiol-17 β and testosterone concentrations, and diameter and atresia of individual ovine follicles. *Journal of Reproduction and Fertility* **72** 153–163.
- Uilenbroek JTI, Woutersen PJA & van der Schoot P** 1980 Atresia of preovulatory follicles: gonadotropin binding and steroidogenic activity. *Biology of Reproduction* **23** 219–229.
- Wolfe MW, Roberson MS, Stumpf TT, Kittock RJ & Kinder JE** 1992 Circulating concentrations and pattern of luteinizing hormone and follicle-stimulating hormone in circulation are changed by the circulating concentration of 17 β -estradiol in the bovine male and female. *Journal of Animal Science* **70** 248–253.
- Wyllie AH** 1980 Glucocorticoid-induced thermocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **339** 625–626.
- Xu ZZ, Garverick HA, Smith GW, Smith MF, Hamilton SA & Youngquist RS** 1995 Expression of messenger RNA encoding cytochrome P450 side chain cleavage, cytochrome P450 17 α -hydroxylase and cytochrome P450 aromatase in bovine follicles during the first follicular wave. *Endocrinology* **136** 981–989.
- Yang MY & Rajamahendran R** 2000 Involvement of apoptosis in the atresia of non-ovulatory dominant follicle during the bovine estrous cycle. *Biology of Reproduction* **63** 1313–1321.

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