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 ovarioectomy 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 ± 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.


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-17β (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 Ca2+/Mg2+-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 × Angus heifers weighing 462 ± 38 kg (mean ± s.d.) following one or two i.m. injections of prostaglandin F$_{2α}$ (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 (Alokina 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 1500 $g$ 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 4750 $g$ 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 μl) 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-μm 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., Burlington, 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 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 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. 1992, Burke et al. 1996). The average intra-assay CV was 2.9% and the average inter-assay CV (11 assays) was 15.4%. Average sensitivity of the assays was 1.0 pg ml\(^{-1}\). Concentrations of oestradiol in plasma were determined in duplicate using a double-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 progesterone in plasma were determined in duplicate using a double-antibody RIA (Anderson et al. 1996). The inter-assay CV 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-log 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, 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-log transformation. Average recovery of testosterone across concentrations ranging from 2.2 to 7 ng ml\(^{-1}\) was 102%. Follicular fluid was diluted 1:10 or 1:100 in Testosterone 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\(^{-1}\).

### 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.

<table>
<thead>
<tr>
<th>Variable (^a)</th>
<th>Control</th>
<th>ODB treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H12</td>
<td>H36</td>
</tr>
<tr>
<td>Observations ((n))</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Concentrations ((ng ml^{-1})) in follicular fluid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>366</td>
<td>258 ± 27(^b)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>38</td>
<td>35 ± 2(^h)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>5.2</td>
<td>2.7 ± 1.3(^h)</td>
</tr>
<tr>
<td>Androsterone</td>
<td>2.9</td>
<td>1.2 ± 0.6(^b)</td>
</tr>
<tr>
<td>Histological characteristics of granulosa cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of layers</td>
<td>8.8</td>
<td>11.1 ± 1.9</td>
</tr>
<tr>
<td>Total cells per field</td>
<td>100</td>
<td>65 ± 11</td>
</tr>
<tr>
<td>% cells apoptotic</td>
<td>4.6</td>
<td>3.8 ± 3.2</td>
</tr>
</tbody>
</table>

\(^a\)Mean comparisons confined to treatment differences among the H36 and NW groups.

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

\(^f\)Treatment \((P < 0.05)\) and time \((P < 0.01)\); see text.

\(^g\)Time \((P < 0.01)\); see text.

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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 used on any data collected from the same animal over consecutive time points. The repeated measures model was used on any data collected from the same animal over consecutive time points. The repeated measures model was used on any data collected from the same animal over consecutive time points. The repeated measures model was used on any data collected from the same animal over consecutive time points.

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 ± 8.4) and C NW (h 89.1 ± 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).

**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 ± 0.3 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 ± 0.3) and the C NW (d 4.4 ± 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 ± 0.1 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 ± 8.4) and C NW (h 89.1 ± 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

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Data were analyzed by ANOVA 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 ± S.E.M.
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 ± 1.7% at h 36 to 25.4 ± 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...
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 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).


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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 h36 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

**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 (×400) is similar for all photomicrographs (note the 25 μm magnification bar in i).
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 17α-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.

Acknowledgements

The authors thank Stuart Morgan, Dr Dave Grum, Allan Robison, Pam Ferrel and Dr Fran Adamski for assistance in data collection. Drs Jim Kinder and Joy Pate for serving as internal reviewers of the manuscript and Dr Normand St-Pierre for statistical support. Donation of Lutalyse (Pfizer, Kalamazoo, MI, USA) is gratefully acknowledged. Salaries and research support were provided by State and Federal funds appropriated to the Ohio Agricultural Resource Development Center (OARDC; Manuscript No. 19-04A5) and through competitive grant funds (OARDC; No. OHOAO628). The authors declare that there is no conflict of interest that would affect the impartiality of this scientific work.

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Received 16 June 2004
First decision 7 September 2004
Revised manuscript received 8 February 2005
Accepted 18 February 2005