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EFFECT OF CHRONIC ADMINISTRATION OF ENDOTHELIN RECEPTOR TYPE A ANTAGONIST (BQ-610) ON FUNCTIONAL LIFESPAN OF THE CORPUS LUTEUM IN SHEEP

Efecto de la administración crónica del antagonista del receptor tipo A (BQ-610) de la endotelina 1 sobre el estado funcional del cuerpo lúteo en ovejas

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ABSTRACT

To test the role of endothelin 1 (END1) in luteolysis, an END receptor type A antagonist (BQ-610) was delivered into the corpus luteum (CL) during spontaneous luteolysis in sheep. On day 9 of the estrous cycle, an osmotic mini-pump containing 2 mg of BQ-610 (n = 12) or vehicle (n = 9) were implanted surgically in the ewes. Corpora lutea were collected 12 h after onset of estrus, or on the afternoon of day 21 in ewes that had not returned to estrus, and from untreated ewes on day 10 to 12 of the estrous cycle (mid-cycle CL). Three of 12 BQ-610-treated ewes did not show estrus before day 21 compared to 0 of 9 vehicle-treated ewes. Estrous cycles in vehicle-treated ewes averaged 15.5 ± 0.2 days. In the three BQ-610-treated ewes, luteal weights on day 21 were greater than in vehicle-treated ewes on the day of estrus (0.62 ± 0.05 versus 0.39 ± 0.03 g, respectively; P < 0.001), as were luteal contents of progesterone (P_4) (20958.2 ± 1830.9 µg/g versus 1291.2 ± 1057.1 µg/g respectively; P<0.0001). Serum concentrations of P_4 in the three BQ-610-treated ewes remained above 1.5 ng/mL through day 21 (P<0.01). Their luteal tissue appeared normal with 53.3 ± 5.8% of apoptotic cells, whereas luteal tissue in vehicle-treated ewes was markedly disorganized and in an advanced stage of structural regression. Expression of mRNA of several genes involved in progesterone production or structural luteolysis was different in CL from vehicle-treated compared to CL of BQ-610-treated ewes or mid-cycle CL. In conclusion, chronic infusion of BQ-610 blocked luteolysis and lengthened the estrous cycle in three of 12 ewes. Furthermore, functional features of CL of those three ewes were similar to mid-cycle CL. Overall this study indicates that END1 might plays mediatory role during spontaneous luteolysis in the ewe via endothelin receptor type A.

Key words: Sheep, spontaneous luteolysis, progesterone, endothelin receptor type A, prostaglandin $F_2\alpha$.

RESUMEN

Con el fin de probar el rol de la endotelina 1 (END1) en la luteólisis, un antagonista del receptor tipo A (BQ-610) de END1 fue administrado dentro del cuerpo lúteo (CL) durante la regresión luteal espontánea en la oveja. En el día 9 del ciclo estrual se implantó quirúrgicamente en el CL de las ovejas una mini bomba osmótica con 2 mg de BQ-610 (n = 12) o un vehículo (n = 9). Los CL fueron colectados 12 horas posteriores al inicio del estro, o en la tarde del día 21 del ciclo estrual en las ovejas que no retornaron en celo, y entre los días 10 a 12 del ciclo estrual en ovejas controles. Tres de 12 ovejas tratadas con BQ-610 (OTBQ-610) no mostraron signos de estro antes del día 21 del ciclo, comparado con 0 de 9 de las que recibieron vehículo (OTV). En las OTV el ciclo estrual promedió 15,5 ± 0,2 días. El peso del CL en las tres OTBQ-610 en el día 21 del ciclo fue mayor que en las OTV en el día del celo (0,62 ± 0,05 versus 0,39 ± 0,03 g, respectivamente; P<0,001); lo cual también ocurrió con el contenido luteal de progesterona (P_4) (20958,2 ± 1830,9 µg/g versus 1291,2 ± 1057,1 µg/g respectivamente; P<0,0001). La concentración sérica de P_4 en las tres OTBQ-610 permaneció

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sobre 1,5 ng/mL hasta el día 21 (P<0,01), y el CL tuvo una apariencia normal, con 53,3 ± 5,8% de células apoptóticas; en las OTV el tejido luteal se observó en un estado avanzado de regresión estructural. La expresión del ARNm de algunos genes involucrados en la producción de P_4 o en la luteólisis estructural fue diferente en los CL de OTV comparado a los de OTBQ-610 o a los colectados en la fase luteal media. En conclusión, la infusión crónica de BQ-610 dentro del CL bloqueó la luteolisis y prolongó la duración del ciclo estrual en tres de 12 ovejas. En general, el presente estudio indica que END1, a través del receptor tipo A, pudiera ejercer un rol mediador durante la luteolisis espontánea en la oveja.

Palabras clave: Oveja, luteólisis espontánea, progesterona, receptor A de endotelial, prostaglandina $F_{2\alpha}$.

INTRODUCTION

In absence of pregnancy, luteolysis is initiated by uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which can be mimicked by an injection of exogenous $PGF_{2\alpha}$ during mid-luteal phase of the estrous cycle [19]. Two phases are currently recognized during luteal regression: functional and structural luteolysis. Initially, the corpus luteum (CL) loses steroidogenic capacity without apparent changes in the structure of the tissue. This is followed by degradation of all cellular and structural components of the CL, with the concomitant reduction of luteal weight and size. The cause of functional luteolysis remains controversial; one of the relatively early changes is a decrease in blood flow to the CL [1, 23, 26], although there is evidence that progesterone (P_4) decreases before changes in blood flow [40]. Once P_4 decreases, structural changes imply degradation of the cellular matrix and apoptosis of all cellular components of the CL [27, 31, 42, 44, 47, 53]. Luteolytic actions of $PGF_{2\alpha}$ might involve direct effects as well as various local mediators such as nitric oxide (NO) [12], angiotensin II [39], tumor necrosis factor (TNF-α), interferon γ (INF-γ), interleukin 1β (IL-1β) [24], $PGF_{2\alpha}$ [47], oxytocin [10] and endothelin 1 (END1) [7,11] secreted by different luteal cells.

Endothelin 1, a 21-amino acid peptide, initially isolated from porcine (*Sus scrofa domesticus*) aortic endothelial cells as a potent vasoconstrictor [52] is involved in a wide variety of physiologic and pathologic roles in numerous mammalian tissues [16, 28]. Substantial evidence demonstrated that END1 is a relevant mediator of luteolytic actions of $PGF_{2\alpha}$ [6, 7, 11, 15, 20]. Two G protein-coupled receptors have been described for END1 [28]. *In vivo* studies in ruminants demonstrated that type A endothelin receptor (ENDRA) mediated luteolytic actions of $PGF_{2\alpha}$ [6, 15]. Moreover, in ruminant luteal cells, END1 reduced both basal and luteinizing hormone (LH)-stimulated P_4 secretion in a dose-dependent manner [7, 11], and the inhibitory effect was prevented by the addition of a selective ENDRA antagonist [6, 7].

Data from *in vivo* studies have confirmed and strengthened the concept that END1 is an important mediator of $PGF_{2\alpha}$-induced luteal regression. Intraluteal injection of BQ-123 (an ENDRA antagonist) partially reversed the luteolytic effect of $PGF_{2\alpha}$ [11]. Intramuscular injection of both END1 (100 μg) and $PGF_{2\alpha}$ (7.5 mg) reduced P_4 concentrations by 48 (hours) h post-treatment, and shortened the length of the cycle, whereas separate administration of either END1 or $PGF_{2\alpha}$ at the same dosage did not induce luteolysis in sheep (*Ovis aries*) [11]. In contrast to the reported luteolytic effects, Weems et al. [50] infused END1 into the ovarian pedicle or the uterus from day (d) 10 to 18 of the estrous cycle in ewes and reported increases in secretion of PGE_2 and maintenance of luteal function and structure.

Intrauterine infusions of BQ-610, a highly specific ENDRA antagonist, every 12 h on day 16 through 18 of the estrous cycle in heifers (*Bos taurus*) delayed luteolysis for about 2 d [15]. Chronic administration of BQ-610 into the sheep CL through a minipump implanted in the ovary during the mid-luteal phase decreased serum P_4 concentration throughout the first 12 h after a single injection of $PGF_{2\alpha}$. However, P_4 progressively increased during the next 36 h, attaining a concentration similar to the control group by 48 h after $PGF_{2\alpha}$ [6]. Apparently, a direct action of $PGF_{2\alpha}$ decreased serum P_4 during early luteolysis, whereas END1 was required in later stages as indicated by the rise of P_4 in BQ-610-treated ewes beginning 12 h after $PGF_{2\alpha}$. In addition, blockade of ENDRA during $PGF_{2\alpha}$-induced luteolysis in cows, prevented the increase of caspase 3 mRNA by 24 h compared with controls [49]. These findings indicate that END1 may participate in structural rather than functional luteolysis, stimulating expression of pro-apoptotic factors.

In the current experiment, BQ-610 was delivered into the CL through an osmotic minipump to examine the effects of the sustained blockade of ENDRA during spontaneous luteolysis on the length of the estrous cycle and functional and structural characteristics of the CL. It was hypothesized that chronic delivery of BQ-610 into the CL would prevent or delay the luteal regression induced by endogenous $PGF_{2\alpha}$, increasing the length of estrous cycle.

MATERIALS AND METHODS

General experimental procedure

Twenty one nonpregnant Suffolk ewes, without apparent reproductive alterations, and with at least one previous estrous cycle of normal length (15 to 19 d) were used in the experiment, conducted in Fall and Winter of 2008 with ewes from the West Virginia University research flock. For observation of estrus (12 h intervals) ewes were penned with a vasectomized ram bearing a harness with a crayon in the area of the brisket; standing estrus was confirmed by teasing with another vasectomized ram. On day nine of the estrous cycle (standing estrus = day 0) ewes were assigned randomly to receive one of the

following treatments: Alzet® mini-osmotic pump (model 2002; Durect Corporation, Cupertino, CA, USA) loaded with either vehicle (200 μL 2:1 methanol:saline solution; n = 9), or 2.5 mg of END receptor A antagonist (n = 12), BQ-610 (Azepane-1-carbonyl-leu-D-Trp(For)-D-Trp-OH) (Bachem Bioscience Inc, King of Prussia, PA, USA), diluted in 200 μL of vehicle. The Alzet® mini-osmotic pumps designed to deliver 0.52 ± 0.02 μL/h for approximately 14 d, were kept overnight in sterile 0.9% saline solution at 37°C prior to implantation in the ovary. The expected delivered dosage was 6.25 μg/h of BQ-610.

Ewes were anesthetized initially with 0.3 mg/kg diazepam (Valium, i.v., 5 mg/mL; Roche Pharmaceuticals, Nutley, NJ, USA) and 7 mg/kg ketamine HCL, i.v., and then placed on a mixture of halothane, oxygen (2.0 L/min), and nitrous oxide gas (1.0 L/min) until mid-ventral laparotomy was finished. Ovaries were examined carefully, and in the presence of multiple CL, one was selected for implantation of the catheter connected to the mini pump. Additional CL in the same ovary were enucleated and an absorbable gelatin sponge was sutured into the remaining cavity (Gelfoam®, Pfizer Inc, New York, NY, USA) as a haemostatic component. The contralateral ovary was removed if it contained additional CL. Each minipump was positioned in the mesoovarium and the attached catheter stabilized within the CL following the surgical procedure previously described [6] (FIG. 1). All ewes received a dose (IM) 3 × 10^6 IU of penicillin G immediately after surgery.

Twenty four hours after surgery, ewes were penned with a vasectomized ram bearing a harness with a crayon in the area of the brisket, and estrous detection was performed as described above. Blood samples were collected (8 mL) by jugular venipuncture at 6 h intervals from day 11 until estrus or until day 21 of the estrous cycle in ewes that did not return to estrus. Blood samples were refrigerated (Whirlpool, OZR134A, Canada) for 24 h to allow them to clot, and then centrifuged (International Equipment Company, IEC, Centra-7R, USA) for 20 minutes at 1400 g. Serum was collected and kept at -20°C (Isotemp Freezer, Fisher Scientific, Suwanee, GA, USA) until radioimmunoassay (Multipurpose Scintillation Counter, USA) for P_4. Approximately 12 h after estrous detection or in the afternoon of day 21, ewes were euthanized, the correct position of the pumps and catheters were verified, and ovaries were collected. All animal procedures were approved by WVU Institutional Animal Care and Use Committee (ACUC # 05-1205).

Luteal tissue processing

Ovaries were refrigerated in ice-cold phosphate buffered saline (PBS), transported to the lab within 2 to 3 h after collection, and each CL was dissected, weighed, sectioned into 5 pieces that were weighed individually and 3 of them were frozen in liquid nitrogen and stored at -96°C. One piece of each CL was homogenized in PBS (100 mg of tissue /1 mL of PBS), centrifuged, and supernatant stored at -20°C, for determination of luteal P_4. One fragment was placed in Bouin's fixative for at least 24 h, and processed later to assess cellular morphology and apoptosis.

Apoptosis detection by immunohistochemistry

Bouin's fixative was removed by rinsing luteal tissue successively in 70% alcohol. Pieces of tissue were embedded in paraffin and cut into 10 µm sections; then about 8 to 14 sections were mounted on each microscope slide. After deparaffinization with xylene and rehydration through descending grades of alcohol, tissue specimens were stained with the terminal deoxynucleotide transferase-mediated deoxy-UTP nick labeling method (TUNEL) for detection of apoptotic cells by immunohistochemistry (APO-BRDU-IHC; Phoenix Flow Systems, San Diego, CA, USA). Slides were observed under a microscope (40x-100x) (Olympus PROVIS AX70, USA) to identify specific brown color indicative of apoptosis. Three fields-of-view from two different sections, at least 100 μm apart, were evaluated to quantify the number of apoptotic cells, and to estimate the rate of apoptosis for three animal samples of each treatment.

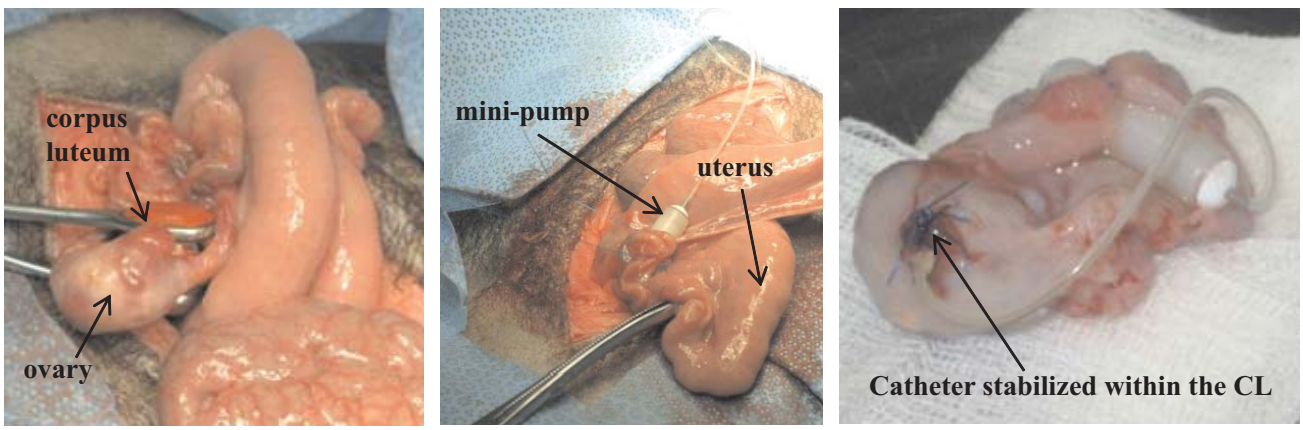

FIGURE 1. VIEW OF CORPUS LUTEUM AND MINI-PUMP DURING THE SURGERY, AND THE CATHETER STABILIZED WITHIN THE CORPUS LUTEUM AFTER THE CORRECT POSITION OF THE PUMPS AND CATHETERS WERE VERIFIED.

Assessing gene expression by real time polymerase chain reaction (RT-PCR)

To corroborate the functional status of the CL, expression of various genes involved in P_4 production or structural luteolysis was determined by quantitative RT-PCR, applying a transverse-transcription real-time PCR procedure. In addition to tissue from BQ-610 and vehicle ewes, luteal tissue from control ewes (no minipump; CL collected on day 10-11 of estrous cycle) was used (n = 3 per group). Accession number, sequence, animal source and additional characteristics of each gene primer are indicated in TABLE I.

Total ribonucleic acid (RNA) was isolated from frozen luteal tissue using Tri-reagent (MRC, Cincinnati, OH, USA) following the recommendations of the manufacturer. Once solubilized in RNAse-free water, the quality and quantity of each RNA sample were assessed by 1% agarose gel electrophoresis and by spectrophotometry (Nano Drop Technologies, Wilmington, DE, USA), respectively. Purified RNA samples were treated with DNAse (Invitrogen, Carlsbad, CA, USA) to remove residual DNA contamination. To assure that low expression of a gene was not negatively affecting the cycle threshold (Ct) values and confirming variability within replicates, the RT-PCR was tested at total RNA concentrations of 0.5, 1.0 and 2.0 μg/μL. The gene expression data reported in this study were from the 2.0 μg of total mRNA concentration.

To generate first-strand cDNA, total RNA was reverse transcribed in a reaction volume of 20 μL containing sample RNA (2.0 μg), dNTP mix (10 nM), oligo dT primer (450 ng) and Superscript II reverse transcriptase (200 U) (Invitrogen, Carlsbad, CA, USA). Real-time PCR was carried out using the approach proposed by Pfaffl [32] and following the procedure described by Goravanahally et al. [9]. Briefly, representative RNA samples from each treatment were pooled and used to produce first-strand cDNA as described above. Five consecutive dilutions (1:10) of cDNA were used to generate a standard curve to estimate the efficiency of the specific primers for each gene. The optimum efficiency of the PCR reactions was standardized between 90 to 110%. Melting curve from optimization reactions of each set of primers assured absence of secondary products.

Oligonucleotide primers were designed with primer3 software [34] based on the mRNA sequences of *Ovis aries*. In the event of mRNA sequences not previously described in sheep, *Bos taurus* mRNA sequences were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the reference gene to normalize the expression values of the genes of interest. Quantitative PCR reactions were carried out in duplicate for each cDNA sample in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using a total reaction volume of 25 µL that included SYBR Green Supermix

TABLE I
CHARACTERISTICS OF PRIMERS USED IN QUANTITATIVE REAL-TIME RT-PCR

Gene *	Accession number	Specie	Primer sequence	Product size (bp)	Annel. Temp. (°C)	Slope Standard curve
StAR	NM_001009243	*O. aries*	F: 5'-TGCTGAGTAAAGTGATCCCTGA-3' R: 5'-AGGACCTTGATCTCCTTGACAC-3'	148	60	-3.631
3βHSD	NM_174343	*B. taurus*	F: 5'-CCACACCAAAGCTACGATGA-3' R: 3'-TGTAAATTGGACTGAGCAGGAA-3'	148	60	-3.419
PPEND1	NM_001009810	*O. aries*	F: 5'-TCTGCAAGTTGTTCCCCTTT-3' R: 3'-ATCTCAATGGCTGTGACCAAC-3'	148	60	-3.331
ENDAR	NM_001009433	*O. aries*	F: 5'-TCTGCAAGTTGTTCCCCTTT-3' R: 3'-ATCTCAATGGCTGTGACCAAC-3'	148	60	-3.208
Bcl-2	DQ152929	*O. aries*	F: 5'-TGGATGACCGAGTACCTGAA-3' R: 3'-CAGCCAGGAGAAATCAAACAG-3'	120	60	-3.314
Bax	AF163774	*O. aries*	F: 5'-GAAGCGCATTGGAGATGAA-3' R: 3'-AAGTAGAAAAGGGCGACAACC-3'	159	60	-3.286
Fas-R	NM_001123003	*O. aries*	F: 5'-CGGAAGAATGGTATGGAGGA-3' R: 3'-TGCAAGAGCTTTTGGGAGAT-3'	162	60	-3.375
Caspase 3	AF068837	*O. aries*	F: 5'-GGATTATCCTGAAATGGGTTTATG-3' R: 3'-GATCGTTTTTAATCCTGACTTCGT-3'	155	60	-3.266
TIMP-1	NM_001009319	*O. aries*	F: 5'-CCAGACATCCGATTCATCTACA-3' R: 3'-GCAGAACTCATGCTGTTCCA-3'	167	60	-3.558
GAPDH	AF030943	*O. aries*	F: 5'-TCTCAAGGGCATTCTAGGCTAC-3' R: 3'-TGTAGCCGAATTCATTGTCG-3'	155	60	-3.254

*StAR: steroidogenic acute regulatory protein; 3βHSD: 3β-hydroxysteroid dehydrogenase; PPEND1: preproendothelin 1; ENDAR: endothelin A receptor; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2 associated protein X; Fas-R: TNF receptor superfamily member 6; TIMP-1: tissue inhibitor of metalloprotease 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

(Bio-Rad, Hercules, CA, USA), 1 μM of each primer and non-diluted target cDNA (2 μL). Real-time PCR conditions were: 30 sec at 95°C (initial denaturation) followed for 40 cycles at 95°C for 30 sec to denature, 30 sec at 60°C to anneal, and 1 min at 72°C for extension.

Radioimmunoassays (RIA) for progesterone

Concentrations of P_4 in serum and luteal tissue were measured in duplicate by RIA as previously described [38]. Assay sensitivity averaged 0.18 ng/mL, and inter- and intra-assay coefficients of variation were 9.5% and 9.8%, respectively.

Statistical analysis

Differences in concentrations of serum and luteal P_4, luteal weight, length of estrous cycle and gene expression among treatments were evaluated by one-way analysis of variance using the General Linear Model (GLM) procedure of SAS [43]. Differences among means were compared by the LSM procedure of SAS. Data were expressed as mean ± SEM. Proportions of ewes in which the length of the estrous cycle was prolonged beyond 20 d were analyzed by Chi-square method of SAS.

RESULTS AND DISCUSSION

Three of 12 (25%) ewes treated with BQ-610 (hereafter designated responsive BQ-610-treated ewes) did not show estrus before day 21 compared to 0 of 9 (0%) vehicle-treated ewes (P = 0.33); the remaining nine ewes treated with BQ-610 had cycles averaging 15.3 ± 0.2 d. Estrous cycles in the responsive BQ-610-treated ewes were at least 5.5 d longer than in vehicle-treated ewes (> 21 d vs. 15.5 ± 0.2 d, respectively; P<0.0001). Differences in size, color and weight of the CL were important aspects to assess the effect of blocking ENDRA during spontaneous luteolysis in sheep (FIG. 2). Corpora lutea from the three responsive BQ-treated ewes were approximately 1.6 times heavier than no responsive BQ-610-treated ewes and vehicle-treated ewes (0.62 ± 0.05 g versus 0.40 ± 0.03 and 0.39 ± 0.03 g, respectively; P<0.01). In addition, CL from responsive BQ-610-treated ewes were pink while CL from control ewes had pale color.

Serum concentrations of P_4 in the three responsive BQ-610-treated ewes remained above 1.5 ng/mL through day 21 of the cycle (P<0.01). In this group, P_4 concentration averaged 2.97 ± 0.08 ng/mL from day 11 through day 16 and decreased to 1.91 ± 0.10 ng/mL from day 17 to day 21 of the cycle (P<0.0001). In non-responsive BQ-610-treated ewes, initial concentration of P_4 was 1.75 ng/mL and decreased consistently from day 11 to minimal values by day 15 of the estrous cycle. In vehicle-treated ewes, P_4 concentration remained above 2 ng/mL from day 11 to day 13 and then decreased progressively until day 15 of the estrous cycle (FIG. 3). Luteal content of P_4 was 10 and 16 fold greater in responsive BQ-610-treated ewes on day 21 than in non-responsive BQ-610-treated

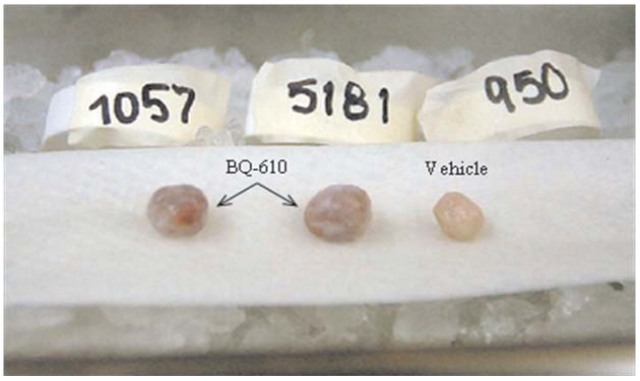

FIGURE 2. REPRESENTATIVE PICTURE OF MACROSCOPIC CHARACTERISTICS OF CORPORA LUTEA COLLECTED 12 H AFTER ONSET OF ESTRUS IN VEHICLE-TREATED EWES, OR ON THE AFTERNOON OF DAY 21 IN BQ-610-TREATED EWES.

and vehicle-treated ewes 12 h after onset of estrus (20958.2 ± 1830.9 µg/g versus 2087.5 ± 1057.1 and 1291.2 ± 1057.1 μg/g of P_4 in homogenized corpus luteum extracts, respectively; P<0.0001). In a similar study, sustained administration of BQ-610 into the CL during $PGF_{2\alpha}$-induced luteolysis in sheep, prevented reduction of luteal P_4 content compared with vehicle-treated group, although the EDNRA antagonist did not completely restore luteal content of P_4 to those of a saline control group [6]. Moreover, blocking of ENDRA by BQ-610 allowed a decrease in peripheral P_4 concentrations during the first 12 h after $PGF_{2\alpha}$ injection; however, P_4 progressively increased during the following 36 h, and attained concentrations similar to the saline control group by around 48 h after PGF2α [6].

Luteal tissue in responsive BQ-610-treated ewes appeared normal with 53.3 ± 5.8% of apoptotic cells, whereas it was not possible to detect apoptotic cells in luteal tissue in vehicle-treated ewes, as the tissue was markedly disorganized and in an advanced stage of structural regression (FIG. 4). Although the EDNRA antagonist prevented luteolysis in responsive BQ-610-treated ewes, the continuous luteolytic effect of $PGF_{2\alpha}$ presumably provoked apoptosis in an important percentage of luteal cells, as indicated above. This finding agrees with the fact that there was a significant increase in the expression of caspase 3 in this group, as well as in vehicle-treated animals, compared with mid-cycle CL. Similarly, serum concentrations of P_4 in BQ-610-treated ewes were about 36% lower from day 16 through 21 (but above 1.5 ng/mL) of the cycle compared with the previous days. During functional luteolysis, the anti-apoptotic effect of P_4 is suppressed and luteal $PGF_{2\alpha}$, directly or via autocrine and paracrine mediators, initiates progressive destruction of all cellular populations of the CL. In the ewe, apoptotic signals appeared after P_4 concentrations declined and structural regression started, and fragmentation of DNA was first observed 12 h after exogenous $PGF_{2\alpha}$ [37]. Endothelial cells with normal nuclei declined significantly at 12 h after $PGF_{2\alpha}$, followed by steroidogenic cells and fibroblasts at 24 and 36 h, respectively [37].

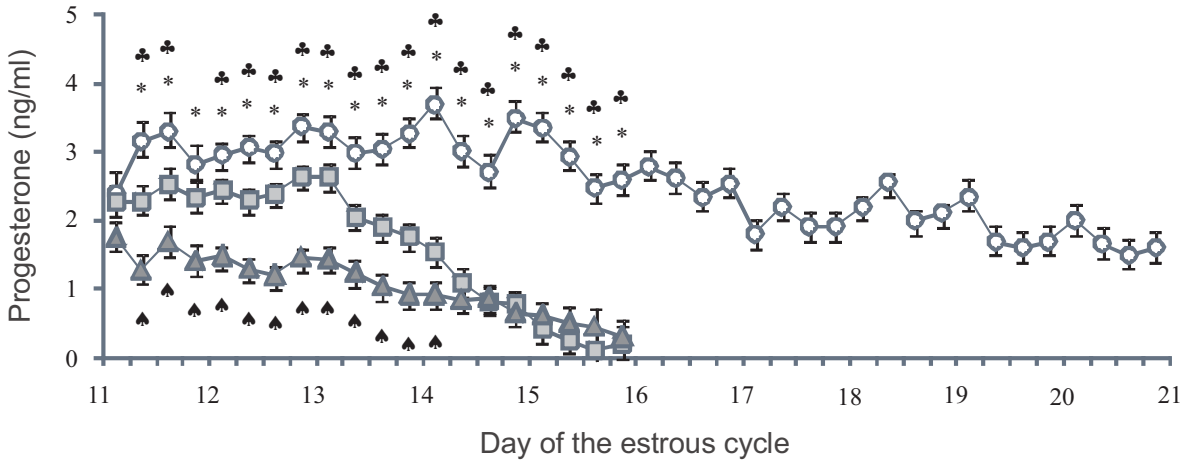

FIGURE 3. EFFECT OF CHRONIC ADMINISTRATION OF EITHER BQ-610 OR VEHICLE DURING SPONTANEOUS LUTEOLYSIS ON SERUM CONCENTRATIONS OF PROGESTERONE IN SHEEP. DATA POINTS REPRESENT MEAN (± SEM) SERUM PROGESTERONE (ng/mL) PER TIME OF SAMPLING FOR RESPONSIVE BQ-610-TREATED (n = 3; o), NON-RESPONSIVE BQ-610-TREATED (n = 9; ▲) AND VEHICLE-TREATED (n = 9;■) EWES. VALUES ARE STATISTICALLY DIFFERENT BETWEEN TREATMENTS AT DESIGNATED TIME POINT (RESPONSIVE BQ-610-TREATED VERSUS NON-RESPONSIVE BQ-610-TREATED EWES P<0.01; RESPONSIVE BQ-610-TREATED VERSUS VEHICLE-TREATED EWES P<0.05; NON-RESPONSIVE BQ-610-TREATED EWES VERSUS VEHICLE-TREATED EWES P<0.05).

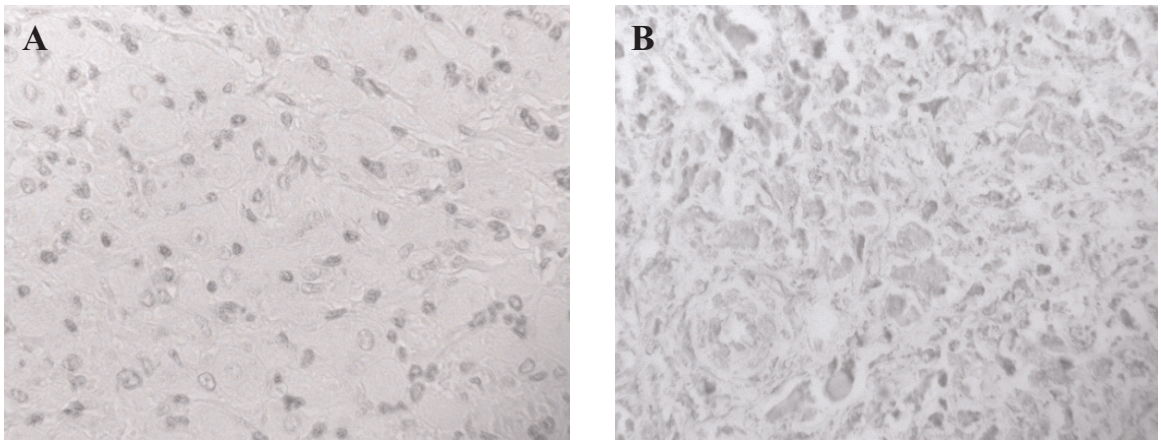

FIGURE 4. REPRESENTATIVE EXAMPLES OF CELLULAR MORPHOLOGY AND APOPTOSIS IN LUTEAL TISSUE AFTER CHRONIC ADMINISTRATION OF EITHER BQ-610 (A) OR VEHICLE (B) DURING SPONTANEOUS LUTEOLYSIS IN SHEEP.

Expression values for selected genes involved in progesterone production or structural luteolysis in ewes treated with BQ-610 or vehicle and in mid-cycle CL are shown in TABLE II. Although StAR mRNA expression was about three to five times greater in mid-cycle CL and responsive BQ-610-treated ewes than in vehicle-treated ewes, differences were not significant. Also, luteal tissue from responsive BQ-610-treated ewes and mid-cycle CL had greater expression of transcript for 3HSD and TIMP-1 than vehicle-treated group. In regressed CL, both ppEND1 and ENDRA mRNA expression were greater ($P \leq 0.05$) than in mid-cycle CL or responsive BQ-610-treated CL. The expression of Bcl-2 was not affected by treatments; nevertheless, it was 3.9 and 1.7 times greater in mid-cycle CL than in CL from responsive BQ-610-treated or vehicle-treated ewes, respectively. Bax mRNA expression was significantly lower in responsive BQ-610-treated and mid-cycle CL than in the vehicle-treated group. The Bcl-2:Bax ratios were 2.25, 0.69 and 0.64 for mid-cycle CL, responsive BQ-610-treated and vehicle-treated ewes, respectively ($P>0.05$). There was no significant effect of treatment on the expression of Fas-R. In contrast, mid-cycle CL had lower expression of caspase 3 than either BQ-610-treated or vehicle-treated CL.

Different methods to assess functional features in luteal tissue treated with BQ-610 or vehicle were used in this study: macroscopic and histological characteristic, steroidogenic capacity and expression of genes related to P_4 synthesis and

TABLE II

EFFECT OF CHRONIC ADMINISTRATION OF EITHER BQ-610 OR VEHICLE DURING EXPECTED SPONTANEOUS LUTEOLYSIS ON EXPRESSION OF GENES INVOLVED IN PROGESTERONE PRODUCTION OR STRUCTURAL LUTEOLYSIS IN SHEEP

Gene *	Treatments [1]			Level of significance
	Mid-cycle CL	BQ-610	Vehicle	
StAR	1.80 ± 1.15 [a]	2.49 ± 1.15 [a]	0.47 ± 1.15 [a]	P > 0.05
3βHSD	0.55 ± 0.08 [a]	1.22 ± 0.08 [b]	0.01 ± 0.08 [c]	[a,b,c] P < 0.01
TIMP-1	1.06 ± 0.11 [a]	0.81 ± 0.11 [a]	0.28 ± 0.11 [b]	[a,b] P < 0.01
ppEND1	0.69 ± 0.27 [a,b]	0.97 ± 0.27 [b]	1.96 ± 0.27 [c]	[a,c] P < 0.04; [b,c] P < 0.01
ENDRA	0.40 ± 0.39 [a]	0.56 ± 0.39 [a]	3.24 ± 0.48 [b]	[a,b] P < 0.01
Bcl-2	2.90 ± 0.86 [a]	0.76 ± 0.86 [a]	1.67 ± 1.48 [a]	P > 0.05
Bax	1.29 ± 0.23 [a]	1.26 ± 0.23 [a]	2.61 ± 0.30 [b]	[a,b] P < 0.02
Fas-R	0.77 ± 0.18 [a]	1.16 ± 0.18 [a]	0.95 ± 0.32 [a]	P > 0.05
Caspase 3	1.47 ± 0.53 [a]	3.16 ± 0.53 [b,c]	3.91 ± 0.65 [c]	[a,b] P < 0.08; [a,c] P < 0.04

[1] Three CL per treatment. Data are expressed as mean ± SEM. Corpora lutea were collected on day 10 to 11 of estrous cycle in mid-cycle ewes, and on day of estrus or in the afternoon of day 21 in no responding and responding BQ-610-treated ewes respectively. *StAR: steroidogenic acute regulatory protein; 3βHSD: 3β-hydroxysteroid dehydrogenase; PPEND1: preproendothelin 1; ENDAR: endothelin A receptor; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2 associated protein X; Fas-R: TNF receptor superfamily member 6; TIMP-1: tissue inhibitor of metalloprotease 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

structural luteolysis. Thus, data from this experiment evaluated both functional and structural aspects of luteolysis.

Blocking of ENDRA prevented endogenous luteolysis for more than 5 d in only 25% of 12 BQ-treated ewes, compared with none of the nine ewes treated with vehicle. Data on macroscopic characteristics of the CL, serum and luteal concentrations of P_4 and gene expression supported this observation, and confirmed previously published *in vivo* studies in ruminants about the role of END1 in mediating luteolytic actions of $PGF_{2\alpha}$ [6,11,15]. Although intraluteal injection of BQ-123 (an ENDRA antagonist) did not reverse completely the luteolytic effect of $PGF_{2\alpha}$, intramuscular injection of both END1 (100 μg) and $PGF_{2\alpha}$ (7.5 mg) effectively reduced P_4 concentrations by 48 h post-treatment, and shortened the length of the sheep estrous cycle [11].

A large body of evidence indicates that END1, a 21-amino acid peptide that regulates vascular function in different tissues, participates in the luteolytic cascade initiated by uterine $PGF_{2\alpha}$ at the end of the luteal phase. In ruminants, a greater ppEND1 and ENDRA mRNA expression was observed at the end of estrous cycle near the time of luteolysis [8,18] and after exogenous $PGF_{2\alpha}$ during mid-luteal phase [4,6,11]. In addition, END1 reduced both basal and LH-stimulated production of P_4 and this anti-steroidogenic effect was reversed by the addition of a selective ENDRA antagonist [6,7,11]. In concordance with the previously cited studies, expression of ppEND1 and ENDRA mRNA in vehicle-treated ewes was greater by the time of estrus than in mid-cycle CL and responsive BQ-610-treated CL, which further supports the relevance of the END system in mediating luteal regression in ruminants.

Different methods for administration or delivery of ENDRA antagonists in vivo have been applied to test the role of END1 in luteal regression. Apparently, intraluteal injection of a selective ENDRA antagonist (BQ-610 or LU-13522) was ineffective to reverse the decline of P_4 production induced by $PGF_{2\alpha}$ in cows [49]. In contrast, intrauterine infusions of 500 μg of BQ-610 every 12 h from day 16 through day 18 of the estrous cycle delayed spontaneous luteolysis in heifers for about 2 d [15]. Moreover, BQ-610 chronically delivered into the CL by means of an osmotic mini-pump effectively reversed the luteolytic effect of $PGF_{2\alpha}$ 12 h after its administration, allowing concentrations of serum P_4 to attain similar values to the control group by 48 h after $PGF_{2\alpha}$ [6]. Apparently, a direct action of $PGF_{2\alpha}$ decreased serum P_4 during early luteolysis, whereas END1 was required in later stages, indicating that END1 might participate more in structural than in functional luteolysis.

This study followed a similar experimental approach to that utilized by Doerr et al. [6], but it seems that the greater dosage of BQ-610 in a vehicle containing more methanol (2:1 vs 1:1 in order to solubilize the greater dosage) prevented spontaneous luteolysis and lengthened the estrous cycle in only 3 of 12 (25%) ewes. Endogenous $PGF_{2\alpha}$ starts to be secreted from the uterus in increased amounts around day 11 to 13 of the sheep estrous cycle [41,54]. The sustained delivery of BQ-610 for more than eight d after the beginning of endogenous $PGF_{2\alpha}$ secretion effectively blocked endogenous END1 in those three ewes. Apparently, a lower initial concentration of P_4 and its sustained reduction from day 11 through day 15 indicated that CL from the non-responsive BQ-610-treated ewes produced less P_4 and were more susceptible to the effect that $PGF_{2\alpha}$ exerts through END1-independent mechanisms. Accordingly, lack of protective actions of P_4 in luteal tissue may allow the luteolytic cascade to occur in those animals [5].

Functional regression of the CL is characterized by a rapid decrease of P_4 production by luteal steroidogenic cells coincident with a transient increased followed by a diminution of blood flow to the CL [1, 22]. During this short period synthe-

sis and secretion of P_4 is inhibited, mostly by interrupting mobilization of cholesterol throughout the cytoplasm and outer mitochondrial membrane [13, 21, 47]. Thus, lacking the substrate to synthesize P_4, steroidogenic activity declines and less P_4 is secreted by large and small luteal cells. Although differences in mRNA expression for StAR were not significant, probably due to the large variation among samples (and few samples per treatment), mean values from responsive BQ-610 and mid-cycle CL were 5.3 and 3.8 times greater than in CL of vehicle-treated ewes. Transcript concentration for 3βHSD was greater in responsive BQ-treated and mid-cycle CL than in vehicle-treated CL. Hence, there were greater serum and luteal concentrations of P_4 in responsive BQ-610-treated ewes. Data from serum and luteal P_4 together with similar expression values for StAR and 3βHDS transcripts in mid-cycle CL and responsive BQ-610-treated CL, strongly indicate the cellular metabolic machinery to synthesize P_4 by luteal steroidogenic cells was completely operative in responsive BQ-610-treated ewes at day 21 of the estrous cycle.

In vehicle-treated ewes, luteal weight 12 h after estrus was about 37% lighter than CL from responsive BQ-610-treated ewes (0.39 ± 0.03 versus 0.62 ± 0.05 g) on day 21 of the cycle. Moreover, judged by color, CL from the three responsive BQ-610-treated ewes were larger and appeared healthier than those collected from vehicle-treated ewes (FIG 2). As several authors reported [2,13,46], CL weight significantly decreased 30 to 50% by 24 to 36 h after exogenous $PGF_{2\alpha}$ in sheep. The decrease in CL weight represents clear evidence that structural aspects of luteal regression have been already induced. However, morphological alterations were not evident until 24 to 36 h after initial exposure to $PGF_{2\alpha}$ [37].

The expression pattern of genes related to structural luteolysis appeared to be less clear. Although the expression of Bax mRNA was significantly lower in BQ-610 and mid-cycle CL compared with vehicle CL, Bcl-2 and Fas-R expression did not differ among treatments. Moreover, the ratio between Bcl-2 and Bax mRNA was around 3.5 times greater in mid-cycle CL than in CL of either responsive BQ-610-treated or vehicle-treated ewes. Bcl-2 and Bax are proteins that prevent and promote apoptosis, respectively, and their relative abundance at a specific luteal stage could favor or oppose apoptosis [45]. While Bcl-2 prevents apoptosis by regulating mechanisms related to Ca^{2+} homeostasis and oxidative stress [14,33], Bax promotes apoptosis by antagonizing anti-apoptotic actions of Bcl-2 [3,29]. Expressions of Bax but not Bcl-2 mRNA and protein increased after $PGF_{2\alpha}$-induced luteolysis in ruminants [36,51]; this change was reflected in Bax/Bcl-2 ratio for both mRNA and protein that was significantly greater at 4 h than immediately before $PGF_{2\alpha}$ injection [51]. In addition, both protein and activity of caspases 3 and 9 increased after $PGF_{2\alpha}$ [51]. Thus, it is evident that $PGF_{2\alpha}$ upregulates proteins that induce apoptosis in luteal cells. The reduction in Bax mRNA in BQ-610 and mid-cycle CL compared with vehicle CL is consistent with blocking the luteolytic effects of endothelin.

Interestingly, there was a significant increased in the expression of caspase 3 in vehicle-treated animals as well as in BQ-610-treated ewes compared with mid-cycle CL. This finding agrees with the fact that luteal tissue from the three responsive BQ-610-treated ewes had 53.3 ± 5.8% of apoptotic cells and that serum concentrations of P_4 in this group were about 36% lower from day 16 through 21 of the cycle compared with the previous days, which is presumed to be an effect of endogenous $PGF_{2\alpha}$. Nevertheless, elevated luteal concentrations of P_4 on day 21 indicated that CL from responsive BQ-610-treated ewes were steroidogenically active. To provide a physiological explanation for this discrepancy, it is important to consider two aspects. First, BQ-610 blocks ENDRA but does not prevent $PGF_{2\alpha}$ actions that are END1-independent. In pregnant ewes [17,23,48], secretion of uterine $PGF_{2\alpha}$ around day 21 post estrus was still elevated to mid range, and consequently $PGF_{2\alpha}$ may be inducing changes in mRNA or even protein by different pathways than those mediated through END1. Second, anti-apoptotic and protective effects of P_4 on luteal function have been documented [25,30,35]. Thus, elevated concentrations of P_4 in BQ-610-treated ewes may counteract the luteolytic actions of $PGF_{2\alpha}$ by inhibiting or reducing activity of pro-apoptotic factors.

CONCLUSION

Chronic delivery of BQ-610 into the CL during mid- to late-luteal phase prevented natural luteolysis and prolonged the estrous cycle for more than five d in only three of 12 ewes. Macroscopic and functional characteristics of those responsive BQ-610-treated CL were similar to a healthy mid-cycle corpus luteum. Overall, although this study indicates that END1 might plays a mediatory role during spontaneous luteolysis in the ewe, the small number of animals in which an effect was demonstrated stresses the participation of END1-independent aspects of luteal regression and animal to animal variability as well.

In vivo and *in vitro* evidences have indicated that END1 is an important mediator of $PGF_{2\alpha}$-induced luteal regression. However, to test the role of END1 in spontaneous luteolysis in sheep using this experimental approach, luteal regression was effectively blocked by BQ-610 in only 25% of ewes in this study. Given the close anatomical relationship between uterine vein and ovarian artery in the sheep, delivery of BQ-610 into the uterine vein may be a more effective method to prevent luteolysis and extend luteal life span of the CL, as was demonstrated elsewhere for a luteotropin.

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