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Role of the Endothelin-1 System in the Luteolytic Process of Pseudopregnant Rabbits

Cristiano Boiti, Gabriella Guelfi, Gabriele Brecchia, Cecilia Dall'Aglio, Piero Ceccarelli, Margherita Maranesi, Chiara Mariottini, Danilo Zampini, Anna Gobbetti, and Massimo Zerani

Dipartimento di Scienze biopatologiche veterinarie, Sezione di Fisiologia, Laboratorio di Biotecnologie fisiologiche (C.B., G.G., G.B., M.M., C.M., D.Z.), and Sezione di Anatomia (C.D., P.C.), Università di Perugia, 06126 Perugia, Italy; and Dipartimento di Biologia molecolare, cellulare e animale (A.G., M.Z.), Università di Camerino, 62032 Camerino, Italy

The aim of this study was to better understand the role of the endothelin-1 (ET-1) system in the process of controlling the corpora lutea (CL) life span in rabbits. ET-1 (10 μg iv) administration at d 9 and 12 of pseudopregnancy induced a functional luteolysis within 24 h of injection, but it was ineffective at both d 4 and 6. Pretreatments with Bosentan, a dual ET_A/ET_B receptor antagonist, or cyclooxygenase (COX) inhibitor blocked the luteolytic action of ET-1 but not that induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In CL cultured *in vitro*, ET-1 increased ($P \leq 0.01$) both $PGF_{2\alpha}$ production and luteal nitric oxide synthase activity but decreased ($P \leq 0.01$) progesterone release. Addition of ET_A receptor antagonist BQ123 or COX inhibitor blocked the ET-1 luteolytic effects. Positive staining for ET-1 receptors was localized in ovarian blood vessels, granulosa cells of large follicles, and luteal cells. Immunoblot analysis of ET-1 receptor protein revealed a strong band of approximately 48 kDa in d-9 CL. Up to d 6 of pseudopregnancy, ET-1 mRNA abundance in CL was poorly expressed but then increased ($P \leq 0.01$) at d 9 and 13. ET_A-receptor transcript increased ($P \leq 0.01$) at d 6, remained at the same level up to d 13, and then declined to the lowest ($P \leq 0.01$) levels at d 22. ET_B-receptor mRNA increased ($P \leq 0.01$) throughout the late-luteal stage from d 13 up to d 18. Our data suggest that the luteolytic action of ET-1 may be a result of $PGF_{2\alpha}$ synthesis from both luteal and accessory cells, via the COX pathways. (*Endocrinology* 146: 1293–1300, 2005)

IN PSEUDOPREGNANT RABBITS, LUTEOLYSIS normally begins around d 14 and is completed a few days later when blood progesterone declines to basal values (1). In this species, as in many others, besides prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), several other factors are now recognized to regulate, via paracrine and/or autocrine mechanisms, the life span of corpora lutea (CL) from formation to regression (2–5).

An increasing amount of compelling evidence supports the hypothesis that endothelin-1 (ET-1), a potent vasoconstrictor synthesized by vascular endothelium, may promote the cascade mechanism of luteolysis triggered by $PGF_{2\alpha}$ (6, 7). ET-1 was found to inhibit progesterone release by granulosa and luteal cells of various species in a number of systems (8–11). In addition, highly specific receptors for ET-1 (ET-1R) were identified on steroidogenic luteal cells, and their abundance as well as ET-1 expression (12) increased during luteal demise (7, 11, 13). Moreover, $PGF_{2\alpha}$ injection rapidly increased ET-1 release within the regressing CL, as well as into the ovarian venous blood, in cattle (14). Conversely, ET-1 was found to enhance intraluteal $PGF_{2\alpha}$ production (15, 16), and this mechanism may have a critical role in complete luteal regression (17).

ET-1 was found to stimulate endothelial nitric oxide (NO) synthase (NOS) in different physiopathological conditions (18, 19), and the NO/NOS system is known to be involved in luteal regression induced by $PGF_{2\alpha}$ (20) as well as by ET-1 (21). However, despite the emerging impact of this vasoactive peptide on luteal function, the underlying ET-1-dependent mechanisms that control the life span of CL, and that are physiologically involved in the luteolytic process of the rabbit, are still unclear. Similarly, little is known about what mechanisms protect the CL from luteolysis until d 6 of pseudopregnancy, when CL shift from complete refractoriness to partial and complete responsiveness to $PGF_{2\alpha}$ treatment (22).

Thus, the main objectives of this study were to 1) localize, within the ovary of rabbits, the cell type distribution of ET-1R, 2) characterize the gene expression of both ET-1 and its receptor subtypes, ET_AR and ET_BR, in CL at different luteal stages throughout pseudopregnancy, and 3) verify the luteolytic action of ET-1 *in vitro* as well as *in vivo* at different days of pseudopregnancy. We further investigated, in the same animal model, the interactions of the ET-1 system with $PGF_{2\alpha}$ and NOS, using cyclooxygenase (COX) enzyme inhibitors and different receptor antagonists for ET-1.

Materials and Methods

Reagents

Random hexamer primers, deoxyribonuclease I (amplification grade), ribonuclease (RNase) H-reverse transcriptase (Superscript II), *Escherichia coli* RNase H, and DNA ladders were obtained from Invitrogen (S. Giuliano Milanese, Milan, Italy) as well as reagent for isolation and purification of total RNA (TRIzol), *Taq* DNA polymerase (Platinum), RNase-free tubes and RNase-free water, and deoxy-NTPs. Primers for 18S rRNA and corresponding competimers (QuantumRNA 18S internal standards) were acquired from Ambion (Austin, TX). Primers for mRNAs of ET-1R, ET_AR, and ET_BR were supplied by Maxim Biothec Inc. (San Francisco, CA). Tritiated hormones and [2,3-^{3}H]L-arginine, having

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Abbreviations: CL, Corpora lutea; COX, cyclooxygenase; DAB, 3,3′-diaminobenzidine tetrachloride; ET-1, endothelin-1; ET-1R, ET-1 receptor; NOS, nitric oxide synthase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; RNase, ribonuclease.

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a specific activity of 30–40 Ci/mmol, were purchased from Amersham Biosciences (Amersham Biosciences Ltd., Little Chalfont, UK), whereas progesterone and $PGF_{2\alpha}$ antisera and nonradioactive hormones came from Sigma Chemical Co. (St. Louis, MO). The NOS detection assay kit was purchased from Alexis Corp. (Läufelfingen, Switzerland). The primary monoclonal antibody anti-ET-1R, used for immunohistochemistry and protein blotting, was supplied by BD Biosciences (Franklin Lakes, NJ). The biotinylated secondary antibody, goat antimouse IgG used for immunohistochemistry, was purchased from Vector Laboratories (Burlingame, CA), whereas the horseradish peroxidase-conjugated chicken antimouse IgG, employed for Western blot, came from Santa Cruz Biotechnology (Santa Cruz, CA). The avidin-biotin complex (ABC, Vector Elite Kit) and the chromogen 3,3′-diaminobenzidine tetrachloride (DAB) were from Vector Laboratories. The kit for the assay of protein was purchased from Bio-Rad Laboratories (Hercules, CA). Nitrocellulose membranes, Protran BA 85, for Western blot analysis were obtained from Schleicher & Schuell (Dassel, Germany).

Incubation wells were obtained by Becton Dickinson Co. (Clifton, NJ). Medium 199 and Earle's balanced salt solution were from GIBCO (Grand Island, NY). HEPES and BSA were purchased from Sigma, whereas all other pure grade chemicals and reagents were obtained locally.

ET-1 was purchased from Calbiochem (San Diego, CA), whereas Bosentan (Ro 47-0203), a dual ET_AR/ET_BR antagonist, was kindly provided by Actelion Pharmaceuticals Ltd. (Allschwil, Switzerland). BQ123 and BQ788, specific receptor antagonists, respectively, for receptor type A and type B of ET-1 as well as COX inhibitors, acetylsalicylic acid, and indomethacin were all obtained from Sigma. The following hormonal preparations were administered via im injection: GnRH analog (Receptal, Hoechst-Roussel Vet, Milan, Italy), pregnant mare serum gonadotropin (Folligon, Intervet, Milan, Italy), and alfaprostol (Gabbrostim, Centralvet, Milan, Italy), a $PGF_{2\alpha}$ analog.

Animals, hormonal regimen, and tissue collection

Unmated New Zealand White rabbits, 5 months of age and weighing 3.5–3.8 kg, were caged individually in quarters of the University of Perugia Central Animal Facility and maintained under controlled conditions of light (14 h light, 10 h dark) and temperature (18 C). The animals were provided commercial rabbit chow and tap water for drinking *ad libitum*. All rabbits were treated with 20 IU pregnant mare serum gonadotropin followed 3 d later by an im injection of 0.8 μg GnRH to induce pseudopregnancy. The day of GnRH injection is designated d 0. This hormonal protocol was effective in inducing pseudopregnancy.

Rabbits were killed by cervical dislocation at the appropriate day of pseudopregnancy. Reproductive tracts, promptly removed from each animal, were washed throughout with saline. Within a few minutes, the CL were excised from ovaries and, after careful dissection of nonluteal tissue by fine forceps under stereoscopic magnification, immediately processed for *in vitro* studies and blotting analysis or, after rinsing with RNase-free PBS, frozen at −80 C for later evaluation of gene expression.

The protocols involving the care and use of the animals for these experiments were approved by the Bioethic Committee of the University of Perugia.

Immunohistochemistry of ET-1R

For the immunohistochemical detection of ET-1R, three animals were killed by cervical dislocation at d 9 of pseudopregnancy. The ovaries, immediately removed after killing, were quickly frozen in liquid nitrogen and then stored at −80 C. Serial 7-μm-thick frozen sections, mounted on poly-L-lysine-coated glass slides, were placed into cold acetone at a temperature ranging from −10 to −25 C for 10 min. All subsequent steps were carried out in a moist chamber at room temperature. The sections were first treated with a 0.5% solution of hydrogen peroxide in methanol for 10 min to inactivate the endogenous pseudoperoxidase activity and then rinsed with PBS solution and incubated for 30 min with normal goat serum to minimize the nonspecific binding of reagent in subsequent steps. The primary antiserum, mouse anti-ET-1R antibody (no. 611252) was diluted 1:50 in PBS and left on sections overnight. The next day, sections were washed in PBS and incubated with a biotinylated secondary antibody (1:200 in PBS) for 30 min. After PBS washes, sections were exposed to avidin-biotin-peroxidase complex (1:2500 in PBS) for 30 min, followed by the chromogen DAB for 5 min, to visualize the site of reaction. After washing in tap water, the ovary sections were dehydrated and mounted in Canada balsam natural. Positive reactions were recognized as reddish-brown precipitates. Sections in which the primary antibody was omitted or substituted by preimmune mouse γ-globulin were used for the negative control of nonspecific staining.

Western blot analysis

For the Western blot analysis of ET-1R, CL were harvested from two rabbits at d 9 of pseudopregnancy and processed as described previously (23). Briefly, total proteins were extracted from a pool of 10 CL that was homogenized in 1 ml ice-cold RIPA buffer (PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) with added protease inhibitor cocktail EDTA-free (Roche, Mannheim, Germany) using an Omni-μU mixer (Analytical Control, Dasit, Cinisello Balsamo, Milan, Italy). After incubation at 4 C for 20 min, the homogenates were centrifuged at 25,000 × *g* for 60 min at 4 C. The supernatants were collected and their protein concentrations measured using the protein assay kit with BSA as standard. After heating at 95 C for 5 min, equivalent amounts of proteins (60 μg) were separated using an electrophoresis system (Mini-Protean; Bio-Rad) by discontinuous 10% SDS-PAGE with 4% staking gel for 40 min at 200 V and 500 mA, after which proteins were transferred onto a nitrocellulose membrane for 1 h at 100 V and 350 mA. After transfer, membranes were blocked with PBS containing 0.5% casein. The membrane was incubated overnight at 4 C with anti ET-1R primary antibody diluted at 1:1000. Subsequently, membranes were probed with a chicken antimouse IgG-peroxidase antibody at 1:1000 dilution for 60 min at room temperature under gentle agitation. All antibody incubations were performed in PBS containing 0.5% casein and washing in PBS with 0.05% Tween 20. Immunoreactive bands were developed using DAB substrate kit for peroxidase at room temperature for 5 min. Prestained protein molecular weight markers (Bio-Rad) were used as molecular weight references. Blot images were acquired with an HP scanner.

RNA extraction and RT

To study the gene expression for ET-1, ET_AR, and ET_BR, CL were collected from five rabbits at d 4, 6, 9, 13, 15, 18, and 22 of pseudopregnancy. For each rabbit, total RNA was extracted from a pool of six to 10 CL, which was homogenized by Omni-μH in 1 ml of the solution provided with Trizol using the procedure as described by the manufacturer. Concentration of total RNA (OD_{260}), and purity ($OD_{260/280}$, $OD_{260/230}$) was determined spectrophotometrically (BioPhotometer, Eppendorf srl, Milan, Italy). The integrity of each sample was assessed by electrophoresis of an aliquot of 3 μg RNA in agarose formaldehyde gel using ethidium bromide staining. Genomic DNA contamination was prevented by treatment with deoxyribonuclease I according to instructions. Total RNA (5 μg; 1 μg/μl) was reverse transcribed into cDNA in a 20-μl final reaction mixture of iSCRIPT cDNA synthesis kit (Bio-Rad). Genomic DNA contamination was checked by carrying samples through the PCR procedure without reverse transcriptase. The RT products were stored at –20 C.

Muliplex RT-PCR amplification

An aliquot (1.0 μl) of cDNA was used as a template for the subsequent semiquantitative PCR amplification reaction. PCR (25.0 μl) was performed with 0.2 μl *Taq* DNA polymerase (5 U/μl), 1.0 μl dNTPs (10 mM), 5.0 μl 10× *Taq* buffer, 1.0 μl (10 μM) of both forward and reverse primers (Table 1) in two sets: the first for ET-1, ET_AR, and housekeeping gene 18S rRNA and the second for ET_BR and 18S rRNA. The semiquantitative PCR were carried out as reported previously (3). Preliminary experiments were carried out to establish the optimal ratio between 18S primers and their competimers. Between 30 and 40 cycles, both target and 18S products were in a linear exponential phase of amplification (data not shown). To minimize errors, within each experiment, the target gene was coamplified with housekeeping 18S primers at the same PCR cycle. Amplification was performed on a thermal cycler (GeneAmp, PCR System, PerkinElmer Biosystems, Foster City, CA). All PCR consisted of a first denaturizing cycle at 94 C for 75 sec, followed by an amplification profile of 35 reaction cycles with a first denaturizing cycle at 94 C for 15

TABLE 1. Primers for ET-1, ET_AR, ET_BR, and 18S used as internal standard

Gene	Length (bp)	Primers (5′–3′)
ET-1	193	
Sense		caa gcg gtg ctc ctg ctc ct
Antisense		gct cgt gca ctg gca cct gtt
ET_AR	263	
Sense		gcg tcg gga agt ggc aaa ga
Antisense		agc agc agc aga ggc acg ac
ET_BR	116	
Sense		cct cct ccg tgt cag aga cc
Antisense		att cgc agc agt gta gag ttc c
18S	489	
Sense		tcaagaacgaaagtcggaggtt
Antisense		ggacatctaagggcatca

sec, followed by annealing at 60 C for 30 sec and extension at 72 C for 45 sec, and a final extension step at 72 C for 10 min. Within each experiment and for each gene analyzed, the complete set of samples was processed in parallel in a single PCR, using aliquots of the same PCR master mix. Each set of determinations was performed in triplicate. The 18S rRNA is an ideal internal control for quantitative RNA analysis because its expression remains invariant across tissues and treatments. However, the relative high abundance of 18S rRNA makes it difficult to use in RT-PCR experiments because target mRNA species are by far less abundant. However, by mixing appropriate amounts of 18S primers with their competimers, primers modified only at their 3′ ends to block extension by DNA polymerase, the PCR amplification efficiency of 18S cDNA can be reduced to a level roughly similar to that of the gene under study.

Analysis of amplification products

The amplified PCR-generated products (20 μl of 25-μl total reaction volume) were analyzed by electrophoresis on 2% agarose gel using ethidium bromide staining. One product for each day of pseudopregnancy was electrophoresed on a single gel together with a negative control that contained no RNA and standard DNA ladder. The images of gels were acquired by using a Kodak DC290 digital camera. The background-corrected band intensities (absolute OD subtracted by the background levels from corresponding lanes) for each PCR product were quantified using Quantity One software (Bio-Rad). To evaluate the temporal changes in relative levels of mRNAs, the band intensities for the target genes of interest obtained from each aliquot of PCR products were normalized against those of the housekeeping 18S mRNA coamplified product in the same aliquot. Values were expressed as arbitrary units of relative abundance of the specific target genes.

In vivo induction of luteolysis

ET-1 was dissolved in saline at a concentration of 10 μg/ml and administered iv via a catheter inserted in the central ear vein. Bosentan was injected in the vein at a dosage of 10 mg/kg. Appropriate dilutions were prepared immediately before use, using sterile, double-distilled water. The solution was gently warmed at 50 C for a few minutes to completely dissolve the compound and then cooled at room temperature before use. The dosage protocol chosen for Bosentan was based on pharmacological data recently published (24). Indomethacin was injected im 1 h before ET-1 or $PGF_{2\alpha}$ administration.

Experiment 1. At different luteal stages, at d 4, 6, 9, and 12 of pseudopregnancy, two groups of rabbits (n = 5 per group per day) were treated with saline or 10 μg ET-1 dissolved in saline (1 ml), administered iv.

Experiment 2. On d 9 of pseudopregnancy, rabbits (n = 5 per group) were randomly assigned to receive one of the following treatments: saline, Bosentan (10 mg/kg), indomethacin (10 mg/kg), ET-1 (10 μg), ET-1 plus Bosentan, ET-1 plus indomethacin, $PGF_{2\alpha}$ (100 μg), $PGF_{2\alpha}$ plus Bosentan, and $PGF_{2\alpha}$ plus indomethacin.

From each rabbit, blood samples were collected by venipuncture of the marginal ear vein every 3 d during pseudopregnancy and just before (basal) and 2, 4, 8, 24, and 48 h after treatment. The samples, collected in EDTA-containing tubes, were immediately centrifuged at 3000 × *g* for 15 min, and the plasma was stored frozen until assayed for progesterone concentrations to assess the functional status of CL. Complete functional luteolysis was defined as a decline of plasma progesterone concentrations to values less than 1.0 ng/ml, which are normally observed in the estrous rabbit (25).

In vitro incubations

For the *in vitro* study, d-9 CL were randomly distributed (one CL per well) into incubation wells in 1 ml of culture medium 199 with Earle's balanced salt solution containing 2.2 mg/ml sodium bicarbonate, 2.3 mg HEPES, and 3% BSA, referred to here as M199. Before treatment, the CL were quartered inside each well using fine forceps. Each incubation set of wells was divided into 13 experimental groups of five wells as follows: 1) medium alone as control, 2) ET-1 (100 μM), 3) ET-1 plus ET_AR antagonist BQ123 (100 nM), 4) ET-1 plus ET_BR antagonist BQ788 (100 nM), 5) ET-1 plus both ET_AR and ET_BR antagonists, 6) ET_AR antagonist, 7) ET_BR antagonist, 8) both ET_AR and ET_BR antagonists, 9) ET-1 plus COX inhibitor acetylsalicylic acid (2 μM), 10) COX inhibitor, 11) $PGF_{2\alpha}$ (3 μM), 12) $PGF_{2\alpha}$ plus ET_AR antagonist, and 13) $PGF_{2\alpha}$ plus COX inhibitor. The culture plates were incubated at 37 C in air with 5% CO_2 as reported elsewhere (26). The media of each well were collected after 4 h of incubation and stored immediately at −20 C for later determination of progesterone and $PGF_{2\alpha}$. Each CL was weighed and stored immediately at −20 C for later determination of NOS activity. Preliminary evidence led to our choosing the incubation conditions and the minimum effective dose for ET-1 used in the present *in vitro* study (data not shown).

Progesterone and $PGF_{2\alpha}$ assays

Progesterone and $PGF_{2\alpha}$ concentrations were determined by RIA, using specific antibodies according to the procedure reported elsewhere (27). Progesterone and $PGF_{2\alpha}$ were extracted from corresponding samples with ethyl ether. For extraction, 0.1 ml plasma and 0.2 ml culture medium were used, and each sample was assayed in duplicate. The assay sensitivity was 0.08 ng/ml for progesterone and 19 pg/ml for $PGF_{2\alpha}$. Intra- and interassay coefficients of variations were 5.3 and 10.2% for progesterone and 8 and 12% for $PGF_{2\alpha}$.

NOS activity determination

NOS activity was determined in the same CL used for *in vitro* incubations by monitoring the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline using a commercial NOS assay kit according to the procedure previously described (27). Briefly, the CL of each well was homogenized in 1 ml of cold fresh homogenizing buffer (50 mM Tris, 1 mM EDTA, and 1 mM EGTA, pH 7.4) and centrifuged at 20,000 × *g* for 60 min at 4 C. Twenty-five microliters of supernatant and 100 μl of incubation buffer (1.5 mM NADPH, 1 mM $CaCl_2$) containing 150,000 dpm [^{3}H]L-arginine were added to the incubation tube. After 30 min of incubation at room temperature, the enzymatic reaction was stopped by the addition of 2 ml blocking buffer (20 mM HEPES, 2 mM EDTA, pH 5.5). The mixture was applied to a preequilibrated column (20 mM sodium acetate, 2 mM EDTA, 0.2 mM EGTA, pH 5.5; 1 cm diameter) containing 1 ml Dowex AG50W-X8 (Sigma), and the material was eluted with 2 ml water. [^{3}H]L-Citrulline was quantified in a liquid scintillation system (LS 1801; Beckman Instruments, Fullerton, CA). Additional determinations were performed in the presence of excess of NOS inhibitor (*N*-ω-nitro-L-arginine methyl ester; Sigma) to verify the specificity of the assay for production of [^{3}H]L-citrulline by NOS (data not shown). Protein concentration was determined by Bio-Rad protein assay kit.

Statistical analysis

The ratios of each PCR product for target genes (ET-1, ET_AR, and ET_BR) normalized against 18S coamplified product were analyzed by two-way ANOVA (day of pseudopregnancy and gel as the two sources of variability) and Newman-Keuls multicomparison posttest. Progesterone, $PGF_{2\alpha}$, and NOS data were evaluated by ANOVA for repeated measures using the general linear model procedure of the Statistical Analysis System Institute (28). Means obtained were compared using a Student's *t* test (option PDIFF).

Results

ET-1R immunolocalization in rabbit ovary and Western blot analysis

Using a monoclonal antibody, evident positive staining for ET-1R was localized in both endothelial and smooth muscle cells of large ovarian blood vessels (Fig. 1A). Clear positive staining was also identified in granulosa cells of large follicles, in smooth muscle cells of the external theca, and in some steroidogenic cells of the interstitial gland (Fig. 1B). Only weak positive reaction was localized in luteal cells and in smooth muscle cells of the luteal wall (Fig. 1C). Staining was completely abolished when the primary antibody was substituted with nonimmune serum (Fig. 1D).

Western blot analysis of ET-1R protein revealed a strong band of approximately 48 kDa from whole protein extracts of all the rabbit CL examined, whereas no band was visible when incubation with the primary antibody was omitted (data not shown).

Gene expression for luteal ET-1, ET_AR, and ET_BR mRNAs

The expression of 18S rRNA in rabbit CL remained fairly constant, independently of the luteal phases. The corresponding base pair amplification products obtained using primers designed for each gene matched the expected size (Figs. 2 and 3, *bottom*s).

Up to d 6 of pseudopregnancy, ET-1 mRNA abundance was poorly expressed but then increased ($P \leq 0.01$) at d 9 and 13 (Fig. 2, *upper left*). Thereafter, ET-1 transcript dropped again to the low levels of the early luteal stage with an increase ($P \leq 0.05$) at d 22.

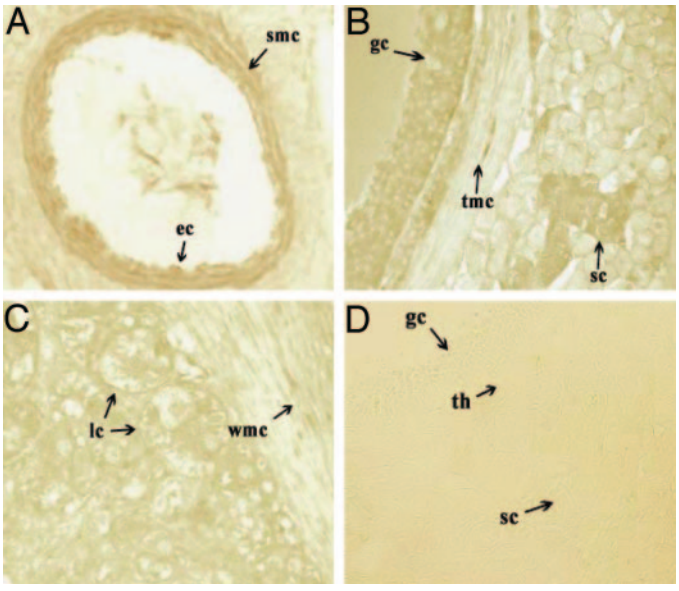

FIG. 1. Cellular localization of ET-1R in the ovary of rabbits obtained at d 9 of pseudopregnancy. A, Strong positive staining in endothelial cells (ec) and in smooth muscle cells (smc) of large ovarian vessels (magnification, ×1000); B, a clear positive reaction in granulosa cells (gc) of a large follicle, in smooth muscle cells of the external theca (tmc), and in some steroidogenic cells (sc) of the interstitial gland (×500); C, a weak expression of ET-1R in the foamy cytoplasm of luteal cells (lc) and in smooth muscle cells of the luteal wall (wmc) (×1000); D, a control section in the absence of primary antibody with no reaction in granulosa cells of a large follicle, theca (th), and steroidogenic cells of the interstitial gland (×500).

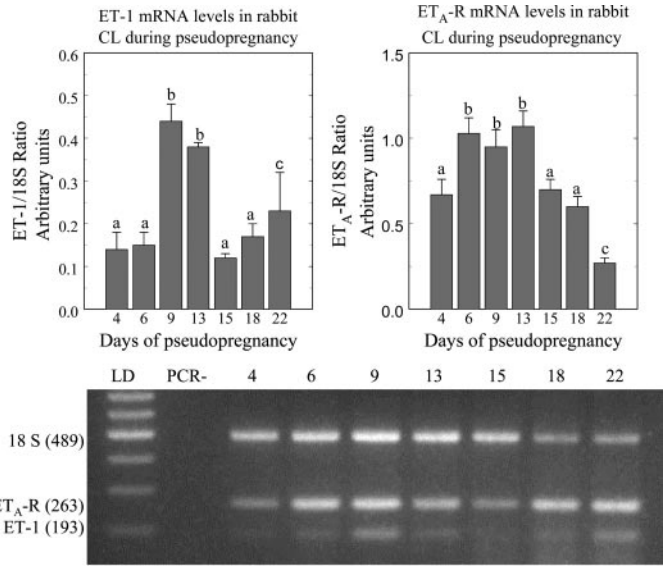

FIG. 2. Gene expression patterns of ET-1 and ET_AR mRNAs in CL of rabbits freshly collected at d 4, 6, 9, 13, 15, 18, and 22 of pseudopregnancy. *Bottom,* Representative photograph of a typical 2% agarose, ethidium bromide-stained gel, showing the presence of the expected base pair products yielded after RT-PCR using primers for target ET-1, ET_AR, and 18S. Lane LD is the kilobase DNA marker, lane PCR– represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification, whereas the other lanes identify the corresponding days of pseudopregnancy. *Top*, Summary of the data (means ± SEM) derived from densitometric analyses of the ET-1 (*left*) and ET_AR (*right*) in CL reported in arbitrary units relative to 18S expression. The values combine the results from five different rabbits for each luteal age. *Different letters above bars* indicate a significantly different value (a and b, $P \leq 0.01$; c, $P \leq 0.05$).

Over the study period, ET_AR transcript in CL increased ($P \leq 0.01$) from d 4 to d 6 of pseudopregnancy, remaining at the same level up to d 13, and then gradually declined to reach the lowest ($P \leq 0.01$) levels at d 22 (Fig. 2, *upper right*). ET_BR mRNA relative abundance did not show any clear pattern in its steady-state up to d 9 of pseudopregnancy when it increased ($P \leq 0.01$) throughout late-luteal stage up to day 18 (Fig. 3, *upper*).

In vivo response to ET-1 treatment

In control rabbits, blood progesterone did not decline during the following 48 h after saline injection, ranging between 6 and 14 ng/ml according to the day of pseudopregnancy (Fig. 4). ET-1 was totally ineffective when administered at d 4 or 6 of pseudopregnancy as evidenced by progesterone profiles closely overlapping those of corresponding controls (Fig. 4, *upper left* and *right*, respectively). Conversely, at both d 9 and 12, ET-1 induced a functional luteolysis within 24 h after injection when plasma progesterone declined ($P \leq 0.01$) to values less than 1 ng/ml (Fig. 4, *lower left* and *right*, respectively).

At d 9 of pseudopregnancy, the time course of the fall in peripheral plasma progesterone evoked by ET-1 (Fig. 5A) closely paralleled that found in $PGF_{2\alpha}$-treated rabbits (Fig. 5B). Pretreatment with Bosentan, an antagonist for both

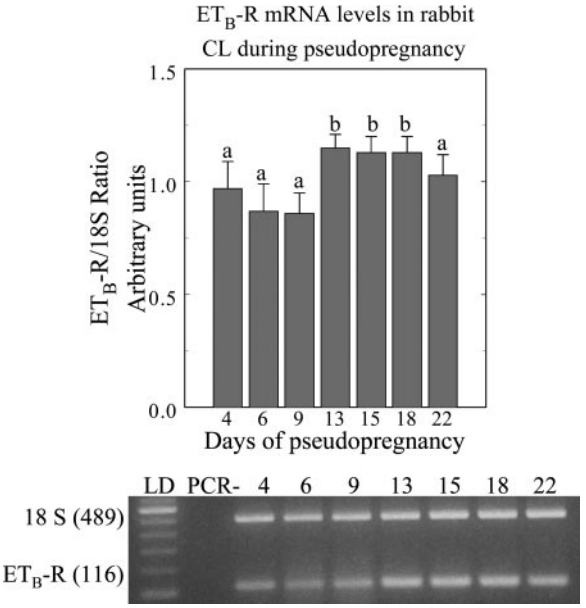

FIG. 3. Gene expression patterns of ET_BR mRNAs in CL of rabbits collected at d 4, 6, 9, 13, 15, 18, and 22 of pseudopregnancy. *Bottom*, Representative photograph of a typical 2% agarose, ethidium bromide-stained gel, showing the presence of the expected base pair products yielded after RT-PCR using primers for target ET_BR and 18S. Lane LD is the kilobase DNA marker, lane PCR– represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification, whereas the other lanes identify the corresponding days of pseudopregnancy. *Top*, Summary of the data (means ± SEM) derived from densitometric analyses of the ET_BR in CL reported in arbitrary units relative to 18S expression. The values combine the results from five different rabbits for each luteal age. Different *letters* above *bars* indicate a significantly different value ($P \leq 0.01$).

ET_AR and ET_BR, completely abolished the luteolytic action of ET-1 at d 9 of pseudopregnancy (Fig. 5A), but did not prevent that evoked by $PGF_{2\alpha}$ (Fig. 5B). The ET-1-induced functional luteolysis at d 9 of pseudopregnancy was also inhibited when rabbits were pretreated with the COX inhibitor indomethacin (Fig. 5A). In contrast, indomethacin pretreatment did not influence the luteolytic action of $PGF_{2\alpha}$ (Fig. 5B).

Treatment with both Bosentan or indomethacin alone did not modify the luteal activity and the life span of CL during the following 48 h (data not shown).

In vitro cultured CL

ET-1 decreased ($P \leq 0.01$) progesterone release in CL cultured *in vitro* (Fig. 6A) but increased ($P \leq 0.01$) $PGF_{2\alpha}$ secretion and NOS activity (Fig. 6, B and C, respectively). Coincubation of ET-1 with the ET_AR antagonist alone (Fig. 6) or with both ET_AR and ET_BR antagonists (data not shown) completely abolished the ET-1-dependent effects on both hormones and NOS activity. Conversely, ET_BR antagonist did not affect the ET-1 actions (Fig. 6). COX inhibitor blocked the luteolytic action of ET-1 on progesterone release, reduced ($P \leq 0.01$) $PGF_{2\alpha}$ secretion, and markedly down-regulated ($P \leq 0.01$) the NOS activity (Fig. 6). Treatment with $PGF_{2\alpha}$ alone or with ET_AR antagonist mimicked the same effects induced by ET-1 on both progesterone production and NOS luteal activity (Fig. 6). These $PGF_{2\alpha}$-dependent effects were not influenced by addition of COX inhibitor (Fig. 6, A and C).

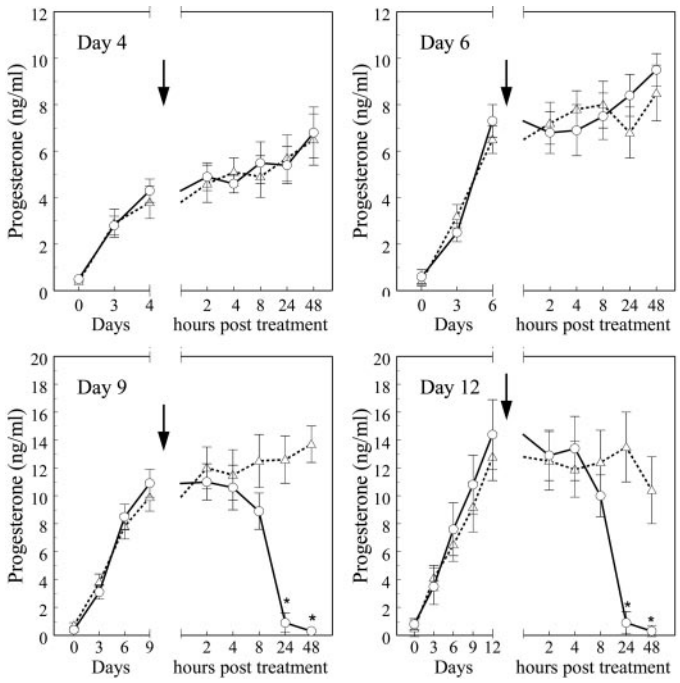

FIG. 4. Plasma progesterone levels after injection (*arrow*) of saline (*dotted line*) or 10 μg endothelin-1 (*solid line*) at d 4, 6, 9, and 12 of pseudopregnancy. Values are mean ± SEM for five animals per group. *, Significantly different from control at $P \leq 0.01$.

COX inhibitor decreased ($P \leq 0.01$) $PGF_{2\alpha}$ release and NOS activity in CL cultured *in vitro* (data not shown). ET_AR and ET_BR antagonists alone or together did not affect hormonal and enzymatic responses (data not shown).

Discussion

The present study demonstrates the cell localization of ET-1R within rabbit ovary, its protein expression in whole CL extracts, and the dynamic regulation of ET-1, ET_AR, and ET_BR genes in the whole course of pseudopregnancy. Moreover, by studying the luteolytic action of ET-1, our *in vivo*

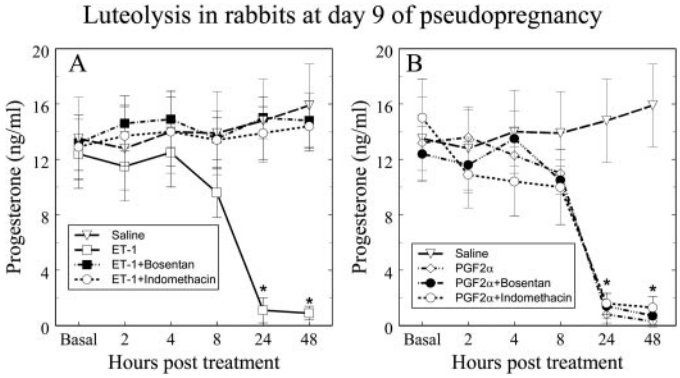

FIG. 5. A, Plasma progesterone levels just before and after injection of saline (control), ET-1 (10 μg), ET-1 plus Bosentan (10 mg/kg), a nonselective ET_AR/ET_BR antagonist, or ET-1 plus indomethacin (10 mg/kg) given at d 9 of pseudopregnancy; B, progesterone concentrations after administration of $PGF_{2\alpha}$ (100 μg of alfaprostol), $PGF_{2\alpha}$ plus Bosentan, or $PGF_{2\alpha}$ plus indomethacin. Values are mean ± SEM for five animals per group. *, Significantly different from control at $P \leq 0.01$.

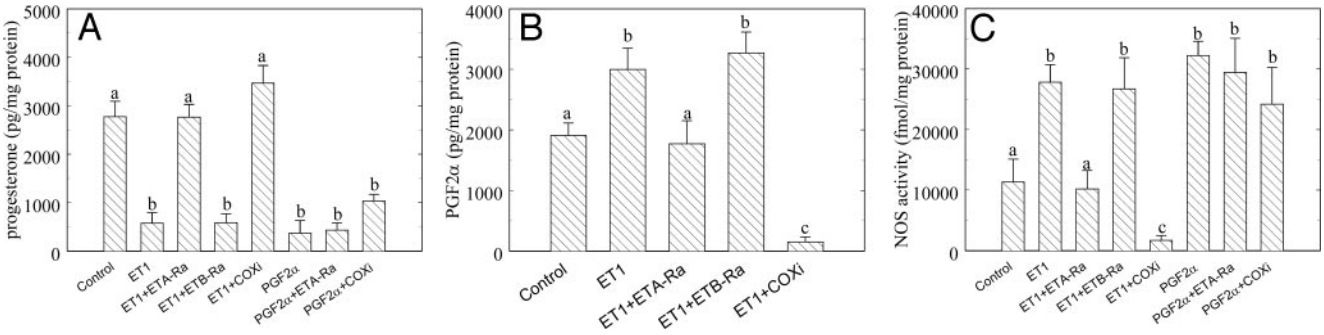

FIG. 6. *In vitro* effects of medium alone, ET-1, ET-1 plus ET_AR antagonist (ET_ARa), ET-1 plus ET_BR antagonist (ET_BRa), ET-1 plus COX inhibitor (COXi), $PGF_{2\alpha}$, $PGF_{2\alpha}$ plus ET_ARa, and $PGF_{2\alpha}$ plus COXi on both progesterone (A) and $PGF_{2\alpha}$ (B) releases and total NOS activity (C) by rabbit CL obtained at d 9 of pseudopregnancy. Results are the means ± SD of five replicates. *Different letters above the bars* indicate significantly different values ($P \leq 0.01$).

results showed that early-luteal-stage CL, at d 4 and 6 of pseudopregnancy, are completely refractory to this vasoactive peptide.

The ET-1 binding in the ovary has been characterized in several species but, to date, not in rabbits. In the present study, using an immunohistochemical technique, strong positive staining was found in the large ovarian blood vessels as well as in the granulosa cells of large follicles and in some steroidogenic cells of the interstitial gland. Conversely, luteal cells showed a weak reaction. By means of autoradiography, ET_AR-binding sites were detected on blood vessels of porcine CL and in the granulosa cell layer of the maturing Graafian follicles (29). Additional work, using RT-PCR on isolated bovine luteal cells, recognized ET_AR expression not only on endothelial cells but also on steroidogenic luteal ones, both large and small, although at a lower level (11). Both ET_AR and ET_BR messengers were found in luteinized human (8) and rat granulosa cells (30). Western blotting of the whole protein fraction derived from luteal extracts yielded a strong band at 48 kDa when probed with a monoclonal antibody generated by rat ET-1R; this was in good agreement with results obtained in rat lungs (31).

Our results indicate that genes for ET-1 and for both its receptor subtypes, ET_A and ET_B, are expressed, although at different levels, in CL of rabbits throughout the course of their life span, from early- to late-luteal stages across CL demise up to d 22 of pseudopregnancy. This observation is not surprising given that, within the rabbit CL, the endothelial compartment contains approximately 30–40% whole cells (32).

Gene expression of ET-1 increased with aging of CL from early- to mid-luteal stages, at d 9 and 13 when a rise occurred. Thereafter, relative ET-1 mRNA abundance dropped, in coincidence with the fall of plasma progesterone concentrations toward basal values, and rose again after luteal regression. In the rat luteal tissue, the highest ET-1 protein levels were found in the mid-luteal phase of pseudopregnancy (33), which is in good agreement with our ET-1 mRNA results. Conversely, in bovine CL, the ET-1 mRNA declined during both mid- and late-luteal phases of the estrous cycle (34).

ET_AR transcript was relatively highly expressed in the mid-luteal phase but then gradually declined in the following days during the transition from late-luteal stage to functional regression. Surprisingly, however, ET_AR mRNA levels were relatively up-regulated also at d 6 of pseudopregnancy, when exogenous administration of ET-1 was ineffective in provoking luteolysis. Thus, there is no clear relationship between the luteal expression of ET-1 type A receptor and its responsiveness to ET-1 *in vivo*. Contrary to what was found in cows (11, 34), in our study there was a direct relationship between ET_AR gene expression and peripheral blood progesterone concentrations, which are a good marker of luteal function. The transiently high ET_AR mRNA levels between d 6 and 13 of pseudopregnancy suggests, although indirectly, that the corresponding receptors for ET-1 may be locally regulated within the CL. It remains to be established, however, what factor is actually involved, whether progesterone, ET-1 itself, or other substances.

In the present study, the ET_BR mRNA increased during late-luteal phase and during luteal regression, which is in good agreement with previous observations in bovine CL (34). Although the role of ET_BR in CL is not well documented, our data suggest that the ET_BR subtype may take part in the whole luteolytic cascade related to structural demise rather than to progesterone decline (13).

In the rabbit, the luteolytic action of ET-1 *in vivo* was clearly dependent on CL age. The 4- and 6-d-old CL remained unaffected by the same luteolytic dose of ET-1 as that which caused a striking reduction of luteal function at d 9 and 12. The reasons for this time-dependent responsiveness are still unclear, given that both ET-1R subtypes are already expressed, at the gene level, from d 4 of pseudopregnancy at approximately the same relative abundance as that found in older CL. By profiling progesterone in blood, ET-1-induced luteolysis, when effective at d 9 and 12, progressed similarly to that triggered by $PGF_{2\alpha}$ (22). Furthermore, ET-1-induced luteolytic action *in vivo* was prevented by antagonizing receptors for ET-1 with Bosentan, an inhibitor of endothelin receptor types A and B, or by blocking COX activity with indomethacin.

In vitro, whereas ET-1 inhibited basal progesterone secretion from CL, it stimulated $PGF_{2\alpha}$ release, similarly to that found in bovine luteal cells (15) and luteinized human granulosa cells (8). All these ET-1-dependent actions were abolished by the ET_AR highly selective antagonist BQ123 and not by the ET_BR antagonist, which is in good agreement with other studies (8, 9). The luteolytic action of ET-1 was also

counteracted by treatment with COX inhibitor, consistently with our *in vivo* findings.

Collectively, the *in vivo* and *in vitro* findings presented here strongly support the hypothesis that ET-1 may be part of an amplifying cascade system that induces and sustains CL regression in rabbits, its antisteroidogenic action being activated via an ET_AR-mediated mechanism involving $PGF_{2\alpha}$ synthesis. In fact, the luteolytic action of ET-1 was prevented *in vivo* and *in vitro*, not only by administration of ET-1R antagonist, as expected, but also by treatments with COX inhibitors. Thus, coherent evidence suggests that the inhibitory action of ET-1 on progesterone release by CL may be a result of an enhancement of $PGF_{2\alpha}$ synthesis both from luteal and accessory cells, via the COX pathways.

Taken together, these results suggest a physiological role for the ET-1 system within this transient organ. In fact, there is increasing evidence that ET-1 can modulate the angiogenesis process both directly and indirectly through the vascular endothelial growth factor (35). The ET-1 system may play a role in the early developing CL by driving luteal cell growth in a paracrine fashion through transcriptional mechanisms (36); in fact, evidence for a cross-talk between endothelial and steroidogenic cells in the control of luteal functions has been found in ruminants (5, 37, 38). ET-1 may also act as a local mediator or promoter of CL regression by governing intraluteal $PGF_{2\alpha}$ production (7, 16), a mechanism that has been recognized to have a critical role for complete luteal demise (17). Thus, ET-1 may be responsible for the increased sensitivity of rabbit CL to luteolytic factors that occur at the proper timing for the spontaneous luteolysis to progress (39).

However, whereas ET-1-induced functional luteolysis appears to be linked to the prostanoid pathway, $PGF_{2\alpha}$, apparently, does not require ET-1 to elicit its luteolytic action, because pretreatment with the ET-1R antagonists did not prevent functional luteal demise from occurring both *in vivo* and *in vitro*. These results differ from those previously found in ewes and cows, which provided evidence for claiming that $PGF_{2\alpha}$-induced luteolysis is mediated by ET-1 (9, 10). In these species, in fact, pretreatment with ET_AR antagonists greatly mitigated the luteolytic effects of $PGF_{2\alpha}$. In ruminants, a strong positive feedback system between luteal ET-1 and $PGF_{2\alpha}$ has been described in which ET-1 increases $PGF_{2\alpha}$ synthesis and release, whereas $PGF_{2\alpha}$ up-regulates luteal gene and protein expression of ET-1 concomitantly with the decline of progesterone production by the CL (7, 10). Although the reciprocal interactions between $PGF_{2\alpha}$ and ET-1 in rabbits remain to be disclosed, the results of the present work suggest, although indirectly, that other mediators, such as angiotensin II (40) and leptin (41), may be involved in the regulation of CL regression. Of note, in our experimental model, both *in vivo* and *in vitro* pretreatments with COX inhibitors did not modify the time course of the luteolytic process primed by the exogenous administration of $PGF_{2\alpha}$. These findings, however, are not in conflict with the current view that an autoamplification pathway, involving $PGF_{2\alpha}$-induced production of $PGF_{2\alpha}$ by the CL, may be responsible for complete luteolysis (5), because in our animal model we employed a high luteolytic dose of $PGF_{2\alpha}$.

The present results show that ET-1 enhanced the enzymatic activity of NOS, via ET_AR activation mediated by $PGF_{2\alpha}$, given that both BQ123 and COX inhibitor abolished its functional up-regulation. Nitric oxide is deeply involved in the regulation of rabbit CL function, because PGE_2 and $PGF_{2\alpha}$ luteotrophic and luteolytic effects are mediated by NOS down- and up-regulation, respectively (27).

Even if the luteolytic outcome of the *in vitro* and *in vivo* experiments here described was the same, the mechanism activated by ET-1 treatment leading to a progesterone fall may not necessarily be identical. The luteolytic effect of ET-1 *in vivo*, for example, can be ascribed to its potent vasoconstrictor action, which may cause a critical reduction in ovarian blood flow and oxygen supply to CL. Therefore, it would be of interest to study the dynamic of blood flow in the ovaries of rabbits treated with ET-1. In fact, the vasoconstrictor action of ET-1 could be blunted or even reversed by the local release of vasodilatory factors, including NO and prostacyclin, induced by ET-1 itself after binding to its receptors localized in both endothelial and vascular smooth muscle cells (42). Interestingly, years ago, $PGF_{2\alpha}$, despite its vasoconstrictor property, was found to cause a vasodilation of the ovarian stroma in rabbits (43, 44). More recently, similar results were also found in CL of mid-cycle cows given that $PGF_{2\alpha}$ *in vivo* induced an acute blood flow increase followed by a decrease (45).

In conclusion, the presence of putative receptors for ET-1 in the vascular components and luteal cells suggests that the ET-1 system is involved in the regulation of ovarian blood flow and in steroidogenesis as well. The luteolytic effect of ET-1 in the rabbit is mediated by a complex interaction with $PGF_{2\alpha}$ and the NO/NOS system, whose up- and downstream pathways after ET-1 binding have not yet been elucidated. At present, taken together, our results suggest that the luteolytic action of ET-1 can be ascribed either to vascular mechanisms, because of its potent vasoconstrictor action that causes a critical reduction in blood flow to ovary and CL, and/or to activation of the $PGF_{2\alpha}$ cascade mechanism mediated by NOS. It remains to be established what factors actually protect CL against undergoing luteolysis prematurely, as well as the intracellular signaling cascade used by ET-1 in rabbit CL.

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Address all correspondence and requests for reprints to: Dr. Cristiano Boiti, Dipartimento di Scienze biopatologiche veterinarie, Università of Perugia, S. Costanzo 4, 06126 Perugia, Italy. E-mail: cristiano.boiti@unipg.it.

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