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Oestrogen and Progesterone Receptors and COX-2 Expression in Endometrial Biopsy Samples During Maternal Recognition of Pregnancy in Llamas (*Lama glama*)

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Contents

Endometrial expression of oestrogen receptor-α (ERα), progesterone receptor (PR) and cyclooxigenase-2 (COX-2) was evaluated in non-pregnant and pregnant llamas during the period when luteolysis/maternal recognition of pregnancy is expected to occur. Females (n = 28) were divided into two groups: non-pregnant llamas were induced to ovulate with a Buserelin injection, and endometrial biopsies were obtained on day 8 (n = 5) or 12 (n = 5) post-induction of ovulation. Animals of the pregnant group (n = 18) were mated with a fertile male. Pregnancy was confirmed by the visualization of the embryo collected by transcervical flushing in 5 of 9 animals on day 8 post-mating and by progesterone profile on day 12 post-mating in 4 of 9 animals, when endometrial biopsies were obtained. An immunohistochemical technique was used to evaluate receptors population and COX-2 expression. Pregnant llamas showed a higher percentage of positive cells and stronger intensity for ERα than for non-pregnant llamas in stroma on day 8 and in the luminal epithelium on day 12 post-induction of ovulation, while a deep decrease in endometrial PR population was reported in pregnant llamas on that day in luminal and glandular epithelia and stroma. In the luminal epithelium, COX-2 expression was lower in pregnant than in non-pregnant animals. Briefly, the increase of ERα in pregnant llamas gives further support to the hypothesis that oestrogens are involved in the mechanism of maternal recognition of pregnancy. Endometrial PR decrease in pregnant llamas might be a necessary event to allow the expression of proteins involved in conceptus attachment, a mechanism widely accepted in other species. Moreover, embryo seems to attenuate maternal $PGF_{2\alpha}$ secretion during early pregnancy by decreasing the endometrial expression of COX-2 in the luminal epithelium of pregnant llamas.

Introduction

Maternal recognition of pregnancy can be considered as the interaction between the maternal unit and the conceptus (embryo and its associated membranes) which involves paracrine regulation by the conceptus of the underlying endometrium that leads to an attenuation of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion (Thatcher et al. 1995). In bovines and ovines, the embryo synthesizes and secretes interferon τ (IFNτ) which acts in a paracrine fashion on the endometrial luminal epithelium and superficial glandular epithelium to suppress the transcription of both oestrogen receptor-α (ERα) and oxytocin receptor (OxR) (ovine: Spencer et al. 1995) or to suppress just OxR expression (bovine: Robinson et al. 1999). The mechanism proposed in pigs is different, as conceptuses secrete increased levels of oestrogens which redirect $PGF_{2\alpha}$ secretion from the uterine venous drainage (endocrine) to the uterine lumen (exocrine) (Bazer and Thatcher 1977). Besides, it has been recently proposed that the pig conceptuses contribute to prevent luteolysis by changing prostaglandin synthesis in favour of the luteoprotective prostaglandin E_2 (Waclawik 2011). The signal of maternal recognition of pregnancy in horses has not been completely defined. Equine conceptuses secrete an appreciable amount of oestrogens which increase with embryo development (Heap et al. 1982; Choi et al. 1997). The presence of the conceptus reduces pulsatile $PGF_{2\alpha}$ secretion by the endometrium during early gestation in the mare, partly attributed to the reduced expression of cyclooxygenase-2 (COX-2) (Boerboom et al. 2004). Besides, embryo mobility has been proved to be essential for the establishment of pregnancy in horses (McDowell et al. 1988).

Female llamas are induced ovulators. After ovulation, a corpus luteum develops and, in non-pregnant llamas, plasma progesterone concentrations start to increase around day 4 and peak at day 8, and around day 9–10 post-induction of ovulation, progesterone levels start to decrease in response to $PGF_{2\alpha}$ pulses (Aba et al. 1995). It has been recently reported that the loss of progesterone receptors (PR) and a concomitant increase in ERα and COX-2 expression occur in the luminal epithelium of the endometrium at the time of luteolysis in llamas (Bianchi et al. 2013).

Pregnancy establishment in camelids requires prolongation of luteal life span and progesterone production (Sumar 1988; Leon et al. 1990). It has been demonstrated that in pregnant llamas, a transient decrease (between days 7 and 15 post-mating) and subsequent recovery in progesterone concentrations occur during the period when maternal recognition of pregnancy is expected to take place (Aba et al. 1995, 2000). The nature of the conceptus signal for the establishment and maintenance of pregnancy has not been completed

elucidated in this species. Unlike ruminant species, camelids conceptus does not produce any interferon-like protein molecules (Leaman et al. 1992) but, like the pigs and horses embryos, it secretes appreciable quantities of oestrogens which are proposed as the signal released by the conceptus during early pregnancy (Powell et al. 2007a). Besides, to our knowledge, there is not information about the regulation of endometrial steroid receptors population and COX-2 expression during the expected time of maternal recognition of pregnancy in llamas. Thus, the aim of the present study was to characterize the population of ERα and PR and COX-2 expression in the endometrium of non-pregnant and pregnant llamas on days 8 and 12 post-induction of ovulation. Furthermore, due to the fact that almost all pregnancies are carried out in the left uterine horn, the expression of the steroid receptors and COX-2 were studied in the right and left horn.

Materials and Methods

Field studies were performed in compliance with animal welfare regulations set by the Faculty of Veterinary Sciences, UNCPBA, where activities were conducted. Facilities are located in Tandil, Argentina, at 37°S, 60°W. Llamas (n = 28) were kept in pens and fed pasture hay and water *ad libitum*. Animals were examined daily by transrectal ultrasonography (Mindray DP-6600 Vet with 5.0/7.5 variable traducer probe) until a follicle with a diameter ≥8 mm, considered ovulatory in this species (Bravo et al. 1991), was observed. At that moment, females were divided into two groups: non-pregnant (n = 10) and pregnant llamas (n = 18). In the non-pregnant group, ovulation was induced with a single intravenous injection of Buserelin (GnRH analogue; 8.4 μg Receptal®, Intervet, Buenos Aires, Argentina), while the animals of the pregnant group were mated twice, 24 h apart, with a fertile male.

Occurrence of ovulation was assessed based on ultrasonographic visualization of the disappearance of the dominant follicle and the later formation of a corpus luteum and further confirmed by the progesterone profiles.

Pregnancy was confirmed by the visualization of the embryo obtained by transcervical flushing on day 8 post-mating (n = 9) as previously reported (Trasorras et al. 2010) or by progesterone profile on day 12 post-mating (n = 9). Plasma progesterone concentrations above 3.2 nmol/l on day 12 post-mating were indicative of pregnancy (Sumar 1996).

Endometrial samples were obtained by transcervical biopsies as previously described (Bianchi et al. 2010). Depending on the day of biopsy obtaining, each group was further divided into two subgroups: non-pregnant day 8 (NP8) and day 12 (NP12) and only animals with confirmed pregnancy conformed the pregnant group day 8 (P8; n = 5) and day 12 (P12; n = 4) post-mating. In each sampling occasion, endometrial samples were taken from the middle of the left and right horn. Immediately after collection, tissue samples were fixed in 4% paraformaldehyde and then embedded in paraffin until analysis.

Blood samples were collected every second day from the day of induction of ovulation (day 0) to the day of endometrial biopsy (day 8 or day 12 post-induction of ovulation). Samples were centrifuged, and plasma was stored at −20°C until plasma progesterone concentrations were determined.

Progesterone determinations

Progesterone was measured using an RIA kit (COAT-A-COUNT®, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) previously validated for its use with llama plasma (Bianchi et al. 2007). The sensitivity of the assay was 0.3 nmol/l, and the intra-assay coefficient of variation was below 14% for concentrations between 0.4 and 128 nmol/l. All samples were measured in duplicates and in one single assay. Hormone concentrations are expressed in SI units. To convert from nmol/l to ng/ml, the following factor should be used: 3.2.

Immunohistochemistry

An immunohistochemical technique (avidin–biotin–peroxidase) previously described (Bianchi et al. 2013) was used to visualize ERα, PR and COX-2 immunostaining. After the paraffin tissue sections (5 μm) were dewaxed and rehydrated, an antigen retrieval procedure was performed. Sections were pre-treated in a microwave oven at 700 watts power and in 0.01 M sodium citrate buffer (pH 6.0) for 10 min and then allowed to cool for 20 min. After washing in buffer, non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 10 min at room temperature (RT). After a 10-min wash in buffer, sections were exposed to a 30-min non-immunoblock using diluted Normal Horse (ERα and PR) or Goat (COX-2) Serum (Vectastain®; Vector Laboratories, Burlingame, CA, USA) in buffer in a humidified chamber at RT. The primary antibody used for the detection of ERα (monoclonal mouse antibody; Cat No. sc-787, Santa Cruz, CA, USA), of PR (monoclonal mouse antibody; Zymed Cat No. 18-0172, South San Francisco, CA, USA) and of COX-2 (polyclonal murine antibody; Cayman Chemical; Cat No. 160106, MI, USA) were diluted 1 : 25, 1 : 100 and 1 : 200 in PBS, respectively, and incubated for 1 h. Negative controls for each receptor were obtained by replacing the primary antibody with normal mouse IgG at equivalent concentration (Santa Cruz, Cat No. sc-2025, CA, USA). After primary antibody binding, the sections were incubated for 60 min at RT with a biotinylated IgG (Vectastain®, Vector Laboratories) diluted in Normal Horse (ERα and PR) or Goat (COX-2) Serum. Thereafter, the tissue sections were incubated for 60 min with a horseradish peroxidase–avidin–biotin complex (Vectastain® Elite ABC-kit, Vector Laboratories, Cat No. PK-6100). The site of the bound enzyme was

visualized by the application of 3,3′-diaminobenzidine (DAB kit; Vector Laboratories, Cat No. SK 4100), a chromogen that produces a brown, insoluble precipitate when incubated with the enzyme. The sections were counterstained with haematoxylin and dehydrated before they were cover slipped with mounting medium (Biopack, Argentina).

Three to six sections from each biopsy (depending on the size of each specimen) were mounted per slide, and there were always a positive and a negative control included for each assay.

Image analysis

After a general inspection of each slide, a subjective image analysis was performed to estimate the expression of ERα, PR and COX-2 in different cell types as previously reported (Bianchi et al. 2007; Sosa et al. 2009). The evaluation was performed by two independent observers who were not aware of assignment to group of animals. Ten fields were analysed for each cell type (luminal and glandular epithelia and stroma) at a magnification of 1000× in all samples. The total area of positively stained cell nuclei (brown reaction product) was measured and expressed as a ratio of the total area of cell nuclei (brown reaction product + blue haematoxylin) (Bianchi et al. 2010). The staining of the nuclei was scored as being negative (−), faint (+), moderate (++) or intense (+++), and the staining of each cell type was in proportion on a scale of 0–10 (Thatcher et al. 2003). The average staining was calculated as $= 1 \times n1 + 2 \times n2 + 3 \times n3$, where n = proportion of cells per field exhibiting faint (1), moderate (2) and intense (3) staining (Boos et al. 1996).

Statistical analysis

Variables from the image analysis that were considered for statistical evaluation of data were the average of total positive area (percentage of the immunoreactive area) and the average staining of the 10 fields. Statistical analysis was conducted using the Statistical Analysis System V9.1 (SAS, Institute Inc., Cary, NC, USA). Immunostaining was analysed by the GLM procedure, and the statistical model included the effects of observers, horns, status (pregnant vs non-pregnant), days and cell types. As no significant differences were observed between observers and horns for ERα, PR and COX-2, data were pooled and only the interactions between status, days and cell types were used.

Data from plasma progesterone concentrations were analysed by one-way analysis of variance (ANOVA) using a repeated measures design to determine differences between means, and the Tukey–Kramer multiple comparison test was performed for the evaluation of significance.

Results are expressed as least square means ± pooled standard errors. The level of significance was always $p < 0.05$.

Results

Pregnancy rates and hormone profiles

Within the group of animals mated with a fertile male, the percentages of pregnant llamas were 55.6% (5/9) for P8 and 44.4% (4/9) for P12 groups, respectively.

Plasma progesterone concentrations were above 4.5 nmol/l in all animals on day 6, in relation to the development of a corpus luteum. Mean plasma progesterone concentrations were 10.2 and 15.4 nmol/l ($p = 0.09$) on day 8 post-induction of ovulation for NP8 and P8 groups, respectively. In NP12 llamas, plasma progesterone concentrations fell below 1.6 nmol/l by day 12 post-Buserelin, while in all animals of P12 group, progesterone levels remained above 6.5 nmol/l.

Mean diameter of the embryos collected on day 8 was 0.74 ± 0.13 mm, and all of them were in the stage of expanded blastocyst.

General observation of receptor immunostaining

Immunoreactive ERα and PR were visualized exclusively in the nuclei of the different studied cell types, while COX-2 immunostaining was recorded exclusively in the cytoplasm of the epithelial cells and it was not observed in stromal cells in any of the sampling days (Figs 3–5).

When monoclonal specific antibodies were substituted by a non-immune mouse IgG, the absence of staining demonstrated the specificity of receptors and COX-2 immunostaining.

Oestrogen receptor α immunostaining

In the luminal epithelium, no statistical differences were observed in the percentage of positive cells neither in the intensity of immunostaining between NP8 and P8 animals. Conversely, a higher percentage of positive cells and stronger intensity for ERα were recorded in the luminal epithelium of pregnant llamas than non-pregnant llamas on day 12 post-induction of ovulation ($p < 0.002$; Figs 1a, 2a and 3).

In the glandular epithelium, no statistical differences were registered in the percentage of positive cells or in the staining intensity between pregnant and non-pregnant animals (Figs 1b and 2b).

In the stroma, the percentage of positive cells was higher and the intensity of immunostaining was greater in P8 than in NP8 animals ($p < 0.006$). Conversely, the number of positive cells was lower and the staining was fainter in the stroma of P12 than in NP12 group ($p < 0.0001$; Figs 1c, 2c and 3).

The number of positive cells and the intensity of the immunostaining in the luminal epithelium decreased by day 12 in non-pregnant and pregnant animals, while in the stroma, this observation was just recorded in pregnant animals ($p < 0.02$).

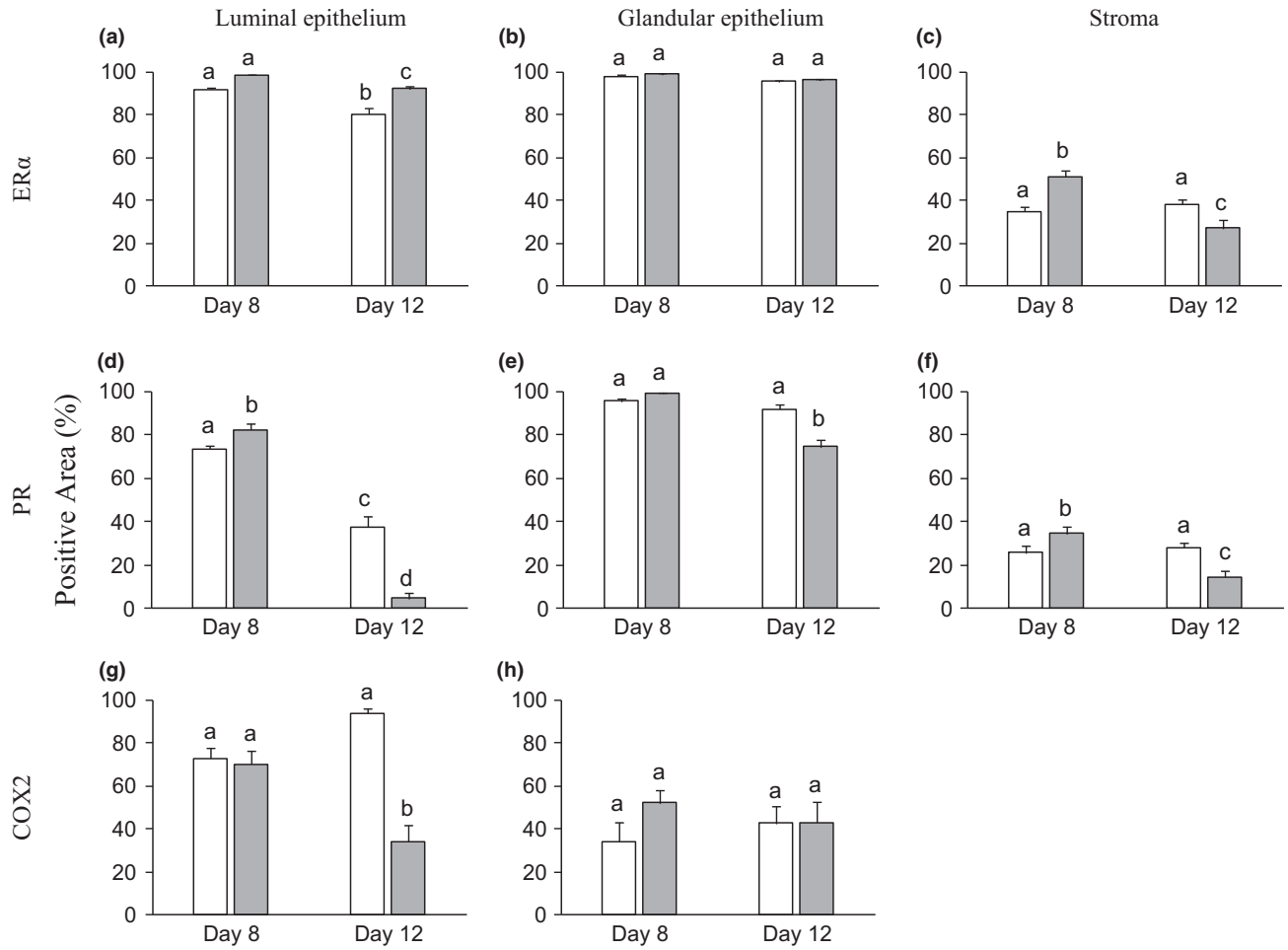

Fig. 1. Positive area for endometrial ERα (a, b, c), PR (d, e, f) and COX2 (g, h) in the luminal (left panel) and glandular epithelium (medium panel) and stroma (right panel) of non-pregnant (white bars) and pregnant (grey bars) llamas on days 8 and 12 post-induction of ovulation. Bars with different letters are significantly different ($p < 0.05$)

Progesterone receptor immunostaining

More positive cells for PR were observed in P8 than in NP8 group in the luminal epithelium and stroma ($p < 0.03$). However, no statistical differences were registered in the intensity of staining between both groups in any of the cell types on this day.

A deep decrease in endometrial PR population was observed in pregnant llamas on day 12 post-mating as the percentage of positive cells and the level of staining intensity were lower in P12 than in NP12 llamas. In addition, the expression of this receptor was lower in P12 than in P8 animals in both epithelial and stromal cells ($p < 0.002$; Figs 1d,e,f, 2d,e,f and 4).

Cyclooxigenase 2 immunostaining

No significant differences were observed in COX-2 immunostaining in the luminal epithelium between P8 and NP8 groups. Yet, the expression of COX-2 was lower in the luminal epithelium of pregnant llamas, being the percentage of positive cells lesser and the intensity of staining fainter in P12 than in NP12 llamas ($p < 0.0002$; Figs 1g,h, 2g,h and 5). Furthermore, there were more positive cells in P8 than in P12.

The number of positive cells and the immunostaining intensity for COX-2 in the glandular epithelium remained at the same levels between both groups.

Discussion

To our knowledge, this is the first study that addresses the effect of the presence of the embryo on endometrial ERα, PR and COX-2 immunostaining during the process of maternal recognition of pregnancy in llamas, providing new insights about their regulation during early pregnancy that might explain corpus luteum maintenance and embryo attachment in this species.

The plasma progesterone concentrations below 1.6 nmol/l registered in non-pregnant llamas on day 12 post-induction of ovulation were expected to occur due to corpus luteum regression in these animals (Aba et al. 1995). In addition, llamas of the P12 group

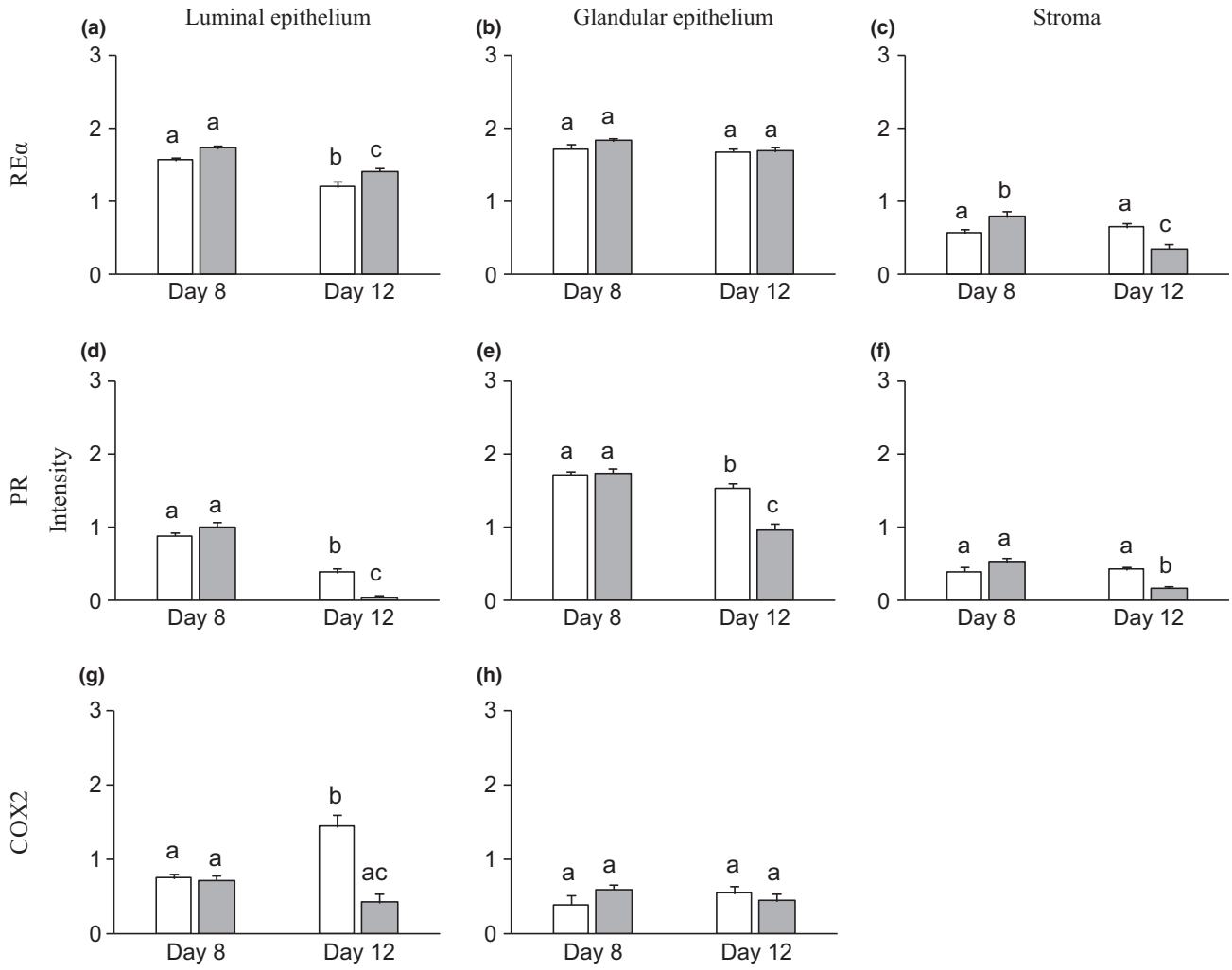

Fig. 2. Average immunostaining for endometrial ERα (a, b, c), PR (d, e, f) and COX2 (g, h) in the luminal (left panel) and glandular epithelium (medium panel) and stroma (right panel) of non-pregnant (white bars) and pregnant (grey bars) llamas on days 8 and 12 post-induction of ovulation. Bars with different letters are significantly different ($p < 0.05$)

maintained plasma progesterone concentrations above 6.5 nmol/l until day 12 post-mating, indicating the persistence of a functional corpus luteum related to pregnancy (Sumar 1996).

The observation that an increase in the expression of ERα occurred in the stromal cells on day 8 and in the luminal epithelium by day 12 in the endometrium of pregnant llamas suggests a possible role of the oestrogens in the process of maternal recognition, as it was previously suggested in this species (Powell et al. 2007a). This latter study has reported that llama blastocysts start to produce estradiol-17β as early as day 7 and a significant raise in the oestrogen production was reported between day 11 and 13 when blastocysts change from an ovoid to elongated morphology (Powell et al. 2007a). Thus, it could be speculated that oestrogen released by the blastocyst during their early development could have a paracrine effect on the endometrium inducing an increase in the population of ERα. Porcine embryos also produce oestrogens during their development; yet, an increase in the endometrial expression of ERα during early pregnancy has not been observed in this species (Geisert et al. 1993). Nevertheless, these authors suggest that the detectable levels of ERα in the surface epithelium after day 12 of pregnancy provides the mechanism by which secretion of oestrogens by the elongating pig conceptus can stimulate changes in uterine function necessary for the maintenance of pregnancy. Similarly, the increase in ERα observed by day 12 post-mating in pregnant llamas in the present study could be a necessary event to maintain pregnancy. Conversely, previous studies in llamas, where the expression of mRNA was evaluated, have observed no significant differences in the endometrial expression of ERα between pregnant and non-pregnant llamas (Powell et al. 2007b). The disclosure might be related to the fact that in the latter study, ERα mRNA was evaluated in total endometrial tissue. On the contrary, the

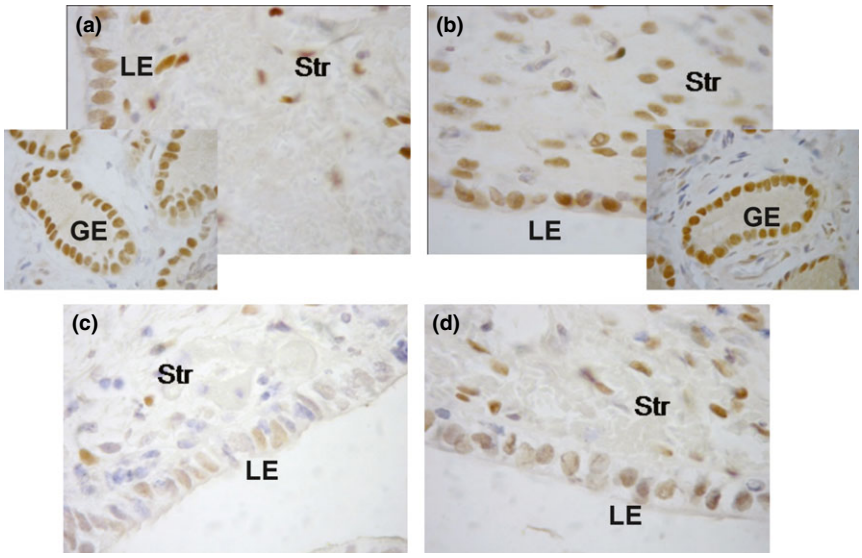

Fig. 3. Immunohistochemical localization of ERα in the endometrium of non-pregnant llamas on days 8 (a) and 12 (c) post-induction of ovulation and of pregnant llamas on days 8 (b) and 12 (d) post-mating. LE = luminal epithelium; GE = glandular epithelium; and Str = stroma (1000×)

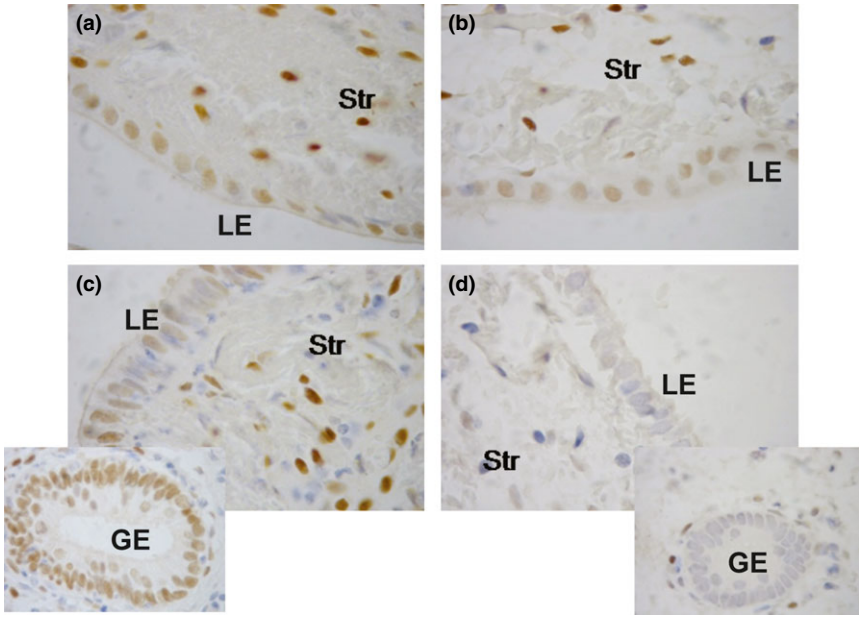

Fig. 4. Immunohistochemical localization of PR in the endometrium of non-pregnant llamas on days 8 (a) and 12 (c) post-induction of ovulation and of pregnant llamas on days 8 (b) and 12 (d) post-mating. LE = luminal epithelium; GE = glandular epithelium; and Str = stroma (1000×)

immunohistochemistry used in the present study is a semiquantitative technique that allows to evaluate the expression of ERα protein in the different cell types. Previous studies in llamas (Bianchi et al. 2010, 2013) and in other species (sheep: Cherny et al. 1991; Sosa et al. 2006; cows: Boos et al. 1996) have demonstrated that endometrial steroid hormone receptors are regulated in a specific manner depending on cell type.

The decrease in the expression of PR in the luminal and glandular epithelium by day 12 post-mating in pregnant llamas fully agrees with the loss of PR in endometrial epithelia prior to implantation reported in most domestic animals (sheep: Spencer and Bazer 1995; cattle: Kimmins and MacLaren 2001; pigs: Geisert et al. 1994 and horses: McDowell et al. 1999; Hartt et al. 2005). Previous studies in sheep have established that the continuous exposure of the endometrium to progesterone down-regulates its own receptor (Spencer and Bazer 1995). During early pregnancy, the loss of PR is required for the expression of different proteins (i.e. uterine milk proteins; osteopontin) in the endometrial glandular epithelium, which are a component of the histotroph and are partially involved in the mechanisms of superficial implantation and placentation in ovines (Spencer et al. 1999; Johnson et al. 2000). Thus, as previously reported in other species, the decrease in the expression of PR in the endometrium of pregnant llamas could be a necessary event to allow the expression of

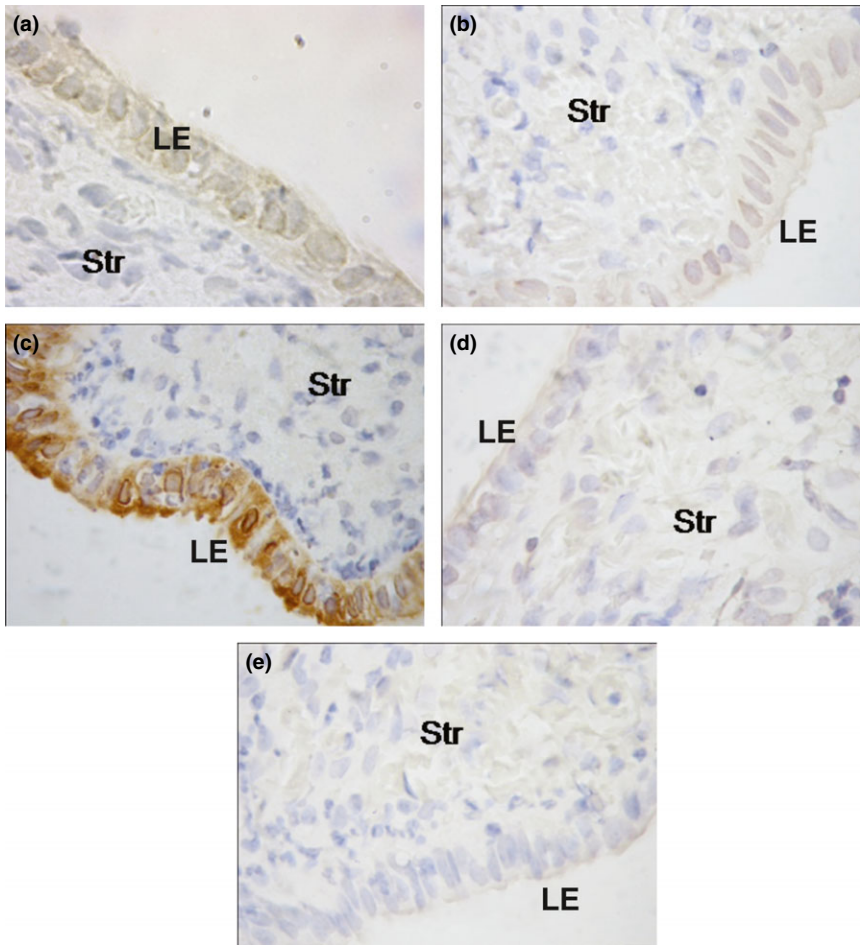

Fig. 5. Immunohistochemical localization of COX-2 in the endometrium of non-pregnant llamas on days 8 (a) and 12 (c) post-induction of ovulation and of pregnant llamas on days 8 (b) and 12 (d) post-mating. Negative control (e). LE = luminal epithelium and Str = stroma (1000×)

those proteins involved in attachment and implantation of embryo, but that have not been still characterized in camelids. Unlike ruminant species, the decrease in the PR population in the endometrium of pregnant llamas is not accompanied by a low expression of ERα as previously reported in ovines (Spencer et al. 1995) and bovines (Robinson et al. 2001). Thus, the declined endometrial expression of PR during early pregnancy occurs in the presence of high ERα levels in the epithelia of the llamas endometrium.

The low expression of COX-2 in the endometrium of pregnant llamas by day 12 post-mating hereby presented could explain the attenuation in $PGF_{2\alpha}$ previously reported in pregnant llamas (Aba et al. 2000). Previous studies have reported a lower magnitude and frequency of $PGF_{2\alpha}$ peaks in pregnant than in non-pregnant llamas during the expected time of maternal recognition of pregnancy (Aba et al. 2000). Thus, these observations provide new evidence that estradiol or other factors released by the embryo during early pregnancy would induce corpus luteum maintenance, at least in part, by reducing COX-2 protein expression in the luminal epithelium of the endometrium. Similarly, in horses, the presence of the conceptus was shown to block the induction of endometrial COX-2 expression, suggesting that this is the mechanism by which it prevents luteolysis during early pregnancy in this species (Boerboom et al. 2004; Ealy et al. 2010).

In agreement to the results hereby reported, previous studies in llamas (Bianchi et al. 2013) and other species (ewes: Spencer et al. 1995; cows: Robinson et al. 1999, 2001) have demonstrated that ERα and PR expressions differ within cell types in the endometrium, being the most pronounced changes recorded in the luminal epithelium. These observations allow to speculate that this cell type is the most sensitive to plasma hormonal changes related to ovarian activity and early pregnancy and thus a key regulator of the endometrial functions (Spencer et al. 1995).

In summary, while the endometrial population of ERα increases during early pregnancy at the time that embryo releases significant amounts of estradiol, a decrease of PR in the endometrium is observed which might be a necessary event to allow the expression of different proteins involved in conceptus attachment, a mechanism widely accepted in other mammals species.

Furthermore, embryo seems to attenuate maternal $PGF_{2\alpha}$ secretion during early pregnancy, at least in part, by decreasing the expression of COX-2 in the luminal epithelium of the endometrium of pregnant llamas.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contribution

C.P. Bianchi conducted field work, collected data, set up the immunohistochemical technique for each receptor and evaluated the immunostaining, analysed and interpreted the data and prepared the manuscript. A. Meikle contributed to the designing of the experiment, collaborated to set up the immunohistochemical technique for each receptor, analysed the data and reviewed critically the manuscript. M.A. Benavente and M.A. Alvarez contributed with field work, evaluated the immunostaining and collaborated preparing the manuscript. V.L. Trasorras contributed with field work and collaborated preparing the manuscript. M.H. Miragaya contributed with field work and reviewed critically the manuscript. E. Rodríguez contributed to analysing the data, interpreting the results and preparing the manuscript. M.A. Aba contributed to the designing of the experiment, collecting data, interpreting the results and reviewed critically the manuscript.

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