Reproduction in Domestic Animals

Reprod Dom Anim **48**, 681–690 (2013); doi: 10.1111/rda.12146
ISSN 0936–6768

Oestrogen, Progesterone and Oxytocin Receptors and COX-2 Expression in Endometrial Biopsy Samples from the Induction of Ovulation to Luteolysis in Llamas (*Lama glama*)

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Contents
Endometrial expression of oestrogen (ERα), progesterone (PR) and oxytocin receptor (OR) and cyclooxygenase-2 (COX-2) was evaluated from the induction of ovulation to luteolysis in llamas. Ovarian activity was daily assessed by ultrasonography in five females. Ovulation was induced immediately after the detection of an ovulatory follicle by a GnRH injection (Day 0). Endometrial samples were obtained by transcervical biopsies from the left and right horns on day 0 and days 4, 8, 10 and 12 post-GnRH. Blood samples were collected daily for progesterone and estradiol-17β determinations by RIA. An immunohistochemical technique was used to study receptors population and COX-2 expression which were then evaluated by two independent observers. The expression of ERα and PR was highest on day 0 in the luminal epithelium and stroma in association with high plasma estradiol-17β concentrations. Thereafter, a decrease in ERα population was registered on day 4 and a new increase of its expression was observed between days 8 and 12 in those cell types. Conversely, PR population was gradually down-regulated until its lowest expression was reached on day 10 post-GnRH in the luminal epithelium. Content of OR was similar throughout the study in all cell types. The expression of COX-2 was highest from day 8 to 12 post-GnRH in the luminal epithelium, in relation to the time of maximal $PGF_{2\alpha}$ release. Both steroid receptors populations and COX-2 expression were similar between horns. Meanwhile, OR expression was higher in the right than in the left uterine horn. In summary, this study showed that the loss of endometrium sensitivity to progesterone by days 8–10 post-induction of ovulation and the concomitant increase of COX-2 expression could play a key role in the mechanism of luteolysis and somehow be related to the short corpus luteum lifespan of llamas.

Introduction

The oestrous cycle of ruminants depends on the endometrium as the source of the luteolysin, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The coordinated effects of oestrogen, progesterone and oxytocin on their respective endometrial receptors drive the production of $PGF_{2\alpha}$ pulses (Spencer and Bazer 1995; Robinson et al. 2001). Cyclooxygenase-2 (COX-2), also known as prostaglandin-endoperoxide synthase 2, is the main enzyme expressed in luminal and glandular epithelium of the ovine and bovine endometrium, and is responsible for the production of the luteolytic pulses of $PGF_{2\alpha}$ (Charpigny et al. 1997; Kim et al. 2003).

Different studies, especially in ewes and cows, have demonstrated that endometrial hormone receptors expression changes during the different stages of the oestrous cycle and between the different uterine compartments (Wathes and Hamon 1993; Spencer and Bazer 1995; Boos et al. 1996; Robinson et al. 2001). Endometrial oestrogen receptor α (ERα), progesterone receptor (PR) and oxytocin receptor (OR) populations are highest around oestrous in response to oestrogens produced by the dominant follicle. Thereafter, during the early and mid-luteal phase, progesterone from the newly formed corpus luteum suppresses ERα and OR expression (Spencer and Bazer 1995; Wathes et al. 1996; Robinson et al. 2001). Exposure of the endometrium to progesterone for 8–10 days down-regulates its own receptor and the loss of PR in luminal and glandular epithelium ends the progesterone suppression of ERα and OR (Flint et al. 1994; Wathes et al. 1996). Thus, the endometrium becomes sensitive to oestrogens which, by acting through ERα, increase the endometrial expression of OR in luminal and glandular epithelium immediately before and during luteolysis (Beard and Lamming 1994; Spencer et al. 1995). Oxytocin, from the pituitary and corpus luteum, stimulates the pulsatile release of $PGF_{2\alpha}$ via OR, resulting in the regression of the corpus luteum (Goff 2004). Furthermore, at the beginning of luteolysis, it has been reported an increase of COX-2 expression in luminal and superficial glandular epithelium, which produces the synthesis and release of $PGF_{2\alpha}$ (Charpigny et al. 1997; Arosh et al. 2002).

Llamas are induced ovulators, requiring copulation when an ovulatory follicle is present, to initiate the ovulatory process (San-Martín et al. 1968; Fernández-Baca et al. 1970; Bravo et al. 1990; Aba et al. 1995). Plasma progesterone concentrations start to increase approximately day 4 post-induction of ovulation, peak at day 8 and approximately day 9–10 post-mating, progesterone levels start to decrease in response to $PGF_{2\alpha}$ pulses (Aba et al. 1995). It has been shown the occurrence of $PGF_{2\alpha}$ pulsatile release as early as day 7, followed by a massive release between days 8 and 11 post-mating (Aba et al. 2000), indicating that the luteal phase of llamas is considerably shorter than those of other ruminant species. Thus, if the luteolytic mechanism is similar to that proposed in bovine and ovine, it should occur, for reasons still unknown, in a shorter time.

More interestingly, different luteolytic activities of both uterine horns have been reported in camelids. It has been demonstrated that the right uterine horn

causes luteolysis only of the corpus luteum located on the right ovary, while the left horn has both local and systemic effects, affecting a corpus luteum located on either ovary (Fernández-Baca et al. 1979). Other particular feature of this species is that almost all pregnancies are carried out in the left horn, although ovulation occurs equally from both ovaries (Fernández-Baca et al. 1973).

Previous studies in llamas have demonstrated high ERα and PR population in the endometrium during the follicular phase when peripheral plasma concentrations of oestrogens are elevated and a decrease of both receptors during late luteal phase (i.e. day 9 post-induction of ovulation) when plasma progesterone concentrations are still high. These results indicate that steroid receptors populations are regulated in a similar manner than in other ruminant species (Bianchi et al. 2007). Information about the expression of OR or COX-2 has not yet been reported in camelids.

The aim of the present study was to evaluate the temporal and spatial changes in ERα, PR and OR populations and COX-2 expression in the endometrium of cyclic llamas in the right and left uterine horn from the induction of ovulation to the time of luteolysis to understand the physiological events occurring in the endometrium during the time of corpus luteum regression.

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. Five adult non-pregnant and non-lactating llamas were kept in pens isolated from males and fed pasture hay and water *ad libitum*. Animals were examined daily by transrectal ultrasonography to evaluate ovarian status (Pie Medical 100 vet with 5.0/ 7.5 variable traducer probe).

When a follicle with a diameter ≥ 8 mm, considered ovulatory in this species (Bravo et al. 1991) was observed, ovulation was induced with a single injection of a GnRH analogue (16.8 μg Receptal®, Intervet). Occurrence of ovulation was assessed based on ultrasonographic visualization of the newly formed corpus luteum and further confirmed by the progesterone profiles. Endometrial samples were obtained by transcervical biopsies as previously described (Bianchi et al. 2010) the day of GnRH injection (Day 0) and on days 4, 8, 10 and 12 post-induction of ovulation. To avoid any carry-over effect of the biopsy on subsequent samples, the biopsies corresponding to different days were collected in the same animals during different luteal phases. A rest period of at least 2 weeks was allowed to the animals between biopsies. 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 daily from the day of induction of ovulation (Day 0) to day 12 post-GnRH by jugular venipuncture. Samples were centrifuged and plasma was stored at −20°C until hormone assays were performed.

Hormone determinations

Estradiol-17β was determined in the samples collected on days of biopsies using an RIA kit (Estradiol Double Antibody, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) reported for use in bovine plasma (Sirois and Fortune 1990) and validated for use with llama plasma after minor modifications (Aba et al. 1995). The sensitivity of the assay was 5.6 pM and the intra-assay coefficient was below 12% for concentrations between 5.6 and 180 pM. Progesterone was measured using an RIA kit (COAT-A-COUNT®; Siemens Medical Solutions Diagnostics) previously validated for its use with llama plasma (Bianchi et al. 2007). The sensitivity of the assay was 0.3 nM and the intra-assay coefficient of variation was below 14% for concentrations between 0.4 and 128 nM. All samples were measured in duplicates and in one single assay for each hormone. Hormone concentrations are expressed in SI units. To convert from pM to pg/ml and from nM to ng/ml, the following factors should be used: estradiol-17β: 3.7 and progesterone: 3.2.

Immunohistochemistry

An immunohistochemical technique (avidin–biotin–peroxidase) previously described (Bianchi et al. 2007; Sosa et al. 2009) was used to visualize ERα, PR, OR and COX-2 immunostaining after minor modifications. 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, 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, PR and OR) or Goat Serum (COX-2), (Vectastain®; Vector Laboratories, Burlingame, CA, USA) in buffer in a humidified chamber at RT. Details of the antibodies used for the detection of each receptor and conditions are specified in Table 1. Negative controls for each receptor were obtained by replacing the primary antibody with normal mouse IgG at equivalent concentration (Cat Nº sc-2025; Santa Cruz, CA, USA). After primary antibody binding, the sections were incubated for 60 min at RT with a biotinylated IgG (Vectastain®, Vector Laboratories, Burlingame, CA, USA) diluted in Normal Horse (ERα, PR and OR) or Goat Serum (COX-2). Thereafter, the tissue sections were incubated for 60 min with a horseradish peroxidase–avidin–biotin complex (Cat Nº PK-6100, Vectastain®, Elite ABC-kit; Vector Laboratories). The site of the bound enzyme was visualized by the application of 3,3′-diaminobenzidine (Cat Nº SK 4100, DAB kit; Vector Laboratories), a chromogen that produces a brown, insoluble precipitate when incubated with the enzyme. The sections were counterstained with

Table 1. List of antibodies used for the determination of endometrial ERα, PR, OR and COX-2 expression by immunohistochemistry

	Laboratory	Antibody	Dilutions with PBS
ERα	Santa Cruz Laboratories (C – 311). Cat # sc-787, CA, USA	Monoclonal	1 : 25
PR	Zymed Laboratories Inc. Cat # ZS18-0172, San Francisco, USA	Monoclonal	1 : 100
OR	Rohto Pharmaceutical CO., LTD MA – 701, Osaka, Japan	Monoclonal	1 : 40
COX 2	Cayman Chemical Co. Ann Arbor. Cat # 160106, MI, USA	Polyclonal	1 : 200

haematoxylin and dehydrated before they were cover slipped with mounting medium (Biopack, Buenos Aires, 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. For each receptor, samples were divided into two separate assays but all tissues of the same animal were processed simultaneously to avoid any systemic error.

Image analysis

After a general inspection of each slide, a subjective image analysis was performed to estimate the expression of ERα, PR, OR 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 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

The variables from the image analysis, which were considered for statistical analysis, 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 version 9.1 (SAS, Institute Inc., Cary, NC, USA). Immunostaining was analysed by the mixed procedure, and the statistical model included the effects of observers, horns, days and cell types. Because no significant differences were observed between observers and horns for ERα, PR and COX-2, data were pooled and only the interaction between days and cell types was used. Similarly, when analysing OR, data between observers and days were pooled and only statistical differences between horns and cell types were considered.

Data from follicular diameters and hormonal 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. Regression analysis was used to determine the relationship between follicular diameter and plasma estradiol-17β concentrations.

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

Results

Follicular dynamics and hormonal profiles

Mean diameter of the largest follicles was higher on days 0, 10 and 12 post-induction of ovulation (8.7 ± 0.20; 7.4 ± 0.36 and 8.7 ± 0.28, respectively) than on days 4 and 8 post-GnRH, (4.7 ± 0.34 and 6.7 ± 0.29 mm, respectively) ($p < 0.05$).

Peak estradiol-17β plasma concentrations were attained on days 0 (28.1 ± 4.67 pM) and 12 (29.5 ± 2.49 pM) ($p < 0.05$). In all cases, a close relationship between the diameter of the largest follicle and plasma estradiol-17β concentrations was observed ($p < 0.05$) (Fig. 1).

Plasma progesterone concentrations remained below 2 nM until day 4 post-GnRH, increased to 3.77 ± 0.07 nM on day 5 and peaked on day 8 (10.65 ± 0.32 nM $p < 0.001$). Plasma concentrations of progesterone start to decrease by day 10–12 post-induction of ovulation ($p < 0.001$) (Fig. 1).

General observation of receptor immunostaining

Immunoreactive ERα and PR were visualized exclusively in the nuclei of the different studied cell types, while OR immunostaining was observed in the cytoplasm of the same cells. Immunostaining for COX-2 was recorded exclusively in the cytoplasm of the epithelial cells and it was not observed in stromal cells in any of the sampling days.

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 (Figs 2f, 3f, 4f and 5f).

Oestrogen receptor α immunostaining

Most positive cells and the greatest immunostaining for ERα were observed on day 0 (day of induction of ovulation) in the luminal epithelium and stroma ($p < 0.01$). By day 4 post-GnRH, the number of positive cells and staining intensity dropped to its lowest levels in both cell types ($p < 0.0005$). An increase of ERα population in the luminal epithelium was recorded between days 8 and 12 post-GnRH as compared to day 4; however, it remained being lower than that

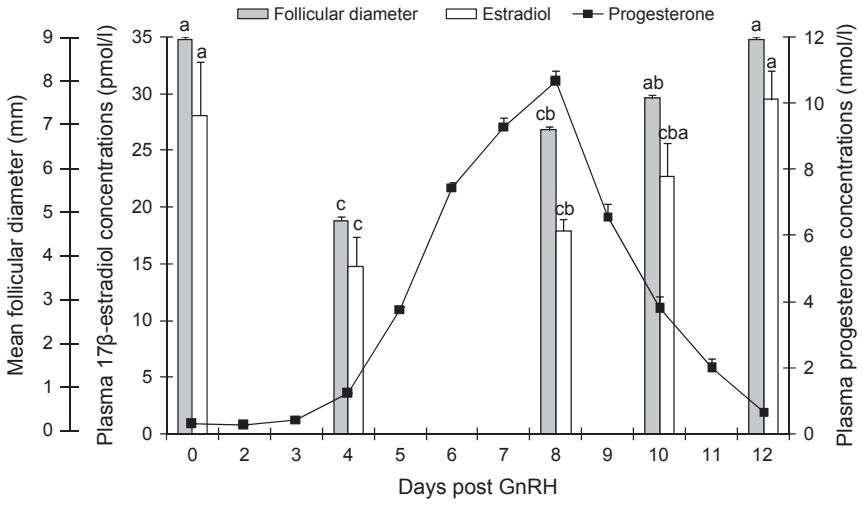

Fig. 1. Mean follicular diameter (grey bars) and plasma 17β-Estradiol concentrations (white bars) on days 0, 4, 8, 10 and 12 post-induction of ovulation and plasma progesterone concentrations (■) throughout the ovarian activity. Different letters mean significant statistical differences ($p < 0.05$)

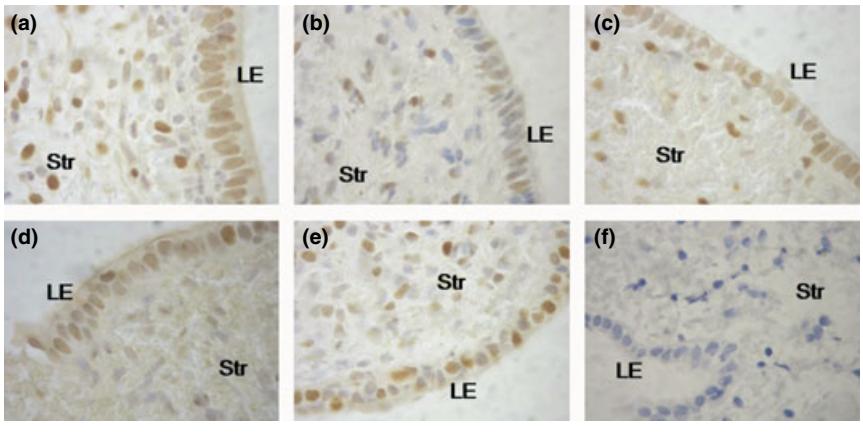

Fig. 2. Immunohistochemical localization of ERα in the endometrium of llamas on days 0 (a), 4 (b), 8 (c), 10 (d) and 12 (e) post-induction of ovulation. (f) Negative control. LE = Luminal epithelium; Str = Stroma (1000×)

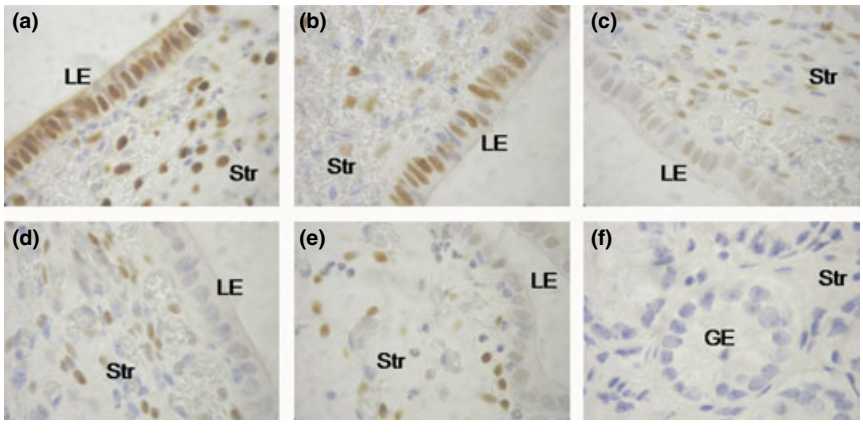

Fig. 3. Immunohistochemical localization of PR in the endometrium of llamas on days 0 (a), 4 (b), 8 (c), 10 (d) and 12 (e) post-induction of ovulation. (f) Negative control. LE = Luminal epithelium; GE = Glandular epithelium; Str = Stroma (1000×)

observed on day 0 ($p < 0.01$). In the glandular epithelium, the percentage of positive area and the immunostaining intensity continued in the same levels throughout the study (Figs 2 and 6a,b).

Luminal and glandular epithelia presented more positive cells and a greater intensity than stroma during all the study ($p < 0.0001$). On days 4, 8, 10 and 12 post-GnRH, more positive cells and intensity were observed in the glandular epithelium than in the luminal epithelium ($p < 0.05$). Nevertheless, on day 0, no significant differences were recorded between both epithelia ($p = 0.6$) (Fig. 6a,b).

Progesterone receptor immunostaining

A greater population of positive cells and stronger immunostaining for PR was visualized in the luminal epithelium and stroma on day 0 than in the other days ($p < 0.01$). Thereafter, a gradual decrease was observed in the luminal epithelium, reaching the lowest percentage of positive cells and the faintest immunostaining on day 10 post-induction of ovulation ($p < 0.05$). By day 12 post-GnRH, PR immunostaining started to increase until similar values to that recorded on day 8 were registered (Figs 3 and 7a,b).

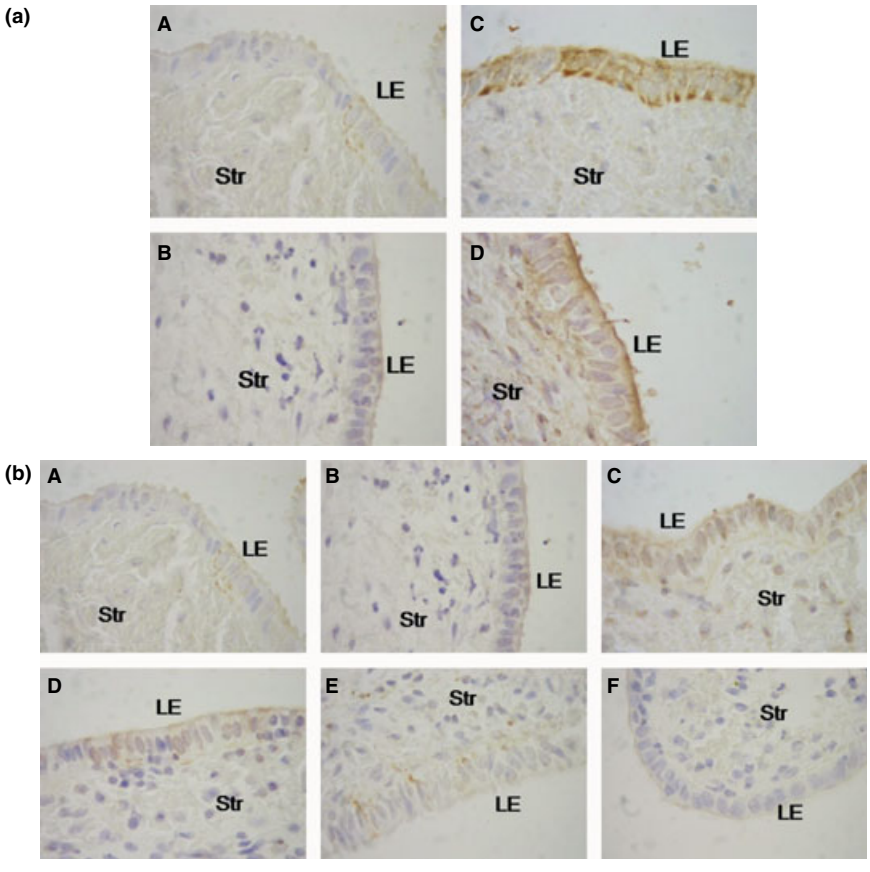

Fig. 4. (a) Immunohistochemical localization of OR in the left horn (a and b) and in the right horn (c and d). (b) Immunohistochemical localization of OR in the endometrium of the left horn of llamas on days 0 (a), 4 (b), 8 (c), 10 (d) and 12 (e) post-induction of ovulation. (f) Negative control. LE = Luminal epithelium; Str = Stroma (1000×)

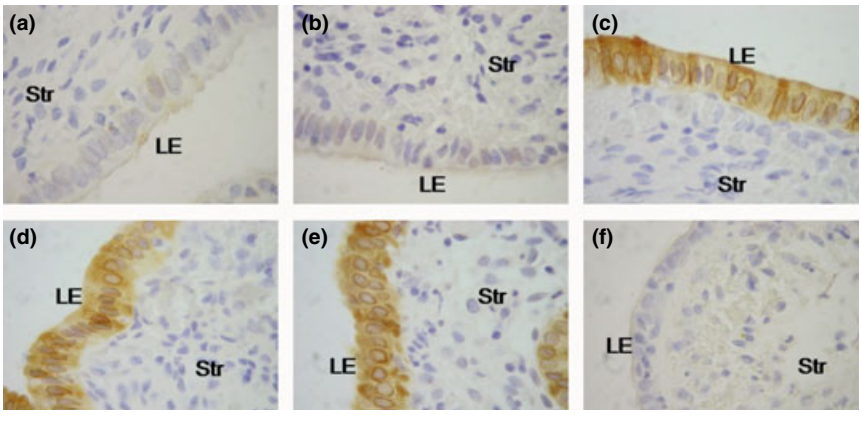

Fig. 5. Immunohistochemical localization of COX-2 in the endometrium of llamas on days 0 (a), 4 (b), 8 (C), 10 (d) and 12 (e) post-induction of ovulation. (f) Negative control. LE = Luminal epithelium; Str = Stroma (1000×)

An abrupt decline of PR population was recorded after day 0 in the stromal cells maintaining low values until the end of the study ($p < 0.01$) (Figs 3 and 7a,b).

Similar to that observed for ERα, the percentage of positive area and the immunostaining intensity for PR in the glandular epithelium remained to the same levels throughout the study. In addition, this cell type presented greater abundance of PR during the study ($p < 0.05$) than the luminal epithelial and stromal cells, except on day 0 when there were no statistical differences in the percentage of positive cells between the luminal and glandular epithelium (Fig. 7a,b).

Oxytocin receptor immunostaining

A greater number of cells positive to OR was observed in the epithelia from the right horn as compared to the left horn (luminal epithelium: 69.4 ± 5.9% vs 44.3 ± 5.0% and glandular epithelium: 65.6 ± 5.1 vs 28.8 ± 4.3 in the right and left horn, respectively). The same observation was recorded for staining intensity (luminal epithelium: 1.02 ± 0.12 vs 0.50 ± 0.06 and glandular epithelium: 0.70 ± 0.06 vs 0.28 ± 0.04 in the right and left horn, respectively) ($p < 0.01$; Figs 4a and 8a,b).

No statistical differences were registered in the percentage of positive cells or in the staining intensity between the different days for any of both horns (Fig. 4b).

On days 4, 8, 10 and 12 post-induction of ovulation, a greater positive area and stronger staining were recorded in the epithelial than in the stromal cells ($p < 0.05$). However, there were no statistical differences between the different cell types on day 0 (data not shown).

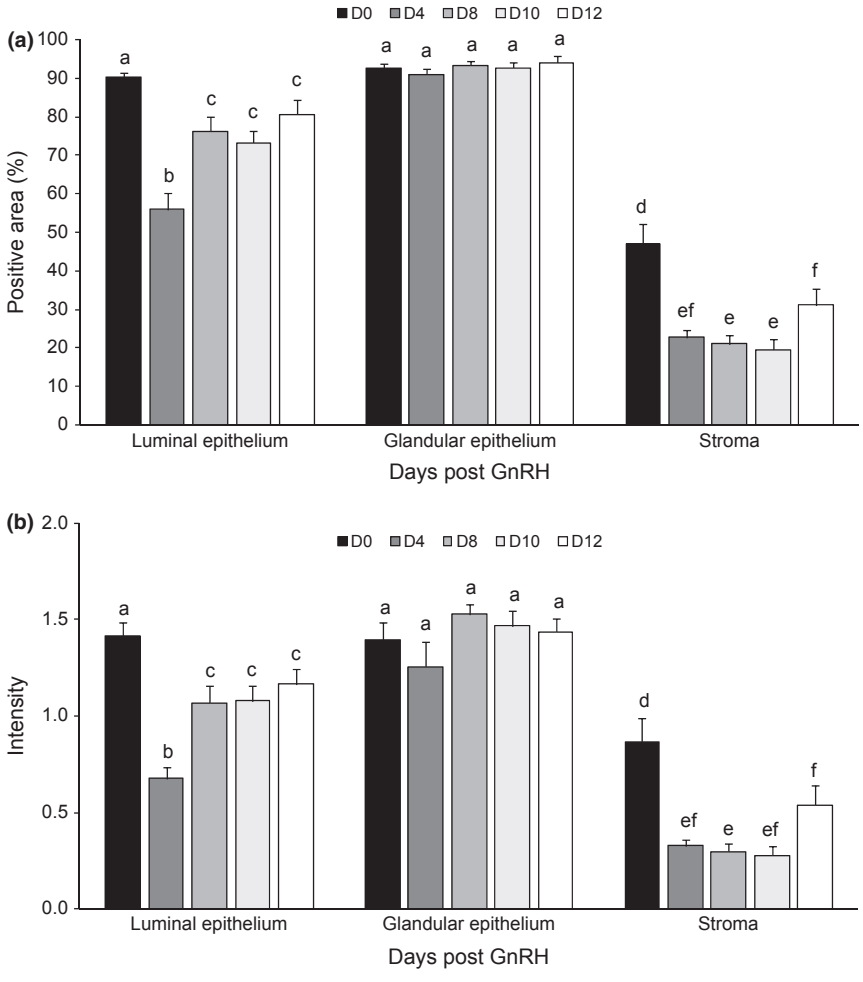

Fig. 6. (a) Positive area and (b) average immunostaining for endometrial ERα on days 0, 4, 8, 10 and 12 post-induction of ovulation in the luminal and glandular epithelium and stroma. Bars with different letters are significantly different ($p < 0.05$)

Cyclooxygenase-2 immunostaining

The greatest percentage of positive cells to COX-2 was observed in the luminal epithelium on days 8, 10 and 12 post-induction of ovulation while less positive cells and less intensity of staining were recorded on days 0 and 4 ($p < 0.005$) (Figs 5 and 9a,b).

In the glandular epithelium, the percentage of positive area and the immunostaining intensity were lower on day 4 post-GnRH than on the other days ($p < 0.05$).

The percentage of positive cells and the staining intensity on day 0 were higher in the glandular epithelium than in the luminal epithelium ($p < 0.05$). However, this latter epithelium presented more positive cells and stronger staining than the glandular epithelium during days 10 and 12 post-induction of ovulation ($p < 0.001$; Fig. 9a,b).

Discussion

To our knowledge, this is the first report where the temporal and spatial changes of the population of ERα, PR, OR and COX-2 were evaluated from the induction of ovulation to the time of luteolysis in different endometrial cell types in llamas. By considering the temporal variations of their expression, this study provides new insights into the possible mechanisms regulating the process of luteolysis in this species.

The observation that the expression of ERα and PR was generally high at the moment when an ovulatory follicle was present, analogue to the time of oestrus in other ruminants, and low during the luteal phase supports the hypothesis that oestrogens have a stimulatory effect on the expression of both receptors while progesterone has the opposite effects. This finding is consistent with those of several studies in other species (Wathes and Hamon 1993; Spencer and Bazer 1995; Robinson et al. 2001). From the results of the present study, it could be speculated that oestrogens released from the ovulatory follicle on day 0 promote an increase in the population of ERα and PR evidenced by a greater percentage of positive area and stronger intensity in the luminal epithelium and stroma. Similar observations have been reported in ewes (Wathes and Hamon 1993; Spencer and Bazer 1995), mares (Hartt et al. 2005), cows (Kimmins and MacLaren 2001; Robinson et al. 2001) and llamas (Bianchi et al. 2007). Thereafter, on day 4 after induction (approximately 2–3 days after ovulation), a sharp decrease on ERα expression was observed in relation to a decline in plasma 17β-estradiol concentrations.

Considering that ovulation occurs approximately 30 h after its induction in llamas (Ratto et al. 2006) and that plasma progesterone concentrations arise above 3.2 nM on day 5 post-GnRH, the sharp decrease in the expression of PR in the luminal epithelium and stroma occurred after 5–6 days of luteal dominance (i.e. day 8 post-induction of ovulation). In sheep, it has been demonstrated that the continuous exposure of the

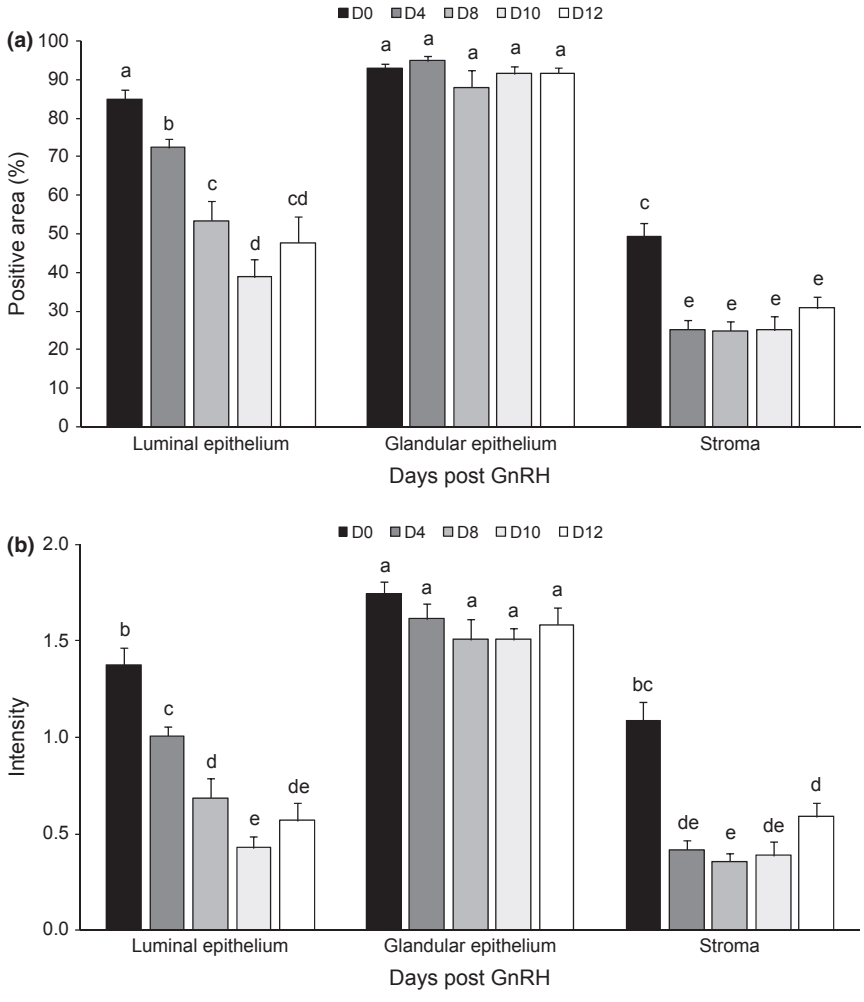

Fig. 7. (a) Positive area and (b) average immunostaining for endometrial PR on days 0, 4, 8, 10 and 12 post-induction of ovulation in the luminal and glandular epithelium and stroma. Bars with different letters are significantly different ($p < 0.05$)

endometrium to progesterone for 10–12 days down-regulates PR (Spencer et al. 1995; Wathes et al. 1996). Additionally, the timing of PR down-regulation by progesterone appears to determine when the luteolytic mechanism develops in the endometrium (Spencer et al. 2004). Thus, it could be speculated that while luteolysis occurs in other ruminants after the endometrium has been under the influence of progesterone action for up of 10 days (Schams and Berisha 2002), the endometrium of llamas requires only 5–6 days of exposure to lose its sensitivity for progesterone (demonstrated by the significant reduction in PR contents) and to trigger the luteolytic process.

Furthermore, COX-2 was highly expressed in the endometrium by day 8 post-induction of ovulation. This observation is in agreement with previous reports showing that the release of $PGF_{2\alpha}$ (estimated by levels of its 13,14-dihydro-15-keto $PGF_{2\alpha}$ metabolite) in llamas starts on day 7 or 8 post-mating and although luteolysis is completed approximately by day 10 after mating, luteolytic pulses of $PGF_{2\alpha}$ are registered until day 12 post-induction (Aba et al. 2000) when COX-2 was still highly expressed in the endometrium. The present findings strongly support the current hypothesis that the expression of COX-2 coincides with the expected time of $PGF_{2\alpha}$ release during luteolysis (bovine: Arosh et al. 2002; equine: Boerboom et al. 2004; porcine: Ashworth et al. 2006). In sheep, it is assumed that the exposure of the uterus to increasing concentrations of progesterone over 10 days is primarily responsible for the expression of COX-2 (Eggleston et al. 1990; Charpigny et al. 1997). However, COX-2 was highly expressed by day 8 post-GnRH (after 5–6 days of elevated plasma progesterone concentrations) in llamas. This is other evidence indicating that the endometrium of llamas seems to require a significantly shorter period of progesterone exposure in comparison with other ruminants, to induce some of the molecular changes that trigger the luteolytic process.

Unlike other ruminant species, an increase (or a cyclical regulation) of endometrial OR population before and during luteolysis could not be observed in llamas. Nevertheless, similar to that observed in ewes, ERα expression was up-regulated at the beginning of the process of luteolysis in llamas, probably in relation to an increase in plasma 17β-estradiol concentrations from the new dominant follicle. In sheep, it has been demonstrated that when the endometrium loses its sensitivity to progesterone, the endometrial epithelium becomes responsive to the effects of oestrogens. The up-regulation of ERα recorded prior to luteolysis would allow oestrogens to act through its receptor to induce OR gene expression in the endometrial epithelium (Spencer and Bazer 1995). This increased sensitivity to

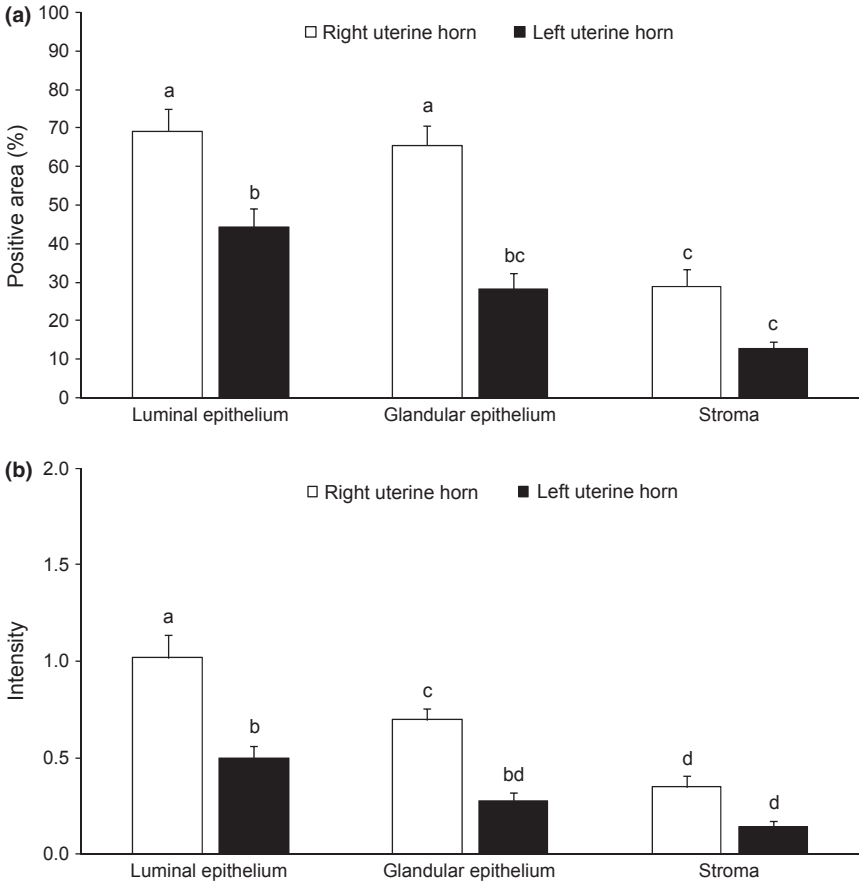

Fig. 8. (a) Positive area and (b) average immunostaining for endometrial OR in the left and right uterine horn in the luminal and glandular epithelium and stroma. Bars with different letters are significantly different ($p < 0.05$)

ovarian and pituitary oxytocin induces $PGF_{2\alpha}$ release and corpus luteum regression (Spencer et al. 1995). In cows, it is suggested that only subtle increases in OR concentration are needed to induce a luteolytic release of $PGF_{2\alpha}$ (Guzeloglu et al. 2004). Nevertheless, changes in the population of OR seem not to be involved in the initiation of luteolysis in llamas. Studies in gilts have indicated that endometrial responsiveness to oxytocin is not regulated by the amount of OR (Ludwig et al. 1998; Franczak et al. 2005). In this species, it has been proposed that the control of endometrial sensitivity to oxytocin may occur through a post-receptor mechanism and an increased affinity of oxytocin to its own receptor during late diestrus (Ludwig et al. 1998). It remains to be determined whether a similar mechanism could be involved in the process of luteolysis in llamas.

Interestingly, while there were no differences in the expression of both steroid receptors (ERα and PR) between the right and left horn, the expression of OR was higher in the right horn as compared to the left uterine horn. Previous studies have demonstrated a distinct luteolytic activity between both horns (Fernández-Baca et al. 1979). Furthermore, it has been reported that despite both ovaries collaborate with oocytes in equal proportion, approximately 98% of pregnancies are carried out in the left horn (Fernández-Baca et al. 1973). These observations indicate the occurrence of early migration of the embryos from the right to the left uterine horn. Thus, the higher expression of OR in the right uterine horn vs the left horn reported in the present study could be related to the necessity of embryos to migrate to the horn with greater survival expectancy. However, more studies are needed to elucidate this issue.

The use of the immunohistochemical technique has allowed to determine the cellular localization of the different endometrial receptors and the regulation within the different cell types (Cherny et al. 1991; Boos et al. 1996). Similar to that reported in other species (sheep: Wathes and Hamon 1993; Spencer and Bazer 1995; cows: Kimmins and MacLaren 2001; Robinson et al. 2001; mares: Hartt et al. 2005) and previously in llamas (Bianchi et al. 2007, 2010), the results of this study demonstrate that the distribution of steroid receptors changes in a cyclic manner and that the individual cell types display differential sensitivities to hormonal stimulus. In the present study, the great variations in the population of ERα and PR were observed in the luminal epithelium and stroma indicating that these cell types are more sensitive to plasma hormonal changes than the glandular epithelium in llamas. A high and constant expression of ERα and PR was detected in the glands throughout the study (always above 90% of positive cells). In cows, the expression of ERα in the deep glands is maintained at high concentrations throughout the oestrous cycle, although the receptor expression shows cyclical variations in the superficial glandular epithelium (Robinson et al. 2001). In the latter study, it was suggested that the greater amount of ERα in the deep glands may be required to

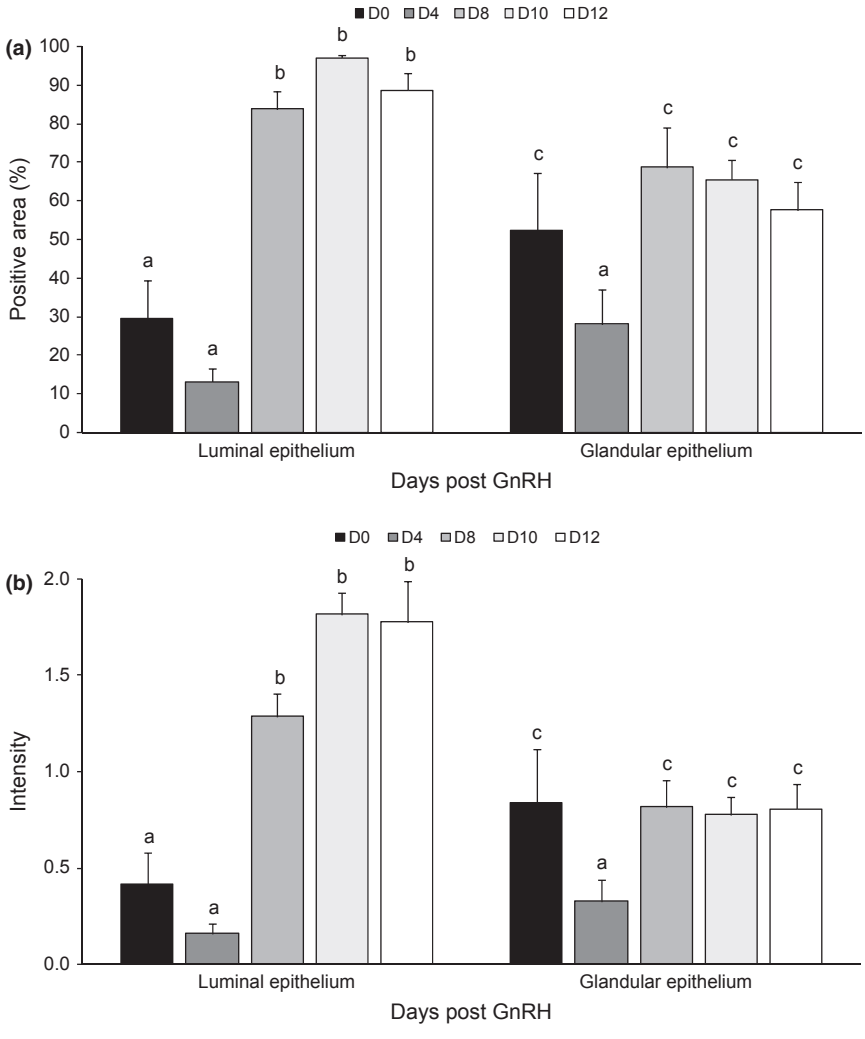

Fig. 9. (a) Positive area and (b) average immunostaining for endometrial COX-2 expression on days 0, 4, 8, 10 and 12 post-induction of ovulation in the luminal and glandular epithelium. Bars with different letters are significantly different ($p < 0.05$)

stimulate glandular secretions or to relay information to the other endometrial cell types via paracrine interactions (Robinson et al. 2001). The observation that COX-2 was exclusively expressed in the cytoplasm of the cells of the luminal and glandular epithelium indicates that these cells are responsible for the production of luteolytic pulses of $PGF_{2\alpha}$ in agreement with previous results in other species (ewes: Kim et al. 2003; mares: Boerboom et al. 2004).

In conclusion, the results of the present study give further support to the hypothesis that the timing of PR down-regulation by progesterone determines the onset of the luteolytic mechanism in llamas. Cyclic changes in the population of OR seem not to be involved in the initiation of the process of luteolysis. Thus, the loss of endometrium sensitivity to progesterone by day 8 post-induction of ovulation and the concomitant increase of COX-2 expression could have a key role in triggering luteolysis and somehow be responsible for the short corpus luteum lifespan of llamas.

Besides, the observation that the populations of ERα, PR and OR and COX-2 change according to cell types, as in other ruminants, indicate that the different compartments of the endometrium display different sensitivity to hormonal stimulus.

Acknowledgement

This research was supported by the grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT Nº 2074). C.P. Bianchi and M.V. Cavilla are holders of fellowships from CONICET and M.A. Benavente from CICPBA.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

Author contributions

C Bianchi conducted field work, collected data, set up the immunohistochemical technique for each receptor and evaluated the immunostaining, analyzed 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, analyzed the data and reviewed critically the manuscript. M Alvarez contributed with field work, evaluated the immunostaining and collaborated preparing the manuscript. M Benavente contributed with field work, evaluated the immunostaining and collaborated preparing the manuscript. MV Cavilla contributed with field work and collaborated preparing the manuscript. E Rodríguez contributed to analyzing the data, interpreting the results and preparing the manuscript. MA Aba contributed to the designing of the experiment, collecting data, interpreting the results and reviewed critically the manuscript.

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Submitted: 10 Dec 2012; Accepted: 31 Dec 2012

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