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Uterine estrogen receptor alpha and progesterone receptor during the follicular and luteal phase in llamas

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Abstract

Estrogen receptor-α (ERα) and progesterone receptor (PR) were characterized in different endometrial cell types as luminal and glandular epithelium and stroma during the follicular (FP) and the luteal phase (LP) in llamas. Animals were examined daily by transrectal ultrasonography for the determination of the presence of an ovulatory follicle and ovulation was immediately induced by a GnRH injection (Day 0). Endometrial samples were obtained by transcervical biopsies from the left uterine horn on Day 0 (FP) and 9 days after the GnRH injection (Day 9, LP). Blood samples were collected on these days for estradiol 17β and progesterone determination by RIA. An immunohistochemical technique was used to visualize ERα and PR immunostaining which was then analyzed by two independent observers. Total positive area and average staining for ERα were affected by the phase of the ovarian activity: in the three cell types there was more positive area and intense staining during the FP than during the LP. Similar findings were observed for PR, more positive stained areas were found during the FP than during the LP in the epithelia. In addition, the three cell types had more intense staining during the FP than during the LP. An effect of the cell type for ERα and PR was observed; epithelia (luminal and glandular) had more positive stained areas and greater intensity than stromal cells.

In conclusion, the results of the present study suggest that in llamas, like in other ruminants, estradiol has a stimulatory effect while progesterone downregulates the ERα and PR and that the receptor is cell type specific.
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Keywords: Llama; Estrogen receptor; Progesterone receptor; Ovarian follicular and luteal phase

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1. Introduction

Estrogens and progesterone play a major role controlling the uterine function by functioning through their respective nuclear receptors. In ruminants, the content of estrogen receptors-α (ERα) and progesterone receptors (PR) in the uterus are greater at estrus and less during the luteal phase (cows—Hendricks and Harris, 1978; Meyer et al., 1988; Boos et al., 1996; sheep—Cherny et al., 1991; Wathes and Hammon, 1993; Spencer and Bazer, 1995). Estradiol has a stimulatory effect on the expression of ERα and PR, whereas progesterone downregulates both receptors (Wathes and Hammon, 1993; Ing and Tornesi, 1997).

The regulation of the number of sex steroid receptors is cell-type specific (Cherny et al., 1991; Spencer and Bazer, 1995; Ing and Tornesi, 1997). Immunohistochemical studies demonstrated that the distribution of receptors in the different uterine compartments varies in a cyclic manner during the ovine estrous cycle in relation to plasma steroid hormone concentrations. However, cell types can display differential sensitivities to estrogens and progesterone, being the overall response of the uterus to steroid stimulation the product of the combined responses of the various cell types (Cherny et al., 1991).

Although llamas are ruminants, they have many unique reproductive features. Female llamas are induced ovulators requiring copulation to induce the ovulatory process (San-Martín et al., 1968; Aba et al., 1995). In the absence of males, ovarian activity occurs in waves of follicular growth and regression. These waves usually overlap; being the estrogens produced, responsible for the prolonged periods of behavioral estrus interrupted by brief episodes of anestrus reported (Bravo and Sumar, 1989; Adams et al., 1990). The luteal phase is shorter than in sheep and cattle. Progesterone secretion from the corpus luteum starts to increase 4–5 days after mating and declines between Days 9 and 11 owing to release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus (Sumar et al., 1988; Aba et al., 1995). Although both ovaries contribute ova in similar proportions, almost all pregnancies are present in the left uterine horn (Fernandez-Baca et al., 1973). Thus, the right uterine horn of South American camelids causes luteolysis in the right ovary, whereas the left horn causes luteolysis in both ovaries (Fernandez-Baca et al., 1979). In other ruminants timing of luteolysis – e.g. $PGF_{2\alpha}$ endometrial secretion – has been reported to be modulated by estrogens and progesterone functioning through their respective nuclear receptors (Flint et al., 1992; Lamming and Mann, 1995). However, the intimate mechanisms regulating the process of luteolysis in llamas remain to be elucidated.

Considering there are no reports describing the population of uterine estrogen and progesterone receptors in South American camelids, the aim of the present study was to characterize ERα and PR in different endometrial cell types as luminal and glandular epithelia and stroma during the follicular (FP) and the luteal phase (LP) of llamas.

2. Materials and methods

2.1. Experimental design

Activities were conducted at the facilities of Faculty of Veterinary Sciences, UNCPBA, Tandil, Argentina, located at 37°S, 60°W. Animal experimentation was performed in compliance with regulations set by the Veterinary Faculty, UNCPBA. Six adult non-pregnant and non-lactating llamas were used in the study. Animals were examined daily by transrectal ultrasonography to asses ovarian status (Pie Medical 100 vet with 5.0/7.5 variable traducer probe).

When a follicle with a diameter ≥ 0.8 cm, considered ovulatory in this species (Bravo et al., 1991) was observed, ovulation was induced with a single injection of GnRH (16.8 μg Receptal®, Intervet). Occurrence of ovulation was assessed based on ultrasonographic visualization of the corpus luteum and further confirmed by the progesterone profiles. Ovulations occurred in all except for one llama, which developed a hemorrhagic follicle and was excluded from the study of the luteal phase.

Endometrial samples were obtained by transcervical biopsies as described previously (Basu et al., 1988) when an ovulatory follicle was detected (Day 0, FP) and 9 days post-GnRH administration (Day 9, LP). All endometrial biopsy samples were taken from the middle of the left horn to avoid any differences in receptors that could be caused by the different functionality of the uterine horns. After the biopsy was taken, tissue samples were immediately fixed with formalin and then embedded in paraffin until analysis.

Blood samples for progesterone (P_4) and estradiol 17β (E_2) determinations were collected by jugular venipuncture at the time of biopsy. Samples were centrifuged and plasma was stored at −20 °C until E_2 and P_4 assays were performed.

2.2. Hormone determinations

Progesterone was measured using an RIA kit (Diagnostic Products Corporation, Los Angeles, CA, USA). Serially diluted llama plasma samples containing relatively greater progesterone concentrations produced curves parallel to the standard curve. The sensitivity of the assay was 0.3 nmol l^{-1} and the intra-assay coefficient of variation was below 13% for concentrations between 0.4 and 128 nmol l^{-1}. Estradiol 17β was determined using an RIA kit (Diagnostic Products Corporation, 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 pmol l^{-1} and the intra-assay coefficient was below 20% for concentrations between 5.6 and 180 pmol l^{-1}. All samples were measured in duplicates and in one single assay for each hormone. Hormone concentrations are expressed in SI units. To convert from pmol l^{-1} to pg ml^{-1} and from nmol l^{-1} to ng ml^{-1} the following factors should be used—estradiol 17β: 3.7 and progesterone: 3.2.

2.3. Immunohistochemistry

An immunohistochemical technique (avidin–biotin–peroxidase) previously described (Meikle et al., 2000) was used to visualize ERα and PR immunostaining. After the paraffin tissue sections (5 μm) were dewaxed and rehydrated, an antigen retrieval procedure was performed. Sections were pretreated in a microwave oven at 900 W power, in 0.01 M sodium citrate buffer (pH 6.0) for 10 min, and then allowed to cool for a further 20 min. After washing in buffer (0.01 M PBS, pH 7.5) nonspecific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Merck) in methanol for 10 min at room temperature (RT). After a 10 min wash in buffer, sections were exposed to a 60 min non-immunoblock using diluted normal horse serum (Vectastain; Vector Laboratories, Burlingame, CA) in PBS in a humidified chamber at RT. The primary anti-PR antibody (monoclonal mouse antibody; Zymed Cat No. 18-0172, South San Francisco, CA, USA) and anti-ERα antibody (monoclonal mouse antibody; Cat No. sc-787, Santa Cruz, CA, USA) were diluted 1:100 and 1:25 in PBS, respectively, and incubated for 1 h. Negative controls for each receptor were obtained by replacing the primary antibody with nonimmune 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 horse anti-mouse IgG (Vectastain, Vector) diluted in normal horse serum. Thereafter, the tissue sections were incubated for 60 min at RT with a horseradish peroxidase–avidin–biotin complex (Vectastain Elite; Vector). The site of the bound enzyme was visualized by the application of 3,3′-diaminobenzidine in H_2O_2 (DAB kit; Vector) a chromogen that produces a brown, insoluble precipitate when incubated with the enzyme. The sections were counterstained with hematoxylin and dehydrated before they were mounted with Pertex (Histolab, Gothenburg, Sweden).

For each receptor, all samples were analyzed in the same immunohistochemical assay.

2.4. Image analysis

After a general inspection of each slide, a subjective image analysis was performed to estimate the expression of ERα and PR in different cell types as previously reported (Sosa et al., 2004). The evaluation was performed by two independent observers who were not aware of assignment to group of animals. The presence of both receptors was studied in three uterine compartments defined by cell type. Ten fields were analyzed for each cell type (the luminal epithelium, glandular epithelium and stromal cells) at a magnification of 1000× in all llamas. 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 $= 1 \times n_1 + 2 \times n_2 + 3 \times n_3$, where n = proportion of cells per field exhibiting faint (1), moderate (2) and intense (3) staining (Boos et al., 1996).

2.5. Statistical analysis

The variables from the image analysis that 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 (SAS Institute Inc., Cary, NC, USA). Nuclei staining was analyzed by the mixed procedure and the statistical model included the effects of observer, phase of ovarian activity, cell type, and the interactions between them. Data were pooled over observer and only the interaction terms that do not include observer were used. Data are presented as least square means ± pooled standard errors. The level of significance was $P < 0.05$.

Data from hormonal concentrations were analyzed using a Student t-test considering variation due to phase of ovarian activity (FP versus LP). The extent of significance was $P < 0.05$. Results are expressed as mean ± S.E.M.

3. Results

There were ovulations in five out of six animals in response to the GnRH treatment. Although differences were not statistically significant ($P = 0.057$), estradiol 17β concentrations during the FP showed a tendency to be greater than during the LP (89.04 ± 12.79 and 60.28 ± 2.55 pmol l^{-1}, respectively). Meanwhile, mean plasma progesterone concentrations during the FP was 1.66 ± 0.06 and had increased to 15.62 ± 1.57 nmol l^{-1} ($P < 0.05$) during the LP, in accordance with the development of the corpora lutea.

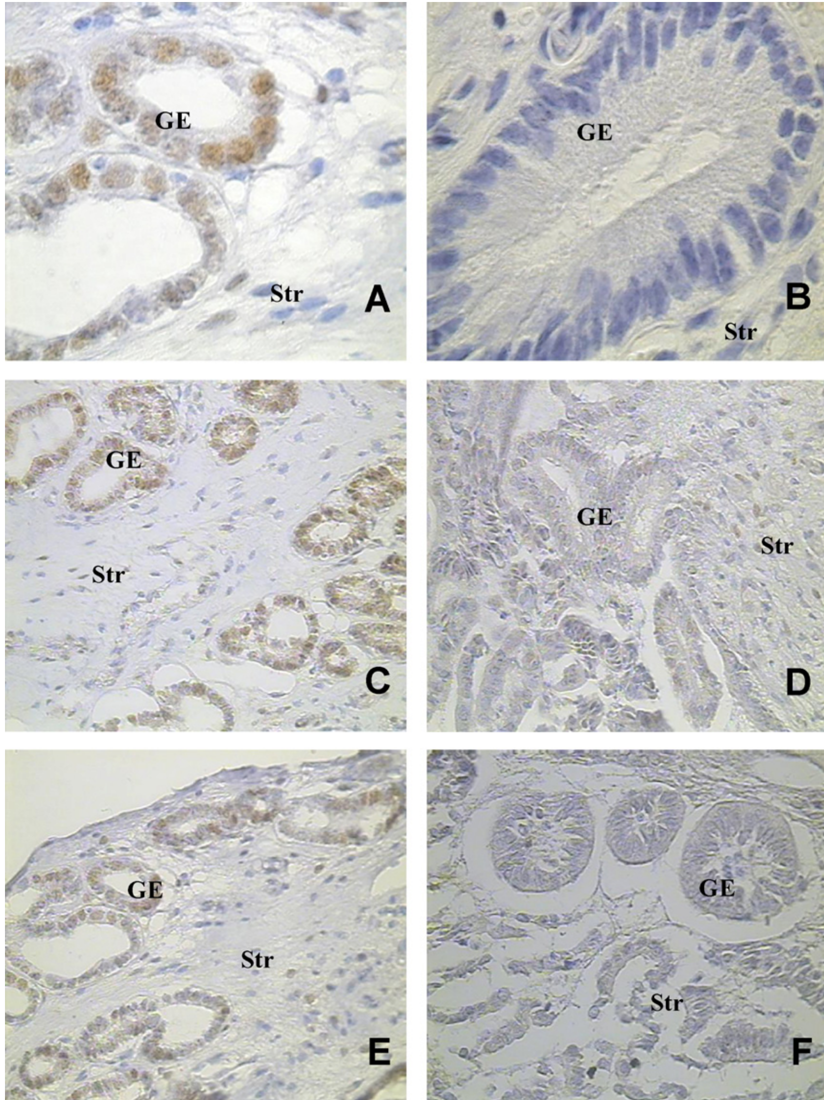

Fig. 1. Immunohistochemical localization of ERα and PR in endometrium in llamas during the follicular (C and E) or luteal (D and F) phase of the ovarian activity (GE = glandular epithelium; Str = stroma). (A) Positive control (1000×); (B) negative control (1000×); (C) ERα: follicular phase (400×); (D) ERα: luteal phase (400×); (E) PR: follicular phase (400×); (F) PR: luteal phase (400×).

3.1. General observations of ERα and PR immunostaining

Estrogen receptor-α (ERα) and progesterone receptor (PR) immunoreactivity was observed exclusively in the nuclei (not cytoplasma) of the studied cell types (Fig. 1A). When monoclonal specific antibodies were substituted by a nonimmune mouse IgG, the absence of staining demonstrated the specificity of both receptors immunostaining (Fig. 1B).

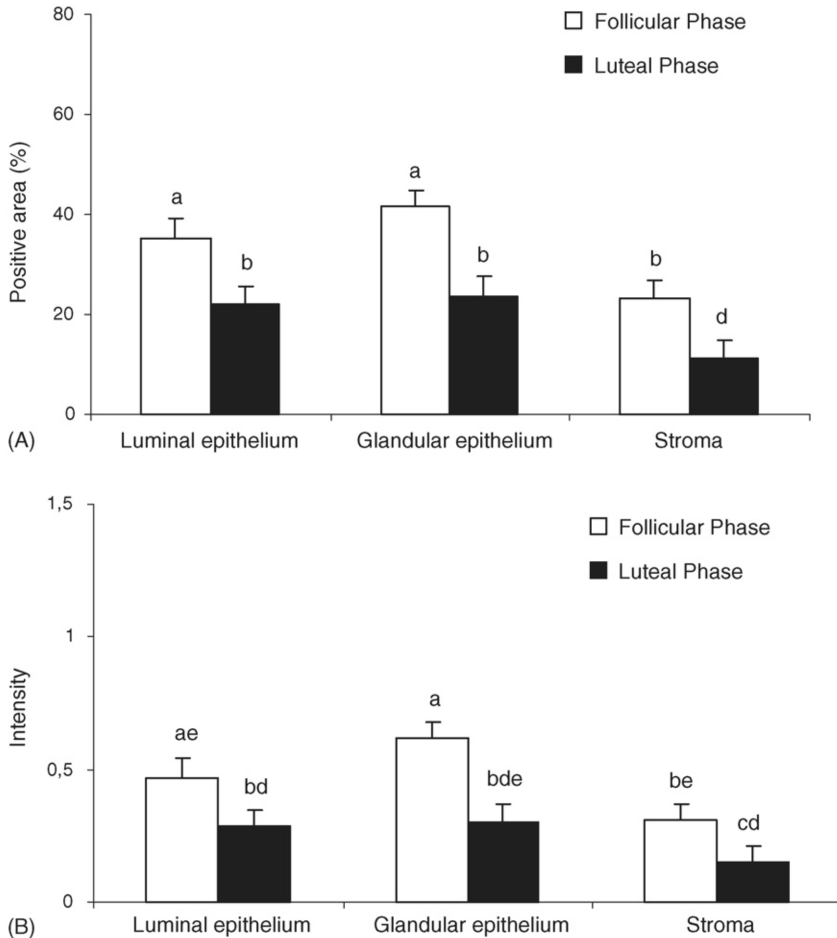

Fig. 2. (A) Positive area and (B) average immunoreactivity for ERα during the follicular (white bars) and luteal (black bars) phases of ovarian activity in llamas. Bars with different letters are significantly different ($P < 0.05$ except, ae vs. bd and be vs. cd, $P = 0.06$).

3.2. Estrogen receptor

In the three cell types, an increase of ERα contents was recorded during the FP. Thus, more positive stained areas were observed during the FP than during the LP ($P < 0.05$) (Fig. 2A), indicating an effect of the phase of ovarian activity. In addition, average staining was also affected by phase of ovarian activity. The greater intensity for ERα in glandular epithelium was found during the FP compared with the LP ($P < 0.05$), while a tendency to be greater was observed in luminal epithelium and stroma ($P = 0.06$) (Fig. 2B).

An effect of the cell type on total positive area was also observed. The epithelia (luminal and glandular) had more positive stained areas for ERα than stromal cells ($P < 0.05$) (Figs. 1C and D and 2A). This difference was more marked during the FP than during the LP (42% and 23%, respectively).

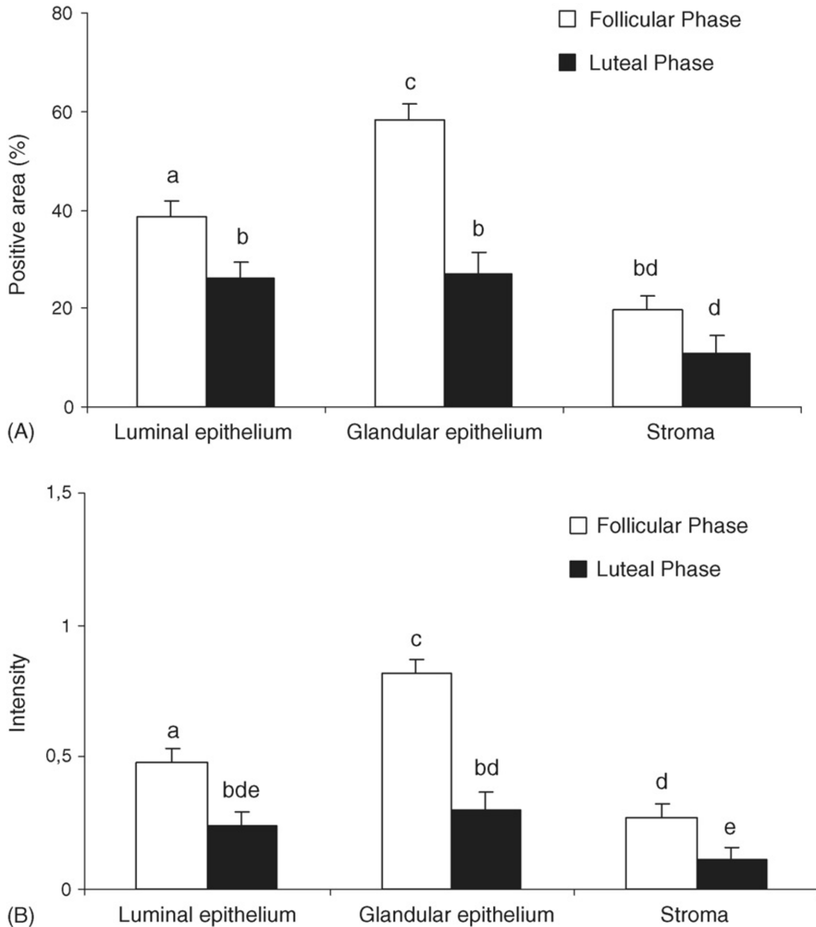

Fig. 3. (A) Positive area and (B) average immunoreactivity for PR during the follicular (white bars) and luteal (black bars) phases of the ovarian activity in llamas. Bars with different letters are significantly different ($P < 0.05$).

3.3. Progesterone receptor

The epithelia (luminal and glandular) had more positive stained areas during the FP than during the LP ($P < 0.05$). This difference was more important for glandular epithelia (58.1% during the FP compared with 27% during the LP). However, this difference between phases was not significant for the stromal cells (Fig. 3A). In addition, a greater intensity was observed during the FP than during the LP in all cell types ($P < 0.05$) (Fig. 3B), indicating greater amounts of PR proteins during the FP.

An effect of the cell type was also observed. During the FP more positive areas and greater intensity of staining was detected in cells of epithelia, while the stroma had lesser positive areas and lesser intensity of staining ($P < 0.05$). During the LP, both epithelia had similar positive areas and intensity of staining and the glandular epithelia showed greater positive areas and staining than stromal cells ($P < 0.05$) (Figs. 1E and F and 3B).

4. Discussion

To our knowledge the present study is the first to assess the presence of ERα and PR in luminal and glandular epithelia and stroma of the llama endometrium during the follicular and the luteal phase. The results indicate that the uterine ERα and PR in llamas vary according to the phase of the ovarian activity and to the cell type.

Plasma concentrations of progesterone were in agreement with the ovarian structures present in each phase. Estradiol 17β concentrations during the FP were not significantly greater than during the LP as was expected, although a tendency was observed. The disclosure could be explained in terms of the important estradiol release from follicles of a new follicular wave emerging before luteolysis that was previously reported (Aba et al., 1995). The observation that one animal failed to respond to the GnRH treatment and developed a follicle that was larger and remained present longer than normal follicles might be explained by the development of a hemorrhagic follicle which had been described to be anovulatory and to occur in 18% of the llamas (Adams et al., 1991).

In the present study, ERα and PR were detectable in all cell types regardless of the phase of ovarian activity. Moreover, in agreement with previous reports in cows (Meyer et al., 1988; Boos et al., 1996; Robinson et al., 2001) and sheep (Cherny et al., 1991; Spencer and Bazer, 1995), endometrial ERα and PR contents were greater during the follicular phase and decreased by the time a corpus luteum was present and consequently plasma progesterone concentrations increased. These findings are consistent with the known regulatory effect of steroid hormones on receptors in other species; estradiol has a stimulatory effect on ERα and PR, whereas progesterone has a suppressing action (Meikle et al., 2004). The extent of presence of a specific receptor in the target tissues has been used as an index of the extent of hormone responsiveness (Katznellbogen, 1980). The greater uterine presence of steroid receptors during the follicular phase hereby reported is consistent with the dramatic changes that occur during this phase (e.g. proliferation induced by estrogens) and the early luteal phase (differentiation induced by progesterone). The decrease in steroid receptor content observed on Day 9 suggest that the activation of the progesterone–receptor complex makes the uterus refractory to further actions of progesterone, timing in this way the termination of progesterone dominance in the uterus at the end of the luteal phase. This has been reported to be one of the mechanisms of the initiation of luteolysis in ruminants (Lamming et al., 1995), but no data as such has been studied in llamas.

Besides the overall receptor presence observed in the uterus during the follicular and luteal phases, it is recognized that cell types respond in a different manner to estradiol 17β and progesterone. Thus, it was demonstrated in the rodent uterus that the response to estrogen stimulation – regarding protein synthesis and proliferation – differs according to cell type (Quarmby and Korach, 1984a,b). In the sheep, humans and mice endometrium, stroma–epithelial interactions have been reported (Brenner et al., 1990; Stevenson et al., 1994; Cunha et al., 2004) showing the importance of the cross-talk between stromal and epithelial cells to regulate uterine function. The immunohistochemical technique allows for evaluation of changes in receptors in specific cell types. In the present study, different amounts of endometrial estrogen and progesterone receptors have been observed in stromal and epithelial cells. Stromal cells had a lesser ERα positive area and immunoreactivity than epithelial cells. Moreover, the intensity observed in the stroma during the FP was similar to the intensity found in the epithelia during the LP. This is consistent with previous reports in rats (Nephew et al., 2000) and ewes (Meikle et al., 2000). Conversely, while in the latter studies a pronounced downregulation of ERα was observed in the epithelial cells when compared with stromal cells, in the present study the ERα downregulation found during the luteal phase was similar among cell types.

The positive areas and the average intensity for PR were also lesser in the stromal cells than in the epithelia, being more marked between glandular epithelium and stromal cells. This observation is in agreement with other report in ewes (Sosa et al., 2004). The downregulation of PR was more pronounced in the glandular epithelium than in the other cell types. Conversely, the downregulation of PR of luminal epithelium was less marked than in the glandular epithelium and stromal cells, while in other species, a similar pattern of downregulation for PR in both epithelia was reported (Sosa et al., 2004).

Although the authors have no obvious explanation for the differences observed in llamas and other species in the process of downregulation, a species-specific mechanism for regulation of ERα and PR can be hypothesized in the llama. However, differences in the stage of the FP and LP at the time biopsies were taken in llamas compared with studies in ewes and cows cannot be neglected. In the latter species, Day 0 is easily defined as day of estrus or spontaneous ovulation. Llamas, however, can remain in estrus for long periods if they are not mated or induced to ovulate (Bravo and Sumar, 1989) thus samples, in the present study were taken the first day that an ovulatory follicle was recorded.

5. Conclusion

In summary, the results of the present study demonstrated that endometrial ERα and PR are greater during the follicular phase than during the luteal phase in llamas, suggesting that, as in other ruminants, estrogens up-regulate both receptors, while progesterone has the opposite effect. In addition, results support the hypothesis that the regulation of reproductive steroid receptors is cell type specific in this species.

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