Livestock Science 155 (2013) 442–453

Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Localization patterns of steroid and luteinizing hormone receptors in the corpus luteum of Nelore (*Bos taurus indicus*) cows throughout the estrous cycle

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ARTICLE INFO

Article history:
Received 25 May 2012
Received in revised form
30 April 2013
Accepted 7 May 2013

Keywords:
Estrous cycle
Corpus luteum
Immunohistochemistry
Nelore
Steroid hormone receptors

ABSTRACT

The aim of the present study was to detect progesterone receptors (A and B isoforms), α and β estrogen receptors, luteinizing hormone receptors and aromatase cytochrome P450 enzymes in the corpus luteum of Nelore (*Bos taurus indicus*) cows using immunohistochemistry. The estrous cycles of 16 Nelore cows were synchronized, and luteal samples were collected via an incision into the vaginal vault. Samples were collected during specific days of the estrous cycle (days 6, 10, 15 and 18) and 24 h after circulating progesterone dropped, after luteolysis had occurred. After each biopsy was taken, all animals were resynchronized so that each biopsy was performed during a different estrous cycle. Our results showed that the concentration of studied proteins vary throughout the bovine estrous cycle. The highest concentration of α and β estrogen receptors and the highest concentration of plasma progesterone were both observed on days 10 and 15 of the estrous cycle. The highest concentration of progesterone receptors was observed on days 6 and 10 of the estrous cycle, and the most intense immunostaining for cytochrome P450 aromatase enzymes was observed on day 10 of the estrous cycle. The highest score of cells with plasma membrane immunostaining for LH receptors was observed on day 15 of the estrous cycle. In conclusion, this study demonstrates the varying concentrations of specific proteins within the corpus luteum of Nelore cows during the estrous cycle. This finding suggests that these receptors and enzymes, and their interactions, are important in regulating luteal viability.

1. Introduction

The corpus luteum (CL) is a transient endocrine gland that develops from the remainder of the ovulatory follicle. It secretes progesterone (P_4) to support pregnancy (Milvae et al., 1996).

Since the 1970s, researchers have hypothesized that steroid hormones produced by the CL act locally to modulate the development, functionality and half-life of

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http://dx.doi.org/10.1016/j.livsci.2013.05.016

this gland. Knobil (1973) suggested that the estrogen produced by primate CLs initiates luteal regression using a local mechanism of self-destruction, which occurs near the end of the menstrual cycle. In domestic animals, autocrine and/or paracrine action of P_4 and estradiol-17β in CL has also been evidenced in the literature (Schams and Berisha, 2002). Rothchild (1981) reported that P4 is a universal luteotropin that promotes its own secretion in many species. Adequate luteal function, allowing for the secretion of progesterone, is crucial for determining the physiological duration of the estrous cycle. Luteal function is also important in maintaining a successful pregnancy. The mechanism controlling the development and secretory function of the CL involves many factors that are produced both within and outside of the CL.

The effects of estrogen and progesterone are mediated through interaction with specific intracellular receptors, which are members of the nuclear receptor superfamily of transcription factors (Conneely, 2001). Receptors for estrogen are expressed as two structurally related subtypes, estrogen receptor α (ERα) and estrogen receptor β (ERβ), which are both encoded by two distinct genes (Couse and Korach, 1999; Kuiper et al., 1996). ERα and ERβ exhibit significant functional differences when examined under similar conditions in cell-based transactivation assays (Conneely, 2001). Selective ablation of ERα and ERβ in mice has provided definitive evidence that these receptor subtypes mediate distinct physiological responses (reviewed by Conneely, 2001). Ablation of ERα, the dominant subtype, results in infertility (Couse and Korach, 1999), and the ablation of ERβ results in a subfertile phenotype (Krege et al., 1998). The expression of ERα and ERβ has already been observed in ovine (Cardenas et al., 2001; Zieba et al., 2000), swine (Slomczynska and Wozniak, 2001), rabbit (Iwai et al., 1991) and bovine (D'Haeseleer et al., 2006; Van Den Broeck et al., 2002a) luteal cells.

In contrast to estrogen, progesterone receptors (PR) are expressed as two distinct isoforms, PR-A and PR-B, which arise from a single gene (Conneely et al., 1989). However, the ratio of the individual isoforms vary depending upon the reproductive tissue. Studies using mutant mice, with selective ablation of PR-A and PR-B, showed that PR-A is necessary and sufficient to elicit progesterone-dependent reproductive responses. PR-B is needed to elicit normal proliferative responses of the mammary gland in response to progesterone (reviewed by Conneely, 2001; Conneely and Lydon, 2000). Progesterone receptors have also been previously observed on swine (Slomczynska et al., 2000), canine (Vermeirsch et al., 2001), rabbit (Iwai et al., 1991; Korte and Isola, 1988) and bovine (D'Haeseleer et al., 2007; Van Den Broeck et al., 2002b) luteal cells.

In addition to steroid receptors, luteinizing hormone (LH) appears to be the most important hormone for triggering progesterone production, mainly via small luteal cells (Alila et al., 1988; Harrison et al., 1987; Niswender et al., 1986; Okuda et al., 1999; Ursely and Leymarie, 1979), confirming their luteotropic activity (Fields and Shemesh, 2004). This action is mediated by the luteinizing hormone receptor (LHR) , which is localized on the plasma membrane of luteal cells (Jones et al., 1992). Additionally, it was demonstrated that PR mRNA expression is upregulated in preovulatory follicles following an LH surge, which may help follicles rupture (Cassar et al., 2002). During the early luteal phase, the number of LHR increased when treated with exogenous progesterone. This may ensure that luteal cells develop a maximal response to gonadotropins (Jones et al., 1992) and indicates a relationship between steroid receptor-hormones and LH-LHR. The expression of LHR has also been verified in bovine (Chegini et al., 1991; Garverick et al., 1985; Jones et al., 1992; Okuda et al., 1999), swine (Meduri et al., 1992), ovine (Harrison et al., 1987), human (Bukovský et al., 1995; Duncan et al., 1996) and murine (Gafvels et al., 1992) luteal cells.

The aim of the present study was to investigate the tissue distribution of steroid receptors, LHR and cytochrome P450 aromatase enzymes during the estrous cycle. The restricted spatiotemporal expression of these factors identifies the tissues targeted by a hormonal response and may be essential for understanding CL function.

2. Materials and methods

2.1. Animals

Sixteen estrous cycling Nelore (*Bos taurus indicus*) cows, at least 2 months post partum and with a body score equal to or greater than 3 (using the 0–5 point scale; Houghton et al., 1990), were used. Animals were maintained in an uncovered paddock, receiving grass, supplemented with hay and concentrate twice daily. Animals had access to mineral supplementation and water "*ad libitum*". The animals had a 30-day adaptation period before the beginning the experiment.

The experimental protocol was approved by the Animal Welfare and Ethics Committee at the Veterinary Medicine and Animal Science College, São Paulo State University, SP, Brazil. The experiment was performed in accordance with international guidelines for the care and use of experimental animals.

2.2. Experimental design

Estrous cycles were synchronized by administering 50 μg of lecirelin (Gestran Plus™, Tecnopec Co, São Paulo, SP, Brazil, IM) and inserting an intravaginal progesterone device (Primer™, Tecnopec Co) that remained in place for 7 days. Six and 7 days after synchronization, 5.0 and 2.5 mg of cloprostenol (Sincrosin™, Vallee Co, São Paulo, SP, Brazil, IM) were administrated, respectively. The ovaries were examined by rectal palpation and ultrasonography (Aloka Co, Tokyo, Japan, with a 5.0 MHz transducer) to determine the day of ovulation (day 1).

Luteal biopsies were performed on specific days of the estrous cycle, days 6 ($n=14$), 10 ($n=13$), 15 ($n=12$) and 18 ($n=13$) of the estrous cycle and 24 h after plasma progesterone concentrations fell below 1 ng/mL, indicating luteolysis had occurred ($n=13$). Each biopsy was performed during a different estrous cycle and the animals were resynchronized for the next procedure, so that all cows were submitted to five biopsies. To determine the appropriate time to take the final biopsy (after luteolysis), blood

samples were collected daily from each animal and submitted to a progesterone assay. Once values dropped below 1 ng/mL, the CL biopsy was scheduled for the following day.

Some cows that were biopsied on day 18 had already entered luteolysis according to their plasma progesterone concentration. Therefore, the biopsies taken on day 18 were divided into two groups: the above 1 ng/mL of plasma progesterone (in diestrus—D18) group or the below 1 ng/mL of plasma progesterone (after luteolysis—D18L) group.

Biopsies were performed via an incision in the vaginal vault using a scalpel blade. Tissue was dissected until it was possible to access the pelvic cavity and retract the ovary inside the vagina. The luteal samples were obtained with a Yomann biopsy nipper. Tissue specimens, which were approximately 1.5 cm long and 0.5 cm wide, were washed in saline, placed in plastic cassettes for inclusion, and fixed in 10% (v:v) buffered formaldehyde for 24 h. Samples were stored in 70% (v:v) ethanol until they were embedded in paraffin. For biopsies, animals were submitted to an anesthetic protocol using both an epidural and a local anesthetic (placed into the vaginal vault). The epidural consisted of 0.14 mg/kg of 2% lidocaine hydrochloride (Xylestesin™ 2%, Cristália Co, Itapira, SP, Brazil) and 0.17 mg/kg of xylazine hydrochloride (Rompum™ Co, Bayer Co, São Paulo, SP, Brazil). In addition, each cow received a local block in the vaginal vault with 20 mL of 2% lidocaine hydrochloride. After waiting 10 min, the vaginal vault was incised (Martin et al., 2010).

Blood samples from the jugular vein were collected into heparinized tubes during days when biopsies were performed. Samples were centrifuged, and the plasma was harvested and frozen at −20 °C for progesterone concentration measurements.

2.3. Hormonal assays

Progesterone concentrations were measured in all plasma samples by RIA using a Count-A-Count kit (Diagnostics Products Corporation Co, Los Angeles, *CA*, USA) according to the manufacturer's instructions. One assay was performed, and the intra-assay coefficient of variation was 1.61%. The sensitivity of the assay was 0.02 ng/mL.

2.4. ERα immunolocalization

Three-micrometer tissue sections were mounted onto glass slides previously treated with Organosilane (Sigma Chemical Co, St. Louis, MO, USA). Sections were deparaffinized with xylene and rehydrated in graded alcohol. For antigen retrieval, sections were microwaved for 5 min, three times each, in a 10 mM sodium citrate (pH=6.0) solution. After heating, slides were allowed to cool over 20 min and were washed in distilled water 10 times.

Sections were then encircled using a DakoPen™ (Dako Co, Carpinteria, CA, USA) and endogenous peroxidase activity was quenched with universal blockage (Dual Endogenous Enzyme Block, Dako Co) for 10 min followed by 10 washes in distilled water. Slides were then washed for 5 min in TRIS buffered solution (pH 7.4) twice. For endogenous biotin blockage, the slides were incubated in the Biotin Blocking System (Dako Co) for 10 min and were then incubated with normal goat serum (1:40, Goat Serum, Dako Co) for 20 min, and protein blockage (Protein Block, Dako Co) for 10 min. These incubations were performed in room temperature and the slides were washed in TRIS buffered solution (pH 7.4) after each blockage.

The next step was incubation with the primary mouse anti-human estrogen receptor α monoclonal antibody (Estrogen Receptor α, clone 1D5, Dako Co), diluted 1:50 in antibody diluent (Antibody Diluent with Background Reducing Components, Dako Co) in a humidified chamber for 18 h at 4 °C.

Then, slides were washed twice for 5 min each in TRIS buffered solution, incubated with the secondary biotinylated anti-mouse antibody for 30 min and streptavidin–biotin (ABC Kit Vectastain, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min each reagent, in a humidified chamber, according to the manufacturer's instructions. Each tissue section was washed in TRIS (pH 7.4), and DAB chromogen (3,3′-diaminobenzidina, Dako Co) was added as a chromogen staining substrate for 5 min. The reaction was stopped by rinsing with Tris (pH 7.4). Tissue sections were counterstained with Mayers hematoxylin for 3 min, dehydrated and preserved using a Permount (Thermo Fisher Scientific, Waltham, MA, USA) mounting medium.

For negative controls, sections of corpus luteum were incubated with mouse immunoglobulin (N-Universal Negative Control Mouse, Dako Co) or 5% BSA replaced the primary antibody; however, the other previously described reaction steps were followed. Other negative controls included incubation with DAB chromogen (endogenous peroxidase blockage negative control) alone, or with the streptavidin–biotin complex and the DAB chromogen (biotin endogenous blockage negative control). For positive controls, sections of endometrium were used (Martin et al., 2008) in the same manner as the CL.

2.5. Immunolocalization of ERβ

Procedures for ERβ detection followed similar procedures as described for ERα. The exceptions were that endogenous peroxidase activity was quenched with a 20% peroxide solution for 30 min, the primary mouse anti-human estrogen receptor β monoclonal antibody (Estrogen Receptor β, clone PPG5/10, Dako Co) was used and diluted to 1:200 in antibody diluents and the secondary antibody EnVision™ (Dako Co) was used for 60 min. For ERβ, the other blockages performed for ERα were not used. For positive controls, sections of endometrium were used (Rosenfeld et al., 1998).

2.6. Immunolocalization of PR

Procedures for PR detection followed similar procedures as described for immunolocalization of ERα, except that the primary anti-human progesterone receptor mouse monoclonal antibody (Progesterone Receptor Ab-8 clone hPRa 2+hPRa 3, Thermo Fisher Scientific Co) was used.

For positive controls, sections of endometrium were used (Martin et al., 2008).

2.7. Immunolocalization of LHR

Procedures for LHR detection followed similar procedures as those described for immunolocalization of ERα. The exception was with respect to antigen retrieval, which was performed with pre-heated (96 °C) Tris–EDTA (pH 9.0) solution in a water bath at 96 °C for 20 min. In addition, a primary anti-human luteinizing hormone receptor rabbit polyclonal antibody (Anti-Luteinizing Hormone Receptor, Novus Biologicals, Littleton, CO, USA) was used and diluted 1:800 in antibody diluent, and the secondary antibody was used (EnVision, Dako Co) for 40 min at room temperature. For LHR immunolocalization, the other blockages performed for ERα were not used.

For negative controls, the procedures followed similar techniques as described for ERα, except that for the polyclonal antibody, a rabbit immunoglobulin (N-Universal Negative Control Rabbit, Dako Co) was used instead of the primary antibody. For positive controls, sections of endometrium were used (Fields and Shemesh, 2004).

2.8. Immunolocalization of cytochrome P450 aromatase

Cytochrome P450 aromatase detection followed similar technique as those described for ERα, with the exception of antigen retrieval, which was performed with pre-heated (96 °C) 10 mM sodium citrate (pH=6.0) solution in a water bath at 96 °C for 30 min. The endogenous peroxidase activity was quenched with a 3% peroxide solution for 20 min, and the primary anti-human cytochrome P450 aromatase rabbit polyclonal antibody (Hauptman-Woodward Medical Research Institute, Buffalo, NY, USA) was diluted 1:100 in antibody diluent. The secondary antibody employed (EnVision, Dako Co) was used for 60 min. For Cytochrome P450 Aromatase, immunolocalization blockages performed for ERα were not used.

For negative controls, a similar technique as that described for ERα was used, except that for the polyclonal antibody, rabbit immunoglobulin (N-Universal Negative Control Rabbit, Dako Co) was used instead of a primary antibody. For positive controls, sections containing an antral follicle were used, and positive immunostaining was observed in the cytoplasm of granulosa cells (Slomczynska and Tabarowski, 2001).

2.9. Evaluation of immunoreactivity

Stained sections were observed and imaged using a Leica DML optic microscope (Leica Microsystems, Wetzlar, Hessen, Germany) equipped with a Leica DFC 500 camera (Leica Microsystems Co). Sections were magnified to 400× with an image analyzer program (Leica QWin Standard V2.5).

For the evaluation of ERα, ERβ and PR immunostaining, the number of luteal cell nuclei that stained positively and negatively was counted in five randomly selected microscopic fields. Any nuclei showing evidence of a reaction were considered to be positive, regardless of intensity. Using this method, a proportion of positive-receptor nuclei were obtained for each of the five different time periods for all animals. For the evaluation of cytochrome P450 aromatase immunostaining, the staining intensity of cytoplasm, predominantly in the field, was scored in 10 randomly selected microscopic fields. Findings were classified in the following categories: 1 (weak), 2 (intermediate) and 3 (strong). Using this method, a mean intensity for each slide was obtained for each of the five different moments time periods for all animals. For the evaluation of LHR immunostaining, five randomly selected microscopic fields were classified in the following categories: 1 (up to 25% of the cell surface with strong immunostaining), 2 (from 25% to 50% of the cell surface with strong immunostaining), 3 (from 51% to 75% of the cell surface with strong immunostaining) and 4 (more than 75% of the cell surface with strong immunostaining), according to the number of cells with their surface positively stained.

2.10. Statistical analysis

Data were analyzed by GLM using SAS version 5.0 (1996). The percentage of nuclei staining for ERα, percentage of nuclei staining for ERβ, percentage of nuclei staining for PR, intensity of staining for cytochrome P450 aromatase, categories for LHR immunostaining and plasma progesterone concentrations were the dependent variables. Cows and days of the estrous cycle were the independent variables.

First, data were reviewed, and their descriptive analysis (mean, standard error, maximum and minimum values) was performed by the MEANS procedure (PROC MEANS). The following variables were compared during the different time periods evaluated (days 6, 10, 15, 18, 18L): percentage of positive nuclei for ERα, ERβ and PR, intensity of staining for cytochrome P450 aromatase, categories for LHR immunostaining and plasma progesterone concentrations, by GLM procedure (General Linear Model Procedure —PROC GLM), using SNK test (Student–Newman–Keuls).

3. Results

All proteins evaluated varied throughout the estrous cycle ($p < 0.01$). ERα, ERβ and PR immunostaining patterns were clearly observed in the nucleus of the luteal cells.

Immunostaining for ERα and ERβ showed a very similar pattern. Both had increased number of positive nuclei at the beginning of the estrous cycle and achieved maximal count during the middle of diestrus, after which, there was a decline during late diestrus. The difference between estrogen receptors appeared after luteolysis, which was after plasma progesterone concentrations felt below 1 ng/mL. At that point, the ERβ fell significantly, and the ERα disappeared, which may indicate the importance of ERα on corpus luteum cell viability. Immunostaining for ERα and ERβ was also observed in endothelial cells (Figs. 1 and 4a and b).

Nuclear immunostaining for PR had its maximum count until day 15 of the estrous cycle, and overall, its staining was consistently weak. Immunostaining for PR

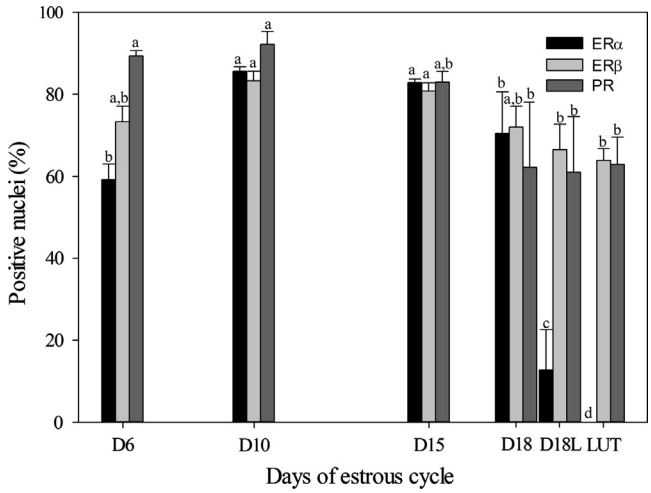


Fig. 1. Percentage of nuclei positive for steroid receptors (mean ± standard error) on days 6, 10, 15, 18, day 18 after luteolysis (D18L) and after luteolysis (LUT) in the corpus luteum of Nelore cows. Bars of the same color with different letters are significantly different ($p < 0.05$).

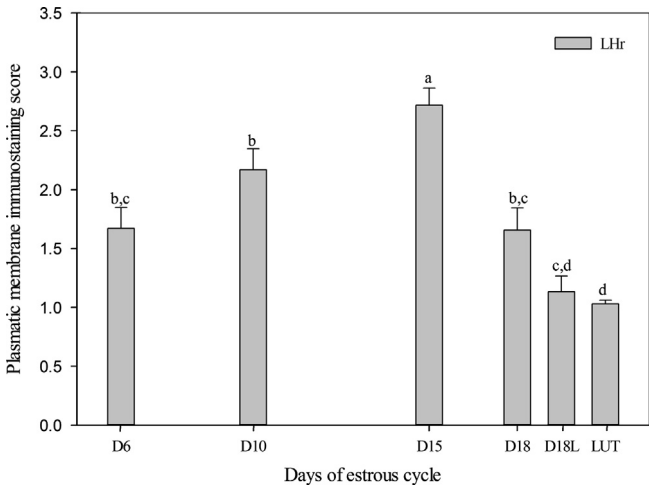


Fig. 2. Score of plasma membrane immunostaining, indicating a LH receptor (mean ± standard error), on days 6, 10, 15, 18, day 18 after luteolysis (D18L) and after luteolysis (LUT) in the corpus luteum of Nelore cows. Bars with different letters are significantly different ($p < 0.05$).

was also prevalent in fibroblast (stromal) cells and in the plasma membrane of some luteal cells (Figs. 1 and 4c).

Immunostaining for LHR was clearly observed on the cell membrane of the both steroidogenic luteal cells and in a multifocal pattern in the cytoplasm, predominantly observed in the large luteal cells. The pattern observed was a gradual increase of the cell surface immunostaining during the early diestrus, achieving maximal at day 15 of the estrous cycle, followed by a significant decrease during late diestrus. Nevertheless the cytoplasmatic multifocal immunostaining pattern decreases from day 6 to day 15 (Figs. 2 and 4d). Cytochrome P450 aromatase immunostaining was verified predominantly in the cytoplasm of luteal cells. However, weak nuclear immunostaining was also present. The maximum intensity for cytochrome P450 aromatase immunostaining was observed on day 10 of the estrous cycle (Figs. 3 and 4e).

Plasma progesterone concentrations also varied throughout the estrous cycle ($p < 0.01$), with maximum values observed on days 10 and 15 (Fig. 5).

4. Discussion

This study is the first one in which corpus luteum biopsies were collected *in vivo* on specific days of the estrous cycle and confirms the complex dynamics of protein expression of steroid and LH receptors and aromatase cytochrome P450 in bovine luteal cells.

The differences in the number of cells expressing estrogen receptors (ERα and ERβ) throughout the estrous cycle, identified using immunohistochemistry, paralleled the protein concentration determined by a western blot (Shibaya et al., 2007). However, we observed an absence of ERα immunolabeling 24 h after the drop of progesterone

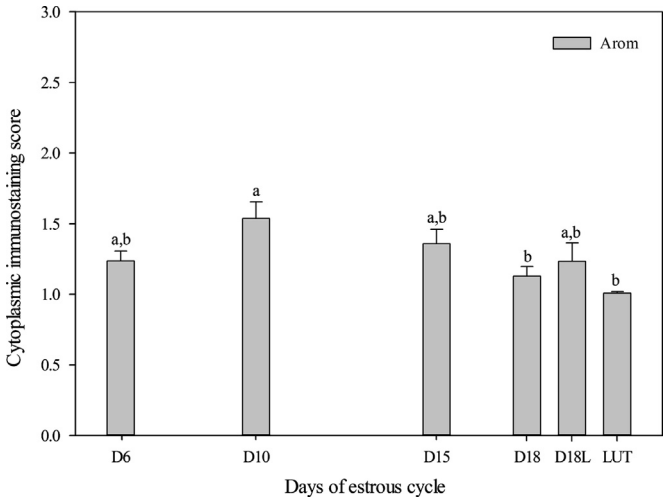


Fig. 3. Score of cytoplasmic immunostaining for aromatase cytochrome P450 (mean ± standard error) on days 6, 10, 15, 18, after luteolysis on day 18 (D18L) and after luteolysis (LUT) in the corpus luteum of Nelore cows. Bars with different letters are significantly different ($p < 0.05$).

levels below 1 ng/mL (luteolysis), whereas Shibaya et al. (2007) reported extremely low levels of this protein (ERα) when using western blot analysis in corpus luteum classified as regressed. Data in the study of Shibaya et al. (2007) was classified by gross luteal observations, not by the specific day of the estrous cycle.

Regarding ERβ our results are similar to the study of Shibaya et al. (2007), which is the only study in the literature concerning ERβ protein detection, in which we observed that the protein level increased at the beginning of the estrous cycle and achieved maximal expression/count during the middle of diestrus and decreased thereafter.

For ERα, the results of the present study are also similar to the study by Van Den Broeck et al. (2002a), which evaluated the expression of ERα by immunohistochemistry in *Bos taurus taurus*. These authors divided the animals into three groups according to plasma progesterone concentration and observed greater immunostaining in animals with progesterone higher than 3.0 ng/mL. There was intermediate immunostaining in the group with progesterone between 0.5 and 3.0 ng/mL and an absence of ERα immunostaining when plasma progesterone concentrations were below 0.5 ng/mL. Our study differs from the ERα study of D'Haeseleer et al. (2006) because these authors only observed the presence of ERα during late diestrus using immunohistochemistry technique.

It has also been shown that a bovine CL produces estradiol-17β in a constant manner throughout the luteal stages (Okuda et al., 2001). Thus, the disappearance of ERα and the decline of ERβ on these cells show that these receptors are very important for maintaining CL cells and that the secretion of PGF2α associated with the drop in progesterone concentrations probably stimulates the decline of ERs. In contrast, estradiol-17β showed no effect on progesterone secretion and had a stimulatory effect on PGF2α secretion (Okuda et al., 2001).

Is widely known that estradiol has several functions in bovine reproduction (Hafez et al., 2000). Further, estradiol also plays an important systemic role in preserving endothelial cells integrity, increasing both migration and proliferation of endothelial cells and contributing to accelerate the reendothelialization. During an acute inflammatory process, estradiol attenuates the endothelial activation by preventing the adhesion of leukocytes to endothelial cells and by the inhibition of proinflammatory chemokines secretion, such as macrophage chemoattractant peptide-1 (MCP-1) and interleukin (IL)-8. So, estradiol also reduces inflammation and antagonizes the deleterious inflammatory action of angiotensin II (Ang II) through mechanisms involving both nitric oxide (NO) and cyclooxygenases (COX). Importantly, endothelial apoptosis in response to various injuries is also prevented by E2 (Arnal et al., 2010).

After induction of luteolysis by endometrial PGF2α, a cascade of events occurs within the CL leading to functional and structural luteolysis. The acute changes in vasoactive factors suggest that modulation of vascular stability is a critical component of luteolysis. Other cascades, after PGF2α-induced luteolysis, that occur in parallel or subsequent to changes in vascularity include up-regulation of inflammatory cytokines (e.g. tumor necrosis factor—TNF, interleukin 1 beta—IL1B, IL8) and luteal cell apoptosis factor (e.g. FAS, Fas-ligand—FASL), endothelial adhesion and invasion by immune cells (monocytes, macrophages, T-lymphocytes) from the blood stream after MCP-1 up-regulation, up-regulation of vasoactive peptides (Ang II, Endothelin 1—EDN1, NO), up-regulation of prostaglandin-endoperoxide synthase 2 (PTGS2—COX2) and prostaglandin F synthase (PTGFS) (Atli et al., 2012; Miyamoto et al., 2010; Pate et al., 2012). These events modulate intraluteal synthesis of prostaglandins and induce apoptosis, initially in endothelial cells and thereafter in steroidogenic luteal cells (Hojo et al. 2010; Sawyer et al. 1990).

Taking all this information in account, estradiol may have a powerful protective role in CL acting as endothelial stabilizer, preventing the adhesion and invasion by immune cells and the intraluteal synthesis of PGF2α, antagonizing the action of angiotensin II (Ang II), nitric oxide (NO) and cyclooxygenases (COX). Therefore, the sudden disappearance of ERα observed in our study interrupts the protective

effects of estradiol and probably allows endothelial and immune components of luteolysis act.

The cytochrome P450 aromatase had higher immunostaining in early and middle luteal stages. In the literature, there is no information regarding cytochrome P450 aromatase protein detection in bovine CLs. The putative role of this enzyme is the conversion of androgens to estrogens (Conley et al., 1995). Therefore, it could be presumed that intraluteal secretion of estradiol may fluctuate in accordance with stages of major or minor cytochrome P450 aromatase expression. Otherwise, it was described by Okuda et al. (2001) that estradiol concentrations from bovine CLs did not vary during luteal phases (initial, medium and late).

Autocrine and paracrine regulation of steroid, steroid receptors, growth factors, peptides and prostaglandins has played an important modulatory role during different stages of bovine CL function (Schams and Berisha, 2004). Considering the angiogenic importance and vascular protective effect of estradiol, it could be hypothesized that the estradiol also has an important role during the formation of corpus luteum (after ovulation) and mid luteal phase (soon after atresia of dominants follicles) when the plasmatic estradiol concentration is low. In this study, the luteal stages with increased expression of ERα and ERβ were coincident with higher cytochrome P450 aromatase immunostaining and reinforce the importance of the steroid hormones on this tissue.

In our study, we observed weak immunostaining for PR on bovine luteal cells. This finding has also been described in the literature (D'Haeseleer et al., 2007; Korte and Isola, 1988; Van Den Broeck et al., 2002b; Vermeirsch et al., 2001). This pattern could reflect a negative effect of local higher progesterone concentrations or the decreased necessity of PRs due to the higher concentration of this hormone. We also observed strong immunostaining around the plasma membrane of some luteal cells, which suggests specific non-genomic membrane-localized binding sites. This finding has been previously described by Rae et al. (1998) and Bramley et al. (2002) and reviewed by Bramley (2003). According to these authors, the membrane binding sites could be a local site for rapid and specific changes induced by progesterone. These sites could also mediate an autocrine and/or paracrine action of progesterone in the CL.

We verified that there is increased PR protein immunostaining in early and middle diestrus, which decreased to reach minor values during late diestrus and after luteal regression. We found similar results to those published by D'Haeseleer et al. (2007) when the initial and late diestrus periods were considered. However, the increased immunostaining described by these authors during proestrus and estrous differs from our results observed after luteolysis.

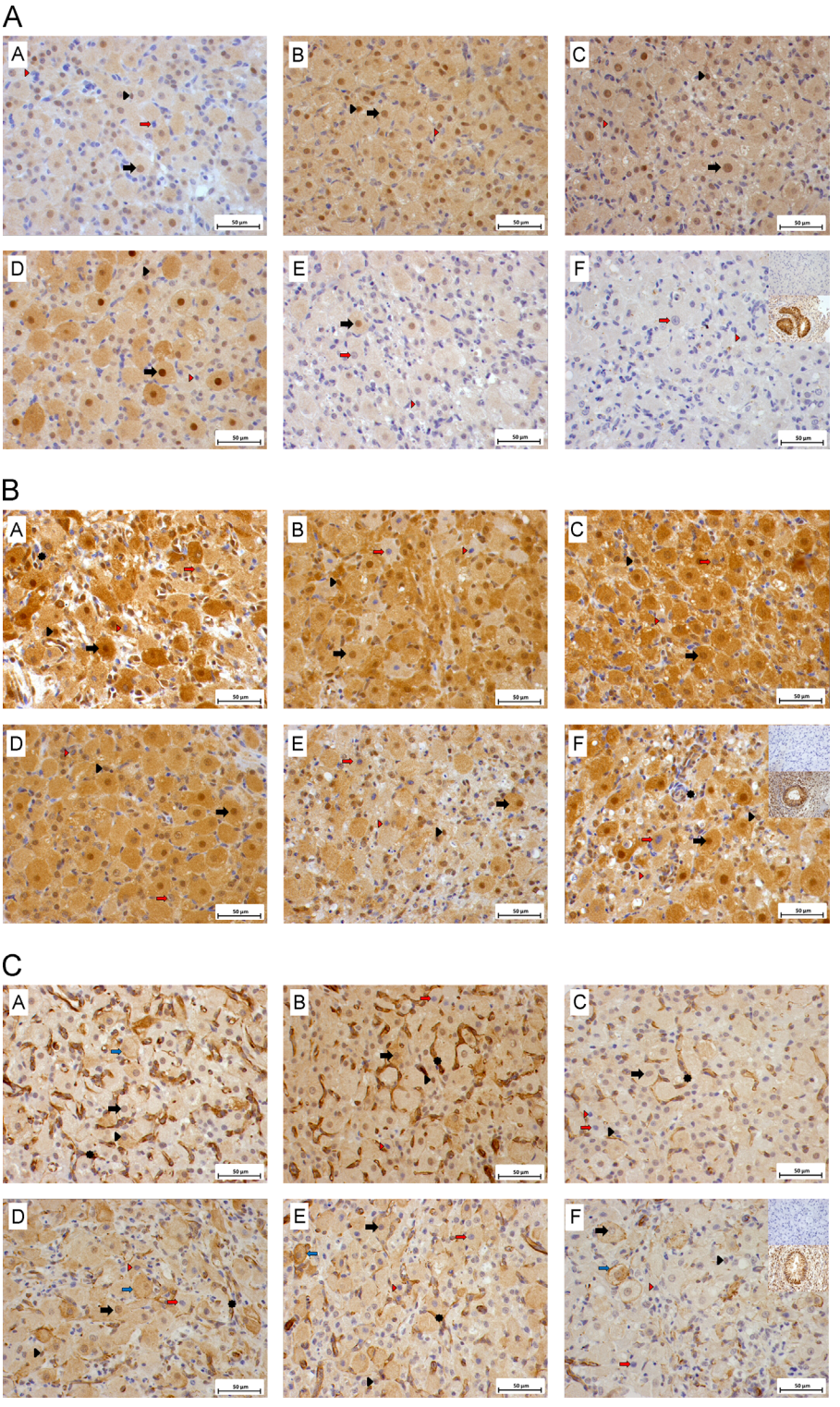
Other study has allocated research animals to various groups according to the plasma progesterone concentration and reported that animals with intermediate plasma progesterone concentrations (0.5–3.0 ng/mL) present higher counts of positive PR cells when compared to animals with concentrations above 3.0 ng/mL and were absent in animals with progesterone below 0.5 ng/mL (Van Den Broeck et al., 2002b). These findings differ from the results observed in the present study, where the immunostaining for PRs was increased at day 6 of the estrous cycle, when the plasma progesterone concentration was increasing. The maximum PR counts were observed on day 10 of the estrous cycle, when the plasma progesterone concentration was above 5 ng/mL. We also observed minor immunostaining at plasma progesterone concentrations at approximately 0.5 ng/mL; however, immunostaining was never absent.

In the present study, it was also possible to verify strong PR immunostaining around the plasma membrane, in the cytoplasm and sometimes in the nucleus of the stromal cells (fibroblast). This result indicates that these cells are also sites for progesterone binding. This observation was also reported in bovine CL stroma, CL perivascular stroma cells and in the ovary stroma (D'Haeseleer et al., 2007), in human corpus luteum fibroblast and perivascular fibroblast (Maybin and Duncan, 2004), and in bitch CL fibroblast and capillary pericytes (Hoffmann et al., 2004). A positive staining for PR was also observed in canine ovarian stromal cells by (Vermeirsch et al., 2001).

The increased immunostaining for PR in stroma cells is coincident with the period of formation of the CL, when the proliferation of steroidogenic and non-steroidogenic cells and the raise of progesterone plasmatic

Fig. 4. (a) Immunolocalization of ERα in bovine corpus luteum throughout the estrous cycle. Day 6 (A), day 10 (B), day 15 (C), day 18 (D), D18 after luteolysis (E) and after luteolysis (F). Negative and positive controls, inset in (F). Positive signal can be observed as the brown color in the nuclei of the large (black arrow) and small (black arrow head) luteal cells. Negative signal can be observed as the blue color in the nuclei of the large (red arrow) and small (red arrow head) luteal cells. Immunohistochemistry, DAB, counterstaining with Mayer's hematoxylin, magnification of 400 ×. (b) Immunolocalization of ERβ in bovine corpus luteum throughout the estrous cycle. Day 6 (A), day 10 (B), day 15 (C), day 18 (D), D18 after luteolysis (E) and after luteolysis (F). Negative and positive controls, inset in (F). Positive signal can be observed as the brown color in the nuclei of the large (black arrow) and small (black arrow head) luteal cells. Negative signal can be observed as the blue color in the nuclei of the large (red arrow) and small (red arrow head) luteal cells. An asterisk highlights the presence of positive and negative endothelial cells. Immunohistochemistry, DAB, counterstaining with Mayer's hematoxylin, magnification of 400 ×. (c) Immunolocalization of PR in bovine corpus luteum throughout the estrous cycle. Day 6 (A), day 10 (B), day 15 (C), day 18 (D), D18 after luteolysis (E) and after luteolysis (F). Negative and positive controls, inset in (F). Positive signal can be observed as the brown color in the nuclei of the large (black arrow) and small (black arrow head) luteal cells. Negative signal can be observed as the blue color in the nuclei of the large (red arrow) and small (red arrow head) luteal cells. A blue arrow highlights the presence of PR in the plasma membrane of some luteal cells and an asterisk the prevalence in stroma cells. Immunohistochemistry, DAB, counterstaining with Mayer's hematoxylin, magnification of 400 ×. (d) Immunolocalization of LHR in bovine corpus luteum throughout the estrous cycle. Day 6 (A), day 10 (B), day 15 (C), day 18 (D), D18 after luteolysis (E) and after luteolysis (F). Negative and positive controls, inset in (F). Positive signal can be observed as the brown color on the cell surface of large (black arrow) and small (black arrow head) luteal cells. An asterisk highlights a multifocal pattern in the cytoplasm of some luteal cells. Immunohistochemistry, DAB, counterstaining with Mayer's hematoxylin, magnification of 400 ×. (e) Immunolocalization of Aromatase Cytochrome P450 in bovine corpus luteum throughout the estrous cycle. Day 6 (A), day 10 (B), day 15 (C), day 18 (D), D18 after luteolysis (E) and after luteolysis (F). Negative and positive controls, inset in (F). Positive signal can be observed as the brown color in the cytoplasm of large (black arrow) and small (black arrow head) luteal cells. Immunohistochemistry, DAB, counterstaining with Mayer's hematoxylin, magnification of 400 ×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrations occur, suggesting a stimulatory effect of progesterone in a paracrine/autocrine manner on the formation and the initial secretory activity of the CL. This finding indicates that progesterone exerts its influence also by an interaction between stromal and steroidogenic cells, which has been suggested between follicles and stromal steroids in humans ovaries (Revelli et al., 1996). This phenomenon is also reported in others reproductive tissues like endometrium where uterine–mesenchyme interactions are responsible for the epithelial proliferation



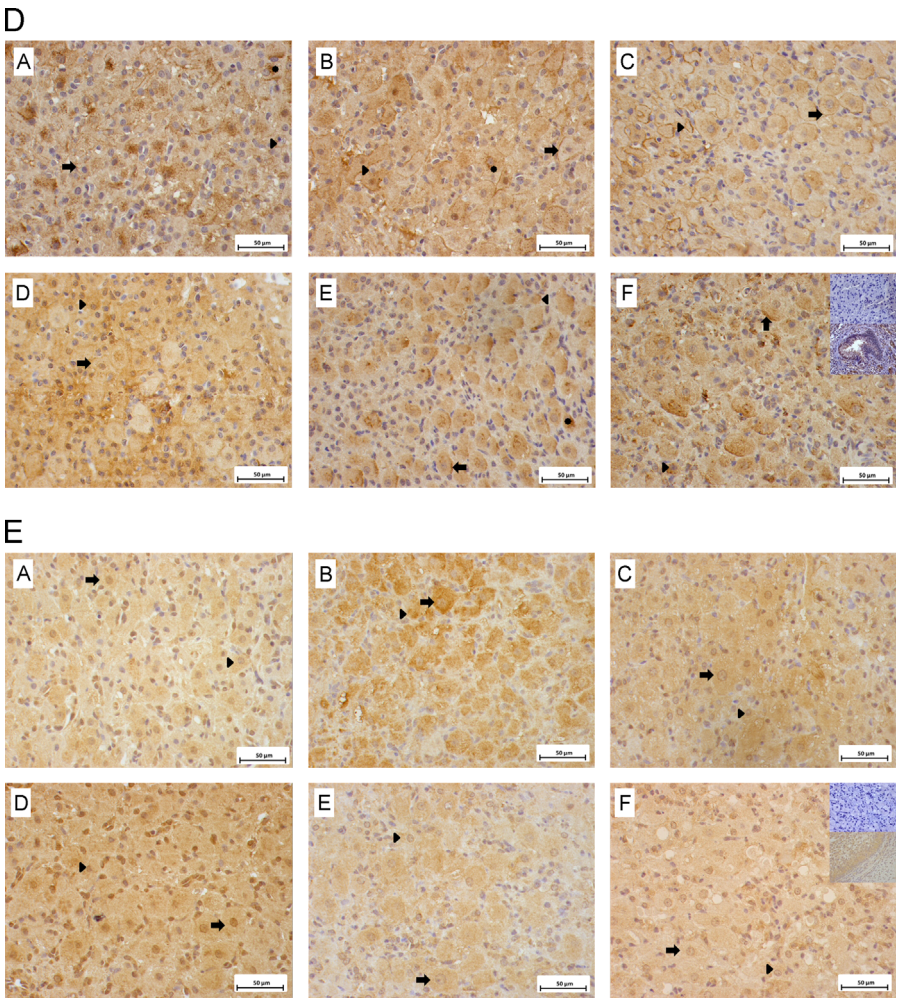


Fig. 4. (*continued*)

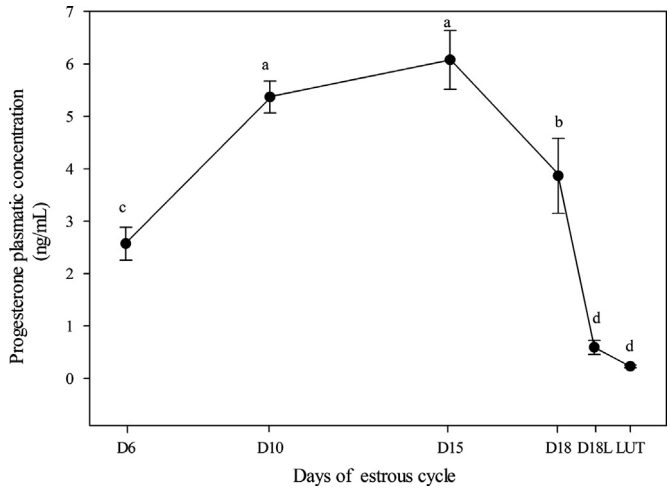


Fig. 5. Plasma progesterone concentration on days 6, 10, 15, 18, day 18 after luteolysis (D18L) of the estrous cycle and after luteolysis (LUT) in Nelore cows.

in response to steroids mediated by stromal receptors in mice (Cooke et al.1997; Inaba et al., 1988) and was apparently also in bovine between epithelium and stroma by Martin et al. (2008).

For LHR, there was clear staining in the plasma membrane, which has been previously reported in the literature (Niswender et al., 1985). This immunostaining increased throughout the estrous cycle, reaching a peak on day 15. At

this point, we noticed a decrease. The lowest values were appreciated after luteolysis, which is in accordance with the results reported by Chegini et al. (1991), Garverick et al. (1985), Jones et al. (1992) and Okuda et al. (1999), who employed binding assays and by Atli et al. (2012) using gene expression.

The increase in LHR immunostaining, accompanied by the rise in plasma progesterone concentration, suggests that the expression of LHR must be important to the stimulatory effects of LH on progesterone secretion. This has been reported by Okuda et al. (1999). Otherwise, *in vitro* studies reported that progesterone increases LHR in bovine luteal cells (Jones et al., 1992; Niswender et al., 1985). Additionally, it was also observed in monkey luteal cells that LH is the most important hormone for inducing the expression of PRs in progesterone secretion (Chandrasekher et al., 1991; Duffy et al., 1996). LH is suspected to be the "start" hormone responsible for this interaction. It may be concluded that the early CL has not reached maturity and that these cells must have undergone changes relating to their functional capacity to secrete progesterone, for example, the development of a full complement of LHR.

According to Rothchild (1981), the ability of progesterone to elevate LHR numbers in early CL may be an example of its luteotropic nature. Furthermore, the addition of progesterone to cultured granulosa cells overcame an aminoglutethimide block to induce LHR (Sheela Rani et al., 1981). Therefore, the increase in progesterone concentrations during bovine CL development must have physiological significance. The lack of a similar response in cells from mature CLs indicates an age-related limit in responsiveness to this mechanism (Jones et al., 1992). After the CL cells reach maturity, the stimulatory effect of progesterone is lost, which could explain the drop in LHR immunostaining observed on day 18 of the estrous cycle. Snook et al. (1969) observed the decline in luteal weight and/or luteal progesterone content after treatment with antisera against LH and our observation of the simultaneous drop in PR and LHR reinforces this hypothesis.

However there are a lot of questions that remain unanswered regarding the specific luteoprotective mechanisms involving LH. The steroidogenic cell types in the CL have a different response to LH stimulation. Small luteal cells respond to LH or cAMP with an increase in secretion of progesterone whereas large luteal cells secrete a high basal concentration of P4 (≥85%) and do not respond to LH or cAMP (Miyamoto et al., 2010; Wiltbank, 1994). However this phenomenon does not seems to be related to the absence of LHR, since in our study the LHR stained in both luteal cells.

During the ruminant CL life, granulosa-derived luteal cells are predominantly non-proliferative while theca-derived luteal cells are proliferative during the early luteal phase and become non-proliferative by the late luteal phase (Farin et al., 1986). In bovines, the withdraw of the granulosa cells from the cell cycle that make them resistant to apoptosis is induced by the preovulatory LH surge and the treatment with P4 seems to amplify this effect (Porter et al., 2001; Quirk et al., 2004). The withdrawal from the cell cycle is associated with development of resistance to apoptosis (Schutte and Ramaekers, 2000; Wang and Walsh, 1996). The reentry into the cell cycle, known to be associated with cell death in pathological situations, is observed in the CL after the treatment with PGF (Quirk et al., 2013).

The mechanism involved in reentry of luteal cells in cell cycle/apoptosis triggered by PGF is related to pro-apoptotic cytokines gene expression (*IL8*, *IL1B*, *TNF* and *FASL*; Atli et al., 2012; Miyamoto et al., 2010). Since intraluteal P4 and cortisol (Kawaguchi et al., 2013) are dependent on LH, and suppress *FAS* and *Caspase-3* mRNA expression (Komiyama et al., 2008; Okuda et al., 2004) the drop of LHR observed at the end of luteal phase in our study may be the key role in luteolysis process.

5. Conclusions

Immunostaining patterns in bovine luteal cells for ERα, ERβ, cytochrome P450 aromatase enzyme, PR and LHR vary throughout the estrous cycle. However, different results were observed according to studies using CLs obtained in slaughterhouses. This finding could reinforce the need to use samples obtained *in vivo*, during specific days of the estrous cycle. The variation of immunostaining throughout the estrous cycle supports the hypothesis that regulation of these proteins depends upon the plasma progesterone concentrations. The ERβ, cytochrome P450 aromatase enzyme, PR and LHR proteins were always present, and ERα protein decreased during the end of diestrus and was undetectable after luteolysis. There was an increase in LHR protein expression, accompanied by an increase in plasma progesterone concentrations. Therefore, we assume that luteal function is at least in part regulated by LH via its specific receptors.

Our data support the idea that the studied variables were present and vary throughout the estrous cycle of Nelore cows, suggesting that these receptors and enzymes are important to the viability and maintenance of this endocrine gland.

Policy and ethics

This work was approved by the Ethics and Animal Experimentation Committee at the School of Veterinary Medicine and Animal Science, UNESP, Botucatu.

Conflict of interest statement

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

Acknowledgments

The authors thank FAPESP, Brazil for their financial support (Grant nos. 2005/53149-5 and 2007/51103-3).

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