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Immuno Localization of Estrogen Receptor (ERα) and Progesterone Receptor (PR) in Uterus of Buffalo during Follicular and Luteal Phases of Estrous Cycle

Devendra Pathak[1*], Neelam Bansal[1], Opinder Singh[1], Kuldip Gupta[2] and S.P.S. Ghuman[3]

[1]Department of Veterinary Anatomy, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, INDIA
[2]Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, INDIA
[3]Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, INDIA

**Corresponding author: D Pathak; Email: drdevendra@gmail.com*

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ABSTRACT

The tissue distribution of estrogen receptor alpha (ERα) and progesterone receptor (PR) was examined using immunohistochemical technique. Image analysis was done to quantify the immune reactivity. The ERα and PR was localized in luminal epithelium, glandular epithelium, stromal cells, endothelial cells and myometrium and few cells in perimetrium. The immuno staining was observed in the nuclei of cells, however, faint cytoplasmic staining for PR was also observed. Variations were seen in the different tissue compartments of the uterus and during the different phases of the estrous cycle. Significantly higher number of ERα positive cells was observed in lamina epithelialis as compared to stromal cells and smooth muscle cells in myometrium. Significantly higher percentage of ERα positive cells was observed in the lamina epithelialis and lining epithelium of endometrial glands during follicular phase as compared to the luteal phases of estrous cycle ($P < 0.05$). Higher number of PR positive cells was observed in lamina epithelialis as compared to stromal cells and smooth muscle cells in myometrium ($P < 0.05$). Higher percentage of PR positive cells was observed in the lamina epithelialis during follicular phase as compared to the luteal phases of estrous cycle ($P < 0.05$). There was no significant difference in the immuno reactivity in stromal cells, lining epithelium of endometrial glands and smooth muscle cells of myometrium during the phases of estrous cycle. The study concluded that ERα and PR expressions were higher during follicular phase as compared to the luteal phase.

Keywords: Buffalo, Estrogen Receptor (ERα), Estrous Cycle, Progesterone Receptor (PR), Uterus

Water buffaloes play an important place and are integral part of Indian rural economy and contribute more than 60% of total milk produced in the country. They are preferred over cattle because of their better disease resistance and feed conversion efficiency. The productivity of an animal is primarily determined by its reproductive efficiency. The uterus is a luminal organ that connects the oviduct and cervix uteri and consisted of two uterine horns. It is instrumental in specialized function of reception of the blastocyst by the endometrium and provides nourishment to the developing fetus. It also plays a vital role in the expulsion of fetus.

It is dynamic organ and undergoes morphological and functional changes under the influence of circulating ovarian steroid hormones mainly estrogen and progesterone (Wood *et al*., 2007). It acts as peristaltic pump during estrous cycle for transport of sperms towards the oviduct (Leyendecker *et al*., 1996). It undergoes extensive morphological and molecular modulation under the influence of these hormones to become receptive to the blastocyst for successful implantation, survival and development of fetus (Spencer and Bazer, 2002).

Estrogens and progesterone play a major role controlling the functions of uterus by acting through their respective nuclear receptors. The content of estrogen receptors alpha (ERα) and progesterone receptors (PR) in the uterus of cows varies during the follicular and luteal phases of estrous cycle (Boos *et al*., 1996). Studies on the immuno localization of ERα and PR on the uterus have been conducted in cows (Boos *et al*., 1996), sheep (Cherny

et al., 1991), human (Noe *et al*., 1998), rat (Nephew *et al*., 2000), canines (Vermeirsch *et al*., 2000) and llamas (Bianchi *et al*., 2007), but meager literature is available to best of our knowledge with regard to buffaloes. Thus the present investigation was designed to study the immunohistochemical localization of estrogen receptor alpha (ERα) and progesterone receptor (PR) in different compartments of uterus during follicular and luteal phases of estrous cycle in relation to the plasma concentration of estradiol and progesterone hormones.

MATERIALS AND METHODS

Animals

Uteri from twelve buffaloes (six each during follicular and luteal phases of estrous cycle) were collected from a local slaughter house immediately after slaughter of the animal. Blood samples from same animals were collected before slaughter for estimation of levels of estrogen and progesterone hormones. The stage of estrous cycle was determined by morphological appearance of ovaries and accordingly the animals were grouped into follicular (n=6) and luteal (n=6) phases.

Processing of tissue for paraffin sectioning

Samples were fixed in 10% neutral buffered formalin for 24 hours at room temperature, washed in running tap water, processed by acetone benzene, schedule embedded in paraffin wax and sectioned at 4-5µ thickness for hematoxylin and eosin staining and immunohistochemical staining (Pathak and Bansal, 2012).

Immunohistochemistry

Immunohistochemistry was performed by one step super sensitive polymer based horse radish peroxidase method (Poly HRP method). Briefly, sections were deparaffinized using AR-common (BioGenex) at 70°C for 10 minutes (one cycle in BioGenex EZ antigen retrieval System). Rinsed with de-ionized water and heat induced antigen retrieval was done in AR-3 solution (BioGenex) at 95 ^{o}C for 15 minutes. Blocking of endogenous peroxidases was done using 3% H_2O_2 in methanol for 20 minutes and after washing in 0.1M phosphate buffer saline (PBS), protein blocking was done using 2% horse serum (Vector's ImmPRESS Universal Kit). Sections were incubated in primary antibody (Santa Cruz Biotechnology, ERα: SC-787 at 1:500 dilutions and PR: SC-538 at 1:2000 dilutions) for one hour at room temperature. After washing with PBS, sections were incubated in ready to use secondary antibody tagged with HRPO (Vector's ImmPRESS Universal Kit) for 30 minutes at room temperature in moist chamber. After washing the sections with PBS, sections were incubated with ImmPACT DAB (Diaminobenzidine) peroxidase substrate from Vector laboratory USA for 60 seconds and DAB reaction was stopped by keeping them in tap water. Sections were counterstained with hematoxylin solution modified according to Gill III for 2 minutes, dehydrated with ethanol, cleared with xylene and mounted in DPX mounting medium.

Image analysis and quantification of immunopositive cells

Immunostained sections were examined and photographed (10 images per slide per animal) using a light microscope (Nikon 80i) attached with a digital camera. For each section, 6–10 photomicrographs were captured at 400-magnification (40× objective lens). Images were processed and counted using multi point cell counter tool of Fiji (ImageJ) (Schindelin *et al*., 2012). The sections were evaluated and quantified by calculating the percentage of positively stained cell nuclei at 400 magnifications. Data obtained on percentage positive cells were subjected to analysis of variance and student's t-test were done between each pair of means to find out significant difference between the means at 95% significant levels (Snedecor and Cochran, 2004).

Estimation of estrogen and progesterone hormone in the blood samples

Estradiol hormone in blood serum was estimated using ELISA kit for estradiol (LabServ, Thermo Fisher Scientific). Plasma progesterone was estimated by liquid phase Radioimmunoassay (RIA) procedure using progesterone antisera (Ghuman *et al*., 2009).

RESULTS AND DISCUSSION

The uterus was histologically comprised of endometrium,

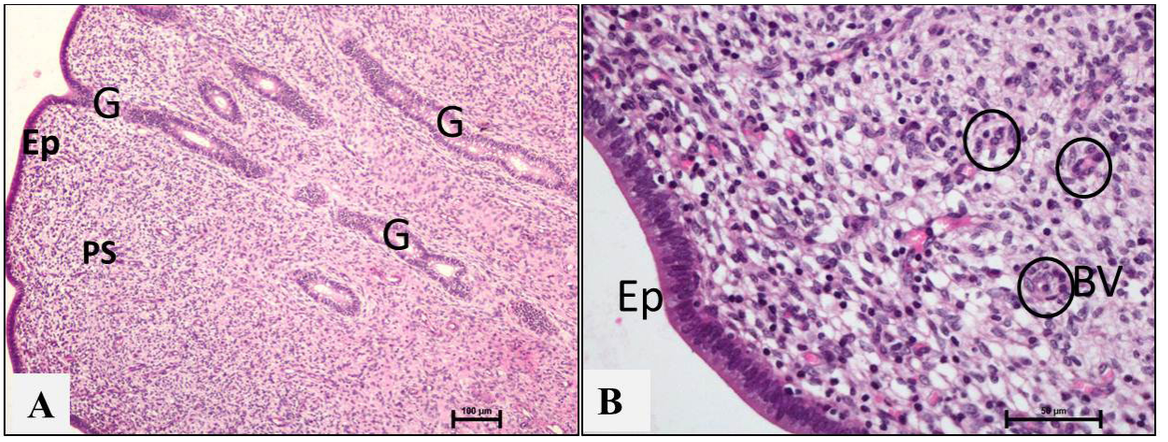

Fig. 1: Uterine horn of buffalo showing, **(A)** Lining epithelium (Ep) of endometrium, propria submucosa (PS) and endometrial glands (G). H&E. x100; **(B)** Endometrium showing lining epithelium (Ep) with columnar cells, propria submucosa with numerous blood capillaries (BV).H&E. x400.

myometrium and perimetrium. Endometrium was consisted of tunica mucosa and submucosa (Fig.1A). The surface epithelium of the tunica mucosa consisted of patches of simple columnar and pseudostratified epithelium (Fig.1B). Similar observations have been recorded by Pathak and Bansal (2011) in buffalo uterus by using transmission electron microscope. The sumucosa layer was richly endowed with endometrial glands as observed earlier by Pathak and Bansal (2012) in buffalo uterus.

Estrogen receptor alpha (ERα)

In the present investigation, ERα and PR was immuno localized using immunohistochemical technique with the use of specific antibodies against ERα and PR in tunica mucosa, submucosa, tunica muscularis and tunica serosa both during follicular and luteal phases of estrous cycle. The ERα was localized in luminal epithelium, glandular epithelium, stromal cells (Fig.2A and 2B) and myometrium (Fig. 2C) and few cells in perimetrium. The endothelial cells lining the large arteries and capillaries present in the stratum vasculare of the myometrium were also strongly positive for ERα (Fig. 2D). The immuno staining was observed in the nucleus of cells. However faint cytoplasmic reactions were also seen at places. The immuno reactivity in lamina epithelialis was higher during follicular phase as compared with luteal phase of estrous cycle. Experiments by Pelletier *et al*. (2000) also showed nuclear reactions in epithelial and stromal cells of rat uterus. Similar to our findings, Silva *et al.* (2014) also recorded immunostaining for ERα in the nucleus and cytoplasm of luminal epithelium, glandular epithelium and stromal nuclei of the endometrium. In our study cytoplasmic staining was rarely observed whereas, Stanchev *et al*. (1990) observed both nuclear and cytoplasmic staining in the pig uterus.

In the connective tissue stroma, the highest number of ER alpha positive cells was found during the follicular phase, which was significantly different compared to luteal phase (Fig. 2). Few endothelial cells and the cells located in peri-vascular area in the stroma were also positive were also positive for ERα (Fig.3A). No staining was observed in negative controls where the primary antibody was replaced with that of washing buffer (Fig. 3B). Subjective assessment made by Silva *et al.* (2014) demonstrated that the ER-α immunostaining appeared to be more intense during estrus than diestrus period in the endometrium of mare. In the llama uterine tissue, Bianchi *et al*. (2007) recorded exclusive nuclear reactions in luminal epithelium, stromal cells and the glandular epithelium. The nuclear reactions in the muscle fibres and endothelial cells in stratum vasculare of myometrium corroborated with the findings of Noe *et al*. (1999) in the human endometrial-subendometrial unit. Winuthayanon *et al*. (2010) studied the ERα expression in mice and established that ERα was required proliferation of epithelium and to prevent uterine epithelial apoptosis. They found that absence of ERα induces the process of

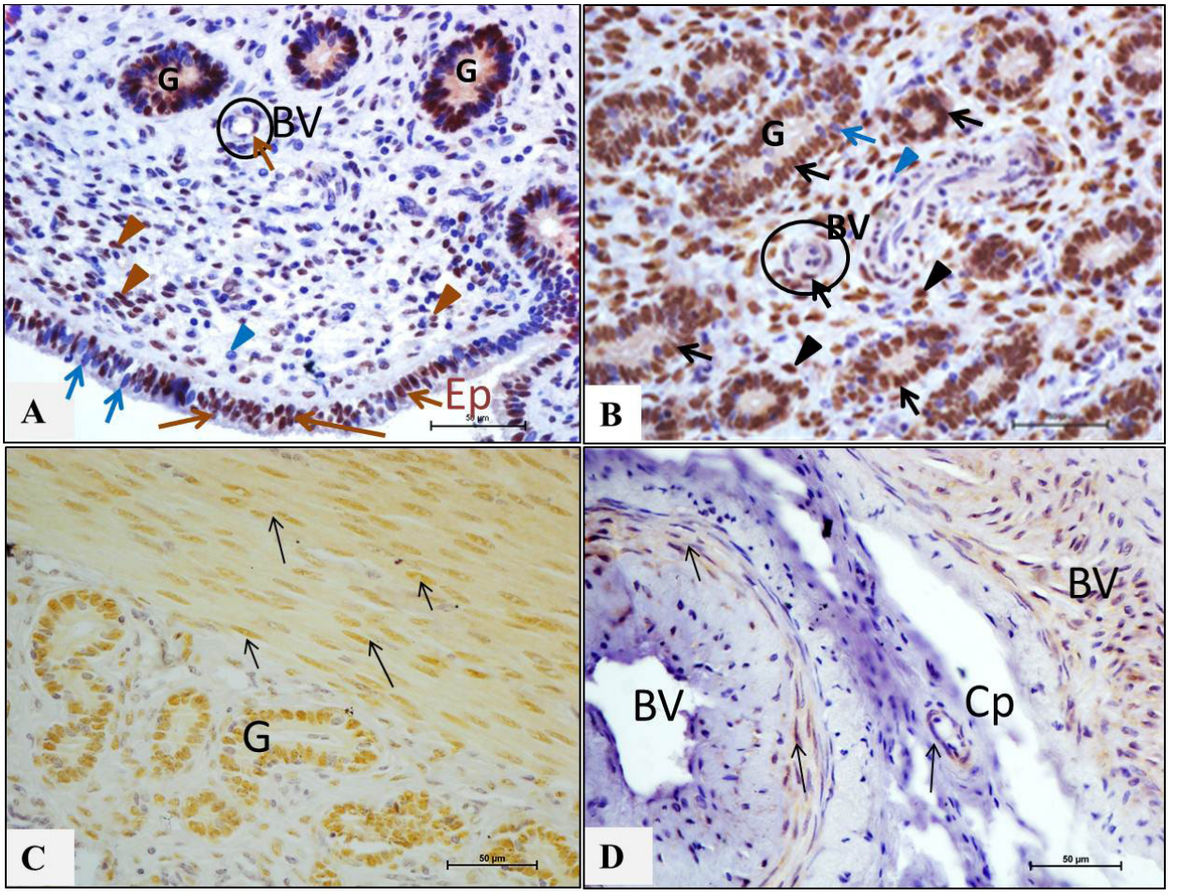

Fig. 2: Immunostaining of uterus during follicular phase with anti-ERα antibody showing nuclear reaction, **(A)** In the luminal epithelium (positive cells with brown arrows, negative cells with blue arrows), stromal cells in Propria submucosa (positive cells with brown arrow heads, negative cells with blue arrow heads), endothelial cells of blood vessels (arrow in circle with BV) and glandular epithelial cells (G); **(B)** in the stromal cells in Propria submucosa (positive cells with black arrow heads, negative cells with blue arrow heads), endothelial cells of blood vessels (arrow in circle with BV) and glandular epithelial cells (G); **(C)** In the smooth muscle cells in tunica muscularis (arrows) and glands in stratum compactum (G); **(D)** In endothelial cells in blood vessels (BV) and capillaries (Cp). Polymer HRP method. Original magnification x400

apoptosis in uterine epithelium and uterine growth was induced after estrogen treatment. The presence of intense reactions in different compartment of uterus indicated that all of the cell types were responsive to the estrogen hormone. The proliferation of the epithelial cells, stromal cells and glandular epithelium during the follicular phase might have been influenced by the estrogen hormone as indicated by presence of its receptors. The presence of ERα in the endothelial cells of the blood capillaries in endometrium and myometrium and larger vessels in the stratum vasculare layer of the myometrium inferred that the estrogen hormone was also controlling the active flow of hormone during the estrous cycle.

The quantitative analysis based on the counting of percentage of ERα positive cells in different compartments of uterus of buffalo has been presented in Fig. 5. Variations were seen in the different tissue compartments of the uterus and during the different stages of the estrous cycle. Significantly higher number of ERα positive cells was observed in lamina epithelialis as compared to stromal cells and smooth muscle cells in myometrium. There was no significant difference in immuno reactivity between

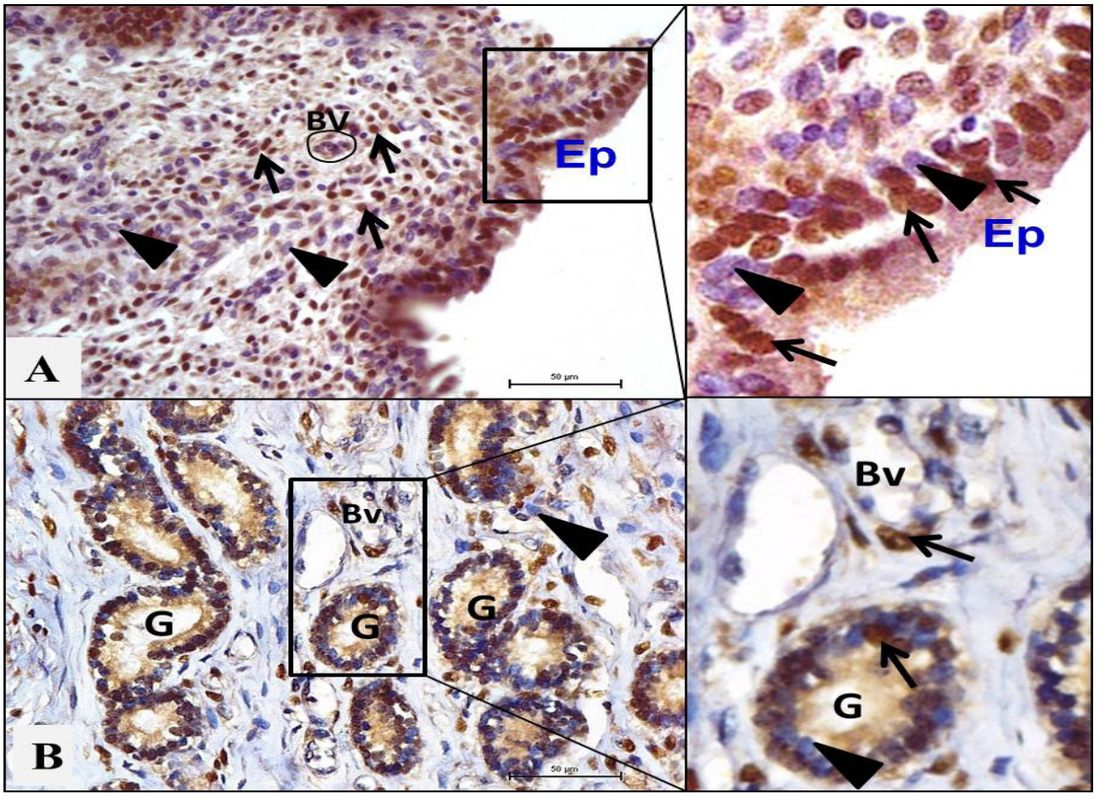

Fig. 3: Immunostaining of uterus of buffalo during luteal phase with anti-ERα antibody showing nuclear positive immunostaining (arrows) and negative reactions (arrow heads), **(A)** In the luminal epithelium (EP) and stromal cells and endothelial cells of blood vessels (BV) present in Propria submucosa; Magnified view of selected area showing distinct nuclear reaction in epithelial lining (Ep). **(B)** In the glandular epithelial cells (G), Endothelial cells of blood vessels (BV), Magnified view of selected area showing distinct nuclear reaction in glandular epithelium (G) and endothelial cells of blood vessels (BV). Polymer HRP method. Original magnification x400

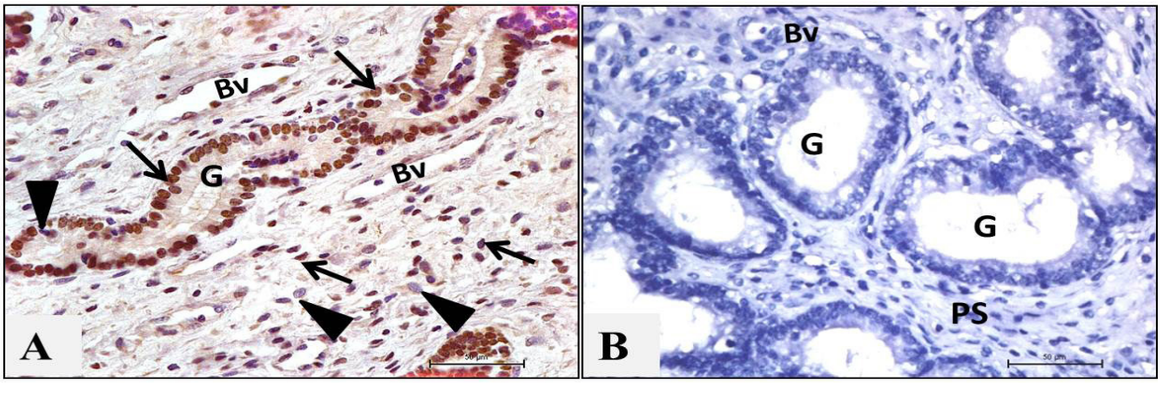

Fig. 4: Immunostaining of uterus of buffalo during luteal phase with anti-ERα antibody showing, **(A)** Nuclear positive immunostaining (arrows) in the glandular epithelial cells (G), endothelial cells of blood vessels (Bv) and stromal cells, negative reactions (arrow heads); **(B)** No staining was observed in glands (G), propria submucosa (PS) and blood vessels (Bv) in negative controls. Polymer HRP method. Original magnification x400

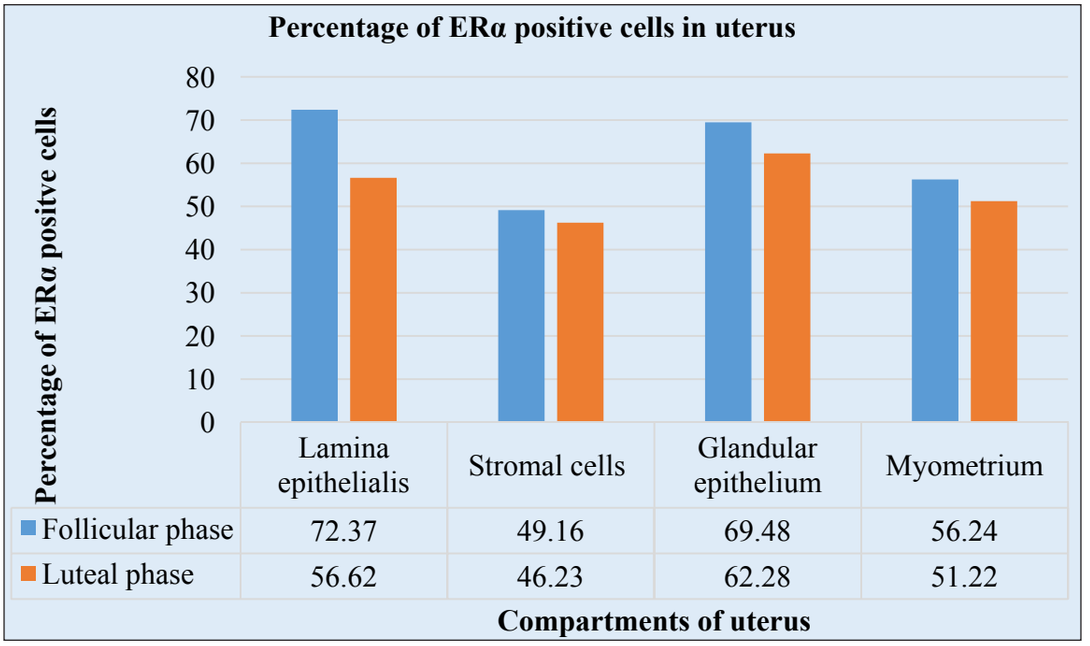

Fig. 5: Quantitative analysis of ERα positive cells in different compartments of uterus during follicular and luteal phases of estrous cycle

lamina epithelialis and lining epithelium of endometrial glands. Higher number of ERα positive cells was found during the follicular phase in all the compartments as compared to the luteal phase of estrous cycle. But there was significantly higher number of ERα cells in the lamina epithelialis and lining epithelium of endometrial glands during follicular phase as compared to the luteal phases of estrous cycle. There was no significant difference in the immuno reactivity in stromal cells and smooth muscle cells of myometrium during the phases of estrous cycle. Similar observations on the quantitative analysis based on positive stained area for ERα have been reported in the endometrium of llamas by Bianchi *et al*. (2007).

Progesterone Receptor (PR)

The PR was localized in luminal epithelium (Fig. 6A), glandular epithelium (Fig. 6B), stromal cells and endothelial cells of blood vessels present in the propria sub mucosa (Fig.6A and 6B) and myometrium and few cells in perimetrium of buffalo uterus during the follicular phase of estrous cycle. The immuno staining was observed both in the nuclei and cytoplasm of cells, however the reactivity in nuclei was strong while in the cytoplasm was weak to moderate as seen as faint staining in the cytoplasm. During the luteal phase of the estrous cycle, intense nuclear reaction was observed in the lamina epithelialis and stromal cells in the propria submucosa (Fig.6C). Intense nuclear reaction was also observed in the lining epithelium of endometrial glands (Fig. 6D). The lining epithelium of endometrial glands in both the zones viz; stratum spongiosum and stratum compactum were immuno positive for PR. Similar to our observations, immunostaining for PR was detected mostly in the nuclei of luminal and glandular epithelium, as well as in the stromal nuclei within the endometrium of mare during estrus and late diestrus (Silva *et al*., 2014). Presence of PR in all the compartments of uterus indicated that the entire uterine tissue must be responsive to the circulating progesterone hormone. In corroboration to our findings, Wathes and Hamon (1993) observed the PR immunostaining in the luminal epithelium and superficial glands in the early luteal phase in the ewe uterus.

In lamina epithelialis and glandular epithelium, immunoreactivity was higher in follicular phase as compared with luteal phase of oestrous cycle. Variations were seen in the different tissue compartments of the uterus and during the different stages of the estrous cycle. Few endothelial cells and the cells located in peri-vascular area in the stroma were also positive for PR. No staining was

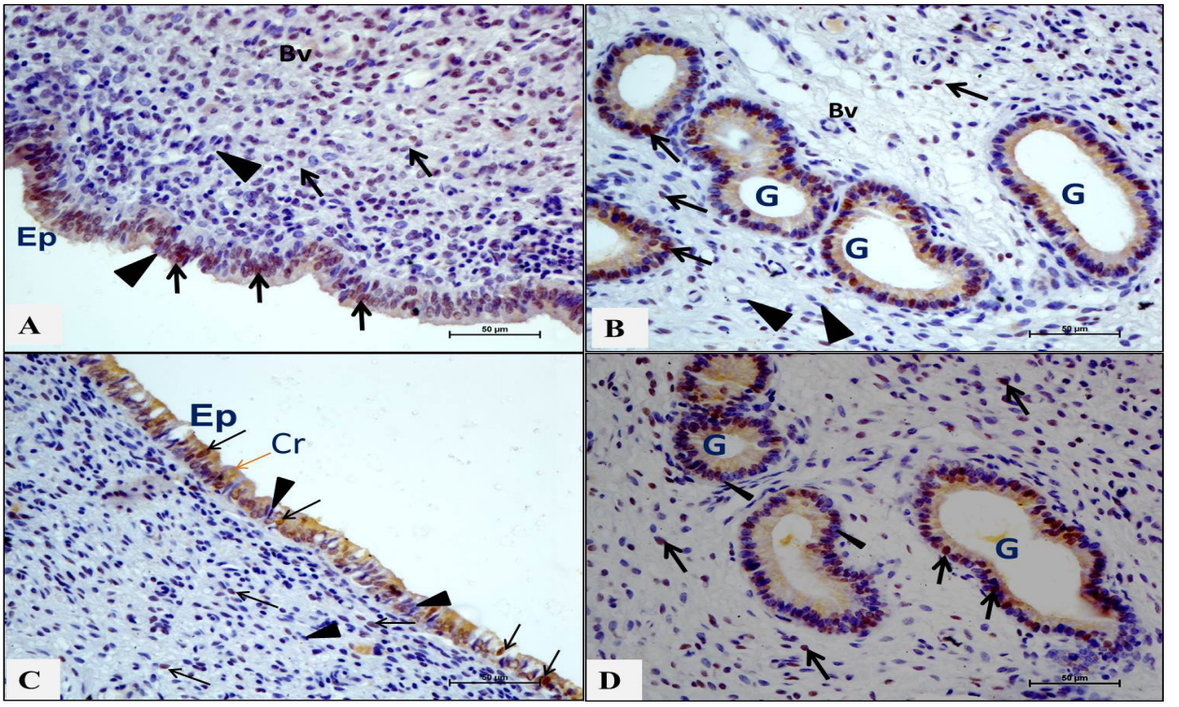

Fig. 6: Immunostaining of uterus of buffalo with anti-PR antibody showing nuclear positive immunostaining (arrows) and negative reactions (arrow heads), **(A)** in the luminal epithelium (EP) and stromal cells and endothelial cells of blood vessels (BV) present in Propria submucosa during follicular phase; **(B)** in the glandular epithelial cells (G), Endothelial cells of blood vessels (BV) and stromal cells during follicular phase; **(C)** in the luminal epithelium (EP) and stromal cells and glandular epithelial cells present in propria submucosa during luteal phase, faint cytoplasmic reaction (Cr with arrow); **(D)** in the glandular epithelial cells (G) and stromal cells during luteal phase. Polymer HRP method. Original magnification x400.

observed in negative controls. The intensity of staining for PR was characteristically remarkable in epithelial lining and the lining epithelium of the glands as compared to that of the stromal cells. Bianchi *et al*. (2007) recorded PR immunoreactivity in epithelia of endometrium and endometrial glands, stromal cells of llama uterus. Similar findings have been reported for the immuno localization of ERα and PR on the uterus of canines by (Vermeirsch *et al*., 2000). It has been postulated that in uterus both epithelial and stromal steroid receptors were required for steroidal regulation of certain aspects of epithelial differentiation such as production of secretory proteins (Cunha *et al*., 2004).

The quantitative analysis based on the counting of percentage of PR positive cells in different compartments of uterus of buffalo has been presented in figure 7. Significantly higher number of PR positive cells was observed in lamina epithelialis as compared to stromal cells and smooth muscle cells in myometrium ($P < 0.05$). There was no significant difference in immuno reactivity between lamina epithelialis and lining epithelium of endometrial glands. Significantly higher number of PR positive cells was observed in the lamina epithelialis during follicular phase as compared to the luteal phases of estrous cycle ($P < 0.05$). There was no significant difference in the immuno reactivity in stromal cells, lining epithelium of endometrial glands and smooth muscle cells of myometrium during the phases of estrous cycle. The content of ERα and PR in the uterus of cows varied during the follicular and luteal phases of estrous cycle (Boos *et al*., 1996). Our findings are also consistent with findings at the mRNA level as the studies in cow uterus by Robinson *et al*. (2001). They recorded higher concentrations of both estrogen receptor α and progesterone receptor mRNA and protein at estrus and low during the luteal phase. Contrary to our findings, Wathes and Hamon (1993) recorded more progesterone receptor concentration in the stroma and

myometrium of ewe in the early luteal phase. Myometrial staining was clearly maintained throughout the luteal phase.

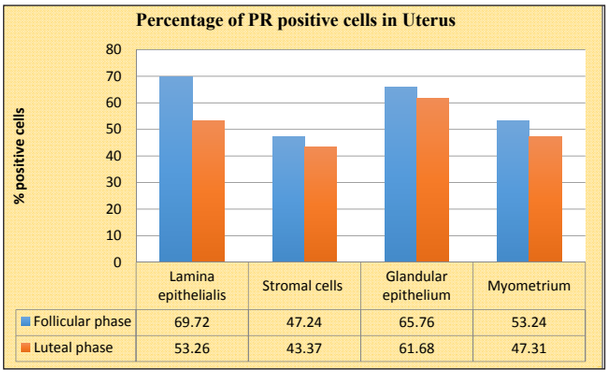

Fig. 7: Quantitative analysis of PR positive cells in different compartments of uterus during follicular and luteal phases of estrous cycle

The average concentrations of plasma estradiol during follicular and luteal phase of the animals under the study were 28.82±1.13 pg/ml and 13.13±1.18 pg/ml respectively. The average concentration of plasma progesterone during follicular and luteal phase of the animals under the study was 0.13±0.42 ng/ml and 2.63±0.62 ng/ml respectively. The hormonal levels recorded were in range as described in the review by Phogat *et al*. (2016) in buffaloes. The data on receptor and hormone analysis showed that the ER and PR expression was higher during the estradiol dominance and lower during the progesterone dominance. Maciel *et al*. (2018) recorded no change in the endometrial expression of progesterone receptors in non-cyclic mares treated only with long-acting progesterone. Our finding corroborated with the hypothesis that estradiol had a stimulatory effect on the expression of ERα and PR (Ing and Tornesi, 1997).

CONCLUSION

It was concluded from the present study that the ERα and PR were localized in luminal epithelium, glandular epithelium, stromal cells, endothelial cells of blood vessels present in the propria sub mucosa and myometrium and myometrium and few cells in perimetrium during the follicular and luteal phases of estrous cycle. The immuno staining was observed in the nuclei of cells. In lamina epithelialis immuno reactivity for both the receptors was higher in follicular phase as compared with luteal phase of estrous cycle. Thus estrogen and progesterone hormones played a major role in controlling the physiology of uterus by acting through their respective nuclear receptors.

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