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Immuno-localization of estrogen receptor (ER) and progesterone receptor (PR) in the buffalo ovary in relation to their plasma hormonal levels

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ABSTRACT

In this study, we examined the tissue distribution of estrogen receptor alpha (ERα) and progesterone receptor (PR) in different compartments of the buffalo ovary during follicular and luteal phases of the estrous cycle. The receptors were localized by immunohistochemistry. Image analysis was done to quantify the immune reactivity. ERα was localized in various cell types of buffalo ovaries differentially during follicular and luteal phases of the estrous cycle. Immunoreactivity of ERα was detected in the primordial, primary, secondary and tertiary follicles, atretic follicles, in cells of the deep and superficial stroma, and the tunica albuginea. Specific immunostaining was observed with anti-ERα antibodies in the nuclei of follicular cells/granulosa cells and theca cells. No reaction was observed in the ovarian surface epithelium. In the growing follicle and secondary follicle, the immunoreaction for these receptors was strong. While in the tertiary follicles weak immunoreactions were recorded in the granulosa cells and theca cells. The progesterone receptors (PR) as revealed by immunohistochemistry were localized in the nuclei of different groups of ovarian cells. It was detected in the primordial, primary, secondary and tertiary follicles, atretic follicles, in cells of the deep and superficial stroma, and the tunica albuginea and surface epithelium. PR was localized in follicular cells of preantral and antral follicles, the stroma of the ovary, endothelial cells of blood vessels. PR positivity was found in one or two granulosa cells of primordial and primary follicles, with moderate immunoreaction, but no staining in oocytes. In the antral follicles, both granulosa cells, as well as theca cells, were immunostained for PR. In the obliterative atretic follicles, the invading stromal cells were highly positive for PR. Follicular cells of the primordial follicle and granulosa cells and theca cells of tertiary follicles had statistically higher percentage positive cells in the follicular phase as compared to the luteal phase. No staining was observed in the negative controls.

Keywords: Buffalo, Estrogen receptor (ER), Estrous cycle, Ovary, Progesterone receptor (PR)

India stands at the top rank in the world in total milk productions. Of the total milk produced in India, water buffaloes contribute the highest share (49.2%) (Basic Animal Husbandry Statistics 2017) and therefore play a vital role in the Indian rural economy. The production capability of an animal is dependent upon the efficiency of its reproduction. The ovary is the primary sex organ in females and its activity is pivotal to reproductive functions. It performs two basic functions of the reproductive cycle, i.e. production of ova and synthesis and secretion of steroid hormones. Estrogen and progesterone are the two steroid hormones produced by the developing ovarian follicles. Estrogen hormone acts inside the follicle and stimulates aromatase activity along with the follicle-stimulating hormone (Adashi and Hseh 1982). Britt and Findlay (2002) concluded that estrogen hormone is obligatory for normal folliculogenesis beyond the antral stage, and they also hypothesized that the ERá might influence the proliferative actions of estrogen, within a follicle. Progesterone is another important steroid hormone of the female reproductive system which regulates follicular development, ovulation, and luteinization and is instrumental in the maintenance of pregnancy and mammary gland development (Conneely *et al*. 2002 and Al-Asmakh 2007). It is established that the physiological actions of steroid hormones are mediated by their interaction with specific hormone-binding proteins called receptors.

Studies on the immunolocalization of ERá and PR on the ovary have been conducted in pigs (Slomczynska *et al*. 2000), canines (Vermeirsch *et al*. 2001), cows (Van den Broeck *et al.* 2002, D'Haeseleer et *al*. 2007) and spiny mice (Hu³as-Stasiak and Gawron 2010) and rabbit (Abd-Elkareem 2017, Abd-Elkareem and Abou-Elhamd 2019), but little information is available to the best of our knowledge concerning buffalo ovaries. Thus, the present investigation was designed to study the immunohisto-chemical localization of estrogen receptor alpha (ERá) and progesterone receptor (PR) in different compartments of

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the ovary during follicular and luteal phases of the estrous cycle with the plasma concentration of estradiol and progesterone hormones.

MATERIALS AND METHODS

Animals: Ovaries from 12 buffaloes (six each during follicular and luteal phases of the estrous cycle) were collected from a local slaughterhouse immediately after the slaughter of the animal. Blood samples from the same animals were collected before slaughter for estimation of levels of estrogen and progesterone hormones. The stage of the estrous cycle was determined by the 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 h at room temperature, washed in running tap water, processed by acetone benzene, schedule embedded in paraffin wax and sectioned at 4–5 µ thickness for immunohistochemical staining.

Immunohistochemistry: Immunohistochemistry was performed by a step super sensitive polymer-based horseradish peroxidase method (Poly HRP method) as per the procedure described earlier by Pathak *et al*. (2019). Briefly, sections were deparaffinized using AR-common (BioGenex) at 70°C for 10 min and heat-induced antigen retrieval was done in the AR-3 solution (BioGenex) at 95°C for 15 min. Blocking of endogenous peroxidases was done using 3% H_2O_2 in methanol for 20 min and after washing in 0.1 M phosphate buffer saline (PBS), protein blocking was done using 2% horse serum. Sections were incubated in primary antibody (Santa Cruz Biotechnology, ERá: 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 for 30 min at room temperature in the moist chamber. After washing the sections with PBS, sections were incubated with DAB (Diaminobenzidine) peroxidase substrate from Vector laboratory USA for 60 sec and the DAB reaction was stopped by keeping them in tap water. Sections were counterstained with hematoxylin solution modified according to Gill III for 2 min, 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 a 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.

Estimation of estrogen and progesterone hormone in the blood samples: Estradiol hormone in blood serum was estimated using an ELISA kit (Shanghai Crystal Day Biotech Co, Shanghai, China). The assay range during the procedure was 5 pg/ml to 1500 pg/ml. The sensitivity of the assay was 2.53 pg/ml for estrogen. The mean intra-assay coefficient of variation was 2.43%. Plasma progesterone was estimated by the liquid phase Radioimmunoassay (RIA) procedure using progesterone antisera (Ghuman *et al*. 2009). The sensitivity of the assay is 0.1 ng/ml for progesterone. The mean intra- and inter-assay coefficients of variance were 6.2 and 9.5%, respectively.

Data obtained on percentage positive cells were subjected to analysis of variance and a student's t-test was done between each pair of means to find out the significant difference between the means at 95% significant levels (Snedecor and Cochran 2004).

RESULTS AND DISCUSSION

Estrogen receptor alpha (*ERα*): ERá was localized in various cell types of buffalo ovaries differentially in a different stage of the reproductive cycle. The qualitative assessment of the intensity of immuno-staining has been represented in Table 1 and the percentage of ERá positive cells in different types of follicles has been presented in Table 2. Immunoreactivity of ERá was detected in the primordial, primary, secondary and tertiary follicles, atretic follicles, in cells of the deep and superficial stroma, and the tunica albuginea and surface epithelium. Similar observations were recorded by Hu³as-Stasiak and Gawron (2007) in the ovary of the spiny mouse. They observed nuclear immunostaining of ERá in surface epithelium cells, interstitial gland cells, granulosa cells of primordial follicles, growing follicles, and preovulatory follicles. In contrast to our findings, Darawiroj *et al*. (2003) observed strong staining in the germinal epithelium, stroma, and faint staining in some luteal cells, however, no positive staining of ERá was detected in ovaries of cats at other stages.

Specific immunostaining was observed with anti-ERá antibody in the nuclei of follicular cells of primordial

Table 1. Average staining intensity of ERα in the ovary at different stages of the estrous cycle

Stage	Surface epithelium	Primordial follicle	Primary follicle	Secondary follicle	Antral follicle
Follicular phase	—	+++	+++	+++	++
Luteal phase	—	+++	++	+++	++

–, No reaction; +, Weak Reaction; ++, Strong Reaction; +++, Intense reaction.

Table 2. Percentage of ERα positive cells (Mean±SE) in the ovary during follicular and luteal phases of the estrous cycle

Stage	Primordial follicle	Primary follicle	Secondary follicle	Antral follicle
Follicular phase	14.28±1.08	5.88±0.78	9.09±1.04	8.33±1.05
Luteal phase	10.62±1.21	3.97±0.63	6.36±0.98	7.72±1.02

follicles (Fig. 1A), primary follicles (Fig.2B), Secondary (Fig. 1C) and tertiary follicles (Fig.1D). No reaction was observed in the ovarian surface epithelium. ER nuclear labeling was observed in the surface epithelium and interstitial tissue, granulosa cells of primary and secondary follicles, in small antral follicles of sheep ovaries (Tomanek *et al*. 1997). In contrast to our observations in buffalo, ER expression has been observed in the surface epithelium of cats (Darawiroj *et al.* 2003) and spiny mice (Hu³as-Stasiak and Gawron 2007).

ERá immunostained cells were observed during the follicular phase and luteal phases of the estrous cycle. In primordial follicles, the nuclear reaction was observed in one or two granulosa cells of the follicle. Like the primordial follicles, the ERá was localized in the granulosa cells in primary follicles. The connective tissue around these follicles both in the superficial and deep stroma showed a strong reaction for ERá antibodies. In the growing follicle and secondary follicle, the reaction was strong. While in the tertiary follicles weak reactions were observed in the granulosa cells and theca cells (Fig. 1D). ERá was weak or absent in the cells of corpora lutea. No staining was observed in the negative control sections where the primary antibodies were replaced with the washing buffer. In contrast to our findings, Hild-Petito *et al*. (1988) recorded the absence of localization of ER in any of the ovarian structures, i.e. stroma, follicles, interstitial tissue, or corpora lutea regardless of the stage of development except for germinal epithelium.

A significantly higher percentage of positive cells were observed during follicular cells of primordial follicles during the follicular phase as compared to the luteal phase ($P<0.05$). But no significant differences were observed in primary follicles, secondary and tertiary follicles during two phases of the estrous cycle. A decreasing trend of the percentage of ER-positive cells was observed from primordial to tertiary follicles.

Progesterone receptor: The progesterone receptors (PR) as revealed by immunohistochemistry were localized in the nuclei of different groups of ovarian cells. It was detected in the primordial, primary, secondary and tertiary follicles, atretic follicles, in cells of the deep and superficial stroma, and the tunica albuginea and surface epithelium. PR was localized in follicular cells of preantral and antral follicles,

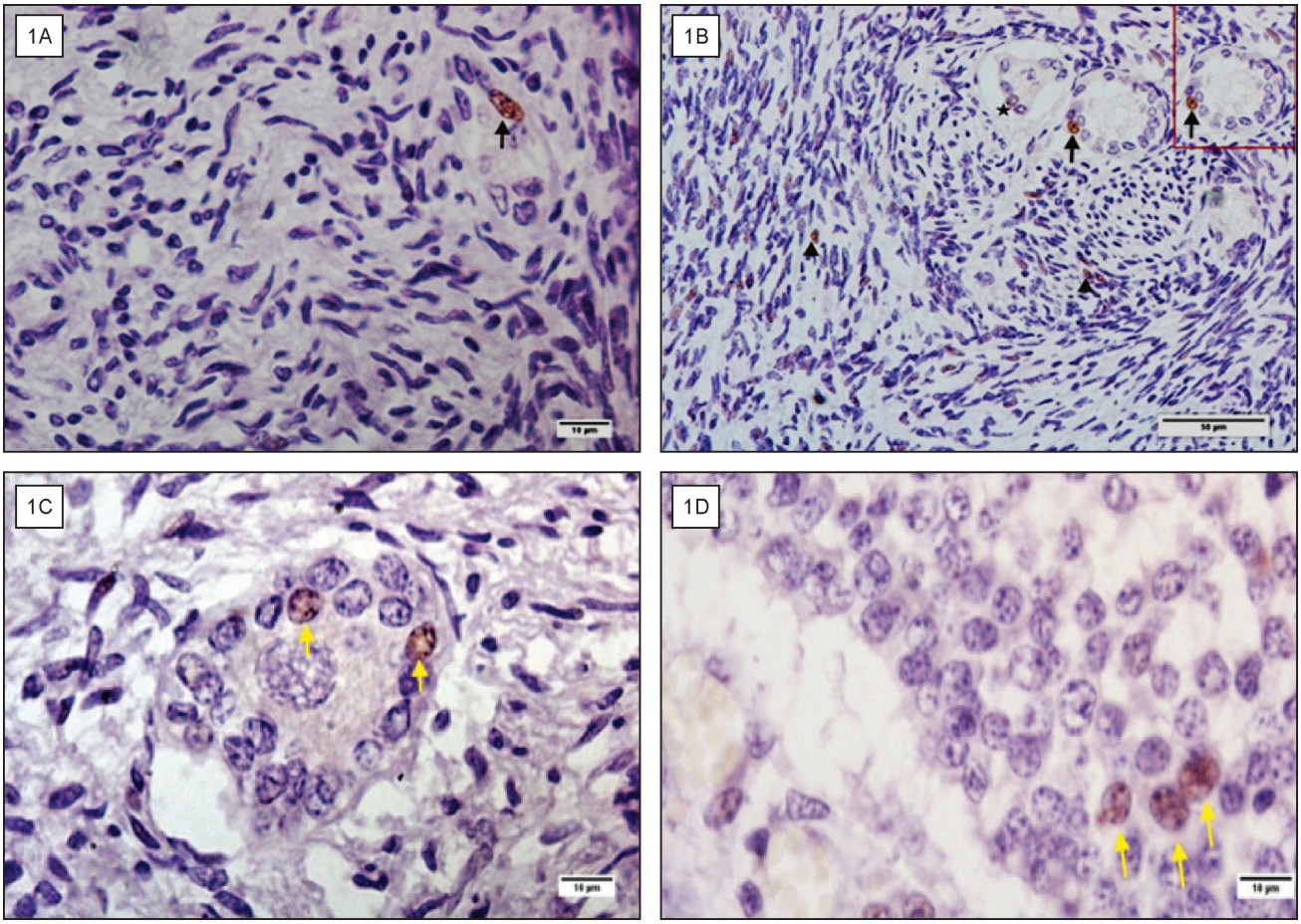

Fig. 1A. Immunostaining of ovary of buffalo with anti-ERα antibody. Nuclear reaction in follicular cells of primordial follicle (arrow); 1B. Immunostaining of ovary of buffalo with anti-ERα antibody. Nuclear reaction in follicular cell of primary follicle (arrow), stromal cells (arrowhead). Weak nuclear reaction in atretic follicle (star); 1C. Immunostaining of ovary of buffalo with anti-ERα antibody. Nuclear reaction in follicular cells of secondary follicle (arrow), stromal cells (arrowhead); 1D. Immunostaining of ovary of buffalo with anti-ERα antibody. Nuclear reaction in granulosa cells of tertiary follicle (arrow). [Polymer HRP method. Original magnification ×400]

the stroma of the ovary, endothelial cells of blood vessels. One or two granulosa cells of primordial and primary follicles were PR positive, and immunoreaction was moderate while no staining was observed in oocytes (Fig. 2A). The moderate nuclear reaction was observed in the stromal cells and endothelial cells of blood vessels in the ovarian cortex (Fig. 2B and 2C). Stage-specific variation was observed both qualitatively and quantitatively. The qualitative assessment of the intensity of immunostaining has been represented in Table 3 and the percentage of PR positive cells in different types of follicles has been presented in Table 4. A similar observation was recorded in the ovary of bovines by D'Haeseleer *et al.* (2007). They observed the highest PR score in primordial, primary and secondary follicles during the oestrus phase, decreased score during metoestrus and lowest levels in early dioestrus, and increased again to moderate scores during the subsequent period of dioestrus and proestrus. In the present study, an increasing percentage of positive cells were observed from primordial to tertiary follicles. Follicular cells of the primordial follicle and granulosa cells and theca cells of tertiary follicles had statistically higher percentage positive cells in the follicular phase as compared to the luteal phase ($P<0.05$). Cell-specific immunostaining of PR was observed in the rabbit ovary in the ovarian surface epithelial and stromal, and granulosa and theca cells of follicles (Abd-Elkareem 2017). D'Haeseleer *et al.* (2007) also recorded PR immunostaining in both deep and superficial stroma and higher immunostaining was recorded during oestrus and metoestrus, very low during early dioestrus and higher again in the subsequent stage of dioestrus and proestrus. Similar to our findings they also recorded immunopositive stromal cells in the perivascular areas. In the present investigation, we did not record immunostaining in the germinal epithelium but stromal cells in tunica albuginea were moderately positive however, Hild-Petito *et al*. (1988) recorded staining in the germinal epithelium and D'Haeseleer *et al.* (2007) recorded low scores for PR in the surface epithelium and the tunica albuginea, with minimal cyclic variations. Durlej *et al.* (2010) also recorded nuclear staining for PR in cells of granulosa and theca layer, corpora lutea, and surface epithelium of the porcine ovary.

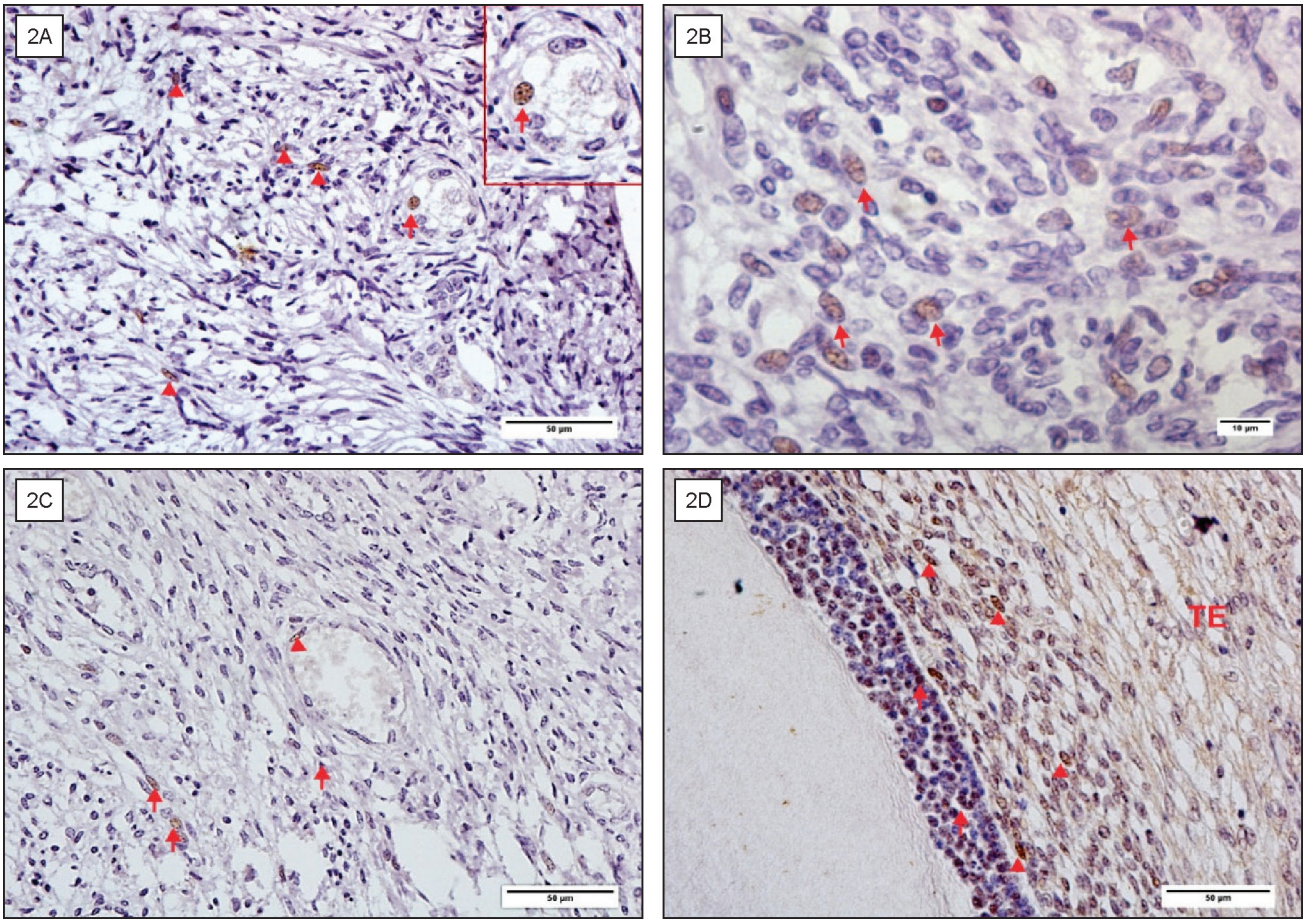

Fig. 2A. Immunostaining of ovary of buffalo with anti-PRA antibody. Nuclear reaction in follicular cells of primordial follicle (arrow), stromal cells (arrowhead). Polymer HRP method. Original magnification ×400 Inset showing magnified view of nuclear reaction in follicular cell (arrow); 2B. Immunostaining of ovary of buffalo with anti-PRA antibody. Nuclear reaction in stromal cell of ovarian cortex (arrows); 2C. Immunostaining of ovary of buffalo with anti-PRA antibody. Nuclear reaction in stromal cells (arrow) and endothelial cells (arrowhead); 2D. Immunostaining of ovary of buffalo with anti-PRA antibody. Nuclear reaction in granulosa cells of tertiary follicles (arrow), theca interna cells (arrow) and theca externa cells (TE). [Polymer HRP method. Original magnification ×400].

Table 3. Average staining intensity of PR in the ovary at different stages of the estrous cycle

Stage	Surface epithelium	Stroma	Primordial follicle	Primary follicle	Secondary follicle	Antral follicle	Atretic follicle
Follicular phase	–	++	++	+++	+++	+++	++
Luteal phase	–	++	++	+++	+++	+++	+++

–, No reaction; +, Weak Reaction; ++, Strong Reaction; +++, Intense reaction.

Table 4. Percentage of PR positive cells in the ovary during follicular and luteal phases of the estrous cycle

Stage	Primordial follicle	Primary follicle	Secondary follicle	Antral follicle		Atretic Follicles
				Granulosa cells	Theca cells	
Follicular phase	15.28±1.38	18.62±2.08	24.23±1.51	78.70±4.5	86.30±6.02	49.41±3.49
Luteal phase	10.62±1.21	15.78±1.34	16.71±1.62	62.78±3.82	71.64±5.32	42.41±2.86

In the antral follicles, both granulosa cells, theca cells as well as surrounding stromal cells were immunostained for PR (Fig. 2D). D'Haeseleer *et al.* (2007) observed a similar staining pattern in the granulosa and theca layers of vital tertiary follicles in bovine ovaries. They recorded slightly lower PR immunoreactivity in theca interna and theca externa cells in proestrus than in late dioestrus. Contrary to our studies, PR was exclusively observed in the theca layers of secondary and tertiary follicles in the monkey ovary (Hild-Petito *et al*. 1988). Slomczynska *et al*. (2000) recorded PR in granulosa cells of early antral follicles. Contrary to our findings they did not observe PR in granulosa cells of small and medium antral follicles, however, they recorded it in theca cells. It was hypothesized by D'Haeseleer *et al.* (2007) that progesterone might regulate follicular development in the bovine ovary via granulosa cells, theca cells as well as stromal cells.

In the obliterative atretic follicles, the invading stromal cells were highly positive for PR (Fig. 2E.). No staining was observed in negative controls (Fig. 2F). The score for PR in the granulosa cells of cystic atretic tertiary follicles of bovines was similar to that of the granulosa cells of vital tertiary follicles, whereas the score in the granulosa cells of obliterative atretic follicles was much lower (D'Haeseleer *et al.* 2007). Higher expression of PR in invading stromal cells in obliterative atresia might be correlated to its role in the formation of atretic follicles.

The average levels of plasma estradiol during the follicular and luteal phase of the animals under the study were 27.38±1.21 pg/ml and 14.25±1.14 pg/ml respectively while the average level of plasma progesterone during the follicular and luteal phase of the animals under the study was 0.15±0.39 ng/ml and 2.97±0.67 ng/ml respectively. The hormonal levels recorded were similar in range as described in the earlier study by Pathak *et al*. (2019) in buffaloes. The data on the receptor and hormone analysis showed that the ER and PR expression was higher during the estradiol dominance and lower during the progesterone dominance. Our findings are in line with the hypothesis that estradiol had a stimulatory effect on the expression of these hormonal receptors (Ing and Tornesi 1997). D'Haeseleer *et al.* (2007) recorded a negative correlation between the plasma progesterone levels and the PR score for all ovarian cell types in bovine ovaries and thus it could be hypothesized that estradiol upregulated the expression of these receptors while progesterone hormone downregulates the same.

Thus it can be concluded that both the estrogen and progesterone hormones act locally through their receptors

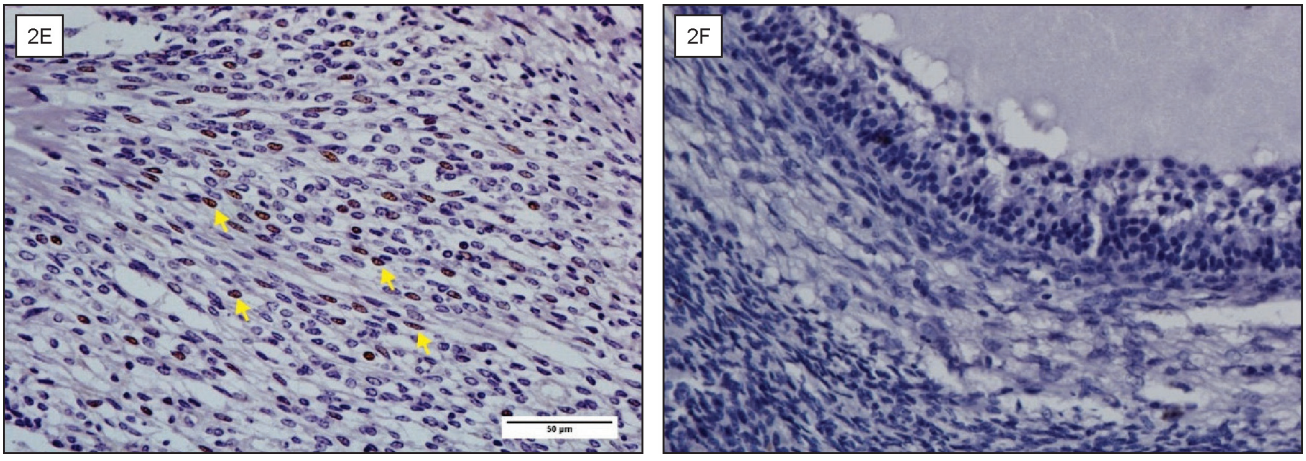

Fig. 2E. Immunostaining of ovary of buffalo with anti-PRA antibody. Nuclear reaction in stromal cells (arrow) in obliterative atretic follicle; 2F. Negative control for immunostaining anti-PRA antibody. [Polymer HRP method. Original magnification ×400].

to modulate the growth and differentiation of ovarian follicles.

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REFERENCES

Abd-Elkareem M. 2017. Cell-specific immuno-localization of progesterone receptor alpha in the rabbit ovary during pregnancy and after parturition. *Animal Reproduction Science* **180**:100–20.

Abd-Elkareem M and Abou-Elhamd A S. 2019. Immunohistochemical localization of progesterone receptors alpha (PRA) in ovary of the pseudopregnant rabbit. *Animal Reproduction* **16**(2): 302–10.

Adashi E Y and Hsueh A J W. 1982. Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured Rat granulosa cells. *The Journal of Biological Chemistry* **257**(11): 6077–83.

Al-Asmakh M. 2007. Reproductive functions of progesterone. *Middle East Fertility Society Journal* **12**(3): 147.

Basic Animal Husbandry and Fisheries Statistics. 2017. Government of India, Ministry of Agriculture and Farmers Welfare, Department of Animal Husbandry, New Delhi.

Britt K L and Findlay J K. 2002. Estrogen actions in the ovary revisited. *Journal of Endocrinology* **175**: 269–76.

Conneely O M, Mulac-Jericevic B, Demayo F, Lydon J P and O'Malley B W. 2002. Reproductive functions of progesterone receptors. *Recent Progress in Hormone Research* **57**(1): 339–55.

D'Haeseleer M, Simoens P and Van Den Broeck W. 2007. Cell-specific localization of progesterone receptors in the bovine ovary at different stages of the oestrous cycle. *Animal Reproduction Science* **98**(3–4): 271–81.

Darawiroj D, Srisuwatanasagul K, Pianchop S and Sukjumlong S. 2003. Immunohistochemical studies of the estrogen receptor alpha in the ovaries and uteri of the domestic cat at different stages of the oestrous cycle. *Thai Journal of Veterinary Medicine* **33**(4):89–95.

Durlej M, Tabarowski Z and Slomczynska M. 2010. Immunohistochemical study on differential distribution of progesterone receptor A and progesterone receptor B within the porcine ovary. *Animal Reproduction Science* **121**(1–2): 167–73.

Ghuman S P S, Dadarwal D, Honparkhe M, Singh J and Dhaliwal G S. 2009. Production of polyclonal antiserum against progesterone for radioimmunoassay. *Indian Veterinary Journal* **86**: 909–11.

Hild-Petito S, Stouffer R L and Bremmer R M. 1988. Immunocytochemical localization of estradiol and progesterone receptors in the monkey ovary throughout the menstrual cycle. *Endocrinology* **123**: 2896–2905.

Hu³as-Stasiak M and Gawron A. 2007. Immunohistochemical localization of estrogen receptors ERα and ERα in the spiny mouse (Acomys cahirinus) ovary during postnatal development. *Journal of Molecular Histology* **38**(1): 25–32.

Hu³as-Stasiak M and Gawron A. 2010. Distribution of androgen and progesterone receptors in the spiny mouse (*Acomys cahirinus*) ovary during postnatal life. *Reproductive Biology* **10**(1): 37–51.

Ing N H and Belen Tornesi M. 1997. Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. *Biology of Reproduction* **56**(5): 1205–15.

Pathak D and Bansal N. 2012. Histomorphochemical and electron microscopic studies on the endometrial gland of buffalo. *Indian Veterinary Journal* **89**(9): 25–28.

Pathak D, Bansal N, Singh O, Gupta K and Ghuman S P S. 2019. Immunohistochemical localization of estrogen receptor alpha (ERα) in the oviduct of Indian buffalo during follicular and luteal phases of estrous cycle. *Tropical Animal Health Production* **51**(6): 1601–09.

Pathak D, Bansal N, Singh O, Gupta K and Ghuman S P S. 2019. Immuno-localization of estrogen receptor (ERα) and progesterone receptor (PR) in uterus of buffalo during follicular and luteal phases of estrous cycle. *Journal of Animal Research* **9**(1): 185–93.

Phogat J B, Pandey A K and Singh I. 2016. Seasonality in buffalo's reproduction. *International Journal of Plant Animal Environmental Sciences* **6**(2): 46–54.

Pelletier C, Labrie C and Labrie F. 2000. Localization of oestrogen receptor α, oestrogen receptor α and androgen receptors in the rat reproductive organs. *Journal of Endocrinology* **165**: 359–70.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J Y, White D J, Hartenstein V, Eliceiri K, Tomancak, P and Cardona A. 2012. Fiji: An open-source platform for biological-image analysis. *Nature Methods* **9**: 676–82

Slomczynska M, Krok M and Pierscinski A. 2000. Localization of the progesterone receptor in the porcine ovary. *Acta Histochemica* **102**:183–91.

Snedecor G W and Cochran W G. 2004. *Statistical Methods*. 8th Edn., East-West Press Pvt. Ltd., New Delhi.

Tienthai, P. and Kriengyot, S. 2009. Morphological studies and immunolabeling of female hormonal receptors in Thai swamp buffalo oviducts at prepuberty. *Thai Journal of Veterinary Medicine* **39**:121–34.

Tomanek M, Pisselet C, Monget P, Madigou T, Thieulant M L and Monniaux D. 1997. Estrogen receptor protein and mRNA expression in the ovary of sheep. *Molecular Reproduction and Development: Incorporating Gamete Research* **48**(1): 53–62.

Van den Broeck W, D'haeseleer M, Coryn M and Simoens P. 2002. Cell specific distribution of progesterone receptors in the bovine ovary. *Reproduction in Domestic Animals* **37**(3): 164–70.

Vermeirsch H, Simoens P, Coryn M and Van den Broeck W. 2001. Immunolocalization of progesterone receptors in the canine ovary and their relation to sex steroid hormone concentrations. *Reproduction-Cambridge* **122**(1): 73–83.