

Functional Characterization of Corpus Luteum and its Association with Peripheral Progesterone Profile at Different Stages of Estrous Cycle in the Buffalo

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ABSTRACT

Corpus luteum (CL) serves as marker to ascertain the stages of cyclicity as well as to diagnose acyclic condition in the farm animals. Accordingly, we studied the cellular components and functional aspects of CL in relation to cyclicity with the buffalo ovaries (n=32) collected from slaughter house. The paired ovary samples (n=8, per stage) were categorized as early luteal (EL, day 1-5), mid luteal (ML, day 6-14) and late luteal (LL, day 15-21) and acyclic stage based on the gross ovarian dating. Histological changes of cellular differentiation of the CL were assessed using H&E staining and progesterone concentration in the luteal extract and peripheral circulation was measured by RIA method. A significantly higher (4.22 fold) proportion of small luteal cells were observed at ML (376.58±11.65) as compared to EL (89.35±5.54) and decreased thereafter in LL (57.40±3.02) stage. However, maximum number of large luteal cells was also found in ML (74.38±2.02) in contrast to EL (15.11±1.86) and LL (22.17±1.68). Further, serum and luteal progesterone was found maximum at ML stage and there was a significant (r=0.998) positive correlation. This study indicates maximum functional activity of CL was at ML stage in the buffalo.

Keywords: Bubaline, Estrous cycle, Corpus luteum, Progesterone

Corpus luteum a temporary and dynamic endocrine gland develops through extensive tissue remodelling, angiogenesis, cellular growth and differentiation during post-ovulation (Davis *et al.*, 1996). It performs several vital functions in the reproductive process namely estrous cycle, embryo survival, implantation of embryo (blastocyst) and maintenance of pregnancy (Niswender *et al.*, 2000). Corpus luteum synthesizes and secretes a variety of hormones, principle of which is progesterone (P_4) in many domestic animals including buffalo (Zain and Omar, 2001; Mondal and Prakash, 2003). Blood P_4 serves as marker, frequently used as a diagnostic variable correlating the ovarian functions and many reproductive diseases. Estimation of progesterone in peripheral blood/

or CL content helps to access the functional status of corpus luteum and its morphology (El-Sheikh *et al.*, 1967; Mondal and Prakash, 2003; Mondal *et al.*, 2004). Progesterone production patterns of CL at various stages of estrous cycle would be helpful in understanding the functioning of cyclic corpora lutea (Memon *et al.*, 1971) and provide a valuable basis for normal CL development in the buffalo. Moreover, the blood progesterone has emerged as a useful tool to determine an appropriate time of insemination, monitoring of cyclicity and pregnancy diagnosis in buffaloes (Batra and Pandey, 1983). Therefore, the present study was investigated to assess the corpus insights and to correlate the serum and luteal progesterone during different stages of development in cyclic buffaloes.



MATERIALS AND METHODS

Ethical procedure

Collection of biomaterials from live and slaughter house animals were approved by Institute Animal Ethics Committee as per prescribed CPSCEA guidelines (No. F.25/33/2016-CPSCEA Part 1 dated 16/02/2017), Ministry of Environment, Forest and Climate Change, Government of India.

Collection of sample

Non-pregnant genitalia from individual buffalo (n=32) were procured from the local slaughter house within 10-20 min after exsanguinations and were transported on ice to the laborartory. The stage of estrous cycle was determined by macroscopic ovarian dating (color, consistency, vasculature of CL, number and size of follicles) and the gross appearance of the endometrial mucosa after ruling out the subclinical endometritis through endometrial cytology (Kasimanickam et al., 2004). The samples (n=8, per stage) were classified retrospectively, as early (day 1-5), mid (day 6-14) and late luteal/ follicular (day 15-21) (Ireland et al., 1980; Ali et al., 2003; Jaglan et al., 2010; Baithalu et al., 2013) and acyclic stage (Khan et al., 2011) with slight modifications (Fig. 1). Simultaneously, blood sample (approx. 8 mL) was also collected from each animal for estimation of serum P₄ concentration to confirm the reproductive stages, retrospectively.

Histology of the corpus luteum (CL)

A piece of ovarian tissue containing CL was fixed in 10% neutral buffered formalin saline (NBFS). The fixed tissue samples were taken out and shifted to freshly prepared 10% NBFS for final trimming of the tissues. The tissue samples were washed overnight under tap water and dehydrated through ascending grades of alcohol and tissue clearing was performed with acetone and benzene. The tissues sections were embedded in paraffin blocks. The paraffin block of the tissue was then cut into 4-5 μ m thick paraffin sections by microtome and stained with Haematoxylin and Eosin (H&E). Luteal cells were counted in ten best fields per slide at random and the mean number of cells was counted. Cellular components of corpus luteum and luteal cell population was observed under microscope (NI-

U, Nikon, Japan) of stained luteal tissue section and was digitally photographed and recorded.



Fig. 1: The buffalo genitalia along with the changes in ovary at different stages of cyclic and acyclic condition

Progesterone Assay

The concentration of serum and luteal progesterone was determined by using RIA kit (Immunotech, France). The P_4 was measured using ¹²⁵I RIA and the radioactivity measured in a gamma counter depends on the ability of an antibody to bind its antigen. To quantitate the antigen, the radioactive and non-radioactive forms of the antigen compete for binding sites on a specific antibody. As more non-radioactive antigen is added, less radioactive antigen remains bound until equilibrium between free and antibody bound antigen occurs. To perform this test, 50 μ L of sample, standards (0, 0.1, 0.5, 2.0, 13.0 and 55.0 ng/mL) or control (0.85-1.41 ng/mL) were added sequentially to their respective tubes. 500 μ L of progesterone I¹²⁵ tracer was added to all the tubes along with a blank tube. Contents

of all the tubes were thoroughly mixed and incubated for 1 h at 27-28°C in the waterbath. After incubation the contents of all the tubes were decanted and blotted by absorbent filter paper. The empty tubes were counted in a gamma counter calibrated for I¹²⁵. Radioactivity of the tubes was measured for 1 minute for counts bound per minute, cpm (B) and total cpm (T) for only tracer in non-antibody coated tube. The final concentration was calculated by interpolation from a standard four parametric logistic sigmoidal curve (Fig. 2) that was drawn using different concentrations of progesterone standard. The intra and inter-assay variations were 8.15% and 8.66%, respectively. Analytical sensitivity of the progesterone assay was 0.03 ng/mL (0.10 nmol/L).



Fig. 2: Sigmoid four parametric logistic curve for standardizing progesterone concentrations (Log_{10} concentration) by Radioimmuno-assay (RIA)

Luteal progesterone (P4) Assay

The CL was enucleated from the ovary and washed thrice with fresh phosphate buffer saline (PBS; 0.05 M, pH 7.4). Luteal tissues were chopped and weighed to yield 100 ± 0.6 mg tissue that was transferred in 1 ml PBS (0.05 M, pH 7.4) and stored at-20°C for hormone assay. On the day of assay, the tissue was thawed and homogenized using glass teflon homogenizer and the total volume of homogenate was made up to 1 mL with PBS. The samples were finally diluted at 1: 40 to make with PBS for progesterone concentration (µg/gm) estimation with the RIA kits.

Statistical analysis

Data obtained in the experiment were analyzed using statistical software SPSS 16.0 (SPSS Corporation, USA). The mean value of examined variable was compared by one-way ANOVA with Tukey's as *post-hoc* test as equality of variance assumed. The difference of mean values for all data analyzed with p<0.01 was considered as significant. Figures were constructed in GraphPad Prism Version 6.0.

RESULTS AND DISCUSSION

Cyclic buffalo CL was composed of mainly small and large luteal cell (Fig, 3).



Fig. 3: Histological changes in the buffalo ovarian tissue during different stages of estrous cycle and acyclicity (H&E staining). LLC: Large luteal cell; SLC: Small luteal cell. Bar10X: 100µm



Stage of cyclicity	Number of luteal cell components (Mean±SEM)				
	Small luteal cells	Large luteal cells	Total luteal cells		
Early luteal	89.35±5.54ª (85.54%)	15.11±1.86 ^a (14.46%)	104.46±3.37 ^a		
Mid luteal	376.58±11.65 ^b (83.51%)	74.38±2.02 ^b (16.49%)	$450.96{\pm}7.82^{b}$		
Follicular	57.40±3.02° (72.14%)	22.17±1.68° (27.86%)	79.57±2.14°		
P value	0.001	0.001	0.001		

Table 1: Cellular component of corpus luteum (stained section) at different stages of estrous cycle in the buffalo

Different superscripts in column differ significantly (p<0.01); Figures in parenthesis indicate proportion of cells

Table 2: Serum and luteal progesterone profile and their correlation at different stages of estrous cycle in the buffalo

Stage of cyclicity	Serum P ₄ (ng/mL)		Luteal P ₄ (µg/gm)		Correlation ^{\$}
	Mean±SEM	Range	Mean±SEM	Range	between serum and luteal P ₄
Early luteal	2.83±0.21a	2.27-2.98	7.40±0.3 ⁵ a	6.68-7.15	0.400
Mid luteal	5.43±0.3 ⁹ b	3.02-8.00	9.99±0.4 ⁰ b	8.06-12.41	0.998**
Follicular	$0.53{\pm}0.0^{8}c$	0.30-0.92	$1.24{\pm}0.4^{0}c$	0.18-4.18	0.189
Acyclic	0.11±0.05°	0.02-0.23	—		—
P value	0.001		0.001		

Different superscripts in column differ significantly (p<0.01); Spearman's rank correlation coefficient's (rho) was calculated. **(p<0.01)



Fig. 4: Hormonal assay (**A**) serum progesterone (ng/mL) and (**B**) luteal progesterone (μ g/g) concentrations by Radio-immuno-assay (RIA)

The mean numbers of small luteal cells (SLC) were 89.35 ± 5.54 , 376.58 ± 11.65 and 57.40 ± 3.02 at early (EL), mid (ML) and late luteal (LL) stage, respectively. Whereas, the number of large luteal cells (LLC) were 15.11 ± 1.86 , 74.38 ± 2.02 and 22.17 ± 1.68 at EL, ML and LL, respectively (Table 1). The number of luteal cells increased significantly (p<0.01) at ML as compared to EL

and thereafter decreased at LL stage. The increase in the number of SLC and LLC at ML stage was consistent with the reports of Schawall *et al.* (1986) and Jaglan (2008). The mean serum progesterone concentration (ng/mL) was 2.83 ± 0.21 , 5.43 ± 0.39 , 0.53 ± 0.08 and 0.11 ± 0.05 at early, mid, late and acyclic group, respectively (Table 2). Significant (p<0.01) difference was observed in the serum

progesterone concentration among groups (Fig. 4A). Our reports are in line with the reported serum progesterone concentration in buffalo cows (Takkar *et al.*, 1982).

Additionally, the mean luteal progesterone concentration (ng/mL) was 7.40±0.35, 9.99±0.40 and 1.24±0.40 at EL, ML and LL stage (Table 2). Significant difference (p<0.01) was observed in progesterone concentration among different stage of CL (Fig. 4B). Further, there was significant (p<0.01) positive correlation of serum progesterone with luteal progesterone concentration. The present trend in P_4 concentration in the luteal tissue further at ML strengthen the earlier findings reported in Indian buffalo (Shah and Mehta, 1992; Mondal et al., 2004) and in Egyptian buffalo (El-Sheikh et al., 1967). The progesterone concentration in the luteal tissue was increased from corpus haemorrhagicum to mature CL however, decrease in the regressive CL, completely undetectable in the corpus albicans in the Egyptian buffalo (El-Sheikh et al., 1967). Shah and Mehta (1992) observed significantly higher luteal progesterone concentrations in the mid luteal CL as compare to other luteal phases. Memon et al. (1971) observed that the progesterone concentration $(62 \mu g/g)$ was highest on day 13 and decreased to 32.3 $\mu g/g$ on day 18 in Surti buffalo. Net progesterone content in the bovine corpus luteum increase progressively from day 2 to day 11 and remained relatively constant up to day 20 and, thereafter, fell at day 0 (Hafs and Armstrong, 1968). Mares et al. (1962) observed that total progesterone content in corpus luteum was increased from day 7 (26.9 $\mu g/g$) to day 15 (50.7 $\mu g/g$) and then declined at day 17 $(9.1 \ \mu g/g)$. Roy and Mullick (1964) also reported that the peak progesterone concentration from buffalo corpus luteum was $41.73\pm6.78 \ \mu g/g$ on day 12 of the cycle in the buffaloes. These findings are in agreement with the present study as maximum P₄ concentration was found in mid CL. As bovine corpus luteum synthesizes more progesterone compared to buffalo corpus luteum (Hafs and Armstrong, 1968), comparatively lower progesterone levels in present study could be due to smaller size and weight of corpus luteum (El-Sheikh et al. 1967). Moreover, the luteal function was significantly lower (P<0.01) in buffaloes compared to cows throughout the cycle (Mondal and Prakash, 2003).

From the present study, it is concluded that the highest peripheral P_4 concentration is associated with more number SLC and LLC present in the CL and positively correlated with the luteal P_4 production at ML stage in buffaloes.

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REFERENCES

- Ali, A., Abdel-Razek, A.K., Abdel-Ghaffar, S. and Glatzel, P.S. 2003. Ovarian follicular dynamics in buffalo cows (*Bubalus bubalis*). *Reprod. Domest. Anim.*, **38**: 214-218.
- Baithalu, R.K., Singh, S.K., Gupta, C., Raja, A.K., Saxena, A., Kumar, Y., Singh, R. and Agarwal, S.K. 2013. Cellular and functional characterization of buffalo (*Bubalus bubalis*) corpus luteum during the estrous cycle and pregnancy. *Anim. Reprod. Sci.*, 140(3): 138-146.
- Batra, S.K. and Pandey, R.B. 1983. Luteinizing hormone and oestradiol-17B in blood plasma and milk during the oestrous cycle and early pregnancy in Murrah buffaloes. *Anim. Repord. Sci.*, 5: 247-257.
- Davis, J.S., May, J.V. and Keel, B.A. 1996. Mechanisms of hormone and growth factor action in the boveine corpus luteum. *Theriogenology*, 45: 1351-1380.
- El-Sheikh A.S., Sakla, F.B. and Amin, S.O. 1967. Changes in the density and progesterone content ofluteal tissue in the Egyptian buffalo during the estrous cycle. *J. Endocrinol.*, **39**: 163-171.
- Hafs, H.D. and Armstrong, D.T. 1968. Corpus luteum growth and progesterone synthesis during the bovine estrous cycle. *J. Anim. Sci.*, 27: 134-141.
- Ireland, J.J., Murphee, R.L. and Coulson, P.B. 1980. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J. Dairy Sci., 63: 155-160.
- Jaglan, P. 2008. Morphologiocal and functional studies of buffalo corpus luteum during estrous cycle. Thesis, M.V.Sc. Deemed University, Indian Veterinary Research Institute, Izatnagar, UP, India.
- Jaglan, P., Das, G.K., Kumar, B.S., Kumar, R., Khan, F.A. and Meur, S.K. 2010. Cyclical changes in collagen concentration in relation to growth and development of buffalo corpus luteum. *Vet. Res. Commun.*, 34(6): 511-518.
- Kasimanickam, R., Duffield, T.F., Foster, R.A., Gartley, C.J., Leslie, K.E., Walton, J.S. and Johnson, W.H. 2004. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology*, 62(1): 9-23.

- Khan, F.A., Das, G.K., Pande, M., Mir, R.A. and Shankar, U. 2011. Changes in biochemical composition of follicular fluid during reproductive acyclicity in water buffalo (*Bubalus bubalis*). *Anim. Reprod. Sci.*, **127(1)**: 38-42.
- Mares, S.E., Zimbelman, R.G. and Casida, L.E. 1962. Variation in progesterone content of the bovine corpus luteum of the estrous cycle. *J. Anim. Sci.*, **21**: 266-271.
- Memon, G.N., Buch, N.C. and Patel, B.M. 1971. A study of follicular and luteal characteristics of the ovary at different stages of estrous cycle in Surti buffalo heifer. J. Reprod. Fertil., 27: 301-312.
- Mondal, S. and Prakash, B.S. 2003. Periphearl plasma progesterone concentration in relation to estrous expression in Murrah buffalo. *Indian J. Anim. Sci.*, **73**: 292-293.
- Mondal, S., Kumar, V., Reddy, I.J. and Singh, K. 2004. Progesterone and nucleic acid contents of buffalo corpus luteum in relation to stages of estrouscycle. *Indian J. Anim. Sci.*, 74: 710-712.
- Niswender, G.D., Juengel, J.L., Silva, P.J., Rollyson, M.K. and McIntush, E.W. 2000. Mechanisms controlling the function and life span of the corpus luteum. *Physiol. Rev.*, 80: 1-29.

- Roy, D.J. and Mullick, D.N. 1964. Endocrine function of corpus luteum of buffaloes during estrous cycle. *Endocrinology*, 75: 284-287.
- Schwall, R.H., Gamboni, F., Mayan, M.H. and Niswender, G.D. 1986. Changes in the distribution of sizes of ovine luteal cells during estrous cycle. *Buffalo J.*, 2: 167-173.
- Shah, R.G. and Mehta, V.M. 1992. Correlated behaviour of blood and corpus luteum progesterone levels with luteal cell types in Surti buffaloes. *Buffalo J.*, 8(2): 167-73.
- Takkar, O.P., Singh, M. and Varman P.N. 1982. Progesterone profile in *buffaloes* during various stages of oestrous cycle using radioimmunoassay technique. *Theriogenology*, 17(5): 565-569.
- Zain, D.E. and Omar, H.M. 2001. Antioxidants activities and changes in lipid peroxide and nitric oxide productions in cyclic corpora lutea and its relation to serum progesterone levels in buffalo cows. *Buffalo J.*, 1: 79-89.