



Changes in phosphatases activities in uterine secretion during estrous cycle: A possible indicator of important biochemical changes in buffaloes uterine microenvironment

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ABSTRACT

Defining biochemical nature of uterine secretion has been the focus of recent research interest due to conception failure after embryo transfer and normal pregnancy in ruminants including buffaloes. Proteins are important as biomolecules for cell function, and phosphatases control the protein function. Therefore identification of changes on secretory proteins and phosphatases activities during early luteal, mid luteal, late luteal and follicular stages of estrous cycle in buffaloes is attempted. The luteal stages uterine secretion contained significantly more protein as compared to the follicular stages of cycle. Two-fold increase in specific and total activities of acid phosphatases during mid-luteal phase and 10-fold specific and 100-fold increase in total alkaline phosphatases activities during late luteal and follicular stages was observed as compared to other stages of the cycle. Corpus luteum growth and regression did not influence the total uterine protein secretion or phosphatases activities. The changes of phosphatases in buffalo uterine secretion during different stages of estrous cycle might be the indicator of the process of endometrial epithelial differentiation and degeneration.

Key words: Alkaline phosphatases, Acid phosphatases, Buffalo, Corpus luteum, Estrous cycle, Uterine secretory proteins

The uterine environment is unique because it is under the control of ovarian steroids which determine the availability of blood constituents by changing the permeability properties of uterine blood vessels (McRae 1988). At the same time it has its own physiology due to endometrial tissues comprising luminal and glandular epithelia, stroma and immune cells. This micro-environment which is crucial for successful establishment of pregnancy might change due to the alteration of external environment and the presence of conceptus. It was seen that even the 1 day asynchrony of uterine environment for the developing embryos are detrimental for survival during transfer in cattle (Rowson *et al.* 1972). Hatched embryo or the trophoblastic vesicle does not elongate *in vitro* unless transferred into the uterus in sheep (Flechon *et al.* 1986). Studies (Guisse and Gwazdauskas 1987, Roy *et al.* 2006) revealed that the protein milieu of uterine secretion changes during different stages of estrous cycle in cattle and buffaloes.

Among the ruminants the buffalo uterine environment is

less characterized although is known to have a low conception rate (45%) as compared to cattle (60%; Jainudeen and Hafez 1987). Our study in buffaloes showed the presence of about 11 uterus specific proteins in uterine secretion during estrous cycle of which some were stage specific and some present in all the stages of the cycle (Roy *et al.* 2006). Phosphatases are the key enzymes in liberating and recycling the phosphate molecules that are necessary for many fundamental biological processes including cell differentiation and proliferation, gene expression and number of metabolic processes. The role of phosphatases is implicated in the process of implantation in rodents (Emadi and Salehnia 2004). The uterine secretory acid phosphatases in pig are speculated to have a role in transferring iron molecule to the early developing conceptus (Zavy *et al.* 1984). We have reported the presence of acid and alkaline phosphatases enzymes in buffalo uterine secretion (Roy *et al.* 2006). However, till date studies available in buffaloes are unable to explain how the overall protein quantities in the uterine luminal secretion vary with the stages of estrous cycle. It also not known how much influence corpus luteum exerts to the total secretory protein content of uterine lumen in buffaloes as the corpus luteum progesterone is known to influence the uterine environment in a major way. Similarly, the changes of these enzyme

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activities during different stages of the cycle are not elucidated in buffaloes which may give clues for some important physiological events in buffalo uterus. Therefore in this study the changes of the protein content in uterine secretion along with the acid and alkaline phosphatases activities during different stages of estrous cycle are correlated with the corpus luteum growth and regression.

MATERIALS AND METHODS

Collection of corpus luteum tissues and uterine secretions

Reproductive tract of cycling buffalo having bilaterally symmetrical uterine horn with no gross ovarian or uterine pathology were collected from corporation slaughterhouse, Bengaluru, immediately after slaughter and brought to the laboratory in ice. The morphology of ovarian functional structures (follicle and CL) was studied for classification of tract into early-luteal ($n = 5$; days 1–4, day 1: ovulation), mid-luteal ($n = 13$; days 5–10), late-luteal ($n = 16$; days 11–17) and follicular phase ($n = 10$; day 18–20) as per Ghosh and Mondal (2006). The diameter of the CL was measured using a divider and scale and the CL was weighed using a weighing balance having sensitivity of 0.1 mg. Uterine lumen of each tract of different categories was washed by introducing 20 mL pre-cooled (4°C) 0.33 M sodium chloride containing 0.02% (wt/vol.) sodium azide using a blunt 18 ga needle and 20 mL syringe through the cut tip of one uterine horn and collected from tip of the other horn. Washed fluid was centrifuged at 10 000 g for 30 min to remove the cell and cell debris and the supernatant was stored frozen at -20°C until further analysis. Uterine washes having cloudiness or with white flakes was discarded and not included for the study.

Determination of protein content and phosphatases activities in buffalo uterine secretions

The protein concentrations in uterine secretion were determined by Lowry's method (Lowry *et al.* 1951) using purified bovine serum albumin as standard (BSA; fraction V). To calculate the total uterine protein and the alkaline and acid phosphatases content in each tract, the concentration (mg/mL) of protein was multiplied by 20 irrespective of the volume of fluid recovered. Since it was assumed that the proteins were dissolved in 20 mL and there were some loss of fluid in the endometrial folds and crevices.

Acid and alkaline phosphatases activity were determined in 0.2 and 0.1 mL samples, respectively as per Linhardt and Walter (1965) by adding 1 mL 5 mM p-nitrophenyl phosphate substrate either in alkaline (0.05 M glycine-NaOH, pH 10.5 with 1 mM magnesium chloride) or acid buffer (0.05 M citrate buffer, pH 4.8) at 37°C for 30 min. The reaction was stopped by adding 10 mL of 0.02N NaOH for alkaline phosphatases or by adding 4 mL 0.04N NaOH for acid phosphatases activity determination and read at 400 nm in

spectrophotometer. Each sample was prepared in duplicate and read against the control tube which was prepared similarly except the samples were added after stopping the enzyme reaction and read against the standard curve prepared using p-nitrophenol in the 11.1 mL volumes. To obtain the unit of alkaline phosphatase micromole value from standard curve was multiplied by 20. Similarly, to obtain the unit of acid phosphatase activity values from the standard curve were multiplied by 4.68 since the standard curve was prepared with the volume of 11.1 mL. The alkaline and acid phosphatases content in each tract were calculated by multiplying the specific activities of enzyme and total uterine protein content in each tract.

Statistical analyses

The means of diameter and weight of the corpus luteum and the uterine protein contents, alkaline and acid phosphatases activities across the stages were compared by SPSS 10.0 using a general linear multivariate analysis model where stage of cycle were kept as fixed factor and all the parameter such as CL diameter and weight, uterine protein concentration and total contents, specific activities and total contents of alkaline and acid phosphatases in uterine secretion were kept as dependant variables. The differences in means across the stages were compared by LSD. The relation of protein and phosphatases content with the CL growth and regression were assessed by correlation analysis at individual stages, pooled data of luteal phase and follicular phases including overall.

RESULTS AND DISCUSSIONS

Diameter weight relationship of buffalo corpus luteum during different stages of estrous cycle

The corpus luteum diameter and weight increased significantly ($P \leq 0.05$) with the progress of cycle starting from early luteal to mid and late luteal phase then decreased at follicular phase of the cycle when it undergoes regression (Table 1). Early luteal phase CL being the earliest period of development, the structure was mostly disorganized with soft tissues therefore was difficult to isolate. The maximum diameter of CL recorded for buffalo CL in this study was 18 mm and the corresponding weight was 2.02 g. As expected a significant correlation ($r = 0.98$; $P \leq 0.05$) existed with corpora lutea diameter and weight.

Gradual increase in diameter and weight of CL coincided with luteal phase of the cycle and decreased with the follicular phase when corpus luteum undergoes regression. The maximum CL weight as noticed in this study was little more compared to our earlier study (Ghosh and Mondal 2006). However the maximum weight of the mature buffalo CL was still less compared to cattle as reported in the literature. This might explain the reason for which buffalo has a less circulating progesterone level as compared to cattle at the peak profile level (Mondal and Prakash 2002).

Table 1. Corpus luteum (CL) diameter and weights side-by-side uterine secretory proteins, alkaline (ALP) and acid (ACP) phosphatases activities during different stages of estrous cycle in buffaloes

Stages of cycle	CL diameter (mm)	CL weight (g)	Total uterine protein (mg)	ALP (U/mg protein)	Total ACP (U)	ACP (U/mg protein)	Total ALP (U)
Early luteal (n=5) (Days 1–4)	10.30±1.08a (7–12.5)	0.3420±0.07a (0.15–0.52)	29.98±5.71a (20–50)	34.88±4.97a (17.16–45.10)	1034.25±263.89a (608.65–2044)	2.09±0.35a (0.95–2.89)	61.78±11.77ac (18.93–85.30)
Mid luteal (n=13) (Days 5–10)	12.57±0.38b (10–15)	0.7454±0.04b (0.49–0.95)	31.50±3.12a (13.13–55.31)	29.99±3.17a (14.14–53.66)	844.59±52.12a (627.86–1346.36)	4.39±0.46b (2.28–7.62)	123.68±6.64b (94.09–175.99)
Late luteal (n=16) (Days 11–17)	14.63±0.51c (9.5–18)	1.2425±0.08c (0.91–2.02)	27.78±2.17a (14.34–43.7)	395.64±28.06b (239.06–578.66)	10995.37±1028.46b (3717.07–17034.04)	2.38±0.19a (1.16–3.70)	66.03±6.58a (16.71–112.03)
Follicular (n=10) (Days 18–20)	11.71±0.48ab (10–14.5)	0.636±0.06b (0.32–0.99)	18.46±2.50b (11.61–55.31)	428.50±51.27b (154.31–731.85)	8330.69±1675.32b (2222.16–16736.86)	1.96±0.18a (1.37–3.02)	36.80±5.99c (16.25–72.01)

The data are mean ± SE. a,b,c indicate significant ($P \leq 0.05$) differences among the stages. Values in parenthesis indicate the ranges.

Protein and phosphatases activities in uterine secretion in relation to the changes in estrous cycle and corpus luteum growth and regression

The changes in CL growth in terms of diameter or weights did not show any association with the total uterine secretory protein, alkaline or acid phosphatases activities levels. However, total protein in uterine secretion was more in each luteal stage as compared to follicular stage samples. Even though the total protein secretion did not change between early and late luteal phase, the acid phosphatases activity in uterine secretion increased by 2-fold at mid luteal phases as compared to early luteal phase, followed by a decrease at late luteal and follicular stages. Most strikingly at late luteal phase the alkaline phosphatases specific activity was found increased by 10-fold and the total activity by 100-folds as compared to the early and mid-luteal phase level without any change in total protein. The activity of alkaline phosphatases remained increased up to the follicular phase and decrease by the start of the next cycle early luteal phase (Table 1).

The total protein of uterine secretion was significantly higher during luteal phase when CL remains functional as compared to follicular phase when CL undergoes regression. However the total uterine protein in secretion found unaffected by changes in CL growth expressed either in terms of changes in diameter and weight of the CL. The reason might be that majority of the proteins in the buffalo uterine secretion is originating from the blood serum components and whatever minor contribution available from the endometrial secretion is not enough to affect changes in the total uterine secretory protein pool. In cattle the contribution of serum protein in uterine secretion is accounted for 98% whereas non-serum protein components accounted less than 2% of the total protein (Roberts and Parker 1974). To have a better picture one needs to explore the changes at the individual protein level as has been reported in our earlier study in buffaloes (Roy *et al.* 2006) and in cattle (Alavi-Shoushtari *et al.* 2008). In our study all the luteal phase secretion had more proteins compared to the follicular phase

of the cycle. This result is in contrast to the report in cattle which described availability of more proteins during pro-estrous and estrous period (Guisse and Gwazdauskas 1987, Roberts and Parker 1974). The pro-estrous period coincides with the follicular phase of our study. The protein recovery as reported in our study for buffalo is more compared to the cattle the reason might be due to the species difference.

Both acid and alkaline phosphatases activities are reported to be present in the endometrial tissues of buffalo (Bugalia and Sharma 1990) and sheep (Zamiri and Blackshaw 1979) by enzyme histochemistry. A small amount of recoverable acid phosphatases has been detected in uterine secretions of progesterone-treated cows along with lactoferrin, the major non-serum protein (Dixon and Gibbon 1979). Porcine uterine fluid also reported to have a major iron containing, progesterone induced, acid phosphatase which transfers iron to the developing conceptus with a molecular weight of 32 kDa (Schlosnagle *et al.* 1974). In our study, the acid phosphatases level in uterine secretion found 2-fold increased ($P \leq 0.05$) during mid luteal phase (day 5–10) when epithelial cells proliferate; culturable and hatching of embryo is expected to occur. Interestingly the acid phosphatase level found decreased at late luteal phase (days 11 to 17) coinciding with 10-fold increase in specific activities and a 100-fold increase in total activities of alkaline phosphatases. The increased activity of alkaline phosphatases coincided with the time when endometrial secretion is discharged through the ruptured apical surface of the endometrial epithelial cells in cattle (Hafez and Ludwig 1977). The sudden increase in alkaline phosphatases activity in uterine secretion during late luteal phase is probably due to the process of epithelial cell secretion. Since the clearance of secretion and the degenerative process is time consuming, the level continues to remain high during follicular phase. Therefore increased activities of alkaline phosphatases in uterine secretion are expected and can be an indicator of epithelial cell secretory and degenerative process. Our studies on the changes of gelatinases, matrix metalloproteinases (MMPs), and tissue inhibitor of metalloproteinases (TIMPs) further proved the

involvement of extensive remodeling of endometrial cells in buffaloes during estrous cycle and early pregnancy (Roy and Ghosh 2010). Evidences in cattle indicated that the secretory and necrotic activities of endometrial cells continue during maternal recognition of pregnancy (day 14) which are considered physiological (Munson *et al.* 1991). The endometrial secretory material undoubtedly serves a role and bathes the rapidly elongating conceptuses. It is expected that the high level of alkaline phosphatases would remain in the secretion even if animal is pregnant and the embryos continue to grow for maternal recognition of pregnancy therefore alkaline phosphatases would confer an important role during this period.

Our study indicated that the changes in phosphatases activities were not associated with the growth and regression of CL suggesting no direct control of CL on the uterine alkaline phosphatases secretion. It has been reported by Eltohamy *et al.* (1990) that buffalo vaginal mucus secretion contained significantly higher activities of phosphatases (alkaline and acid) and peroxidases during late-luteal phase as compared to the estrus. They concluded that the presence of these enzymes might reflect some important reproductive activities of buffaloes. Uterine tissues of sheep (Zamiri and Blackshaw 1979, Roy and Saigal 1987), cattle (Marinov and Lovell 1968) and buffaloes (Uppal and Roy 2000) had shown intense alkaline phosphatase staining in luteal phase endometrium as compared to follicular phase. The enzyme staining was reported to be confined in uterine glands and surface epithelium of endometrium in buffaloes (Uppal and Roy 2000) and sheep (Roy and Saigal 1987) indicating its source in uterine fluid. However none of the studies reported the availability of this enzyme in uterine luminal secretion.

The probable role of this enzyme would be dephosphorylation of the phosphoserine or phosphothreonine residues of protein as all other metallo-phosphatases does in a single reaction step using a metal-activated nucleophilic water molecule, by hydrolyase/transferase reactions on phosphate (Barford *et al.* 1998). The uterine secretory alkaline phosphatases found in this study cannot hydrolyse phosphate esters stored in the cell interior therefore would perform some function by access to some extra cellular phosphate esters. Uppal and Roy (2000) indicated that buffalo uterine tissue phosphatases might be involved in anaerobic glycolysis since alkaline phosphatases are known to promote glucose transfer for endometrial carbohydrate metabolism.

Since the period of increased alkaline phosphatases activity (late-luteal phase) in secretion coincided with the assumed period of maternal recognition of pregnancy in buffaloes. Evidences suggest that there are involvement of multiple pathways activation and deactivations in the process of luteolysis and antiluteolytic mechanism (Binelli *et al.* 2000) during this period. Phosphatases are known for its role in several metabolic pathways activation. Similarly existing evidences indicated that various kinases and phosphatases

are involved in switching the permeability properties of tight junction in varying epithelial cell mono layers (Sallee and Burridge, 2009). Since uterine epithelium also contain the tight junction it is likely that secretory phosphatases may play a role in that process too.

In conclusion, the changing milieu of individual uterine secretory proteins and characterization would throw some light in the events of uterine micro-environment particularly in buffaloes which suffer from many reproductive problems related to conception. The alkaline phosphatases identified in this study indicate some important role at the critical period of maternal recognition of pregnancy and in the process of endometrial control of luteolysis. More experiments on varying aspects of alkaline phosphatases function in uterine epithelium and trophoblast cells would throw some light on these issues which in turn would help in enhancement of reproductive efficiency in buffaloes.

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