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# Histochemical study of river buffalo's uterine endometrium in follicular and luteal phases

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## Summary

This study was conducted to evaluate histochemical alterations of river buffalo's uterine wall acid and alkaline phosphatases (ALP and ACP), lipase and carbohydrate ratio during follicular and luteal phases. Forty apparently healthy and non pregnant river buffalos were considered for removal of the uterus after sacrifice in slaughterhouse. All dissected uteri were divided into two luteal (by observing corpus luteum on the ovary) and follicular phase (by identifying growing follicles) specimens. In order to prepare frozen section slides, the fresh samples of uterus were transferred into chilled normal saline and cut with cryostat. ALP, ACP, lipase and periodic acid-schiff (PAS) staining techniques (paraffin sections) were conducted. Endometrial epithelium, glandular cells, vascular endothelium and macrophages in luteal phase showed positive reaction to both phosphatases. Comparing the luteal and follicular phases showed that, the mentioned cells presented significantly higher reaction to both phosphatases in luteal stage. The number of endometrial macrophages increased in luteal phase. The cytoplasmic carbohydrate ratio of endometrial epithelium and glands were significantly higher in follicular phase. Reactions to PAS staining revealed that, the glandular cells showed remarkably denser PAS stained cytoplasm in comparison to epithelial and endothelial cells. In luteal phase, uterus represented higher lipase ratio in comparison to follicular phase. In conclusion, similar to other bovine and buffalo species, in river buffalos the histochemical alterations in endometrial phosphatases, carbohydrates (mainly glycogen) and lipase depend on cyclic hormonal changes.

**Key words:** Histochemical study, River buffalo, Endometrium, Luteal and follicular phases

## Introduction

In all mammals, the endometrium of the uterine horns plays a critical role in normal fertility and also represents different features in order to adapt through various phases of oestrus cycle (Aitken, 1979). According to previous reports, the uterus not only has an influence on ovarian structure but also can participate in different physiological events by producing uterine milk. Therefore, it can provide an appropriate environment for sperms and/or it can accommodate a vital nutritional condition for blastocyst. Finally, the implantation process can be done completely (Bugalia and Sharma, 1990; Bugalia *et al*., 1991).

As is well known, the uterine horn of the mammals consists of three different layers including: endometrium, myometrium and perimetrium (Mescher, 2013). The endometrium is comprised of two functional and basic layers (Aitken, 1979). During estrus cycle, the functional layer of the endometrium undergoes specific molecular, enzymatic, morphological, and structural changes, which are under control of ovarian hormonal changes (Bugalia and Sharma, 1990; Eurell and Frappier, 2006). ALP and ACP are two different enzymes which show considerable alterations depending on the volume of luminal secretion (those secretions which are essential for embryo development) in luteal phase. Alkaline phosphatase (ALP) is known as a major sialoprotein and is mainly localized on the basal and apical cell membrane and cytoplasm of the epithelial and glandular epithelium (Fleming *et al*., 1995). This enzyme plays very important role in early phases of implantation (Leiser and Wille, 1975a; Lindhard *et al*., 2002). Especially in

luteal phase, the acid phosphatase (ACP) activities are responsible for hydrolysis of organic phosphoesterase (Leiser and Wille, 1975b). According to previous findings, in female genital tract the cytoplasmic carbohydrate and lipid accumulations differ depending on sexual hormones (estrogen and progesterone) alterations (Goding, 1972). However, there are limited studies on the biochemical changes in uterine stromal cells of river buffalos. Thus the current study was aimed to evaluate the cytoplasmic carbohydrate, lipase, acid and alkaline phosphatases alterations during two luteal and follicular phases.

## Materials and Methods

### Collecting specimens

To conduct the current study 40 mature female river buffaloes (RBs) (almost 4–6-year-old) were selected. All animals were non pregnant and apparently healthy. After scarifying, the genital tracts of the animals were sampled very carefully. All specimens were divided into two groups (20 specimens in each group), uteri from those animals that contained corpus luteum on their ovaries were marked as luteal phase specimens or group I and those with growing follicles on their ovaries were considered as follicular phase samples or group II. One half of all the samples were stored in ice deposit and the other half were fixed in 10% formal saline fixative solution and were transferred immediately to the laboratory.

### Histochemical study of carbohydrate

Specimens, which were fixed with 10% formal saline, underwent the paraffin embedding process. Paraffin embedded samples were cut with rotary microtome (Microm HM335E) semi serially (6-7 µm) and stained with periodic acid-shift (PAS) (Fouman Asia Shimi Co., Iran) to investigate the carbohydrate ratio in different endometrial stromal cells, glands, myometrium and perimetrium of uterus.

### Histochemical study of enzymes

The ice deposited samples that immediately transferred to laboratory were sectioned with cryostat microtome (Bright LTD. Huntingdon, England Co.) and the histochemical staining methods (Humson, 1979) including: lipase (LP), acid phosphatase (ACP) and alkaline phosphatase (ALP) staining techniques which were conducted on specimens in order to evaluate the mentioned enzymes ratio in epithelial cells, glands, endometrial stromal cells, myometrium and perimetrium.

The intensity of LP, ACP, ALP and PAS were scored (ELsayed *et al*., 2009) between zero to $3^+$ in the endometrial surface and glandular epithelium, endothelium and trunk of large (>500 µm), medium (100 µm >500 µm) and small (<100 µm) vessels, and the number of macrophages in 1 $mm^2$ with different scores was reported. Obtained data were analysed with paired samples t-test by SPSS software between two groups (luteal and follicular).

## Results

Endometrial and glandular epithelial cells were manifested with ALP stained cytoplasm in two groups. Meanwhile, the glandular cells in group I revealed much more numerous and denser stained cytoplasm in comparison to endometrial lining epithelium. The endometrial macrophages were revealed with dark ALP stained cytoplasm in luteal phase. The connective tissue adjacent to caruncular epithelium represented intensive ALP positive sites in luteal phase in comparison to follicular stage. The ALP reaction was the same in endothelial cells of vessels in both luteal and follicular phases. Although all large and/or medium size blood vessels were presented with ALP positive sites, the ALP reaction was manifested more distinctly in larger blood vessels trunk in comparison to smaller parenchymatous vessels (Figs. 1A, B, C and D). This enzyme was observed much scantier in the blood vessels of the follicular phase specimens. The data for macrophages and vessels ACP and ALP are presented in Figs. 2 and 3.

The endometrial epithelial cells showed dark-brown stained sites for ACP in the apical portion in both luteal and follicular phases. There were no significant differences in endometrial epithelium ACP

reaction during two luteal and follicular phases (Figs. 4A, B, C and D). Comparison between the ACP and ALP reaction in endometrial and glandular epithelium presented in Figs. 5A and B. Similar to endometrial epithelium, the endothelial cells of endometrial vessels were densely stained with ACP. In luteal phase samples, the macrophages in interstitial connective tissue beneath the endometrial epithelium and perivascular zone in caruncles were

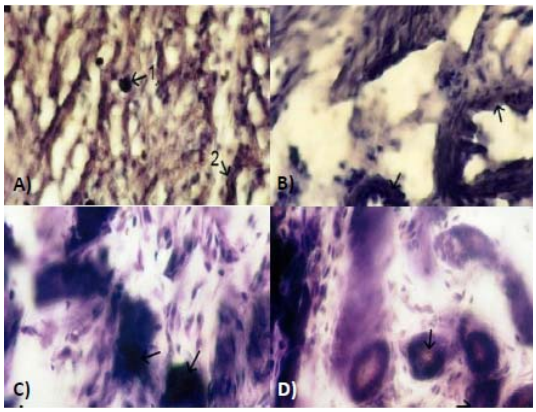


**Fig. 1: Cryosections from endometrium. (A) Note macrophage (1) and capillary epithelium (2) with dark reaction for alkaline phosphatase, (B) The macro-vessels wall are presented with intensive reaction for alkaline phosphatase, (C) Lipase has detected as dark granules in the glandular epithelium of uterus in luteal phase and (D) Note the faint reaction for lipase in follicular phase glandular epithelium, (A&B: Alkaline phosphatase staining and C&D: Lipase staining, ×40)**

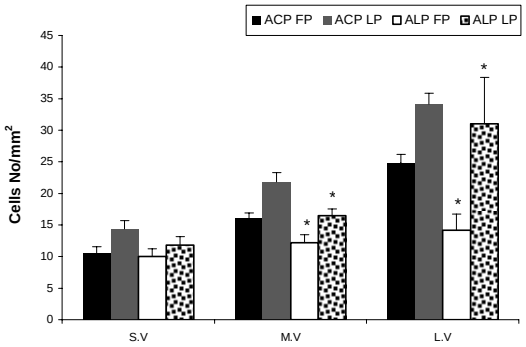


**Fig. 2: Mean average of the different size ACP and ALP positive blood vessels in luteal and follicular phases, there are significant differences (P≤0.05) between all data for ACP positive vessels in two luteal and follicular phases with each other. There are significant differences (P≤0.05) between data marked with stars in two different luteal and follicular phases. ACP: Acid phosphatase, ALP: Alkaline phosphatase, FP: Follicular phase, LP: Luteal phase, S.V: Small vessels, M.V: Medium size vessels, and L.V: Large size vessels**

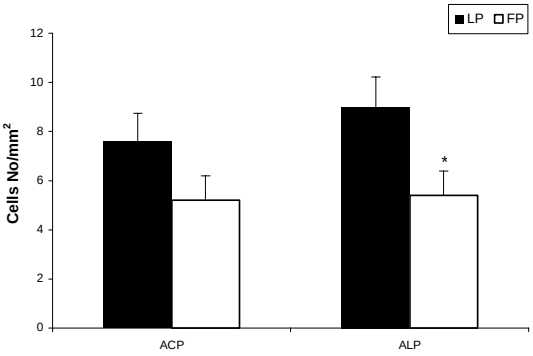


**Fig. 3: Mean average of the acid and alkaline phosphatase and lipid positive macrophages distribution per one mm$^2$ of the tissue in luteal and follicular phases. * Indicating significant difference (P≤0.05) between the number of acid phosphatase positive macrophages number in luteal phase with follicular stage. ACP: Acid phosphatase, ALP: Alkaline phosphatase, LP: Luteal phase, and FP: Follicular phase**

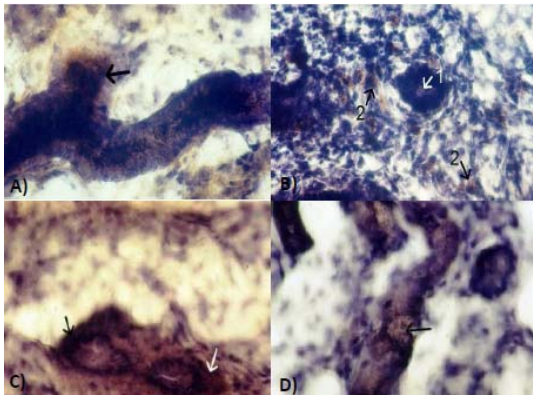


**Fig. 4: Cryosection from uterus. (A) Note uterus in luteal phase on which dark brown acid phosphatase positive granules are presented in glandular epithelium (↑), (B) Glandular epithelium presented with less intensely acid phosphatase positive granules (1) In follicular phase. Note macrophages with light brown granules (2) in Fig. B, (C) Glandular epithelium in luteal phase are presented with intensive alkaline phosphatase reaction and in Fig. (D) The glandular epithelium are manifested with lesser reaction for acid phosphatase in follicular phase, (A&B: Acid phosphatase staining and B&C: Alkaline phosphatase, ×40)**

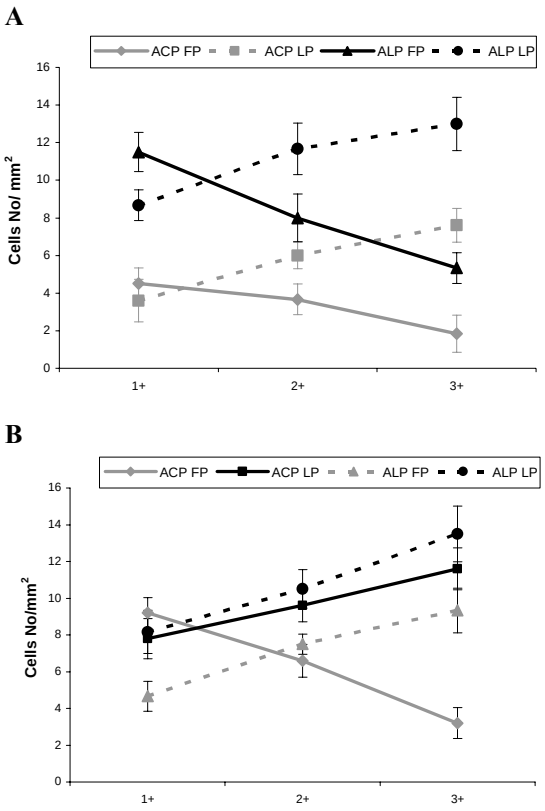


**Fig. 5: Mean distribution of ACP and ALP positive endometrial epithelium (A) and glandular epithelium (B) (in three $1^+$, $2^+$ and $3^+$ scores) per one $mm^2$ of the tissue in luteal and follicular phases. ACP: Acid phosphatase, ALP: Alkaline phosphatase, FP: Follicular phase, and LP: Luteal phase**

illustrated with positive reaction sites for ACP. In luteal phase the distribution of ACP stained macrophages was more in comparison to follicular stage, especially those macrophages located close to the endometrial glands, which exhibited more ACP reaction sites.

Endometrial epithelium was indicated by narrow red strip stained with PAS method in cytoplasm of two apical and basal portions of the cells. The PAS reaction of these cells was significantly higher in follicular phase in comparison to luteal. The active glandular cells, analysed in follicular phase were revealed with denser cytoplasmic PAS positive granules in comparison to the same cells from luteal phase (Figs. 6A, B and C). In the endometrial connective tissue, reticular fibers showed a positive response to PAS staining as very thin and scattered fibers. The fibers were distributed remarkably compact in luteal phase.

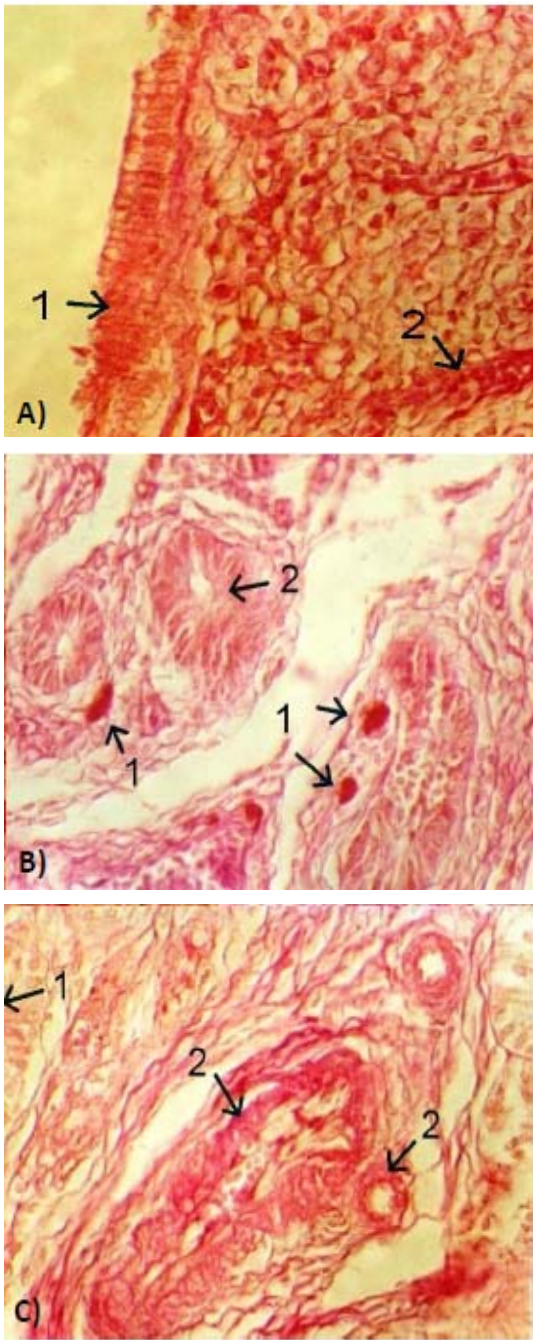


**Fig. 6: Paraffin section of endometrium. (A) Follicular phase endometrial epithelium (1) and capillary wall (2) are presented with PAS positive cytoplasm. (B) Note PAS positive macrophages (1) and PAS positive granules in glandular epithelium of endometrium in follicular phase, (C) See the faint reaction for PAS in glandular epithelium (1) and in capillary wall (2), (periodic acid-schiff (PAS) staining, ×40)**

The glandular cells and in some regions the epithelial cells were manifested with dense reacted sites for lipase staining in

luteal phase. Meanwhile the same cells in the follicular phase exhibited remarkably lower response to lipase staining. On the other hand, in luteal phase the number of cells with lipase positive sites was significantly higher in comparison to follicular phases.

## Discussion

This study showed that during different phases (follicular and luteal) endometrial epithelium and parenchyma, glandular epithelium, microvasculature trunk cells and macrophages show different patterns in chemical contents of their cytoplasm. According to previous reports during luteal phase the ACP and ALP are more active and mostly these enzymes are visible in the apical portion of the epithelial cells of the endometrium (Wordinger *et al*., 1972). Phosphatase discharging was enhanced by high progesterone level in luteal phase. Activity of this enzyme is responsible for hydrolysis of organic phosphatase (Stroband *et al*., 1986; Bugalia and Sharma, 1990). In a good accordance with the previous findings of these histochemical analyses of river buffalo's uterus showed that, cytoplasmic accumulation of ACP and ALP increased in endometrial epithelial cells in luteal phase. This situation suggests that during luteal phase the Golgi apparatus of the endometrial epithelium synthesizes ACP and ALP highly in order to inhibit and hydrolyze organic phosphomonesterase. Consequently, it plays a critical role during advanced luteal phase in order to support implantation (Leiser and Wille, 1975a).

Further to epithelial cells, the endometrial glandular epithelium was manifested with ACP and ALP positive sites in their cytoplasm in both groups, meanwhile the reaction intensified in luteal phase. Thus, can be conclude that the secretory activities of glandular cells seem to be much enhanced in luteal phase in comparison to follicular stage. Comparison of ACP and ALP density in endometrial surface epithelium with glandular cells revealed that, in river buffaloes the glandular cells are the main sources for these two enzymes discharging during luteal phase.

On the other hand, the number of macrophages increased in luteal phase and these cells were manifested with remarkably higher ALP and LP reaction sites in luteal phase in comparison to follicular stage. Recently it has become clear that cytokines are essential factors for insuring pregnancy by enhancing implantation success (Dimitriadis *et al*., 2005). Macrophages are the main source of cytokines in endometrium (Kummer *et al*., 1995). Therefore, according to the current findings it can be concluded that macrophages play a very important role in phosphatase and cytokines production in luteal phase. These cells increase in number and adapt to form enough phosphatase and cytokine to elevate successful implantation. On the other hand, macrophages have an essential role in normal and abnormal conditions by phagocyting abnormal endometrial cells and eliminating pathogens (Cobb and Watson, 1995; Athanasakis *et al*., 1999). Thus, sexual hormones changes in different phases significantly influence the activity and distribution of macrophages (Kaeoket *et al*., 2002).

These light microscopic observations demonstrated that, during luteal phase the endometrial epithelial cells exhibited low and the glandular cells higher amounts of cytoplasmic carbohydrate accumulation. In contrast to luteal stage in follicular phase, the higher number of epithelial cells was identified with PAS positive cytoplasm. There are several reports, which indicate that after LH surge, glycogen begins to accumulate within the cytoplasm, initially in a subnuclear area, but over time large aggregations are seen in the apical region of the cell by increasing progesterone (Murphy and Shaw, 1994). When progesterone surge increases, the glycoproteins are clearly present in the uterine secretions. Therefore PAS positive sites demonstrated in glandular and surface epithelial cells undoubtedly will contribute to these secretions. According to these hypotheses, it appears that similar to other mammals (Duncan *et al*., 1998; Murphy and Turner, 1991), in river buffalos glycogen and carbohydrate elements are very important for embryonic nutrition in the early stages of pregnancy and the majority of these elements are produced from uterine glandular cells, mainly in the

first trimester of pregnancy. Thus, identifying remarkably higher number of carbohydrate supplemented cells (especially glandular cells) in luteal phase is predictable.

The activity of lipase enzymes such as phospholipase A2 and phospholipase C (enzymes participating in prostaglandins synthesis) enhances during luteal phase (Smith, 1989; Bugalia *et al*., 1991). These alterations are expected to reduce the cytoplasmic storage of the lipase foci. On the other hand, there is a positive correlation between serum steroidogenesis and the lipase enzyme distribution on the ovary and in glandular cells of the endometrium as well (Christopher and Adam, 2010). According to previous studies, function of lipase largely depends on corpus luteum bioactivity (Shemesh *et al*., 1976). The current study confirmed these reports. Observations demonstrated that, in river buffalos the lipase synthesis significantly increased in luteal phase. Accordingly, a remarkably higher number of glandular and epithelial cells manifested with intensive reaction for lipase. Therefore, it can be concluded that, the cytoplasmic storage of lipase depends on cyclic alterations. Accordingly, in luteal phase the phospholipases ratio increases in order to participate in prostaglandins synthesis (Shemesh *et al*., 1976; Ahmed and Smith, 1992). Ultimately a significantly higher number of glandular cells exhibit LP positive cytoplasm in comparison to follicular stage.

In conclusion, the results of current study confirmed previous theories and findings about remarkable changes in various biochemical substances in endometrial compartments during different stages of estrous cycle. The histochemical analyses of uterine tissue in river buffalos showed that similar to other mammals, the cytoplasmic ALP, ACP, carbohydrates, and lipase largely change in both groups depending on steroid hormone alterations.

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