GROSS AND HISTOPATHOLOGICAL CHANGES/ AEROBIC BACTERIAL PLATE COUNT AND CYTOLOGICAL ALTERATIONS IN ACUTE, SUBACUTE AND CHRONIC ENDOMETRITIS OF BUFFALOES (*BUBALUS BUBALIS*)

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ABSTRACT

A total numbers of 100 adult buffaloes was selected for the study from Cantonment Board MHOW and Indore Slaughter Houses and screened for infection. The cytology, aerobic bacterial plate count, gross and histopathological changes of positive cases were studied to determine and differentiate acute, subacute and chronic endometritis. Examination of uterus of 100 buffaloes revealed inflammatory changes of endometritis in 78 (78%) cases and classified nine cases under acute endometritis (11.53%), 31 cases of subacute endometritis (39.74), 38 cases of chronic endometritis (48.71%) and 22 cases of non specific affections (28.20%). In most cases of acute endometritis, the uterus was thick, oedematous and high amount of exudate was present. Microscopically, sub endometrium was heavily infiltrated with multilobed inflammatory cells around uterine glands and blood vessels. Endometrial glands were proliferated and cystic at places with haemorrhage in different sites of stroma. MacCallum-Goodpasture staining revealed presence of coccobacillary gram negative bacteria in 2 cases. In subacute endometritis, the uterus was firm, thick and enlarged uterine horns revealed presence of moderate mucinous exudate, denudation, oedema, vascular wall hyalinization, infiltration of mainly lymphocytes. In chronic endometritis, the uterus was thick, firm, indurated and corrugated with severe infiltration of sub endometrium by lymphocytes, plasma cells and macrophages, connective tissue proliferation and vascular hyalinization. Cytology, aerobic bacterial plate count (APC) and histopathology revealed significant changes in types, number of cells and inflammatory reaction. In acute endometritis cytology showed a rise in epithelial cells, neutrophils and degenerated macrophages, low number of lymphocytes and other cells. Aerobic bacterial plate count revealed highest count. In subacute endometritis cytology showed a rise in lymphocytes and low numbers of epithelial cells, neutrophils, macrophages and a fall in other cells. Aerobic bacterial plate count revealed lowest count in subacute as compared to acute endometritis. In chronic endometritis cytology revealed mainly epithelial cell casts, isolated epithelial cells being few, lymphocytes, few macrophages and mucin threads.The research findings indicate that cases of endometritis are common in buffaloes and uterine cytology can be used as a quick sensitive and specific diagnostic tool to assess the status of uterine pathology both in dead and live animals.

Keywords: buffaloes, *Bubalus bubalis*, uterus, endometritis, cytology, APC, MHOW

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INTRODUCTION

Recently, India had emerged as one of the largest buffaloes milk producer in the world. In spite of the huge buffalo population, animal husbandry and dairy sectors do not provide greater percentage of total agricultural income as low productivity of buffaloes is considerably affected by the inherent problems like late maturity, poor oestrus expressiveness in the female particularly during summer, long post partum interval, diseases of genital system and infertility. Investigation on buffalo reproductive abnormalities (metritis) based on abattoir survey of specimens provides information on prevalence of reproductive disorders and their incidence. The annual incidences of uterine infections in postpartum cows range from 10 to 50% in dairy cattle (Lewis, 1997) and 20 to 75% in buffaloes (Usmani *et al*., 2001). Metritis and endometritis are most common problem in buffaloes affecting fertility and productivity.

Clinical metritis may be either acute, appearing quickly and generally affecting the buffalo's appetite and milk production, or chronic, persisting over a long period. Clinical metritis may be detected by rectal palpation as an increase in size and thickness of the uterine wall. A purulent (contains pus) vaginal discharge may or may not be present. Subclinical endometritis is not detectable by rectal palpation. More commonly it occurs in the chronic rather than the acute form. No vaginal discharge is evident. Sometimes examination with a speculum will reveal a purulent discharge, but not always. Cultures of the uterus may or may not verify a microbial infection (Azawi, 2010). Dohmen *et al*. (1995) stated that, clinical endometritis is characterized by the presence of a purulent (>50% pus) or mucopurulent (approximately 50% pus, 50% mucus) discharge detectable in the vagina after 26 days of postpartum. While subclinical endometritis can be defined as endometrial inflammation of the uterus usually determined by cytology in the absence of purulent material in the vagina.

Controversy exists over the effects of metritis and endometritis on fertility in cattle. Metritis and endometritis usually occur between resolution of pituitary sensitivity to GnRH and the first postpartum ovulation. Uterine PGF2α and leukotriene B4 production decrease to basal within a few days after oestrus, when progesterone concentrations begin to increase, and the uterus again becomes susceptible to infections. Prostaglandin F enhances neutrophil chemotaxis and the ability of neutrophils to ingest bacteria and leukotriene B4 enhanced chemotaxis, random migration and antibody independent cell mediated cytotoxicity (Hoedemaker *et al.*, 1992). Postovulatory infections arise during the time between the first ovulation and complete uterine involution. Diseases of the postovulatory period include chronic metritis, endometritis and pyometra. Bacteria, viruses, fungi and protozoa have been cultured from uteri when metritis has been present. Bacterial organisms that cause endometritis are *Streptococci, Staphylococci, Brucella abortus, Corynebacterium pyogenes, Pseudomonase aeruginosa*. Infection in the uterus may result from an infection elsewhere in the body such as infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD) which then spreads to the uterus (systemic infections) (Roberts, 1986).

MATERIALS AND METHODS

The study was conducted in the Department of Pathology, College of Veterinary Science and Animal Husbandry, MHOW, State

Madhya Pradesh, India. A total numbers of 100 adult buffaloes was selected for the study from Cantonment Board MHOW slaughter house and screened for infection.

Organ collection

Immediately after slaughter, the entire uterus in its anatomical continuity (cervix, body of the uterus, right horn, left horn, fallopian tube and overies along with broad ligament) was examined in situ for the gross abnormalities, if any. After evisceration of the cadavers, the uterus as a whole was collected, brought to the laboratory and spread out in the normal position, for a careful examination of pathoanatomical abnormalities, wherever present.

Uterine swab collection and aerobic bacterial plate count of samples

According to the degree of endometritis acute, subacute and chronic, the uterine fluid was collected aseptically from both uterine horns for aerobic bacterial plate count (APC) investigation. For enumeration purpose ten-fold serial dilutions of each sample was prepared in sterile NSS up to 10^{-6}. Subsequently 0.1 ml of each dilution was aseptically transferred with the help of a sterile pipette on a well sterile media plate. The inoculums were then be spread on the surface of the media plates using a sterile spreader. For each dilution different sets of sterile pipettes and spreaders were used. The inoculated plates were kept at room temperature for 30 minutes to allow the inoculums to be absorbed and then incubated at 37^{o}C for 24 h. The bacterial colonies were counted using digital colony counter. For calculation of colony forming units, the plates with 30 to 300 colonies were considered and the counts were further multiplied with the reciprocal of dilution factor (ICMSF 1978). The result was expressed in cfu /gm or cfu / ml of samples (Collee, 1996).

Cytology

Cytology, performed by smears prepared from the uterine fluid, fixed with absolute methyl alcohol and stained by Wright-Giemsa staining and Modified Giemsa staining method to detect acute endometritis, subacute endometritis, chronic endometritis, was diagnostic five microscopic fields at 400X was done to quantitate the degree of inflammation (Couto and Hughes, 1985). Modified Giemsa stain for rapid staining was done by the standared method (Benjamin, 2007). Lactophenol cotton blue staining of impression smear was also done to rule out fungal infections (Lillie, 1976).

Histopathology

After recording the gross lesions the uterine tissue was collected from slaughtered buffaloes and subsequently preserved in 10% neutral buffered formalin for 48 h. Further these tissues were processed by routine method of dehydration in graded acetone, clearing in benzene and embedding in paraffin. Sections of 4 to 5 micron thickness were processed by standard procedures using routine Haematoxyline and Eosin, Verhoeff-Van Gieson stain for collagen (Singh and Sulochana, 1996) and elastic fibers and by MacCallum Goodpasture staining for bacteria in tissues (Sharma *et al*., 2004) for histopathological studies.

RESULTS AND DISCUSSION

Cytology

Cytological examination of nine endometritis cases revealed more number of neutrophils in addition to epithelial cells, several

RBC's and degenerated macrophages indicative of acute endometritis. Similar observations were made by Samantha *et al.* (2013); Oruc *et al.* (2015). Cytological examination of 31 endometritis cases revealed more number of lymphocytes and few neutrophils indicative of subacute endometritis in accordance with findings of Chapwanya *et al.* (2009); Samantha *et al.* (2013); Oruc *et al.* (2015). Endometrial cytology of 38 endometritis cases revealed lymphocytes and few plasma cells indicating chronic endometritis. Similar observations were made by Samantha *et al.* (2013); Oruc *et al*. (2015).

A total of five high-power microscopic fields was counted from each positive case. When the number of cells in the sample was low then an assessment of the ratio of cell type was noted. If there were more than one inflammatory cell per 40 endometrial cells, the sample was classified as inflammed.

Epithelial cells

The mean values of epithelial cells in acute endometritis (40.0±0.44) and non specific affections (39.2±1.25) were higher (P≤0.05) as compared to subacute (7.87±0.61) and chronic endometritis (9.21±0.70) (Table 1).

Neutrophils

The mean values of neutrophil in acute endometritis (9.88±0.30) and subacute endometritis (3.96±0.15) were higher (P≤0.05) as compared to chronic endometritis (0.42±0.08) and non specific affections (0.31±0.10) (Table 1).

Lymphocytes/plasma cells

The mean values of lymphocyte\plasma cells in subacute endometritis (11.90±0.45) were much higher (p≤0.05) as compared to chronic (4.92±0.25), acute endometritis (1.55±0.17) and non specific affections (0.27±0.9) (Table 01).

Table 1. Mean± SE of Cytology in Acute endometritis, Subacute endometritis , Chronic endometritis and Non specific affections in the uterus of buffaloes.

Condition	Epithelial cells	Neutrophils	Lymphocytes	Macrophages	Other inflammatory cells**
Acute endometritis	40.0^{a}±0.44	9.88^{a}±0.30	1.55^{c}±0.17	3.33^{a}±0.16*	1.00±0.23
Subacute endometritis	7.87^{b}±0.61	3.96^{b}±0.15	11.90^{a}±0.45	1.00^{c}±0.16	0.42±0.08
Chronic endometritis	9.21^{b}±0.70	0.42^{c}±0.08	4.92^{b}±0.25	0.13^{c}±0.07	0.70±0.09
Non specific affections	39.2^{a}±1.25	0.31^{c}±0.10	0.27^{d}±0.9	0.81^{c}±0.09	0.09±0.06

Values bearing similar superscripts do not differ significantly (P≤0.05)

*Degenerated macrophages

**Other inflammatory cells including few Eosinophils and Basophils.

Macrophages

The mean values of macrophages in acute endometritis (3.33±0.61) were higher (P≤0.05) as compared to subacute (1.00±0.16), chronic endometritis (0.31±0.07) and non specific affections (0.25±0.09). Several degenerated macrophages were also visible in each field along with many epithelial casts in all cases of chronic endometritis (Table 1, Figure 1, 2 and 3).

Other inflammatory cells

Endometrial cytological smears in nine cases revealed more number of neutrophils along with epithelial cells and other inflammatory cells (eosinophils,basophils) (1.00±0.23) indicating acute endometritis. Lymphocytes and few polymorphonuclear cells were observed in addition to epithelial cells and other inflammatory cells (0.42±0.08) in 31 samples indicating sub acute endometritis cases. 38 endometrial cytology cases revealed lymphocytes, few plasma cells in addition to epithelial cells, mucin strands and other inflammatory cells (0.70±0.09) indicating chronic endometritis. The presence of lymphocytes, macrophages, or plasmatocytes usually indicates that a chronic problem is present, although macrophages may occasionally be present when an acute process is regressing. The polymorphonuclear leucocytes migration to the uterine lumen is one of the first responses of immune system to endometritis, Therefore, determination of PMNs proportion to the other cells (plasma cells, neutrophils and endometrial cells) in uterus helps evalution of endometritis existence and severity in dairy cows and buffaloes. Usually endometrial and inflammatory cells are classified into either fresh or degenerative states. Signs of degeneration would include changes in the appearance of the cell membrane, hypersegmentation of the nucleus, vacuole formation, droplets or inclusions, increased cytoplasmic staining, and cell swelling (Figure 1 and 2). In the normal non specific affections, the sample had normal, healthy endometrial cells with very rare neutrophils. In the inflammatory category, the number of inflammatory cells exceeds defined limits as stated above. The inflammatory category is usually subdivided into acute and chronic, depending on the cell types present. If degenerated cells are present, this may indicate that a chronic process is occurring or that an acute process is regressing. Presence of epithelial casts in chronic cases was indicative of a slow regenerative process of cell cycle and excessive denudation of uterine epithelium (Dascanio *et al*., 1997). Cytological smears with lacto phenol cotton blue staining were found negative. Hence, fungal growth was ruled out.

BACTERIOLOGY

The mean values of aerobic bacterial plate count of acute (276.0±0.97X10^6), subacute endometritis (160.0±2.30X10^6) and non specific affections (148.0±1.27X10^6) were significantly high as compared to chronic endometritis (88.56±2.3X10^6) (Table 2). Staining biopsies from animals harbouring gram negative cocco bacilli in their uterus indicated by standard bacteriological procedures, and using modified gram stain for histological examination revealed massive infection and colonization of a possible *E. coli* infection in the subendometrial region of the uterine tissue.

Similarly, Azawi *et al*., 2008 Identified bacteria using API systems following aerobic and anaerobic cultures, and the bacterial density was scored semi quantitatively. The common predisposing factor for uterine infection was

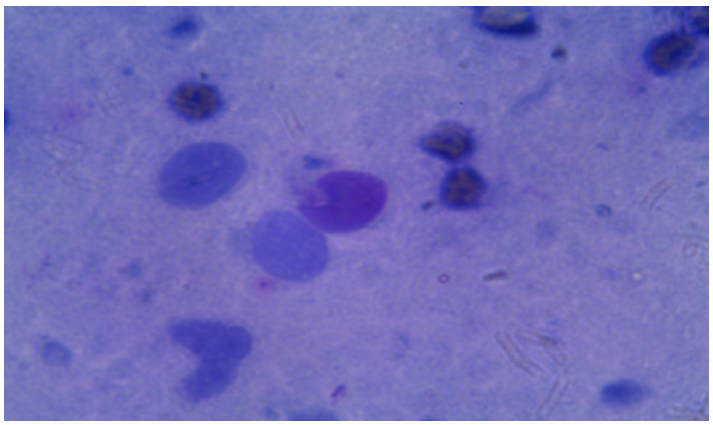

Figure 1. Uterine fluid cytology in acute endometritis showing degenerated macrophages with vacuoles formation, droplets, increased cellular swelling (Modified Giemsa stain100x).

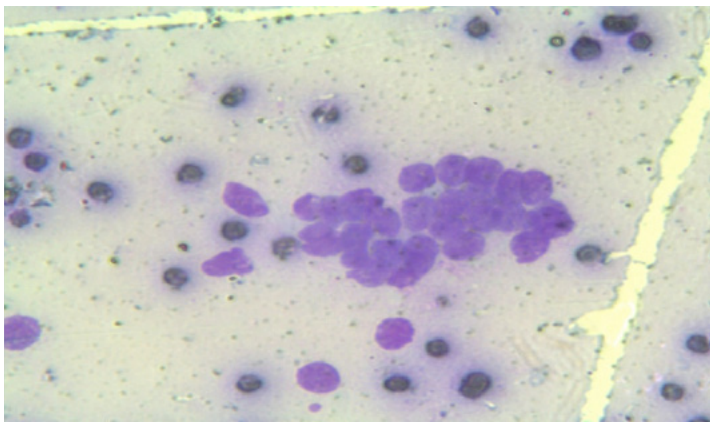

Figure 2. Uterine fluid cytology in acute endometritis showing presence of degenerated macrophage with intranuclear vacuoles formation and increased cellular swelling (Modified Giemsa stain 100x).

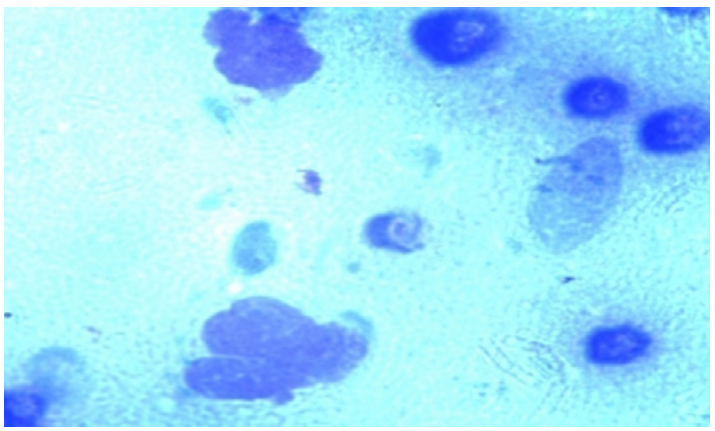

Figure 3. Uterine fluid cytology in chronic endometritis showing a clump of epithelial cells as cast (Modified Giemsa stain 40x).

retained placenta. Concurrently, El-Azab *et al*., 1988 reported that Intra-uterine oxygen reductase potential fell in the presence of infection, and mostly with aerobic bacteria, thereby creating an anaerobic environment. This drop in intrauterine oxygen reductase potential may be associated with either micro-organism metabolism or increased oxygen consumption by polymorphonuclear inflammatory cells. DelVecchio (1994), reported that 64% of the infected dairy cows harbored *A. pyogenes* usually in combination with *E. coli*. Further isolation of organisms was not followed in the present study in buffaloes.

GROSS EXAMINATION AND HISTOPATHOLOGY LESIONS

Acute endometritis was observed in nine buffaloes with moderately thick and oedematous. On cutting, the horns revealed pinkish to reddish sticky exudates, covering the endometrium. The cotyledons appeared enlarged and the mucosa was congested. Os cervix was open. The presence of multiple persistant follicular cysts indicated a possible association with hormonal imbalance as also hypothesized by Hoedemaker *et al*. (1992); Azawi *et al*. (2010); Dwivedi and Singh (1975); Heranjal and Rao (1980), who suggested progesterone deficiency as the etiology factor. The increased production of mineralocorticoide by zone glomerulosa of adrenal can also be one of the factors responsible for the development of follicular cysts (Sharma, 2004). Two cases revealed mucopurulent exudate in horns, uterine body and terminal part of cervix. Os cervix was closed. Right ovary revealed presence of large persistant corpus luteum and perimetrial cyst in one case. The continuous production of progesterone from such ovaries might lead to cystic glandular hyperplasia, hyperaemia, pyogenic bacterial infections and enhance the anoestrous condition in the animals (Roberts, 1986). Microscopically, the epithelial lining was desquamated at few places. Sub endometrium was heavily infiltrated with multilobed inflammatory cells around uterine glands and blood vessels. Endometrial glands were proliferated and cystic at places. There was hyperemia of blood vessels, thrombus formation with haemorrhage in different sites of stroma almost similar to the finding of Mittal *et al.* (2013); Azawi *et al.* (2013); Babu *et al.* (2013) (Figure 4). MacCallum-Goodpasture staining revealed presence of coccobacillary gram negative bacteria

Table 2. Mean±SE of Aerobic bacterial plate count in Acute endometritis, Subacute endometritis, Chronic endometritis and Non specific affections in the uterus of buffaloes.

Condition	Aerobic bacterial plate count
Acute endometritis	$276.0^{a}\pm0.97X10^{6}$
Subacute endometritis	$160.0^{b}\pm2.30\ X10^{6}$
Chronic endometritis	$88.56^{d}\pm2.3\ X10^{6}$
Non specific affections	$148.0^{c}\pm1.27X10^{6}$

Values bearing similar superscripts do not differ significantly ($P\leq0.05$).

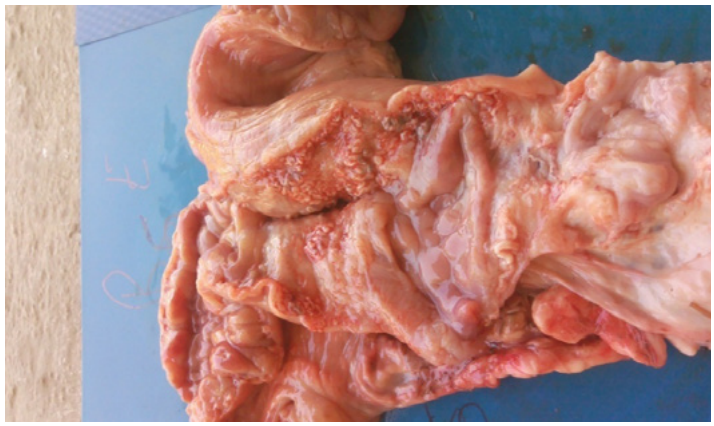

Figure 4. Microphotograph of uterus in acute endometritis showing infiltration of neutrophils in uterine stroma (H and E 40X).

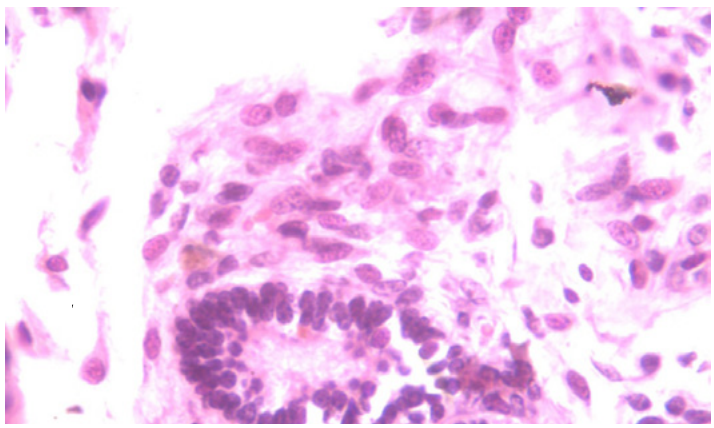

Figure 5. Chronic endometritis: Uterus showing excessive thickening of mucosa, cystic granular, glandular proliferation (arrow), hyperaemia and haemorrhage.

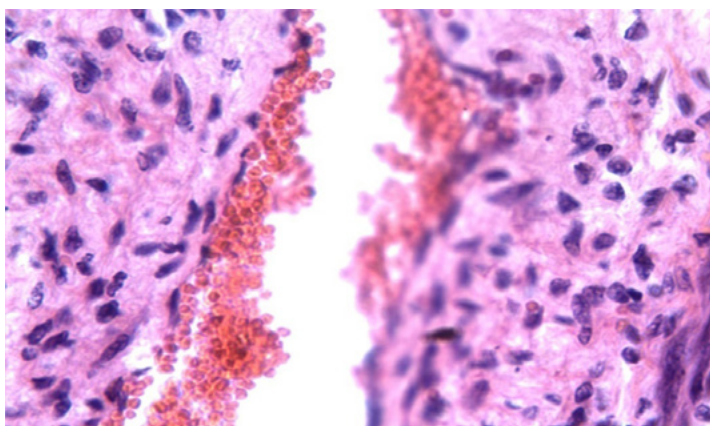

Figure 6. Microphotograph of uterus in chronic endometritis showing excessive infiltration of monocytoid cells (arrow) and few RBCs around cystic uterine glands (H and E 40x).

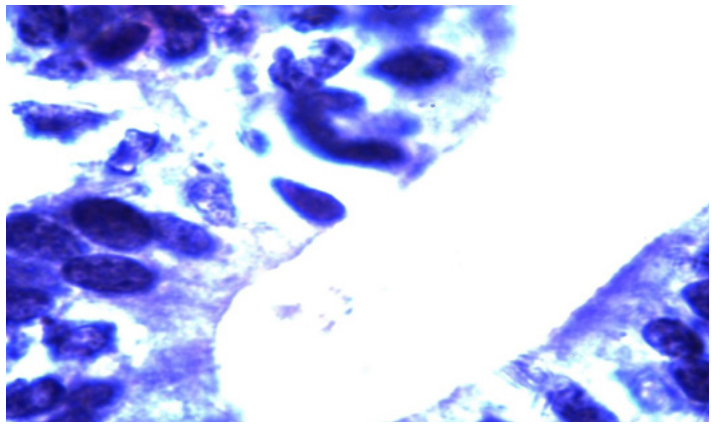

Figure 7. Microphotography of uterus in chronic endometritis showing enlarged cystic uterine gland (H and E 100X).

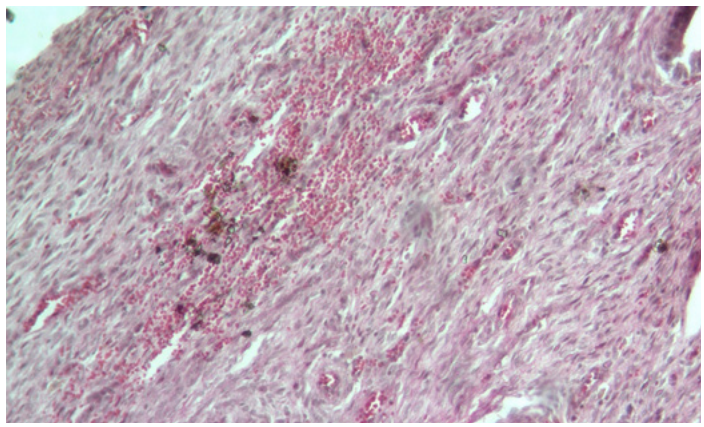

Figure 8. Microphotograph of chronic endometritis showing submucosal haemorrhage and deposition of hemosiderin pigment (arrow) (Verhoff-Van Gieson stain 10x).

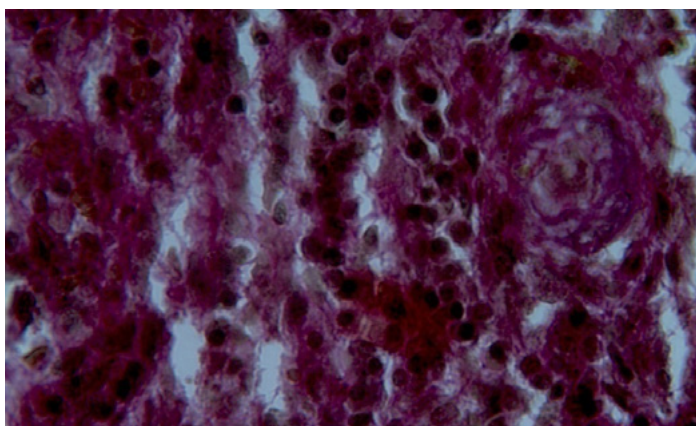

Figure 9. Microphotograph of chronic endometritis showing atrophy of endometrial gland with periglandular lymphocytic infiltration and fibrosis (Verhoff-Van Gieson stain 40x).

in 2 cases. This finding corroborated with the findings of bacteriology and APC.

Subacute endometritis was observed in 31 buffaloes with firm, thick and enlarged uterine horns with presence of moderate mucinous exudate inside. Histopathologically there was mild epithelium denudation, oedema, vascular wall hyalinization, infiltration of mainly lymphocytes. Similarly, Azawi (2010) reported that the microscopic changes of mild endometritis were not striking and consisted of a diffuse, light infiltration of inflammatory cells with slight desquamation and no significant vascular changes. In contrast, Babu *et al.* (2013) reported extensive denudation of the luminal epithelium, stromal oedema and mild infiltrations of neutrophils, lymphocytes and few macrophages in the stratum compactum and stratum spongiosum, vascular hyalinisation, and perivascular oedema in murrah graded buffloes. Chronic endometritis was observed in 38 buffaloes with thick, firm, indurated and corrugated uterus (Figure 5). In chronic endometritis, the lesions were characterized by extensive desquamation of luminal epithelium, severe infiltration by lymphocytes, plasma cells and macrophages, connective tissue proliferation and vascular hyalinization, which were similar to Mittal *et al.* (2013); Babu *et al.* (2013) (Figure 6, 7, 8 and 9).

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