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PATHOLOGICAL STUDIES ON SPONTANEOUS URINARY BLADDER LESIONS IN INDIAN BUFFALOES

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ABSTRACT

The present investigation describes the spontaneous pathological lesions in urinary bladders (n = 177) of slaughtered buffaloes collected from Northern India. Gross examination revealed congestion, haemorrhages, oedematous changes and thickening of mucosa. Microscopically, epithelial abnormalities, inflammatory and neoplastic conditions were noticed. Epithelial abnormalities included epithelial hyperplasia, von Brunn's nests, cystitis cystica and cystitis glandularis. Inflammatory conditions such as congestion, haemorrhages and cystitis were noticed. Cystitis was classified as lymphocytic, plasmo-lymphocytic, follicular and interstitial types. However, only single case of haemangiosarcoma was observed. Polymerase chain reaction revealed the presence of DNA of only BPV-1 in one sample out of 21, while one sample showed the presence of DNA of both BPV-1 and BPV-2. On immunohistochemistry, Ki-67 expression was observed only in haemangiosarcoma and one case of plasmo-lymphocytic cystitis with immunostaining restricted to nuclei. COX-2 immunostaining was observed in one case each of flat hyperplasia, von Brunn's nest, haemangiosarcoma and two cases of plasmo-lymphocytic cystitis. Among epithelial abnormalities, COX-2 immunostaining was restricted to epithelial component of the lesions, and it was diffused cytoplasmic type with occasional perinuclear staining particularly in the cytoplasmic membrane. However, in haemangiosarcoma the COX-2 immunoreactivity was noticed in epithelial as well as connective tissue stroma. In conclusion, it may be stated that urinary bladders of slaughtered buffaloes showed a number of epithelial abnormalities, inflammatory conditions and occasionally neoplasms. Further studies to explore the infectious as well as non-infectious etiologies should be undertaken.

Keywords: *Bubalus bubalis*, buffaloes, epithelial abnormalities, pathology, spontaneous, urinary bladder

INTRODUCTION

In Indian rural economy, livestock sector plays a significant role. Among livestock, buffalo is the premier animal in dairy industry and to

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farmers like a Bank ATM for drawing cash in emergency. It is popularly known as an 'Asian Animal or black gold' in the recent years. Out of total milch population of animals in India, buffaloes share only one third, however, it contributes to more than 50% of India's total milk production. Apart from this, it is also reared for meat, draught purpose, and manure (Banerjee, 1998). Water buffaloes are susceptible to the most of the diseases and parasitic infections that affects cattle but the effects of these conditions on it and its productivity is less evident (Fagiolo *al*., 2005). The systematic investigation on non-infectious and systemic pathological conditions of urinary bladder pathology in buffaloes is either negligible or (Somvanshi *et al*., 2012). As compared to other animal species, pathological abnormalities in urinary tract of buffaloes are less well understood. The reason for this may be due to fewer reported incidences of clinical manifestations of urinary system related affections in buffaloes. Cystitis, the inflammation of urinary bladder has a direct effect on its function. It is caused by infectious as well as non-infectious etiologies. *Escherichia coli* represent the most common cause of infectious cystitis (Radostits *et al*., 2007). Bovine papilloma viruses (BPVs) are responsible for causing lesions and tumours in urinary bladders of cattle and buffaloes (Pathania *et al*., 2012; Kumar *et al*., 2014; Sreelekshmy *et al*., 2017).

Several markers are employed in pathological investigations to evaluate cell growth and proliferation. A nuclear non-histone protein, Ki-67 is typically expressed in G1, G2, M, and S stages of the cell-cycle. It is an excellent marker for the determination of growth fraction (Scholzen and Gerdes, 2000). The cyclooxygenase (COX) is a key enzyme in prostaglandin synthesis. There are three isoforms of COX *viz*., COX-1, COX-2 and COX-3. Of these, COX-1 is normally expressed in a wide variety of cells and tissues. An inducible enzyme, COX-2, is mostly expressed during the pathological alterations, particularly during inflammatory conditions and the development of cancer (Dore, 2011).

Several researchers throughout the world have conducted abattoir studies of urinary bladder affections, mainly in cattle, but also to a lesser extent in buffaloes. (Mckenzie, 1978; Herenda *et al*., 1990; Somvanshi *et al*., 2012). The urinary bladder disorders have received much less attention in buffaloes and cattle in comparison to other animal species. Still there is a lack of knowledge in this field and paucity of literature in urinary tract pathological lesions especially in the urinary bladder of buffaloes than other species. Therefore, keeping above in the view; the present study was carried out with an objective to describe the pathomorphological lesions in the urinary bladder of buffaloes of Northern India.

MATERIALS AND METHODS

Collection of samples

Urinary bladder samples (n=177) of buffaloes were collected from different places. These included 85 samples from Buffalo Slaughterhouse, Bareilly, Uttar Pradesh (UP), 89 from Buffalo Slaughterhouse, Haldwani, Uttarakhand, 2 from Post-Mortem Facility of Division of Pathology and 1 from Referral Veterinary Polyclinic of ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, UP. After thorough gross examination, representative tissue samples for histopathology and immunohistochemistry were collected and preserved in 10% neutral buffered formalin. A small part of the tissue samples from urinary bladders

showing macroscopic lesions was collected and kept in sterile vials at -20°C for polymerase chain reaction (PCR).

Gross and histopathological examination

Systematic opening and detailed observations of urinary bladders was carried out to explore the presence of gross lesions and any abnormal growth. The processing of tissues for histopathology was carried out as per the conventional method and stained with haematoxylin and eosin (Luna, 1968).

DNA extraction, Primers and PCR

Extraction of DNA was carried out from tissues of 21 selected samples with help of DNeasy Blood and Tissue Kit (Qiagen) as per the instructions of manufacturer. Then, PCR assay was performed with the specific primers for amplification of the DNA template specific to L1 genes of BPV-1, BPV-2, BPV-5 and BPV-10. The primer pairs for BPV-1 and BPV-2 were used as per earlier workers (Yaguiu *et al*., 2006). The primer pairs: BPV-5 (forward 5'-actggctctaccaagctcaagg-3', reverse 5'-gacagaagggttaacggtctgca-3') and BPV-10 (forward 5'-tgcattcaataggcttgcagatgc-3', reverse 5'-cacctcgagaccacaaatgc-3') were used. Using previously available sequences, BPV-5 and BPV-10 primers were designed by using primer designing software (DNASTAR software, DNASTAR, Madison, WI, USA). These primer pairs were targeted to amplify the viral DNA segments of L1 gene of sizes 301, 164, 266 and 422 bp of BPV-1, BPV-2, BPV-5, and BPV-10, respectively. The cycling conditions for PCR included an initial denaturation step of 3 minutes (min) at 94°C followed by the amplification of DNA for 35 cycles of denaturation at 94°C for 40 seconds (sec), annealing at 52°C (BPV-1 and BPV-2), 62°C (BPV-5) and 60°C (BPV-10) for 40 seconds, and an extension at 72°C for 50 seconds and then a final extension step at 72°C for 10 minutes. Then PCR products were analyzed by gel electrophoresis by visualizing the ethidium bromide stained 1.5% agarose under Gel documentation system (Geldoc; Alpha Tech, San Leandro, CA, USA).

Immunohistochemistry

Tissue sections of 4 µm thickness were mounted on 3-aminopropyl-triethoxysilane coated glass slides (Sigma Chemicals, Sigma, USA). Sections were deparaffinized in xylene and then rehydrated by dipping them into descending grades of ethanol. Antigen retrieval was achieved by heating in microwave in citrate buffer (0.01 M, pH 6.0), twice for 5 minutes each. The sections were immersed in 3% hydrogen peroxide prepared in methanol for 30 minutes to quench the activity of endogenous peroxidase. The non-specific sites for Ki-67 and COX-2 expression were blocked by incubating the sections with 5% normal goat serum (Sigma Chemicals) and 1.5% donkey serum (Santa Cruz Biotech), respectively in phosphate buffered saline (PBS) for 30 minutes. Thereafter, sections were covered with primary antibodies (mouse monoclonal anti-Ki67, 1:50, mAb PP-67, P6834, Sigma Chemicals, USA; goat polyclonal anti-COX-2 IgG antibody 1:50, SC-1746; Santa Cruz Biotech;) diluted in 1% bovine serum albumin prepared in PBS for overnight at 4°C. This was followed by incubating with secondary antibody and peroxidase as per instructions of manufacturer (Sigma for Ki-67 and Santa Cruz Biotech for COX-2). Between treatments, the sections were rinsed with PBS thrice for 5 minutes each. The sections were then stained with 3-amino-9-ethyl-carbazole (AEC; Sigma Chemicals) for 3 to 10 min and reaction was stopped by dipping sections

in distilled water. Then sections were counter stained lightly with Mayer's haematoxylin (MHS-16; Sigma). Thereafter, the sections were rinsed with the running tap water for 5 minutes and finally mounted with glycerol gelatin. According to the procedure outlined by Woods *et al*. (1991) and Aaltomaa *et al*. (1993) scoring of the Ki-67 immunopositive cells was performed. The staining intensity of cells was classified semi-quantitatively. COX-2 staining distribution score was determined by estimating the percentage of positive stained cells in 5 to 10 high power fields (40x objective) objective as per Dore *et al*. (2003). Percentage of immunopositive cells was determined semi-quantitatively by assessing the entire section. Staining intensity of COX-2 was defined as the strength of signal in immunopositive cells, with no signal (-), weak signal (+), moderate signal (++) and strong signal (+++).

RESULTS AND DISCUSSIONS

Gross and histopathological findings

The detailed account of pathological lesions observed in urinary bladders is presented in Table 1. The gross examination revealed congestion (Figure 1A), haemorrhages, oedematous changes and thickening of mucosa. Haemorrhages were either petechial, ecchymotic (Figure 1B) or extensive. Mucosa of one urinary bladder revealed blackish appearance due to extensive haemorrhages (Figure 1C), while the mucosa of another bladder showed tumourous growth (Figure 1D). A total of 57 bladders failed to show any gross pathological changes.

Histopathologically, the lesions were classified into different categories *viz*. epithelial abnormalities, inflammatory and neoplastic conditions, and nothing abnormally diagnosed or normal. The representative histopathological observations are depicted in Figure 2. Epithelial abnormalities were classified as epithelial hyperplasia, von Brunn's nests, cystitis cystica and cystitis glandularis. Microscopically, the bladder sections which showed increased number of layers of transitional cells, either focal or diffused were considered as hyperplasia and these were further classified as flat epithelial, papillary, or nodular type. In flat epithelial hyperplasia, urothelium showed marked thickening in number of transitional cell layers, usually 8 or more. In papillary urothelial type, the hyperplastic urothelium was arranged in undulating mucosal folds and papillary-like appearances (Figure 2A) whereas, the nodular hyperplasia was characterized by markedly thickened urothelium with downward and generalized increase in number of transitional epithelial layers (Figure 2B). Von Brunn's nests were compact and characterized by the development of round urothelial cell aggregates in the lamina propria, either connected to the surface epithelium or not (Figure 2C). Cystitis cystica and cystitis glandularis were characterized by thickened urothelium and glandular hyperplasia forming acini-like structures. Hyperplasia revealed nodular downward growths of transitional cells which extended into lamina propria. It also showed adenomatous gland-like structures which had slightly thickened epithelium extended into the lamina propria. Few cysts-like structures with presence of pinkish fluid in the lumina were noticed.

The inflammatory lesions included varying degrees of congestion, haemorrhages, and cystitis. Mild perivascular infiltration of mononuclear cells (MNCs) was also noticed but the degree of the vascular changes was more intense than cellular

changes. Moderate to extensive haemorrhages were noticed near the base of urothelium and lamina propria (Figure 2D). In some cases, haemorrhages were also observed in the muscularis layer. Oedematous changes were also evident. Different types of cystitis were noticed and classified as lymphocytic, follicular, plasmo-lymphocytic, and interstitial types. Lymphocytic cystitis was characterized by denudation of urothelium with diffuse infiltration of MNCs predominantly lymphocytes in lamina propria and extending towards muscularis layer (Figure 2E). Mostly, perivascular lymphocytic infiltration was observed. Follicular cystitis showed multiple, prominent large sized lymphoid follicles with germinal centres in lamina propria, and a few lymphoid follicles showed distinct compartmentalization with fibrous connective tissue (Figure 2F). Plasmo-lymphocytic cystitis revealed diffused infiltration of mainly plasma cells and a few lymphocytes in the lamina propria (Figure 2G). Interstitial cystitis revealed dilated and engorged blood capillaries, focal and/or diffused infiltration of MNCs in the muscular layer.

One case of haemangiosarcoma was diagnosed and it was characterized by presence of pleomorphic neoplastic cells varying from spindle shaped to polygonal or ovoid with numerous delicate small blood vessels filled with erythrocytes at many places along with the fibrous connective tissue proliferation (Figure 2H). In many cases, mucosa was lined with several layers of urothelial cells, which had distinctive polarity with palisading arrangement of basal cells. Lamina propria, which lies beneath the urothelium, revealed an interrupted layer of smooth muscles and the muscularis mucosa was at top of the thick bundles of smooth muscles. These were categorized as nothing abnormally diagnosed or normal cases.

These findings were in corroboration with earlier studies on non-neoplastic conditions in bladder in 131 adult cattle and water buffaloes in the Black Sea region of Turkey. This region was known to encompass the bracken fern (Aydin and Ozkul, 1995). In addition, a number of earlier workers from India also described similar histopathological conditions in urinary bladders from enzootic and non-enzootic areas (Pangty, 2009; Nagarajan, 2011; Pathania *et al*., 2012; Somvanshi *et al*., 2012; Sreelekshmy *et al*., 2017). Authors opined that the pathology of buffalo urinary bladders should be studied and compared in both enzootic and non-enzootic areas of different parts of country which may help in understanding the development of the lesions and neoplastic conditions. The almost negligible occurrence of tumour in present study was in agreement with the earlier workers (Ozkul and Aydin, 1996; Carvalho *et al*., 2009; Deshmukh *et al*., 2010; Kumar *et al*., 2014). The endothelial derived bladder tumours *viz*. haemangiomas, haemangio-endothelioma and haemangiosarcoma in cattle have been described earlier (Carvalho *et al*., 2009). The urinary bladder tumours of cattle (Carvalho *et al*., 2009), cattle and buffaloes (Ozkul and Aydin, 1996; Kumar *et al*., 2014) have been described. The sporadic occurrence of haemangiosarcoma in this study was similar to those of Kumar *et al*. (2014). Sreelekshmy *et al*. (2017) observed only three cases of benign urinary bladder tumours, and these were diagnosed as fibroma (2) and leiomyoma (1).

Polymerase chain reaction (PCR)

Out of 21 samples, one sample revealed the presence of DNA of BPV-1, while, in another one sample DNA of both BPV-1 and BPV-2 was detected. None of the samples were positive for DNA of BPV-5 and BPV-10. The detailed results

of the PCR are presented in Table 2. Detection of DNA of either BPV-1/-2 or both was like earlier workers who also detected the DNA of these BPVs in urinary bladder lesions or tumours of cattle and buffaloes (Borzacchiello *et al.,*2003; Leishangthem, 2006; Pangty, 2009; Somvanshi, 2011; Pathania *et al*., 2012; Kumar *et al*., 2014).

Immunohistochemistry

A total of 15 samples were stained for immunohistochemical expression of Ki-67 and COX-2. The details of immunohistochemistry results are presented in the Table 3 and Figure 3. Ki-67 immunoreactivity was mainly restricted to nucleus. Immunopositive reaction for Ki-67 was observed only in haemangiosarcoma and single case of plasmo-lymphocytic cystitis. In haemangiosarcoma, Ki-67 immunoreactivity was noticed in the nuclei of the proliferating fibroblasts, lining endothelial cells of blood vessels and stromal cells (Figure 3A and 3B). Plasmo-lymphocytic cystitis showed weak positive reaction. The remaining samples were negative for Ki-67 immunostaining. The immunoreactivity for COX-2 was mainly restricted to the cytoplasm. COX-2 immunostaining was observed in one case each of flat hyperplasia, von Brunn's nest, haemangiosarcoma and two cases of plasmo-lymphocytic cystitis. In epithelial abnormalities *viz*. flat hyperplasia and von Brunn's nest COX-2 staining was restricted to the epithelial component of the lesions (Figure 3C and 3D) and it was diffused cytoplasmic type with occasional perinuclear staining particularly in the cytoplasmic membrane, but no staining was observed in nucleus. The intensity of staining varied from mild to moderate but none of the cases showed strong COX-2 staining. In plasmo-lymphocytic cystitis, the COX-2 staining was observed in the infiltrating inflammatory cells within inflammatory zone and immunostaining was restricted to the cytoplasm only. The intensity of the staining was mild type. In haemangiosarcoma, the COX-2 immunoreactivity was observed in epithelial as well as connective tissue components. In urothelium only few cells showed COX-2 immunostaining with mild intensity (Figure 3E). The connective tissue stromal cells as well as inflammatory cells also showed COX-2 immunoreactivity (Figure 3F).

The immunostaining for Ki-67 expression in this study was similar to that reported by Kumar *et al*. (2014). In contrary to present study, earlier workers reported that 3 cases of cystitis cystica and cystitis glandularis showed weak positive reaction for Ki-67. The COX-2 immunohistochemical expression in urinary bladder lesions in the present study were in corroboration with that reported by earlier workers (Ristimaki *et al*., 2001; Sledge *et al*., 2015); Li *et al*. (2004); Wadhwa *et al*. (2005) reported the prominent expression of COX-2 in urinary bladder cancers such as transitional cell carcinomas (TCCs) and squamous cell carcinomas. Khan *et al*. (2000) reported mild to marked immunoreactivity of COX-2 within the cytoplasm of macrophages and endothelial cells of blood vessels in the inflammatory lesions of lungs of non-human primates. However, the neutrophils which constituted 90% of the inflammatory cells in lung lesions did not exhibit any COX-2 immunoreactivity. Similar to present study, Ristimaki *et al*. (2001) reported that COX-2 expression was noticed in non-neoplastic lesions in human urinary bladder samples. It was detected in 66% (67/102) carcinomas and 25% (4/16) non-neoplastic lesions. The COX-2 immunoreactivity was restricted to the neoplastic cells in carcinomas. Strongest COX-2 immunostaining intensity was noticed in invading cells of several invasive

Table 1. Spontaneous lesions in urinary bladders of buffaloes.

Pathological conditions	Microscopical lesions	Number (%)
Epithelial abnormalities		37 (20.90)
	Flat type hyperplasia	10 (05.64)
	Papillary type hyperplasia	01 (00.56)
	Nodular type hyperplasia	11 (06.21)
	Von Brunn's nest	12 (06.77)
	Cystitis cystica and cystitis glandularis	03 (01.69)
Inflammatory conditions		82 (46.32)
	Congestion	20 (11.29)
	Haemorrhages	06 (03.38)
	Lymphocytic cystitis	27 (15.25)
	Plasmo-lymphocytic cystitis	17 (09.60)
	Follicular cystitis	11 (06.21)
	Interstitial cystitis	01 (00.56)
Neoplasms		01 (00.56)
	Haemangiosarcoma	01 (00.56)
	Nothing abnormally diagnosed	57 (32.20)
Total		177

Figures in parentheses indicate the per cent of a particular pathological condition out of total samples studied.

Table 2. Detection of BPV-1 and -2 in urinary bladder lesions by PCR.

S. No.	Pathological conditions	Number	PCR Results	
			BPV-1	BPV-2
1	Nodular hyperplasia	2	-	-
2	Von Brunn's nest	1	-	-
3	Cystitis cystica	1	-	-
4	Cystitis glandularis	1	-	-
5	Congestion	1	-	-
6	Haemorrhages	1	-	-
7	Lymphocytic cystitis	1	-	-
8	Plasmo-lymphocytic cystitis	8	+ (1)	-
9	Follicular cystitis	3	-	-
10	Haemangiosarcoma	1	+	+
11	Nothing abnormal diagnosed	1	-	-

Number: Number of bladder samples; Figures in parentheses indicate the number of samples.

Table 3. Scoring of Ki-67 and COX-2 immunostaining in urinary bladder lesions/tumours.

S. No.	Histopathological Diagnosis	Ki-67 Expression		COX-2 Expression	
		Ki-max (%)	Ki-tot (%)	Positive cells (%)	Staining intensity
1	Flat hyperplasia	-	-	12.60	++
2	Nodular hyperplasia	-	-	-	-
3	Nodular hyperplasia	-	-	-	-
4	Plasmo-lymphocytic cystitis	-	-	7.20	+
5	Cystitis glandularis	-	-	-	-
6	Von Brunn's nest	-	-	-	-
7	Von Brunn's nest	-	-	7.33	+
8	Von Brunn's nest	-	-	-	-
9	Lymphocytic cystitis	-	-	-	-
10	Plasmo-lymphocytic cystitis	8.60	5.80	4.20	+
11	Plasmo-lymphocytic cystitis	-	-	-	-
12	Follicular cystitis	-	-	-	-
13	Haemangiosarcoma	27.60	21.33	8.90	+
14	Normal	-	-	-	-
15	Normal	-	-	-	-

- : No reactivity; weak signal (+); moderate signal (++).

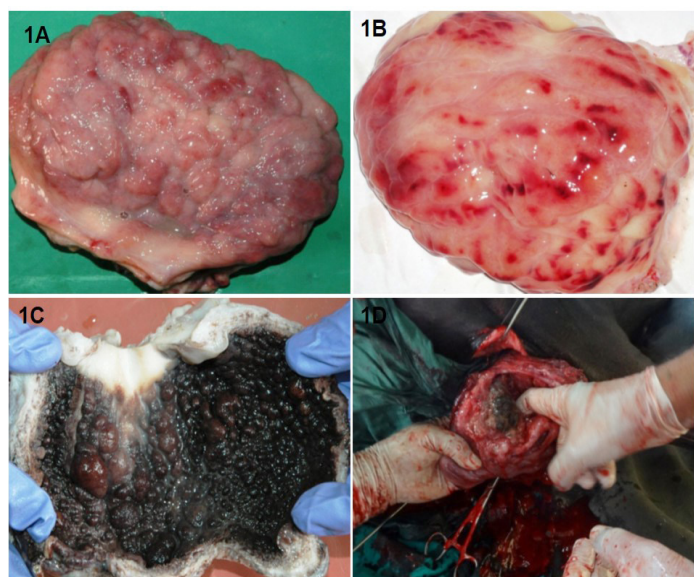

Figure 1. Gross lesions in mucosa of urinary bladders of buffaloes:
1A) Diffused congestion; 1B) Ecchymotic haemorrhages; 1C) Extensive haemorrhages with blackish appearance and 1D) Extensive fragile blackish tumour-like growth in bladder mucosa.

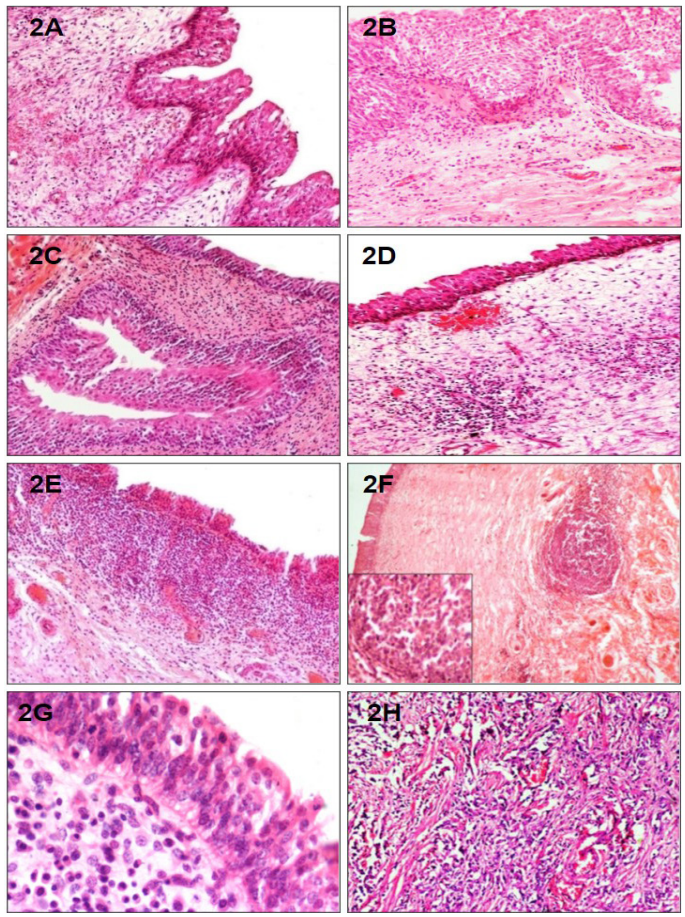

Figure 2. Histopathological lesions in buffalo urinary bladders:

2A) Papillary hyperplasia: Hyperplastic urothelium with undulating mucosal folds and papillary-like projections. H&E×100;

2B) Nodular epithelial hyperplasia: Thickened urothelium with generalized increase in number of transitional epithelial layers. H&E×100;

2C) Von Brunn's nest: Multiple layers of transitional epithelial cells enclosed in lumen. H&E×100;

2D) Focal haemorrhages and mild infiltration of mononuclear cells in lamina propria. H&E×100;

2E) Lymphocytic cystitis: Denuded urothelium with diffused infiltration of mononuclear cells mainly lymphocytes in lamina propria and extending towards muscularis layer. H&E×100;

2F) Follicular cystitis: Well-developed lymphoid follicle with germinal centre (inset). H&E×100;

2G) Plasmo-lymphocytic cystitis: Diffused infiltration of plasma cells (predominantly) and lymphocytes in lamina propria. H&E×400; 2H) Haemangiosarcoma: Thin-walled vessels along with proliferated connective tissue with pleomorphic fibroblasts. H&E×400.

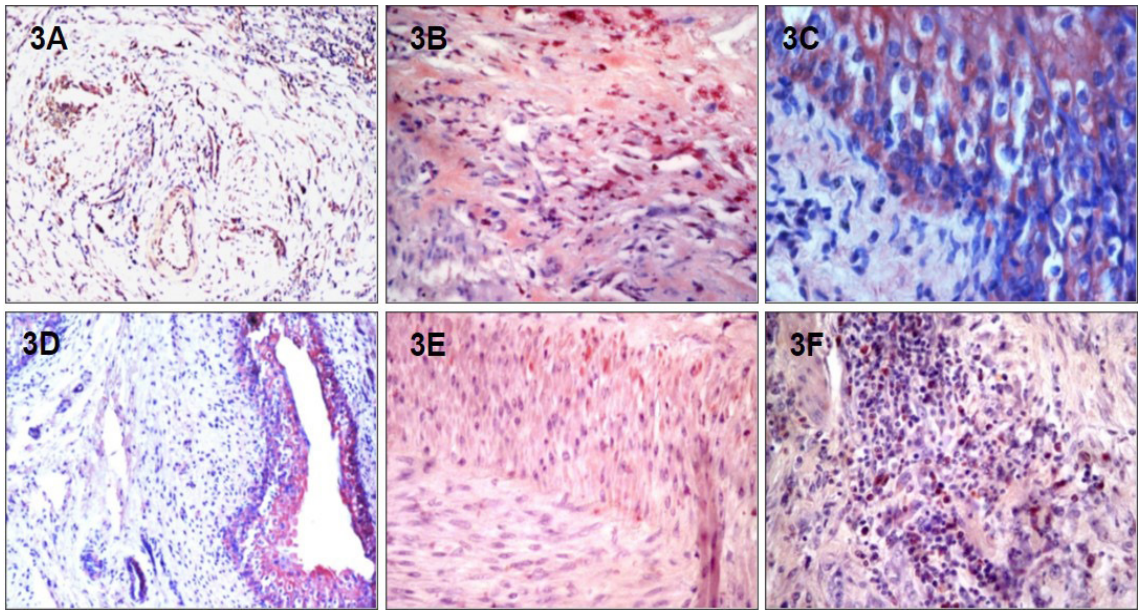

Figure 3. Immunohistochemical expression of Ki-67 and COX-2 in urinary bladder of buffaloes:

3A) Ki-67 immunoreactivity in proliferating fibroblasts and lining epithelium of blood vessels in haemangiosarcoma. IHC×100;

3B) Haemangiosarcoma: Intense intranuclear Ki-67 immunostaining in stromal cells. IHC ×400;

3C) Immunopositive reaction for COX-2 with membranous staining in hyperplastic transitional cells. IHC×400;

3D) Membranous staining for COX-2 in lining transitional epithelium of von Brunn's nest. IHC×100;

3E) Intracytoplasmic COX-2 expression in transitional epithelial cells in hemangiosarcoma. IHC×400 and

3F) Intracytoplasmic COX-2 expression in inflammatory cells and fibroblasts in haemangiosarcoma. IHC×400.

TCCs; however, other parts of tumours revealed no staining or were virtually negative. In addition to it, strong COX-2 positivity was reported in the non-neoplastic ulcerations (2/2) and inflammatory pseudotumours (2/2). The immunoreactivity was localized to the non-epithelial cells. Sledge *et al*. (2015) studied the expression of COX-2 in canine proliferative urothelial lesions and reported that no positive immunoreactivity for COX-2 was observed in normal urothelium, however, superficial layers of non-proliferative urothelium adjoining to proliferative lesions showed COX-2 immunopositivity in <10% cells. COX-2 expression in urothelial polyps and polypoid cystitis was mainly restricted to the superficial layers or was less commonly noticed in full thickness.

CONCLUSION

In conclusion, it may be stated that the urinary bladders of slaughtered Indian buffaloes showed a number of epithelial abnormalities, inflammatory conditions and occasionally neoplasm. Investigation has added pathological findings in the urinary bladder as much data was not available in literature. Further studies to explore infectious as well as non-infectious etiologies should be undertaken.

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