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## Research Article

# Histochemical and Immunohistochemical Observations of Bursa of Fabricius in Nandanam Chicken

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**ABSTRACT**

The study was aimed to provide the basic findings about the histochemical and immunohistochemical details of bursa of Fabricius in Nandanam broiler chickens of four different age groups *viz.*, day-old, two, four and six weeks. Paraffin sections and cryosections of fresh unfixed tissue, tissues fixed in chilled formol-calcium and neutral buffered formaldehyde were used for various histochemical and immunohistochemical studies. The presence of acid and neutral mucopolysaccharides was demonstrated in the epithelial cells lining the bursal mucosa, tunica serosa and epithelium between the cortex and medulla of lymphoid follicles. The presence of tyrosine kinase (Trk) like protein was evident in the epithelium lining the bursal plicae and lymphoid follicles in all the age groups studied. Lipid droplets were noticed only in interfollicular connective tissue in day-old birds. They were also noticed in the cortex and medulla of lymphoid follicle from two weeks onwards and they coalesced to form clumps in the medulla as age advanced. Metachromasia in the epithelium covering the bursal plicae, in mast cells present in the interfollicular connective tissue and in subcapsular area was demonstrated by toluidine blue method. The Bu-1a+ cells were found both in the cortex and medulla, but the intensity of immune-labelling was more in cortex when compared to medulla. CD3+ cells were noticed in the bursa of all age groups except day-old birds.

**Key words:** Nandanam chicken, Bursa of Fabricius, Histochemistry, Immunohistochemistry

## INTRODUCTION

Unlike other vertebrates, avian species have two discrete primary lymphoid organs, the thymus in which T-lymphocytes develop and is responsible for cell mediated immunity (CMI) and the bursa of Fabricius, the primary site for B-lymphopoiesis which is responsible for humoral immunity (HI) (Glick *et al.*, 1956 and Warner, 1967). The bursa of Fabricius in chicken is located at the dorsal portion of cloaca and connects with it by the bursal duct at its junction with the colon (Glick, 1964).

The chicken is a foundational model for immunological research and continues to be a valuable animal model for insights into immune function. In particular, the development of B cells in this unique organ, the bursa of Fabricius, has provided a novel opportunity to study B cell development. Although chicken generate their immunoglobulin (Ig) repertoire in a different way than mammals, there are many striking similarities in the developmental process. In particular, the control of lymphocyte migration and survival is key to the development of an immune system (Funk and Thompson, 1996).

It has been recognized that the bursa also functions as a peripheral gut-associated lymphoid organ. Antigens presented via the cloaca and bursal lumen can stimulate specific antibody production by bursal lymphocytes (Lupetti *et al.*, 1984). Thus, the bursa plays a role in local gut immunologic defence. A critical component of the local bursal response is the surface epithelium of the bursa overlying the medullary region of the lymphoid follicles *viz.* the follicle-associated epithelium (Houssaint *et al.*, 1986).

Although, there are several detailed reports available on histoarchitecture of bursa in chicken, the study on histochemical observation and immunohistochemical details of T and B lymphocytes in Nandanam Chicken is very limited. Nandanam strain of chicken is a dual purpose, coloured variety with good disease resistance, developed at Institute of Poultry Production and Management, TANUVAS. Hence, the present work was designed to carry out the same.

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## MATERIALS AND METHODS

Forty eight healthy Nandanam broiler chickens were procured from Institute of Poultry Production and Management, Tamil Nadu Veterinary and Animal Sciences University and Venkateshwara Hatcheries Pvt. Ltd. Chennai. Bursal tissue samples, from twelve birds of each age group, required for the work were collected from four different age groups viz., day-old, two, four, and six weeks.

Chemicals, substrates and kits required for histochemical staining methods were of analytical grade and procured from Himedia®. Tissue pieces were collected from bursa of Fabricius and were rinsed in normal saline and fixed in 10 percent neutral buffered formalin and chilled formol-calcium. The fixed tissues were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax.

Cryosections of 15 µm thickness were obtained from fresh and formol-calcium fixed tissues for different histochemical studies. Frozen sections from fresh unfixed tissue were used for localisation of lipids. Paraffin sections were used for immunohistochemical localisation of T and B lymphocytes in the present study. The frozen sections were subjected for the following histochemical staining methods:

1. Periodic acid-Schiff's (PAS) method for neutral mucopolysaccharides (Bancroft and Gamble, 2008).
2. Combined Alcian blue-PAS method for acid and neutral mucopolysaccharides (Bancroft and Gamble, 2008).
3. Toluidine blue method for metachromasia of mast cell granules (Bancroft and Stevens, 1996).
4. Millon's reaction for tyrosine containing proteins (Pearse, 1968).
5. Oil red 'O' method for lipids (Bancroft and Stevens, 1996).

### Immunohistochemistry

In the present study, localisation of T and B lymphocytes was done by immunohistochemical staining of paraffin sections as per Bancroft and Gamble, (2008) and Antibody source book (2009/2010). To enhance immunostaining heat mediated antigen retrieval was done by antigen retrieval solution.

Sections were incubated in primary antibody (1:50 dilution) for 1 hour at room temperature in a humid chamber, or overnight at $4^0$C and washed 3 times in PBS. HRP conjugated secondary antibody was added at 1:100 dilution and incubated for at least 30 minutes at room temperature and washed 3 times in PBS. Then sections were incubated for 7-10 min in DAB solution (Dissolve 32.5mg of DAB in 65ml of Tris-HCl buffer (0.05M pH 7.6) and just before use 81µl of 3% $H_2O_2$ was added and the reaction was terminated by transferring slides into PBS (Jeurissen *et al.,* 2000). Wash the sections in water once. The sections were counterstained with haematoxylin for 1 minute. After blueing for one minute, the sections were mounted in aqueous mounting medium or alternatively dehydrated through a graded series of alcohols and xylene and mounted in synthetic mountant.

## RESULTS

### Histochemistry

### Carbohydrates

The epithelial cells lining the bursal mucosa showed PAS positive reaction. These cells were also positive for Alcian blue. In combined Alcian blue-PAS method, the tip of the epithelium was PAS positive and the remaining part of the epithelium showed positive reaction for Alcian blue (Fig.1). The intensity of the reaction to both PAS and Alcian blue increased as age advanced. The cyst in the cortical region of six week-old bursa of Fabricius contained PAS positive material, indicating the mucoid nature of the cystic content (Fig.2). In combined Alcian blue-PAS technique, cyst wall was positive for Alcian blue alone, whereas, the contents of the cyst were positive for both PAS and Alcian blue. The basement membrane of lining epithelium and lymphocytes in the cortico-medullary junction of the lymphoid follicle also showed a positive reaction to Alcian blue (Fig.3).

### Metachromasia

The epithelium covering the bursal plicae and muscular layer showed metachromatic activity. The interfollicular epithelium showed strong metachromasia when compared to follicle associated epithelium and subcapsular mast cell granules also exhibited metachromatic activity (Fig.4). The bursal epithelium of four week-old birds showed intense metachromatic activity when compared to two week-old birds.

### Proteins

In the present study, tyrosine containing proteins were found in lymphoid follicle and epithelium covering the plicae of the bursa of all the age groups studied (Fig.5). The reaction was found to be more in four week and six week age groups when compared to day-old and two-week age groups.

### Lipids

In day-old birds, lipid droplets positive to oil red 'O' were noticed in interfollicular connective tissue. The lipid droplets were also noticed in the cortex and medulla from second week onwards (Fig.6). The amount of lipid substances increased as age advanced. The lipid droplets coalesced to form clumps in the medulla of bursa in six week-old birds (Fig.7).

### Immunohistochemistry

In the present study, T and B lymphocytes were demonstrated in bursal tissue using mouse anti-chicken Bu-1a and CD3 monoclonal antibodies. The Bu-1a+ cells were found both in the cortex and medulla, but the intensity of immune-labelling was more in cortex when compared to medulla (Fig. 8). In day-old birds, the reaction was less and it gradually increased as age advanced. The epithelium between cortex and medulla contained Bu-$1a^-$ cells.

CD3+ cells were found in the epithelium, subepithelial region, near blood vessels of interfollicular connective tissue and in diffusely infiltrated area (DIA) just above the bursal duct opening (Fig. 9). CD3+ cells were noticed in the bursa of all age groups except day-old birds.

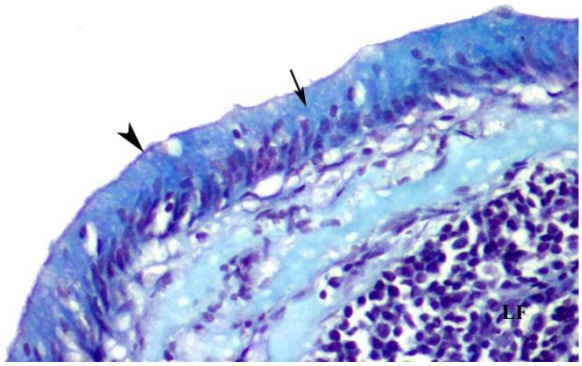


**Fig.1:** Photomicrograph of the bursa of Fabricius of four week-old chicken showing the presence of acid and neutral (arrow and arrow head) mucopolysaccharides. LF-Lymphoid follicle, Alcian blue-PAS X100

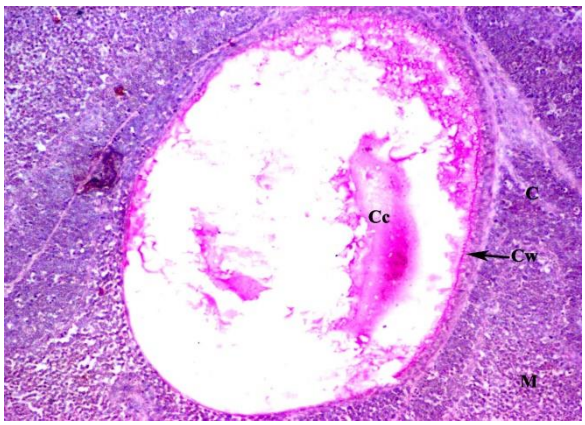


**Fig. 2:** Photomicrograph of the bursa of Fabricius of six week-old chicken showing PAS reaction in the cyst, M-Medulla, C-Cortex, Cc-Cyst content, Cw-Cyst wall PAS x 100

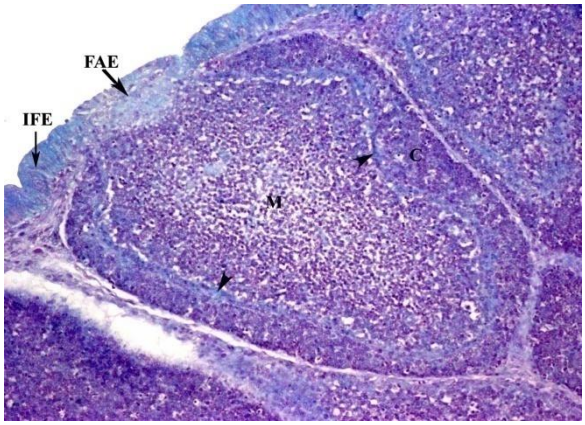


**Fig. 3:** Photomicrograph of the bursa of Fabricius of six week-old chicken showing Alcian blue positive reaction in epithelium (arrows) and cortico-medullary junction (arrow heads). M-Medulla, C-Cortex, IFE-Interfollicular epithelium, FAE-Follicle associated epithelium, Alcian blue-PAS x 100

## DISCUSSION

The observations of this study provided new insights into the histochemical changes within the bursa of fabricius with age. The results of the histochemical techniques indicate that the cytoplasm of the bursal cells contains several chemical substances.

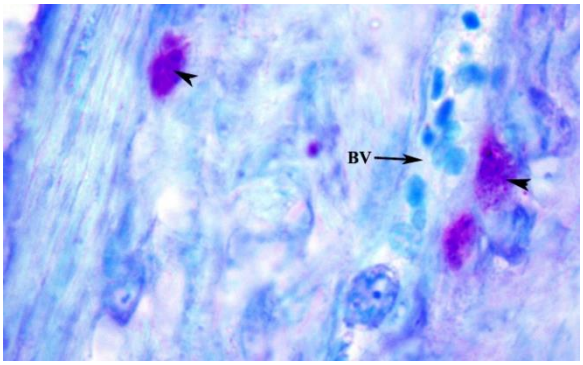


**Fig. 4:** Photomicrograph of the bursa of Fabricius of two week-old chicken showing metachromasia in mast cells (arrow heads). BV-Blood vessel, Toluidine blue x 1000.

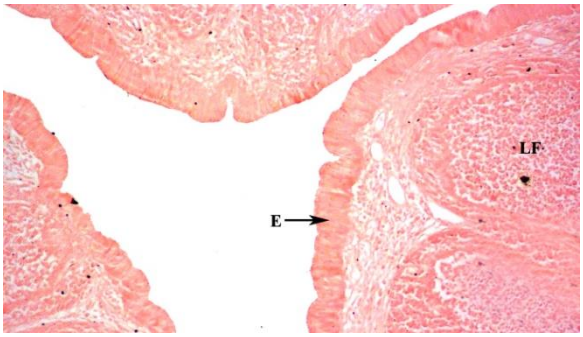


**Fig. 5:** Photomicrograph of the bursa of Fabricius of six week-old chicken showing the presence of Trk like proteins (arrow). E-Epithelium, LF-Lymphoid follicle, Millon reaction x 100.

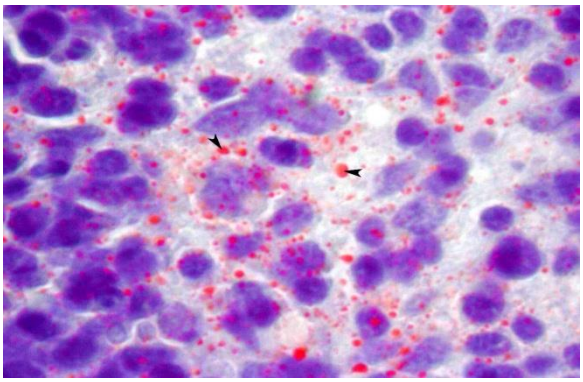

**Fig. 6:** Photomicrograph of the bursa of Fabricius of four week-old chicken showing lipid deposition in the medulla (arrow heads). Oil Red 'O' x 1000.

In combined Alcian blue-PAS method, the luminal surface of the epithelium covering the bursal mucosa was PAS positive and remaining part of the epithelium was Alcian blue positive and as age advanced PAS positive material in the epithelium also increased. Similar findings were noticed by Mazzone *et al.* (2003) in fifteen days and one month-old White Leghorn chicken, Ackerman and Knouff (1959), Bockman and Cooper (1973) and Betti (1989) in domestic fowl. These observations might suggest that due to maturation of the organ, more of mucopolysaccharides are deposited and may act as a source of energy for the developing and maturing lymphocytes as opined by Jagapathi Ramayya (2007) in thymus of buffalo and Mahesh Kumar (2010) in thymus of chicken.

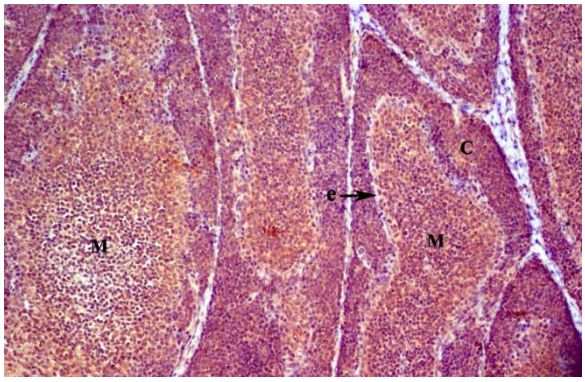


**Fig. 8:** Photomicrograph of the bursa of Fabricius of four week-old chicken showing Bu-1a+ cells. M-Medulla, C-Cortex, e-Epithelium in the cortico-medullary junction, IHC (DAB) x 100.

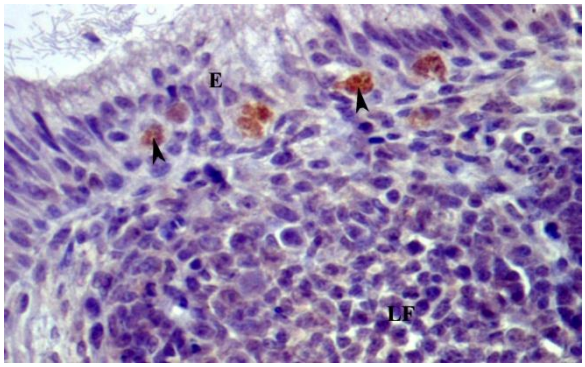


**Fig. 9:** Photomicrograph of the bursa of Fabricius of four week-old chicken showing CD3+ cells (arrows) in the lining epithelium E-Epithelium, LF-Lymphoid follicle, IHC (DAB) x 400.

As bursal regression starts from two month-old White Leghorn chicken, loss of medullary lymphocytes and cyst formation in the lymphoid follicle was observed by Glick (1974) and Franchini and Ottaviani (1999). In the present study, cyst was noticed in the cortex of six week-old birds, containing both acid and neutral mucopolysaccharides as demonstrated by combined Alcian blue-PAS method. The wall of the cyst was also positive for PAS which probably shows the presence of hexose-containing mucosubstances. The positive reaction with alcian blue reflects a content of acid mucopolysaccharides. It may be that both neutral and acid mucosubstances are constituents of the cytoplasm (Pearse, 1968, Magnusson and Majeed, 1978).

The timing of bursal regression probably depends on the strain of chicken (Jolly, 1914), based on the environmental situation (Glick, 1974), and also determined genetically (Bellamy and Mohamed, 1982).

Metachromasia in the present study indicated the presence of mast cells in subcapsular zone and in the tunica muscularis which contained the intracytoplasmic granules viz., heparin and histamine. The release of histamine and other vasoactive mediators increases vascular permeability and local blood flow, and can act on smooth muscle to increase the expulsion of antigens. Mast cells can modulate host innate immune responses through the release of granular and secreted mediators as per Dawicki and Marshall, (2007); Abraham and John, (2010).

In addition, metachromatic activity in the bursal epithelium indicated mucus production by the epithelial cell, which may aid in pathogen immobilisation and cytoprotection (Mirjam Urb and Sheppard, 2012).

The mast cells are believed to play an important role in control of allergic reactions. They have been viewed as key cells in immuno-pathology of immediate-type of hypersensitivity reactions initiated by preformed mediators stored in their secretory granules (Karaca *et al.*, 2006 and Mahesh Kumar, 2010). Similar observations were made by Indu *et al.* (2005) and Karaca *et al.* (2006).

Ciriaco *et al.* (1997), Vega *et al.* (2003) and Jayachitra (2008) demonstrated tyrosine like (Trk) proteins in the FAE and IFE and within the lymphoid follicle in turkey as recorded in the present study. The present results provided evidence for the localization of Trks in the non-lymphoid cells (epithelial and dendritic) of the avian primary lymphoid organs, suggesting a role for neurotrophins in these cells. Moreover, the selective cell localization of each Trk protein, and the absence of apparent overlapping, claims for a differential role of the specific Trk ligands as pointed out by Ciriaco *et al.* (1996).

Increase in Oil Red 'O' reaction as age advanced in the present study, indicated the hallmark of age dependant involuntary change of bursa by depletion of lymphocytes, invasion of collagen fibres and accumulation of adipose tissue in the parenchyma. A similar observation was made by Sabiha.H.Basha (1993) in Japanese quail, Indu *et al.* (2005) in White Pekin ducks and Jayachitra (2008) in turkey.

Distribution of B lymphocytes in the present study confirmed that bursa provides a microenvironment essential for proper B cell diversification and maturation. It became clear that interactions between immature B cells and the bursal epithelium were required for B cell differentiation. This is needed to generate a complete and diverse antibody repertoire for the adult bird as reported by Funk and Palmer (2003). The result also showed that the bursa of chickens was furnished with the distinct types of T-cell subsets at early postnatal stages of development to maintain its local immunity (Zahirul Khan and Hashimoto, 1997).

## Conclusion

In the present study, histochemical details and immunohistochemical localization of T and B lymphocytes were observed in bursa of Fabricius of Nandanam broiler chickens. The presence of acid and neutral mucopolysaccharides was demonstrated in the epithelial cells lining the bursal mucosa, tunica serosa and epithelium between cortex and medulla which indicated the presence of both acid and neutral mucopoly-saccharides in the cytoplasm.

Metachromatic activity in the epithelium covering the bursal plicae, in the interfollicular connective tissue and in subcapsular area confirms the role of host innate immune responses through the release of granular and secreted mediators. Increase in Oil Red 'O' reaction as age advances indicate the age dependant involuntary change of bursa by accumulation of adipose tissue in the parenchyma.

Distribution of B lymphocytes in the present study confirm the role of bursa of Fabricius in providing microenvironment essential for proper B cell diversification and maturation.

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