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Morphological Assessment of the Cecal Tonsil of Pre-hatch and Post-hatch Broiler Chicken

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Abstract

Vascularized ampullar dilatations of the cecal bases, called cecal tonsils, which are key components of avian gut-associated lymphoid tissues, are very strategic for the health of birds. The morphological development of the cecal tonsils of broiler chicken was evaluated using light microscopic and transmission electron microscopic techniques. A total of 10 fertile eggs and 50-day-old chicks of Marshall Broiler chicken were used for the study. On pre-hatch day (PrD) 19 and post-hatch day (PD) 1, the lamina epithelialis mucosae showed maturing enterocytes, goblet cells, and a basal lamina. Apicolateral tight junctions, adherent junctions, and desmosomes were frequently observed in the lamina epithelialis mucosae at PrD 19 and PDs 1, 35, and 56. However, at PDs 7, 14, and 21, the desmosomes were rarely observed. The lamina propria submucosae of the cecal tonsil showed areas of diffuse infiltration of lymphocytes on PDs 3 and 5, whereas areas of dense lymphocytic infiltration occurred on PDs 7, 11, 14, and 21. The population of plasma cells, which was first observed on PD 14, increased with age. In conclusion, the cecal tonsil of the broiler chicken assumes adult histological features through a gradual process that begins from PD 3 and continues until PD 35.

Keywords: Broiler chicken, cecal tonsil, junctional complexes, lymphoepithelium, ultrastructure

Introduction

Cecal tonsil morphology in birds is comparable to avian Peyer's patches, mammalian tonsils, and human appendix (Casteleyn et al., 2011; Gómez Del Moral et al., 1998; Smith et al., 2009). They are made up of specialized lymphoepithelium, sub-epithelial zone, lymphoid follicles with or without germinal centers and interfollicular areas, which are either bursa-dependent or thymic-dependent zones (Oláh et al., 2014; Rezaian and Hamedi, 2007). The bursa-dependent lymphocytes expressing mainly immunoglobulin M and immunoglobulin A are thought to predominate other lymphocyte subsets in the cecal tonsil of an adult chicken at the age of 6 weeks (Gómez Del Moral et al., 1998). However, there are varied reports on the time of appearance and maturation of the gut-associated lymphoid tissues (GALTs) of birds (Bucy et al., 1988; Payne, 1971; Vervelde and Jeurissen, 1993).

It is widely speculated that the cecal tonsils may participate in the maintenance of the gut health, fermentation of undigested nutrients, and modulation of the gut microbiota (Casteleyn et al., 2010; Svihus et al., 2013; Yun et al., 2000); however, some of the processes are not well understood. The cecal tonsils were found to be reservoirs for *Eimeria tenella* in the infected birds (Laurent et al., 2001). The infestation of the organ by these parasites was shown to induce local lymphocyte and macrophage responses as well as production of interferon gamma, an important component of the host immune response to coccidia (Laurent et al., 2001; Yun et al., 2000).

The morphological features of the cecal tonsils have largely been evaluated in adult birds (Akter et al., 2006; Clench, 1999; Kannan et al., 2015; Rezaian and Hamedi, 2007; Udoumoh et al., 2016). However, participation of the cecal tonsils in antigen-specific immune

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responses and other postulated roles may be age related. There have been studies on the appearance and development of lymphoid cells of the cecal tonsil in some birds (Gómez Del Moral et al., 1998; Islam et al., 2012; Nnadozie et al., 2019), but there is a dearth of information on the development and functional morphology of the lymphoid cells of the cecal tonsil in the broiler chicken, a fast-growing breed of chicken with huge production-related challenges. Thus, this study aimed to investigate the age-related morphological changes in the cecal tonsil of the broiler chicken during the pre- and post-hatch periods using light microscopic and transmission electron microscopic techniques.

Materials and Methods

Animals

The experimental animals were 10 fertile eggs (group A) and 50-day-old chicks (group B) of Marshall Broiler chicken that were obtained from a reputable farm in Ibadan, Oyo State, Nigeria. After incubation of the fertile eggs for 21 days, samples of the cecal tonsils were obtained at pre-hatch day (PrD) 19 and post-hatch day (PD) 1. The chicks (group B) were brooded and reared for 56 days under standard conditions. Chick starter mash and water were provided *ad libitum*, and the cecal tonsil

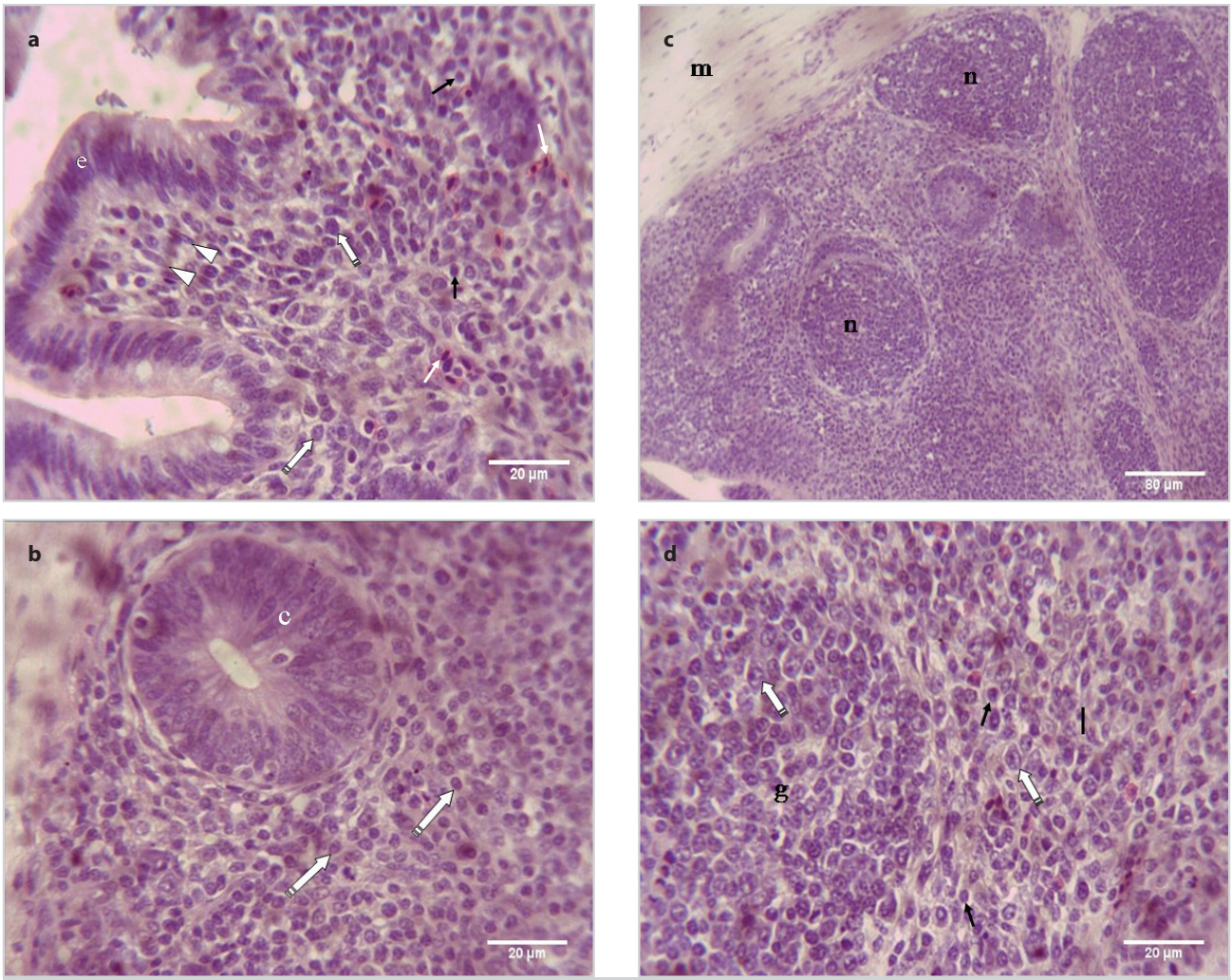

Figure 1. a-d. (a) Photomicrograph of the cecal tonsil of the broiler chicken on post-hatch day 3, showing lamina propria submucosae with an area of lymphocytic infiltration and with lymphocytes (black arrows and segmented white arrows) and nuclei (arrow heads) of reticular cells. Note the epithelium (e) and blood capillaries (white arrows). Hematoxylin and eosin stain x400. (b) Cecal tonsil of broiler chicken on post-hatch day 14, showing an area of dense lymphocytic infiltration with lymphocytes (segmented white arrows). Note the crypt of Lieberkühn (c). Hematoxylin and eosin stain x400. (c) Cecal tonsil of broiler chicken on post-hatch day 28, showing primary lymphoid follicles (n) and tunica muscularis (m). Hematoxylin and eosin stain x100. (d) Cecal tonsil of broiler chicken on post-hatch day 35, showing an area of dense lymphocytic infiltration (l) and secondary lymphoid follicle with lymphocytes (black arrows) and plasma cells (white segmented arrows). Note the germinal center (g). Hematoxylin and eosin stain x400

samples were obtained at PDs 3, 5, 7, 14, 21, 28, 35, 42, and 56. On each of the PrDs and PDs, 4 randomly selected embryos or chicks were used for the study.

The experimental protocols conducted were approved by the ethical committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka (FVM-UNN-IACUC-2019-077). The experimental animals were humanely used and cared for in strict conformity with the ethics and regulations guiding the use of research animals in the University of Nigeria, Nsukka, and in accordance with the guidelines for care and use of experimental animals of the Canadian Council on Animal Care, Ontario, 2017. The embryos and chicks were humanely sacrificed using 13 mg/kg ketamine hydrochloride injection.

Histological procedures

Samples of the cecal tonsils were fixed by immersion in 10% neutral-buffered formalin. The fixed tissues were dehydrated in increasing concentrations of ethanol, cleared in xylene, embedded in paraffin wax, and mounted on wooden blocks for sectioning. Using a rotary microtome, 5–6-µm thick sections were obtained and stained with hematoxylin and eosin for light microscopy. The staining procedure was performed according to the methods of Sheehan and Hrapchak (1980). Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic Group Ltd.) attached to the Motic binocular light microscope.

Transmission electron microscopy procedures

Samples of the cecal tonsils were minced with a sharp blade and fixed by immersion in modified Karnovsky's mixture, containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. The samples were post-fixed in 1% osmium tetraoxide in Millonig's buffer and dehydrated in graded concentrations of ethanol. The dehydrated tissues were infiltrated with propylene oxide, embalmed in epoxy resin, and cured overnight in an embalming oven at 65°C. The 1-µm thick (semi-thin) sections were obtained and stained with toluidine blue for light microscopy. Ultrathin sections of 50–90-nm thick were cut using an ultra-microtome and stained with Reynold's lead citrate and saturated aqueous uranyl acetate. The stained sections were examined using a Philips CM 10 transmission electron microscope. Electron micrographs were captured using an Olympus Megaview III digital camera (Olympus Corporation Japan) attached to the transmission electron microscope.

Results

Light microscopy

The cecal tonsil of post-hatch broiler chicken was made up of 4 tissue layers, namely tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa (Figure 1a-b). The tunica mucosa exhibited several villous projections and crypts of Lieberkühn, both of which were covered by lamina epithelialis mucosae, beneath which was the lamina propria mucosae. Each crypt of Lieberkühn was encircled by concentric ring(s) of the reticular cells. Similarly, the reticular cells, connective tissue fibers, and a network of blood capillaries were observed in the lamina propria mucosae and submucosa (Figure 1a-d). The tunica muscularis exhibited circularly arranged layers of smooth muscle fibers, whereas the tunica serosa showed a single layer of mesothelium (Figure 1c).

On PDs 3 and 5, the lamina propria mucosae was diffusely infiltrated by lymphocytes, whereas the tunica submucosa showed areas of dense lymphocytic infiltration without lymphoid follicles (Figure 1a). However, on PDs 7, 11, 14, and 21, dense infiltration of a mixed population of lymphocytes was evident in both the lamina propria mucosae and submucosa (Figure 1b). On PD 28, several primary lymphoid follicles were observed in the lamina propria mucosae and submucosa, whereas the tunica muscularis showed bundles of circularly arranged smooth muscle fibers (Figure 1c). Furthermore, on PDs 35, 42, and 56, both primary and secondary lymphoid follicles occurred in the lamina propria mucosa and submucosa. The secondary lymphoid follicles showed the characteristics corona and germinal centers (Figure 1c-d). Each germinal center contained a mixed population of lymphocytes and plasma cells. Plasma cells were also present among the lymphocytic aggregations in the interfollicular areas (Figure 1d).

Transmission electron microscopy

On PrD 19, the enterocytes of the lamina epithelialis mucosae of the cecal tonsil showed large basally displaced euchromatic nuclei and few cytoplasmic mitochondria (Figure 2a-d). Microvilli of irregular heights were evident on the apical surfaces of the enterocytes. Goblet cells with large basally displaced nuclei and thin apical cytoplasm were also found within the epithelium (Figures 2a and 3a). The lamina propria mucosae and submucosa of the cecal tonsil showed presence of reticular cells and mesenchymal cells (Figure 2a), macrophages with irregular nuclear outlines, cytoplasmic vacuoles, and plasma membrane indentations.

On PD 1, the microvilli of the enterocytes were long and uniform in outline (Figure 2d). Each goblet cell possessed a small, basally elongated displaced nucleus with apical cytoplasmic vacoules (Figures 2a and 3a). A thin basal lamina separated the epithelium from the subjacent connective tissue (Figure 2b). The lamina propria mucosae and submucosa contained reticular cells, mesenchymal cells, heterophils, and blood vessels (Figure 2a).

On PD 7, microfold cells (M-cells) appeared in the epithelium and occupied an adluminal position between the adjacent enterocytes (Figure 2d). The M-cells showed no microvilli, and the lamina epithelialis mucosae lacked the basal lamina (Figure 2d). Reticular cells, collagen fibers, lymphocytes of various sizes, and network of blood vessels were present in the lamina propria mucosae and submucosa.

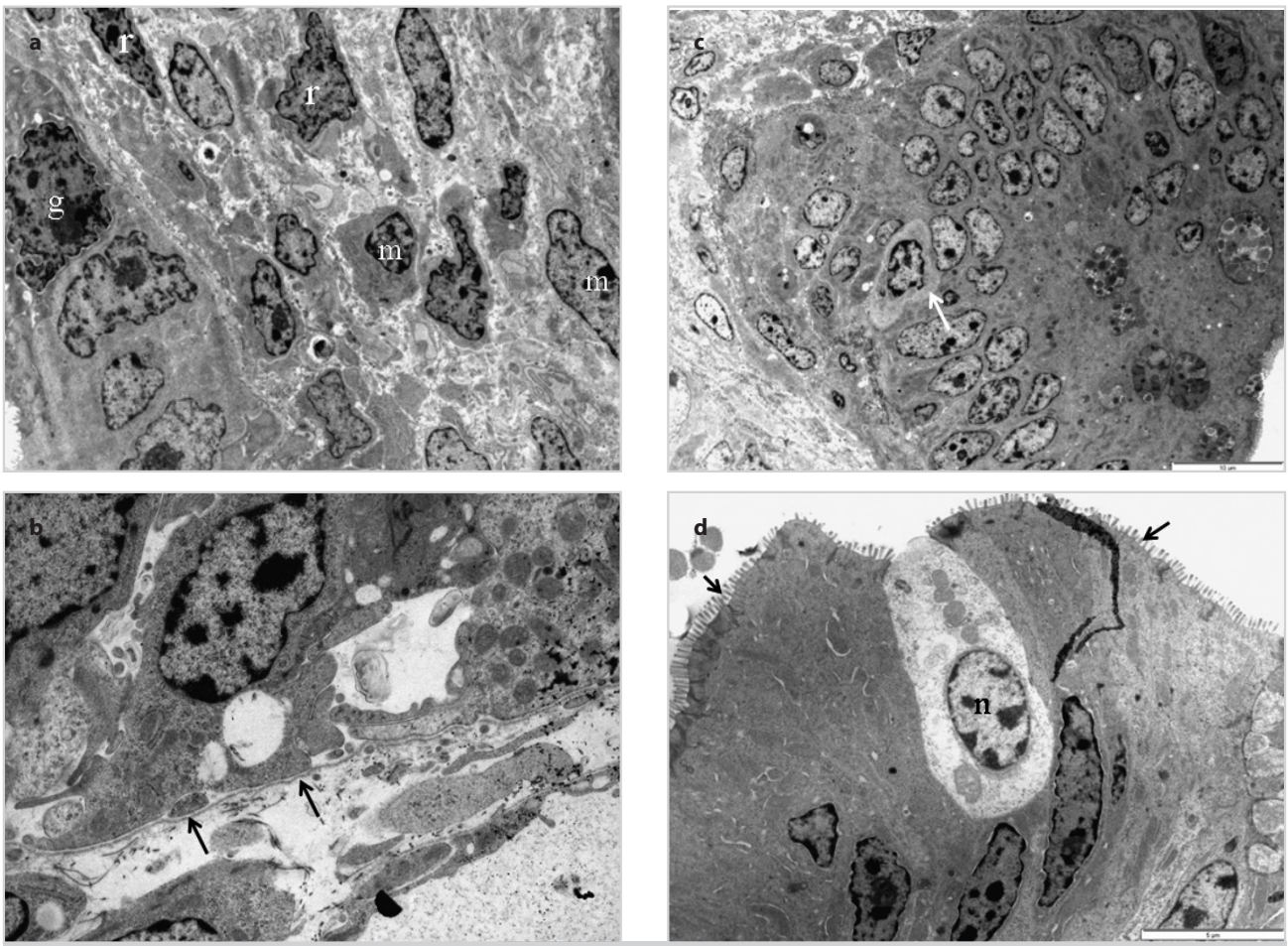

Figure 2. a-d. (a) Cecal tonsil of the broiler chicken on pre-hatch day 19, showing lamina propria submucosae with goblet cell (g), reticular cells (r), and mesenchymal cells (m). Transmission electron microscopy x3,400. (b) Cecal tonsil of broiler chicken on post-hatch day 1, showing basal lamina (arrows). Transmission electron microscopy x8,700. (c) Cecal tonsil of broiler chicken on post-hatch day 1, showing intraepithelial lymphocyte (white arrow). Transmission electron microscopy x1,650. (d) Cecal tonsil of broiler chicken on post-hatch day 7, showing the nucleus of microfold cell (n) and microvilli (arrows). Transmission electron microscopy x4,400.

On PDs 14 and 21, the lamina epithelialis mucosae of the cecal tonsil of the broiler chicken was transformed into a lymphoepithelium characterized by the presence of numerous infiltrated lymphocytes among the enterocytes (Figure 2c). The nuclei of the lymphocytes were euchromatic, with patches of heterochromatin in the nucleoplasm. The cytoplasm contained many polysomes and mitochondria. The M-cells were frequently encountered in the adluminal compartment of the epithelium, whereas the lymphocytes, plasma cells, and heterophils occurred in the lamina propria mucosa and submucosa (Figures 2d and 3b-d).

On PDs 35 and 56, enteroendocrine cells with their characteristic electron-dense cytoplasmic granules were found in the lamina epithelialis mucosae (Figure 3c). Few intraepithelial lymphocytes were also observed within the epithelium; however, many plasma cells and lymphocytes at various stages of differentiation were present in the lamina propria mucosa and submucosa (Figure 3b and d).

Development of junctional complexes

The enterocytes of the lamina epithelialis mucosae exhibited apicolateral tight junctions, adherent junctions, and desmosomes between the lateral surfaces of the adjoining cells on PrD 19 and PD 1 (Figure 4a-d). However, on PDs 7, 14, and 21, the apicolateral tight junctions and adherent junctions occurred consistently, while desmosomes were rarely observed (Figure 4b-c). On PDs 35 and 56, the apicolateral tight junctions, adherent junctions, and desmosomes were evident in the lamina epithelialis mucosae of broiler cecal tonsils (Figure 4d).

Discussion

The strategic anatomical position of the cecal tonsil at the interface between the small and large intestines of chicken highlights its importance. Occurrence of a typical 4-tissue-layered cecal tonsil in the broiler chicken on PrD 19 suggests that

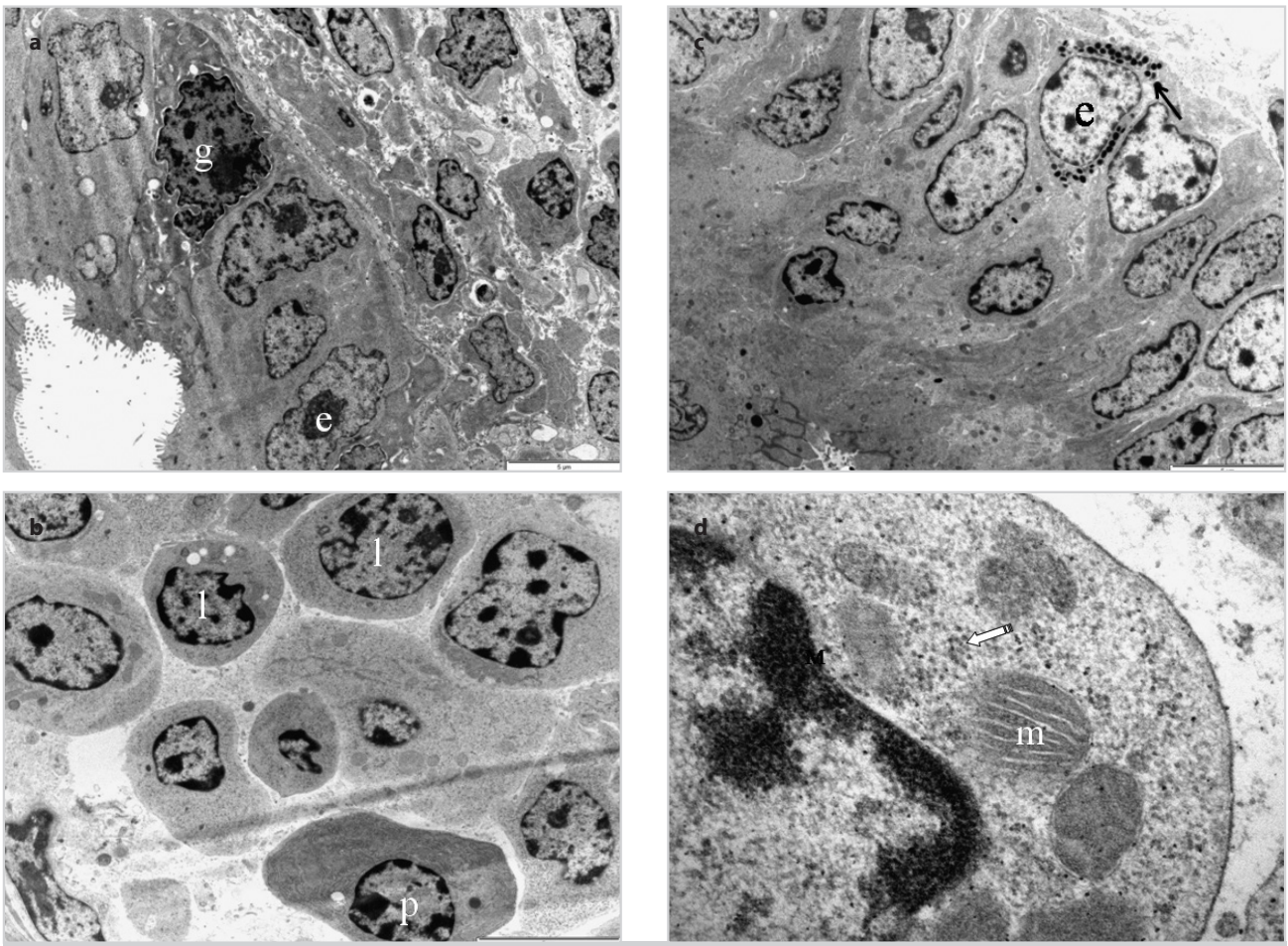

Figure 3. a-d. (a) Cecal tonsil of the broiler chicken on pre-hatch day 19, showing nuclei of goblet cell (g) and enterocyte (e). Transmission electron microscopy x3,400. (b) Cecal tonsil of broiler chicken on post-hatch day 14, showing the nuclei of plasma cell (p) and lymphocytes (l) in the lamina propria submucosae. Transmission electron microscopy x4,400. (c) Cecal tonsil of broiler chicken on post-hatch day 56, showing enteroendocrine cell nucleus (e) and electron-dense cytoplasmic granules (arrow). Transmission electron microscopy x3,400. (d) Cecal tonsil of broiler chicke on post-hatch day 35, showing lymphocyte with mitochondria (m) and polysomes (arrow). Transmission electron microscopy x41,000

the structural architecture of the organ is established in the late embryonic life of the bird. This is similar to the report in partially inbred white Leghorn chicken (Kajiwara et al., 2003). However, there was no significant presence of lymphoid elements in the cecal tonsils of the broiler chicken on PrD 19 and PD 1, and rather a preponderance of mesenchymal cells, reticular cells, and heterophils was demonstrated. This observation differs from that of a previous study that showed lymphoid elements in chicken cecal tonsils on PrD 18 after 24 hours of hatch (Gómez Del Moral et al., 1998). In this study, the lymphoid elements first appeared in the cecal tonsil on PD 3, and the lymphocyte population increased steadily through PDs 7 to 21, resulting in many areas of dense lymphocytic infiltration. Furthermore, the primary lymphoid follicles became organized by PD 28, and the secondary lymphoid follicles with germinal centers appeared on PD 35. Thus, it can be inferred that the cecal tonsil of the broiler chicken assumes adult histological features through a gradual process that occurs after hatch, beginning about 3 days post-hatch and continuing until the 35th day. In a similar manner, the lymphoid follicles were present in the cecal tonsil of the Kasilla Broiler chicken on PD 28, although these were yet unorganized (Akter et al., 2006). *In situ* cell proliferation and massive migration of the lymphocytes in the cecal tonsil were demonstrated in 4-day-old white Leghorn chicken, whose cecal tonsils assumed adult morphology on PD 10 and showed presence of secondary lymphoid follicles on PD 14 (Gómez Del Moral et al., 1998; Hoshi and Mori., 1973). The timing and pattern of development, proliferation, and colonization of the gut by lymphocytes may be influenced by exposure and colonization of the gut by microbiota or pathogenic microorganisms (Dibner et al., 2008; Han et al., 2017).

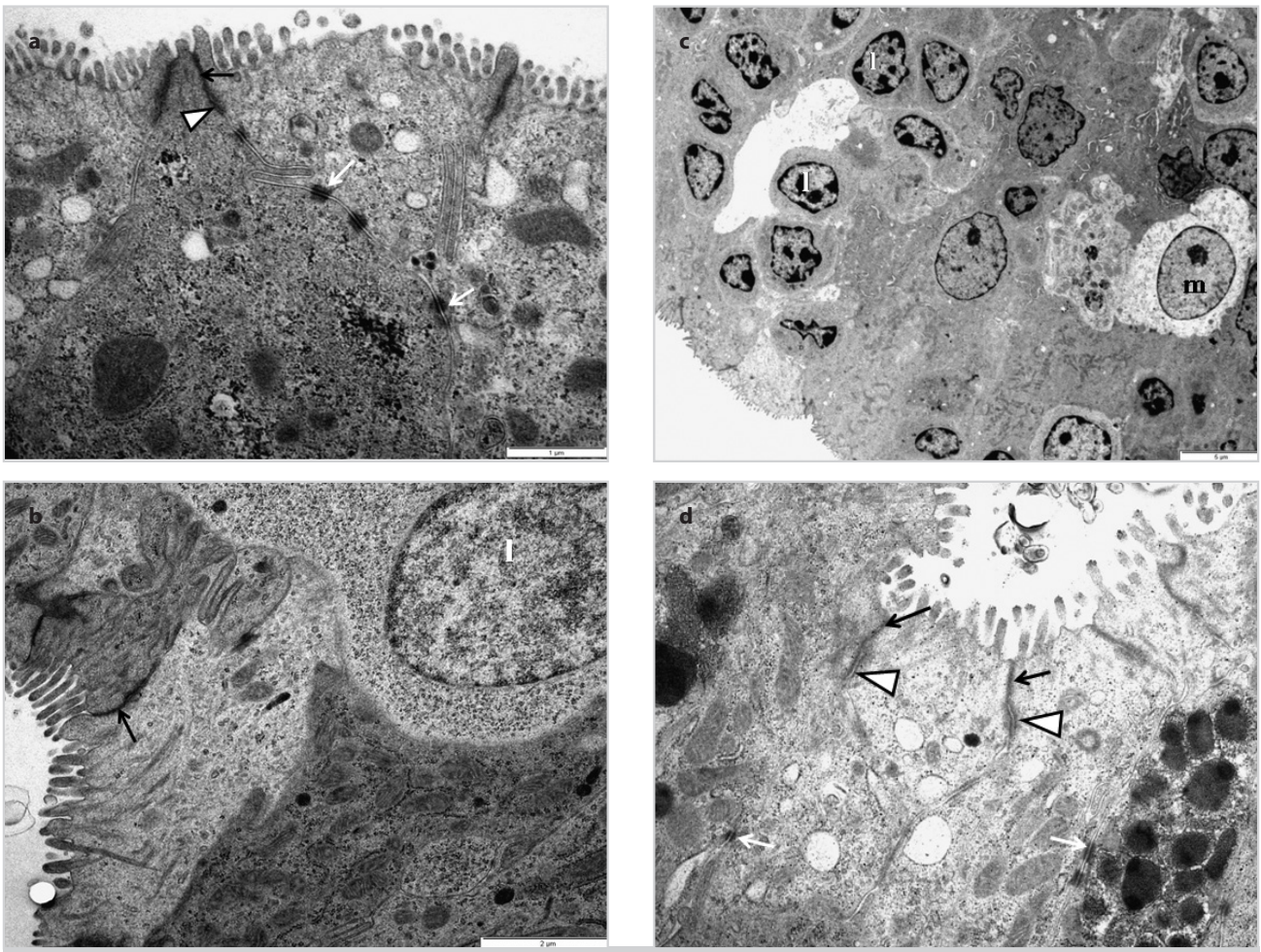

Figure 4. a-d. (a) Cecal tonsil of the broiler chicken on pre-hatch day 19, showing tight junctions (black arrow), adherent junction (arrow head), and desmosomes (white arrows). Transmission electron microscopy x12,500. (b) Cecal tonsil of broiler chicken on post-hatch day 14, showing tight junction (black arrow) and lymphocyte (l). Transmission electron microscopy x8,700. (c) Cecal tonsil of broiler chicken on post-hatch day 14, showing lymphoepithelium with microfold cell (m) and lymphocytes (l). Transmission electron microscopy x2,400. (d) Cecal tonsil of broiler chicken on post-hatch day 35, showing tight junctions (black arrows), adherent junctions (arrow heads), and desmosomes (white arrows). Transmission electron microscopy x12,500

The cecal tonsil is a major component of the GALTs that are very strategic for the immunological responses and health of the birds. Immune-competent cells encountered in the cecal tonsils of the broiler chicken include lymphocytes and plasma cells, both of which showed an increase in population with age. This suggests a steady increase in the potential of the cecal tonsil to participate in immunological surveillance of the distal gut. The lymphoepithelium of enterocytes, intraepithelial lymphocytes, M-cells, goblet cells, and occasional enteroendocrine cells covered the surface of the cecal tonsils of the broiler chicken during the post-hatch period. Occurrence of lymphocytes and M-cells in the lymphoepithelium has been reported by previous studies (Claeys et al., 1996; Kitagawa et al., 2000; Olah and Glick, 1992; Oláh et al., 2014), but goblet cells were previously considered to be absent in the lymphoepithelium (Oláh et al., 2014). The mucous secretions of the cecal tonsilar goblet cells may serve to trap the invading pathogens and expose them to the immune-competent cells of the lymphoepithelium. Demonstration of enteroendocrine cells in the cecal tonsils of the broiler chicken implies that the organ may function in the secretion of some gut hormones in addition to its role in the immunological responses.

The apicolateral tight junctions, adherent junctions, and desmosomes occurred between the adjacent enterocytes of the cecal tonsilar epithelium in the broiler chicken. These junctional complexes may provide firm attachment between the cells, serve as locations for intimate cell-to-cell interaction (Corden et al., 2017; Kowalczyk and Green, 2013), and constitute effective barriers that render the epithelium less permeable to materials within the gut, including pathogens. However, the desmosomes were apparently absent on PDs 7, 14, and 21. This

corresponds to the period of massive infiltration of the epithelium by lymphocytes from the subjacent connective tissue of the lamina propria mucosa (Gómez Del Moral et al., 1998; Hoshi and Mori, 1973). Absence of desmosomes would allow for unhindered migration of the lymphocytes into the epithelium. Moreover, there was no basal lamina between the epithelium and the subjacent connective tissue during this period. On PDs 35 and 56, the desmosomes and basal lamina were encountered in the lymphoepithelium, indicating reduced cellular infiltration of the epithelium after the adult histological features of the cecal tonsil had been established.

In conclusion, the cecal tonsil of the broiler chicken assumes adult histological features beginning from PD 3 and continuing until PD 35. The histological characteristics of the cecal tonsil during this period allow for intense migration of cellular components within the epithelium and the subjacent connective tissue layers.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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