Original Article

Histogenesis and histochemical features of gastric glands of pre-hatch and post-hatch broiler chicken

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Abstract

The objective of this study was to evaluate the morphological development of the stomach of broiler chicken using histological and histo-chemical techniques. Thirty fertile eggs and 50 chicks of broiler chicken were used for the study. The results showed that at embryonic day (ED) 14, the simple tubular glands of the proventriculus were poorly defined while the framework of the compound tubular glands of the proventriculus were fully established. The secretory tubules of the compound tubular glands were lined by simple cuboidal cells at embryonic days 14, 17 and 19. However, at post-hatch days 1, 3, 7, 14, and 21, each proventricular gland contained several secretory tubules that were lined by both cuboid-shaped and columnar-shaped epithelial cells. The simple tubular glands which appeared as short tubes at ED 17, became elongated at ED 19, assuming adult morphology at post-hatch day (PD) 1. Moreover, at ED 14, poorly defined glandular areas were present in the lamina propria mucosae of the ventriculus. However, at ED 17 and ED 19, elongated pits which represented simple tubular glands were observed in the ventriculus. The production of neutral and acidic mucins in the stomach at ED 14 may reflect early secretory roles of glands in the stomach of broiler chicken. Thus, this study has provided morphological evidence of rapid development of broiler stomach glands in the late pre-hatch periods and maturation of the glands at hatch. These modifications may be essential for early food ingestion, food digestion and strong innate immune defense just after hatch.

Keywords: broiler chicken, gastric gland development, histogenesis, mucin histochemistry, proventriculus

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Introduction

The stomach is a very crucial component of the gastrointestinal tract which participates in the second phase of digestion, after mastication in mammals and deglutition in birds (Bacha and Bacha, 2000; Nisa *et al*. 2010). Gastric glands are the key components of the stomach which provide suitable environment to facilitate chemical reduction of the size and molecular complexity of animal feed (Bacha and Bacha, 2000; Svihus, 2011). While the mucous secretions of gastric glands have been implicated in the innate immune defense of most animal species against invading pathogens, its serous secretory products contain substances that can aid the digestion of food particles (Hold *et al*. 2011; Sotolongo *et al*. 2012; Svihus, 2014). The proventriculus is the cranial glandular stomach of birds, similar in function and molecular characteristics as the simple stomach of monogastric animals like dogs and cats. The glands of this stomach segment have been shown to produce digestive enzymes (Bacha and Bacha, 2000). The caudal compartment of avian stomach, the ventriculus, is thought to perform mechanical roles during digestion, due to its muscular nature. However, ventricular glands have been reported in some birds (Bacha and Bacha, 2000; Udoumoh and Ikejiobi, 2017). Adequate knowledge of the morphological development of gastric glands may aid feed modifications with respect to their stages of development and growth, to enhance the efficiency of feed utilization of the widely reared broiler chicken.

Avian stomach develops from a single gastric primordium which has cranial and caudal segments (McGeady *et al*. 2006). The cranial part of this primordium becomes the proventriculus while the caudal part gives rise to the ventriculus (McGeady *et al*. 2006). At first the gastric primordium is lined by a simple cuboidal epithelium and may later be modified into a simple columnar epithelium, with species-specific regional modifications (Grosse *et al*. 2011). Gastric glands are believed to develop in regions of the stomach where simple columnar epithelium persist (McGeady *et al*. 2006). Changes in shape of epithelial cells, epithelial folding and epithelial invaginations were implicated in the formation of gastric glands in human (McCracken and Wells, 2017). Although the developmental events that will lead to the formation of gastric glands in birds are yet to be fully elucidated, it is widely speculated that progressive maturation of gastric glands occur through the post-hatch periods to assume the complex structures found in adult birds. Early developmental processes have future implications in later homoestasis and disease in the adult stomach (Kim and Shivdasani, 2016; McCracken and Wells, 2017).

Information on the morphological features of gastric glands of mature birds are available (Bacha and Bacha, 2000; McLelland, 1990). Available literature contains limited information on the developmental features of glands in avian stomach (Soliman *et al*. 2014; Omonona *et al*. 2014; Gosomji *et al*. 2017; Wang *et al*. 2017). However, there is dearth of information on the developmental morphology of the gastric glands of broiler chicken. The aim of the present study is to investigate the morphological development of glands in the proventriculus and ventriculus of broiler chicken using histological and histochemical techniques.

Materials and Methods

Animals: Thirty fertile eggs and 50 day-old chicks of Broiler chicken used for this study were obtained from a reputable breeder farm at Ibadan, Oyo State, Nigeria. The fertile eggs were incubated for 21 days and tissue samples were collected on embryonic days 14, 17, and 19. The day-old chicks were brooded and reared for 56 days under standard conditions. Chick starter mash and water were provided *ad libitum* and samples were collected at post-hatch days 1, 3, 7, 14 and 21. On each of these days, 5 randomly selected birds were humanely sacrificed by euthanasia, using 6.5 - 13 mg/kg ketamine hydrochloride intramuscular injection. Samples of the proventriculus and ventriculus were collected for further histological procedures.

Histological and histochemical procedures: Samples of the proventriculus and ventriculus collected from the birds were fixed by immersion in 10% neutral buffered formalin. The fixed tissues were dehydrated in graded concentrations of ethanol, cleared in xylene, embedded in paraffin wax and sectioned with a rotary microtome to obtain 5 - 6 μm thick sections. Sectioned tissues were stained with haematoxylin and eosin (H&E) and Periodic Acid Schiff-Alcian blue (PAS-AB) stains as described by Sheehan and Hrapchack (1980). Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic Group Ltd) attached to the Motic binocular light microscope.

Histomorphometry and data analysis: The tubular diameters, heights of the tunica mucosa and widths of the compound tubular glands of the proventriculus were measured using an ocular micrometer calibrated with a stage micrometer. The data obtained were analyzed using one-way analysis of variance (ANOVA) and significant means were separated by the least significant difference (LSD) method. Statistical significance was accepted at $p < 0.05$.

Ethical considerations: The guideline for the use, care and use of the animals were strictly followed in accordance with the Ethics and Regulations guiding the use of research animals in the University of Nigeria, Nsukka and in accordance with guide to the care and use of experimental animals of Canadian Council on Animal Care, Ontario, 1993. The experimental protocols were carried out according to the approval of the ethical committee of Faculty of Veterinary Medicine, University of Nigeria, Nsukka (FVM-UNN-IACUC-2019-077).

Results

Histogenesis of glands in the proventriculus: At embryonic day 14, the proventriculus of broiler chicken exhibited low mucosal folds (Fig 1). Each mucosal fold showed a lamina epithelialis mucosae composed of pseudo-stratified columnar epithelium, and lamina propria mucosae, a mesenchymal connective tissue. The poorly defined glandular areas showed randomly arranged cells in the upper layer of the lamina propria mucosae (Figs 1, 2). At embryonic day 17, the upper layer of the lamina propria mucosae showed short tubular profiles that were lined by simple cuboidal epithelial cells (Fig 3). At embryonic day 19, the simple tubular glands of the lamina propria mucosae were evident and appeared as slightly elongated tubes (Fig 4). At post-hatch day 1, the tunica mucosa was modified into tall folds that contained highly elongated simple tubular glands, morphologically similar to that of mature birds. The simple tubular glands extended through the upper and lower layers of the lamina propria mucosae and were lined by low columnar epithelial cells (Fig 5).

At embryonic day 14, the framework of the compound tubular glands (proventricular glands) were fully established (Fig 1). Their secretory tubules were lined by simple cuboidal cells at embryonic days 14, 17 and 19. However at post-hatch days 1, 3, 7, 14, and 21, each proventricular glands conatined several secretory tubules that were lined by both cuboid-shaped and columnar-shaped epithelial cells (Fig 5). Apical aspects of adjacent epithelial cells were seperated from each other.

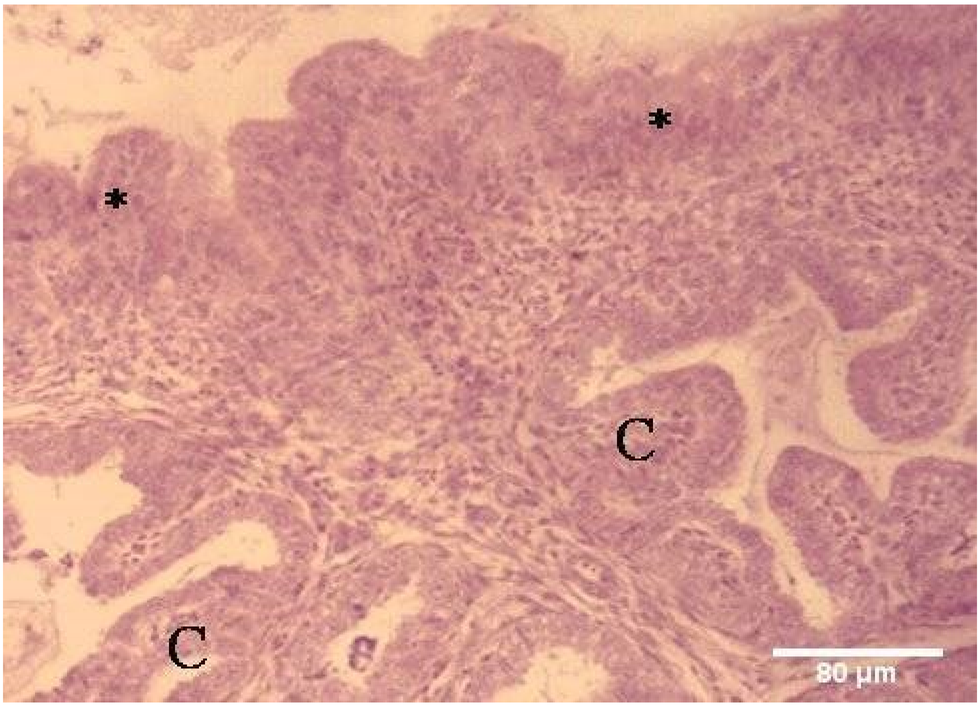

Figure 1 Photomicrograph of the proventriculus of broiler chicken at ED 14 showing locations of forming simple tubular glands (asterisks) and compound tubular glands (C). H&E stain x100.

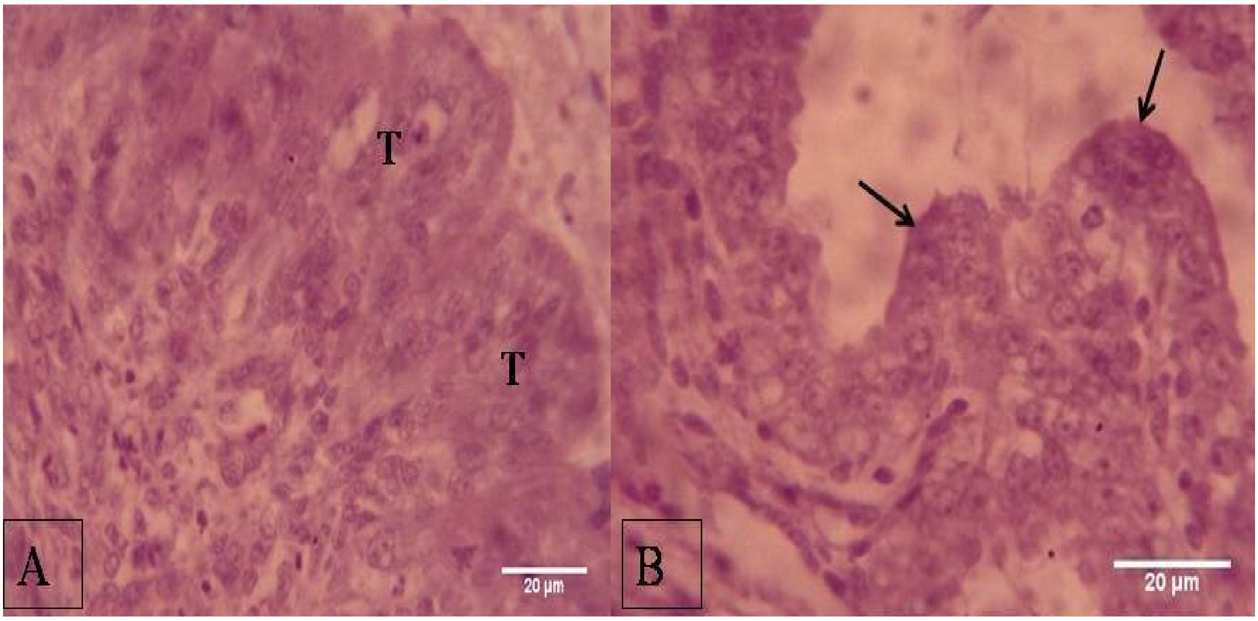

Figure 2 Photomicrograph of the proventriculus of broiler chicken at ED 14: **2A**. Showing location of forming simple tubular glands (T). H&E stain x400. **2B**. Compound tubular gland with simple cuboidal epithelia (arrows). H&E stain x400.

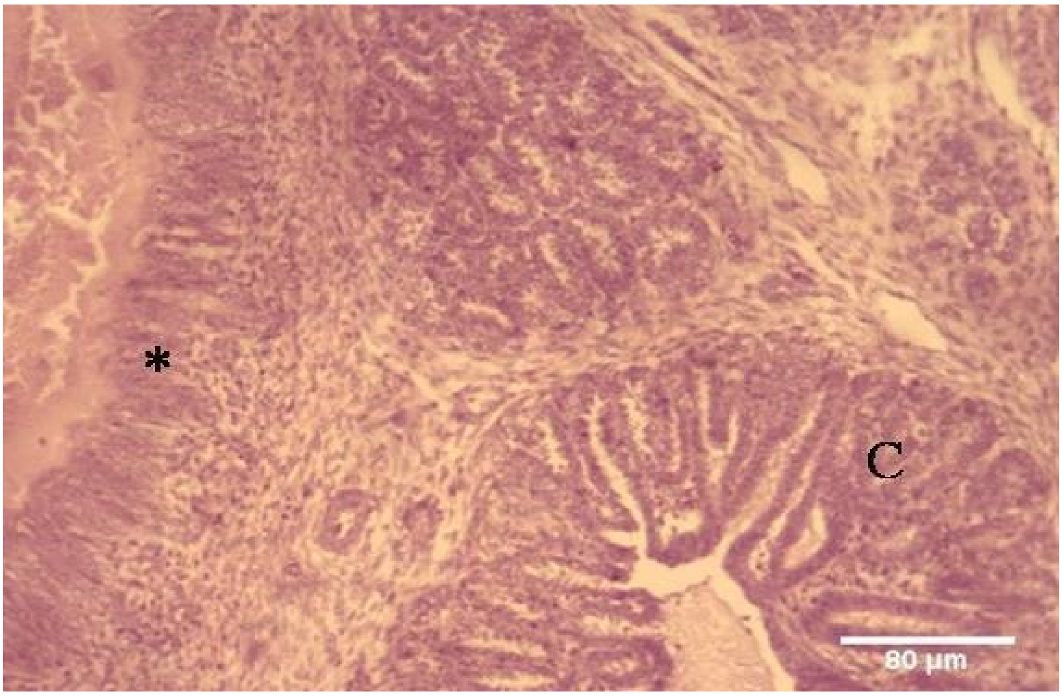

Figure 3 Photomicrograph of the proventriculus of broiler chicken at ED 17 showing location of short tubes (asterisk) in the apical aspect of tunica mucosa and compound tubular gland (C). H&E stain x100.

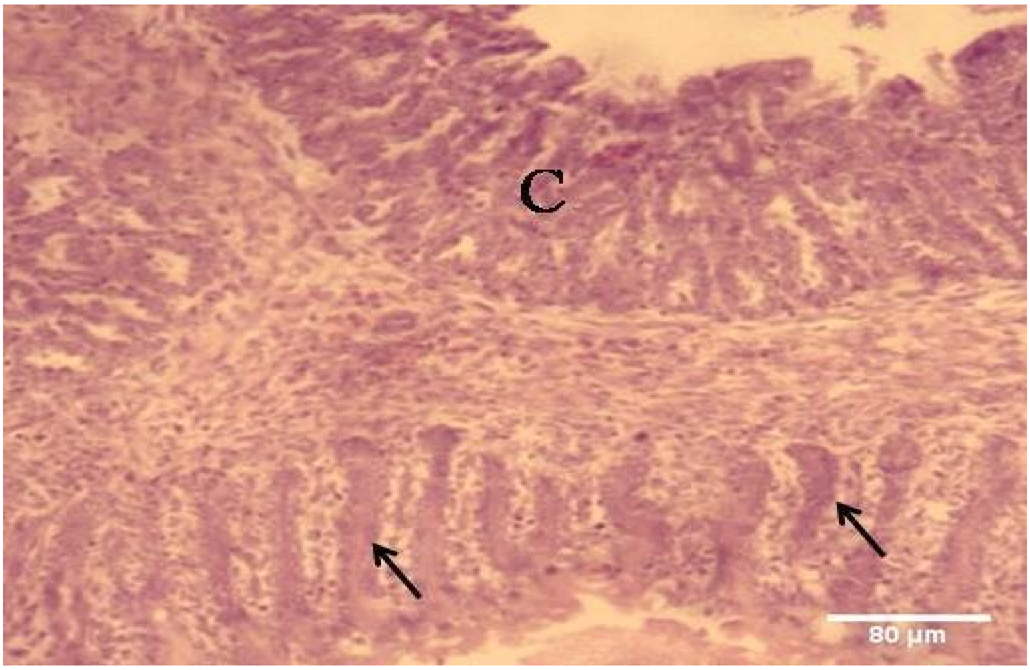

Figure 4 Porventriculus of broiler chicken at ED 19 showing simple tubular glands (arrows) and compound tubular gland (C). H&E stain x100.

Histomorphometric features of the proventriculus: The mean thickness of the proventricular wall increased linearly with age, from embryonic day 17 to post-hatch day 14 (Table 1). At post-hatch day 1, the thickness of the wall of the proventriculus was significantly ($p < 0.05$) higher than the thickness of the wall at embryonic days 14, 17 and 19. The mean thickness of the proventricular wall was significantly higher ($p < 0.05$) at post-hatch days 3 and 14 than at embryonic days 14, 17, 19 and post-hatch day 1. The mean heights of the tunica mucosa of the proventriculus of broiler chicken peaked at post-hatch day 1 with a mean value of 617.8 ± 13.4 µm. Although the width of the compound tubular glands increased with age, significant ($p < 0.05$) increases occurred at embryonic day 19, post-hatch days 1, 3 and 14 (Table 1).

Histogenesis of glands in the ventriculus: At embryonic day 14, the ventriculus of broiler chicken exhibited low mucosal folds that were characterised by the presence of lamina epithelialis mucosae and lamina propria mucosae. The upper layer of the lamina propria mucosae showed areas of poorly formed tubes with randomly arranged cells while the lower layer of the lamina propria mucosae was a mesenchymal connective tissue (Fig. 6). At embryonic day 17 and 19 elongated pits were observed extending through the upper and lower layers of the lamina propria mucosae. Each pit contained uncanalized tubular structures (Fig. 6).

At post-hatch days 1 and 3 numerous simple tubular glands were present in the lamina propria-submucosae. Each tubular gland was lined by a simple cuboidal epthelium (Fig. 7). At post-hatch days 7, 11, 14 and 21, the simple tubular glands of the ventriculus were lined by both simple cuboidal cells and low columnar epithelial cells (Figs 7). The luminal compartments of these tubular glands contained eosinophilic stained materials in H&E stained preparations. At post-hatch day 21, the ventriculus of broiler chicken showed the presence of simple branched tubular glands (Fig 7).

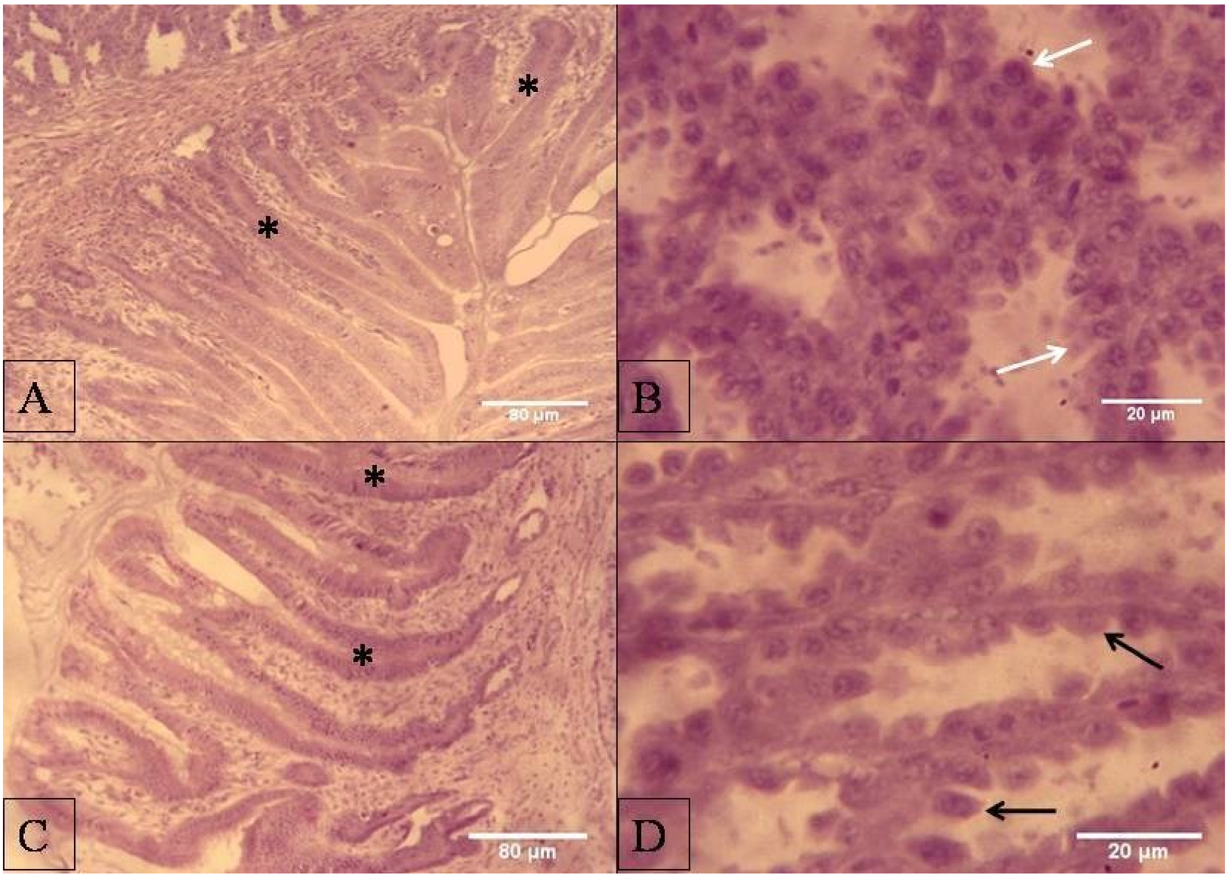

Figure 5 **(5A).** Proventriculus at PD 1 showing elongated simple tubular glands (T). H&E stain x100. **(5B).** Secretory tubules of compound tubular glands at PD 1 showing simple cuboidal and tall columnar epithelial cells (arrows). H&E stain x400. **(5C).** Proventriculus at PD 3 showing simple tubular glands (asterisks). H&E stain x100. **(5D).** Proventriculus at PD 3 showing secretory tubules of the compound tubular glands lines by tall columnar cells (arrows). H&E stain x400.

Table 1 Histomorphometric features of the proventriculus of Marshall broiler chicken (Mean ± SEM)

Prehatch (ED) and posthatch (PD) days	Thickness of Proventricular wall (µm)	Height of Tunica mucosa (µm)	Width of compound tubular glands (µm)
ED14	352.3 ± 10.7^{a}	104.2 ± 9.1^{a}	239.9 ± 15.8^{a}
ED17	313.3 ± 12.4^{a}	98.7 ± 4.9^{a}	253.7 ± 14.8^{a}
ED19	349.7 ± 15.3^{a}	123.9 ± 5.7^{ae}	311.1 ± 3.5^{b}
PD1	991.7 ± 19.9^{b}	617.8 ± 13.4^{b}	421.2 ± 10.1^{c}
PD3	1443.7 ± 128.5^{c}	529.4 ± 7.9^{c}	634.0 ± 23.1^{d}
PD14	1760.0 ± 61.8^{d}	554.8 ± 0.8^{d}	660.6 ± 5.6^{de}

Different superscripts in a column indicate significant differences across the groups ($p < 0.05$).

PAS-Alcian blue histochemistry of the proventriculus: At embryonic days 14, 19 and post-hatch days 1, 3 and 21, the lining epithelium of the proventriculus of broiler chicken demonstrated the presence of neutral mucins (magenta with PAS-AB stains) (Figs. 8A, 8B). At embryonic day 19 and post-hatch days 1 and 3, the simple tubular glands of the lamina propria mucosae showed moderate presence of neutral mucins. The compound tubular glands of the submucosal regions sparingly exhibited neutral mucins at embryonic days 14 and 19. The lining epithelium of the proventriculus at embryonic days 14, 19 and post-hatch day 1 rarely expressed acidic mucins while the lining of epithelium of the proventriculus at post-hatch days 3 and 14 showed moderate presence of acidic mucins . There was no PAS-AB reactions in the compound tubular glands of the proventriculus (Fig. 8B).

PAS-Alcian blue histochemistry of the ventriculus: At embryonic day 14, the lining epithelium of the ventriculus of broiler chicken showed moderate presence of neutral mucins (Fig 8C). This epithelium showed intense reaction with PAS-AB stain at embryonic day 19 and post-hatch days 1, 7, 14, 21, demonstrating the presence of neutral mucins (Fig 8D). The glandular lumina moderately expressed neutral mucins. The cuticle of the ventriculus of 7 and 21 day old post-hatch broiler chicken showed PAS postive reactions. Moreover, the lining epithelium of the ventriculus at embryonic days 14 and 19, post-hatch days 1, 7 and 21 sparingly expressed acidic glycosaminoglycans (Fig 8D).

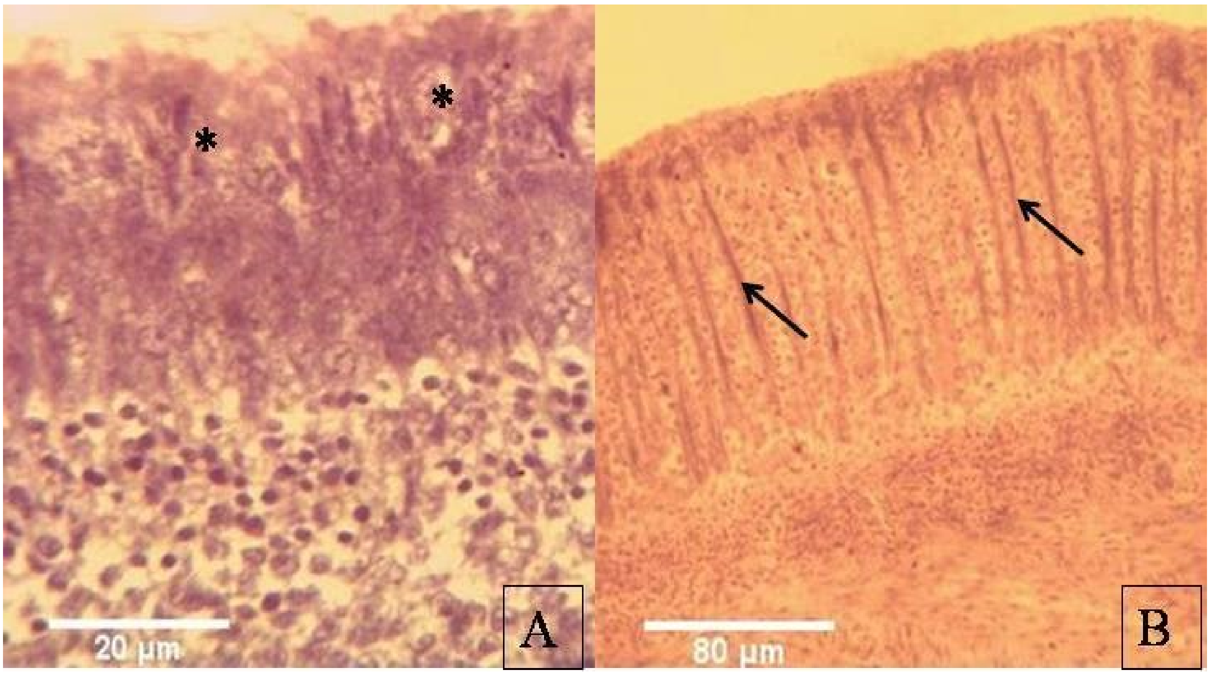

Figure 6 **(6A).** Ventriculus of broiler chicken at ED 14 showing locations of poorly formed simple tubular glands (asterisks). H&E stain x400. **(6B).** Ventriculus of broiler chicken at ED 19 showing elongated simple tubular glands (arrows). H&E stain x100.

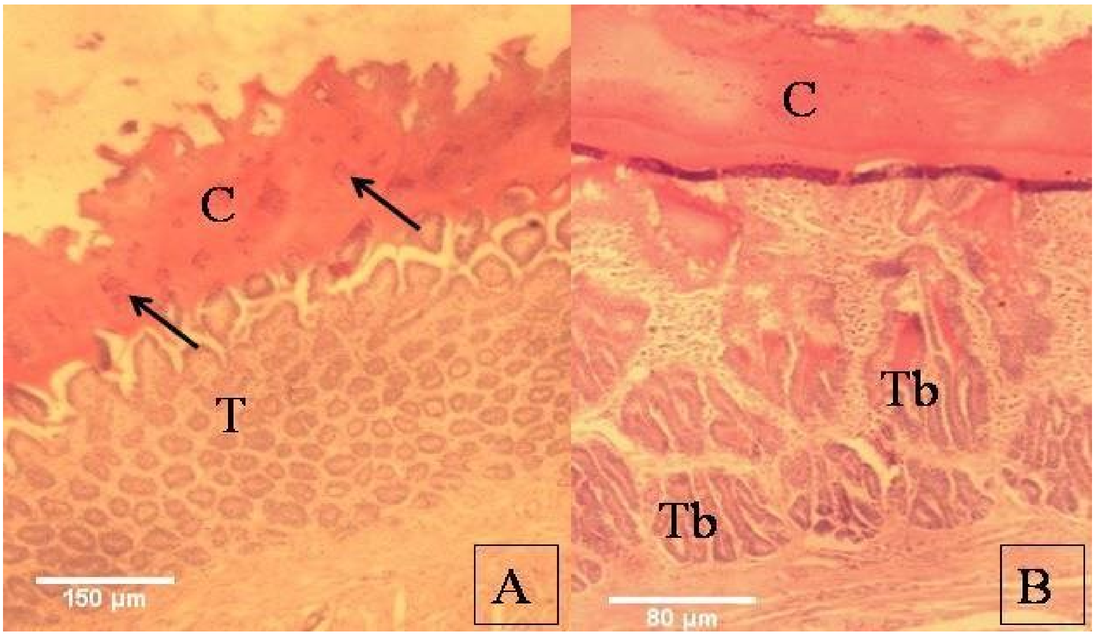

Figure 7 **(7A).** Ventriculus of broiler at PD 1 showing eosinophilic-stained cuticle (C) with areas of tubular degeneration (arrows) and simple tubular glands (T) H&E stain x40. **(7B).** ventriculus at PD 21 showing thick cuticle (C) and simple branched tubular glands (G). H&E stain x100.

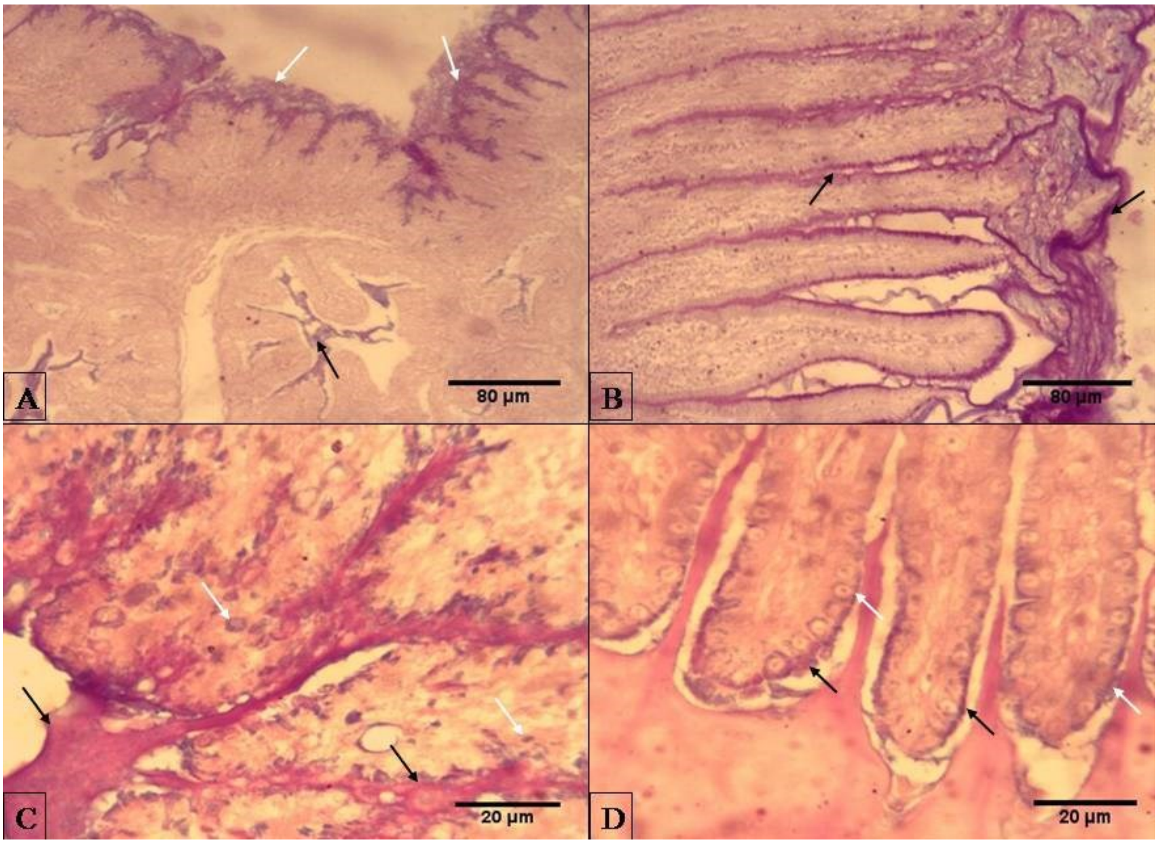

Figure 8 **(8A).** Proventriculus of broiler chicken at ED14 showing lining epithelium of lamina epithelialis mucosae (white arrows) and proventricular gland lumen (black arrow) with glycosaminoglycan reactions. PAS-AL x100. **(8B).** Proventriculus broiler chicken at PD 14 showing areas of glycosaminoglycan reactions (arrows). PAS-AL x100. **(8C).** Ventriculus at ED 14 showing neutral mucin (white arrows) and acidic mucin (black arrows) areas. PAS-AL stain x400. **(8D).** Ventriculus at PD 1 showing areas of neutral (black arrows) and acidic (white arrows) mucins. PAS-AL stain x400.

Discussion

Early developmental events in the stomach of avians could have huge implications on the future digestive functions of the gut. In the current study, although the framework of the proventriculus of broiler chicken were fully established at embryonic day 14, the glandular areas were poorly developed. The areas of randomly oriented cells which were observed in the lamina propria mucosae of the proventriculus at embryonic day 14 may represents areas of forming simple tubular glands. These disoriented cells were organised into short consistent tubes at embryonic days 17 and 19, and assumed adult morphology at hatch. Udoumoh and Ikejiobi (2017) reported the occurrence of two types of glands, simple and compound tubular glands in the proventriculus of mature African pied crow. The morphological features observed in this study suggest that changes in the shape of epithelial cells, re-arrangement of epithelial cells, epithelial invagination and tubular elongation are probably the key developmental modifications that occurred between ED 14 and PD 1 to formed the simple tubular glands in the proventriculus of broiler chicken. In this study, the compound tubular glands may develop earlier than the simple tubular glands of the proventriculus since the framework of the compound tubular glands were fully established at ED 14. Epithelial-mesenchymal interaction with embryonic chicken pepsinogen (ECPg) expresssion signal the embryonic formation of proventricular glands (Yasugi and Fukuda, 2000). However, changes in the shape of the epithelium of secretory tubules of the compound tubular glands from simple cuboidal epithelium in the embryonic ages to cubic-shaped and columnar-shaped secretory cells in the post-hatch periods of this study suggest that further epithelial modifications are required at this stages for maturation of the the secretory cells. The seperated apical ends of adjacent secretory cells of the glandular alveoli is a unique feature of the compound tubular glands of the proventriculus. Oxyntic cells and peptic cells have been reported as the key cellular constituents of the secretory tubules of the proventricular glands (McLelland, 1990; Bacha and Bacha, 2000). The observation of simple tubular glands in the lamina propria mucosae of chicken in the present study coroborates the report of Kadhim *et al*. (2011) in the proventriculus of red jungle fowl. In contrast, Imai *et al*. (1991) reported simple branched tubular glands in the proventriculus of fowl.

Several factors including environmental factors and nutrition can influence the development of glands in the gastrointestinal tract of birds (Yegani and Korver, 2008; Ganjali *et al*. 2015). In this study, the significant increase in the thickness of the wall of proventriculus at post-hatch day 1 may be a morphological adaptation caused by sudden exposure of the birds to food and other environmental factors. The development of simple tubular glands in the lamina propria mucosae may account for the increased thickness of the tunica mucosa in this study. In the present study, the mean height of the tunica mucosa of the proventriculus peaked at post-hatch day 1, suggesting that at hatch the glands may be fully developed. Moreover, the age-dependent increase in the thickness of proventricular wall and width of the compound tubular glands of proventriculus may be due to increased function of the glands. The proventriculus like the stomach of monogastric animals produces hydrochloric acid and pepsinogen (Bacha and Bacha, 2000; Svihus, 2014).

In the present study, the pattern of development of glands in the ventriculus of broiler was similar to that of the simple tubular glands of the proventriculus. The simple tubular glands of the ventriculus which were poorly formed with randomly oriented cells at ED 14, were later organised into short uncanalised tubes that were highly elongated at embryonic day 19. The glands of the ventriculus in this study which formed during the late embryonic and early post-hatch periods as simple tubular glands could be modified into simple branched tubular glands with age. This observation contrasts the reports of simple tubular glands in the ventriculus of most birds by previous authors (Catroxo *et al*. 1997; Batah *et al*. 2012; Nasrin *et al*. 2012). Cells of the lining epithelium of the ventricular glands observed in this study may be chief cells which produces protein-rich secretions that may reacts with carbohydrate moieties to form the cuticle. Areas of dark patches observed within the cuticle may reflect areas of apical degeneration of the simple tubular glands of ventriculus. Hence, the degenerated apical aspects of the the simple tubular glands could contribute to the formation of the cuticle.

Despite the poorly formed nature of the stomach glands in this study at ED 14, the presence of neutral and acidic mucin in the stomach at ED 14 suggests the secretory roles of the randomly oriented cells of the forming glands. The luminal content of neutral mucin in the ventriculus of broiler in this study at ED 14 may act as lubricants as well as protective barriers against pathogens. Ventura *et al.* (2013) reported the production of moderate amount of neutral mucin from embryonic day 6 in the ventriculus of white Leghorn while Gosomji *et al*. (2017) observed early embryonic function of the proventriculus of helmeted guinea fowl. The tubular glands of the ventriculus which sparingly expressed acidic mucin in this study may also participate in the production of neutral mucin. Acidic mucin production occured in the late embryonic life of the broiler chicken, most probably to lower the pH of the ventriculus as low pH is essential for the hardening of the cuticle (Svihus, 2011). In addition, the production of both neutral and acidic mucins may be required to moderate the gizzard environment to prevent gizzard erosion and ulceration syndrome (GEU) which is prevalent in clinically healthy broiler chicken. Overproduction of gastric acid, in other words an acidic environment alone could predispose the birds to GEU (Gjevre *et al*. 2013). The above report is supported by the findings of Selvan *et al*. (2008) who detected both acidic and neutral mucin reactions in the lamina epithelialis mucosae, but constrasts the reports of neutral mucin production only (Shyla *et al*. 1992; Kadhim *et al*. 2011). Moreover, the absence of PAS-Alcian blue reaction in the compound tubular gland of the proventriculus suggest that they are serous secreting glands, that could primarily secrete hydrochoric acid. The presence of cuboidal cells with round nuclei in the secretory tubules of the glands buttresses this fact. Our observations is strongly supported by the reports of previous authors in the stomach of African pied crow and sparrow hawk (Demirbag *et al*. 2015; Udoumoh and Ikejiobi, 2017).

In conclusion, the structural modifications of the stomach of broiler chicken demonstrated in this study provides vivid evidence that stomach glands develop rapidly in the late pre-hatch periods and assume adult morphology at hatch. These modifications may adapt the stomach for food ingestion and digestion as well as strong innate immune defense just after hatch.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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