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Full Length Research

Embryonic and early post-hatch developmental morphology of the Thymus in Nigerian indigenous chickens (*Gallus gallus domesticus*)

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ABSTRACT: The avian thymus is a primary lymphoid organ that produces T-lymphocytes that engage in active cell-mediated immune responses. The pre- and early post-hatch development of this organ in Nigerian indigenous chickens of the south-eastern region was understudied from embryonic incubation day (EID) 10 to day (D) 42 post-hatch to determine the pattern of growth and the timeline of development of the vital immunological structures using gross anatomical, histological, and morphometric techniques. The embryos were harvested from the gravid eggs for the pre-hatch studies. The thymus was collected through a ventral neck incision in both embryos and post-hatch chicks, observed for gross features, weighed and fixed in Bouin's fluid. The fixed tissues were routinely processed and stained with hematoxylin and eosin (H&E) for histological studies. The thymus is composed of a bilateral chain of 7-9 irregular flat and pale-coloured lobes whose sizes vary with age. The absolute and relative weights of post-hatch thymus increased significantly between Day 0 (D 0), Day 14 (D 14) and Day 42 (D 42), and between D 0 and D 14, respectively. The lobation of thymic tissues was not quite significant until EID 18. Between D 0 and D 42 post-hatch, thymic lobes were outstanding. Histologically, the thymus at EID 10 appeared as tissue buds on both sides of the neck. By EID 14, each lobe was enveloped by a connective tissue capsule from which thread-like trabeculae penetrated the thymic substance. At EID 18, the parenchyma showed marked differentiation of tissues into cortical and medullary regions with remarkable cortical lymphocyte accumulation. At hatch (D 0), Hassall's corpuscles were observed in the thymic medulla, and thymic cell density also significantly increased. Subsequent ages were marked by an increase in both thymic cell densities and thickness of the connective tissue capsule and trabeculae, together with the accompanying blood vessels. The early attainment of high relative weight and the high rate of proliferation of thymic lymphocytes, especially at late embryonic and early post-hatch ages, imply early establishment of cell-mediated immunity, which may offer immunological protection prior to vaccination in Nigerian indigenous chicken.

Keywords: Development, indigenous chicken, morphology, post-hatch, pre-hatch, thymus.

INTRODUCTION

In Africa, village poultry contributes over 70 per cent of poultry products and 20 per cent of animal protein intake (Kitalyi, 1998). It was estimated that in the sub-Saharan countries, indigenous chickens constitute 80 per cent of poultry production (Desha *et al*., 2016).

In Nigeria, poultry production was estimated at 165 million birds, which constituted 58.2 per cent of the total livestock production (Akintunde *et al*., 2015). Kperegbeyi *et al*. (2009) reported that 90 per cent of this estimated population of birds was indigenous poultry stock and comprised 91 per cent chickens, 3 per cent ducks, 4 per cent guinea fowl and 2 per cent turkey and others.

In most developing countries of the world, including Nigeria, inclusive poultry production faces numerous challenges ranging from disease influence to poor management practices. Indeed, worldwide disease has been identified as one of the major constraints to poultry production (Udokainyang, 2001; Fathi *et al*., 2016).

Genetic selection for disease resistance to major infectious agents in chicken has been recognised for a long time as an adjunct to non-genetic means of disease control (Boa-Amponsem *et al*., 1997; El-Safty *et al*., 2006; Chatterjee *et al*., 2007; Fathi *et al*., 2016), and as a consequence of natural selection, indigenous breeds of chicken have been shown to possess appreciable resistance to disease (Minga and Gwakisa, 2004). Similarly, experimental demonstrations have also credited high immunocompetence to the local breeds of chicken; for instance, a total score index for immunocompetence traits of CARI-Nirbheek chickens, a cross of CARI Red and Aseel (Indian local) breeds, was considerably better than that of the high-yielding breeds such as Broiler type of chicken and White Leghorn (Singh *et al*., 2004). Horst (1988) and Sonaiya *et al*. (1999) equally stated that the indigenous poultry breeds represent valuable resources for poultry development as the traits of disease resistance in them provide a basis for genetic improvement and diversification to produce breeds that will adapt to local conditions for the benefit of farmers in developing countries.

The ability to resist disease to a large extent depends on the functionality of the lymphoid system. Therefore, appreciation of disease processes and management in poultry requires a sound knowledge of the avian lymphoid system. This system is structurally divided into two distinct components: the primary or central and the secondary or peripheral components (Davison *et al*., 2008). The primary or central component includes the thymus and the bursa of Fabricius, in which the lymphocytes for cell-mediated and humoral immune responses, respectively, develop (Ratcliffe, 1989; Silverstein, 2001; Ciriaco *et al*., 2003). The secondary or peripheral component includes all areas of normal lymphocytic aggregation or proliferation outside the central component. This includes the spleen and all mucosa-associated lymphoid tissues (MALT) such as the caecal tonsil, Harderian gland, Mickel's diverticulum (Davison *et al*., 2008; Islam *et al*, 2012).

The thymus has long been reported as the first clearly recognisable lymphoid organ that is well developed as a functional organ at hatch, but attains its greatest development in sexually immature birds, with involution setting in at the onset of sexual maturity (Miller and Davies, 1964; Payne, 1971). Besides its primary responsibility in active immunity, the thymus also plays an essential role in the development of the peripheral tissues and their associated adaptive immune functions (Muthukumaran *et al*., 2011).

Indeed, several studies have been carried out on various aspects of the avian thymus, but information on the properties of the developing thymus in Nigerian indigenous chicken remains insufficient. Therefore, the primary objective of this study is to determine the pattern of growth and the timeline of the development of vital immunological structures of the thymus in indigenous chickens of Nigeria. Results obtained may contribute to improving management processes in terms of disease control in chicken.

MATERIALS AND METHODS

Eighteen (18) chick embryos and thirty (30) post-hatch chicks of either sex were used for the study. However, one hundred and twenty indigenous chicken eggs were acquired from apparently healthy laying indigenous chickens raised by the free-range backyard method in Ovim community, Isuikwuato Local Government Area of Abia State, Nigeria. The eggs were incubated in an electric egg incubator at 37°C and 55 – 60% relative humidity (Yoshimura *et al*., 2009; Oznurlu *et al*., 2010).

Six (6) chick embryos were harvested on each of Embryonic Incubation Day (EID) 10, 14 and 18 by cracking the fertilised egg shell around the vertical midline using a small knife. The entire transverse diameter of the shell was cut using surgical scissors to expose the embryo. The yolk sac was detached from the embryo by severing the stalk, and foetal debris was removed. The weights of embryos were determined using a SCOUT PRO – 200 X 0.001g (OHAUS Corporation) analytical balance. The remaining eggs were left to hatch for the post-hatch studies.

Following hatching, the chicks were housed in a deep litter pen in the poultry unit of the College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. The chicks were fed commercially compounded feed (Topfeed^R, broiler chick mash), and water was given *ad libitum*. No medications, including vaccinations were given throughout the period of study.

Six (6) randomly selected chicks were sacrificed by cervical subluxation at hatch or day 0 (D 0) and on each of D 7, D 14, D 28 and D 42 post-hatch after the live body weights have been determined using SCOUT PRO – 200 X 0.001g (OHAUS Corporation) analytical balance (for weights < or = 200g) and MEASURE-TECH MB 2610 triple beam balance (for weights > 200g). The thymus was collected through a mid-ventral neck incision using the approach of Alboghobeish and Mayahi (2003), weighed and examined for gross features. The organ body weight index was obtained for each chicken as described using the formula: (Organ body weight index= weight of organ×100/Body weight of the chicken; Lucio and Hitchner, 1979). Data on weight were subjected to one-way analysis of variance (ANOVA) using SPSS version 23. The variant means were separated using Duncan's multiple range test. Significance was accepted at $p<0.05$ (IBM Corporation, Armonk, NY, USA, 2015).

Histological investigation

Slices of the thymus of different ages were fixed in Bouin's fluid (75% picric acid, 20% acetic acid and 5% formaldehyde) and transferred to 70% ethanol after 24 hours to avoid excess tissue hardening. The samples were processed by placing them in ascending grades of ethanol in the following order, 70% for 1 hour, first 95% ethanol for 1 hour and second 95% ethanol for 1¼ hours, first absolute ethanol for 1½ hours, second absolute ethanol for 2 hours and third absolute ethanol for 2 hours to ensure proper dehydration of the tissues. The tissues were transferred to a mixture of equal volumes of ethanol and xylene, where it was left overnight, and later cleared in two changes of xylene for 1 hour each. It was then infiltrated for 1 hour with molten paraffin wax (salsa wax) in the oven at 60°C. The tissues were embedded in paraffin wax, trimmed and mounted on a wooden chuck, and then sectioned at 5 μm thickness. The sections were floated in a floating-out bath from where it was picked with clean albumenised slides. Excess wax was removed by two changes of xylene, followed by hydration through descending grades of ethanol in the following order: absolute ethanol, 95% ethanol and 70% ethanol for 2 minutes each. The sections were stained with Ehrlich hematoxylin for 15 minutes, washed in water for 5 minutes, differentiated in 1% acid alcohol for 3 seconds, and blued in running tap water for 10 minutes. It was then counterstained with eosin for 2 minutes. Excess eosin was removed in ascending grades of ethanol in the following order: 75% ethanol, 95% ethanol and absolute ethanol for 2 minutes each. It was then cleared in two changes of xylene and cover slipped with Depex mountant. The slides were viewed under a light microscope (BestSco, e; China), and selected images were captured using moticam 2.0 digital camera attached to a computer.

RESULTS

The body weights of both embryos and post-hatch chicks increased with age but only varied significantly ($p<0.05$) between EID 10 and EID 18 (Table 1). The mean weight of post-hatch thymus increased between D 0, D 7 and D 14 with a decrease at D 28, followed by an increase at D 42. A significant ($p<0.05$) increase in mean thymic weight occurred between D 0, D 14, and D 42. The mean weight at D 0 and D 42 were 0.13±0.00 g and 0.78±0.15 g, respectively. The relative weights were similar at D 0 and D 7. There was an increase at D 14, followed by a decrease at D 28 and finally an increase at D 42. The relative weight of the thymus at D 0 and D 42 were 0.49±0.01% and 0.45±0.06% respectively. Significant difference ($p < 0.05$) in relative weight only occurred between D 0 and D 14. The maximum relative weight of 0.60±0.03% was attained at D 14 post-hatch (Table 2).

Table 1. Absolute Body Weight of chicks at various ages.

Age of animal (day)	Weight of animal (grams)
EID 10	2.82±0.39^{a}
EID 14	7.40±0.51^{a}
EID 18	22.73±1.05^{bc}
0	27.23±0.38^{c}
7	43.98±1.34^{d}
14	66.89±0.06^{e}
28	90.12±5.06^{f}
42	168.59±0.2^{g}

*Results with different superscripts in a column are significantly different at $p<0.05$.

Gross morphology

The embryonic and post-hatch thymus was observed as pale coloured, flat, chain-like, lobed structure on both sides of the neck parallel to the vagus nerve and the internal jugular vein. It extended from the cranial cervical region to the thoracic inlet. Each lobe, which was more prominent in the post-hatch chicks, was well-separated from the other and lay in the subdermal connective tissue of the neck. The number of lobes ranged from 7-9.

Differentiation of the thymus at EID 10 was relatively poor; hence, its gross features were difficult to identify as it blended in colour with other structural relatives, except the red stripe-like jugular vein.

At EID 14, the thymus showed a marked organisation of its structures into lobes. Each pale-coloured lobe was flat in shape and adhered to the other by connective tissue. By day 18 of incubation, the thymus presented more distinct flat-shaped lobes whose colour remained pale (Figure 1).

At hatch (D 0), the colour and shape of the thymic lobes became more prominent due to an increase in size of the organ. There was a minimum of 7 and 6 lobes on the right and left sides of the neck, respectively. At D 7, D 14, D28 and D 42 post hatch, thymic lobes were still pale in colour and flat in shape, but varied remarkably in size among the respective ages (Figure 2).

Histological observation

At EID 10, the thymus appeared as serial tissue buds on both sides of the neck (Figure 3). At EID 14, each thymic lobe was covered externally by a primitive capsule of connective tissue, but differentiation of thymic parenchyma into cortical and medullary regions was unapparent (Figure 4). However, thymic parenchyma showed a high level of mesenchymal connective tissue network within which were primitive thymic cells. These cells were dominated by round euchromatic reticular cells that possessed prominent nucleoli with few thymocytes. The primitive connective tissue septae had penetrated the superficial regions of the parenchyma, and in it were blood vessels (Figure 5).

By day 18 of incubation, there was a pronounced increase in the thickness of the thymic capsule and the trabeculae from it traversed deeper into the stroma, creating more

Table 2. Weights (absolute and relative) of the thymus at various ages.

Age of Animal(day)	Weight of animal (grams)	Weight of thymus (gram)	Relative weight of thymus (%)
0	27.23±0.38[a]	0.13±0.00[a]	0.49±0.01[a]
7	43.98±1.34[b]	0.20±0.02[a]	0.46±0.03[ac]
14	66.89±0.06[c]	0.40±0.02[b]	0.60±0.03[b]
28	90.12±5.06[d]	0.33±0.05[bc]	0.36±0.03[c]
42	168.59±0.72[e]	0.78±0.15[d]	0.45±0.06[ac]

*Results with different superscripts in a column are significantly different at $p<0.05$.

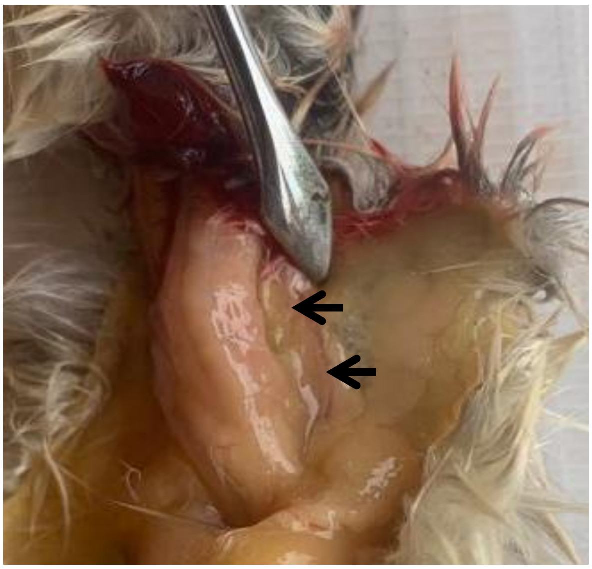

Figure 1. Gross photograph of a dissected section of the cervical region of an 18-day-old embryo showing the embryonic thymic lobes (arrow) *in situ*.

Figure 2. Gross photograph of a dissected section of the cervical region of a 7-day-old chick showing the thymic lobes (arrow) *in situ*. Note the colour and flat shape of each lobe.

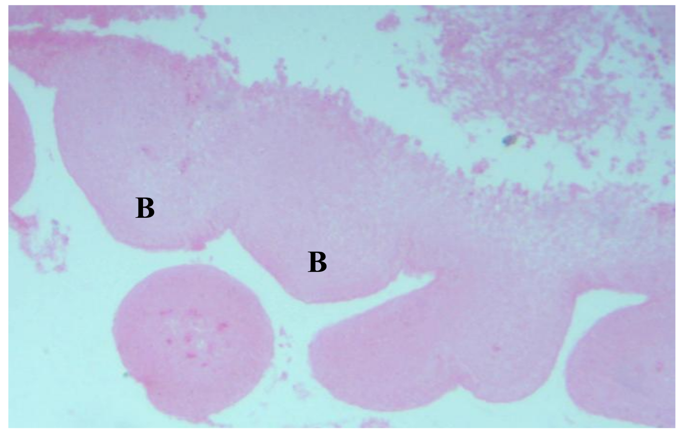

Figure 3. Photomicrograph of a section of the embryonic thymus at incubation day (EID) 10 showing buds, B of primitive thymic tissue (Haematoxylin and Eosin stain; X100).

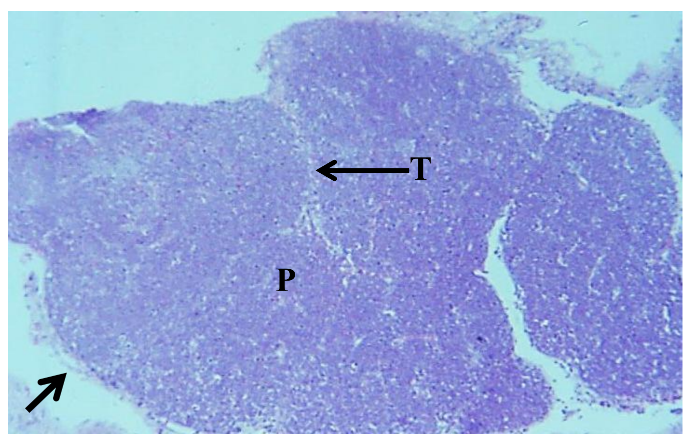

Figure 4. Photomicrograph of a section of embryonic thymus at EID 14 showing a thymic lobe. Note the capsule (arrow) and the undifferentiated or uniformly stained thymic parenchyma, P. T: trabeculae (Haematoxylin and Eosin stain; X100).

visible incomplete lobules, and the cortical and medullary regions of the respective lobules appeared more conspicuous (Figure 6). The thymic cells advanced in development, and equally increased in density, especially in the cortical region. The medulla was composed mainly

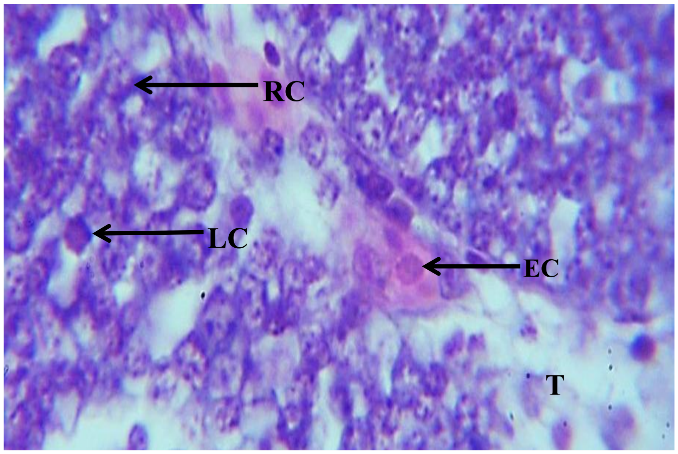

Figure 5. Photomicrograph of a section of the thymus at EID 14 showing the parenchyma. Observe the thymic cells in the mesenchymal tissue and the trabecula, T. LC: lymphocyte; RC: reticular cell; EC: erythrocyte in blood vessel (Haematoxylin and Eosin stain; X1000).

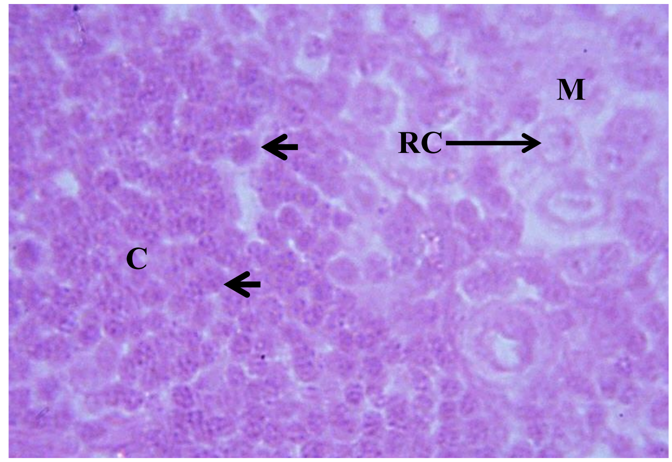

Figure 7. Photomicrograph of a section of the thymus at EID 18 showing the parenchyma. Note the thymic cell density. Lymphocyte: arrow; RC: reticular cell; C: cortex; M: medulla (Haematoxylin and Eosin stain; X1000).

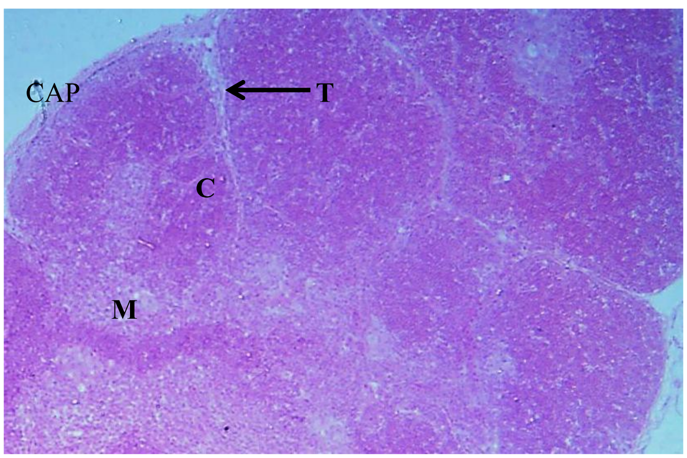

Figure 6. Photomicrograph of a section of the thymus at EID 18 showing the parenchyma with prominent cortex, C and medulla, M. CAP: capsule; T: trabeculae (Haematoxylin and Eosin stain; X100).

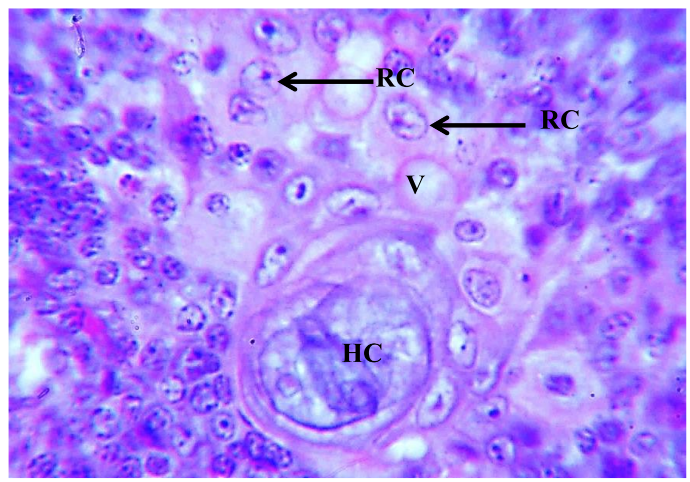

Figure 8. Photomicrograph of a section of the thymus at hatch showing the thymic parenchyma. Note the Hassall's corpuscle, HC, in the medulla. RC: reticular cell; LC: lymphocyte; V: VACUOLE (Haematoxylin and Eosin stain; X1000).

of reticular cells, but no Hassall's corpuscles were identified (Figure 7).

At hatch (D 0), the trabeculae increased in thickness, and the stroma showed more distinct cortex and medulla. There was also a marked increase in lymphocyte density and frequency of plasma cells, especially in the cortical region. Hassall's corpuscle, surrounded by lymphocytes and reticular cells with some vacuoles, had developed in the medulla (Figure 8).

By D 14 post-hatch, the trabeculae traversed the entire stroma and compartmentalised the parenchyma into units with separate cortical and medullary regions and the medulla by proportion appeared larger than the cortex (Figure 9).

At D 28 and D 42, lymphocytes still dominated the thymic cortical cell population, while the medulla possessed, in the neighbourhood of Hassall's corpuscle, a cluster of degenerating cells walled off by epithelial-like reticular cells. Similar cells were equally distributed in single units within the medulla (Figure 10).

DISCUSSION

The location of the thymus is parallel to the jugular vein and vagus nerve on both sides of the neck, and its appearance is as two long chains of lobed structures that extend from the cranial cervical region to the thoracic inlet

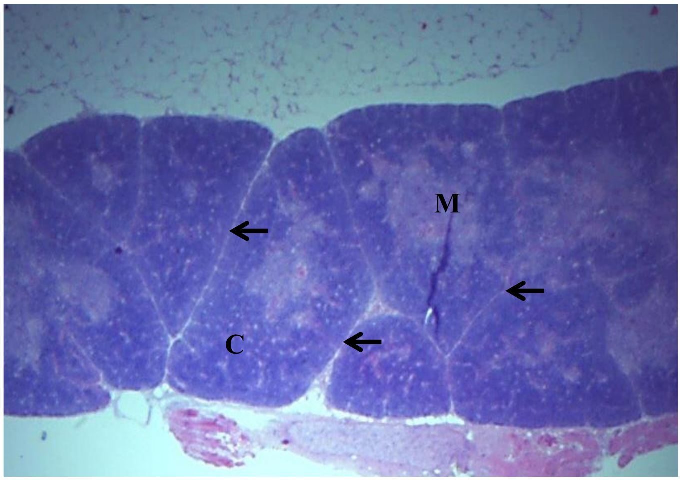

Figure 9. Photomicrograph of a section of the thymus at D7 showing the thymic parenchyma. Note the cortex, C; medulla, M and the trabeculae (arrow, Haematoxylin and Eosin stain; X100).

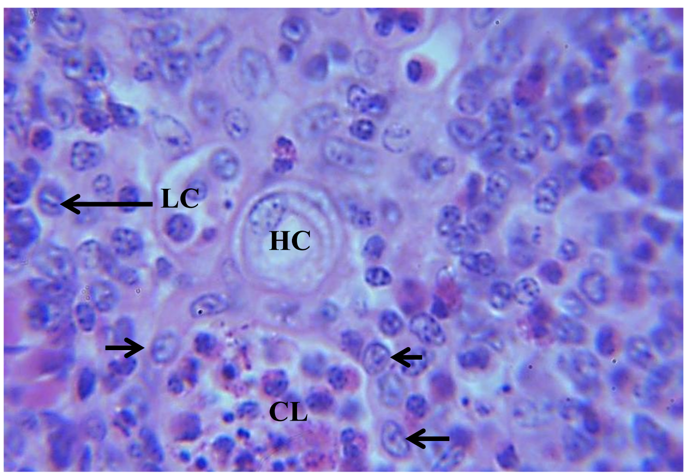

Figure 10. Photomicrograph of a section of the thymus at day 28 showing the parenchyma. Note the cluster of cells, CL surrounded by epithelial reticular cells (arrow). HC: Hassall's corpuscle; LC: lymphocyte (Haematoxylin and Eosin stain; X1000).

in the Nigerian indigenous chicken is in agreement with the reports of Hodges (1974) in white leghorn chicken, Akter *et al*. (2006) in broiler chickens, Muthukumaran *et al*. (2011) in turkeys and Haseeb *et al*. (2014) in Aseel chicken.

The colour of the thymus of Nigerian indigenous chickens was pale at the various ages studied, except the early embryonic thymus, which was pale white. So far, there has been no suggested relationship between the colour of the thymus and its immunological functions; however, our observation on the colour of the thymus in Nigerian indigenous chickens agrees with the report of Akter *et al*. (2006) in broiler chickens. In the Aseel chicken, Haseeb *et al*. (2014) noted that the thymus was pale white to yellowish white. Sultana *et al*. (2011) observed that the thymus in ducklings of Bangladesh appeared similar to that of Aseel chickens. However, Muthukumaran *et al*. (2011) reported that the colour of the thymus varied from pale red to pink in the turkey.

In Nigerian indigenous chicken, the thymus possessed 7 - 9 lobes, with a few individuals having up to 12 lobes on either side of the neck. The number of thymic lobes from reports of previous authors varied in different breeds and even in individuals of the same breed. However, the immunological significance of the number of thymic lobes has not been clearly stated in the literature, but it may indirectly influence immune potentials since the immune-competent cells inhabit the thymic lobes; hence, the more the number of lobes, the higher the number of immune-competent cells. Muthukumaran *et al*. (2011) and Nnadozie *et al.* (2019) reported 6 - 8 lobes and 5 - 6 lobes, respectively, on each side of the neck in the turkey. Sultana *et al*. (2011) observed 5 lobes on each side of the neck in indigenous ducklings of Bangladesh, while in the white Pekin duck and goose, Bahadir *et al*. (1992) recorded 4 - 7 and 6 - 9 lobes on the right side and 4-6 and 5 - 9 lobes on the left side, respectively. Haseeb *et al*. (2014) stated that there were 7-9 lobes on the right side and 6-8 lobes on the left side in the Aseel chicken. Species differences may be the cause of the variations in the number of thymic lobes.

The weights of the thymus increased progressively with age during the post-hatch periods of development, although there was a drop at week 4. The drop in weight of the thymus as observed may be due to the exit of immunological cells from the thymus to the peripheral tissues, where defence activities are required. In Sonali chicken, the mean weight of the thymus increased significantly from day 1 to day 42 post-hatch before regressing at day 56. A similar observation was made in deshi chicken, where the average weight of the thymus increased up to 3 months (Khalil, 2001). Also, Onyeanusi and Onyeanusi (1990) reported an equivalent increase in thymic weight with age in guinea fowl.

Evaluation of the relative weight of the thymus showed that the thymus attained maximum relative weight at the 2nd week of age during the period under investigation. This indicates that the growth rate of the thymus is fast in the indigenous chicken, and by implication, the establishment of the immunologically relevant tools of the thymus might as well have been accomplished. This observation suggests an early development of an active cell-mediated immune defence mechanism in Nigerian indigenous chickens. The observed drop in relative weight at D 28 may be due to an accelerated increase in body weight of the chicks at D 28, rather than a decrease in organ weight. Onyeanusi and Onyeanusi (1990) noted that the thymus in Nigerian indigenous guinea fowl attained its maximum relative weight at 4 weeks of age.

Histologically, budding of thymic tissues at early embryonic incubation day 10 (EID 10), as observed in Nigerian indigenous chicken, is similarly reported in the local chicken of Bangladesh (Islam *et al*., 2012). However, Soliman *et al*. (2014) state that the thymic primordia can be detected on both sides of the neck beside the jugular vein in the 4-day-old chicken embryo. The development of connective tissue capsule and trabeculae and subsequent formation of thymic lobules at EID 14 in Nigerian indigenous chicken are similarly reported in high-yielding chickens (Venzke, 1952). However, compartmentalisation of the parenchyma into cortex and medulla at EID 12 in the high-yielding chicken is not quite obvious in Nigerian indigenous chicken even at EID 14. The developmental pattern of the thymus in native chicken of Bangladesh is similar to that of Nigerian indigenous chicken, where parenchymal differentiation commences around EID 14 (Islam *et al*., 2012). Soliman *et al*. (2014) noted that septae formation as well as the appearance of the first lymphopoietic cells occurred as early as EID 8 in chicken. They also observed that at EID 12, the capsule with their associated septae was well developed, and differentiation of cortex and medulla was equally recognisable. Increase in thymic cell densities with further compartmentalisation of thymic lymphocytes into cortical and medullary regions, as observed in the indigenous chicken at EID 18, may be in preparation for the immunological challenges ahead, since the chicks will hatch in a few days and become exposed to the hazards of the environment. A similar increase in thymic cell density at late incubation day 18 was reported by Islam *et al*. (2012) and Soliman *et al*. (2014) in chicken.

At hatch (D 0), observations from the study indicate that thymus in indigenous chickens advances apparently in development, although the development of thicker capsule and trabeculae with distinct lobules are more of structural changes; but the observed increase in lymphocyte densities and appearance of plasma cells are indication that the thymus may have acquired potentials of eliciting immunological responses capable of protecting the chicks as they become exposed to the environmental microbes.

The typical thymic structure, the Hassall's corpuscles, were first detected in the indigenous chickens' thymic medulla at hatch. However, the functional significance of this structure is not known, but it may be involved in the turnover of epithelial-reticular cells as proposed by Davison *et al*. (2008). Islam *et al*. (2012) first observed this structure in 20 days 20-day-old embryo of native chicken of Bangladesh, but Soliman *et al*. (2014) and Itoi *et al*. (2001) note that the Hassall's corpuscles are detected in 16-day-old chick embryos. The cluster of cells enveloped by epithelial-reticular-like cells in the medulla may be pyknotic cells that probably await elimination from the thymus.

Structural transformations associated with the thymus during the rest of the post-hatch periods include progressive proliferation of immunological cells that may be targeted at stabilising the thymus for enhanced defence activities. The overall appearance of the thymus at day 42 post-hatch signifies a full-grown thymus with features characteristic of avian thymus. The possession of a parenchyma that is apparently compartmentalised into cortical and medullary regions, with a dense lymphocyte population and medulla consisting of numerous epithelial-reticular cells, is an obvious feature observed in the thymus of Nigerian indigenous chickens. Similar findings are documented in various avian species (Ciriaco *et al*., 2003; Sultana *et al*., 2011; Song *et al*., 2012; Soliman *et al*., 2014).

Conclusion

The gross morphology and histology of the embryonic and post-hatch thymus of Nigerian indigenous chickens are relatively similar to those of other studied breeds of chicken. However, the growth rate of the thymus in both embryos and post-hatch chicks indicates a fast-growing lymphoid apparatus in this breed of chicken. Similarly, the appreciable proliferation of thymic lymphocytes at late embryonic and early post-hatch periods of development also suggests early development of cell-mediated immunological tools that can afford protection to the unvaccinated chicks of this breed of chicken.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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