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# Gross and histological study of developing spleen in pre- and post-hatch Nigerian native chicken (*Gallus gallus domesticus*)

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**ABSTRACT:** The avian spleen is a primary peripheral lymphoid organ located in the region dorsal to the gonad, ventral to the liver and lateral to the stomach, just opposite the dorsal surface of the right hepatic lobe. It plays both lymphopoietic and hematopoietic roles, depending on the respective amounts of white pulp and red pulp elements in the parenchyma. The pre- and post-hatch development of the spleen of native chicken ecotype predominant in the south eastern region of Nigeria was studied from embryonic incubation day (EID) 10 to day (D) 42 post hatch using gross anatomical and histological techniques to detect age-related developmental changes associated with the spleen and the relative time of establishment of the basic immunological structures of the spleen. The embryos were harvested from the eggs at different embryonic ages for the pre-hatch studies. The spleen was collected through a ventral abdominal incision in both embryos and post-hatch chicks, weighed and fixed in neutral-buffered formalin. The fixed tissues were routinely processed and stained with haematoxylin and eosin (H&E) for histological studies. The mean weight of the spleen increased significantly ($p < 0.05$) between D 14, 28 and 42, while the relative weight varied significantly between D 14 and 28. The spleen was bean-shaped at EID 10 and throughout the post-hatch periods, but it became triangular at EID 14. The colour was relatively red in both embryos and post-hatch chicks. At EID 10, the parenchyma was undifferentiated into white and red pulp regions, but splenic sinusoids had developed. At EID 14, lymphocytes had infiltrated into the parenchyma and white and red pulp zones fairly distinct. Apparent white and red pulp regions appeared at EID 18, but were more prominent at hatch (D 0). Ellipsoids and periarteriolar lymphoid sheath equally developed at hatch. By D 7, the arterial wall and the capsule increased in thickness. The parenchyma appeared to be dominated by the white pulp elements between D 0 and D 42. There was also a progressive increase in lymphocyte densities during the post-hatch periods, but splenic nodules only developed at D 42. The development of the spleen as a functional peripheral lymphoid organ in native chickens of Nigeria occurred in the embryos but progresses after hatch, and the dominance of the parenchyma by the white pulp during the embryonic and early post-hatch periods of development emphasised lymphopoietic responsibility of the spleen in Nigerian native chicken.

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

## INTRODUCTION

The ability of chickens to resist or manage disease conditions has long been one of the major factors considered in the selection of native chicken strains for smallholder rural poultry flocks rather than the production performances (Moreki *et al*., 2010; Moges *et al*., 2010; Negassa *et al*., 2014). This natural ability depends on the activities of the immune system, whose primary functional units are produced in the lymphoid organs.

The avian lymphoid organs or tissues include the bursa of Fabricius, thymus, spleen, the mucosa-associated lymphoid tissues such as the caecal tonsil, Mikel's diverticulum, Harder's gland and other regions of normal lymphocytic aggregation (Davison *et al*., 2008). Structurally, the chicken lymphoid system is divided into two components: the primary or central and the secondary or peripheral components (Islam *et al*., 2012). The primary component comprises the thymus and the bursa of Fabricius that produce lymphocytes which play significant roles in active immune responses (Ratcliffe, 1989; Silverstein, 2001; Ciriaco *et al*., 2003); while the secondary component such as the spleen, caecal tonsil, Mickel's diverticulum, Harder's gland, Peyer's patches function as reservoirs for the immune competent lymphocytes produced by the bursa of Fabricius and thymus (Davison *et al*., 2008; Islam *et al.,* 2012).

The avian spleen, a major peripheral lymphoid organ, plays a vital immunological role in addition to haemopoietic responsibility. Histology of the spleen showed that the splenic parenchyma is composed of the white pulp and the red pulp, whose proportions may vary among avian species. According to Smith and Hunt (2004), species variation in the proportion of pulp elements at hatch suggests different functional responsibilities of the spleen at this stage of life, since the functions of the embryonic and neonatal spleen in avian species are different. Even during post-hatch periods of life, dominance of splenic parenchyma by white pulp elements no doubt emphasises lymphopoietic responsibility. This is because of the abundance of primary immune-potent lymphocytes in the white pulp. Indeed, avian species show remarkable variations in the proportion of the splenic pulp elements, probably in accordance with the functional demands of the species in question. In the guinea fowl, Onyeanusi (2006) reported the dominance of the red pulp at hatch, while in quail, there was dominance of the parenchyma by the white pulp (Liman and Bayram, 2011). Equal proportions of both pulp tissues at certain stages of development have also been noted in some species (Onyeanusi, 2006; Liman and Bayram, 2011).

However, irrespective of the wide range of studies already carried out on avian lymphoid structures, information on the developmental properties of the spleen in Nigerian native chicken is still insufficient. Therefore, the aim of the study is to characterise the gross morphology and histology of the developing spleen to identify the relative time of establishment of the basic immunological structures of the spleen in Nigerian native chicken from EID 10 to D 42 post-hatch.

## MATERIALS AND METHODS

A total of eighteen chick embryos and thirty post-hatch chickens of either sex were used for the study. However, one hundred and twenty indigenous chicken eggs were acquired from apparently healthy laying native chickens raised by free 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$^{o}$C and 55 – 60% relative humidity (Yoshimura *et al*., 2009; Oznurlu *et al*., 2010).

Six chick embryos were harvested on Embryonic Incubation Day (EID) 10, six at EID 14 and six at EID 18 by cracking the gravid egg shell around the vertical midline using a knife, and the transverse diameter 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 on SCOUT PRO – 200 X 0.01g (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 mesh), and water was given *ad libitum***.** No medications, including vaccination, were given throughout the period of study.

Six randomly selected chicks were sacrificed at hatch or day 0 (D 0), D 7, D 14, D 28 and D 42 post-hatch by inhalation anaesthesia using chloroform soaked in cotton wool in a lid plastic container after the live body weights have been determined using either SCOUT PRO – 200 X 0.01g (OHAUS Corporation) analytical balance or MEASURE-TECH, MB 2610 triple beam balance (for weights > 200g). The spleen of both embryos and post-hatch chicks was collected by ventral abdominal dissection using the approach of Alboghobeish and Mayahi (2003), weighed and examined for gross features. Data on weight were subjected to one-way analysis of variance (ANOVA). Variant means were separated using Duncan's multiple range test, and significance was accepted at $p<0.05$.

### Histological investigation

Slices of the spleen 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 tissues 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. It was then transferred to a mixture of equal volumes of ethanol and xylene, where it was left overnight. It was later cleared in two changes of xylene for 1 hour each. It was 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 taken to the microtome for sectioning at 5 µm thickness. The sections were floated in a floating-out bath from where it was picked with clean albumenised slides. The slides were placed in

**Table 1**. Absolute body weights of embryos and post-hatch chicks at various ages.

| Age of animal (day) | Weight of animal (gram) |
|---|---|
| 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.

**Table 2.** Weights (absolute and relative) of the spleen of post-hatch chicks at various ages.

| Age of animal (day) | Weight of Animal (gram) | Weight of spleen (gram) | Relative weight of spleen (%) |
|---|---|---|---|
| 0 | 27.23±0.38[a] | 0.01±0.00[a] | 0.05±0.01[a] |
| 7 | 43.98±1.34[a] | 0.03±0.00[a] | 0.07±0.01[a] |
| 14 | 66.89±1.06[a] | 0.06±0.00[a] | 0.10±0.00[a] |
| 28 | 90.12±5.06[a] | 0.23±0.02[b] | 0.26±0.02[b] |
| 42 | 168.59±13.72[a] | 0.45±0.08[c] | 0.26±0.03[b] |

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

a staining dish and excess wax was removed by two changes of xylene, hydrated in 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, and then 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 eosine for 2 minutes. Excess eosine 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), and selected images were captured using a Moticam 2.0 digital camera attached to a computer.

## RESULTS

Results showed that 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 the spleen increased progressively between D 0 and D 42. Significant increase ($p < 0.05$) in splenic weight was recorded between D 14, D 28 and D 42. The mean weight at D 0 and D 42, respectively, was 0.01±0.00g and 0.45±0.03g. The relative weight equally increased with age but recorded equal values at D 28 and D 42. Significant difference ($p < 0.05$) in relative weights was recorded only between D 14 and D 28. The relative weights of the spleen were 0.05±0.01% and 0.26±0.03% at D 0 and D 42, respectively. The maximum relative weight of 0.26±0.03% was attained at D 28 (Table 2).

### Gross morphology

The spleen in indigenous chicken was located dorsal to the gonads, ventral to the liver and lateral to the stomach, just opposite the dorsal surface of the hepatic lobe. The relatively bean-shaped embryonic spleen was small in size and reddish in colour at EID 10. At EID 14, the light red-coloured spleen appeared triangular in shape with a slightly convex dorsal free border. By EID 18, there was an increase in the size of the organ, but the shape remained relatively triangular, and the colour was reddish brown (Figure 1).

At hatch (D 0), there was a further increase in size and the shape transformed to a bean-shape while the colour was dark red. The gross morphology of the spleen at D 7, D 14, D 28 and D 42 did not vary significantly both in colour and shape from the spleen at hatch (Figure 2).

### Histological observations

At EID 10, the spleen was already covered by a capsule of

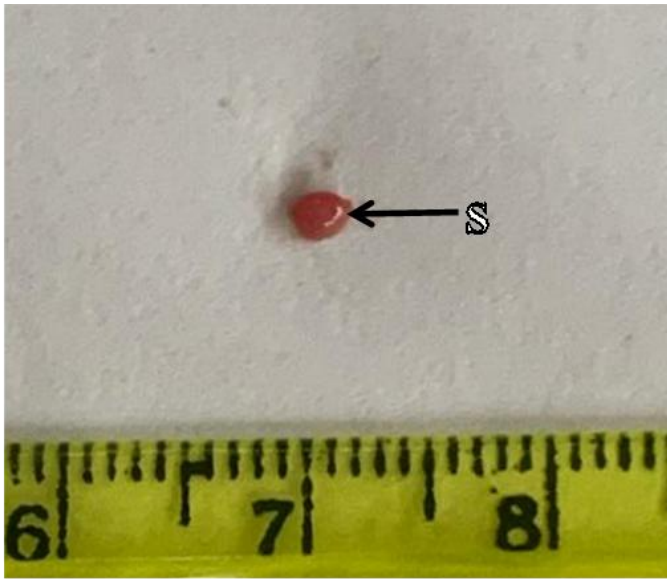


**Figure 1.** Gross photograph of a dissected spleen, S at embryonic incubation day (EID) 14. Note the shape and colour of the organ.

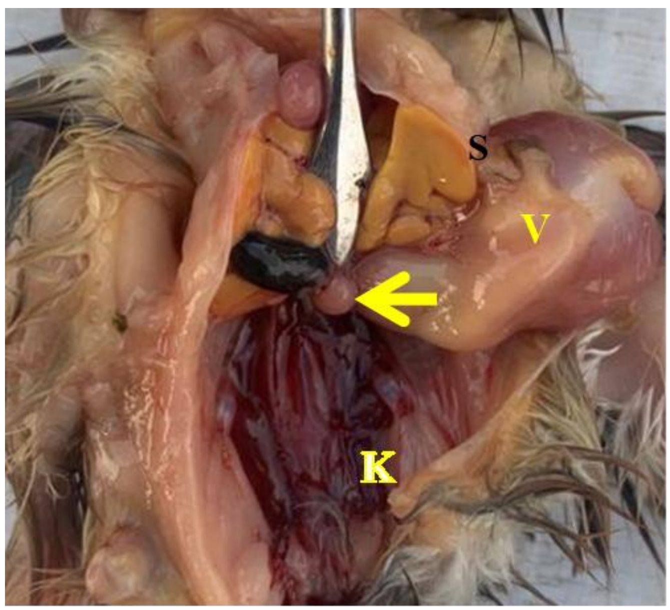


**Figure 2.** Gross photograph of a dissected section of the abdominal cavity at hatch (D 0) showing some abdominal viscera. Note the colour and shape of the spleen (arrow). V: ventriculus, K: kidneys.

connective tissue, and in the mesenchyme were splenic sinusoids, but no blood vessels were observed. The parenchyma showed no apparent differentiation of splenic tissue into white and red pulp regions. Splenic mesenchymal cells appeared quite undifferentiated, but an erythrocyte was identified in the sinusoid (Figure 3).

At EID 14, differentiation of splenic parenchyma into red pulp and white pulp regions was evident. The capsule relatively increased in thickness, but splenic trabeculae were not observed. Apparently, more cells had infiltrated into the spleen (Figure 4).

By EID 18, the capsule significantly increased in

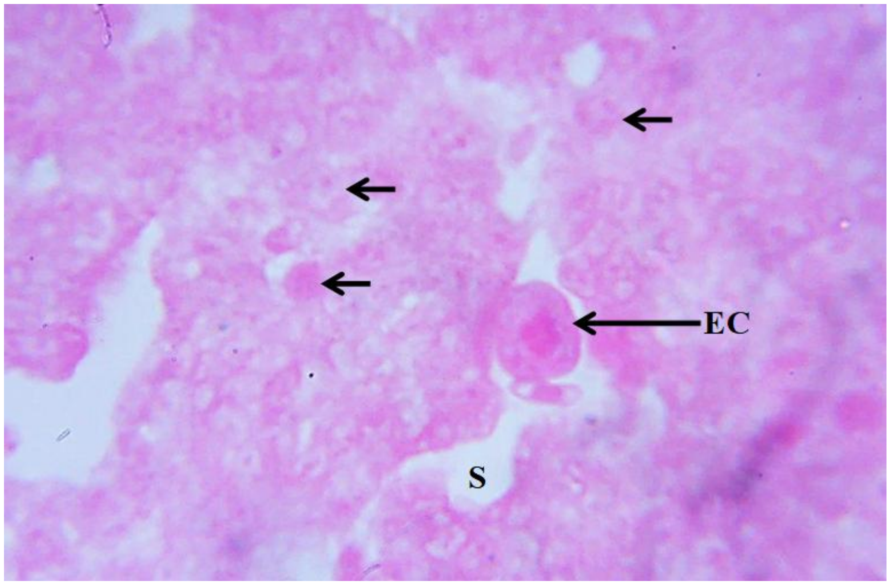


**Figure 3.** Photomicrograph of a transverse section of the spleen at EID 10 showing the splenic parenchyma. Observe some fairly distinct undifferentiated cells, (arrow) and the sinusoid, S containing an immature erythrocyte, EC (Haematoxylin and Eosin stain; X100).

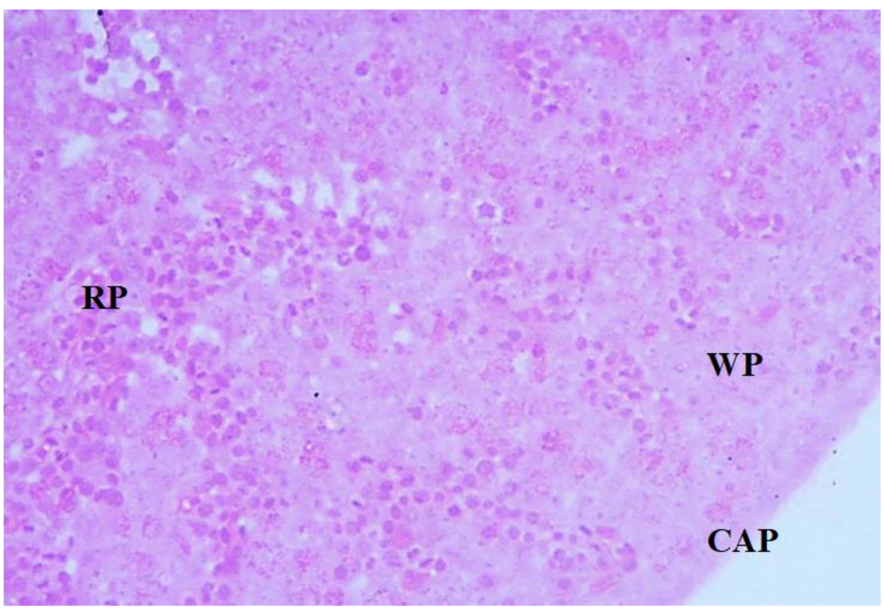


**Figure 4.** Photomicrograph of a transverse section of the spleen at EID 14 showing the parenchyma. Note the white pulp, WP and red pulp, RP. CAP: capsule (Haematoxylin and Eosin stain; X100).

thickness, but no trabeculae was as well observed; however, the pulp regions appeared more conspicuous. The lymphocyte density relatively increased, and ellipsoids with distinct arterial capillaries surrounded by mesenchymal tissues equally developed (Figure 5).

At hatch (D 0), there was clear differentiation of splenic substance into white and red pulp regions with pronounced dominance of the parenchyma by the white pulp. The white pulp was dominated by lymphocytes and characterised by well-defined peri-ellipsoidal white pulp (PWP) that comprised capillaries surrounded by lymphocytes and reticular cells (Figure 6).

At D7, there was still pronounced dominance of the splenic tissue by the white pulp elements and the capsule,

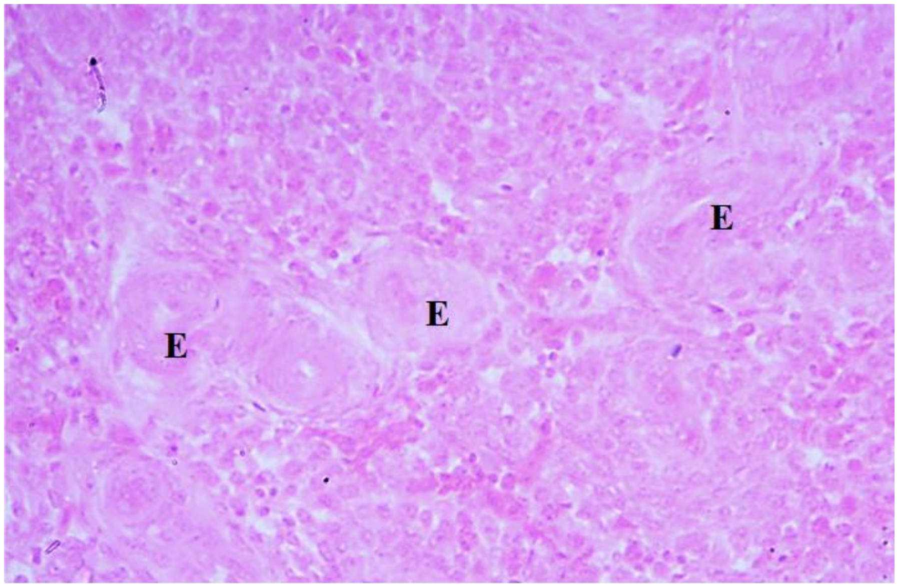


**Figure 5.** Photomicrograph of a transverse section of the spleen at EID 18 showing the splenic parenchyma. Note the ellipsoids, E within the lymphocyte population (Haematoxylin and Eosin stain; X400).

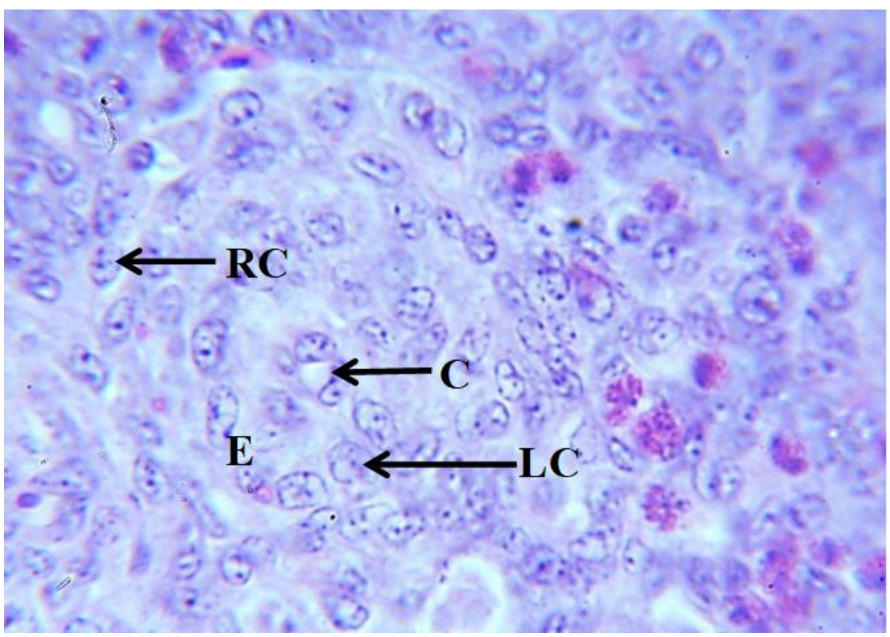


**Figure 6.** Photomicrograph of a transverse section of the spleen at hatch showing the parenchyma. Note the capillary, C surrounded by the ellipsoid. LC: lymphocyte; RC: reticular cell (Haematoxylin and Eosin stain; X1000).

together with the arterial walls, showed a remarkable increase in thickness. Splenic lymphocyte density remarkably increased too, and few plasma cells were equally identified (Fig. 7).

At D14, the more dominant white pulp tissues appeared more confined, leaving their boundaries with the surrounding red pulp tissues more distinct. The thickness of the arterial walls and the splenic capsules increased remarkably (Figure 8).

Between D 28 and D 42, partially encapsulated lymphatic nodules filled predominantly with lymphocytes and a few other splenic cells developed (Figure 9).

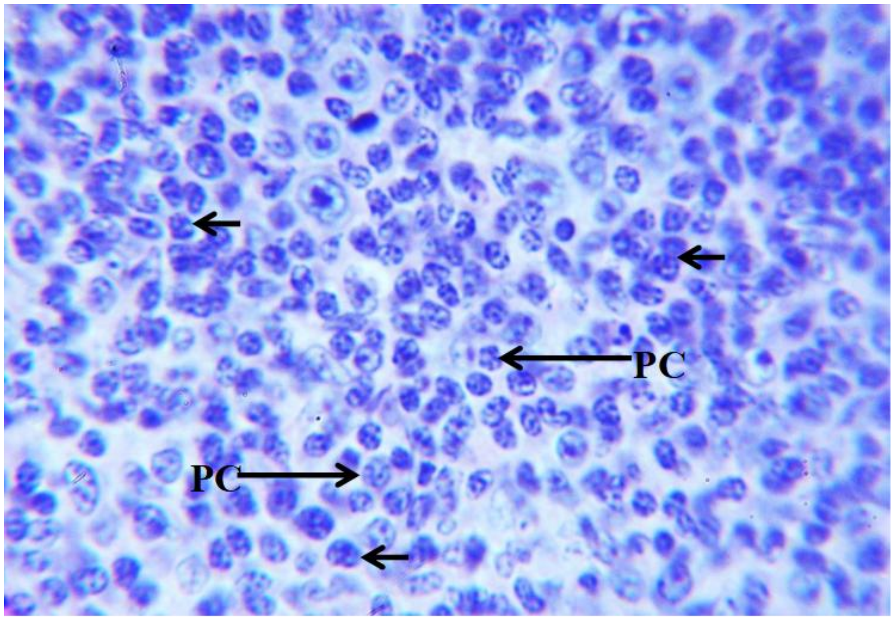


**Figure 7.** Photomicrograph of a transverse section of the spleen at D 7 post-hatch showing the splenic white pulp region. Note the cell density. Lymphocytes: (arrow), plasma cells, PC (Haematoxylin and Eosin stain; X1000).

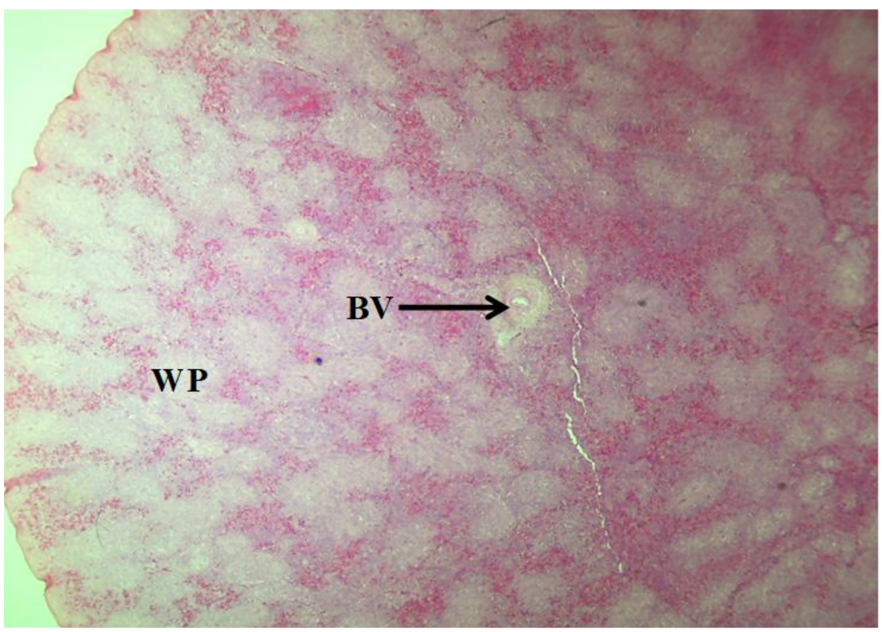


**Figure 8.** Photomicrograph of a transverse section of the spleen at D 14 post-hatch showing the parenchyma. Note the confined white pulp, WP tissue and the thickness of the blood vessel, BV (Haematoxylin and Eosin stain; X100).

## DISCUSSION

The topography of the spleen in indigenous chicken of south eastern Nigeria is similar to that reported for an unspecified breed of chicken (Olah and Vervelde, 2008) and guinea fowl (Onyeanusi, 2006). The shape of the embryonic spleen in Nigerian indigenous chicken was triangular with a smooth convex dorsal free border, but bean-shaped during the post-hatch period. Although such variation in shape of the developing spleen as observed in

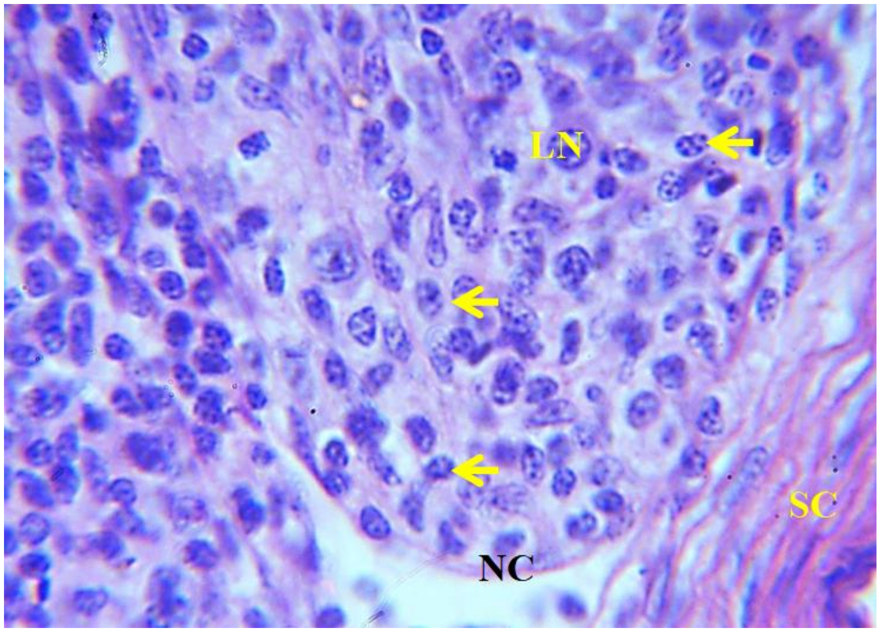


**Figure 9.** Photomicrograph of a transverse section of the spleen at D 42 showing the splenic parenchyma. Note lymphatic nodule, LN filled predominantly with lymphocytes (arrow). NC: nodular capsule; SC: splenic capsule (Haematoxylin and Eosin stain; X1000).

the indigenous chicken has not been reported among domestic chicken species, Onyeanusi (2006) noted that the spleen in guinea fowl is elongated in outline at day 20 of incubation, while in the adult it is bean-shaped without an indented hilus. This variation in shape may be in conformity with the topographic demands, as the relatives of the spleen at some stage had not attained their definitive sizes and contours.

Investigations on the splenic weight showed that the mean weight of the spleen increased progressively with age during the post-natal period of growth. The maximum splenic weight was attained at D 42 post-hatch. Records on relative weight showed that the spleen attained maximum relative weight at D 28 post-hatch. The attainment of maximum relative weight at D 28 shows that the spleen growth in native chicken was rapid. However, histological investigation may be needed to explain the actual growth changes that influenced the splenic weight. Dieter and Breitenbach (1968) observed that the maximum relative weight of the spleen in the cockerels is about 0.3% at 35-77 days. Ogata *et al*. (1977) reported that the relative weight of the spleen in chicken increases gradually from hatching till it reaches its maximum at day 56 of age, while in guinea fowl the spleen attained a maximum mean weight of 1.163g at day 168 but has peak relative weight at 28 days of age (Onyeanusi and Onyeanusi, 1990).

At EID 10, the splenic capsule from the study lacked trabeculae, and the parenchyma was undifferentiated into white pulp and red pulp regions. Although no immunological function is known for the splenic trabecula, splenic blood vessels traverse the splenic tissue through it. The early development of the splenic capsule without trabecular outgrowth at EID 10, as observed in the indigenous chickens, was similar to the findings in the native chicken of Bangladesh (Islam *et al*., 2012). Furthermore, the first wave of splenic cell population at EID 10 was primordial cells that are predestined to transform to definitive cells in the course of development. However, differentiation of the parenchyma into pulp regions at EID 14 indicates early homing or migration of splenic cells from the primary lymphopoietic and haemopoietic organs to the spleen, which signifies embryonic establishment of proactive peripheral lymphoid structures that may be capable of eliciting immunological responses in Nigerian native chicken. Islam *et al*. (2012) stated that differentiation of splenic tissues into white and red pulp was not established in the native chicken of Bangladesh until about EID 14. In guinea fowl, pulp differentiation occurred about incubation day 19 as irregular light and dark staining areas of the parenchyma (Onyeanusi, 2006). The distinction of these pulp elements seemed delayed in the duck where the white and red pulps were only clearly defined after 15 days post hatch (Indu *et al*., 2005), and in quail where distribution of the red pulp was sparse when compared with white pulp and the sharply distinguished areas of red and white pulps not defined at hatch (Liman and Bayram, 2011). Few studies that focused on the emergence of immunocompetent cells, especially lymphocytes in the lymphoid organs, report that the first wave of lymphocytes in the chicken spleen appeared at EID 14 (Islam *et al*., 2012).

One of the elements of the white pulp, the ellipsoid, was detected at EID 18. The development of this principal immunological tool during the embryonic phase of development emphasises the lymphopoietic role of the spleen in native chicken, since the ellipsoids are responsible for cleansing the blood of a wide range of endogenous and exogenous substances. Furthermore, the appearance of these ellipsoids as early as EID 18 as well suggests that the immune potentials of the native chicken spleen are established before hatch. Ontogenic studies show that in the quail, the ellipsoids appeared in the spleen at day 12 of incubation, with the capsule of Schweigger-Seidel sheath forming around it on incubation day 15, followed by the emergence of peri-ellipsoidal white pulp on day 16 of incubation (Liman and Bayram, 2011). However, Mast and Goddeeris (1998) reported that PALS and the ellipsoids appeared on day 20 of incubation in chicken, although Ogata *et al*. (1977) and Kasai *et al*. (1995) had earlier stated that the ellipsoids in chicken were obvious on the 18th day of incubation, as observed in the indigenous chickens.

At hatch, the development of specific pulp regions appeared quite distinct, with the dominance of the parenchyma by the white pulp. This implies that there was a preponderance of lymphocytes in the early post-hatch spleen, suggesting a high level of lymphocyte production in the primary lymphoid organs and ample migration to the spleen. This by implication, means that the spleen in native chicken has the potential of offering immunological protection during the early post-hatch periods of life. According to Smith and Hunt (2004), species variation in the proportion of pulp regions at hatch suggests different functional responsibilities of the spleen at this stage of life, since the functions of the embryonic and neonatal spleen in avian species are different. In quails, Liman and Bayram (2011) observed that the white pulp dominated the spleen at hatch. John (1994) explained that the avian red pulp occupies less space (40-45%) of the splenic parenchyma than the white pulp due to its less prominent contribution to oxygen circulation. The dominance of the white pulp in some avian species like chicken, turkey and quail, therefore, suggests that the spleen in these species plays more of a lymphopoietic role, while dominance of the red pulp as observed in guinea fowl and duck suggests more of a hemopoietic responsibility of the spleen in these species. In the quail, the red and white pulps appear in equal proportions about day 49 (Liman and Bayram, 2011), and day 60 in chicken (Hodges, 1974; Payne, 1971), but much earlier, at day 14 in guinea fowl (Onyeanusi, 2006). The significance of an equal proportion of the white and red pulps is not clear, although Liman and Bayram (2011) suggest that an imbalance of the red pulp and white pulp indicates that the spleen growth is not yet concluded.

The development of lymphatic nodules with a primitive capsule of connective tissue in the indigenous chicken occurred about D 28. However, the immunological significance of this structure is equally clear, but it could be a storage compartment for immunocompetent cells rather than a site for immune cells transformation, since no variation in cell morphology between the intra-nodular and extra-nodular cells has been reported. The time of appearance of this white pulp element varies greatly among avian species. In the chicken, Ogata *et al*. (1977) discovered that germinal centres develop as early as day 10 post hatch, while Onyeanusi (2006) identified the lymphatic nodules in guinea fowl at day 91 post hatch.

## Conclusion

The development of the basic immunological structures of the spleen commenced in the embryos and advanced through the post-hatch periods. The spleen of Nigerian indigenous chicken may play a more lymphopoietic role due to the dominance of the splenic tissue by the white pulp.

## Recommendation

Further investigations before EID 10 and beyond D 42 post-hatch are recommended to add to the existing information for knowledge purposes.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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