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Evaluation of the morphological development of the testes in pre- and post-hatch broiler chicken

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Abstract

Early events during testicular development set the stage for the male reproductive organ development and species propagation. The developmental morphology of the testes was investigated in the broiler chicken using light microscopic techniques. Fifteen (15) fertile eggs and 40 day old chicks of Marshall broiler chicken were used for this study. Samples of the testes of the pre- and post-hatch broiler chickens were collected and subjected to routine histological, histomorphometric and histochemical evaluation, following standard procedures. Results showed that on embryonic day (ED) 17, the testes contained areas of poorly formed primordial genital cords and unorganized cellular aggregations. The seminiferous cords became clearly delineated at ED 19, increasing in population as the birds aged. These cords of cells consisted of a single layer of pre-Sertoli cell and spermatogonia until post-hatch day (PD) 35, after which the seminiferous tubular profile showed several foci of cellular proliferation, including the presence of primary spermatocytes. The mean diameter of the seminiferous cords/tubules increased steadily with age while the epithelial height at PD 42 was significantly higher ($p < 0.05$) than in the previous post-hatch days. Reticulin histochemistry revealed the abundance of reticular fibres in the peritubular tissues and also in the loose connective tissues of the testicular insterstitium in both early and late-stage testes. In conclusion, the morphological features of the testes highlighted in this study reveal that there was comparatively a delay in seminiferous cord formation in the broiler chicken. The seminiferous cords/tubules formed, persisted as pre-pubertal testes until after PD 35, when there was an accelerated spermatogenic activity within Class 1 testes.

Keywords: Broiler chicken; Testes; Developmental morphology; Pre-hatch; Post-hatch..

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Introduction

Several avian species have been used as models in the study of morphological development of several organs of the body (Ainsworth *et al*., 2010; Intarapat and Satayalai, 2014). Chick embryos were commonly employed for such studies. However, recently quail embryos were also utilized. In both models, emphases were on general gonadal development with little recourse to the step-wise embryonal changes which occurs after gonadal differentiation up to the period of initiation of gonadal function and early pubertal changes. In chicken, gonadal development, just as seen in mammals, has been reported to consist of phase of indifferent gonads, phase of gonadal differentiation, and phase of gonadal function (Rizzi and Verdiglione, 2016).

Generally, in avians, gonadogenesis was thought to begin with the migration of primordial germ cells (PGC) derived from the central zone of blastodisc (Fujimoto *et al*., 1976), migrating through an anterior course via blood capillaries to settle in the genital ridge (Tanaka *et al*., 2014). The primordial germ cells, cells of the coelomic epithelium and the mesonephroi have been reported to contribute to the various cell populations of the developing gonads (Dagmar e*t al.*, 2013; Ioannidis *et al*., 2018). At first the developing gonad has a dual potential but later diverges to follow a male (ZZ) or female (ZW) sex course at about 5.5 to 6.5 days of the 21 day incubation period (Clinton, 1998; Smith and Sinclair, 2001; Chue and Smith, 2011). At this period, the gonadal sex is histologically obvious. Following sexual differentiation, specialized cells with supporting and steroidogenic functions form cords around the germ cells. In the testes, the cords of cells in the medulla develop while that of the cortical region is reported to regress (Smith and Sinclair, 2004). Reports in available literature have shown that the entire process of testicular development in chicken, starting from the phase of genital ridge formation to gonadal functions are regulated by genetic factors such as doublesex and mab-3 related transcription factor 1 (DMRT1) (Smith and Sinclair, 2001; Chue and Smith, 2011) and transcription factor 21 (TCF21) (Zhang *et al*., 2017) genes located on the Z chromosome, hormonal factors (Weniger, 1991; Ottinger and Bakst, 1995; Intarapat *et al*., 2014), and environmental factors (Chue and Smith, 2011).

According to Clinton and Haines (1999), Intarapat and Satayalai (2014), three phases of avian gonadogenesis which have been described to include phases of genital ridge formation, gonadal differentiation and gonadal function, have peculiar features. Swift (1916) reported that primordial germ cells first differentiated into spermatogonia on day 13 of incubation, after which the cell does not undergo further cell division until at the 10^{th} week post hatch. This age long findings may not reflect the present trend as the present environmental factors which have changed over the years may impact on the development of embryonic testes. Although it is popularly believed that male germ cells remain at rest prior to sexual maturation (Swift, 1916), available literature does not show the cascades of morphological activities which occur in the testes of pre-pubertal broiler chicken. The present study evaluated the morphological changes in the testes of late pre-hatch and post-hatch broiler chicken using histological, histomorphometric and reticulin histochemical techniques.

Materials and Methods

Animals used for the study: Fifteen fertile male Marshall broiler eggs were obtained from Anke's Breeder farm, Ibadan, Nigeria for the study. They were transported to the Department of Veterinary Anatomy, University of Nigeria, Nsukka for hatching. The temperature of 38^{o}C and humidity values of 58 – 60% was maintained in the incubator except

in the last 3 days of incubation where the humidity was changed to 65%. The guidelines for the care and use of the animals were strictly followed during the study. The experimental protocols were carried out according to the guidelines approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka (Approval Reference Number: FVM-UNN-IACUC-2019-077).

Day old male Marshall broiler chicks (40 in number) for the study were obtained from the same breeder farm were transported to the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Nigeria, where they were kept under standard conditions and fed with broiler chick starter mash. Feed and water were provided *ad libitum.*

Sample collection: Following ketamine euthanasia, samples of the testes of the pre-hatch broiler chicken were obtained on days 14, 17 and 19 of incubation while samples of the testes of post-hatch broiler chickens were obtained on days 1, 5, 8, 11, 21, 35, 42, and 56 post-hatch. These were processed for routine histology.

Histological and histochemical techniques: The testes samples that were collected were fixed in 10% neutral buffered formalin. They were subsequently dehydrated in graded concentrations of ethanol, cleared in several changes of xylene and embedded in paraffin wax. Furthermore, the embedded tissues were sectioned using a rotary microtome to obtain sections of 5µm thick. The sections were mounted on grease free microscope slides and stained routinely with haematoxylin and eosin for light microscopy. Reticulin staining procedures were carried out as described by Lefkowitch (2006). Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic Group Ltd, China) attached to the light microscope.

Histomorphometric analysis: The seminiferous tubule diameters and heights were measured using a standardized ocular micrometer. These were measured by randomly choosing 30 profiles of the seminiferous tubules that were round or nearly round per animal.

Statistical analysis: Quantitative data obtained were subjected to one-way analysis of variance (ANOVA). Variant means were separated post-hoc using the least significant difference (LSD) method. Differences among means were considered statistically significant at $p < 0.05$.

Results

Histological changes in the testes of pre-hatch and post-hatch broiler chickens: The testes of the 14-day old embryo of broiler chicken showed both cortical and medullary regions. The cortical region exhibited oocyte-like germ cells while the medulla regions were characterized by the presence of randomly distributed primary sex cords (Figure 1). At embryonic day (ED) 17, the testes contained areas of poorly formed primordial genital cords and areas of unorganized cellular aggregations (Figure 1). At ED 19, the outlines of the few forming seminiferous cords were becoming well-delineated. However, there were still areas of unorganized cellular aggregations, while cortical and medullary areas were no longer distinct (Figure 2).

Furthermore, at days 1 and 5 post-hatch, the testes contained numerous well-delineated seminiferous cords. Each seminiferous cord consisted of a layer of pre-Sertoli cells surrounding a core of few pre-spermatogonia. Scanty peri-tubular myoid cells encircled the seminiferous cords. The interstitial areas showed abundance of interstitial cells (Figure 2). Moreover, at post-hatch days (PDs) 8 and 11, the population of the seminiferous cords increased and each cord exhibited a single

layer of pre-Sertoli cells with a central core of few large spermatogonia (Figure 3). At post-hatch days 21 and 35, the seminiferous tubules contained small lumina surrounded by a single layer of germinal epithelium, made up of Sertoli cells and spermatogonia. The tubular limits were closely associated with each other resulting in thin interstitial areas that contained few interstitial cells (Figure 3). At PDs 42 and 56, the seminiferous tubular profiles showed several foci of cell proliferation. The germinal epithelium of each seminiferous tubules consisted of more than one layer of cells, with abundance of spermatogonia and Sertoli cells, as well as isolated spermatocytes in the adluminal compartment (Figures 4 and 5). Apart from the observed proliferation of cells of the germinal epithelium of the seminiferous tubules at PD 56, wide interstitial spaces which contained numerous interstitial cells were observed (Figure 5).

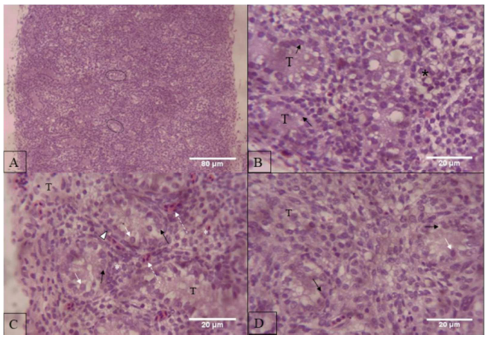

Figure 2. Photomicrograph of the testes (H & E stain); **[A]** – Embryonic day (ED) 19 showing seminiferous cords (circled), ×100; **[B] –** ED 19, the seminiferous cords consisted of a single layer of cells (arrows) with wide interstitial area containing several FLCs (asterisk), ×400; **[C] and [D]**, at PDs 1 and 5, the seminiferous cords were well delineated with a single layer of cells consisting of pre-Sertoli cells (black arrows), and spermatogonia (white arrows). Note the interstitial areas (asterisks) with FLCs and capillaries (segmented white arrows), as well as peritubular myoid cell (arrow head), ×400.

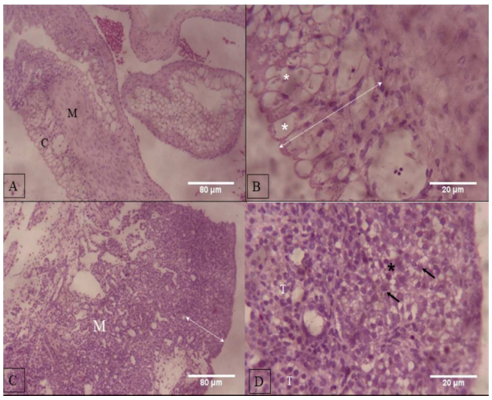

Figure 1. Photomicrograph of the testes (H & E stain); **[A]** – Embryonic day (ED) 14 showing cortical (C), and medullary (M) areas, ×100; **[B]** – ED 14, showing cortical area (dotted arrow) with oocyte-like germ cells (asterisks), ×400; **[C]** – ED 17 showing the testes with cortical area (dotted arrow) and medulla (M) with forming seminiferous cords, ×100; **[D]** – ED 17 showing testes with few forming seminiferous cords (T) and area of unorganized cellular aggregates (asterisk) made up of varied cells (arrows), ×400.

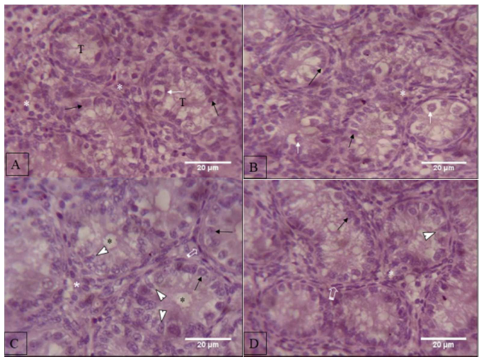

Figure 3: Photomicrograph of the testes (H & E stain); **[A]** and **[B]** – Post-hatch days (PDs) 8 and 11 showing seminiferous cords (T) lined by a single layer of Sertoli cells (black arrows) and spermatogonia (white arrow). Note the interstitial areas (white asterisks), ×400; **[C]** and **[D]** – PDs 11 and 21 showing seminiferous tubules with a germinal epithelium made up of a single layer of Sertoli cells (arrow heads) and spermatogonia (black arrows). Note the seminiferous lumina (black asterisks), narrow interstitial areas (white asterisks), and the peritubular myoid cells (white arrows), ×400.

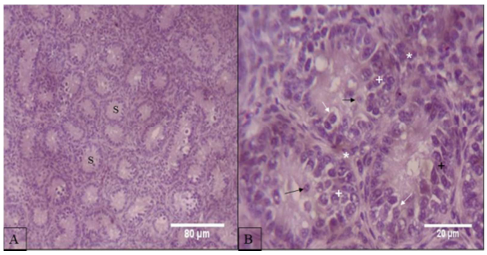

Figure 4. Photomicrograph of the testes at post-hatch day (PD) 42 showing seminiferous tubules (S), focal areas of Sertoli cell (black plus) and spermatogonia (white plus) proliferation. Note the spermatogonia (white arrows), spermatocytes (black arrows), and testicular insterstitium (white asterisks), H & E stain, **[A]** – ×100, **[B]** – ×400.

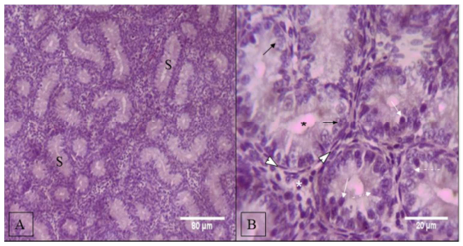

Figure 5. Photomicrographs of the testes of the broiler chicken at post-hatch day (PD) 56 showing class 1 seminiferous tubules with spermatogonia (black arrows), Sertoli cell (dotted white arrow), and spermatocytes (white arrows). Note the seminiferous lumen (black asterisk), and testicular interstitium (white asterisk); H & E stain, **[A]** – ×100; **[B]** – ×400.

Histomorphometric features of the testes of pre hatch and post hatch broiler chicken: The mean tubular diameter of the seminiferous cords/tubules increased steadily and significantly ($p < 0.05$) with age (Table 1). The mean epithelial height of the cords/tubules was significantly lower at pre-hatch day 19 when compared to the post-hatch days (Table 1). However, between post-hatch days 8 and 21, the mean epithelial heights did not significantly ($p > 0.05$) differ, except at PD 42 when it became significantly ($p < 0.05$) higher than previous post-hatch days (Table 1).

Reticulin histochemistry of the testes of post hatch broiler chicken: Following reticulin staining, the testes at hatch showed tiny strands of reticular fibres within the testicular cords. Whereas the thin basement membrane exhibited dense reticular fibres, the interstitial areas demonstrated loose network of reticular fibres (Figure 6). Similarly at day 56 post hatch, tiny strands of reticular fibres were observed within the seminiferous tubules while the thick basement membrane showed dense and more regular reticular fibres. At the interstitial areas, slightly dense randomly organized reticular fibres were observed (Figure 6).

Table 1. Mean tubular diameters (TD) and epithelial heights (EH) of the testes of pre- and post-hatch broiler chickens (± SEM)

Days	Mean tubular diameters (µm)	Mean epithelial height (µm)
Day 19 pre-hatch	41.90 ± 0.90 ^{a}	16.13 ± 0.75 ^{a}
Day 8 post-hatch	54.20 ± 0.72 ^{b}	24.83 ± 1.21 ^{bc}
Day 21 post-hatch	64.60 ± 0.90 ^{c}	24.83 ± 0.23 ^{c}
Day 42 post-hatch	70.83 ± 0.15 ^{d}	29.30 ± 0.10 ^{d}

$^{a\,b\,c\,d}$ Different superscripts in a column indicates significant differences across the groups ($p < 0.05$).

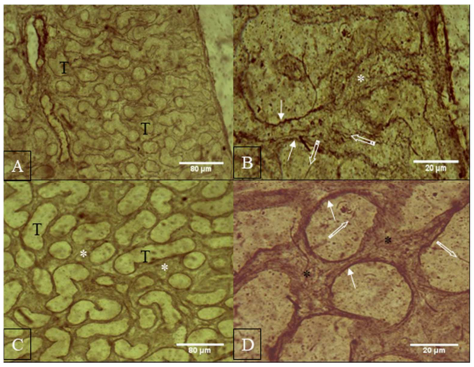

Figure 6: Photomicrograph of the testes of the broiler chicken at post-hatch day (PD) 1 **[A]** and **[B]**, and PD 56 **[C]** and **[D]**, showing testicular cords/seminiferous tubules (T) with tiny strands of reticular fibres (segmented arrows), thin and thick basement with dense reticular fibres (white arrows). Note that loose interstitial tissue areas (asterisks) contained loose reticular fibre networks at PDs 1 and 56 (white and blacks asterisks). Reticulin stain, **[A]** and **[C]** - ×100; **[B]** and **[D]** - ×400.

Discussion

In the present study, the absence of distinct seminiferous cords at ED 14 shows a stage of histologically indistinct testes. The primordial genital cords observed in the medulla suggest that testes development begins from the medulla. Earlier authors opined that the testes develop from the medulla (Smith and Sinclair, 2004), though later, authors disagreed with the above assertion (Piprek *et al*., 2016; Mizia *et al*., 2023). The observation of forming seminiferous cords in both cortex and medulla at ED 19 is indicative of the participation of both regions in testes development. It is most probable that the formation of seminiferous cords which may begin from the medulla, ultimately extends to the cortex. The indistinct cortical and medullary areas, which we observed at this age further highlights the above assertion. Although it was earlier reported that seminiferous cords are apparent in chicken by embryonic day 9 (Esterman *et al*., 2021), this may not be the case in broiler chicken probably because their genetic selection focused on growth rate and food efficiency traits, and not early ability to reproduce.

Seminiferous cords were sparsely distributed in the testes during the late embryonic life of the broiler chicken. As the birds aged, the population of seminiferous cords/tubules increased, and was composed of a single layer of Sertoli and pre-spermatogonia. Based on Rizzi & Verdiglione (2016) classification of testicular growth, the persistence of a single layer of cells within the seminiferous cords/tubules in this study, until post-hatch day 35, shows that the testes were in Class 0 (pre-pubertal testes). The pre-pubertal testes observed in this study persisted much longer than the 3-weeks old post-hatch period that was earlier reported in chicken (Mfoundou *et al*., 2022). The presence of focal areas of cell proliferation in the seminiferous tubules of the testes at PDs 42 and 56 signaled a shift from the pre-pubertal testes. About two layers of cells were observed in the seminiferous epithelia, despite the occasional single-cell layered portions, corresponding to Class 1 of testicular growth. Here, spermatogonia differentiated into a few primary spermatocytes observed in the adluminal compartment of the seminiferous tubules. The spermatocytes formation marked an important time in broiler chicken as the testes prepares for pubertal life.

The age-related increase in the mean tubular diameter of the seminiferous cords/tubules observed in this study may be related to spermatogenic activity. However, these activities was probably much accelerated from PD 42 in this breed of chicken as their mean epithelial height was significantly higher than in the preceding ages.

Pre-pubertal testes were earlier considered quiescent. But it is now obvious from histologic and morphometric data reported in this study that there is massive growth, proliferation of Sertoli cells and

spermatogonia, and according to Rey (1999), the male testis triples its volume between birth and the onset of puberty. These key morphological processes are essential for normal adult testes maturation and functions.

At hatch and also at PD 56, we observed that the peritubular tissues consisted of reticular fibres that could act as supportive framework for the germinal epithelia. The dense deposition of reticular fibres within the basement membrane of the tubules at PD 56 is thought to be due to the age of the birds. From our findings, reticular fibres also contribute to the loose connective tissue framework of the testicular interstitium. Previously, collagen type IV was considered as the most abundant in seminiferous basement membrane and interstitium (Gofur *et al*., 2008; Abreu-Velez and Howard, 2012). However, the current finding reveals reticular fibres as a major constituent of the basement membrane and interstitial tissues. Apart the provision of supportive framework, reticular fibres may also facilitate a cross-talk between the germinal epithelium, peritubular cells and cells of the interstitium (Fakoya, 2002; Siu and Cheng, 2008).

The areas of unorganized cellular aggregation observed in the testis at ED 17 may also contain fetal Leydig's cells (FLCs) in addition to other cells. At hatch, until day 5, the well delineated interstitium of the testis contained abundance of these cells which later became fewer in population as the birds aged. The decreasing population of Leydig's cells in the later post-hatch life of the bird may reflect atrophy of fetal FLCs. Different hypothesis regarding the fate of FLCs have been proposed (Tapanainen *et al*., 1984; Ariyaratne and Chamindrani, 2000). It is most probable that most FLCs may atrophy in post-natal life, while a few may remain to proliferate and complement the Leydig's cell pool in the adult testes. The decreasing pool of the Leydig's cells in the later post-hatch life demonstrated in this study strongly supports the above opinion. Whereas FLC regulates fetal Sertoli cell proliferation which are involved in seminiferous cord elongation and expansion, adult Leydig cells are predominantly responsible for the production of testosterone (Wen *et al*., 2016; Liang *et al*., 2023). Although mature FLCs are thought to also produce androgens, such function may be limited in post-natal life as they had been reported not to express hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 6 (HSD3B6) and HSD17B3 – hydroxysteroid 17 beta dehydrogenase 3 (Shima *et al*., 2015). The HSD3B6 are involved in the steroid biosynthesis process while the HSD17B3 catalyzes the conversion of androstenedione to testosterone (Ota *et al.,* 2014; Cocchetti *et al.,* 2022).

In conclusion, based on the results of our study, the onset of seminiferous cord formation is delayed into the late pre-hatch period in broiler chicken. While the cortical and medullary areas participated in the seminiferous cord formation, the morphological features of these cords/tubules revealed in this study showed the persistence of Class 0 (pre-pubertal) testes until the 35th day post-hatch, with much more accelerated spermatogenic activity at about PD 42. The pre-pubertal testes was characterized by extensive seminiferous cord/tubule formation, within which there were proliferations of Sertoli cells and spermatogonia.

Conflict of interest

The authors declare that there is no conflict of interest

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