Growth of Japanese Quail Testes in Relation to Age: Morphological and Immunohistochemical Observation

Original Article

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ABSTRACT

Objective: The purpose of this research was to observe the age-related growth and histomorphological changes in Japanese quail testes from post-hatching to sexual maturity as well as spermatogenesis.

Materials and Methods: After collection, the testes weight, length, width, and index analysis were performed, and histomorphological, histological and immunohistochemical observations were conducted using hematoxylin-eosin, PAS-hematoxylin, and anti-Hsc70t staining, respectively at different ages.

Results: The weight of the testes increased gradually with age and reached a peak at 70 days. The length and width of the testes were maximal at 70 days. The testis index was positively correlated with age and body weight (BW) toward sexual maturity. Histologically, at 15 days of age, the seminiferous tubules (STs) remained in immature state with undifferentiated spermatogenic cells of only one to two layers of epithelial cells. At 28 days, the spermatogenic cells were differentiated from spermatogonia to spermatocytes. At 42 days, remarkable enlargement of STs with a series of spermatogenic cell development up to the round spermatids and Leydig cells in the interstitial region. Finally, at 70 days, extremely enlarged STs containing all stages of spermatogenic cells. The diameter and height of STs were maximal at 70 days than that of 42 days of age. A strong Hsc 70t immunoreaction was found in the round to elongated spermatids/spermatozoa near to the lumen of STs, clearly indicating well-characterized spermatogenesis in quail testis at 70 days of age.

Conclusion: The findings may contribute to our understanding of quail's spermatology and provide basic knowledge for reproductive toxicology, physiology and pathology studies.

Received: 09 December 2020, **Accepted:** 29 January 2021

Key Words: Immunohistochemistry; japanese quail; sexual maturity; spermatogenesis.

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ISSN: 1110-0559, Vol. 45, No.1

INTRODUCTION

Japanese quails (Coturnix Japonica) are the smallest member of poultry. They have some encouraging traits, such as faster growth, short generation interval, small floor space and fewer feed requirements, and less susceptibility to diseases[1]. They have the immense potentiality to use as a laboratory animal model[2] in research in a variety of disciplines, such as physiology, nutrition, endocrinology, pathology, reproduction and toxicology. Therefore, histochemical and immunohistochemical studies on Japanese quail testes development, gonad size variation and spermatogenesis from hatching to sexual maturity have of great interest to poultry industrialists and researchers.

The testis is the prime gonadal organ of the reproductive system. Similar to mammals, birds have two testes, one on either side of the midline of the body[3]. It is a tubular gland having both exocrine and endocrine functions. The exocrine function is to produce spermatozoa and the endocrine function is to secrete testosterone[4]. The testis is covered by a connective tissue capsule. Through the capsule, the blood vessels and nerves enter into the testis and exit from it. The capsule has three layers, namely the tunica serosa, the outermost layer; the tunica albuginea, the middle layer; and the tunica vasculosa, the innermost layer. The tunica albuginea divides the testis mass into 200 to 250 lobules, each lobule is filled with two to five U-shaped, double-ended seminiferous tubules (STs)[5,6]. The ST includes supporting (Sertoli cells) and spermatogenic cells. The Sertoli cells are located in the basal region of the tubules which metabolically support and physically anchor the adjacent spermatogenic cells; spermatogonia, spermatocytes, and round spermatids. The spermatid undergoes morphological changes into elongated and finally becomes mature motile spermatozoa[7]. In between the three or more adjacent STs, the angular area, interstitium that is filled with Leydig cells[8,9]. These Leydig cells secrete testosterone which maintains spermatogenesis[4,10].

Anatomy and histology of male reproductive organs of different avian species have been studied by many researchers[5,6]. Moreover, Razi *et al*. (2010)[11] described White Rooster testis anatomy and Al-Tememy (2010)[12], the quail testicular histology. Additionally, Shill *et al*. (2015)[13] described the annual cycle on adult

 DOI: 10.21608/ejh.2021.52917.1398

quail testicular histomorphology. The quail is considered to be representative of terrestrial birds and is an accepted model of experimental animals for a variety of disciplines of research. Although the gross anatomy and histology of testis have been studied in many species of birds, its specific development and histomorphometry in Japanese quails are still poorly understood. In this study, we examined the weight, length, width and organ index of the testis of 15 to 70-day-old healthy Japanese quail, and explored the histological development of testis staining with hematoxylin and eosin, and histomorphology of spermatogenesis with Hsc 70t staining. This research may provide basic knowledge for quail reproductive toxicology, physiology as well as pathology studies.

MATERIALS AND METHODS

Quails

Japanese quails (Coturnix japonica) were purchased from a commercial farm at Mymensingh, Bangladesh. The birds were reared under an optimum environment (temperature 26-28ºC and humidity 50-70 % under daily photoperiods of 12 h light and 12 h dark) and balanced feeding conditions with strict biosecurity. They were fed with commercially available feed along with filtered tap water ad libitum. Vaccination and management of the birds were also taken into consideration. They were individually weighed at various ages, from day10 to 70 after hatching. At 10, 15, 20, 28, 35, 42 and 70 days of age, 10 animals (n=10) in each group were killed by cervical subluxation method and kept on an electronic balance to record body weight. The collected birds were free from any developmental disorder or detectable diseases. An incision was given on the abdomen at the midline to open the testes.

The experimental birds were killed humanely under the ethical and welfare guidelines of the Animal Welfare and Experiments of Bangabandhu Sheikh Mujibur Rahman Agricultural University.

Gross morphometry

After collection, the weight of the testes was recorded using an electric balance. Then, the organ index was calculated based on the formula; organ index=(organ weight/body weight)Χ100%. Additionally, the length and width of the testes were calculated with venire calipers and finally, images were taken using a digital camera.

Histomorphometry

After measuring weight, testes were fixed in neutrally buffered 10% formalin for 48 h at room temperature. They were then washed in 0.1M phosphate buffer solution (PBS) for 3 h and dehydrated through a graded series of alcohol followed by cleaning in xylene and embedding in paraffin. The paraffin blocks were cut at 5 µm in thickness. The sections were then stained with Meyer's hematoxylin and eosin and/or periodic acid–Schiff (PAS)–hematoxylin as described previously 10. The samples were studied with a light microscope. The diameter of the STs and height of the stratified layer of the epithelium in the STs were measured by a computer-assisted system with Scion Image software (Scion Co., Frederick, MD, USA).

Immunohistochemistry

The immunohistochemical staining with an anti-Hsc70t antibody was performed according to the original article[14]. Briefly, the testes sections of 70-day-old Japanese quail were cleaned in xylene, washed in PBS, blocked with 10% bovine serum albumin in PBS and then incubated with the primary antibody, anti-Hsc70t at 1:3000 dilution in the blocking solution at 4ºC for 12-18 h (overnight). After washing in PBS, the slices were incubated with secondary antibody, biotinylated goat anti-rabbit IgG (BRL Gaithersburg, MD, USA) at room temperature for 2 h. The samples were washed again in PBS and incubated with Avidin Biotinylated Horseradish Peroxidase complex system (Elite ABC Peroxidase Kit, Vector Laboratories, Burlingame, CA, USA). The immunoreaction was visualized with 0.05% diaminobenzidine (DAB)-H2O2 solution. Finally, the samples were counterstained with hematoxylin. For negative control, the incubation step with the primary antibody was omitted.

Statistical analysis

Bodyweight, testis weight, diameter and epithelial height of STs were represented from 8 birds (n=8) in each group as the mean±S.E.M. Organ index=(organ weight/body weight)Χ100% was calculated. The student's t-test was used to compare the mean values of body weight and testis weight, diameter and epithelial height of STs. Differences in organ index were considered to be statistically significant when the *p-value* was less the 0.05.

RESULTS

Weight, length, width and index analysis of the testicular growth

The bodyweight of Japanese quails from day 10 to 70 was measured with an electric balance and the result was presented in (Figure 1 and Table 1). The body weight was gradually increased up to 42 days and became remarkable after 42 to 70 days of age.

Unlike in most mammals, the testes are situated in the abdominal cavity of the avian species, including quails. The testes weight analysis showed that the weight of Japanese quail testes increased gradually with age from 15 to 28 days and became most prominent from 42 to 70 days of age and reached a peak at 70 days (1986.25±.07 mg) (Table 1 and Figure 2). It was obvious that the growth of the testis was strong from 42 days of age. Similar to weight analysis, the length and width of the testis were gradually increased and was maximum at 70 days than that of 42 days of age (179.97±1.08 mm vs. 17.62±1.9 mm, $p<0.001$ and 35.6±1.07 mm vs. 10.08±0.5 mm, $p<0.05$, respectively) (Table 1). The organ (testis) index was higher at 42 days of age compared to 28 days (0.702568 vs. 0.328875, $p< 0.001$) and reached a maximum (1.382509, $p< 0.001$) at 70 days of age (Table 1).

Histomorphological analysis of the testicular growth

The histomorphological analysis of the quail testes of different age groups is shown in Figure. 3. At 15 days of age, the STs remained in the immature state with 1 to 2 layers of epithelial cells. These cells were undifferentiated spermatogenic cells. The diameter of the STs was 34.57±4.92 µm and the height of the epithelial cell layer was 12.43±1.091 µm (Figure 3A and Figure 4). There was no clear demarcation of the interstitial regions and the cells were undifferentiated. At 28 days of age, the STs underwent further development, such as enlargement and elongation of the STs, stratification of the STs increased that those of 15 days of age (2 to 4 epithelial layers) and differentiation of spermatogenic cells from spermatogonia to spermatocytes were observed. The unclear boundary of interstitium in between the STs, a loose arrangement of Leydig cells and a small number of blood vessels were also observed (Figure 3B). The diameter of the STs was 113.28±29.99 µm and the height of the stratified cells layer was 22.54±3.91 µm (Figure 4). This suggests that the maturation of the testes was stimulated at this period. At 42 days of age, the remarkable enlargement of STs with a series of spermatogenic cell development from the spermatogonia to the round spermatids and increased number of Leydig cells in the interstitial region was observed (Figure 3C). The boundaries of interstitium between the tubules were not clear at that time. (Figure 3C). The diameter of the STs was 179.14±17.65 µm and height of the epithelial cells layer was 59.76±5.09 µm (Figure 4). Finally, at 70 days of age, extremely enlarged STs containing all stages of spermatogenic cells from the basal epithelial (spermatogonia) to elongated spermatids and spermatozoa and boundary of the interstitium, arrangement of Leydig cells and blood vessels were distinctly observed (Figure 3D). The diameter of the STs was 256.42±32.02 µm and height of the epithelial cells layer was 198.6 ±7.71 µm (Figure 4).

Histochemical and immunohistochemical observation of mature testes

For a neat and clear observation of round spermatids and elongated spermatozoa, PAS–hematoxylin, and immunostaining of Hsc70t were performed. In PAS–hematoxylin staining section, testis parenchyma; interstitial cells and the seminiferous epithelium were observed clearly. The interstitial cells were found singly or in clusters and were recognized by their round nucleus and acidophilic cytoplasm in the interstitial space (Figures 2,3).

The stratified epithelium layer of the tubules was made with two cell lineages- somatic cells (Sertoli cells), and germ cells (spermatogenic cells). The spermatogenic cells gave rise to spermatozoa via a complex process named spermatogenesis. Spermatogonia were seen as round cells with dark, rounded nuclei that are located in the basal regions of STs. These cells have undergone divisions and produced spermatocytes, larger cells with distinct chromatin. The spermatocytes were undergone meiotic division and formed round spermatids. The spermatids were round cells with pale nuclei toward the lumen of ST tubule and undergone morphological changes into elongated and were eventually released from the ST as spermatozoa (Figure 5A). The Sertoli cells were elongated, irregularly shaped cells with the prominent nucleolus, and were extended from the base to the lumen of the seminiferous epithelium (Figure 5A).

(Figure 5B) shows the immunohistochemical staining of the testicular section with the Hsc 70t antibody. Hsc 70t protein is expressed in the cytoplasm of round spermatid to the elongated spermatozoa, but not in spermatogonia, spermatocytes and Sertoli cells[14]. A strong Hsc 70t immunological reaction was found in round spermatids and elongated spermatozoa near the lumen of ST, clearly indicated well-characterized spermatogenesis in quail testis at 70 days of age.

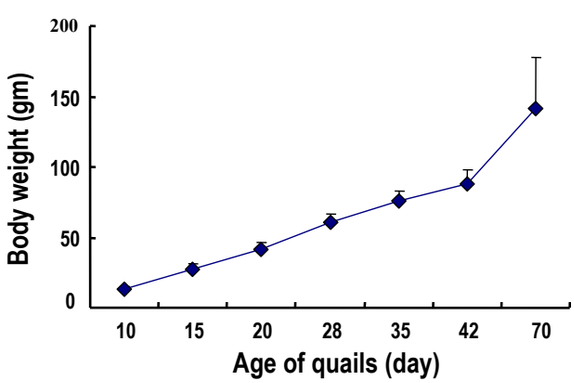

Fig. 1: Japanese quail body weight from post-hatching to sexual maturity (n=6)). The body weight was gradually increased up to 42 days and became remarkable after 42 days to 70 days of age. Differences in organ index were considered to be statistically significant when the p-value was less the 0.05 (*) and 0.001 (**).

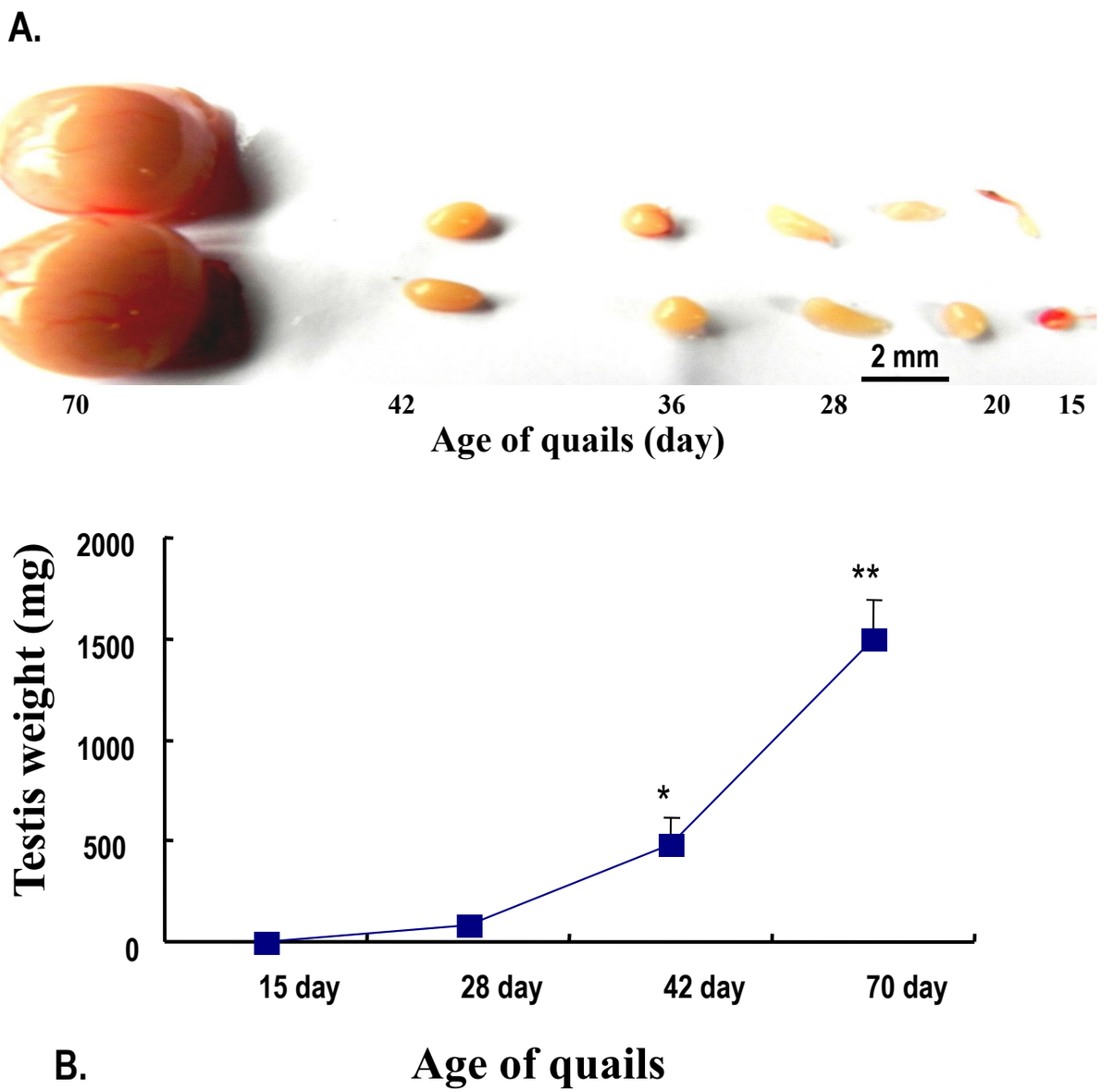

Fig. 2: (A) Images of Japanese quail testes from post-hatching to sexual maturity. (B) Quantification of the testes weight. Note that the testis weight from 15 to 28 days of age increased gradually and became prominent from 42 to 70 days of age. Differences in testes weight were considered to be statistically significant when the p-value was less the 0.05 (*) and 0.001 (**). Scale bar, A=2 mm.

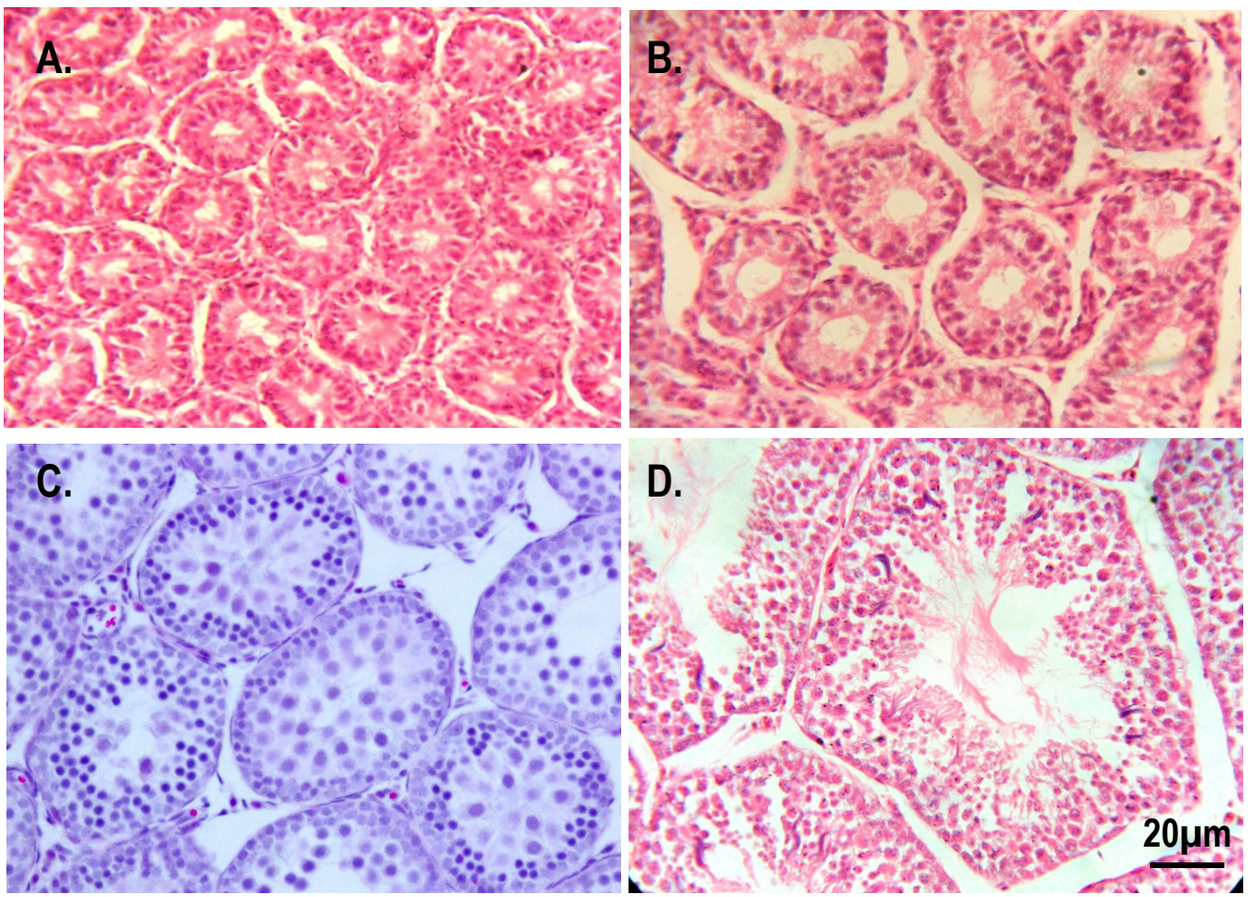

Fig. 3: Histomorphology of testes using hematoxylin and eosin staining. (A) Testis of 15-day-old quail, (B) testis of 28-day-old quail, (C) testis of 42-day-old quail and (D) testis of 70-day-old quail, scale bar, A-B=20 µm; C-D=50 µm.

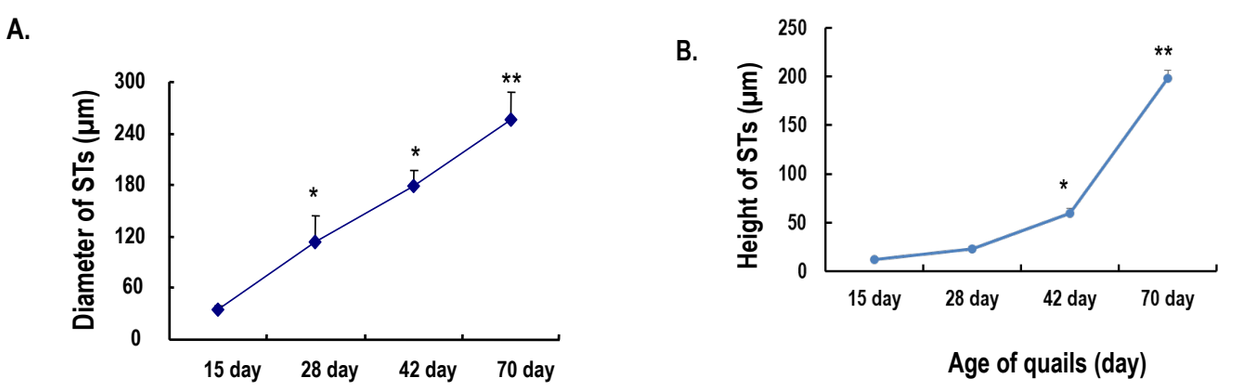

Fig. 4: (A) Diameter of STs at different days of age and (B) height of seminiferous epithelium (n=8). Note that the diameter and height of STs were maximal at 70 days than that of 42 days of age. Differences in ST diameter and ST epithelial height were considered to be statistically significant when the p-value was less the 0.05 (*) and 0.001 (**).

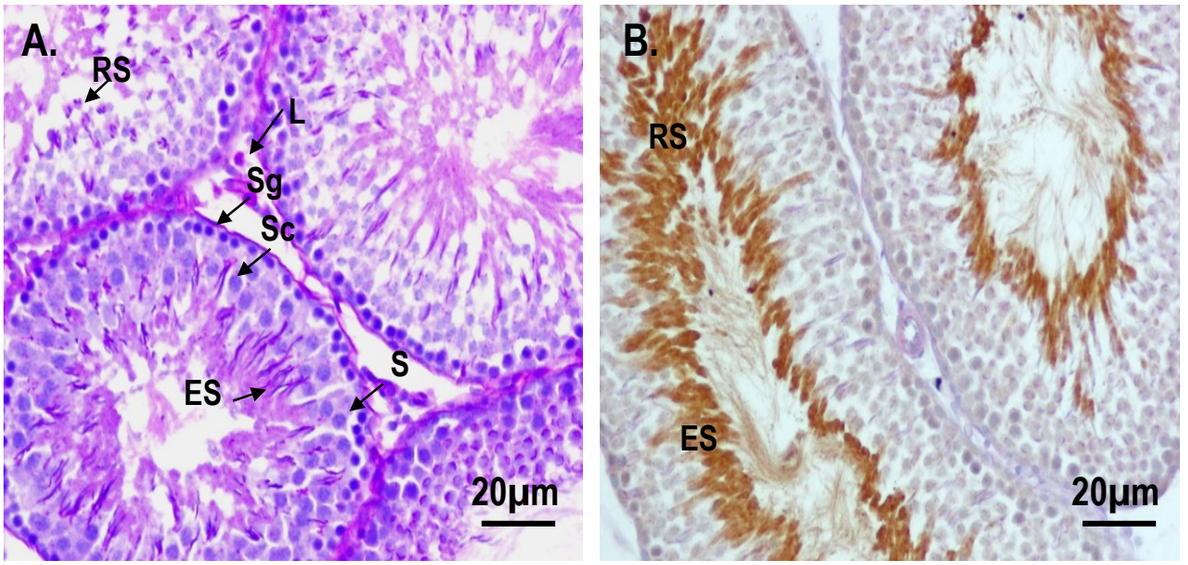

Fig. 5: (A) PAS-hematoxylin staining, (B) Immunohistochemical staining for Hsc 70t protein, a specific marker for the round to elongated spermatid and spermatozoa. The Sertoli cell is denoted by S; spermatogonia, Sg; spermatocyte, Sc; round spermatid, RS; elongated spermatid, ES and Leydig cell, L, etc.

Table 1: Japanese quails body weight, testis weight, length, width and testis index (n=8)

Age	Body weight (g ± SD)	Testis			
		Weight (mg ± SD)	Length (mm ± SD)	Width (mm ± SD)	Testis Index (%)
15 day	27.7±4.05	7.23±1.1	5.6.87±2.4	1.09±1.09	0.026101
28 day	60.26±6.09*	198.18±0.8	11.45±1.7	5.05±0.4	0.328875*
42 day	88.79±10.6*	623.81±0.6*	17.62±1.9*	10.08±0.5*	0.702568**
70 day	143.67±7.8*	1986.25±0.7**	179.97±1.08**	135.6±1.07**	1.382509**

(*p<0.05 and **p<0.001versus preceding group).

DISCUSSION

In the present study, accelerated growth of quail testes was found during day 28-42 and the most significant growth occurred from day 42 onwards to sexual maturity. Vatsalya and Arora (2011) reported that the most significant increase in quail testis occurred at day 36[15]. Although our results differ to some extent from those of Vatsalya and Arora (2011)[15], the Japanese quail begins to secrete LH at 28 to 36 days of age that stimulates the secretion of testosterone which, in turn, stimulates sexual maturity at 42 to 49 day of age[16,17,18]. This explains the reason for the appearance of sexual maturity in the quail around day 42 in this study. Moreover, our observations also indicated that testes weight, length, width, and organ index are positively correlated with age and body weight toward the onset of sexual maturity, i.e. these data tend to increase in increased body weight and age. A similar pattern of relationship was reported in sparrows (Passer domesticus)[19]. A positive relationship among various physiological traits was also published in male Japanese quail[15]. In contrast, it has been reported that domestic fowl testis reaches maximum weight at 160-200 days of age and at 1 month of age the testis become elongated and cylindrical; at 2 months of age and become bean-shaped[20].

Testes are primary sex organs that provide a suitable environment for the production of spermatozoa. Like mammals, the parenchyma of quail testis is divided into lobules by loose connective tissue trabecula (septuli testes). These lobules are composed of STs lined by stratified layers of spermatogenic cells (spermatogonia, spermatocytes, spermatids and spermatozoa) and Sertoli cells. In between the STs, the angular areas, primarily the extra-tubular connective tissue, interstitium which is filled with Leydig cells[21,22]. Blood vessels and lymphatics are also found in the interstitium. The Leydig cells are responsible for testosterone secretion. In reviewing past literature, STs of avian species are slightly different from those of mammals by forming a highly and complexly anastomotic network of tubules and this probably responsible for the lack of connective tissue septa[5]. Unfortunately, the thickness of the connective tissue septa cannot be ascertained at present in routine hematoxylin-eosin staining, further exploration should be needed using special staining. In the present study, the Sertoli cells are tall, columnar and extend from the basal lamina to the luminal border of the STs, and that are well agreed with the report of Aire, (2014)[23] in non-passerine birds and Rajendranath *et al*. (2013)[24] in emu. These suggested that the avian Sertoli cells are almost similar to those of the mammal. The

present results on spermatogenesis are well agreed with the report of William and Linda, (2012)[25] in domestic chicken, where described that the spermatogenesis as in mammals, involves a series of divisions of spermatogonia into primary spermatocytes and secondary spermatocytes, both of which undergo meiotic divisions, results in spermatids and finally, differentiate to form motile, spermatozoa. The well-developed interstitial space of the testis has been reported in mammals[8], the space was found almost similar in the present study, in Japanese quail testis. Moreover, it has been shown similar organization of the interstitial space between other domestic species of birds[5], duck[26], and non-passerine birds[23]. In the present study, the clear boundary of the interstitium, a loose arrangement of connective tissue, and a small number of blood vessels and a single or clusters of Leydig cells in the interstitial space were observed that are well agreed with the reports of others[5,23,26-29], suggesting that the interstitial space has somewhat similar to that of mammals[8].

Quails spermatogenesis is one of the most dynamic processes of cell proliferation, differentiation and morphogenesis and involves numerous cellular and molecular steps. In order to clarify the spermatogenesis, we performed immunohistochemical staining using anti-Hsc 70t antibody. Hsc 70t protein is expressed in the cytoplasm of elongate spermatids, but not in less differentiated spermatogenic cells including spermatocytes and round spermatids[14]. Immunohistochemical staining of Hsc 70t confirmed the well-developed spermatogenesis.

Although the current study on morphological and histomorphological observations of testicular growth and gonads size variation from hatching to sexual maturity, screening of environmental endocrine disruptors (EDCs) toxicity and mechanism of action on the quail testis, has been of great interest over the last decade[10,30-34]. Such a study is now underway in our laboratory to elucidate the mechanism of action of quail testis dysfunction induced by EDCs.

CONCLUSION

The present data revealed the age-related growth and histomorphology of the Japanese quail testes from 15 to 70 days of age and spermatogenesis by histochemical and immunohistochemical staining. These findings may contribute to our understanding of quail's spermatology and provide basic knowledge for reproductive toxicology, physiology as well as pathology studies.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid from the Ministry of Science and Technology, Bangladesh and Research Management Committee/University Grant Commission implemented by BSMRAU (to M. Shah Alam).

ETHICAL STATEMENT

All ethical protocols have been followed in the conduct and preparation of this manuscript.

CONFLICT OF INTERESTS

There was no conflicts of interest.

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