

Morphological characterization of postembryonic development of blood–spleen barrier in duck

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ABSTRACT The spleen is the largest peripheral lymphoid organ and an important site of immune response, in which the blood–spleen barrier (BSB) plays a significant role to resist various pathogens. The BSB structure of duck spleen is different from that of chicken and mammals. However, no information about the development of BSB after the postembryonic age has been reported in ducks. The current study observed the spleen of 1, 7, 14, 21, 35, and 60-day-old ducks by light and electron microscopy to analyze the cellular structural development. The results showed that the spleen index was continuously increased from 1 to 14-day-old ducks. During their early age, the spleen of ducks showed no definite zone of white and red pulp, but the area of the white pulp was large compared to that of the red pulp. The diameter of the ellipsoid was constantly increased in up to 35-day-old duck spleen, while the periellipsoidal

lymphatic sheath (PELS) and periarterial lymphatic sheath continuously developed after 1 D. The reticular fibers developed with age; their branching reached the ellipsoidal wall to show a developed framework in the BSB of 14-day-old ducks. After 7 D, the endothelial cells of the sheathed capillary showed a typical cuboidal shape; between these cells, the gaps increased as age advanced, while the thickness of the basement membrane and collagen fibers increased in 35-day-old ducks. The mechanical filtration function of BSB by intravenous injection showed a 1-layer ring of carbon particles restricted in the white pulp in 1-day-old duck spleen; however, in 14 to 60 D, these particles were restricted in the ellipsoid and PELS, forming 2-layer rings of carbon particles. Collectively, the cellular features of the duck BSB developed up to 35 D of postembryonic age to perform their immune function.

Key words: blood–spleen barrier, high endothelial cell, reticular fiber, duck

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INTRODUCTION

The spleen is the largest peripheral lymphoid organ of the duck, which is located in the blood circulation pathway and plays a major role in hematopoiesis during embryonic development (Linna et al., 1972; Godin and Cumano, 2005). During postembryonic development, with the migration of T and B lymphocytes and the progressive development of red and white pulp, the spleen plays an important role in the body's immune responses, especially to resist and clear blood antigens (Fukuta and Mochizuki, 1982; John, 1994). These functions mainly depend upon the blood–spleen barrier (BSB). The BSB was suggested as a filter bed for red blood cells against microorganisms that lies between the arteries and veins

in rat spleen (Weiss et al., 1986). Later, Zhu et al. (2005) found that the BSB of rat was located in the marginal area of the spleen which was evaluated through carbon injection experiments. They reported that the BSB is composed of splenic sinus endothelial cells, vascular basement membrane, reticular cells, and macrophages. The BSB is a kind of immune barrier, functioning as a mechanical and biological barrier, and presenting antigens to maintain a stable spleen microenvironment (Bao et al., 2009).

The structure of the avian spleen is different from mammalian spleen, which lacks the marginal zone but has a periellipsoidal lymphatic sheath (PELS). The distribution of BSB in avians is different from that of mammals (Zhang et al., 2015). It was suggested that the function of the ellipsoid in avian spleen is identical to the marginal zone of mammalian spleen (Jeurissen et al., 1992). In ducks, the BSB is located in the ellipsoid and PELS, and is mainly composed of high endothelial cells of sheathed capillary (SC), reticular cells, reticular fibers, and macrophages, but lacks ellipsoid-associated cells, compared with chickens (Sun et al., 2019). Unlike

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the mammalian spleen, the duck spleen has high endothelial cells, which play an important role in resisting exogenous substances and lymphocyte homing (Zhang et al., 2017). Including chickens, the avians lack lymph nodes and have a bursa and thymus; however, the bursa and thymus gradually degenerate when the avian matures (Ciriaco et al., 2003). Although the duck has 2 pairs of lymph nodes, there are only a few lymphocytes in the lymph nodes, and there is no reticular tissue in the lymphatic sinus (Rautenfeld and Budras, 1983). The filtration capacity of the lymph nodes is not strong. The BSB has a well-developed reticular fiber network structure with strong filtration, and so it plays a vital role in the peripheral immune system.

The immunological structure and function between avians and mammals are quite different. Although chickens and ducks are both avian, there are some differences between them. Recently, there have been a few studies on the composition and development of chicken BSB, but there is no study examining the development of postembryonic duck BSB. In this study, the development of BSB was investigated in ducks at different ages to further understand the development process of the structure and function of the BSB, which provided a certain morphological theoretical basis for the study of avian immunological mechanisms.

MATERIAL AND METHODS

Experimental Animals

A total of one hundred and eighty, 1-day-old SPF Jinding sheldrakes (purchased from Zhushun Biological Technology Co. Ltd., Nanjing, China) were raised in a sanitized animal room. No vaccine was given during the feeding process. Ducks (1, 7, 14, 21, 35, and 60-day-old) were used in this study ($n = 30$ per age group). The spleen index, hematoxylin and eosin (H&E) staining, silver staining, and transmission electron microscopy values were, respectively, measured in each age group ($n = 5$). The remaining 10 ducks in each age group were used for ink injection experiments (5 ducks were injected with ink; 5 ducks were injected with 0.9% physiological saline). All ducks were euthanized by cervical dislocation after intravenous administration of 3% sodium pentobarbitone (25 mg/kg; Sigma-Aldrich, Darmstadt, Germany). All procedures were approved by the Ethics Committee of Animal Experiments of the Nanjing Agricultural Veterinary College (approval ID: SYXK (SU) 2010-0005).

Spleen Index

Body weights of the ducks were recorded firstly. Then, immediately after euthanization, the spleens were collected, and their weights were measured. Furthermore, the spleen tissue samples were processed for light and ultramicroscopic investigation. The spleen index was calculated by the following formula:

$$\text{Spleen index} = \frac{\text{spleen weight (mg)}}{\text{body weight (g)}} \times 100\%$$

H&E Staining

The spleens were stored in 4% paraformaldehyde (P6148, Sigma-Aldrich) to obtain paraffin sections; a sections thickness of 6 μm was achieved by using a microtome (RM2015, Leica, Solms, Germany). After being dewaxed and dehydrated, they were stained with H&E (Harris's hematoxylin for 2 min and 1% eosin for 30 s). The tissue slides were then observed under a light microscope (DP73, Olympus, Tokyo, Japan).

Injecting Ink

Chinese ink (5 mL/kg of 10% ink; Yidege, Beijing, China) was injected intravenously into ducks at different ages. The ink particles were about 50–100 nm in diameter, and the ink was diluted with 0.9% physiological saline (R21479, Yuanye Biotechnology Co. Ltd., Shanghai, China). The control group was injected with the same volume of 0.9% physiological saline (R21479, Yuanye Biotechnology Co. Ltd.). After 30 min of ink injection, the ducks were euthanized and the spleens were collected. Then, formalin-fixed, paraffin sections (6 μm) were assessed using light microscopy (DP73, Olympus), with or without H&E staining.

Silver Staining

The spleens were stored in 4% paraformaldehyde (P6148, Sigma-Aldrich) to obtain paraffin sections, and sections of 10 μm thickness were obtained. Paraffin sections were assessed by the method of Gordon and Sweets. After dewaxing in water, the sections were rinsed in distilled water for 5 min between incubation steps of being oxidized by 1% potassium permanganate (441244, Sigma-Aldrich) for 3 min, bleached by 1% oxalic acid (247537, Sigma-Aldrich) for 3 min, mordanted by 2.5% ferric ammonium alum (A9750, Solarbio Science & Technology Co. Ltd., Beijing, China) for 14 min, immersed by silver ammonia (R20376, Yuanye Biotechnology Co. Ltd.) for 5 min, reduced by 10% formaldehyde (F8775, Sigma-Aldrich) for 2 min, toned with 0.2% gold chloride (S30172, Yuanye Biotechnology Co. Ltd.) for 10 s, and removing silver by 5% sodium thiosulfate (S24159, Yuanye Biotechnology Co. Ltd.) for 3 min.

Transmission Electron Microscopy

Immediately after euthanization, the spleens were cut into 1 mm^3 blocks, immersed in 2.5% glutaraldehyde (G5882, Sigma-Aldrich) at 4°C overnight, and then immersed in 1% osmium tetroxide solution (41949, Sigma-Aldrich) for 60 min. Then, they were gradient dehydrated in ethanol (117902104, Yonghua Chemical Technology Co. Ltd., Jiangsu, China) and embedded

in Epon 812 (45359, Sigma-Aldrich). Ultrathin sections were obtained and stained with lead citrate and uranyl acetate for 20 min, and the sections were observed with a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan).

Data Analysis

The slices were measured with Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD) and the data were expressed as means \pm SEM. Data were processed using IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY). One-way ANOVA was followed by Duncan test, with significant differences set at P -values < 0.05 .

RESULTS

Spleen Index of 1 to 60-day-Old Ducks

The spleens of ducks of different ages are shown in Figure 1. Results are presented in Table 1. It was found that the spleen index continuously increased between 1 and 14 D of age, while the spleen index tended to decrease after 14 D.

Morphological Characteristics of Spleen of 1 to 60-day-Old Ducks

After hatching, the duck spleen consisted of red and white pulp without any definite demarcation of boundary. The area of white pulp was large compared to that of the red pulp, the PELS was formed, and showed no splenic nodule (Figures 2A and 2B). The diameter of the ellipsoid was continuously increased from 1 to 35 D of age (Table 2). At 7 D of age, the area of red pulp increased, the periarterial lymphatic sheath (PALS) was formed, and the splenic nodule appeared, but the boundary was not clear (Figures 2C and 2D). Until 14 D of age, the splenic nodule was intact and clear, and the lymphocytes around the ellipsoid and central artery increased (Figures 2E and 2F). In 35 and 60-day-old ducks, the diameter of the ellipsoid tended to be stable, and there were more lymphocytes around the PALS and PELS, which is more likely characteristic of a mature spleen (Figures 2I–2L).

Distribution and Development of Reticular Fibers in 1 to 60-day-Old Duck BSB

The 1-day-old duck BSB showed scant and fine reticular fibers in the ellipsoid and PELS areas, while the reticular fibers around the basement membrane of the SC were very thin. There was no clear delineation of the ellipsoidal wall (Figures 3A and 3B). As age advanced, the reticular fibers around the SC became thicker. Less developed network structures of reticular fibers were observed in the ellipsoid and PELS areas of 7-day-old duck BSB. Fibers were arranged radially around the SC (Figures 3C and 3D). The reticular fibers formed the ellipsoidal wall and showed a definite structure of the ellipsoidal area at 14 D of age. Meanwhile, there was a clear border of discontinuous fibers between PELS and the red pulp (Figures 3E and 3F). The reticular fibers became thicker and condensed around the SC to show a well-developed network structure in the spleen in 21 to 60-day-old ducks (Figures 3G–3L). It was observed that the reticular fibers continuously increased from early age to 14 D, as shown in Figure 4. Afterward, the reticular fibers showed no definite changes, due to their developed and condensed structure.

Ultrastructure of the High Endothelium Vessel in 1 to 60-day-Old Duck Spleen

In the 1-day-old duck spleen, the morphology of endothelial cells was relatively round, and the cells were rich in mitochondria, endoplasmic reticulum, and multivesicular bodies. The adjacent endothelial cells were tightly connected with each other and the basement membrane was thin (Figure 5A). At 7 D of age, the high endothelial cells showed a typical cuboidal shape (Figure 5B). The gap between adjacent endothelial cells became wider with the advancement of age. After 35 D of age, the basement membrane was thickened and discontinuous, and the collagen fibers around the high endothelial cells increased with age (Figures 5C and 5D).

Function of Mechanical Filtration of 1 to 60-day-Old Duck BSB

Intravenous ink was injected to investigate the development of BSB in ducks of different ages. In the 1-day-old duck spleen, carbon particles were partly restricted

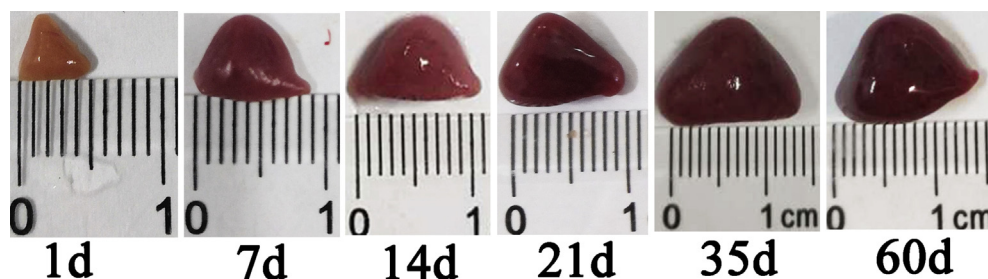


Figure 1. Duck spleens in different ages.

Table 1. The spleen index of 1 to 60-day-old ducks.

Age (D)	Weight of duck (g)	Weight of spleen (mg)	Spleen index (%)
1	34.40 ± 1.50	22.52 ± 0.96	0.6549 ± 0.01 ^a
7	89.97 ± 4.18	96.04 ± 7.24	1.06 ± 0.04 ^{b,c}
14	193.8 ± 10.86	220.8 ± 6.63	1.14 ± 0.05 ^c
21	364.6 ± 15.10	327.3 ± 25.37	0.8945 ± 0.04 ^d
35	710.6 ± 20.31	695.6 ± 20.21	0.9789 ± 0.01 ^{b,d}
60	1,348 ± 59.78	799.46 ± 58.70	0.59 ± 0.02 ^a

^{a-d}Data without the same superscripts differ significantly ($P < 0.05$) in the list of spleen index.

around the ellipsoid in the white pulp, while most carbon particles dispersed in the red pulp. Carbon particles appeared as a 1-layer ring (Figures 6A and 6B). At 7 D of age, 2-layer rings of carbon particles appeared in the spleen with a few carbon particles dispersed in the red pulp (Figures 6C and 6D). At 14 to 60 D of age, 2-layer rings of carbon particles were observed in the ellipsoid and PELs, with almost no carbon particles dispersed in the red pulp (Figures 6E–6L).

DISCUSSION

The spleen is an important immune organ of avians, which performs functions of resisting and presenting antigens, and has an immune response; these functions are highly dependent on the BSB. The increase in spleen weight is an important macroscopic indicator of organ development, which is caused by cell growth, and division and proliferation (Wang et al., 2016). An increase in the spleen index means an increase in its immune

function. The enhancement of the immune function of the spleen has a certain correlation with the development of the BSB. We found that the spleen index of ducks continuously increased between 1 and 14 D of age. Under the H&E results, we found 2 main changes during development. One change is that the ellipsoid area, which is the primary component of the BSB, increased constantly from 1 to 35-day-old duck spleen. The other is related to the lymphocytes. The PELs formed before the PALS in 1-day-old duck spleen, and the visible splenic nodules were not found until 14 D of age. The PALS, PELs, and splenic nodules are mainly composed of lymphocytes, which are important immune cells. The increase in lymphocytes indicates that the immune function of the spleen is gradually developing. Both these changes suggested that the structure and function of the BSB improved. After 14 D of age, the duck weight-gain rate is higher than that of the spleen, and the spleen index decreases. However, this is also consistent with the growth and development of the duck.

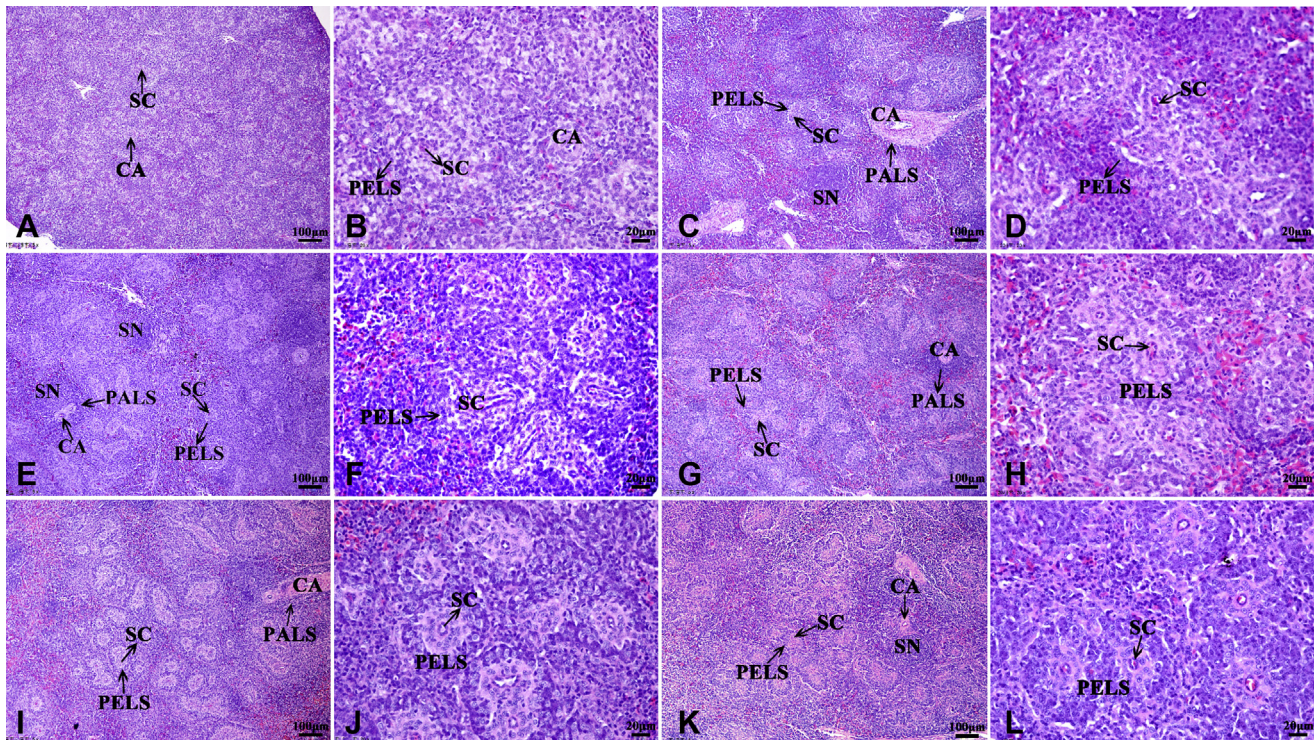


Figure 2. Light microscopy of 1 to 60-day-old duck spleens (hematoxylin and eosin staining). Histological structure of (A, B) 1-day-old, (C, D) 7-day-old, (E, F) 14-day-old, (G, H) 21-day-old, (I, J) 35-day-old, and (K, L) 60-day-old duck spleen. Abbreviations: CA: central artery; PALS: periarterial lymphatic sheath; PELs: periellipsoidal lymphatic sheath; SC: sheathed capillary; SN: splenic nodule. Bar = 100 µm (A, C, E, G, I, K) and 20 µm (B, D, F, H, J, L).

Table 2. The diameter of the ellipsoid of 1 to 60-day-old ducks.

Age (D)	Mean diameter of ellipsoid (μm)
1	$14.41 \pm 0.62^{\text{a}}$
7	$23.91 \pm 1.17^{\text{b}}$
14	$38.56 \pm 1.27^{\text{c}}$
21	$41.02 \pm 0.85^{\text{c}}$
35	$50.89 \pm 1.50^{\text{d}}$
60	$52.31 \pm 1.23^{\text{d}}$

^{a-d}Data without the same superscripts differ significantly ($P < 0.05$).

Our study found that the reticular fibers were sparse in the duck spleen after 1 D, and the ellipsoidal network did not appear. From 1 to 14 D of age, the reticular fibers in the spleen continue to increase, until, at 14 D, the reticular cells and reticular fibers form a stable supporting framework of the BSB. The distribution of reticular fibers in the duck spleen is similar to that of the quail; however, the network of reticular fibers appeared within 10 D after embryo formation in quails (Liman and Bayram, 2011). When compared with chicken (Biro et al., 2011), the distributions of reticular fibers in the duck BSB were different; as well as in the basement membrane of blood vessels and the ellipsoids, there are also discontinuous circular distributions of reticular fibers between the border of PELS and red pulp in ducks. In lymphoid tissues, reticular cells and reticular fibers form a supporting framework that provides channels and specific microenvironments for the migration of T and B lymphocytes (Ewijk and Kwast, 1980; Brelinka et al., 1984; Pellas and Weiss, 1990). Weiss (1991) showed that activated reticulocytes increase their

branches to protect the spleen from parasites by isolating blood. It is obvious that the developed network of reticular fibers plays an important supporting role in the immune function of the spleen.

After the blood antigen invades the spleen, the endothelial cells of the SC are the first that are exposed to the antigen, and then the antigens enter into the ellipsoids (Yassine et al., 1989). The endothelial cells of the SC are the first line of defense of the BSB. The high endothelial structure in the avian spleen is a channel for lymphocyte homing (Buysens et al., 1984; Zhang et al., 2015), which is useful for identifying antigenic substances and enhancing immune function (Girard et al., 2012). During the development of lymphoid organs, high endothelial vessels express vascular addressins that regulate lymphocyte entry into lymphoid organs that initiate and sustain the development of lymphoid tissues (Ager, 2017). Electron microscopy results showed that, in 1-day-old ducks, the shapes of the endothelial cells of the splenic SC were round, and there was no gap between the adjacent cells. Typical cuboid high endothelial cells were observed at 7 D of age. As age increased, the intercellular space gaps increased, which may facilitate the migration of lymphocytes for the development of the spleen. High endothelial cells are rich in multivesicular bodies, which play important roles in cell-to-cell communication (Robbins and Morelli, 2014). With advances in age, the structure of high endothelial cells of the capillary is continuously improved, which is conducive to the strengthening of the immune function of the body.

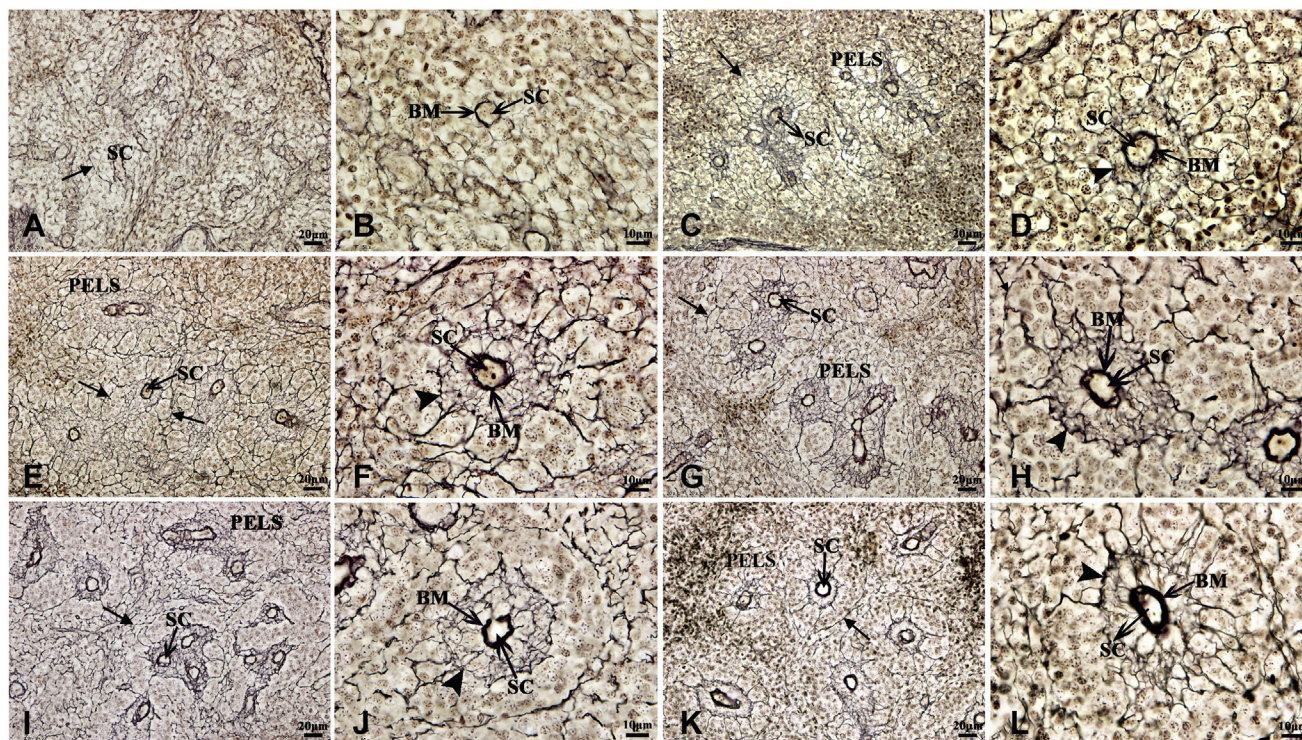


Figure 3. The distributions of reticular fibers in the BSB of 1 to 60-day-old ducks (silver stainings): (A, B) 1-day-old, (C, D) 7-day-old, (E, F) 14-day-old, (G, H) 21-day-old, (I, J) 35-day-old, and (K, L) 60-day-old duck spleen. Abbreviations: BM: basement membrane; PELS: periellipsoidal lymphatic sheath; SC: sheathed capillary. → denotes reticular fibers and ▲ denotes the ellipsoidal wall. Bar = 20 μm (A, C, E, G, I, K) and 10 μm (B, D, F, H, J, L).

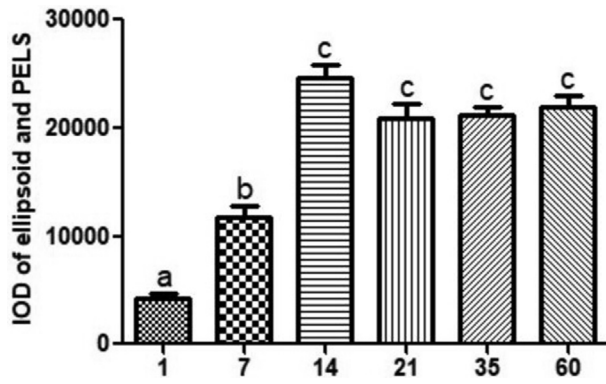


Figure 4. Integrated optical density (IOD) of reticular fibers in the ellipsoid and its surrounding periellipsoidal lymphatic sheath (PELS) in the spleens of 1 to 60-day-old ducks. ^{a-c}Data without the same superscripts differ significantly ($P < 0.05$).

BSB is mainly involved in functioning as a mechanical barrier, biological barrier, and in presentation of antigens. Carbon particles are often used as heterogeneous particles to study the non-specific immune function of the body and the ability of macrophages to clear antigens (Fujita et al., 1983; Bao et al., 2009). In this study, carbon injection tests revealed that, in 1-day-old duck, the carbon particles were partly dispersed in the red pulp, which may be due to the imperfect development of the spleen barrier structure of the duck, due to the

thin basement membrane of the SC and sparse reticular fibers. With the development of ellipsoids, the carbon particles in the spleen were restricted and concentrated around the ellipsoids and PELs at 14 D of age, and the BSB had enhanced ability to block the carbon particles. It is reported that macrophages, which are located at the junction of PELs and the red pulp in duck spleen, are important constituents of the biological barrier (Sun et al., 2019). In our study, it was found that, with the development of the spleen, the area of red pulp gradually increased, and so the biological barrier function also developed. Lu et al. (2016) reported that the duck Tembusu virus pathogenesis is stronger in 1-week-old ducks compared with older ducks. It was also suggested that the structure and function of the BSB of 1-week-old ducks were not developed, and the abilities of virus resistance and antigen presentation were weaker compared with the older ducks. In addition, when adult chickens were injected with ink for 30 min, the carbon particles are concentrated around the ellipsoids, forming only a single layer of carbon ring (Zhang et al., 2019), while the duck spleen forms a double-layer carbon ring at the ellipsoids and PELs after ink injection. This suggests that the mechanical barrier function of the duck is stronger than that of the chicken. Although ducks and chickens are both avians, avian influenza is highly lethal for chicken when compared to duck (Kang et al., 2017).

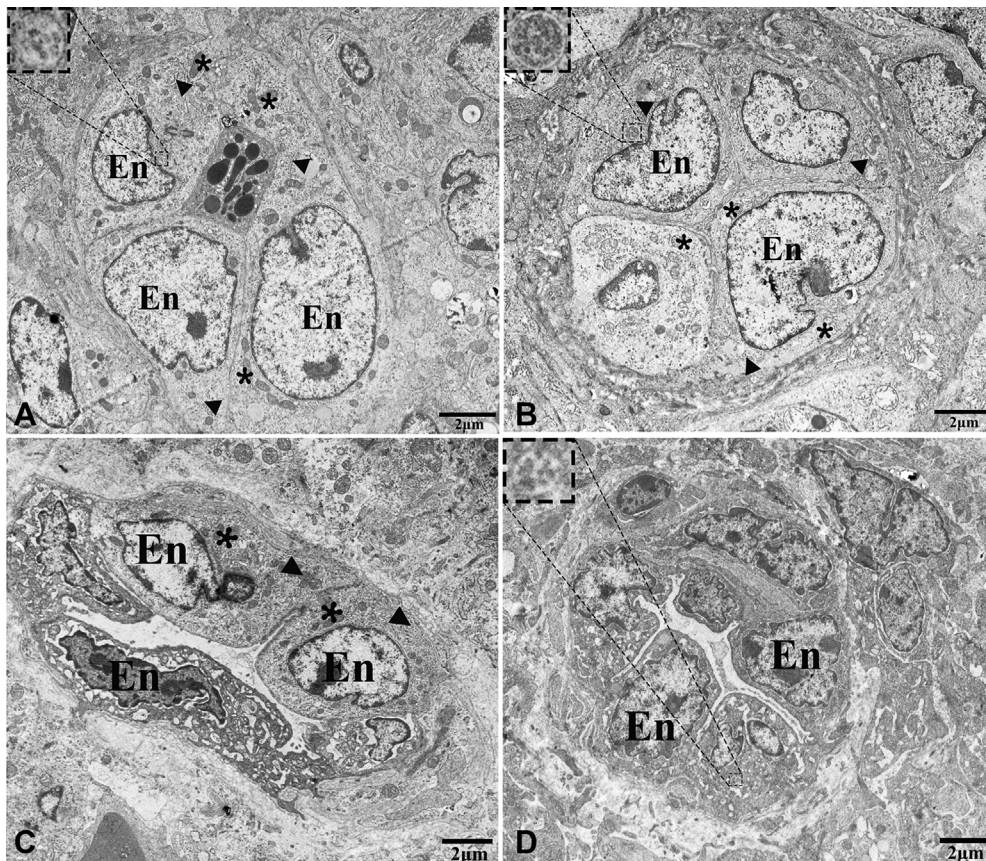


Figure 5. The ultrastructure of high endothelial cells of the sheathed capillary of 1 to 60-day-old ducks: (A) 1-day-old, (B) 7-day-old, (C) 35-day-old, and (D) 60-day-old duck spleen. En: endothelial cell. Black dotted rectangular area (enlarged area) shows the multivesicular bodies, ▲ represents the endoplasmic reticulum, and * denotes mitochondria. Bar = 2 μm (A–D).

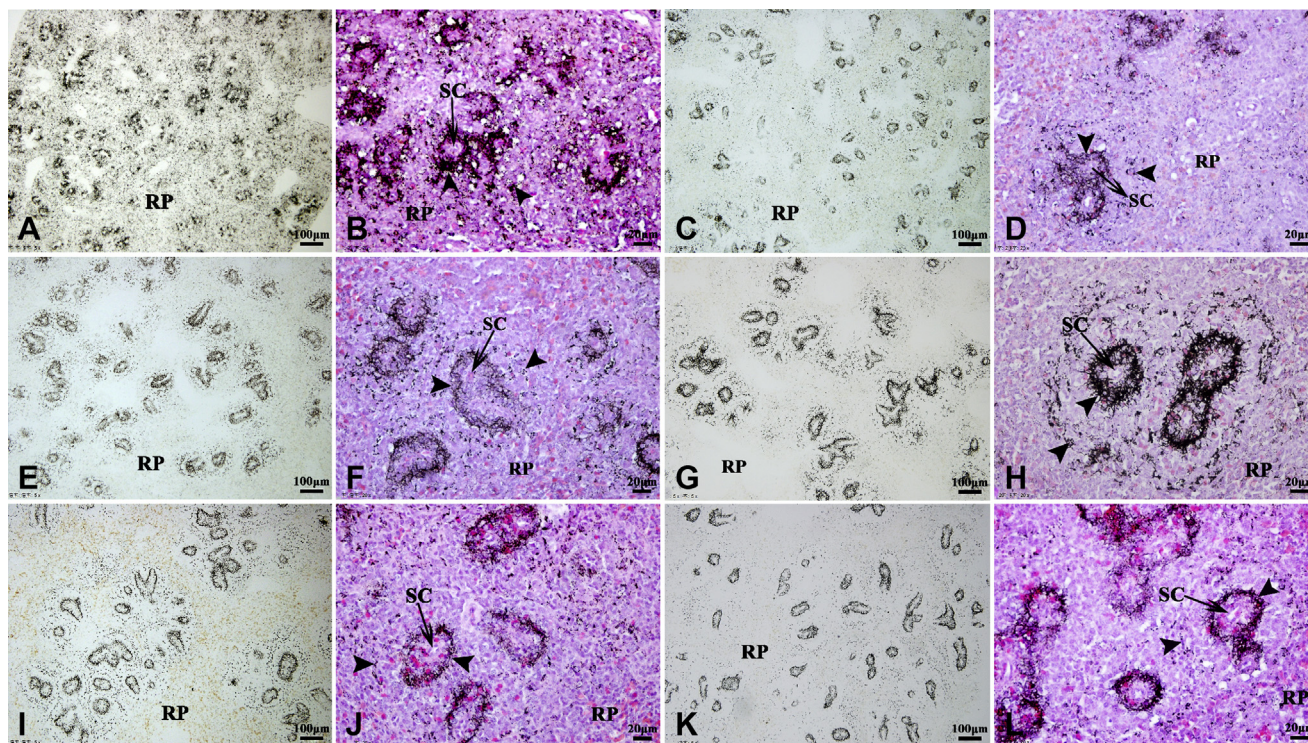


Figure 6. The blood-spleen barrier resisting carbon particles in 1 to 60-day-old duck spleen (hematoxylin and eosin staining). Histological structure of (A, B) 1-day-old, (C, D) 7-day-old, (E, F) 14-day-old, (G, H) 21-day-old, (I, J) 35-day-old, and (K, L) 60-day-old duck spleen. RP: red pulp; SC: sheathed capillary. Bar = 100 μ m (A, C, E, G, I, K) and 20 μ m (B, D, F, H, J, L). \blacktriangle denotes the carbon particles.

This may be related to the differences in the structure and function of the BSB in chickens and ducks.

CONCLUSION

In summary, our study shows details of the morphological characteristics of the development of postembryonic duck BSB. In 1-day-old duck spleen, the reticular fibers were sparse and the function of BSB was not developed. In 7-day-old duck BSB, the endothelial cells of the SC formed a typical cuboidal shape. The reticular fibers developed into a supporting framework of BSB at 14 D of age. Up to 35 D of postembryonic age, the cellular structure and function of the BSB developed. These findings provide a structural theoretical basis for splenic barrier function and will likely be helpful for investigating avian infectious diseases.

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