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Expression of Zonula Occludens-1 and Claudin-1 Proteins in Japanese Quails Testis

Bıldırcın Testisinde Zonula Okludens-1 ve Klaudin-1 Proteinlerinin Ekspresyonu

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ABSTRACT

The aim of this study was to evaluate the general histological structure of testis in prepubertal and postpubertal stages of quails and determine the presence and location of claudin-1 and zonula occludens-1 proteins. In this study, testicular tissues obtained from 6 prepubertal and postpubertal stage quails were used. Tissue samples were fixed in 10% formaldehyde and processed for paraffin embedding. Crossman's triple staining method was used for general histological evaluation. Immunohistochemistry and immunofluorescent staining were performed for the expression of claudin-1 and zonula occludens-1 proteins, respectively. It has also been concluded that the proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity because an active blood–testis barrier is not formed yet in seminiferous tubule epithelium in the testis in the prepubertal period as a result of immunohistochemical and immunofluorescence stainings but show immunoreactivity in the basal area in seminiferous epithelium with the blood–testis barrier formed in the postpubertal period. Immunoreactivity wasn't observed in prepubertal quail's testis. The immunoreactivity of claudin-1 has been distinguished as cytoplasmic and membranous in Sertoli cells and spermatogonia in postpubertal quails' testis. The immunoreactivity of zonula occludens-1 has not been observed in seminiferous tubules in prepubertal stage quail's testis. Immunoreactivity has been observed in the basal half of seminiferous tubules in postpubertal stage quail's testis. It has also been concluded that the proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity because an active blood–testis barrier is not formed yet in seminiferous tubule epithelium in the testis in the prepubertal period as a result of immunohistochemical and immunofluorescence stainings but show immunoreactivity in the basal area in seminiferous epithelium with the blood–testis barrier formed in the postpubertal period.

Keywords: Blood–testis barrier, claudin, Japanese quail, zonula occludens

ÖZ

Bu çalışmanın amacı, bıldırcınların prepubertal ve postpubertal dönemlerinde testisin genel histolojik yapısını değerlendirmek ve klaudin-1, zonula okludens-1 proteinlerinin varlığını ve yerleşimini belirlemektir. Bu çalışmada 6 adet prepubertal ve postpubertal dönemlerdeki bıldırcınlardan elde edilen testis dokuları kullanıldı. Dokular %10'luk formaldehitte fikse edildi. Rutin doku takibi işleminden geçirildikten sonra parafin bloklar elde edildi. Genel histolojik değerlendirme için Crossman'ın üçlü boyama yöntemi kullanıldı. Klaudin-1 ve Zonula Okludens-1 proteinlerinin gösterimi için sırası ile immünohistokimya ve immünfloresan boyamaları gerçekleştirildi. Prepubertal bıldırcın testislerinde immünreaktivite gözlenmemiştir. Claudin-1'in immünoreaktivitesi postpubertal bıldırcın testislerinde Sertoli hücrelerinde ve spermatogonyumlarda sitoplazmik ve membransel olarak ayırt edilmiştir. Zonula occludens-1'in immünreaktivitesi prepubertal bıldırcın testisinde seminifer tübüllerde gözlenmemiştir. Postpubertal dönemdeki bıldırcın testisinde seminifer tübüllerin bazal bölgesinde immünreaktivite gözlenmiştir. Sonuç olarak diğer kanatlılarda olduğu gibi bıldırcınlarda da prepubertal dönemde kan-testis bariyerinin gelişiminin tamamlanmamış olduğu; postpubertal dönemde ise kan-testis bariyerinin oluştuğu ve bu bariyerin oluşumunda klaudin-1 ve zonula okludens-1 proteinlerinin katıldığı sonucuna ulaşılmıştır.

Anahtar Kelimer: Bıldırcın, kan-testis bariyeri, klaudin, zonula okludens

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INTRODUCTION

The reproductive system is one of the most critical factors necessary for the continuity of the generation. The protection of normal male fertility is based on producing healthy sperm. Healthy sperm production requires the differentiation of germ cells in a sheltered environment. The differentiation of the germ cells occurs in the postpubertal period, a long time after the maturation of the immune system. That is why these differentiated germ cells become an inevitable threat to the immune system and are trying to be destroyed by the immune system. The blood–testis barrier occurring with the pubertal period in the testis ensures that the cells are located in a protected medium by keeping the germ cells separate from this adverse situation. The blood–testis barrier among Sertoli cells in seminiferous tubule epithelium was situated around the basal third of the seminiferous tubule. This placement of the blood–testis barrier divides the seminiferous tubule epithelium into 2 regions, apical and basal. Spermatogonia and preleptotene spermatocytes are found in the basal region of the seminiferous epithelium, while primary and secondary spermatocytes, round spermatids, and elongating/elongated spermatids are located in the apical region. Tight junctions have many integral and peripheral membrane proteins. Zonula occludens-1 and claudin-1 proteins are peripheral and integral membrane proteins, respectively.[1-6]

Claudin was discovered by Furuse et al[7] (1998) for the first time. Claudin is a molecule weighing approximately 22 kDa. Claudins are composed of a short amine (NH_2) cytoplasmic area, 2 extracellular areas, 4 transmembrane areas, and a long carboxyl (COOH-) cytoplasmic area. There are 27 different claudin molecules identified in different epitheliums. In the testis, 7 other claudin molecules have been identified as claudin 1, 3, 5, 7, 8, and 11. The cytoplasmic COOH- area of claudin protein binds to Zonula occludens-1's Postsynaptic Density-95 Discs-large zonula occludens-1 (PDZ) in the ratio of 1 : 1.[4,7-11] Claudin-1 is the structural element of epidermal barrier in the epithelium. Claudin-1 takes part in cell motility. The localization of claudin-1 in the testis differs between mammalian species.[12,13]

Zonula occludens, a member of the Membrane Associated Guanylate Kinase homolog protein family, have 3 members zonula occludens-1, -2, and -3. Zonula occludens is structurally composed of 3 different areas: a guanylate kinase–like area, a src-coupling area, and 3 PDZ areas. Zonula occludens control cell reproduction and membrane organization, regulate cell differentiation and polarization, and manage signal transduction pathways. Zonula occludens-1 is synthesized by Sertoli cells but not by germ cells. Zonula occludens-1 provides a mechanochemical binding between the cytoskeleton and integral membrane proteins.[4,14-18]

By detecting these proteins in the structure of the blood–testis barrier, changes in the blood–testis barrier can be detected, and thus, the defects in the blood–testis barrier can be revealed. In the literature review, it was seen that claudin and zonula occludens proteins were not studied in quail testicles before. Therefore, this study aims to conduct the general histological evaluation of prepubertal and postpubertal stage quails' testis and determine the presence and location of claudin-1 and zonula occludens-1 from tight junction proteins forming the basis of the blood–testis barrier.

MATERIALS AND METHODS

This study was approved by the decision no. 2016/54 of the Ethics Committee of Experimental Animals of Reproduction and Research Center of the Faculty of Veterinary of Selçuk University on 29.06.2016. In the study, 30-day-old prepubertal (n = 6) and 70-day-old postpubertal (n = 6) Japanese quails (*Coturnix coturnix japonica*) were used. Testis tissues were obtained from the sacrificed animals. The tissue samples were fixed in 10% formol solution, processed by routine histological techniques, and embedded in paraffin. For the general evaluation, Crossman's triple staining method was used.[19] General histological examinations were made in preparation, and the thickness of the capsule and the diameters of seminiferous tubules were measured.

For the assessment of the claudin-1 protein, the avidin-biotin-peroxidase immunohistochemical staining method was used. Testis samples were left in 1 M citrate buffer (pH = 6) in the microwave (750 W) for antigen retrieval for 15 minutes. Then, the section was incubated with a protein block for 5 minutes to prevent nonspecific antibody binding and ground staining. Then, the section was incubated with claudin-1 primer antibody (Invitrogen, cat. no: 51-900) diluted in the ratio of 1/100 and, after that, with the secondary antibody for 30 minutes. To prevent endogenous peroxidase activity, they were placed into 3% hydrogen peroxidase (H_2O_2) prepared with methanol for 30 minutes. After, they were incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 30 minutes. Finally, they were incubated with diaminobenzidine (DAB). They were counterstained for 2 minutes with Mayer's Hematoxylin. For negative control, phosphate buffered saline (PBS) was dropped on samples instead of primary antibody. Then, the protocol was continued in the same way.[20,21]

For the assessment of the zonula occludens-1 protein, immunofluorescence staining was made. First, the samples were incubated with PBS Triton-X100 normal goat serum for 15 minutes. Then, they were incubated with zonula occludens-1 primary antibody (Abcam, cat. no: ab59720) diluted with antibody dilution solution in the ratio of 1 : 100 and after that with fluorescein isothiocyanate (FITC) conjugate goat anti-Rabbit IgG (Abcam, ab6717) secondary antibody diluted with block solution in the ratio of 1 : 1000 for 3 hours. Finally, the samples were mounted with 4′,6-diamidino-2-phenylindole (DAPI). For negative control, the slides were incubated with PBS instead of primary antibody and the protocol was continued the same way.[22]

Statistical Analysis

Statistical analysis of the data obtained by the measurements was created using the independent sample *t*-test method with MINITAB 14 package program. $P < .05$ was considered as significant.

RESULTS

Macroscopic Results

It was observed that prepubertal and postpubertal stage quails' testis was placed in the abdominal cavity. In addition, it was observed that the ductus deferens of quails were not apparent in the prepubertal period but got apparent in the postpubertal period and opened into the cloaca. In addition, it has been observed that postpubertal stage quails' testis are quite more significant compared to prepubertal stage quails (Figure 1).

Light Microscopic Results

It was observed in tissue sections prepared by Crossman's triple staining method in prepubertal stage quail's testis that

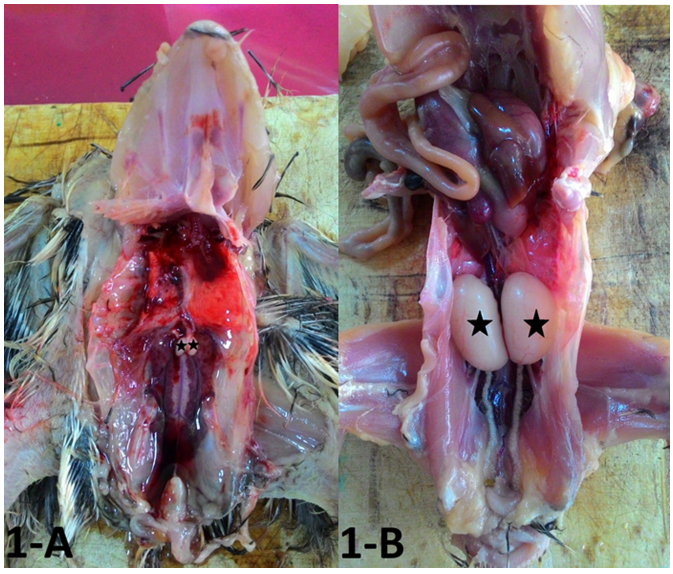

Figure 1. Macroscopic view of the genital organs of man quail. (A) Prepubertal period. (B) Postpubertal period (stars: testis, arrows: ductus deference).

seminiferous tubules are composed of Sertoli cells and spermatogonia. It was observed that Sertoli cells are placed in basal area and in the form of cells having triangle cores sloping toward apical and that there is an apparent basal membrane around seminiferous tubules. Loose connective tissue, blood vessels, and Leydig cells have been encountered in the interstitial area (Figure 2).

In the postpubertal quail testis, it was observed that Sertoli cells and spermatogenic germ cells at various stages of development in seminiferous tubule epithelium were present, and these cells were in the form of columns extending toward the lumen. The presence of sperms in the lumen of the seminiferous tubule has drawn attention. It has been seen that there is an apparent basal membrane around seminiferous tubules. It has been observed that loose connective tissue, blood vessels, and Leydig cells have been encountered in the interstitial area (Figure 3).

Statistical evaluation of the data obtained with measurement of the diameter of seminiferous tubule and thickness of capsule in prepubertal and postpubertal quails' testis has been carried out using the independent sample *t*-test method. It has been found that the diameter of the seminiferous tubule and thickness of the capsule increase in the postpubertal period, and this increase is statistically significant (Tables 1 and 2).

Immunohistochemical Results

As a result of immunohistochemical staining by using a claudin-1 primary antibody, immunoreactivity has not been observed in prepubertal stage quail's testis (Figure 4).

Immunoreactivity in the basal area of seminiferous tubules in postpubertal stage quails' testis sections has been observed. The immunoreactivity of claudin-1 has been distinguished as cytoplasmic and membranous in Sertoli cells and spermatogonia. It has drawn attention that claudin-1 is mainly in the area where the blood–testis barrier is formed. Any immunoreactivity has not been observed in negative control sections (Figure 5).

Immunofluorescence Results

As a result of immunofluorescence evaluation using zonula occludens-1 primary antibody in prepubertal stage quail, the immunoreactivity of zonula occludens-1 has not been observed in seminiferous tubules (Figure 6).

Immunoreactivity has been observed in the basal half of seminiferous tubules in postpubertal stage quail's testis sections

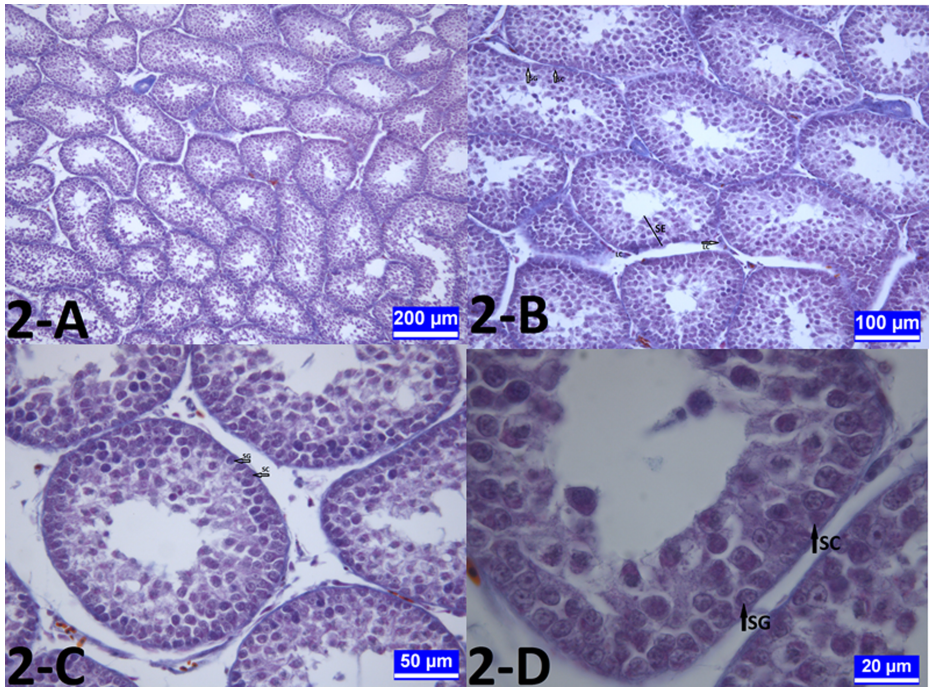

Figure 2. General histological view of prepubertal stage quail's testis in different zoom rates, Crossman's triple staining. LC, Leydig cell; SC, Sertoli cell; SE, seminiferous tubule epithelium; SG, spermatogonia.

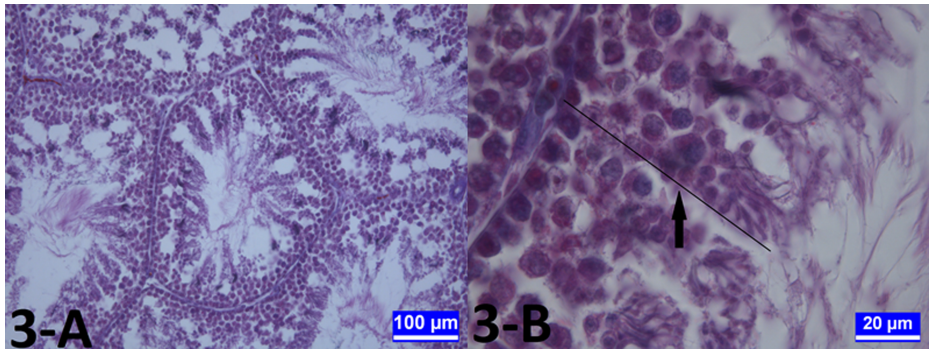

Figure 3. General histological view of postpubertal quail's testis in different zoom rates (arrow: cell cords), Crossman's triple staining.

Table 1. The Diameters of Seminiferous Tubules of Prepubertal and Postpubertal Stage Quails' Testis

	Number of Measurement	Average	Standard Error	Minimum	Maximum
Prepubertal stage	60	158.4	3.7	101.29	234.7
Postpubertal stage	60	294.8[a]	8.9	176.6	472.2

[a]The difference between groups is statistically significant ($P < .05$).

Table 2. The Thicknesses of Capsules of Prepubertal and Postpubertal Stage Quails' Testis

	Number of Measurement	Average	Standard Error	Minimum	Maximum
Prepubertal stage	60	29.04	0.98	16.5	49.75
Postpubertal stage	60	37.92[a]	0.76	26	49,51

[a]The difference between groups is statistically significant ($P < .05$).

(Figure 7-A, B, and C). It has drawn attention that radiation is especially in seminiferous tubules in Sertoli cells. Any immunoreactivity has not been observed in negative control sections (Figure 7-D).

DiscussionThe testis is responsible for reproducing sperms, one of the most critical elements necessary for generation continuity. Sperms are produced in seminiferous tubules, functional units of the testis. There are germ cells and spermatogonia in seminiferous tubules in the prepubertal period.[23] There are specific changes in the postpubertal period. One of these changes is that the germ cells begin to synthesize different surface proteins. Because surface proteins reproduced in germ cells show up a long time after the immune system grows, germ cell is perceived as foreign and want to be destroyed.[1] The formation of the blood–testis barrier occurs in the postpubertal period when germ cells differentiate. This barrier between Sertoli cells keeps spermatogenic cells separate from antigens in systemic circulations and prevents their destruction.[24-26]

Molele et al[27] (2021) reported that spermatogenic germ cells at various stages of spermatogenesis in seminiferous tubule epithelium were present. Correspondingly, in this study, it has been observed that the spermatogenic germ cells at various stages of spermatogenesis in seminiferous tubule epithelium were present.

In their study relating to the determination of the blood–testis barrier in cocks, Bergmann and Schindelmeiser[28](1987) observed that there is no functional barrier in the prepubertal period. Still, an active barrier is formed with the postpubertal period. Osman et al[29] (1980) suggested in their study on matured cocks that there are tight junction ties in the upper part of the area where spermatogonia are between Sertoli cells. They also indicated that

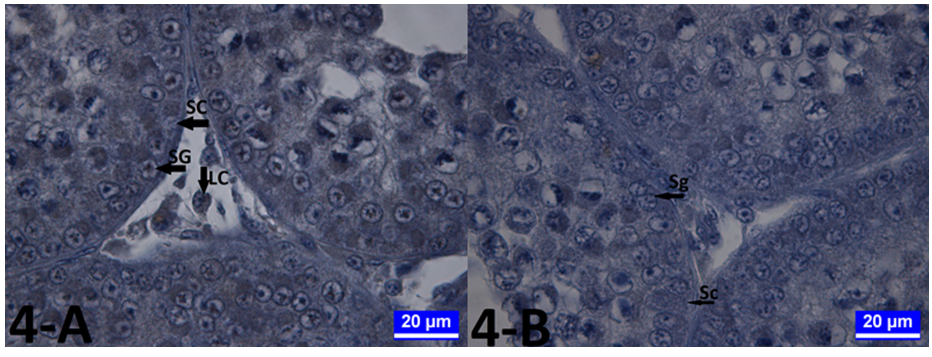

Figure 4. Immunohistochemical evaluation of claudin-1 antibody in prepubertal stage quail's testis. (A) Claudin-1 immunohistochemical staining. (B) Immunohistochemical negative control staining. LC, Leydig cell; SC, Sertoli cell; SG, spermatogonia.

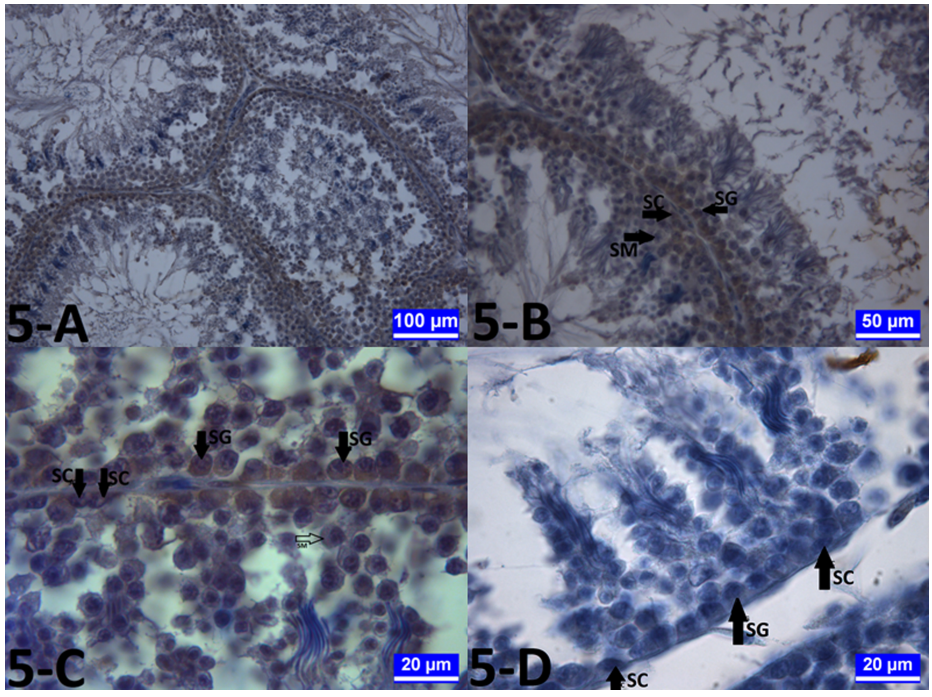

Figure 5. (A, B, C) Claudin-1 immunohistochemical staining of postpubertal stage quail's testis. (D) Negative control. SC, Sertoli cell; SG, spermatogonia; SM, Spermatocyte.

the agent does not go beyond the blood–testis barrier in the testis where coloring agent injection is performed.[14,15] In that study, it has been suggested that claudin-1 and zonula occludens-1 proteins, indicators of the presence of the blood–testis barrier, are present. Therefore, undeveloped blood–testis barrier in the prepubertal period develops in the postpubertal period.

In the study on the testis of a mouse, Gilio et al[30] (2013) reported that the immunohistochemical staining of the claudin-1 protein appears as dark brown in the basal compartment of seminiferous tubules in the testis of mature mice. In the study on the testis of immature and mature pheasants, Park et al[13] (2011) observed that there is weak immunoreactivity in immature (3 and 6 weeks) pheasants and strong immunoreactivity in adult (50 weeks) pheasants as a result of immunohistochemical evaluation. This study also observed that immunoreactivity is in the basal compartment between Sertoli cells in postpubertal stage quail testis by claudin-1's immunohistochemical staining. In Sertoli cells, the immunoreactivity of claudin-1 has been distinguished as cytoplasmic and membranous. It has also been observed that claudin-1 shows immunoreactivity near the basal area of the seminiferous tubule, where the blood–testis barrier is mainly formed. Claudin-1 is a vital protein taking part in the blood–testis barrier, and the presence of this protein couldn't be proven in the prepubertal period when the blood–testis barrier is not formed yet with many studies; it has been shown that the presence of this protein joining the formation of this barrier is seen in the postpubertal period.[282931] In this study, it has not been observed that the immunoreactivity of claudin-1 is in the testis in the postpubertal period. It has been thought to be originated from the fact that the blood–testis barrier is not formed yet in the prepubertal period. The fact that the immunoreactivity of claudin-1 is observed in the area where the blood–testis barrier is formed between Sertoli cells in postpubertal stage quail testis has made us think that the protein of claudin-1 takes part in this barrier. The barrier is formed in this period.

Molele et al[32] (2022) observed that immunoreactivity is in the basal area of seminiferous tubule epithelium. Correspondingly,

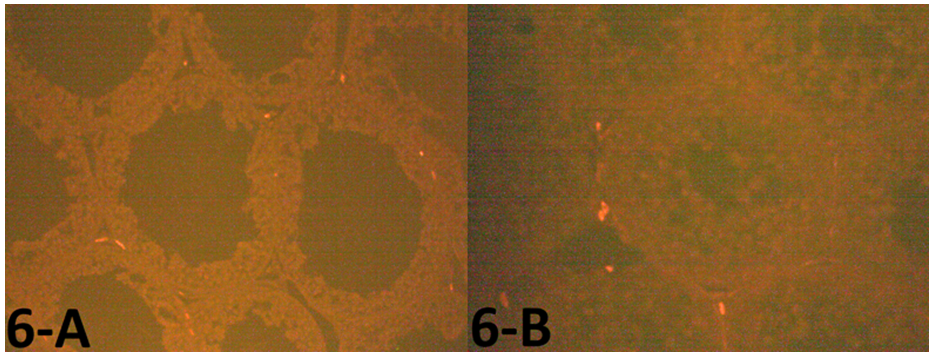

Figure 6. Immunofluorescence designation of prepubertal stage quail's testis. (A) Zonula occludens-1 immunofluorescence staining. (B) Zonula occludens-1 immunofluorescence negative control.

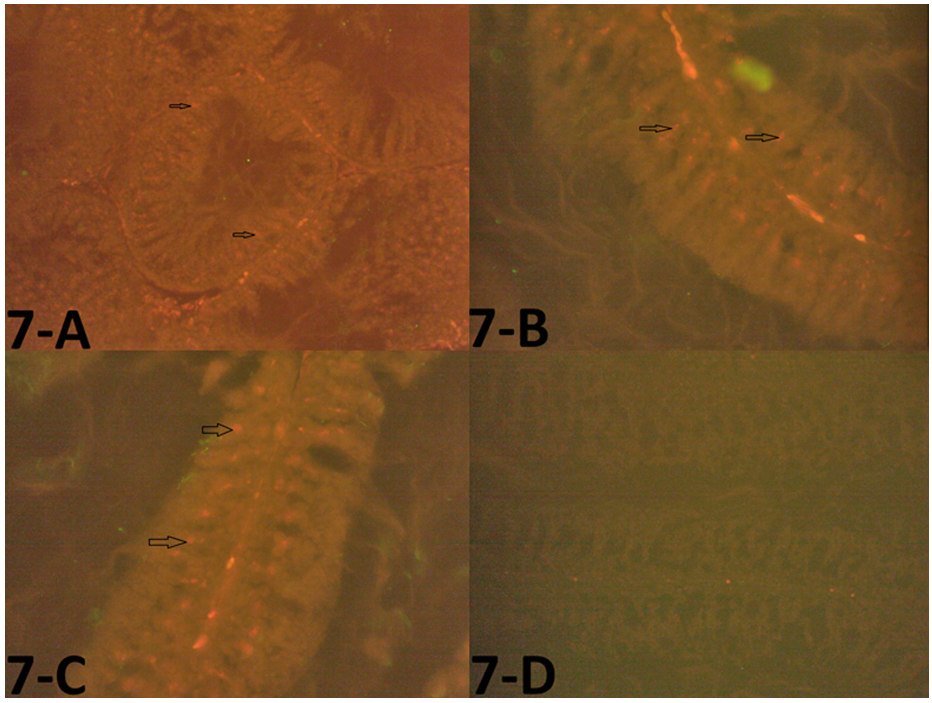

Figure 7. Immunofluorescence designation of postpubertal stage quail's testis. (A, B, C) Zonula occludens-1 immunofluorescence staining. (D) Zonula occludens-1 immunofluorescence negative control (arrow: the immunoreactivity of zonula occludens-1).

in this study, it has been observed that the immunoreactivity of zonula occludens-1 is in the basal area of the seminiferous tubule. Gilula et al[33] (1976) reported in the study on the testis of immature mammals that there is no tight junction in the testis of immature mammals. Stevenson et al[34] (1986) performed immunofluorescence staining of the protein of zonula occludens-1 in the testis of mice. They reported that zonula occludens-1 are immunostained where separated the basal and adlumenal testicular compartments.[34] Fink et al[35] (2006) performed immunohistochemical staining on testis tissues from patients with testicular cancer and healthy people. They suggested that the immunoreactivity of the protein of zonula occludens-1 is present in the blood-testis barrier between Sertoli cells in healthy people. However, they highlighted that the immunoreactivity of zonula occludens-1 decreases in those with testicular cancer and spreads to the cytoplasm. The western blot analyses they performed verified this result, and they suggested that the blood–testis barrier is degraded compared to healthy peoples by lanthanum dye.[35] Byers et al[36] (1991) reviewed the distribution of zonula occludens-1 in the testis of mice and reported that there is no tight junction complex in immature mice. However, they highlighted that zonula occludens-1 is generally distributed as apicolateral in the Sertoli cell membrane in the testis of mature mouse and not in the basal area. In this study, it has been seen that there is radiation in seminiferous tubules in prepubertal stage quail testis as a result of immunofluorescence staining of zonula occludens-1 and in seminiferous tubule epithelium between neighboring Sertoli cells in postpubertal stage quail testis. Based on this result, it has been thought that the blood–testis barrier is not formed or does not complete its formation in the prepubertal period and that the protein of zonula occludens-1 becomes visible as immunofluorescence with the formation of the blood–testis barrier in the postpubertal period.

As a result, diameter of the seminiferous tubule and thickness of the capsule increase in the postpubertal period compared to the prepubertal period. The proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity in the testis in the prepubertal period but show immunoreactivity in the seminiferous epithelium in the postpubertal period.

Prepubertal (30 days) and postpubertal (70 days) stage quails' testis have been evaluated with this study. It has been concluded that general histological views of the testis of quail are similar to those of the testis of poultry and that the diameter of the seminiferous tubule and thickness of the capsule increase in the postpubertal period compared to the prepubertal period.It has also been concluded that the proteins of claudin-1 and zonula occludens-1 do not show immunoreactivity because an active blood–testis barrier is not formed yet in seminiferous tubule epithelium in the testis in the prepubertal period as a result of immunohistochemical and immunofluorescence stainings but show immunoreactivity in the basal area in seminiferous epithelium with the blood–testis barrier formed in the postpubertal period.

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