Junctional complexes of the blood-testis barrier in the Japanese quail (*Coturnix Coturnix japonica*)

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Abstract

This study investigated the developmental changes in the adherens junctions, gap junctions, as well as tight junctions forming the blood-testis barrier (BTB) in Japanese quail (*Coturnix Coturnix japonica*) testis. Testicular tissue from pre-pubertal, pubertal, adult, and aged Japanese quail were examined by immunohistochemistry and transmission electron microscopy (TEM). The tight junction proteins claudin-3, claudin-11, occludin and zonula occludens-1 (ZO-1), were generally localised in the cytoplasm of Sertoli cells, spermatogonia, and spermatocytes of pre-pubertal, pubertal, some adult birds. The adherens junction protein E-cadherin had a similar distribution pattern. During pre-pubertal development, the gap junction protein connexin-43 (Cx43) was only localised between Leydig cells in the testicular interstitium. However, TEM revealed the presence of gap junctions between cells of the seminiferous epithelium as early as the pre-pubertal stage. Furthermore, TEM confirmed the presence of tight and adherens junctions in the seminiferous epithelia of all age groups. The findings of this study document age-related differences in the immunolocalisation and intensity of the junctional proteins and the ultrastructure of the junctional complexes forming the BTB in quail testes. Additionally, the junctional complexes forming the BTB in the Japanese quail are well established prior to puberty. This study provides baseline information for the future evaluation of pathological changes in the BTB of avian species at different developmental stages.

Keywords: Adherens junction; Gap junction; Immunohistochemistry; TEM; Testis; Tight junction

1. Introduction

The seminiferous tubules of vertebrate testes consist of two cell types: germinal and Sertoli cells (Cooksey and Rothwell, 1973). Sertoli cells provide nourishment for developing sperm cells and form the blood-testis barrier (BTB) by means of junctional complexes of varying type and function (Sharma et al., 2018, Sikka and Wang, 2008).

The BTB isolates the antigenically foreign spermatogenic cells from the immune system and is crucial for the process of spermatogenesis and therefore male fertility (Cheng and Mruk, 2012, de Freitas et al., 2016, Lui and Lee, 2009). The junctional complexes forming the BTB are comprised of adherens junctions, gap junctions, as well as tight junctions (Rode et al., 2015), composed of specialised proteins involved in cell attachment, adhesion and communication (Ahmed et al., 2018, Hejmej et al., 2012).

Adherens junctions are responsible for the lateral adhesions between Sertoli and germ cells (Yan et al., 2009) and are formed by three integral protein complexes: cadherin and catenin, integrin and laminin, and nectin and afadin (Yan et al., 2009). In epithelia, the core of the adherens junction includes interactions among transmembrane glycoproteins of the classical cadherin superfamily, such as epithelial cadherin (E-cadherin) and neural cadherin (N-cadherin), and the catenin family members including β-catenin. Together, these proteins control the formation, maintenance and function of adherens junctions (Hartsock and Nelson, 2008, Takeichi, 2014).

Gap junctional communication has an important function in testis development and sperm maturation (Kidder and Cyr, 2016). Testicular gap junctions are composed of several proteins, the most abundant of which is connexin 43 (Cx43) (Batias et al., 1999, Juneja et al., 1999). Cx43 is responsible for initiating and maintaining spermatogenesis. Additionally, this protein is involved in regulation of the proliferation and maturation of Sertoli cells, as well as the coordination of germ cell-Sertoli cell junction communication (Batias et al., 1999, Brehm et al., 2007, Giese et al., 2012, Juneja et al., 1999, Sridharan et al., 2007).

Tight junctions play a crucial role in barrier formation and constitute a major part of the BTB (Bergmann et al., 1984). The tight junctions between adjacent Sertoli cells form intercellular adhesions close to the basal lamina that prevent the diffusion of fluid and molecules through the BTB (Russell et al., 1989). Membrane proteins including claudins, occludin, and zonula occludens (ZO) contribute to the formation of tight junctions in the mammalian testis (Park et al., 2011, Yan and Cheng, 2005, Yan et al., 2008). Claudins and occludin have been identified as the main components of the BTB in the rat (McCabe et al., 2016), and mouse (Morrow et al., 2010). Moreover, claudin-3 and claudin-11 play a crucial role in BTB development (Park et al., 2011). ZO-1 is localised mainly in Sertoli cell tight junctions and at the sites of Sertoli cell ectoplasmic specialisation, which are found near early spermatids, suggesting its involvement in germ cell migration (Byers et al., 1991).

The immunolocalisation of junctional proteins have been described in mammalian as well as turtle testes (Ahmed et al., 2018, Brehm et al., 2007, Byers et al., 1991, Rode et al., 2015, Roscoe et al., 2001), while the ultrastructure of junctional complexes has been investigated in the rat (Dym and Fawcett, 1970), turtle (Ahmed et al., 2018), and the domestic fowl

(Cooksey and Rothwell, 1973, Pelletier, 1990). However, there is limited information on age-related changes in the immunolocalisation of junctional proteins as well as an ultrastructural description of the junctional complexes in birds.

Japanese quail have a small size, which reduces feeding costs and facilitates handling. They have a short life cycle and are less susceptible to disease compared to other avian species (Egbeyale et al., 2013, Quinn, 2012). Furthermore, the Japanese quail is a common laboratory species and a good model for environmental, behavioural, developmental, as well as comparative studies (Ainsworth et al., 2010, Huss et al., 2008, Zakariah et al., 2020).

The objective of the present study was to investigate temporal changes in the immunoexpression of proteins associated with junctional complexes in the Japanese quail testes, as well as the ultrastructural differences in appearance of the junctional complexes at different developmental stages (pre-pubertal, pubertal, adult, and aged) using immunohistochemistry and transmission electron microscopy (TEM), respectively.

2. Materials and methods

2.1. Experimental animals and tissue preparation

A total of 28 male Japanese quail, comprising four age groups, pre-pubertal (4 weeks of age, n = 7), pubertal (6–7 weeks of age, n = 7) adults (10 weeks of age, n = 7) and aged (52 weeks of age, n = 7), were obtained from the Agricultural Research Council of South Africa (Pretoria, South Africa). The birds were sacrificed by CO_2 inhalation euthanasia upon arrival at the University of Pretoria. Testicular tissue was collected from each bird and immediately fixed in 10 % buffered formalin and processed routinely for light microscopy. All experimental procedures were conducted with the approval of the institutional Animal Ethics Committee of the University of Pretoria (certificate number V034–18).

2.2. Immunohistochemical procedures

The immunostaining technique was performed on 3-µm-thick sections using a Biogenex super sensitive one-step polymer-horseradish peroxidase (HRP) detection system kit (Emergo Europe, Hague, Netherlands). The sections were first deparaffinized and rehydrated. For antigen retrieval, slides were microwaved in citrate buffer (pH 6.0) or Tris-EDTA (pH 9.0) for three cycles of 7 min each and then allowed to cool for 20 min. Endogenous peroxidase activity was blocked using hydrogen peroxide (3 %) in phosphate buffer saline (PBS) solution containing bovine serum albumen (pH 7.6) for 5 min. The sections were then incubated with the primary antibodies at 37 °C for 1 h (Table 1). Following incubation with primary antibodies, the slides were rinsed in two changes of PBS for 5 min each and then incubated for 15 min with the one-step polymer-HRP reagent. Slides were then rinsed in three changes of PBS and bound antibody was visualised by the addition of a 3,3’-diaminobenzidine substrate solution. The sections were counter-stained with Mayer’s haematoxylin for 1 min and then dehydrated, cleared, and mounted with a mounting medium.

Table 1. Primary antibodies used in the study.

Antibodies	Product code	Host species	Clonality	Antigen retrieval	Dilution	Source
Adherens junctions						
Anti-β catenin	ab16051	Rabbit	Polyclonal	Citrate buffer (pH 6.0)	1:400	Abcam
	ab32572	Rabbit	Monoclonal	Pronase (pH 9.0)	1:100	Abcam
Anti-E cadherin	ab15148	Rabbit	Polyclonal	Tris-EDTA (pH 9.0)	1:30	Abcam
	SAB5600058	Rabbit	Monoclonal	Pronase (pH 9.0)	1:300	Sigma-Aldrich
Anti-N cadherin	ab18203	Rabbit	Polyclonal	Citrate buffer (pH 6.0)	1:500	Abcam
	ab76011	Rabbit	Monoclonal	Pronase (pH 9.0)	1:100	Abcam
Gap junctions						
Anti-Cx43	ab11370	Rabbit	Polyclonal	Proteinase K	1:1000	Abcam
	ab235585	Rabbit	Monoclonal	Pronase (pH 9.0)	1:100	Abcam
Tight junctions						
Anti-claudin 3	ab15102	Rabbit	Polyclonal	Citrate buffer (pH 6.0)	1:10	Abcam
	ab52231	Rabbit	Polyclonal	Pronase (pH 9.0)	1:100	Abcam
Anti-claudin 11	ab53041	Rabbit	Polyclonal	Citrate buffer (pH 6.0)	1:50	Abcam
	Ab175236	Rabbit	Monoclonal	Pronase (pH 9.0)	1:100	Abcam
Anti-occludin	ab31721	Rabbit	Polyclonal	Citrate buffer (pH 6.0)	1:200	Abcam
	Ab235986	Rabbit	Polyclonal	Pronase (pH 6.0)	1:200	Abcam
Anti-ZO 1	ab216880	Rabbit	Polyclonal	Citrate buffer (pH 6.0)	1:30	Abcam
	HPA001636	Rabbit	Polyclonal	Pronase (pH 9.0)	1:100	Sigma-Aldrich

Details of the dilutions, antigen retrieval and sources of the antibodies used for validation are provided in Table 1. Japanese quail myocardial, brain, as well as dog testicular tissue were used as positive controls, while Japanese quail skeletal muscle was used as negative control. On the basis of visual examination, the relative immunointensities of adherens junction proteins (β-catenin, E-cadherin, N-cadherin), gap junction protein (Cx43) and tight junction proteins (claudin-3, claudin-11, occludin and ZO-1) were classified as absent (—), weak (+), moderate (+ +) and strong (+ + +) as described previously (Madekurozwa and Kimaro, 2006).

2.3. Transmission electron microscopy (TEM)

Tissue specimens (n = 3 per bird) were fixed in 2.5 % glutaraldehyde in 0.075 M phosphate buffer (pH 7.4) for 24 h. Thereafter, specimens were post-fixed in 0.5 % osmium tetroxide for 2 h. Specimens were then rinsed in phosphate buffer (pH 7.4), dehydrated in a series of ethanol concentrations, and embedded in epoxy: resin at a ratio of 1:2 for 1 h, 1:1 for 2 h and pure resin overnight. Semi-thin Section (1 µm thick) were cut using a glass knife and stained with toluidine blue. Ultra-thin (50–90 nm thick) sections of selected areas were cut on a Reichert-Jung Ultracut (C. Reichart AG., Vienna, Austria) using a diamond knife, collected onto copper grids, and stained with lead acetate. The sections were counterstained with uranyl citrate and examined with a Phillips CM10 transmission electron microscope (FEI, The Netherlands), fitted with an Olympus Mega View III imaging system.

3. Results

3.1. Immunohistochemistry

3.1.1. Adherens junction proteins

The adherens junction proteins β-catenin (Fig. 1a–d) and N-cadherin (Fig. 2a–d) were localised between Sertoli cells and spermatogenic cells, while E-cadherin immunostaining was observed in the cytoplasm of Sertoli and spermatogenic cells (Fig. 3a–d) of pre-pubertal, pubertal, adult, and aged birds (Table 2).

Table 2. Scoring of intensity of the adherens junction proteins' immunoreactivity (absent "—", weak "+", moderate "+ +" and strong "+ + +").

Junctional complex proteins	Immunolocalisation	Pre-pubertal	Pubertal	Adult	Aged
β-catenin	Sertoli cell-spermatocyte	+ +	+	+	—
	Sertoli cell-spermatogonia	+ + +	+ +	+	+ +
N-cadherin	Sertoli cell-spermatocyte	+ +	+	—	—
	Sertoli cell-spermatogonia	+ +	+ + +	+ +	+ + +
E-cadherin	Sertoli cells	—	+	+	+
	Spermatogonia	+	+	+	+
	Spermatocyte	+	+	+	+
	Peritubular myoid cells	+	+	+	+

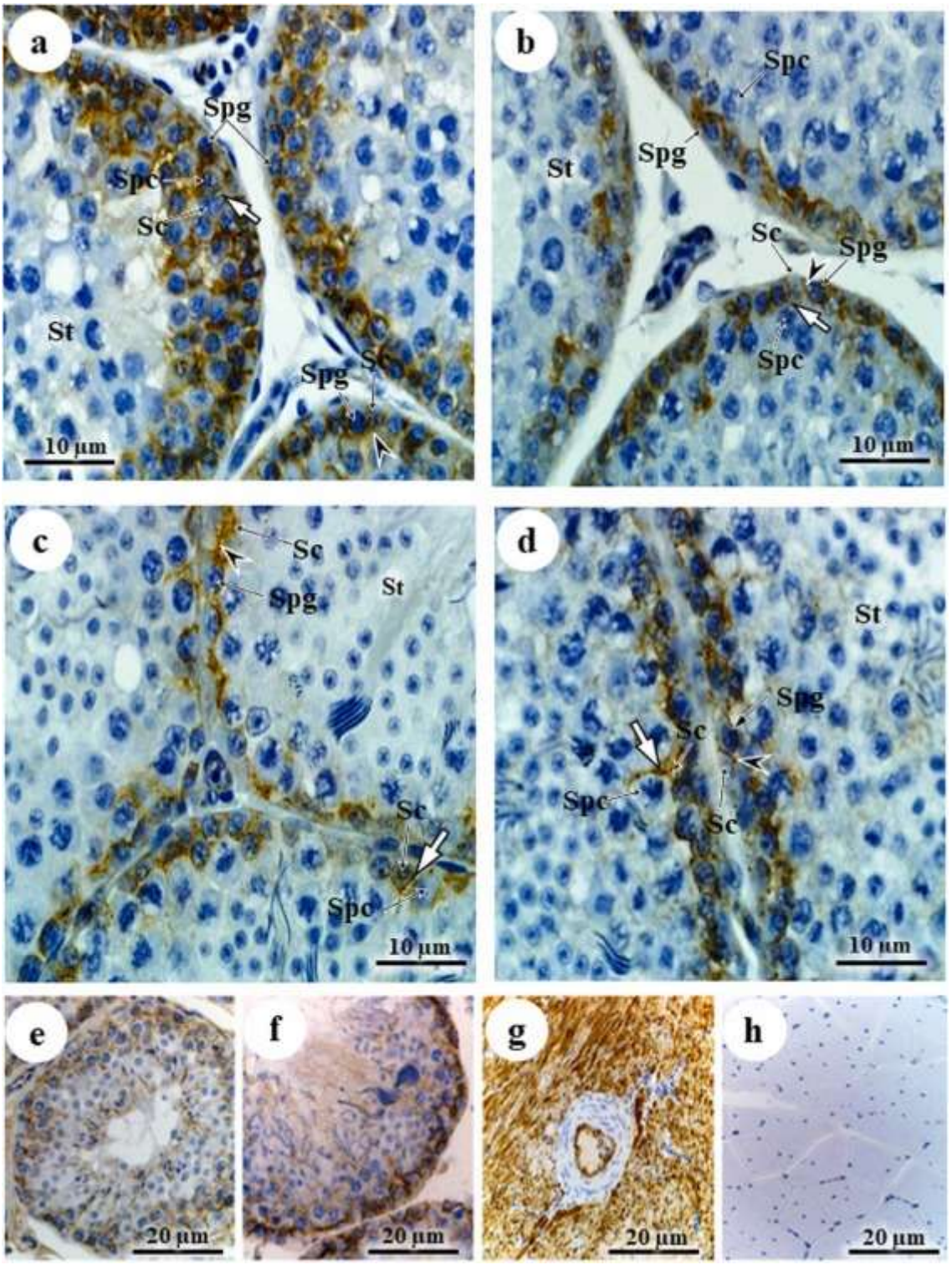

Fig. 1. Immunolocalisation of β-catenin in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. **Thick white arrow**: moderate immunostaining was present between Sertoli cells (Sc) and Spc of pre-pubertal birds **(a)**, weak immunostaining in pubertal and adult birds **(b, c),** but no immunostaining in aged birds **(d)**. **Thin arrowhead**: strong immunostaining was observed between Sc and Spg of pre-pubertal birds **(a)**, moderate immunoreactivity in pubertal and aged birds **(b, d)**, and weak immunostaining in adult birds **(c). (a–d)** Seminiferous tubule epithelium (St). **(e–h)** Validation: dog testis **(e),** Japanese quail testis **(f),** and myocardium **(g).** Dog testis and quail myocardium were used as positive controls for β-catenin. **(h)** Negative control (Japanese quail skeletal muscle).

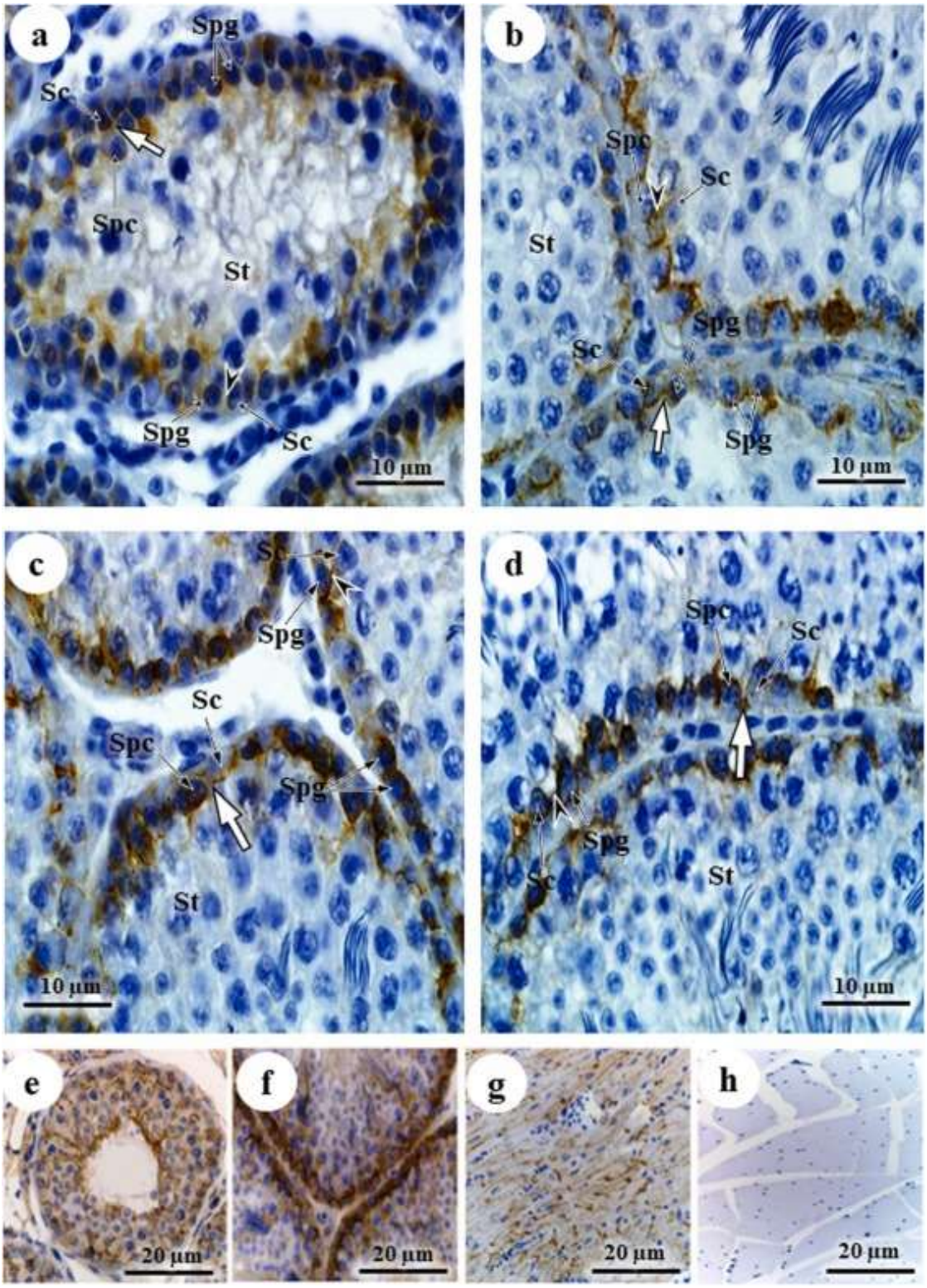

Fig. 2. Immunolocalisation of N-cadherin in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. **(a) Thick white arrow**: moderate immunostaining was present between Sertoli cell (Sc) and spermatocyte (Spc) in pre-pubertal birds **(a)**, weak immunostaining in pubertal birds **(b)**, but no immunostaining in adult and aged birds **(c, d)**. **Thin arrowhead**: moderate immunostaining was localised between Sc and Spg in pre-pubertal and adult birds **(a, c)**, while a strong immunostaining was observed in pubertal and aged birds **(b, d)**. **(a–d)** Seminiferous tubule epithelium (St). **(e–h)** Validation: dog testis **(e),** Japanese quail testis **(f),** and myocardium **(g)**. Dog testis and quail myocardium were used as positive controls for N-cadherin. **(h)** Negative control (Japanese quail skeletal muscle).

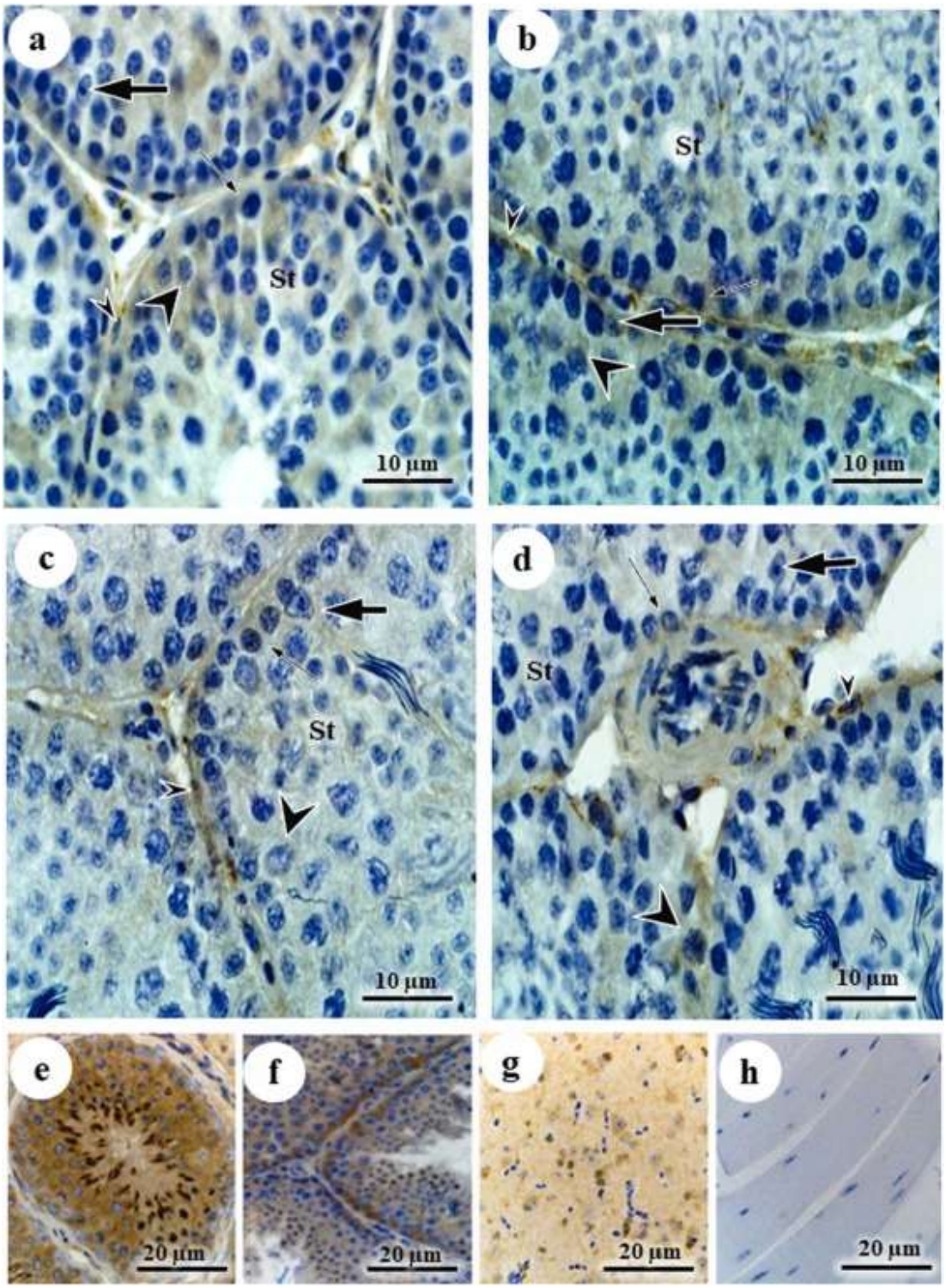

Fig. 3. Immunolocalisation of E-cadherin in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. **Thick arrow**: immunonegative Sertoli cells (Sc) were observed in pre-pubertal birds **(a)**, while weak immunostaining was present in the cytoplasm of the Sc in pubertal, adult, and aged birds **(b–d). Thin arrow**: weak immunostaining was localised in the cytoplasm of the spermatogonia (Spg) of pre-pubertal, pubertal, adult, and aged birds **(a–d). Thick arrowhead**: weak immunostaining was observed in the cytoplasm of the spermatocyte (Spc) of pre-pubertal, pubertal, adult, and aged birds **(a–d). Thin arrowhead**: weak immunostaining was localised in peritubular myoid cells of pre-pubertal, pubertal, adult, and aged birds **(a–d). (a–d)** Seminiferous tubule epithelium (St). **(e–h)** Validation: dog testis **(e),** Japanese quail testis **(f),** and brain tissue **(g).** Dog testis and quail brain were used as positive controls for E-cadherin. **(h)** Negative control (Japanese quail skeletal muscle).

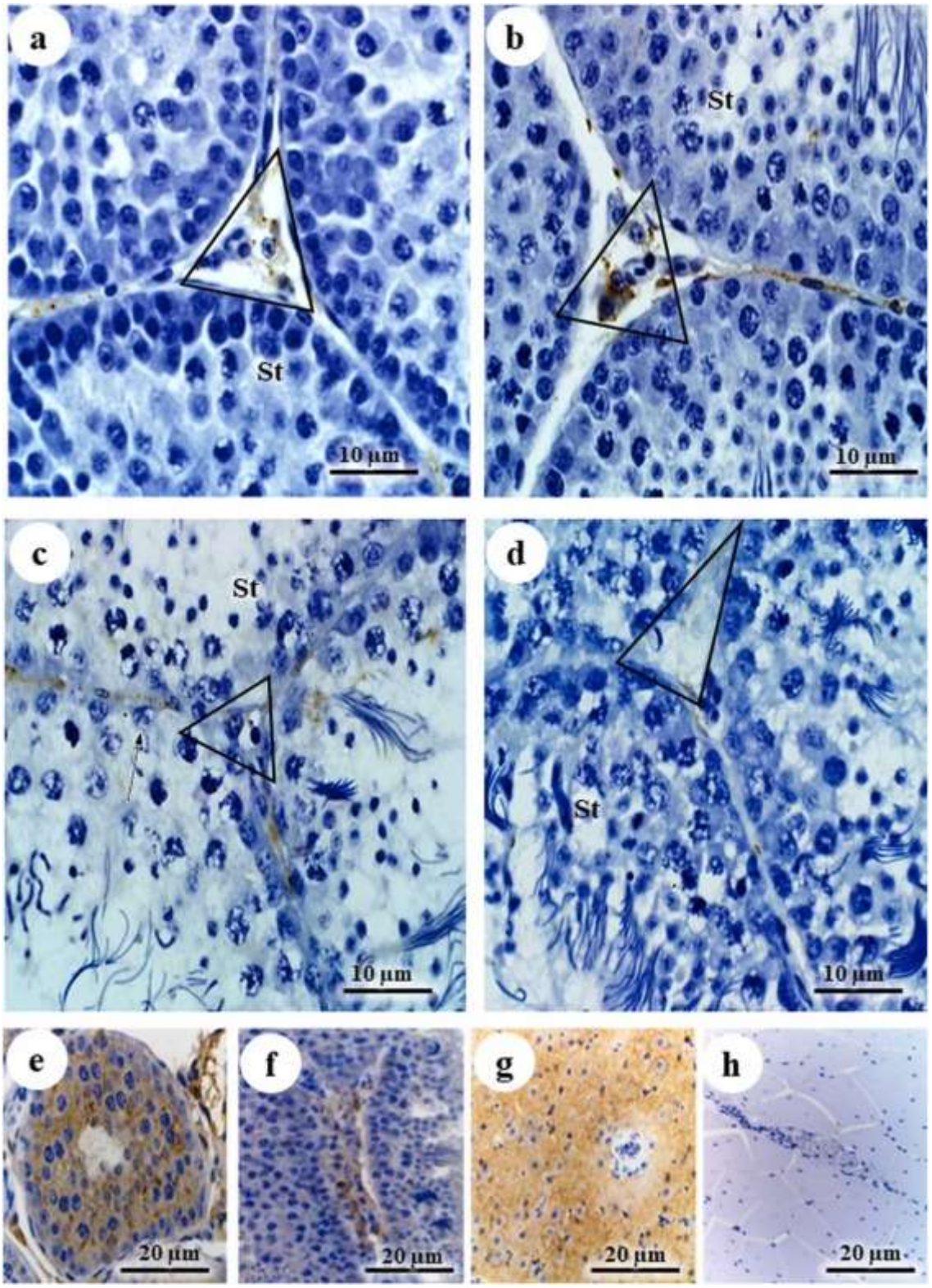

Fig. 4. Immunolocalisation of Cx43 in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. **Triangle**: weak immunostaining was localised in testicular interstitium of pre-pubertal **(a)** and adult birds **(c)**, moderate immunostaining in pubertal birds **(b),** and no immunostaining in aged birds **(d)**. **(a–d)** Seminiferous tubule epithelium (St). **(e–h)** Validation: dog testis **(e),** Japanese quail testis **(f),** and brain tissue **(g).** Dog testis and quail brain were used as positive controls for Cx43. (h) Negative control (Japanese quail skeletal muscle).

3.1.2. Gap junction protein (Cx43)

Cx43 immunostaining was localised in the testicular interstitium of pre-pubertal, pubertal, and adult birds while no immunostaining was detected in the seminiferous epithelium of aged birds (Fig. 4a–d; Table 3).

Table 3. Scoring of intensity of Cx43 immunoreactivity (absent "—", weak "+", moderate "+ +" and strong "+ + +").

Junctional complex proteins	Immunolocalisation	Pre-pubertal	Pubertal	Adult	Aged
Cx-43	Sertoli cells	—	—	—	—
	Spermatocytes	—	—	—	—
	Spermatogonia	—	—	—	—
	Interstitial tissue	+	++	+	—

3.1.3. Tight junctional proteins

The tight junction proteins claudin-3 (Fig. 5a–d), claudin-11 (Fig. 6a–d), occludin (Fig. 7a–d) and ZO-1 (Fig. 8a–d), were generally localised in the cytoplasm of Sertoli cells, spermatogonia, and spermatocyte of pre-pubertal, pubertal, some adult birds (Table 4).

Table 4. Scoring of intensity of the tight junction proteins' immunoreactivity (absent "—", weak "+", moderate "+ +" and strong "+ + +").

Junctional complex proteins	Immunolocalisation	Pre-pubertal	Pubertal	Adult	Aged
Claudin-3	Sertoli cell	++	—	—	—
	Intertubular blood vessel	++	++	—	—
	Spermatocytes	++	—	—	—
	Spermatogonia	++	+	—	—
Claudin-11	Sertoli cell	+	++	++	—
	Spermatogonia	+	++	++	—
	Spermatocyte	+	++	++	—
Occludin	Spermatogonia	++	+	—	—
	Spermatocytes	++	+	—	—
	Sertoli cells	++	—	—	—
ZO-1	Sertoli cells	+	+	+	—
	Spermatogonia	+	+	+	—
	Spermatocytes	+	+	+	—

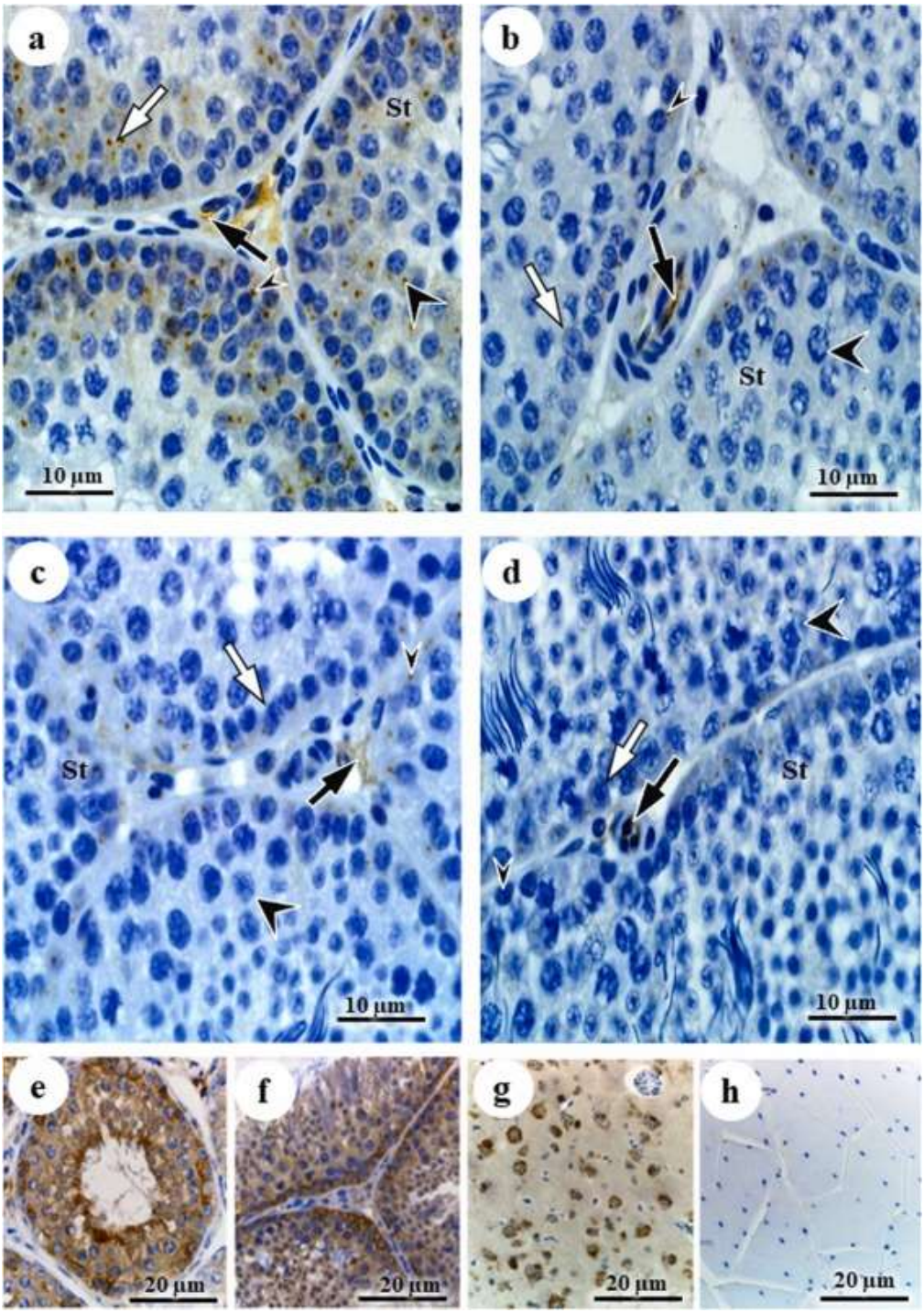

Fig. 5. Immunolocalisation of claudin-3 in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. **White arrow**: moderate focal immunostaining was present in the cytoplasm of Sertoli cells (Sc) in pre-pubertal birds **(a)**, while no immunostaining was observed in pubertal, adult, and aged birds **(b–d). Black arrow**: moderate immunostaining was observed in the endothelial cell of intertubular blood vessel in pre-pubertal and pubertal birds **(a, b)**, while no immunostaining was observed in adult and aged birds **(c, d)**. **Thick arrowhead**: moderate focal immunostaining was localised in the cytoplasm of spermatocyte (Spc) in pre-pubertal birds **(a)**, while Spc in pubertal, adult, and aged birds were immunonegative **(b–d). Thin arrowhead**: moderate focal immunostaining was localised in the cytoplasm of spermatogonia (Spg) in pre-pubertal birds **(a)**, while a weak immunostaining was observed in pubertal **(b),** and no immunostaining was observed in adult and aged birds **(c–d). (a–d)** Seminiferous tubule epithelium (St). **(e–h)** Validation: dog testis **(e),** Japanese quail testis **(f),** and brain tissue **(g)**. Dog testis and quail brain were used as positive controls for claudin-3. **(h)** Negative control (Japanese quail skeletal muscle).

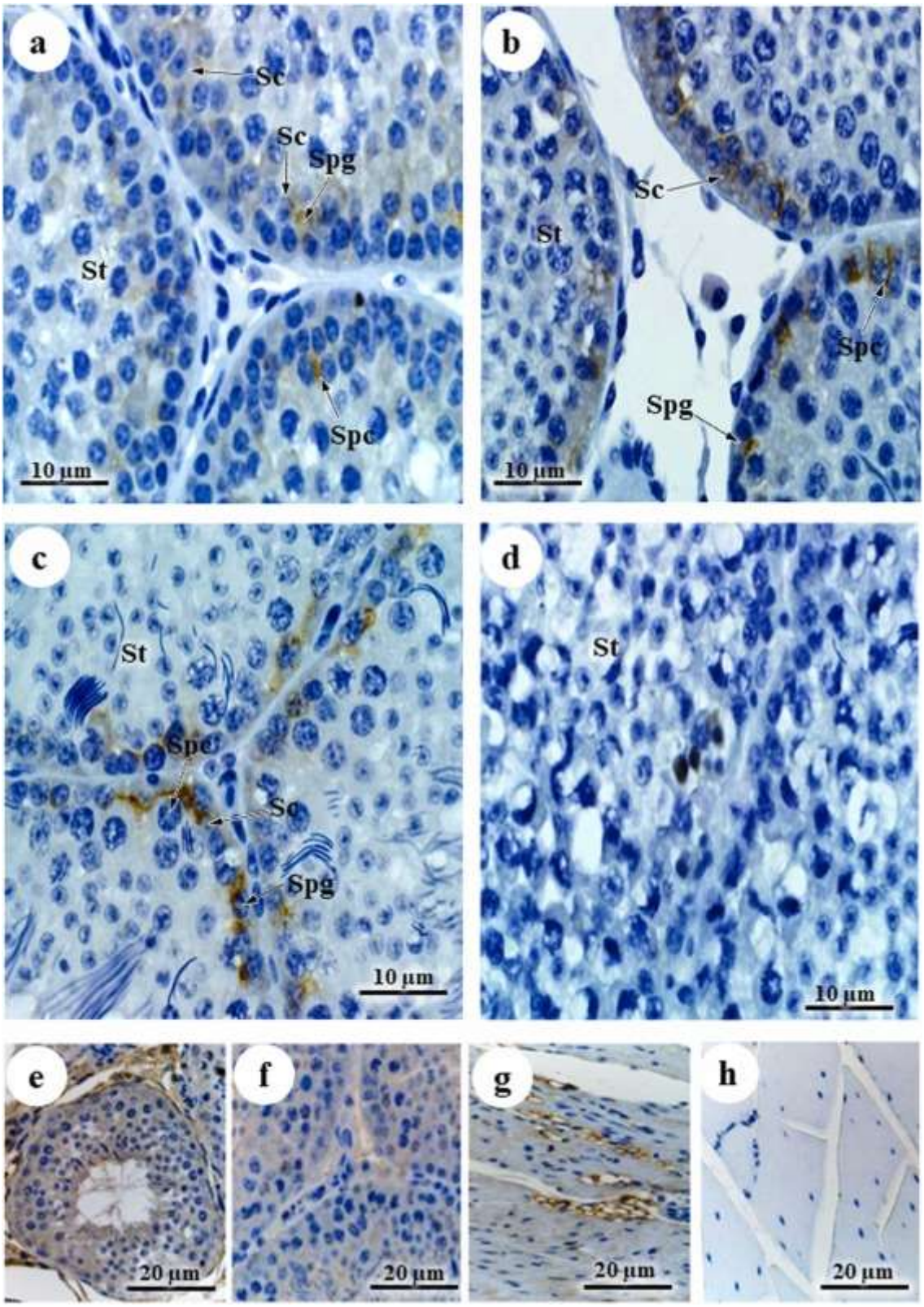

Fig. 6. Immunolocalisation of claudin-11 in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. Weak immunostaining was localised in the cytoplasm of Sertoli cells (Sc), spermatogonia (Spg), and spermatocyte in pre-pubertal birds **(a)**, moderate in the in pubertal and adult birds **(b–c),** but no immunostaining in aged birds **(d). (a–d)** Seminiferous tubule epithelium (St). **(e–h)** Validation: dog testis **(e),** Japanese quail testis **(f),** and myocardium **(g).** Dog testis and quail myocardium were used as positive controls for claudin-11. **(h)** Negative control (Japanese quail skeletal muscle).

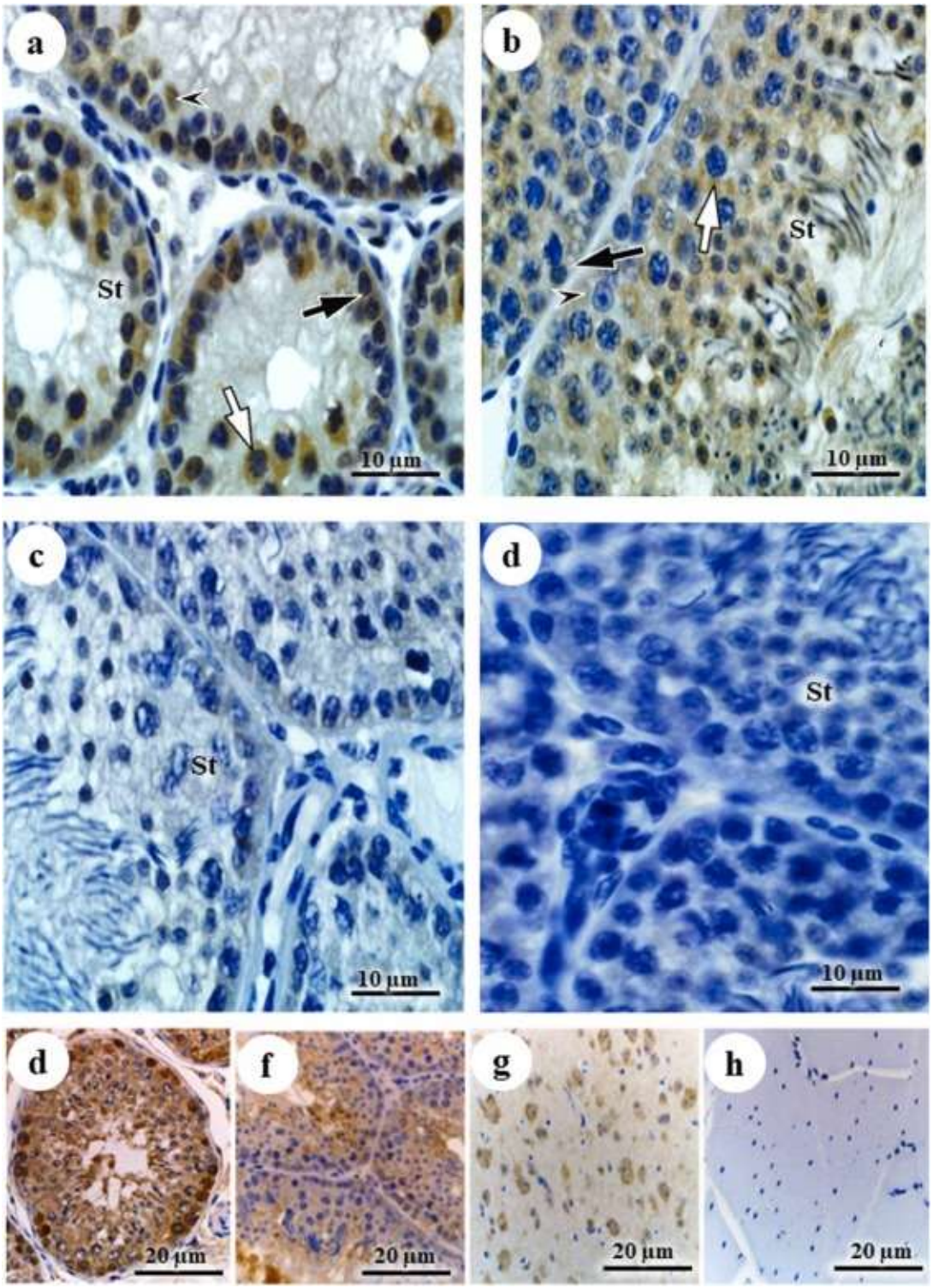

Fig. 7. Immunolocalisation of occludin in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. **Black arrow**: moderate immunostaining was observed in the cytoplasm of the spermatogonia (Spg) in pre-pubertal birds **(a)**, weak immunostaining in pubertal birds **(b)**, but no immunostaining in adult and aged birds **(c–d)**. **White arrow**: moderate immunostaining was detected in the cytoplasm of the spermatocyte (Spc) in pre-pubertal birds **(a)**, weak immunostaining in pubertal birds **(b)**, but no immunostaining in adult and aged birds **(c–d)**. **Thin arrowhead**: moderate immunostaining was localised in the cytoplasm of the Sertoli cell (Sc) in pre-pubertal birds **(a),** while no immunostaining was detected in pubertal, adult, and aged birds **(b–d)**. **(a–d)** Seminiferous tubule epithelium (St). **(e–h)** Validation: dog testis **(e),** Japanese quail testis **(f),** and brain tissue **(g).** Dog testis and quail brain were used as positive controls for occludin. **(h)** Negative control (Japanese quail skeletal muscle).

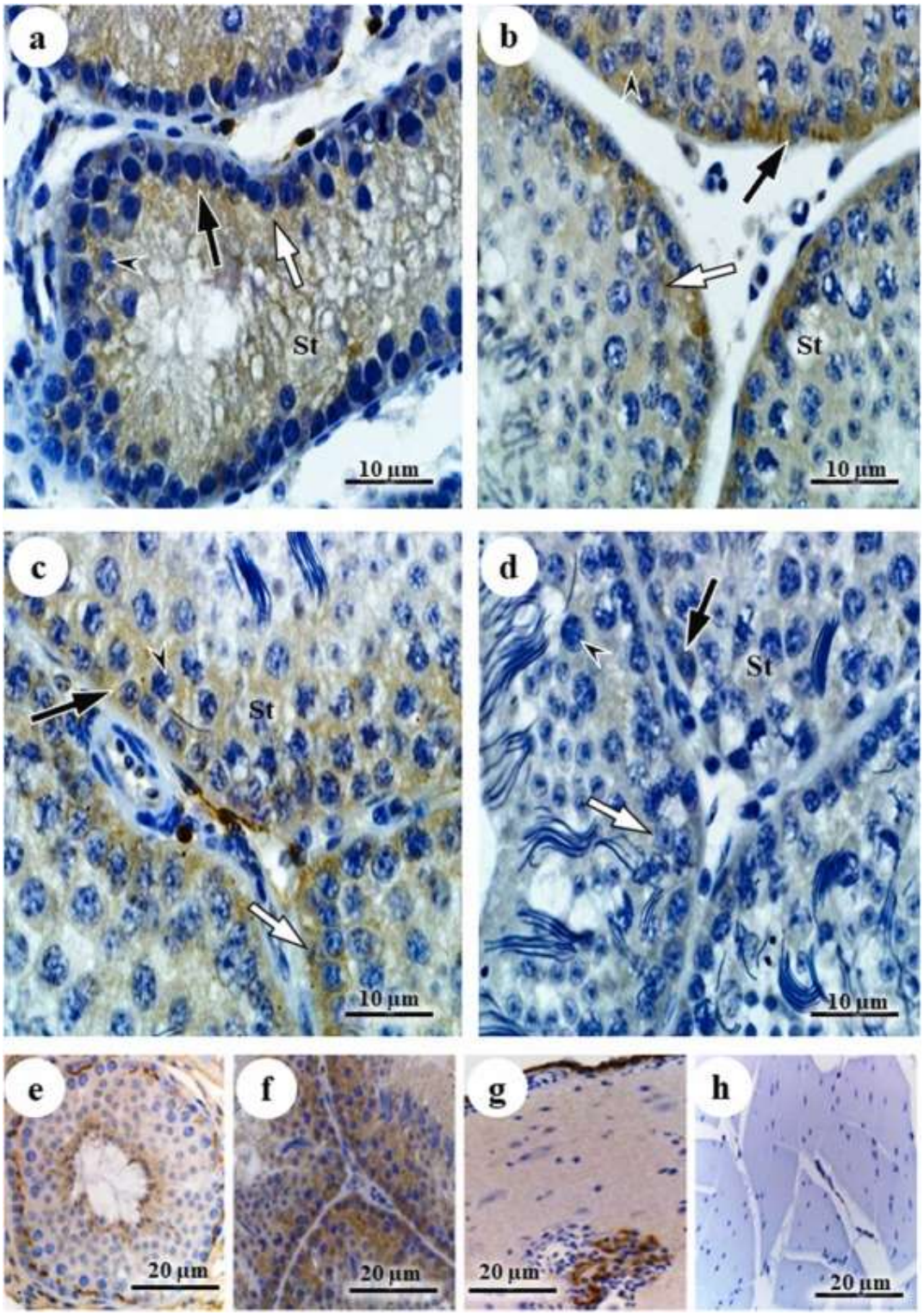

Fig. 8. Immunolocalisation of ZO-1 in the testes of pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails. **White arrow**: weak immunostaining was localised in the cytoplasm of Sertoli cells (Sc) in pre-pubertal, pubertal, and adult birds **(a–c)**, while no immunostaining was observed in aged birds **(d)**. **Black arrow**: weak immunostaining was detected in the cytoplasm of the spermatogonia (Spg) in pre-pubertal, pubertal, and adult birds **(a–c),** while no immunostaining was observed in aged birds **(d)**. **Arrowhead**: weak immunostaining was observed in the cytoplasm of the spermatocyte (Spc) in pre-pubertal, pubertal, and adult birds **(a–c),** but no immunostaining was detected in aged birds **(d)**. **(a–d)** Seminiferous tubule epithelium (St). **(e–h)** Dog testis **(e),** Japanese quail testis **(f),** and brain tissue **(g).** Dog testis and quail brain were used as positive controls for ZO-1. **(h)** Negative control (Japanese quail skeletal muscle).

3.2. Transmission electron microscopy (TEM)

3.2.1. Adherens junctions

In all age groups, adherens junctions were present between adjacent Sertoli cells (Fig. 9a–e). During the pre-pubertal stage, adherens junctions were already observable despite the close apposition of adjacent Sertoli cell membranes (Fig. 9a). Adherens junctions between adjacent Sertoli cells in pubertal, adult, and aged birds were well-developed and sites of membrane apposition were clearly defined (Fig. 9b–e).

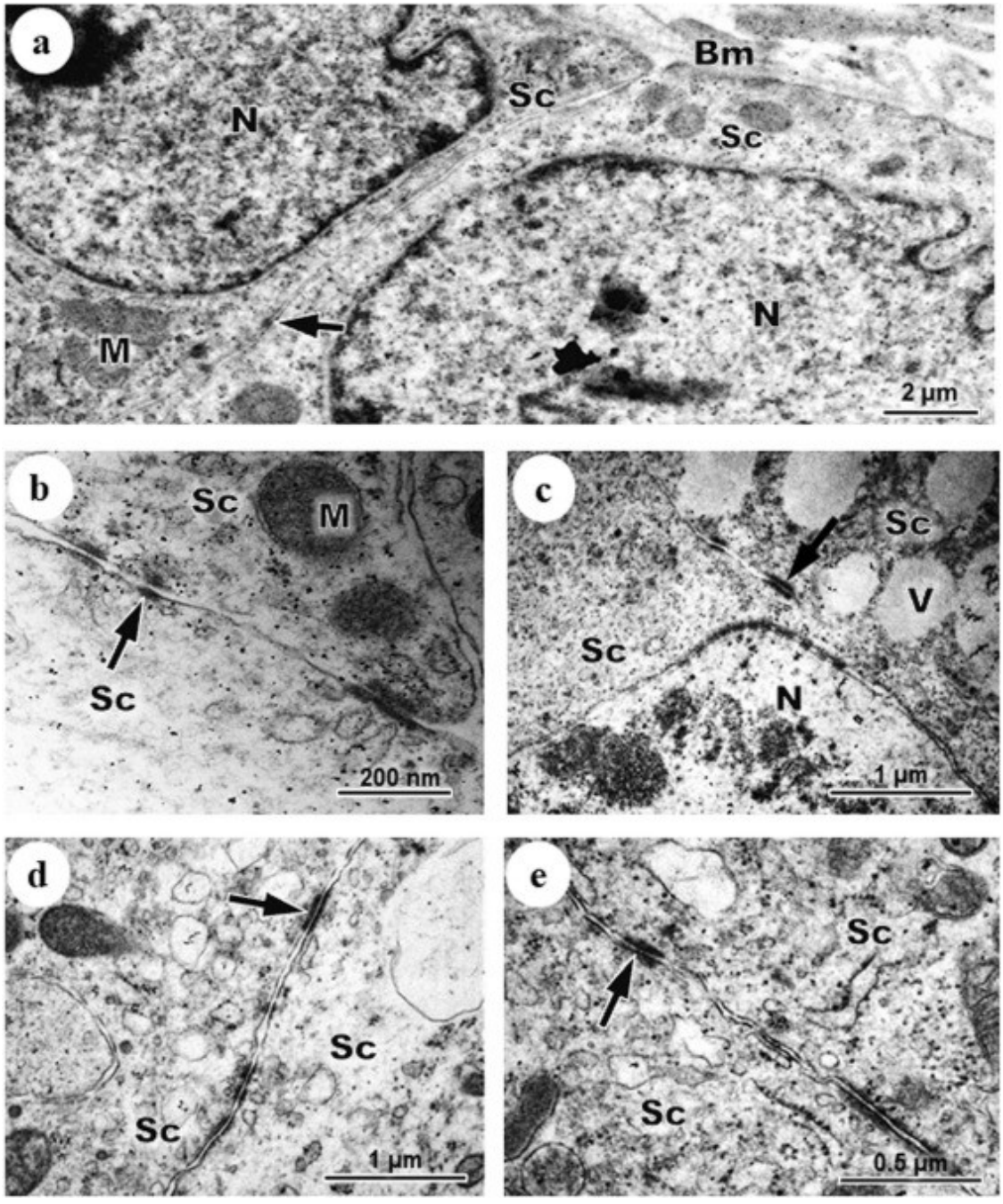

Fig. 9. Electron photomicrographs of adherens junctions between adjacent Sertoli cells in pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d, e)** Japanese quails showing: adherens junction (arrow), Sertoli cell (Sc), Sertoli cell nucleus (N), basement membrane (Bm), mitochondria (M), and Vacuole (V).

Adherens junctions between Sertoli cells and spermatogonia were detected in all studied age groups (Fig. 10a–d). There was a close apposition of membranes of Sertoli cells and spermatogonia in pre-pubertal and aged birds (Fig. 10a, d), while adherens junctions in pubertal and adult birds were well-developed (Fig. 10b, c). Well-developed adherens junctions were also present between Sertoli cells and spermatocytes in all age groups (Fig. 11a–d).

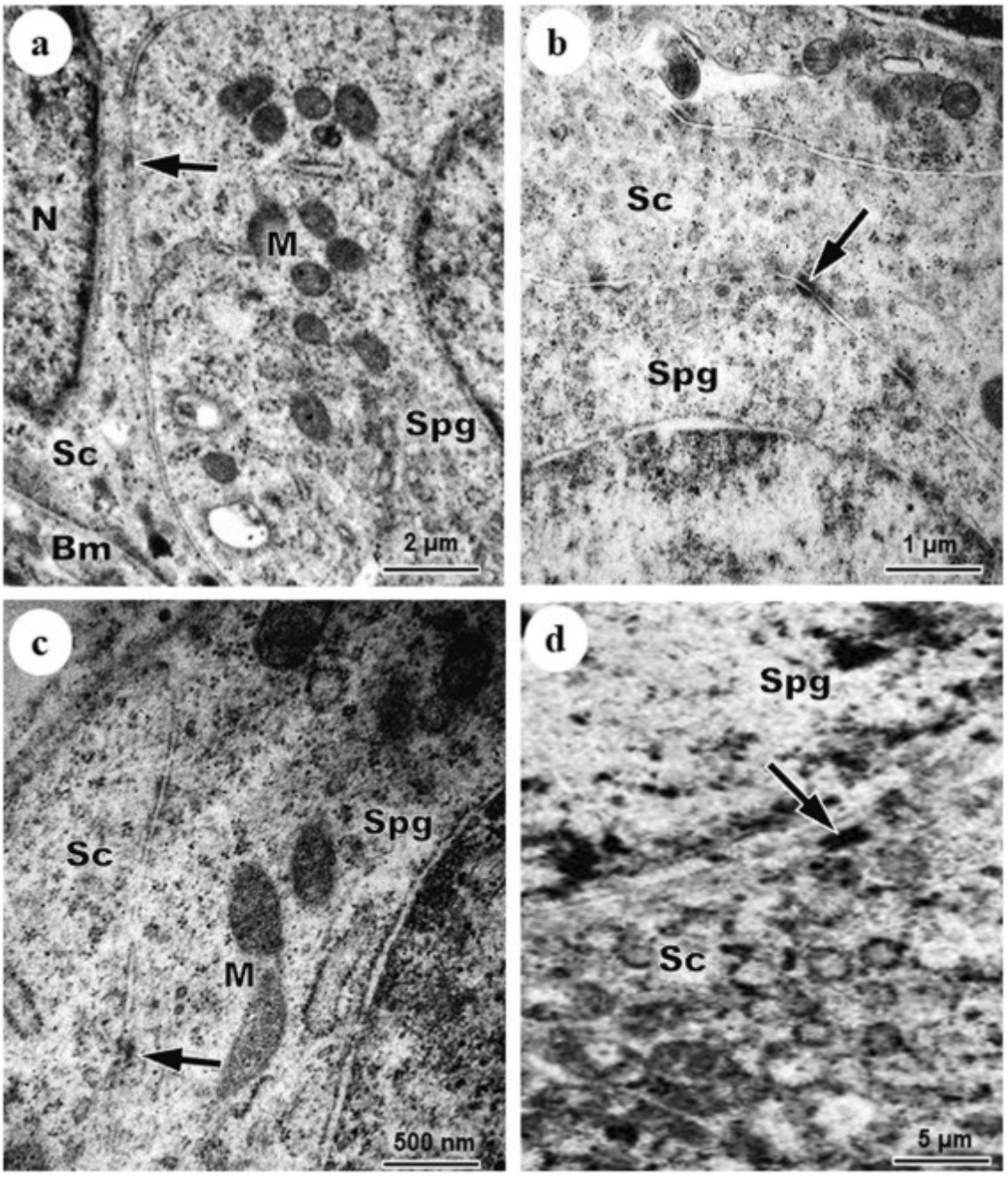

Fig. 10. Electron photomicrographs of adherens junctions between a Sertoli cell and spermatogonia in pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails showing: adherens junction (arrow), Sertoli cell (Sc), Sertoli cell nucleus (N), basement membrane (Bm), mitochondria (M), and spermatogonium (Spg).

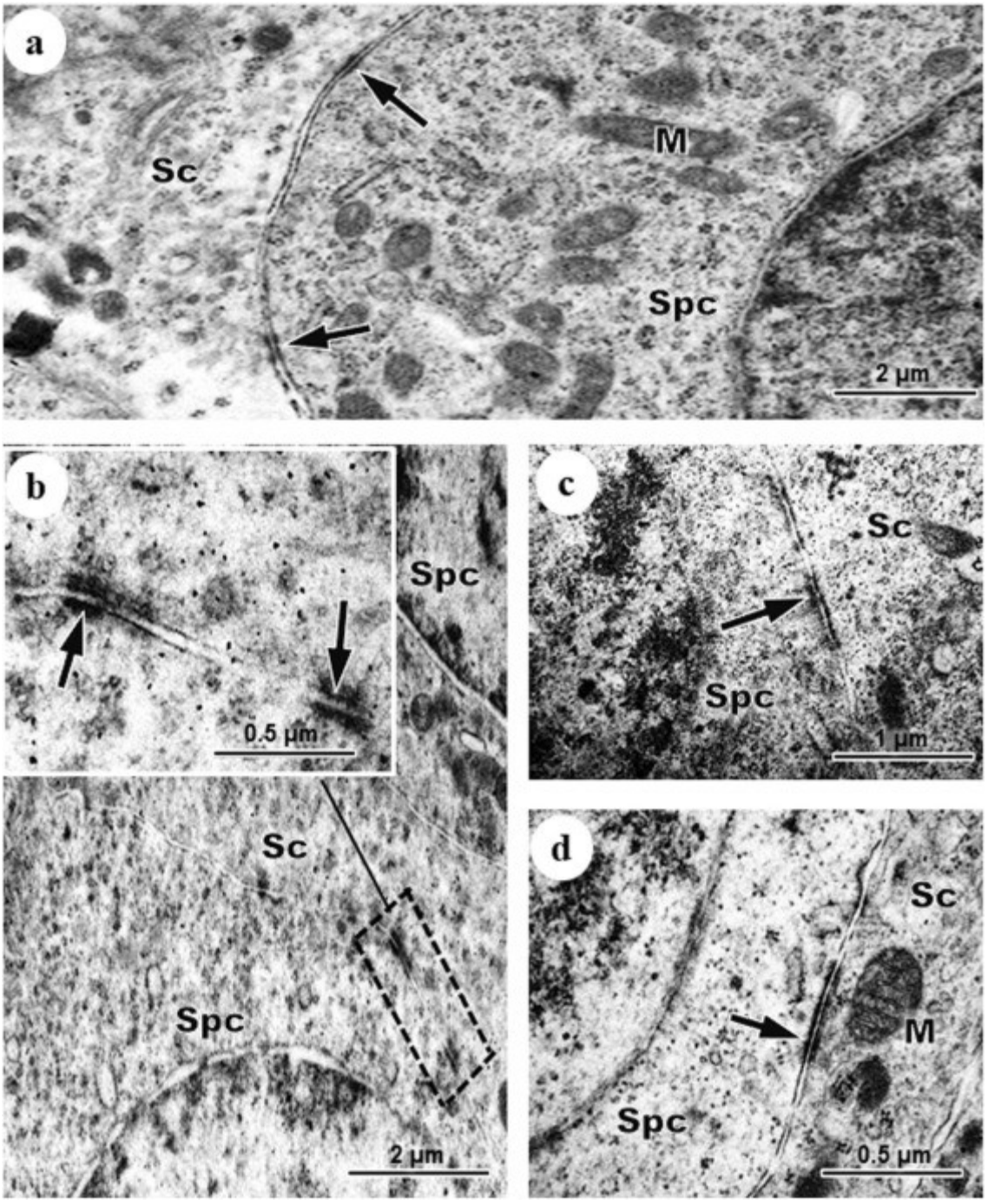

Fig. 11. Electron photomicrographs of adherens junctions between a Sertoli cell and spermatocyte in pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails showing: adherens junction (arrow), Sertoli cell (Sc), mitochondria (M), and spermatocyte (Spc).

3.2.2. Gap junctions

Although sites of membrane apposition of adjacent Sertoli cells in pre-pubertal birds were close, gap junctions were clearly visible in all age groups (Fig. 12a–f). In addition, gap junctions were present between Sertoli cells and spermatogonia in pre-pubertal, pubertal, adult, as well as aged birds (Fig. 13a–d). Gap junctions in pre-pubertal birds were underdeveloped (Fig.

14a) but well-developed between Sertoli cells and spermatocytes in pubertal, adult, and aged birds (Fig. 14b–d).

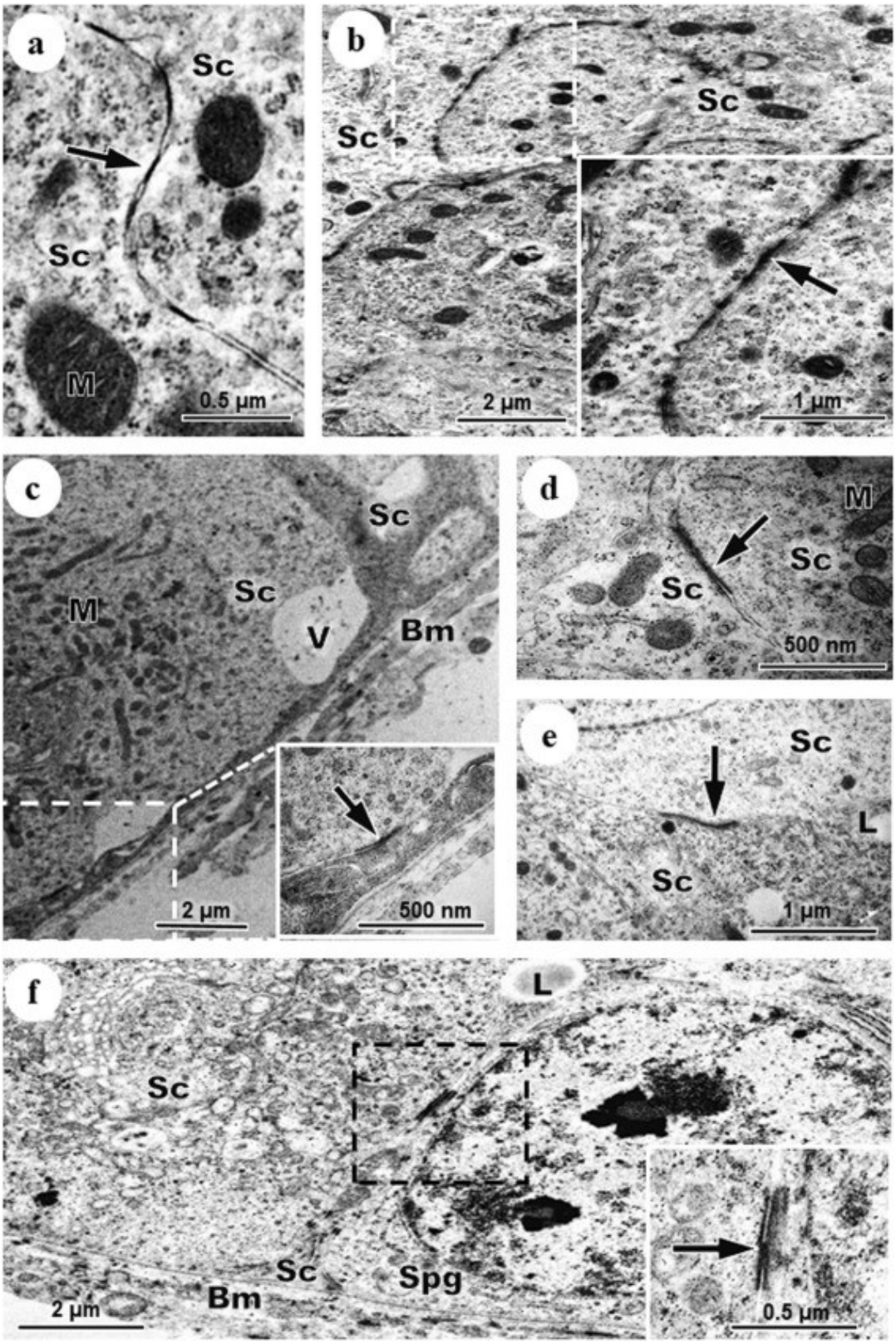

Fig. 12. Electron photomicrographs of gap junctions between adjacent Sertoli cells in pre-pubertal **(a & b)**, pubertal **(c)**, adult **(d & e)** and aged **(f)** Japanese quails showing: gap junction (arrow), Sertoli cell (Sc), mitochondria (M), vacuole (V), basement membrane (Bm), lipid droplet (L), and spermatogonium (spg).

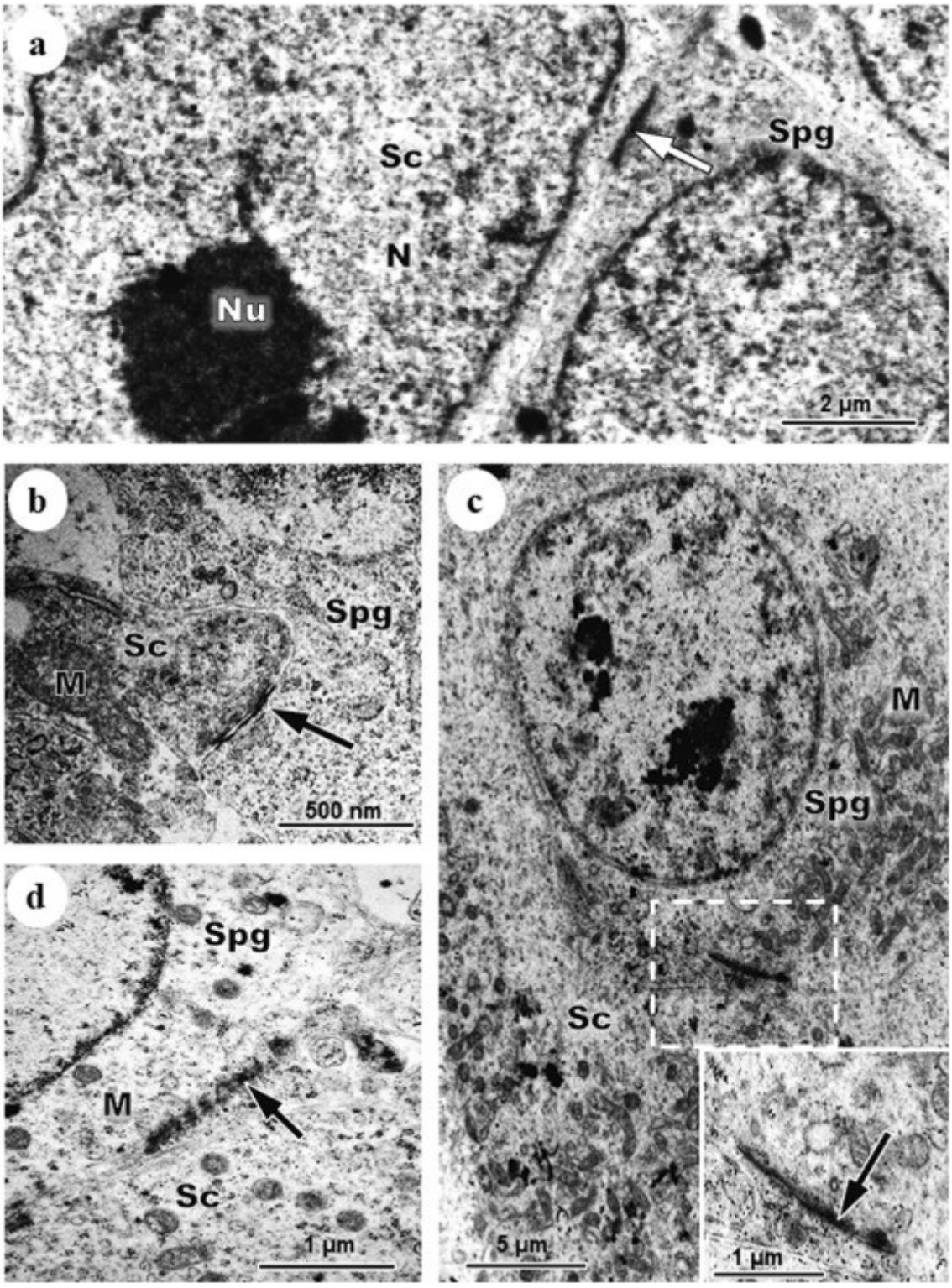

Fig. 13. Electron photomicrographs of gap junctions between a Sertoli cell and spermatogonia in pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails showing: gap junction (arrow), Sertoli cell (Sc), Sertoli cell nucleus (N), nucleolus (Nu), spermatogonium (spg), and mitochondria (M).

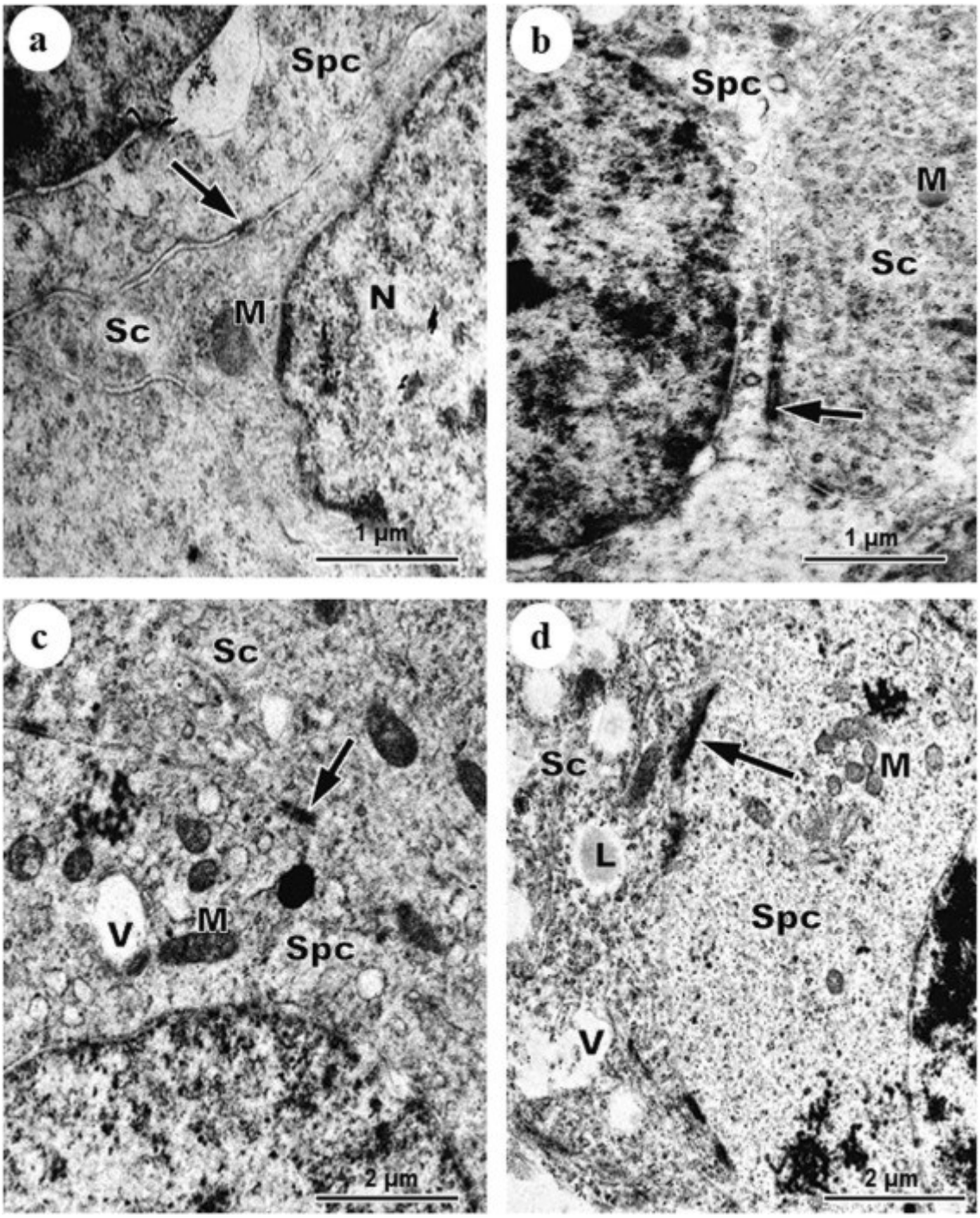

Fig. 14. Electron photomicrographs of gap junctions between a Sertoli cell and spermatocyte in pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails showing: gap junction (arrow), spermatocyte (Spc), Sertoli cell (Sc), Sertoli cell nucleus (N), mitochondria (M), vacuole (V), and lipid droplet (L).

3.2.3. Tight junctions

Tight junctions occurred between adjacent Sertoli cells in all age groups (Fig. 15a–d). Surprisingly, tight junctions in pre-pubertal birds were as clearly defined as those in the pubertal, adult, and aged birds (Fig. 15a–d).

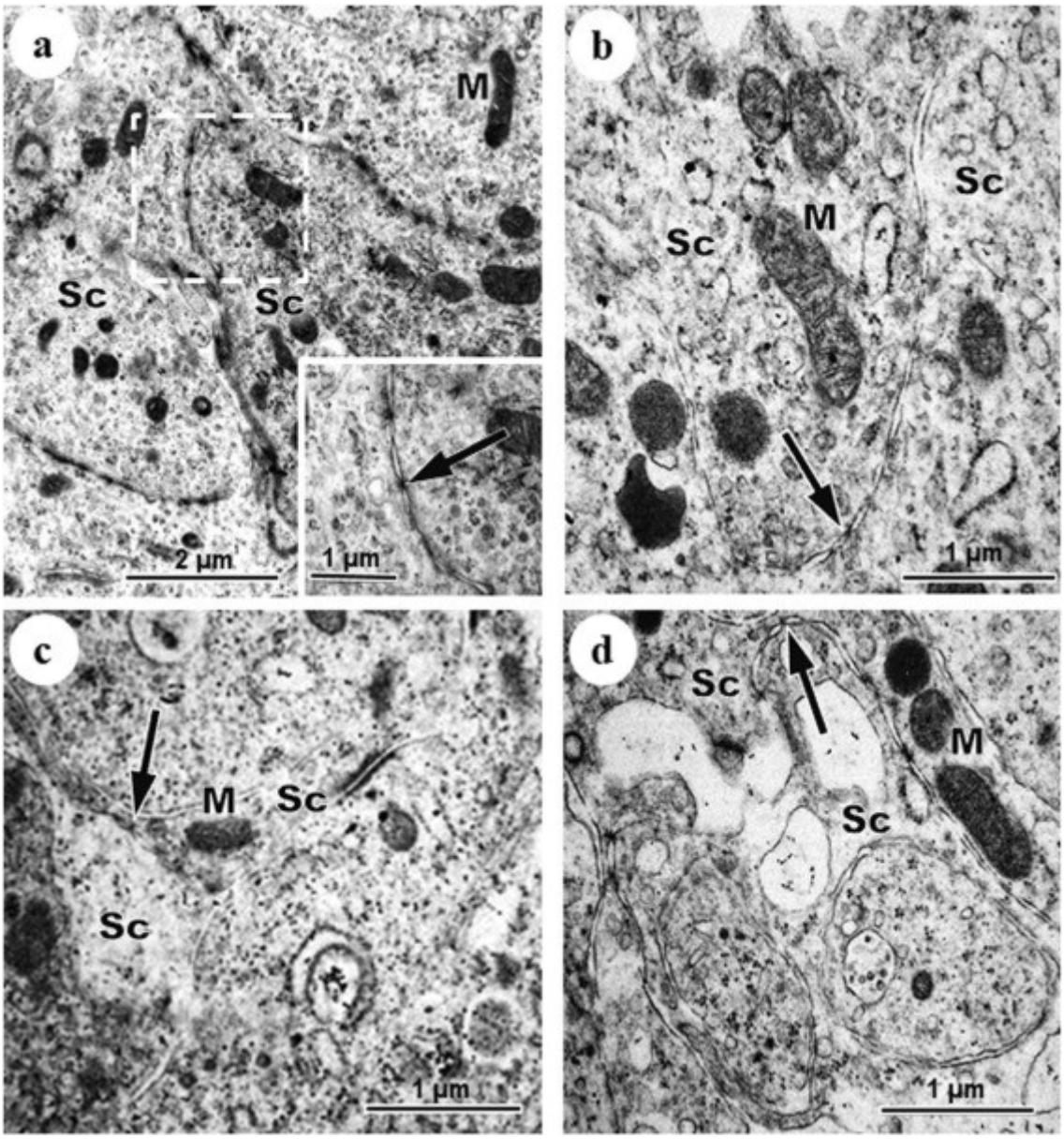

Fig. 15. Electron photomicrographs of tight junctions between adjacent Sertoli cells in pre-pubertal **(a)**, pubertal **(b)**, adult **(c)** and aged **(d)** Japanese quails showing: tight junction (arrow), Sertoli cell (Sc), and mitochondria (M).

4. Discussion

The current study represents the first evaluation of the immunolocalisation of proteins associated with junctional complexes of the BTB, as well as the ultrastructure of the junctional complexes in Japanese quail testes at different developmental stages. The age-related variations in the expression and localisation of the junctional proteins suggest that the proteins play different roles in the development and functioning of the BTB in the Japanese quail at different developmental stages.

This study also demonstrated that the adherens junction proteins β-catenin and N-cadherin in the quail testis are localised at the BTB. This is in agreement with previous literature for N-cadherin in the boar (Hejmej et al., 2012) as well as in the rat testis (Yan and Cheng, 2005). The occurrence of N-cadherin and β-catenin at the same site (BTB) is attributed to the fact

that these proteins contribute to the cadherin and catenin complex (Lee et al., 2003, Yan et al., 2009). This complex is distributed at the site of basal ectoplasmic specialisation and at inter-Sertoli cell junctions where they are involved in the BTB structure (Lee et al., 2003). The cadherin and catenin complex forms a functional unit to regulate adherens junction dynamics between Sertoli and germ cells (Lee et al., 2003).

In the current study, E-cadherin was detected in the cytoplasm of Sertoli cells and spermatogenic cells including spermatogonia and spermatocytes and this expression was observed mainly in pubertal, adult, and aged testes. E-cadherin, in conjunction with β-catenin play a key role in cellular adhesion and signal transduction (Chen et al., 2004, Kobayashi et al., 2018). Moreover, E-cadherin controls β-catenin protein levels and localisation (Chen et al., 2004).

Data from the present study demonstrate that β-catenin is detectable in the quail testis in high amounts during the early stages of development (pre-pubertal). Bae et al. (2013) reported that β-catenin is already present in chicken gonads during embryonic development. The protein was expressed predominantly in the seminiferous cords of the male embryonic gonad on embryonic days E6 to E14 (Bae et al., 2013). This might explain why β-catenin immunostaining was expressed strongly in the quail testes during the pre-pubertal stage.

Consistent with previous literature (Chakraborty et al., 2014), claudin-3 immunoreactivity was observed in the basal compartment of the seminiferous epithelium. This is the first study to record claudin-3 in the Japanese quail testis. The expression of claudins can vary by species. This was demonstrated by Stammler et al. (2016) who observed high amounts of claudin-3 in testes of mice, but did not observe any staining in human and rat testes, even after extended exposure (>1 h).

Interestingly, immunoreactivity for claudin-3 decreased as the testis developed. According to Smith and Braun (2012), claudin-3 is associated with newly formed tight junctions and is then replaced by claudin-11. This association indicates the importance of claudin-3 in the formation of new tight junctions (Smith and Braun, 2012). In mice, claudin-11 occurs in Sertoli cells throughout the entire cycle of the seminiferous epithelium, while claudin-3 is stage-specific and androgen-dependent (Chihara et al., 2010, Meng et al., 2005). According to Meng et al. (2005), claudin-3 is detected by immunostaining when androgen receptor expression in Sertoli cells is strong. Although immunostaining for androgen receptors was not performed in the present study, the levels of claudin-3 suggest that androgen receptors might be present in high amounts in the Sertoli cells of quail testes.

According to prior studies, tight junctions occur at the most apical region of epithelia and endothelia (Powell, 1981, Russell and Peterson, 1985). Contrarily, in the present study ZO-1, a tight junction protein, was detected at the basal parts of the seminiferous tubule. This complements the findings of Park et al. (2011) who observed immunoreactivity of ZO-1 and occludin in the basal compartment of the seminiferous epithelium of pheasant testis (Park et al., 2011). Similarly, Moroi et al. (1998) reported strong immunoreaction of occludin at tight junction strands in the basal region of mouse Sertoli cells. This is expected because of the important role this protein plays as a structural and functional component in well-developed tight junction strands (Moroi et al., 1998). An in vitro study by McCabe et al. (2016),

demonstrated the importance of occludin, along with claudin-11 in contributing to the function of Sertoli cell tight junctions. These researchers conducted an analysis of the contribution of occludin and claudin-11 to tight junction, in vitro, using siRNA-mediated gene silencing. Their findings demonstrated that the silencing of occludin and claudin-11 caused a significant decrease in tight junctions by 51 % and 55 %, respectively (McCabe et al., 2016).

In the present study, adherens junctions occurred between adjacent Sertoli cells, between Sertoli cells and spermatogonia as well as between Sertoli cells and spermatocytes. However, the proteins forming adherens junctions were predominantly expressed in the basal compartment of the seminiferous epithelium. According to Lee et al. (2003), adherens junctions are regulated by the cadherin and catenin complex, which is distributed at the site of basal ectoplasmic specialisation and at inter-Sertoli cell junctions where they are involved in the BTB structure. Generally, adherens junctions are responsible for lateral adhesions between Sertoli cells and germ cells (Yan et al., 2009).

Although Cx43 could not be detected by immunohistochemistry in the seminiferous epithelium of pre-pubertal testis, TEM revealed the presence of gap junctions between cells of the seminiferous epithelium as early as the pre-pubertal stage. Gap junctions between adjacent Sertoli cells might occur because such junctions are responsible for regulating Sertoli cell proliferation (Brehm et al., 2007), while gap junctions between Sertoli cells and spermatogonia have an indirect involvement in regulating the number of germ cells by controlling their survival (Gilleron et al., 2009). Additionally, gap junctions between Sertoli cells and germ cells facilitate Sertoli cell-germ cell metabolic relation and cell communication (Ahmed et al., 2018).

Although TEM documented the presence of tight junctions between adjacent Sertoli cells only, immunohistochemistry identified the presence of tight junction protein (claudin-11) in the cytoplasm of Sertoli cells and spermatogonia within the basal compartment of the seminiferous epithelium. This is attributed to the fact that Sertoli cell tight junctions play a crucial role in facilitating paracellular sealing at the basal lamina, which in turn prevents diffusion of fluid and other molecules through the BTB (Russell et al., 1989). Tight junctions are also known to be the major structural component of the BTB (Bergmann et al., 1984, Byers et al., 1991).

In conclusion, the present study documents the age-related differences in the immunolocalisation of the proteins forming the junctional complexes, as well as the ultrastructure of the junctional complexes forming the BTB in pre-pubertal, pubertal, adult, and aged quail testes as detected by immunohistochemistry and TEM, respectively. Our observations indicate that the junctional proteins are predominantly localised in Sertoli cells and within the basal compartment of the seminiferous tubules, where they form major components of the BTB and play a crucial role in the BTB structure. Moreover, the findings indicate that the junctional complexes forming the BTB in the Japanese quail are well established prior to puberty. Although there was generally a great decrease in the intensity of junctional proteins in the aged testes as shown by immunohistochemistry, TEM identified the presence of well-developed junctional complexes at this stage. The current study provides the first record on the developmental changes in adherens junctions, gap junctions, tight junctions, and the expression of the proteins forming these junctions in Japanese quail testes.

Conflict of interest

There is no conflict of interest.

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Data Availability

Data will be made available on request.

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