



Expression pattern of galectin-1 and galectin-3 in rat testes and epididymis during postnatal development

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ARTICLE INFO

Keywords:

Rat
Testis
Epididymis
Galectin-1
Galectin-3

ABSTRACT

Galectins are a family of lectins-binding beta-galactosides involved in a variety of extracellular and intracellular processes, thereby contributing to homeostasis, cell adhesion, cellular turnover, and immunity. This study aimed to determine the localization and expression of galectin-1 (Gal-1) and galectin-3 (Gal-3) in the testis and epididymis of rats at postnatal [(prepubertal (day 5), pubertal (day 20), postpubertal (day 50) and mature (day 70)] periods by using immunohistochemistry and Western blotting. Gal-1 and Gal-3 were differentially expressed in different types of cells in the testis and epididymis during postnatal development. While we detected Gal-1 expression in some spermatogenic cells and Leydig cells in the testis, not in the epididymal epithelium, Gal-3 was expressed in Sertoli cells, peritubular myoid cells, Leydig cells, smooth muscles and interstitial CD68-positive macrophages. Epithelial cells of the corpus and cauda epididymis showed an intense Gal-3 expression. Gal-1 expression was higher in the testis than in the epididymis on days 50 and 70. The expression of Gal-3 in the testis increased from the prepubertal to mature period. While the expression difference of Gal-3 was not statistically significant in the testis and epididymis until puberty, Gal-3 expression in the postpubertal and mature periods was higher in the epididymis. The expression of Gal-3 in the corpus and cauda epididymis was higher than that in the caput epididymis. In conclusion, our findings suggest that puberty has potential regulatory effect on the expression of galectins in testis and epididymis of rats. Gal-1 and 3 may play a role in the development of the reproductive system and the preservation of the immune-privileged environment in the testis, due to their pro-apoptotic and anti-apoptotic functions. The presence of intense expression of Gal-3 in the corpus and cauda epididymis may contribute to the maturation and storage of spermatozoa.

1. Introduction

The testes are a pair of organs that is highly specialized for the production of spermatozoa and male sex hormones (Russell et al., 1993), are comprised of two main compartments, the seminiferous tubules surrounded by the interstitium. The interstitium contains Leydig cells, leukocytes, fibroblasts, blood and lymphatic vessels. The seminiferous tubules consist of Sertoli cells and germ cells surrounded by one or more layers peritubular myoid cells (Kaur et al., 2013). The epididymis is an accessory organ comprising highly coiled duct extending around the back of the testes. With the beginning of puberty, germ cells turn into spermatozoa after various developmental stages. A large number of intracellular and cell surface proteins are synthesized

during the development and differentiation of germ cells. An immunological tolerance develops for some of these proteins that have autoantigenic potential (Ferguson and Griffith, 1997; Filippini et al., 2001).

Due to the properties of outstanding immune privilege and effective local innate immunity, exceptional immunological environment in the mammalian testis protects immunogenic germ cells against possible systemic immune attacks and is essential for the prevention of microbial infections in the testis (Zhao et al., 2014).

Galectins, a subfamily of β -galactoside-binding lectins, demonstrate a substantial sequence similarity in their carbohydrate-binding sites (Barondes et al., 1994). Galectins composed of 15 members have been divided into three groups based on their molecular architecture: (a)

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<https://doi.org/10.1016/j.acthis.2018.09.006>

Received 1 August 2018; Received in revised form 6 September 2018; Accepted 13 September 2018

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“proto-type” galectins comprising a single polypeptide chain with one carbohydrate recognition domain (CRD) able to dimerize (Gal-1, -2, -5, -7, -10, -11, -13, -14, and -15); (b) “chimera-type” Gal-3 consisting of a C-terminal CRD linked to an N-terminal peptide, and (c) “tandem repeat-type” galectins composed of a single polypeptide chain having two CRDs in tandem connected by a linker peptide (Gal-4, -6, -8, -9, and -12) (Hirabayashi and Kasai, 1993; Rabinovich and Croci, 2012). While some galectins (e.g., Gal-1 and Gal-3) show a wide distribution among different types of cells and species, others have been expressed by few cells (Camby et al., 2006; Dumić et al., 2006).

Galectins can be found in extracellular space and in cellular compartments including cell membrane, cytoplasm, and nucleus. They can bind to proteins via a carbohydrate interaction or via direct protein-protein interactions. Most galectins perform various functions and can mediate (1) receptor crosslinking or lattice formation, (2) cell-extracellular matrix interactions, (3) cell-to-cell interactions, (4) intracellular signaling, and (5) posttranscriptional splicing (Thijssen et al., 2007). Galectins can also control very important biological and physiological functions such as inflammation, cell proliferation, cell cycle, transcription processes, and cell death (Rabinovich et al., 2002).

The expression of Gal-1 is controlled during the spermatogenic process. In rats, the expression of this lectin in Sertoli cells usually occurs in stages 10–12 of the spermatogenic cycle and is upregulated during spermiogenesis (stages VI–VIII) within the luminal pole of the seminiferous epithelium. It was also found on the apical stalks of Sertoli cells and heads of mature spermatids. Following spermiogenesis (stage 8), it begins to be expressed at the basal segment of Sertoli cells and continues to be seen throughout the whole cell as the differentiation of germ cells proceeds (Dettin et al., 2003).

Most members of the galectin family including Gal-1 possess apoptotic activities, whereas Gal-3 has anti-apoptotic activity (Akahani et al., 1997). Therefore, Gal-3 is a structurally unique member of galectin family (Dumić et al., 2006). Gal-3 is mainly found in the cytoplasm, although being detected significantly in the nucleus, on the cell surface or in the extracellular environment depending on cell type and proliferative state (Krześlak and Lipińska, 2004). In immunocompetent/inflammatory cells, Gal-3 is expressed in monocytes, dendritic cells (DCs), macrophages, eosinophils, mast cells, NK cells, and activated T and B cells (Chen et al., 2005).

The limited number of studies have revealed regarding the developmental expressions of galectins in the reproductive organs and their cellular and subcellular localizations (Dettin et al., 2003; Kim et al., 2007, 2008b; Khorsandi and Orazizadeh, 2011). Therefore, investigation of differences in Gal-1 and -3 expressions in the testis and epididymis throughout the postnatal development period would shed further light on their roles in the reproductive physiology. We here aimed to determine the localizations and quantities of Gal-1 and Gal-3 at different stages of maturation in the testis and epididymis of healthy rats.

2. Materials and methods

2.1. Animal materials and experimental design

Testes and epididymis tissues were taken from four groups Wistar albino rats at different stages of postnatal development. Each of the four groups composed of six rats were designed as prepubertal (5 days), pubertal (20 days), postpubertal (50 days) and mature (70 days). The animals were kept at room temperature (20–24 °C) in a 12-h light/dark circle and fed a standard diet and given water ad libitum. At the end of each developmental stage, all rats were euthanized under anesthesia and tissue samples were taken. This procedure was performed between 10:00 and 12:00 to avoid any changes caused by animals' biological rhythm.

This study was approved by the Department of Experimental Animals Ethic Committee, University of Ankara (2014-18-128).

2.2. Tissue processing

Tissue processing was performed as previously reported (Beyaz et al., 2017). Briefly, the testis and epididymis samples were fixed in Bouin's solution, which is prepared by mixing 15 units of saturated picric acid, 5 units of formaldehyde (37–40%), 1 unit of glacial acetic acid. Then, tissues passed through a graded series of alcohols, methyl benzoate and benzole, and then embedded in paraplast.

2.3. Immunohistochemistry

Immunohistochemical staining was undertaken using the streptavidin-biotin-peroxidase technique, as described previously (Beyaz et al., 2017). In brief, sections of 5-micron thickness were placed on Poly-L-Lysine-coated slides (Menzel Microscope slide Polysine, Thermo Fisher Scientific, Fremont, CA, USA). Following deparaffinization and dehydration, the sections were rinsed in PBS (P4417, Sigma-Aldrich, Germany) and incubated in 3% H₂O₂ in 70% methanol/PBS for 20–30 min to eliminate endogenous peroxidase activity. Following rinsing with PBS, the sections were subjected to the necessary antigen retrieval procedures [citrate buffer (AP-9003-500, Thermo Fisher Scientific Lab Vision, Fremont, USA) for Gal-1 and Gal-3, and trypsin for CD68]. After rinsing with PBS again, the sections were incubated in 10% normal goat serum (TP-125-HL, Ultra V Block[®], Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 10 min. Then, they were incubated with primary antibodies (Table 1) at +4 °C for 16 h. After washing in PBS for 15 min, the sections were incubated with biotinylated secondary antibody (TP-125-HL, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 30 min at room temperature (RT) and rinsed again with PBS for 15 min. Afterwards, they were treated with enzyme-conjugated streptavidin (TP-125-HL, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 30 min, followed by rinsing in PBS for 15 min and incubation in an AEC chromogen (TA-060-HA, AEC Chromogen & Substrate System, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 5–10 min (color development observed under a microscope). The sections were washed with distilled water and stained with Gill's hematoxylin for 3–5 min. They were rinsed under running tap water until the color of the sections turned blue. Finally, the sections were mounted with aqueous mounting medium (TA-125-UG, Vision Mount, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) and coverslipped. Immunohistochemical assessment was performed by two blind observers under a light microscope (DM 2500, Leica, Germany) equipped with a digital camera (DFC450, Leica, Germany). Leica Application Suite software was used for image capture. Semi-quantitative scoring was performed as follows: 0 (no staining), + (weak staining), ++ (moderate staining), and +++ (strong staining).

One of the serial sections was treated with the CD68 primary antibody (Table 1) to assess the presence of galectin proteins in macrophages.

All immunohistochemical stainings were carried out in a moisture chamber unless otherwise stated. The primary antibody was replaced with non-specific rabbit immunization serum (sc-2027, Santa Cruz Bio Inc., Dallas, TX, USA) for negative control. Rat colon tissue was used for

Table 1
Primary antibodies used in the immunohistochemical staining.

Primary antibodies	Clonity	Host	Dilution	Source	Code
Galectin-1	Polyclonal	Rabbit	1:250	Novus Biologicals	NBP1-89791
Galectin-3	Monoclonal, Clone A3A12	Mouse	1:300	Novus Biologicals	NB300-538
CD68	Monoclonal, Clone ED1	Rabbit	1:100	AbD Serotec	MCA341R

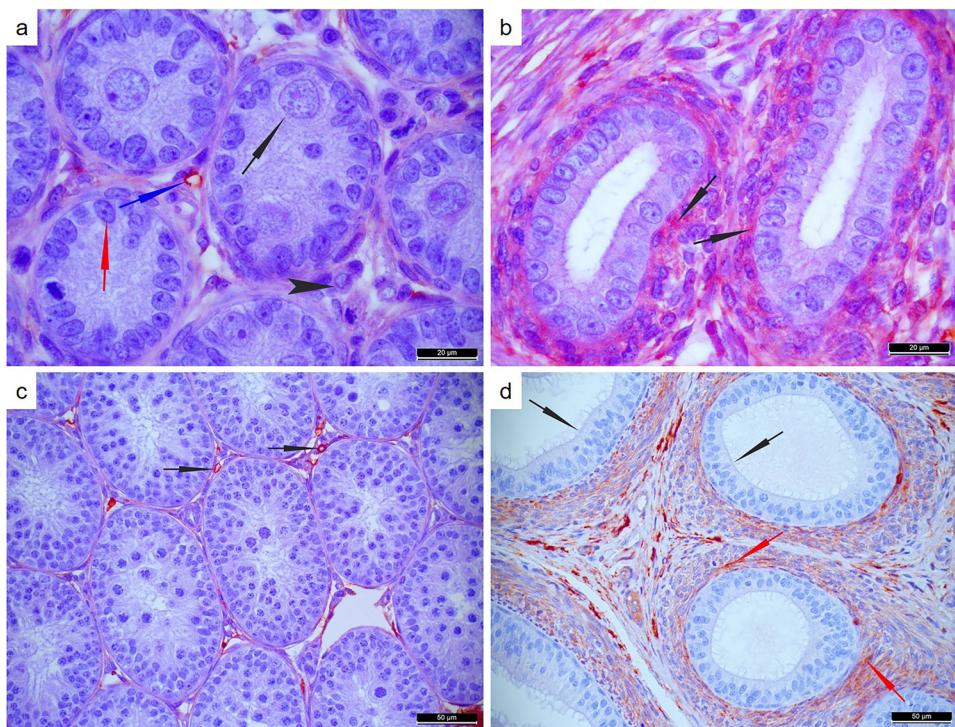


Fig. 1. Gal-1 immunoreactivity in the testis and epididymis of rats on postnatal days 5 (a, b) and 20 (c, d). Strept-ABC, AEC. (a) In the testis, no immunoreaction in Sertoli cells (red arrow) and gonocytes (black arrow). Positive immunoreaction in the smooth muscle cells (blue arrow) in the wall of capillaries. No immunoreaction in Leydig cells (arrow head). Bar: 20 μ m. (b) In the ductus epididymidis, the only positive immunoreaction in the smooth muscle cells at the periphery of the ductal epithelia (arrows) and vessels. Bar: 20 μ m. (c) Positive immunoreaction in the smooth muscle cells (arrows) in the vessel wall in the interstitial area of the testis. Bar: 50 μ m. (d) No immunoreaction in the epithelium of the ductus epididymidis (black arrows), but positive immunolabeling in the smooth muscle cells at the periphery of the ductal epithelia (red arrows). Bar: 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

positive control. The same protocol was followed for all stainings including the positive and negative controls.

2.4. Protein extraction

Freshly collected tissue samples were immediately transferred to liquid nitrogen and kept at -80°C until western blot is performed. Total protein was isolated from dissected testis and epididymis including caput corpus and cauda, as described previously (Yumusak et al., 2018). Briefly, tissues were crushed on ice in mammalian cell lysis buffer (# 17081, PRO-PREPTM Protein Extraction Solution, iNtRON Biotechnology, Kirkland, Washington, USA) containing a final concentration of 1X the protease inhibitor cocktail (#5872, Cell Signaling Technology, Danvers, USA) with a tissue homogenizer (Heidolph homogenizers, SilentCrusher, Germany). The tissue homogenates were cleared by g force at $20,000 \times \text{rpm}$ for 25 min at 4°C , and the supernatant was transferred to tubes for final collection after g force at $15,000 \times \text{rpm}$ for 15 min. Total protein concentrations were determined by using Bradford method (Bradford 1976) with Quick Start Bradford Protein Assay (Bio-Rad Laboratories, California, USA).

2.5. Western blot

Total protein samples (25 μ g, each) were resolved using SDS-PAGE with 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immun-Blot PVDF, Bio-Rad, USA) using Bio-Rad Trans-Blot Turbo for 30 min under constant current of 25 V with 1.0 mA. After blocking the membrane with 5% skimmed milk for 2 h at RT, the blots were incubated with primary antibodies (Rabbit polyclonal anti-Galectin-1: NBP1-89791, 1:1000 dilution, Novusbio; Mouse monoclonal anti-Galectin-3: NB300-538, 1:1000 dilution, Novusbio; Rabbit polyclonal anti-GAPDH: sc-25778, 1:1000 dilution, Santa Cruz Bio Inc., Dallas, TX, USA) overnight at 4°C . Then, the blots were washed and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG: sc-2004, 1:10,000 dilution and goat anti-mouse IgG: sc-2005, Santa Cruz Bio Inc., Dallas, TX, USA) for 2 h at RT, and washed with Tris-buffered saline with Tween solution (TBST, 50 mM Tris-HCl, PH 8.0, 150 mM

NaCl, and 0.1% Tween-20). Signals were detected by chemiluminescence substrate using SignalFire™ ECL Reagent (#6883, Cell Signaling Technology Inc., Danvers, MA, USA). GAPDH was used as an internal control and band intensities were quantified using Image Studio™ Lite (LI-COR Biotechnology, Lincoln, Nebraska, USA).

2.6. Statistical analysis

Quantification of the data presented in bar graphs was created with GraphPad Prism 5 (GraphPad Software, California, USA). All data are presented as mean $\pm$ SEM. Statistical analysis for two groups (testis vs epididymis) was performed using a two-tailed Student's *t*-test. Statistical analysis for multiple groups (testis among 5-20-50-70th days, testis vs epididymal regions [caput (CT), corpus (CS), cauda (CA)] was performed with one-way factorial analysis of variance (ANOVA) and subsequent Tukey's multiple comparison test. Level of significance was accepted as $p < 0.05$.

3. Results

3.1. Immunohistochemical findings

3.1.1. Gal-1

Postnatal day 5: We did not observe any positive immune reaction in Sertoli cells, gonocytes and Leydig cells while labeling was detected in the capillary in the interstitial tissue (Fig. 1a). In the epididymis, immunolabeling was present only in smooth muscles and vessel walls (Fig. 1b).

Postnatal day 20: We detected a positive immunolabeling only in the wall of interstitial vessels (Fig. 1c) and smooth muscle cells in the ductus epididymis (Fig. 1d).

Postnatal day 50: We began to see a positive immunoreaction in Leydig cells, Sertoli cells (Fig. 2a), spermatids at spermatogenic stages 9 (Fig. 2b), 10 (Fig. 2c), 11 (Fig. 2d) and 12 (Fig. 2e). Gal-1 immunoreactivity was also seen in the vascular smooth muscle cells (Fig. 2f). No immunoreaction occurred in the epithelial cells of the ductus epididymis while positive immunoreaction was present in smooth muscle cells at the periphery of the ductal epithelia and the

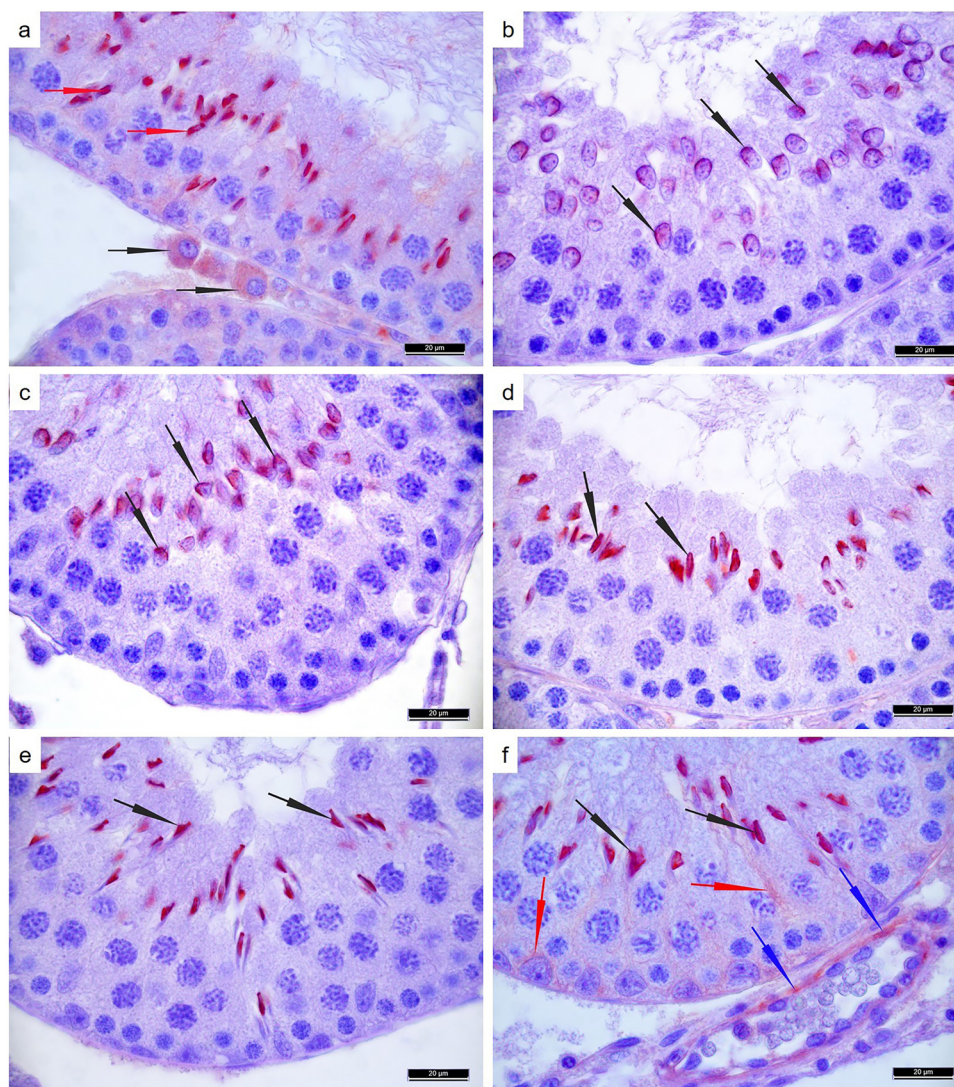


Fig. 2. Gal-1 immunoreactivity in the testis of rats on postnatal day 50. Strept-ABC, AEC. (a) Positive immunoreaction in Leydig cells (black arrow) and spermatids (red arrows). Bar: 20 µm. (b) Positive immunoreaction in step 9 spermatids at spermatogenic stage 9 (arrows). Bar: 20 µm. (c) Positive immunoreaction in step 10 spermatids at spermatogenic stage 10 (arrows). Bar: 20 µm. (d) Positive immunoreaction in step 11 spermatids at spermatogenic stage 11 (arrows). Bar: 20 µm. (e) Positive immunoreaction in step 12 spermatids at spermatogenic stage 12 (arrows). Bar: 20 µm. (f) Positive immunolabeling in the smooth muscle cells in the vessel wall (blue arrows), spermatids (black arrows) and Sertoli cells (red arrows). Bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vessel wall (Fig. 3a–d).

Postnatal day 70: We detected that the positive reaction in the Leydig and Sertoli cells (perinuclear cytoplasm and apical cytoplasm) increased compared to that of the postpubertal period (Fig. 4a, b). In the early stages of the spermatogenic cycle, we observed a positive reaction in extending from the perinuclear region to the apical cytoplasm in Sertoli cells. The positive immunoreaction was also detected in the head of mature spermatids (Fig. 4c, d). In the ductus epididymis, we observed a positive reaction in smooth muscle cells at the periphery of the ductal epithelia and the vessel wall (Fig. 5a–d).

At all stages of postnatal development, peritubular myoid cells and interstitial macrophages in the testis and epididymis did not show any immunoreaction for Gal-1. Epithelial cells of epididymis wall were also negative for Gal-1. The immunohistochemical staining of Gal-1 is summarized in Table 2.

3.1.2. Gal-3

Postnatal day 5: We detected a positive immunoreaction in the macrophages in the connective tissues of the testis and ductus epididymis but not in the remaining cells in these two organs (Fig. 6a, b).

Postnatal day 20: In testes, we observed an intense immunoreaction in the nucleus and cytoplasm of peritubular myoid cells and interstitial macrophages (Fig. 6c). In the ductus epididymis, some epithelial cells showed weakly positive immunoreaction, while interstitial macrophages showed an intense positive immunoreaction (Fig. 6d).

Postnatal day 50: We detected that the positive reaction in the Sertoli cells was weaker compared to that of the mature period. While the immune reaction was intense in the nucleus and cytoplasm of peritubular myoid cells and macrophages, Leydig cells showed weak immunoreaction (Fig. 7a, b). Gal-3 and CD68 immunostaining on serial sections also confirmed specificity of immunoreaction to macrophages (Fig. 7c, d).

We observed a positive immunolabeling in the nucleus and cytoplasm of narrow cells in the initial segment of the caput epididymis but not in principal, basal and apical cells in the initial segment (Fig. 8a). The principal cells in the caput epididymis also showed no immunoreaction. We detected an immunoreaction in macrophages of the connective tissue (Fig. 8b). The majority of principal cells in the corpus and cauda epididymis showed an intense immunoreaction while some of them showed no immunoreaction (Fig. 8c, d).

Postnatal day 70: In the early stages of the spermatogenic cycle, we observed an intense labeling in the perinuclear region of Sertoli cells. Labeling was also apparent in the apical cytoplasm of these cells during spermiogenesis. Moreover, the immunoreaction was intense in the nucleus and cytoplasm of macrophages and peritubular myoid cells, but weak in Leydig cells (Fig. 9a–d).

We identified differences in Gal-3 expression in different parts of the epididymis. Narrow cells and connective tissue macrophages in the initial segment showed a positive immunoreaction (Fig. 10a). There was no reaction in the epithelial cells in the caput epididymis

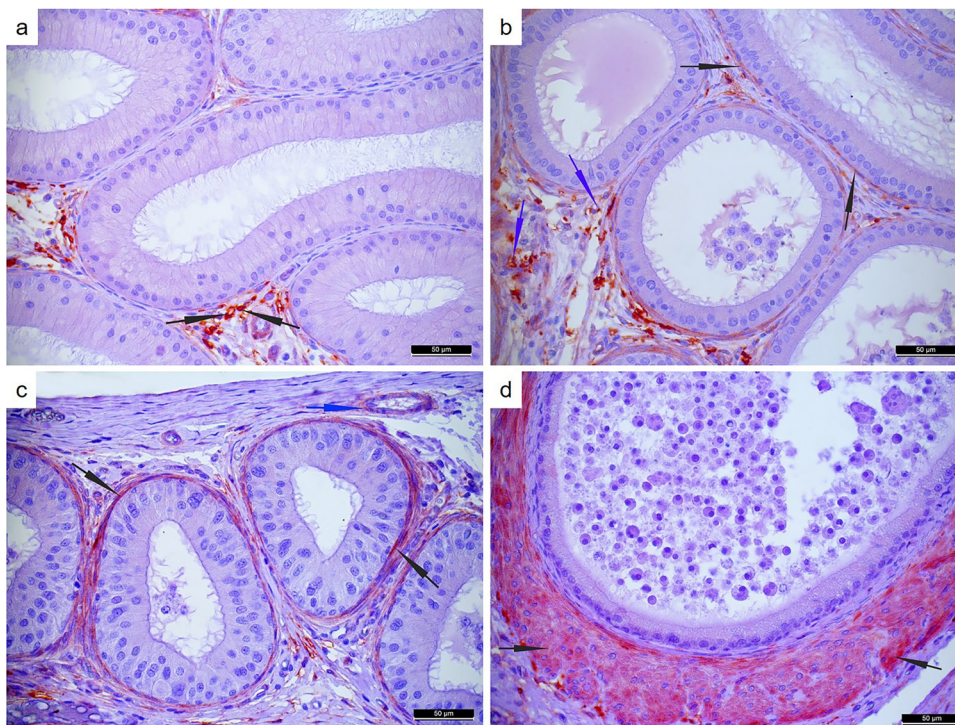


Fig. 3. Gal-1 immunoreactivity in the epididymis of rats on postnatal day 50. Strept-ABC, AEC. Positive immunoreaction in: (a) the smooth muscle cells in the vessel wall in the initial segment (arrows), (b) the smooth muscle cells at the periphery of the ductal epithelia (black arrows) and the smooth muscle cells in the vessel wall (blue arrows) in the caput epididymis, (c) the smooth muscle cells at the periphery of the ductal epithelia (black arrows) and the smooth muscle cells in the vessel wall (blue arrows) in the corpus epididymis, and (d) the smooth muscle cells at the periphery of the ductal epithelia (black arrow) in the cauda epididymis. Bar: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

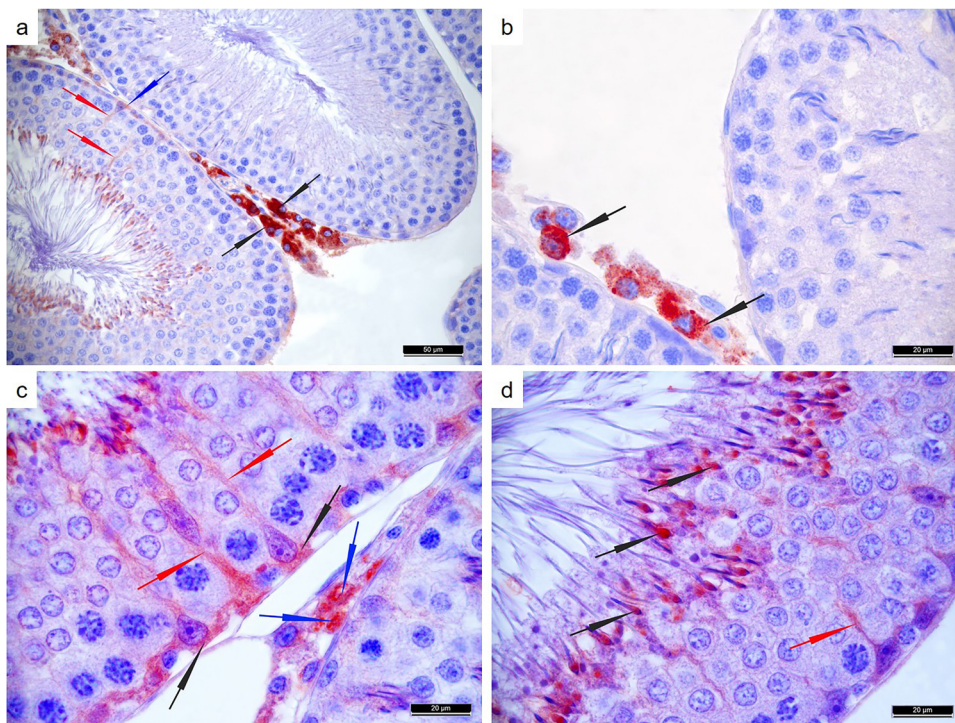


Fig. 4. Gal-1 immunoreactivity in the testis of rats on postnatal day 70. Strept-ABC, AEC. (a) An immunoreaction was present in Leydig cells (black arrows) and perinuclear (blue arrows) and apical (red arrows) regions of Sertoli cells. Bar: 50 µm. (b) The Leydig cells (black arrows) show positive immunoreactions at varying intensities; Bar: 20 µm. (c) At later stages (5 and 6), a positive immunoreaction was observed in the perinuclear (black arrows) and apical cytoplasm (red arrows) of Sertoli cells, as well as in Leydig cells (blue arrows); Bar: 20 µm. (d) During the spermiation stages (6–8), A positive immunoreaction in the apical cytoplasm (red arrow) of Sertoli cells and head of mature spermatids (black arrows); Bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 10b). The reaction was intense in the epithelial cells and their cilia in the corpus region (Figs. 10c, 11a). In the cauda epididymis, we observed a positive immunoreaction in the majority of epithelial cells (Fig. 10d). Furthermore, we detected Gal-3 immunoreactivity in CD68-positive connective tissue macrophages (Fig. 10b) and intraepithelial macrophages in the initial segment, caput and corpus epididymis (Fig. 11b). In the testis, CD68-positive macrophages and Leydig cells were localized close to each other (Fig. 11c, d).

Table 3 presents a summary of the subjective evaluation of Gal-3 immunohistochemical staining.

3.2. Western blotting findings

Expressions of the protein levels of both Gal-1 and Gal-3 were detected in the rat testis and epididymis during postnatal development using Western blot analysis. Both galectin proteins were found in whole testes and epididymis samples and the results confirmed the immunohistochemical data. The comparison of the expression level of these lectins in the developing testis and epididymis is given in the histograms in Figs. 12 and 13.

Gal-1 expression in testis and epididymis increased from the

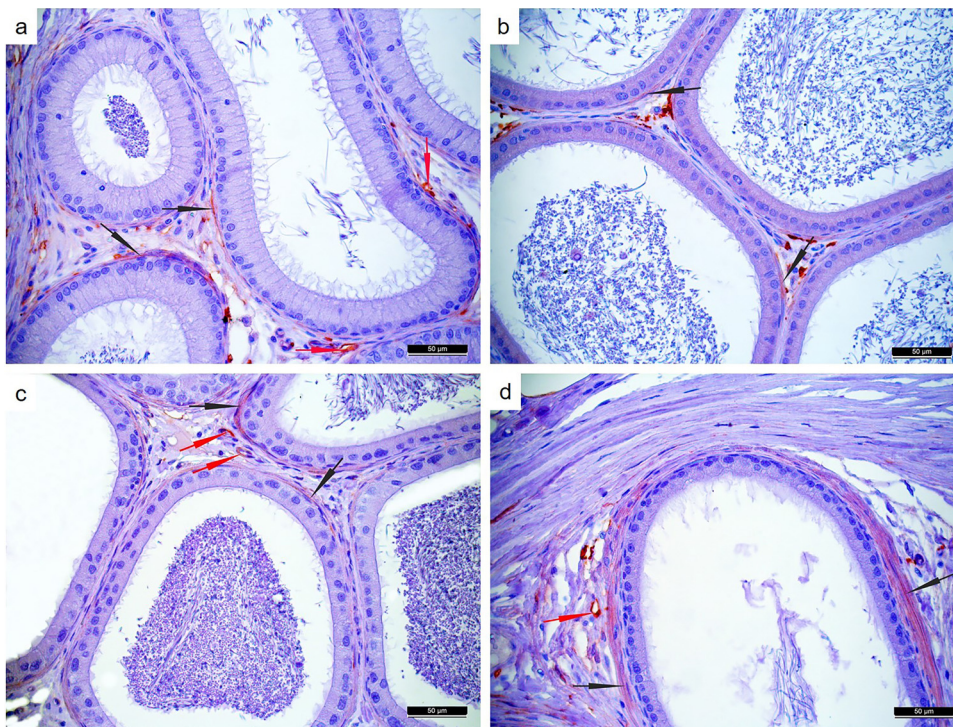


Fig. 5. Gal-1 immunoreactivity in the epididymis of rats on postnatal day 70. Strept-ABC, AEC. A positive immunoreaction in: (a) the smooth muscle cells at the periphery of the ductal epithelia (black arrows) and the smooth muscle cells in the vessel wall (red arrows) in the initial segment, (b) the smooth muscle cells at the periphery of the ductal epithelia in the caput epididymis (arrows), (c) the smooth muscle cells at the periphery of the ductal epithelia (black arrows) and the smooth muscle cells in the vessel wall (red arrows) in the corpus epididymis, and (d) the smooth muscle cells at the periphery of the ductal epithelia (black arrows) and the smooth muscle cells in the vessel wall (red arrows) in the cauda epididymis. Bar: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prepubertal period to the postpubertal period, which was statistically significant after the pubertal period (Fig. 12b). In the prepubertal and pubertal periods, the expression of Gal-1 in epididymis was found to be higher than that in the testis ($p > 0.05$) (Fig. 12a), whereas in the postpubertal and mature periods, its expression was higher in the testis than in the epididymis ($p < 0.05$) (Fig. 12c, d). The difference between the epididymal and testicular expression further increased in the mature period ($p < 0.01$) (Fig. 12d). There was no difference in the expression of Gal-1 among the epididymal regions (Fig. 12c, d). The expression of Gal-3 in the testis and epididymis increased from the prepubertal period to the postpubertal period ($p < 0.05$) (Fig. 13a, b). In the prepubertal and pubertal periods, expression of Gal-3 was greater in the testis than in the epididymis ($p > 0.05$) (Fig. 13a), while, postpubertal ($p < 0.05$) and mature ($p < 0.01$) periods, epididymal Gal-1 expression was higher than the testicular expression (Fig. 13c, d). There was no statistical difference between Gal-3 expression in the corpus and cauda at postpubertal (Fig. 13c) and mature periods ($p > 0.05$) (Fig. 13d). However, Gal-3 expression in the corpus and cauda was higher than that in the caput (postpubertal: $p < 0.05$, mature $p < 0.01$) (Fig. 13c, d).

4. Discussion

We here found that Gal-1 and Gal-3 proteins were differentially expressed in different types of cells in the testis and epididymis during postnatal development.

In rats, Dettin et al. (2003) observed an intense immunological reaction in the luminal pole of the seminiferous epithelium, apical of Sertoli cells, and the head of mature spermatids during the spermiogenesis. They also reported that Gal-1 expression was present in the basal segments of Sertoli cells following spermiogenesis, and that Gal-1 expression was seen throughout Sertoli cells as the differentiation of the germ cells progressed. In the same study, Gal-1 was superficially localized to spermatozoa in the caput epididymis. In the current study, we observed Gal-1 immunoreactivity in the perinuclear region of Sertoli cells in the early phases of the spermatogenic cycle (Fig. 2). As the differentiation of germ cells progressed, this reaction was seen throughout Sertoli cells (Fig. 4). In the sixth, seventh and eighth stages of spermiogenesis, reactions

also occurred in the heads of mature spermatids located in the apical region of Sertoli cells (Fig. 4).

Dettin et al. (2003) did not detect Gal-1 immunoreactivity in spermatocytes and round spermatids in any of the spermatogenic stages. However, we observed a Gal-1-positive reaction in the same-step spermatids at the 9th, 10th, 11th and 12th stages of the spermatogenic cycle (Fig. 2). We consider that Gal-1 may play possible a role in immunological reactions. Furthermore, the localization of Gal-1 to only certain spermatids at specific phases indicates that these proteins may be active in certain events and participate in the differentiation and physiological functions of germ cells.

Chui et al. (2011) found a high concentration of Gal-1 in peritubular myoid cells in human. They reported that these cells surround seminiferous tubules and affect leucocytes in the interstitium. In the present study, peritubular cells and macrophages were negative for Gal-1 while Sertoli, Leydig and smooth muscle cells were positive for Gal-1 (Figs. 1–5).

Dettin et al. (2003) detected Gal-1 expression in the testis of rats on the ninth day of the postnatal period and reported increased expression by age. Huang et al. (2011) found that Gal-1 expression in the porcine testis increased until postnatal month 3 but started to decrease after 1 year of age. A study conducted on the buffalo, it was stated that Gal-1 expression decreased in the testis until puberty but started to increase in the postpubertal period (Zhang et al., 2017). In the current study, we determined that Gal-1 expression in the testis increased until the postpubertal period, and this increase was statistically significant after the first 20 days. While there was no statistically significant difference between testicular and epididymal expression of Gal-1 up to puberty, this protein was observed to be more prominent in the former than in the latter on days 50 and 70 (Fig. 12). We consider that this increase in Gal-1 expression in the testis is due to the increased number of Leydig cells and spermatids after puberty.

Khorsandi and Orazizadeh (2011) observed a positive immunoreaction to Gal-3 in Leydig cells, peritubular myoid cells and seminiferous tubules of mouse testes, but not in Sertoli cells. They concluded that Gal-3 might be a multifunctional function in the testicular tissue of mice. The authors also found that Gal-3 expression was more intense at stages that were susceptible to apoptosis (stages 7 and

Table 2
Evaluation of immunoreaction positivity for Gal-1 in the postnatal development stages of the rats.

Tissue		Day 5	Day 20	Day 50	Day 70
Cell type					
Testis	Spermatogonium	0 (Gonocyte)	0	0	0
	Spermatocyte/spermatid	0(Gonocyte)	0	+++ Spermatids at the same step at stages 9, 10, 11 and 12	+++ Spermatids at the same step at stages 9, 10, 11 and 12
	Sperm	0	0	0	0
	Sertoli cells	0	0	+	++
	Leydig cells	0		++	+++
	Interstitial macrophages	0	0	0	0
	Peritubular myoid cells	0	0	0	0
	Vessel wall muscles	+++	+++	+++	+++
Epididymis					
Initial segment	Principal cells	Columnar cells 0	Columnar cells 0	0	0
	Narrow cells	No differentiation	0	0	0
	Basal cells	No differentiation	No differentiation	0	0
	Intraepithelial macrophages	No differentiation	0	0	0
	Apical cells	No differentiation	No differentiation	0	0
	Interstitial macrophages	0	0	0	0
	Vessel wall muscles	+++	+++	+++	+++
	Muscle layer in the ductal wall	+++	+++	+++	+++
Caput epididymis	Principal cells	Columnar cells 0	Columnar cells 0	0	0
	Narrow cells	No differentiation	NONE	NONE	NONE
	Basal cells	No differentiation	No differentiation	0	0
	Intraepithelial macrophages	No differentiation	0	0	0
	Apical cells	No differentiation	No differentiation	0	0
	Interstitial macrophages	0	0	0	0
	Vessel wall muscles	+++	+++	+++	+++
	Muscle layer in the ductal wall	+++	+++	+++	+++
Corpus epididymis	Principal cells	Columnar cells 0	Columnar cells 0	0	0
	Narrow cells	No differentiation	NONE	NONE	NONE
	Basal cells	No differentiation	No differentiation	0	0
	Intraepithelial macrophages	No differentiation	0	0	0
	Apical cells	No differentiation	No differentiation	0	0
	Interstitial macrophages	0	0	0	0
	Vessel wall muscles	+++	+++	+++	+++
	Muscle layer in the ductal wall	+++	+++	+++	+++
Cauda epididymis	Principal cells	Columnar cells 0	Columnar cells 0	0	0
	Narrow cells	No differentiation	NONE	NONE	NONE
	Basal cells	No differentiation	No differentiation		
	Intraepithelial macrophages	No differentiation	0	NONE	NONE
	Apical cells	No differentiation	No differentiation	0	0
	Interstitial macrophages	0	0	0	0
	Vessel wall muscles	+++	+++	+++	+++
	Muscle layer in the ductal wall	+++	+++	+++	+++

Staining intensity: 0 negative (no staining even at high magnification), + weak (only visible at high magnification), ++ moderate (readily visible at low magnification), +++ strong (strikingly positive at low power magnification) staining.

8), suggesting that Gal-3 might be a significant anti-apoptotic factor. Wollina et al. (1999) reported that Gal-3 showed positive immunoreactivity in Sertoli cells, but this protein was not expressed in germ cells. Deschildre et al. (2007) found that Gal-3 was present in Sertoli cells, but not in spermatocytes or even in spermatids in rat testis. They also observed a Gal-3 reaction in peritubular myoid cells of porcine testis but not in seminiferous tubule cells or Leydig cells. In the current study, we did not find any Gal-3 expression in spermatogenic cells of the seminiferous tubule while we detected an immunoreaction in only peritubular myoid cells and Sertoli cells. We also detected a weak immunoreaction in Leydig cells but very intense in macrophages (Figs. 6, 7 and 9). Khorsandi and Orazizadeh (2011) reported that expression of Gal-3 in the cytoplasm of spermatogenic cells may act as a mechanism that inhibits apoptosis of germ cells caused by changes in both testosterone production and environmental toxic agents. But our findings do not support this result.

Gal-3 can be found in the nucleus, cytoplasm, cell surface or extracellular fluid depending on the cell type and proliferative stage. It has different functions based on intracellular or extracellular expression (Hughes, 1999; Krześlak and Lipińska, 2004; Kim et al., 2006). We

agree that this molecule may have a multifunctional role since we observed Gal-3 positivity in both the nucleus and cytoplasm of peritubular myoid cells and macrophages (Fig. 6). However, an immunopositive reaction was only present in the cytoplasm of Leydig and Sertoli cells (Figs. 7 and 9).

In the prepubertal period, Gal-3 immunoreaction was observed in macrophages but not in Sertoli cells (Fig. 6). However, an increasing expression was detected in Sertoli cells in the postpubertal period. Furthermore, the immunoreaction was strong in peritubular myoid cells but weak in Leydig cells (Fig. 7). Devouassoux-Shisheboran et al. (2006) explained that this may result from mature and differentiated Sertoli cells being susceptible to androgenic hormones. Therefore, we support their results and suggestions.

Testicular macrophages have morphological and functional associations with Leydig cells (Hutson, 2006), and play an important role in the development of Leydig cell and regulation of steroidogenesis in adults (Koeva, 2003). These macrophages have an effect on spermatogenesis through some of the mediators they secrete (Beyaz et al., 2009). In our study performing CD68 staining to identify macrophages, we observed that macrophages and Leydig cells are localized close to each

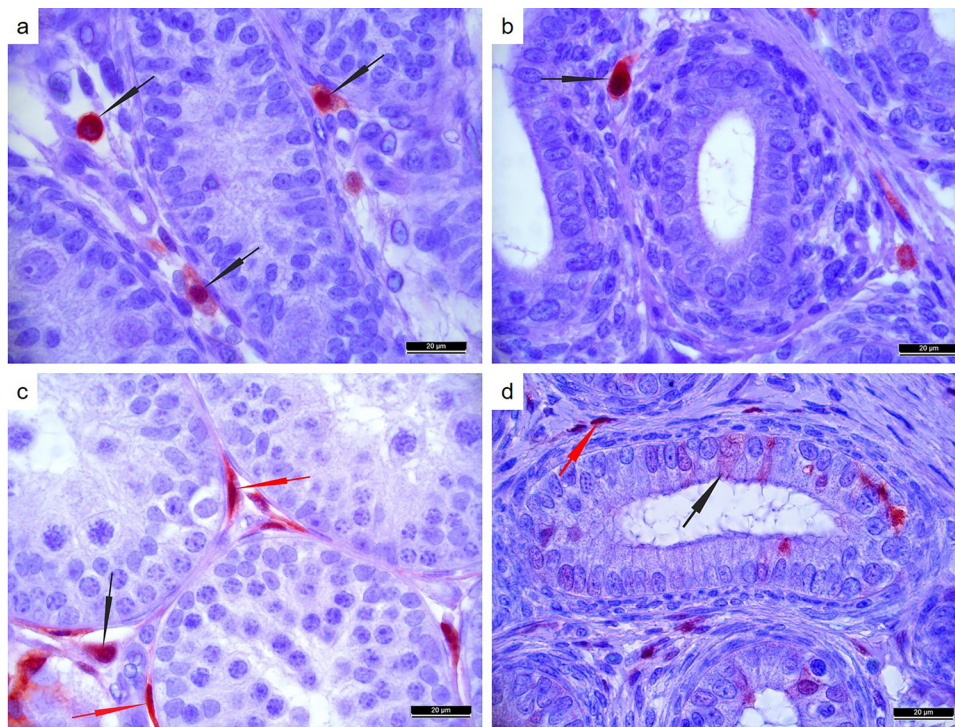


Fig. 6. Gal-3 immunoreactivity in the testis and epididymis of rats on postnatal days 5 (a, b) and 20 (c, d). Strept-ABC, AEC. Positive immunolabeling was present in: (a) nucleus and cytoplasm of macrophages (arrows) in the testis of five-day-old rats, (b) nucleus and cytoplasm of macrophages (arrow) in the epididymis of five-day-old rats, and (c) nucleus and cytoplasm of peritubular myoid cells (red arrows) and macrophages (black arrow) in the testis of 20-day-old rats. Bar: 20 µm. (d) In the epididymis of 20-day-old rats, some epithelial cells (black arrow) showed a weak positive immunoreaction while macrophages (red arrow) showed a positive immunoreaction; Bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

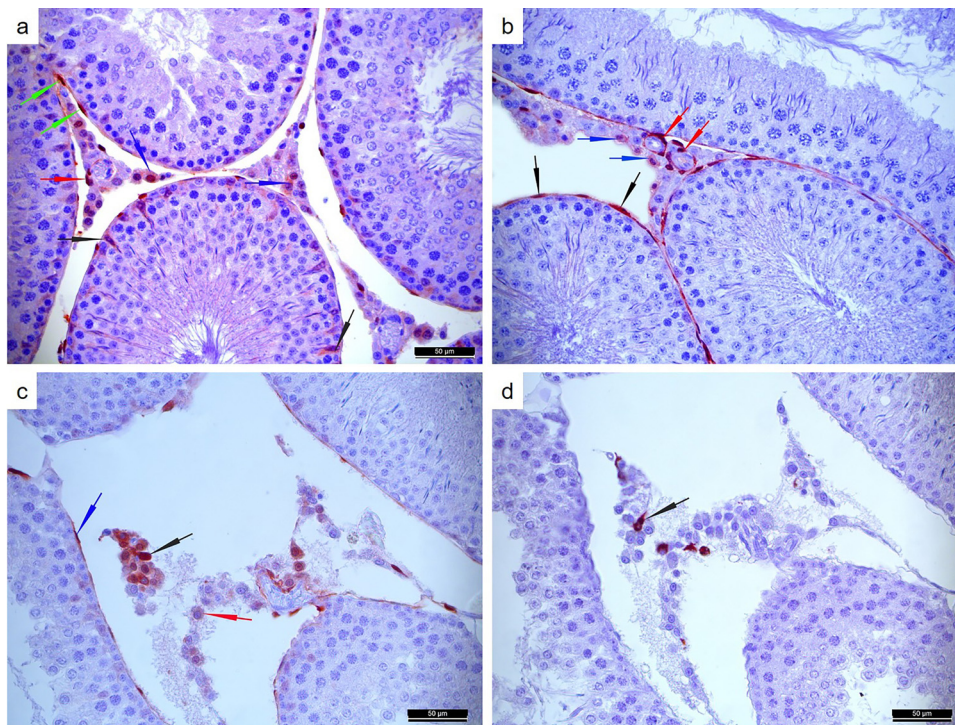


Fig. 7. Gal-3 immunoreactivity in the testis of rats on postnatal day 50 (a, b). Comparison of Gal-3 (c) and CD68 (d) staining in serial testicular sections. Strept-ABC, AEC. (a) Positive immunoreaction in peritubular myoid cells (green arrow), macrophages (red arrow), Leydig cells (blue arrows), and Sertoli cells (black arrows); Bar: 50 µm. (b) Positive immunoreaction in the peritubular myoid cells (black arrows), macrophages (red arrows), and Leydig cells (blue arrows); Bar: 50 µm. (c) Gal-3-positive immunoreaction in peritubular myoid cells (blue arrow), macrophages (black arrow), and Leydig cells (red arrow); Bar: 50 µm. (d) CD68-positive immunoreaction in macrophages (black arrow); Bar: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other. Therefore, these cells are functionally closely related to each other (Fig. 11). However, we cannot explain why macrophages were only positive for Gal-3 while Leydig cells were positive for both Gal-1 and Gal-3.

Some studies showed that Gal-3 has different functions in some immunocytes (Dumic et al., 2006) and intracellular Gal-3 is essential for the survival of macrophages and for performing phagocytic functions (Sano et al., 2003). Based on the strong expression of Gal-3 in both the nucleus and cytoplasm of macrophages in the current study (Figs. 6, 7 and 9), we also agree that this lectin has an important role in

facilitating the functioning of macrophages and protecting against pathogens.

Nurmio et al. (2012) emphasized that peritubular myoid cells play a crucial role in the development and functioning of Sertoli cells in spermatogenesis through their contraction and secretion properties. In the current study, intense Gal-3 expression was seen in peritubular myoid cells (Figs. 6, 7 and 9), suggesting that these cells may be a source of this protein. We also consider that Gal-3 secreted from peritubular myoid cells may somewhat contribute to testicular development in a paracrine fashion.

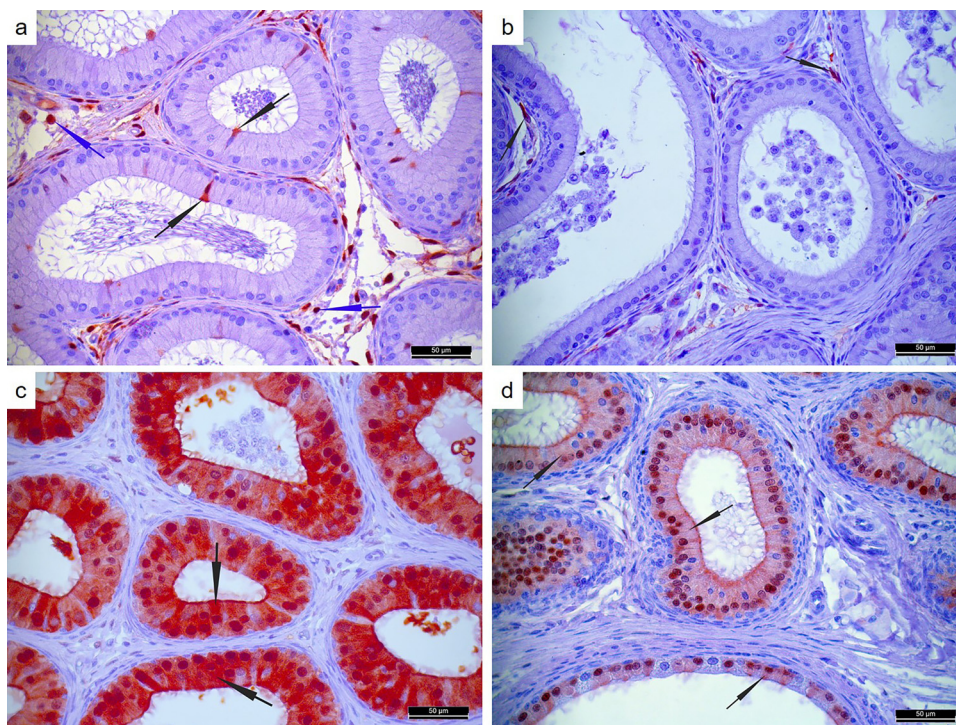


Fig. 8. Gal-3 immunoreactivity in the epididymis of rats on postnatal day 50. Strept-ABC, AEC. Positive immunoreaction in (a) narrow cells (black arrows) and interstitial macrophages (blue arrows) in the initial segment, (b) interstitial macrophages (arrows) in the caput epididymis, (c) epithelial cells in the corpus epididymis (arrows), and (d) epithelial cells in the cauda epididymis (arrows). Bar: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

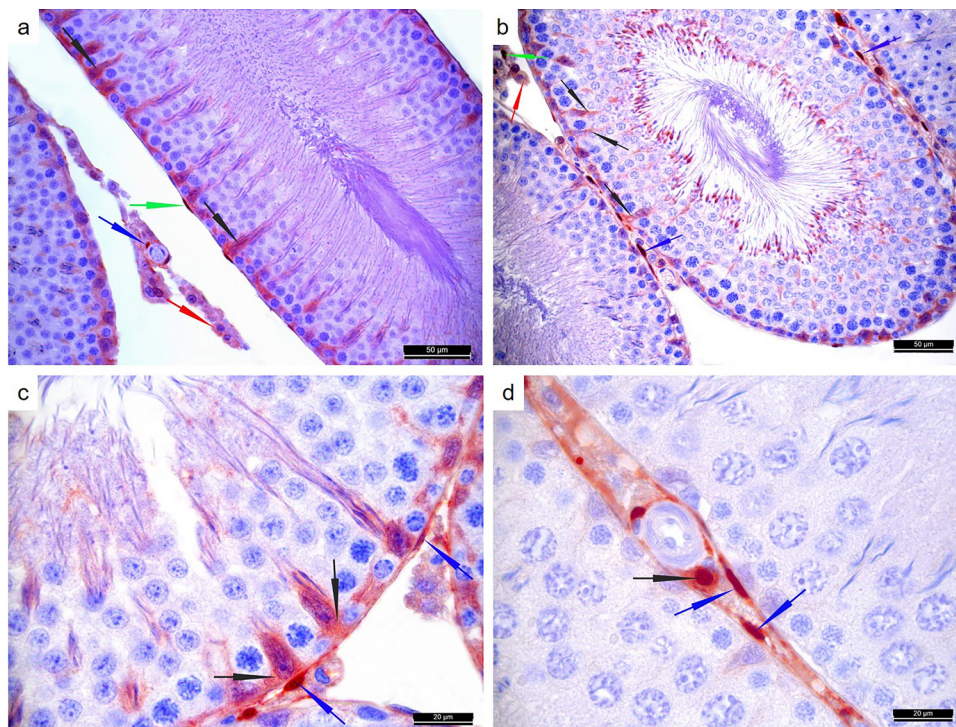


Fig. 9. Gal-3 immunoreactivity in the testis of rats on postnatal day 70. Strept-ABC, AEC. Positive immunolabeling in (a) peritubular myoid cells (green arrow), macrophages (blue arrow), Leydig cells (red arrow), and perinuclear region of Sertoli cells (black arrows), Bar: 50 µm; (b) peritubular myoid cells (blue arrows), macrophages (green arrow), Leydig cells (red arrow), and apical of Sertoli cells (black arrows); Bar: 50 µm, (c) peritubular myoid cells (blue arrows) and perinuclear region of Sertoli cells (black arrows), Bar: 20 µm; and (d) nucleus and cytoplasm of peritubular myoid cells (blue arrows) and macrophages (black arrow), Bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The epididymis gives motility and fertility to a spermatozoon. Mammalian epididymis is macroscopically divided into three different regions; caput, corpus, and cauda. The first two regions are concerned with the maturation of spermatozoa and the last one is responsible for their storage. The ductus epididymis of rats is covered by epithelial cells consisting of two main cell types; principal and basal cells, as well as other less commonly seen apical, narrow and halo cells (Nio et al., 2005). Pöllänen and Cooper (1994) identified immunocytes in the epididymis of many species according to the protective role of this organ. In rats, Deschildre et al. (2007) reported an increase in testicular

Gal-3 expression between the 14th and 20th postnatal days and a decrease after this period. In our study, we observed that expression of Gal-3 in the testis increased from the prepubertal to mature period. While there was no statistically significant difference between the expression of Gal-3 in the testis and epididymis until puberty, Gal-3 was higher in the epididymis in the postpubertal and mature periods. Furthermore, the expression of Gal-3 in the corpus and cauda epididymis was higher than in the caput region during the indicated periods (Fig. 13). Deschildre et al. (2007) have suggested that the increase in the expression of Gal-3 in the testis after puberty may be due to

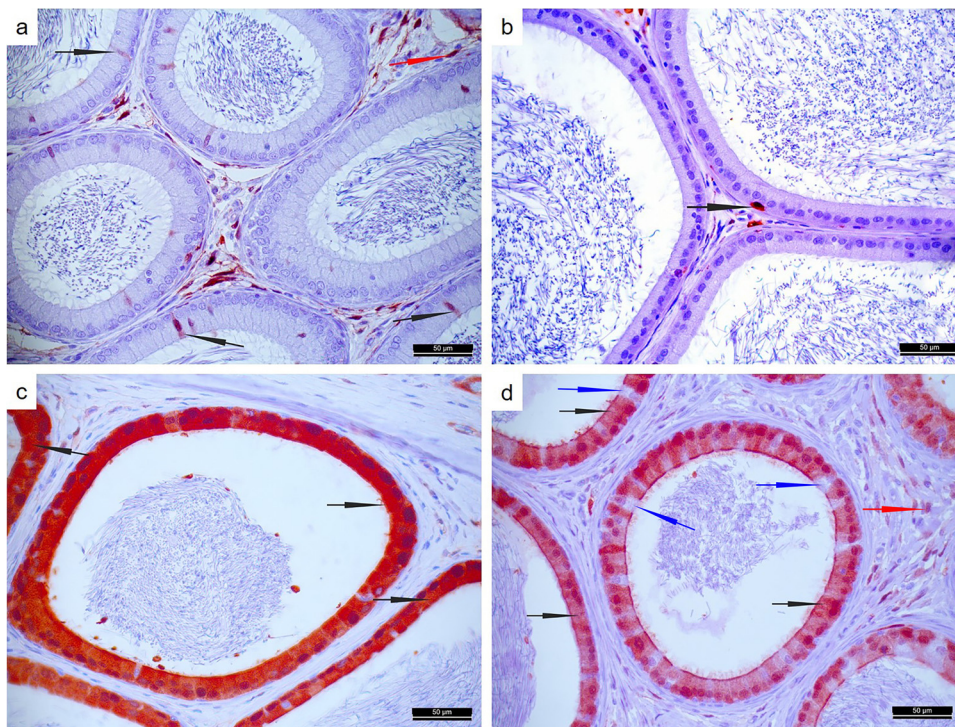


Fig. 10. Gal-3 immunoreactivity in the epididymis of rats on postnatal day 70. Strept-ABC, AEC. Positive immunoreaction in (a) narrow cells (black arrows) and interstitial macrophages (red arrow) in the initial segment, (b) macrophages in the epithelium of the caput epididymis, (c) epithelial cells in the corpus epididymis (arrows), and (d) the majority of epithelial cells in the cauda epididymis (black arrows) with some cells occasionally showing a negative immunoreaction (blue arrows); Bar: 50 µm. A positive immunoreaction in the connective tissue macrophages (red arrow); Bar: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

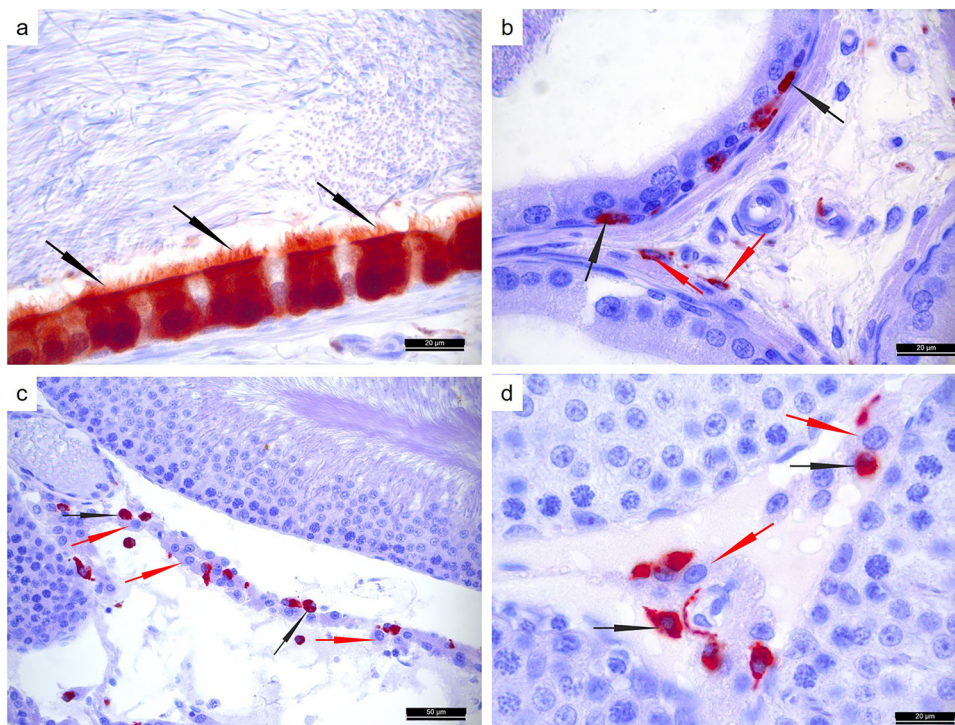


Fig. 11. CD68 immunoreactivity in the testis and epididymis, and Gal-3 immunoreactivity in the corpus epididymis of rats on postnatal day 70. Strept-ABC, AEC. (a) Gal-3 immunoreaction in cilia in the corpus epididymis; Bar: 20 µm. (b) CD68-positive macrophages in the epithelium (black arrows) and connective tissue (red arrows) in the caput epididymis; Bar: 20 µm. (c) CD68-positive macrophages (black arrows) located close to Leydig cells (red arrows) in the testis interstitium; Bar: 50 µm. (d) CD68-positive macrophages (black arrows) located close to Leydig cells (red arrows) in the testis interstitium; Bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hormones such as FSH and testosterone. They also reported that other local signaling factors (e.g., TNF- α and epidermal growth factor) may play a role in Gal-3 expression (Deschildre et al., 2007). We also consider that expressional increase may be due to reproductive hormonal changes.

Kim et al. (2006) detected intense Gal-3 expression in the porcine caudal epididymis and moderate Gal-3 expression in other regions and reported that the presence of this protein in the cauda epididymis contributes to the maturation and activation of spermatozoa. Another study found that Gal-3 immunoreactivity was stronger in mature bull

testis than in immature bull testis (Kim et al., 2008a). Similarly, we observed intense Gal-3 expression in the corpus and cauda epididymis (Figs. 8 and 10); therefore, we agree that this protein plays a role in the maturation and activation of spermatozoa and may also be involved in the mucosal defense system (Kim et al., 2006, 2007; Kim et al., 2008a).

In bulls, Gal-3 expression was observed in the interstitial cells of immature and mature testes, peritubular myoid cells, principal and basal cells in ductus epididymis, and spermatozoa (Kim et al., 2008a). In a study on human, rat and porcine testes, Deschildre et al. (2007) reported Gal-3 expression not only in Leydig cells but also in Sertoli

Table 3
Evaluation of immunoreaction positivity for Gal-3 in the postnatal development stages of the rats.

Tissue		Day 5	Day 20	Day 50	Day 70
Cell type					
Testis	Spermatogonium	0 (Gonocyte)	0	0	0
	Spermatocyte/spermatid	0 (Gonocyte)	0	0	0
	Sperm	0	0	0	0
	Sertoli cells	0	0	+	+++
	Leydig cells	0	0	+	+
	Interstitial macrophages	+++	+++	+++	+++
	Peritubular myoid cells	0	+++	+++	+++
	Vessel wall muscles	0	0	0	0
Epididymis					
Initial segment	Principal cells	Columnar cells 0	Columnar cells +	0	0
	Narrow cells	No differentiation	0	+++	+++
	Basal cells	No differentiation	No differentiation	0	0
	Intraepithelial macrophages	No differentiation	+++	+++	+++
	Apical cells	No differentiation	0	0	0
	Interstitial macrophages	+++	+++	+++	+++
	Vessel wall muscles	0	0	0	0
	Muscle layer in the ductal wall	0	0	0	0
Caput epididymis	Principal cells	Columnar cells 0	Columnar cells +	0	0
	Narrow cells	No differentiation	NONE	NONE	NONE
	Basal cells	No differentiation	No differentiation	0	0
	Intraepithelial macrophages	No differentiation	+++	+++	+++
	Apical cells	No differentiation	No differentiation	0	0
	Interstitial macrophages	+++	+++	+++	+++
	Vessel wall muscles	0	0	0	0
	Muscle layer in the ductal wall	0	0	0	0
Corpus epididymis	Principal cells	Columnar cells 0	Columnar cells +	+++	+++
	Narrow cells	No differentiation	NONE	NONE	NONE
	Basal cells	No differentiation	No differentiation	+++	+++
	Intraepithelial macrophages	No differentiation	+++	+++	+++
	Apical cells	No differentiation	No differentiation	0	0
	Interstitial macrophages	No differentiation	No differentiation	0	0
	Vessel wall muscles	+++	+++	+++	+++
	Muscle layer in the ductal wall	0	0	0	0
Cauda epididymis	Principal cells	0	0	0	0
	Principal cells	Columnar cells 0	Columnar cells +	++	++
	Narrow cells	No differentiation	NONE	NONE	NONE
	Basal cells	No differentiation	No differentiation	++	++
	Intraepithelial macrophages	No differentiation	NONE	NONE	NONE
	Apical cells	No differentiation	No differentiation	0	0
	Interstitial macrophages	+++	+++	+++	+++
	Vessel wall muscles	0	0	0	0
	Muscle layer in the ductal wall	0	0	0	0

Staining intensity: 0 negative (no staining even at high magnification), + weak (only visible at high magnification), ++ moderate (readily visible at low magnification), +++ strong (strikingly positive at low power magnification) staining.

cells; however, Kim et al. (2008a) did not detect Gal-3 expression in mature Sertoli cells in bulls using immunohistochemistry. Since Gal-3 expression was more intense in the testis of mature animals, the researchers concluded that the cellular activity of Gal-3 was hormone-dependent. According to the present findings, Gal-3 was strongly expressed particularly in the corpus and cauda epididymis, as well as in narrow cells of the initial segment of the caput epididymis (Figs. 8 and 10). Our study suggests, therefore, that this protein has a significant role in the maturation and storage of spermatozoa. On the other hand, narrow and apical cells are involved in the acidification of lumen content. This function is very important in protecting the viability of spermatozoa and their maturation. However, we did not observe an immunoreaction in apical cells despite Gal-3 positivity in the narrow cells (Figs. 8 and 10).

In rodents, Hirai et al. (2010) reported that the only initial segment of the caput epididymis has a very dense fenestrated capillary network and a high level of absorption capacity. Therefore, its 90% is

reabsorbed when testicular fluid is passing through efferent duct and initial segment. However, many fenestrated capillaries are located close to the duct formed a fragile area in the blood-epididymis barrier. Thus, macrophages in the initial segment are involved not only in the surveillance of luminal compartment but the monitoring of circulating antigens as well. Da Silva and Barton (2016) also suggested that the initial segment exhibits intense immunological activity. Its lumen is narrow and has a lower number of spermatozoa. It is, therefore, an ideal place for the direct contact between spermatozoa and mucosal macrophages. This idea is supported by the present study (Fig. 11) that indicates a higher number of macrophages in the initial segment compared to the other regions.

Liguori et al. (2015) found that CD68 protein expression was not solely restricted to the monocyte/macrophage system, but it was also prominent in principal cells of the cranial and caudal segments of the epididymis. This may contribute to the maturation process of spermatozoa. In contrast, we did not detect CD68 expression in epithelial cells

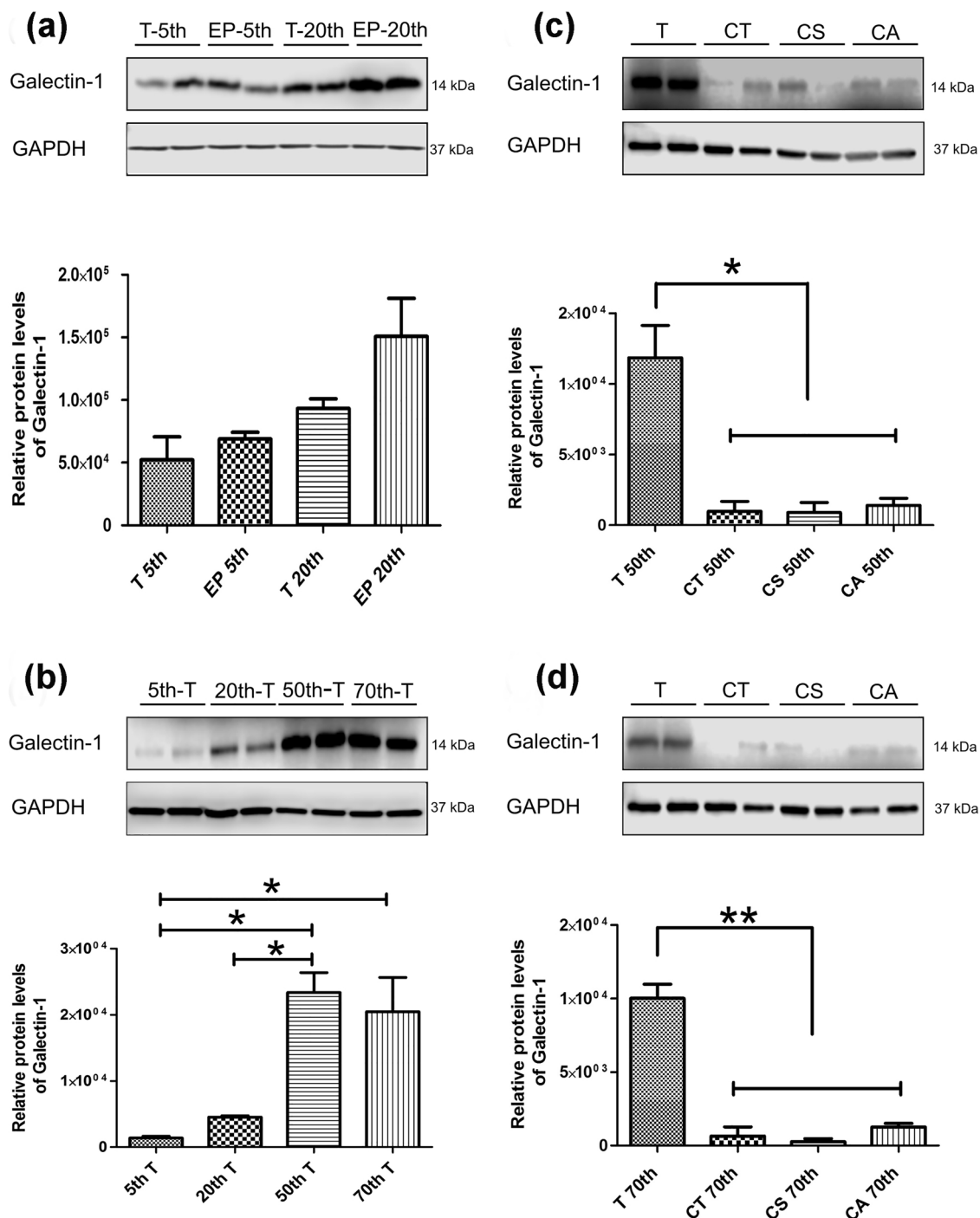


Fig. 12. Representative western blot analyses for Galectin-1 were performed on 5-20-50-70-days old-rats ($n = 5$, 3 replicates/days/testis vs epididymis groups/ with CT-CS-CA/ group, each lane represents 1 rat and 2 rats are shown per group). The relative intensity of each band was quantified by densitometry using the GAPDH as an internal control. The corresponding histogram representing means $\pm$ SEM, of relative arbitrary units of the bands for immunoblots, were conducted with separate experiments in each group and expressed as arbitrary units ("Galectin-1:GAPDH ratio"). T, EP, CT, CS, CA denotes testis, epididymis, caput, corpus, cauda respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

of any region of the epididymis (Fig. 11).

In conclusion, Gal-1 and Gal-3 proteins were differentially expressed in different types of cells in the testis and epididymis during postnatal development. The secretion of both Gal-1 and Gal-3 in Sertoli cells and only Gal-3 in peritubular myoid cells suggests that these two somatic cells may have a key role in testicular immunity. Both galectins play an important role in the protection of the immunity privilege

environment of this organ. Surprisingly, macrophages were not the target cell of Gal-1. CD68 staining showed that macrophages were localized close to Leydig cells. Macrophages were positive for Gal-3 while Leydig cells showed positive for both Gal-1 and Gal-3. Therefore, we consider that both proteins are important for the development of reproductive systems. In particular, the presence of Gal-3 in the corpus and cauda epididymis shows that this protein may play an important

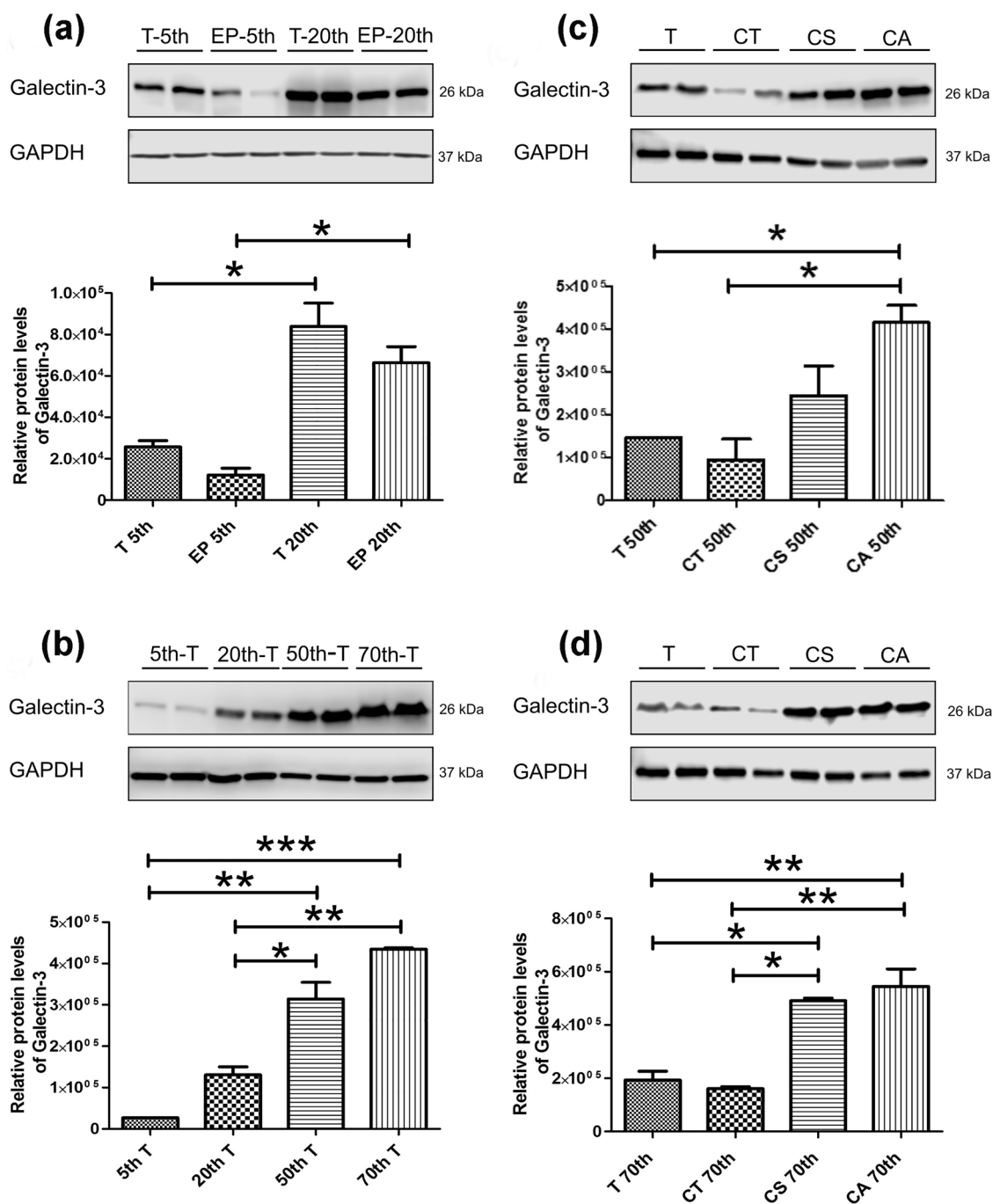


Fig. 13. Representative western blot analyses for Galectin-3 were performed on 5-20-50-70-days old-rats ($n = 5$, 3 replicates/days/testis vs epididymis groups/ with CT-CS-CA/ group, each lane represents 1 rat and 2 rats are shown per group). The relative intensity of each band was quantified by densitometry using the GAPDH as an internal control. The corresponding histogram representing means $\pm$ SEM, of relative arbitrary units of the bands for immunoblots, were conducted with separate experiments in each group and expressed as arbitrary units ("Galectin-3:GAPDH ratio"). T, EP, CT, CS, CA denotes testis, epididymis, caput, corpus, cauda respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

role in the maturation and storage of spermatozoa.

Funding

This study was financially supported by Scientific Research Council of Ankara University (Project No: 15B0239004).

Disclosure information

No potential conflicts of interest were disclosed.

Authors' contribution

Conceived of and designed the experiments: MÖ, EE, LE. Performed the experiments: MÖ, MH, NY, ÖÖ, NK, FB, HA. Analyzed the data: MÖ, EE, MH. Contributed reagents/materials/analysis tools: MÖ, MH, EE, FB, LE. Wrote the paper: MÖ, EE, MH.

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