

Expression profile of Toll-like receptor 4 in rat testis and epididymis throughout postnatal development

Mehmet Özbek¹ | Mustafa Hitit² | Emel Ergün³ | Levent Ergün³ | Feyzullah Beyaz⁴ | Füsün Erhan³ | Nuh Yıldırım³ | Banu Kandil³ | Özge Özgenç³ | Erdoğan Memili⁵

¹Department of Histology and Embryology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Turkey

²Department of Genetics, Faculty of Veterinary Medicine, Kastamonu University, Kastamonu, Turkey

³Department of Histology and Embryology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

⁴Department of Histology and Embryology, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkey

⁵Department of Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS, USA

Correspondence

Mehmet Özbek, Department of Histology and Embryology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Turkey.
Email: mehmetozbek2@gmail.com

Abstract

Toll-like receptors (TLRs) belonging to pattern recognition receptors are involved in maintaining testicular and epididymal immune homeostasis. The purpose of the current study was to investigate TLR4 expression in rat testis and epididymis throughout postnatal development. Weak staining was detected in peritubular myoid cells and immature Sertoli cells while no staining was observed in gonocytes during prepubertal period. However, TLR4 expression began to appear in spermatocytes in pubertal period and gradually increased in spermatids. An intense staining was observed in steps 5–19 spermatids in post pubertal and mature periods. Similarly, TLR4 expression in the testes steadily increased from pubertal period to mature period. Puberty also caused a significant increase in TLR4 expression in epididymis. TLR4 expression in cauda epididymis was lower as compared to those of other epididymal segments. The majority of epididymal epithelial cells exhibited apical TLR4 expression, whereas basal cells showed intense intracytoplasmic immunoreaction. We detected an intense staining in epididymal smooth muscle cells. The expression levels of TLR4 showed dynamic changes in both spermatogenic cells, and entire testicular and epididymal tissues during postnatal development. These results suggest that TLR4 expression contributes not only to inflammation but also to the development of spermatogenic cells.

KEYWORDS

epididymis, postnatal, rat, testis, TLR4

1 | INTRODUCTION

The testis as an immune-privileged organ has some mechanisms that guard neoantigens on meiotic and post-meiotic germ cells against host immune responses (Meinhardt & Hedger, 2011). During mammalian spermatogenesis, a large number of novel antigens are generated by developing spermatogenic cells after immunocompetence is established (Yule, Montoya, Russell, Williams, & Tung, 1988). In spite of testicular immune privilege, these autoantigens do not trigger harmful immune response within the testes under physiological conditions (Schuppe et al., 2008). The epididymis has also immunoprivileged status but immunosuppression in

the epididymis is not as complete as in the testis (Hedger, 2011b). The adult rat epididymis is divided into four regions including the initial segment, caput, corpus, and cauda, which exhibit region-specific characteristics (Robaire, Hinton, & Orgebin-Crist, 2006). The epididymis has a pseudo-stratified epithelium consisting of apical, principal, narrow, basal, clear, and halo cells. Region- and cell-specific gene expression significantly affects composition of the luminal fluid, which enables the sperm to become matured and conserved throughout the epididymal duct (Cornwall, 2009; Dacheux, Gatti, & Dacheux, 2003).

Inflammatory conditions and microbial infections in the male genital tract account for almost 15.0% of male infertility reasons in

developed countries (Fijak et al., 2018), and this ratio can be much higher in undeveloped countries (Ekwere, 1995). Orchitis and epididymitis are more likely to contribute to male infertility than inflammatory conditions in seminal vesicle glands and prostate (Schuppe et al., 2008). Bacterial infections are the most frequent etiological factors of male genital tract. Researchers showed that lipopolysaccharides (LPS), major components of the outer membrane of Gram-negative bacteria, induced epididymitis (Michel, Pilatz, Hedger, & Meinhardt, 2015; Silva et al., 2018). However, autoimmune orchitis can occur under certain pathological conditions, such as physical trauma, and exposure to chemical toxins or high temperature that may damage germ cells (Schuppe et al., 2008). Research on the importance of the Toll-like receptors (TLRs), inflammatory cytokines and their signaling pathways in testicular function have shed new lights on this enigmatic connection between testicular dysfunction and inflammation/infection (Hedger, 2011a). To date, 10 functional TLRs (TLR1–10) have been detected in humans, and additional three mammalian TLRs (TLR11–13) have been identified in rodents (Roach et al., 2005). Of the TLR family, TLR4 is the most studied member (Takeuchi & Akira, 2010). TLR4 detects endogenous danger-associated molecular patterns such as high mobility group box 1 and heat shock proteins. In addition, TLR4 recognizes exogenous pathogen associated molecular patterns including lipopolysaccharide (LPS), as well as microbiome/microbe associated molecular patterns (Akira & Takeda, 2004). TLRs exert functions through binding their ligands which results in a conformational change in TLRs followed by the recruitment of Toll/IL-1 receptor-domain-containing adaptors, including Toll/IL-1 receptor-domain-containing adaptor protein-inducing interferon (TRIF) and the myeloid differentiation protein 88 (MyD88). While TLR4 activates MyD88 and TRIF-dependent pathways, stimulation of TLR3 agonist induces the TRIF-dependent pathway. These two pathways activate IFN regulatory factor 3 to induce the expression of nuclear factor- κ B (NF- κ B) and type 1 IFN to trigger the generation of various proinflammatory cytokines including IL-1, IL-6, and TNF- α (Kawai & Akira, 2006).

In mammals, the blood-testis barrier is established by specialised cell junctions between adjacent Sertoli cells. This barrier starts to assemble by postnatal day 15 (PND15) to PND16 in rat testis and it is completed by PND18 to PND21 (Toyama, Ohkawa, Oku, Maekawa, & Yuasa, 2001). This coincides with the time that immature Sertoli cells become mature and they stop dividing in rat testis (Orth, 1982). The epididymis is fully functional after undergoing morphological changes that take place during its postnatal development, especially before puberty (Sun & Flickinger, 1979). There is a lack of knowledge in TLR4 expression in the testis and epididymis during postnatal development. Better understanding dynamics of TLR4 expression in healthy rat testis and epididymis during postnatal development is vitally important for both fundamental science and clinical pathogenesis of orchitis and epididymitis induced by LPS and endogenous danger-associated molecular patterns. For this purpose, we here sought to examine TLR4 expression in rat testis and epididymis throughout postnatal

development by using western blotting and immunohistochemistry methods.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The maintenance of all animal experimentation in the current study was approved by Department of the Experimental Animals Ethics Committee, at Ankara University. The epididymal and testicular tissues from Wistar albino rats were used in the current study. Twenty-four male rats were obtained from Experimental Research and Application Center of Ankara University. To determine the localization and expression characteristics of TLR4 in the testis and epididymis during the postnatal development, the rats were allocated into four age groups as follows: prepubertal rats (PND5, $n = 6$), pubertal rats (PND20, $n = 6$), post pubertal (PND50, $n = 6$), and mature rats (PND70, $n = 6$), with mean weights of 1,084, 4,255, 12,962 and 28,523 g, respectively. The animals were housed in a temperature-controlled room (20–24°C) with an artificial 12-hr light/dark cycle and fed a standard diet and given water ad libitum. Rats were decapitated 10–15 min following an intraperitoneal injection of 100 mg/kg ketamine (88.9 mg of ketamine/ml) with 10 mg/kg xylazine hydrochloride (11.1 mg of xylazine/ml). The samples were harvested for western blotting and immunostaining. For western blotting, each epididymis was gently separated from the testis. Whole epididymis was used for both western blotting in prepubertal and pubertal rats while caput, corpus and cauda regions of epididymis dissected under stereomicroscope were used in post pubertal and mature rats. In addition, whole testes were used for western blotting in all experimental groups. For immunohistochemistry, we did not separate the epididymis from the testis in all experimental groups. While the samples were kept at -80°C until used for western blotting, they were fixed in Bouin's solution for immunohistochemistry. Following the fixation, the samples were kept in 70% alcohol for 12–15 hr and passed through ascending series of alcohol. Following dehydration, the tissues were incubated in methyl benzoate and benzene solutions and embedded in paraplast.

2.2 | Immunohistochemistry

To detect TLR4 location in testicular and epididymal tissues, we applied immunostaining as described previously (Özbek et al., 2019). Briefly, 5- μm thick sections were placed on slides. The samples were dewaxed and kept in a graded alcohol. The slides were then rinsed in PBS and transferred into 3% H_2O_2 for 20 min. Then, the sections were transferred to citrate buffer (10 mM, pH 6.0) for antigen retrieval, and boiled for 20 min. Then, the slides were encircled using hydrophobic pap pen and treated with 10% goat serum blocking solution for 10 min. Following that, the sections

were incubated with primary antibodies (#NB100-56566, Novus Biologicals) at +4°C overnight. Then, the sections were treated with secondary antibody for 30 min. After rinsing in PBS, the sections were treated with horseradish peroxidase-conjugated streptavidin for 30 min. The 3-amino-9-ethylcarbazole was used as chromogen to visualize the stained and counterstained sections with Gill's hematoxylin II.

To validate primary antibody specificities, nonimmune rabbit serum and PBS were applied instead of the primary antibody for isotype and negative control, respectively. The other immunostaining steps were performed using the same procedures. The negative control slides did not show any nonspecific staining (Figure 1a). In addition, rat colon tissue was used as positive control. Consistent with previous study (Price et al., 2018), intestinal epithelium and some interstitial cell show positive immunoreaction (Figure 1b).

2.3 | Semi quantitative analysis of TLR4 immunostaining

The sections were analyzed using a research microscope (BX51, Olympus), and the slides were imaged with a digital camera (DP74, Olympus). The degree of immunostaining was determined as either negative (-), weak (+), moderate (++) or intense (+++) by two independent observers (MÖ, EE), as previously described (Özbek et al., 2018). Semi-quantitative analysis of TLR4 immunostaining in rat testis and epididymis during postnatal development is presented in Table 1.

2.4 | Western blot analysis

2.4.1 | Protein extraction

Protein extraction was carried out as previously described (Özbek et al., 2018). Briefly, snap-frozen epididymal and testicular tissue samples harvested from each animal were homogenized in cell lysis solutions (# 17081, PRO-PREPTM) containing protease inhibitor cocktail (#Cell Signaling Technology) by using an ultrasonic homogenizer. Following sonication and homogenization, the lysates were centrifuged at 13,000 × g for 25 min at 4°C, and the supernatant was placed into new tubes for final collection after centrifugation at 10,000 × g for 15 min. By using Quick Start Bradford Protein Assay (Bio-Rad Laboratories), we detected the total protein concentrations.

2.4.2 | Western blotting

The proteins (25 µg) from each group were separated in 10% SDS-PAGE-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (PVDF, #1620177, Biorad). Following the transfer, the PVDF membrane (#1620177, Biorad) was blocked in % 5 skimmed milk and treated with primary antibody (#NB100-56566, Novus Biologicals) overnight at 4°C followed by washing. After this step, the

PVDF membrane (#1620177, Biorad) was incubated with goat anti rabbit- (#sc-2004, polyclonal, Santa Cruz Biotechnology) horseradish peroxidase conjugated secondary antibody for two hours, and rinsed in 0.1% Tween-20, 10 mM Tris-HCl pH 7.4, 150 mM NaCl. The TLR4 protein was imaged using chemiluminescence by Clarity™ Western ECL Substrate. For the internal control, we applied GADPH and the TLR4 signal intensities were measured using Image Studio™ Lite (LICOR).

2.5 | Statistical analysis

GraphPad Prism 5 was employed for the data analysis. All of the data were given as mean ± SEM. Two-tailed Student's *t*-test was used to compare two groups (testis and epididymis). Comparison of groups (comparison between 5th, 20th, 50th and 70th, and between testis and epididymal regions [caput (CT), corpus (CS), cauda (CA)] were performed using One-Way Factorial Analysis of Variance (ANOVA), with Tukey's multiple comparison test. *p* < .05 was deemed significant.

3 | RESULTS

3.1 | Immunohistochemistry

3.1.1 | TLR4 immunostaining in testis and epididymis of prepubertal rats

A weak staining in peritubular myoid cells and immature Sertoli cells was detected while no staining was observed in gonocytes in the lumens of the seminiferous tubule. In interstitial spaces, we found an intense labelling in some blood cells and vessel walls but moderate staining in some interstitial cells (Figure 1c). Moreover, epithelium and smooth muscle cell of the epididymis showed a strong staining (Figure 1d).

3.1.2 | TLR4 immunostaining in testis and epididymis of pubertal rats

We found a weak labelling in primer spermatocytes (leptoten/zygote, pakiten), and mature Sertoli cells but a moderate staining in peritubular myoid cells and some interstitial cells. However, an intense staining was observed in some blood cells and vessel walls (Figure 1e). While majority of the cells in epididymal epithelium showed apical staining, an intense intracytoplasmic staining was detected in basal cells (Figure 1f).

3.1.3 | TLR4 immunostaining in testis and epididymis of post pubertal rats

We could distinguish 14 stages of the seminiferous epithelial cycle in this period. Interestingly, we did not observe immunostaining

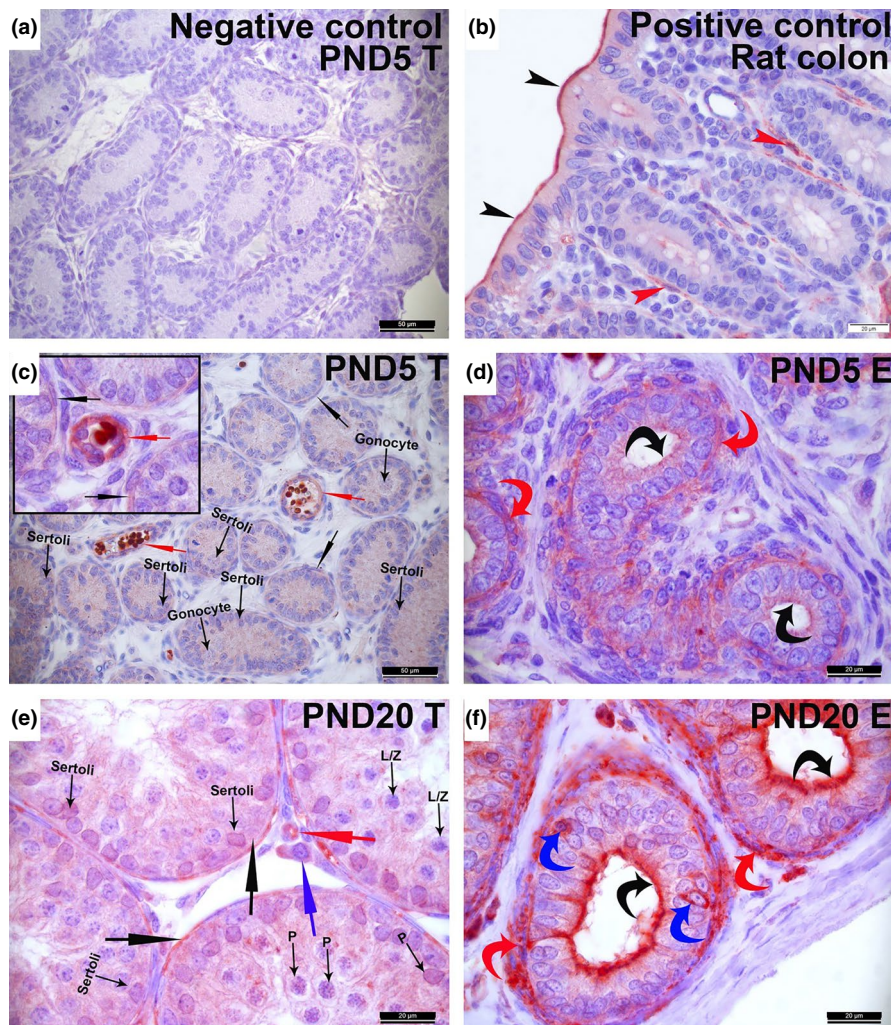


FIGURE 1 Representative images showing negative control (a), positive control (b) and TLR4 expression in testis and epididymis on PND5 (c, d) and PND20 (e, f). TLR4 immunostaining in intestinal epithelium (black arrowheads) and some interstitial cells (red arrowheads) of rat colon. In the PND5, a weak staining in peritubular myoid cells (black arrows) and immature Sertoli cells. No staining in gonocytes in seminiferous tubule lumen. An intense labelling in some blood cells and vessel walls (red arrows). An intense staining in epithelium (black curved arrows) and smooth muscle cells (red curved arrows) of the epididymis. In the PND20, a weak labeling in primer spermatocytes (L/Z; leptotene/zygotene, P; pachytene), and mature Sertoli cells but a moderate staining in peritubular myoid cells (black arrows), and some interstitial cells (blue arrows). An intense apical staining in epididymal epithelium (black curved arrows) and smooth muscle cells (red curved arrows) of the epididymis. An intense intracytoplasmic staining in basal cells (blue curved arrows). Bars: 50 μ m (a, c) and 20 μ m (b, d, e, and f)

in spermatogonia and basal part of the Sertoli cells. While a weak staining was detected in peritubular myoid cells, primary and secondary spermatocytes, and round spermatids in steps 1–4, an intense staining was observed in steps 5–19 spermatids. Furthermore, we found a moderate labelling in some interstitial cells but an intense staining in vessel walls and some blood cells (Figure 2a–d). In all the epididymal segments, we also observed weak to moderate staining in the apical part of the epithelial cells. Interestingly, basal cells of epididymal epithelium showed an intense intracytoplasmic labelling in all of the epididymal segments. Epididymal smooth muscle cells were also strongly positive for TLR4 in all the epididymal segments. In addition, we detected an intense staining in vessel walls and some blood cells in all epididymal interstitia (Figure 3a–d).

3.1.4 | TLR4 immunostaining in testis and epididymis of mature rats

As in the postpubertal period, we did not detect a staining in spermatogonia and basal part of Sertoli cells. However, we observed weak to moderate staining in peritubular myoid cells, primary and

secondary spermatocytes, and round spermatids in steps 1–4. Staining intensity in steps 5–19 spermatids increased compared to those of the post pubertal period. As in the post pubertal period, we found a moderate labelling in some interstitial cells but an intense staining in vessel walls and some blood cells (Figure 4a–i). In all of the epididymal segments, we also detected weak to moderate staining in apical part of the epithelial cells. As in the post pubertal period, the basal cells of epididymal epithelium showed an intense intracytoplasmic labelling in all the epididymal segments. In addition, we found an intense labelling epididymal smooth muscle cells, vessel walls and some blood cells in all epididymal interstitium (Figure 5a–d).

3.2 | Western blotting

In the prepubertal period, there was no difference in TLR4 expression in testis and epididymis. However, TLR4 expression in epididymis was higher than that in the testis in pubertal period ($p > .05$) (Figure 6a). The TLR4 expression in the testis increased from the prepubertal period to the mature period (Figure 6b). In the post pubertal period, we did not observe any differences in

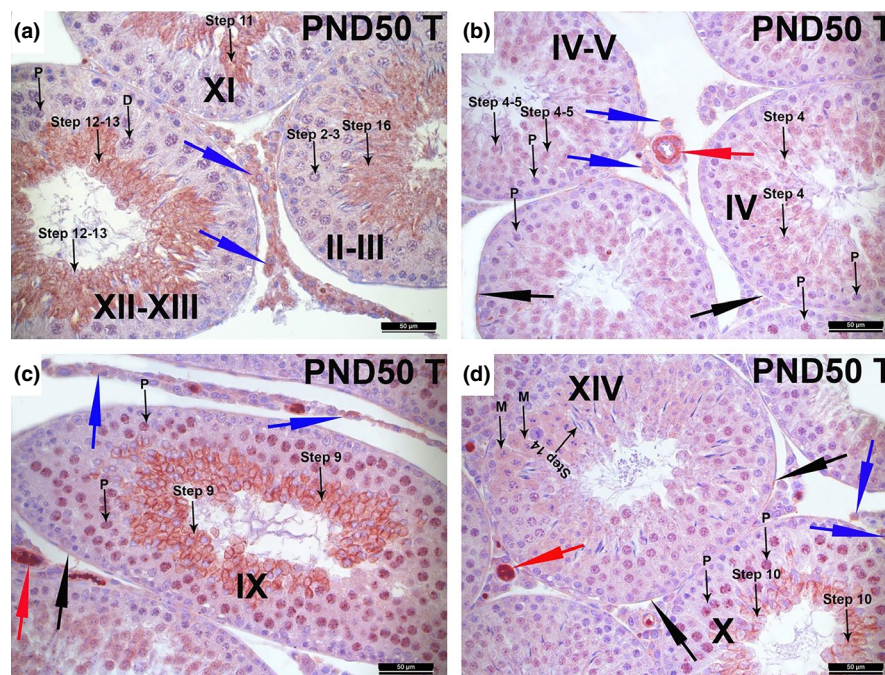
TABLE 1 Semi quantitative analysis of TLR4 immunostaining in rat testis and epididymis during postnatal development

Tissue	Cell type	PND5	PND20	PND50	PND70
Testis	Gonocyte	–	0	0	0
	Spermatogonium	–	–	–	–
	Spermatocyte	0	+	+	+
	Spermatid	0	0	Step 1–4 +	Step 1–4 +/++
				Step 5–19 +++	Step 5–19 +++
	Spermatozoon	0	0	+++	+++
	Sertoli cells	+	+	+	+
	Interstitial cells	++	++	++	++
	Peritubular myoid cells	+	++	+	+/++
	Vessel wall muscles	+++	+++	+++	+++
Epididymis	The majority of epididymal epithelial cells	Apical staining	Apical staining	Apical staining	Apical staining
		+++	+++	+/++	+/++
	Basal cells	0	Intracytoplasmic	Intracytoplasmic	Intracytoplasmic
			+++	+++	+++
	Vessel wall muscles	+++	+++	+++	+++
	Muscle layer in the ductal wall	+++	+++	+++	+++

Notes: The degree of immunostaining was determined as either negative (–), weak (+), weak to moderate (+/++), moderate (++) or intense (+++/+++ or intense (+++) by two independent observers (MÖ, EE), as previously described (Özbek et al., 2018). 0: no cell.

Abbreviation: PND, postnatal day.

FIGURE 2 Representative images showing TLR4 expression in different stages of the seminiferous epithelium cycle in testis on PND50. No immunostaining in spermatogonia and basal part of Sertoli cells. While a weak staining was detected in peritubular myoid cells (black arrows), primary and secondary spermatocytes (P; pachytene, D; Diakinesis, M: Meiosis) and round spermatids in steps 1–4, an intense staining was observed in steps 5–19 spermatids. A moderate labelling in some interstitial cell (blue arrows) but an intense staining in vessel walls (red arrows) and some blood cells. Bars: 50 μ m



the TLR4 expression between testis and epididymis (Figure 6c). However, expression levels in the testis and epididymis differed in terms of TLR4 expression in mature period. In mature period, level of TLR4 expression in testis was higher than that in the cauda epididymis ($p > .05$). In addition, the TLR4 expression in the epididymis decreased from the caput to cauda epididymis (Figure 6d).

4 | DISCUSSION

Here, we report a detailed analysis of TLR4 expression in rat testis and epididymis during postnatal development. We found that TLR4 was expressed in different types of cells in the testis and epididymis. We also captured dynamic changes in TLR4 expression levels of testis and epididymis as postnatal development proceeds.

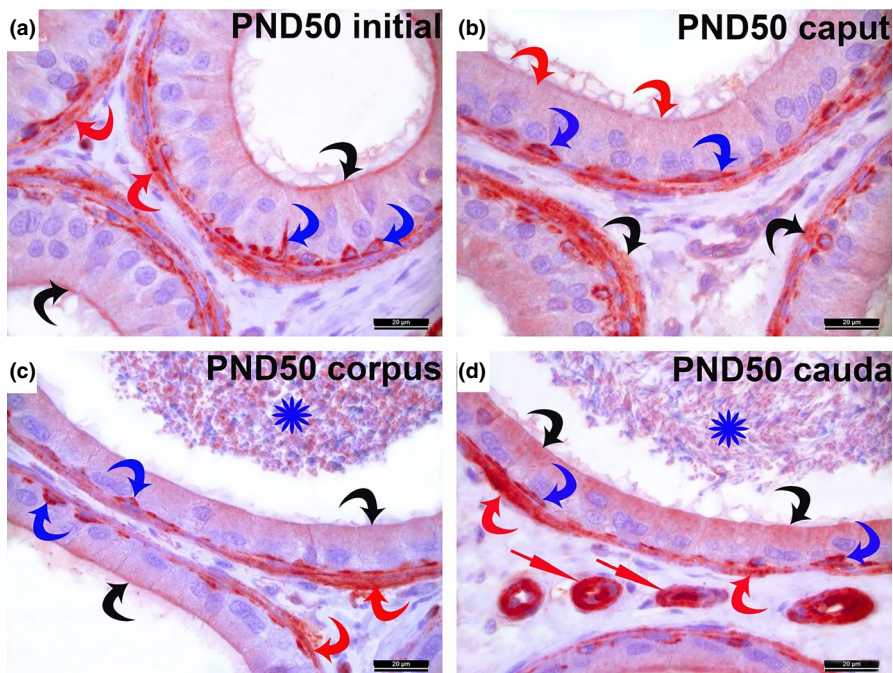


FIGURE 3 Representative images showing TLR4 expression in epididymis on PND50. In all the epididymal segments, a weak to moderate staining in the apical part of epithelial cells (black curved arrows). Basal cells of epididymal epithelium showed an intense intracytoplasmic labelling (blue curved arrows) in all the epididymal segments. Epididymal smooth muscle cells (red curved arrows) were also strongly positive for TLR4 in all the epididymal segments. An intense staining in vessel walls (red arrows) and some blood cells in all epididymal interstitium. An intense staining in luminal spermatozoa (blue asterisk). Bars: 20 µm

Gene expression during spermatogenesis is organized and modulated through transcriptional and posttranscriptional mechanisms. To complete the highly complex development of male germ cells, several specific transcriptional regulators are required (Meikar, Ros, & Kotaja, 2012). In late elongating spermatids, transcription is mostly silenced as a result of chromatin condensation by histone-protamine transition (Tanaka & Baba, 2005). In mice, the transcripts of 1,652 genes significantly increase during or after meiosis (Schultz, Hamra, & Garbers, 2003). Large amounts of active RNA polymerase II are found in round spermatids up to step 3 in humans and up to steps 8–9 in mice (Dottermusch-Heidel et al., 2014). Little is known about TLR4 expression in spermatogenic cells during postnatal development. In the present study, we did not observe immunoreaction in gonocytes and spermatogonia while TLR4 expression appeared in primary spermatocytes. We detected weak to moderate staining in peritubular secondary spermatocytes, and round spermatids in steps 1–4. However, staining intensities were increased in steps 5–19 spermatids. As stated in the previous study (Schultz et al., 2003), the transcripts of TLR4 genes may be activated in spermatogenic cells during meiosis and this may result in increased TLR4 expression in steps 5–19 spermatids. In addition, we also found that puberty had an effect on TLR4 expression in the testis. TLR4 expression in the testis gradually increased from the pubertal period to the mature period.

Previous studies showed the immunological functions of Sertoli cells in activation of testicular immune responses and testicular protection (Riccioli et al., 2006; Starace et al., 2008; Sun et al., 2010). Recent studies have also indicated that Sertoli cells express TLRs (Starace et al., 2008; Winnall, Muir, & Hedger, 2011), and play a significant role in the activation of inflammatory response. Moreover, Sertoli cells are involved in phagocytosis of the spermatid residual bodies (Gérard, Syed, & Jégou, 1992). Hedger (2011a) speculated

that the contents of the released sperm cytoplasm may most likely provoke the response by utilizing pattern recognition receptors, such as the TLRs, found in the Sertoli cells. In the current study, we revealed TLR4 expression in immature and mature Sertoli cells in accordance with previous studies. TLR4 expression of immature and mature Sertoli cells suggest that TLR4 expression contributes not only to inflammation but also to the development of spermatogenic cells.

Testicular interstitial tissue is involved in the first line of defense system against blood-borne pathogens. The immune cells of interstitial tissue, especially macrophages, play crucial roles in the defense against pathogens (Li, Wang, & Han, 2012). Testicular interstitium also contains Leydig cells both fetal and postnatal period. In neonatal period (birth to PND7), fetal Leydig cells are regressed, these cells turn into progenitor cells. In the late infantile period (PND15–PND21), testicular interstitium has progenitor Leydig cells with a smaller population of immature Leydig cells. After PND45, mature Leydig cells are found within interstitial tissue (Picut et al., 2015). Shang et al. (2011) showed that mouse Leydig cells trigger testicular innate immune responses through inducing of TLR3 and TLR4. They also reported that the activation of TLR3 or TLR4 Leydig cells inhibits testosterone synthesis in mice. Considering findings reported in the literature and stained cell morphology, we reason that the majority of the stained cells in the interstitial space were Leydig cells. Interestingly, progenitor, immature and mature Leydig cells are positive for TLR4 immunoreaction. Considering immunosuppression effect of testosterone (Foo, Nakagawa, Rhodes, & Simmons, 2017), we suggest that TLR4 is effective in regulation of steroidogenesis at all stages of the postnatal period.

The appearance and number of individual cell types vary between epididymal segments. The epididymal epithelium at birth is

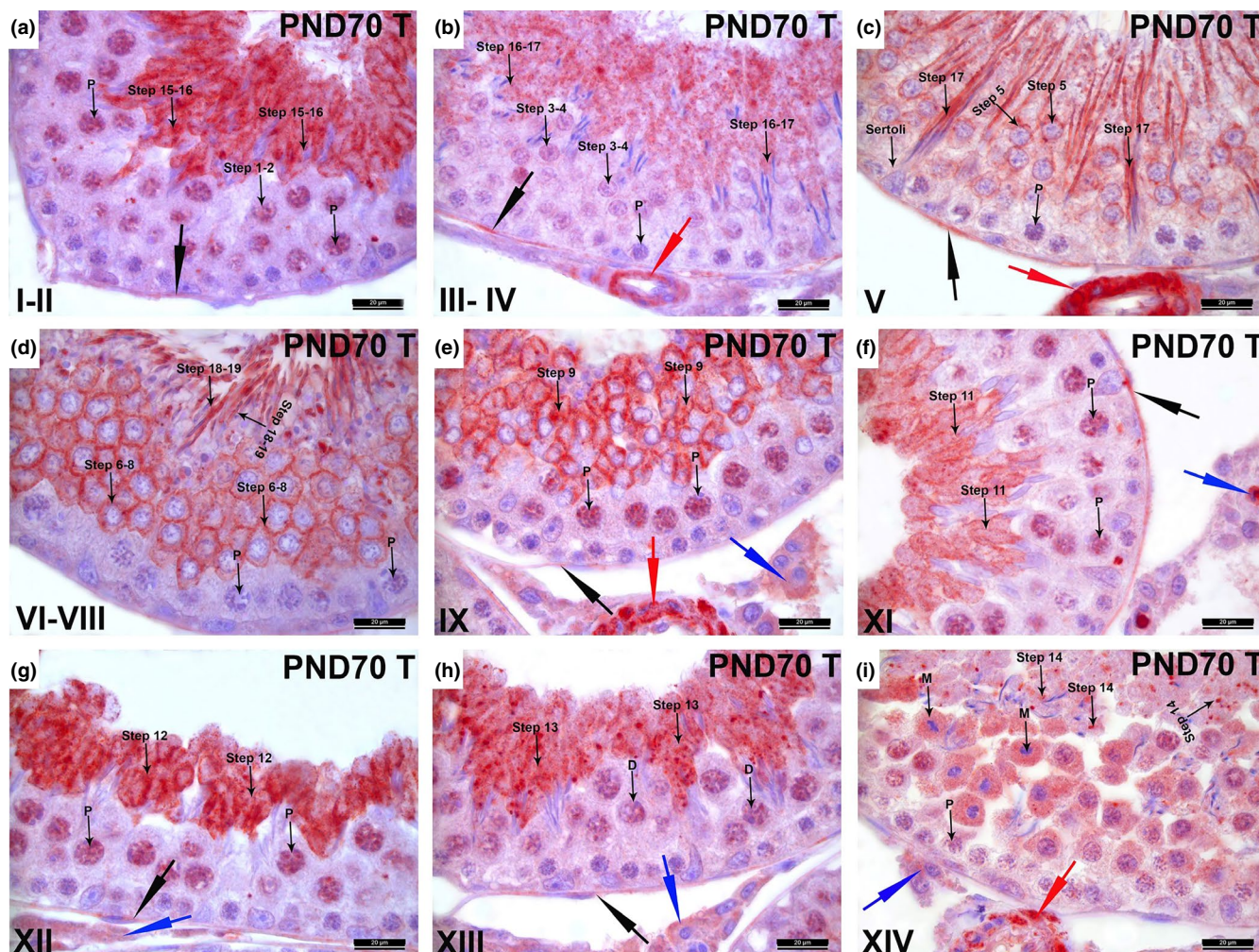


FIGURE 4 Representative images showing TLR4 expression in different stages of the seminiferous epithelium cycle in testis on PND70. No staining in spermatogonia and basal part of Sertoli cells. A weak to moderate staining in peritubular myoid cells (black arrows), primary and secondary spermatocytes (P; pachytene, D; Diakinesis, M: Meiosis) and round spermatids in steps 1–4, an intense staining was observed in steps 5–19 spermatids. A moderate labelling in some interstitial cell (blue arrows) but an intense staining in vessel walls (red arrows) and some blood cells. Bars: 20 μ m

determined by undifferentiated columnar cells in the rat (Dufresne, Finsson, Gregory, & Cyr, 2003; Sun & Flickinger, 1982). This epithelium starts to differentiate around PND16. The basal cell first appears in the cauda epididymis at PND21 and are found throughout the epididymal segments at PND28. They comprise approximately 20% of the total epididymal epithelial cell population (Hermo, Papp, & Robaire, 1994; Sun & Flickinger, 1979) in the adult rat epididymis. Moreover, they may reach the lumen through slender and long cytoplasmic projections (Shum et al., 2008). Studies indicate that basal cells are found in higher number in the corpus epididymis and less numerous towards the cauda epididymis (Arrighi, 2014). Although TLR4 expression in epididymis was investigated before, no information was available on dynamic changes TLR4 expression in rat epididymis during postnatal development. Moreover, reports on TLR4 expression in the rat epididymis are contradictory. Palladino, Savarese, Chapman, Dughi, and Plaska (2008) reported intense TLR4 immunolabeling in epithelial cells from the initial segment to the cauda epididymis in the rats. In mouse epididymis, TLR4 expression

was mainly observed in the basal cells, principal cells, and some interstitial cells (Zhu et al., 2015). In domestic cats, however, TLR4 expression was detected in the apical surface of the epithelial cells with stereocilia. They observed intense TLR4 immunostaining in the apical cytoplasm of the principal cells in regions I and II. They also detected nuclear and cytoplasmic immunolabeling in the narrow cells of the epithelium in region III (Liman, Alan, & Apaydin, 2019). In the current study, we demonstrated an intense staining in apical epithelium and smooth muscle cell of the epididymis in the prepubertal and pubertal periods. In the post pubertal and mature periods, however, we detected weak to moderate staining in the apical part of epithelial cells in all the epididymal segments. Unlike the previous studies, we observed an intense intracytoplasmic labelling in basal cells of all the epididymal segments. We think that this difference is due to methodological errors or to lot-to-lot difference of antibodies. In addition, TLR4 expression significantly increased in the epididymis during pubertal period. We attributed this increase to the appearance of basal cells in the epididymal epithelium. Furthermore,

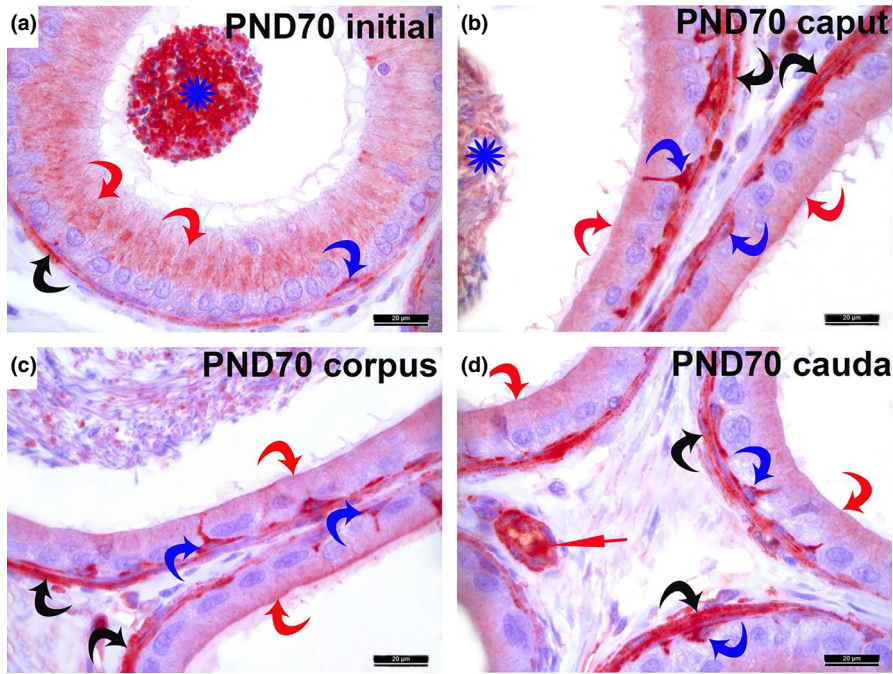


FIGURE 5 Representative images showing TLR4 expression in epididymis on PND70. In all the epididymal segments, a weak to moderate staining in the apical part of epithelial cells (black curved arrows). Basal cells of epididymal epithelium showed an intense intracytoplasmic labelling (blue curved arrows) in all the epididymal segments. Epididymal smooth muscle cells (red curved arrows) were also strongly positive for TLR4 in all the epididymal segments. An intense staining in vessel walls (red arrows) and some blood cells in all epididymal interstitium. An intense staining in luminal spermatozoa (blue asterisk). Bars: 20 µm

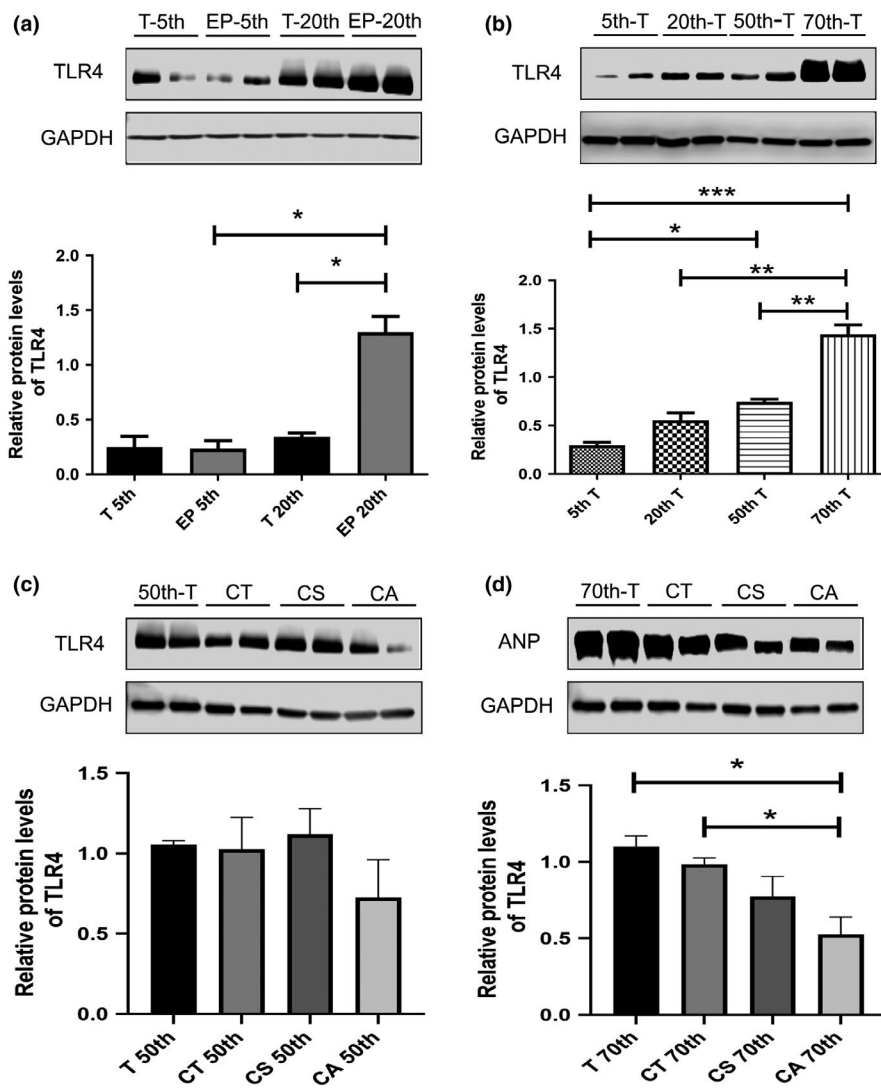


FIGURE 6 Western blot analyses of expression levels of TLR4 in rat testis and epididymis. Tissue samples were harvested from rats on postnatal days 5 (5th), 20 (20th), 50 (50th) and 70 (70th). Lysates from testis (T) and epididymis (E) including caput (CT), corpus (CS) and cauda (CA). Each lane in graph indicates 1 rat and 2 rats per group. Relative band intensity of TLR4 was quantified by densitometry and normalized to GAPDH expression. Data were normalized by dividing arbitrary units of TLR4-GAPDH. The results were presented as means $\pm$ SEM in arbitrary densitometric units ($n = 6$, 3 replicates). * $p < .05$; ** $p < .01$; *** $p < .001$

with the onset of spermatogenesis, this increase in TLR4 expression may be the mechanism by which spermatozoa protect against both pathogenic and autoimmune reactions in the epididymal duct. In the postpubertal and mature periods, TLR4 expression in cauda epididymis was less compared to other epididymal segment. This difference may be due to the fact that basal cells are less numerous towards the cauda epididymis. In addition, we observed that basal cells reached the lumen through their long projections. Therefore, we suggest that basal cells are involved in the recognition of luminal antigens.

In conclusion, TLR4 showed dynamic changes in both spermatogenic cells and entire testicular and epididymal tissues during postnatal development. The TLR4 expression was not detectable in the gonocytes and spermatogonia. However, TLR4 expression began to appear in spermatocytes and gradually increased in spermatids. Similarly, the entire TLR4 expression in the testes steadily increased from pubertal period to mature period. The findings reported in this study are important because TLR4 expression may contribute not only to inflammation but also to the development of spermatogenic cells. Puberty also had a significant effect on the TLR4 expression in epididymis and caused a significant increase in TLR4 expression in epididymis. TLR4 expression in cauda epididymis was less compared to other epididymal segment. The majority of epididymal epithelial cells showed apical TLR4 expression, whereas basal cells showed intense intracytoplasmic immunoreaction. Basal cells may be involved in the immunoregulation of epididymis. The new data and knowledge reported in this study help advance both basic and applied developmental biology and assisted reproductive technologies.

ACKNOWLEDGEMENT

This study was supported by Ankara University Research Fund (Project number: 16H0239005).

REFERENCES

- Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology*, 4, 499–511. <https://doi.org/10.1038/nri1391>
- Arrighi, S. (2014). Are the basal cells of the mammalian epididymis still an enigma? *Reproduction, Fertility and Development*, 26, 1061–1071. <https://doi.org/10.1071/RD13301>
- Cornwall, G. A. (2009). New insights into epididymal biology and function. *Human Reproduction Update*, 15, 213–227. <https://doi.org/10.1093/humupd/dmn055>
- Dacheux, J. L., Gatti, J. L., & Dacheux, F. (2003). Contribution of epididymal secretory proteins for spermatozoa maturation. *Microscopy Research and Technique*, 61, 7–17. <https://doi.org/10.1002/jemt.10312>
- Dottermusch-Heidel, C., Klaus, E. S., Gonzalez, N. H., Bhushan, S., Meinhardt, A., Bergmann, M., ... Steger, K. (2014). H3K79 methylation directly precedes the histone-to-protamine transition in mammalian spermatids and is sensitive to bacterial infections. *Andrology*, 2, 655–665. <https://doi.org/10.1111/j.2047-2927.2014.00248.x>
- Dufresne, J., Finnson, K. W., Gregory, M., & Cyr, D. G. (2003). Expression of multiple connexins in the rat epididymis indicates a complex regulation of gap junctional communication. *American Journal of Physiology-Cell Physiology*, 284, C33–C43. <https://doi.org/10.1152/ajpcell.00111.2002>
- Ekwere, P. D. (1995). Immunological infertility among Nigerian men: Incidence of circulating antisperm auto-antibodies and some clinical observations: A preliminary report. *British Journal of Urology*, 76, 366–370. <https://doi.org/10.1111/j.1464-410X.1995.tb07716.x>
- Fijak, M., Pilatz, A., Hedger, M. P., Nicolas, N., Bhushan, S., Michel, V., ... Meinhardt, A. (2018). Infectious, inflammatory and 'autoimmune' male factor infertility: How do rodent models inform clinical practice? *Human Reproduction Update*, 24, 416–441. <https://doi.org/10.1093/humupd/dmy009>
- Foo, Y. Z., Nakagawa, S., Rhodes, G., & Simmons, L. W. (2017). The effects of sex hormones on immune function: A meta-analysis. *Biological Reviews*, 92, 551–571. <https://doi.org/10.1111/brv.12243>
- Gérard, N., Syed, V., & Jégou, B. (1992). Lipopolysaccharide, latex beads and residual bodies are potent activators of Sertoli cell interleukin-1 α production. *Biochemical and Biophysical Research Communications*, 185, 154–161. [https://doi.org/10.1016/S0006-291X\(05\)80969-6](https://doi.org/10.1016/S0006-291X(05)80969-6)
- Hedger, M. P. (2011a). Toll-like receptors and signalling in spermatogenesis and testicular responses to inflammation—A perspective. *Journal of Reproductive Immunology*, 88, 130–141. <https://doi.org/10.1016/j.jri.2011.01.010>
- Hedger, M. P. (2011b). Immunophysiology and pathology of inflammation in the testis and epididymis. *Journal of Andrology*, 32, 625–640. <https://doi.org/10.2164/jandrol.111.012989>
- Hermo, L., Papp, S., & Robaire, B. (1994). Developmental expression of the Yf subunit of glutathione S-transferase P in epithelial cells of the testis, efferent ducts, and epididymis of the rat. *The Anatomical Record*, 239, 421–440. <https://doi.org/10.1002/ar.1092390409>
- Kawai, T., & Akira, S. (2006). TLR signaling. *Cell Death and Differentiation*, 13, 816–825. <https://doi.org/10.1038/sj.cdd.4401850>
- Li, N., Wang, T., & Han, D. (2012). Structural, cellular and molecular aspects of immune privilege in the testis. *Frontiers in Immunology*, 3, 152. <https://doi.org/10.3389/fimmu.2012.00152>
- Liman, N., Alan, E., & Apaydin, N. (2019). The expression and localization of Toll-like receptors 2, 4, 5 and 9 in the epididymis and vas deferens of a adult tom cats. *Theriogenology*, 128, 62–73. <https://doi.org/10.1016/j.theriogenology.2019.02.001>
- Meikar, O., Da Ros, M., & Kotaja, N. (2012). Epigenetic regulation of male germ cell differentiation. *Sub-cellular Biochemistry*, 61, 119–138. https://doi.org/10.1007/978-94-007-4525-4_6
- Meinhardt, A., & Hedger, M. P. (2011). Immunological, paracrine and endocrine aspects of testicular immune privilege. *Molecular and Cellular Endocrinology*, 335, 60–68. <https://doi.org/10.1016/j.mce.2010.03.022>
- Michel, V., Pilatz, A., Hedger, M. P., & Meinhardt, A. (2015). Epididymitis: Revelations at the convergence of clinical and basic sciences. *Asian Journal of Andrology*, 17, 756. <https://doi.org/10.4103/1008-682X.155770>
- Orth, J. M. (1982). Proliferation of Sertoli cells in fetal and postnatal rats: A quantitative autoradiographic study. *The Anatomical Record*, 203, 485–492. <https://doi.org/10.1002/ar.1092030408>
- Özbek, M., Hitit, M., Öztop, M., Beyaz, F., Ergün, E., & Ergün, L. (2019). Spatiotemporal expression patterns of natriuretic peptides in rat testis and epididymis during postnatal development. *Andrologia*, 51, e13387. <https://doi.org/10.1111/and.13387>
- Özbek, M., Hitit, M., Yıldırım, N., Özgenç, Ö., Ergün, E., Ergün, L., ... Altunay, H. (2018). Expression pattern of galectin-1 and galectin-3 in rat testes and epididymis during postnatal development. *Acta Histochemica*, 120, 814–827. <https://doi.org/10.1016/j.acthis.2018.09.006>
- Palladino, M. A., Savarese, M. A., Chapman, J. L., Dughi, M. K., & Plaska, D. (2008). Localization of Toll-like receptors on epididymal epithelial cells and spermatozoa. *American Journal of Reproductive Immunology*, 60, 541–555. <https://doi.org/10.1111/j.1600-0897.2008.00654.x>
- Picut, C. A., Remick, A. K., de Rijk, E. P., Simons, M. L., Stump, D. G., & Parker, G. A. (2015). Postnatal development of the testis in the rat: Morphologic study and correlation of morphology to neuroendocrine parameters. *Toxicologic Pathology*, 43, 326–342. <https://doi.org/10.1177/0192623314547279>

- Price, A. E., Shamardani, K., Lugo, K. A., Deguine, J., Roberts, A. W., Lee, B. L., & Barton, G. M. (2018). A map of Toll-like receptor expression in the intestinal epithelium reveals distinct spatial, cell type-specific, and temporal patterns. *Immunity*, 49, 560–575. <https://doi.org/10.1016/j.immuni.2018.07.016>
- Riccioli, A., Starace, D., Galli, R., Fuso, A., Scarpa, S., Palombi, F., ... Filippini, A. (2006). Sertoli cells initiate testicular innate immune responses through TLR activation. *The Journal of Immunology*, 177, 7122–7130. <https://doi.org/10.4049/jimmunol.177.10.7122>
- Roach, J. C., Glusman, G., Rowen, L., Kaur, A., Purcell, M. K., Smith, K. D., ... Aderem, A. (2005). The evolution of vertebrate Toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 9577–9582. <https://doi.org/10.1073/pnas.0502272102>
- Robaire, B., Hinton, B. T., & Orgebin-Crist, M.-C. (2006). The epididymis. In E. Knobil & J. D. Neill (Eds.), *Physiology of reproduction* (3rd ed., pp. 1071–1148). St Louis, MO: Academic Press. <https://doi.org/10.1016/B978-012515400-0/50027-0>
- Schultz, N., Hamra, F. K., & Garbers, D. L. (2003). A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. *Proceedings of the National Academy of Sciences*, 100, 12201–12206. <https://doi.org/10.1073/pnas.1635054100>
- Schuppe, H. C., Meinhardt, A., Allam, J. P., Bergmann, M., Weidner, W., & Haidl, G. (2008). Chronic orchitis: A neglected cause of male infertility? *Andrologia*, 40, 84–91. <https://doi.org/10.1111/j.1439-0272.2008.00837.x>
- Shang, T., Zhang, X., Wang, T., Sun, B., Deng, T., & Han, D. (2011). Toll-like receptor-initiated testicular innate immune responses in mouse Leydig cells. *Endocrinology*, 152(7), 2827–2836. <https://doi.org/10.1210/en.2011-0031>
- Shum, W. W. C., Da Silva, N., McKee, M., Smith, P. J., Brown, D., & Breton, S. (2008). Transepithelial projections from basal cells are luminal sensors in pseudostratified epithelia. *Cell*, 135, 1108–1117. <https://doi.org/10.1016/j.cell.2008.10.020>
- Silva, E. J., Ribeiro, C. M., Mirim, A. F., Silva, A. A., Romano, R. M., Hallak, J., & Avellar, M. C. W. (2018). Lipopolysaccharide and lipoteichoic acid differentially modulate epididymal cytokine and chemokine profiles and sperm parameters in experimental acute epididymitis. *Scientific Reports*, 8, 103. <https://doi.org/10.1038/s41598-017-17944-4>
- Starace, D., Galli, R., Paone, A., De Cesaris, P., Filippini, A., Ziparo, E., & Riccioli, A. (2008). Toll-like receptor 3 activation induces antiviral immune responses in mouse sertoli cells. *Biology of Reproduction*, 79, 766–775. <https://doi.org/10.1095/biolreprod.108.068619>
- Sun, B., Qi, N., Shang, T., Wu, H., Deng, T., & Han, D. (2010). Sertoli cell-initiated testicular innate immune response through Toll-like receptor-3 activation is negatively regulated by Tyro3, Axl, and Mer receptors. *Endocrinology*, 151, 2886–2897. <https://doi.org/10.1210/en.2009-1498>
- Sun, E. L., & Flickinger, C. J. (1979). Development of cell types and of regional differences in the postnatal rat epididymis. *The American Journal of Anatomy*, 154, 27–55. <https://doi.org/10.1002/aja.1001540104>
- Sun, E. L., & Flickinger, C. J. (1982). Proliferative activity in the rat epididymis during postnatal development. *The Anatomical Record*, 203, 273–284. <https://doi.org/10.1002/ar.1092030209>
- Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, 140, 805–820. <https://doi.org/10.1016/j.cell.2010.01.022>
- Tanaka, H., & Baba, T. (2005). Gene expression in spermiogenesis. *Cellular and Molecular Life Sciences*, 62, 344–354. <https://doi.org/10.1007/s00018-004-4394-y>
- Toyama, Y., Ohkawa, M., Oku, R., Maekawa, M., & Yuasa, S. (2001). Neonatally administered diethylstilbestrol retards the development of the blood-testis barrier in the rat. *Journal of Andrology*, 22, 413–423. <https://doi.org/10.1002/j.1939-4640.2001.tb02197.x>
- Winnall, W. R., Muir, J. A., & Hedger, M. P. (2011). Differential responses of epithelial Sertoli cells of the rat testis to Toll-like receptor 2 and 4 ligands: Implications for studies of testicular inflammation using bacterial lipopolysaccharides. *Innate Immunity*, 17, 123–136. <https://doi.org/10.1177/1753425909354764>
- Yule, T. D., Montoya, G. D., Russell, L. D., Williams, T. M., & Tung, K. S. (1988). Autoantigenic germ cells exist outside the blood testis barrier. *The Journal of Immunology*, 141, 1161–1167.
- Zhu, W., Zhao, S., Liu, Z., Cheng, L., Wang, Q., Yan, K., ... Han, D. (2015). Pattern recognition receptor-initiated innate antiviral responses in mouse epididymal epithelial cells. *The Journal of Immunology*, 194, 4825–4835. <https://doi.org/10.4049/jimmunol.1402706>

How to cite this article: Özbek M, Hitit M, Ergün E, et al. Expression profile of Toll-like receptor 4 in rat testis and epididymis throughout postnatal development. *Andrologia*. 2020;52:e13518. <https://doi.org/10.1111/and.13518>