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Research Article

Expression Profiles of Toll-like Receptors 2, 7 and 8 in Rat Testis and Epididymis During Postnatal Developmental Period [1]

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Abstract

Toll-like receptors take an essential part in innate immunity in response to invasion of the various harmful pathogens. We aimed to investigate TLR2, 7 and 8 expression in rat testis and epididymis throughout postnatal development. In the prepubertal period, TLR2 and 7 were variably localized to peritubular myoid cells, interstitial cells, blood vessels, epithelial cells, ductal smooth muscle cells in testis and epididymis. In the pubertal period, immunostaining of TLR2 and 7 started to be seen in primary spermatocytes, as well as other cells, in the testis. Narrow cells showed strong intracytoplasmic staining in the epididymis. In the postpubertal period, moderate to strong immunostaining of TLR2 and TLR7 was seen in spermatids at different developmental steps but weak immunoreaction in pachytene spermatocytes. Other cells in testis and epididymis showed variable immunostaining of TLR2 and 7. However, weak to moderate immunoreaction to TLR8 was detected in only interstitial cells in testis. In the mature period, immunostaining of TLR2, 7 and 8 tended to increase in different types of cells in testis and epididymis. Our findings suggest that expression of TLR2, 7 and 8 changed dynamically during postnatal development and increased towards mature period. We consider that TLR2, 7 and 8 might be associated with the regulation of spermatogenesis and the maintenance of innate immunity of testis and epididymis during postnatal development.

Keywords: *Toll-like receptors, Innate immunity, Testis, Epididymis, Postnatal development*

Postnatal Gelişim Döneminde Rat Testis ve Epididimisinde Toll-benzeri Reseptörler 2, 7 ve 8'in Ekspresyon Profilleri

Öz

Toll-benzeri reseptörler, çeşitli zararlı patojenlerin saldırısına yanıt olarak doğal bağışıklıkta önemli bir rol oynamaktadırlar. Postnatal gelişim boyunca rat testis ve epididimisinde TLR2, 7 ve 8 ifadesini araştırmayı amaçladık. Prepubertal dönemde TLR2 ve 7'nin, testis ve epididimisteki perităbüler miyoid hücreler, interstisyal hücreler, kan damarları, epitel hücreleri ve duktal düz kas hücrelerinde değişik şekillerde lokalize olduğu tespit edildi. Pubertal dönemde TLR2 ve 7 immunboyanması, testiste diğer hücrelerin yanı sıra primer spermatositlerde görülmeye başladı. Epididimiste dar hücreler, güçlü intrasitoplazmik boyanma gösterdi. Postpubertal dönemde farklı gelişimsel aşamalardaki spermatidlerde orta-güçlü derecede TLR2 ve 7 immünboyanması görülürken, pakiten spermatositlerde zayıf immünboyanma görüldü. Testis ve epididimisteki diğer hücre tipleri değişken derecelerde TLR2 ve 7 immünboyanması göstermiştir. Ancak testiste sadece interstisyal hücrelerde zayıf-orta derecede TLR8 immünreaksiyonu saptandı. Erişkin dönemde testis ve epididimisteki farklı hücre tiplerinde TLR2, 7 ve 8 immünboyanmasının artma eğiliminde olduğu dikkat çekti. Bulgularımız TLR2, 7 ve 8 ekspresyonunun postnatal gelişim sürecinde dinamik olarak değiştiğini ve erişkin döneme doğru arttığını göstermektedir. TLR2, 7 ve 8'in postnatal gelişim sürecinde spermatogenezin düzenlenmesi ve testis ile epididimisin doğal bağışıklığının sürdürülmesi ile ilişkili olabileceğini düşünüyoruz.

Anahtar sözcükler: *Toll-benzeri reseptörler, Doğal bağışıklık, Testis, Epididimis, Postnatal gelişim*

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INTRODUCTION

Pattern recognition receptors (PRRs) cover structurally divergent proteins, including Toll-like receptors (TLRs). PRRs recognize conserved moieties known as pathogen-associated molecular patterns (PAMPs) that are characteristic of specific pathogens. TLRs take an essential part in innate immunity [1]. TLRs mediate the induction of antimicrobial peptides in response to invasion of the bacterial, fungal and viral pathogens [2]. TLR family is composed of 13 members (TLR1-13) commonly found in many vertebrate species. TLR1-10 have been discovered in humans and TLR11-13 are confined to rodents [3]. Each TLR has been demonstrated to recognize specific components of pathogens. For instance, TLR2 recognizes microbial lipopeptides such as porin, peptidoglycan and lipoteichoic acid [4] while TLR7 and TLR8 recognize single-stranded RNA molecules (ssRNAs) [5-7]. Recognition of specific cell wall components with TLRs initiates a series of events containing various adaptor proteins and protein kinases, ultimately leading to the activation of immune response genes [8,9]. The targeted genes contain those encoding cytokines such as antimicrobial peptides, interleukin-12p40, interferon-beta, adhesion molecules, acute phase proteins, tumor necrosis factor-alpha (TNF-α), chemokines, and cyclooxygenase 2 [10]. The initial host defense against invading pathogens and opportunistic organisms culminates in the production of proinflammatory cytokines during an inflammatory response. They all together protect immediately hosts against pathogens and mount adaptive immune responses to those pathogens as well [8].

Microbial and pathogenic agents such as *Escherichia coli*, *Chlamydia trachomatis*, *Staphylococcus aureus* and *Neisseria gonorrhea* may infect the male reproductive tract and present a challenge for normal reproductive and endocrine functions. Epididymitis, which obstructs sperm movement and leads to inflammation, is due to the backward movement of microorganisms from the vas deferens [11]. Development of infection in the epididymis may form an epididymal abscess. Moreover, progressing infection may result in testicular involvement, eventually causing epididymo-orchitis or a testicular abscess [12,13]. As is the case with other systems, infections occurring in the male reproductive tract is anticipated mounting immune response through the activation of immune receptors, ultimately culminating in the induction of a lot of genes including the antimicrobial peptides and proteins [14]. For example, infection with *S. aureus* increased the expression of TLR2 in rat epididymis, suggesting that epididymal epithelium mounts an innate immune response through the activation of p38 MAPK and NF-κB after the increased expression of TLR2 [15].

Antimicrobial protection of immune-privileged organs such as testis and epididymis has become a recent active area of research. It has been reported that TLRs are expressed in different regions of the male reproductive tract [14,16,17]. Previous studies have focused mainly on mRNA expression of TLRs. However, cellular localization, distribution and expression patterns of TLRs such as TLR2, 7 and -8 have not been investigated in testis and epididymis of the rats at different periods of postnatal development. Therefore, we hypothesized that TLR2, 7 and 8 play essential roles in the pattern recognition during postnatal development of male reproductive tract. We here sought to identify the cell types expressing TLR2, 7 and 8 and to investigate their cellular localization, distribution and expression patterns in testis and epididymis of the rats by using immunohistochemistry.

MATERIAL and METHODS

Animal Materials and Experimental Design

Approval was obtained from Ankara University Local Ethics Committee for Animal Experiments (approval number #2014-18-128). A total of 24 of Wistar albino rats (200-300 g) at different stages of postnatal development were used in this study. Rats were divided into four groups, each of which composes of six rats. Groups were designated as prepubertal (postnatal 5 days, PND5), pubertal (PND20), postpubertal (PND50) and mature (PND70) periods. The animals were maintained at room temperature (20-24°C) in a 12-h light/dark circle and fed a standard diet and water *ad libitum*. By the end of each developmental period, all the rats were euthanized under anesthesia and testicular and epididymal tissues samples were harvested. These processes were done at the same time interval to prevent any alteration resulted from biological rhythm of animals.

Tissue Processing

Tissue processing was made as previously described [18]. Shortly, the testicular and epididymal samples were fixed in Bouin's solution, passed through a graded series of alcohols, methyl benzoate and benzene, and embedded in paraplast.

Immunohistochemistry

Immunohistochemistry was performed employing the streptavidin-biotin peroxidase technique [19]. A 5-µm thick sections were affixed onto Poly-Lysine-coated slides (Thermo Fisher Scientific). The sections were kept in 3% hydrogen peroxide in methanol/PBS for 20-30 min to quench endogenous peroxidase activity. Antigen retrieval was performed with citrate buffer (#AP-9003-500, Thermo Fisher Scientific Lab Vision) to unmask antigenic epitopes. They were then incubated with primary antibodies (TLR2, 1/300, #bs-1019R; TLR7, 1/300, #ab45371; TLR8, 1/300, PA5-20056) at +4°C for 16 h. Following the routine procedure, they were treated with AEC chromogen (#TA-060-HA). The sections were counterstained with Gill's II hematoxylin and coverslipped with water-based mounting medium (#TA-125-UG). Results of immunostaining were analyzed by two blind observers under a light microscope (DM 2500, Leica, Germany) with a digital camera (DFC450, Leica, Germany). Images were captured using Leica Application Suite software.

RESULTS

No staining was observed in negative control sections when normal rabbit serum (#sc-2027) was used instead of the primary antibodies. Representative sections for immunolocalization of TLR2, TLR7 and TLR8 are presented in the testis and epididymis of rats *(Fig. 1, 2, 3, 4, 5, 6)*. Expression pattern of TLRs, especially of TLR2 and TLR7, tended to increase from prepubertal period to mature period. Expression of TLR8 was not detected in testis and epididymis at the prepubertal, pubertal and postpubertal periods, except for interstitial cells at postpubertal period. In general, staining intensity of TLR2 and TLR7 were much more pronounced than that of TLR8 at all the periods.

TLR2 Immunohistochemistry

PND 5: Peritubular myoid cells, some interstitial cells and blood vessels showed weak, moderate and strong immunoreactivity, respectively. However, any immune reaction was not detected in immature Sertoli cells and gonocytes *(Fig. 1-a)*. In epididymis, epithelial cells, ductal smooth muscle

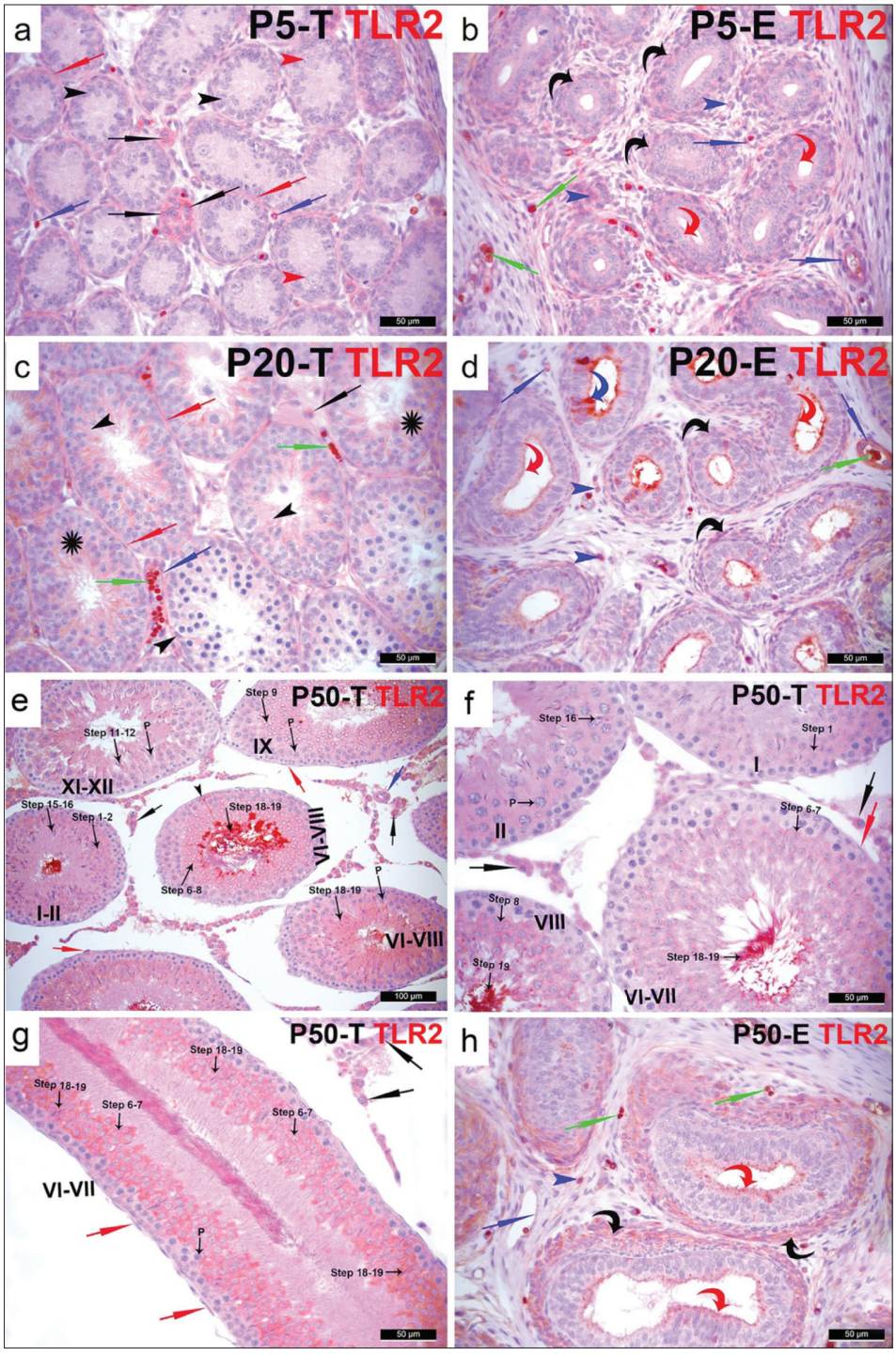


Fig 1. TLR2 immunostaining in the rat testis and epididymis at PND5, 20 and 50. **(a, c)** In the testis, no staining in gonocytes *(red arrowheads)* and Sertoli cells *(black arrowheads)*. Positive staining in primary spermatocytes *(asterisks)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)* and blood vessels *(blue arrows)*. **(b, d)** In the epididymis, positive staining in epithelial cells *(red curved arrows)*, ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)*, vessel walls *(blue arrows)* and blood cells *(green arrows)*. **(e-g)** Positive staining in pachytene (P) spermatocytes *(black arrows)*, spermatids at different steps *(black arrows)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)*. **(h)** In the epididymis, positive staining in epithelial cells (red curved arrows), ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)*, vessel walls *(blue arrows)* and blood cells *(green arrows)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale bars: 100 µm (E), 50 µm (A, B, C, D, F, G, H)

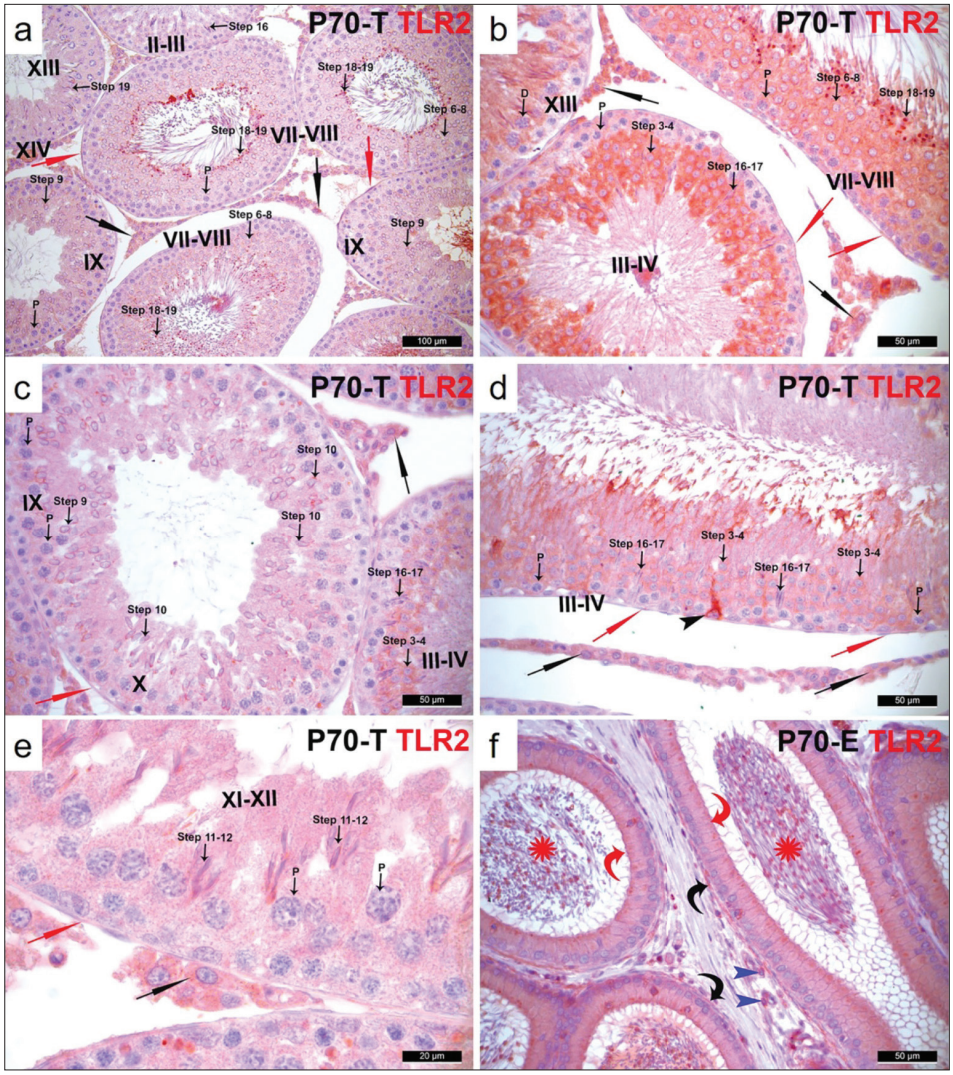


Fig 2. TLR2 immunostaining in the rat testis and epididymis at PND70. **(a-e)** Positive staining in pachytene (P) spermatocytes *(black arrows)*, spermatids at different steps *(black arrows)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)*. **(d)** In the epididymis, positive staining in epithelial cells (red curved arrows), ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)* and spermatozoa *(red asterisks)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale bars: 100 µm (A), 50 µm (B, C, D and F), 20 µm (E)

cells and some interstitial cells had weak immunostaining while vessel walls and some blood cells showed strong staining *(Fig. 1-b)*.

PND 20: In testis, we observed weak staining in primary spermatocytes and some interstitial cells, weak to moderate staining in peritubular myoid cells and apical surface of Sertoli cells, moderate staining on vessel wall and strong staining on some blood cells *(Fig. 1-c)*. In epididymis, apical surface of all epithelial cells had moderate to strong staining while some narrow cells exhibited strong intracytoplasmic staining. Vessel wall and some interstitial cells had moderate immunostaining. Some blood cells showed strong immunoreactivity. Weak staining was detected in ductal smooth muscle cells *(Fig. 1-d)*.

PND 50: We found moderate to strong immunoreaction in elongated spermatids and weak to moderate immunoreaction in round spermatids at different spermatogenic stages. Pachytene spermatocytes exhibited weak immunoreaction. Peritubular myoid cells had weak to moderate immunoreaction. Moderate immunoreaction was detected in some interstitial cells and apical surface of Sertoli cells *(Fig. 1-e,f,g)*. In epididymis, moderate immunoreaction was seen in ductal smooth muscle cells and some interstitial cells and on apical surfaces of epithelial cells. Some blood cells showed strong immunoreaction *(Fig. 1-h)*.

PND 70: Compared to earlier periods, immunostaining tended to increase in testis and epididymis. We observed moderate to strong immunoreaction in elongated and round spermatids at different developmental steps while pachytene spermatocytes exhibited weak to moderate immunoreaction. We found weak immunoreaction in peritubular myoid cells but moderate immunoreaction in interstitial cells *(Fig. 2-a,b,c,d,e)*. In epididymis, moderate to strong immunoreaction was seen in ductal smooth muscle cells, luminal spermatozoa, some interstitial cells and the apical surfaces of epithelial cells *(Fig. 2-f)*.

TLR7 Immunohistochemistry

PND 5: We observed moderate immunostaining in immature Sertoli cells and peritubular myoid cells although no reaction was present in gonocytes. Vessel wall and some interstitial cells showed weak to moderate immunoreaction *(Fig. 3-a)*.

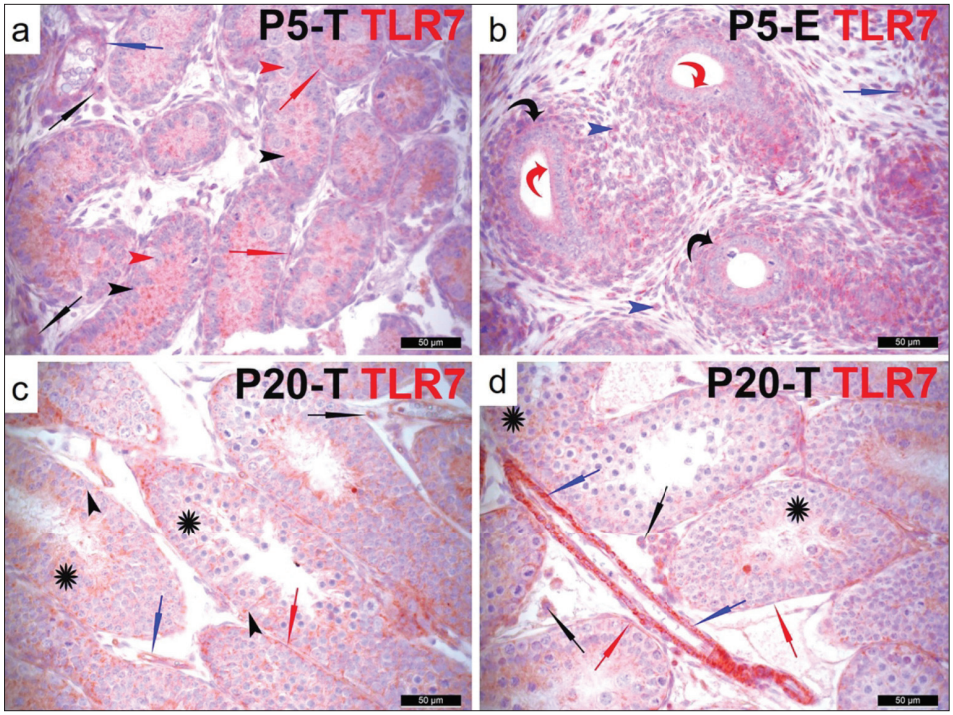


Fig 3. TLR7 immunostaining in the rat testis and epididymis at PND5 and 20. **(a, c, d)** In the testis, no immunoreaction in gonocytes *(red arrowheads)* and Sertoli cells *(black arrowheads)*. Positive staining in primary spermatocytes *(asterisks)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)* and blood vessels *(blue arrows)*. **(b)** In the epididymis, positive staining in epithelial cells *(red curved arrows)*, ductal smooth muscle cells *(black curved arrows)*, interstitial cells *(blue arrowheads)* and vessel walls *(blue arrows)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 50 µm

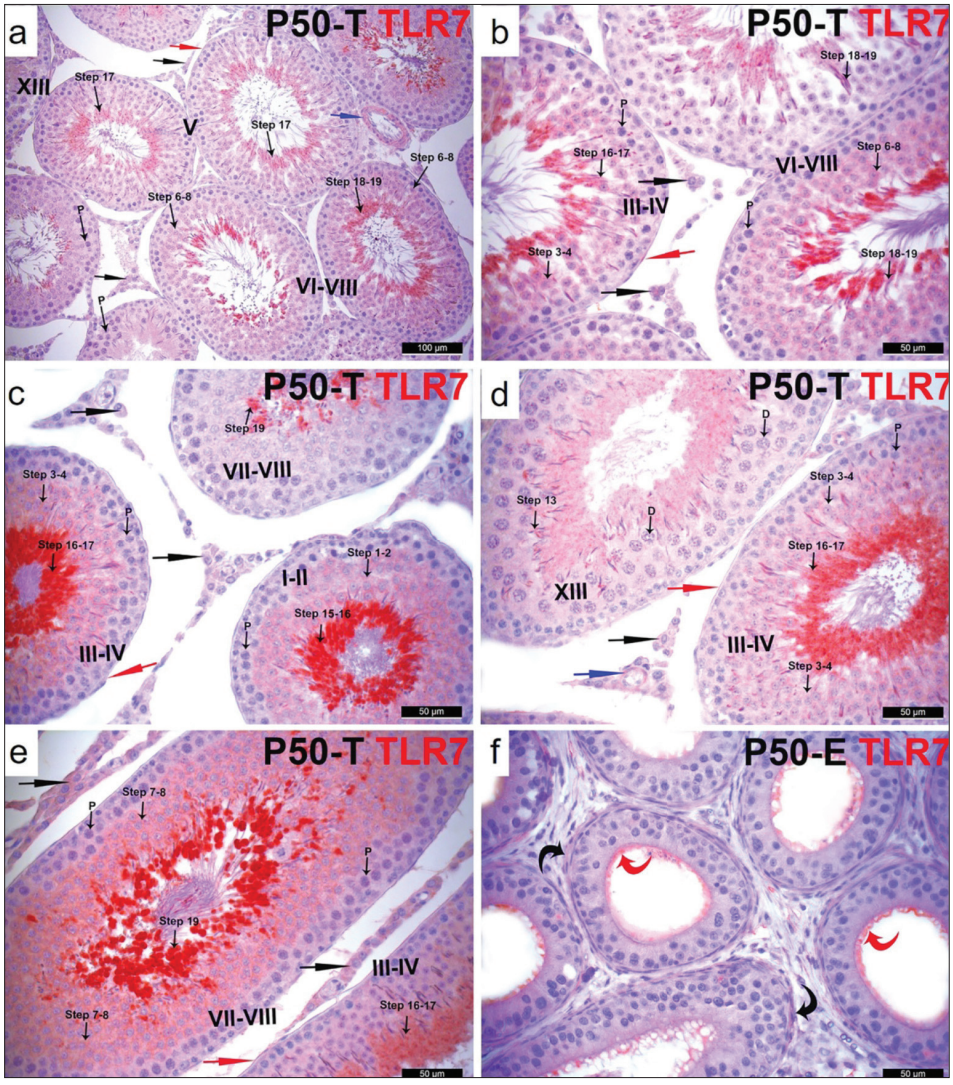


Fig 4. TLR7 immunostaining in the rat testis and epididymis at PND50. **(a-e)** Positive staining in pachytene (P) spermatocytes *(black arrows)*, spermatids at different steps *(black arrows)*, peritubular myoid cells *(red arrows)*, interstitial cells *(black arrows)*. **(f)** In the epididymis, positive staining in epithelial cells *(red curved arrows)* and ductal smooth muscle cells *(black curved arrows)*. Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 100 µm (A), 50µm (B, C, D, E and F)

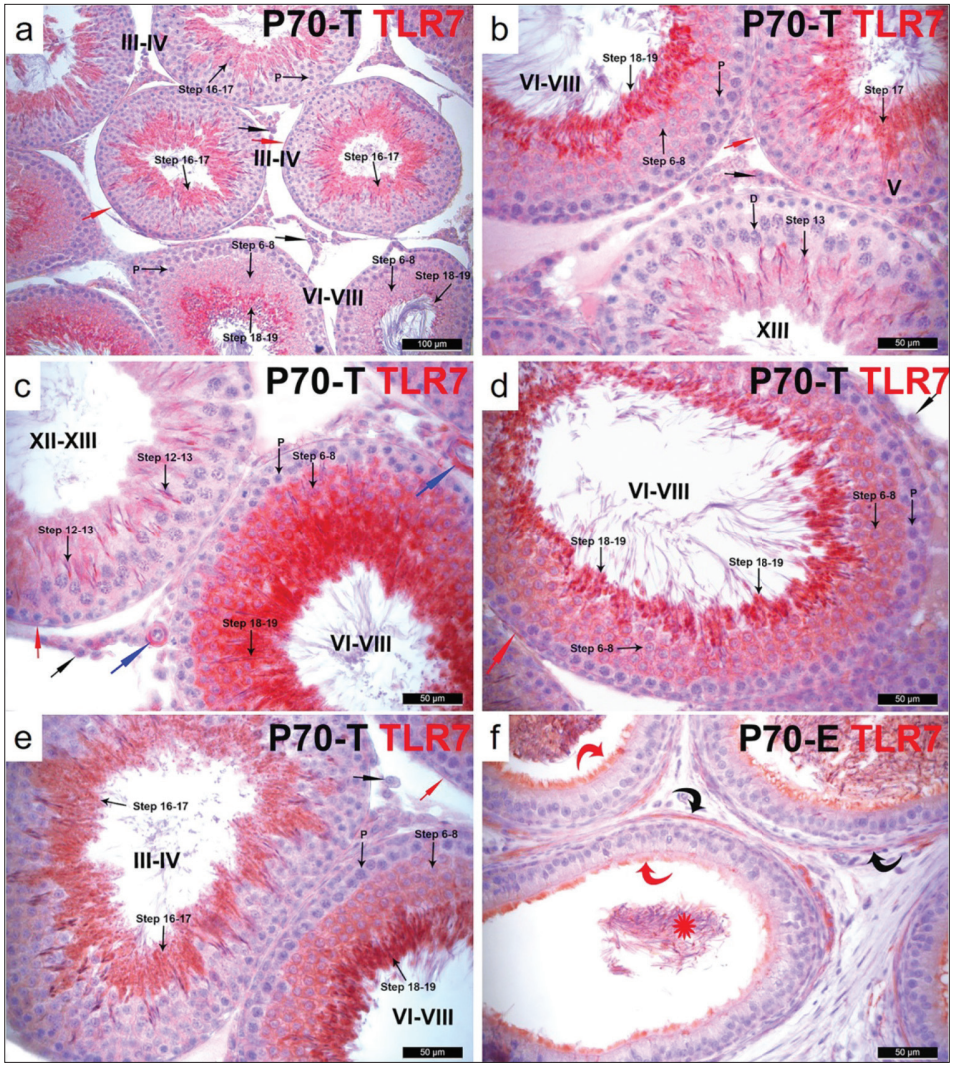


Fig 5. TLR7 immunostaining in the rat testis and epididymis at PND70. **(a-e)** Positive staining in pachytene (P) spermatocytes *(black arrows),* spermatids at different steps *(black arrows),* peritubular myoid cells *(red arrows),* interstitial cells *(black arrows).* **(d)** In the epididymis, positive staining in epithelial cells *(red curved arrows),* ductal smooth muscle cells *(black curved arrows)* and spermatozoa *(red asterisks).* Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 100 µm (A), 50 µm (B, C, D, E, F)

In epididymis, weak to moderate immunoreaction was observed in interstitial cells and vessel wall while ductal smooth muscle cells and apical surface of all epithelial cells exhibited moderate immunostaining *(Fig. 3-b)*.

PND 20: We found moderate immunostaining in Sertoli cells, primary spermatocytes, some interstitial cells, peritubular myoepithelial cells, and strong staining in vessel wall *(Fig. 3-c,d)*. In epididymis, immunoreaction was similar to that of prepubertal period.

PND 50: We detected strong immunoreaction in elongated spermatids and moderate immunoreaction in round spermatids at different developmental steps. Pachytene spermatocytes, peritubular myoid cells and interstitial cells showed weak immunoreaction. We found moderate to strong immunoreaction in the apical surface of Sertoli cells but moderate immunostaining in vessel wall *(Fig. 4-a,b,c,d,e)*. In epididymis, weak immunoreaction was seen in ductal smooth muscle cells while moderate staining was present on the apical surfaces of epithelial cells and vascular smooth muscle cells. On the other hand, interstitial cells stained heterogeneously weak *(Fig. 4-f)*.

PND 70: Compared to earlier periods, we detected the increased TLR7 expression in testis and epididymis. We observed strong immunoreaction in elongated spermatids and moderate to strong immunoreaction in round spermatids at different developmental steps. Pachytene spermatocytes and peritubular myoid cells showed weak immunoreaction. Vessel wall had strong immunostaining. Interstitial cells exhibited weak to moderate immunoreaction *(Fig. 5-a,b,c,d,e)*. In epididymis, moderate immunoreaction was observed in ductal smooth muscle cells, luminal spermatozoa and the apical surfaces of epithelial cells whereas interstitial cells were weakly stained *(Fig. 5-f)*.

TLR8 Immunohistochemistry

PND 5 and *20:* We did not detect any positive immunoreaction in the testis and epididymis *(Fig. 6-a,b,c,d)*.

PND 50: We detected weak to moderate immunoreaction in only interstitial cells in testis but weak staining in epididymis *(Fig. 6-e,f)*.

PND 70: We found weak immunolabelling in elongated

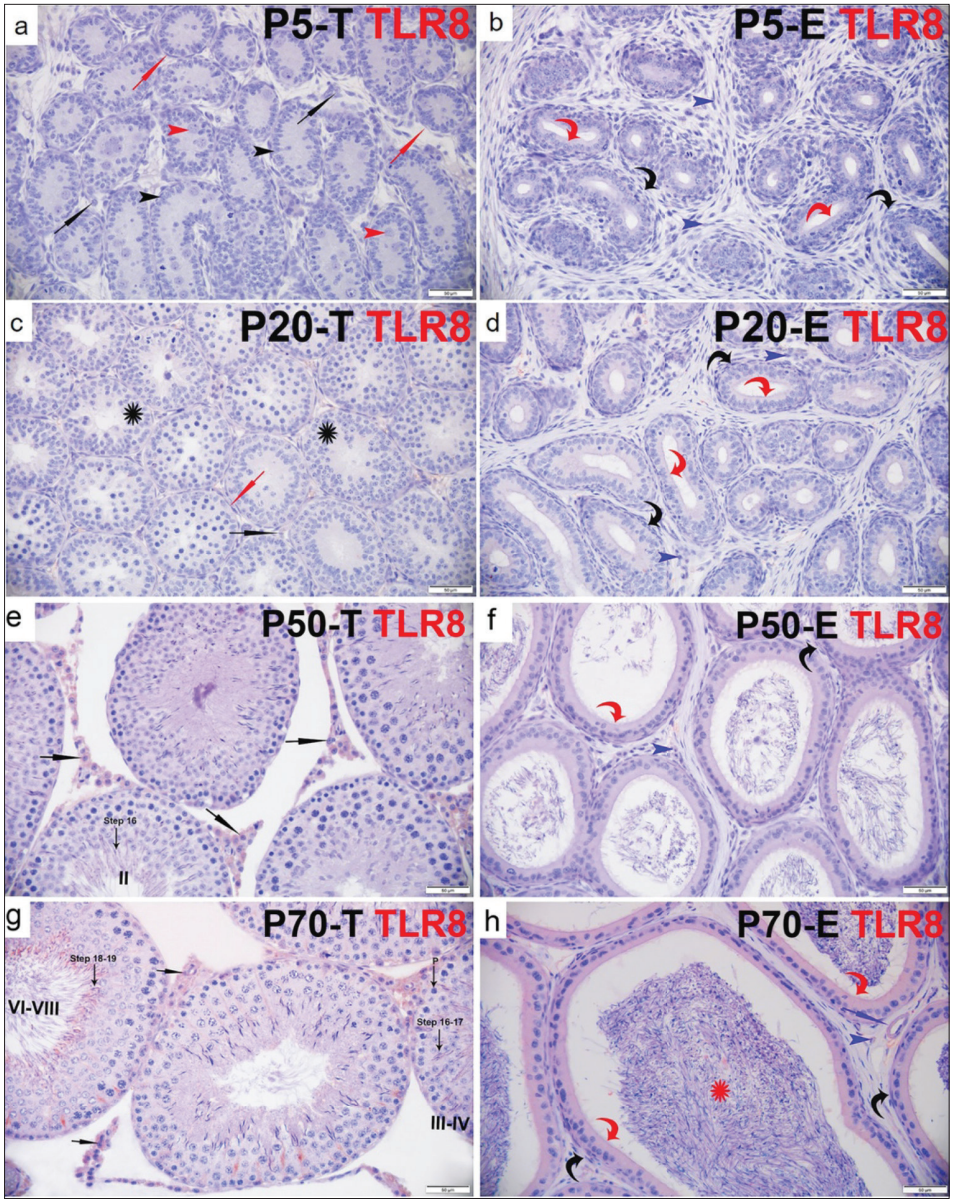


Fig 6. TLR8 immunostaining in the rat testis and epididymis at PND5, 20, 50 and 70. **(a-c)** No staining in gonocytes *(red arrowheads),* Sertoli cells *(black arrowheads),* primary spermatocytes *(asterisks),* peritubular myoid cells *(red arrows)* and interstitial cells *(black arrows).* **(b-d)** In the epididymis, no staining in epithelial cells *(red curved arrows),* ductal smooth muscle cells *(black curved arrows)* and interstitial cells *(blue arrowheads).* (e-g) In testis, positive staining in interstitial cells *(black arrows),* Sertoli cells *(black arrowhead)* and spermatids at different steps *(black arrows).* **(f-h)** In epididymis, positive staining in epithelial cells *(red curved arrows),* ductal smooth muscle cells *(black curved arrows),* interstitial cells *(blue arrowhead),* vessel wall *(blue arrow)* and spermatozoa *(red asterisk).* Strept-ABC, AEC, Gill's II Hematoxylin. Scale Bars: 50 µm

spermatids and interstitial cells but weak to moderate immunolabelling in Sertoli cells. In epididymis, there was weak immunoreaction in epithelial cells, ductal smooth muscle cells and vessel wall *(Fig. 6-g,h).*

DISCUSSION

We here investigated the expression pattern of TLR2, 7 and 8 proteins in rat testis and epididymis during postnatal development. The testicular and epididymal expression of TLRs, notably TLR2 and TLR7, significantly differed according to the developmental periods.

Many molecules such as androgens are required for postnatal differentiation and maintenance of testicular and epididymal structure [20]. In addition, TLRs involving in innate immunity may play key roles during postnatal differentiation. A study has performed a comprehensive analysis of TLR expression and distribution in the rat testis and epididymis [21]. They reported that TLR2 was abundantly expressed in testis and epididymis, TLR7 was mainly present in the testis despite of being weakly expressed in the epididymis, and TLR8 exhibited weak expression in testis and epididymis, which are consistent with our results. In addition, it has been showed that TLR2 mRNA is more produced by human testis than TLR7 mRNA and TLR8 mRNA, with more expression of TLR7 mRNA than that of TLR8 mRNA [22], which are also largely in concordance with the expression of TLR2, TLR7 and TLR8 proteins in our study. Unlike previous researches, it has been reported that testicular expression of TLR2 decreased towards further developmental periods [14]. This difference may arise from a higher rate of TLR2 mRNA destabilization or degradation than those of its protein, presumably through mechanisms that are dictated by miRNAs or from posttranscriptional and/or posttranslational modifications [23,24].

Previous studies reported that the existence of other innate molecules inc luding MYD88 [21] and CD14 [25] in the male tract along with the TLRs [16] gives an indication that an invasion of microbial or pathogenic agents could lead to the activation of immune effector pathways that have the capacity to induce the production and secretion of antimicrobial peptides and cytokines. In the present study, the increased expression of TLR2, TLR7, and TLR8 in the transition from the prepubertal period to mature period might show that the male reproductive tract is ready to respond to any infection that could be developed.

It has been found that, in human testis, TLR2 was expressed in peritubular cells, Sertoli cells and interstitial cells [26] in accordance with our data. In addition, a recent study has shown variable immunostaining for TLR2 in the epididymal epithelial cells, interstitial cells, ductal smooth muscle cells and sperm cells of adult tom cats, with marked staining on the apical surfaces of epithelial cells [17]. They did not observe any difference between epididymal regions. Their findings are consistent with our results of testis and epididymis. However, we observed different from their study that epididymal expression of TLR2 tended to increase towards mature period. Yet another study reported that mouse Sertoli cells express TRL2 at high levels and TLR7 at lower levels, but they do not express TLR8 [27]. Their results are in line with our findings of TRL2 and TRL7 at PND5 and 20 in terms of animal ages since they used Sertoli cells from 3-week old mice. However, we observed TLR8 expression in Sertoli cells at PND70. We speculated that increased TLR expression in Sertoli cells may serve a protective purpose during further developmental periods, suggesting that Sertoli cells provide the defense machinery for developing germ cells against auto- and alloantigens.

There is limited research on the expression of TLR2, 7 and 8 in male germ cells. We observed that expression of TLR2, 7 and 8 showed a tendency to increase after the transition from gonocytes and spermatogonia to spermatocytes and spermatids and their expression was stronger in spermatids than other spermatogenic cell types. This may mean that their expression initiated in response to the formation of functional spermatogenic cells. Their enhanced expression towards the mature period could also provide a protective mechanism for spermatozoa capable of fertilizing ovum against pathogenic microbial agents, and protect the female genital tract against these agents.

In conclusion, our results show that expression of TLR2, 7 and 8 changed dynamically during postnatal development and increased towards the mature period. We consider that TLR2, 7 and 8 might be associated with the regulation of spermatogenesis and the maintenance of innate immunity of testis and epididymis during postnatal development. However, further studies are required to better understand their roles during the postnatal development of testis and epididymis.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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