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RESEARCH ARTICLE

Seasonal Variation in TLR4 Expression in The Testis and Epididymis of Anatolian Ground Squirrels *(Spermophilus xanthoprymnus)*: Insights From Non-Breeding Period of Pre-Hibernation and Hibernation

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ABSTRACT

This study investigates the modulation of Toll-like receptor 4 (TLR4) expression within the testis and epididymis of Anatolian ground squirrels *(Spermophilus xanthoprymnus)* during the non-breeding period of pre-hibernation and hibernation. Immunohistochemical investigation showed that TLR4 was not detected in germ cells, Leydig cells, or Sertoli cells in the testicular tissue during the pre-hibernation period. Nevertheless, there was a presence of TLR4 in the vessel's walls and certain interstitial cells within the intertubular regions. Epithelial cells in the caput, corpus, and cauda regions of the epididymis showed no TLR4 expression. However, it was observed in the vessel walls, smooth muscle layers, and some interstitial cells. TLR4 expression was seen in spermatogonia and primary spermatocytes during hibernation, with strong labeling observed in the vessel walls of the intertubular area. In contrast, TLR4 was detected in the epididymal epithelium, as well as in the smooth muscle layers and vessel walls throughout all segments. A notable upregulation in the expression of TLR4 in the testis was identified through quantitative image analysis during hibernation as compared to pre-hibernation. During pre-hibernation, the cauda segment of the epididymis exhibited the highest expression of TLR4, whereas during hibernation, the corpus segment demonstrated the highest expression. These findings suggest a dynamic modulation of TLR4 in response to hibernation, highlighting its potential role in reproductive function and immune adaptation.
Keywords: Epididymis, *Spermophilus xanthoprymnus,* Testis, *TL*R4

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ÖZ

Anadolu Yer Sincaplarının (*Spermophilus xanthoprymnus)* Testis ve Epididimisinde TLR4 Ekspresyonundaki Dönemsel Değişiklikler: Üreme Dışı pre-Hibernasyon ve Hibernasyon Dönemlerinden Elde Edilen Bulgular

Bu çalışma, üreme dışı dönemde pre-hibernasyon ve hibernasyon süresince Anadolu yer sincaplarının *(Spermophilus xanthoprymnus)* testis ve epididimis dokularında Toll-like reseptör 4 (TLR4) ekspresyonunun regülasyonunu araştırmaktadır. İmmünohistokimyasal inceleme, TLR4'ün pre-hibernasyon döneminde testis dokusundaki germ hücreleri, Leydig hücreleri ve Sertoli hücrelerinde tespit edilmediğini göstermiştir. Bununla birlikte, intertubuler alandaki damar duvarlarında ve bazı interstisyel hücrelerde TLR4 mevcuttur. Epididimisin kaput, korpus ve kauda bölgelerindeki epitel hücrelerinde TLR4 ekspresyonu gözlenmemiştir. Ancak, damar duvarlarında, düz kas ve bazı interstisyel hücrelerde TLR4 mevcuttur. Hibernasyon sırasında, spermatogonyum ve primer spermatositlerde TLR4 ekspresyonu gözlenmiş olup, tübüller arası alanın damar duvarlarında güçlü bir immun reaksiyon görülmüştür. Buna karşılık, epididimisin bütün bölümlerindeki epitelinde, düz kas hücrelerinde ve damar duvarlarında TLR4 tespit edilmiştir. Hibernasyon döneminde, pre-hibernasyona kıyasla testiste TLR4 ekspresyonunda anlamlı bir artış, kantitatif görüntü analizi ile tespit edilmiştir. Pre-hibernasyon döneminde epididimisin kauda segmenti en yüksek TLR4 ekspresyonunu gösterirken, hibernasyon sırasında korpus segmenti en yüksek ekspresyonu göstermiştir. Bu bulgular, hibernasyona yanıt olarak TLR4'ün dinamik bir şekilde düzenlendiğini göstermekte olup, bu reseptörün üreme fonksiyonu ve bağışıklık adaptasyonundaki potansiyel rolüne işaret etmektedir.
Anahtar Kelimeler: Epididimis, *Spermophilus xanthoprymnus,* Testis, *TL*R4

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INTRODUCTION

The innate immune system forms the body's first barrier against infections, utilizing pattern recognition receptors to detect characteristic molecular patterns known as pathogen-associated molecular patterns (Akira et al., 2006; Medzhitov, 2001). Toll-like receptors (TLRs) play a crucial role in identifying pathogen-associated molecular patterns and triggering immune responses (Takeuchi & Akira, 2010). TLRs are membrane-bound proteins that identify microbial components and initiate signaling pathways, resulting in the synthesis of cytokines and other critical mediators for the inflammatory response and subsequent activation of the adaptive immune system (Beutler, 2009; Kawai & Akira, 2010).

TLR4 is a well-researched receptor that plays a crucial role in detecting lipopolysaccharides (LPS). This receptor is essential for initiating the body's response to bacterial infections (Iwasaki & Medzhitov, 2004). TLR4 is extensively distributed across a multitude of tissues and cell types, from pivotal immune cells such as dendritic cells and macrophages to a diverse array of non-immune cells (Janeway & Medzhitov, 2002). The study of TLRs in several tissues, including reproductive organs, has attracted considerable attention due to their involvement in immunological privilege, inflammation, and tissue homeostasis (Kawasaki & Kawai, 2014; O'Neill & Bowie, 2007).

The testis and epididymis serve as fundamental organs in the male reproductive system, overseeing the critical processes of spermatogenesis, sperm maturation, and storage. These organs maintain a state of immunoprivilege, which protects germ cells from being attacked by the immune system. However, these organs also need strong mechanisms to defend against infections (N. Li et al., 2012; Meinhardt & Hedger, 2011). The expression and modulation of TLRs, namely TLR4, in the testis and epididymis play a vital role in maintaining a balance between immune protection and reproductive activities (Hargreaves & Medzhitov, 2005). The testis is considered immunoprivileged due to the presence of the blood-testis barrier and local immunosuppressive settings. These mechanisms are crucial for protecting developing germ cells from potential immune attacks (Hedger, 2011b).

Anatolian ground squirrels (*Spermophilus xanthoprymnus)* are a useful model for investigating hibernation and its impact on several physiological systems, such as the immunological and reproductive systems (Gür & Kart Gür, 2005; Kart Gür et al., 2009). Hibernation elicits significant alterations in metabolic, endocrine, and immune processes. Studying the presence of TLR4 in the testis and epididymis throughout the non-breeding periods of pre-hibernation and hibernation can offer valuable knowledge on the adaptive mechanisms that safeguard reproductive organs during these periods. Hibernation leads to a large decrease in metabolic activity, which can affect the immune system's ability to respond and require specific adjustments to preserve reproductive function and immune defense (Bouma et al., 2010; Giroud et al., 2020; Q. Li et al., 2015; Saeidi et al., 2014).

In this study, we aim to investigate the expression of TLR4 in the testis and epididymis of Anatolian ground squirrels during non-breeding period of pre-hibernation and hibernation. This research will enhance our understanding of the immune-regulatory mechanisms in the reproductive system during hibernation and contribute to the broader knowledge of TLR function in seasonal breeders.

MATERIALS and METHODS

Experiment conditions and an animal ethics statement

Regarding the treatment and utilization of animals, the experimental protocols were carried out in adherence to the standards established by the Ethical Committee of Erciyes University. The Erciyes University Local Ethics Committee for Animal Experiments (HADYEK), situated in Kayseri, granted approval for these protocols under the authorized number 15/140. Twelve male Anatolian ground squirrels *(Spermophilus xanthoprymnus)* weighing between 300 and 380 grams were uniformly distributed as a sample for the research. The exact chronological age of the animals is uncertain, given that they were procured from the steppes near Develi, Kayseri, Turkey in late August via Tomahawk Traps. Preceding their use in the experiments, this was performed to ensure that the rodents were in perfect health. The experimental groups were subsequently divided into two categories: pre-hibernation (comprising six male subjects) and hibernation (late torpor), which also comprised six male subjects. Following intraperitoneal administration of ketamine and xylazine, the pre-hibernation group was euthanized via cardiac puncture, and the hearts were immediately removed (Olson & McCabe, 1986). A series of alcohol solutions were used to dehydrate the testis and epididymis samples after they had been immersed in Bouin's solution for 12 hours. The samples were clarified with benzol and methyl benzoate subsequent to the water removal procedure. Subsequently, they were immersed in paraffin.

Hibernation group

Until their body lipid levels were at an optimal level for hibernation, the squirrels in the hibernation group were supplied with a standard rodent diet, sunflower seeds, fresh produce, and unrestricted access to water. Initially, a temperature of 21.1 ± 1 °C was maintained

in the laboratory. In order to replicate the natural photoperiod from September 2016 to December 2016, an artificial light-dark cycle was established, with values extending from 200 to 0 lx. In order to promote a smooth transition into torpor, the laboratory environment was subsequently adjusted to 6 °C, all food sources were eliminated from the enclosures, and the lights were entirely deactivated. A red safe light, ranging in intensity from 3 to 5 lumens, was employed upon entering the laboratory so as not to arouse dormant rodents. Three months after initially entering torpid and hypothermic conditions, the squirrels were rendered unconscious by intraperitoneal administration of ketamine and xylazine (11.1 mg of xylazine/ml and 88.9 mg of ketamine/ml, respectively) (Olson & McCabe, 1986). Tissue processing procedures were maintained for the experimental hibernation group subsequent to sampling.

Immunohistochemistry

A rotary microtome was employed to section paraffin-embedded tissue to a thickness of 5 µm. The sections were subsequently transferred to slides that had been coated with poly-L-lysine. For the purpose of immunohistochemistry staining, the streptavidin-biotin-peroxidase method was implemented, as described in a 2019 study by Özbek et al (Özbek et al., 2019). The sections were rehydrated through a succession of graduated alcohols following deparaffinization with xylene. Antigen retrieval was accomplished by subjecting the sample to a 20-minute microwave boil in citrate buffer with a pH of 6.0, subsequent to the washing phase in phosphate-buffered saline (PBS). For the purpose of inhibiting the activity of naturally occurring peroxidase, the sections were submerged in a solution containing 3% H_2O_2 in distilled water for-20 minutes. By incubating with Ultra V Block for a period of ten minutes, non-specific binding was averted. Then, they were treated overnight at 4 °C with an unconjugated monoclonal TLR4 primary antibody (Novus Biologicals, cat no: NB100-56566) subsequent to the removal of any surplus serum from the slides. After thirty minutes of exposure to a biotinylated secondary antibody at room temperature, the sections were subjected to the second wash in PBS. Following which, they underwent an additional 15-minute rinse with PBS. Using 3-amino-9-ethylcarbazole (AEC), the resulting signal was produced. Prior to being mounted in an aqueous medium, the slides were stained with Gill's hematoxylin. The primary antibody was substituted with PBS as the negative control; all other procedures were executed in accordance with the provided instructions. Colon tissue was employed as the positive control.

Qualitative Assessment of Immunohistochemical Staining Patterns

The results of the qualitative examination are meticulously documented in Table 1. Examination and imaging of the stained sections were performed with a BX51 microscope (Olympus, Tokyo, Japan). Utilizing a phenomenological intensity scoring system with four tiers, the immunostaining assessments were carried out. A 100x and 400x magnification, respectively, was applied to assess the intensity of TLR4 staining in the testis and epididymis. Öztop et al. (2019) outlined the scoring system employed to determine the intensity of TLR4 staining: "-" denoted the absence of staining, "+" indicated weak staining, "++" moderate staining, and "+++" intense staining (Öztop et al., 2019).

Image analysis

In accordance with the methodology described by Jensen (2017), the staining intensities of TLR4 in images of the epididymis and testes were quantified (Jensen et al., 2017). For the entire image, the intensity of immunostaining was measured. During the pre-hibernating and hibernating phases, images of the and epididymis were testes captured at a 400X magnification. Analyses of each tissue during each period comprised a total of twelve photographs. ImageJ 1.54 was utilized to import the pertinent photographs. The images were then processed using the 'color deconvolution' plug-in, which separated the hematoxylin and AEC stains into three distinct panels: one showcasing the hematoxylin, another the AEC, and the third serving as the background. Specific threshold values were set exclusively for the AEC images. For each image, the statistical analysis concentrated on the area and area fraction metrics, providing a detailed percentage-based assessment of the stained region's extent and staining intensity

Statistical Analysis

Utilizing GraphPad Prizm 8 for Windows, more precisely Version 8.0.2, the statistical analysis was conducted. The means, with or without the standard error of the means (SEM), were utilized to represent the data. In this study, a two-tailed Student's t test was employed to compare two distinct groups, namely the testis and epididymis. Before applying Bonferroni's multiple comparison test, a one-way factorial analysis of variance (ANOVA) was performed to compare the groups. We considered the data significant at a p-value of less than 0.05.

Table 1. Evaluation of TLR4 expression in the testicular and epididymal tissues of Anatolian ground squirrels *(Spermophilus xanthoprymnus)* during non-breeding period of pre-hibernation and hibernation

Tissue		Pre-hibernating	Hibernating
Testis	**Cell type**		
	Spermatogonium	-	+
	Spermatocyte/spermatid	-	+
	Sperm	No differentiation	No differentiation
	Leydig cells	-	
	Sertoli cells	-	-
	Peritubular myoid cells	-	-
	Vessel wall	+++	+++
Epididymis			
Caput epididymis	Principal cells	-	+
	Narrow cells	-	+
	Basal cells	-	+
	Apical cells	-	+
	Vessel wall	++	++
	Interstitial cell in connective tissue	+	+
	Muscle layer in the ductal wall	++	++
Corpus epididymis	Principal cells	-	+
	Narrow cells	-	+
	Basal cells	-	+
	Apical cells	-	+
	Vessel wall	++	++
	Interstitial cell in connective tissue	+	+
	Muscle layer in the ductal wall	+	++
Cauda epididymis	Principal cells	-	+
	Narrow cells	-	+
	Basal cells	-	+
	Apical cells	-	+
	Vessel wall	++	++
	Interstitial cell in connective tissue	+	+
	Muscle layer in the ductal wall	++	+++

RESULTS

Positive and negative control

Colon tissue was chosen as a positive control, and the reaction was detected in the smooth muscle cells in the vessels and mucosa. No reaction was observed from the testicular tissue used as negative control (Figure 1).

Non-breeding period of pre-hibernation

We did not observe TLR4 immunostaining in germ cells within the testicular tissue. Moreover, Leydig and Sertoli cells did not show TLR4 expression. However, we observed a positive immune reaction in vessel wall, and some interstitial cells in the intertubular areas. The epididymis was analyzed in three distinct sections: caput, corpus, and cauda. We did not observe TLR4 expression in the epithelium of all three epididymis segments. Nevertheless, we noticed a TLR4 immunoreaction in vessel wall, the smooth muscle layer in the ductal wall and some interstitial cells in all three segments of the epididymis (Figure 2).

Hibernation

Interestingly, TLR4 expression was observed in spermatogonia and primary spermatocytes. We did not detect TLR4 immunostaining in Sertoli cells, peritubular myoid cells, and Leydig cells in the intertubular area, but intense TLR4 staining was observed in the vessel walls in the intertubular area. In contrast to the pre-hibernation period, TLR4 immune labeling was observed in the epithelial cells of the epididymis. In addition, TLR4 labeling was detected in the smooth muscle layer in the ductal wall, some interstitial cells, and vessel walls in all three segments of the epididymis, similar to the pre-hibernation stage (Figure 3).

Quantitative image analysis of TLR4 immunostaining

Compared to the non-breeding period of pre-hibernation, TLR4 expression in the testis was significantly elevated during hibernation. TLR4 expression variations were identified in epididymis sections during the pre-hibernation. The cauda epididymis exhibited the highest expression of TLR4, whereas the corpus epididymis displayed the lowest TLR4 expression during the pre-hibernation. Curiously, the corpus epididymis had the most pronounced expression of TLR4 during the hibernation period. There was no significant variation observed in TLR4 expression between the caput and cauda epididymis (Figure 4).

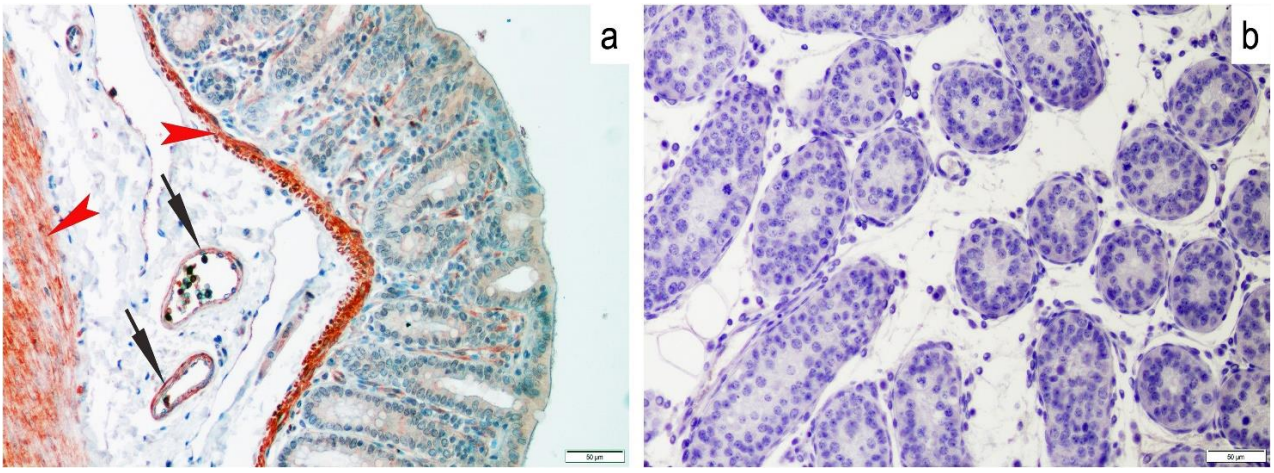

Figure 1: Colon tissue as a positive control for TLR4 (a) and testis as a negative control (b). Red arrowheads: smooth muscle cells. Black arrows: vessel walls.

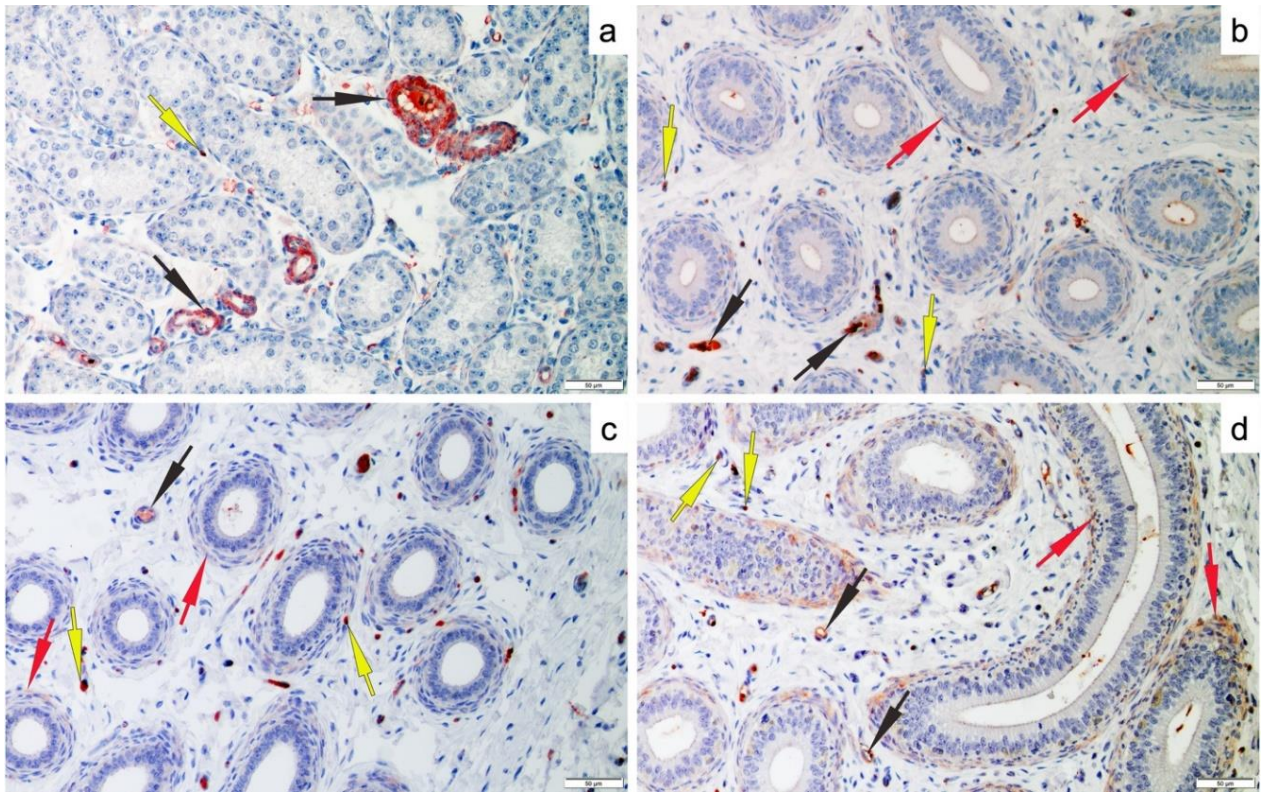

Figure 2: Immunohistochemical localization of TLR4 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during the non-breeding period of pre-hibernation. Black arrows: Vessel walls. Yellow arrows: Interstitial cells. Red arrows: Muscle layer in the ductal wall of epididymis

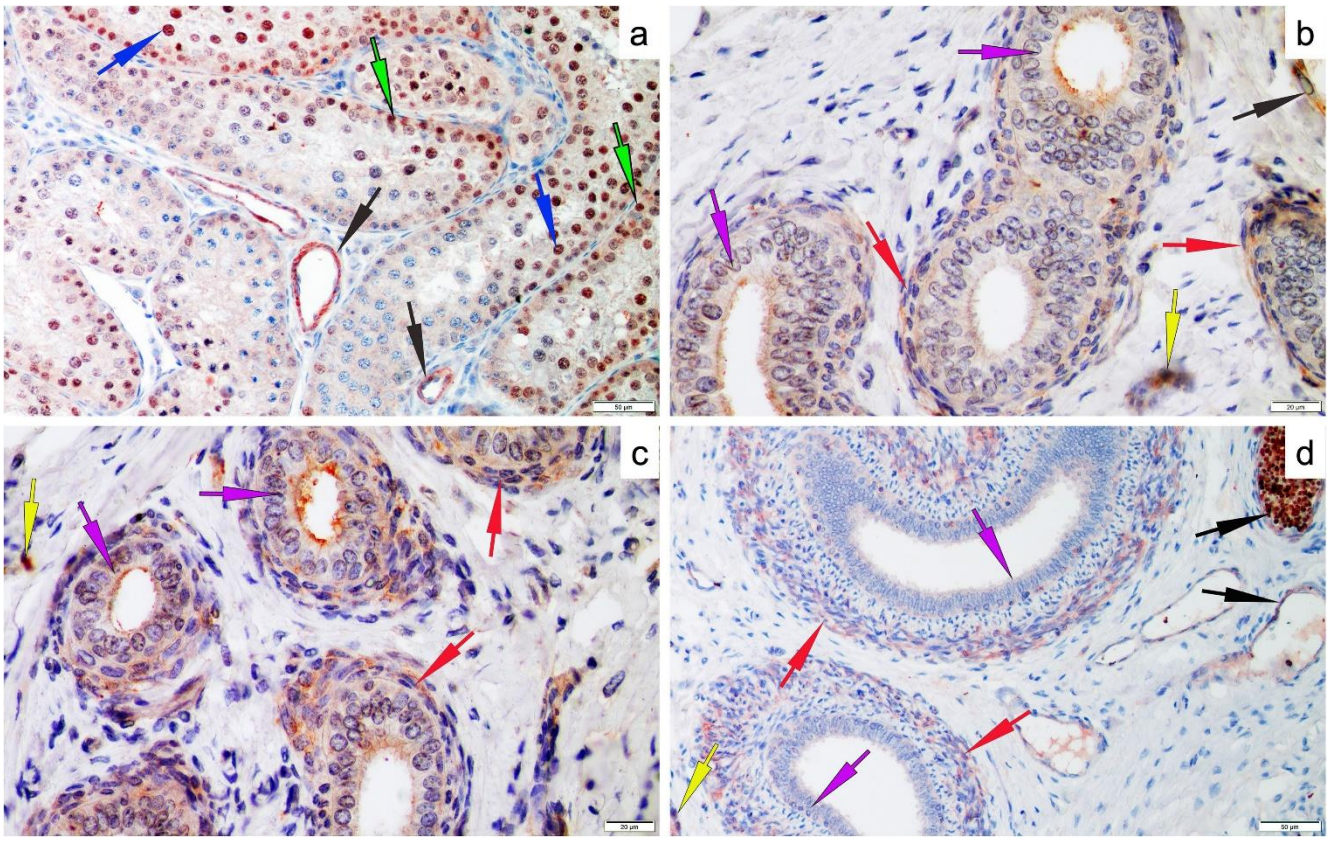

Figure 3: Immunohistochemical localization of TLR4 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during the hibernation. Black arrows: Vessel walls. Yellow arrows: Interstitial cells. Red arrows: Muscle layer in the ductal wall of epididymis. Green arrows: Spermatogonium. Blue arrows: Primary spermatocytes. Purple arrows: Epididymal epithelium

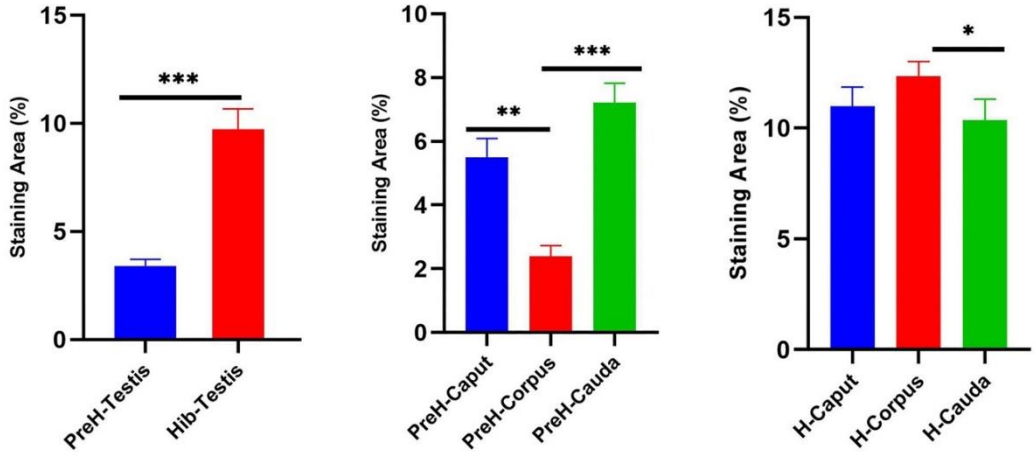

Figure 4: Quantitative evaluation of TLR4 immunostaining during the pre-hibernation (PreH) and hibernation (H) periods. Statistical significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

The present study offers a comprehensive analysis of the dynamic expression patterns of TLR4 in the epididymis and testis of Anatolian ground squirrels *(Spermophilus xanthoprymnus)* throughout the pre-hibernation and hibernation periods, which are devoid of reproductive activity. Significant adaptive changes in immune regulation within the reproductive organs are revealed by the findings; these changes are crucial for comprehending how these animals maintain reproductive function and general health throughout these distinct physiological states.

The absence of TLR4 immunostaining in germ cells, Leydig cells, and Sertoli cells within the testicular tissue was conspicuous during the pre-hibernation period. This is consistent with the well-established notion that the testis functions as an immunoprivileged site, a

location where protective immune responses against pathogens are strictly regulated (Meinhardt & Hedger, 2011; Zhao et al., 2014). The blood-testis barrier and local immunosuppressive environments play a critical role in preserving the testis' immunoprivileged status. These mechanisms safeguard developing germ cells against potential immune assaults (Fijak & Meinhardt, 2006; Hedger, 2015).

Nevertheless, the identification of TLR4 in specific interstitial cells and vessel walls within the testicular intertubular regions indicates that these cells are involved in overseeing the local immune response and regulating inflammation. This aligns with prior research that emphasizes the contribution of interstitial and vascular cells to the regulation of the immune system in the testis Gu et al., 2022; Hedger, 2011a; Heinrich & DeFalco, 2020).

During pre-hibernation, the absence of TLR4 expression in the caput, corpus, and cauda segments of epithelial cells in the epididymis suggests the presence of a protective mechanism that aims to shield maturing sperm from potential immune attacks. On the contrary, TLR4 immunoreactivity was detected in certain interstitial cells, the smooth muscle layer of the ductal wall, and vessel walls, indicating that these anatomical components might participate in local immune reactions that regulate the immune environment in a way that promotes sperm maturation and storage (Hermo & Robaire, 2002; Hu et al., 2016). In contrast, TLR4 expression patterns underwent substantial alterations during the hibernation period. Observations of TLR4 expression in spermatogonia and primary spermatocytes indicate that germ cells enter hibernation with an enhanced immune readiness. Potential infections or stress may be evaded through this mechanism while in the metabolically dormant state of hibernation (Bouma et al., 2010; Carey et al., 2003; van Breukelen & Martin, 2015). The significance of vascular structures in immune regulation during hibernation is underscored by the lack of TLR4 in Sertoli cells, peritubular myoid cells, and Leydig cells, in conjunction with the prominent labeling of TLR4 on the vessel walls.

TLR4 immune labeling was notably observed in the epididymal epithelial cells throughout the hibernation phase, as opposed to the pre-hibernation phase. This implies that in order to safeguard spermatozoa during the susceptible hibernation phase, an adaptive immune mechanism is engaged. This concept is further substantiated by the consistent labeling of TLR4 in the smooth muscle layers, interstitial cells, and vessel walls throughout all segments of the epididymis while it is in hibernation. This suggests that the immune system places greater emphasis on protecting the reproductive tract from potential infections (Tung et al., 2022; Wang et al., 2019).

CONCLUSION

The intricate relationship between reproductive physiology and the immune system is highlighted by the dynamic expression of TLR4 in the testis and epididymis of Anatolian ground squirrels throughout the various seasonal phases. The alterations in TLR4 expression indicate that adaptive modulation of immune regulatory mechanisms safeguards reproductive health and guarantees effective reproduction, in spite of the physiological difficulties presented by hibernation. Future research should focus on clarifying the molecular processes that govern the modulation of TLR4 and its functional consequences in the testis and epididymis during hibernation. In addition, studying the interplay between TLR4 and other immune pathways could offer a more profound understanding of the immunological tactics utilized by hibernating animals to sustain reproductive function and overall well-being.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: Idea, concept and design: MÖ Data collection and analysis: MÖ, Drafting of the manuscript: MÖ, Critical review: MÖ

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