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RESEARCH ARTICLE

Galectin-1 and -3 Expression in the Testis and Epididymis of Anatolian Ground Squirrels *(Spermophilus xanthoprymnus)* during Non-Breeding Periods of Pre-Hibernation and Hibernation

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Abstract

This study aims to investigate the expression patterns of Galectin-1 (Gal-1) and Gal-3 in the testis and epididymis of Anatolian ground squirrel (*Spermophilus xanthoprymnus*) during nonbreeding pre-hibernation and hibernation periods. Hibernation is a physiological state characterized by a reduction in metabolic rate and body temperature. Gal-1 and -3 are implicated in many biological functions. Twelve squirrels were used in this study. Followed by routine tissue processing, tissue samples underwent immunohistochemical procedure. Histological examination and statistical analysis were performed. Immunohistochemical investigation revealed that Gal-1 expression during pre-hibernation was confined to peritubular myoid cells and vascular smooth muscle cells, with no expression observed in Sertoli or spermatogenic cells. Gal-1 in the epididymis was localized to smooth muscle cells encircling the epithelium and within blood vessel walls, exhibiting markedly elevated expression across the caput, corpus, and cauda regions. During hibernation, testicular and epidydimal Gal-1 expression exhibited a considerable reduction. During pre-hibernation, Gal-3 exhibited a unique pattern, with expression noted in the seminiferous epithelium and Leydig cells. Gal-3 was detected in the epithelial cells throughout the epididymis, with greater intensity in specific epithelial cells. During hibernation, Gal-3 expression increased in Sertoli cells, spermatogonia, and spermatocytes within the testis, while exhibiting diminished intensity in the epididymal epithelium across all regions. The findings suggest that Gal-1 and -3 may be involved in seasonal reproductive adaptability during nonbreeding pre-hibernation and hibernation. Further research could clarify their specific molecular functions in hibernating species.

Key Words: Anatolian ground squirrel, epididymis, galectin-1, galectin-3, testis

Üreme Dışı Aktif ve Hibernasyon Dönemlerinde Anadolu Yer Sincabı (*Spermophilus xanthoprymnus*) Testis ve Epididimisinde Galektin-1 ve -3 Ekspresyonu

Öz

Bu çalışma, üreme dışı aktif ve hibernasyon dönemlerinde Anadolu yer sincabı (*Spermophilus xanthoprymnus*) testis ve epididimisinde Galectin-1 (Gal-1) ve Gal-3 ekspresyonunu araştırmayı amaçlamaktadır. Hibernasyon, metabolik hız ve vücut sıcaklığındaki azalma ile karakterize fizyolojik bir durumdur. Gal-1 ve Gal-3 birçok biyolojik fonksiyonda rol oynamaktadır. Bu çalışmada on iki sincap kullanılmıştır. Rutin doku işleme tabi tutulduktan sonra, doku örneklerine immünohistokimyasal boyamalar yapıldı. Boyamalar histolojik olarak incelenip istatistiksel analizleri yapıldı. İmmünohistokimyasal inceleme, aktif dönemde Gal-1 ekspresyonunun peritübüler miyoid hücreler ve vasküler düz kas hücreleriyle sınırlı olduğunu, Sertoli veya spermatogenik hücrelerde ekspresyon gözlemlenmediğini ortaya koymuştur. Epididimisde Gal-1, epiteli çevreleyen düz kas hücrelerinde ve kan damarı duvarlarında lokalize olmuş, kaput, korpus ve kauda bölgelerinde belirgin şekilde yüksek ekspresyon sergilemiştir. Hibernasyon sırasında testis ve epididimal Gal-1 ekspresyonunda önemli bir azalma gözlenmiştir. Aktif dönemde Gal-3 kendine has bir ekspresyon göstermiş olup seminifer epitel ve Leydig hücrelerinde gözlemlenmiştir. Gal-3, epididimis boyunca epitel hücrelerinde tespit edilmiş, belirli epitel hücrelerinde daha yoğun olarak bulunmuştur. Hibernasyon sırasında Gal-3 ekspresyonu testisteki Sertoli hücrelerinde, spermatogonyumlarda ve spermatositlerde artarken, tüm bölgelerde epididimal epitelde yoğunluk azalmıştır. Bulgular, Gal-1 ve Gal-3'ün üreme dışı aktif dönem ve hibernasyon sırasında mevsimsel üreme adaptasyonunda rol oynayabileceğini göstermektedir. Hibernasyona yatan türlerde spesifik moleküler işlevlerini açıklığa kavuşturmak için daha fazla çalışmaya ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Anadolu yer sincabı, epididimis, galektin-1, galektin-3, testis

INTRODUCTION

Hibernation is a distinctive physiological adaptation that involves a profound suppression of metabolic activity, a significant decrease in body temperature, and the downregulation of various physiological processes, enabling animals to conserve energy and endure extended periods of environmental stress, such as cold temperatures and limited food resources (1). Seasonal reproduction is an adaptation strategy observed in numerous wild animals. This technique aligns reproductive efforts with the season most conducive to the survival and growth of progeny. Male seasonal breeders exhibit coordinated phases of testicular maturation and regression during the reproductive cycle. This results from the annual environmental fluctuations and energy constraints they encounter (2-4).

Galectins, a group of β-galactoside-binding proteins, are crucial regulators of diverse biological functions, such as cell growth, programmed cell death, and immune system modulation (5). These proteins possess a conserved carbohydrate recognition domain (CRD) and a core consisting of 130 amino acids (6). The lectin family is characterized by two fundamental traits: a high affinity for galactosides and significant similarity in their amino acid sequences (7). These attributes are the principal distinguishing characteristics. Researchers have identified fifteen different galectins in mammals, labeled gal-1 through gal-15 based on their specificity. Galectins can be categorized into three fundamental types: prototype galectins, chimeric galectins, and tandem repeat galectins. The classifications are predicated on the structural composition of the galectins (8). The expression of Gal-1 undergoes dynamic control throughout the spermatogenic cycle (9,10). At the luminal pole of the rat seminiferous epithelium, this lectin has heightened expression throughout the spermiation stages (VI–VIII) and is predominantly expressed in Sertoli cells during stages X–XII of the cycle (10). This phase is marked by its occurrence on the apical projections of Sertoli cells, the heads of mature spermatids, and the residual cytoplasmic bodies of the spermatids. Upon the conclusion of the eighth phase of spermiation, Gal-1 expression is reinstated at the basal region of Sertoli cells. As germ cell differentiation progresses, its expression progressively extends throughout the entire cell (11,12). Furthermore, the expression of Gal-1 has been identified in the Leydig cells of rats (9). While many galectin family members, such as Gal-1, are known to promote apoptosis, Gal-3 exhibits the opposite effect by acting as an anti-apoptotic molecule (13). It has been demonstrated that in rats, Gal-3 is expressed in Leydig cells, peritubular myoid cells, interstitial CD68-positive macrophages, Sertoli cells, smooth muscle cells, and the epididymal epithelium. Moreover, the expression profile of Gal-3 appears to undergo significant modulation across distinct stages of postnatal development (9).

Anatolian ground squirrels (*Spermophilus xanthoprymnus*) are communal, diurnal rodents that predominantly consume herbivorous diets and engage in burrowing behavior. From late summer to early spring, these rodents hibernate within subterranean burrows. The Anatolian ground squirrel exhibits sexual activity from mid-March to late-April, followed by an extended period of sexual inactivity from late-April to mid-March, and hibernates from late August to mid-March (14-16).

By investigating the expression dynamics of Gal-1 and -3 in the testis and epididymis of Anatolian ground squirrels during the nonbreeding pre-hibernation and hibernation periods, this study aims to uncover the molecular mechanisms underlying reproductive quiescence and subsequent reactivation. Understanding these patterns will provide deeper insights into the adaptive strategies employed by hibernating mammals to synchronize their reproductive cycles with environmental changes, thus enhancing our knowledge of mammalian reproductive biology and potentially informing conservation strategies for hibernating species.

MATERIAL AND METHODS

We collected samples from six animals in the nonbreeding pre-hibernation period and six animals in the hibernation period of the Anatolian ground squirrel (*Spermophilus xanthoprymnus*) to obtain testicular and epididymal tissues. To ensure the preservation of cellular and structural integrity, the tissues were promptly excised and fixed in Bouin's solution following euthanasia. After being dehydrated in a graded series of alcohol concentrations, the fixed specimens were processed through methyl benzoate and benzol for clearing, then embedded in paraffin to enable microtome sectioning.

Immunohistochemistry

The presence and localization of Gal-1 and -3 in the tissue samples were both detected using immunohistochemistry. Deparaffinized and rehydrated sections were exposed to citric acid retrieval solution (pH 6.0) and heated in a microwave to facilitate antigen retrieval. Following the cooling process to 20-25°C, the sections were thoroughly rinsed with PBS to ensure the removal of any residual reagents and to prepare them for subsequent steps. In order to inhibit endogenous peroxidase activity, the samples were rinsed after being treated with 3% H_2O_2 in PBS for 20 minutes in a dark environment. To minimize non-specific binding and enhance the specificity of antibody interactions, the slides were pre-incubated with 10% normal goat serum for 10 minutes at room temperature. Using Gal-1 Polyclonal (1:400, Novus Biologicals, NBP1-89791) and Gal-3 Monoclonal (1:400, Novus Biologicals, NB300-538) antibodies, primary antibody incubation was performed overnight at 4°C. After that, the samples were treated with a secondary antibody, followed by enzyme-conjugated streptavidin to enhance signal detection. In positive cells, the antibody-antigen complexes were visualized using AEC (3-amino-9-ethylcarbazole) chromogen solution, which resulted in a red coloration. Afterward, the samples were counterstained with Gill's II hematoxylin. The primary antibody was replaced with PBS in the negative control, and the same procedures were subsequently adhered to. Colon tissue was employed as a positive control. Images were captured from the sections using an Olympus BX51 research microscope with an integrated DP72 digital camera and then analyzed.

Quantitative Evaluation of Immunohistochemical Staining

This study quantitatively assessed the staining intensities of Gal-1 and -3 using the method outlined by a previous study

(17). Quantitative analyses of staining intensities for Galectin-1 and Galectin-3 were conducted using images acquired from the testis and epididymis. The intensity of immunostaining was quantified for the entire image. The testis and epididymis images of squirrels were obtained at 400X magnification during the nonbreeding pre-hibernation and hibernation periods. Ten random images were utilized for each group. Relevant images were imported into ImageJ (version 1.51, Java 1.8.0_112, https://imagej.nih.gov/ij/) and processed using the "color deconvolution" plug-in, where the staining of hematoxylin and AEC was separated into three distinct panels with the only hematoxylin, with the only AEC image, and with the only background image. Threshold values were set for only the AEC images. Subsequently, parameters for area and area fraction, namely the percentage of staining area referred to as "staining intensity," were set. After that, the area and percentage of the staining area were calculated for each image.

Statistical Analysis

GraphPad Prism 7 for Windows (Version 7.04) was employed to conduct statistical analyses. The software was used to quantitatively evaluate the staining intensity (% staining area) by importing percentage values for the stained areas. Student's t-test was conducted to compare staining intensity of the testis, caput, corpus, and cauda epididymis between the pre-hibernation and hibernation periods. Data are presented as mean ± SEM, and significance was considered at $p<0.05$.

RESULTS

Colon tissues were utilized as positive controls. Gal-1 expression was localized to the smooth muscle cells within the mucosa and vascular structures, whereas Gal-3 was prominently expressed in the intestinal epithelial cells (Figure 1).

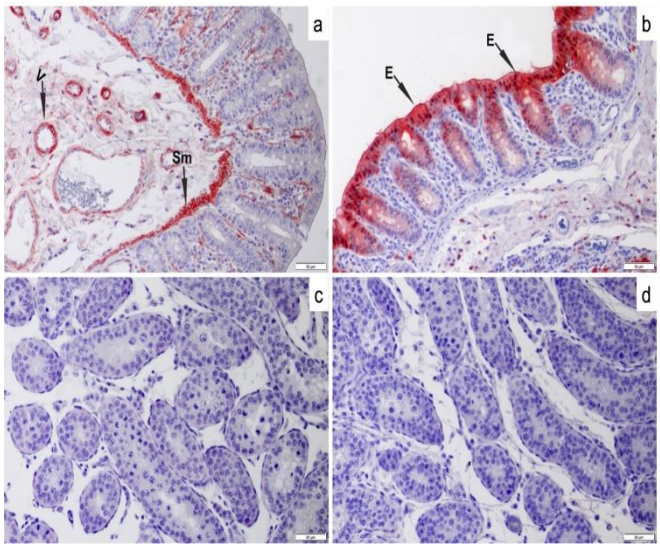

Figure 1. Colon tissue as a positive control for Gal-1 (a) and Gal-3 (b). Testis as a negative control (c, d). E: Intestinal epithelium. Sm: smooth muscle cells. V: vessel walls. Bar: 50 um.

Gal-1 Immunostaining

In the pre-hibernation period, Gal-1 expression was not observed in the seminiferous epithelium of the testis, specifically in either Sertoli cells or spermatogenic cells. A positive reaction was observed in peritubular myoid cells and in the walls of blood vessels within the intertubular area. No positive reaction was detected in the epithelial cells of the caput, corpus, or cauda regions of the epididymis. However, we identified a positive Gal-1 reaction in the smooth muscle cells surrounding the epididymal epithelium and in the walls of blood vessels located within the connective tissue (Figure 2).

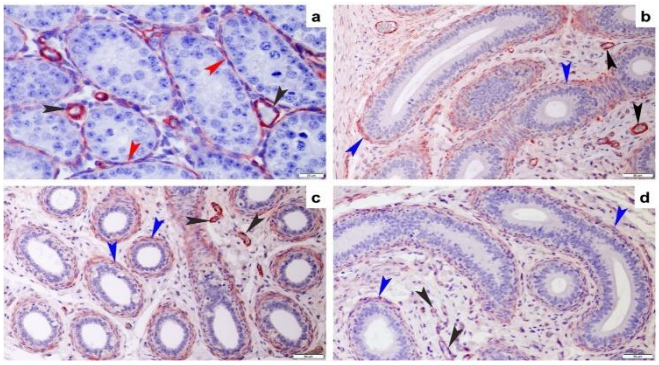

Figure 2. Immunohistochemical localization of Gal-1 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during the non-breeding period of pre-hibernation. Black arrowhead: Vessel walls. Red arrowhead: Peritubular myoid cells. Blue arrowhead: Muscle layer in the ductal wall of epididymis. Bar: 20 um (a), 50 um (b, c, d).

During the hibernation period, the pattern of Gal-1 immunostaining closely resembled that observed during the pre-hibernation phase. In the testis, Gal-1 expression was predominantly localized to the peritubular myoid cells and vascular smooth muscle cells. Similarly, in the epididymis, Gal-1 immunoreactivity was evident in the smooth muscle cells surrounding the epididymal epithelium and in the vascular walls within the connective tissue, consistent with pre-hibernation findings (Figure 3).

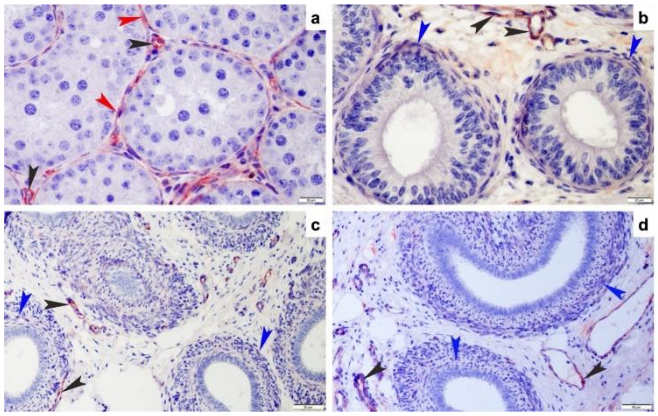

Figure 3. Immunohistochemical localization of Gal-1 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during hibernation. Black arrowhead: Vessel walls. Red arrowhead: Peritubular myoid cells. Blue arrowhead: Muscle layer in the ductal wall of epididymis. Bar: 20 um (a, b), 50 um (c, d).

Analysis of Gal-1 expression in testicular and epididymal tissues revealed significant variations between pre-hibernation and hibernation periods. In the testis, Gal-1 levels were notably elevated during the pre-hibernation phase ($p<0.01$). In the caput region of the epididymis, Gal-1 levels were markedly elevated in pre-hibernation ($p<0.0001$). In

the corpus region, Gal-1 levels were markedly elevated during the pre-hibernation phase (p<0.0001). Similarly, in the cauda region, Gal-1 expression was significantly higher in pre-hibernation compared to the hibernation period (p<0.0001). (Figure 4a).

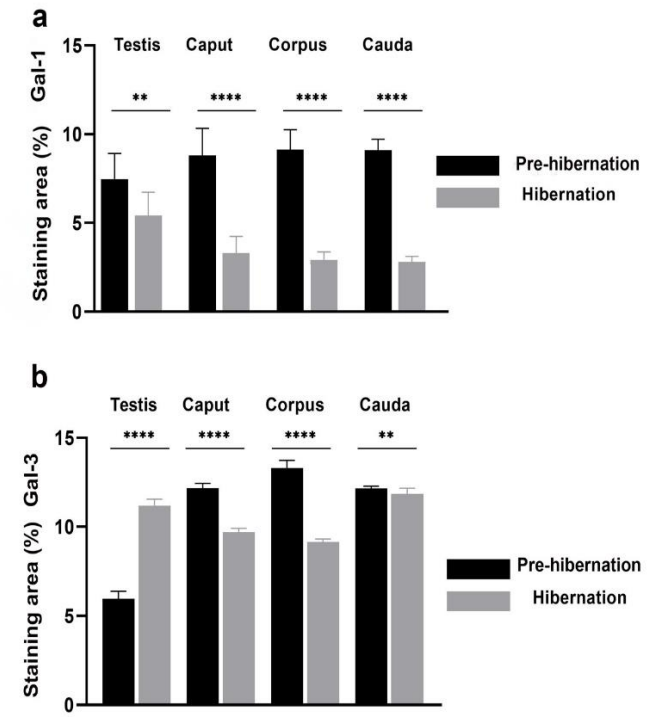

Figure 4. Quantitative evaluation of Gal-1 (a) and Gal-3 (b) immunostaining during the pre-hibernation and hibernation periods. Statistical significance is denoted as follows: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Gal-3 Immunostaining

In the pre-hibernation period, Gal-3 immunostaining showed a distinct pattern from that of Gal-1, with expression observed within the seminiferous epithelium of the testis. Positive Gal-3 expression was also detected in Leydig cells located in the intertubular area. In the epididymis, intense Gal-3 staining was noted in the epithelial cells of the caput, corpus, and cauda regions. Interestingly, intraepithelial some cells within the epididymal epithelium exhibited more intense Gal-3 expression compared to other cell types in this tissue (Figure 5).

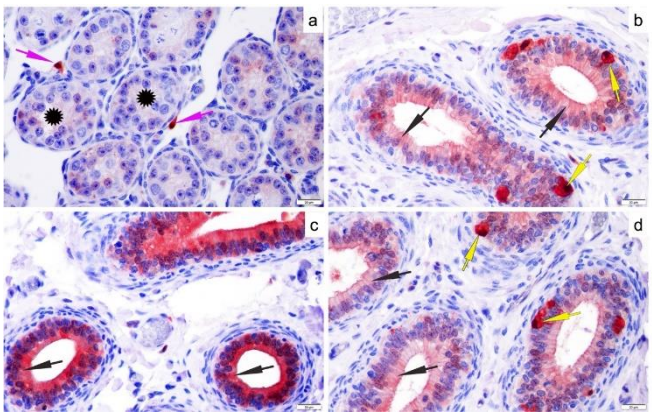

Figure 5. Immunohistochemical localization of Gal-3 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during non-breeding period of pre-hibernation. Asterisk: Seminiferous epithelium. Black arrow: Epididymal epithelium. Yellow arrow: Some intraepithelial cells staining intensely. Purple arrow: Leydig cell. Bar: 20 um.

During the hibernation period, Gal-3 immunoreaction was observed to increase within the seminiferous epithelium of the testis, displaying more intense staining compared to the pre-hibernation period. Gal-3 immunostaining was specifically detected in Sertoli cells, spermatogonia, and spermatocytes. Positive Gal-3 expression was also observed in Leydig cells situated in the intertubular region. In the epididymis, Gal-3 expression was present in the epithelial cells of the caput, corpus, and cauda regions, similar to the pre-hibernation period, but with reduced staining intensity (Figure 6).

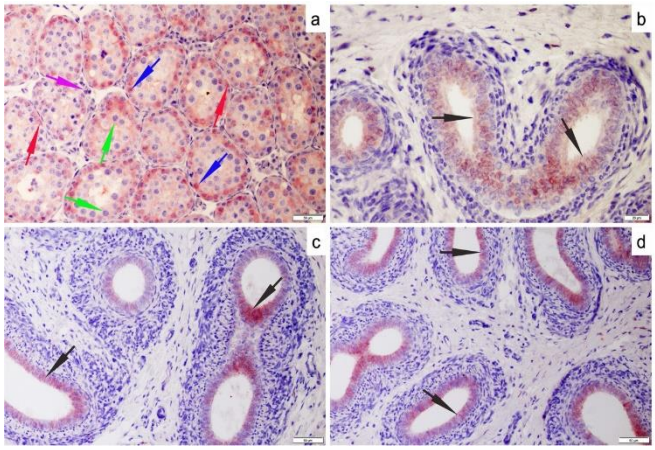

Figure 6. Immunohistochemical localization of Gal-3 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during hibernation. Black arrow: Epididymal epithelium. Red arrow: Sertoli cells. Purple arrow: Leydig cell. Blue arrow: Spermatogonium. Green arrow: Spermatocytes. Bar: 20 um (b), Bar: 50 um (a, c, d).

During the hibernation period, Gal-3 expression in the testis was observed to be significantly higher compared to the pre-hibernation period (p<0.01). In contrast, in the epididymis, Gal-3 expression was significantly higher in the pre-hibernation period across all regions. Specifically, the caput region showed a highly significant decrease in Gal-3 expression during hibernation (p<0.0001), as did the corpus region (p<0.0001), while the cauda region also demonstrated a significant reduction (p<0.01) in expression during hibernation compared to pre-hibernation levels (Figure 4b).

DISCUSSION AND CONCLUSION

This study elucidates the unique expression patterns of Gal-1 and -3 in the testis and epididymis of the Anatolian ground squirrel throughout pre-hibernation and hibernation phases. The findings indicate that Gal-1 and -3 have distinct functions in modulating the adaptive response of reproductive system to hibernation, essential for seasonal breeders.

Our results show that Gal-1 expression in the testis was confined to vascular smooth muscle cells and peritubular myoid cells, with no expression in Sertoli or spermatogenic cells. In the epididymis, Gal-1 was detected in the smooth muscle cells surrounding the epithelium and in blood vessel walls. Quantitative analysis revealed significantly higher expression levels of Gal-1 in the epididymis during the pre-hibernation period across all regions (caput, corpus, and cauda) compared to hibernation (p<0.0001). In a study conduc-

ted on rats, unlike in our findings, Gal-1 expression was observed in Sertoli cells, particularly with intense immunoreactivity in the apical regions of Sertoli cells and in the heads of mature spermatids during spermiation. Following spermiation, Gal-1 expression was detected in the basal segments of Sertoli cells and gradually extended throughout as germ cell differentiation progressed (10). In humans, a high concentration of Gal-1 was observed in peritubular myoid cells, consistent with our findings (12). Özbek et al. (9), in contrast to our findings, reported positive Gal-1 expression in Sertoli and Leydig cells, while observing no Gal-1 reactivity in peritubular myoid cells in rat (9). This expression variation implies that Gal-1 may fulfill species-specific functions within the reproductive system, potentially adapting to the distinct physiological and structural requirements of each species.

The testis and epididymis exhibited divergent patterns of Gal-3 expression during the hibernation period. Gal-3 immunoreactivity was substantially increased in the testis, with intense expression observed in Sertoli cells, spermatogonia, and spermatocytes, suggesting a pronounced presence within the seminiferous epithelium. Conversely, the epididymis exhibited a substantial decrease in Gal-3 expression in all regions-corpus, cauda, and caput-compared to pre-hibernation levels. The quantitative analysis demonstrated that the decrease in Gal-3 immunoreactivity in the epididymis was statistically significant ($p<0.0001$ for caput and corpus, $p<0.01$ for cauda), indicating a substantial decrease in expression as the tissue transitions to a quiescent state. These findings align with certain aspects of previous research, though some differences highlight species-specific variations in Gal-3 expression. Khorsandi and Orazizadeh (18) observed Gal-3 expression in Leydig cells and peritubular myoid cells, but not in Sertoli cells of mouse testes. This contrasts with our findings, where Sertoli cells showed significant Gal-3 immunoreactivity, particularly during hibernation. Similarly, Deschildre et al. (19) observed Gal-3 expression in Sertoli cells in rats, but noted the absence of Gal-3 in spermatocytes and spermatids. Our results, however, revealed Gal-3 immunoreactivity in both spermatogonia and spermatocytes, especially during hibernation. Additionally, Özbek et al. (9) reported no Gal-3 expression in spermatogenic cells, while we detected immunoreactivity in both Sertoli cells and certain spermatogenic cells. Similar to Gal-1, Gal-3 may exhibit species-specific differences in its expression patterns and functional roles within the testis. The increase in Gal-3 immunostaining in Sertoli cells and spermatogenic cells within the seminiferous epithelium during hibernation could be linked to known anti-apoptotic properties of Gal-3 (20). In conditions of reduced energy availability and environmental stress, as seen in hibernation, the role of Gal-3 in inhibiting apoptosis may be essential for cellular survival. This anti-apoptotic activity likely contributes to the preservation of germ cell integrity by protecting against cell death during prolonged metabolic suppression.

The epididymis is categorized into three separate regions: Caput, corpus, and cauda. The caput and corpus are chiefly responsible for spermatozoa maturation, whilst the cauda functions as a reservoir for mature sperm. Moreover, in rats, it is lined by an epithelium consisting primarily of principle and basal cells, along with less prevalent cell types such as apical, narrow, and halo cells (21). It has been reported that Gal-3 is intensely expressed in certain epithelial cells (22). Studies conducted in various species, such as rats (9), and bulls (23), have reported intense Gal-3 expression in the epididymal epithelium, with particularly high expression in the distal regions of the epididymis. Özbek et al. (9) suggest that the presence of Gal-3 in the corpus and cauda regions indicates an important role for this protein in the maturation and storage of spermatozoa. In our study, the observed decrease in Gal-3 expression in the epididymal epithelium during hibernation may reflect a reduction in the need for this function, as there may not be sufficient spermatozoa available for storage during this period, consistent with the hypothesis proposed by Özbek et al. (9).

This study demonstrates distinct expression patterns of Gal-1 and Gal-3 in the testis and epididymis of the Anatolian ground squirrel. Gal-1 was primarily localized in peritubular myoid cells and vessel wall in testis, with higher expression in pre-hibernation, suggesting a structural role adapted to seasonal reproductive demands. Gal-3 showed increased expression in Sertoli and spermatogenic cells during hibernation, likely due to its anti-apoptotic properties. In the epididymis, Gal-3 was more intense in pre-hibernation, particularly in distal regions, indicating a role in sperm maturation and storage, which declines during hibernation as reproductive activity reduces. Further research could clarify their specific molecular functions in hibernating species.

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CONFLICT OF INTEREST

The authors state no conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS

Mehmet ÖZBEK: Writing, editing, methodology, investigation. Mustafa ÖZTOP: Writing, validation, visualization, editing.

ETHICAL STATEMENT

All animal experiments in this study were performed in accordance with the ethical guidelines established by the Erciyes University Animal Experiments Local Ethics Committee. Approval for the procedures was granted under the ethical approval number 15/140, Kayseri.

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