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Expression of the Homeobox Proteins Dlx-5 and TLX1 During the Development of Cat Testis

Expresión de las Proteínas Homeobox Dlx-5 y TLX1 Durante el Desarrollo del Testículo de Gato

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SUMMARY: Recent studies have shown that homeobox proteins play an important role in the formation and development of tissues and organs in the embryonic period. In our study, the distribution of Dlx-5 and TLX proteins, which are members of the homeobox family, in the testis, epididymis and ductus deferens ducts of some cat breeds were investigated. For this purpose, in the study, 18 testes younger than six months (immature) and older than one year (mature) were examined under a light microscope using an immunohistochemical method (indirect streptavidin-biotin complex). While it was determined that Dlx-5 and TLX1 proteins were expressed at varying levels in cells in immature and mature cat testicles, epithelial cells of ductus epididymis and ductus deferens, and smooth muscle cells of ductus deferens, no differences were observed between cat breeds. Dlx-5 immunoreactivity was more intense in the testes, epididymis and deferens ducts of immature and mature compared to TLX1. These results suggested that both proteins play important roles in the development of male feline genital organs and in the secretion and differentiation of cells, and also further observation of Dlx-5 expression suggested that this protein may be more effective than TLX1 in testicular development and physiological processes.

KEY WORDS: Cat; Dlx-5; Immunohistochemistry; Testis; TLX1.

INTRODUCTION

Testicular development and spermatogenesis are both highly complicated processes, which are regulated by several hormones, cytokines and growth factors (Zhao *et al*., 2011). The testes have two main functions: the production of the male sex hormone testosterone and sperm cells. These functions are critical to male reproduction and the preservation of species (Martins-Lara *et al*., 2018). The epididymis is a dynamic organ, which hosts the maturation of spermatozoa. Spermatozoa not only mature by way of the absorptive and secretory functions of the epididymal epithelium, but also gain motility during their transit through the epididymis (Axnér, 2006). The transit of mature and motile spermatozoa to the ejaculatory ducts occurs through the ductus deferens, which is an essential part of the male reproductive system.

The Dlx proteins are the vertebrate homologues of the Drosophila distal-less gene, and act a critical role in regulating the formation of the distal appendage (Nishida *et al*., 2008). The Dlx-5 protein first encodes a DNA-binding homeobox protein, which is expressed in the anterior area of the mouse embryo during early embryonic development. In the later stages of development, it has been reported to occur in the branchial arches, ear, diencephalon and restricted regions of the telencephalon (Kimura *et al*., 2004). Furthermore, Dlx-5 is indicated to be involved in regulating the biosynthesis and activation of certain steroid hormones that control the development and maintenance of male sex characteristics and ensure the attainment of male reproductive health. This protein also positively affects androgen production in the differentiated fetal (Nackley *et al*., 2002; Nishida *et al*., 2008).

Having been initially described as a chromosomal translocation in humans, TLX1 is a potential transcription factor of the homeobox family that may take part in the development of the cranial sensory pathways. TLX1/Tlx1 (T-cell leukemia translocation, aka Hox11) is expressed by the central and peripheral nervous system and is reported to act a crucial role in both embryonic growth and normal spleen development (Logan *et al*., 1998). Described as being necessary for splenic development in mice, TLX1 is also

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reported to be involved in steroid synthesis by affecting the transcription factor, steroidogenic factor-1 (SF-1), which plays a part in steroid synthesis, reproduction and sexual differentiation (Zangen *et al*., 2014).

This study was aimed at investigating the Dlx-5 and TLX proteins, which are subunits of the homeobox proteins that regulate the formation site and development of embryonic tissues and organs, to determine the distribution in the testis, epididymis and deferens ducts in several domestic cat breeds under preservation (Ankara, Van and Iran), ii; to show whether the determined distribution differs between immature and mature cats, and to reveal the possible physiological roles of these proteins in the male cat reproductive system.

MATERIAL AND METHOD

Animals and samples. The research material consisted of 18 testicular tissue specimens collected from Angora, Persian and Van cats. The tissue specimens were obtained from some special veterinary clinics located in the Diyarbakır province and from the Polyclinic of Dicle University, Faculty of Veterinary Medicine. The tissue specimens were assigned to two groups, one including 9 immature testes belonging to juvenile animals younger than 6 months of age, and the other including 9 mature testes belonging to adult animals older than 1 year of age. All tissue specimens were trimmed and immersed in 10 % formaldehyde for 24 h for fixation. Once fixed, the specimens were bathed below flowing fountain water for 12 h. After, they were passed through a ranked alcohol series (70 %-100 %), and then to harden the tissues, through three methyl benzoate series, each lasting 12 h. Subsequently, the specimens were placed twice, for 45 min-periods, in benzole for polishing. After being suspended in a paraffin-benzene mixture for 15 min, the specimens were immersed in fluid paraffin for 3 h. Once the tissues were embedded in paraffin, 5-µm-thick serial sections were part from the paraffin blocks on a microtome (Leica RM-2125, Germany), and were moved onto adhesive slides.

Immunohistochemistry. For the detection of the Dlx-5 and TLX1 proteins, the serial sections were stained with the indirect streptavidin-biotin complex procedure. For this aim, the sections were first placed in xylol for 5 min, then passed through a ranked series of alcohol (100 %, 100 %, 96 %, 80 %, and 70 %; 3 min each), and washed in distilled water. Next, the tissues were immersed in a mixture of 270 ml methanol + 30 ml hydrogen peroxide for 20 min, and washed in distilled water. For antigen retrieval, the sections were seethed in citrate buffer (0.01 M, pH 6) at 95 0C for 20 min, and left to cool. The cooled sections were washed in 0.01 M phosphate buffer saline (PBS) to be subsequently reacted in a protein blocking (Ultra V Blok, Thermo Fisher Scientific, Lab Vision Corporation) for 15 min at room heat. At the end of the incubation period, while the normal sections were reacted with 1:100 diluted antibodies (Table I), the negative control sections were incubated with PBS at +4 oC for 12 h to affirm the correctness of the staining method. Following incubation, the sections were washed in PBS three times, every time for 5 min. Next, they were first placed in a biotinylated secondary antibody (Histostain Plus Bulk Kit, Zymed) and then in a streptavidin-peroxidase (Histostain Plus Bulk Kit, Zymed) for 20 min at room heat. This was followed by other three washings in 0.01 M PBS, each time for 5 min. Next, with an aim to visualize the antigen-antibody reaction, the preparations were suspended in 3'3-diaminobenzidine hydrochloride (DAB) for 5-15 min, and depending on the development of immunoreactions, the sections were transferred into distilled water to end the reaction. After washing all of the sections, background staining was performed with Mayer's hematoxylin for 2-3 min. Next, the sections were washed under flowing fountain water for 5-7 min. This was followed by passing the sections through a graded alcohol series of 96 %, 100 % and 100 %, running each stage for 3 min, and then through xylol I for 5 min and xylol II for 30 min. Lastly, the tissues were mounted in Entellan. Immunoreactions were observed under a Nikon Eclipse E400 (Nikon, Tokyo, Japan) research microscope accoutred with a digital camera (Nikon Coolpix 4500).

Semi-quantitative evaluation. Tissue immunoreactions for Dlx-5 and TLX1 were evaluated with a semi-quantitative scoring method (Saruhan *et al*., 2020), as either negative (-), weak (+), moderate (++) or strong (+++). This evaluation was made independently by two senior histologists (UT and MEA), based on average values. For this purpose, the presence of immunoreactions was investigated in the testis, epididymis and ductus deferens by microscopic examination at x40, x100, x200, and x400 magnification. The findings obtained were separately evaluated for the testis ((spermatogonia, spermatocytes, spermatids, sustentocytes (Sertoli cells), interstitial endocrine cells (Leydig cells), epididymis (epithelium and stroma), and ductus deferens (epithelial and smooth muscle cells)) (Table II).

Table I. Primary antibodies used for immunohistochemistry.

Antibodies	Clonality/Isotype	Host	Reactivity	Dilution	Catalog number
Dlx-5	Polyclonal/IgG	Rabbit	Human, Mouse, Rat	1/100	St John's Laboratory, model no: STJ92725
TLX1	Polyclonal/IgG	Rabbit	Human, Mouse, Rat	1/100	Invitrogen, cat no: PA5-34553

Table II. Intensity of immunoreactivity for Dlx-5 and TLX1 in the testis, epididymis and ductus deferens.

Animals	Organs	Cells	Immunohistochemical semi-quantitative scoring	
			Dlx-5	TLX1
MATURE	Testis	Spermatogonia	++++	++
		Spermatocytes	+/++	++
		Spermatids	++++	+/++
		Sertoli cells	++++	+/++
		Leydig cells	++++	++++
	Epididymis	Epithelial cells	++++	+++
		Stromal cells	+/++	+/++
	D. Deferens	Epithelial cells	+++/++++	+++
		Smooth muscle cells	+++	++/+++
	Testis	Germ cells	++++	++
		Sertoli cells	++++	++
IMMATURE	Epididymis	Epithelial cells	++++	++
		Stromal cells	+/++	+/++
	D. Deferens	Epithelial cells	+++	++
		Smooth muscle cells	+/++	+/++

RESULTS

Positive immunoreactivity for Dlx-5 was observed in both the immature and mature testes. In the immature testis, immunoreactions were strong and localized to the germ and sustentocytes. Similarly, in the mature testis, strong immunoreactions were defined in the sustentocytes, spermatogonia and spermatids within the seminiferous tubules as well as in the interstitial endocrine cells, distributed in between the seminiferous tubules. On the other hand, the spermatocytes displayed a weak immunoreactivity for Dlx-5 (Fig. 1A-B). Positive immunoreactivity for TLX1 was observed in some specific germ cells in the immature feline testis (Fig. 1C). On the other hand, in the mature feline testis, immunoreactions were strong in the Leydig cells, but weak in the cells within the seminiferous tubules. Moreover, some vascular endothelial cells also displayed positive immunoreactivity (Fig. 1D).

Immunoreactivity for Dlx-5 was strong and localized to the nuclei of the epithelial cells lining the adult and juvenile epididymis (Fig. 2A-B). While TLX1 immunoreactivity was scarce in the epithelial cells of the juvenile epididymis, it was of moderate intensity and localized to the cytoplasm in the mature epididymis (Fig. 2C-D).

Dlx-5 immunoreactions, which were of moderate intensity and localized to the epithelial cells of the immature ductus deferens, ranged from moderate to strong and were localized to both the nucleus and cytoplasm in the epithelium of the mature ductus deferens. Immunoreactions seen in the smooth muscle cells were weak in the immature specimens and moderate in the mature specimens (Fig. 3A-B-C). In the immature ductus deferens, immunoreactions for TLX1 were very weak in the epithelial and smooth muscle cells. On the other hand, in the mature ductus deferens, TLX1 immunoreactivity was of moderate intensity and localized to the apical cytoplasm of the epithelial cells, and ranged from weak to moderate in the smooth muscle cells (Fig. 3C-D).

In order to prove the correctness of the immunohistochemistry procedure, no positive immunoreaction was observed in negative controls that were reacted with PBS in place of antibodies (Fig. 4).

DISCUSSION

The male reproductive system is comprised of the testes, which are the main reproductive organs, and the ducts that enable the maturation and transit of the spermatozoa produced in the testes. The testis is a significant organ, owing to the hormones secreted from the interstitial endocrine cells and sustentocytes as well as the spermatozoa produced within (Arı & Kamiloglu, 2015). Several cellular and hormonal interactions as well as certain proteins are required for the physiological functions of the testes. In view of the very limited number of studies available on the expression of the Dlx-5 and TLX1 proteins in the male reproductive system, the present study was aimed at the investigation of the expression of the Dlx-5 and TLX1 proteins in the cells

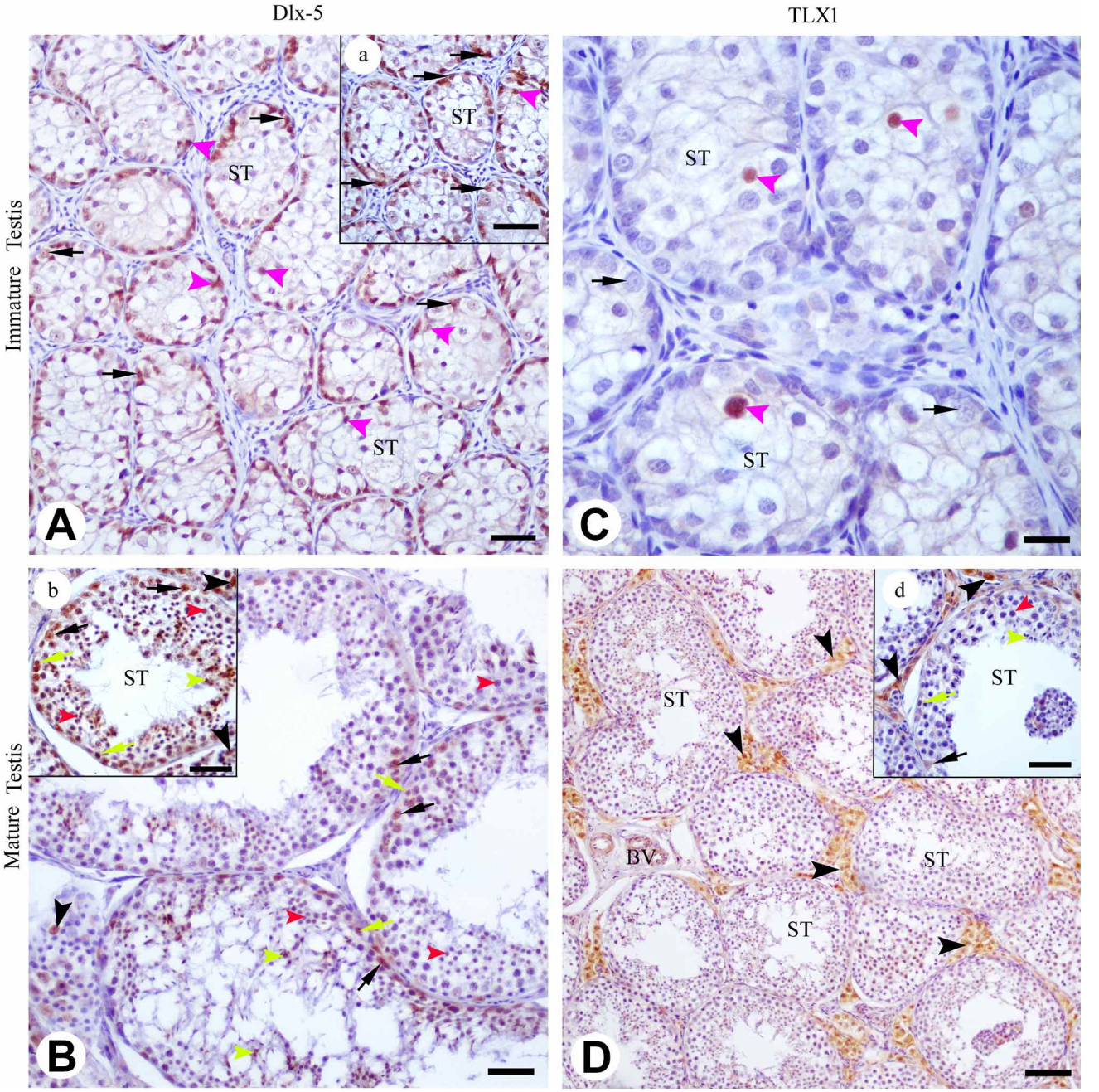

Fig. 1. Dlx-5 and TLX1 immunoreactivity in the immature and mature feline testes. ST: Seminiferous tubule, BV: Blood vessel, Pink arrowhead: Germ cell, Black arrow: Sertoli cell, Black arrowhead: Leydig cell, Yellow arrow: Spermatogonia, Red arrowhead: Spermatocyte, Yellow arrowhead: Spermatid. Bar: 25 µm (C, a, b, d), 50 µm (A, B, D).

found within the testes, as well as in the epididymis and deferens ducts.

Dlx-5 has been reported to be expressed in the Leydig cells of the testes in fetal mice. Thereby, it has been indicated that, in the differentiated fetal testis, Dlx-5 contributes to the secretion and regulation of the steroid hormones that define the male sex characteristics. While the same study has also reported the absence of Dlx-5 expression in the Leydig cells of mature testes, it has demonstrated that Dlx-5 is expressed in mature ovaries, but not in immature ovaries. This suggests that, Dlx-5 contributes to steroidogenesis rather than cell differentiation. Furthermore, reduced testosterone levels and abnormal male characteristics having been determined in Dlx5/6 mutant mice suggests that these proteins may serve multiple functions in the developing tes-

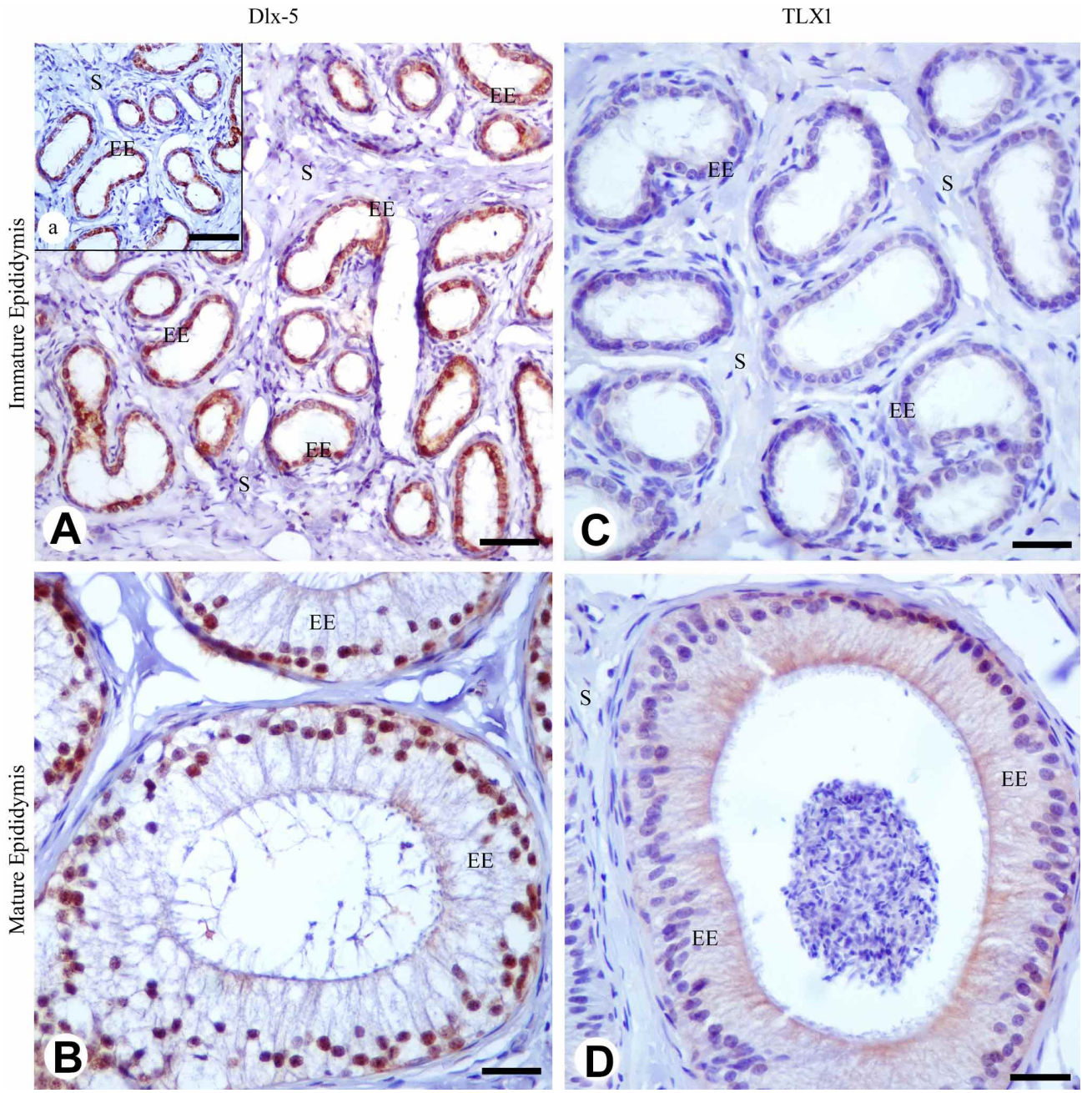

Fig. 2. Dlx-5 and TLX1 immunoreactivity in the immature and mature feline epididymis. EE: Epididymal epithelium, S: Stroma. Bar: 25 µm (a, B, D), 50 µm (A, C).

tes (Nishida *et al*., 2008). To our knowledge, there is no further study on Dlx-5 in the male reproductive system. In general, the presence of Dlx-5 has been reported to be associated with craniofacial development, the genital bud, osteogenesis, the morphogenesis of the sensory organs and placental development. Furthermore, the deficiency of this protein has been reported to be associated with malformations of the limbs (hands and feet) (Merlo *et al*., 2000). In the present study, similar to the case in the testes of mouse fetuses, Dlx-5 was found to be expressed in the Leydig cells of the mature feline testes. Furthermore, Dlx-5 immunoreactivity having been detected to be strong in the cells of both the immature and mature testes suggests that this protein may be involved in the development and differentiation of cells in the feline testes, as well as in the secretion of several hormones and development of

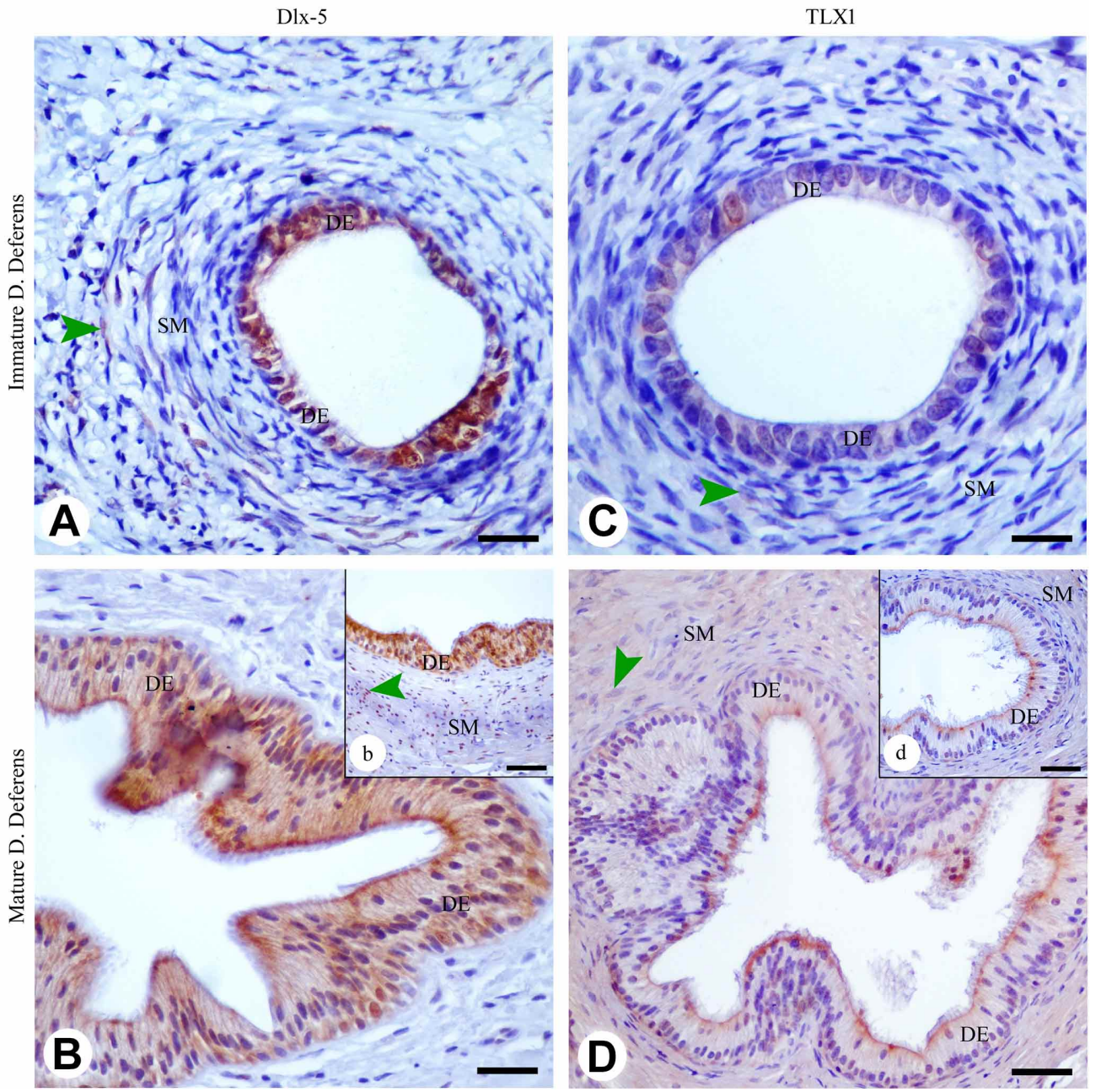

Fig. 3. Dlx-5 and TLX1 immunoreactivity in the immature and mature feline ductus deferens. DE: Deferens epithelium, SM: Smooth muscle layer, Green arrowhead: Smooth muscle cell. Bar: 25 µm (A, b, C, d), 50 µm (B, D).

spermatozoa. Moreover, Dlx-5 having been determined to be strongly expressed in the nuclei of the epithelial cells lining the immature and mature epididymis, indicates that this protein may be involved in the division and proliferation of epithelial cells, and thereby, in the maturation and storage of spermatozoa. In addition, Dlx-5 having been detected in the nuclei of the epithelial cells lining the juvenile ductus deferens suggests that this protein could be involved in the proliferation and division of these cells, whilst the detection of Dlx-5 in the nucleus and cytoplasm of some cells and in part of the surrounding smooth muscle cells of the mature ductus deferens indicates potential critical roles of this protein in the transport of spermatozoa.

TLX1, a member of the homeobox gene family, which is described as being required for splenogenesis and the normal development of some sensory neurons, has also been reported to induce the proliferation and differentiation of these

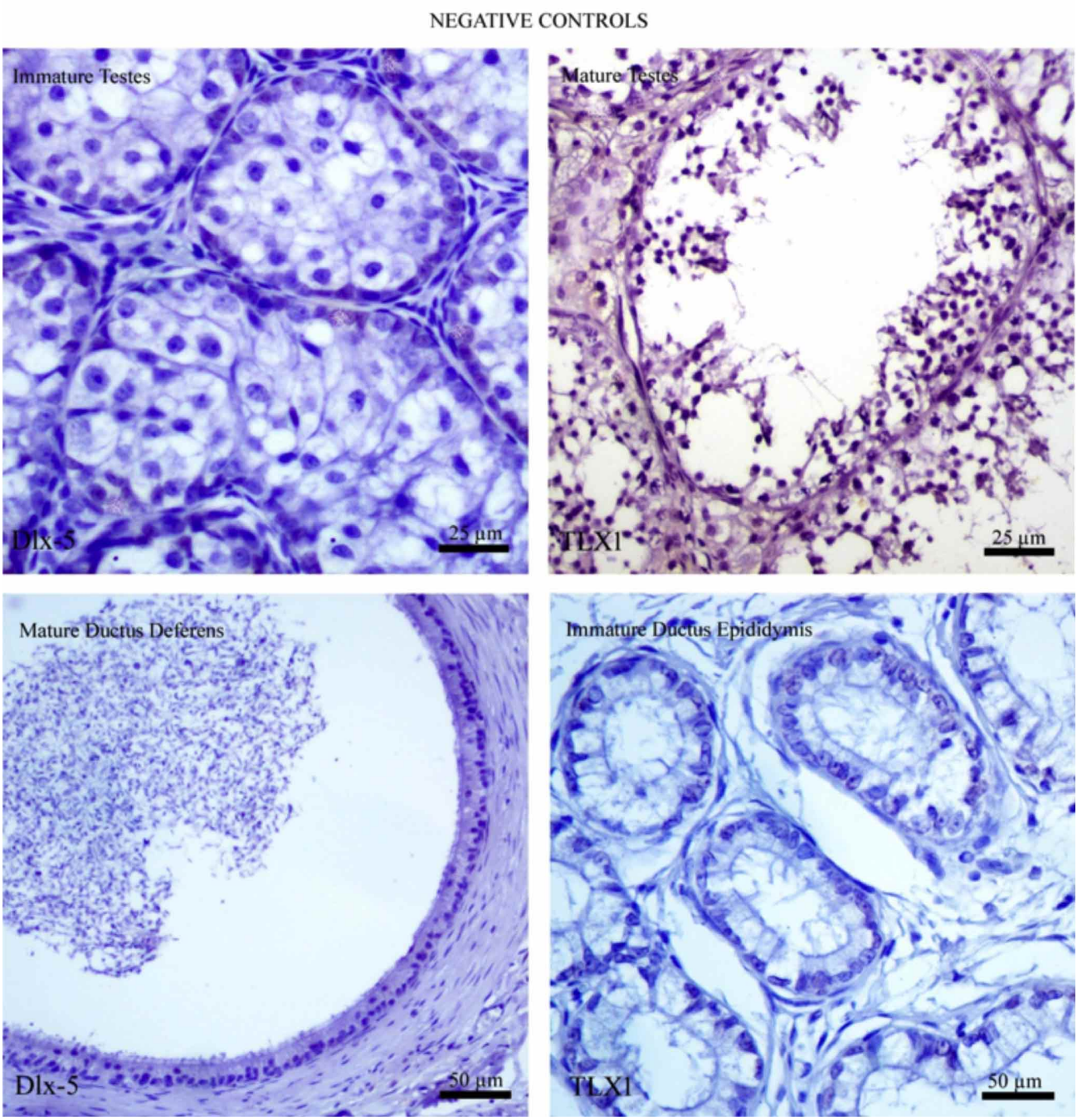

Fig. 4. Negative control immunoreactions for the Dlx-5 and TLX1 proteins in the ductus epididymis and ductus deferens of the adult and juvenile feline testes.

cells (Riz *et al*., 2007). Moreover, it is indicated that TLX1 is localized to the placenta in humans (Novakovic *et al*., 2017) and may contribute to the proliferation, differentiation and apoptosis of trophoblasts. In the present study, while TLX1 produced positive immunoreactions in some specific germ cells of the immature feline testes, immunoreactivity for this protein was strong in the Leydig cells and weak in the cells found within the seminiferous tubules in the mature feline testes. Positive immunoreactivity was also observed in several of vascular endothelial cells. Based on these results, TLX1 is considered not to have much effect on the proliferation and differentiation of testicular cells, but the strong positive immunoreactivity determined in the interstitial endocrine cells of the mature cat testes suggests the possible involvement of this protein in the production of testosterone, and thereby, its contribution to the male sexual development process. Scarcely

any TLX1 immunoreactions were observed in the epithelial cells lining the juvenile epididymis and deferens ducts. On the other hand, mature specimens presented with moderate immunoreactions localized to the cytoplasm in the epithelial cells of the epididymis and to the apical region in the epithelial cells lining the deferens ducts. Immunoreactivity in the smooth muscle cells of the ductus deferens was weak in the immature specimens, and ranged from weak to moderate in the mature specimens. This suggests that the involvement of TLX1 in the physiological functions of the male genital ducts is greater in adult animals, compared to juvenile animals.

In conclusion, it was determined that the Dlx-5 and TLX1 proteins are expressed at varying levels in the testicular cells, epididymal epithelium and deferens ducts, and smooth muscle cells peripheral to the ductus deferens of both immature and mature male cats with no difference being observed between feline breeds. Higher Dlx-5 levels having been determined in both the immature and mature specimens suggested a more active role for Dlx-5, compared to TLX1, in the development and differentiation of the genitalia and the autocrine/paracrine secretion of the genital cells in male cats. This study is hoped contribute to further research on the potential role and functions of homeobox proteins, described as being related in the formation and development of the body structure, in the male reproductive system.

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RESUMEN: Estudios recientes han demostrado que las proteínas homeobox juegan un papel importante en la formación y desarrollo de tejidos y órganos en el período embrionario. En nuestro estudio, se investigó la distribución de las proteínas Dlx-5 y TLX, que son miembros de la familia homeobox, en los testículos, en el epidídimo y en los conductos deferentes de algunas razas de gatos. En el estudio fueron examinados, 18 testículos de animales menores de seis meses (inmaduros) y mayores de un año (maduros) bajo un microscopio óptico utilizando un método inmunohistoquímico (complejo indirecto de estreptavidina-biotina). Si bien se determinó que las proteínas Dlx-5 y TLX1 se expresaron en niveles variables en las células de los testículos de gatos inmaduros y maduros, las células epiteliales del epidídimo y del conducto deferente y las células del músculo liso del conducto deferente, no se observaron diferencias entre las razas de gatos. La inmunorreactividad de Dlx-5 fue más intensa en los testículos, epidídimo y conductos deferentes de gatos inmaduros y maduros en comparación con TLX1. Estos resultados sugieren que ambas proteínas tienen un rol importante en el desarrollo de los órganos genitales felinos masculinos y en la secreción y diferenciación de células, y también la observación de la expresión de Dlx-5 sugirió que esta proteína puede ser más efectiva que TLX1 en el desarrollo testicular y en los procesos fisiológicos.

PALABRAS CLAVE: Gato; Dlx-5; Inmunohistoquímica; Testículos; TLX1

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