A Thesis

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Immunohistochemical Study of Galectin-3 in the Bull Testis and Epididymis

Department of Veterinary Medicine

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CONTENTS

1. Abstract

The expression of galectin-3, a member of the β-galactoside-binding protein family, was examined in the testis and epdidymis of sexually mature and immature bulls. Western blot analysis showed varying levels of galectin-3 expression in the testis and epididymis. Galectin-3 immunoreactivity was higher in the maturetestis and epididymis than in the immature organs. Galectin-3 expression was immunolocalized primarily in interstitial cells in the immature bull testis and in the peritubular myoid cells andinterstitial cells in the mature testis. In the immature epididymis head, galectin-3 was detected in the epithelium and basal cells, but not in the stereocilia and connective tissue. Moderate levels of galectin-3 were detected in the sperm of the mature epididymis head, but low levels were found in the stereocilia, epithelium, and connective tissue. Moderate levels of the protein were detected in the epithelial cells of the immature epididymis body, while lower levels were found in the basal cells, and no staining was detected in the connective tissue. The mature epididymis body showed moderate levels of galectin-3 immunostaining in the stereocilia and epithelium, low levels in the connective tissue, and no staining in the basal cells and sperm. The only galectin-3 staining found in the immature epididymis tail was low levels in the epithelium. In contrast, the mature epididymis tail showed high levels of galectin-3 in the epithelium, moderate levels in the basal cells, and low levels in connective tissue. No staining was found in the stereocilia or sperm. These findings suggest that galectin-3 expression plays a role in the maturation and activation of sperm in bulls.

Keywords: Bull; Epididymis; Galectin-3; Immunohistochemistry; Testis

2. Introduction

Galectin-3, also known as Mac-2, eBP, IgE-binding protein, CBP35, CBP30, L-29, and L-34, is a β-galactoside-binding protein that has been highly conserved throughout animal evolution (Liu et al., 2002; Ravinovich et al., 2002). Approximately 14 members exist in the galectin family (Leffler et la., 1989), and each contains at least one domain of about 130 amino acids designated as the carbohydrate recognition domain (CRD), which is responsible for carbohydrate-binding activity.

Galectin-3 plays a critical role in several functions, including cell growth (Barondes et al., 1994), regulation of apoptotic activity (Dumic et al., 2006), mRNA splicing (Liu et al., 2002), metastasis (Takenaka et al., 2004), angiogenesis (Nangia-Markker et al., 1998), inflammation and adhesion of leukocytes (Almkvist and Karlsson, 2004), and regulation of leukocyte viability and cytokine secretion (Stowell et al., 2008).

Male reproductive potential is based on the ability to deliver spermatozoa to the female genital tract, and the tubular structure of the male reproductive system is well suited for the generation, maturation, and transportation of spermatozoa (Ong et al., 2002).

Several studies have demonstrated that galectin-3 is expressed in the urothelium and excretory tubes of the kidney during the first trimester of human embryogenesis (Van den Brûle et al., 1997), the retina (Uehara et al., 2001),and the bull respiratory and digestive tracts during fetal development (Kaltner et al., 2002). Recent studies have shownthat galectin-3 is differentially expressed in the horse testis (Ha et al., 2003) and in the boar testis and epididymis (Kim et al., 2006). In addition, galectin-3 expression has

been reported in pig, rat, and human Sertoli cells (Deschildre et al., 2007). However, little is known about the presence and distribution of galectin-3 expression in the bull testis and epididymis.

The aim of this study was to determine the distribution of galectin-3 expression in the testis and epididymis of sexually immature and mature bulls.

3. Materials and Methods

Animals and tissue sampling

Testis and epididymis samples (n = 4 samples/group) were collected from immature (5-month-old) and mature (24-month-old) bulls at a local animal farm and slaughterhouse. The samples weredivided into small pieces. Some tissues were fixed in 10% bufferedfor 48 h before being embedded in paraffin wax for histological examination and stored at 70℃ for biochemical analysis.

Antibodies

A rat anti-galectin-3 monoclonal antibody (1 mg/ml) was purified from the supernatants of hybridoma cells (TIB-166; ATCC, Rockville, MD, USA) (Lee et al., 2004). Biotinylated isolectin B4 (IB4) derived from *Griffonia simplicifolia* (Sigma-Aldrich, St. Louis, MO, USA) was used to mark macrophages and mucus-secreting epithelial cells (Kim et al., 2006) because IB4 has a strong affinity for terminal αD-galactosyl residues, which are abundant in macrophages (Judd et al., 1978) and some epithelia (Flint et al., 1986). Mouse monoclonal ant-β-actin antibody (Sigma-Aldrich) was used to detect β-action. β-actin was used as an internal control to ensure that the amount of protein loaded in each lane was similar (Kim et al., 2006).

Western blot analysis

Tissue samples of the testis and three regions of the epididymis were homogenized in lysis buffer(40Tris, 120mM NaCl, 0.1% Nonidet 40, $2Na_3VO_4$, 1mM PMSF, 10μg/ml aprotinin, 10μg/ml leupetin). The homogenate was centrifuged at 14,000for 20min and the supernatant was harvested. For the immunoblot assay, the protein concentration of the supernatant was quantified using the Bradford protein assay (Bio-Rad, Hercules, CA, USA), and equal samples containing 20μg/lane were loaded, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE), and blotted onto nitrocellulose membranes membranes (Schleicher and Schuell, Keene, NH, USA) using standard protocols. The lysate of the B16F10 mouse melanoma cells was used as a positive control for galectin-3 (data not shown)

The residual binding sites on the membrane were blocked by incubation of the blotting membrane in 5% nonfat milk in Tris-buffered saline (TBS; 10 TrisHCl, pH 7.4, and 150mM NaCl)) for 1and then the membrane was incubated for 2h with rat anti-galectin-3 (1:10,000). The blots were washed three times in TBS containing 0.1% Tween 20 and then incubated with horseradish peroxidase-conjugated anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (stock concentration 400μg/ml;working dilution, 1:2000) for 1h. Membranes were developed in the enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL, USA) prepared according to the manufacturer's instructions for 1min and then exposed to AGFA medical X-ray film (Agfa Gevaert N.V., Mortsel, Belgium). After imaging, the membranes were stripped and re-probed using monoclonal anti-β-actin antibody (1:10,000 Sigma-Aldrich) as the primary antibody for 2 otherwise the protocol described

above was followed. The density (OD/mm^2) of each band was measured with a scanning laser densitometer (GS-700 Bio-Rad) and was reported as the mean ± SE. The ratios of the density of the galectin-3 band to that of the β -actin band were compared using Molecular Analyst software (Bio-Rad).

Immunohistochemistry

Section (5-㎛-thick) of paraffin-embedded bull testis and epididymis were deparaffinized using routine protocols before being exposed to citrate buffer (0.01 M, pH 6.0) and heated in an autoclave for 10 min. All subsequent steps were performed at room temperature. The sections were treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes in phosphate-buffered saline (PBS), the sections were blocked with 10% normal rabbit serum (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA), diluted in PBS for 1 h, and then allowed to react with the rat anti-galectin-3 antibody (1:5000) for 1 h. After three washes in PBS, the sections were then reacted with biotinylated rabbit anti-rat IgG (1:100; Vector Laboratories) for 45 min. After three washes in PBS, the sections were incubated with the avidinbiotin peroxidase complex (ABC Elite Kit), prepared according to the manufacturer's instructions, for 45 min. After three washes in PBS, the peroxidase reaction was developed using a diaminobenzidine substrate (DAB Kit, SK-4100; Vector Laboratories), prepared according to the manufacturer's instructions, for 3 min. As a control, the primary antibody was omitted for a few test sections in each experiment. After completion of color development, the sections were counterstained with Harris's hematoxylin for 5 s, washed in running tap water for 20 min, dehydrated through a graded ethanol series, cleared with xylene, and mounted with Canada balsam (Sigma-Aldrich).

To visualize the co-localization of galectin-3 and IB4 in the bull reproductive tissues, the sections were reacted with biotinylated IB4 (Sigma-Aldrich), followed by TRITC-labeled streptavidin (Zymed Laboratories,

San Francisco, CA, USA). The sections were then reacted with the anti-galectin-3 antibody, followed by FITC-labeled goat anti-rat IgG (Zymed Laboratories).

To reduce or eliminate lipofuscin autofluorescence, the sections were washed three times for 5 min each in PBS at room temperature, treated with 10mM $CuSO_4$ in 50mM CH_3COONH_4 buffer (pH 5.0) for 20 min, and returned to PBS. The double-immunofluorescence-stained specimens were examined under a FV500 laser confocal microscope (Olympus, Tokyo, Japan).

4. Results

Histological examination of bull testis and epididymis

The histological examination confirmed that no pathological changes occurred, including inflammation, in the testis or epididymis tissue (Fig. 1). The testis is composed of seminiferous tubules and interstitial cells (Fig. 1A). The three distinct regions of the epididymis, the head, body, and tail, were examined histologically.The epithelium in the epididymis head was thick (Fig. 1B), and gradually became thinner in the body (Fig. 1C) and tail (Fig. 1D). These samples were used for the subsequent Western blot and immunohistochemical analysis.

Expression of galectin-3 in the bull testis and epididymis

Western blot analysis with the anti-galectin-3 antibody allowed detection of changes in galectin-3 expression as a result of sexual maturation of the male reproductive organs. Galectin-3 was detected in all tissues in both the mature and immature testis (Fig. 2).

We found that the intensity of galectin-3 (molecular weight = ~29 kDa) staining increased more than twofold in the mature compared to the immature testis (Fig. 2, lane 1). Galectin-3 was detected in high levels in all three regions of the epididymis. Similar to the results for thetestis, galectin-3 levels in the epididymis head and tail were higher in the mature (Fig. 2, lanes 4 and 8) than in the immature bulls (Fig. 2, lanes 3 and 7).

Immunohistochemical localization of galectin-3 in the bull testis and epididymis

Immunohistochemical staining revealed that galectin-3 expression was higher in

the interstitial and peritubular myoid cells in the mature (Fig. 3B) compared to the immature testis (Fig. 3A). Galectin-3 was detected in the epithelial and basal cells, but not in the stereocilia and connective tissue in the epididymis head of the immature animals (Fig. 3D). Moderate levels of galectin-3 were detected in the sperm of the maturehead of the epididymis, while the protein expression was low in the stereocilia, epithelium, and connective tissue (Fig. 3G). Moderate levels of galectin-3 were detected in the epithelial cells of the immature epididymisbody, while staining in the basal cells was sparse, and no galectin-3 was found in the connective tissue (Fig. 3E). The stereocilia and epithelium of the mature body of the epididymis had moderate levels of galectin-3, but expression of the protein was low in the connective tissue, and no galectin-3 staining was observed in the basal cells and sperm (Fig. 3H). We found low levels of galectin-3 in the epithelium of the immature epididymis tail, but intense immunoreactivity was detected in the epithelium of the mature epididymis tail. Galectin-3 was found in moderate levels in the basal cells, but only low levels were detected in the connective tissue, and no galectin-3 was found in the stereocilia or sperm (Fig. 3I). The immunohistochemical findings are summarized in Table 1. These findings agree with the localization of galectin-3 expression shown by the Western blot analysis.

Double-staining for galectin-3 and *Griffonia simplicifolia* isolectin B4

In the head (Fig. 4A-C), body (Fig. 4D-F), and tail (Fig. 4G-I) of the mature bull epididymis, IB4 was detected in some galectin-3-positive macrophages in the submucosa (Fig. 4A-F, arrows), as well as in some epithelial cells that were also positive for galectin-3 (Fig. 4A-I).

5. Discussion

This is the first study to show that galectin-3 is differentially expressed in the testis and epididymis of immature and mature bulls.

Galectin-3 has previously been detected in pig, rat, and human Sertoli cells (Deschildre et al., 2007), as well as in human and boar interstitial cells (Devouassoux-Shisheboran et al., 2006 Kim et al., 2006). In the present study, we detected high levels of galectin-3 only in the interstitial and peritubular myoid cells of the mature bull testis. In human testes, galectin-3 is specifically expressed in mature Sertoli and Leydig cells and is absent in fetal and prepubertal testes (Devouassoux-Shisheboran et al., 2006). This suggests that galecin-3 is involved in hormonal regulation of the adult testes for spermatogenesis in bulls.

In the three regions of the epididymis, galectin-3 was mainly detected in the sperm, stereocilia, epithelium, and basal cells. In addition, the galectin-3 levels were higher in the mature reproductive tissue than in the immature tissue. Recent studies indicatethat galectin-3 is expressed in a variety of epithelial cells, including the mucosa in the mouse urinary system and the bull respiratory system (Uehara et al., 2001). Our study confirmed the finding that galectin-3 is occasionally present in the mucus-secreting (IB4-postrive cells) epithelial cells in the epididymis (especially, intensively expressed in the epididymis tail). Galectin-3 plays an important role in the mucosal epithelium, as well as in the maturation of sperm in the lumen, as do epididymal secretory proteins in the boar (Dacheux et al., 2005; Kim et al., 2006). We postulate that sperm in the epididymis are influenced by galectin-3 secreted from the mucosal epithelium, and that the protein may play a role in the

activation and maturation of sperm.

In conclusion, in the present study, we demonstrated that galectin-3 is present in the immature and mature bull reproductive organs, and we postulate that galectin-3 plays a role inthe maturation and activation of sperm in bulls.

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7. Table

Table 1. Immunohistochemical staining for galectin-3 in various cell types in the reproductive system of normal immature (5-month-old) and mature (24-month-old) bulls

Tissue	Cell type	Galectin-3	
		Immature bull	Mature bull
Testis	Spermaotogonia	- (gonocyte)	−
	Spermatocyte/Spermatid	- (gonocyte)	−
	Sertoli cell	−	−
	Interstitial cell	+	+++
	Myoid cell	−	++
	Sperm	ND	−
Head of epididymi	Stereocilia	−	+
	Epithelium	+	+
	Basal cell	+	−
	Sperm	ND	++
	Connective tissue	−	+
Body of epididymis	Stereocilia	−	++
	Epithelium	++	++
	Basal cell	+	−
	Sperm	ND	−
	Connective tissue	−	+
Tail of epididymis	Stereocilia	−	−
	Epithelium	+	+++
	Basal cell	−	++
	Sperm	ND	−
	Connective tissue	−	+

Stained sections were scored for the density of positive cells per field. , negative; +, weak; ++, moderate; +++, intense.

ND, not detected.

8. Figures

Figure 1. Histological sections of the (A) testis, (B) head, (C) body, and (D) tail of the epididymis. EP, epithelium. Scale bar = 50 m. The sections were stained with hematoxylin-eosin stain.

Figure 2. Western blot analysis of galectin-3 in the bull reproductive tissue. The differential expression of galectin-3 in the immature and mature testis and epididymis. The lower panel shows the expression of the internal control, β-actin, in the same membrane. T, testis; M, mature; IM, immature E, epididymis.

Figure 3. Immunohistochemical staining of the immature (A, DF) and mature (B, GI) testis and epididymis. (A and B) The expression of galectin-3 was occasionally higher in the interstitial (arrows) and peritubular myoid cells (arrowhead) in both the immature and mature testis. (D) Galectin-3 was detected in the epithelium (arrows) and basal cells (arrowheads) of the immature epididymis head. (G) In the mature head of the epididymis, moderate levels of galectin-3 were detected in the sperm (asterisks), while the stereocilia and epithelium showed sparse staining (arrows). (E) Moderate levels of galectin-3 were found in the epithelial cells of the immature body of the epididymis (arrows) and lower levels of the protein were found in the basal cells. (H) The stereocilia and epithelium of the mature epididymis body showed moderate galectin-3 levels (arrows). (F) Only weak staining for galactin-3 was observed in the epithelium of the immature epididymis tail (arrows). (I) High levels of galactin-3 were detected in the epithelium of the mature tail of the epididymis (arrows), but none was found in the stereocilia or sperm. Cshows a control section in which the primary antibody was omitted. The sections were counterstained with hematoxylin. Scale bars = 50 m.

Figure 4. Immunofluorescent co-localization of galectin-3 with isolectin B4. (AC) The mature head, (DF) body, and (GI) tail of the epididymis. (AF) The arrows show galectin-3 immunoreactivity in isolectin B4-positive macrophages. (GI) The arrowheads indicate galectin-3 immunoreactivity in isolectin B4-positive mucosal epithelium. C, F, and I are merged images. Scale bars =20 m.

Fig. 1. Histological sections of the (A) testis, (B) head, (C) body, and (D) tail of the epididymis. (Kim H et al.)

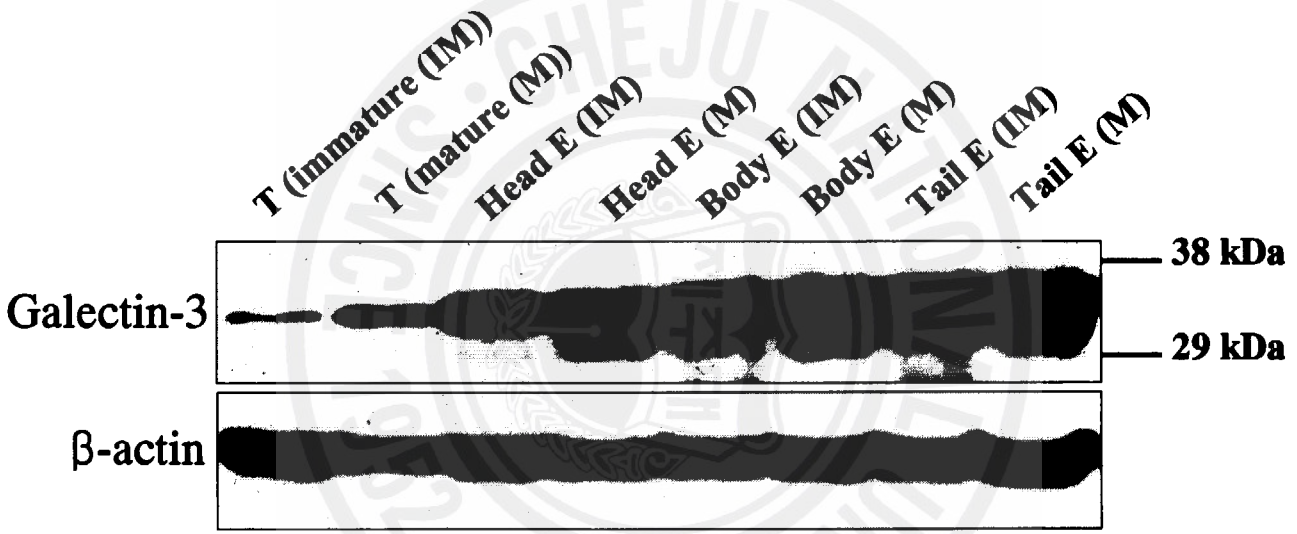
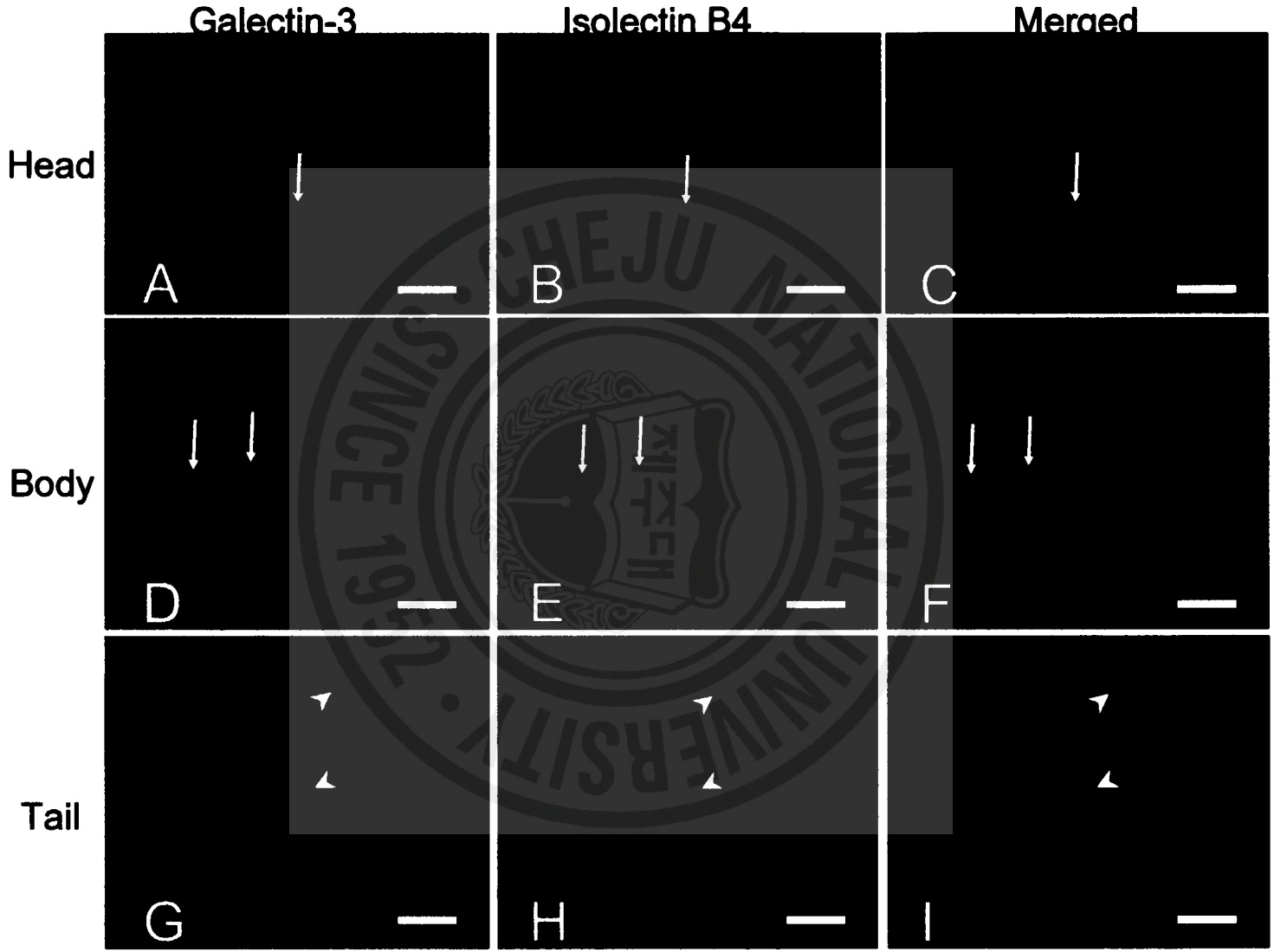


Fig. 2. Western blot analysis of galectin-3 in the bull reproductive tissue. The differential expression of galectin-3 in the immature and mature testis and epididymis. (Kim H et al.)

Fig. 3. Immunohistochemical staining of the immature (A, D–F) and mature (B, G–I) testis and epididymis. (Kim H et al.)



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