

Morphology and Lectin histochemistry of the testes of brown-banded bamboo shark (*Chiloscyllium punctatum*)

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Abstract

The testes of three brown-banded bamboo male sharks collected from Okinawa Churaumi Aquarium, Japan were used in this study. The testes were studied grossly and microscopically. In addition, conventional and lectin histochemistry (panel of 8 HRP lectins, UEA-I, DBA, LCA, PNA, ConA, PHA-E4, WGA and RCA 120) were applied for studying the glycoconjugates.

The testes are suspended to the dorsal wall by mesorchium. They are covered ventromedially by the epigonal organ (Hematopoietic organ). The testis is divided into germinal, spermatogonial, spermatocytes, spermatid, spermatozoal and degenerative zones.

Analysis of the sugar binding-lectins in the shark testes revealed the presence of all sugars under investigation, although they varied in distribution throughout the different zones. The

germinal zone was not labeled to any sugar, while the spermatogonial zone was labeled to galactosamine. The spermatocyte zone was labeled to glucose, galactose and glucosamine within the Leydig cells, while the spermatocysts were labeled only to the galactose and glucoseamine. The spermatocyst of the spermatid zone was similar to that of the spermatocyte zone while the Leydig cells were labeled to all sugars under investigation. The spermatozoal zone was labeled to all sugars under investigation either to the spermatocyst or to the Leydig cells. At the degenerative zone, Leydig cells were labeled to all sugars under investigation while the spermatocyst was labeled to glucose and glucosamine only.

In conclusion, our results indicate that the structure of the testes of the brown-banded bamboo shark simulate that of the cartilaginous fish. There is progressive increase in glycosylation during spermatogenesis, especially at

the embedding of the elongated spermatids to the Sertoli cells (spermatoblasts) in the spermatid spermatozoa step. The Leydig cells are strongly labeled in the spermeiogenesis and degenerative zones than in the germinal, spermatogonial and spermatocyte zones due to its androgen activity.

Key Words

Lectin, testes, bamboo shark

Introduction

The brown-banded bamboo shark is a cartilaginous and tropical species found across the northern coast of Australia and the Indo-pacific north to Japan and west to the east coast of India. The Morphological data of the shark testes are studied in different species (Matthews, 1950; Stanely, 1966; Holestein, 1969; Chen et al. 1973; Wourms, 1977; Dobson and Dodd, 1977; Teshima, 1981; Pudney and Callard, 1984 a,b; Pratt, 1988; Parson and Grier, 1992 and Girard et al. 2000). Testicular structure and spermatogenesis of the cartilaginous fishes are unique in several ways, such as cystic organization, zones arrangement of the spermatocysts and a close association of the testes with hematopoietc epigonal organ (Dodd and Sumpter, 1984; Pratt, 1988; Hamlett et al. 1999 and Liguoro et al. 2004).

Specific carbohydrates expression patterns may exhibit striking changes related to cell differentiation (Roth

1996). Recent advances in glycan research have shown that cell surface proteoglycans are implicated in cell development and differentiation through a role in cell signaling by paracrine and autocrine factors, such as growth factors, chemokines and cytokines (Iozzo, 1998; Bernfield et al. 1999; Lander and Selleck, 2000 and Kassab et al. 2007).

In the field of male fertility, lectin histochemistry is considered as a valuable method for determining changes in the glycoconjugate content during spermatogenesis under normal and pathological conditions (Wine and Chapin, 1997 and Arenas et al., 1998). In addition, lectin histochemical analysis of the testes has allowed the staining patterns of spermatogenic cells to be visualized, thus allowing determination of the sequential glycosylation processes of acrosome development in rats (Martinez-Menargues et al., 1999), mice (Arya and Vanharpertula, 1986), hamsters (Ballesta et al., 1991), dogs (Montkowski, 1992), humans (Arenas et al., 1998), bulls (Ertl and Wrobel, 1992 and Abd-Elmaksoud, 2005), goats (Kurohmaru, 1991), horse (Verinisupplizi et al., 2000), boars (Pinart et al., 2001, 2002) and camel (Abd-Elmaksoud et al., 2007) .

The morphology of the shark testis is studied by several authors, although glycoconjugates of the spotted ray *Torpedo marmorata* was investigated (Liguoro et al. 2004), the brown-banded bamboo shark has not yet according to our knowledge. So the aim of this study is to describe the morphological struc-

ture of the testis and the glycol-conjugates of different structures and its relation to spermatogenesis in brown-banded bamboo shark.

Materials and Methods

Three brown-banded bamboo male sharks were used in this study at winter season (October-January). The sharks were collected from Okinawa Churaumi Aquarium, Japan. The sharks ranged about 100 cm length and 4 kg weight (Fig. 1). The sharks were dissected at the middle of body cavity, eviscerated and the testes were exposed. The length of the testes was measured. Cross section samples were fixed in bouin's solution for 18-24 hours at room temperature. The samples were extensively washed in 70% ethanol. Thereafter, the samples were dehydrated in graded series of ethanol (80%, 90%, 95% and absolute), cleared in lemosol and embedded in paraffin wax. Sections 3-5 microns thickness were mounted on uncoated and coated slides with 3-aminopropyltriethoxy-silane. For general histological studies, selected slides were stained with Haematoxylin and Eosin.

Conventional histochemistry:

For general description of glyco-conjugates, the slides were stained with Periodic Acid Schiff (PAS) for neutral mucopolysaccharides, Alcian Blue pH 2.5 (AB 2.5) AB pH 1.0 and AB pH 0.5 for acidic mucopolysaccharides (Pearse, 1985).

Lectin histochemistry:

Lectin binding sites were demonstrated by means of horse radish peroxidase (HRP). The slides were deparaffinized, then rehydrated using descending grades of ethanol until distilled water. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide (H₂O₂) in methanol for 30 minutes at room temperature, thereafter the sections were incubated in 1% goat serum albumin (DAKO, USA) in phosphate buffer saline (PBS) for 20 minutes to minimize nonspecific staining. Subsequently, the slides were incubated with HRP conjugated lectins (J Oil Mills, Tokyo, Japan) for one hour at room temperature. Lectins were generally used at concentrations 5- 20 µg/ml PBS pH 7.6 (Table 1). All sections were washed in PBS 3 times (3x5min.). The peroxidase activity was visualized by Dako cytomation (Liquid DAB; 3, 3' diaminobenzidine; and substrate chromogen system) (DAKO, USA) for 30 minutes at room temperature. Finally, the sections were washed in distilled water (3x5min.), counterstained with Meyer's haematoxylin, dehydrated, and mounted with Mount-Quick (Daido Sangyo co., Japan). Lectin specificities were adopted to Debray et al. (1981), Spicer and Schulte (1992) and Danguy (1995).

To examine the specificity of lectin staining, control sections were prepared by one of the following: addition of inhibitory sugars to the respective lectin solution, substitution of unconjugated lectins for the horse radish and finally the exposure of sections to Dakocyto-mation without lectins.

The staining intensity was classified by two independent observers into 4 categories: no labeling (-ve), weak labeling (+), moderate labeling (++), and strong labeling (+++).

Table 1: The lectins used and their sugars binding specificities.

Lectin group*		Name	Sugar binding specificity	Concentration	Binding	Inhibitor sugar
Glucose binding lectins	LcA	Lens culinaris	α -Man	5 μ /ml	HRP	Man
	Con A	Concanavalin A	α -D- Man> α -d-Glc	10 μ /ml	HRP	Man
Galactose binding lectins	PNA	Peanut agglutinin	Gal β 1-3GalNAC	20 μ /ml	HRP	Gal
	RCA 120	Ricinus communis 120	Gal β 1-4GlcNAC	5 μ /ml	HRP	Gal
Glucosamine binding	WGA	Wheat germ agglutinin	β -D-GlcNAC	5 μ /ml	HRP	GlcNAC
Galactosamine binding	DBA	Dolicos biflorus	GalNac α 1-3 GalNac	20 μ /ml	HRP	GalNac
Fucose binding lectin	UEA-1	Ulex europaeus -1	α -L-Fuc	20 μ /ml	HRP	Fuc
Non specific	PHA-E4	Phaseolus vulgaris	D-GalNAC	5 μ /ml	HRP	GalNAC

Gal : Galactose, GalNAC: N-acetylgalactosamine, Glc: glucose, GlcNAC: N-acetylglucosamine, Fuc: fucose, Man: mannose,

Table 2: lectins binding specificities to various zones of the testis

Lectin group*	Glucose/ mannose binding lectins		Galactose binding lectins		Glucos-amine binding lectin	Fucose binding lectin	Galactos-amine binding lectin	Non specific
	LcA	Con A	PNA	RCA 120				
Germinal zone	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	-ve	-ve	-ve	-ve	-ve	-ve	++ve	++ve
Spermatogonial zone	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	++ve	++ve	-ve	++ve	++ve	-ve	-ve	++ve
Spermatocyte zone	-ve	-ve	-ve	++ve	++ve	-ve	-ve	++ve
	++ve	++ve	-ve	++ve	++ve	-ve	-ve	++ve
Spermatide zone	-ve	-ve	++ve	++ve	++ve	-ve	-ve	++ve
	++ve	++ve	++ve	++ve	++ve	++ve	++ve	++ve
Spermatozoal zone	-ve	++ve	++ve	++ve	++ve	++ve	++ve	++ve
	++ve	++ve	++ve	++ve	++ve	++ve	++ve	++ve
Degenerative zone	++ve	++ve	-ve	-ve	++ve	-ve	-ve	++ve
	++ve	++ve	++ve	++ve	++ve	++ve	++ve	++ve

Results

The testes were suspended to the dorsal wall by mesorchium in the cranial end of the body cavity, just under the vertebral column. They were covered ventromedially by the epigonal organ (hematopoietic organ). They were measured about 7 cm in length. The left one is slightly longer than the right (Fig.1B). Microscopically, the parenchyma was divided into several lobes.

These lobes were clearly organized dorsally by connective tissue septae. These septae contained intratesticular ducts that lined with cuboidal epithelium. These ducts were prominent in the dorsal and ventral parts of the testis and could not be demonstrated at the middle part. Each lobe showed zones arrangement and contained numerous round- shaped spermatocysts (follicles). The zones were named germinal, spermatogonial, spermatocysts, spermatid, spermatozoal and degenerative zones (Fig.1C, D, E, F and G).

The germinal zone was located in the most dorsal part of the testis containing spermatogonium and undifferentiated Sertoli cells not organized into a definite shape (Fig.1D and E). Once these two cell types form round- shaped spermatocyst it became spermatogonial zone (Fig.1F). The mitotic division of spermatogonium continued, the Sertoli cells were located basally, to form the spermatocysts zone. Then the spermatocysts began meiotic division to form finally the spermatids zone, in which there was increment of the number of the layers in the

spermatocyst, decrement of the size of the cells with dark nuclei. The spermatids then enter the process of spermeiogenesis which started by elongation of the nuclei with the formation of the acrosome, then all the spermatids overlie the Sertoli cells and imbedded in it forming together the spermatoblast which characterized by PAS positive granules (Fig.1G and H). After complete transformation of the spermatids to spermatozoa, the spermatozoal spermatocysts evacuate their spermatozoa into the intratesticular ducts and the remnant of the spermatocysts underwent degeneration with prominent PAS positive materials in the degenerative zone which occupied the most ventral part of the testis in relation to the epigonal organ. All the previous zones were negative to the AB with different pH. The Leydig cells were few in the spermatogonial zone and then increased in numbers to appeared more in the spermatids and spermatozoal zones (Fig. 1C, F, G and H). The glycoprotein histochemistry was expressed mainly on spermeiogenesis zones (spermatids and spermatozoal), and degenerative zone (table 2).

Germinal zone showed no labeling to all lectins under investigation. Spermatogonial zone showed moderate labeling to N-acetyl-galactosamine as observed by DBA and PHA. The reaction was labeled to the nuclei (Fig. 2A).

The spermatocyte zone labeling differed from spermatocyst to the Leydig cells and interstitial tissue. Spermatocysts showed galactose, N-acetyleglucosamine as labeled by RCA,

WGA and PHA, while the interstitial tissue containing Leydig cells showed mannose, galactose and N-acetylglucosamine as described by LCA, ConA, RCA, WGA and PHA.

The spermatids spermatocyst were positive for galactose, N-acetylglucosamine as observed by PNA, RCA, WGA and PHA. The Leydig cells contained mannose, galactose, N-acetylglucosamine, fucose and N-acetylgalactosamine as described by all lectins under investigation (Fig. 2B,C and D).

The spermatozoal zone showed strong labeling to most lectins under investigation either in spermatocysts or Leydig cells. The sperms differentiate according to labeling into head, tail and the junction with the Sertoli cells. The head showed reaction to all lectins except LCA, while the tail was not labeled with LCA and ConA and labeled to the others (Figs. 2 E, F, G, H and 3 A).

In the degenerative zone, where the spermatocysts evacuate their sperms to the intratesticular duct, the remainder of the follicles underwent degeneration and their was high expression to mannose, glucosamine as observed by LCA, ConA, WGA and PHA. The Leydig cells showed labeling to all lectins under investigation (Figs. 3 B, C, D, E and F).

Discussion

The testes of the brown-banded bamboo shark are like the diametric type stated by Pratt (1988), who added that the germinal zone is nested among the origin of the trabeculae. The seminiferous follicles development spreads from this zone across the diameter of the testis toward the opposite wall, where the efferent ductules are present to receive spermatozoa (Stanley, 1966). The displacement of the spermatocysts is not active movement for each, but they are passively replaced by the newly formed ones (Parson and Grier, 1992 and Chatchavalvanich et al. 2005). The intratesticular ducts are clearly visible in the germinal and spermatogonial zones but are rarely seen in the spermatocyte, spermatid and spermatozoal zones because the increases of spermatocysts size obscure them. These ducts can be clearly seen again in the degenerative zone, as the spermatocysts became small and had undergone degeneration even as the duct becomes more developed (Chatchavalvanich et al. 2005).

Apparently the acrosomal structure in spermatid and the Sertoli-spermatid attachment are positive to the neutral mucopolysaccharides as indicated by PAS. These results are in accordance to that of mammalian species, but unlike that of fresh water stingray and other non mammalian species as frog, cobra and avian which are negative to PAS stain (Gunawardana, 1977; Aire et al., 1980 and Chatchavalvanich et al., 2005).

Lectins have a specific binding affinity for the sugar residues of glycol-conjugates, therefore they are used as histochemical reagents to investigate the distribution of glycol-conjugates in various tissues including testis (Arya and Vanha-perttula, 1985, 1986; Lee and Damjanov, 1984; Soderstrom et al., 1984; Wollina et al., 1989; Malmi et al., 1990; Ballesta et al., 1991; Kurohmaru, 1991; Ertl and Wrobel, 1992; Montkowski, 1992; Arenas et al., 1998; Martinez-Menargues et al., 1999; Verinisupplizi et al., 2000, Pinart et al., 2001, 2002; Abd-Elmaksoud, 2005). In our study, the sugar residues of glycoconjugates in bambooshark testis were investigated using eight (ConA, LCA, PNA, DBA, PHA, WGA, UEA-I and RCA) horse radish peroxidase (HRP) lectins. Detection of sugar moieties by lectins was carried out in winter season. Careful analysis of sugar binding-lectins in the shark testes revealed the presence of all sugar under investigation but their distributions were varied according to the zones of the testes. The germinal zone was not labeled to any sugar while the spermatogonial zone was labeled to the galactosamine. The spermatocyte zone was labeled to glucose, galactose and glucosamine in the Leydig cells, while the spermatocyst was labeled only to the galactose and glucosamine. The spermatocyst of the spermatide zone was like that of the spermatocyte zone while the leydig cells were labeled to all sugars under investigation. The spermatozoal zone was labeled to all sugars under investigation either to the spermatocyst or to the Leydig cells. The

Leydig cells in the degenerative zone were labeled to all sugars under investigation, while the remnant of spermatocysts was labeled to glucose and glucosamine only.

Our results showed that, some germ cells in the spermatogonial zone were labeled to galactosamine indicating apoptosis. This reaction could be attributed to, during apoptosis, the cellular surface composition changes like apoptotic hepatocytes with increased GalNAC and galactose, making the cells susceptible to phagocytosis (Dini et al 1992), on the other hand, the presence of galactosamine NAc on the spermatogonia might induce in the onset of meiosis within the cysts and the germ cells lose the ability to divide mitotically and become able to divide meiotically (Liguoro et al. 2004).

After formation of spermatoblasts, positive labeling to most lectins under investigation is evident on the germ and Sertoli cells. The positive reaction may be due to the presence of glycol-proteins, like cadherin, that contain mannosyle chains available to establish interactions between germ and somatic cells (Liguoro et al. 2004). In this regard, it is noteworthy that in mammals a unique oligosaccharide containing mannosyle residues was identified within germ cells to adhere to Sertoli cells (Akama et al. 2002).

Interestingly, after spermiation, the Sertoli cells were positive and strongly labeled more than in the early glandular tissue, indicating that, these

sugar residues could be attributed to either spermiation related functional changes or the start of the degenerative process (Saez et al. 2001a,b). The strong staining of the degenerative zone specially the evacuated cysts may be due to the residual spermatides cytoplasm in the cytoplasm of Sertoli cells by phagocytosis, as reported in mammals (Arya and Vanha-Parttula 1985, Verini-Supplizi et al. 2000).

It is widely accepted that glycolconjugates are involved in cell differentiation (Zaik et al. 1996). Recently it has been shown that glycosaminoglycans of the cell surface proteoglycans are involved in controlling cell differentiation and proliferation by binding to local mediators as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) require heparin sulphate for high affinity binding to their receptors (Bernfield et al. 1999, Lander and Selleck 2000). In addition, binding of transforming growth factor (TGF β) to heparin sulphate prevent its activation (Carreau 1996, Mendis-Handagama 1997, Hedger and de Kretser 2000). Recently it has been shown that some growth factors as insulin like growth factor (IGF) activate, while TGF β 1 inhibit testosterone synthesis and leydig cell differentiation (Bentone et al. 1995, le Roy et al. 1999). It is possible to speculate some possible roles for carbohydrate chains in the differentiation of steroid hormone secreting cells. The distribution of lectins binding sites significantly changes within Leydig cells. The labeling of lectins to the Leydig cells was significant in the spermiogenesis

zones (spermatids, spermatozoal) and degenerative zone than in the spermatogonial and spermatocyte zones, which indicate great changes in spermatogenesis due to its androgen activity.

In conclusion, our results indicate that the structure of the testes of the brown-banded bamboo shark is like that of the cartilaginous fish. There is progressive increase in glycosylation during spermatogenesis, especially at the elongated spermatids and Sertoli cells (spermatoblasts) in the spermatide-spermatozoa step. The Leydig cells are strongly labeled in the spermeiogenesis and degenerative zones than in the germinal, spermatogonial and spermatocyte zones due to its androgen activity.

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Fig. (1):

Panel A, Photograph of the Brown-banded bamboo shark.

Panel B, demonstrates the position of the testes after dissection of the shark (arrow heads).

Panel C, photomicrograph of the testis, showing the Capsule (C), connective tissue septa (T), germinal zone (G) and spermatogonial zone (S), H&E, bar 50 μ .

Panel D, showing germinal (G) and spermatogonial (S) zones, H&E, bar 50 μ .

Panel E, germinal zone of the testis showing spermatogonia (white arrow) and Sertoli cells (black arrow), H&E, bar 5 μ .

Panel F, showing spermatogonial (S) and spermatocyte zone(SC) with connective tissue septa in between the lobes (CT), H&E, bar 50 μ .

Panel G, showing spermatozoal zone with its spermatocysts (SZ), degenerative zone(D), Leydig cells (L) and intratesticular duct (black arrow), PAS, bar 20 μ .

Panel F, high magnification to the previous panel showing spermatocyst (SZ) and elongated sperm nuclei (DS), PAS, bar 20 μ .

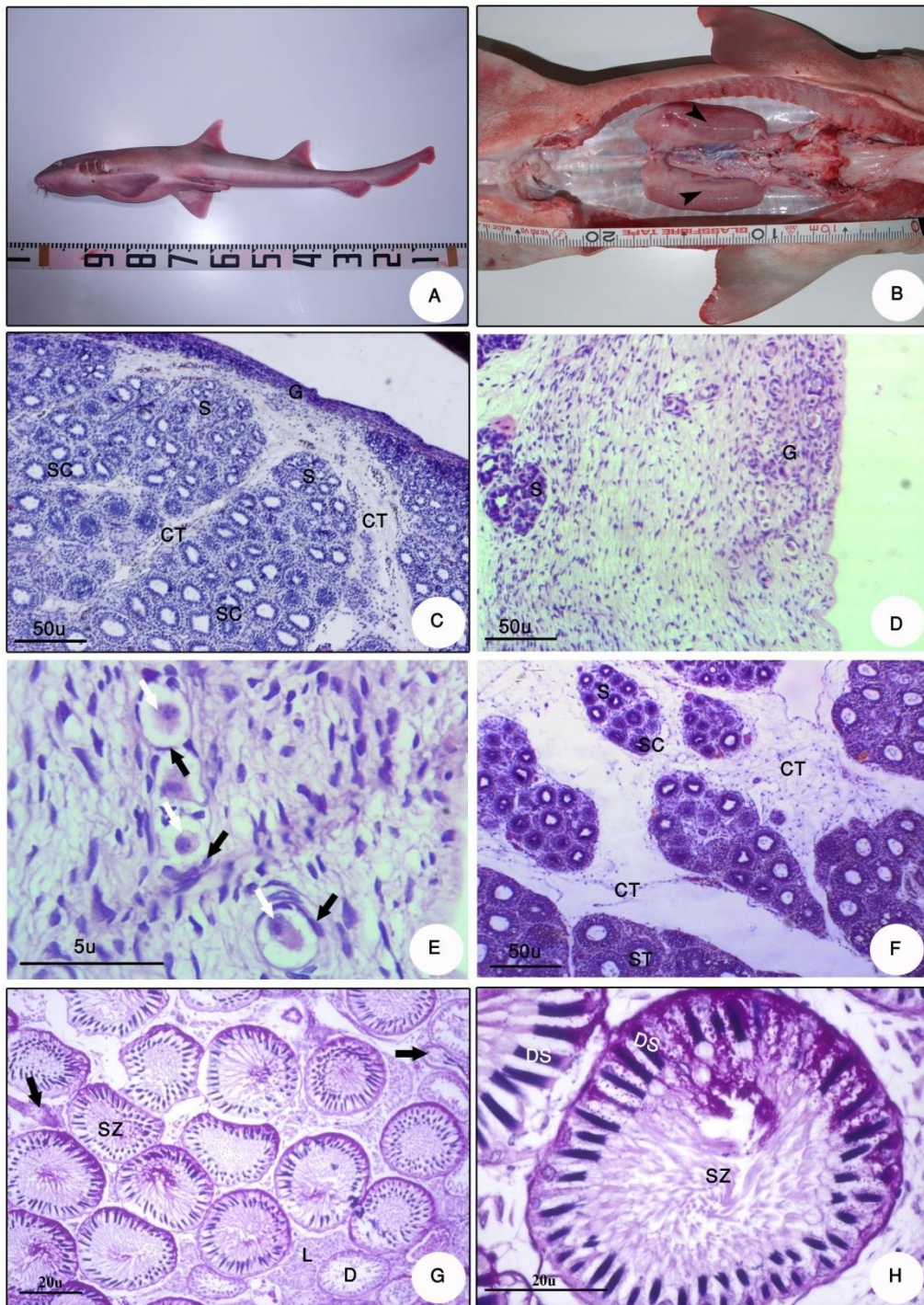


Fig. (2):

Panel A, DBA labeling to the nucleus (black arrows) in the spermatocyst (S) of the spermatogonial zone, bar 5 μ .

Panel B, ConA labeling to the interstitial tissue of spermatide zone (black arrow) in between the spermatocysts (SD), bar 20 μ .

Panel C, ConA labeling to the interstitial tissue of spermatide zone (black arrows) in between the spermatocysts (SD), bar 20 μ .

Panel D, PNA unlabelled to the spermatid zone, bar 20 μ .

Panel E, ConA labeling to the elongated sperm (black arrow) in the spermatocyst (SZ) of spermatozoal zone and leydig cells (L), bar 20 μ .

Panel F, LCA weak labeled the spermatoblast, heads and tails of the sperm in spermatocyst of spermatozoal zone (SZ), although high labeling to the leydig cells (Black arrows) with the presence of unlabelled intratesticular duct (arrow head), bar 20 μ .

Panel G, PNA high labeled to the head of sperm only (Black arrows) in the spermatocyst of spermatozoal zone (SZ), and weak labeling to the leydig cells (L), bar 20 μ .

Panel H, UEA moderate labeled to the tail of sperm (arrow head) and leydig cells (L) in between the spermatocyst (SZ) of spermatozoal zone, bar 20 μ .

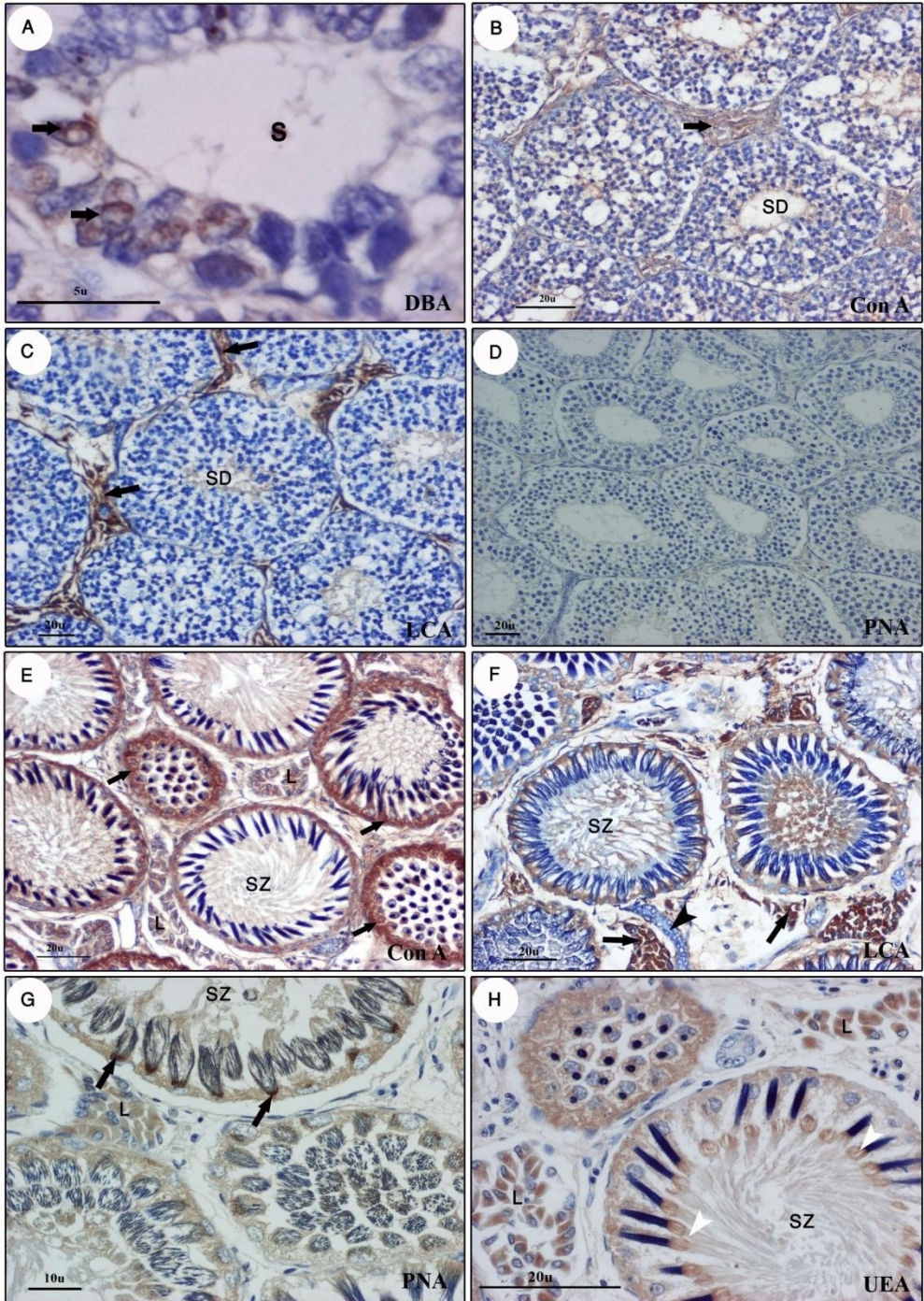


Fig. (3):

Panel A, PHA labeling to the tail of the sperm (white arrow) and spermatoblasts (black arrow) in the spermatocyst (SZ) of spermatozoal zone, also strong labeling to the leydig cells (L), bar 10 μ .

Panel B, LCA labeling to the degenerative zone spermatocysts (DZ) and high labeling to the leydig cells (L), notice the negative labeling of the spermatocyst of spermatozoal zone (SZ) at the junction with the degenerative zone, bar 50 μ .

Panel C, ConA labeling to the evacuated spermatocyst of the degenerative zone (DZ) with high labeling to the degenerated cells and also high labeling to the leydig cells (L), notice their was no labeling to the dilated intratesticular duct (arrow head), bar 20 μ .

Panel D, PNA labeling to the degenerative zone at the junction with the epigonal organ (EO). Notice the labeling was restricted to the leydig cells only (L) but not to the evacuated spermatocyst (DZ) or to the spermatocyst of the spermatozoal zone (SZ), bar 30 μ .

Panel E, High magnification to the previous, bar 10 μ .

Panel F, DBA labeling to the Leydig cells only (L) and negative for evacuated spermatocyst (DZ), bar 20 μ .

