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Morphological and Immunohistochemical Study on the Testis of Brown-Banded Bamboo Shark (*Chiloscyllium punctatum*)

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ABSTRACT

The present study aimed to investigate the morphological changes in the testis of brown-banded bamboo shark using routine histological and PCNA immunohistochemical techniques in order to describe the role of cell division versus cell death in germ cells and Sertoli cells during summer and winter seasons. Four mature brown-banded bamboo male sharks testes were used in this study; the first two were collected during winter season and the other were collected during summer season. Testicular specimens were used for histological and PCNA immunohistochemistry. The obtained results revealed that the testis of brown-banded bamboo shark showed a diametric pattern of development in which the testis was divided into several zones including germinal, spermatogonial, primary spermatocyte, secondary spermatocyte, spermatid, spermatozoal and degenerative zones. Noteworthy, PCNA expression was restricted to the nucleus of spermatogonia, spermatocytes and Sertoli cells in both mitotic and pre-meiotic zones only while it was absent in the post-meiotic zone. Moreover, the intensity of expression was different among the various zones as its expression was prominent in the nucleus of spermatogonia in the germinal zone compared with spermatogonial zone. The intensity of PCNA expression in the testis of shark was different either during summer or winter season. Briefly, the testis of brown-banded bamboo Shark has a polar pattern of development as it started with spermatogonial zone dorsally and ends with degenerative zone ventrally. This pattern of development is the best paradigm to track the cascade of the spermatogenesis process as each zone represents only one type of cells. The seasons under investigation has a non-significant effect on the PCNA expression in the testis of brown-banded bamboo Shark.

1. INTRODUCTION.

Brown-banded bamboo shark *(Chiloscyllium punctatum)* is belonged to the family Hemiscylliidae, order Orectolobiformes. It is a widely spread population in Indian Ocean to Western Pacific. *Chiloscyllium punctatum* is one of the most popular species that characterized by hardiness, small size, and beautiful patterning so it is commonly imported for the pet trade (Dingerkus and DeFino, 1983).The elasmobranch (sharks, skates, rays, and sawfish) testes exhibit three types of structural development; diametric, radial and compound developments that are closely related to hematopoietic epigonal organ. Testes are responsible for the production of spermatozoa

(spermatogenesis) and steroid hormones (steroidogenesis, Pratt Jr, 1988). In the shark, each spermatogonial stem cell enters the process of spermatogenesis is sequestered into anatomically distinct follicle-like units identified as spermatocysts (McClusky, 2005, McClusky, 2006). Each cyst also houses a second clonal population of stage synchronized Sertoli cells. The simple topographical arrangement of cysts in maturational order across the diameter of the testis provides a readily visible zonation of the testis comprising cysts in the premeiotic (spermatogonia and preleptotene spermatocytes), meiotic (spermatocytes), and postmeiotic (spermatids) regions. This particular testicular organization has been described by Pratt Jr (1988) as the diametric elasmobranch testis type which found in carcharhinids for instance Scyliorhinus canicula and Squalus acanthias, bonnethead shark, Sphyrna tiburo, and the blue shark, (Prionace glauca).

The routine histological examination of the normal testes in most vertebrate species reveals invariable degeneration in testicular cells. These cell deaths in multicellular organisms have been ascribed to the orderly removal of abnormal or superfluous cells, that is, apoptosis (Jacobson et al., 1997, Wyllie et al., 1980). The pattern of removal of mitotically and/or the meiotically dividing germ cell population is generally conserved at a lower phyletic levels as well, as seen in bony (Almeida et al., 2008, Andreu-Vieyra et al., 2005, Corriero et al., 2007, McClusky et al., 2009) and in some cartilaginous (McClusky, 2005) fishes, however spermatid death has also been observed in the spotted ray (Prisco et al., 2003). In rodents, the damaged and superfluous or inadequately supported germ cells are phagocytosed by Sertoli cells, which have the responsibility to maintain the integrity of the spermatogenic epithelium (Dym and Fawcett, 1971, Pudney, 1995). However, in lower vertebrates the entire germinal colonies enter apoptosis (Corriero et al., 2007, McClusky et al., 2008, Yazawa et al., 2003). This phenomenon may be attributed to the delayed phagocytic removal of dead germ cells by Sertoli cells and exaggerated death phenomena other than classic apoptosis in a scattering of cells (Elliott et al., 2010).

Proliferating cell nuclear antigen (PCNA) is a highly conserved 36 kDa nuclear protein that serves as an auxiliary protein of DNA polymerase and functions during DNA replication (Bravo and Macdonald-Bravo, 1987, Prelich et al., 1987). The synthesis of PCNA increases during late Gl and S phases (Bravo and Macdonald-Bravo, 1987). It is widely used to provide an immunohistochemical assessment of cell cycle activity in normal tissues (Hall and Woods, 1990), and its expression is used as a marker of granulosa cell proliferation in cultured granulosa cells (the female equivalent of Sertoli cells) following their stimulation with follicle-stimulating hormone (FSH) and activin (El-Hefnawy and Zeleznik, 2001). Although, there are many literatures on large number of shark species but to our best knowledge, there are few literatures on the testes of brown-banded bamboo shark therefore, the present study aimed to investigate the histological structure of the testes of brown-banded bamboo shark using routine histological technique to study the normal structure as well as the PCNA immunohistochemistry in winter and summer of the testis.

2. MATERIALS AND METHODS

2.1. Animals

Four mature brown-banded bamboo male sharks were used in this study; the first two were collected during winter season (December - January) and the other were collected during Summer season (June - August; according to Alimi et al., 2015). These sharks were collected from Okinawa Churaumi Aquarium, Okinawa, Japan. The shark measures about 100 cm length and 4 kg body weight.

2.2.Tissue preparation

The sharks were dissected and eviscerated to liberate the testes. Cross section (4 - 5mm) samples were taken midway along the entire length of the testis and fixed with 10% neutral buffered formaldhyde solution for 48 hours at room temperature. The samples were thoroughly washed in 70% ethanol before it dehydrated in ascending concentrations of ethanol (80, 90, 95 and 100%), then cleared in lemosol and finally immersed in paraffin wax. Sections 3 - 5 µm thickness were collected on poly-L-lysine pre-coated slides. Sections were then stained with hematoxylin and eosin for describing the general morphology of the testis.

2.3.PCNA immunohistochemistry

The immunostaining was carried out according to McClusky and Sulikowski (2014). Briefly, endogenous peroxidase was utilized by treating sections in darkness for 10 min with 3% hydrogen peroxide then rinsing in distilled water. The sections were subjected to an antigen-retrieval procedure which require incubation for 10 min in 'Retrieve-all' pH 8.0 (Signet Laboratories, Dedham, MA, USA) in a steamer bath, followed cooling for 10 min at room temperature (RT). Washing twice in diluted Cadenza buffer wash (Thermo Electron, Waltham, MA, USA) was performed then the sections were incubated with

Omnitags protein blocking solution (Thermo Electron) for 5–10min at RT to reduce non-specific binding, and then overnight at RT with anti-PCNA PC10 (Calbiochem, San Diego, CA, USA) at a final concentration of 0.001 µg/µL. After three buffer washes in Cadenza buffer, sections were incubated at 1: 40 dilution in biotinylated anti-mouse secondary antibody (Thermo Electron) for 30 min at RT followed by three washes in Cadenza buffer. After washing the sections were incubated with the Vectastain streptavidin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for a further 20 min at RT then The antigen was detected by treating the sections with Vector Nova Red substrate kit (Vector Laboratories) for 10min. the sections were counterstained with hematoxylin.

3. RESULTS

3.1. Histological findings

The testis of brown-banded bamboo shark was suspended in vertebral column dorsally and surrounded by the epigonal organ ventromedially (Fig. 1A, B). It was surrounded by a delicate connective capsule from it, arise connective tissue septae that divide the testis into several lobules from the dorsal surface (Fig. 1C). These lobules were not observed in the middle and distal part of the testis. The testicular parenchyma was arranged into a zonation pattern including germinal, spermatogonial, primary spermatocyte, secondary spermatocyte, spermatid, spermatozoal and degenerative zones (Fig. 1C-F and Fig. 2A-H). The germinal zone was present at the germinal ridge and it contained round cells characterized by spherical nucleus with clear nucleolus and dispersed chromatin which is the spermatogonia. Around the spermatogonia, there was a spindle shaped cells with darkly stained nucleus distributed randomly which is the Sertoli cells (Fig. 1D). Due to the mitotic division of spermatogonia and Sertoli cells, there was a cyst like structure which was surrounded by a basement membrane. These cysts were composed of one or two layers of spermatogonia with the same characters of cells of the previously mentioned layer resting on a basement membrane in addition to a Sertoli cells with elongated nucleus arranged toward the luminal part of the cyst. The previously described structure is the spermatogonial zone (Fig. 1D-F). In the primary spermatocyte zone there were several layers (3 - 6 layers) of primary spermatocytes that rested on the basement membrane and also only one layer of Sertoli cells toward the lumen which migrate toward the basement membrane which composed of one or two layers of myoepithelial cells (Fig.2A). All these three aforementioned layers were belonging to the pre-meiotic zone where the mitosis observed.Regarding the meiotic zone it includes both secondary spermatocyte and spermatid zones in which the cells appeared small in size, densely packed with narrow lumen and the Sertoli cells located peripherally (Fig. 2B, C). Finally, round spermatid zone in which the cells were arranged in colonies. These colonies were separated from each other by spaces and each one was rest on a single layer of Sertoli cell (Fig. 2D, E). Noteworthy, the spermatozoal zone in which the process of spermiogenesis takes place were occupied by spermatozoal cyst containing spermatozoa characterized by acidophilic tail cytoplasm near the center and elongated nuclei embedded in the apical part of Sertoli cells which located peripherally at the basement membrane (Fig. 2F, G). In the degeneration zone the spermatozoa were elaborated from the spermatocyst leaving only Sertoli cells which degenerate later (Fig. 2H).Generally, there were interstitial cells among the cysts in all testicular zones but it is predominant in the secondary spermatocyte and spermatozoal zones (Fig. 2C, F). These cells appeared rounded with spherical nucleus and acidophilic cytoplasm. Moreover, there were tubules among spermatocyst lined with cuboidal epithelium through it the spermatozoa were transported to outside the testis (Fig. 2G, H).

3.2. PCNA expression

The main point of interest was that PCNA expression was restricted to the nucleus of spermatogonia, spermatocytes and Sertoli cells in the pre-meiotic and meiotic zones only while it was absent in the post-meiotic zone (Fig. 3A-F). Moreover, the intensity of expression was different among the various zones as its expression was prominent in the nucleus of spermatogonia in the germinal zone compared with spermatogonial zone (Fig. 3A, B). The intensity of PCNA expression in the testis of shark was different either during summer (Fig. 3C, D) or winter season (Fig. 3A, B). Additionally, the intensity of reaction during the two seasons in both spermatogonia and sertoli cells was variable in each testicular zone where some cells were darkly stained and others were moderate as presented in Table 1. On the other hand, the interstitial cells were moderately labeled in all zones except spermatozoal and degenerative zones (Fig. 3E, F).

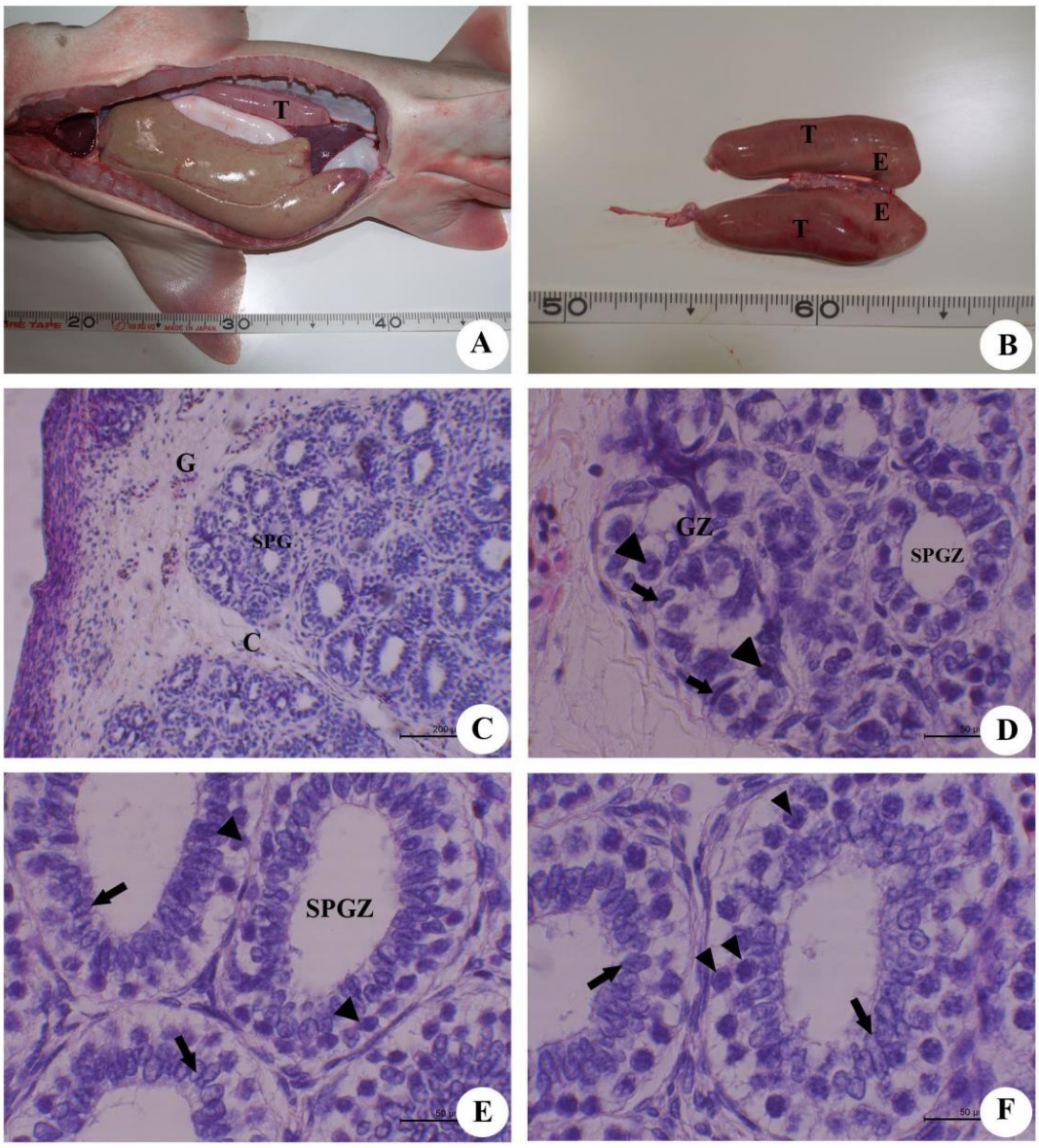

Fig. 1: The position, gross morphology and the histological features of testes in brown-banded bamboo shark.
A: Testicular Position (T) in relation to abdominal viscera; B: Showing the dissected testes (T) with the epigonal organ (E); C: Histological section of testis showing germinal ridge (G), connective tissue septae (C) and spermatogonial cyst (SPG); D: Showing both germinal zone (GZ) containing spermatogonia (arrow head) and Sertoli cells (black arrow) in addition to spermatogonial zone (SPGZ); E: Showing spermatogonial zone (SPGZ) with spermatogonia (arrow head) near the basement membrane and Sertoli cells (black arrow) toward the lumen; F: Double layer of spermatogonia (arrow head) near basement membrane and Sertoli cells (black arrow) toward the lumen.

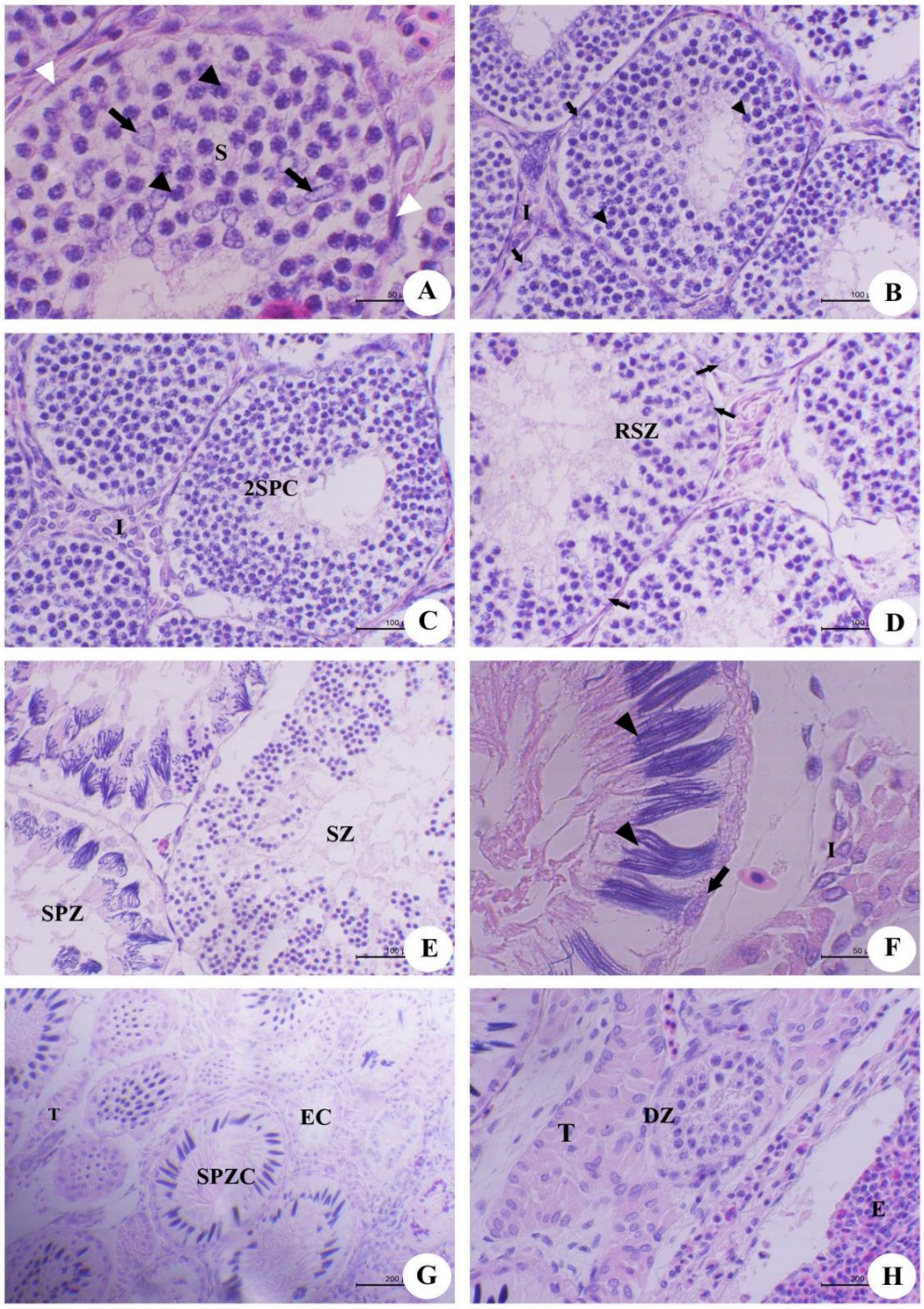

Fig. 2: Photomicrographs of the testicular zones of brown-banded bamboo shark
A: Primary spermatocyte zone showing spermatocyst (S) with migrated Sertoli cells (black arrow) from the lumen toward the basal lamina and several layers of primary spermatocytes (arrow head) in addition to myoepithelial cells (white arrow head); B: Secondary spermatocyte zone containing Sertoli cells (black arrow) at the basal lamina and several layers of secondary spermatocytes (arrow head) in addition to the interstitial cells (I); C: Showing numerous interstitial cells (I) between secondary spermatocysts (2SPC); D: Showing round spermatid zone (RSZ) in which the cells arranged in colonies attached to the Sertoli cells (black arrow); E: Transition zone between spermatid zone (SZ) and spermatozoal zone (SPZ); F: spermatozoal zone showing elongated colonies of spermatozoa (arrow head) over Sertoli cells (black arrow) and acidophilic interstitial cells (I); G: Showing spermatozoal cyst (SPZC) and evacuation (degeneration) cyst (EC) in addition to intra-testicular tubule (T); H: Showing degeneration zone (DZ) and epigonal organ (E) in addition to intra-testicular tubule (T).

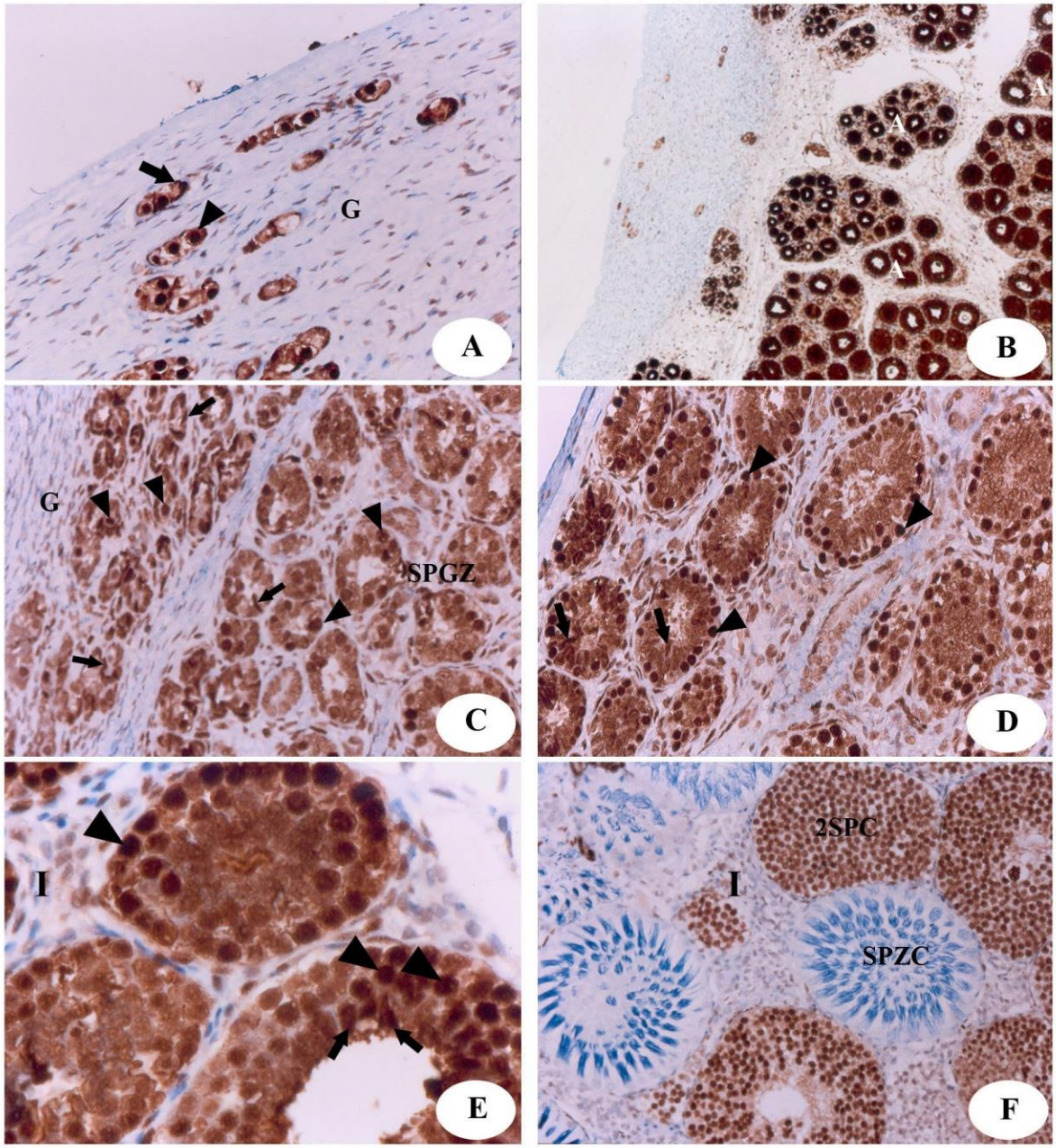

Fig. 3: Photomicrographs of PCNA expression in brown-banded bamboo shark testis during summer and winter seasons .
A: Showing the germinal ridge (G) it has intense reaction in both spermatogonia (arrow head) and Sertoli cells (black arrow) before the formation of the cyst; B: Showing spermatogonial zone containing spermatogonial cysts (A) with intense reaction in both types of cells; C: Showing the testis during summer season revealing intense reaction to some spermatogonial (arrow head) and Sertoli cells (black arrow) in both germinal ridge (G) and spermatogonial zone (SPGZ); D: Showing spermatogonial zone of testis during summer season revealing intense reaction in spermatogonia (arrow head) and moderate reaction in Sertoli cells (black arrow); E: Showing primary spermatocyte zone during winter season revealing intense PCNA expression in both primary spermatocyte (arrow head) and Sertoli cells (black arrow) in addition to interstitial cells (I); F: Showing the transition zone between secondary spermatocyte cyst (2SPC) and spermatozoal cyst (SPZC) in addition to interstitial cells (I) with positive reaction to PCNA in secondary spermatocyte cyst only.

Table 1: The intensity of PCNA expression within the different zones of shark testes during winter and summer seasons

	Testicular zone							
	Germinal zone		Spermatogonial		Meiotic		Post-meiotic	
Season	SPG	Sertoli cell	SPG	Sertoli cell	SPC	Sertoli cell	Spermatozoa	Sertoli cell
Winter	+++	+++	+++	+++	++	++	-	-
Summer	++	++	++	++	++	++	-	-

SPG= Spermatogonia, SPC= Spermatocyte

4. DISCUSSION.

In this study the results showed that the testes of Brown-banded bamboo shark were suspended in the vertebral column dorsally and surrounded by the epigonal organ ventromedially. This finding was different from that of Rêgo et al. (2016) who observed that the epigonal organ of P. glauca, R. lalandii, and M. canis support the posterior end of the testes. On the other hand, Girard et al. (2000) found that there was no epigonal organ nearby the testes along its whole length in Centroscymnus coelolepi and C. squamosus species. The testes of the brown-banded bamboo shark have a diametric pattern of development starting from the dorsal part of the testis wherein, the germinal zone toward the epigonal organ ventrally wherein, the spermatozoal and degenerative zone. Moreover, the spermatozoal cysts evacuate the spermatozoa into the efferent ductules. This unique testicular anatomy is similar to other sharks, skates, rays and saw fish but differ from that of other animals due to their phylogenetic position at the base of vertebrate tree of life (Rêgo et al., 2016). The diametric pattern of testes (germinal, spermatogonial, primary spermatocyte, secondary spermatocyte, spermatid, spermatozoal and degenerative zones) is the best model to track the cascade of the process of spermatogenesis where each zone represent only one type of germinal cells in addition to sertoli cells which is not found in the mammalian testes. This diametric testicular development is characteristic for carcharhinidae, sphyrnidae and triakidae (Rojas, 2013); Somniosidae, Centrophoridae (Girard et al., 2000) and also recently Hemiscyliidae shark have exhibit the diametric type of testicular development (Kassab et al., 2009).The arrangement of sertoli cells in the different testicular zones of brown-banded bamboo shark was different because it was randomly distributed with the spermatogonia in the germinal and spermatogonial zones then it was located toward the lumen in the pre-meiotic zone and appeared at the basal lamina in the post-meiotic zone. These findings might be attributed to the process of spermiogenesis in which the spermatids are attached to the apical part of Sertoli cells and this might be the reason for the colony formation in the spermatid and spermatozoal cyst.The interaction between sertoli cells and spermatogonia to form the spermatocyst was detected in the germinal zone of testis in P. glauca, R. lalandii, and M. canis species. Interestingly, the pattern of interaction observed in the current study is similar to that described by Loir et al. (1995), where the two types of cells were present in the interstitial tissue of the testis and became surrounded by a basement membrane to form the spermatocyst (Parsons and Grier, 1992).It is well known that, PCNA is an auxiliary protein to DNA polymerase-d, which being involved in nucleotide excision repair mechanisms (Hall et al., 1993, Chapman and Wolgemuth 1994). Furthermore, PCNA is located in the nuclear matrix in all stages of cell cycle (Casasco et al., 1988). It considered one of the most applied tools to evaluate the mitotic activity beside (3H) thymidine or bromodeoxyuridine (Miyachi et al., 1978).The main point of interest in the present study is that the expression of PCNA and the intensity of expression were season-dependent. The sertoli cells as well as the spermatogonia showed strong and moderate reaction in both winter and summer seasons respectively. The aforementioned findings is in accordance with that of Garnier et al. (1999) who reported that in S. canicula species there was an uninterrupted spermatogenesis throughout the year. On the other hand, the spermatogonia of the germinal ridge have been observed with an active period from April to August in S. acanthias species (McClusky, 2005).In shark the development of spermatogonial cells within the germinal ridge and early pre-meiotic cysts was gonadotrophins-independent (Dobson and Dodd 1977) and may be attributed to Sertoli cell-mediated seasonally regulated diffusible factors (Loir 1994). The results of this study revealed a moderate affinity of

spermatogonia to PCNA during the summer season which may be attributed to a state of developmental arrest probably Sertoli cell-mediated (McClusky, 2005). In this regard, it is worth noting that both mRNA and PCNA protein are known to be under the effect of FSH in mammals, rat granulosa cells (female equivalent to sertoli cells; El-Hefnawy and Zeleznik 2001), and Sertoli-cell proliferation in foetal and neonatal rodent testis which was FSH-stimulated (Sharpe et al. 2003, Allan et al. 2004)

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5. CONCLUSION

The testis of brown-banded bamboo Shark have a diametric pattern of development starting from the dorsal part of the testis wherein, the germinal zone toward the epigonal organ ventrally wherein, the spermatozoal and degenerative zone. This diametric pattern of development is the best model to track the cascade of the process of spermatogenesis where each zone represent only one type of germinal cells in addition to Sertoli cells which is not found in the mammalian testes. The season has non-significant effect on the PCNA expression on the testis of brown-banded bamboo Shark .

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