Niger. J. Physiol. Sci. 34 (December 2019) 121-124
www.njps.com.ng

Niger. J. Physiol. Sci.

Age Related Histology and Immunohistochemistry of Some Intermediate Filaments in the Testis of the African Catfish (*Clarias Gariepinus*)

Aina O. O.*[1], Ozegbe P. C.[1], Adeyemo O. K.[2]

[1]Department of Veterinary Anatomy, University of Ibadan, Ibadan, Nigeria. [2]Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Nigeria

Summary:

The African Catfish (*Clarias gariepinus*) are important source of protein for local consumers in developing countries in Africa and have also been reported to have enormous commercial potential. Several works have been done on plethora of general histological, biochemical and hormone changes which accompany puberty in African Catfish. Other studies have touched the effects of ecotoxins on the histological and reproductive parameters of the mature African Catfish. This study is an attempt to use immunohistochemical and basic histology to elucidate the baseline information on the general structural differences between the testes of immature and post-pubertal catfish with respect to some intermediate filaments arrangement within the testicular tissue. Ten (10) each of mature male catfish (4-5 months old) and immature male catfish (3 months old) were used in the study. The fish were subjected to cold shock and decapitated before the testes were harvested from both groups. These tissues were fixed in Bouin's fluid for 24 hours and subsequently transferred into 70% Ethanol. Testicular tissues from both groups were processed for paraffin embedding for routine staining with H&E; another set of tissues were fixed in 10% Neutral Buffered Formalin for testicular immunostaining techniques against Vimentin, Desmin, Cytokeratin and Smooth Muscle Actin (SMA) expression using standard methods. There is an increase in seminiferous luminal area/diameter in the mature catfish testis with the presence of mature spermatozoa in the lumen when compared with immature catfish testis which has small size of lumen with absence of mature spermatozoa. Testicular interstitium thickness remain relatively unchanged. SMA was markedly expressed in the cytoplasm of interstitial Leydig cells in the immature catfish testis whereas it was weak in its expression in the mature catfish. However, SMA was not expressed in the connective tissue proper in the testicular interstitium. Cytokeratin expression was also marked in the testicular capsule of immature catfish but was weak to absent in the mature catfish, however, both mature and immature catfish had moderate cytokeratin expression in their seminiferous tubule basement membrane. Desmin was strongly expressed in cytoplasm of immature germinal cells in the immature catfish testis but was moderate in its expression in the mature catfish testis. Vimentin expression was marked in the cytoplasm of immature germinal cells in both immature and mature catfish testis but weak in its expression in the Sertoli cell cytoplasm of both groups. This study infers that ultra-structural and protein changes can be related to age changes alone apart from the contribution of seasonality and external interference by ecotoxins. The age-related changes seen in this study could set ``baseline information. The extent of contribution of season and other external factors will be better understood. Though the age-related difference might be peculiar to the species of current interest, the differences elucidated are a sound background for relational studies, especially on the effect of ecologic toxins on immature testis, as separate from the mature testis.

Keywords*:* *Clarias gariepinus*; Testis; Vimentin; Desmin; Cytokeratin; Smooth Muscle Actin

**Address for correspondence*: ainasanmi@gmail.com

Manuscript Accepted: November, 2019

INTRODUCTION

The African Catfish (*Clarias gariepinus*) are important source of protein for local consumers in developing countries in Africa and also have been reported to have enormous commercial potential. Apart from these, they are currently being used in various biological researches centring on ecological and nutritive events that affect this species. Anatomical data on the structural framework of both developmental and mature features of the fish is very important.

The testis of the African Catfish are paired elongated lobes located within, in the dorsocaudal compartment of the celomic cavity. They are suspended by mesenteries (mesorchia) which attach them to the gas bladder. A thin tunica albuginea, forms the capsule of the testicular lobes. Each lobe is composed of a collecting duct (ductus deferens) aligned with the long axis of the lobes. Seminiferous tubules radiate from sections off the entire length of the ductus deferens.

Spermatic tissue of fish histologically is composed of a tubular compartment which is made up of clusters of large germ cells. These are separated by an interstitial compartment of connective tissue (Lofts and Holmes,1985; Cristiane *et al.*, 2012). Intermediate filaments are relatively a lot more abundant than other structural proteins like microfilaments and microtubules in the skeletal frame of epidermal cells (Lodish *et al.*, 2000). Compared with microfilaments and microtubules, they have a greater stability, a larger assemblage of proteins and also exist in nature as dynamic polymers.

They are structural proteins which laminates the cellular and nuclear membranes, where they associate and interact with other cytoskeletal structures and receptors (Lodish *et al.*, 2000; Alberts *et al.*, 2002). They are most probable to be identified intact in cells even after different types of tissue processing (Lodish *et al.*, 2000; Alberts *et al.*, 2002).Several works has been done on the plethora of general histological, biochemical and hormone changes which accompany puberty in African catfish (Lofts and. Holmes, 1985; Cavaco *et al.*,1999; Nóbrega and Quagio-Grassiotto, 2007). Other studies have also touched the effect of ecotoxins on the histological and reproductive parameters of the mature African catfish (Sayed *et al.*., 2011; Sayed *et al.*., 2012). This study is an attempt to use immunohistochemistry and basic histology to elucidate the baseline information on the general structural differences between the testes of immature and post pubertal catfish with respect to some intermediate filament arrangements within the tissue.

MATERIALS AND METHODS

Ten matured male fish (4-5 months old) and ten immature (3 months old) male fish were used. The fish were subjected to cold shock and decapitated before harvest of the testes

Testicular tissues obtained from both groups and further divided into two portions. The first portion was fixed in Bouin's fluid for 24 hours before being transferred to 70% ethanol. The fixed tissue samples were processed, using routine paraffin embedding technique. 5µm thick testicular sections were prepared and mounted on specimen slides. These were stained with Harris' Hematoxylin and Eosin stain (H&E). The second set of testicular tissues was directly fixed in 10% Buffered Formalin, before the routine paraffin embedding for the immunostaining technique.

LSAB-plus kit (Dakocytomation, Denmark) was used for the immunostaining technique, performed on 5 µm-thick testicular sections. Deparafinization of the sections was done using xylene and standard grades of ethanol concentrations. The slides were immersed in hydrogen peroxide solution in water 3% (v/v) for 5 minutes to block endogenous peroxidase activity. The slides were then rinsed in a 0.01M phosphate buffer saline (PBS), solution 1-1 (pH 7.4) for 5 minutes. Thereafter, the slides were immersed in citrate buffer solution and microwaved at low heat(750 W) for 15 minutes .

After cooling, the sections were rinsed with PBS and incubated for 30 minutes at room temperature with standard dilutions of monoclonal antibodies against vimentin (1:100), desmin (1:300), cytokeratin (1:100) and smooth muscle actin(1:50). The slides were subsequently rinsed with PBS and then incubated for 15 minutes with a ready-to-use biotinylated secondary antibody (LSAB-plus kit, Dakocytomation, Denmark). Thereafter, the slides were rinsed in PBS and subsequently incubated for 15 minutes with the streptavidin component of the LSAB-plus staining kit. Slides were then rinsed in PBS and bound antibody was visualized after the addition of a 3,3' -diaminobenzidine tetrachloride solution (LSAB-plus kit, Dakocytomation, Denmark).

In the negative controls normal mouse serum was used as the primary antibodies. Intestinal tunica mucosa was used as a positive control for both desmin and smooth muscle actin, tissue from the salivary gland was used for cytokeratin whilst tissurom the tonsils was used as a positive control for vimentin.

On examination of the stained slides with a digital photomicroscope (VJ-2005 DN), the relative intensities of vimentin, desmin, cytokeratin and actin immunostaining were designated as absent (-), weak (+), moderate (++) and strong (+++) as described by Madekurozwa and Kimaro (2006).

RESULTS

There is an apparent increase in seminiferous luminal area/diameter in the matured testis which also showed presence of mature spermatozoa in the lumen, as seen in Figure 1.

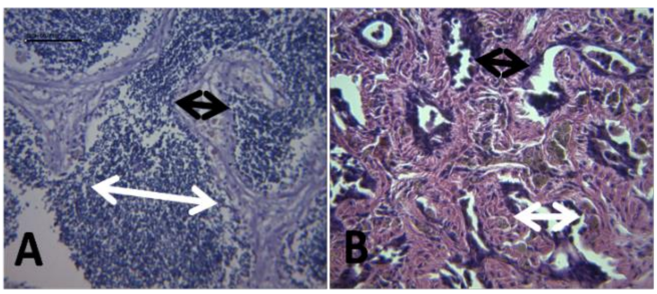

Figure 1

Light microscopy of Hematoxylin and Eosin (H&E) stained *Clarias gariepinus* testis (A) cross-section of the testis of the mature (A) and immature (B). Testicular organization with seminiferous lobes and interstitial tissue. Diameter across seminiferous lobes (white double headed arrows), interstitial thickness (black double headed arrows) MAG: X 400

While the thickness of the interstitium does not appear to increase relative to prepubertal to post pubertal age, the prominence in the immature is accentuated by the small size of the lumen and the absence of matured spermatozoa.

As shown in figures 2-5, Cytokeratin expression is found in the inner recesses of the testicular capsule and the basement membrane underlying the sertoli cells. The immature catfish testis has a more intense expression at these sites relative to the mature. Desmin is intensely expressed in the cytoplasm of immature germinal cell cytoplasm.

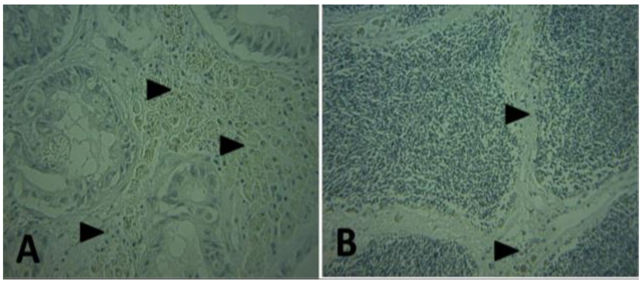

Figure 2:
Localisation of actin filaments in cells at the intertubular connective tissue (arrows) of groups A : Immature Testis ; B: Mature Testis.

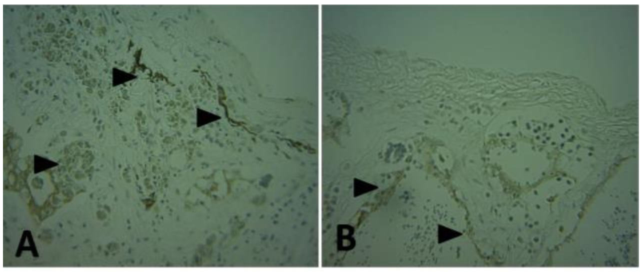

Figure 3:
Localisation of Cytokeratin filaments in cells at the testicular capsule and basement membrane of (arrows) of groups A : Immature Testis ; B: Mature Testis. MAG: X 400

The comparison of the intensities is summarised in Table 1. Actin immunostaining was well expressed in the cytoplasm of cells found in the testicular interstitium (Leydig cells). They are however not seen in the connective tissue that forms the interstitium proper. This expression is relatively higher in the immature testis. The Actin immunostaining is also present but poorly expressed in the sertoli cell cysts and immature germinal cell cytoplasm.

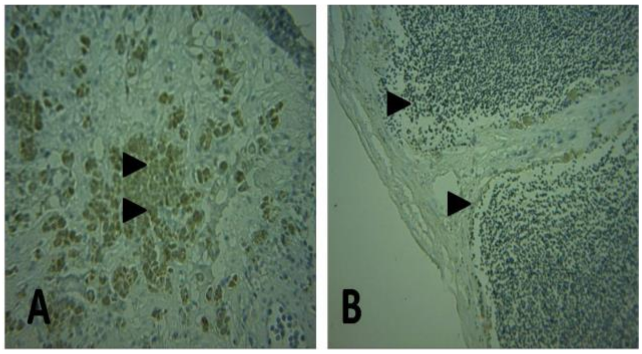

Figure 4:
Localisation of Desmin filaments in Immature germinal cells at the seminferous tubular lumina (arrows) of groups A : Immature Testis ; B: Mature Testis. MAG: X 400

These cells are relatively more abundant in the immature testis; the immune-expression appears more prominent though at the same intensity with the same site in mature testicular tissue. Vimentin expression is limited to the cytoplasm of immature germinal cells and they are also found lightly at the margins of sertoli cysts. The strength of expression appears the same in the two age groups of testicular tissues.

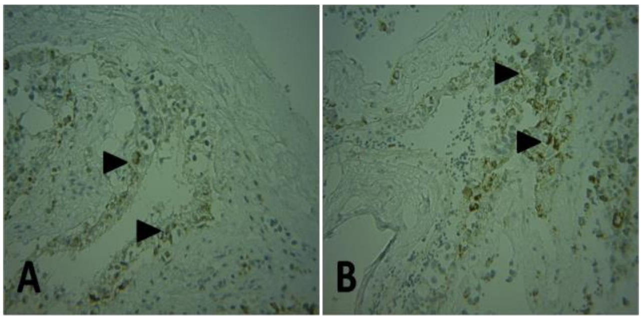

Figure 5:
Localisation of Vimentin filaments in Immature germinal cells at the seminferous tubular lumina and also at the margins of the sertoli cell cysts. (arrows) of groups A : Immature Testis ; B: Mature Testis. MAG: X 400

Table 1:
Summary of the immunohistochemical localization of the intermediate filaments; cytokeratin, vimentin, desmin and the microfilament-actin in the Mature and immature testis of the African Catfish.

Cell/Tissue stained	Actin		Cytokeratin		Desmin		Vimentin	
	A	B	A	B	A	B	A	B
Testicular Capsule	-	-	+++	+/-	-	-	-	-
Testicular interstitium	-	-	-	-	-	-	-	-
Basement membrane			++	++	-	-	-	-
Immature Germinal cell cytoplasm	++	+	-	-	+++	++	+++	+++
Sertoli Cell Cysts	+	+	++	+	-	-	+	+
Leydig cells	++++	+	-	-	-	-	-	-

Intensities of immunostaining: --, absent; +, weak; ++, moderate; +++, strong. A= Immatured, B= Matured.

DISCUSSION

Weak actin immune-expression was seen in interstitial cells, Sertoli cysts and germinal cells similar to what obtains in the study of cytoskeletal proteins in Mosquito fish (Arenas *et al.*., 1995). However, there is poor expression of actin immunostaining in the Sertoli cell cysts and immature germinal cell cytoplasm relative to the Leydig and contractile cells within the interlobular septum supports the assertion that intralobular and interlobular cell population are not homologous (Grier *et al.*., 1989).

There was no immunoreaction to desmin and very mild immunoreaction vimentin observed in the Sertoli cells of both immature and mature testes in this study. This is quite similar to the result of desmin and vimentin assay in Mosquito fish (Arenas *et al.*., 1995). Cytokeratin immunoreaction was clearly seen in the testicular capsule, germinal basement membrane and Sertoli cysts of both mature and immature African catfish, though the reactivity was more pronounced in the immature group.

The results of this study clearly indicate that the intermediate filaments in the testis of prepubertal and post pubertal testis exhibit disproportionate epithelial and non-epithelial tissue distribution pattern. Increase in germinal cell activity with respect to maturity in addition to other cellular and molecular components might be responsible for the enhanced seminiferous luminal area/diameter in the matured testis .The thickness of the interstitium at maturity does not appear to increase relative to pre pubertal age. The prominence of the interstitial connective tissue seen in the immature is accentuated by the small size of the tubular lumen, absence of matured spermatozoa and possible a smaller mass of the testis.

Biochemical and hormone changes have already been proven to accompany puberty in African catfish (Lofts and. Holmes, 1985; Cavaco *et al.*,1999; Nóbrega and Quagio-Grassiotto, 2007). Also, several works have indicated age, reproductive season and treatment related difference in quantity and distribution pattern of some proteins in the testis of African catfish (Raghuveer and Senthilkumaran, 2009; Raghuveer and Senthilkumaran, 2010; Rajakumar *et al.*., 2012). The relationship between age and protein distribution could be related with the effects of ecological factors on age and seasonality.

This study infers that ultra-structural and protein changes can also be related to age changes alone apart from the contribution of seasonality and external interference by ecotoxins. The age related changes seen in this study could set baseline information. The extent of contribution of season and other external factors will be better understood. Though the age related differences might be peculiar to the species of current interest, the differences elucidated are a sound background for relational studies, especially on the effect of ecologic toxins on immature testis, as separate from the mature ones.

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