

Nerve and Glial Cell Expressions in the Testes and Epididymides of Different Age Groups of Cane Rat (*Thryonomys swinderianus*)

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Abstract

Purpose: This study was conducted to examine the variations in the expressions of neuronal and glial cell markers in the testes and epididymides of different age groups of cane rat using histochemical and immunohistochemical techniques. **Method:** Thirty (32) healthy domesticated male cane rats were used for this investigation. The rats were divided into four groups (prepubertal [≤ 4 months], pubertal [$>4 \leq 12$ months], adult [$>12 \leq 30$ months], and aged [>30 months]) of 8 animals each. Subsequent to anesthesia and intracardiac perfusion of the rats with 10% buffered formalin, testes were harvested and preliminary assessment of nervous and glial structures was determined using the Golgi technique. Specific immunolocalization was done using the anti-neurofilament (NF-20) and anti-glial fibrillary acid protein (GFAP) for the expressions of neuronal and astrocyte-like cells, respectively. **Result:** Neuronal and astrocyte-like structures as revealed by the Golgi procedure were demonstrated in the tunica albuginea and interstitium of the testes as well as in the periductal muscle coat and epididymal interstitium of the caput down to the caudal segments. Golgi signal intensities of the expressions in both testes and epididymides increased with age advancement. Immunolocalization of the nerve structures and glial cells tallied with the Golgi results. However, NF signal intensity was significantly higher in the adult relative to others. Similarly, GFAP signal intensity increased with age increment. **Conclusion:** This study has shown that the variation in the expression of neuronal and glial cells in the testis and epididymis of the cane rat could be associated with increased reproductive activity.

Keywords: Astrocytes, cane rat, epididymides, neurons, testes

INTRODUCTION

The male gonad neuronal network is formed by the peripheral nerves (the superior and the inferior spermatic nerve (SSN and ISN) fibers that emanate from the autonomic ganglionic system.^[1] The SSN fibers emanate from the superior mesenteric ganglion coupled with inputs from renal, spermatic, and aortic plexuses and descend bilaterally to approach the testes in the company of testicular artery and gain entrance into the testis at the cranial pole.^[2-4] The SSN can also receive afferent and possibly vagal parasympathetic fibers, whereas the ISN fibers carry mostly sympathetic fibers. It originates from the inferior mesenteric ganglion and pelvic plexus accompanied by the vas deferens and then gain access to the

caudal pole of the testes through the inferior ligament of the tail of epididymis.^[2,3]

The morphological closeness between neuronal elements and testicular cells (Leydig cells, boundary tissue, and vascular cells) make testicular cells direct the targets of catecholamines and neuropeptides and thereby provide strong proof for a functional association.^[5-8] These neurotransmitters in the presence or absence of pituitary hormones are capable of intrinsic triggering of receptors on the Leydig cells, Sertoli cells, and smooth muscle cells of the testis.^[9,10] Several

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neuronal markers have been utilized to localize the testicular nerves around the branches of the testicular artery, interstitial Leydig cells, and seminiferous tubules.^[11-14] Conspicuous evidence of abundant innervations has been reported in the testis of prepubertal (PRE) pigs in a study that investigated age-related changes in the density, distribution pattern, and neurochemical coding of nerve fibers of boars from postnatal period to adulthood.^[15]

Similarly, neuronal fibers in the epididymis have been localized within the perimuscular coat, subepithelial regions, and in the coat of vessels within the interstitium.^[16,17] Nerve distribution pattern in the epididymis has been found to be segment-specific with the cauda segment having the highest nerve ramifications owing to its thick muscular wall.^[18] The neurotransmitters in the epididymal nerves have been suggested to be important in mediating epididymal epithelial cell function of electrolyte transport and protein processing.^[19,20]

Astrocytes are glial cells that are usually situated close to the nerves as support cells in both central and peripheral nervous systems. Functionally, astrocytes have many roles prime of which is the maintenance of blood–brain barrier.^[21] The recognized marker of astrocyte in the central nervous system (CNS) remains an intermediate filament, the glial fibrillary acid protein (GFAP).^[22,23] Recent studies have shown its expression in non-CNS tissues with mesenchymal stellate cells, including the liver, kidney, pancreas, lungs, and testes, which share functional similarity with astrocytes.^[24-26] Astrocytes have been localized in Leydig cells of the rat and human testes.^[27,28]

Cane rat (*Thryonomys swinderianus* Temminck, 1827) is a wild rodent currently being domesticated as an alternative animal protein source and potential animal research model in West African countries.^[29,30] The male cane rat attains sexual maturity at about 8 months of age and can live up to 4 years in captivity.^[31,32] To our knowledge, there is no report on the nerve fiber and glial-cell expressions in the reproductive organ of the different age-category of this animal.

Aim of the work

Therefore, this study seeks to investigate the variations in the testicular nerve fiber and glial-cell expressions in the cane rat of different age groups.

MATERIALS AND METHODS

Animals

Thirty-two (32) healthy male cane rats procured from a commercial farm, Iberekò, Badagry, Lagos state, Nigeria, were used for this study. Birth records of the different rats were obtained at purchase. They were subsequently acclimatized for 7 days in the Experimental Animal Unit of Faculty of Veterinary Medicine, University of Ibadan and provided with dry corn feed daily and water *ad libitum*. The different cane rats were humanely cared for by strict adherence to the ethical considerations of the University of

Ibadan Animal Care and Use Research Ethics Committee Review (UI-ACUREC) and therefore was approved and assigned UI-ACUREC/18/0120.

Experimental design

Using the modified age grouping of Soro *et al.*,^[32] 32 male cane rats were divided into four groups of eight ($n = 8$) rats each as follows:

Group I (PRE; ≤ 4 months), Group II (pubertal [PUB]; $>4 \leq 12$ months), Group III (adult (ADT); $>12 \leq 30$ months), and Group IV (aged (AGD); >30 months). Five (5) out of the animals in each group were used for immunohistochemistry, and the rest (3) were used for Golgi technique. All cane rats were acclimatized for 7 days. On day 8, they were sedated with xylazine and ketamine combination (20:80 mg/kg body weight respectively) injected intramuscularly. The abdominal wall of each rat was dissected open through a ventral midline incision and the thoracic cage opened to expose the heart. This was then followed by the perfusion of 0.5 L of preperfusion (primary) solution 0.9% sodium chloride (Aventra, Fidson, Nigeria) and 25,000IU/ml of heparin. The primary solution was followed with secondary perfusion of 10% buffered formalin. The perfusion persisted till satisfactory change was observed in the testes color. Both the testes and epididymides segments (caput, corpus, and cauda) were excised for further processing.

TISSUE PROCESSING FOR GOLGI-SILVER STAINING PROCEDURE

The demonstration of nerves and glial cells using Golgi-silver staining procedure was carried out in accordance with the method described by Olude *et al.*^[33] Briefly, the excised testes and epididymides were trimmed (1 mm thick) and immersed in sample bottles of 10–30 mL capacity containing 3% potassium dichromate solution for 5 days. During these days, the bottles were wrapped externally with foil paper to prevent the light penetration into the solution. Stale solutions of dichromate were discarded and fresh one added on daily basis within the 5 days. On the 6th day, the tissue blocks were moved into 2% silver nitrate solution for impregnation in the next 3 days at the room temperature. On day 9, the impregnated tissues were removed from silver nitrate into a clean filter paper to remove excess silver precipitates on the tissues. The clean tissues were processed histologically by dehydration through increasing grades of alcohol: 70%, 90%, 100% alcohol, and xylene for 5 min duration each. Dehydrated tissues were then infiltrated in molten wax at 56°C for 30 min. Sections (5 μm thick) were made from the tissue block using microtome (Microm–HM 330, Germany) and then air-dried for 10 min and cover-slipped using DPX. Slides were viewed with light microscope for the presence of glial and neuronal structures.

Tissue processing for immunohistochemical localization of nerve fibers and astrocyte-like cells in the testes and epididymides of cane rat.

Testicular and epididymal sections were dewaxed in oven operated at 60°C and deparaffinized in two changes of

xylene, rehydrated in ascending grades of alcohol. This was followed by antigen retrieval from the sections using 10 mM citrate buffer at pH of 6.0 for 25 min. Nonspecific antibody binding and endogenous peroxidase activities were inhibited by subjecting the testicular sections to 3% H₂O₂/methanol for 15 min. The sections were then washed in phosphate-buffered saline (PBS) and later encircled with PAP pen to create a hydrophobic barrier. This was succeeded by incubating in (2% PBS) containing 5% bovine serum albumin for an hour. Each section was then immunolabeled using primary antibodies; anti-Neurofilament (NF) (Dako 1:100) and anti-GFAP (Dako 1:500). Thereafter, each section was diluted in 1% PBS milk and 0.1% Triton X detergent (for rapid penetration of antibody) and then incubated overnight for 18 h at 4°C. At this stage, horseradish peroxidase-conjugated secondary antibodies were consequently used by strictly adhering to the manufacturer protocol to detect the bound antibody. The end-product of the reaction was enhanced with 3, 3'-diaminobenzidine (DAB; Vectastain ABC kit) chromogen at 1:25 dilution ratio for 5 min. The sections were later dehydrated in the grades of alcohol concentrations, dealcoholized in xylene, mounted with DPX permanent mounting media, cover slipped and allowed to dry. The prepared slides were then viewed and photographed with the light microscope (Olympus BX3-CBH, USA). The captured images were quantified using Image J software (NIH, Bethesda, MD, USA), and results obtained were presented with bar charts.

Statistical analysis

Data obtained from the imageJ quantification of staining

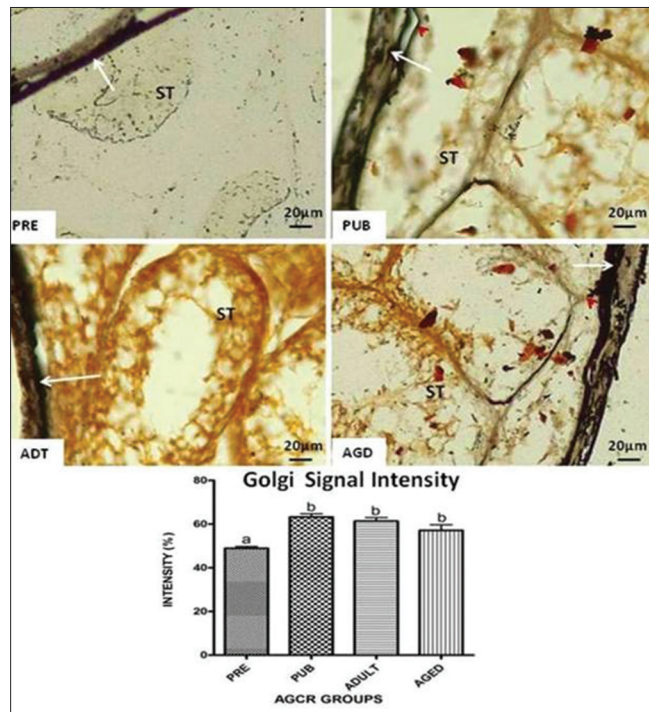


Figure 1: Golgi staining of the testis of different age-groups of the AGCR. Note the display of nerve fibre in the tunica albuginea (arrow) and twigs radiating from it to parenchyma (arrow head)

intensities of the Golgi and the different immunolabelings were analyzed using GraphPad Prism Version 4.00 for Window (GraphPad Software Inc., La Jolla California, USA) statistical package. The variations in the staining intensity of each immunolabelings were compared using the one-way analysis of variance and Tukey test was used for multiple comparisons *post hoc*. Level of significance was at $\alpha_{0.05}$.

RESULTS

Golgi stainings for testicular and epididymal nerves and glial-like cells (Astrocyte-like) in the different age group of cane rat

With the use of Golgi-silver technique, both the neuronal and the astrocyte-like structures were demonstrated in the testicular tunica albuginea [Figure 1], interstitium, and along the seminiferous tubular boundary [Figure 2] in all the AGCR groups. Within the segments of the epididymal duct, neuronal and astrocyte-like structures were remarkably observed in the periductal muscle coat and in the epididymal interstitium of the caput, corpus, and cauda segments [Figures 3-5]. Golgi intensity profile in both the testicular capsule [Figure 1] and interstitium [Figure 2] were significantly lower ($P < 0.05$) in the PRE rat when compared to others. The intensity appeared to increase with age advancement, though an insignificant ($P > 0.05$) decline value was displayed by aged AGCR relative to the PUB and

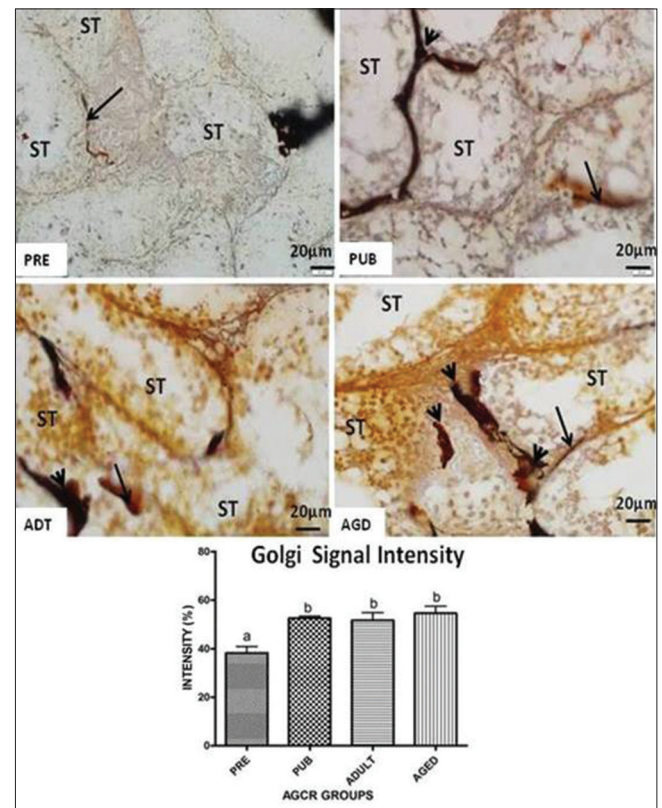


Figure 2: Golgi staining of the testis of different age groups of AGCR. Note the display of fine nerve fibre (long arrow) between seminiferous tubular boundary and thick nerve fibre in the seminiferous tubular interstitium (arrow head)

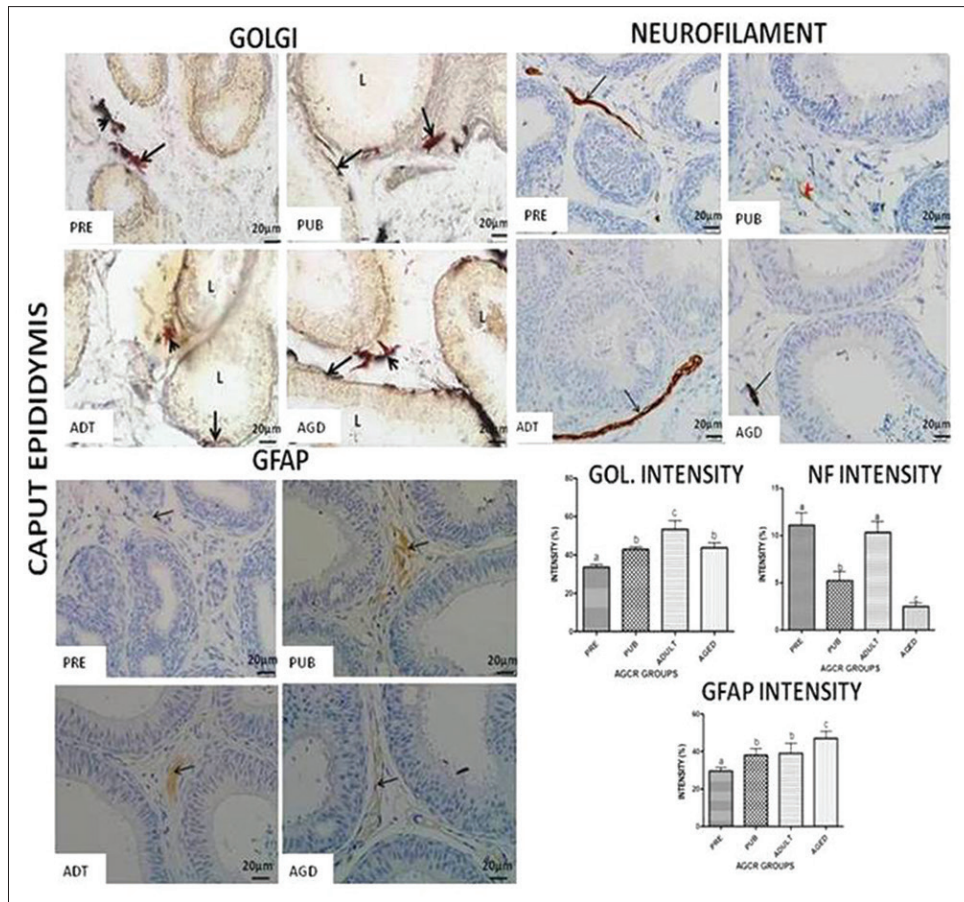


Figure 3: Photomicrographs of the caput epididymis of different age groups of AGCR. Note in Golgi sections, the display of nerve fibres within the periductal muscle coat (long arrow) and the interstitium (arrow head) in all groups. Also, note the positive neurofilament staining of nerve structures in the epididymal ductal interstitium (arrow) and in perivascular area (arrow head). Note also, positive glial fibrillary acid protein staining of astrocyte-like cells in the perivascular area of ductal interstitium (arrow), in all groups

adult values. The intensities of the nerve and glial-like cells of the caput, corpus and cauda epididymal segments consistently displayed significantly higher intensity ($P < 0.05$) in the PUB AGCR relative to others [Figures 3-5].

Immunohistochemical demonstrations of testicular nerves and glial-like cells in the different age groups of cane rat

NF-positive areas for nerve fiber presence in the testes of different age groups of AGCR were localized in the tunica albuginea and peri-albuginea interstitium of the capsule [Figure 6] as well as in the seminiferous tubular interstitium [Figure 7]. For the segments of epididymal duct, conspicuous NF-positive areas were restricted to the periductal muscle coat and epididymal ductal interstitium most, especially in the perivascular part of the caput, corpus, and cauda segments of the epididymis in all AGCR groups [Figures 3-5]. The intensity of NF expression in the testicular capsules [Figure 6] and interstitium [Figure 7] was observed to be significantly higher in the PUB and adult AGCR when compared to others and the intensity increases with age with peak shown in PUB and a subsequent decline. The profile of NF intensity in the caput segment downward consistently revealed significantly higher intensities in the PUB

and adult rats relative to others [Figures 3-5]. In general, the trend of NF expression intensity from the caput to the cauda segment appears to increase with age.

Positive areas for the presence of astrocyte-like cells on using antiGFAP marker in the testes of all AGCR includes the interstitium between seminiferous tubules [Figure 8] and perivascular part of the epididymal ductal interstitium [Figures 3-5]. Regarding the profile of testicular GFAP intensity [Figure 8], significantly higher ($P < 0.05$) intensity was noticed in the aged AGCR relative to others. The intensity seems to increase with advancement in age with both PUB and adult AGCR displaying a non-significant difference ($P > 0.05$) in the value of their intensities. In the epididymis, similar trend described for the testicular GFAP intensity [Figures 3-5] was remarkably observed in the different segments of the epididymis.

Testis

Testicular Golgi staining in the different age group of cane rat

With the use of Golgi-silver technique, both the neuronal and the astrocyte-like structures were demonstrated in the testicular tunica albuginea [Figure 1], interstitium, and along the seminiferous tubular boundary [Figure 2] in all the

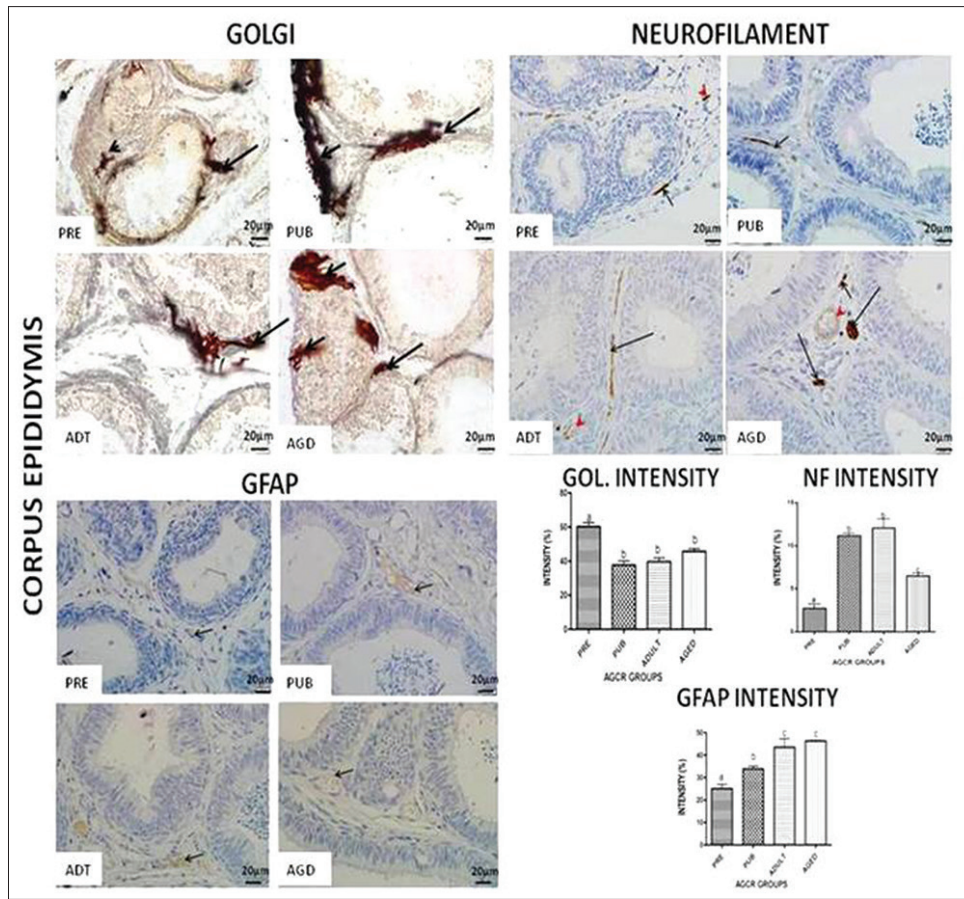


Figure 4: Photomicrographs of the corpus epididymis of different age groups of AGCR. Note in Golgi section, the display of dense nerve fibres within the periductal muscle coat (long arrow), the epididymal sheath (short arrow), and the interstitium (arrow head). Also, observe in neurofilament section, positive neurofilament stained nerve structures in the epididymal ductal interstitium (long arrow), perivascular area (arrow head) and periductal muscle coat (short arrow). For glial fibrillary acid protein section, note the positive glial fibrillary acid protein stained astrocyte-like cells in the perivascular area of ductal interstitium (arrow), in all groups

AGCR groups. Golgi intensity profile in both the testicular capsule [Figure 1] and interstitium [Figure 2] were significantly lower ($P < 0.05$) in the PRE rat when compared to others. The intensity appeared to increase with age advancement, though an insignificant ($P > 0.05$) decline value was displayed by aged AGCR relative to the PUB and adult values.

Immunohistochemical demonstrations of testicular nerves and glial-like cells in the different age groups of cane rat

NF-positive areas for nerve fiber presence in the testes of different age groups of AGCR were localized in the tunica albuginea and peri-albuginea interstitium of the capsule [Figure 6] as well as in the seminiferous tubular interstitium [Figure 7]. The intensity of NF expression in the testicular capsules [Figure 6] and interstitium [Figure 7] were observed to be significantly higher in the PUB and adult AGCR when compared to others and the intensity increases with age with peak shown in PUB and a subsequent decline.

Positive areas for the presence of astrocyte-like cells on using anti GFAP marker in the testes of all AGCR includes the interstitium between seminiferous tubules [Figure 8]

Regarding the profile of testicular GFAP intensity [Figure 8], statistically significantly higher ($P < 0.05$) intensity was noticed in the aged AGCR relative to others. The intensity seems to increase with advancement in age with both PUB and adult AGCR displaying a non-significant difference ($P > 0.05$) in the value of their intensities.

Epididymis

Golgi staining for epididymis in the different age group of cane rat

Within the segments of the epididymal duct, neuronal and astrocyte-like structures were remarkably observed in the periductal muscle coat and in the epididymal interstitium of the caput, corpus, and cauda segments [Figures 3-5]. The intensities of the nerve and glial-like cells of the caput, corpus, and cauda epididymal segments consistently displayed statistically significantly higher intensity ($P < 0.05$) in the PUB AGCR relative to others [Figures 3-5].

Immunohistochemical demonstrations of nerves and glial-like cells in the epididymis of different age groups of cane rat

For the segments of epididymal duct, conspicuous NF

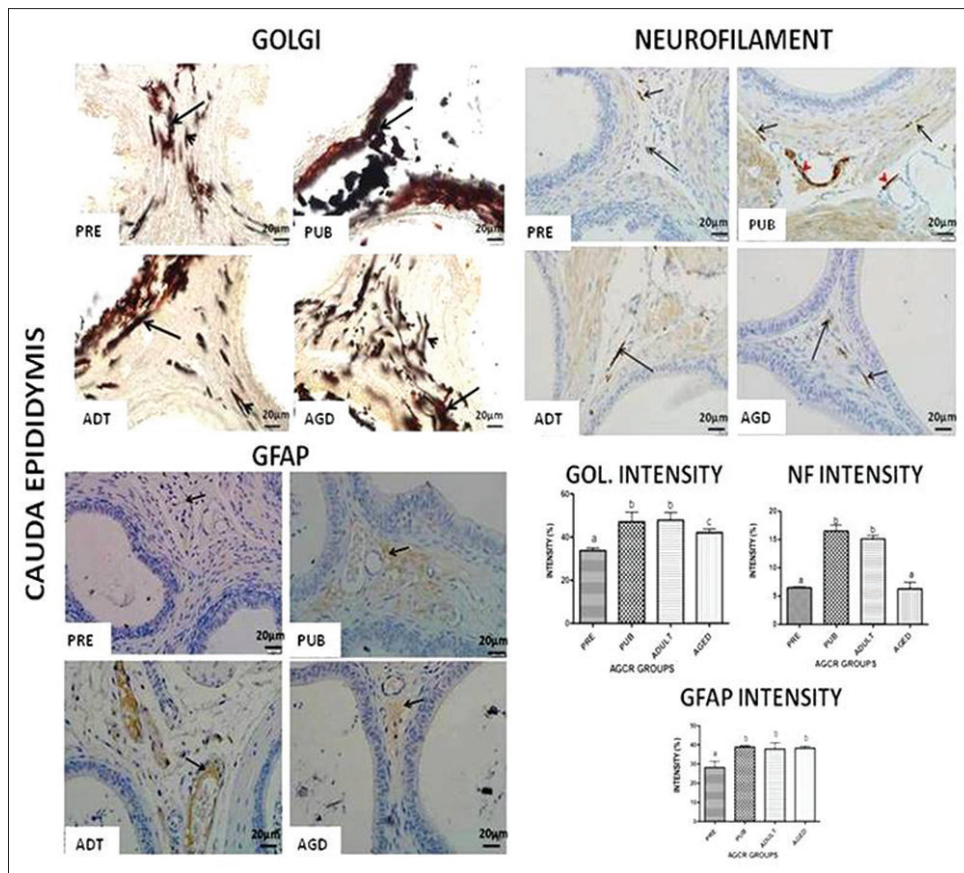


Figure 5: Photomicrographs of the cauda epididymis of different age groups of AGCR Note in the Golgi sections, the display of thick nerve bundles (long arrow) and solitary nerve fibres (arrow head) along the periductal muscle coat and the epididymal interstitium. Also, observe in the neurofilament sections the positive neurofilament stained nerve structures in the epididymal ductal interstitium (long arrow), perivascular area (arrow head) and periductal muscle coat (short arrow). For glial fibrillary acid protein sections, note the positive glial fibrillary acid protein stained astrocyte-like cells in the perivascular area of ductal interstitium

positive areas were restricted to the periductal muscle coat and epididymal ductal interstitium most especially in the perivascular part of the caput, corpus, and cauda segments of the epididymis in all AGCR groups [Figures 3-5]. The profile of NF intensity in the caput segment downward consistently revealed significantly higher intensities in the PUB and adult rats relative to others [Figures 3-5]. In general, the trend of NF expression intensity from the caput to the cauda segment appears to increase with age and perivascular part of the epididymal ductal interstitium [Figures 3-5]. Concerning GFAP immune reaction of the epididymis, similar trend described for the testicular GFAP intensity [Figures 3-5] was remarkably observed in the different segments of the epididymis.

DISCUSSION

The nervous system has been implicated in the extrusion of spermatozoa from the seminiferous tubules of some mammals (rat, dog, and rabbit) that possess smooth muscle cells in their capsules.^[34] Therefore, the age-related increase in the intensities of NF and Golgi expressed capsular nerve fibers in cane rats seem to justify the functional need of innervation

with age advancement. In addition, the higher NF and Golgi intensities expressed in the testicular interstitium of both PUB and adult groups fairly correlate well with the sperm parameters (increased sperm concentration and motility) more particularly in adult cane rats reported in our previous work.^[35] In the same vein, it is logical to assume that the conspicuous reduction in the intensities of expression of both in the PRE rat could be connected to the low reproductive activity. These findings agree with the report of Falade *et al.*^[21] in the African giant rat, but contrast the reports of Prince^[7] in man and Wrobel and Brandl^[15] in pig.

The intense NF and Golgi expressions of nerve fibers in the interstitium and periductal muscle coat aspects of the caput and corpus epididymal segments in PUB and adult cane rats could be suggested to correlate with the developmental and functional states of the epididymal ducts more particularly the adult group with active reproductive activity. The progressive segment-related increase neuronal fiber expressions observed along the epididymal segments in all the age groups corroborates the pattern documented in rats,^[16] camel,^[17] and rabbits.^[36] In addition, the marked NF expression in the cauda

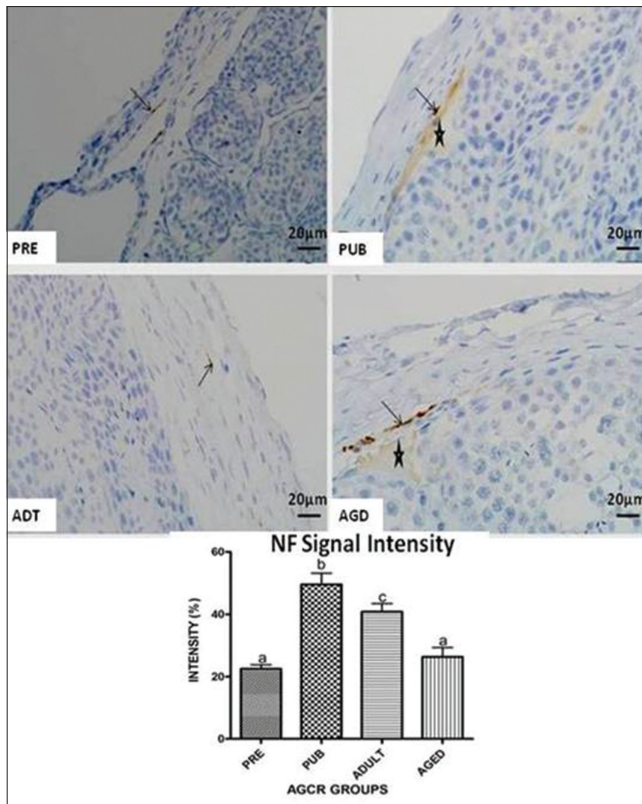


Figure 6: Photomicrographs of neurofilament expression in the testicular capsule of different age groups of AGCR. Note the positive neurofilament staining of nerve structures in the tunica albuginea (arrows) and peri-albuginea interstitium (star)

epididymis of the adult cane rat relative to others could be presumed to mediate the neuromuscular events needed to transport spermatozoa through the duct. Several studies have equally associated the presence of certain neurotransmitters in the nerve fibers supply to the epididymis in regulating certain epithelial cell functions which include electrolyte transport^[19] and protein processing.^[20]

The positive immunolocalization of GFAP in the interstitium of seminiferous tubules of all cane rat groups is in agreement with previous reports of interstitial Leydig cell being immunopositive for astrocyte marker (GFAP).^[21,27,28,37] The functional implication of the localization of astrocyte-like cells in the interstitium has been suggested to be involved in blood-testis barrier (BTB) formation, a prototype of the blood-brain barrier formed by astrocytes in the brain.^[27] In respect of the highlighted function, the increased testicular GFAP expression intensity with age advancement might be correlated with the strengthening of BTB. This finding concurs with the increased testicular GFAP intensity reported by Falade *et al.*^[21] in the African giant rat but contrasts the reports of decrease GFAP profile, especially in the CNS tissues of human^[28] and Africa giant rat.^[33]

GFAP localization in perivascular regions has been assumed to regulate the blood pressure and permeability of vascular walls.^[38] Therefore, the consistent demonstration of varying levels of

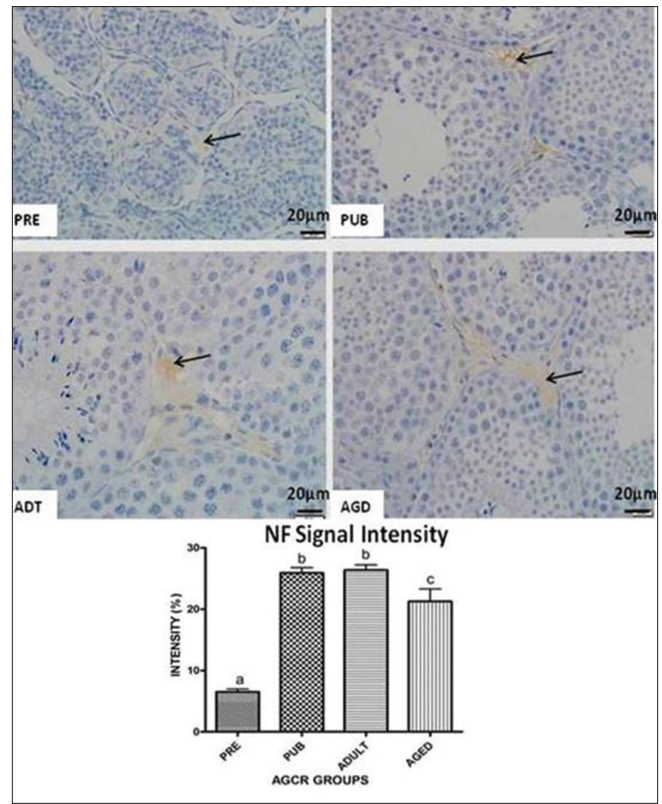


Figure 7: Photomicrographs of neurofilament expression in the testis of different age groups of AGCR. Note the positive neurofilament staining of nerve structures in the seminiferous tubular interstitium (arrows)

strong positive GFAP immunolocalization around the vascular components of the epididymal ductal interstitium in all segments of all rat groups could be connected to the marked exchange of materials between interstitial vessels and the epithelial components in these segments. In addition, the observed age-dependent increase in the intensity of GFAP expression in the epididymal segments (caput, corpus, and cauda) of the cane rat further substantiates the need for the proportionate increase in the permeability regulation with ageing.

In conclusion, this work has brought to fore the description of the variation in the expression of neuronal and glial cells in the testis and epididymis of the cane rat and the possible association of increased reproductive activity to the abundant nerve fibers in the testes and epididymides of adult rat. This work is possibly the first of its kind to report the demonstration of nerve and glial structures in peripheral tissues such as testes and epididymis in the cane rat.

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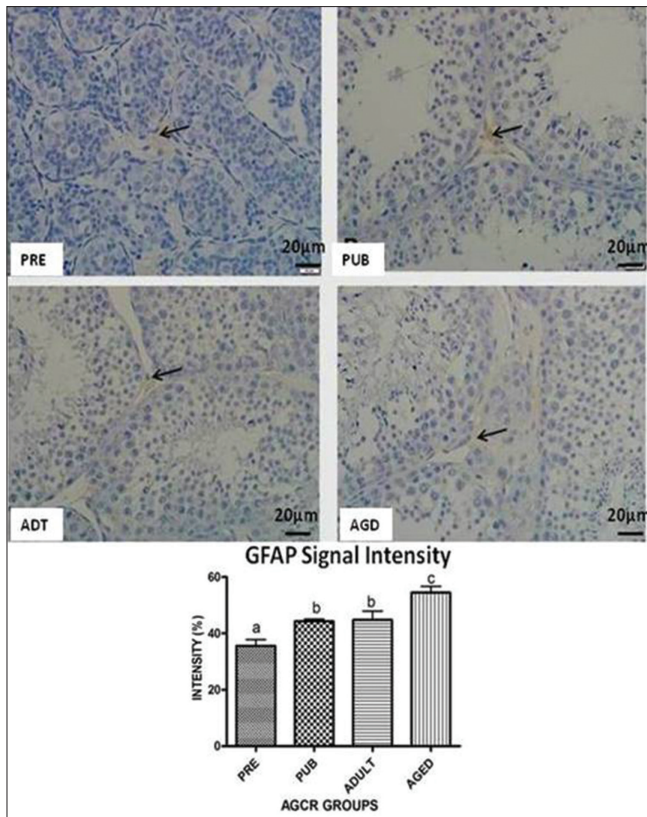


Figure 8: Photomicrographs of glial fibrillary acid protein expression in the testis of different age groups of the AGCR. Note the positive glial fibrillary acid protein staining of astrocyte-like cells in seminiferous tubular interstitium (arrow)

Conflicts of interest

There are no conflicts of interest.

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