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Original Article

The histochemistry of the saccus vasculosus in red-bellied Piranha, *Pygocentrus nattereri* Kner, 1858

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Abstract: The presence of mucopolysaccharides, glycogen, protein, and lipid component in the cellular constituents of saccus vasculosus of *Pygocentrus nattereri* Kner, 1858 were demonstrated histochemically using light microscopy. The saccus vasculosus was richly vascularized and comprised of a number of loculi enclosed by blood sinusoids. The loculi contained predominant coronet cells and supporting cells. Plenty of secretory materials were observed in the lumen. Periodic acid Schiff's reaction in combination with Alcian blue for mucopolysaccharides was positive for the apical protuberances of coronet cells and secretory matters in the lumen. Significant amounts of glycogen and protein were localized in coronet cells and blood cells. The coronet cells along with luminar protrusion contained an appreciable amount of DNA and RNA. Lipid is notably detected through Sudan black reaction in globular protrusion of coronet cells. The silver reaction was employed to investigate the presence and distribution of neurons within the epithelial lining as well as other regions of the saccus vasculosus. These histochemical tests revealed that the saccus vasculosus served dually as both a secretory and sensory organ.

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Introduction

The saccus vasculosus, a reddish sac-like structure situated on the ventral wall of the diencephalon in teleosts, is positioned immediately behind the pituitary gland. The size of this specialized ependymal organ varies considerably among various species of chondrichthyes and osteichthyes, ranging from well-developed structures to rudimentary forms, and in some species, it may be absent (Lanzing, 1970). The structure and function of saccus vasculosus in fishes have been described by many workers (Yáñez, 1997; Sueiro et al., 2007; Nakane et al., 2013; Ghosh and Chakrabarti, 2014; Maeda, 2015; Chakrabarti and Khatun, 2017). The epithelial lining of the saccus vasculosus exhibits diverse characteristics, presenting as either a single-layered or multi-layered structure. Within this lining, two distinct cell types coexist: the distinctive coronet cells, also known as crown cells, and the supporting cells which are referred to as glial cells. Anatomically, the saccus vasculosus reveals a close and intricate relationship between neural tissue and blood vessels (Cid et al., 2015).

A review of the literature reveals that there is a considerable difference of opinion regarding the function of saccus vasculosus. A comprehensive review of the literature underscores the substantial variance in viewpoints concerning the function of the saccus vasculosus. Certain researchers have proposed that the saccus vasculosus primarily serves a secretory function (Galer and Billenstien, 1972; Bhatnagar et al., 1978; Saksena, 1989; Gupta, 2007); others have mentioned that saccus vasculosus may be involved in the transport of low molecular substances (Jansen, 1975; Joy and Sathyanesan, 1979). However, recent studies have shed new light on this matter, suggesting that the coronet cells, connected to liquor-contacting neurons, are predominantly sensory in nature (Ryohi and Keiji, 2001; Chakrabarti and Ghosh, 2009; Rodriguez-Moldes and Anadón, 2010; Nakane et al., 2013; Khatun and Chakarabarti, 2016).

In the present investigation, an attempt has been made to elucidate the chemical composition and

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functional significance of the saccus vasculosus to understand the potential physiological roles of the cells involved. Taking this perspective, carnivorous redbelly pirhana, *Pygocentrus nattereri* (Characiformes: Serrasalmidae: Serrasalminae) has been chosen for this study.

Materials and Methods

Specimen collection: Adult specimens of *P. nattereri* (42±2.06 cm in total length; n = 12) were collected from the freshwater ponds of Itachuna (23.029°N, 88.176°E), Hooghly district of West Bengal. The fish were euthanized with an overdose of tricaine methanesulfonate (MS 222; Sigma-Aldrich Chemical). The brain together with saccus vasculosus was disclosed from the head's ventral side and fixed *in situ* with 10% neutral formalin.

Histochemical analysis: The saccus vasculosus along with the base of the brain was cautiously detached from the cranium and submerged in 10% neutral formalin for 18-20 h. The fixed tissues were washed thoroughly in 70% ethanol and dehydrated in an ascending series of ethanol. The tissues were cleared in benzene and embedded in paraffin wax of 52-54℃ (CDH Fine Chemical).

Serial sections of 8-10 µm thickness was cut using a rotary microtome (Weswox MT-1090A) and prepared for the histochemical tests to examine the chemical nature of cells lining the saccus vasculosus at the light microscopical level. The histochemical tests include (1) Periodic Acid Schiff's (PAS) reaction in combination with Alcian Blue (AB) (PAS-AB) for the detection of neutral and acid mucins (Yamabayashi, 1987), (2) Best's Carmine (BC) reaction for the detection of glycogen (Horobin and Murgatroyd, 1971), (3) Mercuric Bromphenol Blue (MBB) method for the detection of basic protein (Mazia et al., 1953), (4) Methyl Green Pyronin Y (MGP) method for the detection of nucleic acid (Unna, 1902), (5) Sudan Black B (SB) method for the detection of bound lipid (Berenbaum, 1958), and (6) Silver Impregnation (SI) method for the detection of neurons (Marsland et al., 1954). All the slides were examined and microphotographed using a Leica EC3 light microscope at different magnifications.

Results

In *P. nattereri*, the saccus vasculosus is well-developed and highly vascularized, located on the ventral side of the diencephalon, near the third ventricle of the brain (Fig. 2A). It is composed of an enormous number of loculi having irregular shapes and sizes encircled by blood vessels. The saccus epithelium contains two types of cells: specialized coronet cells and basal supporting cells (Figs. 1B, 2B, 3B, 4B, and 5B). The loculi are either simple or branched patterns and the lumen appears as an intercommunicating arrangement of channels which ramify considerably. The distal branches are linked together to form a collecting channel which opens in the diocoel (Fig. 5A).

Periodic acid Schiff's reaction in combination with Alcian blue: Periodic Acid Schiff's (PAS) reaction in combination with Alcian Blue (AB) (PAS-AB) is used for assessment of high molecular weight glycoproteins, mucins. This combined analysis shows a purple colour as a result of the PAS reaction for neutral mucin and blue colour by cause of AB reaction for acid mucin (Fig. 1A). The cytoplasm of coronet cells lining saccus epithelium impart light purple colour whereas the blood vessels exhibit bluish colour. The apical protrusion and globules of coronet cells are deep bluish-purple colour due to the presence of both acidic and neutral mucopolysaccharides (Fig. 1B). However, the luminal border lined with coronet cells and supporting of saccus vasculosus have PAS positive deposition make a brush border looks. The basement membrane exhibits intense blue colour. PAS-AB positive coagulum is observed in the lumen of the loculi.

Best's carmine reaction: Best's carmine investigation shows different shades of glycogen deposition in the coronet cells according to their physiological activity (Figs. 2A-C). The cytoplasmic content and edged protrusion of coronet cells exhibit abundant glycogen content (Fig. 2B). Supporting cells are packed with little glycogen. The bright red coloured basement membrane and blood sinusoids

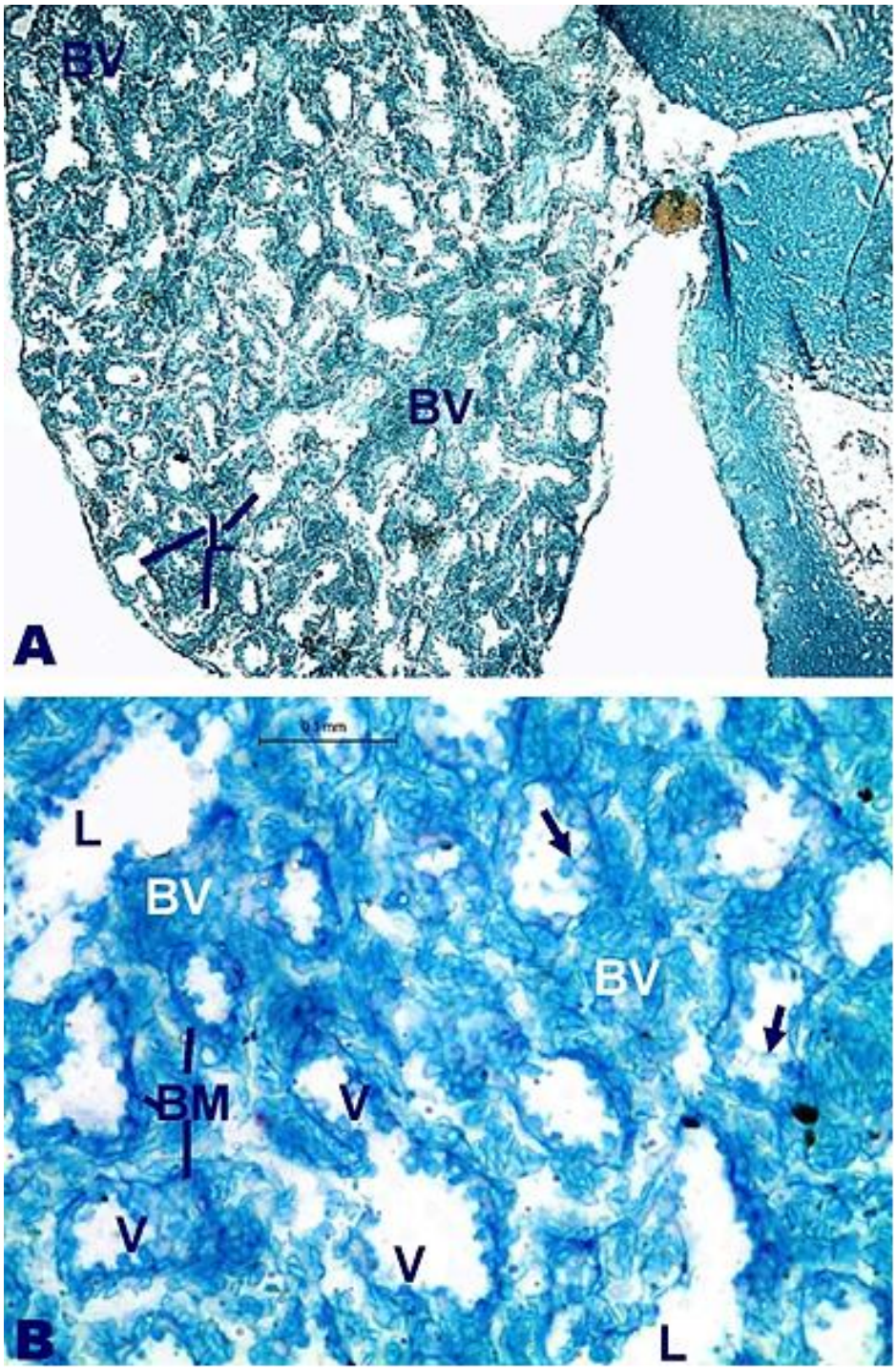

Figure 1. Photomicrographs of the sections of saccus vasculosus of *Pygocentrus nattereri* showing Periodic Acid Schiff's (PAS) reaction in combination with Alcian Blue (AB). **(A)** Coronet cells lining the loculi (L) show purple colour for neutral mucopolysaccharides. Blood vessels (BV) display blue colour because of acidic mucopolysaccharides (10X). **(B)** Apical protrusion of the coronet cells (arrow heads) and basement membrane (BM) exhibit intense purple colour. PAS-AB reaction is also discernible in the BV and coagulum (solid arrows) of the loculi (L) (40X).

surrounding the loculi also display intense carmine stating.

Mercuric bromphenol blue reaction: The saccus vasculosus is deeply stained with acidic mercuric bromphenol blue due to its proteinaceous nature (Fig. 3A). This acidic dye responds to basic groups of protein and furnishes blue colour. The cytoplasm and apical secretion of coronet cells display acute reactions for protein (Figs. 3A-B). A dignified amount of protein is discernible in the blood vessels encircling the loculi. The basal supporting cells and luminal secretion of coronet cells exhibit positive reactions for

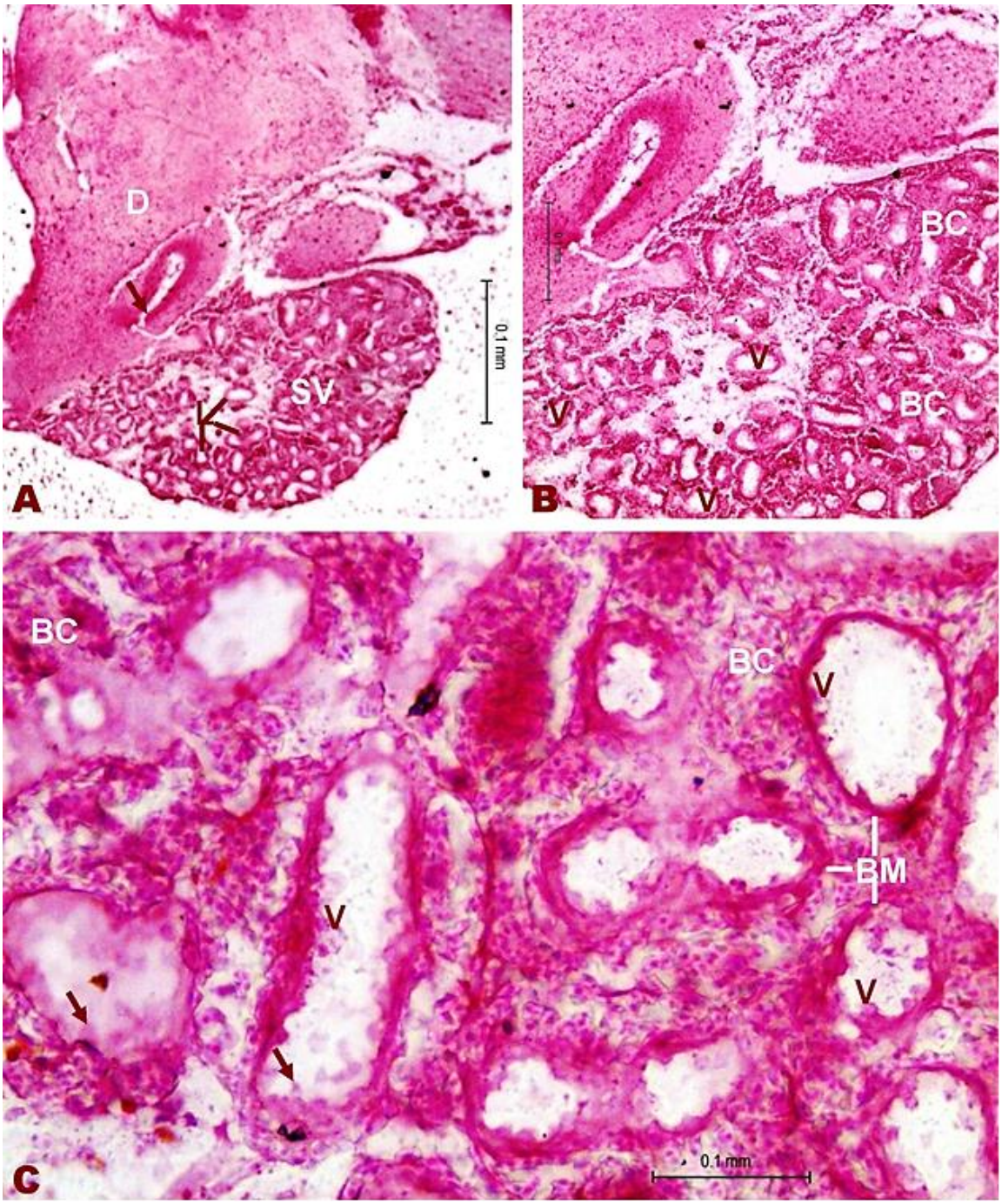

Figure 2. Section of saccus epithelium of *Pygocentrus nattereri* displaying Best's Carmine (BC) reaction. **(A)** Saccus vasculosus (SV) having numerous loculi (L) located on the ventral side of the diencephalon (D), near the third ventricle (arrow) of brain (4X). **(B)** Showing different shades of glycogen in coronet cells (arrow heads) and blood cells (BC) (10X). **(C)** Intense glycogen reaction in the apical protrusion of coronet cells (arrow heads), blood cells (BC) and basement membrane (BM). Note moderate reaction in supporting cells (solid arrows) (40X).

protein.

Methyl green pyronin Y reaction: This classical staining technique employing two basic dyes is used for localization and differentiation of DNA and RNA content in the various cells lining the saccus vasculosus. The combined test affords bluish-green colour for methyl green specifically binds to DNA whereas red colour for pyronin which binds to RNA (Fig. 4A). Strong reaction of DNA is observed in the nucleus of coronet cells while the cytoplasmic content and apical protrusion of coronet cells exhibit purple colour due to appreciable amount of RNA (Fig. 4B). Colloids in the loculi are bluish-purple coloured confirming the mixture of DNA and RNA. The

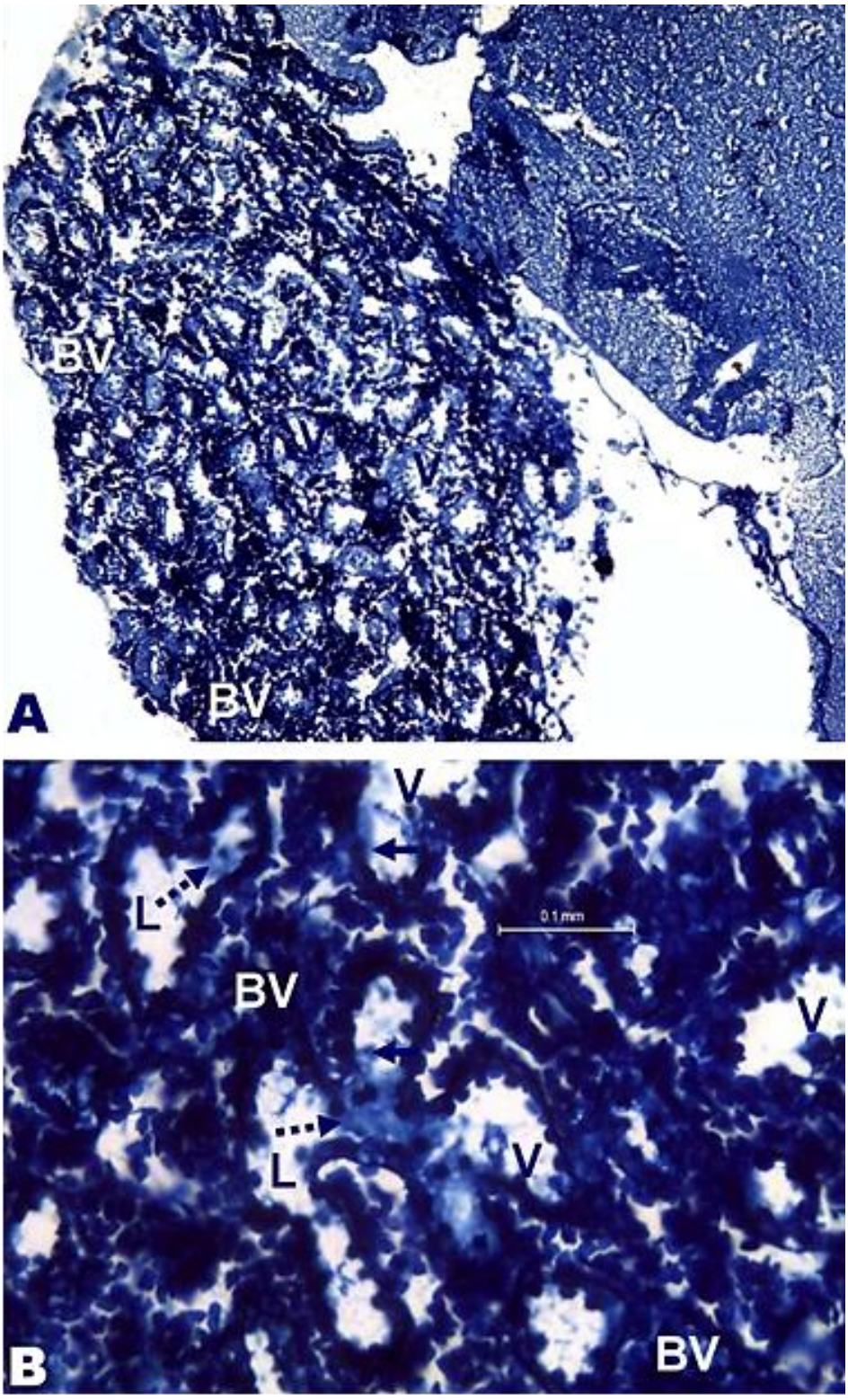

Figure 3. Section of saccus vasculosus of *Pygocentrus nattereri* showing Mercuric Bromphenol Blue reaction. **(A)** Strong protein reaction in coronet cells (arrow heads) and blood vessels (BV) of saccus vasculosus (10X). **(B)** Showing intense protein reaction in the apical processes of coronet cells (arrow heads) and BV encircling the loculi (L). Note positive reaction in supporting cells (solid arrows) and luminal secretions of coronet cells (broken arrows) (40X).

maximum reaction of pyronin for RNA has been noticed in the cytoplasm of blood cells.

Sudan black reaction: Different shades of lipid content are observed in closely arranged coronet cells

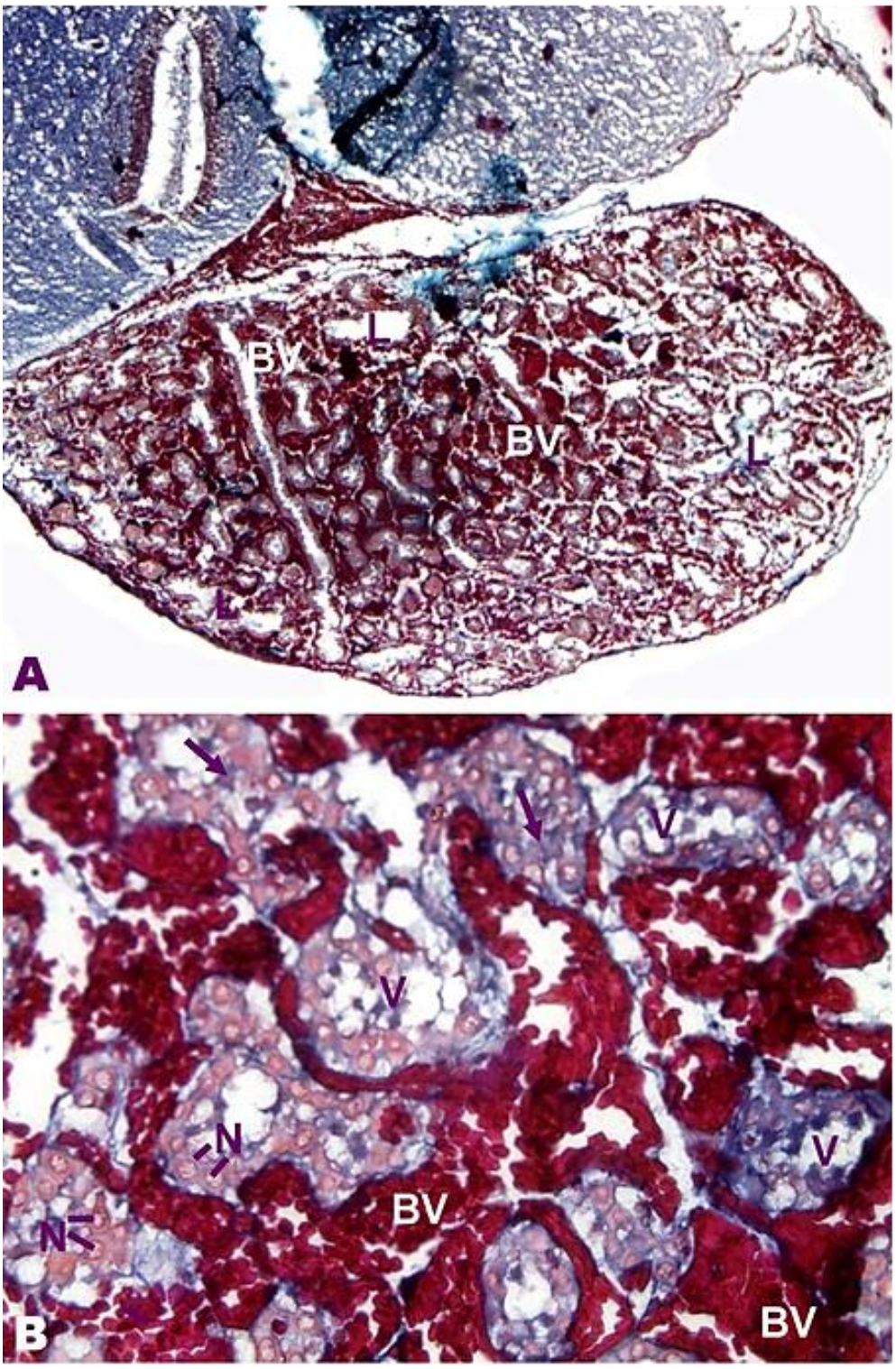

Figure 4. Section of saccus vasculosus of *Pygocentrus nattereri* showing Methyl Green Pyronin Y reaction **(A)** The cellular lining of loculi (L) and blood vessels (BV) of saccus vasculosus show DNA and RNA content (10X). **(B)** Strong methyl green reaction in the nuclei (N) of coronet cells whereas cytoplasmic processes (arrow heads) show pyronin reaction. Note maximum pyronin reaction in BV and luminal secretions (solid arrows) display mixture of DNA and RNA content (40X).

and underlying blood vessels (Fig. 5A). The apical globular protrusion of coronet cells yields an intense reaction of Sudan black. However, the highest lipid reaction is detected in the blood vessels surrounding the loculi (Fig. 5B). Supporting cells are weak in lipid reaction. Varying degrees of sudanophilic droplets in the loculi show lipid in nature.

Silver reaction: The silver reaction is discernible in

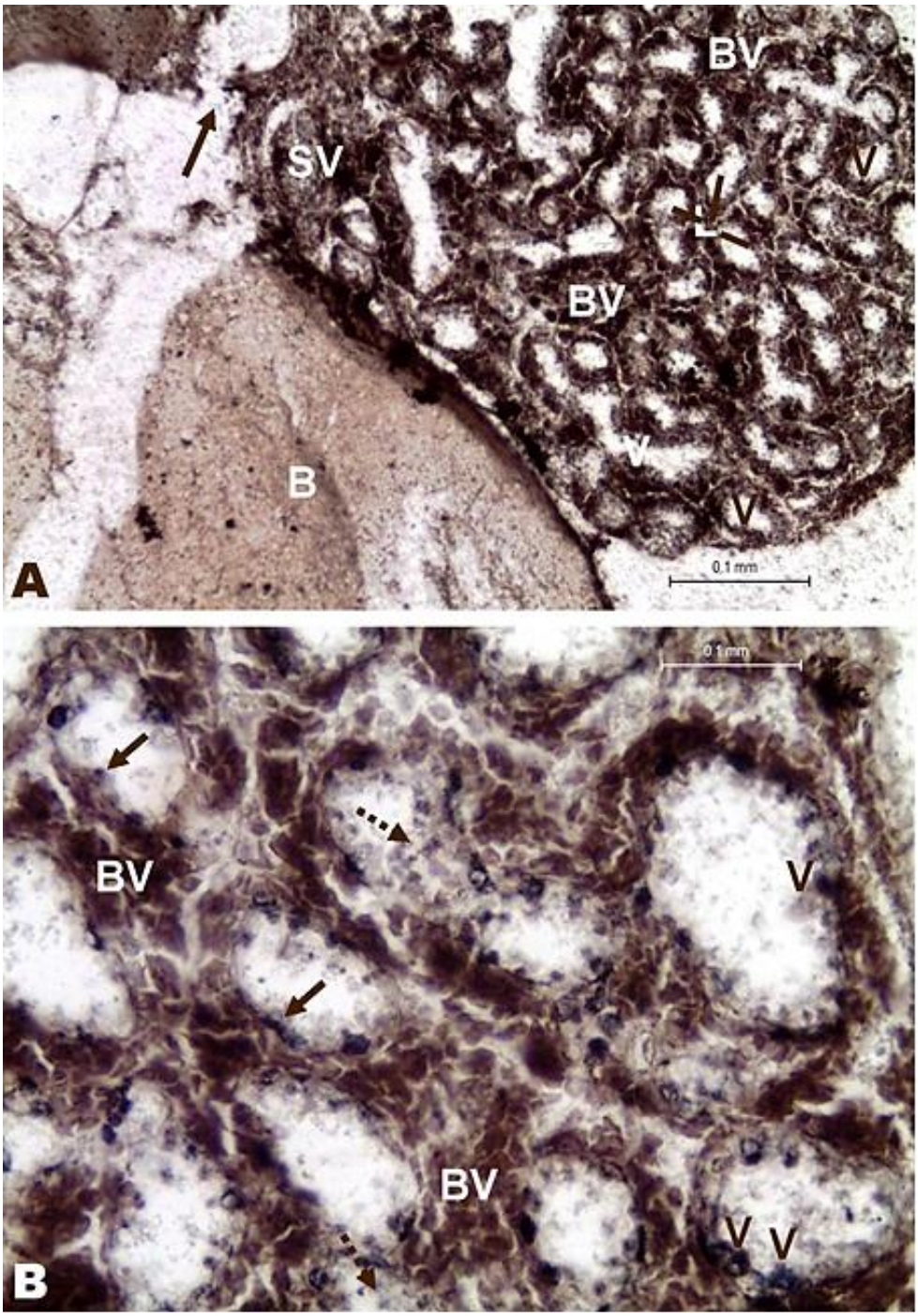

Figure 5. Section of saccus vasculosus of *Pygocentrus nattereri* showing Sudan Black reaction **(A)** Showing sudanophilic materials in coronet cells (arrow heads) of loculi (L) intermingled with blood vessels (BV). Solid arrow marks the connecting channel in between the saccus vasculosus (SV) and diocoel of brain (B) (10X). **(B)** Intense lipid reaction in the apical protrusion (arrow heads) of coronet cells and BV. Note the presence of sudanophilic materials (broken arrows) in L. Solid arrows mark basal cells (40X).

the neural part of saccus vasculosus (Fig. 6A). Enormous silver stain is observed in the ciliary structures on the apical part of coronet cells as well as neurosecretory fibres attached to the basal portion of coronet cells intermingled with blood vessels (Fig. 6B). Some axonic extensions enter and encompass the blood vessels of saccus vasculosus also display positive reaction. Nerve fibres on the outer surface of saccus epithelium are marked with silver stains.

Discussions

The saccus vasculosus is recognized as a

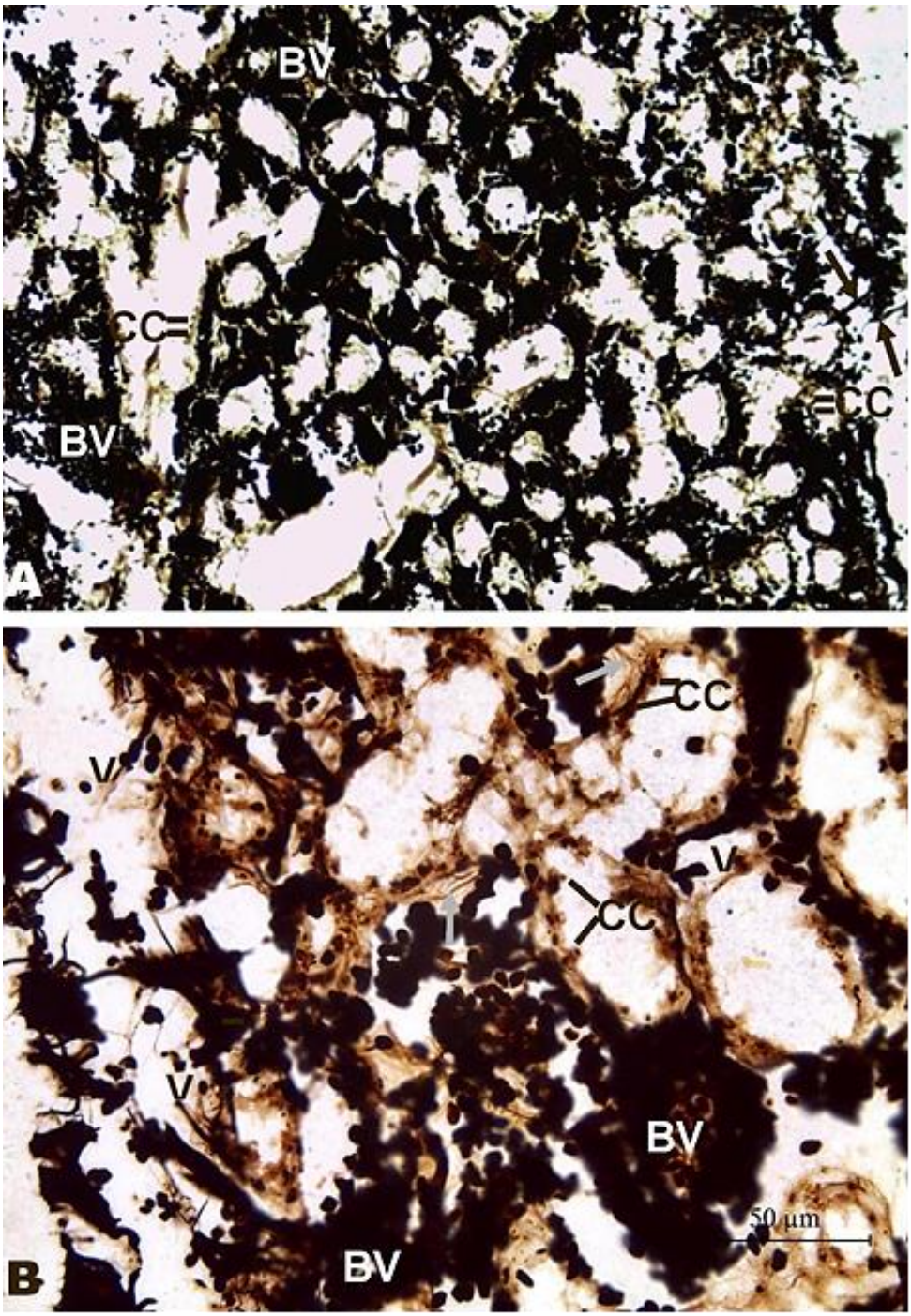

Figure 6. Section of saccus vasculosus of *Pygocentrus nattereri* showing silver reaction **(A)** Showing silver reaction in coronet cells (CC) intermingled with blood vessels (BV). Note intense reaction in nerve fibres (arrows) on outer coating of saccus vasculosus (10X). **(B)** Magnified portion of saccus vasculosus shows silver reaction in the apical ciliary structure (arrow heads) and basal neurosecretory fibres of CC connected with BV. (40X).

circumventricular organ, housing specialized coronet in contact with the venricular cerebrospinal fluid in the absence of a blood-brain barrier (Altner and Zimmerman, 1972). In *P. nattereri*, the well-developed saccus vasculosus is situated on the ventral side of the brain and serves a dual purpose, encompassing both sensory and secretory functions. The saccus vasculosus consists of a vast number of loculi having distinctive cornet cells and supporting cells, which are enveloped by a network of blood sinusoids. The rich vascularization of the saccus vasculosus likely serves to supply nutritive substances

to the various cells lining the epithelium of the saccus. Scharrer (1948) suggested that the blood sinusoids play an important role in the secretion of fluid from the blood stream into the lumen of the saccus vasculosus and they also contribute to adjusting the volume of this fluid. This process helps to equalize differences in intracranial pressure, particularly during vertical movement.

A PAS-positive reaction in the coronet cells signifies the existence of a variety of chemical compounds, including neutral mucopolysaccharides, glycoproteins, mucoproteins, and glycolipids. However, the PAS-AB reaction varies in the different parts of the saccus vasculosus. The apical protrusions of coronet cells and the accumulated material in the loculi lumen are rich in acid and neutral mucopolysaccharides strongly indicating their secretory function. Similar types of findings were also reported in other fishes (Galer and Billenstien, 1972; Bhatnagar et al., 1978; Kulkarni and Sathyanesan, 1982; Ghosh and Chakrabarti, 2014). Lanzing and Lennep (2005) showed the presence of acid mucopolysaccharides in the apical protrusions of coronet cells in teleosts. These mucopolysaccharides are initially stored in globules and subsequently secreted into the brain's ventricle.

The presence of glycogen in the coronet cells of the saccus epithelium in *P. nattereri* likely serves both metabolic and physiological functions. Sundararaj and Prasad (1964) noted that coronet cells exhibit secretory functions and the stored glycogen within their apical protrusions is thought to be released in the form of glucose into the cerebrospinal fluid. Saksena (1989) suggested that the saccus vasculosus serves as a storage site for carbohydrates intended for the brain. Coronet cells are implicated in glycogen metabolism by converting glycogen into acid muco-polysaccharides. Strong glycogen reactions in the blood vessels and basement membrane could generate energy during the transport of various chemicals and the transmission of impulses through the membrane.

The cytoplasmic contents and terminal protrusions of coronet cells are proteinaceous in nature. Protein reaction observed in the coagulum of the lumen strongly implies an extensive secretion of neutral glycoproteins from the coronet cells of the saccus vasculosus. Kulkarni and Sathyanesan (1982) documented an acute protein reaction, further confirming the substantial secretion of glycoproteins from the coronet cells of *Mystus vittatus*. In contrast, the supporting cells present alongside the coronet cells do not exhibit any signs of secretory activity.

The acute response of DNA within the nuclei of coronet cells in the saccus epithelium may be linked to the biochemical differentiation of various stages of coronet cell development. Notably, the cytoplasm of coronet cells displays a strong reaction for RNA, indicating its relatively higher presence. This suggests its essential role in cellular metabolism and the synthesis of proteins which are crucial for augmenting the secretion of products from coronet cells into the lumen. The abundant RNA content in the cytoplasm of coronet cells indicates a significant role in the physiological transformation of electron-dense materials during the formation of secretory products. Bhatnagar et al. (1978) proposed that the RNA and cystine content in the cytoplasm of coronet cells may initiate the production of acid mucopolysaccharides within the coronet cells themselves in the saccus vasculosus of *Cyprinion macrostomus*.

Observations have shown variations of sudanophilic materials among the coronet cells. It is probable that the energy required for the physiological activities and the secretion of the products from the coronet cells primarily originates from the accumulated lipid materials within these cells. Sundararaj and Prasad (1964) documented the localization of phospholipids in the apical protrusions and globules of coronet cells within the saccus vasculosus of *Notopterus chitala*. This observation underscores the potential involvement of the saccus vasculosus in fluid secretion and the extrusion of low molecular weight organic substances into the ventricular system.

The coronet cells in the saccus vasculosus of *P. nattereri* make contact with nerve terminals, indicating a credible sensory role for this neural element. In a study on *Cyprinus carpio*, Ryohi and

Keiji (2001) observed the synaptic vesicles within the terminals on the coronet cells and proposed that the function of these cells in metabolizing cerebrospinal fluid is controlled by cholinergic nerves. Furthermore, Benjamin (1974), in *Gasterosteus aculeatus*, emphasized that the coronet cells are the most prominent feature of the saccus vasculosus and have direct contact with the cerebrospinal fluid. Therefore, it is plausible that coronet cells serve as chemoreceptors and play a role in maintaining the composition of the cerebrospinal fluid.

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