IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

THE HISTOCHEMICAL ANALYSIS OF GLYCOGEN CONTENT IN THE GILLS OF *CHANNA STRIATUS* INFECTED WITH FUNGI CAUSES RED SPOT DISEASE

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Abstract

The present study was investigates fungal infection effects on the gills of the freshwater fish, *Channa striatus*. The following prolonged exposures of polluted water and native fishes stress. The gills of *Channa striatus* face the direct contact stress of decomposed materials. The blood capillaries (BLCs) of the secondary lamellae (SL) of gills showed extensive congestion followed by wear and tear. The ladder like arrangement of blood capillaries and pillar cells (BLCs-PLCs) gets dismantled and haemorrhages regularly take place in the gills. The staining property and density of the mucous cells (MCs) showed extensive periodic alterations. The fungi causes hyperplasia followed by their degeneration frequently noticed. The gills continued to show hyperplasia. The density and staining properties of the MCs continued. Present study was aimed to assess the histochemical analysis of mucosubstances was carried out in the epithelial cells and mucous cells in the gills of control fish and infected fishes exposed to fungal concentration on the basis of histochemical staining reactivities. The histochemical staining reactivities revealed fluctuations in mucosubstances in epithelial cells while neutral mucosubstances, was increased in mucous cells in gills of fishes. From these observations it is concluded that the fungal infection and impurities of the water toxic to fish and brings significant physiological alterations.

Keywords: *Channa striatus,* Mucosubstances, Histochemical staining

Introduction

In the freshwater fishes of gills are the important organs for respiration and osmoregulation. Gills have their greatest development among teleostean fishes considering the lungs in efficiency. The gills in a number of fishes show considerable modification with changes in oxygen environment and function in combination with other respiratory structures in the exchange of gases. The indiscriminate use of insecticides in agriculture on

variety of crops, pollutants the surrounding water resources, such as ponds and rivers and seriously damage of the aquatic fauna, more particularly in the fishes. The intake of pollutants variously affect the fish, the gills become more exposed because of their location and constant intimate contact with the water and are liable to damage by any irritant material, whether dissolved or suspended in water (Lemke and Mount,1963).

C. striatus an indigenous species is of great significance because of its nutritional value. The gills are important organs in fish including *C. striatus* to perform respiration, osmoregulation, acid base balance and nitrogenous waste excretion. The gills in suprabranchial chambers remain in direct contact to surrounding medium hence more suitable organs for toxicity studies. In aquatic environment where the oxygen is more as in surface water, In this paper histochemical analysis of the gills of *C. striatus* were performed to detect the toxic impact of an fungal load and deoxygenated conditions so that the gills can be used as efficient bioindicator. **(**A.K. Singh et., al. 2014). The gill lamellae are supported by gill rays which are partially bony and are connected to the gill arch and with each other by fibrous ligaments. Each gill ray is bifurcated at its proximal end provides a passage for the efferent branchial vessel. Each hole-branch carries two hemi branches or half gills. Each hemi branch comprises a row of long thin filaments or primary gill lamellae. Each primary lamella bears a large number of secondary lamellae on both sides. The secondary lamellae are free from each other but fused at the distal ends of the primary lamellae. The epibranchium of the first gill arch is covered by a thin vascular respiratory membrane. It also bears a folded and highly inched respiratory labyrinthine organ for the purpose of accessory respiration.

Material and Methods

The freshwater fish's material for the present study was collected from different lakes of Hasanparthy, Dharmasagar and Bandham resources in Warangal district, Telangana, India. The *Channa striatus* EUS infected fish samples were collected. The specimens were obtained alive by using fishing net, and they were brought immediately to the laboratory, in plastic containers with oxygen filled water. The specimens were deeply anaesthetized by immersion into 5 ml/L aqueous solution of ethylene glycol monophonylether. To study the gross anatomy of different regions of the dissected tissue parts gills were carefully removed and small pieces were taken to obtain proper fixations. For the histochemical studies the required tissues were removed and the blood vessels and mucous attached to them were scrapped off smoothly without damaging the original stricture. The tissues collected from control and infected fishes were immediately fixed and processed. The fixatives used in the present study were Alcoholic Bouin's, Susa, Carnoy for mucopolysaccharides modified (Bouin'sHarris et al., 1973), and cetyl pyridinium chloride (1% cetylpyridinium chloride in formalin) was used specially. Bouin's and Susa comparatively gave good results.

After fixation in Bouin's for 18-24 hours material was washed in scots tap water. Susa fixed material has been treated with iodine alcohol. Experience proved that Susa formed the choicest fixative for most histological and histochemical reactions with the exception of lipids, nucleic acids and mucopolysaccharides. After fixation and post treatment the material was washed and then dehydrated in graded series of alcohols. Cleared in xylene and embedded in paraffin wax. Depending upon the parts of the systems the infiltration time varied from (1- 4) hours was minimized and then serial transverse or sagittal sectionswere cut 3-6mμ thickness for all the tissues. Excellent staining results for histological studies could be obtained by using Dalafields haematoxylin counterstained with eosin (Gurr 1962 and Heidenhain's Azan Gurr, 1962). For the EUS infection studies the fishes collected from three lakes of Hasanparthy, Dharmasagar and Bhandham of fish like *Channa striatus*. The procedures for determination of infected and control fishes. The tissues from control and infected groups were collected separately and processed individually, for histochemical investigations.

The following various histoichemical methods have been employed to elucidating the chemical nature of viz., the presence of carbohydrates, in tissue like gills of *Channa striatus*. The procedure as outlined in (Pearse, 1968) for the different histochemical tests was adopted in the present study. However, the techniques mentioned in the following books were also referred (Lillie 1965, Humason1967, Bancroft 1975, Kiernan1999), (Shyamasundari and Hanumantha Rao, 2007). Presence of carbohydrates with 1, 2 glycols was demonstrated with PAS staining. Acidic glycans were stained with alcian blue (AB) at pH 2.5 and high iron diamine (HID) stains. AB pH 2.5 stains blue both sialylated and sulphated residuals, whereas only sulphated residuals are stained brown by HID. (Roberto Carlucci et al., 2019). Different lakes of Hasanparthy, Dharmasagar and Bandham resources in Warangal district, Telangana, India. (Fig.1,2,3,4&5).

Fig.1. Hasanparthy Lake

Fig.2. Dharmasagar lake

Fig.3. Bhandham Lake

Fig.4. Control *C. striatus*

Fig.5. Infected *C. striatus*

IDENTIFICATION OF CARBOHYDRATES

For the testing carbohydrates, material fixed in Bouin's and Susa fixatives was used. Periodic acid/ Schiff (PAS) technique (Pearse, 1968) has been employed to detect the presence of carbohydrates and other groups. Periodic acid brings about oxidative cleavage of carbon to carbon bond in 1, 2 glycols to form dialdehydes, which are subsequently coloured by Schiff's reagent. This oxidant does not further oxidize the resulting aldehydes. As a number compounds give PAS positive reaction, the different Pas positive groups that may be present were further characterized by subjecting the sections to various procedures.

POLYSACCHARIDES

The conventional method for detecting polysaccharides by the periodic acid Schiff (PAS) method. As varieties of substances were known to give a positive reaction with PAS technique, suitable controls were employed to determine the actual compound responsible for the positive reaction. It was performed without prior oxidations with periodic acid to know whether the reaction was due to performed aldehydes to detect glycogen, sections were subjected to PAS light green technique in conjunction with diastase digestion. Best's caramine method was also used to determine the presence of glycogen. The PAS reaction was conducted after acetylation (24 hours at room temperature in 16 ml of acetic acid and 24 ml of pyridine) and subsequent deacetylation (45

minutes in 0.1 N potassium hydroxide at room temperature or with 20% ammonia in 70% alcohol for 24 hours) to establish the presence of 1: 2 glycol groups. To determine whether the reactivity is due to lipid, sections were treated with various lipid solvents such as pyridine or methyl alcohol chloroform mixture prior to the application of PAS technique of the substance gives a positive PAS reaction not extractable by diastase or lipid solvents and when it also gives positive reaction for protein tests, then it was considered as either a mucoprotein or glycoprotein. Finally identification was confirmed by employing methylene blue extinction technique which involves the staining of sections in methylene blue at different pH levels.

MUCOS SUBSTANCES

Tissue was fixed in either new Comer's fluid dioxane or 1% acetyl pyridinium chloride in 10% formaldehyde containing 2% calcium acetate are modified (Bouin'sHarris et al.,1973), of all these acetylpyridinimum chloride formalin was the best as judged by the integrity of cells and by the intensity of staining of especially for mucopolysaccharides. A variety of histochemical tests were employed to demonstrate different types of mucous cells and to characterize the mucosubstances elaborated by them in turn of their vicinal hydroxyl groups their carboxyl or sulfate acid groups or both.

To differentiate mucosubstances, sections were subjected to the following testes PAS technique of (McManus, 1946).PAS technique with prior acetylation, PAS (McManus and Cason 1950), PAS after diastase digestion.PAS after phenylhydrazine treatment (5% phenyl hydrazine for 1 hour at 25°C). (Spicer et al., 1967). These are all to demonstrate mucosubstances with vicinal hydroxyls.

Acid mucosubstances were detected by using following techniques; Alcian blue (AB) at pH 2.5 (1%AB 8GX in 3% acetic acid) for 30 minutes AB at pH 1.0 (1% AB in 0.1N HC1) for 30 minutes (Mowry, 1956), (Lev and Spicer 1964), (Mowry's, 1963) modifications of Male's colloidal iron solution for 2 hours.

Acid mucosubstances were distinguished from the neutral mucosubstances by following tests,the combined technique with PAS and AB at pH 2.5 (Mowry and Winkler, 1956) at pH 1.0 (Spicer et al.,1967).

The following procedures were adopted to distinguish sialomutins and sulfomucins. Aldehyde fuchsine (AF) of (Halmi and Davis, 1953), AF / AB (pH 2.5) (Spicer and Mayer 1960).

Most of the above techniques were done concomitantly with supplementary procedures and specific tests involving chemical blockage or enzymate removal of certain reactive groups in the mucosubstances. These include mild methylation (O.40 cc of concentrated HC1 in 50 cc of absolute alcohol for 4 hours at 37°C) followed by Alcian blue staining at pH 2.5 and active methylation (4 Hours at 60°C) followed by AB at pH 2.5 (Fisher and Lillie 1954, Spicer 1960). Mild methylation saponification (1% KOH in 70% alcohol for 20 minutes) followed by AB (pH.2.5) staining, active methylation soponification AB (Spicer and Lillie 1959).These methylation and demethylation treatments were performed in conjunction with Azure A, AB/PASandAF/AB.

RESULTS

COMPARATIVE HISTOCHEMICAL ANALYSIS OF CONTROL AND INFECTED GILLS

The present study of histochemical tests reveals the control freshwater fishes of *C. striatus* have four pairs of gills. Each gill filament or primary gill lamellae bears a series of alternately arranged respiratory (secondary) lamellae on its either side. Secondary lamellae are made up of alternately arranged blood channels and supporting pillar cells, which give them a ladder like configuration. A thin barrier layer of respiratory epithelium covers the pillar cells and blood channels are the components of the secondary lamellae. Usually one or two RBCs can pass through each blood channels. The mucous cells are mostly present in the primary lamellae (Singh et al., 2014).The periphery of these mucous cells stained moderately with PAS and moderately too strongly with AB 2.5 and AB 1.0 taking bluish violet coloration with AB 2.5/PAS. The thin mucous layer when present on the primary lamellae and secondary lamellae stains weakly too moderately with AB 2.5 and AB 2.5/PAS negatively with AB 1.0 and PAS.

The infected gills blood cells of secondary lamellae showed extensive congestion. Increased weight of these RBCs caused stretching out of the respiratory epithelium that resulted in wear and tear often leading to extensive haemorrhage. Due to congestion of the blood cells, the pillar cells got vertically compressed. Haemorrhage from the blood cells of secondary lamellae ceased with bacterial and fungal infection. The gill filaments became compactly formed due to extensive hyperplasia of the epithelial cells of primary lamellae and secondary lamellae when the individual entity of the secondary lamellae was lost at certain places. The ladder like arrangement of the blood cells and pillar cells started losing their shape the space with blood cells decreased. This is followed by partial regaining of the ladder like appearance of the secondary lamellae even though the volume of blood cells remained distinctly shrunken and the pillar cells came very closer to each other.

The damaged secondary lamellae showed lifting from the vascular components causing haemorrhages. However due to subsequent hyperplasia, the secondary lamellae got completely embedded into the primary lamellae which appeared solid. After the seasonal infectious conditions the mucous cells also showed hyperplasia followed by hypertrophy, when a layer of mucous covered the respiratory surface. The subsequent hyperplasia of the epithelial cells caused fusion of neighboring primary lamellae during later stages. Although the chloride cells continued to exhibit periodic hyperplasia, they frequently got degenerated. The density and dimension, secretory activity and staining properties of the mucous cells fluctuated independently of one another at several stages of infection. The fusion of adjacent primary lamellae continued even after seasonal recovery. The distorted histo-morphology of the primary lamellae along with disintegrated blood cells and pillar cells components of the secondary lamellae also persisted at several places. RBCs were invariably present within the scattered blood cells in the presence of infection, marked repair of the gill filaments were observed. The ladder like vascular component of the secondary lamellae re-established with greatly decreased thickness of the epithelial lining. The regeneration of the gills continued during the subsequent stages. However at the inner

layers these cells appear loosely woven. At certain later stages the small sized mucous cells confined mostly to the outer lining of the epithelial layer, showing more affinities for Alcian Blue (AB) staining. Some of the unidentified cell mass/cells or de-generated mucous cells staining strongly with Aldehyde fuchsine (AF) were also noticed in the deeper layer of the primary lamellae primary lamellae.

GLYCOGEN CONTENT

PERIODIC ACID/SCHIFF (PAS) REACTION

This stain moderately too strongly with PAS positive in control gills in mucous cells was noticed in primary lamellae or secondary lamellae. Blood cells of the secondary lamellae became considerably engorged with red blood cells which stained positively with PAS method.

In the EUS infected gills extensive inter-cellular vacuolization with widespread hyperplasia of cells of the epithelial linings of primary lamellae and secondary lamellae resulted in their increased thickness when presence of the infection. Lifting of the epithelial lining both from the primary lamellae as well as secondary lamellae was very commonly observed in infection. And in the density of the mucous cells are both in the primary lamellae and secondary lamellae increased deeply. These cells were stained negatively with PAS.

ALCIAN BLUE (AB) pH 2.5

The AB 2.5 stain moderately too strongly with control gills at this stage a good number of rounds, large vacuoles of uniform size began appearing in the primary lamellae, as well as secondary lamellae. A small amount of basophilic slimy substance stained positively with AB 2.5. And in the bacterial and fungal infected gills showed the density of the mucous cells decreased slightly and basophilic fuzzy substance regularly sloughed from the mucus coated surface of the primary lamellae and secondary lamellae is negative with AB 2.5.

ALCIAN BLUE (AB) pH 1.0

In the control gills the vacuolization aggravated with this stage a good number of rounds, large vacuoles of uniform size began appearing in the primary lamellae as well as secondary lamellae. A small amount of basophilic slimy substance stained positively with AB 1.0 was frequently observed, especially in the inner lining of these vacuoles. The size and density of these vacuoles in the hyperplasic primary lamellae in general and secondary lamellae in particular increased in the subsequent stages. Even though mild wear and tear of the epithelial linings of the primary lamellae and secondary lamellae was noticed at this stage, no haemorrhage or rupture of the blood cells was detected.

The bacterial and fungal infected gills shows the blood cells of the primary lamellae, however, became greatly dilated and engorged with red blood cells after the infection. The densities of the mucous cells are decreased. A slightly basophilic fuzzy substance regularly sloughed from the surface of the secondary

lamellae. However, a thin layer of mucus coated the surface of the secondary lamellae AB 1.0 negative with this several sites the typical ladder-like arrangement of the pillar cells and blood cells are collapsed. However, the gills regenerated partially and regained some of their lost staining properties at several other sites. The blood capillaries running through the gill filament showed extensive congestion and engorgement with a large number of red blood cells. (Fig 6 to 11).

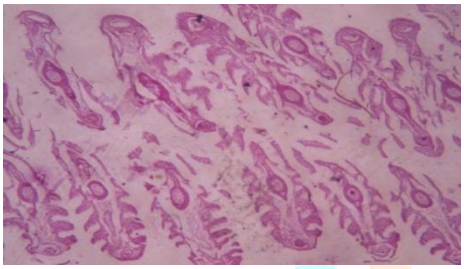
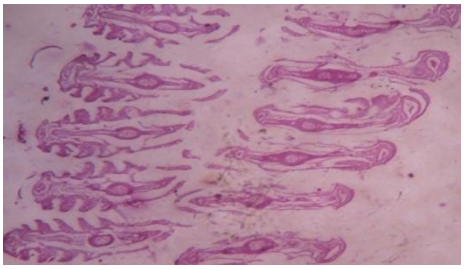


Fig 6



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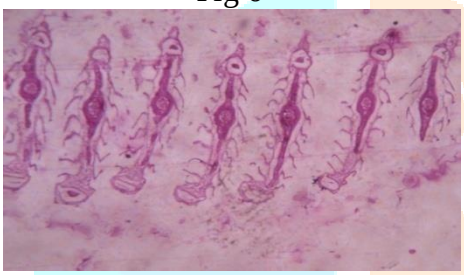

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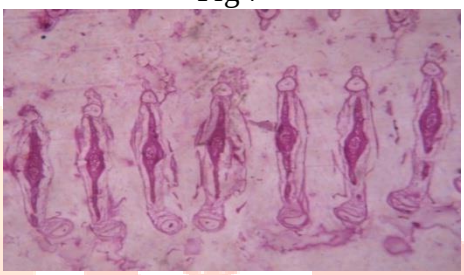

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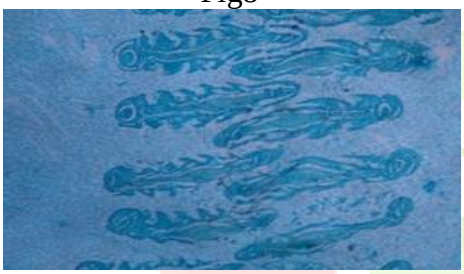

Fig 10

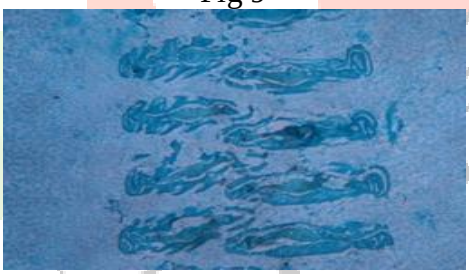

Fig 11

Fig1: T.S of *Channastraitus* Controlgill showsmoderate the presence of Glycogen (PAS). Fig2: T.S of *Channastraitus* Infected gill shows strong the presence of Glycogen substances (PAS). Fig 3: T. S of *Channa straitus* Control gill showing pale blue presence of acid mucin (AB2.5pH). Fig 4:T.S of *Channastraitus* Infectedgill shows light and dark blue presence of sulphatedmucins (AB 2.5 pH). Fig5: T.S of *Channastraitus* Controlgill shows presence of mucin (AB 1.0 pH). Fig6:T.S of *Channastraitus* Infectedgill showing presence of sulfated mucosubstances (ABl.0 pH).

ALCIAN BLUE (AB) pH 2.5/ PAS

The control gill shows vascular components of the secondary lamellae are made up of alternately arranged pillar cells, blood channels that remained covered by a thin respiratory epithelium. The mucous cells are mostly observed in the epithelium of the gill filaments or primary lamellae. A few saucer-shaped mucous cells are also present in the secondary lamellae. The mucous cells take on a dark greenish-black color with AB

2.5/PAS. The mucous cells on the primary lamellae stain light greenish-blue, with their periphery taking on a dark blackish green color with AB 2.5/PAS.

EUS infected *C. striatus* gills extensive inter-cellular vacuolization with widespread hyperplasia of cells of the epithelial linings of primary and secondary lamellae resulted in their increased thickness density of the mucous cells both in primary lamellae and secondary lamellae increased greatly to greenish-blue with AB 2.5/PAS techniques.

ALDEHYDE FUCHSINE

With this aldehyde fuchsine of normal gills have shown vascular components of the secondary lamellae purple and blood channels that remained covered by a thin respiratory epithelium stained with light yellow, the pillar cells show negative reaction. Gills epithelial cells appear in intense purple color. The mucous cells were giving a red color suggested that were likely to be sulfomucins. The EUS infected gill lamellae tissues were strongly positive to Aldehyde fuchsine indicating the presence of sulfated mucosubstances. The other hand the infected gills tissue showed that the epithelium was decreased. (Fig 12 to 15).

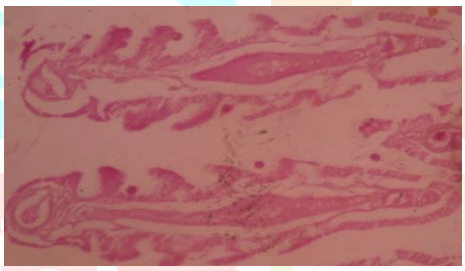

Fig 12

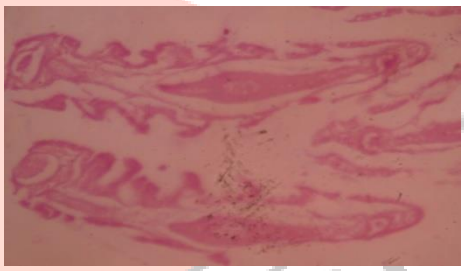

Fig. 13

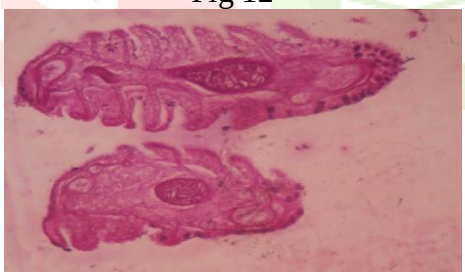

Fig. 14

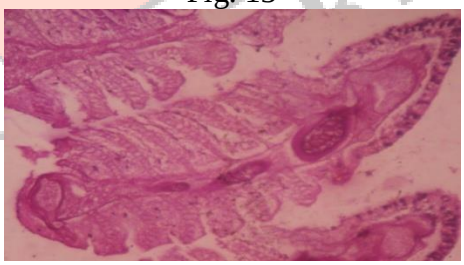

Fig. 15

Fig12: T.S of *Channa straitus* Control gill, displaying presence of Glycogen predominately (pink), less amount of mucin (blue) (AB25pH/PAS). Fig13. T.S of *Channa straitus* Infected gill displaying the presence of mucin predominately (AB 2.5 pH /PAS). Fig.14. T.S of *Channa straitus* Control gill presented elastic fibers purple and collagen stained with light yellow (Aldehyde fuchsine). Fig.15. T.S of *Channa straitus* Infected gill shown elastin decreased (Aldehyde fuchsine).

DISCUSSION

The histochemical studies in the present microscopic investigations of the gills. Histochemical tests carried out on the gills, revealed alcianophilia of the mucus glands when stained with alcian blue at 1.0 pH and 2.5 pH.The alcian blue at 1.0 pH the staining intensity is inconspicuous as compared to alcian blue at 2.5 pH.

This clearly indicated the presence of sulphated mucosubstances, hyaluronic acid and sialic acid in the gills. Alcian blue (1.0 pH)/PAS demonstrated the traces of acidmucopolysaccharidessulphatedmucosubstances, hyaluronicacid and sialomucins. When alcian blue (2.5 pH)/PAS stain was employed, the neutral muco-polysaccharides and strongly acidic mucopolysaccharides revealed their presence.The most remarkable toxic effect of the arsenic salt on the gills of *C. striatus* is periodic fluctuationin their density, percentage of area occupancy and staining properties of the mucous cells. The extensive secretion of sulphated mucosubstances by the mucous cells. A survey of the literature indicates that after severe stages of infection increases, the density and percentage of area occupancy of the mucous cells of the gills decreased significantly in the seasonal condition. However the density remained above the normal level up to end of the seasonal period even though it shows periodic fluctuations. This indicates regeneration of large number of small sized mucous cells. Later on both the parameters fluctuate at different stages of exposure and remain subnormal especially at later stages of EUS infection. Bacteria and fungi have permanently altered the monogenic activity of mucous cells of the gills as evidenced by subnormal density/area occupancy of the mucous cells. Although excessive mucuscoagulation on the respiratory surfaces might cause disturbances in several important physiological processes such as gas exchange, nitrogen excretion, salt balance and circulation of blood (Laurent and Dunel, 1978). It is also prevents the penetration of the ambient arsenic salt temporally.

The gill structure in case in the case of teleost fishes is likely to be markedly alterd resulting into pathological conditions in various ways if subjected to any environmental and chemical pollutants like heavy metals, pesticides and insecticides (Benarjee, 1986).These conditional changes are visible especially at the base of the secondary gill lamellae, respiratory epithelium of primary and secondary gill lamellae, with necrosis of lamellar epithelium causing respiratory and osmoregulatory distress.(Skidmore and Tovell 1972), (Smith and Piper 1975), (Lamke and Mount 1963), (Christae and Battle 1963), (Ashley, 1970).

The present observations points out to the similar conditions as a result of fungal factors were infected to *C. striatus.* However the severity of damage caused was more with the increased in the period of seasonal infection. The major changes were necrosis, rupture of capillaries and atrophy of respiratory gill filaments. These findings of the present investigations thus show a similarity with the observations made (Rao *et al.,* 1983).

Extensive secretion of the mucous in acutely arsenic exposed fishes has been reported to causes death of the fishes because increased mucus production causes suffocation or direct detrimental effects on the gills epithelium. While reviewing the toxicity of arsenic, arsenic exposed fish suffer from difficult breathing due to clogging of gills by coagulated mucus film, vascular collapse in gills and anoxia due to the direct damaging effect of arsenic ions on blood vessels (Irwin, 1997). However, prolonged exposure of *Clarias batrachus* to sub-lethal concentration of sodium arsenate did not cause any death even though all the respiratory organs (including skin) Singh and (Banerjee, 2008a), showed extensive mucous secretion. Similar secretions of mucus by the various respiratory organs of different fishes following exposure to several other heavy metalsalts have

frequently been also noticed (Rajan and Banerjee 1991; 1992), (Hemalatha and Banerjee 1993), (Banerjee and Chandra 2005). These authors also did not observe any death of the fish due to mucus coagulation. Hence death of fishes observed (Sorenson *et al.,* 1979). And others might perhaps be due to damages caused by the arsenic salt on various cellular components of the vital organs including gills of the fishes (Singh et al., 2014).

CONCLUSION

Marked pathological changes in fish gill architecture were observed. The changes include epithelial lifting, bulging of tips in primary gill filaments, degenerated secondary lamella, curling of secondary gill filaments, atrophy in secondary lamella and fusion of secondary gill filaments. The damage of gills in fishes exposed to the high level of disease was severe. Shortened and clubbing of ends of the secondary gill lamellae, fusion of adjacent secondary gill lamellae and necrosis in the primary lamellae were well marked. Hyperplasia and hypertrophy of nuclei were also seen.

The histochemical reactions were determined that the control gill tissues of these fishes contained glycogen, weakly acidic sulfated mucosubstances, hyaluronicacid, sialomucins, carboxlylated mucosubstances. In the EUS infected fishes gill the tissue had shown some variation with the control. The damaged secondary lamellae showed lifting from the vascular components causing haemorrhages. However due to subsequent hyperplasia, the secondary lamellae got completely embedded in the primary lamellae which appeared solid. After the infection the mucous cells also showed hyperplasia followed by hypertrophy, with a layer of mucus covering the respiratory surface.

Acknowledgement: Authors with to thanks Dr. P. Srinivas Plant Pathology and Microbiology laboratory, Department of Biotehnology, Kakatiya University, Warangal and Dr. G. Rajender Department of Zoology Kakatiya University, Warangal for their continuous support and inspiration and providing necessary facilities for the work.

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