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Research Article

HISTOPATHOLOGICAL AND HISTOCHEMICALSTUDY ON GILLS OF THE FRESHWATER WALKING CATFISH *CLARIASBATRACHUS* (LINN.) FOLLOWING EXPOSURE AND WITHDRAWAL OF ARSENIC STRESS.

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ABSTRACT

The present study investigates sublethal (1 ppm) effects of disodium arsenate heptahydrate (DSA) on the gills of the freshwater catfish,*Clariasbatrachus* (Linn.) following prolonged exposure and withdrawal of the arsenate stress. The gills of *Clariasbatrachus* L. face the direct contact stress of arsenic salt following exposure. 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 adjacent primary lamellae (PL) of the damaged gills often fused together. The staining property and density of the mucous cells (MCs) showed extensive periodic alterations showing more affinity for the sulphated moieties known for metal binding. The chloride cells hyperplasia followed by their degeneration frequently noticed. Following withdrawal significant but incomplete recovery takes place. The gills continued to show hyperplasia. The density and staining properties of the MCs continued to remain altered.

Abbreviations: AB 2.5: alcian blue at pH 2.5,AF: aldehyde fuschin, BLCs: blood capillaries, BVs: blood vessels, BB: bismark brown, d: days, h: hours, H/E: Ehrlich's haematoxylin/eosin, MCs: mucous cells, PAS: periodic acid Schiff, PL: primary lamellae, PLCs: pillar cells, RE: respiratory epithelium, RBCs: red blood cells, SL: secondary lamellae, T. S.: transverse section

KEYWORDS: *Sodium arsenate, gills ,accessory respiratory organs, catfish, histopathology, recovery*

INTRODUCTION

Arsenic toxicity in human subjects has been a matter of great concern. However the data related to non-human animals especially those related to the aquatic forms including fishes are meagre even though the toxicity of this metalloid is usually mediated through water. Fishes have widely been used as effective bioindicators of pollution (Singh and Banerjee, 2008abc, 2009; Chandra and Banerjee 2003; Rajan and Banerjee, 1993b; Munshi, 1961). Amongst the various organ systems the gills have widely been used for testing the contaminated waters.

C. batrachus, an indigenous species is of great significance because of its nutritional value. It generally creeps on soil surface of the pond and sometimes it also live into the mud of the pond.The gills are important organs in fish including *C. batrachus* to perform respiration,osmoregulation, acid base balance and nitrogenouswaste excretion. The gills in suprabranchial chambers remain in direct contact to surrounding medium hence more suitable organs for toxicity studies.

Elemental arsenic as such is insoluble but whenever come in contact with oxygen rich environment, it become oxidized and soluble. In aquatic environment where the oxygen is more as in surface water, arsenic always exists in its highest oxidation state in the form of arsenates. Disodium arsenate heptahydrate (DSA) is more soluble, thermodynamically more stable and readily available to aquatic fauna including fishes.

In this paper histopathologicaland histochemicalanalysis of the gills of *C. batrachus* were performed to detect the toxic impact of an

arsenic salt (sodium arsenate heptahydrate; DSA) so that the gills can be used as efficient bioindicator.

MATERIALS AND METHODS

Animals and experimental design

Irrespective of sex, live specimens of *C. batrachus* (15 ± 1 cm in length; 45 ± 5 gm weight) were collected from a local fish market, Chaukaghat, Varanasi, India and acclimated in the laboratory condition for 30 days in plain tap water (dissolved O_2 6.3 mg/l, pH 7.2, hardness 23.2 mg/l and room temperature 28 ± 3^0 C). Regular feeding and renewal of water was done after every 24h. Ten groups of ten fish each were exposed separately to ten litre (10 L) of sublethal concentration (1 ppm) (10 % of LC_{50} value for 96h) of disodium arsenate heptahydrate (DSA) (s. d. fine-chem. Ltd. Mumbai, India) prepared in tap water for 90 days. Control fish were exposed to plain tap water. For withdrawal experiment, the 90 d arsenic salt (DSA) exposed fish were returned to plain tap water. Three fish (N=3) each from experimental (both, arsenic exposed and withdrawal) as well as untreated control aquaria were sacrificed after different intervals and the entire gills from both sides of the fish were dissected out, washed in normal saline to remove blood clots present if any and fixed in 10% neutral formalin (Lillie and Fullmer 1976), aqueous Bouin's fluid (Bancroft and Stevens 1996), 70% alcohol and Helly's fluid (Pearse 1985) for histopathological and histochemical analyses.

Histopathology and Histochemistry

Paraffin sections (6 µm thick) were cut using Lieca semi-motorized rotary microtome (Lieca RM 2145, Lieca Microscopy and Scientific Instruments Group, Switzerland). Serial sections were stained with Ehrlich's haematoxylin and eosin (H/E) (Ehrlich, 1886) for routine histopathology, periodic acid- Schiff (PAS) (Mc Manus, 1948), alcian blue (AB) pH 1.0 (Lev and Spicer, 1964) and pH 2.5 (Mowry, 1956), aldehyde fuchsin (AF) (Pearse, 1985), Bismarck brown (BB) (Gurr, 1958) and combined AB 2.5/PAS (Mowry, 1956) for various carbohydrate moieties. Observations were made under light microscope attached with an imaging system (Lieca DM 2000, Lieca Microscopy and Scientific Instruments Group Germany). The density and area occupancy of the MCs were calculated with help of image analysing software, Motic Images 2000, v. 1.3.

Statistical analysis

One way ANOVA followed by the Dunnett's t test was performed for the statistical analyses. The criterion for significance was set at $p < 0.05$ and $p < 0.01$.

RESULTS

(A) Gills

(a) Control

C. batrachus has four pairs of gills. Each gill filament or primary gill lamellae (PL) bears a series of alternately arranged respiratory (secondary) lamellae (SL) on its either side. SL are made up of alternately arranged blood channels (BLCs) and supporting pillar cells (PLCs), which give them a ladder like configuration (Fig. 2a). A thin barrier layer of respiratory epithelium (RE) covers the PLCs-BLCs components of the SL. Usually one or two RBCs can pass through each BLC (Fig. 2a). The mucous cells (MCs) are mostly present in the PL (Figs 3a and 3d)**.** The periphery of these MCs stained moderately with PAS and moderately to strongly with AB 2.5 and AB 1.0 taking bluish violet colouration with AB 2.5/PAS. The thin mucus layer when present on the PL and SL stains weakly to moderately with AB 2.5 and AB 2.5/PAS negatively with AB 1.0 and PAS.

(b) Experimental

(i) Exposed

After 03h of exposure, the BLCs of SL 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 BLCs, the PLCs got vertically compressed.

Haemorrhage from the BLCs of SL ceased after 06h. The gill filaments became compactly formed due to extensive hyperplasia of the ECs of PL and SL when the individual entity of the SL was lost at certain places. The ladder like arrangement of the BLCs-PLCs started losing their shape after 06h. From 12h onwards the space with BLCs decreased. This is followed by partial regaining of the ladder like appearance of the SL even though the volume of BLCs remained distinctly shrunken and the PLCs came very closer to each other. The RE of the greatly damaged SL showed lifting from the vascular components causing haemorrhage (e.g. after 12h and 24h of exposure). However due to subsequent hyperplasia, the SL got completely embedded into the PL which appeared solid. After 24h the MCs also showed hyperplasia followed by hypertrophy (after 03d) when a layer of mucus covered the respiratory surface. After 07d of exposure the PL as well as SL although showed quite congestion and the respiratory epithelia lining the SL continued to remain lifted upto 30d (Fig. 3c), they (SL) almost returned to their normal configuration. Periodic hyperplasia followed by wear and tear (e.g. after 14 and 21 days) and subsequent regeneration (e.g. 30d, 45d, 60d and 90d) of the PL and SL continued several times during the entire exposure period (Fig. 3d). The

subsequent hyperplasia of the ECs caused fusion of neighbouring PL during later stages (Fig. 2c).

Although the chloride cells continued to exhibit periodic hyperplasia, they frequently got degenerated. The density and dimension, secretory activity and staining properties of the MCs fluctuated independently of one another at several stages of exposure. Their density increased greatly after 24h (Figs. 1a and 3b).

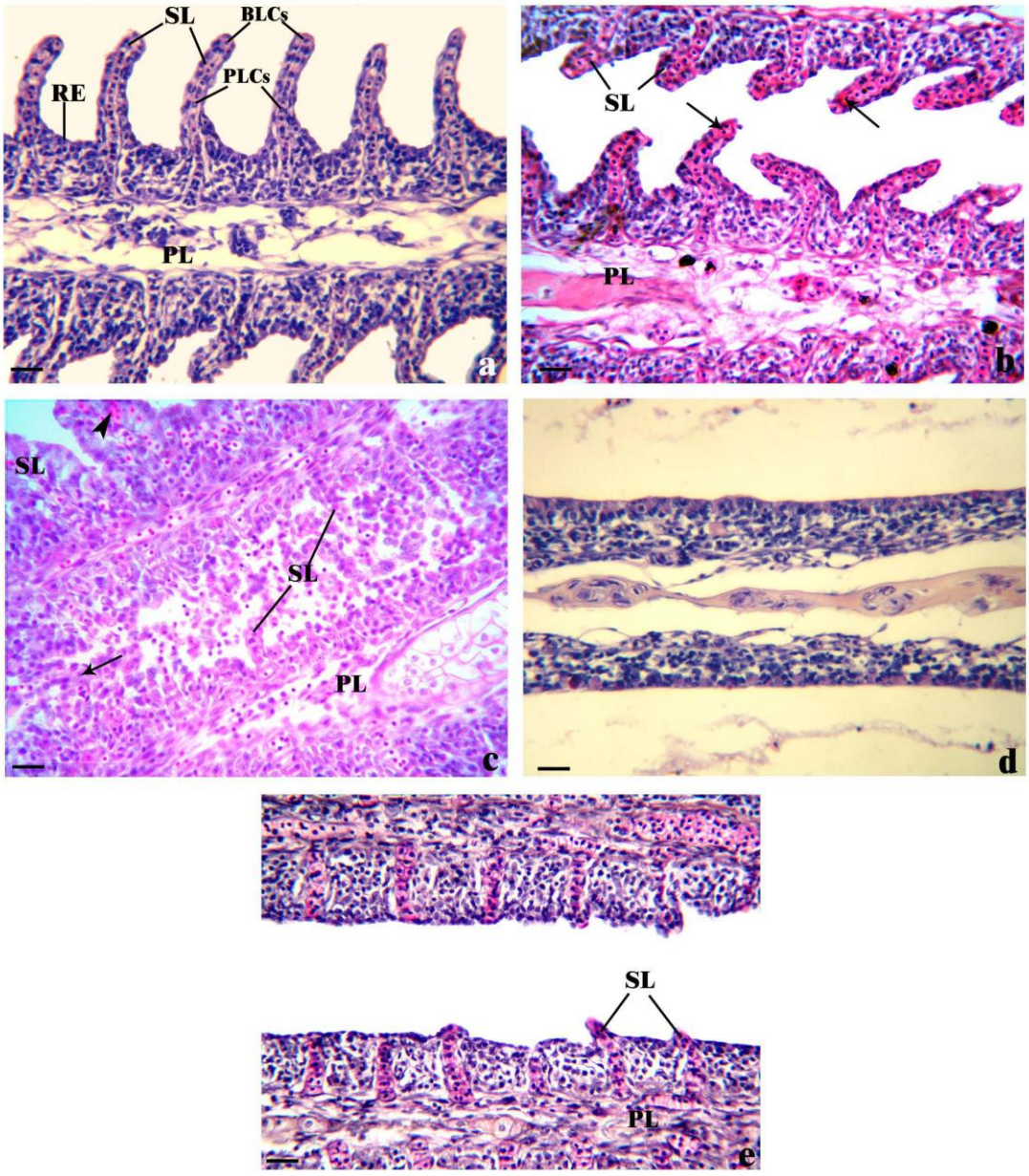

Figure 2

Fig. 3a: T. S. of gill of untreated control C. batrachus showing its structural organisation. (H/E) (bar = 20µm). Fig. 3b: Congestion of BLCs (arrows) of SL of the gill after 07d of exposure. H/E (bar = 20µm). Fig. 3c: Fusion (arrow) of neighbouring SL of severely damaged gills after 21d of exposure. Note the congested BLCs (arrow head) of SL. H/E (bar = 20µm). Fig. 3d: Greatly altered histomorphology of the gill following prolonged (90d) exposure. Note the hyperplastic PL and SL. H/E (bar = 20µm). Fig. 3e: Great congestion of the BLCs of the regenerated SL after 90d of withdrawal following arsenic stress. The hyperplastic epithelial lining of the PL showed vacuolisation and the histomorphology of the gills continued to differ greatly from untreated control after 90d. H/E (bar = 20µm).

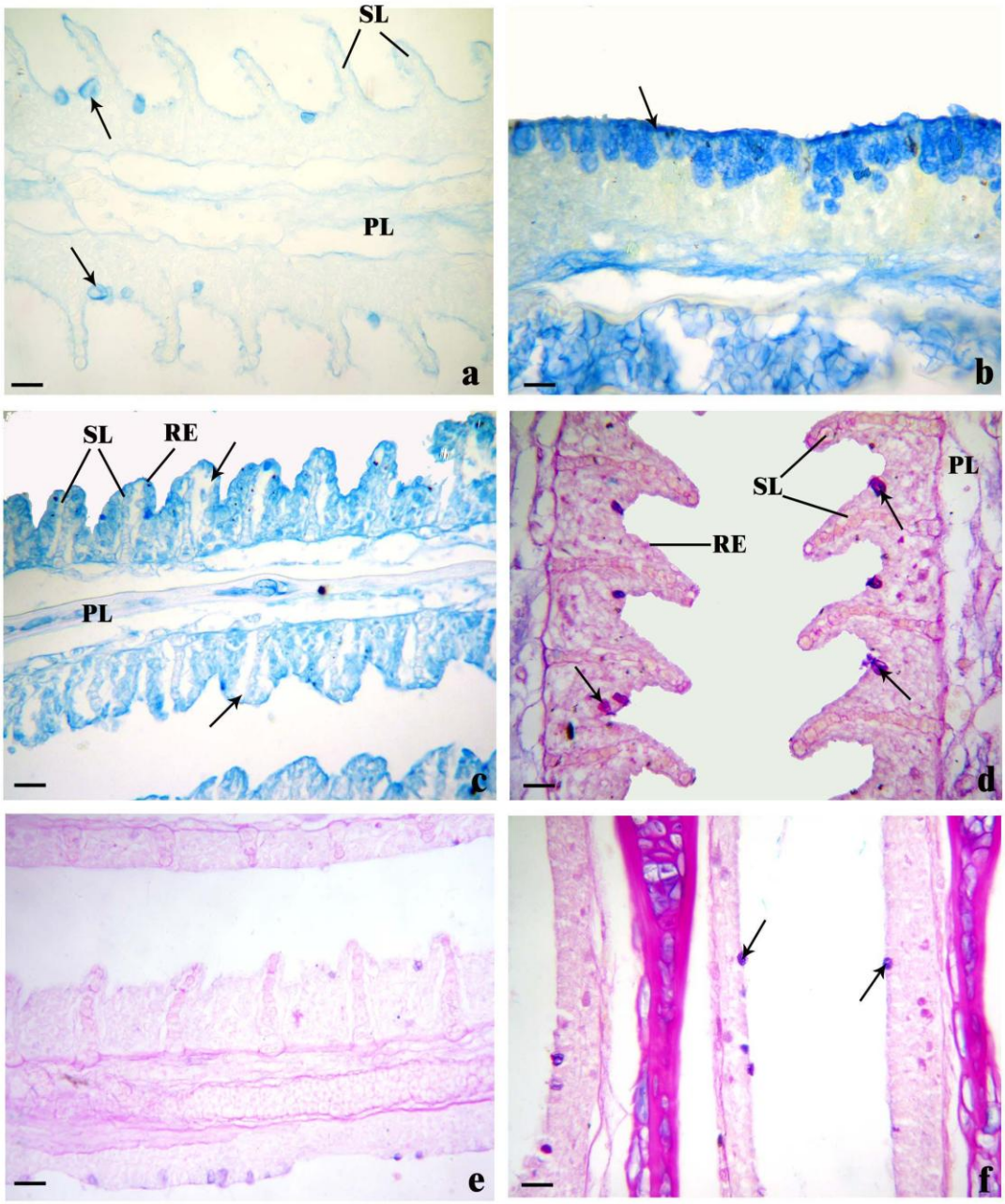

Figure 3

Fig. 4a: Normal distribution of acidic carbohydrates in mucus cells (MCs) (arrows) of the gill of untreated control fish. Note a very thin layer of mucus covering the surface of PL as well as SL. AB 2.5 (bar = 20µm). Fig. 4b: Extensive hyperplasia of the strongly positive MCs in the epithelial layer of PL after 24h of exposure. Note a thick layer of strongly positive mucus (arrow) covering surface of the PL. AB 2.5 (bar = 20µm). Fig. 4c: Lifting (arrow) of detached RE from the vascular component of the SL of the gill after 30d of exposure. Note the decreased density of the MCs. AB 2.5 (bar = 20µm). Fig. 4d: Untreated gills showing normal distribution MCs taking both, red and blue colouration (arrows). AB 2. 5/PAS (bar = 20µm). Fig. 4e: Decreased density as well as staining intensity of MCs after 90d of with-drawal of arsenic stress. AB 2.5/PAS (bar = 20µm).Fig. 4f: Presence of dark bluish violet coloured MCs (arrows) in outer layer of the epithelium after 90d of sodium arsenate exposure. Note a few regenerating red coloured MCs in the inner layers. AB 2.5/PAS (bar = 20µm)

(ii) Withdrawal

The fusion of adjacent PL continued even after 24h of recovery. The distorted histo-morphology of the PL along with disintegrated BLCs-PLCs components of the SL also persisted at several places. RBCs were invariably present within the scattered BLCs.

After 07d, marked repair of the gill filaments were observed. The ladder like vascular component of the SL re-established with greatly decreased thickness of the epithelial lining. The regeneration of the gills continued during the subsequent stages (14d and 30d) also when the BLCs-PLCs components of the SL got re-oriented with subsequent decreased thickness of the epithelial lining of the PL. After 45d the ECs at the surface formed a double layer and appeared compactly arranged**.**However at the inner layers these cells appear loosely woven (Fig. 2e).

At certain later stages the small sized MCs confined mostly to the outer lining of the epithelial layer, showing more affinities for AB staining (Fig. 3e). Some of the unidentified cell mass/cells or degenerated MCs staining strongly with AF were also noticed in the deeper layer of the PL.

DISCUSSION

The most remarkable toxic effect of the arsenic salt on the gills of *C. batrachus* is periodic fluctuation in their density, percentage of area occupancy and staining properties of the MCs. The extensive secretion of sulphated mucins by the MCs perhaps helps to bind the arsenic salt in an attempt to reduce its toxic impact at least partially. A survey of the Fig 1a indicates that after an initial (after 3h exposure) increase, the density and percentage of area occupancy of the MCs of the gills decreased significantly (by about 50%) after 6h. However the density remained above the normal level up to 30d, even though it shows periodic fluctuations. Then after it becomes subnormal till the termination of the experiment. However percentage of area occupancy remains below the control level at most of the stages beyond 07d. This indicates regeneration of large number of small sized MCs. Beyond 30d, the regeneration of MCs perhaps slows down causing their decreased density and percentage of area occupancy. Later on both the parameters fluctuate at different stages of exposure and remain subnormal especially at later stages of exposure.

Arsenic exposure has permanently altered the mucogenic activity of MCs of the gills as evidenced by subnormal density/area occupancy of the MCs (Fig 1a). Although excessive mucus coagulation 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 also prevents the penetration of the ambient arsenic salt temporally. Extensive secretion of the mucus 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 (Sorenson *et.al*. 1979). 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 *Clariasbatrachus* 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 metal salts have frequently been also noticed (Rajan and Banerjee, 1991, 1992, 1993b, 1994b; Hemalatha and Banerjee, 1993, 1997a, 1997b, 1997c; Parashar and Banerjee, 1999a, b, c; Banerjee and Chandra, 2005). These authors also did not observe any death of the fish due to mucus coagulation. Hence death of the fishes observed by Sorenson e*t. 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. Fish exposed to 1 to 2 µg of arsenic/ litre for 2 – 3 days have shown haemorrhagic spheres on gills, necrosis of heart, liver and ovarian tissues (NRCC, 1978).

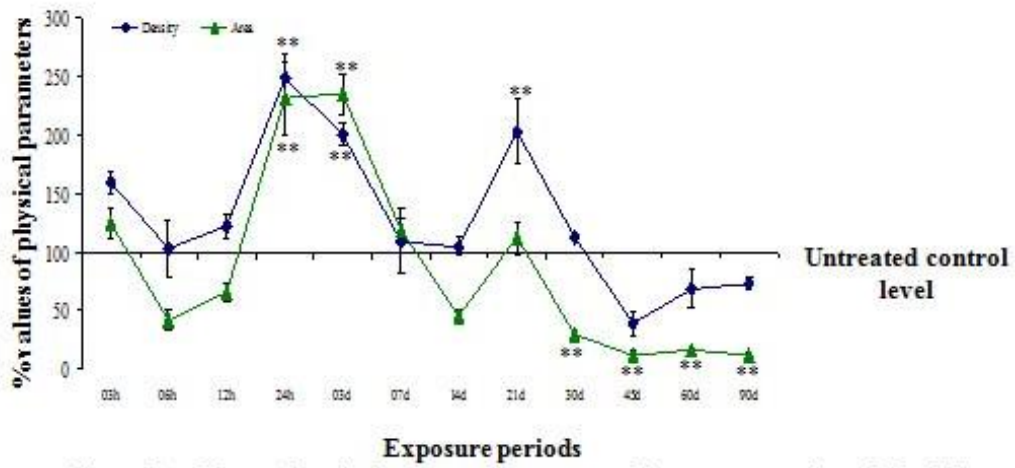

Figure 1a: Fluctuations in density and percentage of area occupancies of the MCs of the Ist gill of *C. batrachus* after different periods of exposure of sodium arsenate. Values (mean ± SEM) are expressed in percentage and untreated control value is taken as 100%. *, $P < 0.05$ and **, $P < 0.01$.

The other important toxic impact of arsenic salt on gills is congestion of BLCs due to engorgement with RBCs. This causes stretching out of the BLCs onto the respiratory surface. This reduces the respiratory barrier distance that compensates the oxygen deficiency rendered by disturbed branchial respiration following gills damage.

The binding capacity of arsenic with sulphydryl group of critical proteins such as GSH and cysteine (Scott *et. al.* 1993; Delnomdedieu*et. al.* 1994) also causes cellular toxicity. This may perhaps be due to oxidative stress resulting from intracellular oxidation- reduction reaction of various forms of arsenic.

Due to wear and tear following prolonged exposure, severe loss of lamellar structure of the gills was noticed at several places. Regeneration of the damaged lamellae often followed especially in the initial stages of exposure. Prolonged exposure causes extensive loss of the lamellar structure when the number of RBCs decreased significantly.

Continuation of arsenic stress although causes more damages, uncontrolled regeneration of the gills is frequently noticed. This resulted in hyperplasia of the ECs of the epithelial linings of the SL/PL leading to fusion of the adjacent SL of the same or neighbouring PL. The ladder like arrangement of the vascular components of the SL also gets very often dismantled. The extensive hyperplasia of the ECs with subsequent fusion of the adjacent gill lamellae in arsenic exposed fish perhaps prevents the entry of arsenic salt into the underlying vital tissue components of the gills thus decreasing accumulation of the arsenic salt in the cells at least for the initial periods of exposure. The chloride cell hyperplasia in the respiratory organs of arsenic exposed fish *C. batrachus*is a compensatory attempt to maintain the internal homoeostasis via regulating at least temporarily the uptake of the toxic ions (Laurent, 1985; Laurent and Perry, 1990) including the arsenic ones.

Following withdrawal, both the respiratory organs show slow but significant recovery in their cellular architecture and regain most of their normal histomorphology. The structure of regenerated gills continues to differ from that of untreated control. The increased thickness of the epithelial layer of gills although persisted at many stages, the re-establishment of normal ladder like arrangement of the vascular (BLCs-PLCs) components of the SL continues up to 90d. The decreased density and percentage of area occupancy of the MCs of the gills also continue to persist during all the periods of withdrawal (Fig 1b). These MCs group mainly in the inner layer of the epithelial lining of the PL where they stain strongly for sulphated GPs

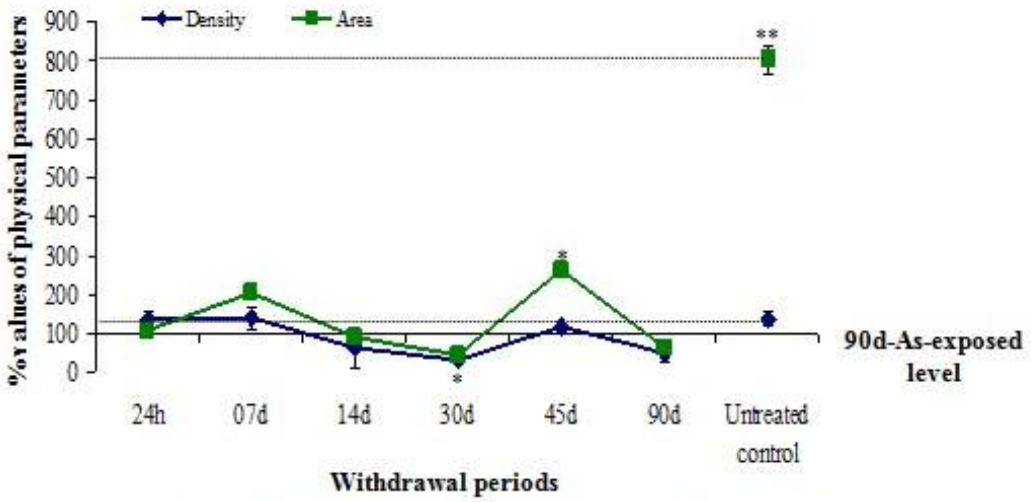

Figure 1b: Fluctuations in density and percentage of area occupancies of the MCs of the Ist gill of *C. batrachus* after different periods of withdrawal of sodium arsenate stress. Values (mean ± SEM) are expressed in percentage and 90d-As-exposed value is taken as 100%. *, $P < 0.05$ and **, $P < 0.01$.

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