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A RESPONSE STUDY ON DENDRITIC ORGANS OF A BOTTOM DWELLING FRESHWATER EDIBLE CATFISH *CLARIAS BATRACHUS* (LINN.) FOLLOWING LONG – TERM EXPOSURE AND WITHDRAWAL OF SODIUM ARSENATE HEPTAHYDRATE ($NA_2HASO_4.7H_2O$) STRESS.

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ABSTRACT
The dendritic organs of *Clarias batrachus* (Linn.), a nutritionally important bottom dwelling freshwater edible catfish of the region, are the important organs adopted chiefly for aerial respiration when the fish are under aquatic stress. The dendritic organs are the accessory respiratory organs and are present in suprabranchial chamber and are equally exposed along with the gills, the main respiratory organs to water-born toxicants including the arsenic. Live and healthy male and female *C. batrachus* L. (length 15 ± 1cm and weight 45 ± 5g) which were belonging to single population were exposed to 1 ppm sublethal concentration ($1/10^{th}$ of LC_{50} for 96h) of sodium arsenate heptahydrate ($Na_2HAsO_4.7H_2O$) for 90 days. The 90 days arsenic exposed fish were returned to plain tap water again for 90 days to study the effects of withdrawal of arsenic stress. The fish were cold anaesthetized and dendritic organs were isolated and analysed histopathologically and histochemically after different periods of exposure and withdrawal of arsenic stress. Following exposure, blood capillaries of the secondary lamellae of dendritic organs showed extensive congestion followed by wear and tear. The ladder like arrangement of blood channels and supporting pillar cells gets dismantled and haemorrhages regularly took place in the dendritic organs. The density of red blood cells in subepithelial blood vessels of dendritic organs decreased. The staining property and density and area occupancy of mucous cells showed extensive periodic fluctuation showing more affinity for acidic glycoproteins containing sulphate and carboxylic groups. The density and area occupancy of mucous cells however decreased significantly ($P < 0.05$ and $P < 0.01$) at all stages of exposures of arsenic salt. Following withdrawal of arsenic stress, significant but incomplete recovery took place. The dendritic organs continued to show mucous cells hyperplasia. The density and staining properties of the mucous cells continued to remain altered.

KEYWORDS: sodium arsenate, dendritic organs, catfish, histopathology, recovery.

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, Linn.: Linnaeus 1958

INTRODUCTION
In many toxicological research studies the fishes are employed as bioindicators against the aquatic pollutants (Rajan and Banerjee, 1993b; Singh and Banerjee, 2006; 2008abc; 2009; 2014; 2016). *Clarias batrachus* (Linn.), an important nutritious and medicinal catfish of the region is found in fresh water aquatic bodies. *C. batrachus* L. have non-scaly smooth body surface and hence easily creep on and inside the bottom soil. *C. batrachus* L. are unique and possess bimodal respiratory mechanisms i.e. aquatic and aerial performed by gills and dendritic organs respectively. Presence of dendritic organs makes the fish very hardy. The dendritic organs are modified gills and are present on second and fourth gill arches of either side in suprabranchial chamber (Munsi 1962; Banerjee 2007). Although the dendritic organs constitute surface organs in *C. batrachus* L. and are equally exposed to any ambient toxicants present in water, they are the less studied organs as compared to the gills. Hence in this paper an approach has been made to investigate whether the dendritic organs apart from the gills can be employed for biomarker studies against water-born toxicants including the arsenic salt.

Arsenic is a potent toxicant found into the nature. In environment, it comes via both natural and anthropogenic sources. The prevalence of arsenic is more at places where there is low annual rain fall and people are dependent on ground water. In earth's crust arsenic is mainly present as insoluble inorganic arsenic pyrites, the main source of arsenic contamination into the environment. The extensive use of submersibles perhaps is provoking the dormant arsenic to come into the environment. In environment, arsenic exists in trivalent and pentavalent forms depending on oxygen available into the water. Arsenic in pentavalent forms such as sodium arsenate heptahydrate, the salt selected for present study, is thermodynamically more stable and remains available to both aquatic flora and fauna including the fishes. In tissues/organs of the animals including the fishes, arsenic metabolizes into harmful metabolites leading to varying degree of toxicity in different animals.

MATERIALS AND METHODS

Live specimens of *C. batrachus* (15 ± 1 cm in length; 45 ± 5 gm weight) (both male and female) were brought from a local fish market and were kept in laboratory for purpose of acclimation for 30 days in plain tap water (dissolved O_2 6.3 mg/l, pH 7.2, hardness 23.2mg/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 10 liter of sublethal concentration (1 ppm) of sodium arsenate (s. d. fine-chem. Ltd. Mumbai, India) dissolved in tap water. Control fish were exposed to plain tap water. For withdrawal experiment, the 90 d arsenic salt 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 time intervals and the dendritic organs from both the sides were fixed in 10% neutral formalin and aqueous Bouin's fluid for histopathological and histochemical analyses. Paraffin sections (6 µm) were stained with Ehrlich's haematoxylin and eosin (H/E) for routine histopathology, periodic acid- Schiff (PAS), alcian blue (AB) pH 1.0 and 2.5, aldehyde fuchsin (AF) and Bismarck brown (BB) for various carbohydrate moieties. The density and area occupancy of mucous cells (MCs) were calculated using software, Motic Images 2000, v. 1.3. One way ANOVA followed by the Dunnett t test was performed for the statistical analyses.

RESULTS

a) Control fish

The dendritic organs in control *C. batrachus* were two pairs (total four) and were located one each on the 2nd and 4th gill arches. The terminals or knobs of dendritic organs were comprised of a vascular region with alternately arranged blood channels (BLCs) and supporting pillar cells (PLCs) giving an appearance of ladder. (figure. 2a). Secretions of MCs (figure. 2e) that stained variously for different carbohydrate moieties regularly lubricated the respiratory surface of the dendritic organs.

(b) Experimental fish

(i) Exposed

Within 03h of exposure, the dendritic organs of *C. batrachus* showed great congestion when their BLCs got engorged with RBCs causing pushing out of their BLCs at the surface. This reduced the gas diffusion distance. The number of RBCs also increased in the subepithelial blood vessels (BVs)**.** The number of MCs in the respiratory epithelium decreased (figure 2b) and stained variously for different carbohydrate moieties throughout the exposure periods.

The BLCs showed further bulging due to increased congestion in the subsequent stages (Figure. 5b). The subepithelial BVs also remained congested.

After 12h, due to continued damage the ladder like arrangement of the PLCs-BLCs components of SL got greatly dismantled. However regeneration also took place. The newly formed ECs filled up most of the spaces of damaged SL haphazardly. The number and size of subepithelial BVs decreased with decreased density of RBCs. However the number of WBC increased at this stage. While the density of AB 2.5 positive MCs remained almost unchanged, the density of PAS positive MCs continued to decrease.

The congestion of the BLCs increased further and disintegration of SL spread to many other areas after 24h. The small regenerating MCs in the lower layer stained darkly with AF. An AF positive slime coated the surface of respiratory epithelium.

The cellular components of the epithelial lining became haphazardly arranged after 03d. At certain other places partial regeneration of SL were noticed with congestion of the BLCs. The subepithelial BVs got further engorgement. There was an overall decrease in the staining properties of the MCs.

The disintegration of the SL continued even beyond 07d. The number of RBCs in the BLCs and in the subepithelial BVs decreased greatly and appeared almost empty. However a large number of WBCs like cells with darkly stained nuclei were often noticed within the BVs. The staining intensity of the MCs continued to decrease greatly. With PAS and AB 2.5, MCs remained almost unstained. Patches of slime continued to cover the surface of the RE.

After 14d, no much alternation was noticed. However a large number of MCs developed in lower layers (fig. 5f). Developing MCs stained strongly for sulphated moieties (fig. 5f). After 21d, the lamellar structures of the SL at the many places were again lost. The randomly distributed BLCs contained RBCs. The SL regenerated once again after 30d with their BLCs engorged RBCs

protruding onto the respiratory surface. The number of AB 2.5 positive MCs increased with increased staining intensity.

The alternately arrangement of PLCs-BLCs components of the SL again got disturbed after 45d even though the blood capillaries engorged with RBCs continued to protrude out. The number of MCs increased (fig. 2a). No much alternation in the SL was observed after 60d (fig. 5c). After 90d the lamellar structure of dendritic organs was greatly lost with substantially decreased congestion. The subepithelial BVs also showed congestion with RBCs. The number of MCs increased substantially (fig. 2a).

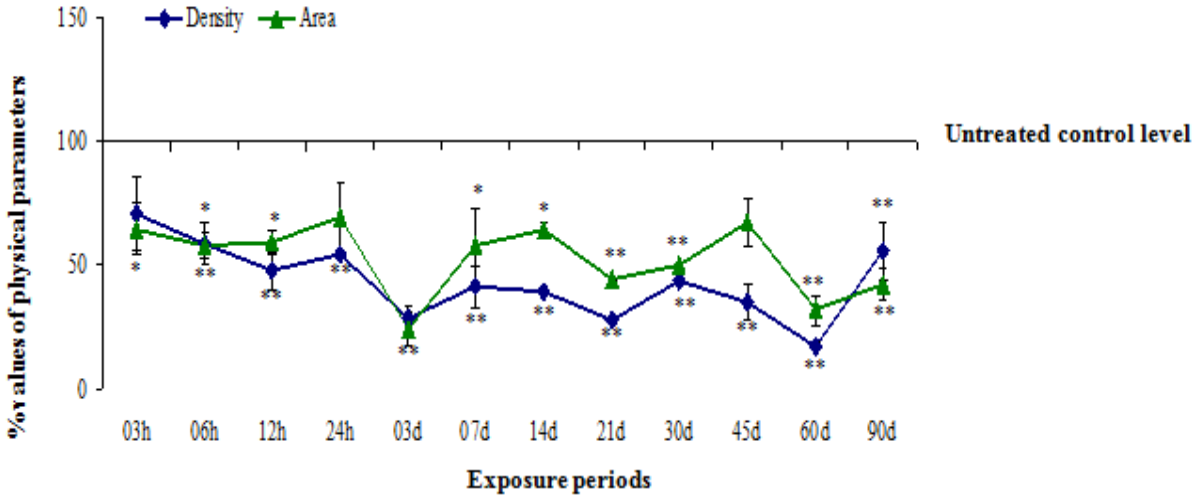

Figure 1a: Fluctuations in the density and percentage of area occupancies of the MCs of the dendritic organs of *C. batrachus* after different periods of exposure of sodium arsenate. Values (mean ± SE) are expressed in percentage and untreated control value is taken as 100%. *, $P < 0.05$ and **, $P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

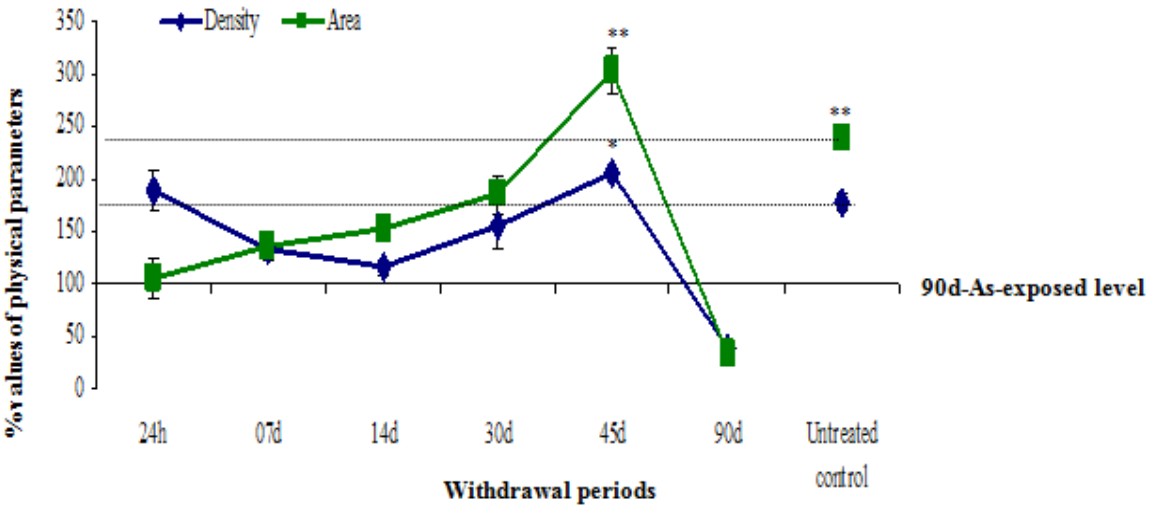

Figure 1b: Fluctuations in density and percentage of area occupancy of the MCs in the dendritic organs of *C. batrachus* after different periods of withdrawal of sodium arsenate stress. Values (mean ± SEM) are expressed in percentage and the 90d-As-exposed value is taken as 100%. *, $P < 0.05$ and **,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

Figure 1

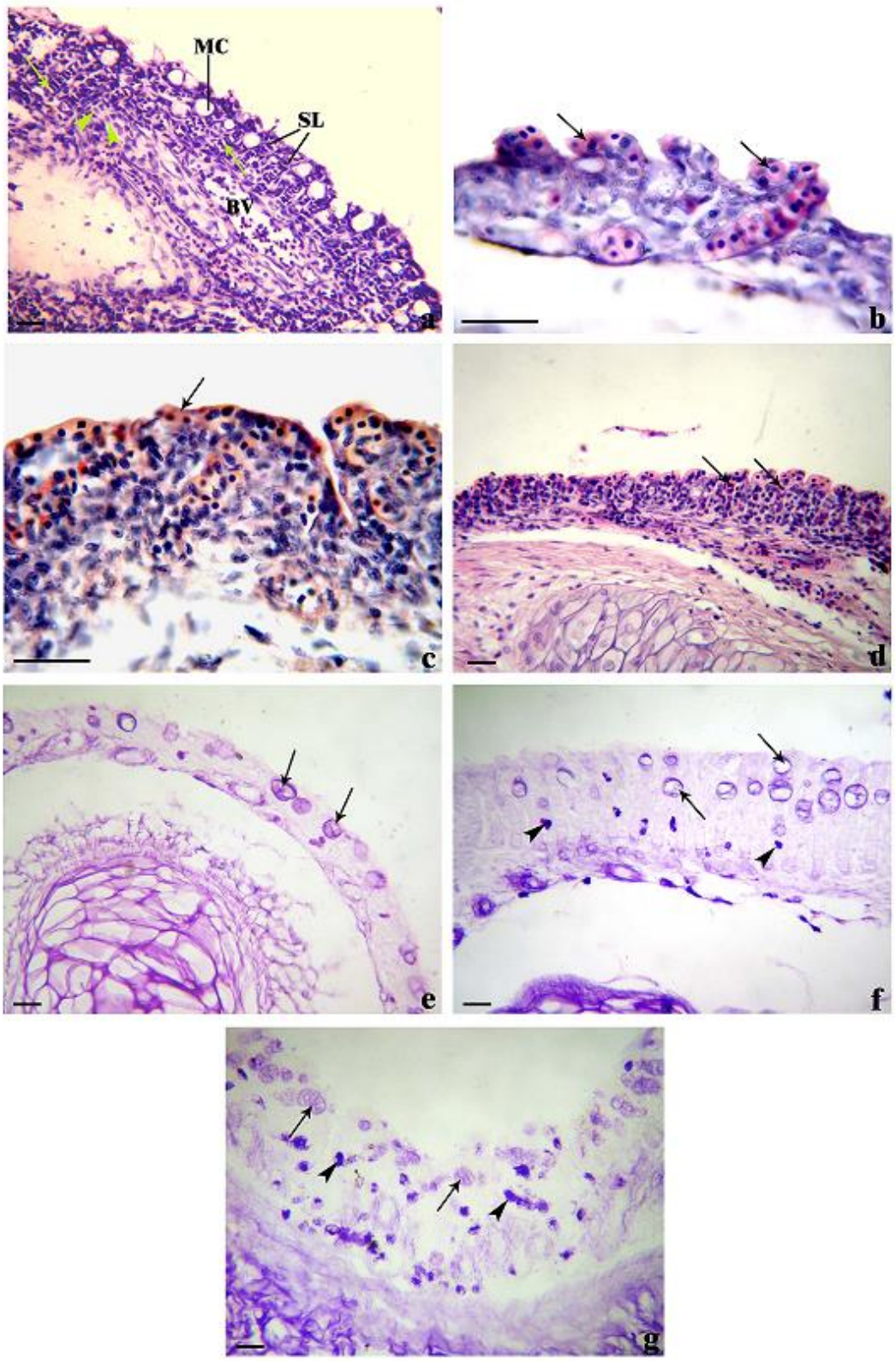

Figure 2

Explanation of photomicrographs

Figure 2a. T. S. of the dendritic of untreated control *C. batrachus* showing its normal histomorphology. Note the alternately arranged pillar cells (arrow head) and blood capillaries (arrow) constituting the vascular components of the secondary lamellae and randomly distributed mucous cells of SL. H/E (bar = 20µm)

Figure 2b. Protrusion of the BLCs with engorgement of RBCs after 06h of exposure causing bulging out (arrows) of BLCs at the surface of dendritic organs. H/E (bar = 20µm)

Figure 2c. Greatly congested BLCs (arrows) of SL after 60d of exposure. Note the badly disturbed vascular components of the SL. H/E (bar = 20µm)

Figure 2d. Greatly regenerated dendritic after 90d of withdrawal of arsenic stress. Note the normal looking SL (arrows) bearing alternately arranged PLCs-BLCs (filled with RBCs). H/E (bar = 20µm)

Figure 2e Dendritic organs of the untreated control *C. batrachus* showing normal distribution of sulphated mucins in its epithelial lining. Note the strong reaction shown by several MCs (arrows). AF (bar = 20µm)

Figure 2f. Regeneration of MCs containing sulphated mucin after 14d of exposure. Note the almost emptied MCs (arrows) in outer most and middle layers. Also note strong reaction shown by the regenerating small sized MCs in inner layers (arrow heads). AF (bar = 20µm)

Figure 2g. Hyperplasia of the MCs distributed throughout the area of the epithelial lining of the dendritic organs after 45d of withdrawal. Note strong reaction (arrow heads) shown by the newly formed smaller MCs in inner and middle layers and comparatively weaker reaction (arrows) given by the larger MCs at outer layers. AF (bar = 20µm)

Abbreviations: AF, aldehyde fuschin; BLCs, blood capillaries; BVs, blood vessels; d, days; h, hour (s) H/E, Ehrlich's haematoxylin/eosin; MCs, mucous cells; PL, primary lamellae; PLCs, pillar cells; RE, respiratory epithelium; RBCs, red blood cells; SL, secondary lamellae; T. S., transverse section.

(ii) Withdrawal

After 24h of withdrawal of arsenic stress no significant alternation in histopathology of the dendritic organs was noticed and the dendritic organs appeared almost identical to that of 90d stress fish. However the size of the heavily loaded MCs increased.

Significant recovery of SL took place after 07d onwards of withdrawal when the vascular (BLCs-PLCs) components of the SL regained their normal ladder like arrangement (07d and 14d). The size of the MCs continued to increase.

Even though marked re-establishment of the respiratory epithelia continued, RBCs on the surface of the respiratory epithelia continued to be present after 30d. After 45d, the SL got well established. Presence of blood cells along with ECs and other secretory material was still noticed on the surface upto 90d (figure 5d). Density and dimension of the fully loaded MCs also increased at this stage (figure 2b). A large number of developing MCs stained strongly for sulphated moieties (figure 5g). After 90d of withdrawal lamellar structure looked identical to that of control fish (figure 5d). The density and dimension of the MCs decreased greatly (figure 2b).

DISCUSSION

Arsenic exposure permanently altered the mucogenic activity of MCs of the dendritic organs as evidenced by subnormal density and area occupancy of the MCs (figure. 1a). The excessive mucus secretion on the surface of dendritic organs is perhaps an attempt to prevent further entry of arsenic salts into the fish body. The mucus coagulation on surface however may disturb the important physiological processes such as gas exchange, nitrogen excretion, salt balance and circulation of blood (Laurent and Dunel, 1978). In many toxicology research studies on fish it has been reported that the extensive secretion of the slime on surface of respiratory organs causes death of the fishes because of either suffocation or direct detrimental effects on the respiratory epithelium (Sorenson *et.al*. 1979). Further studies on gills of arsenic exposed fish, it has been seen that the 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). The prolonged exposure of *Clarias batrachus* to sub-lethal concentration of sodium arsenate heptahydrate however did not cause any death even though all the respiratory organs (including skin and gills) (Singh and Banerjee, 2006; Singh and Banerjee, 2014) showed extensive mucous secretion. In several other research studies, the secretions of mucus by the various respiratory organs of different fishes following exposure to several other heavy metal salts have also been 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). According to NRCC, 1978, the fish exposed to 1 to 2 µg of arsenic/ litre for 2 – 3 days show haemorrhagic spheres on gills, necrosis of heart, liver and ovarian tissues.

The MCs of dendritic organs of *C. batrachus* showed periodic fluctuation in density, percentage of area occupancy and staining properties (figure 1a and table 1a). The amount of sulphated glycoproteins was more (figure 1a). This perhaps helps to bind the arsenic salt in an attempt to reduce its toxic impact at least partially. The density and percentage of area occupancy of the MCs of dendritic organs even though fluctuate at different stages of exposure, remain below the control

level at all stages (figure. 1a). This is perhaps due to less mucogenic activity of the dendritic organs which has cartilaginous support making it less delicate. The MCs however show more affinity for acidic and sulphated moieties well known for metal binding.

The other important toxic impact of arsenic salt on dendritic organs 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. Due to continuation of the exposure, the dendritic organs too get damaged causing disturbed aerial respiration also.

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.

Following withdrawal of arsenic stress the dendritic organs recover promptly in its histomorphology with re-establishment of many of the vascular components. The MCs activity also increases showing hyperplasia as well as hypertrophy at several stages. However the MCs continue to secrete sulphated mucins.

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