Comparative Assay of Different Doses of Arsenic Trioxide (As$_2$O$_3$) on *Channa punctatus* (Bloch) through Light and Scanning Electron Microscopic Observations

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Abstract—*Freshwater air-breathing carnivorous teleost, Channa punctatus (Bloch) was exposed to two different doses, high 30.93 mg/l and low of 15.47 mg/l of As$_2$O$_3$ under congenial laboratory condition of 30 days duration. There were some distinct differences in histopathological observations in most of the cases in the different concerned regions of the fish as viewed in light and scanning electron microscopic studies. In gill, at low dose of arsenic trioxide, the primary and secondary lamellae were damaged at some places showing curling, hypertrophy and hyperplasia; but in high dose, damage was more serious in lamellae also showing distortion, shrinkage and vacuolation of chloride cells. Double-microridged stratified epithelial cells of primary and secondary gill lamellae and distorted concentric arrangement of microridges were the marked lesions as viewed under SEM for both the doses. At low dose, the mucosal layers and mucosal folds, and the gastric glands were undergoing comparatively less degeneration compared to high dose of exposure. Vacuolation in gastric glands, proliferation of mucous pits and mucus secretion, and degeneration of columnar epithelial cells were prominent in high dose. In intestine, there was comparatively less damage in villi and columnar epithelial cells but in high dose it showed degeneration and clumped nuclei, damage in intestinal top plate, and disruption of connective tissue of lamina propia and vacuolation. Intestinal mucosal folds, absorptive columnar epithelial cells and its microvilli resulted into deformation, fragmentations, and excessive secretions of mucus under high dose exposure. Liver is the most vulnerable target organ in fish. There were serious degeneration, vacuolation and conspicuous changes in the perilobular area of liver with scattered hepatopancreas. At high dose, the orientation of cords of hepatocytes, its cytoplasm and connective tissue depicted the significant alterations. In some areas, hepatocytes present a syncytial appearance with reduced nuclear volume and pycnotic conditions. At high dose, the hepatocytes were severely damaged showing completely disguised and disorientation of cords of hepatocytes with severe rupture in appearance. So, the present study has been able to establish a definite pattern of lesions based on dose-response relationship.*

Keywords: *Arsenic trioxide, Channa punctatus, Histopathological & Topological, Gill, Stomach, Intestine, Liver*

1. INTRODUCTION

The heavy metal contamination of water body has now become an inevitable phenomenon owing to the march of industrialization, civilization, and green revolution. Aquatic pollution by arsenical compounds considered as strong biological poisons, is an obvious concomitance of industrial and agricultural activities [1-6] attributed by runoff or other illegal practices. Importance of determining the adverse effects of arsenic on aquatic environment, especially on the main aquatic inhabitants, like plankton [7] and fish species [8] lies in the way they are incorporated in the food chain. Although, arsenic is the natural constituent of the earth's crust [9] a relatively accessible and ubiquitous metalloid [10-13] produces cumulative toxic effects in the different target organs including the organs of distribution [14]. Pesticides and pesticidal industries are the specific contributors of arsenic incorporation into the aquatic environment because it is used as a major constituent of most of the pesticides.

Arsenic are biologically available to aquatic organisms including fish, living in the contaminated environment [15-17] and it produces some toxicological effects [18, 19]. Although an important route of As uptake by benthic-feeding fish is dietary [19-20] the majority of studies on As toxicity in freshwater fish have examined the effects associated with uptake of waterborne As across the gills [19, 21-23]. Fish can metabolize, concentrate and store water borne pollutants [24, 25]. Arsenic was absorbed by the gastrointestinal (GI) tract of Lake White fish (*Coregonus clupeaformis*) and accumulated in different organ like liver, kidney, stomach, intestine, skin and scales [20].

Arsenic is lethal for most of the organisms at high concentration, while chronic intake of low doses like, 5 to 10 ppb can lead to cancer of the skin, lungs, liver fibrosis, cirrhosis, parenchymal cell damage, inflammation, focal

necrosis in addition to liver neoplasia, and hepatocellular carcinoma, melanosis (hyperpigmentation or hypopigmentation or white spots), hyperkeratosis (harden skin), restrictive lung disease, peripheral vascular disease (Blackfoot disease), gangrene, diabetes mellitus, hypertension and ischemic heart disease [12, 26-27]. The microanatomical effects of different organs like gill, liver, kidney, intestine, muscle and brain are also established by [28-29]. The tissue disorientation, peliosis, and vacuolization accompanied by karyolysis, apoptosis, and necrosis of hepatocytes were significant based on different concentrations (from nonlethal to 1/20 and 1/10 LC50 values) of As_2O_3 at different days of exposures, like 1, 2, 7, and 14 on *Channa punctatus* (Bloch) [30]. They observed the shrinkage of the glomerulus and increased space in the Bowman's capsule on days 1, 2, and 7, and irregularities in the renal tubule including apoptotic and necrotic cells. Epithelial hyperplasia, epithelial lifting and oedema, lamellar fusion, aneurism, desquamation and necrosis was observed in gill, whereas, the liver tissue showed focal lymphocytic and macrophage infiltration, congestion, vacuolization and shrinkage of hepatocytes, dilation of sinusoids, cloudy swelling, vacuolar degeneration, focal necrosis and nuclear hypertrophy when *Oreochromis mossambicus* was exposed to 3, 28, and 56 ppm concentrations of $NaAsO_2$ after 48, 96 and 192 hours of exposure[31]. The relationship between period of exposure and concentration of arsenic in selected tissues suggests an adaptive response of *Channa punctatus* to arsenic [32].

Corresponding with the histopathological lesions, the dose-dependent disturbances of arsenic in fish species are essentially an important breakthrough in arsenic toxicosis. The objective of the present study is to compare the responses of different doses of arsenic on the different target organs of fish like gill, liver, stomach and intestine through evaluation of hitopathological lesions at cellular and subcellular levels associated with the mechanism of actions of arsenic and establishment of important biomarkers in the monitoring of aquatic environment.

2. MATERIALS & METHODS

2.1. Chemicals

Arsenic trioxide and all other reagents of analytical grade were purchased from Merck Specialities Private Limited. Osmium tetraoxide was procured from Spectrochem Pvt. Ltd., Mumbai, India.

2.2. Fish

Freshwater air-breathing carnivorous teleost, *Channa punctatus* (Bloch) having the average weight of 32 ± 1.45 g and length of 12 ± 0.63 cm was acquired from the local market and was disinfected with $KMnO_4$ and acclimatized to laboratory conditions for 15 days in 40 L aquarium with continuous aeration. During acclimation, fish were fed once a

day with living fish food *viz., Tubifex* sp. Average value of water parameters during the acclimatization period, were as follows: temperature 20.70 ± 0.129°C, pH 8.43 ± 0.041, electrical conductivity 351.3± 2.59 µS/cm, total dissolved solids 215± 0.69 mg/L, dissolved oxygen 6.88± 0.06 mg/L, total alkalinity 174.4 ± 6.30 mg/L as $CaCO_3$, total hardness 172.4 ± 2.94 mg/L as $CaCO_3$, Chloride 34.04±1.24 mg/L as Cl⁻, sodium 16.8 ± 0.32 mg/L, potassium 7.12 ± 1.01 mg/L, ammoniacal nitrogen 1.68 ± 0.11 mg/L and nitrate-nitrogen 0.50 ± 0.044 mg/L.

2.3. Experimental Design

After 15 days the fish were divided into three groups, containing a total of 15 specimens in each aquarium of one control and other two treatment sets at a dose of 30.93 mg/L (T1) and 15.47 mg/L (T2) for a period of 30 days. During the period of experiment both the treatment and control group of fish were fed with live *Tubifex* sp. on every day. This experimental water was renewed and glass aquaria were also cleaned regularly on every alternate day. The aerator bubble was also provided to avoid any oxygen depletion, and also combat with the possible alteration of carbon-dioxide tension. Experiments were conducted with a natural photoperiod and at an ambient water temperature.

During experimentation period, the average water parameters were as follows: in T1 it was: temperature 20.68 ± 0.123°C, pH 8.76 ± 0.047, electrical conductivity 403 ± 2.59 µS/cm, total dissolved solids 272.15 ± 1.57 mg/L, dissolved oxygen 4.64 ± 0.41 mg/L, total alkalinity 175.2 ± 6.13 mg/L as $CaCO_3$, total hardness 170 ± 1.24 mg/L as $CaCO_3$, Chloride 25.53±0.94 mg/L as Cl⁻, sodium 26 ± 1.62 mg/L, potassium 15.26 ± 1.13 mg/L, ammoniacal-nitrogen 9.46 ± 1.15 mg/L, and nitrate-nitrogen 2.16 ± 0.06 mg/L; in T2 it was temperature 20.60 ± 0.124°C, pH 8.435 ± 0.071, electrical conductivity 471.00 ± 2.99 µS/cm, total dissolved solids 237.25 ± 1.29 mg/L, dissolved oxygen 5.78 ± 0.47 mg/L, total alkalinity 174.26 ± 7.50 mg/L as $CaCO_3$, total hardness 170.16 ± 3.08 mg/L as $CaCO_3$, Chloride 24.53±0.45 mg/L as Cl⁻, sodium 23± 1.52 mg/L, potassium 10.12 ± 1.14 mg/L, ammoniacal-nitrogen 4.48± 1.23 mg/L, and nitrate-nitrogen 1.35 ± 0.026mg/L.

2.4. Sampling

Water quality during experimentation was measured as per APHA [33]. Fish were sacrificed after 30 days carefully. The desired tissues namely gill, stomach, intestine, and liver were collected from both the control and treatment sets groups of fish and were subjected to histological and ultrastructural study.

2.5. Histological study

After dissection of fish the desired tissues like gill, stomach, intestine and liver were fixed in aqueous Bouin's fluid solution for overnight. After fixation, tissues were dehydrated

through a graded series of ethanol, cleared by xylene, and embedded in paraffin. Sections were cut at 4-5 micron using Leica RM2125 microtome and stained with haematoxylin-eosin (H&E). Stained sections were observed under Leica DM2000 light microscope and images were captured.

2.6. Ultrastructural study

For ultrastructural study (SEM) tissues were fixed in 2.5% glutaraldehyde solution and examined in the microscope, HITACHI-S 530. The desired tissues like gill and the representative portions of alimentary canal *viz.*, stomach and intestine were removed immediately after dissection and exposed the luminal surface, through longitudinal incision. Now, the mucosal surface of the incised tissues were spread out and pinned with luminal surface uppermost on the cork sheets. The adhering mucous of the luminal surface was removed by rinsing in heparinized saline to remove excess mucus. After rinsing in 0.1M cacodylate buffer pH 7.5, the tissues were infiltered with 2.5% glutaraldehyde for 24 h at 4^0C. After fixation the tissues were removed, rinsed in buffer, trimmed into 8 mm squares and subjected to post fixation in 1% Osmium tetraoxide (OsO_4) in 0.1M cacocodylate buffer, pH 7.5 for 2 h, dehydrated through graded acetone followed by amyl acetate and subjected to critical point drying (CPD) method with liquid carbon dioxide. The mucosal surface of each tissue was mounted on metal stubs, coated with gold with thickness of approximately 20 nm. The tissues were then scanned in HITACHI -S-530 SEM at University Science Instrumentation Centre the University of Burdwan and images were captured.

2.7. Ethical statement

Fish handling and the experiment was performed following the guideline of the Institutional Animal Care and Use Committee of the University of Burdwan and approved by the ethical committee of this University.

3. RESULTS

3.1. Histopathological Observation

3.1.1. Gill

In the control condition, gill of *Channa punctatus* is usually made up of the primary and secondary gill lamellae and a cartilaginous base (Photo 1.1). A number of chloride cells are present in the secondary gill lamellae.Both the primary and secondary gill lamellae play an important role in gaseous exchange (Photo 1.1). Under arsenic toxicosis at T2 dose the primary gill lamellae of *Channa punctatus* is damaged at some places, and some regions showed curling of secondary gill lamellae. Hypertrophy and hyperplasia of the secondary gill lamellae, and vacuoles were seen in primary gill lamellae (Photo 1.2). Primary gill lamellae were severely damaged in the T1 dose of arsenic trioxide. Curling and distortion of secondary gill lamellae were much prominent. Chloride cells

became shrinked and vacuolated as observed in secondary gill lamellae (Photo 1.3).

3.1.2. Stomach

In the control condition, stomach is made up of usual four layers *viz.*, mucosa, submucosa, muscularis and serosa. The gastric glands are elongated. The gastric mucosa is lined with a single, layer of compactly arranged columnar epithelial cells with single centrally placed nuclei (Photo 1.4). A thin layer of top plate externally covers the gastric columnar epithelium. The well vascularised submucosa is a thick layer of connective tissues (lamina propria), which extends as narrow strip in the mucosal fold and separates the gastric gland. The muscularis layers are made up of an inner circular and outer longitudinal layer. The outer most layer, serosa, is thin and consisted of a compact layer of blood vessels. After the exposure of T2 dose *i.e.,* at high dose the mucosal layer and mucosal folds were damaged, the gastric glands were degenerated but less as compared to high dose of exposure of arsenic trioxide. Vacuolation was found in gastric glands and as well as in columnar epithelial cells (Photo 1.5). In the T1 dose *i.e.,* at high dose, the gastric epithelium showed degeneration and vacuolation in basal region with various mucus secretion by exocytosis from the damaged columnar epithelial cells. Subepithelial vacuolation was also noticed due to toxicity. Erosion and degeneration of the gastric glands were also visualized. Vacuolation was also noticed in tunica propria and submucosa (Photo 1.6)

3.1.3. Intestine

In the control condition, intestine possesses prominent four histological layers *viz.*, mucosa, submucosa, muscularis and serosa. The intestinal villi of *Channa punctatus* are narrow and slender. The mucosa of intestine is madeup of simple, long absorptive columnar epithelial cells each with a basally and centrally placed nucleus. The intestinal villi are covered by means of thin top plate *i.e.,* brush border. The loose connective tissue fibres of submucosa are projected into the mucosal folds forming lamina propria. The muscular layer is composed of outer longitudinal and inner cellular muscle fibres. The serosa is distinct and contains blood capillaries (Photo 1.7).

The effects of T2 dose revealed little damage to villi forming clumped nuclei and small vacuoles appeared in the lining of the columnar epithelial cells (Photo 1.8). The cellular distortion in intestine of *Channa punctatus* was very prominent due to T1 dose. The intestinal top plate of intestinal villi was damaged. The mucosa of intestine was severally damaged with the toxicity and different types of degenerative changes leading to the total destruction of columnar epithelial cells resulting into the formation of clumped nuclei (Photo 1.9).

3.1.4. Liver

In the control condition, *Channa punctatus* liver is made up of polygonal hepatocytes with centrally placed prominent spherical nucleus and heapatopancreas is provided with exocrine acinar cells. Central veins are found randomly throughout the hepatic parenchyma (Photo 1.10). Due to low dose (at T2 dose) the connective tissue showed damage at some points, the hepatopancreas was degenerated and scattered. Vacuolations occurred in cytoplasm and hepatocytes but less in areas (Photo 1.11). But at high dose *i.e.,* T1 dose, the most conspicuous changes were disarray of cords of hepatocytes, damage of connective tissue, and degeneration of hepatocytes with syncytial appearance. The nuclei were reduced in volume and become pycnotic (photo 1.12).
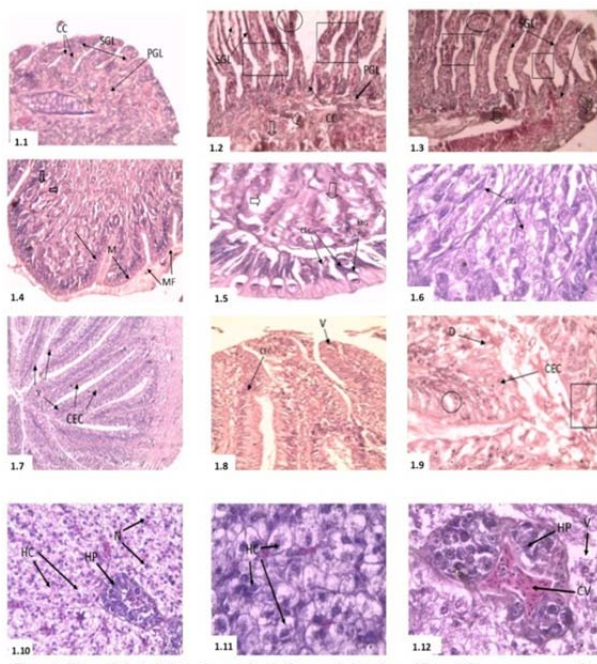

Figure 1: (1.1-1.12) Histopathological photomicrographs of gill, stomach, intestine and liver of *Channa punctatus* under control condition (C), arsenic treated laboratory condition [low dose (T2) and high dose (T1)] (1.1) Showing normal structure of primary gill lamellae (PGL) and secondary (SGL) lamella under light microscopy (C × 400). (1.2) Showing vacuoles in PGL (bold arrow), curling (square) (T2 × 400). (1.3) Showing chloride cells shrinked and vacuolated in SGL (square) (T1 × 400). (1.4) Mucosa, submucosa muscularis serosa are prominent gastric gland are elongated (C × 200). (1.5) Mucosal layer and mucosal fold damaged (arrow) (T2 ×1000). (1.6) Gastric epithelium degenerated (arrow) (T1 × 1000). (1.7) Columnar epithelial cell (arrow) and centrally placed nucleus (C×200). (1.8) Forming clump nucliei and small vaculation (dark arrow) (T2 × 400). (1.9) Showing intestinal villi (arrow) and mucosa damaged (T1 × 1000). (1.10) Hepatocytes centrally placed prominent nucleus found (dark arrow) (C×400). (1.11) Vacuolations occurred in cytoplasm and hepatocytes. (T2×1000). (1.12) Degeneration of hepatocytes and connective tissue damaged (T1×1000).

3.2. Scanning Electron Microscopic Observation

3.2.1. Gill
In the control condition, topographic study depicted that the luminal surface of the secondary gill lamellae made up of stratified epithelial cells which are embaced with concentric arrangements of microridges and double ridged structure forms the boundary (Photo 2.1). After exposure to T1 dose gills were severely damaged showing degeneration in secondary gill lamellae (Photo 2.2) and loss of microridges which were more serious than T2 dose (Photo 2.3).

3.2.2. Stomach
In the control condition, in stomach the mucosal folds are anastomosed with each other to form the deep, shallow and rectangular shaped concavities with prominent gastric pits surrounded by the epithelial cells. The mucosal layers made up of oval and/or round shaped columnar epithelial cells and possess densely packed but short and stubby microvilli and mucous cells (Photo 2.4). At T1 dose *i.e.,* at high dose, mucosal folds and columnar epithelial cells were necroesd and showed severe degenerative changes (Photo 2.6). But in the T2 dose, the toxicity included some changes of columnar epithelial cells and excessive secretion of mucus spreaded over the luminal surface (Photo 2.5).

3.2.3. Intestine
In *Channa punctatus*, the mucosal folds of intestine were irregularly arranged and were supported by regularly packed oval or rounded columnar epithelial cells. The apical surface of the CEC were densely packed with the microvilli (Photo 2.7) in control condition.Due to high dose *i.e.,* T1 dose toxicosis the mucosal folds were severely damaged with excessive mucus secretion over the epithelial cell surface (Photo 2.9). Intestinal absorptive columnar epithelial cells were damaged severely and microvilli showed deformation and disintegration. Under low dose, *i.e.,* T1dose, the mucosal folds and columnar epithelial cells showed less damage but there were some fragmentation of the mucosal folds (photo 2.8).

3.2.4. Liver
In control condition, liver hepatocytes were arranged in regular cords of hepatocytes (photo 2.10). After exposure to T1 dose the hepatocytes were severely damaged and the orientation of hepatic cords were degeneratedand showing vacuolations. Huge amount of mucus were secreted over the hepatocytes (Photo 2.12). But at T2 dose the hepatocytes were not damaged so significantly and also showing low amount of mucus (Photo 2.11).
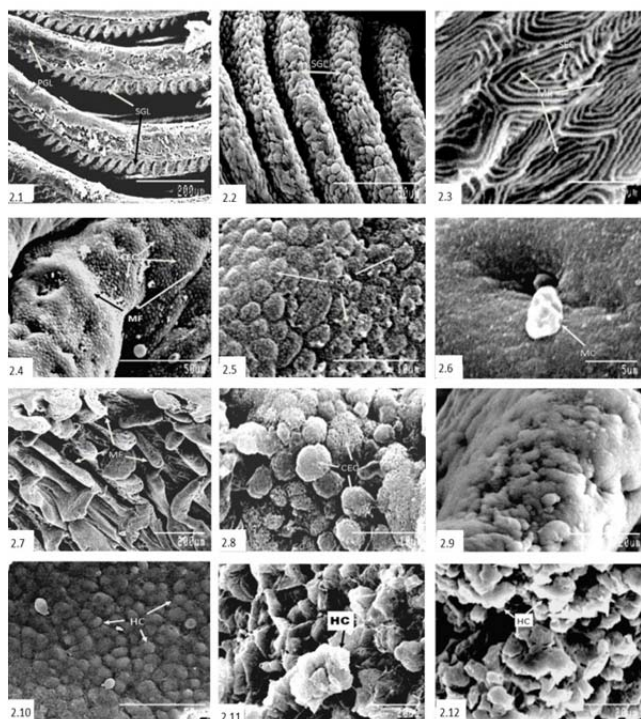
Figure 2: (2.1-2.12) Scanning electron micrograph of gill, stomach, intestine and liver of *Channa punctatus* under control condition (C), arsenic treated laboratory condition [low dose (T2) and high dose (T1)] (2.1) Showing normal arrangement of primary gill lamellae (PGL) and PGL (C × 200). (2.2) Showing degeneration in SGL (T2 ×600). (2.3) Showing loss of microridges (T1 × 6000). (2.4) Showing mucosal folds and columnar epithelial cell in normal structure. (C × 600) (2.5) CEC necroesd and shows sever damage. (T2× 3000). (2.6) Excessive secretion of mucus showed on the surface of lumen (T1× 6000). (2.7) Shows regularly packed oval or rounded CEC and dense microvilli (C× 200) (2.8) Shows fragmentation of mucosal folds (T2× 3000) (2.9) Severely damaged mucosal folds and excessive mucus secretion (T1×1500) (2.10) Regular arrangement of hepatocytes (C×600) (2.11) Showing hepatocytes and mucus (T2 × 200) (2.12) Hepatocytes are severely damaged hepatic cords are degenerated and excessive mucous secretion (T1×1500)

4. DISCUSSION

In the present study, histopathological and ultrastructural effects in gill, stomach, intestine and liver of freshwater air-breathing, fish, *C. punctatus* were investigated and compared between different doses after arsenic intoxication. The study aimed at assessing the suitability of histological and ultrastructural responses degrees of toxicity of arsenic contributing to environmental contamination with special reference to aquatic ecosystem. Cellular biomarkers including

histological and ultrastructural alterations represent an intermediate level of biological organization between lower-level biochemical effects and higher-level population effects [34-35], which mainly occur earlier than reproductive changes and are more sensitive for evaluation of organism health than a single biochemical response [36-37]. In this context, histopathological study through light microscopy is a rapid investigation method to detect the toxic effects of different xenobiotics, especially chronic ones, in various tissues and organs.

Fish gills are referred to perform the functions like respiration, osmoregulation, and excretion. The gills of freshwater fish are in direct contact with the water [38]. Respiratory distress is one of the early symptoms of toxicant poisoning. A high rate of absorption of arsenic through gills also makes the fish a vulnerable target of its toxicity. In this study, necrosis, lifting of the lamellar epithelium and fusion of the secondary lamellae were observed in the gills after exposure to As_2O_3. Similar kind of toxicity effects were noticed earlier in fish exposed to other toxicants including heavy metals [39]. The findings of the surface architecture of gill which was severely damaged with fusion and clumping of middle and distal part of primary lamellae; vacuoles or swelling and deterioration of the cells, inter lamellar space filled by mucosal cell and swelling of primary and secondary epithelial cells[40]. The defensive responses as observed were lifting up of the epithelium, hyperplasia and lamellar fusion. The lifting up of the epithelium increased the distance through which the toxicant has to travel to reach the blood stream. This appearance of the secondary lamellae results from the collapse of the pillar cell system and breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward [41]. Degenerative changes in gills of the *Cyprinus carpio* were also observed due to mercury and cadmium poisoning [42]. Injuries in gill tissues may reduce the oxygen consumption and disruption of the osmoregulatory functions of the fish.

Arsenic penetrates into the gastrointestinal tract though ingestion of food as well as through gill producing toxic effects on different regions of alimentary canal like stomach and intestine *etc*. Cadmium after penetration undergoes accumulation in the gastrointestinal canal and then in kidney [43]. Arsenic can also undergo accumulation in the gastrointestinal tract [20]. Here, lesions in the stomach and intestine were not uniform at different doses, but secretion of mucus in excessive reflects the first hand protection of the mucosal layers from toxicity [44]. Intestinal mucosal surface is mainly responsible for digestion and absorption that supports the findings of densely compact microvilli on the apical surface of the absorptive columnar epithelial cells, but during toxicosis of arsenic the damaged of microvilli may diminish the retention and absorption of nutrient as well as transit the food stuffs. In stomach, the gastric epithelium was seriously affected at high dose which means that the arsenic caused the reduction of ability of protection and cell lysis.

Cellular necrosis, disorientation, and vacuolization were also the marked features of arsenic toxicity at different doses on *Channa punctatus* [30]. Concomitant changes of the columnar epithelial cells, erosion of top plate, damages in lamina propria, and submucosa reflect the different degrees of toxic effects due to different doses. This may cause indigestion or under-digestion of the food stuffs and final absorption of the nutrients through the brush border present in the apical surface of the epithelial cells [32].

The teleostean liver is one of the most sensitive organs with regard to showing alterations in histo-architecture, biochemistry, and physiology following the exposure to various types of environmental pollutants. Liver cord disarray, shrinkage in the liver cells, degenerated nuclei and focal necrosis in *Channa punctatus* due to lead intoxication [45]. The vacuolation within and outside the hepatocytes, severe necrotic changes in liver, breakdown of cellular boundary, vacuolation in liver of *Puntius conchonius* induced by copper and zinc intoxication [46]. Necrosis, hypertrophy and atrophy in the liver tissues, loss of polygonal shape of liver cells, splitting of the cells and formation of spaces in the tissues after exposure of *Cyprinus carpio* to lethal and sublethal concentrations of copper and cadmium [47]. Arsenic-induced liver injury in teleosts has been reported earlier [48, 20] but microanatomical changes have not been documented. During the present investigation intense degenerative changes in the hepatopancreas of *C. punctatus* were found in 30 day exposure to the sublethal levels of Arsenic. The degenerative changes were characterized by vacuolation and apoptosis of the hepatocyte, pycnosis in many of the cells, necrosis of the pancreatic tissue, and disintegration of blood sinusoids.

5. CONCLUSION

The present study revealed that the heavy metal, arsenic is toxic to fish and causes histopathological and ultrastructural changes in gill, stomach, intestine and liver under laboratory condition. The results have clearly been able to establish the degrees of toxicity at different doses which have mentioned the dose- response relationship. In further study the marked differences in alterations may be used as biomarkers in evaluating the toxicological responses.

6. ACKNOWLEDGEMENTS

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