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The Modulation of Oxidative Stress Biomarkers in Assessing Arsenic Induced Toxicity in *Channa punctatus*

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Abstract *Channa punctatus* is a common fresh water fish in India and regularly consumed because of its high nutritional value. Heavy metals are common pollutants of the aquatic environment because of their persistent and tendency to concentrate in aquatic organisms. This freshwater fish is continuously exposed to arsenic toxicity as this metalloid enters the body through gills and arsenic contaminated food. Fresh water murrel, *C. punctatus* were exposed to different sub-lethal concentrations of sodium arsenite for varied span of time in controlled laboratory condition to assess the impact of metalloid toxicity on marker enzymes in gill. Arsenic-induced stress can specifically achieved in fish through elevated level of reactive oxygen species which is responsible for biochemical, cell metabolism and physiological activities. Arsenic induced changes in Acid phosphatase and Alkaline phosphatase activity in gill of freshwater murrel after one week of exposure. Results revealed reduction of Superoxide dismutase and Glutathione-S-transferase activity after 7 days of exposure in sub lethal concentration of sodium arsenite in gill. Result revealed that enzyme assays determination is relevant tool to monitor stress in freshwater ecosystem. The present study is also indicative of immune alteration in *C. punctatus* that may lead to decline population size in its natural habitat.

Keywords: *Arsenic, Channa punctatus, SOA, ACP, ALP, SOD, GST*

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1. Introduction

Channa punctatus is one of the most important fish species of Indian flood plains [1] and has a great demand in market because of its high nutritional value. Heavy metals are common pollutants of the aquatic environment because of their persistent and tendency to concentrate in aquatic organisms [2]. Arsenic, a sulphydryl reactive metalloid is one of the most important and concerned global environment toxicant widely spread in the aquatic environment as a result of both geogenic processes and anthropogenic disturbances [3]. Due to adverse effects on human health, the contamination of aquatic ecosystems with arsenic (As) has been receiving worldwide attention. In aquatic environments several species of microorganism make arsenic biologically available to organisms including fish [4]. Fish appear to be particularly susceptible to arsenic toxicity as they are continually exposed to it through gills and intake of arsenic contaminated food [5]. Enzymes are biochemical macromolecules that control metabolic process of organisms, thus a slight variation in enzyme activities would affect the organism by disturbing its metabolism [6]. Acid phosphatase and Alkaline phosphatase was estimated as a representative of metabolic modulation at the backdrop of metalloid toxicity. Several reactive oxygen species occur as a result of normal oxygen metabolism, but can be produced in large quantities during toxicant-induced interactions, leading to oxidative stress. The extent, to which such biological damage occurs, depends on the effectiveness of antioxidant defenses and detoxification mechanism to remove reactive oxygen species [3]. Thus, oxyradical production ultimately poses a threat to the fitness and health of organisms. In this present study, superoxide anion radical was estimated as a representative of reactive oxygen species. Superoxide dismutase and Glutathione-S-transferase represent the main enzymatic defenses against reactive oxygen species. For the study of xenobiotic induced impairment in relation to biochemical adaptive response, activities of metabolic enzymes like Acid phosphatase and Alkaline phosphatase, antioxidant enzymes like Superoxide dismutase and detoxification enzyme like Glutathione-S-transferase was quantified in gill of *C. pumctatus*. The present study would provide information of arsenic induced oxidative stress and will establish the selected parameters as biomarker of the health of the larger population and community.

2. Materials and Methods

2.1. Collection and Acclimatization of Animal

The small size freshwater fish, *C. punctutas*, weighting 15±2 gram and measuring 11±2 cm were collected with the help of local fisher man from water bodies located in the sub region of Coochbehar district of West Bengal, India. The fish was properly washed in tap water and treated with 0.02% $KMnO_4$ and 0.004% formalin solution to remove external infection of algae, fungi etc. Fishes were separately maintained at temperature ranging between 14ºC-30ºC in aquarium of 20 liter capacity with continuously aerated and dechlorinated tapwater (pH 7.2-7.4; hardness 185-200 mg/l as $CaCO_3$; alkalinity 170-175 mg/l as $CaCO_3$) for 15 days before taken for experimentation. The animals were fed with boiled eggs and earthworms [6].Water was renewed periodically so as to maintain the dissolved oxygen. The specimens were devoid of feeding prior to the test period to reduce the quantum of excretory products in the aquarium to avoid vomiting of the fish.

2.2. Determination of LC_{50}

Prior to treatment, LC_{50} value of sodium arsenite for *C. punctatus* was calculated following the method of [7]. During determination of the median lethal concentration (LC_{50}) of sodium arsenite to *C. punctatus*, the fishes were divided into five equal groups consisting of 10 each and each group was transferred separately to glass aquaria of 20 liter volume. The groups I fish were maintained as control without any treatment, the group II, III, IV and V fishes were exposed to varied concentrations of sodium arsenite for four days to determine the median lethal concentration (LC_{50}) for selection of sublethal dose.

2.3. Treatment of Animal

The experiment was conducted in a static system in glass aquaria of 10 litre capacity. The acclimatized fishes were grouped into four experimental groups each consisting of five fishes. The experimental groups were categorized based on the LC_{50} value and from the reports of highest level of arsenic contamination of natural freshwater bodies.

Group1: Fish subjected to zero arsenic level (control).
Group2: Fish subjected to 3.2 mg/L of sodium arsenite.
Group3: Fish subjected to 2.4 mg/L of sodium arsenite.
Group4: Fish subjected to 1.8 mg/L of sodium arsenite.

The fish were exposed to sublethal concentrations of arsenic for 2, 4 and 7 days. Tissue like gill was isolated from control and arsenic exposed fish for study of enzyme profile.

2.4. Biochemical Parameters in Gill

2.4.1. Superoxide Anion (SOA)

Superoxide anion productions by gill were determined by a modified method of [8] following the principle of NBT reduction. 1ml of freshly collected gill suspension ($1x10^6$cells/ml) was taken in a test tube and allowed to react with 1ml of NBT solution (0. 03%) for 30 mins at 37°C. The reaction was terminated by removing the NBT solution and addition of absolute methanol. After proper washings with 70% methanol, the cells were treated with a solution of KOH (1ml, 2M) and DMSO (1ml) to dissolve the cytoplasmic formazan. The optical density of the dissolved formazan was estimated spectrophotometerically at 630 nm. The Superoxide anion generation was expressed as absorbance (O.D.) at 630 nm/min/10^6 cells.

2.4.2. Preparation of Lysate of Gill

C. punctatus were exposed in batches to sodium arsenite for various span of exposure (refer to section 2.3.) in static water environment. Gills of *C. punctatus* was dissected in cold sterile saline and tissue of uniform weight (100 mg) was homogenized in 0.25 M sucrose (Himedia, India) solution (3%w/v) in mechanical glass homogenizer at 4°C and was centrifuged at 100x g for 15 minutes at cold [9]. Estimation of protein and enzyme were carried out from supernatant.

2.4.3. Acid Phosphatase (ACP)

Acid phosphatase activity was measured after [10]. The principle involved the hydrolysis of p-nitrophenyl phosphate at 37°C to produce p-nitrophenol which was recorded spectrophotometrically at 420 nm. Tissue lysate of 0.1 ml, 0.5 ml of PNPP (5.5 mM) and 0.5 ml of citric acid buffer (0.1 M) were pipetted into a test tube, mixed well and incubated at 37°C for 30 minutes. After incubation, reaction was terminated by adding 8.9 ml of 0.05 (N) NaOH. Optical density was determined spectrophotometrically at 420 nm against blank using a standard curve prepared with p-nitrophenol. Protein was estimated after the method of [11] using bovine serum albumin as standard. The enzyme activity was expressed as µM of p-nitrophenol generated / mg protein/ min.

2.4.4. Alkaline Phosphatase (ALP)

Alkaline phosphatase activity was estimated by the hydrolysis of p-nitrophenyl phosphate at pH 10.8 using glycine-NaOH buffer at 37°C following the method of [10]. The product of hydrolysis was recorded spectrophotometrically at 420 nm. Tissue lysate of 0.1 ml, 0.5 ml of PNPP (5.5 mM) and 0.5 ml glycine- sodium hydroxide buffer (pH 10.8) were pipetted into a test tube and incubated at 37°C for 15 minutes. After incubation, the reaction was stopped by adding 8.9 ml of 0.05(N) sodium hydroxide. Optical density was determined spectrophometrically at 420 nm against a blank using the standard curve prepared with p-nitrophenol. Protein was estimated after the method of [11] using bovine serum albumin as standard. The enzyme activity was expressed as µM of p-nitrophenol generated / mg protein/ min.

2.4.5. Superoxide Dismutase (SOD)

Activity of super oxide dismutase was determined in tissue lysate following the method of [12]. Assay involved reaction of cell suspension with Griess reagent followed by recording of absorbance at 560 nm by spectrophotometer. One unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50% of nitrite

formation under assay condition. Activity of superoxide dismutase was expressed as unit of SOD/ mg of protein / min.

2.4.6. Glutathione-S-transferase (GST)

Glutathione-S transferase activity was estimated in the lysates of gill following the method of [13]. One unit of GST refers to the enzyme amount needed to conjugate 1µM of 1 chloro 2, 4dinitrobenzene (cDNB) per min, per mg of total protein present in the homogenate. The reaction medium consisted of 50 µl of GSH (5mM), 50 µl of tissue lysate and 890 µl of potassium phosphate buffer (0.1M) which was incubated for 2 minutes at room temperature. The reaction was started with addition of 10µl of cDNB and was monitored spectrophotometrically at 340 nm for 300 seconds with an interval of 60 seconds. Protein was estimated after the method of [11] using bovine serum albumin as standard. One unit of GST activity is defined as the amount of enzyme producing 1µM of GS-cDNB conjugate. The enzyme activity was expressed as µM GST/ mg protein/ min.

3. Results

3.1. Quantitative NBT Reduction

The control set showed an average activity of 0.04 O.D./10^6 cells/mL. Arsenic of 3.6 mg/L exposure for 7 days showed a maximum elevation of activity of superoxide anion as 0.99 O.D./10^6 cells/mL (Figure 1). Superoxide anion production expresses a dose dependent response. Superoxide anion production was higher in *C. punctatus* exposed to 3.6 mg/L exposure of arsenic compared to control for all span of exposure (Figure 1).

3.2. Acid Phosphatase (ACP)

The activities of acid phosphatase (ACPase) were estimated in gill of *C. punctatus* (Figure 2). The acid phosphatase activities were significantly decreased in gill under the exposure of arsenic *in vivo* (Figure 2). The degree of inhibition is determined to be a dose dependent one and considerable decrement occurred even in the low concentration of metalloid. Maximum decrement in the activity of acid phosphatase in gill was observed in animals exposed to 3.6 mg/L sodium arsenite/7 days as compared to control (Figure 2). A significant decrement in the activity of acid phosphatase was observed in gill after treatment of 2.4 mg/L and 3.6 mg/L of arsenic for 7 days (Figure 2).

3.3. Alkaline Phosphatase (ALP)

The activity levels of alkaline phosphatase (ALPase) were estimated in gill of *C. punctatus*. The alkaline phosphatase activities were significantly inhibited in gill of *C. punctatus* exposed to 3.6 mg/L and 2.4 mg/L of arsenic for 7 days *in vivo* (Figure 3). A maximum inhibition in alkaline phosphatase activity was observed in the gill of *C. punctatus* treated with 3.6 mg/L arsenic for 7 days in comparison to control (Figure 3).

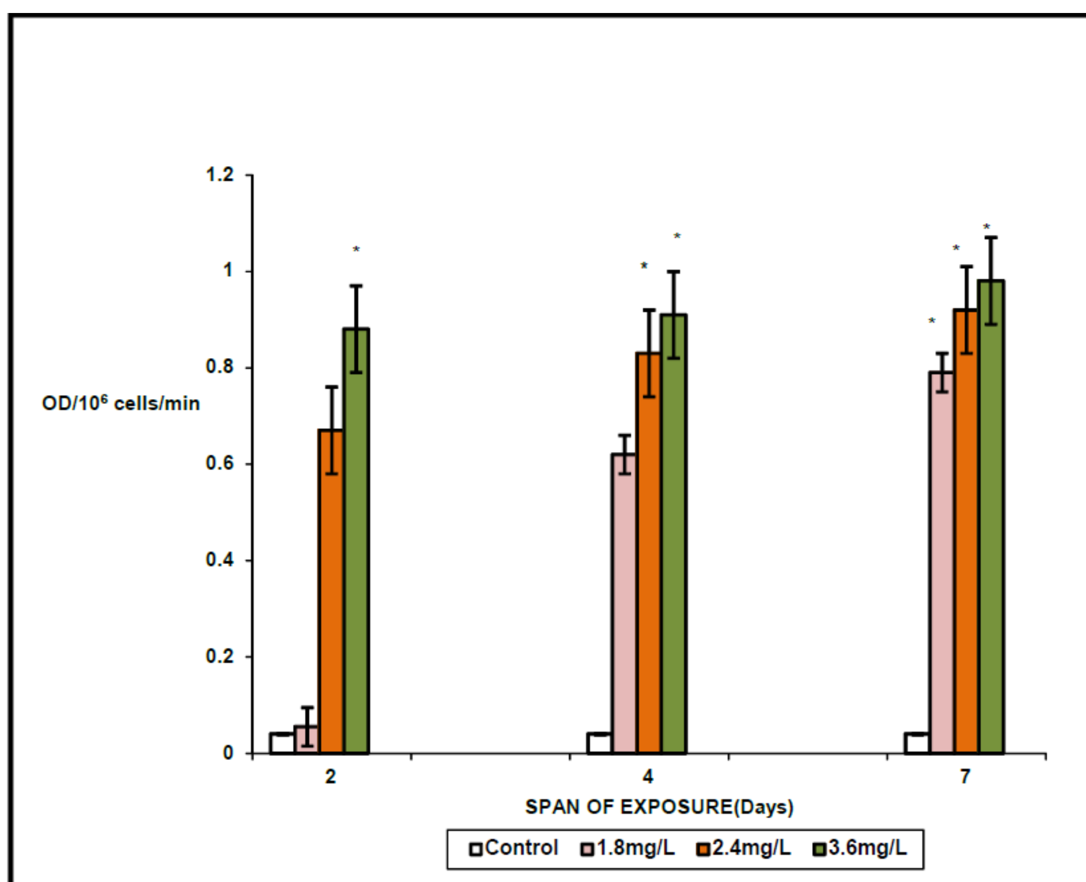

Figure 1. Generation of superoxide anion in gill of *C. punctatus* exposed to sodium arsenite *in vivo*. Data is represented as Mean± S.D. Statistical significance is shown at P<0.05 (n=5).

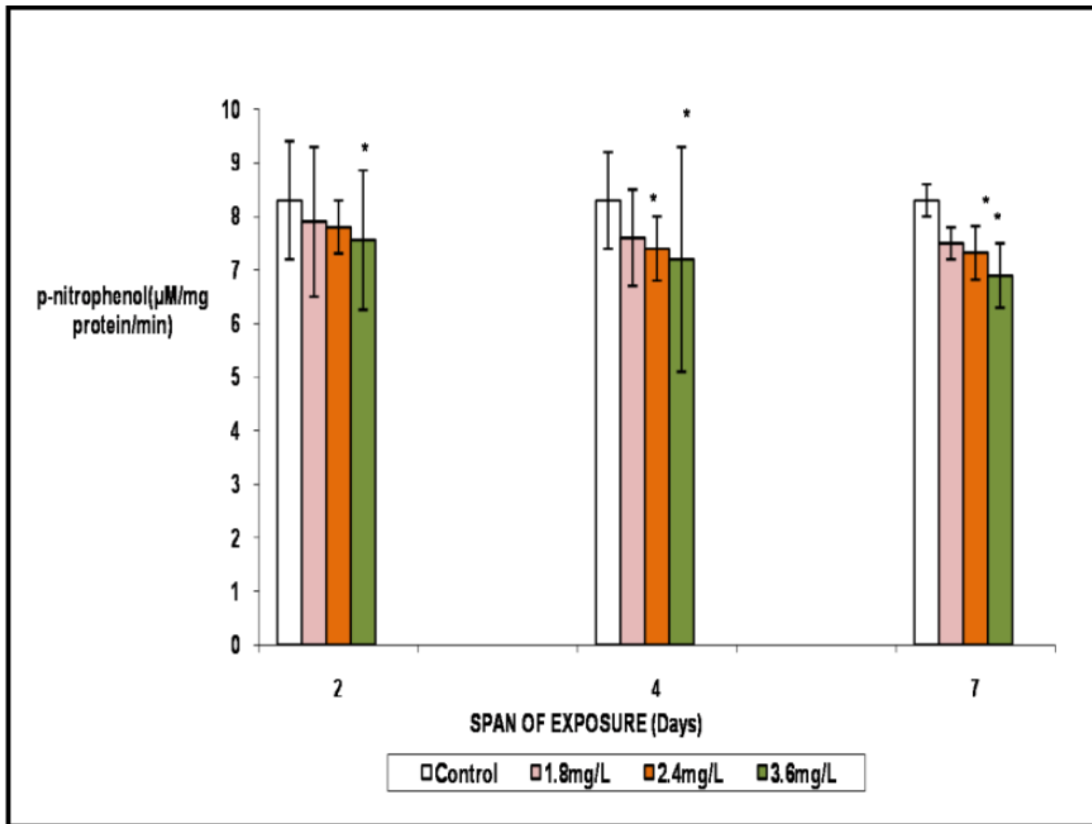

Figure 2. Activity of acid phosphatase of gill of *C. punctatus* exposed to sodium arsenite *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at P<0.05 (n=5).

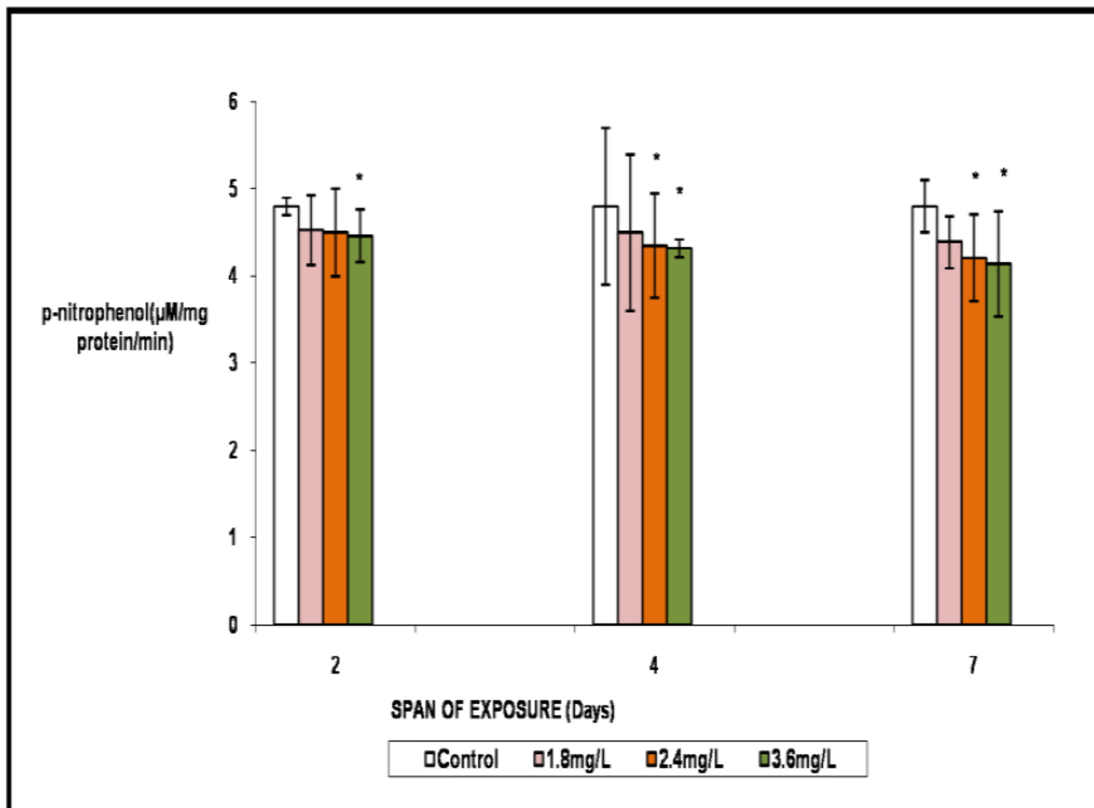

Figure 3. Activity of alkaline phosphatase of gill of *C. punctatus* exposed to sodium arsenite *in vivo*. Data is represented as Mean± S.D. Statistical significance is shown at P<0.05 (n=5).

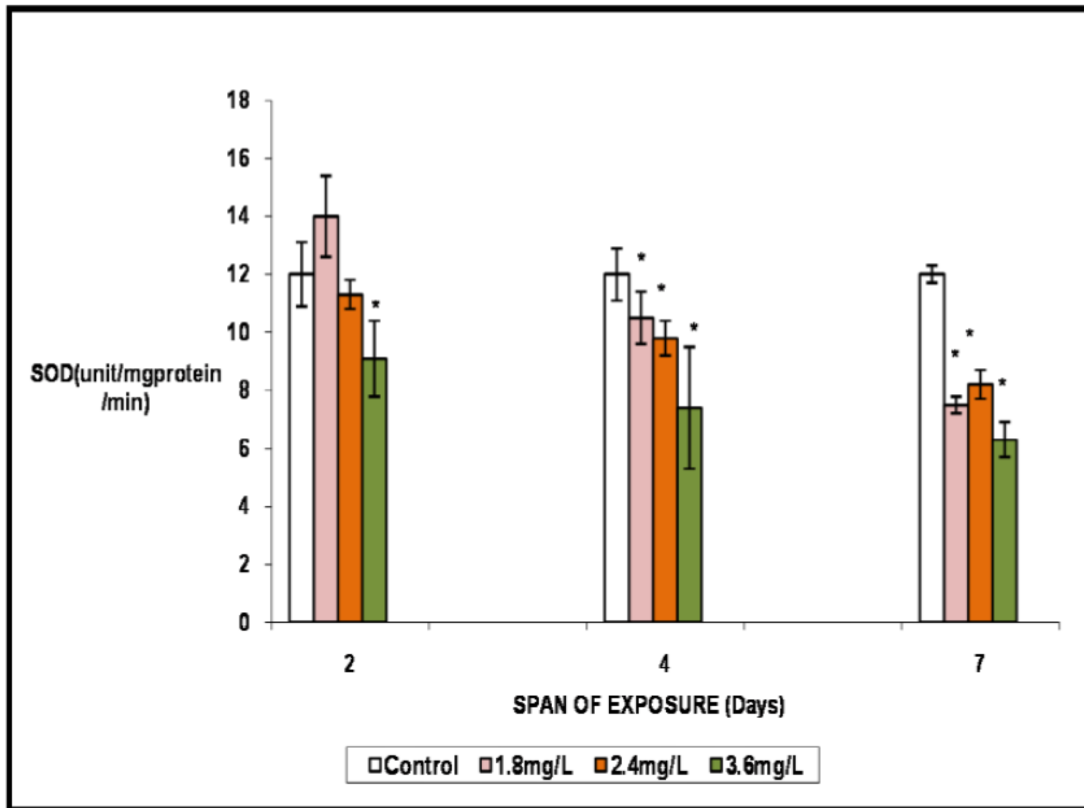

Figure 4. Activity of superoxide dismutase of gill of *C. punctatus* exposed to sodium arsenite *in vivo*. Data is represented as Mean± S.D. Statistical significance is shown at P<0.05 (n=5).

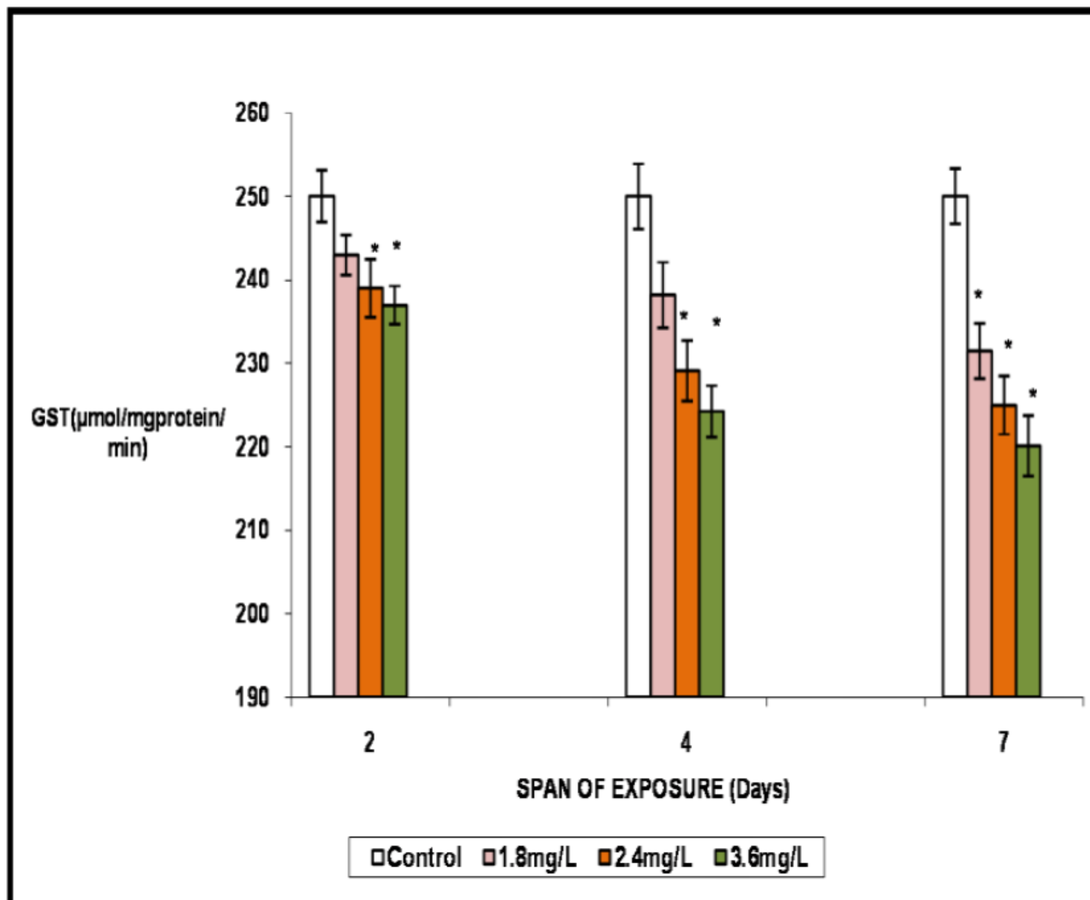

Figure 5. Activity of GST of gill of *C. punctatus* exposed to sodium arsenite *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at P<0.05 (n=5).

3.4. Superoxide Dismutase (SOD)

Antioxidant enzymes are crucial in the effort to counteract oxidative stress caused by toxicants once the supply of other antioxidant compounds is depleted [2]. Most reactive oxygen species (ROS) are generated as superoxide anion ($O_2^{\cdot-}$) and are rapidly dismutated by the action of superoxide dismutase to hydrogen peroxide and oxygen. In this study, generation of superoxide dismutase was estimated in gill in control and treated *C. punctatus*. The activity of antioxidant enzyme namely superoxide dismutase is higher in gills in control set (Figure 4).

3.5. Glutathione-S-transferase (GST)

Glutathione-S-transferase is involved in the detoxification reaction process of various xenobiotic chemicals. In this study, a decrement in GST activity in gill and liver of *C. punctatus* was recorded in arsenic exposure. In tissues of control animal, the activity of GST was higher in gill compared to treat one (Figure 5). The degree of inhibition of GST in the tissue expressed a dose dependent response and considerable inhibition occurred at highest concentrations of arsenic exposure (Figure 5).

4. Discussion

The aquatic environment is continuously being contaminated with chemicals from agriculture and urban activities. Fish constitute a valuable dietary item for human consumption and aquatic pollution affects health and survival status of the organism. In many aquatic systems, metal concentrations are elevated over natural background levels due to a continuous release of metals from industrial and agricultural sources [14]. Arsenic levels are higher in the aquatic environment than in most areas of land as it is fairly water soluble and may be washed out of arsenic bearing rocks [15]. Recently, the anthropogenic activities such as treatment\ of agricultural land with arsenical pesticides, treating of wood using chromated copper arsenate, burning of coal in thermal plants power stations and the operations of gold-mining have increased the environmental pervasiveness of arsenic and its rate of discharge into freshwater habitat [4]. Superoxide anion (SOA) is regarded as cytotoxic agents reported both in invertebrate and vertebrate [16]. A significant alteration in generation of superoxide anion (SOA) in gill of *C. punctatus* were recorded against the exposure of 1.8 mg/L, 2.4 mg/L and 3.6 mg/L of sodium arsenite for 2, 4 and 7days (Figure 1). Pattern of alteration in the generation of superoxide anion (SOA) appear to be a dose independent and indicative to a state of a toxin induced cellular stress. The cell release cytotoxic substances for elimination of phagocytised materials during adverse physicochemical environmental conditions [17]. This result is suggestive to a possible state of immunological alteration due to arsenic induced immunological stress in the gill function of *C. punctatus*. In the present study, the significant decrease in acid phosphatase (ACP) and alkaline phosphatase (ALP) activity in arsenic exposed fish gill with short term sublethal exposure (Figure 2 and Figure 3) indicates gill damage due to arsenic accumulation. In the present study, the decline in activity of gill acid phosphatase (ACP) and alkaline phosphatase (ALP) in *C. punctatus* reflects the arsenic induced toxicity which may be responsible for impairment of phosphorylation or changes in permeability and disruption of lysosomes and mitochondria. Superoxide dismutase (SOD) activities were decreased in gill under toxin exposure (Figure 4). The present data demonstrate the sensitivity of antioxidant enzymes of *C. punctatus* under the exposure of sodium arsenite. Arsenic induced decrement of antioxidant enzyme is indicative to an onset of physiological stress in the specimen distributed in water bodies contaminated with metalloid toxicity. Glutathione-S- transferase (GST) which catalyzes the conjugation of reduced glutathione with nucleophilic xenobiotic cellular components damaged by oxyradical attack in their detoxification reaction [18]. Glutathione S-transferase (GST) is involved in the detoxification of various xenobiotic chemicals [13]. Arsenic moderately suppressed the activity of GST in selected tissue of *C. punctatus* (Figure 5) which is indicative of possible impairment of detoxification reaction as evident from the studies of [19].

5. Conclusion

The findings of the present study reflect that arsenic exposure of *C. punctatus* affect its biochemical profile in gill. These parameters would be effectively used as potential biomarker of arsenic toxicity to the freshwater fish in the field of environmental biomonitoring. The measurement of biochemical responses of individual fish in respect to xenobiotic toxicity indicates the health of the larger population and community, contributing a lot in the field of biomarker research.

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