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Arsenic Induced Oxidative Perturbations in Freshwater Air Breathing Fish *Clarias batrachus*: *In Vivo* Study

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Abstract

Arsenic is ubiquitously prevalent metalloid contaminant released in the aquatic environment as a consequence of geogenic and anthropogenic processes. Bihar is emerging as hot spot of Arsenic toxicity in India and needs immediate environmental concern. In the present study, freshwater walking catfish *Clarias batrachus* (Linnaeus,1758)) was used as an experimental model to investigate oxidative stress related enzyme activities in serum. Fish were procured from Phulwarisharif Fish farm, Patna, Bihar, India, acclimated in the laboratory under ideal physiochemical condition for 15 days, and fed *ad libitum*. LC_{50} of fish for Arsenic trioxide was determined by probit regression analysis and confirmed by pilot test. The fish were exposed to 3.66 mg/L, 5.5 mg/L and 11.0 mg/L of arsenic trioxide for one, two and four week respectively. After schedule exposure, the fish serum of different groups was analysed for lipid peroxidation (LPO), superoxide dismutase (SOD), Catalase (CAT) and reduced Glutathione (GSH). Significant increase in the level of LPO was observed at all levels of arsenic exposure. A dose dependent notable decrease was observed in the activities of SOD, GSH and CAT. Statistical analysis showed a significant positive correlation between elevated levels of LPO and reduced GSH, SOD and CAT of the serum. The results of the present study indicate that sub-acute (two weeks) or chronic exposure (four weeks) of even a sub-lethal dose of arsenic trioxide induces significant oxidative stress in freshwater fishes. The assessment of oxidative parameters may be used as a biomarker for the health status of fish as well as aquatic body.

Keywords: Arsenic trioxide, oxidative stress, *Clarias batrachus*, Lipid peroxidation (LPO) superoxide dismutase (SOD), Catalase (CAT), Reduced Glutathione (GSH).

1. Introduction

Freshwater ecosystem has been subjected to immense toxicants since last two decades with the advent of industrial technology and non-judicious extensive use of different synthetic chemicals like fertilizers, pesticides, insecticides, herbicides *etc.* and generated potential threat for the survival of the aquatic life (Mandour *et al*., 2012; Mashkoor *et al*., 2013; Ghaffar *et al*., 2014; Witeska *et al*., 2014). It serves as major vector for pollutant dispersion acting as sink for environmental contaminants (Amundsen *et al.*, 2011). Undesirable changes are induced in the aquatic biota by these contaminants and ultimately affect the ecological balance (Witeska *et al*., 2014).

Heavy metals and metalloids are a consistent source of pollution of natural aquatic bodies. They contaminate fish via their general body surface, gills or digestive tract and induce disturbances in various physiological and biochemical mechanisms of fish, including alteration in carbohydrate, protein and fat metabolism, reducing cellular adaptive immunity and inducing histopathological anomalies in their vital organs (Abdel-Rahman *et al*., 2011; Mandour *et al*., 2012). Fishes are the best sentinels for determining the health status of an aquatic ecosystem as well as its potential impact on human health. Identification, monitoring and management of these pollutants are crucial in order to minimize their adverse effect on aquatic ecosystem.

Arsenic is a ubiquitous metalloid "pnictides" located on group V of the periodic table (Smith, 1973). It is introduced in the environment from both natural and anthropogenic sources (Abdel Hameid, 2009; Baldissarelli *et al.*, 2012; Rahman and Hasegawa, 2012; Aruljothi *et al*., 2013). Natural resources of Arsenic include withering of rock, leaching, run off, volcanic and biological activities (Rehman and Hasegawa, 2012; Zhang *et al*., 2014). Anthropogenic sources include- extensive mining and geothermal activities, use of metallic arsenic for strengthening alloys as well as in the processing of glass, pigments, textiles, parts, metal adhesive wood preservatives and ammunition (Thompson *et al*., 2007). According to USEPA aquatic life criteria, the acceptable limit of total arsenic concentration is 340 µg/L for acute exposure and 150 µg/L for chronic exposure in freshwater. However, 1.5 – 3.8 mg As^{3+}/L of water has been considered safe for fish (NAS, 1977). The concentration of arsenic for effective weed control (13.6 mg/L) has been reported to be harmful to several fish species (NRCC, 1978; Sorensen *et al*., 1985).

Air breathing fishes can concentrate and metabolize water borne pollutants and heavy metals effectively via antioxidant defense system, thereby considered as an excellent subject for the study of various effects of contaminants present in water sample (Valco *et al.*,2005).

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They have been utilized as biomarker to indicate the existence of toxicant exposure and/or the impact towards the evaluation of molecules cellular to physiological level (Sabullah *et al.*, 2015). Since fishes occupy the apex of the aquatic food chain, they have been considered as suitable bio-indicator of heavy metal contamination. Arsenic is known to induce oxidative stress in different organism. It is linked with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) free radicals viz. hydrogen peroxide (H_2O_2) (Wang *et al*., 1996; Chen *et al*., 1998); hydroxyl radical (OH) species (Wang *et al*., 1996), nitric oxide and super oxide anion (O_2) (Barchowsky *et al*., 1999; Lynn *et al*., 2000), Dimethyl arsenic peroxyl radical ($[CH_3]_2ASOO^-$]) (Yamanaka *et al*., 1991, 1997 & 2001) and dimethyl arsenic radical.

The present research work has been designed to investigate the oxidative biomarkers in the blood sample of freshwater walking catfish *Clarias batrachus* (Linn.) exposed at sub-lethal level of arsenic through LPO induction, and assessment of glutathione dependent antioxidant defense enzymes.

2. Materials and Methods

2.1. Test Chemicals

Arsenic trioxide (AS_2O_3) CAS No.13629 was purchased from Nice Chemicals Private Limited, Cochin. Its commercial grade formulation was white, powdery and insoluble in water. Reduced Glutathione (GSH), L chloro, 2-4 dinitrobenzene (DNB), Hydrogen peroxide (H_2O_2), 5-5'dithio-bis-2 nitrobenzoic acid (DTNB) and Trichloro acetic Acid (TCA) were purchased from Sisco Research Laboratory, Mumbai, while lauryl sulphate, Glacial acetic acid, Thiobarbituric acid (TBA), Pyrogallol were purchased from Hi Media Laboratories Private Ltd., Mumbai, India.

2.2. Test animal

Freshwater walking catfish *Clarias batrachus Linn*. (Siluriformes: Clariidae), commonly known as 'Magur,' was selected as test animal. It is hardy and restraining to most of the adverse ecological condition and mild xenobiotic exposure. It has a fast growth rate & prolific breeding ability. A very high content of protein (20.80 gm/100 gm tissues) in its flesh (Singh *et al*.,2016) and high percentage of haemoglobin in its blood (Maheshwaran *et al*.,2008) have raised its nutritional and therapeutic value. It inhibits all forms of swampy, marshy and derelict water. Nearly 380 Fresh healthy *Clarias batrachus (Linn)* with almost same age group and relatedness of mean body length 18±2 cm and mean body weight 74±6 gm were procured from Phulwarisharif Fish farm, Patna, Bihar, India.

2.3. Fish Care & Maintenance

After procurement fishes were brought to the Aquatic Toxicology laboratory, Department of Zoology, Patna University, Patna, Bihar and were first disinfected with 0.1% $KMnO_4$ solution bath for 20 minutes. The fishes were then maintained in nineteen plexiglass aquaria of 76.2cm x 45.72 cm x 45.72 cm dimension with 30L of de-chlorinated bore-well water @ 20 fishes/aquarium. Any disease or wounded fish, showing abnormal behaviour were removed from the aquaria immediately. The fishes were acclimatized in the laboratory under normal physico-chemical parameter i.e. room temp 27-30^{o}c, pH 7.2-7.5 and dissolved O_2 content 6.0-6.5mg/l as per the protocol followed by APHA(2012). The optimal condition during acclimatization was maintained (Benette and Dooley, 1982). The fishes were fed alternately with minced pellets of suji & egg and goat liver once daily @3-4% of body weight. The water was changed periodically.

2.4. Determination of sub lethal concentration

The 48 hours and 96 hours LC_{50} of *Clarias batrachus* for AS_2O_3 was determined according to graphical interpolation method (Doudoroff *et al*., 1951) and probit regression analysis (Finney, 1971). Later, it was confirmed by pilot test.

For the determination of median tolerance limit (LC_{50}), a group of 10 fishes was kept in six glass aquaria of 30 L capacity. A group of 10 fishes was kept in a separate aquarium and treated as control. After range finding test, six consecutive concentrations of AS_2O_3 *i.e.* 25 mg/l, 50mg/L, 75mg/L, 100mg/L, 125mg/L and 150mg/L respectively were added separately in each aquarium to determine the 48 hours and 96 hours LC_{50}.The test water was removed and fresh solution of AS_2O_3 was added daily to remove faecal wastes and to maintain the concentration of the test solution throughout the experimental duration under ideal condition. The median value was determined by plotting the experimental data on semi-logarithmic coordinate paper with test concentration on logarithmic scale and the percentage of survival on the arithmetic scale. A straight line was drawn between two points which are above or below the 50% survival line. The point of intersection with 50% survival line was considered LC_{50}.The experiment was done in triplicate. In the present study, the 48 hours LC_{50} and 96 hours $LC_{50}$of AS_2O_3 for *Clarias batrachus* was found at 130mg/L and 110mg/L respectively. Nearly similar value of LC_{50} was also calculated by probit regression analysis using SPSS software.

2.5. Experimental design

In the present study, fishes were grouped into four-(i) Control group (without AS_2O_3 exposure), (ii) SL-I group (Fishes treated with 3.66mg/L of AS_2O_3 i.e. 1/30th of LC_{50}), (iii) SL-II group (fishes treated with 5.5mg/L i.e. 1/20th of LC_{50}) and (iv) SL-III (fishes treated with 11 mg/L of AS_2O_3 i. e. 1/ 10th of LC $_{50}$).The stock solution for considered doses was prepared in dilute acidic water (stock solution A- 3.36 mg/L of AS_2O_3 was dissolved in 996.33 ml of distilled water; stock solution B-5.5 mg/L of AS_2O_3 was dissolved in 994.50 ml of distilled water; stock solution C-11 mg/L of AS_2O_3 was dissolved in 989 ml of distilled water).

A batch of 90 healthy fishes was placed in nine separate plexi glass aquaria @ 10 fishes each, and exposed to three sub-lethal concentrations i. e. 3.66 mg/L, 5.5 mg/L and 11mg/L of AS_2O_3 for one, two and four week respectively. The toxicant solution in the text aquaria was replaced after interval of 24 hours with the fresh solution of the same concentration. Three parallel groups of 10 fishes each were also maintained in arsenic free water under similar physico-chemical condition and treated as control throughout the experimental tenure.

At the termination of schedule time exposure, both treated and control group of fishes were collected and

anaesthetized under MS222 @100 mg/L. In each case, blood was collected in a heparinize glass syringe by puncturing caudal vein, allowed to clot and then centrifuged @3000rpm for 20 minutes. Clear supernatant was collected in fresh RIA tube, labeled properly and stored at 4^0c for biochemical analysis. The entire experiment was done in triplicate at three points of time.

2.6. Biochemical estimation:

2.6.1. Lipid peroxidation (LPO):

The level of LPO in serum was estimated by the standard method of Ohkawa *et al*. (1979) with thio-barbituric acid (TBA) and colour reaction for malonaldehyde (MDA). 1 ml of test serum sample was taken with equal volume of 0.67%w/v TBA, vertoxed for two minutes and kept on boiling water bath for 20 minutes, cooled and diluted with 1ml of TDW. The pink colour absorbance was read against blank at 532 nm in Spectronic 20 Bausch & Lamb spectro-photometer. The concentration of MDA was read from a standard calibration curve plotted using 1, 1, 3, 3'tetra methoxy propane (Sigma Aldrich Co. St Louis. USA) as standard, and the results were expressed as µmole of MDA/ml.

2.6.2. Superoxide dismutase (SOD):

The estimation of SOD was done by standard method (Markland & Markland, 1994). The method utilized the inhibition of auto oxidation of pyrogallol by SOD. The results were expressed in Unit/ml. 1 unit of SOD was considered as the amount of enzyme required to cause 50% inhibition of pyrogallol auto oxidation /3 ml of assay mixture.

2.6.3. Catalase (CAT):

The catalase activity was measured as per standard method of Sinha (1972) and Giri *et al*. (1996). Dichromate is reduced to chromic acetate with the formation of per-chromic acid as an unstable intermediate complex when treated with H_2O_2 in the presence of acetic acid. The chromic acid is measured colorimetrically at 620 nm. CAT split H_2O_2 at different time intervals by the addition of a dichromatic acetic acid mixture and remaining H_2O_2 is determined colorimetrically. The results were expressed as µmole of H_2O_2 utilized/ml.

2.7. Reduced Glutathione (GSH):

The estimation of glutathione was done by standard method (Ellman, 1959 modified by Beutler *et al*., 1963). The sulfhydryl group of GSH reacted with Ellman reagent (5-5'dithio-bis-2 nitro-benzoic acid (DTNB) and produced a yellow coloured 5-thio, 2-Nitro benzoic acid (TNB) which was measured on spectro-photometer at 412 nm. The results obtained were expressed as glutathione mg/dl in blood.

2.8. Statistical analysis:

For the estimation of each oxidative parameter six (n=6) observations at random were taken and the arithmetic mean in each case was calculated and subjected to statistical analysis. The standard deviation, standard error of mean and one way standard analysis of variance (ANOVA) was applied to determine the significance difference between the different experimental mean. Paired 't'-test was applied between control and different arsenic treated groups. A value at $p<0.05$ and <0.01 was considered statistically significant. All the statistical analysis was done on SPSS 17.0 version.

3. Results

The present research work was undertaken to assess the arsenic induced oxidative stress and related anomalies in the serum of fresh water air breathing fish *Clarias batrachus (Linn.*).To address our hypothesis, the effect of AS_2O_3 on the four oxidative parameters i.e. LPO, SOD, CAT and GSH was studied at three different concentrations of AS_2O_3 for one, two and four week respectively. The inter-relationship of four different oxidative parameters and their fluctuations in response to arsenic exposure was significantly analyzed.

3.1. Effect of AS_2O_3 on serum LPO:

The data of serum LPO in all the four groups are presented in text table 1 and also illustrated with the help of logarithmic Figure 1A, 2A and 3A. It was observed that the level of serum LPO uniformly mounted in all three test groups at all exposure level.

Table 1. LPO (Mean ± SEM) of control and treated *C. batrachus* exposed to three sub-lethal concentrations of As_2O_3 for one week, two weeks & four weeks

Group	LPO level (µmole/ml) Period of Exposure		
	1 week (Mean±SEM)	2 week (Mean±SEM)	4 week (Mean±SEM)
Control	1.435±0.232	1.435±0. 232	1.435±0. 232
SL-I	1.920±0.185* (+33.79%)	2.363±0.340* (+64.67%)	2.266±0.228* (+57.90%)
SL-II	2.784±0.392* (+94.07%)	3.151±0.236** (+119.587%)	6.820±0.290** (+375.26%)
SL-III	2.848±0.47** (+98.47%)	2.546±0.06* (+77.42%)	4.500±0.228* (+213.58%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired't'-test was applied between control and different arsenic treated groups. Significant response: * $p<0.05$, ** $p<0.01$. One way 'ANOVA test' was done to observe overall variation in LPO. Calculated F-value is 33.341 while table F-value is 2.18. Values in Parenthesis are % increase over control.

3.2. Effect of AS_2O_3 on SOD

A significant decline in serum SOD was marked at higher duration of all the three exposure level. The data of serum SOD in all the four groups at all the level of exposure have been shown in table 2 and illustrated in figure 1B, 2B and 3B.

Table 2. SOD (Mean ± SEM) of control and treated *C. batrachus (Linn.)* exposed to three sub-lethal concentrations of As_2O_3 for one week, two weeks & four weeks respectively.

Group	Serum SOD level (unit/ml) Period of Exposure		
	1 week (Mean±SEM	2 week (Mean±SEM)	4 week (Mean±SEM)
Control	1.379±0.187	1.379±0.187	1.379±0.187
SL-I	1.242±0.090* (–9.93%)	1.387±0.060 (+0.58%)	0.942±0.16** (–31.83%)
SL-II	1.260±0.190* (–8.62%)	0.925±0.11** (–32.92%)	0.856±0.140* (-37.93%)
SL-III	1.280±0.233* (–7.17%)	0.903±0.15* (–34.51%)	0.548±0.023** (–60.26%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired't'-test was applied between control and different arsenic treated groups. Significant response: * $p<0.05$, ** $p<0.01$, NS= Non- significant. One way 'ANOVA test' was done to observe overall variation in serum SOD. Calculated F-value is 15.777 while table F-value is 2.18. Values in Parenthesis are % increase (+) or decrease (–) over control.

3.3. Effect of AS_2O_3 on CAT

Text table 3 represents the concentration of serum catalase in control and all the three experimental groups. Initially serum CAT showed non-significant induction, but in all other exposure level significant reduction were marked, as illustrated in figures 1C, 2C and 3C.

Table 3. CAT (Mean ± SEM) of control and treated *C. batrachus (Linn.)* exposed to three sub-lethal concentrations of As_2O_3 for one, two and four week respectively.

Group	Serum CAT level (µmole/ml) Period of Exposure		
	1 week (Mean±SEM)	2 weeks (Mean±SEM)	4 weeks (Mean±SEM)
Control	135.56±2.47	135.56±2.47	135.56±2.47
SL-I	140±2.60^{NS} (+3.27)	110.65±1.85* (–18.37%)	92.65±2.569** (–31.65)
SL-II	115.65±8.86* (–14.68%)	82.56±7.25** (–39.09%)	65.75±2.02** (–51.49%)
SL-III	86.25±9.55** (–36.375%)	55.92±7.86** (–58.75%)	28.50±5.2** (–78.97%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired‘t’-test was applied between control and different arsenic treated groups. Significant response: * p<0.05; ** p<0.01, NS= Non- significant. One way ‘ANOVA test’ was done to observe overall variation in serum CAT. Calculated F-value is 33.341while table F-value is 2.18. Values in Parenthesis represents percentage increase (+) or decrease (–) over control.

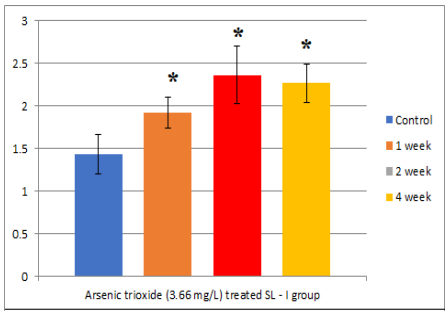
3.4. Effect of AS_2O_3 on GSH:

Text table 4 represents the concentration of blood GSH in control and all the three experimental groups. As it is clearly depicted in figures 1D, 2D and 3D, GSH, an important detoxifying enzyme, significantly decreased with increasing time of AS_2O_3 exposure against control.

Table 4. GSH (mg/dl) of control and treated *C.batrachus (Linn.)* exposed to three sub-lethal concentrations of As_2O_3 for one, two & four week respectively.

Group	Blood GSH level (mg/dl) Period of Exposure		
	1 week (Mean±SD)	2 weeks (Mean±SD)	4 weeks (Mean±SD)
Control	57.409±2.21	57.409±2.21	57.409±2.21
SL-I	38.201±1.236* (–33.45%)	35.449±3.81* (–38.25%)	33.992±2.24* (–40.78%)
SL-II	33.411±1.92* (–41.80%)	24.819±3.10** (–56.76%)	17.163±2.30** (–70.10%)
SL-III	31.337±1.960* (–45.41%)	20.373±1.416** (–64.51%)	13.502±2.11** (–76.48%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired‘t’-test was applied between control and different arsenic treated groups. Significant response: * p<0.05, ** p<0.01, NS= Non- significant. One way ‘ANOVA test’ was done to observe overall variation in blood GSH. Calculated F-value is 55.138 while table F-value is 2.18. Values in Parenthesis represented % decrease (–) in blood GSH over control.



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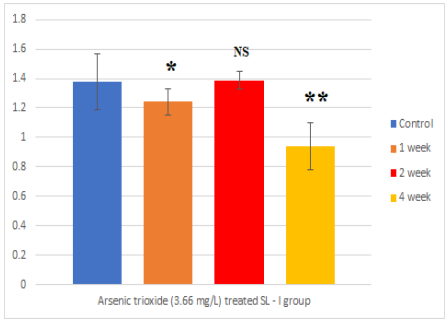

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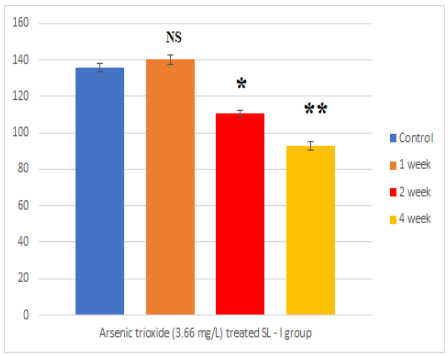

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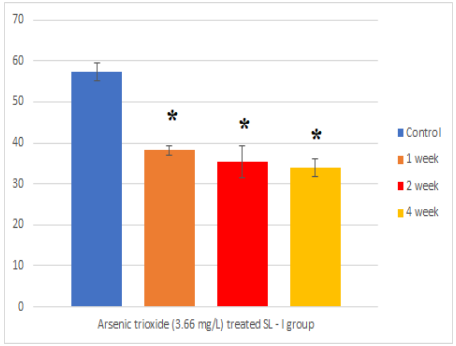
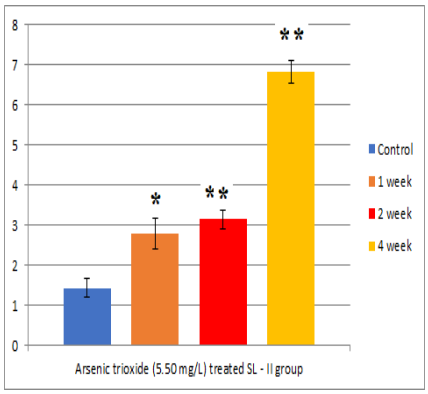
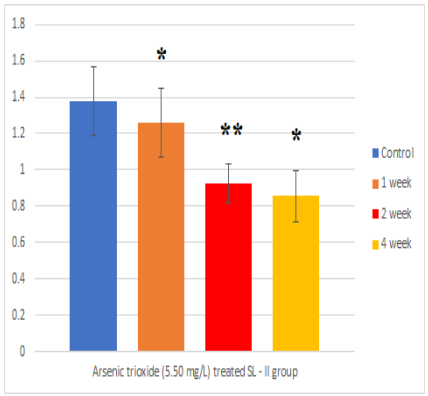


(D)

Figure 1. (SL – I group): Time dependent level of serum Lipid peroxidation-LPO (A), serum superoxide dismutase- SOD (B), Serum Catalase – CAT (C) and reduced Glutathione-GSH (D) in *Clarias batrachus* after exposure to 3.66 mg/L Arsenic trioxide. The results are expressed as mean ± SEM of six replicates (n = 6). * indicates significant differences at $p < 0.05$ and ** indicates significant difference at $p < 0.01$.



(A)



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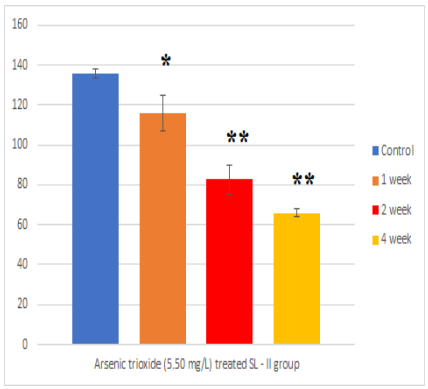

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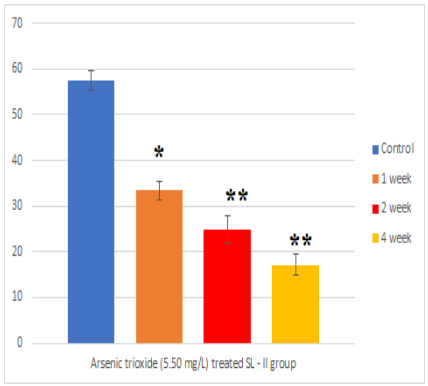
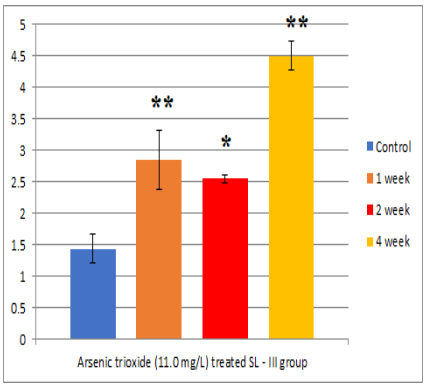
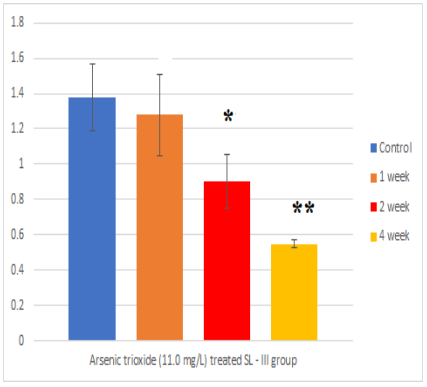


(D)

Figure 2. (SL – II group): Time dependent level of serum Lipid peroxidation-LPO (A), serum superoxide dismutase- SOD (B), Serum Catalase – CAT (C) and reduced Glutathione-GSH (D) in *Clarias batrachus* after exposure to 5.5 mg/L Arsenic trioxide. The results are expressed as mean ± SEM of six replicates (n = 6). * indicates significant differences at $p < 0.05$, and ** indicates significant difference at $p < 0.01$. NS = Not significant.



(A)



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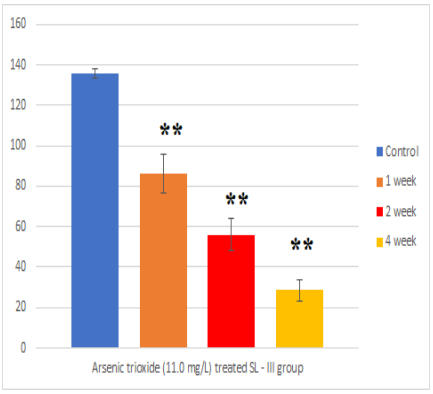

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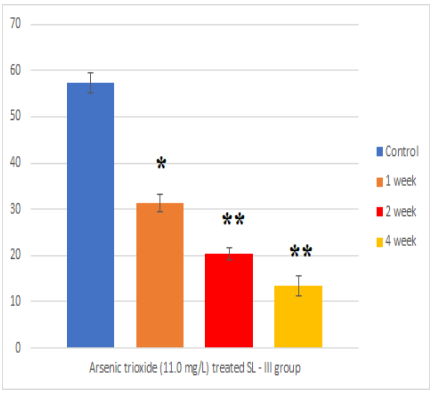

(D)

Figure 3. (SL – III group): Time dependent level of serum Lipid peroxidation-LPO (A), serum superoxide dismutase- SOD (B), Serum Catalase – CAT (C) and reduced Glutathione-GSH (D) in *Clarias batrachus* after exposure to 11.0 mg/L Arsenic trioxide. The results are expressed as mean ± SEM of six replicates (n = 6). * indicates significant differences at $p < 0.05$, and ** indicates significant difference at $p < 0.01$.

4. Discussion

In the present investigation, walking cat fish were exposed to 3.66 mg/L ($1/30^{th}$ of LC_{50}), 5.5 mg/L ($1/20^{th}$ of LC_{50}) and 11 mg/L ($1/10^{th}$ of LC_{50}) of As_2O_3 for one, two and four weeks respectively, and corresponding change in the oxidative parameters was studied. Three concentrations of AS_2O_3 were selected since they were below the lethal range with no mortality till 60 days, and were within the limit of USEPA aquatic life criteria. Similar sub-lethal range of AS_2O_3 has also been considered by several authors viz.3.8 mg/L *i.e.*1/20 LC_{50} and 7.6mg/L *i. e.*1/10LC_{50} (Roy & Bhattacharya, 2006) 42.42µM *i. e.* 1/10 of LC_{50} (Ghosh *et al.* 2006; 2007) and 35 µM *i. e.* 1/10 of LC_{50} (Roy *et al*., 2006).). The reason behind the consideration of duration of exposure in this experiment for one, two and four weeks, is that four weeks was found to be sufficient to reflect the effect of the toxicants and at the same time triggered the up-regulation of the detoxification system in fish (Datta et al.,2007). Review of literature suggests that pathway of xenobiotic metabolism systems in fish is analogous to higher vertebrates and efficient in eliminating a multitude of xenobiotics (Chatterjee and Bhattacharya, 1984; Sarkar,1997 ;Bhattacharya *et al*. 2007).

The reactive oxygen species (ROS) generation is the principal toxico-kinetics of arsenic (Farombi *et al*., 2007; Kitchin, 2001; Flora *et al*., 2005). As (III) exerts its toxic action either directly by attacking –SH group of an enzyme and thereby inhibiting enzyme action or indirectly through generation of ROS (Chen *et al*. 2001). The production of ROS is a normal aspect of cellular metabolism, but increased production of ROS leads to oxidative stress. Enhancement of intracellular ROS modulates the occurrence of cell damage via initiation and propagation of LPO (Gutteridge, 1995).

Lipid peroxidation refers to the oxidative degeneration of lipid. It is the process by which free radicals steal electron from the lipid in cell membrane resulting in considerable cell damage. It includes three basic steps: initiation, propagation and termination. Peroxidation of lipid can disturb the assembly of membrane causing changes in fluidity and permeability, alteration of ion transport and inhibition of metabolic processes. Mitochondrial damage through lipid peroxidation, directs further ROS generation and enhances the extent of oxidative stress (Brazilai and Yamamoto 2004; Varanka, 2004; Patra *et al*, 2011). Peroxidative damage due to AS (III) accumulation has been reported in *Anabas testudineus* (Roy *et al*., 2004), *Channa punctatus* & *Clarias batrachus* (Bhattacharya & Bhattacharya, 2005), *Tilapia mossambica* (Sounderarajan *et al.* 2009). Oxidative stress in fish results in free radicals generation, a reduction in antioxidant protection and failure to repair oxidative damage. In the present study, at lower sub-lethal exposure (SL-I group), LPO showed a significant increase ($P<0.05$) of 64.67% and 57.90% over the control within two week and four week duration. At median sub-lethal exposure (SL-II group), it showed a significant ($P<0.01$) increase of 375.26% over control. At higher sub-lethal concentration (SL-III Group), a significant ($P<0.05$) increase of 98.47%, 77.42% ($P<0.01$) and 213.58% ($P<0.01$) respectively over control has been observed at one, two and four week duration respectively. Similar kind of LPO inductions were found in zebra fish brain due to low dose of arsenic trioxide (Sarkar *et al.,* 2014). The consistent significant increase in serum LPO with the increasing concentration of As_2O_3 at sub acute and chronic exposure level in the present study can be correlated with increased ROS & RNS free radicals generation over time that completely disrupted the anti oxidative system in *Clarias batrachus.* Free radicals may be generated by the oxidation of carbohydrates, fats and proteins through both aerobic and non aerobic processes. ROS inactivates membrane transporters, damage DNA & transcriptional machinery (Pryor and Godber,1991; Vendemiale *et al.,*1999) and initiate a chain reaction that peroxidizes poly unsaturated fatty acids, leading to increased production of LPO (Cand and Verdetti, 1989; Rikans *et al.,*1997).

Antioxidant enzymatic defenses like SOD, CAT, GPX (Glutathione peroxidase) and GST (Glutathione S-transferase) play a massive role in protecting the tissue from oxidative damage (Valvanidis *et al*, 2006). Superoxide dismutase is an important endogenous antioxidant enzyme that act as first line of defense against ROS, catalyses dismutation of the superoxide anion & scavenges superoxide radicals & generates H_2O_2 (Winston & Digiulio, 1991; Gunney *et al*, 2009). Catalase and GPX are both involved in H_2O_2 removal in peroxisomes and cytosol by coupling its reduction to H_2O with oxidation of GSH. The activation of SOD requires concomitant activities of cytosolic GPX and/or catalase activity to protect the cell from oxidative stress (Halliwell and Gutteridge, 1999). In the present study, in SL-I Group SOD initially declines by 9.93% over control after one week exposure but then at 2 week exposure it elevates up to nearly normal value. At 4 week exposure, it again declines by 31.83% over the control. Similarly, in SL-II Group, it showed a consistent decline of 8.62%, 32.92% and 37.93% over the control at three exposure levels. At higher sub-lethal concentration of As_2O_3, a sharp decline of 34.51% and 60.26% over control was recorded at 2 week and 4 week exposure levels. In the present study, a slight elevation in SOD in SL-I group at sub acute exposure level marks the simultaneous activation of antioxidant system in fish, but huge accumulation of free radicals due to constant exposure of arsenic render the level of SOD and various enzymes of antioxidant system declining consistently. Significant decrease in serum catalase was observed at all exposure levels except a non-significant increase of 3.275% over control in one week exposure of SL-I Group. At lower sub-lethal concentration of As_2O_3 (SL-I), a significant decline of 18.37% ($p<0.05$) and 31.65% ($p<0.01$) over control was marked at two week and four week test exposure, while at higher sub-lethal concentration of As_2O_3 (SL-III) a significant decline of 36.37% ($p<0.01$), 58.75% ($p<0.01$) and 79.97 % ($p<0.01$) over control was marked at one, two and four week duration, respectively.

GSH plays a significant role in management of arsenic induced oxidative stress (Valco *et al.,*2005). In the present study, blood GSH showed a significant decline at almost every exposure level when compared with control. At lower sub-lethal concentration of As_2O_3, blood GSH showed a marked reduction by 33.45%, 38.25% and 40.78% over the control in one, two and four week, respectively. While at higher sub-lethal concentration a sharp decline in GSH by 45.41% ($p<0.05$), 65.51%

($p<0.01$) and 76.48% ($p<0.01$) over control was marked in one, two and four week exposure respectively. A similar decrease in reduced GSH in zebra fish gills and livers exposed to Dimethoate (Ansari and Ansari,2014) and cardiac tissues in rats as a consequence of short term arsenic toxicity was reported (Muthumani,2013). Elevation in CAT and GSH activities is usually related with the increased SOD concentration to neutralize toxic H_2O_2 in peroxisomes. But decline in CAT and GSH level in the present study can be related with extra ordinary generation of free radicals and diminished SOD due to consistent sub lethal exposure of AS_2O_3.

Catalase is very pertinent in protecting the cell from toxic effects of H_2O_2 and radical oxygen species (Coban *et al*. 2007, Altikat *et al,* 2006). In the present study, the reduction in the activities of CAT is found to be directly linked with the concentration and duration of As_2O_3 exposure. A similar decrease in CAT activity in the tissues of living animal exposed to arsenic has been reported (Wang *et al.* 2006; Ventura-Lima *et al*. 2009). Our findings are in agreement with the results of Altikat *et al.* (2014), who reported similar decline in CAT activities in all the tissues of *Cyprinus carpio carpio*, exposed to 0.5 and 1.0mg/L of sodium arsenite. A significant decrease in the CAT activities in liver and ovary of zebra fish in response to 0.006 and 0.03 ppm AS_2O_3 exposure has been reported and claimed by the authors that reduced CAT activity may be associated with the diminished level of NADPH in fish (Sunaina *et al.*,2016). Superoxide radicals inhibit CAT enzyme activity (Kono and Fridovich, 1982). The decrease in CAT enzyme activity as a result of arsenic exposure might be due to depression in protein synthesis caused by the free radical damage (Humtose *et al*. 2007; Palaniappan and Vijayasundaram, 2008). However, our findings are in contrast to the earlier reports of increased CAT activity after arsenic exposure (Bhattacharya and Bhattacharya, 2007).

5. Conclusion

The contamination of natural aquatic system with heavy metals released from domestic, industrial and other anthropogenic activities has become a matter of serious concern over the last few decades (Vutukuru, 2005; Conacher *et al*., 1993; Velez and Montoro, 1998). Fishes being at the apex of the aquatic ecosystem food chain, get easily victimized to their adverse effects (Al-Yousuf and El- shaahwi, 1999; Farkas *et al*., 2002). The aquatic bodies in State of Bihar are facing severe arsenic contamination over the last few years (Chakraborty *et al.,* 2003).The toxic effects of nonlethal concentration of arsenic on the various oxidative indices have been demonstrated in the present study. As per National Academy of Sciences, US (NAS, 1977) the approved safe limit of arsenic for fish is 1.5 – 3.8 mg/L of water, but the findings of the present study revealed a significant perturbations in the oxidative parameters of freshwater air breathing fish *Clarias batrachus (Linnaeus*, 1758) even at much lower concentration of arsenic *i.e*. 3.66 mg As^{3+}/L, than the approved limit of NAS. Oxidative stress in Indian major carps (IMC) have been even reported at further lower concentration of arsenic *i.e.* 0.5 mg/L and 1.0 mg/L (Altikat *et al.*, 2014). Induction of oxidative stress through ROS generation is one of the principal toxico-kinetics of As_2O_3 in aquatic organism as marked by significant increase in serum LPO level in the test fish at all levels of arsenic exposure. A dose dependent reduction in antioxidant enzyme system, i.e. SOD, CAT and GSH, clearly marks their failure in repairing oxidative damage in fish generated by arsenic exposure. Hence, the assessment of oxidative parameters of fish may be considered as bio-indicators of heavy metal toxicity of aquatic body. Considering the deleterious impact of arsenic on the aquatic system and their implications on human health, it is highly recommended to monitor the level of arsenic in edible fishes in Bihar consistently. It will be equally important to work on mitigation strategies for the bio-conservation of these fishes.

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Compliance with Ethical Standards Findings:

The study was not funded by any grant funding body.

Conflict of Interest

There is no conflict of interest in the study as declared by both authors.

Ethical Approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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