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Long-Term Exposure of Arsenic Caused Arsenic Bioaccumulation, Imbalance in Hepatic Markers, Oxidative Status, and Histological Changes in the Liver of Zebrafish (*Danio Rerio*)

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***Abstract*-**The present experiment was conducted to analyze the effect of sub-lethal concentration of 1.11mg/l of arsenic (III) oxide over long-term (15, 30, 45, 60 days) exposure on the liver of zebrafish. The arsenic (III) oxide exposed zebrafish liver showed a significant increase in arsenic bioaccumulation with the increase of exposure duration. The activities of lipid peroxidation (LPO) markers like thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide (H_2O_2) were also significantly increased with the increase of arsenic exposure duration, while the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) activities were notably increased during the initial short term arsenic exposure (15 and/or 30 days), but later decreased significantly in the fishes that were exposed to arsenic for an extended period (45 & 60 days) when compared to normal. Further, the hepatic enzymes like alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) levels were significantly increased in serum, and degenerative changes were also found in the histology of the zebrafish liver. Thus, the above results suggest that long-term exposure to arsenic can lead to bioaccumulation of arsenic as well as alter the function and tissue architecture of the zebrafish liver.

***Keywords*-** Arsenic; long-term exposure; lipid peroxidation; zebrafish; liver;

I. INTRODUCTION

Arsenic (As) distribution and toxicology in the environment is a serious issue, with millions of individuals worldwide being affected by As toxicosis. Sources of As contamination are both natural and anthropogenic, and the scale of the contamination ranges from local to regional [1]. The long-term As exposure through drinking-water as well as food can lead to cancer and/or skin lesions. As toxicosis has also been implicated to be a cause of cardiovascular disease as well as diabetes. In utero and/or early childhood exposure to As has also been linked to lowering of cognitive development and mortality in young adults [2]. Arsenic contaminated water used for drinking, food preparation and irrigation of food crops pose the greatest threat to public health [3]. Moreover, consumption of arsenic-contaminated fish is also an important exposure pathway through diet for humans [4].

Among the various As compounds, arsenic (III) oxide (As_2O_3) is a trivalent inorganic compound of arsenic, absorbed fairly rapidly into fish and is more toxic than other arsenic compounds [5]. The liver is one of the vital organs associated with basic metabolism, and is the major organ participating in the accumulation, biotransformation as well as excretion of contaminants/metabolic wastes in fish [6]. Thus, the impact of contaminants on the aquatic ecosystems can be evaluated by estimating various biochemical parameters in the liver of the fish [7]. The liver is susceptible to damage from a vast variety of toxicants. The liver also facilitates in cleaning xenobiotics/pollutants from the blood and thus it is a good indicator of pollutants in the aquatic environment [8]. Previously, Alarifi et al. [9] and Sunaina et al. [10] reported that oxidative stress and DNA damage induced by arsenic occur via production of superoxide and hydrogen peroxide radicals, specifically reactive oxygen species. Therefore, the aim of this study is to observe the long-term exposure of sub lethal concentration of arsenic (III) oxide on the liver of zebra fish, by analyzing the bioaccumulation of arsenic, oxidative status and histological changes in the liver tissues of zebrafish.

II. MATERIALS AND METHOD

Chemicals

Arsenic III oxide was purchased from Hi Media Ltd. and all the remaining chemicals and the reagents used were of the analytical grade and were purchased either from Merck and/or Himedia, Mumbai, India.

Experimental fish

Studies using zebrafish to investigate inorganic arsenic (iAs) exposure in adults have established zebrafish as an excellent model for investigating/analyzing the mechanism of As toxicity [11]. Healthy adult wild-type zebrafish 4±1 cm in average length and approximate weight of 0.78 ±0.05g of both male and female fish were purchased from Red hills fish farm, Chennai, Tamil Nadu. Fishes were separately maintained at 25±1ºC in 150 capacity glass tanks with continuously aerated and dechlorinated tap water (pH 7.1-7.3; hardness 185-200 mg/L as CaCo3; alkalinity 165-170 mg/L as CaCo3) at least one month prior to the experiments. The laboratory photoperiod was 10 hr D; 14 hr L. Fishes were fed with goldfish flake food (or) frozen brine shrimp twice per day *ad libitum*.

Chronic study was conducted by exposing the adult zebrafish to the test solution of a sub-lethal cum safe level concentration of 1.11 mg/L (1/8th of 96 hr LC_{50}) of As (III) oxide for long-term period of 60 days of exposure (Bhavani and Karuppasamy [12]. After the 15^{th}, 30^{th}, 45^{th} and 60^{th} day of exposure of As (III) oxide, 6 fish from each group were sacrificed and dissected to separate the liver to observe the accumulation of As (III) oxide in liver, oxidative status and histopathological analysis.

Bioaccumulation

To determine the quantity of the accumulated As (III) oxide in the liver, the tissues were placed in separate petri dishes to dry at 80ºC until reaching a constant weight. The dried materials were powdered separately using a mortar and pestle. 100 mg of the powdered sample from each tissue was digested separately with a mixture of nitric and perchloric acid in the ratio 4:1 and was heated until the sample was almost dry and colorless. The final products were made up to 25 ml with double distilled water, and the concentration of As (III) oxide was analyzed using a Perkin Elmer AS 3100, an atomic absorption spectro photometer (AAS). All the blanks were also formulated in the same method as those for the samples. The results were represented in microgram per gram dry weight.

Hepatic enzymes

The blood was collected from group of fishes, poured in tubes and allowed to clot under room temperature (22°C) for 30 min. After centrifugation at 1000 rpm serum was removed from the clotted sample after 5 min and then frozen at −80°C until analysis. The samples were then pooled for each of the experimental groups for further analysis. The hepatic enzymes such as AST, ALP and ALT were assayed by Wood et al. [13] in serum of fish.

Estimation of lipid peroxidation and antioxidant markers

The activity of TBARS and lipid hydrogen peroxide in the liver homogenate was measured by the method of Ohkawa et al. [14]. The activity of SOD was evaluated by the method of Kakkar [15]. The activity of CAT and GPx were measured by the method of Sinha [16] and Rotruck [17], respectively. The activity of GSH was assessed by the method of Ellman [18].

Histopathological evaluation

The harvested liver tissues were fixed in 10 % formalin for 48 hrs. It was then followed by dehydration by passing through a series of graded alcohol and was finally embedded in paraffin. Sections of the kidney (5-6 µm thick) were developed using semi-automated rotator microtome. All the tissue sections were then mounted on a glass slide, deparaffinated and stained with Hematoxylin and Eosin (H & E) dye and observed microscopically.

Statistical analysis

All the data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test by using commercially available statistics software package (IBM SPSS Statistics for Windows, version 15). The results were presented as mean ± SD of ten fish in each group. The value of $p < 0.05$ was considered as statistically significant.

III. RESULTS

Bioaccumulation of arsenic

The bioaccumulation of arsenic in the liver of zebrafish after the sub-lethal (1.11 mg/L) exposure of As (III) oxide during different days of exposures, i.e., 15, 30, 45 and 60 days, are given in the figure 1. The level of arsenic in the liver of control fish

was non-detectable. Whereas, a significant increase ($p < 0.05$) in the level of arsenic was observed in sub lethal As (III) oxide treated fish than the control. As the exposure time (15, 30, 45 and 60 days) of As increased, the level of arsenic was also found to be gradually increased in the liver tissues of zebrafish.

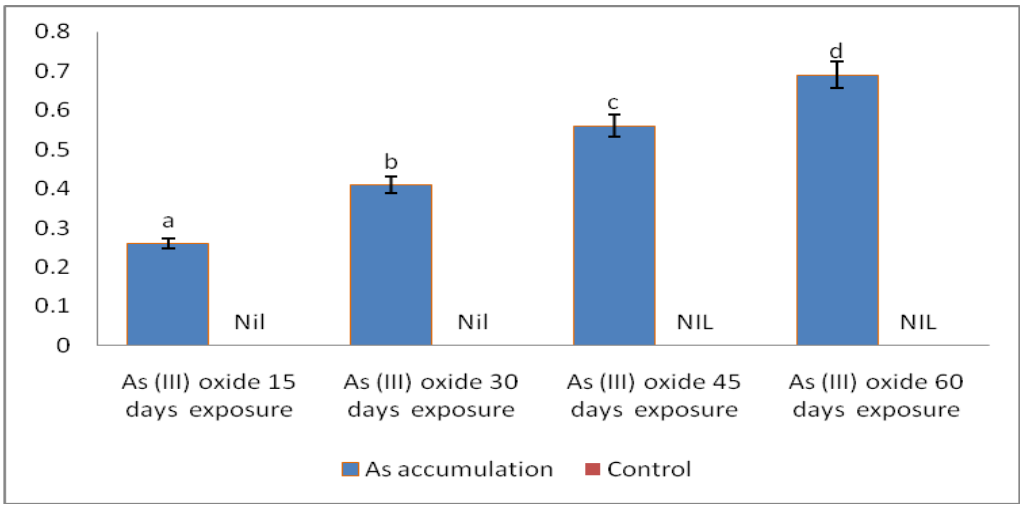

Figure 1: Bioaccumulation of arsenic in the liver of control and experimental zebrafish. Values are mean ± S.D., n = 6/treatment; Different letters denote statistically differences (P < 0.05) between different time interval within the same treatments

Effect of As (III) oxide on hepatic markers

The sub-lethal concentration of As (III) oxide exposed fish showed significantly increased activities of AST, ALT and ALP activities when compared to control fish. Moreover, the activities of AST, ALP and ALT were found to be gradually increasing from 15, 30, 45 and 60 days of sub-lethal As (III) oxide exposure (Figure 2).

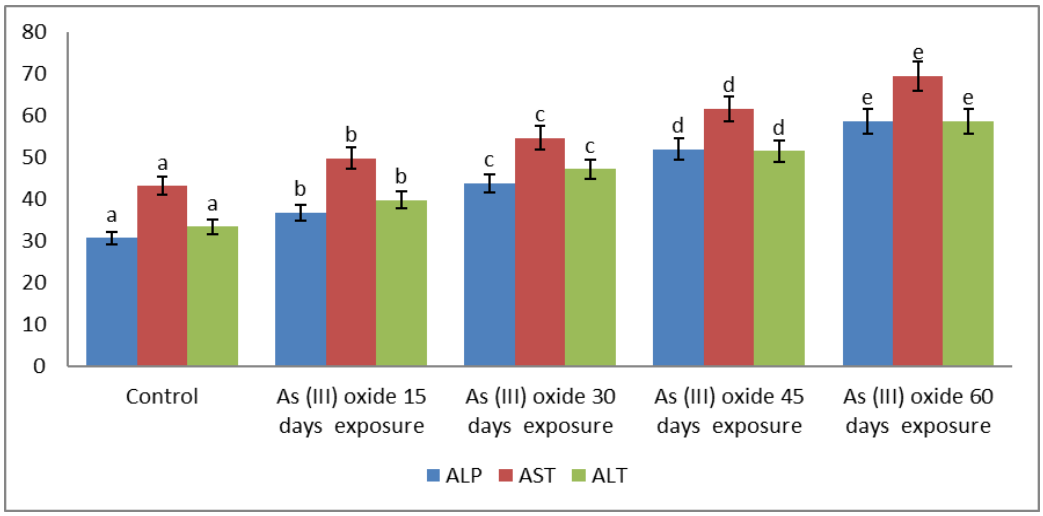

Figure 2: Effect of As (III) oxide on hepatic enzymes of control and experimental zebrafish. Values are mean ± S.D., n = 6/treatment; Different letters denote statistically differences (P < 0.05) between different time interval within the same treatments

Effect of As (III) oxide on lipid peroxidation markers

Table 1 represents the activity of TBARS and H_2O_2 in the liver of experimental fish. Sub-lethal concentration of As (III) oxide exposed fish showed significantly increased activities of TBARS and H_2O_2 in the liver tissues as compared to normal fish. Moreover, a significant ($p<0.05$) gradually increased activity of TBARS and H_2O_2 in the tissues were noticed from 15, 30, 45 and 60 days of As (III) oxide exposure.

Effect of As (III) oxide on enzymatic and non-enzymatic antioxidant markers

The activities of enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH) antioxidant markers of the experimental zebrafish liver are tabulated in Table 1. The enzymatic and non-enzymatic antioxidant activities were notably increased during the initial

As (III) oxide exposure (15 and/or 30 days), but later decreased significantly ($p<0.05$) in the fishes that were exposed to As (III) oxide for a long period (45 & 60 days) when compared to control.

Table 1. Effect of As (III) oxide on lipid peroxidation and antioxidant enzymes of control and experimental zebrafish

Parameters/ markers	Control	As (III) oxide exposure at different time intervals			
		15 Days	30 Days	45 Days	60 Days
TBARS (nmol/mg prot.)	23.6 ± 1.80^{a}	29.4 ± 2.23^{b}	31.2 ± 2.38^{c}	33.4 ± 2.56^{cd}	36.8 ± 2.76^{d}
H_2O_2 (µmol/mg prot.)	1.20 ± 0.09^{a}	1.57 ± 0.12^{b}	1.80 ± 0.14^{c}	1.99 ± 0.15^{cd}	2.08 ± 0.16^{d}
SOD (U/mg prot.)	53.2 ± 4.05^{a}	59.4 ± 4.53^{b}	60.7 ± 4.62^{b}	37.35 ± 2.85^{c}	35.73 ± 2.64^{d}
CAT (U/mg prot.)	46.4 ± 3.53^{a}	56.78 ± 4.28^{b}	57.54 ± 4.29^{b}	34.98 ± 2.62^{c}	31.54 ± 2.40^{d}
GPx (U/mg prot.)	29.6 ± 2.25^{a}	34.90 ± 2.66^{b}	35.76 ± 2.82^{b}	21.34 ± 1.63^{c}	18.76 ± 1.42^{d}
GSH (µg/g wet wt)	2.49 ± 0.20^{a}	3.18 ± 0.24^{b}	$3..92 \pm 0.31^{b}$	1.76 ± 0.14^{c}	1.52 ± 1.2^{d}

Values are mean ± S.D., n = 6/treatment; Different letters denote statistically differences ($P < 0.05$) between different time interval within the same treatments

Histopathological effects of As (III) oxide on liver

The histopathological changes have been noticed in the liver of *D. rerio* at various intervals (30, 45 and 60 days) of As (III) oxide at a sub-lethal concentration of 1.11 mg/L. At all the intervals of observations of As (III) oxide exposure, the liver cords become disarranged, irregular shape of hepatocytes, scattered and disintegrated nucleus, pycnotic nuclei, karyolysis and necrosis were observed. Further, the cytoplasm become granulated and vacuolated and less homogenous compared with the liver of control fish (Figure 3). These changes were more pronounced in the 60 days As (III) oxide exposure period when compared to the 30 days of As (III) oxide exposure. In addition, damaged blood vessels and clumping of hepatocytes were observed prominently at 60 days of As (III) oxide exposure when compared to other intervals of As (III) oxide exposures.

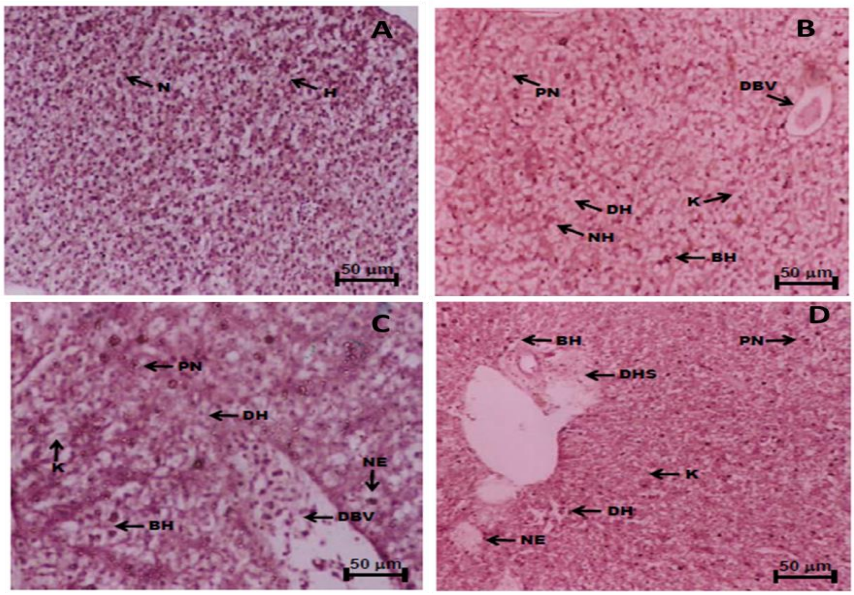

Figure 3: Effect of As (III) oxide on liver histopathology of control and experimental zebrafish. A: Liver of control zebrafish ; B: Liver of zebrafish exposed to 1.11 mg/L of As(III) oxide for 30 days; C: Liver of zebrafish exposed to 1.11 mg/L of As(III) oxide for 45 days; D: Liver of zebrafish exposed to 1.11 mg/L of As(III) oxide for 60 days. H – Hepatocyte, N – Nucleus, DH – Degenerated hepatocyte, BH – Binucleated Hepatocyte, DBV – Degenerated Blood Vessel, NE – Necrosis, PN – Pycnotic Nucleus, K – Karyolysis

IV. DISCUSSION

Fishes due to its feeding and living habit in the aquatic environments are very vulnerable and also heavily exposed to pollutants, as they cannot escape from the water medium leading to detrimental effects induced by the pollutants in the fish body. The present study demonstrates that long-term As (III) oxide exposed liver of zebrafish showed bioaccumulation of arsenic, imbalance of oxidative markers and structural changes in liver when compared to control.

The mechanisms involved in the absorption, distribution, metabolism, and excretion (ADME) of iAs are very important for human health risk assessment [19]. Fish is a very economical source of proteins for the human population. The bioaccumulation of heavy metals in fish gill, muscle, and liver has acquired greater attention because of the potential toxic effects of such substances not only on fish, but also on the human population who are consuming fish in their regular diet [20]. In the present study, as the As (III) oxide exposure time interval increased, i.e., from 15 to 60 days, the bioaccumulation of As was gradually increased in the liver of As (III) oxide treated fish. Bioaccumulation of As in the liver leads to imbalance of liver function and also arsenic passed on to human through food (fish consumption), where it deposited in vital organs leading to biomagnification. This result was supported by Pei et al. [20], who have stated that As accumulated in the liver of Tilapia.

Serum hepatic enzymes such as ALP, ALT and AST are considered to be important markers to analyze the hepatic health of the animal [21]. In the present study, it has been observed that the enzyme activity of ALP, ALT and AST in the serum increased significantly in As (III) oxide treated fish as compared to control group, which may have been due to inflammation and necrosis of the liver caused by the bioaccumulation of As in the liver tissues. Therefore, an increase in the activities of these markers is primarily due to the leakage of these enzymes from the cytosol of hepatocytes into the circulation and are also an indication of hepatocyte damage [22]. These results were similar with Ghaffar et al. [23], who have reported that sub-chronic exposure of As notably increased serum ALP, ALT and AST in *Labeo rohita.*

Lipid peroxidation is one of the well-known causative agents for cellular injury in animals, and is used as an indicator of oxidative stress in tissues. The TBARS and H_2O_2 are commonly used markers to observe the lipid peroxidation status [24]. Reactive oxygen species (ROS) produced due to the toxic effect of metal lead to the lipid peroxidation [25]. Increased level of ROS is also responsible for lipid peroxidation and increased concentration of TBARS and H_2O_2, which are cytotoxic product of lipid peroxidation and is used as an indicator of cellular membrane damage. The present study showed that the TBARS and H_2O_2 activities increased with the increased exposure time of As (III) oxide (sub-lethal concentration of 1.11 mg/L for 15, 30, 45 and 60 days) in the liver of zebrafish. This observation may be contributed by the bioaccumulation of As that induced oxidative stress in the liver, which increased the activities of TBARS and H_2O_2 in the As (III) oxide exposed zebrafish liver [10].

The enzymatic antioxidant system that includes SOD, CAT and GPx plays a coordinating role in the prevention of oxidative damage by ROS in the body [26]. The SOD is the first enzyme to respond against ROS. They are the important endogenous antioxidants that provide protection against oxidative stress [27]. The CAT is the major enzyme involved in hydrogen peroxide (H_2O_2) detoxification and thus also protects organisms against oxidative damage [28]. The GPx, a protein reliant on the micronutrient selenium (Se), assumes a basic part in the lessening of lipids and hydrogen peroxides in the system [29]. Non-enzymatic GSH has an important role in scavenging of cellular ROS. A variety of environmental pollutants are known to change the GSH level in aquatic organisms, including heavy metals [30]. In the present study, initially the enzymatic and non-enzymatic antioxidant activities were notably increased during the As (III) oxide exposure (15 and/or 30 days), but later decreased significantly ($p<0.05$) in the fish liver that were exposed to As (III) oxide for a long period (45 & 60 days) when compared to control group. This initial increase and then decrease in the activities of SOD, CAT and GPx occurred probably as a defense response against H_2O_2 generated by As (III) oxide, where the initial As (III) oxide exposure caused the fish biological system to combat the oxidative stress by increasing the antioxidant enzymes, but later increase (long-term) in the exposure period of As (III) oxide caused increased bioaccumulation of As in tissues leading to cellular damage, resulting in decrease in the antioxidant levels and increase in oxidative stress. Moreover, the decrease in GSH may be partly contributed by the increased accumulation of As leading to the more utilization of GSH to detoxify metals and ROS or due to its oxidation to GSSG. The above results were similar with Sunaina et al. [10], who reported that As caused significant decline in the activities of SOD, CAT and GPx as well as a decrease in the GSH levels in the organs and tissues of zebrafish.

The liver has been proposed as the critical target organ for As toxicity in fish due to the role it plays in metabolism and detoxification [31, 32]. In the current study, As (III) oxide 30, 45 and 60 days sub-lethal exposed zebrafish showed intense degenerative change in the liver when compared to normal. The degenerative changes were characterized by vacuolation of hepatocyte, pycnosis in many of the necrotic cells, necrosis and disintegration of blood sinusoids. This pathological changes in

the liver tissues were well correlated with an increase in As levels during long-term As (III) oxide exposure. The above observation in the present work coincided with the study of Kim et al. [33, 34], who have suggested that pathological changes in liver were caused by cumulative toxicity due to prolonged period of As exposure in fish.

V. CONCLUSION AND FUTURE SCOPE

From the results of the present study, it can be concluded that the long-term exposure to sub-lethal As (III) oxide on zebrafish has the ability to induce alterations in the liver functions, cause imbalance in oxidative status and cellular damage, and induce changes in the liver histological of zebrafish. Thus, it can be evident that the long-term exposure of arsenic (III) oxide imposes a threat on the normal development fish and may also have similar effect on economically important fishes and in turn will affect the economic and health status of the aquaculture industries and fish consumers, respectively. Further work is required to fully enumerate both biochemical as well as molecular pathways affected by arsenic in the liver and other vital organs such as gills, kidney, eye, etc.

Conflict of interest

The authors declare no conflict of interest.

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