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ORIGINAL ARTICLE

STUDIES OF ARSENIC TRIOXIDE ON OXIDNAT AND ANTIOXIDANT ACTIVITY OF LIVER TISSUES OF *Cirrhinus mrigala*.

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ABSTRACT

The environmental pollutants, like heavy metals are of particular concern, due to their potential toxic effect and ability to bioaccumulation in aquatic ecosystems. After reaching the arsenic in the aquatic bodies deteriorated the life sustaining the causes of cell damages of flora and fauna and fishes. In the present experimental investigation, evaluate the oxidant and antioxidant (LPO, GSH, GPx, SOD, and CAT) activity in liver tissue of freshwater fish *Cirrhinus mrigala* exposed by arsenic trioxide. During the treatment of fresh water fish were exposed to sublethal concentration of arsenic to the 96 hrs LC_{50} values of 2.96 ppm for the periods of 7, 14, 21, and 28 days. The results revealed that treatment of arsenic trioxide enhanced level of lipid peroxidation (LPO), and simultaneously decreased in the level of glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), in liver tissues of fish. Present investigation concluded that the arsenic trioxide was affected and significant alteration by the metabolic activity in liver tissues of the fresh water fish *Cirrhinus mrigala*.

Keywords: Arenic, *Cirrhinus mrigala*, LPO, GSH, GPx, SOD, and CAT.

1.INTRODUCTION

Heavy metals are the most prominent dangerous groups of anthropogenic environmental pollutants with high toxicity and persistence in the environment. Heavy metal contamination has been reported in aquatic organisms (Adham *et al*., 2002; Olojo *et al*., 2005; Pandey Govind and Madhuri, 2014). These pollutants build up in the food chain and are responsible for adverse effects and death in the aquatic organisms (Farkas *et al*., 2002). Arsenic may undergo a variety of reactions in the environment including oxidation-reduction reactions, ligand exchange, precipitation and biotransformation (Welch *et al*., 1988). Arsenic belongs to the group of highly toxic heavy metal indeed, long term exposure of arsenic induced impairment of reproductive function and neuro endocrine disruption. Currently accepted opinion of arsenic action is related to protein synthesis and enzyme functioning (Drastichova *et al*., 2004).

Arsenic can produce all three types of toxicity at difference dosages, acute, sub-acute and chronic. One sign of acute exposure is edema of the eyelids and gastrointestinal irritation and both central and peripheral neuropathies frequently occur. During chronic intoxication garlic breath, skin sensitivity, dermatitis and keratitus frequently occur. All types of arsenic exposure can cause kidney, gill and liver damage and in the most serve exposure, there is erythrocyte haemolysis (Selamoglu Talas *et al*., 2012). Aquatic ecosystems are at risk of contamination by the metalloid arsenic leaching form inorganic arsenic compounds used in pesticide sprays, combustion of arsenic containing fossil fucls, smelter runoff and mine tailings.

Arsenic is known to bind to cellular sulfhydryl, particularly vicinal ones, accounting for its ability to interfere with energy generation (Aposhin, 1989; Sohini Singh and Rana, 2007). Once in the tissues, arsenic exerts its toxic effects through several mechanisms, the most significant of which is the reversible combination with sulfhydryl groups. Arsenic inhibits numerous other cellular enzymes, especially those involved in cellular glucose uptake, gluconeogenesis, fatty acid oxidation and produce of glutathione through sulfhydryl group binding. Prolonged exposure of arsenic has detrimental effects in tissues. It may impair the glycolysis as well as the oxidative processes (Tchounwou *et al*., 2003; Shahidul Haque and Swapan Kumar Roy, 2012) and causes different types of pathogenic syndromes in rodents, fishes and other organisms. Exposure of higher concentration of arsenic in water may also cause severe effects in fish and might be

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involved in producing cancer of other cellular effects. However the mechanism underlying the effects of acute arsenic exposure on the regulation of oxidative and glycolytic processes in liver exposed to cold is not known. Arsenic is classified as a human carcinogen based on several epidemiological studies showing an association of arsenic exposure with cancers in lung bladder, kidney and liver (Hughes, 2002; Tchounwou *et al.,* 2003; Shahidul Haque and Swapan Kumar Roy, 2012). Moreover fish have long been used as sentinels for biomonitoring of aquatic environmental pollutants and are good indicators of arsenic toxicity (Abdel-Hameid, 2009). The regulation of metabolic activities in liver in response to changes of temperature is an important aspect in fish and to clarify the role of arsenic in cold-induced liver metabolic functions responsible for the survival of these species of fishes in the environment.

2. MATERIALS AND METHODS

The fresh water fish *Cirrhinus mrigala* were collected from Sathanur Dam Thiruvannamalai District. The collected fish were acclimated to laboratory condition for 15 days. They were checked thoroughly for injury and disease conditions, and only healthy fishes were used for this study. After washing with 0.01% KMnO4 solution for 15 min, they were placed in nine plastic pools (500 L) containing non-chlorinated water. Prior to the start of the experiment, the fishes were acclimatized to the food and laboratory conditions with 12 h dark and 12 h light cycles, pH range of 6.95 to 7.60 and temperature ranging from 16 to 24ºC for 15 days. Fishes were divided into five equal groups each comprising of 50 fishes. Each group was kept in separate plastic tanks. The first group was kept as negative control; the fishes were maintained in water containing normal water without any treatment. The fishes of two groups were exposed to a sub-lethal concentration of 2.96 ppm of arsenic trioxide added in the water for 7, 14, 21 and 28 days respectively. Solutions were renewed once daily after exposure period, animals were sacrificed and the liver tissues were removed, homogenized and stored at −80 °C for further biochemical analyses.

Estimation of Lipid peroxidation (TBARS)

The concentration of TBARS in the kidney tissue was estimated by adopting the method of Nichans and Samuelsen (1968). Known amount of whole kidney tissue homogenate was prepared in Tris-HCl buffer (pH 7.5). 1ml of the tissue homogenate was taken in a clean test tube and 2.0ml of TBA-TCA-HCL reagent was added and then mixed thoroughly. The mixture was kept in a boiling water bath (60ºC) for 15 minutes. After cooling, the mixture was taken to read the absorbance of the chromophore at 535nm against the reagent blank in a UV-visible spectrophotometer (Spectronic-20, Bausch and Lamb). 1, 1', 3, 3' tetra methoxy propane was used to construct the standard graph. Values were expressed as n moles of MDA released / 100 mg.

Estimation Reduced glutathione (GSH) activity

The level of reduced glutathione in kidney tissue was estimated by the method of Ellaman (1959). A known weight of tissue was homogenized in phosphate buffer (0.1 M. pH 7.0) and centrifuged at 2500 rpm for 5 minutes. 0.2 ml of the sample (Supernatant) was taken in a clean test tube and 1.8 ml of EDTA solution was added. To this 3.0 ml of precipitating reagent was added and mixed thoroughly and kept for 5 minutes before centrifugation at 3000 rpm for 10 minutes. In a clean test tube, 2.0 ml of the content mixture was taken and to this 4.0 ml of 0.3M disodium hydrogen phosphate solution and 1.0 ml of DTNB reagents were added. The appearance of yellow colour was read at 412 nm in UV-visible spectrophotometer (Spectronic-20, Bausch and Lamb). A set of standard solution containing 20-100 µg of reduced glutathione was treated similarly. Values are expressed as µg/100mg protein.

Estimation of Superoxide dismutase (SOD) activity

Superoxide dismutase in the kidney tissue was assayed by adopting the method of Kakkar (1984). The kidney tissue was homogenized with 2.0 ml of o.25M sucrose solution and the centrifuged the contents at 10,000 rpm for 30 minutes in a cold centrifuge. After completing the centrifugation the supernatant was taken in a clean test tube and the content was dialysed against the Tris-HCl buffer and then mixed the contents thoroughly. The contents were centrifuged again at 3000 rpm for 15 minutes. The supernatant was taken in a clean test tube and then 1.2 ml of sodium pyrophosphate buffer, 0.1ml of phenazine methosulphate and 0.3ml of nitroblue tetrazolium reagents were added. The sample mixture (enzyme preparation) was kept in water bath at 30ºC for 90 seconds and appropriately diluted enzyme preparation in a total volume of 3 ml with double distilled water. The reaction was started by the addition of 0.2 ml NADH. After completing the incubation period, the reaction was stopped by the addition of 1m1 glaicial acetic acid. The reaction mixture was stirred vigorously and shaken with 4ml n-butanol. The mixture was allowed to stand for 10 min and then centrifuged the contents at 3000 rpm for 5 minutes and n-butanol layer was separated the colour density of the chromogen in n-butanol was measured in an UV spectrophotometer at 510nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit. The specific activity of the enzyme was expressed as unit/min/mg of protein for tissues.

Estimation of Catalase (CAT) activity

The activity of catalase in the kidney tissue was determined by the method of Sinha (1972). Tissue homogenate was prepared by phosphate buffer (0.01 M. pH 7.0) in a clean test tube 0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate and 0.4ml of hydrogen peroxide were added. The reaction was arrested after 15, 30, 45 and 60s by adding 2.0 ml of dichromate – acetic acid mixture. The tubes were kept in a boiling water bath for 10 min.and then cooled with the help of tap running water and the colour developed was read at 620 nm in an UV spectrophotometer. Standards in the concentration range of 20-100µ mol were taken and preceded as for the test. The specific activity was expressed as µ mol of H_2O_2 consumed/ min/mg of protein for tissues.

Estimation of Glutathione peroxidase (GPx) activity

The activity of GP_X in the kidney tissue was measured by the method of Rotruck (1973). The known quantity of whole liver tissue was homogenized with tris buffer. After completing the homogenization the content was centrifuge at 2500rpm for 5 minutes. 0.2 ml of supernatant was taken in a clean test tube and then 0.2 ml of EDTA and 0.1 ml of sodium azide reagents were added. By lateral shaking the test tube the above said reagents were mixed well. To the mixture, 0.2ml of GSH followed by 0.1 ml of H_2O_2 reagents was added. The contents were mixed well thoroughly and incubated at 37ºC for 10 min and then 0.5 ml of 10% TCA was added. Simultaneously reagent blank was also used which is containing all reagents except tissue homogenate. The contents were centrifuged and then supernatant was used for GSH assay by using the method of Ellman (1959). The activity was expressed as µ mol of GSH consumed /min/mg of protein tissues.

Histology and Histopathological study

For the qualitative analysis of tissue histoarchitexture, the tissue sample (kidney) was fixed in 10% buffered formaldehyde for 48hrs and dehydrated by passing successfully in different concentrations of ethyl alcohol and cleaned in xylene and embedded in paraffin. Sections of tissue (5-6µm thick) was prepared by using a rotary microtome and rehydrated and then stained with heamatoxylin and eosin dye (H & E), which was mounted in DPX medium for microscopic observations.

Statistical analysis

Values are given as mean ± S.D. for six rats in each group. The data for various biochemical parameters were analyzed using analysis of 't'-test. Values were considered statistically significant when $p<0.05$ and the values sharing a common superscript did not differ significantly.

3. RESULTS

In this studies of oxidant and antioxidant activity have a significant value of toxicological investigation because of their significant alteration appeared before the clinical symptoms produced by the toxicant. Changes in the activity of certain enzymes in fish exposed to arsenic were recorded at the end of experimental period. The level of LPO were significantly increased ($P<.05$) in sublethal concentration of arsenic exposed freshwater fish liver tissue when compared to control. At the same time level of GSH, GPx, CAT and SOD in the liver tissue was significantly decreased when compared to control at sublethal concentration of arsenic trioxide exposure to duration.

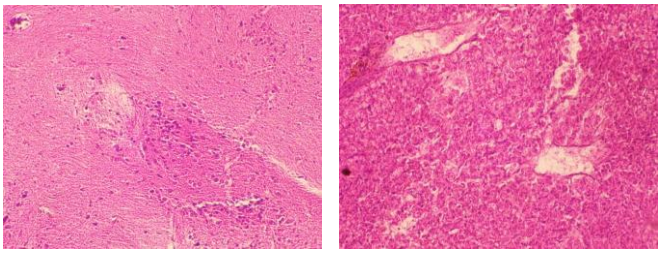

Fig-1 shows histology of experimental *Cirrhinus mrigala* liver tissue Fig- (A) and (B) shows Arsenic trioxide exposed *Cirrhinus mirgala* fish liver.

Examination of liver tissues of *cirrhinus mirgala* fish arsenic showed necrotic changes dilatation of central vein, pyknotic nuclei, vacuolar necrosis, degenerative changes and necrotic changes when compared to control.

4. DISCUSSION

Many classes of environmental pollutants or their metabolites may exert toxicity related to oxidative stress and can cause oxidative damage in fish (Velisek *et al.,* 2012; Alzbeta Stara *et al.,* 2012). One of the consequences of increased oxidative stress is the enhancement of lipid peroxidation (Jinling cao *et al.,* 2013; Ananth and Mathivanan, 2014). Toxicant induced stress at a biochemical level is based on the production of free radicals. Oxidative stress caused by the toxicants in biological system may be involved in a variety of disease states and toxic reaction, thereby contributing indirectly to injury (Bhattacharya and Bhattacharya, 2007). Fish react to xenobiotics by changing and adapting their metabolic functions and their enzymatic systems appear to be very similar as that of the mammalian system (Malarvizhi *et al.,* 2012). The products of saturated and unsaturated lipids resulting from oxidative injuries of lipid hydroperoxides. Lipid peroxidation is a chemical mechanism capable of disrupting the structure and function of the biological membranes that occurs as a result of free radical attack on lipids (Hasan *et al.,* 2013). The ability of Arsenic to produce ROS was indicated in the present study by increased amount of lipid peroxides (LPO). Other studies have reported that intracellular generation of hydrogen peroxides (H_2O_2) could be involved in the initiation of Arsenic hepatotoxicity in mice (Hussain *et al.,* 1999). Arsenic causes cell membrane damage like lipid peroxidation which leads to the imbalance between synthesis and degradation of enzyme protein (Guzzi and La Porta, 2008). The excess production of ROS by Arsenic may be explained by its ability to produce alteration in mitochondria by blocking the permeability transition pore. According to Aruljothi and Sankarsamipillai, (2014) they have reported that, the lipid peroxidation in liver significantly increased in *Labeo rohita* exposed to arsenic

The dynamic equilibrium can be disturbed, leading to enhanced peroxidative processes (ROS) and damage to cellular constituents linked to low antioxidant concentrations (Aruljothi and Sankarsamipillai, 2014; Altikat *et al.,* 2015), resulting in oxidative stress. Under normal conditions, antioxidant systems within the cell minimize the perturbations caused by ROS (Altikat *et al.,* 2015). Our study showed increased ROS, mainly in the liver, following exposure to arsenic at concentrations of 7, 14, 21 and 28 days. To neutralize ROS, animals possess an antioxidant defense pathway comprising antioxidant enzymes such as SOD, CAT, GPx, as well as non-enzymatic antioxidant such as GSH, which prevent oxidative damage (Modesto and Martinez, 2010). Though the generation of H_2O_2 in a normal attribute of cellular mechanism, the increased production of H_2O_2 may lead to oxidative stress when the cellular antioxidant defense system is over whelmed. GSH is an important antioxidant, reduction of hydrogen peroxide (H_2O_2) at the expense of GSH. In the

Table-1 shows the level of Lipid peroxidation (LPO) and reduced glutathione (GSH), superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx) activity of liver tissue of freshwater fish, *Cirrhinus mrigala* exposed to sub-lethal concentration of Arsenic trioxide.

Parameters	Exposure periods				
	Control	7 Days	14 Days	21 Days	28 Days
LPO	1.82±0.07	2.11±0.08 15.94	2.35±0.13 29.12	2.52±0.15 38.47	2.71±0.17 49.01
GSH	4.67±0.17	4.42±0.15 -5.36	4.29±0.12 -8.13	3.99±0.11 -14.56	3.80±0.14 -18.62
GPx	3.69±0.10	3.47±0.09 -5.96	3.40±0.08 -7.85	3.26±0.06 -11.65	2.99±0.05 -18.97
SOD	11.84±0.23	9.41±0.20 -20.53	7.69±0.22 -35.06	6.53±0.21 -44.85	5.56±0.17 -53.05
CAT	4.36±0.14	3.98±0.12 -8.72	3.64±0.12 -16.51	3.20±0.10 -26.62	2.81±0.09 -35.48

current study, As (III) oxide induced a marked time-dependent increase in H_2O_2 content and this elevation in well correlated with increased in GPx activity. The antioxidant enzyme CAT catalyzes the decomposition of endogenously produced H_2O_2. Tanu Allen *et al*. (2004) have reported that, the high rate of GSH reduction recorded in the gill, liver, kidney of freshwater fish, *Channa punctatus* with exposed to arsenic. Aruljothi and Sankarsamipillai, 2014 also reported that, the GSH was reduced in the selected tissues of freshwater fish *labeo rohita* exposed with sublethal concentration of arsenic trioxide.

Glutathione peroxidase activity catalyses the reduction of H_2O_2 and lipid peroxides and is considered an efficient protective enzyme against lipid peroxidation at the expense of GSH (Moreno et al., 2005). GPx enzymes exhibits pivotal role in protection of animals from oxidative damage by reducing lipid hydroperoxides to alcohols. The activity of GPx increased significantly in higher test concentration in liver suggesting elevated levels of hydroperoxides in liver (Venkatramreddy Velma and Tchounwou, (2010).

The GSH induction indicated that the As(III) oxide act as pro-oxidants and cause production of free radicals. The decreased concentration of glutathione in liver at period of exposure may result from either inhibited synthesis or increased consumption in arsenic toxicity.

In aquatic animals, various conditions are reported to induce oxidative stress. For example, it may result as a response to several xenobiotics and hormonal changes (Bainy *et al*., 1996). SOD and GPx activity was reduced in both the liver. Jianga *et al*. (2011) have reported in the SOD, CAT and GSH activities were reduced in copper induced oxidative stress in *Cyprinus carpio* than control fish. The liver is found to be stronger in view of oxidative stress than the other tissues with the highest SOD and CAT activities (Atli *et al*., 2006, 2007). This could be related to the fact that the liver is the site of multiple oxidative reactions and maximal free radical generation; therefore liver tissue was thought to be the best to present the response of CAT activity to metal exposure (Gul *et al*., 2004; Avci et al., 2005). Antioxidant defense enzymes such as CAT and SOD have a remarkable importance for aquatic organisms because these enzymes protect them from free radicals that cause oxidative stress (Neha Pandey and Renu Bhatt, 2015). The present results showed that SOD activity generally decreased though CAT activity increased. CAT was also found to be the most sensitive antioxidant enzyme when compared to the others (Bharathi *et al*., 2014; Aslanturk *et al*., 2014). The increase in CAT activity may be related to cope with the increased oxidative stress caused by metal exposures, while the decrease may be related to possible direct binding of metal ions to -SH groups on the enzyme molecule.

Atli et al. (2006) demonstrated the significant alterations in CAT activity both in vivo and in vitro in different tissues of *O. niloticus* after acute and chronic metal exposures. Sensitivity of SOD and CAT activities to metal exposures were also supported with our previous results (Atli and Canli, 2007, 2010). Decreased SOD activity might be an indicator of damage in the antioxidant mechanisms caused by metal exposure. Thirumavalavan. (2014) also found variations in SOD and CAT activity in *Catla catla* after mercuric chloride exposures depending upon metal concentrations. They concluded that toxicants may induce different antioxidant/prooxidant responses depending on their ability to produce ROS. The response of the antioxidant system could differ when organisms exposed to metals and some other factors.

In the present studies of normal histological structure of fish liver tissues was some alteration when arsenic trioxide exposure. Liver is the main organ responsible for detoxification of harmful substances. Toxicants reach to liver through circulation. Histological the liver is made of roughly hexagonal hepatic lobules consist of cords of polygonal hepatic cells called hepatocytes concentrating towards the central vein. The histological changes on fish is a noteworthy and promising field to understand the extent to which changes in the structural organization are occurring in the organs due to environmental pollution. Toxicants induced changes in the liver of fishes can be regarded as an index for the identification of pollution stress in fishes (Pedlar *et al*., 2002; Figueired-Fernandes *et al*., 2007). Radhakrishnan and Hemalatha, (2010) mentioned cadmium chloride exposed *Channa striatus* freshwater fish, liver section of normal fish the hepatocytes form a rather cord like patter. These cords are arranged around tributaries of the hepatic vein. The liver cells are large in size, polygonal in shape with homogenous eosinophilic cytoplasm and

centrally located and separates the hepatic cords one from another (Roy and Bhattacharya, 2006). Histopathological alterations result depending upon the metal type and concentrations, length of exposure, fish species, and other physico-chemical factors. It is brought about due to increase or decrease in hepatic enzyme activities, (Bharathi *et al*., 2014; Paul *et al*., 2014). Hepatocytes may thus be expected to be the primary targets of toxic substances, providing an excellent biomarker of aquatic pollution (Braunbeck and Volkl, 1993; Paul *et al*., 2014). The acute toxic injury usually includes cloudy swelling or hydropic degenerations and pyknosis, karyorrhexis and karyolysis of nuclei (Jiraungkoorskul *et al*., 2003; Visoottviseth *et al*., 1999; Paul *et al*., 2014).

5. CONCLUSION

Present experiment demonstrated alteration in oxidative and antioxidant in liver tissues of fresh water fish *Cirrhinus mirgala* after exposure arsenic trioxide. During the experimental duration 7, 14, 21, 28 days. The histoarchitectural changes on liver tissues of *Cirrhinus mirgala* on arsenic trioxide exposure to compare to control.

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