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IDENTIFICATION OF HEAVY METAL TOXICITY INDUCED BIOMARKERS AND THE PROTECTIVE ROLE OF ASCORBIC ACID SUPPLEMENTATION IN *CHANNA PUNCTATUS*

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Keywords:

Metal toxicity, Lysosomal markers, Mitochondrial damage, Arsenic, Mercury, Ascorbic acid

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ABSTRACT: Arsenic and mercury are presently the most common pollutants of freshwater bodies. There is a continuous increase in the toxic level of these pollutants with some seasonal changes, affecting the aquatic biota. The present study aimed to identify cellular biomarkers of arsenic and mercury toxicity in freshwater fish *Channa punctatus*. Antioxidant defense like catalase (CAT), superoxide dismutase (SOD), ascorbate, reduced glutathione (GSH), oxidative stress marker lipid peroxidation (LPO), lysosomal marker like acid phosphatases and apoptotic marker namely caspases-3 were employed to check the damage caused to the fish as a result of arsenic and mercury contamination of water. Results indicate that increased lipid peroxidation induced apoptosis in arsenic toxicity. On the other hand, mercury toxicity induced necrosis mediated by lysosomal induction. These results further significantly indicate the protective effects of ascorbic acid that reduces the increased level of hepatic oxidative stress during metal toxicity. This study suggests that ascorbic acid supplementation can be a good option to save fish, which are at high risk of heavy metal-induced damage resulting in the availability of healthy edible fish in the market.

INTRODUCTION: With day by day, in the current scenario of increasing pollution in the environment it has become important to pay attention to long term effects of sub-lethal stress. The challenge is to determine if the individuals can live in a habitat that is critically under stress and also in identifying the impact of that chronic stress on the organism's health. Some organisms develop the possible way out to detoxify or to sequester the effects of toxicants and their combinations and thus exposures to stressors do not always result in adverse effects.

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However, not all the organisms have this capability of ameliorating the effects of stressors and their physiological functions are adversely affected, in fact, this leads towards the point where a particular population, dynamics, and sustainability of that population are endangered. Therefore, effective methods are required which can help in identifying when altered conditions of habitat affect the integrity of a biotic system, before it is very late to reverse the effects.

Cellular biomarker responses are some of the best methods to identify when conditions have exceeded the critical level, and the organisms are under stress, and if the stress is ignored for a prolonged period this may lead to a critical impact at the ecosystem level. Cellular biomarker responses are frequently used as effective diagnostic methods in biomedical applications, as warning signals of pathological conditions and evaluating the efficacy

of preventive tools. Membrane lipid peroxidation has mostly been considered to play an important role in the subcellular as well as tissue grade injury in the liver [1]. Also, various mitochondrial functions, lysosomal enzymes, and their functions have been reported to be affected due to lipid peroxidation [2]. Several workers have reported membrane lipid peroxidation as a major consequence of cell death [3, 4]. Therefore, in present work, the lipid per-oxidative liver damage was studied in freshwater fish intoxicated with arsenic and mercury together, and the efficacy of ascorbic acid against these metal toxicants was also studied.

MATERIALS AND METHODS: Freshwater fish *Chana punctatus* were procured from the fish farm. All fish were acclimatized in glass aquaria in dechlorinated water at laboratory conditions (25 ± 2 °C) for 1 month. Fish were fed with goat liver during acclimatization. For this experimental study, the heavy metals were procured from Fisher Scientific (Arsenic trioxide- Crystalline, Assay 99 %, and mercury chloride II-crystalline, Assay 99.5 %). LC_{50} (96 h) was estimated for the species *C. punctatus* for arsenic trioxide and mercury chloride by using Boyd [5] method.

After acclimatization for 15 days, fish were divided into seven groups for each metal and with 10 fish in each group. The metal concentrations which were given to different groups were as 0, 0.1, 0.25, 0.5, 0.75, 1.0 mg/l and 1.5 mg/l. After the estimation of 96 h LC_{50} values, the sub-lethal concentrations were calculated for the arsenic trioxide, mercury chloride, and in a combination of both metals.

For this experiment, the fish were divided into 7 groups (n=5), group 1served as control (without any metal treatment), group 2, 5 were exposed to the sub-lethal dose of arsenic trioxide (1 mg/l), group 3, 6 were exposed to mercury chloride (0.10 mg/l), group 4, 7 with both arsenic and mercury together for 15 days and then group 2, 3 and 4 were left untreated for next 15 days. Group 5, 6 and 7 are supplemented with ascorbic acid for the next 15 days. During the exposure period, the aquarium water was changed every alternate day and goat liver feed was given every day. There was no mortality during the experimental period.

Acclimatized fish were randomly selected with an average weight of 18 ± 2 gm and size 10 ± 2 cm. They were transferred to the experimental aquariums pretreated with the potassium permanganate solution to remove any kind of infection.

Sample Preparation: After 15 days, no more metal treatment was given and all the fish were sacrificed by using an aqueous solution of tricane methane sulphonate (1:4000). The fish were dissected and the liver tissues were preserved in the respective reagents in the deep freezer till further tests. The protein content in the preserved tissue samples was determined using the colorimetric method as described by the Lowry *et al.* [6]

Analysis:

Estimation of Metals in Tissue: To analyze the concentration of metals in liver tissues, tissues were first digested in pure nitric acid and then after full digestion, tissues were diluted with double distilled water. 100 mg of liver tissue was digested in 1 ml of concentrated nitric acid at 80 °C for 1 h. Atomic Absorption Spectrophotometer was used to calculate the concentration of metals in liver tissue (Perkin Elmer AA800).

Histopathological Study: To measure the degree of cellular damage as a result of arsenic and mercury toxicity in fish liver, small pieces of liver tissues were fixed and processed by the histological method as described by Gurr [7]. Liver tissue dehydration was done in a graded series of alcohol followed by xylene and embedded in paraffin wax. Liver tissues were fixed in formalin and embedded in paraffin. Embedded tissues were cut using a rotary microtome which was adjusted to produce a cutting rhythm at a thickness of 5 µm. After sectioning they were spread out at 45 ºC in water bath thereafter, sections were carefully attached to the slide by using an adhesive like bovine albumen. Sections were fixing at a hot plate set to 50 ºC to prepare the slides for staining. Further, the sections were de-paraffinized in xylene followed by descending grades of alcohol and water. Hematoxylin and counterstained with eosin (HE) was used to stain the liver sections and mounted in DPX then observed under light microscopy.

Electron Microscopic Studies (Transmission Electron Microscopy): Approximately 1 mm^3 of liver tissues were immersed in 2.5% glutar-aldehyde, postfix in 1.0% osmium tetra-oxide,

dehydrated through ethanol and embedded in Epon 812 after several changes of propylene oxide. Uranyl acetate and lead citrate were used to stain the ultra-thin sections followed by observation under a Phillips, CMIO Transmission electron microscope, at Sophisticated Analytical Instrument Facility of All India Institute of Medical Sciences, New Delhi.

Liver pathology severity index was assessed on the following basis: percent of liver cells containing fat (steatosis) was counted as 1 with less than 25% of the cell containing fat, 2 with 26% to 50%, 3 with 51% to 75%, and 4 with more than75% of the cell containing fat. Necrosis was assessed as the number of necrotic foci per square millimeter and inflammation was counted as the number of inflammatory cells per square millimeter. The number of apoptotic bodies per 10 high power fields (HPF) / sample was recorded and the mean no. of apoptotic bodies for each case was calculated. Apoptotic bodies seen in H and E sections of liver tissue were identified by the following features: Nuclear condensation, round to ovoid bodies, and karyorrhexis/karyolysis.

Biochemical Analysis:

Assay of Lipid Peroxidation and Antioxidant Enzymes: Frozen samples were homogenized in chilled phosphate buffer [0.1 M, pH (7.0)] at 11,500 rpm for 20 min at 4º and supernatant were used for further analysis.

Lipid Peroxidation as Oxidative Stress Marker: The lipid peroxidation in the liver tissue was assayed by determining malondialdehyde as described by Jordan and Schenkman [8] with some modifications. The optical density was taken at 532 nm the amount of thiobarbituric acid reactive substances (TBARS) was calculated. The unit expressed as nM MDA per mg protein.

NO in the Liver of Fish: Liver nitrite (NO_2^-) concentration, a stable metabolic product of NO with oxygen were assessed as indirect indicators of tissue NO levels. Alteration of nitrate (NO_3^-) into NO_2^- was carried out in the presence of elementary zinc. NO_2^- concentration in tissues was determined by the classic colorimetric Griess reaction. For the assay, equal volumes of a tissue sample and Griess reagent were mixed and the absorbance was measured at 570 nm. The concentration of $NO2^-$ was determined using sodium nitrite standard curve [9].

Reduced Glutathione (GSH) and Ascorbic Acid in Liver of Fish: Reduced glutathione (GSH) in the liver tissue was analyzed by using Ellman's' reagent [10]. Extract of liver was treated with trichloroacetic acid (TCA, 10% w/v) and centrifuged with rpm 8,900 for 15 min. 50 µl of supernatant was mixed with Tris-HCl buffer (230 µl, 0.8 M Tris/HCl with 0.02M EDTA, pH 8.9) and 0.01 M DTNB (5, 5-dithiobis (nitrobenzoic acid) - Ellman's reagent) 20 µl. The reaction mixture was then incubated at room temperature for almost 5 min. Absorbance was measured at 412 nm, and the concentration of GSH (nM GSH/mg protein) was calculated by using the GSH as the standard. The ascorbic acid concentration in tissue homogenates was measured by its oxidation using Cu^{+2} to form di-hydroascorbic acid, which reacted with acidic 4-dinitrophenyl hydrazine to give a red hydrazone. The red color was measured at 520 nm [11].

SOD and Catalases Activity: The activity of the antioxidant enzymes, SOD and catalases were measured in liver homogenates of fish. SOD activity was measured by generating superoxide radicals using photochemical reduction of phenazinemethosulphate, which reduces nitroblue-tetrazolium into a blue-colored compound, formazone. SOD quenches free oxygen radicals and inhibits reduction of nitroblue tetrazolium, which was measured at 560 nm [12]. Catalases activity was measured by observing the rate of hydrogen peroxide degradation at 240 nm in the presence of liver tissue [13].

Acid Phosphatase Activity as Lysosomal Marker: Acid phosphatase activity was measured using p-nitrophenyl phosphate as a substrate as reported by Ramponi *et al.* [14] One unit of acid phosphatase was defined as the amount of enzyme which liberates 1 Mmol of o-naphthol per min. In assay mixture had 0.4 ml 5 mmol/1 α-naphthyl-phosphate and 0.1 ml enzyme solution both in 0.1 mol/l sodium acetate (pH 5.5). The reaction was stopped after incubation, by adding a 0.1 ml solution of 1.25 g/1 Fast Red B in 5 mmol/1 H_2SO_4 followed by 0.8 ml 0.2 mol/l NaOH. The absorbance was measured at 600 nm.

Caspase 3 Activity in Liver Tissue: Caspases-3 activity in the fish liver was estimated with a DEVD-pNA substrate, using ApoAlert CPP32 protease assay kit [15], procured from CLONTECH Laboratories Inc., Palo Alto, California, USA. Absorbance was recorded at 405 nm.

Statistical Analysis: Statistical analysis was done by SPSS 16.0. Data were analyzed using a Levene test for homogeneity and variance. One way ANOVA followed by posthoc, Scheffe's multiple comparisons tests were applied to compare the difference between only metal, combinations of metal exposed group and control. Difference between mean set at a 5% ($p<0.05$) level was considered significant. Results are expressed as mean ± S.D.

RESULTS:

Metal Bioaccumulation Increased Oxidative Stress in Fish, and Ascorbic Acid Reduced the Changes: Increased concentration of metals was observed in all-metal treated groups, however, in combined metals group, a significant increase of mercury uptake was observed than in comparison to only mercury treated fish liver tissue. Ascorbic acid resulted in a significant reduction in metal content in all liver tissues of metal + ascorbic acid-treated fish ($P<0.05$) **Table 1**.

TABLE 1: SUMMARY OF METAL CONCENTRATION (mg/kg) OBSERVED IN LIVER OF *CHANNA PUNCTATUS* (n=5) EXPOSED TO DIFFERENT METALS AND METALS WITH VITAMIN C AFTER 15 DAYS

	Metal Conc.(mg/Kg)					
Groups	**As**	**Hg**	**As+Hg**	**As+VitC**	**Hg+VitC**	**As+Hg+VitC**
Control	ND	ND	ND	ND	ND	ND
As	0.61±0.04	ND	0.57±0.05	0.30±0.04*	ND	0.37±0.02*
Hg	ND	0.89±0.07	1.11±0.23*	ND	0.54±0.07*	0.65±0.07#

Values are expressed as mean ± SD of three replicate tanks. * Significant difference ($p<0.05$) with individual metal, # Significant difference ($p<0.05$) with metals in combination.

Next, we examined the metal-induced malonyl dialdehyde (MDA) production, a terminal compound of lipid peroxidation that is commonly used as an index of oxidative stress. Fifteen days of metal treatment resulted in a significant ($P<0.05$) increase in hepatic MDA levels in all three metal treatments when compared to control. On the other side, there was a significant ($P<0.05$) increase in lipid peroxidation in only arsenic-treated fish than in comparison to mercury and mercury + arsenic-treated fish **Fig. 1A**. Ascorbic acid treatment normalized the increase in MDA in metal treated fish ($P<0.05$; **Fig. 1B** and **1C**). Furthermore, the upsurge in oxidative stress in metal treated fish was linked with a significant increase in levels of NO in liver ($P< 0.05$; **Fig. 2A**). Ascorbic acid treatment significantly reduced the rise in liver NO levels in metal treated fish as shown in **Fig. 2B** and **2C**.

Ascorbic Acid Improved Levels of Endogenous Antioxidants that was Reduced After Metal Treatment: We next determined the impact of metal accumulation on antioxidant defense which is endogenous. As shown in **Fig. 3A**, metal treatment resulted in significant ($P<0.05$) reduction in reduced glutathione level of liver when compared to control fish. Ascorbic acid supplementation improved reduced glutathione levels significantly **Fig. 3B** and **3C**. Moreover, the metal treatment also reduced catalase activity in the liver when it was compared to the control group fish **Fig. 4A**. However, ascorbic acid supplementation after metal treatment reverted reduction of catalase activity **Fig. 4B** and **4C**. Likewise, ascorbic acid supplementation significantly amended the decrease in SOD activity in the liver in metal treated fish **Fig. 5B** and **5C**.

Ascorbic Acid Reduced the Lysosomal Proliferation and Cell Death Observed in Metal Treatment: Metal treatment raised lysosomal proliferation and acid phosphatase in the fish liver. There was a significant ($P<0.05$) increase in acid phosphatase in mercury and mercury +arsenic treated group than in comparison to only arsenic-treated fish **Fig. 6A**. However, in spite of lysosomal proliferation, acid phosphatases activity significantly ($P<0.05$) decreased in the liver homogenates of arsenic-treated fish **Fig. 6A**.

In arsenic treatment, liver cells underwent an apoptotic type of death, while in mercury and arsenic + mercury, it was necrotic cell death,

including damage to the organelle like mitochondria and lipofuscin formation **Fig. 8**.

The severity of liver pathology in fish treated with metals is shown in **Table 2**. No evidence of pathological changes was observed in control fish. Ascorbic acid supplemented fish showed only fatty liver, fish with only arsenic treatment showed fatty liver with apoptosis, while fish with mercury and mercury + arsenic treatment showed fatty liver and severe necrosis and inflammation **Fig. 9**. A significant increase (P<0.05) in caspases-3 activities were observed in the arsenic treated group than in comparison to rest metal treatment groups **Fig. 7A**, which could be the cause of apoptosis observed in arsenic-treated groups.

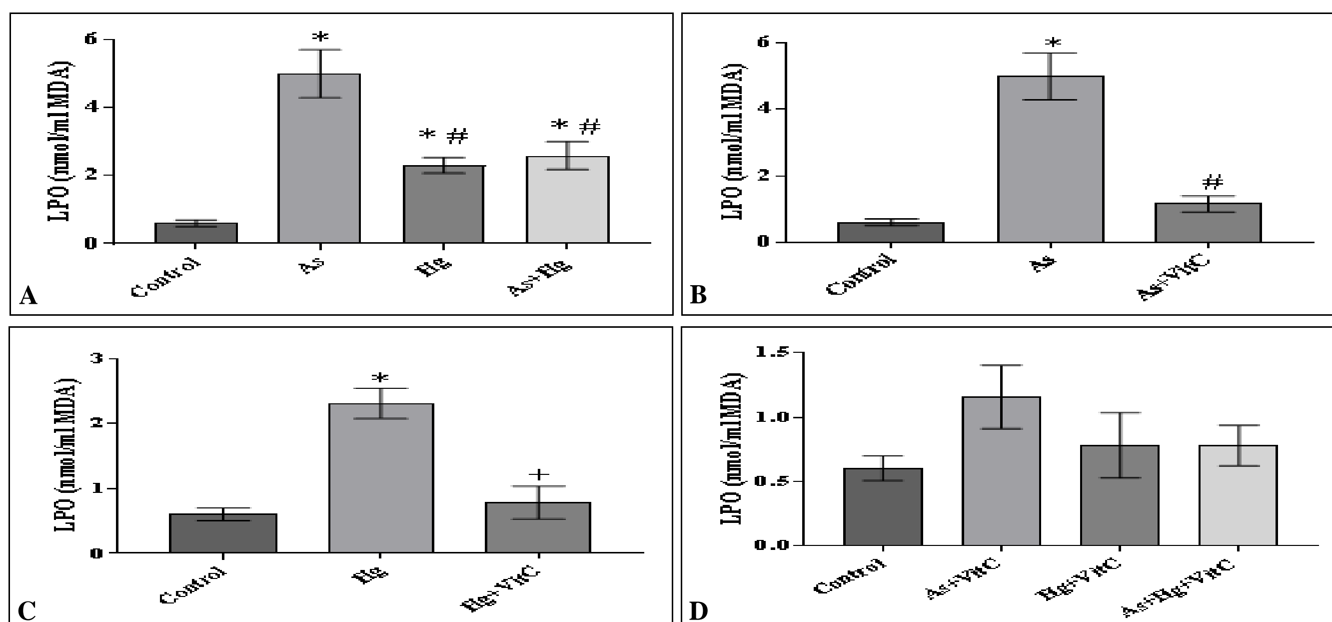

FIG. 1: LIPID PEROXIDATION IN THE LIVER OF *CHANNA PUNCTATUS* AFTER 15-DAYS OF EXPOSURE WITH TWO METALS (INDIVIDUAL AND IN COMBINATIONS) AND METALS WITH ASCORBIC ACID. Data represented as mean± SD (n=5). *Significant difference (p<0.05) with control fish. # Significant difference (p<0.05) with arsenic. + Significant difference (p<0.05) with mercury as compared to Hg + Vit C treated fish.

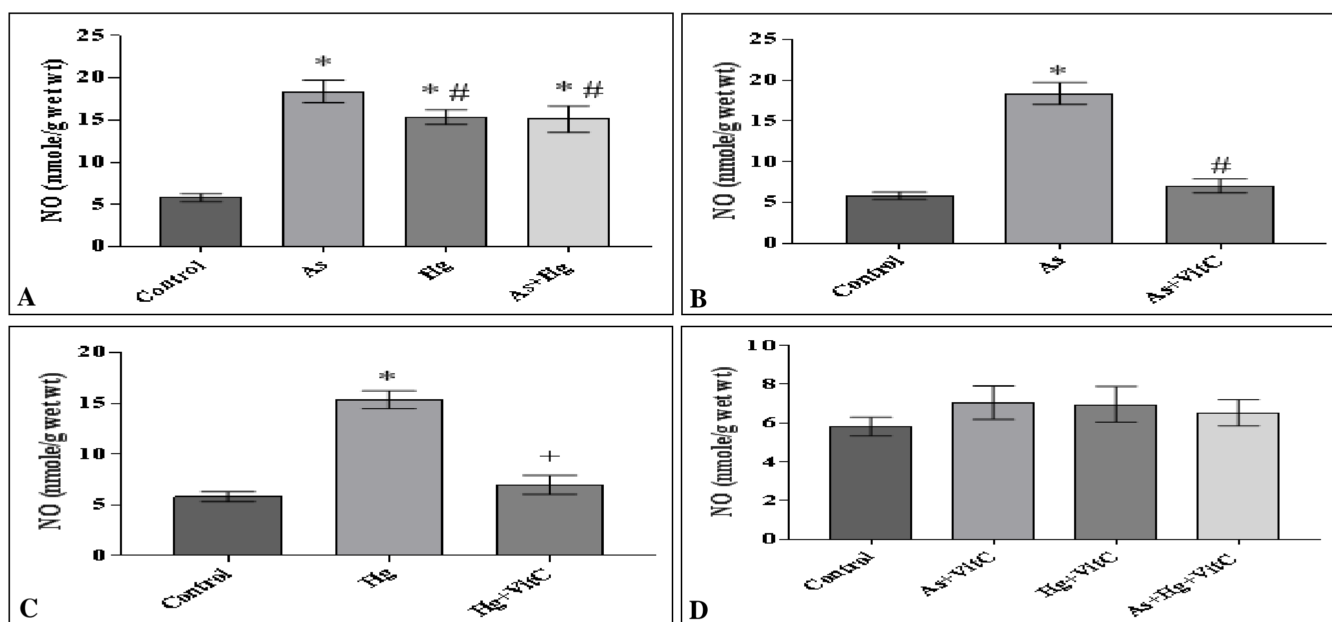

FIG. 2: NO LEVEL IN THE LIVER OF *CHANNA PUNCTATUS* AFTER 15-DAYS OF EXPOSURE WITH TWO METALS (INDIVIDUAL AND IN COMBINATIONS) AND METALS WITH ASCORBIC ACID. Data represented as mean± SD (n=5). *Significant difference (p<0.05) with control fish. # Significant difference (p<0.05) with arsenic. + Significant difference (p<0.05) with mercury as compared to Hg + Vit C treated fish.

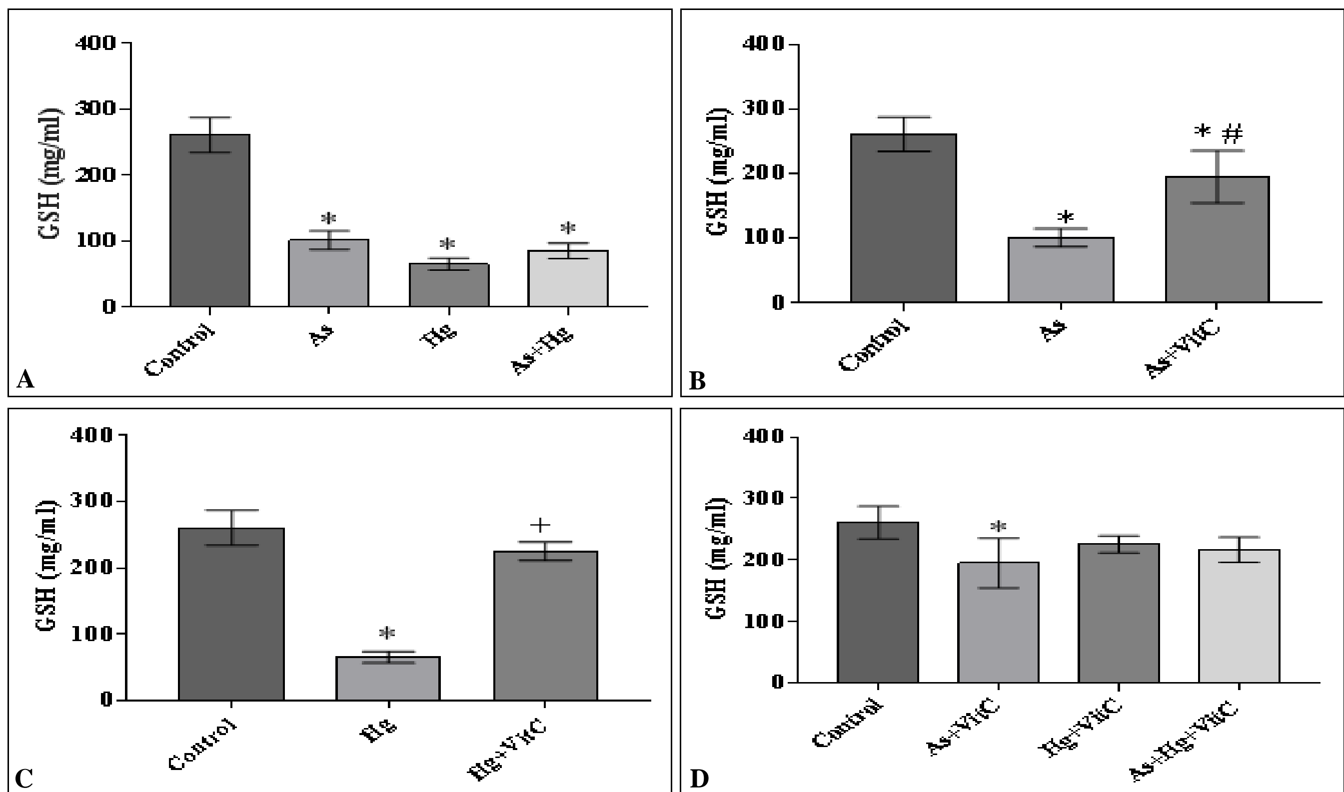

FIG. 3: LEVEL OF REDUCED GLUTATHIONE (GSH) IN THE LIVER OF *CHANNA PUNCTATUS* AFTER 15-DAYS OF EXPOSURE WITH TWO METALS (INDIVIDUAL AND IN COMBINATIONS) AND METALS WITH ASCORBIC ACID. Data represented as mean ± SD (n=5). *Significant difference ($p<0.05$) with control fish. # Significant difference ($p<0.05$) with arsenic. + Significant difference ($p<0.05$) with mercury as compared to Hg + Vit C treated fish.

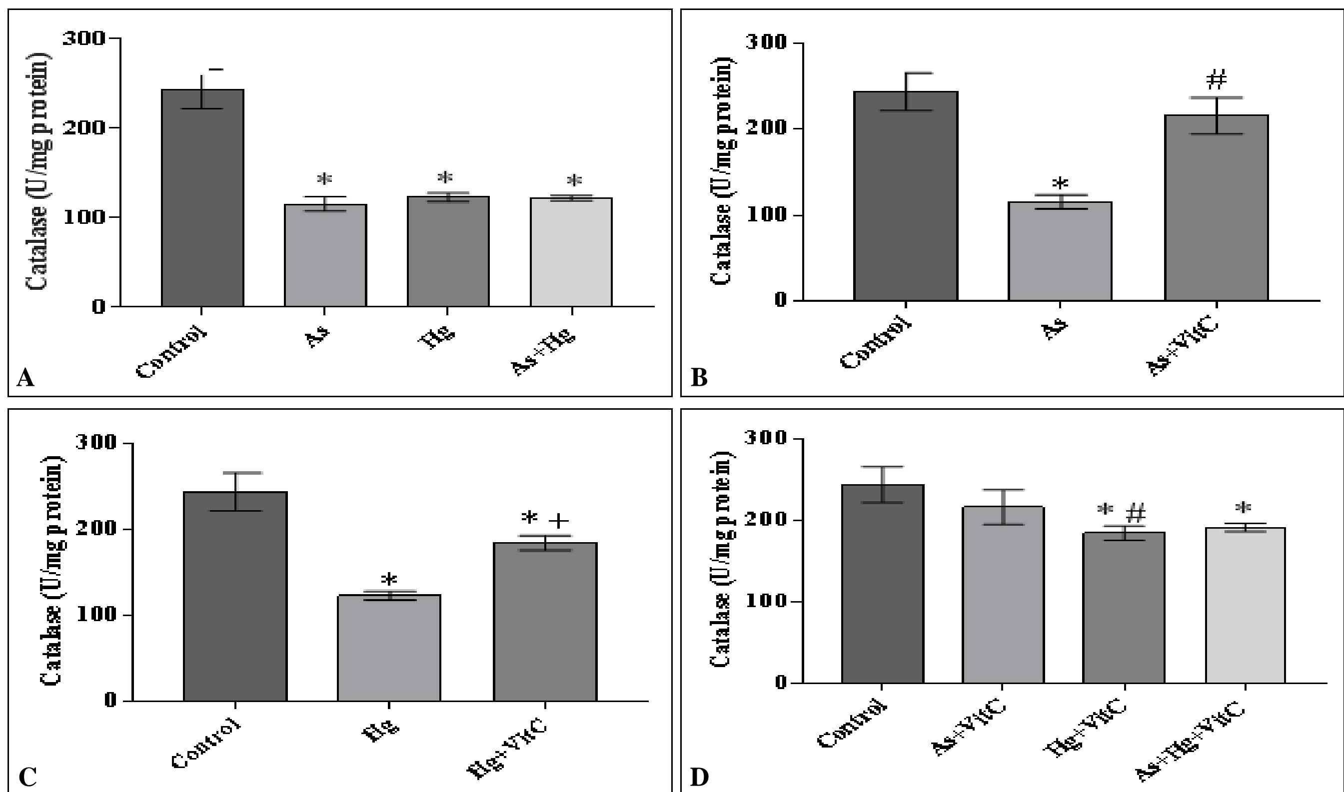

FIG. 4: CATALASE ACTIVITY IN THE LIVER OF *CHANNA PUNCTATUS* AFTER 15-DAYS OF EXPOSURE WITH TWO METALS (INDIVIDUAL AND IN COMBINATIONS) AND METALS WITH ASCORBIC ACID. Data represented as mean ± SD (n=5). *Significant difference ($p<0.05$) with control fish. # Significant difference ($p<0.05$) with arsenic. + Significant difference ($p<0.05$) with mercury as compared to Hg + Vit C treated fish.

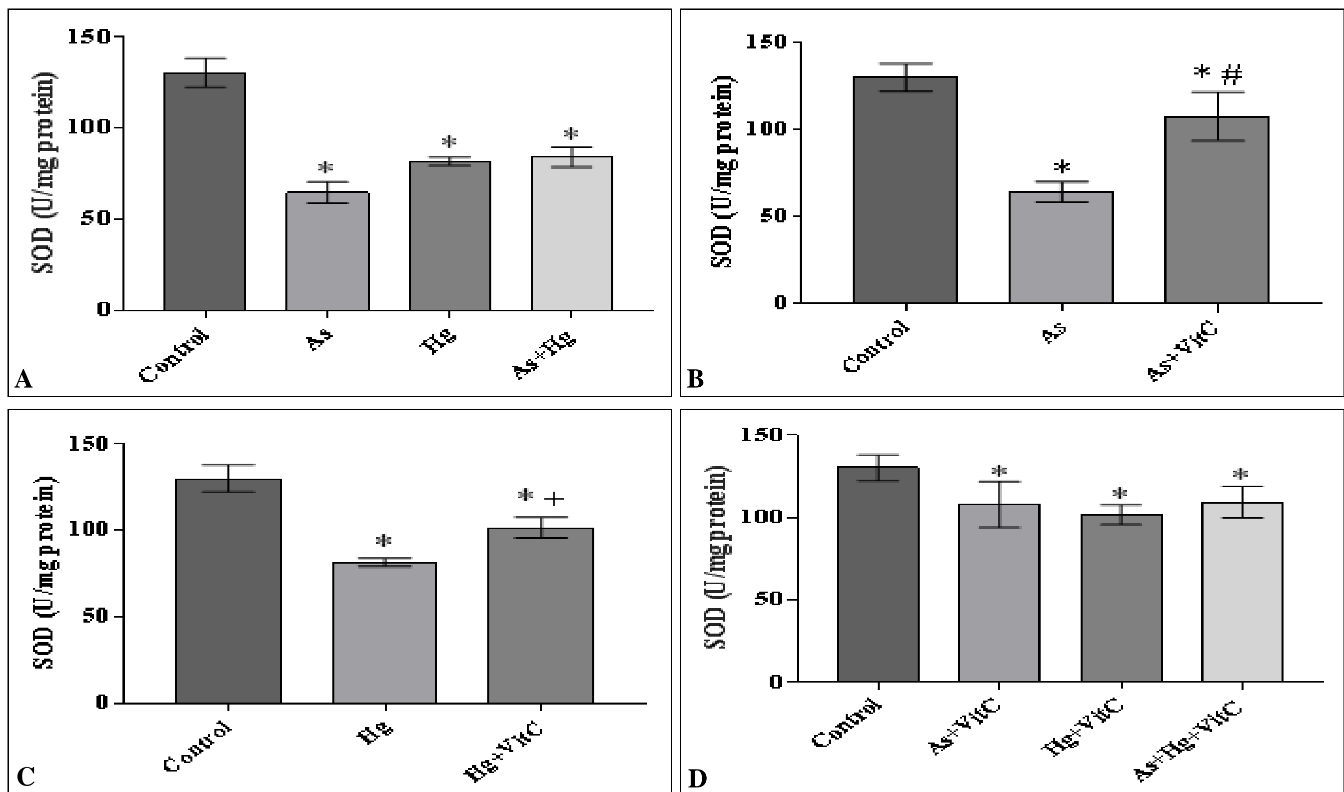

FIG. 5: SUPEROXIDE DISMUTASE (SOD) LEVEL IN THE LIVER OF *CHANNA PUNCTATUS* AFTER 15-DAYS OF EXPOSURE WITH TWO METALS (INDIVIDUAL AND IN COMBINATIONS) AND METALS WITH ASCORBIC ACID. Data represented as mean± SD (n=5). *Significant difference ($p<0.05$) with control fish. # Significant difference ($p<0.05$) with arsenic. + Significant difference ($p<0.05$) with mercury as compared to Hg + Vit C treated fish.

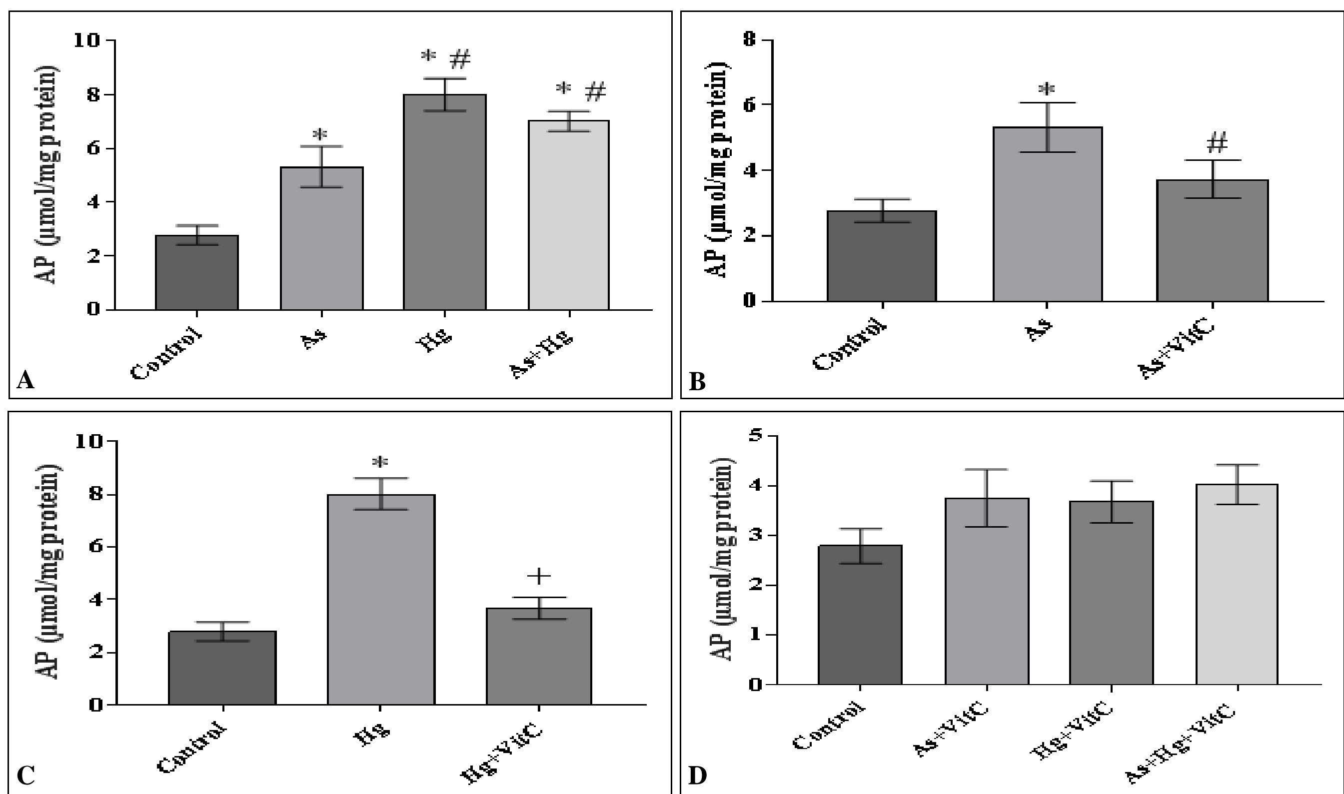

FIG. 6: ACID PHOSPHATASE (AP) ACTIVITY IN THE LIVER OF *CHANNA PUNCTATUS* AFTER 15-DAYS OF EXPOSURE WITH TWO METALS (INDIVIDUAL AND IN COMBINATIONS) AND METALS WITH ASCORBIC ACID. Data represented as mean± SD (n=5). *Significant difference ($p<0.05$) with control fish. # Significant difference ($p<0.05$) with arsenic as compared to As+VitC treated fsh. + Significant difference ($p<0.05$) with mercury as compared to Hg + Vit C treated fish.

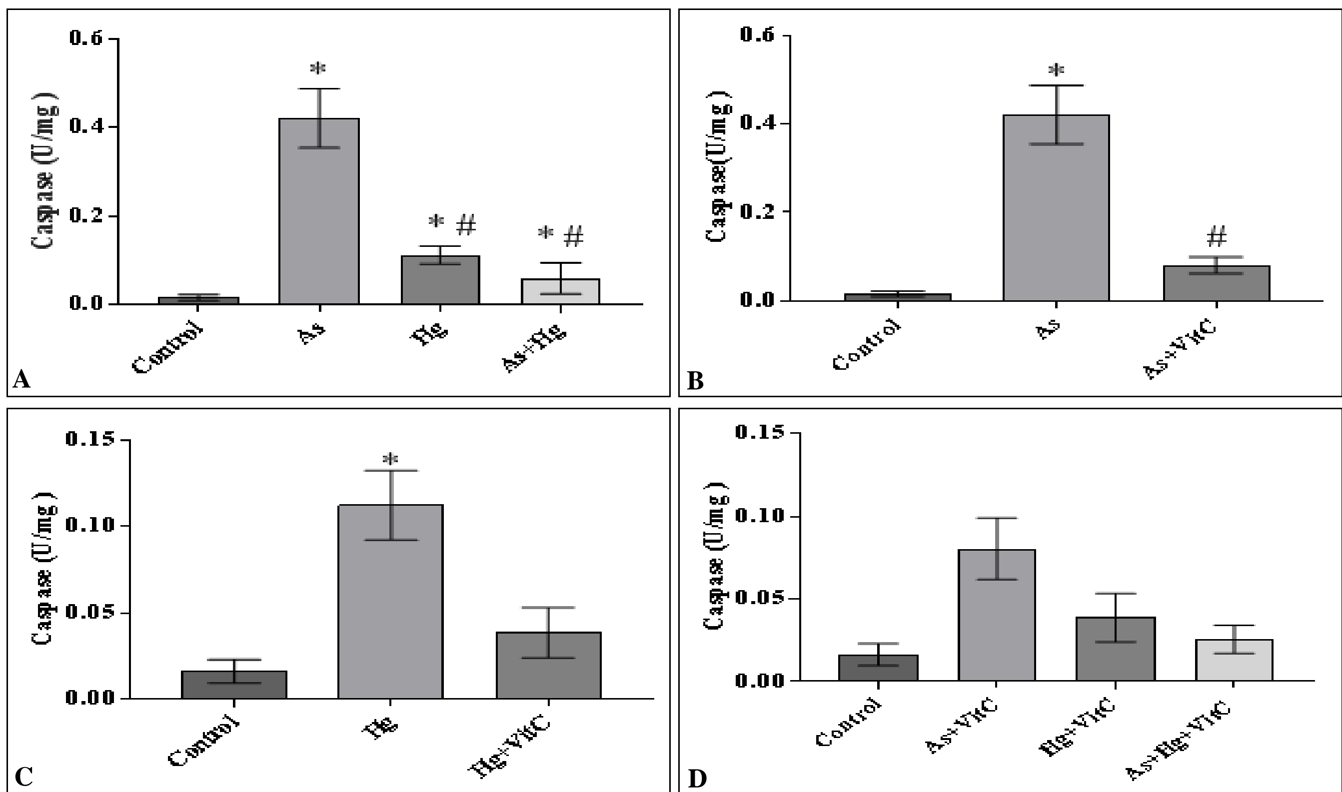

FIG. 7: ACTIVITY OF CASPASE IN THE LIVER OF *CHANNA PUNCTATUS* AFTER 15-DAYS OF EXPOSURE WITH TWO METALS (INDIVIDUAL AND IN COMBINATIONS) AND METALS WITH ASCORBIC ACID. Data represented as mean± SD (n=5). *Significant difference (p<0.05) with control fish. # Significant difference (p<0.05) with arsenic. + Significant difference (p<0.05) with mercury as compared to Hg + Vit C treated fish.

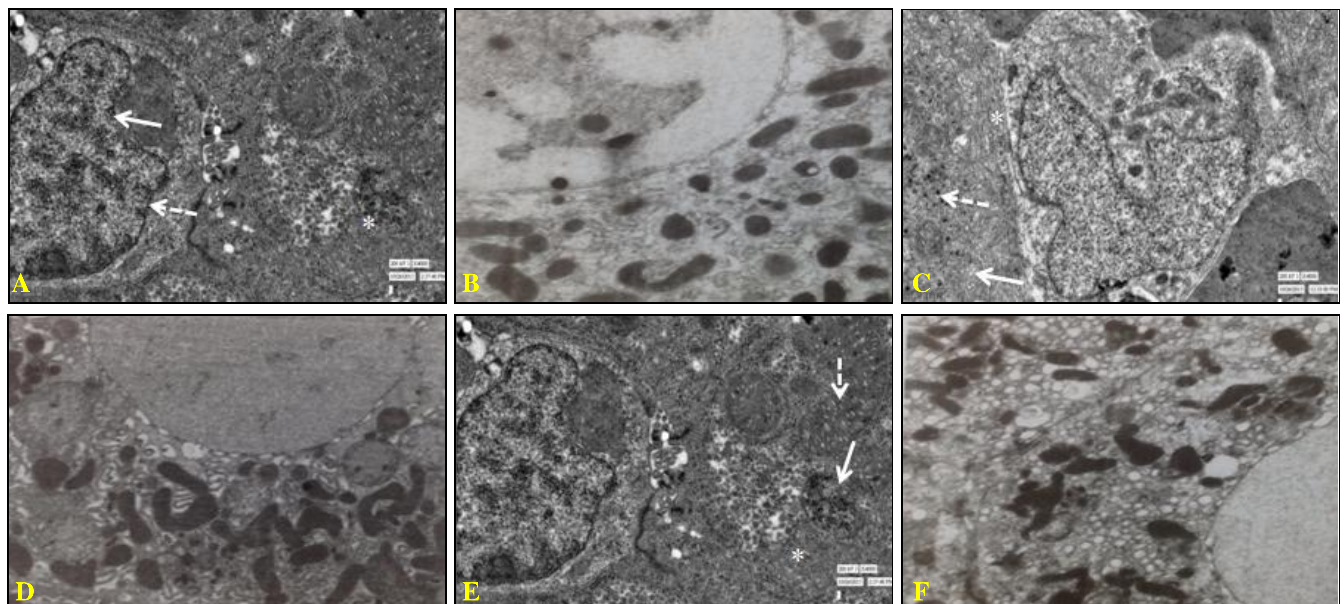

FIG. 8: PHOTOMICROGRAPHS OF LIVER TISSUE FROM *CHANNA PUNCTATUS*. A- LIVER OF FISH EXPOSED TO ARSENIC FOR 15 DAYS, SHOWING DEGENERATED NUCLEUS WITH MARGINALIZED CHROMATIN(DOTTED ARROW), FORMING APOPTOTIC BODY AT ONE END (ARROW), NUMEROUS SMALL DEGENERATING MITOCHONDRIA (*). B- LIVER OF FISH SUPPLEMENTED WITH As + Vit C. C- LIVER OF FISH EXPOSED TO MERCURY FOR 15 DAYS, SHOWING GIANT MITOCHONDRIA WITH CONDENSATION OF THE MATRIX (ARROW) AND MARKEDLY INCREASED ASSOCIATION WITH WIDE CISTERNAE OF ROUGH ENDOPLASMIC RETICULUM (*). INCREASED NUMBER OF PEROXISOMES AND ENDOSOMES (DOTTED ARROW) CAN BE ALSO SEEN. D- LIVER OF FISH SUPPLEMENTED WITH Hg + Vit C. E- LIVER OF FISH EXPOSED TO ARSENIC+ MERCURY FOR 15 DAYS, SHOWINGENDO-PHAGOCYTOSIS OF MITOCHONDRIA (ARROW), ENLARGED MITOCHONDRIA (DOTTED ARROW). LYSOSOMES SURROUNDED THE MITOCHONDRIA AND MITOCHONDRIA UNDERGOING AUTOLYSIS (*). F- LIVER OF FISH SUPPLEMENTED WITH As + Hg + Vit C.

TABLE 2: SUMMARY OF HISTOPATHOLOGICAL EFFECTS OBSERVED IN *CHANNA PUNCTATUS* (N=5) EXPOSED TO DIFFERENT METALS AND METALS WITH VITAMIN C AFTER 15 DAYS

Groups	Fatty liver (0-4)	Necrosis (foci/mm^2)	Inflammation (cells/mm^2)	Apoptosis (apoptotic bodies/10HPF)
Control	0±0	0±0	0.20±0.44	0.20±0.44
As	3.4±0.54*	0.40±.54	1±0.70	3.2±0.83*
Hg	3.4±0.89*	2±0.70*	4.2±1.30*	1.6±1.14
As+Hg	3.4±0.89*	2.2±0.83*	4.2±0.83*	2.4±0.54*
As+VitC	0.6±0.54	0.2±0.44	0.6±0.54	1.4±0.54
Hg+VitC	1±0.70	1.2±0.44	2.6±0.89*	0.6±0.54
As+Hg+VitC	1.4±0.54	1±0.70	1.8±0.83	0.6±0.54

Values are expressed as mean ± SD of three replicate tanks. *$P < 0.05$ versus control

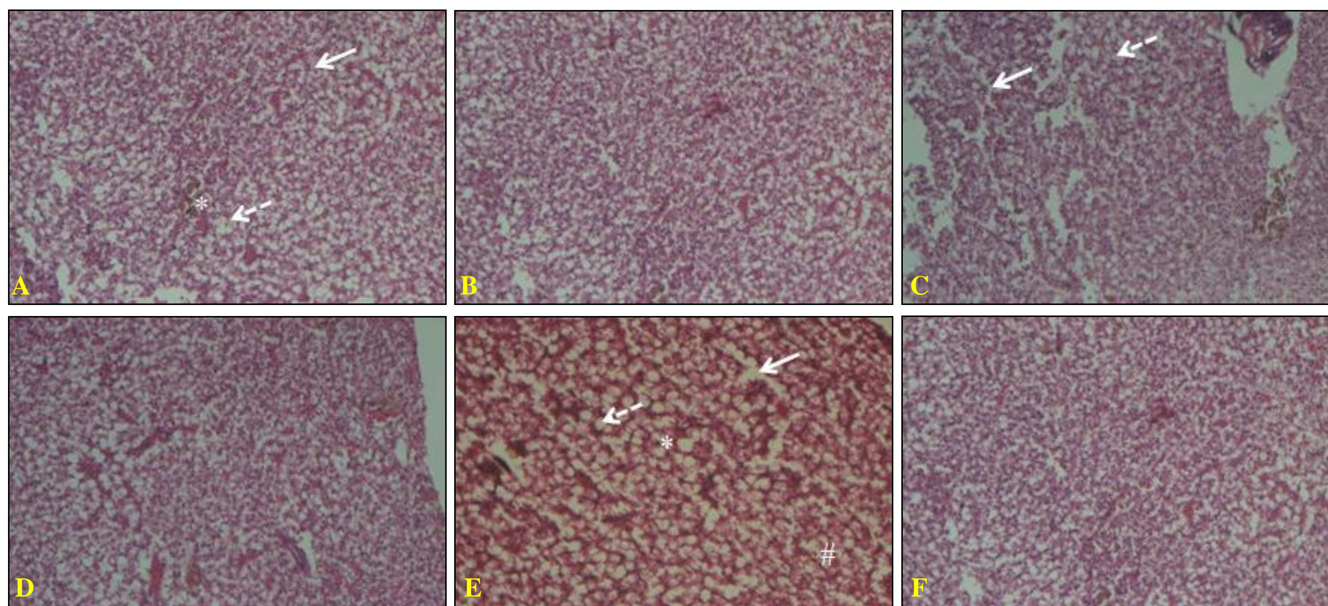

FIG. 9: PHOTOMICROGRAPHS OF LIVER TISSUE FROM *CHANNA PUNCTATUS*. A- LIVER OF FISH EXPOSED TO ARSENIC FOR 15 DAYS, SHOWING DS- DILATED SINUSOIDS (ARROW), E-EOSINOPHILIC GRANULES (*), FC- FOAM CELLS (DOTTED ARROW) (HE 40X). B- LIVER OF FISH SUPPLEMENTED WITH As + Vit C. C- LIVER OF FISH EXPOSED TO MERCURY FOR 15 DAYS, SHOWING DS- DILATED SINUSOIDS (ARROW), FL- FATTY LIVER DEPOSITION (DOTTED ARROW) (HE 40X) D- LIVER OF FISH SUPPLEMENTED WITH Hg + Vit C. E- LIVER OF FISH EXPOSED TO ARSENIC+ MERCURY FOR 15 DAYS, SHOWINGS- DILATED HEPATIC SINUSOIDS (ARROW), FC- FOAM CELLS (DOTTED ARROW) KC- KUFFER CELLS (*), NC- NECROTIC CELLS (#) (HE 40X). F- LIVER OF FISH SUPPLEMENTED WITH As + Hg + Vit C

DISCUSSION: The existing study established that metal accumulation in fish caused damage to antioxidant defense mechanisms and increased lipid peroxidation. Ascorbic acid treatment reduced oxidative stress caused by metal accumulation, at the same time it improved antioxidant defenses. The results suggest that ascorbic acid treatment could be a possible beneficial choice to fight against damages associated with arsenic and mercury toxicity.

In a previous study, the impact of arsenic and mercury on fish health has been reported [16, 17]. Ascorbic acid is commonly used as a chelator of various toxicants. The presence of dienol group in the molecule of the L-Ascorbic acid allows assuming its possible complexation of the molecule with metal ion [18, 19, 20]. Ascorbic acid is also a well-known antioxidant; its ability has been confirmed in various animals for conditions linked with oxidative damage [21, 22]. Increased heavy metal concentration in the cell is quite damaging since it initiates oxidative stress reactions and oxidative stress reactions propagate tissue damage reactions as they act as catalysts for lipid peroxidation [23, 24].

Lipid peroxidative damage is known for one of the molecular mechanisms of cell damage in acute methyl mercury poisoning [24]. High lipid peroxidation in liver, kidneys, lung, testis, and serum in of mercuric chloride intoxication to the rats was also reported in earlier studies [25].

Oxidative stress is well known for the pathogenesis and progression of many tissue damages, as well as metal-induced toxicity is well-known. Arsenic causes cellular injury by inducing oxidative damage [26]. In the earlier study, a tendency for a positive correlation between arsenic concentration and lipid peroxidation level in the liver, kidney, and heart of arsenic-treated rats was also reported [27]. A similar increase in lipid peroxidation was also observed in earlier study [28] as a result of high arsenic levels in the blood, liver, and kidney of rats.

In our study, all three metal treatments; arsenic, mercury, and arsenic + mercury damaged fish liver which was evident in histopathological observations as cell death. However, as per observations, cell death in arsenic-treated liver tissues was apoptotic, while in mercury and arsenic + mercury treated fish liver, it was necrotic cell death with subcellular inflammation, including damage to the organelle like mitochondria and lipofuscin formation. Lipofuscin has been reported as a cellular waste molecule that has lipids and proteins which originate due to incomplete lysosomal degradation of malfunctioning mitochondria [29]. When fish are exposed to environmental contaminants lipofuscin accumulates at higher rates in them [30]; therefore, its quantification may be valuable in conditions of chronic exposure.

The histopathological observations were also supported by caspases result, Caspase 3 activity was more in arsenic-treated fish liver than in mercury and arsenic + mercury treated fish liver. Lysosomal proliferation was observed in all-metal treated fish liver tissues. In a prior study, it was reported that permeability of mitochondria as a result of a partial lysosomal membrane protein can induce reactive oxygen species generation and apoptosis, while massive lysosomal membrane protein can cause cytosolic acidification and necrosis [31].

The current study also confirmed that arsenic accumulation in fish resulted in increased lipid peroxidation and apoptosis in liver tissue, while in mercury and arsenic+ mercury treated fish massive necrosis was observed. Products of lipid peroxidation have been reported to induce lysosomal dysfunctioning [32], and in this study after arsenic treatment in fish, lipid peroxidation inhibited the lysosomal enzymes and that may be the possible reason of apoptosis, while in mercury and mercury + arsenic-treated fish, lysosomal proliferation resulted in necrosis of liver tissue.

The results favor earlier studies as ascorbic acid administration reduced lipid peroxidation during metal toxicity [33]. The elevation in lipid peroxidation during metal treatment was linked with an increase in NO levels which might be one of the compensatory ways to quench the increased oxidative stress. This postulation is favored by the point that ascorbic acid-induced reduction in lipid peroxidation was also linked with a decrease in levels of NO during metal treatment.

This total oxidative stress reduction by ascorbic acid supplementation could also be endorsed to its capability to bring back endogenous antioxidant defense mechanisms in active mode [34]. The rise in oxidative stress in metal treated fish was linked with a decrease of assumed non-enzymatic (GSH) and enzymatic (catalase and SOD) antioxidant defense of liver in this study. Moreover, ascorbic acid supplementation not only reestablished the weakening quantity of GSH but also boosted up the activities of enzymatic antioxidants.

As the reduction of the antioxidant defense was reestablished by ascorbic acid supplementation, the present report proposes, ascorbic acid supplementation could lessen the increased level of oxidative stress in the case of metal toxicity not only by decreasing lipid peroxidation but also by reestablishing the loss of the exhausted endogenous antioxidant defense. Ascorbic acid has the ability to reinstate actions of catalases, reduced glutathione and superoxide dismutase, many reports have backed the finding that ascorbic acid is an outstanding reducing agent, it undergoes two successive oxidations to form the ascorbate radical (Asc˙-). Ascorbate is relatively unreactive owing to the stability of the unpaired electron and ascorbate oxidizes ascorbic acid to DHA; this reducing agent's purpose is to maintain the structure of enzymes, thus permitting the biochemical mechanism of cells and tissues functioning normally [19, 20]. Low electron potential and resonance stability of ascorbic acid make it an antioxidant.

Ascorbic acid plays the role of assembling reactive oxygen species, functioning as an antioxidant for conserving the intracellular redox balance, and reducing the oxidative damage caused by the free radicals [18]. These manifold roles of ascorbic acid envisage its use as a promising treatment for heavy metal toxicity, whether it be anyone, toxicity due to increased lipid peroxidation in arsenic treatment or lysosomal induced necrosis in mercury treatment.

Moreover, the limited ascorbic acid supplementation methods to fish present great hurdles in its application to protect fish from heavy metal pollution. Nonetheless, an upcoming trend will emphasize improving ascorbic acid treatment methods to save fish leading to the availability of healthy edible fish in the market. This is expected to take ascorbic acid as a valued therapeutic preference for fish with high exposure to heavy metal-induced damage.

CONCLUSION: Heavy metal pollution in the rivers deteriorates the fish's health, which severely affects the availability of food fish in the market. The present study indicates the different modes of oxidative stress-induced injury in response to two heavy metals. Moreover, antioxidant protection by ascorbic acid further confirmed the oxidative injury by arsenic and mercury. The study indicates that arsenic-induced lipid peroxidation results in apoptosis while mercury induces lysosomal proliferation that leads to necrosis. The study also reestablishes the protective effects of ascorbic acid against metal-induced toxicity in fish. Finally, it is suggested that ascorbic acid can be a good option to save fish which are at high risk of heavy metal-induced damage. In view of the high demand for healthy edible fish in the market, the study might play a pivotal role.

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