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Research Article

# Antioxidant Status in *Clarias Gariepinus* Challenged With Ammonium Chloride and Mercury Nitrate

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**Abstract:** This work investigated the effect of Ammonium chloride and Mercury nitrate in the liver of Clarias gariepinus. Twenty four (24) fish of Clarias gariepinus were randomly divided into control and experimental groups. Group A (Control) Fish received palm fruits only as diet. Group B Fish received 10 mg/l of Ammonium chloride with palm fruits. Group C Fish received 0.1 mg/l of Mercury (II) nitrate with palm fruits. Group D fishes received 10 mg/l of Ammonium chloride and 0.1 mg/l Mercury II nitrate combined with palm fruits. At the end of experimental period, reduced glutathione (GSH) and lipid peroxidation levels and the activities of Myeloperoxidase (MPO), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) in liver were assayed. The levels of oxidative stress biomarkers and the activities of the enzymes assayed were significantly increased in fish exposed to ammonium, mercury and both. The changes in these parameters were intensified exposed to both ammonium and mercury. The significance of these alterations in enzyme activities is discussed.

**Keywords:** Clarias gariepinus, glutathione S-transferase.

## INTRODUCTION

Contamination of fresh water with a wide range of pollutants has become a matter of concern over last few decades because of population density and industrialization (Vutukuru, 2005). Heavy metals released from domestic, industrial and other man made activities may contaminate the natural aquatic system extensively (Velez, 1998). Heavy metals have devastating effects on ecological balance of the recipient environment and a diversity of aquatic organisms (Farombi *et al.*, 2007). Heavy metals and chemicals are toxic to animals and may cause death or sublethal pathology of liver, kidneys, reproductive system, and respiratory system in both invertebrate and vertebrate animals. Accumulated chemicals may lead to morphological alterations in the tissues of fish (Monteiro *et al.*, 2005).

Environmental exposure to Hg can interfere with physiological as well as biochemical activities through oxidative stress (Verlecar *et al.*, 2007). This stress is generated in cells through an increase in the production of reactive oxygen species (ROS) as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (•OH), or decrease in the antioxidant that modifies ROS to the less reactive intermediate (Miller and Evans-Rice, 1997).

The dominant route of exposure to mercury is through the ingestion of fish. Most fish, both freshwater and saltwater fishes absorbed into the circulation, mercury enters erythrocytes where more than 90% will be found bound to hemoglobin (Clarkson, 2002).

According to our knowledge, there is no or little information on whether the combination of mercury and ammonium induces oxidative stress in fish liver. Therefore, the present study investigated the activities of antioxidant enzymes when exposed to both ammonium and mercury.

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## MATERIALS AND METHODS

### Fish Husbandry

*Clarias gariepinus* were obtained from the fish pond in Niger Delta University. The fish were transported in plastic container. Fish of nearly equal size (24) were distributed in 40 L plastic aquaria. Four fish were placed in every aquarium. The fish were acclimated to the laboratory conditions for one week. Group A (Control) Fish received palm fruits only as diet. Group B Fish received 10 mg/l of Ammonium chloride with palm fruits. Group C Fish received 0.1 mg/l of Mercury (II) nitrate with palm fruits. Group D fish received 10 mg/l of Ammonium chloride and 0.1 mg/l Mercury II nitrate combined with palm fruits. The experiment lasted for 7 days. The fish were then sacrificed by a sharp blow on the head. The fish were immedately dissected and the liver was quickly removed and washed in ice-cold 1.15%, KCl solution blotted and weighed. They were then homogenized in 9 volumes of homogenizing buffer (50 mM Tris - HCl mixed with 1.15% KCl and pH adjusted to 7.4), using Teflon Homogenizer. The resulting homogenate was centrifuged at 10,000g for 20 min in a Beckman centrifuge at – 40 $^0$C. The supernatant was decanted and stored at -20 °C until biochemical analysis.

### Protein Content

Protein precipitated from 0.1 ml of supernantant with an equal volume of 10% ice-cold trichloroacetic acid was solubilized in a known volume of 0.1 N NaOH solution. The quantity of protein in the sample was determined following the method of Lowry et al. (1951) using a standard curve prepared with bovine serum albumin.

### Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was assessed by measuring the $H_2O_2$-dependent oxidation of o-dianisidine according to the method of Bradley et al (1982). An aliquot of 0.1ml of liver sample was mixed with 50 mM phosphate buffer pH 6.0 containing 0.129mg/ml O-dianisidine and 0.005 % $H_2O_2$ in a final volume of 3ml. absorbance change at 460 nm was measured. One unit of MPO activity was defined as that degrading one micro mole of peroxide/minute at 25 $^0$C.

### CALCULATION

MPO (Units/mg protein) = Δ 460 nm / 0.0113 × volume of sample × mg of protein

### Superoxide dismutase (SOD) activity

The SOD activity was measured by the method of Marklund and Marklund (1974). The reaction mixture consisted of 2.875 ml Tris–HCl buffer (50 mM, pH 8.5), pyrogallol (24 mM in 10 mM HCl) and 100 uL of liver homogenate in a total volume of 3 ml. The enzyme activity was measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

### Catalase Activity

Catalase (CAT, EC 1.11.1.6) activity was assayed according to the method of Cohen et al. (1970). One milliliter of 50mM phosphate buffer (pH 7.4) and 10 uL of liver homogenate was added to the cuvette. The reaction was then initiated by the addition of 300 uL of 30mM $H_2O_2$ prepared by diluting 0.34mL of 30% $H_2O_2$ to 100mL of 50mM phosphate buffer (pH 7.4). Specific catalase activities were determined following the changes in the absorbance of $H_2O_2$ at 240nm ($\varepsilon$ = 0.0394 $mM^{-1}$ $cm^{-1}$ at 240 nm).

### Glutathione-S-transferase

Glutathione-S-transferase (GST, EC 2.5.1.18) activity was assayed according to the method of Habig et al. (1974). The final reaction mixture contained 1mM CDNB, 1mM GSH in 50 mM phosphate buffer pH 7.4 and the reaction was initiated by the addition of 50 uL liver homogenate. Specific GST activities were determined following the changes in the absorbance of CDNB per min at 340nm ($\varepsilon$ = 9.6.00 $mM^{-1}$ $cm^{-1}$ at 340 nm).

### Measurement of malondialdehyde (MDA)

The assay for liver homogenate lipid peroxidation was done by the method of Wright et al. (1981) with some modifications. The reaction mixture in a total volume of 3.0 ml contained 1.0 ml tissue homogenate, 1.0 ml of TCA (10%), and 1.0 ml TBA (0.67%). All the test tubes were placed in a boiling water bath for a period of 45 min. The tubes were shifted to ice bath and then centrifuged at 2500g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the nmol MDA formed/min/gram tissue by using a molar extinction coefficient of 1.56 x $10^5$ $M^{-1}$ $cm^{-1}$.

### Glutathione content

The GSH content in liver homogenate was determined by the method of Jollow et al. (1974) in which 1.0 ml of PMS fraction (10%) was mixed with 1.0 ml of sulphosalicylic acid (4%). The samples were incubated at 4 $^0$C for at least 1 h and then subjected to centrifugation at 1200g for 15 min at 4 $^0$C. The assay mixture contained 0.4 ml filtered aliquot, 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (10 mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on spectrophotometer. The GSH content was calculated as nmol of DTNB conjugate formed/g tissue using molar extinction coefficient of 13.6 x $10^3$ $M^{-1}$ cm

**Histopathology**

The liver were excised, flushed with saline, cut open longitudinally along the main axis, and then again washed with saline. These liver sections were fixed in 10% buffered formalin for at least 24 h and after fixation, the specimens were dehydrated in ascending grades of ethanol, cleared in benzene, and embedded in paraffin wax. Blocks were made and 5µm thick sections were cut from the liver. The paraffin embedded liver tissue sections were deparaffinized using xylene and ethanol. The slides were washed with phosphate buffered saline (PBS) and permeabilized with permeabilization solution (0.1M citrate, 0.1% TritonX-100). These sections stained with haematoxylin and eosin and were observed under light microscope at 40X magnifications to investigate the histoarchitecture of liver.

**Statistical Analyses**

Results are presented as means±standard deviation. The statistical evaluation of all data was done using one-way analysis of variance (ANOVA) followed by Dunnett's test. P values < 0.05 were regarded as statistically significant.

## RESULTS

**Table 1 Glutathione and lipid peroxidation levels, Glutathione-S-transferase, catalase, superoxide dismutase and myeloperoxidase activities in liver of clarias gariepinus exposed to ammonium chloride and mercury nitrate.**

| | GSH | LPO | GST | CAT | SOD | MPO |
|---|---|---|---|---|---|---|
| **A** | 5.65 ± 0.11 | 2.37 ± 1.3 | 9.65 ± 0.11 | 12.38 ± 0.73 | 7.53 ± 0.25 | 3.05 ± 0.35 |
| **B** | 2.59 ± 0.76* | 5.13 ± 2.3* | 2.59 ± 0.76* | 3.63 ± 1.38* | 3.12 ± 1.22* | 6.94 ± 0.31* |
| **C** | 2.03 ± 0.10* | 4.02 ± 2.08* | 4.03 ± 0.10* | 4.60 ± 0.16* | 3.39 ± 0.49* | 7.81 ± 0.10* |
| **D** | 0.29 ± 0.03* | 7.17 ± 2.0* | 2.29 ± 0.02* | 5.45 ± 0.12* | 2.82 ± 0.18* | 9.40 ± 0.15* |

Each reading represents mean ± SD of 5 fish.
* Significantly different from the control value, P ≤ 0.05.

**GSH (nmol DTNB formed/mg proten)**
**LPO (nmol MDA formed/mg proten)**
**GST (nmol CDNB formed/mg proten)**
**CAT (Units/mg protein)**
**SOD (Units/mg protein)**
**MPO (Units/mg protein)**

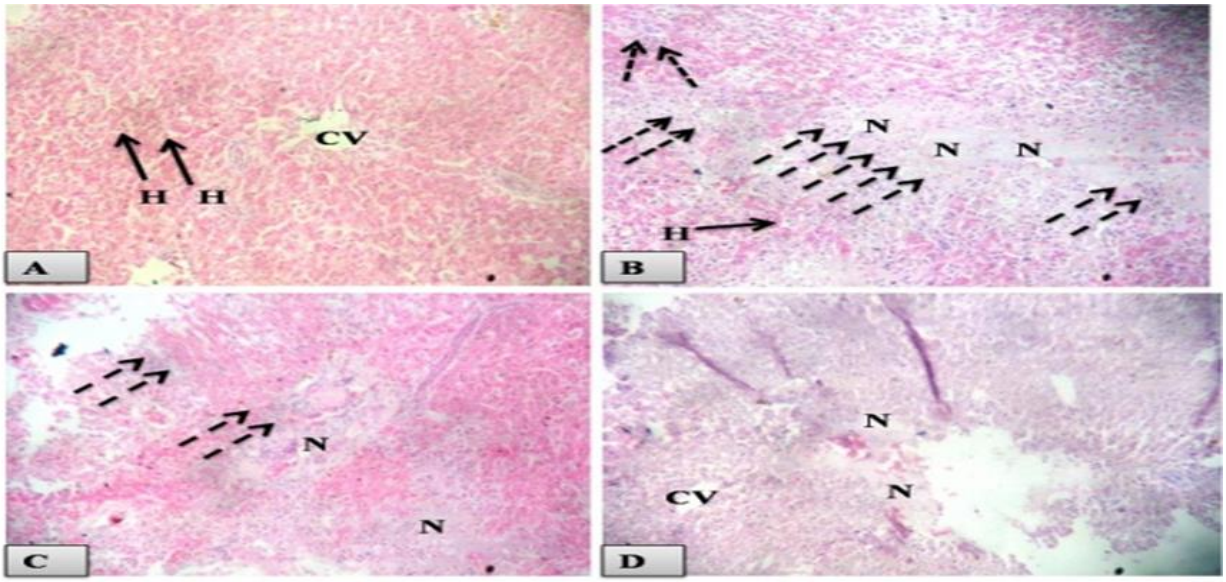


**Figure 1: Photomicrograph of Liver.**

Group A (Control)shows normal central vein (**CV**) with well radiating Hepatocytes (**H**). Groups B,C and D show degenerative hepatic stroma characterized by infiltration of inflammatory cells (**Dash arrows**) and focal necrosis (**N**) when compared with the GroupA (control). H&E x100

## DISCUSSION

The fish liver is a vital organ concerned with basic metabolism and is the major organ of accumulation, biotransformation and excretion of contaminants in fish (Figuiredo-Fernandes *et al.*, 2006). Impact of contaminants on aquatic ecosystems can be evaluated by measuring biochemical parameters in the liver of fish that respond specifically to the degree and

type of contamination. The liver is particularly susceptible to damage from a variety of toxicants. One of the most important functions of liver is to clean pollutants from the blood so it is considered as indicator of aquatic environmental pollution (Figuiredo-Fernandes *et al.,* 2006).

Lipid components of membrane undergo lipid peroxidation as a result of action of free radicals. During lipid peroxidation (LPO) a large number of toxic byproducts are also formed that behave as 'second messengers'. The damage caused by LPO is highly detrimental to the functioning of the cell (Devasagayam *et al.,* 2003). The levels of LPO increase significantly in group B, C, and D as compared to group A. This increase may be due to the toxicity of ammonium chloride or mercury nitrate because both chemicals are linked to oxdative stress (Hegazi *et al.,*2010). The level of LPO in group D is more pronounce because it is the combination of both ammonium and mercury. These results are in line with the results Hegazi *et al.,* 2010 who also reported increase in LPO exposed to ammonium.

GSH is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds (Sapakal *et al.,* 2008). The levels of GSH decrease significantly in group B, C, and D as compared to group A. This decrease may be due to the toxicity of ammonium chloride or mercury nitrate because both chemicals are oxidants in biological systems (Cogun *et al.*, 2012). The decrease in the levels of GSH could be that ROS increase overwhelms the concentration of GSH in cells.

Phase II reactions are conjugation reactions that involve the addition of polar groups to the foreign xenobiotic molecules. GSTs are an important phase II enzyme that catalyzes the reaction between GSH and the hydrophobic or electrophilic compound (Sapakal *et al.,* 2008). CAT is a common antioxidant enzyme present almost in all living tissues that utilize oxygen. The enzyme uses either iron or manganese as a cofactor and catalyzes the degradation or reduction of hydrogen peroxide ($H_2O_2$) to water and molecular oxygen, consequently completing the detoxification process imitated by SOD (Chelikani *et al.,* 2004). Superoxide dismutase is one of the most potent intracellular enzymatic antioxidants and it catalyzes the conversion of superoxide anions to dioxygen and hydrogen peroxide. The hydrogen peroxide is removed by catalase or glutathione peroxidase (Sheng *et al.,* 2014). Our present study reveals significant decrease activities of GST, CAT and SOD in fish exposed to ammonium and mercury as compared to control values. These decrease values of these antioxidant enzymes will increase the levels of ROS these may inhibit the activities of these antioxidant enzymes.

Myeloperoxidase (MPO) is a heme-containing enzyme that catalyzes the hydrogen peroxidase-mediated oxidation of halide ions to hypohalous acid. MPO is a lysosomal protein, highly expressed in neutrophils, that plays a role in the antimicrobial actions that occur as a result of neutrophil stimulation. The actions of MPO may also contribute to the initiation and pathogenesis of inflammatory related disease (Oday *et al.,* 2014). MPO activity was markedly elevated in groups B, C, D, the elevated activity of MPO can be due to the elevated levels of $H_2O_2$ in the ammonium and mercury exposed fish as compared to control.

Histopathological examination of the liver of *Clarias gariepinus* shows specific pathological alteration in the liver especially groups B, C and D as compared with the control.

## CONCLUSION

Our findings indicate that exposure of *Clarias gariepinus* to ammonium and mercury combined induced oxidative stress resulting in significant increase in the levels of LPO and decreased levels of GSH. The use of antioxidant defense systems (SOD, CAT and GST) biomarker for detecting pollution stress in fish can be suggested as a valuable biological indicator.

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