Evaluation of the antioxidant markers in *Clarias gariepinus* heart exposed to mercury and ammonium chloride

Abstract

Reactive Oxygen Species (ROS) parameters and antioxidant enzyme activities in heart of *Clarias gariepinus* weighing (15 ± 1.7 g) in toxic exposure to mercury concentrations, 0.1 mg/ L group B, group C 10 mg/L ammonium chloride and group D 0.1 mg/L mercury and 10 mg/L ammonium chloride for 7 consecutive days at 26 ± 1 ◦C temperature group A was exposed to water alone and serve as control. At the end of the experimental period, Reduced glutathione (GSH), NO levels and the activities of Peroxidase, Superoxide dismutase (SOD) and Alanine Amino Transferase (ALT) in heart were assayed. The result showed a significant increase (P <0.05) in the activities of ALT, Peroxidase, and SOD. However, a significant decrease in the levels of total protein content, GSH and NO were also observed. The results showed that the combined effect of mercury and ammonium had an adverse effect greater than that obtained individually.

Introduction

Fish constitutes an important aspect of human food due to the high level of quality protein and essential amino acids for the proper growth and functioning of body muscles and tissues. Fish are commonly situated at the top of the food chain and therefore, they can accumulate large amount of toxicants (Alaa *et al.,* 2010). Fish are considered as one of the most susceptible aquatic organisms to toxic substances present in water (Enkeleda *et al.,* 2013). Fish are also usually considered an organism of choice for assessing the effects of environmental pollution on the aquatic ecosystem. In nature, aquatic animals are constantly exposed to toxic substances (Ijeoma *et al.,* 2015). Rapid industrial development, as well as the use of metals in production processes, has led to the increased discharges of toxic substances into the environment (Borham and Rahimeh, 2011).

Mercury occurs naturally as a mineral and is widely distributed throughout the environment as a result of natural and human activities. Inorganic mercury is the most common form of metal released by industries in the environment. Mercury (Hg) is a famous contaminant to aquatic life (fish and marine organisms). Hg accumulates in many fish species and causes toxicity to various organs (Bano and Hasan 1990; Jalila *et al.,* 2012). On the other hand Ammonium Chloride (NH_4Cl) is an inorganic, mildly acidic, white crystalline salt compound that is highly soluble in water. The toxicity of ammonia (NH_3) and its compounds such as Ammonium chloride (NH_4CL) have been ascribed to the fact that it can readily diffuse through the gill membranes due to its high solubility (Hegazi and Hasanein 2010). Free radicals and ROS are produced by a variety of oxidative enzymes. Metabolic processes and metal-catalyzed oxidation (Halliwell and Gutteridge, 1990). It is therefore of great significance to evaluate pollution effects on fish for both environmental protection and socio-economic reasons (Farombi *et al.,* 2007; Okomoda *et al.,* 2010). That is why this study investigated the toxicities of mercury and ammonium chloride on some antioxidant markers.

Materials and methods

Chemicals

Trishydroxyaminomethane Hydrochloric acid pyrogallol hydrogen peroxide sulphosalicylic acid DTNB Bovine Serum Albumin

Fish husbandry

Clarias gariepinus were obtained from the Niger Delta University fish farm. The fish were transported in a plastic container. Fish of nearly equal size weighing (15 ± 1.7 g) 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 immediately dissected and the heart 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 supernatant 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.

Peroxidase activity

The peroxidase activity was measured following the method of Wadhwa et al. (1988). In the experimental set, the reaction mixture included 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.0), 1.0 ml pyrogallol (0.005 M), 1.0 ml of a 0.05 M hydrogen peroxide "H_2O_2" solution and 1.0 ml heart supernatant (crude enzyme preparation) in a total volume of 5 ml. The reaction was started by adding 1 ml of supernatant to each of experimental. After 5 min at 25°C, the reaction was stopped by adding 0.5 ml H_2SO_4 (5% v/v). The optical density was measured at 430 nm in a JASCO 7800 UV/visible spectrophotometer (Japan). After suitable corrections with the control, the enzyme activity was expressed as optical density (OD) of purpurogallin formed/mg protein/5 min.

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 heart 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%.

AST Activity

Briefly, 0.1ml of heart was mixed with phosphate buffer (100mmol/L, pH 7.4), L-aspartate (100mmol/L), and α-oxoglutarate (2mmol/L) and the mixture incubated for exactly 30min at 37^{o}C. Then 0.5ml of 2, 4-dinitrphenylhydrazine (2mmol/L) was added to the reaction mixture

and allowed to stand for exactly 20min at 25^0C. Then 0.5 ml of NaOH (0.4mol/L) was added and the absorbance at 546nm read against the reagent blank (Reitman and Frankel 1957).

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 colour 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

Nitrite estimation in plasma

Nitrite assay was done using Griess reagent by the method of Green *et al*.1982 with some modifications. In brief, 100 µL of Griess reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water) was added to 100 µL of heart sample incubate for 10 min at room temperature protected from light. Purple/magenta colour began to form immediately. Absorbance was measured at 546 nm, nitrite concentration was calculated using a standard curve for sodium nitrite, and nitrite levels were expressed as nmol/mg protein.

Histopathology

The heart tissues were excised, flushed with saline, cut open longitudinally along the main axis, and then again washed with saline. These heart 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 heart. 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 a light microscope at 40x magnifications to investigate the histoarchitecture of heart.

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 nitric oxide levels, Peroxidase, superoxide dismutase and ALT activities in heart of *Clarias gariepinus* exposed to ammonium chloride and mercury nitrate.

Groups	Protein (mg/ml)	**ALT (U/L)**	**GSH (nmol DTNB/mg protein)**	Peroxidase O.D purpurogallin/mg protein	**NO (nmol/mg protein)**	**SOD (U/mg protein)**
A	1.03±0.08^a	66.92±7.84^a	4.08±0.107^a	6.13±0.16^a	22.19±3.19^a	13.45±0.10^a
B	0.76±0.16^b	89.46±8.61^b	1.18±0.28^b	6.41±0.70^a	44.48±14.71^b	7.88±0.19^b
C	0.66±0.00^b	92.2±5.83^b	1.17±0.03^b	6.57±0.096^a	44.98±0.62^b	9.56±0.16^b
D	0.49±0.01^b	81.20±5.33^b	0.63±0.06^b	8.52±0.08^b	58.16±1.78^a	5.65±0.03^b

Values are expressed as mean ±standard deviation of triplet determination. Mean with same superscript letters on a column are not significantly different (P <0.05). The result showed a significant change (P <0.05) in the activities of ALT, Peroxidase, and SOD. However, a significant decrease in the levels of total protein content, GSH and NO were also observed

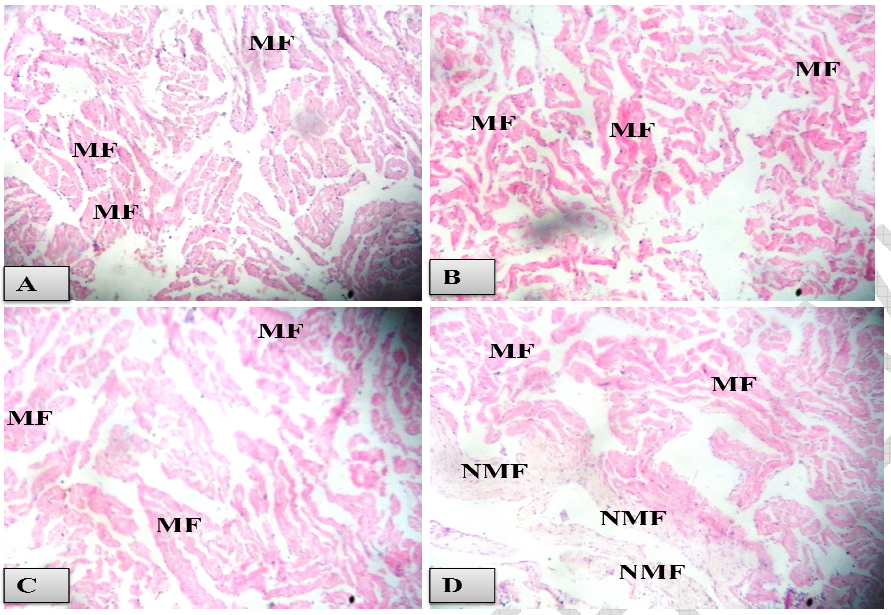

Figure 1: Photomicrograph of Heart: Group A (Control) shows normal cardiac muscle fibres (MF). Similarly, Groups B and C also displayed normal cardiac tissue architecture. On the contrary group D show focal area of necrosis (NMF). H&E x100

Discussion

Due to the industrial development, many dangerous chemicals have been released directly or indirectly which resulted in the polluted water bodies. The African cat fish (*Clarias gariepinus*) shows high sensitivity towards dissolving toxicants, hence this fish has been utilized as a biomarker to indicate the existence of toxicant exposure. The present study revealed a significant variation in the activities of Alanine aminotransferase (ALT), reduced glutathione (GSH), Nitric oxide (NO), peroxidase and Superoxide dismutase (SOD). The changes in these biochemical parameters indicate that they can be used as indicators of related stress in fish on exposure to toxic chemicals such as ammonium chloride and mercury (II) nitrate.

Peroxidases are haem-containing enzymes that use hydrogen peroxide as the electron acceptor to catalyse a number of oxidative reactions in this experiment the activity of peroxidase decreases although catalase is another enzyme that can carry out the same function the results are in line with the work reported by Wadhwa et al., 1988

GSH is a tripeptide, a cellular antioxidant. It protects the heart cells from oxidative stress and plays an important role in the scavenging reactions by acting both as a nucleophilic of various undesired compounds and their toxic metabolites and as a specific substrate for the enzymes GPx and GST. The present study reveals that there was a significantly different ($P < 0.05$) from group A the levels of GSH were high as compared to the other groups but the decrease in the levels of GSH is more pronounced in group D may be the combined effects of mercury and ammonium. These results are in line with the research results of Hegazi et al., 2010

Elevated activities of heart ALT are a common sign of heart disease. The levels of heart enzymes are used as diagnostic indicators of heart injury. ALT is one of the most sensitive tests employed in the diagnosis of heart diseases. The activity of ALT increased significantly from group A –D this shows that there is an injury and since the activity of ALT is more in group D the injury is much in D.

Superoxide anion formed from aerobic respiration is scavenged by Super Oxide Dismutase (SOD) leading to the formation of hydrogen peroxide (H_2O_2) and in the presence of reduced transition metals (such as iron) reactive hydroxyl radical (OH•) is produced through the Fenton reaction. The decreased activity of SOD in groups B-D indicates that there are more ROS in the form of super oxide that has no been scavenged by this enzyme due to the toxicity of mercury or ammonium or both. These findings are also in tune with the recent works of Hegazi et al., 2010

Nitric Oxide (NO) is another important mediator in the pathogenesis of inflammatory diseases. The levels of this molecule are higher in groups B-D these results shows that many inflammatory enzymes are produced but that less of these molecules are produced in group A. this shows that the toxicity of mercury, ammonium or the combination of both is lined with inflammation. These are inconsonant with the work reported by Eboh et al 2016 even though nitric oxide was assayed on the skin of rats.

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