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Alterations in Antioxidant Status of Erythrocytes of African Catfish (*Clarias gariepinus*) Exposed to Cypermethrin: A Possible Involvement of Free Radicals

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Abstract
Different concentrations of cypermethrin have been reported to induce oxidative stress due to generation of free radicals and alteration in antioxidant defense mechanisms. Clarias gariepinus was exposed to various concentrations (0, 5, 10, 15 and 20) µg/L of cypermethrin in dechlorinated water. The most outstanding results of this study were found for superoxide dismutase (SOD) and acetylcholinesterase (AChE) activities in erythrocyte and erythrocyte membrane respectively. Cypermethrin resulted in decreased erythrocyte glucose-6-phosphate dehydrogenase (G6PDH), glutathione peroxidase (GPx) and catalase (CAT), whereas activity of glutathione reductase (GR) was increased. Increased activity of GR was due to induction of natural defense mechanism of erythrocyte against the toxicity of the pesticide. A lower SOD activity favours lipid peroxidation induced membrane damage by cypermethrin which in turn reduced activities of membrane-bound enzymes, such as AChE, Na^+K^+ - ATPase and Ca^{2+} - ATPase. The decrease in antioxidant enzymes (SOD, CAT, G6PDH) activities have been interpreted as an indirect inhibition of the enzymes resulting from the binding of oxidative molecules produced during cypermethrin metabolism. In contrast, increased GR activity might result from an activation of the compensatory mechanism leading to induction of free radical scavenging enzymes to counteract the oxidative stress generated by the pesticides. Overall, altered activities of erythrocyte and erythrocyte membrane enzymes indicate oxidative stress involving free radicals induced by cypermethrin.

Keywords: Cypermethrin, Erythrocyte, Catfish; Free radical, Antioxidant

Introduction

The pollution of rivers and streams with chemical contaminants has become one of the most critical environmental problems of the century. As a result of the pollutants' transport from industrial and agricultural areas into the environment and their chemical persistence, many freshwater ecosystems are faced with spatially or temporally alarming high levels of xenobiotic chemicals (1-2).

Due to the increasing regulatory restrictions on organophosphate pesticides, pyrethroids pesticides have replaced organophosphates for many residential and agricultural uses. Cypermethrin is a synthetic pyrethroid insecticide used to control many pests, such as moth pests attacking cotton, fruit and vegetable crops, including structural pest control, or landscape maintenance. In Nigeria, Cypermethrin is a synthetic pyrethroid widely used alone or in combination with other pesticides (3).

Exposure to low-level of pesticides, in general, is known to produce a variety of biochemical changes, some of which may be responsible for the adverse biological effects reported in human and experimental studies (4-5). Conversely, some biochemical alterations may not necessarily lead to clinically recognizable symptoms, although all the biochemical responses can be used as markers of exposure or effect. Oxidative stress can also be induced by pesticides, either by overproduction of free radicals or by alteration in antioxidant defence mechanisms, including detoxification and scavenging enzymes (6). Oxidative stress has been reported to play an important role in the toxicity of various pesticides, including organochlorines, organophosphates (OPs) (7-8), carbamates and pyrethrods (9).

In blood, normal erythrocyte function depends on the intactness of cell membrane which is the target for many toxic factors including pesticides. Erythrocyte reduced glutathione (GSH) together with glutathione peroxidase (GPx), glutathione reductase (GR), glutathione Stransferase (GST), gamma-glutamyl transferase (GGT), superoxide dismutase (SOD) and catalase (CAT) efficiently scavenge toxic free radicals and are partly responsible for protection against lipid peroxidation due to acute/chronic pesticide exposure (10-11).

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The biochemical changes induced after exposure to pesticides or their active metabolites include target cell/receptor binding, protein and DNA adduct formation, and induction or inhibition of enzymes (12). Hence, the present study was undertaken to evaluate the aquatic toxicity of cypermethrin-based pesticides, with special emphasis on enzymes of erythrocyte and erythrocyte membrane of *C. gariepinus* exposed to various concentrations of cypermethrin-contaminated water.

Materials and Methods

Cypermethrin ([S, R]-N-α-cyno-3-phenoxybenzyl-(IR, IS, cis, trans)-2, 2-dimethyl-3, (2, 2-dichlorovinyl) cylcopropane carboxylate) manufactured by M/S Tropical Agrosystem Pvt. Ltd. Chennai, India, and purchased from local market of Udu, Delta State, Nigeria. Other reagents are of analytical grade.

One hundred and fifty species of C. gariepinus with the mean weight of 61.2±4.5 g and standard length mean length of 15.3±2.1cm were used for the experiment. They were purchased from a reputable fish farm in Delta State, Nigeria. The fish were kept in transparent plastic tanks filled with dechlorinated tap water and made to acclimatize in laboratory conditions for two weeks. The experimental fish were managed in accordance with the guidelines for handling experimental animals approved by the ethics committee of the University. They were fed (3% w/w) with commercial feeds. Water quality was measured according to the method of APHA/AWWA/WEF (13). The temperature of the experimental water was 25.8±0.6°C, pH was 7.3±0.2 dissolved oxygen was 6.8±0.3 mg/L, free carbon dioxide was 5.8±0.5 mg/L and alkalinity was 104.9 mg/L. Water was changed every day.

Five plastic aquaria (56 x 28 x 28cm) with 30 L of dechlorinated water were contaminated with varying concentration of cypermethrin, and designated as follows:

A: dechlorinated tap water free of cypermethrin
B: water contaminated with 5µg/L cypermethrin
C: water contaminated with 10µg/L cypermethrin
D: water contaminated with 15µg/L cypermethrin
E: water contaminated with 20µg/L cypermethrin

After the period of acclimation, the fish were randomly distributed into the five plastic aquaria (A – E) ten fish per aquarium. Each of these treatments had three replicates. The control group of fish were kept in aquarium A while aquaria B-E contained the test group of fish reared in water contaminated with varying concentrations of cypermethrin. The experiment lasted for ten days.

After completion of treatment, the test animals were removed from aquaria, washed with water, and sacrificed. Afterwards, blood was collected from the fish by cardiac puncture using hypodermic needle and syringe.

Preparation of Erythrocyte Hemolysate: Erythrocyte hemolysate was prepared by the method of Lohr and Waller (14). Blood (0.5ml) was added into a graduated centrifuge tube containing 0.5 ml of anticoagulant. Tube was centrifuged at 1000 g, the buffy coat was removed and the cells were washed twice with 5 ml physiological saline. The sediment obtained was suspended in 1 ml physiological saline and was properly mixed by rotation; 1 ml of erythrocyte suspension was pipette into centrifuge tube and was mixed with 1 ml double distilled water, 0.7 ml triethanolamine buffer (pH 7.5) and 0.3 ml digitonin solution (1 g/100 ml). The mixture was allowed to stand for 15 min in a refrigerator at 4^{o}C and was then centrifuged at 1000 g for 15 min. The insoluble material was discarded.

Biochemical Analyses: Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined according to the method developed by Deutsch et al. (15). The final cuvette concentrations of the reagents used in the assay were 0.49 mol/l Tris–HCl buffer pH 7.5, 3.2 mmol/l glucose 6-phosphate, 0.37 mmol/l NADP as cofactor and 61.7 mmol/l MgCl2. Addition of maleimide (0.5 mg/ml in cuvette) inhibited further oxidation of reaction products by 6-phosphogluconate dehydrogenase (6-PGDH). After 3 min incubation at 37oC, the reaction was started with the sample diluted 1:6 in 500 mmol/l Tris–HCl buffer pH 7.5. The rate of increase in absorbance at 339 nm is a measure of G6PDH activity. One unit was equivalent to 1µmol of NADPH oxidized/min.

Glutathione reductase (GR) activity was measured according to the method developed by Goldberg and Spooner (16). The oxidation of NADPH to NADP during the reduction of oxidized glutathione (GSSG) was monitored at 340 nm. The final cuvette concentrations of the reagents used in the assay were 100 mmol/l phosphate buffer pH 7.2, 0.49 mmol/l EDTA, 5.1 µmol/l FAD, and 2.14 mmol/l GSSG. The sample was diluted 1:8 in 0.12 mol/l phosphate buffer pH 7.2 and added to the assay tube. After 5 min incubation at 37^{o}C, the reaction was started with 0.16 mmol/l NADPH. During the assay, reagents were kept in an ice bath with the exception of GSSG which might undergo precipitation.

Glutathione peroxidase (GPx) activity was determined according to the method developed by Paglia and Valentine (17). The final cuvette concentrations of the reagents used in the assay were 0.3 mmol/l GSH, 0.3 mmol/l NADPH,

1.1 U/ml GR from *Saccharomyces cerevisiae* (Sigma, St. Louis, MO), and 44.1 mmol/l phosphate buffer pH 7.4. The sample was diluted 1:50 in 100 mmol/l phosphate buffer pH 7.4 containing 1 mmol/l EDTA and added to the assay tube. After 2 min incubation at 37^{o}C, the change in absorbance was monitored at 340 nm after addition of 1.1 mmol/l (final concentration) tert-butyl hydroperoxide. The unit is µmol of NADPH oxidized/min. Blank assays containing sample but not substrate were run in parallel.

Superoxide dismutase (SOD) activity was measured spectrophotometrically according to the method developed Andersen et al. (18) coupling an $O_2^{\bullet-}$ generator with an $O_2^{\bullet-}$ detector. In the method, xanthine and xanthine oxidase are used to generate $O_2^{\bullet-}$, which further reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye monitored at 500 nm. SOD inhibits the formation of the formazan dye, and the activity is measured as percent inhibition compared with a calibration curve built with purified SOD from bovine liver (Sigma, St. Louis, MO). The final concentrations of the reagents used in the assay were 0.05 mmol/l xantine, 0.01 mmol/l INT and 46.5 mmol/l phosphate buffer pH 7.8. The sample was diluted 1:100 with 50 mmol/l phosphate buffer pH 7.8, containing 0.1 mmol/l EDTA, and incubated with the former reagents at 30oC for 5 min and then the reaction was started with 3.1 U/l (final concentration in cuvette) of xantine oxidase (Sigma, St. Louis, MO). The unit of activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50%.

Catalase (CAT) activity was determined according to the method developed by Aebi (19). The decomposition rate of the substrate H_2O_2 at a final concentration of 10 mmol/l was monitored at 240 nm and 20^{o}C after adding directly the sample diluted 1:50 in 50 mmol/l phosphate buffer pH 7.0.

Preparation of erythrocyte membranes: Erythrocyte membranes were prepared by hemolysing the erythrocytes in distilled water and centrifuging at 20,000 g. Post hemolytic residue was washed twice with 1 mM Tris EDTA (pH 7.4).

Acetylcholinesterase (AChE) enzyme activity was estimated in the membrane preparation by the method of Ellman et al. (20). Na^{+}-K^{+} and Ca^{2+} - ATPases activities were assayed by the method described by Quigley and Gotterer (21). Protein content was measured by the methods of Lowry et al (22).

Statistical Analyses: Data obtained were presented as mean±SEM and subjected to statistical analysis using a one way analysis of variance (ANOVA). Significant difference between the treatment means was determined at 95% confidence level.

Results

Activities of erythrocyte enzymes studied are presented in Table 1. Erythrocyte enzyme activity, except erythrocyte GR, of animal treated with various concentrations of cypermethrin decreased significantly ($p<0.05$) compared with the control. The observed decrease in enzyme activity was found to depend on the concentration of cypermethrin. However, erythrocyte catalase activity of groups D and E was no significantly different ($P>0.05$). Activity erythrocyte GR of fish treated with various concentrations of cypermethrin was significantly ($p<0.05$) higher than control (E>D>C>B>Control).

Table 1: Effect of cypermethrin on erythrocyte enzyme activity (Units/min/mg protein) of *Clarias gariepinus*

Group	G6PDH	GR	GPx	SOD	CAT
A (Control)	0.524±0.013^{a}	0.252±0.001^{a}	0.80±0.029^{a}	75.3±2.5^{a}	1.98±0.02^{a}
B	0.498±0.004^{b}	0.281±0.002^{b}	0.56±0.017^{b}	43.7±2.1^{b}	1.37±0.02^{b}
C	0.427±0.003^{c}	0.301±0.004^{c}	0.43±0.010^{c}	37.8±1.8^{c}	1.04±0.01^{c}
D	0.366±0.003^{d}	0.345±0.003^{d}	0.36±0.012^{d}	33.2±2.0^{d}	0.97±0.01^{d}
E	0.345±0.003^{e}	0.367±0.001^{e}	0.33±0.008^{e}	29.1±1.5^{e}	0.95±0.01^{d}

Values are means ± SEM for 10 determinationss. [a,b,c] Column values with different superscripts are significantly different (p<0.05).

Results of erythrocyte membrane enzyme activities are presented in Table 2. Like erythrocyte enzyme activity, activity of erythrocyte membrane enzyme of fish treated with various concentrations of cypermethrin decreased significantly ($p<0.05$) relative to the control. In the case of Na^+K^+ - ATPase activity, decrease range from 20% (in B) and 80% in E. In case of Ca^{2+} - ATPase activity, the decrease range from 20% (in B) and 64% in (in E) whereas in the case of AChE activity, decrease range from 82% in B and 94% in E.

Table 2: Effect of cypermethrin on erythrocyte membrane enzyme activity (Units/min/mg protein) of *Clarias gariepinus*

Group	Na^+K^+ - ATPase	Ca^{2+} - ATPase	AChE
A (Control)	0.015±0.001 [a]	0.067±0.005[a]	0.551±0.018[a]
B (5 µg/L Cypermethrin)	0.009±0.001 [b]	0.053±0.003[b]	0.097±0.009[b]
C (10 µg/L Cypermethrin)	0.007±0.001 [bc]	0.044±0.003[c]	0.069±0.003[c]
D (15 µg/L Cypermethrin)	0.005±0.001 [cd]	0.030±0.001[d]	0.043±0.001[d]
E (20 µg/L Cypermethrin)	0.003±0.001 [d]	0.024±0.001[e]	0.031±0.001[e]

Values are means ± SEM for 10 determinationss. [a,b,c] Column values with different superscripts are significantly different (p<0.05).

Discussion

This is one of the few studies performed so far addressing changes in erythrocyte antioxidant enzymes and erythrocyte membrane enzymes in *C. gariepinus* exposed to cypermethrin. The most outstanding results of this study were found for SOD (Table 1) and AChE (Table 2) activities in erythrocyte and erythrocyte membrane respectively. This may result from the oxidative stress induced by pesticide exposure (23). A lower SOD activity favours the accumulation of oxygen free radicals in erythrocytes, and other cells, leading to tissue damage as a result of oxidative binding of key intracellular molecules containing thiol groups and lipid peroxidation of biological membranes, which might be of greatest importance in the cytotoxicity of pesticides and can be eventually responsible for cellular death. Concurrent decreased AChE activity is not unexpected since the lipid peroxidation induced by cypermethrin may affect membrane-bound enzymes, such as AChE, Na^+K^+ - ATPase and Ca^{2+} - ATPase (5, 24).

Decreased CAT activity indicated that the higher the concentration of cypermethrin the less enzyme activity. A mechanistic explanation for this finding is that the generated oxygen radicals might inhibit thiol groups of CAT (25) and thus the resultant excess in hydrogen peroxide may further inhibit SOD, although the precise mechanism involved merits further research.

G6PDH is an important enzyme of hexose monophosphate shunt and its function in the mature RBCs is to generate NADPH, which is required for the conversion of oxidized glutathione to reduced glutathione that in turn is necessary for membrane integrity of erythrocyte membranes (26). Earlier “in vitro” studies have found a significant reduction in erythrocyte G6PDH activity after treatment of erythrocytes with orgonophosphate pesticides (27) and phenoxyherbicides (28). A decreased G6PDH activity would lead to lower levels of NADPH inside the erythrocytes and may further challenge the replenishment of GSH by GR, thus favouring the appearance of oxidative stress induced by other sources.

Increased activity of GR observed in this study appears to be a defense against pesticide toxicity in erythrocytes arising from unbalanced antioxidant enzyme cycle. Earlier work showed that in vivo administration of various pesticides results in decrease in the activity of GR (28-30). However, no data on this erythrocyte enzyme has been reported so far in *C. gariepinus* exposed to cypermethrin.

GPx catalyzes the glutathione-dependent reduction of lipid hydroperoxides and hydrogen peroxide for detoxification. In this study, GPx activity significantly decreased in the erythrocyte of fish exposed to cypermethrin (Table 1). It is possible that cypermethrin may exert a direct effect on GPx or, alternatively, the decreased enzyme activity could result from a negative modulation because of an unbalanced antioxidant enzyme cycle.

Conclusion

Based on results from this study, it is concluded that various concentrations of cypermethrin exert differential effect on the activity of antioxidant enzymes and membrane bound enzymes which may be useful in their toxicological evaluation. The results clearly show a relationship of exposure to cypermethrin at various levels and decrease in AChE activity with antioxidant enzymes (SOD, CAT, and GPx). Further research is needed to conclude if these indicators should be considered biomarkers of effect or biomarkers of exposure.

Conflict of interest

The author declares that there are no conflicts of interest.

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