Effect of Butachlor on Antioxidant Enzyme Status and Lipid Peroxidation in Fresh Water African Catfish, (*Clarias gariepinus*)

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Abstract: The present study was undertaken to evaluate the influence of butachlor, a widely used herbicide, on antioxidant enzyme system and lipid peroxidation formation in African cat fish (*Clarias gariepinus*). Fish were exposed to sub-lethal concentrations of butachlor 1, 2, 2.5 ppm and sacrificed 24hrs after treatment. A significant increase in malondialdehyde formation was observed in the liver, kidney, gills and heart of the fish following exposure to different concentrations of butachlor. Superoxide dismutase and catalase activities increased in the liver and kidney but decreased in the gills and heart in a concentration-dependent pattern. Glutathione level and glutathione-S-transferase activities increased ($P<0.05$) in the liver but decreased in the kidneys, gills and heart when fishes were exposed to the three concentrations of butachlor. The results suggest that butachlor induced oxidative stress in the various tissues of the fish particularly in the kidney and as such the organ may be subjected to severe oxidative toxicity due to depressed glutathione detoxification system.

Keywords: Butachlor, African catfish, antioxidant enzymes, oxidative stress, environmental pollution, pesticides

Introduction

The widespread use of chemicals to control pest weeds has been recognized in agricultural practices. Indiscriminate use of these chemicals to improve agricultural production and yield may result in aquatic pollution due to rain and surface runoff.

Butachlor 2-chloro-N-(2,6-diethylphenyl)acetamide is an herbicide that is widely used to control perennial grasses and some broad leaf weeds in Asia, South America and Africa [10]. Butachlor is used in large amounts >100,000,000 lbs/year for economic weed control in certain parts of Asia [2]. It was found to flow out with effluents causing contamination of rivers water [1] and such concentration of 0.163 ppb has been recorded for butachlor in ground water collected from tube wells adjacent to rice field in the Philippines [14]. In spite of the wide application of the pesticide and possible environmental risk attached to its use, there is paucity of toxicological information available on it. However, in

vitro studies have demonstrated the mutagenicity of butachlor in *Salmonella typhimurium* strain TA 100 [11], induction of stomach tumors in rats [21] and induction of micronuclei in the cat fish erythrocytes [2]. The exact mechanism of carcinogenicity of butachlor is not known but the possible mechanism involves the formation of a DNA-reactive metabolite, 2,6-diethybenzoquinone imine [7, 9, 16].

Fish can serve as bioindicators of environmental pollution and therefore can be used for the assessment of the quality of aquatic environment [8, 13] since they are directly exposed to chemicals resulting from agricultural production via surface runoff of water or indirectly through the food chain of ecosystem [2]. Fish are endowed with defensive mechanisms to counteract the impact of reactive oxygen species (ROS) resulting from the metabolism of various chemicals. These systems include various antioxidant defense enzymes such as superoxide dismutases which catalyze the dismutation of superoxide radical to hydrogen peroxide, catalase acting on hydrogen

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peroxide, glutathione S-transferase family possessing detoxifying activities towards lipid hydroperoxides generated by organic pollutants such as heavy metals [22].

African catfish (*Clarias gariepinus*) is of great commercial importance and it is the most common fresh water fish widely consumed in Nigeria [15]. It can therefore be a good model to study responses to various environmental contaminants due to two reasons. First this species of fish exhibits anatomical and physiological changes at the level of both respiratory and circulatory systems, owing to the presence of a ramifying organ in the peribranchial cavity for air-breathing. Secondly, this specie apart from the fact that it is found in Africa rivers also lives in temporary puddles forming in desert areas after rainy inundation, in which a large amount of pollutant rapidly accumulate. To the best of our knowledge, there are no reports on the effects of butachlor on antioxidative enzymes in animals and aquatic species. We therefore report for the first time the influence of butachlor on antioxidant enzyme system and malondialdehyde formation in African catfish (*Clarias gariepinus*) in order to understand further the mechanism of toxicity of this widely used pesticide.

Materials and Methods

Chemicals

Butachlor was obtained from Chem Services (West Chester, PA, USA). All other chemicals were of the highest purity available and were purchased from the British drug houses Dorset, UK.

Fish and Treatment

Clarias gariepinus (20) average weight 275 ± 12.5 g were purchased from Government owned Agodi fish farm in Ibadan, Nigeria. The fishes were kept in an aquarium for some 7 days to acclimatize them to laboratory conditions and had access to feeds (fishmeal). The fishes were maintained at a temperature of $23 \pm 2°C$ (12:12 L: D), pH of 7.6 and an oxygen concentration of 7.02 mg/l. water was changed every other day to minimize contamination from metabolic wastes. After acclimatizing them to laboratory conditions, the animals were divided into 4 groups with 5 fishes in each group. Group 1 served as the control. Fishes in groups 2, 3 and 4 were exposed to sub lethal concentrations of butachlor 1, 2, 2.5 ppm respectively [2]. After 24 hours, the fishes were sacrificed by decapitation, dissected and the liver, gills, kidney and heart were removed. The organs were washed in ice cold 1.15% KCl solution, blotted and weighed. They were then homogenized in 4 volumes of homogenizing buffer (50mM Tris-HCl mixed with 1.15% KCl and pH adjusted to 7.4), using a Teflon homogenizer. The resulting homogenate was centrifuged at 10,000g for 20 minutes in a Beckman L5-50B centrifuge at 4°C to obtain the post mitochondrial supernatant fraction.

Biochemical Assays

Glutathione (GSH) was determined in the 10, 000 g supernatant fraction of the liver, kidney, gills and heart homogenates of *Clarias gariepinus* according to Jollow et al. (1974) at 412 nm using 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). Glutathione *S*-transferase (GST) activity was determined by the method of Habig et al. (1974) using 1 chloro 2, 4 dinitrobenzene as substrate. The specific activity of glutathione S-transferase is expressed as nmoles of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of $9.6mM^{-1}cm^{-1}$. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of autoxidation of adrenaline at pH 10.2 at 30°C as described by Magwere et al. (1997). One unit of SOD activity is the amount of SOD necessary to cause 50% inhibition of adrenaline autooxidation. Activity of catalase (CAT) was determined as described previously [6] by following the absorbance of hydrogen peroxide at 240 nm, pH 7.0 and 25°C. The intra-assay CV for GST, SOD and CAT were 2.5, 1.9 and 1.4% respectively while the inter-assay CV for the enzymes were 4.2, 2.7 and 3.2% respectively. Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation as described previously [4]. All the assays were run in triplicates. The protein content of the various fractions was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistics

All variables were tested for normal distribution using the Kolmogorov-Smirnov test (P>0.05) and for homogeneity of variance among groups using the Levene's test (P > 0.05). ANOVA was used to compare the experimental groups. If significant differences were found (P < 0.05), the treatment groups were compared with the control group using student's test. All the statistics were carried out in SAS (The SAS System for windows, v8; SAS Institute Inc., Cary, NC).

Results and Discussion

The effects of different concentrations of butachlor on MDA formation in the different organs of *Clarias garienpinus* are depicted in Table 1. A significant increase in lipid peroxidation as MDA formation was observed in the liver, kidney, gills and heart of the fish following exposure to butachlor at concentrations of 1, 2 and 2.5 ppm. At a concentration of 2.5 ppm of butachlor, MDA increased by 161%, 151%, 205% and 118 % compared to control in the liver, kidney, gills and heart respectively (Fig.1). The data indicate that reactive oxygen species may be associated with the metabolism of butachlor leading to peroxidation of membrane lipids of the respective organs. Previous investigations have reported the induction of lipid peroxidation by other pesticides such as endosulfan [18] and cypermethrin [24] in fish. The observed lipid peroxidation resulting possibly from ROS

generated by the compound may lead to cell apoptosis. ROS and oxidative stress have been shown to be triggers of apoptosis [20] . Exogenous ROS such as H_2O_2 at moderate levels induce apoptosis in many cell types [23]. Endogenously produced ROS have also been found to be important in the apoptotic cell death triggered by many other stimuli including environmental chemicals [5], requiring the participation of cell death signaling pathways such as c-Jun N-terminal kinases (JNK) [20].

Table 1: The formation of Malondialdehyde (nmol/mg protein) in the liver, kidneys, gills and heart of *Clarias gariepinus* exposed to Butachlor

Groups	Liver	Kidney	Gills	Heart
Control	0.84± 0.09	0.86 ± 0.07	0.39 ± 0.11	0.68±0.17
1ppm Butachlor	1.57±0.10*	1.44±0.16*	0.69±0.08*	0.86±0.12*
2ppm Butachlor	1.97±0.07**	2.06±0.03**	0.95±0.18**	1.29±0.12**
2.5ppm Butachlor	2.19±0.03**	2.16±0.02**	1.19±0.08**	1.48±0.11**

The results are mean $\pm$ SD of 5 fishes in each group. Significantly different from control *$P<0.05$; **$p<0.001$
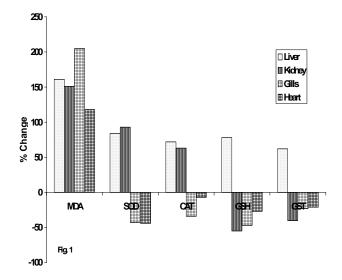

Figure 1: The effect of 2.5 ppm butachlor on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S transferase (GST) and the levels of glutathione (GSH) and malondialdehyde (MDA) in the liver, kidney, gills and heart of *Clarias garienpinus* with respect to controls.

Table 2 shows the profile of SOD activities in the various organs of the fish after 24 hrs of exposure to butachlor at various concentrations. SOD activities increased in a concentration-dependent pattern in the liver and kidney but also decreased in concentration-dependent manner in the gills and heart. At a concentration of 2.5 ppm, a significant increase ($P<0.001$) in SOD activity was

observed in the liver (84%) and kidney (93%) whereas a decrease ($P<0.001$) was seen in the gills (43%) and heart (44%) compared to controls (Fig.1).

Table 2: The activity of Superoxide dismutase (Unit/mg protein) in the liver, kidneys, gills and heart of *Clarias gariepinus* exposed to Butachlor

Groups	Liver	Kidney	Gills	Heart
Control	23.2±2.0	20.6±2.7	25.1 ± 2.4	18.0 ± 1.2
1ppm Butachlor	27.4±1.8*	25.3±2.1*	23.6 ± 3.5§	16.8± 3.9§
2ppm Butachlor	35.5±2.3**	30.2±1.8**	17.9±1.9**	12.9± 2.0*
2.5ppm Butachlor	42.6±2.2**	39.8±2.2**	14.2±2.1**	10.1± 2.7*

The results are mean $\pm$ SD of 5 fishes in each group. Significantly different from control *$P<0.05$; **$p<0.001$. Not significantly different from control §$p>0.05$. 1 unit of superoxide dismutase is the amount that inhibits autoxidation of epinephrine by 50% at pH 10.2 and 30°

Table 3: The activity of Catalase (Units/mg protein) in the liver, kidneys, gills and heart of *Clarias gariepinus* exposed to Butachlor

Groups	Liver	Kidney	Gills	Heart
Control	100.2±2.4	106.4±1.1	110.0±2.7	92.1±3.0
1ppm Butachlor	127.8±2.0*	117.7±2.2*	97.4±3.4§	90.0±3.7§
2ppm Butachlor	164.3±1.9**	167.2±1.5**	85.0±2.3*	88.3±4.6§
2.5ppm Butachlor	172.6±1.4**	174.2±2.0**	72.4±1.1*	85.5±3.7§

The results are mean $\pm$ SD of 5 fishes in each group. Significantly different from control *$P<0.05$; **$p<0.001$. Not significantly different from control §$p>0.05$. 1 unit of catalase decomposes 1µmol of H_2O_2/min at pH 7.0 and 25°

Similarly, CAT activities follow the same profile in all the organs as SOD (Table 3).The activities of CAT increased significantly in the liver and kidney by 72% and 63% respectively while its activity decreased by 34% and 7% in the gills and heart respectively following exposure of fish the butachlor at a concentration of 2.5 ppm (Fig.1). The apparent increase in SOD activities in the liver and kidneys of the fish may be due to the production of superoxide anions which led to the induction of SOD, to convert the superoxide radical to H_2O_2. The increase in CAT activities in these organs may be a response to the hydrogen peroxide produced by SOD activity since CAT is responsible for the detoxification of hydrogen peroxide to water. Increase in the activity of CAT and SOD is usually observed in the face of environmental pollutants since SOD-CAT system represents the first line of defense

against oxidative stress [19]. Furthermore the increase in antioxidant enzymes in the kidney demonstrates that kidney has an important role in the detoxification of butachlor and /or its metabolites.

Table 4: The concentrations of Glutathione (nmol/mg protein) in the liver, kidneys, gills and heart of *Clarias gariepinus* exposed to Butachlor

Groups	Liver	Kidney	Gills	Heart
Control	7.2 ± 0.4	5.6 ± 1.0	4.7 ± 0.5	5.9 ± 1.7
1ppm Butachlor	$9.2 \pm 0.9*$	$4.5 \pm 1.5^{\S}$	$4.3 \pm 1.8^{\S}$	$5.6 \pm 1.4^{\S}$
2ppm Butachlor	$11.6 \pm 0.8**$	$2.9 \pm 0.3**$	$2.6 \pm 0.2**$	$4.3 \pm 2.3^{\S}$
2.5ppm Butachlor	$12.8 \pm 0.6**$	$2.5 \pm 0.5**$	$2.5 \pm 0.3**$	$4.1 \pm 0.4*$

The results are mean $\pm$ SD of 5 fishes in each group. Significantly different from control $*P<0.05$; $**p<0.001$. Not significantly different from control $^{\S}p>0.05$.

In the present study, GSH level increased significantly up to 78% at 2.5 ppm in the liver when fishes were exposed to butachlor, but decreased by 55%, 47% and 27% in the kidneys, gills and heart respectively (Table 4) when fishes were exposed to butachlor (Fig.1). Analogously, GST activity was significantly increased in the liver up to 62% at 2.5 ppm, but decreased significantly by 40%, 23% and 21% in the kidneys, gills and heart of the fish, respectively (Table 5; Fig.1).

Table 5: The activity of Glutathione S-transferase (nmoles /min/mg protein) in the liver, kidneys, gills and heart of *Clarias gariepinus* exposed to Butachlor

Groups	Liver	Kidney	Gills	Heart
Control	122.4 ± 2.2	120.3 ± 3.3	101 ± 3.6	117.5 ± 3.1
1ppm Butachlor	$172.3 \pm 3.5**$	$115.1 \pm 2.4^{\S}$	$96 \pm 5.3^{\S}$	$115.3 \pm 2.2^{\S}$
2ppm Butachlor	$188.3 \pm 2.6**$	$77.1 \pm 2.5**$	$82 \pm 2.5**$	$100.3 \pm 4.9^{\S}$
2.5ppm Butachlor	$197.1 \pm 2.3**$	$72.5 \pm 1.7**$	$78 \pm 2.4**$	$92.8 \pm 1.0*$

The results are mean $\pm$ SD of 5 fishes in each group. Significantly different from control $*P<0.05$; $**p<0.001$. Not significantly different from control $^{\S}p>0.05$.

The apparent increase in GSH level with attendant increase in GST activity in the liver and a decrease in both GSH and GST in the kidney suggest that both liver and kidney are involved in the metabolic detoxification of butachlor. In vitro incubation of liver and kidney fractions with butachlor showed that butachlor was first biotransformed by conjugation with GSH by the enzyme GST to form butachlor glutathione conjugate which was further transported to the kidneys to form mercapturic acid

by N- acetylation [17]. However it was also reported that in the absence of acetyl CoA, the GSH conjugate was metabolized to butachlor cysteine conjugate [17].

Therefore, the apparent decrease in GSH level and GST activity in the kidney suggests an overproduction of reactive species which depleted GSH and inactivated GST enzyme. It has been shown that cysteine conjugate resulting from GSH metabolism can be bioactivated in the kidney by either cysteine β-lyase or the flavin containing monoxygenase to toxic reactive species which can cause nephrotoxicity [12]. The decreased levels of antioxidant enzymes and GSH with lowered level of GST in the gills could account for the marked lipid peroxidation observed. Indeed, the gills of both invertebrates and vertebrates are more exposed to the water being the first area of contact with aquatic xenobiotics like butachlor and as such the compound can penetrate through their thin epithelial cells [3] to produce reactive species, thereby inactivating the antioxidant defenses.

Conclusion

Taken together, the results indicate that butachlor induced oxidative stress in the various tissues of the fish as shown by increase in lipid peroxidation and in response to this, antioxidant defense mechanisms were induced. In addition, the kidney may be subjected to severe oxidative toxicity due to depressed glutathione detoxification system in this organ. These data, together with those related to both mutagenicity and carcinogenicity demonstrate the severe risk butachlor possesses. Therefore, further restrictions on use should be considered and that actual environmental stress from this compound is investigated.

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