inteGrity Research Journals

Journal of Bioscience and Biotechnology Discovery
Volume 4(4), pages 101-107, August 2019
Article Number: 0ED30CD26
ISSN: 2536-7064
https://doi.org/10.31248/JBBD2019.114
https://integrityresjournals.org/journal/JBBD

Full Length Research

Genotoxicity, behaviour and erythrocytic-oxidative stress modulation of clariid catfish to Acetamenophen N-(4-hydroxyphenyl) ethanamide EC number: 203-157-5 | CAS number: 103-90-2

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Received 25th July, 2019; Accepted 28th August, 2019

ABSTRACT: This study was undertaken to evaluate the acute toxicity and sub-acute paracetamol toxicity-induced nuclear aberration, antioxidative stress enzyme activities such as CAT, SOD, LPO, GPX of *Clarias gariepinus*. Three hundred (300) juveniles with average weight of 50.96±1.3g were procured from a reputable fish farm in Enugu to the Fisheries Research Laboratory (latitude 6.4659°N and longitude 7.5762°E). 180 juveniles were exposed to acute concentrations of 5000, 5500, 6000, 6500, 7000mgL^{-1} and control in triplicate replication while remaining 120 fish in similar replication were exposed to 620, 1240, 3100 mgL^{-1}corresponding to 1/10, 1/5 and ½ 96hour LC_{50} = 6200 mgL^{-1}, y = 14.23x - 48.96, R^2 = 0.963, safety dose 1/100 LC_{50} = 62 mgL^{-1}. Blood samples were collected from the caudal vein on day 2, 4, and 8 for erythrocyte-oxidative enzyme activities. Data was subjected to one way analysis of variance to determine the significant differences between treatments and control at $p<0.05$ using computer software SPSS version 20 and was expressed as means plus standard error of means. The exposed fish showed behavioral changes (erratic movement, air gulping, nervous manifestation and imbalance) before death. CAT reduced significantly below the control value of 0.83 to the least value of 0.57 µmol mm^{-1} mg $protein^{-1}$ among the highest group exposed to 3100 mg/L. Similarly, SOD activity in exposed group of fish varied significantly from the control and ranged from the highest control value of 12.31 to the least value of 8.27 U mol mm^{-1} mg $protein^{-1}$among fish to the highest concentration of the drug. GPX ranged from 9 to 4.65 µmol mm^{-1} mg $protein^{-1}$ among group of fish in the control to 3100mg/L of the drug. On the other hand, LPO increased significantly ($p<0.05$) from the control value of 6.83 to 8 mMole/TBARS/mg protein among group of fish to 3100 mg/L of the drug indicative of a dose dependent elevation of micronuclei The use of genotoxic potential and oxidative stress modulation by fish in inland water bodies can be a useful diagnostic tool to measure the health alterations in *C. gariepinus* which may serve as early warning indicators of pollution.

Keywords: Antioxidants, genotoxicity, oxidative stress, paracetamol, peroxidation, toxicity.

INTRODUCTON

Pharmaceuticals are developed and used for specific biological effects, being administered for human and animal health care, and livestock farming. Due to its physiochemical and biological properties, there are concerns about the potential for their impact to non target species (Singh et al., 2011) especially when available in the aquatic ecosystems. Although a variety of pharmaceutical compounds have been detected in the

environment, their potential ecological significance remains largely unknown (Sanderson et al., 2004). Paracetamol (acetaminophen) and by its chemical name Paracetamol overdose is known to cause hepatotoxicity and numerous studies about paracetamol induced hepatotoxicity and its mechanism are available in literature (Kim et al., 2007; Nunes et al., 2014). Aside the United States and Canada, paracetamol is available over the counter (OTC) as a prescription medication either as a single agent or in combination with other pharmaceuticals. It is cited worldwide as a primary drug in over 50 brands or trade name product (e.g. Tylenol, Panadol, Tempra, Mapap, e. t. c). It enters the environment mainly due to improper disposal (Kim et al., 2007) and has been detected in aquatic systems in several countries (Kim et al., 2007; Roberts and Thomas, 2006) causing impact on aquatic animals such as fish (Nunes et al., 2014). Oxidative stress is an inescapable component of life which strikes a balance between the production of reactive oxygen species ROS and the systems that protect cells in a healthy aerobic animal. The elevation in the production of ROS often results in defects that may cause cell or organ's damage or death. This imbalance is referred to as oxidative stress (Kim et al., 2007). The knowledge in oxidative stress in fish has great importance for environmental and aquatic toxicology, because oxidative stress is evoked by many chemicals including paracetamol. Antioxidant factors in fish and other organisms can be used to assess water pollution (Alla et al., 2007; Thomas et al., 2007). Few studies indicate that the blood-oxidative stress is involved in various toxicities associated with paracetamol and various antioxidants in blood have been evaluated in oxidative enzymatic alterations in rodents (Ahmida, 2010, Yousef et al., 2010; Singh et al., 2011), aquatic mollusks *Dreissena polymorpha* (Parolini et al.,2010) and man (Orhan and Sahin, 2010)), but the erythrocyte oxidative stress effects of paracetamol to fish is rare. Fish can serve as an indicator of environmental pollution and therefore can be used for the assessment of the quality of the aquatic environment since they are directly exposed to chemicals resulting from pharmaceutical production via inappropriate discharge or through the food chain of aquatic ecosystem. African catfish *Clarias gariepinus* is an air breeding species well suited for aquaculture and pollution studies biomarker (Sogbanmu et al., 2018). Therefore, the objective of this research was to evaluate the acute toxicity and sub-acute paracetamol toxicity-induced antioxidative stress enzyme activities such as catalase (CAT), super oxidase dismutase (SOD), lipid peroxidation (LPO) and glutathione peroxidase (GPX) of African catfish (*Clarias gariepinus*).

Experimental fish and paracetamol

A total of three hundred (300) juveniles of African catfish (mean weight 50.96±1.3 g were obtained from a local outskirts in Enugu Nigeria and transported to Fisheries Laboratory of the Department of Animal/Fisheries Science and Management, Enugu State University of Science and Technology ESUT, Enugu Nigeria. They were held in four fiber reinforced plastic tanks, containing 500 L of dechlorinated tap water. Aeration was provided to all tanks round the clock in order to maintain dissolved oxygen contents. The fish were acclimatized for two weeks before commencement of the study and they were fed with commercial fish diet composed of 40% crude protein. The faecal matter and other waste materials were siphoned off daily to reduce ammonia content in water. Paracetamol N-(4-hydroxyphenyl) ethanamide obtained from a pharmaceutical shop in Enugu was dissolved in distilled water to make a stock solution that was used in the study. Ethical clearance from the Enugu State University of Science and Technology Committee on Experimental Animal Care was obtained and followed.

MATERIALS AND METHODS

Acute and subacute toxicity tests

Toxicity of paracetamol to *C. gariepinus* was carried out according to the OECD guideline for testing of chemicals No. 203 in a semi-static renewal system by using 200L capacity glass aquaria (OECD, 1992). Five different concentrations (5000, 5500, 6000, 6500, 7000mg/L) and control 0.00 mgL^{-1} were selected and prepared in triplicates for definitive exposures after range-finding test. Ten (10) fish were exposed to each replicate but one group that was exposed to clean freshwater served as the control. Feed was not offered to the fish12 hours before the commencement of the experiment and also during the 96 hours exposure duration. Dead fish were removed immediately after death to prevent deterioration of water quality. The exposure solution was renewed each day and was analyzed using LC–MS/MS to ensure the agreement between nominal and actual concentrations of the pharmaceutical drug in the aquaria (Li et al., 2011). The experiment was conducted under the natural photoperiod of 12:12 light-dark cycle. The physico-chemical parameters of the test water were analysed daily, using standard methodsAPHA (2005) andwere recorded (dissolved oxygen 7.50±0.45 mg L^{-1}, temperature 27.75±0.5^{0}C, pH 7.8±0.13 and free carbon dioxide 4.28±0.6 mg L^{-1}). The test fish were sampled on hours 24, 48, 72 and 96 in each replicate to determine the toxic effects of paracetamol on the fish. The behavioural responses in exposed and control fish were observed and recorded daily. The LC_{50} was determined by using the Probit analysis (Finney, 1971) while the safety level was estimated by applying the safety application factor (AF) suggested by CCREM (1991). Fish were exposed to lower sub acute concentrations of paracetamol corresponding to 1/10, 1/5, and ½ of LC_{50} (620 mg/L, 1240 mg/L, 3100 mg/L

and control) for 8 days in order to ascertain the blood-oxidative stress modulation lipid peroxidation and nuclear aberrations, following exposure for 8 days and were fed at 3% of their body weight once a day with commercial fish food.

Genotoxicity assessment

The genotoxicity assessment/potential of the acetaminophen was assessed by micronuclei assay. Peripheral blood samples were collected from the caudal vein and smeared on clean, grease-free, frosted glass slides. The slides were fixed in methanol for 10mins and left to air dry at room temperature and finally stained with 6% Geimsa in Sorenson buffer (pH 6.9) for 20mins. After dehydration through graded alcohol and clearing in Xylene, slides were mounted in a mixture of Distyrene (Polystyrene), Plasticizer (tricresyl phosphate) and Xylene. From each slide, 1000 erythrocyte cells were scored under light microscope under 100 magnifications. Non refractive circular or ovoid chromatin bodies smaller than one third of the main nucleus and displaying same staining and focusing patterns as the main nucleus were scored as the micronucleus (Al-Sabti and Metcalfe, 1995). The micronuclei frequency was calculated as:

$$\mathrm{MN}\ (\%) = \frac{\text{Number of cells containing micronuclei}}{\text{Total number of cells counted}} x\ 100$$

Anti oxidative stress enzyme assay in erythrocytes of fish blood

Blood samples were collected by incising the caudal vein at the caudal peduncle with a heparinised syringe. The catalase (CAT) in the blood was determined according to the standard methods (Takahara et al., 1960; Orhan and Sahin, 2001) which involved H_2O_2 breakdown, and was measured spectrophotometrically at 240 nm. Enzyme activity was expressed as nanomoles of H_2O_2 decomposed min/L mg/L protein. Superoxide dismutase (SOD) activity was determined using the method of Misra and Fridovich (1972), based on the oxidation of epinephrine- adenochrome transition by the enzymes. Superoxide dismutase activity was assed spectrophotometrically at 420 nm and expressed as the amount of enzyme mg/L of protein required to give 50% inhibition of epinephrine auto-oxidation. Glutathione peroxidase (GPX) activities were assayed according to Paglia and Valentine (1967) which was based on the oxidation of gluthathione in the presence of NaN_3. Lipid peroxidation (LPO) in the liver tissue was determined by estimation of thiobarbituric acid reactive substances (TBARS), according to Sharma and Krishna-Murti (1968).TBARS concentration was measured spectrophotometrically at 535 nm at molar extinction coefficient of 156 Nm cm/L. specific activity was expressed in nanomoles of TBARS mg/L protein.

Statistical analysis

Data obtained was expressed as mean ± standard error and analyzed using the statistical package SPSS 20.0 computer program (SPSS Inc. Chicago, Illinois, USA). Differences in the test concentrations and control were subjected to one-way analysis of variance (ANOVA). The statistical significance was determined by 95% level of probability. Mean difference was separated using Duncan multiple range test.

RESULTS

Cumulative mortalities of *C. gariepinus* (50.96±1.3 g, n = 180) obtained after 96 hours exposure to mg/L concentrations of paracetamol (5000, 5500, 6000, 6500, 7000 and 0.00 mg/L) gave 10, 20, 40, 60, 80 and 0% mortalities respectively (Tables 1a and b). The 96 hours medial lethal concentration gave a value of 6200 mgL^{-1} and safety dose $1/_{100}$ LC_{50} = 62 mgL^{-1} (Figure 1). The exposed fish manifested various behavioral changes including erratic movement, air gulping, nervous manifestation and imbalance before death.

The parameters examined for oxidative stress were catalase (CAT), super oxide dismutase (SOD), lipid peroxidation (LPO) and glutathione peroxide (GPX). CAT reduced significantly below the control value of 0.83 to the least value of 0.57 µmol mm^{-1} mg $protein^{-1}$among the highest group exposed to 3100 mg/L. Similarly, SOD varied significantly from control and ranged from the control value of 12.31 to the least value of 8.27 U mol mm^{-1} mg protein $^{-1}$among fish to the highest concentration of the drug. GPX ranged from 9 to 4.65 µmol mm^{-1} mg $protein^{-1}$among group of fish in the control to the 3100 mg/L of the drug. On the other hand, LPO increased significantly ($p<0.05$) from the control value of 6.83 to 8 mMole/TBARS/ mg protein among group of fish to 3100 mg/L of the drug indicating that the effect was dose dependent (Figure 2). Genotoxicity potential showed dose dependent nuclear aberration represented by significant micronuclei elevation above the control among the treated fish.

DISCUSSION

Fish exposed to the acute doses of paracetamol manifested dose dependent uncoordinated movements, air gulping, and inappropriate swimming, preceding a 96 hours median lethal concentration of 6200 mg/L. The present findings however indicated a less toxic value than the value of 160 mg/L for exposed Oryzias latipes to the same (Kim et al., 2007), possibly due to species and age variations. CAT frequently respond to ROS generation by

Table 1a. Cumulative mortality of *C.gariepinus* exposed to paracetamol for 96 hours.

Concentration (mg/L)	Log Conc.	24	48	72	96	Mortality	% Mortality	Probit
5000	3.69	-	-	1	-	1	10	3.72
5500	3.74	-	-	-	-	2	20	4.16
6000	3.77	-	-	2	2	4	40	4.48
6500	3.81	-	1	1	4	6	60	5.25
7000	3.84	1	2	2	3	8	80	5.84
0	00	-	-	-	-	0	0	-

Table 1b. Behavior of *C.gariepinus* exposed to paracetamol for 96 hours.

Parameters of behaviour	Concentrations (mg/L)					
	0.0	5000	5500	6000	6500	7000
Erratic movement	-	-	x	xx	xxx	xxxx
Air gulping	-	x	x	xx	xxx	xxxx
Nervous manifestations	-	-	x	xx	xxx	xxx
Backward swimming	-	-	-	x	x	xx
Loss of balance	-	-	xx	xx	xxx	xxx

-none, x mild, xx moderate, xxx strong, xxxx very strong.

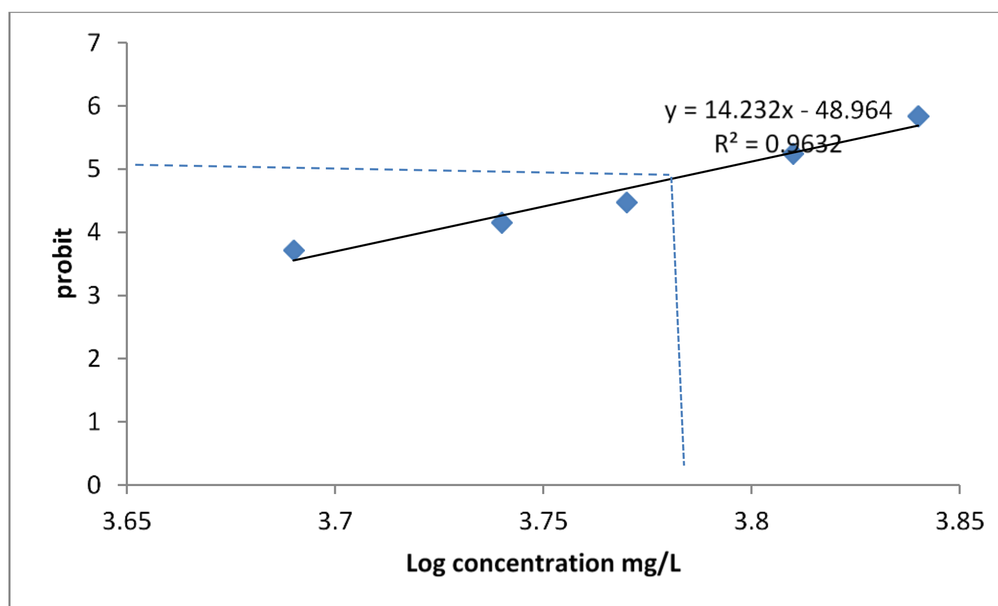

Figure 1. Logarithimic probit line for determination of 96 hours LC_{50} Paracetamol to *C. gariepinus.*

catalyzing the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al., 2004) but when the rate of reactive oxygen species (ROS) generation supersedes that of their removal by CAT as depicted in this findings, oxidative stress was invoked. The result showed that the activity of CAT in the erythrocytes of exposed group of fish was significantly inhibited compared with the control. SOD is known to be one of the major anti oxidant defense enzymes produced in response to oxidative stress which converts the highly toxic superoxide anions to hydrogen peroxide (Azad et al., 2005) by catalyzing the dismutation of the superoxide anion to hydrogen peroxide and molecular oxygen, invariably CAT activity decomposes the hydrogen peroxide into oxygen and water which constitutes an important part of the cellular antioxidant defense mechanism in organisms (Chanu et al., 2014;

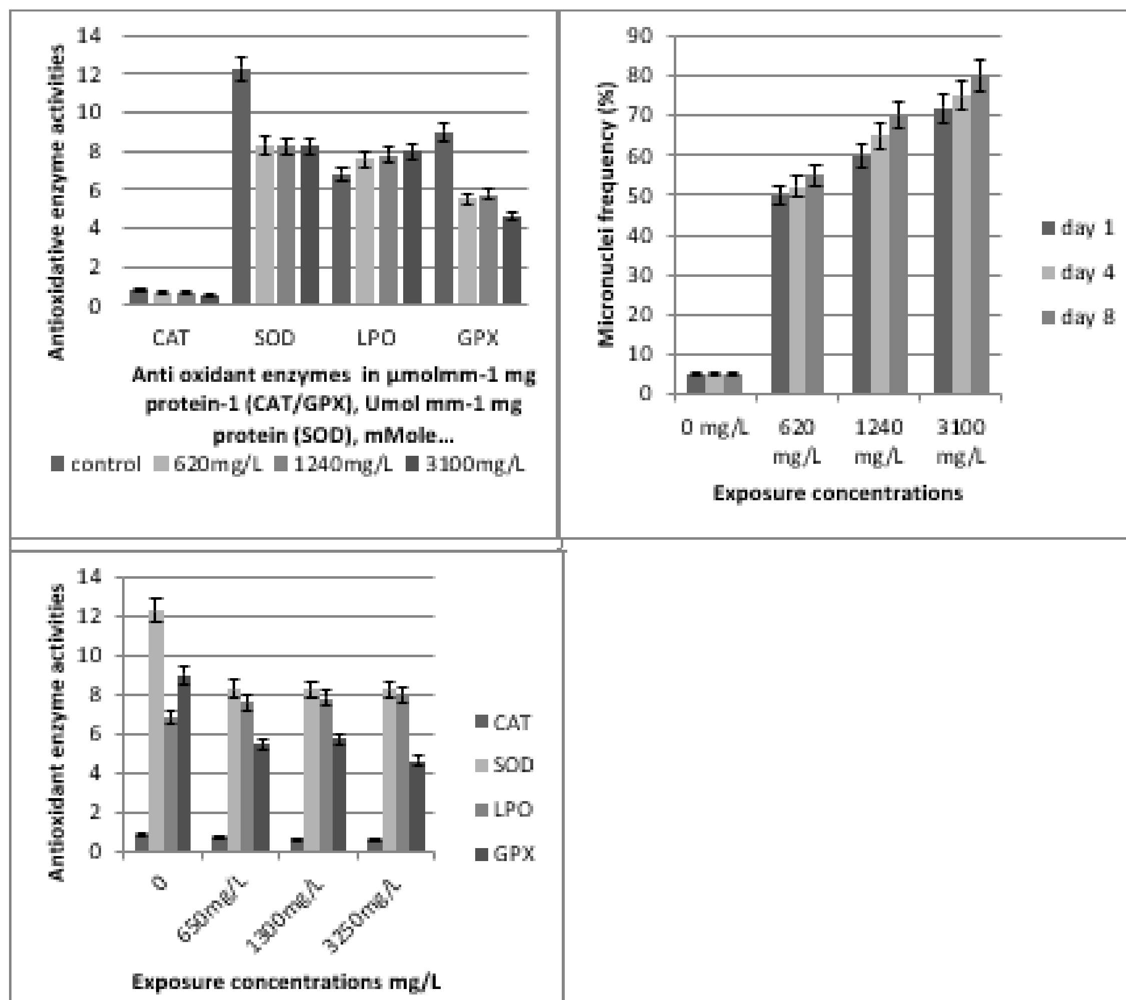

Figure 2. Means of CAT, SOD, LPO, GPX, and nuclear abberation of *C. gariepinus* to paracetamol.

Zheng et al., 2009). The result showed that SOD activity was significantly induced in the blood of the exposed fish in both time and concentration dependent relationship, which demonstrated that the exposure to paracetamol decreased its production of superoxide anion and the enzymes responsible for its metabolism. Similar result was reported by Ghelfi et al. (2015) on exposure of *Rhamdia quelen* to paracetamol. GPX enzyme plays an important role in protection of animals against oxidative dysfunction by reducing lipid hydro peroxides to alcohols (Velma and Tchounwou, 2010). Decrease in the activity of GPX in the erythrocyte can be attributed to decreased level of hydro peroxide in the tissues (Pandey et al., 2001).

Changes in LPO usually indicate oxidative stress and loss of cell function and physiology leading to oxidative damage in aquatic animals (Storey, 1996). The result showed that exposure to paracetamol led to increased oxidative damage by increasing the LPO level in the tissues. The alteration in the level of LPO in response to exposure depicted increased production of ROS, which lead to peroxidation of membrane lipids during metabolism of the drug (Zhang et al., 2004).

The SOD– CAT system has been noted to be the first line of defense against oxygen toxicity, due to the inhibitory effects on the formation of oxygen radicals (Pandey et al., 2003), and these enzymes were frequently used as biomarkers, that indicated the production of reactive oxygen species (ROS) (Monteiro et al., 2006). The reduction in SOD activity following exposure to the drug may be related to the production of oxidants. An excess of hydrogen peroxide may have reduced SOD activity, while the superoxide anion may be responsible for the decrease in CAT activity in response to paracetamol exposure (Bagnyukova et al., 2006; Scandalios, 2005). Thus, it may be reasonable to assume that hydrogen peroxide was responsible for the reduction observed in SOD activity while the reduction of CAT activity was due probably to accumulated superoxide anions not sufficiently neutralized by SOD. The activities of enzymes involved in animal's antioxidant system have been known to be a complex pathway of synergistic interactions among enzymes, due to the fact that the activity of one enzyme may influence

the other. In the present work, the inhibition of CAT and SOD limited the antioxidant defenses of the fish during the first day and progressed to the eight day of exposure to paracetamol in time and concentration dependent manner. Maran et al. (2009) reported GPX involvement in the metabolism of hydrogen peroxide although it was principally associated with the removal of organic peroxides. The significant increased activity of GPX in the fish following exposure to the drug was an indication that the antioxidant pathway was stimulated, probably due to the increased production of peroxides. Thus, the activation of GPX may be an indication of adaptive response to compensate the inhibition of CAT at the period of exposure. Production of lipid peroxidation due to ineffective neutralization of ROS has been considered as the main consequence of oxidative stress (Ansari et al., 2011), which in this investigation progressed throughout the duration of the study. Thus, the defensive activities of the antioxidant enzymes were insufficient to prevent the incidence of LPO in exposed Clarrid catfish to paracetamol at subacute exposure doses. Elicitation of LPO may have resulted to nuclear aberration of the exposed members of fish to the drug.

Conclusion

Exposed *C. gariepinus* to acute doses of paracetamol manifested abnormal behaviours before a 96hours median lethal dose of 6200 mg/L while reduced activities in SOD, CAT and GPX of the same to subacute doses of the drug for 8 days elicited LPO which led to nuclear aberration.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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