

Chronic Effect of Chlorpyrifos on Biochemical, Immunological Changes and DNA Damage in Juvenile Nile Tilapia (*Oreochromis niloticus*)

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Abstract

Tremendous use of organophosphate insecticides especially chlorpyrifos have led to elevation of the residual levels of these insecticides in aquatic system via contaminated runoff and atmospheric deposition. Our study was done to detect the effect of different chronic concentrations of chlorpyrifos ($1/10$ and $1/20$ of 96 h LC_{50}) on fingerlings Nile tilapia, *Oreochromis niloticus*, after 8 weeks of exposure through determine the oxidative potential indices, immunological parameters and hepatic and renal markers in serum. DNA damage was determined by single-cell gel electrophoresis (Comet assay). The marked change in clinical signs and postmortem findings were recorded in chlorpyrifos exposed groups. The results showed significant increase in malondialdehyde (MDA) levels, decline in antioxidant parameters which represented by decreasing in Glutathione (GSH) activity, inhibition in immunological parameters which represented by decrease in lysozyme and nitric oxide levels in the groups that were exposed to $1/10$ and $1/20$ of 96 h LC_{50} of chlorpyrifos. The significant elevation in hepatic and renal markers which represented by increasing in Alanine transaminase (ALT) and creatinine activities and a marked elevation DNA damage were recorded in the groups that were exposed to $1/10$ and $1/20$ of 96 h LC_{50} of chlorpyrifos. We conclude from our study that chronic exposure of juvenile *O. niloticus* to chlorpyrifos has the potential to cause harmful effect.

Keywords: Chlorpyrifos, *Oreochromis niloticus*, Comet assay, Immunological parameters, Antioxidant parameters.

Introduction

Fish, in several parts of the world, is the essential protein source for human mainly in developing countries [1]. Aquaculture is a perfect method to supply the increasing demand for fresh and marine water fish [2]. Tilapias are used in intensive aquaculture, importance of tilapia in this field, is due to its growth and reproduction in a wide range of environmental changes. Tilapia can withstand rough handling and have become one of the most important warm-water aquaculture fish group in the world [3]. Aquaculture field in Egypt grows rapidly. Nile tilapia (*Oreochromis niloticus*) is the most spread farmed fish species in Egypt. Aquaculture needs good health program and perfect collection of data about environmental toxicities such as insecticides [4]. The insecticides represent one of water pollutants, both synthetic and natural, which participate in the environmental problems [5,6]. Insecticides have been used in agriculture for improvement of production of food by killing of unwanted insects and controlling disease causes mainly organophosphorous (OP) [7]. At present,

insecticides water toxicity spread in developing countries, where there is elevation in insecticides uses as in agriculture. Insecticides enter the water surface through several pathways after leaving their places of use and spread throughout the environment, one of them in urban areas is through rainfall runoff and atmospheric deposition and other sources is through municipal and industrial dischargers [8-10]. Insecticides are highly toxic to fish. Insecticides have hazard effects on the survivability, growth rate, reproduction and physiological and biochemical processes activities of fish which are sensitive to insecticides toxicity [11-13].

Chlorpyrifos is a one of most selling, used and highly spread organophosphate insecticide, to kill pests on different types of crops. It is heavily used leads to its accumulation in water surfaces by chlorpyrifos, causing hazard effects on fish [14]. This study aimed to evaluate the consequences of chronic exposure of *Oreochromis niloticus* (*O. niloticus*) to $1/10$ and

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$1/20$ 96-h LC₅₀ of chlorpyrifos on the serum hepato-renal biochemical Markers, antioxidant and immunological parameters and DNA damage of *O. niloticus*.

Materials and Methods

Experimental fish and protocol

Sixty *Oreochromis niloticus* cultured fingerlings were collected from Abbassa fish hatchery, Sharkia, Egypt with average body weight 25 ± 0.5 g. The *Oreochromis niloticus* were kept in a glass aquaria filled with dechlorinated tap water and aerated and allowed to acclimatize to laboratory conditions for two weeks before exposure. *O. niloticus* were divided into 3 equal groups, each group has two replicates (10 fish replicate⁻¹). The first group (G1) was kept as control. The second and third groups (G2 and G3) were exposed to $1/10$ (2.64 $\mu\text{g/L}$) and $1/20$ (1.32 $\mu\text{g/L}$) 96 hrs LC₅₀ of chlorpyrifos, respectively according to Gawish *et al.* [15]. The water of the aquaria was changed every week and fresh solutions were spiked so as to keep water quality with an appropriate chlorpyrifos level. During the exposure period, fish were closely observed and clinical signs, postmortem lesions were recorded. The aquaria were periodically cleaned. Water parameters were periodically measured with average dissolved oxygen (D.O) 7 mg/L, water temperature $28 \pm 2^{\circ}\text{C}$, PH 7.5, Nitrite 0.05 mg/L, Nitrate 10 mg/L, Ammonium 0.02 mg/L. The photoperiod (10 h light: 14 h dark) in the laboratory was controlled. Through the duration of the experiment, *Oreochromis niloticus* were regularly fed with diet commercially available dry fish pellets for 8 weeks. Feeding rate was 3% from total weight. The diet was given three times daily. The food was applied by hand in corresponding aquaria. The experiment was done according to the Care and Use of Laboratory Animals Guidelines of the National Institutes of Health (NIH). The study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Chemicals and reagents

Chlorpyrifos (lorsban) (0,0-diethylO-3,5,6-trichloro 2pyridylphosphorothioate) (organophosphate insecticide), with

commercial name Chlorzane EC® (a.i. chlorpyrifos, 480 g/l) was produced from Kafr El-Ziate for pesticide and chemicals Co., Egypt. The kits used for biochemical measurements of ALT activity and creatinine level were obtained from Biodiagnostic Co., Egypt., nitric oxide level was obtained from Biorad, USA., lysosyme assay were obtained from Sigma Aldrich, USA., MDA and GSH levels were obtained from Thermo Scientific, USA and DNA damage were obtained from Ltd., UK.

Blood samples collection

Blood samples were collected at the end of the experiments from the caudal blood vessels according to Stoskopf [16] for determination of biochemical parameters, put blood samples in plain centrifuge tubes and centrifuged at 3000 R.P.M (round per minute) for 15 minutes for serum separation then stored at -20°C till biochemical analysis.

Serum biochemical measurements

Determination of serum ALT level according to Reitman and Frankel [17]. Estimation of serum creatinine was carried out by photometric colorimetric test for kinetic measurement without depolarization as mentioned by Henry [18].

Determination of oxidative stress indices

MDA concentration was determined according to Ohkawa *et al.* [19]. Measurement of MDA was based on the colorimetric reaction with thiobarbituric acid (TBA) and read spectrophotometrically at 532 nm using a Varioskan Flash Spectral Scanning Multimode Reader. GSH content was determined according to Moron *et al.* [20]. GSH was detected by measuring NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidation at 340 nm spectrophotometrically using a Varioskan Flash Spectral Scanning Multimode Reader.

Determination of immunological parameters

Lysozyme activity was determined according to Parry *et al.* [21]. Forty microliter of fish serum will be added to 3 ml of the bacterial suspension and the reduction in absorbance at 540 nm detected after 0.5 and 4.5 min incubation at temperature 22°C . One unit of lysozyme activity was determined as a

reduction in absorbance of 0.001 per min. The nitric oxide level in each serum sample was determined as the method described by Rajaraman *et al.* [22]. A total amount of 100 µl of serum for each sample was added to the same volume of Griess reagent and incubated in a 96 microtiter plate well for 10 minutes at 27°C then the optical density was measured, spectrophotometrically at 570 nm by ELISA reader.

Preparation of gill tissues homogenates

At the end of the experiment, fish dissected immediately and the gills tissues were taken, homogenized in 10 volumes of phosphate buffer saline (pH 7.4) then the homogenates were centrifuged for 30 min at 664 × g at 4°C and the supernatant kept at -80°C for determination DNA damage by the alkaline comet assay method.

Single cell gel electrophoresis (SCGE); Alkaline Comet assay

The Comet assay was done according to Singh *et al.* [23]. The cells were observed by 400 magnifications in an Optika Axioscope fluorescence microscope (Olympus-Bx60,

excitation filter: 515-560 nm; barrier filter: 590 nm) attached to a Camera of color CCD video and attached to an Images and Statistical Analysis occur through the comet score analysis system (tritek comet score™ freeware v1.5) to determine DNA damage (tail length, tail DNA intensity (%) and tail moment). The length of DNA migration (tail length) was measured in PX from the center of the nucleus to the end of the tail. Tail DNA intensity (%) is the percentage of DNA in the tail. The tail moment was calculated as: tail length X percentage of migrated DNA / 100.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) through the general linear models (GLM) procedure of the Statistical Package for Social Sciences version 21.0 (SPSS for Windows 21.0, Inc., Chicago, IL, USA). The post hoc comparisons of means were performed with Duncan's multiple range tests (DMRT). Results were expressed as mean ± standard errors (SE); the value of P < 0.05 was used to indicate statistical significance.

Table 1: Behavior, mortality and survivability of *Oreochromis niloticus* exposed to chlorpyrifos after 8 weeks

Group n=20	Escape reflex*	Mortality	
		No.	%
G1 (Control)	+	0	0
G2 ¹ / ₁₀ 96 hrs LC ₅₀ of chlorpyrifos (2.64 µg/L)	-	12	60
G3 ¹ / ₂₀ 96 hrs LC ₅₀ of chlorpyrifos (1.32 µg/L)	-	7	35

*The fish respond activities to any external stimulation.

+ The fish respond well to escape reflex.

- The fish not respond to escape reflex.

Results and Discussion

Chronic exposure assay and clinical observations

After 8 weeks of exposure period during this study, no mortalities were observed with positive escape reflex in control group while high mortalities were observed in the ¹/₁₀ 96-h LC₅₀ group (60%) followed by ¹/₂₀ 96-h LC₅₀ (35%) chlorpyrifos group with loss escape reflex so mortality percentage increase with increase chlorpyrifos concentration (Table 1). During the exposure period, control fish showed no clinical signs but fish groups exposed to chlorpyrifos showed decrease in

movement activity, loss of appetite, fin rot, increase in opercular movement and collection of fish near water. Darkness of skin and congestion in liver, kidney and gills were noted during the postmortem investigation (Figure 1). These results agreed with Palanikumar *et al.* [24] who reported that increase in the chlorpyrifos concentration in water lead to increase fish mortality rate. The elevation of mortality rate may be due to toxic effect of chlorpyrifos. Similar results were recorded by El-Bouhy *et al.* [25] who reported that Nile tilapia showed fin rot, rapid movement of operculum with opened mouth and collection of fish near water surface when exposed to chlorpyrifos insecticide.

Table 2: Chronic effects of $1/10$ and $1/20$ 96-h LC₅₀ values of chlorpyrifos exposure on biochemical and immunological parameters of *O. niloticus*

Parameters	G1	G2	G3
	Control	$1/10$ 96-h LC ₅₀ (2.64 µg/L)	$1/20$ 96-h LC ₅₀ (1.32 µg/L)
ALT (U/l)	15.21±0.58 ^c	30.56±0.47 ^a	25.01±0.63 ^b
Creatinine (mg/dl)	0.14±0.008 ^c	0.98±0.04 ^a	0.71±0.05 ^b
GSH (ng/ml)	9.45±0.15 ^a	3.50±0.18 ^c	5.19±0.26 ^b
MDA (nmol/l)	34.25±0.91 ^c	51.61±1.23 ^a	46.23±1.02 ^b
lysozyme (µg /mL)	8.32±0.35 ^a	6.87±0.23 ^c	7.52±0.12 ^b
Nitric oxide (Mmol/L)	0.82±0.02 ^a	0.32±0.04 ^c	0.41±0.07 ^b

Means within the same row bearing different letters (a, b and c) are significantly different (p <0.05).

Table 3: Effects of $1/10$ and $1/20$ 96-h LC₅₀ value of chlorpyrifos exposure on the tail length, tail DNA % and tail moment in *O. niloticus* gills after 8 weeks exposure period

Parameters	G1	G2	G3
	Control	$1/10$ 96-h LC ₅₀ (2.64 µg/L)	$1/20$ 96-h L C50 (1.32 µg/L)
Tail length (px)	2.91±0.07 ^c	4.54±0.13 ^a	4.12±0.08 ^b
% DNA in tail	16.71±0.41 ^c	22.13±0.35 ^a	20.21±0.45 ^b
Tail moment	0.64±0.02 ^c	1.29±0.05 ^a	1.02±0.01 ^b

Means within the same row bearing different letters (a, b and c) are significantly different (p <0.05).

Serum biochemical parameters

Exposure to chlorpyrifos showed significant (P<0.05) elevation in hepato-renal markers (ALT and creatinine levels) than control fish; this observation was significantly highly cleared in the $1/10$ 96-h LC₅₀ exposed fish (Table 2). These results agree with that reported by Ibrahim and Banaee [26] who mentioned that the fish treated by diazinon showed high creatinine level and Jee *et al.* [27] who found that exposure of *Sebastes schlegeli* to cypermethrin showed increase in serum level of ALT. The increase in ALT in serum of fish exposed to chlorpyrifos toxicity may be attributed to the liver cellular damage which causing leakage of ALT enzyme into the blood [28] and the increase in creatinine in serum of fish exposed to chlorpyrifos toxicity may be attributed to decrease in kidney glomerular filtration or tubular dysfunction [29].

Antioxidants and lipid peroxidation indices

Significant (P<0.05) increase in serum MDA contents and decrease in serum GSH levels were noted in groups exposed to

chlorpyrifos than control fish; this observation was high significantly cleared in the $1/10$ 96-h LC₅₀ exposed fish (Table 2). The increase in serum MDA level may be attributed to the high production of ROS as result of insecticide toxicity and cause damage to important cellular macromolecules such as proteins, lipids (lipid peroxidation) and nucleic acids, which lead to release high MDA level [30]. The decrease in serum GSH concentration may be due to insecticides oxidative stress which releases free radicals. GSH molecules remove free radicals through reacting GSH with free radicals so occur oxidation of GSH to glutathione disulfide GSSG which lead to decrease in GSH level [31]. Similar results showed by Olsvik *et al.* [32] who reported that Atlantic salmon exposed to chlorpyrifos showed decline in serum reduced glutathione (GSH) and Hakim *et al.* [33] who showed that serum reduced glutathione content (GSH) significantly decrease and lipid peroxidation biomarker (MDA) significant increase in serum after *Oreochromis niloticus* treated with FNT insecticide.



Figure 1: Clinical signs and post mortem findings of *Oreochromis niloticus* of control group and that exposed to chlorpyrifos. A: Fish of control group and appeared healthy without any external or internal lesions. B: Fish exposed to chlorpyrifos showed congested liver, kidney and gills and darkness of skin.



Figure 2: Nucleus of gill cells of Nile tilapia after exposure to different concentrations of chlorpyrifos.

Immunological parameters

Lysozyme and nitric oxide showed significant ($P < 0.05$) decrease in groups exposed to chlorpyrifos than control fish which be more cleared in the $1/10$ 96-h LC_{50} exposed fish (Table 2). These results agree with that reported by Helali *et al.* [34] who showed decrease in lysozyme and nitric oxide production from macrophages in rats exposed to chlorpyrifos and Wang *et al.* [35] reported that *C. carpio* that exposed to chlorpyrifos (75 $\mu\text{g/L}$) showed decline in lysozyme level in plasma and spleen. El-Murr *et al.* [36] who reported that exposure of *Oreochromis niloticus* to fipronil insecticide lead to decreased serum level of lysozyme. Hibbs *et al.* [37], Umezawa *et al.* [38] and Granger *et al.* [39] showed that macrophages release nitric oxide which is one of non-specific immunity where it plays as an endogenous antibacterial substance during infections and antitumor and require arginine for its production. Sarder *et al.* [40] mentioned that macrophages of head kidney and spleen tissue secrete serum lysozyme, which is nonspecific chemical defense. The decrease in serum nitric

oxide and lysozyme value in fish exposed to chlorpyrifos toxicity may be attributed to decrease in macrophage number in fish as result of water pollution [41].

DNA damage level

DNA damage level in gills of both groups exposed to chlorpyrifos and control group was measured by comet assay. Fish exposed to chlorpyrifos showed Significant ($P < 0.05$) increase DNA damage level which represented by significant increase in tail length (px), tail DNA% and tail moment (TM) than control group; DNA damage was cleared in the $1/10$ 96-h LC_{50} exposed fish (Figure 2 and Table 3). These results agreed with Abu Zeid and Khalil [42] who reported that occurrence of increase in DNA damage in fenitrothion exposed *Oreochromis niloticus* indicated by increase in tail length (px), tail DNA% and tail moment (TM). The increase in DNA damage may be attributed to reactive oxygen species (ROS) which resulted from insecticide metabolism causes DNA damage by reaction between ROS and DNA [43].

Conclusion

The results of this study consider evidence that exposure of *O. niloticus* to sub lethal concentrations of chlorpyrifos lead to biochemical, immunological and DNA damage and interfering with the antioxidant defense.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

التأثير المزمن للكلوربيريفوس على التغيرات الكيميائية الحيوية، والتغيرات المناعية وتلف الحمض النووي في البلطي النيلي

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الزيادة في استخدام المبيدات الحشرية الفسفورية و خاصة الكلوربيريفوس ادى الى زيادة تواجد هذه المبيدات في النظام المائي عن طريق جريان المياه الملوثة و لترسيبات الجوية. تهدف هذه الدراسة الى تحديد تأثير الجرعات تحت المميته من الكلوربيريفوس (١.٠/١ و ٢.٠/١ من الجرعة نصف المميته لمدة ٩٦ ساعة) على اسماك البلطي النيلي بعد ٨ اسابيع من التعرض لهذه الجرعات من خلال قياس مؤشرات الاكسدة (محتوى المالوندايهايد) , مضادات الاكسدة (مستوى الجلوتاثيون المختزل) , المعايير المناعية (الليزوزيم و اوكسيد النيتريك) و علامات الكبد و الكلى (مستوى الالانين امينوترانسفيراز و الكرياتنين) في السيرم. تم تحديد تلف الحامض النووي عن طريق الفصل الكهربائي بالجيل (فحص الكوميت). و لقد تم تسجيل تغير ملحوظ في الاعراض الظاهرية و الصفات التشريحية في المجموعات المعرضة لكلوربيريفوس. كما اوضحت النتائج حدوث زيادة معنوية في مستوى المالوندايهايد , نقص في مضادات الاكسدة الممثلة في نقص نشاط الجلوتاثيون المختزل, انخفاض في المعايير المناعية الممثلة في نقص في الليزوزيم و اوكسيد النيتريك, زيادة في علامات الكبد و الكلى المتمثلة في زيادة مستوى الالانين امينوترانسفيراز و الكرياتنين و زيادة ملحوظة في تلف الحامض النووي في المجموعات التي تعرضت ل ١.٠/١ و ٢.٠/١ من الجرعة نصف المميته لمدة ٩٦ ساعة من الكلوربيريفوس.. نستنتج من دراستنا ان التعرض المزمن لاسماك البلطي النيلي للكلوربيريفوس يسبب لها الضرر البالغ.