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EFFECT OF METHIDATHION ON THE CYTOCHROME P-450 ENZYMES IN THE NILE TILAPIA *OREOCHROMIS NILOTICUS*

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Key words: *Oreochromis niloticus,* EROD, Cytochrome P-450 organophosphate; xenobiotics, hormones, Manzala Lake.

ABSTRACT

An experiment was conduced on adult male and female Nile tilapia to investigate the effect of Methidathion (MD,is one of the more toxic organophosphates) MD at doses 3 μg l^{-1}·10 μg l^{-1}, 20 μg l^{-1}on the microsomal of ethoxy-resrufin o-deethylase activity (EROD). The experimental fish was obtained from Manzala Lake from the areas suffering from pollution. Result revealed that activity of EROD in female livers μg/kg/ fresh liver was (3,33,25,17,12) for MD concentrations 3μgl^{-1};10μgl^{-1};20μgl^{-1}, Control and IC groups, respectively. Analysis of variance indicated that differences among the tested groups were not significant P<o.o5 for females. Whereas for males were significant P<o.o5 (10,153,105,97and 58).The results also indicated that EROD activity in livers of tilapia males was the highest at MD lower concentrations was due to the resistance of fish against MD at the concentrations 10 μg l^{-1} and 20 μg l^{-1}· then it decreased at higher concentrations indicating that resistance against MD decreased which will be reflected on growth performance and fish activity. These results indicated that fish males, in general, showed better resistances against toxins compared to females.

INTRODUCTION

Increasing pollutant concentrations in lake Manzala has affected the different organs systems of fish due to interaction of xenobiotics with metabolizing systems. Investigation of Nile tilapia fish *(Oreochromis niloticus)* liver monooxygenase system has been carrited out, simultaneously, accumulation of different organic

compositions, viruses, heavy metals, pesticides.and xenobiotics.These can provoke genovariations and affect tilapia development. Two different approaches may be used to quantify Cytochrome P-450 enzymes (CYP) induction, the measurement of the catalytic activity was measured as EROD activity.7-ethoxyre-sorufin-O-deethylase activity .The effect of MD has been examined on the EROD activity of microsomes.

Increasing pollutant concentrations in Manzala Lake during the last decades caused harmful effects in fish tissue, in addition to increasing of organic load, viruses, heavy metales, pesticides and xenobiotics (Hong aand Yang, 1997). Mechanisms of accumulation in fish are very carefully investigated. Pollution affects the membrane detoxication systems of freshwater fish, resulting in alteration of its functions and tissue trouble mechanisms. On the other hand, it allows to offer the biological test systems for water biocenoses accumulation analysis.

Among biochemical biomarkers, the cytochrome P-450 (CYP) dependent monooxygenase activity,which is one of the best parameters for measuring the response of aquatic organisms to certain environmental contaminants (Melancon, *et al.*, 1983).

In these fishes, tissues extracts genotoxicity analysis were carried out to detect the organic material present in tissues that can provoke genovariations and affect tilapia posterity development and fish monooxygenase system that fulfils the detoxication and metabolic activation of xenobiotics.(Mark J.Snyder., 2000).

P-450 proteins are found in a diverse array of organisms including bacteria, plants, fungi, and animals. Phylogenetic analysis of this diversity suggests that a common ancestor to all present day P-450 forms existed prior to the evolution of eukaryotes from prokaryotes (Nelson and Strobel, 1987).

Functions of P450s in the metabolism of endogenous compounds (e.g.steroids, fatty acids, eicosanoids) and xenobiotics (e.g. dietary plant chemicals, various aromatic hydrocarbons (PAH, AH), polychlorinated biphenyls (PCB), insecticides, rugs) has been extensively studied during the last 30 years (Gonzalez, 1989). Types of P-450 mediated rreactions include hydroxylation, epoxidation, oxidative deamination,S-,*N*-,and 0-dealkylations, and dehalogenation.

P-450 research, especially the degenerate primer-based RT-PCR procedures, represent the beginning of molecular approaches to reveal potential YP biomarker genes.This approach circumvents he aforementioned problems with P-450 enzyme measurements and

biochemical purifications (Mark J.Snyder, 2000).

Effect of Methidathion (organophosphate) on the P- 450 induction was assessed both by measurements of the induction of the EROD activity and of the immunodetection of the CYP protein in *Gudgeon* (Patrick *et al.*, 1998). Fish were pre-treated with 5 mg Kg^{-1} of B-naphthoflavone (BNF) and exposed to concentration of Methidathion in laboratory. Methidathion proved to inhibit EROD induction in vivo and in vitro. In contrast, levels of immunoquantified CYP were found to be slightly higher in microsomes from fish exposed to 10-30 $\mu g\ l^{-1}$ of Methidathion than in control fish exposed to BNF alone. This suggests that the inhibition of the EROD activity by Methidathion resulted from a catalytic inhibition without decrease of the CYP-450 protein.

Thus, the aim of this work was to verify the inhibition of the EROD induction by Methidathion using Nile tilapia fish (*Oreochromis niloticus*) .In an earlier work, it showed that the exposure of Rainbow Trout (*Salmo gairdneri*) to a mixture of environmentaly relevant concentrations of copper and Methidathion resulted in an additive decrease of the EROD induction (Flammarion *et al.*, 1996). Yet, in rivers contaminated by pesticides, tilapia in particular, was more appropriate sentinel species than trout. Besides, the measurement of the CYP protein might give additional information on the mechanism of inhibition. In that particular case of fish treated with an organophosphate pesticide, it is interesting in our future research to measure the AchE (acetylcholinesterase) activity in muscle to compare the sensitivity of effects, specific (AChE) and non-specific (EROD).

MATERIALS AND METHODS

Chemicals:

Methidathion (MD) (97%) (S-2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-yl-methyl o, o-dimethyl phosphorodithioate) and 7-ethoxyresorufine,Bnicotinamide adenosine-diphosphate reduced (NADPH), *B*-naphthoflavone (BNF),phenylmethylsulfonylfuoride (PMSF), acetylthiocholine iodide (ATCI) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB). Mouse anti-cod CYP IgG monoclonal antibodies (Mab NP7) were obtained from Centeral Lab for pesticides and All other chemicals were of the highest available commercial grade.

Preparation of Microsomal Fractions:

S9 supernatants were pooled and centrifuged at 105000-x g for 1 h at 4-5^{0}C.The microsomal pellets were resuspended in homogenization buffer (100 mM phosphate, pH 7.8, 1mM EDTA, 1 mM DTT. 20% glycerol) (Stien *et al.*,1997)

EROD Assay:

Liver homogenates were thawed at 4^{0}C and centrifuged at 9000 x g.Thesupernatant (S9) was used for enzymatic assay performed at room temperature 20±1°C on a 96-well microplate according to Flammarion *et al.*, (1996) with a slight modification. Enzymatic activities were reported on S9 protein concentrations measured by method of Lowry *et al,* (1951)

Methidathion Analysis :

MD was extracted from the water samples with dichioromethane (3x50 ml) at pH 4. Samples in a final volume of 2 ml isooctane were analyzed according to Flammarion *et al* (1996).

Microcosms from 4-d BNF-induced Nile tilapia were pre-incubated with increasing concentrations of MD. Microsomes were used, instead of the S9 fraction, to obtain higher EROD activities in vitro. After 30 min of pre-incubation, 7-ethoxyresorufin and NADPH were added to the incubation mixture and EROD activity was followed for 4 min. CI_{50} values were estimated on percentages of control EROD activities by a non-linear estimation.The confidence interval of the CI_{50} parameter was calculated according to (Efron, 1981).

The MD concentrations in water were controlled twice during the assay. The observed concentrations of MD were 10-20% lower than the nominal ones (low stability of the MD to light).Fish groups contained ten individuals each.

Experiments:

Nile tilapia specimens were obtained 6 months before conducting of the experiment from Fish Research Center in Ismallia Suez Canal University . First group was obtained from Fish Research Center in Ismallia Suez Canal University as control group, while second group was obtained from Manzala lake induced control pollution locations, the third group was treated with concentrations $3\mu.gl^{-1}$, $10\mu gl^{-1}$and 20 μgl^{-1} of MD. Fish was kept in dark tanks supplied with water flow. Feeding was stopped for 24 hs. prior to i.p.

injection and prior to sampling. Fish were maintained on a 10:14-h dark:light photoperiod (Flammarion *et al* 1996).

Immediately before the exposure to MD, fish were intraperitoneally injected with 5 mg kg^{-1} of BNF in corn oil in order to induce EROD activity to a level comparable to that of the Nile tilapia exposed to environmental induces. This dose leads to about 10 fold induction in tilapia fish. Then, groups of 10 fish each were exposed to continuous flow of MD for 96 hs.

When the fish was sacrificed, liver was removed and rinsed in 150 mM KCl, homogenized in 100 mM phosphate buffer, pH 7.8 with 20% glycerol and PMSF (0.2 mM), poured into 1.8 ml cryotubes. Liver homogenates, as ept rozen n a liquid of nitrogen and kept for several days at- 80^0C before enzymatic assays.

Data-Analysis:

Statistical software was used for all statistical analysis.EROD was log-transformed to conform to the normality test (X^2-test for normality) Geometric ean ive hen eliable osition arameters nd compared by t-tests. All levels of significance were $P< 0.05$ except where otherwise noted. The 95% confidence interval (IC_{95}) were calculated using the critical values of Student's-distributions.

Results and Discussion

The effect of MD was only assessed after four days exposure to 3µg l^{-1}·10µgl^{-1} ,20 µg l^{-1}.A significant decrease was only observed at the highest MD concentration (20 µg l^{-1}) · Methidathion had no significant effect on basal EROD activity. In contrast, the exposure of tilapia fish to MD led to a 60 -70% decrease of the EROD induction when fish had been pre-treated with BNF (Table 1). However, this decrease was significant only in males $P<0.05$). While in females, dispite of a similar decrease (64%), the inhibition was not significant ($P < 0.05$). This could be explained by the greater variability of female EROD activities, probably due to the sexual maturation .

Averages of EROD activity in liver of female and male tilapia as affected with MD concentrations (3µgl^{-1},10µgl^{-1}·20µgl^{-1} (compared with control (C) and induced control (IC) collected from Fish Research Center Lake Manzala are presented in table (1). Results revealed that activity of EROD in female livers µg/kg/ fresh liver was (3,33,25,17,12) for MD concentrations 3µgl^{-1} ; 10µgl^{-1};

$20\mu g l^{-1}$) Control ,IC , and treatment groups, respectively.

Analysis of variance indicated that differences among the tested groups were not significant $p<o.o5$. Results revealed also that EROD activity measured as % of that of the control group for the same female groups cited above was (100, 1100, 833.3, 566.6, 400 %) i.g control, induced control and treated respectively, these results indicate that EROD activity in livers of tilapia females was the highest at MD lower concentrations due to resistance of fish against MD at this concentrations, then it decreased at higher concentrations indicating that resistance against MD decreased which will be reflected on growth performance and fish activity.These results are in accordance with the findings of Simon *et al.* (1984) in carp, Flammarion *et al.* (1996) in rainbow trout and Cebrian *et al* . (1991) in crayfish.

While for EROD activity in male livers were (10,153,105,97,58)for MD concentrations $3\mu g l^{-1}$;$10\mu g l^{-1}$,$20\mu g l^{-1}$) C,IC, and treatmeant groups, respectively. Results revealed that EROD activity measured as % of that of the control group for some female groups cited above was (100,1530,1050,970,580%) respectively, these results indicate that EROD activity in livers of tilapia males was the highest at MD lower concentrations due to resistance of fish against MD at the concentrations $10\mu g l^{-1}$ and $20\mu g l^{-1}$, then it decreased at higher concentrations indicating that resistance against MD decresed which will be reflected on growth performance and fish activity.

These results are in agreement with finding reported by the findings of De–Bruijn *et al* ,(1991) in guppy; Patrick *et al* ., (1998) in gudgeon ; Montgomery .(1993) in rainbow trout. .These results indicate that males in general showed better resistances against toxins compared to females . (Fig . 1).

Flammarion *et al.* (1996) found that MD is an inhibitor to the EROD. Induction in rainbow trout resulting 60 % decrees at 5 $\mu g\ l^{-1}$ of MD when trout had been pre-treated with 0.5 mg kg^{-1} of BNF (resulting in 10-30 times of induction). A higher dose of BNF (5 mg kg^{-1}) was necessary to induce the EROD activity in tilapia to a similar level. Tilapia was also less sensitive to MD than trout, since the EROD induction was reduced (63% for both female and male) after exposure to $30\mu g l^{-1}$of MD. It was noticed that fish exposed to 30 $\mu g\ l^{-1}$ of MD lost their appetite. This was already observed with rainbow trout exposed to 5 $\mu g\ l^{-1}$of MD. At those upper concentrations, it was also observed loss of equilibrium and trouble in swimming, which

corresponds to the pattern of response of fish to chemicals with a narcotic action (De-Bruijn *et al.*, 1991). Then, the inhibition of the EROD activity could also have resulted from non specific toxicity at those concentrations of MD. However, a decrease in the CYP protiein would then have occurred at higher concentrations of exposure. Methidathion is one of the more toxic organophosphates with a rainbow trout (*Salmo gairdneri*) 96-h LC_{50} = 10 μg l^{-1} (Montgomery, 1993), a crayfish 96-hr LC_{50} = 280 μg l^{-1} (Cebrian *et al.*. 1991). The effect of MD was only assessed after 4 days exposure and would need more studies at different durations of exposure to provide a complete analysis of the MD mechanism in vivo. The earlier authors suggested that MD not only block the active center of the enzyme. Some products of P-450 metabolism are however more reactive, leading to cellular damage and in certain cases to the initiation of carcinogenesis (Hong and Yang, 1997) discovering the molecular mechanisms that regulate detoxification enzyme levels, such as multiple genes of the cytochrome P-450 superfamily.

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Table (1) EROD activities in Nile tilapia exposed to increasing concentrations of Methidathion (MD) after a pre- treatment with 5 mg kg^{-1} of BNF

Treatment	Female		Male	
	EROD	%	EROD	%
Control	3(2;7)	100	10(5;21)	100
Induced control	33(11;95)	1100	153(95;244)	1530
MD 3µg l^{-1}	25(9;71)	833.3	105(66;168)	1050
10µg l^{-1}	17(6;47)	566.6	97(77;122)	970
20µg l^{-1}	12(5;27)	400	58(38;90)	580

Geometric means are given with Cl_{95} in parentheses.
% between MD exposed and i control fish .