A STUDY OF BIOCHEMICAL CHANGES IN THE TISSUES OF ZEBRAFISH (DANIO RERIO) ON EXPOSURE TO ORGANOPHOSPHORUS PESTICIDES

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ABSTRACT

The aim of this study was to investigate the acute toxicity of Organophosphate (Malathion and Atrazine), and determined the accumulation of these pesticides in the whole body of zebrafish, and some biochemical responses in its brain and muscle. The results showed that MAL and ATR could be rapidly accumulated in the fish body shortly after being exposed to acute concentrations of the toxicants and exhibited a dose-dependent manner. This was also validated by the response of antioxidant defense, superoxide dismutase and catalase, both exhibited significant increase in activity. Acetylcholinesterase activity of zebrafish was also shown inhibited in a dose-dependent manner.

INTRODUCTION

An increasing number of pollutants currently exist in aquatic ecosystems, pose a seriously threaten to the environment and directly affect the health of organisms, as well as human consumers (1). The important sources of water pollution are industrial effluent, domestic, sanitation, drainage and pesticides that pollute rivers and major water sources(2) and the bioaccumulation of pollutants in aquatic organisms resulting from water pollution cause growing risk to wildlife and humans(3). The organophosphate (OP) compounds are one of the most widely used pesticides in the

field of agriculture and public health, which represents 50% of the global insecticides use because of their biodegradation promptly and non persistent nature (4). Associated mechanism of OP toxicity is showed as the inhibition of acetylcholinesterase (AChE), which resulting in accumulation of acetylcholine in the cholinergic receptors of the peripheral and central nervous system (5). In ecotoxicology, it is strongly recommended to use cellular and biochemical parameters to assess the exposure / effects of chemicals (6). AChE has been used as a bioindicator of exposure to OP pesticides in non-target species (6), However, oxidative stress became increasingly important molecular mechanism in OP-induced toxicity. Some biomarkers of antioxidant more common include superoxide dismutase (SOD) and catalase(CAT) (7). System antioxidant defense plays a critical role in maintaining homeostasis of the cell. Under normal physiological conditions, the reactive oxygen can be removed from the metabolism of extraneous chemicals in the body well by the regime of antioxidant defense. Some of these enzymes can be regarded as good molecular bioindicators for pollutant-mediated oxidative stress and can also indicate the magnitude of the response in populations exposed to pollutants such as pesticides, metals and other xenobiotics (8). Fish are used widely to assess the health of aquatic ecosystems because of the accumulation of contaminants in the food chains, which is responsible for the negative effects and death in aquatic systems (9). Zebrafish is one of the vertebrates that is most important model for studies in basic physiology. Despite its size is small, the analysis at the level of the whole organ, tissue, or intact organism is possible, zebrafish also has several properties that make of vertebrate useful model to study the toxicology(6).

The aim of study was performed to determine the acute toxicity of OP ((Malathion (MAL) and Atrazine (ATR)) on zebrafish, and described important physiological problems associated with the accumulation of toxicants in the body of zebrafish and showed the effect of this toxicant on AChE inhibition. Additionally, antioxidant defense, lipid and protein stores (energy reserves) in muscle and brain, which indicate major physiological responses and biomarkers, were also estimated. The effects of OP (MAL and ATR) on the environment are not well known which mainly resulting from

human discharges and more information is required to better understand the importance of these pollutants.

MATERIALS AND METHODS

Fish samples that used in this experiment was zebrafish, which were collected from various weights (0.295 to 0.847) g and with a total length ranged from (3.1 to 4.6) cm. The fish samples put in aquarium have 4 L dechlorinated tap water of pH (7.6), temperature (25-28)°C and illumination 12:12 light: dark, with the availability of oxygen during the study period. Fish was purchased from the Institute of hydrobiology Chinese academy of Science. Test solution was replaced every two days. Test run for 96 hours, the fish were not fed during the experimental period.

2.1 Experimental water and stock solution

The standard analytic of Atrazine (ATR) (2-chloro -4-(ethylamino) -6-(isopropylamino) - S-triazine) and Malathion MAL) (O, O-dimethyl dithiophosphate of dietyl mercaptosuccinate) with a purity of (97% and 95%) respectively was obtained from Xingyinhe chemical engineering co.ltd. A 1000 mg L-1 stock standard solution of each ATR and MAL was prepared by dissolved in (80% aceton).

2.2 Acute toxicity testing

All experiment was conducted for a period of 96h. Each test concentration was used 3 replicates. Each aquarium contained 4L of exposure solution and ten fish. The exposure solution was always aerated and fully renewed every 48h. Water quality parameters (pH and temperature) were measured daily. During the acute toxicity experiment the number of dead fish was counting every 24h and the dead fish were removed from the aquarium as soon as possible. Fish were divided into two groups: first group without treatment (control group with acetone just for organophosphorus pesticides), whereas the second group were treated with a series of different concentration of organophosporus insecticide and herbicide (0, 2.5, 5, 10 and 20 mg L^{-1}), the mortality rate was determined at the end of the 96h by use of Finney's Probit

Analysis LC_{50} determination method Finney (10) and were recorded daily during the test period. A second test was using tissues organs for biomarker analysis (10) fish per treatment were used). At the end of the test, the live fish were killed by ice water. Heads and muscles were isolated in frozen in 2 ml tubes.

Table (1) LC_{50} values (with 95% confidence limit) of MAL and ATR in (mg L^{-1}) of zebrafish estimated by Finney.

Chemical	LC_{50} value	95% confidence limit	R^2
MAL	5.292	4.8201-5.8104	0.964
ATR	9.567	8.366-10.941	0.922

2.3 Bioacumulation test

Bioaccumulation of MAL and ATR in the whole body tissue of zebrafish, to determine the pesticides in water after filtrated it using 0.22μ m filtration membrane and stored at 4°C before analysis by high-performance liquid chromatography (HPLC). Pesticides were extracted from fish tissue depending on the method described by Zhao (11), use whole body of zebrafish except its fins, after crushed 2g wet weight of sample were extraction the pesticides and the extract was also filtered with 0.22μ m filter and analysis by HPLC, this system contained the SSI 2300-525HPLC: Detector: Variable dual wavelength 525 UV detector; column: Apollo C18 column (250mm × 4.6 mm, 5μm) ALLTECH company; the Series III pump Cschrom Plus chromatography workstation; column temperature: 30°C. Pesticide detections were made in the UV region (λ = 210 nm for MAl and λ = 254 nm for ATR). The composition of mobile phase for MAL consisted of acetonitrile

(70%) plus water (30%) with pH 3.5 (30%) and for ATR it was acetonitrile (35%) plus 0.025 M dipotassium hydrogen phosphate (pH 3.0 with acetic acid) (65%). The flow rate and injection volume for both the pesticides was 1.0 mL/min and 20µL respectively. The retention times for MAL and ATR were 5.2 and 11.08 min, respectively. Under the chromatographic conditions was performed quantification of pesticides by using external standard was described above.

2.4 Biochemical analysis

At end of each exposure period, the muscle and brain tissues of zebrafish were homogenized to 1/10 (w/v) ratio of cold physiological saline solution NaCl (0.86%) using a mortar and pestle, then centrifuged at (10min, 8000r/min) in 4°C, the supernatant was used for biochemical analysis.

AChE activity was determined at 412nm wavelength of spectrophotometer by method of George (12). SOD and CAT activities were determinated by method of Can (13), SOD was use NBT photochemical reduction reaction, then calculate absorbance values in 560nm with a micro plate reader. For quantitative determination of CAT activity by use 30% hydrogen peroxide that was diluted by 7.4 phosphate buffer to a final concentration of 65 micromolar of hydrogen peroxide per milliliter of sodium potassium phosphate buffer (PH7.4, 60mmol L-1) as substrate solution and ammonium molybdate solution (32.4mmol L-1), then measure absorbance at 450nm. The activity of enzymes was defined as units of activity per milligram of protein. Total protein content was measured by dependent on the procedure of Bradford (14), at 595nm and using bovine serum albumin as standard and the standard calibration curve was set as ($y=0.0051x- 0.0013$, $R2=0.9993$). The total lipid content was determined by using 1g dry weight for whole body of zebrafish and extracted the lipid by used the soxhlet for 6h by method of AOAC (15).

2.5 Statistical Analysis

The bioconcentration factor (BCF) of MAL and ATR in zebrafish was estimated using the following equation: $BCF = Cf/Cw$

Where Cf is the concentration of toxicant in fish and Cw is the concentration of MAL and ATR in the exposure solution. One-way analysis of variance (ANOVA) was performed to determine statistical significance among and between groups followed by Dennett's test, using the graphPad program. The criterion for significance was set at ($p < 0.05$).

RESULTS

3.1 Bioaccumulation of toxicants and total lipid of whole body of zebrafish

Based on the results of acute toxicity, zebrafish were exposed to different concentrations of OP (MAL and ATR), and bioaccumulation in whole body of fish were detected during 96h (Figure 1). The results showed that for both treatments, the BFC was increased with increased the toxicants concentration. The maximum values to BCF was in higher MAL and ATR concentration (8.467×10^3 and 2.007×10^4) respectively. Total lipid content was significantly increased in 1st concentration for all toxicants compared with control groups ($p<0.01$), However, there was slight reduction in total lipids content with increased concentration of toxins (Figure 2).

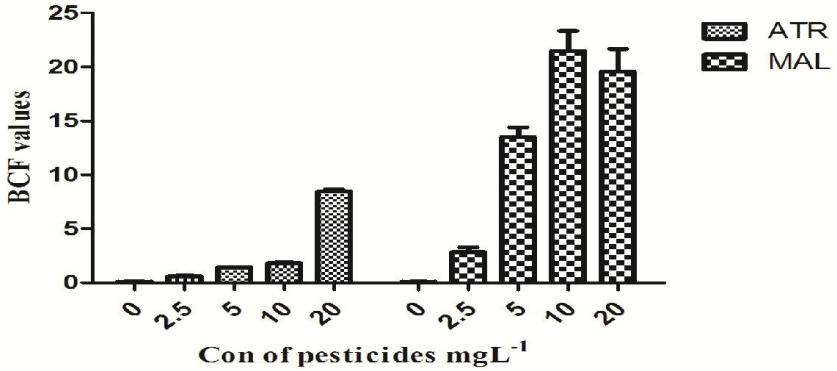

Figure (1) Bioaccumulation factor (BCF) of MAL and ATR exposure to different concentrations to whole body of zebrafish during 96h. Values are presented as a mean ± SD.

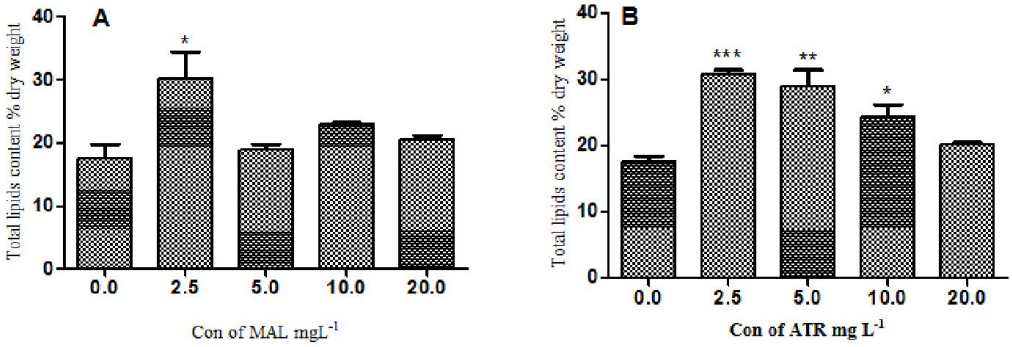

Figure (2) Total lipid contents % dry weight for whole body of zebrafish in different acute concentration of toxicants (A) MAL and (B) ATR mg L-1. Values are presented as a mean ± SD. The asterisk represents a statistically significant difference when compared with control groups; * at $p<0.05$, ** at $p<0.01$ and *** at $p<0.001$ levels.

3.2 Effects of toxicants on AChE activities of zebrafish tissues

The AChE activity in muscle tissue was significantly induction ($p<0.01$) in higher concentration of MAL and ATR by (49.366 and 54.398 %) respectively compared with control groups for 20 mgL^{-1}, after 96h (Table 2). While in the brain tissues of zebrafish were significantly induction ($p<0.05$), the maximum slip was in 5 mg L^{-1} of MAL by (29.114%), while in 20 mg L^{-1} of ATR by (9.653%), compared with control groups (Table 2).

3.3 Effects of toxicants on antioxidant enzymes activities of zebrafish tissues

The result demonstrated significantly increased ($p<0.01$) in CAT activities in muscle tissues after exposure to different acute concentrations for 96h of toxicants. The maximum increased of CAT activities was apparent with a treatment concentration of 20 mgL^{-1}, when CAT activity increased by (502.74 and 433.35 %) respectively, compared to the control groups. as well as observed significantly increased ($p<0.05$) for SOD activities in muscle tissues for both pesticides in the last concentration by (185.454 and 109.463 %) respectively. While it was observed that different apparent with a treatment concentration in brain's tissues, that the maximum increased of CAT in brain tissues for both pesticides by (108.652 and 130.103%) respectively for 5 mgL^{-1} after 96h compared with control groups (Table 2).

3.4 Effects of toxicants on Protein contents in zebrafish tissues

The protein content in muscle tissue was significantly depression ($p<0.01$) with increased of toxins of MAL and ATR, specialties in higher concentration of pesticides by (18.369 and 35.152%) respectively compared with control group for 20 mgL^{-1}, after 96h (Table 2). The significantly decreased of protein content was also observed in brain's tissue of zebrafish ($p<0.01$). But the processes were different from that observed in muscle, Maximum decreased of protein content in 20 mgL^{-1} for ATR, the decreased about (33.683 %) respectively.

Table (2) Changes in biochemical parameters after exposed to different concentrations of MAL and ATR for 96h in muscle and brain of zebrafish

Chemical	con. MgL^{-1}	AChE [a]		CAT[b]		SOD[b]		Protein content [c]	
		Muscle	Brain	Muscle	Brain	Muscle	Brain	Muscle	Brain
MAL	0	1.1526±0.11	0.237±0.02	69.172±1.52	320.502±3.54	8.607±0.36	18.903±0.10	13.294±0.91	8.428±0.66
	2.5	0.477±0.013*	0.506±0.22	166.074±2.17**	250.255±1.94*	11.75±0.53*	10.088±0.64**	7.116±2.814	12.727±4.67
	5	0.303±0.03**	0.069±0.04	109.405±3.11**	348.235±2.28**	8.14±0.45	13.521±0.37*	4.363±2.73	10.577±0.13
	10	0.538±0.11**	0.135±0.05	200.581±1.01*	300.099±2.05**	9.763±0.54	15.723±0.51**	3.992±4.37	9.139±3.77
	20	0.569±0.04**	0.353±0.06	347.758±1.95**	173.536±2.50**	15.962±0.68*	21.567±0.40**	2.442±3.19	9.326±0.83
ATR	0	1.1526±0.107	0.259±0.02	69.152±1.52	320±3.54	8.507±0.35	18.863±0.61	6.819±4.175	17.632±1.67

	2.5	1.011±0.65	0.374±0.25	283.443±2.43**	312.516±1.78	7.005±0.703	9.233±0.52**	3.997±2.73	7.387±3.95
	5	0.716±0.03	0.583±0.34	102.472±1.75**	416.328±3.06**	2.094±0.64***	10.862±0.61**	4.256±2.73	14.766±3.15
	10	0.358±0.04	0.782±0.17	268.195±3.29**	362.806±1.98**	8.046±0.67	22.329±0.47**	4.284±4.37	10.725±0.90
	20	0.627±0.01	0.025±0.39	299.667±2.36**	338.022±2.14**	9.312±0.57	22.986±0.697**	2.397±3.19	5.939±0.31

The standard deviations values in the same row with different significantly compared to the control; * at p<0.05 and ** at <0.01 levels.

[a] Activities are expressed as mmol/min/mg protein.

[b] Activities are expressed as U/mg protein.

[c]Contents are expressed as mg/g .

DISCUSSION

The pesticides and chemical pollutants appears to play a major role in the loss of fish resources and impact on the ecosystem aquaculture and human health risk assessment, and this led to the study of acute toxicity and accumulation of pesticides in the organisms to obtain more and more attention(11). The present study showed that MAL and ATR could be rapidly accumulated in fish shortly after their exposure to acute concentrations of toxicants and the highest BCFs of toxins was in fish were (8.467×103 and 2.007×104) respectively in 20 mgL^{-1} for MAL and ATR after 96h exposure. The results suggested that both pesticides had probably to accumulate rapidly in fish or other organisms, which was in agreement with some previous reports. A study by Zhao(11) showed that exposed zebrafish to pyrimorph fungicide could be rapidly accumulated in fish shortly after their exposure to a sublethal concentration of pyrimorph, and the highest BCFs of its were 1.07×10^2 (144 h) and 23.1 (96 h) after exposure to 2.00 and 0.25 mg L^{-1} of pyrimorph respectively. Sun (16) was determined the accumulation of HC Orange No. 1 in liver of goldfish (*Carassius auratus*), and referred to the accumulation in fish tissue shortly after the start of the exposure and reached the maximum level at a 24h exposure.

Enzymatic changes considered as important signs of a hazardous chemical materials (17). Many studies have shown that inhibition of AChE after OPs exposure, they were also used to measure the activity of this enzyme in the fish to monitor neurotoxicity of the OPs. These pesticides inhibit the activities of this enzyme was dose and time-dependent, as well as the difference in the rate of inhibition depends on

the species and age (18). The inhibition of AChE enzyme activity is widely as a good biomarker for exposure to toxic OPs (19). This investigation in our study for most of tested groups with OP (MAL and ATR) was significant inhibition of AChE activity ($p<0.05$) in brain and muscle tissues of zebrafish with the increase of concentration. In agreement with our results. Senger (6) suggested that pesticides can interact directly with cholinergic receptors at or below the concentrations that inhibit AChE. Feng (20) directly investigated AChE inhibition of trichlorfon with muscle of (*T. niloticain*).

Increase or inhibitions of antioxidant activity under pressure of chemical depend on the severity and duration of stress applied, as well as susceptibility of the species to exposure. In this study, SOD and CAT activities showed an increasing trend in the tissues of zebrafish exposed to MAL and ATR and an increase was more pronounced in muscle with increased the concentration of toxin especially in high concentration for all toxicants. It was noted that this increase in SOD activity may be due to the higher O_2^- production (8). Moreover, mechanism of antioxidant defense has great significant for fish because it works to protect them from free radicals produced by oxidative stress and other factors and it is the first defense mechanism against oxidative stress (7) this was agree with Oruc (21) who investigated the increase in SOD activity caused by chlorpyrifos exposure in adult of (*Oreochromis niloticus*). Most of the increase in CAT activities in the muscles and brain zebrafish observed in this study may be a response to H_2O_2 produced by activity SOD, because the CAT is responsible for detoxification of H_2O_2 in the water. This refers to a positive correlation between of SOD and CAT to the muscles and the brain in this study, should be accompanied by increased activity SOD with increase in the production of H_2O_2, which led to higher activity CAT, and chemicals used in this study cause significant elevation ($P<0.05$) in the activities of the SOD and CAT in zebrafish after exposure. This agreed with the investigation of Sharbidre (7) showed increase in CAT and SOD levels in brain and gills of guppy fish (*Poecilia reticulate*) after 96h exposure of chlorpyrifos in all treatment groups.

Changes in the biochemical parameters such as proteins and lipids are important to

signify the sensitivity of organs to pollutants by changing their function. The lipids are one of the most important sources of energy and structural components in the body of fish. Biological studies have revealed for lipids the importance of lipids during periods of stress. Where that stress increases the overall energy consumption, reduce the availability of energy for other processes strongly expensive. Thus, it invokes compensatory metabolic changes in the tissues of animals through the modulation and modification of the quantity and quality of various metabolites, including the lipids (22). In the present study, it was proved the increase in the total lipids content in the first concentrations for OP (MAL and ATR) in the analyzed tissues of the zebrafish body exposed to acute concentrations compared with control groups. There are some different factors which may effect on the lipid contents of the organisms, such as age, sex, and food supplies. It was noted that the increase in lipid contents may due to the exposure of animals to conditions stressful, and the biotransformation of other organic components such as carbohydrates and proteins to lipids, and also to resist the toxicity made by various chemicals. Nandurkar and Zambare (23) found the increase in lipid contents in both the selected models, (*Lamellidens corrianus* and *Parreysia cylindrical*) after acute and chronic exposure of chloramphenicol. At the same time, the increased lipid content in fish may also cause damage, because the organisms with high lipid content have bio-concentration of these chemicals and this property leads to bioaccumulation and thus, to a detrimental effect on the human food chain(24).

CONCLUSION

The results of the current study showed that (OP) pesticides were highly toxic to zebrafish. Studies indicated that the acute toxicity as a first step in determining the water quality requirements to fish and so reveal toxin concentrations (LC_{50}) that cause fish mortality even in the short exposure. It can be concluded from this study that the zebrafish is very sensitive to these toxicants and their mortality rate was dependent on the concentration of the toxins. The ability of zebrafish to bioaccumulation of these toxins in a dose-dependent manner is a strong indicator of suitability as a model in

biomonitoring programs. As well as the zebrafish has a number of properties that makes them useful sentinels for environmental monitoring. Biological monitoring using a series of assays having different endpoints. It could allow a sensitive approach to predict the potential risk of pesticides which are useful in the formulation of the "safe levels" of such bioaccumulative chemicals. However, this study has been ignored many of the factors in the experiment as fish that live in the environment where they face different circumstances. Therefore, there is a need to further study to answer whether induction of these parameters can be useful as early biomarkers of toxicants in the environment practical for fish.

دراسة التغيرات الكيميائية الحيوية في أنسجة سمكة الصغيرة المخططة بعد تعريضها للمبيدات الفوسفورية العضوية

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الخلاصة

ان الهدف من هذه الدراسة تحقيق السمية الحادة لمبيدي (الملاثيون و الأترازين)، و تحديد تراكم هذه المبيدات في كل جسم السمكة ، و بعض الردود الحيوية في الدماغ و العضلات.

وأظهرت النتائج أن الملاثيون و الأترازين سريعة التراكم في جسم السمكة و بعد فترة وجيزة من التعرض لتراكيز الحادة من المواد السامة و بطريقة تعتمد على الجرعة .وتم تحقيق ذلك من ردود فعل الدفاعات المضادة للأكسدة ، superoxide dismutase و catalase ، نتيجة لزيادة الكبيرة لفعاليتها الدفاعية . وقد تبين تثبيط

نشاط انزيمAcetylcholinesterase للسمكة بطريقة تعتمد على الجرعة .

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