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Increased Apoptosis in the Liver of *Chalcalburnus tarichi* Exposed to Sublethal Concentrations of Methyl Parathion

Ertuğrul KANKAYA[1] Burak KAPTANER[2*]
[1]Yüzüncü Yıl University, Fisheries Faculty, Van, Turkey
[2]Yüzüncü Yıl University, Science Faculty, Department of Biology, Van, Turkey

*Corresponding author:
E-mail: bkaptaner@yahoo.com

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Abstract
In the present study, the effect of methyl parathion (MP), an extensively used organophosphorus insecticide in agriculture, on liver apoptosis in *Chalcalburnus tarichi*, an endemic carp species of the Lake Van Basin of Turkey, was investigated. For this purpose, fish were exposed to sublethal concentrations (1.47, 3.0, and 6.11 mg/L) of MP for 30 days under semistatic conditions. Following exposure, sections taken from the liver were stained using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling method for the examination of apoptosis, and then apoptotic hepatocytes were evaluated as the apoptotic index (AI). According to the results, the AI was significantly enhanced at MP concentrations of 3.0 and 6.11 mg/L. In conclusion, the present study indicates that MP has apoptosis-inducing potential in the liver of *C. tarichi* and MP-dependent hepatotoxicity causally related to the increase in the apoptosis.
Key words: Methyl parathion, *Chalcalburnus tarichi*, liver, apoptosis

INTRODUCTION

Organophosphorus compounds (OPCs) are widely utilized throughout the world in agricultural activities as pesticides [1]. During their applications, they contaminate terrestrial and aquatic environments and affect nontarget organisms such as fish [2]. One of the most important OPCs frequently used in agricultural and household applications is methyl parathion (MP; *O*,*O*-dimethyl *O*-4-nitrophenyl phosphorothioate). It is widely used as an insecticide in agriculture, food storage shelters, and fish culture tanks for the elimination of aquatic larval stages of insects threatening fish larvae [3]. Common and uncontrolled usage of this substance has been reported to cause bioaccumulation and toxicological symptoms in fishes [4]. It has been reported that MP is neurotoxic because it inhibits acetylcholinesterase (AChE) activity [5]. Exposure to MP at sublethal concentrations has been reported to influence the normal function of brain centers and cause behavioral changes in fish [6]. It causes developmental and reproductive disturbances in female fish and their offspring [2]. MP is capable of altering antioxidant defenses and inducing oxidative stress in various tissues [7, 8]. It may also induce histopathology in different tissues and hematological alterations in fish [9, 10].

Apoptosis is a highly organized process characterized by the progressive activation of precise pathways leading to specific biochemical and morphological alterations. Initial changes in the apoptotic process are characterized by caspase activation, alterations in the cellular redox status, cell shrinkage, loss of membrane lipid asymmetry, and chromatin condensation. Later changes are associated with executioner caspases, endonucleases, apoptotic bodies, and chromatin condensation [11]. Stress-induced apoptosis can provide a useful biomarker for monitoring sublethal effects of environmental contaminants [12]. It has been reported that OPCs such as paraoxon, dichlorvos, and chlorpyrifos increased apoptosis in different cultured cell types [13, 16] and MP induced apoptosis in the endometrial tissue of female rats [17].

C. tarichi is an endemic fish species living in the Lake Van basin of Turkey. It displays an anadromous character, migrating to the rivers pouring into the lake for spawning during the reproduction period (April-June) and returning to the lake postspawning [18]. Lake Van is known as the biggest soda lake in the world, having highly alkaline water with a pH of 9.8, and *C. tarichi* is the only vertebrate species living in this lake [19]. Apart from its interesting biological features, it is also an economically important species, with approximately 12000 tons harvested per annum [20]. MP is also commonly used in agricultural facilities in Turkey [21] and because of its wide usage in the Van region, *C. tarichi* is under risk of MP toxicity. Accordingly, recent studies have been focused on MP-related toxicity assays in this species. Kankaya [22] measured the 96 h-median lethal dose (LC_{50}) value and reported it to be 11.44 mg/L for *C. tarichi* exposed to MP and determined histopathological changes in the tissues of the liver, gills, and ovaries, and hematological alterations and inhibited AChE activities at sublethal MP concentrations. In another study, MP was reported to cause the induction of a micronucleus frequency in the erythrocytes of *C. tarichi* [23].

The liver of a fish plays an important role in the uptake, biotransformation, and detoxification of xenobiotics [24]. MP is converted into highly toxic methyl paraoxon by oxygenation in the endoplasmic reticulum of the hepatocytes. Thus, the liver is the organ that suffers serious morphological changes when exposed to pesticides with organophosphorus [9, 25, 26, 27]. In this regard, the aim of the present study is to evaluate MP-related hepatotoxicity in fish through apoptosis. For this purpose, apoptosis was evaluated in liver sections stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) from *C. tarichi* exposed to sublethal concentrations of MP.

MATERIALS AND METHODS

Fish

C. tarichi samples (body weight: 3–7 g, fork-length: 8–10 cm) used in this study were caught from the Karasu River, pouring into Lake Van, by electrofishing. Live fish were transported to the laboratory in aerated containers and placed in a 260×80×70 cm fiberglass tank containing running dechlorinated tap water aerated by air pumps. The fish were acclimatized in this tank for approximately 1.5 months. During this period, the fish were maintained under a natural photoperiod at an average temperature of 17.5 °C, fed commercial pellets, and the tank water was cleaned daily.

Experimental design and MP exposure

Before exposure, the fish in the stock tank were randomly assigned to 60×30×40 cm glass aquaria (10 fish in each aquarium). The sides of the aquaria were covered with papers to prevent visual stress. A stock solution of MP [($C_8H_{10}NO_5PS$) with 80% technical concentration were obtained from an agricultural company in Turkey] was prepared in dimethyl sulfoxide (Sigma). After a 7-day acclimation period in the glass aquaria, the experiment was started and the fish were exposed to sublethal concentrations (1.47, 3.0, and 6.11 mg/L) of MP for 30 days. Test concentrations were chosen on the basis of the 96 h-LC_{50} value of MP for *C. tarichi* reported by Kankaya [22]. Exposures were conducted under a semistatic renewal system. The system water was renewed every 48 h by changing 80% of the aquarium waters to maintain the initial MP concentrations. To observe the effect of the DMSO solvent on the fish, a solvent aquarium was set up in addition to the control aquarium. All of the treatments were tested in duplicate. During the experiments, the water quality criteria were monitored in the aquarium waters (temperature: 17.9 °C; pH: 8.46; dissolved oxygen: 6.04 mg/L; conductivity: 882 µS/cm; hardness as $CaCO_3$: 344 mg/L; and total alkalinity: 518 mg/L).

Histological procedures

After the treatments were complete, the fish were anesthetized with tricaine methanesulfonate. The livers of the fish were dissected and fixed in 10% neutral buffered formalin. The fixed tissue samples were dehydrated in a graded ethanol series (70%, 80%, 90%, and absolute), cleared in xylene, and embedded in paraffin wax. Afterwards, sections (4-µm-thick) were taken from tissues and mounted onto polylysine coated slides.

TUNEL staining

To determine apoptotic hepatocytes in the liver sections, the TUNEL technique was chosen. TUNEL staining was conducted using a commercial TdT-FragEL™ DNA fragmentation detection kit (Cat. No: QIA33, Calbiochem, Merck, USA). A liver section from each fish (totaling 6 sections from each group) was processed via the TUNEL procedures. Briefly, after the deparaffinization and dehydration of the liver sections, they were washed with tris-buffered saline (20 mM Tris, pH: 7.6; 140 mM NaCl). All of the other staining steps were conducted according to the manufacturer's instructions, as described by Kaptaner and Ünal [12]. In the final staining stage, the sections were covered with 3,3′diaminobenzidine to develop a brown color in the nuclei of positive (apoptotic) cells, and the sections were then counterstained with 3% methyl green for the visualization of negative nuclei in green. After completion of the staining procedure, the slides were covered with Entellan and examined under a light microscope (Nikon Eclipse E600) with a Spot digital camera (Nikon, Coolpix 5000).

Apoptotic index

Five fields that were randomly chosen and did not intersect one another from 1 section of liver from each fish were scanned and acquired at 400× magnification, using a Spot camera, and stored as .JPG data files. To estimate the apoptotic index (AI) for each individual, cell counting was performed in these imaged fields containing a total of 2500–3500 cells using Image J software, a cell counting module (National Institutes of Health, USA, http://rsbweb.nih.gov/ij/). Apoptotic (TUNEL-positive) hepatocytes were discriminated by their brown nuclei and their characteristic morphological changes, such as cell shrinkage, membrane blebbing, and chromatin condensation. The AI for each individual was calculated as a percentage of the TUNEL-positive cells using the following formula: AI = (number of TUNEL-positive cell nuclei counted / number of total cell nuclei counted) × 100.

Statistical analysis

Data were statistically analyzed using the Statistical Package for the Social Sciences software, version 16.0. A nonparametric Kruskall-Wallis one-way analysis of variance, followed by the Mann-Whitney U test, was used to compare differences between the groups. The results were expressed as mean ± standard error of the mean. The differences were considered to be statistically significant at $P < 0.05$.

RESULTS

As shown in Figure 1, intensity of the apoptotic hepatocytes was noticeably high in the TUNEL-stained liver sections of fish exposed to MP concentrations of 3.0 and 6.11 mg/L compared to the control sections.

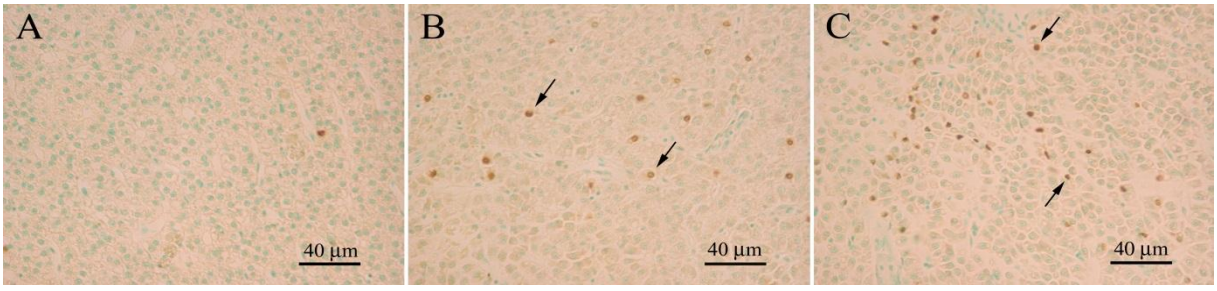

Figure 1. TUNEL-stained liver sections of *C. tarichi* from A) the control group, B) 3.0 mg/L MP exposure, and C) 6.11 mg/L MP exposure. Some apoptotic hepatocytes are indicated with arrows.

AI values belonging to the control and exposure groups are shown in Figure 2. The mean AI was 0.80 ± 0.20% and 0.85 ± 0.22% in the control and solvent control groups, respectively. A slight but not significant increase (AI = 0.96 ± 0.24%) in apoptosis was observed in the fish liver exposed to 1.47 mg/L of MP. Statistically significant increases in the liver AI were observed in the 3.0 mg/L (AI = 2.64 ± 0.39%; $P < 0.01$) and 6.11 mg/L (AI = 2.18 ± 0.44%; $P < 0.05$) exposure groups.

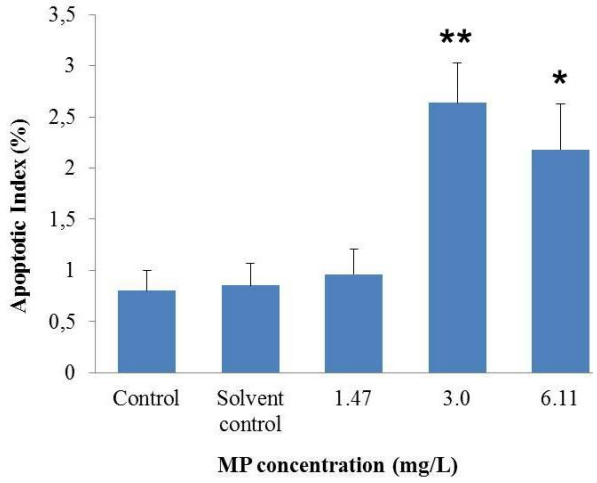

Figure 2. Liver AI values in the *C. tarichi* exposed to sublethal MP concentrations. The values were analyzed in 6 fish for each group. A significant difference from the control group is indicated as $*P < 0.05$; $**P < 0.01$.

DISCUSSION

MP is an organophosphorus insecticide used extensively throughout the world in agriculture, household, and aquaculture applications, due to its high effectiveness against a wide range of insect pests [21]. Excessive and uncontrolled usage of MP has resulted in high concentrations in water bodies and negative impacts on fish species and other aquatic organisms [4]. In fish, the liver is the primary organ responsible for detoxification and excretion of foreign compounds and is highly impacted as a consequence of MP exposure, and numerous histological and biochemical changes in the liver have been reported by several authors [3, 9, 27]. In a recent study conducted on *C. tarichi*, hepatic alterations were also reported after exposure to sublethal MP concentrations [22].

Apoptosis is a fundamental and controlled cell death pathway that occurs under both physiological and pathological conditions [28]. It has been reported that environmental stressors such as heavy metals, pesticides, and toxicants can induce apoptosis [29]. OPCs have also been reported to induce apoptosis, both in in vivo and in vitro laboratory studies [14, 15, 17, 30]. The results of the present study also showed that MP could induce apoptosis in the liver of *C. tarichi* and as far as we know, this is the first report exhibiting the apoptosis-inducing effect of MP in the liver of fish.

In vitro studies have demonstrated that there are various mechanisms underlying OPC-induced apoptosis. Apoptosis may be induced by extrinsic pathways activated by death receptors or intrinsic pathways (mitochondria, DNA damage and/or endoplasmic reticulum stress), which both lead to caspase activation [13, 30]. OPCs such as paraoxon-, parathion-, tri-ortho-tolyl phosphate- and triphenyl phosphite-induced apoptosis in human neuroblastoma cells and it has been reported that OPC-induced cytotoxicity were related to muscarinic receptor activation or an increase in Bcl-2, mitochondrial permeability transition pore closure, and receptor mediated caspase activation [13]. Another OPC, chlorpyrifos, induced apoptosis in a time- and dose-dependent manner in Jurkat T cells by the activation of caspase-3 [31]. The inducing of apoptosis by OPCs, dichlorvos and chlorpyrifos, via a caspase-3 pathway was also reported in human natural killer cells [15]. Paraoxon induced apoptosis in EL4 cells by altering the mitochondrial transmembrane potential, causing cytochrome c release into cytosol, leading caspase-9 activation [14]. Similar mechanisms mentioned above may underlie apoptosis occurring in the liver of *C. tarichi* after MP exposure.

On the other hand, the induction of apoptosis by environmental toxicants is widely associated with alterations in the redox homeostasis, which include altered antioxidant defenses and the generation of reactive oxygen or nitrogen species, leading to oxidative stress. Oxidative stress causes DNA damage and is known to trigger apoptosis [29]. It has been shown that the organophosphorus pesticide, trichlorfon, increased intracellular reactive oxygen species and lipid peroxidation levels and caused cytochrome c release from mitochondria into the cytoplasm leading to caspase-3 activation and induced apoptosis in the cultured hepatocytes of *Carrasius auratus gibelio* [30]. Studies have demonstrated that MP has oxidative stress-inducing potential in the liver of fish [7, 8]. Güney et al. [17] reported that MP caused oxidative stress, increasing the lipid peroxidation levels, and activated caspase-3 and -9 in the endometrial tissue of rats. Thus, increased apoptosis in the liver of *C. tarichi* may arise from the induction of oxidative stress as a consequence of MP exposure.

CONCLUSION

In conclusion, the results of the present study demonstrated that MP has apoptosis-inducing potential in the liver of *C. tarichi* and MP-dependent hepatotoxicity causally related to the increase in the apoptosis. However, mechanisms underlying the apoptosis-inducing effect of MP in the liver of fish should be investigated in more detailed studies.

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