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Chronic toxicity of pesticides to the mRNA expression levels of metallothioneins and cytochrome P450 1A genes in rainbow trout

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Abstract

The hazardous effects of pesticides on various metabolic pathways are a great problem for environmental health and should be well determined. In the present study, the authors treated rainbow trout with 0.6 μg/L deltamethrin for 28 days and 1.6 mg/L 2,2-dichlorovinyl dimethyl phosphate for 21 days. After this time period, the authors observed alterations in mRNA expression levels of MT-A, MT-B and CYP-1A. Chronic exposure to low levels of pesticides may have a more significant effect on fish populations than acute poisoning. While both pesticides caused a significant increase on mRNA levels of MT-A and CYP-1A, MT-B mRNA levels were increased significantly only upon deltamethin administration. The significant increase in mRNA levels of the corresponding genes may be considered as a defence mechanism in addition to the anti-oxidants against oxidative stress, as well as a detoxification mechanism against adverse effects of pesticides.

Keywords

MTs, CYP-450, deltamethrin, DDVP, *Oncorhynchus mykiss*

Introduction

Metallothioneins (MTs) are a superfamily of low-molecular mass (approx 6 kDa) cysteine and metal rich proteins or polypeptides conserved through evolution and present in all eukaryotes and certain prokaryotes (Vašák and Meloni, 2008). They are thought to play roles both in the intracellular fixation of the essential trace elements zinc and copper, in controlling the concentrations of the free ions of these elements, in regulating their flow to their cellular destinations, in neutralizing the harmful influences of exposure to toxic elements and in the protection from a variety of stress conditions (Kägi and Schäffer, 1998). Although MTs have been widely utilized to identify specific responses to heavy metal pollution, there is now a body of evidence demonstrating that in vertebrates MT synthesis is stimulated by different endogenous and exogenous agents (Kägi, 1993), cytokines and in particular compounds leading to production of reactive oxygen species (ROS) (Dalton et al., 1994). Therefore, in mammals and fish, not only inorganic pollutants such as heavy metals but also organic contaminants may activate MT neosynthesis (Viarengo et al., 1999).

Cytochrome P450s are a large and diverse group of enzymes. The function of most cytochrome P450 (CYP) enzymes is to catalyse the oxidation of

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organic substances. The substrates of CYP enzymes include metabolic intermediates such as lipids and steroidal hormones, as well as xenobiotic substances such as drugs and other toxic chemicals. Induction of CYP-1A1 can occur within hours of exposure and when inducing chemicals are cleared from the body, levels of CYP-1A1 decrease. CYP-1A1 induction can therefore be considered an early biological effect of exposure to contaminant inducers.

Dichlorvos or 2,2-dichlorovinyl dimethyl phosphate (DDVP) is a highly volatile organophosphate, widely used as a fumigant to control household pests, in public health, and protecting stored produce from insects. On the other hand, deltamethrin is extensively used as an ectoparasiticide in animals and as insecticides in crop production and public health programmes. Some of the toxic actions of those kinds of pesticide have been reported earlier, but reports on tissue residue level and effects after repeated daily oral administration on cytochrome P450, cytochrome b5, antioxidant status, blood biochemistry and histology of some tissues in rats are scarcely available (Manna et al., 2005).

Pesticides may cause various side effects, for example, change in DNA structure (Lee and Steinert, 2003), cause of sperm malformations (Mathew et al. 1992), generation of ROS (Bagchi et al., 1995), increase in the level of free radicals (Freeman and Crapo, 1982), inhibition of specific enzymes (Ekinci and Beydemir, 2010) influence on antioxidant defense system (Brian et al., 2005; Ceyhun et al., 2010; Senturk et al., 2009), decrease expression levels of growth-related genes (Aksakal et al., 2010) and act as inducers of heat shock protein (Ceyhun et al., 2010) in tissues and cells in different organisms. In this study, we report on the long-term effects of deltamethrin and DDVP on the expression of metallothionein A, B and cytochrome P450 1A genes in rainbow trout (*Oncorhynchus mykiss*) muscle.

Materials and methods

Fish husbandry and maintenance

One-year-old rainbow trout samples were obtained from the Fisheries Department of the Agricultural Faculty at Atatürk University in Erzurum and weighed of 200 $\pm$ 17 g. They were fed twice a day with a commercial pelleted trout feed at a daily ration of 1% of their wet body mass during the study. Trout feed was purchased from Pınar Yem Company, İzmir-Turkey. Feed was given by hand. Fish treatments were conducted according to Applied Research Ethics National Association.

Prior to the experiments, fish in each group were kept in 1 $\times$ 1.2 m (wide-deep) fiber-glass tanks for 1 month. The average water temperature was 9°C during the tests. The water quality parameters were analysed in the Laboratory of the Erzurum Hygiene Institute. Fiber-glass tanks contained 15 fish in 600 m^3 water. The average dissolved oxygen level was adjusted to 8.8 ppm and maintained by means of a ventilation system. One tank was used as a control, and did not contain any pesticide while 0.6 μg/L deltamethrin and 1.6 mg/L DDVP was added to the others. The muscle samples were collected after deltamethrin treatment from each group on the 28th day, and after DDVP treatment on the 21st day. All fish samples were frozen with liquid nitrogen and stored at -80°C until RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated from frozen muscle tissues of both pesticide treated and control fish using TRIzol reagent. RNA was treated with DNase in order to avoid genomic contamination. RNA concentrations and quality were verified by means of spectrophotometer (Nanodrop) and RNA gel electrophoresis, respectively. Following isolation, cDNA synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. All cDNA was stored at -20°C until use.

Primer and TaqMan probe design

Primers and TaqMan probes were designed in Primer3 software (v. 0.4.0) (http://frodo.wi.mit.edu/) using rainbow trout MT-A (GenBank accession number M18103), MT-B (GenBank accession number M18104) and CYP-1A (GenBank accession number AF015660) sequences and blasted to ensure correct mRNA sequences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene (GenBank accession number NM_001124246). In order to perform multiplex real-time polymerase chain reaction (PCR), TaqMan probe of the housekeeping gene was conjugated with Cy5/Blackhole Quencher 2, the fluorophore and quencher molecules, whereas the probes for target genes were conjugated with FAM/TAMRA. The primer and probe sequences are provided in Table 1.

Table 1. Sequence of primers and probes used for real-time PCR

Primers/Probes	Sequence 5′-3′
MT-A Forward	ATCCTGCAAGTGCTCCAAC
MT-A Reverse	ACAAAAGCTATGCTCAAGATGG
MT-A TaqMan Prob	^{FAM}- TGCGTGTGCAAGGGCAAGACT -^{TAMRA}
MT-B Forward	ATCCTGCAAGTGCTCAAACT
MT-B Reverse	ATGACTGCATTGTCACGGTA
MT-B TaqMan Prob	^{FAM}- AAAGTTGCTGCCCCTGCTGTCC -^{TAMRA}
CYP-450 1A Forward	AGTGATGAGTTTGGGCAGGT
CYP-450 1A Reverse	TCACGGATGTTGTCCTTGTC
CYP-450 1A TaqMan Prob	^{FAM}- TCGTTACCTGCCCAACCGCAC -^{TAMRA}
GAPDH Forward	ATCAAAGGGGCTGTCAAGAA
GAPDH Reverse	AGGAGTGGGTGTCTCCAATG
GAPDH TaqMan Prob	^{Cy5}- CGCCGAAGGACCCATGAAGG -^{BQ2}

PCR: polymerase chain reaction.

Multiplex real-time PCR

Quantification of gene expression by real-time PCR analysis was performed using a thermal cycler Stratagene MxPro3000. The PCR was carried out in a reaction volume of 50 µl containing template DNA, 900 nM of both target and reference forward and reverse primers, 250 nM both target and reference TaqMan probes, and 25 µl FastStart TaqMan Probe Master (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase uracil *N*-glycosylase (UNG), dNTP with dUTP and optimized buffer component. Amplification and detection of the samples and the standards were performed using the following thermal cycling conditions: 50°C for 2 min for activation of optical AmpErase UNG enzyme, 95°C for 10 min as hot start to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Real-time PCR data were analysed using the efficiency $(e)^{(-\Delta\Delta Ct)}$ method (Pfaffl, 2001), which is used to determine mean fold changes in gene expression against the control group and housekeeping gene GAPDH. Analytical sensitivity was confirmed by running standard curves. Amplification efficiency (e) was calculated based on the slopes of the curves (slope) using the formula $e = 10^{(-1/\text{slope})}$ (Pfaffl, 2001), and the slope value via Stratagene MxPro3000 software.

Statistical analyses

All experiments were triplicated. Presented data, expressed as mean values ± SE, show differences between the control and treated groups. An independent t test (SPSS 17.00) was used to test the differences between the deltamethrin, DDVP-exposed fish and the control groups.

Results

In the present study, we investigated the effects of DDVP and deltamethrin long-term exposure on MT-A, MT-B and CYP-450 1A gene expression levels in rainbow trout muscle. The results are shown in Figure 1. According to the results, we observed that both pesticides increased CYP-450 1A gene expression levels compared to the control group and reference gene GAPDH. While MT-A expression levels significantly increased ($p < 0.001$) after DDVP exposure, a slight increase was observed after deltamethrin exposure ($p < 0.05$). On the other hand, both pesticide exposures resulted in an increase in MT-B expression levels ($p < 0.05$ for deltamethrin).

The percentages of efficiency values were found as 98.6%, 98.7% and 98.2%, for MT-A, MT-B and CYP-450 1A, respectively, in DDVP exposure and 97.3%, 98.3% and 97.8%, for MT-A, MT-B and CYP-450 1A, respectively, in deltamethrin exposure (Figure 2).

The measured water quality parameters were $O_2 = 8.8$ ppm, pH = 7.9, Mg = 8.83 ppm, Ca = 31.13 ppm, Na = 4.58 ppm, Cl = 6.75 ppm, K = 2.99 ppm, B = 0.17 ppm, Fe = trace, $SO_4^{-2} = 10.33$ ppm, $CO_3^{-2} = 120$ ppm, $HCO_3^{-} = 146.4$ ppm, PO_4^{-3} = trace, $NO_3^{-} = 3.45$ ppm, NO_2^{-} = trace, conductivity = 276 µ s/cm and total hardness = 114 ppm of $CaCO_3$.

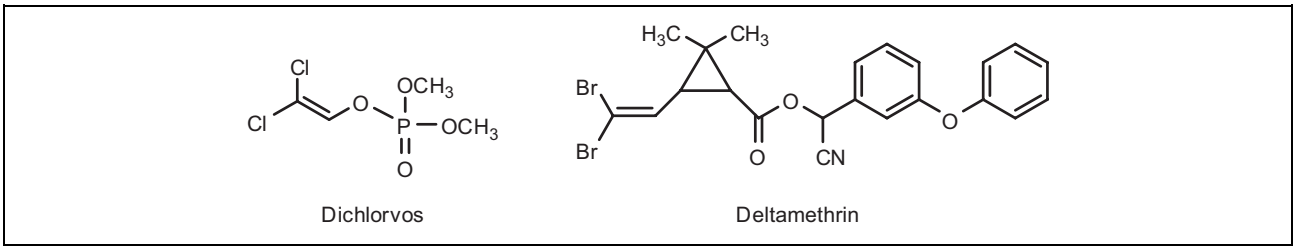


Figure 1. Molecular structures of used pesticides.

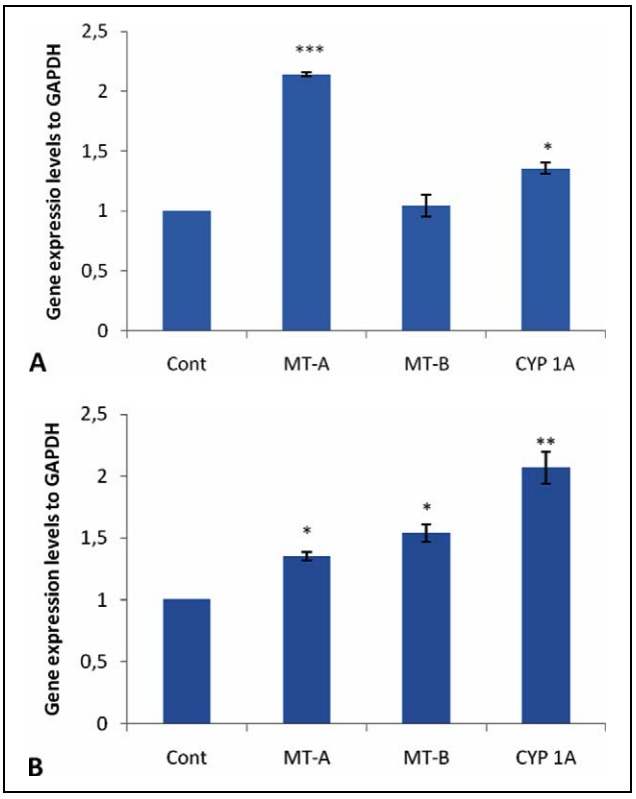


Figure 2. Relative mRNA expression levels of MT-A, MT-B and CYP-1A in the muscles of rainbow trout exposed to 28 days deltamethrin (A) and 21 days DDVP (B). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Discussion

In human and experimental animals, DDVP is rapidly absorbed through the gastrointestinal and respiratory tracts and skin, and easily metabolized by esterases that exist in most tissues. Dichlorvos exposed to rats is distributed in the kidney and adipose tissue at relatively high concentrations. Dichlorvos is mainly metabolized by esterase to dimethyl phosphate and dichloroacetaldehyde. While dimethyl phosphate is excreted in urine, dichloroacetaldehyde is rapidly metabolized via two pathways to dichloroethanol glucuronide, hippuric acid, urea and carbon dioxide.

Deltamethrin is in the chemical class of pyrethroids. Pyrethroids are synthetic chemicals modeled after the pyrethrin components of pyrethrum. Unlike other pyrethroids, deltamethrin consists of one pure compound. Pyrethroids, in general, interfere with normal production and conduction of nerve signals in the nervous system. Pyrethroids act on nerve membranes by delaying the closing of the activation gate for the sodium ion channel. Metabolism of deltamethrin in rats involves rapid ester cleavage and hydroxylation. Deltamethrin has a half-life in the rat brain of 1–2 days, but it is more persistent in body fat, with a half-life of 5 days (Hayes and Laws, 1990). In mammals, the point of death from deltamethrin poisoning is sharply defined by respiratory or cardiac failure (Leahey, 1985). Rats and dogs that were given the oral doses of 10 mg/kg/day for 13 weeks exhibited some motor symptoms but caused no fatalities or pathological changes.

Pesticides and heavy metals produce many physiological and biochemical changes in freshwater organisms by influencing the activities of several enzymes. There are a lot of studies related to changing enzyme activity in several organisms including rainbow trout with exposure to some xenobiotics such as pesticides and heavy metals both *in vivo* and *in vitro* conditions (Ceyhun et al., 2010; Ekinci and Beydemir, 2010; Ekinci et al., 2007; Hisar et al., 2005; Soyut and Beydemir, 2008; Soyut et al., 2008). The best way to understand whether alteration of the enzyme activity results from inhibition or activation of the enzyme or from the changes in expression levels is to check the expression levels of the mRNA levels before translation. Gene expression changes are likely to be the initial response to toxic exposure, and different types of stressors might be affecting different genes and/or pathways.

Most studies related to MT induction have focused on metals, leading to the conclusion that these proteins appear to play a role in the regulation of essential metals as well as in metal detoxification. Although many works showed that MT synthesis was induced by various metals, their accumulation was also induced by other

factors such as exposure to UV, starvation, physical trauma, handling, anoxia, freezing and the presence of antibiotics (English and Storey, 2003; Mosleh et al., 2004; Roesijadi, 1994; Templeton and Cherian, 1991), and by pesticide contamination (Mosleh et al., 2004; Paris-Palacios et al., 2003). Roch et al. (1982) have suggested the use of hepatic MT levels for monitoring the degree of pollution in water. In the present study, we observed that long-term exposure to both DDVP and deltamethrin resulted in an increasing effect on gene expression level of both types of MT gene. Similarly, Olsson et al. (1995) demonstrated that in RTH-149 cells hydrogen peroxide could activate MT gene transcription probably through an antioxidant response element consensus sequence. Similar *in vitro* experiments concerning organic xenobiotics are lacking, but it can be inferred from *in vivo* experiments on fish that polar xenobiotics such as parquet (Pedrajas et al., 1995) , but not apolar xenobiotics such as BaP or PCB-156 (George, 1989; Hylland et al., 1996), can stimulate MT neosynthesis. Moreover, it has been shown that the pretreatment of fish with BaP or PCB-156 prior to heavy metals can inhibit MT induction (Sandvik et al., 1997), showing that the use of MT as a biomarker of exposure to heavy metal can be complicated by interactions with other chemicals (Viarengo et al., 1999). In addition to these, studies in experimental animals and tissue culture studies show that pesticides, especially organophosphate pesticides, induce oxidative stress (Bagchi et al., 1995; Banerjee et al., 2001; Mena et al., 2009; Nasuti et al., 2007; Srivastava and Shivanandappa, 2005; Suke et al., 2006). As known, MTs may reduce the dangerous effects of free radicals and ROS by releasing zinc to the neuronal membrane (Shiraga et al., 1993). Thus, the significant increase in mRNA levels of MTs in response to deltamethrin and DDVP administration may be considered as a defence mechanism in addition to the antioxidants against oxidative stress.

Living beings exposed to pesticide can display several responses, such as the induction of biotransforming enzymes. The metabolism of xenobiotics usually involves two distinct stages, commonly referred to phases I and II. Phase I metabolism involves an initial oxidation, reduction or dealkylation of the xenobiotic by cytochrome P450 monooxigenases (Guengerich, 1991). This step is often needed to provide a molecule with hydroxyl- or amino- groups, which are essential for phase II reactions. Phase II metabolism generally adds hydrophilic moieties, thereby making a toxin more water soluble and less biologically active (Erat et al., 2003).

CYP-1A induction is a sensitive and specific adaptive response of organisms exposed to an important series of environmental pollutants such as planar congeners of polychlorinated dibenzodioxins and dibenzofurans (PCDD, PCDF), polychlorinated biphenyls (PCB), several polycyclic aromatic hydrocarbons (PAH), polychlorinated naphthalenes and others (Fent, 2001). Induction of CYP-1A may be explained as a result of binding of foreign compound to a cellular receptor (Perdew and Poland, 1988). This binding triggers the expression of the gene coding for CYP-1A leading to increased RNA transcription (Okey et al., 1994) and hence increase the synthesis of CYP-1A (Hassanain et al., 2007). Therefore, CYP-1A induction is used as a biomarker indicative for exposure to such pollutants in fish and fish cell systems.

Similarly in various studies, CYP-1A induction has been used as a biomarker of exposure to various pollutants in a wide variety of vertebrate species, including mammals (Qualls et al., 1998; White et al., 1994), fish (Smolowitz et al., 1991; Woodin et al., 1997), reptiles (Rie et al., 2000) and birds (Sanderson et al., 1994).

We demonstrated in our previous study that deltamethrin inhibits antioxidant enzymes, elevates the expression levels of heat shock protein 70 (Ceyhun et al., 2010) and has negative effects of the pesticide on the expression of IGF-I, IGF-II and GH (Aksakal et al., 2010). Therefore, combining the literature work with our previous and current study, we chose deltamethrin. Moreover, to generalize our finding, we supported our data with another pesticide DDVP.

As known, rainbow trout is one of the most important cultivated species in the world. One of the most important criteria for successful aquaculture of this fish is conversion of the feed into meet at maximum level. This conversion mechanism is quite sensitive and can be affected by any environmental factors. Farmed fish are exposed to a range of potentially stressful conditions (e.g. water quality such as low oxygen, high ammonia and CO_2 levels), often in combination with high- or low-chemical contamination, thus affecting the profitability of the aquaculture industry.

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