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Potential Toxic Effects of Mancozeb on Catalase (Cat) Activity and Lipid Peroxidation (Lpo) on Brain Tissue of Zebrafish, *Danio rerio*

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ABSTRACT

The aim of this study is to evaluate the potential toxic effects of mancozeb on stress biomarkers such as catalase (CAT) activity, malondialdehyde (MDA) level and protein level in the brain tissue of zebrafish (*Danio rerio*). Mancozeb, is a synthetic fungicide contaminating aquatic environments as a potential toxic pollutants, was investigated in the present study for acute toxicity. Zebrafish groups were exposed to different doses of mancozeb (5 mg L^{-1} and 7,5 mg L^{-1}) for 120 hours. Catalase (CAT) activity, Malondialdehyde (MDA) level and total protein level were determined with spectrophotometer. The results showed that CAT activity and MDA level decreased in all experiment groups. Protein level increased in the experiment groups when compared to the control group. In conclusion, the changes in the CAT activity and MDA levels were time and as well as mancozeb dose-dependent. Furthermore, the biochemical parameters of mancozeb exposure on zebrafish, showed that mancozeb has significant effect on the zebrafish and/or aquatic organisms

Introduction

The consequence of human population growth is the rise of global agricultural production and hence the increased use, abuse or excessive of pesticides all over the world (Yıldırım et al., 2006; Köhler and Triebskorn, 2013; de la Cruz et al., 2014). Aquatic biota of existing agricultural areas are often exposed to a broad spectrum of pesticides that reach water ecosystems through unintended direct application, spray drift and runoff, thus posing a possible risk for non-target species, such as fish and other aquatic animals (Wagner et al.,2014; Vutukuru and Basani, 2013). Ethylene-bis-dithiocarbamates (EBDCs) are broadly used in agriculture as fungicides, especially on fruit, vineyard, and potato crops. One of the EBDC is; Mancozeb and it is widely used as an active ingredient of fungicides applied worldwide like dithane, fore, manzeb and others (Debbarh et al., 2002; Kubrak et al., 2012). All pesticides are described as critical pollutants for the aquatic environments with the potential to cause harmful effects on the fish (Srivastava and Singh, 2013; Akbulut et al., 2014).

Zebrafish (*Danio rerio*) is a significant vertebrate model organism (Sawlow et al., 2010; Sharma and Ansari, 2014; Yön et al., 2015). Thus to better understand the harmful effects of mancozeb and oxidative stress process induced by mancozeb, this study examined dose-dependent oxidative stress in zebrafish brain tissue by determining the malondialdehyde (MDA) and protein level and catalase (CAT) activity. In this study, we aimed to evaluate the possible harmful effects of mancozeb on zebrafish brain tissue that would be an early indicator of the aquatic pollutants.

Material and Method

Experimental design: Mancozeb is the member of EBDC fungicides, a broad spectrum protectant from fungi for the control of a wide range of diseases in agricultural, horticultural and ornamental crops (EPA, 2005). First, a stock solution of mancozeb was prepared. After animal groups are divided into two experimental groups and a control group, the prepared stock solution was applied in the experimental groups with different doses 5 and 7,5 mg L^{-1}, respectively. Zebrafish is a common and useful model organism for research of vertebrate development and aquatic toxicology.

Zebrafish (weight 4-9 g. length 4-5 cm) obtained from a commercial pet shop, brought to the Marmara University, Department of Biology, Zoology Laboratory, and acclimatized for a week under standard laboratory conditions. Healthy ten-month old female and male zebrafish were selected and put in tanks. Before exposure to different doses of mancozeb, the fish groups were acclimatized in an aquarium filled top water (free chlorine) at ambient temperature 25°C for a week. The fish were fed twice a day and with a photoperiod consisting of 14h light/10h dark period during experiment. After 120 hours, fish were dissected immediately with sterile surgical instruments. The brain tissue of fish were removed from their cranial cavity with aseptic conditions, cold 0,1 M phosphate buffer (pH 7,4) were added to the brain tissue samples and the samples were homogenized by using glass tissue homogenizers under ice-bath cooling. Then homogenates were centrifuged at 10.000 g for 30 min at 4°C to obtain the supernatant for analyzing the enzyme activities. The supernatants were stored at 4°C, and all applications were performed under ice-bath cooling to keep the enzyme activities stable.

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CAT, MDA and total protein levels were detected using spectrophotometric methods.

Catalase (CAT) activity assay (Aebi, 1974): Catalase enzyme activity was determined according to the method of Aebi (Aebi, 1974). The principle of the assay is based on the determination of the rate constant of hydrogen peroxide decomposition by the CAT. Briefly, the activity was determined by measuring the decrease in absorbance at 240 nm of a reaction mixture consisting of H2O2, in phosphate buffer, pH 7,0 and requisite volume of serum sample.

Lipid peroxidation (LPO) estimation assay (Ledwozyw, 1986): The MDA content was measured after incubation at 95°C with thiobarbituric acid in aerobic conditions. The pink color produced by the reactions was measured with spectrophotometer at 532 nm (Ledwozyw et al., 1986). Specific activity was defined as the unit of activity per milligram protein.

The SPSS 23.0 package program was used for analysis, study data were given as arithmetic means and standard deviations. The one way analysis of variance and student t-test were used for the determination of the significance of the differences between the groups. A value of $p<0,05$ was considered statistically significant.

Results

In this study, we determined low CAT activity in the brain tissue of zebrafish after both of mancozeb treatment. Our results demonstrated reduced levels of CAT and MDA in zebrafish brain tissue when compared to the control group. This decrease in CAT activity could be due to the excess production of superoxide radicals. MDA, which is itself responsible for some of the damaging effects of free radicals on cell membranes. Whereas, severe oxidative stress in the cells can cause cell injury and death of cell. The decreased MDA levels may be consequence of cellular-oxidative damage due to pesticide exposure. Total results of this study including MDA and protein levels, CAT activity in brain tissue of zebrafish with or without exposed to mancozeb are in Table 1.

Table 1. MDA, CAT and protein level changes related to after mancozeb exposure (Values were expressed as mean±SD).

	Exposure concentration (mg L^{-1})		
	Control group	**M-Low (5 mg L^{-1})**	**M-High (7,5 mg L^{-1})**
CAT	5,505 ± 0,125	3,162 ± 0,60	2,912 ± 1,07
MDA	0,105 ± 0,018	0,0377 ± 0,003	0,0055 ± 0,0006
Protein (µg/µl)	16,379 ± 1,53	21,972 ± 1,102	22,331 ± 1,095

The changes of the CAT activities in brain tissues exposed to different sublethal concentrations of mancozeb are in Fig. 1. The CAT activities; for 5 mg L^{-1} is 3,162 ± 0,60 (U/ mg protein), for 7,5 mg L^{-1} is 2,912 ± 1,07 (U/ mg protein) and for the control group is 5,505 ± 0,125 (U/ mg protein).

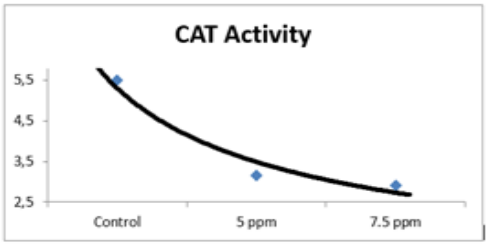

Figure 1: CAT activity in all experiment and control groups (U/ mg protein) (p<0,05) (n=10).

Fig. 2 indicates that the MDA levels in zebrafish were significantly effected by mancozeb. The MDA levels; for 5 mg L^{-1} is 0,0377 ± 0,003 (nmol MDA/ g), for 7,5 mg L^{-1} is 0,0055 ± 0,0006 (nmol MDA/ g), and for the control group is 0,105 ± 0,018(nmol MDA/ g).

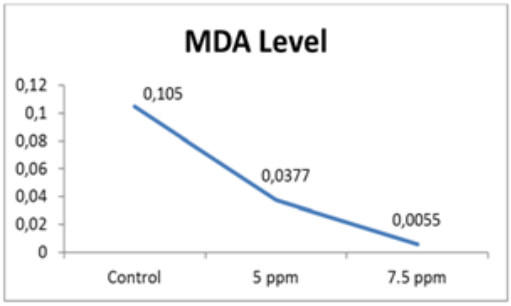

Figure 2. MDA levels in all experiment and control group (nmol MDA/g) (p<0,01) (n=10).

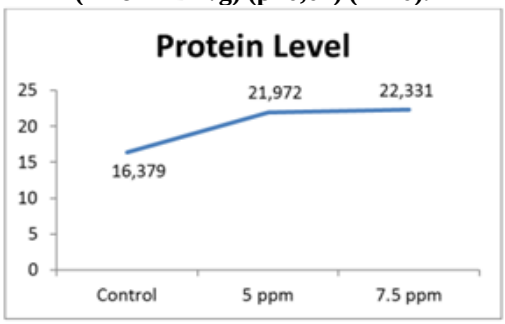

Figure 3: Protein levels in all experiment and control group (µg/µL) (p<0,01) (n=10).

The changes in the protein levels in brain tissues exposed to different sublethal concentrations of mancozeb are in Fig. 3. The protein levels; for 5 mg L^{-1} is 16,379 ± 1,53 (µg/µL), for 7,5 mg L^{-1} is 22,331 ± 1,095 (µg/µL) and for the control group is 16,379 ± 1,53 (µg/µL).

Discussion

Fungicides are a class of pesticides and potentially capable of generating oxidative stress in non-target organisms (Tabassum *et al*., 2016). Mancozeb is mainly used to eliminate fungi which are harmful for agriculture. Unfortunatelly, the indiscriminate use of pesticides may have impacts on non-target organisms especially fish. Little is known about the effects of pesticides on fish physiology and the nervous systems, even though these chemicals are extensively used in the environment (Kaymak *et al*., 2014). Many of these compounds or their metabolites have toxic effects related to oxidative stress. The potential of reactive oxygen species (ROS) in damage tissues, called oxidative stress, in living systems such as fish. Many organisms have unique systems to protect themselves against the harmful effects of ROS. Also, fish like many other organisms, can fight against the reactive oxygen species with antioxidant enzyme systems such as CAT.

CAT plays a significant protective role in tissue against ROS attack (Lushchak, 2011). CAT is one of the primary enzyme which involves in peroxide detoxification and has a special importance for the clearance of H2O2 (Jin *et al*., 2010; Saravanan *et al*., 2011; Xing et al., 2016). In our study, we found low CAT activity in the brain tissue of zebrafish. Jin *et al*., reported that the efficiency of CAT level decreased significantly in the liver tissue of zebrafish exposed to cypermethrin as observed in their study (Jin *et al*., 2011). Therefore, in our study, it is possible that the decreased activity of CAT enzyme contributes to the elimination of ROS in the zebrafish induced by mancozeb exposure.

Pesticide-induced studies, about oxidative stress, catalase activity have been investigated in various fish species. According to Alves *et al*., furadan had induced increase in CAT activity as an adaptive response (Alves *et al*., 2012).

In another study, researchers reported that dichlorves increased CAT activity in *Ictalurus nebulosus* in a concentration-dependent manner (Hai *et al*., 1997). On the contrary, we found low CAT activity in the brain tissue of zebrafish after mancozeb treatment. Similar results have also been reported in various fish species. Blahova *et al*., (2013) reported a considerable decline in CAT activity in all test groups of zebrafish exposed to atrazine (Blahova *et al*., 2013). According to similar to ours, deltamethrin exposure caused considerable decrease in catalase activity in liver, kidney and gill tissues of *Channa punctatus* (Sayeed *et al*., 2003). Another similar study, the treatment of *Ameliurus nebulosus* with menadione led to decrease in catalase activity (Hasspieler *et al*., 1994). However, Paulino (2012) observed no change in catalase efficiency in gills of *Prochilodus lineatus* after acute exposure to various doses of atrazine (Paulino *et al*., 2012). Environmental toxicants, such as over dose pesticides, support oxidative damage by directly increasing the cellular concentration of reactive oxygen species and also by reducing the cellular antioxidant capacity. In our study, CAT activity decreased in brain tissue of zebrafish after exposed to mancozeb for 120 hours. The decreased CAT activity may be as the consequence of cellular-oxidative damage due to pesticide exposure at different concentrations during the experimental study. Reduced CAT activity, after mancozeb exposure, could be as a result of brain damage due to peroxidation of tissue or the flux of superoxide radicals, resulting in an increase in cellular H_2O_2. So, we can infer that oxidative balance might play an important role in prevention of antioxidative enzyme activity due to mancozeb exposure.

Lipid peroxidation (LPO) is one of the most important early events in cell degeneration leading to necrosis and occurs primarily in the cell membrane (Tabassum *et al*., 2016). Pesticides can generally cause oxidative stress and LPO has been widely used as a marker of oxidative stress in living cell (Tsaboula *et al*., 2016). Malondialdehyde (MDA) is one of the most prefered indicator of LPO in all living organisms, including aquatic organisms. MDA, as a significant indicator of free radical injuries, can readily and widely bind or crosslink important biomolecules such as proteins and nucleic acids (Xing *et al*., 2012).MDA is the product formed as a result of LPO and is a parameter extensively used to show the oxidative damage on cells. (Topal *et al*., 2014). It is the indication of the preservative effects of oxidative enzymes that lipid peroxidation either does not occur or it occurs at lower levels (Kaymak *et al*., 2014). In this study it was shown that LPO estimation could provide useful information about the effect of exposure to environmental pollutant such as mancozeb. Oxidative stress induced by high formation of free radicals considered to be crucial in cell injury in a variety of diseases. Brain is noted abnormally sensitive to oxidative stress. Free radicals are produced in the organism both in physiological and pathological circumstances. The membrane lipids of neurons in central nervous system have high content of polyunsaturated fatty acids which are the fundamantal substrates for lipid peroxidation (Tsaboula *et al*., 2016). Disturbance of the antioxidant protection and oxidative damage are the main mechanisms of several xenobiotics toxicity derived of pesticides (Ozden *et al*., 2013). The major findings in our study, reduction of oxidative stress, plays a major role in the mechanism of mancozeb induced toxicity in the brain tissue of zebrafish.

Although proteins are now used generally and widely as a marker of ROS, they infrequently have been investigated in aquatic pollution (Slaninova *et al*., 2009; Itziou *et al*., 2011; Husak *et al*., 2014). Total protein levels are one of the main targets for the explanation of effects of pesticides in aquatic organisms. Miron *et al*., (2008), reported that exposure to clomazoneon *Leporinus obtusidens* for 8 days, resulted as increased protein levels in fish liver (Miron *et al*., 2008). Moraes *et al*., (2011) also reported similar results related with the total protein levels when carps were exposed to imazethapy+imazapic pesticides (Moraes *et al*., 2011). Thus our results, recommend that zebrafish exposed to mancozeb, demonstrated with the increase in protein levels in brain tissue and caused oxidative stress. The oxidative stress as a response to antioxidant system in tissues showed variations from among fish due to the variations in antioxidant potential (Ahmad *et al*., 2000; Xing *et al*., 2012; Toni *et al*., 2013). Although mancozeb has been detected in surface waters, studies addressing the toxic risk to fish arising from the use of mancozeb, testing environmentally realistic concentrations, are absent from the literature. Hence, this work purposed to determine the toxic effects of mancozeb (5 and 7.5 mg L^{-1}) on the brain tissueof zebrafish after exposure (120h). In our study, it was observed that mancozeb induced ROS generation in zebrafish brain tissue in the exposure groups. At the same time, levels of protein had also significantly increased in the brain tissue of zebrafish after mancozeb exposure.

In conclusion, the present study investigated the existence of oxidative stress bio-markers in zebrafish brain tissue, under laboratory conditions, and found that decreased level of MDA and CAT activity with an increased level of protein in relation to oxidative stress. Numerous studies have demonstrated that oxidative stress has become one of the major research field in aquatic toxicology. The results are significant for reporting acute mancozeb toxicity in terms of biochemical changes: mancozeb is substantially toxic to fish. Pollution of the aquatic ecosystems is a very serious and ever growing problem. All pesticides cause non-degredable residues on soil, water and living organisms for a long time. Exposing many environmental stressors such as pesticides have deleterious effects on aquatic animals. Special studies, such as detecting biomarkers in polluted aquatic environments, recommended to ensure continue the sustainability of healthy environment.

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