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Protective role of vitamins C and E in diclorvos-induced oxidative stress in human erythrocytes *in vitro*

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ABSTRACT

Organophosphate (OP) pesticides such as dichlorvos (DDVP) intoxication has been shown to produce oxidative stress due to the generation of free radicals, which alter the antioxidant defense system in erythrocytes. In this study, the effects of DDVP (1, 10, 100 μM) or DDVP + vitamin C (VC; 10 μM) or vitamin E (VE; 30 μM), on the levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities in human erythrocytes were examined *in vitro*. There were no statistical differences between all groups for 1 μM concentration of DDVP. Treatment with DDVP alone produced an increase in the level of MDA and decreased activities of antioxidant enzymes ($P < 0.05$). Groups treated with vitamins and DDVP showed protective effects of vitamins against DDVP-induced changes in antioxidant enzyme activity and lipid peroxidation (LPO) (10 μM). At 100 μM concentration of DDVP vitamins had no effect on DDVP-induced toxicity. The results show that administration of DDVP resulted in the induction of erythrocyte LPO and alterations in antioxidant enzyme activities, suggesting that reactive oxygen species (ROS) may be involved in the toxic effects of DDVP. Also the data show that the plasma level of VC and VE may ameliorate OP-induced oxidative stress by decreasing LPO in erythrocytes at certain doses of OP pesicides.

Key words: antioxidant enzyme, dichlorvos, oxidative stress, vitamin, malondialdehyde.

INTRODUCTION

Pesticides are occasionally used indiscriminately in large amounts causing environmental pollution, and therefore are a cause of concern (John et al., 2001). Environmental pollution by pesticide residues is a major environmental concern due to their extensive use in agriculture and in public health programs (Celik and Suzek, 2009). Organophosphate (OP) pesticides have been detected in the soil, water bodies, vegetables, grains and other food products (IARC, 1983). OP insecticides are some of the most useful and diverse insecticides; they have been in use for almost five decades. However, the uncontrolled use of these insecticides in agriculture and public health operation has increased the scope of ecological imbalance and thus many non-target organisms have become victims (Celik and Suzek, 2009).

Dichlorvos (DDVP) is an OP that has been in use for more than 40 years. It has been evaluated in a wide range of toxicological assays including bioassays for carcinogenicity and mutagenicity (genotoxicity). The genotoxicity evaluations have included a wide range of test systems and endpoints including assays both *in vitro* and *in vivo*. There is general agreement that DDVP is genotoxic *in vitro* (Booth et al., 2007). Literature also cites DDVP toxicity to humans which includes dose-dependent decrease in human erythrocyte cholinesterase activity and sperm motility based on the urinary concentration of dimethyl phosphate, a urine metabolite of DDVP (Okamura et al., 2005).

Recent findings indicate that toxic manifestations induced by OP may be associated with an enhanced production of reactive oxygen species (ROS) (Gultekin et al., 2000, 2001; Durak et al., 2009). Among the ROS, superoxide anions, hydroxyl radicals and hydrogen peroxide enhance the oxidative process and induce lipid peroxidative damage in cell membranes (Altuntas and Delibas, 2002). The cell has several ways to alleviate the effects of oxidative stress, either by repairing the damage or by directly decreasing the occurrence of oxidative damage by means of enzymatic and non-enzymatic antioxidants. Enzymatic and non-enzymatic antioxidants have also been shown to scavenge free radicals and ROS (Gultekin et al., 2001).

As some of the pesticides may be present in tissues of exposed humans and animals, they may produce oxidative stress in tissues. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in tissues may neutralize the oxidative stress (Kalender et al., 2004). Non-enzymatic antioxidants such as vitamin E (VE) and vitamin C (VC) can also act to overcome oxidative stress, being a part of the total antioxidant system. α-Tocopherol (the main form of VE) is considered as a major lipophilic antioxidant (Vatassery, 1998). VE resides mainly in the membranes and thus helps to maintain membrane stability (Baker et al., 1996). VC and VE have been shown to possess anticarcinogenic, anticlastogenic, and antimutagenic properties in a variety of *in vivo* and *in vitro* models of pesticide exposure (Hoda and Sinha, 1993).

VC (L-ascorbic acid) is hydrophilic and a very important free-radical scavenger in extracellular fluids, trapping radicals in the aqueous phase and protecting biomembranes from peroxidative damage (Harapanhalli et al., 1996). The anticarcinogenic, anticlastogenic and even antimutagenic roles of VC have been tested in a variety of *in vivo* and *in vitro* systems exposed to radiation and pesticides (Castillo et al., 2000; Durak et al., 2009). It prevents the increased production

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of free radicals induced by oxidative damage to lipids and lipoproteins in various cellular compartments and tissues (Sies et al., 1992). The antioxidative efficiency of VE can be considerably increased by co-supplementation with VC, which is a co-antioxidant for VE (Stocker, 1994; Durak et al., 2009).

The present study was undertaken to determine the possible negative effects of DDVP on erythrocytes and additionally to investigate whether there is any preventive effect of a combination of plasma level of vitamins C and E *in vitro* after DDVP administration.

METHODS

Erythrocyte preparation

Twenty ml venous blood samples were obtained in heparinized dry tubes from each of six male volunteers (range 21–26 years). All volunteers were healthy, taking no medication, non-smokers, and none of them were farm or agricultural workers. Plasma was separated by centrifugation. Erythrocyte packets were prepared by washing with cold isotonic saline. After the supernatant was removed, packed erythrocytes were suspended in phosphate buffer. The concentration of hemoglobin was determined (Drabkin, 1946).

Treatment of erythrocytes

VC (L-ascorbic acid) was supplied by Carlo Erba (Milano, Italy). VC was dissolved in distilled water (Konopacka et al., 1998). VE (DL-α-tocopherol) was supplied by Merck (Germany). VE was dissolved in corn oil (Kalender et al., 2007). The doses of VC (10 μM) and VE (30 μM) were chosen based on the levels of each vitamin in human plasma (Blasiak and Stankowska, 2001). Other chemicals were supplied by Sigma-Aldrich. DDVP (1, 10 and 100 μM) was supplied by Ankara Agricultural Protection Central. Erythrocytes were divided into non-treated control and experimental groups. The control group was incubated in 0.9% NaCl at 7.4 pH. The experimental group was divided into treatment groups: DDVP (n=6), VC (n=6), VE (n=6), VC+VE (n=6), DDVP +VC (n=6), DDVP+VE (n=6), DDVP +VC+VE (n=6) groups. MDA levels and the activities of SOD, CAT and GPx were measured by spectrophotometer (Shimadzu UV-1700, Japan).

Antioxidant enzyme assays

CAT enzyme activity was measured according to the method described by Aebi (1983) by assaying the hydrolysis of H_2O_2 and the resulting decrease in absorbance at 240 nm. Data are expressed as UCAT/mg hemoglobin. GPx activity was measured using H_2O_2 as substrate according to the method described by Paglia and Valentine (1967) of absorbance at 340 nm. Data are presented as UGPx/mg hemoglobin. Total SOD activity was determined according to the method described by Marklund and Marklund (1974) by assaying the autooxidation and illumination of pyrogallol at 440 nm. Data are expressed as USOD/mg hemoglobin.

Measurement of MDA levels

MDA content was assayed using the thiobarbituric acid (TBA) test as described by Ohkawa (1979). Absorbance was measured at 532 nm to determine the MDA content. Specific activity is presented as nmol/mg hemoglobin.

Statistical analysis

Data were analyzed by software program SPSS 11.0 for Windows. Differences were calculated using one-way analysis of variance (ANOVA), followed by Tukey's procedure for multiple comparisons. $P < 0.05$ value was taken as statistically significant. All data are expressed as means ± standard deviation (S.D).

RESULTS

There were no statistical differences between VC-treated, VE-treated and VC + VE-treated cells compared to control cells (Figs. 1-4). There were no statistical differences between all groups for 1 μM concentration of DDVP.

Antioxidant Enzyme Activities

SOD (Fig. 1), CAT (Fig. 2) and GPx (Fig. 3) activities were significantly ($P < 0.05$) decreased in the DDVP treatment group compared to control group and vitamin groups alone (10 μM and 100 μM). The activities of enzymes were significantly increased in DDVP + VC and DDVP + VE groups compared to the DDVP group (10 μM). In the DDVP + VC + VE group in CAT and GP there were significantly ($P < 0.05$) increased enzyme activities compared to the DDVP, DDVP + VC and DDVP + VE groups (10 μM). These effects were not seen in 100 μM treated groups. For enzyme activities there were no statistical differences between DDVP-treated, DDVP + VE-treated, DDVP + VC-treated and DDVP + VC + VE-treated cells at 100 μM.

MDA levels

MDA levels were significantly increased in the DDVP treatment group compared to the control and groups with only vitamins (10 μM and 100 μM). There were significant decreases in the DDVP + VC and DDVP + VE groups compared to the DDVP group (10 μM). The DDVP + VC + VE group showed a significant decrease compared to the DDVP, DDVP + VC and DDVP + VE groups (10 μM) ($P < 0.05$). These effects were not seen in 100 μM treated groups. There were no statistical differences between DDVP-treated, DDVP + VE-treated, DDVP + VC-treated and DDVP + VC + VE-treated cells at 100 μM (Fig. 4).

DISCUSSION

A large number of xenobiotics have been identified to have the potential to generate free radicals in biological systems (Kehrer, 1993). DDVP acts primarily by irreversibly inhibiting acetylcholinesterase (AChE) at cholinergic junctions of the nervous system (Petroianu et al., 2006), which produces hepatotoxicity in rats and induces oxidative stress (Gupta et al., 2005). DDVP is taken into the human body very rapidly by the lungs, stomach, or skin (Guloglu et al., 2004). DDVP has toxic effects on mammals and also on fish, birds, honeybees and non-target invertebrates (Ural and Köprücü, 2006; Ogutcu et al., 2008). High doses of DDVP stimulate LPO through increasing plasma MDA levels and decreasing erythrocyte CAT activities

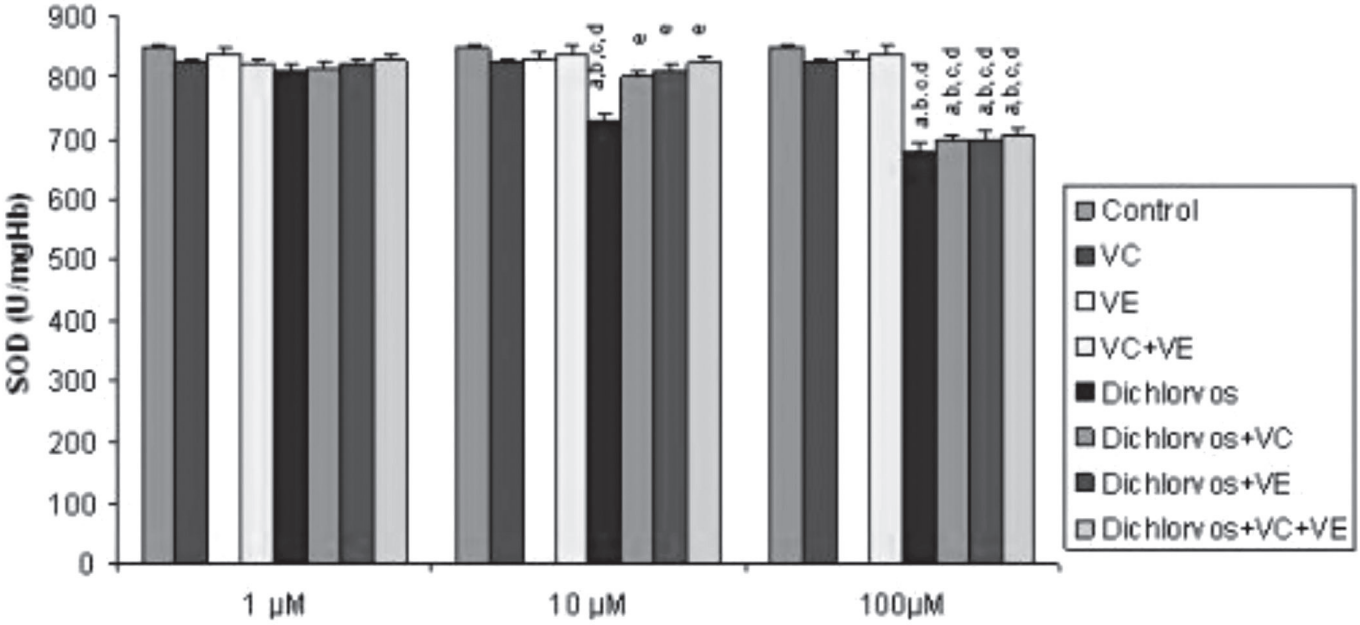

Figure 1. SOD activity in control and experimental groups of erythrocytes with dichlorvos. Comparison of nontreated control cells and other groups. [b]Comparison of VC-treated cells with VE-, VE+VC-, dichlorvos-, dichlorvos +VC-, dichlorvos +VE- and dichlorvos+VC+VE-treated cells. [c]Comparison of VE-treated cells with VE+VC-, dichlorvos-, dichlorvos+VC-, dichlorvos+VE- and dichlorvos+VC+VE-treated cells. [d]Comparison of VC+VE-treated cells with dichlorvos-, dichlorvos+VC-, dichlorvos+VE-dichlorvos+VC+VE-treated cells. [e]Comparision of dichlorvos-treated cells with dichlorvos+VC-, dichlorvos+VE- and dichlorvos +VC+VE-treated cells ($P<0.05$). Data represent the means ±SD of six samples.

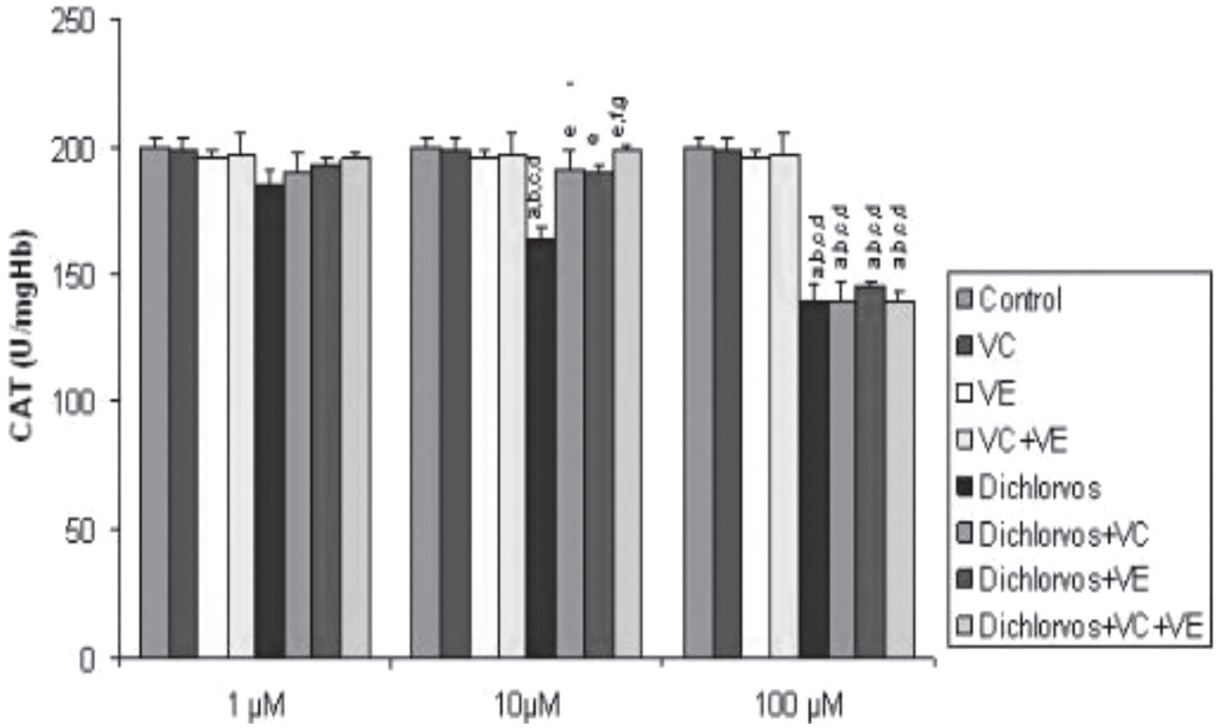

Figure 2. CAT activity in control and experimental groups of erythrocytes with dichlorvos. Comparison of nontreated control cells and other groups. [b]Comparison of VC-treated cells with VE-, VE+VC-, dichlorvos-, dichlorvos +VC-, dichlorvos +VE- and dichlorvos+VC+VE-treated cells. [c]Comparison of VE-treated cells with VE+VC-, dichlorvos-, dichlorvos+VC-, dichlorvos+VE- and dichlorvos+VC+VE-treated cells. [d]Comparison of VC+VE-treated cells with dichlorvos-, dichlorvos+VC-, dichlorvos+VE-dichlorvos+VC+VE-treated cells. [e]Comparision of dichlorvos-treated cells with dichlorvos+VC-, dichlorvos+VE- and dichlorvos +VC+VE-treated cells. [f]Comparision of dichlorvos+VC-treated cells with dichlorvos+VE- and dichlorvos +VC+VE-treated cells. [g]Comparision of dichlorvos+VE-treated cells with dichlorvos+VC+VE-treated cells ($P<0.05$). Data represent the means ±SD of six samples.

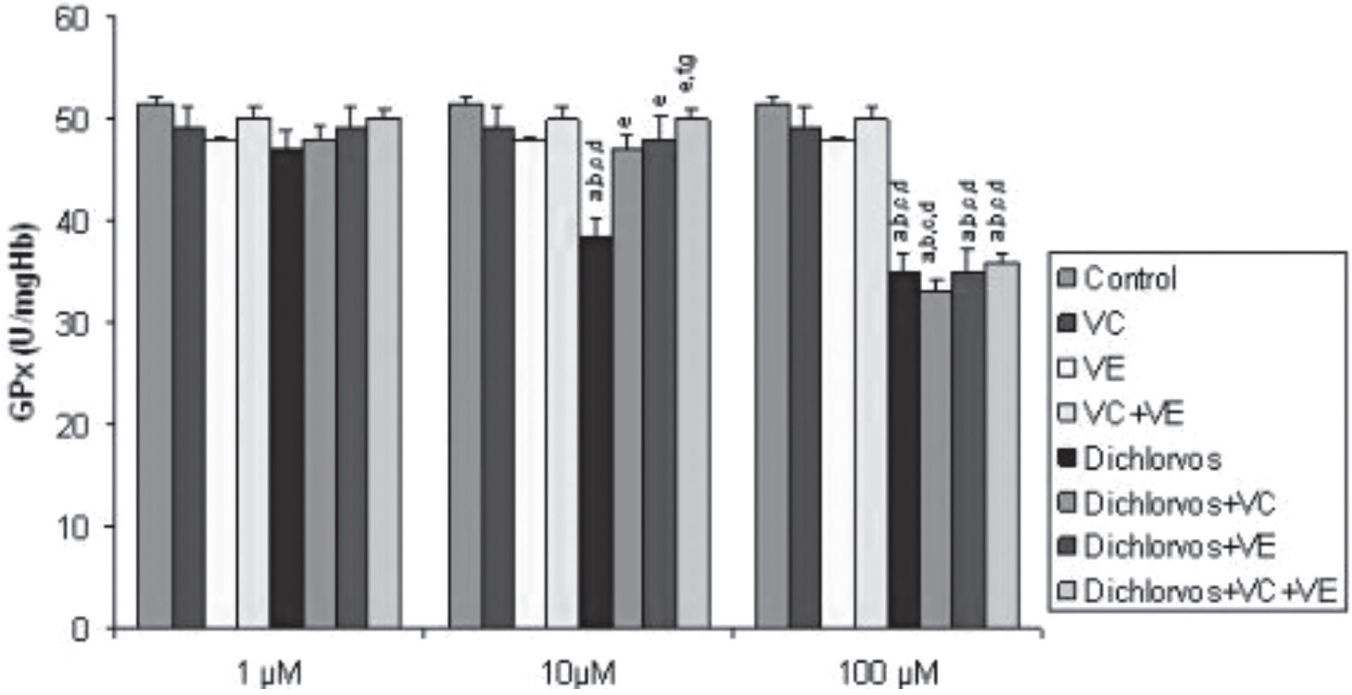

Figure 3. GPx activity in control and experimental groups of erythrocytes with dichlorvos. [a]Comparison of nontreated control cells and other groups. [b]Comparison of VC-treated cells with VE-, VE+VC-, dichlorvos-, dichlorvos +VC-, dichlorvos +VE- and dichlorvos+VC+VE-treated cells. [c]Comparison of VE-treated cells with VE+VC-, dichlorvos-, dichlorvos+VC-, dichlorvos+VE- and dichlorvos+VC+VE-treated cells. [d]Comparison of VC+VE-treated cells with dichlorvos-, dichlorvos+VC-, dichlorvos+VE-dichlorvos+VC+VE-treated cells. [e]Comparision of dichlorvos-treated cells with dichlorvos+VC-, dichlorvos+VE- and dichlorvos +VC+VE-treated cells. [f]Comparision of dichlorvos+VC-treated cells with dichlorvos+VE- and dichlorvos +VC+VE-treated cells. [g]Comparision of dichlorvos+VE-treated cells with dichlorvos+VC+VE-treated cells ($P<0.05$). Data represent the means ±SD of six samples.

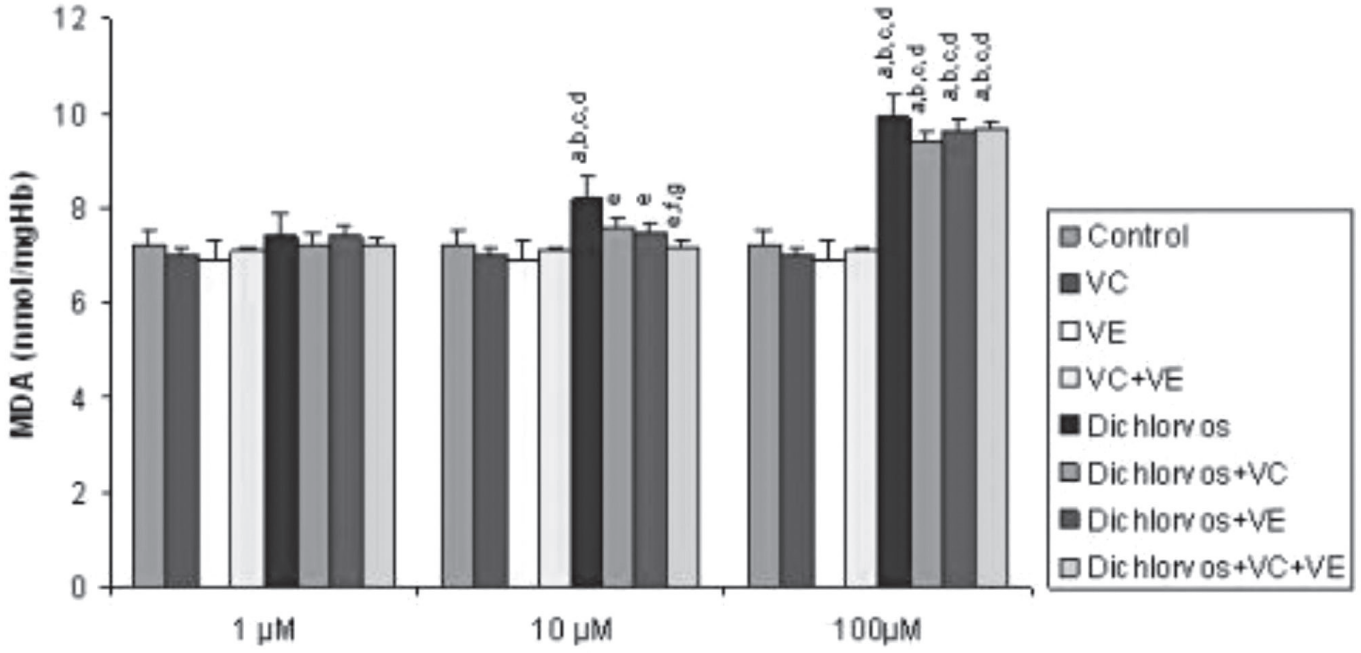

Figure 4. MDA levels in control and experimental groups of erythrocytes with dichlorvos. [a]Comparison of nontreated control cells and other groups. [b]Comparison of VC-treated cells with VE-, VE+VC-, dichlorvos-, dichlorvos +VC-, dichlorvos +VE- and dichlorvos+VC+VE-treated cells. [c]Comparison of VE-treated cells with VE+VC-, dichlorvos-, dichlorvos+VC-, dichlorvos+VE- and dichlorvos+VC+VE-treated cells. [d]Comparison of VC+VE-treated cells with dichlorvos-, dichlorvos+VC-, dichlorvos+VE-dichlorvos+VC+VE-treated cells. [e]Comparision of dichlorvos-treated cells with dichlorvos+VC-, dichlorvos+VE- and dichlorvos +VC+VE-treated cells. [f]Comparision of dichlorvos+VC-treated cells with dichlorvos+VE- and dichlorvos +VC+VE-treated cells. [g]Comparision of dichlorvos+VE-treated cells with dichlorvos+VC+VE-treated cells ($P<0.05$). Data represent the means ±SD of six samples.

in vivo (Yarsan and Cakir, 2006). In this study, treatment with DDVP significantly increased the levels of MDA and decreased the activities of antioxidant enzymes (10, 100 μM).

LPO has been suggested as one of the molecular mechanisms involved in pesticide-induced toxicity (Kehrer, 1993). Some studies have reported that OP cause LPO (Celik and Suzek, 2009; Gultekin et al., 2000; Durak et al., 2009) in vertebrates. The interaction of hemoglobin with redox drugs or xenobiotics is a source of radical production in erythrocytes (French et al., 1978, Winterbourn et al., 1978), giving rise to superoxide radicals, hydrogen peroxide and in some cases peroxy radicals, and inducing membrane LPO and hemolysis (Clemens et al., 1984). MDA is a major oxidation product of peroxidized polyunsatured fatty acids and increased MDA content is an important indicator of LPO. Xenobiotics such as pesticides cause increase of the MDA level in tissues (Banerjee et al., 2001; Hazarika et al., 2003). In this study the MDA level was increased in DDVP group, suggesting that the MDA level could be used as a marker for OP injury. This increase suggested that DDVP can accelerate the increase in free oxygen groups when administrated at 10 μM and 100 μM concentrations. The treatment with plasma levels of VE and VC attenuated the DDVP-induced increase in MDA level at 10 μM. These data show that the plasma level of VE and VC may have significant effects in reducing DDVP-based toxicity in this dose.

Oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species (Davies, 1995); organisms contain intracellular antioxidant enzymes such as SOD, CAT and GPx, that work together to prevent oxidative damage to the cell (Bukowska, 2004). SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, while CAT converts hydrogen peroxide into water (Mansour and Mossa, 2009). The major function of GPx, which uses glutathion as a substrate, is to reduce soluble hydrogen peroxide and alkyl peroxides (Bebe and Panemangalore, 2003). These antioxidant enzymes can, therefore, alleviate the toxic effects of ROS (Mansour and Mossa, 2009). In this study, erythrocyte SOD, CAT and GPx activities were decreased in the DDVP-treated groups. The decrease in the activity of SOD in OP-treated groups may be attributed to the saturation of SOD during the process of converting O_2^- to H_2O_2. The CAT decrease may involve CAT saturation during the breakdown of free radicals and hydrogen peroxide (H_2O_2), or the inhibition of CAT by these radicals (Eraslan et al., 2007). The decrease in GPx activity may be result directly from the decreased levels of GSH following exposure, since the GPx enzyme depends on GSH for activity (Mahaboob and Kour, 2007). In this study, SOD, CAT and GPx activities significantly decreased in the at 10 μM and 100 μM DDVP-treated groups.

The widespread use of OP has long been shown to exert deleterious effects on living organisms. Oxidative damage to biomolecules is inhibited by antioxidants (Ames et al., 1993). A combination of vitamins E and C can reduce LPO caused by toxic substances (Gultekin et al., 2001). There are many studies demonstrating that VE protects cell membranes by preventing LPO. α-Tocopherol reacts with peroxyl radicals depending on its methylation state of the chromanol ring and the saturation grade of the side chain, forming tocopheroxyl radicals (Brigelius-Flohe, 2009). Tocopheroxyl radicals are converted to tocopherols by reacting with ascorbate (May et al., 1998). VE has been shown to play a role in various enzymes' activities by enabling their translocation to the membrane (Kempna et al., 2004) or affecting their transcriptional activation process (Khor and Ng, 2000). In many studies VE neutralizes LPO and unsaturated membrane lipids because of its oxygen scavenging effect (Aldana et al., 2001; John et al., 2001). Frei *et al.* (1989) have shown that VC is a powerful antioxidant preventing LPO in plasma exposed to various types of oxidative stress. VC is a significant water-soluble antioxidant in plasma which helps to reduce the effect of oxidative stress. As it is water soluble, it can easily react with free radical in extracellular body fluids (Bendich, 1990). VC exerts its antioxidant effects in both direct and indirect ways. In the direct way, VC scavenges the free radicals formed as a byproduct of metabolic reactions (Dawson et al., 1990). In an indirect way VC helps recycling of oxidized VE, thus supplying active VE fighting against LPO (Netke et al., 1997). In the present study, plasma level of VC and VE protected against DDVP-induced oxidative stress only in a 10 μM concentration of DDVP *in vitro*.

The results of the present experiment showed that DDVP induces oxidative stress in erythrocytes *in vitro* through the generation of free radicals and alteration of the cellular antioxidant defense system. Plasma levels of VC and VE protected cells against only certain doses of DDVP-induced oxidative stress.

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