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Full Length Research Paper

Mercury chloride-induced oxidative stress in human erythrocytes and the effect of vitamins C and E *in vitro*

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Mercury can exist in the environment as metal, as monovalent and divalent salts and as organomercurials, one of the most important of which is mercuric chloride ($HgCl_2$). It has been shown to induce oxidative stress in erythrocytes through the generation of free radicals and alteration of the cellular antioxidant defense system. The effect of simultaneous pretreatment with vitamins C and E on the toxicity of $HgCl_2$ in human erythrocytes was evaluated. We examined the effect of several different doses of $HgCl_2$ (1.052, 5.262, 10.524 µM), or $HgCl_2$ in combination with vitamin C (VC; 10 µM) and vitamin E (VE; 30 µM), on the levels of malondialdehyde (MDA) and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities in human erythrocytes *in vitro*. Erythrocytes were incubated under various treatment conditions ($HgCl_2$ alone, vitamins alone, or $HgCl_2$ plus vitamin) at 37°C for 60 min and the levels of MDA and SOD, CAT and GPx activities, were determined. Treatment with $HgCl_2$ alone increased the levels of MDA and decreased SOD, CAT and GPx activities in erythrocytes ($P < 0.05$). VC and VE-pretreated erythrocytes showed a significant protection aganist the cytotoxic effects induced by $HgCl_2$ on the studied parameters. There were no statistical differences among VC+VE-treated erythrocytes, as compared to non-treated control cells. These results indicated that the presence of vitamins at concentrations that are similar to the levels found in plasma could be able to ameliorate $HgCl_2$-induced oxidative stress by decreasing lipid peroxidation and altering antioxidant defense system in erythrocytes.

Key words: Mercury chloride, vitamins C and E, oxidative stress, erythrocytes, *in vitro.*

INTRODUCTION

Mercury a highly toxic metal, results in a variety of adverse health effects including neurological, renal, respiratory, immune, dermatological, reproductive and developmental sequela (Risher and Amler, 2005; Sharma et al., 2007). Mercury-induced oxidative stress; make an important contribution to molecular mechanism for liver injury (Farina et al., 2004; Sharma et al., 2007). Recent evidences also show that mercury causes severe oxidative damages (Kim and Sharma, 2005). Mercuric chloride ($HgCl_2$) is a widespread environmental and industrial pollutant, which induces severe alterations in the tissues of both animals and men (Lund et al.,1993; Mahboob et al., 2001). One of the harmful effects of mercury action during its accumulation in a body in a region contaminated by mercury is the excessive release of reactive oxygen species and increased lipid peroxidation in the cells (Lund et al., 1993). Free radicals and intermediate products of peroxidation are capable of damaging the integrity and altering the function of biomembranes, which can lead to the development of many pathological process (Gutteridge, 1993). Various spesicific enzymes that limit free-radical formation, such as superoxide dismutase (SOD), catalase (CAT) and glutation peroxidase (GPx), play an important role in the protection of cell membranes aganist oxidative damage

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(Faix et al., 2003).

The average daily intake of mercury from food is 6.71 µg and may be higher on diets comprising of fish from polluted water (WHO, 1990). Intake of metallic mercury vapour amounts to 3, 9 - 21 µg /day (WHO, 1990). Some irregularity in renal and thyroid function in occupational exposure has been observed at lower mercury (Ellingsen et al., 2000a; Ellingsen et al., 2000b). Bansal et al. (1992) reported the in vitro toxicity by lower concentrations of mercuric chloride on human erthrocytes in relation to their effect on lipid peroxidation and some enzymes which have a protective role in a such condition.

L-Ascorbic acid (VC) and α-tocopherol (VE) have been shown to possess anti-carcinogenic, anti-clastogenic and anti-mutagenic propoerties in a variety of in vivo and in vitro models of mercury exposure (Patil and Rao, 1999; Rao et al., 2001; Rao and Sharma, 2001). Mercury induces oxidative stress in erythrocytes in a dose-dependent manner through the generation of free radicals and alterations in the antioxidant defense system of cells (Bansal et al., 1992; Zabinsky et al., 2000) and these effects are attenuated by VE or VC (Patil and Rao, 1999; Rao and Sharma, 2001). The antioxidant activity of VE is targeted primarily towards the lipid component of cells (Kalender et al., 2006, 2007). Antioxidants such as VE have been shown to inhibit free radical formation (Kalender et al., 2004, 2005; Eraslan et al., 2007) and are effective in minimizing lipid peroxidation in several different biological systems (Aldana et al., 2001). VC is a natural antioxidant and prevents the increased production of free radicals induced by oxidative damage to lipids and lipoproteins in various cellular compartments and tissues (Sies et al., 1992). To date, several studies have examined the effects of VC and VE on pesticide and heavy metals toxicity in different experimental systems (Chorvatovičová, et al., 1991; Kalender et al., 2007; Uzunhisarcıklı et al., 2007; Durak et al., 2008).

In the present study, $HgCl_2$ and plasma-equivalent concentrations of VC and VE were examined for their effect, on MDA levels and the levels of activity of several antioxidant enzymes in vitro in human erythrocytes. The aim of the study was to determine the effect of $HgCl_2$ at several different doses, in combination with VC and VE on MDA levels and the activities of SOD, CAT and GPx in human erythrocytes.

MATERIALS AND METHODS

Chemicals

L-Ascorbic acid (VC) was supplied by Carlo Erba (Milano, Italy). Mercuric chloride ($HgCl_2$) and DL-α- tocopherol (VE) and all other chemicals were supplied by Merck (Germany).

Erythrocyte preparation

Twenty milliliters of fresh blood were collected in dry tubes from six healthy male volunteers by venipuncture. Heparin was used as an anticoagulant. Erythrocytes were separated from blood plasma by centrifugation (1600 rpm at 4°C for 5 min) and then washed three times with a cold isotonic saline solution (0.9% NaCl). The supernatant and the buffy coat were carefully removed after each wash. After separation, packed erythrocytes were suspended in phosphate buffer [170 ml of Na_2PO_4H (1.41 g/l) solution+77 ml of $NaPO_4H_2$ (1.19 g/l) solution+NaCl (8.8 g/l)], at pH 7.40 to obtain a 50% cellular suspension. Erythrocytes that were incubated in buffer without $HgCl_2$ were used as the non-treated control cells. The concentration of hemoglobin was determined using the method of Drabkin (1946).

Treatment of erythrocytes

A solution of $HgCl_2$ was prepared in distilled water (Rao et al., 2001). The doses of $HgCl_2$ that were used in this study [lower concentrations (D1) 1.052 µM; the medium concentration (D2) 5.262 µM and the higher concentration (D3) 10.524 µM] were selected based on the earlier studies (Verschaeve et al., 1984; Rao et al., 2001). VC were dissolved in distilled water (Konopacka et al., 1998). VE were dissolved in corn oil (Kalender et al., 2007). VC (10 µM) and VE (30 µM), in combination, were added to non-treated and $HgCl_2$-treated cultures of erythrocytes. The doses of VC and VE were chosen based on the levels of each vitamin in human plasma (Blasiak and Stańkowska, 2001; Durak et al., 2009).

Erythrocytes were divided into non-treated control and experimental groups. The control group was incubated for 1 h at 37°C in 0.9% NaCl. Erythrocytes in the experimental group were treated with 1.052, 5.262 or 10.524 µM $HgCl_2$ in the presence or absence of 10 µM VC+30 µM VE for 1 hr at 37 °C. After incubation, the cell mixtures were stored - 20°C for 24 h. The mixtures were thawed, the erythrocytes were destroyed by osmotic pressure and then subjected to centrifugation. Supernatants were isolated and MDA levels and the activities of SOD, CAT and GPx were measured by spectrophotometer (Shimadzu UV-1700, Japan).

Antioxidant enzyme assays

Measurement of SOD activity

SOD activity was measured as the inhibition of autoxidation of pyrogallol, according to the method of Marklund and Marklund (1974). Activity was monitored at 440 nm for 180 s (s). Data is expressed as U of SOD/mg hemoglobin.

Measurement of CAT activity

CAT activity was measured according to the method of Aebi (1984) as the rate constant of hydrogen peroxide (H_2O_2) decomposition. Activity was monitored at 240 nm for 60s. Data is expressed as U of CAT/mg hemoglobin.

Measurement of GPx activity

GPx activity was measured according to the method of Paglia and Valentine (1967). Reaction mixtures contained NADPH, reduced glutathione, Tris-HCl and glutathione reductase. Reactions were initiated by the addition of H_2O_2 and GPx activity was measured as the change in absorbance at 340 nm. Data is presented as UGPx/mg hemoglobin.

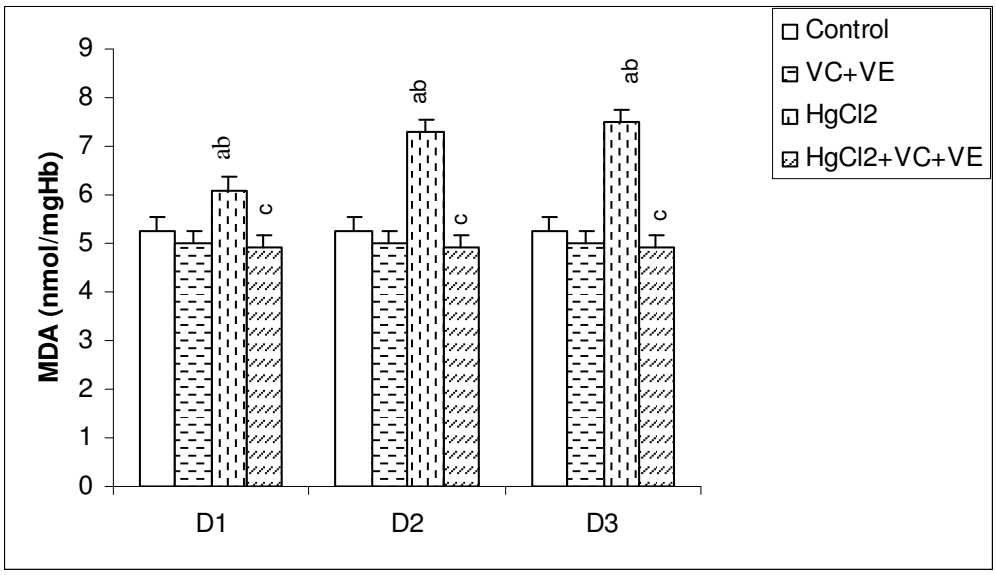

Figure 1. MDA levels in control and experimental groups of erythrocytes. [a]Comparison of non-treated control cells with other groups ($P < 0.05$), [b]Comparison of VC+VE-treated cells with $HgCl_2$- and $HgCl_2$+VC+VE-treated cells ($P < 0.05$). [c]Comparison of $HgCl_2$-treated cells with $HgCl_2$+VC+VE-treated cells ($P < 0.05$). Data represents the means ± SD of six samples.

Measurement of MDA levels

MDA is a secondary product of lipid peroxidation (LPO). Cells were incubated at 95 °C with thiobarbituric acid under aerobic conditions (pH 3.4) and MDA levels were monitored at 532 nm (Ohkawa et al., 1979). Specific activity is presented as nmol/mg hemoglobin.

Statistical analysis

Data was analyzed using the software program SPSS 11.0 for Windows. Differences were calculated using one way analysis of variance (ANOVA), followed by Tukey multiple comparison to calculate significance. A P value of < 0.05 was determined to be statistically significant. All data is expressed as the means ± standard deviation (S.D).

RESULTS

The mean age of the six male volunteers was 24 years (range 21-26 years). All volunteers were healthy, taking no medication, non-smokers and none were farm or agricultural workers. There were no statistical differences between VC+VE-treated cells, as compared to control cells (Figures 1 - 4). The protective effect of VC and VE on $HgCl_2$-induced markers of toxicity was more pronounced with the combination of VC+VE than with $HgCl_2$ alone.

MDA levels

MDA levels were higher in $HgCl_2$-treated erythrocytes than in non-treated and VC+VE treated cells at all concentrations of $HgCl_2$ examined (1.052, 5.262 or 10.524 µM) ($P < 0.05$) (Figure 1). The MDA levels in cells treated with these $HgCl_2$ plus VC+VE were statistically similar to non-treated cells, or cells treated with vitamins alone (VC+VE-treated cells). These results suggested that VC and VE have a protective effect on $HgCl_2$-induced changes in MDA levels in erythrocytes at these doses of $HgCl_2$.

SOD activity

SOD activity was lower in $HgCl_2$-treated erythrocytes than in non-treated and VC+VE treated cells ($P < 0.05$) (Figure 2). There were no differences in SOD activity between cells that were treated with these doses $HgCl_2$ plus VC+VE as compared to non-treated control cells, or cells that were treated with vitamins alone. These results indicated that VC and VE have a protective effect against $HgCl_2$-induced changes in SOD activity at three doses of $HgCl_2$.

CAT activity

CAT activity was lower in $HgCl_2$-treated cells than in non-treated and VC+VE-treated cells ($P < 0.05$) (Figure 3). The levels of CAT activity in cells treated with these concentrations $HgCl_2$ plus VC+VE were similar to nontreated cells, or cells that were treated with vitamins alone. Thus, VC and VE appeared to have a protective effect against $HgCl_2$-induced changes in CAT activity at three doses of

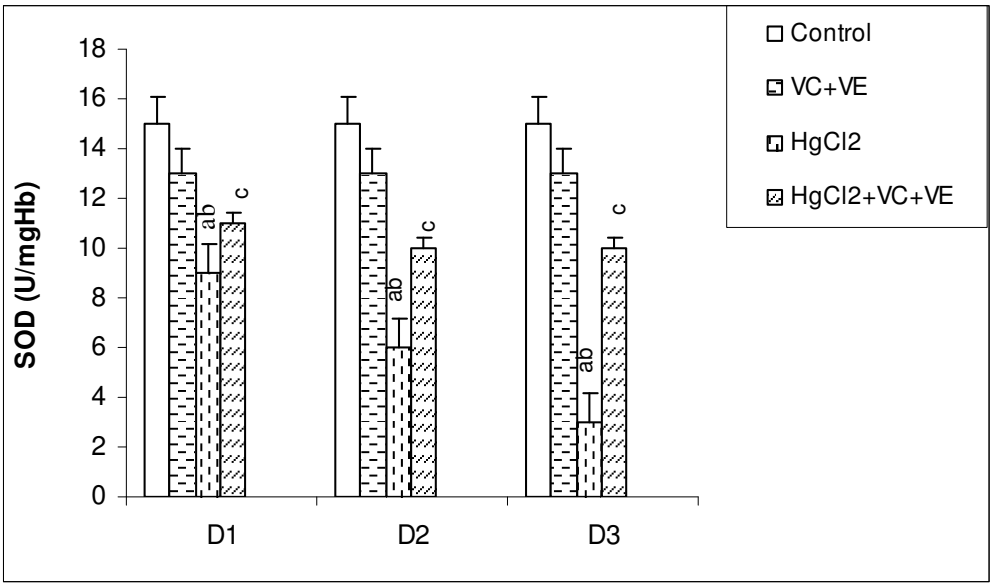

Figure 2. SOD activity in control and experimental groups of erythrocytes. [a]Comparison of non-treated control cells with other groups ($P < 0.05$), [b]Comparison of VC+VE-treated cells with $HgCl_2$- and $HgCl_2$+VC+VE-treated cells ($P < 0.05$). [c]Comparison of $HgCl_2$-treated cells with $HgCl_2$+VC+VE-treated cells ($P < 0.05$). Data represents the means ± SD of six samples.

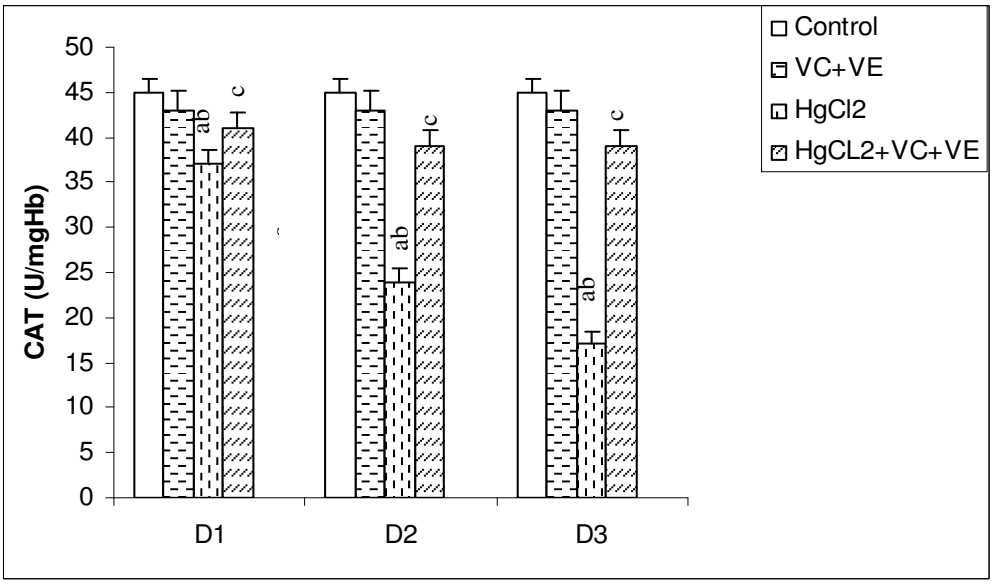

Figure 3. CAT activity in control and experimental groups of erythrocytes. [a]Comparison of non-treated control cells with other groups ($P < 0.05$), [b]Comparison of VC+VE-treated cells with $HgCl_2$- and $HgCl_2$+VC+VE-treated cells ($P < 0.05$). [c]Comparison of $HgCl_2$-treated cells with $HgCl_2$+VC+VE-treated cells ($P < 0.05$). Data represents the means ± SD of six samples.

$HgCl_2$.

GPx activity

GPx activity was lower in $HgCl_2$-treated cells than in non-treated and VC+VE-treated cells ($P < 0.05$) (Figure 4). There were no differences in GPx activity between cells that were treated with these concentrations plus VC+VE as compared to non-treated cells, or cells that were treated with vitamins alone. These results indicated that, similar to their effect on SOD and CAT activity, VC and

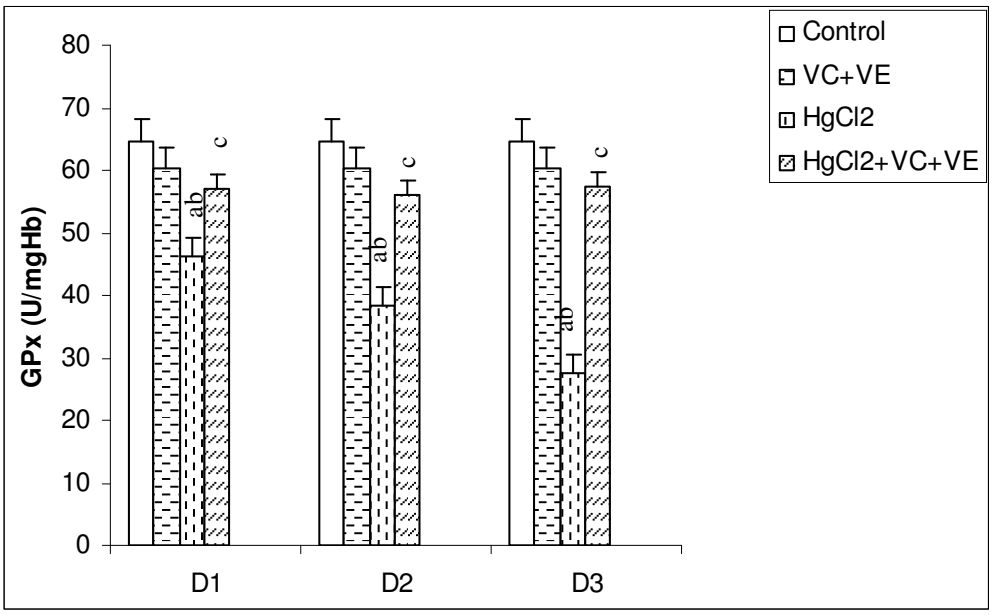

Figure 4. GPx activity in control and experimental groups of erythrocytes. [a]Comparison of non-treated control cells with other groups ($P < 0.05$), [b]Comparison of VC+VE-treated cells with $HgCl_2$- and $HgCl_2$+VC+VE-treated cells ($P < 0.05$). [c]Comparison of $HgCl_2$-treated cells with $HgCl_2$+VC+VE-treated cells ($P < 0.05$). Data represents the means ± SD of six samples.

VE have a protective effect on $HgCl_2$-induced changes in GPx activity at three doses of $HgCl_2$.

DISCUSSION

Normal erythrocyte function is mainly wholly dependent on an intact erythrocyte membrane. The toxic effect of many environmental chemicals and pesticides is largely due in large part to their effect on erythrocyte membranes (Schara et al., 2001; Brandão et al., 2005). Previously, Bansal et al. (1992) showed that $HgCl_2$ induce the production of reactive oxygen species (ROS) leading to oxidative tissue damage. Some works have reported that the activities of enzymes associated with antioxidant defense mechanisms are altered by $HgCl_2$ both *in vivo* and *in vitro* (Kobal et al., 2004; Park and Park, 2007). In this study, we demonstrated that $HgCl_2$ intoxication induces oxidative stress in erythrocytes *in vitro* through the generation of free radicals and alterations in the cellular antioxidant defense system.

Three concentrations (1.052, 5.262 or 10.524 µM) of $HgCl_2$ and durations of exposure were evaluated from various earlier studies and investigated the genotoxic effect of $HgCl_2$ at these concentrations (Verschaeve et al., 1985; Rao et al., 2001), which are comparatively higher concentrations as found in plasmatic concentrations from occupational, accidental or alimentary sources (WHO, 1991). *In vitro* studies on short-term and/or lower concentration that is, within µM range of mercury poisoning suggest that $HgCl_2$ increases m-RNA levels (Mondal et al., 1997), post-transcription elevation of protein (Kumagai et al., 1997), tyrosine phosphorylation of numerous cellular proteins (MacDougal et al., 1996) and uncoupled oxidative phosphorylation in mitochondria (Santos et al., 1997). Hence, $HgCl_2$ at lower concentration and short-term exposure induces cytotoxicity. The concentrations of $HgCl_2$ used in this study were in the range of 1.052-10.524 µM which corresponds to approximately 2 -20 µg/ml.

Various mechanisms have been proposed to explain the biological toxicity of $HgCl_2$, including oxidative stress. The precise mechanism for ROS production by mercury is not known. Hg^{+2} reacts with thiol groups (-SH), thus depleting intracellular thiols, especially glutathione and causing cellular oxidative stress or predisposing cells to it (Gstraunthaler et al., 1983) and forming free radicals which may further increase lipid peroxidation. Molecular damage of the cells in mercury toxicity is by the formation of peroxyl radicals which can also be formed in lipid and non-lipid systems such as proteins (Dean et al., 1993). The scavenger role of antioxygenic enzymes in removing toxic electrophiles helps the cell to maintain its internal enviroment to a limited extent at lower concentration of mercury. An increase in the oxidative stress may be due to a decrease in the antioxidant defences or due to an increase in the proceses that produce oxidants (Hussain et al., 1999; Lukaszewicz-Hussain and Moniuszko-Jakoniuk, 2003). In this study, $HgCl_2$ initiate lipid peroxidation by generating free radicals and thereby interferring with the antioxidant system of the cell. This toxicity may be due to mercury-induced alterations in membrane integrity

via the formation of reactive oxygen species and the perturbation of antioxidant defense mechanisms.

VC (L-ascorbic acid) and VE (α-tocopherol) have a protective effect aganist mercury toxicity. Vitamin C is a strong antioxidant (Rao, 1997) having nucleophilic properties and binds to mercury ions (Hg^{+2}) to reduce mercury-induced damage. It further manifests its detoxification effect by removing or minimizing free radicals produced by mercury (Herbaczynska et al., 1995). Co-treatment with $HgCl_2$ and vitamin E was associated with a control rate of some disease, perhaps due to protection by vitamin E from oxidative tissue damage induced by mercury ion (Basu and Dickerson, 1996; Jha et al., 1995). Vitamin E prevents lipid peroxidation and maintains antioxidant enzyme activity and ascorbic acid levels in damaged tissue by inhibiting free radicals formation (Duval and Poelman, 1994). VC and VE inhibits oxidative damage in the liver and other tissues caused by mercury intoxication (Patil and Rao, 1999; Fukino et al., 1984). Depletion of reduced glutathione, ascorbate and α-tocopherol on short term and long-term exposures were due to binding of mercury, even at low concentrations to the cellular antioxidant components (Anderson et al., 1990). In this study, at these concentration of $HgCl_2$, the vitamins VC and VE protected against $HgCl_2$-induced oxidative stress. These vitamins incline the enzymatic antioxidants levels to the control levels by effectively disposing the free radicals and possibly the mercury from the endogenous -SH groups. At concentrations that were similar to those found in plasma, VC and VE treatment, in combination, had no effect on MDA levels, or the levels of SOD, CAT and GPx activities in erythrocytes as compared to non-treated cells.

MDA is one of the major oxidation products of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of LPO. It has been shown previously that $HgCl_2$ increase MDA level in tissues (Mahboob et al., 2001; Augusti et al., 2008). In this study, MDA levels were increased in $HgCl_2$-treated erythrocytes, which suggests that MDA levels could be used as a marker of $HgCl_2$ injury. Pretreatment of cells with a combination of VC and VE at concentrations that were similar to the levels found in plasma, attenuated the $HgCl_2$-induced increase in MDA levels, which indicates that these vitamins may have a beneficial effect in reducing $HgCl_2$ toxicity. Our results are particularly significant in light of previous results showing that a dietary deficiency in VE and VC results in the peroxidation of subcellular fractions of various organs (Burton et al., 1983; Kalender et al., 2007), whereas dietary supplementation of VE and VC confers protection against increased MDA levels (Uzunhisarcıklı et al., 2007; Durak et al., 2008).

Under physiological conditions, intracellular antioxidant enzymes, such as SOD, CAT and GPx, eliminate ROS, thereby playing an integral role in the anti-oxidative stress defenses of the cell (Bukowska, 2004). We found that when erythrocytes were pretreated with vitamins, $HgCl_2$-induced decreases in the levels of SOD, CAT and GPx activities were prevented. The response was concentration dependent. In view of the parameters examined, the administration of the three compounds at the indicated doses and for the indicated period were considered to alleviate the adverse effects of $HgCl_2$. The mechanism by which plasma level of vitamins increased the levels of SOD, CAT and GPx activities in $HgCl_2$-treated cells most likely involved the dismutation of superoxide anions ($O_2^{\cdot-}$) and the decomposition of H_2O_2 and may represent an aspect of the cellular response to increased levels of ROS induced by $HgCl_2$ toxicity.

In conclusion, treatment of human erythrocytes with $HgCl_2$ increased the levels of MDA in these cells and pretreatment with combination of VC+VE lowered LPO in $HgCl_2$ intoxicated blood cells. Erythrocyte SOD, CAT and GPx activities were decreased in $HgCl_2$-treated erythrocytes and this effect was prevented by pretreatment with combination of VC and VE. Our results indicate that at these levels of $HgCl_2$, the levels of ascorbic acid and α-tocopherol that are present in plasma prevent $HgCl_2$ induced-oxidative stress in vitro, most likely due to their nucleophilic and detoxifying properties and that the combination of VC and VE has a synergistic effect. Thus, dietary supplementation of VC and VE might be useful in populations that are occupationally exposed to $HgCl_2$ poisoning.

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