The Effects of Cadmium on Enzymatic Antioxidant System and Lipid Peroxidation of Human Erythrocytes *in vitro* and the Protective Role of Plasma Level of Vitamins C and E

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Abstract

In our study, the effect of simultaneous pretreatment with plasma levels of vitamins C and E on the toxicity of $CdCl_2$ in human erythrocytes was evaluated. We aimed to examine how several different doses of $CdCl_2$ or $CdCl_2$, in combination with vitamins, affects lipid peroxidation and the antioxidant defense system *in vitro*. Levels of malondialdehyde and the activities of antioxidant enzymes changed in $CdCl_2$-treated groups in different concentrations. Vitamin-pretreated erythrocytes showed significant protection aganist the cytotoxic effects induced by low and moderate $CdCl_2$ concentrations on the studied parameters.

Keywords: antioxidant enzymes, cadmium, vitamins C and E, erythrocytes, *in vitro*

Introduction

The heavy metal cadmium (Cd) is used for industrial purposes throughout the world, and has been discharged into the environment in large quantities [1-3]. Cd has great environmental health effects and all Cd compounds have been classified as human carcinogens [4, 5]. It can induce cell death either by necrotic or by apoptotic mechanisms [6, 7].

Cd is a contaminant that may enter the food chain from a number of natural and industrial sources. Cd was last reviewed in 1972 [8] when a provisional tolerable weekly intake of 400-500 µg per person (6.7- 8.3 µg/kg bw/week) was allocated. Results from more recent surveys demonstrated that most foods contain, on average, less than 0.02 µg/g cadmium. Dietary intake estimates range from 13-35 µg/day or 0.2 to 0.7 µg/kg bw for an adult. For infants and children, cadmium intake on a body-weight basis is generally higher than that estimated for adults and, in some countries, for the younger age groups have been reported to exceed 1 µg/kg bw [9, 10].

Cd is absorbed into blood circulation through metal transporters, the hematological system being one of the major targets of Cd^{2+} toxicity. It has been reported that Cd pollutant can induce anemia, alteration of antioxidant and metabolic status of erythrocytes, increase lipid peroxidation (LPO), decrease hemoglobin content, and alter the membrane skeleton [11]. Although the toxicity of this metal has been well established *in vivo* [12] and *in vitro*, the mechanisms involved in membrane physiology remain unclear [6].

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 processes [13, 14]. Malondialdehyde (MDA) is a marker of membrane LPO resulting from the interaction of reactive oxygen species (ROS) and the cellular membrane [15]. Various specific enzymes that limit free-radical formation, such as superoxide dismutase

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(SOD), catalase (CAT), and glutathione peroxidase (GPx), play an important role in the protection of cell membranes against oxidative damage [16].

Nonenzymatic antioxidants such as vitamin E (VE), vitamin C (VC), and melatonin can also act to overcome oxidative stress, being a part of the total antioxidant system. VE is an important biological free radical scavenger in the cell membrane [17]. This has been shown to povide protection against superoxides as well as H_2O_2 [18]. It has also been shown that VC, a watersoluble vitamin and known antioxidant, can react with VE radicals to regenerate VE [19]. To date, several studies have examined the effects of VC and VE on pesticide and heavy metal toxicity in different experimental systems [20-22]. But the protective effect of plasma levels of these vitamins in erythrocytes *in vitro* have been examined in a few studies [20, 23].

We aimed to determine the effects of various doses of $CdCl_2$ on lipid peroxides and the activities of SOD, CAT, and GPx in erythrocytes and the protective role of plasma-equivalent concentrations of VC and VE *in vitro*.

Materials and Methods

Chemicals

VC (L-ascorbic acid) was supplied by Carlo Erba (Milano, Italy), cadmium ($CdCl_2$) was supplied by Sigma Aldrich (Germany), and VE (DL-α-tocopherol) 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 centrifuge 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 to obtain a 50% cellular suspension. Erythrocytes that were incubated in buffer without $CdCl_2$ were used as the non-treated control cells. The concentration of hemoglobin was determined using the method of Drabkin [24].

Treatment of Erythrocytes

A solution of $CdCl_2$ was prepared in distilled water [25]. The doses of $CdCl_2$ that were used in this study (lower concentrations of 1 μM; a moderate concentration of 50 μM, and a higher concentration of 150 μM) were selected based on the earlier studies [26, 27]. VC were dissolved in distilled water [28]. VE were dissolved in corn oil [21]. VC (10 μM) and VE (30 μM), in combination, were added to non-treated and $CdCl_2$-treated cultures of erythrocytes. The doses of VC and VE were chosen based on the levels of each vitamin in human plasma [20, 29].

To determine the effects of various doses of $CdCl_2$ on the antioxidant enzymes SOD, CAT, GPx activities and MDA levels, erythrocytes were divided into non-treated control and experimental groups. Plasma was separated. Erythrocyte packets were prepared by washing the erythrocytes three times with cold isotonic saline. And the control group was incubated for 1 hr at 37ºC in 0.9% NaCl. Erythrocytes in the experimental group were treated with 1, 50, or 150 μM $CdCl_2$ in the presence or absence of 10 μM VC + 30 μM VE for 1 hr at 37ºC. After incubation, the cell mixtures were stored at -20ºC for 24 hr. The mixtures were thawed, the erythrocytes were destroyed by osmotic pressure, and then subjected to centrifuge. Supernatants were isolated, and MDA levels, plus the activities of SOD, CAT, and GPx were measured by spectrophotometer (Shimadzu UV – 1800, Japan).

Antioxidant Enzyme Assays

SOD activity was measured as the inhibition of autoxidation of pyrogallol, according to the method of Marklund and Marklund [30]. Activity was monitored at 440 nm for 180 seconds (s). Data is expressed as USOD/mg hemoglobin. CAT activity was measured according to the method of Aebi [31] as the rate constant of hydrogen peroxide (H_2O_2) decomposition. Activity was monitored at 240 nm. Data is expressed as UCAT/mg hemoglobin. GPx activity was measured according to the method of Paglia and Valentine [32]. 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. MDA is a secondary product of LPO. Cells were incubated at 95ºC with thiobarbituric acid under aerobic conditions, and MDA levels were monitored at 532 nm [33]. Specific activity is presented as nmol/mg hemoglobin.

Statistical Analysis

Data was analyzed using SPSS 11.0 for Windows. Differences were calculated using oneway 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 (SD).

Results

Blood was collected in heparinized vials from male volunteers. The mean age was 25 years (range 23-29 years). All of them were healthy and taking no medication; none of them was a farmer or agricultural worker. There were no statistical differences between VC+VE treated cells, as compared to control cells (Figs. 1-4). The protective effect of VC and VE on $CdCl_2$ induced markers of toxicity was more pronounced with the combination of VC+VE than with $CdCl_2$ alone.

The MDA production in erythrocytes treated with $CdCl_2$ showed significantly high LPO for different increasing doses (1, 50 or 150 μM) as compared to untreated erythrocytes and VC+VE treated cells (P< 0.05) (Fig. 1). The MDA levels in cells treated with low and moderate concentrations of $CdCl_2$ plus VC+VE were statistically similar to nontreated cells, or cells treated with vitamins alone (VC+VE treated cells), but not similiarity at high concentrations of $CdCl_2$.

The erythrocyte CAT activity decreased significantly in moderate and high doses of $CdCl_2$-treated cells as compared to untreated cells and VC+VE-treated cells (P< 0.05) (Fig. 2). The treatment of low concentration of $CdCl_2$-exposed erythrocytes has no significant effect on CAT activity compared to untreated cells and VC+VE-treated cells. Thus, VC and VE appeared to have a protective effect against $CdCl_2$-induced changes in CAT activity at only medium doses of $CdCl_2$, but a protective effect was not seen at high concentration. The protective effect of VC and VE wasn't significant at low concentrations.

The erythrocytes SOD and GPx activities decreased significantly in all doses of $CdCl_2$-treated cells as compared to untreated cells and VC+VE-treated cells (P< 0.05) (Figs. 3 and 4). The levels of SOD and GPx activity in cells treated with low and moderate concentrations of $CdCl_2$ plus plasma level of 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 at only two doses of $CdCl_2$-induced changes in SOD and GPx activities, but no protection at high concentrations.

Discussion of Results

Erythrocytes have an efficient antioxidant mechanism to scavenge ROS and maintain integrity. This detoxifying system includes enzymatic (including SOD, CAT, and GPx) and nonenzymatic antioxidant systems. Many studies have investigated the effects of Cd on antioxidant systems and LPO [34, 35], but only a few studies have investigated the combined effects of $CdCl_2$ and plasma level of

vitamins C and E on LPO and antioxidant systems. In this study, we investigated the effects of plasma level of vitamins C and E and three-dose Cd administration in human erythrocytes on LPO levels and antioxidative enzymes *in vitro*.
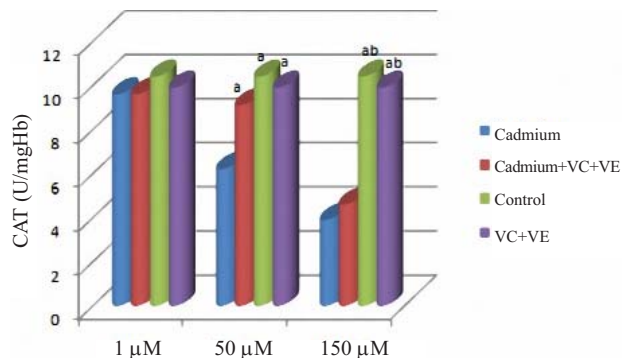

Fig. 2. CAT activity in control and experimental groups of erythrocytes. a – Comparison of Cadmium-treated cells with other groups (P<0.05), b – Comparison of Cadmium+VC+VE-treated cells with control-, and VC+VE-treated cells (P<0.05). Data represents the means±SD of six samples.
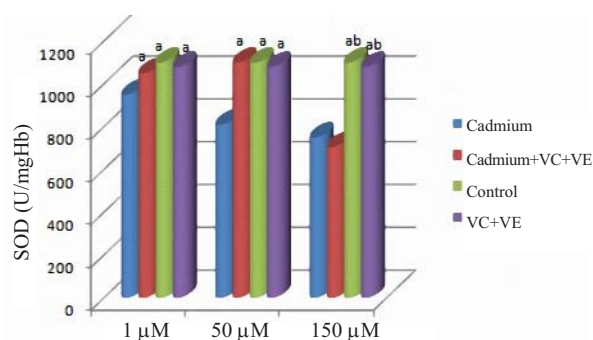

Fig. 3. SOD activity in control and experimental groups of erythrocytes. a – Comparison of Cadmium-treated cells with other groups (P<0.05), b – Comparison of Cadmium+VC+VE-treated cells with control-, and VC+VE-treated cells (P<0.05). Data represents the means±SD of six samples.
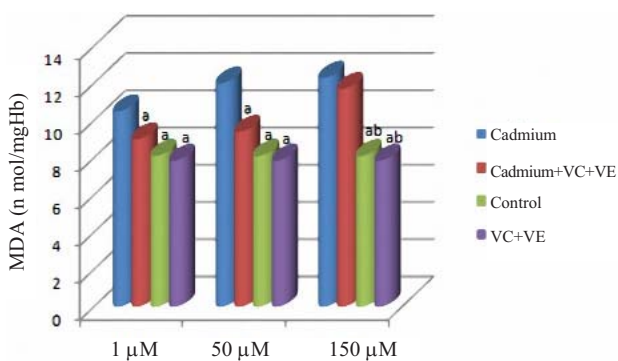

Fig. 1. MDA levels in control and experimental groups of erythrocytes. a – Comparison of Cadmium-treated cells with other groups (P<0.05), b – Comparison of Cadmium+VC+VE-treated cells with control-, and VC+VE-treated cells (P<0.05). Data represents the means±SD of six samples.
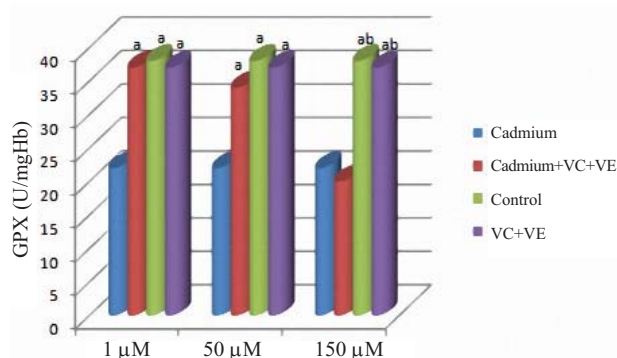

Fig. 4. GPx activity in control and experimental groups of erythrocytes. a – Comparison of Cadmium-treated cells with other groups (P<0.05), b – Comparison of Cadmium+VC+VE-treated cells with control-, and VC+VE-treated cells (P<0.05). Data represents the means±SD of six samples.

CdCl$_2$ is known to induce LPO, but its effect on antioxidant enzymes is controversial. It has been reported that Cd may induce oxidative damage in a variety of tissues by enhancing peroxidation of membrane lipids due to inhibition of the antioxidant enzymes [36]. Other authors have noted that Cd exposure might lead to LPO, causing an increase in antioxidant enzyme activities [37]. LPO is considered the primary mechanism for Cd toxicity, despite its inability to directly generate free radicals under physiological conditions [38]. This causes oxidative stress through the Fenton reaction, producing hydroxyl radical species that are believed to initiate LPO [39]. Decomposition products of lipid hydroperoxide such as MDA and 4-hydroxynonenal can cause chaotic cross-linkage with proteins and nucleic acids, which plays an important role in the process of carcinogenesis. ROS may lead to cellular damage when the rate of its generation surpasses the rate of its decomposition by antioxidant defence systems, such as the enzymes SOD, CAT, or reduced GSH [40-42]. MDA level and CAT, GPx, and SOD activities were used as markers of oxidative stress in human erythrocytes *in vitro*. Significantly higher lipid peroxidation and lower activities of CAT, GPx, and SOD in human erythrocytes were observed with the increasing concentrations of CdCl$_2$ treatment. In our study, genotoxically effective doses of CdCl$_2$ were selected [26].

SOD detoxifies superoxide radicals and thus provides cytoprotection against free-radical-induced damage. Reports about SOD activity in Cd-treated rats are contradictory; some studies report an increase [43, 44] and some others report a decrease [45, 46] in activity. The alterations in SOD activity may depend on several factors such as Cd dose, Cd exposure time, and type of Cd administration. In this study, a significant reduction in SOD activity of erythrocytes was observed in all concentrations of the CdCl$_2$ group compared to the control group and VC+VE. Plasma level of vitamins is not protective aganist CdCl$_2$ in high concentrations. CAT activity has not changed in low concentrations while its activity decreased in moderate and high concentrations. So, in this low concentration, GPx may play a major role in H$_2$O$_2$ detoxification.

VC is considered the most important antioxidant of plasma and also acts as an anti-stress factor [47]. It scavenges the free radicals produced by Cd [48]. VE, the lipid soluble chain-breaking antioxidant, also plays a critical role in detoxifying Cd toxicity [48, 49]. VE prevents LPO and maintains antioxidant enzyme activity and ascorbic acid levels in damaged tissue by inhibiting free radicals formation [17]. While both VC and VE serve as scavengers of free radical ions, VC exerts its effects primarily in the extra cellular space, whereas VE exerts its effects within cells, where reactive metabolites are produced. In addition, it has been suggested that VC removes free radicals that are bound to VE, thus serving to regenerate VE [50, 51]. In this study enzymatic activities of erythrocytes that exposed CdCl$_2$ increased markedly when treatments with vitamins C and E were applied. These antioxidant enzyme activities of human erythrocytes have not combated Cd toxicity, giving the doses.

The molecular mechanism responsible for the toxic effect of Cd is not well understood. Various studies connect Cd with oxidative stress, since this metal can alter the antioxidant defence system in several animal tissues. This causes depletion in the levels of reduced glutathione, as well as an alteration in the activity of antioxidant enzymes, and a change in the structure of the cellular membrane through a process of LPO [52]. As a consequence, enhanced LPO, DNA damage, altered calcium, and sulfhydryl homeostasis, as well as marked disturbances of the antioxidant defence system, occurred [40, 53, 54]. Also, in this study, Cd induces the formation of superoxide anion radicals in erythrocytes and this may increase free-radical-mediated LPO and decreased the enzymatic antioxidant system. With vitamins C and E administration during exposure to Cd, only partial corrective effects on Cd-induced oxidative stress in the erythrocytes have been observed, while vitamins C and E together assured more efficient protection against the observed oxidative stress. In agreement, Durak et al. [23] have reported that simultaneous treatment with vitamins C and E may prevent HgCl$_2$-induced oxidative impairment in the erythrocytes.

The World Health Organization has fixed the provisional tolerable weekly intake of Cd to 7 mg/kg of Cd per person [55]. Furthermore, the carcinogenicity of Cd was evaluated by IARC [56] and recently reviewed by Waalkes [57]. However, results are often contradictory and generally obtained from experiments performed for short time periods with high Cd concentrations on a single cell [58, 59]. Depault et al., [26] investigated and confirmed the genotoxic effect of CdCl$_2$ at different concentrations (0-150 μM). Three low concentrations (1, 50 and 150 μM) of CdCl$_2$ evaluated MDA production and enzyme activities at different time periods in human erythrocytes *in vitro* [34]. The concentrations of CdCl$_2$ used in this study were in the range of 1-150 μM, which corresponds to approximately 0.2-32.9 μg/ml.

In conclusion, the combined treatment of low and moderate CdCl$_2$-exposed human erythrocytes with plasma level of vitamins C and E were more effective than high concentrations in reversing Cd-induced decreases in SOD, CAT, and GPx activities to the control values. This treatment also partially restored Cd-induced depletion of GSH. For CAT activity no statistically significant changes were observed at the lowest concentrations, but the moderate and highest Cd concentrations produced a decrease of CAT acivity.

CAT activity of erythrocytes' antioxidant defense system of the human have not significantly changed alone or with plasma level of vitamins C and E in low concentration of CdCl$_2$. It suggested that in this concentration antioxidant defense systems overcome Cd toxicity.

Acknowledgements

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