Effect of Ascorbic Acid and Thiamine Supplementation at Different Concentrations on Lead Toxicity in Liver

CHUNHONG WANG1*, JIANCHENG LIANG1, CHUNLIAN ZHANG1, YONGYI BI1, XIANGLIN SHI2 and QUN SHI1

1Department of Toxicology, School of Public Health, Wuhan University, DongHu Road 115, Wuhan 430071, People’s Republic of China; 2Institute for Nutritional Sciences, SIBS, CAS, Shanghai 20032, People’s Republic of China

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Objective: To investigate the effect of ascorbic acid [vitamin C (VC)] on liver damage parameters in the lead-exposed mice, when given in combination with thiamine [vitamin B1 (VB1)] at different concentrations.

Methods: Sixty-six male mice were randomly assigned into 11 groups (n = 6). Mice in Group I were supplied with only the tap water as the drinking water; mice in Group II were provided with a tap water containing 0.2% lead acetate; mice in Group III–XI were given different dose of VC (140, 420, 1260 mg kg⁻¹ bw) and VB1 (10, 30, 90 mg kg⁻¹ bw) according to 3 × 3 factorial design by oral gavages, along with ingestion of 0.2% lead acetate. After 42 test days, DNA damage of liver cells was assessed using single-cell gel electrophoresis. The apoptosis rate of liver cells was determined by flow cytometry. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in blood and the level of reduced glutathione (GSH) in liver cells were measured based on individual biochemical reactions.

Results: Compared with the Group I, sub-chronic lead ingestion (Group II) resulted in a significant decrease of Hb, GSH-PX, SOD in blood and GSH level in liver cells; lead exposure induced also a significant increase in DNA damage and apoptosis of liver cells (P < 0.05). Supplementation with VC and VB1, however, reversed these effects. The best effective combination was VC (420 mg kg⁻¹ bw) and VB1 (10 mg kg⁻¹ bw), followed by the combination of VC (420 mg kg⁻¹ bw) and VB1 (30 mg kg⁻¹ bw). But no reversion was shown in the combination of the highest combination of VC (1260 mg kg⁻¹) and VB1 (90 mg kg⁻¹).

Conclusions: These findings strongly indicated that combination of VC and VB1 can lessen the damage to liver cells from oxidative stress induce by lead, but the antioxidant effects are dependent on their concentrations.

Keywords: ascorbic acid; DNA damage; lead; liver; thiamine

INTRODUCTION

Lead-induced oxidative stress in blood and other soft tissues has been postulated to be one of the possible mechanisms of lead-induced toxic effects (Pande et al., 2001). Disruption of pro-oxidant/antioxidant balance might lead to the tissue injury. It was reported that lead increased the level of lipid peroxidation (Upasani et al., 2001) and brain thiobarbituric acid-reactive substances and altered the antioxidant defense system (Adanaylo and Oteiza, 1999). Similar effects were also reported in the hepatic tissues (Sandhir and Gill, 1995). A number of recent studies confirmed the possible involvement of reactive oxygen species (ROS) in lead-induced toxicity (Gurer and Ercal, 2000).

Several antioxidant enzymes and molecules have been used to evaluate lead-induced oxidative damage in animal and human studies. Reduced glutathione (GSH) and glutathione disulfide (GSSG) concentrations, as well as modifications in superoxide dismutase (SOD) activity are the most frequently used markers in tissues or in blood.

*Author to whom correspondence should be addressed.
Tel/fax: +86-27-68758648;
e-mail:chwang027@yahoo.com.cn
Based on the observation that free radical was generated during the pathogenesis processes induced by lead exposure, it was presumed that supplementation of antioxidants could be an alternative method for chelation therapy (Flora et al., 2003). Specifically, ascorbic acid, the known chelating agent with antioxidant features, was widely reported with the capability of protecting cells from oxidative stress (Ramanathan et al., 2002; Patra and Swarup, 2004). Besides, additional protective effect of vitamin C (VC) on cell apoptosis was revealed in a recent study (Gruss-Fischer and Fabian, 2002).

Thiamine, the endogenous –SH containing molecule, was recognized as one of the protective agents for lead exposure (Ghazaly, 1991). Additional investigations have confirmed that the simultaneous administration of vitamin B1 (VB1) and VC is more efficient in protecting or treating the experimental lead intoxication than either of them individually (Flora and Tandon, 1986; Dhawan et al., 1988).

Although the investigations about prevention of lead toxicity utilizing VC and VB1 are fairly well defined and previously been considered, reports on the impacts of simultaneous administration with VC and VB1 at different concentrations on lead exposure are novel. Moreover, some authors have raised questions pertaining to the potential risk for side effects of with intake of large doses of vitamins such as VC or tocopherol (Aiguo, 2001). In fact, some people in China are intaking vitamin supplement largely. These also deserve deeper investigations. It was presumed that these vitamins might act as pro-oxidant for the introduction of apoptotic cells in the presence of oxygen and the generation of H2O2. This study was therefore conducted to investigate whether the combinations of VC and VB1 at various concentrations are able to protect the target organs (liver) from lead-associated oxidative stress.

MATERIALS AND METHODS

Animals and experimental design

Animal used were Kunming albino male mice (n = 66) at the initial age of 5–6 weeks, weighting 15 ± 4 g. Animals were purchased from the Laboratory Animal Center of Wuhan University, China. Animals were accommodated in polypropylene cages in the laboratory animal house, with a 12-h dark/light cycles, at the temperature of 20–25°C and relative humidity of 30–70%. Mice were provided with the standard rodent feeds procured from the Laboratory Animal Center of Wuhan University. Water was supplied ad lib.

After 7 days acclimatization period, mice were randomly assigned into 11 groups (n = 6). Mice in Group I served as negative control and received tap water alone; mice in Group II served as positive control and received 0.2% lead acetate in drinking water; mice in Group III–XI were given different doses of VC (140, 420, 1260 mg kg−1 bw) and VB1 (10, 30, 90 mg kg−1 bw) according to 3 × 3 factorial design by oral gavage daily, along with intaking of the 0.2% lead acetate as shown in Table 1. All experimental animals were under the described treatments for continuous 42 days. For each mice, the average water consumption was 5.6 ml day−1, so total lead intake was about 470.4 mg during experiment.

At the end of treatment, blood samples were collected in heparinized vials and the liver was removed and kept in a container with the ice jacket for following analysis.

Biochemical analysis

**Activity of SOD and GSH-Px in blood.** The SOD activity in blood was determined using the method modified from the colorimetric NADH–phenazine-methosulphate–nitroblue tetrazolium formazan inhibition reaction assay and was expressed in U g−1 of hemoglobin (Kakkar et al., 1984).

The glutathione peroxidase (GSH-Px) activity in blood erythrocyte hemolysates was measured using the coupled method described by Paglia and Valentine (Paglia and Valentine, 1967; Hatanaka et al., 2000), with the t-butylhydroperoxide as the substrate. The enzyme activity was expressed in international units per gram of hemoglobin.

**Assay of GSH content in liver.** About 0.2 g liver was trimmed in ice-cold saline (0.85% w/v NaCl) and gently homogenized in the ice-cold 1.17% KCl of the 0.10 mol l−1 phosphate buffer (pH 7.4) at a ratio of 10% (w/v), using a glass Potter-type homogenizer at 500–800 rpm. The homogenates were filtered through a muslin cloth and were centrifuged at 20,000 g for 30 min at 4°C to obtain the supernatants. The GSH was measured in deproteinized supernatant fractions from liver homogenates, employing 0.04% 5,5′-dithiobis-(2-nitrobenzoic acid) in 10%

<table>
<thead>
<tr>
<th>Group</th>
<th>Lead acetate (%)</th>
<th>VC (mg kg−1 bw)</th>
<th>VB1 (mg kg−1 bw)</th>
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<tbody>
<tr>
<td>I</td>
<td>0</td>
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<tr>
<td>II</td>
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<tr>
<td>III</td>
<td>0.2</td>
<td>140</td>
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<tr>
<td>IV</td>
<td>0.2</td>
<td>140</td>
<td>30</td>
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<tr>
<td>V</td>
<td>0.2</td>
<td>140</td>
<td>90</td>
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<tr>
<td>VI</td>
<td>0.2</td>
<td>420</td>
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<tr>
<td>VII</td>
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<td>VIII</td>
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<tr>
<td>IX</td>
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<td>1260</td>
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<td>X</td>
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<td>XI</td>
<td>0.2</td>
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sodium citrate and recording absorption at 412 nm on a spectrophotometer (Meng and Bai, 2004).

Assessment of DNA damage

The single-cell gel electrophoresis assay (SCGE) or comet assay was used for the detection of single-strand DNA breaks and reparation in individual cells. The method was modified from that reported by Wang et al., (2006) as follows.

Preparation of liver cell suspensions. A liver specimen was cut into pieces with the addition of a phosphate-buffered saline (PBS) solution, followed by a cleaning through a filter. The cellular suspension was then centrifuged at 1000 rpm for 10 min; the supernatant was discarded and the cell pellets were resuspended in an EP tube. The concentration of suspension was adjusted to 10^6–10^7 cells ml^-1 for each sample.

SCGE assay. Slides. Slides were cleaned with acid wash and spread with 40 μl of 0.6% agarose, then stood for ~5 min at room temperature for the gel to set. Onto the first gel layer, a mix of a 20-μl live cell suspension and a 80-μl 1.1% low-melting agarose was laid. The slide was then immersed in a cold lysis buffer (2.5 mol l^-1 NaCl, 100 mmol l^-1 Na2EDTA, 10 mmol l^-1 Tris, pH 10, 1% Triton X-100 and 10% dimethyl sulfoxide) for at least 1 h at 4°C. At completion of those procedures, slides were arranged on the electrophoresis chamber and fresh prepared electrophoresis buffer (1 mmol l^-1 Na2EDTA and 300 mmol l^-1 NaOH, pH 12) was added to the chamber. After rinsing the slides with PBS for ~20 min, electrophoresis was performed at 25V, 300 mA for 30 min. All procedures were performed in the absence of strong light. At the completion of the electrophoresis, slides were neutralized by washing with a 0.4-mol l^-1 Tris–HCl buffer (pH 7.5) for three times, 5 min each. Slides were then covered with 40 μl of 0.1 mg ml^-1 propidium iodide (PI) for the DNA dyeing. Using a fluorescence microscope (Olympus, Japan) configured with the wavelengths of excitation (510–560 nm) and filtration (690 nm), 100 cells from the randomly selected filed were counted and the photograph was taken in each slide. The images were scored for each sample using an image analysis software system (IMI 1.0). Parameters for assessing DNA single-strand breaks included the percentage of cells with tail and tail length by visual estimation.

Apoptosis analysis

Hepatic cells apoptosis were determined with the appearance of the DNA fragments in the apoptotic cells using flow cytometry (FCM). The method used for the detection of the DNA condensation and fragmentation was modified from the subdiploid DNA peak assay described by Iavicoli et al., (2001). Genetic materials can be bound by the DNA binding fluorochrome, such as PI and ethidium bromide. Vi-able cells resist the intake of the fluorochromes due to the integration of the cell membrane. Cells stained with these fluorescence indicate the occurrences of the late-stage apoptosis or the appearances of necrosis. On the DNA histogram figures acquired from the FCM, the diplod DNA peak (G1 cells) depressed in apoptotic cells and the subdiploid DNA peak (SD peak) appeared ahead of the G1 peak. The appearance of an SD peak represents cells apoptosis. Necrosis cells do not have SD peak, but it exhibits consecutive small DNA fragment curves.

Subdiploid DNA peak assay: (i) Aliquot 1 × 10^6 cells in suspension and centrifuge at 500 rpm for 5 min. Discard the supernatant; (ii) Fix cells in 70% alcohol (−20°C) for 2 ~ 24 h until analysis; (iii) Add PI to a final concentration of 20 μg ml^-1. Mix well, and let stand at 4°C for 30 min in order to allow the dye to equilibrate; (iv) Analyze with a flow cytometer with an argon–ion laser tuned to 488 nm. Record the light emitted into the red fluorescence photomultiplier (PMT) using a 620-nm long pass filter in front of the PMT and (v) Analyze the percentage of SD peak which represents the percentage of apoptotic cells.

Statistical analysis

The analyses were performed with the SPSS 11.5 software package. The analysis of variance was applied for the comparison of the means of the different treatment groups. Post hoc testing was performed for inter-group comparison applying the least significant difference. Values of $P < 0.01$ and $P < 0.05$ were considered to be significant.

RESULTS

Biochemistry

Chronic lead ingestion (Group II) resulted in a significant decrease in blood Hb, GSH-PX, SOD and in liver GSH levels after 42 days of treatment (Table 2). Supplementation with VC and VB1, however, reversed these effects. Moreover, the biochemistry parameters investigated showed no significant differences in Group III, VI and VII with that of the negative control (Group I) ($P > 0.05$).

DNA damage of liver cells

As shown in Table 3 and Fig. 1 A,B, animals in the negative control (Group I) exhibited low but measurable levels of DNA migration in liver cells assessed by the comet assay. The analysis of the data showed that lead intake increased percentage of cells with tail and tail length. Significant difference was observed in animals in positive control (Group II) after 6 weeks of lead exposure in comparison with the negative control ($P < 0.01$). Combined administration
with VC and VB₁ resulted in a significant reduction in the tail length and in the rate of DNA damage, especially in Group VI and VII, in comparison with that of Group II (P < 0.05). From Table 3, we observed the lower values of DNA damage at Group VI.

The percentage of liver apoptotic cells was shown in Table 3 and Fig. 2. In the negative control group (Group I), only about 5% apoptotic cells were detected. After lead intake, the percentage of liver apoptotic cells (11.9%) in positive control (Group II) was significantly higher than that in Group I (P < 0.01). Except Group XI, the percentage of liver apoptotic cells was markedly decreased in all other groups administered with combinations of VC and VB₁, as compared with that of Group II (P < 0.01 or P < 0.05). In consistent with the finding in biochemistry, the percentage of apoptotic cells in Group VI and VII was also lowest (5.8%, 5.2%).

The Olive tail length resulted from SCGE in Table 3 showed similar reversing effect induced by the supplementation of vitamins. Factorial analysis showed that the best effective combination was 420 mg kg⁻¹ bw VC and 10 mg kg⁻¹ bw VB₁, the second best was 420 mg kg⁻¹ bw VC and 30 mg kg⁻¹ bw VB₁. However, the combination of the highest dose VC (1260 mg kg⁻¹) and VB₁ (90 mg kg⁻¹) had no effect on lead toxicity of oxidative damage (Fig. 3).

**DISCUSSION**

Lead poisoning has been among the most studied health problems over the years. One of the ways that
lead exerts its toxic effects is through oxidative stress that may be an important contributor to the negative pathogenesis observed after lead exposure.

This study clearly showed that chronic lead ingestion results in a significant decrease in blood Hb, GSH-Px, SOD and in liver GSH levels. Under normal conditions, cells possess enzymatic and non-enzymatic defenses to cope with free radicals, such as SOD, GSH-Px, catalase and GSH (Shalana et al., 2005). Oxidative damage, however, may occur when antioxidant potential is decreased and/or when oxidative stress is increased. Some investigators had indicated that lead exposure increased the production of ROS in the liver (Patra et al., 2001; Pande and Flora, 2002). Patra reported that lead exposure is indeed associated with significant increases in lipid peroxide level in the liver.

Free radical-induced oxidative damage has been implicated in the pathogenesis of a number of injury and disease states. We have previously found that ROS played a pivotal role in apoptosis of testis cells in lead-exposed mice (Wang et al., 2006). In the present study, it was exhibited that a significant increase in DNA damage and apoptosis in liver cells occurred via ROS as it played a very important role in apoptosis induction under both physiological and pathological conditions. Interestingly, mitochondria were both the source and target of ROS. ROS, which was
predominantly produced in the mitochondria, led to the free radical attack of membrane phospholipids and loss of mitochondrial membrane potential, which caused the intermembrane proteins, such as cytochrome c, to be released out of the mitochondria and ultimately triggered caspase-3 activation. Caspase-3 activation led to DNA breakage, nuclear chromatin condensation and cell apoptosis (Li et al., 2006; Wang et al., 2006).

After coadministrated with vitamins, the lead toxicity was normalized effectively. The levels of GSH-PX, SOD in blood and GSH in the liver had a significant increase when compared with lead-exposed group ($P < 0.01$). Namely, free radicals induced by lead may oxidize GSH and the vitamin treatment reversed lead-induced decreases in GSH levels. It is well known that ascorbic acid directly reacts with lipid hydroperoxides and restores the antioxidative potency of $\alpha$-tocopherol. Production of GSH is considered to be the first line of defense against oxidative damage and free radical generation where GSH functions as a scavenger and a cofactor in metabolic detoxification of ROS (Tandon et al., 2003). As discussed previously, ROS played a pivotal role in apoptosis. So lead-exposed mice co-treated with ascorbic acid could directly react with lipid hydroperoxides or/and increase the levels of GSH and then prevented liver destruction from apoptosis.

The exact mechanism of thiamine in antagonizing lead toxicity has not been clearly elucidated until now. It might be attributed to the formation of complexes between thiamine and lead followed by its excretion. Thiamine also has been found to protect against lead-induced lipid peroxidation in rat liver and kidney (Senapati et al., 2000). It may scavenge $O_2^•$ and $^*OH$ directly and thus affect the cellular response to oxidative stress (Jung and Kim, 2003). Anna and Wiglo (2006) reported that thiamine may act as a potent antioxidant as it scavenges free radicals such as 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation relatively slowly. Its radical-scavenging activity, expressed as the trolox equivalent antioxidant capacity value, is comparable to such natural antioxidants as some carotenoids. The fact that simultaneous supplementation of ascorbic acid and thiamine ameliorated the liver damage in lead-treated mice, indicating that prevention was attributed to apoptotic inhibition by increased levels of GSH-PX, SOD and GSH.

At the highest dose of ascorbic acid and thiamine, the results differed from the low and middle doses. When compared with the only lead exposure group, the combination of high dose VC (1260 mg kg$^{-1}$) and VB$_1$ (90 mg kg$^{-1}$) had no effect on lead toxicity of oxidative damage. We also previously found that this combination caused much more severe injury in testis than that in lead-treated group (Wang et al., 2006). Although ascorbic acid was a well-known antioxidant, it might possess pro-oxidant property under certain conditions. When the concentration of ascorbic acid is higher than normal, ascorbic acid level still kept normal in plasma by way of self-metabolism indeed continued to act as antioxidant scavenging free radicals. However, excessive concentration led to accumulation of ascorbic acid, and then an increased level of lipid peroxidation (Childs et al., 2001). The pro-oxidant character of ascorbic acid was due to its high reactivity with iron (Halliwell and Gutteridge, 1999). Ascorbic acid, as an electron donor of chemical reaction both in intracellular and extracellular states, provided an electron to Fe$^{3+}$ while the concentration of ascorbic acid was excessive. The reduction potentials of Fe$^{3+}$ and ascorbic acid easily allow for the formation of the ascorbate radicals and Fe$^{2+}$ iron, which generated $O_2$, $OH$ and $H_2O_2$. The free radicals increased lipid peroxidation, oxidize GSH and subsequently promoted liver cell apoptosis. Additionally, thiamine is a soluble vitamin and has been assumed to be generally non-toxic mainly due to the ready excretion of excess thiamine from the body. In many multi-vitamin supplements for human and mice that were commercially available at present, $>100$ mg of thiamine per 1 kg of body weight were added for everyday intake (Franca et al., 2001; Zhou et al., 2003). Thus, we thought that VC had no effect on lead toxicity of oxidative damage at higher dose.
CONCLUSION

In summary, combination of VC and VB\textsubscript{1} can lessen the damage to liver cells from oxidative damage induced by lead, but the antioxidant effects are dependent on their concentrations.

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