Long-Term Intake of High Doses of Vitamin C Down-regulates Anti-oxidant Enzymes in Human Erythrocytes

Hee Joon Kim¹, Min-Kyung Park²*, Kwang-Ho Rhee³, Hee-Sang Youn⁴, Seong-Hee Ko⁵,⁶, Hyun-Sook Kim⁵, and Myung-Hee Chung⁶†

¹Department of Pharmacology, Dankook University College of Medicine, Cheonan 330-714, Korea
²Department of Human Nutrition and Food Science, Chungwoon University, Chungnam 350-701, Korea
³Department of Microbiology, and ⁴Department of Pediatrics, Gyeongsang University College of Medicine, Chinju 660-751, Korea
⁵Major in Food and Nutrition, College of Human Ecology, Sookmyung Women’s University, Seoul 140-742, Korea
⁶Department of Pharmacology, Seoul National University School of Medicine, Seoul 110-799, Korea

Abstract

We located a group of healthy young males (aged 20 ~ 30) who had been taking a high dose (more than 5 g) of vitamin C daily for more than one year. We observed that this vitamin C group had plasma levels of vitamin C that were more than three times that of the control group. The control group had not taken any additional vitamin C except for that included in their diets. But the vitamin C group showed significantly lower amounts of Cu/ZnSOD, catalase and glutathione-s-transferase and lower activities of glutathione peroxidase and glutathione reductase in erythrocyte lysates than the control group. However, there was no difference in the plasma levels of lipid peroxides between the two groups. These results suggest that vitamin C offsets its own contribution to anti-oxidant activity by repressing the expression of anti-oxidant enzymes and also excludes the possibility that vitamin C acts as a pro-oxidant in vivo.

Key words: vitamin C, anti-oxidants, anti-oxidant enzymes, lipid peroxidation, anti-oxidant capacity

INTRODUCTION

Reactive oxygen species (ROS) are constantly formed in aerobic cells by various mechanisms. Because of their high reactivity, they can damage major cellular components such as lipids (1), proteins (2) and nucleic acids (3). Aerobic organisms remove ROS by using enzymes (4) such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd) and glutathione-s-transferase (GST) and non-protein anti-oxidants (5) such as vitamins C, E, β-carotene and polyphenols. Normally, organisms are not harmed by ROS since production and removal of ROS are in equilibrium; however, ROS can harm organisms and even cause diseases when this equilibrium is shifted far toward production. Various inflammatory disorders (6), diabetic complications (7) and ischemic diseases (8) are known as conditions in which ROS are involved as major pathogenic factors. Various anti-oxidants to remove ROS have been tried in treating these disorders. Vitamin C is one of the anti-oxidants that have been used most frequently for this purpose. However, the results appear to be inconsistent as some studies report beneficial effects (9) and some show negative effects (10). Some reports even warn that vitamin can be detrimental since it can act as a pro-oxidant (11). It is not clear why the results of vitamin C studies are inconsistent, but the inconsistencies are probably due to variables such as differences in doses and duration of intake from study to study and, more importantly, to the difference in the extent of ROS involvement in the pathogenesis of the diseases on which vitamin C was tested.

Recently, the numbers of people who are taking high doses of this vitamin at 50 ~ 100 times the recommended daily requirement (60 ~ 100 mg) seem to be increasing. Despite many studies of its therapeutic effect on various diseases, studies on the effects of such high doses of vitamin C in normal healthy subjects have not been performed. We located a group of healthy young males who had been taking a high dose (more than 5 g) of vitamin C daily for more than one year. These young males provide suitable conditions to investigate the effects of vitamin C in healthy humans without the influences of the variables mentioned above. We found that this vitamin C intake group had higher plasma concentrations of vitamin C (more than three times the control)
and significantly lower amounts and activities of anti-oxidant enzymes in erythrocyte lysates than the control subjects. However, there were no differences in plasma levels of lipid peroxides between the two groups.

**MATERIALS AND METHODS**

**Human subjects**

Two groups of healthy males aged 20–30 participated in this study. One group (vitamin C group) consisted of 16 subjects who had taken 5–10 g of vitamin C for more than one year. The other group (control group) consisted of 19 subjects who had not taken additional vitamin C during the same period except for that included in their diets. The subjects participating in this study were male students of Gyeongsang University College of Medicine and thus, the conditions such as health and age were quite similar and we could obtain accurate histories of vitamin C intake. Medical examinations revealed no abnormalities or diseases in the subjects.

**Preparation of erythrocyte lysates**

Erythrocyte lysates were prepared in order to measure the amounts and activities of anti-oxidant enzymes therein. Blood was sampled into tubes containing heparin and centrifuged at 3,000 ×g for 10 min at 4°C. Supernatants (plasma) were collected for assays of vitamin C and lipid peroxides. Erythrocyte pellets were washed twice with saline and subjected to hemolysis by mixing with 1.5 volumes of distilled water. To remove hemoglobin, as previously described (12), the suspension of hemolyzed erythrocytes was mixed with 1.5 volumes of distilled water (3.5 mL), ethanol (1 mL) and chloroform (0.5 mL); vortexed for 1 min, and centrifuged at 3,000 ×g for 10 min. The supernatants obtained were concentrated in Centriprep concentrators (Amicon, Bervely, MA, USA) to a final concentration of about 2 mg protein/mL and kept at -70°C until use.

**Immunoblotting of anti-oxidant enzymes**

In order to measure the amounts of Cu/ZnSOD, CAT and GST in the erythrocyte lysate, each of these enzymes was immunoblotted as described previously (13). Briefly, the erythrocyte lysates prepared above were subjected to SDS-PAGE. For analysis of Cu/ZnSOD and GST, 40 and 25 μg of the lysate, respectively, were loaded onto 12% gel, and for CAT analysis, 10 μg of the lysate loaded onto 10% gel. Purified Cu/ZnSOD (0.5 μg) from human erythrocytes, GST (0.5 μg) from human placenta and CAT (0.2 μg) from human erythrocytes (Sigma, St. Louis, MI, USA) were also loaded as controls. After electrophoresis, proteins in the gels were transferred to nitrocellulose membranes. The nitrocellulose membranes containing the proteins were incubated with PBS containing 5% non-fat dry milk and 0.2% Triton X-100 for 1 hr at 37°C and then with the following antibodies (Biodesign, Kennebunk, ME, USA): sheep anti-human erythrocyte Cu/ZnSOD (1:200 dilution), rabbit anti-human erythrocyte CAT (1:500 dilution) or rabbit anti-human placental GST-π (1:500 dilution). After washing with PBS, the membranes were treated with NBT solution (Vector Laboratory Inc., Burlingame, CA) to detect Cu/ZnSOD or with secondary antibody conjugated with alkaline phosphatase (Biodesign, Kennebunk, ME, USA) and then with BCIP solution (Vector Laboratory Inc., Burlingame, CA) to detect CAT and GST. The densities of the visualized protein bands were analyzed by Receptor Autoradiography Analysis System (Model 3000 series, Amersham Co., USA) equipped with band analysis software. The band density of each enzyme in the erythrocyte lysate of the control or vitamin C group was compared to that of the standard enzyme and a density ratio (%) was calculated. The density ratios of the two groups were then compared and presented as % of the control group. The mean ± SD obtained from four randomly selected samples are presented.

**Activities of glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd)**

The erythrocyte lysates were also used for GSH-Px and GSH-Rd assays. GSH-Px was assayed as described previously (14). Briefly, the erythrocyte lysates were incubated with GSH-Rd (Sigmna, St. Louis, MI, USA), GSH and nicotinamide adenine dinucleotide phosphate (NADPH) for about 3 min to induce hydroperoxide-independent consumption of NADPH, thereafter t-butyl hydroperoxide (substrate) was added and the mixture incubated for about 5 min. During the second incubation, GSH was oxidized to GSSG, which was then reduced to GSH again by GSH-Rd and NADPH. The amount of NADPH used to reduce GSSG to GSH was measured by the decrease of absorbance at 340 nm using a spectrophotometer equipped with a thermostat (Hewlett Packard, Omaha, NE, USA). The activity of GSH-Px was expressed as μmoles of NADPH consumed/mg protein. GSH-Rd was assayed as described previously (15). The erythrocyte lysates were incubated with GSSG and NADPH and then NADPH was oxidized to reduce GSSG to GSH. The amount of NADPH used for reduction of GSSG to GSH was measured spectrophotometrically as described above. The activity of GSH-Rd is expressed as μmoles of NADPH consumed/mg protein.

**Plasma vitamin C**

The concentrations of vitamin C in plasma of the vitamin C (n=16) and control (n=19) groups were measured
Long-Term Intake of High Doses of Vitamin C Down-regulates Anti-oxidant Enzymes in Human Erythrocytes

as previously described (16) and expressed as μg/mL plasma.

**Plasma lipid peroxides**
The concentration of lipid peroxides in plasma was estimated by measuring malondialdehyde (MDA) as previously described (17) and expressed as nmoles/mL plasma.

**Proteins in erythrocyte lysates**
The protein concentrations in erythrocyte lysates were determined by the bicinchoninic acid method, as described by Smith et al. (18). Bovine serum albumin was used as a standard.

**Statistics**
The data obtained from each group were expressed as mean ± SD and the significance of difference between groups was tested by Student’s t-test at p<0.05.

**RESULTS**

**Vitamin C levels in plasma**
First, plasma vitamin C concentrations in the control and vitamin C groups were compared. As shown in Fig. 1, the concentrations of the control and vitamin C groups were 11.93 ± 1.91 and 28.80 ± 13.95 μg/mL plasma, respectively. The concentration in the vitamin C group was more than three times higher than that in the control group (p<0.01).

**Amounts of Cu/ZnSOD, CAT and GST in erythrocyte lysates**
Anti-oxidant enzymes, Cu/ZnSOD, CAT and GST, in erythrocyte lysates of the two groups were compared by immunoblotting. Fig. 2 shows the results for Cu/ZnSOD.

![Fig. 2. Immunoblotting analysis of Cu/Zn superoxide dismutase (SOD) in human erythrocytes.](image)

The bands of this enzyme in the two groups and its pure form as a standard are shown in Fig. 2A. The band density for each group was compared to that of the pure enzyme and a ratio (%) was calculated. The ratios obtained were compared and presented as % of the control group. Asterisks (***p<0.01) indicate the significance of the difference between the two groups at p<0.01 by Student’s t-test.

![Fig. 1. Plasma vitamin C levels. The vitamin C group consisted of 16 healthy males aged 20–30 who had taken 5–10 g of vitamin C for more than one year. The control group consisted of 19 healthy males aged 20–30 who had not taken additional vitamin C during the same period, except for that included in their diets. The data presented are means ± SD. The plasma level difference was significant at p<0.01.](image)
Activities of GSH-Px and GSH-Rd in erythrocyte lysates

The two groups were compared in terms of GSH-Px and GSH-Rd activities in erythrocyte lysates and the results are shown in Fig. 5. GSH-Px activities of the control and vitamin C groups were 1.84 ± 0.56 and 1.35 ± 0.29 μmoles NADPH oxidized/mg protein, respectively. The activity of the vitamin C group was significantly lower than that of the control group (p<0.05). GSH-Rd activities of the control and vitamin C groups were 0.09 ± 0.02 and 0.07 ± 0.01 μmoles NADPH oxidized/mg protein, respectively. The activity of the vitamin C group was also significantly lower than that of the control group (p<0.02).

MDA levels in plasma

In order to estimate the level of oxidative stress in the two groups, lipid peroxides in plasma were measured as MDA in plasma. As shown Table 1, MDA levels in the control and vitamin C groups were 3.3 ± 0.6 and 3.9 ± 0.9 nmoles/mL plasma, respectively, and were not significantly different (p>0.05).

DISCUSSION

In the present study, we observed the effects of long-term (more than 1 year) intakes of high dose vitamin C (5~10 g/day) on the protein amounts or activities of several anti-oxidant enzymes in healthy men aged 20~30. The level of vitamin C in plasma was three times higher in the vitamin C group than in the control group (Fig. 1). This result is consistent with the report showing that plasma vitamin C levels correlated with the amount of intake (19). In contrast, the amounts of Cu/ZnSOD, CAT and GST in erythrocytes were significantly lower in the vitamin C group than in the control group (Figs. 2~4), as were the activities of GSH-Px and GSH-Rd.
Long-Term Intake of High Doses of Vitamin C Down-regulates Anti-oxidant Enzymes in Human Erythrocytes

143

Fig. 5. Activities of glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd) in human erythrocytes. The erythrocyte lysates were used to assay GSH-Px and GSH-Rd as described in Materials and Methods. Open and filled columns indicate the control and vitamin C groups, respectively. Asterisks (*) or (**) indicate the significance of the difference between the two groups at *p<0.05 or **p<0.02, respectively, by Student’s t-test. The numbers of subjects of the control and vitamin C groups are 19 and 16, respectively.

Table 1. Plasma malondialdehyde (MDA) levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmoles/mL plasma)</td>
<td>3.3 ± 0.6 (n=19)</td>
<td>3.9 ± 0.9 (n=16)</td>
</tr>
</tbody>
</table>

1) The results are presented as means ± SD.
2) Numbers of subjects are shown in parentheses. The MDA levels of the two groups were not different at p>0.05 by Student’s t-test.

(Fig. 5). Interestingly, there was no difference in the plasma MDA levels of the two groups (Table 1), suggesting that the two groups have the same anti-oxidant capacity.

Aerobic organisms may survive an environment with ROS or toxic substances by inducing anti-oxidant enzymes or detoxifying enzymes through a mechanism involving the transcription factor, Nrf2, and an anti-oxidant response element (ARE) in the promoters of the genes of these enzymes (20). The increase in ROS or toxic chemicals in cells triggers an increase in Nrf2, which promotes its translocation into the nucleus where it binds to ARE and initiates the transcription of these enzymes. Induction of Cu/ZnSOD in tissues or cells by hyperoxia (21), treatment with paraquat (22) or xanthine/xanthine oxidase (23) and induction of Cu/ZnSOD, CAT and GSH-Px in human mononuclear cells by exposure to light stress (24) are examples of this. In an environment with low ROS levels, however, binding of Nrf2 and ARE is decreased to lower the expression of these enzymes. Decreased induction of γ-glutamylcysteine synthetase, an enzyme that synthesizes GSH in cancer cells (KCL22) treated with vitamin C (25), and suppression of exercise-induced Cu/ZnSOD and GSH-Px by vitamin C in rats and humans (26) may be examples of this phenomenon. The result observed in this study is another example, which shows that, even in healthy individuals, long-term intake of vitamin C can suppress the induction of anti-oxidant enzymes by shifting the balance between the production/removal of ROS to the removal side. Therefore, we know that increases in anti-oxidant capacity by vitamin C are limited by feed-back inhibition of anti-oxidant enzyme expression. It is thought that the down-regulation of the enzymes observed in the erythrocytes of the vitamin C group occurred during the stages from proerythroblast to normoblast because nuclei of erythrocytes are retained up to normoblast and excluded when normoblast is transformed into reticulocyte (27).

Vitamin C has been tested on various ROS-associated diseases such as motor dysfunction by cerebral ischemia (28), lung cancer risk (29), H. pylori-induced gastritis and gastric carcinoma (30), and degenerative osteoarthritis (31), but the results were negative. One of the reasons for the negative effects may be this limitation in the anti-oxidant capacity. On the other hand, there are reports showing that vitamin C enhances the activities of anti-oxidant enzymes and has positive effects on some diseases. Examples include the increase of CAT activity and the inhibition of lipid peroxidation in the brains of rats subjected to pilocarpine-induced convolution (32), and the increase of CAT and Cu/ZnSOD activities and the inhibitions of sperm number decrease, abnormal sperm formation and lipid peroxidation in cadmium-treated mice (33).

As discussed above, the effects of vitamin C on pathological conditions are inconsistent. These inconsistencies
may be caused by differences in the doses and duration of use or, most importantly, by differences in ROS involvement in the pathogenesis of the diseases on which vitamin C was tested. In order to eliminate these variables, the effect of vitamin C was tested on young healthy males using high doses (5–10 g/day) and long-term administration (more than one year). Under these conditions, we can more accurately assess the effects of vitamin C and obtain information regarding the action of high-dose vitamin C in healthy individuals. In this study, three findings were obtained: significantly higher plasma concentrations of vitamin C and significantly lower protein amounts and activities of anti-oxidant enzymes in the vitamin C intake group than in the control group and the same level of lipid peroxides in both groups. These results imply two facts. One is that long term intake vitamin C does not enhance anti-oxidant capacity in proportion to the dose and duration of intake since it offsets its anti-oxidant contribution by repressing the expression of anti-oxidant enzymes. Another is that vitamin C does not act as a pro-oxidant in vivo. As an electron donor, vitamin C in the presence of metal ions in vitro promoted the formation of ROS. For example, vitamin C together with Fe ++ or Cr(VI) induced lipid peroxidation in milk (34) and severe DNA damage in cultured cells (35,36). Thus, the possibility of a pro-oxidant action in vivo was raised. However, this study shows that the plasma MDA levels of the vitamin C group were the same as those of the control group.

ACKNOWLEDGEMENTS

This work was supported by the grant from the Korean Ministry of Science and Technology (Grant 2006-02293).

REFERENCES

Long-Term Intake of High Doses of Vitamin C Down-regulates Anti-oxidant Enzymes in Human Erythrocytes

835-841.


(Received June 25, 2008; Accepted July 9, 2008)