Vitamin C augments lymphocyte glutathione in subjects with ascorbate deficiency

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ABSTRACT

Background: Ascorbate and glutathione play central roles in the defense against free radicals and oxidants that are implicated in chronic diseases.

Objective: The objective was to determine the ability of vitamin C supplements to modulate the concentration of glutathione in human lymphocytes.

Design: The effect of vitamin C supplements was determined in a sequential study with time points before supplementation, after 13 wk of vitamin C supplements (500 or 1000 mg/d), and after 13 wk of matching placebo. The supplementation group was selected on the basis of low plasma ascorbate (< 33 mmol/L) and consisted of 48 healthy men and women, smokers and non-smokers, aged 25–64 y. Ascorbate and glutathione were measured in purified lymphocytes.

Results: At baseline, the mean (± SD) concentration of plasma ascorbate was 19.5 ± 7.2 μmol/L, 22.5 μmol/L below the median of normal distribution. The ascorbate concentration in plasma was linearly associated with that in lymphocytes (r = 0.53, P < 0.001). On supplementation with vitamin C, lymphocyte ascorbate increased by 51% (from 16.7 ± 4.9 to 25.3 ± 6.9 nmol/mg protein; P < 0.001) and was accompanied by an increase of lymphocyte glutathione by 18% (from 22.5 ± 4.5 to 26.6 ± 6.5 nmol/mg protein; P < 0.001). After placebo, the ascorbate and glutathione concentrations fell to near baseline concentrations (17.1 ± 5.4 and 23.5 ± 6.4 nmol/mg protein, respectively). No significant interaction was observed for sex and smoking status. Finally, the changes in lymphocyte ascorbate after supplementation were strongly associated with changes in lymphocyte glutathione (r = 0.71, P < 0.001). The association suggests that every 1-mol change in ascorbate is accompanied by a change of ~0.5 mol in glutathione.


INTRODUCTION

Several studies have pointed to an association between vitamin C intake and chronic diseases. In a 4-y prospective study with 30,466 men and women, the risk of mortality due to all causes for those in the highest quintile of plasma ascorbate concentration was about one-half that for those in the lowest quintile of plasma ascorbate concentration (1). Similar results were also reported for men but not for women in the 12-y follow-up of the second National Health and Nutrition Examination Survey (2). Another recent study indicates that free-living elderly who consume citrus fruit twice a week have reduced risks of dying that are half those for elderly who consume citrus fruit less than once a week (3). Diseases that have been associated with mild vitamin C deficiency include cardiovascular diseases, cancer, and cataracts. Two studies reported that vitamin C supplements lower blood pressure (4, 5). Most epidemiologic studies of vitamin C and cancer point to an inverse association, particularly for cancer of the oral cavity, pharynx, esophagus, stomach, colon, and lung (as reviewed in 6); however, there are also studies that do not show an effect (7, 8). A likely reason for these inconsistencies is that plasma and tissue ascorbate is saturated when the consumption of vitamin C exceeds 100 mg/d (7, 9).

Ascorbate is an excellent antioxidant in cells, not only because of its relatively high concentration, but also because of the high rate constant of its reaction with free radicals. It even reacts with glutathione radicals in the aqueous phase and with vitamin E at the aqueous-lipid interface. The relative concentration of ascorbate has been suggested to be an indication of systemic oxidative stress. For example, plasma and lymphocyte ascorbate concentrations decline ~20% during aging (10). Plasma ascorbate is also significantly lower in smokers, alcoholics (11), and persons with cancer (12, 13), diabetes (14), hepatitis (15), HIV (16), cystic fibrosis (17), or β thalassemia major (18). It is difficult to delineate the effects of vitamin C in vivo because ascorbate has prooxidant properties under certain conditions, eg, in the presence of free iron or copper metal ions. Nevertheless, the analyses of various markers of oxidative damage strongly suggest that ascorbate acts as an antioxidant in vivo without any clearly proven prooxidant activity (19).

The possibility of mutual sparing by ascorbate and glutathione was proposed by Meister (20, 21) on the basis of studies in animals that do not synthesize ascorbate, including...
guinea pigs and newborn rats. Meister showed that ascorbate rescues glutathione-deficient animals and that glutathione esters alleviate the symptoms of ascorbate deficiency. Although there are several examples of ascorbate glutathione synergy in rodents, the exact nature of this interaction remains unknown. Moreover, there is very little evidence for a similar effect in humans. Previously, we reported a strong positive association between intracellular ascorbate and glutathione in healthy persons (10).

SUBJECTS AND METHODS

Recruitment of subjects

Potential candidates were recruited by newspaper and publicity pamphlets, which targeted hospital staff and local residents. The candidates were rapidly screened for plasma ascorbate concentrations by finger-prick blood samples and HPLC analyses (see below). Subsequently, an experienced nurse contacted the candidates who had low plasma ascorbate concentrations (<33 μmol/L) to determine their eligibility on the basis of a detailed medical questionnaire and consultation with a physician. Eligible candidates were those who were apparently healthy, without signs of serious illnesses, such as heart disease or cancer, or of diabetes, hepatitis, and arthritis.

Clinical protocol

Three blood samples were obtained from each subject. The first blood sample was taken at the beginning of the study (baseline), the second was taken after the subjects ingested vitamin C supplements for 13 wk, and the third after subjects ingested placebo for an additional 13-wk period. The supplementation study extended over 9 mo (May through January), including an initial 13-wk period during which the first blood donation was obtained from each member of the subject group. The entire subject group was uniformly sampled over the 13-wk periods at a rate of 3–5 subjects/d. Blood donations of 100 mL were obtained from fasting subjects by venipuncture, and the blood was processed immediately (see below). Subjects ingested vitamin C in doses of either 500 or 1000 mg/d, by taking one or two 500-mg tablets of vitamin C daily. Vitamin C supplements and matching placebo were provided in sealed bottles of 100 tablets (Roche Vitamins Inc, Nutley, NJ). According to the number of tablets remaining after each period, the average compliance for taking supplements and placebo was 95%. Written informed consent was obtained from all subjects, and the ethics committee at the Sherbrooke University Institute of Geriatrics approved the protocol.

Laboratory water and buffers

Water was extensively purified in 2 steps: first, by double distillation through a Fi-stream II glass still and then by ion exchange and filtration through an Easypure RF water system equipped with ultrapure and high-purity Low Toc cartridges giving a final resistivity of at least 18.3 MΩ cm (Barnstead Thermolyne, Dubuque, IA). Buffers were made from chemicals with the highest available purity (either Sigma, St Louis or Aldrich, Milwaukee). Phosphate-buffered saline (150 mmol/L, pH 7.4) was treated with 1 g/L of Chelex 100 (Bio-Rad, Richmond, CA) to remove trace metal ions and filtered before use. The presence of transition metal ions in buffers and filtered before use. The presence of transition metal ions in buffers was negligible, as inferred from the ascorbate-degradation test (22).

Rapid screening of plasma ascorbate

Plasma ascorbate concentrations were measured by pricking the fleshy fingertip of candidates with a lanceting device (SoftflexPro; Roche Diagnostics, Laval, Canada). Twelve milliliter of blood was collected with the use of a 200-μL pipette and a sterile pipette tip. The sample was immediately mixed with an equal volume of 10 mmol EDTA/L in phosphate-buffered saline and centrifuged at 5000 × g for 3 min at room temperature to separate the plasma from the red blood cells. A 10-μL volume of the supernatant fluid was added to an equal volume of ice-cold 10% (wt:vol) metaphosphoric acid, mixed by vortex for 10 s, and centrifuged at 15 000 × g for 3 min at 4°C. The resulting supernatant fluid was then stored at −80°C. Subsequently, the analysis of ascorbate in plasma samples was carried out by HPLC with electrochemical detection. Ascorbate was separated by HPLC using a 5-micron octadecylsilyl column with a 150 × 4.6-mm internal diameter (Inertsil; CSC, Montreal) with 40 mmol sodium acetate/L at pH 4.75, 0.54 mmol sodium EDTA/L, and 1.5 mmol dodecyltriethylammonium phosphate/L as the mobile phase. The HPLC system consisted of a pump (LC-600; Shimadzu, Kyoto, Japan) and a detector (Coulchem II; ESA Associates, Chelmsford, MA) with an electrochemical cell (model 5010; ESA Associates) set at 450 mV for the palladium reference electrode.

Analysis of lymphocyte glutathione and ascorbate

Blood was collected in five 20-mL tubes containing sodium citrate as an anticoagulant. Lymphocytes were isolated from whole blood by density centrifugation with a mixture of Ficoll (Sigma, St Louis) and Hypaque (Picker International Canada, Inc, Brampton, Canada) subsequently separated from monocytes by adherence to plastic flasks coated with defibrinated plasma, as described previously (10, 23). The resulting lymphocytes were >90% viable as determined by trypan blue exclusion, and the purified cell pellets contained no visual trace of red blood cells. The analysis of glutathione and ascorbate in lymphocytes was carried out by a method adapted from Rose and Bode (24). Ten million cells were washed 3 times with ice-cold phosphate-buffered saline, suspended in 200 mmol phosphoric acid/L containing 0.1 mmol EDTA/L, subjected to 3 freeze-thaw cycles (from 4°C to −80°C), and centrifuged at 12 000 × g for 10 min at 4°C. Samples were stored at −80°C before analysis. Glutathione and ascorbate were separated by HPLC using the Inertsil column with 200 mmol phosphate/L at pH 3.0 as the mobile phase. The HPLC system was the same as described above. With this protocol, there was no indication of ascorbate or glutathione degradation during sample storage or processing. The oxidation of glutathione was negligible as inferred by the high ratio (>100) of reduced glutathione to oxidized glutathione. Furthermore, multiple analyses of the same sample at different times throughout the study resulted in the same concentration of antioxidant, within experimental error. The concentrations of ascorbate and glutathione are expressed in nmol/mg protein, in which protein represents total protein.

Analysis of protein

The concentration of protein in cell lysates was determined by the Bradford assay, which was automated with an HPLC system. This consisted of a primary pump to deliver a carrier buffer [10% (vol:vol) methanol in water] at a flow rate of 1.2 mL/min; 80-μL protein samples were injected into the pump with the use of an autoinjector (WISP 710B; Waters, Milford, MA). A solution of 15% (vol:vol) Bradford assay reactant (Bio-Rad) and 10%
TABLE 1
Plasma ascorbate concentrations in male and female smokers and nonsmokers

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma ascorbate concentration* µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
</tr>
<tr>
<td>Nonsmokers (n = 228)</td>
<td>49.6 ± 19.1</td>
</tr>
<tr>
<td>Smokers (n = 77)</td>
<td>37.1 ± 18.5</td>
</tr>
<tr>
<td>Males</td>
<td></td>
</tr>
<tr>
<td>Nonsmokers (n = 122)</td>
<td>38.9 ± 17.0</td>
</tr>
<tr>
<td>Smokers (n = 63)</td>
<td>26.8 ± 19.4</td>
</tr>
<tr>
<td>Total (n = 490)</td>
<td>42.0 ± 20.1</td>
</tr>
</tbody>
</table>

* ± SD.

Significantly different from female nonsmokers, *P < 0.001.

Significantly different from male smokers (paired t test): *P = 0.011,

Statistical analysis

The distributions of plasma ascorbate in different subject groups were compared by analysis of variance (ANOVA) and multiple unpaired t tests. The effect of supplementation was studied by one-, two-, or three-factor ANOVAs with repeated measurements on the same variable, either ascorbate or glutathione, at 3 time points. All data displayed nearly normal distributions. Changes of variables between time points were determined by multiple paired t tests. Linear regression analyses were used to determine the association between variables. P values <0.05 were considered significant. For multiple t tests, the P value was adjusted according to Bonferroni’s correction, which takes into account a lower threshold of significance depending on the number of comparisons. Statistical analyses were performed on a personal computer with the use of SPSS software, version 10.1.0 (SPSS Inc, Chicago).

RESULTS

Four hundred ninety subjects were screened for plasma ascorbate concentrations. The distribution of plasma ascorbate was normal, with a range of 7 to 112 µmol/L and a mean (±SD) of 42.0 ± 20.1 µmol/L. The concentration of plasma ascorbate was significantly different between men and women as well as between smokers and nonsmokers (Table 1). No interaction between sex and smoking status was observed (two-factor ANOVA). The concentrations of ascorbate are consistent with those of a meta-analysis by Brubacher et al (25), who reported average plasma concentrations of 33.6 µmol/L in smokers and 42.4 µmol/L in nonsmokers. It is surprising that 15 (3%) of the 490 persons in our study had plasma ascorbate concentrations (<10 µmol/L) that are considered to indicate a high risk of scurvy.

The subject group selected for vitamin C supplementation consisted of 15 female nonsmokers, 10 female smokers, 15 male nonsmokers, and 8 male smokers (n = 48). The mean (±SD) concentration of plasma ascorbate for this group was 19.5 ± 7.2 µmol/L, and the range was 7.4–32.9 µmol/L. The concentration of plasma ascorbate was linearly associated with that of lymphocyte ascorbate (Figure 1). In addition, there was no significant difference in the association of plasma and lymphocyte ascorbate between subjects with above-median plasma ascorbate and those with below-median plasma ascorbate. This suggests that the concentration of ascorbate in lymphocytes does not become saturated at the relatively low concentration of plasma ascorbate within the reference group.

A sequential design was used to examine the effect of vitamin C supplementation. Blood samples were taken before supplementation (baseline), after supplementation for 13 wk with either 500 or 1000 mg vitamin C/d, and after a further 13 wk of matching placebo. To determine the ability of vitamin C supplements to increase the concentration of ascorbate in lymphocytes, the intracellular concentration of ascorbate was monitored at the 3 time points. Because there was no significant difference between 500- and 1000-mg/d doses of vitamin C, these data were treated as a single supplemented dose. Furthermore, the change in lymphocyte ascorbate at the 3 time points did not significantly depend on sex (P = 0.10), smoking status (P = 0.23), or sex and smoking status (P = 0.57), as inferred by three-factor repeated-measures ANOVA. Therefore, men, women, smokers, and nonsmokers were combined into a single group. The changes in lymphocyte ascorbate concentrations between the 3 time points were significant (P < 0.001; one-factor repeated-measures ANOVA). The mean (±SD) concentration of lymphocyte ascorbate increased by 51% (16.7 ± 4.9–25.3 ± 6.9 nmol/mg protein) and then returned to baseline values after removal of the supplement (17.1 ± 5.4 nmol/mg protein) (Figure 2). The differences between baseline and supplement were highly significant (P < 0.001), as were those between supplement and placebo (P < 0.001), but not those between baseline and placebo (P = 1.00). The concentrations of ascorbate in lymphocytes attained by vitamin C supplementation are comparable to values reported in the literature. For example, Levine et al
different from A and C, these groups by two-factor repeated-measures ANOVA (median age groups, there was no significant difference between
When the subjects were divided into below-median and above-
association of age with vitamin C supplementation was studied.
seasonal variation, the mean (± SD) concentrations of lymphocyte ascorbate were assumed to vary with the
phocyte ascorbate concentrations were assumed to vary with the
lymphocyte ascorbate concentration with age, the average of ascor-
lymphocyte glutathione concentrations between baseline and supplement was significant (P < 0.001). The difference in
baseline and placebo time points was not significant. In conclusion,
these analyses imply that seasonal variations in vitamin C may partly explain the changes in lymphocyte ascorbate during sup-
plement, but that the contribution is minor compared with the
effect of vitamin C supplements.
The effect of vitamin C supplements on lymphocyte glutathione was examined. As in the case of ascorbate, no significant interaction was observed for sex or smoking status, and, thus, the subjects were pooled into a single group. The changes in lymphocyte glutathione between the 3 time points were significant (P < 0.001; one-factor repeated-measures ANOVA). On supplementation with vitamin C, the mean (± SD) concentration of glutathione in lymphocytes increased from 22.5 ± 4.5 to 26.6 ± 6.5 nmol/mg protein (18%), and the concentration dropped to 23.5 ± 6.4 nmol/mg protein when subjects received placebo (Figure 2). The difference in glutathione concentrations between baseline and supplement was significant (P < 0.001), as was that between supplement and placebo (P = 0.010), but there was no difference between baseline and placebo (P = 0.84). Finally, note that the concentrations of lymphocyte glutathione observed in this study are in the range of concentrations reported in previous studies [26.4 ± 9.0 nmol/mg protein, n = 240 (10); and 17.2 ± 2 nmol/mg protein, n = 144 (28)].

To investigate an association between ascorbate and glutathione, the changes in lymphocyte ascorbate were plotted against the changes in lymphocyte glutathione (Figure 3). These analyses found a strong and positive association between the changes in antioxidants from baseline to vitamin C supplement (r = 0.71, P < 0.001). The slope of the linear regression suggests that for every 1-mol change in ascorbate, there is a 0.5-mol change in glutathione.

**FIGURE 3.** Association between the change in glutathione and the change in ascorbate in the subjects (n = 48) from baseline to supplement (●) and in the same subjects from supplement to placebo (○). A linear regression for changes from baseline to supplement (— — — ) gave y = 0.54x − 0.6 (r = 0.71, P < 0.001), where y is the concentration of glutathione and x is the concentration of ascorbate in lymphocytes. Similarly, analysis of the changes from supplement to placebo (—) gave y = 0.68x + 2.5 (r = 0.69, P < 0.001).
Previously, we reported a linear association between lymphocyte glutathione and ascorbate by \( y = 0.78x + 10 \) \((r = 0.62, P < 0.001; n = 240)\), where \( x \) is the concentration of ascorbate and \( y \) is the concentration of glutathione in lymphocytes in nmol/mg protein \((10)\). The same association was observed for subjects in the present study. The fitting expressions were \( y = 0.77x + 7 \) \((r = 0.82, P < 0.001; n = 48; x \) and \( y \) same as above) for subjects at the supplement time point and \( y = 0.80x + 10 \) \((r = 0.67, P < 0.001; n = 48)\) for subjects at the placebo time point. In contrast, there was no significant association at baseline \((y = 0.17x + 20; r = 0.18, P = 0.21; n = 48)\).

**DISCUSSION**

Meister \((20, 21)\) showed a close association between ascorbate and glutathione in newborn rats or guinea pigs, which, as humans, do not synthesize ascorbate but must obtain it from the diet. When these animals were treated with buthionine sulfoximine, which reduces tissue glutathione to lethal concentrations, dietary supplementation of ascorbate was found to increase glutathione and prevent tissue damage and mortality. Similarly, the administration of glutathione ester to animals without dietary ascorbate increased tissue ascorbate and delayed the onset of scurvy and death. These studies, therefore, established the possibility that ascorbate and glutathione mutually spare each other. In addition, Meister proposed that glutathione was directly involved in the regeneration of ascorbate from its oxidized form, because induced glutathione deficiency was accompanied by increases in dehydroascorbate, and supplementation with dehydroascorbate did not rescue animals with low concentrations of tissue glutathione. Several studies have highlighted ascorbate glutathione synergy in rodents. Treatment of rats with \((R)\)-\(\alpha\)-lipoic acid was shown to restore ascorbate in oxidatively stressed cardiac myocytes of old rats to the concentrations observed in myocytes of young rats \((29)\). Vitamin C administration alleviated the loss of renal and hepatic glutathione in cadmium chloride–intoxicated rats \((30)\). Severe oxidative stress induced by ischemia-reperfusion in isolated rat hearts resulted in well-correlated losses of glutathione and ascorbate \((31)\). Finally, a 30% elevation in tissue ascorbate was reported in knockout mice with glutamate-cysteine ligase \((EC\ 6.3.2.2)\) deficiency and, consequently, low tissue glutathione \((32)\).

Evidence for ascorbate and glutathione synergy is lacking in humans. In general, ascorbate and glutathione tend to change in the same direction, in several human conditions. For example, ascorbate and glutathione in lymphocytes decrease during aging, and low concentrations appear to render elderly persons more susceptible to disease \((2, 33)\). In diabetes or HIV infection, below-average concentrations of plasma ascorbate correlate with low concentrations of erythrocyte or leukocyte glutathione \((34, 35)\). The concentrations of both ascorbate and glutathione in bronchoalveolar lavage in smokers are double those in non-smokers \((36, 37)\). On the other hand, cancer-affected tissues have higher concentrations of intracellular glutathione \((12)\), probably because of the induction of glutamate cysteine ligase, the rate-limiting enzyme in glutathione synthesis \((38)\). The ability of vitamin C supplements to increase erythrocyte glutathione was reported in 2 studies with small subject groups. In one study, vitamin C supplements of 500 or 2000 mg/d given for 2 wk increased glutathione in red blood cells by \(\approx 2\)-fold, even though no significant association of the 2 antioxidants was observed \((39)\). The other supplementation study reported a significant \((28%)\) increase in glutathione in red blood cells \((40)\). It is important to cite the study of a 45-mo-old girl with hereditary glutathione synthetase \((EC\ 6.3.2.3)\) deficiency and severe glutathione deficiency \((41)\). After receiving vitamin C supplementation, she had dramatic increases in the concentrations of plasma \((8\)-fold) and lymphocyte \((4\)-fold\) glutathione. Indeed, the observed increases in glutathione after vitamin C supplementation were greater than those observed with high doses of \(N\)-acetylcysteine.

The close association of ascorbate and glutathione in lymphocytes may be attributed to overlapping antioxidant functions. Ascorbate and glutathione are highly favored targets for free radicals because they exist at such a high concentration in cells and react relatively efficiently with free radical species. Assuming that ascorbate and glutathione compete for free radicals in cells, it is logical that an increase in the concentration of ascorbate will spare glutathione because ascorbate scavenges a greater percentage of free radicals. This will reduce radical-mediated losses of glutathione and thus increase its steady state concentration. In addition, ascorbate readily reacts with glutathione radicals arising from the reaction of glutathione and free radicals. Thus, ascorbate spares glutathione first by competing with glutathione for free radicals and second by converting thiol radicals back to glutathione.

Each 1-mol change in ascorbate is associated with a change of \(\approx 0.5\) mol in glutathione. The wasting of glutathione by its reaction with free radicals is consistent with the high reactivity of thiol radicals, particularly with oxygen, and the lack of efficient biochemical systems to regenerate glutathione from these species. In contrast, semidehydroascorbic radicals, resulting from reactions of free radicals with ascorbate, are relatively nonreactive and efficiently reduced to ascorbate by specific reductases \((42)\). Alternatively, the association between ascorbate and glutathione may be explained by changes in the percentage of glutathione disulfide, the oxidized form of glutathione. For example, a high concentration of ascorbate may decrease the amount of glutathione disulfide and thus lead to apparent increases in reduced glutathione. However, this possibility is unlikely because glutathione is predominantly \((>99%)\) in the reduced state in lymphocytes, and there is no association between ascorbate and oxidized glutathione \((10)\).

The association of ascorbate and glutathione in lymphocytes may in part explain the potential beneficial effects of vitamin C on health. The intracellular concentration of glutathione determines to a large extent the cellular thiol-disulfide redox potential, which regulates a variety of cell processes through disulfide bridge formation and protein glutationylation. In particular, proliferation and apoptosis are very sensitive to changes in the redox potential \((43)\). Glutathione deficiency can seriously impair immune function in lymphocytes. For example, glutathione deficiency in lymphocytes is associated with reduced immune function in HIV-infected patients, and the administration of \(N\)-acetylcysteine, which enhances intracellular glutathione, restores glutathione immune function \((16)\). The ability of vitamin C to increase lymphocyte glutathione suggests that vitamin C may also be valuable in the treatment of diseases or conditions involving glutathione deficiency. Although the effect of vitamin C on immune function is usually minor, it may be accentuated in persons with below-average...
ascorbate concentrations. For example, an association between vitamin C deficiency and delayed hypersensitivity to antigens was observed in young men receiving <20 mg vitamin C/d, which corresponds to ≈20% of the vitamin C needed to saturate the concentration of ascorbate in lymphocytes (44). In addition, improvement in immune function has been reported in elderly receiving supplementation with antioxidant cocktails containing vitamin C (45). The effect of vitamin C on the immune system may also depend on oxidative stress. In a study of ultramarathon runners (46), vitamin C supplementation reduced the number of upper respiratory tract infections from the number seen in control subjects, who did not receive supplementation. In view of the large percentage of the population that consumes <2 daily servings of fruit and vegetables and consequently has below-average concentrations of ascorbate, the potential health benefits of increasing vitamin C intake are inestimable. More studies are needed to focus on persons with below-average vitamin C concentrations.

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REFERENCES


