Vitamin C Supplementation Affects Oxidative-Stress Blood Markers in Response to a 30-Minute Run at 75% VO$_{2\text{max}}$

Allan H. Goldfarb, Stephen W. Patrick, Scott Bryer, and Tongjian You

Vitamin C supplementation (VC) (either 500 or 1000 mg/d for 2 wk) was compared to a placebo treatment (P) to ascertain if VC could influence oxidative stress. Twelve healthy males (25 ± 1.4 y) were randomly assigned in a counter-balanced design with a 2-wk period between treatments. Data were analyzed using repeated measures ANOVA. Exercise intensity measures (VO$_2$, RER, RPE, HR, lactate) were similar across treatments. Resting blood oxidative-stress markers were unaffected by treatment. Exercise decreased total blood glutathione (TGSH) and reduced glutathione (GSH) and increased oxidized glutathione (GSSG) ($P < 0.01$) independent of treatment. Protein carbonyls (PC) increased 3.8 fold in the P ($P < 0.01$). VC attenuated the PC exercise response in a dose-dependent manner ($P < 0.01$). Thiobarbituric acid reactive substances (TBARS) was not influenced by exercise ($P = 0.68$) or VC. These data suggest that VC supplementation can attenuate exercise-induced protein oxidation in a dose-dependent manner with no effect on lipid peroxidation and glutathione status.

**Key Words:** antioxidants, ascorbic acid, oxidative stress, glutathione

Aerobic exercise of sufficient intensity and duration has been reported to result in oxidative stress in numerous studies (11, 27). The increase in oxidative stress is related to a greater production of reactive oxygen species (ROS) during the aerobic production of adenosine triphosphate as well as other processes. One of the sites that can generate ROS is the mitochondria where approximately 2 to 5% of the oxygen can escape from the ubiquinone step as singlet oxygen (5). These ROS molecules can interact with cellular components that can result in cellular damage and might lead to health-related problems (13). ROS can also increase as a result of inflammatory mediated process (27, 28) and other processes (13) such as ischemia reperfusion that occurs with an acute exercise bout.

The scavenger antioxidant enzymes and antioxidants such as vitamins C and E, beta carotene, and glutathione could protect cellular components from ROS. An increase in oxidative stress markers has been reported with aerobic exercise of sufficient intensity and duration despite these protective antioxidants (27, 28).
Oxidative stress has been reported in response to exercise based on changes in antioxidant enzyme activity (15, 17), glutathione status (6, 10, 15), increased lipid peroxidation (1, 4, 15), and increased protein oxidation (18, 24, 25). These results suggest that the normal defense mechanisms in the body can be insufficient to adequately handle the increased production of ROS.

Nutritional antioxidants have been proposed to help augment the normal antioxidant protection level and help prevent damage to cellular components from ROS. Several studies have reported that pretreatment of the antioxidant vitamin E can partially protect the exercise-induced increase in markers of oxidative stress especially in lipid markers of oxidative stress (11, 12, 28). Vitamin E is a lipidsoluble antioxidant. In contrast, some of the oxidative stress markers that are in the aqueous compartment will have protection from ascorbic acid and other soluble antioxidants.

Vitamin C (ascorbic acid), an important aqueous antioxidant (8, 9), can reduce oxidants via the donation of a hydrogen ion. Plasma exposed to free radicals in vitro resulted in oxidation of lipids, proteins, thiols, and vitamin E. When vitamin C was added to the plasma, however, oxidation to these molecules was prevented or delayed (8).

Studies that examined the role of vitamin C in preventing exercise-induced oxidative stress in humans are limited. Vitamin C supplementation (1 g/d) for 2 wk minimally altered plasma thiobarbituric acid reactive substances (TBARS) concentration after 30 min of exercise at 80% VO2max compared to placebo treatment (1). In addition, the vitamin C treatment for 1 d appeared to have similar effects. Antioxidant capacity in blood at rest and after exercise, however, was unchanged with this treatment (1). Vitamin C (0.5 g/d for 8 wk) treatment was reported to increase baseline lymphocyte superoxide dismutase and catalase activity as well as attenuating the exercise-induced response to 45 min of cycling at 70% VO2peak (17).

Vitamin C (2 g/d), compared to a placebo treatment, had minimal effect on lipid peroxidation markers in 9 athletes performing a 10.5 km run (32). Vitamin C supplementation for 2 wk (0.4g/d) showed only a modest attenuation in plasma MDA compared to placebo in response to an intermittent shuttle run (30). Interestingly, all of these studies only examined plasma lipid peroxidation markers in response to the vitamin C treatment. Vitamin C supplementation (1 g) given 2 h before exercise to VO2max was shown to prevent exercise-induced oxidative stress (3). The acute vitamin C treatment prevented an increase in lipid peroxidation as measured by lipid hydroperoxides and malondialdehyde (MDA) in plasma.

In contrast, vitamin C supplementation and its role in muscle damage and influencing oxidative stress might have potential beneficial effects although this is controversial and appears to be related to dose of vitamin C and extent of muscle damage. Several studies suggest an advantageous effect (3, 16, 17, 23) whereas others report no effects (1, 10, 31). Studies that demonstrated beneficial effects, however, often did not control for the relative intensity of exercise.

The acute effect of vitamin C has had mixed results on oxidative stress to exercise. Thompson et al. reported that acute vitamin C supplementation (0.4 g for 1 d) had no effect on muscle damage and oxidative stress (30). Alessio et al. also reported that 1 d of vitamin C treatment (1 g) had a minimal effect on plasma TBARS (1). Ashton et al., however, reported that vitamin C (1 g) given 2 h before
Vitamin C Supplementation Effects with Exercise

an exercise to VO$_{2\text{max}}$ attenuated lipid peroxidation (3). Because different doses of vitamin C have been used it is unclear what dose of vitamin C would be appropriate for preventing exercise-induced oxidative stress. Levine et al. reported that plasma saturation of vitamin C occurred between 0.2 to 1g depending on the duration of the treatment and that bioavailability would decrease and increased urinary excretion would occur at the higher dose (19). It seems reasonable to examine the higher dose indicated in the Levine et al. study which also coincides with the dose in two of the previous acute studies. In addition, the dose of 0.5 g was used in the Khassaf et al. study (17) and is similar to the 0.4g dose in the Thompson et al. acute study (30).

Therefore, this study was designed to ascertain if 2 wk of vitamin C supplementation (either 0.5 or 1g/d) could influence oxidative-stress markers in response to a specific relative intensity of exercise (75 to 80% VO$_{2\text{max}}$). The secondary purpose was to ascertain if the dose of vitamin C was a factor as a potential protective agent for exercise-induced oxidative stress. A second study was conducted to confirm the vitamin C plasma levels in response to the 2 wk supplementation of vitamin C in 10 males of similar age, etc. as there were technical problems with the original samples.

**Methods**

**Subjects**

Twelve males age 18 to 35 y volunteered as subjects and gave their written and oral consent as approved by the University of North Carolina Institutional Review Board. Subjects indicated they were free from cardiovascular problems and had not taken dietary vitamin supplements for the previous 6 months. They were nonsmokers nor did they use tobacco products, or use medications known to affect the systems measured. The volunteers completed a physical activity and lifestyle questionnaire and a health history questionnaire prior to participation.

**Testing Procedures**

Each subject performed a graded maximal oxygen consumption test (VO$_{2\text{max}}$) on a motorized treadmill (model Q3000, Quinton Cardiology, Bothell, WA) using an open circuit system using Ametek O$_2$ and CO$_2$ analyzers (Ametek, Pittsburgh, PA) calibrated to known gases. Subjects reported to the laboratory in a post-absorptive state (10 to12 h), rested for at least 20 min, and were given a 5-min warm up with HR monitored by ECG rhythms. Workload was increased every 2 min and the test was stopped when the VO$_2$ or HR leveled off or the subject was fatigued. The peak VO$_2$ obtained was considered the VO$_{2\text{max}}$.

All subjects were blocked across all treatment conditions and were randomly assigned to either the placebo or vitamin C treatment in a counter-balanced order. Each subject performed 30 min of running at 75 to 80% of VO$_{2\text{max}}$ at the end of 2 wk of each treatment. Prior to and immediately after exercise, blood was drawn. Subjects exercised in a control condition prior to any treatment to familiarize them to the procedure. No difference between control and P data was obtained (data not given). The supplementation for all treatments was 2 wk taking 2 pills/d (one in the AM and one in the PM). Vitamin C (VC) was taken either 250 mg/twice per d
or 500 mg/twice per d with a minimum of 2 wk between treatments. The supple-
ments were supplied by CIBA Pharmaceuticals (Edison, NJ) and the VC was > 99%
ascorbic acid. The last treatment was taken the day before the exercise. Researchers
and subjects were blinded to the treatment.

All exercise sessions were between 0800 and 1000 h to control for diurnal
variations. Subjects abstained from caffeine, alcohol, and heavy exercise for at least
24 h preceding the tests. All subjects rested at least 20 min prior to the start of the
exercise. Heart rate, relative perceived exertion (RPE), and oxygen consumption
were monitored and recorded every 5 min. If more than 4 wk occurred prior to the
next exercise test, another VO2 max test was performed.

Three-day food records were analyzed (Nutritionist III, N-Squared Comput-
ing, Salem, OR) to see if diets were similar across treatments. Verbal instruction
and written examples concerning the procedures for recording food intake were
given to all subjects (4). Following the control test, subjects received a copy of
their food intake record and were asked to repeat that diet for the 3 d preceding
the subsequent 2 sessions. Water was provided ad libitum during the submaximal
exercise sessions. Skin folds were measured to predict the subject’s body fat percent-
age and were taken at 3 sites on the right side of the body before their maximum
aerobic capacity test (14).

**Blood Sampling and Analysis**

Blood was collected by a trained phlebotomist prior to and immediately (2 to 3 min)
after the runs from an antecubital vein into Vacutainers containing 0.1 microliters
of 15% EDTA. The blood was immediately processed and the treated samples were
stored in a –80 °C freezer until subsequently analyzed.

Whole blood (5 mL) was immediately treated with 50 mg of Chelex 100,
centrifuged at 1500 × g for 15 min at 4 °C and plasma stored for subsequent analysis
of lactate, protein carbonyls, and TBARS. Whole blood was immediately processed
for glutathione status by adding 10% 5-sulfosalicylic acid solution containing 1 mM
BPDS. Oxidized and total glutathione were determined using Anderson’s procedures
(2) as previously reported (5). Protein carbonyls were determined in plasma using
the dinitrophenylhydrazine spectrophotometric method (29) as previously described
(18). The amount of protein was determined by the Lowry assay (20).

Hemoglobin was determined (procedure no. 525, Sigma-Aldrich, St. Louis,
MO) spectrophotometrically and hematocrit measured and these values placed into
the Dill and Costill equation to adjust samples for hemoconcentration if it occurred
(7). All values are expressed as corrected for fluid shifts.

Plasma lactate was determined spectrophotometrically at 340 nm (procedure
no. 826, Sigma-Aldrich). Plasma TBARS were analyzed according to the proce-
dures by Ohkawa et al. (22).

Vitamin C was analyzed from heparinized plasma and determined immedi-
ately by the method of Lynch et al. (21). All assays were analyzed in duplicate and
compared to known standards.

**Statistical Analysis**

Variables that were measured during each exercise session (HR, RPE, VO2, RER)
were analyzed using a 1 × 4 repeated measures analysis of variance (AÑOVA).
All blood measures were analyzed using a repeated measures analysis of variance (2 times × 3 conditions). Each variable was compared across the three different conditions (P, VC500, and VC1000). The data were analyzed by an SPSS statistical package (version 11.5, SPSS, Inc., Chicago, IL). The level of significance was set at an alpha level ≤ 0.05. Post hoc analysis was performed using the Tukey HSD analysis where appropriate. The data are presented as means ± standard error.

Results

The characteristics of the subjects are presented in Table 1. The subjects were aerobically fit and slender, and of normal height and weight. Analysis of the 3-d food records indicated no significant difference across treatments (P > 0.05). The subjects ingested approximately 2470 ± 378 Kcals/d with similar percent of the macronutrients [carbohydrate (57 ± 6%), fat (29 ± 4%) and protein (15 ± 2%)] across treatments. Vitamin C content in their diet was similar across treatments (189 ± 84 mg).

The subjects had almost 100% compliance with the C treatment, with only 1 subject missing 3 P pills during the first week of supplementation. Only 2 subjects needed to have their VO2max reassessed and these subjects had no change in their VO2max.

Table 2 presents the physiological variables measured during the different exercise sessions averaged over time and the post-exercise lactate level. No differences were detected for any variable with condition. Post-exercise plasma lactate, HR, RPE, RER, and relative intensity during the exercise were similar across treatments. Therefore, vitamin C supplementation had no influence on these parameters during and immediately following the exercise.

The oxidative stress markers in the blood in response to treatment and exercise are presented in Figures 1 through 3. The ratio of oxidized glutathione to total glutathione is presented in Figure 1. There was a similar significant increase in the ratio of oxidized to total glutathione after exercise independent of treatment. Total blood glutathione significantly decreased with exercise to a similar extent across treatments.

Table 1 Characteristics of Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± standard error</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td>25.0 ± 1.4</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176.3 ± 2.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.4 ± 2.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.05 ± 0.7</td>
</tr>
<tr>
<td>% body fat</td>
<td>9.48 ± 0.9</td>
</tr>
<tr>
<td>VO2max (mL · kg⁻¹ · min⁻¹)</td>
<td>50.17 ± 1.8*</td>
</tr>
</tbody>
</table>

Note. N = 12 for all variables. Values were obtained during first VO2max for all subjects. *average value for all subjects (2 subjects averaged measured twice).
Table 2  Physiological Variables During Each Exercise Bout

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>500 mg VC</th>
<th>1000 mg VC</th>
</tr>
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<tbody>
<tr>
<td>% VO_{2max}</td>
<td>75.3 ± 1.1</td>
<td>75.3 ± 1.0</td>
<td>75.99 ± 1.0</td>
</tr>
<tr>
<td>RER</td>
<td>0.90 ± 0.01</td>
<td>0.90 ± 0.01</td>
<td>0.89 ± 0.01</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>174.1 ± 2</td>
<td>172.9 ± 2</td>
<td>172.0 ± 2</td>
</tr>
<tr>
<td>RPE</td>
<td>14.0 ± 0.6</td>
<td>13.7 ± 0.5</td>
<td>13.6 ± 0.5</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>3.47 ± 0.69</td>
<td>3.59 ± 0.62</td>
<td>3.56 ± 0.65</td>
</tr>
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</table>

*Note. N = 12. Values are means ± standard error. Values were averaged each 5-min during the 30-min exercise except the lactate value (post-exercise value).*

**Figure 1** — Glutathione ratio response to vitamin C supplementation at 75% VO_{2max} for 30 min. GSSG, oxidized glutathione; TGSH, total glutathione, * = significant difference compared to rest for same treatment.

Blood Glutathione ratio in response to vitamin C supplementation

Figure 1 — Glutathione ratio response to vitamin C supplementation at 75% VO_{2max} for 30 min. GSSG, oxidized glutathione; TGSH, total glutathione, * = significant difference compared to rest for same treatment.

Independent of condition. Reduced glutathione (GSH) decreased significantly after exercise independent of condition (range, 41 to 47% for condition). Oxidized glutathione (GSSG) significantly increased in response to the exercise independent of condition. Vitamin C did not alter the exercise-induced response.

Plasma TBARS response to treatment and exercise is presented in Figure 2. Plasma TBARS were similar at rest independent of treatment. There was no
change in plasma TBARS with exercise and there was no treatment effect. Protein carbonyl response to treatment and exercise is presented in Figure 3. Plasma protein carbonyls at rest were similar across treatments. Exercise significantly increased plasma protein carbonyls for all treatments. There was an attenuated PC response to exercise with the 2 VC treatments. Exercise increased protein carbonyls about four-fold with P. There was a significant attenuation of PC at both VC doses after
exercise with each VC dose significantly different from each other. The increase in PC with the higher VC treatment was only 27% of that compared to the P trial.

In the second study, that aimed to confirm changes in plasma vitamin C levels associated with vitamin C supplementation, vitamin C concentration at rest for the P was 30.4 ± 2.1 uM whereas plasma vitamin C concentration was 50.5 ± 5.8 and 63.2 ± 7.1 uM for the 0.5 and 1 g treatments, respectively.

Discussion

The present investigation is the first to our knowledge to demonstrate that vitamin C supplementation at 0.5 and 1 g/d for 2 wk can attenuate oxidative stress as indicated by plasma protein carbonyls in response to a specific exercise intensity in a dose-dependent manner. This study also reports that this same amount of VC supplementation did not significantly influence two other oxidative stress markers (TBARS and glutathione). The exercise bout increased the amount of glutathione in the oxidized state independent of treatment. It also reports that the indirect marker of lipid peroxidation (TBARS) was unaffected by either the exercise or the treatment. Therefore, this study suggests that 2 wk of VC supplementation can protect protein oxidation in what appears to be a dose-dependent manner to a 30 min exercise run at 75% VO2max without influencing blood glutathione status. It also reports that the VC treatment had no influence on the physiological measures obtained during the exercise and immediately after the exercise. It should be noted, however, that the dietary level of vitamin C for these subjects was well above the recommended dietary allowances (RDA).

Several studies have suggested that vitamin C is an important antioxidant in vitro (8, 9). There are equivocal findings with vitamin C supplementation and its potentially beneficial antioxidant role in reducing exercise-induced oxidative stress, in part because the supplement dose has varied and because the intensity of the exercise has typically not been controlled (3, 17, 23, 30). Another potential confound that could influence the results of vitamin C supplementation is the dietary consumption of vitamin C by the subjects. We tried to control this in the present study by asking the subjects to maintain their normal diets and reproducing their diets. The subjects in the present investigation had similar vitamin C in their diets across treatments but their vitamin C consumption was well above the RDA.

Vitamin C supplementation over a period of 1 to 2 wk has ranged from 200 to 3000 mg/d to attenuate exercise-induced oxidative stress (28). In general, 1 d of vitamin C supplementation does not appear to be beneficial. Equivocal results with vitamin C supplementation for a longer time period, however, have been noted with exercise-induced oxidative stress. Sastre et al. reported that vitamin C supplementation in rats could attenuate the exercise-induced decrease in GSSG without any change in GSH (26). The results of the present investigation do not support this finding in humans. It is possible that rats might respond to the vitamin C supplementation differently than humans and the rats ran to exhaustion whereas our subjects ran at a specific relative intensity for 30 min. In addition, rats can synthesize vitamin C whereas humans cannot.

Alessio et al. supplemented their subjects with 1 g of vitamin C and reported a modest attenuation in plasma TBARS in response to a similar but slightly higher exercise intensity (1). The present study does not confirm their findings with
TBARS. In fact, TBARS, which is a nonspecific marker of lipid peroxidation, did not change independent of treatment as well as exercise in the present study. Thompson et al. reported that a dose of 400 mg of ascorbic acid given twice per day for 12 d attenuated the MDA response to a shuttle run which elicited muscle damage (31). The authors noted that the vitamin C supplementation was stopped 36 h prior to the exercise to avoid the acute effects of vitamin C. Ashton et al. reported that an acute dose of 1 g given 2 h prior to a maximal VO2 test was able to reduce lipid hydroperoxides and MDA (3). It should be noted there was a modest increase in the MDA with this exercise and that the acute vitamin C dose prevented this increase. Subjects in the present study did not report muscle soreness associated with the exercise. Therefore, the MDA rise in Thompson et al. study (30) might have been related to muscle damage/soreness processes as opposed to the metabolic oxidative stress in the present study. In addition, the Ashton et al. study used a higher intensity and a larger dose of vitamin C (3). Vitamin C supplementation of 2 g/d did not, however, alter serum-conjugated diene concentration after a 10.5 km run compared to placebo (33). These studies taken together suggest that the type and intensity of exercise as well as the dose of vitamin C can vary the response of lipid peroxidation.

It is interesting to note that the plasma protein carbonyls were increased in response to this type of exercise and that the vitamin C attenuated this response in what appears to be a dose response. Plasma protein carbonyls were significantly elevated in response to this exercise-induced stress. This response was reproducible in comparing the control and placebo trials (data not shown). Previous studies have reported increases in protein carbonyls in response to damaging exercise in humans (18) and in animals (25). This is the first study that we are aware of that reports increased plasma protein carbonyls without significant muscle damage/soreness in humans in response to exercise.

The higher vitamin C treatment significantly attenuated this response but did not completely prevent protein oxidation in response to this exercise. In addition, the lower dose of vitamin C also attenuated the exercise-induced oxidation of proteins but to a lesser extent. It is possible that the vitamin C protected the oxidation of the proteins but it is unclear where this protection occurred. It is possible that some of the proteins originated from exercised muscles, but future research is needed to confirm the origin of these oxidized proteins. It is unknown if the muscle vitamin C concentration was a factor. Based on the data by Levine et al. (19), however, the concentration of vitamin C as measured in circulating immune cells plateaus at doses of 100 mg or higher. It is unclear if the concentration of circulating immune cells reflects intracellular vitamin C status in other tissues. Based on the second study, the plasma vitamin C levels were significantly elevated in the subjects after the 2 wk of vitamin C supplementation. Although the 1000 mg treatment was slightly higher than the 500 mg treatment this was not significantly different. The values obtained from our subjects are similar but slightly lower than the values reported by Levine et al. (19). This could be related to the fact that our subjects stopped taking supplements the day before the exercise and some of the vitamin C in the circulation should have been reduced over this time frame.

The performance factor results demonstrated that vitamin C supplementation in healthy well-nourished males did not alter heart rate, oxygen consumption, and blood lactate responses at this specific exercise intensity. This supports the findings
by van der Beek et al. that acute vitamin C deficiency did not influence physical performance (32). This study suggests that chronic vitamin C ingestion will not alter the exercise response to heart rate, oxygen consumption, perceived exertion, and post-exercise blood lactate levels if exercise intensity is controlled.

In the present study, the diets of the subjects were monitored and the subjects were told to reproduce their diets. The subjects consumed similar amounts of macronutrients across treatments. The subjects had similar vitamin C consumption across conditions but had greater than the suggested RDA under all 3 conditions. It is possible that the higher dietary vitamin C level could have attenuated the exercise-induced oxidative-stress response. The glutathione and protein carbonyl data suggest, however, that these subjects were not adequately protected without the additional vitamin C supplementation despite consuming vitamin C greater than the RDA.

Numerous investigations have reported a significant increase in blood GSSG levels following exercise (6, 12, 15, 27). The decline in reduced glutathione and the increase in oxidized glutathione were similar to the results in women exercising for a similar time and intensity (6). Other studies have reported variable responses in the glutathione changes depending on the intensity, duration, and type of subject (27). The glutathione results in the present study are similar to other studies which utilized similar workloads (6, 12).

It is unclear why the vitamin C supplementation had no effect on the glutathione system. Our results in humans and the results in rats suggest that vitamin C supplementation does not prevent the exercise-induced changes in glutathione with exercise (10, 26). It has been reported that vitamin C could protect plasma components in vitro and can also regenerate oxidized vitamin E (8). Protein thiols and other substances were swiftly oxidized in the absence of vitamin C in the in vitro study, however, when vitamin C was added these substances were protected from ROS. It should be noted that proteins were protected from ROS with the vitamin C supplementation in the present study. The absorption and distribution of vitamin C in vivo might be a factor to consider as to why proteins and not the glutathione system were protected. The reason for the protection of proteins by vitamin C against ROS as opposed to the glutathione system in blood remains to be elucidated.

In conclusion, the present study demonstrates that 2 wk of vitamin C supplementation can partially protect against exercise-induced oxidative stress in humans as indicated by an attenuated increase in plasma carbonyls. In addition, there appears to be a dose response in the protection of these proteins. This protection in plasma proteins to oxidation occurred without similar changes in blood glutathione status.

References