SUPPLEMENTATION WITH VITAMIN C AND N-ACETYLCYSTEINE INCREASES OXIDATIVE STRESS IN HUMANS AFTER AN ACUTE MUSCLE INJURY INDUCED BY ECCENTRIC EXERCISE

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Abstract—There has been no investigation to determine if the widely used over-the-counter, water-soluble antioxidants vitamin C and N-acetyl-cysteine (NAC) could act as pro-oxidants in humans during inflammatory conditions. We induced an acute-phase inflammatory response by an eccentric arm muscle injury. The inflammation was characterized by edema, swelling, pain, and increases in plasma inflammatory indicators, myeloperoxidase and interleukin-6. Immediately following the injury, subjects consumed a placebo or vitamin C (12.5 mg/kg body weight) and NAC (10 mg/kg body weight) for 7 d. The resulting muscle injury caused increased levels of serum bleomycin-detectable iron and the amount of iron was higher in the vitamin C and NAC group. The concentrations of lactate dehydrogenase (LDH), creatine kinase (CK), and myoglobin were significantly elevated 2, 3, and 4 d postinjury and returned to baseline levels by day 7. In addition, LDH and CK activities were elevated to a greater extent in the vitamin C and NAC group. Levels of markers for oxidative stress (lipid hydroperoxides and 8-iso prostaglandin F$_{2\alpha}$; 8-Iso-PGF$_{2\alpha}$) and antioxidant enzyme activities were also elevated post-injury. The subjects receiving vitamin C and NAC had higher levels of lipid hydroperoxides and 8-Iso-PGF$_{2\alpha}$ 2 d after the exercise. This acute human inflammatory model strongly suggests that vitamin C and NAC supplementation immediately post-injury, transiently increases tissue damage and oxidative stress. © 2001 Elsevier Science Inc.

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INTRODUCTION

It is well established by in vitro experiments that vitamin C is reactive with free iron and produces the ascorbate radical [1–3], while causing oxidative damage to biomolecules [1,4,5]. In humans, scientists have claimed increases in DNA damage in healthy humans supplemented with vitamin C and iron salts [6], as well as ascorbyl radical formation in subjects with sepsis following ascorbate loading [7]. Podmore et al. [8] supplemented subjects with 500 mg/d of ascorbate and showed a drop in the content of 8-hydroxyguanine in lymphocyte DNA, however, there was a concurrent increase in 8-hydroxyadenine, a mutagenic base that causes adenine → guanine transitions and adenine → cytosine transversions. Although not as well documented as vitamin C, several reports suggest a pro-oxidant or adverse effect from N-acetyl cysteine (NAC) in vitro and in vivo [9–12]. However, other studies performed show that vitamin C and NAC are protective antioxidants that can prevent oxidative stress [1,13–19]. Therefore, there is still debate on whether supplements such as vitamin C and NAC could act as pro-oxidants in vivo. Moreover, no human or animal investigations have determined if immediately after an acute inflammation—characterized by release of free iron from its normal sequestration sites—these water-soluble antioxidants could cause oxidative stress.

Under normal physiological circumstances, metals are bound to circulating proteins and are rendered redox inactive [1,20,21]. However, levels of free metal ions, including iron, may be elevated during acute inflamma-
tion and sepsis, becoming redox-active [1,22]. Inflammation stimulates polymorphonuclear leukocytes and macrophages that produce large amounts of superoxide (O$_2^{-}$) and hydrogen peroxide (H$_2$O$_2$) [1,24]. The detrimental effects of these species may be amplified in the presence of iron by the subsequent formation of other reactive intermediates, such as the hydroxyl radical (HO•). The hydroxyl radical has been widely postulated to cause significant damage to several biomolecules in vivo. The relevance of the hydroxyl radical in biology has been questioned because of the requirement of redox-active catalytic iron [21]. However, Biemond et al. have shown iron release from ferritin during inflammation [22]. In addition, Gutteridge et al. [23] show release of iron from hemoglobin following treatment with hydrogen peroxide.

The reduction potentials of Fe$^{+3}$ (-0.4 V) and vitamin C (-0.17 V) easily allow the formation of the ascorbate radical and Fe$^{+2}$ iron. Therefore, in vitro vitamin C can exert pro-oxidant effects by converting Fe$^{+3}$ into Fe$^{+2}$, which reacts with H$_2$O$_2$ to generate HO• [1,25]. Iron-ascorbate mixtures have been shown to stimulate free radical damage to DNA, lipids, and proteins in vitro [21]. In vivo, iron [26] supplementation and ascorbate-copper supplementation [27] to rats have been reported to stimulate HO• generation. In addition, mixtures of thiols with transition-metal ions can be cytotoxic because of reactions that produce O$_2^{-}$, H$_2$O$_2$, HO•, and sulphur-containing radicals such as RS$^-$ and RSO• [1,28]. It is feasible that the release of iron and the presence of vitamin C and NAC during acute inflammation could lead to HO•, ascorbate•, and thiol radical generation.

Inflammation can be induced by acute exercise in unadapted subjects and can increase the levels of free iron [29]. Several types of exercise damage enzymes and lipid membranes, increase DNA damage, stimulate oxidative stress, and increase plasma markers of cell damage [30–37]. Specifically, eccentric exercise leads to a condition characterized by severe inflammation and edema. Examples of eccentric exercises are downhill running and eccentric arm exercises, which have been shown to increase neutrophil migration into the skeletal muscle after such injury [33–35,37]. In the present study, we used an eccentric exercise protocol to induce a severe injury to a human arm muscle. Severe inflammation, pain, and a decrease in range of motion characterize this injury. This condition provides an informative experimental human model for evaluation of compounds believed to exhibit antioxidant properties. Supplements increased lipid peroxidation products, superoxide dismutase, glutathione peroxidase, markers of muscle damage, and increased bleomycin-detectable iron in the serum above levels of subjects receiving a placebo. Therefore, intake of these two supplements immediately following acute inflammatory conditions may not be advisable.

**Materials and Methods**

**Subjects and supplementation**

Fourteen healthy, nonsmoking, untrained young males from the University of Florida (24.4 ± 3.6 years, 178.3 ± 6.5 cm, 78.0 ± 10.8 kg) were recruited for participation in this study. Subjects were free of vitamin/mineral supplementation for 6 weeks prior to the study. Persons involved in a regular weight-training program or with a prior history of injury to the biceps brachii or elbow region were excluded. This was a double-blind, placebo-controlled study in which subjects received either 12.5 mg vitamin C and 10 mg of NAC per kg body mass in a powdered drink mix not containing any minerals and/or supplements (Powerade, Atlanta, GA USA), or the drink mix alone immediately after and each day following the exercise for 7 d.

**Exercise protocol**

The Cybex arm curl machine (Cybex International, Inc., Medway, MA, USA) was used for the eccentric exercise protocol. Subjects performed three sets of 10 repetitions using 80% of their eccentric 1 repetition maximum using only the nondominant arm. Subjects were given 2 min rest periods between sets. Severe pain and edema characterize this type of exercise-induced injury for several days post-injury. The University of Florida’s institutional review board approved of all methods and procedures used in this study and subjects signed an informed consent before participation.

**Blood collection**

Blood was taken from the cubital vein of the uninjured arm just before the exercise (Day 0) and on days 2, 3, 4, and 7 following the eccentric injury. Blood was not collected on day 1, because preliminary experiments showed peaks of LDH and CK between days 2 and 4. Blood was collected into Vacutainer tubes containing ethylenediaminetetraacetic acid (K$_2$EDTA: 8.4 mg/Vacutainer) or into serum collection tubes. Blood was then centrifuged at 4°C at 1500 × g for 5 min. Plasma was allocated to storage tubes containing 100 μM butylated hydroxytoluene (BHT) and 100 μM diethylenetriamine pentaacetic acid (DTPA). EDTA serves as a calcium and metal chelator, DTPA serves as a metal chelator, and BHT acts as a chain-breaking antioxidant to prevent lipid peroxidation ex vivo. Samples were stored immediately at −80°C in multiple aliquots. Previous experiments
show that samples stored during the same time period in the presence of antioxidants had lower baseline levels compared to samples not containing these antioxidants. We stored samples in multiple aliquots (~0.25 ml) and each sample was thawed only once and immediately analyzed for lipid peroxidation content. This precaution was taken because prior experimentation indicated that samples subjected to freeze-thaw even once, showed increases in baseline lipid peroxidation products.

**Bleomycin detectable iron**

The method of Evans and Halliwell [38] was used for measurement of bleomycin-detectable iron (BDI) present in the serum. Bleomycin in the presence of ferrous iron degrades DNA to form thiobarbituric acid-reactive products. Degradation by bleomycin is dependent on the concentration of total chelatable redox-active loosely bound, or “free” iron. Therefore, the rate of degradation of DNA by bleomycin can be used to measure the concentration of catalytic iron in biological fluids.

**Total antioxidant status**

Total antioxidant status of the serum was measured using methods developed by Randox laboratories (Crumlin, UK). The method is based on the formation of 2’-2’-Azino-di-[3-ethylbenzthiazoline sulphonate] radical (ATBS). This has a stable blue-green color, which is measured at 600 nm. The levels of antioxidants in the serum cause a suppression of this color to a degree, which is proportional to their concentration.

**Inflammatory markers**

To ensure that inflammatory cells were increased in the blood, we measured myeloperoxidase and the cytokine interleukin-6. Myeloperoxidase was measured using an enzyme-linked immunosorbent assay (ELISA) system (OXIS International, Portland, OR, USA). Interleukin-6 was determined using an ELISA assay (Endogen Laboratories, Woburn, MA, USA).

**Markers of cellular damage**

Both creatine kinase (CK-Kit-DG-1340-K) and lactate dehydrogenase (LDH-Kit-DG147-K) were measured spectrophotometrically at 340nm (Sigma Chemicals, St. Louis, MO, USA). Myoglobin was measured using an ELISA (Bethyl Laboratories, Montgomery, TX, USA). Human myoglobin was used to establish a standard curve.

**Antioxidant enzymes**

Superoxide dismutase (SOD) was measured in the serum following the technique described by Oyanagui [39]. Selenium-dependent glutathione peroxidase (GPX) was measured in the serum following the method described by Flohe and Gunzler [40] using t-buty! hydroperoxide and GSH as substrates.

**Markers of lipid peroxidation**

Lipid hydroperoxides were measured with a spectrophotometric assay from Cayman Chemicals (Ann Arbor, MI, USA). Lipid hydroperoxides were extracted into chloroform and detected at 500 nm. 8-iso prostaglandin F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$) level was measured by a commercially available kit from Cayman Chemical. 8-iso-PGF$_{2\alpha}$ was measured using an extraction process followed by the ELISA procedure. The antibody was highly specific for 8-iso-PGF$_{2\alpha}$, currently classified as 15-F$_{2\alpha}$-IsoP.

**Protein determination**

Protein concentration was determined using the Bradford method [41].

**Statistical analysis**

All biochemical analyses were performed in duplicate or triplicate and the mean was used for statistical analysis. A two-way analysis of variance (ANOVA) with repeated measures for the time component of the experiment was performed. Bonferroni post-hoc analysis was used where appropriate. Statistical significance was set at $p < .05$. Data were analyzed using a statistical package from Prism (San Diego, CA, USA).

**RESULTS**

**Serum free iron levels are increased after eccentric injury**

Bleomycin detectable iron (BDI)—potentially catalytic for free radical reactions—was measured in the serum immediately postexercise (Day 0) and for 7 d following the injury (Fig. 1). Day 0 indicates blood taken immediately before the eccentric exercise. Levels of BDI were significantly elevated above preinjury levels in both groups ($p < .01$) on days 2, 3, and 4. Day 7 showed that levels of BDI returned to baseline. Importantly, the amount of bleomycin-detectable iron in the serum of supplemented subjects (Vit C + NAC) was significantly higher compared to the placebo group ($p < .05$).
Plasma total antioxidant status is increased with vitamin C and NAC supplementation

To test whether the supplements did lead to increased levels of antioxidants in the plasma, total antioxidant status was quantified (Fig. 2). Total antioxidant status was measured prior to the injury and for 7 following the eccentric exercise-induced injury. Supplemented subjects showed a significant increase \((p < .0001)\) in their total antioxidant status on days 2, 3, 4, and 7. The placebo group show no significant change in the total antioxidant status.

Markers of inflammation in the plasma are increased after eccentric injury

In order to quantify the severity of inflammation, we measured interleukin-6 (IL-6) and myeloperoxidase (MPO) in plasma (Table 1). Myeloperoxidase was significantly elevated postinjury on day 2 \((p < .0001)\) in the placebo group. The group receiving the supplement also showed a significant increase in MPO on day 2, however, levels were significantly \((33\%)\) lower \((p < .05)\) than the placebo group. In addition, MPO levels in the placebo group remained above baseline around day 7, whereas MPO levels in subjects receiving the supplements returned to baseline around day 7. Furthermore, interleukin-6 was significantly elevated in both groups above preinjury levels on day 2 \((p < .001)\) and returned close to baseline levels by day 7. There were no significant differences in the levels of IL-6 between the placebo and supplemented groups.

Plasma markers of lipid peroxidation are elevated in the vitamin C and NAC group after eccentric injury

Two markers of lipid oxidation were quantified in the plasma: lipid hydroperoxide (LOOH), and 8-iso prostaglandin F\(_{2\alpha}\) (8-Iso-PGF\(_{2\alpha}\)) (Fig. 3). LOOH is a marker formed in the initial stages of lipid peroxidation [1] and 8-iso-PGF\(_{2\alpha}\) is a specific marker formed by nonenzymatic mechanisms from arachidonic acid oxidation [42]. LOOH levels were significantly elevated postinjury in both groups on days 2, 3, and 4 \((p < .0001; \text{Fig. 3A})\). Supplementation with vitamin C + NAC yielded significantly higher levels of LOOH on days 2 and 3 \((p < .001)\). LOOH levels were not significantly different on day 7 compared to baseline levels (Day 0). 8-Iso-PGF\(_{2\alpha}\) was significantly increased \((p = .050)\) after the injury in both the placebo and vitamin C + NAC group (Fig. 3B). In addition, vitamin C and NAC supplementation tended to exacerbate the levels of 8-iso-PGF\(_{2\alpha}\) in the plasma \((p = .07)\). In summary, both markers of lipid peroxidation were elevated with time after the eccentric injury, whereas Vit C + NAC supplementation increased levels of LOOH significantly, and 8-Iso-PGF\(_{2\alpha}\) also tended to be increased in this group.

Plasma antioxidant enzymes are elevated in the vitamin C + NAC group after the injury

We measured the activity of plasma superoxide dismutase (SOD) and glutathione peroxidase (GPX) because they have been reported to increase after acute exercise and in response to chronic oxidative stress [43, 44]. Plasma SOD activity (Fig. 4A) was significantly elevated in both groups after the injury \((p < .05)\).
Furthermore, SOD activity was significantly higher in the vitamin C and NAC group \((p < .05)\). Plasma GPX activity (Fig. 4B) was not significantly elevated above baseline levels, however, on day 2, there were significantly higher activity levels in the supplemented group \((p < .05)\) compared to placebo.

Enzymatic and nonenzymatic markers of muscle damage in the plasma are elevated post-injury

All markers used to determine cellular damage showed an expected increase after the injury (Fig. 5). Plasma CK was significantly elevated above pre-injury levels in both groups on days 2, 3, and 4 \((p < .0001)\), and levels tended to be higher on days 2, 3, and 4 in the Vit C + NAC group \((p = .0981;\) Fig. 5A). Plasma LDH activity levels (Fig. 5B) in both groups was significantly elevated from pre-injury levels on days 2, 3, and 4 \((p < .0001)\). In addition, the group receiving antioxidant supplementation had significantly higher levels of LDH than the group receiving the placebo treatment \((p = .0326)\). Both CK and LDH returned to baseline values by day 7.

We determined the levels of myoglobin (Fig. 5C), a nonenzymatic marker indicative of muscle damage, in the serum. Myoglobin was significantly elevated post-injury in both groups on days 2, 3, and 4 \((p < .0001)\). Levels of myoglobin returned to baseline by day 7 postinjury. In contrast to enzymatic markers of muscle damage, myoglobin tended to be decreased in the supplemented groups \((p = .13)\). Myoglobin’s release compared to LDH and CK appears different with vitamin C and NAC supplementation. It may be that this injury affected multiple cell types, such as smooth muscle cells, endothelial cells, and muscle cells.

Pain and range of motion in injured arm

We also assessed subjective pain perceived by the subjects and the range of motion of the injured arm before and after the injury. Pain was measured using a visual analogue scale and active arm flexion was determined using standard goniometry. Pain was significantly increased after the injury and peaked after 2 d, but minimal pain was perceived after 7 d (data not shown). Range of motion of the injured arm was significantly reduced after the injury and returned to similar degrees of initial range of motion after 7 d. There were no differences between groups in either pain or range of motion.

<table>
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<tr>
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<th>Day 0</th>
<th>Day 2</th>
<th>Day 7</th>
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<td><strong>Myeloperoxidase</strong></td>
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<tr>
<td>Placebo</td>
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<td>36.2 ± 4.3*</td>
<td>17.5 ± 4.1*</td>
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<tr>
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<td>25.2 ± 4.8†</td>
<td>10.4 ± 3.3†</td>
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<tr>
<td><strong>Interleukin-6</strong></td>
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<tr>
<td>Placebo</td>
<td>1.1 ± 0.16</td>
<td>4.4 ± 1.25*</td>
<td>2.62 ± 0.55*</td>
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<tr>
<td>Vitamin C + NAC</td>
<td>1.6 ± 0.24</td>
<td>4.6 ± 0.63</td>
<td>1.97 ± 0.30</td>
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Mean ± SEM of myeloperoxidase (ng/ml) and interleukin-6 (pg/ml). \(*p < .0001\) different from day 0. †\(p < .05\) different from placebo.

Fig. 3. Levels of plasma (A) lipid hydroperoxides and (B) 8-iso prostaglandin F\(_{2\alpha}\) (8-Iso-PGF\(_{2\alpha}\)), of healthy human volunteers receiving either a placebo \((n = 7)\) or an antioxidant supplement \((n = 7)\).

*Significant differences from baseline (Day 0) in both placebo group and Vit C + NAC group \((LOOH p < .001; 8-Iso-PGF_{2\alpha}; p = .05)\).
†\(p < .01\); significant differences between groups. Data are represented as the mean ± SEM.
motion assessment (data not shown). In summary, pain and range of motion were significantly increased after the injury, but the supplement intervention did not affect these basic physiological parameters.

DISCUSSION

The well-established antioxidant vitamin C (ascorbic acid) has been credited with benefits in many human diseases such as atherosclerosis, cancer, and cataract [45–47]. However, it has been suggested that under certain conditions, vitamin C may act as a pro-oxidant due to the high reactivity of vitamin C with transition metals, including iron [1,7,20,23,49]. N-acetyl-cysteine, a thiol antioxidant, has also been documented in vivo and in vitro to act as a pro-oxidant, although in most studies it is an effective antioxidant [1,9–19]. The main findings in this study indicate that supplementation with vitamin C and N-acetyl-cysteine (NAC), after severe and acute inflammation caused by eccentric exercise, increases oxidative stress and cell damage above levels induced by the injury alone. Increases in free metals, immediate intake of supplements, and activation of peroxide-producing cells could be responsible for the increased levels of oxidative stress and cell damage in the subjects receiving the supplements.

Eccentric exercise has been shown to increase the amount of redox-active free iron [29]. Tissues and blood have evolved to avoid iron toxicity by producing several
proteins that tightly bind iron. Transferrin has a high affinity for iron and ferritin has a high storage capacity [1,20,49]. Besides transferrin and ferritin, there are a wide variety of other proteins, such as albumin, which bind to iron [1,49]. We found a significant increase in serum bleomycin-detectable catalytic iron in both exercise groups after the injury. The activation of inflammatory cells might be responsible for the observed increases in serum catalytic iron. Indeed, it has been shown that O2•− derived from polymorphonuclear leukocytes increases the release of catalytic iron from ferritin and myoglobin [22,48,50,51]. We found that myoglobin (released from damaged muscle cells) was elevated in the serum in both treatment groups. In vitro oxidant systems release approximately 1 and 10% of the iron from ferritin and myoglobin, respectively, which suggests that myoglobin is susceptible to oxidant stress [22,50,52,53]. The greater levels of bleomycin-detectable catalytic iron in the vitamin C + NAC subjects could be due to a direct effect of vitamin C in helping to mobilize iron from its stores (i.e., ferritin) [1,49].

It is also well known that eccentric exercise causes severe damage to muscle cells and consequently stimulates an inflammatory response [34,36,54–56]. In addition, muscle injury induced by exercise has been shown to increase levels of myeloperoxidase (MPO) in blood and muscle tissues [33,37], as well as markers of lipid peroxidation [36]. This leads to acute necrotic myopathy, edema, and severe pain in skeletal muscles. In our study, MPO was significantly increased in both treatment groups after the injury confirming that the injury did stimulate a significant neutrophilic response. Although the supplements significantly reduced the amount of MPO in the plasma, it appears that the oxidants generated from various sources interacted with the increases in free iron and the available vitamin C and NAC to cause oxidative stress. Increases of interleukin-6 (IL-6) seen in our study demonstrate the increased activity of immune cells, such as macrophages, which may be partly responsible for the observed injury to skeletal muscle. Others also show increases in IL-6 in plasma and mRNA for IL-6 in muscle biopsies after a marathon race [56]. The activity of IL-6 producing cells, such as macrophages, does not appear to be affected by the supplement.

Lipid hydroperoxide levels were significantly higher in the supplemented group around days 2 and 3, whereas 8-isoprostanates F2α (8-Is-PGF2α) showed a delayed (after 3 d) increase in the supplemented group. Increases in both LOOH and 8-Is-PGF2α demonstrate that there is a significant amount of oxidative stress, which is reduced after 7 d. It is possible that the supplements accelerate LOOH and 8-Is-PGF2α formations due to the interaction of vitamin C with free iron and oxidants. 8-Is-PGF2α are found at very low baseline levels and originate by nonenzymatic oxidation from arachidonic acid residues [42]. In addition, these products are inflammatory mediators and may contribute to further tissue injury. We also measured malondialdehyde (MDA) levels—a nonspecific marker of lipid peroxidation using the thiobarbituric acid reactive substance test—and found significant increases after the injury, but no alteration due to the supplements (data not shown). The baseline levels of MDA were much greater in plasma compared to 8-Is-PGF2α and lipid hydroperoxides. The respective ratios of 8-Is-PGF2α to LOOH to MDA were approximately 1 to 25,000 to 7,000,000 parts, markedly decreasing the specificity and sensitivity of MDA.

Besides increases in LDH and CK levels in the plasma after the acute injury, there were also increases found in superoxide dismutase and glutathione peroxidase activity. In addition, it appears that vitamin C and NAC supplemented subjects increased the enzyme activity to a greater extent as compared to the placebo group. Increased levels of these enzymes could have been directly due to the injury and/or oxidative stress of membranes causing increased enzyme leakage.

The increased levels of lipid peroxidation products and enzymatic markers of muscle damage in the supplemented subjects after the injury may be significant. These subjects exhibit severe edema, pain, and loss of range of motion in the injured arm, but when comparing parameters of subjective pain and arm range of motion each day after the injury, no significant differences between the two treatment groups were found. Therefore, it is unclear if the greater amount of oxidative stress and muscle damage had short-term physiological consequences in this human model. Additional indices for physiological function, such as noninvasive measurements of ATP levels using NMR techniques and muscle strength tests need to be assessed in future studies.

Intake of these supplements for long periods of time may have harmful effects in certain chronic disease conditions characterized by increased levels of free iron and inflammation. There are mixed results regarding the effect of vitamin C and iron in human studies. One study [57] investigated whether the naturally occurring high levels of ascorbic acid in preterm infants, along with the presence of detectable levels of iron, would increase oxidative stress. This study showed no concurrent increases in either lipid hydroperoxides or protein carbonyls in the plasma of these infants. In contrast, others suggest that cosupplementing healthy volunteers with iron and vitamin C increased levels of oxidative DNA damage in white blood cells [6]. They concluded that the increased levels of DNA damage evident in well-nourished subjects after iron/ascorbate supplementation were disturbing in view of the frequent use of dietary supplements containing both iron salts and ascorbate [6].
In summary, this in vivo human model holds promise to further test the interactions of antioxidants and/or supplements during acute inflammatory conditions. This study does not argue to discontinue supplementation with either vitamin C or NAC in healthy humans, however, further studies are required to study the effects of chronic use of these supplements with disease conditions characterized by increased levels of free iron and inflammation.

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REFERENCES


