

## AN ABSTRACT OF THE THESIS OF

Dawn Marie Weseli Hopkins for the degree of Master of Science in Human Performance presented on February 19, 2003. Title: Inflammation, Immune Suppression, and Iron Status in Endurance Athletes and the Effects of Antioxidant Supplementation.

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Abstract approved: \_\_\_\_\_

Maret G. Traber

During extreme exercise, athletes experience increased inflammation that is similar to the acute phase response. Endurance athletes, distance runners in particular, are also more susceptible to compromised iron stores. This study evaluated inflammation, immune function and iron status in athletes completing a 50K ultramarathon. Twenty-two well-trained distance runners, 11 males and 11 females, were randomized in a double blind manner into—1) those who consumed 300 mg vitamin E and 1000 mg vitamin C (500 mg twice daily) or 2) placebos—for six weeks before and one week following a 50K ultramarathon race. Blood samples were obtained on 13 separate occasions throughout the study: before supplementation, during supplementation, the day before the race, pre-race, mid-race, immediately post-race, 2 hours following the race, and daily for six days following the race. Plasma levels of ascorbic acid and  $\alpha$ -tocopherol were measured by HPLC with electrochemical detection. Inflammatory cytokines, interleukin-6

(IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-1 $\beta$  (IL-1 $\beta$ ) were measured using standard clinical assays. Each subject recorded immune function in an activity log and incidence of illness was tabulated as number of days ill. Ferritin was measured by enzyme immunoassay. Hemoglobin, hematocrit, and total-iron binding capacity (TIBC) and serum total iron were analyzed by standard procedures.

Plasma concentrations of ascorbic acid and  $\alpha$ -tocopherol increased significantly in supplemented subjects ( $p < 0.0001$ ). Although the ultramarathon race elicited an inflammatory response, antioxidant supplementation did not alter the responses of IL-6 and TNF- $\alpha$ , which both increased from pre-race to mid-race, post- and post-2 h (Scheffe post-hoc analysis,  $p < 0.0001$ ) and returned to pre-race concentrations by 1 day after the race. Male supplemented subjects had lower IL-1 $\beta$  concentrations compared to females consuming the supplement or to males consuming the placebo (ANCOVA, gender/time/treatment interaction;  $p < 0.01$ ) at mid-race ( $p < 0.05$  females,  $p < 0.005$  males), post 1 and 2 days (all  $p < 0.002$ ). Males had significantly higher ferritin levels than the female subjects (ANOVA,  $p < 0.0001$ ); supplementation resulted in lower ferritin concentrations at post-5 days ( $p < 0.02$ , ANCOVA treatment time interaction,  $p < 0.005$ ). Supplementation did not reduce the days illness among those consuming antioxidants compared to those consuming the placebos.

Ferritin not only increases during inflammation, it also is a measure of iron stores. Females had significantly lower levels of iron than the male subjects for each of the iron parameters measured (hemoglobin and hematocrit both  $p < 0.0001$ , ferritin  $p < 0.001$ , TIBC  $p < 0.02$ ) excluding serum total iron. The ferritin concentrations measured in the women were indicative of depleted iron stores ( $< 12 \mu\text{g/l}$ ), and antioxidant supplementation increased hematocrit levels in the female subjects ( $p < 0.05$ ). This investigation indicates that female distance runners need to be aware of an increased susceptibility to iron depletion compared to their male counterparts. Antioxidant supplementation improved hematocrit levels ( $p < 0.05$ ) among female runners and may improve iron status among females with depleted stores.

Although other investigations have suggested that antioxidant vitamins decrease exercise induced inflammation, no profound benefit of supplementation was found in this investigation though a response similar to the acute phase response was elicited by the ultramarathon race. Improvements in IL-1 $\beta$  and ferritin in response to antioxidant supplementation may indicate that the supplementation was beneficial, but more research is needed to draw definitive conclusions.

INFLAMMATION, IMMUNE SUPPRESSION, AND IRON STATUS IN  
ENDURANCE ATHLETES AND THE EFFECTS OF ANTIOXIDANT  
SUPPLEMENTATION

by

Dawn Marie Weseli Hopkins

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Dawn Marie Weseli Hopkins, Author

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## CONTRIBUTION OF AUTHORS

Dr. Maret G. Traber was involved in the design, analysis, and writing of this thesis. Angela Mastaloudis was involved with the protocol design, and grant proposal writing, data collection, analysis, and design of the study. Scott Leonard was involved with the method design, sample collection and data analysis for the study. Dr. Ishwarlal Jialal and Dr. Sridevi Devaraj were involved in the measurement and analysis of plasma cytokines. All involved aided in the interpretation of data. Michael Harms performed the vitamin C analysis and assisted with the vitamin E analysis.

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## DEDICATION

My work is dedicated to my parents and to Karl, the half that makes me whole. Without their support and love, I would not be where I am today.

# Inflammation, Immune Suppression, and Iron Status in Endurance Athletes and the Effects of Antioxidant Supplementation

## INTRODUCTION

### HYPOTHESIS

Extreme endurance exercise can induce muscle damage, increase hemolysis and augment respiration, all of which can contribute to oxidative stress that may lead to inflammation and further oxidative stress. Endurance exercise also increases the risk of infections that may be due to impaired immune function and compromised antioxidant status. Extreme endurance exercise training may alter iron status among ultra-marathon participants. Antioxidant supplementation may reduce inflammation and the incidence of illness while improving iron status among endurance athletes completing a 50K ultramarathon.

### SPECIFIC AIMS

Aim 1: Does endurance exercise increase markers of inflammation?

The inflammatory markers, interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were measured before, during and after a 50-kilometer ultramarathon race. Specifically, blood was collected from each subject six weeks before the race, three weeks before the race, the morning of the race, at kilometer 27 during the race, immediately after the race finish, two hours after

finish, and for six mornings following the race (See Appendix A). Iron status, including ferritin, which is an acute phase protein, hemoglobin, hematocrit, and total-iron binding capacity were also assessed at the same time-points.

**Aim 2:** Does antioxidant supplementation decrease exercise-induced inflammation and poor iron status commonly found in ultramarathon runners?

The study population was stratified into two groups in a double-blind manner—one group consumed antioxidants 300-mg vitamin E and 1000-mg vitamin C for seven weeks and the other group consumed identical placebos. Indices of inflammation and iron parameters were assessed among the two study groups at the time-points mentioned previously.

**Aim 3:** Does antioxidant supplementation influence the incidence of illness associated with ultra-endurance exercise?

An activity log was provided to each study participant. In the log, each subject was to rate his or her physical well being on a one-to-five Likert scale each day for six weeks before the race and for one week following the race. If the subjects rated their wellness below average, they were asked to explain their symptoms. The incidences of illness were evaluated between supplemented and non-supplemented subjects by comparing the number of days that each subject experienced illness between the two groups.



## LITERATURE REVIEW

### INTRODUCTION

Following participation in endurance exercise, athletes are at an increased risk of illness, particularly upper respiratory tract infections (2-4). The susceptibility to illness may be due to increased inflammation common after extreme endurance events such as a marathon or ultramarathon (5-10), and the response to extreme exercise may be analogous to the acute phase response (11-13). The increased susceptibility to illness may also be due to compromised levels of antioxidants causing the oxidant/antioxidant balance to be tipped in the direction of oxidative stress (14). Muscle damage may be the initiating event in the inflammatory response (15, 16). Oxidative stress may also contribute to inflammation by the up-regulation of redox sensitive signal transduction pathways that control inflammatory responses (17).

During normal respiration, reactive oxygen species (ROS) are formed. The body is protected from ROS by antioxidant defenses (17). During endurance exercise, respiration increases in response to physical activity to fulfill oxygen requirements. With increased respiration, the production of free radicals is also enhanced. During exercise neutrophil activation and the xanthine/xanthine oxidase pathways are accelerated (18, 19). Antioxidant supplementation may alleviate the endurance exercise-induced oxidative stress by deactivating ROS, and in turn protect muscle tissue, thereby decreasing the inflammatory response that may cause immune suppression.

## IMMUNE SUPPRESSION IN ENDURANCE ATHLETES

Although mild aerobic activity such as brisk walking, is protective against upper respiratory tract infections (URTI) (20), extreme endurance exercise such as training and running a marathon or an ultramarathon appears to increase the risk of experiencing URTI in elite and recreational athletes (2-4, 21-23). Endurance athletes tend to be more susceptible to URTI than the average populous (3, 4). The increased incidence of UTRI in endurance athletes appears to be related to several factors, such as over training, poor nutrition, psychological stress, and environmental stress that lead to immune dysfunction (24). Heath et al. (21) utilized a self-report diary and found that runners in the upper three quartiles for total mileage (486-865, 866-1388, > 1388 miles) for a year were twice as likely to experience UTRI symptoms than the lowest quartile for mileage (< 486 miles). In a large epidemiological study of the Los Angeles Marathon, Nieman et al. (2) found that runners who completed the marathon were nearly six times more likely to experience UTRI symptoms than runners of similar ability that did not participate in the race for reasons other than illness. Furthermore, runners who ran more than 97 kilometers per week for training were twice as likely to experience an infection than runners who completed less than 32 kilometers of training distance per week (2). In the two-week period following a 35 mile ultramarathon, participants were twice as likely to experience URTI than controls that did not participant in the race (3). A J-shaped model of exercise volume has been developed by Nieman (25) that suggests that moderate exercise is protective while extreme exercise may compromise overall immune function.

## THE INFLAMMATORY RESPONSE TO EXERCISE

Elite and recreational endurance athletes appear to experience immune suppression, increasing their risk of URTI. Endurance athletes also experience increased inflammation after completing long distance events. This inflammatory response has been compared frequently to the acute phase response (5, 8, 9, 11).

The acute phase response is the systemic reaction to localized infection or tissue damage. During the acute phase response at the site of tissue insult, a number of local reactions transpire, such as platelet aggregation and clot formation, dilation of blood vessels with subsequent leakage into adjacent tissue, and the aggregation and activation of granulocytes and mononuclear white blood cells. The activated leukocytes release acute phase cytokines, which are protein mediators that are able to relay messages to specific receptor site leading to a systemic reaction. Cytokines are effective at very low concentrations and have extremely complex interactions (17, 26). Cytokines are secreted by cells and can effect the actions of the secreting cells (autocrine) or other near by cells (paracrine) (26).

The systemic reaction is characterized by a variety of indicators, such as fever, leukocytosis, increased secretion of adrenocorticotrophin hormone and glucosteroids, and dramatic alterations in the concentrations of plasma acute phase proteins (27). Inflammation is essential because it allows an organism to return to homeostasis after infection or injury, but inflammation can be harmful to an organism (26). Chronic inflammation is observed in diseases such as atherosclerosis (28, 29) and diabetes (30).

Fallon (11) compared the acute phase response to the inflammatory response elicited by ultramarathon participation. Eleven acute phase reactants were measured in participants and six responded as if an acute phase response was elicited. Significant increases were measured in C-reactive protein (CRP), ferritin, and erythrocyte sedimentation rate, while significant decreases were measured in percent transferrin saturation and serum iron. Haptoglobin also responded as if an acute phase response was present, first decreasing significantly then increasing significantly over time. The other five acute phase reactants measured in participants, transferrin, albumin, alpha-1-antitrypsin, and complement proteins 3 and 4, did not respond in a manner analogous to an acute phase response. The acute phase response is not completely equivalent in different inflammatory states; therefore, the variation is not surprising and provides evidence that endurance exercise produces some aspects of an acute phase response.

## THE CYTOKINE RESPONSE TO EXERCISE

It is well recognized that cytokine levels are altered with endurance exercise, although results of specific studies vary. IL-1 $\beta$  increases in response to exercise but the response is of low magnitude in comparison to IL-6 (7-9). Endurance exercise consistently increases IL-6 levels (5-9, 15, 31). CRP, which is produced in response to IL-6, also increases after endurance exercise (5, 10, 11, 32). The pro-inflammatory cytokine, TNF- $\alpha$ , has been reported to increase in some studies (8, 9) and not change in other studies (5, 10). Anti-inflammatory cytokines, IL-10 and interleukin 1-receptor agonist, also increase after endurance exercise (6-

8, 31, 33). IL-2 has also been found to decrease in response to endurance exercise (12).

The inconsistency of results of cytokine response to exercise may be due to several factors including differences in duration, intensity, and type of physical activity. Other differences may be due to different assays used to detect the cytokines or the fact the cytokines have short half-lives and the time points when samples are collected may have a great deal of influence on results (34).

## MUSCLE DAMAGE AND ECCENTRIC EXERCISE

Endurance exercise elicits an inflammatory response in athletes, but what is the initiating factor of this response? Muscle damage may be the initiating event that leads to subsequent inflammatory responses (15, 16, 34-36). Eccentric exercise, which is the loading of a muscle while it is being lengthened, may cause more damage than concentric exercise as demonstrated by Bruunsgaard et al. (15). They examined the enzymatic responses of nine young healthy men following two bouts of exercise (30 minutes each). The eccentric exercise bout, when compared with the concentric exercise, caused more damage, as indicated by increases in plasma creatine kinase, aspartate aminotransferase and alanine aminotransferase. Creatine kinase increased 40-fold 4 days after the eccentric exercise, while no change was found after the concentric exercise. Similarly, IL-6 significantly increased after eccentric exercise, but no significant difference was detected after the concentric bout. Additionally, plasma creatine kinase and IL-6 concentrations

were correlated, suggesting that muscle damage may elicit an invasion of white cells that produce cytokines, resulting in an inflammatory response.

Fielding et al. (16) studied neutrophil infiltration in muscle following eccentric exercise in nine healthy, young, untrained men, who ran downhill for 45 minutes. Muscle biopsies were performed before, 45 minutes after and 5 days after the exercise bout. Neutrophil infiltration significantly increased after the exercise bout and remained increased at five days. The ratio of Z-bands damaged to total Z-bands increased significantly at 45 minutes but was no longer elevated at five days. Furthermore, a significant correlation was found between neutrophil infiltration and Z-band damage. Muscle levels of IL-1 $\beta$  also increased and were significantly higher by day 5. The eccentric exercise caused structural damage to the muscle fibers, resulting in the release of intracellular material. The release of the intracellular material may have caused chemotaxis that attracted the neutrophils to the site of damage.

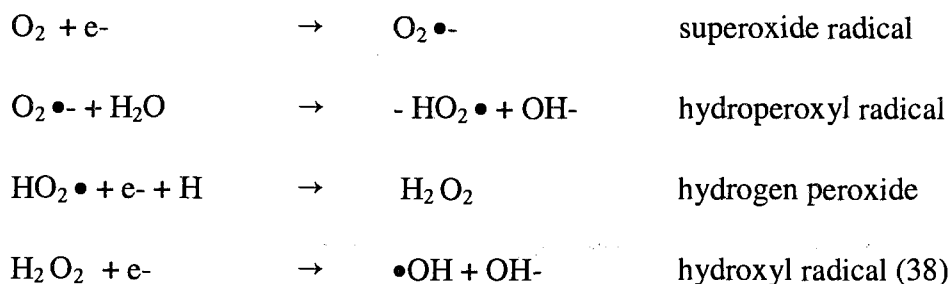
## RADICAL OXYGEN SPECIES AND ENDURANCE EXERCISE

In addition to muscle damage induced by endurance exercise and the release of acute phase cytokines, reactive oxygen species (ROS) production is accelerated during endurance exercise (19), leading to increased levels of oxidative stress after endurance exercise (37). Superoxide, hydroxyl radical, and hydrogen peroxide are typical ROS (17) that can be responsible for adverse physiological effects (19). Hydrogen peroxide, although it is not an oxygen free radical, can undergo a

reaction with iron (II) to form a hydroxyl radical through the Fenton reaction (17), explaining its classification as a ROS.

#### Mitochondrial reactive oxygen species production

During normal respiration, oxygen molecules are reduced to water in the electron transport chain of the mitochondria. Molecular oxygen is a diradical with two unpaired electrons and does not accept all four electrons required for complete reduction all at one time. The reduction of oxygen to water happens in a stepwise manner:



Halliwell (17) suggests that 1-3% of electrons could escape and become free radicals during the step-wise process. During maximal exercise, whole body oxygen consumption can increase up to 20-fold, while oxygen consumption at the level of the muscle may be increased as much as 100-fold compared to resting values, proportionally increasing the production of ROS (18).

### Activated polymorphoneutrophil release of ROS

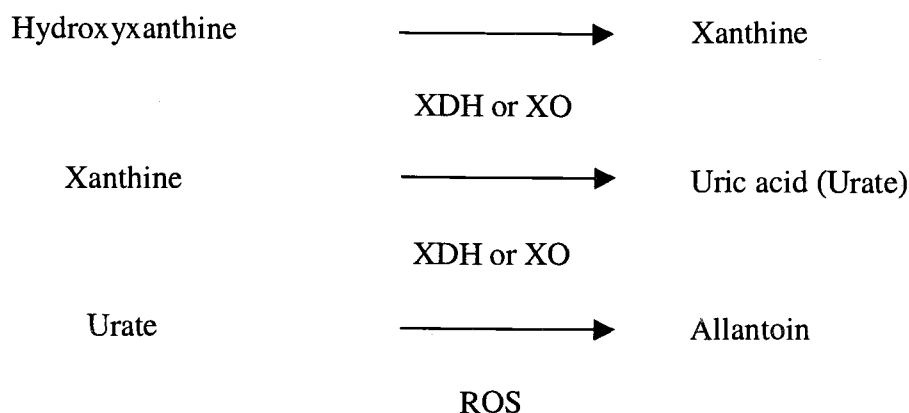
Neutrophilia, which is an increase in the mobilization of neutrophils, has been consistently reported as a response to exercise (24), and polymorphoneutrophil (PMN) activation has been proposed to contribute to ROS production in heavy eccentric exercise and extreme endurance exercise (18). Hessel et al. (39) examined the neutrophil response in marathon runners and found that circulating PMNs increased 435% after the race compared with pre-race values. The capacity of PMNs to produce ROS was measured using luminol-enhanced chemiluminescence. Chemiluminescence intensity increased by 142% after the race and considering the increase in the number of PMNs that occurred during the race, the total capacity for ROS production increased by approximately 640%. These data suggest that ROS production is increased during endurance exercise due to increased neutrophil production and activation.

### Xanthine/xanthine oxidase pathway of ROS production

Xanthine oxidase catalyzes reactions that produce ROS in the ischemic reperfused heart. Xanthine oxidase formation of ROS is a conditional pathway dependent upon three factors. (1) Sufficient amounts of the substrates hypoxanthine and xanthine must be present. (2) Xanthine dehydrogenase (XDH) must be present in its oxidized form, xanthine oxidase (XO). XO uses oxygen and produces ROS, while XDH, the reduced form, utilizes NAD<sup>+</sup> and does not produce ROS. (3) Oxygen must be present to act as an electron acceptor. There is some evidence that endurance exercise produces these conditions (18). Xanthine degradation leads to the production of uric acid, which when attacked by ROS, is converted to allantoin. Allantoin accumulation in the muscle would indicate that



urate has reacted with ROS because allantoin is not very diffusible across the muscle cell sacrolemma. The hypothesis that ROS production is increased in the muscle during endurance exercise would occur through the following reactions:



Where XDH is xanthine dehydrogenase and XO is xanthine oxidase (40). Hellsten et al. (40) examined two consecutive high intensity bouts of cycling exercise; allantoin levels in the muscle tissue increased similarly in both bouts. These data support the hypothesis that ROS are available during exercise to cause the formation and accumulation of allantoin.

## REACTIVE OXYGEN SPECIES ACTIVATION SIGNALING PATHWAYS

Many genes, including inflammatory, immune response, cell proliferation, and apoptosis (programmed cell death) are regulated by the signal transduction factor, nuclear factor-kappa B (NF- $\kappa$ B) (41). NF- $\kappa$ B-I $\kappa$ B is a protein complex in

the cytosol that is inactive. Cytokines or ROS can cause the disassociation and degradation of the inhibitory subunit (I $\kappa$ B) and the subsequent migration of NF- $\kappa$ B complex into the nucleus. Once inside the nucleus, NF- $\kappa$ B binds to DNA and stimulates the translation of several genes, including those programmed for the production of cytokines, cytokine receptors, acute phase proteins, and adhesion molecules (17). NF- $\kappa$ B can also be activated by stimuli other than ROS, such as bacterial LPS, certain viruses, or certain inflammatory cytokines, especially TNF- $\alpha$ . Therefore, ROS and cytokines are intimately related and can cause further damage through the potentiation of this viscous cycle (17, 18, 41).

## ANTIOXIDANTS

An antioxidant is "any substance that when at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (17). Many substances act as physiological antioxidants, such as vitamins E, vitamin C, beta carotene and other substances, such as ubiquinol, and glutathione (17, 42). Humans also possess enzymatic antioxidants such as catalase, superoxide dismutase, and the selenium-containing enzymes of the glutathione peroxidase family (41). Vitamin E is the most potent chain breaking, lipid soluble antioxidant found in humans and has the ability to protect membranes from oxidation (43). Vitamin C has many functions, including the ability to scavenge oxygen free radicals and regenerate  $\alpha$ -tocopherol from  $\alpha$ -tocopheryl radicals (17).

## Vitamin E

Vitamin E is the name given to a collection of four tocopherols and four tocotrienol derivatives that have the same antioxidant functions. Although there are eight naturally occurring forms of vitamin E,  $\alpha$ -tocopherol has the greatest biologic activity. The different forms of vitamin E are all absorbed with the same affinity and incorporated into the chylomicrons that enter circulation. The action of lipoprotein lipase upon the chylomicrons causes vitamin E to be incorporated into all types of circulating lipoproteins. When the lipoproteins reach the liver, the tocopherol transfer protein preferentially incorporates  $\alpha$ -tocopherol into the plasma (43).

As mentioned previously, vitamin E is a chain-breaking antioxidant that prevents free radical damage through its abilities to quench peroxy radicals ( $\text{ROO}\bullet$ ) in cell membranes. If a peroxy radical forms in a membrane it reacts 1000 times faster with vitamin E than a polyunsaturated fatty acid, hence protecting the membranes and lipoproteins from oxidation (43). Vitamin E is considered a chain breaking antioxidant because it breaks the chain of oxidation. One peroxy radical could oxidize a polyunsaturated fatty acid and create another peroxy radical, which in turn could oxidize another polyunsaturated fatty acid, but vitamin E blocks this chain of events.

Because vitamin E has the ability to quench free radicals it may be useful in the protection of athletes participating in endurance exercise that cause the production of greater amounts of ROS (37). Vitamin E has other properties beyond its antioxidant value that may reduce the inflammatory response that is common

after endurance exercise. Vitamin E may also inhibit IL-1 $\beta$  production, cell adhesion molecule expression, and platelet aggregation (44).

Vitamin E has improved immune function, but results are not completely conclusive. Meydani and associates have conducted several studies on the efficacy of vitamin E supplements in immune function. One such study (45) examined the effects of 800-mg dl- $\alpha$ -tocopheryl acetate for 30 days upon immunity in healthy elderly subjects. Thirty-two subjects were housed in a metabolic research unit and several immune measures were analyzed. The supplemented group experienced a significant increase in circulating polymorphonuclear white cells, while prostaglandin E2 and plasma lipid peroxides significantly decreased. Overall, immune responsiveness increased significantly in response to short term vitamin E supplementation in healthy elderly subjects.

In a similar study performed by De Waart et al. (46), 3 months of 100 mg dl- $\alpha$ -tocopheryl acetate did not increase overall immune responsiveness in elderly subjects in good health. The dose of 100 mg may have been too small to improve immune function.

In another study conducted by Meydani et al. (47), elderly volunteers were randomized into one of four groups. For 235 days, the subjects received 800 mg, 200 mg, 60 mg, or a placebo of vitamin E. Several immune parameters such as delayed-type hypersensitivity skin response (DTH), antibody response to hepatitis B, tetanus and diphtheria, and pneumococcal vaccines, and autoantibodies to DNA and thyroglobulin were measured before and after supplementation. Subjects who

received 200 mg vitamin E had significantly more positive responses to the DTH test than control subjects, but there were no differences found between the 60 mg and 800 mg compared to the placebo. The group receiving 200 mg of vitamin E also had a significant enhancement in antibody titer to tetanus vaccine, as well as to parameters such as antibody titer to diphtheria and immunoglobulin levels of T- and B-cells, and autoantibody levels. Subjects in the upper tertile for serum  $\alpha$ -tocopherol after the supplementation phase had significantly higher antibody response to hepatitis B and DTH. The investigators concluded that 200 mg of vitamin E is the therapeutic dose for elderly subjects because the immune response was the most positively enhanced in the 200 mg dose group.

In a large, randomized, controlled trial, the effect of vitamin E or multivitamin mineral supplementation on acute respiratory tract infections in elderly non-institutionalized persons was investigated (48). Subjects were divided into four groups: 163 subjects received a multivitamin supplement, 164 subjects received 200 mg of  $\alpha$ -tocopherol acetate, 172 subjects received both, and 153 subjects received a placebo each day. The median observation period was 441 days and no differences in the mean incidence of infections among any of the four groups were observed. The severity of symptoms was significantly worse among the subjects supplemented with vitamin E, compared with those who did not receive the supplement. The severity of symptoms was measured by total duration of illness, number of symptoms, presence of fever, and if the illness restricted activity levels. Although the plasma vitamin E increased significantly in the groups receiving the  $\alpha$ -tocopherol supplements, the plasma concentrations after supplementation were not reported.

Lee and Wan (49) examined the effects of 233 mg dl- $\alpha$ -tocopherol on cell-mediated immunity and oxidative stress in Asian men and women. Thirteen men and thirteen women ages 25 to 35 were recruited and measures of immunity and oxidative stress were measured before and after supplementation. Vitamin E supplementation significantly decreased urinary 8-hydroxy-2'-deoxyguanosine, a marker of oxidative DNA damage. Vitamin E supplementation also significantly decreased lymphocyte hydrogen peroxide production, indicating that vitamin E reduces the production of ROS. Vitamin E also significantly enhanced the CD4/CD8 ratio, which are different leukocytes used as an indicator of immune function. A ratio of less than one is a marker of possible viral infection or other cause of immune system compromise. After supplementation the subjects experienced a significant increase in this ratio from  $1.59 \pm 0.08$  to  $1.85 \pm 0.09$ , which indicates an improvement in immune function.

The investigation of the effects of vitamin E supplementation upon immune function warrants further research because the results of various trials do not provide definitive conclusions. Several well-designed studies show that vitamin E supplementation enhances immune function especially in the elderly, yet the same number of well-designed studies does not support the efficacy of vitamin in the improvement of immune function.

#### Vitamin C

Vitamin C, or ascorbic acid, is a water-soluble vitamin that is readily absorbed in the gut through active transport. Vitamin C is an essential nutrient for the synthesis of collagen, carnitine, and certain neurotransmitters (42). Vitamin C

also has antioxidant activity in aqueous solution such as in blood or intercellular fluid and it is considered the most efficient water-soluble antioxidant in the plasma (50). Vitamin C has the ability to neutralize ROS, such as the extremely reactive peroxy radical, hydroperoxy radical, and superoxide. Vitamin C has the ability to reduce tocopherol radicals in cell membranes to regenerate  $\alpha$ -tocopherol.

Vitamin C has long been recognized to abbreviate the length of the common cold (51, 52), but these findings have also been questioned (53). Some studies have investigated the relationship of antioxidant supplementation in runners. Krause (54) examined high intensity exercisers and found that supplementation with 2 g vitamin C for one week in a cohort of 10 runners did not correct the neutrophil dysfunction that is common after exercise. The length of supplementation may not have been long enough to sufficiently increase tissue concentrations to the level that is necessary for the benefits of vitamin C supplementation to be detected.

Peters and associates have conducted several studies concerning the immune function of ultramarathon athletes. In 1990, Peters et al. (55) studied 92 runners and the same number of non-running controls supplemented with 600-mg vitamin C or an identical placebo for 21 days prior to a 90-km ultramarathon race. The vitamin C supplementation significantly reduced the risk of upper respiratory tract infection in runners, but not in the non-running control subjects, although infection duration was significantly reduced in the controls supplemented with vitamin C. Runners who trained the most were the most susceptible to URTI symptoms.

Peters et al. (4) conducted a similar study on the same 90-km ultramarathon race. For this study, 178 runners and 162 sedentary controls were recruited and divided randomly into four groups receiving (1) 500 mg vitamin C (2) 500 mg vitamin C and 400 IU vitamin E (3) 300 IU vitamin E plus 300 mg vitamin C and 18 mg Beta Carotene, or (4) a placebo. The subjects were supplemented for the 21 days prior to the race. The two groups that received vitamin C or vitamin C, E and Beta-Carotene both had significantly lower incidence of URTI symptoms than the runners receiving placebo.

#### EFFECTS OF ANTIOXIDANT SUPPLEMENTATION UPON OXIDATIVE STRESS AND INFLAMMATION IN EXERCISE

Several investigators have examined the effects of various antioxidants upon exercise-induced inflammation and oxidative stress; divergent results have been published. Of these studies, one demonstrated a protective effect of antioxidants (56), two found no significant effects of antioxidant supplementation, and yet another found an increase in oxidative stress with antioxidant supplementation (57). The different studies each have strengths and weaknesses, which make a definitive conclusion difficult.

Kawaii and associates (56) found a beneficial effect of vitamin E supplementation in sedentary collegiate women. Ten women were supplemented with 200 mg d- $\alpha$ -tocopherol for one week after an initial 9-minute "exhaustive" exercise bout. Blood was drawn before and after the first exercise bout and then again after a week of supplementation, as well as before and after a second exercise



bout. Vitamin E concentrations increased significantly in serum and red blood cells (RBC) after supplementation. Unfortunately, measures of oxidative stress and muscle damage were not carried out. Before supplementation, exercise significantly decreased RBC vitamin E and had no effect on serum vitamin E. After supplementation the exercise significantly decreased serum but not RBC vitamin E. The authors concluded that the vitamin E in the serum decreased to conserve the vitamin E in the RBC thus protecting the RBC from oxidation. The supplementation and the bout of exercise were both of short duration making comparisons to other studies difficult.

Two different studies utilizing vitamin C and a combination of low dosage  $\alpha$ -tocopheryl acetate and coenzyme Q10 both found no significant reduction in oxidative stress after exercise due to supplementation (58, 59). Vasankari et al. (59) carried out a study with vitamin C supplementation at specific intervals during the trial. In this study, nine endurance athletes were supplemented with a total of 2 g of vitamin C during the study. Each subject ingested 500 mg 15 minutes before the 5 km treadmill run and another 500 mg was ingested immediately following the maximal run. Another 1000 mg was ingested 5 to 10 minutes after the completion of the maximal exercise bout. Although vitamin C is a water-soluble vitamin that is readily absorbed by the body, the timing of the administration of the vitamin C may have not allowed the vitamin to increase at sites where increased oxidative stress occurred. In another study, Kaikkonen et al. (58) found no benefit of d- $\alpha$ -tocopheryl acetate (12.5 mg) and coenzyme Q10 (90 mg) administration in the reduction of oxidative stress in subjects who consumed the supplements for the three weeks prior to a marathon race. Although plasma concentrations increased

due to supplementation, the doses may be below the optimal dosage for beneficial effects to be detected or delivery to oxidative stress sites were not optimal.

Childs et al. (57) examined the effects of vitamin C and N-acetyl-cysteine (NAC) supplementation on a single bout of eccentric exercise. Vitamin C (12.5 mg per kg body weight) and NAC (10 mg per kg) were administered for seven days after the bout of 3 sets of 10 eccentric contractions. Oxidative stress increased significantly compared to the placebo group, as quantized by an increase of bleomycin detectable iron. The authors concluded that supplementation caused the increase of the oxidative marker. The amount of vitamin C and NAC were not measured in the blood, only total antioxidant status, which significantly increased with the supplementation. Supplementation was also administered after the exercise bout, reducing the ability of the antioxidants to prevent oxidative stress. Additionally, NAC is converted to cysteine, which is a readily oxidizable thiol, and may increase oxidative stress.

Similar to the examination of oxidative stress, studies evaluating the effects of antioxidant supplementation on inflammation induced by endurance exercise have yielded inconsistent results. Some studies found that antioxidant supplementation attenuated the pro-inflammatory response (8, 60). Supplementation did not change the inflammatory response in other studies (6, 31, 61). Yet in other studies, the anti-inflammatory response was suppressed (33) and the pro-inflammatory response was enhanced by supplementation (10). More research is warranted because of these varying results in investigations concerning inflammation in endurance exercise.

Nieman et al. (8) conducted an investigation of the effects of vitamin C supplementation on the cytokine response following a 90-km ultramarathon. Subjects were allocated to one of three experimental groups: placebo, 500 mg, or 1500 mg vitamin C. Each subject was supplemented for a week prior to the race. Supplementation did not alter cytokine responses in the placebo or 500 mg groups, but benefited the group consuming 1500 mg vitamin C. When the placebo and 500 mg groups were combined, immediate post-race concentrations of IL-10 and IL-1ra were significantly lower in the 1500 mg group. Subjects consuming 1500 mg vitamin C had a trend for lower IL-6 and IL-8 levels than the two other groups combined, but the trend did not reach significance. The study suffers from a small number of subjects (placebo, n=7; 500 mg, n=10 and 1500 mg, n=12). The supplementation was for one week, and increasing the duration of supplementation and the number of subjects may produce more significant results.

Cannon et al. (60) examined the response of several cytokines to eccentric exercise after vitamin E supplementation (800 IU/day) for 48 days. Twenty-one male subjects in two different age groups were separated into a supplement and placebo group in a double blind manner. Each subject participated in an exercise session that consisted of three 15-minute periods of downhill running with a 5-minute rest between bouts. After 24-hours, mononuclear cells of the supplemented group stimulated with lipopolysaccharide secreted significantly less IL-1 $\beta$  than the cells from the placebo group treated in the same manner. The mononuclear cells of the vitamin E supplemented group also secreted significantly less IL-6 than the placebo group over the 12-day period of observation. There were no differences in TNF- $\alpha$  secretion between the placebo and supplemented groups.

Singh et al. (61) studied the effects of 5-days of 400 IU vitamin E or 25 mg zinc on exercise-elicited responses. Women ran at 65-70% of their maximal oxygen consumption until exhaustion (about 90 minutes). The protocol was reproduced three times implementing a different supplement in same menstrual (follicular) stage each time. No significant differences were found between the vitamin E, zinc, and placebo trials in the hormonal or inflammatory responses measured. In particular, IL-6 rose linearly with all of the trials; neither zinc nor vitamin E supplementation significantly attenuated the cytokine increases. Because of the short duration of supplementation, further investigation is necessary to examine the effects of longer duration supplementation on inflammatory markers.

Petersen et al. (31) examined the cytokine response after 1.5 hour bout of downhill treadmill running at 75%  $\text{VO}_{2\text{max}}$  in male recreational runners. Twenty runners were recruited and assigned to one of two groups in a double-blind manner. For two weeks before and for one week after the exercise bout, one group received 500 mg vitamin C and 400 mg vitamin E, and the other group received a placebo. Although the plasma levels of both antioxidants increased in the supplemented group, there were no differences in cytokine response between the two groups despite the significant cytokine response over time.

Nieman et al. (6) have studied athletes' immune function and inflammation in response to extreme endurance exercise. Recently, the group observed the effects of vitamin C supplementation on oxidative, inflammatory and immune parameters following an 80 km ultramarathon race. During the seven-day period before the race, the subjects were given 1500 mg vitamin C in 3 pills of 500 mg to

be ingested at meal times or an identical placebo; the subjects were randomized into the two groups in a double blind manner. The supplementation significantly increased plasma ascorbic acid, and the plasma concentration in the supplementation group was higher at all time points compared to the placebo group. Although the plasma cytokines increased over the time from pre-race to km 32 to post race, the supplementation did not have a significant effect upon plasma cytokine levels. No baseline levels of vitamin C were measured; therefore, the differences between the two groups could have existed prior to the study.

Peters et al. (10) observed the effect of vitamin C supplementation upon the inflammatory response of ultramarathon runners completing a 90 km ultramarathon race. Subjects were allocated into supplemented and placebo groups, 10 and 6 subjects in each group respectively. The supplemented group was given 500 mg of vitamin C twice daily for seven days prior to running. Serum CRP levels were significantly higher in the supplemented group immediately after the race, 24 and 48 hours after the race. No significant differences were found between the two groups and TNF- $\alpha$  was not elevated significantly at any time after the race in either experimental group. Once again, the duration of supplementation may need to be extended to observe benefits. Vitamin C was the sole supplement; vitamin E and C together may have a greater benefit than merely vitamin C alone due to the synergistic qualities of the two vitamins (42).

Peters et al. (33) studied the same ultramarathon race a few years later. Forty-five athletes were divided randomly into three groups, but only 29 complied with the study requirements. Subjects received either 500 mg per day ( $n = 10$ ),

1500 mg per day (n = 12) or a placebo (n = 7) for 7 days before the race, on race day, and on two days following the race. Blood samples were collected 15-18 hours before the race, immediately post-race, 24 hours post race and 48 hours post race. Plasma IL-10 and IL-1Ra, which are anti-inflammatory cytokines, increased significantly over time. The supplementation caused attenuation in the rise of the anti-inflammatory cytokines; significant differences were found between each of the three groups. The increase in plasma anti-inflammatory cytokines tended to be smaller than the increase in cytokines for the other two groups. Anti-inflammatory cytokines would seem important for modulating the endurance exercise induced inflammation.

Exercise-induced oxidative stress and inflammation have been previously examined, yet the body of literature does not provide definitive evidence to the efficacy antioxidant supplementation in the reduction of these related states. More research is needed to elucidate the mechanisms causing oxidative stress and inflammation and define the role that antioxidant supplementation may play in the reduction of oxidative stress and inflammation.

#### FERRITIN AS AN INFLAMMATORY MARKER

Ferritin, the storage form of iron, increases in response to infections, inflammatory conditions, cancers, and liver disease, among others. Because ferritin responds to such states, it is one of a class of proteins deemed acute phase proteins (1, 62). In response to inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-2 (IL-2), ferritin production increases from numerous cells,

including mesenchimal cells, hepatocytes, monocytes and macrophages. Ferritin production is mediated by a ferritin response element that contains a binding site for the transcriptional factor, nuclear factor  $\kappa$ B (NF $\kappa$ B) (62). NF $\kappa$ B is a redox sensitive signaling factor (41) thus may increase ferritin production in response to the oxidative stress of endurance exercise (37).

Iron is an essential mineral for humans and nearly two-thirds of total body iron is found in the hemoglobin contained within the erythrocytes. Four classes of proteins contain iron: heme proteins, iron sulfur enzymes, proteins for iron storage and transport, and other iron-containing or activated enzymes (1). The iron enzymes account for only 1 % of total body iron (63), therefore, the heme proteins and the proteins of iron storage and transport will be the focus of this literature review.

## IRON AND ENDURANCE ATHLETES

Oxygen binds to hemoglobin within the erythrocytes, and the erythrocytes carry oxygen to the peripheral tissues of the body; therefore, adequate iron levels are important to athletes because of the increased demand for the transport of oxygen to muscle tissues (63). Physiologically, it is of great importance for endurance athletes to possess adequate iron stores, but endurance athletes, runners in particular, are at greater risk than the general population to have inadequate iron stores (1, 63, 64). According to Beard and Tobin (63), "three groups appear to be at greatest risk for developing altered body iron: female athletes, distance runners and vegetarian athletes."

There are several reasons that runners have a greater risk of possessing inadequate iron stores than the sedentary population. Endurance athletes appear to have higher iron turnover and subsequently lower iron stores than the sedentary population. Erythrocytes appear to have a shorter half-life and higher turnover than in sedentary subjects (65). Greater blood loss was also found in the urine of marathon runners after the race than before the event. Eighteen percent of the marathon participants studied experienced hematuria that subsided in all cases 72 hours after the running event (66). Iron losses in the feces also appear to increase after running when compared to sedentary controls (67). Endurance athletes also lose iron in the sweat in greater amounts and have an increased demand for myoglobin and iron-containing respiratory enzymes, which may also be contributing factors to insufficient iron stores (64). It is estimated that total iron losses increase from the average 1 mg per day for males and 1.4 mg per day for females to approximately 1.75 mg per day and 2.3 mg per day for the two genders of endurance-trained athletes, respectively (64). Females also have the added iron loss of menses each month, which contributes to greater iron depletion among women endurance athletes (63).

Iron insufficiencies are classified into three groups: depleted stores, early functional iron deficiency, and iron deficiency anemia and different laboratory measurements are implemented at these different stages. Table 1.1 summarizes the laboratory techniques and the values that indicate problematic iron status.



Table 1.1 Laboratory Measurements Used to Evaluate Iron Status

Stage of Iron Deficiency	Indicator	Diagnostic Range
Depleted Store	Stainable bone marrow iron	Absent
	TIBC- (total iron binding capacity)	> 400 µg/dl
	Serum ferritin concentration	<12 ng/ml
Early functional iron	Transferrin saturation	<16%
	Free erythrocyte protoporphyrin	70 µg/ dl erythrocyte
	Serum transferrin receptor	>8.5 mg/l
Iron deficiency anemia	Hemoglobin concentration	<13 g/dl (male) <12 g/dl (female)
	Mean cell volume	< 80 fl

Table adapted from Dietary Reference Intakes (1).

## FERRITIN

Cellular iron is stored in the form of ferritin when it is not needed for other physiological processes. Ferritin is a protein designed to contain and retain up to a few thousand iron atoms, which would otherwise be available for oxidative reactions (62). Although most iron in the body is found within the circulating erythrocytes, surplus iron is stored as ferritin primarily in the liver, but also is found within the bone marrow and the reticuloendothelial system (68). Small amounts of ferritin also circulate through the bloodstream (1). Ferritin, along with transferrin, which transports iron in the circulation, maintains iron homeostasis and the two proteins function in an inverse manner. When iron stores increase, the expression of ferritin is up-regulated in order to sequester the free iron. Conversely, when iron stores are depleted ferritin levels decrease and expression of transferrin receptors that mediate iron uptake by internalizing transferrin are increased (69).

The amount of ferritin found in the plasma is an indicator of the total body stores of iron. In adult humans, the plasma ferritin concentration is directly proportional to the size of body stores; each 1  $\mu\text{g/l}$  of plasma ferritin indicates the presence of about 8 mg of storage iron. Iron stores are virtually depleted when the ferritin levels fall below 12  $\mu\text{g/l}$  (1).

Endurance exercise has often been compared to the acute phase response common in such conditions as infections, inflammatory states, and surgical procedures (11-13). Although endurance athletes generally have reduced levels of ferritin due to a myriad of factors, after endurance exercise plasma ferritin concentrations increase and remain elevated for several days following an the exercise bout (11, 68, 70, 71). These data suggest that extreme endurance exercise elicits an "acute phase response".

## TOTAL IRON BINDING CAPACITY

Free iron may be harmful to an organism because redox active iron could participate in Fenton chemistry, which produces hydroxyl radicals. These radicals are so reactive that they attack biologically significant molecules near their sites of production. Therefore, iron is transported in extracellular fluids and in circulating plasma bound tightly to transferrin. Transferrin is a metalloprotein with high affinity for iron. Total iron-binding capacity (TIBC) is a simple measure that indirectly quantifies plasma transferrin concentrations. TIBC is the total amount of iron bound to transferrin after the addition of known amounts of exogenous iron to the plasma. An elevated TIBC level ( $> 400 \mu\text{g/dl}$ ) is indicative of depleted iron

stores. TIBC, however, is a less sensitive measure than plasma ferritin concentrations. Individuals can have iron deficiency anemia without elevated TIBC levels, but low ferritin levels are usually indicative of anemia. (1).

With respect to the use of TIBC as a measure of immune function, infections and inflammatory states, TIBC generally decreases (1). TIBC has been measured in studies examining the effect of ultra-distance running on different iron parameters with varying results. After a 160 km triathlon consisting of canoeing, cycling, and running, TIBC in men was not significantly changed (72). In two other studies, TIBC was measured in participants of two different 1600-km ultramarathons, in which the participants attempt to cover the longest distance on a 400 m track in a six day period. TIBC significantly decreased on day 4 in one study and on day 11 in the other study (70, 71). The distance run by the athletes in both of the studies was extremely long and may not be comparable to the results found in the study of the 160 km triathlon. Between the two studies conducted by Fallon (70, 71), three women were included and as to date, TIBC has not been widely measured in women, especially those completing more "reasonable" distances.

## HEMOGLOBIN AND HEMATOCRIT

Hematocrit is calculated as the percentage of erythrocytes to whole blood, while hemoglobin is the oxygen-carrying component of these cells and quantifies the oxygen carrying capacity of the circulating blood (73). Normal values for hemoglobin are 40-54% and 37-47% for males and females, respectively. Values

below the acceptable range may indicate iron deficiency (74). Hemoglobin values <13 g/dl for males and <12 g/dl for females may also indicate iron deficiency anemia. Hemoglobin and hematocrit are clinical measures of iron deficiency anemia, but the diagnosis of iron deficiency anemia should not be based entirely upon the results of hemoglobin and hematocrit measures (1).

The effects of an acute bout of endurance exercise on hemoglobin and hematocrit have not been studied extensively. Moreover, measures of hematocrit and hemoglobin are confounded by plasma volume changes during exercise. Fallon and Bishop (71) studied the effects of six day track race on several blood parameters. Hemoglobin and hematocrit decreased significantly by day 4 and continued to decrease for the remainder of the study. Balaban and colleagues (75) examined whether selected blood parameters were modified by 115 days of training, which included running 40 miles a week for 50 days, followed by two months of long, paced runs mixed with short distance interval training. The final hemoglobin value after the 115 days of training was not significantly lower than the baseline value. Serum ferritin and percent TIBC saturation decreased significantly with the training protocol, while a red blood cell ferritin presented a similar but statistically insignificant trend.

## IRON ABSORPTION

Iron is highly conserved and regulated within the body. Intestinal iron absorption is modulated by several factors. Heme iron, derived mainly from hemoglobin and myoglobin in meat, is more readily absorbed than non-heme iron

(1, 63). Even though heme iron may account for only 10% of the iron found in the diet, it may account for a third of the iron absorbed from the diet. Heme iron absorption is not readily affected by dietary factors. Non-heme iron is not absorbed as readily from the diet; absorption can range from 2% to 20%, which is generally lower than the 5% to 35% absorption of heme iron. Non-heme iron absorption is enhanced by dietary factors such as ascorbic acid intake (63). Although ascorbic acid improves the absorption of iron from a single meal (76), studies examining the long-term effects of ascorbic acid intake on iron absorption (77, 78) and improving iron stores has been less positive (78).

## IRON AND ANTIOXIDANT SUPPLEMENTATION

Although it is well documented that different iron parameters are sensitive to inflammation induced by endurance exercise (32, 70, 71), investigators have not examined the effects of antioxidant supplementation on iron changes in endurance exercise. It can be hypothesized that antioxidant supplementation may reduce the inflammatory response by quenching ROS (18), thus attenuating the rise in ferritin and the fall TIBC levels.

Antioxidant supplementation may increase hemoglobin and hematocrit among runners. Ascorbic acid may increase the absorption of non-heme iron (63, 76) especially among runners with reduced iron stores. With increased non-heme iron absorption, hemoglobin and hematocrit levels may increase. Vitamin E supplementation may also increase the fluidity of the erythrocyte membranes (43) increasing the longevity of red blood cells in circulation.

## SUMMARY

Studies investigating endurance athletes have found with fair certainty that extreme exercise increases production of cytokines and ROS. On the other hand, studies investigating oxidative stress and inflammation have not produced a consensus of the benefits of antioxidant supplementation in the reduction of these parameters and warrants further investigation. Iron parameters, ferritin in particular, change with the acute phase response, but no studies have researched the effects of antioxidant supplementation upon these parameters during and after an extreme endurance event. Distance runners, females in particular, are more likely to have compromised iron status than the average populous.

This study examined the differences between the genders and antioxidant influences upon inflammatory markers and iron status before, during, and after an ultramarathon race. Vitamin E and C have been studied in regards to inflammation induced by exercise. These antioxidants have been found to reduce inflammation in exercise (8, 79) and other disease states (30, 80). This study tracked the inflammatory cytokines and iron responses before, during and for a week after an ultramarathon race. Several biological indices were measured to test the hypothesis that supplementation with vitamin E and C for 6 weeks prior to and for one week after an ultramarathon race would reduce inflammation and immune suppression and enhance iron status in endurance athletes.

INFLAMMATION AND IMMUNE SUPPRESSION IN ENDURANCE  
ATHLETES AND THE EFFECTS OF ANTIOXIDANT SUPPLEMENTATION

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## ABSTRACT

This study evaluated whether antioxidant supplementation would decrease inflammatory markers and improve immune function in athletes completing a 50K ultramarathon. Twenty-two well-trained distance runners, 11 males and 11 females, were randomized in a double blind manner into—1) those who consumed 300 mg vitamin E and 1000 mg vitamin C (500 mg twice daily) or 2) placebos—for six weeks before and for one week following a 50 km ultramarathon race. Blood samples were obtained on 13 separate occasions: before supplementation, during supplementation, the day before the race, pre-race, mid-race, post-race, 2 hours post race and for six days following the race. In response to antioxidant supplementation, plasma ascorbic acid and  $\alpha$ -tocopherol concentrations increased significantly from baseline ( $p < 0.0001$ ) and remained elevated for the duration of the study. IL-6 and TNF- $\alpha$  both increased from pre-race to mid-race ( $p < 0.0001$ ) and were not significantly different from pre-race levels by 1 day post-race. Neither antioxidant supplementation nor gender altered these responses. Antioxidant-supplemented males compared with supplemented females or placebo-treated males had significantly lower IL-1 $\beta$  responses at mid-race and on the two days following the race (gender/time/treatment interaction,  $p < 0.01$ ). Males had significantly higher ferritin levels than the females (ANOVA,  $p < 0.0001$ ). After adjustment for baseline differences, ferritin concentrations were lower in antioxidant treated subjects on post-day 5 (ANCOVA,  $p < 0.005$ ). Overall, the exercise elicited an acute phase response, and significant treatment effect for



ferritin and IL-1 $\beta$  concentrations suggesting that the supplementation enhanced the recovery from the inflammation.

## INTRODUCTION

Following participation in endurance exercise, athletes are at an increased risk of illness, particularly upper respiratory tract infections (2-4). The susceptibility to illness may be due to increased inflammation common after extreme endurance events such as a marathon or ultramarathon (5-10), and the response to extreme exercise may be analogous to the acute phase response (11-13). The increased susceptibility to illness may be caused by a dysregulation of the immune system, and to compromised levels of antioxidants causing the oxidant/antioxidant balance to be tipped in the direction of oxidative stress (14). Muscle damage may be the initiating event in the inflammatory response (15, 16). Oxidative stress may also contribute to inflammation by the up-regulation of redox sensitive signal transduction pathways that control inflammatory responses (17).

During normal respiration, reactive oxygen species (ROS) are formed (17). During endurance exercise, respiration increases to fulfill the oxygen requirement of the physical activity. With increased respiration, the production of free radicals is also enhanced. During exercise neutrophil activation and the xanthine/xanthine oxidase pathways both occur at accelerated rates (18, 19). Antioxidant supplementation may alleviate the endurance exercise oxidative stress by quenching ROS, thus providing protection to muscle tissue decreasing the inflammatory response that may cause immune suppression.

The purpose of the present study was threefold. The first aim of the study was to evaluate whether endurance exercise would increase markers of

inflammation. Secondly, if exercise elicited an inflammatory response, would antioxidant supplementation with vitamins E and C reduce inflammation in these athletes? Finally, the investigation examined the ability of antioxidants to ameliorate the incidence of illness among the ultra-endurance athletes completing a 50K ultramarathon.

## MATERIALS AND METHODS

### Study Design

Twenty-two subjects, eleven women and eleven men were randomly assigned to one of two treatment groups—1) those who consumed 300 mg vitamin E and 1000 mg vitamin C (500 mg twice daily) or 2) placebos—for six weeks before and for one week following a 50 km ultramarathon race. The effects of participation in a 50 km ultramarathon run on markers of inflammation and immune function were measured and compared in trained individuals consuming either placebos or antioxidants. The study investigated these indices in a group of subjects whose oxidative stress and vitamin E utilization was examined as a part of a Ph.D. thesis project conducted by Angela Mastaloudis and reported separately.

Inflammatory markers were assessed at thirteen different time-points during the study (See Appendix A). In addition, subjects reported incidence of illness in an activity log for six weeks prior to the race and for one week subsequent to the race. In the log, each subject was to rate his or her physical well being on a one-to-five Likert scale each day (See Appendix B). In addition to their wellness

rating, subjects recorded daily physical activity in the same log. If the subjects rated their wellness below normal, they were asked to explain their symptoms. The incidences of illness were evaluated between supplemented and non-supplemented subjects by comparing the number of days that each subject experienced illness between the two groups.

### Subject Recruitment

The Oregon State University Institutional Review Board for the Protection of Human Subjects approved the protocol for the research project (Appendix C). Subjects were recruited from the pool of ultramarathon participants in the Corvallis, OR area intending to participate in the 2002 McDonald Forest Ultramarathon. Approval to recruit subjects from the athletes registered for the ultramarathon was given by the race promoter, Clem LaCava. Each potential subject received a copy of the informed consent, which outlined the purpose, procedures, risks, and benefits of the study (Appendix D).

### Inclusion Criteria, Exclusion Criteria, and Subject Screening

Several criteria were established for study participation. The subjects were required to be non-smokers between the ages of 18 and 60 years of age with a  $\text{VO}_{2\text{max}}$  classified as excellent fitness by the stratification presented by Powers and Howley (81) ( $\text{VO}_{2\text{max}} \geq 47, 45, 44, \text{ or } 41 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  for males 21-29, 30-39, 40-49 and 50-60 years, respectively or  $\geq 37, 36, 33, \text{ or } 32 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  for females aged 21-29, 30-39, 40-49 and 50-60 years, respectively). Several exclusion criteria were established, including unwillingness to cease supplement use prior to the study (antioxidant or performance-enhancing supplements), a pre-screen total cholesterol

level above 7.758 mmol/l (300 mg/dl), triglyceride level above 3.387 mmol/l (300mg/dl), or fasting glucose level greater than 7.77 mmol/l (140mg/dl). Subjects were excluded if they were pregnant or pregnancy was suspected, and those with chronic upper respiratory infections were also excluded. Of the 24 potential subjects screened, two subjects were excluded because they did not meet the study criteria.

To better define the subject population and ensure excellent fitness among the study participants, percent body fat and submaximal oxygen consumption were measured at the Oregon State University Human Performance Laboratory. The BOD POD® Body Composition System (Life Measurement Instruments, Concord, CA) was used to assess percent body fat. The Bod Pod has been tested for test-retest reliability and has been compared to hydrostatic weighing for validity (82).

The subjects also completed a submaximal treadmill test to 85% of their age predicted heart rate maximum by the following equation:

$$\text{Age Predicted Maximum Heart Rate} = 220 - \text{age in years.}$$

Respiratory gases were collected by indirect calorimetry and analyzed by the Sensor Medics Metabolic cart. During the test, each subject wore a POLAR a<sub>1</sub> series heart rate monitor (Oulu, Finland), and heart rates were recorded every minute during the test. The linear relationship between heart rate and oxygen

consumption was implemented in order to extrapolate each subject's maximal oxygen consumption ( $\text{VO}_{2\text{max}}$ ) from the submaximal test. Using the average heart rate output from each individual's heart rate monitor during the race, energy expenditure was estimated by examining the oxygen consumed at the given heart rate and the corresponding calories per minute consumed at that heart rate from submaximal treadmill test.

### Study protocol

Subjects were randomly assigned to one of two groups (placebos or supplements) based on age, gender, body mass index (BMI) and cholesterol level. At the outset of the study, each subject received supplement packets that were labeled by month and day to clearly show the subjects if they had consumed their supplements for that given day. Twice per day, once in the morning and once in the evening, subjects were asked to consume based on their randomization category either an orange-colored pill containing either 500 mg of ascorbic acid, or citric acid as the placebo. The ascorbic acid tablets and placebos were a gift from BASF. In the evening, the subjects were to consume a brown capsule containing either 300-mg *RRR*- $\alpha$ -tocopheryl acetate in soybean oil, or soybean oil alone as the placebo. The *RRR*- $\alpha$ -tocopherol capsules and matching placebos were a gift from Jim Clark, (Cognis Nutrition and Health, La Grange, IL).

The subjects were asked to adhere to a prescribed low antioxidant diet (Appendix E) for 6 weeks prior to the race and for one week subsequent to the race. Subjects were also provided low antioxidant training foods, such as energy bars, carbohydrate pastes (Clif shot gels were donated by the Clif Corporation) and

drinks (Gatorade powder was donated by the Gatorade Corporation). Athletes were asked to use only the ergogenic aids provided at the onset of the study; additional items were provided on demand. The subjects were also required to consume a controlled diet for 2 days: the day prior to the race and race day. Meals were prepared and provided to the participants in the Metabolic Feeding Unit in the Human Nutrition Research Laboratory at Oregon State University. The day before the race, breakfast was consumed after a fasting blood draw. Lunch was given to the subjects at breakfast as a sack lunch. The subjects returned to the Metabolic Lab that evening where they were served dinner. All foods were weighed and recorded for each meal. The foods consumed by the subjects were analyzed for nutrient and caloric content by ESHA's food processor (Salem, OR).

On race day, the controlled diet continued before, during, and immediately following the race. Food consumption was recorded and analyzed for each of these meals. Breakfast was available after a fasting blood draw on the morning of the race. Volunteers were stationed at seven different points on the racecourse with food and sport fuels low in vitamin E and C available for the study participants. The volunteers recorded food consumption at each of the stations. After the race food was provided for all the study participants, and registered dietician recorded the post-race intakes.

The subjects were asked to refrain from using aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) including naproxen sodium and ibuprofen throughout the seven weeks of the study. As an alternative to these drugs, subjects were asked to use acetaminophen. Although this was requested, several subjects

consumed aspirin and NSAIDs during the 6 week period before the race ( $n = 3$ ), during the race ( $n = 2$ ), and the day after the race ( $n = 1$ ). The data was not eliminated for these subjects because it did not differ from the other subjects.

Samples were obtained at baseline six weeks prior to the race, 3 weeks prior to the race, the morning before the race, the morning of the race 1 to 2 hours before start, at kilometer 27 during the race, immediately following the race, and 2-hours following the race. Additional blood draws were collected on the six mornings following the race (See Appendix A). The exact time of each blood collection was recorded for each subject. Four different vacutainers were filled at each draw; two heparin, one  $K_3EDTA$ , and one serum tube. All the blood draws were fasting except mid-race, at the end of the race and 2-hours after the race.

The MacDonald Forest Ultramarathon is an annual event that takes place in Corvallis, OR. It is a 50-kilometer (31-mile) trail run over rugged terrain, with a total elevation gain and loss of approximately 12,000 feet. Before the race, subjects were weighed using a Health O Meter (Chicago, IL) sliding scale before and after the race. During the race, subjects were asked to wear a Polar Heart Rate Monitor (Oulu, Finland) with memory. Using the average heart rate from the entire race, energy expenditure was estimated by examining the oxygen consumed at the given heart rate and the corresponding calories per minute consumed at that heart rate from the information from their submaximal treadmill test.



Subjects were asked to refrain from all physical activity, especially running, for the six days following the ultramarathon allowing investigation of the effects of antioxidant supplementation on post-race inflammation.

### Analytical Techniques

Plasma  $\alpha$ -tocopherol was measured by high pressure liquid chromatography (HPLC) using electrochemical detection according to Podia et al. (83), with the exception that only the isocratic mobile phase was used for the HPLC system. Plasma tocopherol concentrations are expressed as  $\mu\text{mol/l}$ .

To analyze vitamin C, fifty  $\mu\text{l}$  of freshly drawn EDTA plasma was mixed with 50  $\mu\text{l}$  of freshly prepared chilled 5% (wt/vol) met phosphoric acid in 1mM diethylenetriamine pentaacetic acid (DTPA). The mixture was then centrifuged, and a portion of the supernatant was frozen at  $-80\text{ }^{\circ}\text{C}$  until the day of analysis. The analysis was completed by paired-ion reversed-phase HPLC coupled with electrochemical detection according to Kutnink et al. (84). Ascorbate standards in 1mM of DTPA with concentrations from 1.2  $\mu\text{mol/l}$  to 5  $\mu\text{mol/l}$  were run before and after each of the samples. Results are expressed as  $\mu\text{mol/l}$  plasma.

An enzyme immunoassay (EIA) 96-well plate from Ramco Laboratories (Stafford, TX) was implemented to measure plasma ferritin levels. Plasma (approximately 1 ml) was frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  until the time of analysis. On the day of analysis, the samples and the necessary reagents were brought to room temperature. First, the standards were added in duplicate to the designated wells. Ten  $\mu\text{l}$  of the test plasma was added in duplicate to the

designated wells along with 200  $\mu$ l of conjugated antihuman ferritin. The samples were then incubated for 2 hours at room temperature on a rotator table. The temperature at the incubation was recorded each time the assay was performed. The 96-well plate was then washed 3 times using an autowasher. After the plate was washed and dried, 200  $\mu$ l of the substrate solution was pipetted into each well. The plate was then incubated for another 30 minutes. After the incubation, 100  $\mu$ l of the 0.24% potassium ferricyanide was added to each well in order for a color to develop. The plate was then read at 500 nm and the absorbance for each sample was recorded and data were analyzed according to instructions provided by Ramco. Ferritin values are reported as ng/ml.

Cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were measured using immunoassay as reported previously by Devaraj et al. (30). The cytokine values are reported as pg/ml.

## STATISTICAL ANALYSIS

A repeated measures ANOVA was used to test between and within subject differences using SAS version 8.0 (SAS Institute, Cary NC). Results were compared with repeated measures ANOVA with Scheffe post hoc analysis using Statview version 5.0.1 (SAS Institute, Cary NC) statistical software. SPSS version 11.0 (SPSS Inc., Chicago IL) was implemented to examine the normality of data. The data for IL-1 $\beta$  and ferritin were natural log transformed to better meet the assumption of normality of the repeated measures ANOVA. One subject was excluded from the ferritin analysis because the subject had a stomach illness the

day before the race and was still nauseous on race day. It is well known that ferritin is responsive to infection. Consistent with that, the subject's ferritin levels were extremely elevated at pre-race ( $> 4$  S.D. outside the mean) and were not consistent with the study population. Immune function was analyzed using a non-parametric Chi-squared test. Data are reported as mean  $\pm$  standard error of the mean.

## RESULTS

### Subject Characteristics

Participant characteristics are presented in Table 2.1. None of the subjects had abnormal lipid values and all participants included met the oxygen consumption criteria for inclusion in the study. Several expected differences were detected between the genders. The females had higher HDL levels and percent body fat than the males, while males were taller, heavier, and had greater BMI than the female subjects, but no differences were detected between the two treatment groups.

The average weight loss during the race was  $0.82 \text{ kg} \pm 1.32 \text{ kg}$  with a range from a loss of 3.18 kg to a gain of 2.73 kg. During the race, the average study participant expended  $6000 \pm 1200 \text{ kcal}$ .

A controlled diet was provided to the subjects the day before the race and on race day, and the food intakes were analyzed using ESHA food software. Table 2.2 displays the composition of the foods consumed during the two-day controlled diet.

### Plasma Ascorbic Acid and $\alpha$ -Tocopherol Concentrations

Plasma ascorbic acid concentrations increased significantly ( $p < 0.0001$ ) with supplementation and were significantly higher in the supplemented group

Table 2.1 Subject Characteristics

	Female		Male		Differences	p value
	Antioxidant	Placebo	Antioxidant	Placebo		
Number of Subjects	6	5	6	5		
Age (years)	41 ± 4	34 ± 4	41 ± 5	39 ± 7	NS	
Cholesterol (mg/dl)	184 ± 10	174 ± 15	180 ± 20	193 ± 19	NS	
LDL (mg/dl)	104 ± 9	100 ± 11	106 ± 17	127 ± 16	NS	
HDL (mg/dl)	64 ± 6	61 ± 5	46 ± 7	47 ± 4	Females > Males	< 0.02
% Body Fat	19 ± 2	22 ± 2	16 ± 3	13 ± 2	Females > Males	< 0.03
VO <sub>2max</sub> (ml/kg/min)	57 ± 3	54 ± 3	58 ± 2	62 ± 1	NS	
Calculated max HR (bpm)	179 ± 4	185 ± 4	179 ± 5	181 ± 7	NS	
Height (cm)	166 ± 2	167 ± 2	182 ± 1	176 ± 2	Males > Females	< 0.0001
Weight (kg)	56 ± 1	63 ± 2	81 ± 4	75 ± 4	Males > Females	< 0.0001
BMI (kg/m <sup>2</sup> )	20 ± 1	23 ± 1	25 ± 1	24 ± 1	Males > Females	< 0.01
Finish Times (hours:mins)	6:59 ± 0:16	7:13 ± 0:31	6:43 ± 0:46	7:07 ± 0:62	NS	

\*Calculated maximum heart rate (beats per minute)

Table 2.2 Controlled Diet Composition

	Total kcal	% CHO	% PRO	% Fat	Vitamin C (mg)	Vitamin E (mg)
Day before the Race	3210 ± 930	54 ± 16	13 ± 5	33 ± 11	63 ± 21	7 ± 5
Race Day Breakfast	540 ± 225	66 ± 29	11 ± 5	23 ± 17	9 ± 9	2 ± 1
During the Race	1280 ± 440	9 ± 32	3 ± 2	90 ± 7	14 ± 13	0
Post Race	420 ± 217	59 ± 30	8 ± 5	32 ± 22	7 ± 5	1 ± 0.5

compared to the placebo group (Figure 2.1). Plasma  $\alpha$ -tocopherol concentrations were significantly higher in the supplemented compared with the placebo group (Figure 2.2,  $p < 0.0001$ ). No gender differences were detected for plasma ascorbic acid or for  $\gamma$ -tocopherol concentrations.

### Inflammatory Markers

Inflammatory responses were assessed by measuring cytokines, IL-6, TNF- $\alpha$  and IL-1 $\beta$ , over the course of the study. IL-6 concentrations were unaffected by either gender or supplementation; therefore, all groups were combined for analysis by repeated measures ANOVA. IL-6 concentrations changed over the course of the study (main effect,  $p < 0.0001$ ). Serum IL-6 increased nearly seven-fold from pre-race to mid-race and remained elevated until 2 h after the race (repeated measures ANOVA,  $p < 0.0001$ , Scheffe post hoc analysis, comparisons of mid-, post-, post 2 h to pre-race were all  $p < 0.0001$ ; Figure 2.3). By the morning after the race, IL-6 levels plummeted to pre-race levels demonstrating the transient nature of this cytokine. Because IL-6 was disappeared from the bloodstream so quickly, it is not surprising that IL-6 elevation was only found on race day.

TNF- $\alpha$  changes were nearly identical to IL-6 changes, but were smaller in magnitude with a four- to five-fold increase from pre-race to mid-race compared to the seven-fold increase of IL-6 (Figure 2.3). By mid-race, TNF- $\alpha$  was significantly elevated compared to pre-race levels and remained significantly elevated at 2h post-race (repeated measures ANOVA,  $p < 0.0001$ , Scheffe post hoc analysis, comparisons of mid-, post-, post 2 h to pre-race were all  $p < 0.0001$ ). As with IL-6,

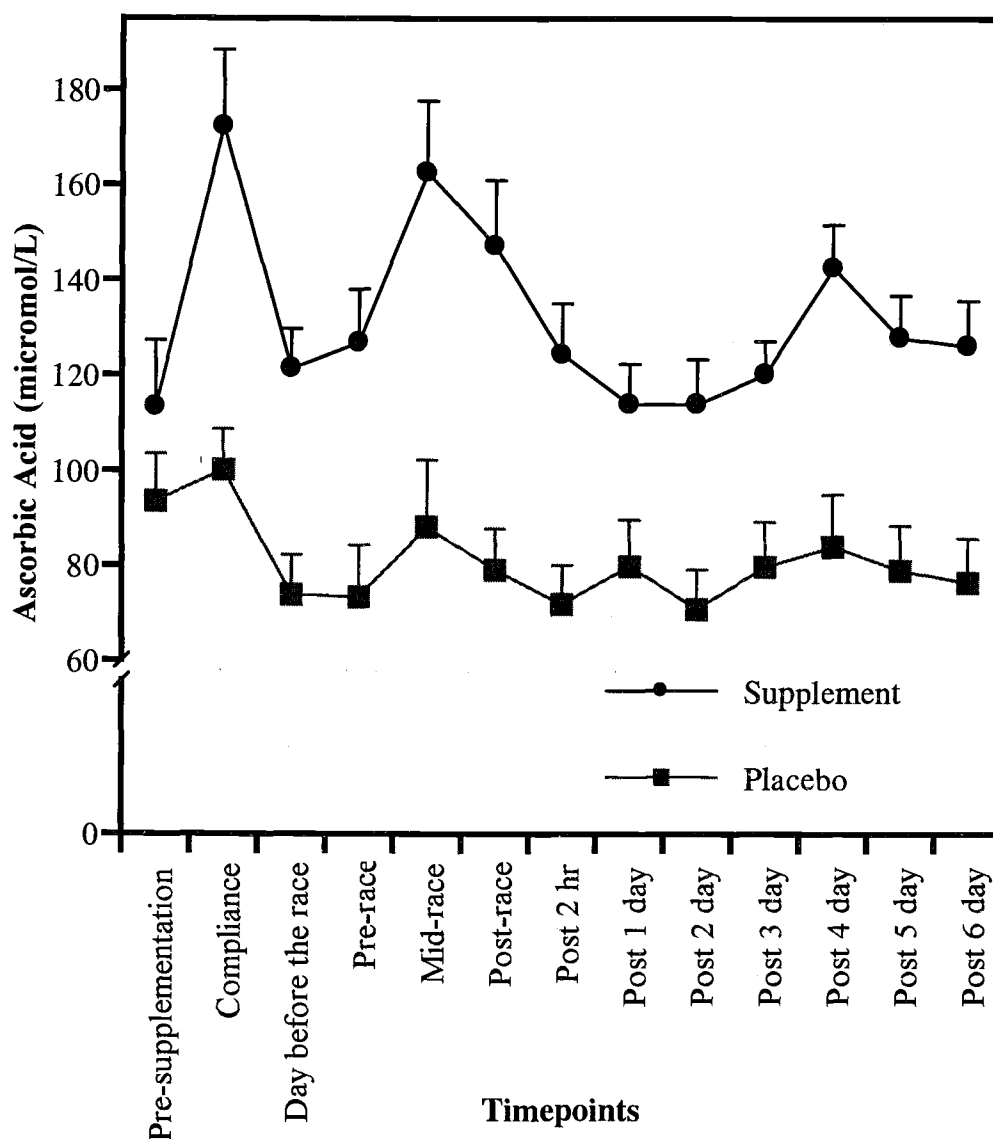


Figure 2.1 Plasma Ascorbic Acid Concentrations Before and After Supplementation

Plasma ascorbic acid concentrations (mean + SE) for the placebo ( $n = 10$ ) and antioxidant supplemented ( $n = 12$ ) groups are shown. In response to supplementation, plasma ascorbic acid concentrations increased ( $p < 0.0001$ ) from baseline and remained elevated for the duration of the study.

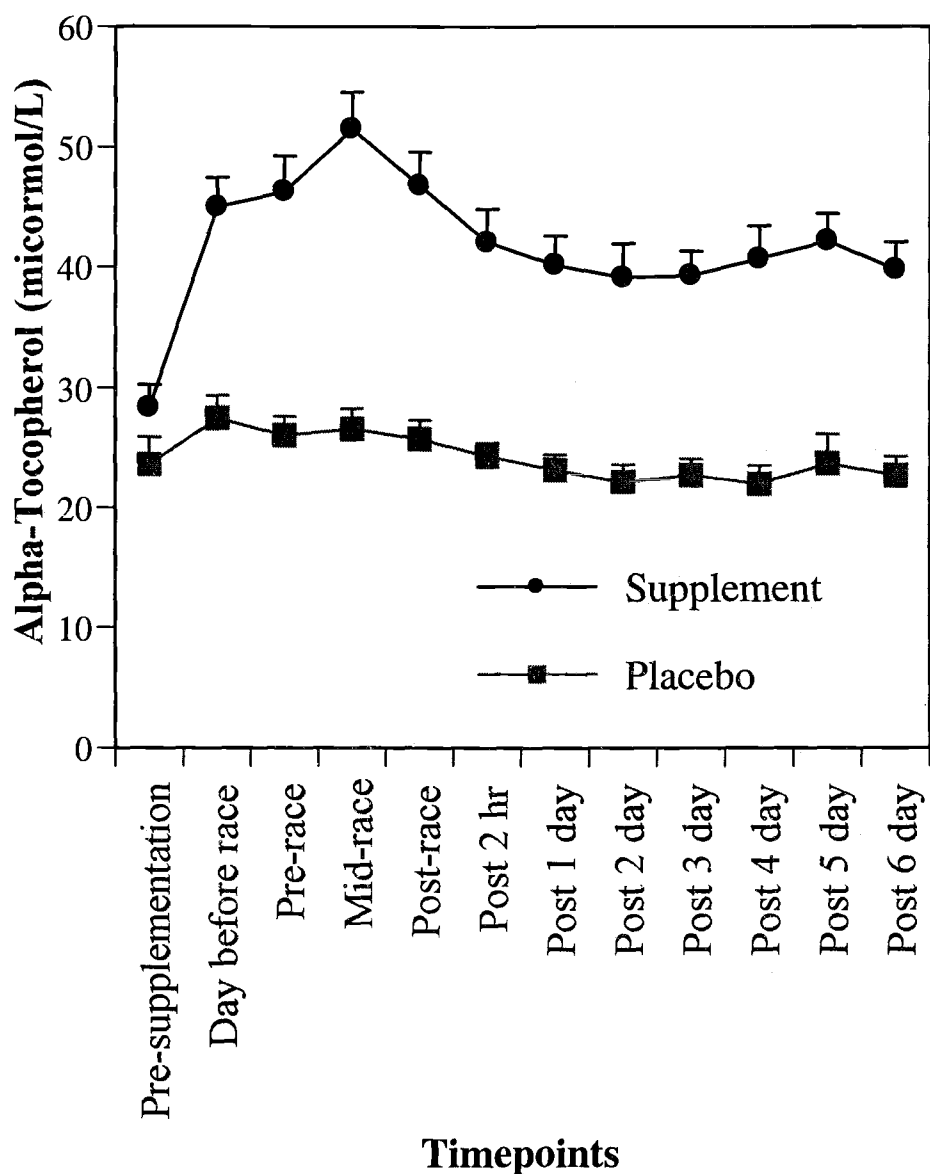


Figure 2.2 Plasma  $\alpha$ -Tocopherol Concentrations Before and After Supplementation

Plasma  $\alpha$ -tocopherol concentrations (mean + SE) for the placebo ( $n = 10$ ) and antioxidant ( $n = 12$ ) supplemented groups are shown. In response to supplementation, plasma  $\alpha$ -tocopherol concentrations increased ( $p < 0.0001$ ) from baseline and remained elevated for the duration of the study.



the inflammatory response of TNF- $\alpha$  was transient in nature and the TNF- $\alpha$  reached pre-race levels the morning after the race.

In contrast to IL-6 and TNF- $\alpha$  concentrations, baseline mean IL-1 $\beta$  levels (the mean of pre-supplementation, compliance, and the day before the race) were different between groups ( $p < 0.0001$ ). Therefore, baseline values were used as a covariate. Adjusted IL-1 $\beta$  concentrations changed differently in males and females with respect to the supplements over time (ANCOVA gender/treatment/time interaction,  $p < 0.01$ ). Supplemented males compared with males taking placebos had significantly lower IL-1 $\beta$  concentrations at mid-race ( $p < 0.005$ ), post 1 day supplemented ( $p < 0.002$ ), post 2 days ( $p < 0.002$ ) (Figure 2.4. top). Compared to the supplemented females, supplemented males had significantly lower IL-1 $\beta$  concentrations at mid-race ( $p < 0.03$ ), post 1 day ( $p < 0.002$ ), and post 2 days ( $p < 0.002$ ) (Figure 2.4 bottom).

Ferritin was the final inflammatory marker assessed in the study and is known to increase response to inflammation and infection. Initially at baseline, the female subjects had significantly lower ferritin levels compared to the males ( $p < 0.001$ , Figure 2.5). After controlling for baseline differences, the repeated measures ANCOVA revealed time as a main effect ( $p < 0.0001$ ), a time/treatment interaction ( $p < 0.005$ ) and a time/gender interaction ( $p < 0.05$ ). The supplemented subjects had lower ferritin concentrations at post 5 days ( $p < 0.02$ , Figure 2.6).

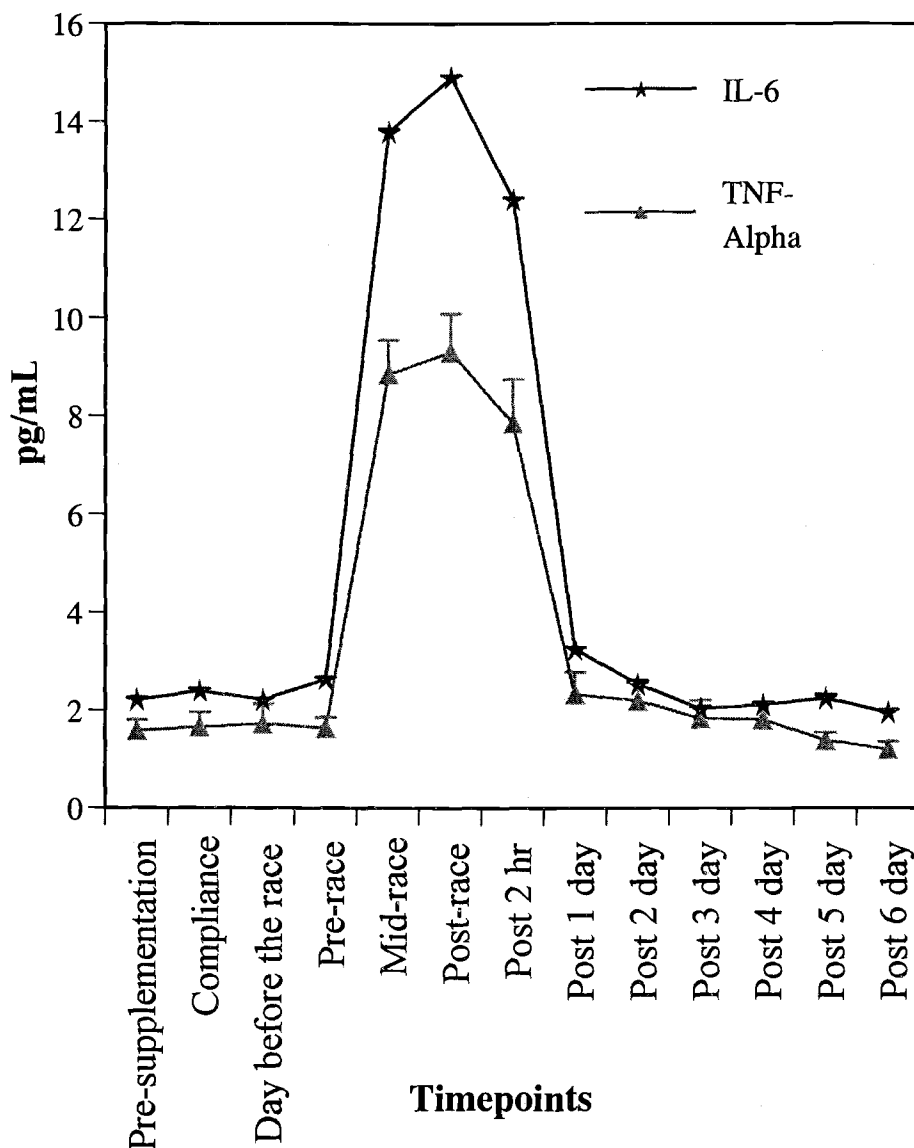


Figure 2.3 Serum IL-6 and TNF- $\alpha$  Concentrations Before, During, After the Ultramarathon Race

Serum IL-6 and TNF- $\alpha$  concentrations (mean + SE) are shown. Supplemented and placebo groups, as well as males and females, were combined because these parameters had no statistically significant effects on these cytokines. By Scheffe post hoc analysis, both serum IL-6 ( $n = 22$ ) and TNF- $\alpha$  ( $n = 22$ ) were found to increase significantly compared pre-race at mid, post and post-2h ( $p < 0.0001$ ) and returned to baseline by one day after the race.

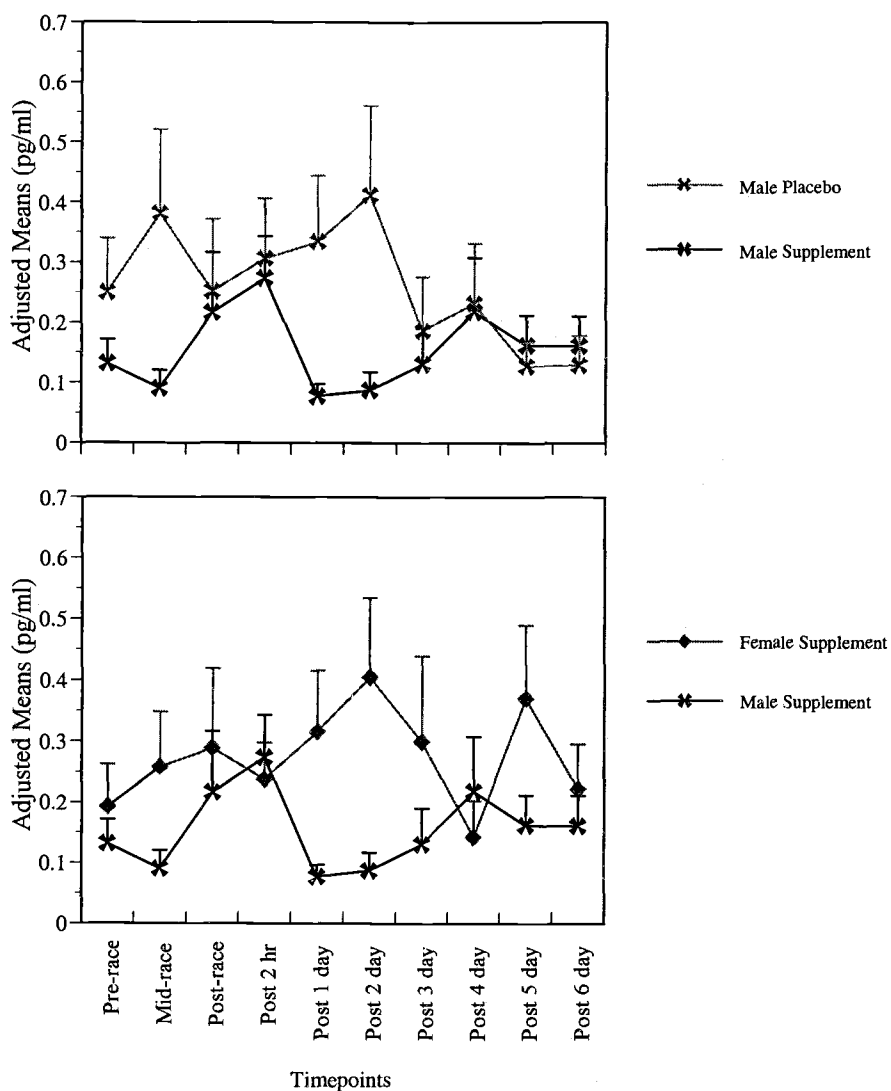


Figure 2.4 IL-1 $\beta$  Differences in Response to Supplementation and Gender

Baseline-adjusted serum IL-1 $\beta$  concentrations (mean + SE) are shown. After adjustment for difference between the groups, a significant gender/treatment/time effect for IL-1 $\beta$  was detected by ANCOVA ( $p < 0.01$ ). Supplemented males ( $n = 6$ ) had significantly lower IL-1 $\beta$  concentrations than the placebo males ( $n = 5$ ) at mid-race ( $p < 0.005$ ), post 1 day ( $p < 0.002$ ), post 2 days ( $p < 0.002$ ) (top). Supplemented males ( $n = 6$ ) had significantly lower IL-1 $\beta$  concentrations at mid-race ( $p < 0.03$ ), post 1 day ( $p < 0.002$ ), and post 2 days ( $p < 0.002$ ) than the supplemented females ( $n = 6$ ) (bottom).

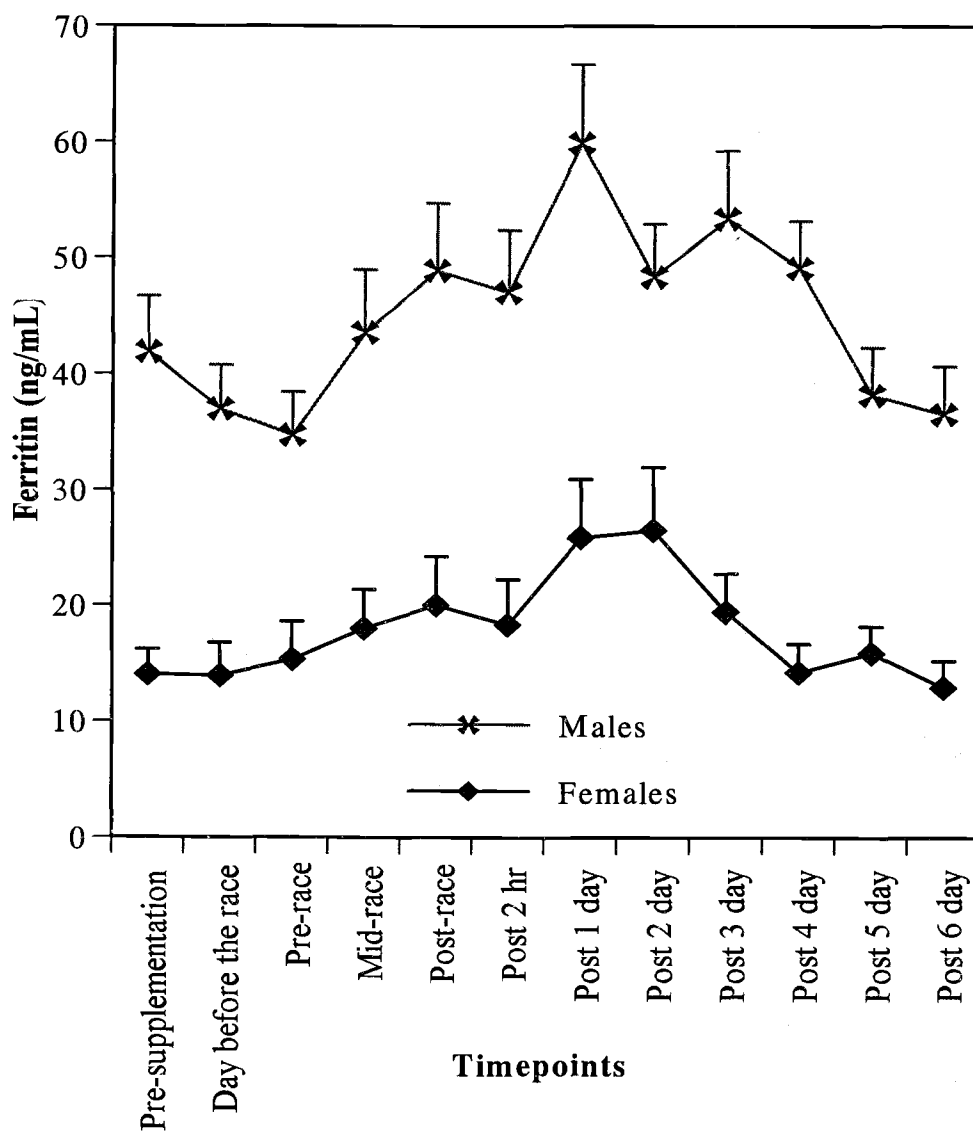


Figure 2.5 The Ferritin Values for Males and Females Over the Course of the Study

Ferritin concentrations (mean + SE) in males ( $n = 10$ ) and females ( $n = 11$ ) over the course of the study are shown. Males had significantly higher ferritin levels than did females (main effect repeated measures ANOVA,  $p < 0.0001$ ).

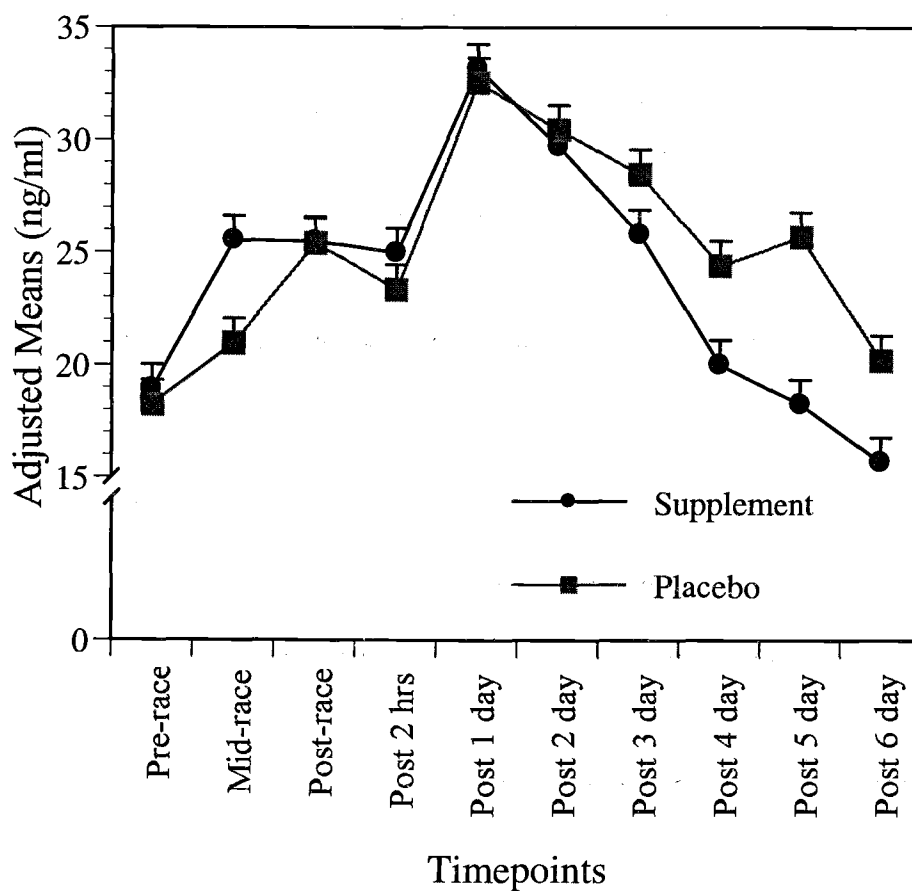


Figure 2.6 Ferritin Treatment Differences

Baseline adjusted-serum ferritin concentrations (mean + SE) are shown (ANCOVA significant treatment/time interaction,  $p < 0.005$ ). At 5 days after the race, ferritin concentrations were lower in the antioxidant ( $n=11$ ) compared with the placebo group ( $n = 10$ ) ( $p < 0.02$ ).

## Immune Function

Immune function was assessed by counting the number of days that each subject rated their physical well being as below normal and reported symptoms such as a cold, runny nose, nasal congestion, flu, vomiting, and/or diarrhea. Illness was not considered to be tiredness, menstrual or pre-menstrual symptoms, or soreness from activity. One subject was excluded from the analysis because the subject lost the activity log; therefore, a total of 21 subjects were analyzed. Although the placebo group has more than twice the incidence of illness compared to the supplemented group, the number of days ill was not statistically significant (Table 2.3).

Table 2.3 Comparison of "illness days" in supplemented and placebo subjects

	Placebo (n = 9)	Supplemented (n = 12)
Total Number of Days Ill	16 $\pm$ 0.64	7 $\pm$ 0.36
Number of Subjects	9	12
Number of Days Ill Per Person	1.78 $\pm$ 0.056	0.67 $\pm$ 0.071

## DISCUSSION

This study assessed whether extreme exercise would increase inflammation, and if inflammation did increase, would antioxidant supplementation decrease markers of inflammation. The extreme endurance exercise bout elicited an inflammatory response as documented by increases in IL-6, TNF- $\alpha$ , IL-1 $\beta$ , and ferritin. Nonetheless, the response to the antioxidant supplementation was variable among the inflammatory markers measured. Although plasma ascorbic acid and  $\alpha$ -tocopherol concentrations increased dramatically in the subjects consuming the antioxidant supplements, supplementation did not alter the athletes' IL-6 or TNF- $\alpha$  responses. These findings are in contrast to previous studies of exercise induced inflammation that have demonstrated an attenuating effect of antioxidant supplementation (8, 60, 79).

Unlike the cytokines TNF- $\alpha$  and IL-6, IL-1 $\beta$  was responsive to the supplementation, at least in the male subjects. At mid-race and post 1 and 2 days the IL-1 $\beta$  levels of the supplemented males were significantly lower than the males in the placebo group, suggesting that the supplementation attenuated the cytokine response during the race and enhanced recovery. At those same three time points, females in the supplemented group had significantly higher levels of IL-1 $\beta$  than the males in the supplemented group, which highlights the differences in gender response to endurance exercise and antioxidant supplementation. The reason for the differences between the genders and the differential response to treatment is difficult to pinpoint and warrants further investigation.

Although ferritin increased in response to the extreme exercise, the trend for ferritin was dissimilar to the trend for IL-6 and TNF- $\alpha$ . These cytokines peaked at post race, yet ferritin did not peak until post one day. Ferritin synthesis increases in response to cytokines, TNF- $\alpha$  in particular (62). The elevation of TNF- $\alpha$  subsequent to the spike in ferritin provides evidence for this interaction.

Ferritin levels between the two treatment groups were found to be significantly different (ANCOVA  $p < 0.005$ , figure 2.6), especially 5 days after the race, where there was a statistically significant difference between supplemented and placebo subjects ( $p < 0.02$ ). Antioxidant supplementation appeared to improve clearance of ferritin from the blood stream. Ferritin is known to increase with inflammation and the greater clearance of ferritin in the antioxidant group may indicate a beneficial effect of the supplementation in endurance athletes.

The ferritin concentrations in the female athletes at baseline are noteworthy. The female subjects had ferritin levels that were significantly lower than the male subjects. These data suggest that the female subjects had depleted iron stores; ferritin levels below 12  $\mu\text{g/ml}$  demonstrate depleted stores of iron. Distance runners may have increased needs for iron due to several factors including footstrike hemolysis (85). The female subjects in the study were not only endurance athletes who may be prone to iron deficiency (64) but many of them were “pseudo” vegetarians who routinely do not consume a great deal of heme iron in their diets increasing the likelihood of compromised iron status. Iron is essential for humans, but even more important the endurance athletes. Although sports anemia, the physiological adaptation to endurance exercise caused by an expanded



plasma volume, often accounts for the anemia measured in distance runners (86), the stark differences between the males and females of the same approximate training level indicated that the females had true iron depletion. Female distance runners may want to consider increasing intake of iron especially heme iron that is more readily absorbed in the gut (1).

Ferritin levels between the genders followed the same general pattern for most of the study. The ferritin response in the female subjects appeared to be more acute than the male subjects where the ferritin levels remained high 2 days after the race and the ferritin levels in the male subjects began to decrease 2 days after the race. After the acute increase in ferritin, females appeared to experience a greater rate of ferritin clearance than the male subjects.

Extreme exercise has also been correlated with increased risk of illness (25), and the present study assessed the efficacy of antioxidant supplementation in the improvement of immune function in ultramarathon runners. Although the supplementation appeared to decrease days of illness, no statistical differences were found between the groups. To perform a study examining immune function, more subjects should be recruited because our study lacked statistical power to assess these small changes.

Among the strengths of the present study was the controlled diet, which decreased variability among the study participants. The subjects were allowed to eat as many calories as desired but the types of foods that were provided were low in vitamins C and E.

Another strength of the study was the length of the post-race recovery follow-up. Changes in recovery in ferritin were detected between the supplement and placebo groups that would not have been detected with a shorter examination period. The daily blood collections for 6 days after the ultramarathon race are unique to this study.

The examination of equal numbers of males and females was also strength of the study. In many extreme endurance exercise investigations, males were predominantly included in the studies. Differences between males and females were detected in response to supplementation for IL-1 $\beta$  and for ferritin. Without equal number of males and females, these differences may not have been detectable. The inclusion of female athletes increases the knowledge of a population that has not been as readily represented in the literature.

One major limitation of the study was that the placebo group never achieved low concentrations of plasma ascorbic acid, which may be a major confounding factor in the study. In the placebo group, plasma ascorbic acid concentrations were higher than expected considering that a low antioxidant diet was prescribed during the study. The results provide reasonable doubt about the subjects' adherence to the prescribed diet and this may be a confounding factor for the study. Marginal vitamin C status, which indicates a moderate risk of developing clinical signs of deficiency, is indicated by a plasma level between 11 to 23  $\mu\text{mol/l}$  (74). All the subjects in the study at baseline were well above the marginal level; the level of vitamin C in both supplemented and placebo groups was nearly four times the plasma concentration that is considered "marginal" at

baseline. Because the plasma levels of vitamin C in the placebo group did not reach lower levels, the attenuating effect of antioxidant supplementation upon cytokines TNF- $\alpha$  and IL-6 may have been experienced in both groups thus masking the antioxidant effect.

Another limitation of the study was that the supplementation of vitamin E may not have been the length or dosage needed to decrease cytokine levels in inflammatory states. Recently, experts in the field of cytokines and vitamin E therapy have reported that the doses of  $\alpha$ -tocopherol must be 1000 mg and taken for 3 months in order significantly decrease the level of inflammation in human subjects (30).

Another factor that may have influenced the cytokine results was the high level of fitness in the study population. In a study examining untrained, sedentary persons, the cytokine response to exercise was attenuated with antioxidant supplementation (79). The lack of response in the trained population may be due to regular training, which possibly enhances natural antioxidant defenses (79).

In summary, extreme endurance athletes, such as ultramarathon runners, are susceptible to exercise induced inflammation and immune suppression. Although other investigations have suggested that antioxidant vitamins decrease exercise induced inflammation, no profound benefit of supplementation was found in this investigation though a response similar to the acute phase response was elicited by the ultramarathon race. Antioxidants may improve chronic inflammation common

with diabetes and cardiovascular disease, but antioxidants do not appear to improve the inflammation or immune dysfunction common after extreme endurance exercise in trained athletes. Improvements in IL-1 $\beta$  and ferritin in response to antioxidant supplementation may indicate that the supplementation was beneficial, but more research is needed to draw definitive conclusions.

IRON STATUS IN ENDURANCE ATHLETES AND THE EFFECTS OF  
ANTIOXIDANT SUPPLEMENTATION

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## ABSTRACT

This study evaluated whether antioxidant supplements would improve iron status in athletes completing a 50K ultramarathon because ascorbic acid may increase absorption of non-heme iron and vitamin E supplementation may increase the fluidity of erythrocyte membranes increasing their resistance to hemolysis. Twenty-two well-trained distance runners, 11 males and 11 females, were randomized in a double blind manner into—1) those who consumed 300 mg vitamin E and 1000 mg vitamin C (500 mg twice daily) or 2) placebos—for six weeks before and for one week following a 50 km ultramarathon race. Blood samples were obtained on 13 separate occasions throughout the study: before supplementation, during supplementation, the day before the race, pre-race, mid-race, post-race, 2 hours post race and for six days following the race. In response to the supplementation, plasma levels of ascorbic acid and  $\alpha$ -tocopherol in the subjects consuming the antioxidants increased significantly from baseline (both  $p < 0.0001$ ) and remained elevated for the duration of the study. Females had significantly lower levels of iron than the male subjects for each of the iron parameters measured ( $p < 0.0001$ ). The ferritin concentrations measured in several of the women were indicative of depleted iron stores. Antioxidant supplementation increased hematocrit levels in the female subjects ( $p < 0.05$ ). This investigation indicates that female distance runners need to be aware of an increased

susceptibility to iron depletion and that antioxidant supplementation may improve iron status in athletes with compromised iron stores.

## INTRODUCTION

Iron is an essential mineral for humans and nearly two-thirds of total body iron is found in the hemoglobin contained within the erythrocytes. Four classes of proteins contain iron: heme proteins, iron sulfur enzymes, proteins for iron storage and transport, and other iron-containing or activated enzymes (1). The iron-containing enzymes account for only 1 % of total body iron (63).

Oxygen binds to hemoglobin within the erythrocytes, and the erythrocytes carry oxygen to the peripheral tissues of the body; therefore, adequate iron levels are important to athletes because of the increased demand for the transport of oxygen to muscle tissues (63). Physiologically, it is of great importance for endurance athletes to possess adequate iron stores, but endurance athletes, runners in particular, are at greater risk than the general population to have inadequate iron stores (1, 63, 64). According to Beard and Tobin (63), "three groups appear to be at greatest risk for developing altered body iron: female athletes, distance runners and vegetarian athletes."

There are several reasons that runners have a greater risk of possessing inadequate iron stores than the sedentary population. Endurance athletes appear to have higher iron turnover and subsequently lower iron stores than the sedentary population. Erythrocytes appear to have a shorter half-life and higher turnover than in sedentary subjects (65). Endurance athletes may experience blood loss in the urine (66) and the feces (67) that is uncommon in sedentary controls. Endurance



athletes also lose iron in the sweat in greater amounts and have an increased demand for myoglobin and iron-containing respiratory enzymes, which may also be contributing factors to insufficient iron stores (64). Footstrike hemolysis may also be a contributor to the increased iron turnover in distance runners (85). It is estimated that total iron losses increase from the average 1 mg per day for males and 1.4 mg per day for females to approximately 1.75 mg per day and 2.3 mg per day for the two genders of endurance-trained athletes, respectively (64). Females also have the added iron loss of menses each month, which contributes to greater iron depletion among women endurance athletes (63).

Antioxidant supplementation may have a positive influence upon endurance athlete's iron status. Ascorbic acid may increase the absorption of non-heme iron (63, 76, 87) especially among runners who are more susceptible to reduced iron stores. With increased non-heme iron absorption, hemoglobin and hematocrit levels may increase. Vitamin E supplementation may also improve the fluidity of erythrocyte membranes (43) increasing the longevity of red blood cells in circulation thus maintaining iron within the body. The focus of this study is to determine if antioxidant supplementation can improve iron status in athletes completing a 50K ultramarathon.

## MATERIALS AND METHODS

### Study Design

Twenty-two subjects, eleven women and eleven men were randomly assigned to one of two treatment groups—1) those who consumed 300 mg vitamin E and 1000 mg vitamin C (500 mg twice daily) or 2) placebos-- for six weeks before and for one week following a 50 km ultramarathon race. The effects of participation in a 50 km ultramarathon run on markers of iron status were measured and compared in trained individuals consuming either placebos or antioxidants. Iron markers were assessed at thirteen different time-points during the study (See Appendix A). Inflammation and immune function were also assessed in the subjects as reported in the previous chapter. The present study investigated these indices in a group of subjects whose oxidative stress and vitamin E utilization was examined as a part of a Ph.D. project conducted by Angela Mastaloudis and reported separately.

### Subject Recruitment

The Oregon State University Institutional Review Board for the Protection of Human Subjects approved the protocol for the research project (Appendix C). Subjects were recruited from the pool of ultramarathon participants in the Corvallis, OR area intending to participate in the 2002 McDonald Forest Ultramarathon. Approval to recruit subjects from the athletes registered for the ultramarathon was given by the race promoter, Clem LaCava. Each potential subject received a copy of the informed consent, which outlined the purpose, procedures, risks, and benefits of the study (Appendix D).

## Subject Screen and Inclusion, Exclusion Criteria

Each subject was tested in the Oregon State University Human Performance Laboratory as part of the screening process. Additional screening information is given in the previous chapter.

## Study protocol

Subjects were randomly assigned to one of two groups (placebos or supplements) that based on age, gender, body mass index (BMI) and cholesterol level. At the outset of the study, each subject received pill packets that were labeled by month and day to promote compliance. Additional study protocol details are outlined in the previous chapter.

The subjects were asked to adhere to a prescribed low antioxidant diet (Appendix E) for 6 weeks prior to the race and for one week subsequent to the race. Subjects were also provided low antioxidant training foods, such as energy bars, carbohydrate pastes (Clif shots were donated by the Clif Corporation) and drinks (Gatorade powder was donated by the Gatorade Corporation). Athletes were asked to use only the ergogenic aids provided at the onset of the study. The subjects were also required to consume a controlled diet for 2 days: the day prior to the race and race day. Additional details concerning the meals are provided in the previous chapter.

On race day, the controlled diet continued before, during, and immediately following the race. Food consumption was recorded and analyzed for each of these meals. Breakfast was available after a fasting blood draw on the morning of the

race. Volunteers were stationed at seven different points on the racecourse with food and sport fuels low in vitamin E and C available for the study participants. The volunteers recorded food consumption at each of the stations. After the race food was provided for all the study participants, and a registered dietician recorded the post-race intakes.

Samples were obtained at baseline six weeks prior to the race, 3 weeks prior to the race, the morning before the race, the morning of the race 1 to 2 hours before start, at kilometer 27 during the race, immediately following the race, and 2-hours following the race. Additional blood draws were collected on the six mornings following the race (See Appendix A). The exact time of blood drawing was recorded for each subject. Additional details concerning the race course and energy monitoring are provided in the previous chapter.

Subjects were asked to refrain from all physical activity, especially running, for the six days following the ultramarathon allowing investigation of the effects of antioxidant supplementation on post-race iron alterations.

#### Analytical Techniques

Plasma  $\alpha$ -tocopherol and ascorbic acid were measured by high pressure liquid chromatography (HPLC) using electrochemical detection, as described in the previous chapter.

An enzyme immunoassay (EIA) 96-well plate from Ramco Laboratories (Stafford, TX) was implemented to measure plasma ferritin levels, as described in the previous chapter.

The determination of TIBC was completed through colorimetric method with reagents from Sigma Diagnostics (St. Louis, MO). The measurement of TIBC is a two step process, the measurement of serum total iron and serum unsaturated iron-binding capacity (UIBC). The first step (measurement of total serum iron) measures the transferrin-bound iron that disassociates from transferrin to form ferrous ions in an acid pH. The second step (UIBC) measures the amount of unbound ferrous ions after adding a specific amount of ferrous ions at an alkaline pH. These two values are calculated and summed to calculate TIBC. Serum (approximately 1 ml) was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until the time of analysis. To measure total iron, 160  $\mu\text{l}$  of hydroxylamine hydrochloride, 1.5% (wt/vol), in acetate buffer, pH of 4.5 was pipetted into each well of a 96 well plate; then 30  $\mu\text{l}$  of each sample was pipetted in duplicate into the plate; the initial absorbance was read at 560 nm. To each well 5  $\mu\text{l}$  of ferrozine, 0.85% (wt/vol) in hydroxylamine hydrochloride was added to each well and incubated at  $37^{\circ}\text{C}$  for 10 minutes to develop color. The absorbance of each well was read at 560 nm, and the total serum iron was calculated from a linear standard curve.

UIBC was measured in a similar manner as serum total iron. After bringing reagents and samples to room temperature, 130  $\mu\text{l}$  of TRIZMA® 0.5 mol/L of a pH of 8.1 was added to each well of a 96-well plate. To the standard wells, 30  $\mu\text{l}$  of iron-free water was added and to the sample wells 30  $\mu\text{l}$  the iron standard (500

$\mu\text{g/dl}$  iron in hydroxylamine hydrochloride solution) was added. Then the 30  $\mu\text{l}$  of the standards and samples were added to the designated wells; the samples were added in duplicate. The absorbance was then read at 560nm as the initial values. To each well 5  $\mu\text{l}$  of ferrozine, 0.85% (wt/vol) in hydroxylamine hydrochloride was added to each well and incubated at 37° C for 10 minutes to develop color. The absorbance of each well was read at 560 nm, and the UIBC was calculated by equations provided. TIBC was the sum of serum total iron and UBIC, and data was reported as  $\mu\text{g/dl}$ .

Hemoglobin and hematocrit were also measured for each subject over the course of the study. Hemoglobin was measured on whole blood using a kit according to manufacturer's instructions (Sigma Diagnostics; Procedure No. 525). The principle of this kit is based on the oxidation of hemoglobin to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin may then react with potassium cyanide to form cyanmethemoglobin to form cyanmethemoglobin. This end product has a maximum absorbance at 540 nm.

A 20  $\mu\text{l}$  sample of whole blood was added 5 ml of Drabkin's solution and allowed to incubate at room temperature for 15 minutes before measuring the absorbance. Results were then compared against a standard curve prepared from cyanmethemoglobin to quantify g/dl of hemoglobin.

Blood hematocrit was measured using standard techniques. In short, whole blood was collected into a heparinized micropipette (40mm) and then spun for 5 minutes in a microhematocrit centrifuge (Statspin; Norwood, MA). Hematocrit,

expressed as a percentage of total blood volume, was then obtained by comparison to a microhematocrit tube reader.

## STATISTICAL ANALYSIS

A repeated measures ANOVA was used to test between and within subject differences using SAS version 8.0 (SAS Institute, Cary NC). Results were confirmed with a repeated measures ANOVA with Scheffe post hoc analysis using Statview version 5.0.1 (SAS Institute, Cary NC) statistical software. SPSS version 11.0 (SPSS Inc., Chicago IL) was implemented to examine the normality of data. The data were natural log transformed to better meet the assumption of normality of the repeated measures ANOVA. One subject was excluded from the ferritin analysis because the subject had a stomach illness the day before the race and was still nauseous on race day. It is well known that ferritin is responsive to infection. Consistent with that, the subject's ferritin levels were extremely elevated at pre-race ( $> 4$  S.D. outside the mean) and were not consistent with the study population. The same subject had several missing samples for TIBC and was also dropped from that analysis.

## RESULTS

### Subject Characteristics

Participant characteristics are presented the previous chapter. None of the subjects had abnormal lipid values and all participants met the oxygen consumption criteria for inclusion in the study.

A controlled diet was implemented the day before the race and on race day, and the food intakes were analyzed using ESHA food software. The previous chapter displays the composition of the foods consumed during the two-day controlled diet.

### Plasma Ascorbic Acid and Tocopherol Concentrations

Plasma ascorbic acid concentrations increased significantly ( $p < 0.0001$ ) with supplementation and were significantly higher in the supplemented compared with the placebo group (see previous chapter Figure 2.1). Plasma  $\alpha$ -tocopherol concentrations were significantly ( $p < 0.0001$ ) higher in the supplemented compared with the placebo group (see previous chapter Figure 2.2). In the supplemented group, plasma  $\alpha$ -tocopherol concentrations increased from baseline levels and remained elevated at each subsequent time point ( $p < 0.0001$ ). These data demonstrate that the subjects took the supplements, or placebos, and are correctly identified.



## Iron Profile

Because endurance athletes, especially distance runners, have increased susceptibility to compromised iron status defining iron deficiency in this population is of great importance for performance and health. Several measurements of iron status were measured at several different time points over the course of the study. Examination of the measurements before supplementation can help define particular subjects that may be in danger of depleted iron stores or iron deficiency anemia (Table 3.1).

Table 3.1 Iron Status Parameters at Baseline Prior to Supplementation

Females						
	Group	Ferritin	Hemoglobin	Hematocrit	TIBC	total serum iron
Units		ng/ml	g/dl	%	$\mu\text{g/dl}$	$\mu\text{g/dl}$
JH11	P	5.2	13.37	38.5	340	57
LW10	S	5.5	14.59	42.5	419	29
JC15	S	7.0	14.57	40.5	383	66
PK5	S	8.5	14.20	41.0	388	40
KB21	S	12.1	13.74	39.0	338	92
JL17	P	12.5	14.66	40.0	290	83
KC8	S	13.9	15.12	40.5	289	57
KG7	P	17.0	16.45	38.5	368	34
DJ9	P	22.0	13.08	40.0	350	103
MS6	P	23.9	14.64	40.0	304	70
VI16	S	26.6	15.97	43.0	314	74
Mean		$14.0 \pm 2.3$	$14.58 \pm 0.31$	$40.3 \pm 0.4$	$344 \pm 13$	$64 \pm 7$
Males						
MP22	P	13.3	16.25	42.0	292	61
JR4	P	22.0	15.15	42.5	237	104
JP12	P	24.7	16.86	46.0	289	49
SA1	S	28.9	18.98	49.0	337	127
DB2	P	29.5	17.87	50.0	359	120
CB3	S	38.8	15.99	46.0	304	289
BKP13	S	42.5	15.99	43.5	220	73
JDR18	P	52.4	16.42	45.0	271	98
JM14	S	53.4	15.94	48.5	306	66
SH20	S	60.8	16.86	45.5	317	93
PB19	S	66.4	16.47	45.5	330	65
Mean		$39.3 \pm 5.2$	$16.62 \pm 0.31$	$45.8 \pm 0.8$	$296 \pm 13$	$104 \pm 20$

Assignment S = supplement and P = placebo

## Ferritin

Ferritin is the storage form of iron found in the body. Overall, the female subjects had significantly lower ferritin concentrations than did the males (ANOVA  $p < 0.001$ ) (for data, see previous chapter, Figure 2.5). Baseline ferritin means were calculated from blood samples obtained at pre-supplementation, compliance, and the day before the race. After this covariate adjustment, there was a main effect of time ( $p < 0.0001$ ) and interactions of gender with time ( $p < 0.05$ ) and treatment (supplement vs. placebo) with time ( $p < 0.005$ ). By 5 days after the race, the antioxidant supplemented subjects had significantly lower ferritin concentrations ( $p < 0.02$ ), than placebo subjects.

## Total-Iron Binding Capacity (TIBC) and Serum Total Iron

TIBC approximates concentrations of transferrin, the transport form of iron, and increases with depletion of iron stores. The genders were significantly different at baseline; the females had significantly higher TIBC levels ( $344 \pm 13$ ) than the males ( $296 \pm 13$ ) ( $p < 0.02$ , Figure 3.1). After the baseline covariate adjustment, TIBC changed with time ( $p < 0.0001$ ) and changed differently in supplemented compared with placebo-treated subjects (interaction with time  $p < 0.03$ , Figure 3.2). At post race, the placebo group had significantly lower TIBC levels compared to the supplemented subjects ( $p < 0.05$ ).

TIBC measurement is the sum of serum total iron and unsaturated iron-binding capacity. Serum total iron indicates the amount of iron bound to a transferrin molecule. Serum total iron changed over time ( $p < 0.0001$ ) and changed

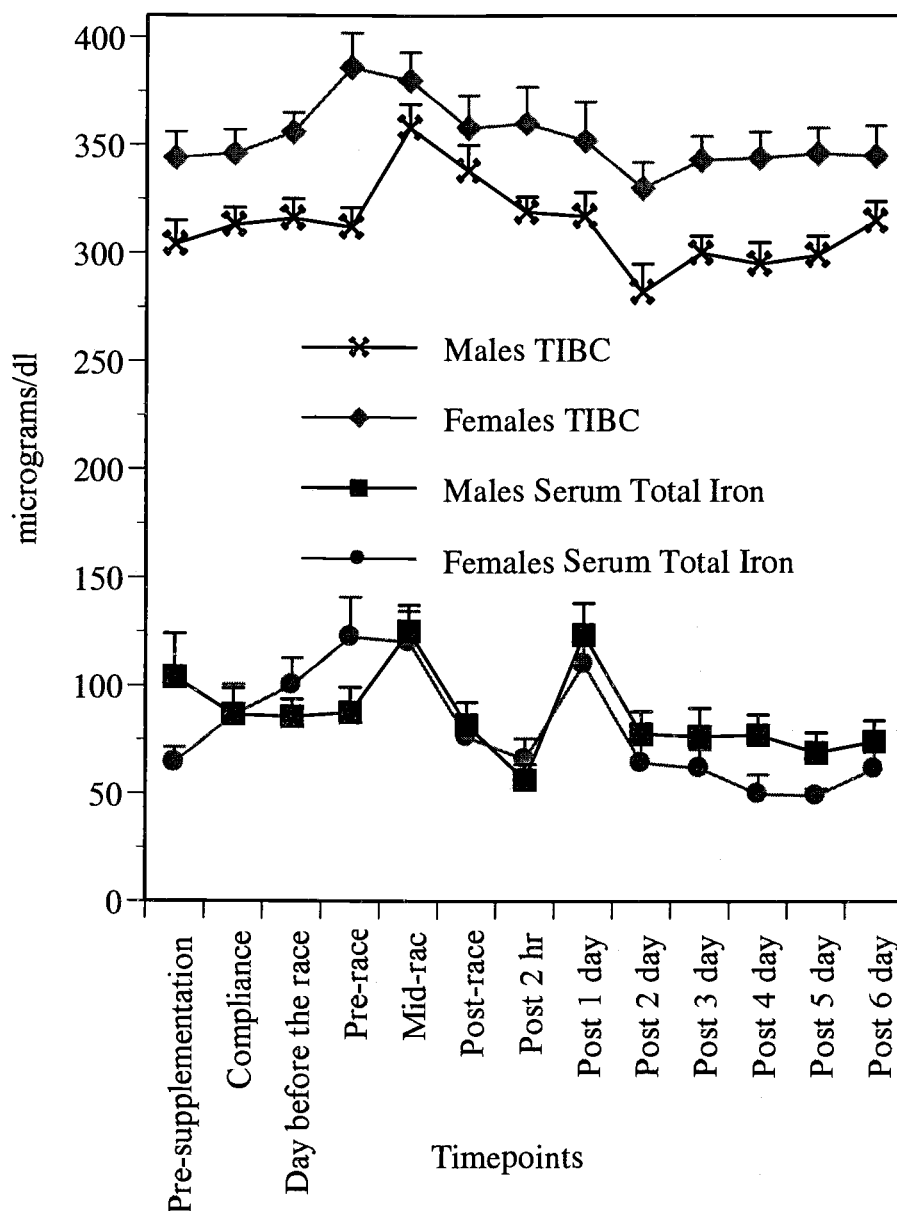


Figure 3.1 Gender Differences in TIBC and Serum Total Iron over the Course of the Study

TIBC and serum total iron (mean + SE) are shown. For TIBC, the genders were significantly different at baseline; the females ( $n = 11$ ) had significantly higher TIBC levels than the males ( $n = 10$ ) ( $p < 0.02$ ). Serum total iron changed differently for males and females over the course of the study (ANOVA, gender/time interaction  $p < 0.02$ ).

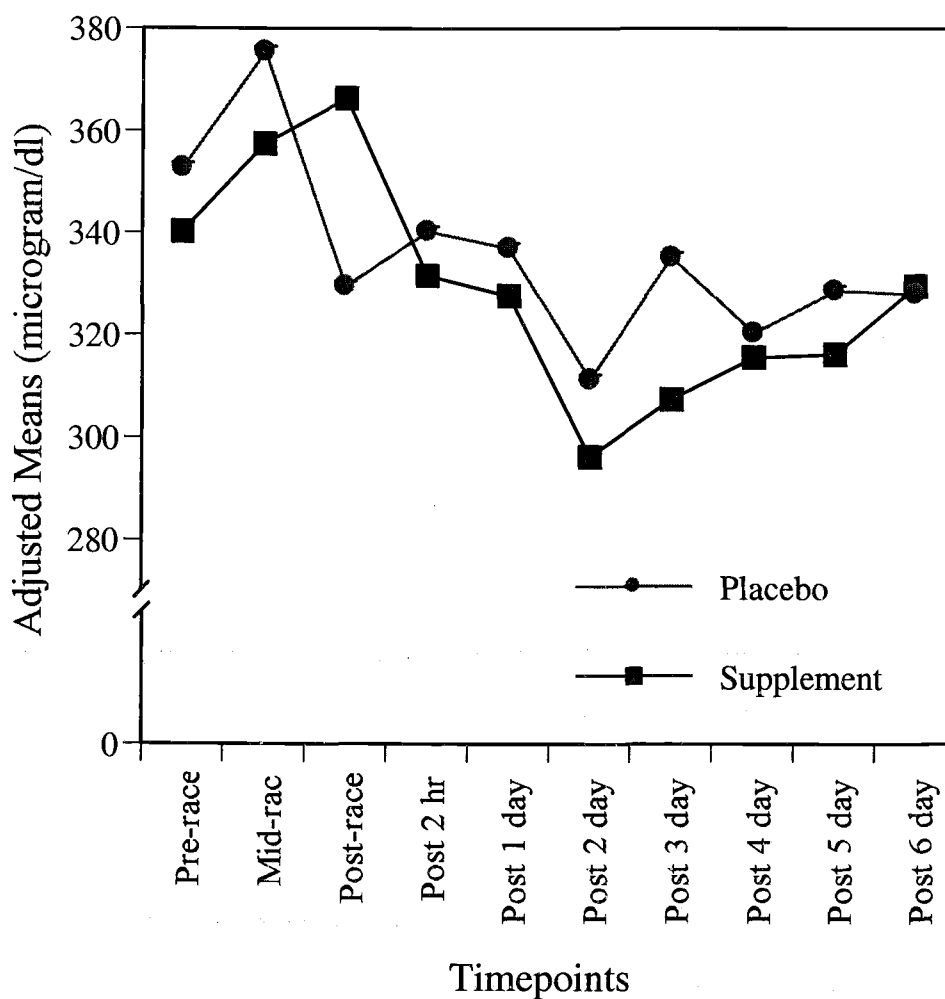


Figure 3.2 TIBC Treatment Differences

TIBC (mean + SE) are shown. After baseline covariate adjustment, TIBC changed with time ( $p < 0.0001$ ) and changed differently in supplemented ( $n = 11$ ) compared with placebo-treated ( $n = 10$ ) subjects (interaction with time  $p < 0.03$ ). At post race, the placebo group had significantly lower TIBC levels compared to the supplemented subjects ( $p < 0.05$ ).

differently for males and females over the course of the study (ANOVA, gender/time interaction  $p < 0.02$ , Figure 3.1).

### Hemoglobin

Hemoglobin assessment approximates the blood's oxygen carrying capacity and can be used to assess iron status. Overall, females had lower hemoglobin concentrations than the males (main effect,  $p < 0.005$ ) (Figure 3.3). Time was also a significant main effect ( $p < 0.0001$ ) and baseline average was a significant covariate ( $p < 0.03$ ). Following correction for the baseline covariate, both main effects gender ( $p < 0.005$ ) and changes over time ( $p < 0.0001$ ) remained significant. Hemoglobin concentrations increased from pre-race to post-race ( $p < 0.0001$ ) and to post-2 h ( $p < 0.003$ ) but returned to pre-race values by post-day 1.

### Hematocrit

Hematocrit was also measured as a marker of iron status. Females displayed significantly lower hematocrit values than the males (Figure 3.4,  $p < 0.0001$ ), which is consistent with the other iron parameters. A significant treatment/gender interaction was also detected ( $p < 0.05$ ). Treatment significantly increased hematocrit levels in the female subjects (Figure 3.5,  $p < 0.001$ ), compared to the females on placebos, but appeared to have no effect in the male subjects. In all subjects, hematocrit decreased significantly from pre-race to post-race ( $p < 0.05$ ) and remained depressed for the rest of the study ( $p < 0.0001$ , except post 1 day,  $p < 0.01$ ).

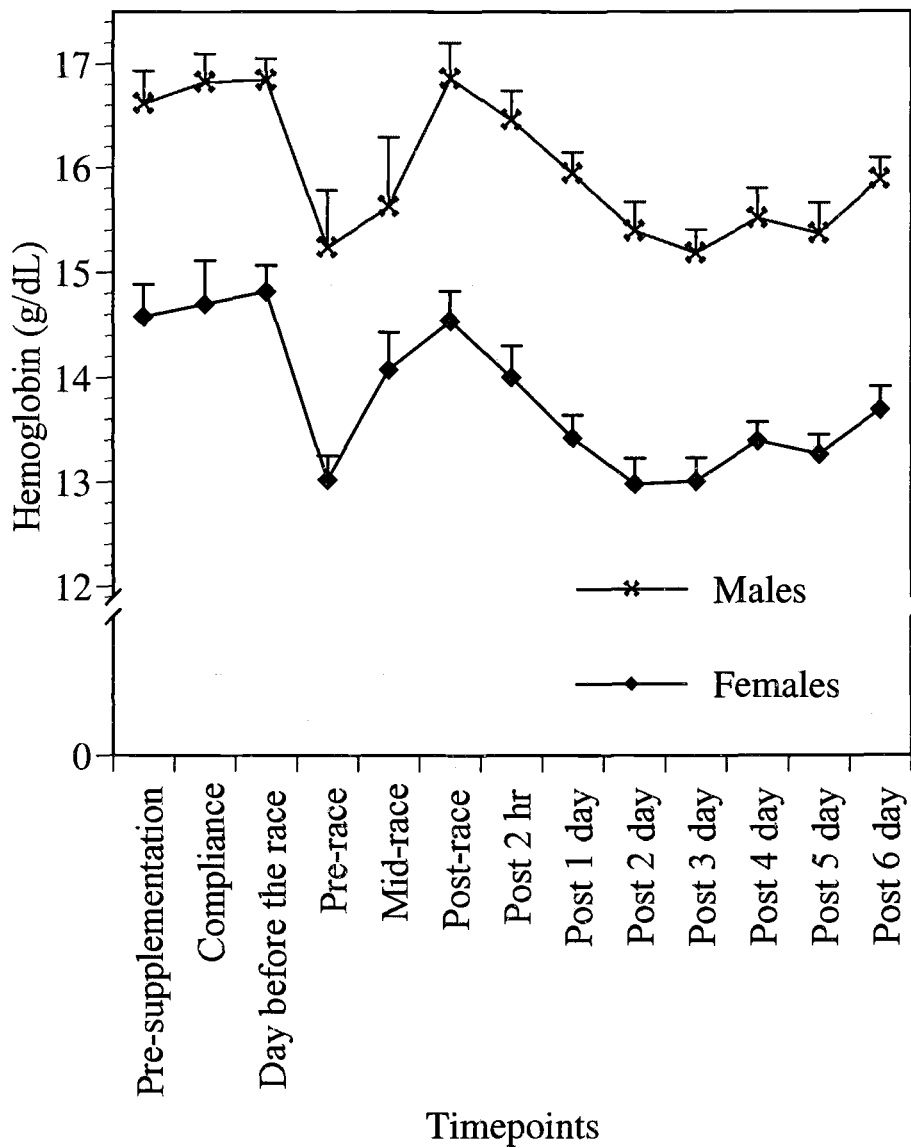


Figure 3.3 Hemoglobin Concentrations Over the Course of the Study

Hemoglobin concentrations (mean + SE) were unaffected by supplementation. Gender and time were both main effects of the repeated measures ANOVA before covariate correction (both  $p < 0.0001$ ), males ( $n = 11$ ) possessing higher hemoglobin concentrations than females ( $n = 11$ ).

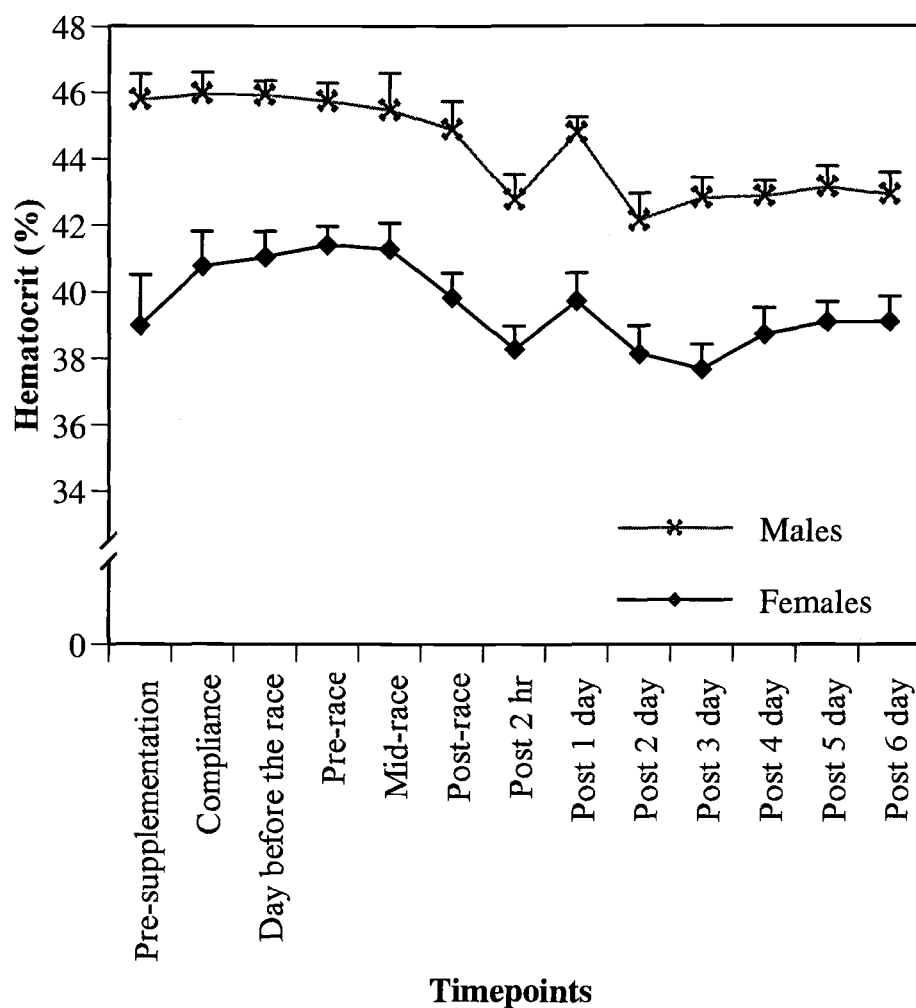


Figure 3.4 Gender Differences in Hematocrit over the Course of the Study

Percent hematocrit (mean + SE) are shown. The female subjects have significantly lower hematocrit levels than the male subjects ( $p < 0.0001$ ). Compared to pre-race, hematocrits decreased significantly at post-race ( $p < 0.05$ ) and remained depressed for the remainder of the study ( $p < 0.0001$ , except post 1 day,  $p < 0.01$ ). A significant gender/treatment interaction was also detected in the analysis ( $p < 0.05$ ).

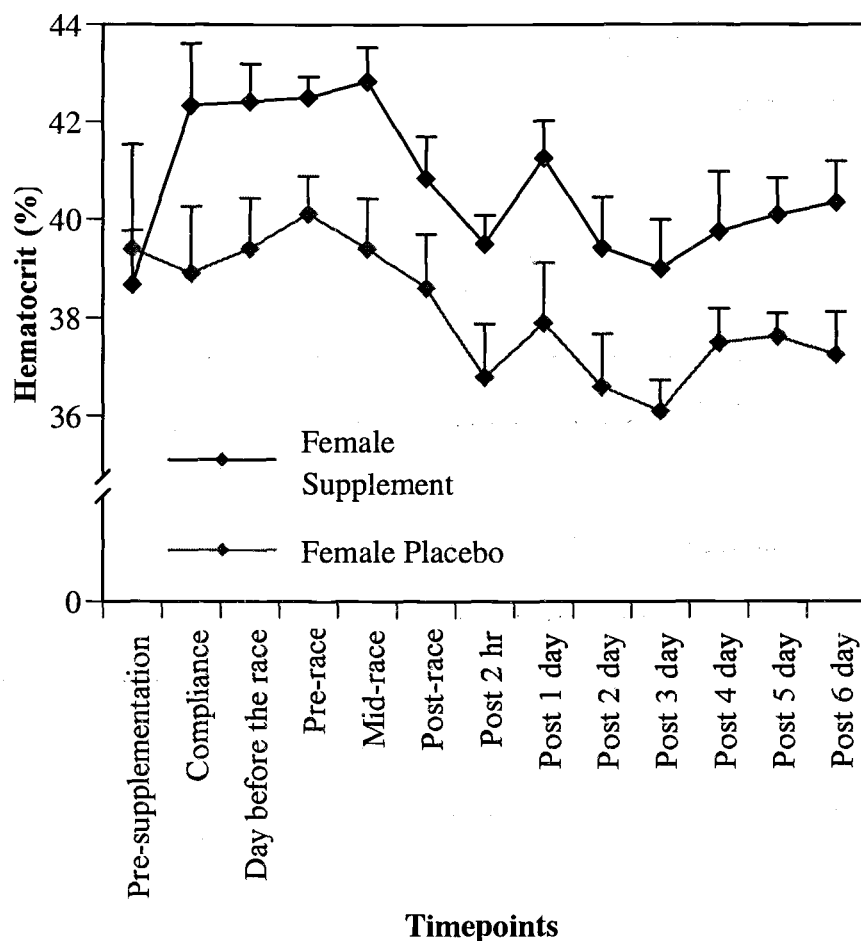


Figure 3.5 Antioxidant Treatment Increases Hematocrit Levels in Female Ultramarathon Runners

Percent hematocrit (Mean + SE) in female runners is displayed. The antioxidant treatment significantly increased the levels of hematocrit in the female subjects ( $n = 6$ ) compared to the females consuming placebos ( $n = 5$ ) ( $p < 0.001$ ).



## DISCUSSION

This study assessed iron status in a group of distance runners and the influence of antioxidant supplementation before, during, and after a 50K ultramarathon. Plasma ferritin, TIBC, serum total iron, hemoglobin and hematocrit responses indicate that the female runners have a compromised iron status that may be improved with antioxidant supplementation.

The male subjects had significantly higher plasma concentrations of ferritin than the female subjects ( $p < 0.0001$ ). In fact, the female runners had ferritin values that are near levels that indicate depleted iron stores ( $< 12 \mu\text{g/l}$ ). Iron is essential for humans, but even more important the endurance athletes. Although sports anemia, the physiological adaptation to endurance exercise caused by an expanded plasma volume, often accounts for the anemia measured in distance runners (86), the stark differences between the males and females of the same approximate training level indicated that the females had true iron depletion.

Ferritin levels increased with the stress of the extreme endurance event because it also functions as an acute phase reactant (11). Nonetheless, antioxidant supplementation had only a marginal effect upon ferritin levels. At five days after the race, ferritin concentrations were lower in the supplemented subjects than those who consumed placebos. Antioxidant supplementation appears to improve clearance of ferritin from the blood stream. Ferritin increases with inflammation and the greater clearance of ferritin in the antioxidant group may indicate a beneficial effect of the supplementation in endurance athletes.

To better understand the iron status, especially of the female athletes, TIBC, serum total iron, hemoglobin, and hematocrit levels were measured in the study population. The TIBC levels in the females were significantly higher than in the males ( $p < 0.02$ ). This finding is consistent with the results of the ferritin analysis, which show the female subjects to have a more compromised iron status. TIBC levels  $> 400 \mu\text{g/dl}$  indicate depleted iron stores. At baseline, the TIBC in the female subjects was  $344 \pm 13 \mu\text{g/dl}$ , which is within the normal range, but two subjects were near or above  $400 \text{ TIBC } \mu\text{g/dl}$  indicating depleted iron stores. TIBC is a less sensitive marker of iron deficiency than other clinical measures such as ferritin.

At post-race, the placebo subjects had lower TIBC concentrations (covariate-adjusted) than those consuming supplements (Figure 3.1). TIBC generally decreases with inflammation (70, 71); therefore, the suppressed levels of TIBC in the placebo group at post-race suggests a more severe inflammatory response than experienced by the antioxidant group.

Hemoglobin and hematocrit were also lower in females than males ( $p < 0.0001$ ). Once again this is an indication that the female athletes studied may have compromised iron status. The level of hemoglobin that is consistent with iron deficiency anemia is  $< 12 \text{ g/dl}$ , while the baseline value observed in our female subjects is approximately  $14.58 \pm 0.31$ , therefore some of the subjects may be iron deficient (by ferritin and TIBC measures) but not anemic. Following correction for the baseline covariate, hemoglobin concentrations increased from pre-race to post-

race ( $p < 0.0001$ ) and to post-2 h ( $p < 0.003$ ) but returned to pre-race values by post-day 1 in both genders. The differences detected over the course of the study are most probably due to different hydration levels at various time points. With increased water intake, hemoglobin concentrations decrease.

Compared to pre-race, hematocrits were significantly depressed at post-race and remained low for the rest of the study. The decrease in hematocrit after the race may be due to red cell damage and loss caused by footstrike hemolysis. Plasma volume expansion may also contribute to the decreased levels of hematocrit after the race (86).

The females also had a significantly depressed hematocrit when compared with the males ( $p < 0.0001$ ). Although antioxidant supplementation did not alter hematocrit levels in the male subjects, a beneficial effect was observed in females. The antioxidants may be more beneficial to individuals with depressed total body iron (88), and the females had a more compromised iron status than the male subjects. Because females in the placebo and antioxidant group had high plasma ascorbic acid levels from the onset of the study, the increase in hematocrit may be influenced more by vitamin E's protection of the red blood cells from oxidation and hemolysis than enhanced iron absorption due to vitamin C consumption. The beneficial qualities of the vitamin C may be due to the bolus of vitamin C consumed with meals in the supplement group rather than the plasma levels of ascorbic acid (87). The bolus of vitamin C may improve non-heme iron absorption and account for the increased hematocrits. Despite the increase in hematocrit levels

in the females consuming supplements, serum total iron did not change significantly with supplementation; therefore, protection of the erythrocytes by vitamin E may better explain the increase in hematocrit than improved vitamin C absorption. In summary, vitamin C supplementation can improve iron absorption (63, 76, 87) and vitamin E effectively protects erythrocytes membranes (43) and may contribute to the increase in hematocrit observed in the females consuming antioxidants.

In summary, antioxidant supplementation improved hematocrit levels ( $p < 0.05$ ) among female runners. Several iron status parameters indicated that female runners have decreased iron stores as compared with their male counterparts. The mean ferritin concentrations in females were near the level indicative of depleted stores ( $< 12$  ng/ml). All of the other markers of iron that were measured showed that female distance runners may be prone to compromised iron status. The results of the hematocrit measurement indicate that antioxidant supplementation may improve iron status in this population. Most of the females in the study were borderline vegetarian and such a diet may not be suitable for female distance runners without special dietary considerations.

## CONCLUSIONS

Extreme endurance athletes, such as ultramarathon runners, are susceptible to exercise induced inflammation and immune suppression. Although other investigations have suggested that antioxidant vitamins decrease exercise-induced inflammation, no profound benefit of supplementation on markers of inflammation was found in this investigation. It should be noted that a response similar to the acute phase response was elicited by the ultramarathon race. Antioxidants may improve chronic inflammation common with diabetes and cardiovascular disease, but antioxidants do not appear to improve the inflammation common after extreme endurance exercise in trained athletes. Improvements in IL-1• and ferritin in response to antioxidant supplementation may indicate that the supplementation was beneficial, but more research is needed to draw definitive conclusions.

Antioxidant supplementation improved hematocrit levels among female runners. This finding could be a result of the functions of both or either vitamins C and E. Vitamin C supplementation has been shown to improve iron absorption and Vitamin E effectively protects erythrocyte membranes. Vitamin E increases membrane fluidity, which may contribute to the increase in hematocrit observed in the females consuming antioxidants. Through analysis of several iron parameters, it was clear that female runners have decreased iron availability when compared to their male counterparts. The mean ferritin for females was near the level indicative of depleted stores. All of the other markers of iron that were measured showed that female distance runners may be prone to compromised iron status. The results of

the hematocrit measurement indicate that antioxidant supplementation may improve iron status in this population.. Antioxidants may be more beneficial to those with depleted iron stores than those with adequate total body iron.

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## APPENDICES

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## APPENDIX A. OUTLINE OF STUDY EVENTS

DATE	TIME-POINT	DESCRIPTION OF EVENTS
March 15	6 weeks before the race	Fasting blood draw, prescribed diet began, supplementation began
April 3	3 weeks before the race	Fasting blood draw
April 26	1 day before the race	Fasting blood draw, controlled diet began
April 27	2 hours before the race	Fasting blood draw
April 27	27 km of race	Blood draw (non-fasting)
April 27	Immediately post-race	Blood draw (non-fasting)
April 27	1-2 hours after race finish	Blood draw (non-fasting)
April 28	1 day post race	Fasting blood draw, prescribed diet resumed
April 29	2 days post race	Fasting blood draw
April 30	3 days post race	Fasting blood draw
May 1	4 days post race	Fasting blood draw
May 2	5 days post race	Fasting blood draw
May 3	6 days post race	Fasting blood draw, supplementation ceased



## Likert Scale Explanation

Each day you will be asked how are you feeling physically on a scale of 1 to 5 as seen below:

**Extremely Poor ① ② ③ ④ ⑤ Excellent**

- ◆ Where ① corresponds to your health being **extremely poor**
- ◆ Where ② corresponds to your health being **below average**
- ◆ Where ③ corresponds to your health being **average**
- ◆ Where ④ corresponds to your health being **above average**
- ◆ Where ⑤ corresponds to your health being **excellent**

Please fill out this section by marking one number each day assessing your personal physical health status.

## APPENDIX B. LIKERT SCALE (CONTINUED)

# Wednesday March 20, 2002

## Reminders for today:

Cardio Exercise	Time/Distance	Notes

Strength training	Wt	Sets	Reps

YES NO

Did you take your supplements today?

☐☐

How are you feeling physically?

Extremely Poor ① ② ③ ④ ⑤ Excellent

If you are not feeling well please describe your symptoms

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APPENDIX C.  
APPROVAL

## INSTITUTIONAL REVIEW BOARD PROTOCOL

**Report of Review by the Institutional Review Board**

December 19, 2001

TO: Maret Traber and Angela Mastaloudis, Linus Pauling Institute


RE: Inhibition of Endurance Exercise Generated Oxidative Stress by Antioxidant  
Supplementation

The referenced project was reviewed under the guidelines of Oregon State University's Institutional Review Board (IRB) and the U.S. Department of Health and Human Services. The IRB has **approved** your application. The approval of this application expires upon the completion of the project or one year from the approval date, whichever is sooner. The informed consent form obtained from each subject should be retained in program/project's files for three years beyond the end date of the project.

Enclosed with this letter please find the original informed consent document for this project, which has received the IRB stamp. The informed consent document has been stamped to ensure that only current, approved informed consent forms are used to enroll participants in this study. All participants are to receive the IRB stamped informed consent document. Please make copies of this original as needed.

Any proposed change to the protocol, the informed consent form, or testing instrument(s) that is not included in the approved application must be submitted using the MODIFICATION REQUEST FORM. Allow sufficient time for review and approval by the committee before any changes are implemented. Immediate action may be taken where necessary to eliminate apparent hazards to subjects, but this modification to the approved project must be reported immediately to the IRB. Any happening not connected with routine expected outcomes that result in bodily injury and/or psychological, emotional, or physical harm or stress must be reported to the IRB within three days of the occurrence using the ADVERSE EVENT FORM. Please use the included forms as needed.

If you have any questions, please contact the IRB Coordinator at [IRB@orst.edu](mailto:IRB@orst.edu) or by phone at (541) 737-3437.

  
\_\_\_\_\_  
Anthony Wilcox, IRB Chair  
Langton Hall 214  
[Anthony.Wilcox@orst.edu](mailto:Anthony.Wilcox@orst.edu); 737-6799

Date: 12/19/01

APPENDIX C. INSTITUTIONAL REVIEW BOARD APPROVAL OF  
PROTOCOL (CONTINUED)



OREGON STATE  
UNIVERSITY

INSTITUTIONAL REVIEW BOARD  
REPORT OF REVIEW

TO: Maret G. Traber,  
Linus Pauling Institute

RE: Inhibition of Endurance Exercise Generated Oxidative Stress by Antioxidant  
Supplementation

Protocol No. 1481

The referenced proposed modification to a previously approved project was reviewed under the guidelines of Oregon State University's Institutional Review Board (IRB) and the U.S. Department of Health and Human Services. The IRB has **approved** the modification. This approval is valid through 12/18/02.

Enclosed with this letter please find the original informed consent document for this project, which has received the IRB stamp. The informed consent document has been stamped to ensure that only current, approved informed consent forms are used to enroll participants in this study. All participants are to receive the IRB stamped informed consent document. Please make copies of this original as needed.

Any proposed change to the protocol, the informed consent form, or testing instrument(s) that is not included in the approved application must be submitted using the MODIFICATION REQUEST FORM. Allow sufficient time for review and approval by the committee before any changes are implemented. Immediate action may be taken where necessary to eliminate apparent hazards to subjects, but this modification to the approved project must be reported immediately to the IRB. Any happening not connected with routine expected outcomes that result in bodily injury and/or psychological, emotional, or physical harm or stress must be reported to the IRB within three days of the occurrence using the ADVERSE EVENT FORM. Please use the included forms as needed.

If you have any questions, please contact the IRB Coordinator at [IRB@orst.edu](mailto:IRB@orst.edu) or by phone at (541) 737-3437.

for *Anna K. Gindon*  
Anthony Wilcox, IRB Chair  
Langton Hall 214  
[Anthony.Wilcox@orst.edu](mailto:Anthony.Wilcox@orst.edu); 737-6799

Date: 2/1/02

cc: IRB Coordinator

## APPENDIX D. INFORMED CONSENT

LINUS PAULING INSTITUTE



OREGON STATE UNIVERSITY  
571 Weniger Hall, Corvallis, Oregon 97331-6512  
Telephone 541-737-7977, Fax 541-737-5077

Date \_\_\_\_\_

OSU IRB Approval Date: 2/1/02  
Approval Expiration Date: 12/15/02

## INFORMED CONSENT DOCUMENT

**A. Title of the Research Project:** Inhibition of Endurance Exercise Generated Oxidative Stress by Antioxidant Supplementation.

**B. Principal investigators:**

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**C. Purpose of the Research Project.**

You are being asked to participate in a research study. We plan to study the effects of running a 50 km ultramarathon in twenty trained individuals consuming one of the following daily for 7 weeks: placebos, or 300 mg vitamin E and 1000 mg vitamin C (500 mg twice daily). In addition, vitamin E turnover will be evaluated during the ultramarathon run using labeled vitamin E. The evening prior to the race, subjects will consume, with dinner, capsules containing vitamin E labeled with deuterium. It should be noted that deuterium is a stable isotope; it is not radioactive, there are no known hazards or risks to humans by deuterium consumption. Prior to admittance into the study, a screening test will be administered involving a blood chemistry and a submaximal

## APPENDIX D. INFORMED CONSENT (CONTINUED)

oxygen consumption ( $\text{VO}_{2 \text{ submax}}$ ) treadmill test. The purpose of this study is to evaluate whether oxidative stress generated during ultra-endurance exercise is affected by prior supplementation with the antioxidants vitamins E and C.

**D. Procedures.**

I was interviewed over the phone to determine whether I am a suitable candidate for this study. I have received an oral and a written explanation of this study and I understand that as a participant in this study the following things will happen:

**1. Pre-study questionnaires.**

I will complete a health/diet history questionnaire and provide a blood sample for a pre-screening blood chemistry. After completion of this screening, I may or may not be asked to participate in the remainder of the study. Whether or not I am selected as a participant in this study, I will receive a copy of my pre-screening blood chemistry. In the event that I am not chosen to participate in this study, all of my other records will be destroyed by shredding.

*Blood Chemistry.* An initial blood screening will be used to determine if I have abnormal triglyceride, cholesterol or glucose levels. My blood vitamin E and vitamin C levels will also be measured in order to verify that I am not a supplement user. I will receive the results of my individual blood chemistry. In the event that I have an abnormal result I will be notified and advised to seek further evaluation from my regular physician.

**2. In Person Screening**

One to two months prior to the trial, I will be tested in the laboratory as part of the screening process. I will report to the Human Performance Laboratory in the Dept. of Exercise and Sport Science and baseline information including:  $\text{VO}_{2 \text{ submax}}$  and a body composition assessment.

*Treadmill run to determine my submaximal aerobic capacity.* The submaximal oxygen consumption test is conducted during moderate exercise to estimate the greatest amount of oxygen taken up by the body; it is commonly used as an indicator of cardiorespiratory fitness.

The test consists of running on a motorized treadmill until the technician asks me to stop. This will be when my heart rate reaches ~ 85% of my age-predicted maximal heart rate. I will be given instructions on how to run on the treadmill, how to step off the treadmill, and how to operate the emergency shut-off switch. Before the test begins, I will be given sufficient practice so that I feel comfortable running, stepping off, and stopping the treadmill.

I will be given 5-10 minutes of warm-up exercise consisting of walking/jogging on the treadmill. The initial work rate of the aerobic capacity test will be very mild. Every 2-3 minutes, the treadmill grade and/or speed will be increased by a moderate amount. Thus, at each stage, it will become harder and harder to run. My goal is to run until the technician asks me to stop; I realize that because this is a submaximal exercise test I may or may not feel tired at this point. When instructed by the technician, I will either stop or step off the treadmill. I can expect the test to last approximately 12-15 minutes.

During the test, ECG electrodes will be applied to my chest in order to monitor my heart's performance. A nose clip will be attached to my nose and I will breath through a mouth piece so that my oxygen consumption can be determined.

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## APPENDIX D. INFORMED CONSENT (CONTINUED)

*Determination of my height, weight, and body composition.* My height and weight will be recorded. A Bod Pod will be used to determine my body composition (% body fat). The Bod Pod is an apparatus that estimates my % body fat based on changes in pressure created by the displacement of air by my body. I understand that this is a non-invasive technique, but that I must be comfortable sitting quietly in a small space, approximately the size of a phone booth, for 1-3 minutes.

*Upon completion of in person screening.* If results from my blood chemistry profile reveal that I have abnormal triglyceride, cholesterol or glucose levels or that I am currently taking vitamin E and/or vitamin C supplements, I will be asked to withdraw from the study at this point. If my  $\text{VO}_{2\text{submax}}$  does not classify me as having excellent fitness based on the following criteria:  $\text{VO}_{2\text{max}} \geq 47, 45, 44$  or  $41 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ , for males aged 18-29, 30-39, 40-49 and 50-60 years, respectively, or  $\geq 37, 36, 33$  or  $32 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ , for females aged 18-29, 30-39, 40-49 and 50-60 years, respectively, I will also not be accepted into the study. In the event that I am not asked to participate in the remainder of the study, all of my records, except my individual blood chemistry which will be mailed to me, will be destroyed by shredding.

\*Although my weight, height and body composition will be determined for my subject record during the initial screening, these criteria will not effect whether or not I am asked to participate in the study.

3. What I will be asked to do during the study.

*Randomization Criteria:* I will be randomly assigned to one of two treatment groups (placebos, or vitamin E and vitamin C supplements). I realize that because this is a placebo controlled study, I will not be informed of which treatment group to which I have been assigned.

*Antioxidant supplementation:* Twice per day, each morning and each evening for seven weeks, I will be required to consume, based on my randomization category:

- 1) An orange capsule containing 500 mg of ascorbic acid, or citric acid as the placebo (for a total of 1000 mg/day).

Subjects per Group	
Placebos	Vitamins C & E
N=10	N=10

Once per day, each evening, with dinner, for seven weeks, I will be required to consume, based on my randomization category:

- 2) A brown capsule containing 300 mg *RRR*- $\alpha$ -tocopheryl acetate in soybean oil, or soybean oil alone as the placebo.

*Labeled Vitamin E Administration:* 12 hours prior to the race I will consume a single gelatin capsule containing deuterium labeled vitamin E (300 mg  $\text{d}_6$ -*RRR*- $\alpha$ -ocopheryl acetate) with dinner.

*Ultramarathon:* I am already familiar with the McDonald Forest Ultramarathon Race as I have participated in the race in past years and/or I was already registered for the race when I was recruited to participate in this research project. I realize that it is a 50 km (30 mile) trail run over rugged terrain; with a total elevation gain and loss of ~12,000 ft.

*Diet:* I will be required to consume a prescribed diet for 6 weeks prior to the race and 1 week following the race. As part of the prescribed diet, I will be asked to avoid foods high in antioxidants especially vitamin E and vitamin C, but I will not be asked to restrict my caloric intake.

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## APPENDIX D. INFORMED CONSENT (CONTINUED)

I will also be required to consume a controlled diet for 2 days: 1 day prior to the race and the day of the race. I will be required to eat only the foods provided to me at the Human Metabolic Kitchen. Both diets will be made up of 10-15% protein, 55-65% carbohydrate, and 25-30% fat; the macronutrient breakdown recommended for optimal athletic performance. My compliance with the prescribed diet will be monitored by 3-day food records and a blood screening 3 weeks (21 days) after the start of supplementation with the antioxidants (or placebos). A staff member will go over with me how to fill out the food records, including food descriptions and instructions on how to best estimate portion size.

For the controlled diet, all foods will be prepared and provided for me in the Metabolic Feeding Unit in the Human Nutrition Research Laboratory. I will consume the evening meal in the Metabolic Lab at which time study personnel will provide me with the supplements and supervise consumption of the capsules. With dinner, the evening before the ultramarathon race, I will be provided with a single capsule containing the deuterated vitamin E. This capsule will replace the unlabeled vitamin E capsule (or placebo) I will be taking the other six weeks of the study. Breakfast and lunch will be provided for me in the form of pre-packaged meals that I will pick up at the Metabolic Lab each morning of the controlled diet.

I will be provided with energy bars, carbohydrate pastes and carbohydrate drinks that are not fortified with antioxidants. I will be asked to use only these specific sport fuels both for my training the six weeks prior to the run and during the actual race.

*Blood Draws:* Experienced phlebotomists will draw blood. A screening sample will be obtained one to two months prior to the start of the study. In addition to the pre-screening draw, samples will be obtained at baseline March 15<sup>th</sup> (day 0), April 3<sup>rd</sup> (3 weeks prior to the race), April 26<sup>th</sup> (the day prior to the race), April 27<sup>th</sup> (1 hour prior to the race, mid-race at kilometer 27, at race end and immediately before dinner). Additional blood draws will be taken Sunday April 28<sup>th</sup> through Friday May 3<sup>rd</sup> (36, 60, 84, 108, 132 and 156 hours after consumption of the labeled vitamin E (time=0)).

In addition to the pre-screening draw, the total amount of blood to be drawn will be 30 ml (2 Tablespoons) each time, for a total of 13 blood draws equaling 390 ml or 1 1/2 cups. All blood draws except three of the draws on race day (mid-race, race end and dinner draw) will be fasting a.m. draws; I will not be allowed to eat or drink anything except water after 8 p.m. the previous night.

*Activity Log:* For the seven weeks I am involved in the study, I will record daily the amount, type, duration and intensity of exercise performed each day. This data will be used to identify inter-subject variations in training regimen.

*Physical Activity:* I will be asked to refrain from all physical activity, especially running, for the six days following the ultramarathon so that the researchers are able to investigate the effects of antioxidant supplementation on recovery.

*Non-steroidal anti-inflammatory drugs (NSAID's):* I will be asked to avoid the use of aspirin and NSAID's including naproxen sodium (brand name Alleve) and ibuprofen (brand names Advil, Motrin, Nuprin, Pamprin, Bayer Select Ibuprofen, Excedrin IB) throughout the seven weeks of the study as these anti-inflammatory drugs may confound the results of the study. As an alternative to these drugs, I may use acetaminophen (brand name Tylenol).

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## APPENDIX D. INFORMED CONSENT (CONTINUED)

Study Schedule

Days	Pre-screening: 1-2 months prior to trial	March 15 (Day 0)	April 3 (Day 21)	April 26 (Day 41)	April 27 (Day 42) <b>RACE DAY</b>	April 28 (Day 43)	April 29 (Day 44)	April 30 (Day 45)	May 1 (Day 46)	May 2 (Day 47)	May 3 (Day 48)
Diet		Start Prescribed Diet		Controlled Diet	Controlled Diet	Resume Prescribed Diet					
Vit E + C (or placebos)		Supplements	Supplements	Supplements	Supplements	Supplements	Supplements	Supplements	Supplements	Supplements	Supplements
Labeled Vitamin E				time=0 h, ~6 PM							
	Info Mtg ~1-2 hrs										
Blood Draws	Pre- screening: (fasting)	Draw 1 Baseline (fasting)	Draw 2 (fasting)	Draw 3 (fasting)	Draw 4 Pre-race (fasting) Draw 5 Mid-race Draw 6 Post-Race Draw 7 Dinner-6 PM	Draw 8 *Post 36hr (fasting)	Draw 9 *Post 60hr (fasting)	Draw 10 *Post 84hr (fasting)	Draw 11 *Post 108hr (fasting)	Draw 12 *Post 132hr (fasting)	Draw 13 *Post 156hr (fasting)
	Submax Testing/ BodPod ~1 hr										

OSU IRB Approval Date: 2/1/02  
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## APPENDIX D. INFORMED CONSENT (CONTINUED)

*Race Day Schedule:*

**WEIGHT:** I will be weighed immediately before and after the race to estimate losses in body water.

**BLOOD DRAWS:** On the day of the race, blood samples will be taken 1 hour prior to the race, mid-race at kilometer 27, at race end and prior to dinner (~6 PM).

**FOODS AND BEVERAGES:** Selected foods and beverages will be provided for me at check points along the race course.

**HEART RATE MONITOR:** I will wear a heart rate monitor during the run; this data will be used to determine the amount of oxygen I consumed during the race in order to calculate my energy expenditure. The monitor is a small plastic device weighing only a few ounces. It will be secured to my chest by an elastic strap, thus it will not bounce or disrupt my pace during the run.

**E. Risks and Benefits**1. Foreseeable risks or discomforts.

- a) *Submaximal oxygen consumption test.* I realize that I may feel tired immediately after the submaximal oxygen consumption test. I may experience some tiredness or muscle soreness after this testing. This is a normal response and will subside within a few hours to a few days.

I understand that tests of submaximal oxygen consumption have a very remote chance of triggering a cardiac event (such as abnormal heart rhythms) or even death. However, the possibility of such an occurrence is very slight (less than 1 in 10,000), since I am in good physical condition with no known symptoms of heart disease. Furthermore, trained personnel will be monitoring me for signs of exercise intolerance during the test.

- b) *Blood sampling.* I understand that I may experience some discomfort while my blood is being drawn and that this procedure could result in a small amount of bleeding, bruising, and slight soreness at the site of needle insertion. I have been informed that my blood will be drawn by trained personnel who will apply a bandage to my arm in order to minimize bleeding or bruising as a result of the blood drawing procedure.
- d) *Treadmill Exercise.* There is the small risk that I may trip while running on the treadmill. To minimize this risk, I will be familiarized with treadmill running and provided practice time before the actual testing. Testing will not begin until I feel confident of my ability to exercise safely on the treadmill. Finally, I will be taught how to operate the emergency shut-off button on the treadmill.
- e) *Run Time.* I realize that because I will be required to stop during the race to have my blood drawn, my run time may be affected, as much as 15 minutes may be added to my time as a result.

f) Benefits to be expected from the research.

- a) I will benefit by learning several important aspects about my body, physiological responses to exercise, and my fitness level. These include my body composition, and my maximal aerobic capacity.

## APPENDIX D. INFORMED CONSENT (CONTINUED)

- b) I will receive monetary compensation for participation in the study. I will be paid \$10.00 for each blood draw. Not including the pre-screening draw, there will be 13 blood draws for a total of \$130.00. If for any reason I am unable to complete the study, I will be compensated for all blood draws taken prior to my withdrawal from the study.
- c) I will be provided with energy bars, carbohydrate pastes and carbohydrate drinks for the six weeks prior to the run to use during my training and the run. I will also be provided with meals and snacks for the two days of the controlled diet including specific foods that will be provided for me at check points along the race course on race day.

**F. Confidentiality.** Any information collected from me will be kept confidential. The only persons who will have access to this information will be the investigators. A code number will be used to identify test results or other information provided by the investigators. All files with any of my personal information will be stored in a locked file cabinet in the principal investigator's office. Files linking my identity and code number will be kept indefinitely. My name will never be used in any data summaries or publications.

**G. Compensation for injury.** I understand the University does not provide a research subject with compensation or medical treatment in the event that the subject is injured as a result of participation in the research project.

**H. Voluntary participation statement.** I understand that my participation in this study is completely voluntary and that I may either refuse to participate or withdraw from the study at any time without penalty or loss of benefits to which I am otherwise entitled.

**I. If I have questions.** I understand that any questions I have about the research study and/or specific procedures should be directed to: Maret G. Traber, (541) 737-7977; 571 Weniger Hall, Oregon State University or Angela Mastaloudis, (541) 737-8004; 571 Weniger Hall, Oregon State University. If I have questions about my rights as a research subject, I should contact the IRB Coordinator, OSU Research Office, (541) 737-3437.

My signature below indicates that I have read and I understand the procedures described above and give my informed and voluntary consent to participate in this study. I understand that I will receive a signed copy of this consent form.

\_\_\_\_\_  
Signature of subject

\_\_\_\_\_  
Name of subject

\_\_\_\_\_  
Date signed

\_\_\_\_\_  
Subjects present address

\_\_\_\_\_  
Subjects phone number

\_\_\_\_\_  
Signature of Investigator

\_\_\_\_\_  
Date signed

OSU IRB Approval Date: 2/1/02  
Approval Expiration Date: 12/1/02

# APPENDIX E.      PRESCRIBED DIET EXPLANATION

Thank you for participating in our research project. In order to obtain the most accurate results possible from this project, we need your help and cooperation! We are asking you to pay attention to your diets and avoid specific foods during the seven weeks of the study including the six days after the race.

<b>Please Choose</b>	<b>Please Avoid</b>
	Foods that provide 100% the Daily Value (DV) vitamin E or vitamin C
Apple, grape, lemonade, juices, gatorade, soda, tea, coffee	Grapefruit juice, orange juice, Kool aid, pineapple juice, cranberry juice, all other juices with added vitamin C
Apples, apricots, avocados, bananas, blueberries, cherries, grapes, figs, dates, cranberry sauce, kiwi, honeydew melons, nectarines, peaches, pears, plums, pineapple, pomegranate, raisins, rhubarb, raspberries	Grapefruit, oranges, lemons, limes, mangoes, cantalope, papayas, strawberries, tangarines
All other vegies	Broccoli, kohlrabi, sweet peppers
Gatorade	Carbohydrate Drinks (ie Hydrafuel)
Energy/meal replacement bars provided	Energy/meal replacement bars (ie Clif Bars, Power Bars)
Clif Shots	Carbohydrate pastes (ie Gu)
	Total breakfast cereal (contains 100% the Daily Value (DV) vitamin E)