Antioxidant Supplementation Enhances Neutrophil Oxidative Burst in Trained Runners Following Prolonged Exercise

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The influence of an antioxidant vitamin supplement on immune cell response to prolonged exercise was determined using a randomized, double-blind, placebo-controlled, cross-over study. Twelve healthy endurance subjects ($n = 6$ male, $n = 6$ female; mean ± SD for age, $30.1 ± 6.2$ yr; height, $1.76 ± 7$ m; body mass, $72.2 ± 10.2$ kg; $V_{O_{2max}}$, $63.7 ± 12$ ml · kg$^{-1}$ · min$^{-1}$) participated in the study. Following a 3-week period during which subjects ingested a multivitamin and -mineral complex sufficient to meet the recommended daily allowance, they took either a placebo or an antioxidant vitamin supplement (containing $18$ mg β-carotene, $900$ mg vitamin C, and $90$ mg vitamin E) for 7 days prior to a 2-h treadmill run at $65\%$ $V_{O_{2max}}$. Blood samples were drawn prior to and immediately following exercise. These were analyzed for neutrophil oxidative burst activity, cortisol and glucose concentrations, and white blood cell counts, as well as serum anti-oxidant vitamin concentrations. Plasma vitamin C, vitamin E, and β-carotene concentrations significantly increased following 7-day supplementation ($p < .05$). In comparison to the placebo group, neutrophil oxidative burst was significantly higher following exercise ($p < .05$), but no differences were found in any other parameter following the 7-day supplementation period. Although the impact of exercise on neutrophil function is multifactorial, our data suggest that antioxidant supplementation may be of benefit to endurance athletes for the maintenance of this particular function of the innate immune system following the 7-day supplementation period.

Key Words: phagocytes, running, micronutrients, auto-oxidation

Introduction

Epidemiological studies have concluded that athletes experience an increased risk of infection when undergoing heavy training and especially following long-duration
competitive events (10, 37, 45). One of the reasons for the increase in the incidence of infection may be a prolonged suppression of the innate immune system function (51). This effect is multifactorial and may be mediated by the actions of stress hormones, cytokines, hematological factors as well as by an increase in free radical production by leucocytes.

There is evidence that exercise leads to an increased formation of reactive oxygen species (ROS; 7, 19, 27) as a consequence of augmented metabolic oxidative metabolism (1) but also from immune-competent cells that are stimulated during exercise (53). The ROS formation, such as superoxide ($O_2^-$), hydroxyl radicals (OH$^-$), and hypochloric acid (HOCL), during exercise can potentially inhibit immune system function (40, 42). They can impair phagocyte function by inhibiting microbicidal functions by inactivating oxidant-generating enzymes (i.e., auto-oxidation; 9). Neutrophils are phagocytic cells and are one of the first lines of defense of the innate immune system. The majority of studies have reported a suppressed neutrophil microbicidal function following prolonged exercise (29, 34, 36, 47, 51). It has been previously hypothesized that the observed exercise-induced fall in phagocyte oxidative burst activity may be caused, at least in part, by increased oxidative stress on these cells (9, 40, 42).

As comprehensively reviewed by Peters-Futre (40), an increased intake of nutritional antioxidants such as vitamin C, vitamin E, and β-carotene, may reduce the exercise-induced auto-oxidative stress on immune cells (42), since they are effective ROS scavengers (14, 21, 48). These micronutrients can directly deactivate ROS (6), which accumulate during prolonged exercise and oxidative metabolism. Hence, it may be speculated that the increased quenching of ROS by these micronutrients may attenuate the observed fall in neutrophil function following prolonged exercise.

Supplementation with antioxidant micronutrients has been associated with a reduced incidence of URTI following an ultra-distance marathon (41, 46) as well as a lower adrenal stress hormone response (39, 43, 44). In these studies, it has been proposed that the antioxidant supplementation caused an increased quenching of ROS, which attenuated the exercise-induced immune-suppression, culminating in the maintenance of host defense (42).

Some athletes adopt unbalanced dietary regimens, which can cause poor nutritional status—for example, high carbohydrate diets may lead to zinc deficiencies (20, 52) and vegetarian diets can cause deficiencies in heme-iron (54). Micronutrient deficiencies may predispose some athletes to immune-suppression (reviewed elsewhere; 11). It has previously been recommended that athletes should take a multivitamin-/mineral supplement sufficient to meet the recommended daily allowance (RDA) to maintain optimal immune system function (22). Hence, for the duration of the present study, subjects took a single daily multivitamin and -mineral complex (no greater than RDA) to ensure against micronutrient insufficiencies.

The purpose of the present study was to examine the effect of a daily antioxidant supplement taken for 7 days on neutrophil responses to a single bout of controlled prolonged exercise in trained endurance runners. Leucocyte count and neutrophil oxidative burst as well as changes in serum concentrations of cortisol and glucose were measured. We hypothesized that the prolonged exercise bout would induce a suppression of neutrophil oxidative burst, which would be attenuated by antioxidant supplementation.
Methods

Subjects

Twelve healthy endurance subjects ($n = 6$ male, $n = 6$ female) were recruited (mean ± SD for age, $30.1 ± 6.2$ yr; height, $1.76 ± 7$ m; body mass, $72.2 ± 10.2$ kg; VO$_{2\max}$, $63.7 ± 12$ ml · kg$^{-1}$ · min$^{-1}$). All subjects completed health and physical training questionnaires. None were receiving medication or had suffered from an infection in the preceding 2 weeks. They were informed of the experimental procedures and gave informed consent in writing. University ethics committee approval was obtained. All subjects completed three specified exercise tasks on three different occasions.

Incremental VO$_{2\max}$ Test

Subjects performed a continuous incremental exercise test on a treadmill to volitional exhaustion to determine VO$_{2\max}$. Subjects began running at a workload of $12$ km · hr$^{-1}$ with increments of $1$ km · hr$^{-1}$ every 60 s. Expired air was analyzed using an online metabolic cart (Oxycon Pro, Jaeger, Berlin, Germany). Heart rate was measured using a telemetric heart rate monitor (Polar Electro, Kempele, Finland). From the VO$_2$–work rate relationship, the work rates equivalent to $65\%$ VO$_{2\max}$ were interpolated.

Exercise Trials

Subjects ran on the treadmill at $65\%$ VO$_{2\max}$ (mean treadmill speed: $11.6 ± 1.8$ km · hr$^{-1}$) for 2 hours on two occasions. On each occasion, subjects reported to the laboratory at precisely the same time of day following an overnight fast. Subjects refrained from exercise in the 24-hour period prior to each exercise trial. To standardize procedures, subjects were instructed to keep an accurate diary of their exercise training and dietary intake in the week prior to the first exercise trial so that they could replicate these factors in the week prior to the second exercise trial. All subjects completed both exercise trials. During each trial subjects could drink water ad libitum. Venous blood samples were obtained by venepuncture from an antecubital vein before and immediately after exercise. Heart rate and rating of perceived exertion (13) was recorded throughout each exercise trial at 15-min intervals.

Intervention

Venous blood samples were obtained by venepuncture from an antecubital vein before any supplement was ingested at the onset of the study (see Figure 1). A baseline supplement (BASE) in a tablet form consisting of a multivitamin/-mineral complex was taken daily for the duration of the study (see Table 1 for composition). After 21 days on BASE, a further blood sample was drawn. Subjects then started the intervention treatment (an antioxidant-based supplement, INT) or took a placebo (PLA) on a daily basis for 7 days; both INT and PLA were supplied in a slow-release tablet form. There were no visible differences between the antioxidant-based tablets and the placebo tablets. All tablets were supplied by Pharma Natura Pty. (Johannesburg, South Africa). After 7 days on INT or PLA, subjects returned to the...
laboratory (at precisely the same time of day for each trial), tablets were ingested following the pre-exercise blood draw, after which subjects completed a 2-hour run at 65% $\text{VO}_{2\text{max}}$. Immediately after exercise, a further blood sample was drawn. Subjects were instructed to ingest all tablets with breakfast except for days when blood samples were drawn, when tablets were ingested following the blood draw. This procedure was standardized throughout the trials. The exercise trials were randomly assigned in a counterbalanced manner. Following the first exercise trial and prior to the next 7-day supplementation period, subjects completed a 2-week washout period during which only the baseline supplement was taken. After BASE, the study was of a randomized, double-blind, crossover, placebo-controlled design. Codes were not broken until all sample analyses had been completed.

### Blood Analysis

Hematological analysis was performed on the blood samples collected into K$_3$EDTA vacutainers by a clinical hematology laboratory using an automated hematology analyzer (Cell-Dyn 3700, CA, USA). Total and differential leucocyte counts were measured.
Within 4 hours of collection, neutrophil oxidative burst was stimulated in whole blood collected into heparinized vacutainers using the Bursttest kit (Orpegen Pharma, Heidelberg, Germany). The Bursttest kit permits the quantitative determination of neutrophil oxidative burst in heparinized whole blood. The evaluation of oxidative burst activity was performed using flow cytometry (FACSCalibur, Becton Dickinson, NJ, USA), a technique capable of assessing individual cells. The neutrophils were activated by a bacterial stimulant (E.coli). Dihydrorhodamine-123 was the fluorogenic substrate, which determined the percentage of neutrophils that produced reactive oxidants and the extent of the oxidative burst. Oxidizing neutrophils convert enzymatically dihydrorhodamine-123 to rhodamine-123, and the extent of their enzyme activity is reported as mean fluorescence intensity per neutrophil. The intra-assay coefficient of variation was 4.6%. Results were expressed as either percentage of cells positive for the fluorescent oxidized substrate or as mean fluorescent channel of the cell preparation.

Plasma glucose was measured from blood collected into fluoride vacutainers and analyzed by the oxygen rate method (Beckman Instruments Inc., CA, USA).

Blood collected into serum clot activating vacutainers was centrifuged at 1500 g for 10 min. The supernatant was aspirated and collected into eppendorf tubes on ice and immediately frozen at −70 ºC until later analysis. Aliquots of serum were analyzed to determine the concentrations of cortisol using a competitive immunoassay by direct chemiluminescence (Chiron Diagnostics, Suffolk, UK).

Blood was also collected for determination of plasma concentrations of vitamin C, vitamin E, and β-carotene prior to and following 7-day supplementation with INT and PLA. Blood was collected into foil-covered, K3EDTA vacutainers and centrifuged immediately at 1500 g for 10 min for the analysis of vitamin C, vitamin E, and β-carotene. For vitamin E and β-carotene determination, the supernatant was collected into opaque eppendorfs to minimize exposure to light before they were frozen at −70 ºC until later analysis. Due to the sensitivity of β-carotene and vitamin E to UV light, sample preparation was performed under yellow light with isocratic C18 reverse-phase high-performance liquid chromatography (HPLC), using a modification of the method published by Milne and Botnen (33). For vitamin C determination, a precipitating agent (10% of HCLO4 containing 1% of metaphosphoric acid) was added to the supernatant and a second centrifugation step was included to remove the protein precipitate; the supernatant was then stored at −70 °C for later analysis. For vitamin C analysis, the analyses were performed by HPLC according to the method of Liau et al. (32).

Statistics

Data in the figures and tables are presented as mean values and standard errors of the mean (SEM) unless specified. Statistical evaluation of results was carried out using either a 2-way (exercise trial × time) or 1-way analysis of variance (BASE × time) with post hoc Tukey tests (Statistica 5.5, Statsoft Inc., OK, USA). The accepted level of significance was p < .05.

Results

Prior to the start of the study, hematological variables were within normal ranges for all subjects. The baseline supplement (BASE) had no effect on resting leucocyte,
After 21 days on BASE, neutrophil oxidative burst increased compared to pre-supplementation values and was still elevated by day 42 of the study ($p < .01$; Table 2).

Plasma vitamin C, vitamin E, and $\beta$-carotene concentrations were significantly greater following 7 days of INT than pre-INT. A difference between post-INT and post-PLA plasma vitamin E and $\beta$-carotene concentrations was found (interaction between trials, $p < .05$; Table 3).

Heart rate and rating of perceived exertion during exercise increased similarly during the exercise on both trials (mean heart rate for each trial: $158 \pm 12, 157 \pm 13$ beats·min$^{-1}$, RPE: $4 \pm 1, 4 \pm 1$; INT, PLA, respectively; $p < .01$). As neither heart rate nor rating of perceived exertion was significantly different between exercise trials, it may be concluded that each trial imposed a similar magnitude of stress on subjects, despite the separation of 3 weeks.

Table 2  Effect of BASE on Resting Neutrophil Oxidative Burst and Percentage of Oxidizing Neutrophils After 3 Weeks on BASE (Day 21) and After the 2-Week Antioxidant (or Placebo) Wash-Out Period (Day 42)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 0 (pre-BASE)</th>
<th>Day 21 of BASE†</th>
<th>Day 42 of BASE†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean fluorescent channel</td>
<td>265 ± 29</td>
<td>603 ± 67*</td>
<td>706 ± 97*</td>
</tr>
<tr>
<td>Percentage oxidizing</td>
<td>48 ± 3</td>
<td>43 ± 2</td>
<td>45 ± 2</td>
</tr>
</tbody>
</table>

*Note.* Data are mean ± SEM. †$n = 12$ (6 subjects: pre-INT and 6 subjects: pre-PLA); *$p < .01$ vs. Day 0.

Table 3  Effect of 7 Days Antioxidant Supplementation or Placebo on Resting Plasma Vitamin C, Vitamin E, and $\beta$-Carotene Concentrations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-INT</th>
<th>Post-INT</th>
<th>Pre-PLA</th>
<th>Post-PLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C (µmol · L$^{-1}$)</td>
<td>69.9 ± 3.3</td>
<td>90.1 ± 4.1*</td>
<td>74.1 ± 5.0</td>
<td>81.6 ± 2.9</td>
</tr>
<tr>
<td>Vitamin E (µmol · L$^{-1}$)</td>
<td>23.7 ± 1.8</td>
<td>36.5 ± 3.1*#</td>
<td>26.5 ± 1.2</td>
<td>27.4 ± 1.7</td>
</tr>
<tr>
<td>$\beta$-carotene (µmol · L$^{-1}$)</td>
<td>1.9 ± 0.2</td>
<td>2.5 ± 0.2*#</td>
<td>2.2 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>

*Note.* Data are mean ± SEM. *$p < .05$ versus Pre-INT; #*$p < .05$ versus Post-PLA. Blood samples were drawn prior to ingestion of tablets.
Immediately after the exercise bouts, circulating leucocyte, neutrophil, and lymphocyte counts increased similarly in both trials (main effect of time, \( p < .01 \); Figure 2). Neutrophil oxidative burst was significantly higher in INT compared to PLA post-exercise (interaction between trials, \( p < .05 \); Figure 3). The percentage of oxidizing neutrophils increased following exercise, but there were no differences between trials (main effect of time, \( p < .05 \); pre-exercise: 43 ± 5, 42 ± 4%; post-exercise: 51 ± 3, 48 ± 4%; INT, PLA, respectively).

Figure 2 — Changes in leucocyte, neutrophil, and lymphocyte counts after exercise at 65% \( \text{VO}_{2\text{max}} \). Data are mean and SEM. Filled symbols: INT trial; open symbols: PLA trial; *main effect of time: \( p < .05 \).

Figure 3 — Changes in neutrophil oxidative burst (mean fluorescent channel) during exercise trials. Data are mean and SEM. *Interaction between trials: \( p < .05 \).
Plasma cortisol concentration was not changed following exercise, and there were no differences between trials (pre-exercise: $639 \pm 38, 670 \pm 47$ nmol·L$^{-1}$; post-exercise: $709 \pm 62, 719 \pm 45$ nmol·L$^{-1}$; INT, PLA, respectively). Plasma glucose concentration was not different between trials but increased post-exercise (main effect of time, $p < .01$; pre-exercise: $4.4 \pm 0.1, 4.6 \pm 0.1$ mmol·L$^{-1}$; post-exercise: $4.8 \pm 0.2, 5.1 \pm 0.1$ mmol·L$^{-1}$; INT, PLA, respectively). Although not different between trials, plasma volume fell post-exercise compared to pre-exercise (main effect of time, $p < .01$; INT: $3.9 \pm 0.9$, PLA: $3.3 \pm 0.7$%)

**Discussion**

The main finding of the present study is that a daily antioxidant supplement taken for 7 days prior to a bout of prolonged exercise is associated with an enhanced innate immune response to a bacterial stimulant in vitro. The pattern of change in circulating white blood cell, neutrophil, and lymphocyte count following exercise was not different between the antioxidant supplemented and placebo trials. An additional finding was that a comprehensive vitamin and mineral supplement (BASE) also improved neutrophil oxidative burst in samples taken at rest.

Several studies have investigated the effect of nutritional interventions on immune response to prolonged exercise. The studies indicate that glutamine (35), zinc (52), carbohydrate (25, 38) and, most recently, vitamin C (39, 44) can attenuate some of the factors associated with negative immune system changes during exercise.

To our knowledge, no studies have investigated the effect of a combination of antioxidant nutrients on neutrophil oxidative burst following prolonged exercise. In the 7 days prior to the exercise bouts, the daily amount of antioxidant supplementation was 960 mg vitamin C, 100 mg vitamin E, and 27 mg $\beta$-carotene (including BASE), which significantly elevated plasma antioxidant concentrations. Previously, Peters et al. (41) found that a combination of antioxidant nutrients (300 mg vitamin C, 200 mg vitamin E, 18 mg $\beta$-carotene) significantly reduced symptoms of URTI in the days after a 90-km marathon, but no immune measures were taken during that study to illuminate possible mechanisms.

In agreement with our findings, Nieman et al. (36) found no changes in plasma cortisol concentrations or circulating immune cell counts post-exercise following supplementation with 1 g of vitamin C for 8 days prior to the 2.5-h run. However, in contrast to our results, they reported no changes in post-exercise neutrophil oxidative burst between groups. However, caution should be taken when interpreting results from the latter study. First, subjects consumed carbohydrate drinks during the exercise trial, which may have masked the potential effect of vitamin C on post-exercise immune and hormonal responses. Later research by the same group (25) has shown that carbohydrate ingestion during exercise suppresses neutrophil function post-exercise. Second, large inter-individual variability inherent in immune parameters may necessitate the use of either a crossover designed study to elucidate treatment effects or large subject numbers. Nieman et al. (36) did not use a crossover design and had low subject numbers ($n = 6$ in each group). This criticism may also be applied to Krause et al. (29), who reported that 2 g of vitamin C in the week prior to a biathlon did not attenuate the post-exercise fall in neutrophil function ($n = 4$ and $n = 6$). Hence, we considered it prudent to use larger subject numbers and a study with a crossover design to rigorously test our hypotheses. Finally, the antioxidant effect
of vitamin C is enhanced in the presence of vitamin E (49). Hence, the combination of antioxidants taken in the present study may have a greater effect on ROS quenching and neutrophil function following exercise than supplementation with a single antioxidant nutrient.

Increased plasma concentrations of neutrophil-derived enzymes following exercise (15, 51) provide evidence that neutrophils are activated during prolonged exercise. It has been suggested that this exercise-induced neutrophil stimulation would also result in an increased release of neutrophil-derived ROS, such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH$^-$), and hypochloric acid (HOCL), into the circulation (40, 42). In support of this, an increased neutrophilic production of ROS has been reported immediately following exercise (26). Previous research has shown that phagocyte-derived ROS are cytotoxic to neutrophils and can lead to auto-oxidation and impaired function of these cells (3, 8, 9). If ROS are not neutralized, they can inactivate the oxidant-generating cellular enzymes causing inhibition of the neutrophil microbicidal functions (2, 4, 6). Furthermore, the production of reactive nitrogen species (RNS) such as nitric oxide also increases during exercise (31). Nitric oxide can suppress neutrophil oxidative burst by inhibiting an enzyme (nicotinamide adenine dinucleotide phosphate-oxidase) essential for this function (17, 18).

Extracellular neutralization of ROS and RNS is largely attributed to the ability of non-enzymatic mechanisms such as antioxidant micronutrients (vitamin C, vitamin E, and β-carotene). These vitamins can directly deactivate ROS and RNS, which accumulate during prolonged exercise and oxidative metabolism. It is well established that vitamin C can scavenge O$_2^-$, OH$^-$, and HOCL (4, 6, 55). It is theorized that this action may reduce auto-oxidative effects on neutrophil function during exercise (40, 42). Administration of vitamin E reduces the suppressive effects of H$_2$O$_2$ on neutrophil oxidative burst (9), and β-carotene is an effective singlet oxygen scavenger (48). Furthermore, there is some evidence to suggest that antioxidant supplementation can suppress the production of some cytokines during exercise (16). It may be speculated that reduced cytokine production during exercise may result in a reduced stimulation of neutrophils during exercise thereby reducing the phagocyte-derived ROS, but further research is required before this conclusion can be drawn.

Although endurance training appears to be accompanied by an upregulation of the antioxidant defense system (23, 50), it has been suggested that this adaptation may be insufficient to protect athletes who are training intensively (42). In the present study, prior to supplementation with INT and PLA, the subjects were ingesting the multivitamin and -mineral BASE supplement, and plasma concentrations of antioxidant micronutrients (vitamin C, vitamin E, and β-carotene) were within the normal physiological range. Antioxidant supplementation with INT for 7 days significantly elevated these values, suggesting that the antioxidant defense system may be improved by an increased intake of antioxidant micronutrients (50).

After 21 days on BASE, neutrophil oxidative burst increased compared to pre-supplementation values. The exact mechanisms underpinning the action of BASE on neutrophil oxidative burst are unclear, but we propose two reasons. First, subjects may have had a micronutrient deficiency prior to the study that caused a suppressed neutrophil function but that was rectified by BASE. For example, zinc deficiency inhibits neutrophil function, whereas this is facilitated by normal physiological concentrations (24). Furthermore, low zinc concentrations have been
previously reported in endurance athletes due to high carbohydrate diets, which are typically low in zinc (20). Second, as the subjects were regularly exercising throughout the study, the antioxidant constituents in BASE may have attenuated chronic exercise-induced immune-suppression (by protecting neutrophils from oxidative stress) that occurs not only as a result of an acute bout of prolonged exercise but also with chronic exercise training (12, 47). Third, the constituents in BASE enhanced neutrophil function either independently of each other (e.g., selenium which at low doses stimulates neutrophil function; 28), or as a combined effect (e.g., zinc and copper supplementation in trained runners inhibited the exercise-associated increase in superoxide formation by neutrophils; 52). Nutrient-nutrient interactions can have additive effects on immune function above that of supplementation with an individual nutrient (reviewed elsewhere; 30). Confirmation of our finding—that BASE taken daily by endurance athletes for a prolonged period was associated with an increased neutrophil oxidative burst—by further, more specific investigations would promote the recommendation that athletes should take a multivitamin/-mineral supplement that is sufficient to meet the RDA for maintenance of normal innate immune system function (22).

Although the impact of exercise on neutrophil function is multifactorial, our data suggest that antioxidant supplementation may be of benefit to endurance athletes for the maintenance of this function of the innate immune system. We propose that the significant increase in post-exercise neutrophil oxidative burst in the antioxidant supplemented condition compared to the placebo condition was due to a reduction in oxidative stress—more specifically, auto-oxidative stress on neutrophils as a result of an increased quenching of neutrophil-derived reactive oxygen species during exercise.

References


**Acknowledgments**

This work was funded by Pharma Natura (Pty.) Ltd., Johannesburg, South Africa, the Technology and Human Resources for Industry Programme of the National Research Foundation, and a Postdoctoral Fellowship from Stellenbosch University.