Antioxidant Status and Oxidative Stress in Elite Alpine Ski Racers

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Key Words: exercise, free radicals, glutathione, lipid peroxidation, training, uric acid

The goal of this field study was to assess antioxidant status and markers of oxidative damage in elite alpine ski racers during routine training. Subjects included 12 members of the U.S. Men's Alpine Ski Team attending a 10-day summer training camp. Blood draws were collected at rest and after exercise: (a) prior to training, (b) following 2 days of dry land training, and (c) after 4 days of on-snow skiing. Seven measures of antioxidant status were determined using colorimetric and HPLC methods (Trolox® equivalent antioxidant capacity, uric acid, α-tocopherol, γ-tocopherol, total glutathione, cytosolic glutathione peroxidase, and superoxide dismutase). Oxidative stress was assessed using 2 markers of lipid peroxidation (malondialdehyde and lipid hydroperoxides) and 2 markers of protein oxidation (carbonylated total proteins and carbonylated hemoglobin). The results of this study suggest that antioxidant status of elite alpine skiers may decline over a period of intense training. However, elevations in markers of oxidative stress were not evident.

Introduction

By definition, free radicals are molecules or molecular fragments that contain unpaired electrons in their outer orbitals (36). Such species may seek to stabilize their electron configuration by oxidizing neighboring molecules. Evidence is accumulating regarding the role of free radicals in cellular signaling processes (5, 12, 19), yet free radicals have also been associated with irreversible oxidative stress. In relationship to exercise, free radicals have been hypothesized to affect muscle contractility (11, 42), energy production (22), and subsequently physical performance (16).

Evidence of free radical production during exercise has been demonstrated in vivo through the use of electron spin resonance techniques (1, 10). The rate of

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superoxide formation has been directly correlated with mitochondrial oxygen utilization (4). During periods of heightened aerobic metabolism, it has been estimated that 0.1–2% of the total electron flux through the electron transport system may leak from the cytochrome chain and reduce molecular oxygen to the superoxide radical (2). This mechanism of free radical generation has led investigators to study the effects of aerobic exercise-induced oxidative stress (15, 31, 32, 40).

Free radical production during exercise may not be limited to aerobic metabolism. During periods of intense anaerobic exercise, free radicals may be generated by: (a) increased xanthine oxidase activity (41); (b) exercise–induced ischemia/reperfusion (24); (c) increased cyclooxygenase activity (7); (d) increased activity of nitric oxide (NO) synthase (44); and (e) activation of invading leukocytes (30). In addition, environmental factors, such as altitude related hypobaric hypoxia, low ambient temperatures, and increased UV light exposure may also play roles in oxidative stress (6, 34).

Due to repetitive, high intensity efforts in extreme environments, it may be hypothesized that alpine skiers exhibit compromised antioxidant status and concomitant elevations in markers of oxidative stress during daily training. The aim of this study was to evaluate antioxidant status and markers of oxidative stress in elite alpine ski racers during routine training.

Methods

Subjects

Subjects included 17 male members of the U.S. National Alpine Ski Team attending a 10-day summer training camp. Subjects reported no use of antioxidant supplementation for the 3 months prior to and during the study. The experimental procedures were approved by the Intermountain Health Care Institutional Review Board (Salt Lake City, UT). All subjects provided written informed consent prior to participation in the study.

Experimental Procedures

The training camp included 4 days of dry land training at sea level (Portland, OR), 1 day of travel and rest, and 5 days of on-snow training between 2,200 and 2,900 m (Mt. Hood, OR). During the camp, all athletes lived, ate, and trained together. Three 1-day dietary records were obtained on days 1, 2, and 10. During all meals, dietary intakes were observed, while serving sizes were estimated and recorded by the investigators. With respect to physical training, day 1 consisted of the U.S. Ski Team Alpine Medals Test (v. 3.1, ©1997, United States Ski Association), a standardized battery of tests designed to evaluate fitness levels of alpine skiers. Day 2 included tests of maximal (IRM) power clean, back squat, and bench press lifting ability, and maximal number of dips and sit ups loaded with 25 lbs. In addition, a peak lactate squat test was performed. The squat test included three sets of back squats (as many repetitions as possible in 30, 45, and 60 s with 125, 100, and 75% of body weight, respectively) with 2 min of standing rest between sets. Finger prick blood samples were taken 105 s after the completion of the test to measure peak lactate concentrations with an Accusport™ portable lactate analyzer (Boehringer Mannheim, Indianapolis, IN). Days 3 and 4 were comprised of sporting activities and games designed to allow recovery before the on-snow portion of the camp. Day 5 was scheduled as a
rest and travel day. Days 6–10 included on-snow slalom and giant slalom training, during which athletes averaged 15 runs per day.

Blood samples (20 ml) were collected in two 10-ml tubes, containing EDTA and sodium heparin anticoagulants, at three times during the training camp: (a) upon waking on day 1 (PRE); (b) immediately following the peak lactate test on day 2 (LAND); and (c) immediately following the last on-snow training session on day 10 (SNOW).

**Biochemical Analyses**

Immediately after collection, $2 \times 500 \mu l$ aliquots of whole blood from each tube were placed on dry ice. The remaining portions were centrifuged for 15 min at 2,300 × g. The plasma samples from each tube were divided into $4 \times 900 \mu l$ aliquots and frozen on dry ice. The cellular fractions were harvested, diluted with equal volume of phosphate buffered saline (PBS), and stored on ice. All samples were sent to OXIS Health Products (Portland, Oregon) for the respective analyses.

**Antioxidant Status**

*Trolox® equivalent antioxidant capacity* (TEAC) was used as an overall measure of plasma antioxidant capacity. The method has been previously described (27). Briefly, the method introduced a radical cation (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to the sample. This radical had a stable color, which was measured at 600 nm. Antioxidants in the sample caused suppression of the color development proportional to antioxidant concentration.

*Uric acid* (UA), a measure of plasma antioxidant status, was assayed using a commercial kit purchased from Sigma Diagnostics (St. Louis, MO). The assay was based on two enzymatic reactions, catalyzed by uricase and a peroxidase, respectively. The first reaction oxidized uric acid to allantoin, $H_2O_2$, and $CO_2$. The $H_2O_2$ reacted with 4-aminopyridine and 3,5-dichloro-2-hydroxybenzene sulfonate to form a quinoneimine dye that had maximal color absorbency at 520 nm. The color development was directly proportional to the uric acid concentration in the sample.

*$\alpha$-Tocopherol* and *$\gamma$-tocopherol* were used to assess plasma antioxidant vitamin status. The vitamins were extracted in hexane after deproteinization with ethanol. The extracts were then concentrated by evaporation and analyzed by HPLC at 292 nm and 298 nm for $\alpha$- or $\gamma$-tocopherol, respectively. Briefly, 0.5 ml of plasma, 0.5 ml of an ethanol based standard (1mg/ml $\alpha$- or $\gamma$-tocopherol), and 1.0 ml of hexane were added to a clean tube, mixed, and centrifuged at 2,100 rpm for 6 min. The hexane layer was transferred to a clean tube and evaporated. Next, 15 ml of chloroform and 50 $\mu l$ of a mobile phase reagent (containing 70% acetonitrile, 25% chloroform, and 2% deionized water by volume, plus 15 mg/L butylated hydroxytoluene) were added to the tube and mixed prior to the HPLC analysis.

*Glutathione* (GSH) was assayed as an indication of endogenous antioxidant status. The method was based on a two-step chemical reaction. The first step led to the formation of substitution products (thioethers) between 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate (R1) and all mercaptans in the sample. The second step was a beta-elimination reaction mediated by a second reagent, 30% NaOH (R2), which specifically transformed the substitution product obtained with
GSH into a chromophoric thione having maximal absorbency at 400 nm. Specifically, erythrocytes from heparinized blood samples were resuspended in 4 volumes of 6% metaphosphoric acid (1/5 dilution, v/v) at 4 °C. The sample was thoroughly mixed and centrifuged at 3,000 g and 4 °C for 10 min. 300 µl of upper aqueous layer was collected and added to 600 µl of 200 mM potassium phosphate buffer, containing 0.2 mM diethylenetriamine pentaacetic acid and 0.025% (w/v) lubrol. To the sample, 50 µl of R1 was added and mixed thoroughly. Additionally, 50 µl of R2 was added and mixed before incubation at 25 °C for 10 min under dark conditions. Absorbency was measured at 400 nm. GSH concentrations were determined from a standard curve.

Cellular glutathione peroxidase (cGPx) activity was used as a measure of antioxidant enzyme status. The assay was an indirect measure of the activity of cGPx as described by Paglia and Valentine (33). Briefly, erythrocytes were added to a solution containing glutathione, glutathione reductase, and NADPH. An enzymatic reaction was initiated by adding the substrate, tert-butyl hydroperoxide. The absorbency was recorded at 340 nm for 3 min. The rate of decrease in absorbency was directly proportional to the cGPx activity in the sample.

Superoxide dismutase (SOD) activity was used as an indication of antioxidant enzyme status. A commercial kit from Randox Laboratories (Antrim, UK) was used to assay SOD activity. The method was based upon the competition between p-iodonitrotetrazolium salts and SOD for superoxide radicals. Superoxide dismutase catalyzes the reaction of superoxide radicals to oxygen and hydrogen peroxide, whereas p-iodonitrotetrazolium reacts with superoxide to form a formazan dye. SOD activity was inversely proportional to the dye formation. Specifically, 1.7 ml of a mixed substrate (xanthine and p-iodonitrotetrazolium) was added to a sample containing 0.05 ml of lysed erythrocytes (25-fold dilution). The enzyme xanthine oxidase (0.25 ml) was added to generate superoxide radicals. The rate of formazan dye production was calculated based on the change in absorbency over 3 min at 505 nm and 37 °C.

**Oxidative Stress**

Malondialdehyde (MDA), a marker of lipid peroxidation, was assayed in plasma samples based on its the reaction with a chromogenic reagent, N-methyl-2-phenylindole. Specifically, 200 µl of plasma, 10 µl of 0.5 M butylated hydroxytoluene, and 650 µl of N-methyl-2-phenylindole were added to a clean tube and mixed gently. Additionally, 150 µl of 12 N hydrochloric acid was added to the tube, mixed, and incubated for 60 min at 45 °C. The sample was centrifuged at 15,000 × g for 10 min. The clear supernatant was transferred to a clean tube and the absorbency was measured at 586 nm. MDA concentrations were determined from standard curves in conjunction with third derivative spectroscopy (3).

Lipid hydroperoxides (LOOH) were assayed based on the oxidation of ferrous ions to ferric ions by hydroperoxides under acidic conditions. The ferric ions were bound to a dye indicator, xylene orange, to form a stable, colored complex with maximal absorbance at 560 nm. Specifically, 10 µl of catalase (3800 units/ml in 10 mM PBS) and 90 µl of heparinized plasma were mixed in a clean tube and incubated at room temperature for 2 min. Additionally, 10 µl of a reducing agent, 20 mM tris (2-carboxyethyl)phosphineHCl, was added to the tube and incubated for 30 min at
room temperature. Next, 900 µl of a 25 µM ferrous ammonium sulfate and 125 µM xylenol orange mixture was added, mixed, and incubated for 60 minutes at room temperature. The sample was then centrifuged at 12,000 × g for 10 minutes. The supernatant was transferred to a clean cuvette and the absorbance was measured at 560 nm. Concentrations were calculated from a standard curve.

Carbonylated total proteins (CBP) and hemoglobin (CBHb) were assayed according to the methods reported by Levine et al. (21). Briefly, carbonylated proteins were incubated with dinitrophenylhydrazine to produce protein hydrazones. Samples were washed to remove free dinitrophenylhydrazine and any lipid contamination. Measures of absorbency were noted at 280 nm for protein and 370 nm for hydrazone. Using the molar extinction coefficient of dinitrophenylhydrazone, the concentrations of carboxyls were calculated.

Statistical Analyses

Descriptive statistics were used to quantify the results of the dry land testing sessions. Repeated measures ANOVAs were performed on each variable using a multiple univariate approach. Post hoc paired t tests were used to assess differences between sampling periods (α = .05).

Results

During the camp, 1 skier was injured, 2 missed the PRE draw, and 2 missed the SNOW draw due to scheduling conflicts. Data from the 12 skiers (age: 22.6 ± 2.6 years, height: 181.1 ± 4.2 cm, weight: 89.0 ± 6.6 kg) who participated in all scheduled activities and blood draws were used in the analyses. Three-day dietary information is shown in Table 1. All nutrient intakes were noted to exceed the RDA standards.

Findings with respect to antioxidant status and oxidative stress are reported in Tables 2 and 3, respectively. Antioxidant measures of TEAC and UA values were elevated at LAND compared to both PRE and SNOW time points. Measures of α-tocopherol, γ-tocopherol, and SOD were highest at PRE and declined over the training camp. GSH values at LAND were significantly lower than those measured at PRE. With respect to markers of oxidative stress, LOOH values were lower at SNOW compared to both PRE and LAND sampling points.

Discussion

Training for alpine ski racing involves high intensity, strenuous exercise. On-snow evaluations suggest that top skiers function at 90–200% of their maximal oxygen consumption and reach lactate levels above 10 mmol/L (43). Based on these evaluations, anaerobic metabolism has been estimated to meet 60% of the energy demands during alpine skiing.

Nonmitochondrial sources of free radicals are increased with intense exercise through various mechanisms (41). Intense intervals of exercise may increase oxidative stress due to transient hypoxia and reoxygenation occurring in exercising muscle (24). Under hypoxic conditions, reducing equivalents may accumulate within the mitochondrial electron transport chain, resulting in a phenomenon known as reductive stress (17). Upon reoxygenation, a burst of one-electron reductions may convert molecular oxygen to superoxide radicals.
Table 1  Dietary Intake

<table>
<thead>
<tr>
<th>Variable</th>
<th>M</th>
<th>SD</th>
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<tbody>
<tr>
<td>Total Calories</td>
<td>5517</td>
<td>722</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>711</td>
<td>61</td>
</tr>
<tr>
<td>(Kcal)</td>
<td>51</td>
<td>7</td>
</tr>
<tr>
<td>(g)</td>
<td>207</td>
<td>67</td>
</tr>
<tr>
<td>(%)</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>Fats</td>
<td>227</td>
<td>43</td>
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<tr>
<td>(g)</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>2936</td>
<td>561</td>
</tr>
<tr>
<td>(μg RE)</td>
<td>294</td>
<td>56</td>
</tr>
<tr>
<td>(%RDA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td>600</td>
<td>180</td>
</tr>
<tr>
<td>(mg)</td>
<td>1000</td>
<td>299</td>
</tr>
<tr>
<td>(%RDA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>16.72</td>
<td>2.93</td>
</tr>
<tr>
<td>(mg α-TE)</td>
<td>167</td>
<td>29</td>
</tr>
<tr>
<td>(%RDA)</td>
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Table 2  Antioxidants

<table>
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<tr>
<th>Measure</th>
<th>PRE</th>
<th>SD</th>
<th>LAND</th>
<th>M</th>
<th>SD</th>
<th>SNOW</th>
<th>M</th>
<th>SD</th>
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</thead>
<tbody>
<tr>
<td>TEAC (mmol/L)</td>
<td>1.93</td>
<td>.18</td>
<td>2.30*</td>
<td>.17</td>
<td></td>
<td>1.93</td>
<td>.11</td>
<td></td>
</tr>
<tr>
<td>UA (μg/mL)</td>
<td>63.0</td>
<td>9.2</td>
<td>103.8*</td>
<td>23.2</td>
<td></td>
<td>66.9</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol (mg/L)</td>
<td>11.17†</td>
<td>1.13</td>
<td>8.16</td>
<td>2.77</td>
<td></td>
<td>7.46</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>γ-tocopherol (mg/L)</td>
<td>3.26‡</td>
<td>.92</td>
<td>2.36</td>
<td>.78</td>
<td></td>
<td>2.08</td>
<td>.52</td>
<td></td>
</tr>
<tr>
<td>GSH (nmol/mg Hb)</td>
<td>5.78‡</td>
<td>.92</td>
<td>5.45</td>
<td>.75</td>
<td></td>
<td>5.58</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>cGPX (mU/mg Hb)</td>
<td>28.57</td>
<td>6.58</td>
<td>26.67</td>
<td>5.88</td>
<td></td>
<td>26.61</td>
<td>5.45</td>
<td></td>
</tr>
<tr>
<td>SOD (U SOD/mg Hb)</td>
<td>1.53†</td>
<td>.15</td>
<td>1.43</td>
<td>.13</td>
<td></td>
<td>1.41</td>
<td>.15</td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different from the PRE and SNOW conditions (p < .05).
† Significantly different from the LAND and SNOW conditions (p < .05).
‡ Significantly different from the LAND condition (p < .05).

Table 3  Oxidative Stress Markers

<table>
<thead>
<tr>
<th>Measure</th>
<th>PRE</th>
<th>SD</th>
<th>LAND</th>
<th>M</th>
<th>SD</th>
<th>SNOW</th>
<th>M</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (μmol/L)</td>
<td>0.16</td>
<td>.10</td>
<td>0.12</td>
<td>.08</td>
<td></td>
<td>0.15</td>
<td>.11</td>
<td></td>
</tr>
<tr>
<td>LOOH (mmol/L)</td>
<td>4.65</td>
<td>2.16</td>
<td>6.06</td>
<td>3.40</td>
<td></td>
<td>2.67*</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>CBP (nmol/mg protein)</td>
<td>1.19</td>
<td>.66</td>
<td>.89</td>
<td>.41</td>
<td></td>
<td>1.09</td>
<td>.49</td>
<td></td>
</tr>
<tr>
<td>CBHB (nmol/mg protein)</td>
<td>6.30</td>
<td>3.75</td>
<td>5.74</td>
<td>2.96</td>
<td></td>
<td>3.82</td>
<td>2.07</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from PRE and LAND conditions (p < .05).
Temporary disruptions of ATP-dependent Ca\(^{++}\) pumps, leading to increased intracellular Ca\(^{++}\) concentrations, during exercise may activate the xanthine oxidase pathway. Under normal skeletal muscle conditions, the enzyme xanthine dehydrogenase aids in the conversion of hypoxanthine to uric acid. Increased intramuscular Ca\(^{++}\) concentrations during periods of high intensity exercise may activate calcium dependent proteases, which convert xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase uses molecular oxygen instead of NAD\(^{+}\) as an electron acceptor and thus generates the superoxide radical (18).

Activation of invading leukocytes may stimulate free radical production to enhance host defense mechanisms in response to exercise-induced muscle damage. In particular, neutrophils may reduce molecular oxygen to the superoxide radical via NADPH oxidase, which is dormant in resting cells (30). Similar processes have also been observed in monocytes and eosinophils.

Two other potential mechanisms for free radical generation during high intensity exercise include increases in the activities of cyclooxygenase and nitric oxide synthase (NOS). Increased Ca\(^{++}\) concentrations may activate the enzyme phospholipase A\(_{2}\), which releases arachidonic acid from phospholipids. Cyclooxygenase reacts with arachidonic acid to generate the hydroxyl radical (7). Hypoxic conditions have also been shown to increase NOS activity leading to the formation of nitric oxide radicals. These radicals may exert a weak pro-oxidant effect by themselves or combine with superoxide to form a more potent oxidant, peroxynitrite.

Despite possible free radical production from aerobic metabolism, anaerobic metabolism, and environmental factors, no elevations in markers of lipid (MDA & LOOH) or protein (CBP & CBHb) oxidation were found in these skiers following a period of intense training. Although LOOH values were highest following the LAND training session, the values were not statistically different from the PRE measures. The drop in LOOH values following SNOW is difficult to explain but may merely reflect a normal range of LOOH values. These findings are in accord with those of Sahlin et al. (37) and Saxton et al. (39), who did not demonstrate increases in markers of oxidative stress following repetitive contractions of knee flexors and forearm flexors, respectively. These findings are contrary to the findings of McBride et al. (24) and Marzatico et al. (23), who found increased markers of oxidative stress following total body resistance and sprint training, respectively. The fact that markers of oxidative stress were not elevated following intense training may indicate enhanced antioxidant protective mechanisms in elite alpine ski racers.

Habitual high intensity training necessary for elite level competition may increase antioxidant defenses (20, 35). Enhanced antioxidant levels may provide protection to muscles during repetitive contractions and reduce the rate of fatigue (28). In this study \(\alpha\)- and \(\gamma\)-tocopherol values were highest at the PRE condition and decreased over the course of the camp. The decrease in antioxidants following LAND and SNOW may be indicative of their sacrificial nature. Although the decrease in tocopherols was significant, dietary antioxidant intake exceeded the RDA standards, and plasma values were not considered to be deficient (29). GSH values were depressed immediately following the LAND training session. Similar findings have been reported following exercise to exhaustion (38). Fortunately, oxidized GSH (GSSG) may be enzymatically reduced back to GSH. The fact that GSH values following SNOW were not different from PRE indicates that total GSH status was not compromised over the course of the camp.
Physical training has been shown to affect antioxidant enzyme activities in muscle and erythrocytes (13, 25). In this study, cGPX activity was maintained during the course of the camp, indicating a relative stability of the glutathione system during intense training. Maintenance of this mechanism may have limited the damaging effects of free radicals produced during the training period helping to explain the lack of measured oxidative stress (32). Training studies examining SOD activity have reported contradictory results (14, 32). The decline in SOD activity across the training camp supports the notion that SOD is a training responsive antioxidant enzyme needing further study.

Intense training may also stimulate production of potent non-enzymatic antioxidants (9). UA is an important free radical scavenger in vivo and has been found to increase following intense exercise (26) due to heightened hypoxanthine metabolism and/or decreased renal clearance of UA (9). In this study, UA was increased at the LAND condition. The increase in UA concentration was mimicked by a concomitant increase TEAC. The correlation between UA and TEAC was significant ($r = 0.85, p < .001$) accounting for approximately 72% of the variance between the two measures. These data support the notion that uric acid is a major contributor to the TEAC assay (8). Recently, increases in UA concentrations following exercise have been associated with decreased markers of lipid peroxidation (26). Although a small decrease in MDA was noted to coincide with the increase in TEAC at LAND, the correlation between these two values was not significant ($r = -0.35, p < .05$).

Lack of oxidative stress suggests adequate antioxidant capacity in alpine skiers during summer training. Maintaining a diet that met the RDA standards may have provided sufficient antioxidant protection and may negate the need for antioxidant supplementation in highly trained skiers. However, the decrease in antioxidants such as α- and γ-tocopherol may be an area of interest for future investigators. Future studies should investigate the potential depletion of antioxidant capacity over longer periods of training to determine if oxidative stress becomes more evident.

References


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