Influence Of Carbohydrate Ingestion On Oxidative Stress And Plasma Antioxidant Potential Following A 3 Hour Run

By: Steven R. McAnulty, Lisa McAnulty, David Nieman, Jason D. Morrow, Alan C. Utter, Debra M. Vinci, Charles L. Dumke, and Dru A. Henson

Abstract
Concentrations of reactive oxygen species (ROS) increase during exercise secondary to increased oxygen uptake, xanthine oxidase activity, and immune system activation. Carbohydrate compared to placebo beverage ingestion is associated with an attenuated cortisol and catecholamine response. Catecholamines can undergo autooxidation to form ROS. We hypothesized that during intense exercise, ingestion of carbohydrate compared to placebo would diminish oxidative stress. Sixteen experienced marathoners ran on treadmills for 3 h at approximately 70% VO2(max) on two occasions while receiving carbohydrate or placebo beverages (11/h, double-blinded) in a randomized, counterbalanced order. Blood samples were collected before and immediately after exercise, snap frozen in liquid nitrogen, and stored at -80 degrees C until analysis. Plasma samples were analyzed for F2-isoprostanes (FIP) and lipid hydroperoxides (ROOH) as measures for lipid peroxidation, ferric reducing ability of plasma (FRAP) as a measure of plasma antioxidant potential and for cortisol. The pattern of change in cortisol was significantly different between carbohydrate and placebo conditions (P = 0.024), with post-exercise levels higher in the placebo condition. Under both carbohydrate and placebo conditions, significant increases in FIP, ROOH, and FRAP were measured, but the pattern of increase was not different (FIP, interaction effect, P = 0.472; ROOH, P = 0.572; FRAP, P = 0.668). Despite an attenuation in the cortisol response, carbohydrate compared to placebo ingestion does not counter the increase in oxidative stress or modulate plasma antioxidant potential in athletes running 3 h at 70% VO2(max).

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Concentrations of reactive oxygen species (ROS) increase during exercise secondary to increased oxygen uptake, xanthine oxidase activity, and immune system activation. Carbohydrate compared to placebo beverage ingestion is associated with an attenuated cortisol and catecholamine response. Hypothesized that during intense exercise, ingestion of carbohydrate compared to placebo would diminish oxidative stress. Sixteen experienced marathoners ran on treadmills for 3 h at ~70% VO2max on two occasions while receiving carbohydrate or placebo beverages (11/h, double-blinded) in a randomized, counterbalanced order. Blood samples were collected before and immediately after exercise, snap frozen in liquid nitrogen, and stored at -80°C until analysis. Plasma samples were analyzed for F2-isoprostanes (FIP) and lipid hydroperoxides (ROOH) as measures for lipid peroxidation, ferric reducing ability of plasma (FRAP) as a measure of plasma antioxidant potential and cortisol. The pattern of change in cortisol was significantly different between carbohydrate and placebo conditions (P = 0.024), with post-exercise levels higher in the placebo condition. Under both carbohydrate and placebo conditions, significant increases in FIP, ROOH, and FRAP were measured, but the pattern of increase was not different (FIP, interaction effect, P = 0.472; ROOH, P = 0.572; FRAP, P = 0.668). Despite an attenuation in the cortisol response, carbohydrate compared to placebo ingestion does not counter the increase in oxidative stress or modulate plasma antioxidant potential in athletes running 3 h at 70% VO2max.

Keywords: Carbohydrate; Cortisol; Gatorade; Oxidative stress; Exercise

INTRODUCTION

Studies conducted over the last 15 years have identified reactive oxygen species (ROS) as agents in cellular differentiation, aging, mutagenesis, carcinogenesis, pathophysiology of many diseases, and muscle soreness and damage during exercise. Aerobic exercise improves cardiovascular fitness and health but at intense levels increases oxidative stress. Our research group has found significant increases in lipid oxidation markers, such as biologically active F2-isoprostanes (FIP) and lipid hydroperoxides (ROOH), following prolonged and intensive exercise. FIP are of biological interest not only as a specific and sensitive index of lipid peroxidation but also for potential involvement in activation of innate immunity. FIP have been found to increase platelet aggregation and smooth muscle constriction and are present in atherosclerotic plaques.

Exhaustive long duration exercise increases plasma concentrations of stress hormones, neutrophils, monocytes, and inflammatory cytokines, decreases lymphocyte proliferation and natural killer cell activity, and causes many other changes in immunity. Exercise-induced alterations in immune function may contribute to otherwise modulate oxidative stress. Furthermore, exhaustive exercise dramatically increases plasma levels of catecholamines which can undergo autooxidation to...
form ROS. Production of ROS and oxidative stress products during exercise may further propagate release of inflammatory cytokines, most likely from activation of transcription factors, such as NF-kB.

Given the potential involvement of ROS and inflammatory cytokines in detrimental cellular processes, much research has focused on the potential beneficial effects of antioxidant consumption such as vitamin C and vitamin E. Interestingly, very few studies have examined the effect of macronutrient ingestion such as carbohydrate on ROS production and antioxidant status, particularly during exercise. Carbohydrate compared to placebo ingestion has been shown to improve maintenance of blood glucose levels, causing a decrease in release of AC1H, cortisol, and epinephrine. Thus, carbohydrate supplementation might result in attenuation of oxidative stress by decreasing stress hormone levels. Lord-Fontaine et al. reported, for example, that glucose administration protected Chinese hamster ovary cells from heat shock and hydrogen peroxide administration by increasing NADPH from the hexose monophosphate shunt. Therefore, we hypothesized that carbohydrate supplementation (11/h, 6% solution) would attenuate elevations in oxidative stress markers and increase or maintain plasma antioxidant capacity during long duration exercise.

MATERIALS AND METHODS

Subjects

Sixteen experienced marathon runners were recruited through a letter of invitation. Male and female runners ranging in age from 25 to 60 years were accepted into the study if they had run at least one competitive marathon, had been training for marathon races for 2 years or more, and were willing to adhere to all aspects of the research design. Informed consent was obtained from each subject, and the experimental procedures were in accordance with the policy statements of the institutional review board of Appalachian State University (ASU).

Research Design

Three to six weeks prior to the first test session, subjects reported to the ASU Human Performance Lab for orientation and measurement of body composition and cardiorespiratory fitness. Body composition was assessed by hydrostatic weighing, and VO2max was determined using a graded maximal protocol adapted for runners as described in an earlier study. Oxygen uptake and ventilation were measured using the MedGraphics CPX metabolic system (MedGraphics Corporation, St Paul, MN). Heart rate was measured using a chest heart rate monitor (Polar Electro Inc., Woodbury, NY). Basic demographic and training data were obtained through a questionnaire. Subjects functioned as their own controls and returned to the lab for 2–3 h runs on the treadmill. Subjects ingested carbohydrate or placebo beverages in a randomized, counterbalanced-double-blind design.

Subjects reported to the lab at 6:00 a.m. not having ingested energy in any form for at least 8 h. The two runs were 4–6 weeks apart, and three to four marathoners ran on treadmills in the same room at the same time on the same day of the week (7:00–10:00 a.m., Saturday), with metabolic measures collected every 30 min to verify workload (using the same equipment used during baseline testing). On the test days, subjects received 6% carbohydrate or placebo beverages 15–30 min pre-run (12 ml/kg) and during the 3 h run (4 ml/kg 15 min−1). The beverages were supplied by the Gatorade Sports Science Institute (Barrington, IL) as in an earlier study. The carbohydrate and placebo beverages were identical in appearance and taste. The two fluids were identical in sodium (~190 mEq l−1) and potassium (~30 mEq l−1) concentration and pH (~3.0). No other beverages or food were ingested during the exercise.

Blood and skeletal muscle biopsy samples were collected ~30 min pre-run and immediately post-run. Runners agreed to avoid the use of large-dose vitamin/mineral supplements (above 100% of Recommended Dietary Allowances), herbs, and medications known to possibly affect oxidative stress for 1 week prior to each run. During orientation, a dietician instructed the runners to follow a diet high in carbohydrate during the 3 days prior to each 3 h run (through use of a food list) and record intake using a food record. The food records were analyzed using a computerized dietary assessment program, and no unusual intakes of antioxidants were found (data not shown) (Food Processor, ESHA Research, Salem, Oregon).

Skeletal Muscle Biopsies

Skeletal muscle biopsy samples were acquired before and after exercise, immediately following blood sample collection. The exact same procedures were utilized pre and post-exercise, with incisions made in the same thigh ~3 cm apart. During the second 3 h treadmill run, samples were collected from the opposite thigh. Local anesthesia (1% xylocaine) was injected subcutaneously and into the vastus lateralis. A muscle biopsy sample was then obtained using the percutaneous needle biopsy procedure modified to include suction. Muscle biopsy samples were divided into two pieces and immediately frozen in liquid nitrogen. Samples were stored at −80°C until...
subsequent analysis. Muscle cytokine mRNA and plasma cytokine data are published elsewhere.

**Muscle Glycogen Analysis**

Samples were dissected free of connective tissue, blood, and other non-muscle constituents and then freeze dried and powdered. A portion of the muscle was extracted with acid, neutralized, and glucosyl units were analyzed enzymatically in triplicate using a spectrophotometer.\(^{[20]}\)

**Oxidative Measures**

Plasma FIP were determined using gas chromatography mass spectrometry according to the methodology of Morrow.\(^{[5]}\)

ROOH were determined with a kit obtained from Cayman Chemical (#705002 Cayman Chemical, Ann Arbor, MI) using extraction and spectrophotometric analysis. Briefly, 100 µl of plasma sample were pipetted into duplicate test tubes, and 100 µl of extract R saturated methanol (Fisher Scientific, Pittsburgh, PA) were added to each tube. All tubes were then vortexed, and 750 µl of cold chloroform (Fisher Scientific, Pittsburgh, PA) were added to each test tube and vortexed. All tubes were then centrifuged at 1500g, at 4°C for 5 min. Then, 500 µl of the bottom chloroform layer were extracted from each tube and immediately placed in ice. At this point, 450 µl of a 2:1 ratio of deoxygenated chloroform–methanol mixture were added to each tube, and the tubes were vortexed. The chloroform–methanol mixture was deoxygenated by bubbling with nitrogen gas for 30 min. A standard curve was prepared using a hydroperoxide standard and varying amounts of the 2:1 ratio deoxygenated chloroform–methanol mixture giving a range of 0–5 nmol and a total volume of 950 µl. Lastly, 50 µl of chromogen were added to each sample and standard in duplicate, and the tubes were vortexed. All tubes were incubated at room temperature for 5 min, and then 300 µl of each standard and sample were removed and placed in a 96-well glass plate and read at 500 nm ROOH concentration was determined from a linear regression line generated from the standard curve.

Total plasma antioxidant potential was determined by the ferric reducing ability of plasma (FRAP) assay according to the methodology of Benzie\(^{[21]}\). Working FRAP reagent was prepared as required by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml iron trichloride hexahydrate solution. The working FRAP solution was placed in a water bath and warmed to 37°C. Then, 100 µl of either standard, sample, or blank (deionized water) were added to glass test tubes containing 30 µl of warmed FRAP reagent and vortexed. All tubes were then incubated at 37°C for 4 min and read at 593 nm. Samples and standards were analyzed in duplicate, and FRAP values were expressed as ascorbic acid equivalents as determined by linear regression from an ascorbic acid standard curve (0–1000 µmol).

Intra-assay and inter-assay coefficients of variation for ROOH and FRAP assays were <10% and <6%, respectively. Plasma volume shift according to the method of Dill and Costill was calculated, but plasma volume shift changes were not significant from pre-exercise\(^{[22]}\).

**Statistical Analysis**

SPSS version 11.0 was utilized for all statistical analysis. Statistical significance was set at the \( P \leq 0.05 \) level, and values were expressed as means ± SEM. Performance measures were compared under carbohydrate and placebo conditions using paired \( t \)-tests. All other data and figures were analyzed using a 2 (carbohydrate and placebo conditions) × 2 (times of measurement) repeated measures ANOVA. If the condition × time interaction \( P \)-value was \( \leq 0.05 \), the change from pre- to post-exercise value was calculated and compared between conditions using a Bonferroni correction. Pearson product-moment correlations were used to test the relationship between changes in plasma markers and the muscle measure.

**RESULTS**

Table I summarizes subject characteristics for the 16 runners (12 males, 4 females) completing all phases of the study. Data for the male and female runners were

<table>
<thead>
<tr>
<th>TABLE I Subject characteristics (n = 16) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Stature (m)</td>
</tr>
<tr>
<td>Body mass (kg)</td>
</tr>
<tr>
<td>Body composition (% fat)</td>
</tr>
<tr>
<td>Heart rate, maximal (beats/min)</td>
</tr>
<tr>
<td>( VO_{2\text{max}} ) (ml kg⁻¹ min⁻¹)</td>
</tr>
<tr>
<td>Respiratory exchange ratio, maximal</td>
</tr>
<tr>
<td>Minute ventilation, maximal (l/min)</td>
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<tr>
<td>Training distance (km/week)</td>
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</table>
combined because no significant differences were measured for the hormonal data reported in this paper. The marathon runners in this study were highly experienced and committed to regular training and racing but still well below elite status. The treadmill test data indicate a high degree of cardiorespiratory fitness for this age group. Carbohydrate intake during the 3 days prior to the 3 h treadmill runs did not differ significantly between carbohydrate and placebo conditions (58.8 ± 3.1 and 61.8 ± 2.1% of total energy intake, respectively, P = 0.271, data not shown). Oxygen consumption was measured six times during the 3 h treadmill runs, and averaged 30.9 ± 10 (69.1 ± 1.2% VO₂max) and 31.4 ± 0.9 ml·kg·min⁻¹ (70.3 ± 1.3% VO₂max) in the carbohydrate and placebo conditions, respectively (P = 0.176, data not shown). Heart rates during the 3 h treadmill runs averaged 81.4 ± 11 and 81.5 ± 1.3% HRmax in the carbohydrate and placebo conditions, respectively (P = 0.922). Plasma volume shift was negligible and did not differ between carbohydrate and placebo conditions (1.9 ± 0.3 and 1.5 ± 0.6%, respectively P = 0.597, data not shown). Table II illustrates the effect of carbohydrate during the exercise on plasma glucose, insulin, and cortisol values. Plasma glucose and insulin values were significantly higher in the carbohydrate supplemented group post-run compared to the placebo group, while plasma cortisol was significantly lower in the carbohydrate supplemented group post-run compared to the placebo group. Plasma cortisol decreased 24 and 34% in the runners during carbohydrate and placebo conditions, respectively [F(1,15) = 6.26, P = 0.024].

The skeletal muscle biopsy procedure was initiated immediately post-run, with samples removed from the vastus lateralis 141 ± 0.8 and 131 ± 1.0 min post-run in the carbohydrate and placebo conditions, respectively (P = 0.419). Muscle glycogen decreased significantly under both carbohydrate (28%) and placebo (34%) conditions at a similar rate [F(1,15) = 1.46, P = 0.246] (Data not shown). Table III illustrates a significant time effect for FIP, ROOH, and FRAP as a result of exercise but no significant interaction effects between treatments. There were no significant correlations between variables, with the exception of a positive correlation between FIP and cortisol in the placebo group post-exercise (data not shown).

### DISCUSSION

These data demonstrate that 3 h of treadmill running exercise significantly increases oxidative stress, as evidenced by an increase in lipid peroxidation markers. These findings are consistent with observations from other studies which have used long-duration exercise, although the absolute post-exercise values observed in this study were not as high as those found after ultra-racing. The acute and chronic physiological and biochemical ramifications of increased FIP during and after exercise are not currently known. High concentrations of FIP are found after cardiac arrest, in individuals with diabetes, and in smokers. FIP may also play a role in innate immune response.

### TABLE II

<table>
<thead>
<tr>
<th>Variable</th>
<th>Carbohydrate (Pre-run)</th>
<th>Carbohydrate (Post-run)</th>
<th>Placebo (Pre-run)</th>
<th>Placebo (Post-run)</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol·l⁻¹)</td>
<td>3.84 ± 0.2</td>
<td>5.17 ± 0.2*</td>
<td>3.75 ± 0.1</td>
<td>4.02 ± 0.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Insulin (pmol·l⁻¹)</td>
<td>63.6 ± 6.7</td>
<td>126 ± 26*</td>
<td>77.0 ± 10.6</td>
<td>67.2 ± 8.8</td>
<td>0.008</td>
</tr>
<tr>
<td>Cortisol (ng·ml⁻¹)</td>
<td>237 ± 12.1</td>
<td>180.9 ± 16.9*</td>
<td>231.8 ± 16.9</td>
<td>223.8 ± 25.5</td>
<td>0.024</td>
</tr>
</tbody>
</table>

*Significant difference in change in carbohydrate compared to placebo condition, P ≤ 0.05

### TABLE III

<table>
<thead>
<tr>
<th>Effect</th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
<th>Treatment: time: interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIP (pg·ml⁻¹)</td>
<td>CHO</td>
<td>38.8 ± 2.2</td>
<td>43.2 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>39.3 ± 3.8</td>
<td>48.0 ± 4.1</td>
</tr>
<tr>
<td>ROOH (umol·l⁻¹)</td>
<td>CHO</td>
<td>44 ± 14</td>
<td>91 ± 33</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>35 ± 0.6</td>
<td>117 ± 35</td>
</tr>
<tr>
<td>FRAP (umol·l⁻¹)</td>
<td>CHO</td>
<td>380.8 ± 49.2</td>
<td>452 ± 26.1</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>317.2 ± 39.5</td>
<td>416.3 ± 18.2</td>
</tr>
</tbody>
</table>

*P ≤ 0.05
modulation [24] Whereas FIP are thought to originate from extravascular tissue,[25] ROOH originate primarily from oxidation of omega-3 and omega-6 fatty acids found in plasma lipoproteins.[26] In the present study, there was no significant interaction of carbohydrate administration on either FIP or ROOH, indicating that neither tissue nor plasma compartments were affected by any influence of the carbohydrate.

Few studies exist which have examined the effect of macronutrient supplementation on lipid peroxidation and oxidative stress. KarolKiewicz et al.[14] examined the effect of a carbohydrate and protein supplement on reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) in 19 teenage track and field athletes divided into supplement and placebo groups. Carbohydrate and protein supplementation had no effect on plasma GSH or TBARS Vasankari et al.[16] examined eight athletes who ran 27 km on two separate occasions with random assignment to carbohydrate (105 g of carbohydrate during exercise) or placebo conditions. This study found no effect of carbohydrate supplementation on serum diene conjugation compared to placebo after exercise. These studies are in support of our findings of no attenuation by carbohydrate on oxidative stress.

We had hypothesized that a carbohydrate supplement during exercise would diminish oxidative stress through increased blood glucose levels and lowered stress hormone levels.[27] A low compared to high carbohydrate diet might also be important, particularly in regards to hepatic and muscle glycogen content and the ability to maintain blood glucose levels during exercise. As far as we are aware, hepatic glycogen content and oxidative stress during exercise have not been examined. Although there was no difference in muscle glycogen content between groups, there was a significant difference in blood glucose and insulin levels. Despite the increase in blood glucose in the carbohydrate group, there was no difference in lipid peroxidation. In support of our findings in relation to muscle glycogen content, Hellsten et al.[27] concluded that changes in muscle glycogen level do not affect levels of oxidative stress.

Carbohydrate ingestion has been shown to blunt catecholamine and inflammatory cytokine release.[6,7,27] Carbohydrate ingestion did significantly suppress post-exercise cortisol values in our study; therefore, it is likely that catecholamine values responded similarly based on previous data published by our team.[6,7] Catecholamines may undergo autooxidation to ROS,[10] but may not contribute substantially to the overall oxidative stress, as reflected in our FIP or ROOH data. Autooxidation of catecholamines is believed to be involved in certain disease states such as progression of Parkinson’s disease.[28] Additionally, increases in cortisol concentration are linked to mobilization of neutrophils and inflammatory responses. In our study, post-exercise IL-6, IL-10, and IL-1ra were significantly lower in the carbohydrate compared to placebo condition (data published elsewhere). Therefore, it seems reasonable to conclude that inflammatory responses did not significantly contribute to formation of our oxidative stress markers. Conversely, other researchers have found relationships between immune system activation and oxidative stress.[8,9]

Plasma FRAP values were significantly elevated in both groups following exercise, although there was no significant interaction between groups. The increase in plasma antioxidant potential is primarily due to increasing uric acid and vitamin C in the blood during exercise.[14,23] The reasons for failure of an increased plasma antioxidant status to attenuate plasma ROOH is somewhat surprising and not completely understood, particularly given that ROOH predominately arise from plasma LDL and HDL.[26] Carbohydrate has not been reported to directly increase or contribute to antioxidant status, although carbohydrate can be oxidized to form carbon derivatives.[29] We did not observe any significant effects of carbohydrate administration on plasma antioxidant potential versus placebo. Total plasma antioxidant potential has been found to be inversely related to oxidative stress in some disease states.[30]

CONCLUSION

In conclusion, 3 h of treadmill running at 70% \( \text{V}_{\text{O}_{2\text{max}}} \) significantly increased oxidative stress in experienced marathon runners, despite an increased plasma antioxidant potential. Carbohydrate ingestion during exercise did not affect oxidative stress or increase plasma antioxidant potential compared to placebo, despite significantly attenuating plasma cortisol levels.

Acknowledgements

This work was funded by the Gatorade Sports Science Institute and National Institutes of Health Grants (to J. D. Morrow) DK-48831, GM-15431, CA-77839, and DK-26657. J. D. Morrow is the recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research. Fisher/Nycom Laboratory.

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