Effect of a special carbohydrate–protein cake on oxidative stress markers after exhaustive cycling in humans

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Abstract

Exercise has been associated with oxidative stress that is correlated with muscle fatigue and reduced exercise performance. The aim of this study was to examine the effects of a special cake (consisting of carbohydrate to whey protein 3:5:1) vs an isocaloric carbohydrate cake on biomarkers of oxidative stress in 9 males after exhaustive cycling. A randomized single-blind cross-over study was completed. They performed one trial involving a 2-h exercise on a cycle ergometer at 60–65% VO2max followed by a 4-h recovery and then a second trial involved an 1-h exercise at 60–65% VO2max which was increased at 95% VO2max (time trial). The subjects received 4 experimental or placebo cakes after the first trial (the first immediately after and then one every hour). Blood samples were collected at eight time intervals: pre-exercise, 30 min, 1.5 h and 4 h post-exercise, post time Trial, 1 h, 24 h and 48 h post time Trial. ThioBarbituric Acid Reactive Substances (TBARS), protein carbonyls, total antioxidant capacity (TAC), catalase and glutathione (GSH) were determined spectrophotometrically. The mean time to exhaustion did not differ upon cake consumption. Consumption of the special cake reduced TBARS significantly, but had no effect on other oxidative stress markers.

1. Introduction

It is well established that exercise induces excessive production of reactive species. An amount of reactive species is necessary for physiological processes, namely signal transduction (Ji, 2007; Wells et al., 2009), gene expression (Ji et al., 2006) and useful adaptations during exercise (Gomez-Cabrera et al., 2005). However, excessive production of reactive species leads to oxidative stress, which has been implicated in oxidative damage of macromolecules (Veskoukis et al., 2008), immune dysfunction (Schneider and Tiidus, 2007), muscle damage (Nikolaidis et al., 2007) and fatigue (Betters et al., 2004). It has also been observed that muscle-damaging exercise also induces oxidative stress and its detrimental effects last for a few days after the end of it (Nikolaidis et al., 2008; Urso et al., 2003). Therefore, recovery of athletes after severe exercise could be slow and athletic performance is usually not optimal. Several investigators have tried to improve athletic performance via supplementation of beverages rich in carbohydrates and proteins, sometimes whey with contradictory effects on...
performance (Baty et al., 2007; Betts et al., 2007; Ferguson-Steggall et al., 2010; Haff et al., 2000; Ivry et al., 2002). To the authors’ knowledge, solid or other than liquid forms of supplementation have not been studied in the literature.

Milk has been recently recognized as functional food, suggesting that its constituents have direct and measurable effects on health (Nagendra, 2000). Its main sources of protein are caseins and whey. After cheese manufacturing, caseins are the proteins responsible for making curds, while whey remains in an aqueous environment. The components of whey include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lacto-peroxidase enzymes, glycomacropeptides and lactose (Madureira et al., 2007; Walzem et al., 2002). Whey is considered as by-product of cheese manufacturing, but it is also described as functional food (Marshall, 2004; Morr and Ha, 1993), as it is involved in preventing or improving several pathogenic conditions. Whey is a dietary supplement providing antimicrobial activity, immune modulation and or improving several pathogenic conditions. Whey seems to participate in the prevention of cardiovascular diseases (Marshall, 2004). It contains high concentration of branched-chain amino acids such as leucine, isoleucine and valine, which play a key role in protein metabolism and are important factors in muscle hypertrophy (Ha et al., 2003) and strength, in tissue growth and repair (Anthony et al., 2001). Whey is also rich in sulfur-containing cysteine and methionine, which contribute to the enhancement of antioxidant defense through intracellular conversion to glutathione (Marshall, 2004).

Relative studies of the literature usually examine the effects of the supplementation of liquid beverages rich in carbohydrates or proteins on performance. However, studies investigating the effects of carbohydrates and proteins administered in other forms than liquid on oxidative stress are lacking. The main objective of the present study was to examine the effects of a special cake consisted of a specific ratio of carbohydrates and whey protein on oxidative stress in athletes after exhaustive exercise.

2. Materials and methods

2.1. Subjects

Nine physically active men (age, 28 ± 2 yr; height, 184 ± 3 cm; weight, 77 ± 2 kg; body fat, 11 ± 2%; body mass index, 23 ± 1 kg/m2; VO2max, 695 ± 21 L/min ± SEM) participated in the present study. The subjects were training at least 3 times per week for at least 3 h and had a training history of at least 2 years. They were nonsmokers and were not receiving anti-inflammatory medication or nutritional supplements. VO2max measurement ensured that the subjects exercised at similar intensities. A written informed consent to participate in the study was provided by all participants after they had been informed of all risks, discomforts and benefits involved in the study. The procedures were in accordance with the Helsinki declaration of 1975 and approval was received by the human subjects committee of the University of Thessaly.

The subjects visited the laboratory for the first time for a screening of anthropometric parameters and they completed a health and activity questionnaire. Each participant reported to the laboratory in the morning after an overnight fast and abstained from alcohol and caffeine for 24 h. Body mass was measured to the nearest 0.5 kg (Beam Balance 710, Seca, UK) with the subjects lightly dressed and barefoot. Standardized training protocol was repeated to the nearest 0.5 kg (Radometer 208, Seca). Percentage body fat was calculated from seven skinfold measures (average of two measurements of each site), using a Harpenden caliper (John Bull, UK) according to published guidelines (American College of Sports Medicine, 2000). Body mass index was calculated as the ratio of body weight (kg)/height (m)2. VO2max was determined after a maximal consumption test on a cycle ergometer (Monark 834E, Sweden) was performed. The protocol began at 1.5 kg (~70RPM) for 1 min and was increased by 0.5 kg every 2 min until VO2max was reached. Respiratory gas variables were measured using a metabolic cart (Vmax29, Sensormedics, USA), which was calibrated before each test using standard gases of known concentration. Exercise heart rate was monitored by telemetry (Polar Tester, SE10TM, Electro Oy, Finland).

2.2. Diet and activity before the experiment

The subjects were instructed to follow their usual eating habits during the days before the experiment. They were also asked to record on a dietary record sheet their diet 3 days before the first exercise bout and for 2 days after it. All volunteers were instructed to stay away from strenuous physical activity for 2 days preceding and 2 days following the experiment. The subjects received a copy of their dietary record sheets and were asked to follow exactly the same food intake patterns (as recorded in their dietary record sheets) before their second experimental session.

2.3. Design

Each subject participated in two trials in a counterbalanced fashion (same subjects received both the experimental cake and placebo cake in a random order) using a crossover, double-blind, repeated-measures design. The subjects visited the laboratory for a second time 5 days after their VO2max determination (08:00~09:00 h in the morning) and were participated either in the experimental or placebo trial. Each subject participated in two experimental sessions separated with wash out period of one week. During each session the subjects consumed either an experimental cake providing 0.9 g carbohydrate/kg body weight/h and 0.28 g protein/kg body weight/h, providing a ratio between carbohydrates and whey protein of 3.5:1, or a placebo cake providing 1.1 g carbohydrate/kg body weight/h and 0.1 g protein/kg body weight/h. The experimental protocol consisted of the following phases: (I) 2 h of continuous cycling on a cycle ergometer (Monark 834E, Sweden) at an intensity corresponding to 60–65% of their established VO2max, (II) 4 h of recovery, (III) 1 h of continuous cycling at 60–65% of their VO2max, (IV) cycling speed was increased to 95% of their VO2max until exhaustion (time Trial), (V) 1 h of recovery (Fig. 1). Exercise was performed at a temperature of 21 ± 2 ℃ and 45 ± 4% relative humidity. To attenuate subjects’ discomfort, water was available. A blood sample of lithium heparin (Vacutainer, USA) was collected.Expired gas samples were collected every 15 min to ensure the prescribed exercise intensity. Perceived fatigue of the subjects was recorded every 15 min using Borg scale during phases I and III and at the end of phase IV. Blood samples were collected pre exercise (T1), 30 min post exercise (T2), 1.5 h post exercise (T3), 4 h post exercise (T4), immediately post time Trial (T5), 1 h post time Trial (T6), 24 h post time Trial (T7) and 48 h post time Trial (T8) (Fig. 1). One experimental or placebo cake was consumed by the subjects immediately post exercise and three more experimental or placebo cakes were consumed every 1 h after the first one. Exercise testing, cake administration and blood sampling was repeated at the same time of day and in the same order before and after the first trial (experimental, Fig. 1A) as well as before and after the second trial (placebo, Fig. 1B).

2.4. Fat and carbohydrate oxidation

Fat and carbohydrate oxidation rates (g min-1) during submaximal exercise were calculated indirectly by monitoring the rate of O2 consumption (L min-1) and CO2 production (L min-1) using the following stoichiometric equations (Jeukendrup and Wallis, 2005), assuming that protein oxidation during exercise was negligible:

\[ \text{fat oxidation} = \frac{1.165 \times \text{O}_{2}}{3.76} \]
\[ \text{carbohydrate oxidation} = \frac{2.410 \times \text{CO}_{2}}{2.96} \]

2.5. Blood collection and handling

Blood samples (10 mL) were drawn from a forearm vein with subjects in a seated position. Blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes, centrifuged immediately at 1370 g for 10 min at 4 ℃ and the plasma was collected and used for the measurement of TAC and the determination of TBARS and protein carbonyl concentrations. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020 g for 15 min at 4 ℃ and the erythrocyte lysate was collected for measurement of catalase activity. A portion of erythrocyte lysate (500 μL) was treated with 5% trichloroacetic acid (TCA) (1:1 v/v), vortexed vigorously, and centrifuged at 28,000g for 5 min at 4 ℃. The supernatants were removed, treated again with 5% TCA (1:3 v/v) and centrifuged again at 28,000g for 5 min at 4 ℃. The clear supernatants were transferred to eppendorf tubes and were used for the determination of GSH concentration. A blood aliquot (1 mL) was immediately mixed with EDTA to prevent clotting for hematology. Plasma and erythrocyte lysate were then stored at −80 ℃ until biochemical analyses.

2.6. Assays

For TBARS determination, a slightly modified assay of Keles et al. (2001) was used. According to this method, 100 μL of plasma was mixed with 500 μL of 35% TCA and 500 μL of trishydroxymethylaminomethane hydrochloride (Tris–HCl) (200 mM, pH 7.4) and incubated for 10 min at room temperature. One milliliter of 2 M Na2SO4 and 55 mM thiobarbituric acid solution was added and the samples were incubated at 95 ℃ for 45 min. The samples were cooled on ice for 5 min and were vortexed after adding 1 mL of 70% TCA. The samples were centrifuged at 15,000g for 3 min and the absorbance of the supernatant was read at 532 nm. A baseline absorbance was taken into account by running a blank along with all samples during the measurement. Calculation of TBARS concentration was based on the molar extinction coefficient of malondialdehyde. The intra- and inter-assay coefficients of variation (CV) for TBARS were 3.9% and 5.9%, respectively.
Protein carbonyls were determined based on the method of Patsoukis et al. (2002). Briefly, 20 μL of 20% TCA was added to 50 μL of plasma and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000g for 5 min at 4 °C. The supernatant was discarded and 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) [in 2.5 N hydrochloric (HCl)] for the sample, or 500 μL of 2.5 N HCl for the blank, was added in the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min and were centrifuged for 3 min at 20,000g. The supernatant was discarded and 1 mL of ethanol–ethyl acetate (1:1 v/v) was added, vortexed and incubated at 37 °C for 15 min. The samples were centrifuged at 15,000g for 5 min at 4 °C. The supernatant was discarded and 1 mL of ethanol–ethyl acetate (1:1 v/v) was added, vortexed and centrifuged at 15,000g for 5 min at 4 °C. This washing step was repeated twice. The supernatant was discarded and 1 mL of 5 M urea (pH 2.3) was added, vortexed and incubated at 37 °C for 15 min. The samples were centrifuged at 15,000g for 3 min at 4 °C and the absorbance was read at 375 nm. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH. The intra- and inter-assay CV for protein carbonyls were 4.3% and 7.0%, respectively. Total plasma protein was assayed using a Bradford reagent from Sigma–Aldrich.

Catalase activity was determined using the method of Aebi (1984). Briefly, 4 μL of erythrocyte lysate (diluted 1:10) were added to 2991 μL of 67 mM sodium potassium phosphate (pH 7.4) and the samples were incubated at 37 °C for 10 min. Five microliters of 30% hydrogen peroxide (H2O2) were added to the samples and the change in absorbance was immediately read at 240 nm for 130 s. Calculation of catalase activity was based on the molar extinction coefficient of H2O2. The intra- and inter-assay CV for catalase were 6.2% and 10.0%, respectively.

The determination of TAC was based on the method of Janaszewska and Bartosz (2002). Briefly, 20 μL of plasma were added to 480 μL of 10 mM sodium potassium phosphate (pH 7.4) and 500 μL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical and the samples were incubated in the dark for 30 min at room temperature. The samples were centrifuged for 3 min at 20,000g and the absorbance was read at 520 nm. The intra- and inter-assay CV for TAC were 2.5% and 5.4%, respectively. TAC is presented as mmol of DPPH• reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH•H) by the antioxidants of plasma.

The hematological parameters were measured in a Sysmex K-1000 (TOA Electronics, Japan) autoanalyzer. Hematocrit and hemoglobin were determined on the basis of calibration curve made using commercial standards. The intra- and inter-assay CV for hemoglobin were 6.2% and 10.0%, respectively. White blood cells and platelets main effects of time were found. In mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) neither significant main effects nor interactions were observed.

3.2. Performance and RPE

Performance was defined as the time of cycling at the 95% of VO2max (time Trial) until exhaustion. No significant difference in performance between the two groups was observed (P > 0.05). The subjects of the experimental group reached exhaustion after 169.4 ± 47.6 s and the subjects of the placebo group reached exhaustion after 153.4 ± 19.5 s. Mean RPE for the 2 h exercise (experimental: 12.4 ± 0.48, placebo: 12.3 ± 0.69) and for the 1 h exercise (experimental: 14.3 ± 0.40, placebo: 13.9 ± 0.50) was not significantly different between the two conditions.

3.3. Fat and carbohydrate oxidation

There were no significant differences in fat and carbohydrate oxidation during the 2 h submaximal exercise (Fig. 2A) and during the 1 h submaximal exercise following the cake ingestion (Fig. 2B).

3.4. Oxidative stress markers

In plasma TBARS (Fig. 3A), main effect of treatment and interaction of treatment × time (P < 0.05) were found. In plasma protein carbonyls (Fig. 3B), in erythrocyte GSH (Fig. 3C), in erythrocyte catalase (Fig. 3D) and in plasma TAC (Fig. 3E) neither significant main effects nor interactions were observed.
4. Discussion

The present study examined the effect of a cake containing carbohydrates and whey protein in a specific ratio (3.5:1) on oxidative stress in a protocol of muscle damaging exercise (cycling) until exhaustion. The oxidative stress markers examined were TBARS, protein carbonyls, GSH, catalase and TAC. The main finding of the study is that the experimental cake reduced TBARS, namely lipid peroxidation, compared to the placebo cake containing carbohydrates only. However, it did not affect exercise performance as it was calculated by the time until exhaustion after cycling at an intensity corresponding to 95% of the volunteers established VO2max (time Trial). Some reservations exist with respect to the validity of the TBARS assay in detecting lipid peroxidation, as this measure is criticized for the lack of specificity (Halliwell and Gutteridge, 2007). Although this may be considered as a limitation of the present work, yet many studies from our and other research groups (Fischer et al., 2011; Nikolaidis et al., 2007, 2008) have repeatedly shown that TBARS concentrations have been consistently increasing after exercise. In addition, it has been found that TBARS concentrations followed similar changes to F2-isoprostane concentrations (currently considered the reference method) after exercise (Margonis et al., 2007).

Previous studies have shown that supplementation of a carbohydrate or a carbohydrate–protein mixture does not diminish exercise-induced oxidative stress. More specifically, carbohydrate compared to placebo ingestion did not counter the increase in oxidative stress as determined via F2-isoprostanes and lipid hydroperoxides or modulate plasma antioxidant potential measured by ferric reducing ability of plasma in athletes running 3 h at 70% of VO2max (McAnulty et al., 2003). Similar results were obtained as exhaustive resistance exercise and carbohydrate ingestion did not modulate oxidative stress (F2-isoprostanes concentration) or plasma antioxidant potential (ferric reducing capacity of plasma) in trained subjects (McAnulty et al., 2005). Karolkiewicz et al. (2001) examined the effect of a carbohydrate and protein supplement on GSH and 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. Vasanikari et al. (1998) examined 8 athletes who ran 27 km on two separate occasions with random assignment to carbohydrate or placebo conditions. This study found no effect of carbohydrate ingestion on serum diene conjugation compared to placebo after exercise. Finally, a drink containing carbohydrates, proteins and vitamins had no effect on plasma lipid hydroperoxides compared with an isocaloric drink of carbohydrates (Goldfarb et al., 2009).

There are several studies in the literature examining the effects of carbohydrate–protein supplements on exercise performance and the majority of them have observed ergogenic effects of carbohydrate–protein beverages. More specifically, it has been found that the addition of protein to a carbohydrate supplement enhanced aerobic endurance performance in cycling of different intensities (Ivy et al., 2003). In addition, a carbohydrate beverage with additional protein calories produced significant improvement in time to fatigue and reduction in muscle damage in athletes of endurance cycling (Saunders et al., 2004, 2007), and an improvement of exercise capacity was also demonstrated after treadmill running at 70% of VO2max (Bettis et al., 2007). Other investigators also observed that a mixture of carbohydrates plus a moderate amount of protein can improve aerobic endurance at exercise intensities between 45 and 70% of VO2max, despite containing lower total carbohydrate and caloric content (Ferguson-Stegall et al., 2010). Niles et al. (2001) showed that a carbohydrate–protein drink improves time to exhaustion after recovery from endurance exercise. Recently, it has been suggested that the inclusion of small amounts of protein (typically 20% of total calories) in a carbohydrate beverage may produce benefits over traditional carbohydrate-only beverages (Ivy et al., 2002, 2003).

However, this is not always the case. Interestingly, it has also been shown that a carbohydrate–protein beverage had no effect.
on exercise performance on isokinetic leg exercise (Haff et al., 2000) and on resistance exercise (Baty et al., 2007) compared to supplementation of beverages containing carbohydrates only.

In addition, Betts et al. (2005) revealed that including protein in a carbohydrate solution had no effect on treadmill run time to exhaustion at 85% VO₂max. These results are in line with the finding of the present study which has shown that the experimental cake containing a mixture of carbohydrates and whey protein did not enhance exercise performance.

Whey protein used in the present study is referred to as a bioactive compound. Its amino acid profile makes it ideal for improving body composition via supporting protein synthesis, body fat decrease and muscle growth. Other bioactive components found in whey might benefit additional aspects of health in active people and trained athletes by improving immune function and gastrointestinal health and exhibiting anti-inflammatory activity. In this line of reasoning, it has been demonstrated that men engaged in resistance training programs while supplemented with whey protein showed greater improvements in strength than men who trained without receiving whey protein as supplement (Burke et al., 2001). Additionally, supplementation of whey protein resulted in greater muscle performance and lower percentage of body fat compared to casein supplementation (Lands et al., 1999).

One question arising is why whey protein protects only lipids from oxidative stress? It is known that whey protein contains many times more cysteine than the placebo cake. This probably leads to preconditioning with high GSH levels, that the cell prefers...
as an antioxidant source (Elia et al., 2006). This is a probable expla-
nation for the lower lipid peroxidation since the cell has a higher pool of GSH to consume and protect other macromolecules. Our explanation needs further investigation.

The main finding of this study is that a cake consisted of a spe-
cific ratio of carbohydrates and whey protein decreases lipid per-
oxidation in volunteers after cycling. This is the first study to administrate in athletes a supplement in a cake form compared to others examining mainly the effects of liquid beverages. The re-
search about this novel product needs to continue in order to be elucidated if the cake could probably be used as a supplement for the improvement of recovery and for the amelioration of the exercise detrimental effects on redox status of athletes.

5. Conflict of Interest

The authors declare that there are no conflicts of interest

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As previously mentioned, the cake formulation provides a specific ratio of carbohydrates and whey protein that decreases lipid peroxidation in volunteers after cycling. This is the first study to administer a supplement in cake form compared to studies examining mainly liquid beverages. Further research is needed to elucidate if this cake could be used as a supplement for improving recovery and ameliorating exercise detrimental effects on redox status.

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