The Thesis Committee for Benjamin Dessard

Certifies that this is the approved version of the following thesis:

The Effects of Carbohydrate-Protein Supplementation on Glycogen Utilization and Fatigue During a Simulated Soccer Match

APPROVED BY

SUPERVISING COMMITTEE:

Supervisors:

_____

John L. Ivy

_____

Hirofumi Tanaka

The Effects of Carbohydrate-Protein Supplementation on Glycogen Utilization and Fatigue During a Simulated Soccer Match

by

Benjamin Dessard, B.S.

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Abstract

The Effects of Carbohydrate-Protein Supplementation on Glycogen Utilization and Fatigue during a Simulated Soccer Match

Benjamin Dessard, M.S. Kin.

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Supervisor: John L. Ivy

The purpose of this study was to examine if the addition of protein to a carbohydrate supplement (CHO+PRO), provided during a simulated soccer match, would reduce fatigue and muscle glycogen utilization in comparison to an isocaloric carbohydrate only supplement (CHO). Two female and eight male (n = 10) trained soccer players performed a modified version of the Loughborough Intermittent Shuttle Test (LIST) on two separate occasions, followed by a run to exhaustion (RTE). Supplements were provided 10 minutes before the simulated match and at the beginning of half-time, but not during exercise in order to create real-match conditions. Supplements were composed of 2.8% protein + 7% carbohydrate (CHO+PRO) or 9.8% carbohydrate (CHO). Muscle biopsies were performed before and at the end of the LIST, after which

participants ran to exhaustion. No differences were found between treatments for RTE (489 ± 121 sec for CHO and 589 ± 186 sec for CHO+PRO) or glycogen utilization (37.9 ± 7.6 μmol·g wet wt^{-1} during the CHO and 29.1 ± 6.0 μmol·g wet wt^{-1} during the CHO+PRO). No differences were found for the other measurements such as sprint times, heart rate, RPE, blood glucose, lactate, and insulin. Blood Creatine kinase (CK), and overall muscle soreness were measured 24 hours after each trial in order to evaluate muscle damage but no differences between treatments were found. In accordance with these findings, the phosphorylation state of the protein FOXO3a was not altered differently by the treatments. These results suggest that the addition of protein to a traditional carbohydrate-only supplement provided immediately prior to and at the half of a simulated soccer match does not further improve the benefits of a CHO supplement.

KEY WORDS: soccer, fatigue, muscle glycogen, carbohydrate, protein.

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INTRODUCTION

In soccer, the settlement of fatigue during a match leads to reduced performance. Deterioration in soccer-specific skills as well as a reduction in the amount of high-intensity running have been observed from the first to the second half of a match, especially during the last 15 minutes (1-4). The ingestion of carbohydrates during a game has been shown to reduce fatigue in soccer players as indicated by more frequent sprints performed and an overall greater distance covered during the second half (5-9). More recently, various well-controlled laboratory studies involving intermittent high-intensity exercise have been performed to determine the effects of carbohydrate ingestion on protocols simulating a soccer match (10-14). The Loughborough Intermittent Shuttle Test (LIST) was designed as a controlled field test to simulate the activity patterns observed during a game of soccer (15). It was found that ingesting carbohydrate before and every 15 minutes during this protocol reduced fatigue as it took longer for participants to run to exhaustion at the end of the trial (11, 13, 14). More interestingly, Nicholas et al. found that glycogen utilization during this same protocol was reduced by 22% when their trained soccer player participants ingested a carbohydrate-electrolyte beverage compared to a placebo control (10).

These studies support the widespread consensus that glycogen depletion towards the end of a soccer game is the primary cause of fatigue (2, 4, 16-18), and that carbohydrate supplementation during a game reduces fatigue through a mechanism that allows players to reduce muscle glycogen utilization (10, 19, 20). Our laboratory as well

as others have shown that the addition of protein to a carbohydrate (CHO+PRO) supplement can further enhance performance beyond that of carbohydrate (CHO) alone (21-23), while some have reported no differences (24, 25). Reduction in post-exercise muscle damage was also noted as an advantage of the addition of protein to a carbohydrate supplement (23, 26). However, all of these were cycling studies, and no one has yet investigated the effects of a carbohydrate-protein drink on fatigue and muscle damage during an intermittent high-intensity running protocol such as the LIST.

Therefore, the purpose of this study was to investigate if the addition of protein to a carbohydrate supplement (CHO+PRO) would reduce fatigue by affecting glycogen utilization during a simulated soccer match, as well as attenuate post-exercise muscle damage, when compared to a carbohydrate alone supplement (CHO). It was hypothesized that performance would be enhanced when consuming CHO+PRO in comparison to CHO due to sparing of intramuscular glycogen throughout the protocol.

METHODS

Participants

Two female and eight male trained soccer players completed the study. Their trained state was determined by relative VO_{2max} of at least 40 and 45 ml/kg/min for females and males, respectively. Subject characteristics including mean age, height and body mass are found in table 1. A detailed explanation of the experimental procedures and the potential risks of the study were given both verbally and in writing to all subjects prior to initial testing. Subjects were given the opportunity to ask questions before signing the informed consent, according to the protocol described in The University of Texas at Austin's "Institutional Review Board Procedures Manual for Faculty, Staff and Student Researchers with Human Participants". The University of Texas at Austin Institutional Review Board approved the study before it commenced.

Preliminary testing

Prior to beginning the study, each subject reported to the laboratory for determination of their maximal oxygen consumption (VO_{2max}). The VO_{2max} test was performed on treadmill, using the Fitness Institute of Texas (FIT) maximal treadmill ramped protocol. The protocol started at a speed of 1.7 mph and a grade of 6%, and both gradually increased every minute until fatigue. Subjects were breathing through a Hans Rudolph valve, with expired gases directed to a mixing chamber for analysis of oxygen (O_2) and carbon dioxide (CO_2) (ParvoMedics TrueOne 2400, ParvoMedics, Sandy, UT, USA). Outputs from this system were directed to a laboratory computer for calculation of

ventilation, O_2 consumption (VO_2), CO_2 production, and respiratory exchange ratio (RER) every 30 s. The criteria used to establish VO_2max was a plateau in VO_2 with increasing exercise intensity and RER > 1.10.

On the same day as the VO_{2max} tests, the subjects performed a practice trial to familiarize them with the laboratory environment and the experimental protocol to be used. The practice trials simulated the protocol, but were performed without blood samples or biopsies being taken. The practice trial was also used to adjust and/or verify appropriate running speeds for the experimental trials.

Experimental design

This study followed a randomized, double-blinded, repeated measures design. After initially completing a VO_{2max} test and familiarization trial, subjects performed two experimental trials in order to test the effects of a 2.8% protein + 7% carbohydrate (CHO+PRO) supplement compared to a 9.8% carbohydrate alone (CHO) supplement (Table 2). During each trial, a total of two supplements were provided. The first one 10 minutes before the beginning of the first half of the simulated soccer match, and the second one at the beginning of the half-time break. The rationale for this supplementation timing was to reproduce match conditions as closely as possible. In fact, soccer players can drink before the game and during the 15-minute half-time, but are usually unable to do so during the continuous playing periods. Supplement composition for each treatment are shown in table 2. For each of the two supplements given during a CHO+PRO trial, a volume that provides 0.5g of CHO and 0.2g of PRO per kg body weight were ingested. For example, a 70-kg subject was to ingest 500ml of the mixture at both time points,

which represents 35g CHO and 14g PRO each time. For the CHO trial, the same volume of an isocaloric 9.8% CHO drink was ingested, which represents 49g of CHO each time for the 70-kg subject. Both treatments contained the same amount of electrolytes. The order of testing was randomly assigned.

The subjects were asked to refrain from strenuous exercise for 2 days before each trial and record their diet for 2 days preceding the first trial. They were required to reproduce their activity and diet as closely as possible for the same period of time prior to the second trial. The two main trials were separated by at least 7 days.

Protocol

On the day of an experimental trial, the subjects reported to the laboratory 30 min before the start of exercise having fasted for 12 h. They were weighed and fitted with a heart rate monitor (Polar Beat, Polar Electro, Finland) secured in place around their chest. Subsequently, the participants underwent their first muscle biopsy and were then asked to ingest the first supplement.

Following consumption of the initial supplement, participants completed a standardized warm-up and stretching protocol and then started the experimental protocol exactly 15 minutes after ingestion on the first drink. Additional amounts of water ingested during this warm-up period and half-time were recorded. Equal quantities were provided during the second trial.

The exercise protocol is illustrated in Figure 1. Participants completed three 15-min blocks of the LIST for the first half, rested for a 15-min half time, and reproduced a second half. Each 15-min block of exercise during a half was separated by a 2-min rest

period. A second muscle biopsy was taken right after the second half. The biopsy site was closed using steristrips and secured with a pressure pack as well as a spandage sleeve. The subjects then performed their run to exhaustion in order to evaluate fatigue.

Each 15-min block consisted of a set pattern of intermittent high-intensity running that was designed to be similar to the activity pattern typically recorded for soccer match play(15). The participants were going back and forth at different intensities on a 20-m track. This pattern was repeated approximately 11 times per block and is as follows:

- 3 × 20 m at walking pace (13 seconds to complete)
- 1 × 20 m at maximal running speed
- 3 × 20 m at a running speed corresponding to approximately 55% of individual VO_{2max}
- 3 × 20 m at a running speed corresponding to approximately 95% of individual VO_{2max}

Using a free audio editor (Audacity 1.3.8), a recording was played during the protocol with a countdown from 3 followed by a "beep" to inform the participant each time a line must be reached. The audio track was matched to the individual's VO_{2max} in order to provide the appropriate jogging and running speeds.

Time to cover the 20 m at maximal running speed was measured using the Brower wireless timing system mounted with a reflective beam (Brower Timing Systems, USA). Average sprint time per 15-min block was calculated.

The original Borg Rating of Perceived Exertion Scale (RPE) was used to assess perceived exertion during exercise and was administered every 15 min during exercise (during the last walking stage of each 15-min block of the LIST). The scale ranges from 6 to 20, with anchors ranging from ‘‘very, very light’’ to ‘‘very, very hard.’’

Heart rate was monitored during the main trials by short-range telemetry using Polar heart rate monitor units and stored in memory mode. Average heart rate per 15-min block was calculated.

After blood collection and the last biopsy, the participants started the run to exhaustion (RTE) exactly 15 minutes from the end of the second half. It consisted of an open-ended period of intermittent shuttle running, designed to exhaust the participants within approximately 15 min. The participants were required to produce 2 runs (95% VO_{2max}) followed by a jog (55% VO_{2max}) on the same 20-m track, and reproduce this pattern continuously until they were unable to maintain the required speed (reach the line) for two consecutive shuttle runs.

On the day following each experimental trial, subjects were asked to report to the laboratory in order to obtain a 24h-post blood draw to evaluate muscle damage by measuring blood levels of creatine kinase. At the same time, they rated their overall muscle soreness from 1 to 10 (1 being no soreness at all and 10 too much pain to walk).

Blood sampling

Blood samples (8ml) were collected pre-exercise, at the beginning as well as at the end of the 15-min half-time, after the second half, following the run to exhaustion, and 24 hours post-trial. 0.3 ml of sample was transferred into a tube containing 0.6 ml of

10% perchloric acid (PCA). The remaining sample was divided into two tubes and mixed with 0.3 ml of EDTA (24mg·ml^{-1}, pH 7.4) to prevent coagulation. Tubes were centrifuged for 10 minutes at 3,000 rpm with a HS-4 rotor in a Sorvall RC6 centrifuge (Kendro Laboratory Products, Newtown, CT). Plasma and PCA extracts were separated into aliquots and all tubes were stored at -80°C until analysis.

Muscle biopsy

The thigh was cleansed with 10% betadine solution and then 1.8 ml of a local anesthesia (1% Lidocaine Hydrochloride Injection, Elkins-Sinn, Inc., Cherry Hill, NJ) was injected to prepare the leg for the muscle biopsy. A 5-8 mm incision was made through the skin and fascia, 2 inches from the midline of the thigh on the lateral side and 4 inches above the patella. Once the bleeding was stopped, the muscle biopsy was taken and pressure reapplied to the incision to stop bleeding. The biopsy was then trimmed of adipose and connective tissue and frozen in liquid nitrogen at -80° C for subsequent analysis. Once bleeding stoped, the incision was closed with steristrips and a Band-Aid and a pressure pack was affixed over the incision. A spandage sleeve was then wrapped around the thigh to support the pressure pack during running.

Blood analysis

Blood glucose concentrations were measured using a modified Trinder procedure at 37°C (27). Samples were read at 500 nm using a Beckman DU640 Spectrophotometer (Coulter, Fullerton, CA) and had a coefficient of variation (CV) of 3.7%.

Blood lactate concentrations were measured from the PCA extracts using enzymatic analysis according to Hohorst (28). The assay was run in duplicate and had a

CV of 1.2%. Samples were read at 340 nm using a Beckman DU640 Spectrophotometer (Coulter, Fullerton, CA).

Plasma insulin was analyzed via radioimmunoassay based on the principles of Goetz (Goetz et al., 1961) (MP Biomedicals ^{125} I RIA, Solon, OH, USA) and had a CV of 6.0%. Duplicate tubes were prepared and counted in a Wallac 1470 Wizard Gamma Counter (Wallac 1470 Wizard Gamma Counter, PerkinElmer Life and Analytical Sciences, Boston, MA), which had been calibrated for insulin ^{125} I.

Creatine kinase (CK) is an indirect marker of muscle damage. It was determined by enzymatic analysis (Diagnostic Chemicals Limited, Charlottetown, Canada). The conversion of creatine phosphate plus adenosine diphosphate to creatine plus adenosine triphosphate by creatine kinase is linked to several enzyme reactions to produce nicotinamide adenine diphesphopyridine nucleotide-reduced form (NADPH). The rate of NADPH is a measure of creatine kinase activity. The rate of NADPH was measured on a Beckman DU640 spectrophotometer (Beckman Bioanalytical Systems Group) at 30°C. The %CV for CK was 2.3.

Muscle glycogen

Intramuscular glycogen utilization throughout the simulated game was evaluated by subtracting glycogen levels at the end of the second half from the pre-exercise levels. Muscle glycogen was determined using muscle biopsies (~50 mg wet wt) taken from the vastus lateralis. Glycogen content was determined by enzymatic degradation with amyloglucosidase in a modified method of Passonneau and Lauderdale (29). The muscle sample was weighed, digested in 1N KOH while incubated at 70°C for 20 minutes,

mixed, then incubated for an additional 10 minutes. One hundred µl of homogenate was added to 250 ml of 0.3 M sodium acetate (pH 4.8) then mixed. Ten µl of 50% glacial acetic acid and 250 ml of sodium acetate (containing 10 mg/ml amyloglucosidase, pH 4.8) were then added to the tubes. Tubes were sealed and incubated overnight at room temperature. The glucose reagent was prepared using a Raichem Glucose Color Reagent Kit (Hemagen Diagnostics, San Diego, CA). One hundred µl of muscle homogenate solution and 1.5 ml of reagent were added to clean tubes then incubated for 10 minutes at 37°C. Samples were read with a Beckman DU640 Spectrophotometer (Coulter, Fullerton, CA) at 500 nm.

Metabolic regulation

Using the muscle tissue samples obtained from the muscle biopsies, we measured the phosphorylation state of the protein FOXO3. This protein is a transcription factor that leads to muscle atrophy, and is therefore used as an indicator of muscle damage. As FOXO3 is dephosphorylated, it can move from the cell's cytoplasm into the nucleus, where it promotes muscle atrophy.

Muscle samples were weighed and homogenized in ice-cold buffer, containing 20 mM Hepes, 2 mM EGTA, 50 mM sodium flouride, 100 mM potassium chloride, 0.2 mM EDTA, 50 mM glycerophosphate, 1mM DTT, 0.1 mM PMST, 1 mM benzamidine, and 0.5 mM sodium vanadate (pH 7.4). Homogenization was performed on ice using Caframo RZR1 Stirrer (Caframo Limited, Wiarton, Ontario, Canada). The homogenate was centrifuged at 14,000 g for 10 minutes at 4°C and the supernate was then aliquoted to

several test tubes and stored at -80°C for later analysis. A modified version of the Lowry assay (30) was used to determine protein concentration.

Equal amounts of muscle proteins (80 µg) were separated by gel electrophoresis, using a Sodium Dodecyl Sulphate (SDS)-Page, 10% resolving gel. The proteins were then transferred to special blotting paper, Polyvinylidene fluoride (PVDF) membranes and blotted in freshly prepared TBS containing 5% nonfat dry milk and 0.06% Tris-Buffered Saline Tween-20 (TBST-MLK) for 1 hour at room temperature with agitation. The PVDF membranes were then incubated with a primary antibody in fresh 5% BSA-TBS + 0.06% Tween-20 over night with gentle agitation at 4°C. A rabbit anti-phospho-FoxO3a (Ser318/321) antibody from Cell Signaling was used (1:500 dilution; Cell Signaling Technology, Inc., Beverly, MA). After the membranes were washed in 0.06% Tris-Buffered Saline (TBS) solution, they were incubated with a secondary reagent for 1h40min at room temperature with agitation. An anti-rabbit IgC HRP-linked antibody (Cell Signaling Technology, Inc., Beverly, MA) was used at a dilution of 1:1500. The PVDF membranes were then washed in 0.06% TBS solution, and the anti-body-bound proteins were visualized by means of Western Lightning Chemiluminescence Reagent Plus (PerkinElmer LAS, INC., Boston, MA) according to manufacturer's protocol. Images were scanned using Adobe Photoshop and quantified using Scion Image (Scion Corporation, Frederick, Maryland. All samples were run with a standard from rat muscle and the phosphorylation states of our subject's samples were measured as % of standard. The protein alpha-tubulin was also measured as a control for equal loading.

Statistical Analysis

Data were analyzed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL). Run time to exhaustion (RTE) and 24 hour-post muscle soreness were analyzed using a one-way analysis of variance (ANOVA). Glycogen utilization was analyzed using a one-way ANOVA, with glycogen difference from pre to post as the dependent variable and the pre measures as a covariate in order to control for the variability of these pre measures. All other variables were measured using a two-way (treatment × time) repeated measures ANOVA. Significance was determined at $p < 0.05$. Data were expressed as mean ± SE.

RESULTS

Time to exhaustion

Run times to exhaustion for both treatments are shown in figure 2. With the CHO treatment, subjects ran for 489 ± 121 seconds, while they ran for 589 ± 186 seconds on the CHO+PRO treatment. No significant difference was found between treatments.

Sprint times

Average sprint times over the 6 exercise block are shown in figure 3. For both groups, average sprint times significantly increased over time ($p < 0.05$) but no significant difference was found between treatments. The average sprint time over the entire protocol was 3.25 ± 0.02 seconds for CHO and 3.22 ± 0.02 for CHO+PRO.

Muscle glycogen

Changes in intramuscular glycogen levels from pre-exercise to the end of the second half are shown in figure 4. Glycogen levels significantly decreased in both groups ($p < 0.05$) but no significant difference was found between treatments.

RPE and heart rate

Changes in RPE and heart rate over the entire exercise protocol are shown in figure 5 and 6, respectively. A significant time effect was observed for both variables ($p < 0.05$) but again it was not the case for treatment × time. The average RPE over the entire protocol was 15.1 ± 0.7 and 14.7 ± 0.6 for CHO and CHO+PRO, respectively. The average heart rate was 174.6 ± 1.7 and 174.9 ± 1.3 beats per minute (bpm) for CHO and CHO+PRO, respectively.

Glucose, insulin, and lactate

Changes in glucose, insulin, and lactate over the entire protocol are shown for both groups in figure 7, 8, and 9, respectively. None of these variables showed significant treatment differences. Plasma glucose peaked at the end of half-time for both treatments, to reach 129 ± 8 and 142 ± 7 mg/dL for CHO and CHO+PRO, respectively.

Plasma insulin also peaked at the end of half-time, to reach 60.4 ± 5.0 and 65.0 ± 10.2 pmol/L for CHO and CHO+PRO, respectively.

Blood lactate levels reached their highest levels at the end of the run to exhaustion, with values of 5.68 ± 0.7 and 5.83 ± 0.7 mmol/L for CHO and CHO+PRO, respectively.

CK, FOXO3 and muscle soreness

CK levels were measured pre-exercise, at the end of the run to exhaustion, and 24 hours post-exercise. Changes in absolute values are found in figure 10. CK levels significantly increased over time for both treatments ($p < 0.05$) but there was no treatment × time effect. At 24 hours post-exercise, CK levels were 951.9 ± 254.8 and 896.4 ± 239.0 U/L for CHO and CHO+PRO, respectively.

Phosphorylation of FOXO3 was not significantly affected over the course of the match in both treatments. See figure 11.

Subjects rated their overall muscle soreness from 1 to 10 (1 being no soreness at all and 10 representing extreme soreness) 24 hours after each trial. No significant difference between treatments was found as CHO reported soreness of 5.4 ± 0.3 and CHO+PRO of 5.2 ± 0.4.

DISCUSSION

The primary purpose of this study was to determine if the addition of protein to a carbohydrate supplement could further enhance the performance improvements observed when carbohydrate alone was provided during intermittent high-intensity running. It is now widely recognized that carbohydrate supplementation during exercise improves aerobic performance, and several studies have suggested that CHO supplementation during a simulated soccer match may improve endurance and performance. The addition of protein with CHO has been found to be of additional benefit when provided during prolonged endurance exercise (21-23, 31). In the present study, we sought to compare the effects of a CHO and a CHO+PRO supplement on endurance performance during a simulated soccer match. To keep the conditions as realistic as possible, subjects only received supplements before the start of the match and at half-time.

No significant differences between the CHO and CHO+PRO treatments were found for our main measures which were run time to exhaustion and glycogen utilization. When on the CHO treatment, subjects ran for 489 ± 1 seconds, while they lasted 589 ± 2 seconds before reaching exhaustion on the CHO+PRO treatment. This similarity in times, even though slightly longer in CHO+PRO, is in accordance with the glycogen levels at the beginning of the run to exhaustion. Indeed, subjects finished the second half of the simulated soccer match and started the run to exhaustion with 76.7 ± 4 μmol·g wet wt^{-1} during the CHO trial and 78.0 ± 6 μmol·g wet wt^{-1} on the CHO+PRO trial. No muscle biopsies were taken after the run to exhaustion, which makes it impossible to claim that muscle glycogen depletion was responsible for the occurrence of fatigue. However, the

similar glycogen levels prior to the run and the similar times to reach exhaustion suggest a correlation. Considering the high-intensity at which the subjects were running, it is possible that selective depletion of certain muscle fibers caused the inability to further maintain the high running speeds. This rational was previously suggested by Krustrup et al. (3) who performed histochemical analysis of individual muscle fibers after a soccer match. It was found that total glycogen concentration at the end of the game was reduced to 30-70 μmol·g wet wt^{-1} but that half of the individual muscle fibers of both types were completely or almost depleted for glycogen. They concluded that even if glycogen was still available, such a depletion of glycogen in some fibers is responsible for fatigue when high-intensity performance is required.

This is the first study that compared CHO and CHO+PRO supplements on an intermittent high-intensity running protocol. However, different cycling studies have reported contradictory results regarding the effects of the protein addition. Some investigators have found that supplementation with CHO+PRO during exercise improves endurance performance in comparison with CHO only supplements (21-23). In our laboratory, we have observed a 36% improvement in cycling time to exhaustion when subjects consumed a CHO+PRO supplement during prolonged exercise of varying intensity (21). In addition, Saunders et al. (22) reported a 29 and 40% performance improvement for subjects cycling at 75% as well as at 85% VO_{2max} to fatigue, respectively. In a subsequent study, Saunders et al. (23) looked at the efficacy of a CHO+PRO gel and again found a performance benefit of 13% during a ride to exhaustion at 75% VO_{2max} when compared to a CHO-only gel. In these studies, the CHO+PRO

supplements had a 20-25% higher caloric content than the CHO-only supplement since the PRO was added, but the CHO content was the same in both drinks. Therefore, it was suggested that the benefits observed with the CHO+PRO supplements were simply due to a higher caloric content rather than a specific protein-mediated mechanism. In this regard, several studies found no performance improvements when a CHO+PRO supplement was compared to an isocaloric CHO-only supplement (25, 26).

Van Essen and Gibala (25) found no difference in 80-km cycling time trial performance when comparing a 6% CHO + 2% PRO drink with a 6% CHO-only drink. However, they provided carbohydrate at a rate of 60 $g \cdot h^{-1}$, suggesting that when CHO is ingested at levels that approach the optimal rate of CHO oxidation of 60-70 $g \cdot h^{-1}$, the addition of PRO to a CHO supplement does not further enhance performance. While this might be true, 60 g $CHO \cdot h^{-1}$ is a significant amount of CHO to ingest during exercise. Some individuals who are concerned with their caloric intake in regards to their body composition might be reticent to such a nutritional supplement. Carbohydrate overload can also be a problem in this situation. Indeed, two subjects reported gastro-intestinal discomfort during the CHO treatment in the present study. For a 70-kg subject, each CHO drink contained 50g of CHO, which indeed might be practically too much to ingest during exercise. Recent studies might have found a solution to this problem.

In fact, the debate over a protein-dependent mechanism as a performance enhancer during exercise recently received new arguments. Martinez et al. (32) found no differences in time to fatigue when participants ingested a CHO-only supplement or CHO+PRO, despite a lower carbohydrate and total caloric content in the CHO+PRO

drink. The author suggested that the efficacy of a sports drink containing less calories can be maintained with the addition of a small amount of protein. More interestingly, Ferguson-Stegall et al. (31) recently showed that a supplement containing a mixture of carbohydrates plus a moderate amount of protein improves aerobic endurance by 28.7%, despite containing 50% less total carbohydrate and 30% fewer calories relative to a higher carbohydrate beverage. Some methodological differences between Ferguson-Stegall's study and ours might explain why we did not observe performance improvements. First, the Ferguson-Stegall (31) investigation took into account the relative intensity at which the subjects were exercising, recognizing that a CHO+PRO supplement was beneficial when cycling at or below the ventilator threshold. Considering that our participants were running at intensities alternating from 55 to 95% VO_{2max}, it is possible that the addition of protein might only match, rather than improve, the benefits of a CHO-only supplement for this type of exercise. Second, and unlike the study by Ferguson-Stegall (31), a single carbohydrate (dextrose) rather than a mixture of different carbohydrates was used in the present study. Previous investigation has demonstrated that, compared to a single-CHO supplement, those containing a mixture of carbohydrates can increase exogenous CHO oxidation and decrease endogenous CHO oxidation (33). Finally, frequency of supplementation in our study might have caused these contradictory results. Where Ferguson-Stegall provided supplements every 20 minutes, our participants only ingested two drinks, nearly separated by one hour. Nonetheless, this study shows that replacement of CHO by PRO maintains the performance benefits observed with a CHO-only supplement during a simulated soccer match, and further research should

examine if this replacement allows a reduction of the total caloric content of the drink by reducing the CHO component.

Sparing of muscle glycogen has been proposed as a mechanism responsible for the performance improvements. Indeed, CHO supplementation alone has been found to spare intramuscular glycogen stores during variable intensity exercise (20). During an actual soccer match, Leatt and Jacobs (5) examined players who were given either 500 ml glucose polymer solution or placebo 10 min pre-game and at half-time. Glycogen reduction was greater in the placebo group than in those subjects given the glucose polymer, demonstrating that glucose ingestion decreased the net muscle glycogen utilization during the game (5). Most interestingly, Nicholas et al. (10) found that the amount of muscle glycogen utilized during the LIST protocol was reduced by 22% when a carbohydrate-electrolyte solution was consumed immediately before and at frequent intervals during exercise. Moreover, it appears that a CHO+PRO beverage can accelerate glycogen resynthesis post-exercise when compared to a CHO-only supplement (34, 35). Considering that low-intensity exercise accounts for about 55% of total exercise during the LIST (15), and our modification of the protocol to add a 15-minute half-time to create real-match conditions, it seemed possible that glycogen resynthesis could occur at a greater rate with the CHO+PRO supplement during those low-intensity and resting periods, especially in type II fibers which are totally at rest during the walking and jogging sections of the protocol. However, we were not able to observe this phenomenon as the CHO+PRO group used slightly less muscle glycogen but not to a significant extent. Therefore, it seems that the short time allowed for glycogen resynthesis

throughout the game and the intermittence at which it might occur do not allow the addition of PRO to a CHO supplement to cause a greater sparing of muscle glycogen during intermittent high-intensity exercise when supplementing infrequently.

In order to confirm that glycogen sparing still happened in both treatments, we compared our results with those of Nicholas et al. (10), who found glycogen sparing during the LIST with CHO supplementation, compared to a placebo. Nichloas et al. (10) reported that total glycogen utilization over the 90 minutes of exercise was significantly lower during the CHO (38.5 ± 5.3 μmol·g wet wt^{-1}) compared with the placebo trial (49.1 ± 4.6 μmol·g wet wt^{-1}). In the present study, total glycogen utilization was 37.9 ± 7.6 μmol·g wet wt^{-1} during the CHO and 29.1 ± 6.0 μmol·g wet wt^{-1} during the CHO+PRO. These results would suggest that intramuscular glycogen sparing occurred in both treatments during our investigation. Moreover, it is important to note that our protocol was slightly modified by adding a 15-minute half-time and by only providing two supplements, one before the first half and the other at half-time. While this completely reproduces real match conditions, Nicholas et al. (10) was providing supplements every 15 minutes throughout the entire simulated match, which would be impossible during an actual soccer game. Therefore, our study confirms and assures that CHO or CHO+PRO supplements have the potential to spare muscle glycogen during a soccer match.

Another benefit of CHO+PRO supplementation has also proposed regarding reduction of exercise-induced muscle damage. Creatine kinase (CK), LDH, and myoglobin are usually used to indirectly assess muscle damage from blood samples. Saunders et al. (22) compared isocarbohydrate treatments and reported that CPK values

at 12-15 hours post-exercise were 83% lower after the CHO+PRO trial than during the CHO-only trial. Romano-Ely et al. (26) compared isocaloric supplements and reported that LDH (9%) and CK (53%) postexercise levels at 24 hours were significantly lower in the CHO+PRO trial compared with the CHO-only trial. Others have used a resistance exercise model to demonstrate a significant reduction in myoglobin levels 6 hours after a strenuous resistance exercise bout when subjects ingested a CHO+PRO supplement compared to a placebo. Recently, Valentine et al. (36) showed that a CHO+PRO beverage, along with reducing post-exercise muscle damage, could improve muscle function when compared with an isocaloric and an isocarbohydrate supplement. Compared with the CHO-only trials, subjects on the CHO+PRO treatment performed significantly more knee extensions at 70% 1-repetition maximum 24 hours after the experimental trial. The authors suggested that the replacement of some CHO by PRO could induce a mechanism by which the integrity of the muscle tissue was better maintained than when a CHO-only supplement was ingested.

In the present study, pre-exercise, post-second half, and 24 hours post-exercise CK levels were measured, along with a subjective rating of overall muscle soreness by the subjects 24 hours after each experimental trial. No significant differences were found for these variables. These findings might be due to the fact that supplementations were given pre and half-way through exercise but no recovery supplements were provided. In fact, once the trial was over, subjects were free to leave and no restrictions were placed on their subsequent eating. Therefore, it is a limitation of this study that should be considered regarding such exercise-recovery data. The analysis of the phosphorylation

state of the protein FOXO3a is in accordance with the CK values regarding muscle damage. In fact, this protein is responsible for promoting muscle atrophy when it is dephosphorylated and therefore muscle damage can be estimated from this measurement. Similarly to CK, no differences between treatments were observed for phospho-FOXO3a.

Finally, it has been proposed that the ability of a CHO+PRO supplement to increase performance above a traditional CHO supplement may be related to the central fatigue hypothesis (21, 37). In short, this hypothesis is based on the regulation of arousal, mood, motivation and fatigue in humans by brain 5-hydroxytryptamine (5-HT, serotonin) and its precursor free tryptophan (Trp). Trp and many other amino acids share the same blood transporter as plasma free fatty acids. As duration of exercise increases, free fatty acids concentration in the blood rises, resulting in more competition for their common transporter. This leads to higher levels of free Trp potentially crossing the blood brain barrier, increasing serotonin production, and creating feelings of fatigue (38, 39). CHO supplementation during exercise decreases levels of plasma free fatty acids and free Trp (40). Using the soccer simulating LIST protocol, Backhouse et al. (12) found that CHO ingestion during intermittent high-intensity exercise appears to elicit an enhanced perceived activation profile that may impact upon task persistence and performance. Branched-chain amino acids supplementation during exercise was shown to decrease mental fatigue (37), and improve endurance performance (41). We theorized that the CHO+PRO might prevent a greater rise in serotonin production through decreased free Trp levels during exercise in comparison to CHO supplementation alone, allowing individuals to exercise longer without the sensation of exhaustion. However, once again

the slightly lower RPE and quicker average sprints over the entire simulated match while on the CHO+PRO treatment were not significantly different from the CHO-only treatment. Methodological differences such as the amino acid content of the beverages and the exercise protocol might explain the contradictory results.

In summary, the CHO+PRO supplement was not found to improve performance or spare muscle glycogen to a greater extent than the CHO-only supplement. However, it appeared that every single measurement, from sprint time and run to exhaustion to RPE, CK, and glycogen utilization, showed better results with the CHO+PRO treatment, but not to a significant extent. While this should not be regarded as a scientific finding, it suggests that more participants and a greater power should be used in future studies. In addition, further investigation regarding the potential of PRO addition to reduce CHO and caloric intake during soccer while maintaining the performance benefits, is warranted.

REFERENCES

1. Mohr M, Krustrup P, Bangsbo J. Match performance of high-standard soccer players with special reference to development of fatigue. *Journal of Sports Sciences* 2003;21:519-528.
2. Bangsbo J. The physiology of soccer--with special reference to intense intermittent exercise. *Acta Physiol Scand Suppl* 1994;619:1-155.
3. Krustrup P, Mohr M, Steensberg A, Bencke J, Kjaer M, Bangsbo J. Muscle and blood metabolites during a soccer game: Implications for sprint performance. *Med Sci Sports Exerc* 2006;38(6):1165-1174.
4. Mohr M, Krustrup P, Bangsbo J. Fatigue in soccer: A brief review. *Journal of Sports Sciences* 2005;23:593-599.
5. Leatt PB, Jacobs I. Effect of glucose polymer ingestion on glycogen depletion during a soccer match. *Can J Sport Sci* 1989;14(2):112-116.
6. Ostojic SM, Mazic S. Effects of a carbohydrate-electrolyte drink on specific soccer tests and performance. *Journal of Sports Science and Medicine* 2002;1(2):47-53.
7. Guerra I, Chaves R, Barros T, Tirapegui J. The influence of fluid ingestion on performance of soccer players during a match. *Journal of Sports Science and Medicine* 2004;3(4):198-202.
8. Muckle DS. Glucose syrup ingestion and team performance in soccer. *Br J Sports Med* 1973;7(3-4):340-343.
9. Kirkendall DT. Effects of nutrition on performance in soccer. *Medicine & Science in Sports & Exercise* 1993;25(12):1370-1374.
10. Nicholas CW, Tsintzas K, Boobis L, Williams C. Carbohydrate-electrolyte ingestion during intermittent high-intensity running. *Med Sci Sports Exerc* 1999;31(9):1280-1286.

11. Welsh RS, Davis JM, Burke JR, Williams HG. Carbohydrates and physical/mental performance during intermittent exercise to fatigue. *Med Sci Sports Exerc* 2002;34(4):723-731.

12. S. H. Backhouse, A. Ali, S. J. H. Biddle, C. Williams. Carbohydrate ingestion during prolonged high-intensity intermittent exercise: Impact on affect and perceived exertion. *Scandinavian Journal of Medicine & Science in Sports* 2007;17(5):605-610.

13. Foskett A, Williams C, Boobis L, Tsintzas K. Carbohydrate availability and muscle energy metabolism during intermittent running. *Med Sci Sports Exerc* 2008;40(1):96-103.

14. Nicholas CW, Williams C, Lakomy HKA, Phillips G, Nowitz A. Influence of ingesting a carbohydrate-electrolyte solution on endurance capacity during intermittent, high-intensity shuttle running. *Journal of Sports Sciences* 1995;13(4):283 - 290.

15. Nicholas CW, Nuttall FE, Williams C. The loughborough intermittent shuttle test: A field test that simulates the activity pattern of soccer. *Journal of Sports Sciences* 2000;18(2):97.

16. Bangsbo J, Iaia FM, Krustrup P. Metabolic response and fatigue in soccer. *Int J Sports Physiol Perform* 2007;2(2):111-127.

17. Balsom PD, Gaitanos GC, Söderlund K, Ekblom B. High-intensity exercise and muscle glycogen availability in humans. *Acta Physiologica Scandinavica* 1999;165(4):337-345.

18. Reilly T. Energetics of high-intensity exercise (soccer) with particular reference to fatigue. *Journal of Sports Sciences* 1997;15:257-263.

19. Hargreaves M, Costill DL, Coggan A, Fink WJ, Nishibata I. Effect of carbohydrate feedings on muscle glycogen utilization and exercise performance. *Medicine & Science in Sports & Exercise* 1984;16(3):219-222.

20. Yaspelkis BB, Patterson JG, Anderla PA, Ding Z, Ivy JL. Carbohydrate supplementation spares muscle glycogen during variable-intensity exercise. *J Appl Physiol* 1993;75(4):1477-1485.

21. Ivy JL, Res PT, Sprague RC, Widzer MO. Effect of a carbohydrate-protein supplement on endurance performance during exercise of varying intensity. *Int J Sport Nutr Exerc Metab* 2003;13:382-395.

22. Saunders MJ, Kane MD, Todd MK. Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Medicine & Science in Sports & Exercise* 2004;36(7):1233-1238.

23. Saunders MJ, Luden ND, Herrick JE. Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents post-exercise muscle damage. *Journal of Strength & Conditioning Research* 2007;21(3):678-684.

24. Osterberg KL, Zachwieja JJ, Smith JW. Carbohydrate and carbohydrate plus protein for cycling time-trial performance. *Journal of Sports Sciences* 2008;26(3):227 - 233.

25. Van Essen M, Gibala MJ. Failure of protein to improve time trial performance when added to a sports drink. *Medicine & Science in Sports & Exercise* 2006;38(8):1476-1483.

26. Romano-Ely BC, Todd MK, Saunders MJ, St.Laurent T. Effect of an isocaloric carbohydrate-protein-antioxidant drink on cycling performance. *Medicine & Science in Sports & Exercise* 2006;38(9):1608-1616.

27. Trinder P. Determination of blood glucose using 4-amino phenazone as oxygen acceptor. *J Clin Pathol* 1969;22(2):246.

28. Hohorst H. Determination of l-lactate with ldh and dpn. *New York: Academic* 1965:266-270.

29. Passonneau J, Lauderdale V. A comparison of three methods of glycogen measurement in tissue. *Anal Biochem* 1974;60:405-412.

30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193(1):265-275.

31. Ferguson-Stegall L, McCleave EL, Ding Z, Kammer LM, Wang B, Doerner PG, Liu Y, Ivy JL. The effect of a low carbohydrate beverage with added protein on cycling

endurance performance in trained athletes. *Journal of Strength & Conditioning Research*;24(10):2577-2586.

32. Martinez-Lagunas V, Ding Z, Bernard JR, Wang B, Ivy JL. Added protein maintains efficacy of a low-carbohydrate sports drink. *Journal of Strength & Conditioning Research* 2010;24(1):48-59.

33. Jentjens RLPG, Achten J, Jeukendrup AE. High oxidation rates from combined carbohydrates ingested during exercise. *Medicine & Science in Sports & Exercise* 2004;36(9):1551-1558.

34. Ivy JL, Goforth HW, Jr., Damon BM, McCauley TR, Parsons EC, Price TB. Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *J Appl Physiol* 2002;93(4):1337-1344.

35. Zawadzki KM, Yaspelkis BB, 3rd, Ivy JL. Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *J Appl Physiol* 1992;72(5):1854-1859.

36. Valentine R, Saunders M, Todd M, St. Laurent T. Influence of carbohydrate-protein beverage on cycling endurance and indices of muscle disruption. *Int J Sports Nutr Exerc Metab* 2008;18:363-378.

37. Blomstrand E, Hassmen P, Newsholme E. Effect of branched-chain amino-acid supplementation on mental performance. *Acta Physiol Scand* 1991;143:225-226.

38. Struder H, Hollman W, Platen P, Donike M, Gotzmann A, Weber K. Influence of paroxetine, branched-chain amino acids and tyrosine on neuroendocrine system responses and fatigue in humans. *Horm Metab Res* 1998;30:188-194.

39. Wilson W, Maughan R. Evidence for a possible role of 5-hydroxytryptamine in the genesis of fatigue in man - administration of paroxetine, a 5-ht reuptake inhibitor, reduces the capacity to perform prolonged exercise. *Exp Physiol* 1992;77:921-924.

40. Blomstrand E. A role for branched-chain amino acids in reducing central fatigue. *J Nutr* 2006;136(2):544S-547.

41. Blomstrand E, Hassmen P, Ekblom B, Newsholme E. Admininstration of branched-chain amino-acids during sustained exercise - effects of performance and

plasma concentration of some amino acids. *Eur J Appl Physiol Occup Physiol* 1991;63:83-88.

TABLE 1: Subject Characteristics (n=10)

Measure	Mean
Age (years)	22.2 ± 0.73
Height (cm)	177 ± 2.1
Body Mass (kg)	71.7 ± 3.1
VO2max (L/min)	3.74 ± 0.24
VO2max (ml/kg/min)	51.9 ± 2.2

Data presented as mean ± SE

TABLE 2: Supplement composition

	CHO	CHO+PRO
% CHO	9.8	7
% PRO	0	2.8
Ratio of CHO:PRO	-	2.5 : 1
CHO g/kg BW	0.7	0.5
PRO g/kg BW	0	0.2
Kcals/kg BW	2.8	2.8
Electrolytes (mg/100ml):		
Magnesium	12.7	12.7
Sodium	35.2	35.2
Sulfate	5.6	5.6
Chloride	109.9	109.9
Potassium	36.6	36.6

Per drink. Two drinks were given during the protocol, one pre-exercise and one at the beginning of half-time. Both treatments contained the same amounts of electrolytes.

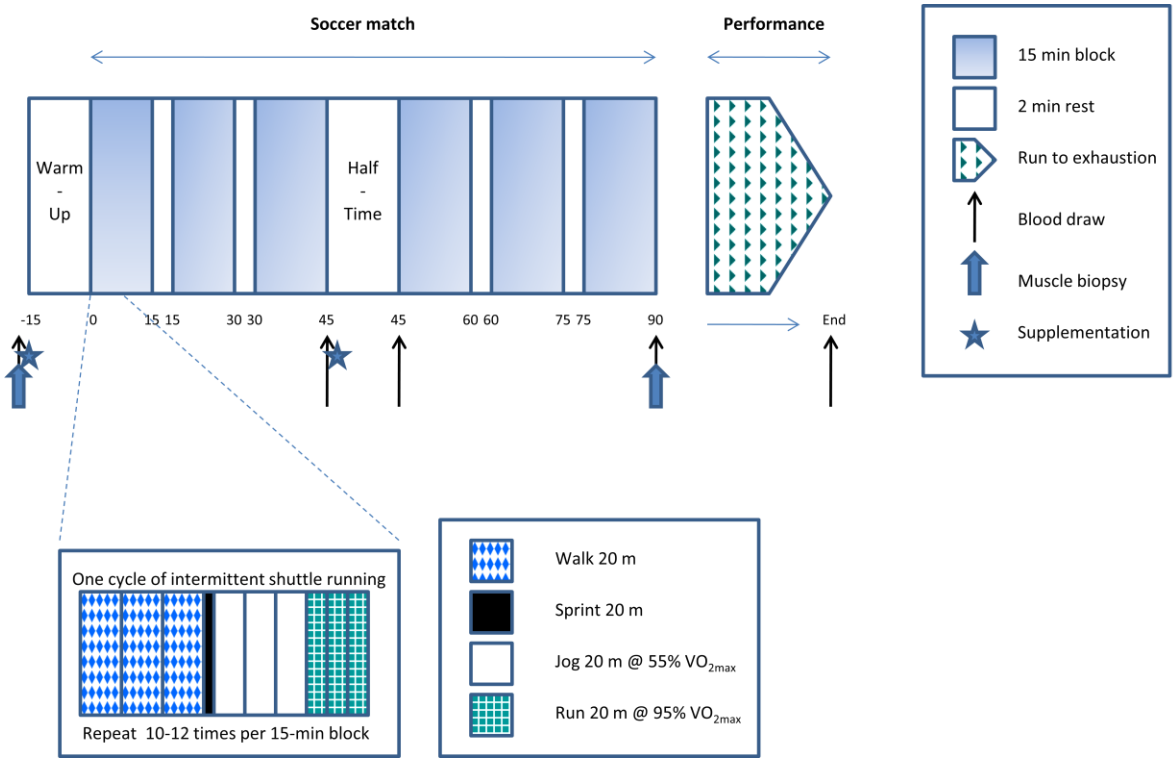


Figure 1: Exercise protocol (modified LIST)

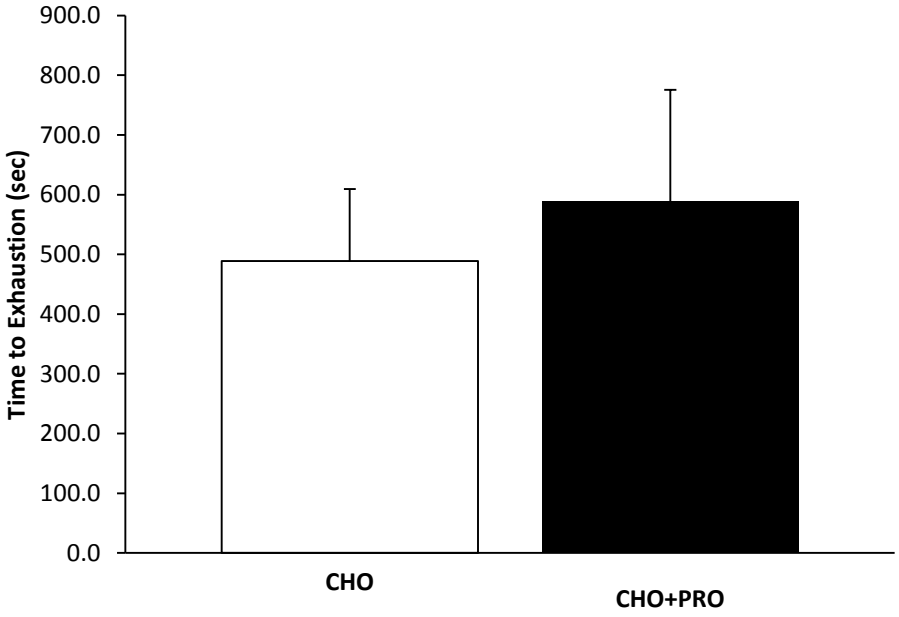


Figure 2: Run time to exhaustion (RTE). Subjects were considered exhausted when unable to maintain the required speed for two consecutive shuttle runs. No significant difference between treatments.

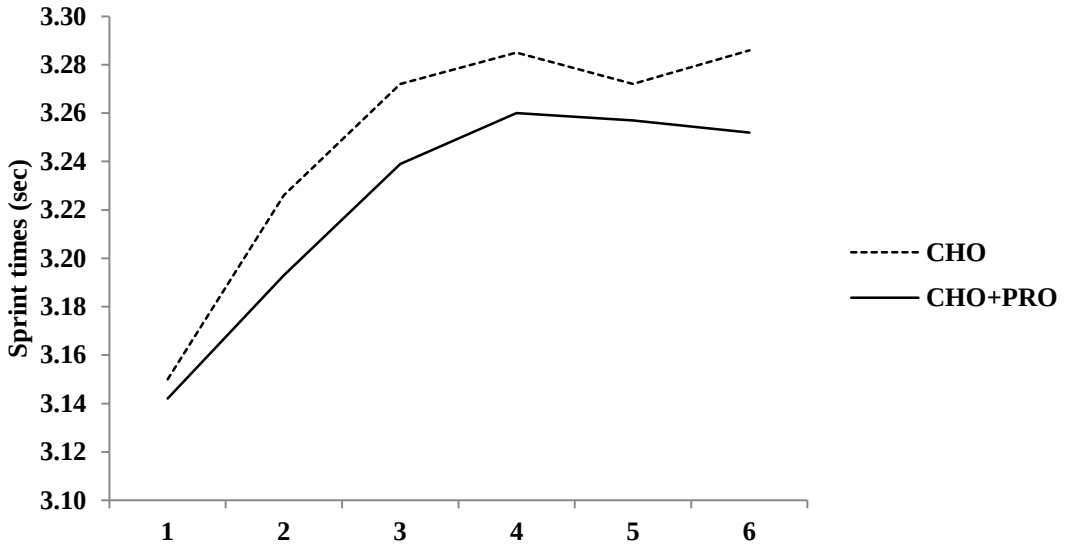


Figure 3: Changes in average sprint times over the 6 exercise blocks of the simulated soccer match. A significant time effect was observed as sprint times increased throughout the protocol ($p < .05$). No significant difference between treatments.

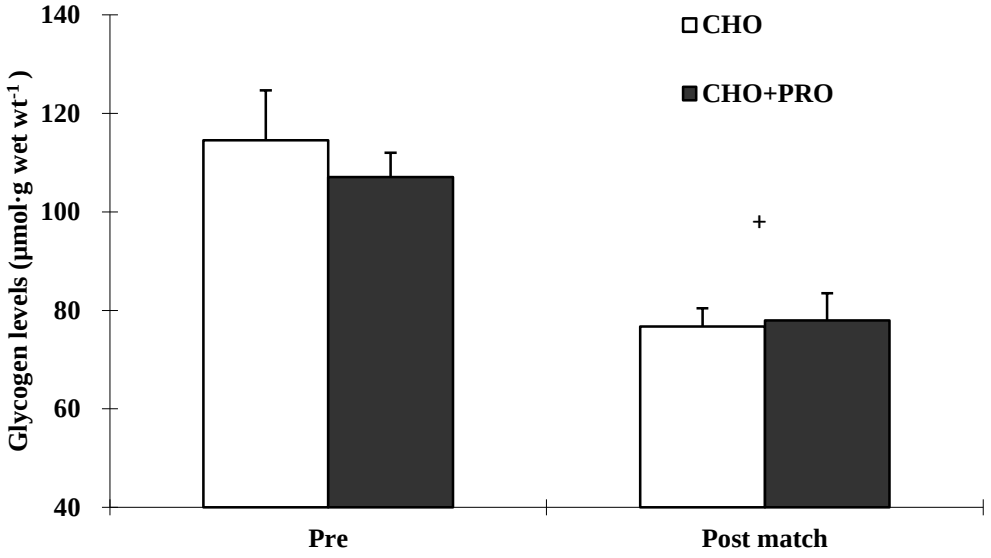


Figure 4: Intramuscular glycogen levels at pre-exercise (Pre) and end of second half (Post match). $^{+}$ A significant time effect was observed in both treatments as glycogen levels decreased from Pre to Post ($p < .05$). There was no treatment × time effect.

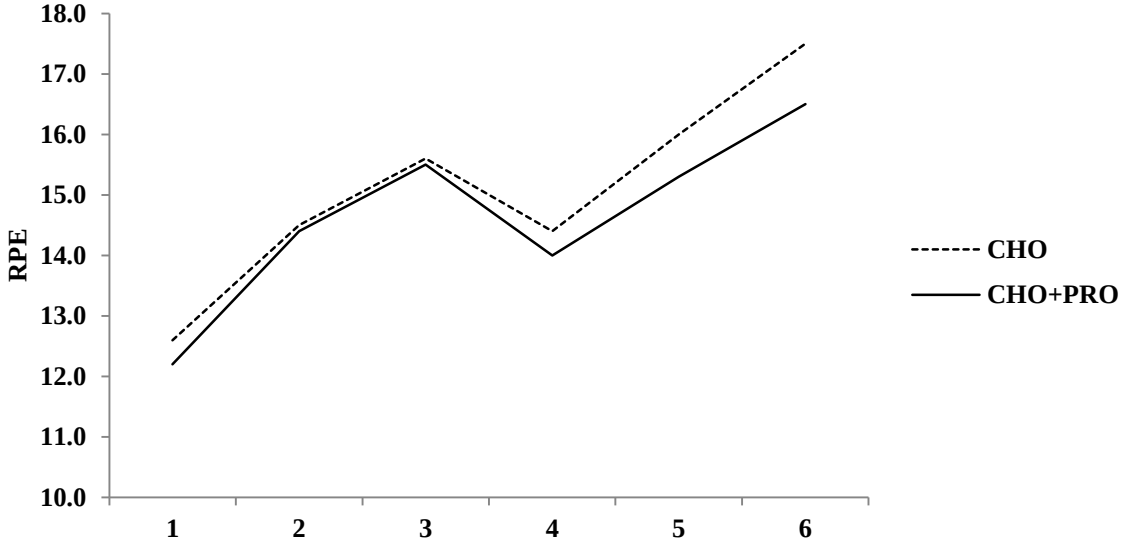


Figure 5: Changes in ratings of perceived exertion (RPE) over the 6 exercise blocks of the simulated soccer match. No significant difference between treatments.

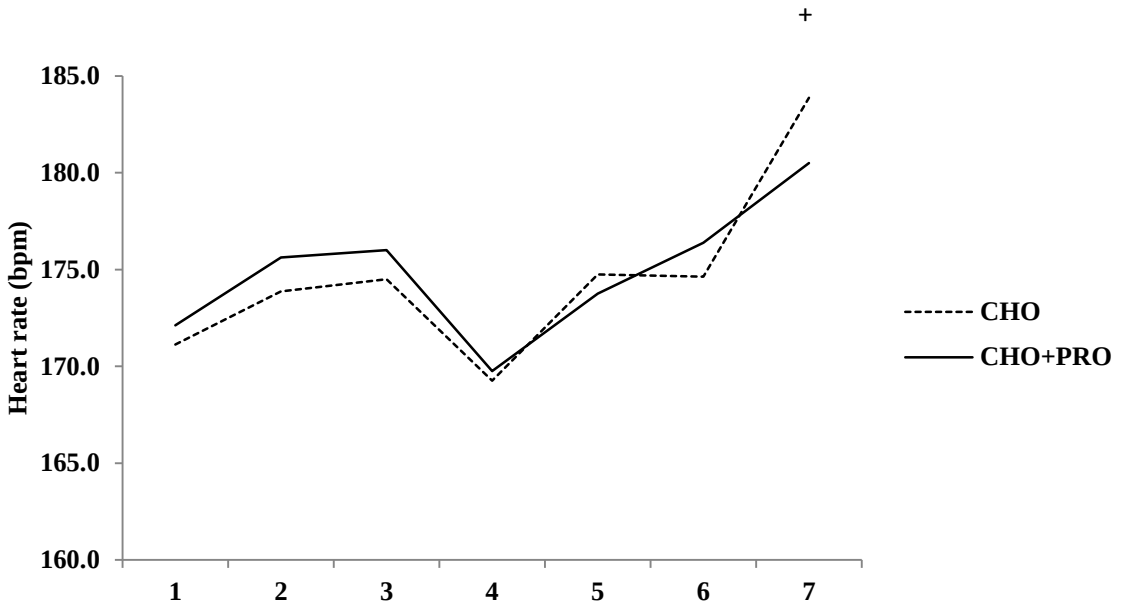


Figure 6: Changes in average heart rate (bpm) over the 6 exercise block of the simulated soccer match and during the run to exhaustion (7). A significant time effect was observed ($p < .05$). + Significantly increased from 1 to 7.

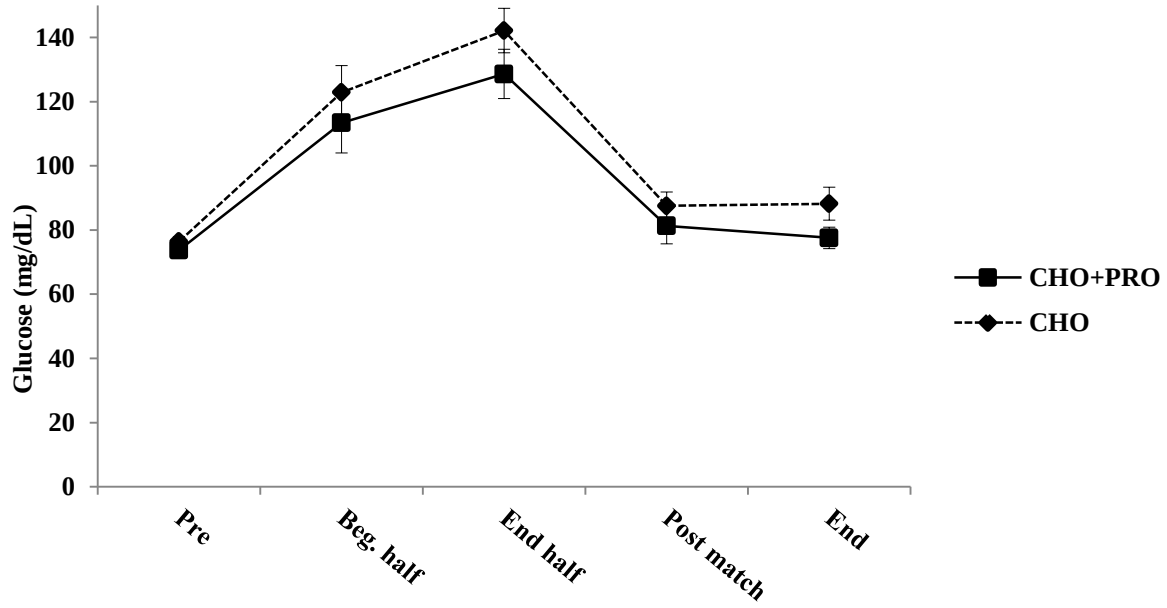


Figure 7: Blood glucose levels at pre-exercise (Pre), beginning of half-time (Bag. Half), end of half-time (End half), end of second half (Post match), and at exhaustion (End). No significant difference between treatments.

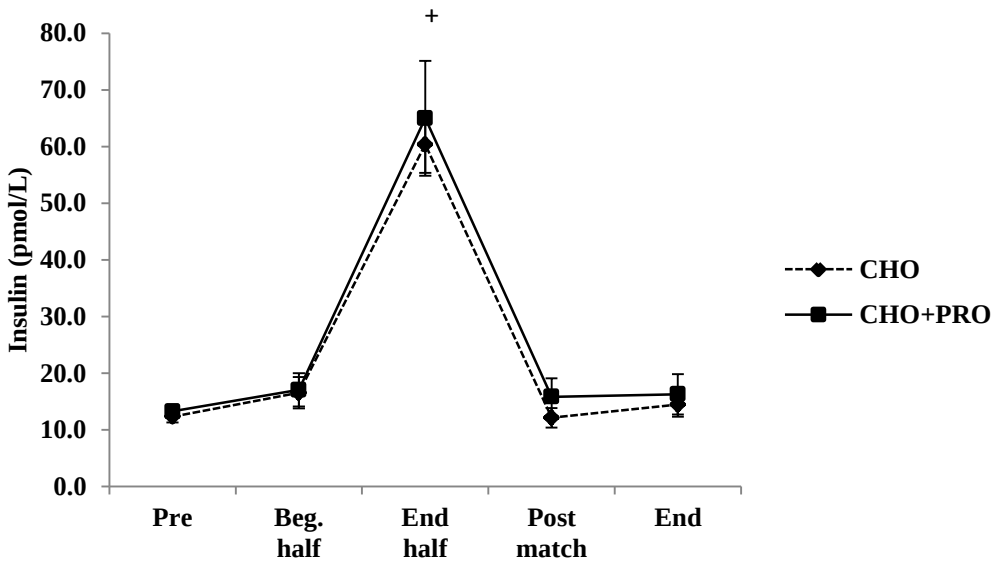


Figure 8: Plasma insulin levels at pre-exercise (Pre), beginning of half-time (Bag. Half), end of half-time (End half), end of second half (Post match), and at exhaustion (End). In both treatments, insulin levels significantly rose during half time ($p < .05$).

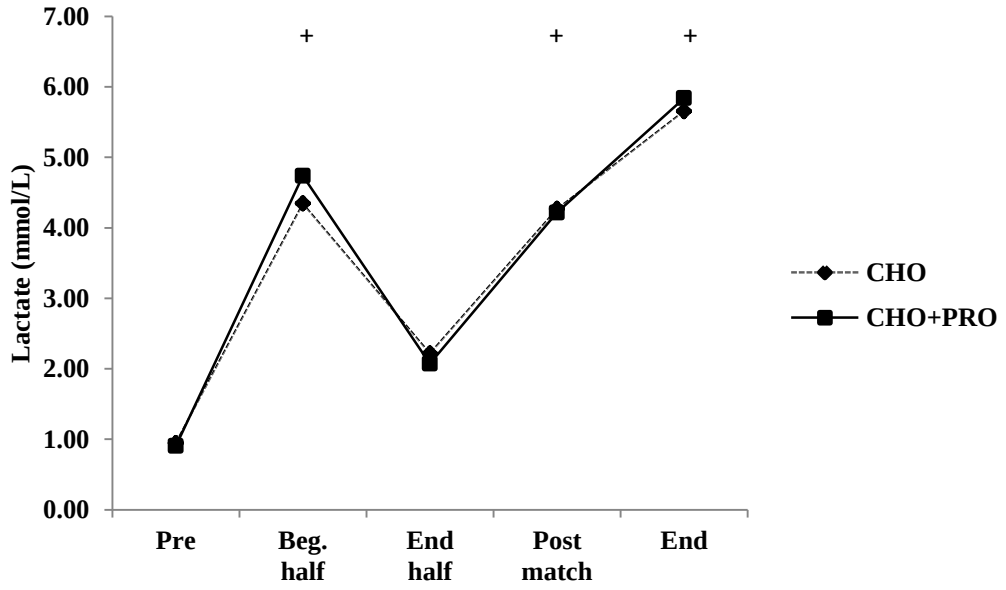


Figure 9: Plasma insulin levels at pre-exercise (Pre), beginning of half-time (Bag. Half), end of half-time (End half), end of second half (Post match), and at exhaustion (End). In both treatments, lactate levels significantly rose during the exercise periods from pre-exercise and the end of half-time, to peak at the end of the run to exhaustion ($p < .05$).

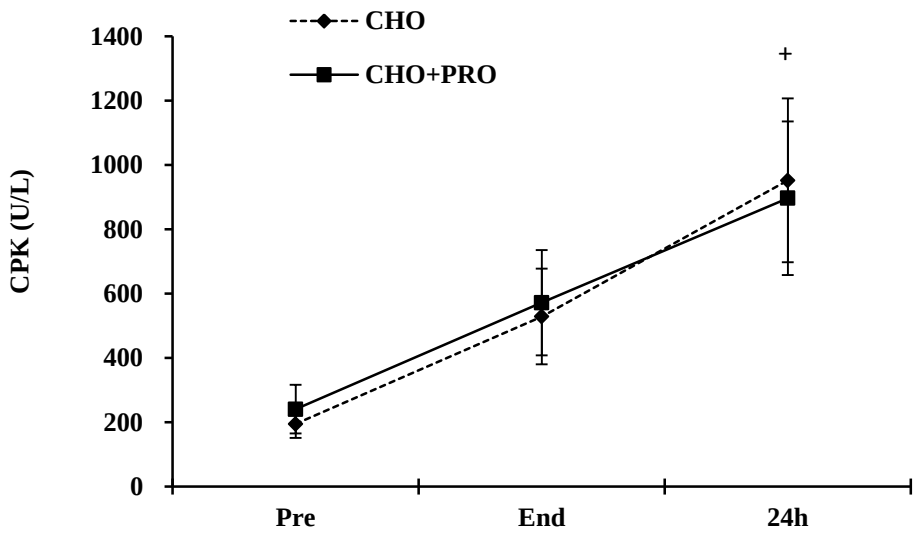
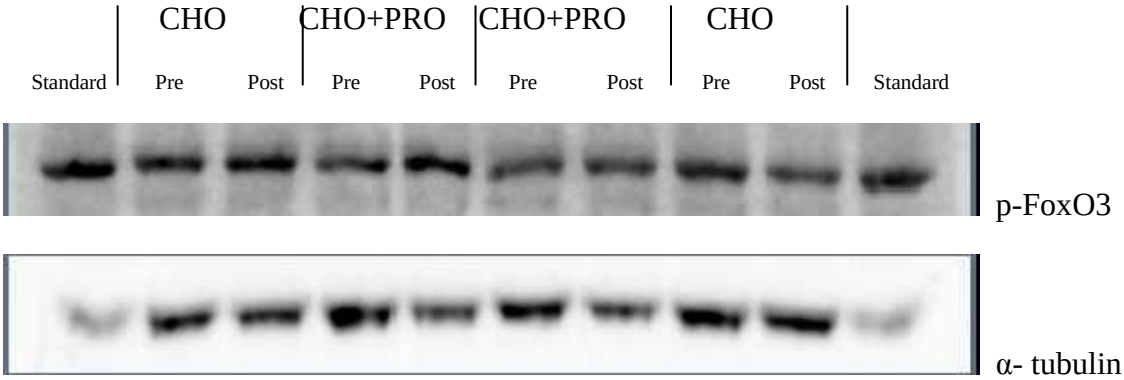


Figure 10: Plasma CPK levels at pre-exercise (Pre), end of RTE (End), and 24 hours post-exercise (24h). A significant time effect was observed ($p < .05$). + Significantly different from PRE to END.



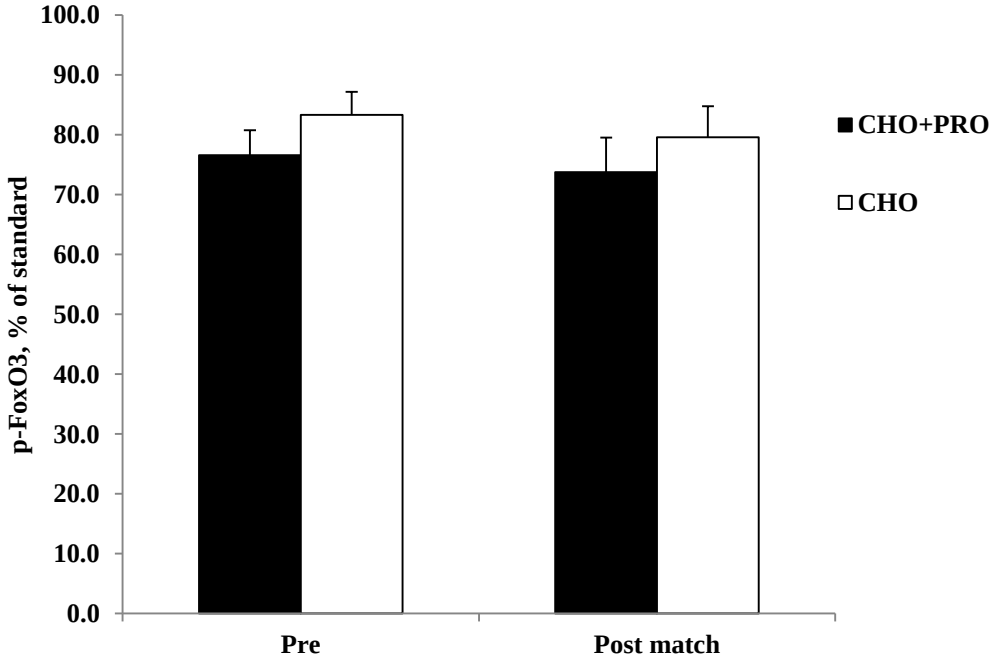


Figure 11: Phosphorylation of FOXO3 as % of standard.

REVIEW OF LITERATURE

Introduction

Soccer is the most popular sport worldwide. Millions of people regularly go to football stadiums to follow their favorite teams, while billions more watch the game on television or on the internet. According to a survey conducted by FIFA published in 2001, over 240 million people from more than 200 countries regularly play soccer (1). As soccer has the highest global television audience in sport (2), professional players are constantly required to perform at their best level, despite an usually heavy schedule. As a result, an increased awareness of the importance of nutritional supplementation and a demand for nutritional products among both professionals and recreational athletes has developed. Over the past decade, many researchers have focused on the optimization of aerobic endurance performance and the reduction of fatigue through nutritional strategies to support this interest.

Soccer is a complex team sport in which many factors can affect a player's performance. Fatigue during a match can occur for different reasons, temporarily following short intense bouts of exercise such as sprints, or towards the end of the game when the body is unable to provide sufficient fuel to the working muscle. New technologies such as time-motion analysis have increased our understanding of the physical demands placed on soccer players during a 90-minute match. As a result,

researchers have been able to comprehend the physiological responses and mechanisms that govern a player's performance during a match. It is now widely recognized that nutritional supplementation during exercise, in particular carbohydrates, will delay the onset of fatigue (3-7) and improve a team's results (8)

Recently, the addition of protein to carbohydrate (CHO+PRO) has been proposed to increase the ergogenic effect of a traditional carbohydrate-only (CHO) supplement. Several investigators have found supplementation with CHO+PRO during exercise to improve endurance performance in comparison to CHO (9-11). However, these studies were done on cycling subjects, but no one has yet explored the potential of CHO+PRO supplementation on soccer players.

Following a description of the physical and physiological demands placed on a soccer player during a match, this literature review will investigate the potential of nutritional strategies in improving performance. The benefits of CHO supplementation prior to and during a game will be reviewed, as well as the underlying mechanisms responsible for this ergogenic effect. The new research on CHO+PRO supplementation will also be presented, followed by its possible applications to soccer.

Soccer characteristics, metabolic responses, and the development of fatigue

***Soccer**:*

The typical distance covered by top-class outfield soccer players during a match is 10-13km and most of this distance is covered by walking and low-intensity running (12). Soccer is considered an intermittent type of exercise as players change activity every 4-6 s. Game-related energy demands not only include running but other activities such as dribbling, passing, shooting, tackling and heading(13). Mean and peak heart rates observed during matches are around 85 and 98% of HRmax(14). Taken into account dehydration, hyperthermia, and mental stress that can elevate heart rate without affecting oxygen uptake during a match, heart rate measurements during a game seem to suggest that the average oxygen uptake is around 70% VO_{2max} (12). Core temperatures of 39-40°C also suggest average aerobic loading around 70% VO_{2max} (15).

Elite soccer players perform 150-250 brief intense actions during a game (16). These cause high rates of creatine phosphate breakdown, which resynthesizes during the following low-intensity periods. They also cause high rates of muscle lactate production, leading to blood lactate concentrations of moderate to high levels (average blood lactate concentrations of 2 to 10 $mmol.l^{-1}$ (12, 17)), with individual values recorded above 12mM.

***Substrate utilization**:*

In order to optimize nutritional strategies and enhance performance of a soccer player, it is important to understand substrate utilization and energy demands during a game. During high intensity exercise, such as when sprinting, creatine phosphate and muscle glycogen are the preferred sources of fuel. The role of plasma free fatty acids as a fuel source appears to increase as the game goes on.

Blood concentration of free fatty acids (FFA) increases during the game, especially during the second half (18). Low insulin and progressively high catecholamine concentrations stimulate a high rate of lipolysis, and significant blood flow to adipose tissue during low-intensity exercise allows for the increase in blood concentrations of FFA. Therefore higher uptake and oxidation of blood FFA may occur in the exercising muscles, especially during the recovery periods of the game(19). Mostly during second half and toward the end of the game, an increase in blood catecholamine levels might also lead to higher utilization of muscle triglycerides. An increase in plasma FFA and muscle triglyceride utilization throughout the game compensate for the progressive lowering of the main substrate: muscle glycogen.

Saltin observed that the muscle glycogen stores were almost depleted at halftime when the prematch values were low (45 mmol/kg wet weight). For the players with normal initial levels (100 mmol/kg wet weight), values were still rather high at halftime but below 10 mmol/kg wet weight at the end of the game (20). However, others have found concentrations ranging from 40 to 65 mmol/kg wet weight after a game (18, 21),

indicating that muscle glycogen levels are not always depleted in a soccer game. Krustrup et al. (18) reported muscle glycogen concentrations at the end of a game to be reduced to 30-70 mmol/kg wet weight, indicating glycogen availability. However, histochemical analysis of single muscle fibers showed that 47% of the individual fibers of both types I and II were completely or almost depleted of glycogen at the end of the game. The authors concluded that while some glycogen was still available, selective depletion of certain muscle fibers was responsible for fatigue when high-intensity performance such as sprints were required. To summarize, the intermittent, high-intensity characteristics of a soccer match leads to significant glycogen utilization and possible depletion towards the end of the game, with partial compensating increases in plasma FFA and muscle triglyceride oxidation. However, with significant glycogen reduction soccer performance is compromised.

Fatigue:

During a soccer match, fatigue can occur at 3 different stages: (1) after short-term intense periods in both halves; (2) in the initial phase of the second half; and (3) towards the end of the game (22). We will focus on fatigue as it develops towards the end of the game.

Studies have shown that the amount of sprinting, high-intensity running and distance covered are lower in the second half when compared to the first half of a game (13, 16). Particularly, reduced ability to perform high-intensity exercise towards the end

of games (16, 18, 22) and reduction in the amount of high-intensity running in the last 15 minutes of a top-class soccer game (16, 23) were observed. A review of the possible causes of this fatigue is provided below.

An increase in muscle lactate and a subsequent lowering of muscle pH has been studied as a possible cause of fatigue. Krustrup et al. found a weak relationship between reduction in performance and muscle lactate (18) but the changes in muscle lactate were moderate, and it has been shown that accumulation of lactate does not cause fatigue (24, 25). Low muscle pH has also been suggested as a cause for fatigue(26) but values were found to be only moderately reduced during a game (>6.8)(27) and no relationship with lowered performance has been observed (18). Therefore, high muscle lactate and acidosis are not likely to be responsible for the fatigue observed towards the end of a game.

Muscle ATP and IMP have also been studied during a soccer match (18). Muscle ATP was only moderately reduced (15%) during the game, with a corresponding accumulation of muscle IMP considerably lower than during exhaustive exercise(28). Therefore, fatigue is not likely to be due to the inability of the contracting muscle fibers to maintain ATP levels either.

Hypoglycemia is unlikely to be responsible for fatigue during a soccer game as blood glucose does not reach critical values (13, 29). Dehydration and hyperthermia have also been proposed as factors causing fatigue in the later stages of a soccer game(30). Indeed, players can lose more than 3 liters of fluid during a game played in a normal thermal environment (13, 30). Hoffman et al. have shown that loss in body mass of only

1-2% can contribute to elevation in core temperature as well as cardiovascular strain (31). However, along with average core temperatures ranging from 39.0 to 39.5°C, Mohr et al. found no differences in core temperatures between the end of the first and the second halves(15). Therefore, unless a game takes place in a hot and humid environment, dehydration and hyperthermia will not be key factors in the development of fatigue towards the end of the game.

Blood samples taken during soccer games have shown that blood lactate concentration declines towards the end of the game, along with an increase in plasma free fatty acids (13). This trend reflects the reduced exercise intensity and the previously mentioned change in substrate utilization in the later stages of a game (22), most likely induced by low muscle glycogen together with elevated concentrations of catecholamine (13). The main cause of fatigue remains unclear but reduction of muscle glycogen stores towards the end of the game has been associated with fatigue during prolonged intermittent exercise. In fact, dietary manipulations to lower muscle glycogen have caused development of fatigue during long-term intermittent exercise (29, 32). Conversely, elevating muscle glycogen before exercise through a carbohydrate diet enhances high-intensity exercise performance (29, 33). Some investigations (3, 18, 21) found that glycogen levels during a soccer match would not decrease below the required value to maintain a maximal glycolytic rate (50mmol/kg wet weight). For example, Krustrup et al. (18) found muscle glycogen concentrations between 40 and 60 mmol/kg wet weight at the end of a game, indicating glycogen availability. However, as mentioned

earlier, histochemical analysis revealed that about half the individual muscle fibers of both types were almost or completely depleted of glycogen. In addition, this reduction was associated with a decrease in sprint performance immediately after the game (18). Therefore, fatigue towards the end of a soccer game could be due to depletion of glycogen in some but not all muscle fibers, preventing maximal output during sprints and other high-intensity activities.

Nutritional strategies:

The main cause of fatigue observed in soccer players towards the end of a game seems to be low glycogen levels in selective muscle fibers. Indeed, it appears that an important number of muscle fibers are almost or completely depleted of glycogen at the end of the game. Therefore, strategies to prevent low glycogen levels must be used to improve performance and increase the amount of sprinting, high-intensity running and distance covered in the later stages of a soccer game. Two strategies have been used: optimizing glycogen levels prior to and after the game and carbohydrate supplementation during the game.

CHO supplementation prior to and post-exercise

Many have focused on developing nutritional strategies such as a high-carbohydrate diet in order to optimize glycogen stores prior to a competitive event. Some

have tried to promote fuel availability and enhance glycogen storage by nutritional practices on the day of the event (34), but it appears that efforts to restore or even super-compensate muscle glycogen content must commence in the 24 – 48 h before a game. In the study of Balsom et al. (29), participants followed 48 h of either a high or low-carbohydrate diet before short-term (<10 min) and prolonged (>30min) protocols of intermittent exercise, which consisted of 6-s bouts of high-intensity exercise performed at 30-s intervals on a cycle ergometer. Muscle glycogen concentrations were reduced by at least 50% in the low-carbohydrate trial compared to the high-carbohydrate trial, and were associated with a dramatic reduction in the work performed in both exercise protocols. Elite atheltes' playing schedules are very busy and recovery between games can be as short as two days; therefore researchers have investigated the effects of a high-carbohydrate diet during the recovery period. Akermark et al. (35) found that elite ice hockey players who ''carbohydrate-loaded'' (8.4 g/kg/day) during the 3 day recovery between two games were able to skate for longer distances and at higher intensities than when their normal diet plan (6.2 g/kg/day) was followed. Muscle glycogen concentrations were reduced after the first game for all players (43mmol/kg wet weight), but restoration levels were 45% higher in the carbohydrate-loaded players before the next game (99 vs. 81mmol/ kg wet weight). During the second game, distance skated, number of shifts skated, amount of time skated within shifts, and skating speed were all increased in the carbohydrate-loaded players compared with the control group, with the differences being most marked in the third period. Overall, it is clear that glycogen recovery in between soccer matches is a key component to optimize a player's performance during

consecutive games. However, only a few studies have specifically investigated dietary carbohydrate needs to achieve optimal refueling of soccer players. Results from early studies suggested that soccer players were unable to fully replenish their muscle glycogen stores during the 48h after a match, with an increase in glycogen concentrations during the first 24h but no further replenishment during the second 24h recovery period (21).Even though muscle damage due to the nature of a soccer match was used as a possible explanation, it should be noted that the glycogen concentrations reported in this study were abnormally low in comparison to other values usually found for well-trained athletes, and are in contrast with more recent research that suggests well-trained muscle can recover or even super-compensate glycogen stores within 24-36h post-exercise (36).

The International Olympic Committee Consensus on Nutrition for Athletes discussed daily carbohydrate guidelines to optimize muscle glycogen storage and were summarized by Burke et al. (37). A key factor in post-exercise glycogen refueling is the amount of carbohydrate consumed, which should match the fuel needs of the athlete's training and competitive schedule. Depending on a player's energy demands in function of his mobile role in the team as well as his personal schedule, a reasonable target range for carbohydrate intake is 5-7g/kg/day for less active players and 7-10g/kg/day for more active players during an intense training schedule (37). Along with the amount of carbohydrate, the timing of intake and type of carbohydrate are crucial components for optimal refueling (38). Carbohydrates with a moderate or high glycemic index (GI) seem to be more efficient than low-GI choices in promoting glycogen synthesis post-exercise

(39). Timing is also very important as the highest rates of glycogen synthesis occur during the first hour post-exercise. During this period, glucose delivery and enzyme activity within the muscle are enhanced to produce an insulin-independent followed by an insulin-dependent phase. Carbohydrate intake immediately post exercise results in greater glycogen synthesis rates (7.7μmol/g wet weight) than if carbohydrate intake occurs 2h post-exercise (4.1 μmol/g wet weight) (40). Rapid glycogen replenishment during the first hours following strenuous exercise may be achieved by a total carbohydrate intake of approximately 1.0-1.2g/kg/hr (41).

CHO+PRO supplementation post-exercise

The effects of adding protein to carbohydrate ingestion on glycogen resynthesis has been debated (37). Probably due to differences in experimental designs, some have found increased glycogen storage when protein is added to a carbohydrate feeding (42-45), and others observed no differences between treatments (46-50). Zawadzki et al. (42) was the first to study the question and found that the rate of muscle glycogen storage was 38% greater when protein had been added in the recovery supplement. However, it could not be established if this was solely due to the protein addition since the CHO+PRO treatment contained more calories and it is known that the energy content of a supplement influences the rate of muscle glycogen storage post-exercise (47). In a study by van Loon et al. (44), a CHO supplement was compared to a CHO+PRO matched for carbohydrate content and to a high CHO supplement that was isoenergetic to the CHO+PRO. It was

established that the rate of muscle glycogen synthesis during recovery from exhaustive cycling can be increased with equal effect whether protein or additional carbohydrate are added to an existing solution that provides moderate amounts of CHO (≤ 0.8g/kg/h). More recent evidence also suggests that the addition of protein to a moderate amount of carbohydrate similarly increases post-exercise glycogen resynthesis (43, 45). Other studies such as the one by van Hall et al. (49) and Howarth et al. (50) assessed if the maximal rate of glycogen resynthesis observed in responses to ingesting ~ 1.2g/kg/h CHO could be exceeded when adding protein. Despite some reporting a significant increase in plasma insulin with the CHO+PRO treatment (49), none found the rate of glycogen resynthesis to be increased with the added protein. However, it is important to note that in these studies (49, 50), participants were provided supplements every 15 minutes, whereas investigations finding a benefit to the protein addition were supplementing at least 1h apart (42, 43, 45). Taking into consideration the differences between these studies, it seems that co-ingestion of protein with carbohydrate will increase the efficiency of glycogen storage when feeding intervals are more than 1h apart or when the amount of carbohydrate ingested is below the threshold for maximal glycogen synthesis (37). After all, it appears that the key effect of a CHO+PRO supplement is the enhanced muscle glycogen synthesis rate during the first hour of recovery (43). Indeed, Ivy et al. observed that of the total glycogen utilized during exercise, the amount recovered in the first 40 min was 22% for a CHO+PRO treatment but only 11.5 and 5.5% for the high (isolcaloric) and low (isocarbohydrate) carbohydrate treatments, respectively. This represents rates of glycogen resynthesis that are twice and

four times greater with the CHO+PRO feeding than with high and low carbohydrate feedings. These findings are very interesting as they suggest possible applications for intermittent high-intensity types of sports with short recovery periods, such as soccer.

CHO supplementation during exercise

While some have focused on glycogen recovery and supercompensation, others have investigated carbohydrate ingestion during a soccer match as another way to reduce fatigue and enhance performance (3-5, 8, 51). Results indicated that carbohydrate ingestion during the game allowed the players to perform more sprints (5) and to cover a greater distance in the second half (51). Also, Muckle (8) found that the ingestion of glucose syrup prior to the game improved both team and individual performance as assessed by goals scored and conceded, as well as the total number of scoring efforts and ball-contact. In a study by Ostojic et al., supplementation with a carbohydrate-electrolyte solution improved soccer-specific skill performance and recovery after an on-field soccer match play, compared with ingestion of a placebo (4). Finally, Leatt and Jacobs examined players who were given either 500 ml glucose polymer solution or placebo 10 min pre-game and at half-time. Glycogen reduction was greater in the placebo group than in those subjects given the glucose polymer, demonstrating that glucose ingestion decreased the net muscle glycogen utilization during the game (3).

More recently, various well-controlled laboratory studies involving intermittent high-intensity exercise have been used to observe the effects of carbohydrate ingestion on protocols simulating a soccer match (6, 7, 52-54). The Loughborough Intermittent Shuttle Test (LIST) was designed as a controlled field test to simulate the activity patterns observed during a game of soccer (55). Throughout the 90 minutes of exercise, participants are required to run between two lines, 20-m apart, at various speeds related to individual VO_{2max}, and then perform a run to fatigue. It was found that ingesting carbohydrates before and every 15 min during exercise improves endurance running capacity during prolonged intermittent exercise as it took longer for the subjects to run to exhaustion (7, 52, 54). Also, Backhouse et al. found that in addition to the physiological and metabolic benefits of the energy supply, carbohydrate ingestion during prolonged high-intensity exercise appears to elicit an enhanced perceived activation profile that may improve the athlete's performance, especially towards the end of the game (53). The most interesting finding was by Nicholas et al. who found that the amount of glycogen utilized during prolonged, intermittent, high-intensity exercise was reduced by 22% when a carbohydrate-electrolyte solution was consumed immediately before and at frequent intervals during exercise (6). In fact, it was reported that total glycogen utilization over the 90 minutes of exercise was significantly lower during the CHO (38.5 ± 5.3 μmol·g wet wt^{-1}) compared with the placebo trial (49.1 ± 4.6 μmol·g wet wt^{-1}) (6). Others had also observed this apparent sparing of muscle glycogen with a carbohydrate-electrolyte solution during intermittent exercise (56, 57).

Suggestions have been made to explain this decreased utilization of muscle glycogen. First, direct utilization of the ingested carbohydrate could spare the endogenous store of muscle glycogen. Indeed, higher serum insulin and blood glucose concentrations could facilitate an increased glucose uptake by the exercising muscle (56), and reduce the contribution of intramuscular glycogen stores to energy production (58). The alternative explanation for the glycogen sparing is that glycogen resynthesis may occur during the periods of low intensity, especially in the type II muscle fibers. Indeed, low-intensity exercise accounts for about 55% of total exercise duration during the LIST (6). Some have previously reported that the elevated blood glucose and serum insulin levels caused by the carbohydrate ingestion would facilitate glycogen resynthesis in type II fibers (59, 60). Therefore, ingesting carbohydrates before the game and at half time should facilitate glycogen resynthesis during the low-intensity periods of the game, as well as during the half-time rest, especially in the non-active type II muscle fibers. This would result in a reduced net glycogen use over the 90 min of exercise and improved performance towards the end of the game.

CHO+PRO supplementation during exercise

Unlike the ergogenic effects of CHO supplementation that have been studied extensively, the addition of PRO to a sports drink only recently received attention. The investigations that looked at the effects of a CHO+PRO supplement during exercise have only been cycling studies, but no one has yet explored the potential of CHO+PRO on

running or soccer performance. Therefore, this section will review the existing literature on CHO+PRO and cycling performance, further suggesting applications for the soccer player.

Several studies have demonstrated that the addition of PRO to CHO improves endurance performance as compared to CHO-only (9-11, 61). However, contradicting results have also been found (62-64). In the first study to demonstrate the benefits of the PRO addition (9), subjects cycled for three hours at intensities varying between 45% and 75% $VO_{2\ max}$, followed by a performance ride to exhaustion at 85% $VO_{2\ max}$. Every 20 minutes throughout the ride, subjects received 200 ml of either a placebo, a 7.75% CHO, or a 7.75% CHO + 1.94% PRO supplement. The CHO+PRO treatment significantly improved time to exhaustion above CHO-only (26.9 and 19.7 min, respectively). The addition of 2% PRO therefore led to a 36% improvement in performance. In addition, Saunders et al. (10) reported a 29 and 40% performance improvement for subjects cycling at 75% as well as at 85% VO_{2max} to fatigue, respectively. In a subsequent study, Saunders et al. (11) looked at the efficacy of a CHO+PRO gel and again found a performance benefit of 13% during a ride to exhaustion at 75% VO_{2max} when compared to a CHO-only gel. In these studies, the CHO+PRO supplements had a 20-25% higher caloric content than the CHO-only supplement since the PRO was added, but the CHO content was the same in both drinks. Therefore, it was suggested that the benefits observed with the CHO+PRO supplements were simply due to a higher caloric content rather than a specific protein-mediated mechanism. In this regard, several studies found

no performance improvements when a CHO+PRO supplement was compared to an isocaloric CHO-only supplement (63, 64).

Van Essen and Gibala (64) found no difference in 80-km cycling time trial performance when comparing a 6% CHO + 2% PRO drink with a 6% CHO-only drink. However, they provided carbohydrate at a rate of 60 $g \cdot h^{-1}$, suggesting that when CHO is ingested at levels that approach the optimal rate of CHO oxidation of 60-70 $g \cdot h^{-1}$, the addition of PRO to a CHO supplement does not further enhance performance. While this might be true, 60 g $CHO \cdot h^{-1}$ is a significant amount of CHO to ingest during exercise. Some individuals who are concern with their caloric intake in regards to their body composition might be reticent to such a nutritional supplement. Carbohydrate overload can also be a problem in this situation. Recent studies might have found a solution to this problem.

In fact, the debate over a protein-dependent mechanism as a performance enhancer during exercise recently received new arguments. Martinez et al. found no differences in time to fatigue when participants ingested a CHO-only supplement or CHO+PRO, despite a lower carbohydrate and total caloric content in the CHO+PRO drink. The author suggested that the efficacy of a sports drink containing less calories can be maintained with the addition of a small amount of protein. More interestingly, Ferguson-Stegall et al. (61) recently showed that a supplement containing a mixture of carbohydrates plus a moderate amount of protein improves aerobic endurance by 28.7%, despite containing 50% less total carbohydrate and 30% fewer calories relative to a

higher carbohydrate beverage. These findings suggest that the addition of protein could be used to reduce the carbohydrate and caloric contents of a sport drink, while maintaining its ergogenic effect.

Mechanisms responsible for CHO+PRO performance improvements

It is still not clearly understood how the addition of PRO improves endurance performance, but a few mechanisms have been suggested. Sparing of muscle glycogen, maintenance of Krebs Cycle intermediates, reduction in exercise induced muscle damage, and maintenance of plasma amino acids (AA) in regard to the central fatigue hypothesis will be discussed.

Supplying CHO+PRO during exercise may enhance performance via its ability to decrease the reliance on endogenous CHO stores. Ivy et al. (9) proposed that a sparing, or more efficient use of muscle glycogen, may have resulted in the 36% improvement in time to exhaustion with CHO+PRO in comparison to the isocarbohydrate CHO treatment. However, the authors could not make direct conclusions regarding the issue since no muscle biopsies were taken. A strong argument for the glycogen sparing ability of CHO+PRO is that it was found that the PRO addition to a CHO supplement further enhances the insulin response (42, 65). In accordance with this finding, it was demonstrated that a CHO+PRO treatment increases blood glucose uptake into the muscle as compared to a CHO-only supplement. In a study conducted by Levenhagen et al. (66),

subjects consumed one of three treatments immediately after cycling for 60 min at 60% $VO_{2\ max}$. Supplements were composed of 8g CHO, 10g PRO + 8g CHO, or a placebo. CHO alone did not significantly increase leg glucose uptake above placebo, but the CHO+PRO supplement significantly increased glucose uptake 3.5 fold above both CHO and placebo. Therefore, through increasing exogenous glucose uptake, a CHO+PRO supplement can increase fuel availability for the working muscles, as well as decrease the reliance on endogenous carbohydrate stores such as liver or muscle glycogen.

The Krebs cycle is a crucial system in the oxidative phosphorylation process of carbohydrates, fats and protein in the body. Krebs cycle intermediates, including citrate, oxaloacetate and malate, increase at the onset of exexrcise and progressively decline as exexrcise continues (67, 68). It has been proposed that a decrease in CHO availability may further decrease Krebs cycle intermediates, causing them to be a limiting factor in the aerobic energy production process in the mitochondria (68). As ingested proteins are broken down into amino acids, they increase the availability of precursors for anaplerotic reactions needed to maintain Krebs cycle intermediates. Ivy et al. (9) proposed the maintenance of Krebs cycle intermediates as a possible mechanism by which CHO+PRO could improve performance above that of CHO-only in their study. In this regard, Cermak et al. (69) measured the Krebs cycle intermediates citrate and malate before and after a 90-min ride at 69% $VO_{2\ max}$. Every 15 minutes, subjects consumed a 6% CHO + 2% PRO or an isocarbohydrate CHO supplement. No differences in Krebs cycle intermediates changes nor in performance were found between treatments. However,

performance was measured as a 20-km time trial 24 hours later, which might not be the best design to evaluate the effects of the supplement. In addition, α-ketogluterate is most likely to be the rate limiting intermediate in these conditions, but only citrate and malate were measured during this experiment. Clearly, more research is required to confirm the role of this proposed mechanism in regard to performance.

A mechanism by which a CHO+PRO supplement would attenuate exercise-induced muscle damage has been suggested. Creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and myoglobin are usually used to indirectly assess muscle damage from blood samples. Saunders et al. (10) compared isocarbohydrate treatments and reported that CPK values at 12-15 hours post-exercise were 83% lower after the CHO+PRO trial than during the CHO-only trial. Romano-Ely et al. (63) compared isocaloric supplements and reported that LDH (9%) and CPK (53%) postexercise levels at 24 hours were significantly lower in the CHO+PRO trial compared with the CHO-only trial. Others have used a resistance exercise model to demonstrate a significant reduction in myoglobin levels 6 hours after a strenuous resistance exercise bout when subjects ingested a CHO+PRO supplement compared to a placebo. Recently, Valentine et al. (70) showed that a CHO+PRO beverage, along with reducing post-exercise muscle damage, could improve muscle function when compared with an isocaloric and an isocarbohydrate supplement. Compared with the CHO-only trials, subjects on the CHO+PRO treatment performed significantly more knee extensions at 70% 1-repetition maximum 24 hours after the experimental trial. The authors suggested that the replacement of some CHO by

PRO could induce a mechanism by which the integrity of the muscle tissue was better maintained than when a CHO-only supplement was ingested.

Finally, it has been proposed that the ability of a CHO+PRO supplement to increase performance above a traditional CHO supplement may be related to the central fatigue hypothesis (9, 71). In short, this hypothesis is based on the regulation of arousal, mood, motivation and fatigue in humans by brain 5-hydroxytryptamine (5-HT, serotonin) and its precursor free tryptophan (Trp). Trp and many other amino acids share the same blood transporter as plasma free fatty acids. As duration of exercise increases, free fatty acids concentration in the blood rises, resulting in more competition for their common transporter. This leads to higher levels of free Trp potentially crossing the blood brain barrier, increasing serotonin production, and creating feelings of fatigue (72, 73). CHO supplementation during exercise decreases levels of plasma free fatty acids and free Trp (74). Using the soccer simulating LIST protocol, Backhouse et al. (53) found that CHO ingestion during intermittent high-intensity exercise appears to elicit an enhanced perceived activation profile that may impact upon task persistence and performance. Branched-chain amino acids supplementation during exercise was shown to decrease mental fatigue (71), and improve endurance performance (75). Given these results, it appears that CHO+PRO may not only be associated with reducing peripheral fatigue, but also reduces central nervous system factors involved with the perception of fatigue.

Conclusion

Soccer is a complex sport during which many different activities such as dribbling, jumping, tackling, or shooting the ball are occurring randomly throughout the game. For this reason, it is hard to study and isolate the factors that are responsible for performance and fatigue in a soccer player. However, soccer fits in the category of intermittent high-intensity type of exercise, which means that sprints bouts and high speed runs are separated by longer periods of low-intensity running or walking. With recently developed protocols such as the LIST, researchers have been able to observe the physiological responses to a match, as well as the effects of nutritional strategies, in a controlled setting. Carbohydrate ingestion is crucial during recovery in order to replenish intramuscular glycogen storage (29, 35), which appears to be the main cause of fatigue towards the end of a game, when muscle fibers become depleted (18, 20). Carbohydrate supplementation during a match has also been associated with increased performance (3-5, 8). It was suggested that the mechanism responsible for this ergogenic effect was the sparing of muscle glycogen as less utilization was observed with CHO supplementation compared with a placebo (6).

Recently, some research has shown benefits of adding protein to carbohydrate in terms of accelerating the glycogen resynthesis process post-exercise (42-45). This finding could find applications in sports such as soccer since glycogen resynthesis during low-intensity and resting periods could be responsible for the glycogen sparing effect of CHO supplementation (6, 59, 60). Most interestingly, several investigations have found

CHO+PRO supplementation during exercise to improve endurance performance (9-11, 61). Further sparing of muscle glycogen, maintenance of Krebs cycle intermediates, reduction of exercise-induced muscle damage, and decreased central fatigue have been proposed as mechanisms potentially creating this ergogenic effect of CHO+PRO supplementation. Further research in the application of such nutritional strategy on soccer performance and fatigue is warranted.

REFERENCES

1. Fifa survey: Approximately 250 million footballers worldwide. 2006.

2. 2006 fifa worldcup broadcast wider, longer and farther than ever before. 2006.

3. Leatt PB, Jacobs I. Effect of glucose polymer ingestion on glycogen depletion during a soccer match. *Can J Sport Sci* 1989;14(2):112-116.

4. Ostojic SM, Mazic S. Effects of a carbohydrate-electrolyte drink on specific soccer tests and performance. *Journal of Sports Science and Medicine* 2002;1(2):47-53.

5. Guerra I, Chaves R, Barros T, Tirapegui J. The influence of fluid ingestion on performance of soccer players during a match. *Journal of Sports Science and Medicine* 2004;3(4):198-202.

6. Nicholas CW, Tsintzas K, Boobis L, Williams C. Carbohydrate-electrolyte ingestion during intermittent high-intensity running. *Med Sci Sports Exerc* 1999;31(9):1280-1286.

7. Nicholas CW, Williams C, Lakomy HKA, Phillips G, Nowitz A. Influence of ingesting a carbohydrate-electrolyte solution on endurance capacity during intermittent, high-intensity shuttle running. *Journal of Sports Sciences* 1995;13(4):283 - 290.

8. Muckle DS. Glucose syrup ingestion and team performance in soccer. *Br J Sports Med* 1973;7(3-4):340-343.

9. Ivy JL, Res PT, Sprague RC, Widzer MO. Effect of a carbohydrate-protein supplement on endurance performance during exercise of varying intensity. *Int J Sport Nutr Exerc Metab* 2003;13:382-395.

10. Saunders MJ, Kane MD, Todd MK. Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Medicine & Science in Sports & Exercise* 2004;36(7):1233-1238.

11. Saunders MJ, Luden ND, Herrick JE. Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents post-exercise muscle damage. *Journal of Strength & Conditioning Research* 2007;21(3):678-684.

12. Bangsbo J, Mohr M, Krustrup P. Physical and metabolic demands of training and match-play in the elite football player. *Journal of Sports Sciences* 2006;24:665-674.

13. Bangsbo J. The physiology of soccer--with special reference to intense intermittent exercise. *Acta Physiol Scand Suppl* 1994;619:1-155.

14. Ali A, Farrally M. Recording soccer players' heart rates during matches. *Journal of Sports Sciences* 1991;9(2):183 - 189.

15. Mohr M, Krustrup P, Nybo L, Nielsen JJ, Bangsbo J. Muscle temperature and sprint performance during soccer matches - beneficial effect of re-warm-up at half-time. *Scandinavian Journal of Medicine & Science in Sports* 2004;14(3):156-162.

16. Mohr M, Krustrup P, Bangsbo J. Match performance of high-standard soccer players with special reference to development of fatigue. *Journal of Sports Sciences* 2003;21:519-528.

17. Roi GS, Sisca G, Perondi F, Diamante A, Nanni G. Post competition blood lactate accumulation during a first league soccer season [abstract]. *Journal of Sports Sciences* 2004;22(6):560.

18. Krustrup P, Mohr M, Steensberg A, Bencke J, Kjaer M, Bangsbo J. Muscle and blood metabolites during a soccer game: Implications for sprint performance. *Med Sci Sports Exerc* 2006;38(6):1165-1174.

19. Turcotte LP, Kiens B, Richter EA. Saturation kinetics of palmitate uptake in perfused skeletal muscle. *FEBS Lett* 1991;279(2):327-329.

20. Saltin B. Metabolic fundamentals in exercise. *Med Sci Sports* 1973;5(3):137-146.

21. Jacobs I, Westlin N, Karlsson J, Rasmusson M, Houghton B. Muscle glycogen and diet in elite soccer players. *European Journal of Applied Physiology and Occupational Physiology* 1982;48(3):297-302.

22. Mohr M, Krustrup P, Bangsbo J. Fatigue in soccer: A brief review. *Journal of Sports Sciences* 2005;23:593-599.

23. Di Salvo V, Baron R, Tschan H, Calderon Montero FJ, Bachl N, Pigozzi F. Performance characteristics according to playing position in elite soccer. *Int J Sports Med* 2007;28(03):222-227.

24. Bangsbo J, Graham T, Johansen L, Strange S, Christensen C, Saltin B. Elevated muscle acidity and energy production during exhaustive exercise in humans. *Am J Physiol* 1992;263(4 Pt 2):R891-899.

25. Lamb GD, Stephenson DG, Bangsbo J, Juel C. Point:Counterpoint: Lactic acid accumulation is an advantage/disadvantage during muscle activity. *J Appl Physiol* 2006;100(4):1410-1412.

26. Sahlin K. Metabolic factors in fatigue. *Sports Med* 1992;13(2):99-107.

27. Bangsbo J, Iaia FM, Krustrup P. Metabolic response and fatigue in soccer. *Int J Sports Physiol Perform* 2007;2(2):111-127.

28. Y. Hellsten, E. A. Richter, B. Kiens, J. Bangsbo. Amp deamination and purine exchange in human skeletal muscle during and after intense exercise. *The Journal of Physiology* 1999;520(3):909-920.

29. Balsom PD, Gaitanos GC, Söderlund K, Ekblom B. High-intensity exercise and muscle glycogen availability in humans. *Acta Physiologica Scandinavica* 1999;165(4):337-345.

30. Reilly T. Energetics of high-intensity exercise (soccer) with particular reference to fatigue. *Journal of Sports Sciences* 1997;15:257-263.

31. Hoffman JR, Maresh CM, Armstrong LE, Gabaree CL, Bergeron MF, Kenefick RW, Castellani JW, Ahlquist LE, Ward A. Effects of hydration state on plasma testosterone, cortisol and catecholamine concentrations before and during mild exercise at elevated temperature. *Eur J Appl Physiol Occup Physiol* 1994;69(4):294-300.

32. Bangsbo J, Norregaard L, Thorsoe F. The effect of carbohydrate diet on intermittent exercise performance. *Int J Sports Med* 1992;13(2):152-157.

33. Bangsbo J, Graham TE, Kiens B, Saltin B. Elevated muscle glycogen and anaerobic energy production during exhaustive exercise in man. *The Journal of Physiology* 1992;451(1):205-227.

34. Williams C, Serratosa L. Nutrition on match day. *Journal of Sports Sciences* 2006;24:687-697.

35. Akermark C, Jacobs I, Rasmusson M, Karlsson J. Diet and muscle glycogen concentration in relation to physical performance in swedish elite ice hockey players. *Int J Sport Nutr* 1996;6(3):272-284.

36. Bussau V, Fairchild T, Rao A, Steele P, Fournier P. Carbohydrate loading in human muscle: An improved 1 day protocol. *European Journal of Applied Physiology* 2002;87(3):290-295.

37. Burke L, Kiens B, Ivy JL. Carbohydrates and fat for training and recovery. *Journal of Sports Sciences* 2004;22:15-30.

38. Burke L, Loucks A, Broad N. Energy and carbohydrate for training and recovery. *Journal of Sports Sciences* 2006;24:675-685.

39. Burke LM, Collier GR, Hargreaves M. Muscle glycogen storage after prolonged exercise: Effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* 1993;75(2):1019-1023.

40. Ivy JL, Katz AL, Cutler CL, Sherman WM, Coyle EF. Muscle glycogen synthesis after exercise: Effect of time of carbohydrate ingestion. *J Appl Physiol* 1988;64(4):1480-1485.

41. Jentjens. Determinants of post-exercise glycogen synthesis during short-term recovery. *Sports Medicine* 2003.

42. Zawadzki KM, Yaspelkis BB, 3rd, Ivy JL. Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *J Appl Physiol* 1992;72(5):1854-1859.

43. Ivy JL, Goforth HW, Jr., Damon BM, McCauley TR, Parsons EC, Price TB. Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *J Appl Physiol* 2002;93(4):1337-1344.

44. van Loon LJ, Saris WH, Kruijshoop M, Wagenmakers AJ. Maximizing postexercise muscle glycogen synthesis: Carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am J Clin Nutr* 2000;72(1):106-111.

45. Berardi JM, Price TB, Noreen EE, Lemon PW. Postexercise muscle glycogen recovery enhanced with a carbohydrate-protein supplement. *Med Sci Sports Exerc* 2006;38(6):1106-1113.

46. Tarnopolsky MA, Bosman M, Macdonald JR, Vandeputte D, Martin J, Roy BD. Postexercise protein-carbohydrate and carbohydrate supplements increase muscle glycogen in men and women. *J Appl Physiol* 1997;83(6):1877-1883.

47. Roy BD, Tarnopolsky MA. Influence of differing macronutrient intakes on muscle glycogen resynthesis after resistance exercise. *J Appl Physiol* 1998;84(3):890-896.

48. Carrithers JA, Williamson DL, Gallagher PM, Godard MP, Schulze KE, Trappe SW. Effects of postexercise carbohydrate-protein feedings on muscle glycogen restoration. *J Appl Physiol* 2000;88(6):1976-1982.

49. van Hall G, Shirreffs SM, Calbet JAL. Muscle glycogen resynthesis during recovery from cycle exercise: No effect of additional protein ingestion. *J Appl Physiol* 2000;88(5):1631-1636.

50. Howarth KR, Moreau NA, Phillips SM, Gibala MJ. Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. *J Appl Physiol* 2009;106(4):1394-1402.

51. Kirkendall DT. Effects of nutrition on performance in soccer. *Medicine & Science in Sports & Exercise* 1993;25(12):1370-1374.

52. Welsh RS, Davis JM, Burke JR, Williams HG. Carbohydrates and physical/mental performance during intermittent exercise to fatigue. *Med Sci Sports Exerc* 2002;34(4):723-731.

53. S. H. Backhouse, A. Ali, S. J. H. Biddle, C. Williams. Carbohydrate ingestion during prolonged high-intensity intermittent exercise: Impact on affect and perceived exertion. *Scandinavian Journal of Medicine & Science in Sports* 2007;17(5):605-610.

54. Foskett A, Williams C, Boobis L, Tsintzas K. Carbohydrate availability and muscle energy metabolism during intermittent running. *Med Sci Sports Exerc* 2008;40(1):96-103.

55. Nicholas CW, Nuttall FE, Williams C. The loughborough intermittent shuttle test: A field test that simulates the activity pattern of soccer. *Journal of Sports Sciences* 2000;18(2):97.

56. Yaspelkis BB, Patterson JG, Anderla PA, Ding Z, Ivy JL. Carbohydrate supplementation spares muscle glycogen during variable-intensity exercise. *J Appl Physiol* 1993;75(4):1477-1485.

57. Hargreaves M, Costill DL, Coggan A, Fink WJ, Nishibata I. Effect of carbohydrate feedings on muscle glycogen utilization and exercise performance. *Medicine & Science in Sports & Exercise* 1984;16(3):219-222.

58. Tsintzas OK, Williams C, Boobis L, Greenhaff P. Carbohydrate ingestion and glycogen utilization in different muscle fibre types in man. *J Physiol* 1995;489 (Pt 1):243-250.

59. Constable SH, Young JC, Higuchi M, Holloszy JO. Glycogen resynthesis in leg muscles of rats during exercise. *Am J Physiol* 1984;247(5 Pt 2):R880-883.

60. Kuipers H, Keizer HA, Brouns F, Saris WH. Carbohydrate feeding and glycogen synthesis during exercise in man. *Pflugers Arch* 1987;410(6):652-656.

61. Ferguson-Stegall L, McCleave EL, Ding Z, Kammer LM, Wang B, Doerner PG, Liu Y, Ivy JL. The effect of a low carbohydrate beverage with added protein on cycling endurance performance in trained athletes. *Journal of Strength & Conditioning Research*;24(10):2577-2586.

62. Martinez-Lagunas V, Ding Z, Bernard JR, Wang B, Ivy JL. Added protein maintains efficacy of a low-carbohydrate sports drink. *Journal of Strength & Conditioning Research* 2010;24(1):48-59.

63. Romano-Ely BC, Todd MK, Saunders MJ, St.Laurent T. Effect of an isocaloric carbohydrate-protein-antioxidant drink on cycling performance. *Medicine & Science in Sports & Exercise* 2006;38(9):1608-1616.

64. Van Essen M, Gibala MJ. Failure of protein to improve time trial performance when added to a sports drink. *Medicine & Science in Sports & Exercise* 2006;38(8):1476-1483.

65. Spiller G, Jensen C, Pattison T, Chuck C, Whittam J, Scala J. Effect of protein dose on serum glucose and insulin response to sugars. *Am J Clin Nutr* 1987;46(3):474-480.

66. Levenhagen DK, Carr C, Carlson MG, Maron DJ, Borel MJ, Flakoll PJ. Postexercise protein intake enhances whole-body and leg protein accretion in humans. *Medicine & Science in Sports & Exercise* 2002;34(5):828-837.

67. Gibala MJ, Tarnopolsky MA, Graham TE. Tricarboxylic acid cycle intermediates in human muscle at rest and during prolonged cycling. *Am J Physiol Endocrinol Metab* 1997;272(2):E239-244.

68. Sahlin K, Katz A, Broberg S. Tricarboxylic acid cycle intermediates in human muscle during prolonged exercise. *Am J Physiol Cell Physiol* 1990;259(5):C834-841.

69. Cermak NM, Solheim AS, Gardner MS, Tarnopolsky MA, Gibala MJ. Muscle metabolism during exercise with carbohydrate or protein-carbohydrate ingestion. *Medicine & Science in Sports & Exercise* 2009;41(12):2158-2164.

70. Valentine R, Saunders M, Todd M, St. Laurent T. Influence of carbohydrate-protein beverage on cycling endurance and indices of muscle disruption. *Int J Sports Nutr Exerc Metab* 2008;18:363-378.

71. Blomstrand E, Hassmen P, Newsholme E. Effect of branched-chain amino-acid supplementation on mental performance. *Acta Physiol Scand* 1991;143:225-226.

72. Struder H, Hollman W, Platen P, Donike M, Gotzmann A, Weber K. Influence of paroxetine, branched-chain amino acids and tyrosine on neuroendocrine system responses and fatigue in humans. *Horm Metab Res* 1998;30:188-194.

73. Wilson W, Maughan R. Evidence for a possible role of 5-hydroxytryptamine in the genesis of fatigue in man - administration of paroxetine, a 5-ht reuptake inhibitor, reduces the capacity to perform prolonged exercise. *Exp Physiol* 1992;77:921-924.

74. Blomstrand E. A role for branched-chain amino acids in reducing central fatigue. *J Nutr* 2006;136(2):544S-547.

75. Blomstrand E, Hassmen P, Ekblom B, Newsholme E. Admininstration of branched-chain amino-acids during sustained exercise - effects of performance and plasma concentration of some amino acids. *Eur J Appl Physiol Occup Physiol* 1991;63:83-88.

DETAILED METHODS

Participants

Two female and eight male trained soccer players completed the study. Their trained state was determined by relative VO_{2max} of at least 40 and 45 ml/kg/min for females and males, respectively. A detailed explanation of the experimental procedures and the potential risks of the study were given both verbally and in writing to all subjects prior to initial testing. Subjects were given the opportunity to ask questions before signing the informed consent, according to the protocol described in The University of Texas at Austin's "Institutional Review Board Procedures Manual for Faculty, Staff and Student Researchers with Human Participants". The University of Texas at Austin Institutional Review Board approved the study before it commenced. Subjects' characteristics are found in Table 1.

Preliminary testing

Prior to beginning the study, each subject reported to the laboratory for determination of their maximal oxygen consumption (VO_{2max}). The VO_{2max} test was performed on treadmill, using the Fitness Institute of Texas (FIT) maximal treadmill ramped protocol. The protocol started at a speed of 1.7 mph and a grade of 6%, and both gradually increased every minute until fatigue. Subjects were breathing through a Hans Rudolph valve, with expired gases directed to a mixing chamber for analysis of oxygen (O_2) and carbon dioxide (CO_2) (ParvoMedics TrueOne 2400, ParvoMedics, Sandy, UT, USA). Outputs from this system were directed to a laboratory computer for calculation of

ventilation, O_2 consumption (VO_2), CO_2 production, and respiratory exchange ratio (RER) every 30 s. The criteria used to establish VO_2max was a plateau in VO_2 with increasing exercise intensity and RER > 1.10.

On the same day as the VO_{2max} tests, the subjects performed a practice trial to familiarize them with the laboratory environment and the experimental protocol to be used. The practice trials simulated the protocol, but were performed without blood samples or biopsies being taken. The practice trial was also used to adjust and/or verify appropriate running speeds for the experimental trials.

Experimental design

This study followed a randomized, double-blinded, repeated measures design. After initially completing a VO_{2max} test and familiarization trial, subjects performed two experimental trials in order to test the effects of a 2.8% protein + 7% carbohydrate (CHO+PRO) supplement compared to a 9.8% carbohydrate alone (CHO) supplement. During each trial, a total of two supplements were provided. The first one 10 minutes before the beginning of the first half of the simulated soccer match, and the second one at the beginning of the half-time break. The rationale for this supplementation timing was to reproduce match conditions as closely as possible. In fact, soccer players can drink before the game and during the 15-minute half-time, but are usually unable to do so during the continuous playing periods. Supplement composition for each treatment are shown in table 2. For each of the two supplements given during a CHO+PRO trial, a volume that provides 0.5g of CHO and 0.2g of PRO per kg body weight were ingested. For example, a 70-kg subject was to ingest 500ml of the mixture at both time points, which represents

35g CHO and 14g PRO each time. For the CHO trial, the same volume of an isocaloric 9.8% CHO drink was ingested, which represents 49g of CHO each time for the 70-kg subject. Both treatments contained the same amount of electrolytes. The order of testing was randomly assigned.

The subjects were asked to refrain from strenuous exercise for 2 days before each trial and record their diet for 2 days preceding the first trial. They were required to reproduce their activity and diet as closely as possible for the same period of time prior to the second trial. The two main trials were separated by at least 7 days.

Protocol

On the day of an experimental trial, the subjects reported to the laboratory 30 min before the start of exercise having fasted for 12 h. They were weighed and fitted with a heart rate monitor (Polar Beat, Polar Electro, Finland) secured in place around their chest. Subsequently, the participants underwent their first muscle biopsy and were then asked to ingest the first supplement.

Following consumption of the initial supplement, participants completed a standardized warm-up and stretching protocol and then started the experimental protocol exactly 15 minutes after ingestion on the first drink. Additional amounts of water ingested during this warm-up period and half-time were recorded. Equal quantities were provided during the second trial.

The exercise protocol is illustrated in Figure 10. Participants completed three 15-min blocks of the LIST for the first half, rested for a 15-min half time, and reproduced a second half. Each 15-min block of exercise during a half was separated by a 2-min rest

period. A second muscle biopsy was taken right after the second half. The biopsy site was closed using steristrips and secured with a pressure pack as well as a spandage sleeve. The subjects then performed their run to exhaustion in order to evaluate fatigue.

Each 15-min block consisted of a set pattern of intermittent high-intensity running that was designed to be similar to the activity pattern typically recorded for soccer match play(1). The participants were going back and forth at different intensities on a 20-m track. This pattern was repeated approximately 11 times per block and is as follows:

- 3 × 20 m at walking pace (13 seconds to complete)
- 1 × 20 m at maximal running speed
- 3 × 20 m at a running speed corresponding to approximately 55% of individual VO_{2max}
- 3 × 20 m at a running speed corresponding to approximately 95% of individual VO_{2max}

Using a free audio editor (Audacity 1.3.8), a recording was played during the protocol with a countdown from 3 followed by a "beep" to inform the participant each time a line must be reached. The audio track was matched to the individual's VO_{2max} in order to provide the appropriate jogging and running speeds.

Time to cover the 20 m at maximal running speed was measured using the Brower wireless timing system mounted with a reflective beam (Brower Timing Systems, USA). Average sprint time per 15-min block was calculated.

The original Borg Rating of Perceived Exertion Scale (RPE) was used to assess perceived exertion during exercise and was administered every 15 min during exercise (during the last walking stage of each 15-min block of the LIST). The scale ranges from 6 to 20, with anchors ranging from ‘‘very, very light’’ to ‘‘very, very hard.’’

Heart rate was monitored during the main trials by short-range telemetry using Polar heart rate monitor units and stored in memory mode. Average heart rate per 15-min block was calculated.

After blood collection and the last biopsy, the participants started the run to exhaustion (RTE) exactly 15 minutes from the end of the second half. It consisted of an open-ended period of intermittent shuttle running, designed to exhaust the participants within approximately 15 min. The participants were required to produce 2 runs (95% VO_{2max}) followed by a jog (55% VO_{2max}) on the same 20-m track, and reproduce this pattern continuously until they were unable to maintain the required speed (reach the line) for two consecutive shuttle runs.

On the day following each experimental trial, subjects were asked to report to the laboratory in order to obtain a 24h-post blood draw to evaluate muscle damage by measuring blood levels of creatine phosphokinase. At the same time, they rated their overall muscle soreness from 1 to 10 (1 being no soreness at all and 10 too much pain to walk).

Blood sampling

Blood samples (8ml) were collected pre-exercise, at the beginning as well as at the end of the 15-min half-time, after the second half, following the run to exhaustion,

and 24 hours post-trial. 0.3 ml of sample was transferred into a tube containing 0.6 ml of 10% perchloric acid (PCA). The remaining sample was divided into two tubes and mixed with 0.3 ml of EDTA (24mg·ml^{-1}, pH 7.4) to prevent coagulation. Tubes were centrifuged for 10 minutes at 3,000 rpm with a HS-4 rotor in a Sorvall RC6 centrifuge (Kendro Laboratory Products, Newtown, CT). Plasma and PCA extracts were separated into aliquots and all tubes were stored at -80°C until analysis.

Blood glucose concentrations were measured using a modified Trinder procedure at 37°C (2). Dry powder reagent was dissolved in 100 ml deionized water. Tubes were run in duplicates, with 1.5 ml of reagent added to each tube (blank, standard and blood samples). Twenty microliters of standard (50, 100, 200, 300 mg·dL^{-1}) and blood samples were added to their respective tubes, vortexed, and incubated in shaking water bath for 10 minutes at 37°C. Samples were read at 500 nm using a Beckman DU640 Spectrophotometer (Coulter, Fullerton, CA) and had a coefficient of variation (CV) of 3.7%.

Blood lactate concentrations were measured from the PCA extracts using enzymatic analysis according to Hohorst (3). Glycine-hydrazine buffer (Fisher G-46 glycine, Fisher Scientific, Pittsburg, PA; Sigma H-9507 Hydrazine, Sigma-Aldrich, Inc., St Louis, MO) totaling 1000 milliliters was mixed, and used to prepare the cocktail reagent prior to beginning the assay. Added to each tube was 1 ml glycine-hydrazine buffer, 0.83 mg β-Nicotinanide adenine dinucleotide (NAD) (Sigma N-7004, Sigma-Aldrich, Inc., St Louis, MO) and 5 µl L-Lactate Dehydrogenase (LDH) (Sigma 826-6 or L-3916, Sigma-Aldrich, Inc., St Louis, MO). Fifty µl 10% PCA was added to the blank,

two tubes were designated for low and high lactic acid standards (1.1 mM and 2.2 mM, respectively). Fifty µl of blood PCA samples was added to each remaining tube. Following vortexing, tubes were incubated at 37°C for 45 min in a shaking water bath. Samples were read at 340 nm using a Beckman DU640 Spectrophotometer (Coulter, Fullerton, CA). The assay was run in duplicate and had a CV of 1.2%.

Plasma insulin was analyzed via radioimmunoassay based on the principles of Goetz (4). The radioimmunoassay kit used was MP Biomedicals ^{125} I RIA, Solon, OH, USA and had a CV of 6.0%. Duplicate tubes were prepared and incubated at room temperature for 18 hours. Tubes were decanted, blotted with absorbant paper, and rinsed with 4 ml of deionized water before a second decantation. Empty tubes were counted in a Wallac 1470 Wizard Gamma Counter (Wallac 1470 Wizard Gamma Counter, PerkinElmer Life and Analytical Sciences, Boston, MA), which had been calibrated for insulin ^{125} I.

Creatine kinase (CK) is an indirect marker of muscle damage. It was determined by enzymatic analysis (Diagnostic Chemicals Limited, Charlottetown, Canada). The conversion of creatine phosphate plus adenosine diphosphate to creatine plus adenosine triphosphate by creatine kinase is linked to several enzyme reactions to produce nicotinamide adenine diphesphopyridine nucleotide-reduced form (NADPH). The rate of NADPH is a measure of creatine kinase activity. 1 ml of reagent was pipetted into appropriate tubes and pre-warmed at 37°C for 5 minutes. The Beckman DU640 spectrophotometer (Beckman Bioanalytical Systems Group) was then blanked with water at 340 nm. 25 µl of sample was added to the reagent, mixed and incubated at 37°C for 2

minutes. The absorbance was then recorded every minute for 3 minutes. The average absorbance difference per minute (ΔAbs/min) was calculated and multiplied by the factor 6592 (see calculations) to yield results in U/L. The %CV for CK was 2.3.

Calculations

Glucose:

Abs sample / Abs standard * [C]std = mg/dl

[C]std: mg/dl in standard

Lactate:

(Abs sample/6.22) * (1.05/0.05) *(3/1) = mM

or Abs sample * 10.13 = mM

6.22 = millimolar absorptivity of NADH

1.05/0.05 = cuvette dilution

3/1 = blood dilution (0.3 ml blood in 0.6 ml of 10% PCA)

Insulin:

%B/Bo = CPM / CPM (0µlU/ml) * 100

CPM = average counts of a duplicate, sample or standard

0µlU/ml = 0 tube (also known as Bo or total binding tube)

A standard curve was generated using this equation, and insulin concentration for each sample was determined using this standard curve.

Creatine Kinase:

(ΔAbs/min * 1.025 * 1000) / (1 * 6.22 * 0.025) = U/L

Or ΔAbs/min * 6592 = U/L

ΔAbs/min = average absorbance change per minute

1.025 = total reaction volume

1000 = conversion of U/ml to U/L

1 = light path in cm

6.22 = millimolar absorptivity of NADH

0.025 = sample volume in ml

Muscle glycogen

Intramuscular glycogen utilization throughout the simulated game was evaluated by subtracting glycogen levels at the end of the second half from the pre-exercise levels. Muscle glycogen was determined using muscle biopsies (~50 mg wet wt) taken from the vastus lateralis. The thigh was cleansed with 10% betadine solution and then 1.8 ml of a local anesthesia (1% Lidocaine Hydrochloride Injection, Elkins-Sinn, Inc., Cherry Hill, NJ) was injected to prepare the leg for the muscle biopsy. A 5-8 mm incision was made through the skin and fascia, 2 inches from the midline of the thigh on the lateral side and 4 inches above the patella. Once the bleeding was stopped, the muscle biopsy was taken and pressure reapplied to the incision to stop bleeding. The biopsy was then trimmed of adipose and connective tissue and frozen in liquid nitrogen at -80° C for subsequent analysis. Once bleeding stoped, the incision was closed with steristrips and a Band-Aid

and a pressure pack was affixed over the incision. A spandage sleeve was then wrapped around the thigh to support the pressure pack during running.

Glycogen content was determined by enzymatic degradation with amyloglucosidase in a modified method of Passonneau and Lauderdale (5). The muscle sample was weighed, digested in 1N KOH while incubated at 70°C for 20 minutes, mixed, then incubated for an additional 10 minutes. One hundred µl of homogenate was added to 250 ml of 0.3 M sodium acetate (pH 4.8) then mixed. Ten µl of 50% glacial acetic acid and 250 ml of sodium acetate (containing 10 mg/ml amyloglucosidase, pH 4.8) were then added to the tubes. Tubes were sealed and incubated overnight at room temperature. The glucose reagent was prepared using a Raichem Glucose Color Reagent Kit (Hemagen Diagnostics, San Diego, CA). One hundred µl of muscle homogenate solution and 1.5 ml of reagent were added to clean tubes then incubated for 10 minutes at 37°C. Samples were read with a Beckman DU640 Spectrophotometer (Coulter, Fullerton, CA) at 500 nm.

Metabolic regulation

Using the muscle tissue samples obtained from the muscle biopsies, we measured the phosphorylation state of the protein FOXO3a. This protein is a transcription factor that leads to muscle atrophy, and is therefore used as an indicator of muscle damage. As FOXO3a is dephosphorylated, it can move from the cell's cytoplasm into the nucleus, where it promotes muscle atrophy.

Muscle samples were weighed and homogenized in ice-cold buffer, containing 20 mM Hepes, 2 mM EGTA, 50 mM sodium flouride, 100 mM potassium chloride, 0.2 mM

EDTA, 50 mM glycerophosphate, 1mM DTT, 0.1 mM PMST, 1 mM benzamidine, and 0.5 mM sodium vanadate (pH 7.4). Homogenization was performed on ice using Caframo RZR1 Stirrer (Caframo Limited, Wiarton, Ontario, Canada). The homogenate was centrifuged at 14,000 g for 10 minutes at 4°C and the supernate was then aliquoted to several test tubes and stored at -80°C for later analysis. A modified version of the Lowry assay (6) was used to determine protein concentration. A standard curve was generated using bovine serum albumin (BSA) serially diluted with deionized water. Duplicate tubes were made with 0.1 ml of standards or samples and 1 ml of solution A (48 ml of sodium carbonate, 1 ml of 2% sodium potassium tartrate and 1 ml of 1% cupric sulfate). After vortexing, tubes were incubated at room temperature for 10 min. After 10 min incubation, while vortexing each tube, 0.1 ml of phenol solution was pipeted into the center of each tube. Tubes were then incubated at room temperature for 30 min. Samples and standards were read with a Beckman DU640 Spectrophotometer (Coulter, Fullerton, CA) at 750 nm.

Equal amounts of muscle proteins (80 µg) were separated by gel electrophoresis, using a Sodium Dodecyl Sulphate (SDS)-Page, 10% resolving gel. The proteins were then transferred to special blotting paper, Polyvinylidene fluoride (PVDF) membranes and blotted in freshly prepared TBS containing 5% nonfat dry milk and 0.06% Tris-Buffered Saline Tween-20 (TBST-MLK) for 1 hour at room temperature with agitation. The PVDF membranes were then incubated with a primary antibody in fresh 5% BSA-TBS + 0.06% Tween-20 over night with gentle agitation at 4°C. A rabbit anti-phospho-FOXO3a (Ser318/321) antibody from Cell Signaling was used (1:500 dilution; Cell

Signaling Technology, Inc., Beverly, MA). After the membranes were washed in 0.06% Tris-Buffered Saline (TBS) solution, they were incubated with a secondary reagent for 1h40min at room temperature with agitation. An anti-rabbit IgC HRP-linked antibody (Cell Signaling Technology, Inc., Beverly, MA) was used at a dilution of 1:1500. The PVDF membranes were then washed in 0.06% TBS solution, and the anti-body-bound proteins were visualized by means of Western Lightning Chemiluminescence Reagent Plus (PerkinElmer LAS, INC., Boston, MA) according to manufacturer's protocol. Images were scanned using Adobe Photoshop and quantified using Scion Image (Scion Corporation, Frederick, Maryland). All samples were run with a standard from rat muscle and the phosphorylation states of our subject's samples were measured as % of standard. The protein alpha-tubulin was also measured as a control for equal loading.

Statistical Analysis

Data were analyzed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL). Run time to exhaustion (RTE) and 24 hour-post muscle soreness were analyzed using a one-way analysis of variance (ANOVA). Glycogen utilization was analyzed using a one-way ANOVA, with glycogen difference from pre to post as the dependent variable and the pre measures as a covariate in order to control for the variability of these pre measures. All other variables were measured using a two-way (treatment × time) repeated measures ANOVA. Significance was determined at $p < 0.05$. Data were expressed as mean ± SE.

REFERENCES

1. Nicholas CW, Nuttall FE, Williams C. The loughborough intermittent shuttle test: A field test that simulates the activity pattern of soccer. *Journal of Sports Sciences* 2000;18(2):97.

2. Trinder P. Determination of blood glucose using 4-amino phenazone as oxygen acceptor. *J Clin Pathol* 1969;22(2):246.

3. Hohorst H. Determination of l-lactate with ldh and dpn. *New York: Academic* 1965:266-270.

4. Goetz FC, Greenberg BZ. A simple immunoassay for small amounts of insulin. *J Lab Clin Med* 1961;58:819-822.

5. Passonneau J, Lauderdale V. A comparison of three methods of glycogen measurement in tissue. *Anal Biochem* 1974;60:405-412.

6. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193(1):265-275.

APPENDIX A

Individual Physiological Data

Individual Subject Data

Subject	Gender	Age (years)	Height (cm)	Body Weight (kg)	VO2max (L/min)	VO2max (ml/kg/min)
1	M	24	188	82.6	4.83	58.5
2	M	27	175	74	3.63	49
3	M	20	173	83.9	4.54	54.1
4	M	24	188	85.6	4	46.8
5	M	21	178	72.5	3.91	53.9
6	M	21	183	67.2	3.76	55.9
7	F	22	172	60.8	2.73	44.9
8	F	19	173	59	2.43	41.3
9	M	22	170	65.8	3.3	50.3
10	M	22	174	65.8	4.24	64.4
Average		**22.20**	**177.40**	**71.72**	**3.74**	**51.91**
SE		**0.73**	**2.10**	**3.05**	**0.24**	**2.17**

Performance: Run Time to Exhaustion (sec)

Subject	**CHO**	**CHO+PRO**
1	438	502
2	477	366
3	594	830
4	509	481
5	578	692
6	187	352
7	279	234
8	288	203
9	80	89
10	1459	2140
Average	**488.9**	**588.9**
SE	**120.5**	**186.3**

Sprint times (sec)

	Subject	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6
CHO	1	3.11	3.16	3.19	3.25	3.24	3.2
	2	3.19	3.25	3.3	3.38	3.32	3.33
	3	3.36	3.47	3.51	3.55	3.51	3.52
	4	3.09	3.12	3.16	3.2	3.18	3.17
	5	2.91	2.95	3.01	2.98	2.97	2.95
	6	3.28	3.33	3.34	3.4	3.42	3.47
	7	3.17	3.34	3.43	3.41	3.46	3.54
	8	3.04	3.05	3.07	3.07	3.04	3
	9	3.15	3.37	3.49	3.33	3.31	3.4
	10	3.2	3.22	3.22	3.28	3.27	3.28
	Average	**3.15**	**3.23**	**3.27**	**3.29**	**3.27**	**3.29**
	SE	**0.04**	**0.05**	**0.05**	**0.05**	**0.05**	**0.07**
CHO+PRO	1	3.11	3.16	3.11	3.2	3.24	3.23
	2	3.1	3.21	3.28	3.24	3.32	3.25
	3	3.3	3.34	3.38	3.44	3.37	3.32
	4	3.07	3.11	3.14	3.16	3.12	3.1
	5	2.92	2.97	3.07	3.02	2.97	2.93
	6	3.3	3.35	3.4	3.38	3.47	3.53
	7	2.94	3	3.03	3.06	3	3.04
	8	3.38	3.39	3.46	3.59	3.61	3.6
	9	3.19	3.3	3.4	3.38	3.37	3.4
	10	3.11	3.1	3.12	3.13	3.1	3.12
	Average	**3.14**	**3.19**	**3.24**	**3.26**	**3.26**	**3.25**
	SE	**0.05**	**0.05**	**0.05**	**0.06**	**0.07**	**0.07**

Heart rate (bpm)

	Subject	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Exhaustion
CHO	1	161	163	169	161	164	164	176
	2	165	167	164	160	165	164	172
	3	182	183	184	175	185	188	191
	4	165	169	168	165	170	170	178
	5	170	176	179	171	175	176	183
	6	170	173	176	173	176	176	188
	7	174	173	173	168	171	171	188
	8	182	187	183	181	192	188	195
	9	173	174	173	178	175	178	183
	10	180	178	179	182	184	182	188
	Average	**172.2**	**174.3**	**174.8**	**171.4**	**175.7**	**175.7**	**184.2**
	SE	**2.6**	**2.5**	**2.3**	**2.8**	**3.2**	**3.1**	**2.5**
CHO+PRO	1	164	167	169	163	165	165	176
	2	168	170	170	165	167	170	172
	3	180	186	184	177	183	186	187
	4	168	170	170	163	167	170	174
	5	172	178	177	170	174	176	182
	6	170	172	176	173	177	176	185
	7	176	174	175	169	172	179	189
	8	179	188	187	178	185	189	179
	9	172	175	173	178	178	178	182
	10	180	180	181	184	184	183	186
	Average	**172.9**	**176.0**	**176.2**	**172.0**	**175.2**	**177.2**	**181.2**
	SE	**2.0**	**2.5**	**2.2**	**2.5**	**2.6**	**2.7**	**2.0**

RPE (Borg scale)

	Subject	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6
	WJ	11	13	14	14	15	17
	MH	13	15	17	16	17	19
	JB	14	16	16	15	17	18
	PM	13	14	15	16	16	17
CHO	JG	13	15	17	14	15	17
	NC	12	13	14	13	15	17
	SD	13	14	15	16	17	17
	BV	14	16	17	14	17	19
	CW	13	15	16	14	16	17
	MK	13	14	14	14	14	15
	Average	**12.9**	**14.5**	**15.5**	**14.6**	**15.9**	**17.3**
	SE	**0.3**	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**
	WJ	8	12	13	13	13	13
	MH	14	15	17	15	17	17
	JB	13	14	15	14	15	17
	PM	11	13	13	14	15	16
CHO+PRO	JG	13	14	15	13	14	15
	NC	11	13	15	13	16	17
	SD	14	15	16	16	16	17
	BV	14	17	19	14	15	19
	CW	13	15	16	14	16	17
	MK	13	14	15	14	15	16
	Average	**12.4**	**14.2**	**15.4**	**14.0**	**15.2**	**16.4**
	SE	**0.7**	**0.5**	**0.6**	**0.3**	**0.4**	**0.6**

APPENDIX B

Individual Biochemical Data

Glucose (mg/dL)

	Subject	Pre	Beg. half	End half	Post match	End
CHO+PRO	1	79.51	86.36		52.14	61.90
	2	69.33	77.84	95.75	78.27	80.43
	3	77.41	126.96	135.78	108.90	94.39
	4	76.17	165.43	146.52	97.92	71.28
	5	75.29	81.64	114.59	76.07	83.98
	6	70.40	105.77	131.16	77.93	79.32
	7	71.00	117.39	109.37	85.97	79.49
	8	61.41	100.14	108.65	61.52	60.33
	9	70.66	116.33	152.30	72.49	86.24
	10	86.28	156.35	163.51	101.61	78.15
	AVE	**73.75**	**113.42**	**128.62**	**81.28**	**77.55**
	SE	**2.14**	**9.43**	**7.64**	**5.62**	**3.33**
CHO	1	79.58	100.30	98.20	84.84	79.92
	2	71.16	81.54	132.41	68.85	80.40
	3	72.05	147.09	164.54	100.44	101.51
	4	85.37	158.70	149.16	108.63	90.54
	5	75.98	137.47	167.20	77.71	72.47
	6	79.04	95.55	132.07	86.47	110.35
	7	75.64	141.63	146.88	97.14	90.02
	8	75.50	99.19	118.66	75.17	56.63
	9	70.46	126.60	158.72	74.74	104.79
	10	79.42	140.57	153.54	101.55	95.09
	AVE	**76.42**	**122.86**	**142.14**	**87.55**	**88.17**
	SE	**1.45**	**8.36**	**6.90**	**4.31**	**5.13**

Lactate (mmol/L)

	Subject	Pre	Beg. half	End half	Post match	End
CHO	1	0.67	2.2	1.08	2	4.42
	2	0.83	4.13	2.98	5.92	4.96
	3	0.29	3.09	1.83	2.21	6.12
	4	0.76	2.63	1.41	2.97	2.63
	5	1.14	4.55	2.57	3.23	3.64
	6	1.48	4.53	2.7	3.59	6.91
	7	1.17	5.32	2.49	5.46	7.98
	8	0.91	4.45	2.3	4.12	5.52
	9	0.99	4.21	2.02	4.57	6.03
	10	1.23	8.30	2.68	8.75	8.57
	Average	**0.95**	**4.34**	**2.21**	**4.28**	**5.68**
	SE	**0.1**	**0.6**	**0.2**	**0.7**	**0.7**
CHO+PRO	1	0.52	2.5	0.73	1.43	3.82
	2	0.94	6.54	3.82	7.25	6.73
	3	0.93	3.05	1.59	2.1	3.68
	4	0.83	2.93	1.69	3.48	3.07
	5	1.22	4.62	2.04	3.64	6.04
	6	1.41	3.58	2.58	4.12	8.37
	7	0.5	5.19	2.05	4.47	6.56
	8	0.89	4.75	2.33	4.49	5.39
	9	1.12	4.43	2.72	4.76	6.23
	10	0.86	9.48		7.22	8.43
	Average	**0.92**	**4.71**	**2.17**	**4.30**	**5.83**
	SE	**0.1**	**0.7**	**0.3**	**0.7**	**0.7**

Insulin (pmol/L)

	Subject	Pre	Beg. Half	End half	Post match	End
	1	9	7.487	37.252	7.074	5.3875
	2	16.989	35.672	50.938	19.893	14.175
	3	10.676	15.222	85.284	11.32	20.426
	4	7.359	10.257	69.356	9.119	11.587
	5	10.152	7.981	40.617	6.529	11.624
CHO	6	14.22	21.163	80.816	12.716	19.639
	7	12.1	10.924	54.641	7.022	9.532
	8	11.43	13.88	53.867	15.563	12.912
	9	14.1	18.64	66.714	10.102	10.72
	10	17.4	24.331	64.325	22.014	28.612
	Average	**12.3**	**16.6**	**60.4**	**12.1**	**14.5**
	SE	**1.0**	**2.8**	**5.0**	**1.7**	**2.1**
	1	10.669	8.437	36.8	8.567	5.133
	2	13.294	19.291	44.572	10.567	7.95
	3	13.667	19.511	98.937	37.28	21.999
	4	9.509	5.952	52.001	5.893	8.987
	5	11.175	10.303	47.609	7.795	7.893
CHO+PRO	6	16.596	34.661	133.743	22.144	39.383
	7	8.407	8.454		6.574	7.395
	8	12.198	15.187	60.542	13.662	28.772
	9	17.178	20.871	70.761	19.76	15.009
	10	19.955	27.812	39.919	26.178	20.317
	Average	**13.3**	**17.0**	**65.0**	**15.8**	**16.3**
	SE	**1.2**	**2.9**	**10.2**	**3.3**	**3.6**

Creatine kinase (U/L)

	CHO			CHO+PRO		
Subject	**Pre**	**End**	**24h**	**Pre**	**End**	**24h**
1	124.8	431.2	1014.4	535.1	872.2	1448.2
2	211.4	685.6	2724.6	700.5	1799.1	2604.0
3	231.3	419.6	639.9	137.7	567.9	990.9
4	80.0	168.0	329.4	98.4	221.4	456.9
5	105.4	305.3	340.4	68.0	208.1	298.4
6	574.9	1854.7	2202.4	392.1	684.6	1095.4
7	225.3	459.5	482.8	66.2	282.2	287.7
8	152.2	537.6	871.7	101.0	337.3	554.7
9	62.3	109.6	135.2	64.1	171.4	330.9
10	180.0	312.9	777.7			
Ave	**194.7**	**528.4**	**951.9**	**240.3**	**571.6**	**896.4**
SE	**43.9**	**148.7**	**254.8**	**75.6**	**163.4**	**239.0**

Glycogen (μmol/g wet wt^{-1})

	CHO		CHO + PRO	
Subject	**Pre**	**Post**	**Pre**	**Post**
1	85.6	66.1	115.2	92.1
2	153.3	93.9	94.2	73.5
3	88.7	69.0	98.7	70.2
4	164.1	79.0	103.4	103.3
5	119.6	86.5	120.2	56.5
6	83.1	60.0	132.8	95.4
7	107.8	76.9	104.6	67.8
8	114.4	82.1	87.6	64.9
Ave	**114.6**	**76.7**	**107.1**	**78.0**
SE	**10**	**4**	**5**	**6**

p-FOXO3a (% of standard)

	CHO		**CHO+PRO**	
Subject	**Pre**	**End**	**Pre**	**End**
1	64	64	67	67
2	86	91	91	84
3	83	59	64	48
4	88	87	63	59
5	93	100	87	95
6	95	81	86	84
7	74	75	78	79
Ave	**83**	**80**	**77**	**74**
SE	**4**	**6**	**4**	**6**

COMPREHENSIVE BIBLIOGRAPHY

1. Mohr M, Krustrup P, Bangsbo J. Match performance of high-standard soccer players with special reference to development of fatigue. *Journal of Sports Sciences* 2003;21:519-528.
2. Bangsbo J. The physiology of soccer--with special reference to intense intermittent exercise. *Acta Physiol Scand Suppl* 1994;619:1-155.
3. Krustrup P, Mohr M, Steensberg A, Bencke J, Kjaer M, Bangsbo J. Muscle and blood metabolites during a soccer game: Implications for sprint performance. *Med Sci Sports Exerc* 2006;38(6):1165-1174.
4. Mohr M, Krustrup P, Bangsbo J. Fatigue in soccer: A brief review. *Journal of Sports Sciences* 2005;23:593-599.
5. Leatt PB, Jacobs I. Effect of glucose polymer ingestion on glycogen depletion during a soccer match. *Can J Sport Sci* 1989;14(2):112-116.
6. Ostojic SM, Mazic S. Effects of a carbohydrate-electrolyte drink on specific soccer tests and performance. *Journal of Sports Science and Medicine* 2002;1(2):47-53.
7. Guerra I, Chaves R, Barros T, Tirapegui J. The influence of fluid ingestion on performance of soccer players during a match. *Journal of Sports Science and Medicine* 2004;3(4):198-202.
8. Muckle DS. Glucose syrup ingestion and team performance in soccer. *Br J Sports Med* 1973;7(3-4):340-343.
9. Kirkendall DT. Effects of nutrition on performance in soccer. *Medicine & Science in Sports & Exercise* 1993;25(12):1370-1374.
10. Nicholas CW, Tsintzas K, Boobis L, Williams C. Carbohydrate-electrolyte ingestion during intermittent high-intensity running. *Med Sci Sports Exerc* 1999;31(9):1280-1286.

11. Welsh RS, Davis JM, Burke JR, Williams HG. Carbohydrates and physical/mental performance during intermittent exercise to fatigue. *Med Sci Sports Exerc* 2002;34(4):723-731.

12. S. H. Backhouse, A. Ali, S. J. H. Biddle, C. Williams. Carbohydrate ingestion during prolonged high-intensity intermittent exercise: Impact on affect and perceived exertion. *Scandinavian Journal of Medicine & Science in Sports* 2007;17(5):605-610.

13. Foskett A, Williams C, Boobis L, Tsintzas K. Carbohydrate availability and muscle energy metabolism during intermittent running. *Med Sci Sports Exerc* 2008;40(1):96-103.

14. Nicholas CW, Williams C, Lakomy HKA, Phillips G, Nowitz A. Influence of ingesting a carbohydrate-electrolyte solution on endurance capacity during intermittent, high-intensity shuttle running. *Journal of Sports Sciences* 1995;13(4):283 - 290.

15. Nicholas CW, Nuttall FE, Williams C. The loughborough intermittent shuttle test: A field test that simulates the activity pattern of soccer. *Journal of Sports Sciences* 2000;18(2):97.

16. Bangsbo J, Iaia FM, Krustrup P. Metabolic response and fatigue in soccer. *Int J Sports Physiol Perform* 2007;2(2):111-127.

17. Balsom PD, Gaitanos GC, Söderlund K, Ekblom B. High-intensity exercise and muscle glycogen availability in humans. *Acta Physiologica Scandinavica* 1999;165(4):337-345.

18. Reilly T. Energetics of high-intensity exercise (soccer) with particular reference to fatigue. *Journal of Sports Sciences* 1997;15:257-263.

19. Hargreaves M, Costill DL, Coggan A, Fink WJ, Nishibata I. Effect of carbohydrate feedings on muscle glycogen utilization and exercise performance. *Medicine & Science in Sports & Exercise* 1984;16(3):219-222.

20. Yaspelkis BB, Patterson JG, Anderla PA, Ding Z, Ivy JL. Carbohydrate supplementation spares muscle glycogen during variable-intensity exercise. *J Appl Physiol* 1993;75(4):1477-1485.

21. Ivy JL, Res PT, Sprague RC, Widzer MO. Effect of a carbohydrate-protein supplement on endurance performance during exercise of varying intensity. *Int J Sport Nutr Exerc Metab* 2003;13:382-395.

22. Saunders MJ, Kane MD, Todd MK. Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Medicine & Science in Sports & Exercise* 2004;36(7):1233-1238.

23. Saunders MJ, Luden ND, Herrick JE. Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents post-exercise muscle damage. *Journal of Strength & Conditioning Research* 2007;21(3):678-684.

24. Osterberg KL, Zachwieja JJ, Smith JW. Carbohydrate and carbohydrate plus protein for cycling time-trial performance. *Journal of Sports Sciences* 2008;26(3):227 - 233.

25. Van Essen M, Gibala MJ. Failure of protein to improve time trial performance when added to a sports drink. *Medicine & Science in Sports & Exercise* 2006;38(8):1476-1483.

26. Romano-Ely BC, Todd MK, Saunders MJ, St.Laurent T. Effect of an isocaloric carbohydrate-protein-antioxidant drink on cycling performance. *Medicine & Science in Sports & Exercise* 2006;38(9):1608-1616.

27. Trinder P. Determination of blood glucose using 4-amino phenazone as oxygen acceptor. *J Clin Pathol* 1969;22(2):246.

28. Hohorst H. Determination of l-lactate with ldh and dpn. *New York: Academic* 1965:266-270.

29. Passonneau J, Lauderdale V. A comparison of three methods of glycogen measurement in tissue. *Anal Biochem* 1974;60:405-412.

30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193(1):265-275.

31. Ferguson-Stegall L, McCleave EL, Ding Z, Kammer LM, Wang B, Doerner PG, Liu Y, Ivy JL. The effect of a low carbohydrate beverage with added protein on cycling

endurance performance in trained athletes. *Journal of Strength & Conditioning Research*;24(10):2577-2586.

32. Jentjens RLPG, Achten J, Jeukendrup AE. High oxidation rates from combined carbohydrates ingested during exercise. *Medicine & Science in Sports & Exercise* 2004;36(9):1551-1558.

33. Ivy JL, Goforth HW, Jr., Damon BM, McCauley TR, Parsons EC, Price TB. Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *J Appl Physiol* 2002;93(4):1337-1344.

34. Zawadzki KM, Yaspelkis BB, 3rd, Ivy JL. Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *J Appl Physiol* 1992;72(5):1854-1859.

35. Valentine R, Saunders M, Todd M, St. Laurent T. Influence of carbohydrate-protein beverage on cycling endurance and indices of muscle disruption. *Int J Sports Nutr Exerc Metab* 2008;18:363-378.

36. Blomstrand E, Hassmen P, Newsholme E. Effect of branched-chain amino-acid supplementation on mental performance. *Acta Physiol Scand* 1991;143:225-226.

37. Struder H, Hollman W, Platen P, Donike M, Gotzmann A, Weber K. Influence of paroxetine, branched-chain amino acids and tyrosine on neuroendocrine system responses and fatigue in humans. *Horm Metab Res* 1998;30:188-194.

38. Wilson W, Maughan R. Evidence for a possible role of 5-hydroxytryptamine in the genesis of fatigue in man - administration of paroxetine, a 5-ht reuptake inhibitor, reduces the capacity to perform prolonged exercise. *Exp Physiol* 1992;77:921-924.

39. Blomstrand E. A role for branched-chain amino acids in reducing central fatigue. *J Nutr* 2006;136(2):544S-547.

40. Blomstrand E, Hassmen P, Ekblom B, Newsholme E. Admininstration of branched-chain amino-acids during sustained exercise - effects of performance and plasma concentration of some amino acids. *Eur J Appl Physiol Occup Physiol* 1991;63:83-88.

41. Fifa survey: Approximately 250 million footballers worldwide. 2006.

42. 2006 fifa worldcup broadcast wider, longer and farther than ever before. 2006.

43. Bangsbo J, Mohr M, Krustrup P. Physical and metabolic demands of training and match-play in the elite football player. *Journal of Sports Sciences* 2006;24:665-674.

44. Ali A, Farrally M. Recording soccer players' heart rates during matches. *Journal of Sports Sciences* 1991;9(2):183 - 189.

45. Mohr M, Krustrup P, Nybo L, Nielsen JJ, Bangsbo J. Muscle temperature and sprint performance during soccer matches - beneficial effect of re-warm-up at half-time. *Scandinavian Journal of Medicine & Science in Sports* 2004;14(3):156-162.

46. Roi GS, Sisca G, Perondi F, Diamante A, Nanni G. Post competition blood lactate accumulation during a first league soccer season [abstract]. *Journal of Sports Sciences* 2004;22(6):560.

47. Turcotte LP, Kiens B, Richter EA. Saturation kinetics of palmitate uptake in perfused skeletal muscle. *FEBS Lett* 1991;279(2):327-329.

48. Saltin B. Metabolic fundamentals in exercise. *Med Sci Sports* 1973;5(3):137-146.

49. Jacobs I, Westlin N, Karlsson J, Rasmusson M, Houghton B. Muscle glycogen and diet in elite soccer players. *European Journal of Applied Physiology and Occupational Physiology* 1982;48(3):297-302.

50. Di Salvo V, Baron R, Tschan H, Calderon Montero FJ, Bachl N, Pigozzi F. Performance characteristics according to playing position in elite soccer. *Int J Sports Med* 2007;28(03):222-227.

51. Bangsbo J, Graham T, Johansen L, Strange S, Christensen C, Saltin B. Elevated muscle acidity and energy production during exhaustive exercise in humans. *Am J Physiol* 1992;263(4 Pt 2):R891-899.

52. Lamb GD, Stephenson DG, Bangsbo J, Juel C. Point:Counterpoint: Lactic acid accumulation is an advantage/disadvantage during muscle activity. *J Appl Physiol* 2006;100(4):1410-1412.

53. Sahlin K. Metabolic factors in fatigue. *Sports Med* 1992;13(2):99-107.

54. Y. Hellsten, E. A. Richter, B. Kiens, J. Bangsbo. Amp deamination and purine exchange in human skeletal muscle during and after intense exercise. *The Journal of Physiology* 1999;520(3):909-920.

55. Hoffman JR, Maresh CM, Armstrong LE, Gabaree CL, Bergeron MF, Kenefick RW, Castellani JW, Ahlquist LE, Ward A. Effects of hydration state on plasma testosterone, cortisol and catecholamine concentrations before and during mild exercise at elevated temperature. *Eur J Appl Physiol Occup Physiol* 1994;69(4):294-300.

56. Bangsbo J, Norregaard L, Thorsoe F. The effect of carbohydrate diet on intermittent exercise performance. *Int J Sports Med* 1992;13(2):152-157.

57. Bangsbo J, Graham TE, Kiens B, Saltin B. Elevated muscle glycogen and anaerobic energy production during exhaustive exercise in man. *The Journal of Physiology* 1992;451(1):205-227.

58. Williams C, Serratosa L. Nutrition on match day. *Journal of Sports Sciences* 2006;24:687-697.

59. Akermark C, Jacobs I, Rasmusson M, Karlsson J. Diet and muscle glycogen concentration in relation to physical performance in swedish elite ice hockey players. *Int J Sport Nutr* 1996;6(3):272-284.

60. Bussau V, Fairchild T, Rao A, Steele P, Fournier P. Carbohydrate loading in human muscle: An improved 1 day protocol. *European Journal of Applied Physiology* 2002;87(3):290-295.

61. Burke L, Kiens B, Ivy JL. Carbohydrates and fat for training and recovery. *Journal of Sports Sciences* 2004;22:15-30.

62. Burke L, Loucks A, Broad N. Energy and carbohydrate for training and recovery. *Journal of Sports Sciences* 2006;24:675-685.

63. Burke LM, Collier GR, Hargreaves M. Muscle glycogen storage after prolonged exercise: Effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* 1993;75(2):1019-1023.

64. Ivy JL, Katz AL, Cutler CL, Sherman WM, Coyle EF. Muscle glycogen synthesis after exercise: Effect of time of carbohydrate ingestion. *J Appl Physiol* 1988;64(4):1480-1485.

65. Jentjens. Determinants of post-exercise glycogen synthesis during short-term recovery. *Sports Medicine* 2003.

66. van Loon LJ, Saris WH, Kruijshoop M, Wagenmakers AJ. Maximizing postexercise muscle glycogen synthesis: Carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am J Clin Nutr* 2000;72(1):106-111.

67. Berardi JM, Price TB, Noreen EE, Lemon PW. Postexercise muscle glycogen recovery enhanced with a carbohydrate-protein supplement. *Med Sci Sports Exerc* 2006;38(6):1106-1113.

68. Tarnopolsky MA, Bosman M, Macdonald JR, Vandeputte D, Martin J, Roy BD. Postexercise protein-carbohydrate and carbohydrate supplements increase muscle glycogen in men and women. *J Appl Physiol* 1997;83(6):1877-1883.

69. Roy BD, Tarnopolsky MA. Influence of differing macronutrient intakes on muscle glycogen resynthesis after resistance exercise. *J Appl Physiol* 1998;84(3):890-896.

70. Carrithers JA, Williamson DL, Gallagher PM, Godard MP, Schulze KE, Trappe SW. Effects of postexercise carbohydrate-protein feedings on muscle glycogen restoration. *J Appl Physiol* 2000;88(6):1976-1982.

71. van Hall G, Shirreffs SM, Calbet JAL. Muscle glycogen resynthesis during recovery from cycle exercise: No effect of additional protein ingestion. *J Appl Physiol* 2000;88(5):1631-1636.

72. Howarth KR, Moreau NA, Phillips SM, Gibala MJ. Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. *J Appl Physiol* 2009;106(4):1394-1402.

73. Tsintzas OK, Williams C, Boobis L, Greenhaff P. Carbohydrate ingestion and glycogen utilization in different muscle fibre types in man. *J Physiol* 1995;489 (Pt 1):243-250.

74. Constable SH, Young JC, Higuchi M, Holloszy JO. Glycogen resynthesis in leg muscles of rats during exercise. *Am J Physiol* 1984;247(5 Pt 2):R880-883.

75. Kuipers H, Keizer HA, Brouns F, Saris WH. Carbohydrate feeding and glycogen synthesis during exercise in man. *Pflugers Arch* 1987;410(6):652-656.

76. Martinez-Lagunas V, Ding Z, Bernard JR, Wang B, Ivy JL. Added protein maintains efficacy of a low-carbohydrate sports drink. *Journal of Strength & Conditioning Research* 2010;24(1):48-59.

77. Spiller G, Jensen C, Pattison T, Chuck C, Whittam J, Scala J. Effect of protein dose on serum glucose and insulin response to sugars. *Am J Clin Nutr* 1987;46(3):474-480.

78. Levenhagen DK, Carr C, Carlson MG, Maron DJ, Borel MJ, Flakoll PJ. Postexercise protein intake enhances whole-body and leg protein accretion in humans. *Medicine & Science in Sports & Exercise* 2002;34(5):828-837.

79. Gibala MJ, Tarnopolsky MA, Graham TE. Tricarboxylic acid cycle intermediates in human muscle at rest and during prolonged cycling. *Am J Physiol Endocrinol Metab* 1997;272(2):E239-244.

80. Sahlin K, Katz A, Broberg S. Tricarboxylic acid cycle intermediates in human muscle during prolonged exercise. *Am J Physiol Cell Physiol* 1990;259(5):C834-841.

81. Cermak NM, Solheim AS, Gardner MS, Tarnopolsky MA, Gibala MJ. Muscle metabolism during exercise with carbohydrate or protein-carbohydrate ingestion. *Medicine & Science in Sports & Exercise* 2009;41(12):2158-2164.

82. Goetz FC, Greenberg BZ. A simple immunoassay for small amounts of insulin. *J Lab Clin Med* 1961;58:819-822.