Title

The efficacy of protein supplementation during recovery from muscle-damaging concurrent exercise

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Abstract

This study investigated the effect of protein supplementation on recovery following muscle-damaging exercise, which was induced with a concurrent exercise design. Twenty-four well-trained male cyclists were randomised to three independent groups receiving 20 g protein hydrolysate, iso-caloric carbohydrate or low-calorific placebo supplementation, per serve. Supplement serves were provided twice daily, from the onset of the muscle-damaging exercise, for a total of four days and in addition to a controlled diet (6 $g \cdot kg^{-1} \cdot d^{-1}$ carbohydrate, 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ protein, remainder from fat). Following the concurrent exercise session at time-point 0 h; a simulated high-intensity road cycling trial and 100 drop-jumps, recovery of outcome measures was assessed at 24, 48 and 72 h. The concurrent exercise protocol was deemed to have caused exercise-induced muscle damage (EIMD), owing to time effects ($p<0.001$), confirming decrements in maximal voluntary contraction (peaking at 15 ± 10%) and countermovement jump performance (peaking at 8 ± 7%), along with increased muscle soreness, creatine kinase and C-reactive protein concentrations. No group or interaction effects ($p>0.05$) were observed for any of the outcome measures. The present results indicate that protein supplementation does not attenuate any of the indirect indices of EIMD imposed by concurrent exercise, when employing great rigour around the provision of a quality habitual diet and the provision of appropriate supplemental controls.

Keywords: Concurrent training, exercise recovery, protein supplementation, EIMD, muscle soreness

Introduction

Performing a novel or unaccustomed bout of exercise can result in exercise-induced muscle damage (EIMD) that can negatively affect the ability to meet a high intensity demand during subsequent exercise. Specifically, a temporary reduction in maximal force production, increased muscle soreness, muscle swelling, passive tension, and the appearance of intramuscular proteins in the blood (Howatson and van Someren, 2008) are observed following the exercise. Of the indirect indices utilised in research, those that measure the functional capacity of the muscle, such as force production, appear to be the most meaningful index of EIMD (Warren et al., 1999). The presence of EIMD and hence, a decline in the force generating capacity of the muscle, poses obvious implications for athletic populations.

Performing strenuous exercise bouts that precipitate EIMD is associated with an increase in both muscle protein degradation and synthesis (Phillips et al., 1997). The provision of adequate nutrition is required to confer a positive protein balance following the exercise stimulus to increase myfobrillar protein synthesis rates (Moore et al., 2009, Tipton et al., 2003). Therefore, the consumption of sufficient protein following the exercise stimulus is a pre-requisite for muscle anabolism and hence, muscle remodelling following mechanical stress (Levenhagen et al., 2002). Further, this critical adaptive process of skeletal muscle might facilitate an attenuation in the indirect markers of EIMD following protein consumption (Saunders, 2007). Willoughby et al., (2003) reported an up-regulation of the ubiquitin-proteolytic pathway 48 h following an eccentric exercise bout; indicative of conditions that result in a reduced myofibrillar protein content (Hobler et al., 1998). Hence, efforts to alter protein metabolism and support myofibrillar protein synthesis as soon as possible following EIMD might facilitate recovery.

Additional protein is frequently consumed by elite and recreational athletes (Petroczi and Naughton, 2008, Tsitsimpikou et al., 2011) with the intention to improve exercise recovery (Erdman et al., 2007).

The evidence is equivocal, with research suggesting efficacy (Cockburn et al., 2008, Etheridge et al., 2008, Hoffman et al., 2010) and a lack of benefits (Green et al., 2008, White et al., 2008, Wojcik et al., 2001) for protein or coingested-protein supplementation to attenuate indices of EIMD. Fractional synthesis rates and fractional net balance are still elevated at 48 h following resistance exercise (Phillips et al., 1997). Hence, muscle remodelling can be stressed for at least 48 hours following a resistance exercise stimulus. Therefore, efforts to investigate the efficacy of a protein supplement following EIMD might benefit from providing additional protein across this timeframe of remodelling, increasing bioavailability compared to single bolus supplementation models. Further, it is suggested that study designs where participants are in negative nitrogen and/ or energy balance will present the greatest potential for ergogenic effects associated with protein supplementation (Pasiakos et al., 2014). It is therefore critical that research in this field is conducted with rigorous dietary control, whereby an intervention is assessed against appropriate controls and in addition to a standardised habitual diet.

Beyond the limitations of the literature, there is scant research concerning recovery from concurrent exercise. Concurrent exercise involves the simultaneous incorporation of both endurance and resistance exercise, (Fyfe et al., 2014) and is a prevalent training method for elite and recreational athletes that are aiming to elicit divergent physiological adaptations in parallel. Previous work examining recovery from EIMD has used resistance (Harrison and Gaffney, 2004, Miyama and Nosaka, 2004) or endurance (Bell et al., 2014, Betts et al., 2009) stimuli in isolation. Hypotheses exist for both mechanical and metabolic processes to determine the initial event of muscle fibre injury (Armstrong et al., 1991). Eccentric muscle action protocols have historically proved an effective means to impose damage, with sarcomere length inhomogeneity leading to mechanical disruption of the cell membrane (Morgan, 1990, Proske and Morgan, 2001). Further, exercise of a prolonged nature can result in degenerative changes to the muscle fibre, which lead to fibre necrosis, evidenced by the accumulation of macrophages and phagocytes (Armstrong, 1986). Hence, a concurrent exercise stimulus,

incorporating both eccentric muscle actions through resistance exercise and prolonged endurance activity incorporating high-intensity efforts, could prove an effective method to elicit muscle damage. Beyond the rationale of investigating concurrent exercise in the context of the hypotheses of muscle fibre injury, it is of interest to observe the profile of recovery from the combination of demanding resistance and endurance stimuli.

It is therefore of interest to observe whether indices of EIMD imposed by a concurrent exercise stimulus can be attenuated with protein supplementation. Beyond this, increasing our understanding of recovery from EIMD within such a pertinent training paradigm would be of importance, particularly so, given recent requests for greater specificity and context in post-exercise recovery recommendations (Minett and Costello, 2015). We hypothesised that the magnitude of EIMD would be reduced with protein supplementation compared to controls. Consequently, the aim of the study was to investigate the effect of protein supplementation on recovery following muscle-damaging exercise, induced with a concurrent exercise paradigm, in the context of a quality habitual diet which meets current guidelines (Thomas et al., 2016).

Materials and methods

Participants

Twenty-four male, well-trained endurance cyclists (age 27 ± 4 years; height 177.5 ± 7.9 cm; mass 73.7 ± 8.9 kg; $\dot{V}O_{2peak}$ 61.2 ± 5.8 ml·kg^{-1}·min^{-1}) who were regularly competing (at least a Category 3 British Cycling licence holder or an estimated 16.1 km TT of ≤23 min) volunteered to take part in the study. Following completion of a questionnaire to assess for eligibility and contraindications to the study, all participants provided written, informed consent. Prior to data collection, all procedures were given institutional research ethics approval and subsequently registered as a clinical trial (ClinicalTrials.gov, www.clinicaltrials.gov, NCT02458599). All study procedures were conducted in a laboratory accredited by the British Association of Sport and Exercise Sciences.

Experimental Design

The study utilised an independent group design that was double-blind, randomised and placebo-controlled (Figure 1). Following two preliminary trials for familiarisation to performance tests and collection of demographic characteristics, participants attended the laboratory on five consecutive days at the same time of day (± 1 h) to minimise the effects of diurnal variation. At each visit, participants were deemed fit for testing if they could confirm that they were free from concomitant medications/ vitamins and had refrained from external exercise, caffeine and alcohol since their last visit to the laboratory (24 h). Participants also refrained from consumption of nutritional supplements for the duration of the study period. In order, baseline data were collected for body mass (Seca 704 r, Seca, Hamburg, Germany), indices of muscle damage (creatine kinase)/ inflammation (C-reactive protein), perceived muscle soreness, isometric maximal voluntary contraction (MVC) of the dominant knee extensor, maximal countermovement jump (CMJ) height and cycling time trial (TT) performance.

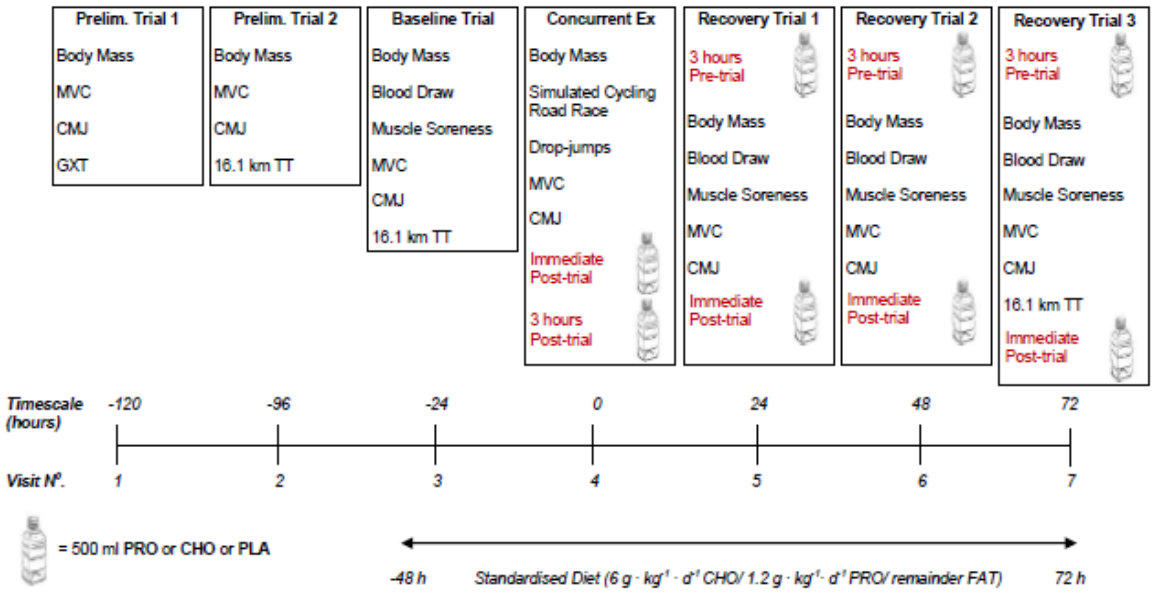

Figure 1. Study schematic. MVC = maximal voluntary contraction; CMJ = countermovement jump; GXT = graded exercise test; PRO = protein; CHO = carbohydrate; PLA = placebo.

Participants were then randomly assigned in a block fashion, using computer software, to one of three supplement groups; (1) whey protein hydrolysate, (2) iso-caloric carbohydrate, (3) low-calorific placebo. Supplements were consumed twice daily, for a period of four days, commencing at the end of the concurrent exercise visit, following the CMJ (Figure 1). Non-supplemental dietary intake was standardised (6 $g \cdot kg^{-1} \cdot d^{-1}$ carbohydrate, 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ protein, remainder from fat) and controlled during the trial (-48 to 72 h). Each group completed the concurrent exercise session at time-point 0 h; a simulated high-intensity road cycling trial and 100 drop-jumps. Recovery from the concurrent exercise bout was assessed by repeating baseline measures at 24, 48 and 72 h. Exceptions were TT performance (repeated at 72 h only) and MVC/ CMJ (also assessed at 0 h). TT performance has previously been reported to be impaired at 48 h following damaging exercise (Burt and Twist, 2011). However, TT performance was repeated only at 72 h, in a bid to improve ecological validity, by better mimicking the amount of time that would be allowed for recovery from a heavy training stimulus prior to a competitive effort.

Nutritional Supplement

The nutritional content of the supplements are listed in Table 1. Participants ingested 500 ml of either HYDRO.365 Berry (Arla Foods Ingredients Group P/S, Viby, Denmark), Powerade ION4 Berry and Tropical (Coca-Cola Enterprises, Middlesex, UK) or Powerade Zero Berry and Tropical (Coca-Cola Enterprises, Middlesex, UK) on each supplement occasion. The supplement choice was such that the PRO condition provided 20 g of whey protein hydrolysate as an experimental intervention, with the CHO condition providing an iso-caloric control and the PLA condition a low-calorific placebo. This approach would help to establish whether potential ergogenic effects of protein supplementation were owing to protein or increased energy consumption. A total of eight supplemental beverages were consumed and these occurred at 3 h preceding, directly following, or 3 h following a laboratory visit during visits 4-7 (Figure 1). Each serving was in a ready-to-drink format, which was blinded to both participant and investigator, and dispensed by a scientist that was independent to the study. Beverages were dispensed on a daily basis, with one consumed immediately following the CMJ (visits 4, 5 and 6) or 16.1 km TT protocols (visit 7) and the other(s) at a time detailed in the daily food plan. In order to test the blinding process at study completion, the dispensing scientist asked participants which supplemental condition they perceived to have been assigned to. The blind was not broken until all statistical analyses were complete.

Table 1. Nutritional content of the supplements.

	PRO	**CHO**	**PLA**
Energy (kcal)	90	90	5
Protein (g)	20	Nil	Nil
Carbohydrate (g)	1.85	20.5	Nil
Fat (g)	0.05	Nil	Nil

Quantities are per 500 ml serve. PRO = protein; CHO = carbohydrate; PLA = placebo

Dietary Control

Non-supplemental dietary intake was standardised (6 $g \cdot kg^{-1} \cdot d^{-1}$ carbohydrate, 1.2 $g \cdot kg^{-1} \cdot d^{-1}$ protein, remainder from fat) and controlled during the trial (-48 to 72 h). The diet was sourced from a tailored menu provider (Soulmatefood Ltd, Lancashire, UK) and designed in line with ACSM recommendations (Thomas et al., 2016). In detail, carbohydrate and protein were prescribed relative to body mass, before selecting a range (20 - 27%) for the contribution of energy from fat, which was within the range recommended by the ACSM guidelines; 20 - 35%. These criteria resulted in categories for daily total kcal, which were a result of body mass; 2,500 kcal = 63.0 - 69.4 kg/ 2,750 kcal = 69.5 - 76.4 kg/ 3,000 kcal =76.5 - 83.3 kg/ 3,250 kcal = 83.4 - 90.3 kg/ 3,500 kcal = 90.4 - 97.2 kg. Food was delivered to participants' home address on two occasions and labelled such that four meals were provided daily, to be consumed at times specified in the participants' daily food plan. This plan ensured that food distribution throughout the day was standardised and that food was not consumed for at least 1 h following a supplement serving. Food content was analysed using dietary analysis software (Nutritics v4.108, Nutritics Ltd., Co. Dublin, Ireland), in order to confirm content against request.

Assessment of Peak Oxygen Uptake ($\dot{V}O_{2peak}$ ***)***

Following a graded 20 min warm-up (4 min incremental stages), participants cycled at a power output of 200 W using a magnetically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, USA). Power output was subsequently increased by 4 W every 10 s (24 $W \cdot min^{-1}$) until volitional exhaustion. Expired gas and heart rate were collected throughout the test, with the test being terminated when the participant was unable to maintain the workload. Expired gas data were averaged across 30 s intervals using online gas analysis software (MetaSoft_Studio, Cortex., Leipzig, Germany), before downloading for subsequent assessment. $\dot{V}O_{2peak}$ was calculated as the highest 30 s average collected during the maximal test. An online gas analyzer (Metalyzer 3B, Cortex., Leipzig, Germany) was used throughout the protocol to measure oxygen and carbon dioxide fractions and volume of gas in inspired and expired air. The analyzer was calibrated for oxygen and carbon dioxide fractions and gas volume (3 L

syringe), as per manufacturer's instructions. During the test, participants breathed through a low dead space (70 mL) mouth piece, low resistance turbine, while inspired and expired gas was sampled continuously at 50 Hz. Routine QA records demonstrated a laboratory coefficient of variation for $\dot{V}O_2$ data of 1.8% in the range of 2.05–3.94 $L \cdot min^{-1}$.

Muscle Damaging Concurrent Exercise

The concurrent exercise session was performed in the fed state, with meal timing standardised at 2 h prior to exercise, and consisted of a high-intensity simulated cycling road race and a drop-jump protocol, separated by a 15 min rest period. The cycling protocol (Table 2) was performed using a magnetically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, USA) and has previously been used to simulate the demands of cycling road racing (Vaile et al., 2008). In brief, participants completed a 10 min self-selected warm-up including 3 × 3 s sprints at 7, 8 and 9 min, before completing a total of 66 sprints of a 5, 10 or 15 s duration, with a work (W) to rest ratio of 1:6, 1:3 or 1:1. Sprints were divided into 9 sets, with a period of active recovery completed between sprints and between sets. Intensity during active recovery was maintained at 40%–50% W achieved at $\dot{V}O_{2peak}$. Furthermore, 9 min of sustained effort was incorporated into the trial through the performance of time trials of 2 min (after sets 3 and 6) and 5 min (after set 9) duration. During all sprints and time trials, participants were encouraged to complete as much work as possible and the total duration of the trial was 109 min. Heart rate was continually recorded throughout each trial using wireless telemetry (T31 transmitter, Polar Electro Ltd., Kempele, Finland) and participants were cooled with an electric fan on a standardised setting. Strong standardised verbal encouragement was provided throughout and water was made available to participants *ad libitum*.

Table 2. High-intensity simulated cycling road race; sprint frequency/ duration and work to rest ratio composition (Vaile et al., 2008).

10 min warm up (self-selected pace)		
Set Number	**Sprint Frequency x Duration**	**Work : Rest Ratio**
1	12 x 5 s	1 : 6
2	12 x 5 s	1 : 3
3	12 x 5 s	1 : 1
4 min Active Recovery - *2 min TT* - 4 min Active Recovery		
4	6 x 10 s	1 : 6
5	6 x 10 s	1 : 3
6	6 x 10 s	1 : 1
4 min Active Recovery - *2 min TT* - 4 min Active Recovery		
7	4 x 15 s	1 : 6
8	4 x 15 s	1 : 3
9	4 x 15 s	1 : 1
5 min Active Recovery - *5 min TT* - 5 min Active Recovery		

The allotted rest period of 15 min comprised passive rest (5 min), standardised dynamic stretching (5 min) and a drop-jump briefing (5 min). Participants then performed a total of 100 drop-jumps from a height of 0.63 m, whereby they were encouraged to jump vertically with maximal force immediately upon landing. The protocol was separated into five sets of 20 drop-jumps, with 10 s rest between each jump and 120 s between each set. Strong standardised verbal encouragement was provided throughout. This protocol has consistently demonstrated a muscle damage response (Goodall and Howatson, 2008, Miyama and Nosaka, 2004) and was selected for the endurance trained cohort, due to a lower skill/ technical demand compared to more applied resistance stimuli.

Performance Tests

Isometric MVC was assessed following a standardised warm up of 5 min at 200 W on a cycle ergometer (Wattbike, Wattbike Ltd., Nottingham, UK). Participants completed three, 3 s MVCs of the knee extensors, separated by 60 s rest, using a Cybex isokinetic dynamometer (Cybex Humac Norm, Computer Sports Medicine Inc., CA, USA). In a seated position, participants initiated a leg extension action against a fixed arm, secured proximal to the ankle joint, immediately above the malleoli. Peak torque was assessed for the participants' dominant leg, at a knee joint angle of 70° from horizontal, assessed with a goniometer (Bodycare Products, Warwickshire, UK). A knee joint angle of 70° has previously been reported to be susceptible to significant loss of isometric strength following a single bout of eccentric exercise (McHugh and Tetro, 2003). CMJ performance was assessed using the OptoJump system (OptoJump, Microgate S.r.l., Bolzano, Italy), with three maximal jumps performed on each testing occasion, each separated by 60 s rest. Participants were instructed to place their hands on their hips, descend rapidly to ~90° knee joint angle, and then jump as high as possible. Standardised verbal encouragement for each effort and the peak value generated across the three repetitions was used for data analysis, in both assessments. The intra-individual reliability of these measures returned a coefficient of variation of 3.8% and 0.9% for the MVC and CMJ protocols, respectively.

Following a standardised 5 min warm up at an intensity of 50% $\dot{V}O_{2peak}$, participants completed a 16.1 km TT using a magnetically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, USA). The assessment required participants to complete a distance of 16.1 km in as short a time as possible, while being blinded to time elapsed. The trial started with the ergometer set in the lowest possible gear ratio, whereby after a 3 s count-down, the participant was responsible for manipulating gearing to a desired level. Feedback of performance data was withheld, except distance elapsed, which was communicated every 1.6 km and participants were permitted to change gears as and when they felt necessary. Heart rate was continually recorded throughout each trial, using wireless telemetry (T31 transmitter, Polar Electro Ltd., Kempele, Finland) and participants were cooled with an electric fan at

a standardised setting, with water available *ad libitum*. The intra-individual reliability of these measures returned a coefficient of variation 1.1% for this protocol.

Muscle Soreness Assessment

Participants were asked to hold a fixed squat position at an ~90° joint angle, while rating perceived muscle soreness on a 20 cm visual analogue scale, consisting of a line from 0 cm (no pain) to 20 cm (pain as bad as it could be). Using similar scales, previous research reports significantly increased soreness following EIMD protocols (Goodall and Howatson, 2008, Mohr et al., 2016).

Blood Sampling and Analysis

A venous blood sample was collected from a branch of the basilica vein in the anti-cubital fossa region using venepuncture method, for assessment of indices of muscle damage (creatine kinase) and inflammation (C-reactive protein). A total of ~10mL of blood was collected into a silica additive serum vacutainer (367896, BD Diagnostics, Dubai, UAE) and rested for 60 min to clot. The vacutainer was then centrifuged at 1300x g, 25°C for 10 min (Heraeus Multifuge 3SR Plus, Thermo Fisher Scientific Inc., MA, USA). The resultant supernatant was pipetted into aliquots and immediately stored at −80 °C, for later analysis.

Serum CK was analysed using a CK NAC-activated ELISA kit (Randox Laboratories Ltd., County Antrim, UK). Hence, a 10 μL serum sample was mixed with 500 μL of reagent and measured at 37°C at a 340 nm wavelength, using an Rx Monza clinical chemistry analyser (Randox Laboratories Ltd., County Antrim, UK). The manufacturer reports intra-assay and inter-assay coefficients of variation for this protocol at 1.6-2.3% and 3.4-3.9%, respectively. Serum C-reactive protein was analysed in duplicate using a Human CRP ELISA kit (R&D Systems Europe Ltd., Abingdon, UK). Hence, a 10 μL serum sample underwent a 100-fold dilution with calibrator diluent, before being processed in accordance with the manufacturer's instructions. Optical densities were determined at 540 nm and 450 nm, using a Fisher

Scientific Multiskan FC Microplate Reader (Thermo Fisher Scientific Inc., MA, USA). Blank subtracted values were averaged and 450 nm readings were corrected with 540 nm values. Standard and controls were plotted as a standard curve with data linearised by producing log scales of both axes. The manufacturer reports intra-assay and inter-assay coefficients of variation for this protocol at 3.8-8.3% and 6.0-7.0%, respectively.

Statistical Analysis

Data are presented as mean ± standard deviation (SD), with statistical significance set at $p \leq 0.05$ *a priori*. Sphericity was assumed if Mauchly's test score returned $p \geq 0.05$, with Greenhouse-Geiser adjustments made where appropriate. All criterion measures were analysed using a group (PRO vs. CHO vs. PLA) by time-point (-24, 0, 24, 48, 72 h) repeated measures analysis of variance (ANOVA). MVC and CMJ analysis included five time-points (-24, 0, 24, 48, 72 h), while four time-points (-24, 24, 48, 72 h) were assessed for muscle soreness and blood markers, with just two time-points (-24 and 72 h) used for analysis of TT performance. Significant main effects were further investigated using LSD *post-hoc*, pair-wise comparisons. All data analysis was performed using statistical software (IBM SPSS 22 for Windows, New York, USA). Where appropriate, data were normalised using percentage change from baseline, to account for differences in baseline measures. Statistical power of the study was calculated using G*Power statistical software (v3.1.9, Düsseldorf, Germany) on the basis of research investigating the effect of protein-based supplementation *vs.* carbohydrate control on maximal muscle function (lower-body 1 repetition maximum or peak torque during extension/flexion) at 48 h post-damage (Cockburn et al., 2008, Hoffman et al., 2010, Rankin et al., 2015), returning a hypothesised effect size of 0.3 and a subsequent sample size of 21 subjects i.e. 7 subjects per group, with α set at 0.05 and sufficient statistical power of 0.8 (Cohen, 1992).

Results

No differences in demographic descriptive variables between groups were observed ($p>0.05$), upon initial presentation (Table 3). There were no significant differences in the prescribed energy intake, or contribution of macronutrients to daily energy intake between groups ($p>0.05$). Daily prescribed energy intake per group is listed in Table 4. The concurrent exercise protocol was deemed to have caused EIMD, owing to time effects ($p<0.001$), confirming decrements in MVC and CMJ performance, along with increased muscle soreness, CK and CRP. The only measure devoid of time effects was the 16.1 km TT, which was assessed at baseline and 72 h only.

Table 3. Baseline demographic descriptive data.

Supp. Group	Age (yr)	Height (cm)	Body Mass (kg)	$\dot{V}O_{2peak}$ (ml·kg^{-1}·min^{-1})	MVC (Nm)
PRO	27 ± 3	178.8 ± 9.7	75.9 ± 8.9	62.3 ± 4.7	271.9 ± 55.9
PLA	28 ± 5	174.9 ± 5.1	72.7 ± 8.4	60.0 ± 9.0	229.8 ± 32.2
CHO	26 ± 5	178.9 ± 8.7	72.4 ± 10.0	61.2 ± 2.2	261.8 ± 50.1

Values presented as mean ± SD. PRO = protein; CHO = carbohydrate; PLA = placebo

Table 4. Daily prescribed energy intake.

Supp. Group	Body Mass (kg)	TOTAL (kcal)	CHO (kcal)	PRO (kcal)	FAT (kcal)
PRO	75.9 ± 8.9	2813 ± 320	1821 ± 213	364 ± 43	627 ± 88
PLA	72.7 ± 8.4	2750 ± 327	1745 ± 201	349 ± 40	656 ± 111
CHO	72.4 ± 10.0	2750 ± 327	1738 ± 241	348 ± 48	665 ± 73

Values presented as mean ± SD. PRO = protein; CHO = carbohydrate; PLA = placebo

Performance

A time effect was observed for both MVC ($F_{(2.7,57.6)} = 30.305$, $p<0.001$) and CMJ ($F_{(2.8,57.7)} = 7.342$, $p<0.001$), with no group or interaction effects ($p>0.05$) (Figures 2 and 3). Peak detriment for MVC was at 0 h for all groups; 82.6 ± 7.9, 84.5 ± 11.1 and 88.9 ± 11.4 % of baseline for PRO, PLA and CHO, respectively, with significant decrements from baseline at 0 and 24 h ($p<0.001$). This was consistent

with CMJ performance, with all groups registering lowest values at 0 h; 93.4 ± 7.5, 91.3 ± 8.3 and 92.5 ± 5.6 % of baseline for PRO, PLA and CHO, respectively, with significant decrements from baseline at 0 ($p<0.001$), 24 ($p=0.032$) and 48 h ($p=0.036$). No time effect was observed for TT time or average $W \cdot kg^{-1}$ sustained during the TT ($p>0.05$) and neither of these measures displayed group or interaction effects ($p>0.05$).

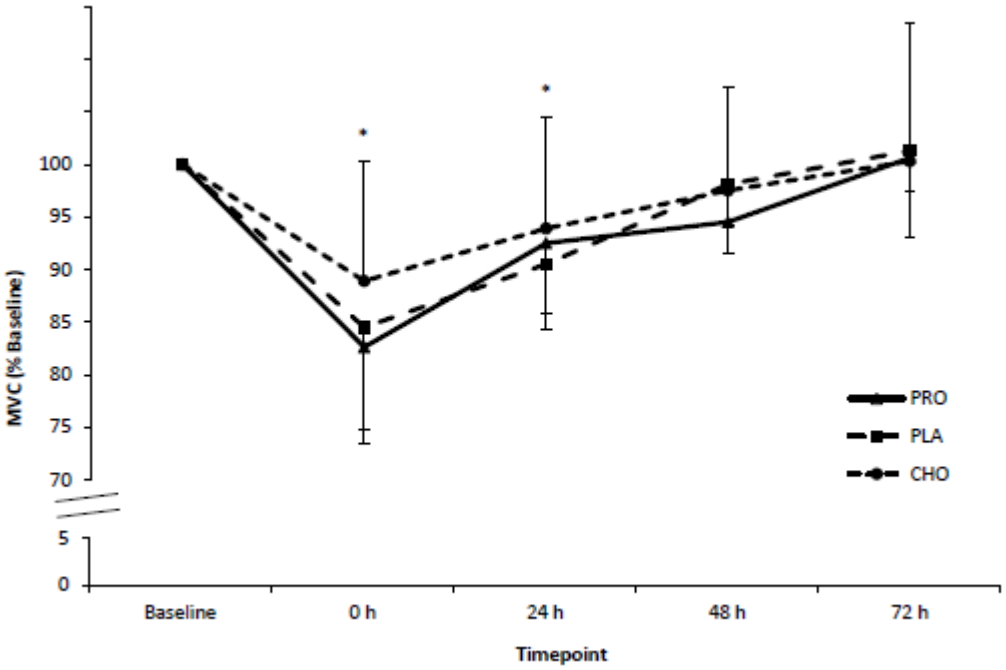

Figure 2. Isometric maximal voluntary contraction response (% change from baseline) to the concurrent exercise protocol in the PRO (n = 8), PLA (n = 8) and CHO (n = 8) groups. Absolute baseline values were 271.9 ± 55.9, 229.8 ± 32.2 and 261.8 ± 50.1 Nm for PRO, PLA and CHO, respectively. *, significantly different from baseline ($p<0.05$). Values presented as mean ± SD. PRO = protein; CHO = carbohydrate; PLA = placebo.

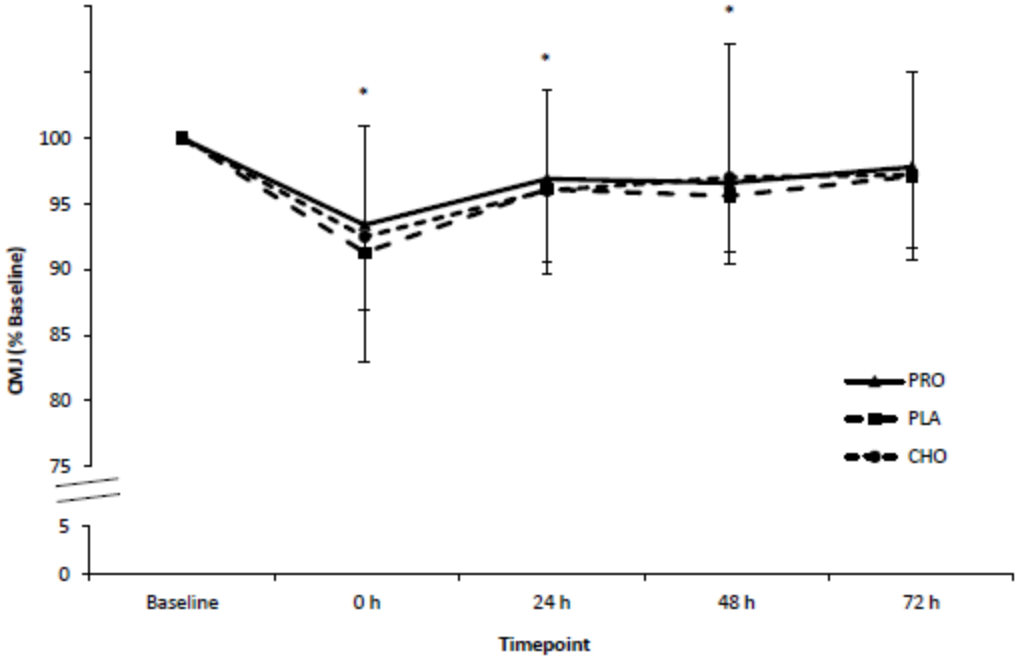

Figure 3. Countermovement jump response (% change from baseline) to the concurrent exercise protocol in the PRO (n = 8), PLA (n = 8) and CHO (n = 8) groups. Absolute baseline values were 33.4 ± 4.3, 33.9 ± 2.8 and 32.7 ± 3.5 cm for PRO, PLA and CHO, respectively. *, significantly different from baseline ($p<0.05$). Values presented as mean ± SD. PRO = protein; CHO = carbohydrate; PLA = placebo.

Muscle Soreness

A time effect, $F_{(1.7,36.6)} = 73.609$, $p<0.001$ was observed (Figure 4), but similar to performance measures, there was no difference between groups ($p>0.05$), nor was there an interaction between group and time ($p>0.05$). Muscle soreness was significantly elevated from baseline at all time-points ($p<0.001$).

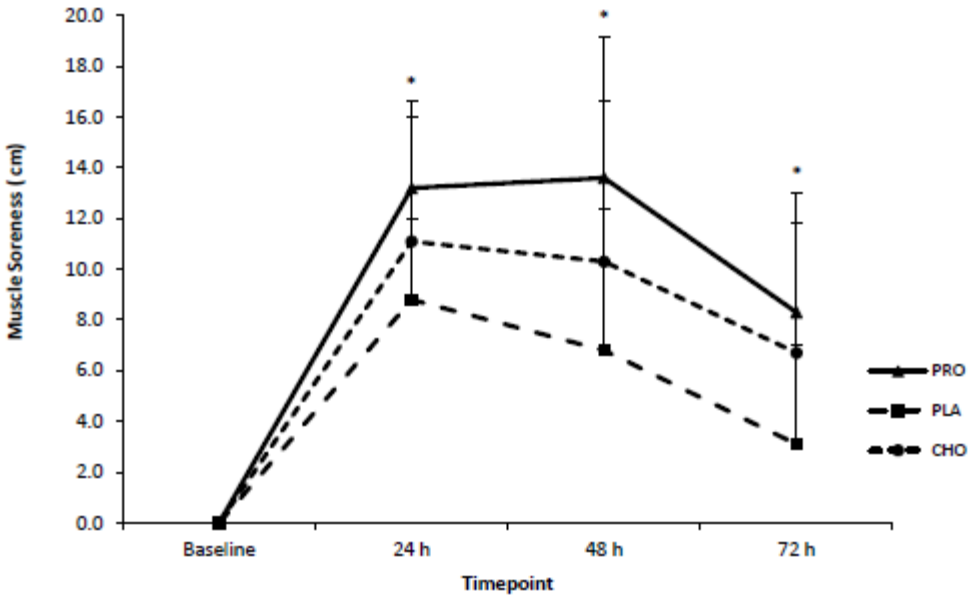

Figure 4. Muscle soreness response to the concurrent exercise protocol in the PRO (n = 8), PLA (n = 8) and CHO (n = 8) groups. *, significantly different from baseline ($p<0.05$). Values presented as mean ± SD. PRO = protein; CHO = carbohydrate; PLA = placebo.

Serum Proteins

Both CK and CRP displayed time effects, $F_{(1.5,27.9)} = 27.867$, $p<0.001$ and $F_{(1.1,20.4)} = 19.765$, $p<0.001$, respectively. However, there were no group or interaction effects ($p>0.05$) for either of these biomarkers of muscle damage and inflammation. Absolute baseline values for CK were 258.4 ± 103.1, 185.3 ± 103.9 and 218.1 ± 56.7 $IU \cdot L^{-1}$ for PRO, PLA and CHO, respectively. All groups displayed a similar CK profile, with peak values experienced at 24 h; 282.4 ± 166.2, 340.7 ± 167.2 and 291.1 ± 177.1 % of baseline for PRO, PLA and CHO, respectively. Both CK and CRP were significantly elevated from baseline at 24 and 48 h ($p<0.05$). Absolute baseline values for CRP were 1.13 ± 0.66, 2.04 ± 2.92 and 0.66 ± 0.54 $mg \cdot L^{-1}$ for PRO, PLA and CHO, respectively. The time course of CRP was similar to that of CK, with peak values experienced at 24 h; 205.6 ± 110.4, 260.4 ± 206.9 and 240.7 ± 177.9 % of baseline for PRO, PLA and CHO, respectively.

The body mass reduction experienced across the trial period for the pooled participants was significant $F_{(3.8,79.2)} = 9.399$, $p<0.001$. However, there was no difference in the body mass reduction between groups ($p>0.05$); -0.75 ± 0.66, -1.08 ± 0.70 and -0.36 ± 0.47 kg for PRO, PLA and CHO, respectively. Upon exit questioning, none of the participants perceived to be receiving protein supplementation and only three participants noted to be unsure of which supplement condition they were receiving. Across all conditions, there was a group majority to select carbohydrate as the perceived treatment condition received (Table 5).

Table 5. Participant selection of perceived treatment condition at the conclusion of study participation.

Supplement Group	PLA Count	PRO Count	CHO Count	Unsure Count
PRO	3	0	4	1
PLA	0	0	7	1
CHO	2	0	5	1

PRO = protein; CHO = carbohydrate; PLA = placebo

Discussion

The primary finding was that protein supplementation did not attenuate any of the indirect indices of EIMD. Beyond this, the research profiled the recovery from EIMD imposed within a concurrent training paradigm.

In order to assess the efficacy of protein supplementation, it was of primary importance that the intense bout of concurrent exercise was of sufficient design to elicit a response in indirect indices of EIMD, which was suggestive of muscle damage. All five indices assessed in the 48 h following the concurrent exercise session displayed significant time effects, indicating that the concurrent exercise bout was effective in eliciting a muscle damage response. Isometric MVC performance was reduced by a peak magnitude of 15% from baseline across the three groups, with time effects at 0 and 24 h. A review by (Clarkson and Hubal, 2002) suggests that eccentric-biased exercise protocols result in force decrements of 10-65% for between 24 h to 2 wk, depending on whether a lower or higher-force eccentric exercise protocol has been utilised. Hence, the decrement in magnitude of force and speed of return to baseline values observed in this study are at the lower end of the spectrum of reported values, compared to EIMD imposed exclusively through eccentric-biased muscle action protocols.

The muted performance decrements observed in this study could have been influenced by the metabolic challenge of the cycling protocol, with high demand on the glycolytic pathway, performed prior to the drop-jump protocol. Endurance exercise is known to significantly deplete intra-muscular glycogen levels (Noakes et al., 1988), which is associated with a concomitant reduction in the capacity to produce optimal muscular strength (Jacobs et al., 1981). Similarly, 2 h of cycling at 65% maximal aerobic power reduced eccentric muscular peak torque by 14% in well-trained cyclists, with these outcomes ascribed to a decline in the neural input to the muscle and peripheral mechanisms (Lepers et al., 2000). If the maximal force output of the muscle was compromised due to the cycling protocol, it is feasible that this would compromise the subsequent drop-jump performance. Further, the lack of

time effect at 48 and 72 h for MVC performance might contribute to the lack of efficacy for protein supplementation. Previous research concerning eccentric muscle damage has reported the magnitude of EIMD to rise progressively from 0 to 48 h, with significance established at 48 h (Cockburn et al., 2008). Authors postulate that thier observations may be related to protein metabolism research in animals; Lowe et al., (1995) displayed that the increase in protein degradation is not significant until 48 h following eccentric contraction-induced injury, with protein balance declining from 24-336 h, resulting in a negative state from pre-damage values. If this were to hold true in human skeletal muscle, it is tenable to expect protein supplementation to display efficacy, if any, when protein metabolism is most stressed, possibly supporting recovery from 48 h onwards. As the deleterious effects in force output from our exercise stimulus were not significant and diminishing at 48 h post-exercise, we may not have imposed damage of a sufficient length of time in order to investigate the benefit of protein in the recovery process of this outcome measure.

CMJ performance declined by a peak magnitude of 8% from baseline across the three groups, with an effect for time at 0, 24 and 48 h. Using an identical drop-jump protocol, others have reported varying peak decrements in CMJ performance, lasting up to 96 h; ~8% (Howatson et al., 2012) and ~25% (Miyama and Nosaka, 2004). It is feasible that the discrepancies in magnitude and resolution of CMJ performance might be explained in part by participant demographics, with Howatson et al., (2012) using a population regularly competing and Miyama and Nosaka, (2004) observing an un-trained population. The training history of the participants may represent a conditioning bout of exercise and lead to a reduction in the resolution and magnitude of EIMD indices in subsequent bouts, as has been discussed previously (McHugh, 2003). The repeated bout effect, facilitates a faster recovery of muscle function following a superseding bout of damaging exercise for up to 9 months (Nosaka et al., 2001), and may help to explain these findings. This is especially true given that evidence suggests a prophylactic effect even when less-severe damaging exercise is performed prior to a more severe bout of damaging exercise (Clarkson and Tremblay, 1988). It should however be noted, that the participants

in our research were a well-trained cohort of endurance cyclists and as such, are unlikely to have benefited from the repeated bout effect. Exclusion criteria restricted individuals that had previously participated in a study involving activity to elicit EIMD, however participants were not excluded on the basis of strength training history. A more tenable explanation for the muted decrement in CMJ performance could be the preceding concentric work in the cycling trial. Nosaka and Clarkson, (1997) reported an attenuation of muscle damage from 12 maximal eccentric actions, when preceded by 100 repetitions of concentric muscle actions. This attenuation was evident in several indices of muscle damage; including force output, muscle soreness, and CK activity.

Perceived muscle soreness was significantly elevated from baseline at all time-points, with peak values expressed at 24 to 48 h. This response, with peak soreness of ~55% of maximum across all groups, is similar to that observed in the literature. Cockburn et al., (2008) reported a peak soreness of ~70% of maximum with time effects still apparent at the final time-point of 48 h, while others have reported peak values of ~60% of maximum soreness and time effects through to 72 h (Jackman et al., 2010, Rankin et al., 2015). An average peak CK value of 610 $IU \cdot L^{-1}$ was observed at 24 h, across the three groups. This response in CK is consistent with other literature profiling recovery from EIMD with an eccentric muscle action bias; 539 $IU \cdot L^{-1}$ (Cockburn et al., 2008), 757 $IU \cdot L^{-1}$ (Green et al., 2008), 974 $IU \cdot L^{-1}$ (Miyama and Nosaka, 2004). The range in these reported values is likely explained by variation in the participant characteristics and protocol used to induce muscle damage, with the highest CK values observed in work by Miyama and Nosaka, (2004) which used a protocol of the highest volume, in a large muscle mass, in a student cohort with little or no training experience.

The elevated levels of CK in blood is indicative of disruption to the muscle membrane, with peak values normally evident no earlier than 24 h post-exercise (Warren et al., 1999). This increase in 'leakage' of intramuscular proteins into the bloodstream is just one of the events that are observed following EIMD, which appear as part of the cascade of events following the disruption of the intracellular Ca^{2+}

homeostasis (Gissel and Clausen, 2001). The increased CRP values, which peaked at 24 h, suggest that the exercise stimulus was also sufficient to elicit an inflammatory response. Observations from biopsied muscle samples that have undergone eccentric muscle damage, suggest the addition of new sarcomeres and de novo synthesis in response to the stimulus (Yu et al., 2004, Yu et al., 2003). This remodelling of the muscle, with the associated reduction in contractile protein content (Warren et al., 2002, Willoughby et al., 2003) is suggestive of an environment that would drive a requirement for protein uptake. Hence, the response in both CK and CRP subsequent to the concurrent exercise bout is suggestive of a stimulus that would place demands on protein availability, despite the lack of group effects reported in this study.

It seems logical that the smaller responses in EIMD indices in this study, particularly MVC and CMJ performance, provide a smaller window for an intervention to display efficacy. For example, if the magnitude of damage is small, then the opportunity to observe the effect of an intervention is small. Similarly, if the duration of performance decrement is acute, when investigating a process (EIMD) which possibly stresses protein metabolism maximally from 48 h onwards (Lowe et al., 1995), the opportunity to observe the affect of supplementation is reduced. Hence, the muted responses in this study, regardless of reason, might act to reduce the possibility of observing any potential beneficial effect of protein supplementation on recovery from EIMD. Further, we chose to provide protein supplements across the observed recovery period of 72 h, with the aim of increasing bioavailability on subsequent days, whereby fractional synthesis rates remain elevated following resistance exercise (Phillips et al., 1997). This design ensured an increased quantity of supplemental protein in comparison to single bolus models in the literature, of which some have displayed efficacy for protein in supporting recovery from muscle damage (Cockburn et al., 2008, Etheridge et al., 2008). It therefore seems unlikely that the quantity of protein supplementation provided in this study would explain the lack of efficacy in supporting the recovery process.

Much of the research relating to EIMD suffers from a limitation, which is often acknowledged, in that there is a lack of control concerning habitual diet (Cockburn et al., 2008, Howatson et al., 2012, White et al., 2008). This is problematic on two counts; with underlying discrepancies in macronutrient intake affecting bioavailability of a given substrate and a likely variance in the energy balance within or between experimental groups. Activating the mTORC1 signaling pathway is a pertinent outcome of resistance exercise, owing to its regulatory role in muscle protein remodelling (Drummond et al., 2009, Philp et al., 2011). A recent review has highlighted the direct or indirect affect that nutrition can have on the known inputs that regulate mTORC1; amino acids, glucose, and growth factors (Drummond et al., 2009). This is supported by evidence that essential amino acids have a stimulatory effect on muscle protein synthesis (Groen et al., 2015) and the observation that carbohydrate ingestion post-resistance exercise acts to reduce protein breakdown (Borsheim et al., 2004). Hence, control of nutrient intake is of the utmost importance when conducting research involving muscle remodelling as part of the recovery process. Great effort was taken in an attempt to account for some of the limitations existing in this field of research, with a habitual diet designed in line with ACSM recommendations provided to participants' home addresses. A small, but significant reduction in body mass across all subjects was observed in the current study, despite the prescription of a diet that was intended to facilitate energy balance. It is not possible to compare the response in body mass change with data from the literature, as this outcome is seldom reported. It could be argued that this data would be insightful in designs possessing discrepancies in energy content between supplemental conditions (Cockburn et al., 2008, Hoffman et al., 2010). Such designs, where participants may have experienced a significant energy deficit and one experimental group has not been allocated to an iso-caloric supplement control, would likely damage the construct validity of research aiming to investigate the efficacy of protein supplementation.

The only outcome measure devoid of a significant time effect was 16.1 km TT performance, which was only repeated at 72 h, in order to mimic the applied scenario of completing a heavy mid-week training

session (0 h) prior to weekend competition (72 h). Hence, any negative effects of the concurrent damaging exercise are resolved by 72 h post-exercise and do not negatively affect 16.1 km time trial performance. Given the desire to increase ecological validity with the experimental design, it was imperative that a well-trained cohort were recruited ($\dot{V}O_{2peak}$ of 61.2 ml·kg^{-1}·min^{-1}). However, with regards to application, it should be acknowledged that although the 16.1 km TT performance measure was assessed at time-points to mimic the applied scenario of completing a heavy mid-week training session prior to competition, the exercise events from baseline through to 72 h would not constitute a regular training week. This should be taken into consideration when deciding whether the observations from this research should inform an athlete's approach to recovery from an intense bout of concurrent exercise.

In summary, these data fail to support the efficacy of protein supplementation in attenuating the relatively modest indices of EIMD imposed by concurrent exercise, when employing great rigour around the provision of a quality habitual diet which met protein intake recommendations and the provision of appropriate supplemental controls. They also offer new information regarding the recovery from muscle damaging exercise imposed with a concurrent exercise paradigm, in a well-trained cohort.

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