The Effect of Post-Exercise Carbohydrate and Protein Ingestion on Bone Metabolism

Rebecca Townsend[1,2], Kirsty J. Elliott-Sale[1], Kevin Currell[2], Jonathan Tang[3], William D. Fraser[3], Craig Sale[1]

[1]Musculoskeletal Physiology Research Group, Sport, Health and Performance Enhancement Research Centre, School of Science and Technology, Nottingham Trent University, UK.

[2]The English Institute of Sport, EIS Performance Centre, Loughborough University, UK.

[3]Norwich Medical School, University of East Anglia, UK, Norfolk and Norwich University Hospital Norfolk UK.

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Corresponding author: Professor Craig Sale, Musculoskeletal Physiology Research Group, Sport, Health and Performance Enhancement Research Centre, School of Science and Technology, Nottingham Trent University, NG11 8NS, UK. E-mail: craig.sale@ntu.ac.uk.

Abstract

Purpose

To investigate the effect of feeding carbohydrate and protein (CHO+PRO), immediately or 2 h after an exhaustive run, on the bone turnover response in endurance runners.

Methods

10 men (age 28±5 y, height 1.74±0.05 m, body mass 69.7±6.3 kg) performed treadmill running at 75%VO_{2max}, until exhaustion, on three occasions. Blood was collected before and immediately, 1, 2, 3, 4 and 24 h post-exercise, for measurement of β-CTX, P1NP, PTH, PO_4, ACa and Ca^{2+}. This was a randomised, counterbalanced, placebo-controlled, single-blinded, cross-over study. The three trials were; i) placebo (PLA), PLA solution was ingested immediately and 2 h post-exercise, ii) immediate feeding (IF), CHO+PRO (1.5 $g \cdot kgBM^{-1}$ dextrose and 0.5 $g \cdot kgBM^{-1}$ whey) were ingested immediately post-exercise and PLA 2 h post-exercise, and iii) delayed feeding (DF), PLA was ingested immediately post-exercise and CHO+PRO solution 2 h post-exercise. Data were analysed using repeated measures ANOVA and *post-hoc* Tukey's HSD.

Results

At 1 and 2 h post-exercise, β-CTX concentrations were lower in the IF trial than the DF and PLA trials ($P \leq 0.001$). At 3 h post-exercise, β-CTX concentrations were higher in the PLA trial than the IF ($P \leq 0.001$) and DF trials ($P = 0.026$). At 4 h post-exercise, β-CTX concentrations were lower in the DF trial than the IF ($P = 0.003$) and PLA trials ($P \leq 0.001$). At 4 h post-exercise, P1NP was higher in the IF trial than in DF ($P = 0.026$) and PLA trials ($P = 0.001$). At 3 h post-exercise, PTH was higher in the IF trial than the DF trial ($P \leq 0.001$).

Conclusions

Following exhaustive running, immediate ingestion of CHO+PRO may be beneficial, as it decreases bone resorption marker concentrations and increases bone formation marker concentrations; creating a more positive bone turnover balance.

Key words: Bone resorption, bone formation, post-exercise feeding, endurance athletes

Introduction

Dietary practices can influence both acute bone turnover and long-term bone health (38) and feeding influences the diurnal rhythm of bone turnover markers at rest (31). Feeding of a mixed nutrient meal suppresses all markers of bone turnover (4) and feeding of individual nutrients; glucose, fat, protein and calcium, also suppresses bone resorption at rest (2, 3, 5, 14). However, previous studies have only investigated the response in resting, non-athletic participants, who have not performed any prior exercise; it is therefore not known whether there is a similar suppressive effect of nutrient ingestion on bone resorption, after exercise in athletic individuals.

Prolonged and intense exercise causes increased bone resorption, as shown by increases in C-terminal telopeptide of type 1 collagen (β-CTX) (19, 22, 33), although markers of bone formation, such as N-terminal propeptides of type 1 procollagen (P1NP), are less responsive to acute bouts of exercise (13, 32, 34). Increases in bone resorption, without concomitant increases in bone formation, have been observed for up to four days after a bout of exhaustive running (32). Although not definitive, this suggests that prolonged and intense exercise could lead to an uncoupling or imbalance in bone turnover, favouring increased bone resorption, which may have detrimental effects on bone mass and health (15). This uncoupling has been implicated in the formation of stress fracture injuries (30, 39), which are debilitating injuries for athletes and on average result in 169 days (with a range of 90 to 270 days) of lost training (23, 27). Therefore maintaining coupled bone turnover and anabolic conditions for bone during and after exercise is important for athletes. Given that endurance athletes train multiple times a day preventing bone loss and stress fracture injury will help maximise available training time.

Pre-exercise feeding has been investigated as a potential means for attenuating the bone resorption response to exercise. Scott *et al*. (34) showed that feeding a mixed nutrient breakfast prior to exercise had no effect on post-exercise β-CTX concentrations compared to fasting and there were no changes in markers of bone formation. This implies that the mechanical loading experienced during exercise over-rides any responses caused by pre-exercise feeding. Scott *et al.* (34) also suggested that the stimulatory effect of PTH on β-CTX may override the effect of pre-exercise feeding, therefore other exercise feeding practices, the subsequent PTH response and related metabolites (calcium and phosphate) require investigation.

Sale *et al*. (29) showed that carbohydrate (CHO) feeding during exercise attenuated the β-CTX and P1NP responses in the hours following exercise, indicating an acute effect of CHO feeding on bone turnover; however, feeding during intense running is not always well tolerated and is limited by time and practicality. Post-exercise feeding provides a practical opportunity to feed multiple nutrients and in the correct amounts, thus allowing athletes to reach other sports nutrition goals, such as aiding muscle glycogen resynthesis, protein synthesis and maintaining adequate hydration status (16, 36), without the restrictions of gastrointestinal discomfort, which commonly limits nutrient ingestion during exercise. Post-exercise feeding also allows for investigation of the bone turnover response to acute feeding without the confounding effect of mechanical loading. It is not known whether the acute bone turnover response to post-exercise feeding is the same as at rest and whether this varies with different timings of post-exercise nutrient ingestion. The aim of this study was to investigate the effect of feeding carbohydrate and protein (CHO+PRO) immediately or 2 h after a prolonged intense running bout, on the bone turnover response in trained endurance runners.

Markers associated with exercise and bone were also measured to explore possible mediating and mechanistic factors.

Methods

Participants

10 men ([mean ± 1SD] age 28 ± 6 y, height 1.74 ± 0.05 m, body mass 69.7 ± 6.3 kg, VO_{2max} 63.0 ± 5.0 $mL \cdot kgBM^{-1} \cdot min^{-1}$, weekly running distance 49.9 ± 12.5 km) completed this study that was approved by Nottingham Trent University's Ethical Advisory Committee. All participants were trained endurance runners who had been competing and training consistently for a minimum of 2 years in 10 km, half marathon, marathon or ultra-distance races, without a significant break. Participants had recorded at least one of the following times in the past 2 years; ≤35 minutes for 10 km, ≤1:25:00 for half marathon or ≤3:00:00 for marathon. Participants were recruited from local running and triathlon clubs and local races, via posters, flyers and posts on club websites. Consent was obtained by the primary researcher. Participants were non-smokers, had not suffered a fracture in the last 12 months, were free from musculoskeletal injury and did not suffer from any condition known to affect bone metabolism. Compliance with these inclusion criteria was confirmed in the initial visit to the laboratory where health screening was completed and written informed consent was provided.

Experimental Design

This was a randomised (Latin Square Design), counterbalanced, placebo-controlled and single-blinded, crossover study. Participants completed a preliminary visit for habituation with trial procedures and measurement of VO_{2max}. Participants then completed three, four-day experimental trials, each separated by 1 week. On days 1 and 2, participants refrained from all exercise and followed a prescribed diet. On day 3, participants performed a bout of treadmill running, at a speed equal to 75% of their previously determined VO_{2max}, until volitional exhaustion. Blood samples (20 mL) were collected before exercise, immediately

after exercise and every hour after exercise for four hours. On day 4, participants returned to the laboratory for a fasted follow up blood sample. The three trials consisted of; i) a placebo (PLA) control trial, where the PLA solution was ingested both immediately and 2 h post-exercise, ii) an immediate feeding (IF) trial, where the CHO+PRO solution was ingested immediately post-exercise and the PLA solution 2 h post-exercise, and iii) a delayed feeding (DF) trial where the PLA solution was ingested immediately post-exercise and the CHO+PRO solution 2 h post-exercise. In the PLA trial, the CHO+PRO solution was ingested after the final blood sample to ensure that the energy content and the composition of the diet was identical between trials. This meant that a final PLA solution also needed to be ingested in the IF and DF trials to ensure participant blinding to the trial conditions (Figure 1).

Assessment of VO_{2max}

Participants performed an incremental treadmill test to determine lactate threshold, as per Jones and Doust (17), followed by a ramp test to determine VO_{2max}. Level running velocities corresponding to 75%VO_{2max} (13.0 ± 0.8 $km \cdot h^{-1}$) were calculated based on the regression of VO_2 and velocity.

Experimental Dietary Provision

Participants completed a three-day food diary for the measurement of habitual energy intake and macronutrient composition. A diet consisting of 55% CHO, 30% fat and 15% PRO, and isocaloric with habitual diets was designed using dietary analysis software (Nutritics, Dublin, Ireland), for each participant to consume on days 1 and 2 of each trial. Participants provided their own food but were given written and verbal instructions for the preparation of meals, including timings of meals and snacks. Any deviations from prescribed diets were confirmed verbally on day 3 and recorded; there were no significant deviations from prescribed diets.

Experimental Trial Procedure

Participants were asked to maintain their habitual training and record this throughout the study to help maintain consistency across trials. Participants refrained from all exercise on days 1 and 2. Participants arrived at the laboratory on day 3, after fasting from 20:00 the previous evening and consuming 500 mL of water upon awakening. Shortly after arriving, body mass was measured and the first 20 mL blood sample was taken via venepuncture after 10 minutes of semi-recumbent rest.

Participants then ran to volitional exhaustion at 75%VO_{2max}, which was preceded by a 5-minute warm-up and volitional stretching. At exhaustion a cannula was inserted into a prominent forearm vein, which was kept patent by flushing with saline, a second 20 mL blood sample was taken, with further blood samples taken at 1, 2, 3 and 4 h into recovery. Exact times of exercise commencement, time to exhaustion and blood samples were recorded and were repeated exactly in each trial within-participants to reduce the impact of circadian variation on the results. Due to differences in individual run times to exhaustion between participants, post-exercise blood sample timings vary between participants, but were controlled for within-participants. The baseline blood sample was taken at 08:40 and exercise commenced at 08:50, the blood sample at exhaustion was taken at 10:10 ± 13 min and blood samples 1 – 4 hours post-exercise were taken at 11:10 ± 13 min, 12:10 ± 13 min, 13:10 ± 13 min and 14:10 ± 13 min.

Depending on the trial, participants were given either the CHO+PRO or PLA solution to consume immediately after exhaustion. Two and four hours after exhaustion participants were given further solutions to consume. After the final solution was consumed, participants

were provided with food and were free to leave the laboratory. Participants consumed a snack at 15:00 and an evening meal at 18:00 and then remained fasted from 20:00 until the next morning. On day 4 participants arrived in the laboratory after consuming 500 mL of water upon awakening and a final 20 mL blood sample was taken.

Recovery Solutions and Evening Meal Composition

The CHO+PRO solution contained 1.5 $g \cdot kgBM^{-1}$ of CHO (dextrose) and 0.5 $g \cdot kgBM^{-1}$ of PRO (unflavoured whey isolate) that was made up to a 12.5% CHO solution with water. The whey isolate and dextrose mix was tested for banned substances by LGC Supplement Screening (Cambridgeshire, UK; UKAS Testing Laboratory 1187; Certificate of Analysis 91530). Preliminary testing ensured that the PLA solution was taste matched to the CHO+PRO solution using artificial sweetener and flavouring; it consisted of 12 $mL \cdot kgBM^{-1}$ of water, making this the same volume as the CHO+PRO solution. Participants were blinded to the solutions that they were consuming throughout trials. The total volume of fluid consumed in the three recovery solutions was 2,509 ± 227 mL.

On day 3 the overall diet composition was 2,000 kcal, 55% CHO, 30% fat and 15% PRO. The recovery solution contained approximately 500 kcal depending on individual body mass, therefore the snack and evening meal contained approximately 1,500 kcal. Deviations from prescribed diets were confirmed verbally on day 4 and recorded; there were no significant deviations from prescribed diets. Participants were allowed to ingest plain water on an *ad libitum* basis throughout the recovery periods, although none of the participants did this during any of the trials.

Treatment and Storage of Blood Samples

Blood was transferred into precooled tubes and gently inverted 5–8 times; 15 mL of blood was transferred into tubes containing 15%, 0.12 mL of K3E EDTA (Becton Dickinson Vacutainer System, USA) and then centrifuged immediately at 3000 $rev \cdot min^{-1}$, for 10 minutes at 5°C, generating plasma. The remaining 5 mL of blood was transferred into standard serum tubes (Becton Dickinson Vacutainer System, USA), left to clot at room temperature for 60 min before being centrifuged at 2000 $rev \cdot min^{-1}$, for 10 minutes at 5°C. Plasma and serum was subsequently stored at -80°C until analysis.

Biochemical Analysis

β-CTX, P1NP and parathyroid hormone (PTH) were measured by electro-chemiluminescence immunoassay on an fully automated COBAS c501 system (Roche Diagnostics, Mannheim, Germany) in blood plasma and were measured in singlicate. The inter-assay CV for β-CTX was ≤3% between 0.2 and 1.5 $\mu g \cdot L^{-1}$, with sensitivity of 0.01 $\mu g \cdot L^{-1}$. The inter-assay CV for P1NP was ≤3% between 20 and 600 $\mu g \cdot L^{-1}$, with sensitivity of 8 $\mu g \cdot L^{-1}$. The inter-assay CV for PTH was ≤4% between 1 and 30 $pmol \cdot L^{-1}$, with sensitivity of 0.8 $pmol \cdot L^{-1}$. Phosphate (PO_4), total calcium (Ca) and albumin were measured in serum, using standard colorimetric assays and spectrophotometric methods, performed on an ABX Pentra 400 (Horiba ABX, Montpellier, France). PO_4 was measured using phosphomolybdate, with an inter- and intra-assay CV of ≤3.6% between 0.09 and 7.80 $mmol \cdot L^{-1}$. Total Ca was measured using ortho-cresolphtalein complexone, with an inter- and intra-assay CV of ≤1.7% between 0.04 and 5.00 $mmol \cdot L^{-1}$. Albumin was measured using bromocresol green, with an inter- and intra-assay CV of ≤1.9% between 0.02 and 5.99 $g \cdot dL^{-1}$. Because fluctuations in protein, particularly albumin, may cause total Ca levels to change independently of the ionised calcium (Ca^{2+}) concentration, total Ca concentrations were corrected to give an albumin-adjusted Ca (ACa) value: 0.8 $mg \cdot dL^{-1}$ was subtracted from the total Ca concentration for

every 1.0 $g \cdot dL^{-1}$ by which the serum albumin concentration was greater than 4 $g \cdot dL^{-1}$ or 0.8 $mg \cdot dL^{-1}$ was added to the total Ca concentration for every 1.0 $mg \cdot dL^{-1}$ by which the serum albumin concentration was less than 4 $mg \cdot dL^{-1}$. *I.e.* $(([\text{Albumin}] - 4) * -0.8) + [\text{Total Ca}]$. Ca^{2+}, glucose and lactate were measured in whole blood using a blood gas analyser (Radiometer ABL90 FLEX, Copenhagen, Denmark). Ca^{2+} is estimated directly between pH 7.2 and 7.6 with no pH correction applied. The inter- and intra-assay CV for Ca^{2+} was ≤3% between 0.2 and 9.99 $mmol \cdot L^{-1}$, for glucose was ≤5% between 0 and 60 $mmol \cdot L^{-1}$ and for lactate was ≤26.7% between 0.1 and 31 $mmol \cdot L^{-1}$.

Statistical Analysis

The study sample size was calculated to detect changes in β-CTX from pre- to post-exhaustive exercise, with 85% power at an alpha level of $P \leq 0.05$, based on the study by Scott *et al*. (32). Statistical significance was accepted at an alpha level of $P \leq 0.05$. All statistical analyses were performed on raw data. Baseline concentrations were compared using a one-way ANOVA. Parametric assumptions of normality and sphericity were confirmed using the Shapiro-Wilks test and Maulchy's test of Sphericity and where assumptions were violated, a transformation was applied to the data so that the assumptions were satisfied. Normality and homogeneity were achieved following log transformations for ACa and PO_4 data. All data were subsequently analysed using a repeated measures ANOVA, with *Trial* (PLA *vs* IF *vs* DF) and *Time* (of sampling) as within participant factors. Tukey's HSD *post-hoc* test was used to compare each time point against baseline and to compare trials at each time point. Effect size for multiple comparisons was calculated using partial (η_p^2) eta-squared (21). *Post-hoc* comparisons are reported with Cohen's *d* effect sizes, with d=0.2 considered as a small effect, d=0.5 considered as a medium effect and d=0.8 considered as a large effect (6). These

250 statistical analyses were performed with Statistica (StatSoft, Tulsa, OK) and SPSS (IBM
251 SPSS Statistics 22, Armonk, NY).

Results

Exercise variables

The average time to exhaustion (exercise duration) was 01:15:00 ± 00:13:00. There was a significant decrease in body mass from pre-exercise (69.4 ± 6.1 kg) to post-exercise (68.9 ± 5.9 kg) (P=0.001).

Baseline biochemistry

Baseline concentrations of β-CTX, P1NP, PTH, ACa, Ca^{2+}, PO_4 and albumin were not significantly different between trials (Table 1).

Habitual diet and experimental dietary provision

There were no significant differences between the diets prescribed for days 1 and 2 of each trial and the diets that were actually consumed by participants, for overall energy content or macronutrient composition. Participants' habitual diets were not different from the diet provided on day 3 of trials, for overall energy content, CHO content, fat content and calcium content (P=0.101 to 0.523). However, PRO content was significantly higher in the habitual diets compared to the experimental trial diet (P=0.049) (Table 2).

Bone turnover markers

C-terminal telopeptide of type 1 collagen (β-CTX)

There was a significant main effect of *Trial* ($P \leq 0.001$; $\eta_p^2 = 0.581$) *Time* ($P \leq 0.001$; $\eta_p^2 = 0.744$) and a significant *Trial x Time* interaction ($P \leq 0.001$; $\eta_p^2 = 0.630$) for β-CTX. β-CTX concentrations were increased from baseline by the end of exercise in all trials (+8 to +12%). In the PLA trial, β-CTX concentrations remained increased above baseline at 1 h post-exercise (+7%), before decreasing thereafter, being significantly lower than baseline

concentrations 3 and 4 h post-exercise (-31 to -42%; $P \leq 0.001$) and 24 h later (-3%). In the IF trial, β-CTX concentrations were significantly lower than baseline at 1 h post-exercise and remained below baseline until the end of the trial (-22 to -61%; $P \leq 0.01$). In the IF trial, β-CTX concentrations were increased above baseline 24 h later (+8%). In the DF trial, β-CTX concentrations were increased above baseline at 1 h post-exercise (+15%), then began to decrease and were significantly lower than baseline concentrations 3 and 4 h post-exercise (-44 to -65%; $P \leq 0.001$). In the DF trial, β-CTX concentrations were increased above baseline 24 h later (+8%) (Figure 2A).

At 1 and 2 h post-exercise, β-CTX concentrations were significantly lower in the IF trial than the DF ($P \leq 0.001$, $d=0.76$) and PLA trials ($P \leq 0.001$, $d=0.84$). At 3 h post-exercise, β-CTX concentrations were significantly higher in the PLA trial than the IF ($P \leq 0.001$, $d=1.13$) and DF trials ($P=0.026$, $d=0.54$). At 4 h post-exercise, β-CTX concentrations were significantly lower in the DF trial than the IF ($P=0.003$, $d=0.82$) and PLA trials ($P \leq 0.001$, $d=1.09$) (Figure 2A). All other time points were not significantly different between trials. The overall β-CTX response was significantly lower in the IF trial than the DF trial ($P=0.019$, $d=0.37$) and the PLA trial ($P \leq 0.001$, $d=0.84$).

N-terminal propeptides of type 1 procollagen (P1NP)

There was no main effect of *Trial* for P1NP, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.621$) and there was a significant *Trial x Time* interaction ($P \leq 0.001$; $\eta_p^2 = 0.292$). P1NP concentrations were significantly increased from baseline by the end of exercise in all trials (+32 to +33%; $P \leq 0.001$) and by 1 h post-exercise P1NP had decreased below baseline concentrations in all trials (-3 to -7%). In the PLA trial, P1NP concentrations remained below baseline until the end of the trial (-7 to -9%), but were increased above baseline 24 h later

(+4%). In the IF trial, P1NP began to increase and reached concentrations above baseline at 3 and 4 h post-exercise (+1 to +3%) and 24 h later (+5%). In the DF trial, P1NP concentrations continued to decrease and by 3 and 4 h post-exercise were significantly lower than baseline (-10 to -11%; $P \leq 0.05$), but were increased above baseline 24 h later (+4%) (Figure 2B). At 4 h post-exercise, P1NP was significantly higher in the IF trial than the DF ($P=0.026$, $d=0.20$) and PLA trials ($P=0.001$, $d=0.25$) (Figure 2B). All other time points were not significantly different between trials.

Calcium metabolism

Parathyroid hormone (PTH)

There was no main effect of *Trial* for PTH, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.791$) and there was a significant *Trial x Time* interaction ($P \leq 0.001$; $\eta_p^2 = 0.428$). PTH concentrations were significantly increased from baseline by the end of exercise in all trials (+124 to +131%; $P \leq 0.001$) but by 1 h post-exercise had decreased significantly below baseline concentrations in all trials (-17 to -37%; $P \leq 0.05$). In the PLA trial, PTH concentrations remained below baseline until the end of the trial (-3 to -15%) but were increased above baseline 24 h later (+4%). In the IF trial, PTH then began to increase and reached concentrations above baseline 3 and 4 h post-exercise (+2 to +7%) and 24 h later (+1%). In the DF trial, PTH continued to decrease and remained below baseline concentrations for the remainder of the trial (-13 to -27%) and 24 h later (-4%) (Figure 3A). At 3 h post-exercise, PTH was significantly higher in the IF trial than the DF trial ($P \leq 0.001$, $d=1.33$) (Figure 3A). All other time points were not significantly different between trials.

Albumin-adjusted calcium (ACa)

There was no main effect of *Trial* for ACa, but there was for *Time* (P=0.003; η_p^2 = 0.290) and there was a significant *Trial x Time* interaction (P=0.020; η_p^2 = 0.191). ACa concentrations were increased from baseline by the end of exercise in all trials (+2 to +3%). In the PLA trial, ACa concentrations remained above baseline until the end of the trial (+2 to +4%) but had decreased below baseline 24 h later (-1%). In the IF trial, ACa remained above baseline (+2 to 3%) until 3 h post-exercise when ACa decreased below baseline (-3%), ACa then increased above baseline 4 h post-exercise (+1%) and remained there 24 h later. In the DF trial, ACa remained above baseline until the end of the trial (+2 to +4%) and returned to baseline 24 h later (Figure 3B). At 3 h post-exercise, ACa was significantly lower in the IF trial than the DF (P=0.008, d=0.79) and PLA trials (P=0.001, d=0.98) (Figure 3B). All other time points were not significantly different between trials.

Ionised calcium (Ca^{2+})

There was no main effect of *Trial* for Ca^{2+}, but there was for *Time* ($P \leq 0.001$; η_p^2 = 0.771) and a significant *Trial x Time* interaction ($P \leq 0.001$; η_p^2 = 0.321). Ca^{2+} concentrations were significantly decreased below baseline by the end of exercise in all trials (-5 to -7%; $P \leq 0.001$). In the PLA trial, Ca^{2+} concentrations were still significantly below baseline by 1 h post-exercise (-4%; P=0.002) and remained below baseline until the end of the trial and 24 h later (-3%; P=0.006). In the IF trial, Ca^{2+} concentrations had returned to baseline by 1 h post-exercise (+1%) and remained at concentrations similar to baseline until the end of the trial and 24 h later (-1%). In the DF trial, Ca^{2+} concentrations had almost returned to baseline by 1 h post-exercise (-1%) and remained at concentrations similar to baseline until the end of the trial and 24 h later (-1%) (Figure 3C). At 1 h post-exercise, Ca^{2+} concentrations were significantly lower in the PLA trial than the IF trial (P=0.010, d=1.41) (Figure 3C). All other time points were not significantly different between trials.

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353 *Phosphate (PO_4)*

354 There was no main effect of *Trial* for PO_4, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.581$) and
355 there was a significant *Trial x Time* interaction ($P=0.007$; $\eta_p^2 = 0.207$). PO_4 concentrations
356 were significantly increased above baseline by the end of exercise in all trials (+21 to +26%*;*
357 $P \leq 0.001$). By 1 h post-exercise, PO_4 concentrations decreased below baseline in all trials (-5
358 to -13%). In the PLA trial, PO_4 concentrations continued to decrease at 2 h post-exercise (-
359 8%), then increased and returned to baseline 3 h post-exercise. In the PLA trial, PO_4
360 concentrations were increased above baseline at 4 h post-exercise (+14%) and 24 h later
361 (+3%). In the IF trial, PO_4 concentrations started to increase at 2 h post-exercise and
362 increased above baseline 4 h post-exercise (+8%). In the IF trial, PO_4 concentrations were
363 below baseline 24 h later (-2%). In the DF trial, PO_4 concentrations continued to decrease at 2
364 h post-exercise (-8%), concentrations started to increase thereafter, but remained below
365 baseline until the end of the trial and 24 h later (-4%) (Figure 3D). At 1 h post-exercise, PO_4
366 concentrations were significantly lower in the IF trial than the DF trial ($P=0.049$, $d=1.03$)
367 (Figure 3D). All other time points were not significantly different between trials.

368

369 *Albumin*

370 There was no main effect of *Trial* for albumin, but there was for *Time* ($P \leq 0.001$; $\eta_p^2 = 0.372$)
371 and there was no *Trial x Time* interaction ($P=0.054$; $\eta_p^2 = 0.167$). Overall mean albumin
372 concentrations were significantly increased from baseline by the end of exercise (+3 to +4%;
373 $P=0.011$). There were no other significant changes in albumin concentrations (Figure 4).

374

Discussion

The main findings of the study are that: 1) ingestion of the CHO+PRO solution containing 1.5 $g \cdot kgBM^{-1}$ of CHO and 0.5 $g \cdot kgBM^{-1}$ of PRO suppressed β-CTX concentrations following an exhaustive run, with a greater overall suppression when the CHO+PRO solution was ingested immediately; 2) immediate ingestion of the CHO+PRO solution resulted in small increases in P1NP concentrations at 3 and 4 h post-exercise; 3) delayed ingestion of the CHO+PRO solution (2 h post-exercise) also resulted in a large suppression of β-CTX concentrations. These findings are novel and have the potential to directly influence an athlete's dietary and/or training practices.

The response in the PLA trial, showed that the exhaustive running bout caused an immediate increase in bone turnover at the end of exercise, indicated by increased β-CTX and P1NP concentrations above baseline. This was followed by decreased bone turnover during recovery, indicated by decreased β-CTX and P1NP concentrations below baseline. Ingestion of the CHO+PRO solution immediately post-exercise caused a rapid and prolonged (at least 4 h) suppression of β-CTX concentrations below baseline levels (-22 to -61%), whereas ingesting the PLA solution immediately post-exercise meant that β-CTX concentrations were increased above baseline by between +7 and +15%. When ingestion of the CHO+PRO was delayed by 2 h, it caused suppression of β-CTX concentrations below baseline (-44 to -65%), which is similar to the suppression caused by immediate ingestion of the CHO+PRO solution and it occurred within the same timeframe, *i.e.*, 1 – 2 h after ingestion.

This rapid response is important because elite athletes habitually train multiple times a day, meaning that there is often only a few hours in between training sessions and therefore limited time for recovery and food consumption. Although the participants in the present

400 study are not elite athletes, their trained nature means that the results are relevant and may be
401 interpreted and used by elite athletes or practitioners. The results indicate that post-exercise
402 nutrient ingestion or exercise commencement can be timed so that the subsequent training
403 session occurs when bone resorption is at its lowest and bone formation at its highest, *i.e.*, 3 –
404 4 hours after the first exercise bout with immediate ingestion of the CHO+PRO solution. This
405 may maximise the anabolic and minimise the catabolic bone response to the subsequent
406 training session, however further research is needed to investigate whether this intervention
407 does indeed produce a more anabolic environment for bone.
408
409 The significant increase in P1NP concentrations (+32 to +33%) and the larger relative
410 increase in P1NP compared to β-CTX concentrations at the end of exercise is interesting, as
411 markers of bone formation are usually less responsive to acute bouts of exercise than markers
412 of bone resorption (13, 32, 34). Similarly, de Sousa *et al.* (7) reported a 77% increase in
413 P1NP after a high-intensity, interval running session (10 x 800m). In the present study, P1NP
414 concentrations then decreased to below baseline levels at 1 h post-exercise in all trials, but the
415 ingestion of the CHO+PRO solution immediately post-exercise caused P1NP to increase
416 above baseline at 3 and 4 h post-exercise by between +1 to +3%, whereas ingesting the PLA
417 solution immediately post-exercise meant that P1NP remained below baseline concentrations
418 by between -7 and -9%. When the CHO+PRO solution was ingested 2 h post-exercise, P1NP
419 concentrations were suppressed further below baseline concentrations (-10 to -11%). It is
420 possible that P1NP could have increased after the last measurement was taken but was missed
421 by the sampling protocol, therefore it would be useful for future research to examine a longer
422 post-exercise period to investigate the longer term response. The significantly increased
423 P1NP concentrations at 4 h post-exercise in the IF trial compared to the DF and PLA trials is
424 novel, and taken together, these results advocate the feeding of a CHO+PRO solution

immediately post-exercise in order to reduce bone resorption marker concentrations and increase bone formation marker concentrations in the short-term recovery from intense exercise.

The effects of the CHO+PRO solution did not persist to the morning following exercise and β-CTX concentrations were increased in the IF and DF trials (+8%) compared to suppressed β-CTX concentrations in the PLA trial (-3%). P1NP was increased 24 h post-exercise in all trials (+4 to +5%). This increased bone turnover in the IF and DF trials may reflect the bones adapting to a possible hormonal response that is mediated by feeding. It is unlikely that the bones are adapting to the mechanical loading from the running bout alone, as β-CTX concentrations were not increased 24 h post-exercise in the PLA trial. The hormonal mediators of this response are currently unknown; Scott *et al.* (34) and Sale *et al.* (29) recently showed that GLP-2, leptin and ghrelin are unlikely mediators of the effect of CHO or mixed meal feeding on bone turnover. Subsequently, this requires further research including the measurement of other gastro-intestinal hormones.

Although this increased bone turnover response may be positive in sub-elite populations, elite athletes that train multiple times a day with minimal recovery time and rest days are more likely to suffer from consistently increased bone remodelling, which may have detrimental effects on bone health and enhance the stress fracture risk (25, 26, 28, 30). The trained runners and triathletes in the present study have mean resting bone turnover marker concentrations that are at the upper end of the reference ranges for the non-active, healthy population (7, 11, 12). Further, unpublished data from our laboratory show that elite triathletes have mean resting bone turnover marker concentrations that are higher than the trained runners and triathletes. This is supported by Oosthuyse *et al.* (25) who showed that

bone resorption and bone formation markers were significantly elevated each morning after four successive 3 h cycling bouts in well-trained cyclists. Although this is speculative, elite athletes may experience an imbalance between whole-body rates of resorption and formation or, defective coupling (26), meaning that neither bone resorption or bone formation is performed adequately and the quality of the bone may be poorer. Or, athletes may experience accelerated remodelling, which can increase bone microdamage accumulation (30), all of which can increase stress fracture risk (1, 9, 28, 30). Indeed it should be noted that in a normal, healthy basic multicellular unit, the suppression of bone resorption may not always be desired, if the function of bone resorption is to breakdown and remove damaged bone at areas of microdamage accumulation to allow the area to be repaired and strengthened. Therefore, it is crucial for future research to investigate the long term effects of post-exercise suppression of bone resorption on different athletic and non-athletic populations.

Ingestion of the CHO+PRO solution post-exercise is not sufficient to cause a decrease in bone resorption marker concentrations and/or an increase in bone formation marker concentrations 24 h post-exercise. However, as elite athletes rarely go 24 h without a training session and often have a second session within four hours of finishing the first session, the bone turnover response 24 h post-exercise is less important than the immediate response as it does not reflect real life athlete practice. The more important time point is therefore, 4 h post-exercise, as this may be around the same time that the second training session would start. As we have now investigated the effect of post-exercise feeding after a single acute bout of exercise, future studies should investigate the effect of post-exercise nutrient ingestion on repeated bouts of exercise occurring on the same day.

The responses of Ca^{2+} and PO_4 to exercise are in line with previous research (37) and the responses are only significantly different between trials at 1 h post-exercise; Ca^{2+} concentrations were lower in the PLA trial compared to the IF trial, suggesting that IF augments the recovery of Ca^{2+} to baseline concentrations, and PO_4 is lower in the IF trial compared to the DF trial. Transient peaks in PTH concentrations, as shown in the present study, are shown to be anabolic for bone (10) and Townsend *et al.* (37) showed that PTH secretion during exercise and recovery is controlled by both Ca^{2+} and PO_4, therefore these metabolites are likely to be mediating any anabolic effect of increased PTH concentrations. The fact that PTH and P1NP follow the same response could suggest that PTH is mediating an anabolic response in the IF trial, however this response needs to be confirmed.

At 3 h post-exercise, PTH concentrations were greater in the IF trial than in the DF trial (+7% *vs* -27%). This response coincides with significantly lower ACa concentrations at 3 h post-exercise in the IF trial compared to the DF and PLA trials (-3% *vs* +3 to +4%). β-CTX concentrations were at their lowest at 3 h post-exercise in the IF trial. Considering that the action of increased PTH secretion is to increase calcium through mobilisation of the bone reservoir via activation of bone resorption (and also by increasing renal tubular reabsorption and intestinal calcium absorption) (24, 35, 40), this suggests that changes in PTH and calcium metabolism are unlikely to mediate the acute suppression in bone resorption seen with post-exercise CHO+PRO feeding. However, ACa has been shown to be unsuitable when investigating the rapid response of calcium metabolism to exercise (37), which may also be true when investigating CHO+PRO ingestion around exercise. Although Ca^{2+} (non-protein bound calcium) decreased at the end of exercise, because albumin concentrations increased, ACa was normalised and remained fairly unchanged throughout exercise. Changes in albumin could have been effected by the ingestion of dietary protein throughout the recovery

period, which has previously been shown to increase circulating albumin concentrations (18, 20), however albumin did not change significantly throughout the recovery period. The increase in albumin at the end of exercise could have been to encourage more calcium to be transported around the body, due to the tissues requiring additional Ca^{2+} to keep up with the demand in energy consumption, although the increase in albumin might also just reflect haemoconcentration as a result of the running bout. Transient haemoconcentration can occur rapidly following the onset of acute exercise, possibly even occurring in advance of any significant losses of fluid through sweating or respiration, and it might be argued that significant haemoconcentration would mean that changes in plasma solutes simply reflect shifts in plasma volume. However, one might argue that the level of a plasma solute, irrespective of plasma volume shifts, is more important, since it is this that the body responds to. The data presented herein are uncorrected for plasma volume changes, which could influence the interpretation of the biological data obtained during the recovery period and this should be considered when interpreting results. It is recommended that future studies take this into consideration and correct bone turnover marker data for plasma volume shifts, where appropriate, perhaps even presenting these data both corrected and uncorrected for plasma volume changes.

In conclusion, following exhaustive running, immediate ingestion of a CHO+PRO recovery solution may be beneficial, as it decreases bone resorption marker concentrations and increases bone formation marker concentrations; creating a more positive bone turnover balance. The mechanisms underlying the acute changes in bone turnover remain unknown, but a change in calcium metabolism is unlikely to fully mediate the response.

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Figures

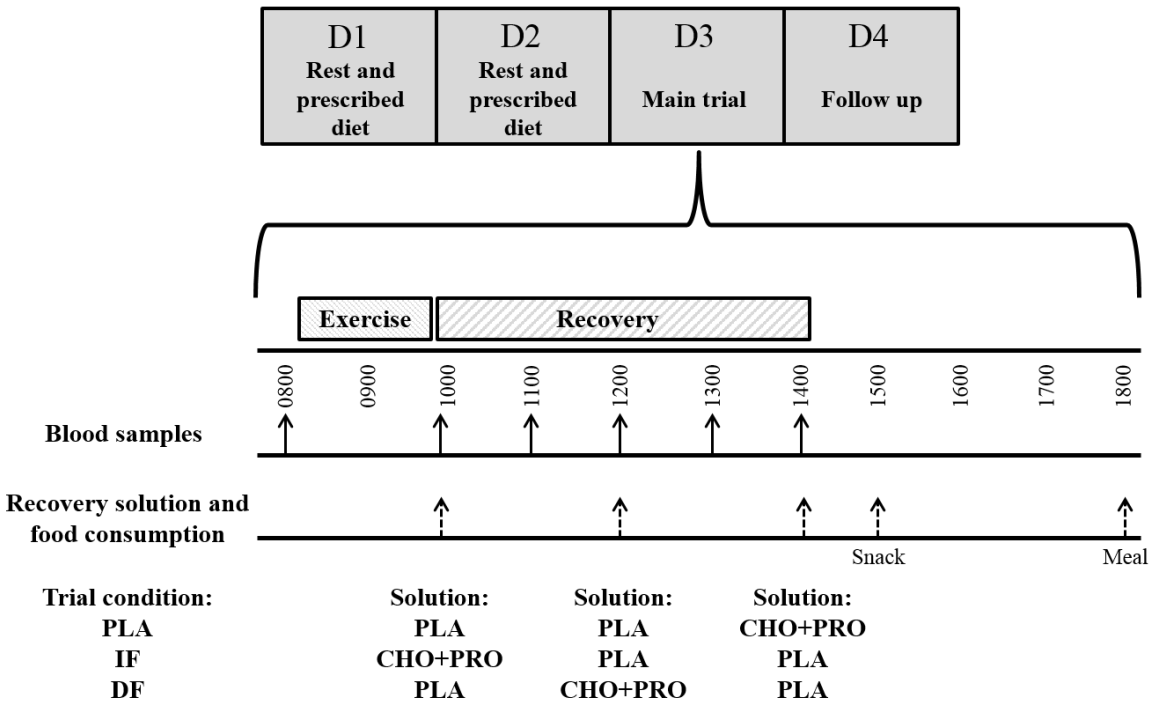

Figure 1. Experimental protocol. Exercise was treadmill running at 75%VO_{2max}, followed by 4 hours of rested recovery. PLA = Placebo trial, IF = Immediate feeding trial and DF = Delayed feeding trial. Participants departed from the laboratory at the end of the recovery period. Solid vertical arrows denote blood samples. Dashed vertical arrows denote recovery solution and food consumption.

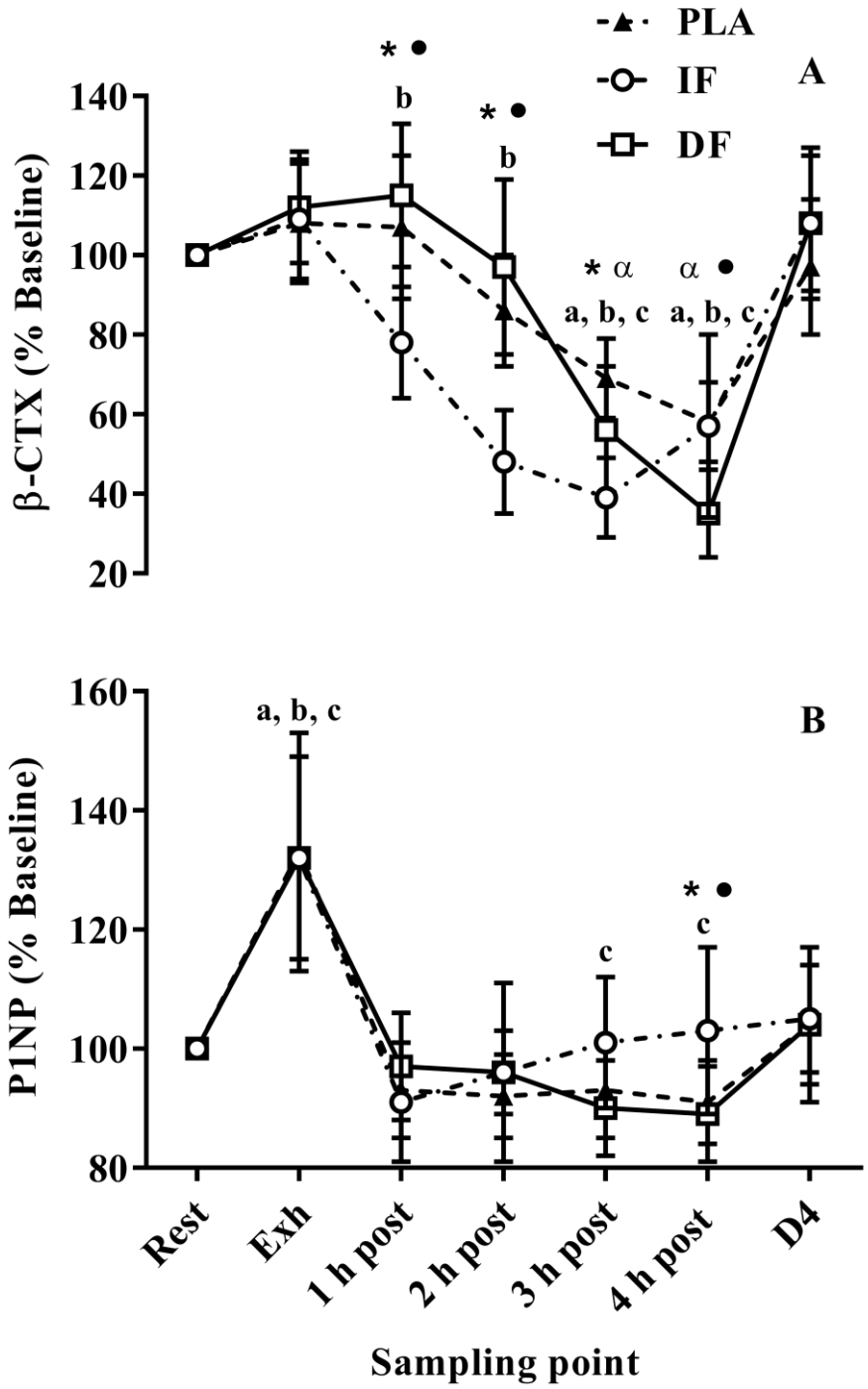

Figure 2. The percentage change in baseline concentrations of β-CTX (A) and P1NP (B), at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4 (follow up sample on day 4), for PLA (filled triangles), IF (open circles) and DF (open squares). Data are mean ± 1SD. [a] different ($P \leq 0.05$) from baseline (PLA) [b] different ($P \leq 0.05$) from baseline (IF), [c] different ($P \leq 0.05$) from baseline (DF). [*] IF different ($P \leq 0.05$) from PLA, [α] DF different ($P \leq 0.05$) from PLA, [•] IF different ($P \leq 0.05$) from DF

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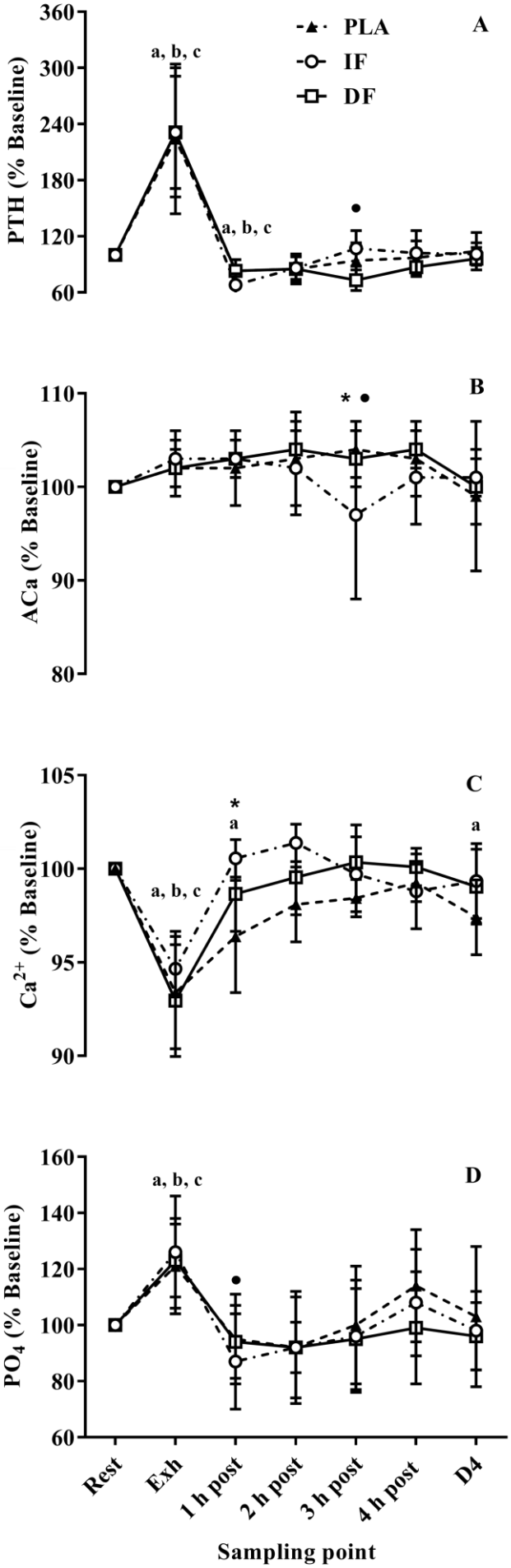
PLA
IF
DF
A
B
C
D
PTH (% Baseline)
ACa (% Baseline)
Ca2+ (% Baseline)
PO4 (% Baseline)
a, b, c
a
*
•
Rest
Exh
1 h post
2 h post
3 h post
4 h post
D4
Sampling point

672 **Figure 3.** The percentage change in baseline concentrations of PTH (A), ACa (B), Ca^{2+} (C)
673 and PO_4 (D) at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4
674 (follow up sample on day 4), for PLA (filled triangles), IF (open circles) and DF (open
675 squares). Data are mean ± 1SD. [a] different ($P \leq 0.05$) from baseline (PLA) [b] different ($P \leq 0.05$)
676 from baseline (IF), [c] different ($P \leq 0.05$) from baseline (DF). [*] IF different ($P \leq 0.05$) from PLA,
677 [α] DF different ($P \leq 0.05$) from PLA, [•] IF different ($P \leq 0.05$) from DF.

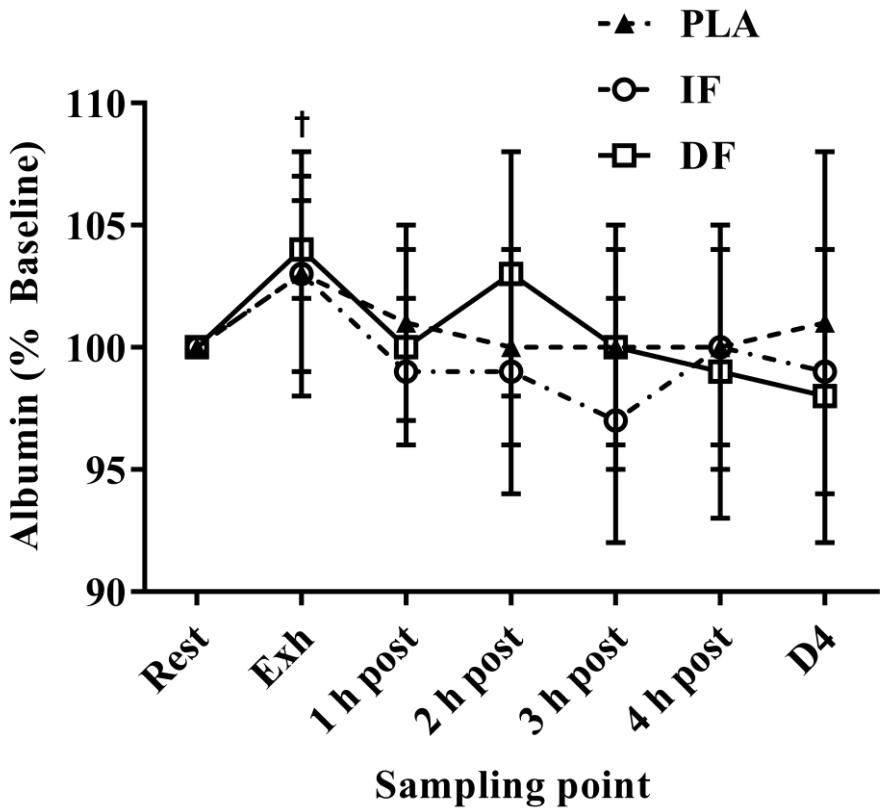

Figure 4. The percentage change in baseline concentrations of albumin at Rest (Baseline), Exh (at exhaustion), 1 to 4 hours post-exercise and D4 (follow up sample on day 4), for PLA (filled triangles), IF (open circles) and DF (open squares). Data are mean ± 1SD. † overall mean concentrations different from baseline ($P \leq 0.05$).