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ORIGINAL ARTICLE

Effect of ingesting carbohydrate only or carbohydrate plus casein protein hydrolysate during a multiday cycling race on left ventricular function, plasma volume expansion and cardiac biomarkers

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Abstract

Purpose Multiday racing causes mild left ventricular (LV) dysfunction from day 1 that persists on successive days. We evaluated ingesting casein protein hydrolysate–carbohydrate (PRO) compared with carbohydrate-only (CHO) during a 3-day mountain bike race.

Methods Eighteen male cyclists were randomly assigned to ingest 6.7% carbohydrate without (CHO) or with 1.3% casein hydrolysate (PRO) during racing (~4–5 h/day; 68/71/71 km). Conventional LV echocardiography, plasma albumin content, plasma volume (PV) and blood biomarkers were measured before day 1 and post race on day 3.

Results Fourteen cyclists ($n=7$ per group) completed the race. PV increased in CHO (mean increase (95% CI), 10.2% (0.1 to 20.2)%, $p=0.045$) but not in PRO (0.4% (−6.1 to 6.9)%). Early diastolic transmitral blood flow (*E*) was unchanged but deceleration time from peak *E* increased post race (CHO: 46.7 (11.8 to 81.6) ms, $p=0.019$; PRO: 24.2 (−0.5 to 48.9) ms, $p=0.054$), suggesting impaired LV relaxation. Tissue Doppler mitral annular velocity was unchanged in CHO, but in PRO septal early-to-late diastolic ratio decreased ($p=0.016$) and was compensated by increased lateral early ($p=0.034$) and late ($p=0.012$) velocities. Systolic function was preserved in both groups; with increased systolic lateral wall velocity in PRO ($p=0.002$). Effect size increase in serum creatine kinase (CK) activity, CK-MB and C-reactive protein concentrations was less in PRO than CHO (Cohen's *d* mean ± SD, PRO: 2.91 ± 2.07; CHO: 7.56 ± 4.81, $p=0.046$).

Conclusion Ingesting casein hydrolysate with carbohydrate during a 3-day race prevented secondary hypervolemia and failed to curb impaired LV relaxation despite reducing tissue damage and inflammatory biomarkers. Without PV expansion, systolic function was preserved by lateral wall compensating for septal wall dysfunction.

Keywords Endurance exercise · Sports nutrition · Serum albumin content · Secondary hypervolemia · Echocardiography · Inflammation · Membrane stability

Abbreviations

Δ	Change calculated as post minus pre race
A	Late diastolic transmitral blood flow velocity
a'	Late diastolic mitral annular velocity
Ao	Aortic diameter
ALB	Plasma albumin
AV_Vmax	Peak systolic trans-aortic valve blood flow velocity
BV	Blood volume
CHO	Carbohydrate-only supplement
CI	Confidence interval
CK	Creatine kinase
CK-MB	Creatine kinase-MB isoform
cTnI	Cardiac troponin I
d	Cohen's effect size
DBP	Diastolic blood pressure
E	Early diastolic transmitral blood flow velocity
e'	Early diastolic mitral annular velocity
EDV	End diastolic volume
EF	Ejection fraction

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ESV	End systolic volume
Hct	Haematocrit
Hb	Haemoglobin
hsCRP	High sensitivity C-reactive protein
LA	Left atrium
LV	Left ventricular
LVIDd	Left ventricular chamber diameter in diastole
PRO	Casein protein hydrolysate plus carbohydrate supplement
PV	Plasma volume
PWD	Pulsed-wave Doppler
s'	Peak systolic mitral annular velocity
SBP	Systolic blood pressure
SD	Standard deviation
SV	Stroke volume
TDI	Tissue Doppler imaging

Introduction

Prolonged strenuous endurance exercise is known to cause mild, temporary suppression of cardiac function that is mostly restored within 48 h (Eijsvogels et al. 2016; Lord et al. 2018). Ingesting nutritional supplements during exercise may help to lessen left ventricular (LV) dysfunction following exercise. For example, we have found greater evidence for LV dysfunction when ingesting placebo-water compared with carbohydrate during a single 2.5 h bout of strenuous laboratory cycling (Oosthuyse and Millen 2016). Ingesting casein protein hydrolysate with carbohydrate produced even less evidence for LV dysfunction following 2.5 h strenuous cycling (Oosthuyse and Millen 2016) and favoured a carbohydrate-sparing metabolic response compared with ingesting carbohydrate alone (Oosthuyse et al. 2015). Interestingly, these benefits may be specific to the casein hydrolysate protein-source, which includes only short di- and tripeptides, as the same findings were not clearly evident when ingesting carbohydrate plus whey protein hydrolysate, which includes a mix of short and longer peptides (Oosthuyse et al. 2015; Oosthuyse and Millen 2016). Moreover, previously we reported LV dysfunction to occur daily after 4 consecutive days of 3 h strenuous laboratory cycling, where diastolic dysfunction persisted following overnight recovery before each subsequent exercise session, despite ingesting a carbohydrate-only supplement during exercise (Oosthuyse et al. 2012). Previous benefits observed with carbohydrate–casein protein hydrolysate may become increasingly relevant during intense multiday endurance races to promote the well-being of athletes who regularly participate in these popular events.

Reducing oxidative stress and inflammation during exercise may curb exercise-induced LV dysfunction (La Gerche et al. 2015; Vitiello et al. 2011). Cysteine-rich dietary supplements and milk proteins exert antioxidant effects by increasing glutathione synthesis (Mariotti et al. 2004; McLeay et al. 2017) and ingesting short-chain protein hydrolysate peptides reduce exercise-induced oxidative stress more than whole protein isolates (Liu et al. 2014). Post-exercise increases in serum cardiac troponins have a temporal association with increases in lipid peroxidation (Nie et al. 2010) and the appearance of cardiac troponins, and certain other cardiac biomarkers such as creatine kinase-MB, in plasma after exercise may be a consequence of reactive oxygen species-induced cardiomyocyte membrane-leakage and instability (Eijsvogels et al. 2016; Middleton et al. 2008; Nie et al. 2016; Scherr et al. 2011). Moreover, only athletes who suffer myocardial dysfunction following prolonged strenuous exercise display increased serum pro-inflammatory cytokines, not elevated in athletes where cardiac function remains unaffected (La Gerche et al. 2015). However, a serum inflammatory marker which persists for some time after the initiation of inflammation, such as C-reactive protein, may be best to evaluate differences in the inflammatory response over a longer time frame (Scherr et al. 2011; Stewart et al. 2016), of multiday racing.

Secondary hypervolemia is a well-documented phenomenon that occurs during strenuous multiday endurance racing and typically occurs already after 2–4 days of racing (Garvican et al. 2010; Schumacher et al. 2008). Such a physiological response may be functional in partially compensating for further progression of exercise-induced cardiac dysfunction by increasing cardiac preload (Oosthuyse et al. 2012). Secondary plasma volume (PV) expansion appears to be a counter-regulatory measure to the occurrence of notable post-exercise hypotension that is exacerbated with the heightened stress and inflammatory response to strenuous exercise above the level routinely experienced during daily training. Briefly, upright exercise is associated with a redistribution of interstitial and lymph albumin into the vascular space resulting in an increase in colloid oncotic pressure that causes an associated fluid shift (Nagashima et al. 2000). This increase in lymph return is supported by the muscle pump during exercise and persists during recovery only in the incidence of post-exercise hypotension (Hayes et al. 2000; Nagashima et al. 1999). Post-exercise hypotension is a consequence of local inflammatory histamine secretion that maintains vasodilation in previously active muscles (Romero et al. 2017). Other than maintaining increased lymph return, post-exercise hypotension results in reduced atrial natriuretic peptide secretion and thus a reduced transcapillary escape rate of albumin, ensuring extended maintenance of an increased plasma albumin content post exercise (Haskell et al. 1997; Nagashima et al. 1999). Moreover, the rate of hepatic albumin synthesis is increased in

recovery with the provision of calories (Nagashima et al. 2000; Yang et al. 1998; Wada et al. 2018), further adding towards the increase in plasma albumin content and thus PV expansion.

The potential benefit of an increased PV for cardiac function during multiday racing can be appreciated from an increased cardiac preload by a postural intervention of leg raising immediately following a marathon running race that restored the rate of passive early diastolic filling and flow propagation velocity to pre-race levels despite persistent evidence of reduced LV relaxation and thus diastolic dysfunction following the marathon (Hart et al. 2007). Furthermore, induction of 16% PV expansion by intravenous infusion of a dextran-based solution enhanced stroke volume and cardiac output during exercise (Roy et al. 2000). Therefore, an exogenous factor (independent of additional physiological strain) that accentuates secondary PV expansion during multiday racing would be favourable. Interestingly, ingesting protein together with carbohydrate in recovery from strenuous exercise increases plasma albumin content and thus PV by a greater extent than carbohydrate-only (Hobson and James 2015; Kataoka et al. 2016) and results in an increased stroke volume during subsequent exercise (Okazaki et al. 2009). The ingested protein provides the necessary amino acids to increase the rate of hepatic albumin synthesis by hepatocyte nuclear factor-1 and mTOR signalling (Wada et al. 2018). No study has previously evaluated the effect of ingesting protein plus carbohydrate compared with carbohydrate-only during exercise on plasma albumin content and PV expansion following multiday endurance racing. The rate of whole body protein synthesis is increased during exercise when ingesting carbohydrate–casein protein hydrolysate compared with ingesting carbohydrate-only (Beelen et al. 2011). Therefore, it seems reasonable to expect that ingesting carbohydrate–casein hydrolysate during daily racing of a multiday race may magnify the expected PV expansion compared with ingesting carbohydrate-only.

Therefore, the aim of the current study was to compare ingestion of a carbohydrate plus casein protein hydrolysate and carbohydrate-only supplement during a real-life 3-day multiday mountain bike race on pre-day 1 to post-race day 3 changes in PV, LV function and blood biomarkers of membrane instability and inflammation. The race-village setup, which provides constant sleeping and meal conditions for all participants, presents an ideal arrangement to test the efficacy of an intervention under real-life racing conditions. We hypothesize that ingesting casein-protein hydrolysate with carbohydrate compared with carbohydrate-only during a 3-day mountain bike race will reduce the increase in blood biomarkers of membrane instability and inflammation and reduce the evidence for LV dysfunction following completion of the 3-day race. Furthermore, we hypothesize that ingesting casein-protein hydrolysate with carbohydrate each day during racing will increase plasma albumin content and thus the magnitude of PV expansion compared with ingesting carbohydrate-only.

Methodology

Participants

Healthy, well-trained male cyclists who had signed up to participate in a local 3-day mountain bike stage race in the Western Cape, South Africa were recruited to volunteer. Eighteen cyclists enrolled in the study, two cyclists withdrew the day before the race and a further two cyclists withdrew after the first day of racing due to injury or illness. Fourteen cyclists successfully completed the 3 days of racing and all measurements (Table 1). The cyclists were eligible to participate if they: were between 19 and 45 years old, trained at least 6 h/week with a minimum of 5 years cycling history and had prior multiday racing experience, were non-smokers, not taking medication, and had no known history of cardiovascular disease including: hypertension (having a resting brachial blood pressure of < 140/90 mmHg), hypercholesterolemia, or any previously diagnosed structural or functional cardiac defect, and never experienced symptoms, such as, irregular heartbeats or palpitations, exertional chest pain, syncope, shortness of breath. Echocardiograph measures of LV geometry and function were required to fall within the accepted normal limits for athletes (Oxborough et al. 2018). All cyclists provided written consent to take

Table 1 Anthropometry (mean ± SD)

	PRO	CHO	*p*
Sample size (*n*)	7	7	
Age (years)	35.9 ± 5.4	40.1 ± 4.8	0.14
Height (cm)	183 ± 4	182 ± 9	0.84
Body mass (kg)	84.3 ± 8.0	83.1 ± 9.4	0.80
Body mass index (kg/m^2)	25.1 ± 2.3	25.0 ± 2.4	0.91
Body fat (%)	18.7 ± 2.7	19.9 ± 4.0	0.53
SBP (mmHg)	126 ± 13	125 ± 12	0.95
DBP (mmHg)	76 ± 7	81 ± 7	0.24
MAP (mmHg)	93 ± 8	96 ± 8	0.52
LV mass (g)	175 ± 28	168 ± 22	0.58
LV mass (g/m^2)	84 ± 11	82 ± 8	0.62
LV wall thickness (mm)	9 ± 1	9 ± 1	0.56
LV wall thickness (mm/m$^{0.5}$)	6.5 ± 0.6	6.4 ± 0.7	0.73
LV relative wall thickness	0.36 ± 0.05	0.36 ± 0.06	0.96
Cycling history (years)	10.3 ± 3.8	12.3 ± 6.7	0.50
Cycling training (h/week)	8.0 ± 1.7	8.3 ± 2.0	0.78

SBP systolic blood pressure, *DBP* diastolic blood pressure, *MAP* mean arterial pressure, *LV* left ventricular

part after the study procedures, purpose and risks had been explained. The study protocol was conducted in accordance with the 1964 Helsinki declaration and was granted ethical clearance by the Human Research Ethics Committee (Medical) at the University of the Witwatersrand, Johannesburg, South Africa (M160814).

Study design

Cyclists attended a pre-race measurement session at the laboratory 2–7 days before participating in a 3-day mountain bike race. In this double blind study, cyclists were randomly assigned to either ingest a carbohydrate-only supplement (CHO) or a carbohydrate plus casein protein hydrolysate mixed supplement (PRO) during each day of racing. Based on prior racing and training history, the cyclists were pair-matched so that one of each pair would be placed into each treatment group. Briefly, cyclists were ordered according to their weekly training volume and assigned to alternate groups in sequence of order, where the order of group designation was also alternated between successive pairs. Only the investigator TO was aware of the randomisation method and group assignments. Investigator AM who performed all echocardiograph assessments remained blind to the group assignments until completion of data collection. Post-race measurements were recorded at the finish-line.

Pre- and post-race measurements

For 24 h before their first visit and for the duration of their participation, cyclists were told to abstain from ingesting pharmaceutical antioxidant supplements and nutraceuticals. Cyclists were told to avoid hard exercise 48 h before their pre-race measurement and no exercise was permitted within 24 h of the visit. Pre-race measurements took place at the Division of Exercise Science and Sports Medicine at the University of Cape Town. Post-race measurements took place in a covered area at the race finish, 42 ± 18 min and 33 ± 15 min after completing the race in the PRO and CHO groups, respectively. On arrival, participants changed into light shorts and T-shirt and emptied their bladders. Height, body mass and sum of 4-skinfolds (bicep, tricep, subscapular, supra-iliac) were recorded. Seated blood pressure recordings were taken following a 20 min resting period (Medic Elite 1219, Shanghai International Trading Corp. GmbH, Hamburg, Germany).

Blood analyses

Twelve millilitres of blood was collected from an antecubital vein into 3×4 mL vacutainers containing ethylenediaminetetraacetic acid, lithium heparin, and serum separating gel, respectively. Haematocrit and haemoglobin concentration were determined using an automated blood cell counter (DxH800 Coulter, Beckman Coulter Inc, CA, USA). The remaining blood samples were centrifuged and used for analysis of serum albumin concentration, total creatine kinase activity (CK), and high sensitivity C-reactive protein (hsCRP) concentration (with lower detection limit of 0.08 mg/L) on the AU5800 Beckman Coulter analyser (Beckman Coulter Inc, CA, USA). Serum creatine kinase-MB isoform (CK-MB) and plasma cardiac troponin I (cTnI) (with lower detection limit of 0.03 ng/mL) concentrations were measured on the Access Beckman Coulter analyser (Beckman Coulter Inc, CA, USA) and Stratus CS STAT fluorometric analyser (Siemens Healthcare Diagnostics, Camberley, UK), respectively. Blood volume (BV) in litres was estimated from height and body mass using the equation of Nadler et al. (1962),

$$\mathrm{BV} = 0.3669 \times \mathrm{Height\ (m)}^3 + 0.03219 \times \mathrm{Body\ mass\ (kg)} + 0.6041.$$

Plasma volume (PV) and percentage change in plasma volume (%ΔPV) from pre to post race were determined from haematocrit (Hct) (fraction of packed red blood cells) and haemoglobin (Hb) concentration (g/dL) using the equations of Dill and Costill (1974),

$$\mathrm{PV} = \mathrm{BV} - (\mathrm{BV} \times \mathrm{Hct}),$$

$$\%\Delta\mathrm{PV} = \left[100 \times (\mathrm{Hb_{pre}}/\mathrm{Hb_{post}}) \times (1 - \mathrm{Hct_{post}})/(1 - \mathrm{Hct_{pre}})\right] - 100.$$

Post-race plasma and serum protein concentrations were corrected for PV change. Serum albumin content was determined by multiplying serum albumin concentration by PV and expressing it relative to body mass.

Echocardiography

Left ventricular cardiac function was evaluated by two-dimensional echocardiography and tissue Doppler imaging by a single trained operator (AM) who was blind to the respective group designations of the cyclists. Echocardiograph measurements were performed using a SonoSite M-Turbo ultrasound device (Sonosite Inc., Bothell, WA, USA) according to current guidelines (Lang et al. 2015; Oxborough et al. 2018), with the cyclists in the partial left decubitus position.

Two-dimensional, M-mode images were obtained from the parasternal long axis view with measurements taken at the tips of the mitral valve leaflets to record left ventricular posterior wall and inter-ventricular septal wall thickness, left ventricular internal diameter at end diastole (LVIDd) and systole to calculated end diastolic (EDV) and

systolic volume (ESV) (Teichholz et al. 1976). Left atrial (LA) anterior–posterior diameter (D) was measured from the parasternal long axis view and LA transverse (T) and longitudinal (L) dimensions were acquired from averaged measurements from both 4- and 2-chamber apical views during LV systole (Canciello et al. 2017). LA volume was then calculated using the validated ellipsoid model where, LA volume $= \pi/6 \times D \times T \times L$ (Canciello et al. 2017). Left ventricular mass (g) and left ventricular mass indexed to body surface area (g/m^2) were calculated (Lang et al. 2015). Relative wall thickness was derived as the ratio of the sum of inter-ventricular septal wall and posterior wall thickness in diastole and LVIDd, to assess for eccentric or concentric hypertrophy (Oxborough et al. 2018).

Left ventricular systolic function was evaluated by deriving the following indices: stroke volume (SV), as the difference between EDV and ESV; Ejection fraction, as SV divided by EDV converted to a percentage; pressure–volume relationship (systolic blood pressure/ESV); and ventricular–arterial coupling (SV/ESV) (Claessen et al. 2014).

Left ventricular diastolic function was evaluated from an apical four-chamber view. Pulsed-wave Doppler (PWD) early (*E*) and late (*A*) peak transmitral blood flow velocities during filling were measured at the mitral valve leaflet tips and the *E*/*A* ratio calculated. As a further measure of systolic function, the peak rate of blood flow from the LV into the aorta was recorded with the sample cursor placed at the level of the aortic valve (AV_Vmax). Tissue-Doppler imaging (TDI) of the septal and lateral wall at the level of the mitral annulus was used to record peak early (e') and late (a') diastolic tissue velocities and e'/a' ratio was calculated. Peak systolic mitral annular tissue velocity (s') was also recorded at the septal and lateral wall. The E/e' ratio was derived using septal e' and lateral e' as an index of ventricular filling pressure (Nagueh et al. 2016).

Multiday race

The cyclists participated in an official 3-day mountain bike stage race in the Western Cape, South Africa that included: stage 1–68 km and 1650 m ascent; stage 2–71 km and 1350 m ascent; stage 3–71 km and 1200 m ascent. The mean (± SD) weather conditions for each stage were: ambient temperature 19 ± 3; 20 ± 4; 19 ± 2 °C; relative humidity 63 ± 6%, 58 ± 13%, 55 ± 10%; and wind speed 20 ± 4, 13 ± 3, 18 ± 3 km/h, respectively. All participants stayed at the race venue for their full participation where they slept and ate under the same conditions. The camp site was prepared so that each cyclist slept in a standard two-man sized tent fitted with a standard mattress. The camp was woken each morning at 05h30 with floodlights and lights-out at night occurred at 22h00. A fixed menu for breakfast, lunch and supper was supplied each day, with an additional meal supplied immediately on finishing each stage. The cyclists were provided with a tabulated-sheet to record details of all that they ate each day: they were advised on the method of reporting quantities by volume or weight if known or by cup, tablespoon or fixed portions and to list meal components in detail or by product if known. The daily total energy and macronutrient composition (carbohydrate: protein: fat) of nutrients ingested were determined using calorie counter software (MyFitnessPal, San Francisco, CA, USA). Each stage of racing began at 07h00.

Supplied supplement beverages

Sachets of the experimental supplements were prepared by TO by weighing aliquots consisting of: 46 g of a carbohydrate–electrolyte mix (CarboFuel, Optimum Cadence, Cape Town, South Africa) into re-sealable plastic pockets for the CHO group; and 46 g of the same carbohydrate–electrolyte mix plus 10 g of a casein protein hydrolysate supplement (PeptoPro Litely Fruity; DSM Nutritional Products, Johannesburg, South Africa) mixed together into re-sealable plastic pockets for the PRO group. Each weighed aliquot of carbohydrate–electrolyte provided an equal 40 g/sachet of carbohydrate as part maltodextrin: fructose (3:2) plus electrolytes in both the CHO and PRO groups. In addition, the weighed aliquot of casein protein hydrolysate supplement provided 8 g/sachet of casein protein hydrolysate in the PRO group.

Each cyclist was provided with 4 × sachets of supplement powder for each of the 3 days of racing; where sachets were labelled by participant number only and cyclists were blind to the drink-type. They were instructed to mix a full sachet with 600 mL of water in a standard size cycling bottle resulting in a 6.7% carbohydrate solution with 19 mmol/L concentration of electrolytes (CHO group) plus 1.3% protein solution (PRO group); producing a drink osmolality of 231 mOsm/kg H_2O and 288 mOsm/kg H_2O (Model 3320 Osmometer, Advanced Instruments Inc, MA, USA), respectively. They were told to have two reconstituted bottles in the bottle cages on their bikes at the start of each stage. The remaining two sachets of supplement powder were carried in their cycling jersey pocket for reconstituting at designated refuelling points on the route, should they require it. Each stage had 3–4 refuelling points at 15–20 km intervals. The cyclists were told to drink the supplement *ad libitum* depending on thirst with a rough guideline of aiming to ingest one full bottle each hour. They were told that they were free to drink samples of the other fluids provided at the refuelling tables as needed (which included Coca Cola, water, and a plain 5% glucose and electrolyte drink). They were also free to ingest snacks at will that included: jelly babies, bananas, boiled potatoes, marmite sandwiches, watermelon, carbohydrate-based energy gels, selected sports bars. The

cyclists recorded detailed descriptions of all that they had ingested during exercise on a tabulated-sheet on completing each stage of the race.

Rating of perceived exertion, fatigue and muscle soreness

The cyclists were provided with a tabulated-sheet on which they recorded their rating of perceived exertion for each race stage on a scale of 1–10; where each numeral corresponded to a word descriptor, varying from 1—no effort to 10—extreme/maximal effort. Similarly, leg and upper-body fatigue and soreness were ranked following each stage on a scale of 1–10, with word descriptors varying from 1—none to 10—totally exhausted or unbearable pain.

Statistical analysis

A Student's paired t test was used to compare measurements recorded before and after the race within a group and the mean change (Δ, post minus pre-race) and the 95% confidence interval (CI) of the change is presented to describe the magnitude of effect. An unpaired t test was used to compare whether the magnitude of the change in a variable (post minus pre-race) varied between groups. The False Discovery Rate with Benjamini–Hochbery correction was applied to control for multiple comparisons and only outcomes that were classed as a "discovery" are reported as significant. Cohen's standardised mean change/effect size (d) was calculated to describe the effect within a group as the mean change (post-pre)/SDpre, where SDpre is the overall group pre-race SD from $n = 14$. To describe the between group-effect, Cohen's d was calculated as the mean difference of the calculated changes between groups, (ΔPRO minus ΔCHO)/$\sqrt{((SD_{\Delta PRO}{}^2 + SD_{\Delta CHO}{}^2)/2)}$. Cohen's d is described as follows: 0–0.2 trivial; 0.2–0.6 small; 0.6–1.2 moderate; 1.2–2.0 large; 2.0–4.0 very large; > 4.0 extremely large effect. Considering intra-individual day-to-day variability for Hb and Hct of 4.6% (Borel et al. 1991), a sample size of $n = 14$ (or $n = 7$ per group) provided 80% statistical power of identifying a 6% change in PV within a group and a 7% difference in the change in PV between groups. Analysis of variance with repeated measures for time and Bonferroni post hoc test was used to identify effects when more than two measurement points were recorded. Data were tested for normality before analysis and when required was normalized by log-transformation. $p < 0.05$ was considered significant and data are presented as mean ± SD or mean change (and 95% CI).

Results

All cyclists presented with normal resting brachial blood pressure, and normal LV geometry and function—with reference to accepted athlete norms (Oxborough et al. 2018). Groups were matched for anthropometry and training history (Table 1). Body mass did not change significantly from pre-to-post race in either group (mean change (95% CI), PRO: − 0.5 kg (− 1.4 to 0.4 kg), $p = 0.22$; CHO: − 0.6 kg (− 1.6 to 0.4 kg), $p = 0.18$). The daily total energy and macronutrient composition (carbohydrate: protein: fat) of nutrients ingested, excluding nutrients ingested during exercise, were similar between groups (mean ± SD, PRO: 12,907 ± 1389 kJ/day as 40 ± 5%: 23 ± 2%: 36 ± 5%; CHO: 13,194 ± 2586 kJ/day as 41 ± 5%: 23 ± 2%: 36 ± 5%, respectively). The time taken to complete each stage of the race, total volume of fluid, carbohydrate and fat ingested during exercise on each day were similar between groups (Table 2). As expected, the total protein ingested during exercise was significantly greater in the PRO group than CHO group.

Plasma volume and albumin content

In the CHO group, PV increased by 10.2% (95% CI 0.1 to 20.2%) and was associated with a similar 8.6% (95% CI − 1.1 to 18.3%) increase in serum albumin content after 3 days of racing, which was identified as a large effect and a moderate effect, respectively (Table 3). No significant change to PV (0.4%, 95% CI − 6.1 to 6.9%) or serum albumin content (− 0.1%, 95% CI − 5.8 to 5.7%) occurred in the PRO group. The difference of the change in these variables between the PRO and CHO groups tended to be significant and was described as a moderate effect.

Markers of inflammation and cellular membrane instability

Indices of membrane instability including total serum CK activity and cardiac-specific serum CK-MB concentration increased after 3 days of racing with very large–extremely large effect sizes (Table 3). Statistical comparison of effect sizes revealed a trend or significantly smaller effect in the PRO group compared with the CHO group (difference in Cohen's d (95% CI), CK − 5.7 (− 12.1 to 0.7), $p = 0.075$; CK-MB − 6.45 (− 12.58 to − 0.32), $p = 0.042$). Plasma cardiac troponin I (cTnI) concentration was below detection in all cyclists before the race (< 0.03 ng/mL) and only increased modestly above the detection limit (0.04–0.05 ng/mL) in four cyclists following the race ($n = 2$ per group). High sensitivity C-reactive protein (hsCRP) concentration increased following 3 days of racing only in the CHO

Table 2 Nutrition during exercise on each day of racing (mean ± SD)

	Total nutrients ingested during exercise			Nutrients ingested from supplied supplement		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Riding time (h)						
PRO	4.9 ± 0.7	4.3 ± 0.5	4.0 ± 0.5			
CHO	5.1 ± 0.8	4.5 ± 0.5	4.2 ± 0.6			
Fluid volume (mL/h)						
PRO	550 ± 315	663 ± 312	606 ± 256	364 ± 136	342 ± 171	317 ± 128
CHO	472 ± 122	634 ± 239	642 ± 149	317 ± 102	377 ± 161	417 ± 106
Carbohydrate (g/h)						
PRO	45.0 ± 13.5	47.5 ± 14.5	45.8 ± 20.0	23.1 ± 8.6	21.7 ± 10.8	20.1 ± 8.1
CHO	45.6 ± 14.8	50.9 ± 14.6	49.0 ± 13.4	20.2 ± 6.5	23.9 ± 10.2	26.5 ± 6.7
Protein (g/h)						
PRO[a]	6.0 ± 1.7	5.9 ± 1.6	5.4 ± 1.8	4.6 ± 1.7	4.3 ± 2.2	4.0 ± 1.6
CHO	1.7 ± 1.2	1.6 ± 1.0	1.4 ± 1.1	–	–	–
Fat (g/h)						
PRO	1.8 ± 1.4	0.9 ± 1.0	0.5 ± 0.4			
CHO	1.2 ± 1.1	1.0 ± 1.4	0.9 ± 1.1			

[a]Protein ingestion was significantly greater in the PRO group compared with the CHO group on all days, $p < 0.00001$

group with a large effect but did not increase in the PRO group (Table 3). A statistical comparison of effect sizes for hsCRP revealed a trend for a smaller effect in PRO compared with CHO (difference in Cohen's *d* (95% CI), hsCRP − 1.2 (− 2.53 to 0.13), $p = 0.074$).

Left ventricular diastolic function

Pulsed-wave Doppler measured rate of peak blood flow into the LV at the level of the mitral valve during diastole showed that while the rate of early filling (*E*) was preserved, late filling due to atrial contraction (*A*) tended to be increased in the PRO group and was significantly increased in the CHO group resulting in a decrease in the *E/A* ratio in both groups following 3 days of racing (Table 4). That together with the observed prolonged deceleration time from peak *E*, indicates mild suppression of diastolic function as a result of reduced LV relaxation in both the CHO and PRO groups. However, TDI measures of peak mitral annular velocities during early (*e′*) and late (*a′*) LV diastolic filling, show a significantly decreased *e′/a′* ratio only at the septal wall in the PRO group but not the CHO group (Table 4). Interestingly, both lateral wall peak *e′* and *a′* mitral annular velocities were significantly increased in the PRO group only, resulting in no significant change to lateral wall *e′/a′* in the PRO or CHO groups. This may suggest an uneven disturbance of diastolic function in the LV in PRO group following 3 days of racing, with a notable mild suppression of diastolic function in the septal wall and an effort to compensate with increased tissue velocities in the lateral wall.

Left ventricular preload and afterload

The notable PV expansion in the CHO group may be expected to augment LV preload. A small significant increase in LA volume occurred from pre-to-post race in the CHO group (Table 5). Further measures of LV preload, such as LVIDd and EDV, did not change significantly possibly owing to impaired LV relaxation and mild LV diastolic dysfunction. All echocardiograph measures of preload remained unchanged in the PRO group. Systolic blood pressure (mean change (95% CI) − 4.6 (− 16.8 to 7.7) mmHg and 1.3 (− 12.4 to 15.0) mmHg), diastolic blood pressure (− 4.6 (− 11.7 to 2.6) mmHg and − 7.1 (− 15.6 to 1.3) mmHg) and mean arterial pressure (− 4.6 (− 11.2 to 2.0) mmHg and − 4.3 (− 10.6 to 1.9) mmHg) did not change significantly from pre to post race in PRO and CHO, respectively.

Left ventricular systolic function

General LV contractility did not change from pre to post race as indicated by no change in EF or pressure–volume ratios in both groups (Table 6). Interestingly, however, while TDI measured septal wall velocity during systole (septal *s′*) did not change in either group, lateral wall velocity during systole (lateral *s′*) did increase significantly with a large effect from pre to post race in the PRO group only. This again supports the non-uniform effect across LV wall function in the PRO group following 3 days of racing.

Table 3 Change in markers of secondary hypervolemia, membrane instability and inflammation before and after a 3-day mountain bike race when ingesting a carbohydrate–casein protein hydrolysate (PRO) or carbohydrate-only (CHO) beverage during racing

	Pre	Post	Post minus Pre	Within-group		Between-group (ΔPRO versus ΔCHO)	
	Mean ± SD	Mean ± SD	Mean Δ (95% CI)	*p*	*d*	*p*	*d*
ALB (g/dL)							
PRO	4.8 ± 0.2	4.8 ± 0.4	− 0.0 (− 0.3; 0.3)	0.98	− 0.01		
CHO	4.6 ± 0.1	5.0 ± 0.4	0.4 (− 0.04; 0.8)	0.07	1.9	0.089	− 0.99
ALB (g/kg)							
PRO	1.86 ± 0.14	1.85 ± 0.15	− 0.0 (− 0.11; 0.11)	0.95	− 0.025		
CHO	1.74 ± 0.11	1.89 ± 0.22	0.15 (− 0.01; 0.32)	0.067†	1.14	0.083	− 1.01
Hct (%)							
PRO	41.9 ± 1.8	42.1 ± 2.1	0.3 (− 0.1; 1.6)	0.60	0.004		
CHO	43.3 ± 1.8	41.6 ± 2.2	− 1.7 (− 4.0; 0.5)	0.11	− 0.02	0.083	1.01
Hb (g/dL)							
PRO	14.2 ± 0.9	14.1 ± 0.8	− 0.1 (− 0.8; 0.6)	0.73	− 0.12		
CHO	14.9 ± 0.6	14.0 ± 0.7	− 0.9 (− 1.7; − 0.1)	0.033*	− 1.12	0.085	1.01
PV (mL/kg)							
PRO	38.6 ± 2.0	38.7 ± 2.3	0.08 (− 2.5; 2.6)	0.94	0.04		
CHO	37.7 ± 1.9	41.6 ± 5.1	3.9 (0.1; 7.7)	0.045*	2.03	0.063†	− 1.10
CK (IU/L)							
PRO	172.3 ± 114.9	515.5 ± 410.5	343.3 (− 56.3; 742.9)	0.08	4.03		
CHO	128.4 ± 37.7	957.7 ± 803.8	829.3 (92.8; 1566.0)	0.033*	9.73	0.18	− 0.76
CK-MB (ng/mL)							
PRO	2.36 ± 1.02	6.06 ± 2.43	3.71 (1.84; 5.58)	0.003*	4.08		
CHO	2.37 ± 0.87	11.95 ± 8.86	9.58 (1.72; 17.43)	0.025*	10.53	0.10	− 0.95
hsCRP (mg/L)[a]							
PRO	2.07 ± 0.99	6.86 ± 9.77	4.79 (− 4.25; 13.82)	0.33	0.61		
CHO	1.68 ± 1.43	8.73 ± 5.64	7.05 (1.36; 12.74)	0.032*	1.81	0.19	− 0.74

ALB serum albumin concentration (g/dL) and content (g/kg), *Hct* haematocrit, *Hb* haemoglobin concentration, *PV* plasma volume, *CK* Total serum creatine kinase activity, *CK-MB* heart-dominant creatine kinase isoform concentration, *hsCRP* high sensitivity C-reactive peptide concentration, Δ change in a variable as post minus pre race, *d* Cohen's effect size

† and * denotes a difference from pre-to-post race within a group or in the pre-to-post change between groups as a trend and as significant, $p < 0.05$, respectively

[a]Data that is not normally distributed was log transformed before analysis and Cohen's *d* was calculated on the log transformed data, group values are raw mean ± SD and mean (95% CI), respectively

Rating of perceived exertion, fatigue and soreness

Rating of perceived exertion (heavy–very heavy; PRO: 7.2 ± 1.4, CHO: 7.6 ± 1.6) and leg soreness (mild–moderate; PRO: 5.3 ± 1.4, CHO: 4.6 ± 1.8) were ranked similarly following each day of racing in each group and did not differ significantly between groups. However, the following variables were ranked as less severe after day 3 compared with after day 1 of racing only in the CHO group: leg fatigue (mean ± SD, day 1: 6.1 ± 2.0, day 3: 5.1 ± 1.5, $p = 0.06$), upper body fatigue (day 1: 4.9 ± 2.3, day 3: 3.6 ± 1.5, $p < 0.01$) and upper body soreness (day 1: 5.0 ± 2.6, day 3: 3.3 ± 1.4, $p < 0.01$). Conversely, in the PRO group, the rankings of these latter variables (6.4 ± 1.3, 3.9 ± 1.9 and 3.8 ± 1.7, respectively) were maintained at a similar level after all 3 days.

Discussion

Contrary to the hypothesis, ingesting a carbohydrate plus casein protein hydrolysate drink during exercise on each day of a 3-day mountain bike race prevented the increase in plasma albumin content and PV expansion that occurred when ingesting a carbohydrate-only drink each day during racing. Nevertheless, despite evidence of compromised LV relaxation in both groups and evidence for impeded diastolic function in particularly the septal wall in the

Table 4 Change in LV diastolic function before and after a 3-day mountain bike race with a carbohydrate plus casein protein hydrolysate (PRO) or carbohydrate-only (CHO) supplement

	Pre	Post	Post minus Pre	Within-group		Between-group (ΔPRO versus ΔCHO)	
	Mean ± SD	Mean ± SD	Mean Δ (95% CI)	*p*	*d*	*p*	*d*
Pulsed-wave Doppler							
E (cm/s)							
PRO	117.2 ± 21.4	119.5 ± 20.1	2.4 (− 16.5; 21.2)	0.76	0.07		
CHO	118.4 ± 49.8	121.2 ± 52.2	2.8 (− 29.7; 35.3)	0.83	0.08	0.98	− 0.02
A (cm/s)							
PRO	81.7 ± 23.1	103.1 ± 7.6	21.4 (− 3.9; 46.7)	0.081	0.95		
CHO	73.0 ± 25.2	102.3 ± 25.4	29.3 (16.0; 42.6)	0.002**	1.30	0.49	− 0.41
E/A							
PRO	1.48 ± 0.24	1.16 ± 0.16	− 0.32 (− 0.57; − 0.07)	0.022*	− 1.28		
CHO	1.58 ± 0.29	1.17 ± 0.40	− 0.41 (− 0.67; − 0.16)	0.009**	− 1.63	0.53	0.37
E deceleration time (ms)							
PRO	168.3 ± 28.6	192.5 ± 28.2	24.2 (− 0.5; 48.9)	0.054†	1.01		
CHO	146.7 ± 15.1	193.3 ± 26.6	46.7 (11.8; 81.6)	0.019*	1.95	0.21	− 0.78
Tissue Doppler imaging							
Septal wall *e′* (cm/s)							
PRO	11.2 ± 2.0	9.9 ± 1.6	− 1.3 (− 3.3; 0.7)	0.16	− 0.56		
CHO	10.7 ± 2.7	9.8 ± 1.0	− 0.9 (− 2.9; 1.2)	0.35	− 0.37	0.71	− 0.20
Septal wall *a′* (cm/s)							
PRO	6.8 ± 1.9	7.3 ± 1.1	0.5 (− 1.4; 2.3)	0.55	0.29		
CHO	8.0 ± 1.1	8.0 ± 1.9	− 0.02 (− 1.7; 1.6)	0.98	− 0.01	0.63	0.26
Septal wall *e′/a′*							
PRO	1.69 ± 0.30	1.36 ± 0.22	− 0.32 (− 0.56; − 0.09)	0.016*	− 1.04		
CHO	1.32 ± 0.21	1.29 ± 0.29	− 0.03 (− 0.28; 0.22)	0.75	− 0.11	0.063†	− 1.10
Lateral wall *e′* (cm/s)							
PRO	12.0 ± 1.5	15.7 ± 3.5	3.6 (0.4; 6.9)	0.034*	1.38		
CHO	14.2 ± 3.2	13.3 ± 4.3	− 0.9 (− 4.5; 2.7)	0.55	− 0.35	0.04*	1.23
Lateral wall *a′* (cm/s)							
PRO	6.6 ± 1.5	9.7 ± 2.2	3.1 (1.0; 5.3)	0.012*	1.55		
CHO	7.9 ± 2.3	8.2 ± 2.2	0.3 (− 2.0; 2.7)	0.74	0.16	0.052†	1.15
Lateral wall *e′/a′*							
PRO	1.94 ± 0.59	1.70 ± 0.55	− 0.24 (− 0.89; 0.41)	0.40	− 0.39		
CHO	1.92 ± 0.70	1.71 ± 0.60	− 0.83 (− 0.83; 0.41)	0.44	− 0.34	0.93	− 0.05
E/septal *e′*							
PRO	10.4 ± 1.4	12.2 ± 1.8	1.8 (− 0.4; 4.1)	0.09	0.74		
CHO	10.6 ± 3.5	11.9 ± 4.5	1.3 (− 1.3; 3.9)	0.26	0.53	0.71	0.22
E/lateral *e′*							
PRO	10.0 ± 2.9	8.2 ± 2.7	− 1.8 (− 5.4; 1.8)	0.25	− 0.61		
CHO	8.1 ± 3.3	9.6 ± 3.8	1.6 (− 3.1; 6.2)	0.43	0.52	0.17	− 0.85

E early diastolic transmitral blood flow velocity, *A* late diastolic transmitral blood flow velocity due to atrial contraction, *E/A* ratio of early to late diastolic transmitral blood flow velocity, *E deceleration time* the deceleration time from the peak of early diastolic transmitral blood flow, *e′* early diastolic mitral annular tissue velocity, *a′* late diastolic mitral annular tissue velocity due to atrial contraction, *e′/a′* ratio of early to late diastolic mitral annular tissue velocity, *E/septal e′* and *E/Lateral e′* ratio of early diastolic transmitral blood flow velocity to early diastolic mitral annular velocity at the septal wall and lateral wall, respectively, Δ change in a variable as post minus pre race, *d* Cohen's effect size

† and * or ** denotes a difference from pre-to-post race within a group as a trend and as significant, $p < 0.05$ or $p < 0.01$, respectively

Table 5 Change in measures of left ventricular preload before and after a 3-day mountain bike race with a carbohydrate plus casein protein hydrolysate (PRO) or carbohydrate-only (CHO) supplement

	Pre	Post	Post minus Pre	Within-group		Between-group (ΔPRO versus ΔCHO)	
	Mean ± SD	Mean ± SD	Mean Δ (95% CI)	*p*	*d*	*p*	*d*
LA vol (mL)							
PRO	34 ± 7	32 ± 6	−2 (−7; 2)	0.25	−0.38		
CHO	36 ± 6	39 ± 7	3 (1; 5)	0.007*	0.49	0.018*	−1.46
LVIDd (cm)							
PRO	5.21 ± 0.56	5.23 ± 0.53	0.02 (−0.36; 0.40)	0.90	0.04		
CHO	5.11 ± 0.54	5.27 ± 0.61	0.16 (−0.40; 0.71)	0.52	0.29	0.62	−0.26
EDV (mL)							
PRO	128 ± 32	133 ± 29	5 (−17; 27)	0.59	0.03		
CHO	123 ± 30	136 ± 37	13 (−1; 4)	0.29	0.42	0.60	−0.44
Heart rate (bpm)							
PRO	61 ± 12	81 ± 11	20 (14; 26)	0.0002*	1.65		
CHO	60 ± 13	84 ± 13	24 (16; 32)	0.0003*	1.98	0.34	−0.53

LA vol left atrial volume, *LVIDd* left ventricular internal chamber diameter at end diastole, *EDV* end diastolic volume. Δ change in a variable as post minus pre race, *d* Cohen's effect size

*Significant difference from pre-to-post race within a group, $p < 0.05$

Table 6 Change in LV systolic function before and after a 3-day mountain bike race with a carbohydrate plus casein protein hydrolysate (PRO) or carbohydrate-only (CHO) supplement

	Pre	Post	Post minus Pre	Within-group		Between-group (ΔPRO versus ΔCHO)	
	Mean ± SD	Mean ± SD	Mean Δ (95% CI)	*p*	*d*	*p*	*d*
SV (mL)							
PRO	82 ± 23	79 ± 19	−3.6 (−15.4; 8.2)	0.48	−0.17		
CHO	79 ± 21	86 ± 23	7.4 (−2.0; 16.9)	0.10	0.35	0.10	−0.95
EF (%)							
PRO	62 ± 6	59 ± 5	−2.9 (−8.7; 2.9)	0.27	−0.55		
CHO	64 ± 5	64 ± 9	0.4 (−9.8; 10.7)	0.92	0.08	0.51	−0.37
SBP/ESV (mmHg/mL)							
PRO	2.7 ± 0.6	2.4 ± 0.7	−0.3 (−0.8; 0.2)	0.24	−0.38		
CHO	3.0 ± 0.8	3.0 ± 1.5	0.06 (−1.7; 1.8)	0.93	0.09	0.67	−0.24
V–A coupling (SV/ESV)							
PRO	1.7 ± 0.4	1.5 ± 0.3	−0.2 (−0.6; 0.2)	0.22	−0.57		
CHO	1.8 ± 0.4	2.0 ± 0.7	0.1 (−0.6; 0.9)	0.67	0.40	0.34	−0.53
AV_Vmax (cm/s)							
PRO	142.8 ± 30.4	149.1 ± 35.0	6.3 (−29.2; 41.9)	0.68	0.26		
CHO	151.8 ± 17.8	192.6 ± 54.8	40.9 (−7.8; 89.5)	0.086	1.67	0.19	−0.75
TDI septal *s′* (cm/s)							
PRO	7.7 ± 1.9	7.9 ± 0.9	0.2 (−1.5; 1.8)	0.81	0.10		
CHO	7.6 ± 1.4	9.1 ± 2.0	1.6 (−1.4; 4.5)	0.24	0.98	0.33	−0.54
TDI lateral *s′* (cm/s)							
PRO	8.0 ± 0.8	11.1 ± 1.9	3.1 (1.7; 4.5)	0.002**	1.50		
CHO	9.2 ± 2.8	10.5 ± 1.9	1.3 (−2.0; 4.7)	0.37	0.65	0.26	0.64

SV stroke volume, *EF* ejection fraction, *SBP/ESV* ratio of systolic blood pressure to end systolic volume, *V–A coupling* ventricular–arterial coupling as measures of general LV contractility, *AV_Vmax* peak rate of blood flow through the aortic valve, *TDI Septal s′* and *Lateral s′* tissue Doppler imaging measures of peak systolic septal and lateral wall mitral annular velocities, respectively, Δ change in a variable as post minus pre race, *d* Cohen's effect size

**Significant difference from pre-to-post race within a group, $p < 0.01$

carbohydrate-plus casein hydrolysate group following 3 days of racing, diastolic filling volume and systolic function were preserved. However, ingesting the carbohydrate plus casein hydrolysate drink tended to reduce the effect size increase in serum biomarkers for membrane instability and inflammation following 3 days of racing compared with ingesting carbohydrate-only.

In elite endurance athletes PV increases of 11–15% and 13–18% have been reported during 3 week grand tours (Chicharro et al. 2001; Grasso et al. 2015) and during 5–6 day UCI stage races (Garvican et al. 2010; Schumacher et al. 2008), respectively. This agrees with the 10% PV expansion observed following 3 days of racing in the CHO group. Conversely, the absence of PV expansion in the PRO group is contrary to expectation. Therefore, ingesting protein during exercise appears to result in a different response compared with previous reports of accentuated PV expansion when protein is ingested in recovery only (Hobson and James 2015; Kataoka et al. 2016; Okazaki et al. 2009). When protein is ingested only during recovery, the mandatory stimulus created during exercise to promote post-exercise hypotension and the counter-regulatory response of PV expansion (Hayes et al. 2000) is preserved and the provision of protein in recovery magnifies this response by increasing amino acid availability, which enhances the rate of hepatic albumin synthesis (Wada et al. 2018). Conversely, when protein is ingested during exercise, the mandatory stimulus created during exercise for post-exercise hypotension may be suppressed. First, ingested casein protein hydrolysate during exercise is rapidly absorbed and increases circulating amino acid concentrations (Beleen et al. 2011) and plasma osmolality (Oosthuyse and Millen 2016) that may result in increased secretion of osmoregulation hormones, such as vasopressin and angiotensin II, which act vasoactively to increase blood pressure (Dickson et al. 2007). Previously, we found that plasma osmolality was greater and the magnitude of post-exercise hypotension was reduced following 2.5 h of strenuous cycling when ingesting a carbohydrate–casein protein hydrolysate drink during exercise compared with placebo-water (Oosthuyse and Millen 2016). Likewise, in the current study we did not find significant evidence of post-exercise hypotension after 3 days of racing in the PRO group. Furthermore, the current study supports a tendency for protein ingestion during exercise to reduce the inflammatory effect of exercise and it is known that the occurrence of post-exercise hypotension is dependent on a local secretion of inflammatory histamines to maintain vasodilation in previously active muscles (Romero et al. 2017). Thus, increased serum osmolality and reduced inflammation when ingesting casein protein hydrolysate with carbohydrate during multiday racing in the current study may have prevented the mandatory post-exercise hypotension, which is necessary to support counter-regulatory PV expansion (Hayes et al. 2000). While following 3 days of racing, there was also no evidence of post-exercise hypotension in the CHO group, this occurred coincident to notable PV expansion on day 3, which may have cancelled evidence for hypotension in this group on day 3. It is probable that notable post-exercise hypotension likely did occur following racing on days 1 and 2 in the CHO group to support the counter-regulatory response and resulting observed outcome of secondary hypervolemia recorded on day 3.

Second, ingesting casein protein hydrolysate–carbohydrate compared with carbohydrate-only or an amino acid mix compared with placebo during exercise has previously been shown to shift substrate partitioning away from carbohydrate oxidation and in favour of fat oxidation (Oosthuyse et al. 2015; Ueda et al. 2016). In fact, the mechanism by which amino acids induce cellular signalling and metabolic regulation in favour of fat oxidation is being uncovered (White et al. 2018). Such a substrate shift may better preserve endogenous glycogen stores and thereby reduce the stress hormone response to exercise. The rate of hepatic albumin synthesis is dependent on stress hormone concentrations (McNurlan et al. 1996). Thus, it is possible that greater fat oxidation during exercise and a lower stress hormone response in PRO may also explain the lack of an increase in serum albumin content and PV following the 3-day race.

Evidence of PV expansion in the CHO group was observed in echocardiograph measures of preload with a small increase in LA volume. However, evidence of impaired LV relaxation was observed in both the CHO and PRO group following 3 days of racing as noted by the longer deceleration time from the peak of early filling. Impaired LV relaxation may explain why, despite PV expansion and thus increased preload in the CHO group, an associated increase in passive early filling and EDV did not occur. Instead, PV expansion preserved the peak rate of early filling and EDV despite mildly compromised LV relaxation and tissue Doppler imaging (TDI) measures showed no effect of 3 days of racing on septal and lateral mitral annular velocities during LV filling in the CHO group. Conversely, the PRO group did demonstrate signs of diastolic dysfunction in TDI measures after the 3-day race with a decrease in the ratio of septal wall early-to-late diastolic tissue velocities but interestingly an increase in both early and late diastolic velocities at the lateral wall. This uneven ventricular wall diastolic impairment following prolonged strenuous exercise has previously been demonstrated in detailed measures of strain and strain rate (George et al. 2009; Stewart et al. 2017) and in fact may involve specific septal wall segments (Rothwell et al. 2018). The points of inter-ventricular attachment appear to be the most vulnerable to the long-term effects of regular prolonged strenuous exercise (Rothwell et al. 2018). The compensatory increase in lateral wall tissue velocities in the

PRO group after racing is a novel finding and may account for the preserved LVIDd and EDV in this group without PV expansion. Likewise, in the current study, we observed a significant increase in LV tissue velocity during systole only at the lateral wall but not at the septal wall in the PRO group after the 3-day race and again this increased contractility in the lateral wall may have supported the preserved systolic function noted in this group.

However, the outcome of the current study following 3 days of racing, likely does not represent the effects that may be recorded following days 1 and 2 of racing. The benefits for LV function noted when ingesting carbohydrate-only in the current study likely only occur following the realisation of secondary hypervolemia. Previously, we have found LV systolic function to be reduced following exercise on days 1 and 2 but to be preserved following exercise on days 3 and 4 during laboratory-simulated multiday cycling with a carbohydrate-only supplement (Oosthuyse et al. 2012). Future studies should investigate whether ingesting carbohydrate-plus-casein protein hydrolysate compared with carbohydrate-only during endurance racing would better support preservation of LV function following days 1 and 2 before the incidence of PV expansion, as previously suggested (Oosthuyse and Millen 2016).

The findings of the current study tend to agree with frequent reports of reduced markers of tissue damage and inflammation when ingesting carbohydrate–protein compared with carbohydrate-only during exercise (Saunders 2007; Skillen et al. 2008). The post-exercise increase in certain tissue damage markers may reflect membrane-leakage and does not necessarily represent structural tissue damage (Eijsvogels et al. 2016). In particular, cardiac troponins have been shown to increase in a biphasic pattern during and following exercise with rapid return to pre-exercise levels (Middleton et al. 2008) supporting a membrane functional change rather than cardiac myocyte damage. This is further supported by no histological evidence of irreversible cardiomyocyte damage following strenuous exercise despite increases in serum cardiac biomarkers (Nie et al. 2016; Trivax et al. 2010). Improving antioxidant capacity has been shown to attenuate the cardiac biomarker response to exercise and thus reflect better maintenance of membrane stability and function by controlling oxidative stress (Gao et al. 2014). Milk proteins are high in thiol-containing amino acids, which are donors for glutathione synthesis (Mariotti et al. 2004) and this may explain the tendency in the current study for the PRO group to display a smaller effect for increases in general biomarkers of membrane instability (CK and CK-MB) and inflammation (hsCRP) following 3 days of strenuous endurance exercise. Cardiac-specific biomarker, cTnI, has previously been increased post marathon or cycling race well above the current study's detection limit (La Gerche et al. 2015; Neumayr et al. 2002; Trivax et al. 2010; Williams et al. 2009), but in the current study, cTnI was only found to increase above detection in four cyclists (two per group) after day 3 of racing. Previous multiday exercise studies have found cTnI or T to be increased after exercise on certain days only (Middleton et al. 2007; Neumayr et al. 2002; Williams et al. 2009) and therefore, the finding of the current study does not exclude the possibility of differences between groups to have occurred on earlier days or differences that may have occurred below the detection limit. However, despite the previous strong association between cardiac myocellular oxidative stress (Vitiello et al. 2011) or the increase in serum inflammatory cytokine concentration (La Gerche et al. 2015) and the occurrence of exercise-induced cardiac dysfunction, casein protein hydrolysate ingestion during exercise in the current study failed to prevent evidence for diastolic dysfunction linked to reduced LV relaxation. It could be that a higher dose of protein or possibly an alternative antioxidant or thiol substrate source for glutathione synthesis, which is more readily available to cardiac myocytes, may be more effective, such as, melatonin (Leonardo-Mendonça et al. 2017), alpha-lipoic acid (Khanna et al. 1999), or polyphenols like quercetin (Gao et al. 2014).

The current study was conducted during a real-life race, which provides merits that cannot be simulated in a laboratory. While rating of perceived exertion was ranked consistently high as may be expected under racing conditions, the CHO group provided possible evidence that they were feeling better as the race progressed from the lower ranking for leg fatigue, upper-body soreness and fatigue after day 3 compared to day 1 of racing. This occurred coincident to the PV expansion and the recorded benefits to preserve systolic function. Other than supporting cardiac function, PV expansion will also favour better thermoregulation (Kataoka et al. 2016) and could also possibly explain the suggested improvement in introspective ranking. On-the-other-hand, the PRO group who did not experience PV expansion produced a constant rank for perceived bodily fatigue and soreness after each day.

Limitations

Use of linear dimensions to estimate volumes are not recommended for unhealthy individuals with a history of cardiac injury and non-elliptical chamber geometry. To this end, current recommendations support use of Simpson's biplane area measurements (Lang et al. 2015). However, LV volumes measured by Teichholz equation and Simpson's biplane methods are both highly correlated to invasive three-dimensional measures of LV volume and the mean error incurred when using two-dimensional echocardiography is similar for both methods (Grossgasteiger et al. 2014). Thus, in healthy individuals with normal cardiac geometry and where indices based on linear cardiac

dimensions are evaluated as pre-to-post race changes within individuals, such calculated changes represent valid effects. Furthermore, in the current study, findings based on changes in linear dimensions are verified by changes recorded by PWD peak blood flow, TDI and measured haemodynamic changes in PV. Further studies measuring LV mechanics including LV segment-specific strain, strain rate and rotation are encouraged.

The general biomarkers, total CK, CK-MB and C-reactive protein are not exclusively cardiac specific but are nevertheless routinely measured in cardiac patients and in previous studies evaluating cardiac function in athletes (Scherr et al. 2011; Stewart et al. 2016). Future studies including cardiac-specific inflammatory and oxidative stress biomarkers would be informative.

Conclusion

Adding casein protein hydrolysate to a carbohydrate drink ingested during 3 days of racing in a strenuous mountain bike stage race prevented the occurrence of secondary hypervolemia that was noted when ingesting carbohydrate-only, but did tend to reduce the effect for biomarkers of membrane instability and inflammation. Nevertheless, LV function was hampered by impaired LV relaxation in both conditions. However, PV expansion in the CHO group supported preservation of systolic function and was associated with reduced perception of bodily fatigue as the race progressed. Lack of PV expansion in the PRO group resulted in an uneven change in LV wall function with decrements seen in the septal wall and compensatory enhanced function in the lateral wall that resulted in preserved systolic function. Therefore, ingesting casein protein hydrolysate with carbohydrate compared with carbohydrate-only during racing, did not provide additional benefits for the preservation of cardiac function following a 3-day multiday race. Further studies are necessary to confirm the explanation whereby casein protein hydrolysate ingestion during daily racing suppresses the counter-regulatory response of PV expansion.

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Author contributions TO, AB and AM conceived and designed the study. TO and AM conducted data collection and analysis. TO prepared the manuscript. AB and AM reviewed the manuscript. All authors approved the final version.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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