Effects of Protein vs. Carbohydrate Supplementation on Markers of Immune Response in Master Triathletes: A Randomized Controlled Trial

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Abstract

Objective: This study examines the long-term effects of ingesting hydrolyzed beef protein versus carbohydrate on indirect markers of immunity during 10-week of endurance training in master-aged triathletes (n=16, age 35–60 years old).

Methods: Participants were randomly assigned to either hydrolyzed beef protein (PRO, n=8) or non-protein isoenergetic carbohydrate (CHO, n=8) condition, which consisted of ingesting 20 g of each supplement, mixed with water, once a day immediately post-workout, or before breakfast on non-training days. Salivary Human Neutrophil Peptides (HNP1-3) were measured before and after performing an incremental endurance test to volitional exhaustion at both pre and post intervention. Additionally, baseline levels of platelets, neutrophils, eosinophil basophils, monocytes, and lymphocytes were determined at pre and post intervention.

Results: No significant changes in baseline concentration and secretion rate of salivary HNP1-3 were observed either treatment. The CHO group showed a non-significant decrease in resting HNP1-3 concentrations following the intervention (p=0.052, effect size d=0.53). Protein supplementation demonstrated a significant reduction in lymphocyte counts pre-to-post intervention (mean [SD]: 2.30 [0.57] vs. 1.93 [0.45] $10^3/mm^3$, p=0.046, d=0.77) along with a moderate but not statistically significant increase (d=0.75, p=0.051) of the Neutrophil-to-Lymphocyte ratio.

Conclusions: In master-aged triathletes, post-workout ingestion of only protein, with no carbohydrate, may not be as effective as carbohydrate alone to attenuate negative long-term changes of some salivary and cellular immunological markers. Future studies should consider the co-ingestion of both macronutrients.

INTRODUCTION

Multiple factors influence an athlete‘s resistance to illness inclusive of one’s genetic predisposition, immune competence, nutrition status, physical, psychological and environmental stresses and alterations in normal sleep schedule [1]. Athletes undertaking regular intensive and high-volume endurance exercise increase their susceptibility to upper respiratory tract infections (URTI) for up to two weeks following endurance events [2]. Several strategies, including nutritional interventions have been proposed to maintain immunocompetence and to avoid illness [3, 4].

Defensins, including alpha-defensins, are antimicrobial peptides (AMP) that contribute to the mucosal host defense acting as the first line of action against invading pathogens [5]. Salivary human neutrophil peptides (mainly HNP1-3) encompass a specific group of alpha-defensins that increases in response to the respiratory distress syndrome [6]. However, their response to an acute or chronic exercise stimulus is still unclear. Acute increases in the concentration of HNP1–3 and other AMP (i.e. LL-37, LL-37, L Lactoferrin, and Lysozyme) have been reported after 2.5 h [7, 8] or 45 minutes [9] of submaximal (60% to 75% of $\dot{V}O_{2peak}$) endurance exercises. Conversely, decreases in basal levels and secretion rates of HNP1-3 were also observed after an 8-week resistance-training program in young males who regularly consumed a post-workout beverage containing hydrolyzed beef proteins and carbohydrates [10]. As lower basal levels of HNP1-3 have been associated with reduced risks against virus and infections [11], along with higher performance productions [6], the decreased concentrations in athletes have been connected with a better capacity to overcome the degree of stress determined by a given exercise protocol [10].

While the ingestion of carbohydrates during and after heavy exercises to counteract the exercise-induced immune dysfunction is a consistent recommendation [3], the potential benefits of nutrients containing high concentration of essential amino acids and micronutrients need to be established [12, 13]. Due to the critical role of vitamins, minerals

and sulphur-containing amino acids (e.g. cysteine and taurine) in supporting anti-oxidant, muscle recovery and immune responses [12], the effect of special food preparations providing high-quality proteins, fats and elevated proportion of micronutrients (e.g. heme-iron, zinc and vitamin B12,) on acute and long term immunity in athletes requires further investigation. Additionally, as the ingestion of carbohydrates has been proven to attenuate the exercise-induced immunosuppression in endurance athletes [2, 3], the purpose of this study was to compare the long-term induced immunological effects of ingesting a commercially-available post-workout hydrolyzed beef protein (100% All Beef, Crown® Sport Nutrition, Spain) vs. a non-protein, isoenergetic carbohydrate-only supplement on indirect markers of humoral and cellular immunity in recreationally trained master-age endurance athletes.

The primary outcome was resting/basal HNP1-3 concentration and secretion rate measured after completing a 10-week intervention period. Secondary outcomes included changes in HNP1-3 induced by an incremental test to volitional exhaustion, leukocyte count and the Neutrophil-to-Lymphocyte ratio, as the latter has been associated with performance fluctuations and immune system related changes in athletes [1]. We hypothesize that the regular ingestion of a post-workout beef protein drink will attenuate changes associated with the immune dysfunction in a similar way to the ingestion of carbohydrates.

MATERIALS AND METHODS

Participants

Twenty-four recreationally master-age male triathletes, not involved in regular official federative competitions, met the requirements to participate in this study. Inclusion criteria included: (a) being 35–60 years of age, (b) participating in regular endurance training history for at least two years with a minimum of 3 sessions and 6 h of endurance training exposure per week (c) having a normal health history, (d) being free from musculoskeletal limitations, (e) agreeing not to ingest any other nutritional supplements or non-prescription

drugs/medication that can affect blood markers of health as well as muscle growth and the ability to train intensely during the study, and (f) fluent in English in order to receive instructions regarding the study. Participants were excluded if they presented with: (a) a history of metabolic conditions and/or diseases, (b) use of a variety of medications including, but not limited to, those with androgenic and/or anabolic effects and/or nutritional supplements known to improve strength and/or muscle mass such as creatine, essential amino acid, whey protein, glutamine, dehydroepiandrosterone (DHEA), multi-vitamin or iron-supplement within 8 weeks prior to the start of the study, (c) current use of tobacco products, and (d) the presence of any soft tissue or orthopedic limitations.

Participants were informed of the potential risks of the intervention before agreeing to comply with the intervention protocol and signed an informed consent. All experimental procedures were conducted in accordance with the Declaration of Helsinki and approved by the University Research Ethics Committee. After determining eligibility, 24 participants were recruited for the study. Due to reasons not related to the intervention protocols, 8 participants withdrew from the study (Figure 1). Consequently, sixteen participants (n = 8 per treatment) successfully completed the intervention. The study was conducted during the winter and spring period. Trial Registration: ClinicalTrials.gov, U.S. National Institutes of Health (Identifier: NCT02675348).

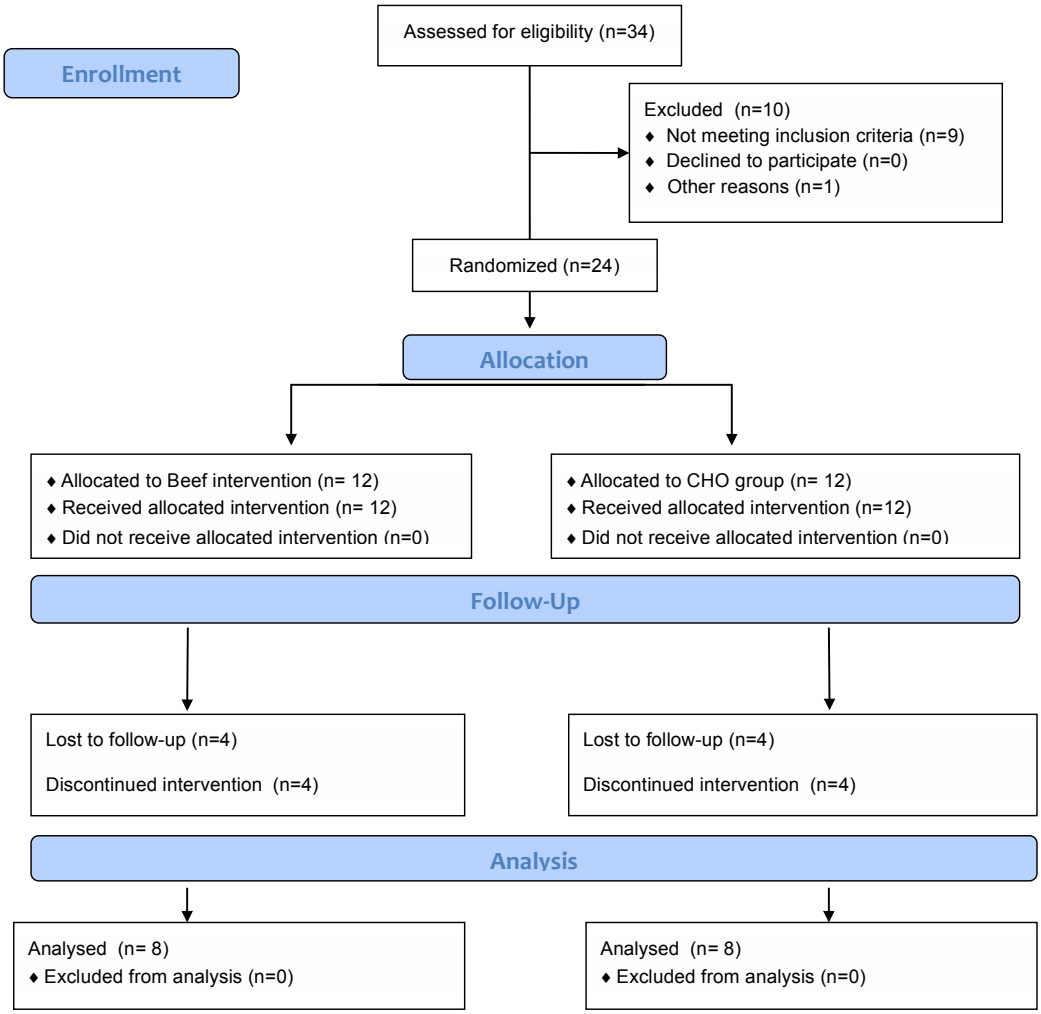

Figure 1. Flow diagram of participants throughout the course of the study

Experimental design

This study was a randomized, parallel group controlled trial employing a double blind between-participant design. Participants were randomly allocated into two treatment groups: beef protein (PRO, n = 8) or maltodextrin (CHO, n = 8). The primary outcome was resting/basal HNP1-3 concentration and secretion rate. Secondary outcomes included the following: (i) changes in HNP1-3 measured after performing the incremental test to volitional exhaustion in order to examine the acute response to exercise, (ii) leukocyte count (iii), the

Neutrophil-to-Lymphocyte ratio, (iv) peak oxygen uptake ($\dot{V}O_{2peak}$), and (v) total white blood cells, involving granulocytes (neutrophils, eosinophil, and basophils) and agranulocytes (monocytes and lymphocytes). All tests were performed at baseline and follow-up. To assess the long-term effect on the basal levels and the exercise-induced changes in concentration and secretion rates of salivary HNP1-3, saliva samples were collected four times, pre and post an incremental laboratory exercise test to volitional exhaustion performed before (test 1) and after (test 2) the 10-week intervention period. Following a pre-intervention screening, the participants were matched for $\dot{V}O_{2peak}$ and age, and then assigned to their respective treatments by block randomization, using a block size of two, generated by a computer tool.

Measurements

Anthropometry and $\dot{V}O_{2peak}$: Body mass and height were assessed on a standard scale and stadiometer according to the methods described by Ross and Marfel-Jones [14]. Peak oxygen consumption was examined after a standardized warm-up where participants completed an incremental laboratory exercise test to volitional exhaustion on a Cyclus2 ergometer (RBM Electronics, Leipzig, Germany). The test commenced at a work rate of 90 W. Thereafter, intensity increased at a rate of 25 W min^{-1}. Participants were instructed to maintain a cadence between 70 and 80 rev min^{-1} throughout the test. When cadence dropped by more than 10 rev min^{-1} for more than 10 s despite strong verbal encouragement, tests were terminated. Expired gases were collected continuously during the test using a Cortex MetaLyzer 3B gas analyzer (Cortex Biophysik, Leipzig, Germany). Heart rate (HR) was continuously monitored using a Polar Sport Tester™ (Polar Electro, Finland). We calculated $\dot{V}O_{2peak}$ as the highest mean oxygen consumption over a 30-s period [15]. This incremental test was chosen because it was the only standardized assessment protocol routinely performed for the participants that volunteered to take part in the study. Furthermore, similar protocols have been recommended to objectively determine physiological performance and

quantify relative work intensity based on laboratory-derived parameters associated with maximal exercise testing [16, 17].

Blood Sampling and Analysis: On two separate occasions (one day before and one day after completing the 10-week intervention period), participants arrived at the physiology laboratory after fasting for 8 h and with a minimum rest period of 24 h from the last exercises session. Two vacutainer venous blood collection tubes (BD Vacutainer® Blood Collection Tubes) were used to collect 8 mL of venous blood from the antecubital vein. An aliquot of the whole blood was used to perform leukocyte (Total, Neutrophils, Lymphocytes, Monocytes, Eosinophil and Basophils) counts and the Neutrophils-to-Lymphocyte ratio, as general markers of cell immunity using an automated hematology analyzer (ABX Pentra 60C+, Horiba Medical, Montpellier, France).

Saliva Collection: Saliva was collected before (test 1) and after (test 2) the 10-week intervention period before and after the incremental $\dot{V}O_{2peak}$ test, four times in total. At baseline (pre test), saliva samples were collected following the blood samples just a few minutes before starting the assessment. For the post-test sample, saliva was collected 60 min after the exercise cessation [9]. Participants remained seated for 10 min (pre test) or about 60 min (post test) prior to each saliva collection. No fluid or food was permitted until after the final 1-hour post-exercise saliva sample had been collected. For all saliva samples, the mouth was rinsed with water 5 min before collection. The participant was requested to swallow in order to empty the mouth before each sample collection. Unstimulated whole saliva was collected by the spitting method while the participant remained seated, leaning forward and with the head tilted down [18]. Saliva was collected for 1 min. To avoid circadian variation, saliva samples were collected between 2-7 pm. The collected saliva was weighed in order to obtain precise flow rate (g/min) [19]. The saliva was stored at -80°C until further sample treatment and analysis.

Saliva analysis: Saliva samples were centrifuged (12,000 g, 10 min 4° C) and the supernatant diluted 1000x with sample dilution buffer. Each sample was analyzed in duplicate with ELISA (Hycult biotech, Netherlands) following the manufacturer's instructions. The calibration curve consisted of eight standards, ranging from 0.15 to 10 µg/mL HNP1-3. Absorbance (450 nm) values for the saliva samples were interpolated from calibration standards with a 4-parameter logistic curve (My assays, version 2015). In addition, the alpha-defensins HNP1-3 secretion rates were determined by multiplying their concentration by the flow rate (mL min^{-1}).

Dietary Monitoring

Each participant's baseline diet (3 days, 2 weekdays, and 1 weekend day) was analyzed using Dietplan 6 software (Microsoft Forestfield Software Ltd. 14). Participants were instructed to maintain their normal diet throughout the intervention. In order to evaluate differences caused by the supplementation protocol, diet was analyzed again during the last week of the intervention.

Control of Training

The participants were master triathletes and at the time of the study had consistently trained between 6-10 h per week (four to seven weekly training sessions) for the past three years. Heart rate (HR), Rate of Perceived Exertion (RPE) using a 15-point, 6 to 20 Borg scale [20], oxygen consumption, and the identification of the first (VT1) and second (VT2) ventilatory thresholds were recorded during the testing procedures. Following the pre-screening tests, participants committed to follow a training intervention period consisting of a 10-week polarized endurance-training intensity distribution model including three intensity zones based on the VT1 and VT2 localization [21]. HR and the associated RPE values assessed during the incremental test were used to quantify the training performed within each intensity zone. The polarized endurance-training intensity distribution model involves significant proportions of both high- and low-intensity training and only a small proportion

of moderate-intensity training [22]. The intensity zones were calculated as Zone 1, low intensity: ≤ 75% of VT2 or below the VT1, ≤ 72% of maximum heart rate (HRmax), rate of perceived exertion (RPE) 6 to 11; Zone 2, moderate intensity: above VT1, between 76 and 95% of VT2, 73 to 82% HRmax, RPE 12 to 14; and Zone 3, high intensity: between 96 and 120% of VT2, 83 to 97% HRmax, RPE 15 to 18 [21]. Participants trained 6 sessions per week with a distribution of 75–80% in Zone 1, ~5% in Zone 2, and 15–20% in Zone 3. The weekly training program included three bouts of running, involving an average of 25 to 30 km, two bouts of swimming involving an average of 2 km, and three bouts of cycling involving an average of 55 km. HR and RPE data files were checked weekly and used to quantify training intensity. In addition, participants were required to complete a training diary, recording average HR, training mode, and duration and distance of training sessions throughout the study. Continuous monitoring of the training load was conducted using two methods: (i) Strava [23] and (ii) face-to-face/telephone interviews after every session. These procedures allowed us to maintain permanent contact with all the participants and adjust the training configuration to ensure similar training load in both groups. The adjustments in training intensity during the intervention period were determined through RPE and HR. All the participants performed their training during the afternoon (12:00 to 6:00 pm).

Dietary Supplementation and Control of the Intervention Compliance

The two supplements under investigation were presented as 20 g sachets of vanilla-flavored powder diluted in ~300 mL of plain water for each intake. The diluted drinks were similar in appearance, texture and taste, and were isoenergetic. The nutritional composition of each product and the amino acid profile of the hydrolyzed beef proteins are shown in Table 1. Supplements were taken once a day during 10 weeks for a cumulative total of 70 doses. On training days, supplements were ingested just after the training, whereas on non-training days supplements were self-administered in the morning before breakfast.

Table 1

After completing the first assessment session, each participant was given a batch of one of the two products, assigned according to randomization. Tolerance, collected from any adverse events and compliance with supplement intake (determined by an individual follow-up), was evaluated continuously during the intervention. Only participants who completed the 70 days of supplementation intake with a minimum training frequency of 4 sessions per week (40 workouts in total) were included in the analysis.

Statistical Analysis

Sample size estimations were calculated based on antimicrobial markers (Alpha-defensins HNP1-3 concentration and secretion rates), assuming a two group by four repeated measures model, where 0.05 was chosen as α-error probability and 0.80 was chosen for statistical power (1-β). A descriptive analysis was performed, and the Kolmogorov-Smirnov and Shapiro-Wilk's tests were applied to assess normality. Sample characteristics at baseline were compared between conditions (PRO vs. CHO) using two-tailed independent means Student's t test**.** Changes pre to post treatment in the baseline levels of the salivary markers, $\dot{V}O_{2peak}$ and leukocytes were assessed using a 2 (treatments) × 2 (times) repeated measures ANOVA. As suggested by Castañeda et al., changes in the primary outcome (e.g., HNP1-3 concentration and secretion rate) from pre intervention baseline (pre-exercise/resting test 1) to post intervention baseline (pre-exercise/resting pre test 2) were analyzed using a priori Bonferroni-adjusted pairwise comparisons [24]. Additionally, as changes in the concentration and secretion rate of the HNP1-3 after the incremental test would be also dependent on each individual's baseline value [7], one-way Analysis of Covariance (ANCOVA) models were used to compare differences in raw change between groups, using the pre-assessment values as covariates. Confidence intervals (CI) of the adjusted differences were calculated and plotted. Those CIs not crossing zero were considered statistically significant. Additionally, two-tailed one sample student's tests were used to test for a null hypothesis. Generalized eta squared (η_G^2) and Cohen's d values were reported to provide an

estimate of standardized effect size (small d=0.2, η_G^2=0.01; moderate d=0.5, η_G^2=0.06; and large d=0.8, η_G^2=0.14 values were used as reference) [25]. Significance level was set to p<0.05, but p values between 0.05 and 0.1 were considered indicative of a trend [26]. Data are presented as Mean (SD) and mean change (95% confidence intervals) unless otherwise noted. All statistics were performed using the Statistical Package for the Social Sciences (IBM statistics, version 20.0.0.1. for Windows).

RESULTS

The statistical power of the study was assessed by performing correlations among repeated measures (pre to post) of the salivary alfa defensins HNP1-3 and leukocytes. Results ranged from r=0.63 to r=0.74. Assuming the most conservative value, significance level 0.50 and power 0.80, the sample size was determined to be large enough to detect moderate group-time interactions effects (η_G^2=0.09) through a sensitivity power analysis.

The baseline characteristics for each group were as follows: PRO, age 48 (8) years, height 1.76 (0.07) m, body mass 78.50 (8.48) kg, $\dot{V}O_{2peak}$ 43.25 (4.97) ml/kg/min^{-1}. CHO, age 46 (6.89) years, height 1.80 (0.04) m, body mass 78.62 (5.22) kg $\dot{V}O_{2peak}$ 42.88 (4.31) ml/kg/min^{-1}. No significant differences were observed between treatments at baseline. Participants confirmed that they maintained their regular diet throughout the trial period. Table 2 shows the average daily dietary characteristics for carbohydrate, protein, fat (g·kg^{-1}·d^{-1}), and energy (kcal·kg^{-1}·d^{-1}) consumption before and after intervention for the two treatment groups. At baseline, no between-group differences were observed for the amount of the macronutrients or energy intake. However, during the course of the nutritional intervention, protein and carbohydrate consumption increased relative to the two respective treatments. No other differences in diet were noted.

Table 2

All the participants reported the completed training diary at the end of the intervention. Training load quantification demonstrated the following training load (volume and intensity) distribution for all the participants included in the final analysis:

(i) total volume (km) completed during the 10-week intervention period in zone 1, 2 and 3 respectively PRO: 671 (3.1), 50 (2.8), 124 (6.5). CHO: 659 (3.9) 56 (2.9), 136.5 (5.6). no significant differences were observed between treatment for each of the three intensity zones ($p>0.05$).

(ii) The training-time distribution was as follows: (i) (ii) 73 (2.1) % - 7 (0.3)% - 20 (1.0) % for Zone 1; Zone 2 and Zone 3 respectively.

Primary Outcome*: Salivary alpha-defensins HNP1-3*

No main time or treatment effects were observed for the HNP1-3 concentrations (Time: $F[1,14]=0.83$, $p=0.377$, $\eta_G^2=0.01$; Treatment $F[1,14]=0.04$, $p=0.850$, $\eta_G^2= 0.01$) or saliva flow rates (Time: $F[1,14]=0.00$, $p=0.953$, $\eta_G^2=0.01$; Treatment: $F[1,14]=0.25$, $p=0.625$, $\eta_G^2=0.03$). Furthermore, no interaction effects between treatments and time for HNP1-3 concentrations ($F[1,44]=4.38$, $p=0.055$, $\eta_G^2=0.06$) and secretion rate ($F[1,14]=1.82$, $p=0.198$, $\eta_G^2=0.04$) were found. To have a closer look at the interaction effect observed for the HNP1-3 concentration, post hoc analysis revealed a trend ($p=0.052$, $d=0.53$) to decrease the concentration of salivary alpha-defensins HNP1-3 in the CHO group at post intervention (pre test 2 vs. pre test 1), Figure 2.

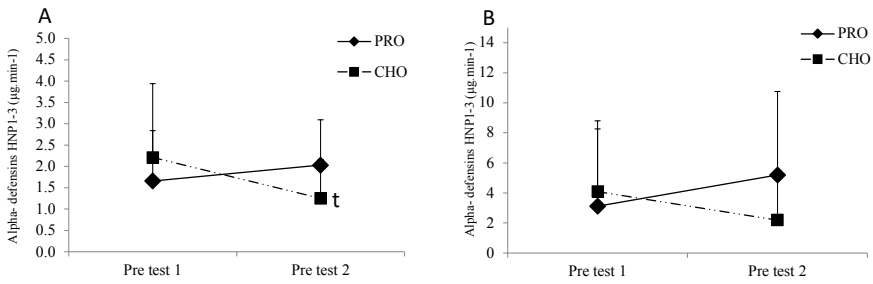

Figure 2. Long-term changes in the concentration (A) and secretion rates (B) of salivaryalpha-defensins HNP1-3 for the two treatment conditions (mean and SEM). ^{T}, p=0.055 for the CHO at pre test 2 (post intervention) vs. pre test 1 (pre intervention)

The adjusted covariate model revealed a significant decrease of the HNP1-3 salivary flow rate for both treatment conditions PRO, (p=0.039, d=0.55) and CHO (p=0.030, d=0.58). Furthermore, the PRO condition showed a moderate effect size (p=0.052, d=0.51) to decrease the concentration of HNP1-3 after performing the incremental test at post intervention respect to the values measured at baseline pre intervention (Figure 3).

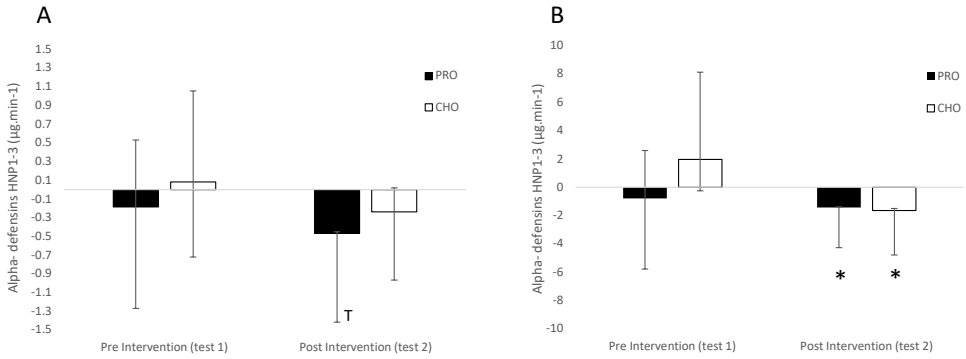

Figure 3. Estimated marginal means and 95% confidence intervals of changes in the concentration (A) and secretion rate of the salivary alpha-defensins HNP1-3. Analysis of covariance (ANCOVA) models were used to compare differences in raw change between groups, using the pre-assessment values as covariates. ^{T}, p = 0.052; *, p < 0.05 from the baseline values.

***Secondary Outcomes**: $\dot{V}O_{2peak}$ and white blood cell*

No differences in $\dot{V}O_{2peak}$ or white blood cell counts were observed at baseline. Pre and post values of $\dot{V}O_{2peak}$ and Leukocytes counts, main time and group effects, as well as interactions between treatments and time, are provided in Table 3. Only the PRO treatment showed a statistically significant main time effect on Lymphocytes. Post hoc analysis revealed a significant decrease in Lymphocytes (p=0.046, d=0.77) and a very close to significant p-value with a moderate effect size (p=0.051, d=0.75) to increase Neutrophil-to-Lymphocyte ratio in the PRO condition. Furthermore, a moderate effect size (d=0.60) with no statistically significant rise (p=0.112) in Neutrophil count was also observed in PRO. No other differences were observed.

Table 3

DISCUSSION

The main finding of the present investigation is that the ingestion of a post-workout drink providing only protein (from beef) or carbohydrate from maltodextrin during 10 weeks of endurance training was not associated with significant changes in the basal concentration and secretion rate of salivary HNP1-3. Nonetheless, ingesting carbohydrates for 10 weeks, produced a moderate effect size (d=0.53) to reduce the HNP1-3 (Figure 2A) and helped to attenuate the exercise-induced depression of this marker after the incremental test (Figure 3A). Even though both groups decreased the HNP1-3 secretion rate measured after performing the incremental test post intervention (Figure 3B), only the PRO group exhibited a likely higher depression of the HNP1-3 concentration after the incremental test measured at post intervention (Figure 3A). In addition, a decrease in post-intervention lymphocyte count and a moderate to high effect size to increase the Neutrophil-to-Lymphocyte ratio was also observed for the participants allocated in the PRO group. Conversely, those participants who ingested a beverage containing only maltodextrin demonstrated no significant changes

in the analyzed cellular markers of immunity. Based on these findings, and within the confines of the study procedures, we reject our research hypothesis that the regular ingestion of a post-workout supplement providing only beef protein concordant to endurance training will attenuate negative changes of some components associated with immune dysfunction in a similar way to the ingestion of carbohydrates in master triathletes.

Although an initial reduction in baseline concentration of some salivary AMP may be an adaptive, chronic outcome to endurance training [27], according to the present results, further decreases would be elicited by the ingestion of a post-workout carbohydrate beverage in trained athletes. Considering that HNP1-3 represents the combined response of three peptides (HNP1, HNP2 and HNP3), which are markers associated with exercise stress [6], showing low levels of these markers after a 10-week intervention period would be interpreted as a positive adaption that would allow individuals to better tolerate the stress imposed by a given exercise protocol. This rationale would support the hypothesis that the chronically reduced HNP1-3 level observed in trained athletes is an expected adaptations to endurance training [10], which in the present study is observed in the participants allocate in the CHO group.

The analysis of the longitudinal individual changes between baseline values measured before performing test 1 (pre-intervention) and test 2 (post-intervention) revealed that six participants in the CHO group depicted a consistent decrease in their levels of HNP1-3, while one showed an increase and the other one produced almost no change. Conversely, the participants allocated to the PRO group showed a different pattern of response with two individuals showing a consistent decrease, four an increase, and two producing no change in the concentration of HNP1-3 at post intervention (see supplementary material). Similarly, Naclerio et al [10], reported a similar post intervention decrease of HNP1-3 levels in young resistance trained participants that ingested a post-workout beverage containing hydrolyzed beef protein and carbohydrates, for eight weeks. Despite the differences in the exercise

protocol and the characteristics of the participants, it could be speculated that the ingestion of admixtures including carbohydrates and high-quality animal-based proteins such as beef would represent a suitable immune protective nutritional countermeasure for athletes. Indeed, the ineffectiveness of the PRO treatment to attenuate the observed non-desired changes in some markers of immunity could be associated with the non-ingestion of carbohydrate, being proteins still considered a synergic nutrient to optimize training adaptations including, performance, muscle repair, and remodeling, etc. [28]. As the objective of the present study was to compare the effect of protein vs. carbohydrate alone, the inclusion of a group consuming a carbohydrate protein mixture was not considered.

Although not significantly different at the beginning of the study, compared to the PRO condition, the CHO group consumed a lower amount of daily carbohydrates from their diet (2.98±1.18 $g \cdot kg^{-1} \cdot d^{-1}$ vs. 3.21±1.19 $g \cdot kg^{-1} \cdot d^{-1}$ respectively). However, the significant rise in total daily carbohydrates experienced by the CHO group during the intervention, increased the daily carbohydrate intake to a similar level as determined in the PRO group [3.23 (1.19) $g \cdot kg^{-1} \cdot d^{-1}$ and 3.46 (1.14) $g \cdot kg^{-1} \cdot d^{-1}$ for CHO and PRO respectively]. Consequently, the overall more favorable immune response observed in the CHO cohort could be mainly caused by the addition of a post workout CHO supplement. On the other hand, individual responses to training may explain the dissimilar results observed between participants allocated to the PRO condition and the opposite changes showed by one of the CHO consumers who tended to increase their levels of HNP1-3 at post intervention.

The decreased after-intervention lymphocyte levels along with observed moderate effect size for increasing neutrophils and the concomitant rise in Neutrophils-to-Lymphocytes ratio for the PRO condition together with the absence of changes in cellular immune markers in the CHO group would reinforce the aforementioned statement that carbohydrates would be an effective nutrient to effectively counteract exercise-induced cellular immune dysfunction in endurance athletes [3, 29]. Nonetheless, as previously

discussed, both groups consumed a similar amount of total daily carbohydrates during the intervention. Consequently, the regular ingestion of 20 g (0.20 to 0.29 $g \cdot kg^{-1} \cdot d^{-1}kg$) of maltodextrin administered as a post-workout supplement or in the morning during the non-training days would represent an appropriate immune-protective nutritional strategy in master endurance athletes.

Contrary to our results, previous studies reported acute increases in the concentration of HNP1-3 determined immediately after submaximal constant intensity long duration protocols [7, 9] or until 1 h compared to pre-exercise values [9]. The post exercise increase in salivary HNP1-3 levels may, to the same extent, be related to an exercise-induced muscle inflammatory response. Prolonged (> 45 min) dynamic exercise may induce airway inflammation and damage to airway epithelial cell. The aforementioned mechanism will activate neutrophils and elicit the release of their contents into saliva resulting in a rise of salivary levels of AMP after such as a type of exercise [30]. Even though the endurance exercise test was performed until volitional exhaustion, the characteristics of the incremental protocol used, starting at very low intensities (< VT1) to progressively reach the high intensity exercises zones (> VT2) where the participants only exercised for 4 to 7 minutes would not be enough to cause an acute meaningful rise in the post exercise salivary AMP. Indeed, the transient exercise-induced immune-depression has been mainly associated with low to moderate intensity and long continuous [1] or intermittent [13, 31] exercise but not with a singular, 12 to 18 min, incremental to volitional exhaustion protocol as performed in the present study. Only Allgrove et al. [32] reported a temporary increase in the secretion rate of both salivary immunoglobulin A and salivary lysozyme with no effect on saliva flow rate after a short-duration (~ 22 min) incremental exercise test to exhaustion in active young men. In this way, the observed responses could also be associated with the cohort's characteristics. Participants in the present investigation were master-age triathletes, while previous studies have analyzed the acute response of young recreationally active participants [7, 9].

Additionally, both conditions (PRO and CHO) showed almost the same concentration of HNP1-3 after performing the endurance test at post intervention (see supplementary material). Consequently, the trend to decrease HNP1-3 levels within our study for the PRO group may not be related to a higher post-exercise immunosuppression observed at the end of intervention but a consequence of the individual variability of the HNP1-3 concentration as reported by others [7], who have recognized the variance as a limitation for the detection of intervention-induced changes in mucosal parameters, particularly in parallel groups designs [33]. When individual responses were compared, it is worth noting that at test 1 (before intervention) a mixed pattern of responses with 8 participants increasing and 8 decreasing the concentration of HNP1-3 measured post exercise was observed (See supplementary material). After the 10-week intervention, all participants but one, who produced almost no change, allocated in the PRO group decreased the level of salivary HNP1-3 after exercise. On the other hand, participants in the CHO group showed a very similar, although more attenuated, pattern of response compared to the previously observed at test 1, with two participants increasing, 2 decreasing and 4 showing no change in the concentration of HNP1-3. Likely the absence of carbohydrates, administered alone or added to the post-workout protein beverage, could be influencing the observed trend to acutely reduce the concentration of salivary HNP1-3 after exercise. Nonetheless, participants in the CHO treatment attenuated changes in HNP1-3 concentrations at test 2.

The unexpected after-intervention fall in salivary flow rate exhibited by the two treatment groups could be explained by the limited access to water [34] during the first hour post exercise that was implemented during the 10-week intervention period for all the participants. Even though testing conditions were replicated at pre and post intervention, the regular ingestion of only 300 ml solution providing ~7% of proteins or carbohydrate with limited access to water could have caused an adaptive response to a transitory limited post exercise re-hydration, which resulted in a reduced saliva flow during the first hour post

exercise.

Our study is not without limitations. Given the observed different patterns of AMP secretion between men and women [30], studies using female population would be needed. Nonetheless, a recent investigation examining a mixed sample of young resistance trained males and females observed no influence of sex on levels of salivary HNP1-3 measured before and after 8 weeks of a combined exercise and nutrition protocol [10]. Dehydration has been shown to decrease saliva flow rate secretion of AMP without affecting its concentrations [6]. Thus, maintaining hydration should be considered to preserve mucosal immune integrity during heavy endurance exercise. Unfortunately, while participants were asked to hydrate prior to each training and testing sessions, hydration status was not assessed. Although the used incremental to exhaustion test is useful for identifying changes in the V̇o2peak and designing endurance training zones in endurance athletes [17], a specific protocol reflecting triathlete performance during competitions would be useful to assess how the conducted intervention impact on their performance. The current study examined singular nutrients; thus, future research should consider mixed supplementation combining carbohydrate, protein, fats and micronutrients. Finally, further research using a larger sample size is required to confirm if nutritional interventions through a post workout animal protein drink administered alone or combined with carbohydrate can attenuate negative changes in immune markers in a similar way as carbohydrates do.

In conclusion, considering the research design and sample size limitations this investigation suggests that a post-workout drink containing only protein with no carbohydrate, may not be as effective as the ingestion of carbohydrate alone to attenuate negative long-term changes on some salivary and cellular markers associated with immune dysfunction in master-aged triathletes. Nonetheless, given the demonstrated positive effect of post-workout protein supplementation to optimize recovery process, including muscle repair and glycogen replenishment in endurance athletes [28], the use of admixture

combining both high quality protein extract and carbohydrate deserves special considerations.

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The Authors declare that the results of the current study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. CPE has consulting relationships with ACAP Health (Dallas, TX), Catapult (Dallas, TX), Naturally Slim (Dallas, TX) and Nutrabolt (Bryan, TX), a nutrition supplement company; however, the current study does not represent a product held by said company.

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References

1. Gunzer W, Konrad M, Pail E. Exercise-Induced Immunodepression in Endurance Athletes and Nutritional Intervention with Carbohydrate, Protein and Fat—What Is Possible, What Is Not? Nutrients 4:1187-212, 2012.

2. Moreira A, Kekkonen RA, Delgado L, Fonseca J, Korpela R, Haahtela T. Nutritional modulation of exercise-induced immunodepression in athletes: a systematic review and meta-analysis. Eur J Clin Nutr. 61:443-60, 2007.

3. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, et al. Position statement. Part two: Maintaining immune health. Exerc Immunol Rev. 17:64-103, 2011.

4. Gleeson M, Lancaster GI, Bishop NC. Nutritional strategies to minimise exercise-induced immunosuppression in athletes. Can J Appl Physiol. 26 Suppl:S23-35, 2001.

5. Wang G. Human antimicrobial peptides and proteins. Pharmaceuticals (Basel). 7:545-94, 2014.

6. Kunz H, Bishop NC, Spielmann G, Pistillo M, Reed J, Ograjsek T, et al. Fitness level impacts salivary antimicrobial protein responses to a single bout of cycling exercise. Eur J Appl Physiol. 115:1015-27, 2015.

7. Davison G, Allgrove J, Gleeson M. Salivary antimicrobial peptides (LL-37 and alpha-defensins HNP1-3), antimicrobial and IgA responses to prolonged exercise. Eur J Appl Physiol. 106:277-84, 2009.

8. Ashrafi N, Lapthorn C, Pullen FS, Naclerio F, Nielsen BV. High performance liquid chromatography coupled to electrospray ionisation mass spectrometry method for the detection of salivary human neutrophil alpha defensins HNP1, HNP2, HNP3 and HNP4. Anal Methods. 9:6482-90, 2017.

9. Gillum TL, Kuennen MR, Castillo MN, Williams NL, Jordan-Patterson AT. Exercise, but not acute sleep loss, increases salivary antimicrobial protein secretion. J Strength Cond Res. 29:1359-66, 2015.

10. Naclerio F, Larumbe-Zabala E, Ashrafi N, Seijo M, Nielsen B, Allgrove J, et al. Effects of protein-carbohydrate supplementation on immunity and resistance training outcomes: a double-blind, randomized, controlled clinical trial. Eur J Appl Physiol. 117:267-77, 2017.

11. Mehlotra RK, Zimmerman PA, Weinberg A. Defensin gene variation and HIV/AIDS: a comprehensive perspective needed. J Leukoc Biol. 99:687-92, 2016.

12. Cruzat VF, Krause M, Newsholme P. Amino acid supplementation and impact on immune function in the context of exercise. J Int Soc Sports Nutr. 11:61, 2014.

13. Naclerio F, Larumbe-Zabala E, Cooper R, Allgrove J, Earnest CP. A multi-ingredient containing carbohydrate, proteins L-glutamine and L-carnitine attenuates fatigue perception with no effect on performance, muscle damage or immunity in soccer players. PloS one. 10:e0125188, 2015.

14. Ross WD, Marflel-Jones MJ. Kineanthropometry. In: MacDougal JC, Wenger HA, Green HJ, editors. Physiological Testing of High Performance Athlete. Champaign, IL: Human Kinetics, pp 223-308 1991.

15. Karsten B, Jobson SA, Hopker J, Stevens L, Beedie C. Validity and reliability of critical power field testing. Eur J Appl Physiol. 115:197-204, 2015.

16. Kasch FW, Boyer JL, Schmidt PK, Wells RH, Wallace JP, Verity LS, et al. Ageing of the cardiovascular system during 33 years of aerobic exercise. Age Ageing. 28:531-6, 1999.

17. Esteve-Lanao J, San Juan AF, Earnest CP, Foster C, Lucia A. How do endurance runners actually train? Relationship with competition performance. Med Sci Sports Exerc. 37:496-504, 2005.

18. Navazesh M. Methods for collecting saliva. Ann N Y Acad Sci. 694:72-7, 1993.

19. Dawidson IJ, Ar'Rajab A, Melone LD, Poole T, Griffin D, Risser R. Early use of the Gore-Tex Stretch Graft. Blood Purif. 14:337-44, 1996.

20. Borg GA. Psychophysical bases of perceived exertion. Med Sci Sports Exerc. 14:377-81, 1982.

21. Esteve-Lanao J, Foster C, Seiler S, Lucia A. Impact of training intensity distribution on performance in endurance athletes. J Strength Cond Res. 21:943-9, 2007.

22. Seiler KS, Kjerland GO. Quantifying training intensity distribution in elite endurance athletes: is there evidence for an "optimal" distribution? Scand J Med Sci Sports. 16:49-56, 2006.

23. Musakwa W, Selala KM. Mapping cycling patterns and trends using Strava Metro data in the city of Johannesburg, South Africa. Data Brief. 9:898-905, 2016.

24. Castañeda MB, Levin JR, Dunham RB. Using planned comparisons in management research: A case for the Bonferroni procedure. Journal of Management. 19:707–24, 1993.

25. Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed: Mahwah, NJ: Lawrence Erlbaum., 1988.

26. Reidy PT, Fry CS, Igbinigie S, Deer RR, Jennings K, Cope MB, et al. Protein Supplementation Does Not Affect Myogenic Adaptations to Resistance Training. Med Sci Sports Exerc. 49:1197-208, 2017.

27. West NP, Pyne DB, Kyd JM, Renshaw GM, Fricker PA, Cripps AW. The effect of exercise on innate mucosal immunity. Br J Sports Med. 44:227-31, 2010.

28. Doering TM, Reaburn PR, Phillips SM, Jenkins DG. Postexercise Dietary Protein Strategies to Maximize Skeletal Muscle Repair and Remodeling in Masters Endurance Athletes: A Review. Int J Sport Nutr Exerc Metab. 26:168-78, 2016.

29. Buonocore D, Negro M, Arcelli E, Marzatico F. Anti-inflammatory Dietary Interventions and Supplements to Improve Performance during Athletic Training. J Am Coll Nutr. 34 Suppl 1:62-7, 2015.

30. Pyne DB. Regulation of neutrophil function during exercise. Sports Med. 17:245-58, 1994.

31. Naclerio F, Larumbe-Zabala E, Cooper R, Jimenez A, Goss-Sampson M. Effect of a carbohydrate-protein multi-ingredient supplement on intermittent sprint performance and muscle damage in recreational athletes. Appl Physiol Nutr Metab. 39:1151-8, 2014.

32. Allgrove JE, Gomes E, Hough J, Gleeson M. Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. J Sports Sci. 26:653-61, 2008.

33. Jones AW, Thatcher R, March DS, Davison G. Influence of 4 weeks of bovine colostrum supplementation on neutrophil and mucosal immune responses to prolonged cycling. Scand J Med Sci Sports. 25:788-96, 2015.

34. Costa RJ, Fortes MB, Richardson K, Bilzon JL, Walsh NP. The effects of postexercise feeding on saliva antimicrobial proteins. Int J Sport Nutr Exerc Metab. 22:184-91, 2012.

Tables

Table 1.

Nutritional composition of drinks per intake (20 g of powder plus ~300 ml of plain water)

Nutrient	PRO	CHO
Energy value (kcal)	82	82
Carbohydrates (maltodextrin g)	0	20
Lipids (g)	1.54	0
Proteins (g)	16.40	0
Alanine	1.04	-
Arginine	1.06	-
Aspartic acid	1.50	-
Cysteine	0.16	-
Glutamic acid	2.58	-
Glycine	1.07	-
Histidine	0.55	-
Isoleucine	0.75	-
Leucine	1.32	-
Lysine	1.44	-
Methionine	0.39	-
Phenylalanine	0.65	-
Proline	0.81	-
Serine	0.65	-
Threonine	0.73	-
Tryptophan	0.187	-
Tyrosine	0.52	-
Valine	0.80	-
Total EEA	**6.82**	**-**
Heme Iron (mg)	3.25	-
Zinc (mg)	3.81	-
Potassium (mg)	241.96	-
Phosphorus (mg)	154.67	-
Selenium (µg)	4.86	-
Folic Acid (µg)	16.84	-
Vitamin B 12 (µg)	0.625	-

Notes: EEA, essential amino acids; CHO, Carbohydrates.

Table 2. Descriptive analysis of the participants' diet composition

Treatment	PRO (n=8)		CHO (n=8)	
	pre	post	pre	post
Proteins ($g \cdot kg^{-1} \cdot d^{-1}$)	1.29 (0.25)	1.52* (0.24)	1.35 (0.24)	1.40 (0.25)
Carbohydrates ($g \cdot kg^{-1} \cdot d^{-1}$)	3.36 (1.21)	3.46 (1.14)	2.98 (1.18)	3.23* (1.19)
Fat ($g \cdot kg^{-1} \cdot d^{-1}$)	1.07 (0.26)	1.09 (0.26)	1.37 (0.80)	1.34 (0.60)
Energy ($kcal \cdot kg^{-1} \cdot d^{-1}$)	28.58 (4.40)	30.54 (4.17)	27.94 (7.97)	31.44 (9.28)

Notes: Pre and post intervention values are presented as mean (SD). The diet analysis includes the amount of macronutrients added by the supplements (beef protein or maltodextrin).

*$p<0.01$ from pre to post-intervention (last week of intervention)

Table 3. Descriptive analysis of the performance and blood immunological variables

Variables	PRO (n=8)		CHO (n=8)		Repeated Measure ANOVA (2 groups x 2 times)
	Pre	Post	Pre	Post	
$\dot{V}O_{2peak}$ (ml/kg/min)	48.9 (5.2)	48.4 (5.1)	49.1 (5.2)	49.2 (5.9)	Time: F(1,14)=0.233, p=0.637, η_G^2=0.01 Group: F(1,14)=0.03, p=0.856, η_G^2=0.01 Group x Time: F(1,14)=0.476, p=0.856, η_G^2=0.01
Platelets (10^3/mm^3)	247.38 (56.84)	231.38 (34.17)	245.00 (48.41)	243.13 (38.81)	Time: F(1,14)=0.93, p=0.351, η_G^2=0.01 Group: F(1,14)=0.04, p=0.837, η_G^2=0.01 Group x Time: F(1,14)=0.58, p=0.459, η_G^2=0.01
Total Leukocytes (10^3/mm^3)	5.25 (1.04)	5.33 (1.02)	5.39 (0.92)	5.51 (1.04)	Time: F(1,14)=0.18, p=0.676, η_G^2=0.01 Group: F(1,14)=0.11, p=0.742, η_G^2=0.01 Group x Time: F(1,14)=0.01, p=0.917, η_G^2=0.01
Neutrophils (10^3/mm^3)	2.37 (0.77)	2.86 (0.74)	2.98 (0.91)	3.19 (0.99)	Time: F(1,14)=2.92, p=0.110, η_G^2=0.04 Group: F(1,14)=1.30, p=0.273, η_G^2=0.07 Group x Time: F(1,14)=0.48, p=0.499, η_G^2=0.01
Lymphocytes (10^3/mm^3)	2.30 (0.57)	1.93* (0.45)	1.85 (0.56)	1.74 (0.33)	Time: F(1,14)=4.06, p=0.064, η_G^2=0.057 Group: F(1,14)=1.94, p=0.185, η_G^2=0.10 Group x Time: F(1,14)=1.16, p=0.301, η_G^2=0.02
Monocytes (10^3/mm^3)	0.38 (0.15)	0.34 (0.09)	0.38 (0.10)	0.39 (0.11)	Time: F(1,14)=0.12, p=0.736, η_G^2=0.01 Group: F(1,14)=0.26, p=0.619, η_G^2=0.01 Group x Time: F(1,14)=0.41, p=0.533, η_G^2=0.01
Eosinophil (10^3/mm^3)	0.16 (0.07)	0.16 (0.07)	0.15 (0.10)	0.19 (0.09)	Time: F(1,14)=1.02, p=0.329, η_G^2=0.01 Group: F(1,14)=0.07, p=0.790, η_G^2=0.01 Time: F(1,14)=0.82, p=0.379, η_G^2=0.01
Basophils (10^3/mm^3)	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.03 (0.02)	Time: F(1,14)=0.18, p=0.675, η_G^2=0.01 Group: F(1,14)=0.07, p=0.800, η_G^2=0.01 Group x Time: F(1,14)=0, p=0.990, η_G^2=0.01
Neutrophils:Lymphocytes ratio	1.13 (0.46)	1.55 ^{t} (0.45)	1.81 (0.80)	1.94 (0.75)	Time: F(1,14)=3.91, p=0.068, η_G^2=0.04 Group: F(1,14)=2.92, p=0.110, η_G^2=0.15 Group x Time: F(1,14)=1.07, p=0.318, η_G^2=0.01

Note: Pre and post intervention values are presented as mean (standard deviation)

Pairwise comparisons: *p<0.05 respect to pre intervention values, ^{t} p=0.051 respect to baseline values.