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Original Article

Improvement of the metabolic profile in young overweight adults using high doses of branched chain amino acids during sprint interval training

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Abstract:
The ingestion of branched-chain amino acids (BCAAs) has been pointed as having controversial effects on cardiovascular diseases (CVD) and energy metabolism, is unknow if sprint interval training (SIT) has a synergist effect when associated with BCAA supplementation. The purpose of this study was to evaluate the effects of BCAA supplementation associated with SIT on energy metabolism and CVD biomarkers. Were randomly distributed forty overweight participants in to 4 groups: sedentary plus placebo (S-PLA); sedentary plus BCAA (S-BCAA); SIT plus placebo (SIT-PLA); SIT plus BCAA (SIT-BCAA). During 8 weeks, the S-BCAA and SIT-BCAA groups were supplemented with 300 mg·kg·day^{-1} of BCAA plus 200 mg·kg·day^{-1} of maltodextrin while S-PLA and SIT PLA were supplemented with 500 mg·kg·day^{-1} of waxy maize (placebo). Also, the SIT-PLA and SIT-BCAA groups undergone on a 3 days/week cycle SIT training. Pre- and post-treatment were evaluated: VO_{2peak}, total cholesterol (TC) and their fractions (HDL-C and LDL-C), triglycerides, protein C-reactive (PCR), HbA1c and body fat percentage (BF%) as CVD biomarkers; respiratory exchange ratio (RER), blood glucose and lactate concentration as energy metabolism. After treatment, VO_{2peak} increased only in the SIT groups ($p<0.05$); %BF, TC and PCR did not change in all the four groups ($p>0.05$). Triglycerides and LDL-C decreased only in S-BCAA and SIT-BCAA ($p<0.05$) groups. SIT-BCAA group also decreased HbA1c and increased HDL-C ($p<0.05$). SIT and BCAA supplementation seem to be exert a synergistic effect on decreasing both RER and blood glucose concentrations, however, higher BCAA supplementation appear to blunt the SIT's lowering effect on blood lactate concentration. In conclusion, the SIT was effective to increase VO_2peak, while associating SIT with BCAA supplementation was the most effective treatment to promote positive synergistic changes on CVD biomarker and on energy metabolism.
Key words: BCAA, Sprint interval training, lactate, insulin resistance, fat oxidation, energy metabolism.

Introduction

Prospective studies are controversial on the effect of high branched-chain amino acids (BCAA) intake. Actually, BCAA has been pointing as benefic to improve the cardiovascular diseases (CVD) metabolic profile (Nagata et al. 2013), but also as adverse to the metabolic health profile inducing dyslipidemia, type 2 diabetes (T2D) and obesity (T2D) (Zheng et al. 2016).

In a cross-sectional study, elevated serum BCAAs level was positively associated with the incidence of metabolic dyslipidemia and insulin resistance (IR) (Yang et al. 2016). However, several animal model studies have demonstrated that high dietary BCAA intake induce a decrease in adipose tissue and an improvement on the profile of variables associated with this tissue i.e., decrease in IR, hyperlipidemia and inflammation (Zhang et al. 2007; Holecek et al. 2016; Zhao et al. 2016) and these changes also occur at the genes level (Bruckbauer et al. 2015). To our knowledge, few studies with humans evaluated whether the high intake (supra-physiological doses) of BCAA would be beneficial for the metabolic syndrome (Takeshita et al. 2012). In humans, both high plasma BCAA concentrations (with absence of BCAAs oxidation) (Lynch et al. 2014) and low gene expression of enzymes related to BCAA oxidation (Tiffin et al. 2006; Taneera et al. 2012) are considered predictors and biomarkers of IR, T2D and obesity incidence.

Animal studies have demonstrated that deficiency in BCAA metabolism, including low activity and low mRNA expression of branched-chain α-ketoacid dehydrogenase (BCKDH) complex is associated with obesity, hyperlipidemia and IR (Bajotto et al. 2009; Lackey et al. 2013). In fact, the BCKDH complex is the limitation rate in BCAA metabolism (Shimomura et al. 2004). It is well know that obese humans have low activity and low gene expression of BCKDH complex (Lackey, Lynch et al. 2013). For example, homozygous humans (with low

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expression of the BCKDH complex) when compared with their heterozygotic peers have difficulties in losing fat in hypocaloric diets, for example when they engage in a high fat, low carb type diet, (Xu et al. 2013). Thus, these works suggest that low BCAA catabolism plays a key role in obesity development and its associated factors, namely IR, T2D, hyperlipidemia and inflammation. Moreover, increases in gene expression and BCKDH complex activation, and the consequent BCAA catabolism are significantly potentiated by physical exercise (Shimomura et al. 1995; Van Hall et al. 1996; Xu et al. 2001; Howarth et al. 2007; Shimomura et al. 2012). Contrarily, the muscle BCAA enzymes catabolism expression can be downregulated due to the lack of physical exercise (Lerin et al. 2016). In animal studies it was demonstrated that the BCAA metabolism increased fat metabolism (Kainulainen et al. 2013; Lerin, Goldfine et al. 2016) and energy expenditure (Masaki 2015b; Masaki 2015a). Early studies have demonstrated that BCKDH complex is further activated when physical exercise is combined whit high doses of BCAA supplementation (Van Hall, MacLean et al. 1996; Shimomura, Murakami et al. 2004). However, to date, there are no studies in humans on the chronic effects of exercise combined with BCAA supplementation on fat metabolism.

To our knowledge only one human study (Gualano et al. 2011) showed changes in energy metabolism (increase fat oxidation) after three days of high doses of BCAA supplementation. The changes occurred when participants run 30min, however, after a glycogen depletion protocol that consisted of 1 hour run followed by a 10-hour overnight fast. However, this protocol does not apply to the reality of the general population. Thus, a study design with easy application (to the general population) is necessary to observe if the BCAA supplementation associated with physical training can promote changes in the energy metabolism and if they improve CVD biomarkers related to adipose tissue. Therefore, this investigation aims to study the effects of SIT training plus BCAA supplementation on CVD biomarkers and on the energy metabolism.

Materials and methods

Overall design

We recruited 40 volunteers (20 men-M and 20 women-W) who were randomized, in a double-blind manner, into 4 groups with 10 volunteers (5M and 5W) in each group: (1) S-PLA, sedentary plus placebo; (2) S-BCAA, sedentary plus BCAA; (3) SIT-PLA, sprint interval training (SIT) plus placebo, and; (4) SIT-BCAA, SIT plus BCAA. Before beginning the treatment, the participants visited the laboratory twice: (1) for blood collection and anthropometric assessments and (2) evaluation of the energy metabolism and VO_{2peak}. These evaluations were replicated at the end of the training program. All tests were performed 2-4 days both, before and after the intervention.

Participants

The participants were included in this study if their age ranged between 20-40 years, if they were sedentary (≤ 2 day of exercise per week, ≤ 30min per session, in the last year prior to study). For the male participants, body mass index (BMI) had to be ≥ 25 ≤39 associated with fat percentage ≥22 ≤30%; for the female participants BMI were supposed to be ≥ 25≤39 associated with fat percentage ≥25 ≤40%. All participants had to be medically approved to start a physical activity program. The profile of participants who engaged in the study is described in Table 1.

We did not include participants with: renal dysfunction; maple syrup urine disease; hypo or hyperthyroidism; hypertension; individuals who were unable to perform the exercise protocol suggested in this study (for example, problems in knee joints that limited flexion and extension with overload). Individuals taking medications to control IR, diabetes, dyslipidemia or high blood pressure. The intervention began after the approval of the experimental protocol by the ethics committee of the São Judas Tadeu University (Issue Number: 749.562 CEP/USJT CAAE: 28714314.2.0000.0089) and the signing of the free and informed consent form by the participants.

Supplementation

During 8 weeks, participants' intake 300 mg·kg·day^{-1} of BCAA [4:1:1 ratio, Leucine, isoleucine and valine, respectively (plus 200 mg·kg·day^{-1} of maltodextrin with lemon flavor- to mask BCAA taste)] or 500 mg·kg·day^{-1} of waxy maize whit lemon flavor (placebo), divided into three daily doses (morning, afternoon and night). Each dosage was diluted in 500 ml of water. For the SIT-PLA or SIT-BCAA groups one of the three daily doses had to be ingested 30min prior to the training sessions. All participants received a spreadsheet with guidelines to control supplementation ingestion frequency. In addition, it was provided to all participant a quick and direct contact (email of cell phone) with the researcher to report any adverse effects promoted by supplementation; This communication form was also used by the researcher to ask participants how they were feeling with supplementation (training was conducted under the supervision of two physical education professionals (E.F and A.P.X) engaged in this project). Supplementation was donated by Max Titanium- Brazil. Supplementation blindness was made by a pharmacy compounding (Orion Lab- Brazil); Participants randomly was made using the site www.randomization.com.

Training

Participants in groups S-PLA and S-BCAA did not train. After being familiarized with the training session, the SIT-PLA and SIT-BCAA participants completed 8 weeks of SIT program on the cycle ergometer

(Biotec 2100 CEFISE, Brazil) three times a week (Monday, Wednesday and Friday or Tuesday, Thursday and Saturday). Is know that this protocol was able to activate BCKD and increase VO_{2peak} (Howarth, Burgomaster et al. 2007). Briefly, the training sessions were composed of 6 sets of 30s of maximum efforts (all-out). However, the training program initially (at weeks 1-2) was composed of 4 sets of all-out efforts, followed by 5 sets at weeks 3-4 and 6 sets at weeks 5-8. All sets were performed against a resistance of 0.075 kg/kg of body weight. Rest interval between sets were 4.5min at 50 rpm, at 30 W intensity or passive recovery (the participant could choose). All sessions were preceded by a 10min warm-up and finished with a 5min calm-down at a load of 50 rpm at 30 W (Howarth, Burgomaster et al. 2007).

Physical activity and feeding control

For the control of these variables we followed the same steps described on elsewhere (Howarth, Burgomaster et al. 2007). Briefly, during the experiment participants were instructed to continue their regular diet and physical activity practice and to abstain from alcohol and exercise 48 hours before each test. The graded exercise test was performed 3 hours after a standardized meal (subjects were given a meal plan to be followed on the days of the exercise tests (VO_{2peak}) consisting of 25% fat, 60% carbohydrate and 15% protein.

Anthropometric evaluation

The anthropometric evaluation was performed on the same day of fasting blood collection.

An experienced technician (A.P.X; >500 evaluations) executed the skinfold protocol with a Lange skinfold caliper. All skinfold values were collected in triplicates, then the mean of the three values was used for the body fat percentage calculation (%BF). The %BF values were obtained by the Jackson and Pollock protocol (7 folds), as described in Heyward and Gibson (2014). Besides, we measured both, abdomen (at the umbilical scar) and hip circumferences (on the largest portion of the hip); body weight (measured with a digital scale, with precision of 100g) and; height (measured with a wooden stadiometer).

Blood collection and analysis

Blood collection was performed after a ≥10h fast, in the morning. The collection was performed in a period of 2-4 days both, before and after the intervention. After blood collection, part of the blood was centrifuged (10,000 RMP at 4°C) for serum or plasma separation and stored (-70°C) for further analysis of total cholesterol and its fractions (LDL-C, Low-density lipoprotein; HDL-C, high-density lipoprotein), triglycerides (TAG) and C-reactive protein (CRP) values. Whole blood was also stored for further hemoglobin A1c (HbA1c) analysis. For total cholesterol, its fractions (LDL-C, HDL-C) and plasma TAG we used commercial kits *Liquiform* (from Labtest, Brazil). For CRP analysis, we used *Ultra-Turbiquest* commercial kits (from Labtest, Brazil). For HbA1c analysis we used commercial kits *Doles* (from Doles, Brazil). All sample values were measured in duplicates and read on a Molecular Device (Spectra Max 190, USA), then the average of the two absorbance values was used in each analysis formulas.

Assessment of VO_{2peak} and energy metabolism

The VO_{2peak}, respiratory exchange ratio (RER), blood lactate concentration [La]b and blood glucose concentration were collected in the same physical exercise test. The RER, [La]b and the blood glucose concentration were collected simultaneously in order to compare their behavior during the test. To compare properly the RER, blood glucose and [La]b values before and after the intervention protocol, the participants had the same meal (described above) 3 hours before the graded exercise tests. Moreover, in the post-assessment all subjects took their third daily dosage of 100 $mg \cdot kg^{-1}$ of BCAA (plus ~66 $mg \cdot kg^{-1}$ of maltodextrin) or Placebo (waxy maize) diluted in 500ml of water, 30min before the VO_{2peak} evaluation.

VO_{2peak} assessment: was previously described (Howarth, Burgomaster et al. 2007). Briefly, the participants performed a graded exercise test until volitional exhaustion on a cycle ergometer (Biotec 2100 CEFISE, Brazil) at 50-70 RPM, with an incremental load of 500kp (for men) or 250kp (women), every 2min. To determine the VO_{2peak} during the test we used a gas exchange analyzer (VO2000, Inbrasport), calibrated at each test and programmed to provide the average breath by breath value. We considered the test valid when the individuals reached one of the following criteria: HRmax predicted for his age reached (HR recorded by S810i Polar device); RER> 1.10; unable to pedal (exhaustion) at a rate higher than 50 RPM. We considered as VO_{2peak} the average values of the last 30s of the test. All VO_{2peak} assessments were performed in the evening (between 16-18 h). In all graded exercise tests, participants were verbally stimulated to reach their maximum work capacity.

*RER at rest and during exercise***:** The RER values (used for comparisons) were collected in three moments: (1) before starting the graded exercise test, in which the subject remained seated for 5min (the values from the last minute of rest were used); (2) during the test (values from 8th minute of the test) and; (3) at the last minute (~15min of test) before the end of graded exercise test. It is important to mention that time to exhaustion (~15 min) was not statistically different ($p>0,05$) when compared in both situations (1) between groups and (2) within the groups (pre- *vs.* post-treatment).

[La]b and blood glucose concentration: Blood collection for [La]b and blood glucose concentration analysis was carried out at the same time point of RER collection (at rest, 8min and in immediately at the end of the graded exercise test). The [La]b analysis was performed on a Accutrend Lactate (Roche Accusport) analyzer and the blood glucose analysis was made on an Accu-Chek® Active Blood Glucose device. Blood collection

was obtained from the right-hand ring finger (through an automatic puncture lancet equipment, Roche softclix II AccuCheck) to the devices for immediate analysis.

Statistical analysis

Data are presented as means and standard deviations (±). After assessment of data normality (by Shapiro-Wilk test) a homogeneity (by Levene test) for VO_{2peak} anthropometric data CVD biomarkers, data were analyzed using a repeated measure analysis of variance (ANOVA) test (4 treatment groups: S-PLA, S-BCAA, SIT-PLA and SIT-BCAA × 2 time points: pre- and post-treatment), when ANOVA assumption was violated we use non-parametric test (indicated in results section). The energy metabolism data were analyzed using the ANOVA test (4 treatment groups: S-PLA, S-BCAA, SIT-PLA and SIT-BCAA × 3 moments: Rest, 8 min and ~15 min of exercise) with repeated measure on time (pre- *vs.* post-treatment). A LSD post hoc test was used for pairwise comparisons. Significance was set at $p<0.05$. Moreover, the magnitude of the effects of key variables (blood markers of cardiovascular disease) were further performed by Cohen's effect size *(d*) (Pre- vs. Post-treatment) followed by the Confidence Interval at 95% (CI 95%). Statistical analysis was performed using IBM SPSS Statistics 20.0 software for windows.

Results

At the end of the study 10 participants remained in both SIT-BCAA and S-PLA groups (5 M-men, 5 W-women) and 9 subjects in both SIT-PLA and S-BCAA groups (5M, 4W). 1 subject of the SIT-PLA group did not adapt to the training protocol due to abrupt reductions in blood pressure and then preferred to leave the study. The other two participants left because of personal reasons not related to the study protocol. Training adherence were 90-100% in both SIT-PLA and SIT BCAA.

Anthropometry and VO_{2peak}

Table 1 shows participants anthropometric and VO_{2peak} profiles before (pre-) and after (post) 8-weeks of BCAA supplementation or placebo associated with SIT or sedentary lifestyle. There were no differences among experimental groups in anthropometric data, both in pre- and post-treatment (treatment*time interaction, $p>0.05$). The SIT intervention associated or not with supplementation (BCAA or placebo) did not promote alteration in the anthropometric variables evaluated (main effect of time, $P> 0.05$). There was treatment*time interaction for VO_{2peak} ($p<0.01$). In both SIT groups VO_{2peak} increased significantly (95% CI: 1 to 7.7 $mL.kg^{-1}.min^{-1}$, SIT-BCAA; 2 to 7.6 $mL.kg^{-1}.min^{-1}$, SIT-PLA) in response to SIT program (main effect of time, $P<0.001$), but did not change significantly in both sedentary groups ($p>0.41$). However, there were no difference between groups at post-treatment ($p> 0.11$).

Table 1. Participant description of pre- and post-treatment.

Groups	S-PLA		S-BCAA		SIT-PLA		SIT-BCAA	
Time point	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Height (Cm)	170.0 ± 5.0		167.0 ± 6.4		168.2 ± 6.8		169.1 ± 8.1	
Age (year)	31 ± 5		29 ± 7		34± 8		32± 7	
Weight (kg)	77.9 ± 11.4	78.5 ± 12.0	69.2 ± 9.1	69.4 ± 9.5	75.9 ± 11.1	77.1 ± 11.0	74.3 ± 8.1	75.9 ± 7.4
% Fat	32.9 ± 4	33.1 ± 6	31.8 ± 11	31.9 ± 11	27.0 ± 9.0	27.6 ± 9.6	29.5 ± 6.1	29.3 ± 9.0
Sum of Skinfolds (mm)	200 ± 60	215 ± 85	199 ± 96	193 ± 112	166 ± 92	172 ± 8.0	199 ± 24	192 ± 29
HC (Cm)	104.0 ± 8.8	105.0 ± 9.2	100.2 ± 6.6	100.5 ± 8.1	102.1 ± 4.6	104.1 ± 6.2	103.0 ± 5.8	103.5 ± 6.2
WC (Cm)	93.3 ± 13.4	93.5 ± 15.6	89.2 ± 8.9	88.9 ± 10	86.4 ± 5.4	87.5 ± 4.7	90.0 ± 4.7	90.2 ± 3.5
BMI	26.9 ± 3.4	26.0 ± 5.5	25.0 ± 4.6	25.0 ± 4.5	25.6 ± 3.1	26.1 ± 2.8	26.2 ± 2.7	26.8 ± 2.4
VO_{2peak} ($mL.kg^{-1}.min^{-1}$)	26.3 ± 5.3	26.5 ± 8.2	25.4 ± 6	24.2 ± 7.6	25.8 ± 8.5	30.6 ± 10.4 * †	27.2 ± 10.1	31.5 ± 10.5 * †

Note: Kruskal-Wallis was performed in WC, weight, sum of skinfolds and %Fat variables for multi-comparisons. Abbreviations: HC, hip circumference; WC, waist circumference; BMI, body mass index; S-PLA, sedentary plus placebo; S-BCAA, sedentary plus branched-chain amino acids (BCAA); SIT-PLA, training plus placebo; SIT-BCAA, training plus BCAA; *, $p<0.01$ vs. pre-treatment; †, $p<0.11$ vs. both sedentary groups in the post-treatment.

Metabolic markers of cardiovascular disease risk

On the variables presented in Table 2, there were significant main effects of treatment ($p<0.05$) for LDL-C, HDL-C, TAG and HbA1c, but not for total cholesterol and CRP ($p>0.16$). Time*group interaction ($p<0.05$) were observed for HbA1c and TAG, but not for total cholesterol, LDL-C, HDL-C and CRP ($p>0.16$). LSD *pos-hoc* reveal that at pre-treatment the SIT-PLA had lower ($p\leq0.05$) LDL-C and TAG when compared to all others group and trend to be lower ($p\leq0.10$) HbA1c and higher HDL-C when compared to S-PLA and SIT-BCAA groups. At post-treatment, S-PLA had higher HbA1c and TAG, when compared to all other groups ($p<0.05$). Also, at post-treatment S-PLA had lower HDL-C when compared to all other groups; and higher ($p<0.05$) LDL-C when compared to SIT-PLA group (see Table 2).

Table 2. Metabolic markers of cardiovascular disease risk with 95% CI (delta change from serum concentrations, between pre- vs. post-treatment) followed by effect size (Cohen's d).

Groups	S-PLA		S-BCAA		SIT-PLA		SIT-BCAA	
Time point	Pre	Post	Pre	Post	Pre	Post	Pre	Post
LDL-C (mg/dl)	132.66 ±14.75	131.33 ±13.07	127.69 ±42.70	103.38 ±38.85 *	93.54 ±32.42 ‖	81.16 ±36.82 §	126.51 ±36.73	104.54 ±53.10 *
(CI 95%) ES	(-6.96 to 4.30) 0.09		(-47.85 to -0.77) 0.59		(-51.11 to 19.83) 0.35		(-42.90 to -1.03) 0.51	
HDL-C (mg/dl)	48.95 ±09.28	48.40 ±06.97 ‡	60.55 ±20.11	62. 68 ±16. 44	61.56 ±12.41 ¶	69.48 ±20.97	55.35 ±10.15	63.86 ±07.47 *
(CI 95%) ES	(-1.85 to 2.96) 0.09		(-16.75 to 21.03) -0.11		(-13.00 to 28.09) -0.45		(2.91 to 18.85) -1.24	
TAG (mg/dl)	239.91 ±70.41	238.00 ± 71.70 ‖	241.68 ±110. 37	144.71 ±60.13 *	162.44 ±50.78 ‖	149.96 ±64.61	235.52 ±84.36	143.76 ±55.35**
(CI 95%) ES	(-5.84 to 2.01) 0.01		(-185.96 to -7.95) 1.09		(-89.02 to 33.19) 0.21		(-119.68 to -28.74) 1.19	
Total Col. (mg/dl)	181.36 ±26.54	178.33 ± 17.67	192.50 ±22.40	185.81 ±38. 33	176.59 ±25.60	209.90 ± 68.65	176.45 ±23.34	201.75 ±53.14 †
(CI 95%) ES	(-12.06 to 5.99) 0.13		(-42.58 to 29.20) 0.21		(-30.00 to 125.00) -0.64		(-7.61 to 58.21) -0.62	
CRP (mg/L)	2.54 ±0.34	2.52 ±0.35	2.35 ±0.18	2.14 ±0.92	2.67 ±0.42	2.56 ±0.23	2.66 ±0.39	2.26 ±0.93 †
(CI 95%) ES	(-0.03 to 0.07) 0.05		(-0.54 to 0.24) 0.31		(-0.46 to 0.18) 0.32		(-1.44 to 0.43) 0.56	
HbA1c (%)	7.46. ±2.07	7.77 ±2.15 ‖	6.68 ±3.17	5.09 ±2.22	5.74 ±2.72 ¶	5.02 ±1.52	7.58 ±3.08	5.10 ±2.7**
(CI 95%) ES	(-0.15 to 0.79) -0.14		(-8.89 to 4.41) 0.58		(-2.47 to 0.94) 0.32		(-5.03 to -0.88) 0.85	

Note: LDL-C, Low-density lipoprotein; HDL-C, high-density lipoprotein; TAG, triacylglycerol plasmatic; Col. Total, cholesterol total; CRP, C-reactive protein; HbA1c; hemoglobin A1c or glycated; * $p < 0.05$ when compared to pre-treatment; ** $p \leq 0.01$ when compared to pre-treatment; † $p \leq 0.10$ when compared to pre-treatment; ‡ $p < 0.05$ when compared to post-treatment of all other groups; § $p < 0.05$ when compared to S-PLA group; ‖ $p \leq 0.05$ when compared to all other groups at the same moment; ¶ $p \leq 0.10$ when compared to pre-treatment of S-PLA and SIT-BCAA

Energy metabolism

Figures 1, 2 and 3 (and Table S1) shows, respectively, the RER, blood glucose concentration and [La]b values from a maximal graded exercise test in a cycle ergometer until volitional exhaustion.

After repeated ANOVA analysis in Fig 1, LSD *pos-hoc* reveal that at pre-treatment SIT-BCAA and S-BCAA had lower RER value at rest and 8min moment, respectively, when compared to all other groups. At post-treatment S-PLA had higher RER values at 8 min and at the last minute of test (~15 min) when compared to all other groups (see Fig 1 and Table S1). There was a main effect of test on RER increase ($P < 0.01$) in all groups (at pre- and post-treatment) for the graded exercise test. The main effect for treatment (RER decrease) was significant for SIT-BCAA groups ($p < 0.01$) and SIT-PLA ($p=0.05$), but this change does not occur for S-BCAA ($p=0.39$) and S-PLA ($p=0.68$).

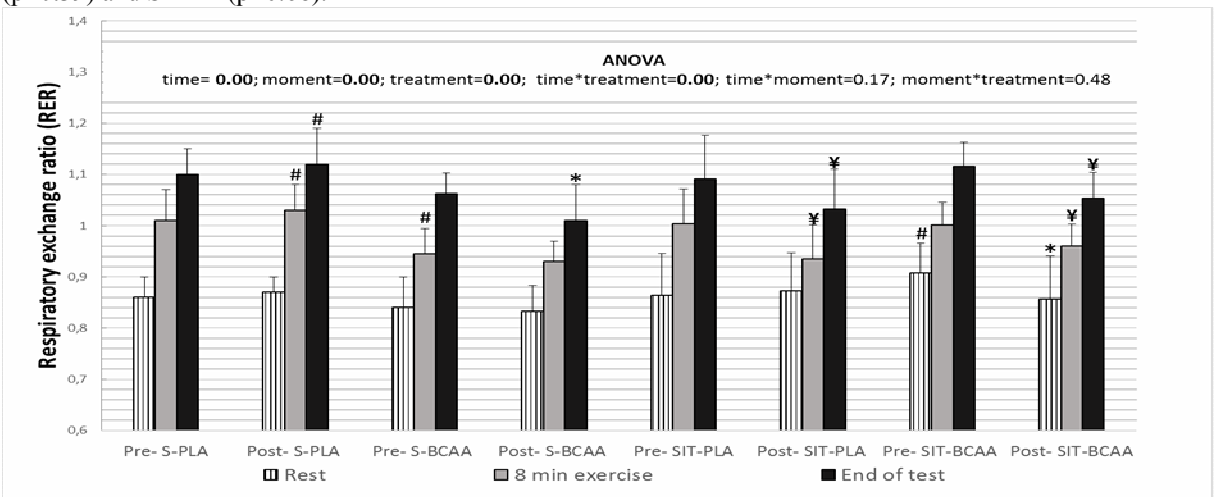

Fig. 1. Respiratory exchange ratio changes from a physical graded exercise test before (Pre-) and after (Post-) treatment with branched-chain amino acids (BCAA) or Carbohydrate (PLA) supplementation associated with a sprint interval training program (SIT) or sedentary lifestyle (S); * $p \leq 0.05$ lower than correspondent pre-treatment; ¥ $p=0.09$ lower than correspondent pre-treatment; # $p < 0.05$ vs. all other groups from correspondent moment (pre- or post-treatment) and condition (PLA or BCAA treatment) of the test.

At pre-treatment, there were no difference between groups for blood glucose concentration at rest, 8min and immediately after the graded exercise test, but at post-treatment LSD *pos-hoc* reveal that S-PLA had higher blood glucose concentration values at rest, at 8 min and immediately after the graded exercise test when compares to all other groups, see Fig 2 and Table S1. Also, LSD *pos-hoc* reveal the main effect for treatment (decrease in blood glucose concentration) was observed in S-BCAA, SIT-PLA and SIT-BCAA groups only ($p < 0.01$). There is no main effect of test on blood glucose concentration decrease ($P > 0.05$) in response to the graded exercise test at pre-treatment for all groups, however, at post-treatment a decrease in SIT-BCAA ($p=0.01$) and a trend in S-BCAA ($p=0.07$) occur, but not in SIT-PLA ($p=0.44$) or S-PLA ($p=0.95$) group.

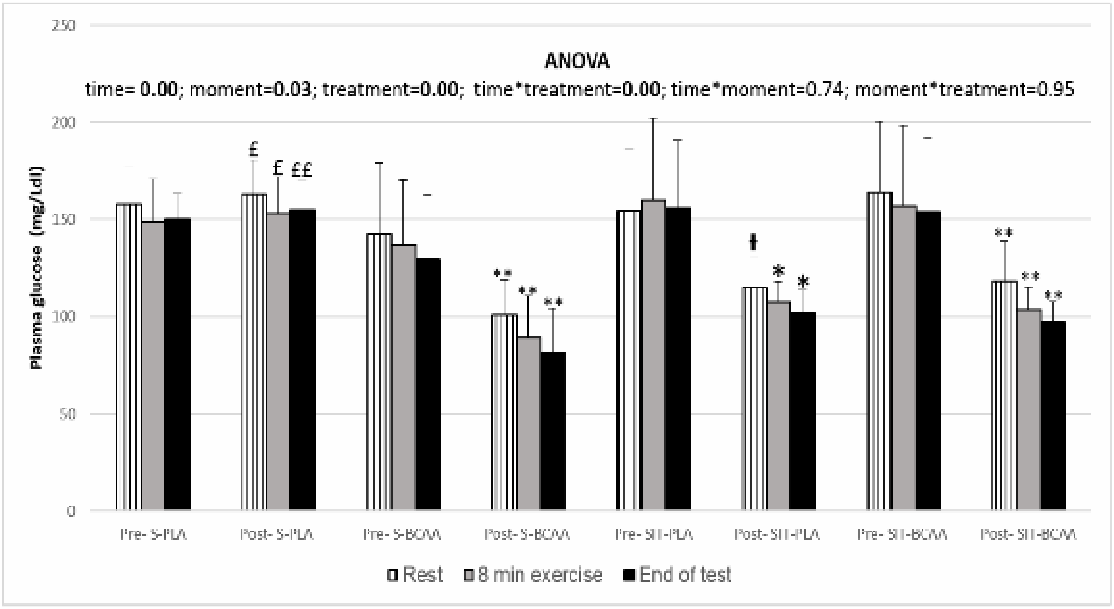

Fig. 2. Response of plasma glycaemia concentration from a physical graded exercise test before (Pre-) and after (Post-) intervention with branched-chain amino acids (BCAA) or Carbohydrate (PLA) supplementation associated with a sprint interval training program (SIT) or sedentarism (S). * p≤0.05 vs. corresponding pre-treatment moment. ** p≤0.01 vs. corresponding pre-treatment moment. p=0.08 vs. corresponding pre-treatment. £, p<0.05 vs. post-treatment (from respective situations) of the other groups. ££ p<0.01 vs. post-treatment (from respective situations) of the other groups.

There was no difference between groups (at pre- or post-treatment) for [La]b at rest, during or after graded exercise test (Fig 3 and Table S1). There was main effect of test on [La]b production (P< 0.01) in all groups for the graded exercise test at pre- and post-treatment. The only main effect for treatment (decrease in [La]b) was for SIT-PLA group (p=0.04), see Fig 3 and Table S1.

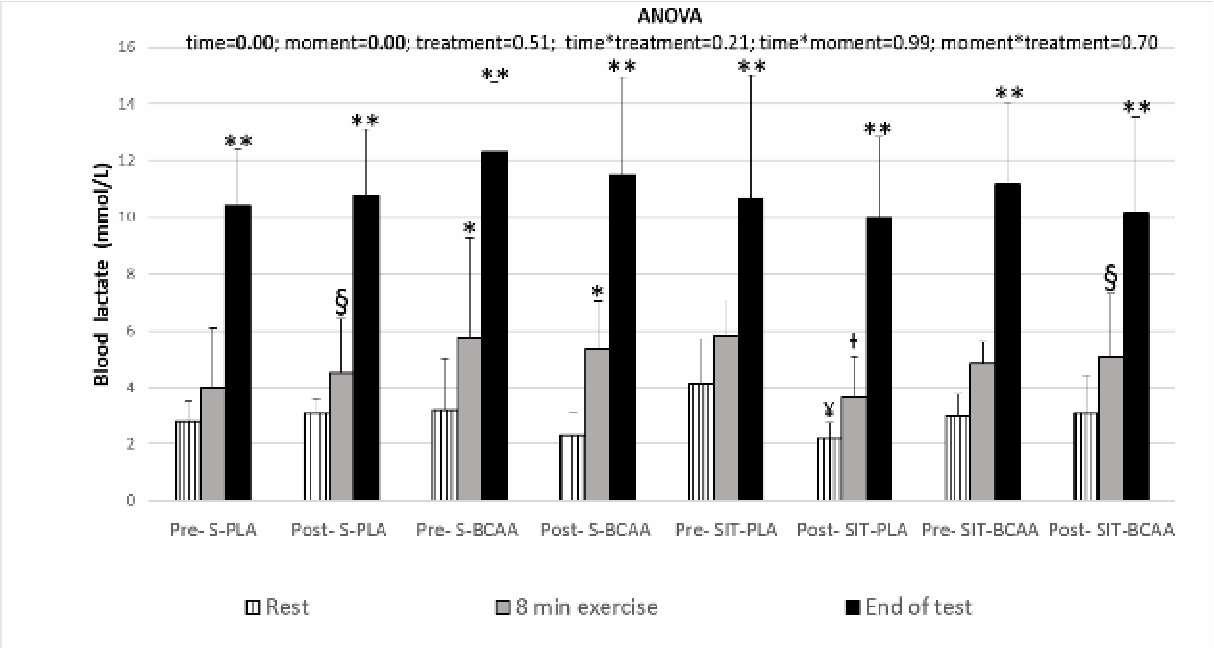

Fig. 3. Changes in blood lactate concentration from a physical graded exercise test before (pre-) and after (post-) intervention with branched-chain amino acids (BCAA) or Carbohydrate (PLA) supplementation associated with a sprint interval training program (SIT) or sedentarism (S). **p≤0.01 vs. previous moments from respective treatment. * p≤0.05 vs. previous moments from respective treatment. § p≤0.07 *vs.* previous moments from respective treatment; p=0.03 vs. pre-treatment. ¥ p=0.09 vs. pre-treatment.

Table S1. Response of Respiratory exchange ratio (RER), plasma glucose concentration and blood lactate concentration from a physical graded exercise test before (Pre-) and after (Post-) treatment with branched-chain amino acid (BCAA) or Carbohydrate (PLA) supplementation associated with a sprint interval training program (SIT) or sedentarism (S).

RER	Groups	Pre- Mean	SD ()	Post-Mean	SD
At rest	S-PLA	0.86	0.04	0.87	0.03
	S-BCAA	0.84	0.06	0.83	0.05
	SIT-PLA	0.86	0.08	0.87	0.07
	SIT-BCAA	0.91 *	0.06	0.86	0.08
8 min test	S-PLA	1.01 ‖	0.06	1.03 ‖ ‡	0.05
	S-BCAA	0.95 ‖	0.05	0.93 ‖	0.04
	SIT-PLA	1.00 ‖*	0.07	0.94 ‖	0.07
	SIT-BCAA	1.00 ‖*	0.06	0.96 ‖	0.04
End of the test	S-PLA	1.10 ‖	0.05	1.10 ‖‡	0.07
	S-BCAA	1.06 ‖ *	0.04	1.01 ‖	0.07
	SIT-PLA	1.09 ‖ *	0.08	1.03 ‖	0.08
	SIT-BCAA	1.11 ‖ †	0.05	1.05 ‖	0.05
Blood glucose concentration (mm/dl)					
At rest	S-PLA	158.22	20.20	163.16 ‡	18.25
	S-BCAA	142.71 **	36.63	100.71	18.700
	SIT-PLA	154.50 †	32.21	114.83	16.77
	SIT-BCAA	163.85 **	36.59	118.55	20.41
8 min test	S-PLA	149.00	22.31	153.20 ‡	19.16
	S-BCAA	137.03 **	33.67	89.14	21.73
	SIT-PLA	159.85 *	42.61	107.67	10.64
	SIT-BCAA	157.22 **	41.24	103.77 ‖	11.42
End of the test	S-PLA	150.43	13.15	155.31 ‡	15.40
	S-BCAA	129.57 **	33.09	81.71	22.59
	SIT-PLA	155.87 *	35.31	102.33	11.58
	SIT-BCAA	154.22 **	37.61	97.62 ‖	10.39
Blood lactate concentration (mmol/l)					
At rest	S-PLA	2.81	0.70	3.13	0.52
	S-BCAA	3.23	1.83	2.31	0.83
	SIT-PLA	4.11†	1.61	2.21	0.55
	SIT-BCAA	2.98	0.76	3.10	1.32
8 min test	S-PLA	4.12	2.16	4.51	1.92
	S-BCAA	5.76	3.52	5.37 ‖	1.67
	SIT-PLA	5.81 *	1.27	3.66 ‖	1.42
	SIT-BCAA	4.86	0.72	5.07	2.24
End of the test	S-PLA	10.43 ‖	2.14	10.80 ‖	2.31
	S-BCAA	12.31 ‖	2.47	11.47 ‖	3.46
	SIT-PLA	10.65 ‖	4.35	9.98 ‖	2.86
	SIT-BCAA	11.16 ‖	2.90	10.16 ‖	3.37

Note: S-PLA, sedentary plus placebo; S-BCAA. sedentary plus BCAA; SIT-PLA, sprint interval training (SIT) plus placebo, and; SIT-BCAA, SIT plus BCAA. * $p<0.05$ when compared to post-treatment; ** $p\leq0.01$ when compared to post-treatment; †$p\leq0.12$ when compared to post-treatment; ‡$p<0.10$ when compared to post-treatment of SIT-PLA, S-BCAA and SIT-BCAA groups; ‖ $p\leq0.05$ when compared to rest moment in the same treatment. Values are mean and standard deviation (SD).

Discussion

The major finding of this study was to demonstrate that chronic BCAA supplementation (300 mg·kg·day^{-1}) promoted significant metabolic changes and these changes were more evident when associated with a SIT program. After a period of 8 weeks, albeit we did not identify a decrease in body fat (measured by skinfolds), the variables associated with fat metabolism (i.e. serum LDL-C, HDL-C, TAG and HbA1c) were significantly altered by SIT when associated with BCAA supplementation. This data suggests that when BCAA supplementation is associated with our proposed physical training model (which increased cardiorespiratory fitness and decreased hypercholesterolemia) these strategies may act synergistically decreasing the relative risk of CVD (Farrell et al. 2012).

Metabolic markers of cardiovascular disease risk

In this study, the SIT-PLA group did not showed significant changes (at p values) in lipid markers, HbA1c or PCR, this results are in agree to the literature of SIT training, on intervention period and population characteristic tested in this study (Nalcakan 2014; Fisher et al. 2015; Jelleyman et al. 2015b; Tenório et al. 2015). Studies with longer periods (with the same training protocol) have demonstrated significant alterations on lipid profile (Bagley et al. 2016a).

To our knowledge, this is the first study with humans that used high doses of BCAA supplementation associated with short term SIT intervention and found changes in serum lipid markers. It is well known, from studies with rats that high dietary BCAA has a hypolipidemic effect. For example, several studies have already shown that BCAA supplementation (Holecek, Siman et al. 2016) or leucine alone (Zhang, Guo et al. 2007; Zhao, Dai et al. 2016), can lower total cholesterol, LDL-C, HDL-C and TAG concentrations (Zhang, Guo et al. 2007;

Holecek, Siman et al. 2016; Zhao, Dai et al. 2016). Our study, demonstrated that for LDL-C, HDL-C and TAG these changes seem to be more pronounced when BCAA supplementation was associated with physical training. For example, HDL-C was virtually unchanged in S-BCAA group (p=0.75, ES=-0.11) elevated in SIT-PLA group Training (p=0.24, ES=-0.45) and further increased in SIT-BCAA group (p=0.05, ES=-1.24). This suggests a synergistic effect of BCAA supplementation and physical training (see Table 2). On the other hand, LDL-C was reduced similarly in both SIT-BCAA and S-BCAA groups, but in SIT-PLA group LDL-C decrease was not significant (at p-value), so it is not evident a synergistic effect of SIT and BCAA supplementation on LDL-C (see Table 2).

Interestingly, both SIT-BCAA and SIT-PLA groups had a trend to increase total cholesterol (p=0.19, ES= -0.62; p=0.08, ES = -0.64, respectively), possibly as a response to physical training (Bagley, Slevin et al. 2016a) by raising HDL-C cholesterol (Williams et al. 1994) and a transient increase in VLDL-C cholesterol (we did not measure VLDL-C in this study) (Shojaee-Moradie et al. 2015). We suggest that the decrease in TAG observed in this study seems to be a response to a decrease in blood glucose concentration, which induces a decrease in insulin secretion, thus allows an increase in VLDL-C production (since that higher insulin suppresses the hepatic production of VLDL-C, regardless of TAG concentrations) (Badaloo et al. 2015). It is known that IR is positively correlated with TAG concentrations (Badaloo, Reid et al. 2015). This hypothesis can be confirmed by our results, on which TAG concentrations from our participants with their respective HbA1c values has significant and positive correlations (pooled data of all groups: pre-treatment, ρ=0.45, p=0.04; post-treatment, ρ=0.43, p=0.05).

Animal experiments have shown that BCAA supplementation decreases TAG concentrations in liver and muscle, suggesting higher fatty acid oxidation in these tissues (Masaki 2015b). However, human studies are necessary to confirm the TAG fate in response to BCAA supplementation (comparing effects associated with both training or sedentary lifestyle). Three possible destinations are: (1) increase in TAG muscle concentrations or (2) stimulation of VLDL-C production in the liver (associated with the respective increase in liver TAG) and (3) decrease in TAG concentrations due to increased fatty acid oxidation. According to our data (decrease in RER on SIT-BCAA group), it is more likely that there is also a decrease in both muscle and hepatic TAG, as a response to increased fatty acid oxidation in these tissues (Masaki 2015b). In other words, we believe that the serum TAG decrease observed (due to BCAA supplementation) happened because it was oxidated.

Chronic BCAA supplementation changes several genes expression regarding cholesterol production and metabolism, such as a decrease in 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Bruckbauer and Zemel 2015) and an increase in enzymes related to cholesterol efflux (i.e. ABCG5 and ABCG8) from liver to biliary vesicle (Zhao, Dai et al. 2016). HMG-CoA reductase is a key enzyme in the metabolic pathway to converts mevalonate to sterol (e.g. cholesterol) (Gazzerro et al. 2012). Statins (HMG-CoA reductase inhibitors) are the most prescribed drugs for lowering LDL-C cholesterol. In a dose-dependent manner it has also an effective effect of decreasing serum TAG concentrations and increasing HDL-C, but in a sharp contrast, one of the adverse effects of statins is muscle loss (Knapik-Czajka 2014). Recently, it has been demonstrated that BCAA supplementation prevents muscle mass loss due to statins treatment (D'Antona et al. 2016). The significant muscle mass loss due to statins treatment come from BDK inactivation, that activates BCKDH (Knapik-Czajka 2014). BDK can be down-regulated by KIC (a leucine metabolite) or structures similar to fibrate (e.g. statins), molecules structurally similar to KIC. So, as a result from statin treatment, can be detected an increase in KIC concentrations (due to inhibition of HMG-CoA reductase, i.e., KIC downstream metabolism impairment) and a decrease in plasma BCAA concentrations (due to increased BCKDH activity) (Gazzerro, Proto et al. 2012; Knapik-Czajka 2014). We can speculate that BCAA might exert its lipid-lowering effect through BCKDH activation (thus providing substrate to HMG-CoA down regulated), however, further investigations need to be made to confirm this hypothesis. Taken together, our data suggest that the high plasma BCAA concentrations found in diabetic and hypercholesterolemic individuals (Yang, Hu et al. 2016) may be related to the lack of BCAA catabolism and not as a consequence of higher dietary BCAA ingestion(Zheng, Li et al. 2016) .

Our results (decrease in HbA1c in SIT-BCAA group) corroborates with the data from Takeshita et al. (Takeshita, Takamura et al. 2012) and the vast body of evidence (Jakicic et al. 2013; Lynch and Adams 2014; Jelleyman et al. 2015a) which suggest that both training and BCAA supplementation should be used to decrease plasma HbA1c (see Table 2). BCAA supplementation *per se* promotes a decrease in HbA1c concentrations both in animal model (Guo et al. 2010; Xie et al. 2015) and in IR humans (Takeshita, Takamura et al. 2012). Takeshita et al. (Takeshita, Takamura et al. 2012) showed that 13g/day of BCAA supplementation improved HbA1c in individuals who had IR in muscle tissue. Moreover, a recently study (Daniel et al. 2017) has been demonstrated that an acute higher BCAA intake (20g/day), increase muscle glucose uptake, throughout independent insulin secretion mechanism. Our results demonstrate great potential of SIT plus BCAA supplementation for T2D treatment and the importance of the muscle tissue in HbA1c modulation, since the literature suggest that decreases in BCAA degradation (low BCKDH activity) (Lynch and Adams 2014) and low cardiorespiratory fitness (Jakicic, Egan et al. 2013) are inversely correlated to HbA1c plasma concentration.

Data from the present study demonstrated that there was a trend towards CRP decrease in the SIT-BCAA group. Then, we can speculate that there might be a possible synergistic effect of BCAA supplementation associated with physical training in this variable, since the ES value is higher in SIT-BCAA group, when compared to the other three groups (see Table 2). A longer physical training period (associated with a specific diet) is necessary to promote a reduction in the concentrations of CRP (Imayama et al. 2012). A similar but longer study would help to answer this question because leucine supplementation has been shown to reduce inflammation, possibly as a consequence from lipid profile improvement (Zhao, Dai et al. 2016). Also, regular physical training promotes an anti-inflammatory effect (Puggina et al. 2016). In particular, short term SIT intervention (same time period tested in this study) exerts little influence on CRP concentrations in healthy subjects (Tenório, Sá et al. 2015).

Energy metabolism

Our study demonstrated that 8-wk of 300 mg·kg·dia^{-1} of BCAA supplementation (when associated with SIT) resulted in a significant decrease on RER at rest and tend to decrease during exercise, without the previously glycogen stores depletion or the need to perform the exercise protocol in the fasting state (as reported in Gualano et al. (Gualano, Bozza et al. 2011) study, that demonstrated that short term (3-days) 300 mg·kg·dia^{-1} BCAA supplementation significantly decreased RER of healthy young men with low muscle glycogen reserves. RER decrease also has been reported in a rat model due to BCAA supplementation (Zhang, Guo et al. 2007).

Our model (fed-state assessment), also demonstrated that even with the glycogen reserve apparently intact after the experimental protocol (although we did not actually evaluated muscle glycogen stores), the RER from S-BCAA group decreased, at 15min of continuous graded exercise test (Fig 1), although the data presented by Gualano et al. (Gualano, Bozza et al. 2011) showed significant changes already at 10min of exercise. We believe that these differences may be related to the participation of glycogen in the energy metabolism. The decrease in RER suggests that there was an increase in the fatty acid (FA) oxidation. This alteration in RER (due to BCAA supplementation associated with SIT) may partially explain the significant decrease in serum TAG levels found in both S-BCAA and SIT-BCAA group (Bajotto, Murakami et al. 2009; She et al. 2010), a fact that did not occur significantly in both S-PLA and SIT-PLA group (as evidenced by the effect size in the TAG variable, see Table 2).

The SIT-PLA tend to decrease ($p=0.07$) RER during exercise. The short term SIT cycle training performed in this study promotes a decrease in RER during physical exercise, but not in resting state (Astorino et al. 2013; Shook et al. 2014; Arad et al. 2015). The increase in CRF also influences the decrease in RER and plays a key role in increasing fat oxidation (Shook, Hand et al. 2014; Gavarry et al. 2015). Thus, our data agree with literature.

Interestingly, despite the decrease in RER in the SIT-BCAA group, there was no decrease in [La]b production, as expected (De Palo et al. 2001; She, Zhou et al. 2010). This probably occurred as a response to the excess BCAA in the body, which decreased pyruvate dehydrogenase (PDH) activity (Jia et al. 2013; Li et al. 2017). In contrast, the SIT-PLA group showed a significant main effect of treatment ($p=0.04$, decrease in [La]b production), possibly as an effect of physical training in decreasing glycogenolysis (Howarth, Burgomaster et al. 2007). Thus, it is likely that increasing BCAAs degradation in β-oxidation (which increases the supply of acetyl-CoA and succinyl-CoA to tricarboxylic acid cycle) promotes an inhibition of PDH enzyme complex (Kainulainen, Hulmi et al. 2013). Also, is described in a mouse study which demonstrates that the PDH4 gene expression is mitigated against chronic BCAA supplementation (Jia, Takahashi et al. 2013). Thus, it is probably that we observed not only a change in substrate utilization (which justifies the RER decrease observed in our study), but also a reduction of pyruvate (to acetyl-CoA) in to mitochondria, thus promoting a consequent increase in lactate production. This can explain the non-decrease in the [La]b production, found in our study on SIT-BCAA group. Another possible explanation (for non-decrease of [La]b on SIT-BCAA group), also supported by our data, is that the muscle was more sensitive to glucose uptake in SIT-BCAA group (a fact corroborated by the significant decrease in HbA1c, see Table 2), data also observed in rats (Zhang, Guo et al. 2007; Jia, Takahashi et al. 2013) and human (Takeshita, Takamura et al. 2012) studies. Also, our acute glucose kinetic assessment demonstrated that the SIT-BCAA group experienced a pronounced decrease (main effect of test, $p=0.01$) in blood glucose concentration in response to exercise (which did not happen at the baseline, see Fig 2), therefore making glycolysis possible. This phenomenon (higher lactate production post-supplementation) was also observed in a study with creatine (Cr) supplementation (Roberts et al. 2016), on which demonstrated that Cr increases both glucose uptake and muscle glycogen stores. Thus, due to the increase in muscle glucose uptake (on Cr group) an increase in lactate production occurred during a physical exercise test, when compared to the placebo group (Roberts, Fox et al. 2016).

Higher [La]b production also suggests a lower glucose phosphorylation. If BCAA actually decreases the rate of glucose phosphorylation (Jia, Takahashi et al. 2013), this may have significant implications in terms of athletic performance, particularly in endurance exercise (>60min) with intensity >75% VO_{2max}, whereas carbohydrate phosphorylation (for energy supply) is a limiting step in the athletic performance (Jeukendrup 2014). If, on one hand, athletes with depleted glycogen stores run longer distances when supplemented with high

doses of BCAA, with similar doses used our study (Gualano, Bozza et al. 2011). On the other hand, evidences from animal studies (rats with intact glycogen reserves, when supplemented with high doses of BCAA) showed ergolytic effect on performance, when compared to the control group (even presenting an increase on tricarboxylic acid cycle activity, supplied by the BCAA supplementation) (Campos-Ferraz et al. 2013). Therefore, future human experiments (with similar BCAA doses, as tested in this study) need to be done to verify if these alterations have any relevance to endurance performance.

Anthropometry and VO_{2peak}

Contrary to a preview studies with SIT training (Nalcakan 2014), our program that consisted of three times per wk^{-1} during 8-wk was not sufficient to change anthropometric variables, even when combined with BCAA supplementation. A previous study (Nalcakan 2014) with young subject (~15% of body fat associated to 24 BMI and VO_2max: ~40.3 $ml \cdot min^{-1} \cdot kg^{-1}$) demonstrated that the SIT protocol used in our study was sufficient to promote a decrease in %BF, sum of skinfolds and waist circumference. However, very recent meta-analyses with obese or overweight individuals did not observe anthropometric changes (%BF decrease) after a 4-12 weeks of HIIT cycle ergometer (Wewege et al. 2017), although the literature presents results in which there is fat mass decrease of greater magnitude with SIT (which used the same training volume that our protocol), both in healthy and unhealthy lean individuals (De Feo 2013; Bagley et al. 2016b). Other studies (Astorino, Schubert et al. 2013; Keating et al. 2014; Shepherd et al. 2017) and our results, indicates that overweight or obese individuals did not lose fat efficiently with short term (4-12 week) HIIT or SIT cycle ergometer training. The discrepancies between these results can be linked to the participants' profile. One hypothesis is that our participants (young adults whit overweight and obesity) have not been able to train intense enough like eutrophic young people (De Feo 2013). However, HIIT run training promotes a higher muscle mass recruitment (when compared to cycle) and can decrease fat mass in overweight or obese individuals in the short term (Wewege, Berg et al. 2017). Thus, is possible that overweight individual with lower CRF capacity (e.g. VO_2max: ~20 $ml \cdot min^{-1} \cdot kg^{-1}$) has a lower fatty acid oxidation capacity, due their impaired metabolic flexibility (Goodpaster et al. 2002; Rynders et al. 2017), which might demand HIIT/SIT run training or a higher cycle training volume (to increase energy expenditure during exercise). For instance, Keating et al. (Keating, Machan et al. 2014) compared HIIT *vs.* continuous training (CT) a observed that %BF was significantly improved in the CT group, whereas in the HIIT group there was a worsening in body composition. These data also raising the training volume on cycle ergometer (to increase energy expenditure during training) as another hypothesis that may explain this lack of change in body composition (Montero et al. 2017). So, Additional studies are needed to check for mechanisms related to lack of response in obese individuals to SIT cycle ergometer, whether it is a matter of metabolic inflexibility (Goodpaster, Wolfe et al. 2002; Rynders, Blanc et al. 2017).

The VO_{2peak} of our participants increased significantly in both trained groups (with no difference between them). The increase in VO_{2peak} of our participants was similar to studies that also used this training protocol (Howarth, Burgomaster et al. 2007; Nalcakan 2014). CRF has a strong correlation with CVD biomarkers (Farrell, Finley et al. 2012; Solomon et al. 2015). Although, the SIT-PLA (that only trained) group did not show any significant changes (at p-value level) on CVD risk markers assessed in this study, we observed by ES values that such changes were taking place (see Table 2). In this sense, if the intervention were to be carried out over a longer period of time, we might have observed strong changes in the CVD biomarkers in the SIT-PLA group (Gillen et al. 2013). The changes in CVD biomarkers observed in the SIT-BCAA group were exciting, especially due to the lack of evidence with human studies (showing a synergistic effect on some CVD biomarkers, between exercise training and higher BCAA intake), however, is well know that both exercise and BCAA supplementation induces gene alterations that strongly influence lipid, glycemic and energy metabolism profile (Jia, Takahashi et al. 2013; Lerin, Goldfine et al. 2016).

Limitations

Some of the limitations that could make this study stronger are: (1) We instructed the participants to maintain their diet during the experiment. Although we do not know of any humans studies (with a similar dose as used in this study), other study models show that high doses of BCAA supplementation have the potential to suppress appetite (Wessels et al. 2016), and we don´t know if the same occurs in humans. So, we cannot discard the hypnosis that part of the changes observed in our study might have been influenced by changes in appetite and a consequent decrease in food intake; (2) Another important limitation was the evaluation of energy metabolism (RER, glycemic level and [La]b response to exercise) in the fed state. More consistent data on energy metabolism in response to BCAA supplementation could have been provided in the fasted state. However, data from the fasted state is already published by Gualano et al. (Gualano, Bozza et al. 2011), so we chose the fed-state model; (3) It is important to note that women who participated in this study had a regulated menstrual cycle (although some women use contraceptives and some do not). With this care was taken to ensure that reevaluations always occur close to the same dates (to avoid individual variation), however, we do not rule out the hypothesis that due to the variability of the cycle between the participants may have affected the results.

Conclusion

In conclusion, the present study demonstrated that eight weeks of SIT associated (or not) with high doses of BCAA intake did not promote anthropometric changes. We also concluded that they have a and synergistic effect on fatty acid oxidation (energy metabolism), LDL-C, HDL-C, TAG and HbA1c concentrations (CVD biomarkers). In summary, BCAA supplementation associated with physical training have a synergistic effect on the improvement of metabolic health.

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