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Randomized control trials

Effects of branched-chain amino acids supplementation on both plasma amino acids concentration and muscle energetics changes resulting from muscle damage: A randomized placebo controlled trial

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SUMMARY

Background & aims: Branched-chain amino acids promote muscle-protein synthesis, reduce protein oxidation and have positive effects on mitochondrial biogenesis and reactive oxygen species scavenging. The purpose of the study was to determine the potential benefits of branched-chain amino acids supplementation on changes in force capacities, plasma amino acids concentration and muscle metabolic alterations after exercise-induced muscle damage.

Methods: ³¹P magnetic resonance spectroscopy and biochemical analyses were used to follow the changes after such damage. Twenty six young healthy men were randomly assigned to supplemented branched-chain amino acids or placebo group. Knee extensors maximal voluntary isometric force was assessed before and on four days following exercise-induced muscle damage. Concentrations in phosphocreatine [PCr], inorganic phosphate [Pi] and pH were measured during a standardized rest-exercise-recovery protocol before, two (D2) and four (D4) days after exercise-induced muscle damage.

Results: No significant difference between groups was found for changes in maximal voluntary isometric force (−24% at D2 and −21% at D4). Plasma alanine concentration significantly increased immediately after exercise-induced muscle damage (+25%) in both groups while concentrations in glycine, histidine, phenylalanine and tyrosine decreased. No difference between groups was found in the increased resting [Pi] (+42% at D2 and +34% at D4), decreased resting pH (−0.04 at D2 and −0.03 at D4) and the slower PCr recovery rate (−18% at D2 and −24% at D4).

Conclusions: The damaged muscle was not able to get benefits out of the increased plasma branched-chain amino acids availability to attenuate changes in indirect markers of muscle damage and muscle metabolic alterations following exercise-induced muscle damage.

Abbreviations: BCAAs, branched chain amino acids; EIMD, exercise-induced muscle damage; MRS, magnetic resonance spectroscopy; BCAA, group supplemented with branched chain amino acids; PLAC, group supplemented with placebo; MVC, maximal voluntary contraction; PCr, phosphocreatine; Pi, inorganic phosphate; TCA, tricarboxylic acid; CK, creatine kinase; DOMS, delayed onset muscle soreness; NMES, neuromuscular electrostimulation; VAS, visual analog score; MIF, maximal isometric force; ATP, adenosine triphosphate; V_{cons} PCr, rate of PCr consumption; V_{recov} PCr, rate of PCr recovery; $[\text{PCr}]_{\text{end}}$, end-exercise [PCr]; $\Delta[\text{PCr}]_{\text{exo}}$, total change in [PCr] during the dynamic exercise; $[\text{PCr}]_{\text{endrec}}$, end-recovery [PCr]; $\Delta\text{pH}_{\text{ini}}$, transient change in pH at the beginning of the dynamic exercise; pH_{end} , end-exercise pH; $\Delta\text{pH}_{\text{exo}}$, total change in pH during the dynamic exercise; $\text{pH}_{\text{endrec}}$, end-recovery pH; pH_{ini} , start-recovery pH; V_{recov} pH, rate of pH recovery; pH_{min} , minimal pH during recovery.

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1. Introduction

Branched chain amino acids (BCAAs) – *i.e.*, leucine, isoleucine and valine – account for almost 50% of the dietary essential amino acids and 35% of the total content of essential amino acids in muscle proteins [1,2]. BCAAs are mainly metabolized in skeletal muscle [1] whereas other essential amino acids are catabolized in liver [3]. They are important precursors of tricarboxylic acid (TCA) cycle intermediates *via* acetyl-CoA and Succinyl-CoA [4] and can be involved in energy production through the modulation of exercise-induced serum BCAAs oxidation [5]. In addition to their involvement as constitutive elements of the structural and contractile proteins synthesis [6], BCAAs are also considered as signaling molecules [7–9]. It has been suggested that they could enhance mitochondrial biogenesis and reactive oxygen species scavenging [4,10]. The corresponding beneficial effects on mitochondria integrity and biogenesis have been mainly demonstrated in isolated muscles [4] and a few studies have been conducted in humans [11–13]. Using ³¹P-magnetic resonance spectroscopy (³¹P-MRS), a reduced submaximal exercise-induced muscle acidosis has been measured in young healthy adults [13] and in elderly patients with liver cirrhosis [12] as a result of BCAAs supplementation.

On the basis of these positive effects on muscle structure integrity, muscle energetics and mitochondrial function attributed to BCAAs, several studies have addressed the issue of using BCAAs supplementation in order to counteract the deleterious effects of exercise-induced muscle damage (EIMD). So far, the effects of BCAAs supplementation on force loss, increased plasma creatine kinase (CK) activity and delayed onset muscle soreness (DOMS) have been controversial [14–18]. Indeed, several studies have illustrated beneficial effects on muscle soreness [14,16,18,19], force [16,18,19] and CK activity [18,20,21], whereas others have not [15,17,22] and the accounting factors of these differences remain still unknown. It should be noticed that most of the studies illustrating positive effects of BCAAs also reported moderate muscle damage.

We recently reported that a single session of isometric neuromuscular electrostimulation (NMES) induced severe muscle damage [23]. NMES involves the application of intermittent stimuli using surface electrodes in order to evoke muscle contraction through the activation of intramuscular nerve branches [24]. The spatially fixed, temporally synchronous and random recruitment pattern of motor units during NMES leads to large structural alterations [23,25]. NMES exercise-induced muscle damage led to an acidotic shift in resting muscle and an impaired mitochondrial function in exercising muscle [26]. Considering the beneficial effects of BCAAs previously reported on mitochondrial function and integrity [4,10,11,13], it would be of interest to determine whether BCAAs supplementation could reverse or alleviate the mitochondrial impairment we previously quantified within the frame of a damaging exercise. So far, changes in plasma amino acids concentration and the corresponding effects of BCAAs supplementation have been mainly investigated throughout submaximal endurance exercise [27–33] whereas very few studies have been related to strength exercise [34]. It has been reported that BCAAs and other amino acids can be used by exercising muscle [34,35]. Interestingly, the corresponding effects of BCAAs supplementation on exercise-induced muscle damage have never been investigated.

The aim of the present study was to determine the effects of BCAAs supplementation on muscle energetics changes and indirect markers of muscle damage (*i.e.*, force, DOMS and plasma CK activity) after a single session of isometric NMES exercise and to characterize the corresponding plasma amino acids dynamics. We

hypothesized that a BCAAs supplementation could attenuate metabolic alterations associated with severe EIMD.

2. Materials and methods

2.1. Subjects

Twenty six healthy men volunteered to participate in this study and were randomly assigned (Fig. 1) to BCAAs supplemented (BCAA, $n = 13$, age: 22 ± 1 yr, height: 179 ± 5 cm, body mass: 70 ± 8 kg) or placebo group (PLAC, $n = 13$, age: 23 ± 2 yr, height: 177 ± 6 cm, body mass: 68 ± 6 kg). Subjects were recreationally active (36 ± 42 min/wk) but none of them were involved in any regular training or strength exercise programs. Subjects were instructed to avoid any intense or unaccustomed physical activities throughout the study period. Subjects were asked to keep their diet habits, limit their alcohol consumption throughout the duration of the protocol and to avoid smoking and consuming caffeine before the experiment. Drug treatment was prohibited during six days before and four days after the damaging exercise. Volunteers were fully informed about the nature and the aims of the study and gave their informed written consent to participate. The study was conducted in conformity with the latest revision of the Declaration of Helsinki and was approved by the Local Human Research Ethics Committee *Sud Méditerranée V* (2012-04 A00449-34). The double blind randomized placebo controlled trial was referenced on clinicaltrials.gov (NCT01687361).

2.2. Study design

The experiment included six sessions performed at the same time of the day aiming at assessing muscle function and energetics before and after the damaging exercise (Fig. 2). Dietary analysis was performed throughout the experimental period.

2.2.1. Initial assessment (D-6)

The first session was performed six days before (D-6) the damaging exercise in order to assess the baseline values of metabolic variables using ³¹P-MRS during a standardized rest-exercise-recovery protocol as previously described [36].

2.2.2. Exercise inducing muscle damage (D0)

Two blood samples were collected to determine plasma CK activity and amino acids concentrations in fasting conditions. A standardized breakfast (*i.e.*, excluding milk, coffee and anything with a high proteins content) was taken by the subject in addition to one BCAAs or placebo supplementation (*i.e.*, 30 min before the damaging exercise [37]). Muscle soreness and maximal voluntary contraction (MVC) of the knee extensor muscles were assessed. Thereafter, a second supplementation was drunk by the subject just before the damaging exercise. Immediately after, MVC was measured, muscle soreness was assessed and two blood samples were drawn. Then, a third supplementation was drunk by the subject (*i.e.*, 10 min after the end of the NMES exercise).

2.2.3. Follow up of muscle function

One (D1), two (D2), three (D3) and four days (D4) after the damaging exercise, two blood samples were collected and muscle soreness together with MVC were assessed. A single BCAAs or placebo supplementation was drunk before MVC assessment. ³¹P-MRS measurements were performed at D2 and D4 similarly to what was performed at D-6.

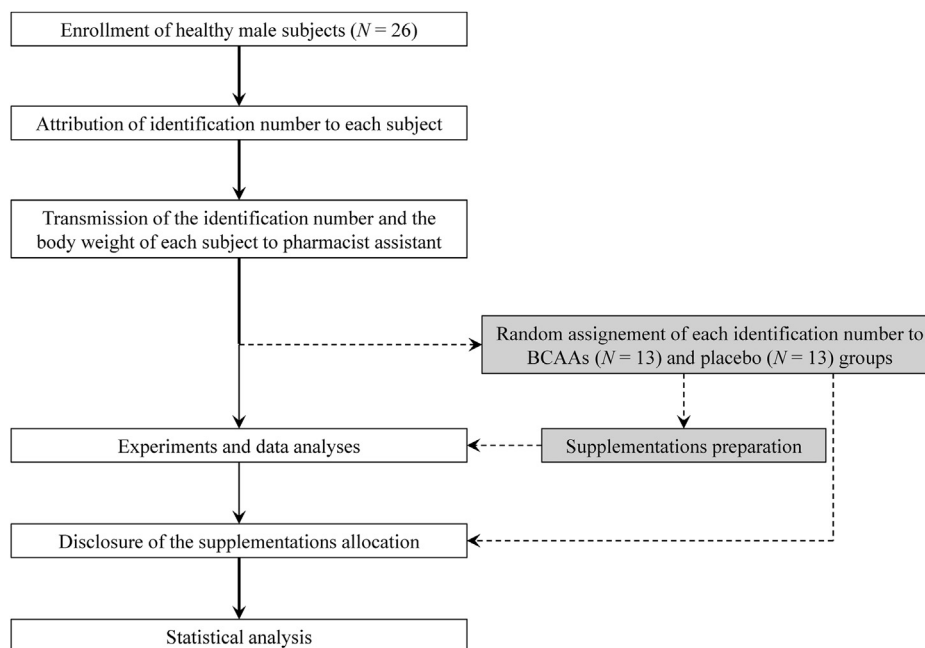


Fig. 1. Random allocation sequence and concealment.

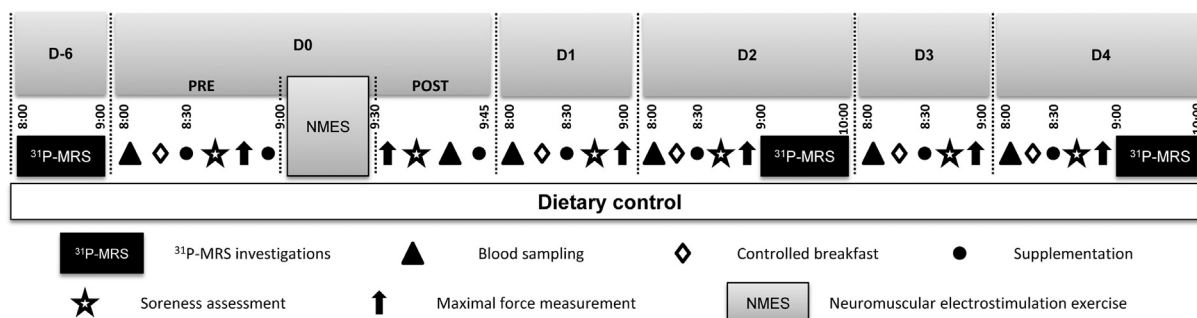


Fig. 2. Schematic representation of the experimental design.

2.3. Neuromuscular electrostimulation (NMES) protocol

Subjects were seated on a chair (Multi-Form', La Roque d'Anthéron, France) customized with a force sensor. Adjustable belts secured hip and ankle joints to hold the hip and knee angles at $\sim 90^\circ$ and $\sim 100^\circ$ respectively (0° corresponding to the joint fully extended). Both legs were stimulated simultaneously using three stimulation electrodes placed over the thigh, a 5×10 cm on the proximal part of the thigh (*i.e.*, placed ~ 5 cm below the inguinal ligament) and two 5×5 cm on the *vastus lateralis* and *vastus medialis* muscle bellies. Biphasic symmetric rectangular pulses were delivered at a frequency of 100 Hz with a pulse duration of $400 \mu\text{s}$ (40 contractions, 5 s on and 35 s off throughout the NMES exercise) using a portable battery-powered stimulator (Compex[®] Performance, DjoGlobal, France). Stimulation intensity was gradually increased in order to reach the highest tolerated (considering the pain threshold) level of evoked force for each subject as previously described [23,38]. This type of NMES protocol has been shown to induce a prolonged MVC force loss and increased DOMS and plasma CK activity [23,38]. Force signal sampled at 1000 Hz was recorded using a Powerlab system and Labchart software (ADInstruments, Colorado Springs, CO, USA) and analyzed with a customized Matlab routine (v7.11, Mathworks, Natick, MA, USA).

For each NMES-evoked contraction, the peak force was measured and averaged over the forty contractions.

2.4. Muscle soreness

Level of muscle soreness was assessed by the same investigator during squat isometric contraction, passive stretching and palpation of *vastus lateralis* and *vastus medialis* using a visual analog scale (VAS) with a 100 mm horizontal line including "no pain" at the one end (0 mm) and "extremely painful" at the other (100 mm). Squat movement was performed from a standing position to a 90° knee flexion angle with a constant movement velocity (*i.e.*, 2 s for movement from standing position). During palpation of *vastus lateralis* and *vastus medialis*, knee was flexed at $\sim 100^\circ$. Soreness was assessed using the mean value obtained from the measurements on the four tests (*i.e.*, isometric squat, passive stretching, *vastus lateralis* and *vastus medialis* palpation).

2.5. MVC force

Subjects were in the position described above for the NMES protocol. After a 5–10 min warm-up session including a set of sub-maximal isometric knee extensions for both legs simultaneously,

subjects were instructed to perform the maximal isometric knee extension force. This measurement was repeated three times with a 30 s resting period between attempts and the MVC was determined as the highest value among the three trials. A similar measurement was performed 30 s after the last electrically evoked isometric contraction of the NMES exercise.

2.6. Dietary analysis

Subjects were instructed to maintain their normal dietary habits and to fill a 10-day food log between D-6 and D4. This was done to monitor macronutrient intakes (especially for proteins) and to ensure that there was no significant difference between the experimental groups. Subjects were instructed to mention all food and drink intakes with the most complete description (e.g., brand, nutritional supplies, information provided by manufacturers). The dietary food logs were evaluated using the "Ciquel Table 2013" database established by the French Agency for Food, Environmental and Occupational Health & Safety to determine the average daily macronutrient breakdown of carbohydrates, proteins and lipids. Intakes were averaged on two separate periods considering the six days before and the four days after the NMES exercise.

2.7. Supplementations

The BCAAs supplementation was a mixture (2:1:1) of leucine, isoleucine and valine (Ajinomoto Co. Inc., Tokyo, Japan). The placebo material was composed of microcrystalline cellulose. The amount of BCAAs (100 mg/kg) was chosen on the basis of previous studies [5,16] and the mixture was blended with 30 mL of InOrpha® (Paddock Laboratories, Inc., Minneapolis, MN, USA) in a 100 mL dark glass bottle so that neither subjects nor experimenters could distinguish the BCAAs from the placebo solution. A total of seven supplementations were taken by each subject: three doses at D0 (i.e., 30 min before, just before and immediately after NMES exercise) and a single dose in each following sessions. BCAAs or placebo solutions were assigned randomly for each subject in a double-blind fashion.

2.8. Blood markers

Two 5 mL blood samples were withdrawn from the forearm vein and used for the plasma CK activity and amino acids assays as described below:

2.8.1. Plasma CK activity

Plasma was immediately separated after blood centrifugation (15 min at 2500 rpm). Plasma CK activity was determined enzymatically at 37 °C using a chemistry analyzer (UniCel DxS 800 Synchron Clinical System; Beckman Coulter, Inc., Brea, CA, USA).

2.8.2. Amino acids concentration

Blood sample was placed in a tube with sodium heparin. Heparinized blood was centrifuged at 4 °C for 10 min at 3,500 rpm and plasma was frozen and sent to the specific in-hospital laboratory for amino acids concentration assays. Plasma was then unfrozen, deproteinized using sulfosalicylic acid (400 g/L) and centrifuged at ambient temperature for 3 min at 10,000 rpm. The supernatant was then analyzed using ion-exchange high-performance liquid chromatography with ninhydrin detection using AminoTac JLC-500/V (JEOL, Tokyo, Japan). The analysis total duration was 120 min. The analyzer was calibrated using standard amino acid solutions before the first run on the day of analysis and an external standard was added to each sample.

2.9. ³¹P-MRS

Subjects lay prone with the right leg centered within a 1.5-T super-conducting magnet (MAGNETOM Avanto, Siemens AG, Healthcare Sector, Erlangen, Germany). A ³¹P-¹H double-tuned surface coil (Rapid Biomedical GmbH, Rimpfing, Germany; Diameter: 18 cm) was positioned under the right *quadriceps femoris* muscle. In order to ensure a stable position during the dynamic exercise and to avoid contraction of back muscles, subjects were firmly fixed to the bed of the magnet with three non-elastic straps positioned over the leg and the hip as previously described [26,36]. A dedicated ergometer was positioned at the back of the magnet so that the horizontal bars were located at the vicinity of the ankle joint. The knee of the subjects was flexed at ~40° in the resting position. A strain gauge (N2A-13-T026P-350; Vishay Micro-Measurements, Germany) was positioned along each bar in order to accurately quantify force output during dynamic exercise. A potentiometer (35905-002-102; Bourns, Mexico) was mounted on each pulley axis located at the opposite side of the ankle in order to continuously record weight displacement. Both signals were amplified using a custom-made device, analog-to-digital converted (PCI-6023E; National Instruments, France) and recorded on a personal computer (WinATS software 6.5; SYMA, France). The position of the thigh over the coil and the distance between the magnet and the ergometer were measured to ensure reproducible measurements for each ³¹P-MRS session.

Maximal isometric force (MIF) of the knee extensor muscles was measured during two 5 s trials separated by 1 min. After a 2-min resting session, a set of multi-slice fast proton magnetic resonance images was acquired in order to determine the position of the leg with respect to the surface coil. Then, an automatic localized map shimming procedure was performed. Full relaxed spectra were acquired at rest with a repetition time of 15 s ($N_{EX} = 4$). Then, ³¹P-MRS spectra were recorded at rest (80 s), during a standardized dynamic exercise (360 s) and during recovery (360 s) with the following parameters (500 μs rectangular pulse, 2 s repetition time, 4 excitations, 32 kHz sweep width, 4096 data points, 128 ms dwell time, 60° flip angle). Dynamic exercise was performed at a rate of 30 repetitions per min with the subjects' lifting the mass corresponding to 25% of the MIF over a distance of ~17 cm. Repeated leg extensions were gated to the MR recordings using an audible signal so that spectra were always recorded at the same time of the contraction-relaxation process. Mechanical signals of force and displacement were computed *a posteriori*. The instantaneous power output (product of the displacement rate and the corresponding force) and the total work during the dynamic exercise were calculated.

Relative concentrations of phosphocreatine (PCr), inorganic phosphate (Pi) and adenosine triphosphate (ATP) were obtained by a time-domain fitting routine using the AMARES algorithm [39] interfaced by CSIPO [40] with a time resolution of 8 s and appropriate prior knowledge of the ATP multiplets as described previously [36]. The resting concentrations were calculated from the full relaxed spectra considering a 8.2 mM ATP muscle concentration at rest [41]. The rate constants ($1/\tau$) of both the PCr dynamics during exercise and throughout the recovery period was determined by fitting the [PCr] changes to a single exponential curve described by the following equation:

$$[PCr](t) = A_1 + A_2 \times e^{-(t/\tau)}$$

where A_1 represent the final concentration (at the end of the exercise or end of the recovery periods) and A_2 the amplitude of the corresponding changes. Model parameters (i.e., A_1 , A_2 and $1/\tau$) were determined with an iterative process by minimizing the squared

errors sum. The rate of PCr consumption ($V_{\text{cons}} \text{ PCr}$) and recovery ($V_{\text{recov}} \text{ PCr}$) were calculated as the product of the rate constant ($1/\tau_{\text{exo}}$ and $1/\tau_{\text{recov}}$, respectively) and the absolute value of A_2 coming out from the fitting procedures for dynamic exercise and recovery data, respectively. $\Delta[\text{PCr}]_{\text{exo}}$ refers to the extent of PCr consumption during the exercise period while $[\text{PCr}]_{\text{end}}$ and $[\text{PCr}]_{\text{endrec}}$ refer to the corresponding values at the end of the exercise and recovery periods, respectively.

Intracellular pH was calculated from the chemical shift of the Pi relative to the PCr signal [42]. The potential transient alkalosis in the early phase of the dynamic exercise ($\Delta\text{pH}_{\text{ini}}$) was calculated as the difference between the resting value and the peak value within the first 24 s of the dynamic exercise. In addition, the total acidosis during the dynamic exercise ($\Delta\text{pH}_{\text{exo}}$) was calculated as the difference between the pH value at rest and at the end of the dynamic exercise (pH_{end}).

During recovery, pH changes including the initial acidification phase and the later phase of continuous pH increase was modeled using the following equation adapted from Walter et al. [43]:

$$\text{pH}(t) = C_0 + (C_1 + C_2 \times t) \times e^{C_3 \times t}$$

where C_0 refers to the pH value at the end of the recovery period ($\text{pH}_{\text{endrec}}$), C_1 is the difference between the pH value at the beginning of the recovery period (pH_{ini}) and $\text{pH}_{\text{endrec}}$, C_2 and C_3 are rate constants. Model parameters (*i.e.*, C_0 , C_1 , C_2 and C_3) were determined with an iterative process by minimizing the squared errors sum. Rate of pH recovery ($V_{\text{recov}} \text{ pH}$) was calculated as the product of the model rate constants C_3 and the amplitude of the corresponding pH change calculated as the difference between $\text{pH}_{\text{endrec}}$ and the lowest pH value (pH_{min}). Initial values used for the iterative process were determined from previous studies [36,44].

2.10. Statistical analysis

Data distribution was initially investigated using Shapiro-Wilk test. Then two-way (group \times time) repeated measures ANOVA were performed (Statistica, Statsoft, Tulsa, USA) to assess the difference for MVC, muscle soreness, plasma amino acid concentrations and ^{31}P -MRS parameters between both conditions. A Tukey's HSD post hoc analysis was performed when appropriate. A non-parametric Friedman's two-way ANOVA was performed to assess the statistical difference for plasma CK activity. The level of significance was set to $P < 0.05$. Results are reported as mean \pm SD. Error bars in the figures represent SD. The number of subjects in each group was determined on the basis of a statistical power calculation ($\alpha = 0.05$ and $1 - \beta = 0.9$) considering an alleviated effect of 30% due to the BCAAs supplements on the 25% MVC force loss previously determined [26] corresponding to a diminished MVC force loss by at least 7.5% for the BCAAs-supplemented group.

3. Results

For two subjects (*i.e.*, one subject in each group), recordings of the mechanical signals related to the dynamic exercise were not possible due to a technical problem. As a consequence, analyses of ^{31}P -MRS parameters were performed in 24 subjects.

3.1. NMES session

Stimulation intensity was gradually and similarly increased throughout the NMES session in both groups (from 33 ± 6 mA to 65 ± 16 mA). The corresponding evoked force increased during the first contractions and reached a plateau after the 20th contraction. Despite the continuous increase in stimulation intensity, no further

significant force change was measured. The relative averaged peak evoked force over the 40 contractions was similar between the two groups ($31 \pm 8\%$ MVC).

3.2. Muscle soreness

We did find neither a group effect nor a group \times time interaction ($P > 0.05$). However, the ANOVA analysis disclosed a significant time effect. Accordingly, a significant increase was measured in both groups at D1 (BCAA: 14 ± 9 mm and PLAC: 12 ± 9 mm) and the highest averaged value was measured at D2 (BCAA: 18 ± 8 mm and PLAC: 17 ± 11 mm). At D3, muscle soreness was still higher (BCAA: 17 ± 11 mm and PLAC: 14 ± 11 mm) than the baseline value (Fig. 3-A).

3.3. MVC force

Similarly to what we observed for muscle soreness, we did not observe a group effect or a group \times time interaction ($P > 0.05$) but identified an effect of time ($P < 0.05$). For both groups, MVC decreased immediately after the NMES exercise in both groups (-31% and -29% for BCAA and PLAC, respectively). Although we measured a slow recovery towards the initial values starting from D3 (Fig. 3-B), the MVC values quantified at D1 (BCAA: 577 ± 141 N and PLAC: 593 ± 112 N), D2 (BCAA: 568 ± 148 N and PLAC: 598 ± 99 N), D3 (BCAA: 561 ± 158 N and PLAC: 608 ± 111 N) and D4 (BCAA: 585 ± 173 N and PLAC: 632 ± 99 N) were still lower than the baseline values (BCAA: 760 ± 177 N and PLAC: 779 ± 111 N).

3.4. Dietary analysis

Dietary analysis illustrated similar proteins, lipids and carbohydrates nutritional intakes for both groups between D-6 and D0. A significantly larger protein intake was found for the BCAA group between D0 and D4 ($P < 0.05$, Table 1).

3.5. Blood markers

3.5.1. Plasma CK activity

A significant group \times time interaction was found ($P < 0.05$). Plasma CK activity significantly increased in both groups at D3 (BCAA: 8640 ± 7848 IU/L and PLAC: 5380 ± 6905 IU/L) and D4 (BCAA: $17211 \pm 22,252$ IU/L and PLAC: 7062 ± 6843 IU/L). As illustrated in Fig. 3-C, the plasma CK activity was significantly ($P < 0.05$) higher in the BCAA group as compared to PLAC.

3.5.2. Amino acids concentration

A significant group \times time interaction was found ($P < 0.05$) for the three BCAAs. As illustrated in Fig. 4, the plasma concentration of leucine (+282%), isoleucine (+152%) and valine (+296%), transiently and significantly increased in the BCAA group immediately after the NMES session. Considering this very large increase and in order to avoid a potential type II statistical error, the BCAAs time-dependent changes in the PLAC group was analyzed using one-way repeated measures ANOVA. On that basis, we found that leucine (-30%); isoleucine (-30%) and valine (-17%) transiently decreased immediately after the NMES exercise ($P < 0.05$). For the other amino acids, we did find neither a group effect nor a group \times time interaction ($P > 0.05$). However, the statistical analysis disclosed a significant effect of time ($P < 0.05$) corresponding to an increased plasma alanine concentration (+25%) ($P < 0.05$, Fig. 5-A), and significantly decreased citrulline (-23%), glycine (-10%), histidine (-5%), phenylalanine (-18%) and tyrosine (-19%) concentrations in both groups immediately after the NMES exercise (Fig. 5B-F). At D1, serine was significantly decreased (-10%) in both

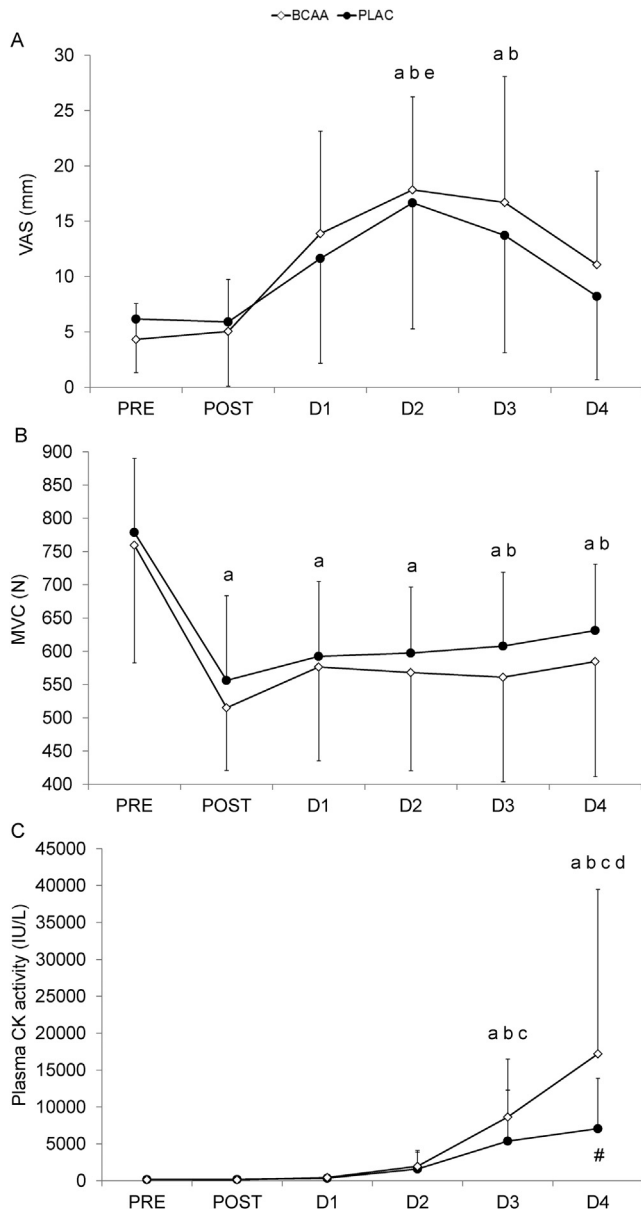


Fig. 3. **A** – Muscle soreness assessed with visual analog scale (VAS), **B** – maximal isometric voluntary force (MVC) and, **C** – plasma creatine kinase (CK) activity before (PRE), immediately after (POST) and one-to-four days (D1, D2, D3 and D4) after NMES exercise-induced muscle damage for subjects supplemented with branched-chain amino acids (BCAA, empty diamonds) or placebo (PLAC, full circles). a: significantly different from PRE for both groups, b: significantly different from POST for both groups, c: significantly different from D1 for both groups, d: significantly different from D2 for both groups, e: significantly different from D4 for both groups, #: significant difference between PLAC and BCAA, $P < 0.05$.

groups (Supplementary material) whereas no change was observed for the other amino acids (Supplementary material).

3.6. ^{31}P -MRS

In both groups, MIF was unchanged at D2 (BCAA: 269 ± 68 N and PLAC: 283 ± 47 N) and D4 (BCAA: 267 ± 52 N and PLAC: 277 ± 46 N) as compared to the baseline values (BCAA: 273 ± 53 N and PLAC: 298 ± 55 N) indicating that the dynamic exercise was performed at the same absolute and relative intensity for the three sessions.

Table 1

Averaged daily intakes of proteins, lipids and carbohydrates including supplementation in the six days before the first supplementation and in the four days after for subjects supplemented with branched-chain amino acids (BCAA) or placebo (PLAC).

		Before supplementation	With supplementation
Proteins (g/kg)	BCAA	1.07 ± 0.26	$1.50 \pm 0.21^{a,b}$
	PLAC	1.07 ± 0.24	1.07 ± 0.27
Lipids (g/kg)	BCAA	1.09 ± 0.35	0.94 ± 0.28
	PLAC	1.07 ± 0.34	0.98 ± 0.32
Carbohydrates (g/kg)	BCAA	3.00 ± 0.66	3.22 ± 1.13
	PLAC	3.25 ± 0.96	3.24 ± 1.17

^a Significantly different from before ($P < 0.05$).

^b Significantly different from PLAC group ($P < 0.05$). Values are mean \pm SD.

Similarly, the mean power and the total work over the 180 sub-maximal voluntary contractions were unchanged in both groups at D2 and D4.

As indicated in Fig. 6, the time-dependent changes in [PCr], [Pi], [Pi]/[PCr] and pH during the standardized protocol were qualitatively similar in both groups. At D2 (+41%) and D4 (+35%), the resting [Pi] was significantly increased in both groups whereas the resting [PCr] was unchanged. Accordingly, an elevated [Pi]/[PCr] ratio was quantified at D2 (+36%) and D4 (+29%) (Table 2). In addition, for both groups, the resting pH value was significantly more acidotic at D2 (7.05 ± 0.04) and D4 (7.06 ± 0.03) as compared to the initial value (7.09 ± 0.02).

Exercise performed before the supplementation resulted in a Δ [PCr]_{exo} of 10.1 ± 3.0 mmol/L and a Δ pH_{exo} of 0.17 ± 0.12 . A slight alkalosis was detected in the early stage of the dynamic exercise ($+0.01 \pm 0.03$) and pH_{end} was 6.92 ± 0.12 . A significant increase in the rest-to-exercise alkalosis was found at D2 ($+0.05 \pm 0.02$) and D4 ($+0.03 \pm 0.03$) for both groups. At the end of exercise, pH_{end} was significantly increased in both groups at D2 (6.97 ± 0.11) and D4 (6.97 ± 0.09) as compared to baseline value resulting in a significantly decreased Δ pH_{exo} at D2 (0.08 ± 0.11) and D4 (0.09 ± 0.08) (Table 3). Additionally, a significant decrease in Δ [PCr]_{exo} was found at D2 (–9%) and D4 (–9%) while [PCr]_{end} increased at D2 (+15%) and D4 (+13%) in both groups. No significant change was observed for $1/\tau_{\text{cons}}$ and V_{cons} PCr in both groups (Table 3). No significant difference was found between both groups.

During the post-exercise recovery period, both PCr and pH displayed slower recovery kinetics after the NMES session. A significant effect of time was found for $1/\tau_{\text{recov}}$ (–6% at D2 and –18% at D4) leading to a slower V_{recov} PCr at D2 (–15%) and at D4 (–22%). The pH recovery kinetics (V_{recov} pH) was significantly slower at D2 only (–48%, Table 3). In addition, a significant group \times time interaction was found for [PCr] and pH during the recovery period ($P < 0.05$) so that the [PCr] values were significantly higher at D2 and D4 in PLAC as compared to the baseline conditions whereas no change occurred in the BCAA group (Fig. 6-A and -B). Regarding pH, significantly lower values were measured at D2 and D4 as compared to the baseline conditions for PLAC whereas a similar difference was only measured at D2 in the BCAA group (Fig. 6-C and -D). At D4, the BCAA group exhibited a full pH recovery.

4. Discussion

The present study intended to determine whether BCAAs supplementation could alleviate functional and metabolic alterations we previously described as a result of a single isometric NMES session [26]. We also assessed changes in plasma amino acids concentration resulting from a damaging NMES exercise and the impact of BCAAs supplementation on these changes. We found no

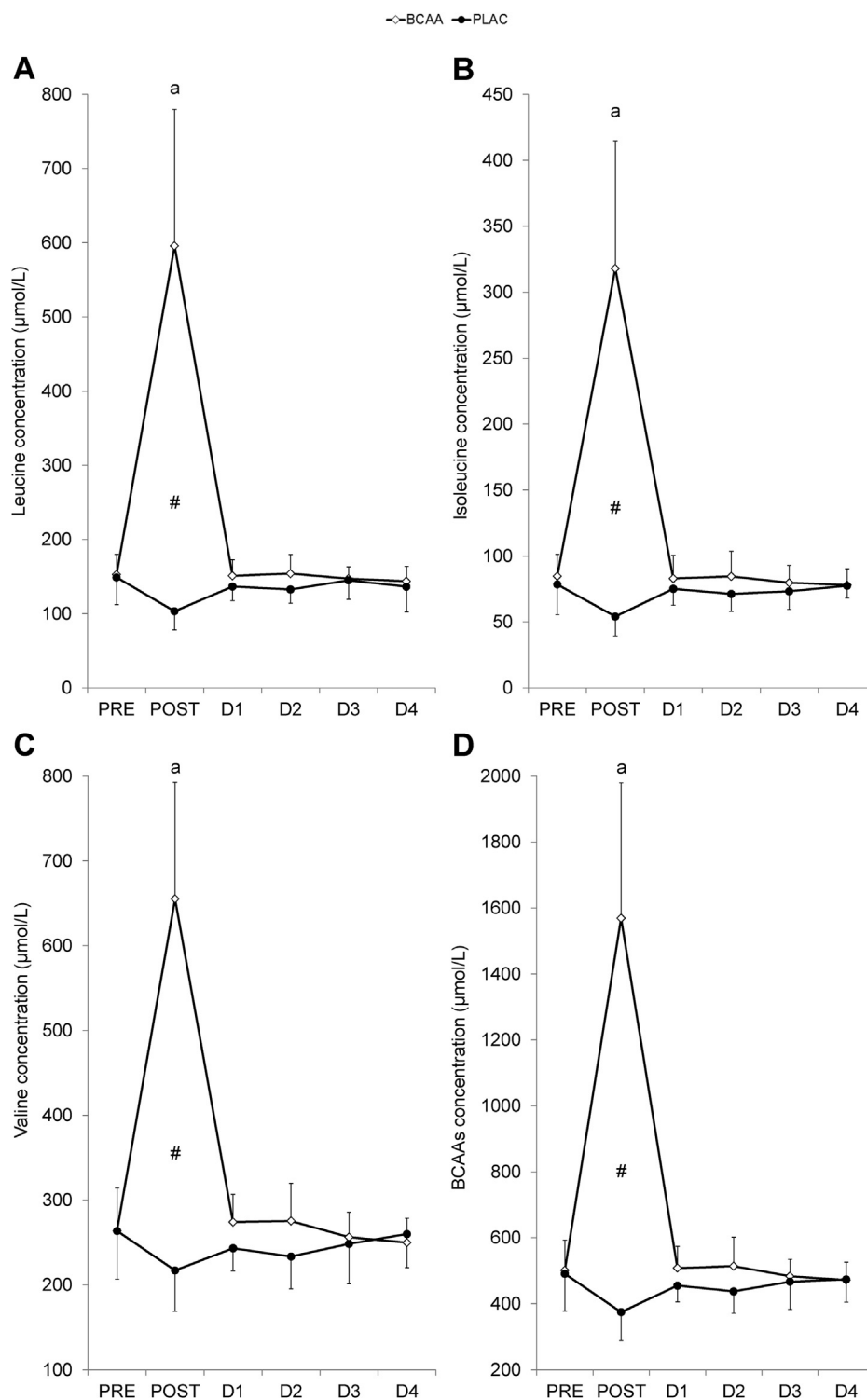


Fig. 4. A – leucine, B – isoleucine, C – valine and D – branched-chain amino acids (BCAAs) concentrations determined 30 min before (PRE), immediately after (POST) and one-to-four days (D1, D2, D3 and D4) after NMES exercise for subjects supplemented with branched-chain amino acids (BCAA, empty diamonds) or placebo (PLAC, full circles). a: significantly different from PRE for both groups, #: significant difference between PLAC and BCAA, $P < 0.05$.

evidence indicating that the increased BCAAs availability could alleviate alterations of muscle function (*i.e.*, MVC, DOMS) and the related metabolic alterations (*e.g.*, pH homeostasis, mitochondrial function). In addition, the BCAA supplementation did affect neither the plasma CK activity nor the amino acids concentrations changes resulting from the damaging exercise.

The time course of MVC, DOMS and plasma CK activity we reported in the present study are in agreement with previous studies [38,45,46]. We observed no significant positive effect of BCAAs supplementation on these commonly reported indirect markers of muscle damage, either immediately after the NMES exercise or during the subsequent recovery period. It is noteworthy that

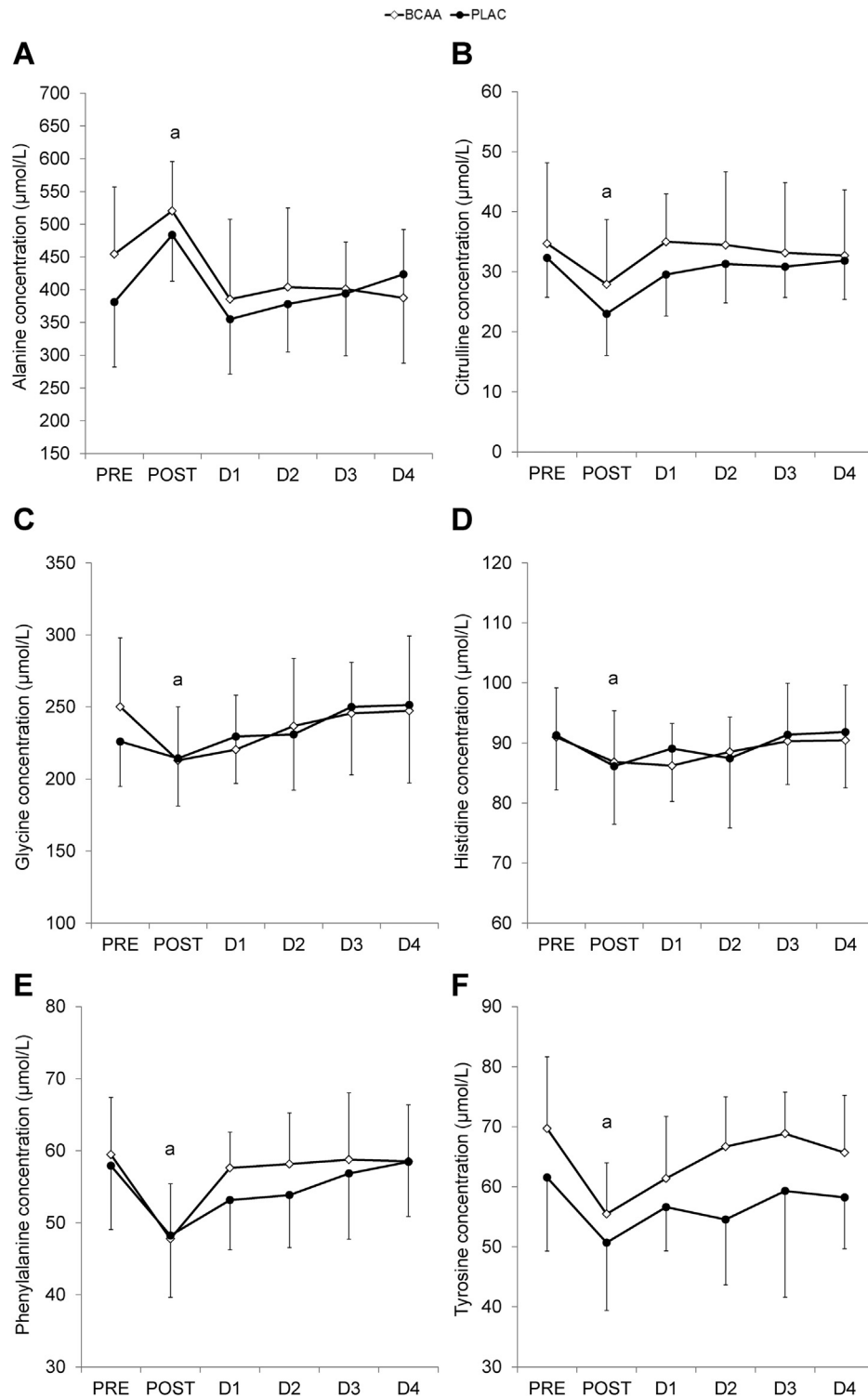


Fig. 5. A – Alanine, B – citrulline, C – glycine, D – histidine, E – phenylalanine and F – tyrosine concentrations determined 30 min before (PRE), immediately after (POST) and one-to-four days (D1, D2, D3 and D4) after NMES exercise for subjects supplemented with branched-chain amino acids (BCAA, empty diamonds) or placebo (PLAC, full circles). a: significantly different from PRE for both groups, $P < 0.05$.

plasma CK activity was significantly higher in the BCAA as compared to the PLAC at D4. However, this significant difference likely results from the large between-subject variability in the CK measurements and the larger fraction of high-CK responders within the BCAA group [47]. For a similar BCAAs intake and an identical mixture, beneficial effects of BCAAs supplementation on

DOMS [14,18] and muscle force [16,18] have been reported after EIMD. While we provided 300 mg/kg of BCAAs to the subjects the day of the damaging exercise and 100 mg/kg/day for the following recovery period, approximately 100–750 mg/kg of BCAAs were given the day of the damaging exercise and between 0 and 250 mg/kg/day for the following recovery period in other previous studies

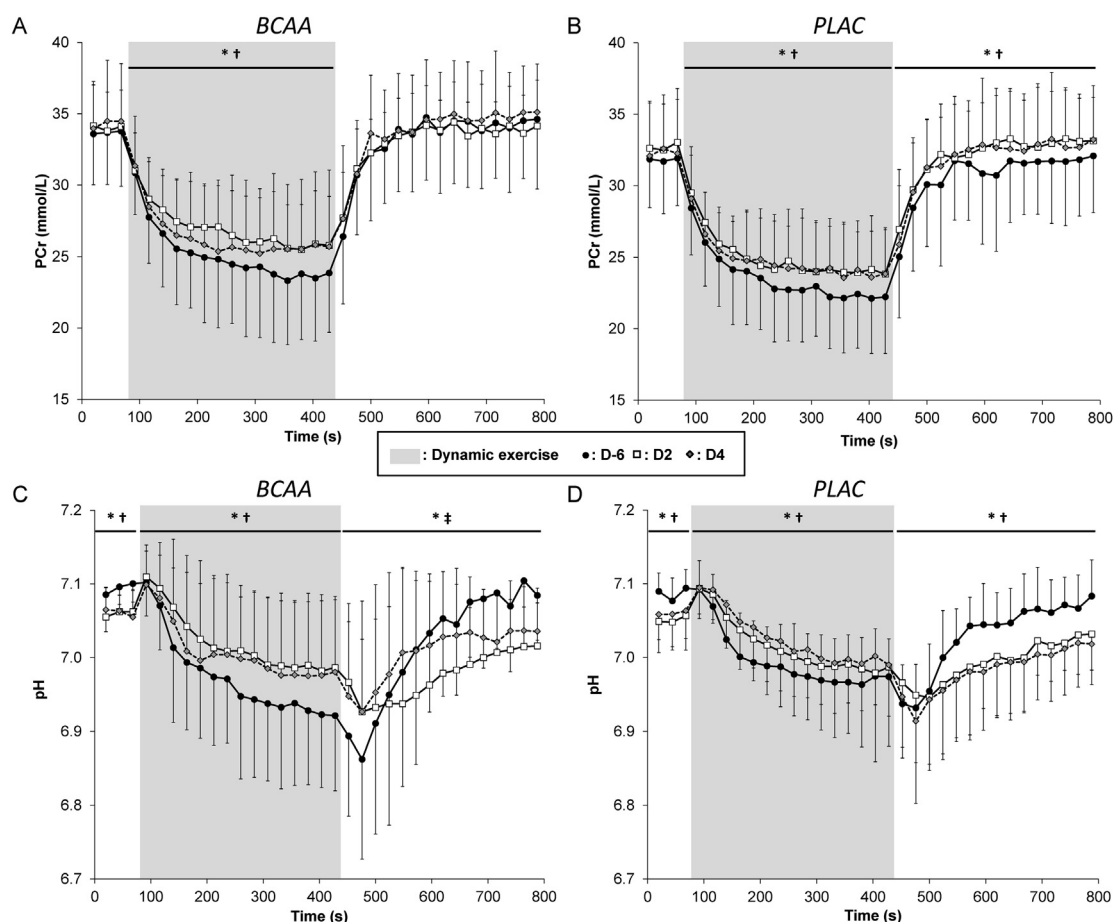


Fig. 6. PCr-time (top) and pH-time (bottom) relationships during the rest-exercise-recovery protocol for the subjects supplemented with branched-chain amino acids (BCAA, A and C) or placebo (PLAC, B and D) before (D-6, black circles – solid line), two days (D2, white squares – dotted line) and four days (D4, gray diamonds – solid line) after NMES exercise. Gray area represents time for dynamic voluntary exercise. *: significant difference between D-6 and D2, †: significant difference between D-6 and D4, ‡: significant difference between D2 and D4, $P < 0.05$.

[14,16,18]. Interestingly, the plasma CK activity values we measured in the present study were very high (peak at D4 for BCAA: $17211 \pm 22,252$ IU/L and PLAC: 7062 ± 6843 IU/L) as compared to those reported in previous studies (i.e., peak values ranged from 80 to 400 IU/L) [14,16,18]. We previously discussed the extent of muscle damage resulting from our experimental protocol [26] and, on that basis, it could be suggested that the large extent of muscle

damage induced in the present study might have limited the potential beneficial effects of BCAAs supplementation. It can then be speculated that BCAAs might alleviate the MVC force loss and the increased plasma CK activity resulting from a damaging exercise as long as the damage remain moderate. Overall, we found negligible effect of a daily 100 mg/kg BCAAs supplementation on indirect markers of muscle damage resulting from NMES exercise.

We also investigated the free plasma amino acids concentrations time-dependent changes after the damaging NMES exercise. As expected, immediately after NMES EIMD, plasma leucine, isoleucine and valine concentrations were higher in the BCAA group indicating an imbalance between the supply and a potential muscle uptake (Fig. 4). These results are in agreement with the plasma BCAAs concentration kinetics reported after an oral consumption showing a progressive increase peaking 30 min after absorption followed by a progressive decrease until baseline level in the next two hours [37]. Comparable changes i.e., a 2-fold increase in valine and a 4 times increase in leucine and isoleucine have been reported using a 100 mg/kg BCAAs supplementation after a damaging exercise [16]. On the contrary to what we measured in the BCAA group, we quantified a 23% decreased plasma BCAAs concentration in PLAC thereby suggesting that BCAAs are metabolized at the muscular level during a damaging NMES exercise. A similar consumption i.e., –21% has been reported for non-supplemented subjects in the context of a squat damaging exercise [5] whereas a lower consumption i.e., –4.9% have been

Table 2

^{31}P magnetic resonance spectroscopy variables measured at rest six days before (D-6), two days (D2) and four days (D4) after NMES exercise for subjects supplemented with branched-chain amino acids (BCAA) or placebo (PLAC).

		D-6	D2	D4
[PCr] (mmol/L)	BCAA	32.9 ± 3.6	33.9 ± 2.4	34.2 ± 3.4
	PLAC	31.6 ± 3.3	32.7 ± 3.5	32.2 ± 3.4
[Pi] (mmol/L)	BCAA	5.3 ± 1.3	7.4 ± 1.5^a	7.0 ± 1.7^a
	PLAC	4.9 ± 1.5	6.6 ± 1.7^a	6.2 ± 1.2^a
pH	BCAA	7.10 ± 0.03	7.05 ± 0.03^a	7.06 ± 0.03^a
	PLAC	7.09 ± 0.02	7.05 ± 0.04^a	7.06 ± 0.04^a
PCr/ATP	BCAA	4.8 ± 0.4	5.3 ± 0.5	4.9 ± 0.5
	PLAC	4.7 ± 0.5	4.8 ± 0.5	4.7 ± 0.5
Pi/ATP	BCAA	0.6 ± 0.2	0.9 ± 0.2^a	0.8 ± 0.2^a
	PLAC	0.6 ± 0.2	0.9 ± 0.2^a	0.8 ± 0.1^a
Pi/PCr	BCAA	0.16 ± 0.03	0.22 ± 0.04^a	0.20 ± 0.05^a
	PLAC	0.15 ± 0.04	0.20 ± 0.05^a	0.19 ± 0.04^a

PCr: phosphocreatine, Pi: inorganic phosphate, ATP: adenosine triphosphate.

^a Significantly different from D-6 ($P < 0.05$). Values are mean \pm SD.

Table 3
Metabolic variables measured during dynamic voluntary exercise and recovery six days before (D-6), two days (D2) and four days (D4) after NMES exercise for subjects supplemented with branched-chain amino acids (BCAA) or placebo (PLAC).

			D-6	D2	D4
Exercise	$\Delta[\text{PCr}]_{\text{exo}}$ (mmol/L)	BCAA	10.7 ± 3.9	8.6 ± 3.6 ^a	9.3 ± 3.9 ^a
		PLAC	9.6 ± 1.8	8.7 ± 2.6 ^a	8.4 ± 2.3 ^a
	$1/\tau_{\text{exo}}$ (min ⁻¹)	BCAA	1.7 ± 1.3	2.0 ± 1.7	1.8 ± 0.8
		PLAC	1.8 ± 1.0	1.9 ± 1.2	2.2 ± 0.9
	$V_{\text{cons}} \text{PCr}$ (mmol/L/min)	BCAA	15.3 ± 7.9	15.8 ± 13.9	16.4 ± 6.9
		PLAC	16.1 ± 9.2	16.5 ± 11.0	16.9 ± 8.8
	$[\text{PCr}]_{\text{end}}$ (mmol/L)	BCAA	22.2 ± 5.1	25.3 ± 3.5 ^a	24.9 ± 4.8 ^a
		PLAC	22.1 ± 3.8	24.0 ± 2.8 ^a	23.8 ± 3.5 ^a
	$\Delta\text{pH}_{\text{exo}}$	BCAA	0.22 ± 0.15	0.10 ± 0.10 ^a	0.11 ± 0.09 ^a
		PLAC	0.12 ± 0.04	0.07 ± 0.12 ^a	0.08 ± 0.07 ^a
	pH_{end}	BCAA	6.88 ± 0.15	6.96 ± 0.12 ^a	6.95 ± 0.11 ^a
		PLAC	6.96 ± 0.06	6.98 ± 0.11 ^a	6.98 ± 0.07 ^a
Recovery	$1/\tau_{\text{recov}}$ (min ⁻¹)	BCAA	1.6 ± 0.5	1.7 ± 0.6 ^a	1.3 ± 0.4 ^a
		PLAC	2.0 ± 0.4	1.7 ± 0.6 ^a	1.6 ± 0.4 ^a
	$V_{\text{recov}} \text{PCr}$ (mmol/L/min)	BCAA	18.4 ± 9.2	14.4 ± 5.3 ^a	13.4 ± 6.4 ^a
		PLAC	19.9 ± 4.8	15.8 ± 6.3 ^a	14.7 ± 3.5 ^a
	$[\text{PCr}]_{\text{endrec}}$ (mmol/L)	BCAA	33.2 ± 4.8	34.0 ± 2.5	34.6 ± 2.9
		PLAC	31.7 ± 3.9	33.2 ± 3.2	32.8 ± 3.5
	$V_{\text{recov}} \text{pH}$ (U/min)	BCAA	0.27 ± 0.24	0.08 ± 0.08 ^a	0.16 ± 0.15
		PLAC	0.15 ± 0.11	0.06 ± 0.03 ^a	0.14 ± 0.18
	$\text{pH}_{\text{endrec}}$	BCAA	7.02 ± 0.26	7.02 ± 0.05	7.04 ± 0.06
		PLAC	7.06 ± 0.03	7.02 ± 0.04	7.01 ± 0.05

$\Delta[\text{PCr}]_{\text{exo}}$: change in phosphocreatine concentration throughout exercise, $1/\tau_{\text{exo}}$: rate constant determined during exercise, $V_{\text{cons}} \text{PCr}$: rate of PCr consumption during exercise, $[\text{PCr}]_{\text{end}}$: PCr concentration at the end of exercise, $\Delta\text{pH}_{\text{exo}}$: change in pH throughout exercise, pH_{end} : pH at the end of exercise, $1/\tau_{\text{recov}}$: rate constant determined during recovery, $V_{\text{recov}} \text{PCr}$: rate of PCr recovery, $[\text{PCr}]_{\text{endrec}}$: PCr concentration at the end of recovery, $V_{\text{recov}} \text{pH}$: rate of pH recovery, $\text{pH}_{\text{endrec}}$: pH at the end of recovery.

^a Significantly different from D-6 ($P < 0.05$). Values are mean ± SD.

reported when muscle exercise was not linked to damage [34] thereby indicating that BCAAs would be more metabolized in damaged muscle. It could then be assumed that BCAAs uptake from the plasma also occurred in the supplemented group during the damaging exercise despite the increased plasma BCAAs concentration associated to the oral BCAAs intakes before NMES exercise. The decreased plasma BCAAs we reported immediately after the NMES exercise likely illustrates an increased uptake at the muscle level and can be considered as specific of the damaging conditions given that on the one hand muscle BCAAs concentration has been reported as unchanged during a non-damaging exercise [48] and on the other hand these changes transiently occurred in the early phase of recovery i.e., immediately after NMES EIMD. Thus, oral BCAAs supplementation led to an acute increase of plasma BCAAs concentration but there was no significant difference in the use of the plasma amino acids during the damaging exercise between both PLAC and BCAA groups.

The BCAAs utilization by the damaged muscle was also accompanied by a significant production of alanine in both groups. The 25% increase was in the same range as compared to the 37% increase reported after a leg press resistance exercise [34]. It has been previously described in humans that alanine can be produced by the alanine aminotransferase catalyzed reaction involving both pyruvate and glutamate [49]. The production of nitrogen as a result of BCAAs degradation during NMES exercise in both BCAA and PLAC groups could also have contributed to the alanine synthesis [50] through an intermediary pyruvate formation [51]. We also measured a higher diminished tyrosine and phenylalanine plasma concentration after NMES exercise (~20%) to what has been described in a previous study (~5%) after a single bout of knee extensors resistance exercise [34]. On that basis, one can suggest that glycine, histidine, phenylalanine and tyrosine might have provided TCA intermediates thereby promoting oxidative energy production [Fig. 1 from 4]. Overall, increased plasma BCAAs availability had no significant effect on plasma amino acids concentration changes resulting from severe EIMD. However, potential BCAAs muscle uptake could have been expected, leading to potential benefits on

muscle metabolic alterations observed after severe NMES EIMD [26].

We identified the commonly reported signs of metabolic alterations i.e., the increased resting [Pi] and the corresponding increased [Pi]/[PCr] [26,52–54] which have been interpreted as illustrating an additional energy requirement related to the repairing process of damaged tissues [54–56] associated to the initiation of an inflammatory process. In addition to the changes related to the [Pi], we measured an acidotic shift at rest for both conditions. This decreased resting pH likely illustrates an abnormal intracellular pH regulation which could be related to a disruption of cellular membranes and a corresponding impaired activity of the Na^+/H^+ exchanger [57]. This abnormality is further supported by the slower pH recovery kinetics at D2 and D4 for the control condition. During the post-exercise recovery period, the rate of PCr resynthesis has been commonly associated with the mitochondrial activity [41] for given end-exercise pH conditions [58,59]. Although pH_{end} was significantly increased at D2 (6.97 ± 0.11) and D4 (6.97 ± 0.09) as compared to the baseline value (6.92 ± 0.12), the change in pH_{end} was assumed to have negligible effect on PCr resynthesis rate. In addition, a decrease in pH_{end} was associated to a diminished τ_{recov} [58]. Therefore, the reduced τ_{recov} and $V_{\text{recov}} \text{PCr}$ we measured in both groups after the damaging exercise clearly illustrates an impaired mitochondrial function in the damaged muscle and a lack of BCAAs supplementation effect. However, it should be pointed out that previous animal studies reporting benefits of BCAAs intake were performed on a long time supplementation period [4,10]. Thus, a short BCAAs supplementation period could have been ineffective to reduce the acute deleterious effect of NMES exercise on mitochondrial function. Overall, metabolic alterations were not alleviated or reversed by BCAAs supplementation.

4. Conclusions

In the present study, we determined whether oral BCAAs supplementation can alleviate muscle function and metabolic

alterations resulting from severe muscle damage on the basis of ^{31}P -MRS measurements and biochemical analyses. We did observe the effects of NMES EIMD on the plasmatic amino acids concentration but did not demonstrate any effects of the oral BCAAs supplementation. Highly damaged muscle was not able to get benefits out of the increased plasma BCAAs availability in order to attenuate changes in indirect markers of muscle damage and muscle metabolic alterations following NMES EIMD. Further studies are needed in order to determine whether a potential association of BCAAs with free organic acid (e.g., taurine) intake could attenuate loss of muscle function and metabolic alterations associated with such a severe EIMD. Additional investigations are also required to deeply explore time course of amino acids changes within muscle with or without supplementation following severe EIMD.

Author contributions

AF, KN, JPM, HB, MGu DB and JG contributed to the conception and the design of the experiments. AF, MGa, YLF, CV and JG contributed to the collection, analysis and interpretation of the data. AF, KN, DB and JG were drafting the article and revising it for important intellectual content. All authors approved the final version of the manuscript, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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Data are available on request that should be submitted to the Delegation for Clinical Research from the APHM who meet the criteria for access to confidential data.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.clnu.2015.03.014>.

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