Basic nutritional investigation

Distinct effects of leucine or a mixture of the branched-chain amino acids (leucine, isoleucine, and valine) supplementation on resistance to fatigue, and muscle and liver-glycogen degradation, in trained rats

Patricia L. Campos-Ferraz Ph.D. a,b,*, Thomas Bozza B.Sc. a, Humberto Nicastro B.Sc. a, Antonio Herbert Lancha Jr. Ph.D. a

a Laboratory of Applied Nutrition and Metabolism, School of Physical Education and Sports, University of São Paulo, São Paulo, Brazil
b Institute of Biology, State University of Campinas, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 27 July 2012
Accepted 7 May 2013

Keywords:
BCAAs supplementation
Tricarboxylic acid cycle intermediates
Intense exercise
Leucine
Liver and muscle glycogen

ABSTRACT

Objective: The aim of this study was to evaluate the effects of the mixture of branched-chain amino acids (BCAAs) supplementation compared with leucine administered orally on muscle biochemical parameters of trained rats submitted to an exercise-induced protocol of glycogen depletion.

Methods: After 6 wk of swimming exercise, 8 wk-old (250 g, adult) male Wistar rats were randomly divided into three experimental groups (n = 8 per group): the mixture of BCAAs (BCAAs), leucine (LEU), and placebo (PLA). All groups were submitted to swimming exercise for 6 wk and supplemented with either the mixture of BCAAs, leucine, or placebo during the last week of training. At week 7 of the protocol, the rats were submitted to an intermittent, progressive swimming test until exhaustion and sacrificed. Muscle gastrocnemius and liver were depicted to determine total glycogen, tricarboxylic acid cycle (TCA) intermediates, and enzymatic activities. Statistical evaluation was performed by one-way analysis of variance with Tukey post hoc test.

Results: Both muscle and liver glycogen degradation ratio were significantly higher in the mixture of BCAAs group compared to the PLA group (P < 0.05) and the LEU group presented decreased liver glycogen degradation ratio compared with the mixture of BCAAs group (P < 0.05). Both muscle and liver glycogen content were significantly spared in the mixture of BCAAs and LEU groups compared to the PLA group (P < 0.01). A performance test demonstrated that LEU supplementation enhanced resistance to exhaustion compared to the mixture of BCAAs (P < 0.001), however, no difference was found when LEU supplementation was compared to PLA (P > 0.05). Muscle citrate content was significantly higher in the mixture of BCAAs group compared with the PLA group (P < 0.001).

Conclusion: Leucine supplementation improved performance compared with the mixture of BCAAs supplementation, sparing muscle glycogen stores despite the augmentation of some TCA intermediate concentrations on the left side of the TCA cycle.

© 2013 Elsevier Inc. All rights reserved.

Introduction

Since the studies of Bergstrom and Hultman in 1960s [1,2], the relationship between skeletal muscle glycogen content and the capacity for exercise (i.e., muscle fatigue) is well known. The regulation of carbohydrate provision during prolonged exercise and recovery is a profound challenge for the human body. Glucose is an important fuel for exercising muscles; the lack of carbohydrate reserves in liver result in reduced pyruvate levels, a substrate for both acetyl-coenzyme A (acetyl-CoA) formation and for anaplerotic reactions, which are necessary for continuous oxidation of free fatty acids and amino acids [3]. However, muscle carbohydrate metabolism does not happen in isolation but requires integration across tissues as well as regulation with the other major substrates, such as fats and amino acids [4,5].

In this context, the supplementation of amino acids, especially the glucogenic types, may play an important role (e.g., the increase in muscle alanine production during exercise and the
increased liberation of alanine in the bloodstream result in its higher contribution to glucose synthesis in liver) [6,7], thus, the mixture of BCAAs (isoleucine, leucine, and valine) supplementation has been demonstrated to enhance glycogen reserves in trained rats, in rested state [8] and enhance mitochondrial biogenesis [9].

The mixture of BCAAs comprises three of the nine essential amino acids present in animal food sources (e.g., milk proteins). Of the three BCAAs, isoleucine and valine are glucogenic, and leucine is ketogenic [10]. More than 50% of BCAAs are catabolized in muscle; in liver this capacity is relatively small [10,11].

Muscle is not a gluconeogenic tissue; therefore, if valine and isoleucine are to be converted to glucose, they cannot be completely oxidized in this tissue [12]. However leucine appears to regulate oxidative use of glucose by skeletal muscle through stimulation of glucose recycling via the glucose-alanine cycle [13]. This occurs through the transamination process, which involves the reversible transfer of the amino group to α-ketoglutarate, generating glutamate, by the enzyme branched-chain amino transferase. Glutamate is then transaminated by pyruvate amino transferase, which transfers the amino group from glutamate to pyruvate generating alanine. Then, alanine is metabolized out of muscle tissue (liver) to produce glucose (Fig. 1) [11,12]. These mechanisms produce protein sparing and contribute to a stable glucose environment with low insulin responses during energy-restricted periods [14,15]. As an insulin secretagogue, leucine can modulate glucose uptake through this hormonal response; furthermore, leucine is strongly involved in the protein translation initiation pathway, which is also related with glucose homeostasis through Glut4 translocation to the sarcolemma. Thus, leucine can modulate glucose metabolism through insulin-dependent and insulin-independent pathways [16] (Fig. 1).

During the first minutes of exercise, there is an accumulation of tricarboxylic acid cycle intermediates (TCAIs) in muscle, which could be attributed to a few mechanisms, such as the reactions catalyzed by alanine aminotransferase (ALT); the TCAIs that augment the most during the first minute of exercise are succinate, fumarate, and malate ([17,18]. In prolonged exercise, the main evidence supporting the hypothesis linking muscle TCAIs pool alterations with glycogen utilization is that, in exhaustion, TCAIs levels are lower than in the first minutes of exercise [17,19]. In a deprived glycogen situation, in addition to a mixture of BCAAs supplementation, it has been hypothesized that α-ketoglutarate would be drained off tricarboxylic acids cycle to oxidize BCAAs, which is named “the carbon drain hypothesis” [12]. Some studies have demonstrated that muscle TCAIs pool is not necessarily linked to aerobic energy provision mechanisms [17,20]. However, to address this question, few studies focused on performance during intense exercise on glycogen depletion comparing leucine supplementation against a mixture of BCAAs supplement in trained condition.

In a double-blind crossover design, our group recently demonstrated that healthy humans supplemented with the mixture of BCAAs (300 mg·kg\(^{-1}\)·d\(^{-1}\)) for 3 d and submitted to an exercise–induced glycogen depletion on day 2 of supplementation presented a greater resistance to fatigue, reduced respiratory exchange ratio (RER), and higher plasma glucose during an exhaustive exercise test performed on the third [21]. However, muscle measurements (i.e., enzymatic activity) and its relation with exercise performance were not investigated [21]. One study [7] supplemented BCAAs (both 300 and 450 mg·kg\(^{-1}\)·d\(^{-1}\)) in trained rats to investigate resistance to fatigue in animals performing moderate exercise for more than 100 min. The results found in this study indicate that the lower BCAAs dose supplementation (BCAAs 300 mg·kg\(^{-1}\)·d\(^{-1}\)) improved performance related to peripheral fatigue compared with control and higher BCAAs supplementation groups. The latter (BCAAs 450 mg·kg\(^{-1}\)·d\(^{-1}\)) featured an impaired resistance to fatigue compared with the two other groups; however, this study did not verify either TCAIs concentrations or liver enzymatic activity. Of note, the animals in this study were not glycogen depleted before exercise.

The present study aimed to evaluate effects of the use of supplementation with leucine or a mixture of BCAAs in trained rats submitted to an exercise-induced protocol of glycogen depletion. Furthermore, we attempted to investigate muscle and liver biochemical parameters that were not performed in the previous study in order to elucidate the role of BCAAs in glycogen depletion. Our hypothesis is that the mixture of BCAAs supplementation would impair performance because they would be used mostly in muscle to yield acetyl-CoA, reducing the activity of the glucose-alanine cycle and thus gluconeogenesis in liver from amino acids, whereas leucine alone could feature the opposite effect in this experimental model.

### Materials and methods

#### Animals

The Ethics Committee of the Institute of Biology—State University of Campinas (UNICAMP) approved the Experimental Protocol. Twenty-four male Wistar SPF rats (90 g), aged 4 wk, provided by the Central Biotechnology of the State University of Campinas (CEMIB - UNICAMP. Campinas, Brazil) were kept in our facility until...
they were 8 wk old (250 g, adult). They were housed (four animals per cage) under controlled environmental conditions (22 °C, 12-h dark period starting at 0700) and fed with appropriate diet (NUVILAB CR1, NUVITAL, Curitiba, Brazil), containing approximately 52% carbohydrates, 21% protein, and 24% lipids. Water and food were provided freely. The animals were treated and exercised always in the dark phase of the 12-h cycle, and infrared lights were used in the facility, so that they would not be bothered by ultraviolet light.

Experimental design

Randomization
After the animals reached the age of 8 wk, they were randomly divided into three experimental groups (n = 8), as described in Figure 2.

Exercise training protocol

After randomization, they started the exercise program in a swimming device specially designed for rats [22] as described in Table 1, always between 1400 and 1700. This training protocol was published previously [8]. After 6 wk of training, the rats were able to swim for 1 h with a 5% of their body weight load attached to their bodies, which corresponded to a moderate intensity exercise [23]; afterward, a lactate threshold test was performed, as described below, to establish the intensity of the final sub maximal exercise test (Fig. 2).

Lactate threshold test

In order to precisely adjust the exercise intensity of the final performance experiment, after 6 wk of physical training, the rats were submitted to an intermittent, progressive swimming test to determine lactate threshold in the beginning of week 7 of the experiment (Fig. 2). This test consisted on determinations of lactate blood, by puncture of the tail vein, either before exercise, or after a 3-min exercise bout with a determined lead load attached to its body (Table 1), until exhaustion, which was defined as a period of 10 sec without emerging their noses out of the water. Tail-vein blood was collected by capillary; NaF 2% solution was used to prevent coagulation, and the samples were read immediately in a Yellow Springs Lactometer (ES 800). Blood lactate levels were plotted into curves and lactate threshold was determined at their inflexion points as described previously [24] (data not shown).

Supplementation protocol

One day after the lactate threshold test and still during week 7 of the experiment, the supplementation protocol began. Animals were supplemented orally by intragastric gavage throughout the following 7 d with either the mixture of BCAAs, leucine, or placebo (Fig. 2). 166 mg of the corresponding amino acids/kg daily diluted in 2 mL distilled water. This dose has been determined based on the calculations of leucine flux in humans as 88 mg/g daily [25] and BCAAs oxidation as 144 mg/g daily [26]. In order to make an isonitrogenous supplementation between the LEU and BCAAs groups, the amount of 166 kg LEU/kg, which corresponded to two times the determined LEU flux dose, as described above, was applied and equalized in the BCAAs group. All amino acids were provided by Ajinomoto (Tokyo, Japan).

Liver glycogen depletion protocol

On day 6 of supplementation, rats were kept fasted after their swimming session, until complete 24 h before the final experiment, in order to deplete liver glycogen. This protocol of glycogen depletion has been previously determined on a pilot study from our group.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Swimming training protocol</strong></td>
</tr>
<tr>
<td><strong>Week of training</strong></td>
</tr>
<tr>
<td>Adaptation</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>

BW, body weight

Submaximal performance test

On day 7 of supplementation, and already on week 8 of the experiment (Fig. 1), rats were submitted to a more intense swimming protocol [24]: They swam gently for the first 10 min, then a 7% or 8% of body weight load was attached to their bodies, according to the animal’s lactate threshold test, until they achieved two load level increments than its lactate curve’s inflexion point, previously determined by lactate threshold test to characterize a submaximal exercise. They swam until exhaustion (defined as the moment where the animal was not able to maintain its nose nose out of water for more than 10 sec), when they were sacrificed by decapitation. Muscles gastrocnemius and liver were depicted and rapidly frozen in liquid nitrogen, until further analysis.

Muscle and liver glycogen concentration

Radioactive isotopes and all the enzymes were purchased from Sigma Co. (St. Louis – MO). Kits for enzyme activity determinations, when used, were obtained from Bioclin (QUIBASA, Belo Horizonte, Brazil). Muscle and liver glycogen were determined according to the method described elsewhere [24]: 1-g piece of frozen muscle was removed from each sample. Tissues were heated in 6N KOH solution and then submitted to glycogen extraction with ethanol 70% solution. Glycogen was then hydrolyzed to glucose with a sulfuric acid plus anthrone solution, which produced a green color. Colorimetric determinations were made at 650 nm, in a Perkin-Elmer Lambda 25 Spectrophotometer.

Muscle and liver glycogen degradation ratio

Muscle and liver glycogen degradation ratio were determined according to a previous study [24] where tissue glycogen concentration (mg/100 mg wet tissue) were plotted against time to fatigue (min) in the submaximal swimming test described previously.

Muscle TCAs

All muscle tissue preparation and TCAs were performed according to a previous study [27]. A 1-g piece of frozen muscle was removed from each sample. The samples were diluted in 10 mL of 0.6 M perchloric acid buffer. They were homogenated gently with a pestle. The homogenates were centrifuged at 10 000g, 10 °C, and a sonicator was used to improve tissue homogenization. Supernatants were separated, neutralized with 60 μL TRIS/KOH buffer and 10μL of Universal Indicator, so that a green color was formed. Homogenates were frozen in liquid nitrogen until further analysis. They were assayed enzymatically for α-ketoglutarate, citrate, malate, 2-oxoglutarate, by spectrophotometry, and fumarate was determined using a fluorometric assay (Hitachi F-2000 fluorescence spectrophotometer, Hitachi Instruments, Japan) [17,27]. Gastrocnemius muscle homogenate neutralized on basic pH (20 ul) was added to 160 ul hydrazine hydrate buffer until the final volume of 100 mL and maintained in freezer. Additionally, 20 mL EDTA were added (0.1 mol/L) and 20 mL reduced acetylated pyruvateamine-dinitrochloride (0.1 mol/L). The samples were analyzed in the primary wavelength of 360 nm and secondary wavelength of 440 nm. Afterward, malate dehydrogenase (MDH) (EC 1.1.1.37) was added (500 U) in a final concentration of 1ul MDH to 5mL TRIS buffer (0.02 mol/L) plus 0.02% bovine serum albumin to determine malate concentration. A 20-min pause was performed until the next analysis in room temperature. Then fumarase (EC 4.2.1.2) was added (1μk/L) in a concentration on 10 μL enzyme to 9.990 mL TRIS buffer (0.02 mol/L) to determine fumarate levels. Another 20-min pause was performed before the second fluorimetric analysis in room temperature. Standard curve was made with fumarate standard solution (1 mol/L) and a solution of perchloric acid, KHCO3 and distilled water was used as a blank.

Enzymes activity

Maximum citrate synthase activity (EC 4.1.3.7) was determined as described previously [28], which is based on reaction between oxaloacetate and acetyl-CoA, forming citrate and CoA. Dithiobisoozonic acid (DTNB), when reacting with CoA, yields a yellow-colored complex whose formation is proportional to the enzyme’s activity. Tissues were added to a TRIS-HCl 50 mM buffer and EDTA1 mM, pH 7.4. Assay buffer was the following: TRIS-HCl 100 mM, DTNB 0.4 mM, TRITON X-100 0.05%, and acetyl-CoA 15 mM; the substrate added was oxaloacetate, which is based on reaction between oxaloacetate and acetyl-CoA, forming both glutamate and oxaloacetate. The latter reacts with nicotinamide adenine dinucleotide hydrogen (NADH), in the presence of MDH, forming malate,
and NADH is oxidized to NAD⁺. ALT catalyzes the transfer of alanine amine group to α-keto-glutarate, forming pyruvate and glutamate. Pyruvate, in the presence of lactate dehydrogenase, reacts with NADH, forming lactate and NAD⁺ oxidizes to NAD⁺. The speed of these two reactions is measured in spectrophotometer at 340 nm and is proportional to AST and ALT activity in the samples, respectively.

Branched-chain transaminase (BCAT) (EC 2.6.1.42) was isotopically assayed as described previously [30], modified. The assay was performed at 37°C and the final volume was set to 400 μL; the assay medium contained 154 mM manitol, 49 mM sucrose, 78 mM HEPES buffer, pH 7.4 at 37°C, 25 mM potassium dihydrogen phosphate, 8 mM sodium carbonate, 2 mM MgCl₂, 1.1 mM EGTA. Nonidet P-40, pyridoxal 5-phosphate, and NAD⁺ were added to the assay medium up to the final concentration of 8 mM. Reaction was interrupted with 0.1 mL 4M sulfuric acid. 0.5 mL of 0.2M eric sulphate in 2 M H₂SO₄ was used to decarboxylate α-keto[14C]isocaproate produced during the incubation. All assays were run in duplicate and they featured linear correlation with time and amount of enzyme. Radioactive carbon dioxide was collected in 1 mL methylcellulose-ethanolamine mixture (2:1, v/v) and determined by liquid scintillation.

Statistical analysis
Values are presented as means ± SD. Statistical evaluation was performed by one-way ANOVA to analyze the effects of supplementation type. When a significant overall effect was detected, differences among statistical means were assessed with Tukey post hoc test. The level of significance was set at P < 0.05 for all statistical tests. All statistical analysis were performed using the software SPSS 20.0 for Windows (IBM SPSS Statistics version 17.0 for Windows, Somers , NY).

Results
Our data demonstrated that liver glycogen degradation ratio was significantly higher in the mixture of BCAAs group compared with the PLA group (Fig. 3A; PLA 3.6 ± 2.5 μg/100 mg/min versus the mixture of BCAAs 54.9 ± 28.8 μg/100 mg/min; P < 0.001). However, the LEU group presented decreased liver glycogen degradation ratio compared to the mixture of BCAAs group (Fig. 3A; LEU 25.8 ± 11.4 μg/100 mg/min versus the mixture of BCAAs group; P < 0.05). Liver glycogen content was significantly spared in the mixture of BCAAs and LEU groups compared with the PLA group (Fig. 3B; The mixture of BCAAs 0.19 ± 0.10 μg/100 mg versus PLA 0.02 ± 0.17 μg/100 mg/min; P < 0.05; LEU 0.23 ± 0.09 μg/100 mg versus PLA group; P < 0.01).

The muscle glycogen degradation ratio was significantly suppressed in the LEU group compared with both PLA and the mixture of BCAAs groups (Fig. 3C; LEU 7.9 ± 3.2 μg/100 mg/min versus PLA 23.6 ± 4.6 μg/100 mg/min; P < 0.05; LEU group versus the mixture of BCAAs 29.07 ± 5.16 μg/100 mg/min, P < 0.01). However, muscle glycogen content was significantly reduced in the LEU group compared with the PLA group (Fig. 3D; LEU 0.08 ± 0.02 μg/100 mg versus PLA 0.18 ± 0.03 μg/100 mg; P < 0.05). In the mixture of BCAAs group, muscle glycogen did not differ compared with the others (Fig. 3D; the mixture of BCAAs 0.11 ± 0.02 μg/100 mg; P > 0.05).

Muscle citrate content was significantly higher in the mixture of BCAAs group compared with the PLA group (Fig. 4A; the mixture of BCAAs 9.8 ± 1.0 μmol/mg versus PLA 3.5 ± 0.6 μmol/mg; P < 0.001). Fumarate, oxaloacetate, and 2-oxo-glutarate content in muscle did not differ among groups (Fig. 4A; P > 0.05). Muscle malate content was significantly elevated in the mixture of BCAAs group compared with both the PLA (Fig. 4A; the mixture of BCAAs 64.8 ± 59.5 μmol/mg versus PLA 29.2 ± 46.0 μmol/mg; P < 0.001) and LEU groups (Fig. 4A; the mixture of BCAAs group versus LEU 317.4 ± 43.0 μmol/mg; P < 0.001).

Use of muscle citrate was significantly increased in the mixture of BCAAs group compared with both the PLA and LEU groups (Fig. 4B; the mixture of BCAAs 0.52 ± 0.13 nmol·mg·min⁻¹ versus PLA 0.08 ± 0.03 nmol·mg·min⁻¹ and LEU 0.09 ± 0.05 nmol·mg·min⁻¹; P < 0.01). This same result pattern was observed in α-keto-glutarate (Fig. 4C; the mixture of BCAAs 0.09 ± 0.03 nmol·mg·min⁻¹ versus PLA 0.01 ± 0.003 nmol·mg·min⁻¹ and LEU 0.009 ± 0.005 nmol·mg·min⁻¹; P < 0.05) and malate utilization (Fig. 4E; the mixture of BCAAs 12.41 ± 2879 nmol·mg·min⁻¹ versus PLA 4076 ± 8651 nmol·mg·min⁻¹ and LEU 2585 ± 1079 nmol·mg·min⁻¹; P < 0.05 and P < 0.01, respectively). Oxaloacetate utilization was statistically different in the mixture of BCAAs group only when compared...
with the PLA group (Fig. 4F); the mixture of BCAAs 0.60 ± 0.19 nmol·mg·min⁻¹ versus PLA 0.06 ± 0.01 nmol·mg·min⁻¹; *P < 0.05). Fumarate utilization was not significantly different among the three groups (Fig. 4D; *P > 0.05).

Muscle citrate synthase (EC 4.1.3.7) activity, AST (EC 2.6.1.1), and ALT (EC 2.6.1.2) concentration did not differ among groups (Fig. 4G; *P > 0.05). However, the mixture of BCAAs supplementation significantly decreased branched-chain amino acids transaminase (EC 2.1.6.42) activity when compared with both PLA (Fig. 4G; the mixture of BCAAs 28.0 ± 4.0 nmol·mg·min⁻¹ versus PLA 39.7 ± 6.0, nmol·mg·min⁻¹; *P < 0.001) and the LEU group (Fig. 4B; the mixture of BCAAs group 28.0 ± 4.0 nmol·mg·min⁻¹ versus LEU 40.0 ± 3.0 nmol·mg·min⁻¹; †P < 0.001).

The performance test demonstrated that LEU supplementation enhanced resistance to exhaustion when compared with the mixture of BCAAs (Fig. 5; LEU 9.4 ± 0.9 min versus the mixture of BCAAs 3.6 ± 0.1 min; *P < 0.001), however, no difference was found when LEU supplementation was compared with PLA (Fig. 5; LEU 9.4 ± 0.9 min versus PLA 8.4 ± 1.2 min; *P > 0.05).

Discussion

To our knowledge, this is one of the few studies comparing the mixture of BCAAs effects against leucine supplementation in low-carbohydrate availability, and one of the few showing that leucine alone can prevent impaired endurance exercise performance compared with the mixture of BCAAs in glycogen depletion.

As stated previously, liver glycogen is fundamental to glucose homeostasis [31]. In our results, leucine supplementation had no improving effect on absolute liver glycogen content compared with the mixture of BCAAs or placebo, as expected, because all the animals were fasted and exhausted. However, we observed that leucine supplementation decreased both liver and muscle glycogen degradation and improved performance. The hepatic glycogen in the leucine group data is in accordance with other studies indicating that supplementation of some amino acids preserves liver glycogen [6,7,24]. The following results lead to the initial hypothesis that the main effect of leucine is improving muscle glucose availability because leucine has important effects to glucose homeostasis [14]. According to a previous study [13], increased serum leucine concentration and its metabolite alpha-ketoisocaprate (α-KIC) inhibit branched-chain keto acids dehydrogenase complex (BCKD) kinase and hence stimulate BCKD dephosphorylation (activation), enhancing the oxidation of other essential amino acids (e.g., isoleucine and valine), which could contribute indirectly to muscle and liver glycogen sparing. Also, amino acids act as primary sources of carbon for hepatic gluconeogenesis [32] and when intracellular levels of leucine are elevated, a decrease in pyruvate dehydrogenase (PHD)
It is possible to metabolize more rapidly BCKA from the portal veins, whereas in the liver, BCKD activity is almost inactivated in order to spare BCAAs for protein synthesis [34].

In conclusion, in the present experimental conditions, the mixture of BCAAs and LEU supplementation in glycogen-depleted rats and before intense physical activity featured distinct effects on performance: LEU supplementation improved performance compared with the mixture of BCAAs supplementation, sparing muscle glycogen stores. Taken together, these data suggest that enhancing the glucose alanine cycle in liver through leucine supplementation might have an interesting use in physical performance in prolonged or submaximal exercise, where muscle glycogen stores are more likely to be depleted.

**Acknowledgments**

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for supporting their studies.

This study is dedicated to Professor Dr. Luis Fernando Bicudo Pereira Costa Rosa (in memoriam), Institute of Biomedical Sciences, University of São Paulo, Brazil.

**References**


Layman DK. The role of leucine in weight loss diets and glucose homeostasis. J Nutr 2003;133:2615–7S.


Mortzakis M, Graham TE, Gonzalez-Alonso J, Saltin B. Glutamate availability is important in intramuscular amino acid metabolism and TCA cycle intermediates but does not affect peak oxidative metabolism. J Appl Physiol 2008;105:547–54.


Dekota S, Layman DK. Increased ratio of dietary carbohydrate to protein shifts the focus of metabolic signaling from skeletal muscle to adipose. Nutr Metab (Lond) 2011;8:13.