Nutrition 65 (2019) 131–137

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Effects of glutamine and alanine supplementation on muscle fatigue parameters of rats submitted to resistance training

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ARTICLE INFO

Article History:
Received 24 July 2018
Accepted 29 September 2018

Keywords:
L-alanyl-L-glutamine
Amino acids
Physical exercise
Peripheral fatigue
Muscle damage
Oxidative stress
Glycogen

ABSTRACT

Objective: Although glutamine and alanine have properties that could delay fatigue, recent evidence showed that these amino acids impaired central fatigue markers. Nevertheless, the effect of this intervention on muscle fatigue is unknown. The aim of this study was to investigate the effects of glutamine and alanine supplementation on muscle fatigue parameters in rats submitted to resistance training (RT).
Methods: Wistar rats were distributed into the following groups: sedentary (SED), exercised (CON), exercised and supplemented with alanine (ALA), glutamine and alanine in their free form (G+A) or L-alanyl-L-glutamine (DIP). Trained groups underwent a ladder-climbing exercise for 8 wk. In the last 3 wk of RT, supplementations were offered in water with a 4% concentration.
Results: G+A and DIP supplementation increased the muscle content of glutamine and glutamate. DIP administration increased glycogen and lactate dehydrogenase (LDH) concentrations in muscle, whereas ALA and G+A supplementation reduced plasma LDH and creatine kinase levels. All trained groups presented higher levels of muscle glutathione (GSH) than SED. There was no difference between groups in lactate, xanthine, hypoxanthine, thiobarbituric acid reactive substances, 8-isoprostane and GSH in plasma; adenosine monophosphate deaminase, citrate synthase and monocarboxylate transporters 1 and 4 in muscle; and glycogen and GSH in the liver. Moreover, physical performance did not differ between groups.
Conclusion: Glutamine and alanine supplementation improved muscle fatigue markers without affecting exercise performance.

Introduction

Muscle fatigue designates biochemical changes occurring within the skeletal muscle cell, which reduce strength and power, impairing physical performance [1,2]. One of the main causes of this phenomenon is the reduction of energy substrates for the continuity of the exercise, evidenced by increased concentrations of enzymes and metabolites from the adenosine triphosphate (ATP) metabolism, such as adenosine monophosphate deaminase (AMPD), xanthine, and hypoxanthine, and by the decrease of energy stores, such as glycogen. Moreover, oxidative stress and muscle damage are considered important causes of fatigue and performance impairment [1,3–5]. Although fatigue is reversible and attenuated after rest [6], the development of this state during exercise reduces muscle activity; therefore, nutritional strategies are applied to delay the onset of fatigue and to improve athletic performance [2].

Glutamine and alanine could attenuate fatigue markers as these amino acids are the most glycogenic in humans and animals, having a significant influence on the anaplerosis of the Krebs cycle and gluconeogenesis [7–10]. Additionally, glutamine activates the enzyme glycogen synthase, stimulating glycogen synthesis [7]. Glutamine also could attenuate muscle damage through its immunomodulatory and anti-inflammatory functions, in addition to improving body antioxidant defenses by increasing glutathione (GSH) synthesis [3,11–14]. Despite these promising roles, we recently observed that glutamine and alanine supplementation impaired central fatigue parameters such as serotonin

The present study was supported by grants from the São Paulo Research Foundation (FAPESP, projects 2016/04910-0 and 2016/22789-3), the Brazilian National Council for Scientific and Technological Development (CNPq), and the Coordination for the Improvement of Higher Education Personnel (CAPES).

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https://doi.org/10.1016/j.nut.2018.09.025
0899-9007/

concentration and the serotonin/dopamine ratio in the hypothalamus of rats submitted to resistance training (RT) [15]. However, the effects of these amino acids on muscle fatigue markers are still unclear in the literature.

Glutamine supplementation is popular among athletes; nevertheless, the efficacy of its administration in free form is controversial in the literature [16–19]. Studies in our laboratory showed that dipeptide L-alanyl-L-glutamine supplementation was more effective in increasing glutamine in plasma and tissues than free glutamine administration [20], and that a solution containing glutamine and alanine in their free forms presented similar effects of L-alanyl-L-glutamine in increasing body glutamine concentrations [3,11]. Notwithstanding, we observed that alanine supplementation alone also increased plasma and tissue glutamine, albeit supplements containing both glutamine and alanine were better at improving immune parameters [12,13]. Nonetheless, the role of alanine, directly or indirectly (by increasing body glutamine concentrations) in muscle fatigue markers is unknown.

Thus, the aim of the present study was to evaluate the effects of glutamine and alanine supplementation, in their free or conjugated form, on muscle fatigue parameters of rats submitted to progressive RT. We hypothesized that these amino acids could be used as energy substrates during exercise, avoiding glycogen depletion, and could also attenuate oxidative stress and muscle damage induced by RT. Finally, we hypothesized that the improvement of these parameters could delay fatigue and increase physical performance.

Material and methods

Animals

This study began with 40 adult male Wistar rats, ages 60 d, weighing ~300 g, and obtained from the Animal House of the University of São Paulo. During the experimental period, one animal from the group exercised and supplemented with alanine died; thus, the study was conducted with the 39 remaining rats. The total experimental period was 9 wk, including 1 wk of adaptation of animals to the cages and laboratory conditions. Throughout the experimental period, rats were housed two per cage in a controlled environment at 22°C ± 2°C and relative air humidity of 55% ± 10%, under a 12-h light/12-h dark cycle (lights on at 1800).

Animals were distributed into five groups: sedentary and without supplementation (SED: n = 8), exercised and without supplementation (CON: n = 8), exercised and supplemented with L-alanine (ALA: n = 7), exercised and supplemented with L-glutamine and L-alanine in their free forms (G+A: n = 8), and exercised and supplemented with the dipeptide L-alanyl- L-glutamine (DIP: n = 8).

Rats had free access to water and were fed ad libitum with standard chow for adult rats (AIN-93 M), composed of 22% protein (NUVILAB CR1, Nuvital Nutrients, Curitiba, Brazil), according to the American Institute of Nutrition [21]. Food consumption, fluid intake, and body weight (BW) were registered three times per week. All procedures were approved by the Ethics Committee on Animal Use of the University of São Paulo (protocol: CEUA/FCF/532).

Resistance training

RT protocol was adapted from Scheffer et al. [22], Raizel et al. [12] and Leite et al. [13], originally published by Hornberger and Farrar [23], and consisted of climbing a vertical ladder (1.1 × 0.18 m, 2 cm grid, 80-degree inclined) with a load affixed to the base of the rat's tail, using plastic insulation tape, for 8 wk. Each set consisted of eight climbs and rests of 2 min between sets.

In the adaptation period, animals repeated only one set, for 2 wk, with a load equal to 5% of BW. After this period, RT was conducted with three sets carrying 25% of BW; four sets with 50% of BW; five sets with 75% of BW, and, finally, animals performed six sets carrying 100% of BW. The progression of loads and sets occurred every 1.5 wk. Animals were trained three times per week (commencing from 0800), during the dark cycle, and with 48 h of rest between sessions. RT protocol and experimental design are presented in Figure 1.

Tests of maximum carrying capacity

Three maximum carrying capacity (MCC) tests were performed during the experimental period—after adaptation, during the load of 50% of BW, and before euthanasia. The test consisted of a first climb with 75% of BW and an addition of 15% of BW for each climb until exhaustion (MCC), with 2-min rest between climbs [24].

Supplementation

Supplements were given chronically in the last 3 wk of the experiment (the most exhaustive period of the RT) [3,14,17,20], diluted to 4% in drinking water, and provided ad libitum. Administration in water was selected in an attempt to increase the frequency of amino acid intake throughout the day and to avoid the stress of manipulation in oral gavages [12,13,15,25].

The amino acid amount was calculated based on commercial dipeptide concentration (Dipeptiven solution consists of 20 g of L-alanyl-L-glutamine dissolved in 100 mL of water, which equals 8.21 g of L-alanine and 13.46 g of L-glutamine). Free L-glutamine and free L-alanine were manufactured by Labsynth (Synth, São Paulo, SP, Brazil), and L-alanyl-L-glutamine was manufactured by Fresenius Kabi S. A (Bad Homburg, HE, Germany). Supplements were offered until the day before euthanasia.

Plasma parameters

Lactate was determined before and after training in the fourth exercise session of each load: 25%, 50%, 75%, and 100% of BW. Samples were collected from the tail vein into tubes containing a sodium fluoride solution (17 mg/mL) and assayed by enzymatic kit (Labtest, Lagoa Santa, Brazil).

Rats were fasted 1 h before the last RT to reduce the influence of food consumption on energy metabolism parameters, such as liver and muscle glycogen. One hour after RT, animals were anesthetized with xylazine hydrochloride (20 mg/mL) and ketamine hydrochloride (70 mg/mL) and then sacrificed by decapitation. Blood was centrifuged for plasma separation, and samples were stored at −80 °C for further analyses.

Glutamine, glutamate, and alanine were measured through the high-performance liquid chromatography method by the CBO company (Análises Laboratoriais Ltda., Valinhos, Brazil). Xanthine, hypoxanthine, and GSH were measured by colorimetric kits (Sigma-Aldrich, St. Louis, MO, USA). Creatine kinase (CK), lactate dehydrogenase (LHD) (Labtest, Lagoa Santa, Brazil), and 8-isoprostane (Cayman Chemical, Ann Arbor, MI, USA) were measured by enzymatic kits. Thiobarbituric acid reactive substances (TBARS) were measured by fluorometric kit (Cayman Chemical).

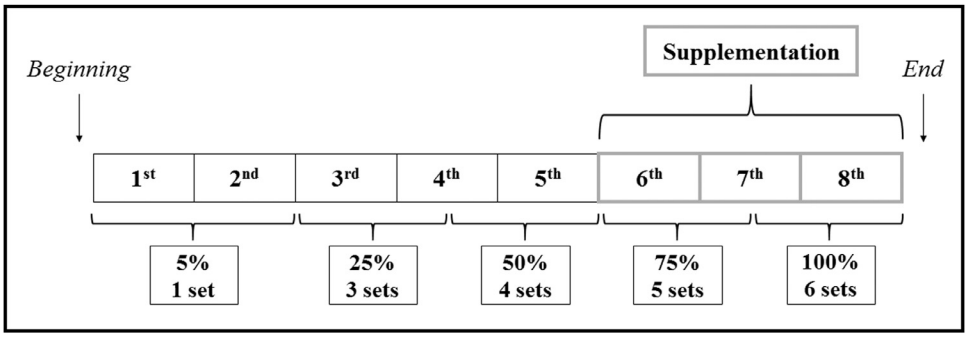

Fig. 1. Resistance training protocol and experimental design.

Tissue measurements

Liver, heart, and the skeletal muscles (extensor digitorum longus [EDL], tibialis anterior, and soleus) were surgically excised after the euthanasia, weighed and immediately frozen in liquid nitrogen. Tissue samples were stored at −80°C for subsequent analyses. Amino acids (glutamine, glutamate, and alanine) were assayed in tibialis anterior muscle by enzymatic kits (Sigma-Aldrich). Glycogen in liver and muscle (soleus) was measured according to Hassid [26]. GSH was measured in liver, heart, and tibialis anterior muscle by enzymatic kit (Sigma-Aldrich).

Western blot analysis

Tibialis anterior and EDL muscles were homogenized with radioimmunoprecipitation buffer, which contains protease and phosphatase inhibitors. Tissue lysates were combined with sample buffer containing 240 mM-Tris (pH 6.8), 40% glycerol, 0.8% sodium dodecyl sulfate (SDS), 200 mM-β-mercaptoethanol, and 0.02% bromophenol blue. Amounts of protein (40 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK).

Membranes were blocked with 0.5% of bovine serum albumin (BSA; Sigma-Aldrich), that was diluted in phosphate-buffered saline with Tween (PBST) for 1 h. After this procedure, membranes were washed with PBST and incubated at 4°C with gentle shaking overnight with primary antibody AMPD1 (1:1000 Abcam Discover More, Cambridge, UK), LDHA (1:1000 Cell Signaling Technology, Beverly, MA, USA), citrate synthase (1:1000 Abcam Discover More, Cambridge, UK) or monocarboxylate transporters (MCT)-1 and MCT-4 (1:1000 Santa Cruz Biotechnology, Dallas, TX, USA). After three washes, membranes were incubated for 1 h with peroxidase-labeled antirabbit immunoglobulin G antibodies (Cell Signaling Technology) diluted 1:5000 in PBST and 2% BSA.

Blots were visualized on ImageQuant 400 (GE Healthcare, Little Chalfont, UK) with 100 μL of each solution (A and B) of ECL-Advance Western Blotting Reagent (GE Healthcare, Little Chalfont, UK). Monoclonal anti-β-actin-peroxidase antibody (Sigma-Aldrich) at a ratio of 1:25000 was used for gel-loading control and protein normalization. The densitometric analysis of the Western blot was performed by the protein level average, normalized to β-actin.

Statistical analysis

Normality was measured by the Shapiro–Wilk test. Comparisons between groups were carried out by one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference as a post hoc test. Analyses over multiple time points were performed with two-way ANOVA with Tukey's Honest Significant Difference as a post hoc test. A significance level of 5% was applied for all comparisons. Data were analyzed using GraphPad Prism software, version 6.0 (GraphPad Software, San Diego, CA, USA), and expressed as means + standard deviation (SD).

Results

Food consumption, fluid intake, and body weight gain

Food consumption was higher in SED animals than in the exercised groups, especially ALA and DIP ($P = 0.019$). Supplemented groups ingested more fluid than SED and CON, especially the animals in the G+A group ($P < 0.001$). As expected, BW gain of SED animals was higher by ~33% compared with the trained groups ($P = 0.015$; Table 1).

Plasma and muscle concentrations of glutamine, glutamate, and alanine

Although supplemented groups ingested more fluid, there was no statistically significant difference in plasma glutamine, glutamate, and alanine between animals. Nevertheless, glutamine-containing supplements (G+A and DIP) increased muscle glutamine concentrations by 34% and 43%, respectively, compared with the SED, CON and ALA groups ($P < 0.001$). Similar results were obtained in muscle glutamate because the G+A and DIP groups presented higher concentrations (13% and 17%, respectively) than SED, CON, and ALA animals ($P = 0.001$). Interestingly, RT and G+A supplementation increased muscle alanine; however, contrary to expectations, the administration of free alanine did not increase its muscular content ($P = 0.012$; Table 2).

Plasma lactate and MCTs

Plasma lactate was slightly increased after exercise compared with a pretraining period, although without a statistically significant difference in almost all loads. There was no difference between groups, indicating that amino acid supplementation did not affect this parameter (Table 3).

Corroborating the results of plasma lactate, there was no difference between groups in the muscle concentrations of the lactate transporters—MCT-1 in EDL ($P = 0.925$; Fig. 2A) and MCT-4 in tibialis ($P = 0.805$; Fig. 2B).

Energy metabolism parameters

There was no difference between groups in the ATP metabolism parameters, such as muscle concentrations of AMPD ($P = 0.847$; Fig. 3A) and plasma concentrations of hypoxanthine and xanthine ($P = 0.347$; Table 4). Nonetheless, the DIP group presented higher muscle concentrations of lactate dehydrogenase (LDH) than the SED, CON, and ALA groups ($P = 0.015$; Fig. 3C). Neither RT nor the glutamine and alanine supplementation affected the muscle concentrations of citrate synthase (CS; $P = 0.871$; Fig. 3B).

RT reduced the liver glycogen content by 52% (SED versus trained groups), although without a statistically significant difference ($P = 0.086$). Similarly, muscle glycogen concentrations of the CON group were lower (~22%) than SED animals, whereas all supplementations prevented the reduction of muscle glycogen induced by RT, and DIP administration increased this parameter by 72% (DIP versus CON; $P = 0.039$; Table 4).

Oxidative stress and muscle damage parameters

Herein, neither RT nor amino acid supplementation affected the oxidative markers plasma TBARS and 8-isoprostane levels. Interestingly, interventions did not affect plasma, liver, and heart GSH, but all trained groups presented higher levels of muscle GSH compared with the SED group ($P = 0.003$; Table 4).

RT also increased (~90%) plasma LDH concentrations (SED versus CON), indicating muscle damage. Supplemented animals presented lower levels of plasma LDH, especially those supplemented with free amino acids. Compared with the CON group, ALA and G+A animals presented lower plasma LDH by 38% and 43%,

Table 1
Food consumption, fluid intake and body weight gain

	SED	CON	ALA	G+A	DIP	*P*-value
Food consumption (kg)	1.52 ± 0.14^{a}	$1.38 \pm 0.06^{a,b}$	1.36 ± 0.10^{b}	$1.39 \pm 0.09^{a,b}$	1.36 ± 0.07^{b}	0.019
Fluid intake (L)	2.16 ± 0.22^{a}	$2.36 \pm 0.21^{a,b}$	$2.61 \pm 0.19^{b,c}$	2.84 ± 0.21^{c}	2.48 ± 0.12^{b}	<0.001
Body weight gain (g)	138.1 ± 29.80^{a}	99.69 ± 33.91^{b}	$114.7 \pm 19.47^{a,b}$	102.1 ± 19.15^{b}	99.08 ± 15.08^{b}	0.015

ALA, exercised and supplemented with alanine; CON, exercised group; DIP, exercised and supplemented with L-alanyl-L-glutamine; G+A, exercised and supplemented with glutamine and alanine in their free forms; SED, sedentary group.
Different letters mean statistically significant differences. Data are presented as mean ± SD.

Table 2
Plasma and muscle glutamine, glutamate and alanine

	SED	CON	ALA	G+A	DIP	*P*-value
Plasma glutamine (μmol/L)	841.3 ± 276.6	1044 ± 263.8	982.9 ± 157.1	850 ± 218.6	886.3 ± 187.7	0.327
Plasma glutamate (μmol/L)	1598 ± 213.8	1440 ± 187.2	1473 ± 220.2	1614 ± 138.5	1683 ± 156.9	0.073
Plasma alanine (μmol/L)	810 ± 155.1	751.3 ± 93.87	792.9 ± 119.4	828.8 ± 95.68	823.8 ± 138.9	0.725
Muscle glutamine (mmoles/L of tissue homogenate)	18.58 ± 1.48[a]	16.56 ± 1.41[a]	18.75 ± 2.29[a]	24.06 ± 2.86[b]	25.75 ± 2.08[b]	<0.001
Muscle glutamate (mmoles/L of tissue homogenate)	19.75 ± 2.70[a]	20.48 ± 1.7 [a,b]	21.80 ± 2.94[a,b,c]	23.33 ± 0.95[b,c]	24.16 ± 2.45[c]	0.001
Muscle alanine (nmoles/μL of tissue homogenate)	9.86 ± 5.42[a,b]	14.23 ± 3.08[a]	7.46 ± 3.99[b]	13.39 ± 1.75[a]	10.69 ± 3.44[a,b]	0.012

ALA, exercised and supplemented with alanine; CON, exercised group; DIP, exercised and supplemented with L-alanyl-L-glutamine; G+A, exercised and supplemented with glutamine and alanine in their free forms; SED, sedentary group.
Different letters mean statistically significant differences. Data are presented as mean ± SD.

Table 3
Plasma lactate before and after training

Lactate (mg/dL)	CON	ALA	G+A	DIP	*P*-value
Pretraining 25% of BW	9.22 ± 2.34	9.57 ± 1.59	9.76 ± 1.54	12.59 ± 6.17	0.213
Post-training 25% of BW	11.52 ± 3.56	12.17 ± 3.07	12.87 ± 3.32	15.07 ± 3.89	0.225
Pretraining 50% of BW	9.89 ± 3.29	9.32 ± 2.21	7.78 ± 0.60*	8.98 ± 1.94	0.303
Post-training 50% of BW	10.59 ± 2.42	11.22 ± 2.88	12.83 ± 4.42*	10.02 ± 1.71	0.303
Pretraining 75% of BW	9.74 ± 1.44	11.53 ± 2.00	10.77 ± 2.49	9.88 ± 1.64	0.278
Post-training 75% of BW	10.12 ± 1.61	10.99 ± 2.18	9.53 ± 1.10	10.76 ± 1.76	0.349
Pretraining 100% of BW	8.85 ± 1.11	9.45 ± 1.09*	9.15 ± 1.40	8.90 ± 1.40	0.792
Post-training 100% of BW	10.38 ± 2.73	12.21 ± 3.34*	9.84 ± 1.98	12.10 ± 1.76	0.212

ALA, exercised and supplemented with alanine; BW, body weight; CON, exercised group; DIP, exercised and supplemented with L-alanyl-L-glutamine; G+A, exercised and supplemented with glutamine and alanine in their free forms; SED, sedentary group.
Data are presented as mean ± SD.
*Significant difference between times—before and after training 50% (only G+A group) and 100% of BW (only ALA group).

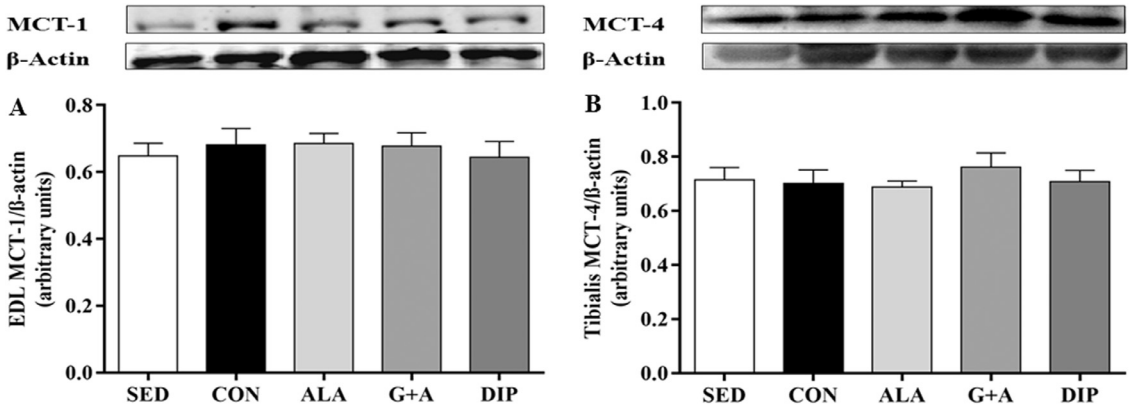

Fig. 2. Muscle concentrations of MCT-1 (extensor digitorum longus - EDL) (A) and 4 (tibialis) (B) of rats submitted to resistance training and supplementation with L-glutamine and L-alanine. ALA, exercised and supplemented with alanine; CON, exercised group; DIP, exercised and supplemented with L-alanyl-L-glutamine; EDL, extensor digitorum longus; G+A, exercised and supplemented with glutamine and alanine in their free forms; MCT, monocarboxylate transporter; SED, sedentary group. Data are presented as mean ± standard deviation.

respectively ($P = 0.001$). Similarly, plasma CK was reduced by ALA and G+A supplementations by 26% and 33%, respectively, compared with the CON group ($P = 0.001$). DIP supplementation did not affect plasma LDH and CK (Table 4).

MCC tests

In the first MCC test, animals from the CON group presented a higher weightlifting capacity than ALA and G+A animals ($P = 0.005$); however, in the second and third tests, there was no difference between groups. The performance of all groups improved in the MCC2 and MCC3 compared with MCC1, by ~22% and 36%, respectively ($P < 0.001$; Fig. 4).

The delta of MCC tests (MCC3−MCC1) showed that, during all the experimental period, the performance improvement of G+A group was almost 200% higher than the CON group ($P = 0.030$; Fig. 5).

Discussion

In the present study, we observed that trained animals had lower food consumption and BW gain than the SED group. Exhaustive physical exercise may suppress appetite, and, consequently, food intake, by increasing the release of specific cytokines, such as IL-1β and IL-6, which activate several hypothalamic nuclei associated with behavioral aspects. In addition, physical exercise increases the energy expenditure, which, associated with insufficient energy intake, results in BW reduction [19,27,28].

Supplemented animals, especially G+A, ingested more fluid than the SED and CON groups; nevertheless, there was no

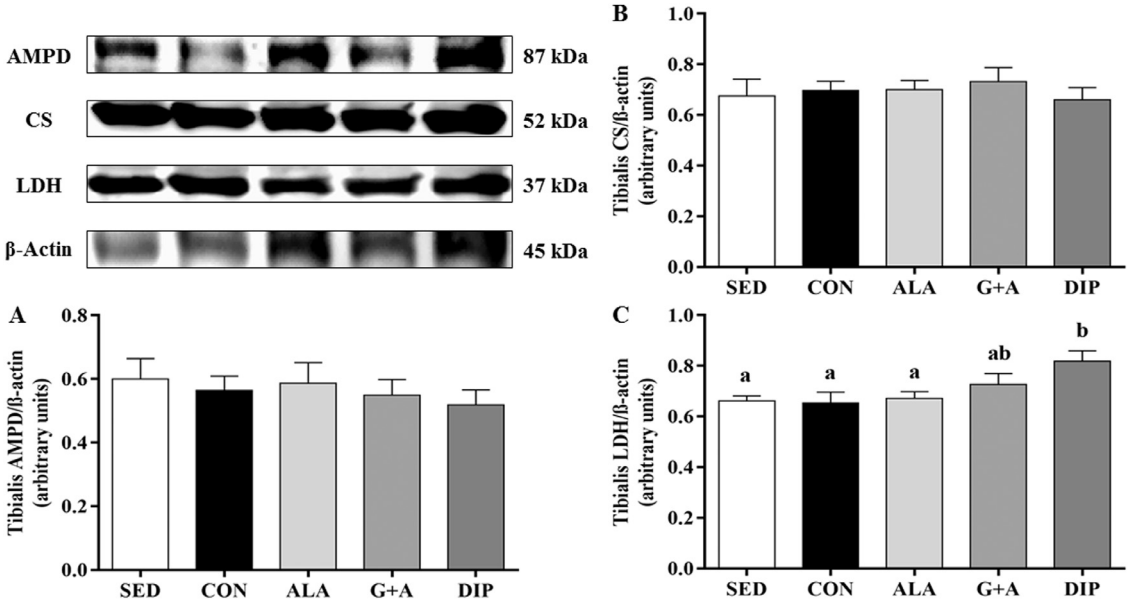

Fig. 3. Muscle concentrations of AMPD (**A**), CS (**B**) and LDH (**C**) of rats submitted to resistance training and supplementation with L-glutamine and L-alanine. ALA, exercised and supplemented with alanine; AMPD, adenosine monophosphate deaminase; CON, exercised group; CS, citrate synthase; DIP, exercised and supplemented with L-alanyl-L-glutamine; G+A, exercised and supplemented with glutamine and alanine in their free forms; LDH, lactate dehydrogenase; SED, sedentary group. Data are presented as mean ± standard deviation.

statistically significant difference between groups in the concentrations of plasma glutamine, glutamate, and alanine. Probably, increased plasma glutamine and alanine levels after supplementation stimulate the tissue uptake of these amino acids, increasing their stores and maintaining plasma concentrations at basal values [17]. Corroborating this hypothesis, in the present study, supplements containing glutamine (G+A and DIP) increased the levels of muscle glutamine and glutamate.

Interestingly, alanine supplementation did not increase its muscular content, and the ALA group presented lower concentrations of muscle alanine than the CON and G+A groups. The main role of alanine is its conversion to glucose in the alanine–glucose cycle that occurs in the liver. During physical exercise, skeletal muscle releases alanine to the liver to increase glucose production by alanine, reducing the muscle content of this amino acid as there is no alanine store [10,29]. In this study, it is possible that immediately after ALA supplementation the plasma and liver content of this amino acid were increased, decreasing the need for muscle release of alanine and, consequently, the synthesis of this amino acid by the skeletal muscle, which could explain the lower content of muscle alanine in the ALA group.

RT did not drastically affect plasma lactate, muscle concentrations of lactate transporters—MCT (parameters of anaerobic metabolism) and muscle levels of CS, an enzyme involved in the first reaction of the Krebs cycle, and considered as an aerobic metabolism marker. Studies that applied a high-intensity interval training (4–6 wk) to endurance athletes observed similar results—unchanged or even decreased blood lactate and muscle CS activity [30,31]—possibly suggesting that when the exercise recruits both anaerobic and aerobic energy systems, some parameters, such as lactate and CS activity, may remain unchanged.

Moreover, Siu et al. [32] observed an increase of CS activity in certain types of muscles (soleus), but not in others (ventricle muscles), 1 and 48 h after an endurance training on the treadmill. Therefore, in the present study, the CS results might have been influenced by the muscle evaluated, and also because we measured the concentrations and not the activity of this enzyme. It is worth mentioning that the effect of physical exercise, especially of the

Table 4
Parameters of energy metabolism, oxidative stress and muscle damage

	SED	CON	ALA	G+A	DIP	*P*-value
Plasma hypoxanthine (ng/μL)	20.14 ± 5.39	19.33 ± 2.84	20.54 ± 3.29	17.66 ± 4.57	21.91 ± 3.63	0.347
Plasma xanthine (ng/μL)	22.50 ± 6.03	21.60 ± 3.18	22.96 ± 3.68	19.73 ± 5.11	24.48 ± 4.06	0.347
Liver glycogen (mg/100 mg of tissue)	0.48 ± 0.23	0.29 ± 0.22	0.24 ± 0.20	0.18 ± 0.08	0.23 ± 0.14	0.086
Soleus glycogen (mg/100 mg of tissue)	0.23 ± 0.11[a,b]	0.18 ± 0.03[a]	0.22 ± 0.06[a,b]	0.25 ± 0.09[a,b]	0.31 ± 0.03[b]	0.039
Plasma TBARS (μM/100 μL)	9.21 ± 2.22	8.77 ± 1.88	9.97 ± 2.41	10.15 ± 1.42	9.46 ± 1.32	0.598
Plasma 8-isoprostane (pg/mL)	371.2 ± 42.97	401.9 ± 25.67	346.9 ± 82.9	366.8 ± 47.49	390.2 ± 32.05	0.245
Plasma glutathione (nmoles/μL)	0.006 ± 0.002	0.006 ± 0.002	0.007 ± 0.004	0.005 ± 0.001	0.005 ± 0.002	0.289
Liver glutathione (nmoles/μL of tissue homogenate)	0.034 ± 0.016	0.026 ± 0.005	0.029 ± 0.007	0.026 ± 0.004	0.027 ± 0.006	0.367
Heart glutathione (nmoles/μL of tissue homogenate)	0.059 ± 0.003	0.060 ± 0.002	0.059 ± 0.004	0.060 ± 0.001	0.059 ± 0.003	0.956
Muscle glutathione (nmoles/μL of tissue homogenate)	0.039 ± 0.018[a]	0.054 ± 0.003[b]	0.055 ± 0.003[b]	0.054 ± 0.002[b]	0.053 ± 0.002[b]	0.003
Plasma LDH (U/L)	535 ± 243[a]	1017 ± 181[b]	631 ± 124[a]	576 ± 168[a]	723 ± 162[a,b]	0.001
Plasma CK (U/L)	1983 ± 414[a,b]	2292 ± 259[a]	1693 ± 175[b]	1547 ± 422[b]	2301 ± 371[a]	0.001

ALA, exercised and supplemented with alanine; CON, exercised group; DIP, exercised and supplemented with L-alanyl-L-glutamine; G+A, exercised and supplemented with glutamine and alanine in their free forms; LDH, lactate dehydrogenase; SED, sedentary group; TBARS, thiobarbituric acid reactive substances.
Different letters mean statistically significant differences. Data are presented as mean ± SD.

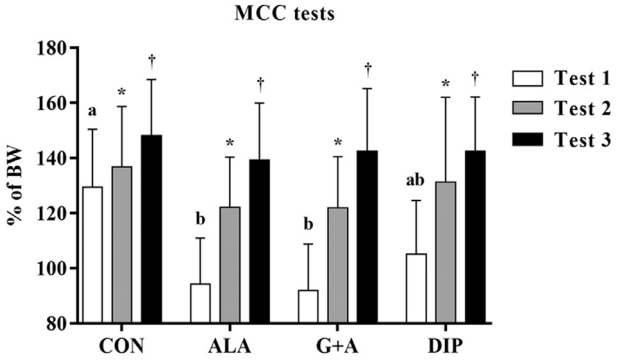

Fig. 4. Maximum carrying capacity tests of rats submitted to resistance training and supplementation with L-glutamine and L-alanine. ALA, exercised and supplemented with alanine; BW, body weight; CON, exercised group; DIP, exercised and supplemented with L-alanyl-L-glutamine; G+A, exercised and supplemented with glutamine and alanine in their free forms; SED, sedentary group. $^{*}P < 0.05$ vs test 1. †$P <$ 0.05 vs tests 1 and 2. Data are presented as mean ± standard deviation.

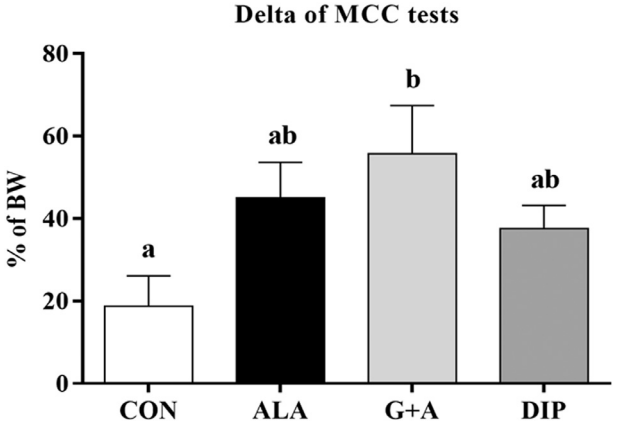

Fig. 5. Delta of MCC tests (MCC3−MCC1) of rats submitted to resistance training and supplementation with L-glutamine and L-alanine. ALA, exercised and supplemented with alanine; BW, body weight; CON, exercised group; DIP, exercised and supplemented with L-alanyl-L-glutamine; G+A, exercised and supplemented with glutamine and alanine in their free forms; MCC, maximum carrying capacity, SED, sedentary group. Different letters mean statistically significant differences. Data are presented as mean ± standard deviation.

high-intensity training, on muscle CS levels and activity is conflicting in the literature [33–36].

Although there was no statistically significant difference between groups on plasma lactate levels, animals supplemented with DIP presented higher muscle LDH concentration than the SED, CON, and ALA groups, but not the G+A group. Similarly, Kohn et al. [31] verified a reduction in blood lactate levels, but an increase in muscle LDH activity after a high-intensity interval training for endurance athletes. LDH converts pyruvate to lactate and vice versa, being considered as a system of lactate clearance [31]. Evidence suggests that LDH level and activity increase to favor the oxidation of carbohydrate [31,37]; therefore, the highest concentration of muscle LDH in the groups supplemented with glutamine and alanine (G+A and DIP) is consistent as these are the most glycogenic amino acids in animals and humans [7,38,39].

Besides being an important energy substrate, glutamine is also a direct stimulator of glycogen synthesis via the activation of glycogen synthase and the diversion of carbon from glutamine to glycogen [7]; for this reason, its supplementation was seen to restore muscle glycogen at the same level as glucose administration in a glycogen-depleting exercise protocol [40]. In the present study, all supplementations (ALA, G+A, and DIP) prevented the reduction of muscle glycogen by RT, whereas the DIP group presented higher muscle glycogen content than the CON animals. However, there was no difference between groups in the concentration of liver glycogen, indicating that supplementation affected glycogen in muscle but not in the liver.

In the present study, RT acted as an antioxidant stimulus as it increased the muscle concentrations of GSH (the most important non-enzymatic antioxidant in the cell) in all trained animals compared with the SED group and did not impair the oxidative parameters plasma TBARS and 8-isoprostane. Leeuwenburgh and Ji [41] also observed an increase of GSH muscle content after endurance training, suggesting that skeletal muscle might take up GSH from the blood during exercise, which augments the muscle levels of GSH to protect this tissue against oxidative stress. Studies indicate that the depletion of glutamine and glutamate concentrations in plasma and tissues promoted by exhaustive exercise compromises the GSH synthesis [11,42–44]. In the present study, RT did not reduce the levels of these amino acids, and consequently did not decrease GSH concentrations. Moreover, evidence shows that even with the high availability of glutamine and glutamate, GSH may not be affected because this is not the only factor that influences the GSH synthesis [44].

Otherwise, RT increased plasma LDH and CK concentrations (CON versus SED), whereas supplementation with free amino acids (ALA and G+A) reduced these parameters, evidencing a protective role of these interventions against muscle damage. Exhaustive exercise culminates in muscle injury, resulting in the release of LDH and CK from muscle tissue to the blood and in the inflammatory response [3,45]. Glutamine presents important immunomodulatory and anti-inflammatory effects [12,13], explaining the present results. In addition, this amino acid is absorbed through a sodium-dependent transport, increasing the intracellular concentration of sodium ions and promoting water retention, which increases cell hydration and its resistance to lesions [46,47]. Alanine supplementation was seen to increase plasma and tissue glutamine [12,13], and, for this reason, could indirectly present the effects mentioned here.

Interestingly, DIP supplementation did not affect plasma LDH and CK levels. Corroborating our results, Cruzat et al. [3] verified that L-alanyl-L-glutamine administration did not influence plasma LDH and CK concentrations after prolonged endurance exercise in rats. Several results obtained in the present study differed according to the supplementation form. This might be associated with the fact that free amino acids and dipeptides are absorbed by different pathways in the luminal membrane. Dipeptides are absorbed in their intact form by the oligopeptide transporter PepT-1; thus, the dipeptide form (L-alanyl-L-glutamine) may be present in blood and certain tissues, playing different roles than free amino acids [20,48].

Finally, despite improving some fatigue markers, such as muscle glycogen and plasma LDH and CK, glutamine and alanine supplementation, in their free or conjugated form, did not improve exercise performance, and similar findings were found in other studies [49–54]. On the other hand, physical performance increased as an adaptation to RT, especially in the G+A group. It is worth highlighting that fatigue is a complex and multifaceted phenomenon because several factors may limit exercise performance; therefore, the improvement of single markers does not necessarily delay fatigue [55].

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