

## L-Glutamine Supplementation Associated With Moderate Aerobic Training Improves Biometric, Glycemic Profile and the Antioxidant Defense

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### ABSTRACT

**Introduction:** L-glutamine is a non-essential amino acid, whose pool appears to be depleted during catabolic conditions, such as intense or high duration exercise, and to avoid the exercise-related benefits. Therefore, its supplementation could provide an additional source of L-glutamine and prevent these effects. However, the oral intake of its free form has been discouraged, despite of some evidences reporting positive effects. **Objective:** to verify whether the L-glutamine supplementation (in its free form) could provide an additional improvement in biometric, glycemic and redox parameters, in animals undergoing moderate aerobic training (MAT). **Methods:** 28 Swiss male mice were divided into four groups: Cont (n=7), Ex (n=7), Glut (n=8), and Ex+Glut (n=6). Glut and Ex+Glut received gastric gavage of L-glutamine (1g/kg), while Cont and Ex groups received 100 µL of PBS one hour before exercising, five days/week, six weeks. Ex and Ex+Glut underwent moderate swimming, while Cont and Glut remained sedentary, for the same period. Mice started swimming with 2% of body weight attached to the tail during 20 min, and ended the experiment with 4% during 60 min. **Results:** L-glutamine supplementation increased the gastrocnemius mass and improved the glucose tolerance in animals submitted to MAT. It improved the antioxidant status in gastrocnemius, liver and pancreas, and declined it in adipose tissue in animals undergoing MAT. The drop of adipose antioxidant defense was associated with adiposity, while pancreas antioxidant activity was inversely associated with the glucose intolerance. **Conclusion:** L-glutamine (free form) improves biometric and glucose parameters, and enhances antioxidant activities.

**Keywords:** Glucose intolerance. Oxidative stress. HSP70. Skeletal muscle. Adiposity.

### SUPLEMENTAÇÃO DE L-GLUTAMINA ASSOCIADA AO TREINAMENTO AERÓBICO MODERADO MELHORA PERFIL BIOMÉTRICO E GLICÊMICO E A DEFESA ANTIOXIDANTE

### RESUMO

**Introdução:** L-glutamina é um aminoácido não essencial, cujo 'pool' intracelular parece ser depletado durante condições catabólicas, como exercício intenso ou de longa duração, e assim inibir os benefícios relacionados ao exercício. Portanto, a sua suplementação pode fornecer uma fonte adicional de L-glutamina e prevenir esses efeitos. No entanto, a ingestão oral da sua forma livre tem sido desencorajada, apesar de algumas evidências reportando efeitos positivos. **Objetivo:** verificar se a suplementação de L-glutamina (na sua forma livre) pode proporcionar uma melhora adicional em parâmetros biométricos, glicêmicos e redox, em animais submetidos ao treinamento aeróbico moderado (MAT). **Métodos:** 28 camundongos Swiss machos foram divididos em quatro grupos: Cont (n=7), Ex (n=7), Glut (n=8), e Ex+Glut (n=6). Glut e Ex+Glut receberam gavagem gástrica de L-glutamina (1g/kg), enquanto os grupos Cont e Ex receberam 100 µL de PBS uma hora antes do exercício, cinco dias/semana, seis semanas. Ex e Ex+Glut foram submetidos a natação moderada, enquanto Cont e Glut permaneceram sedentários, durante o mesmo período. Os camundongos iniciaram a natação com 2% do peso corporal acoplado a cauda durante 20 min, e terminaram o experimento com 4% durante 60 min. **Resultados:** A suplementação de L-glutamina aumentou a massa do gastrocnêmio e melhorou a tolerância à glicose nos animais submetidos ao MAT. Ela melhorou o estado antioxidante no gastrocnêmio, fígado e pâncreas, e reduziu no tecido adiposo branco em animais passando por MAT. A redução da defesa antioxidante do tecido adiposo esteve associada a adiposidade, enquanto a atividade antioxidante pancreática foi inversamente associada a intolerância a glicose. **Conclusão:** L-glutamina (forma livre) melhora parâmetros biométricos e glicêmicos, e aumenta as atividades antioxidantes.

**Palavras-chave:** Intolerância a glicose. Estresse oxidativo. HSP70. Músculo esquelético. Adiposidade.

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## INTRODUCTION

The dietary supplementation L-glutamine has been discussed worldwide regardless its efficacy in preventing muscle fatigue (COQUEIRO; ROGERO; TIRAPEGUI, 2019), in catabolic conditions such as hind limb immobilization (PETRY *et al.*, 2019), sepsis (SULZBACHER *et al.*, 2018) and aerobic physical training (PETRY *et al.*, 2014). L-glutamine is one of the most abundant amino acids in the circulation, and can act in the glycemic control by increasing the insulin production and secretion (CARLESSI *et al.*, 2019; COQUEIRO; ROGERO; TIRAPEGUI, 2019). Besides, the most important role of glutamine is the indirect antioxidant activity through enhancing glutathione availability and the 70 kDa-heat shock protein (HSP70) expression (PETRY *et al.*, 2019). The HSP70 is a high evolutionarily conserved chaperone, expressed under stressful conditions, with a fundamental antioxidant, anti-inflammatory role (MIRAGEM; HOMEM DE BITTENCOURT, 2017), and sensible to exercise (HECK *et al.*, 2017), diabetes (BITTENCOURT *et al.*, 2020) and glutamine levels (PETRY *et al.*, 2014, 2015, 2019).

The main endogenous source of L-glutamine is the skeletal muscle under activity, besides also being produced by adipose tissue and liver (COQUEIRO; ROGERO; TIRAPEGUI, 2019). By challenging our body through an increase of oxidative metabolism and the production of reactive oxygen species (ROS), the exercise can deplete the L-glutamine pool (CRUZAT *et al.*, 2018) and overload the antioxidant defense depending on its intensity and duration. This depletion is followed by an increased release of oxidative stress biomarkers, which can affect other metabolic tissues, and compromise the exercise-related benefits. Moderate physical aerobic training is a non-pharmacological strategy to treat or prevent metabolic conditions, which can, at long-term lead to type II diabetes mellitus (DM2) (PEDERSEN, 2017; WEDELL-NEERGAARD *et al.*, 2018a).

The moderate training-related benefits are related to the amelioration of body composition, with a significant reduction of the adiposity and a stabilization or increase of lean mass, both related to the improvement of glycemic homeostasis (WEDELL-NEERGAARD *et al.*, 2018a). By enhancing the muscle content, exercise helps in contra balancing the inflammatory and oxidative phenotype displayed by adipocytes, in a 'yin-yang' relationship (PEDERSEN, 2013, 2017). Therefore, and can be considered a "real poly-pill" (FIUZA-LUCES *et al.*, 2013), since also enhances the antioxidant defense, which depends on the L-glutamine availability.

L-glutamine deprivation induces metabolic adaptations, such as reduction of glucose uptake, insulin production and release, as well as a drop in the mitochondrial respiration, both associated with a  $\beta$ -cells dysfunction (CARLESSI *et al.*, 2019). Therefore, the L-glutamine supplementation could provide an exogenous source of this amino acid, and improve the glycemic, glutathione and inflammatory profile. However, although some studies report benefits (MEDRAS *et al.*, 2018; ABOUD *et al.*, 2019; ALMEIDA *et al.*, 2020), recently the oral intake of L-glutamine in its free form has been discouraged, due to its utilization for the enterocytes supply (CRUZAT *et al.*, 2018). Nevertheless, if it works in stimulating other antioxidant enzymes than the glutamine-glutathione-HSP70 axis (PETRY *et al.*, 2014, 2015, 2019), and if it has a synergistic effect with moderate aerobic training these parameters remains unclear.

Here, we propose to associate the L-glutamine supplementation in the moderate aerobic training, to verify if the supplementation could provide an additional improvement in biometric, glycemic parameters, by protecting metabolic tissues from oxidative damage and enhancing the muscle HSP70 expression in animals undergoing moderate aerobic training.

## MATERIAL AND METHODS

### Animals

We utilized 28 Swiss male mice, with 90 days old ( $35 \pm 3$  grams), obtained from the Animal Facility of the Regional University of Northwestern of Rio Grande do Sul State (Unijuí). During the experimental period, the animals were maintained in semi-metabolic boxes (5-6 animals/box), with controlled ambient temperature ( $22 \pm 2^\circ\text{C}$ ) and a light-dark cycle of 12 hours. All animals received water and standard diet (Nuvilab CR-1) *ad libitum*.

This study followed all ethical rules established by Arouca's Act (Federal Law 11794/2008) and the Guide for Care and Use of Experimental Animals, published by the National Institutes of Health (NIH publication no. 85-23, revised in 1996) and was approved by the Animal Ethics Committee of Unijuí (Ceua-Unijuí, protocol nº 017/2013).

### Experimental design

Animals were divided into four experimental groups: Control group (Cont,  $n = 7$ ), Exercise (Ex,  $n = 7$ ), Glutamine (Glut,  $n = 8$ ), and Exercise + Glutamine (Ex + Glut,  $n = 6$ ). Glut and Ex + Glut received gas-

tric gavage of L-glutamine (1g/kg), while Cont and Ex groups received the same volume of PBS, adjusted by the body weight, one hour before exercising. Ex and Ex + Glut were submitted to moderate swimming, while Cont and Glut remained physical inactive (sedentary), in the shallow water, five days/week, for six weeks (Figure 1A).

### Swimming protocol

Prior to experiment, all animals were allowed to accustom to the water environment to avoid any stress response related to the new situation and environment. Thus, for three consecutive days, all animals received gastric gavage of 100  $\mu$ L PBS pH 7.4 one hour before were submitted to the water environment. The animals were kept for 10 min in individual swimming pool chambers (10 cm  $\times$  10 cm  $\times$  30 cm) filled with water at  $31 \pm 1^\circ\text{C}$  (20 cm depth), for three consecutive days, without any overload, to avoid stress behavior during exercise training sessions. Afterwards, animals remained three days without manipulation and were further assigned into each experimental groups (Sedentary: Cont and Glut or Trained: Ex and Ex+Glut i.g.).

During the swimming protocol, as in the acclimatization, all animals were maintained in individual swimming pool chambers (10 cm  $\times$  10 cm  $\times$  30 cm) filled with water at  $31 \pm 1^\circ\text{C}$ . The sedentary (Cont and Glut) groups remained at rest in shallow water (2 cm depth), while the trained (Ex and Ex+Glut) groups swam in 20 cm depth, as previously described (MAI *et al.*, 2017; KOSTRYCKI *et al.*, 2019) (Figure 1B). The individual pool chambers with 20 cm of water prevents jumping and diving behavior and allows energy expenditure higher than three metabolic equivalents (METs) (KREGEL *et al.*, 2006; KOSTRYCKI *et al.*, 2019).

In the first week, Ex and Ex + Glut swam with 2% of body weight (b. w.) of workload attached to the tail during 20 min. In the second week, the workload increased to 4% b. w., but the duration remained unchanged. From the third to the sixth week, the animals continued swimming with 4% b. w. workload, but the time increased 10 min per week, ending the experiment with 60 min of swimming with 4% b. w. workload (Figure 1C). Mice were weekly weighted and the workload adjusted according to it, respecting the training protocol.

All experiments were carried out between 7:00 and midday, and the room temperature was kept at  $24^\circ\text{C}$ . They were monitored by an experienced researcher to prevent drowning, in agreement with The American Physiological Society's Resource Book for

the Design of Animal Exercise Protocols (KREGEL *et al.*, 2006). Fatigue was considered when animal stayed eight seconds with the snout underwater, as previously described (MAI *et al.*, 2017; KOSTRYCKI *et al.*, 2019). A previous study of our lab already demonstrated that mice trained with 4% of workload reached a lactate concentration of  $4.54 \pm 0.21$  mmol/L in the 5<sup>th</sup> week (SCHOLER *et al.*, 2016; MAI *et al.*, 2017). It is hypothesized that 4.0–4.6% workloads in swimming would reach a moderate intensity range of 60–75% of  $\text{VO}_2\text{max}$  (KREGEL *et al.*, 2006; MAI *et al.*, 2017).

### Diet and L-Glutamine supplementation

All experimental animals received a standard diet (Nuvilab CR-1, commercially obtained from Nuvital Nutrientes SA), which contains crude protein, mineral material, fibrous matter and minerals. The standard diet presents a total metabolizable energy of 16.6 MJ/kg, being 11.4 % as fats, 62.8 % as carbohydrates, and 25.8 % as proteins (GOETTEMES-FIORIN *et al.*, 2016). A more detailed description of the diet is present in the Supplementary Table S1.

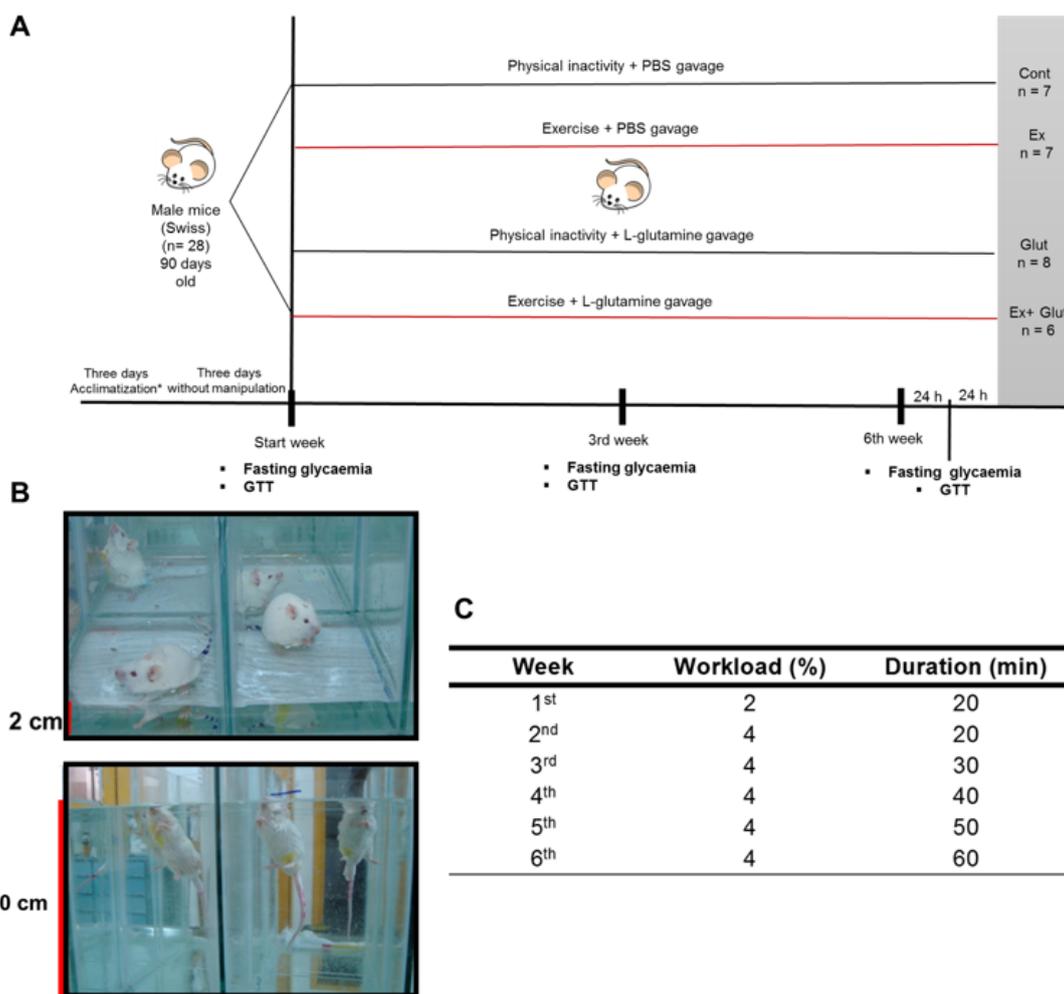
L-Glutamine (m. w. 146 g/mol, Ajinomoto, Brazil) was administered in the dose of 1 g/kg b. w. For this, we prepared a solution in the concentration of 1 g/10 mL in phosphate-buffered saline (PBS) pH 7.4. Each animal received the volume corresponding to its body weight, by gastric gavage administration, one hour before exercising. We weekly monitored the animal's body weight and adjusted the L-glutamine volume. Therefore, every week, animals received a different volume of the L-glutamine solution, respecting the original dose.

The time of one hour before exercising was considered due to the fact that approximately 30 minutes are required until the increase of glutamine blood concentration after oral intake, which returns to baseline about two hours later (CASTELL; POORTMANS; NEWSHOLME, 1996). Besides, the dose of 1 g/kg b. w. combined to L-alanine in its free form or in the dipeptide form (L-alanyl-L-glutamine) was recently reported to prevent oxidative damage and muscle loss in models of hindlimb immobilization-induced disuse muscle atrophy (PETRY *et al.*, 2019) and in aerobic exercise (PETRY *et al.*, 2014), and the immune dysfunction in sepsis in its free and isolated form (SULZBACHER *et al.*, 2018).

### Biometric Profile

Body weight was monitored weekly with a semi-analytical scale. We also verified the naso-anal length (cm) to perform Lee Index at the 3<sup>rd</sup> and 6<sup>th</sup> weeks.

Figure 1 – Experimental design



A) Animals underwent three days of acclimatization to the water environment. They received 100  $\mu$ L of PBS by gastric gavage and one hour later were kept for 10 min in individual swimming pool chambers filled with water, for three consecutive days, without any overload, to avoid stress behavior during exercise training sessions. Animals remained three days without any manipulation and were further assigned into four experimental groups: Glut and Ex + Glut received glutamine (1g/kg), diluted in PBS pH 7.4 by gastric gavage, while Cont and Ex groups received 100  $\mu$ L of PBS one hour before exercising. Ex and Ex + Glut were submitted to moderate swimming, while Cont and Glut remained physical inactive (sedentary), in the shallow water, five days/week, for six weeks. Body weight was verified weekly, fasting glycaemia and glucose tolerance at the baseline, third and sixth weeks. At the end of the sixth week, after the last glucose tolerance test (GTT) and 48 hours without intervention, animals were euthanized with guillotine. B) During experimental design, the sedentary animals (Cont and Glut groups) were kept in shallow water (2 cm depth;  $31 \pm 1^\circ\text{C}$ ) during each training session, while the active animals (Ex and Ex + Glut) were kept in a water (20 cm depth;  $31 \pm 1^\circ\text{C}$ , 20 min). C) Schematic protocol of moderate swimming. The animals started the protocol with 2% b. w. workload attached to the tail during 20 min. In the second week, the workload increased for 4% b. w., but the duration remained stable. From the third to the sixth weeks, the time enhanced 10 min per week with the 4% b. w. workload, ending the experiment swimming with 4% b.w. attached to the tail during 60 min.

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Lee Index was calculated by dividing the cube root of the animal's body weight (g) by naso-anal distance (cm) (LEE, 1929). At the end of the experiment, we evaluated adiposity [% of epididymal white adipose tissue (Ewat)/body weight] and % of liver, pancreas, gastrocnemius and soleus muscle relative to body weight.

### Glycaemia and Glucose Tolerance Test (GTT)

Blood glucose levels were monitored at the baseline, at 3<sup>rd</sup> and 6<sup>th</sup> weeks, in the morning after the last exercise session. Blood glucose was measured by

Glucometer Optium Xceed (Abbott Diabetes Care, Alameda, USA) (5  $\mu$ L of blood) after 12 hours of fasting. The glycaemia results were expressed in mg/dL.

Food was withdrawn in the night before experiments (12 hours before). Glycaemia was measured as described above immediately before and at 30 and 120 min after glucose (1 g/kg in saline solution, *i.p.*) administration. The glycemic response during GTT was evaluated by the area under the curve (AUC) and incremental area under the curve (IAUC) method. AUC and IAUC results were expressed in min.mg/dL.

## Tissue preparation

At the end of the six weeks of treatment, 48 hours after the last session to avoid its acute effects, animals were euthanized by guillotine for rodents. Metabolic tissues were collected and weighted (grams): liver, gastrocnemius and soleus muscle, pancreas and epididymal white adipose tissue (EWAT). We choose gastrocnemius and soleus muscle due to the different metabolism; gastrocnemius mainly composed by fast twitch fiber and glycolytic, and soleus a predominantly slow twitch, oxidative-fiber-type muscle (PETRY *et al.*, 2019). The EWAT, as a visceral depot, presents a higher metabolic rate and is closely associated with insulin resistance and cardiometabolic risks (OIKONOMO; ANTONIADES, 2018; COSTA BEBER *et al.*, 2020). Mainly the EWAT redox status can provide important information about the systemic redox profile and glucose intolerance (COSTA BEBER *et al.*, 2020).

Tissues were frozen by liquid nitrogen and maintained at -20°C until the biochemical analysis. Then, still frozen tissues were homogenized in potassium phosphate buffer (KPI, pH 7.4), containing proteases inhibitor PMSF (Phenyl Methyl Sulfonyl Fluoride, Sigma® P7626, FW = 174.19 g/mol; 1.74 mg/mL = 100 mM) to oxidative stress parameters. Gastrocnemius muscle was also homogenized in sodium dodecyl sulfate (SDS, 0.1% (w/v)) buffer to determine HSP70 expression by Western blotting. We used buffer in a proportion of 9 mL/g of tissue, except for adipose tissue, that we used 5 mL/g. Blotting and oxidative stress preparations were further centrifuged into CentriBio centrifuge for 10 minutes at 4000 rpm. The supernatants were collected.

## Evaluation of Oxidative Stress

### Determination of Lipid Peroxidation

The lipid peroxidation was analyzed using the thiobarbituric acid reactive substances method (TBARS) (BUEGE; AUST, 1978). Homogenates were precipitated with 10% trichloroacetic acid (TCA) in the proportion of 3:1 (540 µL TCA and 180 µL homogenate) centrifuged and incubated with thiobarbituric acid (TBA) in the proportion of 1:1 (300 µL TBA and 300 µL supernatant) for 15 minutes at 100° C. After, the absorbance was measured in a spectrophotometer at 535 nm. The malondialdehyde (MDA) standard was prepared from 1.1.3.3-Tetramethoxypropane (points from 0.0005 – 0.016 mg/mL). Results were expressed in mmol MDA/mL, normalized for by protein concentration, measured by the Bradford method, at 595 nm,

using albumin curve as standard (BRADFORD, 1976). Further, they were normalized by the control and represented as Arbitrary Units.

### Determination of superoxide dismutase (SOD) and catalase (CAT) activity

The total SOD activity was performed by inhibition of the auto-oxidation of pyrogallol (Marklund e Marklund, 1974). Briefly, in a cuvette, 970 µL of 50 mM Tris/ 1mM EDTA buffer (pH 8.2), 4 µL of CAT (CAT; 30 µM), 10 µL of homogenate were added, the equipment was cleared and 16 µL of pyrogallol (24 mM in HCl 10 mM) were added. The SOD activity was determined at 36° C in a spectrophotometer (420 nm), every 20 seconds, during 120 seconds. The results were expressed in USOD/mg prot.

The CAT activity was performed by the decomposition of hydrogen peroxide (Aebi, 1984). In a quartz cuvette, 955 µL of phosphate buffer (50 mM, pH 7.4) and 10 µL of homogenate were added, the equipment was cleared and 35 µL of hydrogen peroxide (0.01 M) were mixed. The CAT activity was determined at 36° C in a spectrophotometer (240 nm), every 15 seconds, during 120 seconds. The results were expressed in pmol/mg prot.

SOD and CAT activities were normalized by protein concentration. Further, they were normalized by the control and represented as Arbitrary Units.

### HSP70 muscle expression (iHSP70)

The iHSP70 expression was evaluated in gastrocnemius tissue by western blotting analyses. Equivalent amounts of protein from each sample (~ 40 µg) were prepared in sample buffer [Tris 50 mM, SDS 10%, glycerol 10%, 2-mercaptoethanol 10% and 2 mg/ml bromphenol blue]. The samples were boiled for 10 min and electrophoresed in a 10% polyacrylamide gel (5h in 15 mA/gel). After, the proteins were transferred to a nitrocellulose membrane (GE HealthCare) by electrotransfer (1h in 100 V) and subsequently, transferred bands were visualized with 0.3% (w/v) Red Ponceau S (Sigma-Aldrich).

Membranes were washed with washing buffer [TEN-Tween 20 solution (0.1% w/v): TEN is 50 mM Tris, 5 mM EDTA, 150 mM NaCl, pH 7.4]; and then blocked in 5% (w/ v) nonfat dry milk in washing buffer. Membranes were incubated for 12 hours with monoclonal anti-HSP70 antibody (Sigma-Aldrich H5147, 1:1000). After three consecutive washings with washing buffer, peroxidase-labeled rabbit anti-mouse IgG (Sigma-Aldrich A9044, Lot # 018M4899V, 1:15000)

was utilized as secondary antibody. Membrane were incubated for one hour, and after this, were washed three times on washing buffer. As a gel loading control, we used Coomassie Blue (0.1% Coomassie blue, 40% methanol, 10% acetic acid) to detect the 43 kDa  $\beta$ -actin region. Blot visualization was performed using ECL-Prime Western Blotting Reagent (GE Healthcare®). Quantification of bands was performed using Image J® software. The data were presented in arbitrary units of iHSP70, relative do control.

### Statistical analyses

First, we verified the Gaussian distribution of the data, by performing the D'Agostino-Pearson and Shapiro-Wilk normality tests. Further, assuming the normality, we used two-way ANOVA followed by Tukey to test the effect of time and of the interventions on body weight and GTT. Area under the curve (AUC) and incremental area under the curve (IAUC) were calculated to GTT curve. One-way Anova, followed by Tukey for comparison between groups. We also performed

Pearson's correlation between some variables. All data were expressed in mean  $\pm$  SD. We considered a significance level of 95% and a P 0.05.

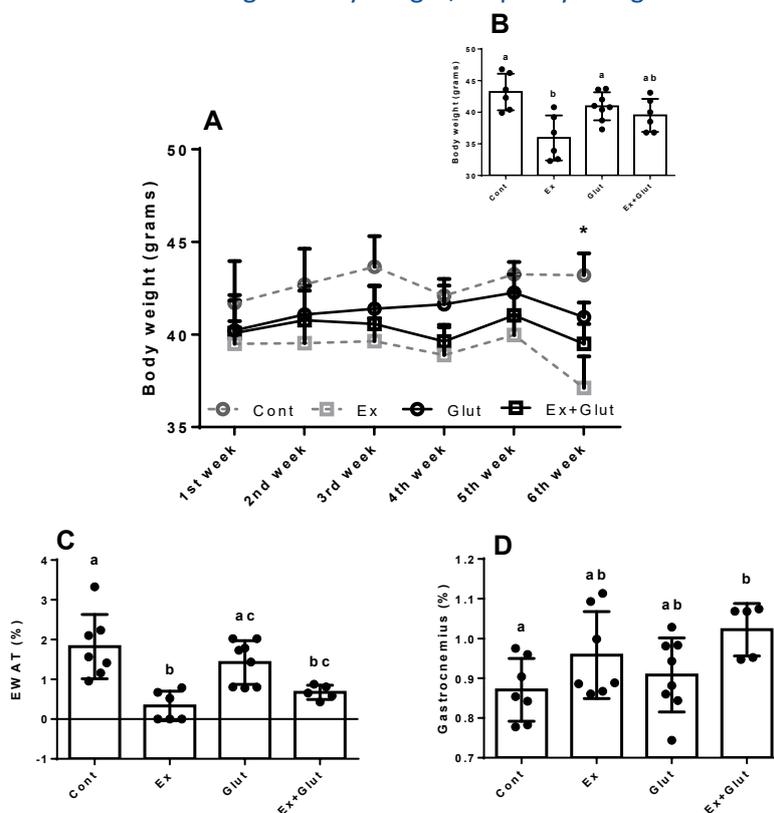
## RESULTS

L-glutamine does not affect the moderate training-induced reduction of adiposity and enhances the gastrocnemius mass in trained animals

Six weeks of moderate aerobic training did not affect the weight during the experiment ( $P = 0.248$ ) (Figure 2A), except in the 6<sup>th</sup> week, when the Ex presented lower body weight than Cont and Glut groups ( $P = 0.001$ ,  $F_{3,22} = 7.143$ ) (Figure 2B). Neither moderate training nor the L-glutamine changed the weight gain during the experiment ( $P = 0.215$ ,  $F_{3,24} = 1.601$ ) (Figure S1A), Lee Index in the 3<sup>rd</sup> ( $P = 0.689$ ,  $F_{3,26} = 0,493$ ) (Figure S1B), and 6<sup>th</sup> weeks ( $P = 0.718$ ,  $F_{3,24} = 0.451$ ) (Figure S1C).

Ex and Ex+Glut presented lower EWAT mass compared to Cont group, while Glut presented higher adiposity than Cont group ( $P = 0.0003$ ,  $F_{3,22} = 9.518$ )

Figure 2 – Effect of association of L-glutamine supplementation in animals submitted to moderate aerobic training on body weight, adiposity and gastrocnemius mass



A) Body weight during the 12 weeks. Two-way ANOVA with repeated measures, followed by the Tukey test. Time  $P = 0.024$ , Interventions  $P = 0.248$ , Interaction between factors  $P = 0.591$ , \* Ex vs Cont. B) Final body weight. One-way ANOVA, followed by Tukey test ( $P = 0.001$ ,  $F_{3,22} = 7.143$ ). C) Epididymis white adipose tissue (EWAT). One-way ANOVA, followed by Tukey test ( $P = 0.0003$ ,  $F_{3,22} = 9.518$ ). D) Gastrocnemius mass. One-way ANOVA, followed by Tukey test ( $P = 0.045$ ,  $F_{3,23} = 3.117$ ). Data presented as mean  $\pm$  SD ( $n = 6-8$  p/ group). a–c Means within a row with different superscripts are different at  $P < 0.05$ . Different letters represent different means while equal letters represent equal means.

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Table 1 – Metabolic tissue's weight of animals submitted to moderate aerobic training and L-glutamine supplementation

Tissue mass (%)	Cont	Ex	Glut	Ex + Glut	P value
Soleus muscle	0.044 ± 0.006	0.052 ± 0.010	0.045 ± 0.004	0.042 ± 0.004	0.100 ( $F_{3,24} = 2.326$ )
Pancreas	0.521 ± 0.042	0.565 ± 0.040	0.555 ± 0.050	0.555 ± 0.052	0.379 ( $F_{3,23} = 1.075$ )
Liver	4.118 ± 0.341	3.960 ± 0.537	3.825 ± 0.213	4.020 ± 0.530	0.591 ( $F_{3,24} = 0.648$ )

Data expressed as mean ± SD. One-way ANOVA, followed by Tukey (n = 6-8 p/ group).

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Table 2 – Fasting glycaemia of animals submitted to moderate aerobic training and L-glutamine supplementation

Glycaemia	Cont	Ex	Glut	Ex + Glut	P value
Baseline	135.00±17.76	121.70±12.68	115.10±47.38	114.80±29.64	0.590 ( $F_{3,24} = 0.650$ )
3 <sup>rd</sup> week	117.30±28.92	96.71±27.22	124.40±28.79	110.30±40.70	0.393 ( $F_{3,24} = 1.040$ )
6 <sup>th</sup> week	123.00±21.27	111.40±27.46	114.10±14.75	108.50±23.67	0.655 ( $F_{3,24} = 0.545$ )

Data expressed as mean ± SD. One-way ANOVA, followed by Tukey (n = 5-6 p/group).

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(Figure 2C). Ex+Glut presented a slight higher gastrocnemius mass than Cont group ( $P = 0.045$ ,  $F_{3,23} = 3.117$ ) (Figure 2D). Therefore, the moderate aerobic training reduces the adiposity independent of supplementation; however, L-glutamine helps in maintaining the lean mass, which explains the lack of effect in the total body weight. Neither the moderate training nor the L-glutamine supplementation affected the mass of the other metabolic tissues, such as soleus muscle, pancreas and liver (Table 1). The biometrical effects were independent of changes in the food ( $P = 0.744$ ,  $F_{3,44} = 0.413$ ) (Figure S1D) and water intake ( $P = 0.3614$ ,  $F_{3,43} = 1.095$ ) (Figure S1E).

### Both moderate aerobic training and L-glutamine supplementation enhances the glucose tolerance

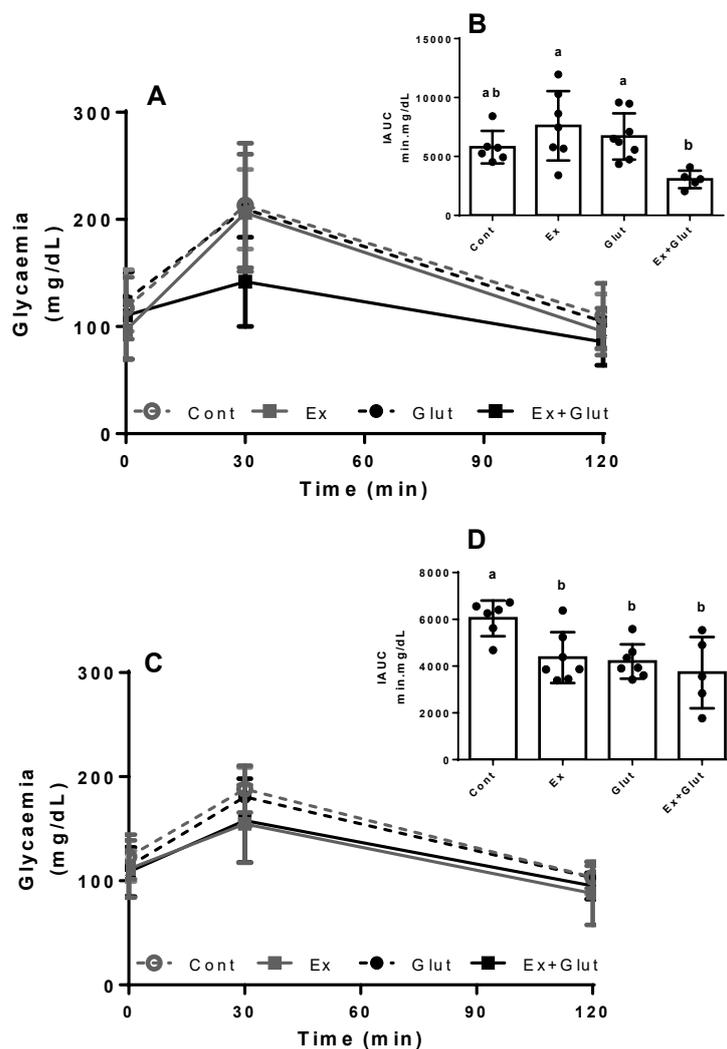
Neither moderate training nor the L-glutamine supplementation changed the fasting glycaemia during the experiment (Table 2). However, to verify if the L-glutamine could improve the glucose tolerance in trained animals, we performed GTT at the 3<sup>rd</sup> and 6<sup>th</sup> weeks of experiment. At the 3<sup>rd</sup> week, the GTT was not affected by the interventions ( $P = 0.199$ ,  $F_{3,24} = 1.674$ ) (Figure 3A), neither was the GTT-AUC ( $P = 0.100$ ,  $F_{3,24} = 2.321$ ) (Figure S2A). However, Ex+Glut presented a lower IAUC than Ex and Glut groups ( $P = 0.006$ ,  $F_{3,22} = 5.290$ ) (Figure 3B). At the 6<sup>th</sup> week, the GTT was also not affected by the interventions ( $P = 0.205$ ,  $F_{3,24} = 1.643$ ) (Figure 2D), neither the GTT-AUC ( $P = 0.168$ ,  $F_{3,24} = 1.833$ ) (Figure S2B). However, the IAUC was lower in all groups compared to Cont group ( $P = 0.005$ ,  $F_{3,21} = 5.534$ ) (Figure 3D). Taking together, these results shows that the association between L-glutamine supplementation and moderate aerobic exercise improves the glucose tolerance, without affecting the glucose overload-induced glycemic peak. Besides, the association anticipates the IAUC improvement compared to the effects each intervention isolated.

Ex+Glut group presented higher lipoperoxidation in the gastrocnemius muscle than Cont and Ex groups ( $P = 0.022$ ,  $F_{3,23} = 3.854$ ) (Figure 4A). The association of L-glutamine supplementation and moderate aerobic training slightly increased the SOD activity in Ex+Glut animals compared to Cont ( $P = 0.044$ ,  $F_{3,24} = 3.125$ ) (Figure 4B). L-glutamine also increased the CAT activity in Ex+Glut compared to Ex group ( $P = 0.027$ ,  $F_{3,22} = 3.678$ ) (Figure 4C). Neither moderate aerobic training nor the L-glutamine supplementation affected the lipoperoxidation in the pancreas tissue ( $P = 0.062$ ,  $F_{3,22} = 2.826$ ) (Figure 4D). L-glutamine supplementation also increased the SOD activity in trained animals, as verified by the higher SOD activity presented by the Ex+Glut compared to Ex group ( $P = 0.031$ ,  $F_{3,21} = 3.581$ ) (Figure 4E). L-glutamine, moderate ae-

### L-glutamine supplementation induces the antioxidant defense in metabolic tissues of animals submitted to moderate aerobic training

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Figure 3 – Effect of association of L-glutamine supplementation in animals submitted to moderate aerobic training in the GTT response and AUC



**A) GTT response at 3<sup>rd</sup> week**, Two-way ANOVA, followed by Tukey ( $P = 0.199$ ,  $F_{3,24} = 1.674$ ). **B) IAUC at 3<sup>rd</sup> week**, One-way ANOVA, followed by Tukey ( $P = 0.006$ ,  $F_{3,22} = 5.290$ ). **C) GTT response at 6<sup>th</sup> week**, Two-way ANOVA, followed by Tukey ( $P = 0.205$ ,  $F_{3,24} = 1.643$ ). **D) IAUC at 6<sup>th</sup> week**, One-way ANOVA, followed by Tukey ( $P = 0.005$ ,  $F_{3,21} = 5.534$ ). Data expressed in mean  $\pm$  SD ( $n = 6-8$  p/group). a-c Means within a row with different superscripts are different at  $P < 0.05$ . Different letters represent different means while equal letters represent equal means.

Font: The authors.

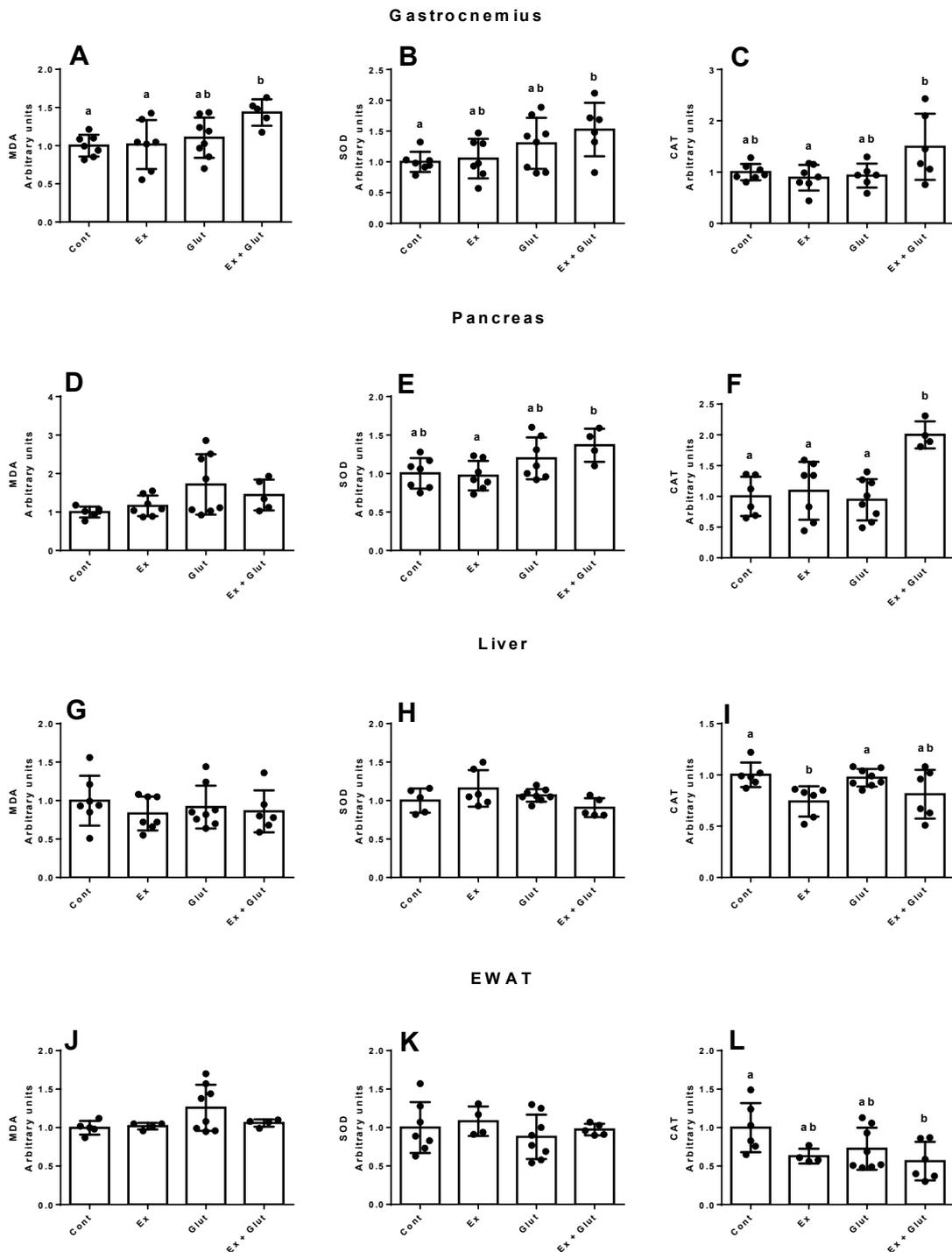
robic training and both associated increased the CAT activity compared to Cont group ( $P = 0.0007$ ,  $F_{3,21} = 8.514$ ) (Figure 4F).

Neither the lipoperoxidation ( $P = 0.697$ ,  $F_{3,24} = 0.483$ ) (Figure 4G) and SOD activity ( $P = 0.086$ ,  $F_{3,20} = 2.526$ ) (Figure 4H) were affected by the interventions in the liver tissue. However, Ex group presented lower hepatic CAT activity than Cont, while Glut group presented higher CAT activity than Ex group ( $P = 0.016$ ,  $F_{3,22} = 4.266$ ) (Figure 4I). Together, these results suggest that the moderate aerobic training reduced the CAT activity in the liver, while the L-glutamine supplementation attenuated this decrease in the Ex+Glut group. In the epididymis white adipose tissue (EWAT), nei-

ther the lipoperoxidation ( $P = 0.106$ ,  $F_{3,17} = 2.376$ ) (Figure 4L), nor the SOD activity ( $P = 0.622$ ,  $F_{3,20} = 0.600$ ) (Figure 4K) were affected by the interventions. However, the association of L-glutamine supplementation and moderate aerobic training had a slight significant effect in reducing the CAT activity in this tissue compared to Cont group ( $P = 0.049$ ,  $F_{3,20} = 3.101$ ) (Figure 4L). Therefore, these results suggest that L-glutamine supplementation enhances the antioxidant defense in metabolic tissues, except the adipose tissue.

Furthermore, we also found interesting correlations between some variables. Adiposity was weakly and positively associated with the GTT-AUC at the 6<sup>th</sup> week ( $r = 0.433$ ,  $P = 0.021$ ) (Figure 5A). Ex+Glut group

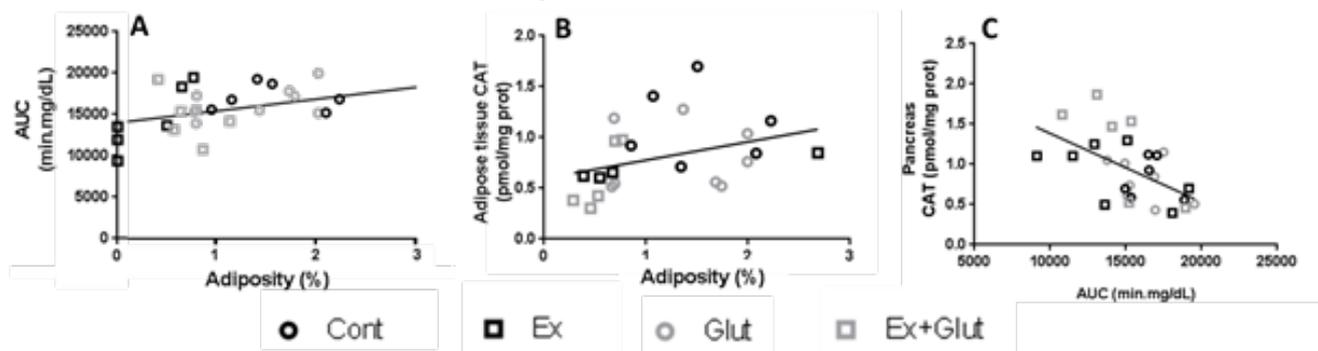
Figure 4 – Effect of association of L-glutamine supplementation in animals submitted to moderate aerobic training on the oxidative parameters in metabolic tissues, gastrocnemius (A-C), pancreas (D-F), liver (G-I) and EWAT (J-L)



**A)** Lipoperoxidation ( $P = 0.022$ ,  $F_{3,23} = 3.854$ ). **B)** SOD activity ( $P = 0.044$ ,  $F_{3,24} = 3.125$ ). **C)** CAT activity ( $P = 0.027$ ,  $F_{3,22} = 3.678$ ). **D)** Lipoperoxidation ( $P = 0.062$ ,  $F_{3,22} = 2.826$ ). **E)** SOD activity ( $P = 0.031$ ,  $F_{3,21} = 3.581$ ). **F)** CAT activity ( $P = 0.0007$ ,  $F_{3,21} = 8.514$ ). **G)** Lipoperoxidation ( $P = 0.697$ ,  $F_{3,24} = 0.483$ ). **H)** SOD activity ( $P = 0.086$ ,  $F_{3,20} = 2.526$ ). **I)** CAT activity ( $P = 0.016$ ,  $F_{3,22} = 4.266$ ). **J)** Lipoperoxidation ( $P = 0.106$ ,  $F_{3,17} = 2.376$ ). **K)** SOD activity ( $P = 0.622$ ,  $F_{3,20} = 0.600$ ). **L)** CAT activity ( $P = 0.049$ ,  $F_{3,20} = 3.101$ ). Data expressed in mean  $\pm$  SD, relative to Cont group. One-way ANOVA, followed by Tukey ( $n = 5-8$  p/group). a-c Means within a row with different superscripts are different at  $P < 0.05$ . Different letters represent different means while equal letters represent equal means.

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Figure 5 – Correlations



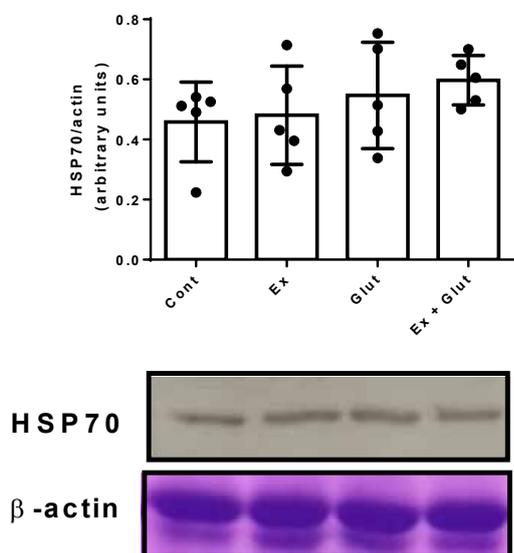
A) Adiposity and GTT-AUC at the 6<sup>th</sup> week ( $r = 0.433$ ,  $P = 0.021$ , all animals together). B) Adiposity and adipose tissue's CAT activity ( $r = 0.350$ ,  $P = 0.092$ , all animals together;  $r = 0.881$ ,  $P = 0.048$  for Ex+Glut). C) GTT-AUC at the 6<sup>th</sup> week and pancreas' CAT activity ( $r = -0.544$ ,  $P = 0.003$ ). Pearson's correlation.

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also presented a positive association between adiposity and adipose tissue CAT activity ( $r = 0.881$ ,  $P = 0.048$ ) (Figure 5B). Besides, pancreas antioxidant status (CAT activity) was inversely related with the GTT-AUC at the 6<sup>th</sup> week ( $r = -0.544$ ,  $P = 0.003$ ) (Figure 5C).

Neither the physical moderate training nor the L-glutamine supplementation affected the HSP70 expression in the gastrocnemius muscle ( $P = 0.999$ ,  $F_{3,16} = 5.278e-013$ ) (Figure 6).

Figure 6 – Effect of association of L-glutamine supplementation in animals submitted to moderate aerobic training on gastrocnemius HSP70 expression



Data expressed in mean  $\pm$  SD, relative to Cont group. One-way ANOVA, followed by Tukey ( $P = 0.999$ ,  $F_{3,16} = 5.278e-013$ ) ( $n = 5-8$  p/group).

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## DISCUSSION

In our study, the L-glutamine supplementation helped in maintaining the gastrocnemius mass, and improved the glucose tolerance of animals submitted

to moderate aerobic training. These effects might be due to the indirect effect of L-glutamine supplementation in the enhancement of the antioxidant activity in metabolic tissues, in a HSP70-independent way.

L-glutamine is one of the most abundant amino acids in the circulation, with a fundamental role in the amino acids and glycemic metabolism. Therefore, the exogenous source of L-glutamine by oral supplementation associated with L-alanine in its free forms, or in alanyl-glutamine dipeptide form can help in the muscle upkeep during catabolic conditions (PETRY *et al.*, 2014, 2019). In our study, we showed that the L-glutamine (without additional alanine) supplementation combined to moderate aerobic training increased the gastrocnemius mass, which explains why this experimental group did not present a decrease in the body weight, induced by the exercise in non-supplemented animals. These biometric effects were independent of food and water intake. Besides, this training protocol reduced the adiposity, independent on L-glutamine supplementation, which contradicts the recent reported possibility of glutamine being lipogenic (COQUEIRO *et al.*, 2019).

Independent of body mass index, low-fitness is associated with central obesity, low-grade inflammation, metabolic diseases and cardiovascular outcomes (WEDELL-NEERGAARD *et al.*, 2018a). Therefore, moderate training can act as a non-pharmacological strategy to prevent and treat metabolic conditions in physically inactive subjects (PEDERSEN, 2017). L-glutamine is a non-essential amino acid, which helps in stimulating insulin secretion and glucose uptake (MEDRAS *et al.*, 2018). Therefore, by helping in glucose uptake and muscle performance (COQUEIRO; ROGERO; TI-RAPEGUI, 2019), by reducing about 25% of the ammonium content (PETRY *et al.*, 2015), we investigated whether the L-glutamine supplementation could also

exert a synergistic effect in the exercise-induced glucose sensibility. We found that three- and six- weeks of L-glutamine supplementation associated with the moderate aerobic training helped in improve the glucose intolerance, which was related with the decrease of adiposity. This effected preceded the effect of the interventions isolated, which was only perceptible after the 6-weeks following. The protective effect of L-glutamine in the glucose tolerance may be due to the enhancement of the insulin production and secretion (MEDRAS *et al.*, 2018), which helps in augmenting the glucose uptake (CARLESSI *et al.*, 2019). Therefore, these positive effects of L-glutamine may be considered with caution before extrapolating it for obese subjects, since it could be related with a hyperinsulinemia (MEDRAS *et al.*, 2018). Since metabolic disorders or its improvement generally follow oxidative and inflammatory signaling, with HSP70 playing a fundamental role, we investigated whether the L-glutamine could prevent oxidative damage.

In our study, L-glutamine associated to moderate aerobic training increased the lipoperoxidation in the gastrocnemius muscle tissue compared to that observed in the non-supplemented trained animals. Nevertheless, it also increased the muscle SOD and CAT antioxidant activities, the last one compared to non-supplemented trained animals. CAT acts on the SOD-generated  $H_2O_2$ , while HSP70 inhibits one of the  $O_2^-$  and  $H_2O_2$  producers, the NADPH oxidase (NOX) (CHEN *et al.*, 2012). Therefore, the increase of SOD and CAT activity may provide enough antioxidant defense to upkeep its mass during moderate training, explaining why the HSP70 remained unchanged. Moreover, L-glutamine upregulated the SOD and CAT antioxidant activities in pancreas tissue of animals undergoing physical training, which is in agreement with its protective role in diabetic rats' pancreatic islets (MEDRAS *et al.*, 2018; CARLESSI *et al.*, 2019). An increased antioxidant activity in the pancreas may neutralize the ROS production, and suppress the ROS-derived neutralization of insulin (MUNHOZ *et al.*, 2016). Thus, the increased pancreas antioxidant activity may explain the L-glutamine-related enhancement in the glucose tolerance, and this is also supported by the inverse correlation between pancreas CAT activity and the GTT-AUC at the final week.

We also found that, in the adipose tissue (EWAT), the association of L-glutamine and moderate aerobic training reduced the CAT activity. Adipose tissue's antioxidant defense is indirectly related to systemic antioxidant status, marks a metabolic impairment (NEWSHOLME; DE BITTENCOURT, 2014; COSTA

BEBER *et al.*, 2020) and is positively related with the adiposity. Thus, the reduction of its antioxidant defense, together with a reduction of central obesity triggered by the association of exercise and glutamine may represent a better prognostic. The reduced antioxidant defense present by the adipose tissue, contrarily to the increased defense presented by the other metabolic tissues may be associated with the adipose insulin resistance triggered by L-glutamine supplementation, while muscle and liver present an L-glutamine-induced amelioration in these aspects (ABBOUD *et al.*, 2019). In this sense, L-glutamine could contribute to reducing the adiposity, by injuring the adipocytes, through insulin resistance and oxidative stress.

As the central metabolic tissue, liver exerts an essential role supporting glycemic, lipid and protein metabolism, especially under intense exercise. However, if the effects of the L-glutamine supplementation are similar in liver and muscle remained unclear until this moment. A previous study showed that L-glutamine associated to L-alanine in its free form or in the dipeptide form could enhance liver glutamine and GSH content, without affecting the HSP70 expression (PETRY *et al.*, 2015). In our study, we tested if the L-glutamine in its free form could improve the hepatic oxidative status in trained animals. We found that moderate training reduced the CAT activity, while L-glutamine supplementation increased it. Thus, this result show an important target of investigation concerning to the hepatic effects of moderate exercise. Nevertheless, this effect was neutralized when the L-glutamine was combined to training.

L-glutamine has an essential role in maintaining the antioxidant system. However, high-duration or intense exercises deplete the L-glutamine pools and lead to an oxidative condition, here perceptible in the CAT down regulation in the hepatic tissue. Thus, the supplementation could provide an exogenous source of L-glutamine. L-glutamine (1g/kg) combined to L-alanine supplementation increased plasma L-glutamine by approximately 60% (PETRY *et al.*, 2014, 2015), muscle GSH content by 100% (PETRY *et al.*, 2014), and liver GSH content by 90% compared to non-supplemented trained animals (PETRY *et al.*, 2015). However, the oral administration of L-glutamine in its free form has been criticized concerning to its efficacy (CRUZAT *et al.*, 2018), since only half of L-glutamine load supplied by the gastrointestinal route escapes the splanchnic bed (MATTHEWS, 1990; DECHELOTTE *et al.*, 1991). Despite the loss of L-glutamine to en-

terocytes provision, our results shows that the oral administration is still an efficient way when combined to physical training.

Here, we used a six-weeks moderate aerobic training protocol, which was already validated in previous studies (HECK *et al.*, 2017; MAI *et al.*, 2017; KOSTRYCKI *et al.*, 2019). Since rodents feed with standard diets and remained sedentary tends to develop weight gain and obesity because of the free access to food (MARTIN *et al.*, 2010; SEO *et al.*, 2014), and present an innate swimming ability, the swimming represents a replicable protocol, very similar to the human condition. Besides, eight-weeks training leads to physiological adaptations, such as an improved physical performance, reduction of adiposity and cardiac hypertrophy, whilst enhances the skeletal muscle mass (ARAUJO *et al.*, 2015). In addition, it also improves glycemic and lipid parameters in high fat diet-fed animals (JANG *et al.*, 2014; ARAUJO *et al.*, 2015). Some of these effects are also evident in standard diet-fed animals, following a twelve-weeks swimming protocol (MAI *et al.*, 2017). Taking together, these data enhances the replicability of swimming protocol and highlights the possibility of extending it for longer periods, with a guarantee of physical adaptation.

However, our study has some limitations. Swimming requires a high tissue demand and response, which can be different between animals and humans, because of the size and movement patterns (SEO *et al.*, 2014). Besides, in this study, we only used male mice, and female could respond differently to the training protocol and L-glutamine supplementation, since estrogen per se has a powerful antioxidant activity. Finally, our data concerning to glucose tolerance may be carefully considered before extrapolating these results to an insulin resistant model, since we used standard diet-fed animals.

Taking together, our results show that L-glutamine oral supplementation, even when not associated with L-alanine, improves the biometrical, glycemic and antioxidant status in male mice undergoing moderate aerobic training. These findings are in agreement with a recent report of the benefits of combined exercise training and L-glutamine supplementation in oral redox and inflammatory parameters (ALMEIDA *et al.*, 2020). It is known that L-glutamine supplementation can improve the muscle and liver health by enhancing the glutamine-glutathione-HSF1-HSP70 regulation and protection (PETRY *et al.*, 2014, 2015, 2019). Furthermore, our study show that L-glutamine exerted the improvement in biometric and glycemic metabolism in animals undergoing aerobic training, by an

indirect antioxidant activity, enhancing muscle, pancreas and liver antioxidant defenses, but not in EWAT, even without changes to the HSP70 content yet.

## CONCLUSION

In conclusion, the L-glutamine supplementation in its free form, helps in increasing the gastrocnemius mass and improving the glucose tolerance in animals submitted to moderate aerobic training. These benefits are related to the improvement of the antioxidant status verified in the metabolic tissues, gastrocnemius muscle, liver and pancreas in animals undergoing moderate aerobic training. Moreover, our study shows that the L-glutamine-related antioxidant effect extrapolates the already known glutamine-glutathione-HSP70 axis, and enhances other antioxidant enzymes, such as SOD and CAT activities.

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## SUPPLEMENTARY MATERIAL

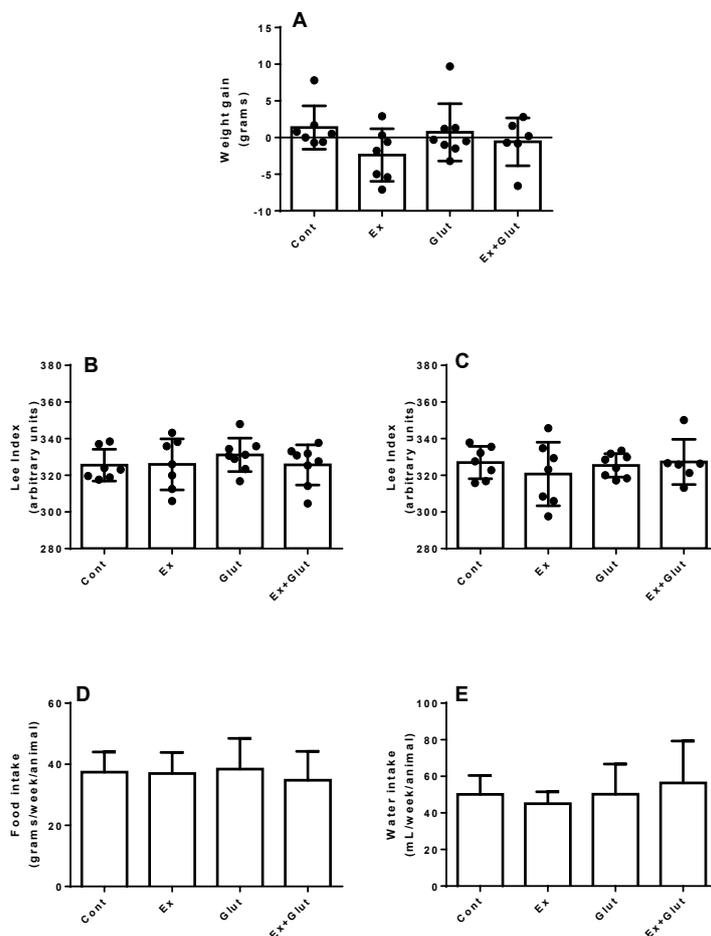
Table S1 – A detailed description of the standard diet (Nuvilab CR-1, commercially obtained from Nuvital Nutrientes SA)

Component	Content
Crude protein	220 g/kg
Ethereal extract	40 g/kg
Mineral material	90 g/kg
Crude fiber	70 g/kg
<b>Vitamins</b>	
Vit A	13000 U/kg
Vit D3	2000 U/kg
Vit E	34 U/kg
Vit K3	3 mg/kg
Vit B1	5 mg/kg
Vit B2	6 mg/kg
Vit B6	7 mg/kg
Vit B12	22mcg/kg
Niacin	60 mg/kg
Calcium pantothenate	20 mg/kg
Folic acid	1 mg/kg
Biotin	0.05 mg/kg
Choline	1900 mg/kg
Sodium minerals	2700 mg/kg
Manganese	60 mg/kg
Zinc	60 mg/kg
Copper	10 mg/kg
Iodine	2 mg/kg
Selenium	0.05 mg/kg
Cobalt	5 mg/kg
Fluorine	80 mg/kg
Iron	50 mg/kg
<b>Amino acids</b>	
Lysine	12 g/kg
Methionine	4000 mg/kg
<b>Additions</b>	
BHT	100 mg/kg

U – International Unit

Font: The authors.

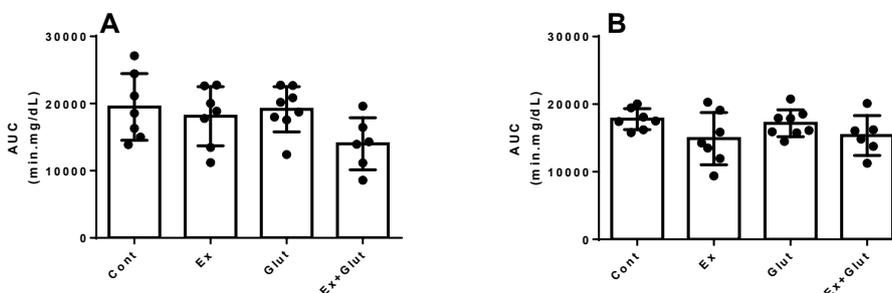
Figure S1 – Effect of association of L-glutamine supplementation in animals submitted to moderate aerobic training on weight gain, Lee Index, food and water intake



A) Weight gain ( $P = 0.215$ ,  $F_{3,24} = 1.601$ ). B) Lee Index at the 3rd week ( $P = 0.689$ ,  $F_{3,26} = 0.493$ ). C) Lee Index at the 6th week ( $P = 0.718$ ,  $F_{3,24} = 0.451$ ). D) Food intake ( $P = 0.744$ ,  $F_{3,44} = 0.413$ ). E) Water intake ( $P = 0.3614$ ,  $F_{3,43} = 1.095$ ). Data presented as mean  $\pm$  SD. One-way ANOVA, followed by Tukey ( $n = 6-8$  p/ group).

Font: The authors.

Figure S2 – Effect of association of L-glutamine supplementation in animals submitted to moderate aerobic training on GTT-AUC



A) AUC-GTT 3<sup>rd</sup> week ( $P = 0.100$ ,  $F_{3,24} = 2.321$ ). B) AUC-GTT 6<sup>th</sup> week ( $P = 0.168$ ,  $F_{3,24} = 1.833$ ). Data presented as mean  $\pm$  SD. One-way ANOVA, followed by Tukey ( $n = 6-8$  p/ group).

Font: The authors.