**CARBOHYDRATE SUPPLEMENTATION ATTENUATES
DECREMENT OF PERFORMANCE IN OVERTRAINED RATS**

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2 **PERFORMANCE IN OVERTRAINED RATS**

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Abstract

Although carbohydrate ingestion at the end of single exercises is recognized to delays fatigue and accelerates recovery, if chronic ingestion can prevent overtraining during periods of intense training is something not yet elucidated. The present study aimed to determine whether carbohydrate supplementation minimize overtraining in Wistar rats. The animals underwent 11 weeks of training (running) on a treadmill, and the last 3 weeks were designed to induce overtraining. One group was supplemented with carbohydrates (EX-CHO; n=13), one group had no supplementation (EX; n=10), and another group remained inactive (C; n=9). Performance tests (Pr) were given before training (Pr1) and at the 8th (Pr2) and 11th training week (Pr3). Food intake, body weight, testosterone, cortisol, malondialdehyde, creatine kinase (CK) and activities of PI3-K, Akt-1, mTOR and GSK-3 enzymes were measured. In the EX group, there was a significant 32.6% performance decrease at Pr3 compared to Pr2. Additionally, at protocol completion, the EX-CHO group had a greater gastrocnemius weight than the C group (p=0.02), which did not occur in the EX group. Training caused anorexia, decreased testosterone (p=0.001) and increased malondialdehyde (p=0.009) in both exercise groups compared to C, without an influence of carbohydrate supplementation on these variables (p>0.05). The activity of Akt-1 was higher in the EX-CHO group but not the EX group compared to the C group (p=0.013). Carbohydrate supplementation promoted an attenuation in the performance decrement and maintained gastrocnemius muscle mass in animals that had undergone overtraining protocols, which was accompanied by increased activity of the Akt-1 molecular indicator.

Keywords: Carbohydrate, Exercise, Performance, Overtraining, Akt-1, Rats.

49 **1 Introduction**

50

51 Overtraining syndrome (OTS) is a phenomenon that has increased in prevalence
 52 among high-performance athletes (Smith 2000). It is estimated that 37% of elite athletes from
 53 various sports have suffered from OTS symptoms at some point in their careers (Kenttä et al.
 54 2001), specifically, 65% of distance runners (Morgan et al. 1987), 50% of semi-professional
 55 soccer players and 21% of swimmers on the Australian national team (Smith 2000).

56 OTS results from an imbalance between excess training and inadequate recovery
 57 (Armstrong and VanHeest 2002). Nutrition participates in the etiology of OTS, as nutrients
 58 strongly influence the recovery time between exercise sessions (Kreider 2010) and provide
 59 energy to muscles during subsequent training sessions. In this sense, the use of nutritional
 60 resources to accelerate post workout recovery has grown considerably (Mitchell 2013).

61 Among various nutritional resources, carbohydrates are recognized as a very
 62 important effective ergogenic aid. Supplementation has been shown to be effective in
 63 delaying fatigue in prolonged exercise (Borges et al. 2012), increasing the rate of glycogen
 64 resynthesis (Van Loon et al. 2000), accelerating the recovery of physical capacity (De Sousa,
 65 et al. 2010) and elevating insulinemia during exercise (McAnulty et al. 2007). As
 66 carbohydrates are capable of accelerating post-exercise recovery, this phenomenon supports
 67 the hypothesis that carbohydrate supplementation can minimize or prevent the development
 68 of OT over a season of intense training. However, most studies that have provided support for
 69 this hypothesis have done so only after one or few sessions (De Sousa et al. 2010; Karelis et
 70 al. 2010) and it is not wise to extrapolate the ergogenic effect of carbohydrates to a whole
 71 training season.

72 The only investigations that have tested the chronic effects of carbohydrates on the
 73 prevention of OT were conducted with cyclists (Halsen et al. 2004) and runners (Achten et al

2004). However, these interventions lasted less than 11 days and had short recovery intervals. Thus, we believe that this short intervention precludes the prevention premise within a more chronic context, as OT is most often experienced.

Thus, this study was conducted to assess the influence of carbohydrate supplementation on the prevention or minimization of overtraining in an experimental animal model of intense physical training with short recovery intervals. The aim of this study was to assess whether carbohydrate supplementation can prevent or minimize OT in adult male Wistar rats by observing a performance test, which is the main indicator OT (Smith 2000). In addition, we aimed to assess whether changes in enzymatic OT markers, hormonal OT markers, oxidative stress and the activity of enzymes involved in the muscular anabolism/catabolism process are involved in the possible protective effects of carbohydrates in OT.

2 Materials and Methods

Animals: A total of 32 Wistar male rats (*Rattus norvegicus albinus*) were used; they were 12 weeks old and procured from the “Professor Dr. Thomas Georgel” vivarium at the Biotechnology Center (CBiotec), Federal University of Paraíba (UFPB). The rats were divided into three groups: Sedentary control (C: n = 9); exercise group without supplementation (EX: n = 10) and exercise group supplemented with carbohydrates (EX-CHO: n = 13). Animals were kept under standard lighting conditions (light/dark cycle, 12/12 hours); the environment was kept at $22 \pm 1^\circ \text{C}$ and 65% humidity, and they were housed in groups of five. They received fresh water and food daily ad libitum. The procedures were in accordance with the laboratory animals’ handling and care principles recommended by the Brazilian College of Animal Experimentation (COBEA). The research project was approved

99 by the Ethics Committee on Animal Use (CEUA) – UFPB under the protocol number
100 1303/12.

101

102 ***Adaptation to training and gavage:*** Rats underwent two weeks of adaptation to the gavage
103 procedure and treadmill running. They were subjected to running 10 minutes per day at a
104 speed of 12 m/min five consecutive days per week. Using a scale described by Lira et al.
105 (2010) the animals were ranked on a daily basis according to the behavior during the
106 treadmill session: 1) refuses to run; 2) below average runner (runs and stops; runs in the
107 wrong direction); 3) average runner; 4) above average runner (runs constantly, occasionally
108 running below the treadmill speed); and 5) good runner (runs consistently at the treadmill
109 speed). Animals that had an average of 3 or more ($n = 32$) were included in the study. After
110 the exercise, they underwent a process of gavage with water.

111

112 ***Training protocol:*** The training protocol started the very next week after adaptation. It was
113 performed according to the previously characterized procedure to induce overtraining in
114 Wistar rats by Hohl et al. (2009). The first stage (T1) consisted of four weeks of running at
115 speeds varying from 15 to 25 m/min and lasting 25 to 60 minutes five consecutive days per
116 week. In the second phase (T2), which was four weeks long, we kept the same running speed
117 and session's duration that were achieved at the end of T1, also five consecutive days per
118 week. Stages T1 and T2 were performed between 01:00PM and 05:00PM. The third and final
119 training stage (T3) lasted three weeks and was characterized by an increased frequency of
120 training to up to four sessions per day. As the number of daily sessions increased, the
121 recovery time between sessions was reduced from 4 hours to 3 hours and then to 2 hours. The
122 last session of the day always ended at 05:00PM.

Performance Tests (Pr): Performance tests were performed 48 hours after the adaptation period, during the 8th week of training and during the 11th week of training (always between 01:00PM and 05:00PM). After the performance test, the rats were allowed 48 hours of rest in order to recover before the next week of training. Tests started with animals running on a treadmill without an incline and an initial speed of 12 m/min for five minutes. Then, the speed was increased 2 m/min every 3 minutes until exhaustion, which was defined when the animals could not maintain the speed despite being encouraged three times by researchers. Performance was quantified with an equation described by Hohl et al. (2009). By considering all the forces proportional to body weight and all the speeds proportional to the running speed, mechanical energy becomes proportional to mass × speed. This assumption allows one to measure the rat's performance with a quantity that is proportional to the mechanical work, i.e., force × duration, as shown in the equation below.

$$Pr = \sum Pri = \sum mViTi = \sum mDi = mD$$

where Pr = rat's performance in kilograms – meters (Kg.m); Pri = rat's performance in each stage, m = body mass index; Vi = stage speed; Ti = running time in each stage; Di = distance achieved in the stage; and D = total distance covered on the test.

The procedures performed throughout the experimental protocol for induction of overtraining are described in figure 1.

Insert Figure 1

Supplementation Protocol: Solutions with 30% carbohydrate in the form of dextrose were administered to the animals by gavage before and immediately after each exercise session during the 9th week and on the 1st and 3rd exercise session of each one of the 10th and 11th weeks. The dose was 1.0 mL per 100 g body weight, as described by Morifuji et al. (2010) and Morifuji et al. (2011).

Animals Sacrifice: Exactly 36 hours after the last performance test and after a 12-hour fast, the animals were intraperitoneally anesthetized with ketamine (75 mg/kg) and xylazine (20 mg/kg). Subsequently, they were sacrificed by exsanguination according to the Brazilian College of Animal Experimentation (COBEA) ethical principles.

Biochemical analyses: Blood was collected by cardiac puncture and placed in test tubes with and without EDTA. Then, the samples were centrifuged at 3,000 rpm for 15 minutes and stored at -80° C until analysis. Biochemical assays were performed on serum creatine kinase (CK) using a commercial kit (Labtest, Minas Gerais, Brazil) and on testosterone and cortisol by chemiluminescence using an automatic biochemical analyzer Elecsys 2010 (Roche). With plasma samples, malondialdehyde (MDA) was determined.

Protein activity of GSK-3, PI3-K, Akt-1 and mTOR: The gastrocnemius muscle was homogenized in hypotonic lysis buffer containing 50 mM potassium phosphate (pH 7.0), 0.3 M sucrose, 0.5 mM DTT, 1 mM EDTA (pH 8.0), 0.3 mM PMSF, 10 mM NaF, and a cocktail of protease and phosphatase inhibitors (1:100). The homogenate was centrifuged at 12,000 rpm for 10 minutes at 4° C, transferred to 1.5 ml tubes and stored at -80° C. The protein concentration was determined using the Bradford method (1976) (Bradford 1976).

Aliquots (30 mg) of the homogenate were diluted in sample buffer (Tris-HCl, 240 mM (pH 6.8), 0.8% SDS, 200 mM β -mercaptoethanol, 40% glycerol and 0.02% bromophenol blue). The analysis of protein levels was performed with western blotting using polyacrylamide gel electrophoresis (SDS-PAGE 6-12%, depending on the protein molecular weight) (Towbin 1992). Subsequently, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) and incubated with casein and PI3K p110 α primary antibodies (rabbit polyclonal, 1:1000, Santa Cruz, CA, USA), Akt1 (rabbit polyclonal, 1:1000, Santa Cruz, CA, USA), p-Akt1 (Ser473) (rabbit polyclonal, 1:1000, Santa Cruz, CA, USA), mTOR (rabbit polyclonal, 1:1000, Cell Signaling Tech., MA, USA), p-mTOR (Ser2448) (rabbit polyclonal, 1:1000, Cell Signaling Tech., MA, USA), GS3Kb (rabbit polyclonal, 1:1000, Cell Signaling Tech., MA, USA), and p-GS3Kb (Ser9) (rabbit polyclonal, 1:1000, Cell Signaling Tech., MA, USA). Unbound antibody was removed by washing, and the membrane was exposed to the secondary antibody (conjugated to horseradish peroxidase (HRP)), which was directed to species-specific portions of the primary antibody. Then, they were washed 3 times for 10 min each in TBS-T and incubated for 2 hours with the secondary antibodies (all anti-rabbit, Amersham Biosciences, NJ, USA) conjugated to peroxidase. Subsequently, the complex was detected by electrochemiluminescence (ECL). The protein concentration was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The images were visualized using the Chemi-doc Gel Quantification System (Bio-Rad, Hercules, CA, USA). Protein concentrations were quantified from the blots using ImageJ software (Image J based on NIH image). The ratio of phosphorylated protein/total protein (P/T) was calculated in order to measure changes in the proteins' activity.

Statistical Analyses: Data are presented as the mean and standard deviation. The Shapiro-Wilk and Levene tests were initially performed in order to verify normality and compare differences between the variables' standard deviations. We used analysis of variance (two-way or one-way ANOVA, as appropriate) with Tukey's post hoc test or, for a non-parametric analysis, the Kruskal-Wallis or Friedman test with Dunn's post hoc test. We adopted a confidence level of 5% ($p < 0.05$) to determine statistical significance for all tests. These procedures were performed using InStat 3.0.1 software (Graph Pad InStat, San Diego, CA, USA).

3 Results

Performance: Results of the three performance tests (Pr) conducted over 11 weeks of training are shown in Figure 2. Prior to training (Pr1), animals in the three groups exhibited similar performance. At the end of the eighth week of training, in (Pr2), the two exercise groups showed significantly better performance compared to the beginning of the training and in relation to the C group ($p < 0.01$), but with no differences between them. The increase in daily training sessions between weeks 9 and 12 resulted in a significant 32.6% decrease in performance in the EX group (616.86 ± 215.46 Kg.m to 415.35 ± 103.08 Kg.m, $p < 0.01$). Meanwhile, the performance of rats in the EX-CHO group decreased, but not to a significant degree (24.1%; 687.80 ± 233.17 Kg.m to 521.95 ± 145.81 Kg.m). However, the final performance of the rats in the EX and EX-CHO groups did not differ significantly, despite a trend towards higher performance in the EX-CHO group ($p = 0.063$).

Insert Figure 2

Food Consumption: The three groups maintained similar food consumption during the first eight weeks of training (Figure 3). While the animals that remained sedentary consumed the same amount of food between the 8th and 11th weeks, the increased training load during this period was accompanied by a drastic reduction of food intake in both the EX ($p<0.01$) and EX-CHO groups ($p<0.001$) as early as the 9th week of the training protocol. Specifically, the rats in the EX and EX-CHO groups consumed less in the 9th week compared to the 8th week and compared to the C group. This anorexic behavior persisted until the 11th week in both groups. The reduction in food consumption both for the EX and CHO-EX groups was of the same magnitude throughout the entire overtraining period.

Insert Figure 3

Mass of Animals, Organs and Peritoneal Fat: The rats in all three groups maintained a similar body weight until the 8th week (data not shown). Then, the animals that underwent the overtraining protocol achieved a lower body weight compared to the C group ($p<0.001$). The rats in the exercise groups also exhibited a lower amount of absolute peritoneal fat and peritoneal fat corrected for body weight ($p<0.001$). There were no differences between the EX and EX-CHO groups. (Table 1), although EX-CHO completed the protocol with body weight 2% higher than EX group.

Insert Table 1

Trained animals presented a reduction in gastrocnemius weight compared to the C group ($p<0,001$) with no differences between groups ($p>0,05$). However, when the gastrocnemius weight was corrected for body weight, an inverse behavior was observed so that the weight of this muscle in the EX-CHO group was significantly higher than in the C group ($p <0.05$). The normalized gastrocnemius muscle mass of the rats in the EX group was not significantly different than that of the C group.

The animals in the three groups presented exactly the same thymus weight in the 8th week. At the end of the experimental protocol, the trained animals presented a significantly lower thymus weight in relation to the C group. When comparing only the trained animals, it was noted that carbohydrate supplementation exerted a protection against the reduction of thymus weight. Indeed, the animals in the EX-CHO group presented a significantly greater thymus weight in relation to animals in the EX group. This same behavior was reproduced in the analysis of thymus weight corrected for body weight.

Biochemical analyses: Parameters related to hormones, muscle damage and oxidative stress at the end of the experimental protocol are described in Table 2. Concentrations of creatine kinase (CK) and cortisol levels were not statistically different between groups. However, EX-CHO group presented cortisol concentration 9,9% lower compared to EX. Meanwhile, the groups that were exposed to training showed increased oxidative stress and reduced serum testosterone concentrations compared to the C group. The carbohydrate supplementation resulted in no difference in these variables, as the rats in the EX and EX-CHO groups completed the experiment with similar results.

Insert Table 2

Molecular Aspects: Analyses of the activities of muscle proteins related to glycogen synthesis and protein synthesis are shown in Figure 4. Training led to an increase in GS3Kb activity compared to the C group ($p < 0.01$), but no differences were noted between the EX and EX-CHO groups, although EX-CHO has finished protocol with values 8% lower than EX. Similarly, PI3K was more active in the EX and EX-CHO groups ($p < 0.001$) compared to the C group. EX-CHO showed PI3k levels 18% greater than EX group but without statistical difference noted between these two groups. Meanwhile, Akt-1 presented significantly higher activity in the EX-CHO group compared to the C group ($p < 0.05$); however, Akt-1 activity was not greater in the EX group compared to the C group. Contrasting with these results, mTOR activity showed no differences between groups.

Insert Figure 4

4 Discussion

The main finding of this study is that carbohydrate supplementation promoted a discrete attenuation OT in rats by minimizing the deterioration in a physical performance test, which is the main indicator of overtraining (Smith 2000). This phenomenon was accompanied by an attenuation in the reduction of food consumption, thymus weight and loss of gastrocnemius muscle mass which are also indicators of OT, in addition to increased activity of Akt-1. However, the carbohydrates had no influence on several other markers of OT: biochemical markers, hormone activity, oxidative stress, body composition and the activity of other enzymes involved in protein synthesis (besides Akt-1).

The exercise protocol used to induce OT was based on a previously validated protocol for Wistar rats (Lira et al. 2010). The performance reduction in the performance test confirmed OT occurrence, and this phenomenon was accompanied with deleterious alterations in classical markers of overtraining: a reduction in food consumption, a reduction in serum testosterone levels and an increase in MDA concentration (Meeusen et al. 2013). In addition to the negative alterations in these markers, the present study also demonstrated thymus atrophy, an indicator of compromised immune activity (Woods et al. 2003) and, in particular, compromised immunity due to stress induced by physical exercise in rats (Sapin et al. 2005; Rogero et al. 2005).

In addition to delaying fatigue by mechanisms involved in glucose metabolism (Mitchell 2013; Coletta et al. 2013; Toone and Betts 2010) carbohydrates prevents exercise-induced immunosuppression (Walsh et al. 2011), reduces pro-oxidant activity (McAnulty et al. 2007), provides a less catabolic hormonal profile during and after the workout (Gleeson 2006) and promotes a greater rate of glycogen resynthesis after training sessions (Alghannam 2011). Although these effects may directly contribute to prevention of OT, the studies that presented these effects were acute in character, i.e., measurements occurred during a single exercise session or in protocols with two daily exercise sessions. These data do not allow extrapolation to a possible prevention OT over a season of several weeks or months of training, as is the reality for athletes. These phenomena can only be demonstrated with a chronic training and supplementation protocol.

Longer studies have been conducted (Halsen et al. 2004; Achten et al. 2004). However, data are insufficient to answer whether carbohydrate supplementation can prevent or minimize OT either because the study was too short (less than 11 days) or because only mood-related variables were evaluated, although an attenuation in the performance decrease has been observed.

Attenuation in the performance decrement as indicator of overtraining prevention during performance test by carbohydrate supplementation in rats corroborates studies' results with humans (Halsen et al. 2004; Achten et al. 2004). In these studies, runners and cyclists who consumed supplemental carbohydrates experienced an attenuation in performance decrease after a strenuous physical training period. However, the protocols lasted only eight or eleven days.

Anorexia is consensually associated with OT. It is an indicator of hypothalamic dysfunction induced by overproduction of cytokines, a phenomenon often observed in the presence of overtraining (Smith 2004). We were not able to quantify the levels of any cytokines, but the observation that supplementation induced a milder anorexic profile is of practical importance, as it alleviates the complications associated with OT-induced anorexia.

Xiao et al. (2012) and Dong et al. (2011) observed inhibition of the growth of the gastrocnemius muscle using the same protocol for OT induction as our study. Similarly, we found that carbohydrate supplementation was able to mitigate this muscle's loss of mass. This protective function of carbohydrates can be attributed to increased glycogen storage due to more food consumption in rats supplemented with carbohydrates. Indeed, muscle glycogen is essential to prevent catabolism from hard physical training (Yan et al. 1992). However, GS enzyme activity was not affected by carbohydrates, despite the observation by Morifuji et al. (2011) who demonstrated that carbohydrate supplementation is associated with higher GS activity and higher glycogen stores. On the other hand, the activity of Akt-1, one of the key enzymes involved in muscle protein synthesis, was increased in supplemented animals, indicating maintenance of anabolic and anti-catabolic activity.

It was expected that the increase in Akt-1 activity was accompanied with a concomitant increase in mTOR enzyme activity, but this did not happen in animals treated with carbohydrates. This result may be due to two reasons: 1) mTOR activation can occur

through an independent pathway through Akt-1, due to the contractile stimulus of exercise (Parkington et al. 2003) and 2) mTOR activation appears to be greater in fiber type IIa, (Deldicque et al. 2005) while the gastrocnemius muscle presents a mixed composition (Song et al. 1963). The activity of FOXO, the primary regulator of muscle catabolism, was not assessed in this study but may lend more insight into the relationship between carbohydrate supplementation and catabolic/anabolic pathways.

The weight of the lymphoid organs have been used as an indicator of the impact of exercise on the immune system (Woods et al. 2003). Ideally, the lymphoid organs react positively to an exercise stimulus, resulting in an improvement in the immune system (Terra et al. 2012). However, in response to chronic and exhausting protocols, these positive effects are minimized (Alghannam 2011). The hindered strengthening of the immune system was confirmed in an experiment in which a 16-week training protocol resulted in thymic atrophy (Woods et al. 2003), suggesting apoptosis from chronic stress. Our data from the two groups of animals subjected to OT also show thymic atrophy. Fortunately, carbohydrate supplementation was able to mitigate this deleterious effect, indicating an ergogenic action of carbohydrates in attenuating the decrease in immunocompetence. The observation of this interesting phenomenon guides prospects for future studies. For example, our data suggest a hypothesis that immune mediators are preserved in response to carbohydrate supplementation in the presence of chronic stress induced by intense and voluminous physical training. Indeed, there is evidence that carbohydrate supplementation preserves immunocompetence in response to single sessions of intense exercise (Gleeson 2006; McNulty et al. 2007).

It is well established that increased oxidative stress, systemic inflammation and cortisol production as well as reduced plasma concentrations of testosterone are found in the OT state (Hohl et al. 2009; Meeusen et al. 2013). Our data regarding pro-oxidant activity of malondialdehyde and testosterone confirm these previous findings. However, carbohydrate

supplementation was not able to prevent these deleterious effects of OT. Antioxidant activity has been reported for carbohydrates in some studies (Nieman et al. 2005; Scharhag et al. 2006). However, all these studies used acute strenuous exercise protocols. Thus, their results can only be cautiously extrapolated to chronic situations. It seems that the large magnitude of oxidative stress caused by strenuous consecutive training that leads to OT surpasses the antioxidant capacity previously reported for post-workout carbohydrates.

Taken together, the data from this study demonstrated that carbohydrate supplementation promotes a slight attenuation in the decrease of performance which is the most direct indicator of OT, a phenomenon that is accompanied by attenuation of some, but not all other physiological indicators of OT. Although there were no statistical differences found, the EX-CHO group completed the protocol with activity PI3k 18% higher than the EX group as well as cortisol 9.9% lower, activity GS3 8% more body weight and 2% above the EX group. Accordingly, these variables should not be discarded in future studies. Added to the markers that statistically minimize OT promoted by carbohydrate (lower decline in physical performance, smaller reduction in food consumption, less loss of lower thymus weight muscle loss), can express more evidently the protection of carbohydrates in minimizing or preventing OT. These data encourage the implementation of studies with athletes undergoing exhaustive training and competition sessions that characterize modern sports competitions.

A limitation of the study is the fact that adopted training protocol was the only validated to induce OT in rats. This protocol provides a training frequency that usually is not seen in humans. However, to meet the objectives of the study, we would have to adopt a protocol which will surely promote OT. Therefore, in this study it was found that if the carbohydrates minimize the OT, so that could not be observed if the carbohydrate prevents the OT.

394 Considering this study's limitations, we recommend extensions of this study that
395 include measurement of the contents of muscle and liver glycogen, the activity of the muscle
396 catabolic enzyme FOXO, and immunological agents responsive to physical training in
397 animals and, when possible, humans. We also recommend that this study be replicated with
398 more similar training loads to athletes. These data will help elucidate how carbohydrates can
399 prevent or minimize the development of OT syndrome.

400
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403 **Conflict of Interest:** none

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Table 1 – Weight of Animals, Organs and Peritoneal Fat after the OT induction protocol.

		C	EX	EX-CHO
Weight	G	436,7±74,9	316,0±22,4**	323,6±30,03**
Gastrocnemius	G	2,06±0,27	1,67±0,06**	1,74±0,23**
Gastrocnemius/ Body Weight	mg/g	4,89± 0,27	5,32±0,36	5,39±0,48*
Thymus	mg	0,339±0,14	0,124±0,03**	0,179±0,05**#
Thymus/ Body Weight	mg/g	0,79±0,22	0,39±0,11**	0,56±0,21**#
Peritoneal Fat	G	30,03± 11,44	7,46± 2,89**	8,29± 3,86**
Peritoneal Fat / Body Weight	g/g	0,06±0,01	0,02±0,0**	0,025±0,01**

Data are presented as the mean ± standard deviation. Weights of gastrocnemius, thymus and peritoneal fat are expressed as an absolute weight (g) and relative to the total body weight (mg/g or g/g). *p<0.05 compared to C; **p<0.001 compared to C in a one-way ANOVA; # p<0.05 between groups EX and EX-CHO, Student's t test.

Table 2 – Muscle damage markers, catabolic/anabolic hormonal activity and oxidative stress after the overtraining induction protocol.

		C	EX	EX-CHO
CK	U/L	903,1±767	1090,7±671,1	1264±745,7
Cortisol	ng/mL	30,33±9,35	30,66±8,3	27,63±10,21
Testosterone	ng/mL	1,70±0,8	0,65±0,42 ^{***}	0,55±0,3 ^{***}
Testosterone/Cortisol	-	0,05±0,03	0,02±0,01	0,02±0,01
MDA	μM	0,5±0,10	0,8±0,10 ^{**}	0,8±0,14 ^{**}

Data are presented as the mean ± standard deviation. C = sedentary control (n = 6); EX = exercise group (n = 9); EX-CHO = exercise group with carbohydrate supplementation (n = 11); CK = creatine kinase; MDA = malondialdehyde * p = 0.01 compared to C; *** p = 0.001 compared to C (one-way ANOVA with Tukey's post hoc test).

Figure 1 – Procedure for the experimental protocol for overtraining.

Figure 2 – Results of performance tests conducted along the overtraining induction protocol.

Pr1, Pr2 and Pr3 = Performance of animals before experimental protocol, at the 9th week and at the 11th week, respectively. C = control group (n = 9); EX = exercise group (n = 10); EX-CHO = exercise group with carbohydrate supplementation (n = 13). Data are mean and standard deviation. * = p<0.05 compared to C in the same Pr; \$ = p<0.05 compared to EX in Pr2. Two-way ANOVA with Tukey's post hoc analysis.

Figure 3 – Food consumption during the overtraining induction protocol.

C = control group (n = 9); EX = exercise group (n = 10); EX-CHO = exercise group with carbohydrate supplementation (n = 13). Data are mean and standard deviation. * = p< 0.01 compared to C in the 8th week; ** = p< 0.001 compared to C in the 8th week. Friedman test with Dunn's post hoc analysis.

Figure 4 – Activity of enzymes associated with glycogen synthesis (GS3Kb, panel A) and protein synthesis in the gastrocnemius muscle (PI3K, mTOR, and Akt 1; panels B, C and D, respectively) after the overtraining induction protocol.

C = control group (n = 7); EX= exercise group (n = 7); EX-CHO= exercise group with carbohydrate supplementation (n=7). Data are shown as the mean ± standard deviation. *p=0.01 compared to C; **p=0.0001 compared to C, *** p<0.0001 compared to C. One-way ANOVA with Tukey's post hoc test.

Figure 1 – Procedure for the experimental protocol for overtraining.
95x54mm (300 x 300 DPI)

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101x55mm (300 x 300 DPI)

Figure 4 – Activity of enzymes associated with glycogen synthesis (GS3Kb, panel A) and protein synthesis in the gastrocnemius muscle (PI3K, mTOR, and Akt 1; panels B, C and D, respectively) after the overtraining induction protocol.

C = control group (n = 7); EX= exercise group (n = 7); EX-CHO= exercise group with carbohydrate supplementation (n=7). Data are shown as the mean $\pm$ standard deviation. *p=0.01 compared to C; **p=0.0001 compared to C, *** p<0.0001 compared to C. One-way ANOVA with Tukey’s post hoc test.

94x56mm (300 x 300 DPI)