The Effects of Resistance Training Volume on Skeletal Muscle Proteome

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ABSTRACT

International Journal of Exercise Science 10(7): 1051-1066, 2017. Studies are conflicting to whether low volume resistance training (RT) is as effective as high-volume RT protocols with respect to promoting morphological and molecular adaptations. Thus, the aim of the present study was to compare, using a climbing a vertical ladder, the effects of 8 weeks, 3 times per week, resistance training with 4 sets (RT4), resistance training with 8 sets (RT8) and without resistance training control (CON) on gastrocnemius muscle proteome using liquid chromatography mass spectrometry (LC-MS/MS) and cross sectional area (CSA) of rats. Fifty-two proteins were identified by LC-MS/MS, with 39 in common between the three groups, two in common between RT8 and CON, one in common between RT8 and RT4, four exclusive in the CON, one in the RT8, and four in the RT4. The RT8 group had a reduced abundance of 12 proteins, mostly involved in muscle protein synthesis, carbohydrate metabolism, tricarboxylic acid cycle, anti-oxidant defense, and oxygen transport. Otherwise one protein involved with energy transduction as compared with CON group showed high abundance. There was no qualitative protein abundance difference between RT4 and CON groups. These results revealed that high volume RT induced undesirable disturbances on skeletal muscle proteins, while lower volume RT resulted in similar gains in skeletal muscle hypertrophy without impairment of proteome. The CSA was significantly higher in RT8 group when compared to RT4 group, which was significantly higher than CON group. However, no differences were found between trained groups when the gastrocnemius CSA were normalized by the total body weight.

KEY WORDS: Histological analysis, liquid chromatography-tandem mass spectrometry, gastrocnemius proteome, resistance training, training volume

INTRODUCTION

Exercise has been widely recommended as an important tool to improve muscle strength, muscle cross-sectional area and general health parameters. Different exercise protocols, such as resistance training (RT), jumping, swimming, wheel running and treadmill running in rodents results in positive adaptations in skeletal muscle (1,6,15,16). Although the phenotypic alterations induced by exercise training have been widely described, the molecular mechanisms, mainly proteomics muscle profile, responsible for skeletal muscle adaptations are not fully understood.

Molecular networks of exercise-induced changes in skeletal muscle have been characterized by proteomics, which is a technique based on mass spectrometry (12,13). One advantage of this method is the ability to quantify a large number of proteins at the same time, whereas immunoblotting can only be utilized to analyze individual target proteins previously identified (or speculated) to be of importance to a particular physiological adaptation (25).

In order to progressively promote training adaptations during RT, various factors can be manipulated including a) the number of exercises, b) repetitions performed per set, or c) sets per exercise. Moreover, the increase of RT volume results in positive neural adjustments, increased muscle mass, and improved metabolic and endocrine responses (17). However, excessive training, including an increased volume, may result in lipid peroxidation in skeletal muscle and underperformance related to overtraining syndrome (4).

Furthermore, it has been demonstrated that four weeks of endurance or resistance training differently modified individual muscle protein expression, and that these responses may be associated with specific muscular adaptations to RT in humans (20). Thus, the investigation of different RT volume on muscle proteomics may contribute to new insights into training adaptations. The aim of the current investigation was to determine the effects of eight weeks of low volume RT (4 sets of climbing a vertical ladder) and high volume (8 sets) on gastrocnemius muscle proteome and muscle cross sectional area (CSA) of rats, and compare with a control group. The initial hypothesis was that a higher RT volume will increase muscle CSA to a greater extent without negative changes in muscle proteomics.

METHODS

Animals
Fourteen male *Wistar* rats aged 5 months were divided into 3 groups: resistance training with 4 sets (RT4, n=5), resistance training with 8 sets (RT8, n=5) and control group (CON, n = 4). All animals came from the Central vivarium of the *Faculdade de Educação Física da Universidade Católica de Brasília*. The animals were housed in polypropylene cages (4 animals per cage) at a temperature of 23 ± 2 °C with 12:12h dark:light cycle and food and water provided ad libitum. All procedures were approved by the Institutional Animal Care and Uses Local Committee (CEUA UCB) and were in accordance with international ethical standards.

Protocol

The RT protocol was adapted from Hornberger and Farrar (6), and procedures are described elsewhere (16,17,22). Briefly, the duration of resistance training lasted 8 weeks and was performed 3 times per week. A initial adaptation period was provided which consisted of the animals climbing a vertical ladder (1.1 m, 0.18 m, 2-cm grid, 80 ° incline, 8-12 movements per climb) with a load secured to their tails. When necessary, a stimulus was applied to the animal's tail to initiate movement. At the top of the ladder, the animals rested for 120 s and the procedure was repeated until the animal voluntarily climbed the ladder 3 consecutive times without a stimulus applied.

Training sessions began 3 days following the familiarization protocol, and involved 4 (RT4) or 8 (RT8) ladder climbs with progressively heavier loads. The initial climb was equivalent to 75 % of the body mass. Subsequently, an additional 30 g weight was added until a load was reached which the animal could not climb the entire ladder length. Failure was determined when the animal could not complete the ladder climb after 3 consecutive stimuli to the tail. The greatest load successfully carried the entire ladder length was deemed the maximal carrying capacity for that training session and used for calculate the training load for the next session. Training sessions for the RT4 group consisted of a total of four ladder climbs in increasing intensity (50%, 75%, 90%, and 100% of maximal carrying capacity). The RT8 group performed two climbs at each intensity for a total of 8 climbs. Total volume training (total ladder climbs completed × load) was adapted from Tibana, et al.(7) according to the experimental model.

Animals were anesthetized using an intraperitoneal injection with a solution of xylazine (12 mg/Kg of body weight) five days after the last training session and further euthanized using ketamine (95 mg/Kg of body weight) and weighed. Five days after the last RT session, the central part of the gastrocnemius muscle from all experimental groups (4 animals for CON; 5 animals for RT4 and 5 animals for RT8) were dissected and immediately washed in saline solution. Samples were weighed, frozen in liquid nitrogen and conserved at -80°C. Subsequently, samples were fixed in the metallic supports of a cryostat. (Micron HE 505, Jena, Germany) at -20°C with small stickers (OCT – Tissue Tek Compound) and oriented with slices of 5 m that allowed a transversal observation of muscle fibers adjusted in glass laminas. Six histological laminas of each animal were than stained with hematoxylin-eosin (HE) to evaluate cross sectional area (CSA).

Images from five different muscle regions were obtained by a capture system and computerized image analysis with a digital camera Axioplan (Carl Zeiss, Oberkochen, Germany) at 10X amplification coupled to a binocular microscope (Olympus® BX51). The CSA of 100 fibers was randomly chosen from each image and measured by using the software Image J. The normalization of these values was calculated by the mean CSA divided by the animal body mass (CSA / body mass). All images were analyzed by the same researcher in a blinded design, in which the analyzer was not aware of the experimental group.

The gastrocnemius was carefully removed, weighed and partitioned into two at the middle of the belly: with the proximal fragment used for the CSA analysis and the distal segment for proteomic assessment. With regard to CSA evaluation, the muscle segment was frozen immediately in isopentane, pre-cooled in liquid nitrogen and stored in a freezer at -80°C (Forma Scientific, Marietta, Ohio). Histological cross-sections (10 µm) from the middle belly of each gastrocnemius muscle were obtained in cryostat (Micron HE 505, Jena, Germany), stained with Touluidine Blue/1% Borax (TB) and accessed by light microscopy (Axiolab, Carl Zeiss, Jena, Germany). The CSA of 100 randomly chosen fibers from each picture was measured using the Axiovision 3.0.6 SP4 software (Carl Zeiss, Jena, Germany) totalizing 600 muscle fibers per animal. Data relating to CSA is shown in distribution levels.

The frozen distal muscle segment was macerated in liquid nitrogen and mechanically homogenized with bicarbonate ammonia 50 mM and PIC (Cocktail protease inhibitor), until samples were completely homogenized. Samples were then sonicated for 30 minutes and incubated overnight in a refrigerator (4°C). Subsequently, the sample was centrifuged for 30 min at 14,000 x g. The supernatant was removed and assayed by Qubit® and the remaining samples were used in the LC-MS analysis.

Samples were prepared for LC-MS analysis by tryspin digestion. For this procedure, 20uL of 50mM $NH4HCO2$ (Vetec) and 50 µl of solution RapiGEST™ (Waters, Milford, MA, USA) was added to each sample, and incubated at 80°C for 15 min and subjected to centrifugation for 15 sec. Subsequently, the supernatant was added to 5 µl of 100 mM dithiothreitol (GE Healthcare) and then the sample was stirred and incubated at 60 °C for 30 min. After the incubation period, the samples were left to cool to room temperature and centrifuged for 15 sec. Subsequently, samples were added to 5 µl 300 mM iodoacetamide (GE Healthcare), shaken and incubated for 30 min at room temperature and protected from light. Then, the sample was added to 40 µL of trypsin solution (Promega Corporation, Brazil) in 50 mM NH_4HCO_2 (Vetec) at a concentration of 1:1000; and samples were again shaken and incubated at 37°C for 18 h to digestion. After digestion, 20 µl of trifluoroacetic acid (TFA) 5% (Mallinckr) was added, shaken and samples incubated for 37°C for 90 min. Then, they were centrifuged at 14,000 x g, at 6°C for 30 min. The supernatant was transferred to a microcentrifuge tube (1.5ml) and again evaporated in a lyophilize (LIOTOP). Finally, the samples were resuspended in 0.1% formic acid in LC-MS degree water, mixed, centrifuged for 5 min at 14,000 x g and supernatant then removed and used for analysis.

The LC-MS/MS experiments were performed utilizing high-efficiency liquid chromatography, Shimadzu (Kyoto, Japan) linked to a micrOTOF ESI-Q III (Bruker Daltonics, Germany) mass spectrometer and the peptide separation was performed by making use of an XR-ODS C18 column (2.0 mm x 30 mm x 2.2 microns; Kyoto, Japan). The solvents used were LC-MS degree water (A) and acetonitrile (B) in both solvents were added 0.1% formic acid. The method construed was a gradient of 5% B for 5 min; 5-50% B for 50 min; 50- 95% B for 10 min; 95% B for 8 min; 95-5% B for 1 min and 5% B for 7 min, adding up to 80 min under a flow of 400 µL.min-1. The LC-ESI-MS/MS analyses were performed under positive linear mode, in the following parameters: spray voltage, 4.5 kV, by using nitrogen (N_2) as a source of ionization,

nebulizer 4.0 Bar, Drygas 8.0, drytemperature 200 °C. Ions were scanned in a range of 300-3000 m/z followed by five MS/MS scans. The fragmentation (MS/MS) raw data, which were generated by the equipment, were analyzed by using Data Analysis software (Bruker Daltonics, Germany), in all ions that were fragmented according to the parameters mentioned above were separated by means of the AutoMs (n) tool; as a result, the spectra were deconvoluted by using the deconvolution tool in order to determine the peptide levels fragmentation. The data were imported into the Biotools software (Bruker Daltonics, Germany) where the searches were conducted by using the MASCOT algorithm (Matrix Science, London, United Kingdom, version 2.3). The database used in this study included the UniProt, SwissProt, (Switzerland, Geneva), and searches focused on the Rattus spp. taxonomy (546, 439 sequences and 194, 445 396 waste). Trypsin was selected as a cleavage enzyme, followed by possible changes: cysteine carbamidomethylation (C*), as fixed modification, and methionine oxidation (Mox), as variable modification. The masses tolerance for precursor ions (MS) and for fragments of ions (MS/MS) was ± 0.2 Da. The inclusion criteria for identification of the protein was due to the presence of the protein in at least two of triplicates and are present in three of the five animals from trained groups and two of the four animals from control group analyzed with detection of 1 to 2 ion traces of protein and at least one peptide per protein. The protein identifications considered were those for which the peptides score was greater than the minimum score for the 95% confidence level ($p < 0.05$), which in turn was obtained by matching each peptide to its theoretical fragmentation profile.

Protein quantification was performed in triplicate and calculated by considering the peak area of the ions found. Two ions were extracted by means of an extracted ion chromatogram tool (XICs), which were associated with the identified protein. The area of each ion was calculated by Integrate Only Chromatogram tool (Bruker Daltonics, Germany) and these values were taken into consideration for the intensity calculations and subsequent comparison with the other groups.

Statistical analysis
The results are expressed as means ± standard deviation (SD). Shapiro-Wilk tests were applied to check for normality distribution of study variables. A two-way mixed model ANOVA was used to compare the training load differences between RT4 and RT8 groups. For comparison of study variables between groups a one-way ANOVA with Bonferroni post hoc test was used. The power of the sample size was determined using G*Power version 3.1.3 (3), based on the differences of CSA of the gastrocnemius between groups. Considering the sample size of this study and an alpha error of 0.05, the power ($1 - \beta$) achieved in this research was 1.0 for the CSA variable. For the inter-group comparative analysis of protein abundance levels, only proteins with up-regulation of Student t test $p \geq 0.95$ and down-regulation of $p \leq 0.05$ were considered, with a delta (Δ) of at least ($\geq$) 0.5-fold change in Log(e) ratio between the treatments. Among these proteins, p-values less than 0.05 were considered significant (13). The SPSS version 20.0 (Somers, NY, USA) software was used.

RESULTS

Pre and post-training body mass was 384.5 ± 42.6 g versus 396.2 ± 36.1 g for CON group, 349.2 ± 32.2 g versus 365.4 ± 36.5 g for RT4 group and 368.8 ± 32.7 g versus 379.0 ± 33.0 g for RT8 group, with no differences between groups (p > 0.05). Post-training body mass was significantly higher than pre-training for RT4 (p = 0.004) and RT8 (p = 0.009) groups. Post-training gastrocnemius mass was 2.29 ± 0.32 g for CON group, 2.18 ± 0.37 g for RT4 and 2.39 ± 0.43 g for RT8 group (with no differences between groups; p > 0.05).

Training load volume progression is presented in figure 1. RT8 group performed significantly higher (p < 0.001) training volume than RT4 group during the 8 weeks of training. A significant increase (p < 0.05) occurred in the training volume until week 5 for RT4 and until week 7 for RT8. The training loads of the groups submitted to training are represented in the Figure 1.
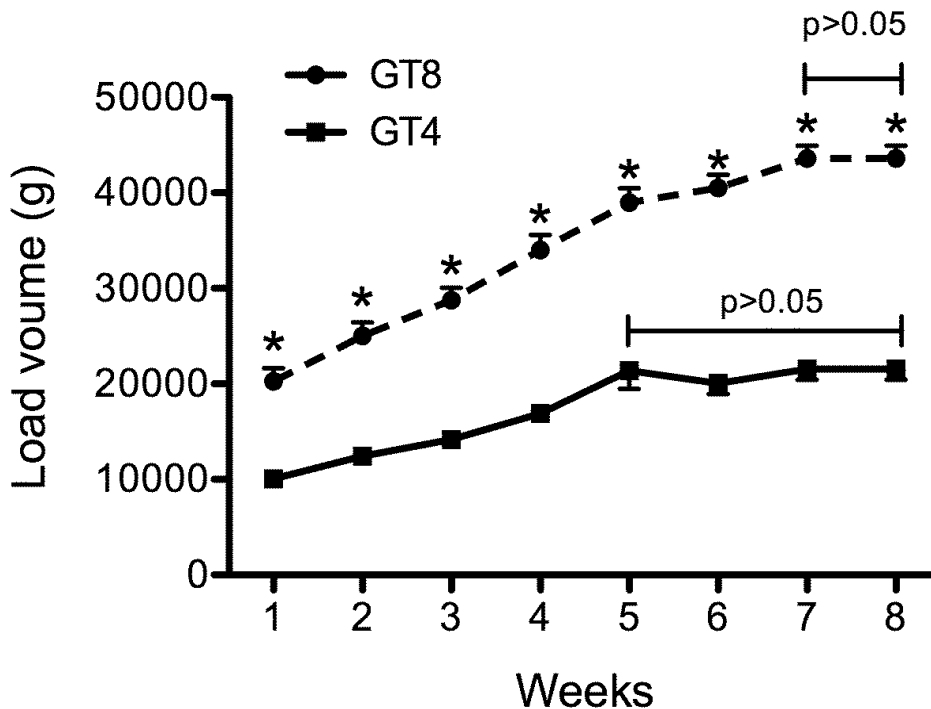

Figure 1. Mean ± SD of training load volume progression during the 8 weeks for GT4 and GT8 groups. * p < 0,05 for GT4 group.

By using LC-MS/MS analysis, 52 proteins were identified in gastrocnemius muscle (supplementary table 1). Thirty-nine appeared in all groups; 2 in CON and RT8 groups; 1 in RT4 and RT8 group; 4 appeared only in CON group; another 4 in RT4 group; and 2 in only the RT8 group (Figure 2).

Supplementary table 1. LC-MS/MS results. Identified proteins from gastrocnemius muscle extract.

PROTEIN NAME	PRIMARY NAME	MASS (Da)	Mascot Score	SEQUENCE	GROUP
Fructose-bisphosphate aldolase A	ALDOA_RAT	39,352	38	FSNEEIAMATVTALR	CON, RT4, RT8
Creatine kinase M-type	KCRM_RAT	43,045	29	FCVGLQK 2: Carbamidomethyl (C)	CON, RT4, RT8
Actin, alpha skeletal muscle	ACTS_RAT	42,051	17	IIAPPER	CON, RT4, RT8
Beta-enolase	ENOB_RAT	47,014	27	TLGPALLEK	CON, RT4, RT8
Glyceraldehyde-3-phosphate dehydrogenase	G3P_RAT	35,828	119	WGDAGAEYVVESTGVFTTMEK	CON, RT4, RT8
Glycogen phosphorylase, muscle form	PYGM_RAT	97,273	29	LPAPDEKI	CON, RT4, RT8
Tropomyosin alpha-1 chain	TPM1_RAT	32,681	63	QLEDELVSLQK	CON, RT4, RT8
Serum albumin	ALBU_RAT	68,731	18	LVQEVTDFAK	CON, RT4, RT8
Pyruvate kinase PKM	KPYM_RAT	57,818	51	VNLAMNVGK	CON, RT4, RT8
L-lactate dehydrogenase A chain	LDHA_RAT	36,451	22	LVIITAGAR	CON, RT4, RT8
Phosphoglycerate mutase 2	PGAM2_RAT	28,755	38	FLGDEETVR	CON, RT4, RT8
Phosphoglycerate kinase 1	PGK1_RAT	44,538	64	AEPAKIDAFR	CON, RT4, RT8
Triosephosphate isomerase	TPIS_RAT	26,849	20	FFVGGNWK	CON, RT4, RT8
Carbonic anhydrase 3	CAH3_RAT	29,431	18	VVFDDTFDR	CON, RT4, RT8
Adenylate kinase isoenzyme 1	KAD1_RAT	21,584	34	ATEPVISFYDKR	CON, RT4, RT8
Phosphoglucomutase-1	PGM1_RAT	61,403	8	QQFDLENK	CON, RT4, RT8
Myoglobin	MYG_RAT	17,157	6	YKELGFQG	CON, RT4, RT8
Hemoglobin subunit alpha-1/2	HBA_RAT	15,329	47	MFAAFPTTK	CON, RT4, RT8
Glucose-6-phosphate isomerase	G6PI_RAT	62,827	3	VKEFGIDPK	CON, RT4, RT8
Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	AT2A1_RAT	109,409	22	NMLFSGTNIAAGK	CON, RT4, RT8
Hemoglobin subunit beta-1	HBB1_RAT	15,979	52	VINAFNDGLK	CON, RT4, RT8
Malate dehydrogenase, cytoplasmic	MDHC_RAT	36,483	55	LGVTADDVK	CON, RT4, RT8
Myosin light chain 1/3, skeletal muscle M isoform	MYL1_RAT	20,680	65	ITLSQVGDVLR	CON, RT4, RT8
Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	GPDA_RAT	37,453	54	KLTEIINTQHENVK	CON, RT4, RT8
ATP-dependent 6-phosphofructokinase, muscle type	PFKAM_RAT	85,560	48	IGLIQGNR	CON, RT4, RT8
Protein deglycase DJ-1	PARK7_RAT	19,974	42	APLVLKD	CON, RT4, RT8
Heat shock cognate 71	HSP7C_RAT	70,871	44	TTPSYVAFTDTER	CON, RT4, RT8
ATP synthase subunit beta, mitochondrial	ATPB_RAT	56,354	74	VLDSGAPIKIPVGPETLGR	CON, RT4, RT8
Aspartate aminotransferase, cytoplasmic	AATC_RAT	46,429	29	APPSFFAQVPQAPPVLVFK	CON, RT4, RT8
Troponin T, fast skeletal muscle	TNNT3_RAT	30,75	72	KPLNIDHLSDDKLR	CON, RT4, RT8
Malate dehydrogenase, mitochondrial	MDHM_RAT	35,684	44	MIAEAIPELK	CON, RT4, RT8
14-3-3 protein gamma	1433G_RAT	28,303	24	TAFDDAIAELDTLNEDSYKDSTLIMQLLR	CON, RT4, RT8
Superoxide dismutase [Cu-Zn]	SODC_RAT	15,912	44	VISLSGEHSIIGR	CON, RT4, RT8
Myosin regulatory light chain 2, skeletal muscle isoform	MLRS_RAT	15,912	46	GADPEDVITGAFK	CON, RT4, RT8
Elongation factor 1-alpha 1	EF1A1_RAT	50,114	29	IGGIGTVPVGR	CON-RT4, RT8
Aconitate hydratase, mitochondrial	ACON_RAT	85,425	62	IVYGHLDDPANQEIER	CON, RT4, RT8
Heat shock protein beta-1	HSPB1_RAT	22,893	28	LFDQAFGVPR	CON, RT4, RT8
Aldose reductase	ALDR_RAT	35,797	23	AIGVSNFNPLQIER	CON, RT4, RT8
Galectin-1	LEG1_RAT	14,857	17	SFVLNLGK	CON, RT4, RT8
Troponin I, fast skeletal muscle	TNNI2_RAT	21,328	22	MSADAMLK	CON
Alpha-crystallin B chain	CRYAB_RAT	20,089	5	HFSPEELKVK	CON, RT4, RT8
Citrate synthase, mitochondrial	CISY_RAT	51,867	22	AALPSHVVTMLDNFPTNLHPMSQLSAAITALNSESNFAR	CON, RT4, RT8
Isocitrate dehydrogenase [NADP], mitochondrial	IDHP_RAT	50,967	35	LNEHFLNTTDFLDTIKSNLDR	CON, RT4, RT8
Filamin-C	FLNC_RAT	290,986	23	EAMQQADDWLGVPQVIAPEEIVDPNVDEHSVMTYLSQFPK	CON
Electron transfer flavoprotein subunit alpha, mitochondrial	ETFA_RAT	34,951	61	TIVAINKDPEAPIFQVADYGIVADLFK	CON, RT4, RT8
Cysteine and glycine-rich protein 3	CSRP3_RAT	20,803	44	SLESTNVTDKDGELYCK 16: Carbamidomethyl (C)	CON, RT4, RT8
Calsequestrin-1 OS	CASQ1_RAT	46,449	57	AYEDAAEEFHPYIPFFATFDSK	CON, RT4, RT8
Microtubule-associated protein 4	MAP4_RAT	110,301	15	LATTVSAPDLK	CON, RT4, RT8
Serotransferrin	TRFE_RAT	76,395	28	TVLPADGPR	CON
Heat shock protein beta-6	HSPB6_RAT	17,505	15	FGEGLLEAELASLCPAAIAPYYLR 14: Carbamidomethyl (C)	CON, RT4, RT8
PDZ and LIM domain protein 3	PDLI3_RAT	39,106	41	LSGGIDFNQPLVITR	CON, RT8

| Tropomyosin beta chain	TPM2_RAT	32,837	28	AISEELDNALNDITSL	CON, RT4, RT8
Actin, aortic smooth muscle	ACTA_RAT	42,009	33	LDLAGRDLTDYLMK	CON, RT4, RT8
14-3-3 protein epsilon	1433E_RAT	29,174	38	AAFDDAIAELDTLSEESYKDSTLIMQLLR	CON, RT4, RT8
Creatine kinase S-type, mitochondrial	KCRS_RAT	47,385	27	LIDDHFLFDKPVSPLLTCAGMAR 18: Carbamidomethyl (C)	CON, RT4, RT8
Elongation factor 1-alpha 2	EF1A2_RAT	50,454	44	VETGILRPGMVVTFAPVNITTEVK	CON, RT4, RT8
Desmin	DESM_RAT	53,457	1	LEEEIRHLK	CON, RT4, RT8
Histone H2B type 1	H2B1_RAT	13,99	74	AMGIMNSFVNDIFER	CON, RT4
Tripartite motif-containing protein 72	TRI72_RAT	52,832	37	MFLAALESSLDREAER	CON, RT4, RT8
Four and a half LIM domains protein 1	FHL1_RAT	31,904	46	AIVAGDQNVEYK	CON, RT4, RT8
Nucleoside diphosphate kinase A	NDKA_RAT	17,193	23	VMLGETNPADSKPGTIR	CON, RT4, RT8
Myosin-binding protein C, slow-type (Fragment)	MYPC1_RAT	68,737	47	FTITGLPTDAK	CON, RT4, RT8
Myosin-4	MYH4_RAT	222,88	33	LQDLVDKLQTK	CON, RT4, RT8
ATP synthase subunit alpha, mitochondrial	ATPA_RAT	59,754	30	TGAIVDVPVGDELLGR	CON, RT4, RT8
Myc box-dependent-interacting protein 1	BIN1_RAT	64,533	38	LVDQALLTMDTYLGQFPDIK	CON, RT4
Vimentin	VIME_RAT	53,733	40	ILLAELEQLKGQGK	CON
Peroxiredoxin-1	PRDX1_RAT	22,109	38	ATAVMPDGQFK	CON, RT8
Nestin	NEST_RAT	208,797	21	AVGNEQMAVSPPEK 7: Oxidation (M)	CON
Acetyl-CoA acetyltransferase, mitochondrial	THIL_RAT	44,695	49	IAAFADAAVDPIDFPLAPAYAVPK	CON
Peroxiredoxin-5, mitochondrial	PRDX5_RAT	22,179	17	ALNVEPDGTGLTCSLAPNILSQL 13: Carbamidomethyl (C)	CON, RT4, RT8
Nucleoside diphosphate kinase B	NDKB_RAT	17,283	25	NIIHGSDSVESAEKEIGLWFKPEELIDYK	CON, RT4, RT8
Myosin-binding protein H	MYBPH_RAT	52,656	47	VTAVNSAGAGPPAVLDQPVHIQEITEAPK	CON, RT8
Phosphatidylethanolamine-binding protein 1	PEBP1_RAT	20,801	41	VDYGGVTVDELGK	CON, RT4
Glycogen phosphorylase, brain form (Fragment)	PYGB_RAT	96,174	9	NLAENISR	CON, RT4, RT8
Long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADL_RAT	47,873	1	ELWEKAGK	CON
Histidine triad nucleotide-binding protein 1	HINT1_RAT	13,777	27	AQVAQPGGDTIFGK	CON
Glutathione S-transferase Mu 2	GSTM2_RAT	25,703	62	LGLDFPNLPYLIDGSHK	CON, RT4, RT8
Histone H2A type 1-C	H2A1C_RAT	14,077	38	VTIAQGGVLPNIQAVLLPK	RT4
Tubulin beta-5 chain	TBB5_RAT	49,671	29	LTTPTYGDLNHLVSATMSGVTTCLR 23: Carbamidomethyl (C)	RT4
NEUREXIN-2	NRX2A_RAT	185,169	31	ESADTLR	RT4
Meiosis arrest female protein 1	MARF1_RAT	192,642	15	NAADDKLR	RT4, RT8
Myosin-3	MYH3_RAT	223,858	82	MKGTLEDQIISANPLLEAFGNAK	RT4, RT8
Centrosomal protein CEP57L1 1	CE57L_RAT	50,274	17	DRSFQPVK	RT4
IQ DOMAIN-CONTAINING PROTEIN G	IQCG_RAT	48,757	19	QLVDVKR	RT4, RT8
MICAL-like protein 1	MILK1_RAT	92,644	16	MAGPRGALLAWCR 12: Carbamidomethyl (C)	RT4
F-box only protein 43	FBX43_RAT	73,228	33	GTPTVGDLVRQPK	RT4
DNA endonuclease RBBP8	COM1_RAT	100,705	30	MDVTVIDTK 1: Oxidation (M)	RT4, CON
Tubulin alpha-4A chain	TBA4A_RAT	49,924	54	AVFVDLEPTVIDEIR	RT4, CON
Alpha-actinin-1	ACTN1_RAT	102,96	25	TFTAWCNSHLR 6: Carbamidomethyl (C)	RT8
NON-POU DOMAIN-CONTAINING OCTAMER-BINDING PROTEIN	NONO_RAT	54,925	20	GTFPDAR	RT8
Receptor tyrosine-protein kinase erbB-3	ERBB3_RAT	147,546	38	IPALDPEK	CON, RT8
Heat shock 70	HS71A_RAT	70,185	45	TTPSYVAFTDTER	RT8
HISTONE ACETYLTRANSFERASE	KAT7_RAT	70,514	15	ASEDLEK	CON, RT8
Lymphocyte activation gene 3 protein	LAG3_RAT	57,988	27	QLSAAVTLAVITVTPK	RT8
Endothelial lipase	LIPE_RAT	55,924	13	LVSALQTREK	RT8
ATP-binding cassette sub-family B member 6, mitochondrial	ABCB6_RAT	93,305	40	AIQASLAK	RT8
TRIPARTITE MOTIF-CONTAINING PROTEIN 55	TRI55_RAT	59,98	19	STSLNYK	CON
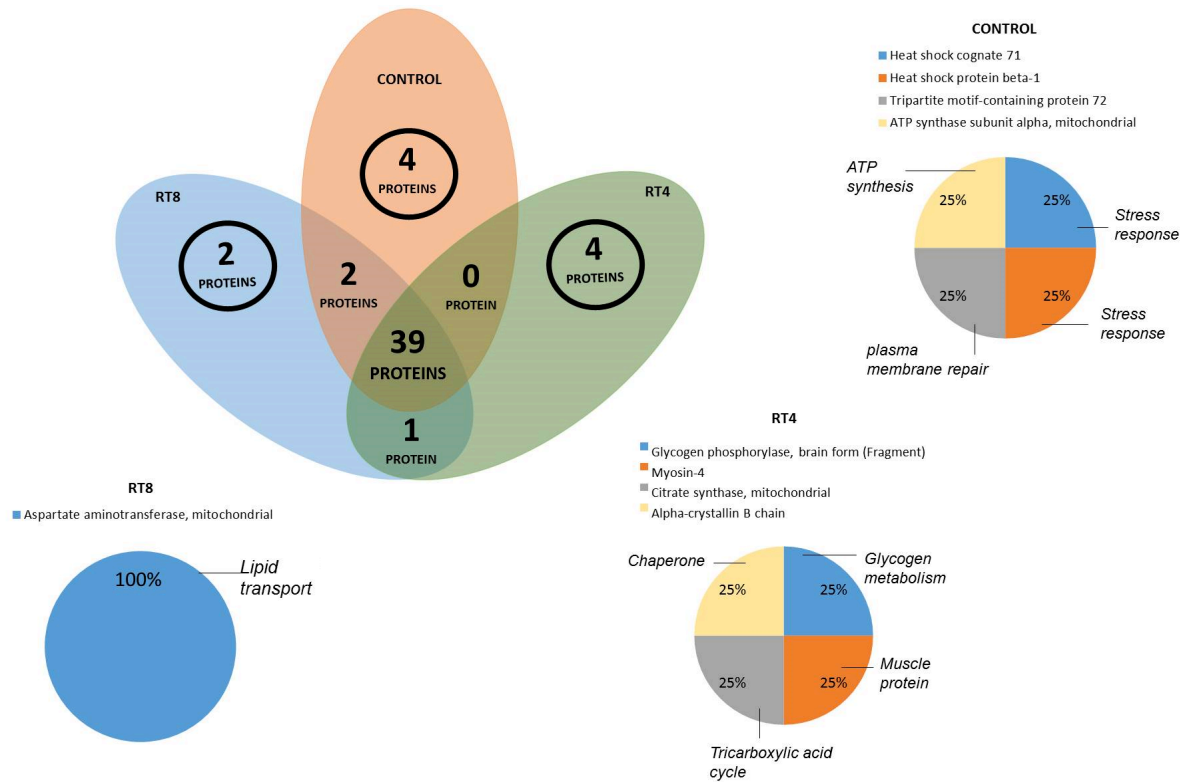
Figure 2. Venn diagram analysis of identified proteins interplayed in RT8 (blue), CON (red) and RT4 (green) groups. Exclusive proteins are itemized and assigned into a biological process according to Gen Ontologies (GO) annotations.

The ratio of protein abundance levels in the RT4: CON and the ratio of RT8: CON were evaluated. This portion of the analysis aimed to assess the effect of high and low volume RT over non-exercised gastrocnemius muscle proteome (CON). From the 13 proteins altered after RT8 intervention, the concentration of 12 of them were reduced (down-regulation) and only 1 was enhanced (up-regulation; figure 3). When the RT4 group was compared to the CON group, no differences were found regarding the ratio of protein abundance.

Figure 4 presents the CSA of the gastrocnemius muscle after the intervention. The CSA was significantly higher ($p < 0.001$) in RT8 group when compared to RT4 and CON groups. RT4 group also had a significantly higher ($p < 0.001$) CSA than CON group. However, no differences ($p = 0.970$) were found between RT4 and RT8 groups when the CSA of the gastrocnemius muscle were normalized by the total body weight.

In this sense, the main objectives of this study were (i) to compare the effects of eight weeks of lower volume and higher volume of RT on gastrocnemius muscle proteome (ii) and to assess the efficacy of two different RT volumes on muscle cross sectional area (CSA) of rats. Proteomic analysis (LC-MS/MS) identified 51 proteins, with 39 in common between groups, two in common between RT8 and CON groups (nucleoside diphosphate kinase B and troponin T, fast skeletal muscle), one in common between RT8 and RT4 (myosin regulatory light chain 2, skeletal muscle isoform), four exclusive in the CON (heat shock cognate 71 protein, heat

shock protein beta-1, tripartite motif-containing protein 72, ATP synthase subunit alpha, mitochondrial), one in the RT8 (aspartate aminotransferase, mitochondrial), and four in the RT4 (glycogen phosphorylase, brain form, myosin-4, citrate synthase, mitochondrial and alpha-crystallin B chain). The RT8 group presented a decrease in the abundance of 12 proteins (involved in muscle protein synthesis, carbohydrate metabolism, tricarboxylic acid cycle, anti-oxidant defense, and oxygen transport) and 1 increased (involved with energy transduction) as compared with CON, while there was no difference between RT4 and CON groups (figure 5). Moreover, the CSA was higher in RT8 group as compared with RT4 and CON groups. RT4 group presented a higher muscle CSA as compared with CON group. However, no differences were found between RT4 and RT8 groups when the CSA of the gastrocnemius muscle were normalized by the total body weight. Taken together, the results revealed that higher volume of RT induced a disturbance with respect to skeletal muscle proteins. In addition, lower volume RT resulted in similar muscle hypertrophy gains without negative effects on skeletal muscle proteomics. Thus, the initial hypothesis, that a higher RT volume should increase muscle CSA to a greater extent without negative changes in muscle proteomics, was not confirmed.
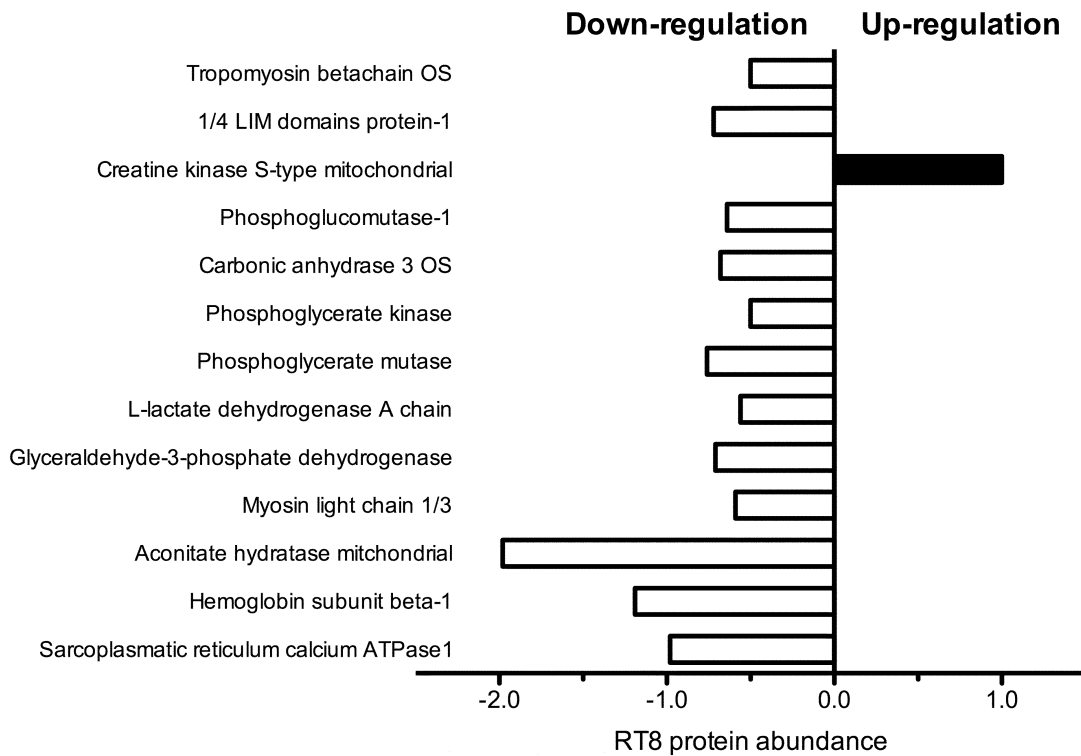

Figure 3. Histogram of protein abundance levels due to exercise training on gastrocnemius muscle proteome from intergroup analyses utilizing only proteins with up-regulation (p ≥ 0.95) and down-regulation (p ≤ 0.05) with at least (≥) 0.5-fold change. The x-axis represents the Log(e) ratio between the RT8 and CON groups. Reduction in protein abundances are represented in negative scale (white bars) on the left side and the improvement in protein abundance, in positive scale (black bars) on the right side of the y-axis. No proteins with up- or down-regulation were seen in RT4 group.
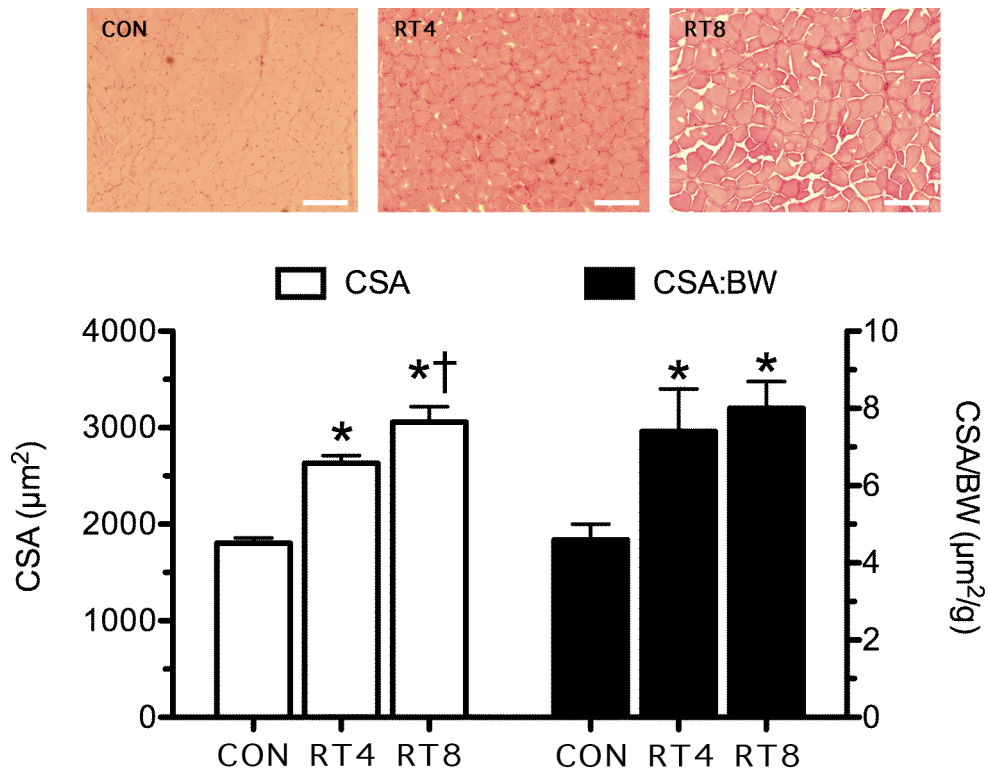
Figure 4. Histological sections representing the gastrocnemius muscle cross sectional area (CSA) are exemplified by the images at the top. Below in the graphic, Mean ± SD of muscle CSA and CSA normalized by body weight (CSA:BW) for CON, RT4 and RT8 groups. * p < 0,05 for CON group; † p < 0,05 for RT4 group. (10x). Bar: 100 um.

DISCUSSION

To the author's knowledge, this was the first study to evaluate the effects of eight weeks of low volume RT and high volume on gastrocnemius muscle proteome and muscle cross sectional area (CSA) of rats, and compare with a control group. The key findings were that high volume RT induced a disturbance on skeletal muscle proteins, while lower volume RT resulted in similar gains in skeletal muscle hypertrophy without impairment of skeletal muscle proteins.

It has been proposed that a single bout of strenuous exercise in untrained animals is more stressful when compared to chronic training. Interestingly, different endurance exercise intensity, volume, and type result in distinct muscle proteomic modulations, where isolated bouts of exercise generally result in reduced alteration in protein expression (14), a finding consistent with the results of the present study performed with resistance exercise. Hody et al. (5) investigated the proteomic response from untrained and trained rectus femoral muscle to two acute intense eccentric exercises (composed of three sets of 30 maximal contractions utilizing an isokinetic dynamometer). Large quantities of myosin heavy chain isoforms as well as several other contractile proteins were decreased after both eccentric sessions in trained and untrained subjects, which was demonstrated in the RT8 group of the present study.
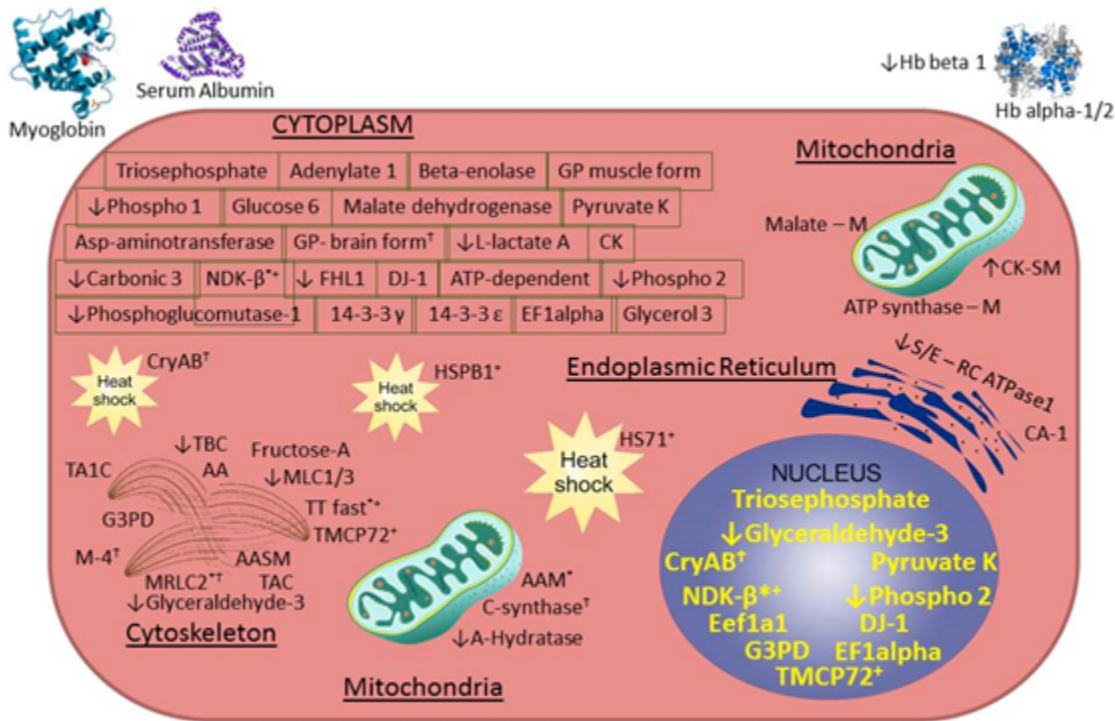
Figure 5. Modulation in gastrocnemius muscle proteome. The main results found in this study are presented in this muscle cell representation. *proteins found exclusively to RT8 group; [†]proteins found exclusively to RT4 group; [+]proteins found exclusively to CON group (↑): increased abundance levels in contrast to the CON, (↓): reduced abundance levels in contrast to the CON. Protein nomenclature: 14-3-3 γ= 14-3-3 protein gamma; 14-3-3 ε= 14-3-3 protein épsilon; A-Hydratase= Aconitate hydratase, mitochondrial; AA = Actin, alpha skeletal muscle; AASM = Actin, aortic smooth muscle; AAM= Aspartate aminotransferase, mitochondrial; Adenylate 1 = Adenylate kinase isoenzyme 1; Asp-aminotransferase = Aspartate aminotransferase, cytoplasmic; ATP-dependent= ATP-dependent 6-phosphofructokinase, muscle type; ATP synthase – M = ATP synthase subunit alpha, mitochondrial; C-synthase = Citrate synthase, mitochondrial; CA-1; Calsequestrin-1; Carbonic 3 = Carbonic anhydrase 3; CK = Creatine kinase M-type; CK-SM= Creatine kinase S-type, mitochondrial; CryAB= Alpha-crystallin B chain; DJ-1 = Protein deglycase DJ-1; EF1alpha = Elongation factor 1-alpha 1; FHL1= Four and a half LIM domains protein 1; Fructose-A = Fructose-bisphosphate aldolase A; G3PD= Glyceraldehyde-3-phosphate dehydrogenase; Glucose 6 =Glucose-6-phosphate isomerase; GP-brain form = Glycogen phosphorylase, brain form; GP muscle form = Glycogen phosphorylase, muscle form; Glycerol 3 = Glycerol-3-phosphate dehydrogenase (NAD(+)), cytoplasmic; Glyceraldehyde-3= Glyceraldehyde-3-phosphate dehydrogenase; Hb alpha ½ = Hemoglobin subunit alpha-1/2; Hb beta 1 = Hemoglobin subunit beta-1; HS71 = Heat shock cognate 71 kDa protein; HSPB1 = Heat shock protein beta-1; L-lactate A = L-lactate dehydrogenase A chain; Malate dehydrogenase = Malate dehydrogenase, cytoplasmic; Malate – M = Malate dehydrogenase, mitochondrial; MLC1/3= Myosin light chain 1/3, skeletal muscle isoform; MRLC2 = Myosin regulatory light chain 2, skeletal muscle; M4= Myosin-4; NDK-B = Nucleoside diphosphate kinase B; Phospho 1 = Phosphoglycerate kinase 1; Phospho 2 = Phosphoglycerate mutase 2; Pyruvate K = Pyruvate kinase PKM; S/E – RC ATPase1 = Sarcoplasmic/endoplasmic reticulum calcium ATPase 1; TA1C= Tropomyosin alpha-1 chain; TAC= Tropomyosin alpha-1 chain; TBC = Tropomyosin beta chain; TMCP72; Tripartite motif-containing protein 72; Triosephosphate = Triosephosphate isomerase; TT fast= Troponin T, fast skeletal muscle.

A number of meta-analyses have shown a clear dose-response relationship to be present between the volume of resistance training and muscular adaptations, whereby increased volume correlates with greater gains in muscle strength and hypertrophy, at least up to a

certain adaptation ceiling (10,11,18,19). Otherwise, excessive training may result in lipid peroxidation in skeletal muscle and underperformance related to overtraining syndrom, which can be characterized by the time needed to re-establish performance following endurance training. For example, Zoppi and Macedo (26) analyzed the behavior of oxidative stress markers in rats that performed an overreaching (OVR) protocol of 8 weeks of endurance training (ET) and 3 weeks of OVR. The study revealed that the OVR protocol induced higher levels of thiobarbituric acid reactive substances, reactive carbonylated derivatives and the stress protein HSP72 compared with the ET only in the soleus muscle. Thus, an elevated activation of the CrP/creatine kinase system and alterations in creatine kinase expression in the higher RT volume (RT8) could be an earlier indicator of oxidative and bioenergetic stress to the cell, which was not observed in the lower RT volume group (RT4).

Heat shock proteins (HSPs) were also identified exclusively in the control (heat shock cognate 71 protein and heat shock protein beta-1) and RT4 (CRYAB) groups. Sedentary behavior is a condition related to a decreased mobilization of the intramyocellular triglycerides, which results in an increased synthesis of toxic fatty-acid-delivered metabolites (the control group had higher body mass when compared with the trained groups). These metabolites then cause an increased production of reactive oxygen and nitrogen species (ROS and RNS), resulting in oxidative/nitrosative stress, mitochondrial dysfunction, and the activation of stress associated transcription factors, such as tripartite motif-containing protein 72 (TRI72, identified exclusively in the control group). TRI72 acts to sense the oxidative environment that occurs with membrane damage, resulting in disulfide bond formation and homooligomerization at the site of injury. Further, oligomerization functions as a nucleation site for recruitment of TRI72-containing vesicles to the injury leading to membrane repair, and HSP is increased as a danger cellular signal and to combat plasma oxidative damage (9). On another hand, the HSP (Alpha-crystallin B chain) identified exclusively in the RT4 group, has been associated with anti-apoptotic properties, preventing cell death and damage caused by the inflammation response itself in response to diseases, such as stroke or infection, and acts in the maintenance of cytoskeletal integrity (24). This HSP was not identified in the sedentary or RT8 groups, demonstrating that the activation of HSPs seems to be different depending on the environmental stimuli, such as RT or sedentary state, which determines a positive or negative cellular effect.

In terms of muscle hypertrophy, the results indicate an increase in CSA for both RT8 and RT4, when gastrocnemius muscle CSA was normalized by the total body weight. Only one protein was common between RT8 and RT4 (myosin regulatory light chain 2, skeletal muscle isoform). Myosin cross-bridges, which contain both the actin-binding surface as well as the ATP pocket, taper to a helical neck connecting the myosin rRLSod region responsible for self-assembly into thick filaments. Two small protein subunits, the essential light chain and regulatory light chain, circumnavigate each helical neck to provide stability. Regulatory light chain phosphorylation has no noticeable effect on actin-activated myosin ATPase activity but changes myosin cross-bridge properties, resulting in modulation of Ca^{+2}/troponin-dependent contractions (8). Previous studies indicated that activity-dependent increases in regulatory light chain phosphorylation could enhance concentric force, work and power during

submaximal contractions in vivo (22). However, the discussion of these data is not an easy task, as literature has provided little evidence concerning RLC and muscle hypertrophy induced by RT in rats.

Some limitations of the present study should be considered. The tissue collection occurred 5 days after the last RT session, thus sedentary behavior could result in a loss of a number of proteomic adaptions, while we also wanted to avoid an acute effect of the last training session on several proteins (muscle damage) that could last several days. Additional performance tests and also the inclusion of targeted immunoblot analysis of proteins changing in abundance could be employed. Moreover, the only muscle analyzed was the gastrocnemius, limiting the conclusion regarding other muscle types.

Resistance training provides substantial health benefits and is very effective in improving muscle hypertrophy and strength (17). Quantifying the underlying molecular mechanisms responsible for these favorable effects are in their infancy. Proteomics has been successfully applied for the characterization of the skeletal muscle proteome, representing a relevant approach to characterize the molecular networks of exercise-induced changes in skeletal muscle (12,13). Previous studies that analyzed the effects of exercise on muscle proteome in rats were performed utilizing treadmill exercise (13), running wheels (2), and swimming (23). To the best of our knowledge, this is the first study to analyze the effects of higher and lower RT volume on gastrocnemius muscle proteome in rats.

To note, utilization of the muscle proteome presents some limitations that should be highlighted. For example, approximately half of the muscle fiber protein content is related to the contractile mechanism and the high volume of these proteins produced considerable difficulties in detecting and quantifying proteins that were less abundant (such as signaling proteins, transcription factors, and mitochondrial proteins) (14). Finally, most studies with RT were acute and conducted with human biopsies, which may limit the comparisons and data interpretation.

The present study provides evidence that eight weeks of higher volume RT in rats modulates numerous biological pathways that may result in different adaptations of skeletal muscle cell induced by chronic training. Nonetheless, the sedentary state of the control group modulates biological pathways associated with cellular stress, demonstrating the importance of exercise for cellular health. Finally, lower volume RT increased gastrocnemius muscle CSA to a similar degree compared with higher volume, without disturbance of important skeletal muscle proteins. The application of higher volume RT should be followed by sufficient recovery periods to avoid excessive cellular stress, which could lead to undesired long-term training results.

ACKNOWLEDGEMENTS

REFERENCES

1. Allen DL, Harrison BC, Maass A, Bell ML, Byrnes WC, Leinwand LA. Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. J Appl Physiol 90(5): 1900–1908, 2001.

2. Alves RM, Vitorino R, Figueiredo P, Duarte JA, Ferreira R, Amado F. Lifelong physical activity modulation of the skeletal muscle mitochondrial proteome in mice. J Gerontol A Biol Sci Med Sci 65(8): 832–884, 2010.

3. Erdfelder E, Faul F, Buchner A. GPOWER: A general power analysis program. Behavior Research Methods, Instruments, & Computers 28(1): 1–11, 1996.

4. Gandra PG, Valente RH, Perales J, Pacheco AG, Macedo DV. Proteomic profiling of skeletal muscle in an animal model of overtraining. Proteomics 12(17): 2663-2667, 2012.

5. Hody S, Leprince P, Sergeant K, Renaut J, Croisier JL, Wang F. Human muscle proteome modifications after acute or repeated eccentric exercises. Med Sci Sports Exerc 43(12): 2281-2296, 2011.

6. Hornberger TA, Farrar RP. Physiological hypertrophy of the FHL muscle following 8 weeks of progressive resistance exercise in the rat. Can J Appl Physiol 29(1): 16-31, 2004.

7. Tibana RA, Vieira DC, Tajra V, Bottaro M, de Salles BF, Willardson JM. Effects of rest interval length on Smith machine bench press performance and perceived exertion in trained men. Percept Mot Skills 117(3): 682–695, 2013.

8. Kamm KE, Stull JT. Signaling to myosin regulatory light chain in sarcomeres. J Biol Chem 286(12): 9941-9947, 2011.

9. Krause M, Heck TG, Bittencourt A, Scomazzon SP, Newsholme P, Curi R. The chaperone balance hypothesis: the importance of the extracellular to intracellular HSP70 ratio to inflammation-driven type 2 diabetes, the effect of exercise, and the implications for clinical management. Mediators Inflamm 2015:1-12, 2015.

10. Krieger JW. Single versus multiple sets of resistance exercise: a meta- regression. J Strength Cond Res 23(6):1890-1901, 2009.

11. Krieger JW. Single vs. multiple sets of resistance exercise for muscle hypertrophy: a meta-analysis. J Strength Cond Res 24(4):1150-1159, 2010.

12. Padrão AI, Ferreira R, Amado F, Vitorino R, Duarte JA. Uncovering the exercise-related proteome signature in skeletal muscle. Proteomics 16(5):816-830, 2016.

13. Petriz BA, Gomes CP, Rocha LA, Rezende TM, Franco OL. Proteomics applied to exercise physiology: a cutting-edge technology. J Cell Physiol 227(3):885-898, 2012.

14. Petriz BA, Gomes CP, Almeida JA, de Oliveira GP, Ribeiro FM, Pereira RW, Franco OL. The effects of acute and chronic exercise on skeletal muscle proteome. J Cell Physiol 232(2):257-269, 2017.

15. Prestes J, Leite RD, Pereira GB, Shiguemoto GE, Bernardes CF, Asano RY. Resistance Training and Glycogen content in ovariectomized rats. Int J Sports Med 33(7):550-554, 2012.

16. Prestes J, de Cássia Marqueti R, Shiguemoto GE, Leite RD, Pereira GB, Selistre-de-Araújo HS. Effects of ovariectomy and resistance training on MMP-2 activity in skeletal muscle. Appl Physiol Nutr Metab 34(4):700-706, 2009.

17. Ratamess NA, Alvar BA, Evetoch TK, Housh TJ, Kibler WB, Kraemer WJ. Progression models in resistance training for healthy adults. Med Sci Sports Exerc 41(3):687-708, 2009.

18. Rhea MR, Alvar BA, Burkett LN. Single versus multiple sets for strength: a meta-analysis to address the controversy. Res Q Exerc Sport 73(4):485-488, 2002.

19. Rhea MR, Alvar BA, Burkett LN, Ball SD. A meta-analysis to determine the dose response for strength development. Med Sci Sports Exerc 35(3):456-464, 2003.

20. Son HJ, Kim HJ, Kim CK. The effect of resistance and endurance exercise training on muscle proteome expression in human skeletal muscle Biol Sport 29(1):121-127, 2012.

21. Sousa Neto IV, Tibana RA, Nascimento DC, Durigan JLQ, de Cássia Marqueti, Pereira GB. Effects of resistance training volume on MMPs in circulation, muscle and adipose tissue. Int J Sports Med, 38(4):307-313, 2017.

22. Stull JT, Kamm KE, Vandenboom R. Myosin light chain kinase and the role of myosin light chain phosphorylation in skeletal muscle. Arch Biochem Biophys 15(1):120-128, 2011.

23. Takahashi M, Kubota S. Exercise-related novel gene is involved in myoblast differentiation. Biomed Res 26(2):79-85, 2005.

24. Thornell E, Aquilina A. Regulation of αA- and αB-crystallins via phosphorylation in cellular homeostasis. Cell Mol Life Sci 72(21):4127-4137, 2015.

25. Vercauteren FG, Arckens L, Quirion, R. Applications and current challenges of proteomic approaches, focusing on two-dimensional electrophoresis. Amino Acids 33(3):405-414, 2007.

26. Zoppi CC, Macedo DV. Overreaching-induced oxidative stress, enhanced HSP72 expression, antioxidant and oxidative enzymes downregulation. Scand J Med Sci Sports 18(1):67-76, 2008.