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. An 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) totaling 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 trypsin 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 NH4HCO2 (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% (Mallinckrodt) 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 (N2) 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 - β) 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 ≥ 0.95 and down-regulation of p ≤ 0.05 were considered, with a delta (Δ) of at least (≥) 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.

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).

Figure 1. Mean ± SD of training load volume progression during the 8 weeks for GT4 and GT8 groups. * p < 0.05 for GT4 group.
## 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 | FSNEEIAMATVTA1R | 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 | IAIPPER | CON, RT4, RT8 |
| Beta-enolase | ENOB_RAT | 47,014 | 27 | TLGPALEK | CON, RT4, RT8 |
| Glyceraldehyde-3-phosphate dehydrogenase | G3P_RAT | 35,828 | 119 | WGDGAEYVVSTGVFTTEMK | CON, RT4, RT8 |
| Glycogen phosphorylase, muscle form | PYGM_RAT | 97,273 | 29 | LPADPEKI | CON, RT4, RT8 |
| Troponymosin alpha-1 chain | TPM1_RAT | 32,681 | 63 | QLEDELVSLQK | CON, RT4, RT8 |
| Serum albumin | ALBU_RAT | 68,731 | 18 | LQVEVTDFAK | CON, RT4, RT8 |
| Pyruvate kinase PKM | KPYM_RAT | 57,818 | 51 | VNLAMNVLK | 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 | TPI_RAT | 26,849 | 20 | FFGVGNWK | CON, RT4, RT8 |
| Carbonic anhydrase 3 | CAH3_RAT | 29,431 | 18 | VVFDFTFDR | CON, RT4, RT8 |
| Adenylate kinase isozyme 1 | KAD1_RAT | 21,584 | 34 | ATPVISEYDKR | CON, RT4, RT8 |
| Phosphoglucomutase-1 | PGM1_RAT | 61,403 | 8 | QQPDLNKR | CON, RT4, RT8 |
| Myoglobin | MYG_RAT | 17,157 | 6 | YKELFGQG | 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 | VKEFQIPK | CON, RT4, RT8 |
| Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 | A2A1_RAT | 109,409 | 22 | NMLFGSUINXAGK | CON, RT4, RT8 |
| Hemoglobin subunit beta-1 | HBB1_RAT | 15,979 | 52 | VINAFDNGLK | 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 | ITLSGQVDVLK | CON, RT4, RT8 |
| Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic | PGPD_RAT | 37,453 | 54 | KLEIINNTQHENVK | CON, RT4, RT8 |
| ATP-dependent 6-phosphofructokinase, muscle type | PFKM_RAT | 85,560 | 48 | IGLIQGNR | CON, RT4, RT8 |
| Protein deglycase DF-1 | PKR7_RAT | 19,974 | 42 | APLVLKDK | CON, RT4, RT8 |
| Heat shock cognate 71 | HSP7C_RAT | 70,871 | 44 | TTPSVAFDTDER | CON, RT4, RT8 |
| ATP synthase subunit beta, mitochondrial | ATPB_RAT | 56,354 | 74 | VLGDSAPIPVG5PTELGR | CON, RT4, RT8 |
| Aspartate aminotransferase, cytoplasmic | AATC_RAT | 46,429 | 29 | APPSFQAQVPOAPPVLFKR | CON, RT4, RT8 |
| Troponin 1, fast skeletal muscle | TNN1_RAT | 30,75 | 72 | KPNIDHLSDDKLKR | CON, RT4, RT8 |
| Malate dehydrogenase, mitochondrial | MDHM_RAT | 35,684 | 44 | MIAEAIPELK | CON, RT4, RT8 |
| 14-3-3 protein gamma | 143Q_RAT | 28,303 | 24 | TAFDDIAEALDNLNSDYKSTLIMQLRL | CON, RT4, RT8 |
| Superoxide dismutase [Cu-Zn] | SODC_RAT | 15,912 | 44 | VILSGHSIHGR | CON, RT4, RT8 |
| Myosin regulatory light chain 2, skeletal muscle isoform | MLRS_RAT | 15,912 | 46 | GAPDPEDVITAFAK | CON, RT4, RT8 |
| Elongation factor 1-alpha 1 | EF1A1_RAT | 50,114 | 29 | IGGITVPYGR | CON, RT4, RT8 |
| Aconitase hydratase, mitochondrial | ACON_RAT | 85,425 | 62 | IVYGHLDDPAQNER | CON, RT4, RT8 |
| Heat shock protein beta-1 | HSP1_RAT | 22,893 | 28 | LFQAGFVPVR | CON, RT4, RT8 |
| Aldolase reductase | ALDR_RAT | 35,797 | 23 | AIGVSNFNPLQIER | CON, RT4, RT8 |
| Galectin-1 | LEG1_RAT | 14,857 | 17 | SFVLNLGK | CON, RT4, RT8 |
| Troponin 1, fast skeletal muscle | TNN1_RAT | 21,328 | 22 | MSADAMLK | CON, RT4, RT8 |
| Alpha-crystallin B chain | CRYAB_RAT | 20,089 | 5 | HFSPEELVK | CON, RT4, RT8 |
| Citrate synthase, mitochondrial | CISY_RAT | 51,867 | 22 | AALPSHVTRLDMNPTLHMPQLS | CON, RT4, RT8 |
| Isocitrate dehydrogenase [NADP], mitochondrial | IDHP_RAT | 59,967 | 35 | LNEHFLNTTDLDFTKSLNLDR | CON, RT4, RT8 |
| Filamin-C | FLNC_RAT | 290,986 | 23 | EAMQOADDLWGVQPVQIAPEEI/DPN VDEHSVMTLYSOFPK | CON, RT4, RT8 |
| Electron transfer flavoprotein subunit alpha, mitochondrial | ETFAT_RAT | 34,951 | 61 | TIAKINDPEAPIFIQVDVYIDLDFKL | CON, RT4, RT8 |
| Cysteine and glycine-rich protein 3 | CSR8P3_RAT | 20,803 | 44 | SLSTNVTKDGELYK16: Carbamidomethyl (C) | CON, RT4, RT8 |
| Calnexin-1 OS | CASQ1_RAT | 46,449 | 57 | AYEDAHEEFHPYP FelPFATFSDK | CON, RT4, RT8 |
| Microtubule-associated protein 4 | MAP1_RAT | 110,301 | 15 | LATTYSAPIK | CON, RT4, RT8 |
| Serotonin | TPK1_RAT | 76,395 | 28 | VLPDADGPR | CON, RT4, RT8 |
| Heat shock protein beta-6 | HSPB6_RAT | 17,505 | 15 | FGEGLLEAASLCPAIAAPYPLL14: Carbamidomethyl (C) | CON, RT4, RT8 |
| PDZ and LIM domain protein 3 | PDL13_RAT | 39,106 | 41 | LSGGIFNDQOLVITR | CON, RT4, RT8 |
| Gene Name                        | Accession | Score | Reference(s) |
|---------------------------------|-----------|-------|--------------|
| Tropomyosin beta chain          | TPM2_RAT  | 3283 | AISEELNDNSLTL_RAT, RT4, RT8 |
| Actin, aortic smooth muscle     | ACTA_RAT  | 4209 | LRLDLGRLTDLVYLMK_RAT, RT4, RT8 |
| T4-3-3 protein epsilon          | 1433E_RAT | 2917 | AAFDDAIADLSDSEYKSTLLLML_RAT, RT4, RT8 |
| Creatine kinase S-type, mitochondrial | KCRS_RAT  | 4738 | LIDDHFLFKPSLTLCTAGMAR_RAT, RT4, RT8 |
| Elongation factor-1 alpha 2     | EF1A2_RAT | 5045 | VETGILPRQVMDTAPV_TNFIEVK_RAT, RT4, RT8 |
| Desmin                          | DESM_RAT  | 5345 | LEEIEIRHLK_RAT, RT4, RT8 |
| Histone H2B type 1              | H2B1_RAT  | 1399 | AMGMI5NFVNDIFER_RAT, RT4 |
| Tripartite motif-containing protein 72 | TRJ72_RAT | 5282 | MFLAALSSLDREAR_RAT, RT4, RT8 |
| Four and a half LIM domains protein 1 | FH1L1_RAT | 31904 | AVIAQIDQVYEK_RAT, RT4, RT8 |
| Nucleoside diphosphate kinase A | NDKA_RAT  | 17193 | VMLGETNPADKPGITR_RAT, RT4, RT8 |
| Myosin-binding protein C, slow-type (Fragment) | MYPC1_RAT | 68737 | FFITGILPDAD_RAT, RT4, RT8 |
| Myosin-4                        | MYH4_RAT  | 22288 | LQDLVDKLQTK_RAT, RT4, RT8 |
| ATP synthase subunit alpha, mitochondrial | ATPA_RAT | 59754 | TGAIVDVPVDQDGELGR_RAT, RT4, RT8 |
| Myc box-dependent-interacting protein 1 | BIN1_RAT | 64533 | LVDQALLMTMDLYLQPDIDK_RAT, RT4, RT8 |
| Vimentin                        | VIME_RAT  | 53733 | ILLAELEQQLGGQKQ_RAT, RT4, RT8 |
| Peroxiredoxin-1                 | PRDX1_RAT | 22109 | ATAVMPDGQFK_RAT, RT4, RT8 |
| Nestin                          | NEST_RAT  | 208797 | AVGNEQMAVSPPEKE7_RAT, RT4, RT8 |
| Acetyl-CoA acetyltransferase, mitochondrial | THIL_RAT | 44695 | IAIFADAADVPIDEPLAYAVPK_RAT, RT4, RT8 |
| Peroxiredoxin-5, mitochondrial  | PRDX5_RAT | 22179 | ALNVEPDGTGLTCSLAPNILSQL_RAT, RT4, RT8 |
| Nucleoside diphosphate kinase B | NDKB_RAT  | 17283 | NIHDSDSVEASEEEGLWFKPEELIDYK_RAT, RT4, RT8 |
| Myosin-binding protein H         | MYBPH_RAT | 52656 | VTAVNSAGAGPVALDQVHQIEITEAPK_RAT, RT4, RT8 |
| Phosphatidylethanolamine-binding protein 1 | PEBP1_RAT | 20801 | VDYGPVTVDYL_RAT, RT4 |
| Glycogen phosphorylase, brain form (Fragment) | PYGB_RAT | 96174 | NLAENSIR_RAT, RT4, RT8 |
| Long-chain specific acyl-CoA dehydrogenase, mitochondrial | ACADL_RAT | 47873 | ELWEKAGK_RAT, RT4, RT8 |
| Histidine triad nucleotide-binding protein 1 | HINT1_RAT | 13777 | AOVAQPGDQTFPK_RAT, RT4, RT8 |
| Glutathione S-transferase Mu 2  | GSTM2_RAT | 25703 | LGLDFPNLPLYDGHK_RAT, RT4, RT8 |
| Histone H2A type 1-C            | H2A1C_RAT | 14077 | VTIAQGGVLPNQAVLPLPK_RAT, RT4 |
| Tubulin beta-5 chain            | TBB5_RAT  | 49671 | LTTPTYGLDNHLVSATMSGVTTCLR_RAT, RT4 |
| NEUREXIN-2                      | NR2A_RAT  | 185169 | MAGRPGLAWCR_12:RAT, RT4 |
| Meiosis arrest female protein 1  | MARF1_RAT | 192642 | Carbamidomethyl (C) |
| Myosin-3                        | MYH3_RAT  | 223858 | RT4, RT8 |
| Centrosomal protein CEP57L1 1   | CEP57L1_RAT | 50274 | DRSFPVPK_RAT, RT4 |
| IQ DOMAIN-CONTAINING PROTEIN G   | IQCG_RAT  | 48757 | OLYSDVRK_RAT, RT4, RT8 |
| MICAL-like protein 1            | MILK1_RAT | 92644 | MACGRGLAWCR_12:RAT, RT4 |
| F-box only protein 43           | FBX43_RAT | 73228 | Carbamidomethyl (C) |
| DNA endonuclease RBBP8           | COMT1_RAT | 100705 | RT4, RT8 |
| Tubulin alpha-4A chain          | TBAAA4_RAT | 49924 | RT4, RT8 |
| Alpha-actinin-1                 | ACTN1_RAT | 10296 | TFAWCMNSHLR_6:RAT, RT8 |
| NON-POU DOMAIN-CONTAINING OCTAMER-BINDING PROTEIN | NONO_RAT | 54925 | GTPPDAR_RAT, RT8 |
| Receptor tyrosine-protein kinase erbB-3 | ERBB3_RAT | 147546 | IPIALDEK_RAT, RT4, RT8 |
| Heat shock 70                   | HS71A_RAT | 70185 | TPTSVYAFDTDER_RAT, RT4 |
| HISTONE ACETYLTRANSFERASE       | HIST1A_RAT | 70514 | ASEDLEK_RAT, RT4, RT8 |
| Lymphocyte activation gene 3 protein | LAG3_RAT | 57988 | QLSAAVTLAVTVPK_RAT, RT8 |
| Endothelial lipase               | LIPE_RAT  | 55924 | LVSAQLTREK_RAT, RT8 |
| ATP-binding cassette sub-family B member 6, mitochondrial | ABCB6_RAT | 93305 | AIQASLAK_RAT, RT8 |
| TRIPARTITE MOTIF-CONTAINING PROTEIN 55 | TRI55_RAT | 5998 | STSRLNYK_RAT, RT8 |
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, antioxidant 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](http://www.intjexersci.com)

**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.
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 adaptations, 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

The authors would like to acknowledge the financial support provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).
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