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Excess of eccentric exercise, mechanical load on the knee joint and osteoarthritis in C57BL/6 mice

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Original version

A thesis presented to the School of Physical Education and Sport of Ribeirão Preto, University of São Paulo, Brazil, to obtain the degree Master of Sciences, Graduate Program Physical Education School.

Concentration area: Physical Activity and Sport

Advisors:

Prof. Dr. Adelino Sanchez Ramos da Silva

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DEDICATION

I grateful to God, who gave me the gift of life and for had the opportunity to do my master's degree in this great university, the University of São Paulo. I thank my family for all the love and support they gave through the way until here, and I dedicate this work to them. I thank my girlfriend and future wife, Luciana, to be with me and give me support in all I needed. I also thank professor Adelino for the orientations, advice and help me to grow in my knowledge and my academic life. Thanks to my friends from the laboratory, who helped me every time I need. I am fortunate to be surrounded by such good people. I grate to professor Walter Herzog, which allowed me to go abroad and do part of my thesis in his laboratory, it was an amazing experience and was pivotal for my professional and personal growth. I thank to São Paulo Research Foundation (processes: 2016/25766-4, 2017/13251-2) and to CAPES (Coordination of the Improvement of Higher Education Personnel) for the financial support. I'm grateful to all the people who made part of my life since I was born. I am here, for sure, because the people I met in my life, no matter if with a great role or a small role, it led me where I am today. Thank you.

"Every time you make a choice you are turning the central part of you, the part of you that chooses, into something a little different than it was before."

C.S. Lewis

RESUMO

MORAIS, G. P. **Excesso de exercício excêntrico, carga mecânica na articulação do joelho e osteoartrite em camundongos C57BL/6.** 2019. 69f. Dissertação (Master) – Escola de Educação Física e Esporte de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2019.

A osteoartrite (OA) é uma doença crônica degenerativa que induz a degradação da cartilagem sendo muito comum na articulação do joelho. A progressão da OA ocorre por conta do excesso de carga na articulação, assim como inflamação sistêmica ou local. O exercício físico é uma ferramenta muito utilizada na prevenção e tratamento dessa doença, atenuando os sintomas, no entanto, ainda existem estudos mostrando resultados controversos, o que poderia ser explicado pelo volume, intensidade e o tipo de exercício utilizado. Investigações científicas demonstram que o excesso de exercício pode ocasionar um quadro inicial de osteoartrite, que pode ser explicado pela sobrecarga articular, e/ou aumentando enzimas relacionadas a degradação da cartilagem. Além disso, nosso grupo de estudos verificou que camundongos submetidos ao excesso de treinamento em declive apresentaram um quadro de inflamação sistêmica de baixo grau, evidenciado pelo aumento de citocinas pró-inflamatórias. Uma hipótese para o maior nível de inflamação nesse tipo de exercício são as alterações mecânicas, histológicas e bioquímicas no músculo, como o aumento no número de sarcômeros em série. Sendo assim, o objetivo do presente estudo foi verificar as diferenças na progressão da osteoartrite, inflamação muscular, e mudanças na estrutura dos sarcômeros entre camundongos C57BL/6 treinados moderadamente e excessivamente com diferentes predominâncias de contração muscular. Os camundongos foram divididos em 5 grupos: sedentário (S; camundongos sedentários), treinado em declive (TRD; camundongos submetidos ao protocolo de treinamento em declive), treinado em aclive (TRA; camundongos submetidos ao protocolo de treinamento em aclive), excesso de treinamento em declive (ETD; camundongos submetidos ao protocolo de overtraining em declive), excesso de treinamento em aclive (ETA; camundongos submetidos ao protocolo de overtraining em aclive). A metodologia empregada, assim como os resultados e a discussão dos achados serão apresentadas nessa dissertação no formato de dois manuscritos. O nível de significância adotado foi $p \leq 0.05$.

Palavras chave: osteoartrite; inflamação; treino excessivo; exercício excêntrico; sarcômeros

ABSTRACT

MORAIS, G. P. **Excess of eccentric exercise, mechanical load on the knee joint and osteoarthritis in C57BL/6 mice.** 2019. 69p. Thesis (Master) – Physical Education School and Sports of Ribeirão Preto, University of São Paulo, Ribeirão Preto, 2019.

Osteoarthritis (OA) is a chronic degenerative disease that induces cartilage degradation and is very common in the knee joint. OA progression occurs due to excess load in the joint as well as systemic or local inflammation. Physical exercise is a commonly used therapy to prevent and treat this illness, attenuating the symptoms. However, studies that exhibit controversial results still exist, which could be explained by the volume, intensity, and type of exercise used. Scientific investigations demonstrate that excessive exercise may cause an onset of osteoarthritis, which can be explained through joint overload, and/or increasing enzymes related to cartilage degradation. Furthermore, our study group has verified that mice submitted to excessive downhill training presented low-grade systemic inflammation, evidenced by an increase in pro-inflammatory cytokines. The mechanical, histological and biochemical changes in the skeletal muscle, such as the increase in the number of sarcomeres in series, can explain the highest level of inflammation in this type of exercise. Therefore, the aim of the present study was to verify the differences in osteoarthritis progression, muscular inflammation, and shift in sarcomere structure among C57BL/6 mice moderately trained in downhill and uphill, and excessively trained in downhill and uphill. The mice were divided into 5 groups: sedentary (S; sedentary mice); trained downhill (TRD; mice submitted to downhill training protocol); trained uphill (TRU; mice submitted to uphill training protocol); excessive downhill training (ETD; mice submitted to downhill overtraining protocol); excessive uphill training (ETU; mice submitted to uphill overtraining protocol). The methods, results, and discussion regarding these findings are presented in the format of two manuscripts. The level of significance adopted was $p \leq 0.05$.

Keywords: osteoarthritis; inflammation; excessive training; eccentric exercise; sarcomeres

SUMÁRIO

1. INTRODUCTION

Osteoarthritis (OA), also known as degenerative arthritis, is a chronic disease which induces cartilage injury, being very common in the knee joint (ZHANG; JORDAN, 2010). Increasing evidence supports that chronic metabolic disorders such as increased levels of proinflammatory cytokines induce to a development of a systemic inflammatory state contributing to the OA pathogenesis (ZHUO et al., 2012; WANG et al., 2015).

Excessive exercise has been related to metabolic inflammation after downhill and uphill running, with increased levels of serum proinflammatory cytokines (DA ROCHA et al., 2019; PEREIRA et al., 2015). Also, excessive downhill running increased levels of proinflammatory cytokines, IKK (IkappaB kinase) and SOCS3 (Suppressor of cytokine signaling 3) proteins in skeletal muscle of mice (PEREIRA et al., 2013; PEREIRA et al., 2013b). Downhill and uphill running have different type of contraction predominance, and eccentric contraction (EE) are known to be more detrimental to the cartilage due to increased load on the joint that can increase chondrocyte death (HORISBERGER et al., 2012).

During eccentric contractions, skeletal muscle produces force while stretching and may induce sarcomere remodeling. Lynn and Morgan (1994) observed increased number of serial sarcomeres after downhill running in rats. This result could be a protective effect, once increased serial sarcomere number would lead to a lower sarcomere stretch to the same joint angle (LYNN, TALBOT AND MORGAN, 1998). In contrast, uphill running led to serial sarcomere number decrease. Therefore, the aim of the present thesis was to verify the changes in serial sarcomere number and the early onset of OA after different protocols of excessive and moderate training with predominance of different type of contractions. The thesis was divided into two manuscripts.

2. MANUSCRIPT I:

Original Article: Chronic uphill and downhill exercise protocols do not lead to sarcomerogenesis in mouse skeletal muscle.

ABSTRACT

It has been suggested that eccentric contraction (EC) is associated with increases in serially arranged sarcomeres (sarcomerogenesis), while concentric contraction (CC) has been associated with serial sarcomeres decrease. Sarcomerogenesis following EC is thought to be a protective muscle adaptation, preventing muscle injury in future eccentric exercise bouts (repeated bout effect). However, the mechanisms underlying sarcomerogenesis in EC remain unknown, and the sarcomerogenic responses observed in response to EC and CC are contradictory. We measured sarcomere length, sarcomere length uniformity, serial sarcomere number, and fascicle length in gastrocnemius medialis, tibialis anterior, vastus medialis and vastus lateralis in sedentary (SED) mice, and in mice following protocols of moderate uphill (TRU) and downhill (TRD) training and uphill (OTU) and downhill (OTD) overtraining. We found pain sensitivity after the first bout of EC exercise on TRD and OTD followed by a normalized sensory response after four weeks of training, indicating a repeated bout effect. However, these findings were not associated with sarcomerogenesis, as serial sarcomere numbers did not increase in TRD and OTD skeletal muscle samples compared to controls (SED). However, we found a decrease in serial sarcomere number in VL and TA in OTU group mice, which was associated with a decrease in fascicle length and no change of sarcomere length at the tested joint configuration. We conclude that excessive concentric muscle contraction (OTU group mice), leads to a decrease in serial sarcomere number, while moderate or excessive eccentric training, did not result in sarcomerogenesis, as reported in the literature.

Keywords: sarcomerogenesis; eccentric contraction; concentric contraction; overtraining; repeated bout effect; muscle soreness

INTRODUCTION

Skeletal muscles provide mechanical energy for movement. They have a hierarchical structure of fascicles, which in turn are composed of muscle fibers. Fibers are made up of hundreds of individual myofibrils, and myofibrils are composed of serially arranged sarcomeres. Sarcomeres are the smallest contractile structures in muscles, and they contain the contractile filaments myosin and actin.

Dynamic muscle contractions are divided into concentric and eccentric actions (Hill, 1938; Huxley, 1969; Mccully and Faulkner, 1985). Eccentric contractions (EC) and concentric contractions (CC) lead to distinct mechanical, metabolic and neuronal adaptations (Huxley and Simmons, 1971; Bigland-Ritchie and Woods, 1976; Westing et al., 1988; Aagaard et al., 2000). EC cause greater muscle damage than CC (Armstrong et al., 1983). During EC, muscles may be stretched onto the descending limb of the length-tension curve, causing "sarcomere instability" (Hill, 1953; Gordon et al., 1966). This instability is thought to result in some sarcomeres being overstretched, thereby losing the ability to contribute to active muscle force (Morgan, 1990).

Eccentric or concentric contractions have been associated with remodeling of serial sarcomeres. Specifically, it has been found that the number of serially arranged sarcomeres (i.e., sarcomerogenesis) is increased in response to EC but decreased in response to CC. For example, Lynn and Morgan (1994) observed that rats exposed to downhill running for five days had an increased number of serial sarcomeres in the vastus intermedius muscle compared to rats exposed to five days of uphill running. They argued that the increase in serial sarcomeres of the downhill trained rats prevented sarcomeres from being overstretched, thereby causing muscle damage (Lynn et al., 1998). Sarcomerogenesis is also thought to be part of the repeated bout effect that makes muscles less susceptible to EC-induced damage after previous EC exposure (Fridén et al.,

1983). In contrast to the downhill running rats, rats submitted to uphill running had a decrease in the number of serial sarcomeres compared to sedentary rats (Lynn and Morgan, 1994). A decrease in serial sarcomere number was also observed in the rat vastus lateralis and vastus medialis muscles after ten days of uphill walking exposure compared to rats who were kept sedentary (Butterfield et al. 2005).

Although EC training is supposed to prevent muscle injury in the long term, muscles of mice submitted to EC downhill running had higher levels of pro-inflammatory cytokines (i.e., IL-1beta, IL-6, and SOCS3) than mice submitted to CC uphill running (Da Rocha et al., 2017). Also, exhaustive downhill training upregulated myostatin, a protein inhibiting muscle hypertrophy (Da Rocha et al., 2016) and possibly sarcomerogenesis. Therefore, sarcomerogenesis regulation in the short-term, as observed by Lynn et al. (1998), might be distinctly different from that in the long-term, when muscle adaptations have reached a steady-state. Therefore, the primary purpose of this investigation was to determine if chronic moderate or excessive training based on EC and CC leads to serial sarcomere number adaptations in skeletal muscles. We hypothesized that moderate and excessive EC (downhill running) training leads to an increase in serial sarcomere number, and CC (uphill running) moderate and excessive training leads to a decrease in serial sarcomere number.

METHODS

Animals

Eight-week-old C57BL/6 mice were divided into five groups: SED (sedentary; n=8); TRU (moderate uphill training; n=8); TRD (moderate downhill training; n=8); OTU (uphill overtraining; n=7); OTD (downhill overtraining; n=6). All experimental procedures were

performed according to the Brazilian College of Animal Experimentation and were approved by the Ethics Committee of the University of Sao Paulo (I.D. 2016.5.84.90.0). The number of animals was based on a power sample analysis based on previous studies (Lynn and Morgan 1994; Lynn, Talbot and Morgan, 1998; Butterfield et al. 2005) with the aim to reach a power of >0.8 for a level of significance of 0.05.

Performance test

An incremental load test (ILT) was performed to prescribe specific training intensities that was appropriate to the performance capacity of each mouse, and to evaluate the adaptations in aerobic capacity due to the training interventions (Ferreira et al. 2007). The ILT protocol started with a speed of 6 m/min and a 0% inclination. The speed was increased incrementally by 3 m/min every 3 minutes until voluntary exhaustion. Voluntary exhaustion was defined when the animals touched the end of the treadmill five times in a 1 min period. The exhaustion velocity for each mouse was used for determining the individual training intensities for the TRU, TRD, OTU, and OTD protocols. The ILT was performed with no inclination 48h before the beginning of the intervention protocols at week 0 and was used specifically to prescribe the training intensities for the first four weeks. At the end of week 4 and week 8, the ILT was performed at +14 degrees for the uphill exercise groups and -14 degrees for the downhill exercise groups. Testing at these time points was used to compare the (over-) training adaptations that occurred between weeks 4 and 8 within a given training group.

Evaluation of mechanical pain sensitivity

Forty-eight hours after the ILT, mice were placed on an elevated meshed grid that allowed access to the ventral aspect of the hind paws. Animals were adapted to the experimental

environment for at least 60 min. Mechanical pain sensitivity was assessed before the intervention protocol before and at the end of weeks 4 and 8 by measuring the paw withdrawal threshold in response to probing using Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL) (Chaplan et al., 1994). Nine filaments were applied to the left hind paw to determine the threshold stiffness required for 50% paw withdrawal (Dixon, 1980). A single, blinded experimenter performed all tests to reduce variability.

Moderate Training and Overtraining protocols

The intensities of the moderate and overtraining protocols were prescribed according to the ILT results. Ferreira et al. (Ferreira et al. 2007) demonstrated that the intensity corresponding to 60% of the exhaustion velocity obtained in the ILT was similar to the maximal lactate steady state (MLSS) intensity. The MLSS can be defined as the highest exercise intensity in which balance between the production and removal of blood lactate occurs, and is used as the gold standard to determine exercise intensity (Ferreira et al. 2007). While the physical exercise sessions below the MLSS intensity are related to the moderate domain (Da Silva et al. 2010), the physical exercise sessions above the MLSS intensity are related to the intense domain. The moderate uphill and downhill training protocols were performed for eight weeks. Each experimental week consisted of three days of training (Monday, Wednesday, and Friday) followed by two days of rest over the weekend (Table 1). The average total intensity across the eight weeks was 52.5% of the exhaustion velocity, characterizing a moderate protocol, with a total volume of 1080 min, resulting in a total workload (intensity x volume) of 56.700 a.u. The uphill and downhill overtraining protocols consisted of five consecutive days of training (Monday – Friday) followed by two days of rest (weekend, Table 2). The average total intensity

across the eight weeks was 65% of the exhaustion velocity, characterizing an intense protocol, with a total volume of 2475 minutes, resulting in a total workload of 160.875 a.u.

Skeletal muscle extraction

At the end of the 8-week intervention protocols, mice fasted for 12 hours. Animals were anesthetized by intraperitoneal administration of xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). The pedal reflex was used to control anesthesia (Arras et al., 2001). Once animals were under deep anesthesia, they were decapitated, the hind limbs, including the sacrum, were harvested and then pinned and stored with the knee and ankle joint at 90º in a 10% buffered Formalin solution for at least four weeks. Following formalin fixation, the vastus lateralis (VL), vastus medialis (VM), gastrocnemius medialis (GM) and tibialis anterior (TA) muscles were carefully removed from their attachment sites and weighed using a precision scale (d = 0.1mg; Max 220g) (Mettler Toledo, Singapore). The skeletal muscle samples were placed individually in a petri dish containing 30% nitric acid (VWR International USA) for at least 7 hours to partially digest connective tissues. Muscles were then transferred to phosphate-buffered saline (PBS) solution for 12 hours, and subsequently placed in a petri dish (Pyrex®, Germany) with glycerol (Fisher Scientific, USA) (Figure 1)

Sarcomere Analysis

After digestion of the connective tissues, a small number of fascicles was carefully teased out from each muscle and placed on glass microslides (VRW International, USA). Sarcomere lengths were measured at five different locations along the length of the fascicle using laser diffraction (beam diameter 0.8 mm, wavelength 633 mm) (Thorlabs Inc. Newton, NJ, USA) (Koh

and Herzog, 1998b). All fascicles spanned the entire distance from the origin to insertion of the muscles. Fascicle lengths were measured using commercially available software (Matrox Inspector, Matrox Systems, Dorval, PQ, Canada). Serial sarcomere numbers were obtained by dividing the fascicle length by the average sarcomere length. Sarcomere length variation was measured using the width of the first order laser diffraction beam diameter.

Statistics

Statistical analyses were performed using GraphPad Prism version 5.01 (San Diego, CA). Results are expressed as means ± standard error of the mean. Mann-Whitney nonparametric testing was used to analyze the differences of the ILT between weeks 4 and 8 for each experimental group. Kruskal-Wallis with Dunns post-hoc analysis was used to examine the differences of the ILT between the experimental groups at week 0. Also, this statistical test was used for comparing sarcomere length, sarcomere number differences, and sarcomere non-uniformities across groups when data were not normally distributed. Mann-Whitney nonparametric testing was used to verify differences in sarcomere length between two specific ILT conditions. Two-way ANOVA was used to determine differences in pain sensitivity. The level of significance was chosen as $p<0.05$.

RESULTS

Performance. OTU group performance was higher compared to OTD group at week 0 ($p=0.01$ Figure 2A). There was no difference in performance at 4 and 8 weeks for the TRU (Figure 2B) and TRD (Figure 2C) groups. Performance in the OTU and OTD group animals decreased by

35% and 44% at week 8 compared to week 4 (p=0.0008; Figure 2D; and p=0.022; Figure 2E), respectively).

Pain sensitivity. Four weeks after training initiation, TRD and OTD mice presented greater pain sensitivity compared to TRU and OTU group mice (p=0.0001) (Figure 3). However, after eight weeks of training, TRD and OTD mice had similar pain sensitivity as TRU and OTU group mice.

Skeletal muscle weights. VM weights at week 8 from TRU group were significantly heavier than those of SED (+ 22%) and OTD (+ 20%) group mice (p=0.006). VL weights from TRU group mice were 23% heavier than those from OTU and were heavier for TRD group mice than OTU (24%) and OTD (20%) group mice, respectively (p=0.0007). GM muscles from SED group mice were significantly heavier (17%) than those of OTD group mice (p=0.05). TA samples were similar across all experimental groups.

Skeletal muscle adaptations. There were no significant differences in sarcomere lengths (Figure 5) of GM, VL, and VM between experimental groups. However, TA sarcomere lengths were increased for TRU group compared to TRD group mice (p=0.011).

Sarcomere length non-uniformity (Figure 6) was similar across experimental groups for VM and TA. However, GM sarcomere lengths were less uniform for the TRU group mice compared to OTD group mice (p=0.02). VL sarcomere lengths were less uniform for TRU compared to OTU group mice (p=0.01).

VL fascicle lengths (Figure 7) were shorter for OTU compared to TRU group mice (p=0.01). There were no differences in fascicle lengths for any of the muscles or any of the experimental groups otherwise.

VL serial sarcomere number (Figure 8) was smaller for the OTU compared to the TRD, TRU, and OTD group mice ($p=0.005$). Similarly, TA serial sarcomere number was smaller for the OTU compared to the TRD group mice ($p=0.05$). There were no differences in serial sarcomere number between any of the experimental groups for GM and VM.

DISCUSSION

The main findings of this study were: (i) the OTU and OTD group mice had a decrease in performance after the exercise intervention protocol, achieving a non-functional overreaching (NFOR) state (Pereira, et al., 2012; Meeusen et al., 2013); (ii) the OTD and OTU group mice had reduced muscle mass; (iii) moderate and excessive EC training did not lead to shortened sarcomeres compared to moderate and excessive CC training; (iv) OTU group mice had a decreased number of serial sarcomeres compared to the other groups.

The decrease in performance for the OTD and OTU group mice (Figure 2) supports previous findings (Da Rocha et al., 2015; Pereira, et al., 2015; Pereira, et al., 2015; Da Rocha et al., 2016; Pinto et al., 2017) using the same overtraining protocol as used here. Pereira et al. (2012; 2015) showed that mice subjected to these same overtraining protocols reached an NFOR state from which they did not fully recover within a two-week rest period (Meeusen et al., 2013).

OTD group mice had less muscle mass in GM, VM, and VL compared to SED, TRU, and TRD group mice (Figure 3), and OTU group mice had less muscle mass for VL compared to TRD and TRU group mice. These results suggest that excessive training leads to muscle atrophy. Da Rocha et al. (2016) found an increase in myostatin content in skeletal muscles of mice subjected to a downhill overtraining protocol. Myostatin regulates muscle growth, and an increase in myostatin is associated with an inhibition of muscle hypertrophy (Amirouche et al., 2008). Da Rocha et al. (2016), also found a decrease in Ribosomal Protein S6 Kinase (S6K), an

essential hypertrophic pathway protein (Bodine, 2006), and an increase in Tuberous Sclerosis Complex 2 (TSC-2) expression, a protein related to inhibition of muscle hypertrophy (Ma and Blenis, 2009) in mice subjected to the uphill overtraining protocol used in this study. The activation of these proteins may explain why the over-trained mice exhibited lower muscle mass compared to trained animals.

Sarcomere adaptations:

Lynn and Morgan (1994) performed a classic study where they exposed rats to five days of uphill or downhill running. They reported an increase in serial sarcomere number of about 7% in the vastus intermedius (VI) for the downhill running compared to the uphill running rats and an increase in sarcomere number of about 2% compared to the sedentary group animals. The uphill group rats had about 5% less serial sarcomeres than the sedentary group animals. The authors also reported that VI sarcomere lengths were shorter for the downhill compared to the uphill running group (Lynn and Morgan, 1994; Lynn et al., 1998).

Our results are distinctly different in some aspects compared to the findings by Morgan and colleagues (Lynn and Morgan, 1994; Lynn et al., 1998). GM, VM, and VL had similar sarcomere lengths across all experimental groups in our mice (Figure 4). TA had increased sarcomere lengths for the TRU compared to the TRD group mice, but the serial sarcomere numbers were the same for these two groups. This general pattern of sarcomere length and serial sarcomere number changes was different from those described by Morgan (1990) for the VI samples. Lynn and Morgan (1994) measured sarcomere lengths only in the center of fibers, while we measured sarcomere lengths at five different locations along the fiber. It has been shown that sarcomere lengths vary substantially from the mid-section to the end regions of fibers (Huxley and Peachey, 1961). These variations in sarcomere lengths have also been observed in entire

muscles (Llewellyn et al., 2008) in general, and specifically for the mouse TA used here (Moo et al., 2016). Therefore, variations in sampling procedures might explain some of the differences in sarcomere lengths and serial sarcomere number. Furthermore, we performed a long-term protocol (8 weeks) compared to 5 days in the Lynn and Morgan (1994) study, using a different animal species. These differences in the protocol may explain some of the different adaptations observed by us.

Butterfield et al. (2005) subjected rats to uphill and downhill walking protocols for five and ten days and analyzed VI and VL sarcomere lengths and sarcomere number. They observed significant changes only after ten days, but not after five days, like Lynn and Morgan (1994). In their study, VI had more serial sarcomeres for the downhill compared to the uphill walking and the control group rats. Serial sarcomeres for VL were the same for the downhill, uphill, and control group rats, corroborating our findings for the VL samples of the TRD and OTD compared to the SED and TRU group mice. However, serial sarcomeres in VL were decreased in the OTU compared to the TRD, TRU, and OTD groups, and TA serial sarcomeres were increased in TRD compared to OTU group mice (Figure 8) without a corresponding change in sarcomere lengths (Figure 5), agreeing with findings of serial sarcomere number adaptations found by Lynn and Morgan (1994). These results support the hypothesis that different muscles can have different adaptations to specific types of contractions (Butterfield et al., 2005; Koh and Herzog, 1998a).

OTU mice had fewer serial sarcomeres than TRD group mice in TA (Figure 8). This was primarily associated with the shorter fascicle length for OTU compared to TRD group mice ($p<0.009$). Also, the difference in the TA fascicle lengths between OTU and TRD group mice (8.6%) was greater than the difference in sarcomere lengths between TRD and TRU group mice

(4.9%), reinforcing that fascicle length had a greater influence on serial sarcomere number than sarcomere length.

Lynn et al. (1998) reported average fiber lengths of 8.11 mm for the downhill and 7.69 mm for the uphill trained rats, totalizing a difference of 0.42 mm (5.4%). The authors assumed fascicle lengths to be uniform. They had 3475 serial sarcomeres for the downhill and 3193 for the uphill trained rats. Lynn et al. (1998) did not provide sarcomere lengths, but sarcomere length can be computed from the reported fascicle length and serial sarcomere number. The sarcomere lengths were about 2.33 (8.11 mm /3475) for downhill and 2.41 µm (7.69 mm /3193) for uphill. Considering a fascicle length difference of 0.42 mm, that means 174 (0.42 mm /2.33 µm) serial sarcomere difference to downhill and 180 (0.42 mm /2.41µm) to the uphill group, 177 serial sarcomeres in average. So, the difference of 282 sarcomeres in series (i.e., 3475-3193) between the downhill and uphill training groups was associated with fiber shortening (177 sarcomeres accounting for 63% of the observed difference), and due to changes in sarcomere length (105 sarcomeres or 37%), which suggests that fascicle length has a greater influence on serial sarcomere number in this case as well.

The VL fascicles of OTU group mice were shorter than those for TRU group mice (Figure 7). Running velocity in TRU group was lower than the OTU group (Table 1). Thus, this difference may have occurred because during fast galloping VL fascicles shorten more than for fast walking (Gillis and Biewener, 2002).

Some studies have shown that eccentric resistance training increases fascicle lengths, while concentric resistance training produces changes in the angle of pennation (Reeves et al., 2009; Franchi et al., 2014; Franchi et al., 2015; Franchi et al., 2017). Franchi et al. (2017) suggested that increases in fascicle length mean more serial sarcomeres, and increases in angle of

pennation mean more parallel sarcomeres. However, these suggestions need to be tested quantitatively before they can be fully embraced. The muscle weight loss observed in our study (Figure 3) may be explained in part with a decrease in serial sarcomeres for VL of the OTU group mice. There was also a loss of VL muscle mass for the OTD group mice. However, this atrophy was not associated with a decrease in serial sarcomeres, suggesting this atrophy may be caused by the loss of parallel sarcomeres. To our best knowledge, muscle atrophy regulation in overtrained muscles has not been studied systematically, and the results of this study might provide the impetus to do so.

Eccentric exercise has been associated with injury to muscles, instabilities of sarcomere lengths on the descending limb of the force-length relationship (Hill, 1953; Gordon et al. 1966), and the development of sarcomere length non-uniformities (Morgan, 1990; Lynn and Morgan, 1994). Therefore, we expected an increase in sarcomere length non-uniformities in TRD and OTD group mice, because of the eccentric nature of these activities for the target muscles (Hill, 1953; Gordon et al., 1966; Lynn and Morgan, 1994; Lynn et al., 1998). However, we did not find support for this idea. Possibly, the lack of sarcomere damage and an associated increase in sarcomere length non-uniformity was due to the chronic nature of our experiments, and associated reduction in muscle injury with eccentric contraction due to the so-called repeated bout effect (Fridén et al., 1983; Faulkner et al., 1993). Our results on pain sensitivity corroborate this theory. Mechanical pain sensitivity was increased at week 4 compared to week 0 but returned to pre-exercise intervention levels at week 8 for OTD and TRD group mice (Figure 2). The repeated bout effect has been associated with sarcomerogenesis (Fridén et al., 1983), which would reduce sarcomere length non-uniformities, as the damage is reduced by the repeated bout effect.

Lieber et al. (2017) discussed plasticity in sarcomerogenesis, citing the study of Takahashi and coworkers (2010), in which the extensor digitorum II was sutured to the extensor retinaculum. They found that sarcomere length increased in the first week following the intervention, and then decreased to the original length over eight weeks. They expected that the serial sarcomere number increased once sarcomeres were back at their original length. However, this happened only in the first-week post-intervention. In the second week, the number of serial sarcomeres decreased, and the additional length was accounted for by an elongation of the tendon, while fascicle lengths decreased. Lieber et al. (2017) commented that the interactions between muscle and tendon adaption are not well understood, and we would like to add that it will likely be different for different muscles. Furthermore, sarcomere lengths appear to adapt to the functional demands, while muscle adaptations occur in ways that are still not fully understood. Serial sarcomere adaptations in chronic studies have been studied extensively, but mostly for static studies with the muscle fixed in some elongated or shortened configuration (Goldspink et al., 1974; Hayat et al., 1978; Williams and Goldspink, 1978) while dynamic changes in muscle lengths or muscle excursions are rare (Koh and Herzog, 1998b; Lieber et al., 2017).

LIMITATIONS

Regarding the significant differences between OTU and OTD group mice for the ILT results at week 0, it is important to point out that all mice were randomly assigned to the experimental groups to prevent selection bias. However, since the training intensity for each mouse was prescribed based on the initial ILT test, each mouse performed the same absolute training load, and thus, any adaptation would be expected to be similar for all mice, even if they

started the experiment with different performance capacities. This was a cross-sectional study based on comparisons at a single time point following the training interventions. Ideally, studies on sarcomerogenesis should be done longitudinally with multiple time points of evaluation. However, we are not aware of a technology that would allow such an approach at present. We did not analyze the VI muscle, as had been done in previous studies (Lynn and Morgan, 1994; and Butterfield et al., 2005), because VI in mice inserts all along the front part of the femur bone. To harvest VI, it must be scraped from the bone, leaving fibers damaged, and it is impossible to quantify the amount of damage. Therefore, we decided not to use the VI because we would not trust the results.

In contrast to Lynn and Morgan (1994) and Butterfield et al. (2005) who used rats, we used mice. There is a distinct possibility that different species have different sensitivities to sarcomerogenesis when exposed to a given intervention. Even if sarcomerogenesis was similar across species, and was regulated by the same mechanism, the mechanics of downhill walking, and how muscles in downhill walking might differ between species, and thus the same intervention might provide different mechanical stimuli to the target muscles. Finally, the downhill walking/running interventions by Lynn and Morgan (1994) and Butterfield et al. (2005) were short-term (5 to 10 day) protocols, whereas our interventions were 8 weeks in total and 4 weeks of uphill/downhill exposure. There is the possibility that sarcomerogenesis might occur in the short-term, and disappear when an exercise intervention becomes a chronic exposure. This is partly supported by Butterfield et al. (2005) who did not see sarcomerogenesis in their target muscles at 5 days but observed it at 10 days. It would have been interesting if they had also looked at sarcomerogenesis after long-term, chronic exposure, as done here. But to the best of our knowledge, such chronic studies on exercise-induced sarcomerogenesis have not been performed to date.

CONCLUSION

In summary, our overtraining protocols induced the NFOR state and led to muscle atrophy in VL and VM compared to the moderate training protocols. Serial sarcomere number of VL decreased in OTU group animals. This result was primarily associated with a decrease in fascicle length. Our study corroborates the results of Butterfield et al. (2005), who found that different muscle architectures and fiber lengths may influence how serial sarcomere adaptations occur. Also, our findings show that serial sarcomere adaptations may differ between short-term and long-term up/downhill running interventions, and thus, a single time point of serial sarcomere analysis might miss important aspects of how serial sarcomere numbers are controlled.

GRANTS

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DISCLOSURE

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTION STATEMENT

G.P.M., A.S.R.S., and W.H. conceived and designed research; G.P.M., and A.L.R., performed chronic protocols; G.A.L. and A.C., performed the pain sensitivity tests; G.P.M., and L.M.N., performed sarcomere experiments; G.P.M., T.R.L., A.S.R.S., and W.H. analyzed data; G.P.M., A.L.R., T.R.L., G.A.L., A.S.R.S., and W.H. interpreted results of experiments; G.P.M. prepared figures; G.P.M. drafted manuscript; G.P.M., A.L.R., T.R.L., G.A.L., A.S.R.S., and W.H. edited and revised manuscript; G.P.M., A.L.R., L.M.N., T.R.L., G.A.L., A.C., A.S.R.S., and W.H. approved final version of manuscript.

FIGURES AND TABLES

Table 1. Characteristics of the uphill and downhill training protocols.

Week	Intensity	Volume (min)	Daily sessions	Recovery between sessions (h)	TRU treadmill grade (%)	TRD treadmill grade (%)
1	60	15	1	48	0	0
2	60	30	1	48	0	0
3	60	45	1	48	0	0
4	60	60	1	48	0	0
5	30	45	1	48	14	-14
6	45	45	1	48	14	-14
7	45	60	1	48	14	-14
8	60	60	1	48	14	-14

Table 2. Characteristics of the uphill and downhill overtraining protocols.

Week	Intensity	Volume (min)	Daily session	Recovery between session (h)	OTU treadmill grade (%)	OTD treadmill grade (%)
1	60	15	1	24	0	0
2	60	30	1	24	0	0
3	60	45	1	24	0	0
4	60	60	1	24	0	0
5	60	60	1	24	14	-14
6	70	60	1	24	14	-14
7	75	75	1	24	14	-14
8	75	75	2	4	14	-14

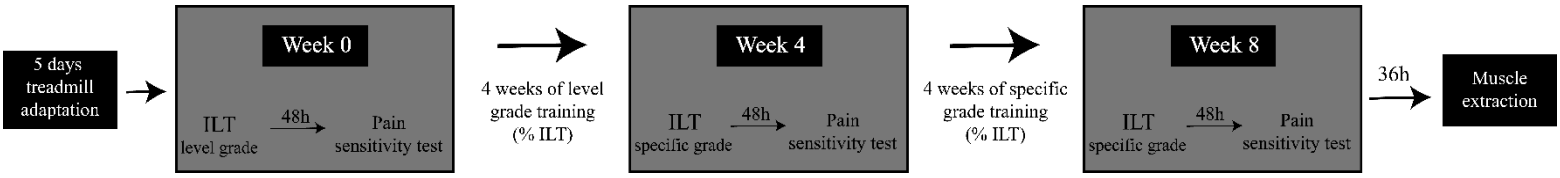

Figure 1. Schematic design of the experiment.

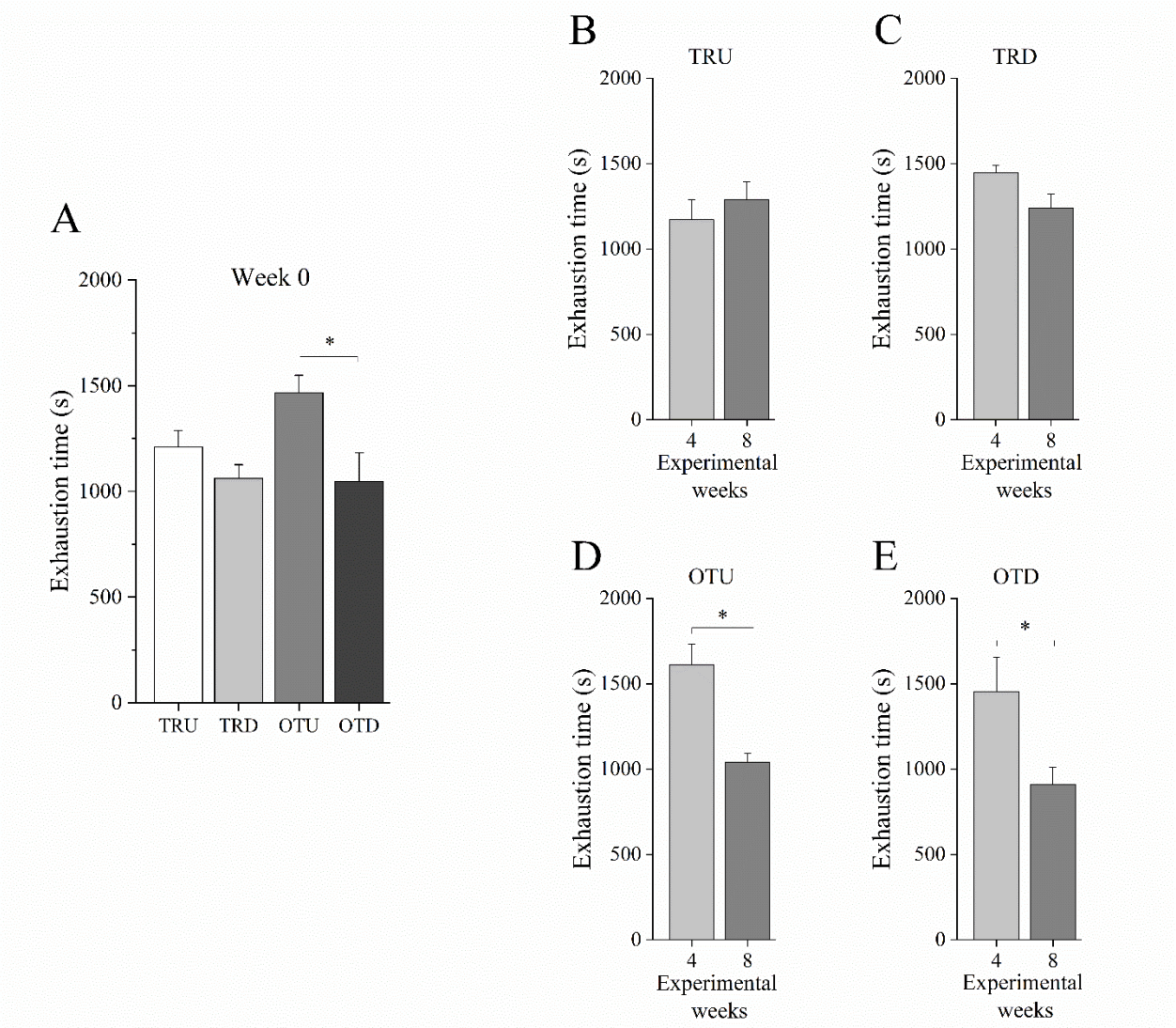

Figure 2. Incremental Load Test (ILT) performance in seconds. Figure A represents data performance at week 0 between groups. Figures B-E represents data performance after four weeks of training (week 4) and at the end of the protocols (week 8) for the same group. (B) TRU group data; (C) TRD group data; (D) OTU group data; (E) OTD group data. *Significant different. $p < 0.05$.

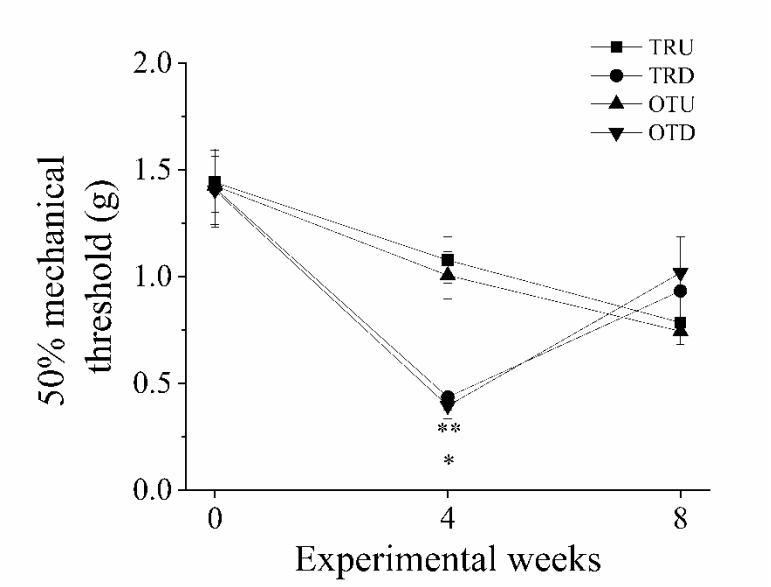

Figure 3. Mechanical pain sensitivity. Nine filaments were applied to determine the threshold stiffness required for 50% paw withdraw, with the pressure applied represented in grams (g). When smaller the threshold, higher mechanical pain sensitivity. *Significant different of TRU group. **significant different of OTU group. $p < 0.05$.

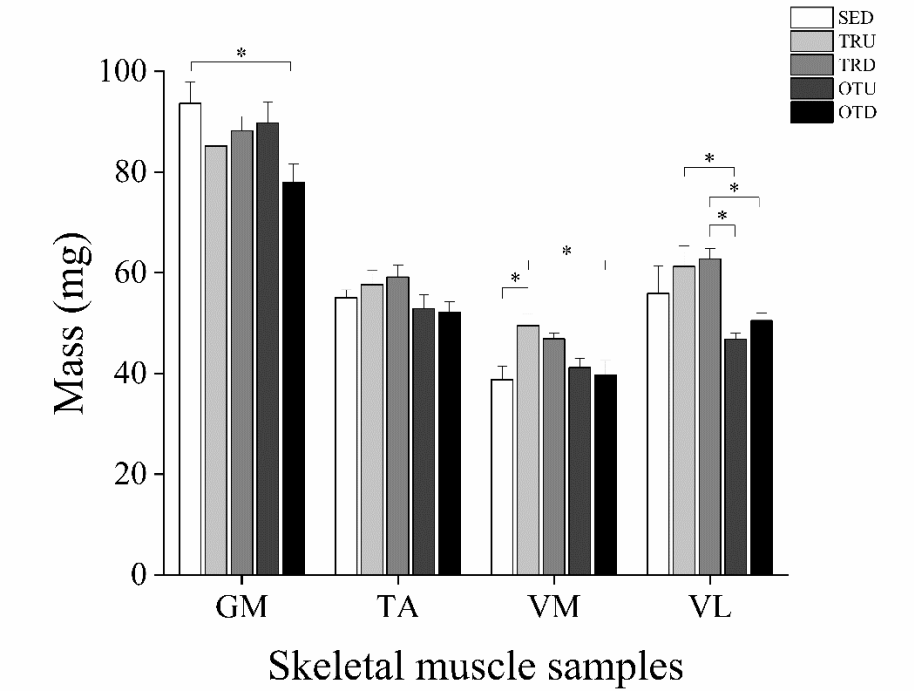

Figure 4. Muscle mass average in milligrams. GM (Gastrocnemius medialis); VM (Vastus medialis); VL (Vastus lateralis); TA (Tibialis anterior). *Significant different. $p < 0.05$.

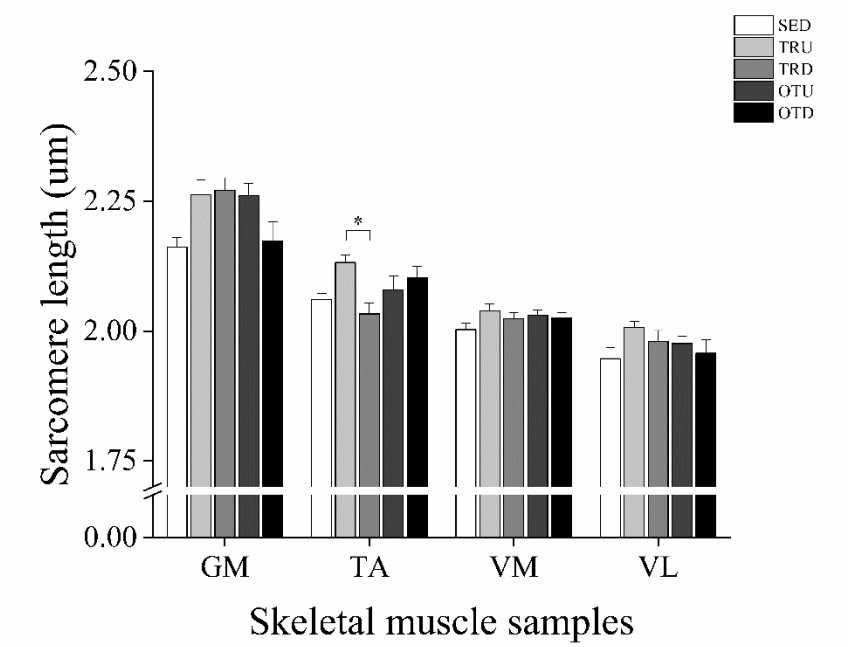

Figure 5. Sarcomere length average in µm. GM (Gastrocnemius medialis); VM (Vastus medialis); VL (Vastus lateralis); TA (Tibialis anterior). *Significant different. $p < 0.05$.

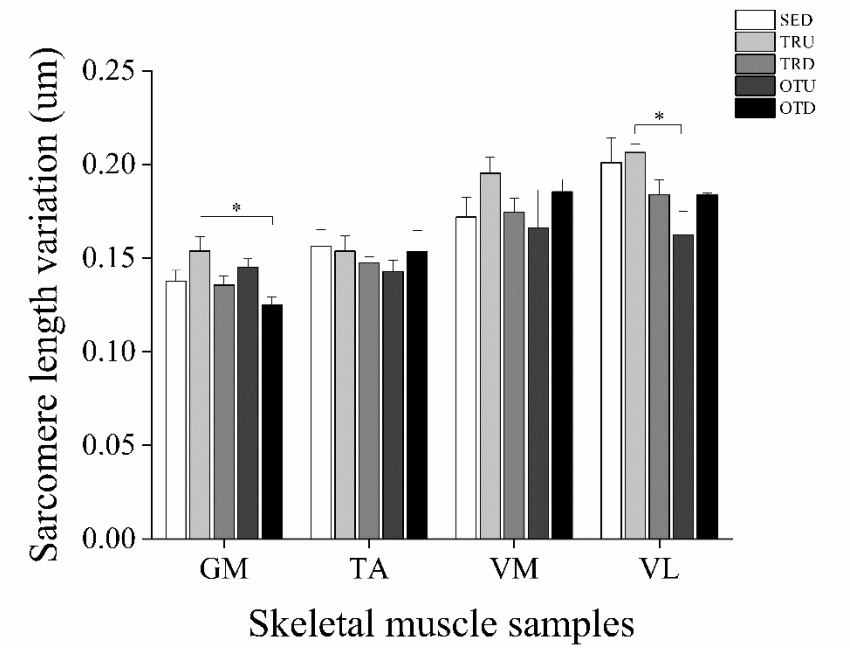

Figure 6. Sarcomere length variation average in µm. GM (Gastrocnemius medialis); VM (Vastus medialis); VL (Vastus lateralis); TA (Tibialis anterior). *Significant different. $p < 0.05$.

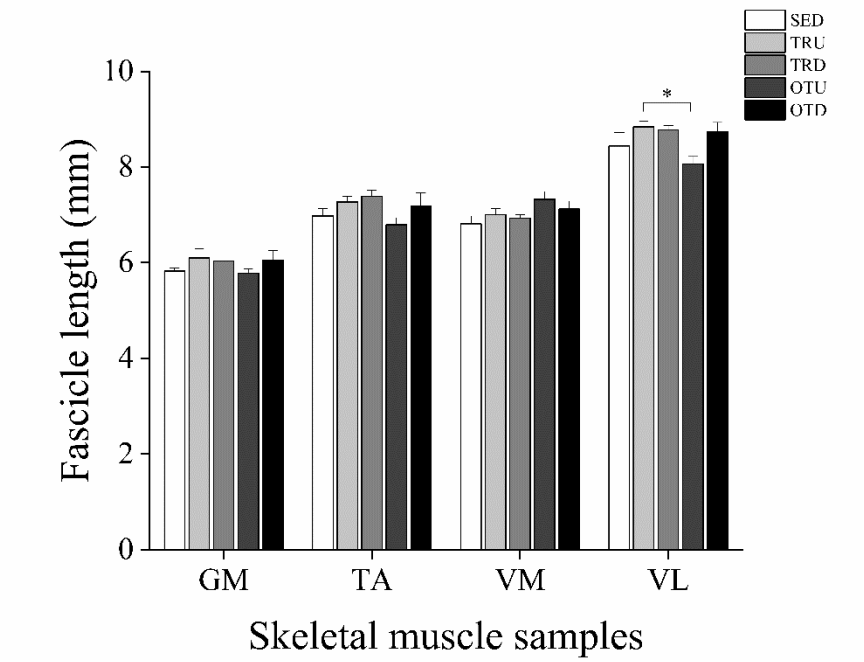

Figure 7. Fascicle length average in mm. GM (Gastrocnemius medialis); VM (Vastus medialis); VL (Vastus lateralis); TA (Tibialis anterior). *Significant different. $p < 0.05$.

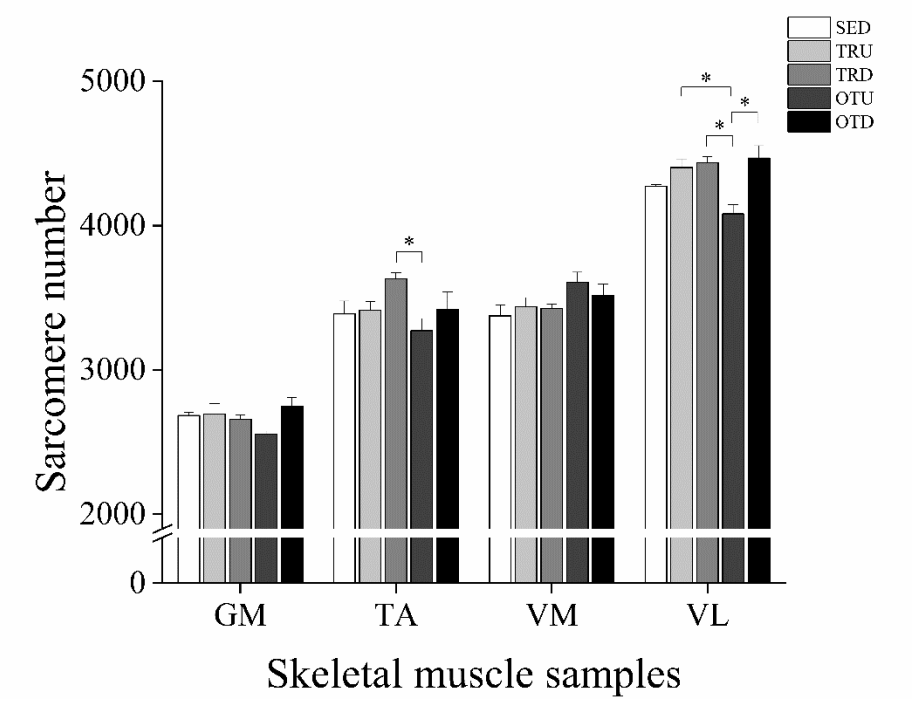

Figure 8. Sarcomere number in series. GM (Gastrocnemius medialis); VM (Vastus medialis); VL (Vastus lateralis); TA (Tibialis anterior). *Significant different. $p < 0.05$.

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3. MANUSCRIPT II

Original Article: Excessive downhill training triggers systemic inflammation leading to the early onset of osteoarthritis.

ABSTRACT

Osteoarthritis (OA) is a chronic degenerative disease characterized by cartilage injury and subchondral bone changes. There is increasing evidence associating chronic metabolic inflammation to the development of OA. Increased levels of proinflammatory cytokines are observed in this pathology, which is capable of releasing cartilage degradative enzymes such as MMP-3 and Adamts-5, and the apoptotic protein Caspase-3. In contrast, PRG-4, a tissue homeostasis player, is decreased in OA. Physical exercise (PE) has been shown as a non-pharmacological approach to prevent and treat OA. However, there is conflicting data in the literature, in which exercise workload and predominance of muscle contraction could explain the divergence of the results, where excessive exercise and eccentric contractions predominance could trigger more pro-inflammatory cytokines leading to the early onset of OA. Therefore, our study aimed to verify whether excessive training with distinct predominance of muscle contractions would lead to an inflammatory state and the early onset of OA. Mice were divided into 5 groups: sedentary (SED); uphill training (TRU); downhill training (TRD); excessive uphill training (ETU); and excessive downhill training (ETD). While the ETD group presented a systemic inflammation, elicited by increased proinflammatory cytokines levels in serum and VL and VM muscles, the ETU group presented an inflammation only in VL and VM muscles. Histology analysis verified increased total OARSI score in ETD group compared to TRU and medial and lateral tibial OARSI score compared to SED, eliciting the early onset of OA. Also, immunohistochemical analysis of MMP-3, Adamts-5, and Caspase-3 showed increased chondrocyte levels in ETD animals. In contrast, TRU presented PRG-4 increased levels in chondrocytes compared to ETU and ETD. Furthermore, low bone volume and trabecular volume and mostly low cortical thickness in ETD group appear to indicate subchondral bone maladaptations, apparently related to the early onset of OA. In summary, excessive downhill training induced a proinflammatory state due to increased mechanical load, leading to an increase in cartilage degradative enzymes, subchondral bone changes, and the early onset of OA.

Keywords: Osteoarthritis; inflammation: excessive training; Adamts-5; MMP-3.

INTRODUCTION

Osteoarthritis (OA) is a chronic degenerative disease characterized by cartilage injury and degeneration, subchondral bone alteration, and synovial joint structural modifications[1]. Metabolic OA emerged as a new phenotype of this illness and was associated with metabolic syndrome and obesity. Increasing evidence supports that chronic metabolic disorders such as increased levels of proinflammatory cytokines and adipokines develop a systemic inflammatory state contributing to the OA pathogenesis[2; 3].

The proinflammatory cytokines Interleukin 1-beta (IL-1beta), Interleukin 6 (IL-6), and Tumor necrosis factor-alpha (TNF-alpha) are important cytokines associated with OA. Increased levels of IL-1beta were observed in OA cartilage[4]. Also, circulating serum levels of IL-6 and TNF-alpha have been associated with cartilage loss[5], with IL-1beta inducing IL-6 release synergistically[6]. Furthermore, IL-1beta is capable of promoting the release of MMP-3 (Matrix Metalloproteinase-3) and the aggrecanase Adamts-5 (a disintegrin and metalloproteinase domain with thrombospondin motifs-5)[4]. The MMP family, including MMP-3, are major enzymes involved in the extracellular matrix degradation in OA[7]. The Adamts-5 is a key player in the progression of OA since its deletion in an inflammatory arthritis model prevented progression of cartilage degeneration[8]. Metabolic inflammation is related to the increase in chondrocyte apoptosis in the cartilage matrix through Caspase-3 activation[9; 10]. In contrast PRG-4 (Proteoglycan 4/Lubricin) reduces friction in biological surfaces promoting tissue homeostasis, while its absence is associated with tissue damage and OA[11]. Finally, PRG-4 may play a regulatory role in inflammatory responses[12].

Physical exercise (PE) reduces proinflammatory cytokine levels and is associated with functional capabilities improvement, decreasing the pain in OA patients[13]. International guidelines [14] recommend the regular practice of PE as a nonpharmacological strategy for prevention and treatment of this illness. However, in general society, the PE is considered as deleterious for the joints[15]. Indeed, there are some conflicting data about this question. Competitive athletes, as hockey players, have high rates of early-onset of knee OA[16]. Otherwise, White et al.[17] observed lower rates of hip and knee OA in age female physical education teachers compared to a control population.

In mice submitted to a moderate training protocol over 16 months, Lapvetelainen et al.[18] observed the progression and severity of OA compared to control. On the other hand, rats induced to OA and submitted to a 4-week moderate PE protocol reduced cartilage degeneration compared to control[19]. Regarding the contraction types, eccentric exercise (EE) may cause a predisposition to cartilage degeneration due to increased cell death as a result of increased load and strength in the knee extensor muscles compared to the concentric exercise load[20]. In contrast, Hamann et al. [21] observed a trend to increase cartilage height on the knee joint after moderate downhill running (predominantly eccentric) compared to a group trained at level. They used the same intensity for downhill and level running; however, downhill and level running are metabolically different.

These contradictory results can probably be explained by total training workload, type of contraction, and training individualization. Excessive running may be responsible for early OA stages. Using intracranial stimulation in rats, Pap et al. (1998) exceeded the normal running activity of the animals by approximately 100 times, concluding that OA degree was related to the distance performed. Animals who ran 15 km by the end of the experiment presented a mild degree of OA, and animals who ran 30 km presented a moderate degree of OA. Furthermore, compared to the control group, MMP-3 was increased by 70.4 and 89.9% on average in 15 and 30 km groups, respectively.

Excessive exercise has been associated with metabolic inflammation[22]. Mice submitted to excessive downhill and uphill running along eight weeks presented increased serum levels of IL-6, IL-1beta, and TNF-alpha[23]. Also, mice submitted to excessive downhill running increased the levels of IL-6, TNF-alpha and the proinflammatory proteins IKK (IkappaB kinase) and SOCS3 (Suppressor of cytokine signaling 3) in skeletal muscle samples, [24; 25]. Herein, using individualized workloads with different inclinations, we verified whether excessive training-induced inflammation would lead to the early onset of OA. Based on the greater damage caused by eccentric contractions[26], we hypothesize that this specific excessive training protocol would lead to a higher inflammation and early onset of OA.

METHODS

Animals: Eight-week-old C57BL/6 mice were divided into five groups: SED (sedentary), TRU (moderate uphill training), TRD (moderate downhill training), ETU (excessive uphill training), ETD (excessive downhill training). All experimental procedures followed the Brazilian College of Animal Experimentation and were approved by the Ethics Committee of the University of São Paulo (I.D. 2016.5.84.90.0). Figure 1 illustrates the schematic representation of the experimental procedures.

Performance test: Animals were adapted in a treadmill (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) for five days, 10 minutes/day at a speed of 3 m/min. For aerobic capacity evaluation and training intensity prescription, an incremental load test (ILT) was performed [27]. The ILT was performed 48h before the beginning of the intervention protocols at week 0 and the end of weeks 4 and 8. The test started at 6 m/min velocity with speed increments of 3 m/min every 3 minutes until voluntary exhaustion, defined by the animals touching the end of the treadmill 5 times in a 1 min interval. The treadmill grades at week 0 were at level for all groups. At weeks 4 and 8, the treadmill inclination was +14 degrees for the uphill groups and -14 degrees for the downhill groups. The individual exhaustion velocity of each mouse at weeks 0, 4 and 8 was determined to prescribe the individual training intensities for the TRU, TRD, ETU, and ETD protocols.

Training protocols: Training protocols were performed for eight weeks. For TRU and TRD, each experimental week consisted of three days of training (Monday, Wednesday, and Friday) followed by two days of rest over the weekend (Table 1). For ETU and ETD, each experimental week consisted of five consecutive days of training (from Monday to Friday), followed by two days of rest over the weekend (Table 2).The intensity of the training protocols was based on the ILT.

Euthanasia and tissue collection: After the 8-week intervention protocols, mice fasted for 12 hours and were anesthetized by intraperitoneal administration of xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). The pedal reflex was used to control anesthesia[28]. Once animals were under deep anesthesia, they were decapitated, total blood was collected and centrifuged at 1100 G for 15 minutes at 4ºC, and stored at -80ºC for subsequent cytokine analysis by Luminex. The knee joints were trimmed cutting the femur and tibia/fibula

approximately 0.5 cm above and below the joint line. Vastus lateralis (VL) and vastus medialis (VM) muscles were carefully removed from its attachments and stored at -80ºC.

Micro-Computed Tomography (µCT): Micro CT scans were performed using the vivaCT 40 – in vivo µCT (Scanco Medical AG, Bassersdorf, Switzerland). A 30.7 mm diameter sample holder was used, and the knee joints were fixed with low-density material. All joints were scanned at an isotropic resolution of 15µ with 55 kVp tube voltage, an integration time of 200 ms and tube current of 145 µA. Image Processing Language (IPL V5.08b, Scanco Medical, Brüttisellen, Switzerland) was used to evaluate and analyze the scans via segmentation [29]. Region of interest (ROI) was generated cropping femur and tibia/fibula at the metaphyseal growth plate or consisting 120 slices. Having the growth plate as a landmark, changes of the bone near the cartilage region were evaluated. Trabecular bone morphology consisting of total volume (TV), bone volume (BV), bone volume fraction (BV/TV), trabecular spacing (TBS), trabecular thickness (TBT), connectivity density (CD), bone mineral density (BMD) and cortical bone morphology consisting of mean cortical thickness (CT) were calculated from ROI after segmentation [29].

Histological and Osteoarthritis Research Society International (OARSI) Scoring Assessment: After µCT analysis, the intact knee joints were stored in formalin for two weeks. Posteriorly, samples were decalcified using Cal-Ex II decalcifying solution (Fisher Scientific, Hampton, NH) for 14 days, with the solution changed daily. Samples were then washed with purified water and underwent tissue processing and paraffin embedding for sectioning. Joints were sectioned on a Leica RM2255 (Wetzlar, Germany) microtome and stained with Hematoxylin, Fast green and Safranin-O using a Leica ST5010 Autostainer XL (Wetzlar, Germany) to visualize cell nuclei, collagen, and proteoglycans. Sections were imaged with an Olympus BX53 (Tokyo, Japan) using cell-sens standard 1.18 (Olympus, Tokio, Japan). Joint sections were assessed using OARSI scoring system [30] and scored by two blinded individual scorers. The region of interest of the knee joints was tibia, femur, patella, and groove divided into medial and lateral sagittal sections. Total scoring describes the sum of all regions for each animal.

Immunohistochemistry: Four not stained joint sections from the histological process of each group were separated and placed in a histological bath dish to start the deparaffinization and rehydration. Afterward, the slides were blocked in a solution using goat serum 1:500 dilution for

one hour. The slides were washed for 5 min in a TBS-T (tris buffered saline with tween 20) solution. The specific antibodies anti-Adamts-5 (PA5-27165), anti-Caspase-3 (PA5-23921), anti-PRG-4 (MABT 400), and anti-MMP-3 (AB_2566077) were conjugated using DyLight fast conjugation kit [AB_201803 (650); AB_201800 (550), Abcam, MA, USA]. Adamts-5 was conjugated with MMP-3 and Caspase-3 was conjugated with PRG-4. The slides were labeled, and 20ul of the conjugation were placed in each section. In the control section, 20ul of TBS-T was used. Slides were placed in a humidity chamber and stored at -4°C overnight. Slides were then washed three times for 10 min with TBS-T. After dry, EverBrite Mounting media with DAPI (Biotium) was placed in the slides, covered with a coverslip and left to dry in room temperature. Slides were scanned with Axion Scan.Z1 (Carl Zeiss) microscope and analyzed on the Zen 2.5 lite software (Carl Zeiss, Germany). A region of interest (ROI) was selected from the images, and cell staining was counted. The quantification refers to the total scoring, the sum of lateral and medial compartments. The antibodies were acquired from the companies Thermo Fisher Scientific (MA, USA), EMD Millipore (MA, USA) and BioLegend Way (CA, USA).

Immunoblotting: Muscle samples were homogenized with a Tissue-Tearor (Variable Speed model 985370; Biospec Products, Inc.) operated at maximum speed for 30s in an extraction buffer (1% Triton X-100, 100 mMTris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg.ml-1 aprotinin) at 4°C. The extracts were centrifuged (9900g) for 40 minutes at 4°C to remove insoluble material, and the supernatants of these homogenates were used for quantification of proteins using the method of Bradford [31]. The proteins were denatured by boiling in Laemmli sample buffer [32] containing 100mM DTT, run on SDS-PAGE gel, and transferred to nitrocellulose membranes (GE Healthcare Hybond ECL, RPN303D). The efficiency of transfer to the nitrocellulose membranes was checked by Ponceau staining of bands. These membranes were blocked with Tris-buffered saline (TBS) with 0.1% Tween-20 containing 5% BSA or 5% milk (BioRad) for 1 hour at 4ºC. Subsequently, the membranes were incubated overnight with a specific antibody [anti-IL-6 (AB9324), anti-IL-10 (sc-JES5-2S5), anti-TNF-alpha (60291), anti-IL1-beta (OAPA 00159), anti-IL-15 (sc-7889), anti-IKK (sc-34674), phospho-IKK (sc-23470), anti-SOCS3 (sc-518020), and anti-GAPDH (14C10)]. After 16h of incubation with the primary antibodies, three washes were conducted for 20 min with TBS containing 0.1% Tween-20. Subsequently, all membranes were incubated for 1 hour at room temperature with anti-mouse or

anti-rabbit secondary antibody (1:10000 dilution) (GE Healthcare). The specific immunoreactive bands were detected through chemiluminescence (GE Healthcare, ECL Plus Western Blotting Detection System, RPN2132). Images were acquired by C-DiGitTM Blot Scanner (LI-CORR, Lincoln, Nebraska, USA) and quantified using Image Studio software for C-digit Blot Scanner. The antibodies were acquired from the companies Cell Signaling Technology (MA, USA), Abcam (MA, USA), Aviva Systems Biology Corporation (CA, USA), Santa Cruz Biotechnology (CA, USA) and Proteintech Group (IL, USA) according to availability. Routine chemical reagents are from Sigma Chemical Corporation (St. Louis, MO, USA).

Blood cytokine analysis: The serum concentrations of IL-1beta, IL-6, and TNF-alpha were evaluated using Luminex TM multiplex reagents according to the instructions of the manufacturer (Millipore, St Charles, MO). For the measurements of cytokines, a MILIPLEX MAP Mouse Cytokine Panel – 3 Plex was used. Samples were collected on the Luminex MAP200 instrument and were analyzed using the 3.1 xPONENT System.

Statistical Analysis: Analysis was performed using the IBM SPSS software version 25 (Chicago, IL, USA). Data are expressed as mean ± standard error of the mean. Normality of the data was assessed using the Shapiro-Wilk test. When normality was confirmed, data was assessed through One-way ANOVA with Bonferroni correction. When the data were not normal non-parametric Kruskal-Wallis with Dunns post-hoc test was performed. $P < 0.05$ was adopted as significant differences.

RESULTS

Performance: All experimental groups increased performance at week 4 compared to week 0 (TRU p=0.02; TRD p=0.009; ETU p=0.03; ETD p=0.01) (figure 2). The TRU (p=0.002) and TRD (p=0.04) groups increased performance at week 8 compared to week 0 but maintained performance at week 8 compared to week 4 (Figures 2A and 2B). The ETU group decreased performance at week 8 compared to week 4 ($p<0.0001$) and 0 (p=0.006) (Figure 2C). The ETD group also decreased performance at week 8 compared to week 4 (p=0.0003) (Figure 2D).

Serum cytokines: The TRU, TRD, and ETU groups presented lower serum levels of IL-1β compared to the SED group (p=0.04, p=0.001, and p=0.005, respectively), while the ETD group

presented higher serum levels of IL-1β compared to the TRD (p=0.01) and ETU (p=0.03) groups (Figure 3A). The IL-6 serum levels for the ETD group were increased compared to the SED (p=0.003), TRU (p=0.01) and TRD (p=0.006) groups (Figure 3B). There was no difference for TNF-alpha serum levels between the experimental groups (Figure 3C).

Immunoblotting:

Vastus lateralis (VL): There were no differences between the experimental groups for IKKβ and IL-15 (Figures 4A and 4E). The ETU group presented higher levels of IL-1β compared to the SED (p=0.04) (Figure 4B), higher levels of IL-6 compared to the SED (p=0.01), TRU (p=0.01) and TRD (p=0.02) groups (Figure 4C), and higher levels of TNF-α compared to the SED (0.001), TRU (p=0.001), and TRD (p=0.004) groups (Figure 4G). The ETD group presented higher levels of IL-1β compared to the SED (p=0.04 and p=0.003) group (Figure 4B), lower levels of IL-10 compared to the TRU (p=0.004) group (Figure 4D), and lower levels of SOCS3 compared to the SED (p=0.04) group (Figure 4F).

Vastus medialis (VM): There were no differences between the experimental groups for IKKβ, IL-10, IL-15, and SOCS3 (Figures 5A, 5D, 5E, and 5F). The ETU group presented higher levels of IL-1β compared to the SED (p=0.01) group (Figure 5B) and higher levels of TNF-α compared to the SED (p=0.03) and TRU (p=0.04) groups (Figure 5G). The ETD group presented higher levels of IL-1β compared to the SED (p=0.01) group (Figure 5B), higher levels of IL-6 compared to the SED (p=0.02) and TRU (p=0.04) groups (Figure 5C), and higher levels of TNF-α compared to the SED (p=0.02) and TRU (p=0.03) groups (Figure 5G).

Cartilage histology (OARSI score): No significant differences were observed in Femur, Patella and Groove in lateral and medial regions of the knee joint between the experimental groups (Supplementary files 1AS, 2AS, and 3AS). However, histological cartilage lesion was observed in the medial (p=0.008) and lateral tibial plateau (p=0.04) of the ETD group compared to the SED group in the OARSI score (Figure 6B). Also, total OARSI score was higher for the ETD group compared to the TRU group (p=0.02) (Figure 6A). Scoring the slides, the chondral defects scored were only in the articular cartilage and did not reach the calcified cartilage. A healthy knee joint showed no disruptions of cartilage and presented good staining (Figure 6C, 6D, 6H and 6I), which was often viewed in medial and lateral tibial plateau of the SED and TRU groups, although in some cases the superficial zone did not show a smooth structure compared to healthy knee

joint. Frayed zones of the cartilage were more common than vertical fissures generally and, in most cases, the superficial layer was disrupted or started to be separated from mid zone, which was often observed for the TRD and OTU groups, pointed out by arrows in figures 6E, 6F, 6J, and 6K. The worst lesions were observed for the ETD group, which presented a cracked surface with an erosion about 2/3rd of depth as indicated by arrows in figures 6G and 6L.

Micro CT scans: We verified a reduction of the TV (p=0.006) and BV (p=0.003) for the ETD group compared to the SED group (Figures 7A and 7B). A reduction in cortical thickness for the ETD group was also observed compared to the SED group (p=0.03) (Figure 7E). For the other parameters (BV/TV, TBS, TBT, CD, and BMD), no differences were observed between the experimental groups. We observed negative correlations of the knee joint degeneration with TV (r = -0.349; p=0.047) and BV (r = 0.374; p=0.031) (Spearman correlation).

Immunohistochemistry: We observed an increase in total cartilage of MMP-3 content for the ETD group compared to the SED (p=0.02) and TRU (p=0.03) groups, in which can be observed more stained chondrocytes pointed by arrows (Figure 8B). Although the ETU group may present some stronger staining, it was not observed a statistical difference. Also, the ETD group presented higher levels of Caspase-3 in the chondrocytes compared to the SED (p=0.002) and TRU (p=0.03) groups, pointed by arrows (Figure 9A). The TRD may show more Caspase-3 staining (pointed by arrows); however, this result was not statistically different. Besides, we verified increased levels of Adamts-5 in the chondrocytes of the ETD group compared to the SED group, pointed by arrows (p=0.02) (Figure 8A). Regarding PRG-4 in the chondrocyte, the arrows indicate that the TRU group presented increased levels compared to the ETD and ETU groups (p=0.008; p=0.01) (Figure 9B). Although the SED group may present some staining in lateral compartment, this result was not significant.

DISCUSSION

The main findings of the study were: (i) ETU and ETD groups decreased performance at week 8; (ii) ETD group increased pro-inflammatory cytokines in serum and skeletal muscle, and ETU group increased pro-inflammatory cytokines in skeletal muscle; (iii) ETD group developed the early onset of OA; (iv) ETD group increased MMP-3, Adamts-5 and Caspase-3 in

chondrocytes and TRU increased PRG-4 in chondrocytes; (v) ETD presented lower bone volume, trabecular volume and lower cortical thickness. These findings confirm our hypothesis that excessive downhill training would induce the early onset of OA.

The performance increase for all groups from week 0 to week 4 may be related as a natural training adaptation[25]. In contrast, at the end of week 8, mice submitted to the excessive training protocols decreased their performance compared to week 4. These protocols were previously linked to the non-functional overreaching state[33], which is characterized by a performance decrease that can last for weeks[34]. Indeed, other investigations using these excessive models verified that mice performance was decreased even after 2 weeks of total recovery[23; 33].

Local and systemic inflammation:

Exercise increases the circulating levels of IL-6 during and after muscle activity, within 1 hour returning to resting levels[35]. This increase appears to be responsible for inducing a rise in the circulating levels of the anti-inflammatory cytokines IL-10 and IL-1RA right after exercise, which is known to inhibit the proinflammatory role of IL-1beta[36]. In our study, the samples were collected 36 h after the last training sessions and the TRU, TRD, and ETU groups displayed lower levels of IL-1beta. These results may be due to the IL-6 effects after exercise. For the ETU, we observed an IL-6 increase in the VL muscle samples, which could impair the IL-1beta circulating levels to rise. Also, we verified increased levels of IL-1beta and TNF-alpha in the VL and VM muscle samples. These results corroborate previous findings of our research group, which verified increased levels of IL-6 and TNF-alpha in soleus and gastrocnemius samples after excessive uphill training[23; 37], presenting a local level of inflammation. However, this local muscle inflammation was not sufficient to induce a rise in the proinflammatory circulating levels in our study.

In contrast, we verified increased serum levels of IL-1beta and IL-6 for the ETD group, characterizing an inflammatory state, and reinforcing previous data from our research group[23]; [37]. IL-10 is known as an anti-inflammatory cytokine, and obese individuals with insulin resistance presented lower circulating levels of IL-10. Also, IL-10 can increase muscle insulin sensitivity and protect against inflammation[38]. Our previous findings verified an impairment in insulin signal transduction in skeletal muscles of mice after the excessive downhill training protocol[25]. Here, we

verified a decrease in the IL-10 levels for the VL skeletal muscle samples after the ETD protocol, supporting the increased status of inflammation in this group.

Moreover, the ETD group increased the IL-1beta levels in VL and VM samples, and IL-6 and TNF-alpha levels in VL samples, which could contribute to the rise in the IL-1beta and IL-6. Also, our previous investigations highlighted a rise in the TNF-alpha, IL-1beta, and IL-6 levels for gastrocnemius, as well as IL-1beta and IL-6 levels for EDL and soleus after excessive downhill training[23; 37]. Besides, Da Rocha et al.[37]observed micro-injuries with macrophage infiltration in EDL and soleus samples with neutrophil infiltration in EDL after this protocol corroborating Fielding et al.[39], who verified damage caused by eccentric contractions in muscle associated with IL-1beta accumulation and neutrophil infiltration. These findings suggest a more detrimental and inflammatory nature of eccentric contractions (e.g., downhill) compared to concentric (e.g., uphill), mostly when performed in excess, which can be observed, for example, in elite alpine skiers[40].

Interestingly, we observed a decrease of SOCS3 in VL samples for the ETD group compared to sedentary, which do no corroborate with other studies demonstrating increased levels of SOCS3 after exercise[41;42]. However, these previous data were obtained after acute exercise. Also, one function of SOCS3 is to inhibit cytokines signaling, being considerate as an anti-inflammatory protein[42]. Thus, decreased SOCS-3 in VL muscle samples could be one of the factors involved in the increased levels of IL-1beta and IL-6 in the serum of the ETD group. Furthermore, IL-10 was shown to be one of the SOCS-3 activators[43]. Therefore, the decreased levels of IL-10 in the VL may be related to the lower levels of SOCS-3 in this skeletal muscle sample.

Early-onset of OA and mechanical loading:

Mechanical loading in the joint was shown to be important to elicit anabolic or catabolic responses depending on the duration, intensity, type of contraction, and age[44]. Experiments in young animals verified that moderate exercise-induced mechanical loading led to better development of cartilage properties and thickness[45; 46]. In adulthood, physiological loading in the joint is pivotal to maintain normal functions and improve conditions of articular cartilage and subchondral bone[44]. In contrast, excessive mechanical loading, as observed in long-distance running[47] and high impact sports[16], can directly damage the extracellular cartilage matrix (ECM),

leading to cartilage degradation and eventually OA[48]. Here, we used individual relative intensities of running for the experimental training groups and observed the early onset of OA in the cartilage of the excessive downhill training, corroborating with findings that elicit the OA development as a consequence of excessive running.

Although the ETU group also ran excessively, they do not present significant differences for the cartilage injuries. The predominance of eccentric contraction for the ETD group may be responsible for their greater damage. Horisberger et al.[20] verified increased death of chondrocytes after eccentric loading compared to concentric loading in the rabbit knee joint. Gottschall et al.[49] observed an increase of 54% in ground impact peaks at downhill running with -9º of inclination compared to level running, and a decrease of 22% in ground impact peaks at uphill running with 6º of inclination. Furthermore, skeletal muscle absorbing force for the joint and muscle dysfunction and/or weakness are also a factor for OA development[50]. Da Rocha et al.[51] verified an upregulation of myostatin, an inhibitor of hypertrophy, after the same excessive downhill training protocol that we used here. Moreover, we observed lower muscle weight in VL and VM after excessive downhill protocol (data in publishing). Thus, the increased impact due to downhill running in combination with excessive running and probable muscle dysfunction are the major factors for the early onset of OA for the ETD group. The current findings suggest that the relationship between workload and the predominance of type of contraction influence the development of OA pathology.

Inflammation leads to the early onset of OA through degradative enzyme activation and apoptosis:

Increasing evidence demonstrates a close relationship between proinflammatory cytokines and OA[52]. These cytokines inhibit the synthesis of ECM components, accelerating cartilage degradation[3]. Chondrocytes in OA cartilage express and synthesize IL-1beta in concentrations capable of inducing MMP-3 expression contributing to the degeneration of ECM[5]. Pap et al.[53] observed increased levels of MMP-3 and OA development after excessive exercise. Herein, we also verified increased chondrocyte staining of MMP-3 for the ETD group, which was associated with increased levels of proinflammatory cytokines. Chondrocyte of the ETD group presented increased Adamts-5 staining. Adamts-5 is considered the major aggrecan-degrading enzymes related to cartilage degeneration in OA, changing physiological homeostasis between matrix

synthesis and degradation resulting in an enhanced proteolysis[54], and having its mRNA expression increased by TNF-alpha in chondrocytes[55]. Echtermeyer et al[56] verified increased Syndecan-4, an Adamts-5 inductor, in cartilage of animals submitted to forced exercise, and data suggesting Adamts-5 activation mediated by MMP-3 release. Thus, increased Adamts-5 in the ETD group may be related to the increased serum inflammatory status, increased muscle TNF-alpha and cartilage MMP-3. To the best of our knowledge, this is the first study highlighting the early onset of OA associated with Adamts-5 increase in response to excessive downhill training.

Proinflammatory cytokines can induce chondrocyte apoptosis through Caspase-3 signaling mediation[57]. Chondrocytes incubation with IL-1beta activates Caspase-3; however, this effect was inhibited through resveratrol treatment blocking IL-beta action[10]. Also, moderate exercise was capable of attenuating OA through Caspase-3 reduction modulating inflammatory process[58]. Furthermore, mice lacking PRG-4 may experience elevated friction due to inflammation and display chondrocyte Caspase-3 activation[59]. Ni et al.[60] observed an intensity-dependent activation of Lubricin (PRG-4), where animals who performed low and moderate exercise along 8 weeks presented increased levels, while animals performing intense exercise displayed lower levels of Lubricin. Our PRG-4 data corroborate these findings, once moderate uphill training enhanced PRG-4 in chondrocytes compared to excessive downhill and uphill training protocols. The decrease in PRG-4 of the ETU group may be related to the observed increase in skeletal muscle proinflammatory cytokines. Besides, for the ETD group, the rise in Caspase-3 may be mediated by the increased levels of proinflammatory cytokines in serum and muscle, decreasing PRG-4 levels.

Subchondral bone adaptations:

Here, we observed lower bone volume, trabecular volume, and cortical thickness in mice submitted to excessive downhill training. Although the increase in BV/TV relation is observed in OA[61], we did not verify changes in this parameter since BV and TV were equally reduced. Also, BV/TV reflects trabecular bone connectivity indicating bone quality; however, BV/TV is unlikely to indicate bone formation and resorption[62]. Although we did not verify changes in bone connectivity (BV/TV), we observed a relationship of cartilage lesions with BV and TV, where the increase in total OARSI score was associated with the decrease of BV and TV. Cortical bone mass is a better determinant of bone strength[62], and we observed lower cortical thickness for the

ETD group, which probably indicates a weaker bone. Furthermore, during the initiation or progression of OA, proinflammatory cytokines such as IL-1beta and IL-6 are shown in osteoblasts. Finally, compression increases IL-6 and MMP-3 release, indicating a possible relationship with subchondral bone sclerosis[63], which may be associated with the lower cortical thickness observed in ETD.

In summary, we verified that excessive downhill training led to the early onset of OA, which was probably influenced by the relationship between training workload and type of contraction predominance. The ETD group presented an association of OA with systemic inflammation, which probably occurred due to the higher impact caused for the conjunction of excessive running and eccentric contractions. The systemic inflammation status seems to be related to the chondrocyte increase of MMP-3, Adamts-5, and Caspase-3, and the chondrocyte decrease of PRG-4. Also, the excessive downhill running protocol led to lower cortical thickness, probably indicating bone weakness. Cartilage lesions were related to lower bone and trabecular volumes, although no alteration was observed for the BV/TV. To our knowledge, this is the first study to induce early onset of OA and verify increased levels of Adamts-5 in chondrocytes after excessive training associated.

FIGURES AND TABLES

Table 1. Characteristics of the training protocols.

Week	Intensity	Volume (min)	Daily sessions	Recovery between sessions (h)	TRU treadmill grade (%)	TRD treadmill grade (%)
1	60	15	1	48	0	0
2	60	30	1	48	0	0
3	60	45	1	48	0	0
4	60	60	1	48	0	0
5	30	45	1	48	14	-14
6	45	45	1	48	14	-14
7	45	60	1	48	14	-14
8	60	60	1	48	14	-14

Table 2. Characteristics of excessive training protocols.

Week	Intensity	Volume (min)	Daily session	Recovery between session (h)	ETU treadmill grade (%)	ETD treadmill grade (%)
1	60	15	1	24	0	0
2	60	30	1	24	0	0
3	60	45	1	24	0	0
4	60	60	1	24	0	0
5	60	60	1	24	14	-14
6	70	60	1	24	14	-14
7	75	75	1	24	14	-14
8	75	75	2	4	14	-14

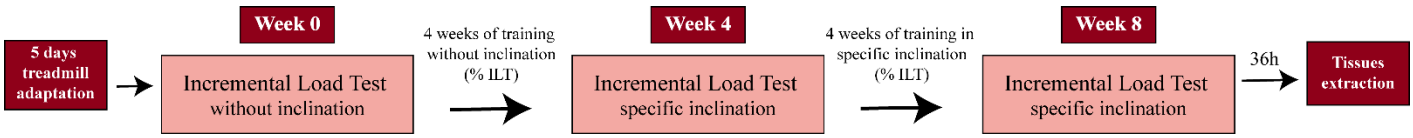

Figure 1. Experimental design of the study.

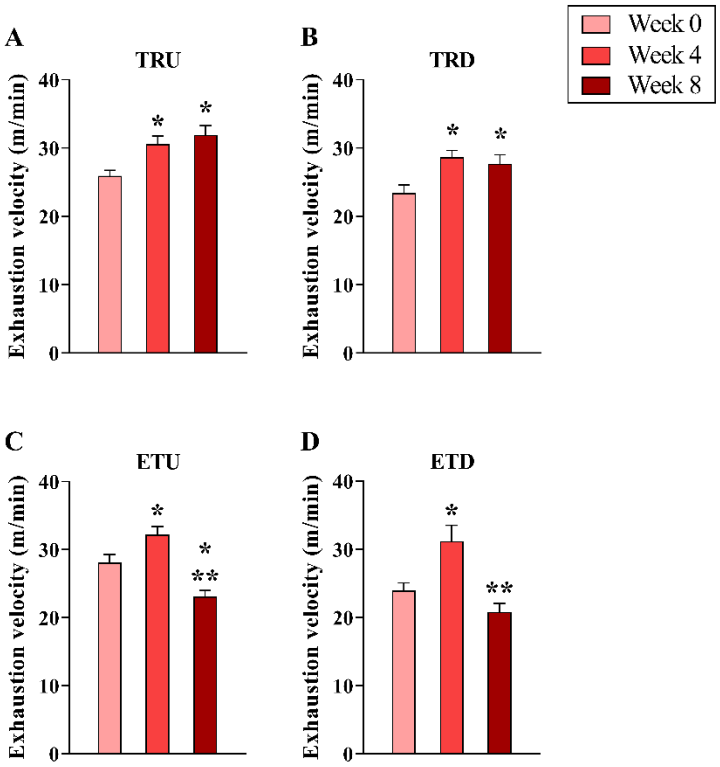

Figure 2. Incremental load test (ILT). (A) TRU group, n=14; (B) TRD group, n=14; (C) ETU group, n=13; (D) ETD group, n=12. *significant different from week 0. **significant different from week 4. $p < 0.05$.

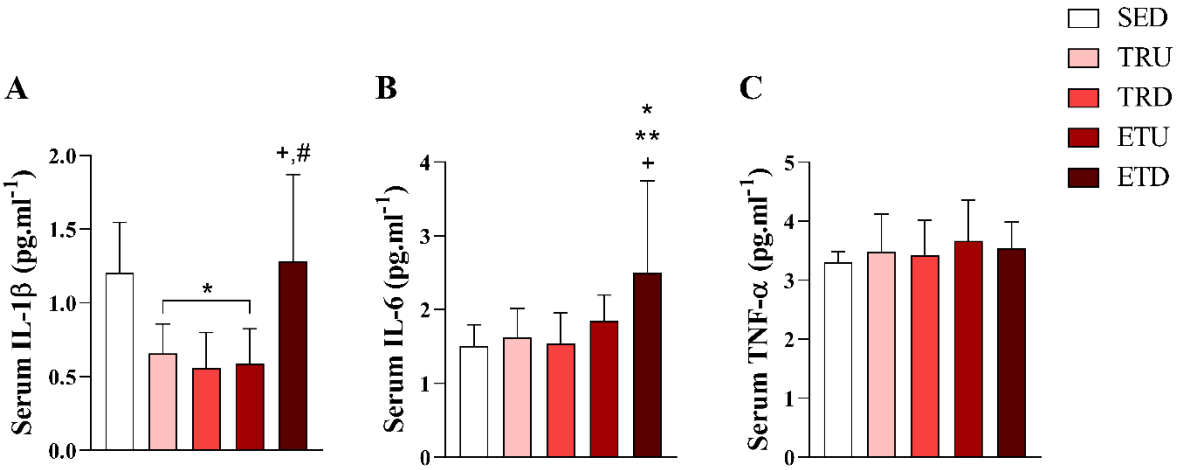

Figure 3. Serum cytokines in $pg.ml^{-1}$. (A) serum IL-1β; (B) serum IL-6; (C) TNF-alpha. SED n=14; TRU n=14; TRD n=14; ETU n=13; ETD n=12. *significant different from SED. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

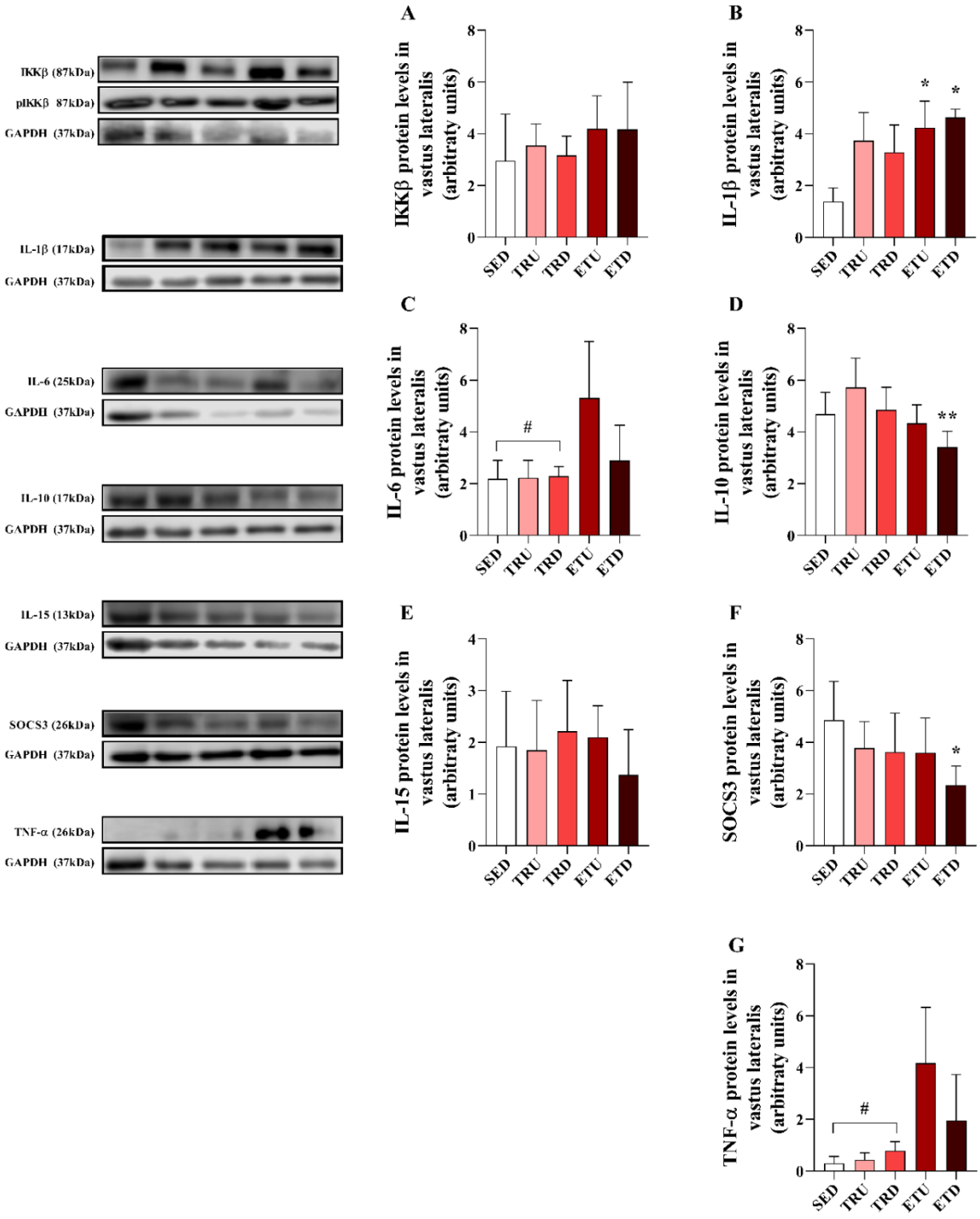

Figure 4. Vastus lateralis muscle immunoblotting proteins. n=6 per group. (A) IKKβ; (B) IL-1β; (C) IL-6; (D) IL-10; (E) IL-15; (F) SOCS3; (G) TNF-α. *significant different from SED. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

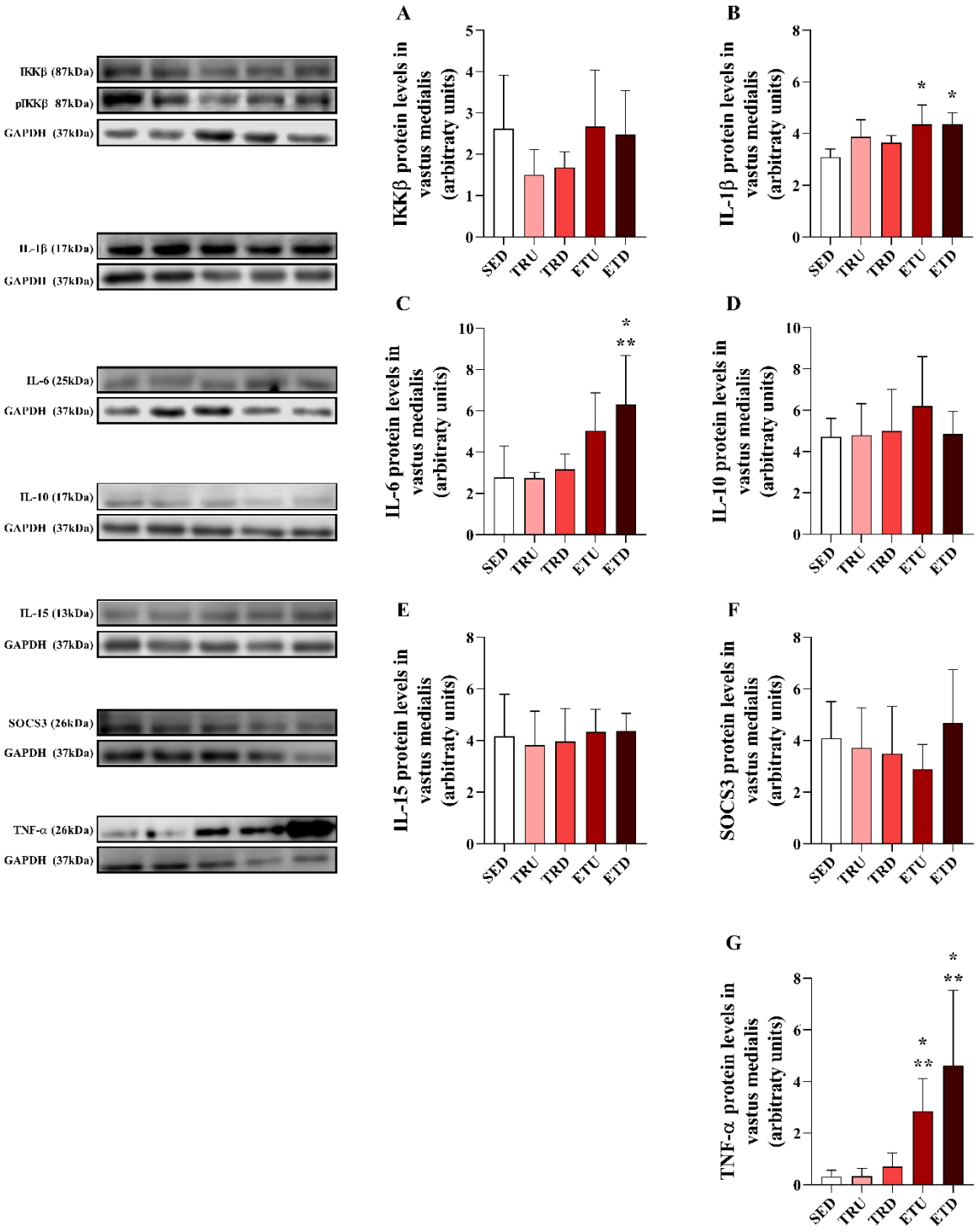

Figure 5. Vastus medialis muscle immunoblotting protein. n=6 per group. (A) IKKβ; (B) IL-1β; (C) IL-6; (D) IL-10; (E) IL-15; (F) SOCS3; (G) TNF-α. *significant different from SED. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

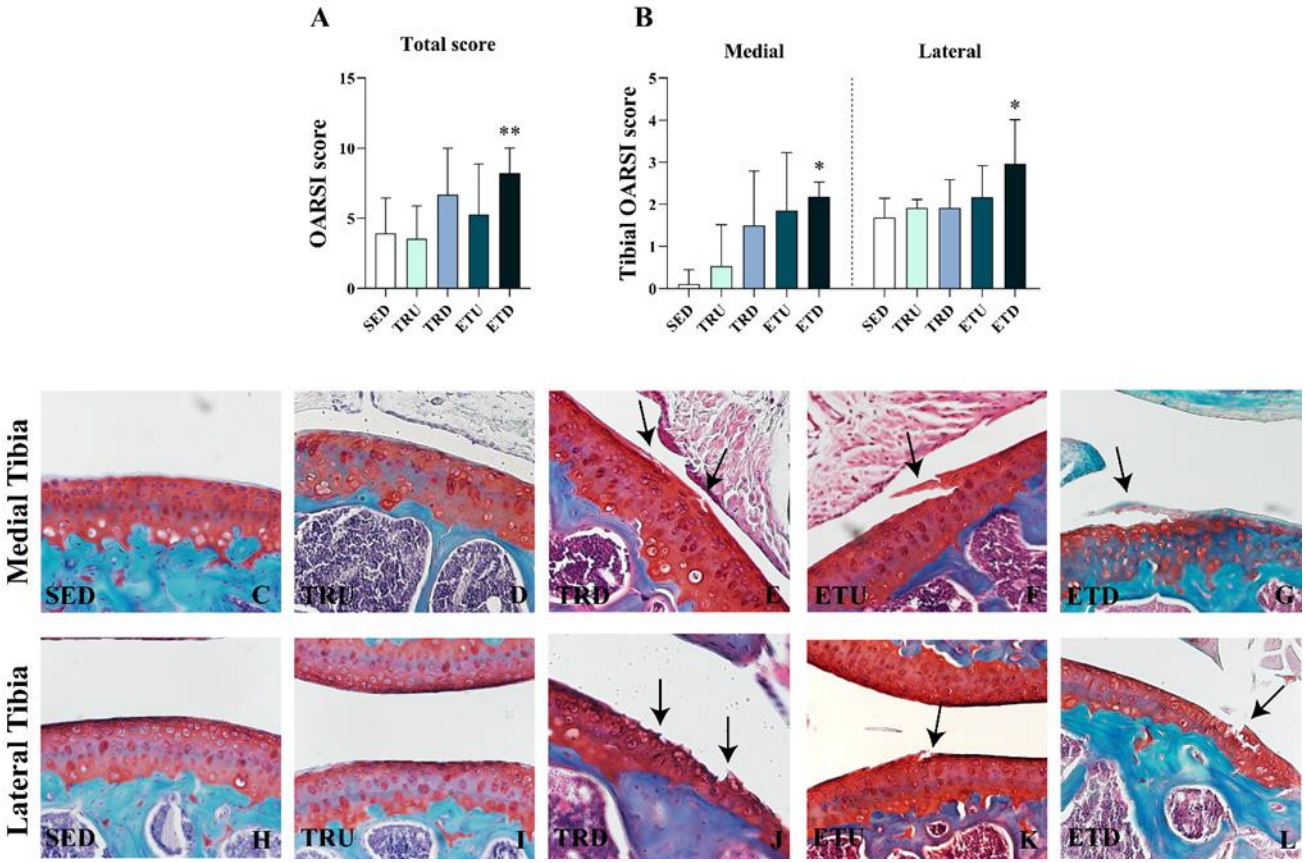

Figure 6. Histological cartilage analysis. (A) Total OARSI score; (B) Tibial OARSI score. Representative medial Tibia compartments: (C) SED; (D) TRU; (E) TRD; (F) ETU; (G) ETD. Representative lateral Tibia compartments: (H) SED; (I) TRU; (J) TRD; (K) ETU; (L) ETD. SED n=8; TRU n=8; TRD n=8; ETU n=7; ETD n=6. *significant different from SED. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

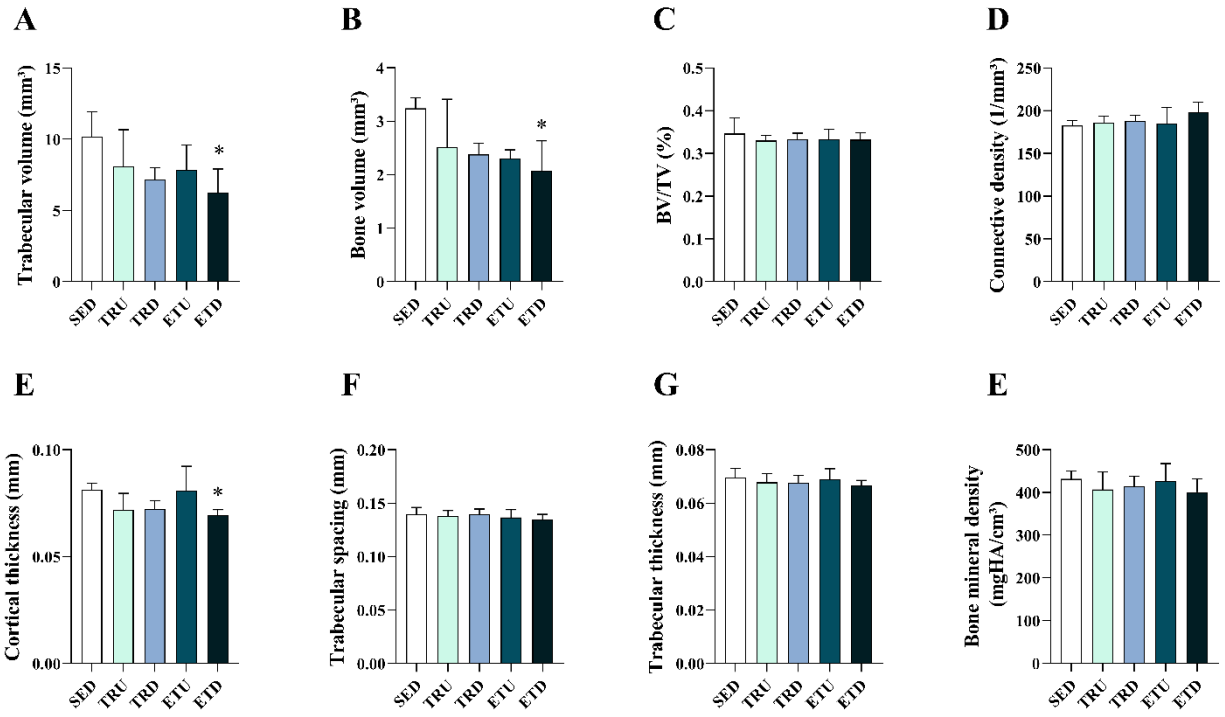

Figure 7. Micro-computed tomography scans of mice knee joint. (A) Trabecular volume; (B) Bone volume; (C) BV/TV %; (D) Connective density; (E) Cortical thickness; (F) Trabecular spacing; (G) Trabecular thickness; (H) Bone mineral density. *significant different from SED. SED n=8; TRU n=8; TRD n=8; ETU n=7; ETD n=6. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

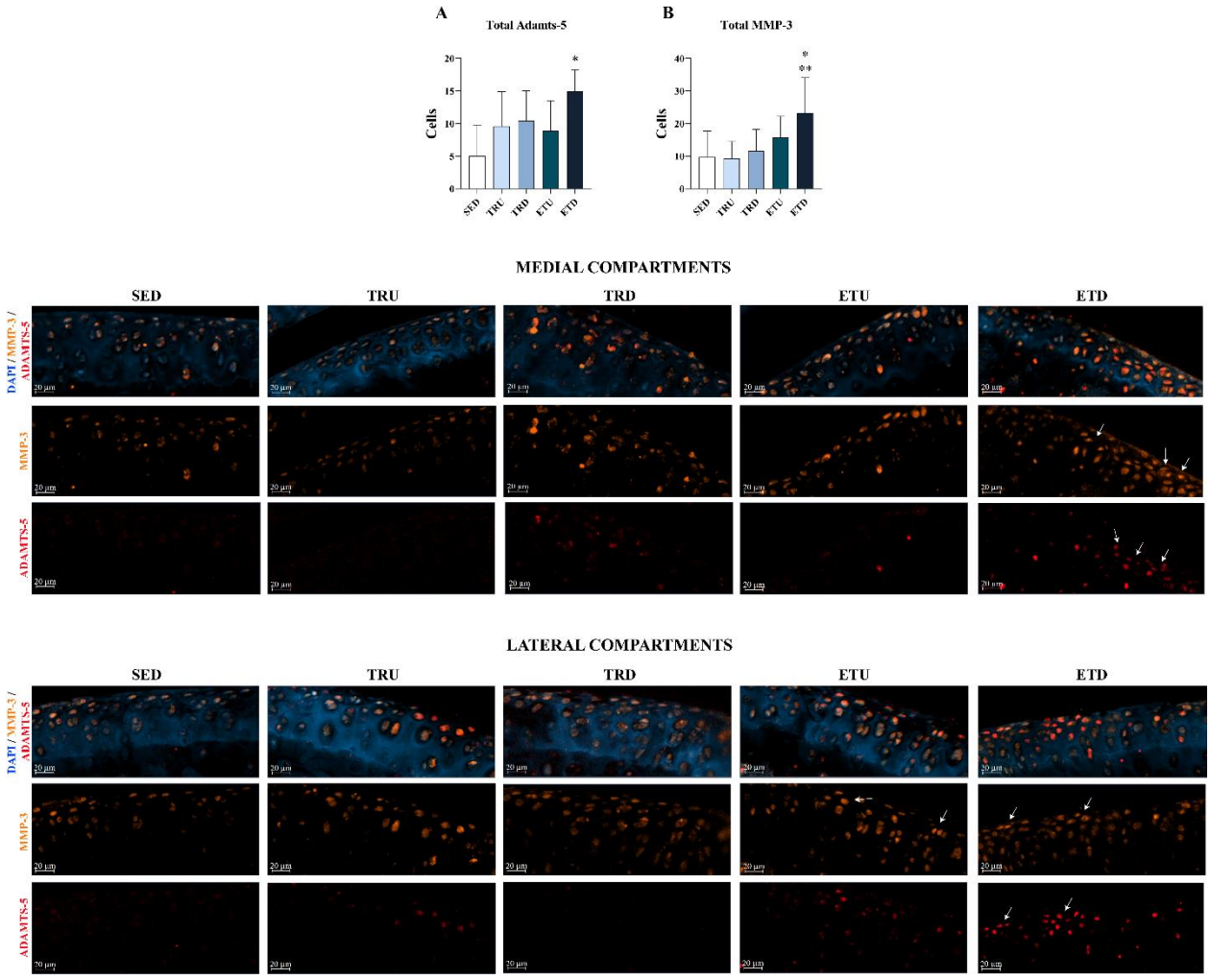

Figure 8. Immunohistochemical analysis of articular cartilage and representative images of medial and lateral cartilage compartments of each group. (A) Total Adamts-5; (B) Total MMP-3. n=7 per group. *significant different from SED. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

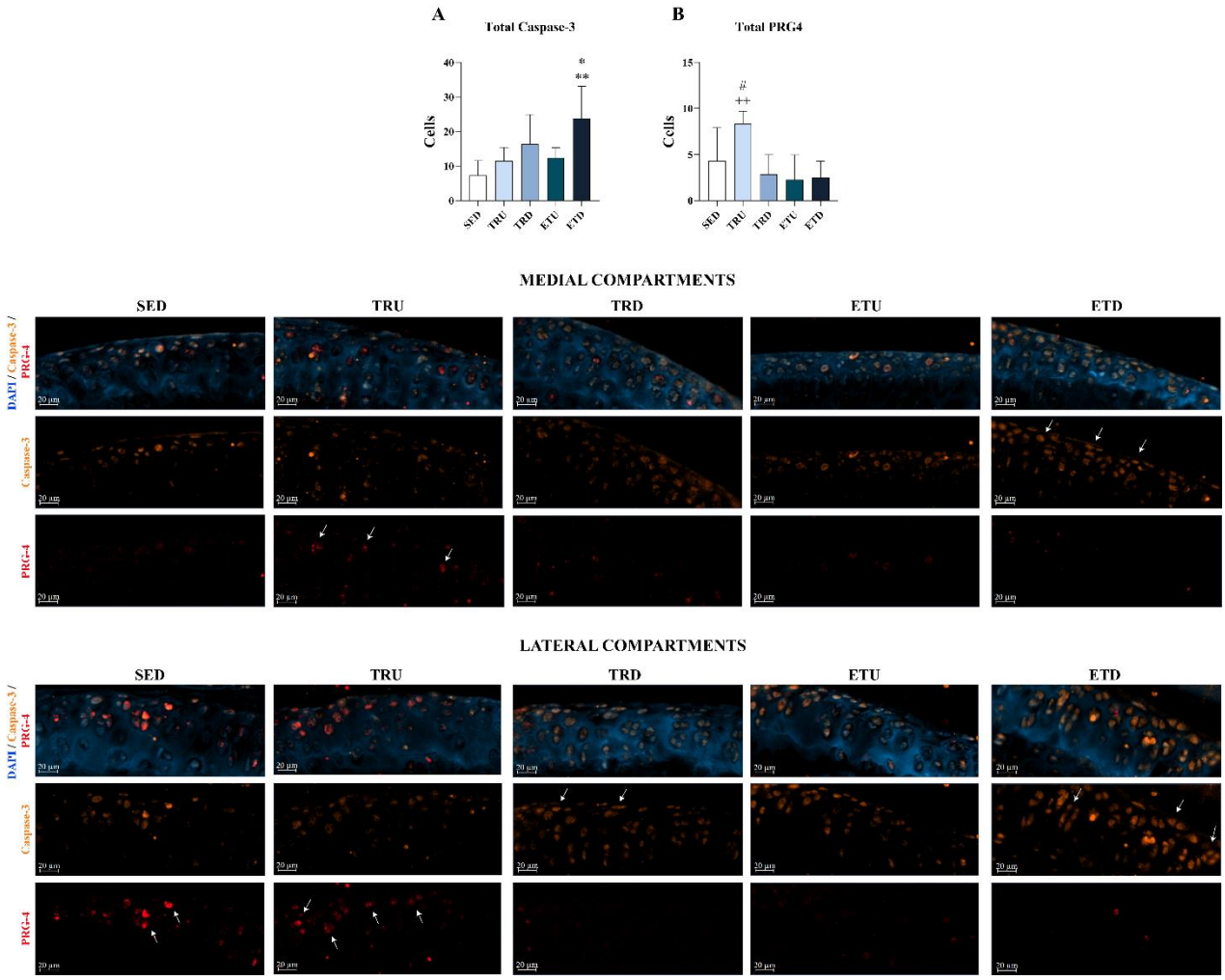

Figure 9. Immunohistochemical analysis of articular cartilage and representative images of medial and lateral cartilage compartments of each group. (A) Total Caspase-3; (B) Total PRG-4. n=7 per group. *significant different from SED. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

Supplementary file.

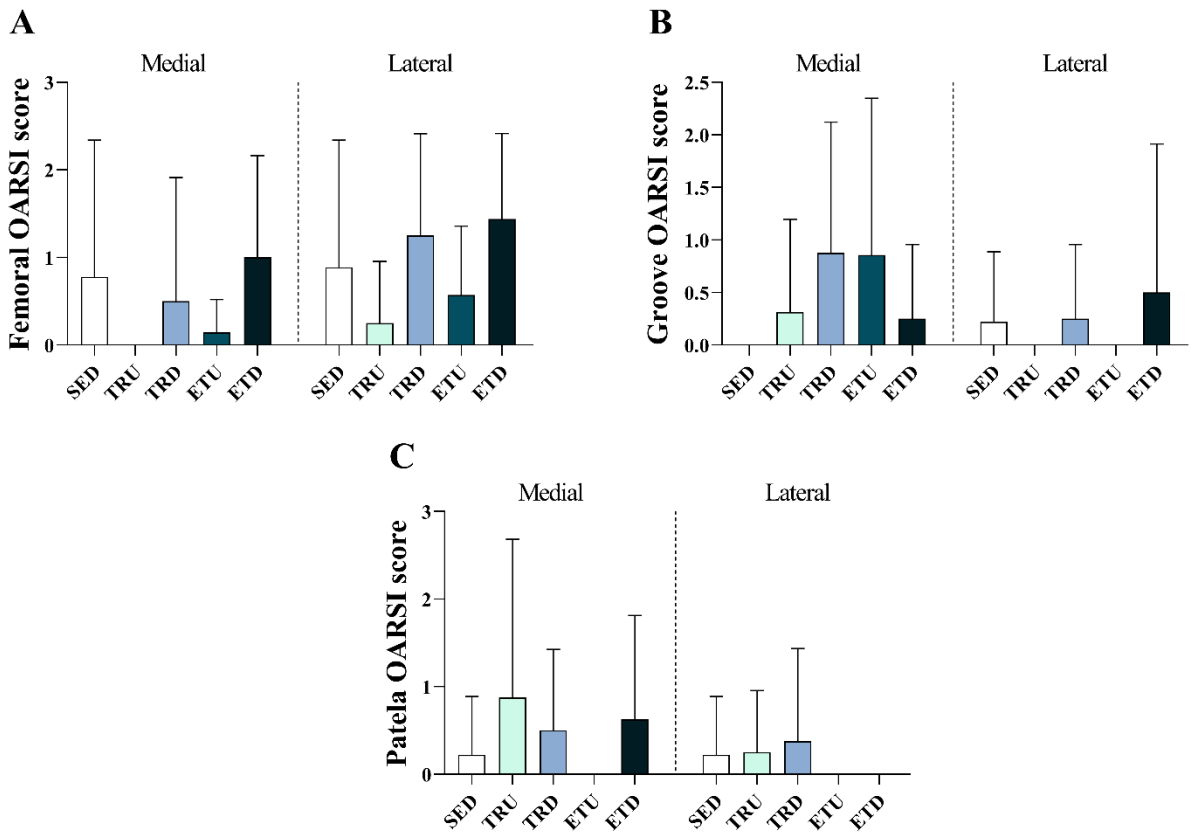

Figure 1S. Histological cartilage analysis. (A) Femoral OARSI score; (B) Groove OARSI score; (C) Patela OARSI score. SED n=8; TRU n=8; TRD n=8; ETU n=7; ETD n=6. *significant different from SED. **significant different from TRU. +significant different from TRD. #significant different from ETU. ++significant different for ETD. $p < 0.05$.

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4. CONCLUSIONS

In summary, the downhill and uphill excessive training protocols induced de NFOR state and lead to muscle atrophy in VL and VM compared to moderate training protocols. Mice submitted to excess of downhill training developed the early onset of OA associated with systemic inflammation. We also observed increased levels of degradative enzymes in chondrocytes of ETD animals, which probably are related to the inflammatory state. In contrast TRU group increased levels of PRG-4 in chondrocytes eliciting a good cartilage adaptation to the training. Subchondral bone adaptations in ETD group presented decreased bone and trabecular bone volumes and lower cortical thickness which is associated with OA. Therefore, workload and predominance of type of contraction can influence in the development of OA. Regarding sarcomere adaptations we observed decrease in serial sarcomere number in ETU group which was associated with increase in fascicle length. Also, our findings show that serial sarcomere adaptations may differ between short-term and long-term up/downhill running interventions.

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