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Remodeling process in bone of aged rats in response to resistance training

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ABSTRACT

Aims: We investigate the effects of RT on the mechanical function, gene, and protein expression of key factors involved in bone remodeling during aging. **Main Methods:** Male rats of 3 and 21 months of age were randomly allocated into four groups (8 per group): young sedentary (YS), young trained (YT), old sedentary (OS), and old trained (OT). RT was performed three times per week (12 weeks). Bone tenacity and stiffness were measured by biomechanical tests and mRNA levels of COL1A1, MEPE, SOST, OPG, BMP-2, PPAR- γ , MMP-2-9-13, and TIMP-1 were evaluated by quantitative PCR. COL1A1 protein and MMP-2 activity were detected by western blotting and zymography assays. **Key findings:** Aging increased stiffness, while BMP-2, OPG,

COL1A1 and MMP-2 mRNA levels reduced (OS vs YS; $p \leq 0.05$). RT increased the tenacity of the femur and reduced PPAR- γ regardless of age (YT vs. YS; OT vs. OS; $p \leq 0.05$). RT downregulated SOST mRNA levels only in the OT group (vs. OS group, $p \leq 0.05$). RT mitigated the age-associated increase in MMP-9 mRNA levels ($p \leq 0.05$). In young animals, upregulation in MEPE, MMP-13, TIMP-1 were observed after RT, as well an increase in COL1A1 protein and MMP-2 activity ($p \leq 0.05$). **Significance:** RT improved bone tenacity independent of aging, which is relevant for mechanical function, while, at protein levels, RT upregulated MMP-2 activity and collagen 1 only in young rats. This study highlights the importance of exercise on bone health and identifies specific molecular changes in response to RT. Our findings provide insights into the mechanisms involved in age-related changes.

Keywords: aging, resistance training, bone homeostasis, function.

Introduction

Aging is a complex and multifactorial process characterized by deleterious changes in various tissues and considered one of the major risk factors for osteoporosis^[1, 2]. Osteoporosis has been characterized as deterioration of bone composition, structure, and function, with an increased risk of fractures^[3]. This overwhelming skeletal fragility syndrome causes more than 9 million fractures worldwide per year^[4]. In terms of quality of life, osteoporosis constitutes the most common chronic metabolic bone disease and therefore may decline functional status, social function, health, and quality of life^[5]. Bones undergo a constant self-regeneration process called remodeling^[5], which leads to the replacement of old elements of the tissue in response to microdamage and mechanical loadings^[6]. However, bone remodeling is reduced with aging, inducing a gradual and progressive decline in the properties of bones^[5]. Although studies show that aging adversely affects the elastic and mechanical properties of bones^[7, 8], the underlying mechanisms involved in these processes have not yet been fully investigated.

Evidence suggests that some proteins are critical to the functioning of bone cells, including osteoblasts, osteocytes, and osteoclasts in the onset of osteoporosis^[9]. The key elements related to bone remodeling may arise as potential candidates to provide more insights into the molecular events within bone tissue homeostasis. RANKL, a ligand of receptor activator of nuclear factor kappa B, has been shown to regulate osteoclast activation both in normal bone remodeling and in pathologic conditions characterized by increased bone turnover^[10]. Osteoprotegerin (OPG), in turn, prevents the RANKL

binding to its receptor RANK, protecting bone from excessive resorption^[10]. An elegant study demonstrated that reducing mechanical loading exerts diminished effects on osteoblasts, resulting in decreased osteoblast secretion of OPG and increased expression and secretion of RANKL^[11]. The reduction in OPG also allows greater binding of RANKL to its receptor (RANK), which further facilitates the resorption^[12]. Moreover, bone morphometric proteins (BMPs) induce osteoclastogenesis by enhancing the expression of RANKL^[10], while sclerostin (SOST) is a potent inhibitor of osteoblastogenesis, reducing bone formation^[5]. SOST mediates responses of bones to mechanical unloading^[5]. Metalloproteinases and their inhibitors (MMPs and TIMPs) also regulate the resorption, remodeling, and repair of bones^[13], particularly cleaving native and undenatured collagen^[14]. Type I collagen plays a substantial role in structural support and bone mechanical stability, which contributes to bone matrix quality^[15]. Conversely, matrix extracellular phospho-glycoprotein (MEPE) appears to inhibit bone resorption and alters bone vascularization^[16].

Resistance training (RT) is a crucial strategy to counteract the intrinsic age-induced changes. RT has been shown to play a pivotal role in counteracting intrinsic age-induced changes either in the musculoskeletal system (muscle-tendon-bone) of elderly individuals^[17-19] or animal experimental models^[20]. Previously, it was demonstrated that RT induced beneficial effects on tendon^[21, 22], skeletal muscle^[23, 24] and heart tissue^[25] of aged rats. Moreover, RT affected the gene expression of molecules associated with intramyocellular lipids (PPAR γ : peroxisome proliferator-activated receptor gamma, an adipogenic factor) and fiber size in old rats^[26]. However, the extent to which RT leads to molecular changes in bone homeostasis of aged rats has not been well documented.

Bone plasticity is a complex process involving cross-talk between multiple mechanisms and signaling pathways. Biophysical, biomechanical, and molecular approaches might aid understanding of the complete picture of cellular adaptation in response to exercise in aged bone. A mechanistic framework of these effects could provide crucial clues for the development of therapeutic approaches to mitigate bone remodeling disorders, as well as mapping of relevant biomarkers in physiological responses inherent to aging. Therefore, we aimed to investigate the effects of an RT model on mechanical function, as well as gene and protein expression of key factors involved in ECM bone remodeling during the aging process. We hypothesized that RT upregulates molecules involved in biological pathways related to protection and damage

recovery and that this regulation is accompanied by enhanced biomechanical properties independent of aging.

Material and methods

Animals and experimental groups

All procedures were conducted in agreement with the Guide for the Care and Use of Laboratory Animals (U.S. National Research Council, Washington D.C., USA). The research protocol received approval from the Animal Research Ethics Committee of the local University (protocol number: 044/2011). Thirty-two (8 per group) male Wistar rats (*Ratus norvegicus albins*) between the ages of 3 months (young) and 21 months (old) at the start of the experiment were randomly separated into four experimental groups: YS (young sedentary), YT (young trained), OS (old sedentary), and OT (old trained). During the experiments, the rats were placed in collective home-cages with members of the same group (4 animals/cage) with water and standard rodent chow (Purina®, Descalvado, São Paulo, Brazil) offered *ad libitum*, and exposed to 12 h light/dark cycles with a temperature of 20–22 °C. The study experimental design is presented in Figure 1.

Resistance Training

Familiarization: The rats were adapted to an RT protocol of climbing a vertical ladder (1.1 m; 0.18 m, 2-cm grid, 80° incline) for two non-consecutive days (48-hour rest interval) with no weight on the load apparatus. The load apparatus was fixed to the tail by wrapping the proximal portion with a self-adhesive foam strip. The rats were placed at the bottom of the ladder and familiarized with climbing. Finger pinching was used on the animal's tail as a stimulus to initiate climbing. At the top of the ladder, the rats reached a house chamber where they rested for 2 minutes. This procedure was repeated until they would voluntarily climb the ladder for three consecutive turns without stimulus.

Determination of maximum carrying capacity: Two days after the familiarization procedure, each animal was evaluated to determine its maximum carrying load, which consisted of 4-9 ladder climbs with progressively heavier loads. The initial climb was performed with 75% of the animal's body mass. Upon successful completion of this load, an additional 30g weight was added to the load apparatus. The highest load that the animal successfully carried through the entire length of the ladder was considered the maximal carrying capacity for that training session. Failure was determined when the animal could not progress up the ladder after three successive stimuli to the tail.

Resistance training period: RT was performed three times per week (Mondays, Wednesdays, and Fridays) for 12 weeks. The length of the ladder permitted the animals to perform 8-12 dynamic movements per climb. The climbs comprised carrying progressive loads of 65, 85, 95, and 100% of the maximum carrying capacity of each animal. RT sessions consisted of 5-8 movements per climb over 6-8 seconds. If a rat reached 100% of its carrying capacity, an additional 30g load was added until a new maximum carrying capacity was determined. The resting period between each climb was 2 minutes. The RT protocol was adapted from Hornberger and Farrar^[27].

Tissue preparation:

One at a time, 48 h after the end of the experimental period, the animals were euthanized using an intraperitoneal injection of xylazine solution (24 mg/ kg of body weight) and ketamine (100 mg/ kg of body weight). The right femur was immediately dissected from each posterior hindlimb and either; frozen in RNase-free tubes using liquid nitrogen (for qPCR, zymography and western blotting) and then stored at -80°C; or kept in saline solution 0.9% and for biomechanical and biophysical analyses. For biometric and biomechanical analysis we used 8 animals per group. For biophysical, gene expression, zymography, and western blotting analysis we used 5 per group.

Biometrical, biochemical, biomechanical, and biophysical analyses

Biometrical analyses: The body was measured during the 12 weeks of RT. The animals were weighed three times per week on a precision digital weighing machine (KERN, model 573, Balingen, Germany). The right femurs (n=8/group) were measured using a digital caliper Carbon Fiber Composites Western® PRO (precision of 0.05 mm). The measurement reference was the distal region of the femoral condyles (articular face with the tibia) and the greater trochanter. Following the biometrical analysis, the femurs were placed in distilled water and placed in a desiccator for 24 h to remove the air present in bone pores. Subsequently, the immersed (m_{imm}) and wet mass (m_{wet}) were measured. The bones were maintained wrapped in saline-soaked gauzes except during measurements.

Biochemical analyses: In order to obtain mineral material related to calcium and phosphorus content, the femurs were solubilized in 2N hydrochloric acid. Subsequently, the calcium and phosphorus content was determined using a colorimetric assay kit Labtest® (Diagnóstica, SA, Brazil).

Biophysical analyses: After the biomechanical test the femurs were placed in an incubator at 100 °C for 24 h to dehydrate and the dry mass (m_{dry}) was obtained. Next, the samples were kept in a muffle (800 °C) for 24 h to obtain the ash mass (m_{ash}). Using the Archimedes Principle, the following results were obtained: Bone density (BD), % of organic material, and % of mineral material with the following formulas: Bone volume (BV) = $m_{wet} - m_{imm} / \rho$ (water density) (cm^3), $BD = m_{wet} / BV$ (mg/cm^3), % of organic material = $100 \times (m_{dry} - m_{ash}) / m_{wet}$ and % of mineral material = $100 \times m_{ash} / m_{wet}$.^[28]

Biomechanical properties: The femurs were measured by the three-point bending test using a computer-controlled universal test machine Instron 4444 (Instron, Canton, Massachusetts, USA) with a 1 kN load cell. Femur extremities were supported on two rollers (3 mm of diameter, 21.70 mm of distance) and a downward vertical load was applied in the central region of each bone^[29]. Initially, a 5 N pre-load was applied, to avoid specimen sliding, in the posterior–anterior direction (perpendicular to the longitudinal axis). After a one-minute accommodation and stabilization period a force was applied at a constant velocity of 0.5 cm/min until fracture. Through the analysis of the graphic, the main biomechanical properties were obtained: maximal load (N), stiffness (N/mm), and resilience (J)^[28, 30]

Gene expression

RNA extraction from femur samples: The head and neck of the femur from rats of each group (n=5/group) were used for this analysis. Initially, the samples were macerated by hand using liquid nitrogen with a pistol and homogenized in TRIZOL reagent (Invitrogen Corporation, California, USA) with a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, USA). Subsequently, total RNA was extracted according to the TRIZOL manufacturer's protocol. The RNA pellet was resuspended in nuclease-free water and stored at -80 °C. The Nanodrop 2000 (Thermo Scientific, Wilmington, USA) was used to measure the RNA concentration and purity (ratios 260/280 nm and 260/230 nm).

qRT-PCR Reverse Transcription: Before reverse transcription, total RNA (1 µg) from each sample was treated with deoxyribonuclease I (DNase I; Invitrogen Corporation, California, USA) according to the manufacturer's instructions in order to remove genomic DNA. Subsequently, treated RNA was reverse-transcribed into cDNA with M-MLV reverse transcriptase (Promega Corporation, Madison, USA) and oligo (dT) (Promega Corporation, Madison, USA). After the reaction the samples were stored at -20°C in a freezer.

Quantitative real-time polymerase chain reaction (qRT-PCR): qRT-PCR was performed using a CFX 96 real-time PCR detection system (Bio-Rad, San Francisco, USA). For each reaction, 20 ng of cDNA, 0.2 µg of each primer, and 5 µg of SsoFastTMEvagreene Supermix (Bio-Rad, San Francisco, USA) were used in a total volume of 10 µL. The gene specific primers were purchased from Sigma Aldrich (Missouri, EUA) or from Bioneer (Republic of Korea) and are listed in Table 1. The thermal cycling program was 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72 °C. After running, the melting curve was analyzed to confirm the specificity of the amplification products. Ribosomal protein S18a (RPS18A) was used as a reference gene. The relative expression of the qRT-PCR products was calculated through the $\Delta\Delta C_t$ method, which uses the following equation: fold induction = $2^{-\Delta\Delta C_t}$ ^[31].

Detection of type I collagen level by Western blot method

After the RNA isolation from the head and neck of the femur, the remaining Trizol was stored at -80 °C. The protein fraction was isolated using the Trizol method according to the manufacturer's protocol with some modifications. Trizol extracts were thawed and centrifuged, and the residual aqueous phase was removed. The protein precipitate was washed twice with 0.3 M guanidine hydrochloride in 95% ethanol. The precipitate was centrifuged in each wash (7,500g for 5 minutes at 4 °C). The pellet was washed twice in 2 mL of 100% ethanol for 20 minutes at room temperature, after which the sample was centrifuged again (7,500g for 5 minutes at 4 °C). The protein precipitate was resuspended in 1% SDS and 1 mL of Protease Inhibitor Cocktail (Cod: P8340 Sigma-Aldrich) and incubated at 60°C for 30 minutes. Next, the samples were centrifuged (10,000g for 10 minutes at 4 °C). The supernatant was transferred to a new tube and the protein concentration was determined using the BCA protein assay. The protein was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). The membranes were blocked with 5% non-fat milk in TBST buffer (200 mM Tris-Cl [pH 7.5], 1.5 M NaCl, 0.1% Tween 20) for 2 hours and then incubated with the primary antibody, type I collagen (Sigma Aldrich, C2456), overnight at 4 °C. Subsequently, the membrane was incubated with secondary antibody goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Sc-2005) for 1 hour at room temperature. Chemiluminescence was detected with an ECL western blotting detection kit (GE Healthcare, Chicago, IL, USA). Densitometry was carried out using Image

Master 2D Platinum 7.0 software (GE Healthcare). The intensity of the protein analysed was normalized to the β -actin band (A1978, Sigma Aldrich).

Gelatin Zymography

The right diaphyseal femur was macerated in liquid nitrogen using a mortar and pestle. To attain complete tissue homogenization, samples were homogenized posteriorly in a tube containing 10mM cacodylic acid, 1uM NaCl, 20mM CaCl₂, 1.5mM NaN₃, 0.01% Triton X-100% ZnCl 1uM with five stainless steel balls (diameter, 2.3 mm) (BioSpec Products, Bartlesville, OK, USA) and three silicon-carbide sharp particles (1 mm) (BioSpec Products) by being shaken in a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA). Next, the solution was centrifuged for 30 min (13,000g at 4°C) and the supernatant was reserved. A NanoDrop® spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA) was used to quantify protein concentrations. Thirty mg of total protein were loaded into each lane.

The samples were resolved by electrophoresis in a polyacrylamide gel containing SDS 10 % and gelatin (1 mg/ ml). Posteriorly, the gels were washed twice in 2.5 % Triton X-100 to remove SDS and incubated in substrate buffer (50 mmol.L – 1 Tris-HCl (pH 8.0), 5 mmol.L – 1 CaCl₂ and 0.02 % NaN₃) at 37 °C for 20 h. The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) for 1½ h and destained with acetic acid, methanol, and water. MMP-2 activity was determined by densitometric scanning of the isoform bands (Image Scanner III, Lab- Scan 6.0). The averages of the band intensities were measured using Image Master 2D Platinum 7.0 software and conducted by a blinded researcher, attenuating possible bias related to this process^[32]. Pro, intermediate, and active isoform bands were identified via standard techniques using molecular weight criteria. The bands found in all groups were 72–62 kDa, as proposed by previous study^[28].

Statistical analysis

The results are expressed as means $\pm$ the standard deviation (SD). The Shapiro-Wilk test was applied to check for normality of variables and the Levene's test to analyze homogeneity of variance. All variables presented normal distribution and homogeneity. A two-way independent measures ANOVA with “training” (2 levels: training and sedentary) and “age” (2 levels: aging and young animals) as factors was used to analyze changes in physical and biomechanical properties, collagen expression levels, and MMP-2 activity in the bone between groups. When a significant difference

was detected, a Tukey post hoc test was applied to identify the differences. An alpha level of $p \leq 0.05$ was considered significant. The software GraphPad Prism 8.3 (San Diego, CA, USA) was used for statistical analysis and graphics design. The power of the sample size for main outcomes was verified by post hoc using G*Power version 3.1.9 (Kiel University, Kiel, Germany) (supplementary data 1).

RESULTS

Body weight, biometric and biochemical parameters in response to aging and resistance training.

At the start of the experiment, the body-weight was greater in old rats (OS and OT) when compared to young animals (YS and YT) ($p = 0.01$). Regarding body weight, the young groups showed a significant increase ($p = 0.01$), while the old animals showed a significant decrease ($p = 0.01$) at the end of the exercise training protocol. Old animals demonstrated an increase in right femur Length, wet, dry and ash weight when compared with young animals (Table 2).

Effects of aging and RT on biophysical properties

Aging and RT did not affect organic material and water (%) in the femur ($p > 0.05$; Fig 2A-B), however, the mineral material and bone mineral density were reduced (OS vs. YS; $p = 0.04$). There were no differences between sedentary and trained groups in right femur length, wet weight, dry weight, and ash weight ($p > 0.05$).

Effects of aging and RT on biomechanical properties

The aging process and RT did not affect maximum load and resilience in the femur ($p > 0.05$; Fig 3A and D). Aging increased stiffness in the old groups when compared to the YS group ($p = 0.01$; Fig 3B). On the other hand, tenacity decreased in the OS group when compared to the young groups ($p = 0.001$). Finally, RT increased tenacity regardless of age ($p = 0.03$, Fig 3C).

mRNA levels in response to aging and RT in the femur neck.

Bone resorption regulatory factors: OPG mRNA levels were downregulated with aging (OS vs. YS; $p = 0.04$). No changes were observed in mRNA levels of RANKL and OPG in response to RT ($p > 0.05$; Fig 4A-B).

Adipogenic factor: No changes were observed in mRNA levels of PPAR- γ in response to the aging process ($p > 0.05$; Fig 4C). On the other hand, RT downregulated PPAR- γ mRNA levels in the femur, in both young and old groups (OT vs. OS and YT vs. YS; $p = 0.01$).

Bone formation regulatory factors: BMP-2 mRNA levels were significantly downregulated with aging (OS vs. TS; $p=0.001$). Differently, SOST mRNA levels were significantly upregulated with aging ($p=0.01$). RT downregulated BMP-2 mRNA levels in the OT group when compared to the OS group ($p=0.0001$, Fig 4D). On the other hand, MEPE mRNA levels were increased after RT in young rats ($p=0.001$; Fig 4D). Interestingly, SOST transcript was lower in OT rats in relation to OS rats ($p=0.004$; Fig 2F).

Structural matrix protein: COL1A1 mRNA levels were significantly downregulated with aging in both old groups when compared with the young groups ($p=0.001$). However, COL1A1 mRNA levels were not affected by exercise training ($p>0.05$; Fig 4G).

Matrix remodeling enzymes: MMP-2 and MMP-13 and TIMP-1 mRNA levels were significantly downregulated with aging (OS vs. YS; Fig 4H-K). Likewise, the aging process upregulated MMP-9 mRNA levels (OS vs. YS; $p=0.01$). Moreover, MMP-13 and TIMP-1 mRNA levels were increased after RT in young rats (YT vs. YS; Fig 4J-K). Finally, RT was effective at mitigating the age-associated increase in MMP-9 mRNA levels in the femur neck (OT vs. OS; $p=0.001$).

Aging and RT on Col1 protein level in the femur

Aging did not modulate Col1 protein level in the head and neck of the femur. However, RT increased the Col1 protein level in the YT group when compared to the YS group ($p=0.001$; Fig 5).

Effects of Aging and RT on MMP-2 activity in the femur

Aging did not affect MMP-2 activity in the femur. Relevantly, the total MMP-2 activity was higher in the YT group when compared to the YS group ($p=0.001$; Fig 6). MMP-9 activity was not detected by zymography.

Discussion

This study demonstrated that aging increases stiffness in the femur, while reducing bone mineral density, tenacity, mRNA levels of bone formation factors, COL-1, and MMPs. Together, these effects are related to detrimental effects on the maintenance of bone functional integrity. We demonstrated that RT increased tenacity regardless of aging, contributing to significantly improve mechanical functions and structural support. However, at the molecular level, the effects of RT on bone remodeling were more pronounced in young rats, demonstrating an increase in COL-1 protein level and MMP-2 activity. Our findings highlight the importance of RT on bone

plasticity in young and older rats and provide insight into their underlying mechanisms. A schematic representation of the femur was used to clarify the multiple mechanisms related to bone remodeling identified in this study (Fig. 7).

The old rats displayed higher body weight when compared to the young group, which could be related to increased content of adipose tissue with advancing age. It has been demonstrated that with longitudinal bone growth, the bone volume increases, explaining the greater wet, dry, and ash weights of the femurs of old rats in the present study^[33]. On the other hand, the limited effects of RT on biometric parameters could be associated with a similar body weight gain between sedentary and trained rats (table 2).

In addition, understanding the mechanical properties is crucial for rehabilitation of osteoporosis and represents a phenomenon of major clinical significance. Byrjalsen et al.^[34] explained that changes in cross-link (PYD, DPD, and AGEs) and in the degree of β -isomerization of carboxy-terminal telopeptide of collagen modulates bone mechanical properties resulting from the aging process. For instance, non-enzymatic collagen cross-links (AGEs) make the tissue more brittle and susceptible to fractures and an excess leads to increased stiffness. Collectedly, these mechanisms might elucidate higher stiffness in old rats when compared with the young animals presented here. We expected stiffness modulation in both trained groups due to the potential of exercise to reduce the amount of glycation in the bone. Nevertheless, no increase in stiffness was observed after RT which could be explained by the possibly of dynamic collagen turnover in the bone^[35].

An exciting result of our study was that RT induces increased tenacity independent of aging. Our results corroborate the Gomes et al.^[36] study, which indicates that exercise training (swimming training, 12 weeks, 5 days/week) exerts crucial regulatory roles in bone tenacity in a diabetic rat model, likely as a protective effect. Tenacity improvements are relevant for energy absorbed efficiency and resistance to injury, which might protect aged bone from weakness and consequent possible fractures. A high degree of tenacity enables bones to be more suitable for several daily activities and support load increases. Gomes et al.^[36] reported that collagen affords bone its ductility and ability to absorb energy (tenacity) before fracturing, however herein there was no difference in collagen 1 expression between the OS and OT groups. Only one type of collagen was assessed, which is a limitation of the present study.

The constant remodeling of the bone matrix is necessary to maintain both skeletal strength and a reservoir for hematopoiesis. RANKL–RANK signaling was

shown to be a potent driver of osteoclast differentiation and animals genetically deficient for any of these factors exhibit osteopetrosis, a thickening of the bones, due to reduced osteoclast numbers and activity^[10, 37]. On the other hand, OPG is a negative regulator of RANK signaling and is capable of inhibiting osteoclastogenesis, and of inducing osteoporosis when overexpressed. In the current study, both markers were reduced with aging^[10]. These changes can be explained by the fact that signaling pathways that govern osteoclast development and function are dysregulated, which could be partially explained by lower bone mineral density in old sedentary animals^[38].

On the other hand, RT did not modify mRNA levels of RANKL/OPG, or important mechanical properties, such as maximum load and resilience in young and old groups. In a recent review, Hughes et al.^[39] reported that pro-osteoblastic signaling inhibition is attributed to signal cellular accommodation due to mechanosensitive cells adjusting cytoskeletal stiffness in response to fluid flow, linked to compensatory mechanisms. One of the essential aspects of the practical application is that the development of effective exercise programs on bone remodeling will require creative solutions based on the physiology of bone mechanosensitivity^[39].

SOST is a protein containing amino acids with two N-glycosylation sites which is secreted by osteocytes and inhibits the genesis of osteoblasts, thereby resulting in a decrease in bone formation. According to Pflanz et al.^[40] long-term SOST deficiency influences remodeling processes and downregulation is a mandatory step in mechano-adaptation. Interestingly, we found a decrease in SOST gene expression of old rats in response to RT, which may play an important role in the anabolic response, enhancing *bone formation*. Optimal bone formation provides resistance to tension and structural integrity. Therefore, an increase in SOST may be reflected in bone tenacity improvements. On the other hand, SOST was originally characterized as a BMP-2 antagonist because of its sequence homology to other members of the DAN family of cysteine-knot proteins^[41]. However, we did not find mitigation of BMP-2 reduction in the OT group, which may suggest that other adjacent molecular pathways or post-translational regulation was involved in bone formation regulatory factors.

Evidence from experimental and clinical studies demonstrated that a reduction in osteoblastogenesis from mesenchymal stem cells at the expense of adipogenesis, as well as the deleterious effects of adipocyte-derived signaling, contribute to the etiology of osteoporosis and bone loss in aging^[42-45]. Moreover, PPAR γ is a critical factor for the regulation of adipogenesis and contributes to the persistent expression of pro-

inflammatory cytokines and MMP-9 in the bone^[46, 47]. Most significantly, in the current study RT downregulated mRNA levels of PPAR- γ and MMP-9 in old rats. Considering these molecular findings, RT may attenuate lipid storage in the adipocyte, as well as adipose cell differentiation in bone and, consequently, modulate MMP-9 activation. These changes might result in a less inflammatory phenotype linked to adipogenesis attenuation in bone. Finally, this result is in agreement with the decrease in mRNA levels of OPG observed in the OT group, since it has been demonstrated that OPG also plays an important role in regulating bone marrow adipogenesis^[48].

The results of the present study are in agreement with Singulani et al.^[49], who demonstrated that RT reduced the expression of Ppar- γ and tumor necrosis factor- α (TNF- α) in bone marrow mesenchymal stromal cells (BMSCs) of old female rats. Additionally, RT increased gene expression of runt-related transcription factor 2 (Runx2) and osterix (Osx), improving bone mechanical properties (maximum load, elastic, modulus, and energy to maximum load) in the femur. These data reinforce that exercise triggers physiological responses that result in changes in the bone microenvironment, which could reduce the risk of fractures during senescent. Nevertheless, these authors did not address the responses of young rats or protein expression of key factors involved in ECM bone remodeling (structural matrix protein and matrix remodeling enzymes), which obscure the mechanisms underlying bone biomechanical adaptations reported in this previous study^[49].

It is well established that the aging process significantly decreases collagen content and impairs matrix remodeling enzymes, which has been correlated with reduced bone fracture resistance^[13]. Indeed, old rats showed a reduction in mRNA levels of COL-1, MMP-2, MMP-13, and TIMP-1, indicating a panel of unfavorable remodeling inherent to aging-associated changes. However, mRNA changes were not reflected *at the level of* proteins, probably due to delayed synthesis between mRNA and protein during state transition or proteins disconnected from the transcripts^[50]. In addition, the effects of RT at protein levels were more evidenced in young rats, demonstrating upregulation in COL-1 expression and MMP-2 activity. A plausible explanation is that optimal bone remodeling is distinct between ages and mechanical-molecular set points may exist for beneficial adaptations.

The responsiveness to RT includes characteristics of the exercise regimen or dose as well as characteristics of the response or effect. Presumably, different ages have distinct physiological adaptations, and, possibly, remodeling is not the same after the

same mechanical loading application. In this same experimental model we demonstrated relevant adaptations observed in terms of function, structure, and molecular level in the tendons^[22], left ventricle^[25], and ECM of skeletal muscle^[51]. Resistance training exerted a potential mechanism in the prevention of aging-related loss in ECM and attenuated the detrimental effects of aging in all the tissues cited. In the same experimental model, Guzzoni et al.^[25] and Sousa Neto et al.^[24] showed that young rats displayed higher load capacity in response to 12-weeks of RT when compared to old rats. This factor may contribute to differences in molecular changes between the OT and YT groups when compared to OS and YS groups, respectively. The local increase in MMP-2 activity in bone observed only in the YT group, might be related to changes in type I collagen (Figure 4), which is fundamental to bone strength development after mechanical loading, linked to substantial mechanisms of bone adaptation following RT.

Finally, it is important to point out some limitations of the present study, such as the absence of histological and immunohistological analysis of ECM markers and bone formation. These factors are relevant for physiological status modulation in response to RT since they involve molecular processes mediating osteoblast and osteoclast survival. Moreover, different bone regions were analyzed, restricting the conclusion regarding specific femur region adaptations. It is important to emphasize that although RT is recommended for elderly individuals there is no evidence to suggest which intensity (light, moderate, or intense), frequency (days per week), and duration (session time) are more effective in a weighted stair climbing model for old animals. Future studies may investigate the variables modulated by training in order to potentiate the effects of exercise and highlight several cornerstone mechanisms involved in bone adaption. This approach might clarify whether the exercise protocol directly influences protein expression of key factors involved in bone remodeling during the aging process.

Conclusion

Aging imposes drastic and negative changes in bone mineral density, tenacity, and gene expression related to ECM remodeling. RT was effective in improving tenacity, independent of aging, which is relevant for mechanical function, while at protein levels, RT upregulated MMP-2 activity and collagen 1 levels only in young rats. This study highlights the importance of exercise training on bone health and identifies specific molecular changes in response to RT.

Supplementary Materials: Supplementary data 1- power analyses

Author Contributions:

GCFJ, IVSN, HSSA, RCM. designed the study. FHB, KON, JLQD, HSSA, RCM funding acquisition, GCFJ, IVSN, VG, GDP, CR, CLL, FARN, conducted the experiments and analyzed the data. GCFJ, IVSN, HSSA, RCM drafted the manuscript. JLQD, HSSA, RCM. critically reviewed and improved the manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1. Experimental design. Schematic illustration of the methodological steps in the following study

Figure 2. Biophysical property modifications of the femur in response to aging and resistance training. The data are mean $\pm$ SD. The experimental groups are represented as young sedentary (YS), young trained (YT), old sedentary (OS), and old trained (OT).

Statistically significant differences compared to: ^a YS, ^bYT, ^cOS, $p < 0.05$. (n = 5 per group).

Figure 3. Aging and resistance training affect bone biomechanical properties. The data are mean $\pm$ SD. The experimental groups are represented as young sedentary (YS), young trained (YT), old sedentary (OS), and old trained (OT). Statistically significant differences compared to ^a YS, ^bYT, ^cOS, $p < 0.05$. (n = 8 per group).

Figure 4. Aging and resistance training modify the gene expression related to bone remodeling. (A-B) Bone resorption regulatory factors (blue): ligand of receptor activator of nuclear factor kappa B (RANKL), Osteoprotegerin (OPG). (C) Adipogenic factor (yellow): Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ). (D-F) Bone formation regulatory factors (purple): Bone morphogenetic protein 2 (BMP-2), Matrix extracellular phospho-glycoprotein (MEPE), Sclerostin (SOST). (G) Structural matrix protein (grey): collagen, type I, alpha 1 (COL1A1). (H-K) Matrix remodeling enzymes (beige): Matrix metalloproteinase-2 (MMP-2), Matrix metalloproteinase-9 (MMP-9), Matrix metalloproteinase-13 (MMP-13), tissue inhibitor of matrix metalloproteinases-1 (TIMP-1). (n = 5 per group).

Figure 5. Bone collagen type I expression in response to aging and resistance exercise. The data are mean $\pm$ SD. The experimental groups are represented as young sedentary (YS), young trained (YT), old sedentary (OS), and old trained (OT). Statistically significant differences compared to ^a YS, ^bYT, $p < 0.05$. (n = 5 per group).

Figure 6. Effect of training and aging on pro (latent) (72 kDa) and active MMP-2 (62 kDa) bands by zymography in 1% gelatin SDS-PAGE on the bone. The data are mean $\pm$ SD. The experimental groups are represented as young sedentary (YS), young trained (YT), old sedentary (OS), and old trained (OT). Statistically significant differences compared to: ^a YS, ^bYT, $p < 0.05$. (n = 5 per group).

Figure 7. Overview of aging process and resistance training on femur remodeling. Ligand of receptor activator of nuclear factor kappa B (RANKL); Osteoprotegerin (OPG); Bone morphogenetic protein 2 (BMP-2); Collagen, type I, alpha 1 (COL1A1); Ligand of receptor activator of nuclear factor kappa B (RANKL); Matrix extracellular

phospho-glycoprotein (MEPE); Matrix metalloproteinase-13 (MMP-13); Matrix metalloproteinase-2 (MMP-2); Matrix metalloproteinase-9 (MMP-9); Osteoprotegerin (OPG); Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ); Sclerostin (SOST); Tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), ($\uparrow$ red) upregulated and ($\downarrow$ blue) downregulated.

Table 1. List of tested genes. and primers sequences

Genes	Sense Primer	Antisense Primer	Access Number	Company
BMP-2	TATATGCTAGATCTGTACCGC	TAAATTGAAGAAGAAGCGCC	NM_053304.1	Sigma Aldrich
COL-1	ATCAGCCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG	NM_053304.1	Bioneer
MEPE	ACTGTTCTCTTCAGTATGAC	TGATATTTCTGAGGAGGGTG	NM_024142.1	Sigma Aldrich
MMP-13	CGTGTGGAGTTATGATGATG	ATCTACTTTGTCGCCAATTC	NM_133530.1	Sigma Aldrich
MMP-2	GATCTTCTTCTTCAAGGATCG	TACACGGCATCAATCTTTTC	NM_031054.2	Sigma Aldrich
MMP-9	TACTTTGGAAACGCAAATGG	GTGTAGAGATTCTCACTGGG	NM_031055.1	Sigma Aldrich
PPARγ	AAGACAACAGACAAATCACC	CAGGGATATTTTTGGCATACTC	NM_013196.1	Sigma- Aldrich
RANKL	TGAGGTTTCCAGAGGACCAC	GGAAAGGTTTCTGGGTGT	NM_012870.2	Sigma- Aldrich
RPS18	ACTGCCATTAAGCGTGTG	GTCAGGGATCTTGTATTGTC	NM_213557.1	Sigma- Aldrich
SOST	ATGATGCCACAGAAATCATC	CACGTCTTTGGTGTGATAAG	NM_030584.1	Sigma Aldrich
TIMP-1	ATAGTGCTGGCTGTGGGGTG	TGATGCCTCTGGTAGCCCTT	NM_053819.1	Bioneer
OPG	AAGGAAATGCAACACATGAC	TCTCTACACTCTCTGCATTC	NM_012870.2	Sigma Aldrich

Table 2. Body weight, bone biometric parameters of experimental groups.

	YS	YT	OS	OT
Body weight				
Initial Body weight (g)	299 $\pm$ 5.44	301 $\pm$ 6.95	507 $\pm$ 12.62 ^{a,b}	508 $\pm$ 4.61 ^{a,b}
Final Body weight (g)	484 $\pm$ 15.40*	414 $\pm$ 6.59*	469 $\pm$ 17.34*	469 $\pm$ 8.70*
Gain (%)	185 $\pm$ 12.75	113 $\pm$ 8.25	- 38 $\pm$ 8.11	- 39 $\pm$ 7.70
Biometric parameters				
Right femur Length (cm)	4.03 $\pm$ 0.01	3.99 $\pm$ 0.03	4.17 $\pm$ 0.03 ^{a,b}	4. 24 $\pm$ 0.53 ^{a,b}
Wet weight (g)	1.16 $\pm$ 0.02	1.11 $\pm$ 0.03	1.37 $\pm$ 0.03 ^{a,b}	1.40 $\pm$ 0.03 ^{a,b}
Dry weight (g)	0.70 $\pm$ 0.01	0.67 $\pm$ 0.02	0.81 $\pm$ 0.02 ^{a,b}	0.83 $\pm$ 0.02 ^{a,b}
Ash weight (g)	0.49 $\pm$ 0.03	0.43 $\pm$ 0.02	0.57 $\pm$ 0.01 ^{a,b}	0.57 $\pm$ 0.02 ^{a,b}

Highlights

- Aging alters the bone mineral density and gene expression related to ECM remodeling
- RT was effective in improving bone tenacity, independent of aging process
- At protein levels, RT upregulated MMP-2 activity and collagen 1 only in young rats
- Understand the bone ECM is crucial for rehabilitation purposes of osteoporosis.