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Ilex paraguariensis decreases oxidative stress in bone and mitigates the damage in rats during perimenopause

Camila Scacco Pereira [a,1], Camila Tami Stringhetta-Garcia [a,1], Lilian da Silva Xavier [a], Keny Gonçalves Tirapeli [a], Ariana Aparecida Ferreira Pereira [a], Giselli Mitsuy Kayahara [b], José Marcelo Tramarim [b], Marcelo Macedo Crivelini [b], Karina Stringhetta Padovani [c], Andréia Machado Leopoldino [c], Mário Jefferson Quirino Louzada [a,d], Adriane Belló-Klein [e], Susana Francisca Llesuy [f], Edilson Ervolino [g], Rita Cássia Menegati Dornelles [a,g], Antonio Hernandes Chaves-Neto [g], Ana Cláudia de Melo Stevanato Nakamune [a,g,*]

[a] Programa Multicêntrico de Pós-Graduação em Ciências Fisiológicas-SBFis, São Paulo State University (Unesp), School of Dentistry, Araçatuba, SP, Brazil
[b] Department of Pathology and Clinical Propaedeutics, São Paulo State University (Unesp), School of Dentistry, Araçatuba, São Paulo, Brazil
[c] Department of Clinical, Toxicological and Bromatological Analysis, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil
[d] Department of Support, Animal Production and Health, São Paulo State University (Unesp), Veterinary Medicine School, Araçatuba, SP, Brazil
[e] Department of Physiology, Health Basic Sciences Institute, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[f] Department of Analytical Chemistry and Physical Chemistry -School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina
[g] Department of Basic Sciences, São Paulo State University (Unesp), School of Dentistry, Araçatuba, São Paulo, Brazil

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ABSTRACT

During perimenopause, oxidative stress increases, which may result in disruption of bone turnover, and consequently in osteoporosis. The use of antioxidants may be an effective nutritional approach to reducing osteoporosis in this period of life. Mate tea (MT) (*Ilex paraguariensis*), a typical and inexpensive beverage consumed in the Brazilian south-east, Argentina and Uruguay, increases antioxidant defense. Our hypothesis was that MT would decrease oxidative stress and mitigate bone deterioration. To test this, we analyzed oxidative stress markers of bone turnover, and local and systemic markers of bone metabolism of rats during natural perimenopause. Female Wistar rats (aged 16 months) in proven perimenopause period received 20 mg/kg BW/day of mate tea, by gavage (PM + MT Group, $n = 10$) or water (PM Group, $n = 10$). Female rats aged 4 months (AD Group, $n = 10$) received water. The treatment period was four weeks. MT minimized the deterioration of rat microarchitecture, characterized by increase in the bone trabecular area, number of osteocytes and areal bone mineral density. These results were accompanied by a lower level of malondialdehyde, an oxidative stress marker, in femoral tissue homogenate. Plasmatic tartrate-resistant acid phosphatase, a typical osteoclastic function marker, decreases after treatment, indicating a decrease in osteoclastic function. MT also modified the immunostaining pattern of bone metabolism markers, decreasing the receptor activator of nuclear factor kappa-B ligant (RANKL), superoxide dismutase isoform 2 (SOD2) and increasing osteoprotegerin (OPG), a decoy receptor for the RANKL, which positively modulates bone mass. These results suggested MT was capable of decreasing bone resorption by inhibiting the osteoclastogenesis in a RANKL-dependent signaling pathway activated by oxidative stress. Taken together, the results indicated that MT minimized bone loss in perimenopause and this effect is at least partly due to the decrease in oxidative stress, confirming our hypothesis.

1. Introduction

Perimenopause precedes menopause and is characterized by physiological changes associated with the end of the reproductive phase (Zitňanová et al., 2011). In this period of a woman's life, hormonal changes are associated with increased oxidative stress, as result of the higher level of reactive oxygen species (ROS) generated, decreased antioxidant defenses (Ramírez-Expósito et al., 2014; Pereira et al., 2017),

* Corresponding author at: Department of Basic Sciences, Building 31, UNESP, Rodovia Marechal Rondon Km 527, 16018-805 Araçatuba, SP, Brazil.
E-mail address: anacmsn@foa.unesp.br (A.C.M.S. Nakamune).
[1] These authors contributed equally to this work.

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and consequently elevated lipid oxidative damage (Zitňanová et al., 2011; Pereira et al., 2017).

Oxidative stress plays a key role in osteoporosis (Syed and Ng, 2010), a degenerative, chronic and progressive disease that affects approximately 200 million women worldwide. One in three women over 50 years old suffers from osteoporotic fractures (Cooper et al., 1992). This bone disease results from the disruption of bone turnover, a delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts (Masi and Brandi, 2001). ROS accumulation enhances RANKL expression (receptor activator of nuclear factor kappa-B ligant) that participates in osteoclast differentiation/activation (Baek et al., 2010). It also enhances TRAP (tartrate-resistant acid phosphatase), an osteoclast activity marker (Garnero et al., 2000). Excess ROS also culminates in accretion of lipid peroxidation products, such as malondialdehyde (MDA), a compound that decreases osteoprotegerin (OPG), an osteoclastogenesis inhibitory factor, favoring bone reabsorption (Huang et al., 2007). All the molecular changes in bone lead to reduced areal bone mineral density (aBMD) (Tantikanlayaporn et al., 2013). Therefore, a deterioration of bone microarchitecture is observed, with a decline in the bone trabecular volume (BTV) and diminution of osteocyte numbers (N·Ot) and increased risk of fragility and fracture (Portal-Núñez et al., 2016).

Osteoporosis is a huge health and social problem (Lane, 2006) and there is an urgent demand to seek potential non-pharmacological alternative therapy for it (Fahmy et al., 2015). Recently a chronic consumption of mate tea (MT) was associated with increased BMD in the femoral neck in postmenopausal women. However, the mechanisms involved in this effect have not been investigated (Conforti et al., 2012). MT is a typical, inexpensive beverage prepared by infusion of *Ilex paraguariensis* (Yerba Mate), consumed in South-eastern Brazil, Argentina and Uruguay, which has a high antioxidant power (Heck and de Mejia, 2007). Regular and prolonged consumption of MT by healthy young women may decrease lipid peroxidation and increase the gene expression of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Matsumoto et al., 2009). A recent publication of our group demonstrated that MT may be an effective nutritional approach to reducing oxidative damage in erythrocytes of rats during perimenopause, by increasing enzymatic antioxidant defense (Pereira et al., 2017).

The present study was proposed in this context. Our hypothesis was that MT would decrease oxidative stress and mitigate bone deterioration. To test this, we analyzed oxidative damage, enzymatic antioxidant defense and bone metabolism markers of rats during perimenopause, after four weeks of treatment.

2. Materials and methods

2.1. Mate tea

MT was prepared using instant powder (Leão Jr. ®, Curitiba, PR, Brazil), batch 7,891,098,000,088, in pure water (0.05 g/mL) at room temperature, as described by Pereira et al. (2017). Total polyphenol (113.37 ± 0.34 mg of gallic acid per g powder), total antioxidant capacity (534.67 Fe^{2+} antioxidant capacity/g powder) and the presence of phenolic compounds, associated with antioxidant activity and some pharmacological properties (Heck and de Mejia, 2007), have previously been analyzed by our group (Pereira et al., 2017).

2.2. Animals

The local Ethics Committee on Research Involving Animals of the São Paulo State University (Unesp), School of Dentistry, Araçatuba, SP, Brazil approved all procedures performed in this study (Permission Number: 00462-2013). Female Wistar rats aged 4 months (Adult group - AD), and 16 months, were housed in a sanitary and controlled room (22 ± 2 °C; 12/12 h light/dark cycle; lights on at 7:00 h; 55 ± 10% of relative humidity) with free access to feed and water. After the acclimation period (15 days) aged rats were randomly distributed into Group PM (perimenopause) and Group PM + MT. The animals of Group PM + MT received 20 mg/kg BW/day of MT, by gavage (0.5 mL); Groups AD and PM received an equal volume of water. The treatment period was four weeks. Estrous cycle was verified daily, as described by Nicola et al. (2016) and Pereira et al., (2017). Only adult rats showing normal estrous cycles, and aged rats (16 months old) with cycles of six or more days, characterizing periestropause, which resembles perimenopause in women (Chen et al., 2013), remained in the Group PM or Group PM + MT. The final number of animals was 10/group.

Body mass was followed-up weekly, and water and feed consumption, daily. To avoid variation in antioxidant intake, the same batch of standard rodent chow (Purina- Labina®, Brazil) was used right from the acclimation period through to the end of the experiment.

2.3. Sample acquisition

Blood was obtained by means of cardiac puncture after an overnight fast; after anesthesia with sodium pentobarbital (50 mg/kg BW). Blood (5.0 mL) was transferred to heparinized tubes, centrifuged at 1000 × *g* at 4 °C for 15 min. Plasma was removed and frozen at −80 °C. Both femurs were removed immediately after the animals were sacrificed, and cleaned by removing adhering soft tissues. Left femurs were kept in NaCl (0.9% w/v) at −20 °C, until the areal BMD (aBMD) analysis was performed. Immediately after this, the structures were used for the preparation of homogenate. The right femurs were prepared for histomorphometry and Immunohistochemistry analyses.

2.4. Plasma assays

TRAP was determined spectrophotometrically by using *p*-nitrophenyl phosphate (*p*-NPP) as the substrate, in pH 5.0 (Chaves-Neto et al., 2011). Alkaline phosphatase (ALP), a marker of bone formation by osteoblasts (Fahmy et al., 2015) was also measured by using *p*NPP as the substrate, in pH 9.4 (Chaves Neto et al., 2011). For both enzymes, one unit of enzyme activity was defined as the amount of enzyme that was required to hydrolyze 1 μmol of *p*NPP per min at 37 °C.

2.5. Areal bone mineral density

aBMD (g/cm^2) of the entire left femur ($n = 10$/group) was measured by Dual-Energy Absorptiometry by means of a Lunar DPX Alpha, WI, USA, with special software for small animals, as described by Stringhetta-Garcia et al. (2016).

2.6. MDA in bone homogenate

Before the analysis, the left femurs ($n = 10$/group) bone was homogenized (150 mmol/L KCl, 10% w/v) with the aid of dry ice and centrifuged at 1.000 × *g* at 4 °C for 10 min (Abujazia et al., 2012). Supernatant was used for MDA as described by Buege and Aust (1978). Protein concentration was determined by the Lowry method (Lowry et al., 1951). Results were expressed in nmol/mg protein.

2.7. Histomorphometry, osteocyte number and immunohistochemistry

Immediately after the animals had been sacrificed, the right femurs ($n = 10$/group) were cleaned by removing soft tissue and fixed in 4% buffer formaldehyde at room temperature for 24 h; and then decalcified in Plank-Rychlo solution (Plank and Rychlo, 1952) for eight to nine days. Decalcified samples were processed in the usual manner, embedded in paraffin, and cut into 3 μm thick sections with a microtome, so that the sections were obtained along the coronal plane of the proximal femur to access the femoral neck. For histomorphometry (trabecular area) and osteocyte number the slices were stained with hematoxylin-eosin.

Table 1
Shows the entire left femur areal bone mineral density (aBMD) and plasmatic bone markers in adult (AD), perimenopause (PM) and perimenopause Groups treated with MT (PM + MT).

Parameter	AD	PM	PM + MT
aBMD (g/cm^2)	0.227 ± 0.004	0.162 ± 0.006*	0.183 ± 0.006$^\#$
TRAP (U/L)	246.8 ± 6.31	273.4 ± 8.72*	237.8 ± 8.8$^\#$
ALP (U/L)	431.1 ± 8.93	507.4 ± 28.72*	416.6 ± 11.41$^\#$

Data are the mean ± SEM. (n = 10/group). $^*p < 0.05$ *vs.* Group AD; $^\#p < 0.05$ *vs.* Group PM. Student's *t*-test.

Images were captured at 100 X magnification (LEICA DM4000B) and analyzed in an area of 100 μm^2 with help of the a LAS V4.2 software tool (Leica Application Suite), to obtain the number of osteocytes (N·Ot) and bone trabecular area (BTA).

For immunohistochemical evaluation, histology slices with samples of all experimental groups (n = 6/group) were submitted to the indirect immunoperoxidase technique for the following primary antibodies: anti-TRAP (1:100, SC-30833), anti-RANKL (1:100; SC-7628), anti-OPG (1:150; SC-8468) and anti-SOD2 (1:200; ab13534). The dilution of primary antibodies was based on a titration test. Immunohistochemical processing followed the protocol described by Stringhetta-Garcia et al. (2016).

Histological sections (femoral neck bone) were examined under bright field illumination of a light microscope (Optiphot-2, Nikon, Japan) by the investigator who was blind to treatment. The scores were adapted from Stringhetta-Garcia et al. (2016). For the TRAP immunolabeling pattern, the following scores were considered: score 3 (over 8 immunoreactive (IR) cells per area, indicated a high pattern), score 2 (3 to 7 IR cells per area, indicated a moderate pattern), score 1 (<3 IR cells per area, indicated a low pattern) and score 0 indicated the absence of immunolabeling. For RANKL, OPG and SOD2 the following scores were considered: score 3 (high pattern, approximately 75% of IR cells per area), score 2 (moderate pattern, approximately 50% IR cells per area), score 1(low pattern, approximately 25% IR cells per area) and score 0, absence of immunolabeling. These immunolabeling scores were compared among the experimental groups.

2.8. Statistical analysis

All data, presented as the mean ± standard error of the mean (SEM), showed normal distribution (Kolmogorov-Smirnov test). Comparisons between two groups were made using the Student's-*t*-test. Analysis was performed with the GraphPad Prism 6 program (La Jolla, CA, USA). Immunohistochemistry analysis was performed with nonparametric Kruskal-Wallis test followed by the Newman–Keuls post-hoc test, by using Biostat V5.3. A value of $p < 0.05$ was considered significant for all analysis.

3. Results and discussion

Only adult female Wistar rats with a regular estrous cycle and aged rats during natural perimenopause, either treated with MT, or not, participated in this study. The inclusion criteria were the same as those we used previously (Pereira et al., 2017). Feed intake, water consumption and body weight were not changed by MT (data not shown), indicating that changes in oxidative stress markers were associated with perimenopause, instead of variations in calorie intake or body mass reduction promoted by MT treatment, in agreement with Pereira et al. (2017). The dose used (20 mg/BW/day) was equivalent to human consumption of 300 mL (1½ Brazilian cup) per day of MT, and it minimized the oxidative stress in erythrocytes during perimenopause, by modulating antioxidant defense (Pereira et al., 2017). For the choice of treatment time, a pilot study was conducted to evaluate aBMD, a property correlated with fracture risk, considered an essential parameter for monitoring and diagnosis of osteoporosis (Cummings, 2002), after four, six and

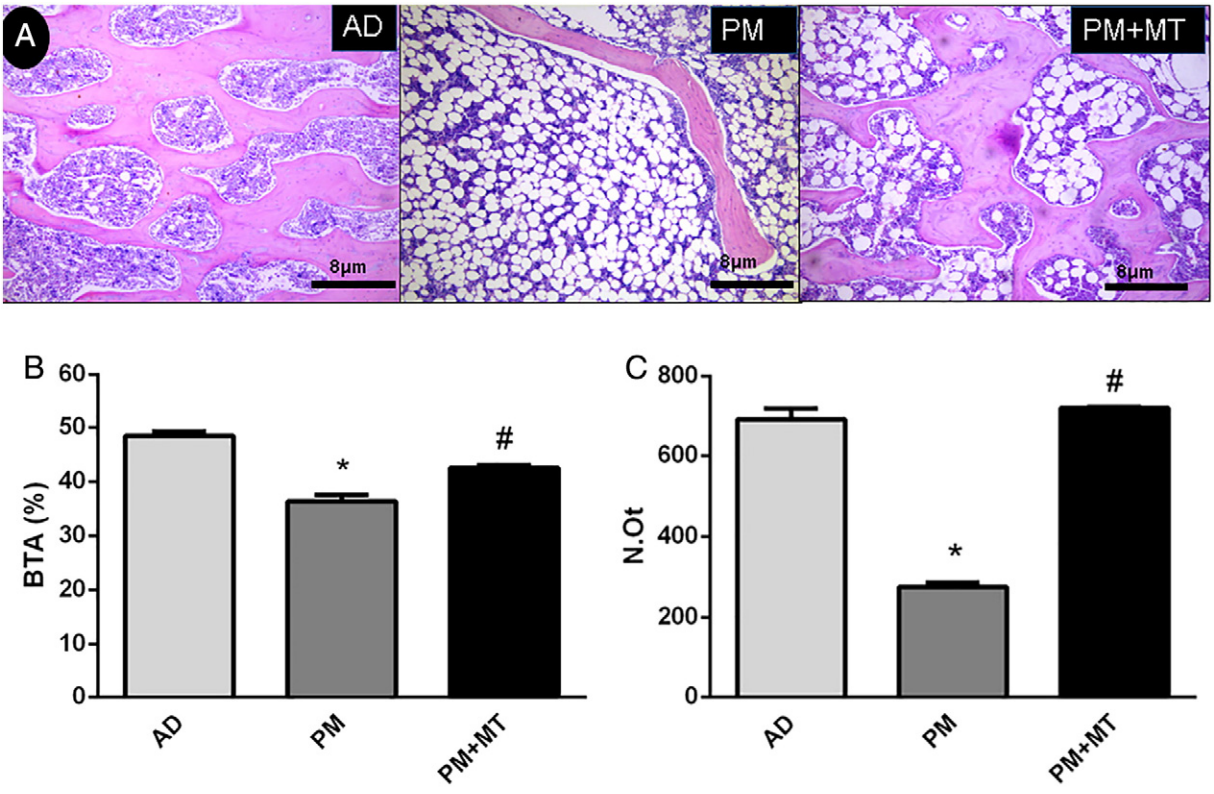

Fig. 1. A - Histological section of femur neck stained with hematoxylin and eosin, B – bone trabecular area (BTA) and C - osteocyte number (N·Ot) in adult (AD), perimenopause (PM) and perimenopause Groups treated with MT (PM + MT). Data are the mean ± SEM. (n = 10/group). $^*p < 0.05$ *vs.* Group AD; $^\#p < 0.05$ *vs.* Group PM. Student's-*t*-test.

eight weeks of MT treatment. The results showed an increase in aBMD after four weeks of MT (PM: 0.162 ± 0.006; PM + MT: 0.183 ± 0.006, $p < 0.05$); after six weeks (PM: 0.149 ± 0.008; PM + MT: 0.222 ± 0.005, $p < 0.0001$) and after eight weeks (PM: 0.151 ± 0.001; PM + MT: 0.216 ± 0.006, $p < 0.0001$). The significant increase in aBMD after a short period of treatment (four weeks), motivated us to characterize the changes at the structural and molecular level. This MT effect on BMD was not observed in femur of normal, young (30 days old) female rats (Brun et al., 2015). This disagreement of results can be attributed to different live phases of females, different breeds (they used Sprague-Dawley) and a difference of route of administration (gavagem *vs. ad libitum*) and dose of MT.

The effect of MT on the bone of perimenopause rats was analyzed by local and systemic markers. Plasmatic TRAP, a typical osteoclastic function marker, showed 9.39% increase ($p < 0.05$) in PM Groups when compared with AD (Table 1). After MT, this parameter was reduced by 10.78% ($p < 0.01$), indicating a decrease in osteoclastic function. Plasmatic ALP, an osteoblastic function marker (Masi and Brandi, 2001) that may be increased in menopause, because of a compensatory mechanism to induce greater osteoclastic activity (Garnero et al., 2000), increased (13.02%, $p < 0.05$) in PM group compared with AD. However, in the group that received MT, there was reduction in ALP ($p < 0.05$) (Table 1).

The entire left femur aBMD of PM Group showed decline of 28.63% ($p < 0.0001$) compared with AD group. This parameter was 12.96% higher in PM ± MT compare to MT group.

Two-dimensional histomorphometric analysis (Fig. 1A) showed a decrease in BTA (25%, $p < 0.0001$) and N·Ot (60%, $p < 0.05$, Fig. 1C). These parameters were changed after MT, as can be seen from the increase in N·Ot (61.7%, $p < 0.05$) and BTA (14.6%, $p < 0.05$). Possibly, the reduction in aBMD verified in PM rats resulted from disruption of the delicate balance between bone resorption and bone formation, reflecting in a decline in the BTA and N·Ot, and the MT minimized the deterioration of rat microarchitecture in this period.

MT minimized bone deterioration during perimenopause, by reducing oxidative stress, as can be observed by bone MDA levels. A 29.54% increase in this parameter in bone homogenate was observed in PM Group (44.95 ± 2.11), compared with AD (34.70 ± 0.82). PM rats treated with MT showed a reduction of 25.42% (33.52 ± 2.33, $p < 0.05$) in bone MDA compared with PM Group. A similar result was observed after Virgin Coconut oil treatment in the tibia of ovariectomized rats (Abujazia et al., 2012), or after *Camellia sinensis* tea (Shen et al., 2009). Increased MDA favored bone absorption by reducing osteoprotegerin (OPG), an osteoclastogenesis inhibitory factor (Huang et al., 2007).

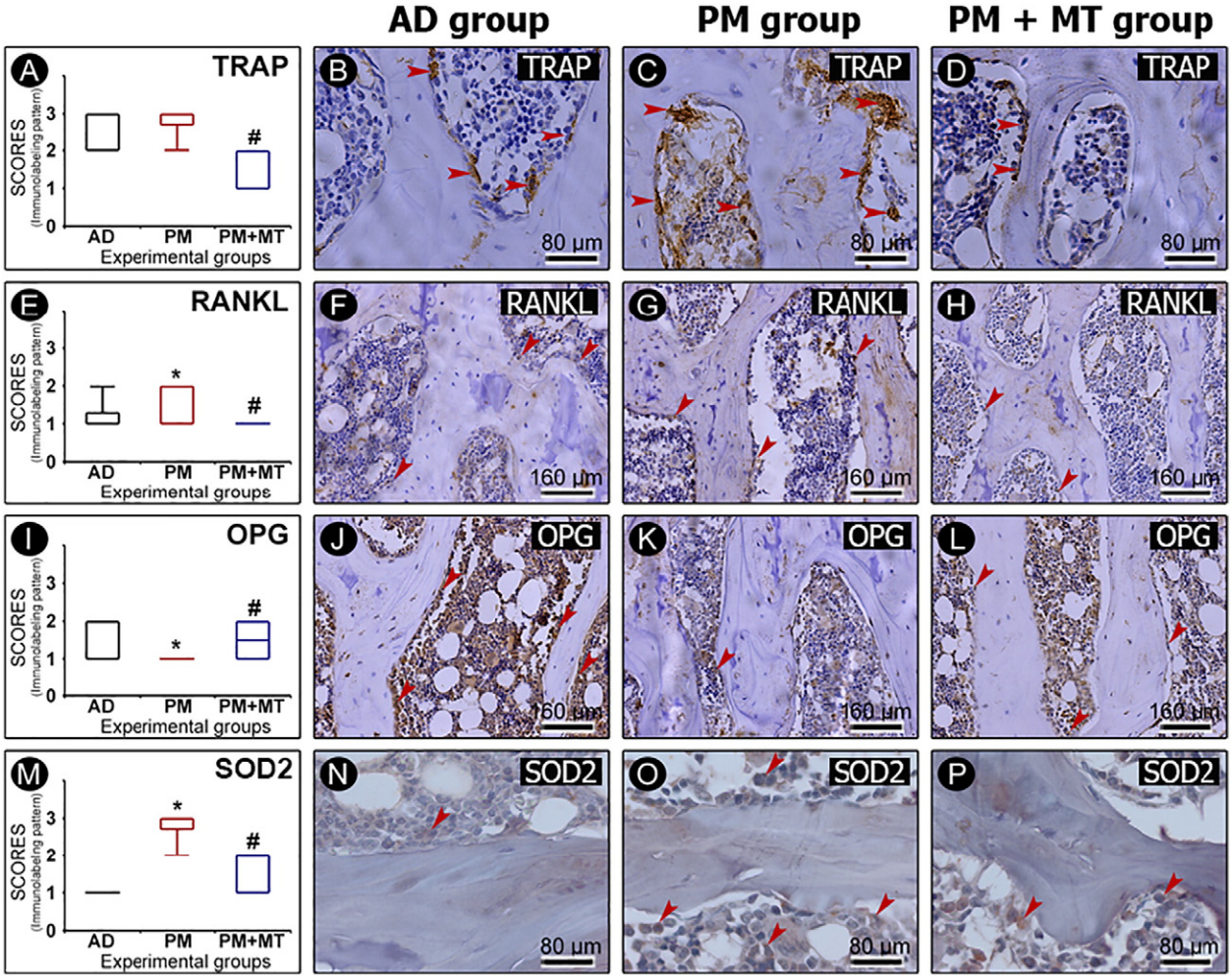

Fig. 2. Graphics and photomicrographs showing the immunoreactivity pattern of TRAP (A, B, C and D), RANKL (E, F, G and H), OPG (I, J, K and L) and SOD2 (M, N, O and P) on trabecular bone of femoral neck of adult (AD), perimenopause (PM) and perimenopause treated with MT (PM ± MT) groups. Data are the mean ± SEM, $n = 6$/group. Statistical analysis was performed by the non-parametric Kruskal-Wallis test followed by the Newman–Keuls post-hoc test (*$p \leq 0.05$ *vs.* Group AD; #$p \leq 0.05$ *vs.* Group PM). p-values <0.05 were considered statistically significant. Hematoxylin counterstaining for TRAP, RANKL and OPG; Mayer Hematoxylin for SOD2. Scale bars: TRAP and SOD = 80 µm; RANKL and OPG = 160 µm. Original magnification, ×1000. Abbreviations: OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B; TRAP, tartrate-resistant acid phosphatase; and SOD2, superoxide dismutase 2.

The immunohistochemical protocol used to detect TRAP, RANKL, OPG and SOD2 showed high specificity for the immunolabeling of this protein, which was confirmed by the total absence of immunolabeling in the negative control. The photomicrography and patterns of immunolabeling and for all biomarkers are shown in Fig. 2.

MT modified the pattern of immunostaining of bone metabolism markers. The perimenopause period in PM group was marked by increased RANKL, SOD2 and decreased OPG immunolabeling when compared with AD group. The perimenopause rats treated with MT (PM + MT Group) showed decreased TRAP, RANKL and SOD2 and increased OPG immunolabeling compared with PM group. These results suggested that MT was capable of decreasing bone resorption by inhibiting the osteoclastogenesis in a RANKL-dependent signaling pathway, a key factor for osteoclast differentiation and activation, activated by oxidative stress. Furthermore, MT also increased OPG, a decoy receptor for the RANKL that positively modulates bone mass.

The cycle of bone remodeling is strongly influenced by oxidative stress, and has been described as an important factor that affects bone and may cause osteoporosis (Abdollahi and Larijani, 2005). Oxidative stress has a negative effect on osteoblasts, inhibiting osteoblastic differentiation (Mody et al., 2001), at the same time it stimulates osteoclastic activity; one of the extremely relevant factors for osteoclast activity seems to lie in the fact that osteoclast produces the superoxide anion, which has a direct effect on its resorptive capacity (Key et al., 1994). Based on this information, we noted in the immunohistochemical analysis that there was increase of SOD2 in animals in perimenopause, corroborating the literature; we could confirm that during aging, oxidative stress increases (Finkel and Holbrook, 2000), which leads to a change in the cycle of bone remodeling, with increase in RANKL and decrease in OPG, culminating in the deterioration of bone tissue. For the first time in the literature, the present study demonstrated that during reproductive physiological aging in animals, MT consumption decreased oxidative stress by reducing the immunolabeling of SOD2, which culminated in a change in the bone remodeling cycle, with decreased TRAP, RANKL and increased OPG. This was reflected in an increase in bone mineral density and showed MT to be a non-pharmacological and nutritional strategy in evidence in the field of bone and mineral research, as well as being a great ally in the prevention of bone loss.

This study was limited to investigating bone aBMD by Dual-Energy Absorptiometry. We did not assess the effects of TM in the other treatment times. Lastly, initially, we chose to use MT, and not its bioactive forms. Taken together, the results of this investigation indicated that MT minimized bone loss in perimenopause and this effect was consequently, at least in part, due to the decrease in oxidative stress, which confirmed our hypothesis.

Disclosures

There are no conflicts of interest that need to be disclosed by any author.

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