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Telmisartan use in rats with preexisting osteoporotics bone disorders increases bone microarchitecture alterations via PPARγ

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ABSTRACT

Aims: Telmisartan (TEL), an angiotensin II type I receptor blocker and PPARγ partial agonist, has been used for to treat hypertension. It is known that PPARγ activation induces bone loss. Therefore, we evaluate the effects of telmisartan on PPARγ protein expression, biomechanics, density and bone microarchitecture of femurs and lumbar vertebrae in SHR ovariectomized animals, a model of hypertension in which preexisting bone impairment has been demonstrated.

Main methods: SHR females (3 months old) were distributed into four groups: sham (S), sham + TEL (ST), OVX (C) and OVX + TEL (CT). TEL (5 mg/kg/day) or vehicle were administered according to the groups. After the protocol, blood pressure was measured and density, microarchitecture and biomechanics of bone were analyzed. Western blotting analysis was performed to evaluate PPARγ protein expression in the bones.

Key findings: Castration induced a deleterious effect on mineral density and trabecular parameters, with telmisartan enhancing such effects. Telmisartan increased PPARγ levels, which were at their highest when the treatment was combined with castration. As to biomechanical properties, telmisartan reduced the stiffness in the castration group (CT vs. S or C group), as well as resilience and failure load in ST group (vs. all others groups).

Significance: These results demonstrated that telmisartan compromised bone density and microarchitecture in animals that shows preexisting osteoporotic bone disorders, probably via mechanisms associated with increased PPARγ. If this translates to humans, a need for greater caution in the use of telmisartan by patients that have preexisting bone problems, as in the postmenopausal period, may be in order.

1. Introduction

Osteoporosis is associated with loss of bone mass due to decreased tissue continuity, with the rupture of the trabecular microarchitecture leading to reduced connectivity, increased bone fragility and fracture risk. The concomitant imbalance between osteoclastic and osteoblastic activities is exacerbated in the postmenopausal phase, being a determining factor for osteoporosis during this period [1]. There is evidence that both men and women – particularly those over 50 years old – may suffer from osteoporotic fractures during their lifetime and in the Caucasian population 40% of women and 13% of men are thus affected [2].

Among the many determinants playing an important role on bone structure that may lead to osteoporosis are renin angiotensin system (RAS), PPARγ (peroxisome proliferator activated receptor gamma) protein expression levels in the bone and the presence of female sex hormones.

The relationship between bones and the renin angiotensin system

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has been investigated, with evidence showing that increased RAS activity has a detrimental role on the bone tissue structure and metabolism, increasing fracture risk [3,4]. PPARγ is a superfamily of nuclear receptors for ligand-activated transcription factors involved in the regulation of the metabolism of glucose and lipids, in the immune response and promotion of cell differentiation [5]. It has been found that increase PPARγ protein expression induced microarchitecture deterioration in the bone structure [6–8]. The reduction of estrogens in aging female is usually associated with reduced bone mass and changes in bone microarchitecture, along with a high risk for fractures due to the increased bone fragility associated with osteoporosis [9,10].

Hypertension is a chronic disease in which the prevalence increases with age, with the postmenopausal reduction of female sex hormones being considered a risk factor for higher blood pressure [11–15]. The coexistence of hypertension and significant bone loss in the femoral neck has been described, suggesting an association between osteoporotic fractures and arterial hypertension [16]. Therefore, the evaluation of how antihypertensive agents can affect the bone is highly relevant. Even more so because the osseous effects of antihypertensive agents are controversial in the literature: they have been shown to preserve the tissue [17–24] or favor its resorption, leading to bone loss [25,26].

Osteoporotic bone disorders have been previously detected in spontaneously hypertensive rats (SHR) [27], an animal model of postmenopausal hypertension [28]. These animals were the chosen as a postmenopausal osteoporosis model for the study of the effects of the antihypertensive drug telmisartan in both intact and ovariectomized female [29]. Telmisartan, a dual drug, work as an angiotensin II receptor blocker (ARB) and also as a partial agonist for PPARγ, having been selected for our study because little is known regarding its effect on osteoporosis associated with hypertension. Thus, in the present study we propose to evaluate the microarchitecture, biomechanics and bone density, as well as PPARγ protein levels, in bones of ovariectomized SHR females treated with telmisartan for 8 weeks.

2. Methods

Ethical approval

Adult female SHRs (3 months old) were provided by the animal care facility of the central laboratory of the Federal University of Espírito Santo (UFES) and were maintained on 12-h light/dark cycles at controlled conditions (temperature and humidity), receiving water and a standard rat diet (Purina Labina, SP-Brazil) *ad libitum*. All procedures were performed according to biomedical research guidelines for the ethical care and use of animals in scientific research, having been approved by our local Ethical Committee for Animal Use (Protocol 64/2012).

2.1. Experimental animals

At the time of ovariectomy, the animals were randomly assigned into the following four groups: sham + vehicle (S), sham under telmisartan (ST), castration + vehicle (C) and castration under telmisartan (CT). S and C groups received daily oral gavage for 8 weeks with vehicle (0.5% carboxymethylcellulose sodium-CMC-Na) and ST and CT groups received a telmisartan dose dissolved in vehicle (5 mg/kg/day). All animals were weighed weekly on a digital scale in order to adjust the doses of both drug and vehicle by body mass, as well as to enable biometric analysis over the treatment period. After the experimental protocol the animals were euthanized by a ketamine (90 mg/kg) and xylazine (10 mg/kg) combination; the bones were then removed (left and right femur, left tibia and fifth lumbar vertebra) and dissected from soft tissues, stored in saline and kept at -80 °C for subsequent analysis. Hearts were excised and weighed tibia length was measured. Weight of heart were related to tibia length to determine heart weight to tibia length ratio.

2.2. Ovariectomy surgery

The surgical procedure for castration (ovariectomy) was performed according to the standard set by previous studies from our group, consisting of abdominal incision in the midline under anesthesia with ketamine (70 mg/kg) and xylazine (10 mg/kg). Animals belonging to the control groups (S and ST) underwent a sham surgery. The onset of telmisartan or vehicle treatment occurred fifteen days after the surgeries.

2.3. Non-invasive systolic blood pressure recordings

Forty-eight hours after the end of the treatment period, systolic blood pressure (SBP) measurements were taken by the tail-cuff method coupled to an electro-sphygmomanometer recorder (IITC Life Science - 23924 Victory Blvd, Woodland Hills, CA). Results were expressed as the mean from three independent measurements [30].

2.4. Analysis by bone densitometry by dual energy X-Ray absorptiometry (DXA)

Dual-energy X-ray absorptiometry (DXA) analysis was used to assess the BMD of the spine vertebrae (L5) and total femur (total femur length including diaphysis and epiphyses). The experiment was performed with the Discovery-A SN: 80999 Hologic device (Bedford, MA, USA) in the high-resolution mode, with the aid of the small animal software provided by the same manufacturer. The accuracy of the DXA for assessing BMD was previously analyzed by measuring the coefficient of variation, expressed as a percentage of the mean. The coefficient of variation was 1.9% for the spine and 0.6% for the total femur, indicating that the measurements were highly accurate [31,32].

2.5. Biomechanical analysis

Bones were frozen at −80 °C until the time of testing and then were thawed and kept fully moist before the mechanical testing To evaluate biomechanical properties, the left femora were tested in three-point bending using a universal test machine (Instron, model 4444, Canton, Massachusetts, USA) with 100 kgf capacity. The bones were supported by the ends in two rolls of 3 mm diameter and at a distance of 21.7 mm. The central region of each bone underwent tension loading [33].

A preload of 10 N was applied perpendicularly to the longitudinal axis of the femur and, after a 1-min period of accommodation and stabilization, force was applied at a constant speed of 0.5 cm/min until bone involvement and fracture. The stress generated on the bone was determined by plotting the amount of force applied over time with the aid of the software Instron (series IX). The mechanical properties visualized in the graph were maximum load (N), stiffness (N/mm), resilience (J) and failure load (N).

2.6. Morphometric analysis the trabecular structure by micro-computed tomography (μCT)

The evaluation of the femur trabecular structure parameters was done using Bruker's micro CT equipment (model SkyScan 1174, Kontich, Belgium). The system configurations were as follows: electric current of 730 μA and operating voltage set at 45 kV. Several X-rays of the bone were taken at different angles, generating measurements of the intensity of X-rays transmitted through the bone sample. Femur samples were rotated 180°, with an angular step of 0.8°, thus producing 225 radiographs (projections) per image, each containing 1024 × 1024 pixels with a spatial resolution of 9.8 μm. A 0.5 mm-thick aluminum filter was used at the outlet of the X-ray source. CT scans with the same spatial resolution as the 2D radiographs were established.

Three-dimensional (3D) renderings of the proximal right femur region were generated from the two-dimensional (2D) projections using appropriate algorithms. Thus, for each femur sample the volume of data generated was isotropic in relation to spatial resolution. The following parameters were analyzed: bone and tissue volumes, bone superface, trabecular thickness, trabecular number and trabecular separation, porosity, number of closed pores and connectivity.

Reconstruction of tomographic images was done with the aid of an appropriate algorithm and of Bruker's NRecon ™ software (version 1.6.9.4, Kontich, Belgium), which gathered all the radiographic projections at each angular position [34].

2.7. Western blot analysis

PPARγ protein expression levels in the total femur of rats from the different groups were detected by Western Blot. Femurs were excised, cleaned of all muscle and connective tissue, and kept at -20 °C until processing. The samples were homogenized in lysis buffer [Tris-HCL pH 7.4 (10 mM), PMSF (1 mM), NaVO3 (1 mM), SDS (1%), DTT (0.5 mM), EDTA (5 mM) and protease inhibitor cocktail (1:100 dilution)]. Total protein content was determined using the Bradford method. Samples containing 50 μg of protein were fractionated on a 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) nonfat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with Mouse anti-PPAR-γ ([1:200], Santa Cruz, Inc., USA) or Mouse anti-β-actin ([1:5000], Santa Cruz, Inc., USA). Membranes were washed five times with TBST and incubated with the peroxidase-conjugated secondary antibodies ([1:5000], Sigma, USA). Western blot signals were quantified using Bio-Rad Image Lab 5.2.1 software, and protein expression levels were normalized to β-actin expression.

2.8. Statistical analysis

Data are expressed as means ± SEM and the statistical analysis was performed using GraphPad Prism 6. The data were compared using two-way ANOVA followed by post-hoc Fisher LSD's test for inter-groups comparison. The data were considered significant at $p < 0.05$.

3. Results

3.1. SBP and ponderal data

Baseline physiological parameters and the effects of telmisartan are presented in Table 1. SBP was found to be reduced in both telmisartan groups after the experimental protocol period, with the CT group showing a greater reduction than the ST group. The ovariectomy surgery led to the expected changes in total body weight and uterine weight, with a significant increase in body weight and a reduction in uterus weight having been observed.

Treatment with telmisartan reduced the final body weight of castrated and intact animals when compared to their respective counterparts (ST vs. S and CT vs. C). Heart weight and tibia length ratio were reduced upon treatment of both castrated (CT) and intact (ST) groups when compared to intact sham animals (S), with animals from the CT group presenting lower ratios than those who went through castration but remained untreated (C).

3.2. Biometrical and biomechanical parameters

In relation to the effects of telmisartan on femoral biometrical and biomechanical parameters (Table 1), its use was found to cause damage to the femur's length, especially in ST group. The maximal load was reduced in the animals from ST when compared to those of the non use telmisartan groups (S and C). There was a reduction in stiffness in the animals from the ST and CT groups when compared to those of the S group. Finally, resilience and failure load were lower in animals from the ST group compared to those belonging to the others groups. No differences were found in others parameters analyzed (data no shown).

3.3. Bone mineral density

On the whole, when the BMD of femur and the fifth lumbar vertebra was evaluated by DXA, a density reduction was observed in the bones of castrated animals when compared to intact ones (Fig. 1). Notably, castration was able to reduce bone density on its own, which was evidenced by the reduction in the BMD of the animals in the C group when compared to those in the S group (panels A, B and C). The treatment with telmisartan led to the worst outcome for the proximal femur and the lumbar vertebra, regardless of castration. Nevertheless, the combination of castration and treatment with telmisartan resulted in a larger reduction in BMD when compared to animals who did not receive the drug (CT versus S or C groups in panels A, B and C).

The use of telmisartan in sham animals resulted in reduced femoral BMD, but no alterations were observed in the lumbar vertebra (ST vs. S group).

BMD measurements from the proximal femoral region were also obtained using the μCT method (Fig. 1, panel D). Yet again, although this parameter was obtained through a technique other than DXA, a clear impairment was observed in castrated animals (C group) in relation to sham ones (S group). Animals treated with telmisartan (ST or CT) showed a reduction in BMD when compared to their respective untreated groups (S or C). Finally, the association of castration with telmisartan use (CT group) resulted in the lowest BMD when compared to all others groups.

Table 1

Effect of treatment with telmisartan on ponderal and morphometric data, SBP, and biometric and biomechanical parameters. Animals groups: S (sham), ST (sham with telmisartan use), C (castration), CT (castration with telmisartan use).

	S	ST	C	CT
Ponderal and morphometrics data and SBP (n = 9)				
Final body weight (g)	199.66 ± 3.87	188.85 ± 2.34[a,c]	244.63 ± 5.03[a]	215.13 ± 2.96[a,b,c]
Uterine weight (g)	0.426 ± 0.035	0.360 ± 0.038[c]	0.122 ± 0.032[a]	0.093 ± 0.024[a,b]
Heart/tibia (g/cm)	0.236 ± 0.010	0.209 ± 0.007[a,c]	0.253 ± 0.005	0.203 ± 0.006[a,c]
Systolyc Blood Pressure (mmHg)	190.8 ± 2.3	122.8 ± 2.8[a,c]	191.2 ± 3.4	111.2 ± 3.7[a,b,c]
Biometric data (n = 7)				
Femur, cm	3,28 ± 0,01	3,19 ± 0,02[a,c]	3,34 ± 0,01[a]	3,32 ± 0,0[b]
Biomechanical data (n = 7)				
Maximum Load (N)	0,092 ± 0,004	0,081 ± 0,001[a,c]	0,092 ± 0,003	0,087 ± 0,003
Stiffness (N/mm)	227,52 ± 20,51	192,67 ± 7,38[a]	208,01 ± 7,93	173,83 ± 12,74[a]
Resilience (J)	0,049 ± 0,007	0,034 ± 0,003[a,c]	0.043 ± 0.004	0.047 ± 0.004[b]
Failure Load (N)	0,076 ± 0,004	0,054 ± 0,005[a,c]	0.072 ± 0.004	0.070 ± 0.003[b]

Data are expressed as the mean ± SEM. [a]$p < 0.05$ *vs.* S group; [b]$p < 0.05$ *vs.* ST group; [c]$p < 0.05$ vs. C group. (two-way ANOVA and post hoc Fisher's test).

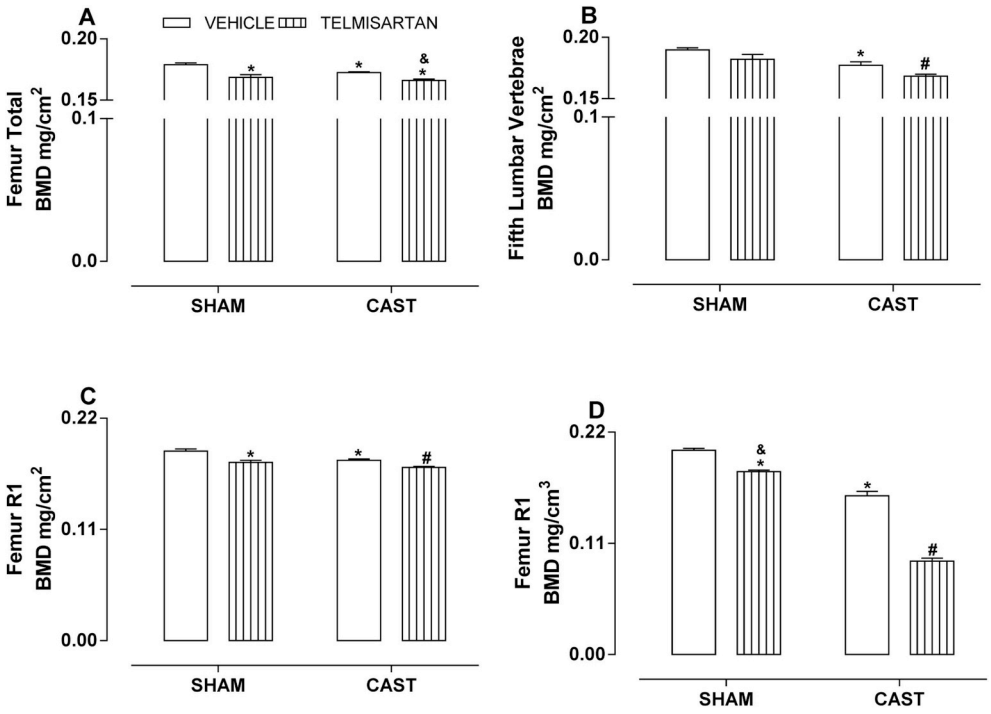

Fig. 1. Effects of the 8-week treatment with telmisartan on bone mineral density (BMD). (A) total femur, (B) fifth lumbar vertebrae, (C) femur proximal region (R1), (C1) femur proximal region (R1) by micro-CT. Data are expressed as the mean ± SEM (n = 7). $^{*}p < 0.05$ vs. S group, $^{\&}p < 0.05$ vs. CT group, $^{\#}p < 0.05$ vs. all others groups (two-way ANOVA and post hoc Fisher's test).

3.4. Morphometric analysis of trabecular microarchitecture

The analysis of bone parameters related to the integrity of the trabecular microarchitecture by μCT is shown in Figs. 2 and 3. Porosity, total pore volume, bone surface density, percent bone volume, number of closed pores, trabecular number and connectivity were parameters that were affected by the interventions.

On the whole, we observed that ovariectomy (C group) caused a marked deterioration in the microstructure of the trabecular region of proximal femur when compared to intact animals (S group). Treatment with telmisartan increased the deterioration in bone architecture of ovariectomized animals (CT group) even further. Also, its use in sham animals (ST group) usually caused damage in the bone microarchitecture when compared to intact, untreated animals (S group).

In general, there was an increase of porosity and a reduction in the closed number pores, bone surface density, percent bone volume, trabecular number and in bone connectivity in animals from the castrated group treated with telmisartan (CT versus all other groups).

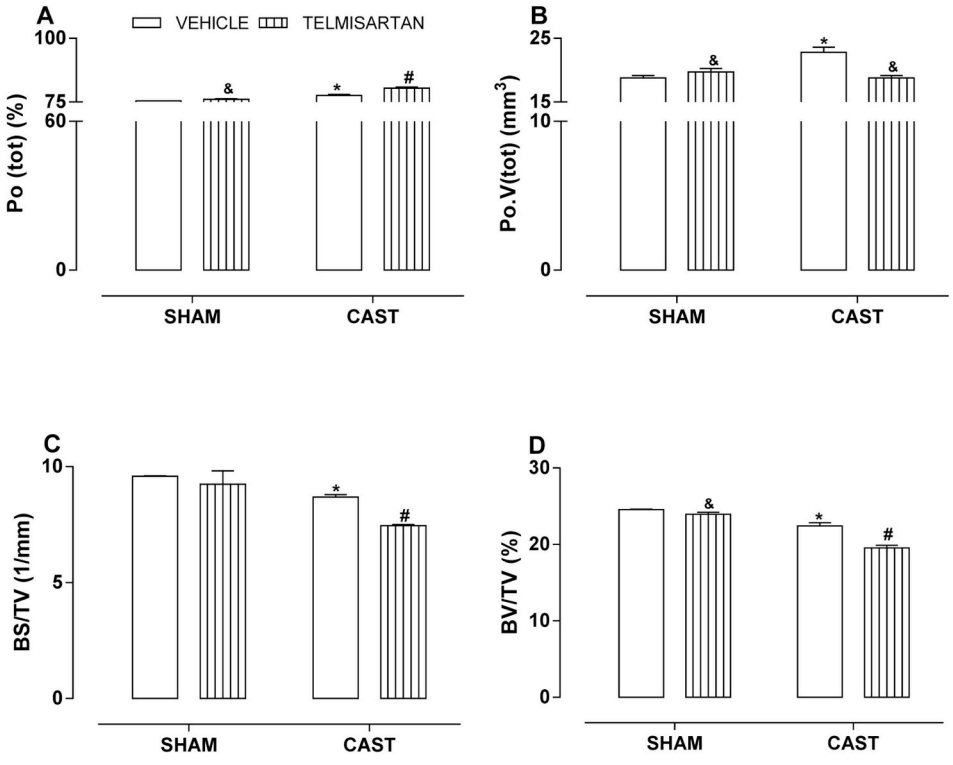

Fig. 2. Effects of 8-week treatment of the animals with telmisartan on the micro-architectural characteristics. (A) Total porosity, (B) total volume of pore space, (C) bone surface density, (D) bone volume percentual. Data are expressed as the mean ± SEM (n = 5). $^{\&}p < 0.05$ vs. CT group, $^{*}p < 0.05$ vs. S group, $^{\#}p < 0.05$ vs. all others groups. (two-way ANOVA and post hoc Fisher's test).

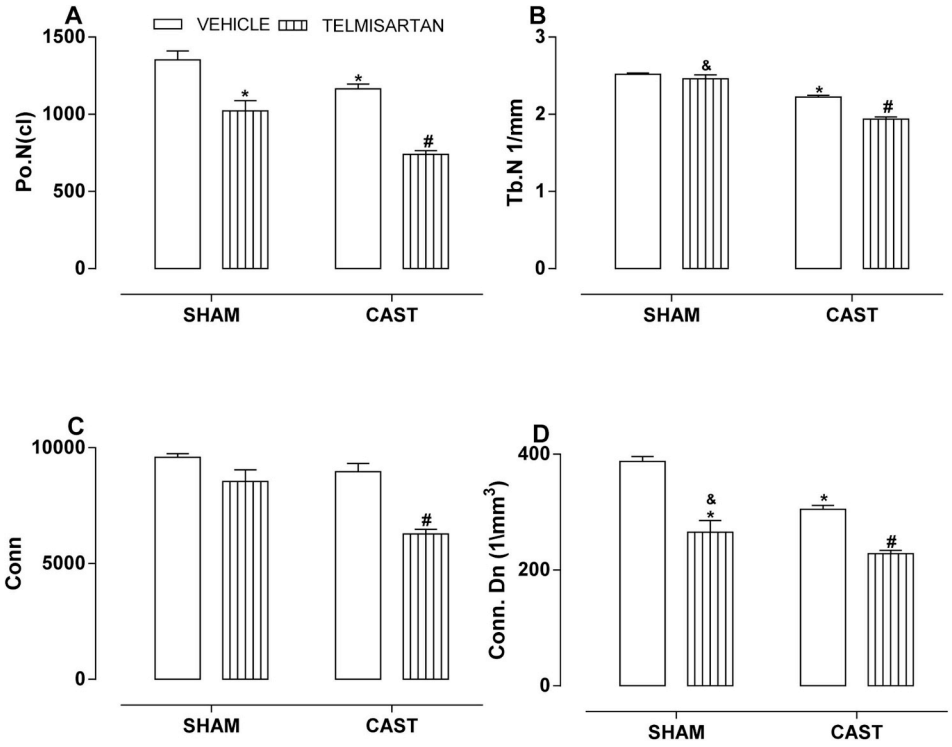

Fig. 3. Effects of 8-week treatment of the animals with telmisartan on microarchitectural characteristics. (A) Number of closed pores, (B) trabecular number, (C) connectivity, (D) connectivity density. Data are expressed as the mean ± SEM (n = 5). $^{*}p < 0.05$ vs. S group, $^{\&}p < 0.05$ vs. C group, $^{\#}p < 0.05$ vs. all others groups (two-way ANOVA and post hoc Fisher's test).

3.5. Western blot analysis (PPARγ protein expression)

Fig. 4 shows the analysis of PPARγ protein expression, quantified by densitometry and normalized by the expression of β-actin. PPARγ levels were found to be increased in castrated animals (C group) and in those who went through telmisartan treatment (ST and CT group), when compared to intact, untreated animals (S group). It is worthy of note that the combination of castration and telmisartan use yielded the higher PPARγ expression levels (CT group vs. ST and C groups).

4. Discussion

In the present study, we provide evidence of increased expression of the protein PPARγ associated with the occurrence of biomechanical and morphologic defects in bones – femur and lumbar vertebrae – in intact and ovariectomized SHR females (an animal model of both postmenopausal osteoporosis and hypertension) by telmisartan use. Interestingly, rats from the ST group resembled those from the castrated group (C) regarding bone loss and increased PPARγ protein expression, and one should note that these detrimental changes were even more pronounced in ovariectomized animals treated with telmisartan (CT group). In spite of negative bone effects, telmisartan efficiently reduced blood pressure and cardiac hypertrophy in animals that were underwent treatment, which corroborates others studies conducted in hypertensive rats [35,36]. Such findings are quite important, adding to the overall relevance of the data present here.

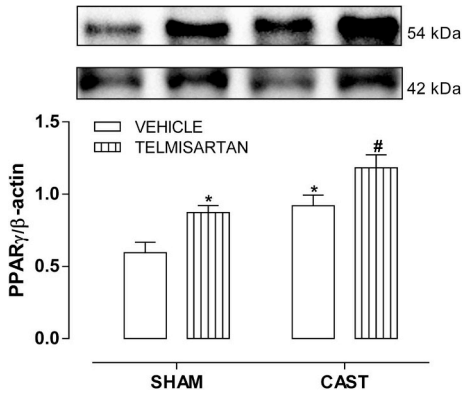

Fig. 4. Effects of 8-week treatment with telmisartan on PPARγ protein (54 kDa) expression in total femur. The signal from each protein was normalized by the respective amount of β-actin (42 kDa) even before calculating the ratios. Data are expressed as mean ± SEM (n = 5). $^{*}p < 0.05$ vs. S group, $^{\#}p < 0.05$ vs. all others groups (two-way ANOVA and post hoc Fisher's test).

Bone microarchitecture deterioration (trabecular destruction and reduction in the connectivity), which can happen as a result of drastic reductions on female sex hormone levels, is considered a sensitive parameter to demonstrate trabecular disconnectivity, therefore reducing the strength of the tissue and its ability to resist to stress, increasing the risk of fractures [37]. The increased risk of fractures observed during the postmenopausal phase is a consequence of the rapid bone loss caused by an increase in bone turnover and a decrease in tissue formation [38]. The consequent reduction in estrogen levels that necessarily follow the castration performed in our model could account for the worsening in the quality of bone microarchitecture observed here, corroborating the aforementioned reports.

It has been put forward that the renin angiotensin system acts in bones through the regulation of structural and metabolic aspects of this tissue by angiotensin II [3,4]. Specifically regarding the use of ARBs the data are controversial and not conclusive [25,39–41]. It has been reported that Angio II activates osteoclasts decreasing bone density in ovariectomized SHRs, and the use of olmesartan reverses this effect [39]. In contrast, telmisartan use in SHR male increased bone detrimental effects [25] or did not change bone turnover markers in patients with mild hypertension [40]. Also, losartan was unable to prevent bone derangement in both hypertensive and normotensive male rats, suggesting a lower participation of AT1 receptors in the effects of angiotensin II on bone tissue [41]. These controversial results from different ARBs may be due to their mode of action and the experimental doses and experimental design used.

Even though the mechanisms involved are not fully understood, it is

known that PPARγ agonists can cause bone loss *in vitro* and in rodent models [6–8,42–44]. Treatment with PPARγ agonists – thiazolidinediones (TZDs) – has side effects such as a significant risk of adverse effects on human bone, and PPARγ activation by TZDs causes bone loss by suppressing differentiation and activity of osteoblasts, while reducing bone formation and enhancing osteoclasts differentiation [42,44]. Another possible mechanism underlying the bone loss related to the action of PPARγ agonists involves adipogenic cells differentiation from mesenchymal stem cells (MSCs) at the expense of osteoblastogenesis [45,46]. Taken together, these data suggest that PPARγ agonists may increase the risk of bone loss.

Our results suggest that the use of telmisartan, a PPARγ partial agonist, may have induced bone loss mediated by the activation of PPARγ in this tissue, in view of the higher levels of this protein found in bone of ovariectomized animals and in those treated with telmisartan (ST and CT). However, one cannot rule out th participation of other mechanisms.

In summary, telmisartan in ovariectomized SHRs – a model of oestrogen deficiency in hypertensive rats – increased the levels of femoral PPARγ protein expression and compromised trabecular bone microarchitecture by reducing bone mass as evidenced by the shape and connectivity parameters observed by DXA and micro-CT analysis.

5. Conclusion

In conclusion, these results demonstrated that the treatment of animals showing preexisting osteoporotic bone disorders with telmisartan compromised bone density and microarchitecture, probably via mechanisms associated with increased PPARγ. Actual data may reveal a need for greater caution in the use of telmisartan in patients that have preexisting bone alterations, as in the postmenopausal period.

Whether these experimental findings translate into human clinical situations is not yet known and additional studies employing specific imaging techniques, such as densitometry and/or tomography, may be required to follow up patients taking telmisartan.

Author contributions

The contributions of each author to the study were:

Antonio Marcos Birocale[a,b], Antonio Ferreira de Melo Jr[a,b], Pollyana Peixoto[a], Phablo Wendell Costalonga Oliveira[a], Leandro Dias Gonçalves Ruffoni[a], Liliam Masako Takayama[a], Breno Valentim Nogueira[a,b], Keico Okino Nonaka[a,b], Rosa Maria Rodrigues Pereira[a,b], José Martins de Oliveira Jr.[a,b], Nazaré Souza Bissoli[a,b]

a Acquisition of data.

b Substancial contributions to conception and design; analysis and interpretation of data; drafting the article; revising it critically for important intellectual content; and final approval of the version to be published.

Declaration of competing interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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