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Mechanical Vibration Associated With Intermittent PTH Improves Bone Microarchitecture in Ovariectomized Rats

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Abstract

Introduction: Intermittent 1-34 parathyroid hormone (iPTH) administration, a bone-forming treatment, is widely used as a therapy for severe osteoporosis. It can only be used for a maximum of 24 mo and must be followed by an antiresorptive drug to retain the new formed tissue. Mechanical load, in the form of low-intensity and high-frequency vibration, has received considerable attention due to its ability to prevent bone loss. Aim: To investigate the ability of whole body mechanical vibration (MV) to potentiate the anabolic effects of iPTH and to inhibit bone resorption following discontinuation of iPTH treatment in estrogen-deficient rats. Methodology: Fifty-four 6-month-old female *Wistar* rats were ovariectomized (OVX) or sham-operated. After 5 mo, they were divided into 7 groups: **Sham** – non-OVX; **Control** – OVX, vehicle for 60 d; **MV** – OVX, submitted to MV for 60 d; **PTH60d** – OVX, injected with iPTH for 60 d; **PTH+MV** – OVX, injected with iPTH combined with MV for 60 d; **PTH30d** – OVX, injected with iPTH for 30 d, and untreated for 30 d; **PTH30d/MV30d** – OVX, injected with iPTH for 30 d, followed by MV for 30 d. Bone mineral density (BMD) and body composition (lean mass and fat) were evaluated at OVX (T0), the beginning (T1), and at the end (T2) of treatments by dual X-ray absorptiometry (DXA). Femurs were processed for histomorphometry (bone volume - BV/TV and cortical thickness - Ct.Th) and tibias for biomechanical test. Results: Body composition and BMD were similar among the groups at T0. In T2, MV presented higher fat than other groups (except PTH60d) and PTH30d/MV30d showed greater lean mass than Control. At T1, Sham presented the highest BMD, but between T1 vs T2 there was an increase in all iPTH-treated groups. At T2, BMD was higher in PTH60d and PTH+MV than in the Control and MV groups. The highest BV/TV was observed in the PTH +MV group, followed by PTH60d. Cortical thickness was increased in PTH60d and PTH+MV compared to Sham. Vibration applied post-iPTH (PTH30d/MV30d) improved the force at failure in tibias when compared to Sham and Control groups. Conclusion: MV potentiated iPTH anabolic effects in cancellous bone; however, MV was unable to maintain bone mass after stopping iPTH in ovariectomized rats.

Key Words: Bone densitometry; bone histomorphometry; intermittent PTH; mechanical vibration; ovariectomized rats.

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Introduction

Osteoporosis is a disease with global incidence characterized by decreased bone strength leading to an increased risk of fracture. Worldwide, it has been estimated that 8.9 million fractures occur annually, resulting in one osteoporotic fracture every 3 s *(1)*.

Recent studies show that the numbers of hospitalizations for osteoporotic fractures (43%) exceeds those for heart attacks (25%), strokes (26%) and breast cancer (6%) *(2)*. Postmenopausal women are most affected due to an estrogen-deficiency, which leads to increased bone resorption compared to bone formation *(3)*. Therefore, the prevention and treatment of osteoporosis in postmenopausal women have relevant aspects to the economy and health. This justifies the need for improved treatments to prevent loss of bone mass and strength.

Intermittent parathyroid hormone administration (iPTH, PTH 1-34, or teriparatide) is an anabolic treatment currently approved by the Food and Drug Administration (FDA) to increase bone mass in patients suffering from severe osteoporosis *(4)*. Controlled trials have shown that iPTH significantly reduces the incidence of fractures *(4,5)*. In ovariectomized rodent models, this drug has been shown to increase bone formation, mineral apposition, and enhance cancellous and cortical bone strength *(6–8)*. Additionally, several studies have indicated that iPTH also sensitizes bone cells to mechanical stimuli *(9,10)*.

Treatment using iPTH is approved for a limited duration of 18–24 mo *(11,12)* and the bone mineral density achieved with this treatment is lost if an antiresorptive agent is not immediately administered following discontinuation of iPTH *(13,14)*. In addition, iPTH administered over a prolonged period and in large doses induced osteosarcoma in rats *(15)*.

Physical activity plays an important role in skeletal health once the bone mass becomes responsive to the mechanical loads placed on the skeleton *(16)*. This high sensitivity to mechanical signals may provide a basis for nonpharmacological interventions able to increase bone mass *(17)*. Mechanical vibration (MV) can be considered as a surrogate for physical exercise possibly by improving balance, motility, and muscle strength in the upper and lower limbs *(18,19)*. Low-intensity and high-frequency whole-body vibration has prevented bone loss in rats caused by the lack of estrogen or induced by glucocorticoids *(20–22)*.

The combined effects of an anabolic agent such as iPTH and a nonpharmacological anabolic intervention (MV) can be potentially important to maximize the treatment response. Nevertheless, the action of these combined therapies is still controversial *(23,24)* and has not been tested in estrogen-deficient mature rats. Furthermore, the limited time-course of iPTH administration requires an agent post-treatment, for which MV therapy could be a sequential nonpharmacological option.

In this regard, the present study aimed to evaluate if mechanical vibration therapy can potentiate the effects of low dose of iPTH, and whether MV is able to maintain bone mass following the treatment with iPTH in estrogen-deficient rats. Herein, we compared the effects of these therapies on BMD, bone volume, cortical thickness, mechanical properties, and body composition in OVX rats.

Materials and Methods

Experimental Groups and Treatments

Animals procedures were conducted according to the "Guiding Principles for the Care and Use of Laboratory Animals", approved by the Animal Care Committee guidelines at the Federal University of São Paulo (protocol 6580150716).

Fifty-four 6-month-old female *Wistar* rats (270 g) were bilaterally ovariectomized (OVX, n = 46) to induce estrogen deficiency or Sham-operated (non-OVX, n = 8). Vaginal smears were collected during four consecutive days to confirm the success of the OVX procedure.

Treatment was initiated 5 mo following OVX surgery (T1) and the animals were randomized into 7 groups: **Sham** (non-OVX; n = 8) without treatment for 60 d; **Control** (OVX, n = 6) injected with vehicle solution (0.9% NaCl) for 60 d; **MV** (OVX, n = 7) submitted to MV for 60 d; **PTH60d** (OVX, n = 9) injected with iPTH for 60 d; **PTH+MV** (OVX, n = 9) injected with iPTH in combination with MV for 60 d; **PTH30d** (OVX, n = 7): injected with iPTH for 30 d and then 30 d without treatment, **PTH30d/MV30d** (OVX, n = 8): injected with iPTH for 30 d and subsequently submitted to MV for 30 more days (Fig. S1).

The rats were submitted to MV for 20 min/day, 5 d/wk. The vibration was provided by an adjustable vibratory platform adapted to a special cage, which was set to impose a vertical acceleration of 0.6 g at a frequency of 60 Hz with amplitude less than 1.0 mm. The parameters used in the present experiment were selected based on previous bone and cartilage studies *(21,22,25,26)*. The PTH 1-34 (Teriparatide -Forteo® Eli Lilly & Co., Indianapolis) was diluted in saline solution and administered by subcutaneous injections, 7 d/wk, at a dose of 5 μg/kg/d. The adopted dose was based on previous studies *(8,27)*. Body weight was recorded every 2 wk since the OVX procedure was performed and continued until the end of the study.

Densitometry Measurements (DXA)

Bone mineral density (BMD, g/cm^2) and body composition (lean mass and fat, g) measurement were performed at the day of OVX (T0), at the beginning (T1), and at the end (T2) of treatments by dual X-ray absorptiometry (DXA, Hologic Model 4500A, Waltham, MA),

using a specific small animal software (Version 610-0691 for QDR XP).

Animals were anesthetized intraperitoneally with ketamine (80 mg/kg) and xylazine (12 mg/kg), then placed in a prone position for whole body densitometry measurements. Examinations were performed by an investigator blinded to the treatment regimens.

The rats were euthanized after the last densitometry (T2) measurements by anesthesia overdose. Femurs and tibias were dissected and aimed at the following techniques.

Bone Mechanical Properties: Maximum Force, Force at Failure, and Stiffness

Biomechanical properties of the left tibia were measured using the 3-point bending test in a blinded manner, using an Instron mechanical testing machine (Instron Corp., Model 4444, Canton, MA) interfaced to a computer with Instron Series IX Automated Materials Tester - version 8.09.00 software.

Tibias were placed on two rollers and a 5 Newton (N) preload was applied to stabilize the specimen. A force, with a constant velocity of 0.5 cm/min, was then applied until fracture occurred *(28)*. As a result of the force applied, a graphic load strain was obtained using maximum force (the highest load supported by the tibias, N), force at failure (the load at which bone fracture occurs, N), and stiffness (the slope of the elastic part of the curve, N/mm).

Histological Preparations

The distal femurs were fixed in 4% formaldehyde (freshly derived from paraformaldehyde) in phosphate buffer (pH 7.2) for 4 d, then decalcified in ethylenediaminetetraacetic acid (EDTA) 10%, (pH 7.0) for 90 d, and subsequently dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Consecutive sections (5 μm thick) were obtained using a Minot-type microtome (Leica, Model RM 2145).

Histomorphometry: Cancellous Bone Volume and Cortical Bone Thickness

Bone sections from each animal were stained with hematoxylin and eosin (H&E) and submitted to histomorphometric analysis to quantify the cancellous bone volume (BV/TV, %) and cortical bone thickness (Ct.Th, μm) as previously described by Pacheco-Costa et al *(8)*. Histomorphometric procedures were carried out using a semi-automatic image analysis system (AxioVision Rel. 4.9., Carl Zeiss, Germany). At least 5 consecutive bone sections from each animal were examined. The histomorphometric indices were reported according to the standardized nomenclature recommended by the American Society of Bone and Mineral Research *(29)*.

Statistical Analysis

Data were analyzed using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA) and STATISTIC 12.0 (Stat Soft Inc.). The groups were compared using one-way ANOVA or repeated measures ANOVA followed by Tukey's test. Data are expressed as mean ± standard deviation and significance was established at $p < 0.05$.

Results

Body Composition Is Altered During Treatments

At T0 there were no differences between groups regarding body weight, lean mass, and fat. Lean mass increased in all groups between T0 and T1, but the body weight and fat increased only in OVX groups. At T1, no difference was observed among the groups in body weight. On the other hand, body fat was higher in the MV, PTH60d, and PTH30d/MV30d groups than in the Sham, while lean mass was higher in the Control group compared to the PTH+MV group. During the treatment period (T1 vs T2), there was an increase of body weight in the MV, PTH60d, PTH+MV, PTH30d, and PTH30d/MV30d groups. In addition, body fat increased in the Control and MV groups, while lean mass decreased in the Control, remained unaffected in the MV group, and increased in all other groups (Table 1).

After treatment (T2), MV group was significantly heavier (body weight) than the Sham. Furthermore, the Sham and PTH30d groups had lower body fat compared to the Control, MV, PTH60d and PTH30d/MV30d groups, while the MV group exhibited a higher body fat compared to Sham, Control, PTH+MV, PTH30d, and PTH30d/MV30d. Moreover, iPTH administration for 30 d followed by MV for an additional 30 d (PTH30d/MV30d) resulted in a higher lean mass compared to the Control group (Table 1).

iPTH Increases Bone Mineral Density Independently if Applied Alone or Combined With Mechanical Vibration

The BMD results showed no differences among the groups at T0. Comparing the interval between T0 and T1, the Sham and PTH+MV groups showed an increase. At T1, the highest BMD was observed in the Sham group (Table 1, Fig. 1). iPTH administered for 30 and 60 d (PTH30d and PTH60d), as well the combined treatment (PTH+MV), and the iPTH for 30 d followed by MV (PTH30d/MV30d) resulted in a BMD increase during the treatment time (T1 vs T2). At the end of treatment (T2), the PTH60d and PTH+MV were higher than the Control and MV groups, while the MV group exhibited a lower BMD than the Sham (Table 1, Fig. 1).

Table 1
Densitometry Measurements (DXA) and Body Weight

		Bone mineral density						
		Sham	Control	MV	PTH60d	PTH+MV	PTH30d	PTH30d/MV30d
BMD (g/cm^2)	T0	0.148±0.003	0.150±0.006	0.150±0.004	0.151±0.006	0.146±0.006	0.148±0.007	0.151±0.005
	T1	0.161±0.005^{b,c,d,e,f,g,h}	0.151±0.002	0.151±0.004	0.151±0.004	0.151±0.006^{h}	0.151±0.008	0.151±0.005
	T2	0.159±0.004^{c}	0.152±0.004	0.151±0.004	0.161±0.005^{b,c,i}	0.160±0.005^{b,c,i}	0.157±0.008^{i}	0.157±0.005^{i}
		Body composition						
Fat (g)	T0	45.3±12.1	29.2±8.1	44.8±10.8	47.9±12.3	47.2±11.2	43.0±12.1	42.7±15.6
	T1	44.2±17.6	52.8±12.0^{h}	81.2±29.1^{a,h}	81.4±22.9^{a,h}	69.7±24.2^{h}	59.6±20.9^{h}	80.0±22.6^{a,h}
	T2	44.2±23.0	75.0±12.5^{a,c,f,i}	105.4±41.4^{a,b,e,f,g,i}	79.0±26.8^{a,f}	69.1±28.3	44.8±22.4	72.9±21.7^{a,f}
Lean mass (g)	T0	213±16	221±11	220±14	213±12	205±17	212±18	220±15
	T1	236±17^{h}	247±8^{e,h,j}	242±15^{h}	233±16^{h}	224±20^{h}	230±2^{h}	240±19^{h}
	T2	245±20^{i}	234±8	239±24	245±19^{i}	240±18^{i}	255±18^{i}	260±22^{b,i}
Body weight (g)	T0	250±20	247±9	255±18	251±13	242±16	246±18	252±24
	T1	274±26	295±11^{h}	314±26^{h}	304±22^{h}	283±31^{h}	281±19^{h}	310±34^{h}
	T2	292±34	307±13	353±25^{a,i}	335±41^{i}	315±37^{i}	312±18^{i}	340±42^{i}

Data are expressed as the mean ± standard deviation (n= 6–9 per group). $p < 0.05$. 95%. To compare all groups at the same period were used the symbols: "a" different from Sham; "b" different from Control; "c" different from MV; "d" different from PTH60d; "e" different from PTH+MV; "f" different from PTH30d, "g" different from PTH30d/MV30d. To compare the same group at the different periods were used the symbols: "h" different from T0 (Baseline); "i" different from T1 (beginning of the treatment); "j" different from T2 (end of treatment). The groups were compared by repeated measures ANOVA.
Abbr: BMD, bone mineral density; MV, mechanical vibration; PTH, intermittent 1-34 parathyroid hormone.

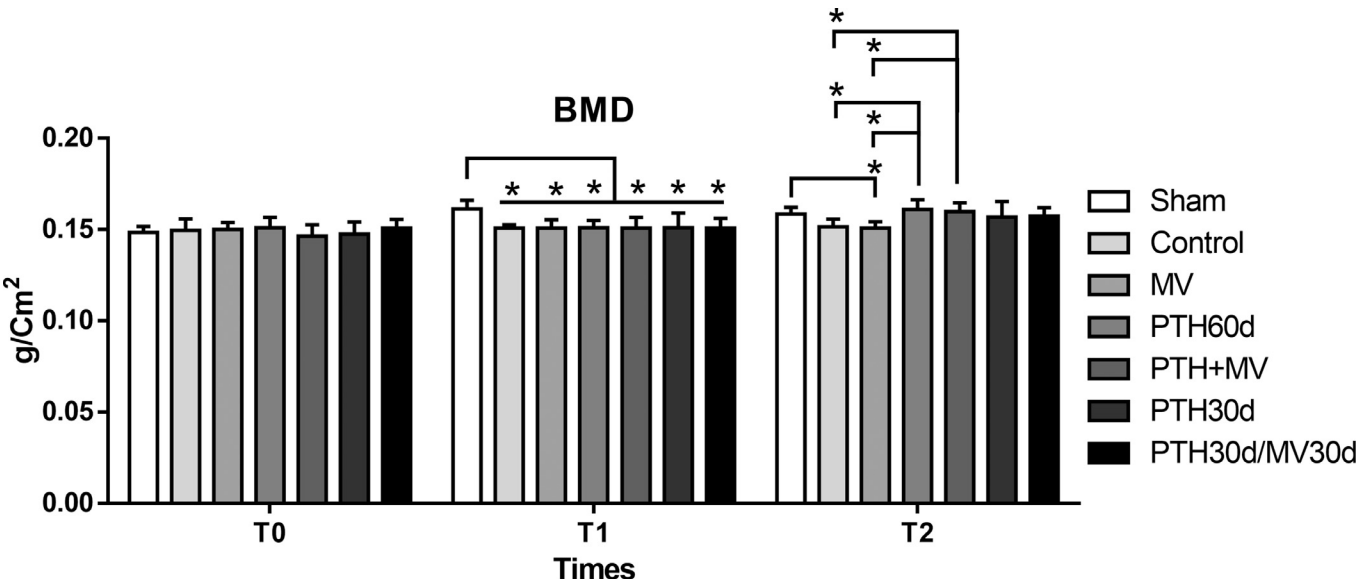

Fig. 1. Graph of BMD comparing all groups in each period. Note that in T0, all groups were homogenous; at T1, the Sham group was the highest; while at T2, PTH+MV and PTH60d were higher than the Control and MV groups, while Sham was higher than MV. Data are expressed as mean ± SD. N = 6–9. *$p < 0.05$ by repeated measures ANOVA. BMD, bone mineral density; MV, mechanical vibration; PTH, intermittent 1-34 parathyroid hormone.

Mechanical Vibration Potentiates the iPTH Effect in the Cancellous Bone

Analysis of bone microarchitecture from rats subjected to MV in combination with iPTH (PTH+MV) showed an increase in BV/TV compared to the other OVX groups. The BV/TV of the combined group (PTH+MV) was 53% higher than the Control, 51% higher than the MV, 47% higher than the PTH30d, 45% higher than the PTH30d/MV30d, and 27% higher than the PTH60d group (Fig. 2).

The group treated with iPTH for 60 d showed an increase of 36% compared to the Control group and 32% in relation to MV in BV/TV. Moreover, there was a decrease of 42% in the Control group, 39% in the MV, 34% in PTH30d, and 31% in the PTH30d/MV30d in relation to the Sham (Fig. 2).

The PTH60d and PTH+MV groups increased Ct.Th 23% compared to the Sham group (Fig. 2-I).

Mechanical Vibration After iPTH Treatment Improves Bone Force at Failure

The PTH30d/MV30d group showed a higher force at failure in the three-point bending strength test compared to the Sham and Control groups. There were no differences in stiffness and maximum load among the groups (Fig. 3).

Discussion

We show, herein, that MV when combined with a low dose of iPTH is able to potentiate the effect of this drug on the cancellous bone volume of estrogen-deficient rats. However, MV therapy was not able to maintain the bone mass due to iPTH treatment in ovariectomized rats with pronounced bone loss.

In the current study, we used a low dose of iPTH (5 μg/kg/d), based on a previous study performed by our group *(8)*. The approved PTH 1-34 dose for patients is 20 μg/d *(4)*, which approximates ~0.3 μg/kg/d *(8)*. Although, our dose (5 μg/kg/d) is 17× higher than usual, it is still considered a low dose when compared to other studies that usually administer daily doses above 20 μg in rodent models *(7,23,24)*. The whole-body MV was applied with a frequency of 60 Hz, an intensity of 0.6 g, and an amplitude of less than 1 mm based on previous studies that showed efficacy in rodents which had bone loss due to glucocorticoids and OVX *(21,25)*.

The body composition analyses revealed that MV applied as a single therapy led to body-fat increase, while the lean mass remained unaffected. However, when iPTH was administered before MV therapy (PTH30d/MV30d group), we observed a higher lean mass suggesting that, perhaps, in the MV group, the body fat increase had impaired the lean mass. Previous literature has demonstrated that the loss of ovarian function promotes an increase in fat tissue *(30)*. Ovariectomy procedure in our study also induced an increase in fat tissue in all groups at T1, which was not observed in the Sham group.

BMD results showed that all groups were homogenous at the beginning of the experiment (T0), but after 5 mo (T1), the Sham group presented the highest BMD, as expected. Our data did not show any significant decrease

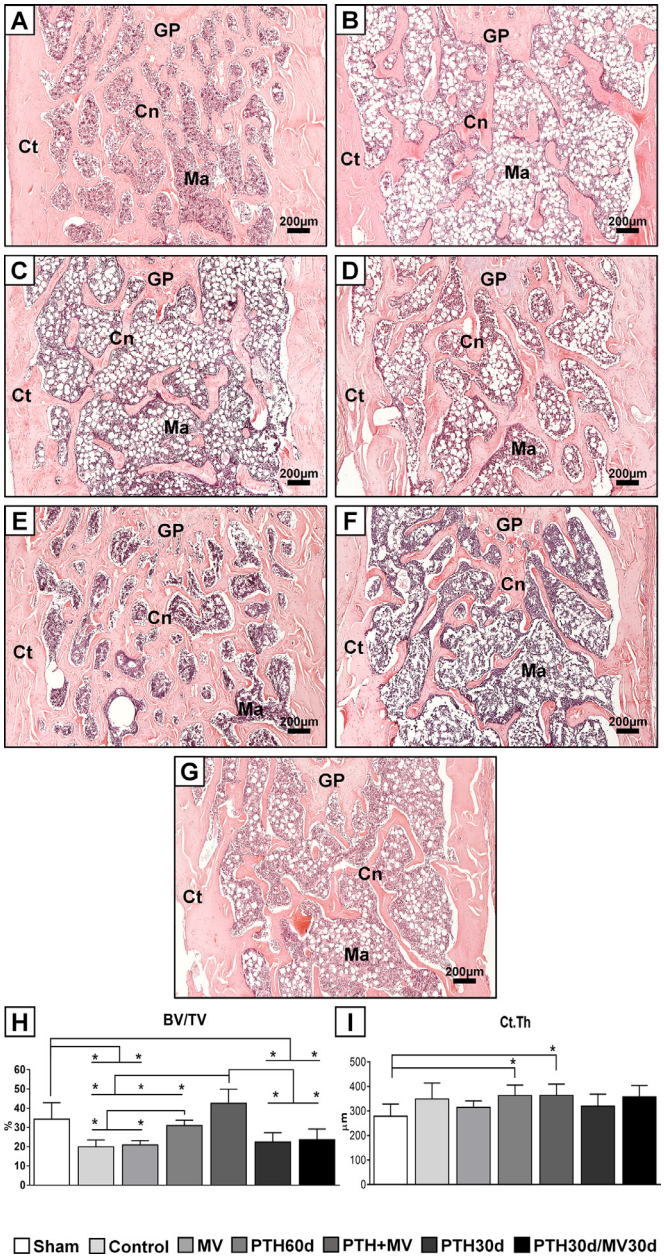

Fig. 2. Histomorphometric analysis of the cancellous bone volume (BV/TV) quantification and cortical bone thickness (Ct.Th) from distal femurs of 13-month-old *Wistar* rats. A-G are representative light micrographs showing the cancellous bone: (A) Sham, (B) Control, (C) MV, (D) PTH60d, (E) PTH+MV, (F) PTH30d and (G) PTH30d/MV30d. Note the higher BV/TV in the PTH +MV (E) compared to Control, MV, PTH60d, PTH30d and PTH30d/MV30d. H&E stain. Scale bars represent 100 μm. Gp: growth plate; Cn: cancellous bone; Ct: cortical bone and Ma: bone marrow. (H) Graph of BV/TV (I) Graph of Ct.Th. Data are expressed as mean ± SD. N= 6–9. *$p < 0.05$ by one-way ANOVA. MV, mechanical vibration; PTH, intermittent 1-34 parathyroid hormone.

of BMD in the OVX groups between T0 and T1, probably due to the continuous growth of the rodents *(31)*, since after a long period of 5 mo, an increase of body composition parameters in all groups was observed after OVX. In addition, the BMD growth rate (T0 vs T1) in the Sham group was 8.8% and in the Control group was only 0.6%. Although both groups showed a BMD increase, the effect of OVX surgery on the Control group limited the BMD improvement.

To identify possible changes in trabecular and cortical compartments, we investigated the treatment effects on trabecular BV/TV and Ct.Th by histomorphometry.

We found a pronounced deterioration in the bone microarchitecture (BV/TV) in the Control compared to the Sham group. According to Li et al *(32)*, cancellous bone loss in OVX rats is significant 30 d post-OVX and remains moderate for the first 90 d, becoming more pronounced over time. In our study, we had a time gap of 150 d between the OVX and the start of treatments, resulting in an intense loss of cancellous bone.

Treatments with iPTH independent of MV combination or administration time (PTH60d, PTH+MV, PTH30d, and PTH30d/MV30d) culminated in an increase in BMD. Nevertheless, we observed that iPTH had a greater effect on the bone microarchitecture (BV/TV) when administered within 2 mo. Thus, both groups (PTH60d and PTH+MV), when injected with iPTH for 60 d, exhibited an increase of BV/TV and Ct.Th suggesting an effect regardless of MV therapy. However, MV was able to enhance the anabolic effect of iPTH on cancellous bone.

A previous study from our group demonstrated an improvement of BV/TV with 1 mo of treatment at the same dose of iPTH (5 mg/kg/d) *(8)*. However, we did not observe significant alterations in the bone microarchitecture in the PTH30d group. Differences in the experimental time frame or OVX-induced estrogen status may be responsible for the variable anabolic efficacy of iPTH in OVX rats. Pacheco-Costa et al *(8)*, started the treatment 3 mo post-OVX, while in the present study there was an interval of 5 mo post-OVX before the beginning of treatments. Furthermore, in our study the animals were injected with iPTH for 1 mo followed by one additional month without treatment before euthanasia (13-month-old) which may have resulted in the different findings.

We dedicated part of the current study to acquire evidences regarding the combined effects of MV with iPTH. Remarkably, the combined treatment demonstrated an enhancement in BV/TV when compared with other treatments.

In the combined treatment, the BV/TV was 27% higher than in the iPTH treatment alone, suggesting that MV can potentiate the anabolic effects of iPTH on bone microarchitecture in ovariectomized old rats. This makes low-intensity mechanical whole-body vibration a potential alternative for improving the results of anabolic

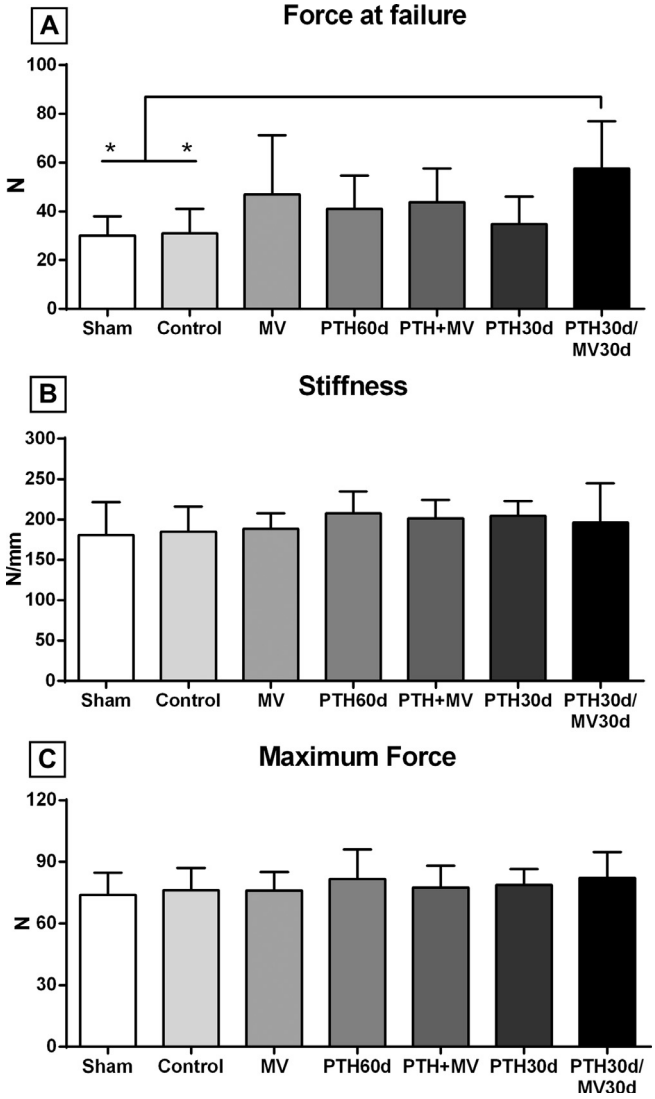

Fig. 3. Graphs of the biomechanical properties in tibias: (A) Graph of force at failure, (B) Graph of stiffness, (C) Graph of maximum force. Note, PTH30d/MV30d supported the higher force at failure compared to Sham and Control groups (A). Data are expressed as mean ± SD. N = 6–9. *$p < 0.05$ by one-way ANOVA. ANOVA, analysis of variance; MV, mechanical vibration; PTH, intermittent 1-34 parathyroid hormone.

treatments in severe osteoporosis. Actually, there is a clinical trial designed to investigate this therapy combination (teriparatide plus vibration) in osteoporotic women, which will be published soon *(33)*. Low-intensity whole-body vibration can also be explored as a surrogate tool for physical activity in disabled patients, once it is established as a safe method of transmitting mechanical signals to bone and muscle in patients who cannot undergo impact exercise *(19,34)*. Further studies are needed to elucidate the molecular targets as well as understand the mechanisms involved in this combined therapy.

In relation to cortical bone, the PTH60d and PTH +MV groups presented an increase in Ct.Th. compared to the Sham, but no difference was observed between them, suggesting that MV potentiates the iPTH effect only on cancellous bone, at least in the treatment model established in this study. It is well known that cortical and cancellous bone remodeling can be attributed to different metabolic turnover rates, which is faster for cancellous bone which also supported our findings *(35)*.

Our results showed that MV applied alone for 60 d (0.6 g/60 Hz) was not sufficient to avoid the progression of bone loss after OVX. The BMD and BV/TV data of the MV group were similar to those obtained in the Control group, suggesting a failure of MV in attempt to recover the bone tissue. It appears that there is currently no consensus regarding the advantages and disadvantages of vibration therapy as a single treatment for osteoporosis.

In this study, we examined whether MV could have a positive effect subsequent to the administration of a potent anabolic drug as iPTH on osteopenic bone, for this reason, it was investigate the PTH30d/MV30d group. Meanwhile, we noted that only 1 mo of treatment with 5mg/kg/days of iPTH was not enough to reverse 5 mo of cancellous bone loss, and therefore MV had a reduced effect in this model. It is possible that if iPTH administration had been extended for a longer period of time before applying MV, we might have obtained better results favoring bone formation. In support of this notion, the PTH30d/MV30d group did demonstrate an increase in BMD and improved the force at failure.

Considering a relation between cortical thickness and an improvement in biomechanical parameters *(23)*, we noted that although the Ct.Th of PTH30d/MV30d was not statistically significant, this group showed an increasing trend for this parameter ($p = 0.09$ vs Sham). Moreover, additional important factors, such as organic bone matrix components, can also affect bone strength *(36)*, which we will investigate in future studies.

Although there are few studies which have examined the effects of MV in combination with higher doses of iPTH, the current study, to the best of our knowledge, is the first to investigate the association of MV with a low dose of iPTH in mature rats with intense cancellous bone loss in an estrogen deprivation model.

In conclusion, our results showed that whole-body low-intensity mechanical vibration therapy potentiated the anabolic effect of iPTH in cancellous bone, increasing bone volume. Vibration alone, however, was unable to prevent bone loss following the interruption of iPTH in estrogen-deficient rats with severe impairment of bone structure.

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Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jocd.2018.09.003.

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