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Evaluation of modal damping factor as a diagnostic tool for osteoporosis and its relation with serum osteocalcin and collagen I *N*-telopeptide for monitoring the efficacy of alendronate in ovariectomized rats

G.E. Christopoulou [a], A. Stavropoulou [a], G. Anastassopoulos [b], S.D. Panteliou [b], E. Papadaki [a], N.K. Karamanos [c], E. Panagiotopoulos [d,*]

[a] *School of Medicine, Laboratory of Anatomy, University of Patras, Patras, Greece*
[b] *Department of Mechanical Engineering and Aeronautics, University of Patras, Patras, Greece*
[c] *Department of Chemistry, Laboratory of Biochemistry, University of Patras, Patras, Greece*
[d] *School of Medicine, Department of Orthopedics, Patras Univesity Hospital, University of Patras, Rio, 26500 Patras, Greece*

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Abstract

Osteoporosis is a metabolic bone disease characterized by reduced bone mass and deterioration of bone microarchitecture. It results from the shift of the osteoblast–osteoclast activity equilibrium in favor of the later. Although, a number of biochemical markers, such as collagen I *N*-telopeptide (NTx) and osteocalcin (OC), have been used for monitoring bone remodeling, a new, monitoring, non-invasive method, which is based on the measurement of the dynamic characteristic of bone and is known as modal damping factor (MDF), has not been evaluated as a diagnostic tool for osteoporosis. Bisphosphonates, such as alendronate, have an established role in the treatment of osteoporosis. The aim of the present study was, therefore, to evaluate the effects of alendronate on the levels of MDF, serum NTx and OC on osteoporosis induced by ovariectomy in rats. Furthermore, the effects of alendronate on osteoporosis have been histologically evaluated. Fifteen adult female Wistar rats were bilaterally ovariectomized and osteoporosis was histologically confirmed and by the use of peripheral quantitative computerized tomography (pQCT). MDF was applied to assess the bone structural integrity.

The serum levels of NTx (37.4 ± 0.5 nM bone collagen equivalents, BCE) and OC (111.0 ± 8.2 ng/mL) were found to significantly increase following ovariectomy (72.0 ± 2.9 nM BCE and 213.5 ± 12.1 ng/mL, respectively, $p < 0.001$). As assessed by histology and the levels of NTx and OC in sera, animals treated with alendronate presented a statistically significant deceleration in the progression of the disease in comparison to the no-therapy control group (alendronate group NTx levels: 146.3 ± 8.9 nM BCE versus no-therapy control group NTx levels: 265.3 ± 14.0 nM BCE, $p < 0.001$, alendronate group OC levels: 205.6 ± 18.2 ng/mL versus no-therapy group OC levels: 353.9 ± 26.1 ng/mL, $p < 0.001$). Data obtained from the vibration analysis performed illustrated that the change in damping was equal or greater to the change in total and trabecular density, respectively. Damping increased with decreasing bone density, as expected, given that damping accounts for the structural integrity of bone (MDF value before ovariectomy: 0.058 ± 0.003 versus MDF value after ovariectomy: 0.098 ± 0.003, $p < 0.001$). The higher damping values correspond to more deteriorated structures. In particular, both total and trabecular density were significantly decreased following ovariectomy (total density before ovariectomy: 702.4 ± 19.0 versus total density after ovariectomy: 542.2 ± 12.8, $p < 0.001$, trabecular density before ovariectomy: 445.3 ± 13.0 versus trabecular density after ovariectomy: 396.7 ± 8.4, $p < 0.05$). MDF value of the alendronate group (0.07 ± 0.002) was significantly lower ($p < 0.001$) as compared to MDF value after ovariectomy (0.098 ± 0.003) and that of the no-therapy group (0.1 ± 0.004, $p < 0.001$). The administration of alendronate seemed to have no effect on either total or trabecular density, since both parameters continued to decrease (alendronate group total density: 549.4 ± 12.3, alendronate group trabecular density: 368.4 ± 14.7). However, when this was compared to the no-therapy group, a statistically significant difference of total density at the 0.05 level was observed (no-therapy total density: 464.8 ± 9.1).

The results of this study suggest that combined measurements of MDF, NTx and OC may be a potential diagnostic tool for osteoporosis and monitoring bone integrity during treatment with bisphosphonates. Furthermore, administration of alendronate showed to offer a critical deceleration in the progression of osteoporosis.

Keywords: Osteoporosis; Collagen I *N*-telopeptide; Osteocalcin; Modal damping factor; Alendronate

* Corresponding author. Tel.: +30 2610 999551; fax: +30 2610 994579.
E-mail address: ecpanagi@med.upatras.gr (E. Panagiotopoulos).

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1. Introduction

Osteoporosis is the leading cause of serious morbidity and functional loss in the elderly [1]. It is a bone disease characterized by reduced bone mass and deterioration of bone microarchitecture [2,3].

Bone is a dynamic tissue, undergoing a continuous process known as bone remodeling. This is a coupled action of osteoclasts and osteoblasts [1,4], the former being the cells responsible for bone resorption and the latter for bone formation. In osteoporosis, this equilibrium of osteoclastic–osteoblastic activity is compromised in favor of osteoclasts. Since osteoporosis is mostly caused by accelerated bone resorption by osteoclasts, most therapeutic agents available today are anti-resorptives, acting to inhibit osteoclast development, maturation and/or function. Anti-resorptive drugs that are approved osteoporosis treatment include bisphosphonates. These are analogs of pyrophosphate (P–O–P) in which the bridging oxygen has been replaced by carbon [5]. This structure protects bisphosphonates from enzymic cleavage and facilitates strong binding to bone due to high affinity to calcium phosphate [6–8]. Alendronate (ALD) is a nitrogen-containing, second generation bisphosphonate. At the cellular level, ALD shows preferential localization to sites of bone resorption, specifically under osteoclasts. This leads to intracellular uptake of ALD by osteoclasts during resorption, which results, eventually, to disruption of osteoclast cytoskeleton, loss of ruffled border, inhibition of lysosomal enzymes, loss of resorptive activity and, finally, apoptosis of osteoclasts [9].

Markers of bone metabolism are, nowadays, used as auxiliary prognostic and diagnostic means for osteoporosis. Biochemical markers of bone turnover are products released from osteoblasts and osteoclasts or collagen breakdown products [10] and can be used as molecular tools both for the detection of high bone turnover, as well as for monitoring the efficacy of the administered drug [11]. Among the various biochemical markers, bone resorption markers are considered to provide stronger evidence than bone formation markers [4,12] and, particularly, osteocalcin (OC) and collagen I *N*-telopeptides (NTx) appear to be the most reliable markers of bone metabolism [13].

Although, bone mineral density (BMD) is recognized as the most important single determinant of fracture risk in osteoporotic populations [14,15], modal damping factor (MDF), which is a new analytical, arithmetic and experimental method, is considered as equal or better predictor of bone strength than BMD [16,17]. This method measures material damping using sweeping sound excitation of bone in the acoustic range. The calculation of quality factor (Q) based on material damping can, in theory, be applied in clinical settings to estimate the biomechanical competence of bone, and thus, it may be used as a diagnostic tool in osteoporosis.

The aim of the present study was, therefore, to evaluate the non-invasive MDF methodology as a diagnostic tool for osteoporosis in relation to serum levels of NTx, which is a specific marker of bone collagen breakdown, and OC, which is an indicator of bone formation activity. Peripheral quantitative computed tomography (pQCT), NTx, OC and MDF were also used to evaluate the anti-resorptive response of ALD on a histologically confirmed osteoporotic model of ovariectomized rats.

2. Biologic material and methods

2.1. Experimental design

Fifteen adult female Wistar rats, weighing 230–250 g, were maintained at 22 ± 1 °C, 12-h light/12-h dark cycle, fed with standard diet and water ad libitum [18]. After a month of acclimatization, the animals were bilaterally ovariectomized according to the guidelines of the Animal Welfare Committee, University of Melbourne [19]. Atropine (DEMO S.A., Athens, Greece) was used as a premedication agent (0.05 mg/kg), and 15 min later, a mixture of (10 mg/kg) xylazine (Rompun, Bayer Hellas, Athens, Greece) and (75 mg/kg) ketamine (IMALGENE® 1000, MERIAL Inc., NY, USA) were administered, intramuscularly, as anaesthetic agents.

The animals were allowed to develop osteoporosis for 60 days after the ovariectomy, and they were, then, randomly divided into two sub-populations ($n_1 = 10$, $n_2 = 4$ due to death of one test animal), randomized as two cases to one control. Sub-population 1 (test population) was administered alendronate (4 mg/kg/day) in 5% Arabic Gum and sub-population 2 was the no-therapy group (control population). The medication was given 5 days/week, from day 60 up to day 145. Drug administration was oral by gavage (University of Iowa Animal Care Unit).

Blood withdrawals were performed on all animals, under the surgical anaesthesia mentioned above, by tail vein venipuncture (University of Iowa Animal Care Unit), before the ovariectomy (day 0), after the ovariectomy (day 60), to verify the development of osteoporosis, as well as, at the end of therapy (day 145), in order to measure the levels of biochemical markers. Accordingly, pQCT and MDF were applied at the same time points. At the end of therapy, the animals were euthanized and bone tissues were collected and maintained at −80 °C for histological studies.

2.2. Biochemical markers

In order to determine the levels of NTx and OC in serum, blood was collected at days 0, 60 and 145, centrifuged at 3000 rpm for 10 min at 20 °C and was stored at −20 °C until further use.

A competitive-inhibition enzyme-linked immunosorbent assay (ELISA/EIA) was performed for the estimation of NTx in rat serum (Osteomark® NTx Serum, Ostex International Inc., Seattle, USA). The absorbance was determined with a microwell plate reader at 450 nm (reference filter 630 nm). Osteomark NTx serum values were expressed in nanomoles bone collagen equivalents per liter (nM BCE). The concentrations of the calibrator solutions (purified NTx) for plotting the standard calibration curve was 0, 5, 10, 20 and 40 nM BCE. The assay values of NTx controls were: level I control = 7.1 nM BCE and level II control = 30.3 nM BCE.

A rat-specific enzyme-linked immunosorbent assay was also used for the quantitative determination of osteocalcin in serum

samples (Rat–MID™ Osteocalcin ELISA, The Nordisc Bioscience Diagnostics A/S, Denmark). This assay is based upon the competitive binding of soluble osteocalcin in serum to a monoclonal antibody raised against human osteocalcin, which recognizes the mid molecular part (amino acids 21–29) of the molecule. Synthetic human osteocalcin is used for standardization. Parallelism is observed with purified rat osteocalcin and rat serum. All required reagents were included in the kit and the experimental procedure was performed according to the instructions of the manual. The absorbance was measured at 450 nm with a 650 nm filter as reference. The levels of osteocalcin in serum samples were expressed in nanograms per milliliter (ng/mL), based on the plotting of a fourth degree polynomial fit calibration curve, using the standards concentration samples given by the kit (0, 66.8, 132.6, 273.1, 529.4 and 901.4 ng/mL). The assay value of osteocalcin control was 183.0 ng/mL.

2.3. Bone densitometry–pQCT

In order to validate the osteoporotic state of animals, pQCT measurements were carried out before ovariectomy (day 0), after ovariectomy (day 60) and at the end of the administration of alendronate (day 145).

The bone mineral density was measured by a pQCT XCT 960 X-ray bone densitometer (Stratec), using software Version 5.2 Research multiple measurements Mask. Each rat was positioned in a special purpose device for placing its tibia in the centre of the tomographic field of view (FOV) and three cross-sectional scans (1 distally and 2 proximally) were performed. The two proximal scans were located 4 and 5 mm apart from the articulate surface of the knee where trabecular bone structure is the dominant volume, whereas the distal scan was located 15 mm apart from the above reference line, where cortical shell and density can accurately be assessed. Analyses of these scans produced measurements of volumetric total density (vTotBMD), total area (CSAtot), trabecular density (vTrabBMD), trabecular area (CSAtrab), subcortical density and area (vSubcrtBMD, CSAsubcrt, respectively), volumetric cortical density (vCtBMD), cortical area (CSAcrt), periosteal and endosteal perimeter (peri, endo), cortical shell thickness (THKcrt), cross-sectional moments of inertia about anatomical axes (I_x, I_y), maximum and minimum moments of inertia (I_{max}, I_{min}), polar moment of inertia (I_p) and stress/strain index in different axes (SSI_x, SSI_y, SSI_p). Relatively high I_y values compared to I_x, imply relatively greater resistance to bending in the medial–lateral direction than in the anterior–posterior direction. For the analysis procedure the contour mode was set at 1 and the peel mode was set at 3. The values for the two proximal scans were averaged. Coefficient of variation (%CV) obtained for vCtBMD and CSA of the same tibia in vivo, after repositioning the rat three times, was found to be 1 and 1.2%, respectively.

2.4. Determination of MDF

Natural frequency of any structural member is defined as the frequency at which the member vibrates if displaced from equilibrium. Usually several frequencies coexist and signal analysis techniques are available for the simultaneous measurement of these frequencies. Material damping factor γ is defined as the energy dissipated throughout the medium in one cycle of deformation, normalized with respect to the elastic energy stored during that cycle, representing the fraction of strain energy loss in one full cycle [20]. The vibration modal damping factor ζ, which defines MDF, and quality factor (Q) are defined as $\zeta = \sqrt{(\gamma^2/(4+\zeta^2))}$, $Q = 1/(2\zeta)$ [20,21].

The modal damping factor was obtained experimentally in vivo by applying the half-power bandwidth method [21]. Each rat tibia was set to free vibration from an initial displaced position and the signal of the accelerometer was then amplified and relayed to an analog-to-digital converter. The damping factor was calculated from the fast Fourier transform (FFT) analysis of time response data. The damping factor is proportional to the energy absorbed per cycle of vibration.

MDF measurements were carried out before ovariectomy (day 0), after ovariectomy (day 60), in order to verify the osteoporotic state of animals, and at the end of the administration of alendronate (day 145).

2.5. Histological studies

Tibia tissues of the alendronate-treated animals and no-therapy controls were isolated for histological studies. The tibiae were fixed in neutral formaline for 12 h, decalcified in 11% hydrochloric acid (TBD-1, Thermoshandon, Pittsburgh, PA, USA) for another 12 h, and dehydrated in ascending alcohol solutions (70, 80, 96 and 100%, 2 h each). Then, the tissues were embedded in paraffin (O/N) and 3 μm sections of each specimen were taken. The paraffin-embedded sections were mounted onto slides and deparaffinized (xylene, 56 °C) when further used. Finally, the sections were stained in heamatoxylin-eosin staining and observed under light microscopy.

2.6. Statistical analysis

All data are presented as means ± S.E. Statistical analysis was performed by *t*-test. Values lower than 0.05 considered to indicate statistically significant differences. All calculations were carried out using the Origin 7.0 statistical software package.

3. Results

As shown in Fig. 1A and B, following ovariectomy serum levels of NTx and OC presented statistically significant increases (NTx serum levels before ovariectomy: 37.4 ± 0.5 nM BCE versus NTx serum levels after ovariectomy: 72.0 ± 2.9 nM BCE, $p < 0.001$, OC serum levels before ovariectomy: 111.0 ± 8.2 ng/mL versus OC serum levels after ovariectomy: 213.5 ± 12.1 ng/mL, $p < 0.001$). Histological examination of the tibia tissues of the ovariectomized rats (Fig. 2A) showed that the trabecular network was severely damaged. The network had lost its continuality and the trabeculae were broken, presenting the "button" phenomenon. Additionally, the bone marrow

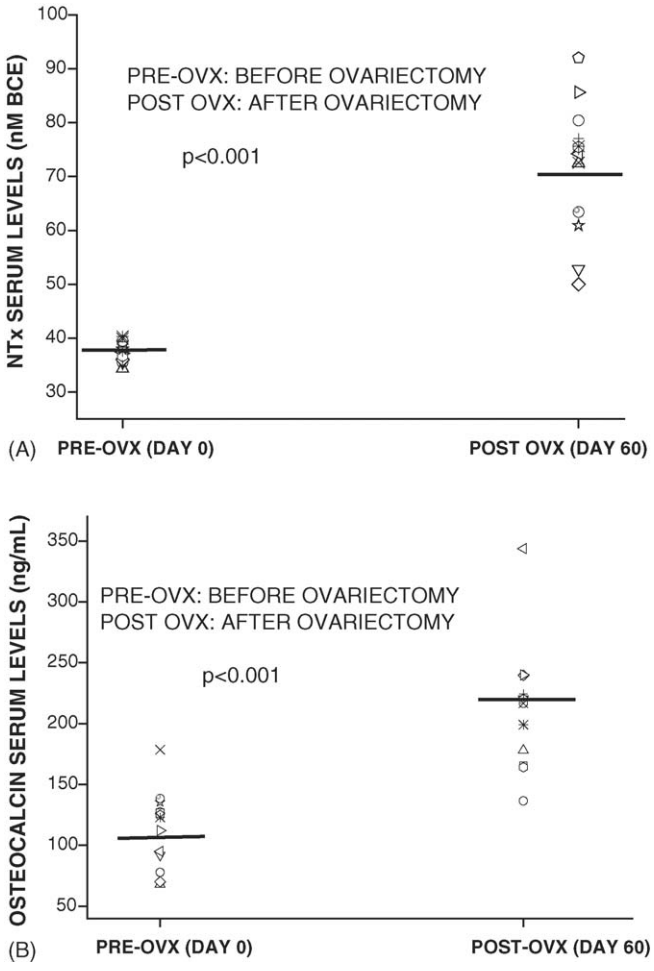

Fig. 1. NTx (A) and OC (B) levels of each individual test animal before and after the ovariectomy (ovx). Horizontal bars show the mean values in each group.

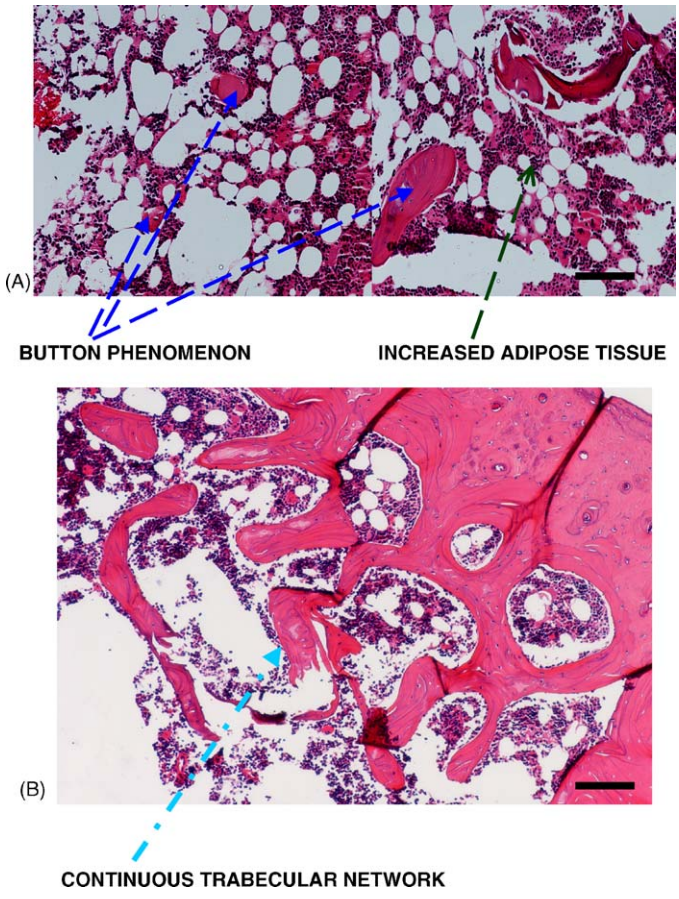

Fig. 2. Paraffin-embedded sections (3 mm) of decalcified tibiae of no-therapy control test animals (A) (▬: M × 20) and decalcified tibiae of alendronate-treated test animals (B) (▬: M × 10).

was hypocellular, with increased amounts of adipose tissue. Taking into account the obtained histological pattern, as well as, the values for both biochemical markers, the induction of osteoporosis within 60 days after ovariectomy, in the model used in this study, was illustrated.

In order to evaluate the dynamic characteristics of bone, MDF was determined by vibration analysis and total and trabecular densities by pQCT methodology. Data obtained from the vibration analysis performed illustrated that the change in damping was equal or greater to the change in total and trabecular density, respectively (Fig. 3A and B and Table 1). Damping increased with decreasing bone density, as expected, given that damping accounts for the structural integrity of bone (MDF value before ovariectomy: 0.058 ± 0.003 versus MDF value after ovariectomy: 0.098 ± 0.003, $p < 0.001$). The higher damping values correspond to more deteriorated structures. Furthermore, both pQCT and MDF measurements followed the changes in bone metabolism and were in accordance to the measurements of bone biochemical markers, NTx and OC. In particular, as shown in Table 1, both total and trabecular density were significantly decreased following ovariectomy (total density before ovariectomy: 702.4 ± 19.0 versus total density after ovariectomy: 542.2 ± 12.8, $p < 0.001$, trabecular density before ovariectomy: 445.3 ± 13.0 versus trabecular density after ovariectomy: 396.7 ± 8.4, $p < 0.05$).

In order to examine the effect of alendronate, ovariectomized rats were divided in two groups: one received alendronate (4 mg/kg/day) and the other was the control population (no-therapy control). The administration of alendronate to the test population seemed to have no apparent effect, since NTx serum levels continued to escalate (Fig. 4A, NTx before ALD treatment: 72.0 ± 2.9 nM BCE versus NTx after ALD treatment: 146.3 ± 8.9 nM BCE, $p_1 < 0.001$). However, when the test population was compared to the control population (no-therapy control), in terms of NTx serum levels, there was a statistically significant difference between them (Fig. 4A, no-therapy population NTx serum levels: 265.3 ± 14.0 nM BCE, $p_2 < 0.001$). OC serum levels were not affected by the administration of alendronate in the test population (Fig. 4B, OC serum levels before therapy: 213.5 ± 12.1 ng/mL versus OC serum levels after therapy: 205.6 ± 18.2 ng/mL, $p_1 > 0.05$). On the other hand, the control population showed a dramatic increase of OC serum levels, which was statistically significant as compared to the test population (Fig. 4B, control population OC serum levels: 353.9 ± 26.1 ng/mL, $p_2 < 0.001$).

Histological examination of the tibia sections of the alendronate-treated test population (Fig. 2B) presented the

Table 1
Densitometric results obtained from pQCT and MDF methodology

Groups	Total density (mg/cm^3)	Trabecular density (mg/cm^3)	MDF
Before ovariectomy (day 0, $n=15$)	702.4 ± 19.0	445.3 ± 13.0	0.058 ± 0.003
After ovariectomy (day 60, $n=15$)	542.2 ± 12.8*	396.7 ± 8.4**	0.098 ± 0.003*
Alendronate-treated group (day 145, $n=10$)	549.4 ± 12.3§§	368.4 ± 14.7§§	0.070 ± 0.002†,§
No-therapy control (day 145, $n=4$)	464.8 ± 9.1	341.9 ± 20.7	0.100 ± 0.004

Note: Data are given as mean values ± S.E.
* Show the statistically significant differences between the group after ovariectomy and that before ovariectomy at the levels of $p<0.001$.
** Show the statistically significant differences between the group after ovariectomy and that before ovariectomy at the levels of $p<0.05$.
† Show the statistically significant differences ($p<0.001$) between the group after ovariectomy and the alendronate-treated one.
§ Show the statistically significant differences between the alendronate-treated group and the no-therapy control group at the levels of $p<0.05$.
§§ Show the statistically significant differences between the alendronate-treated group and the no-therapy control group at the levels of $p<0.001$.

development of a continuous trabecular network, with intact trabeculae. Furthermore, the bone marrow was characterized as of normal cellularity.

As shown in Table 1, the MDF value of the alendronate group (0.07 ± 0.002) was significantly lower ($p<0.001$) as compared to MDF value after ovariectomy (0.098 ± 0.003) and that of the no-therapy group (0.1 ± 0.004, $p<0.001$). The administration of alendronate seemed to have no effect on either total or trabecular density, since both parameters continued to decrease (alendronate group total density: 549.4 ± 12.3, alendronate group trabecular density: 368.4 ± 14.7). However, when this was compared to the no-therapy group, a statistically significant difference of total density at the 0.05 level was observed (no-therapy total density: 464.8 ± 9.1).

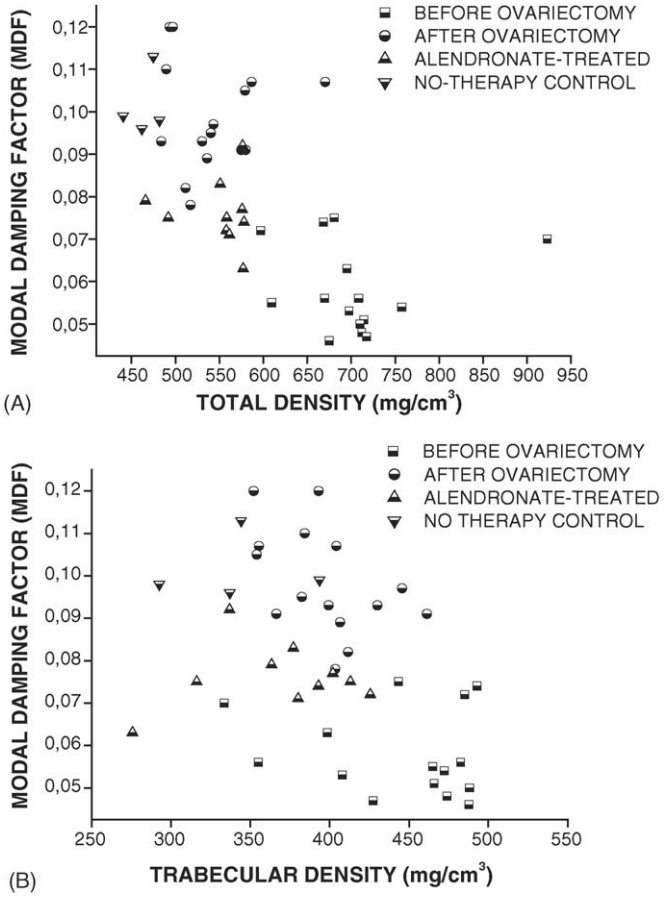

Fig. 3. Variations of modal damping factor (MDF) vs. total density measurements (A) and MDF vs. trabecular density (B), before and after ovariectomy and after administration of alendronate.

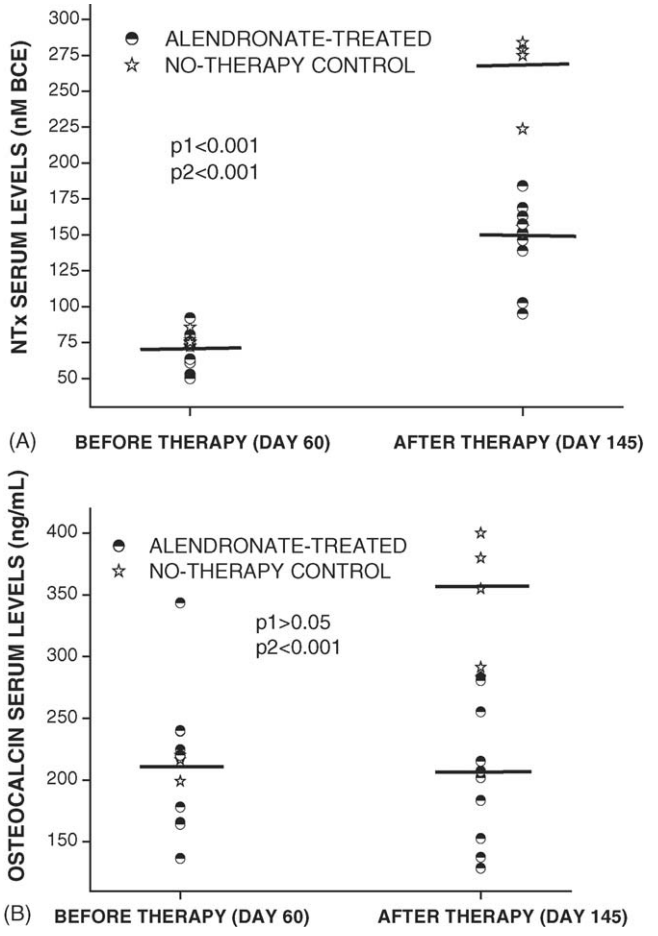

Fig. 4. NTx (A) and OC (B) levels before and after the administration of alendronate in the osteoporotic animals. Ovariectomized rats were divided in two random groups. One population ($n_1=10$) received alendronate and the other ($n_2=4$) was the no-therapy control group (p_1, statistical difference between the population before ALD treatment and the population after ALD treatment; p_2, statistical difference between the population after ALD treatment and the no-therapy population).

4. Discussion

The aim of the present study was to evaluate the potential of MDF as a diagnostic tool for osteoporosis and to examine the effects of alendronate on MDF and the bone biochemical markers NTx and osteocalcin.

Ovariectomy-induced osteoporosis in rats mimics the postmenopausal osteoporosis in women, caused by estrogen depletion. In this study, ovariectomy-induced osteoporosis was confirmed by histological examination. NTx and osteocalcin responded to ovariectomy-induced osteoporosis since their serum levels were significantly increased. These findings are in agreement with previous suggestions [9] that NTx increases because collagen I is broken down, and osteocalcin is released in the blood circulation, since no new bone is formed. Furthermore, the obtained data indicate that osteocalcin and NTx are very sensitive markers for monitoring bone metabolism.

Although, ALD administration appeared to have no significant effect on the serum levels of both NTx and osteocalcin, the values of NTx and OC in the control population (no-therapy group) were significantly higher than the ALD-treated group values. The histological pattern obtained following ALD treatment suggests that alendronate inhibited osteoclast resorption, allowing, thus, osteoblasts to produce new bone. Taking into account the histological observations it can be concluded that NTx and OC levels in serum can be used to evaluate the blockage in the progression of osteoporosis upon alendronate treatment. Furthermore, it is plausible to suggest that the administration of ALD, at the specific dose used and for the particular duration of treatment, results in a critical deceleration in the progression of osteoporosis.

With regard to MDF application, it should be clarified that MDF methodology, used as a bone-condition-monitoring tool, does not give information for the distribution of porosity throughout the bone. It consists, though, an accurate and objective physical property, which accounts for changes in the content of the inclusions of bone structure, as well as, for changes in the porosity value. Determination of the damping factor at one of the modes of vibration results in a weighed average of the material damping, the weight function being the corresponding natural vibration mode. Different modes can give information for the concentration of porosity at different places throughout the bone, but such attempt was not made in this study. Changes in damping factor, due to loss of mineral in the bone, are much more predominant in the trabecular than in the cortical bone or the surrounding soft tissue. However, the correlation found with the conventional density measurements of pQCT, without constituting a proof, is, nevertheless, in support of the assumptions made.

In particular, MDF values significantly elevated upon ovariectomy, following the changes in total and trabecular densities, which both presented a statistically significant descent. Furthermore, this significant increase of MDF values is in accordance to the histologically confirmed osteoporotic test animal population. Additionally, MDF values significantly declined following ALD treatment, presenting a higher sensitivity than total and trabecular densities in detecting the changes in bone remodeling upon therapy. However, it seems that both pQCT and MDF measurements followed the changes in bone metabolism and are in agreement with the serum measurements of bone biochemical markers, NTx and OC.

The animal studies conducted in this project showed that MDF is directly related with stress concentration due to discontinuities in the bone material, such as the change in porosity accompanying osteoporosis. In turn, stress concentration is known to cause fracture in materials with such discontinuities. Thus, MDF is shown to be the most accurate and objective indicator and measure of the tendency of the osteoporotic bone to fracture [22,23]. The fact that the damping measurements have been proved much earlier sensitive during the progress of the change in structure of the bones may render damping a tool for diagnosis of changes in bone architecture much earlier than the conventional method responds. This methodology might be further extended to study the effects of bone morphology on its dynamic behavior and resistance to fracture.

Concluding, evaluation of MDF showed that it might be a potentially valuable assessment tool for monitoring bone integrity and osteoporosis, whereas administration of alendronate resulted in a critical deceleration in the progression of osteoporosis.

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