CLIMACTERIC 2014;17:478–485

Sequential treatment with monofluorophosphate and zoledronic acid in osteoporotic rats

M. L. Brance[*,†], *L. R. Brun*[*], *V. E. Di Loreto*[*], *M. Lupo*[*,†] *and A. Rigalli*[*,†,‡]

[*]Bone Biology Laboratory, School of Medicine, Rosario National University, Rosario; [†]National Council of Scientific and Technical Research (CONICET); [‡]Rosario National University Research Council, Argentina

Key words: MONOFLUOROPHOSPHATE, ZOLEDRONIC ACID, OSTEOPOROSIS, BONE MASS, BONE STRENGTH

ABSTRACT

Objective Osteoporosis is the consequence of an imbalance in bone remodeling caused by excessive resorption or inappropriate bone formation. This paper proposes a sequential treatment with monofluorophosphate (MFP) and zoledronic acid (Z), together with changes in the calcium content in the diet.

Method Seven-week-old female Sprague Dawley rats were divided into five groups ($n = 21$ per group): (1) sham-operated rats (Sham); (2) ovariectomized (OVX) rats fed with a normal calcium diet (OVX); (3) OVX rats fed with a normal calcium diet and treated sequentially with monofluorophosphate and zoledronic acid (OVX.G1); (4) OVX rats sequentially fed with a low calcium diet and then a high calcium diet, without treatment (OVX.G2); (5): OVX rats fed with a low calcium diet and then a high calcium diet, treated sequentially with monofluorophosphate and zoledronic acid (OVX.G3).

Results After 150 days, the OVX.G3 group showed a similar bone volume to that of the Sham group due to an increase in trabecular number. Dual X-ray absorptiometry bone analysis showed an increase of 9.8% compared with OVX rats. Additionally, an increase in the fracture load at the cortical bone and higher fracture load, ultimate load and stiffness in the compression test were found.

Conclusion The sequential treatment with monofluorophosphate and zoledronic acid increases trabecular bone mass, bone mineral density and bone strength.

INTRODUCTION

Osteoporosis is a serious public health problem with multiple mechanisms involved in its pathogenesis. It is characterized by low bone mass and microarchitectural deterioration, resulting in an increased risk for fractures.

Fluoride is a bone cell mitogen that increases bone mass[1]. Its mechanism of action is based on its inhibitory activity on osteoblastic tyrosine acid phosphatase[2]. It has been demonstrated that fluoride only acts on precursors of osteoblasts if growth factors are present[2]. The drugs most commonly used are sodium fluoride[3] and sodium monofluorophosphate (MFP)[4-6]. In patients, sodium fluoride has been shown to increase spine and hip bone mineral density (BMD) but concerns have been raised regarding the strength and quality of the new bone formed[7]. MFP increases lumbar spine BMD[8–10] and even low doses of MFP reduce the incidence of vertebral fractures[11]. On the other hand, aminobisphosphonates are antiresorptive drugs that inhibit the bone-resorptive activity of osteoclasts by selective inhibition of the farnesyl pyrophosphate synthase enzyme[12] and also act positively on the viability of osteoblasts and osteocytes[13].

There are previous studies that used drug combinations for osteoporosis treatment. It has been demonstrated that the combination of a bisphosphonate with estrogen or raloxifene increases BMD compared with bisphosphonate alone[14,15]. The use of alendronate in combination with parathormone (PTH) in postmenopausal women may reduce the anabolic effects of PTH[16]. However, the combination of PTH followed

Correspondence: Dr M. L. Brance, Cátedra de Química Biológica, Facultad de Ciencias Médicas, Santa Fe 3100 (2000) Rosario, Argentina; E-mail: lorenabrance@gmail.com

ORIGINAL ARTICLE

DOI: 10.3109/13697137.2013.860117

Received 20-08-2013
Revised 22-10-2013
Accepted 24-10-2013

by alendronate significantly increases the BMD at the lumbar spine[17]. In postmenopausal women, it was demonstrated that the combined therapy of raloxifene plus MFP showed better results compared to MFP alone[18].

Most published works have used only one drug (antiresorptive or anabolic) rather than a combination of both. Here, we propose not only a sequential treatment with MFP as anabolic bone therapy and zoledronic acid as antiresorptive treatment, which has proved to be effective in the treatment of osteoporosis in humans and animals[19,20], but also changes in the calcium content of the diet in an attempt to act on bone remodeling in order to increase bone mass. The low-calcium diet was used to cause an increase in bone resorption to stimulate the release of cytokines that increase osteoblast proliferation under MFP treatment. The change to a high-calcium diet reduced osteoclast activity and provided appropriate calcium concentration during the treatment with MFP. Finally, zoledronic acid was used to maintain low levels of bone resorption.

Table 1 Experimental groups

	Sham	*OVX*	*OVX.G1*	*OVX.G2*	*OVX.G3*
Ovariectomy	−	+	+	+	+
Low-calcium diet (0.2%) from day 0 to day 30	−	−	−	+	+
High-calcium diet (2%) from day 31 to day 150	−	−	−	+	+
MFP (400 μmol/kg. day) from day 31 to day 90	−	−	+	−	+
Z (1.5 μg/kg. month) from day 91 to day 150	−	−	+	−	+

+ indicates the treatment applied
Sham, sham-operated rats; MFP, sodium monofluorophosphate; Z, zoledronic acid

MATERIALS AND METHODS

Animals and study design

Seven-week-old female Sprague-Dawley rats were divided into five groups ($n = 21$ per group) and were pre-anesthetized with a mixture of xylazine (12 mg/kg body weight, Alfasan, Woerden, Holland) and ketamine (30 mg/kg body weight, Holiday, Scott SA, Buenos Aires, Argentina) administered subcutaneously. After reaching an appropriate level of narcosis and sedation, 60 mg/kg of lidocaine hydrochloride (Indican, Sidus SA, Buenos Aires, Argentina) was administered subcutaneously in the right leg and 5 min later 30 mg ketamine/kg body weight was injected intramuscularly in the same site. Diclofenac subcutaneously (2.5 mg/100 g of body weight) was used as analgesic[21]. Bilateral ovariectomy was performed as an osteoporosis experimental model in four groups of rats[22,23]. The fifth group of rats was subjected to simulated surgery (Sham). Rats were housed in a room with alternate 12-h periods of light and dark, at a constant temperature of 24 ± 1°C. All experiments were carried out according to the international rules for animal care[24] and the project was approved by the Bioethics Committee of the School of Medicine of the National University of Rosario, Argentina.

Following ovariectomy, animals were randomized to be fed with a normal calcium diet (NCaD: calcium 1%, phosphorus 0.5%), or a sequential diet with a low-calcium diet (LCaD: calcium 0.2%, phosphorus 0.2%) from day 0 to day 30 followed by a high-calcium diet (HCaD: calcium 2%, phosphorus: 0.5%) from day 31 to day 150 and tap water *ad libitum*. The LCaD with low phosphorus content was administered to cause an increase in bone resorption without an increase in PTH levels. The five experimental groups are shown in Table 1.

It is important to point out that, since the OVX and OVX.G1 groups received NCaD until day 30, in the Results they are shown as only one group, called OVX_{30}.NCaD ($n = 14$). Similarly, since the OVX.G2 and OVX.G3 groups received LCaD until day 30, they are shown as one group (OVX_{30}.LCaD) ($n = 14$). Then, both groups were divided into two: with and without pharmacological treatment.

The experiment lasted for 150 days and seven rats in each group were euthanized at intervals of 30, 90 and 150 days. Previous to euthanasia, animals were placed in individual metabolic cages for 24 h. Then, 24-h urine samples were collected and blood samples were obtained from the tail vein. Rats were euthanized by intracardiac injection of saturated KCl under deep volatile anesthesia and analgesia. The uteri were obtained and wet weights were determined to verify the success of the surgery. Both tibias and femurs were excised for bone analysis. The proximal epiphysis of the right tibia was processed for histological analysis. BMD was measured in the left tibia and biomechanical tests were performed in the femurs.

Biochemical determinations

Plasma calcium levels (mg/dl) were spectrophotometrically measured with a commercial kit (Ca-Color, Wiener Lab, Argentina) on Perkin Elmer Lambda 11 (Perkin Elmer Corporation Norwalk, CT, USA). Urinary (mg/24-h) and plasma phosphate levels (mg/dl) were spectrophotometrically measured with a commercial kit (Fosfatemia UV AA, Wiener Lab, Argentina). Total fluoride was measured by direct potentiometry with an ion selective electrode (Orion 94-09, MA, USA) after isothermal distillation[25]. Total plasma alkaline phosphatase (AP) activity (U/l) was spectrophotometrically measured with a kinetic method (ALP 405, Wiener Lab, Argentina). Bone alkaline phosphatase (bAP) activity (U/l) was estimated as the difference between total and remnant AP

activity after germ wheat lectin treatment[26]. Calciuria was measured by atomic absorption spectroscopy (Arolab MK II, Buenos Aires, Argentina). Calciuria and 24-h urinary volume were used to calculate 24-h urinary calcium excretion (mg/24-h). Urinary deoxypyridinoline (Dpd, nmol/mmol creatininuria) was measured by radioimmunoassay (DPD RIA, Immunodiagnostic Systems, Boldon, UK) in a solid scintillator (Alfanuclear-Cmos, Buenos Aires, Argentina). 24-h creatininuria was spectrophotometrically measured with a commercial kit (Creatinina cinética AA líquida, Wiener Lab, Argentina). Serum PTH (pg/ml) was measured with an immunoradiometric assay kit for rat intact PTH (Immunotopics, Inc. San Clemente CA, USA).

Bone histomorphometry

Right proximal tibias were fixed in 10% phosphate buffered formaldehyde and decalcified in 10% EDTA before being embedded in paraffin. Five-µm longitudinal sections were stained with hematoxylin & eosin and permanent slides were examined using a light microscope (Leitz, Wetzlar, Germany). Digital images were obtained at a $4\times$ magnification (Olympus SP-350, China) of the proximal epiphysis of the tibia. A 2-mm^2 area – considered as total area (TA) – at 1 mm from the growth plate–metaphyseal junction was selected. As described by Parfitt and colleagues[27], the following measurements were performed (ImageJ 1.40, NIH, Maryland, USA): (1) total tissue volume, TV (µm^2); (2) trabecular bone area, BV (µm^2); and (3) trabecular bone surface, BS (µm). With these values, the following variables were calculated: (1) bone volume, BV/TV (%) = [BV*100/TV]; (2) trabecular thickness, Tb.Th (µm) = [2/(BS/BV)]; (3) trabecular number, Tb.N (1/mm) = [(BV/TV)/(Tb.Th)]; and (4) trabecular separation, Tb.Sp (µm) = [(1/Tb.N) − Tb.Th].

Four micrographs were randomly taken at a $40\times$ magnification from the selected area to measure: (1) bone surface covered by osteoblasts, Ob.S (µm); (2) eroded surface, ES (µm); and (3) osteoclast number, N.Oc. With these values, the following variables were calculated: (1) bone surface (BS) covered by osteoblasts, Ob.S/BS (%) = [Ob.S*100/BS]; (2) eroded surface, ES/BS (%) = [ES*100/BS]; and (3) osteoclast number, N.Oc/mm^2 (1/mm^2) = [N.Oc/TA].

Dual X-ray absorptiometry bone analysis

BMD (mg Ca^{2+}/cm^2) and bone mineral content (BMC, mg Ca^{2+}/g body weight) in the left tibia were measured by dual X-ray absorptiometry (DXA) at the end of the treatment for each experimental group (DPX Alpha 8034, Small Animal Softer, Lunar Radiation Corp., WI, USA)[28].

Mechanical testing

The cortical bone strength in the midshaft was determined in femurs by using a three-point bending test and the trabecular bone strength was determined by a compression test[29,30]. The two-bar distance for the three-point bending test was 11–12 mm. The compression test used a compression cone surface of 7.07 mm^2 on a 2.5-mm thick epiphyseal transversal section of the same bone. In both tests, the speed was 0.01 mm/s.

Statistical analysis

Data are expressed as mean ± standard error of the mean. Unpaired Student's *t*-test was used to compare the OVX group with the Sham group and one-way analysis of variance (ANOVA) and Neuman–Keuls *post-hoc* analysis to compare more than two groups. Differences were considered significant if $p < 0.05$. Statistical analyses were performed with GraphPad Prism 2.0 (GraphPad, San Diego, USA).

RESULTS

As expected, the weights of uteri were significantly lower in OVX rats (25.6 ± 2.7 mg/100 g body weight) than in the Sham group (171.3 ± 13.1 mg/100 g body weight) (unpaired Student's *t*-test, $p < 0.05$).

Biochemical determinations

As expected, on day 30 we found that the OVX_{30}.LCaD group showed a high bone resorption, confirmed by the data of Dpd (Table 2). bAP decreased in the OVX_{30}.LCaD group compared to the OVX_{30}.NCaD group. Although the differences in Dpd data were not statistically significant, the bAP/Dpd ratio was evaluated to estimate the states of BMU. The OVX_{30}.LCaD group showed a bAP/Dpd ratio 15 times lower than that of the $Sham_{30}$ and OVX_{30}.NCaD groups, ensuring an increase in the proportion of BMU in the resorption state. There were no differences in calcemia and calciuria. The phosphatemia in the OVX_{30}.LCaD group was lower than in the $Sham_{30}$ and OVX_{30}.NCaD groups.

Fluoremia at 90 days was lower in the $OVX.G3_{90}$ group (145.2 ± 35.80 µmol/l) than in the $OVX.G1_{90}$ group (216.3 ± 48.72 µmol/l) because of a high fluoride bone uptake consistent with high bone formation (see below).

At 150 days (Table 3), bAP and Dpd showed a slight decrease in the $OVX.G1_{150}$ and $OVX.G3_{150}$ groups with regard to the effect of the treatment, thus indicating a decrease in bone remodeling. The bAP/Dpd ratio in the $OVX.G3_{150}$ group was two-fold higher than in the $OVX.G1_{150}$ group. This result indicates an increase in the relationship between bone formation and bone resorbing cells in OVX.G3 animals compared with OVX.G1 animals. Calciuria was higher in the groups that received HCaD ($OVX.G2_{150}$ and $OVX.G3_{150}$) or pharmacological treatment alone ($OVX.G1_{150}$). There were no differences in calcemia, phosphatemia or PTH at day 150.

Table 2 Biochemical determinations at Day 30. The same superscript letters in each row indicate significant differences ($p < 0.05$)

	$Sham_{30}$	OVX_{30}.NCaD	OVX_{30}.LCaD
Calcemia (mg/dl)	9.75 ± 0.36	10.41 ± 0.56	10.57 ± 0.74
Phosphatemia (mg/dl)	8.52 ± 0.09^{a}	8.70 ± 1.18	6.58 ± 0.41^{a}
24-h urinary calcium excretion (mg/24-h)	0.30 ± 0.08	0.44 ± 0.09	0.52 ± 0.11
Dpd (nmol/mmol creatininuria)	339.6 ± 135.2	290.9 ± 57.7	689.2 ± 282.8
bAP (U/I)	93.96 ± 34.42^{a}	71.45 ± 24.43^{b}	$12.24 \pm 6.63^{a,b}$
bAP/Dpd ratio	0.28	0.24	0.017
PTH (pg/ml)	18.52 ± 4.05^{a}	26.84 ± 9.24^{b}	$10.58 \pm 1.56^{a,b}$

Dpd, Urinary deoxypyridinoline; bAP, bone alkaline phosphatase, PTH, parathormone

Bone histomorphometry

As expected, on day 30, a decrease in BV/TV was observed in the OVX groups (OVX_{30}.NCaD and OVX_{30}.LCaD) compared with the Sham group, due to a decrease in the trabecular number (Table 4). Consistent with biochemical markers, the histomorphometry analysis in OVX_{30}.LCaD animals revealed a high bone resorption state, with a statistical increase in the percentage of eroded surface (ES/BS) and osteoclast number. Although the OVX_{30}.LCaD group increased bone resorption, the BV/TV data indicate that the OVX_{30}.LCaD group did not produce higher bone loss compared to the OVX_{30}.NCaD group.

On day 90 (data not shown), BV/TV continued to decline in OVX_{90} and OVX.$G1_{90}$ animals compared with the Sham group, whereas it showed a small increase in the OVX.$G3_{90}$ group. This increase was due to a higher trabecular number without changes in trabecular thickness between the OVX groups.

After 150 days, BV/TV in the OVX.$G3_{150}$ group increased, reaching values similar to those of the Sham group due to an increase in trabecular number. This increase could be a consequence of a decrease in osteoclast number observed on days 90 (two-fold) and 150 (three-fold) compared with the OVX group (Table 5). The OVX.$G1_{150}$ and OVX.$G2_{150}$ animals showed no increase in BV/TV, indicating that the combination of sequential treatment with MFP/Z and different calcium intake has a favorable response on trabecular bone, and that the bone effect is not only the consequence of calcium content in the diet or the pharmacological treatment alone.

DXA bone analysis

The DXA analysis showed a decrease of 7.5% in the BMD of the OVX_{150} group compared with the $Sham_{150}$ group. The groups that received a pharmacological treatment displayed a statistical increase in BMD compared with the OVX_{150} group. BMD in OVX.$G1_{150}$ animals increased by 16.2% and in OVX.$G3_{150}$ animals by 9.8%.

BMC (mg Ca^{2+} in the tibia/g body weight) showed a similar result: in the OVX_{150} group BMC (0.90 ± 0.08) decreased compared with that in the Sham group (1.31 ± 0.06). The OVX.$G1_{150}$ animals (BMC = 1.28 ± 0.04) and OVX.$G3_{150}$ animals (BMC = 1.14 ± 0.08) showed an increase of 11% compared with the OVX_{150} group. The BMD and BMC of the OVX.$G2_{150}$ group were similar to those of the OVX_{150} group.

Mechanical testing

On day 30 (data not shown), a lower trabecular fracture load and ultimate load were observed in OVX_{30}.LCaD animals

Table 3 Biochemical determinations at Day 150. The same superscript letters in each row indicate significant differences ($p < 0.05$)

	OVX_{150}	OVX.$G1_{150}$	OVX.$G2_{150}$	OVX.$G3_{150}$
Calcemia (mg/dl)	9.62 ± 0.50	10.32 ± 0.35	9.52 ± 0.32	10.34 ± 0.53
Phosphatemia (mg/dl)	5.69 ± 1.04	5.84 ± 0.94	8.23 ± 0.48	7.42 ± 0.53
24-h urinary calcium excretion (mg/24-h)	0.59 ± 0.14^{a}	1.35 ± 0.29	1.81 ± 0.36	1.61 ± 0.26^{a}
Dpd (nmol/mmol creatininuria)	97.44 ± 34.34	40.34 ± 4.06	52.23 ± 4.01	34.00 ± 4.23
bAP (U/I)	$110.90 \pm 25.79^{a,b,c}$	23.34 ± 13.61^{a}	28.70 ± 14.21^{b}	44.37 ± 21.87^{c}
bAP/Dpd ratio	1.14	0.58	0.55	1.30
PTH (pg/ml)	11.69 ± 6.57	11.81 ± 1.93	22.30 ± 7.35	35.67 ± 13.27

Dpd, Urinary deoxypyridinoline; bAP, bone alkaline phosphatase, PTH, parathormone

Table 4 Bone histomorphometry measurements at Day 30. The same superscript letters in each row indicate significant differences ($p < 0.05$)

	$Sham_{30}$	OVX_{30}.NCaD	OVX_{30}.LCaD
BV/TV (%)	$23.77 \pm 1.81^{a,b}$	16.75 ± 1.50^{a}	16.53 ± 1.69^{b}
Tb.Th (μm)	39.75 ± 1.93	37.61 ± 2.10	41.65 ± 1.65
Tb.N (1/mm)	$6.02 \pm 0.54^{a,b}$	4.46 ± 0.31^{a}	3.91 ± 0.32^{b}
Ob.S/BS (%)	13.45 ± 1.33	18.41 ± 2.76	20.00 ± 2.79
ES/BS (%)	6.38 ± 2.36	7.45 ± 1.05^{a}	12.41 ± 1.90^{a}
N.Oc/TA (1/mm^2)	1.06 ± 0.49	0.19 ± 0.19^{a}	3.06 ± 1.08^{a}

BV/TV, bone volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Ob.S/BS, percentage of trabecular bone surface covered with osteoblasts; ES/BS, percentage of trabecular bone surface covered with eroded surface including active lacunae with osteoclasts and lacunae in reversal phase; N.Oc/TA, number of osteoclasts in the total area

compared with the $Sham_{30}$ group, which could be explained by the decrease found in trabecular bone, as assessed by histomorphometry. In the cortical bone of the OVX groups, the fracture load and ultimate load were higher than those in the $Sham_{30}$ group. An increase in cross-sectional moment of inertia (CSMI) was also found, indicating a more efficient distribution of material in the OVX groups as a defense mechanism by decreasing trabecular bone, as demonstrated previously[31].

On day 150, the OVX_{150} group showed a decrease in fracture load, ultimate load, stiffness, toughness and Young's modulus in the compression test compared with $Sham_{150}$ animals (data not shown), all of which confirm the negative effect of ovariectomy on trabecular bone.

The combination of sequential treatment with MFP and Z, and the different calcium contents in the diet ($OVX.G3_{150}$) after 150 days (Table 6) showed an increase in the fracture load at the cortical bone compared with the OVX_{150}, $OVX.G1_{150}$ and $OVX.G2_{150}$ groups, indicating a greater resistance to fracture, which can be explained by better material properties assessed by the Young's modulus with similar material distribution evaluated by the CSMI. $OVX.G1_{150}$ and $OVX.G2_{150}$ animals showed no statistical differences compared with OVX_{150} animals.

The evaluation of the trabecular bone by the compression test showed that the $OVX.G3_{150}$ group had a higher fracture load, ultimate load and stiffness, which can be explained by the increase in trabecular bone assessed by histomorphometry. At the same time, an increase in Young's modulus was observed.

DISCUSSION

Similar to Black and colleagues[17], we propose a sequential treatment with an anabolic drug followed by an antiresorptive therapy. Instead of PTH, we used MFP, which has proved to be effective as an anabolic drug[8–10], and instead of alendronate, we used zoledronic acid as an antiresorptive drug. Additionally, we proposed sequential changes in the calcium content of the diet, with LCaD followed by HCaD.

As expected, on day 30, we found that the OVX_{30}.LCaD group showed a high bone resorption and low bone formation, confirmed by an increase in Dpd and a decrease in bAP, respectively. Also, the bAP/Dpd ratio was 15-fold lower in the OVX_{30}.LCaD group than in the $Sham_{30}$ and OVX_{30}.NCaD groups. Consistent with biochemical determinations, statistically significant increases in the percentage of eroded surface (ES/BS) and osteoclast number were observed by histomorphometry. However, the decrease in bAP did not correlate with the absence of changes observed in Ob.S/BS. This may indicate that osteoblasts are not as active as expected. Subsequently, we administered MFP as an anabolic drug for 60 days, together with HCaD. The HCaD reduces osteoclast activity, provides appropriate calcium concentration for mineralization and increases gastrointestinal absorption of MFP[32]. Finally, the treatment was changed to zoledronic acid as an antiresorptive drug. We found that bAP and Dpd showed a slight decrease in the $OVX.G1_{150}$ and $OVX.G3_{150}$ groups by pharmacological treatment and that the bAP/Dpd ratio in the $OVX.G3_{150}$ animals was two-fold higher than that in the $OVX.G1_{150}$ animals. This result indicates that there is a predominance of bone formation. BV/TV, Tb.Th and Tb.N in the $OVX.G3_{150}$ group showed values similar to those of the $Sham_{150}$ group. The

Table 5 Bone histomorphometry measurements at Day 150. The same superscript letters in each row indicate significant differences ($p < 0.05$)

	OVX_{150}	$OVX.G1_{150}$	$OVX.G2_{150}$	$OVX.G3_{150}$
BV/TV (%)	15.31 ± 0.96^{a}	13.38 ± 0.95^{b}	17.81 ± 2.37^{c}	$22.49 \pm 3.29^{a,b,c}$
Tb.Th (μm)	50.86 ± 4.02	47.05 ± 1.16	50.26 ± 3.86	53.62 ± 9.98
Tb.N (1/mm)	3.09 ± 0.21^{a}	2.83 ± 0.24^{b}	3.48 ± 0.26^{c}	$4.34 \pm 0.28^{a,b,c}$
Ob.S/BS (%)	16.17 ± 2.71	13.39 ± 3.41	24.16 ± 2.72	19.34 ± 4.48
ES/BS (%)	5.09 ± 0.96^{a}	9.21 ± 3.08	6.49 ± 1.78	10.45 ± 2.54^{a}
N.Oc/TA (1/mm^2)	0.90 ± 0.25	2.06 ± 0.74	2.45 ± 1.23	0.28 ± 0.18

BV/TV, bone volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Ob.S/BS, percentage of trabecular bone surface covered with osteoblasts; ES/BS, percentage of trabecular bone surface covered with eroded surface including active lacunae with osteoclasts and lacunae in reversal phase; N.Oc/TA, number of osteoclasts in the total area

Table 6 Mechanical testing at Day 150. The same superscript letters in each row indicate significant differences ($p < 0.05$)

	OVX_{150}	$OVX.G1_{150}$	$OVX.G2_{150}$	$OVX.G3_{150}$
Three-point bending test				
Fracture load (N)	184.20 ± 4.87^{a}	194.60 ± 5.87^{b}	195.8 ± 4.27^{c}	$220.50 \pm 4.13^{a,b,c}$
Ultimate load (N)	197.10 ± 4.40^{a}	206.30 ± 7.77^{b}	200.60 ± 4.18^{c}	$227.00 \pm 5.04^{a,b,c}$
Stiffness (N/mm)	966.6 ± 119.3	1290.0 ± 271.6	983.8 ± 178.1	1300.0 ± 245.5
Toughness (mJ)	113.9 ± 9.9^{a}	95.8 ± 13.3	77.4 ± 9.5^{a}	83.3 ± 7.6
CSMI (mm^4)	6.76 ± 0.38	7.25 ± 0.50	6.32 ± 0.29	6.70 ± 0.34
Ultimate stress (MPa)	161.8 ± 8.39	154.9 ± 6.71	170.40 ± 5.68	183.70 ± 9.42
Young's modulus (GPa)	5.43 ± 0.76	6.35 ± 1.12	5.73 ± 1.02	7.47 ± 1.74
Compression test				
Fracture load (N)	32.44 ± 4.23	26.45 ± 2.49	26.08 ± 4.37^{a}	44.30 ± 5.57^{a}
Ultimate load (N)	55.71 ± 5.47	46.13 ± 3.72^{a}	55.85 ± 5.86	71.77 ± 6.00^{a}
Stiffness (N/mm)	350.90 ± 50.66^{a}	300.10 ± 62.96	388.30 ± 141.90	639.70 ± 105.30^{a}
Toughness (mJ)	2.29 ± 0.49	1.79 ± 0.21	1.73 ± 0.45	2.25 ± 0.46
Young's modulus (GPa)	0.12 ± 0.02^{a}	0.11 ± 0.02	0.13 ± 0.05	0.22 ± 0.03^{a}

CSMI, cross-sectional moment of inertia

maintenance of these histomorphometrical parameters should be the consequence of a decrease in the osteoclast number observed on days 90 (two-fold) and 150 (three-fold). $OVX.G1_{150}$ and $OVX.G2_{150}$ animals showed no increase in BV/TV. This seems to indicate that the bone effect is not only the consequence of HCaD or pharmacological treatment alone.

Despite the fact that calcium supplementation in the OVX.G2 group was not enough to prevent bone loss compared with the Sham group, the OVX.G2 group showed a slight increase in bone volume compared with the OVX_{150} group.

The decrease in Dpd and N.Oc/TA in the $OVG.G3_{150}$ group was consistent with the osteoclast inhibition by zoledronic acid. The discrepancy of ES/BS has been interpreted as it is known that in BMUs the osteoblast action is slower than the osteoclast action[33], so it would take more time to observe a decrease in ES/BS. In $OVX.G3_{150}$ animals, the values of ES/BS were due to inactive resorptive area (data not shown) consistent with the low N.Oc/TA.

Bone strength depends on the material properties, the tissue mineral content and structural characteristics such as size, shape, and three-dimensional architecture. The increase observed in the fragility of bone with age is regarded, predominantly, as the result of the loss of bone density, but changes in bone structure may also influence bone strength.

Consistent with the histomorphometry analysis, the mechanical testing showed a more resistant bone in the $OVX.G3_{150}$ group compared with the OVX_{150} group. The combination of sequential treatment and the different calcium content in the diet after 150 days showed an increase in fracture load at the cortical bone, indicating a greater resistance to fracture which can be explained by better material properties assessed by the Young's modulus with similar material distribution evaluated by the CSMI. Interestingly, the evaluation of the trabecular bone showed that the $OVX.G3_{150}$ group had a higher fracture load, ultimate load and stiffness, which can be explained by the increase in bone volume and trabecular number assessed by histomorphometry.

Treatment without changes in the calcium content in the diet ($OVX.G1_{150}$) showed an increase in BMD (16.16%) greater than in the $OVX.G3_{150}$ group (9.76%). The lower BMD in the $OVX.G3_{150}$ group than in the $OVX.G1_{150}$ group could be explained by the higher urinary calcium excretion observed in $OVX.G3_{150}$ animals (Table 3). However, this result, far from being detrimental to the bone, was reflected in an increased trabecular bone volume (BV/TV in $OVX.G1_{150} = 13.38\%$ vs. BV/TV in $OVX.G3_{150} = 22.49\%$) and especially in the strength of cortical and trabecular bone (cortical fracture load in $OVX.G1_{150} = 193.9$ N vs. 227.3 N in $OVX.G3_{150}$; trabecular fracture load in $OVX.G1_{150} = 26.63$ N vs. 42.42 N in $OVX.G3_{150}$).

Zoledronic acid has been effective in the treatment of osteoporosis in OVX rats by increasing BMD, bone formation rate, histomorphometric and biomechanical parameters[34,35]. In postmenopausal women, treatment with zoledronic acid during 36 months produced a BMD increase of 6.02% in total hip, 5.06% in femoral neck and 6.71% in lumbar spine[19]. Although the data for sequential treatment showed a greater increase in BMD than the previous cited paper with zoledronic acid alone, we cannot attribute this difference to the sequential treatment. The group with sequential treatment without changes in the calcium content in diet also showed an increase in BMD. However, in this group the bone histomorphometric and biomechanical properties were lower than in the $OVX.G3_{150}$ group.

Although the results of our paper do not have an immediate clinical impact, they suggest that a sequential treatment with an anabolic drug followed by an antiresorptive drug is a promising treatment for postmenopausal osteoporosis.

In summary, this paper demonstrates that the sequential treatment with MFP and zoledronic acid, combined with appropriate changes in the calcium content of the diet, increases trabecular bone mass, bone mineral density and

bone biomechanical properties. The implementation in patients will require further studies.

ACKNOWLEDGEMENTS

We thank H. Moreno, D. Vicente, B. Garcia and L. Arias for their technical assistance.

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Source of funding This work was funded by grants from National Council of Scientific and Technical Research (CONICET), Argentina (pip 462) and Alberto J. Roemmers Fundation.

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