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Cancer Therapy: Preclinical

High-Dose Zoledronic Acid Impacts Bone Remodeling with Effects on Osteoblastic Lineage and Bone Mechanical Properties

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Abstract

Purpose: The increasing incidence of osteonecrosis of the jaw and its possible association with high cumulative doses of bisphosphonate led us to study the effects of high doses of zoledronic acid (ZA) on bone remodeling.

Experimental Design: Five-week-old C57BL6 mice were treated with saline or ZA weekly for 3 weeks at increasing doses (0.05-1 mg/Kg). Effects of ZA on bone remodeling were studied using standard assays.

Results: We observed an increase in bone mineral density and content in treated animals at doses of 0.05 mg/Kg, which was not further enhanced at higher doses of ZA. Trabecular bone volume at the proximal tibia and the distal femur assessed by histomorphometry and microCT, respectively, increased significantly in ZA-treated groups. There was however no difference between 0.5 and 1 mg/kg, suggesting a ceiling effect for ZA. ZA led to decreased numbers of osteoclasts and osteoblasts per bone perimeter that paralleled a significant reduction of serum levels of TRAC5b and osteocalcin *in vivo*. Effects on osteoblasts were confirmed in *in vitro* assays. Mechanical testing of the femur showed increased brittleness in ZA-treated mice.

Conclusions: High doses of ZA inhibit both osteoclast and osteoblasts function and bone remodeling *in vivo* interfering with bone mechanical properties. No dose response was noted beyond 0.5 mg/kg suggesting that lower doses of ZA may be adequate in inhibiting bone resorption. Our data may help inform future studies of ZA use with respect to alternate and lower doses in the treatment of patients with cancer bone disease. (Clin Cancer Res 2009;15(18):5829–39)

Bisphosphonates are pyrophosphate analogues characterized by high affinity for hydroxyapatitite (1). They are extensively used in clinical practice for the treatment of diseases with high bone catabolism, mainly osteoporosis, Paget's disease, cancer-related bone disease (2, 3), and some skeletal pediatric diseases such as osteogenesis imperfecta (4). Bisphosphonates target the bone, linking to hydroxyapatite, especially in areas of high bone turnover (5) and are released by osteoclasts, their main cellular target. Their action is mediated by the inhibition of osteoclast recruitment and impairment of osteoclast function as evidenced by morphologic changes on osteoclast cytoskeleton, disruption of ruffle borders, and apoptosis (6, 7). Bisphosphonates are stable in bone for prolonged times, and *in vivo* animal studies have shown a long half-life in the skeleton, with a terminal phase of ~300 days (8, 9). Their effects on cell types other than osteoclast have not been completely elucidated. In particular, interest is focused on delineating the effect of these compounds on cancer cells, osteoblasts, osteocytes, endothelial, and epithelial cells.

Bisphosphonates are broadly divided into two main groups based on their chemical structure: nitrogen and non-nitrogen containing bisphosphonate. Pamidronate and zoledronic acid (ZA), two of the most potent bisphosphonates, are both nitrogen-containing bisphosphonates and are the two main compounds used for the treatment of cancer-related bone disease. Their use is supported by prospective randomized placebo-controlled studies (10, 11) that have shown their ability at reducing skeletal-related events as well as cancer-induced hypercalcemia. Their use is recommended by the American Society of Clinical Oncology for cancer-related bone disease (12, 13). However, despite several decades of intensive use in both malignant and nonmalignant diseases, the duration

Translational Relevance

The use of bisphosphonates in cancer-related bone disease has changed the management of bone disease in patients with significant reductions in skeletal-related events and improvement in bone-related morbidity. Emerging data on the development of bisphosphonate-associated osteonecrosis of the jaw, however, raises new concerns about the duration and dose of aminobisphosphonate administration, prompting the need to better understand their effects on bone remodeling and their targets beyond osteoclasts. Our data suggest that high doses of zoledronic acid failed to show additional beneficial effects when compared with lower doses, but resulted in impairment of bone mechanical properties. These observations form the preclinical rational to test the use of lower doses or shorter duration of bisphosphonate use in cancer-related bone disease.

of therapy and the total dosage required for effective therapy has not been defined. Consequently, with improved outcome and survival of patients despite associated bone involvement, cancer patients are now exposed to two of the most potent bisphosphonates on a monthly schedule for prolonged durations.

These uncertainties were brought to light in 2003 when a new concern with the use of bisphosphonate emerged. The use of bisphosphonates, in particular Pamidronate and ZA, was associated with increasing number of jaw complications, now known as bisphosphonate-associated osteonecrosis of the jaw (ONJ; refs. 14–16). ONJ refers to a nonhealing exposed area of the mandible or maxilla that persists for >8 weeks after its identification, in patients exposed to bisphosphonate, not associated with radiotherapy of head and neck (17). Although this is not a common event in cancer patients (<10%, with a higher prevalence in multiple myeloma compared with solid tumors; ref. 18), a strong association has been described with the use of nitrogen containing bisphosphonate. Several concomitant cofactors may contribute to the pathogenesis of this complication and these are a subject of investigation. Although the underlying pathophysiology is unknown, the relationship between prolonged exposure to bisphosphonate and the development of jaw complications has emerged in many case series (14–16, 18), prompting a revision of current guidelines for the administration of bisphosphonate (19–21). These revised guidelines now suggest limiting treatment with bisphosphonate. The two main concerns related to the prolonged treatment with bisphosphonate are the long half-life of the drug in the skeleton and its possible prolonged effect on bone remodeling.

For these reasons, the 2006 Mayo Clinic consensus statement (19) and the 2007 American Society of Clinical Oncology guidelines (21) suggest 2 years of treatment with either Pamidronate or ZA in multiple myeloma and a careful evaluation for its continued use beyond this time point until more data are available. However, these recommendations are not supported by laboratory or clinical data.

To better characterize the effects of high-dose ZA on bone modeling and remodeling, we studied its effects in preclinical models focusing mainly on the osteoblast lineage and bone mechanical properties. Our data suggest that high-dose ZA, in addition to its inhibition of osteoclast activity, has inhibitory effects on the osteoblast lineage resulting in altered bone remodeling, leading to increased strength but reduced ductility. Thus, high-dose bisphosphonate may adversely affect bone deposition and remodeling and result in brittle bone. Randomized clinical trials to evaluate the appropriate dosing schedule for bisphosphonates are thus warranted.

Materials and Methods

Animals and drug administration. Five-week-old female C57BL6 mice were divided into four groups, and housed in the animal care facility. The Dana-Farber Cancer Institute animal ethics committee and scientific review committee approved the care and experimental protocol. ZA (Novartis) from commercial 4-mg vials was diluted with sterile saline solution before administration and injected i.p.

To determine the dose for treatment of mice, we extrapolated the amount of drug received by patients for cancer-related bone disease. Assuming that a multiple myeloma patient of ~70 Kg receives 4 mg of drug every 4 wk corresponding to a dose of 0.05 mg/Kg/mo (0.65 mg/Kg/y or ~3 mg/Kg in 5 y), we studied the effect of ZA in three arms at doses of 0.05, 0.5, and 1 mg/Kg compared with control (saline solution). Animals were injected i.p. with either saline solution or ZA weekly for 3 wk; the highest dose recapitulating a life time dose of ZA over a 5-y period in an adult multiple myeloma patient. The experiment was repeated twice for a total of eight animals per group. Blood was collected at baseline and every week before treatment. To study the dynamic histomorphometry, calcein was injected i.p. (40 µg/Kg), 10 and 3 days before sacrifice. After 3 wk of treatment, the animals were sacrificed, and bones from both hind limbs were evaluated with additional studies.

Bone mass and microarchitecture assessment. Bone mineral density (BMD, g/cm^2) and bone mineral content (BMC, grams) were evaluated immediately after sacrifice using dual-energy X-ray absorptiometry (DXA; GE-Lunar PIXImus). BMD and BMC measurements of the whole body (minus the head), and isolated femur and tibia were obtained for control and at doses of 0.05 and 0.5 mg/Kg; DXA was not done at 1.0 mg/Kg dose as we did not see any added benefit by histomorphometrty at this dose of ZA.

To assess bone morphology and microarchitecture, femurs were scanned using a high-resolution desktop microtomographic imaging system (µCT40; Scanco Medical AG), as previously described (22–24). Briefly, trabecular bone morphology was analyzed in the distal femur. Trabecular bone outcomes included bone volume fraction (BV/TV; %), trabecular thickness (Tb.Th; µm), trabecular number (Tb.N; mm^{-1}), trabecular separation (Tb.Sp; µm), and connectivity density (1/mm^3). Cortical bone morphology was studied at the midfemoral diaphysis. Outcome assessments include total cross-sectional area (TA, mm^2); cortical bone area (BA, mm^2); bone area fraction (BA/TA, %); cortical thickness (µm); and the maximum, minimum, and polar moments of inertia (Imax, Imin, and Ipolar, mm^4), which describe the shape/distribution of cortical bone (larger values indicate a higher bending strength).

Histology and histomorphometry. Tibias were fixed in 4% formalin for 48 h, followed by ethanol 70% and embedded in methyl methacrylate resin as previously described (25). Samples were cut into 4-µm sections and stained with modified Masson-Goldner's trichrome for static histomorphometry. Dynamic histomorphometry measurements were done on unstained 8-µm-thick sections. Static and dynamic histomorphometric analysis were done on the tibial secondary spongiosa at 40×, with a bone histomorphometry measure system version 4.10 (Osteometrics, Inc.), using standard procedures published by the American Society for Bone and Mineral Research (26).

Serum osteocalcin and TRAC5b levels. Sera from weekly blood collection was separated by centrifugation and kept at -80°C until

further use. Levels of osteocalcin and TRAP5b were measured according to manufacturers' guidelines using commercially available kits: Mouse TRAP Assay (Immunodiagnostic System, Inc.) and Mouse Osteocalcin (Biomedical Technologies, Inc.).

Bone strength testing. To determine the effects of ZA on bone strength, femurs were tested to failure in a three-point bending configuration, as previously described (27). Force-displacement data were used to define ultimate moment (N·mm), bending stiffness (N/mm), work-to-failure (N*mm), and postyield displacement (mm), as previously described (28). In addition, cortical cross-sectional geometry was used to compute the estimated elastic modulus (GPa) and ultimate strength (MPa).

Cell culture. To study the *in vitro* effects of ZA, we tested it on cells derived from bone marrow aspirates of multiple myeloma patients that were obtained after informed consent and approval from the Institutional Review Board. Cells were separated by Ficoll-Hypaque density gradient centrifugation and cultured in flasks containing αMEM (Mediatech, Inc.), supplemented with 20% heat-inactivated fetal bovine serum, and 2% of penicillin and streptomycin (Mediatech, Inc.).

Bone marrow stromal cells. Adherent cells were long-term cultured and expanded in αMEM, supplemented as described above. When cells reached near 80% confluence, they were washed with PBS, trypsinized (Trypsin EDTA 1×; Mediatech, Inc.) and resuspended in αMEM supplemented as previously described, and plated in 96-well plates at 10,000cells per well in triplicate. ZA was diluted just before the experiment in the same culture medium and added to the cells at increasing concentrations (0.1-10 µmol/L). Medium with drug was replaced twice per week. Cell viability was evaluated at 7, 14, and 21 d of treatment by AlamarBlue assay (BioSource International, Inc.) as previously described (29).

Osteoblast. Bone marrow stromal cells (BMSC) were harvested as described above. Medium was replaced with osteoblast differentiation medium [αMEM containing 20% fetal bovine serum, 2% penicillin-streptomycin (Mediatech, Inc.), β-glycerol phosphate at 2.16 mg/mL, ascorbic acid at 0.05 mg/mL, and Dexamethasone at 10 nmol/L (Sigma-Aldrich]. To evaluate the effect of ZA on osteoblast differentiation, cells were plated in triplicate in 96-well plates at 10,000 cells per well and ZA at increasing concentrations. Medium and drug were replaced twice per week. Cell viability was evaluated at 7, 14, and 21 d of treatment with AlamarBlue assay as previously described. The experiment was repeated thrice in triplicates.

To evaluate the effect of the drug on differentiated osteoblast, BMSC obtained as described above were plated in 96-well plates at 10,000 cells per well, with osteoblast differentiation medium for 21 d. Medium was replaced twice per week. After 21 d, cells were treated with ZA at increasing concentrations and cell viability was evaluated with alamar blu at 7, 14, and 21 d of treatment. Experiments were repeated at least thrice in triplicates.

Alkaline phosphatase and alizarin red staining. Following cell viability analysis, wells were washed with PBS once, fixed and washed with distilled water, and stained with alkaline phosphatase staining (Takara Bio).

To evaluate calcium deposition, osteoblasts were washed with PBS and fixed after 14 and 21 d of treatment and assayed by alizarin red staining that specifically stains mineralization area according to the manufacturer's guidelines (Millipore Corporation).

Statistical analysis. Statistical analysis was done using the Graph-Pad Prism 4 software. Control and treated animals were compared with one way ANOVA test, adjusted for multiple comparisons using the Tukey method, with a minimal level of significance of <0.05. Unpaired t test was used when ANOVA test was not applicable.

Results

ZA leads to increased BMD and BMC. Treatment was well tolerated by the animals. No acute toxicities were observed during the 3 weeks of treatment with ZA for all concentrations tested. At the end of the 3 weeks, the animals were sacrificed and the skeletal effects of ZA were analyzed using DXA. Compared with controls, both BMD (grams/cm^2) and BMC (grams) were increased in the whole body, femur, and tibia in the ZA-treated animals. In particular, BMD was increased 13%, 27%, and 24% in whole body, tibia, and femur, respectively, in the 0.05 mg/Kg group compared with control; and 18%, 31%, and 25%, respectively, in the 0.5 mg/Kg group compared with control. These increases were statistically significant when compared with controls ($P < 0.01$); no difference between the two tested doses of ZA was noted, with a maximum effect noted at 0.05 mg/Kg. DXA scans were not done in animals treated with 1 mg/kg of ZA as histomorphometry did not reveal added benefits.

ZA increased trabecular bone at the proximal tibia evaluated by static histomorphometry. Both tibia and femur showed increased mineralization by DXA (Fig. 1). To better define the effect of ZA on bone architecture, we first did static histomorphometry on the proximal tibia to evaluate the trabecular bone.

ZA treatment led to increased trabecular structure as shown in Fig. 1A compared with control. This was related to increased BV/TV, Tb.N, and decreased Tb.Sp compared with controls, with less evident effect on Tb.Th (Fig. 1B and C). The difference was statistically significant ($P < 0.05$) for all the tested doses (0.05, 0.5, and 1 mg/Kg) compared with control, without any difference between the two higher doses of 0.5 and 1 mg/Kg.

ZA impacts osteoclast and osteoblast number in the treated animals. To define the effect of ZA on osteoclast and osteoblast, we counted the number of osteoclast and osteoblast per bone perimeter (N.Oc/B.Pm, N.Ob/B.Pm). The N.Oc/B.Pm (mm-1) decreased in the treated animals compared with control (1.49 versus 1.34 versus 1.67 in 0.05 versus 0.5 versus 1 mg/Kg compared with 1.74 in controls). The N.Ob/B.Pm (mm-1) decreased as well (30.07 versus 24.97 versus 25.16 in 0.05 versus 0.5 and 1 mg/Kg versus 32.50 in controls). But in both cases, this difference did not reach statistical significance (Fig. 2A and B).

We attempted to quantify the functional impact of ZA on osteoblast using dynamic histomorphometry. However, due to the profound increase in trabecular bone and the resultant distortion of trabecular architecture, measurement of calcein double labeling was technically difficult and results not conclusive. We therefore confirmed our *in vivo* observations by *in vitro* assays.

Serum concentration of osteoclast and osteoblast markers: both TRACP5b and osteocalcin were significatively decreased following ZA treatment. To confirm our observations from histomorphometry that showed a slight reduction of both Oc.N/B.Pm and Ob.N/B.Pm, we evaluated the concentration of their serum markers after 3 weeks of treatment. Our data showed a significant decrease in TRACP5b, a marker of osteoclast activity, as well as osteocalcin levels, a marker of osteoblast activity, in the treated animals versus controls (Fig. 2C). Although age-related decrease in TRACP5b levels was noted in all animals, treated and not treated, ZA resulted in a further decrease of TRACP5b compared with controls, to 47% in 0.05 mg/Kg, 33% in 0.5 mg/Kg, and 49% in 1 mg/Kg, respectively, compared with a 64% decrease in the control group compared with week 0 (100%). The fold decrease of TRAC5b for the two doses of 0.5 and 1 mg/Kg was statistically significant ($P < 0.01$) compared with controls. The osteocalcin serum level at week 3 decreased as
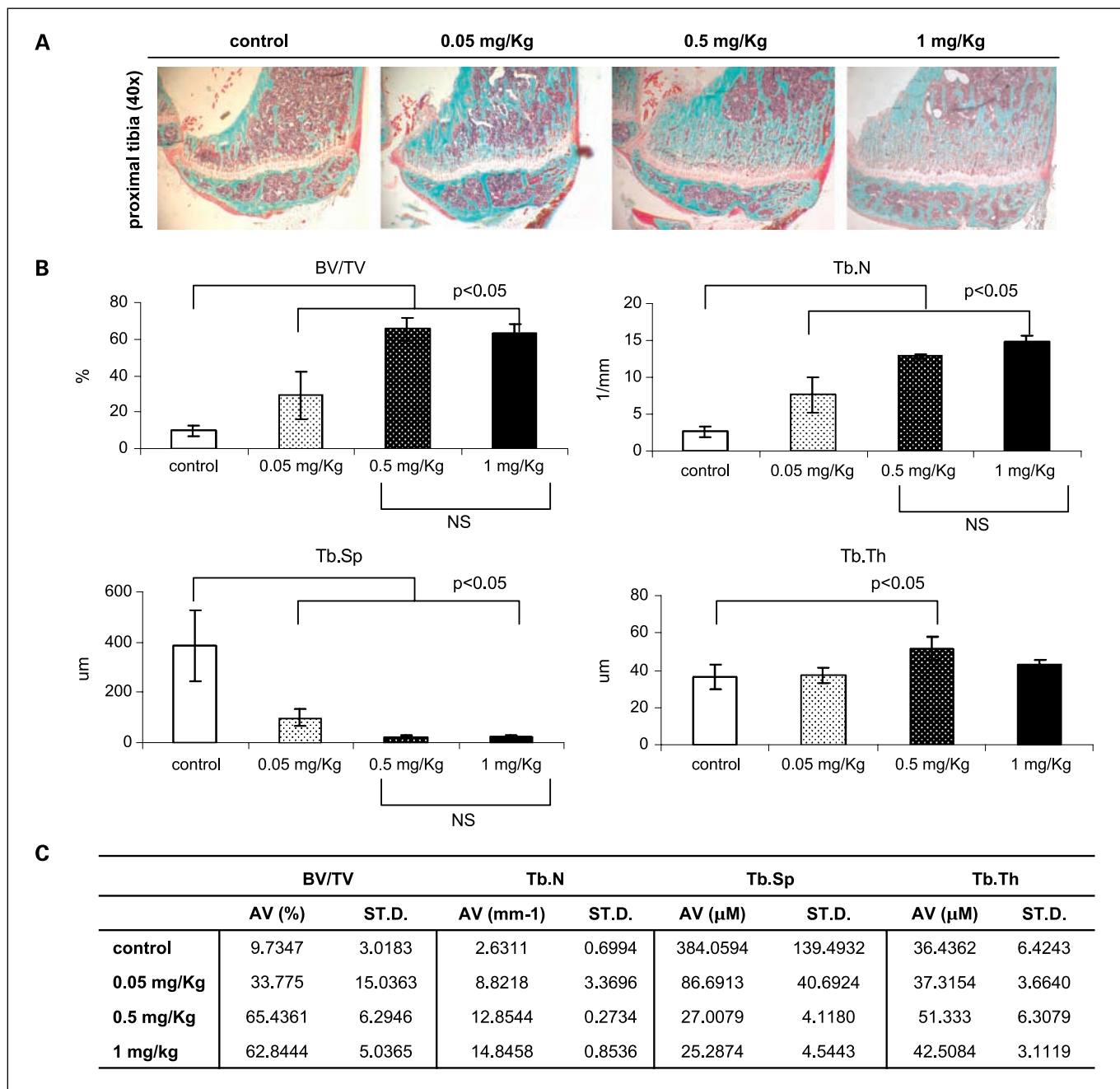
Fig. 1. Static histomorphometry at proximal tibia confirmed increased trabecular bone with ZA. Microphotograph of proximal tibia (modified Masson-Goldner's trichrome assay; ×40) of control, ZA 0.05, 0.5, and 1 mg/Kg (*A*). *B* and *C*, BV/TV, Tb.N., Tb.Sp., and Tb.Th assessed by static histomorphometry. AV= average; ST.D.= standard deviation.

well, to 78% in the 0.05 mg/Kg group, to 74% in the 0.5 mg/Kg, and to 72% in the 1 mg/Kg, compared with control (100%). The decrease between the two higher doses of 0.5 and 1 mg/Kg compared with control was statistically significant ($P < 0.05$) by the unpaired *t* test.

MicroCT analysis of distal femur confirmed the increased trabecular bone observed in the proximal tibia, with no difference between 0.5 and 1.0 mg/Kg. The histomorphometric analysis of the trabecular bone of the tibia showed increased BV/TV related to increased Tb.N and reduction of Tb.Sp. without any difference between 0.5 and 1 mg/Kg. To confirm these observations,

we analyzed the trabecular architecture of distal femur for these two doses of ZA, using the microCT. Compared with controls, trabecular bone at the distal femur increased with increasing doses of ZA (Fig. 3A), with a 5.68-fold and 5.04-fold increase for the 0.5 mg/kg and 1 mg/kg groups, respectively ($P < 0.01$).

This increase in trabecular bone volume was accompanied by an increase in Tb.N and Tb.Th and a reduction in the Tb.Sp, as already described for the tibia. In particular, in the 0.5 mg/Kg group, Tb.N and Tb.Th were increased 1.93- and 2.32-fold, respectively, with a reduction of Tb.Sp of 0.65-fold, compared with control. In the 1 mg/Kg group, the increase of Tb.N and

Tb.Th was 1.83- and 2.31-fold versus control, with a reduction of Tb.Sp of 0.64-fold. Connectivity density was also increased 1.64- and 1.83-fold, respectively, in the 0.5 and 1 mg/Kg groups versus control. The differences for all the parameters were statistically significant in the treated animals compared with control ($P \leq 0.01$), with no differences between the two ZA-treated groups. Treatment with ZA also affected the trabecular architecture, as evidenced by the structure model index (SMI). This is a geometric parameter that describes the characteristics of the trabecular bone, quantifying plate-like versus rod-like structures (30). Our data suggest increased plate-like structures (Fig. 3B).

High doses of ZA affected the cortical bone morphology of the femur, without any difference between 0.5 and 1 mg/Kg. Both histomorphometry at proximal tibia and microCT at distal femur confirmed an architectural change due to ZA treatment in the trabecular region, without any difference between 0.5 and 1 mg/Kg. We next studied the femoral midshaft by microCT of control and the two higher doses of ZA (0.5 and 1 mg/Kg) to evaluate any possible effect of ZA on cortical bone. Although

less pronounced than the effects on trabecular bone, cortical bone was affected as well. In the ZA-treated groups, TA, BA, and BA/TA were 7% to 8%, 12% to 14%, and 4% to 6% higher, respectively, than controls (Fig. 4). Cortical thickness was 9% to 12% higher than control in the ZA-treated groups. Differences between the treated animals and the controls reached statistical significance ($P < 0.01$), without any difference between the two tested doses.

Mechanical testing showed increased bone stiffness associated with a decrease in postyield displacement following ZA treatment. After observing the effects of ZA on cortical bone morphology, we next determined whether the changes observed in bone mass and architecture affected bone biomechanical properties. Three-point bending test was applied to femurs of animals in control, 0.5, and 1 mg/Kg groups. The maximum and minimum moment of inertia (Imax, Imin) that represent resistance to bending of the bone cross-sections (31) were both increased in treated animals versus control (Fig. 5A and B). Specifically, the Imax increased from 12%

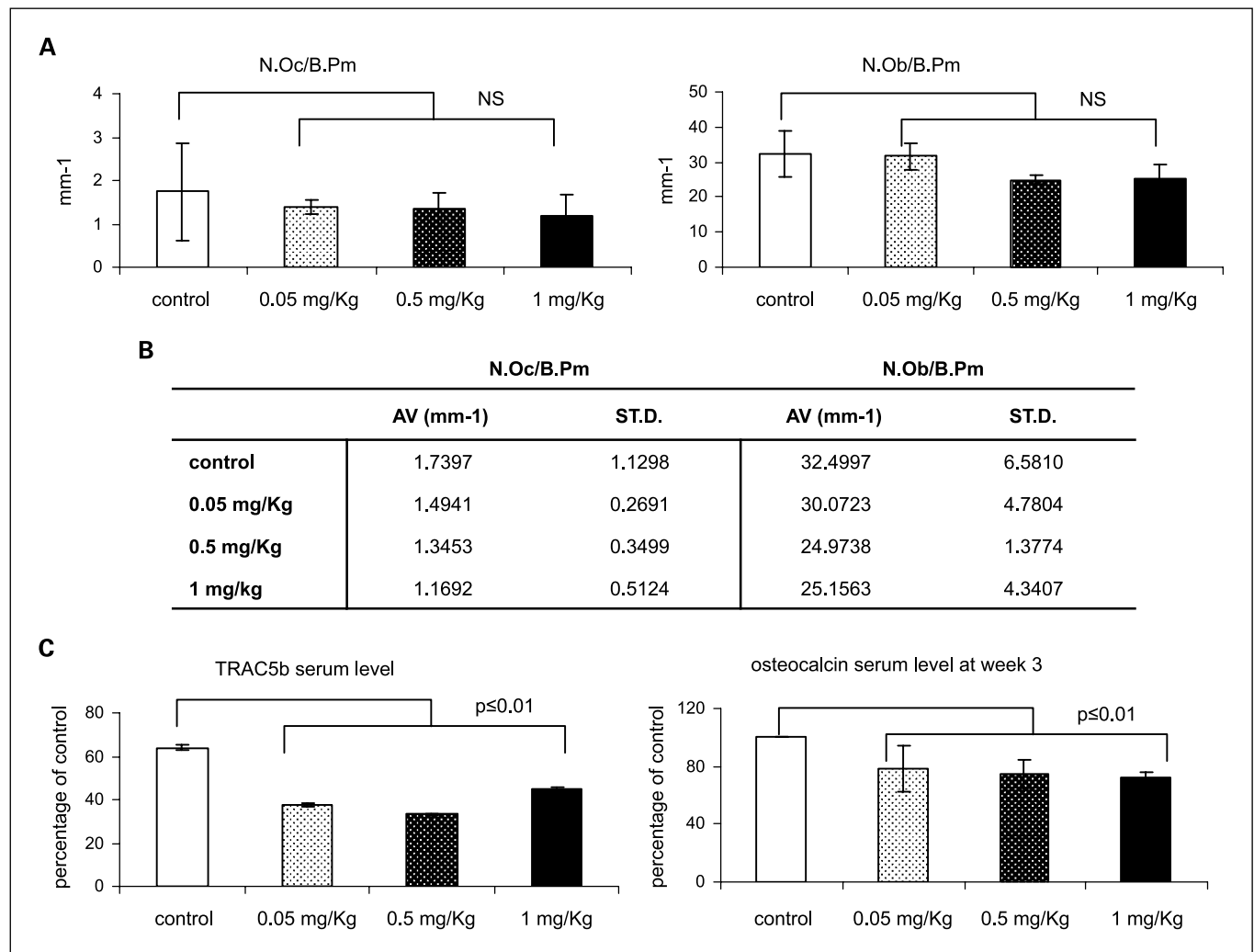|	N.Oc/B.Pm		N.Ob/B.Pm	
	AV (mm-1)	ST.D.	AV (mm-1)	ST.D.
control	1.7397	1.1298	32.4997	6.5810
0.05 mg/Kg	1.4941	0.2691	30.0723	4.7804
0.5 mg/Kg	1.3453	0.3499	24.9738	1.3774
1 mg/kg	1.1692	0.5124	25.1563	4.3407

Fig. 2. Effects on osteoclast and osteoblast numbers and TRACP5b and osteocalcin serum levels after treatment with ZA. Osteoclast number per bone perimeter (*Oc/B.Pm*) and osteoblast number per bone perimeter (*Ob/B.Pm*) were decreased after treatment with ZA (*A* and *B*). After 3 wk of treatment with ZA, TRAC5b decreased in all the animals compared with week 0. This decrease was more pronounced in the ZA-treated animals (37% in 0.05 mg/Kg, 33% in 0.5 mg/Kg, and 45% in 1 mg/Kg) compared with control (60%), which was statistically significant. Osteocalcin level decreased as well to 78% in 0.05 mg/Kg, 74% in 0.5 mg/Kg, and 72% in 1 mg/Kg group, compared with control (100%), suggesting a possible inhibition of osteoblast activity (*C*). AV=average; ST.D.: standard deviation.
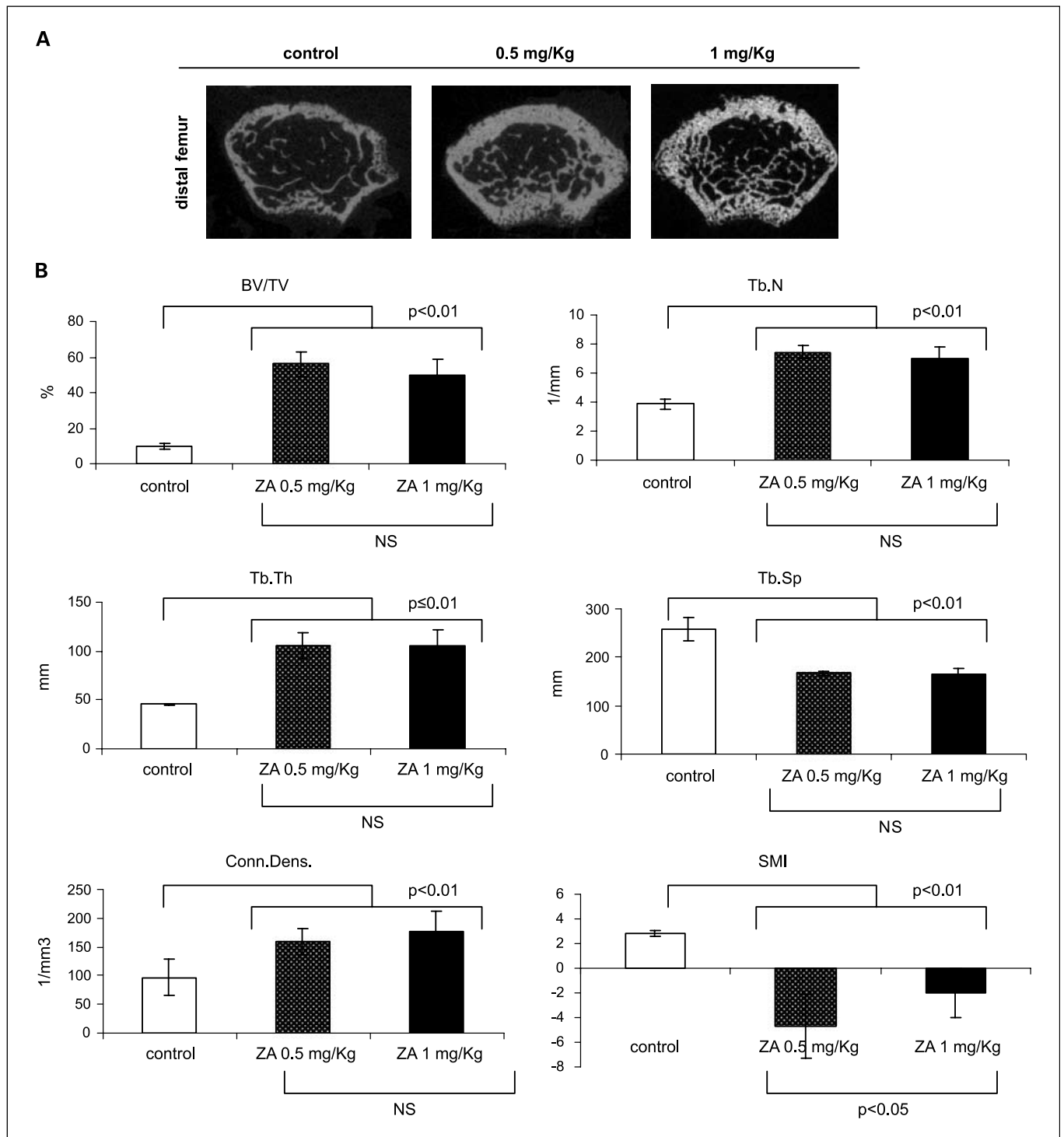
Fig. 3. MicroCT of distal femur confirmed increase of trabecular bone in ZA-treated animals. MicroCT images of distal femur (*A*). *B* demonstrates an increased BV/TV related to increased Tb.N and Tb.Th and reduction of Tb.Sp. Connective density (*conn. dens.*) increased in the treated animals compared with controls. The structure model index was decreased, suggesting prevalent plate-like trabeculae versus rod-like trabeculae in the treated animals compared with control.

to 14% and the Imin from 27% to 34% in the ZA-treated versus control. Even the polar moment of Inertia (Ipolar; Fig. 5C) that measures the resistance of the bone to torsion (31) increased from 18% to 19% in the ZA-treated versus control. The three-point bending test revealed an increased stiffness in ZA-treated mice versus controls (*P* < 0.05; Fig. 5D) and

the postyield displacement was significantly decreased both in 0.5 and 1 mg/Kg cohorts compared with controls (Fig. 5E), suggesting increased brittleness of ZA-treated bones, as expected in highly mineralized bone tissue. Ultimate moment was also increased in ZA-treated groups, but did not reach statistical significance (Fig. 5F). Estimated ultimate strength was higher in

ZA-treated mice than controls ($P < 0.05$), whereas estimated elastic modulus was decreased in the 0.5 mg/kg group (Fig. 5G and H).

Prolonged exposure to ZA** in vitro **induced direct cytotoxicity on osteoblasts compared with bone marrow stromal cells. Given that our dynamic histomorphometry was technically difficult to interpret because of the profound effects of ZA on mice bones, we sought to confirm effects on osteoblasts by *in vitro* assays. We studied the possible direct effect of ZA on osteoblasts using increasing concentrations of ZA *in vitro*, on osteoblasts derived from BMSC of patients affected by multiple myeloma. BMSC similarly treated served as controls (Fig. 6A). Cell viability was tested at 7, 14, and 21 days of treatment. BMSC showed minimal cytotoxicity for concentrations of ZA lower than 10 µmol/L. In contrast, the viability of differentiated osteoblasts showed a dose and time-dependent toxicity starting

from doses of 10 µmol/L at day 7, and 1 µmol/L at longer time points. These data were further confirmed using alkaline phosphatase staining (Fig. 6B) and alizarin red staining (Fig. 6C) that showed reduced osteoblast function with increasing doses of ZA. The results were similar both during osteoblast differentiation as well as in fully differentiated osteoblasts, suggesting that ZA affected viability of osteoblast.

Discussion

The use of bisphosphonate has changed the management of bone disease in patients with a significant reduction in skeletal-related events and improvement in bone-related morbidity (10, 11). Emerging data on the development of bisphosphonate-associated ONJ, however, raises new concerns about the duration and dose of amino bisphosphonate administration,
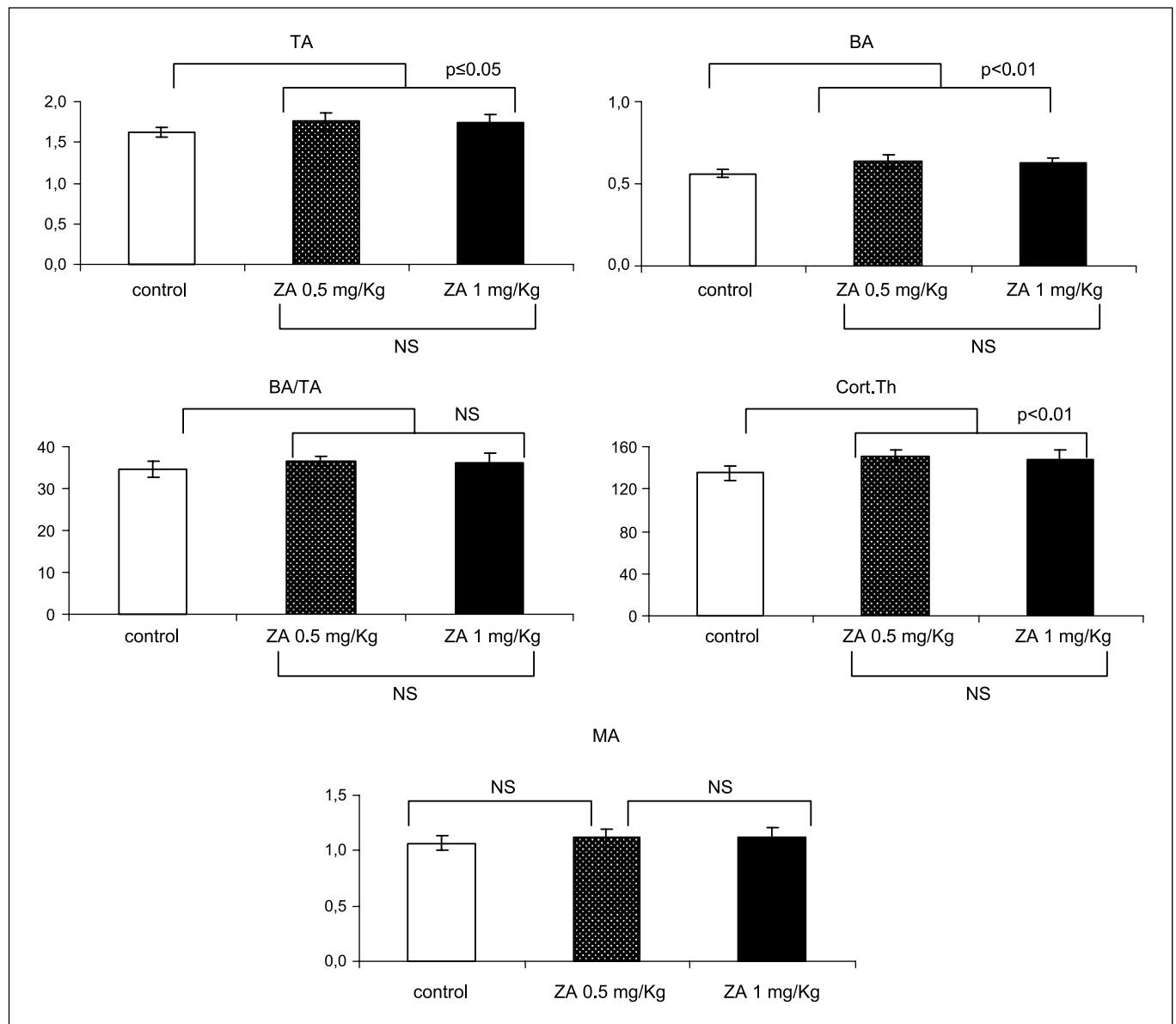

Fig. 4. High doses of ZA affected the cortical bone morphology as evidenced by MicroCT at the femoral midshaft. MicroCT analysis evidenced increased TA and BA in the treated animals compared with control but the BA/TA did not reach the statistical significance. Cortical thickness (*Cort.Th.*) was increased compared with control ($P < 0.01$), without any difference between the two tested doses. Marrow area (*MA*) was increased in the treated animals.
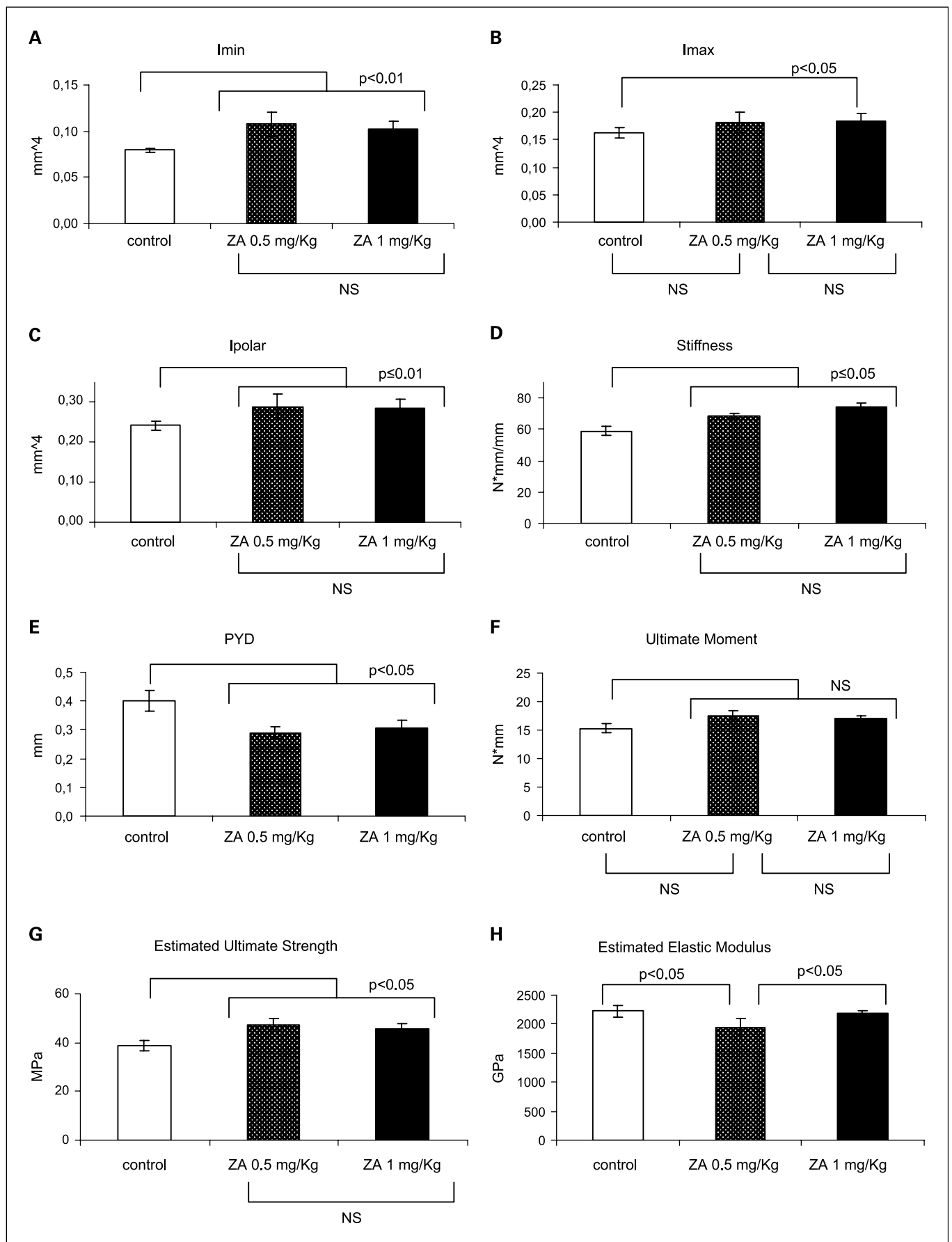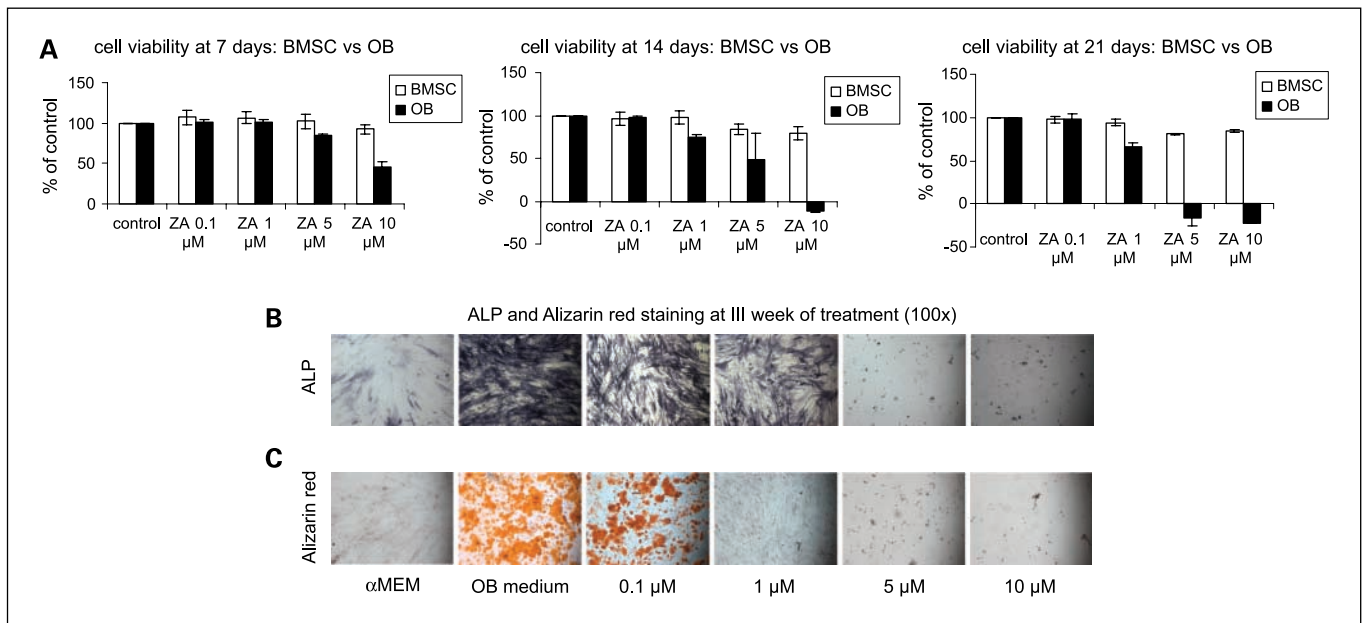
Fig. 6. ZA decreased osteoblast viability and function in *in vitro* assays. Cell viability of osteoblasts (*OB*) derived from BMSC of multiple myeloma patients versus undifferentiated BMSC treated with increasing concentrations of ZA were tested. Viability was evaluated with alamar blue assay at 7, 14, and 21 d of treatment (*A*). After 21 d, alkaline phosphatase (*ALP*; *B*) and Alizarin red staining (*C*) were done in plain αMEM, or with osteoblasts differentiation medium (*OB medium*) with or without ZA. As shown in the figure, concentrations of 0.1 and 1 μmol/L of ZA inhibited osteoblast viability and function.

prompting the need to better understand their effects on bone remodeling and their targets beyond osteoclast. Although the mechanism underlying this complication has not yet been established, we hypothesized that prolonged treatment with bisphosphonate may induce an extreme suppression of bone remodeling and possibly interfere with osteoclast and osteoblast function and bone properties. We have previously characterized ONJ in 11 multiple myeloma patients clinically, radiographically, and biochemically (32). Our data showed a suppression of bone formation markers both at the gene transcript and protein level in patients treated with bisphosphonate. The gene expression profiling of patients with multiple myeloma treated with bisphosphonate, with and without ONJ, compared with healthy volunteers highlighted a downregulation of genes implicated in osteoblast signaling, differentiation, and activation, and was more pronounced in patients who developed ONJ (32). Given that myeloma patients' already have profoundly altered bone remodeling, due to increased osteoclast activity and reduced osteoblast function, prolonged bisphosphonate use likely induces marked suppression, altering bone homeostasis, predisposing them to development of ONJ in the presence of other cofactors. Based on our previous results, we evaluated the effects of high doses of ZA on bone modeling and remodeling, focusing especially on osteoblast lineage and bone mechanical properties.

Bisphosphonates linked to bone mineral matrix are released in the phase of bone resorption (33, 34). The effects of nitrogen-bisphosphonate on osteoclast are well established and rely on

a dual mechanism. The nitrogen containing bisphosphonate affect the mevalonate pathway (35) inhibiting the enzyme farnesyl diphosphate synthase, with consequent inhibition of the prenylation of proteins involved in cell survival (35, 36), proliferation, and cytoskeleton organization (37). They also induce the intracellular production of Apppi [triphosphoric acid 1-adenosin-5-yl ester 3-(3-methylbut-3-enyl) ester], an ATP analogue (38) that induces cell apoptosis. However, the effects of bisphosphonate on osteoblast are not completely elucidated, and data from literature are controversial (39–42).

Extrapolating data from the management of cancer patients who receive the most intensive bisphosphonate therapy, we treated mice with ZA, the most potent bisphosphonate widely used in cancer-related bone disease. The doses used correspond to duration of treatment of 3 months, 2.5, and 4.5 years in cancer patients. Our *in vivo* studies suggest an increase of BMD and BMC in treated animals compared with controls, without any further benefit for doses higher than 0.05 mg/Kg. Increased BMD is a known consequence of bisphosphonate treatment, and it seems to be related to the osteoclast inhibition that induces prolongation of the life span of basic structural units and consequent increased secondary mineralization (43). Studying the effect of ZA in more detail, we saw that high doses of ZA not only affected mineralization, but also the bone architecture. First, we evaluated the effect of escalating doses of ZA on the trabecular bone of the proximal tibia, using static histomorphometry. Our data showed increased trabecular bone with similar effects noted at the two higher doses tested, suggesting a ceiling

Fig. 5. Mechanical testing (three-point bending to failure) of the femur showed increased stiffness and reduced postyield displacement in ZA-treated animals, suggesting increased brittleness. Imin (*A*), Imax (*B*), and polar moment of inertia (*C*) as well as stiffness (*D*) were increased in the treated animals compared with control, showing increased resistance to deformation. However, ZA-treated samples showed reduced postyield displacement (*PYD*, mm; *E*) and the same ultimate moment (*F*) as the control, suggesting increased brittleness induced by the high doses of ZA. Estimated ultimate strength (*G*) was increased in 0.5 and 1 mg/Kg groups, whereas estimated elastic modulus (*H*) was decreased just in 0.5 mg/Kg group.

effect. ZA also induced a decrease in both osteoclast and osteoblast number per bone perimeter, although this decrease was not statistically significant. Importantly, both TRA5b and osteocalcin were significantly decreased in the treated animals compared with controls, suggesting a functional impairment of both osteoclast and osteoblast.

To clarify a possible direct effect of ZA on osteoblast lineage, we tested increasing concentrations of ZA *in vitro* on osteoblast derived from BMSC of multiple myeloma patients. Our data showed that prolonged exposure of differentiated osteoblast *in vitro* to ZA resulted in a cytotoxic effect compared with undifferentiated BMSC, confirming our *in vivo* observation, and suggesting a possible direct toxic effect of ZA on osteoblast.

Changes in tibial trabecular architecture were confirmed by microCT analysis of the femur. Again, no difference between the two tested doses was observed. Furthermore, microCT at the midshaft of the femur showed increased cortical thickness in the treated animals compared with control, without any difference between the two higher doses.

Because of the changes induced by ZA on BMD and on cortical morphology, we used mechanical testing to evaluate if these changes translated into alterations of bone mechanical properties. Our data confirmed increased stiffness associated with increased brittleness of the bones.

Although the direct translation of animal model systems to human studies are not necessarily reproducible, our preclinical studies suggest effects on the osteoblast compartment that warrant studies in prospective human clinical trials. These data along with observations of patients treated with bisphosphonate for a prolonged period experiencing low-energy or spontaneous bone fractures and delayed healing likely related to an extreme suppression of bone turnover (44–46) serves as a cautionary to the use of prolonged and high doses of bisphosphonate in the treatment of bone disease. It is possible that the increased bone fragility is related to accumulation of microdamage, as postulated by some authors (47, 48), although not confirmed (49). It is also possible that this increased fragility is a result of a supraphysiologic increase in the degree of mineralization of the bone matrix.

In conclusion, despite the limitations of our study including the use of young mice, and relatively short treatment duration, we have shown that high doses of ZA interfere with both osteoblast and osteoclast lineages, resulting in a possible inhibition of both cell types. Most importantly, high doses of ZA have effects on trabecular and cortical bone morphology and function. Exposure to ZA results in increased bone stiffness associated with increased brittleness resulting in altered biomechanical properties. These observations will prompt the use of lower doses or shorter duration of bisphosphonate, and/or their combination with novel anabolic agents to counter their purely catabolic effects with the goal of restoring bone homeostasis in the treatment of cancer bone disease in the future.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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