

Research Paper

Bisphosphonate Modulation of the Gene Expression of Different Markers Involved in Osteoblast Physiology: Possible Implications in Bisphosphonate-Related Osteonecrosis of the Jaw

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Abstract

The aim of the present study was to elucidate the role of osteoblasts in bisphosphonates-related osteonecrosis of the jaw (BRONJ). The specific objective was to evaluate the effect on osteoblasts of two nitrogen-containing BPs (zoledronate and alendronate) and one non-nitrogen-containing BP (clodronate) by analyzing modulations in their expression of genes essential for osteoblast physiology. Real-time polymerase chain reaction (RT-PCR) was used to study the effects of zoledronate, alendronate, and clodronate at doses of 10^{-5} , 10^{-7} , or 10^{-9} M on the expression of Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-1, BMP-2, BMP-7, TGF- β 1, VEGF, TGF- β 1, TGF- β 2, and TGF- β 3 by primary human osteoblasts (HOBs) and MG-63 osteosarcoma cells. Expression of these markers was found to be dose-dependent, with no substantive differences between these cell lines. In general, results demonstrated a significant increase in TGF- β 1, TGF- β 1, TGF- β 2, TGF- β 3, and VEGF expressions and a significant reduction in RUNX-2, Col-1, OSX, OSC, BMP-2, BMP-7, ALP, and RANKL expressions, while OPG expression varied according to the dose and cell line. The results of this *in vitro* study of HOBs and MG-63 cell lines indicate that low BP doses can significantly affect the expression of genes essential for osteoblast growth and differentiation and of genes involved in regulating osteoblast-osteoclast interaction, possibly by increasing TGF- β 1 production. These findings suggest that osteoblasts may play an important role in BRONJ development, without ruling out other factors.

Key words: bisphosphonates, osteoblast, BRONJ, gene expression, TGF- β 1

Introduction

Bisphosphonates (BPs) are the first-line treatment for osteoporosis, Paget's disease, multiple myeloma, and malignant hypercalcemia, among other bone disorders [1]. Randomized controlled clinical trials have demonstrated the effectiveness of these drugs, but they have also been implicated in the

development of BP-related osteonecrosis of the jaw (BRONJ) [2, 3].

Among other possible etiologies, BRONJ has been associated with reduced bone turnover and consequent accumulation of microfractures, avascular necrosis due to anti-angiogenic effects, and impaired

viability of fibroblasts and oral keratinocytes [2, 4]. Our group previously demonstrated that high doses of BPs exert toxic effects on osteoblasts [5] and that low doses of these drugs reduce their differentiation capacity [6, 7].

There are two major types of BPs, those that contain nitrogen and those that do not, with distinct molecular action mechanisms and different therapeutic indications [8]. BRONJ development has been related to both nitrogen-containing (e.g. alendronate, zoledronate, or ibandronate) and non-nitrogen-containing (e.g., clodronate) BPs [9, 10].

Osteoblasts play an essential role in bone physiology through their participation in bone formation and turnover and in bone tissue repair. The maturation and function of this cell population is highly complex, involving autocrine, paracrine, and endocrine factors [11].

Despite 25 years of clinical research on BPs, the mechanism of their action on osteoclasts and osteoblasts remains unclear, although evidence has emerged that BPs may interact with them by modulating the expression of osteoblast-synthesized osteoclastogenic factors [12]. Adequate bone metabolism requires the correct functioning of the osteoblast-osteoclast relationship. Among other mechanisms, this process involves the complex formed by the receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) and the release of matrix-derived osteogenic growth factors, e.g., transforming growth factor β 1 (TGF- β 1) [13–16], all of which can be altered by BP administration.

The objective of this study was to evaluate the effect of two nitrogen-containing BPs (zoledronate and alendronate) and one non-nitrogen-containing BP (clodronate) on osteoblasts by analyzing their gene expression of bone morphogenetic protein 2 (BMP-2), and BMP-7, vascular endothelial growth factor (VEGF), and TGF- β 1 and TGF- β receptors (TGF- β R1, TGF- β R2; TGF- β R3). The role of the osteoblast in BRONJ development was also explored by analyzing the effects of these BPs on the expression of the following osteoblast differentiation markers: runt-related transcription factor 2 (Runx-2), alkaline phosphatase (ALP), type I collagen (Col-I), osterix (OSX), OPG, RANKL, and osteocalcin (OSC).

Material and Methods

Osteoblast isolation and culture

Osteoblasts were isolated, characterized, and cultured from bone sections obtained in the course of mandibular surgery from three Caucasian patients (2 female and 1 male) aged between 20 and 30 yrs. The independently processed sections were thoroughly

washed in phosphate-buffered saline (PBS, pH 7.4) to remove the marrow and were then seeded onto culture dishes (Falcon Labware, Oxford, UK) in Dulbecco's modified Eagle medium (Sigma Chemical Co., St. Louis, MO) containing 20% fetal calf serum (FCS). Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Confluent monolayers were obtained after 3-6 weeks. These cultures allowed three highly pure human osteoblast (HOB) cell lines to be obtained (one per patient), with proliferating osteoblastic cells overgrowing other possible contaminant cells. Cells were detached from the culture flask with a solution of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA), and they were washed and suspended in complete culture medium with 20% FCS. Finally, the cells were characterized as described by Reyes-Botella et al. [17] and García-Martínez et al. [18]

Human MG-63 osteosarcoma cell line (MG-63) was purchased from American Type Cultures Collection (ATCC, Manassas, VA) and maintained as described above with 10% FCS. This cell line is commonly used as an osteoblast model because it shares the same characteristics with osteoblasts.

All procedures performed in this study involving human participants were in accordance with the ethical standards of the ethical committee of the University of Granada (reference no. 721) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Treatments

HOB and MG-63 cell lines were treated with two nitrogen-containing BPs, zoledronate (Sigma-Aldrich, St. Louis, MO) and alendronate (Sigma), and one non-nitrogen-containing BP, clodronate (Sigma-Aldrich) at doses of 10⁻⁵, 10⁻⁷, or 10⁻⁹ M, which are within the therapeutic dose range [7], for 24 h.

RNA extraction and cDNA synthesis (reverse transcription)

After 24 h of culture with BP treatment (untreated cells served as controls), cells were detached from the culture flask using 0.05% trypsin-EDTA solution (Sigma) and individually harvested. mRNA was extracted using a silicate gel technique in the QiagenRNeasy extraction kit (Qiagen Inc., Hilden, Germany), which includes a DNase digestion step. The amount of extracted mRNA was measured by UV spectrophotometry at 260 nm (Eppendorf AG, Hamburg, Germany), and contamination with proteins was determined according to the 260/280 ratio. An equal amount of RNA (1 μ g of total RNA in 40 μ l of total volume) was reverse-transcribed to cDNA and amplified by PCR

using the iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA), following the manufacturer's instructions.

Real-time polymerase chain reaction (RT-PCR)

Primers (Table 1) were designed using NCBI-nucleotide library and Primer3-design to detect mRNA of Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-1, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF-βR2, and TGF-βR3. All were matched to the mRNA sequences of target genes (NCBI Blast software).

Final results were normalized using ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as stable housekeeping genes [19, 20].

Quantitative RT-PCR (q-RT-PCR) was conducted using the SsoFast™ EvaGreen® Supermix Kit (Bio-Rad laboratories) and following the manufacturer's instructions. Samples were amplified in 96-well microplates in an IQ5-Cycler (Bio-Rad laboratories) at a specific annealing temperature for each gene, ranging from 60 to 65 °C, and at an elongation temperature of 72 °C over 40 cycles. PCR reactions were carried out in a final volume of 20 µL, with 5 µL of cDNA sample and 2 µL of each primer. Ct values were plotted against log cDNA dilution to construct standard curves for each target gene. After each RT-PCR, a melting profile was created and agarose gel electrophoresis was conducted in each sample to rule out nonspecific PCR products and primer dimers. The comparative Ct method was employed for the relative quantification of gene expression. The mRNA concentration for each gene was expressed as ng of mRNA per average ng of housekeeping mRNAs. The cDNA from individual

cell experiments was analyzed in triplicate RT-PCR studies.

Statistical analysis

SPSS 22.0 (IBM, Chicago, IL) was used for the data analyses. mRNA levels were expressed as means ± standard deviation (SD). A two-tailed unpaired Student's t test was used for comparisons. At least three experiments were performed for all assays. P < 0.05 was considered statistically significant in all tests.

Results

Effect of BPs on gene expression of TGF-β1 and its receptors (TGF-β R1, TGF-β R2, and TGF-β R3)

Figure 1 displays q-RT-PCR results for the gene expression of TGF-β1 and its receptors (TGF-β R1, TGF-β R2, and TGF-β R3). In the HOB cell line, TGF-β1 expression was significantly decreased *versus* the control group after 24 h of treatment with the highest dose of alendronate (10⁻⁵ M) but not with zoledronate or clodronate at this dose. TGF-β1 expression was significantly increased in both cell lines at the lowest doses (10⁻⁷ and 10⁻⁹ M) of each BP. Results in figure 1 also show that the BP treatment of both cell lines produced a significantly increase in TGF-β R1 and TGF-β R2 expressions that was directly proportional to the reduction in dose. However, the expression of TGF-β R3 varied as a function of the BP, dose, and cell line, generally showing a significant increase at doses of 10⁻⁷ and 10⁻⁹ M in the MG-63 cell line and a significant decrease *versus* controls at the highest dose (10⁻⁹ M); in contrast, the only significant change in HOB cells was a post-treatment increase in TGF-β R3 expression at a dose of 10⁻⁹ M.

Table 1. Primer sequences for the amplification of cDNA by real-time PCR

Gene	Sense Primer	Antisense Primer	Amplicon (bp)
TGF-β1	5'-TGAACCGGCCTTTCCTGCTTCTCATG-3'	5'-GCGGAAGTCAATGTACAGCTGCCGC-3'	152
TGF-β R1	5'-ACTGGCAGCTGTCATTGCTGGACCAG-3'	5'-CTGAGCCAGAACCTGACGTTGTCATATCA-3'	201
TGF-β R2	5'-GGCTCAACCACCAGGGCATCCAGAT-3'	5'-CTCCCCGAGAGCCTGTCCAGATGCT-3'	139
TGF-β R3	5'-ACCGTGATGGGCATTGCGTTTGCA-3'	5'-GTGCTCTGCGTGCCTGCCGATGCTGT-3'	173
RUNX-2	5'-TGGTTAATC1CCGCAAGGCAC-3'	5'-ACTGTGCTGAAGAGGCCTGTTG-3'	143
VEGF	5'-CCTTGCTGCTCTACCTCCAC-3'	5'-CACACAGGATGGCTTGAAGA-3'	197
OSX	5'-TGCCTAGAAGCCCTGAGAAA-3'	5'-TTTAACTTGGGGCCTTGAGA-3'	205
BMP-2	5'-TCGAAATTC1CCCGTGACCAG-3'	5'-CCACTTCCACCAGCAATCCA-3'	142
BMP-7	5'-CTGGTCTTGTCTGCAGTGG-3'	5'-GTACCCCTCAACAAGGCTTC-3'	202
ALP	5'-CCAACGTGGCTAAGAATGTCATC-3'	5'-TGGGCATTGGTGTGTACGTC-3'	175
COL-1	5'-AGAACTGGTACATCAGCAAG-3'	5'-GAGTTTACAGGAAGCAGACA-3'	471
OSC	5'-CCATGAGAGCCCTCACACTCC-3'	5'-GGTCAGCCAACTCGTCACAGTC-3'	258
OPG	5'-ATGCAACACAGCACAACATA-3'	5'-GTTGCCGTTTTATCCTCTCT-3'	198
RANKL	5'-ATACCCCTGATGAAAGGAGGA-3'	5'-GGGGCTCAATCTATATCTCG-3'	202
UBC	5'-TGGGATGCAAATCTTCGTGAAGACCCTGAC-3'	5'-ACCAAGTGCAGAGTGGACTCTTCTGGATG-3'	213
PPIA	5'-CCATGGCAAATGCTGGACCCAACACAAATG-3'	5'-TCCTGAGCTACAGAAGGAATGATCTGGTGG-3'	256
RPS13	5'-GGTGTGACACAAGTACGTTTTGTGACAGGC-3'	5'-TCATATTTCCAATTGGGAGGGAGGACTCGC-3'	251

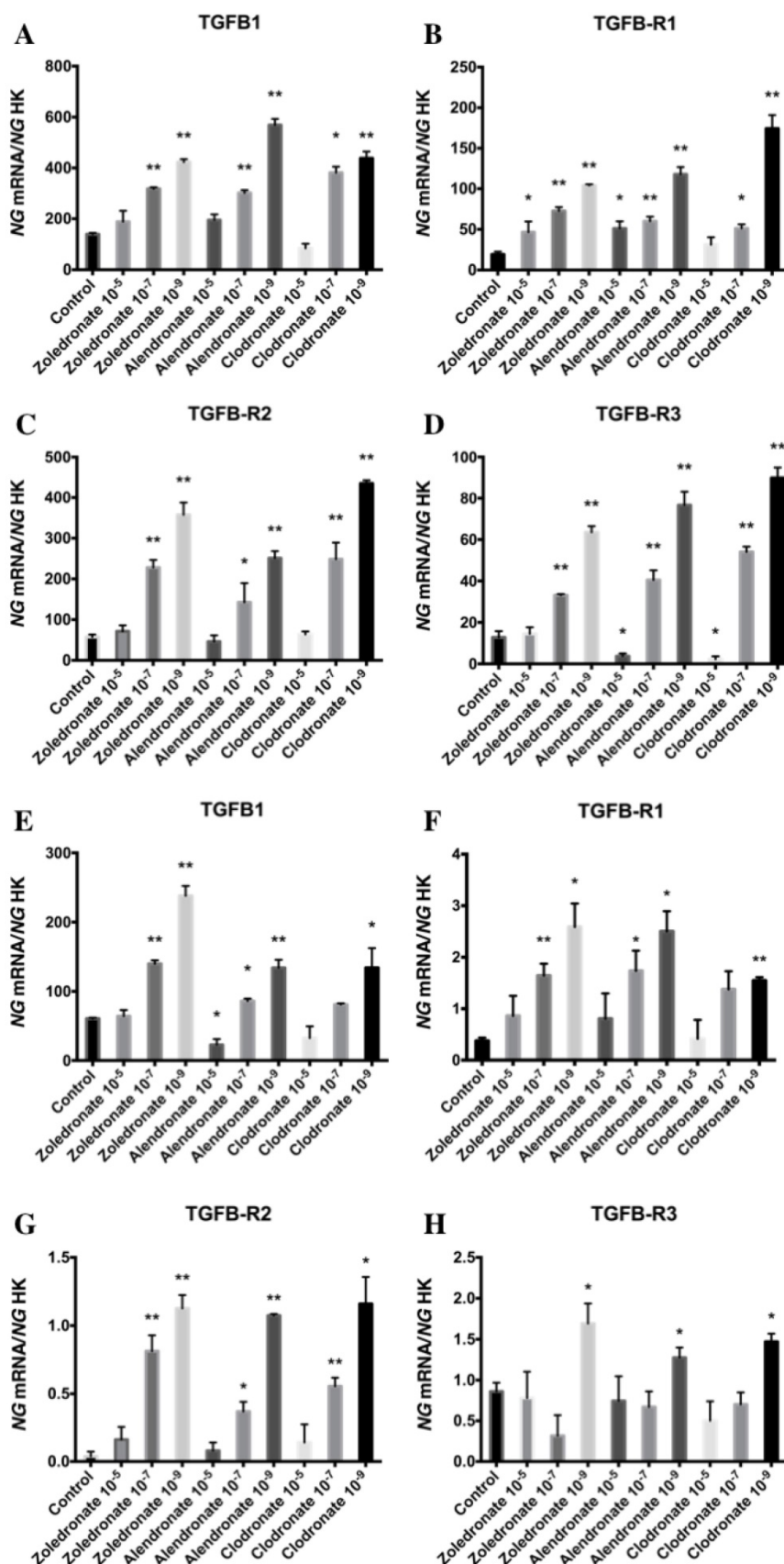


Figure 1. Expression of osteoblast genes (TFG-β1, TFGβR1, TFGβR2, and TFGβR3) treated for 24 h with zoledronate, alendronate or clodronate at doses of 10⁻⁵, 10⁻⁷, or 10⁻⁹ M. MG-63 cell line (A,B,C,D); HOB (E,F,G,H). Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. *p < 0.05, **p < 0.001

Effect of BPs on gene expression of RANKL-OPG complex

Figure 2 depicts q-RT-PCR results for the gene expression of RANKL and OPG. In the HOB cell line, RANKL expression was significantly decreased at higher doses of each BP, with a more marked reduction at 10⁻⁵ M. This decrease was also observed in the MG-63 cell line, although it varied according to the BP and dose used.

In the HOB cell line, OPG expression was significantly reduced at a BP dose of 10⁻⁵ M but was not affected at lower doses (10⁻⁷ and 10⁻⁹ M). In the MG-63 cell line, however, OPG expression was significantly increased by treatment with all three BPs (zoledronate, alendronate, and clodronate) at all three doses (10⁻⁵, 10⁻⁷, and 10⁻⁹ M).

Effect of BPs on the gene expression of Runx2, ALP, Col-I, OSX, and OSC

Figures 3 and 4 reports the q-RT-PCR results for the expression of osteoblast differentiation makers

Runx2, ALP, Col-I, OSX, and OSC. Treatment with each NP for 24 h reduced the expression of all of these genes, more markedly at the lowest doses (10⁻⁷ and 10⁻⁹ M). No differences in these results were observed between HOB and MG-63 cell lines.

Effect of BPs on gene expression of BMP-2, BMP-7 and VEGF

Figure 5 exhibits the q-RT-PCR results for the gene expression of BMP-2, BMP-7, and VEGF. In both cell lines, 24 h of treatment with each BP significantly reduced the expression of BMP-2 and BMP-7, with a more marked reduction at the lowest dose (10⁻⁹ M), and significantly increased the expression of VEGF *versus* controls at 10⁻⁷ and, more markedly, at 10⁻⁹ M.

Discussion

The results of this study demonstrate that the BPs zoledronate, alendronate, and clodronate can modulate the expression of genes involved in osteoblast growth and maturation and in

osteoblast-osteoclast interaction (RANKL-OPG). In general, low doses of these drugs increased the gene expression of important molecules for osteoblast growth (TGF-β1, TGF-βR1, TGF-βR2, TGF-βR3, and VEGF) and decreased the gene expression of molecules directly related to cell maturation (RUNX-2, Col-1, OSX, OSC, BMP-2, BMP-7, or ALP).

The TGF-β superfamily comprises more than 40 members, including TGF-βs, Nodal, Activin, and BMPs [21]. TGF-β signaling is critical for the regulation of osteoblast differentiation and

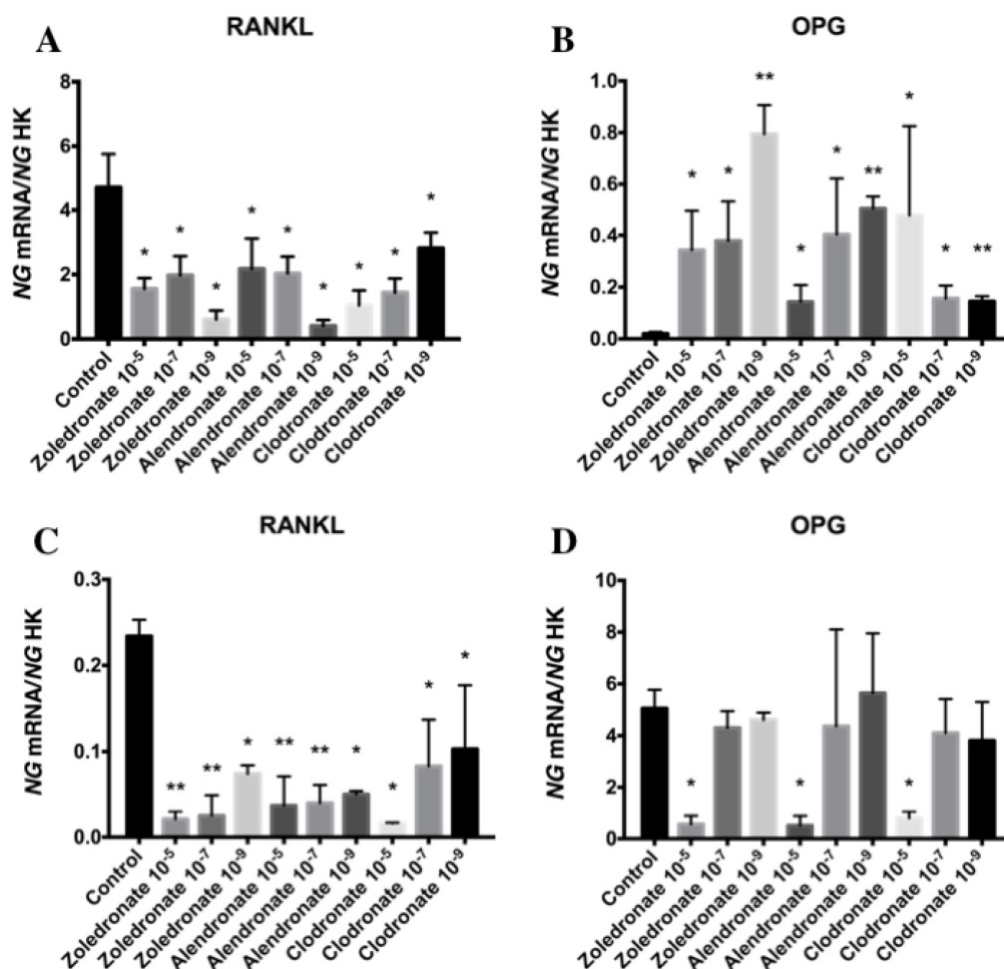


Figure 2. Expression of osteoblast genes (RANKL and OPG) treated for 24 h with zoledronate, alendronate or clodronate at doses of 10⁻⁵, 10⁻⁷, or 10⁻⁹ M. MG-63 cell line (A,B); HOB (C,D). Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. *p < 0.05, **p < 0.001

bone formation, and signaling relays in each stage are responsible for the final target gene expression [22, 23]. At low to moderately elevated levels, TGF- β 1 was reported to stimulate early osteoblast proliferation but inhibit terminal differentiation and mineralization [24–27]. Furthermore, the inhibition of TGF- β 1 signaling has been found to increase bone mass and improve bone quality [24, 27, 28]. Animal studies showed that a reduction in TGF- β 1 signaling enhanced bone stiffness in the 3-point bending test [24, 29, 30] and that inhibition of TGF- β 1 type I receptor kinase had anabolic and anticatabolic effects on bone, increasing both the mineral density and stiffness of bone [28]. According to the above data, TGF- β 1 signaling has detrimental effects on bone quality. In the present study, the expression of TGF- β 1 and its receptors was significantly increased after BP treatment at low doses (10^{-7} and 10^{-9} M), confirming previous reports that low BP doses increase the proliferation of osteoblasts and decrease their differentiation capacity [6, 7]. Taken together, these data indicate a relationship between the effect of BPs on osteoblasts and the development of BRONJ. In agreement with our findings, it was previously observed that treatment of osteoblasts with low doses of alendronate produced an early increase in their TGF- β 1/Smad3 expression, which may contribute to

the bone-preserving effects of BPs by maintaining osteoblast proliferation [31–33]. TGF- β 1 is also involved in the synthesis of RANKL, a member of the tumor necrosis factor (TNF) superfamily, which is produced and secreted by osteoblasts. RANKL stimulates osteoclasts *via* its receptor RANK, a membrane-bound protein present in osteoclasts and their precursors. The interaction between RANKL and RANK can be inhibited by OPG, a soluble protein also produced by osteoblasts [34–36]. TGF- β 1 is known to enhance matrix production and osteoblast differentiation while reducing the ability of osteoblasts to secrete RANKL; hence, TGF- β 1 indirectly limits further osteoclast formation and may reduce bone mass. According to the present results, BP treatment of osteoblasts leads to a reduction in their RANKL expression, which is probably related to the increased expression of TGF- β 1. In contrast, the effects on OPG expression differed between the cell lines, and a significant post-treatment increase was only observed in MG-63 cells. Our results suggest that BP treatment of osteoblasts may severely alter the RANKL-OPG complex, which would reduce bone resorption and turnover, giving rise to the accumulation of non-renewed and hypermineralized bone.

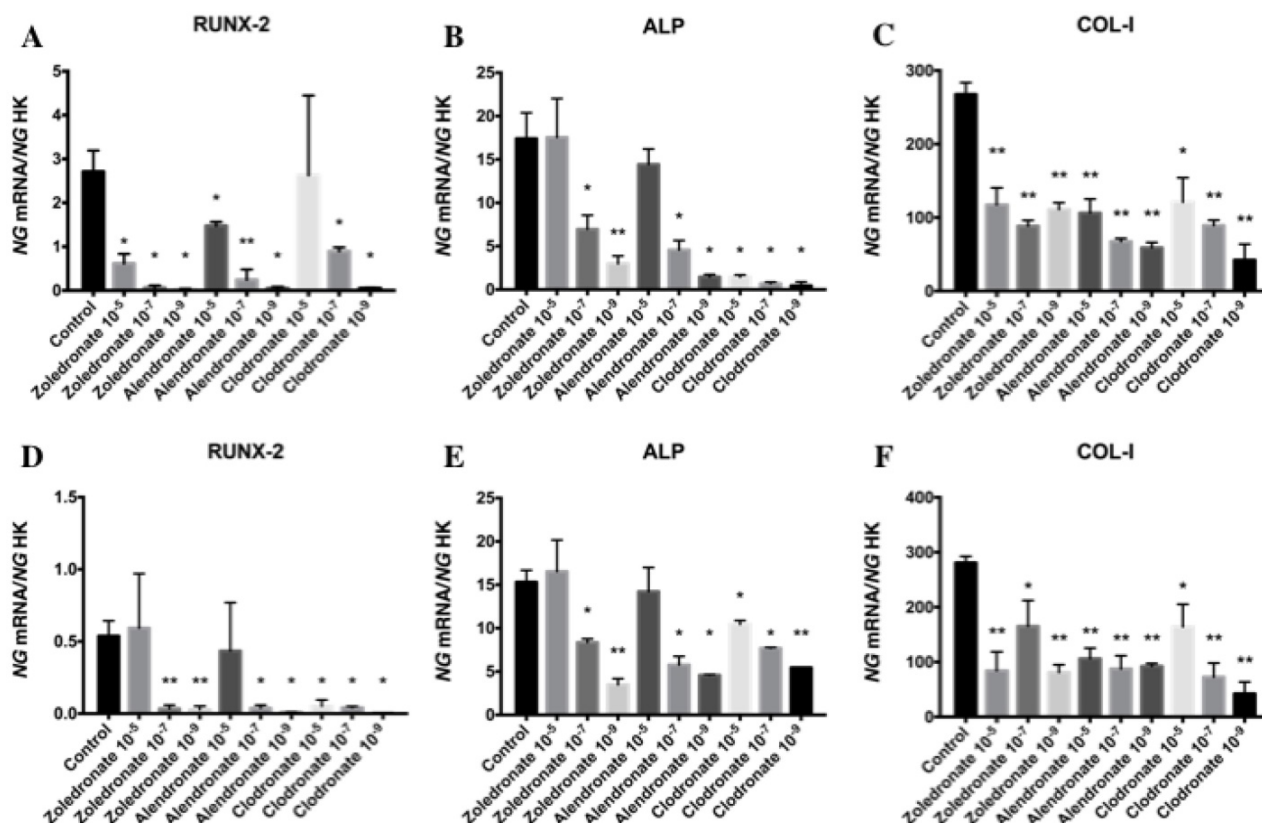


Figure 3. Expression of osteoblast genes (RUNX-2, ALP and Col-1) treated for 24 h with zoledronate, alendronate or clodronate at doses of 10^{-5} , 10^{-7} , or 10^{-9} M. MG-63 cell line (A,B,C); HOB (D,E,F). Data are expressed as ng of mRNA per average ng of housekeeping mRNAs \pm SD. Data are expressed as ng of mRNA per average ng of housekeeping mRNAs \pm SD. * $p < 0.05$, ** $p < 0.001$

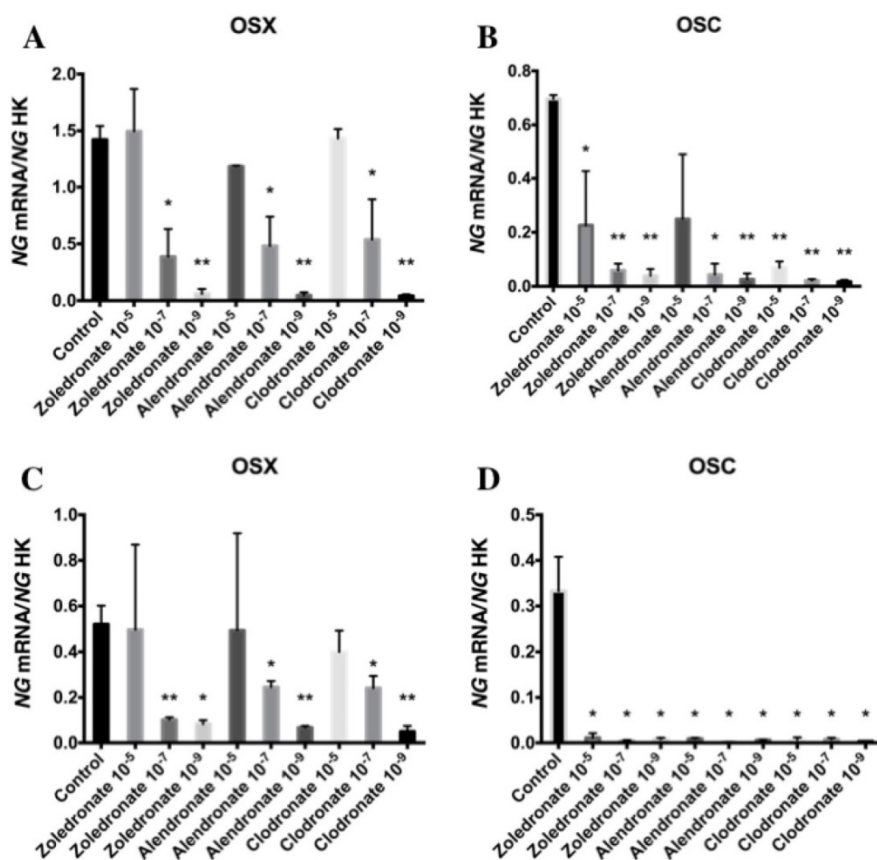


Figure 4. Expression of osteoblast genes (OSX and OSC) treated for 24 h with zoledronate, alendronate or clodronate at doses of 10⁻⁵, 10⁻⁷, or 10⁻⁹ M. MG-63 cell line (A,B); HOB (C,D). Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. *p < 0.05, **p < 0.001

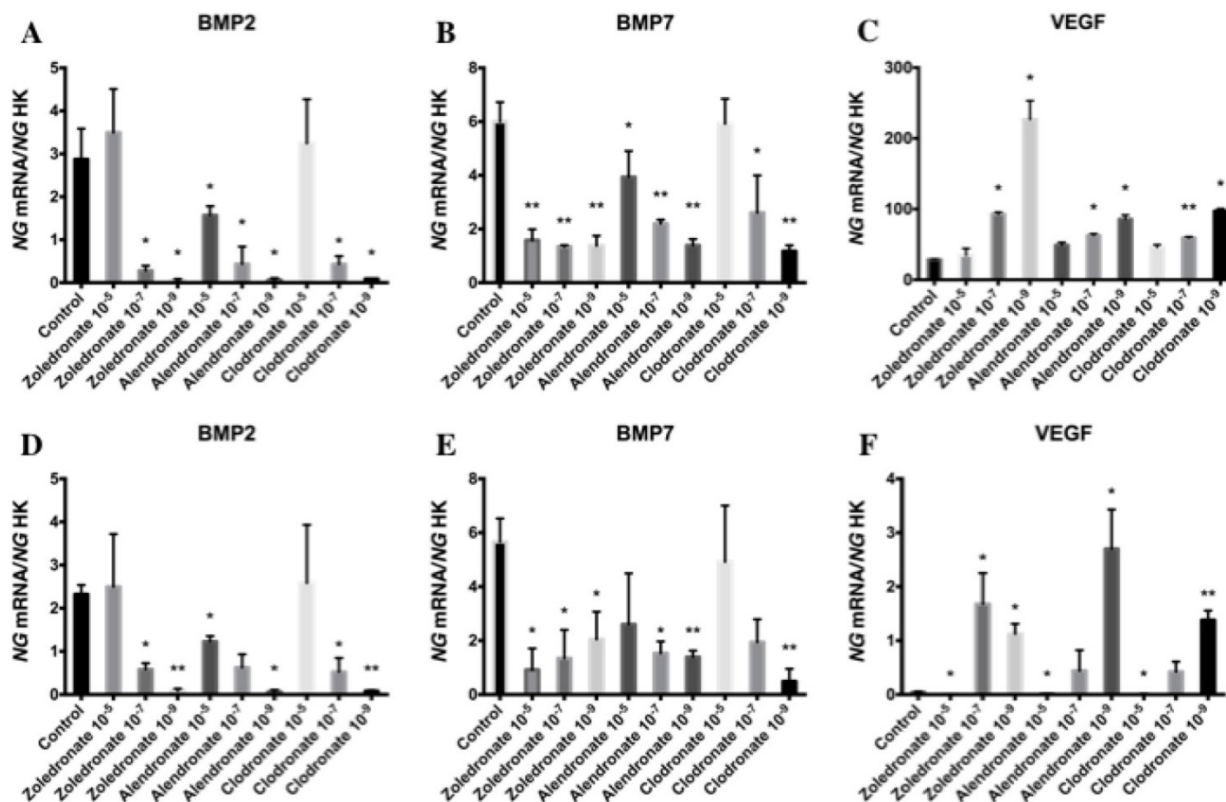


Figure 5. Expression of osteoblast genes (BMP-2, BMP-7 and VEGF) treated for 24 h with zoledronate, alendronate or clodronate at doses of 10⁻⁵, 10⁻⁷, or 10⁻⁹ M. MG-63 cell line (A,B,C); HOB (D,E,F). Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. *p < 0.05, **p < 0.001

The differentiation/maturation of osteoblastic cells follows a linear succession from osteoprogenitors to preosteoblasts, osteoblasts, and osteocytes. Osteoblasts pass through three functional stages *in vivo* and *in vitro*: proliferation, bone matrix synthesis/maturation, and mineralization. The membrane expression of specific function-related proteins (markers) has been observed on precursor cells during their differentiation [37, 38]. In the present study, low-dose BP treatment reduced the expression of BMP-2 and BMP-7. BMP-2 plays a major role in bone formation/remodeling and development and in osteoblast differentiation [39], inducing the expression of ALP and other osteoblastic markers and promoting calcium mineralization [40, 41]. The reduced expression of genes encoding these proteins would help to explain our previous observations on the capacity of BPs to inhibit osteoblast differentiation [6, 7].

Runx-2 is the prime marker of osteoblast differentiation. TGF- β 1 represses RUNX-2 expression through Smad3 to control extracellular matrix elastic modulus, a key determinant of bone material properties [24, 42]. Col-I and ALP expression is observed in the early stage of osteoblast differentiation and persists in early and mature osteoblasts [43]. Another essential transcription factor for osteoblast differentiation and bone formation is OSX, whose expression implies the loss of bipotentiality from preosteoblast to osteoblast and chondrocyte [44]. Our treatment of osteoblasts with low BP doses produced a significant reduction in the gene expression of these early differentiation markers. This treatment also reduced the expression by osteoblasts of OSC, a late differentiation marker, which appears at the start of mineralization and is an osteogenic marker of the final stages of osteoblast differentiation [43,45-47].

Osteonecrosis of the jaw related to alterations in angiogenesis was recently described in cancer patients treated with bevacizumab, an anti-angiogenic agent that inhibits VEGF, leading the American Association of Oral and Maxillofacial Surgeons (AAOMS) to update the term "BRONJ" to "medication-related osteonecrosis of the jaw" (MRONJ)[48]. The expression and production of pro-angiogenic factors such as VEGF and angiopoietin (ANG), which affect endothelial cell growth, migration, and vessel formation in many tissues, also play an important role in regulating vascular growth in the skeleton [49, 50]. These factors are produced by osteoblasts and osteocytes, among other bone cells [51, 52]. Although some authors have reported reduced VEGF expression in BP-treated osteoblasts [53, 54], we observed a significant increase

in VEGF production after 24 h of treatment with low BP doses, which may be related to the increased TGF- β expression observed. Thus, it was recently reported that TGF- β 1 stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells *via* Smad-independent pathways, including p38 mitogen-activated protein (MAP) kinase, p44/p42 MAP kinase, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathways, [55, 56].

These data and previous reports demonstrate the importance of dosage in the effect of BPs on osteoblasts [4-7]. Thus, low doses increase the proliferation and reduce the differentiation capacity of osteoblasts [6, 7]; whereas high doses lead their death by apoptosis [5]. Although therapeutic doses of BPs are low, high concentrations can accumulate in bone over long-term treatments but are inactivated by binding with hydroxyapatite; hence, medium acidification from infection can favor a new release and activation of BP that remains bound to hydroxyapatite crystals [57, 58], with the aforementioned consequences.

Within the limitations of an *in vitro* study, the results of gene expression in this study are relevant, although it would be interesting to perform more studies to show the effect of bisphosphonates on the protein expression of the genes studied.

In conclusion, the results of this *in vitro* study of HOBs and MG-63 cell lines indicate that low BP doses can significantly affect the expression of genes essential for osteoblast growth and differentiation and of genes involved in regulating osteoblast-osteoclast interaction, possibly by increasing TGF- β 1 production. These findings suggest that osteoblasts may play an important role in BRONJ development, without ruling out other factors.

Competing Interests

The authors have declared that no competing interest exists.

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