

RANK/RANKL/OPG signaling pathways in necrotic jaw bone from bisphosphonate-treated subjects

C. Di Nisio,¹ V.L. Zizzari,¹ S. Zara,¹
M. Falconi,² G. Teti,² G. Tetè,¹ A. Nori,³
V. Zavaglia,³ A. Cataldi¹

¹Dipartimento di Farmacia, Università di Chieti-Pescara G. d'Annunzio

²Dipartimento di Scienze Biomediche e NeuroMotorie (DiBiNeM), Università di Bologna

³S.O.D. di Odontostomatologia Chirurgica e Speciale, Azienda Ospedaliera Universitaria Ospedali Riuniti, Ancona, Italy

Abstract

Osteonecrosis of the jaw (ONJ) is a chronic complication affecting long-term bisphosphonate-treated subjects, recognized by non-healing exposed bone in the maxillofacial region. The pathophysiological mechanism underlying ONJ has not been fully elucidated. The aim of the present study was to investigate the role of RANK/RANKL/OPG signaling pathway and, in parallel, to evaluate angiogenic and matrix mineralization processes in jaw bone necrotic samples obtained from bisphosphonate-treated subjects with established ONJ. Necrotic bone samples and native bone samples were processed for Light and Field Emission in Lens Scanning Electron Microscope (FEISEM) analyses, for Real-Time RT-PCR to evaluate the gene expression of TNFRSF11A (RANK), TNFSF11 (RANKL), and TNFSF11B (OPG) and for immunohistochemical analyses of VEGF and BSP expression. Morphological analyses performed by Light microscope and FEISEM show empty osteocytic lacunae and alteration of lamellar organization with degradation of the mineralized bone matrix in necrotic bone samples. A significant increase in TNFRSF11A, TNFSF11, TRAF6 and NFAT2 gene expression, and a reduction of TNFSF11B gene transcription level compared is also showed in necrotic bone compared to control samples. No significant difference of VEGF expression is evidenced, while lower BSP expression in necrotic bone compared to healthy samples is found. Even if the pathogenesis of bisphosphonate-associated ONJ remains unknown, a link between oral pathogens and its development seems to exist. We suppose lipopolysaccharide produced by bacteria colonizing and infecting necrotic bone and the surrounding viable area could trigger RANK/RANKL/OPG signaling path-

way and, in this context, osteoclasts activation could be considered as a protective strategy carried out by the host bone tissue to delimitate the necrotic area and to counteract infection.

Introduction

Bisphosphonates (BPs) are synthetic analogues of pyrophosphate compounds, showing direct anti-tumor capabilities.¹⁻⁴ BPs are currently administered as anti-resorptive medications to manage hypercalcemia of malignancy, skeletal-related events accompanying bone metastases in the context of solid tumors, lytic lesions in multiple myeloma, Paget's disease of the bone, osteogenesis imperfecta, osteoporosis, and osteopenia.⁵⁻¹² BPs have high affinity for sites of active bone remodeling, where they suppress, both directly and indirectly, osteoclast-mediated bone resorption.¹³⁻¹⁵ The inhibitory effects of BPs on the bone-resorbing activity and on the development, proliferation and viability of macrophage lineage cells have been also reported.^{16,17} The specific type of BP administered, the dose of the medication, and the time over which the drug is prescribed, that is its final cumulative dose, play a role in the occurrence and the development of adverse reactions and complications, like the osteonecrosis of the jaw (ONJ), a chronic complication of BPs therapy.^{18,19} ONJ is clinically defined as an area of exposed bone in the maxilla or in the mandible that has failed to heal within a period of six to eight weeks in a patient currently or previously exposed to bisphosphonates and not subjected to radiation therapy in the craniofacial region.²⁰

Reports indicate a higher incidence of ONJ when high dose intravenous BPs are administered in patients with multiple myeloma (3.8%), in breast cancer patients (2.5%), or in prostate cancer patients (2.9%), while ONJ onset is rare in patients treated with oral BPs for osteoporosis.²¹ In non advanced stages, non-operative approach to lesions is preferred.²² In patients with advanced staged ONJ lesions, exposed and non-vital bone may be sequestered by surrounding bone tissue following bone infection.²³ Treatment strategies in these patients consist in eliminating pain, in controlling soft and hard tissue infection and minimizing the progression of bone necrosis. However, symptomatic patients, classified as Stage 3 according to Ruggiero *et al.*, may require bone resection or removal of bone sequestrum when present, in combination with antibiotic therapy, in order to reduce acute infection and pain and to facilitate soft tissue healing.^{24,25}

The pathophysiological mechanism underlying ONJ has not been fully elucidated,

Correspondence: Dr. Vincenzo Luca Zizzari, Dipartimento di Farmacia, Università di Chieti-Pescara G. d'Annunzio, via dei Vestini 31, 66100 Chieti, Italy.

Fax: +39.0871.3556368.

E-mail: vincenzoluca.zizzari@unich.it

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Key words: Bisphosphonate, osteonecrosis of the jaw, gene expression, RANK, RANKL, OPG.

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although its spontaneous occurrence suggests a multifactorial pathogenesis. Altered bone remodeling, over-suppression of bone resorption, angiogenesis inhibition, soft tissue BPs toxicity, occurrence of constant microtrauma, inflammation or infection, dento-alveolar surgery are hypothesized to have a role in the genesis and developing of ONJ.²⁶⁻³²

Bone remodeling is regulated by local and systemic stimulation of osteoclast differentiation and activation. The discovery of the role in osteoclastogenesis of the molecular system consisting of receptor activator nuclear factor kappa B (RANK), also known as tumor necrosis factor receptor superfamily, member 11a, NFKB activator (TNFRSF11A), of receptor activator nuclear factor kappa B ligand (RANKL), also known as tumor necrosis factor (ligand) superfamily, member 11 (TNFSF11), and of osteoprotegerin (OPG), also known as tumor necrosis factor receptor superfamily, member 11b (TNFRSF11B), has been crucial in order to elucidate several important processes regulating bone biology.³³ RANK, RANKL and OPG are essential for the regulation of different fea-

tures of osteoclast functions, including proliferation, differentiation, fusion, activation, and apoptosis.³⁴ In particular, the balance between OPG and RANKL has been already demonstrated to modulate bone formation and resorption.³⁵ In addition, Vascular Endothelial Growth Factor (VEGF) and Bone Sialoprotein (BSP) play an important role. VEGF is produced by endothelial cells and osteoblasts and is involved in healthy initial bone remodeling phases because it regulates osteoblast maturation, while BSP is a component of the extracellular matrix regulating healthy bone remodeling.^{36,37} The aim of the present study was to investigate the role of RANK/RANKL/OPG signaling pathway and, in parallel, to evaluate angiogenic and matrix mineralization processes in jaw bone necrotic samples obtained from BP-treated subjects with established ONJ.

Materials and Methods

Necrotic bone samples and native bone samples were obtained at S.O.D. di Odontostomatologia Chirurgica e Speciale, Azienda Ospedaliera Universitaria Ospedali Riuniti, Ancona, Italy. Necrotic bone samples were withdrawn from bone resections in three BP-treated subjects with established ONJ (age range 64-74 yrs), characterized by symptomatic, exposed, infected and necrotic bone, classified as Stage 3, according to Ruggiero *et al.*,²⁵ while native bone samples were obtained from three healthy patients (age range 35-42 yrs) undergoing impacted third molar extraction, as bone regularization prior suturing. The subjects with established ONJ included in this study had in common a history of at least two years of endovenous zolendronic acid assumption for preventing skeletal-related events accompanying bone metastases following surgical treatment of prostatic cancer. The healthy subjects included in this study, were male patients, showing no evidence of chronic systemic pathology after routinely clinical examinations and laboratory examinations, made prior the scheduled surgical intervention, no chronic drug assumption, no alcohol abuse, less than 10 cigarettes/die consumption. The study design was reviewed and approved by the local Ethical Committee. All patients received sufficient information about the inclusion in this study and gave written consent in accordance with Italian Legislation and with the code of Ethical Principles for Medical Research involving Human Subjects of the World Medical Association (Declaration of Helsinki).

After withdrawn, bone tissue samples were

processed for Light microscope and FEISEM analyses, for Real-Time RT-PCR to evaluate TNFRSF11A/TNFSF11/TNFRSF11B gene expression and for immunohistochemical analyses of Vascular Endothelial Growth Factor (VEGF) and Bone Sialoprotein (BSP).

Light microscopy and immunohistochemistry

Once withdrawn, bone tissues samples were fixed in 10% phosphate-buffered formalin for 72 hours, and decalcified in 10% tetrahydrated EDTA, according to data sheet (MIELODEC kit, Bio-Optica, Milan, Italy). Subsequently, they were dehydrated through ascending alcohols and xylene, and then paraffin embedded. Samples were then dewaxed (xylene and alcohol at progressively decreasing concentrations) and sliced 5 μ m thick and processed for haematoxylin-eosin staining and for immunohistochemical analyses. In order to detect VEGF and BSP proteins, immunohistochemical analysis was performed by means of Ultravision Detection System Anti-Polyvalent Alk-Phos/BCIP/NBT (Lab Vision Thermo, Fremont, CA, USA). Slides were incubated in the presence of rabbit VEGF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse BSP monoclonal antibody (Calbiochem, Darmstadt, Germany), and successively in the presence of specific secondary antibody. Phosphatase was developed using BCIP/NBT chromogen and nuclei were Nuclear Fast Red counterstained. Negative controls were performed by omitting the primary antibody. Randomly selected slides belonging to each sample were then observed by means of Leica DM 4000 light microscopy (Leica Cambridge Ltd., Cambridge, UK) equipped with a Leica DFC 320 camera (Leica Cambridge Ltd.) for computerized images.

Computerized morphometry measurements and image analysis

After digitizing the images obtained from the immunohistochemical stained sections, QWin Plus 3.5 software (Leica Cambridge Ltd.) was used to evaluate VEGF and BSP expression. Image analysis of protein expression was performed through the quantification of threshold area for immunohistochemical brown color, as an average value per ten fields, randomly chosen, for each sample at light microscope observation. Negative control images were randomly chosen. The statistical significance of the results was evaluated by the Wilcoxon, Mann-Whitney Test, using R Software, version 2.12.1 for Mac and setting $P=0.05$. After collecting results, the mean data were reported and showed in an histogram using Excel 2008 for Mac.

Field Emission in Lens Scanning Electron Microscope (FEISEM) analysis

Bone samples for SEM analysis were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4°C, washed in 0.1 M phosphate buffer, dehydrated in ascending graded series of ethanol, paraffin embedded and sliced 15 μ m thick. Paraffin was removed by xylene treatment and the samples were subsequently rehydrated by a descending graded series of ethanol. Then they were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 30 min, post fixed in 1% OsO₄ in 0.1M phosphate buffer for 30 min at room temperature, dehydrated in ascending graded series of ethanol and critical point dried (Critical point dryer CPD 030, Bal-Tec AG, Lichtenstein). The analysis was carried out with a FEISEM Jeol JSM 890 (Jeol LTD., Tokyo, Japan) at 70 kV accelerating voltage and 1×10^{-11} A probe current.

Total RNA extraction

To evaluate gene expression, a fragment from each bone sample was quickly washed with saline, frozen in liquid nitrogen and then crushed to the finest possible powder. The powder deriving from each sample was homogenized in 1 mL of TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C to remove the insoluble material. The supernatant was added to 200 μ L of chloroform, then shaken vigorously, incubated on ice for 15 min and centrifuged at 13,200 rpm for 20 min at 4°C. RNA in aqueous phase was precipitated with 500 μ L of isopropanol, stored for 30 min at -20°C and pelleted by centrifugation at 13,200 rpm for 20 min at 4°C. RNA pellet was washed with 75% ethanol, air dried and resuspended in RNase-free water. Contaminating DNA was removed using DNA-free kit (Life Technologies, Carlsbad, CA, USA). RNA concentration was determined by spectrophotometer reading at 260 nm and its purity was assessed by the ratio at 260 and 280 nm readings (BioPhotometer Eppendorf, Hamburg, Germany). In order to evaluate the quality of extracted RNA, the samples were tested by electrophoresis through agarose gels and visualized by staining with ethidium bromide, under UV light.

RT and real-time RT-PCR

High Capacity cDNA Reverse Transcription kit (Life Technologies) was used to reverse transcribe 1 μ g of bone RNA in a reaction volume of 20 μ L. Reactions were incubated in a 2720 Thermal Cycler (Life Technologies) initially at 25°C for 10 min, then at 37°C for 2 h and finally at 85°C for 5 min.

Gene expression was determined by quanti-

tative PCR using TaqMan probe-based chemistry. Reactions were performed in 96-well plates on an ABI PRISM 7900 HT Fast Real-Time PCR System (Life Technologies). TaqMan probes and PCR primers were obtained from Life Technologies (TaqMan Gene Expression Assays (20X): Hs00187192_m1 for TNFRSF11A; Hs00243522_m1 for TNFSF11; Hs00371512_g1 for TRAF6; Hs00542678_m1 for NFAT2; Hs00900358_m1 for TNFRSF11B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Life Technologies, Part No. 4333764F) was used as the housekeeping gene. Each amplification reaction was performed with 10 μ L of TaqMan Fast Universal PCR Master Mix (2X), No AmpErase UNG (Life Technologies), 1 μ L of primer-probe mixture, 1 μ L of cDNA and 8 μ L of nuclease-free water.

No-template control was used to check for contamination. A reverse transcriptase minus control was included for TRAF6 gene Assay-on-Demand.

Thermal cycling conditions were: 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 1 s and 60°C for 20 s. Sequence Detection System software, ver. 2.3 (Life Technologies) elaborated gene expression data. The comparative 2^{-MMCT} method was used to quantify the relative abundance of mRNA (relative quantification).³⁸ This method uses a calibrator sample to enable a comparison of gene expression levels in different samples. The obtained values indicate the changes in gene expression in the sample of interest by comparison with the calibrator sample, after normalization to the housekeeping gene. Real-time PCR analysis was performed in three independent experiments. In each experiment, we included one cDNA sample for each experimental condition. Amplification was carried out in triplicate for each cDNA sample in relation to each of the investigated genes.

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Gene expression data were collected from each sample used in the experimental procedure and means \pm SEM were determined for each experimental group. Values were analyzed by one-sample *t*-test. 1 (calibrator sample) was obviously considered the theoretical mean for the comparison. The level of statistical significance was set as $P < 0.05$.

Results

Light and Field Emission in Lens Scanning Electron Microscope (FEISEM) analysis

Morphological analysis, performed by light

microscopy after hematoxylin-eosin staining (Figure 1) shows empty osteocytic lacunae and partial alteration of lamellar organization in necrotic bone specimens compared to native bone samples. In fact, native bone samples showed a large trabecular net, with evidence of blood vessels and Haversian canals. By FEISEM observation, native bone section shows a compact structure (Figure 2A), where mineralized matrix components are not detected (Figure 2C). Necrotic bone shows a degraded mineralized matrix (Figure 2B) where irregular fibrillar structures and areas of different diameters are detected, suggesting an aspecific degradation of the mineralized bone matrix due to the necrotic process (Figure 2D).

Gene expression

Necrotic jaw bone deriving from BP-treated subjects shows a significant increase in TNFRSF11A, TNFSF11, TRAF6 and NFAT2 gene expression compared to control jaw bone. In fact, in necrotic bone, TNFRSF11A and TNFSF11 mRNA levels are about seven- and fourfold higher than in control bone, respectively ($P < 0.0001$ *vs* control) (Figure 3). TRAF6 gene expression increases of about 60% in necrotic bone when compared to control ($P = 0.0024$ *vs* control). NFAT2 mRNA levels are about tenfold higher in necrotic bone compared to control ($P < 0.0001$ *vs* control) (Figure 3). On the other hand, TNFRSF11B gene transcription

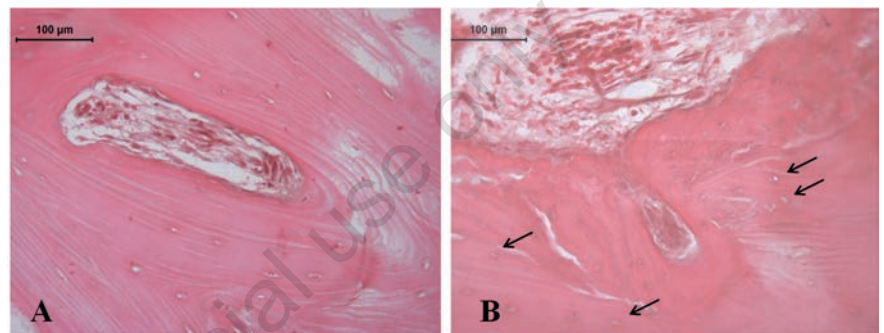


Figure 1. Hematoxylin and eosin staining of native (A) and necrotic (B) bone samples. Arrows indicate empty lacunae in necrotic bone samples.

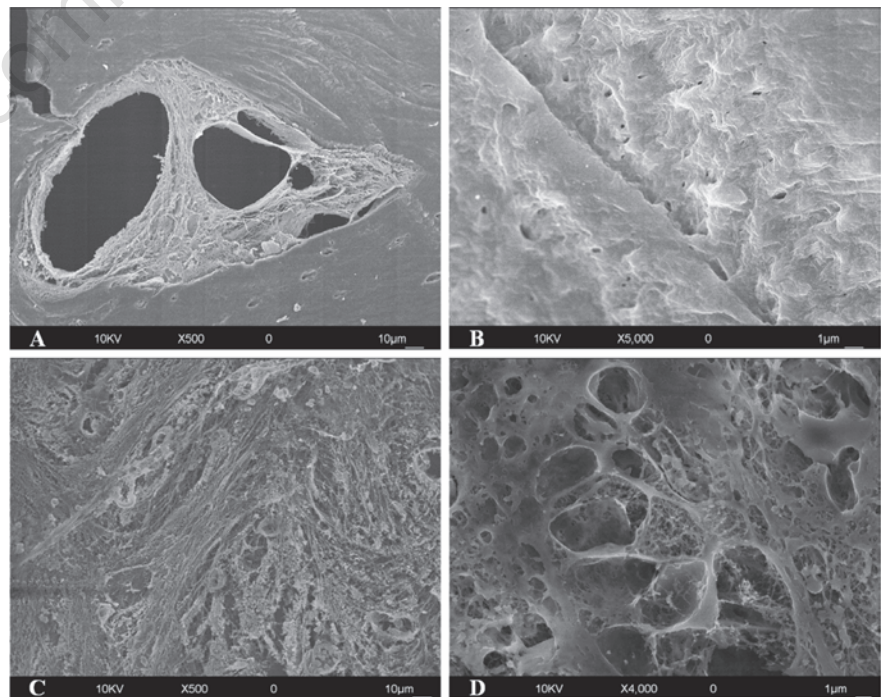


Figure 2. FEISEM analysis of native (A, B) and necrotic (C, D) bone samples, at different magnification.

level in necrotic bone is about twenty-three-fold lower than in control bone ($P < 0.0001$ vs control) (Figure 3).

Immunohistochemical analysis

No significant difference in VEGF expression between necrotic and native bone samples is noticed by immunohistochemical analysis. When BSP expression is evaluated, a lower positivity for this molecule in necrotic bone samples and strong BSP immunolabeling in healthy bone samples is found (Figure 4) ($P < 0.05$).

Discussion

Occurrence of ONJ in BP-treated patients is a side-effect that involves a multifactorial etiopathogenesis.¹⁹ It has been already reported that BPs act directly and indirectly, as bone resorption inhibitors, on osteoclastogenesis. However, to our knowledge, no data suggest the role of RANK/RANKL/OPG signaling pathways in the occurrence of necrosis in bone tissue.

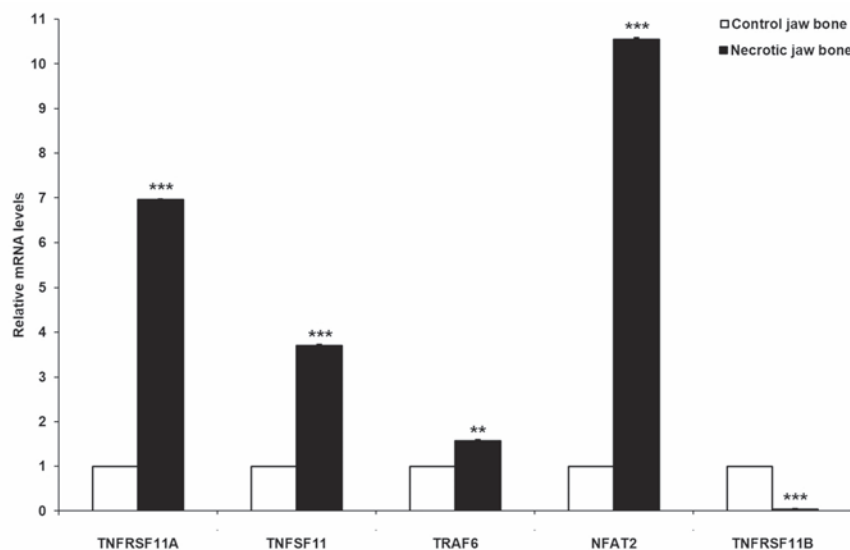


Figure 3. Relative gene expression of TNFRSF11A, TNFSF11, TRAF6, NFAT2 and TNFRSF11B in jaw bone, as determined by real-time RT-PCR. Bar graph represents the means \pm SEM of three independent experiments (** $P < 0.01$, *** $P < 0.001$ vs control). Y-axis, fold change.

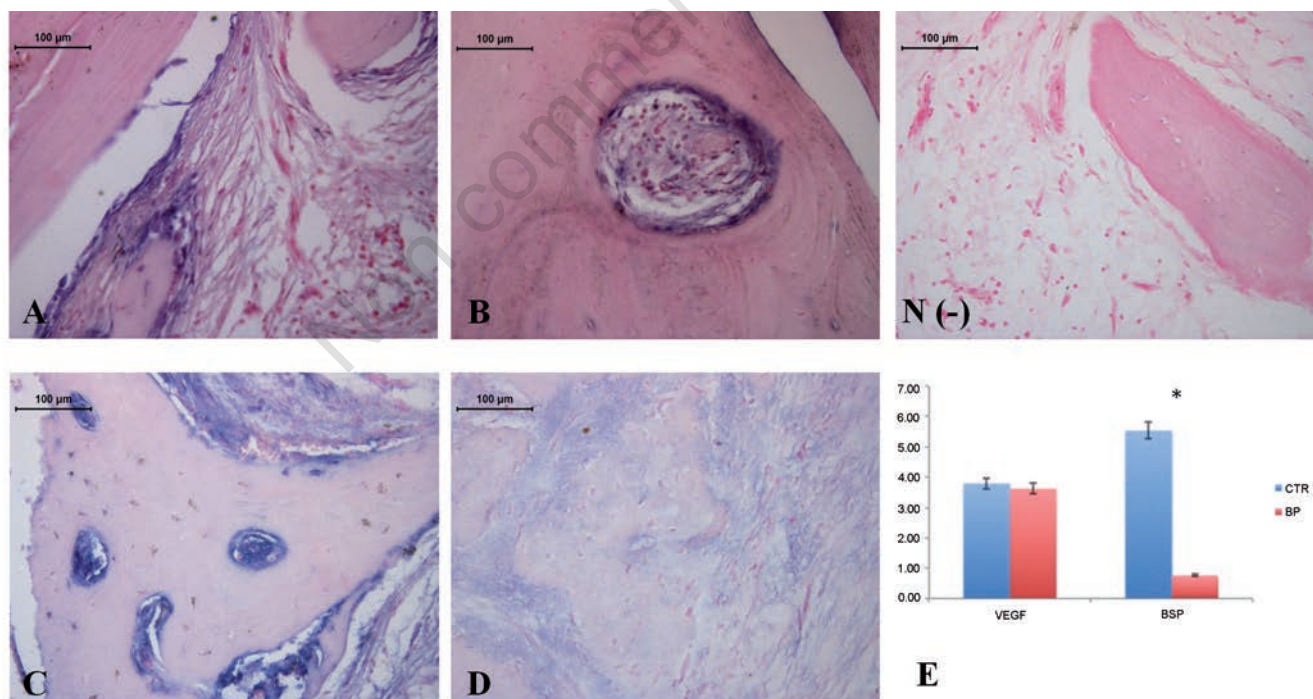


Figure 4. Immunohistochemical analysis of VEGF and BSP expression in native and necrotic bone samples. A) VEGF expression in bone tissue specimens obtained from healthy donors. B) VEGF expression in bone tissue specimens obtained from BP-treated donors. C) BSP expression in bone tissue specimens obtained from healthy donors. D) BSP expression in bone tissue specimens obtained from bisBP-treated donors. N(-) negative control. E) Graphic representation of VEGF and BSP positive area % (\pm SD) densitometric analysis determined by direct visual counting of ten fields (mean values) for each of five slides per specimen at 20x magnification; blue bars, native bone samples; red bars, necrotic bone samples; * $P < 0.05$.

TNFRSF11A is a central activator of nuclear factor kappa B (NF- κ B) transcription factor and is the signaling receptor for TNFSF11. TNFSF11/TNFRSF11A binding stimulates the development and activation of osteoclasts.^{39,40} Like other TNF family receptors, TNFRSF11A has no intrinsic protein kinase activating activity to mediate signaling. NF- κ B and c-Fos interact with the nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFAT2) promoter to trigger the auto-amplification of NFAT2.³⁹ TNFRSF11B is a molecule expressed by osteoblasts and regulates bone homeostasis by inhibiting osteoclastogenesis and bone resorption.⁴¹ TNFRSF11B, binding to TNFSF11, blocks TNFSF11/TNFRSF11A interaction thus inhibiting osteoclast precursor differentiation and reducing osteoclast production.⁴² Moreover, in such processes an important role is also played by angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF) and Bone Sialoprotein (BSP).

In our experimental model, necrotic jaw bone shows a significant increase in TNFRSF11A, TNFSF11, TRAF6 and NFAT2 gene expression compared to control bone. Such a result suggests an enhanced osteoclast differentiation and activation, probably in viable areas in the context of necrotic bone, which could induce a bone resorption increase. In particular, NFAT2 mRNA levels are about tenfold higher in necrotic bone compared to control: this might confirm the NF- κ B- and c-Fos-mediated auto-amplification of NFAT2 in the presence of ONJ, at least in part contributing to elucidate several molecular mechanisms underlying ONJ in BP-treated patients. Moreover, TNFRSF11B gene transcription level in necrotic bone is significantly lower than in control bone, allowing us to hypothesize an osteoclastogenesis induction, probably modulated also by a TNFRSF11B reduction. Osteoclasts have been detected by Hanses *et al.*⁴³ in bone tissue specimens derived from a group of patients treated with BP and showing ONJ. Osteoclasts are surrounded by lacunae as sign of bone resorption. The presence of osteoclasts at the site of bone resorption strongly suggests that these cells are involved in the osteolysis mechanisms.⁴³ The presence of osteolytic lacunae, visible both at light and electron microscope, and data related to the gene expression in necrotic bone samples could underlie, at least in part, Hansen's results. It might be surprising that increased osteoclasts number and histological evidence of osteoclastic activity are found in patients treated with inhibitors of osteoclastic function and osteoclast recruitment.⁴⁴ However, this could be explained as an effort of the host tissue towards the necrotic tissue, addressed to delimitate it. In the above mentioned study, authors have found *Actinomyces* colonies at

the site of necrotic bone exhibiting remarkable signs of erosion. In fact, the presence of *Actinomyces* colonies in contact with vital bone represents a consistent histological finding in bone specimens from patients with BP-associated osteonecrosis.^{43,44} Infection by bacterial colonies is considered both as a contributing factor the genesis of ONJ and a complication. A recent study has highlighted that, in rats injected with zoledronate, local stimulation with freeze-dried *Aggregatibacter actinomycetemcomitans* induces an extensive mandible osteonecrosis.⁴⁵ Tiranathanagul *et al.* have shown that *A. actinomycetemcomitans* lipopolysaccharide increases RANKL expression, but not OPG levels, in human periodontal ligament cells.⁴⁶ We could therefore hypothesize that, in our experimental model, the activation of RANK/RANKL signaling pathway in necrotic jaw bone deriving from BP-treated subjects leads to osteoclast differentiation and activation. Even if the pathogenesis of BP-associated osteonecrosis of the jaw remains unknown, a link between oral pathogens and development of bone pathology of the jaws seems to exist.⁴⁵ We suppose lipopolysaccharide produced by bacteria colonizing and infecting necrotic bone and the surrounding viable area could trigger, at least in part, RANK/RANKL/OPG signaling pathway. In this context, osteoclast activation could be a protective mechanism of the host bone tissue to delimitate the necrotic area and to eliminate infection. This could be confirmed by the expression of VEGF and BSP, detected through immunohistochemistry.

In our study, while the expression of VEGF is similar in bone samples obtained from BP-treated and healthy subjects, indicating the vascular support of the area does not modify, the reduction in BSP expression could be further associated to a reduction in osteoblastic activity in the necrotic area, consequent to osteoclast activation, and in general to an impairment of the physiological remodeling mechanisms. However, further studies are necessary in order to better understand the molecular mechanisms underlying RANK/RANKL/OPG signaling pathway activation in the bone resorption related to the osteonecrosis of the jaw.

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