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ORIGINAL ARTICLE

Immune cellular profile of bisphosphonate-related osteonecrosis of the jaw

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OBJECTIVES: Characterize the cell profile and immunostaining of proinflammatory markers in an experimental model of bisphosphonate-related osteonecrosis of the jaw (BRONJ).
MATERIALS AND METHODS: Male Wistar rats (n = 6–7) were treated chronically with saline solution or zoledronic acid (ZA) at 0.04, 0.20, and 1.00 mg kg^{-1} (1.4×10^{-7}, 6.9×10^{-6}, and 3.4×10^{-5} mol kg^{-1}), and subsequently, the first left inferior molar was extracted. Were performed counting of viable and empty osteocyte lacunae, viable and apoptotic osteoclasts, polymorphonuclear neutrophil, mast cells (toluidine blue), and the positive presence cells for CD68, tumor necrosis factor-alpha (TNF-α), IL (interleukin)-1β, inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF-kB) and IL-18 binding protein (IL-18 bp).
RESULTS: BRONJ was showed in ZA treated with 0.20 and 1.00 mg kg^{-1}. There is a dose dependent increase in percentage of empty osteocyte lacunae ($P < 0.001$) and apoptotic osteoclasts ($P < 0.001$), counting of total osteoclasts ($P = 0.003$), polymorphonuclear neutrophil cells ($P = 0.009$), cytoplasmic-positive cells of CD68 ($P < 0.001$), TNF-α ($P = 0.001$), IL-1β ($P = 0.001$), iNOS ($P < 0.001$), NF-kB ($P = 0.006$), and nuclear-positive cells of NF-kB ($P = 0.011$). Consequently, there is no difference in mast cells ($P = 0.957$), and IL-18 bp immunostaining decreases dose dependently ($P = 0.005$).
CONCLUSIONS: BRONJ is characterized by increases in immunostaining for proinflammatory markers and NF-kB and inversely associated with cells exhibiting IL-18 bp.

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Introduction

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a complicated adverse effect of treatment with bisphosphonates (BF), especially in patients undergoing oncology therapeutics. Some characteristics of this condition have been discovered and elucidated, but the pathogenesis is still uncertain (Allen and Burr, 2009).

The main studied mechanisms of BRONJ involve inhibitory effects of BF on bone turnover and angiogenesis. However, these purported mechanisms *per se* do not adequately explain the pathogenesis of BRONJ (Allen and Burr, 2009). BF have anti-angiogenic and antivasculogenic effects *in vitro* and *in vivo* (Ziebart *et al*, 2011; Misso *et al*, 2012), and this could contribute to the deficient prognosis of BRONJ (Lescaille *et al*, 2014). However, experimental murine models are not consistent with an association between BRONJ and the inhibition of vascularization or vascular proliferation markers (Sonis *et al*, 2009; Aguirre *et al*, 2010; Kobayashi *et al*, 2010; Marino *et al*, 2012).

On the other hand, infection is strongly associated with BRONJ, and antimicrobial approaches are generally utilized by dentistry clinics in association with surgical debridement. Although BF increase the adhesion of bacteria to the hydroxyapatite bone (Ganguli *et al*, 2005; Kobayashi *et al*, 2010), the isolated use of antimicrobial agents without removal of necrotic bone does not promote healing of BRONJ (Hoefert and Eufinger, 2011). The participation of infection in the pathogenesis of BRONJ has not been observed in any experimental model addressing

this parameter (Sonis *et al*, 2009; Kobayashi *et al*, 2010; Maahs *et al*, 2011; Conte Neto *et al*, 2013). Despite the use of antimicrobial agents shows conflicting efficacy in the modification of serum and the local microbial profile of patients with BRONJ (Ji *et al*, 2012), why does antibiotic prophylaxis seems to be an acceptable option to prevent BRONJ in patients treated with high doses of BF (Montefusco *et al*, 2008;)?

Lopez-Jornet *et al* (2011) did not observe a relationship between BRONJ and infection, but the authors reported that infected animals had intense inflammatory infiltrated in exodontic locations, implying the development of BRONJ. Acute inflammation induces osteocyte apoptosis and inhibits the vitality and activity of osteoblasts (Gilbert *et al*, 2000; Cheung *et al*, 2011; Huang *et al*, 2011; Kawai *et al*, 2012). Proinflammatory cytokines also induce osteoclastogenesis (Assuma *et al*, 1998) and increase important modulators of the vitality of bone cells such as reactive oxygen species (ROS) (Halleen *et al*, 1999; Wimalawansa, 2010; Almeida and O'Brien, 2013).

Several experimental models (Sonis *et al*, 2009; Ali-Erdem *et al*, 2011; López-Jornet *et al*, 2011; Maahs *et al*, 2011; Marino *et al*, 2012) and clinical studies (Lesclous *et al*, 2009) have demonstrated associations between inflammation and BRONJ. BF increase production of acute inflammatory mediators *in vitro* (Muratsu *et al*, 2013) and *in vivo* (Norton *et al*, 2012) and modify the immunological and cellular profile of patients undergoing chronic treatment (Rossini *et al*, 2012; Welton *et al*, 2013). Therefore, a consistent characterization of the immune and cellular profile of BRONJ has not been described (Vasconcelos *et al*, 2012).

Thus, the aim of this study was to conduct histomorphometric and immunohistochemical studies to characterize the cellular profile and immunoreactivity of certain chemical mediators.

Materials and methods

Animals, doses, and experimental protocols

Male Wistar rats (n = 6–7) were treated (three consecutive weeks) with intravascular (penile access) saline or zoledronic acid (ZA, molar mass = 290.1) at 0.04, (1.4×10^{-7} mol kg^{-1}), 0.20 mg kg^{-1} (6.9×10^{-6} mol kg^{-1}), and 1.00 mg kg^{-1} (3.4×10^{-5} mol kg^{-1}) (days 00, 07, 14, respectively). Four weeks after the last infusion of ZA or saline (day 42), the first left inferior molar was extracted (using Hollemback 3s and Lecron Zalle spatulas). During the following week (day 49), an additional ZA dose was infused. The animals were euthanized on the 70th experimental day, and their hemimandibles were fixed in neutral formalin 10% (Silva *et al*, 2015).

The approval for experimental use of laboratory animals was obtained from the local Ethics Committee on Animal Use (CEUA, former CEPA) (protocol 26/2013) and is in compliance with the Federal Law No. 11794 of October 8, 2008, and the Decree n° 6689, July 15, 2009, that regulated the law in 11 794, available from http: www.planalto.gov.brccivil03Ato2007–20102008LeiL11794.htm.

Histomorphometric and histochemical analysis

After decalcification (ethylenediaminetetraacetic acid 10%, pH 7.3), microscopic slides (4 μm) were prepared (conventional hematoxylin–eosin (HE) method) and qualitatively and histomorphometrically analyzed. Afterward, ten microscopic fields (400x) were used for counting of viable and empty osteocyte lacunae, total osteoclasts, viable and apoptotic osteoclasts, and polymorphonuclear neutrophil cells (Yamashita *et al*, 2011; Shaker *et al*, 2013).

Hydrated tissue sections (4 μm) were immersed in 0.1% toluidine blue solution (in 0.9% sodium chloride) per 60s for histochemical assessment (Gurgel *et al*, 2013) and mast cell counting using the same methodology (Shaker *et al*, 2013).

Immunohistochemical analysis

After deparaffinization and rehydration, the tissue sections (2.5 μm) were also submitted to immunohistochemical assessment. Antigenic recovery was performed by heating in citrate pH 6.0 solution. After cooling, the slides were submitted to peroxidase blocking with H_2O_2 3% solution diluted in PBS (phosphate-buffered saline) or with methanol solution according to the antibody's specificity (30 min).

After protein blocking (albumin) (1 h), we performed incubation with primary antibody CD68 (Dako®, Dopenhage, Denmark), tumor necrosis factor-alpha (TNF-α) (Abcam®, Cambridge, UK), IL (interleukin)-1β (Abcam®), inducible nitric oxide synthase (iNOS) (Abcam®), nuclear factor-kappa B (NF-kB) (SantaCruz®, Finnell Street Dallas, Texas, USA), or IL-18 binding protein (IL-18 bp) (SantaCruz®) using the dilutions and times shown in Table 1.

Envision System Plus-HRP (Dako®) (ready to use), Simple Stain Rat MAX PO (Multi) Universal Immuno-peroxidase Polymer (anti-mouse and anti-rabbit) (Histofine®) (ready to use) or secondary biotinylated anti-rabbit polyclonal IgG and ABC (avidin–biotin–peroxidase) System (SantaCruz Biotechnology®) (in diluted solutions) were utilized for the secondary antibody incubation. The visualization system used was 5,5-diaminobenzidine tetrahydrochloride (DAB) (Dako®) (Table 1).

Ten microscopic fields (400x) were used to count CD68 immunostaining mononuclear cells; cells exhibiting cytoplasmic positivity for TNF-α, IL-1β, iNOS, IL-18 bp, or NF-kB; and nuclear positivity for NF-kB in exodontic sites (Kim *et al*, 2012).

Statistical analysis

Kolmogorov–Smirnov normality testing was performed, and we utilized analysis of variance (ANOVA) followed by Tukey's post-test for group comparisons. The data were expressed as the mean and standard error of the mean (Mean±s.e.m.).

Results

ZA-induced experimental BRONJ

Twenty-eight days after tooth extraction, the saline solution group showed viable osteocytes and rare osteoclasts at the site of the exodontia's new bone (Figure 1a and 1e). The group treated with 0.04 mg kg^{-1} ZA presented delayed deposition of osteoid; however, the development of BRONJ was not observed, nor were there any inflammations signs or damaged osteoclasts (Figure 1b and 1f). The dose of 0.20 (Figure 1c and 1g) or 1.00 mg kg^{-1} (Figure 1d and 1h) ZA led to large bone sequesters associated with intense inflammatory infiltrate, osteoclasts with intracytoplasmic vacuolization, nuclear hyperchromatism, and necrotic bone with irregular peripheral resorption. No signs of healing or deposition of the mineralized bone matrix were observed.

Cellular profile of BRONJ

The groups treated with 0.20 ($32.5 \pm 1.1\%$) or 1.00 ($26.8 \pm 1.8\%$) mg kg^{-1} ZA showed a statistical increase in percentage of empty osteocyte lacunae compared with the saline group ($6.4 \pm 1.0\%$) and 0.04 ($14.2 \pm 3.1\%$) mg kg^{-1} ZA groups ($P < 0.001$) (Figure 1).

The total osteoclast number was greater in these two groups. Groups treated with 0.20 (31.4 ± 7.9) or 1.00 (39.0 ± 3.5) mg kg^{-1} ZA had a higher osteoclast number

Table 1 Parameters and methodologies of immunohistochemistry evaluation

Antibody	*Production*	*Antigenic recuperation Methodology (solution)*	*Peroxidase blocking H_2O_2 concentration (solution dilution)*	*Primary antibody*		*Secondary antibody*		*Revelation System (time)*	*Cell count*
				Dilution	*Time of incubation*	*System and/or dilution (Production)*	*Time of incubation*		
CD68	Dako®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:500	Overnight	Envision System Plus-HRP (Dako®)	30′	DAB (5′)	Mononuclear-positive cells
TNF-α	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:50	1 h	Universal Immuno-peroxidase Polymer (Histofine®)	30′	DAB (5′)	Positive cells
IL-1β	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:100	1 h	Universal Immuno-peroxidase Polymer (Histofine®)	30′	DAB (5′)	Positive cells
iNOS	Abcam®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:200	Overnight	Universal Immuno-peroxidase Polymer (Histofine®)	30′	DAB (5′)	Positive cells
NF-kB	SantaCruz®	Heat (Citrate pH 6.0)	H_2O_2 3% (Methanol)	1:200	Overnight	Secondary biotinylated anti-rabbit IgG 1:500 + ABC System (SantaCruz®)	30′ + 30′	DAB (10″)	Positive cells in cytoplasm and nucleus
IL-18 bp	SantaCruz®	Heat (Citrate pH 6.0)	H_2O_2 3% (PBS)	1:100	Overnight	Secondary biotinylated anti-rabbit IgG 1:200 + ABC System (SantaCruz®)	30′ + 30′	DAB (45″)	Positive cells

TNF-α, Tumor necrosis factor-alpha; IL-1β, interleukin-1beta; iNOS, induced nitric oxide synthase; NF-kB, nuclear factor-kappa B; IL-18 bp, interleukin-18 binding protein; HRP, horseradish peroxidase; ABC, avidin–biotin–complex; DAB, 5,5-diaminobenzidine tetrahydrochloride.

than the saline group (6.3 ± 1.3) ($P = 0.003$). There was no significantly difference between the saline and 0.04 mg Kg^{-1} (20.5 ± 3.2) groups. This augmentation was accompanied by the number of osteoclasts with apoptotic signs. Both groups with 0.20 (87.4 ± 2.0%) and 1.00 (79.8 ± 1.1%) mg kg^{-1} ZA showed a higher percentage of apoptotic osteoclasts than the saline (5.0 ± 5.0%) or 0.04 mg Kg^{-1} (11.8 ± 5.7%) group ($P < 0.001$) (Figure 1).

Regarding the cellular inflammatory profile, an increase in number of polymorphonuclear neutrophils was observed in the groups treated with 0.20 (213.3 ± 122.4) or 1.00 mg kg^{-1} (255.0 ± 45.0) ZA compared with the saline (0.0 ± 0.0) and 0.04 mg kg^{-1} ZA groups (9.5 ± 8.3) ($P = 0.009$). The number of mononuclear CD68-positive cells was significantly higher in the 0.20 (205.0 ± 31.3) and 1.00 (240.7 ± 11.3) mg kg^{-1} ZA groups than in the 0.04 mg Kg^{-1} ZA (86.5 ± 18.7) or saline (66.7 ± 5.8) group ($P < 0.001$). There was no statistically significant difference in mast cell number among the groups (saline: 30.5 ± 6.7, 0.04 mg kg^{-1} ZA: 30.3 ± 7.0, 0.20 mg kg^{-1} ZA: 34.0 ± 5.93, 1.00 mg kg^{-1} ZA: 29.8 ± 3.7) ($P = 0.957$) (Figure 1).

Immune inflammatory profile of BRONJ

There was a significant increase in cells exhibiting immunostaining for Th1 inflammatory markers (Figure 2).

The immunostaining for TNF-α was greater in the groups treated with 0.20 (3312.0 ± 180.8) or 1.00 (2912.0 ± 179.7) mg kg^{-1} ZA than in the saline group (1811.0 ± 145.0) ($P = 0.001$). However, there was no difference between the 0.04 (2325.0 ± 141.8) mg kg^{-1} ZA and saline groups (Figure 2).

The number of positive cells for IL-1β was higher in the 0.20 (5386.0 ± 459) and 1.00 (6359 ± 512.4) mg kg^{-1} groups than in the saline (2695.0 ± 104.2) group ($P = 0.001$). Nevertheless, a difference between the saline and 0.04 mg Kg^{-1} (3589.0 ± 477.6) groups was not observed. Similarly, positive cells for iNOS were augmented in the 0.20 (5436.0 ± 827.5) and 1.00 (6356.0 ± 523.7) mg kg^{-1} ZA groups compared with the saline (933.3 ± 396.1) or 0.04 ZA mg Kg^{-1} (1825.0 ± 159.4) group ($P < 0.001$) (Figure 2).

Cytoplasmic and nuclear positivity for NF-kB were higher in the groups with BRONJ. In the cytoplasm, the groups treated with 0.20 mg Kg^{-1} ZA (576.5 ± 86.7) or 1.00 mg kg^{-1} ZA (597.7 ± 135.6) showed elevated numbers of cells exhibiting positivity compared with the saline group (52.5 ± 45.5) ($P = 0.011$). There was no difference in immunostaining for cytoplasmic NF-kB between the saline and 0.04 mg Kg^{-1} ZA (157.0 ± 77.7) groups. Nuclear staining for NF-kB was significantly increased in the groups treated with 0.20 mg kg^{-1} ZA (84.3 ± 9.9) or 1.00 mg Kg^{-1} ZA (285.7 ± 115.4) compared with the saline group (8.7 ± 4.3) ($P = 0.006$). There was no significant difference between the saline group and the 0.04 mg Kg^{-1} (19.7 ± 7.1) or 0.20 mg Kg^{-1} ZA (84.3 ± 9.9) groups (Figure 2).

Immunostaining for IL-18 bp was increased in the group treated with 0.04 mg kg^{-1} ZA (418.6 ± 119.1) compared with the saline group (12.5 ± 6.6). A difference between the groups with 0.04 mg kg^{-1} ZA and 0.20 mg kg^{-1} ZA (203.3 ± 93.5) was not observed. Nevertheless, there was a significant decrease in positive cells for IL-18 bp in the group treated with 1.00 mg kg^{-1} ZA (105.8 ± 31.7) compared with the

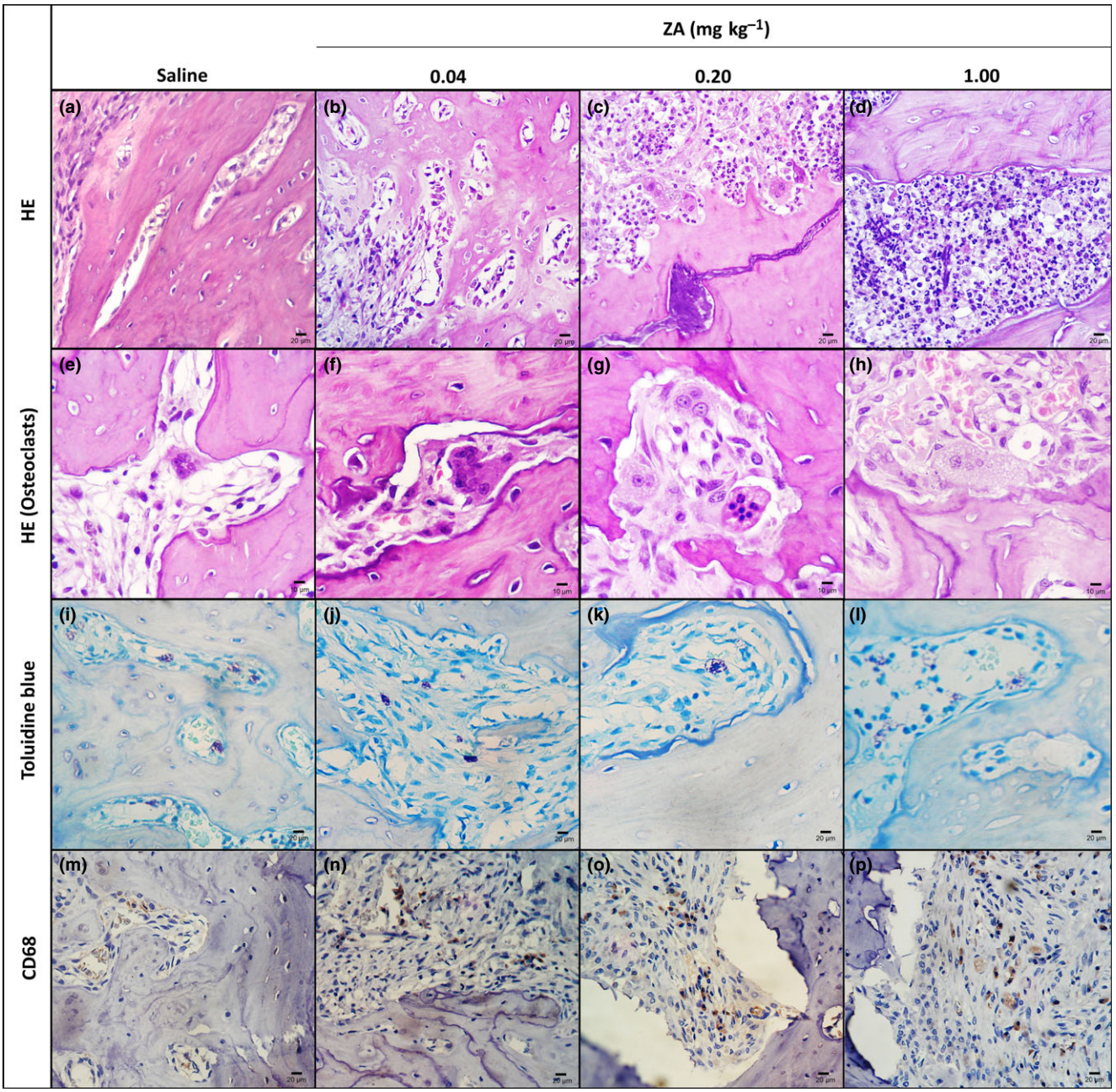

Figure 1 Cellular profile of BRONJ showing reduction in bone formation and viable osteocytes (**a**–**d**), increased number of osteoclasts (**e**–**h**) with apoptotic signs, no alteration in mast cells (**i**–**l**), and increased number of mononuclear CD68-positive cells (**m**–**p**) dose dependently (400×)

0.04 mg kg^{-1} ZA (418.6 ± 119.1) group ($P = 0.005$) (Figure 2).

Discussion

The participation of inflammation in BRONJ has been described since the first reports and research on the disease. However, the focus of the studies has been centered on the role of bacterial infection (Hinson *et al*, 2014). Although infection is importantly associated with BRONJ, antimicrobial therapy has a low level of success compared with the surgical approach in the treatment of this condition (Rupel *et al*, 2014).

More than 81.6% of cases of BRONJ are associated with inflammation. This is a value discreetly higher than the association between BRONJ and infection (80.3%) (Hinson *et al*, 2014). In experimental models with rats, BRONJ was not associated with infection but with intense inflammation (López-Jornet *et al*, 2011). Thus, immune dysregulation may play a key role in BRONJ pathogenesis.

BRONJ is directly associated to proinflammatory condition. This is a dose-dependent pathology associated with leukocytosis and changes of erythrocytes (Silva *et al*, 2015). It is demonstrated that low doses (0.0225 mg kg^{-1}) of ZA not induce BRONJ (Sonis *et al*, 2009) despite

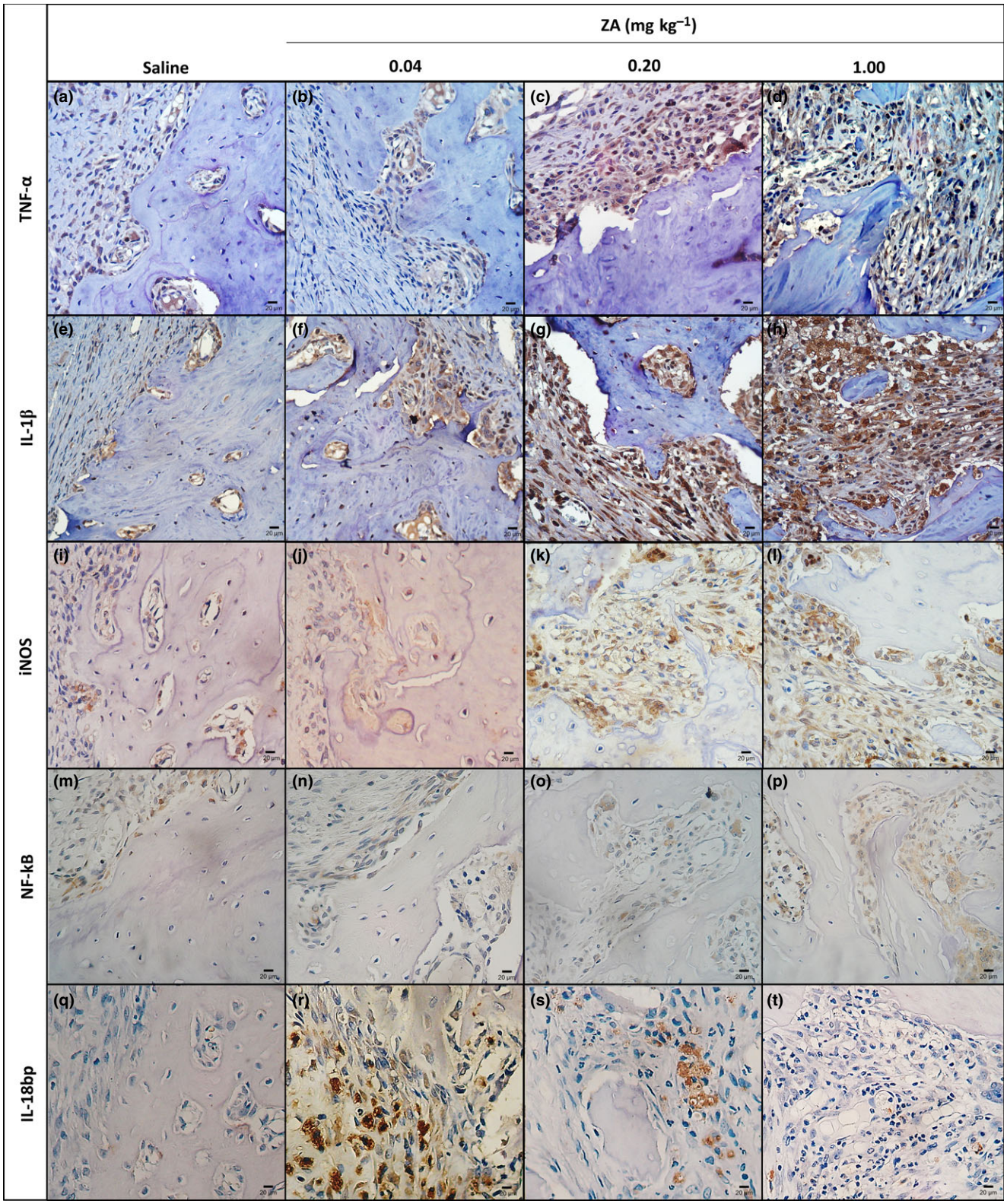

Figure 2 Cytokine profile showing dose-dependent increases in the number of TNF-α (**a**–**d**), IL-1β (**e**-**h**), iNOS (**i**–**l**), and NF-kB (nucleus and cytoplasm) (**m**–**p**)-positive cells. An increase in the number of IL-18 bp-positive cells was seen in the 0.04 mg kg^{-1} ZA group in comparison with the saline group, and a significant reduction was seen with a higher ZA dose (**q**–**t**) (400×)

proinflammatory stimulus is added (Marino *et al*, 2012). *In vivo* (classical peritonitis experiment) amino-bisphosphonates acutely upregulate proinflammatory cytokine production mediated by mast cells and the IL-1 receptor (IL-1R) (Norton *et al*, 2012). This cytokine, IL-1, is directly associated with BRONJ in the salival and gingival

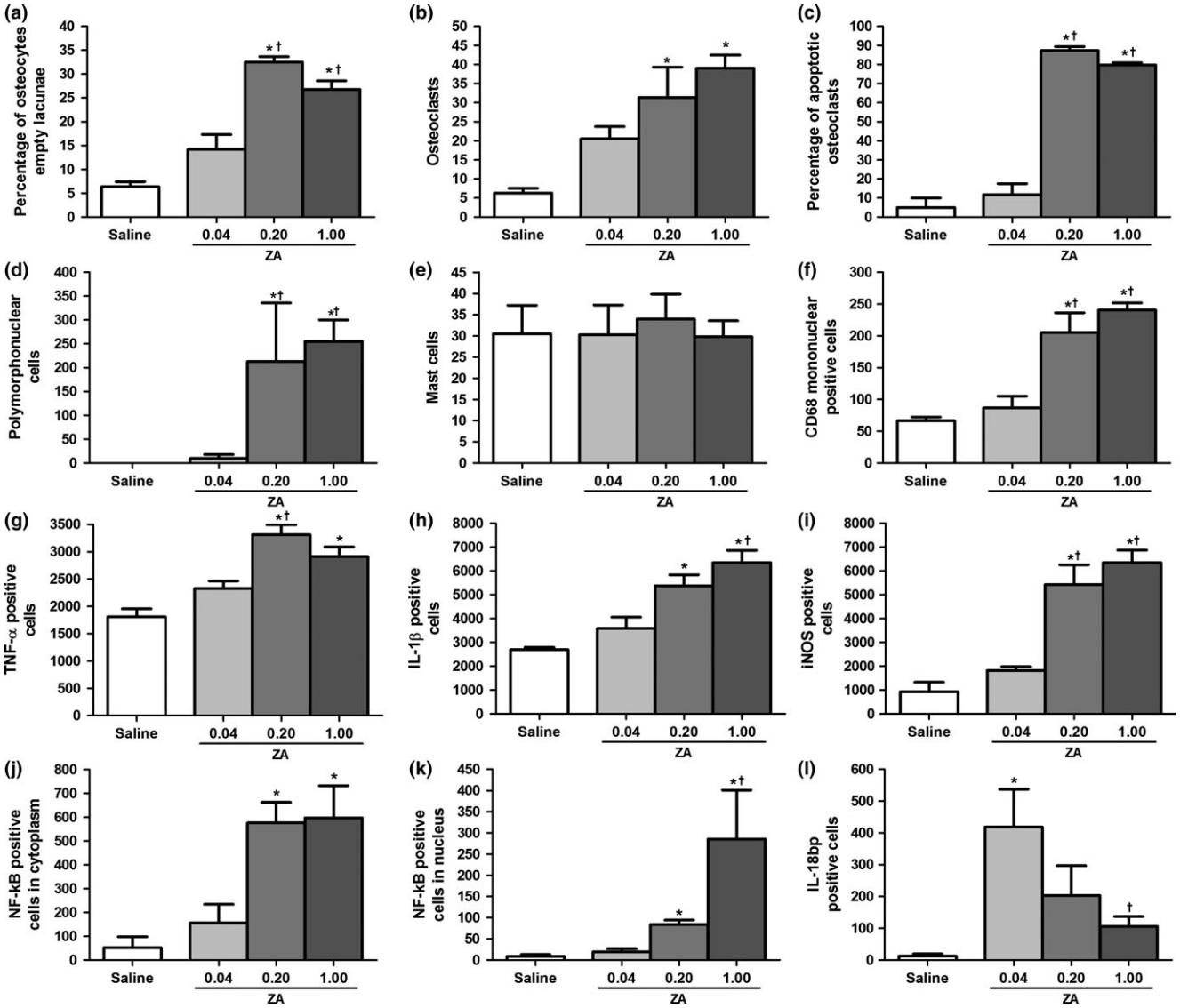

Figure 3 Cellular and cytokine profile analyses. *$P < 0.05$ versus saline, †$P < 0.05$ versus 0.04 mg kg^{-1} ZA (ANOVA test followed by Tukey's post-test; Mean±s.e.m.)

fluid of patients (Bagan *et al*, 2013; Tsao *et al*, 2013) such as oxidative stress markers (Bagan *et al*, 2014).

In this experimental model, mast cells were not augmented in number (Figure 3). In bone, these cells can release proinflammatory cytokines by degranulation, but they are not necessarily increased in number (Huang *et al*, 2013). On the other hand, there were increases in some acute inflammatory markers such as TNF-α, IL-1β, iNOS, NF-kB activation (Figure 2), and numbers of neutrophils and macrophages (positive CD68 cells) but not of mast cells. ZA is a strong myelotoxic drug (Kuiper *et al*, 2012), but it increases cytokine production by macrophages through NF-kB activation (Muratsu *et al*, 2013).

TNF-α (Cheung *et al*, 2011), IL-1β (Bonewald, 2004; Bakker *et al*, 2009), and high oxidative stress from iNOS activity (Almeida and O'Brien, 2013) modulate the increase in osteocyte apoptosis. The formation of apoptotic bodies in osteocytes is a stimulus for the recruitment and activation of macrophages and osteoclasts (Kogianni *et al*, 2008), which are important scavenger cells involved in recognition of these apoptotic bodies for phagocytosis and empty bone resorption (Oizumi *et al*, 2009; Harre *et al*, 2012).

In this study, the increase in empty osteocyte lacunae was accompanied by increases in macrophages, osteoclasts, TNF-α, IL-1β, and iNOS-positive cells, supporting these previous studies. However, as ZA strongly induces osteoclast apoptosis, interrupting physiological bone resorption (Oizumi *et al*, 2009), there was also an increase in apoptotic osteoclasts.

ZA and other BF (alendronate) inhibit TRAP function and infiltration of CD68-positive cells in bone (Xiong *et al*, 2010; Yamashita *et al*, 2011). However, BRONJ is a chronic condition strongly associated with tooth extraction. After a tooth is extracted, it increases TNF-α expression, osteoclastogenesis, bone resorption, and bone matrix deposition occur with a reduction in proinflammatory cytokines to basal levels after 10 days (Kim *et al*, 2012).

Nevertheless, the osteoclast apoptosis induced by ZA adds proinflammatory effects in connective tissue cells and seems to perpetuate and upregulate these proinflammatory mediators, leading to deregulated osteocyte apoptosis (Bonewald, 2004; Bakker *et al*, 2009; Cheung *et al*, 2011; Almeida and O'Brien, 2013), macrophage infiltration and activation (Kogianni *et al*, 2008; Harre *et al*, 2012), and more cytokine production (Muratsu *et al*, 2013).

Conversely, IL-18 bp shows an inverse association with the dose-dependent proinflammatory effects of ZA in bone of rats submitted to tooth extraction (Figure 2). IL-18 bp inhibits IL-8, a chemokine involved in the migration of neutrophils (Novick *et al*, 1999), which is a cellular group that can increase cytokine levels and cause damage to some tissues when it is augmented in number (Lima-Júnior *et al*, 2012).

IL-18 bp has antineutrophil effects and modulates the immune response. This mediator is a member of a binding protein family with anti-inflammatory and anti-osteoclastogenic effects, such as OPG. IL-18 bp prevents binding to the IL-18R receptor (31% similarity with IL-1R (Nakanishi *et al*, 2001), a receptor directly involved in BRONJ patients (Bagan *et al*, 2013) that mediates the immune transition of the Th1/Th2 response (Novick *et al*, 1999). In Th2 responses, osteoclastogenesis and bone resorption are inhibited (Horwood *et al*, 2001; Yamada *et al*, 2002; Morita *et al*, 2010). This process can attenuate bone diseases such as periodontitis (Orozco *et al*, 2007) and decrease inflammatory mediators and high oxidative stress (Shaker *et al*, 2013).

IL-18 bp is produced by plasma cells and B lymphocytes. It inhibits NF-kB activity (Novick *et al*, 1999) by interrupting the interaction between IL-18 and IL-18R. Afterward, there is a breakdown activation cascade of MyD88, IRAK1 and IRAK2, TRAF6, NIK, and IKK and a dissociation of IkB and NF-kB in the cytoplasm (Nakanishi *et al*, 2001). This is the major mechanism of ZA-induced proinflammatory cytokine production in macrophages (Muratsu *et al*, 2013).

There are no experimental models demonstrating an association between IL-18 and IL-18 bp in BRONJ. However, the late mediators that modulate the immune response seem to participate in BRONJ pathogenesis, such as overexpression of IL-17 (Li *et al*, 2013). IL-17 is a proinflammatory cytokine related to increases in some others mediators such as IL-1β, TNF-α, and iNOS produced by macrophages and other connective tissue cells (Lee, 2013). Other experimental models showed no alterations in inflammatory bone markers (RANK/RANKL/OPG axis) in animals treated with ZA (Vasconcelos *et al*, 2012). Thus, ZA seems to modify most strongly general inflammatory markers (TNF-α, IL-1β, IL-6 (Muratsu *et al*, 2013), and IL-17 (Li *et al*, 2013)) than inflammatory bone markers. Accordingly, the role of inflammatory cells and their general mediators seems to constitute a possible direction for understanding the pathogenesis of BRONJ.

In conclusion, BRONJ is characterized by increases in empty osteocyte lacunae; osteoclast numbers (total and apoptotic); polymorphonuclear neutrophils; CD68 mononuclear-positive cells; immunostaining for TNF-α, IL-1β, iNOS, and NF-kB (nuclear and cytoplasmic) but not mast cells. BRONJ is also inversely associated with cells exhibiting IL-18 bp. More studies are needed to show that BRONJ is dependent on immune modulation.

Author contributions

Paulo Goberlânio de Barros Silva and Camila Carvalho de Oliveira performed experiments, statistical analysis, and writing of the paper. Luiz André Cavalcante Brizeno and Deysi Viviana Tenazoa Wong were responsible for histochemical and immunohistochemical reactions. Roberto César Pereira Lima Júnior and Romélia Pinheiro Gonçalves were responsible for the pharmacological study design, review of the statistical analysis, and writing of the paper. Fabrício Bitú Sousa and Mário Rogério Lima Mota were responsible for the pathological study design, review text, and writing of the paper. Ronaldo de Albuquerque Ribeiro and Ana Paula Negreiros Nunes Alves designed the model, guided experiments, and reviewed the text. All the authors read and approved the final version.

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