



Influence of pH on osteoclasts treated with zoledronate and alendronate

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Abstract

Objectives The objectives of this study were to analyze the effect of pH on the growth and activity of osteoclasts treated with different doses of two nitrogen-containing BPs, zoledronate and alendronate.

Materials and Methods Murine osteoclasts cultured on dentine disks were treated with zoledronate (50 or 500 nM) or alendronate (500 or 5 μ M) at two different pH values (7.4 or 7.0). Osteoclasts were counted with transmitted light microscopy, apoptosis/necrosis was studied with flow cytometry and confocal microscopy, and resorption pit number and depth were calculated using reflected light and scanning electron microscopy.

Results The osteoclast count on dentine disks was significantly ($p < 0.001$) reduced by zoledronate or alendronate treatment at pH 7.0 in comparison to treatment with the same doses at pH 7.4 and untreated disks (controls). The percentage of apoptotic cells was significantly increased by treatment with 500 nM zoledronate or 5 μ M alendronate at pH 7.0 in comparison to the same doses at pH 7.4. The number and depth of resorption pits were significantly lower in disks treated at each BP dose studied than in untreated controls at pH 7.0.

Conclusions Zoledronate and alendronate at therapeutic doses have an adverse effect on the viability and resorptive activity of osteoclasts when the local medium pH is reduced.

Clinical relevance These findings suggest that periodontal or peri-implant oral cavity infection may be a key trigger of the cascade of events that lead to BRONJ.

Keywords Bisphosphonates · Osteonecrosis · Jaw · Activity · Growth · Apoptosis

Introduction

Bisphosphonates (BPs) are the first-line treatment for osteoporosis, Paget's disease, multiple myeloma, and malignant hypercalcemia, among other bone disorders [1], and randomized controlled trials have demonstrated their effectiveness. However, these drugs have also been implicated [2, 3] in the development of BP-related osteonecrosis of the jaw (BRONJ).

There are two major types of BP, those that contain nitrogen and those that do not, with distinct molecular action mechanisms and therapeutic indications [4]. BRONJ development has been related mainly to nitrogen-containing BPs, e.g., alendronate, zoledronate, or ibandronate [5, 6]. BRONJ has been associated with various possible etiologies, including reduced bone turnover and the consequent accumulation of microfractures, avascular necrosis due to antiangiogenic effects, impaired viability of fibroblasts and oral keratinocytes, and osteoblast physiology disorders [7–10].

Bone homeostasis is strongly influenced by the local pH. It has long been recognized that the skeleton contains a large

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reserve of alkaline mineral (hydroxyapatite), which is available to neutralize metabolic H^+ if the acid–base balance is not maintained within narrow limits. Bone cells are extremely sensitive to the direct effects of pH; thus, acidosis inhibits mineral deposition by osteoblasts but activates resorption of bone and other mineralized tissues by osteoclasts [11]. Cell culture experiments demonstrated that protons exerted a direct stimulatory effect on bone resorption by cultured rat osteoclasts [12]. Mature rat osteoclasts were almost inactive at pH 7.4, i.e., physiologic (blood) values, but resorption pit formation markedly increased at lower pH values, reaching a plateau at around pH 6.8. Subsequent studies showed that avian [13, 14] osteoclasts also exhibit acid-activation responses.

The fact that infectious conditions often precede BRONJ onset is consistent with recent proposals that local inflammation and associated pH changes may trigger the release and activation of nitrogen-containing BPs, ultimately resulting in necrosis [15]. BPs bind to bone at circumneutral pH and are released in an acidic milieu, as occurs in resorption lacunae during bone resorption, when acid pH increases the dissociation between BP and hydroxyapatite [16, 17]. In humans, an acid environment is frequently found in infections and inflammatory processes after surgery. In particular, jaw bones are constantly exposed to periodontal, periapical, and peri-implant infections and to surgical procedures that produce inflammation, such as dental extraction or implantation. All of these procedures may lower pH values, activating osteoclasts, initiating bone resorption, and thereby releasing BPs bound to hydroxyapatite in patients treated with these drugs. Hence, the pH may play a major role in the pathogenesis of BRONJ [17].

The objective of this study was to analyze the effect of pH on the growth and activity of osteoclasts treated with different doses of two nitrogen-containing BPs, zoledronate and alendronate.

Material and methods

Osteoclast isolation and culture

Murine osteoblasts were obtained by isolation and culture of bone marrow from MF1 mice aged 6–8 weeks provided by the Animal Experimentation Center of the University of Granada, following the methodology published by Orriss and Arnett [18].

Extremities of the mice were dissected to obtain the long bones, from which the bone marrow was extracted using an insulin syringe with 25-gauge needle and sterile PBS. Cells obtained were gathered by centrifugation, suspended in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL

penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin B (hereafter CMEM) and with 2.5 ng/mL M-CSF (R&D Systems Europe Limited, Abingdon, UK), and were then cultured in a 75-cm² culture flask for 24 h at 37 °C and 5% CO₂ to allow adherence of stromal cells to the flask. After 24 h, non-adhered cells were gathered by centrifugation and suspended in CMEM supplemented with 10 ng/mL M-CSF and 3 ng/mL RANKL (R&D Systems Europe Limited) at a final density of 5×10^6 cells/mL.

In vitro treatment

Murine osteoclasts were treated with 50 or 500 nM zoledronate (Sigma-Aldrich, St. Louis, MO) or with 500 nM and 5 μ M alendronate (Sigma-Aldrich). In brief, 200 μ L of the osteoclast suspension (at 5×10^6 cells/mL) was distributed in 96-well plates, whose well bases contained a 5-mm diameter dentine disk cut transversally with a microdrill from elephant ivory (donated by HM Revenue and Customs, Heathrow Airport, UK). Plates were incubated for 24 h at 37 °C to adhere osteoclast precursors to the dentine disks. The disks were then transferred to a 6-well plate with CMEM medium supplemented with 10 ng/mL M-CSF and 3 ng/mL RANKL and treated with the corresponding BP, adding alendronate (final dose of 5 μ M or 500 nM) or zoledronate (final dose of 500 or 50 nM). Wells without BPs were cultured as controls. The cultures were incubated for 6 days at 37 °C with 5% CO₂, renewing the culture medium (supplemented with the appropriate concentration of the corresponding BP) every 3 days. The culture medium was maintained at pH 7.4 for the first 6 days to enable osteoclast growth and was then reduced to pH 7.0 using 6 M HCl.

Effect of BPs on osteoclast growth

Effect on osteoclast count

Eight days after starting treatment with the corresponding BP and 48 h after acidifying the culture medium, the disks were gathered, washed with PBS, and fixed with glutaraldehyde at 2.5% for 5 min. Disks were stained for TRAP using a leukocyte acid phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions, and TRAP-positive multinucleate cells were then counted blindly on coded disks using transmitted light microscopy.

Effect on apoptosis and/or necrosis induction

Flow cytometry and immunofluorescence were used to determine the induction of apoptosis and/or necrosis in BP-treated osteoclasts by acidification of the culture medium.

Apoptosis and/or necrosis by flow cytometry analysis

Apoptosis and necrosis studies were performed as described by De Luna-Bertos et al. [19]. At 8 days of treatment with zoledronate or alendronate and 48 h after acidifying (pH 7.0) the corresponding culture medium, osteoclasts were detached from the culture flask, washed, suspended in 300 μ L PBS, and labeled with annexin V and propidium iodide (PI) (Immunostep S.L., Salamanca, Spain), incubating 100 μ L aliquots of the cell suspension with 5 μ L annexin V and 5 μ L PI for 30 min at 4 °C in the dark. Cells were then washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer with argon laser (Facs Vantage Becton Dickinson, Palo Alto, CA) at a wavelength of 488 nm to determine the percentage of fluorescent cells. The percentage of annexin-positive (apoptotic) cells and PI-positive (necrotic) cells was then calculated.

Immunofluorescence

At 8 days after treatment with zoledronate or alendronate and 48 h after acidifying the culture medium (pH 7.0), cells fixed in ice-cold methanol-acetone (1:1) were immunostained with annexin V and PI for 10 min and then washed with PBS for the detection of apoptotic cells, visualizing the immunostaining with a Leica Spectral confocal laser microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Effect of pH on the resorptive activity of osteoclasts treated with BPs

The effect of culture medium acidification (pH 7.0) on the resorptive activity of BP-treated osteoclasts was evaluated using reflected light and scanning electron microscopy (SEM) to determine the number and depth of resorption pits.

Number and depth of resorption pits

Eight days after treatment with zoledronate or alendronate and 48 h after acidifying the corresponding culture medium (pH 7.0), experiments were ended by washing disks in PBS, followed by their fixation in 2.5% glutaraldehyde for 5 min. Disks were then stained for TRAP using a leukocyte acid phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions. The number of resorption pits was estimated by using reflected light microscopy with a $\times 10$ objective, and the pit depth was estimated by adjusting the fine focus control (calibrated in microns) of the reflected light microscope. Both analyses were performed on coded disks in a blinded manner.

Scanning electron microscopy

The culture medium was maintained at pH 7.4 for the first 6 days to enable osteoclast growth and was then reduced to pH 7.0 using 6 M HCl for 48 h. Osteoclasts seeded on the dentine disks were dehydrated (50% ethanol, 2 h; 70% ethanol, 2 h; 100% ethanol, overnight) before being left to air dry. Images from the osteoclasts were taken using a JEOL 7401 scanning electron microscope (JEOL, Tokyo, Japan) at University College London.

Statistical analysis

SPSS 22.0 (IBM, Chicago, IL) was used for statistical analyses. Experimental data were analyzed with one-way ANOVA using the Bonferroni method to correct for multiple comparisons. Results are presented as means $\pm$ standard deviation. At least three experiments were performed for all assays. $P < 0.05$ was considered statistically significant in all tests.

Results

Effect of BPs on osteoclast growth

Effect on osteoclast count

Figure 1a shows a SEM image of BP-treated acid-activated mouse osteoclasts with resorption trails. In addition, Fig. 1b shows non-treated osteoclasts isolated from mouse bone marrow grown on dentine disks with M-CSF and RANKL for 8 days (pH 7.4). Cells were TRAP-stained and viewed by transmitted light (100 $\times$). Osteoclasts isolated from mouse bone marrow grown on dentine disks with M-CSF, RANKL, and zoledronate 500 nM for 8 days (pH 7.0) are shown in Fig. 1c. Cells were TRAP-stained and viewed by reflected light (100 $\times$).

Osteoclast count was lowest with the highest zoledronate dose (500 nM) at pH 7.0, being significantly lower than with the same dose at pH 7.4 ($p < 0.001$), with the dose of 50 nM zoledronate ($p < 0.001$), and with non-treatment (controls) ($p < 0.001$). Likewise, the osteoclast count after treatment with 50 nM zoledronate was significantly lower at pH 7.0 than at pH 7.4 ($p < 0.001$) and was significantly lower than after treatment with 500 nM zoledronate at pH 7.4 ($p < 0.001$) or non-treatment ($p < 0.001$), although it was significantly higher than after treatment with 500 nM zoledronate at pH 7.0 ($p < 0.001$) (Fig. 2).

Figure 2 also depicts that osteoclast count was lowest with 5 μ M alendronate at pH 7.0, being significantly lower than with the same dose at pH 7.4 ($p < 0.001$), the dose of 500 nM alendronate at pH 7.0 or 7.4 ($p < 0.001$), and

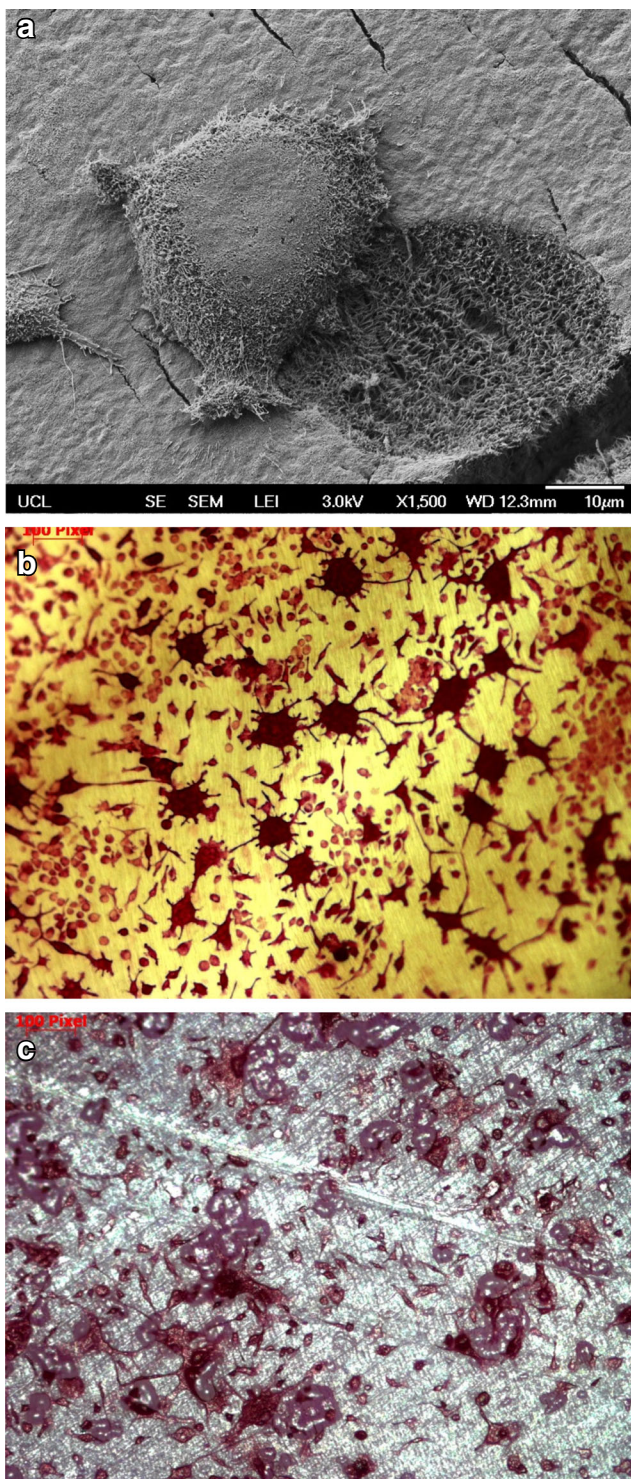


Fig. 1 SEM image of BP-treated acid-activated mouse osteoclasts with resorption trails. Note collagen fibers exposed after dissolution of the mineral component of the dentine disk by osteoclast action (a). Non-treated osteoclasts isolated from mouse bone marrow grown on dentine disks with M-CSF and RANKL for 8 days (pH 7.4). Cells were TRAP-stained and viewed by transmitted light (b, 100 $\times$). Osteoclasts isolated from mouse bone marrow grown on dentine disks with M-CSF, RANKL and zoledronate 500 nM for 8 days (pH 7.0). Cells were TRAP-stained and viewed by reflected light (c, 100 $\times$)

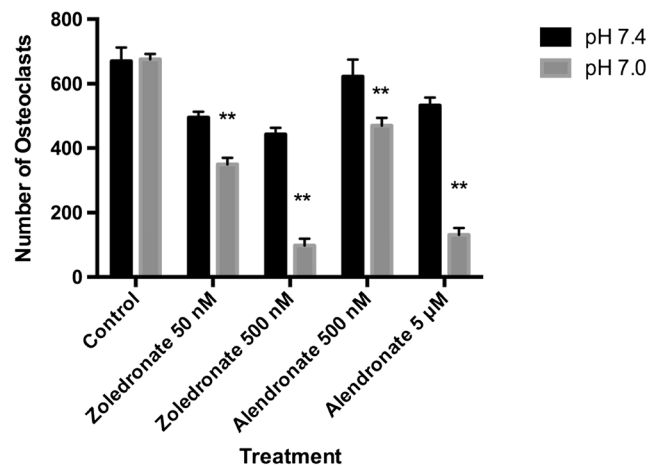


Fig. 2 Number of osteoclasts on dentine disks after culture with different doses of zoledronate and alendronate at pH 7.4 and 7.0. ** $p < 0.001$

with non-treatment ($p < 0.001$). Likewise, the osteoclast count after treatment with 500 nM alendronate was significantly lower at pH 7.0 than at pH 7.4 ($p < 0.001$) and in comparison to non-treatment ($p < 0.001$).

Apoptosis and necrosis analysis

Flow cytometry analysis

Flow cytometry with annexin V and PI labeling were used to discriminate between apoptotic and necrotic cell death. Figure 3 exhibits the percentage of apoptotic cells after culture with different doses of zoledronate and alendronate at pH 7.4 or 7.0. In all cases, treatment with each dose at pH 7.4 served as control for the same dose at pH 7.0.

Osteoclast treatment with 500 nM zoledronate at pH 7.0 significantly increased the percentage of apoptotic cells ($p = 0.003$) and reduced the percentage of viable cells with respect to the same dose at pH 7.4 ($p = 0.006$). However, no significant differences were found between pH 7.0 and pH 7.4 after treatment with 50 nM zoledronate.

Osteoclast treatment with 5 μ M alendronate at pH 7.0 significantly increased the percentage of apoptotic cells ($p = 0.03$) and reduced the percentage of viable cells with respect to the same dose at pH 7.4 ($p = 0.03$). However, no significant differences were found between pH 7.0 and pH 7.4 after treatment with 500 nM alendronate.

Immunofluorescence

Immunofluorescence was used to observe the apoptotic effect of zoledronate 500 nM and alendronate 5 μ M on osteoclasts. Annexin V expression was intense on the membrane of osteoclasts treated with zoledronate and

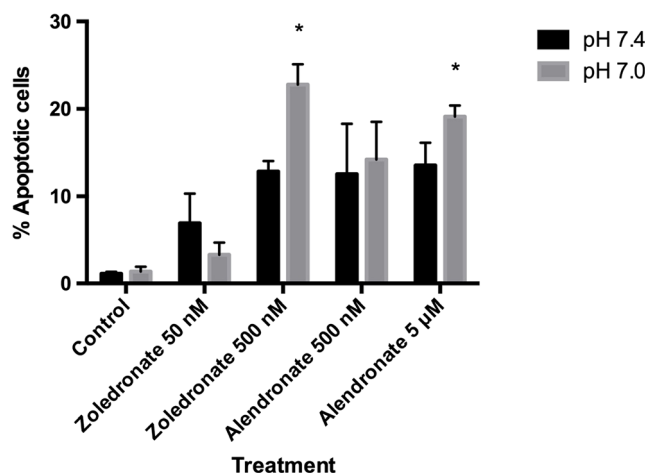


Fig. 3 Percentage of apoptotic cells after culture with different doses of zoledronate and alendronate at pH 7.4 and 7.0. * $p < 0.05$

alendronate at pH 7.0 with respect to controls (untreated osteoclasts) (Fig. 4).

Effect of BPs on osteoclast activity

Number and depth of resorption pits

All studied treatments significantly reduced the number of pits created by osteoclasts in the dentine disks with respect to controls ($p < 0.001$) at pH 7.0. The lowest number of pits was observed with the higher zoledronate dose (500 nM), followed by the higher alendronate dose (5 µM), lower zoledronate dose (50 nM), and lower alendronate dose (500 nM) at pH 7.0 (Fig. 5a).

At pH 7.0, all treatments significantly reduced the depth of the resorption pits created by osteoclasts on the dentine disks with respect to controls ($p < 0.001$) (Fig. 5b). The pit depth was lowest in disks treated with the higher zoledronate dose (500 nM), followed by the higher alendronate dose (5 µM), lower zoledronate dose (50 nM), and lower alendronate dose (500 nM).

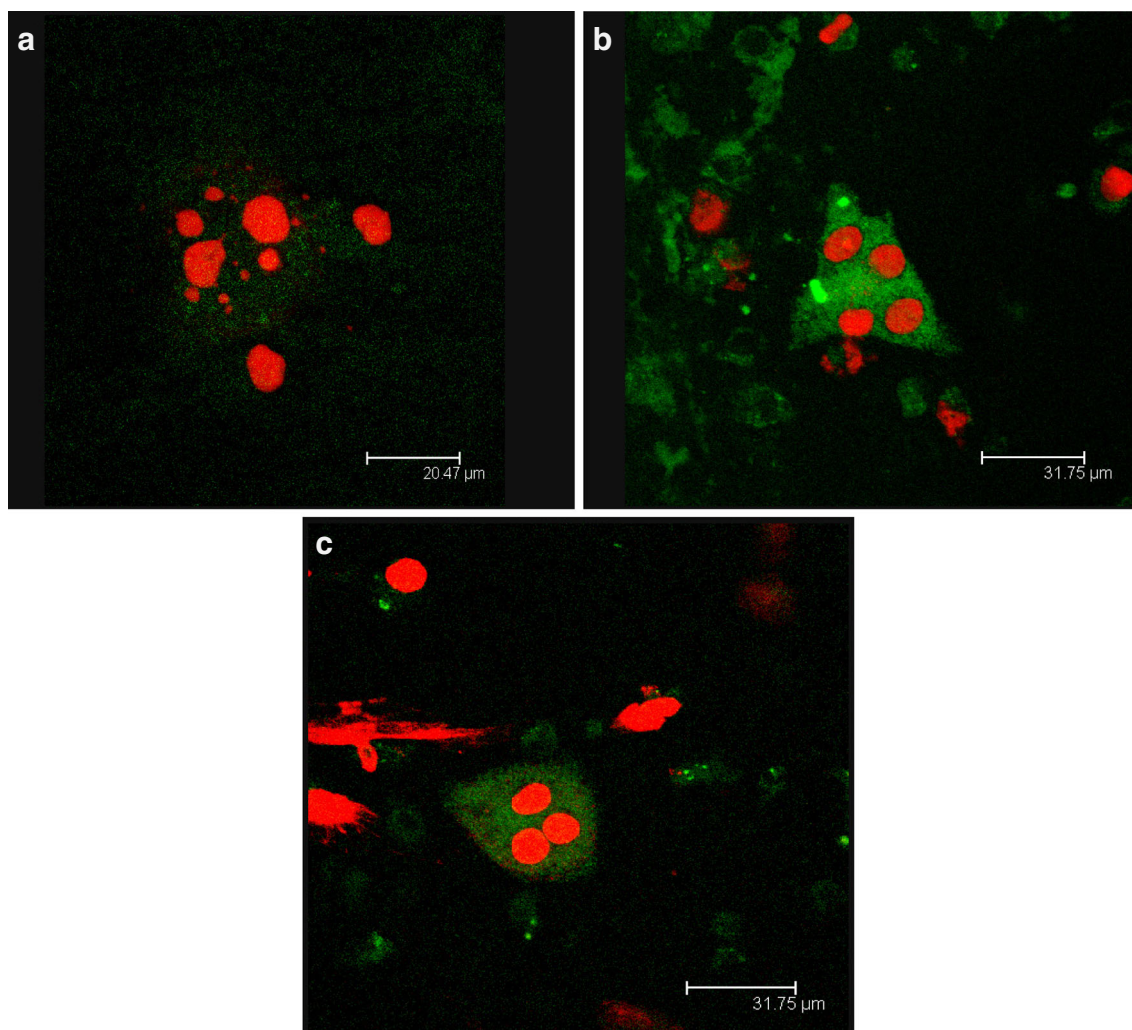


Fig. 4 Immunostaining of osteoclasts with annexin V and PI after culture with zoledronate 500 nM and alendronate 5 µM vs controls at pH 7.0. **a** Controls; **b** zoledronate 500 nM; **c** alendronate 5 µM

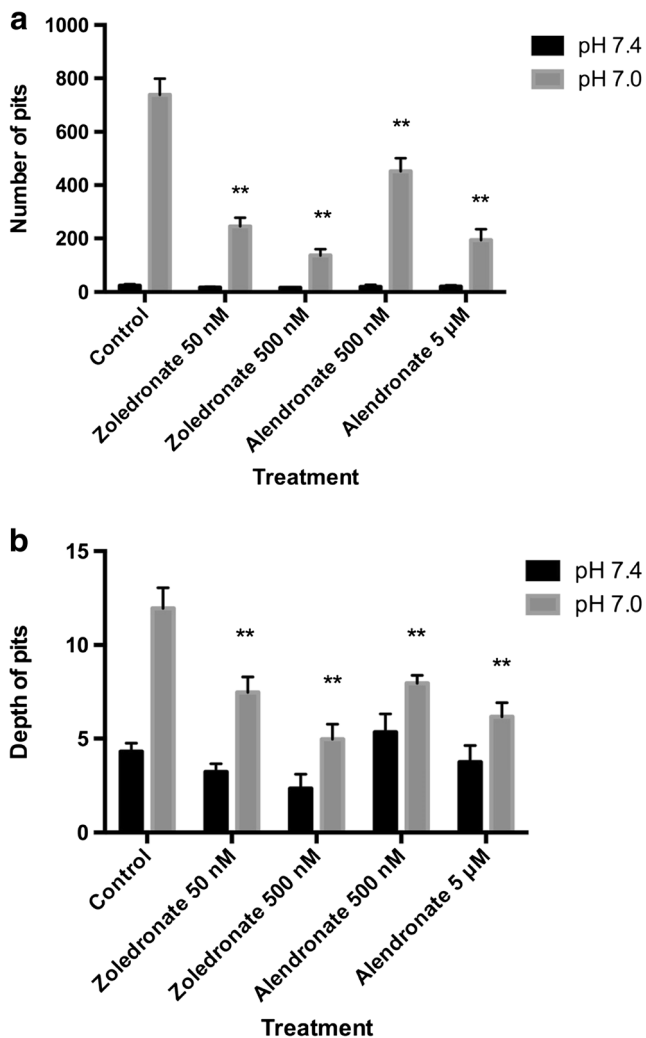


Fig. 5 Number (a) and depth (c) of resorption pits created by osteoclasts in dentine disks after culture with different doses of zoledronate and alendronate at pH 7.4 and 7.0. ** $p < 0.001$

Scanning electron microscopy

Figure 1a depicts the SEM image of BP-treated acid-activated mouse osteoclasts with resorption trails, showing the exposure of collagen fibers after dissolution of the mineral component of the dentine disk by osteoclast activity.

Discussion

The results of this study demonstrate that zoledronate and alendronate exert a pH-dependent effect on osteoclasts. Based on previous studies, the doses in all assays were zoledronate 50 or 500 nM and alendronate 500 or 5 µM which are within the therapeutic dose range [10, 20]. All treatments with different doses of these nitrogen-containing BPs reduced the osteoclast count in comparison to controls at pH 7.0,

observing a greater reduction at higher doses. The percentage of apoptotic cells detected after treatment with the same doses of zoledronate or alendronate was significantly higher at pH 7.0 than at pH 7.4. In this context, Arnett and Dempster [12, 21] reported that acidosis is necessary for the initiation of bone resorption and that osteoclasts, once activated, can be further stimulated by factors such as RANKL, parathyroid hormone [12], 1,25 (OH)₂ vitamin D [14], and ATP [22]. In the present study, the reduced pH would activate resorption of the BP-loaded dentine disk by osteoclasts and thereby release BP into the culture medium, producing their death.

Likewise, the BP treatments reduced the number and depth of resorption pits created by osteoclasts in comparison to untreated controls, and this decrease was again more marked at lower pH (pH 7.0). This reduced resorptive activity of the osteoclasts is also attributable to the effects of BP after activation of its resorption. As noted in the “Introduction,” it is known that BP bound to bone at circumneutral pH is released in bone resorption lacunas at acid pH, increasing the dissociation between BP and hydroxyapatite [16]. Indeed, Sato et al. [23] demonstrated in rats that bone-bound alendronate is released at acid pH. This event has not been linked to the pathogenesis of BRONJ to date but may prove to be the missing piece of the multifactorial puzzle. Acidic milieus are common in human infections, with a typical pH of around 6.2, and in postsurgical wounds [24, 25], while the jaw is frequently exposed to infection, including marginal/apical periodontitis, extended caries with endodontic involvement, and surgical procedures (e.g., tooth extraction or implantation). The resulting localized tissue acidification would increase the release of BP and also produce the protonated activation of nitrogen-containing groups, increasing the transformation of their derivatives to potentially toxic levels [16, 26, 27]. It can therefore be postulated that toxic levels of these derivatives due to prolonged or localized acidification may trigger the cascade of pathways that end in BRONJ. These processes may also occur after minor events (e.g., microtrauma or pressure sore) or may even be spontaneous, depending on the local concentration and type of BP and on the comorbidities and other medication of the patient, among other risk factors.

In addition, reduced oxygen tension is known to stimulate the formation or activation of cells from marrow precursors, including cells of the monocyte–macrophage lineage [28, 29]. Arnett et al. [30] found that hypoxia markedly augmented the number and size of osteoclasts formed in RANKL/M-CSF-treated 7-day mouse marrow cultures, producing a major increase in resorption pit formation. Hypoxia also caused moderate acidosis in calvarial cultures, presumably due to increased anaerobic metabolism, which is a relevant finding because osteoclast activation depends on extracellular acidification. There are multiple potential causes of hypoxia, which occurs when the blood supply to tissues is reduced or disrupted, and many have

been associated with bone loss, including the presence of infection or inflammation [30, 31]. Hypoxia due to local infection plays a major role in activating bone resorption by osteoclasts, and this activation would lead to the release and activation of BP in patients treated with this drug.

Authors have addressed the effect of BPs on other bone tissue cell populations. A study by Otto et al. [32] on mesenchymal stem cells showed that increasing acidity amplifies the concentration-dependent cytotoxic effects of nitrogen-containing BPs. Our group studied too the effect of BPs on osteoblasts [9, 10] and demonstrated the importance of the BP dose, finding that low doses increase the proliferation and reduce the differentiation capacity of osteoblasts, whereas high doses lead to their death by apoptosis. Although therapeutic doses of BPs are low, long-term treatments can produce the accumulation of high concentrations in bone, inactivated by binding with hydroxyapatite crystals. Subsequent medium acidification by infection can therefore favor the release and activation of hydroxyapatite-bound BPs [16, 17], with the aforementioned consequences.

Although the BPs most frequently related to BRONJ are those containing nitrogen [2, 33], it has also been associated with non-nitrogen-containing BPs that strongly bind to hydroxyapatite crystals, such as clodronate [34]. The lesser or greater binding of the BP to hydroxyapatite may explain differences among these drugs in their adverse effects on bone tissue after prolonged treatment. Non-nitrogen-containing BPs are less potent and bind more weakly to hydroxyapatite, and their effects on osteoclasts involve distinct pathways. However, long-term treatments with both types of BP can result in BRONJ, as confirmed by the present and previously published data on nitrogen-containing BPs [10, 35].

Recently, some authors have analyzed the effect of local alendronate as adjunct to mechanical therapy in the treatment of chronic periodontitis based on that bisphosphonate inhibits osteoclastic bone resorption [36, 37]. However, we trust the use of bisphosphonates for the treatment of periodontal disease should be taken with caution because there is some risk of BRONJ development since as we have shown in previous studies, bisphosphonates not only inhibit osteoclast activity but also negatively affect the physiology of osteoblastic cells [10, 38].

In conclusion, our results show that therapeutic doses of nitrogen-containing BPs, zoledronate and alendronate, have adverse effects on the viability and resorptive activity of osteoclasts when the pH of the medium is reduced. This may result from the release and activation of BP bound to hydroxyapatite crystals after prolonged treatment. Periodontal or peri-implant infection in the oral cavity may therefore be a key factor in triggering the cascade of events that produce BRONJ development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study involving animals were in accordance with the ethical standards of the ethical committee of the University of Granada.

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