Journal of Cranio-Maxillo-Facial Surgery 44 (2016) 1387–1394

Contents lists available at ScienceDirect

Journal of Cranio-Maxillo-Facial Surgery

journal homepage: www.jcmfs.com

Severe compromise of preosteoblasts in a surgical mouse model of bisphosphonate-associated osteonecrosis of the jaw

Luis A. Córdova [a, b, c, *], Florian Guilbaud [a, b], Jérôme Amiaud [a, b], Séverine Battaglia [a, b], Céline Charrier [a, b], Frédéric Lezot [a, b], Benoît Piot [d, e], Françoise Redini [a, b], Dominique Heymann [a, b, e, f]

[a] INSERM, UMR 957, Equipe Ligue Contre le Cancer 2012, 1 rue Gaston Veil, Nantes Cedex 1, 44035, Nantes, France
[b] University of Nantes, Nantes Atlantique Universities, Pathophysiology of Bone Resorption and Therapy of Primary Bone Tumours Laboratory, 1 rue Gaston Veil, Nantes Cedex 1, 44035, Nantes, France
[c] Department of Oral and Maxillofacial Surgery, San Borja Arriarán University Hospital – Faculty of Dentistry, University of Chile, Sergio Livingstone Polhammer 943, Independencia, Santiago, Chile
[d] Department of Stomatology and Maxillofacial Surgery, Nantes University Hospital, 1 Place Alexis-Ricordeau, 44093, Nantes Cedex 1, France
[e] Nantes University Hospital, 1 Place Alexis-Ricordeau, 44093, Nantes Cedex 1, France
[f] Department of Oncology and Metabolism, Medical School, Beech Hill Road, S10 2RX, Sheffield, UK

ARTICLE INFO

Article history:
Paper received 14 February 2016
Accepted 15 July 2016
Available online 22 July 2016

Keywords:
Osteonecrosis of the jaw
Zoledronic acid
Osteoclast
Osteoblast
Alveolar bone
Basic multicellular unit

ABSTRACT

Objectives: The effect of amino-bisphosphonates on osteoblastic lineage and its potential contribution to the pathogenesis of bisphosphonate-associated osteonecrosis of the jaw (BONJ) remain controversial. We assessed the effects of zoledronic acid (ZOL) on bone and vascular cells of the alveolar socket using a mouse model of BONJ.
Material and methods: Thirty-two mice were treated twice a week with either 100 μg/kg of ZOL or saline for 12 weeks. The first left maxillary molar was extracted at the third week. Alveolar sockets were assessed at both 3 weeks (intermediate) and 9 weeks (long-term) after molar extraction by semi-quantitative histomorphometry for empty lacunae, preosteoblasts (Osterix), osteoclasts (TRAP), and pericyte-like cells (CD146). Also, the bone microarchitecture was assessed by micro-CT.
Results: Osteonecrotic-like lesions were observed in 21% of mice. Moreover, a decreased number of preosteoblasts contrasted with the increased number of osteoclasts at both time points. In addition, osteoclasts display multinucleation and detachment from the endosteal surface. Furthermore, the number of pericyte-like cells increased at the intermediate time point. The alveolar bone mass increased exclusively with long-term ZOL treatment.
Conclusion: The severe imbalance between bone-forming cells and bone-resorbing cells shown in this study could contribute to the pathogenesis of BONJ.
© 2016 European Association for Cranio-Maxillo-Facial Surgery. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Bisphosphonate-associated osteonecrosis of the jaw (BONJ) is characterized by the persistent jaw bone exposure (>8 weeks) after a surgical procedure in patients with a history of use of bisphosphonates and without previous exposure to head and neck radiotherapy (Ruggiero et al., 2009). The long-term use of intravenous third-generation amino-bisphosphonates (risedronate and zoledronic acid [ZOL]), the most powerful antiresorptive agents, is considered a critical risk factor related to the development of BONJ (Wessel et al., 2008; Basso et al., 2013). The pathogenesis of BONJ

* Corresponding author. Division of Plastic & Reconstructive Surgery, Stanford School of Medicine, 257 Campus Dr, GK211, Stanford, CA 94035, United States. Fax: +1 650 736 4374.
E-mail addresses: lcordova@stanford.edu (L.A. Córdova), florian.guilbaud@univ-nantes.fr (F. Guilbaud), jerome.amiaud@univ-nantes.fr (J. Amiaud), severine.battaglia@univ-nantes.fr (S. Battaglia), celine.charrier@univ-nantes.fr (C. Charrier), frederic-lezot@univ-nantes.fr (F. Lezot), benoit.piot@chu-nantes.fr (B. Piot), francoise.redini@univ-nantes.fr (F. Redini), dominique.heymann@sheffield.ac.uk (D. Heymann).

http://dx.doi.org/10.1016/j.jcms.2016.07.015
1010-5182/© 2016 European Association for Cranio-Maxillo-Facial Surgery. Published by Elsevier Ltd. All rights reserved.

remains unknown and several hypothesis have been proposed; nevertheless, the suppression of bone remodeling induced by bisphosphonates seems to be the most consistent with their intrinsic mechanism of action (Mawardi et al., 2011; R. H. Kim et al., 2011; Allen and Burr, 2009).

Bone remodeling is the coupled process initiated by osteoclastic bone resorption followed by osteoblastic new bone formation (Natalie A. Sims and Martin, 2014). This process occurs in the entire skeleton throughout life and it takes place in the basic multicellular units (BMUs) of cortical and trabecular bone (Natalie A. Sims and Martin, 2014). Tight control of bone remodeling in each BMU is essential for maintaining normal bone mass. This control is regulated by dynamic interactions between the cellular components and coupling factors released during bone resorption (N. A. Sims and Ng, 2014). The former includes osteoclast precursor and mature osteoclasts, osteoblastic lineage, endothelial cells and pericytes, macrophages and dendritic cells (Natalie A. Sims and Martin, 2014). On the other hand, the coupling factors are protein molecules released during the osteoclasts differentiation: cardiotrophin 1, sphingosine-1-phosphate (S1P), bone morphogenetic protein (BMP)-6 and Wnt10b, collagen triple helix repeat containing 1 (CTHRC1) and Sema4D. Also, the coupling factors include bone matrix proteins released during bone resorption: insulin growth factor (IGF)-1 and transforming growth factor (TGF)-β (N. A. Sims and Ng, 2014).

The clinical and preclinical benefits of blocking osteoclast differentiation and activity with subsequent increase of bone density using amino-bisphosphonates have been extensively reported (D Heymann, 2010; Le Goff et al., 2010; D Heymann et al., 2005). However, their effects on the osteoblastic lineage remain poorly understood (Sakagami et al., 2005). Human biopsies show that the terminal stage of bisphosphonate-associated osteonecrotic lesions (bone sequestra) is characterized by the absence of the endosteal osteoblasts, empty osteocyte lacunae and damage in the canalicular system (Lesclous et al., 2009). These findings confirm the compromise of the entire osteoblastic lineage including preosteoblasts, osteoblasts, and osteocytes (Koch et al., 2011; Manzano-Moreno et al., 2015). On the other hand, *in vitro* studies report cytotoxic effects of bisphosphonates on osteoblastic cells, decreasing their viability and osteogenic ability in a dose-dependent manner (Pozzi et al., 2009; Basso et al., 2013). Therefore, the understanding of the effect of amino-bisphosphonates on both osteoblastic lineage and bone remodeling in *in vivo* models is a crucial step to further understand the pathogenesis of BONJ. We postulated that osteoblastic cells are sensitive to the effect of amino-bisphosphonates after a surgical stimulus in alveolar bone. The aim of this study was thus to assess – at the cellular level – the intermediate and long-term effects of clinically relevant high doses of ZOL on the bone and vascular cell components of alveolar socket BMU using a surgical mouse model for BONJ.

2. Material and methods

2.1. Animals, drug administration and surgical procedure

Thirty-two C57BL/6 male mice (Janvier, Le Genest-Saint Isle, France) aged 10 weeks were randomly divided into two groups and treated intra-peritoneally (i.p.) with either 100 μg/kg of ZOL (kindly provided by Novartis, Switzerland) (experimental group; n = 16) or saline solution (control group; n = 16) twice a week for 12 weeks (Supplementary Appendix 1). The drug tolerance of the mice was assessed daily by clinical examination. The total dose of ZOL administered was the equivalent of a lifetime dose of the drug over 4 years of therapy in a 70 kg adult multiple myeloma patients (Pozzi et al., 2009). At the end of the third week, the first left maxillary molar was surgically extracted from all the animals (Supplementary Appendix 1). After 6 weeks of treatment with ZOL (or saline solution), and 3 weeks after the molar extraction, 50 % of the animals were sacrificed to assess the situation at an intermediate time point (the equivalent of 2 years according to Pozzi et al., 2009). The remaining 50 % of the animals was sacrificed at the end of the protocol, after 12 weeks of treatment with ZOL (or saline solution) (the equivalent of 4 years according to Pozzi et al., 2009) and 9 weeks after the molar extraction, for long-term assessment.

2.2. Histology analysis

Harvested maxillae were fixed in 4% buffered formaldehyde for 48 h and then decalcified with 4.13% ethylenediaminetetraacetic acid (EDTA) and 0.2% paraformaldehyde in phosphate-buffered saline (PBS) for 96 h using the KOS microwave histostation (Milestone, Kalamazoo, MI, USA) before embedding in paraffin. Two 4 μm-thick sagittal sections were obtained from 2 levels of the alveolar socket site (each one separated by 50 μm). All slides were stained with Masson trichrome to assess the bone matrix and empty lacunae in both, bone sequestra and submucosal bone. Furthermore, all slides were stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts (Supplementary Appendix 1). The immunostaining for osteoblastic cells was performed using rabbit monoclonal anti-osterix antibody (1/800; Abcam). The immunostaining of the pericytes was carried out using rabbit monoclonal anti-CD146 antibody (1/200; Abcam).

Histological images were acquired using a NanoZoomer 2.0-RS slide scanner (Hamamatsu, Japan). The region of interest (ROI) corresponded to a rectangular area of alveolar bone comprising the full length of the alveolar socket. Static histomorphometric analysis of the number of empty lacunae, percentage of osteoclasts (Gobin et al., 2014a; Gobin et al., 2014b; Lamoureux et al., 2014), number of osterix and $CD146^+$ cells in their defined ROIs, were performed using ImageJ software (NIH, Bethesda, MD, USA).

2.3. Micro-computed tomography assessment

The analysis of alveolar bone microarchitecture was performed at the time of necropsy (6 and 12 weeks) using the high-resolution X-ray micro-computed tomography (micro-CT) system for small-animal imaging SkyScan-1076 (SkyScan, Kontich, Belgium) (Supplementary Appendix 1). The assessment of alveolar bone density was performed by measuring the mineralized bone detected within the VOI (Bone Volume; BV) and expressed in cubic millimeters (mm^3).

2.4. Statistical analysis

All analyses were performed using GraphPad InStat Version 3.02 software (GraphPad Software, La Jolla, CA, USA). The histological and micro-CT results were analyzed by comparisons between experimental and controls groups with unpaired parametric two-tailed t-test. Results were considered significant at p-value < 0.05.

3. Results

3.1. Zoledronic acid and molar extraction induce clinical osteonecrotic-like changes in alveolar bone

A 12-week administration of high doses of ZOL was well tolerated by all mice demonstrated by their conservation of body weight (data not shown). In addition, 21 % of the ZOL-treated mice exhibited osteonecrotic-like changes, characterized by both exposed and necrotic bone (sequestra) in the operative site at the

intermediate time point (3 weeks after molar extraction). The aspect of the sequestra was opaque and yellowish bone, slightly attached to the local mucosa (Fig. 1A). Normal healing of oral mucosa was observed in mice assessed at the long-term time point (9 weeks after molar extraction).

We next analyzed the alveolar socket by histology at two levels: bone sequestra and submucosal bone. All sequestrated bone displayed both the absence of osteocytes and empty lacunae in their whole surface (Fig. 1B). On the other hand, the submucosal bone exhibited empty lacunae exclusively in the superficial layer (Fig. 1B). Their number was significantly higher at long-term (12 weeks) time point in the ZOL-treated group compared with the control (Fig. 1C, $p < 0.01$).

3.2. Zoledronic acid and molar extraction decrease the number of osteoblastic cells in alveolar bone

To reveal the effect of ZOL on alveolar osteoblastic cells, we performed first an histologic qualitative analysis followed by a semi-quantitative assessment of osterix positive cells using immunohistochemistry. We observed new trabecular bone in both the ZOL and saline-treated groups at the intermediate time point (Fig. 2, upper panels). Otherwise, in the long-term, the alveolar site exhibited a large surface of a calcified bone matrix with narrow marrow spaces compared with controls (Fig. 2, lower panels). The osterix positive cells were detected in the superficial layer of the trabecular bone at both time points (Fig. 2, upper and lower panels). Interestingly, ZOL-treated mice significantly decreased the number of osterix positive cells at both intermediate ($p < 0.05$) and long-term ($p < 0.01$) time points (Fig. 2, upper and lower histograms).

3.3. Zoledronic acid and molar extraction increase the number of aberrant giant multinucleated osteoclasts in alveolar bone

Since it has been admitted that bisphosphonates, and particularly ZOL, increase the apoptosis of osteoclasts, thus decreasing bone remodeling, we next assessed the effect of our protocol on the osteoclasts in the alveolar bone. At intermediate and long-term time points, we observed clear changes in the morphology of $TRAP^+$ cells between ZOL-treated mice and controls (Fig. 3, upper and lower panels). In the former group, the shape of the osteoclasts was dramatically modified and the treatment resulted in the formation of large, multinucleated osteoclasts compared to those observed in the control group (Fig. 3, upper and lower left panels). In addition, some of these cells were detached from the endosteal bone surface and located within the bone marrow spaces. Interestingly, the number of $TRAP^+$ cells increase significantly in the mice that received both the intermediate ($p < 0.01$) and long-term bisphosphonate treatments ($p < 0.05$) (Fig. 3, upper and lower histograms).

3.4. Zoledronic acid and molar extraction increase the bone volume of the post-extraction alveolar socket

Considering the high impact of ZOL on bone remodeling through its inhibition of osteoclastic bone resorption, we next assessed the bone mass of trabecular bone in the post-extraction alveolar socket using a volumetric analysis by micro-tomography (micro-CT). We observed a significant increase in the percentage of alveolar bone volume (BV) of mice treated with long-term ZOL compared to controls (Fig. 4, right histogram) ($p < 0.05$). In contrast, no difference was observed at the intermediate time point of this protocol.

3.5. Intermediate treatment with zoledronic acid and molar extraction increases the number of pericyte-like cells ($CD146^+$) in alveolar bone

Given the potentially anti-angiogenic effects of ZOL, we assessed the presence of $CD146^+$ peri-vascular cells (pericytes-like) within the alveolar bone using immunohistochemistry. $CD146^+$ positive cells located in the alveolar bone marrow spaces were clearly identified. Interestingly, a significant increase in the $CD146^+$ pericyte-like cell number ($p < 0.05$) was detected in mice treated with ZOL compared to controls at the intermediate time point (Supplementary Appendix 2). On the contrary, no difference was detected in long-term ZOL-treated mice (data not shown).

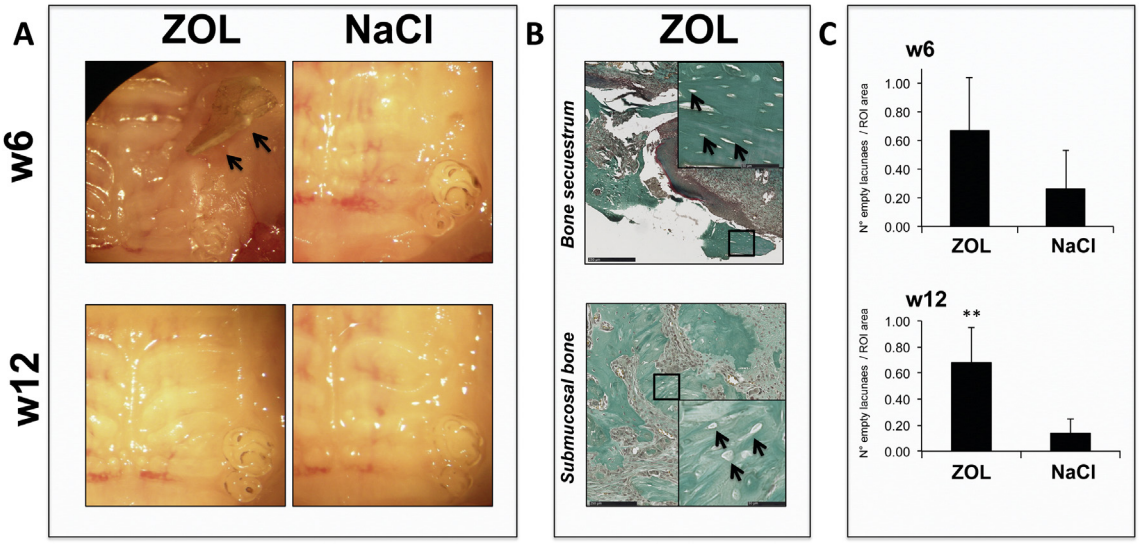

Fig. 1. Zoledronic acid and a surgically-induced mouse model of osteonecrosis of the jaw (BONJ) assessed at intermediate and long-term treatment time points. (A) Clinical view of the sequestra after the intermediate term treatment (black arrows); **(B)** Masson's trichrome stained slides showing empty lacunae (black arrows) in the sequestra and submucosal bone of alveolar BMUs and **(C)** number of empty lacunae within the assessed area. (BMUs, basic multicellular units; w6, intermediate assessment; w12, long delay assessment; ZOL, zoledronic acid; NaCl, sodium chloride; ROI, region of interest; $^{**}p < 0.01$).

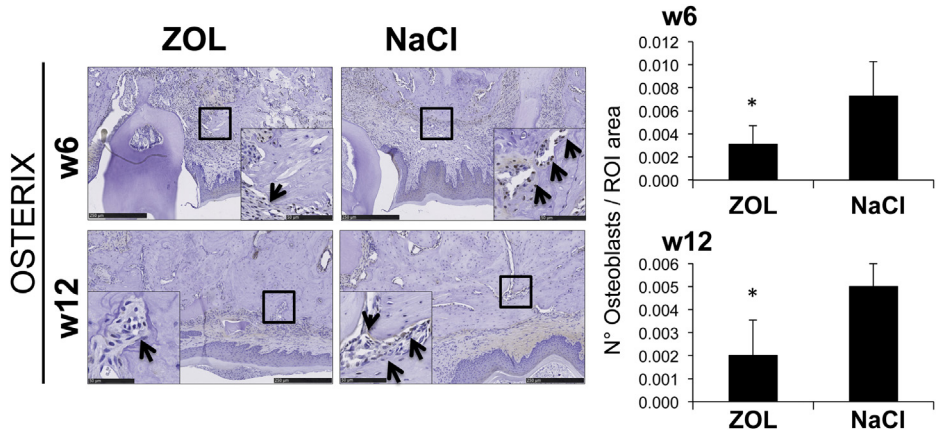

Fig. 2. Osteoblast number decreases in alveolar BMUs after zoledronic acid treatment and molar extraction. Immunostaining of osteoblasts (osterix^{+} cells) confirms that ZOL-treated mice show a significant decrease in the number of osteoblastic cells in alveolar BMUs at both time points assessed. (BMUs, basic multicellular units; week 6 (w6), intermediate assessment; w12, long-term assessment; ZOL, zoledronic acid; NaCl, sodium chloride; ROI, region of interest; $^{*}p < 0.05$).

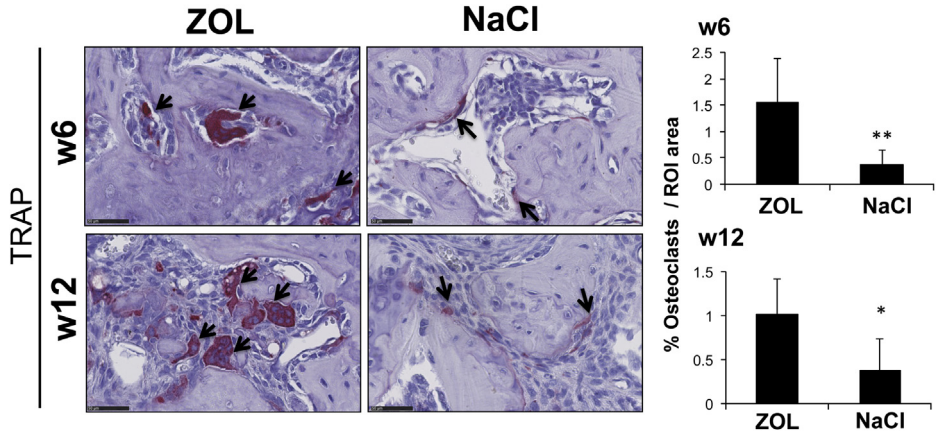

Fig. 3. An increased number of aberrant osteoclasts were observed in alveolar BMUs after zoledronic acid treatment and molar extraction. TRAP-stained slices showing the aberrant morphology of the osteoclasts and a significant increase in the percentage of TRAP^{+} cells observed in alveolar BMUs after intermediate and long-term administration of ZOL. (BMUs, basic multicellular units; week 6 (w6), intermediate assessment; w12, long-term assessment; ZOL, zoledronic acid; NaCl, sodium chloride; $^{*}p < 0.05$ and $^{**}p < 0.01$).

4. Discussion

Maxillomandibular alveolar bone is a particular unit of the skeleton that undergoes periodic stimulus (e.g. facial and dental development, chewing, etc.), exhibiting a higher bone turnover than non-alveolar bone sites (Allen and Burr, 2008). Bone turnover depends on the coupling activities of osteoblasts and osteoclasts in each BMUs (Natalie A. Sims and Martin, 2014; N. A. Sims and Ng, 2014). Otherwise, ZOL markedly decreases bone turnover by apoptosis of the osteoclasts, blocking the bone resorption and subsequently, increasing the bone mass (Dominique Heymann, 2010). The powerful anti-resorptive effect is the main advantage for the treatment of human osteolytic diseases (Dominique Heymann et al., 2004; Dominique Heymann 2010; Le Goff et al., 2010). While the effects of bisphosphonates on bone tissue have been well-described in BMUs of the axial and appendicular skeleton, the specific effects of bisphosphonates on the maxillomandibular alveolar bone, the precise site affected by osteonecrosis, is still less understood. In addition, the effect of bisphosphonates on other cell components of BMUs such as osteoblastic, vascular and immune cells remain still misunderstood (N. A. Sims and Ng, 2014; Pazianas, 2011). We, therefore, assessed the effects of a human equivalent protocol of intermediate and long-term intravenous high doses of ZOL on bone and vascular cells involved in the bone remodeling cycle in alveolar BMUs using an adapted surgical mouse model of osteonecrosis of the jaw (Bi et al., 2010).

We first confirmed that our protocol induced the major features of BONJ, reported in human series (Raje et al., 2008; Marx, 2003). We showed osteonecrotic-like lesions characterized by the formation of sequestra and empty lacunae in the alveolar bone at the operative site. Bone sequestra were observed in a small number of samples at the intermediate time point of the treatment. Consequently, most samples showed normal healing at the operative site. The variable reproduction of osteonecrotic-like changes have been also reported in different murine models of ONJ and seems to be associated with the degree of surgical trauma (Marino et al., 2011). Otherwise, empty lacunae, the other key feature in human and experimental osteonecrotic diseases (Okazaki et al., 2009; Aghaloo et al., 2011), were recognized widely in the bone sequestra and selectively in the superficial layer of submucosal bone in the alveolar socket. Interestingly, the number of empty lacunae in the submucosal bone significantly increased after long-term treatment, suggesting that this finding may be associated with the cumulative doses fixed in the alveolar bone. This fact is in agreement with previous clinical and experimental reports (Ruggiero et al., 2009; Marx et al., 2005; Allen, 2008; Aguirre et al., 2012), supporting

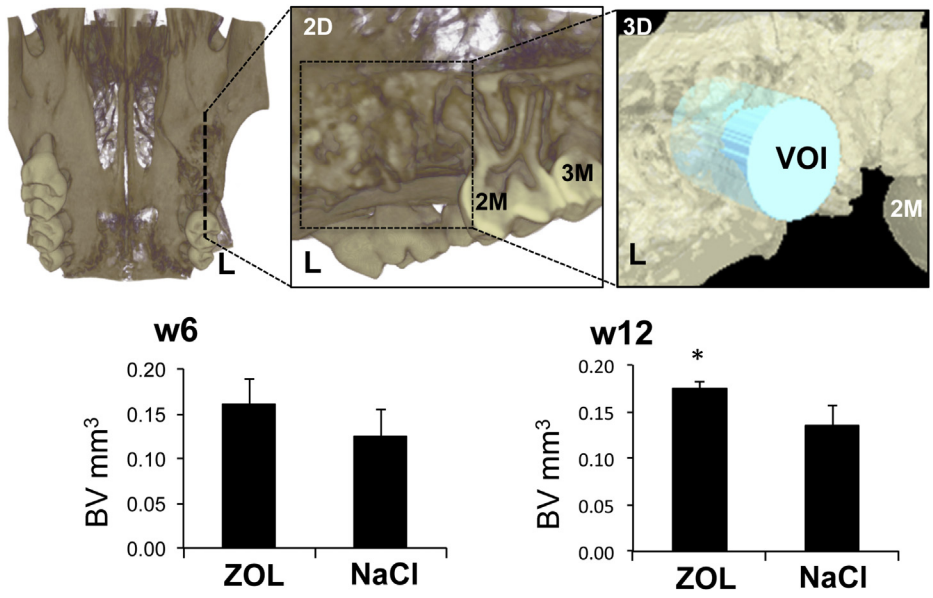

Fig. 4. Bone volume of the extraction socket is upmodulated by bisphosphonate treatment and molar extraction. Volumetric assessment of the alveolar BMU shows an increase in the bone volume (BV) at the long-term time point. (2D, two dimensional view; 3D, tridimensional view; 2 M, second maxillary left molar; 3 M, third maxillary left molar; BMUs, basic multicellular units; week 6, (w6) intermediate assessment; w12, long delay assessment; ZOL, zoledronic acid; NaCl, sodium chloride; VOI, volume of interest; *$p < 0.05$).

the hypothesis that long-term exposure to high doses of amino-bisphosphonates determines their accumulation in alveolar BMUs, inducing local changes and constituting a potential first step in the development of osteonecrosis of the jaw (Allen, 2008; Hoff et al., 2008; Daubiné et al., 2007; Pozzi et al., 2009).

Interestingly, our study demonstrated that ZOL significantly decreased the number of osteoblastic cells in the alveolar BMUs. This observation was in agreement with the down-regulation of gene expression implicated in osteoblast signalization, osteoprogenitor cell differentiation and activation that has been observed in patients treated with high doses of ZOL with and without BONJ by multiple myeloma (Raje et al., 2008). The same study showed that the suppression of bone formation markers was most pronounced in BONJ patients (Raje et al., 2008). In addition, a decrease in osteoblasts number was observed in the long bones after 3 weeks of systemic treatment with increasing doses of ZOL (Pozzi et al., 2009). Moreover, the absence of woven bone in the alveolar socket after tooth extraction in mice treated with bisphosphonate and denosumab, two agents associated with osteonecrotic-lesions, has recently been demonstrated (Williams et al., 2014). In this study, seric levels of bone-specific alkaline phosphatase, a biomarker of osteoblastic cell activity, was also decreased (Williams et al., 2014). Similarly, a cytotoxic effect characterized by the inhibition of viability, bone matrix secretion and mineralization was observed in osteoblasts after prolonged exposure to ZOL under *in vitro* conditions (Pozzi et al., 2009). While the main action of bisphosphonates occurs by the direct effect on osteoclasts in the bone matrix resorption phase of the remodeling cycle, the reduction in the number of osteoblastic cells in alveolar BMUs strongly suggests that ZOL has a potentially additional effect in the apposition phase of this cycle. Accordingly, these clinical and experimental data might be related to the successful use of human recombinant parathyroid hormone (rhPTH), a bone anabolic strategy, as a therapeutic approach for BONJ in the clinic (Doh et al., 2015; Khan et al., 2015).

Otherwise, ZOL induced an increase in the number of osteoclasts and a severe disruption in osteoclast morphology after both intermediate and long-term treatment. Indeed, we reported a significant increase in the percentage of $TRAP^+$ cell observed in ZOL-treated mice at both time points and the detachment of them from the bone trabecule surface. Taken together, these findings suggest a paradoxical effect of ZOL on osteoclasts, primarily supposed to decrease the number and activity of them. Osteoclasts with altered morphology were also reported in biopsies of patients under long-term of amino-bisphosphonate therapy, highlighting their dose-dependence (Weinstein et al., 2009; Jobke et al., 2009). The cytoskeletal reorganization of osteoclasts through inhibition of the protein prenylation induced by amino-bisphosphonates was proposed as an explanation for these facts (Jobke, 2009; Roelofs et al., 2006). Similar data were observed in biopsies of patients after treatment with teriparatide and who had previously been treated with bisphosphonates (Jobke et al., 2009). These aberrant osteoclasts may be subject to prolonged apoptosis or be functionally inhibited by ZOL (Weinstein et al., 2009). Our study shows consistent findings to support the lack of osteoclast bone-resorptive function in these aberrant osteoclasts.

We also observed that ZOL increased the number of $CD146^+$ pericyte-like cells exclusively after intermediate-term treatment. Pericytes are peri-endothelial cells that participate in normal tissue repair by secreting cytokines and growth factors promoting revascularization (Forbes and Rosenthal, 2014). During aberrant tissue repair, activated pericytes become scar-producing myofibroblasts, which are considered a balance among fibrotic or full regenerative response (Forbes and Rosenthal, 2014). Thus, we can hypothesize that the increased number of $CD146^+$ pericyte-like cells contributed to the osteonecrotic-like changes observed in zoledronic acid-treated mice after a surgical injury (Bouacida et al., 2012). Pericytes may be increased in response to bisphosphonate in order to contribute to the bone remodeling. Indeed, pericytes are able to differentiate into osteoblast-like cells, nevertheless, pericytes show high immaturity and we can hypothesize that the differentiation process of pericytes towards osteoblastic lineage may be altered resulting of ONJ (Bouacida et al., 2012). On the other hand, our results are controversial considering the generic compromise of the vasculature in osteonecrotic diseases (e.g. femoral osteonecrosis and osteoradionecrosis) (H. K. W. Kim, 2007;

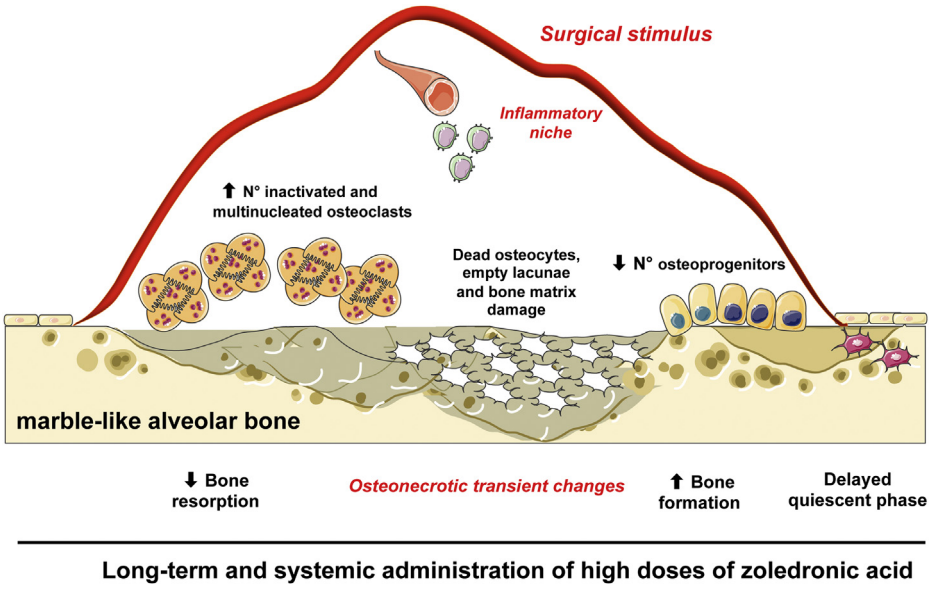

Fig. 5. Scheme representing the disruption of cell components of alveolar BMUs induced by zoledronic acid.

Hansen et al., 2006). Specifically, BONJ patients have shown vascular compromise through decreases in serum level of vascular-endothelial-growth-factor (VEGF) (Santini et al., 2003). In addition, case report studies show an increase in the incidence and severity of osteonecrosis of the jaw after a single administration of bisphosphonates or associated with bevacizumab, a recombinant human monoclonal antibody that targets VEGF (Estilo et al., 2008; Lescaille et al., 2014). There are also numerous *in vitro* studies demonstrating the considerable impact of nitrogen-containing bisphosphonates over non nitrogen-containing bisphosphonates, decreasing the viability and migration of endothelial cells, as well as increasing their apoptosis (Ziebart et al., 2011; Walter et al., 2011). Despite this, only a restricted number of *in vivo* studies have shown the anti-angiogenic effects of nitrogen-containing bisphosphonates (Wood et al., 2002; Fournier et al., 2002; Stresing et al., 2011; Pabst et al., 2014). We hypothesize that our results are strongly influenced by the inflammatory and reparative response triggered following the molar extraction.

The regulation of the bone mass is the product of the coupled phases of the bone remodeling cycle in each BMU: bone resorption is driven by mature osteoclasts, and formation is driven by pre- and mature osteoblasts. The increased alveolar bone mass at the long-term time point showed in our study, confirms the inactivation of osteoclasts and subsequent osteolysis. Interestingly, it occurs despite the decreased number of osteoblastic cells. We propose that the long-term treatment with ZOL affect both, the osteoclastic bone resorption for a long period and, transiently, the osteoblastic bone formation. Thus, a decreased number of osteoblastic cells were thus capable of synthesizing the bone matrix and increasing the alveolar bone mass. This hypothesis might be related to the reported increased bone turnover rate of alveolar bone rather than that of non-alveolar bone sites (Allen and Burr, 2008). Also, this finding could be explained by the bone anabolic effect of the early inflammatory stage in the alveolar socket after the molar extraction. The link between inflammation and bone repair was recently proposed and it may be regulated by oncostatin M-signaling produced by monocyte/macrophage cells (Guihard et al., 2012). A STAT3 pathway activation in mesenchymal stem cells has also been reported (Nicolaidou et al., 2012).

5. Conclusion

Following administration of long-term high doses of ZOL and molar extraction in a mouse model of bisphosphonates-related osteonecrosis of the jaw, we confirm that the cell components of alveolar BMUs were significantly disrupted (Fig. 5). The number of osteoblastic cells was dramatically reduced. In addition, the osteoclasts were inactivated, increased in number and exhibiting an aberrant morphology. The vascular precursors increased significantly after the intermediate-term treatment. Despite this evident cell imbalance, the alveolar bone mass increased, confirming that the effect of ZOL is mostly anti-resorptive rather than anti-anabolic in the alveolar operative site. In short, consistent histological and micro-architectural findings support the disruption of the normal homeostasis of alveolar BMUs induced by the administration of ZOL, with an additional surgical dental stimulus.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Financial support for this research was provided by the Gueules Cassées Foundation, project N°FGC 14-2012, the Agence Nationale de la Recherche, Grant 2007 “Pathophysiology of Human Diseases” 2007 Project N°RO 7196N, the Ligue National Contre le Cancer “Equipe Ligue 2012” and University of Chile and CONICYT- Becas Chile (CONICYT PAI/INDUSTRIA 79090016).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcms.2016.07.015.

References

Aghaloo Tara L, Kang Ben, Sung Eric C, Shoff Michael, Ronconi Matthew, Gotcher Jack E, et al: Periodontal disease and bisphosphonates induce osteonecrosis of the jaws in the rat. J Bone Miner Res 26(8): 1871–1882. http://dx.doi.org/10.1002/jbmr.379, 2011

Aguirre J Ignacio, Akhter Mohammed P, Kimmel Donald B, Pingel Jennifer E, Williams Alyssa, Jorgensen Marda, et al: Oncologic doses of zoledronic acid induce osteonecrosis of the jaw-like lesions in rice rats (*Oryzomys palustris*) with periodontitis. J Bone Miner Res: Off J Am Soc Bone Miner Res 27(10): 2130–2143. http://dx.doi.org/10.1002/jbmr.1669, 2012

Allen Matthew R: Skeletal accumulation of bisphosphonates: implications for osteoporosis treatment. Expert Opin Drug Metab Toxicol 4(11): 1371–1378. http://dx.doi.org/10.1517/17425255.4.11.1371, 2008

Allen Matthew R, Burr David B: Mandible matrix necrosis in Beagle dogs after 3 years of daily oral bisphosphonate treatment. J Oral Maxillofac Surg: Off J Am Assoc Oral Maxillofac Surg 66(5): 987–994. http://dx.doi.org/10.1016/j.joms.2008.01.038, 2008

Allen Matthew R, Burr David B: The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. J Oral Maxillofac Surg: Off J Am Assoc Oral Maxillofac Surg 67(5 Suppl): 61–70. http://dx.doi.org/10.1016/j.joms.2009.01.007, 2009

Basso Fernanda G, Silveira Turrioni Ana Paula, Hebling Josimeri, de Souza Costa Carlos A: Zoledronic acid inhibits human osteoblast activities. Gerontology 59(6): 534–541. http://dx.doi.org/10.1159/000351194, 2013

Bi Yanming, Gao Yamei, Ehirchiou Driss, Cao Chunzhang, Kikuiri Takashi, Le Anh, et al: Bisphosphonates cause osteonecrosis of the jaw-like disease in mice. Am J Pathol 177(1): 280–290. http://dx.doi.org/10.2353/ajpath.2010.090592, 2010

Bouacida Amina, Rosset Philippe, Trichet Valérie, Guilloton Fabien, Espagnolle Nicolas, Cordonier Thomas, et al: Pericyte-like progenitors show high immaturity and engraftment potential as compared with mesenchymal stem cells. PloS One 7(11): e48648. http://dx.doi.org/10.1371/journal.pone.0048648, 2012

Daubiné Florence, Le Gall Céline, Gasser Juerg, Green Jonathan, Clézardin Philippe: Antitumor effects of clinical dosing regimens of bisphosphonates in experimental Breast Cancer bone metastasis. J Natl Cancer Inst 99(4): 322–330. http://dx.doi.org/10.1093/jnci/djk054, 2007

Doh Re-Mee, Park Hye-Jeong, Rhee Yumie, Sil Kim Hyun, Huh Jisun, Park Wonse: Teriparatide therapy for bisphosphonate-related osteonecrosis of the jaw associated with dental implants. Implant Dent 24(2): 222–226. http://dx.doi.org/10.1097/ID.0000000000000232, 2015

Estilo Cherry L, Fornier Monica, Farooki Azeez, Carlson Diane, Bohle George, Huryn Joseph M: Osteonecrosis of the jaw related to bevacizumab. J Clin Oncol: Off J Am Soc Clin Oncol 26(24): 4037–4038. http://dx.doi.org/10.1200/JCO.2007.15.5424, 2008

Forbes Stuart J, Rosenthal Nadia: Preparing the Ground for tissue regeneration: from mechanism to therapy. Nat Med 20(8): 857–869. http://dx.doi.org/10.1038/nm.3653, 2014

Fournier Pierrick, Boissier Sandrine, Filleur Stéphanie, Guglielmi Julien, Cabon Florence, Colombel Marc, et al: Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. Cancer Res 62(22): 6538–6544, 2002

Gobin Bérengère, Battaglia Séverine, Lanel Rachel, Chesneau Julie, Amiaud Jérôme, Rédini Françoise, et al: NVP-BEZ235, a dual PI3K/mTOR inhibitor, inhibits osteosarcoma cell proliferation and tumor development in vivo with an improved survival rate. Cancer Lett 344(2): 291–298. http://dx.doi.org/10.1016/j.canlet.2013.11.017, 2014a

Gobin Bérengère, Baud' Huin Marc, Lamoureux François, Ory Benjamin, Charrier Céline, Lanel Rachel, et al: BYL719, a new α-specific PI3K inhibitor: single administration and in combination with conventional chemotherapy for the treatment of osteosarcoma. Int J Cancer. http://dx.doi.org/10.1002/ijc.29040, 2014b (June)

Guihard Pierre, Danger Yannic, Brounais Bénédicte, David Emmanuelle, Brion Régis, Delecrin Joël, et al: Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling. Stem Cells 30(4): 762–772. http://dx.doi.org/10.1002/stem.1040, 2012

Hansen Torsten, Kunkel Martin, Weber Achim, James Kirkpatrick C: Osteonecrosis of the jaws in patients treated with bisphosphonates – Histomorphologic analysis in comparison with infected osteoradionecrosis. J Oral Pathol Med: Off Pub Int Assoc Oral Pathol Am Acad Oral Pathol 35(3): 155–160. http://dx.doi.org/10.1111/j.1600-0714.2006.00391.x, 2006

Heymann D, Ory B, Blanchard F, Heymann M-F, Coipeau P, Charrier C, et al: Enhanced tumor regression and tissue repair when zoledronic acid is combined with ifosfamide in rat osteosarcoma. Bone 37(1): 74–86. http://dx.doi.org/10.1016/j.bone.2005.02.020, 2005

Heymann Dominique: Bisphosphonates and bone diseases: past, present and future. Curr Pharm Des 16(27): 2948–2949, 2010

Heymann Dominique, Ory Benjamin, Gouin François, Green Jonathan R, Rédini Françoise: Bisphosphonates: new therapeutic agents for the treatment of bone tumors. Trends Mol Med 10(7): 337–343. http://dx.doi.org/10.1016/j.molmed.2004.05.007, 2004

Hoff Ana O, Toth Béla B, Altundag Kadri, Johnson Marcella M, Warneke Carla L, Hu Mimi, et al: Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. J Bone Miner Res: Off J Am Soc Bone Miner Res 23(6): 826–836. http://dx.doi.org/10.1359/jbmr.080205, 2008

Jobke Björn: Giant osteoclast formation and long-term oral bisphosphonate therapy. New Engl J Med 360(16): 1676. http://dx.doi.org/10.1056/NEJMc090167, 2009 author reply 1677–1678

Jobke Björn, Pfeifer Michael, Minne Helmut W: Teriparatide following bisphosphonates: initial and long-term effects on microarchitecture and bone remodeling at the human iliac crest. Connect Tissue Res 50(1): 46–54. http://dx.doi.org/10.1080/03008200802412462, 2009

Khan A, Morrison A, Ruggiero S, Tetradis S, Davison KS, Peters E, et al: Response to comments on "diagnosis and management of osteoporosis of the jaw: a systematic review and international consensus". J Bone Miner Res. http://dx.doi.org/10.1002/jbmr.2524, 2015 (May)

Kim HKW: Osteonecrosis and osteonecrosis of the jaw (ONJ). J Musculoskelet Neuronal Interact 7(4): 348–349, 2007

Kim RH, Lee RS, Williams D, Bae S, Woo J, Lieberman M, et al: Bisphosphonates induce senescence in normal human oral keratinocytes. J Dent Res 90(6): 810–816. http://dx.doi.org/10.1177/0022034511402995, 2011

Koch Felix Peter, Merkel Christina, Al-Nawas Bilal, Smeets Ralf, Ziebart Thomas, Walter Christian, et al: Zoledronate, ibandronate and clodronate enhance osteoblast differentiation in a dose dependent manner—a quantitative in vitro gene expression analysis of Dlx5, Runx2, OCN, MSX1 and MSX2. J Cranio-Maxillo-Facial Surg: Off Pub Eur Assoc Cranio-Maxillo-Facial Surg 39(8): 562–569. http://dx.doi.org/10.1016/j.jcms.2010.10.007, 2011

Lamoureux François, Baud'huin Marc, Rodriguez Calleja Lidia, Jacques Camille, Berreur Martine, Rédini Françoise, et al: Selective inhibition of BET bromodomain epigenetic signalling interferes with the bone-associated tumour vicious cycle. Nature Commun 5. http://dx.doi.org/10.1038/ncomms4511, 2014 (March)

Le Goff B, Guillot P, Glémarec J, Berthelot JM, Maugars Y: A comparison between bisphosphonates and other treatments for osteoporosis. Curr Pharm Des 16(27): 3037–3044, 2010

Lescaille Géraldine, Coudert Amélie E, Baaroun Vanessa, Ostertag Agnès, Charpentier Emmanuel, Javelot Marie-José, et al: Clinical study evaluating the effect of bevacizumab on the severity of zoledronic acid-related osteonecrosis of the jaw in Cancer patients. Bone 58: 103–107. http://dx.doi.org/10.1016/j.bone.2013.10.002, 2014 (January)

Lesclous Philippe, Abi Najm Semaan, Carrel Jean-Pierre, Baroukh Brigitte, Lombardi Tommaso, Willi Jean-Pierre, et al: Bisphosphonate-associated osteonecrosis of the jaw: a key role of inflammation? Bone 45(5): 843–852. http://dx.doi.org/10.1016/j.bone.2009.07.011, 2009

Manzano-Moreno Francisco Javier, Ramos-Torrecillas Javier, De Luna-Bertos Elvira, Ruiz Concepción, García-Martínez Olga: High doses of bisphosphonates reduce osteoblast-like cell proliferation by arresting the cell cycle and inducing apoptosis. J Cranio-Maxillo-Facial Surg: Off Pub Eur Assoc Cranio-Maxillo-Facial Surg 43(3): 396–401. http://dx.doi.org/10.1016/j.jcms.2014.12.008, 2015

Marino Karen L, Zakhary Ibrahim, Abdelsayed Rafik A, Carter Jared A, O'Neill Jack C, Khashaba Rania M, et al: Development of a rat model of bisphosphonate-related osteonecrosis of the jaw (BRONJ). J Oral Implantol. http://dx.doi.org/10.1563/AAID-JOI-D-11-00057, 2011 September, 110909140919004

Marx Robert E: Pamidronate (aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. J Oral Maxillofac Surg: Off J Am Assoc Oral Maxillofac Surg 61(9): 1115–1117, 2003

Marx Robert E, Sawatari Yoh, Fortin Michel, Broumand Vishtasb: Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. J Oral Maxillofac Surg 63(11): 1567–1575. http://dx.doi.org/10.1016/j.joms.2005.07.010, 2005

Mawardi H, Giro G, Kajiya M, Ohta K, Almazrooa S, Alshwaimi E, et al: A role of oral bacteria in bisphosphonate-induced osteonecrosis of the jaw. J Dent Res 90(11): 1339–1345. http://dx.doi.org/10.1177/0022034511420430, 2011

Nicolaidou Vicky, Wong Mei Mei, Redpath Andia N, Ersek Adel, Baban Dilair F, Williams Lynn M, et al: Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation. PLoS One 7(7): e39871. http://dx.doi.org/10.1371/journal.pone.0039871, 2012 Edited by Dimas Tadeu Covas

Okazaki S, Nishitani Y, Nagoya S, Kaya M, Yamashita T, Matsumoto H: Femoral head osteonecrosis can be caused by disruption of the systemic immune response via the toll-like receptor 4 signalling pathway. Rheumatology 48(3): 227–232. http://dx.doi.org/10.1093/rheumatology/ken462, 2009

Pabst AM, Ziebart T, Ackermann M, Konerding MA, Walter C: Bisphosphonates' antiangiogenic potency in the development of bisphosphonate-associated osteonecrosis of the jaws: influence on microvessel sprouting in an in vivo 3D matrigel assay. Clin Oral Invest 18(3): 1015–1022. http://dx.doi.org/10.1007/s00784-013-1060-x, 2014

Pazianas M: Osteonecrosis of the jaw and the role of macrophages. J Natl Cancer Inst 103(3): 232–240. http://dx.doi.org/10.1093/jnci/djq516, 2011

Pozzi Samantha, Vallet Sonia, Mukherjee Siddhartha, Cirstea Diana, Vaghela Nileshwari, Santo Loredana, et al: High-dose zoledronic acid impacts bone remodeling with effects on osteoblastic lineage and bone mechanical properties. Clin Cancer Res 15(18): 5829–5839. http://dx.doi.org/10.1158/1078-0432.CCR-09-0426, 2009

Raje Noopur, Woo Sook-Bin, Hande Karen, Yap Jeffrey T, Richardson Paul G, Vallet Sonia, et al: Clinical, radiographic, and biochemical characterization of multiple myeloma patients with osteonecrosis of the jaw. Clin Cancer Res 14(8): 2387–2395. http://dx.doi.org/10.1158/1078-0432.CCR-07-1430, 2008

Roelofs Anke J, Thompson Keith, Gordon Sharon, Rogers Michael J: Molecular mechanisms of action of bisphosphonates: current status. Clin Cancer Res 12(20): 6222s–6230s. http://dx.doi.org/10.1158/1078-0432.CCR-06-0843, 2006

Ruggiero Salvatore L, Dodson Thomas B, Assael Leon A, Landesberg Regina, Marx Robert E, Mehrotra Bhoomi: American association of oral and maxillofacial surgeons position paper on bisphosphonate-related osteonecrosis of the jaws–2009 update. J Oral Maxillofac Surg: Off J Am Assoc Oral Maxillofac Surg 67(5 Suppl): 2–12. http://dx.doi.org/10.1016/j.joms.2009.01.009, 2009

Sakagami Naoko, Amizuka Norio, Li Minqi, Takeuchi Kiichi, Hoshino Masaaki, Nakamura Midori, et al: Reduced osteoblastic population and defective

mineralization in osteopetrotic (op/op) mice. Micron (Oxford, Engl: 1993) 36(7–8): 688–695. http://dx.doi.org/10.1016/j.micron.2005.06.008, 2005

Santini Daniele, Vincenzi Bruno, Dicuonzo Giordano, Avvisati Giuseppe, Massacesi Cristian, Battistoni Fabrizio, et al: Zoledronic acid induces significant and long-lasting modifications of circulating angiogenic factors in cancer patients. Clin Cancer Res 9(8): 2893–2897, 2003

Sims NA, Ng KW: Implications of osteoblast–osteoclast interactions in the management of osteoporosis by antiresorptive agents denosumab and odanacatib. Curr Osteoporos Rep 12(1): 98–106. http://dx.doi.org/10.1007/s11914-014-0196-1, 2014

Sims Natalie A, John Martin T: Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. BoneKEy Reports 3. http://dx.doi.org/10.1038/bonekey.2013.215, 2014 (January)

Stresing Verena, Fournier Pierrick G, Bellahcène Akeila, Benzaïd Ismahène, Mönkkönen Hannu, Colombel Marc, et al: Nitrogen-containing bisphosphonates can inhibit angiogenesis in vivo without the involvement of farnesyl pyrophosphate synthase. Bone 48(2): 259–266. http://dx.doi.org/10.1016/j.bone.2010.09.035, 2011

Walter C, Pabst A, Ziebart T, Klein Mo, Al-Nawas B: Bisphosphonates affect migration ability and cell viability of HUVEC, fibroblasts and osteoblasts in vitro. Oral Dis 17(2): 194–199. http://dx.doi.org/10.1111/j.1601-0825.2010.01720.x, 2011

Weinstein Robert S, Roberson Paula K, Manolagas Stavros C: Giant osteoclast formation and long-term oral bisphosphonate therapy. New Engl J Med 360(1): 53–62. http://dx.doi.org/10.1056/NEJMoa0802633, 2009

Wessel John H, Dodson Thomas B, Zavras Athanasios I: Zoledronate, smoking, and obesity are strong risk factors for osteonecrosis of the jaw: a case-control study. J Oral Maxillofac Surg 66(4): 625–631. http://dx.doi.org/10.1016/j.joms.2007.11.032, 2008

Williams Drake W, Lee Cindy, Kim Terresa, Yagita Hideo, Wu Hongkun, Park Sil, et al: Impaired bone resorption and woven bone formation are associated with development of osteonecrosis of the jaw-like lesions by bisphosphonate and anti– receptor activator of NF-κB ligand antibody in mice. Am J Pathol. http://dx.doi.org/10.1016/j.ajpath.2014.07.010, 2014 Accessed September 22

Wood Jeanette, Bonjean Karine, Ruetz Stephan, Bellahcène Akeila, Devy Laetitia, Foidart Jean Michel, et al: Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. J Pharmacol Experiment Ther 302(3): 1055–1061. http://dx.doi.org/10.1124/jpet.102.035295, 2002

Ziebart Thomas, Pabst Andreas, Oliver Klein Marcus, Kämmerer Peer, Gauss Leonie, Brüllmann Dan, et al: Bisphosphonates: restrictions for vasculogenesis and angiogenesis: inhibition of cell function of endothelial progenitor cells and mature endothelial cells in vitro. Clin Oral Invest 15(1): 105–111. http://dx.doi.org/10.1007/s00784-009-0365-2, 2011