
Article:
Cordova, L.A., Guilbaud, F., Amiaud, J. et al. (6 more authors) (2016) Severe Compromise of Preosteoblasts in a Surgical Mouse Model of Bisphosphonate-Associated Osteonecrosis of the Jaw. Journal of Craniomaxillofacial Surgery, 44 (9). pp. 1387-1394. ISSN 1010-5182

https://doi.org/10.1016/j.jcms.2016.07.015

Elsevier Editorial System(tm) for Journal of Cranio-Maxillofacial Surgery

Manuscript Draft

Manuscript Number: JCMS-D-16-00155R2

Title: Severe Compromise of Preosteoblasts in a Surgical Mouse Model of Bisphosphonate-Associated Osteonecrosis of the Jaw

Article Type: Original Paper

Keywords: Osteonecrosis of the jaw, zoledronic acid, osteoclast, osteoblast, alveolar bone, basic multicellular unit.

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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 showed in this study could contribute to the pathogenesis of BONJ.

June 6st, 2016

Jörg Wiltfang, MD, DMD, PhD
Editor-in-Chief
Journal of Cranio-Maxillofacial Surgery

Dear Editor-in-Chief,

Thank you for your email dated on June 6th, 2016. We appreciate the opportunity to submit this second revised version of our manuscript entitled *"Severe Compromise of Preosteoblasts in a Surgical Mouse Model of Bisphosphonate-Associated Osteonecrosis of the Jaw"* (JCMS-D-16-00155R1).

As requested by reviewers, we included:

- Highest academic degree for each full-named co-author
- The full name and address for each author's institutional affiliation
- The updated address for the corresponding author (from June, 1st, 2016)

All these requirements were added in the "Title Page" and modified accordingly in the First page and Acknowledgements subsection of the main text.

We look forward to hearing from you regarding our submission. We would be happy to respond to any further questions and comments you may have.

Sincerely,

Dr. Luis A. Córdova,
DDS, PhD, Oral and Maxillofacial Surgeon
First and corresponding author

*Title Page

Severe Compromise of Preosteoblasts in a Surgical Mouse Model of Bisphosphonate-Associated Osteonecrosis of the Jaw

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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"
- University of Chile and CONICYT- Becas Chile (CONICYT PAI/INDUSTRIA 79090016).

Severe Compromise of Preosteoblasts in a Surgical Mouse Model of Bisphosphonate-Associated Osteonecrosis of the Jaw

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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 showed in this study could contribute to the pathogenesis of BONJ.

KEYWORDS: Osteonecrosis of the jaw, zoledronic acid, osteoclast, osteoblast, alveolar bone, basic multicellular unit.

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, Dodson, and Zavras 2008; Basso et al. 2013). The pathogenesis of BONJ 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). A 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 formers include 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 another 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 the long-term assessment.

2.2. Histology analysis

Harvested maxillae were fixed in 4% buffered formaldehyde for 48 hours and then decalcified with 4.13% ethylenediaminetetraacetic acid (EDTA) and 0.2% paraformaldehyde in phosphate-buffered saline (PBS) for 96 hours 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, Battaglia, et al. 2014a; Gobin, Huin, 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 $p\text{-value} < 0.05$.

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 (Figure 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 (Figure 1B). On another hand, the submucosal bone exhibited empty lacunae exclusively in the superficial layer (Figure 1B). Their number was significantly higher at long-term (12 weeks) time point in the ZOL-treated group compared with the control (Figure 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 (Figure 2, upper panels). Otherwise, after long-term, the alveolar site exhibited a large surface of a calcified bone matrix with narrow marrow spaces compared with controls (Figure 2, lower panels). The osterix positive cells were detected in the superficial layer of the trabecular bone at both time points (Figure 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. (Figure 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 (Figure 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 (Figure 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 ($p<0.05$) (Figure 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 (Figure 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).

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 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 trabeculae 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, Roberson, and Manolagas 2009; Jobke, Pfeifer, and Minne 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, Pfeifer, and Minne 2009). These aberrant osteoclasts may be subject to prolonged apoptosis or be functionally inhibited by ZOL (Weinstein, Roberson, and Manolagas 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 another 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; 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 (Figure 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.

Aknowledgements

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).

References

Aghaloo, Tara L, Ben Kang, Eric C Sung, Michael Shoff, Matthew Ronconi, Jack E Gotcher, Olga Bezouglaia, Sarah M Dry, and Sotirios Tetradis. 2011. 'Periodontal Disease and Bisphosphonates Induce Osteonecrosis of the Jaws in the Rat'. *Journal of Bone and Mineral Research* 26 (8): 1871–82. doi:10.1002/jbmr.379.

Aguirre, J Ignacio, Mohammed P Akhter, Donald B Kimmel, Jennifer E Pingel, Alyssa Williams, Marda Jorgensen, Lakshmyya Kesavalu, and Thomas J Wronski. 2012. 'Oncologic Doses of Zoledronic Acid Induce Osteonecrosis of the Jaw-like Lesions in Rice Rats (Oryzomys Palustris) with Periodontitis'. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research* 27 (10): 2130–43. doi:10.1002/jbmr.1669.

Allen, Matthew R. 2008. 'Skeletal Accumulation of Bisphosphonates: Implications for Osteoporosis Treatment'. *Expert Opinion on Drug Metabolism & Toxicology* 4 (11): 1371–78. doi:10.1517/17425255.4.11.1371.

Allen, Matthew R, and Burr, David B. 2008. 'Mandible Matrix Necrosis in Beagle Dogs after 3 Years of Daily Oral Bisphosphonate Treatment'. *Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons* 66 (5): 987–94. doi:10.1016/j.joms.2008.01.038.

Allen, Matthew R, and Burr, David B. 2009. 'The Pathogenesis of Bisphosphonate-Related Osteonecrosis of the Jaw: So Many Hypotheses, so Few Data'. *Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons* 67 (5 Suppl): 61–70. doi:10.1016/j.joms.2009.01.007.

Basso, Fernanda G, Ana Paula Silveira Turrioni, Josimeri Hebling, and Carlos A de Souza Costa. 2013. 'Zoledronic Acid Inhibits Human Osteoblast Activities'. *Gerontology* 59 (6): 534–41. doi:10.1159/000351194.

Bi, Yanming, Yamei Gao, Driss Ehirchiou, Chunzhang Cao, Takashi Kikuiri, Anh Le, Songtao Shi, and Li Zhang. 2010. 'Bisphosphonates Cause Osteonecrosis of the Jaw-like Disease in Mice'. *The American Journal of Pathology* 177 (1): 280–90. doi:10.2353/ajpath.2010.090592.

Bouacida, Amina, Philippe Rosset, Valérie Trichet, Fabien Guilloton, Nicolas Espagnolle, Thomas Cordonier, Dominique Heymann, Pierre Layrolle, Luc Sensébé, and Frédéric Deschaseaux. 2012. 'Pericyte-like Progenitors Show High Immaturity and Engraftment Potential as Compared with Mesenchymal Stem Cells'. *PloS One* 7 (11): e48648. doi:10.1371/journal.pone.0048648.

Daubiné, Florence, Céline Le Gall, Juerg Gasser, Jonathan Green, and Philippe Clézardin. 2007. 'Antitumor Effects of Clinical Dosing Regimens of Bisphosphonates in Experimental Breast Cancer Bone Metastasis'. *Journal of the National Cancer Institute* 99 (4): 322–30. doi:10.1093/jnci/djk054.

Doh, Re-Mee, Hye-Jeong Park, Yumie Rhee, Hyun Sil Kim, Jisun Huh, and Wonse Park. 2015. 'Teriparatide Therapy for Bisphosphonate-Related Osteonecrosis of the Jaw Associated with Dental Implants'. *Implant Dentistry* 24 (2): 222–26. doi:10.1097/ID.0000000000000232.

Estilo, Cherry L, Monica Fornier, Azeez Farooki, Diane Carlson, George Bohle, and Joseph M Huryn. 2008. 'Osteonecrosis of the Jaw Related to Bevacizumab'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 26 (24): 4037–38. doi:10.1200/JCO.2007.15.5424.

Forbes, Stuart J., and Nadia Rosenthal. 2014. 'Preparing the Ground for Tissue Regeneration: From Mechanism to Therapy'. *Nature Medicine* 20 (8): 857–69. doi:10.1038/nm.3653.

Fournier, Pierrick, Sandrine Boissier, Stéphanie Filleur, Julien Guglielmi, Florence Cabon, Marc Colombel, and Philippe Clézardin. 2002. 'Bisphosphonates Inhibit Angiogenesis in Vitro and Testosterone-Stimulated Vascular Regrowth in the Ventral Prostate in Castrated Rats'. *Cancer Research* 62 (22): 6538–44.

Gobin, Bérengère, Séverine Battaglia, Rachel Lanel, Julie Chesneau, Jérôme Amiaud, Françoise Rédini, Benjamin Ory, and Dominique Heymann. 2014. 'NVP-BEZ235, a Dual PI3K/mTOR Inhibitor, Inhibits Osteosarcoma Cell Proliferation and Tumor Development in Vivo with an Improved Survival Rate'. *Cancer Letters* 344 (2): 291–98. doi:10.1016/j.canlet.2013.11.017.

Gobin, Bérengère, Marc Baud' Huin, François Lamoureux, Benjamin Ory, Céline Charrier, Rachel Lanel, Séverine Battaglia, et al. 2014. 'BYL719, a New α-Specific PI3K Inhibitor: Single Administration and in Combination with Conventional Chemotherapy for the Treatment of Osteosarcoma'. *International Journal of Cancer*, June, n/a-n/a. doi:10.1002/ijc.29040.

Guihard, Pierre, Yannic Danger, Bénédicte Brounais, Emmanuelle David, Régis Brion, Joël Delecrin, Carl D. Richards, et al. 2012. 'Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages Depends on Oncostatin M Signaling'. *STEM CELLS* 30 (4): 762–772. doi:10.1002/stem.1040.

Hansen, Torsten, Martin Kunkel, Achim Weber, and C James Kirkpatrick. 2006. 'Osteonecrosis of the Jaws in Patients Treated with Bisphosphonates - Histomorphologic Analysis in Comparison with Infected Osteoradionecrosis'. *Journal of Oral Pathology & Medicine: Official Publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 35 (3): 155–60. doi:10.1111/j.1600-0714.2006.00391.x.

Heymann, D, B Ory, F Blanchard, M-F Heymann, P Coipeau, C Charrier, S Couillaud, J P Thiery, F Gouin, and F Redini. 2005. 'Enhanced Tumor Regression and Tissue Repair When Zoledronic Acid Is Combined with Ifosfamide in Rat Osteosarcoma'. *Bone* 37 (1): 74–86. doi:10.1016/j.bone.2005.02.020.

Heymann, Dominique. 2010. 'Bisphosphonates and Bone Diseases: Past, Present and

Future'. *Current Pharmaceutical Design* 16 (27): 2948–49.

Heymann, Dominique, Benjamin Ory, François Gouin, Jonathan R Green, and Françoise Rédini. 2004. 'Bisphosphonates: New Therapeutic Agents for the Treatment of Bone Tumors'. *Trends in Molecular Medicine* 10 (7): 337–43. doi:10.1016/j.molmed.2004.05.007.

Hoff, Ana O, Béla B Toth, Kadri Altundag, Marcella M Johnson, Carla L Warneke, Mimi Hu, Ajay Nooka, et al. 2008. 'Frequency and Risk Factors Associated with Osteonecrosis of the Jaw in Cancer Patients Treated with Intravenous Bisphosphonates'. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research* 23 (6): 826–36. doi:10.1359/jbmr.080205.

Jobke, Björn. 2009. 'Giant Osteoclast Formation and Long-Term Oral Bisphosphonate Therapy'. *The New England Journal of Medicine* 360 (16): 1676; author reply 1677-1678. doi:10.1056/NEJMc090167.

Jobke, Björn, Michael Pfeifer, and Helmut W. Minne. 2009. 'Teriparatide Following Bisphosphonates: Initial and Long-Term Effects on Microarchitecture and Bone Remodeling at the Human Iliac Crest'. *Connective Tissue Research* 50 (1): 46–54. doi:10.1080/03008200802412462.

Khan, A., A. Morrison, S. Ruggiero, S. Tetradis, K. S. Davison, E. Peters, J. Compston, and O. N. J. Task Force International. 2015. 'Response to Comments on "Diagnosis and Management of Osteoporosis of the Jaw: A Systematic Review and International Consensus"'. *J Bone Miner Res*, May. doi:10.1002/jbmr.2524.

Kim, H K W. 2007. 'Osteonecrosis and Osteonecrosis of the Jaw (ONJ)'. *Journal of Musculoskeletal & Neuronal Interactions* 7 (4): 348–49.

Kim, R. H., R. S. Lee, D. Williams, S. Bae, J. Woo, M. Lieberman, J.- E. Oh, et al. 2011. 'Bisphosphonates Induce Senescence in Normal Human Oral Keratinocytes'. *Journal of Dental Research* 90 (6): 810–16. doi:10.1177/0022034511402995.

Koch, Felix Peter, Christina Merkel, Bilal Al-Nawas, Ralf Smeets, Thomas Ziebart, Christian Walter, and Wilfried Wagner. 2011. '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'. *Journal of Cranio-Maxillo-Facial Surgery: Official Publication of the European Association for Cranio-Maxillo-Facial Surgery* 39 (8): 562–69. doi:10.1016/j.jcms.2010.10.007.

Lamoureux, François, Marc Baud'huin, Lidia Rodriguez Calleja, Camille Jacques, Martine Berreur, Françoise Rédini, Fernando Lecanda, James E. Bradner, Dominique Heymann, and Benjamin Ory. 2014. 'Selective Inhibition of BET Bromodomain Epigenetic Signalling Interferes with the Bone-Associated Tumour Vicious Cycle'. *Nature Communications* 5 (March). doi:10.1038/ncomms4511.

Le Goff, B, P Guillot, J Glémarec, J M Berthelot, and Y Maugars. 2010. 'A Comparison between Bisphosphonates and Other Treatments for Osteoporosis'. *Current*

Pharmaceutical Design 16 (27): 3037–44.

Lescaille, Géraldine, Amélie E. Coudert, Vanessa Baaroun, Agnès Ostertag, Emmanuel Charpentier, Marie-José Javelot, Rafael Tolédo, et al. 2014. 'Clinical Study Evaluating the Effect of Bevacizumab on the Severity of Zoledronic Acid-Related Osteonecrosis of the Jaw in Cancer Patients'. *Bone* 58 (January): 103–7. doi:10.1016/j.bone.2013.10.002.

Lesclous, Philippe, Semaan Abi Najm, Jean-Pierre Carrel, Brigitte Baroukh, Tommaso Lombardi, Jean-Pierre Willi, René Rizzoli, Jean-Louis Saffar, and Jacky Samson. 2009. 'Bisphosphonate-Associated Osteonecrosis of the Jaw: A Key Role of Inflammation?' *Bone* 45 (5): 843–52. doi:10.1016/j.bone.2009.07.011.

Manzano-Moreno, Francisco Javier, Javier Ramos-Torrecillas, Elvira De Luna-Bertos, Concepción Ruiz, and Olga García-Martínez. 2015. 'High Doses of Bisphosphonates Reduce Osteoblast-like Cell Proliferation by Arresting the Cell Cycle and Inducing Apoptosis'. *Journal of Cranio-Maxillo-Facial Surgery: Official Publication of the European Association for Cranio-Maxillo-Facial Surgery* 43 (3): 396–401. doi:10.1016/j.jcms.2014.12.008.

Marino, Karen L., Ibrahim Zakhary, Rafik A. Abdelsayed, Jared A. Carter, Jack C. O'Neill, Rania M. Khashaba, Mohammed Elsalanty, Mark R. Stevens, and James L. Borke. 2011. 'Development of a Rat Model of Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ)'. *Journal of Oral Implantology*, September, 110909140919004. doi:10.1563/AAID-JOI-D-11-00057.

Marx, Robert E. 2003. 'Pamidronate (Aredia) and Zoledronate (Zometa) Induced Avascular Necrosis of the Jaws: A Growing Epidemic'. *Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons* 61 (9): 1115–17.

Marx, Robert E., Yoh Sawatari, Michel Fortin, and Vishtasb Broumand. 2005. 'Bisphosphonate-Induced Exposed Bone (Osteonecrosis/Osteopetrosis) of the Jaws: Risk Factors, Recognition, Prevention, and Treatment'. *Journal of Oral and Maxillofacial Surgery* 63 (11): 1567–75. doi:10.1016/j.joms.2005.07.010.

Mawardi, H, G Giro, M Kajiya, K Ohta, S Almazrooa, E Alshwaimi, S-B Woo, I Nishimura, and T Kawai. 2011. 'A Role of Oral Bacteria in Bisphosphonate-Induced Osteonecrosis of the Jaw'. *Journal of Dental Research* 90 (11): 1339–45. doi:10.1177/0022034511420430.

Nicolaidou, Vicky, Mei Mei Wong, Andia N. Redpath, Adel Ersek, Dilair F. Baban, Lynn M. Williams, Andrew P. Cope, and Nicole J. Horwood. 2012. 'Monocytes Induce STAT3 Activation in Human Mesenchymal Stem Cells to Promote Osteoblast Formation'. Edited by Dimas Tadeu Covas. *PLoS ONE* 7 (7): e39871. doi:10.1371/journal.pone.0039871.

Okazaki, S., Y. Nishitani, S. Nagoya, M. Kaya, T. Yamashita, and H. Matsumoto. 2009. 'Femoral Head Osteonecrosis Can Be Caused by Disruption of the Systemic Immune Response via the Toll-like Receptor 4 Signalling Pathway'. *Rheumatology* 48 (3): 227–32. doi:10.1093/rheumatology/ken462.

Pabst, A. M., T. Ziebart, M. Ackermann, M. A. Konerding, and C. Walter. 2014. 'Bisphosphonates' Antiangiogenic Potency in the Development of Bisphosphonate-Associated Osteonecrosis of the Jaws: Influence on Microvessel Sprouting in an in Vivo 3D Matrigel Assay'. *Clinical Oral Investigations* 18 (3): 1015–22. doi:10.1007/s00784-013-1060-x.

Pazianas, M. 2011. 'Osteonecrosis of the Jaw and the Role of Macrophages'. *J Natl Cancer Inst* 103 (3): 232–40. doi:10.1093/jnci/djq516.

Pozzi, Samantha, Sonia Vallet, Siddhartha Mukherjee, Diana Cirstea, Nileshwari Vaghela, Loredana Santo, Eyal Rosen, et al. 2009. 'High-Dose Zoledronic Acid Impacts Bone Remodeling with Effects on Osteoblastic Lineage and Bone Mechanical Properties'. *Clinical Cancer Research* 15 (18): 5829–39. doi:10.1158/1078-0432.CCR-09-0426.

Raje, Noopur, Sook-Bin Woo, Karen Hande, Jeffrey T. Yap, Paul G. Richardson, Sonia Vallet, Nathaniel Treister, et al. 2008. 'Clinical, Radiographic, and Biochemical Characterization of Multiple Myeloma Patients with Osteonecrosis of the Jaw'. *Clinical Cancer Research* 14 (8): 2387–95. doi:10.1158/1078-0432.CCR-07-1430.

Roelofs, Anke J., Keith Thompson, Sharon Gordon, and Michael J. Rogers. 2006. 'Molecular Mechanisms of Action of Bisphosphonates: Current Status'. *Clinical Cancer Research* 12 (20): 6222s–6230s. doi:10.1158/1078-0432.CCR-06-0843.

Ruggiero, Salvatore L, Thomas B Dodson, Leon A Assael, Regina Landesberg, Robert E Marx, and Bhoomi Mehrotra. 2009. 'American Association of Oral and Maxillofacial Surgeons Position Paper on Bisphosphonate-Related Osteonecrosis of the Jaws--2009 Update'. *Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons* 67 (5 Suppl): 2–12. doi:10.1016/j.joms.2009.01.009.

Sakagami, Naoko, Norio Amizuka, Minqi Li, Kiichi Takeuchi, Masaaki Hoshino, Midori Nakamura, Kayoko Nozawa-Inoue, Nobuyuki Udagawa, and Takeyasu Maeda. 2005. 'Reduced Osteoblastic Population and Defective Mineralization in Osteopetrotic (Op/op) Mice'. *Micron (Oxford, England: 1993)* 36 (7–8): 688–95. doi:10.1016/j.micron.2005.06.008.

Santini, Daniele, Bruno Vincenzi, Giordano Dicuonzo, Giuseppe Avvisati, Cristian Massacesi, Fabrizio Battistoni, Michele Gavasci, et al. 2003. 'Zoledronic Acid Induces Significant and Long-Lasting Modifications of Circulating Angiogenic Factors in Cancer Patients'. *Clinical Cancer Research* 9 (8): 2893–97.

Sims, N. A., and K. W. Ng. 2014. 'Implications of Osteoblast-Osteoclast Interactions in the Management of Osteoporosis by Antiresorptive Agents Denosumab and Odanacatib'. *Curr Osteoporos Rep* 12 (1): 98–106. doi:10.1007/s11914-014-0196-1.

Sims, Natalie A., and T. John Martin. 2014. 'Coupling the Activities of Bone Formation and Resorption: A Multitude of Signals within the Basic Multicellular Unit'. *BoneKEy Reports* 3 (January). doi:10.1038/bonekey.2013.215.

Stresing, Verena, Pierrick G Fournier, Akeila Bellahcène, Ismahène Benzaïd, Hannu Mönkkönen, Marc Colombel, F Hal Ebetino, Vincent Castronovo, and Philippe Clézardin. 2011. 'Nitrogen-Containing Bisphosphonates Can Inhibit Angiogenesis in Vivo without the Involvement of Farnesyl Pyrophosphate Synthase'. *Bone* 48 (2): 259–66. doi:10.1016/j.bone.2010.09.035.

Walter, C., A. Pabst, T. Ziebart, Mo Klein, and B. Al-Nawas. 2011. 'Bisphosphonates Affect Migration Ability and Cell Viability of HUVEC, Fibroblasts and Osteoblasts in Vitro'. *Oral Diseases* 17 (2): 194–99. doi:10.1111/j.1601-0825.2010.01720.x.

Weinstein, Robert S., Paula K. Roberson, and Stavros C. Manolagas. 2009. 'Giant Osteoclast Formation and Long-Term Oral Bisphosphonate Therapy'. *New England Journal of Medicine* 360 (1): 53–62. doi:10.1056/NEJMoa0802633.

Wessel, John H., Thomas B. Dodson, and Athanasios I. Zavras. 2008. 'Zoledronate, Smoking, and Obesity Are Strong Risk Factors for Osteonecrosis of the Jaw: A Case-Control Study'. *Journal of Oral and Maxillofacial Surgery* 66 (4): 625–31. doi:10.1016/j.joms.2007.11.032.

Williams, Drake W., Cindy Lee, Terresa Kim, Hideo Yagita, Hongkun Wu, Sil Park, Paul Yang, et al. 2014. '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'. *The American Journal of Pathology*. Accessed September 22. doi:10.1016/j.ajpath.2014.07.010.

Wood, Jeanette, Karine Bonjean, Stephan Ruetz, Akeila Bellahcène, Laetitia Devy, Jean Michel Foidart, Vincent Castronovo, and Jonathan R. Green. 2002. 'Novel Antiangiogenic Effects of the Bisphosphonate Compound Zoledronic Acid'. *The Journal of Pharmacology and Experimental Therapeutics* 302 (3): 1055–61. doi:10.1124/jpet.102.035295.

Ziebart, Thomas, Andreas Pabst, Marcus Oliver Klein, Peer Kämmerer, Leonie Gauss, Dan Brüllmann, Bilal Al-Nawas, and Christian Walter. 2011. 'Bisphosphonates: Restrictions for Vasculogenesis and Angiogenesis: Inhibition of Cell Function of Endothelial Progenitor Cells and Mature Endothelial Cells in Vitro'. *Clinical Oral Investigations* 15 (1): 105–11. doi:10.1007/s00784-009-0365-2.

Figure legends

Figure 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).

Figure 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).

Figure 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).

Figure 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; 2M, second maxillary left molar; 3M, 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).

Figure 5: Scheme representing the disruption of cell components of alveolar BMUs induced by zoledronic acid**.**

Figure legend of supplementary appendix

Supplementary appendix 2: CD146^{+} perivascular cells are affected in alveolar BMUs by the intermediate zoledronic acid treatment associated with molar extraction. Immunodetection of CD146^{+} cells confirms that ZOL-treated mice show a significant increase in the number of perivascular cells (black arrows) in alveolar BMUs at the intermediate delay. (BMUs, basic multicellular units; week 6 (w6), intermediate assessment; w12, long delay assessment; ZOL, zoledronic acid; NaCl, sodium chloride**;** ROI, region of interest; *p<0.05).

Figure 1

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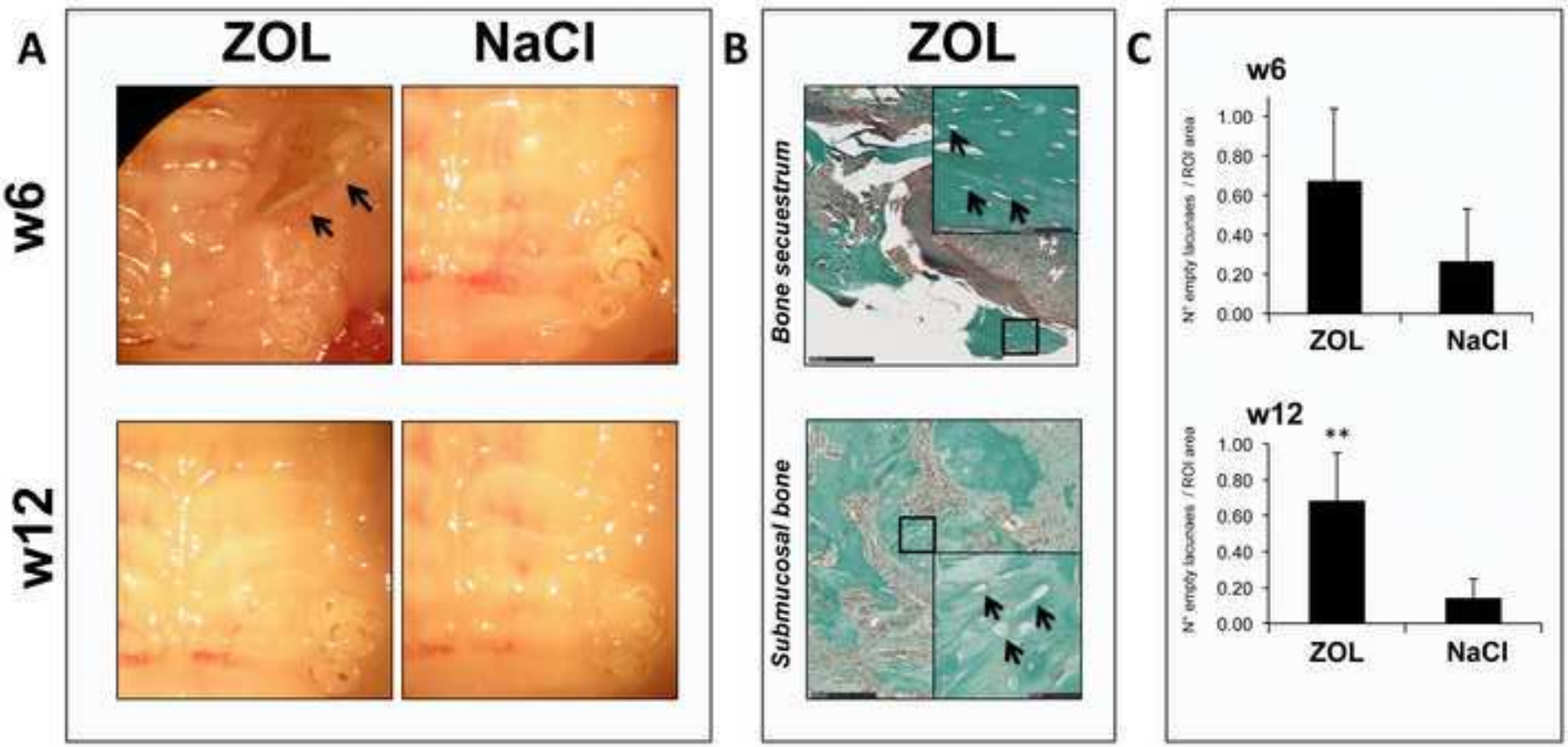

Figure 2

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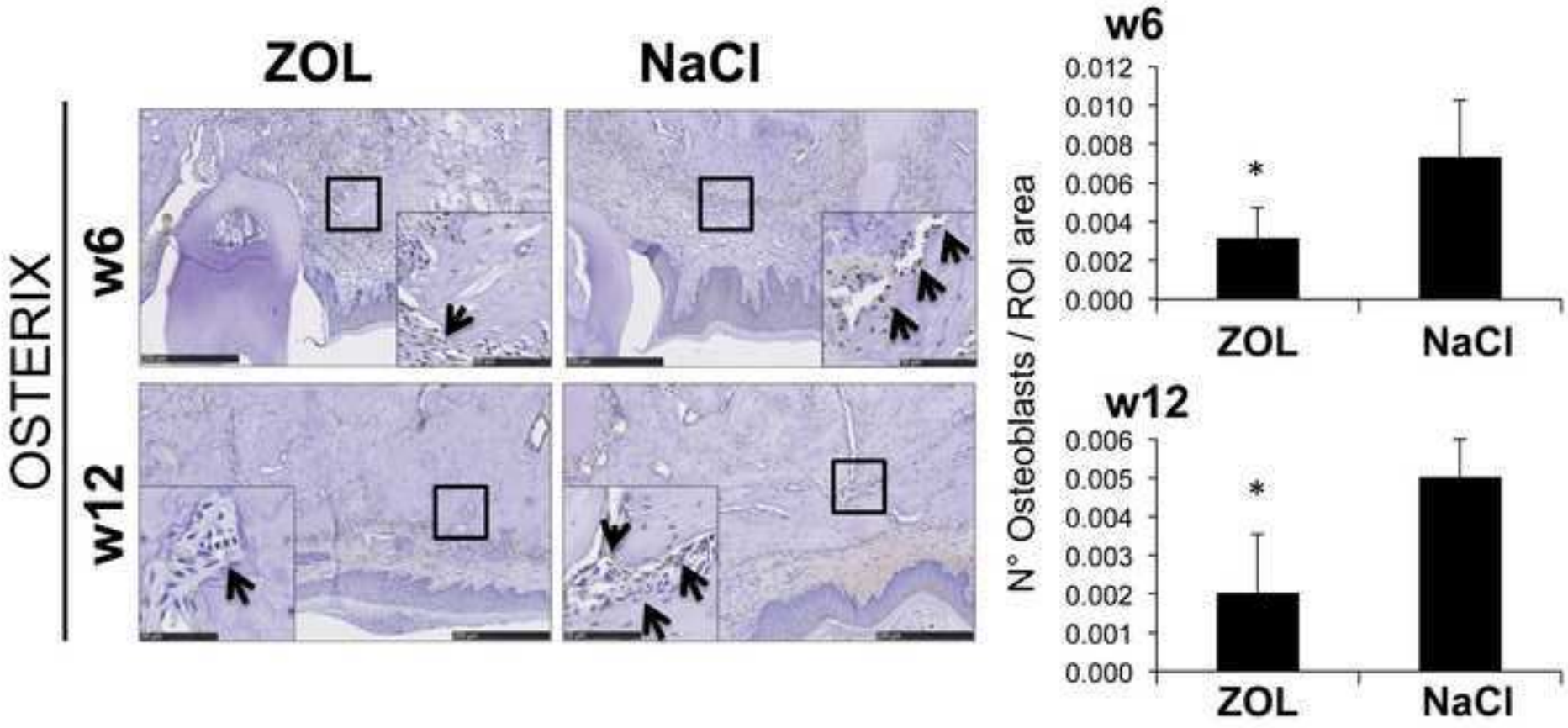

Figure 3

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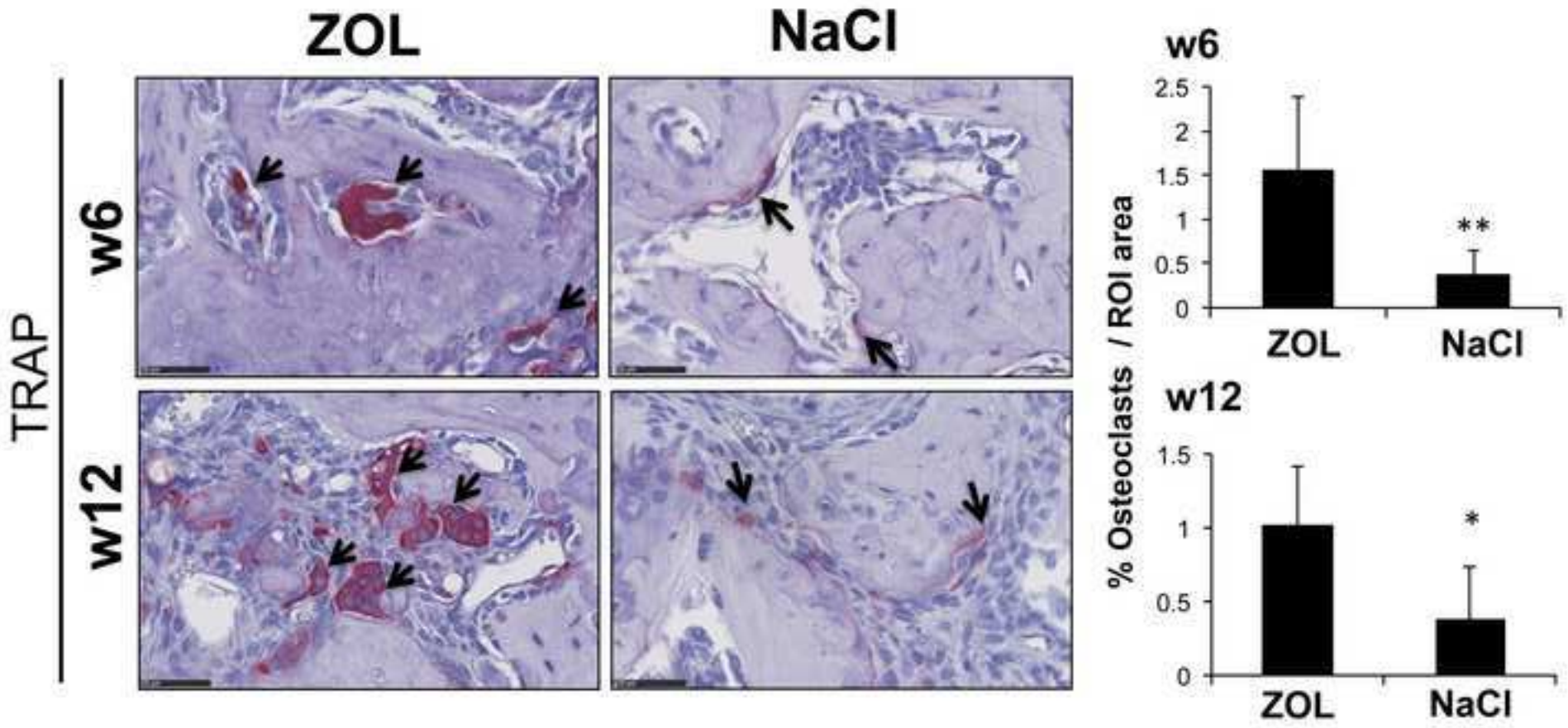

Figure 4

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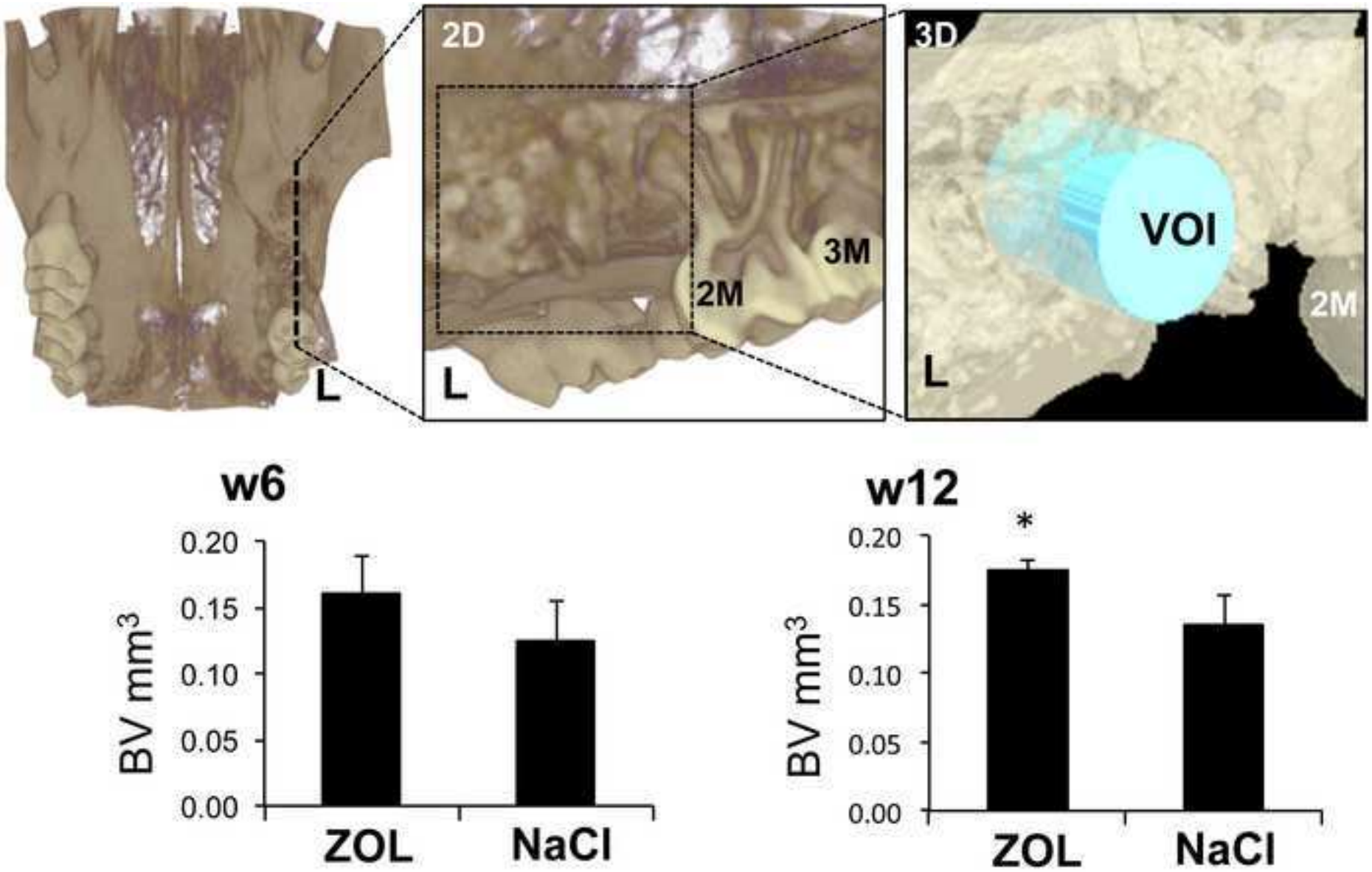

Figure 5

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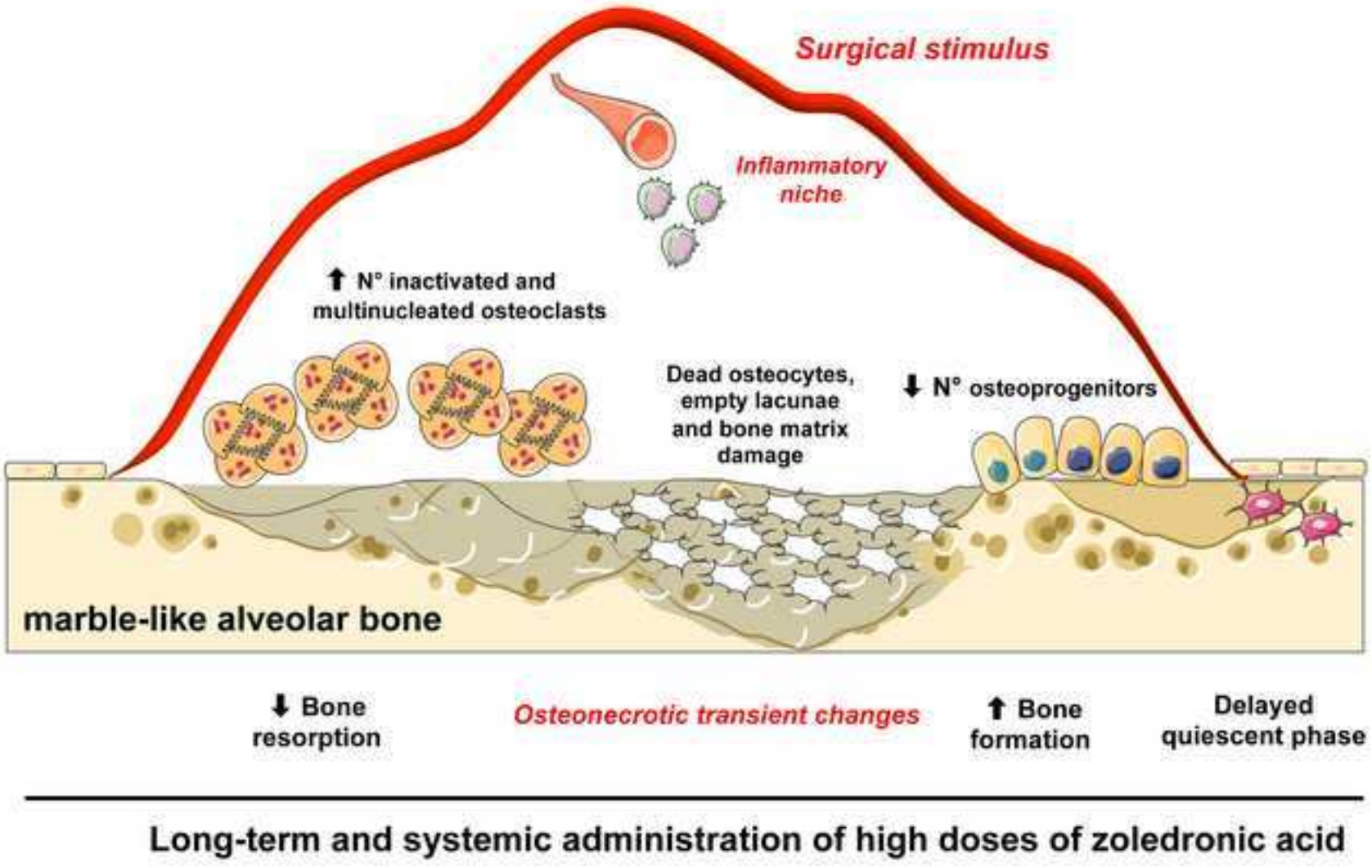

Supplemental appendix 1

Material and methods

Ethical issues of animal housing and animal procedures

The mice (Elevages Janvier, Le Genest Saint Isle, France) were housed in accordance with the institutional guidelines of the French Ethical Committee (CEEA PdL 06 ethical committee, authorization number: 1280.01) and the procedures were performed according to international ethical guidelines for animal care (authorization number: 00568.01).

Surgical procedure – molar extraction

Under general anaesthesia (an intra-peritoneal [i.p.] solution of ketamine 100 mg/kg and xylazine 10 mg/kg), the mice were placed in dorsal decubitus. The molar was luxated and then its three roots were carefully removed using an adapted dental luxator in a previously decontaminated intraoral area (Betadine, France). A subcutaneous injection of Buprenorphine (Buprecar 0.1 mg/Kg) was performed after the surgical procedure (Palier 1 protocol).

TRAP staining technique

All slides were stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts with 1 hour of incubation in a 1 mg/mL naphthol AS-TR phosphate, 60 mmol/L N,Ndimethylformamide, 100 mmol/L sodium tartrate, and 1 mg/mL Fast red TR salt solution (Sigma Aldrich, Saint Quentin Fallavier, France) and counterstained with haematoxylin.

Micro-computed tomography assessment

All maxillae were scanned using the same parameters (pixel size 9 μm, 50 kV, 0.5 mm Al filter and 0.8 degree of rotation step). Three-dimensional reconstruction and quantification of bone parameters were performed in a cylindrical volume of interest (VOI) using ANT and CTvol software (Skyscan).

Supplementary material 2

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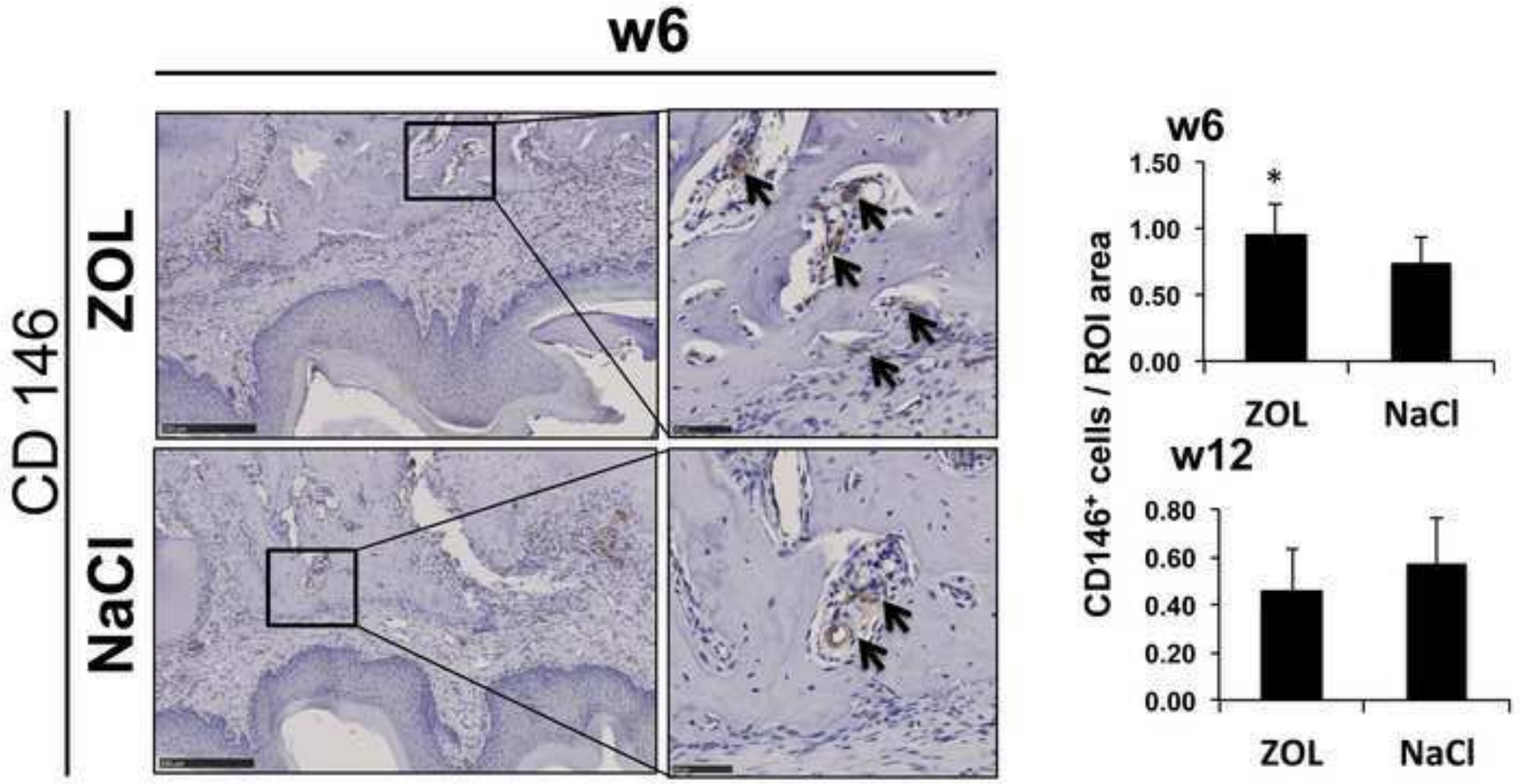

Severe Compromise of Preosteoblasts in a Surgical Mouse Model of Bisphosphonate-Associated Osteonecrosis of the Jaw

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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.

KEYWORDS: Osteonecrosis of the jaw, zoledronic acid, osteoclast, osteoblast, alveolar bone, basic multicellular unit.

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, Dodson, and Zavras 2008; Basso et al. 2013). The pathogenesis of BONJ 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 hours and then decalcified with 4.13% ethylenediaminetetraacetic acid (EDTA) and 0.2% paraformaldehyde in phosphate-buffered saline (PBS) for 96 hours 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, Battaglia, et al. 2014a; Gobin, Huin, 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 $p\text{-value} < 0.05$.

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 (Figure 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 (Figure 1B). On the other hand, the submucosal bone exhibited empty lacunae exclusively in the superficial layer (Figure 1B). Their number was significantly higher at long-term (12 weeks) time point in the ZOL-treated group compared with the control (Figure 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 (Figure 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 (Figure 2, lower panels). The osterix positive cells were detected in the superficial layer of the trabecular bone at both time points (Figure 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. (Figure 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 (Figure 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 (Figure 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 ($p<0.05$) (Figure 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 (Figure 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).

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 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 trabeculae 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, Roberson, and Manolagas 2009; Jobke, Pfeifer, and Minne 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, Pfeifer, and Minne 2009). These aberrant osteoclasts may be subject to prolonged apoptosis or be functionally inhibited by ZOL (Weinstein, Roberson, and Manolagas 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; 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 (Figure 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.

Aknowledgements

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).

References

Aghaloo, Tara L, Ben Kang, Eric C Sung, Michael Shoff, Matthew Ronconi, Jack E Gotcher, Olga Bezouglaia, Sarah M Dry, and Sotirios Tetradis. 2011. 'Periodontal Disease and Bisphosphonates Induce Osteonecrosis of the Jaws in the Rat'. Journal of Bone and Mineral Research 26 (8): 1871–82. doi:10.1002/jbmr.379.

Aguirre, J Ignacio, Mohammed P Akhter, Donald B Kimmel, Jennifer E Pingel, Alyssa Williams, Marda Jorgensen, Lakshmyya Kesavalu, and Thomas J Wronski. 2012. 'Oncologic

Doses of Zoledronic Acid Induce Osteonecrosis of the Jaw-like Lesions in Rice Rats (Oryzomys Palustris) with Periodontitis'. Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research 27 (10): 2130–43. doi:10.1002/jbmr.1669.

Allen, Matthew R. 2008. 'Skeletal Accumulation of Bisphosphonates: Implications for Osteoporosis Treatment'. Expert Opinion on Drug Metabolism & Toxicology 4 (11): 1371–78. doi:10.1517/17425255.4.11.1371.

Allen, Matthew R, and Burr, David B. 2008. 'Mandible Matrix Necrosis in Beagle Dogs after 3 Years of Daily Oral Bisphosphonate Treatment'. Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons 66 (5): 987–94. doi:10.1016/j.joms.2008.01.038.

Allen, Matthew R, and Burr, David B. 2009. 'The Pathogenesis of Bisphosphonate-Related Osteonecrosis of the Jaw: So Many Hypotheses, so Few Data'. Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons 67 (5 Suppl): 61–70. doi:10.1016/j.joms.2009.01.007.

Basso, Fernanda G, Ana Paula Silveira Turrioni, Josimeri Hebling, and Carlos A de Souza Costa. 2013. 'Zoledronic Acid Inhibits Human Osteoblast Activities'. Gerontology 59 (6): 534–41. doi:10.1159/000351194.

Bi, Yanming, Yamei Gao, Driss Ehirchiou, Chunzhang Cao, Takashi Kikuiri, Anh Le, Songtao Shi, and Li Zhang. 2010. 'Bisphosphonates Cause Osteonecrosis of the Jaw-like Disease in Mice'. The American Journal of Pathology 177 (1): 280–90. doi:10.2353/ajpath.2010.090592.

Bouacida, Amina, Philippe Rosset, Valérie Trichet, Fabien Guilloton, Nicolas Espagnolle,

Thomas Cordonier, Dominique Heymann, Pierre Layrolle, Luc Sensébé, and Frédéric Deschaseaux. 2012. 'Pericyte-like Progenitors Show High Immaturity and Engraftment Potential as Compared with Mesenchymal Stem Cells'. PloS One 7 (11): e48648. doi:10.1371/journal.pone.0048648.

Daubiné, Florence, Céline Le Gall, Juerg Gasser, Jonathan Green, and Philippe Clézardin. 2007. 'Antitumor Effects of Clinical Dosing Regimens of Bisphosphonates in Experimental Breast Cancer Bone Metastasis'. Journal of the National Cancer Institute 99 (4): 322–30. doi:10.1093/jnci/djk054.

Doh, Re-Mee, Hye-Jeong Park, Yumie Rhee, Hyun Sil Kim, Jisun Huh, and Wonse Park. 2015. 'Teriparatide Therapy for Bisphosphonate-Related Osteonecrosis of the Jaw Associated with Dental Implants'. Implant Dentistry 24 (2): 222–26. doi:10.1097/ID.0000000000000232.

Estilo, Cherry L, Monica Fornier, Azeez Farooki, Diane Carlson, George Bohle, and Joseph M Huryn. 2008. 'Osteonecrosis of the Jaw Related to Bevacizumab'. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology 26 (24): 4037–38. doi:10.1200/JCO.2007.15.5424.

Forbes, Stuart J., and Nadia Rosenthal. 2014. 'Preparing the Ground for Tissue Regeneration: From Mechanism to Therapy'. Nature Medicine 20 (8): 857–69. doi:10.1038/nm.3653.

Fournier, Pierrick, Sandrine Boissier, Stéphanie Filleur, Julien Guglielmi, Florence Cabon, Marc Colombel, and Philippe Clézardin. 2002. 'Bisphosphonates Inhibit Angiogenesis in Vitro and Testosterone-Stimulated Vascular Regrowth in the Ventral Prostate in Castrated Rats'. Cancer Research 62 (22): 6538–44.

Gobin, Bérengère, Séverine Battaglia, Rachel Lanel, Julie Chesneau, Jérôme Amiaud, Françoise Rédini, Benjamin Ory, and Dominique Heymann. 2014. 'NVP-BEZ235, a Dual PI3K/mTOR Inhibitor, Inhibits Osteosarcoma Cell Proliferation and Tumor Development in Vivo with an Improved Survival Rate'. Cancer Letters 344 (2): 291–98. doi:10.1016/j.canlet.2013.11.017.

Gobin, Bérengère, Marc Baud' Huin, François Lamoureux, Benjamin Ory, Céline Charrier, Rachel Lanel, Séverine Battaglia, et al. 2014. 'BYL719, a New α-Specific PI3K Inhibitor: Single Administration and in Combination with Conventional Chemotherapy for the Treatment of Osteosarcoma'. International Journal of Cancer, June, n/a-n/a. doi:10.1002/ijc.29040.

Guihard, Pierre, Yannic Danger, Bénédicte Brounais, Emmanuelle David, Régis Brion, Joël Delecrin, Carl D. Richards, et al. 2012. 'Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages Depends on Oncostatin M Signaling'. STEM CELLS 30 (4): 762–772. doi:10.1002/stem.1040.

Hansen, Torsten, Martin Kunkel, Achim Weber, and C James Kirkpatrick. 2006. 'Osteonecrosis of the Jaws in Patients Treated with Bisphosphonates - Histomorphologic Analysis in Comparison with Infected Osteoradionecrosis'. Journal of Oral Pathology & Medicine: Official Publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 35 (3): 155–60. doi:10.1111/j.1600-0714.2006.00391.x.

Heymann, D, B Ory, F Blanchard, M-F Heymann, P Coipeau, C Charrier, S Couillaud, J P Thiery, F Gouin, and F Redini. 2005. 'Enhanced Tumor Regression and Tissue Repair When Zoledronic Acid Is Combined with Ifosfamide in Rat Osteosarcoma'. Bone 37 (1): 74–86. doi:10.1016/j.bone.2005.02.020.

Heymann, Dominique. 2010. ‘Bisphosphonates and Bone Diseases: Past, Present and Future’. Current Pharmaceutical Design 16 (27): 2948–49.

Heymann, Dominique, Benjamin Ory, François Gouin, Jonathan R Green, and Françoise Rédini. 2004. ‘Bisphosphonates: New Therapeutic Agents for the Treatment of Bone Tumors’. Trends in Molecular Medicine 10 (7): 337–43. doi:10.1016/j.molmed.2004.05.007.

Hoff, Ana O, Béla B Toth, Kadri Altundag, Marcella M Johnson, Carla L Warneke, Mimi Hu, Ajay Nooka, et al. 2008. ‘Frequency and Risk Factors Associated with Osteonecrosis of the Jaw in Cancer Patients Treated with Intravenous Bisphosphonates’. Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research 23 (6): 826–36. doi:10.1359/jbmr.080205.

Jobke, Björn. 2009. ‘Giant Osteoclast Formation and Long-Term Oral Bisphosphonate Therapy’. The New England Journal of Medicine 360 (16): 1676; author reply 1677-1678. doi:10.1056/NEJMc090167.

Jobke, Björn, Michael Pfeifer, and Helmut W. Minne. 2009. ‘Teriparatide Following Bisphosphonates: Initial and Long-Term Effects on Microarchitecture and Bone Remodeling at the Human Iliac Crest’. Connective Tissue Research 50 (1): 46–54. doi:10.1080/03008200802412462.

Khan, A., A. Morrison, S. Ruggiero, S. Tetradis, K. S. Davison, E. Peters, J. Compston, and O. N. J. Task Force International. 2015. ‘Response to Comments on “Diagnosis and Management of Osteoporosis of the Jaw: A Systematic Review and International Consensus”’. J Bone Miner Res, May. doi:10.1002/jbmr.2524.

Kim, H K W. 2007. ‘Osteonecrosis and Osteonecrosis of the Jaw (ONJ)’. Journal of Musculoskeletal & Neuronal Interactions 7 (4): 348–49.

Kim, R. H., R. S. Lee, D. Williams, S. Bae, J. Woo, M. Lieberman, J.- E. Oh, et al. 2011. ‘Bisphosphonates Induce Senescence in Normal Human Oral Keratinocytes’. Journal of Dental Research 90 (6): 810–16. doi:10.1177/0022034511402995.

Koch, Felix Peter, Christina Merkel, Bilal Al-Nawas, Ralf Smeets, Thomas Ziebart, Christian Walter, and Wilfried Wagner. 2011. ‘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’. Journal of Cranio-Maxillo-Facial Surgery: Official Publication of the European Association for Cranio-Maxillo-Facial Surgery 39 (8): 562–69. doi:10.1016/j.jcms.2010.10.007.

Lamoureux, François, Marc Baud’huin, Lidia Rodriguez Calleja, Camille Jacques, Martine Berreur, Françoise Rédini, Fernando Lecanda, James E. Bradner, Dominique Heymann, and Benjamin Ory. 2014. ‘Selective Inhibition of BET Bromodomain Epigenetic Signalling Interferes with the Bone-Associated Tumour Vicious Cycle’. Nature Communications 5 (March). doi:10.1038/ncomms4511.

Le Goff, B, P Guillot, J Glémarec, J M Berthelot, and Y Maugars. 2010. ‘A Comparison between Bisphosphonates and Other Treatments for Osteoporosis’. Current Pharmaceutical Design 16 (27): 3037–44.

Lescaille, Géraldine, Amélie E. Coudert, Vanessa Baaroun, Agnès Ostertag, Emmanuel Charpentier, Marie-José Javelot, Rafael Tolédo, et al. 2014. ‘Clinical Study Evaluating the Effect

of Bevacizumab on the Severity of Zoledronic Acid-Related Osteonecrosis of the Jaw in Cancer Patients'. Bone 58 (January): 103–7. doi:10.1016/j.bone.2013.10.002.

Lesclous, Philippe, Semaan Abi Najm, Jean-Pierre Carrel, Brigitte Baroukh, Tommaso Lombardi, Jean-Pierre Willi, René Rizzoli, Jean-Louis Saffar, and Jacky Samson. 2009. 'Bisphosphonate-Associated Osteonecrosis of the Jaw: A Key Role of Inflammation?' Bone 45 (5): 843–52. doi:10.1016/j.bone.2009.07.011.

Manzano-Moreno, Francisco Javier, Javier Ramos-Torrecillas, Elvira De Luna-Bertos, Concepción Ruiz, and Olga García-Martínez. 2015. 'High Doses of Bisphosphonates Reduce Osteoblast-like Cell Proliferation by Arresting the Cell Cycle and Inducing Apoptosis'. Journal of Cranio-Maxillo-Facial Surgery: Official Publication of the European Association for Cranio-Maxillo-Facial Surgery 43 (3): 396–401. doi:10.1016/j.jcms.2014.12.008.

Marino, Karen L., Ibrahim Zakhary, Rafik A. Abdelsayed, Jared A. Carter, Jack C. O'Neill, Rania M. Khashaba, Mohammed Elsalanty, Mark R. Stevens, and James L. Borke. 2011. 'Development of a Rat Model of Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ)'. Journal of Oral Implantology, September, 110909140919004. doi:10.1563/AAID-JOI-D-11-00057.

Marx, Robert E. 2003. 'Pamidronate (Aredia) and Zoledronate (Zometa) Induced Avascular Necrosis of the Jaws: A Growing Epidemic'. Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons 61 (9): 1115–17.

Marx, Robert E., Yoh Sawatari, Michel Fortin, and Vishtasb Broumand. 2005. 'Bisphosphonate-Induced Exposed Bone (Osteonecrosis/Osteopetrosis) of the Jaws: Risk Factors, Recognition,

Prevention, and Treatment'. Journal of Oral and Maxillofacial Surgery 63 (11): 1567–75. doi:10.1016/j.joms.2005.07.010.

Mawardi, H, G Giro, M Kajiya, K Ohta, S Almazrooa, E Alshwaimi, S-B Woo, I Nishimura, and T Kawai. 2011. 'A Role of Oral Bacteria in Bisphosphonate-Induced Osteonecrosis of the Jaw'. Journal of Dental Research 90 (11): 1339–45. doi:10.1177/0022034511420430.

Nicolaidou, Vicky, Mei Mei Wong, Andia N. Redpath, Adel Ersek, Dilair F. Baban, Lynn M. Williams, Andrew P. Cope, and Nicole J. Horwood. 2012. 'Monocytes Induce STAT3 Activation in Human Mesenchymal Stem Cells to Promote Osteoblast Formation'. Edited by Dimas Tadeu Covas. PLoS ONE 7 (7): e39871. doi:10.1371/journal.pone.0039871.

Okazaki, S., Y. Nishitani, S. Nagoya, M. Kaya, T. Yamashita, and H. Matsumoto. 2009. 'Femoral Head Osteonecrosis Can Be Caused by Disruption of the Systemic Immune Response via the Toll-like Receptor 4 Signalling Pathway'. Rheumatology 48 (3): 227–32. doi:10.1093/rheumatology/ken462.

Pabst, A. M., T. Ziebart, M. Ackermann, M. A. Konerding, and C. Walter. 2014. 'Bisphosphonates' Antiangiogenic Potency in the Development of Bisphosphonate-Associated Osteonecrosis of the Jaws: Influence on Microvessel Sprouting in an in Vivo 3D Matrigel Assay'. Clinical Oral Investigations 18 (3): 1015–22. doi:10.1007/s00784-013-1060-x.

Pazianas, M. 2011. 'Osteonecrosis of the Jaw and the Role of Macrophages'. J Natl Cancer Inst 103 (3): 232–40. doi:10.1093/jnci/djq516.

Pozzi, Samantha, Sonia Vallet, Siddhartha Mukherjee, Diana Cirstea, Nileshwari Vaghela, Loredana Santo, Eyal Rosen, et al. 2009. 'High-Dose Zoledronic Acid Impacts Bone

Remodeling with Effects on Osteoblastic Lineage and Bone Mechanical Properties'. Clinical Cancer Research 15 (18): 5829–39. doi:10.1158/1078-0432.CCR-09-0426.

Raje, Noopur, Sook-Bin Woo, Karen Hande, Jeffrey T. Yap, Paul G. Richardson, Sonia Vallet, Nathaniel Treister, et al. 2008. 'Clinical, Radiographic, and Biochemical Characterization of Multiple Myeloma Patients with Osteonecrosis of the Jaw'. Clinical Cancer Research 14 (8): 2387–95. doi:10.1158/1078-0432.CCR-07-1430.

Roelofs, Anke J., Keith Thompson, Sharon Gordon, and Michael J. Rogers. 2006. 'Molecular Mechanisms of Action of Bisphosphonates: Current Status'. Clinical Cancer Research 12 (20): 6222s–6230s. doi:10.1158/1078-0432.CCR-06-0843.

Ruggiero, Salvatore L, Thomas B Dodson, Leon A Assael, Regina Landesberg, Robert E Marx, and Bhoomi Mehrotra. 2009. 'American Association of Oral and Maxillofacial Surgeons Position Paper on Bisphosphonate-Related Osteonecrosis of the Jaws--2009 Update'. Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons 67 (5 Suppl): 2–12. doi:10.1016/j.joms.2009.01.009.

Sakagami, Naoko, Norio Amizuka, Minqi Li, Kiichi Takeuchi, Masaaki Hoshino, Midori Nakamura, Kayoko Nozawa-Inoue, Nobuyuki Udagawa, and Takeyasu Maeda. 2005. 'Reduced Osteoblastic Population and Defective Mineralization in Osteopetrotic (Op/op) Mice'. Micron (Oxford, England: 1993) 36 (7–8): 688–95. doi:10.1016/j.micron.2005.06.008.

Santini, Daniele, Bruno Vincenzi, Giordano Dicuonzo, Giuseppe Avvisati, Cristian Massacesi, Fabrizio Battistoni, Michele Gavasci, et al. 2003. 'Zoledronic Acid Induces Significant and Long-Lasting Modifications of Circulating Angiogenic Factors in Cancer Patients'. Clinical

Cancer Research 9 (8): 2893–97.

Sims, N. A., and K. W. Ng. 2014. ‘Implications of Osteoblast-Osteoclast Interactions in the Management of Osteoporosis by Antiresorptive Agents Denosumab and Odanacatib’. Curr Osteoporos Rep 12 (1): 98–106. doi:10.1007/s11914-014-0196-1.

Sims, Natalie A., and T. John Martin. 2014. ‘Coupling the Activities of Bone Formation and Resorption: A Multitude of Signals within the Basic Multicellular Unit’. BoneKEy Reports 3 (January). doi:10.1038/bonekey.2013.215.

Stresing, Verena, Pierrick G Fournier, Akeila Bellahcène, Ismahène Benzaïd, Hannu Mönkkönen, Marc Colombel, F Hal Ebetino, Vincent Castronovo, and Philippe Clézardin. 2011. ‘Nitrogen-Containing Bisphosphonates Can Inhibit Angiogenesis in Vivo without the Involvement of Farnesyl Pyrophosphate Synthase’. Bone 48 (2): 259–66. doi:10.1016/j.bone.2010.09.035.

Walter, C., A. Pabst, T. Ziebart, Mo Klein, and B. Al-Nawas. 2011. ‘Bisphosphonates Affect Migration Ability and Cell Viability of HUVEC, Fibroblasts and Osteoblasts in Vitro’. Oral Diseases 17 (2): 194–99. doi:10.1111/j.1601-0825.2010.01720.x.

Weinstein, Robert S., Paula K. Roberson, and Stavros C. Manolagas. 2009. ‘Giant Osteoclast Formation and Long-Term Oral Bisphosphonate Therapy’. New England Journal of Medicine 360 (1): 53–62. doi:10.1056/NEJMoa0802633.

Wessel, John H., Thomas B. Dodson, and Athanasios I. Zavras. 2008. ‘Zoledronate, Smoking, and Obesity Are Strong Risk Factors for Osteonecrosis of the Jaw: A Case-Control Study’. Journal of Oral and Maxillofacial Surgery 66 (4): 625–31. doi:10.1016/j.joms.2007.11.032.

Williams, Drake W., Cindy Lee, Terresa Kim, Hideo Yagita, Hongkun Wu, Sil Park, Paul Yang, et al. 2014. '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'. The American Journal of Pathology. Accessed September 22. doi:10.1016/j.ajpath.2014.07.010.

Wood, Jeanette, Karine Bonjean, Stephan Ruetz, Akeila Bellahcène, Laetitia Devy, Jean Michel Foidart, Vincent Castronovo, and Jonathan R. Green. 2002. 'Novel Antiangiogenic Effects of the Bisphosphonate Compound Zoledronic Acid'. The Journal of Pharmacology and Experimental Therapeutics 302 (3): 1055–61. doi:10.1124/jpet.102.035295.

Ziebart, Thomas, Andreas Pabst, Marcus Oliver Klein, Peer Kämmerer, Leonie Gauss, Dan Brüllmann, Bilal Al-Nawas, and Christian Walter. 2011. 'Bisphosphonates: Restrictions for Vasculogenesis and Angiogenesis: Inhibition of Cell Function of Endothelial Progenitor Cells and Mature Endothelial Cells in Vitro'. Clinical Oral Investigations 15 (1): 105–11. doi:10.1007/s00784-009-0365-2.

Figure legends

Figure 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).

Figure 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).

Figure 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).

Figure 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; 2M, second maxillary left molar; 3M, 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).

Figure 5: Scheme representing the disruption of cell components of alveolar BMUs induced by zoledronic acid**.**

Figure legend of supplementary appendix

Supplementary appendix 2: CD146^{+} perivascular cells are affected in alveolar BMUs by the intermediate zoledronic acid treatment associated with molar extraction. Immunodetection of CD146^{+} cells confirms that ZOL-treated mice show a significant increase in the number of perivascular cells (black arrows) in alveolar BMUs at the intermediate delay. (BMUs, basic multicellular units; week 6 (w6), intermediate assessment; w12, long delay assessment; ZOL, zoledronic acid; NaCl, sodium chloride**;** ROI, region of interest; *p<0.05).