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ORIGINAL ARTICLE

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Oncologic doses of zoledronic acid induce site specific suppression of bone modelling in rice rats

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Structured Abstract

Objectives: To examine the effect of zoledronic acid (ZOL) on cortical bone modelling and healing of extraction sockets in the jaw bones of a rodent model. We hypothesized ZOL suppresses both the bone formation in the modelling mode in the jaw bones and alters the extraction site healing.

Material & Methods: Rice rats were administered saline solution and two dose regimens of ZOL: 0.1 mg/kg, twice a week, for 4 weeks (n=17, saline=8 & ZOL=9) and a higher dose of 0.4 mg/kg, weekly, for 9 weeks (n=30, saline=15 & ZOL=15). Two pairs of fluorochrome bone labels were administered. Extraction of maxillary teeth was performed in maxilla. Mineral apposition rate, mineralizing surface and bone formation rate (BFR) were quantified on periodontal (PDL), alveolar and basal bone surfaces, and in the trabecular bone of proximal tibia. Bone volume (BV) was evaluated at extraction sockets. Multivariate Gaussian models were used to account for repeated measurements, and analyzes were conducted in SAS V9.3.

Results: ZOL suppressed bone modelling (BFR/BS) at the PDL surfaces in the mandible ($P<.05$), but its effect was not significant at the periosteal surfaces of both jaws. BV for the healing sockets of ZOL treated animals was not significantly different ($P=.07$) compared to the saline group. ZOL suppressive effect was higher in the tibia compared to the jaws.

Conclusion: ZOL severely suppresses coupled remodelling in the tibia, and the suppression of bone formation in the modelling mode in the jaws demonstrates the site specific effects of ZOL in rice rats.

KEYWORDS
animal model, bisphosphonates, bone modelling, zoledronic acid

1 | INTRODUCTION

The association between the use of bisphosphonates (BP) and the subsequent development of osteonecrosis of the jaw (ONJ) in humans was first documented in 2003.[1] Despite the knowledge gained in the past decades, the pathophysiology of ONJ remains elusive. Studies cite infection, alterations in vascularity, genetic predisposition and suppression of bone turnover as possible factors involved in the development of ONJ.[2] The suppressive effect of BP on secondary osteonal remodelling is well documented.[3,4] However, it is less clear how bisphosphonates alter bone modelling.

To study the effects of BP several animal models have been described. Some demonstrated ONJ like lesions especially when the bisphosphonates are concurrent with dental extractions[5,6] or given

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a combination of additional drugs such as dexamethasone.[7] Larger canine models have been used to study ONJ primarily because the jaw bones of these animals possess secondary osteonal cortical bone remodelling, which is not typically observed in smaller rodent models.[4] The rice rat (*Oryzomys palustris*) has been demonstrated to be an animal model for study of destructive periodontal disease.[8] Because periodontitis is a co-factor for ONJ in humans,[9] the extensive bone loss due to naturally occurring periodontal disease (Fig. S1A,B) together with the previous incidental finding of bone exposure provided a strong rationale for utilizing a rice rat animal model.[10] We hypothesized zoledronic acid (ZOL) suppresses bone formation in the modelling mode in the jaw bones and alters extraction site healing.

2 | MATERIAL AND METHODS

2.1 | Animal care and drug administration

Institutional animal review board approval was obtained for all procedures related to this study. To address the issue of equal gender inclusion recently outlined by the National Institute of Health (NIH)[11] both male and female rice rats (*Oryzomys palustris*) were included in this study. We used two different ZOL (ZOMETA®, Novartis, Switzerland) dose regimens to mimic an earlier and a later stage of treatment for patients with cancer.[12] We administered a lower dose of 0.1 mg/kg, twice a week, for 4 weeks to a cumulative dose of 0.8 mg/kg (study 1) and a higher dose of 0.4 mg/kg, weekly, for an extended period of 9 weeks to a cumulative dose of 3.6 mg/kg (study 2).

2.2 | Study 1 – Male rice rats and lower ZOL oncologic dose

Seventeen male rice rats were obtained. Animals were divided into two groups, the control group receiving *i.v.* saline (n=8), and the experimental group receiving ZOL (n=9). Injections of two equivolume doses/week were administered for 4 weeks. Each animal received a pair of alizarin labels (Sigma, St. Louis, MO, USA), at a dose of 20 mg/kg, 1 week apart prior to the experimental treatments (ZOL or saline) and a pair of calcein labels (Sigma, St. Louis, MO, USA), at a dose of 5 mg/kg, 1 week apart prior to sacrifice and after the completion of the experimental treatments. Surgical extraction of maxillary teeth was performed one and a half weeks after the last ZOL injection, under anaesthesia. Average age at sacrifice was 13 months. (Figure 1A)

2.3 | Study 2 – Female rice rats and higher ZOL oncologic dose

Thirty female rice rats were randomly divided into two groups, the saline group receiving *i.v.* saline (n=15), and the experimental group receiving ZOL (n=15), with the experimental design as per study 1 with the exception of the timeline/dosage of 9 weeks. Average age at sacrifice was 13 months. (Figure 1B)

2.4 | Sample preparation

At sacrifice the maxilla, mandible and tibia were obtained from each rat. The tissues were fixed and infiltrated in methylmethacrylate (Polysciences, Inc., Warrington, PA). Thin (~5 microns) unstained sections from the proximal tibiae were obtained using a Leica Microtome (RM 2065, Buffalo Grove, IL) with the aim of examining trabecular bone remodelling. Maxillary and mandibular plastic bone blocks were sectioned bucco-lingually, at the molar regions, with a Diamond Wire Saw (Delaware Diamond Knives, Wilmington, DE), to obtain multiple unstained thick (60-80 microns) sections to study epifluorescent histomorphometry. To evaluate the extraction sites, in study 1 decalcified and paraffin embedded alveolar sockets were stained with haematoxylin-eosin (H&E) for qualitative analysis; in study 2, thick (60-80 μm) bucco-lingual sections of the maxilla were obtained for histomorphometric analyzes.

2.5 | Bone histomorphometry of tooth bearing regions and tibia bones

Sections from the tooth bearing regions of the maxilla and mandible were selected. Data in the maxilla were obtained from two regions: periodontal

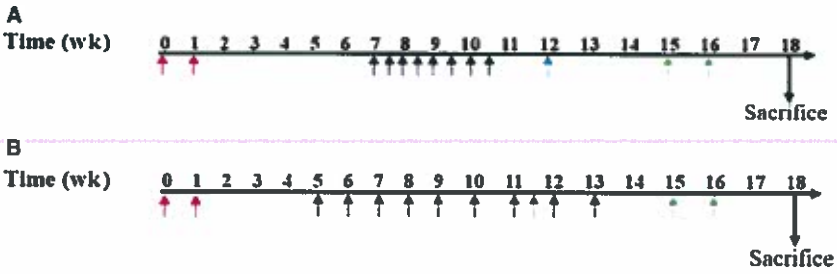

FIGURE 1 A, Study 1 – Male rice rats and lower ZOL oncologic dose (0.1 mg/kg). Rats received *i.v.* injections of ZOL/saline (black arrows) twice a week for 4 wk. Extractions (blue arrow) were performed approximately one and a half week after the last injection. Calcium chelating bone labels were administered prior (alizarin: red arrows) to ZOL/saline administration and just before sacrifice (calcein: green arrows). B, Study 2 – Female rice rats and higher ZOL oncologic dose (0.4 mg/kg). Rats received *i.v.* injections of ZOL/saline (black arrows) weekly for 9 wk. Extractions (blue arrow) were performed approximately one and a half week before the last injection. Calcium chelating bone labels were administered prior (alizarin: red arrows) to ZOL/saline administration and just before sacrifice (calcein: green arrows)

ligament (PDL) supporting bone and periosteal (PO) bone (Figure 2A). In the mandible the PO region was divided into basal periosteal bone (PO B) and alveolar periosteal bone (PO A) (Figure 2B). Sections from the proximal tibial metaphyses were also selected for histomorphometric analyzes. The method has been described previously.[13,14] The following histomorphometric parameters were calculated as follows: mineral apposition rate (MAR, μm/d), mineralizing surface/bone surface (MS/BS) and bone formation rate/bone surface (BFR/BS, $\mu m^3/\mu m^2/d$).[15]

2.6 | Bone histomorphometry and histology of maxillary healing sockets

At the maxillary extraction healing sites of study 2 (n=5 and ZOL, n=7), the following histomorphometric parameters were evaluated as follows: bone volume/total volume (BV/TV [%]), trabecular number (Tb.N.), trabecular thickness (Tb.Th.) and trabecular separation (Tb. Sp.). Extraction healing sites were further evaluated under polarized light for visualization of bone architecture. At the maxillary extraction healing sites of study 1 (sections obtained from 16 animals, saline, n=7 and ZOL=9), the decalcified thin sections were stained with H&E and qualitatively evaluated for histological bone exposure.

2.7 | Static and osteoclast parameters of tibia

Sections of tibia from the female rats (study 2) were evaluated for static BV/TV (%), Tb.Sp., Tb.Th., Tr. and were TRAP (tartrate resistant acid phosphatase) stained and examined for osteoclasts numbers (Oc#). Two tibial sections from each animal (saline, n=9 and ZOL, n=7) were selected and analyzed.

2.8 | Statistical analyzes

Statistical tests were two sided at the 5% significance level (alpha value of 0.05).

2.8.1 | Study 1

Due to the hierarchical structure of the data, mixed effect models were used to analyze relationships between the histomorphometric variables for the groups, skeletal sites and labels. Interaction effects were included in all models, and pairwise comparisons were performed when the interaction effects were significant. For the static trabecular parameters, two-sample t-tests were used to compare their differences between the two groups.

2.8.2 | Study 2

Differences at baseline (alizarin labelling period) were noted and, to account for it, differences in change were analyzed. Multivariate Gaussian models were used to account for repeated measurements from each rat, and analyzes were conducted in SAS V9.3.

3 | RESULTS

3.1 | Bone histomorphometry of tooth bearing regions and tibia bones

3.1.1 | ZOL effect in the trabecular bone of the tibia and different regions of the jaws on bone formation rate (BFR/BS)

In both studies, the effect of ZOL was not significant at the periosteal surfaces of both maxilla and mandible. In study 1, ZOL significantly ($P<0.05$) suppressed the BFR/BS at the tibia, and PDL surfaces of both mandible and maxilla. In study 2, ZOL significantly ($P<0.05$) suppressed the BFR/BS at the tibia and PDL surface of the mandible. In the maxilla, the PDL surfaces were not significantly ($P=0.08$) suppressed; however, a tendency for suppression was observed (Figure 3A-D, and

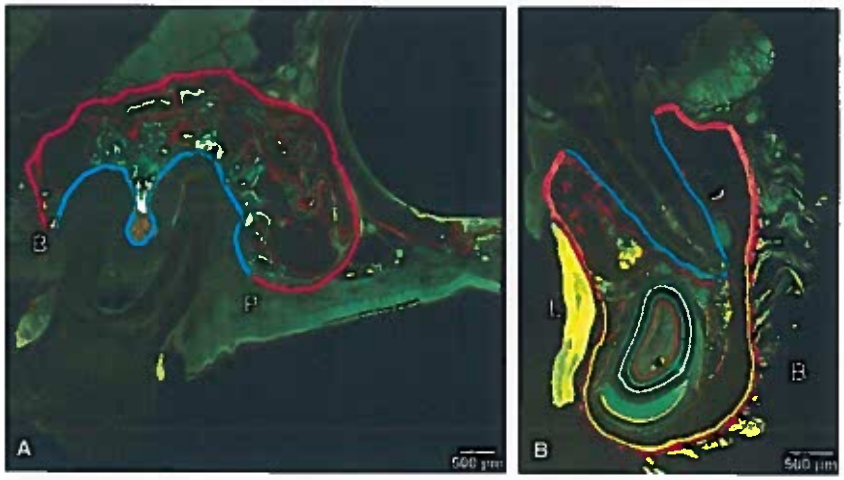

FIGURE 2 Epifluorescent images showing outlined surfaces of interest for histomorphometric data collection of tooth bearing sites in the maxilla (A) and mandible (B). A, Data in the maxilla were collected from two regions: periodontal ligament (PDL) supporting bone (blue outline) and periosteal (PO) bone (red outline). B, In the mandible the PO region (red outline) was divided into basal periosteal bone (PO B) (yellow outline) and alveolar periosteal bone (PO A) (orange outline) and, therefore, data were separately collected for three regions. Buccal (B), palatal (P), lingual (L)

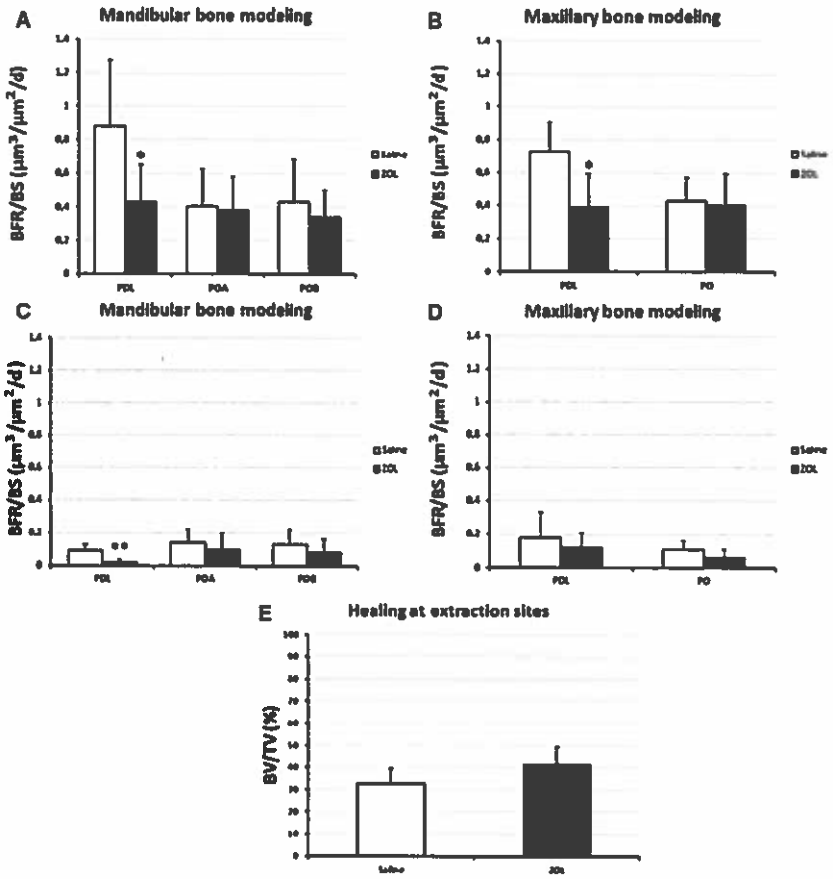

FIGURE 3 Bone formation rate (BFR/BS) for study 1 (A,B) and study 2 (C,D). In study 1, ZOL significantly suppressed ($P<0.05$) (*) the BFR/BS at the PDL surfaces of both mandible (A) and maxilla (B). Study 2 results suggest suppression of bone modelling (BFR/BS) at the PDL surfaces in the mandible (C) ($P<0.001$) (**), and a non-significant tendency in the maxilla (D) ($P=0.08$). In both studies, the effect of ZOL was not significant at the periosteal surfaces of both maxilla and mandible. E. Bone volume (BV/TV) within the sockets of the ZOL treated animals was not significantly decreased ($P=0.07$) when compared to the saline group

TABLE 1 Means and standard deviations (Mean / SD) of static histomorphometric parameters from the tibia

	Study 1		Study 2	
Static Variables	**Mean / SD**	***P*-value**	**Mean/SD**	***P*-value**
Tr. Sp. (nm)	S - 1861 / 468	0.04	S - 420 / 110	0.17
	ZOL - 1479 / 553		ZOL - 570 / 250	
Tr. N /mm	S - 2.9 / 2.2	0.11	S - 1.87 / 0.35	0.62
	ZOL - 5.4 / 5.5		ZOL - 1.7 / 0.82	
Tr. Th. ((4,m)	S - 178 / 54	0.03	S - 131 / 21	0.03
	ZOL - 148 / 15		ZOL - 105 / 21	
BV/TV (%)	S - 9.7 / 4.4	0.07	S - 4.9 / 5.9	1.00
	ZOL - 12.8 / 4.9		ZOL - 3.9 / 2.3	

S, saline; ZOL, zoledronic acid; Tr.Sp., trabecular separation; Tr.N, trabecular numbers; Tr.Th., trabecular thickness; BV/TV, bone volume / tissue volume.

Fig. S2A,B). MAR overall was not suppressed, and MS/BS alterations were very similar to BFR/BS.

3.1.2 | Comparison of skeletal sites: tibia, maxilla and mandible

The overall suppressive effect of ZOL on the three skeletal sites (tibia, maxilla and mandible) was similar in both studies. Results show the suppression of bone formation to be higher in the tibia than in the jaws: In study 1, each jaw of the ZOL treated animals had significantly ($P\leq0.0001$) higher BFR/BS than the tibia site. Similarly, in study 2, the suppressive effect of ZOL was higher in the tibia when compared to the mandible and maxilla ($P\leq0.005$).

3.2 | Static and osteoclast parameters from Tibia

The BV/TV and other parameters (Table 1) were not significantly different between the saline treated animals and the ZOL group for both study 1 ($P=0.07$) and 2 ($P=1.00$). In study 2, the mean (SD) for Oc#/bone surface (#/mm) in the saline (0.904 [1.502]) and the ZOL (0.195 [0.38]) groups were not significantly different ($P>0.05$). High variability was observed between animals for Oc#.

TABLE 2 Means and standard deviations (Mean / SD) of static histomorphometric parameters from maxillary extraction sites

	Extraction Sites	
Static Variables	**Mean / SD**	***P*-value**
Tr. Sp. (ij.m)	S - 5.8 / 2.3	0.08
	ZOL - 1.7 /1.4	
Tr. N /mm	S - 6.9 / 2.28	0.004
	ZOL - 2.7 / 1.9	
Tr. Th. (ji.m)	S - 10.2 / 3.4	0.025
	ZOL - 44.8 / 30.8	
BV/TV (%)	S - 32.7 / 6.8	0.07
	ZOL - 41.2 / 8.1	

S, saline; ZO, zoledronic acid; Tr.Sp., trabecular separation; Tr.N, trabecular numbers; Tr.Th., trabecular thickness; BV/TV, bone volume / tissue volume

3.3 | Bone Histomorphometry and histology of maxillary healing sockets

The mean±SD values of BV/TV (%) for the saline and ZOL groups were 32.7±6.8 and 41.2±8.1, respectively, showing that bone volume within the sockets of ZOL treated animals was not significantly (P=0.07) decreased when compared to the saline group (Figure 3E). The trabecular parameters results are in Table 2. Observation under polarized light shows different patterns of bone organization between the two groups (Fig. S3A,B). Histological, H&E staining, and examination of extraction sites showed both well covered teeth sockets, as areas of possible bone (Fig. S4A,B).

4 | DISCUSSION

The overall results of this study suggest that ZOL produces site specific suppression of bone remodelling in the tibia and bone modelling in the jaw bones in a rodent model. The tibia trabecular compartment remodelling was suppressed by ~82%. The suppression occurred after administration of ZOL to different cumulative doses in two separate studies. In addition, sites of active bone formation, such as the mandibular tooth socket, which is lined by periodontium, had a ~50% (study 1) and ~78% (study 2) decrease in bone formation. However, suppression by ZOL of the bone formation was not seen on the periosteal surfaces of the jaw bones, which undergo bone modelling. The bone volume in the extraction socket was not altered at the time point examined.

In the current study, we did not observe spontaneous ONJ like lesions or protrusion of bone in the oral cavity. It is possible that there was histological evidence of exposed bone, as demonstrated in the past, and as we observed in H&E histological sections. However, in rodent studies on bisphosphonates and ONJ, even animals in the control group have developed ONJ-like lesions with a higher incidence than is seen in humans.[16]

The periodontal surfaces of the mandible showed significant suppression in bone formation. This bone formation is independent of bone resorption as it is occurring in the modelling mode. This suggests an effect of ZOL on the activity of the osteoblasts at a tissue level. Bisphosphonates have been shown to reduce MAR by 40%-50% on periosteal surfaces of female Sprague Dawley rats.[17] However, other studies indicated that bisphosphonates may alter proliferation and differentiation of osteoblasts.[18,19] Thus, the effect of bisphosphonates on osteoblasts remains controversial. In rats that were administered alendronate to examine extraction site healing, suppression of osteoblasts surface, MS/BS and BFR/BS were observed especially in the mandible at an early time point of 10 days after surgery, but not at the 21 daytime point.[20] In the current study, the suppressive effect of ZOL in the jaw was only seen on the periodontal surfaces.

5 | CONCLUSION

Alteration in bone remodelling and modelling is site specific and not uniform throughout the skeleton in the rice rat. ZOL did not alter bone volume in healing extraction sites.

CONFLICT OF INTERESTS

The authors have no conflict of interests to report.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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