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Ovariectomy stimulates and bisphosphonates inhibit intracortical remodeling in the mouse mandible

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Structured Abstract

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Objective – The pathophysiology of osteonecrosis of the jaw (ONJ) is thought to be linked to suppression of intracortical remodeling. The aim of this study was to determine whether mice, which normally do not undergo appreciable amounts of intracortical remodeling, could be stimulated by ovariectomy to remodel within the cortex of the mandible and if bisphosphonates (BPs) would suppress this intracortical remodeling.

Material and Methods – Skeletally mature female C3H mice were either ovariectomized (OVX) or SHAM operated and treated with two intravenous doses of zoledronic acid (ZOL, 0.06 mg/kg body weight) or vehicle (VEH). This ZOL dose corresponds to the dose given to patients with cancer on a mg/kg basis, adjusted for body weight. Calcein was administered prior to sacrifice to label active formation sites. Dynamic histomorphometry of the mandible and femur was performed.

Results – Vehicle-treated OVX animals had significantly higher (eightfold) intracortical remodeling of the alveolar portion of the mandible compared to sham – this was significantly suppressed by ZOL treatment. At all skeletal sites, overall bone formation rate was lower with ZOL treatment compared to the corresponding VEH group.

Conclusions – Under normal conditions, the level of intracortical remodeling in the mouse mandible is minimal but in C3H mice it can be stimulated to appreciable levels with ovariectomy. Based on this, if the suppression of intracortical remodeling is found to be part of the pathophysiology of ONJ, the ovariectomized C3H mouse could serve as a useful tool for studying this condition.

Key words: BRONJ; jaw necrosis; osteonecrosis; osteonecrosis of the jaw; zoledronic acid

Introduction

Bisphosphonates (BPs) are a drug class that is widely and effectively used in the treatment of osteoporosis, Paget's disease, and metastatic cancers involving bone. BPs are associated with a rare condition known as osteonecrosis of the jaw (ONJ) (1, 2). Zoledronic acid (Zometa), the BP most often used in treating patients with cancer, is most often associated with ONJ. This condition is characterized by regions of exposed, necrotic bone in the mandible and/or maxilla (3, 4).

Unfortunately, little is known about the pathogenesis of ONJ due in part to the absence of an animal model for this condition (3, 5).

The mandible undergoes intracortical remodeling at a faster rate than other cortical bone regions in the body (6, 7). Many have suggested that ONJ is related to remodeling suppression (1, 5). Consistent with this theory, BPs have been shown to impart near complete suppression of intracortical remodeling in the mandible of beagle dogs (7) and this is associated with an accumulation of necrotic bone matrix (6).

Unlike humans and other larger animals, rodents do not routinely undergo appreciable amounts of intracortical remodeling (8). This potentially limits the use of rats and mice as an animal model for ONJ if suppression of intracortical remodeling is a part of the disease etiology. Recent data have documented that adult C3H mice undergo significant amounts of intracortical remodeling in the femur mid-diaphysis following ovariectomy (9). Whether or not similar increases in intracortical remodeling occur in the mandible following ovariectomized (OVX) is not clear. If they do, it is possible this mouse strain could be valuable as a model to study ONJ.

Therefore, the goal of this study was to determine whether or not the C3H mouse undergoes significant intracortical bone remodeling in the mandible following ovariectomy. We hypothesized that the mandible of C3H mice would undergo higher levels of intracortical remodeling with ovariectomy and that intravenous zoledronic acid (ZOL) would suppress this increased remodeling.

Materials and methods

Skeletally mature female C3H mice (n = 33) were obtained from Harlan at 16 weeks of age and divided into two groups; OVX (n = 17) and SHAM operated (n = 16). Within each group, some of the animals were treated with ZOL and the remaining animals were treated with physiologic saline vehicle (VEH). Ovariectomy surgeries were performed at Harlan, where the mice were housed for 7 days post-OVX prior to shipment to IU School of Medicine (IUSM) were they were housed 7 days prior to any intervention. Animals were housed four per cage (within treatment group) in the LARC facility and monitored daily for general health and any abnormal effects of treatment. All animal procedures were approved prior to the study by the IUSM Animal Care and Use Committee.

Animals in the ZOL treatment group received the drug via intravenous infusion at weeks 3 and 6 post-OVX. ZOL concentrations were based on the 4 mg drug dose given to patients with cancer assuming a 60 kg individual, adjusted on a mg/kg basis (0.06 mg/kg body weight dissolved in 0.2 ml sterile saline). VEH animals received 0.2 ml IV physiologic saline vehicle at the same time points as ZOL treatment. All IV infusions were administered through the tail vein using a 27-gauge needle over a 3–5 min period while the animal was contained within an animal restrainer. Calcein (0.2 ml/kg at 30 mg/kg) was administered intraperitoneally at 14 and 4 days prior to sacrifice to label active formation sites. The animals were euthanized at 24 weeks of age (CO_2 inhalation with death confirmed by inducing bilateral pneumothorax). The mandibles and femurs were collected at necropsy and fixed in 10% neutral-buffered formalin. The absence/presence of ovaries was confirmed at necropsy.

Histology

Mandible and femoral tissues were processed undecalcified for histological analyses using standard methods (10). To determine whether any bone matrix necrosis existed in these tissues, all specimens were stained with 1% basic fuchsin dissolved in increasing ethanol concentrations (70, 95, and 100%) each for 4 h (6). Mandibles were sectioned (~100 μm thick) throughout the molar region by making successive buccal–lingual cuts in the frontal plane using a diamond wire saw (Histosaw; Delaware Diamond Knives, Wilmington, DE, USA) (Fig. 1). Femurs were sectioned (~100 μm thick) at the mid-diaphysis.

Histological measurements were made using a semiautomatic analysis system (Bioquant OSTEO 7.20.10, Bioquant Image Analysis Co., Nashville, TN, USA) attached to a microscope (Nikon Optiphot 2 microscope; Nikon, Tokyo, Japan) with a fluorescent light source. For the mandible, dental landmarks were used to choose one section for analysis at a similar rostral/caudal region among all animals. Specifically, we chose the section that contained the deepest root of

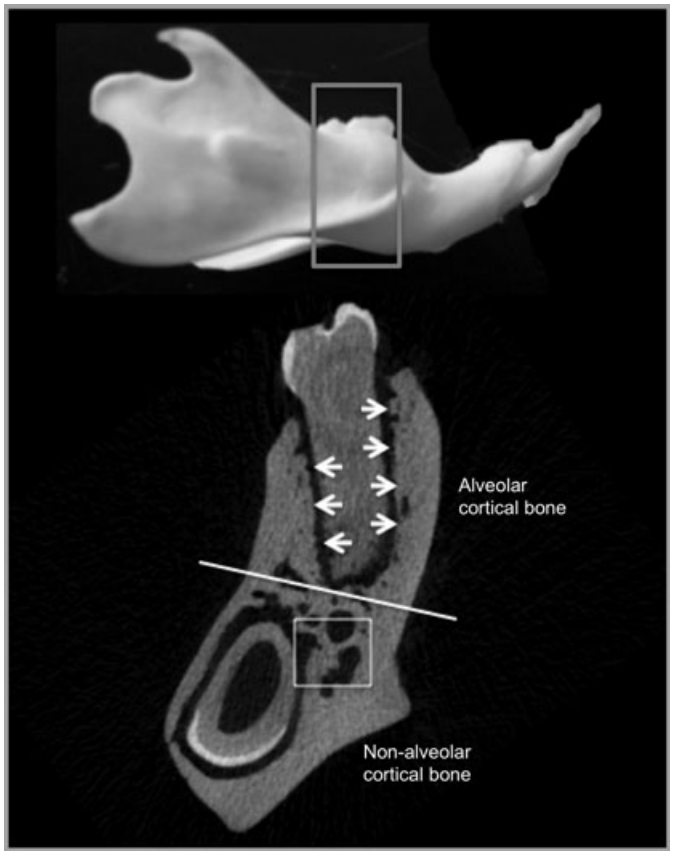

Fig. 1. Region of assessment in mouse mandible. Upper panel is a photograph of the mouse hemimandible showing molar region where histological sections were obtained. Lower panel shows a CT image (for illustration purposes only, no CT was performed in this study) of the region similar to that assessed histologically. The line at the base of the tooth root separates the alveolar from the non-alveolar bone, the two regions of intracortical bone analyses. Arrows show the periodontal ligament bone surface on which assessment of calcein-labeled surface was made. The box represents the region for trabecular bone analyses.

the first molar tooth as this provided us the opportunity to assess the greatest amount of alveolar bone in our analysis. This method of section selection was chosen to provide consistency in the anatomy of the section across all animals. For the mandible sections, data were collected separately for alveolar cortical bone (defined as cortical bone above the most distally observed portion of the tooth root) and the non-alveolar cortical bone (the cortical tissue inferior to the apex of the tooth root) (Fig. 1). In the alveolar mandible, the total surface of bone immediately adjacent to the tooth root where attachment of the periodontal ligament occurs was measured for the amount of calcein labeling (Fig. 1). In addition to the cortical bone of the non-alveolar mandible, the trabecular bone surfaces (BS) (found just below the apex of the molar) were also measured for amount of calcein labeling within the region of interest. The cortical bone of the entire cross section of femur was assessed for intracortical bone label.

For measures of intracortical remodeling, total bone area (B.Ar), number of labeled osteons (L.On.#, osteons with either single or double label), the total length of osteon labeled surface (LS), and the mean interlabel distance (Ir.L.Wi) were determined. Osteons were defined as structures within the cortical bone that contained single or double label (Fig. 2A, B), – resorption spaces without label were not counted in this analysis. For surface-based assessments, total BS, surfaces with either single or double label (sLS and dLS, respectively), and Ir.L.Wi were measured. As calcein labels were injected on two different occasions, measurements were taken separately for surfaces having single label surface (indicative of active bone formation at one of the two times of calcein injection) and double label surface (indicative of active bone formation at both times of calcein administration). Mineral apposition rate (MAR, μm/day) was calculated as Ir.L.Wi/10, where 10 is the number of days between labels. Intra-cortical bone formation rate (BFR, %/year) was calculated as $[\text{MAR} \times (\text{LS}/2)/\text{B.Ar} \times 100] \times 365$. Surface-based mineralizing surface (MS/BS, %) was calculated as $[(0.5 \times \text{sLS}) + (\text{dLS})/\text{BS}] \times 100$. Surface-based BFR ($\mu m^3/\mu m^2$/year) was calculated as $\text{MAR} \times (\text{MS}/\text{BS}/100) \times 365$. For animals with only singly labeled osteons, a value of 0.3 was used for MAR (11); if no label was present, MAR was considered to be a missing value and BFR was considered to be 0 to reflect the absence of any bone formation activity. All measures and calculations are in accordance with ASBMR recommended standards (12).

Statistics

All statistical analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC, USA). Two-way ANOVA tests were used to determine the main effects of surgery (SHAM vs. OVX) and treatment (VEH vs. ZOL) as well as their interaction. Significant interactions were further assessed by one-way ANOVA to determine specific group differences. *Post hoc* comparisons were made using Fisher's PLSD tests. For all tests, $p < 0.05$ was used to determine statistical significance.

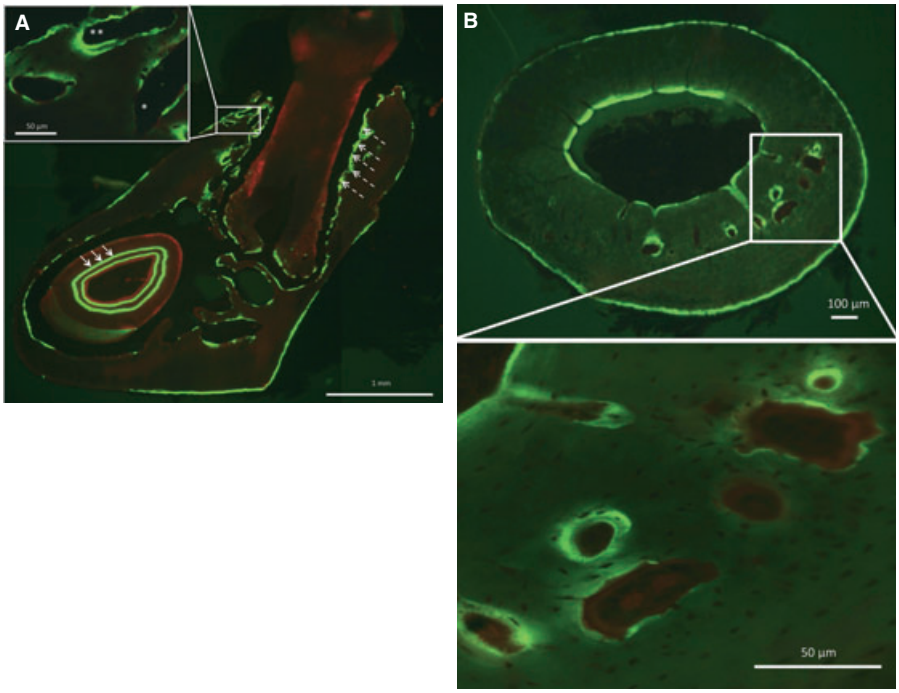

Fig. 2. Photomicrograph of basic fuchsin–stained mandible (A) and femur (B) viewed with fluorescent light. Insets show actively remodeling intracortical osteons (**). Dotted arrows in (A) show the periodontal ligament bone surface on which assessment of calcein-labeled surface was made; solid arrows show the location of dentin mineral apposition rate measures.

Results

Bone matrix necrosis

No areas of bone matrix necrosis were noted in either the mandible or femur of any animal. This assessment consisted of examining five cross sections of the mid-diaphysis femur and between 8 and 15 sections of mandible for each animal.

Mandible: alveolar and non-alveolar cortical bone

In the alveolar bone of the mandible, there was no significant difference in intracortical MAR or labeled osteon number among the four treatment groups (Table 1). There was a significant interaction between surgery and treatment for alveolar bone BFR (Table 1 and Fig. 3A). In VEH-treated animals, BFR was significantly higher in OVX (>sevenfold) compared to SHAM, while in ZOL-treated animals, BFR was 75% lower in OVX compared to SHAM. There was no significant difference between VEH-SHAM and ZOL-SHAM. In the non-alveolar cortical bone of the mandible, there was no significant difference in any of the parameters measured among groups (Table 1).

Table 1. **Effects of ovariectomy surgery and ZOL on intracortical remodeling of the mandible**

	VEH		ZOL		*p*-values		
	Sham	OVX	Sham	OVX	Surgery	Drug	Int.
Alveolar cortical bone							
Mineral apposition rate (µm/day)	0.35 ± 0.05	0.62 ± 0.15	0.33 ± 0.03	0.30 ± 0.00	NS	NS	NS
Labeled osteon number (per mm^2)	2.19 ± 0.80	3.81 ± 0.90	2.69 ± 0.93	1.52 ± 0.51	NS	NS	NS
Non-alveolar cortical bone							
Mineral apposition rate (µm/day)	0.52 ± 0.22	0.30 ± 0.00	–	0.30 ± 0.00	NS	NS	NS
Labeled osteon number (per mm^2)	0.72 ± 0.39	0.33 ± 0.33	0	0.69 ± 0.29	NS	NS	NS
Bone formation rate (%/year)	0.48 ± 0.27	0.11 ± 0.11	0	0.20 ± 0.10	NS	NS	NS

NS, non-significant; OVX, ovariectomized; VEH, vehicle; ZOL, zoledronic acid.

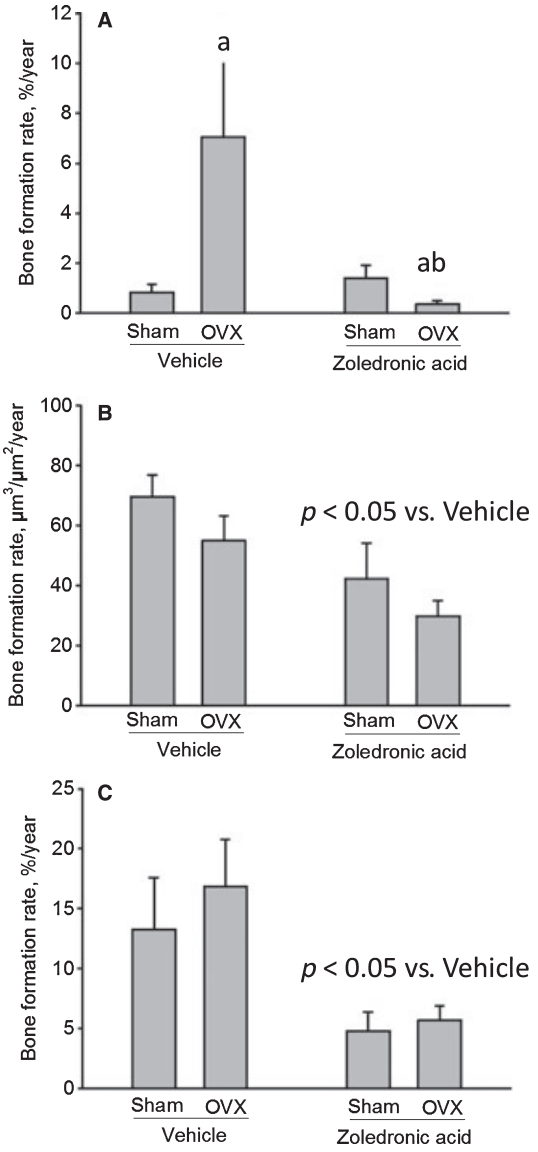

Fig. 3. Ovariectomy stimulates and zoledronic acid (ZOL) reduces remodeling activity in C3H mouse. (A) In alveolar cortical bone of the mandible, vehicle (VEH)-treated ovariectomized animals had >7-fold higher bone formation rate (BFR) compared to SHAM and this was significantly suppressed by ZOL. (B) Trabecular bone of the mandible showed ZOL-treated animals had a 58% lower BFR compared to VEH. (C) Femur intracortical remodeling was significantly suppressed in ZOL-treated animals compared to VEH-treated animals. Data presented as mean + SE. $p < 0.05$ vs. Sham within treatment (a), within surgery between drug (b).

Mandible: periodontal ligament & trabecular bone surfaces

On the periodontal ligament attachment surface of the alveolar cortical bone, there was no significant effect of surgery, drug treatment, or an interaction between the two interventions for MAR, MS/BS, or BFR (Table 2). On the trabecular BS of the mandible, there were significant effects of both surgery and ZOL treatment (Table 2). Trabecular MS/BS showed both a significant surgery and treatment effect with no significant interaction between the main effects. ZOL-treated animals had a 59% lower MS/BS and a 58% lower BFR compared to VEH-treated animals (Table 2 and Fig. 3B).

Tooth dentin mineralization rate

During our measures of calcein labeling in the mandible, we observed clear and consistent double calcein labeling within the incisor tooth dentin (Fig. 2A). We therefore measured mineral apposition rate to determine whether there was any effect of either surgery or treatment in dentin mineralization rate. There was no significant difference because of surgery or ZOL treatment on dentin MAR (Table 2).

Femur

Femoral intracortical bone parameters were all significantly affected by ZOL treatment, but not surgery. There was also no interaction between the two interventions. MAR and labeled osteon number were both significantly lower in ZOL-treated animals compared to VEH (−27 and −37%, respectively). These changes combined to produce a significantly lower intracortical BFR (−65%) in ZOL compared to VEH-treated animals (Table 3 and Fig. 3C).

Discussion

For several years now, BPs have been linked to ONJ (1, 2). Although recent studies have emerged showing potential models of ONJ in mice (13), rats (14–20), and dogs (21, 22), there remains no clear animal model that parallels ONJ etiology and pathophysiology. The most prominently proposed mechanism for ONJ involves remodeling suppression. This hypothesis, as opposed to a specific effect of BPs on the bone/soft tissue, is further supported by the recent case reports of ONJ in patients treated with denosumab (23). Denosumab, a human monoclonal antibody that inhibits osteoclast development by targeting RANK ligand, acts through a different mechanism than BPs and does not accumulate in the skeleton. One prominent similarity between BPs and denosumab is that they both significantly suppress bone remodeling.

Table 2. **Effects of ovariectomy surgery and ZOL on mandible trabecular bone, periodontal ligament cortical bone surface, and dentin formation activity**

	VEH		ZOL		*p*-values		
	Sham	OVX	Sham	OVX	Surgery	Drug	Int.
Mandible trabecular bone							
Mineral apposition rate (μm/day)	0.73 ± 0.03	0.74 ± 0.03	0.68 ± 0.07	0.75 ± 0.03	NS	NS	NS
Mineralizing surface/bone surface (%)	25.75 ± 1.94	19.89 ± 2.34	16.14 ± 4.13	10.89 ± 1.78	<0.05	<0.05	NS
Periodontal ligament surface							
Mineral apposition rate (μm/day)	0.48 ± 0.04	0.60 ± 0.03	0.64 ± 0.11	0.60 ± 0.05	NS	NS	NS
Mineralizing surface/bone surface (%)	14.20 ± 1.30	14.61 ± 2.46	11.39 ± 1.49	11.88 ± 1.75	NS	NS	NS
Bone formation rate ($\mu m^3/\mu m^2$/year)	24.52 ± 3.31	33.12 ± 7.11	26.39 ± 4.41	27.36 ± 5.29	NS	NS	NS
Dentin							
Mineral apposition rate (μm/day)	4.17 ± 0.05	4.33 ± 0.07	4.07 ± 1.34	4.13 ± 0.08	NS	NS	NS

NS, non-significant; OVX, ovariectomized; VEH, vehicle; ZOL, zoledronic acid.

Table 3. **Effect of ovariectomy surgery and ZOL treatment on intracortical remodeling of the femur**

	VEH		ZOL		*p*-values		
	Sham	OVX	Sham	OVX	Surgery	Drug	Int.
Mineral apposition rate (μm/day)	1.58 ± 0.11	2.13 ± 0.19	1.22 ± 0.20	1.48 ± 0.23	NS	<0.05	NS
Labeled osteon number (per mm^2)	4.45 ± 0.82	4.23 ± 0.95	2.46 ± 0.57	2.93 ± 0.49	NS	<0.05	NS

NS, non-significant; OVX, ovariectomized; VEH, vehicle; ZOL, zoledronic acid.

It is generally accepted that rodents do not naturally undergo significant amounts of intracortical remodeling in their long bones or the mandible (8) and therefore their utility as a model for ONJ has been questioned (24, 25). Previously, Li et al. (9) have shown that ovariectomy in C3H mice elicits a significant increase in intracortical remodeling of the femur. Our study expands this work by showing that intracortical remodeling occurs also within the mandible, specifically within the alveolar portion, of C3H mice following ovariectomy. In VEH-treated animals, we showed over a sevenfold higher alveolar BFR because of OVX compared to SHAM-operated animals. Consistent with the low, but measurable, intracortical alveolar BFR in our SHAM group, osteons were noted in the mandible/maxilla of 17- week-old C3H mice although these existed in only some animals and were low in number (26). Our study also documents that ZOL treatment prevented the OVX-induced elevation in intracortical remodeling of the alveolar cortical bone. This is consistent with the suppression of intracortical remodeling at various sites in humans (27, 28) and in the alveolar bone of dogs (6, 7).

Histomorphometry is the gold standard method for the assessment of bone remodeling. Although other methods, such as serum or urine biomarkers, can provide whole body estimates of remodeling, only histology can provide site-specific remodeling data. In the current work, we used the standard technique of fluorochrome labeling to denote actively forming BS. By injecting calcein, which binds to calcium being incorporated into hydryoxyapatite during mineralization, it is possible to determine parameters, such as mineral apposition rate (the rate at which bone is formed at an individual site), mineralizing surface (the number of actively forming surface), and BFR (the rate bone is forming at a tissue level). As mature animals predominantly undergo remodeling (the coupled process of resorption and formation) on trabecular surfaces and exclusively undergo remodeling within the cortex, assessment of BFR in these bone envelopes gives insight into the rate of remodeling. As formation-

based modeling occurs on periodontal ligament surfaces, BFR on this surface is directly related to osteoblast-mediated bone formation.

The effect of ZOL was also evident in the current study by the lower remodeling of the mandible trabecular bone, with ZOL-treated animals having a 42% lower BFR compared to VEH. We did not find a significant difference in OVX vs. SHAM animals for trabecular BFR. Previous work has shown that the OVX-induced increase in remodeling in rodents occurs acutely but is then lost over time (29). Thus, it is possible that our assessment 8 weeks post-ovariectomy missed the period when BFR was stimulated. Alternatively, it is possible that the response to ovariectomy differs in this trabecular bone in the mandible compared to more traditionally assessed sites (vertebra or proximal tibia).

The surface adjacent to the periodontal ligament primarily undergoes bone modeling (30). We found no significant effect of either surgery or drug treatment on this surface. Recent reports from our laboratory have shown that BPs, including ZOL, do not affect formation bone modeling in ovariectomized animals (31). Previous work in B6 mice showed ZOL significantly suppressed mandible periosteal surface BFR, a surface that also primarily undergoes modeling activity (32). The discrepancy between these previous data and the current study is not clear although it is likely because of either the differences in mouse strain (B6 vs. C3H) or the ZOL dosing schedule. The current study administered two doses ZOL, while the previous study administered weekly ZOL (nine total doses); it is possible that these higher doses could produce a suppressive effect on osteoblast activity.

In accordance with Li et al. (9), our study showed a 27% higher intracortical BFR in the femur of OVX-VEH compared to SHAM-VEH, although this was not statistically significant. The discrepancy between the previous and current work could be the result of the technique used to assess intracortical remodeling where Li et al. counted both labeled osteons and resorption spaces while we excluded the latter. We opted to only assess labeled osteons to provide the most conservative assessment of intracortical activity as there existed regions in the mandible in which distinguishing between an unlabeled resorption space and a trabecular surface proved difficult. Intracortical remodeling of the femur was significantly reduced with ZOL treatment, consistent with studies in larger animals treated with either ZOL or other BPs (7, 33).

In both the femora and mandibles, prior to sectioning, all specimens were stained en bloc with basic fuchsin to elucidate regions of bone matrix necrosis, defined as regions of bone devoid of stain, demarcating regions of non-viable bone (34, 35). No regions of bone matrix necrosis were noted in this study, likely because of the short treatment duration. We have previously shown significant accumulation of necrotic matrix existed within the mandible of beagle dogs treated for 1 or 3 years of daily oral alendronate (6, 22) yet not in animals treated for 3 or 6 months (7). This suggests a time-dependent factor in the development of matrix necrosis, which makes some sense as the region would need to have time for the local canalicular network to fill with mineral. We hypothesize that longer duration of bisphosphonate treatment following ovariectomy in this mouse strain would result in accumulation of necrotic bone matrix.

Nearly 20 years ago, prolonged treatment with clodronate in young growing rats was shown to produce necrotic bone fragments projecting through the oral mucosa (20). More recently, several publications have suggested ONJ can be produced in rodents. These studies have involved mainly treatment of rats with high doses of ZOL with or without concomitant treatment with dexamethasone or dental surgery (14–16, 18). One study in B6 mice has also shown ZOL and/or dexamethasone combined with tooth extraction revealed ONJ-like lesions (13). Although these rodent models necessitate further validations, the emerging evidence suggests the rodent may serve as an important animal model for studying ONJ. Unfortunately, none of these studies assessed intracortical bone remodeling. Significant suppression of intracortical remodeling and formation of exposed bone and sequestra has been recently documented in beagle dogs treated with ZOL and subjected to dental extraction (21).

Conclusion

In conclusion, we show that ovariectomy of skeletally mature C3H inbred mice stimulates intracortical remodeling in the mandible, especially in the alveolar region. Furthermore, we show that bisphosphonate

treatment significantly suppresses this intracortical remodeling. If suppression of intracortical remodeling proves to be important in the etiology of ONJ, then the ovariectomized C3H mouse may prove useful as an animal model to study this condition.

Clinical relevance

Osteonecrosis of the jaw is a rare but serious side effect of bisphosphonate treatment. The pathophysiology of this condition remains unclear due in part to the absence of an animal model. A key factor in the etiology of the condition is thought to be the suppression of intracortical remodeling. We show here for the first time that the mandible of mice can be stimulated to undergo intracortical remodeling by ovariectomy and this increased remodeling is suppressed with BPs. These results suggest this mouse strain may be useful in the development of an animal model for ONJ.

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