

Adverse mandibular bone effects associated with kidney disease are only partially corrected with bisphosphonate and/or calcium treatment

Running title: CKD effects on mandible bone

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ABSTRACT

Background/Aims: Patients with chronic kidney disease (CKD) have high prevalence of periodontal disease that may predispose to tooth loss and inflammation. The goal of this study was to test the hypotheses that a genetic rat model of progressive CKD would exhibit altered oral bone properties and that treatment with either bisphosphonates or calcium could attenuate these adverse changes. **Methods:** At 25 weeks of age, rats were treated with zoledronate, calcium gluconate, or their combination for 5 or 10 weeks. Mandible bone properties were assessed using micro-computed tomography to determine bone volume (BV/TV) and cement-enamel junction to alveolar crest distance (CEJ-AC). **Results:** Untreated CKD animals had significantly lower BV/TV at both 30 (-5%) and 35 (-14%) weeks of age and higher CEJ-AC (+27 and 29%) compared to normal animals. CKD animals had significantly higher PTH compared to normal animals yet similar levels of C-reactive protein. Zoledronate-treatment normalized BV/TV over the first 5 weeks but this benefit was lost by 10 weeks. Calcium treatment, alone or in combination with zoledronate, was effective in normalizing BV/TV at both time points. Neither zoledronate nor calcium was able to correct the higher CEJ-AC caused by CKD. Calcium, but not zoledronate, significantly reduced serum parathyroid hormone (PTH) while neither treatment affected C-reactive protein. **Conclusions:** 1) this progressive animal model of chronic kidney disease shows a clear mandibular skeletal phenotype consistent with periodontitis, 2) the periodontitis is not associated with systemic inflammation as measured by C-reactive protein, and 3) reducing PTH has positive effects on the mandible phenotype.

INTRODUCTION

Chronic kidney disease (CKD) is often accompanied by disturbances in mineral metabolism which are classified as their own clinical entity known as CKD-mineral and bone disorder (CKD-MBD) (1). CKD-MBD is hallmarked by altered bone remodeling and loss of bone mass throughout the skeleton, including the oral cavity (2,3). Periodontal disease, including gingivitis and periodontitis are more prevalent in CKD populations compared to healthy individuals (4-6) and have been documented in rodent models of CKD (7). Periodontitis is associated with alveolar bone loss, thought to be secondary to local inflammation and change in the bacterial environment. Secondary hyperparathyroidism is common in CKD, and oral bone remodeling is similar to that of cortical bone remodeling. Therefore, the etiology of periodontitis in CKD may be due to inflammation and/or secondary hyperparathyroidism (8). If the latter is true, then therapies that lower PTH may also have a beneficial effect, as may other bone-sparing treatments that reduce remodeling such as bisphosphonates.

Bisphosphonates have clear efficacy in reducing bone loss in non-CKD patients (9). International clinical practice guidelines recommend bisphosphonates use in patients with CKD stages 1-3 and normal parathyroid hormone levels, but recommended not using bisphosphonates in patients with CKD stages 3-5 with biochemical evidence hyperparathyroidism (1). The main concern for this latter population is the potential of severely suppressed bone turnover although the limited clinical data does not universally support such an effect (10-13). Recently, using an animal model of progressive kidney disease we have documented that the reduction in remodeling of the tibia with zoledronic acid is similar that that of normal animals (14) although this dose failed to normalize biomechanical properties.

The effects of bisphosphonates on the oral skeleton have been extensively reviewed and discussed in recent years do to the condition of osteonecrosis of the jaw (15,16). Although cases of ONJ have been documented in persons treated with oral bisphosphonates, the vast majority of cases have occurred in association with high dose intravenous bisphosphonates. Indeed, oral bisphosphonates have actually been shown to benefit the oral skeleton. Oral alendronate and risedronate have each showed efficacy in attenuating periodontal-induced bone loss in the general population (17,18).

The goal of this study was to characterize the oral cavity skeletal changes in this progressive kidney disease animal model. Specifically, we aimed to test the hypothesis that skeletal properties in the oral cavity would be adversely affected in animals with CKD and that bisphosphonates would attenuate these effects. We also aimed to understand the respective roles of hyperparathyroidism versus inflammation in these periodontal changes.

METHODS

Animal model and experimental design. A rat colony with an autosomal dominant polycystic kidney disease, maintained at the Indiana University School of Medicine, were used for this study. Male heterozygous rats (Cy/+) develop characteristics of CKD (azotemia, anemia, hypertension, secondary hyperparathyroidism) around 10 weeks of age. BUN analyses were conducted in all animals at 10 weeks of age and animals with values over 40 mg/dl were considered to have CKD. These animals have a progressive rise in PTH as they age, (19-21), and all animals had elevations in PTH at the time of treatment.. The normal littermates of the colony were used as non-affected (normal) controls.

At 25 weeks of age (roughly a glomerular filtration rate of 25 ml/min, equivalent to human stage 4 CKD), animals were assigned to treatment groups within two different experiments that differed somewhat in drug dosages and also treatment duration (**Figure 1**).

Experiment one: CKD animals were treated with a single dose of vehicle, ZOL (one group at 20 and another group at 100 µg/kg BW) or given 3% calcium gluconate in the drinking water.

Normal animals injected with either vehicle (saline) or a single intraperitoneal injection of zoledronic acid (ZOL, 100 µg/kg body weight) served as controls. These single doses of ZOL have been shown to significantly suppress remodeling in the tibia in this model over the five week timeframe, and this dose of calcium significantly lowers PTH (14). Animals were sacrificed at 30 weeks of age (5 weeks after treatment initiation).

Experiment two: CKD animals were treated with a single dose of vehicle, ZOL (20 µg/kg BW), calcium gluconate, or calcium gluconate plus ZOL. Normal animals injected with either vehicle (saline) or a single intraperitoneal injection of zoledronic acid (ZOL, 20 µg/kg body weight) served as controls. Animals were sacrificed at 35 weeks of age (10 weeks after treatment initiation)

In both studies, all animals were euthanized by an overdose of sodium pentobarbital. At necropsy, blood was collected by cardiac puncture. The right hemi-mandible was wrapped in saline-soaked gauze and frozen for imaging. All procedures were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Computed tomography. Morphological parameters of the mandible were assessed using high-resolution micro-CT (Skyscan 1172). Bones were wrapped in parafilm to prevent drying during the scanning. Scans were obtained using an x-ray source, set at 60kV with a 12-µm pixel size. Images were reconstructed and analyzed using standard Skyscan software (NRecon and CTAn, respectively). A single slice from the central region of the first mandible molar was analyzed for total bone volume (excluding the molar and incisor) and lingual cementum-enamel to alveolar bone crest distance (CEJ-AC) as previously described (22,23). This distance is roughly

equivalent to the clinically assessed periodontal pocket, which is believed the nidus of inflammation (**Figure 2**).

Biochemical analyses: Serum intact PTH, C-reactive protein, and TNFalpha were measured by ELISA (Alpco, Salem NH) according to the manufacturer's instruction. Calcium and phosphorous were measured from plasma using colorimetric methods (14).

Statistics. All analyses were run using SAS software. All data were compared using a one-way ANOVA with Fisher's LSD post-hoc tests when appropriate. A p value of < 0.05 was used to determine statistical significance. Data are presented as mean and standard error.

RESULTS

Detailed long bone tissue mass and biochemical data from experiment one (14) and two (24) have been previously published. In both experiments one and two, there was no significant difference among groups for body mass within either experiment while all CKD animals had significantly larger kidney masses and elevated BUN indicative of progressive kidney disease. There were no significant differences in serum calcium or phosphorous at sacrifice in experiment 1 (30 weeks) (14). However, in experiment two (treated for 10 weeks), phosphorus was lower and calcium was higher in the calcium treated CKD animals sacrificed at 35 weeks compared to other groups. At both time points PTH was significantly higher in CKD animals (3x higher at 30 wks; 13x higher at 35 wks) while C-reactive protein was not different at either time point (Tables 1 and 2). TNFalpha levels were undetectable in all animals. In both experiments, the calcium treated animals had significant suppression of PTH compared to control treated CKD animals and Normal animals. Thus, the animals had progressive kidney disease and secondary hyperparathyroidism. Similar to human disease, the animals developed progressive secondary hyperparathyroidism with frank hyperphosphatemia late in the disease course.

Treatment with calcium suppressed PTH and lowered phosphorus, but also increased calcium levels when the treatment was given for 10 weeks.

Across both experiments, CKD-vehicle animals displayed a clear and consistent mandibular phenotype compared to normal-vehicle animals (**Figures 3**). Mandible bone volume/tissue volume was 6% lower and cementum-enamel junction to alveolar crest (CDJ-AC) distance 27% larger in the CKD animals compared to normal animals at 30 weeks of age. At 35 weeks of age, BV/TV was 14% lower and CDJ-AC 30% higher in CKD animals compared to normal. Zoledronic acid did not significantly alter either periodontal assessment in normal animals of either experiment (**Figure 3**). Zoledronic acid treatment in experiment 1 (5 weeks of treatment) significantly improved trabecular BV/TV, at both doses with no dose response, relative to CKD-VEH. Conversely, there was no effect of ZOL on BV/TV relative to CKD-VEH in experiment two (10 weeks of treatment and more severe disease). ZOL was ineffective in normalizing CEJ-AC distance in both experiments PTH levels (Table 1) were significantly lower with the high dose, but not low dose, ZOL in experiment 1 relative to CKD-VEH animals yet was still 2x higher than normal. Low dose ZOL in experiment 2 significantly reduced PTH relative to CKD-VEH yet it remained nearly 10-fold higher than normal. C-reactive protein was significantly lower in ZOL-treated animals of experiment two relative to CKD-VEH for unclear reasons.

Calcium supplementation normalized BV/TV in both experiments (Figure 3). Combining calcium with zoledronic acid in experiment two produced BV/TV values comparable to calcium alone. In experiment one, CEJ-AC was not different from normal but was also not different from CKD-VEH. In experiment two, neither calcium alone nor calcium combined with zoledronic acid affected CDJ-AC relative to CKD-VEH. Calcium, either alone or in combination with ZOL significantly lowered serum PTH to below normal vehicle levels. Calcium alone, in experiment two, resulted in C-reactive protein levels that were significantly higher than normal (Table 1). In

the CKD animals, the PTH level was inversely correlated with the BV/TV ($r = -0.77$, $p < 0.001$), although not significantly correlated with the CEJ-AC.

DISCUSSION

Previous work describing the skeletal properties in this progressive, genetically-based CKD animal model has focused on the long bones and has documented increased bone remodeling rates, loss of bone mass, and reduced biomechanical properties (14,21,25). Due to the increasingly prevalent recognition of dental co-morbidities associated with CKD (5,6) and recent documentation of mandibular phenotype in a mouse model of CKD (7), we examined properties of the mandible that are known to be factors in the etiology of periodontal disease in our model. Our results demonstrate that this animal model that develops progressive CKD, has a mandible phenotype consistent with clinical periodontal disease. Specifically, vehicle-treated CKD animals had a modest, but significantly lower mandible bone volume in the region of the first mandible molar compared to normal vehicle animals. CKD animals also had significantly greater cementum-enamel junction to alveolar crest distance compared to normal animals, indicative of alveolar crest bone resorption. These skeletal differences existed in the context of significantly higher serum PTH but no difference in serum C-reactive protein, compared to normal animals. The morphological changes observed in this CKD animal model is consistent with those shown previously for both experimentally- (ligature placement) and pharmacologically-induced periodontal disease (26,27) as well as those for another animal model of CKD (7). Alterations in facial bones, including the mandible, have also previously been documented in a small cohort of dialysis patients where PTH is elevated (28).

Bisphosphonates as a therapy for oral bone loss have been explored, and shown to be clinically effective in non-CKD populations(17,18). In our study, zoledronate treatment, at both

low and high doses, corrected difference in mandible bone volume in the short-term (5 week experiment) but not the long term (10 week experiment). Enhanced bone volume was expected given that the mechanism of action for bisphosphonates is to reduce bone remodeling. These positive effects are consistent with the effects of zoledronate at other skeletal sites in these same animals (14), as well as a wide-array of other animal model conditions of bisphosphonate treatment (29). The inability of zoledronic acid to normalize bone volume in the 10 week study was unexpected. Previous work has shown a single 20 µg/kg dose of zoledronic acid maintained beneficial effects on tibia BV/TV bone up to eight months post-dose in rats (30). We interpret this as evidence that CKD alters the long-term efficacy of a single zoledronic acid dose, likely related to the high PTH that leads to resorption of bone that is covered with the zoledronic acid, leading to less efficacy of the drug. This could be potentially overcome by more frequent dosing – although a theoretical concern exists that with compromised renal function, increased dosing will lead to increased accumulation of drug in the skeleton and the potential adverse effects this could manifest.

The clinical utility of bisphosphonates for treating/preventing oral bone loss has been curtailed by osteonecrosis of the jaw, a rare but significant side effect of potent remodeling suppressive drugs (31). The mechanism underlying osteonecrosis of the jaw remains unclear and although dramatic suppression of remodeling appears to play a role there are also a number of other co-factors that likely are involved (16). Pre-clinical data show that bisphosphonates do not suppress remodeling differently in a high-turnover model of CKD compared to unaffected normal animals (14). This would suggest that risk of osteonecrosis of the jaw, if it's related to level of turnover suppression, would not be expected to be higher in patients with high-turnover kidney disease.

The adverse effects of CKD on the cementum-enamel junction to the alveolar crest distance were not altered with zoledronate treatment. Trabecular and intracortical bone

envelopes undergo bone remodeling, the coupled process of resorption followed by formation, similar to that in long bones. In these situations, inhibition of remodeling with bisphosphonates results in a small but meaningful increase in bone mass because 1) those sites that were in the process of remodeling fill in and 2) no new remodeling sites are initiated. The cellular activity on the alveolar bone surfaces is mostly modeling, the process of resorption or formation (but not both) at a given spatial location. High PTH induced by CKD potently stimulates resorption and this is likely the mechanism underlying the greater CEJ-AC in CKD animals compared to normal (7,28). Suppression of osteoclast-based modeling, as occurs with zoledronic acid, would slow the increase of CEJ-AC distance, but would not be expected to reverse it. Non CKD-models of periodontal disease have documented that when bisphosphonate treatment is initiated before the induction of periodontal disease the CEJ-AC distance can be maintained (22). We interpret the lack of effect in our study as evidence that the increase in CEJ-AC occurred prior to the zoledronic acid dosing (25 weeks of age). Given that PTH levels were already elevated at this age, this is a plausible hypothesis. Modifications of CEJ-AC, a clinically-relevant parameter, would therefore necessitate either earlier treatment with an anti-resorptive or treatment with an anabolic therapy, such as parathyroid hormone (32) or anti-sclerostin antibody (33).

Calcium supplementation, utilized clinically to lower phosphorus when taken with meals, and reduce elevated PTH, provided a prolonged effect on mandible BV/TV maintenance. At both 5 and 10 week time points, animals treated with calcium had BV/TV that was comparable to normal animals, suggesting the control of PTH has a greater effect on bone preservation than the potent but acute treatment with bisphosphonate. Yet similar to zoledronic acid, calcium supplementation was not able to restore CEJ-AC distance, again likely because changes to this parameter occurred prior to the initiation of treatment.

In vehicle-treated animals, serum PTH was significantly higher in CKD animals compared to normal while there was no difference in C-reactive protein, an outcome measure

related to inflammation (34). Further supporting that systemic inflammation was not an etiology in the changes were the undetectable levels TNF-alpha. In the calcium treated groups, serum PTH and bone volume were maintained; in contrast PTH was not controlled, nor bone volume maintained in animals treated with zoledronate. These observations confirm previous observations that hyperparathyroidism was associated with mandibular bone changes in a different animal model of CKD and in a small cohort of dialysis patients (7,28). We also found a significant negative correlation between PTH and bone volume, similar to that observed in the mouse model of CKD-MBD (7). Inflammation, globally assessed by CRP, is also associated with periodontal disease. However, inflammation may be a result, rather than a cause of periodontitis. This induced systemic inflammation may be a cause of CKD, rather than CKD itself leading to periodontal disease (8,35). In the present study, the effects of treatment on C-reactive protein were modest and did not track with response of bone. Based on these outcomes and measurements, we conclude that PTH, not systemic inflammation, drives the bone alterations observed in this CKD model. This highlights the need to control PTH in order to reign in the oral skeletal manifestations of the disease in addition to the long bone changes.

These results should be interpreted in the context of study limitations. Our study did not include baseline controls, animals that are sacrificed at the time of treatment initiation. This would have allowed us to determine if measures such as CEJ-AC were indeed different at the start. We also did not have a group of animals that were dosed more frequently with zoledronic acid (in experiment 2). This would have directly addressed whether controlling PTH was essential to preserving bone volume or if simply controlling osteoclasts through repeated dosing would also have been effective. We also did not have normal animals treated with calcium or calcium plus zoledronic acid as the main reason to include the normal animals was to define the basal phenotype of the rat model.

In conclusion we have documented that this model of progressive chronic kidney disease presents a skeletal phenotype in the oral cavity consistent with clinically observed periodontal disease and that zoledronate and calcium each have mixed effects as a treatment for correcting this phenotype. We also provide evidence that the bone loss of periodontal disease is related more to PTH levels than inflammation markers.

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FIGURE LEGENDS

Figure 1. Experimental design. All animals began treatment at 25 weeks of age. Experiment 1 lasted five weeks and experiment 2 lasted ten weeks. Animals were dosed with a single bolus of saline vehicle (VEH) or zoledronic acid (ZOL). Animals in calcium groups were fed 3% calcium gluconate in their water throughout the experimental duration.

Figure 2. CT-based morphological assessment of mandible bone. Bone volume per tissue volume (BV/TV) was calculated as the fraction of tissue that was mineralized within the entire section, excluding the dental tissue (area encompassed by the white dotted line). The cementum-enamel junction to alveolar crest distance was calculated at the lingual surface as noted by the arrow.

Figure 3. Effects of chronic kidney disease and its treatment with bisphosphonate, calcium, or their combination on mandible trabecular bone volume (A, C) and cementum-enamel junction to alveolar crest distance (B, D). Dotted line notes level of normal-vehicle animals for reference across groups. VEH = vehicle, ZOL = zoledronate, Ca = calcium. Data as mean \pm SE. $p < 0.05$ versus normal vehicle (#), CKD-Vehicle (*), and (^) CKD-ZOL.

TABLE 1. Serum biochemistry (30 weeks of age – 5 weeks of treatment)

	Normal		CKD				1way ANOVA
	Vehicle	ZOL 100 µg	Vehicle	ZOL 20 µg	ZOL 100 µg	Ca	
Parathyroid hormone, pg/ml (range)	251 ± 34 (104-382)	233 ± 16 (174-303)	853 ± 227 # (445-1870)	593 ± 95 # (238-950)	558 ± 203 * (217-4662)	162 ± 69 *^ (22-420)	0.0009
Serum c-reactive protein, µg/ml	375 ± 15	376 ± 15	371 ± 8	353 ± 21	382 ± 20	350 ± 9	0.655

Data as mean ± SE. p < 0.05 versus normal vehicle (#), CKD-Vehicle (*), and (^) CKD-ZOL.

TABLE 2. Serum biochemistry (35 weeks of age – 10 weeks of treatment)

	Normal		CKD				1way ANOVA
	Vehicle	ZOL 20 µg	Vehicle	ZOL 20 µg	Ca	Ca + ZOL	
Parathyroid hormone, pg/ml (range)	230 ± 50 * (108-613)	213 ± 17 * (155-304)	3031 ± 332 # (972-4760)	2119 ± 574 ** (109-4769)	55 ± 14 *^ (20-4760)	69 ± 20 *^ (10-176)	<0.0001
Serum c-reactive protein, µg/ml	459 ± 21	449 ± 17	519 ± 26	427 ± 16 * (109-4769)	528 ± 29 #	525 ± 29	0.0072

Data as mean ± SE. p < 0.05 versus normal vehicle (#), CKD-Vehicle (*), and (^) CKD-ZOL.

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