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A pharmacodynamic investigation into the efficacy of osteoprotegerin during aseptic inflammation

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Background: Osteoprotegerin (OPG), as an osteoclast antagonist, limits mineralised tissue resorption under physiological conditions. Previous work investigating OPG in a rat periodontal ligament (PDL) ankylosis model found no inhibitory effect on osteoclasts when OPG was administered at a dosage of 2.5mg/kg.[1,2]

Aims: The object of this study was to determine whether dosages higher than 2.5 mg/kg of OPG were required to limit osteoclastic activity in an aseptic inflammatory model in rats.

Materials and methods: Dry ice was applied for 15 minutes to the upper right first molar crown of eighteen, 8-week-old, male Sprague-Dawley rats. Three groups of 3 were injected with OPG at dosages of 2.5, 5.0 and 7.5 mg/kg of body weight immediately following the thermal insult. After 7 days, the rats were sacrificed and each maxilla processed for histological examination and stained for osteoclastic activity using tartrate-resistant acid phosphatase (TRAP). Osteoclast population numbers were estimated via light microscopy and results were analysed using a comparative mixed model statistical analysis.

Results: Results showed OPG inhibited osteoclastic activity in a dose-dependent manner. From 2.5 mg/kg to 7.5 mg/kg, osteoclast populations were linearly reduced by 39.8% ($p < 0.05$). OPG did not appear to affect the inflammatory process and had varied efficacy in different regions of individual teeth.

Conclusion: Although osteoclastic activity reduced, it was not completely eliminated, perhaps because dosages were still inadequate, or additional factors might influence OPG and osteoclast activation in the aseptic inflammatory model.

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Introduction

Orthodontic treatment aims to move teeth as efficiently as possible with the least amount of dental and periodontal damage. However, orthodontically induced inflammatory root resorption has been increasingly recognised as an iatrogenic consequence of treatment. Tooth movement relies on the interaction between osteoblasts and osteoclasts which govern bone apposition and resorption. Osteoclasts are recruited to specific resorptive sites while osteoblasts repair resorbed bone by the deposition of matrix and mineral in a coupled process. However, osteoclast-derived bone resorption required for orthodontic tooth movement often has the adverse consequence of external root resorption.[3]

The OPG/RANK/RANKL system has been considered a breakthrough in the understanding of bone biology.[4] It has been accepted that osteoclastic bone resorption precedes apposition by osteoblasts in the normal bone remodelling cycle.[5] Osteoclast differentiation is initiated through direct cell-to-cell communication between osteoblasts and osteoclast

precursors. The initiating factors have been identified as the RANK ligand produced by osteoblasts and the RANK receptor on the osteoclast precursor.[6] Furthermore, OPG, as a protein produced by osteoblasts and stromal cells in the PDL,[7,8] has been identified as a decoy receptor for RANKL.[9,10] OPG competitively binds to RANKL, thereby inhibiting osteoclastogenesis and subsequent bone resorption. Fused or recombinant OPG (Fc-OPG) has been demonstrated to increase bone density and strength in rodents.[4,11] The administration of Fc-OPG is capable of compensating for a reduction of endogenous OPG in OPG-knockout animals.[12]

The inhibitory effects of OPG during external inflammatory root resorption have been previously investigated.[1] Results indicated that an OPG dosage of 2.5 mg/kg did not prevent root resorption in an aseptic inflammatory model. Therefore, the dosage required to completely inhibit osteoclastogenesis during pathological conditions is yet to be clarified and forms the basis of the present study.

Materials and methods

Experimental animals

Eighteen, 8-week-old, male Sprague-Dawley rats were divided into experimental groups of 6 and treated under ethical regulations for animal experiments as approved by the Ethics Committee of The University of Adelaide (M-4-2004). Each animal weighed between 250-300 g at the start of the experiment and increased in weight normally during the experimental timeline. Within each group of 6, rats were equally divided into experimental and control groups.

Experimental protocol

Each rat was anaesthetised with a combination of Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/mL) and Hypnovel (midazolam hydrochloride 5 mg/mL) in a 1:1 ratio with sterile water and administered at a dosage of 2.7 mL/kg of body weight. The anaesthetised rats were placed on a specially constructed rack which stretched the mouth open through the use of metal rings looped around both the upper and lower incisors. With cheek and tongue retraction, the upper right first molar was carefully frozen for a period of fifteen minutes by the application of customized pellets of dry ice (CO_2 at -81°C). Following the application of cold, the tissues were allowed to thaw slowly under anaesthesia and the animals recovered under a heat lamp to prevent hypothermia. The upper left first molar was left unfrozen and served as a control. Immediately following the experimental freezing, each group of experimental animals had single doses of OPG administered intramuscularly (IM), in concentrations of 2.5 mg/kg, 5.0 mg/kg or 7.5 mg/kg.

TRAP staining

Seven days after the thermal insult, all animals were sacrificed under anaesthesia via the intracardiac perfusion of 30 mL of 4% paraformaldehyde fixative. Each maxilla was dissected out and immersed in the same fixative for 24 hours, rinsed in phosphate-buffered saline (0.4M pH 7.4) for 24 hours and decalcified using 4% EDTA in phosphate buffer. Subsequently, the tissues were placed in 70% alcohol before processing through graded alcohols and paraffin wax impregnation in a Shandon Citadel 2000 automatic processor. Coronal plane wax sections of 5 µm were cut and mounted in sequential order on aminopropyltriethoxysilane (APT) coated slides.

For orientation purposes every tenth slide was stained with Mayer's haematoxylin and eosin (H&E) and viewed under a light microscope. The staining protocol for demonstrating TRAP activity in paraffin sections on adjacent slides was based on a modified technique of Goldberg and Barka[13] with basic fuchsin allowed to mature and characterised by a silver lining on the solution surface. A suitable staining solution displayed a dark rust-coloured precipitate. To allow comparison of variations of the technique, a rating was given to each slide based on the clarity of structures associated with the PDL and the intensity of the TRAP staining present.

Cell counts were conducted using a consistent grid, which subdivided the tooth roots into palatal, furcal and buccal columns via lines of best fit orientated through the long axis of the root. Totals for each column were recorded for each slide for further evaluation. Osteoclasts were identified by positive TRAP staining which appeared as an intense red colour on a green background. During cell counts, 50 slides were randomly revisited and recounted by one observer with blinding. Results were recorded and analysed for accuracy and consistency between

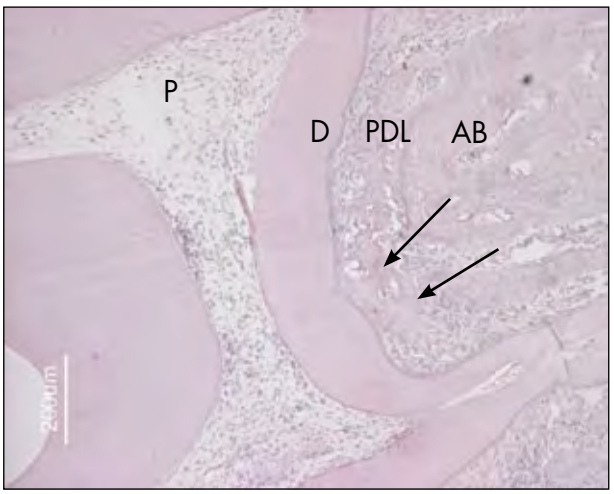


Figure 1. H and E staining of frozen tooth in 5.0 mg/kg OPG administered rat (Scale bar equals 200 µm). Arrows show areas of ankylosis.

P, Pulp; D, Dentine; PDL, Periodontal Ligament; AB, Alveolar Bone.

counts. Cell numbers for the 3 areas were recorded for control and test teeth and analysed statistically using a mixed model.

Results

Haematoxylin and Eosin

Histological stains showed marked differences between frozen (test) and unfrozen (control) teeth via the orientation of the odontoblasts in the pulpal cavities, regardless of the dosage of OPG given. The odontoblasts in the frozen test teeth were disorientated and obliterated, while, in the unfrozen control teeth, they showed parallel alignment close to the walls of the pulp chamber. Increased numbers of resorptive pits were seen on the root surface of frozen test teeth and necrosis of surrounding tissues was evident. Two rats in the 5.0 mg and 7.5 mg/kg groups exhibited ankylosis (Figure 1). This finding appeared incidental and no characteristic common to both and different from the remaining 16 animals was determined.

TRAP staining

The frozen test teeth exhibited higher average osteoclastic populations than the unfrozen control teeth regardless of the OPG dosage administered. The reductions in the osteoclastic populations are represented in Figure 2 (Dose vs osteoclastic population). From 2.5 mg/kg to 7.5 mg/kg, there was

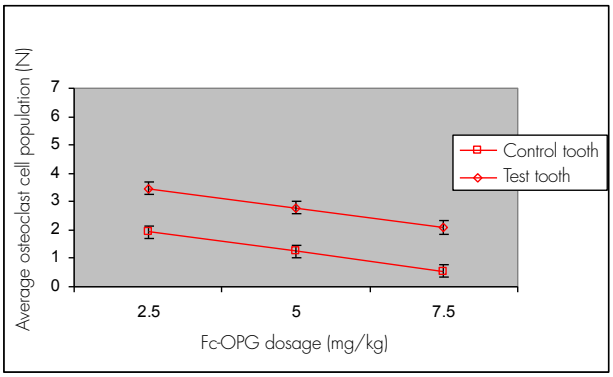
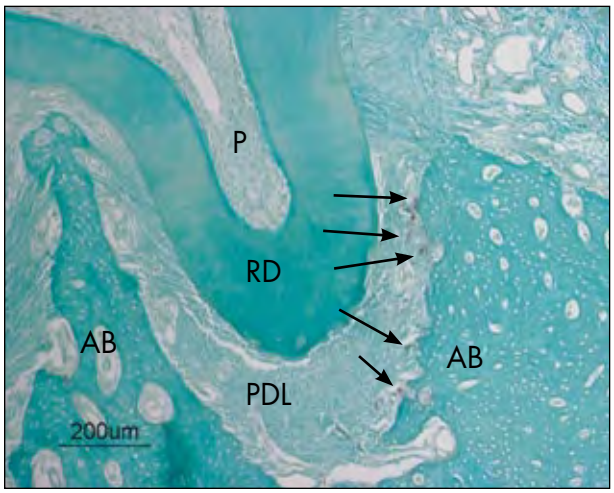


Figure 2. Dose vs osteoclastic population.

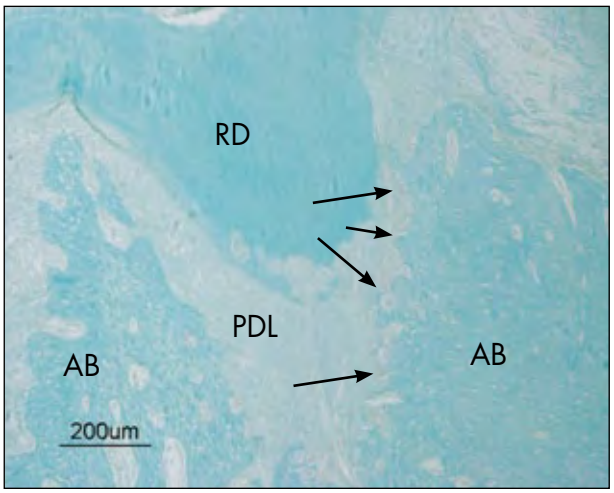
a linear reduction of 39.8% ($p < 0.05$). The osteoclastic populations present at 7.5 mg/kg dosage in frozen test teeth averaged 2.09 cells per slide (average osteoclastic cell population $p < 0.05$ compared to control teeth). The difference in the osteoclastic populations between test and control teeth was statistically insignificant ($p > 0.05$). The test teeth and control teeth parallelled each other during the linear decline of osteoclast populations as the single dose of Fc-OPG increased from 2.5 to 7.5 mg/kg (Figures 3 a-c). The osteoclastic cell populations are presented in Figure 4 (Dose vs. osteoclastic population per area). The reduction was greatest in the palatal region, followed by the furcation region and finally the buccal region. There was no obvious correlation observed between the areas.

Discussion

A frozen tooth model was used to produce aseptic necrosis and subsequent root resorption in an attempt to mimic inflammation caused by orthodontic tooth movement.[1] Previous dental studies using cryotherapy by Wesselink et al.[14] and Tal et al.[15] showed resorption of alveolar bone and cementum. In the present study, it was histologically evident that the application of cold to the rat molar crowns caused necrosis of the underlying pulpal and periodontal tissues. Osteoclast activity was apparent on the bone and cementum surfaces in areas affected by the freezing process. This model of tooth freezing was found to be safe, predictable and correlated with previously studied peak serum concentrations for Fc-OPG.[16] The use of endogenous OPG is difficult due to its limited availability. The use of the recombinant Fc-OPG has been advocated; however, there are structural differences between the endogenous and recombinant



(a)



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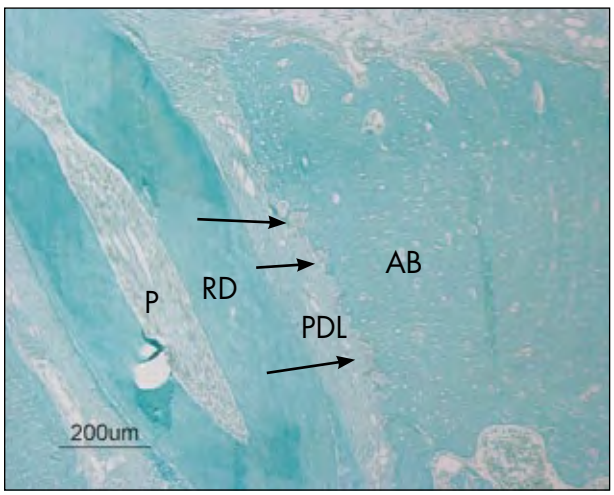

(c)

Figure 3. (a) Histological TRAP stain of 2.5 mg/kg test tooth at 10x magnification (Scale bar equals 200 µm). Arrows show TRAP positive cells. AB, Alveolar Bone; RD, Dentine; P, Pulp; PDL, Periodontal Ligament

(b) Histological TRAP stain of 5.0 mg/kg test tooth at 10x magnification (Scale bar equals 200µm). Arrows show TRAP positive cells. AB, Alveolar Bone; PDL, Periodontal Ligament; RD, Root Dentine

(c) Histological TRAP stain of 7.5 mg/kg test tooth at 10x magnification (Scale bar equals 200µm). Arrows show TRAP positive cells. AB, Alveolar Bone; P, Pulp; PDL, Periodontal Ligament; RD, Root Dentine

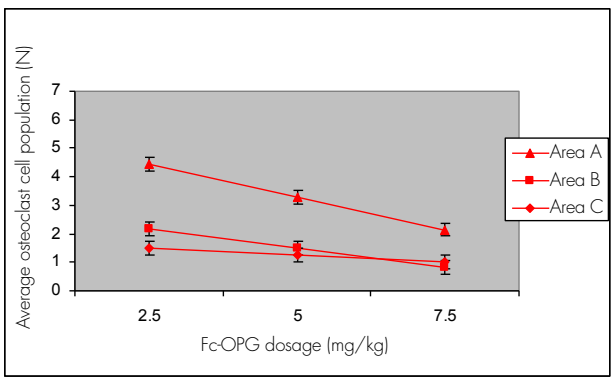

Figure 4. Summary graph of dose versus osteoclast population per area. Area A = Palatal root, Area B = Furcation region, Area C = Buccal root

types. The OPG used in this study consisted of a genetically engineered fusion molecule.

TRAP labelling enables the detection of tartrate-resistant acid phosphatase in multinucleate cells. Andersson and Marks considered the enzyme histochemistry of TRAP staining to be a useful marker of osteoclast ontogeny and function,[17,18] while Modderman et al. indicated that macrophages also attained TRAP properties, suggesting that the TRAP labelling in osteoclasts was not an exclusive marker.[19,20]

The analysis of cell numbers in specified areas was performed using a mixed model analysis. This proved satisfactory, although the use of appropriate statistical analysis in biological models is always open to debate. The raw data showed clusters of higher cell populations in distinct peaks interpreted as the areas in which roots were present. In the future, it may be beneficial to incorporate this observed variable into the analysis. The present study indicated that from 2.5 mg/kg to 7.5 mg/kg, the specific reduction of osteoclast cell populations occurred in a linear dose-dependent manner, with an average reduction of approximately 39.8%. This agrees with the results of other regimes involving single or multiple dosage protocols in both in vivo and in vitro models which have indicated that the principal pharmaco-dynamic action of OPG is the specific, rapid and sustained reduction of osteoclast numbers, along with other indices of bone resorption.[1,10,21,22] The observation of TRAP activity in resorption bays of the control

unfrozen molars was an occasional finding and consistent with previous studies.[1] It is likely that this surface resorption was physiologic in nature and normal for these animals. It was considered that the experimental teeth were sensitive after the application of cold, and that the contralateral side was preferred for mastication. Hence, the stressed animal may have placed higher loads on control teeth, resulting in the unexpected resorption pattern with a short-term increase in osteoclast population.

The present study found that OPG did not appear to affect any tissues or cells associated with the PDL other than osteoclasts. This suggests that OPG is highly specific for RANKL which is in turn highly specific for osteoclasts.[4,23,24] Capparelli et al. found a rapid 24 hour reduction in osteoclast numbers on the surface of rat tibiae with numbers reaching a low at 5 days and a sustained inhibitory effect after the administration of 5 mg/kg of OPG.[25] Lane reported similar findings to indicate that a single dose of OPG had an inhibitory effect on total osteoclast numbers and on the recruitment of osteoclasts during orthodontic tooth movement.[26] Bekker et al. in a randomised, double-blind, placebo-controlled single dose study, used a 3 mg/kg dose of OPG on post-menopausal women and observed an 80% decrease in bone resorption assessed by biochemical markers, indicating that, in this model, the 3 mg/kg dose was effective in rapidly and profoundly reducing bone turnover.[27]

Bolon investigated the efficacy of OPG in adjuvant-induced arthritis (AIA) and found that, if given at the onset of disease, 4 mg/kg of Fc-OPG was sufficient to eliminate all osteoclastic activity.[28] Bolon also reported that Fc-OPG given at increasing dosages of 1-30 mg/kg at the peak of disease progression could eliminate all osteoclastic activity but there was no impact on bone integrity due to pre-existing bone loss. This indicated that OPG inhibited osteoclastic activity, but was both dose and schedule-dependent.[28] In addition, Bolon found that single adenoviral delivery of Fc-OPG dosages had an immediate effect within 24 hours and efficacy peaked after 4 days.[29] Previous studies using single doses of various forms of OPG indicated the principal efficacy of OPG is its rapid inhibition of osteoclastic activity.

Osteoclast populations were present at the 7.5 mg/kg dosage in test teeth (Average osteoclastic cell population = 2.09, $p > 0.05$), and in smaller population numbers in the control teeth at all doses. This unexpected finding may be explained by insufficient OPG available to neutralise the RANKL following extensive necrosis during inflammation, or other mechanisms possibly exist that directly stimulate osteoclastic activity. Atkins et al. demonstrated that OPG completely inhibits the formation of osteoclasts from precursors within osteolytic giant cell tumours at 50 ng/mL OPG with 50% inhibition observed at 6.25 ng/mL.[30] However, the move from in vitro work to in vivo models has not been as comprehensive. Transgenic models have confirmed the initial in vivo results, in so far as transgenic mice, which over express OPG, systemically developed osteopetrosis in association with a near total lack of osteoclasts.[4,31]

Bolon et al., after testing at dosages up to 30 mg/kg, suggested that a dosage of 2.5 mg/kg in the murine model may be insufficient to cause total osteoclast inhibition.[28] Whilst RANKL, RANK and OPG play a significant role in the activity of osteoclasts, other hormonal and inflammatory mediators have the potential to possibly influence the OPG/RANK/RANKL interaction.[2,26]

Conclusion

The reduction in mature osteoclastic population numbers from single doses of 2.5, 5.0 and 7.5 mg/kg OPG occurred in a dose-dependent manner. The three current dosage regimes were either insufficient for complete inhibition of osteoclast activity or other factors were involved in stimulating osteoclasts. The current investigation reported the reduced occurrence of osteoclasts at the 7.5 mg/kg dosage level without complete inhibition as originally hypothesised. By extrapolation of the data, if the reduction of osteoclast numbers continued to decrease in a linear dose-dependant manner, there should be complete inhibition of osteoclasts at 15 mg/kg in this model of aseptic inflammation. This hypothesis warrants further investigation and a study of osteoclastic responses at higher dosages, along with single or multiple dose regimes, would be valuable and critical in understanding the true efficacy of osteoprotegerin.

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