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Research Article

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Vitamin K2 Affects Bone Remodeling Gene Expression in a Rat Model of Bisphosphonate-related Osteonecrosis of the Jaw

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Abstract

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a serious adverse effect seen in some individuals following the administration of bisphosphonate medications used to treat bone density disorders such as osteoporosis and Paget's disease. These medications are also used to prevent bone loss from metastatic osteolytic bone lesions. BRONJ is most often characterized by necros is of osseous tissue secondary to inappropriate healing of the mandible. Bisphosphonates promote BRONJ through inhibition of bone resorption. Bisphosphonates also promote BRONJ by a direct anti-angiogenic effect of the drug on the vasculature leading to a diminished blood supply and subsequent ischemia of the bone. The limited collateral circulation and mechanical environment found in the mandible makes this tissue particularly susceptible. The aim of this study was to investigate the effects of 6 weeks of vitamin K2 supplementation vs. controls without Vitamin K2 on the development of BRONJ in rats receiving first molar extractions and 13 weeks of bisphosphonate treatment. After 6 weeks of treatment with vitamin K2 or carrier, all rats were sacrificed. Tissue samples were evaluated for bone markers with microarray gene analysis and histology of the extraction sites on the rat jaws. Results indicate that vitamin K2 supplementation increases expression of bone-forming genes, increases tissue healing of extraction sites, and overall improves wound healing. Our study suggests that vitamin K2 may be a promising and safe medicament to assist in the prevention of BRONJ development in patients taking bisphosphonate medications for other systemic health issues.

Background/ Introduction

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a well-established, serious adverse effect following the administration of bisphosphonate medications used to treat bone density disorders such as osteoporosis and Paget's disease.[1,2] Intravenous bisphosphonates are also administered to prevent bone loss from metastatic osteolytic bone lesions and are associated with an even greater risk than oral bisphosphonates for the induction for BRONJ[3,4] BRONJ is most often characterized by necrosis of osseous tissue secondary to inappropriate healing of the mandible. Figure 1

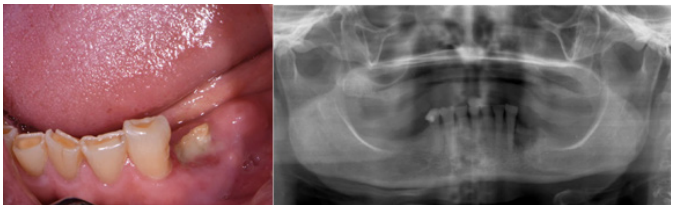

Figure 1 Example of BRONJ in a human patient treated with intravenous injections of bisphosphonate zoledronic acid. A. Intraoral image showing non-healing bone exposure. B. Panoramic radiograph showing resorption of the mandible.

[5]Bisphosphonates promote BRONJ through inhibition of bone resorption which over time may cause a diminished blood supply or ischemia of the bone. Bisphosphonates can also promote BRONJ by a direct anti-angiogenic effect of the drug on the vasculature.[6,7] The limited collateral circulation and mechanical environment found in the mandible makes this tissue particularly susceptible to BRONJ.

The development of BRONJ in an individual taking lower doses of bisphosphonates to prevent post-menopausal osteoporosis, may occur only after several years of exposure to the medication.[1,5] To study this disorder, we have previously created animal models of BRONJ where high doses of bisphosphonates (equivalent to those used in cancer treatment) and wound creation in the oral cavity can shorten the time required for development of BRONJ in a rat model to a few weeks.[8,9]

In our study we attempt to improve healing of the surgically created bone lesions by using a novel treatment strategy involving vitamin K2. Vitamin K2 (menaquinone-7; MK-7) is a fat-soluble essential vitamin that plays a critical role in a wide variety of organ systems for proper homeostasis.[5,10] MK-7 also promotes osteoblast proliferation, inhibits osteoclast activation, and plays a role in the conversion of osteocalcin into its active form, leading to an overall increase in bone formation relative to bone resorption.[11,12]

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The modern Western diet is characteristically low or absent in dietary vitamin K2, the absence of which may play a significant role in the development of various systemic conditions such as osteoporosis and cardiovascular disease.[13] Our study was designed to determine if vitamin K2 administration in our rat model of BRONJ alters expression of osteogenic markers in a manner suggesting an improvement in bone healing.

Methods

Preparation of the BRONJ Model

For this study, we used a rat model of BRONJ, modified from a model previously described by Howie et al.[9] Sprague-Dawley rats of both sexes (age 10–12 months-old) weighing approximately 200 grams were used for this study. At this age and weight, the size of the animals allows for the optimization of the surgical procedures. The experimental procedures for preparation of the model have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Western University of Health Sciences as part of a larger study. To create the BRONJ model, each rat received 13 weekly intravenous injections of 80ug/kg zolendronic acid (ZA) (Enzo LifeSciences, Farmingdale, NY, USA) in 0.3ml saline via the tail vein. After 13 weeks, the first molar teeth on both sides of the mandible of each rat were extracted. Weekly injections were carried out under isoflurane anesthesia.

Dental Extraction

All tooth extractions were supervised by experienced investigators. Extractions were performed using Adson surgical forceps after two weeks acclimatization to the vivarium. Extractions of both first molars were conducted under anesthesia by intraperitoneal injections of ketamine (100 mg/mL) and xylazine (20 mg/mL).

Each extraction socket was cleaned using a 1.0 mm round bur at 15,000 RPM. The use of a bur served to standardize the extraction defect. The procedure was created to represent a surgical extraction, as opposed to a simple, dental extraction. Buprenorphine analgesia was also available post-operatively if stress behaviors were noted. Animals were closely observed for signs of bleeding, discomfort, or lack of feeding and were fed a soft diet for 3 days after extractions.

Euthanasia and Sample Collection

For this study, only BRONJ rats were used. Two groups of 10 animals were euthanized after 13 weeks of BP treatment followed by 6 weeks of gavage with either vitamin K2 in sunflower oil, or with sunflower oil alone as a control.

Rats were euthanized using an overdose of CO_2 in a closed plastic container followed by exsanguination. This method meets the recommendations of The American Veterinary Medical Association. Blood for serum analysis was drawn via an intra cardiac catheter. Immediately following euthanasia, the animals were carefully dissected to collect the mandibles. Samples were coded at that time for subsequent blind analysis. Two mandibles from each group were placed in a formalin-EDTA decalcification solution for histology. Eight mandibles from each group were also frozen in liquid nitrogen and stored at -80°C for RT-PCR marker analysis.

Vitamin K2 administration

This investigation studied the effects of vitamin K2 on the healing of surgical extraction sites in the zoledronic acid (ZA)-treated rats. Vitamin K2 as menaquinone-7 was provided by Gnosis by Lesaffre, Lesaffre, France. We selected 10 rats to receive a 5mg/kg dose of Vitamin K2 in sunflower oil administered daily by gavage. An additional group of 10 rats was administered sunflower oil by gavage of the same volume as the treated rats.

For this study, we used mandibles isolated from all rats that had undergone the protocol to induce BRONJ. The expression of each marker for osteogenesis was identified by an RT-PCR marker array.

RT-PCR

After the 19 weeks of the study (13 weeks following molar extraction to create the BRONJ model and 6 weeks of dosing with vitamin K2 or carrier) eight rats from each group were sacrificed and the mandibular bone tissue was harvested and frozen in liquid nitrogen before storing at -80°C prior to RT-PCR analysis. Frozen bone samples from both groups (vitamin K2-treated and controls) were placed in 1mL of TRIzol (ThermoFisher Scientific Waltham, MA), prechilled in liquid nitrogen. To disrupt bone tissue a Polytron® homogenizer set at maximum speed was activated for 45 seconds on ice. Soluble bone extract was separated from mineralized bone fragments by centrifugation at room temperature for 15 seconds at 8,600 X g. RNA was purified from the extracts using the RNeasy-Plus® Universal Mini protocol following manufacturer's directions. (QIAGEN, Valencia, CA). A NanoDrop® spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE) was used to determine the amount of RNA in each sample.

The experimental RNA and control RNA samples were converted to first strand cDNA using the RT First Strand Kit® (QIAGEN, Germantown, MD). The cDNA was placed in RT SYBR Green qPCR Mastermix and mildly agitated before aliquoting into wells of the RT Profiler PCR Arrays for "Rat Osteogenic Factors". PCR was performed using the ABI Step One Plus Real Time PCR System. Relative expression of genes was compared using data obtained from the real-time cycler and the ΔΔCT method.

Histology

Two bone samples from each treatment group (vitamin K2 and controls) were preserved in 10% phosphate-buffered formalin (1:10 by volume) for 48 hours followed by demineralization with EDTA (17%) for 10 weeks. During demineralization, the containers were placed on a continuous slowly rotating shaker at 4°C. The EDTA was replaced every 2–3 days. X-ray analysis was used to confirm demineralization. Demineralized samples were processed overnight before embedding in paraffin. Each paraffin-embedded sample was sectioned in the sagittal plane through the extraction site. Sections were cut 5 microns thick and stained with hematoxylin and eosin using a standard protocol.

An oral pathologist examined the sections for changes in bone cellularity, vascular presence, inflammatory cell infiltrate, osteoblastic and osteoclastic activity, and necrosis of the bone.

Multiple 20x and 40x fields were examined in each section focusing on the areas of molar extraction. The degree of tissue healing was assessed by comparing vitamin K2-treated and control rats.

Results

RT-PCR

Osteogenesis array data was collected, filtering out only gene expression that was labelled "A" or "OKAY" based on a gene's average threshold being significantly different from the control group, and for relevant bone-turnover genes for this study. The genes in Table 1 were shown to have significant differences in gene expression between groups

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Table 1 RT-PCR results showing relevant genes with 2-fold or greater changes in the expression

Gene Abbreviation	Gene Name	Relative Change	Interpretation
Dlx5	Distal-less homeobox 5	↑	When increased Dlx5 promotes bone development and fracture healing
Runx2	Runt-related transcription factor 2	↑	When increased the number of osteoblasts increases
Smad5	SMAD family member 5	↓	Decreased SMAD5 removes inhibition of BMP2-induced osteoblast differentiation.
Spp1 (OSP)	Osteopontin	↓	Initiates ruffled border on osteoclasts when increased, therefore when decreased resorption is decreased
Tgfb2	Transforming growth factor, beta 2	↓	Slows growth when elevated therefore growth increases when Tgfb2 decreases
Tnfsf11 (RANKL)	RANK ligand	↓	When RANKL decreases bone resorption decreases

Histology

Representative sections of mandibles from BRONJ rats treated with vitamin K2 in sunflower oil (Figure 2) and BRONJ rat controls treated with sunflower oil alone (Figure 3) were compared. Both the control and treated sections showed healing at the extraction site. The vitamin K2-treated tissue however, appeared to regain a more normal tissue epithelium, lamina propria, and bone structure than the BRONJ tissue without vitamin K2 treatment.

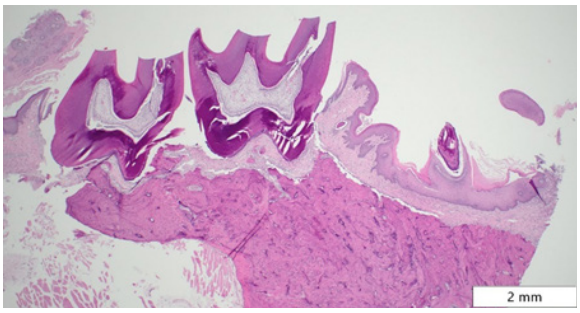

Figure 2 H&E-stained sagittal section of the rat maxilla from a BRONJ rat treated by gavage with vitamin K2 in sunflower oil. This image shows the healing of the site of first molar extraction.

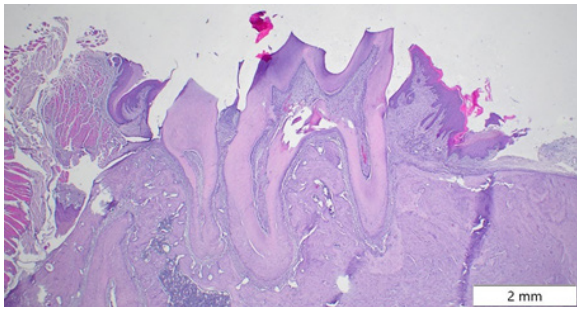

Figure 3 H&E-stained sagittal section of the rat maxilla from a BRONJ rat treated by gavage with sunflower oil only as a control. This image shows less healing of the site of first molar extraction than in the vitamin K2-treated BRONJ rats.

Discussion

The presence of vitamin K2 activates bone matrix and vascular matrix formation and bone GLA proteins, leading to an increase in bone formation and an inhibition of calcium deposits within vascular walls.[14,15] These intimate interactions suggest that vitamin K2 may hold promise as asupplementary treatment option for osteoporosis and for mitigating BRONJ and other disorders disrupting bone turnover. Utilizing RT-PCR analysis, we quantified and evaluated the changes in expression of molecular markers of osteogenesis. Additionally, we examined samples of the bone tissue from mandibles of the sacrificed rats to evaluate histological changes following vitamin K2 administration.

Our results (see Table 1) show an increase in the bone marker genes Runx2 and Dlx5 with the administration of vitamin K2. These transcription factors are known to promote bone development and healing of damaged bone.[16,17] Up-regulation of these genes would be supportive of bone repair and may explain the healed extraction sites seen in our vitamin K2-treated BRONJ model. Our results also show suppression of Smad5 and Tgfb2. These genes code for proteins that are members of the transforming growth factor beta superfamily of secreted proteins that regulate growth and development of a wide variety of tissues including bone.[18] Smad5 is one of the main signal transducers for receptors of transforming growth factor. The down-regulation of these genes by vitamin K2 administration also suggests support for the bone repair seen in our study.

Spp1 was also suppressed in our BRONJ rat model. Spp1 is a gene that has been shown to be associated with osteosarcoma but is not necessary for tumor progression.[19] It has been suggested that Spp1 may play a role in the inflammatory response and in bone resorption.[20] Suppression of Spp1 suggests that vitamin K2 may promote the decrease in inflammation and bone breakdown seen in our study which could be helpful for patients with BRONJ. Similarly, RankL gene expression was also suppressed in our study. RankL is produced by osteoblasts to communicate bone formation signals to osteoclasts for coordination of bone turnover.[21,22] A decrease in RankL is associated with an increase in bone formation suggesting an uncoupling of the bone formation and bone resorption processes that may be of value as an alternative treatment in patients taking bisphosphonates. Table 1 the results of our study are summarized in the drawing in Figure 4.

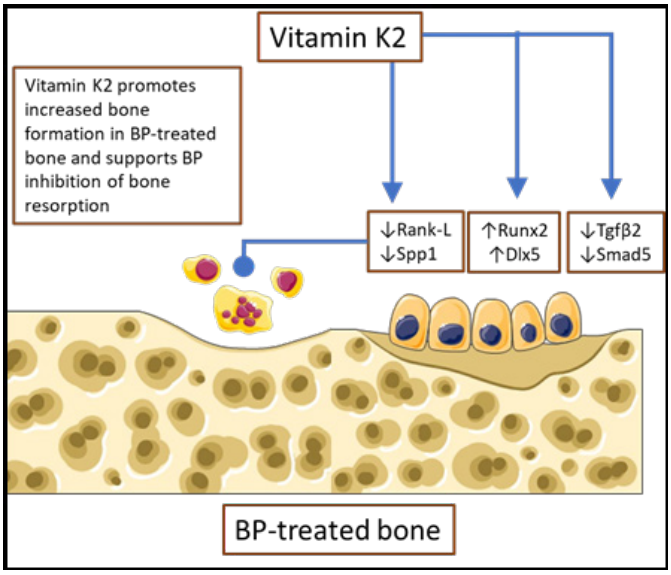

Figure 4 The effects of vitamin K2 on gene expression in osteoblasts promote bone formation in bisphosphonate-treated bone. (Figure generated in part using Servier Medical Art, licensed under a Creative Commons Attribution 3.0 license).

In conclusion, this study suggests that vitamin K2 supplementation may assist in the formation of bone, the inhibition of bone resorption, and the reduction of inflammation in patients taking bisphosphonates.

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Conflicts of Interest

KM is a consultant for Gnosis by Lesaffre.

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