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Sexual Dimorphism in Periapical Inflammation and Bone Loss from MAP Kinase Phosphatase-1 Deficient Mice

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Sexual Dimorphism in Periapical Inflammation and Bone Loss from MAP Kinase Phosphatase-1 Deficient Mice

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A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Master of Science in Dentistry in the College of Dental Medicine.

Department of Oral Rehabilitation

Division of Endodontics

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Abstract

Introduction: Mitogen Activating Protein (MAPK) kinase phosphatase-1 (MKP-1) has been shown to be a key negative regulator of the MAP kinase pathways of the innate immune system. The impact of MKP-1 in an endodontic model has yet to be studied. Thus, the purpose of this study was to determine the role of MKP-1 in a bacterial-driven model of pathological endodontic bone loss.

Methods: Pulps were exposed in both lower 1st molars of 10-week old *Dusp-1*$^{+/+}$/MKP-1$^{+/+}$ and *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice and left open to the oral environment for either 3 or 8 weeks. At sacrifice, mandibles were harvested and scanned by microcomputed tomography (microCT) to determine periapical bone loss. Histopathological scoring was then performed on the samples to determine the amount of inflammatory infiltrate within the periapical microenvironment.

Results: Significant bone loss and inflammatory infiltrate were found in all experimental groups when compared to control. No statistical difference was found between *Dusp-1*$^{+/+}$/MKP-1$^{+/+}$ and *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ at either time point with respect to bone loss or inflammatory infiltrate. At 8 weeks, male DUSP-*1*$^{-/-}$*/MKP-1*$^{-/-}$ mice were found to have significantly more bone loss and inflammatory infiltrate when compared to female *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice. There was also a significant correlation between an increase in bone loss and increase in inflammatory infiltrate.

Conclusions: A sexual dimorphism exists in the periapical inflammatory process, where male *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice have more inflammation than female *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice. The increase in inflammatory infiltrate correlates to more bone loss in the male mice.

Introduction

Etiology and Pathogenesis of Endodontic Disease

The etiology of endodontic disease and ultimately apical periodontitis is microbial in origin, primarily initiated by a carious lesion of tooth structure causing infection of the root canal system. It has been proven that without bacteria there can be no pulpal necrosis or subsequent apical periodontitis (Kakehashi 1965.) In germ-free mice, pulp exposed teeth were able to repair themselves to health, whereas mice with normal oral flora developed apical periodontitis. Another study was able to show similar results in a population of monkeys with either bacterially infected root canals or non-infected root canals (Moller 1981.) Caries is the primary origin of bacteria causing necrosis, but the process could also be initiated by trauma injury, fracture of tooth structure or iatrogenic exposure of the pulp tissue. Fungi and viruses have also reportedly been found in infected root canal systems, but bacteria remain the primary causative factor for pulpal necrosis (Gomes 2010, Sabeti 2012.)

Because the normal root canal system is a sterile environment, any bacterial species that is able to reach these confines is potentially harmful to the pulp. Primary intraradicular infection can be caused by bacteria that initially entered the root canal system through the carious process or could be opportunistic pathogens that took advantage of the necrotic environment. The root canal system is an ideal space for bacteria to colonize, as there are plentiful nutrients from the necrotic tissue, there is protection from the immune response

and the root canal walls are conducive to setting up a biofilm colony. These infections have a high proportion of anaerobic bacteria, characteristic of the occupied environment. It is a mixed infection with many species types having been identified. Bacterial load has been quantified and ranges from 10^3 to 10^8 per root canal (Sakamoto 2007, Siqueira 2007, Sundqvist 1976) with a mean of 10 to 20 species per root canal (Munson 2002, Rocas 2008, Siqueira 2005.) It has also been shown that the size of the bony lesion is directly proportional to the number of species present in the canal with some canals having over 40 species identified (Rocas 2008.) Specific species as identified by molecular methods are both gram negative and gram positive. Most prevalent species present in primary infections include *Dialister invisus, Bacteroidetes* clone X083, *Pseudoramibacter alactolyticus, Porphyromonas endodontalis, Treponema denticola, Dialister pneumosintes, Filifactor alocis* and *Tannerela forsythia* (Siqueira FEMS Micro Lett 2005, Siqueira JOE 2004, Siqueira JOE 2005, Siqueira J Clin Micro 2005.) Roughly half of the microbiota species in infected root canals are still uncultivated and may actually be some of the most prevalent species involved in root canal infection (Sakamoto 2006.)

Bacterial invasion into the pulp stimulates initiation of a pulpal immune response. Pulpal response begins with antigen recognition by immunocompetent macrophages and dendritic cells. Pulpal blood flow is increased allowing for the infiltration of polymorphonuclear neutrophils (PMNs) and monocytes in the early stages of inflammation (Bergenholtz 1985.) The number of inflammatory cells

increases as the infection progresses, activating cells of the adaptive immune system. These are primarily T cells but also B cells (Hahn 1989.) The immune system has a difficult time eradicating an infection of the root canal system due to its confined space and limited blood supply. These limitations of the pulpal immune response lead to progressive necrosis of the pulp in focal areas and eventually total tissue necrosis.

Bacteria and their by-products gain access to the periodontium through the primary apical foramen, as well as lateral portals of exit. The immune response in the periapex is similar to that of the pulp proper with the primary difference being the destruction of osseous tissue in the area surrounding the infected root apex. It has been proposed that the periapical lesion is a teleological response to odontogenic infection to prevent systemic infection from occurring (Stashenko 1990.)

Endodontic Inflammation and Bone Loss

Periapical inflammation progresses in a manner similar to that of pulpal inflammation, with the addition of bone destruction. Bacterial constituents reach the periapex and initially cause an influx of PMN's and monocytes (Okiji 1994.) In rodent models, the innate immune response alone has been sufficient to cause an increase in osteoclastogenesis and subsequent bone destruction in the periapex (Rittling 2010.) In this early stage, chemical mediators (chemokines)

such as interleukin (IL)-8 and monocyte chemoattractant peptide-1, are produced in the periapex. These mediators serve to regulate the influx of PMN's and monocytes. The innate immune response is activated in multiple cell types associated with endodontic lesions, including monocytes/macrophages, granulocytes, pulpal fibroblasts, osteoclast precursors and mesenchymal cells (Hirao 2009, Bar-Shavit 2008.) Lipopolysaccharide (LPS) is the major inducer of the innate immune response in endodontic disease, but other bacterial constituents causing an immune response could be lipoteichoic acid, peptidoglycans, flagellins, and RNA or DNA fragments. These are collectively known as pathogen-associated molecular patterns (PAMPs) that interact with pathogen recognition receptors (PRRs) on immune cells, namely toll-like receptors (TLRs.) TLR's on the cell surface recognize the extracellular bacterial signals and begin the conversion to a cellular response. TLR-4 specifically recognizes LPS after LPS has already bound to LPS-binding protein (LBP) and a macrophage cell-surface receptor, CD-14. Intracellular molecules bind to the trans-membrane components of the TLR and activate a cascade of events that include the stress kinase pathway and the nuclear factor-κB (NF-κB) inflammatory cascade (Liu 2009). These cascades lead to the production of a number of inflammatory cytokines. Notable cytokines produced in endodontic disease are IL-1α, IL-1β, and TNF-α and are derived mostly from PMN's and macrophages (Stashenko 1995.) IL-1β seems to be primarily associated with the pathogenesis of periapical disease, as its levels have been shown to decrease after endodontic therapy, whereas IL-1α levels increase after treatment (Matsuo

1994.) IL-6 has been identified in periapical lesions and is associated with osteoclast formation (Hutter 1998.)

The production of proinflammatory cytokines by macrophages as well as fibroblasts, osteoblasts, and neutrophils, leads to tissue destruction in the periapical region. IL-1, and to a lesser extent TNF, induce the expression of receptor activator of nuclear factor-κB ligand (RANKL) by osteoblasts, causing activation of osteoclasts and resulting bone resorption (Dewhirst 1985, Bertolini 1986.) IL-1, TNFα, and IL-6 have been associated with increased bone resorption, including RANKL production and osteoclastogenesis. IL-1 has been shown to play a crucial role in inducing bone loss by using an IL-1 receptor antagonist in an experimental model. Lesion development was decreased by 60% when IL-1 was antagonized (Stashenko 1994.) In humans IL-1β and TNFα cause an increase in expression of matrix metalloproteinases (MMPs.) MMP's cause degradation of the extracellular matrix, enhancing the development of periapical lesion formation and bone loss. Cytokines can also enhance osteoclastogenesis directly or indirectly through stimulation of RANKL expression. RANKL's interaction with receptor activator of nuclear factor-κB (RANK) induces osteoclast differentiation and activation, also enhancing formation of the periapical osseous lesion. Osteoprotegrin (OPG) acts as an inhibitor of the interaction between RANK and RANKL, thus decreasing osteoclast activation and bone destruction. During an inflammatory process, RANKL concentration is higher than OPG, allowing bone destruction to occur

(Graves 2011.) Adaptive immunity is also important in periapical lesion formation. Adaptive cells were found to be primarily T cells and to a lesser extent B cells (Stashenko 1992.) T_H1 associated cytokines, especially IFNγ, have been associated with cell mediated immunity (Nair 2004) and in general are proinflammatory (Graves 2011.) T_H2 cells produce cytokines IL-4, 5, and 6 and are associated with production of antibodies by plasma cells (Nair 2004) and in general are considered anti-inflammatory (Graves 2011.) IL-1β and TNF-α also cause an increase in prostaglandin E_2 (PGE_2) production (Saito 1990), as well as increases in destructive proteinases (Meikle 1989) and an inhibition of new bone formation (Barkhordar 1999, Stashenko 1994.) The root canal and periapical environment are such that the root canal system provides a protected source of bacteria and bacterial toxins. Once the pulp tissue has necrosed, immune cells are unable to reach the confines of the root canal system to resolve the infection (Nair 2004.) A continuous source of inflammation causes bone resorption in the periapex to go on indefinitely as the immune system battles this barrage of insults.

Innate Immunity and Signal Transduction

The production of inflammatory cytokines is the consequence of activation of key intracellular pathways, including MAPK pathways (Yang 2003, Whitmarsh 2007, Whitmarsh, Davis 2007.) The stress kinase cascade involves multiple

phosphorylations of mitogen activated protein (MAP) kinases. Activation of MAP kinase kinase kinase (MAPK3) phophorylates MAP kinase kinase (MAPK2) which in turn phophorylates MAP kinase (MAPK.) MAPK can then phosphorylate a number of downstream targets. These include activation of activating protein (AP)-1, enhancing cytokine gene transcription, and help regulate protein expression through alterations in mRNA transcripts containing AU-rich elements (Liu 2009.) This process localizes to the cell nucleus where specific MAP kinases, including extracellular signal related kinases (ERK), c-jun N-Terminal kinases (JNK) and p38 relay, amplify and integrate signals, modulating a series of physiological responses including cellular proliferation, differentiation, development, inflammatory responses and apoptosis (Keyse 2000.) Production of cytokines includes pro-inflammatory IL-1, IL-6 and TNF and anti-inflammatory IL-10. This cascade also can result in enhanced RANKL production, osteoclastogenesis and bone resorption (Hirao 2009, Bar-Shavit 2008.) The MAP kinases also interact with proteins that negatively regulate the inflammatory cascade. As MAP kinases are activated by phosphorylation, they can be inactivated by dephosphorylation. The primary phosphatases in mammalian cells considered responsible for MAP kinase dephosphorylation are a group of dual specificity protein phosphatases known as MAP kinase phosphatases (MKPs), of which at least 10 are presently known (Liu 2009.) The first of these phosphatases to be discovered is known as MKP-1, with DUSP-1 being the gene encoding for the protein MKP-1. MAP kinases not only activate MKP's but can also affect MKPs' stability. ERK enhances the stability of MKP's, while JNK

causes degradation of the protein. MAPK's also enhance the catalytic activity of the phosphatases. MKP's dephosphorylate MAPK's, with preference for JNK and p38, attenuating the inflammatory cascade (Liu 2009.) Although the activation of MAPK pathways is critical to initiate an innate immune response against proliferating pathogens, sustained production of pro-inflammatory cytokines can result in extensive bone resorption. A number of studies have demonstrated that LPS from gram-negative bacteria was capable of inducing bone resorption *in vivo* (Orcel 1993, Nishida 2001, Rogers 2007.) Therefore, modulating MAPK immune response to an appropriate level is essential to attenuate bone resorption associated with bacterial infection.

Sexual Dimorphism in Innate Immunity

Gender differences have been well documented in innate immunity. In general it has been shown that males have a more pronounced immune response with more sequela in comparison to females. With respect to systemic inflammation, females have been shown to have a 30% lower innate immune response when the level of cytokine TNF was measured after LPS challenge (Moxley 2002.) In a similar study, females produced significantly less LPS challenged TNF and IL-1β than males and also had lower overall MAPK phosphorylation (Imahara 2005.) Females have a more responsive and protective cell-mediated and humoral response to antigenic challenge (Marriott,

Huet-Hudson 2006) whereas males have been shown to have an increase in macrophage TLR-4 and CD-14, possibly causing an increase in cytokine production and more intense immune response (Marriott, Bost 2006.) The mechanisms for the apparent sexual dimorphism in innate immunity are generally unknown, but there are several theories that may contribute to the biological basis for the differences. It has been proposed that sex hormones help regulate immune cell function (Wyle 1977.) Estrogen may be an important regulator of the immune system as it has been shown to affect the synthesis and release of pro-inflammatory cytokines (Angele 2000, Frink 2007.) In trauma and sepsis studies, premenopausal women have higher survival rates than either men or postmenopausal women, showing a relationship between estrogen and immune function (George 2003, Schroder 1998.) In the dental literature, estrogen was shown to have a protective role in inflammation. Estrogen deficient female rats had a significantly greater systemic response to periapical lesion formation, showing an increase in serum concentrations of IL-1, TNFα, IL-6 and MMP-9 (Zhang 2011.) The sexual dimorphism in innate immunity is most likely multi-factorial. Another explanation is the genetic difference between males and females. There is an X-linked gene base responsible for cytokine receptors and proteins and immune related transcription factors that may be different between genders (Fish 2008.)

Studies have shown that *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice are more susceptible to endotoxic shock and exhibited a marked increase in production of pro-

inflammatory cytokines TNF-α, IL-6, and an anti-inflammatory cytokine IL-10 as compared with wild-type animals (Zhao 2006.) *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice also had a marked increase in both the incidence and severity of experimentally induced autoimmune arthritis (Chi 2006, Salojin 2006) and exhibited more periodontal bone loss after LPS challenge as compared with wild-type animals (Sartori 2009.) These results highlight the significance of MAPK/MKP-1 regulation on innate immune response to LPS-induced inflammation and maintaining bone homeostasis, suggesting that restriction of activated MAPKs is a potential therapeutic strategy for diseases associated with exaggerated MAPK responses. Recent studies confirmed that MKP-1 is a key negative regulator of periodontal disease progression in LPS-driven models of experimental periodontal disease (Sartori 2009, Yu 2010,) but its role in a bacterial-driven environment has not been determined. Thus, the purpose of this study was to determine the role of MKP-1 in a bacterial-driven model of pathological endodontic bone loss.

Material and Methods

Animals

The Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina approved all experimental protocols. Mice used for this application were initially obtained through a material transfer agreement (MTA) from Bristol Myers Squibb. Generation of homozygous *Dusp-1* knockout

mice (KO) was done through mating *Dusp-1*$^{+/-}$ mice to obtain *Dusp-1* null mice and maintained on a mixed C57/129 genetic background.

Periapical Bone Loss Model

Pulp exposure was obtained as described in previous endodontic models (Kawashima 1999, Hou 2000.) Briefly, 10-week old male and female mice were anesthetized using intraperitoneal injection of Ketamine (80mg/kg) and xylazine (10mg/kg) in sterile phosphate-buffered saline after induction with inhalational isoflurane. For experimental groups, lower first molars were accessed and pulps exposed under surgical operating microscope (Olympus Highlight 3100/SZ61; Olympus Imaging America, Center Valley, PA) with a dental handpiece (Aseptico; Woodinville, WA) and ¼ round bur. Access allowed penetration of #6 endodontic hand file to the mesial and distal root canals. The pulp was then left exposed to the oral environment for three or eight weeks. Euthanization occurred by CO_2 asphyxiation. Mandibles were harvested and sectioned into two halves at the midline for analysis. Control mice were mixed gender and did not receive exposure.

MicroCT Analysis

Anatomic sections were performed to include the mandibular 1st through 3rd molars, as well as surrounding osseous tissues. Samples were placed in 10% formalin for 24 hours and then stored in 70% ethanol. Cone-beam microcomputed tomography (μCT) scans were obtained using high-resolution

desktop μCT (μCT40 scanner; Scanco USA, Inc., Wayne, PA). Initial data reconstruction was performed using Scanco Medical Open VMS software (Scanco USA, Inc.) For visualization, samples were digitally reconstructed so that a two-dimensional slice could be obtained showing a patent mesial and distal canal in the first molar.using GE MicroView software (GE Healthcare BioSciences, Chalfont St. Giles, UK). Data was exported into ImageJ for further analysis. Periapical regions of interest were obtained by digital cropping and measured by the software in square pixels. All measurements were performed by the same trained examiners and repeated at separate time intervals. Mean bone loss cross-sectional area was compared in wild-type and knockout mice.

Histology and Inflammation Indices

Following μCT analysis, specimens were decalcified in a 0.5 M EDTA solution pH 8.0 for 4 weeks at 4°C. Mandibular sections were paraffin-embedded, and 7 μm sagittal sections prepared. Some sections were stained with hematoxylin and eosin (H&E) for descriptive histology. Scoring of inflammatory infiltrate was performed with a trained pathologist (HY). Scoring of inflammation corresponded with the following index: 0 = No neutrophils near the root; 1 = sparse (<5%) neutrophils adjacent to the root apex; 2 = 5-20% neutrophil infiltration adjacent to the root apex; 3 = 20-50% neutrophils in the area limited adjacent to root apex; 4 = numerous (>50%) neutrophils in area greater than 50% adjacent to root apex.

Statistical analysis

Non-parametric Wilcoxon (Mann-Whitney) rank-sum test was used with an *alpha*=0.05 two-sided significance level to evaluate potential differences between knockout and wild-type mice in bone loss and histological scoring measures per hemi-mandible. To accommodate for the within-cluster dependence of the data, the corrected variance formula for the Wilcoxon rank-sum statistics developed by Rosner and colleagues (Rosner 2006) was applied. Mean and standard error results were estimated utilizing mixed effect regression models. Multivariable mixed effect regression models were used to assess associations between bone loss and histological scoring, while adjusting for time, gender, and *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ versus *Dusp-1*$^{-/-}$/MKP-1$^{+/+}$ status. All possible 3- and 2-way interactions were tested in this multivariable model. All statistical analyses were performed using SAS® Proprietary software, 9.2, © 2002-2008, SAS Institute Inc., Cary, NC, USA.

Results

Post-endodontic exposure, μCT analysis of mandibular samples revealed that a significant amount of periapical bone loss was achieved using this experimental protocol compared to untreated controls in all groups (Fig. 1.) Although analysis of *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice showed numerically greater amounts of bone loss than bone loss observed in age-match wild-type littermate

controls, significant differences in bone loss were not detected at either 3- or 8-week time points. However, among *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice at 8 weeks, significantly more bone loss occurred in males compared to age-match female mice (P<.05), a result that was not observed among wild-type mice (Fig. 2.) Figure 3 shows representative μCT splines of control, 3 and 8 week treatment samples demonstrating periapical bone loss.

To determine if the degree of inflammation was consistent with μCT data, inflammatory infiltrate was evaluated and quantitated in the periapical area. Similar to data from μCT analysis, there was not a significant difference in the amount of neutrophil infiltrate between *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ and *Dusp-1*$^{+/+}$/MKP-1$^{+/+}$ mice at either 3- or 8-week time points (Fig 4), although the mean scoring for *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice was higher than that of age-match wild-type control mice. As with bone loss, there was a significantly higher histopathology score in Dusp-*1*$^{-/-}$*/MKP-1*$^{-/-}$ males compared to *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ females at 8 weeks (P<.05) (Fig. 4). Figure 5 shows representative H&E slides corresponding to periapical areas used for histopathologic analysis of control group, 3 and 8 week treatment group demonstrating areas of periapical inflammation. There was a significant correlation found to exist between histopathology score and amount of bone loss among all groups (P<.05.)

Discussion/Conclusions

Invasion of the dental pulp system by bacterial constituents, such as LPS, leads to the stimulation of the innate immune system through multiple intracellular signaling pathways, including the MAPK cascade. Innate immune cells (i.e., neutrophils and macrophages) within the periapical microenvironment are activated to both produce and respond to pro-inflammatory cytokines leading to enhanced osteoclastogenesis and bone resorption. In previous studies, MKP-1 has been shown to be a key component in the attenuation of bone loss through a role as negative regulator of the MAPK system.

The endodontic model in this experiment was shown to be effective in producing a measurable amount of bone loss. Previous endodontic studies used injections of human bacterial pathogens into the root canal system to induce an inflammatory response and bone loss (Kawashima 1999, Hou 2000.) In this study, the animal's own commensal oral bacteria were the cause of the bone loss outcome due to the root canal access remaining open to the oral cavity. It is unknown what bacteria are present in this murine model, but the end result of the inflammatory process was more important in this study than which bacteria were the causative agents. Further studies could determine the bacteria present in the murine model. It is also important to note that the mice were maintained in a healthy state. If the mice had lost more than 10% of their initial body weight they would have been sacrificed per IACUC protocol. All mice stayed within the parameters of healthy weight over the course of the experiment (Figures 6 &7.)

Although in this study a trend existed where *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ mice had more bone loss than wild-type counterparts, this did not reach significance. There was, however, a significant difference in bone loss and histological scoring between male and female genders of the 8-week *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ group. Males showed an increased periapical inflammatory response as compared to females with respect to bone loss and histological score.

There have been previous studies showing a sexual dimorphism related to inflammation similar to that observed in this study. The protective role of estrogen has been shown in a study of ovariectomized rats with induced periapical lesions. This protective effect has been hypothesized to be due to effect on the synthesis and release of pro-inflammatory cytokines (Zhang 2011.) In the periodontal literature, it has been shown that males are more prone to periodontal infection than females (Desvarieux 2004.) Elevation of LPS binding protein, CD14 and TLR-4 in males, was noted as a plausible explanation for the gender difference (Marriot, Bost 2006.)

The clinical significance of sexual dimorphism in periapical inflammation has not been fully elucidated. In a previous endodontic outcome study, it was found that male patients were more likely to be associated with preoperative radiolucency than were female patients. The same study showed that presence or absence of a preoperative radiolucency was the most significant predictor for success in initial root canal therapy (Marquis 2006.) One could potentially make the conclusion that males, especially those with preoperative apical periodontitis,

could have a lower success rate in endodontics. A direct comparison for gender and success was not shown in this study, so other factors could come into play. Male patients may wait longer to seek treatment where females could tend to take care of tooth problems sooner. In one large population study, it was shown that males had a significantly lower success rate of initial root canal treatment when compared to females. Male success was 84% where root canals in females had a success rate of 90%. It was also noted that association with apical periodontitis decreased the success rate (Swartz 1983.) Few endodontic studies mention a difference in healing that is associated with gender. Based upon findings in this study, further investigation is necessary to determine whether sexual dimorphism associated with periapical inflammation is clinically significant.

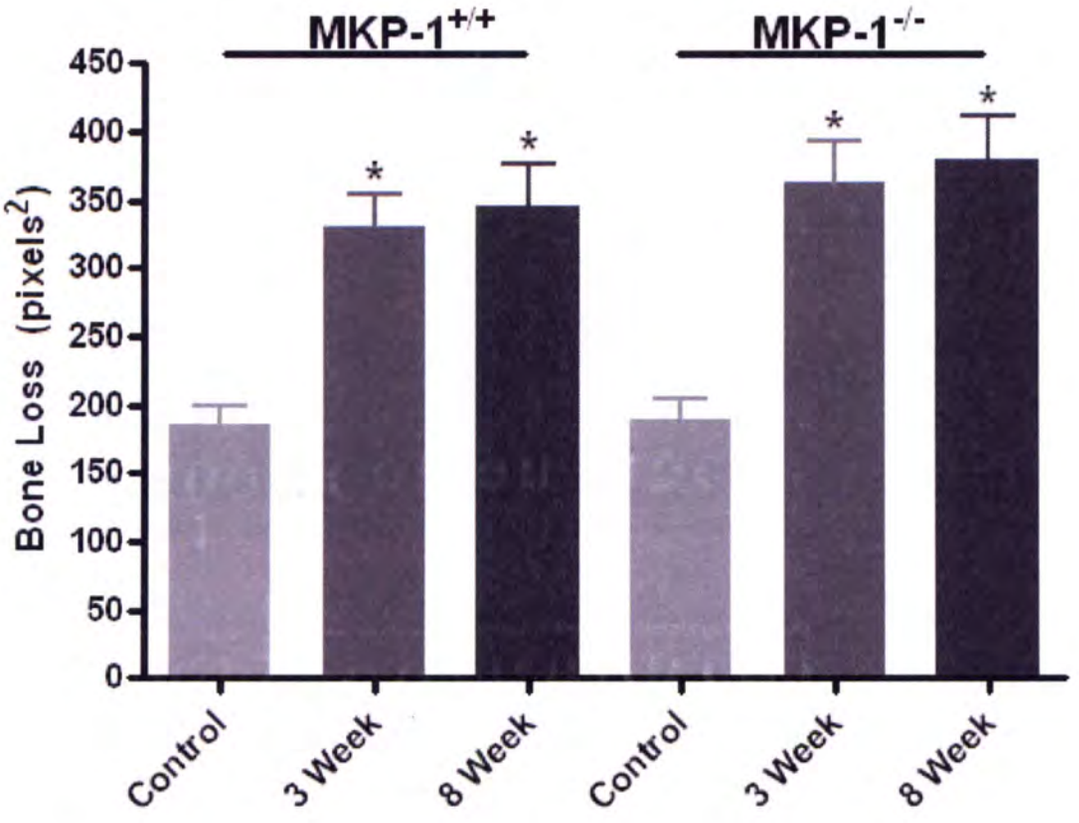
Future Work

With samples that have already been obtained several follow-up studies could be performed. Unstained histological sections could be used for immunohistochemical (IHC) staining of cytokines present in the periapical area. IHC can be performed on previously collected samples to determine levels and types of cytokines present at different time periods. The presence of IL-1 and TNF would indicate active inflammation and bone resorption. Other cytokines, such as IL-8 and monocyte chemoattractant peptide are produced early in the inflammatory process and are important in regulation. Flow cytometry could also be implemented in determining the hematopoietic population of immune responsive cells and pre-osteoclastic cells. Brown and Brenn staining could be

performed on slides already obtained. This stain will allow recognition of gram positive or gram negative bacteria present in the sample. Along the lines of microbiology, in a future study using the same model, bacteriological samples could be obtained from the mouse's open root canal system to determine what species are present in the disease process. In this experiment, the species of bacteria were not important, but future studies could use germ-free mice that receive bacteria injections in different combinations. In this way, the effect of varying bacteria on MKP-1 could be determined.

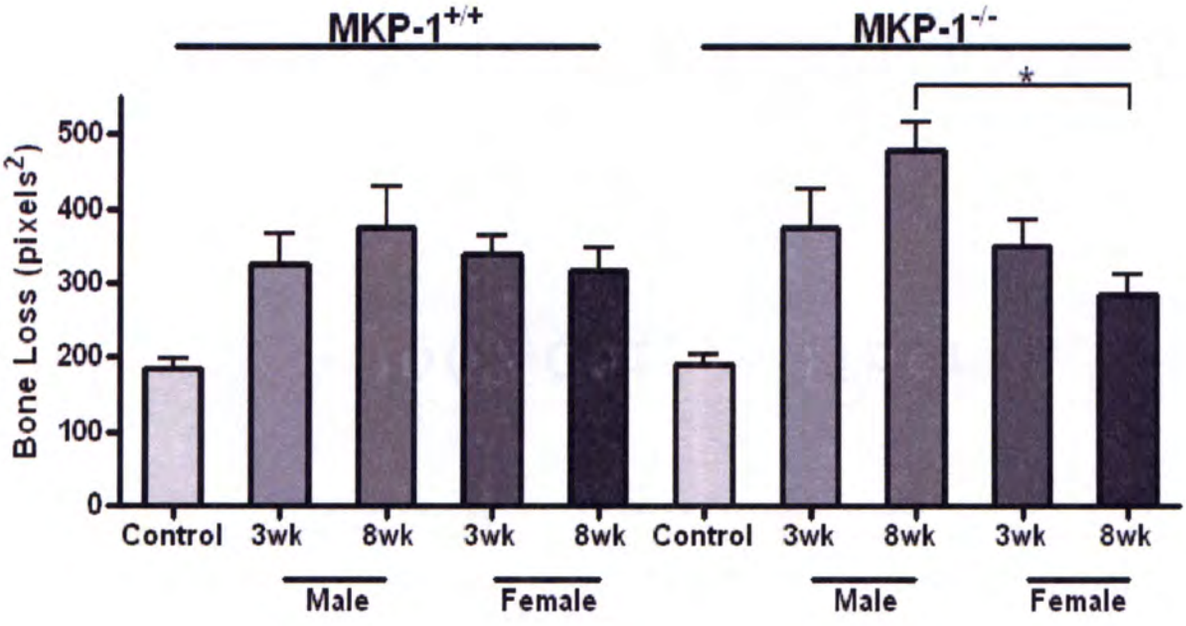
In the future, the issue of gender could be addressed several different ways. Overiectomized mice could be used to remove the possible confounding affect of estrogen in females. Sex hormones play a crucial role in development of the immune response and removal of estrogen along with other hormones could affect periapical disease. Sex linked genes also play a role in the immune response of these animals as different genes can affect expression of inflammatory cytokines involved in disease. A microarray could be established to determine which sex linked cytokines are expressed in male versus female. This could be used to determine which genes may cause any differences in periapical inflammation.

Figure 1



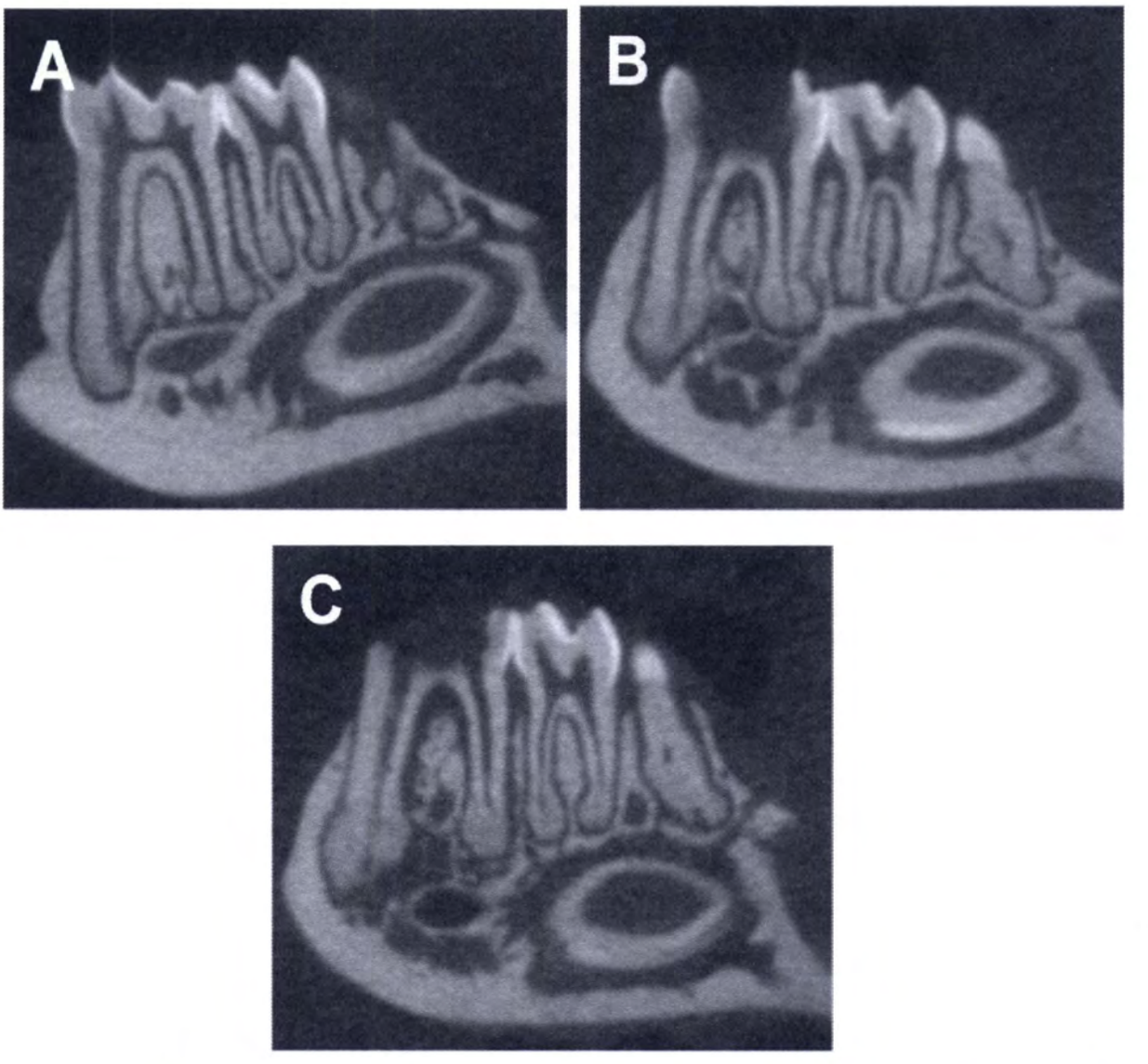
Microcomputed tomography reveals significant periapical bone loss for all experimental groups compared to control. Mean periapical bone loss area as determined by μCT analysis from control and 3-and 8-week exposed molar groups in wild type and MKP-1 deficient mice. (N= 10/group; *P<.05)

Figure 2



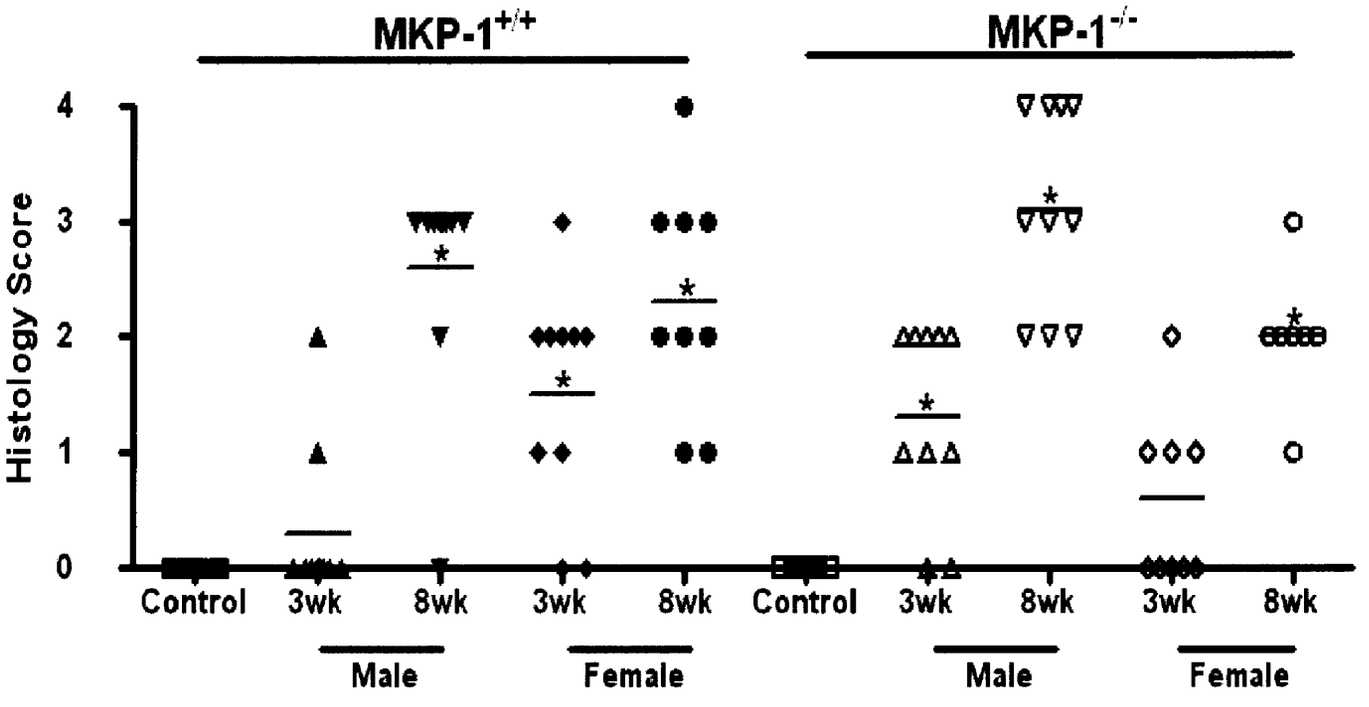
Microcomputed tomography indicates significant periapical bone loss from *Dusp-1*$^{-/-}$/MKP-1$^{-/-}$ male mice. Average periapical bone loss separated by gender. (N= 10/group; *P<.05)

Figure 3



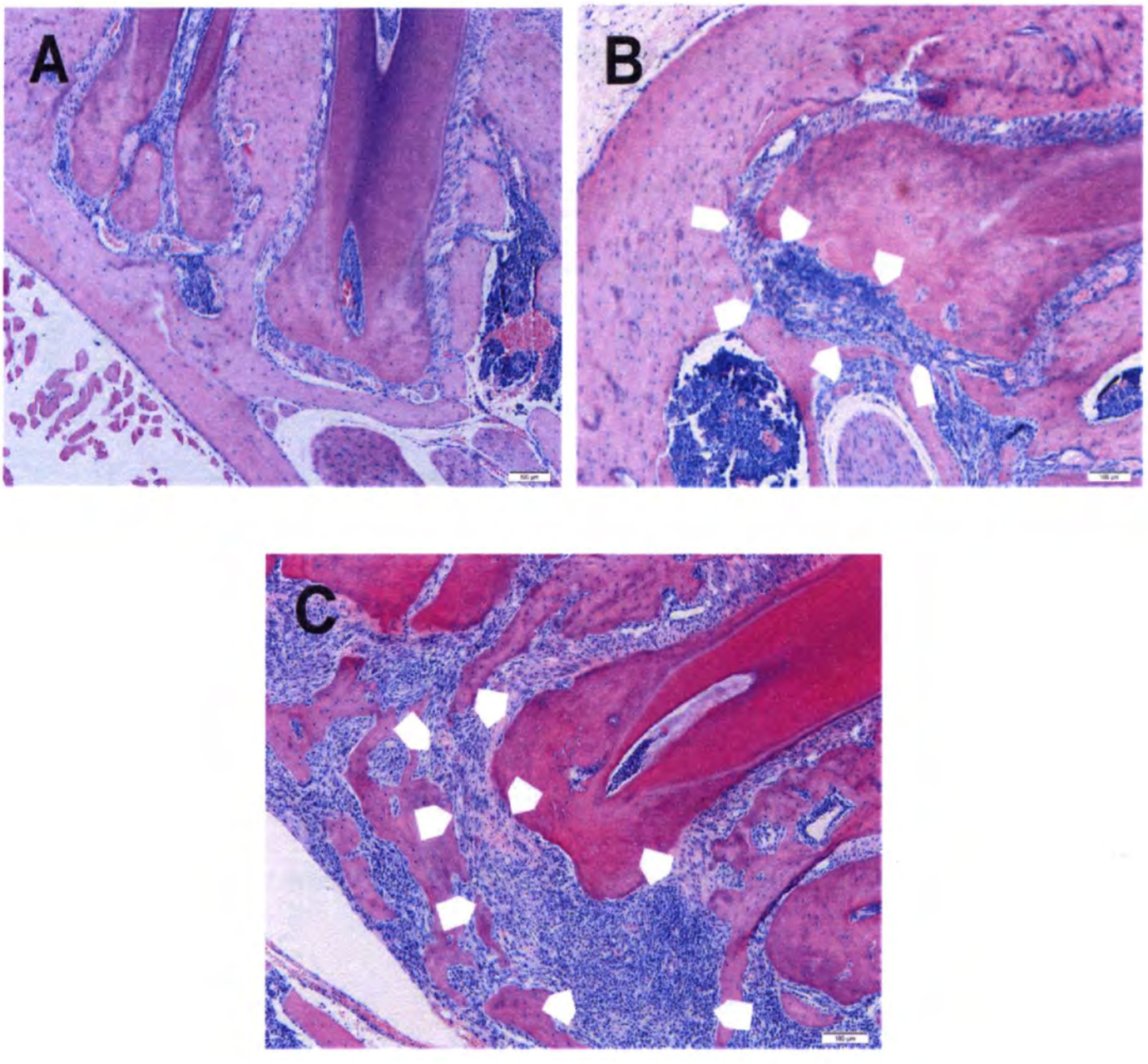
Representative μCT splines of control group (*A*), 3-week treatment group (*B*), and 8-week treatment group (*C*). All μCT images are from MKP-$1^{-/-}$ male mice.

Figure 4



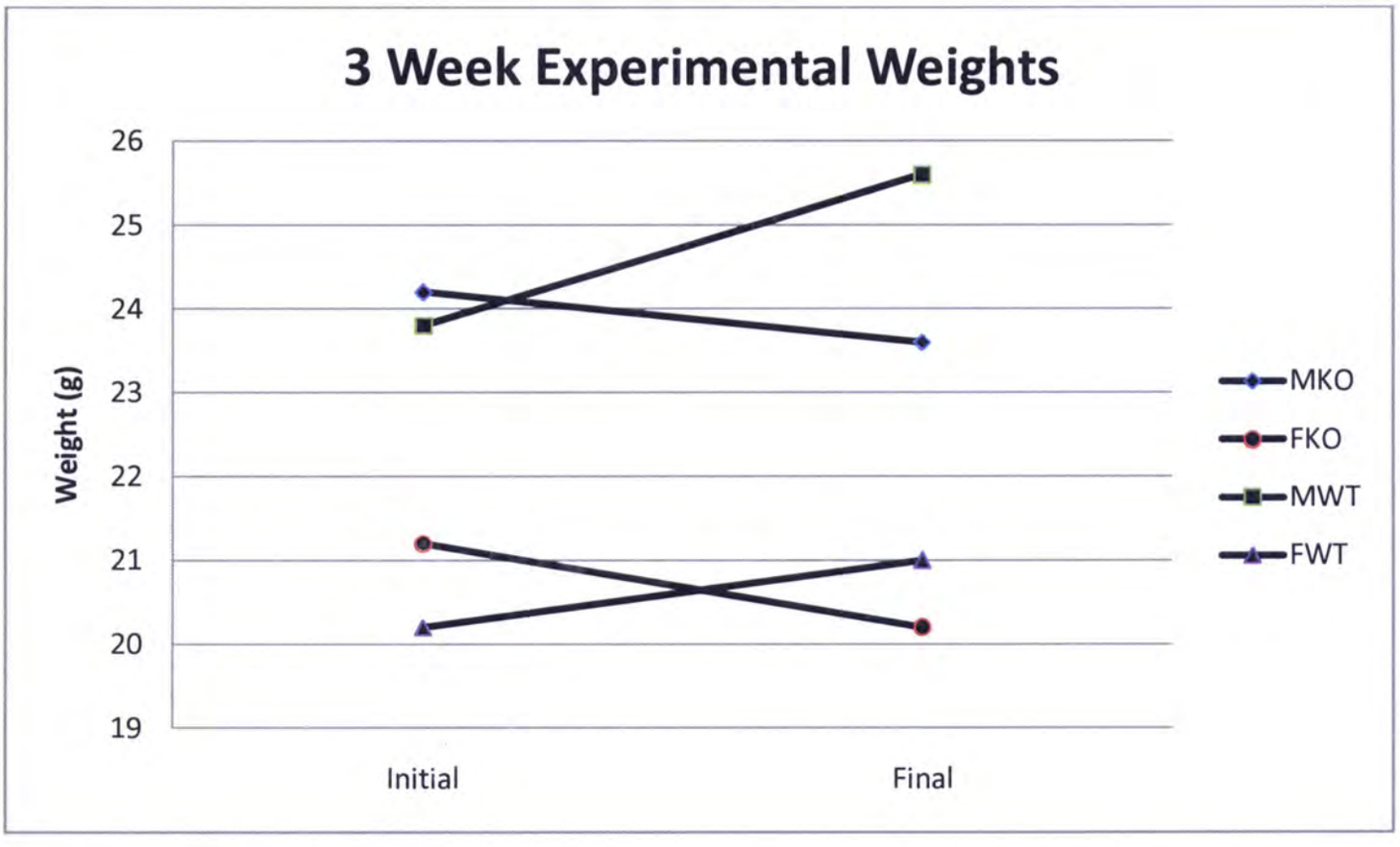
Histopathology indicates that male MKP-1$^{-/-}$ male mice have increased neutrophil infiltrate. Scatter plot analysis with mean histopathologic scoring of neutrophil infiltrate indicated by horizontal line from control and 3- and 8-week treated groups in wild type and MKP-1$^{-/-}$ mice. (N= 10/group; *P<.05)

Figure 5



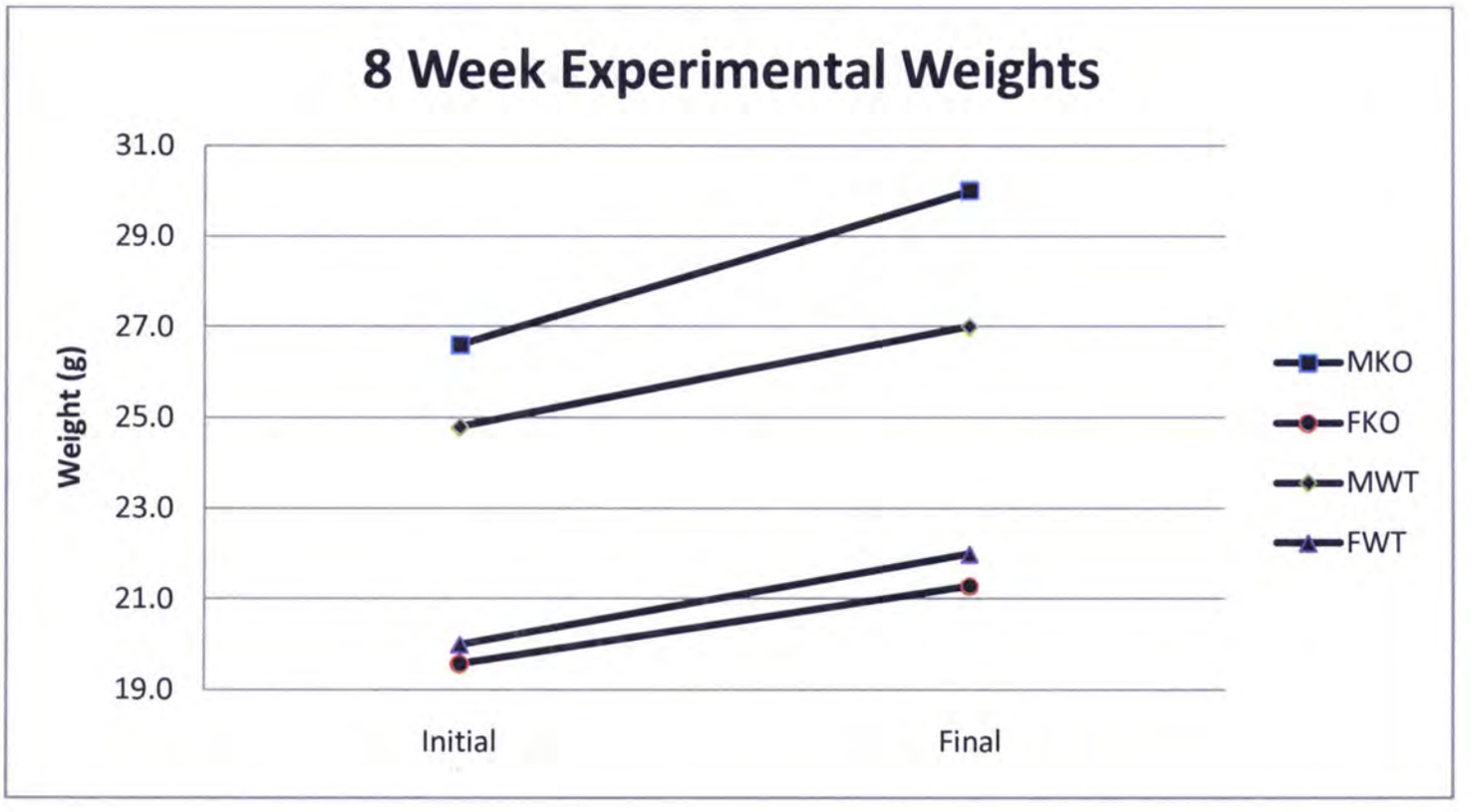
Representative H&E slides corresponding to periapical areas used for histopathologic analysis of control group (*B*), 3-week treatment group (*C*), and 8-week treatment group (*D*). All histological images are from MKP-$1^{-/-}$ male mice. *Arrowheads* indicate neutrophil infiltrate; scale bar is 100μM.

Figure 6



Average weight of mice over experimental period. Results show that mice maintained healthy weight in relation to start weight over 3 weeks.

Figure 7



Average weight of mice over experimental period. Results show that mice maintained healthy weight in relation to start weight over 8 weeks.

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