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Prevention of Experimental Periodontitis in Rats with a Simvastatin Prodrug

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PREVENTION OF EXPERIMENTAL PERIODONTITIS IN RATS WITH A SIMVASTATIN PRODRUG

by

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PREVENTION OF EXPERIMENTAL PERIODONTITIS IN RATS

WITH A SIMVASTATIN PRODRUG

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Introduction: Simvastatin (SIM) is a hypolipidemic drug that has been shown to have anti-inflammatory and bone anabolic properties. The aim of this study was to examine the effects of novel locally-applied SIM conjugated with methoxypolyethylene glycol to form micelles (SIM/SIM-mPEG) in an experimental periodontitis model in rats using micro-computed tomography (μ-CT) and histologic evaluation.

Methods: Experimental periodontitis was induced using silk ligatures around the maxillary right second molar (M2) in 40 Sprague-Dawley rats. Rats were divided into five groups using SIM/SIM-mPEG at 0.5, 1.0, or 1.5 mg SIM doses, mPEG-alone or no drug (ligature-alone) injected into the palatal M1-M2 gingiva at baseline and 1- and 2-weeks post ligature-placement. Rats were euthanized three weeks after baseline and linear measurements were taken using μ-CT from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) between M1-M2. Using hematoxylin and eosin histology, cell counts and area of inflammation were recorded in the connective tissue (CT) of the papilla between M1-M2 interproximally. One-way ANOVA and Pearson correlations were calculated.

Results: All three doses of the micelle resulted in significantly less bone loss compared to the ligature-alone group ($p \leq 0.007$). Significantly greater percentages

of lymphocytes were found only in the ligature-alone and mPEG groups compared to contralateral controls ($p \leq 0.05$). 1.0 and 1.5 mg SIM/SIM-mPEG groups showed significantly more uninflamed CT than all other treatment groups ($p \leq 0.05$).

Conclusions: SIM/SIM-mPEG significantly decreased the amount of bone loss and inflamed tissue in experimental periodontitis near the injection site.

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LIST OF ABBREVIATIONS

% INF	ratio of the area of inflammatory infiltrate to the total area of connective tissue above the alveolar bone crest
μ-CT	micro-computed tomography
ABC	alveolar bone crest
ANOVA	analysis of variance
BMP	bone morphogenic protein
CAL	clinical attachment level
CEJ	cemento-enamel junction
CO_2	carbon dioxide
CT	connective tissue
ELVIS	extravasation through leaky vasculature and inflammatory cell-mediated sequestration
FDA	Food and Drug Administration
FO	flap and osseous surgery
H&E	hematoxylin and eosin histology
HMG-CoA	3-hydroxyl-3-methylglutaryl-coenzyme A
IACUC	Institutional Animal Care and Use Committee
ICC	intra-class correlation coefficient
IL	interleukin
LPS	lipopolysaccharide
M1	maxillary 1st molar
M2	maxillary 2nd molar

M-CSF	macrophage colony stimulating factor
MMP	matrix metalloproteinase
mPEG	methoxy polyethylene glycol
MPO	myeloperoxidase
MW	modified Widman surgery
NHANES	National Health and Nutrition Examination Survey
NSAIDs	non-steroidal anti-inflammatory drugs
OPG	osteoprotegrin
OVX	ovariectomized
PD	probing depth
PGE_2	prostaglandin E_2
PMN	polymorphonuclear leukocyte
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
SDD	subantimicrobial dose doxycycline (Periostat®)
SEM	standard error of the mean
SIM	simvastatin
Smad3	mothers against decapentaplegic homolog 3
SRP	scaling and root planning
TGF-β	transforming growth factor-β
UNMC	University of Nebraska Medical Center

CHAPTER 1: INTRODUCTION

Periodontitis is an inflammatory disease characterized by the loss of the supporting tissues of the teeth. This disease is one of the primary causes of permanent tooth loss in man (Albandar et al. 1999). Based on data collected as part of the Center for Disease Control's 2009-2010 National Health and Nutrition Examination Survey (NHANES), periodontitis affects approximately 47.2% of the adult population in the United States, equivalent to 64.7 million people (Genco et al. 2012). The primary etiological factor in the initiation of periodontitis is the bacterial biofilm collecting between the root and sulcular or pocket epithelium. However, the periodontopathic bacterial flora is necessary, but not sufficient in and of itself for disease activity to occur. It is the initiation of the individual host's inflammatory response to the bacterial challenge that leads to the destruction of the supporting tissues of the teeth (Offenbacher 1996).

Most periodontopathic bacteria are gram-negative anaerobic rods and spirochetes (Thelaide 1986). The bacteria invade the host connective tissue (Saglie & Elbaz 1983), where a massive accumulation of plasma cells and lymphocytes are observed (Allenspach-Petrzilka & Guggenheim 1983). These cells, among others, release a myriad of pro- and anti-inflammatory cytokines. These cytokines prove to be both protective and destructive to the host's tissues. When left untreated, this process can become primarily pro-inflammatory and lead to increases in exudate and probing depths, the apical migration of the junctional epithelium, gingival recession, and the loss of alveolar bone and attachment (Schroeder & Lindhe 1975).

Traditional treatment of periodontitis has been aimed at reducing the levels and proportions of periodontal pathogens and increasing the proportions of beneficial species through both surgical and non-surgical methods (Socransky & Haffajee 2002). Scaling and root planing is the non-surgically based therapy with the goal of removing biofilm plaque and calculus from periodontal pockets and smoothing the tooth root to remove bacterial toxins, thereby reducing the host-inflammatory response. Surgical methods of treatment are aimed at both reducing the bacterial load and recontouring or regenerating bone to proper physiologic form.

Due to the difficulty in adequately reducing bacteria using mechanical means alone, antimicrobial agents may be used as adjunctive therapy. Systemic antibiotics have been shown to provide beneficial effects as adjunctive therapy in the treatment of chronic and aggressive periodontitis (Sgolastra et al. 2012; Sgolastra et al. 2012). These agents are taken orally and affect pathogens via multiple routes, such as through accumulation in connective tissue, saliva, and gingival crevicular fluid. Local antibiotics are placed into the periodontal pocket and released over time with the intention of directly affecting periodontal pathogens and the host response at the site(s) of drug delivery only.

Host modulation is another method of treatment in periodontal therapy. This method aims at affecting the host's response to the bacterial challenge, specifically through agents that inhibit inflammation or affect bone metabolism, thereby decreasing the progression of the disease. These agents include non-steroidal anti-inflammatory drugs, subantimicrobial dose doxycycline (the only

FDA-approved drug for treating periodontitis; Caton et al. 2000), omega-3-fatty acids, bisphosphonates, bone morphogenic proteins (BMPs), growth factors, and statins.

Statins are a class of drugs that have gained research interest in periodontal treatment recently. Simvastatin (SIM) is a hypolipidemic drug that has been found to have anti-inflammatory properties (Xu et al. 2012) as well as cause the local up-regulation of bone growth (Stein et al. 2005). Despite its findings to be bone anabolic, however, its lack of skeletal specificity and low water-solubility via the systemic route have prevented its clinical application (Jia et al. 2015). To address these issues, researchers at the University of Nebraska Medical Center have designed and prepared a novel macromolecular prodrug of SIM. The amphiphilic macromolecule was prepared by "clicking" an alkyne containing SIM trimer with an azido polyethylene glycol monomethylether (mPEG), thus creating the product SIM-mPEG. SIM-mPEG can self-assemble into polymeric micelles in water, and free SIM can be easily incorporated into the hydrophobic cores of SIM-mPEG micelles to produce a SIM/SIM-mPEG micelle.

A recent study (Jia et al. 2015) found that the SIM/SIM-mPEG micelle could boost the differentiation and proliferation of preosteoblasts. In addition, the micelles were used systemically to treat femoral fractures in mice and resulted in enhanced callus formation, calcification, and organization. The study proved that the SIM/SIM-mPEG micelle formulation selectively localized to the fracture site and was internalized and retained by inflammatory cells present via the extravasation through leaky vasculature and inflammatory cell-mediated sequestration (ELVIS)

mechanism during the initial inflammatory phase of fracture repair, where it exerted a potent and locally sustained bone anabolic effect. The effects of this prodrug in treating periodontitis are unknown, and the hypothesis was formulated that SIM/SIM-mPEG would have bone preservation and anti-inflammatory effects in experimental periodontitis. The aim of this study was to examine the effects of locally-delivered SIM/SIM-mPEG in an experimental periodontitis model in rats using micro-computed tomography (μ-CT) and histologic evaluation.

CHAPTER 2: LITERATURE REVIEW

Alveolar Bone Resorption

Resorption of the alveolar bone in periodontitis is dependent on osteoclastogenesis from hematoprogenitor cells. An essential molecule in osteoclastogenesis is receptor activator of nuclear factor kappa-B ligand (RANKL) (McCauley & Nohutcu 2002). Other mediators playing a role in bone resorption include IL-1, IL-6, TNF- α, PGE_2, and macrophage colony stimulating factor (M-CSF). RANKL is expressed on the cell surface of osteoblasts, stromal cells, or fibroblasts or is secreted as a soluble ligand (Belibasakis & Bostanci 2012). RANKL activates its cognate RANK receptor on the surface of pre-osteoclasts and triggers their differentiation into mature osteoclasts. Osteoprotegrin (OPG) is a soluble decoy receptor for RANKL. When OPG is present to bind RANKL, the cell-to-cell signaling between osteoblasts and osteoclast precursors is inhibited and osteoclasts are unable to differentiate from their precursors (Mogi et al. 2004). In chronic periodontitis the RANKL/OPG ratio demonstrates a 2.2-fold increase compared to health, accounting for the alveolar bone resorption seen during disease activity (Belibasakis & Bostanci 2012).

Treatment of Periodontitis

Traditional treatment of periodontitis has been aimed at reducing the levels and proportions of periodontal pathogens and increasing the proportions of beneficial species through both surgical and non-surgical methods (Socransky & Haffajee 2002). Scaling and root planning (SRP) is the nonsurgical-based therapy

with the goal of removing plaque and calculus from periodontal pockets and smoothing the tooth root to remove bacterial toxins, thereby reducing the host-inflammatory response. Cobb (2002) reported decreases in the probing depth (PD) and gains in the clinical attachment level (CAL) following therapy. For 1-3 mm pockets treated with SRP a mean 0.03 mm reduction in PD and a mean net loss of CAL of 0.34 mm can be expected, for 4-6 mm pockets a mean 1.29 mm PD reduction and a mean net gain of CAL of 0.55 mm can be expected, and for pockets 7 mm in depth or greater a 2.16 mm PD reduction and a mean net gain in CAL of 1.19 mm can be expected.

When bone morphology or the persistence of deep pockets following non-surgical therapy continues to promote a state of disease in an individual, the patient may be entered into a surgical phase of therapy. These surgical methods of treatment are aimed at both reducing the bacterial load and recontouring or regenerating bone to a proper physiologic form. In a long-term evaluation of periodontal therapy, Kaldahl et al. (1996) reported that flap and osseous surgery (FO) produced the greatest PD reduction in deep (>5 mm) sites when compared to coronal scaling, SRP, and modified Widman surgery (MW). It was also reported that similar CAL gains over time were produced in sites with an initial PD of 7 mm or greater treated with SRP, MW, and FO. The authors concluded that both non-surgical and surgical periodontal therapy greatly improves the clinical parameters of periodontal disease and sustains them long term.

Host Modulation

In addition to mechanical removal of bacteria with SRP and surgery, host modulating agents aim to affect the host response-aspect of the disease process. These agents inhibit inflammation or alter bone metabolism, decreasing the progression of the disease and include non-steroidal anti-inflammatory drugs (NSAIDs), subantimicrobial dose doxycycline (SDD), and statins.

NSAIDs have been used as an adjunct to periodontal therapy on the basis of inhibiting cyclooxygenase and thus prostaglanding synthesis, including PGE_2. A clinical trial reported that long-term use of NSAIDs was shown to decrease alveolar bone loss in patients with periodontitis (Williams et al. 1989). One of the disadvantages of long-term NSAID use is potential side effects to the liver, kidney, and gastrointestinal system. In a review on the effects of NSAIDs, Salvi & Lang (2005) concluded that although some studies present promising results, no data from long-term, multicenter prospective clinical trials are available for determining whether these therapeutic effects can be retained on a long-term basis.

Periostat®, or SDD, is available in a 20 mg formulation and has been FDA approved in the treatment of chronic periodontitis (Golub 2001). The regimen consists of 20 mg doxycycline taken twice daily for a duration of three to nine months. The clinical efficacy of SDD has been shown to be due, in part, to its ability to inhibit collagenolytic matrix metalloproteinases (MMPs) in gingival tissues and fluid, specifically MMP-8 and MMP-13 (Golub et al. 1997). It has been proven that long-term treatment with SDD exerts no antibacterial effect on the subgingival microflora associated with chronic periodontitis (Walker et al. 2000). The

adjunctive use of SDD with SRP has been shown to be more effective than SRP alone in terms of gain in CAL and reduction in PDs in patients with chronic periodontitis (Caton et al. 2000). The major disadvantages to SDD therapy are related to patient compliance and the cost associated with prolonged dosage scheduling.

Statins

Statins are a class of drugs that have gained research interest in the treatment of periodontal disease. These drugs are prescribed for the treatment of cardiovascular disease, specifically to suppress cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the cholesterol metabolism pathway. In addition to the cholesterol-lowering effect, statins have a series of pleiotropic effects, including bone anabolic and anti-inflammatory properties (Mundy 2001). The bone anabolic properties associated with statins can be summarized into three major mechanisms: the promotion of osteogenesis, the suppression of osteoblast apoptosis, and the inhibition of osteoclastogenesis (Zhang et al. 2014). Statins' promotion of osteognesis relates to their ability to stimulate the expression of BMP-2, their anti-osteoblast apoptosis effects relate to their role in altering the transforming growth factor-beta (TGF-β)/mothers against decapentaplegic homolog 3 (Smad3) signaling pathway, and their anti-osteoclastic effect seems to be due to their effect on the OPG/RANKL/RANK signaling pathway. Locally-applied BMP-2 has been used to stimulate craniofacial bone formation (Triplett et al. 2009), but it is not approved specifically for periodontitis and costs/morbidity may be prohibitive. Although these effects would be beneficial in the treatment of bone-catabolic diseases such as

osteoporosis or periodontitis, their lack of skeletal specificity, low water-solubility, and first pass effect that takes place in the liver has prevented their clinical application. Systematic reviews of human studies of orally administered statins and bone effects over the past 10 years generally have come to the conclusion that their impact is modest (Zhang et al. 2014).

Simvastatin

Simvastatin (SIM) is a statin formulation that has been studied in the treatment of periodontitis. In a study (Vaziri et al. 2007) where SIM was injected subperiosteally following ligature-induced bone-resorption around mandibular teeth in ovariectomized rats, simvastatin groups developed significantly less periodontal breakdown. Another study (Seto et al. 2008) using ligatures around maxillary molars of rats to induce periodontitis found that local SIM topically injected into periodontal defects recovered the ligature-induced alveolar bone resorption, showing a 46% reversal of bone height loss. Another study (Price et al. 2013) used a SIM/alendronate-β-cyclodextrin complex that was injected prior to the induction of experimental periodontitis in rats. Results showed that this SIM complex has the potential to prevent episodes of periodontitis bone loss. The authors also noted that both the increase in osteoclasts and subsulcular inflammation seen in the experimental periodontitis model were reduced when preceded by treatment with the SIM complex. However, Powell (2011) found that neither prophylactic nor therapeutic local injections of SIM provided beneficial effects on alveolar bone or CT attachment levels in the treatment of lipopolysaccharide (LPS) injection-induced experimental periodontitis in rats.

A limited number of human clinical studies have also reported on the effect of simvastatin in the treatment of periodontitis. In a study of 60 patients receiving SRP plus either a locally delivered placebo or SIM, there was a greater decrease in gingival inflammation and significantly more intrabony defect fill at sites treated with SRP plus SIM (Pradeep & Thorat 2010). Another similarly designed study by the same group examining SIM's effect in treating molar Class II furcation defects in 72 patients resulted in a similar reduction in gingival inflammation and greater mean percentage of bone fill in SIM-treated subjects (Pradeep et al. 2012). The authors concluded that locally delivered SIM provides a comfortable and flexible method to improve clinical parameters and also enhance bone formation. Also, 38 subjects with type 2 diabetes similarly treated with SIM resulted in the same inflammation reduction with significant intrabony defect fill at sites treated with SRP plus locally delivered SIM compared to SRP alone (Pradeep et al. 2013). While the human trials above are supportive of the use of statin treatment of periodontitis and other craniofacial defects, Zhang et al. (2014) states that future confirmation of these results by other investigators is crucial.

SIM/SIM-mPEG

To address statins' lack of skeletal specificity, low water solubility, and first pass effects, investigators at the University of Nebraska Medical Center College of Pharmacy have developed a novel macromolecular prodrug of SIM designed and prepared by chemically conjugating multiple SIM molecules to the chain terminus of methoxy polyethylene glycol (mPEG). This amphiphilic macromolecular prodrug (SIM-mPEG) can be further formulated into micelles loaded with free SIM (SIM/SIM-

mPEG). When tested to examine the effects of intravenously administered SIM/SIM-mPEG on femoral fractures in mice, the results showed that the SIM/SIM-mPEG-treated mice exhibited a potent and locally sustained bone anabolic effect with consolidated calcified callus formation, while control and conventional SIM-treated mice were found with foci of immature callus with relatively low density (Jia et al. 2015). The micro-computed tomography (μ-CT) analysis suggested that the SIM/SIM-mPEG formulation enhanced callus formation, calcification, and organization.

Experimental Periodontitis Models

It has been demonstrated that the structure and organization of the periodontal tissues of the molars in rats are similar to those of humans (Listgarten 1975). However, rats are not susceptible to the development of natural periodontitis and periodontal destruction must be induced experimentally (Pellegrini et al. 2009). The most commonly used methods to induce periodontitis include the placement of ligatures into the sulci around teeth or the injection of LPS directly into the periodontium. Currently there is no universally accepted, proven method that acts as a standard in the creation of an experimental periodontitis defect in rats.

The direct injection of LPS into the gingiva has been reported to induce biochemical changes that parallel natural adult periodontitis. Ramamurthy et al. (2002) reported that 10 μL of *Escherichia coli* LPS (1 mg/mL) given through three injections (every other day for five days) resulted in a loss of alveolar bone in a short time frame. Dumitrescu et al. (2004) reported that the tightly bound tissue of

the gingiva in rats would not expand to accommodate an injection of 10 μg LPS in a volume of 10 μL saline and much of the injected volume was lost along the needle tract. To adjust for this, the authors reported that 10 μg LPS in a volume of 1 μL saline resulted in significant gingival and periodontal inflammation with inflammatory infiltrate, apical migration of the JE, interdental bone loss, and activation of osteoclasts at the site of injection. Price et al. (2013) demonstrated that 10 μL of *E coli* LPS injected into the palatal/interproximal gingiva of rat molars resulted in a reduction of bone volume, density, and thickness. These LPS injections also gave histologic evidence of increased osteoclasts and subsulcular inflammation.

The ligature model of experimental periodontitis is considered to be more representative of periodontitis in humans mainly because of the participation of live microorganisms with diverse virulent factors other than LPS. This greater diversity of antigens may result in a more complex host response, which may have an effect on the cytokine and inflammatory mediator network (de Aquino et al. 2009). However, the physical pressure of the ligature may induce mechanical stripping of periodontal attachment, not consistent with naturally occurring periodontitis. It has been reported (Spolidorio et al. 2014) that the use of a ligature around the maxillary 2nd molar results in a significant increase in IL-1β, TNF-α, and IL-6 levels at days 8 and 15 in the gingival tissues surrounding ligated teeth compared to non-ligated teeth. Another study (Furlaneto et al. 2014) where ligatures were placed around maxillary 2nd molars reported greater alveolar bone loss in experimental periodontitis groups at buccal, palatal, furcation, and interproximal sites compared

to control groups with attachment loss and a moderate-to-severe inflammatory infiltrate.

Micro-computed Tomography

μ-CT is a three-dimensional (3-D) reconstruction technique that can image specimens on the micron level and allow for computer-aided reorientation following image scanning (Park et al. 2007). Specifically, this method allows for the production of 3-D images of mineralized tissue (bone, teeth) to analyze the architecture and slight deviations in alveolar bone loss (Mengel et al. 2005). Due to the ability to maintain the tissues during and after μ-CT, histology can be performed later. μ-CT has been used to analyze alveolar bone loss in ligature-induced periodontitis in rats (Furlaneto et al. 2014, Spolidorio et al. 2014), and results obtained from μ-CT were found to be virtually identical to those obtained from histology in the analysis of periapical bone loss in mice (Balto et al. 2000).

CHAPTER 3: MATERIALS AND METHODS

Pilot Study

The hypothesis was that the ligatures would attract bacteria and allow build-up in the gingival sulcus. In addition, ligatures would also help create inflammation and bone loss and allow for an increased volume of LPS (and/or experimental drug in the main study) to be injected into the periodontium to further produce inflammation. The aim of the pilot study was to determine the most effective method to induce experimental periodontitis, specifically testing whether ligatures alone, LPS injections alone, or a combination of the two methods produce the most periodontitis-like bone loss in rats. The most destructive model, measured by alveolar bone loss, was desired for use in the core study.

Three groups of five mature Sprague-Dawley female rats were used for the pilot study. All animals were treated and housed in the University of Nebraska Medical Center (UNMC) College of Dentistry Animal Facility under the auspices of the UNMC Institutional Animal Care and Use Committee (IACUC #13-006-03-FC). These rats were allowed to acclimate one week prior to the first procedure. Rats were divided into groups as shown in Table 1. Groups 1 and 3 had 4-0 silk ligatures tied around the maxillary right 1st molars at the initiation of the study. *E coli* LPS injections (10 μg in a volume of 1 μL saline) were delivered to the distopalatal aspect of the maxillary right first molar (M1) every other day for five days during the first week for Group 2, and during the second week for Group 3 (following ligature placement). The contralateral maxillary first molars served as untreated

controls. All animals were euthanized via CO_2 asphyxiation on the first day of the second week following the final treatment of experimental periodontitis induction—week three for groups 1 and 2, and week four for Group 3. The palates with all three bilateral maxillary molars and their associated periodontium were removed via block resection and stored in 10% formalin. Specimens were scanned and analyzed with μ-CT using methods described in the core study. The sole purpose of using μ-CT in the pilot was to become familiar with the methods for the core study. Sections were then prepared for hematoxylin and eosin (H&E) histology using methods described in the core study. Using both μ-CT and histology, the amount of bone loss was determined using a linear measurement from the CEJ to the alveolar bone crest (ABC) at the distopalatal aspect of M1, with the histologic measurements used as the primary outcome. In addition, inflammation was categorized using a scoring system described by Coimbra et al. (2011): 0, no inflammatory cells; 1, slight inflammation (few inflammatory cells); 2, moderate inflammation (remarkable inflammatory cells scattered throughout the connective tissue above the bone crest); 3, severe inflammation (predominance of inflammatory cells). To assess differences of measurements among groups one-way ANOVA was completed.

Core Study

The core study consisted of 40 mature retired-breeder female Sprague Dawley rats (Harlan Teklad, Madison, WI). All animals were treated and housed in the University of Nebraska Medical Center (UNMC) College of Dentistry Animal Facility under the auspices of the UNMC Institutional Animal Care and Use

Committee (IACUC #13-006-03-FC). These rats were allowed to acclimate one week prior to the first procedure.

Synthesis of SIM/SIM-mPEG

The SIM/SIM-mPEG micelles were suspended in a PBS solution as a releasing medium. Synthesis of the micelle has been described previously (Jia et al. 2015, Figure 1). Briefly, the dendritic amphiphilic SIM prodrug was designed by conjugating a cluster of three SIMs covalently to the chain terminus of mPEG. In designing the hydrophobic SIM trimer block, an ester bond was selected as the chemical linkage as it can not only hold the three SIMs together, but also can be hydrolyzed in vivo by esterases to release the conjugated SIM. Of the 3 SIMs conjugated to mPEG, two are in the prodrug form with an intact lactone ring and the other SIM is in the form of simvastatin acid. When exposed to in vivo environmental factors (esterases, water, acidity, elevated temperature, etc.), the lactone ring will open to produce the active form of the drug simvastatin acid. Therefore, all of the drug released will be in the bioactive form. Click chemistry was then used to conjugate the SIM trimer to the mPEG terminus to form the amphiphilic macromolecule, which self assembles into micelles. Because of the structural similarity between SIM and the SIM trimer, free SIM can be incorporated into the hydrophobic core to form SIM/SIM-mPEG micelles, which permits additional drug-loading capacity.

Anesthesia

All animals were weighed following anesthesia induction and prior to all procedures to monitor weight gain/loss. Each animal was initially placed into an induction chamber attached to an isofluorane anesthetic vaporizer and anesthesia was induced to effect with 1-4% isofluorane/100% O_2 (1-3 L/min), followed by application of a nose cone with 0.5-2% isofluorane/100% O_2 (0.5-1 L/min) to maintain anesthesia during the experimental procedures. Following injections and anesthesia, rats were monitored until awake and normal movement was noted (hourly for the entire day).

Experimental Periodontitis and Experimental Groups

Experimental periodontitis was induced using 4-0 silk ligatures around the maxillary right second molar in all 40 Sprague-Dawley rats (Figure 2). Ligatures were selected because a pilot study determined experimental periodontitis to be induced most effectively using ligatures compared to *Escherichia coli* lipopolysaccharide (LPS) injections alone as well as *E coli* LPS injections in combinations with ligatures. Rats were divided into five groups of eight rats each as outlined in Table 2. The local injections of the drug or carrier were delivered to the palatal gingiva between the maxillary right first (M1) and second molars (M2) using an insulin syringe with a 30-gauge needle (Becton Dickinson, Franklin Lakes, NJ) (Figure 3). All ligatures were removed after one week and injections were delivered at baseline following ligature placement (week one) and on the first day of weeks two and three. The contralateral maxillary molars received no treatment and

served as untreated controls of the preceding treatment group (Groups 2, 4, 6, 8, & 10).

Euthanasia

All animals were euthanized on the first day of week four by CO_2 asphyxiation. Animals were weighed following euthanasia and the maxilla was then separated from the rest of the skull. The entire palate was placed in 10% formalin for storage prior to scanning with the μ-CT.

Micro-computed Tomography Measurements

The palates were scanned using a high-resolution μ-CT system (Skyscan 1172;Skyscan, Kontich, Belgium). Each maxilla was scanned and reconstructed into a 3D-structure with a pixel size of 8.71 μm. The X-ray tube voltage was 70 kV and the current was 141 μA, with a 0.5 mm thick aluminum filter. Exposure time was 580 ms. The X-ray projections were obtained at 0.7° intervals with a scanning angular rotation of 180° and eight frames were averaged for each rotation. 3D reconstructions were performed using NRecon software. Using Data Viewer (v. 1.5.0, Bruker), the generated 3D models were rotated into a standard position according to the following criteria: 1) in the transaxial plane, the maxillary first molar had its axis vertically positioned; 2) in the coronal plane, the roots in cross section were positioned vertically; 3) in the sagittal plane, all teeth were positioned horizontally with the first molar (M1) positioned to the right (Figure 4). The sagittal dataset was analyzed using software (CT-Analyzer, v. 1.13, Bruker) and the distance from the CEJ to the alveolar bone crest (CEJ-ABC) was measured at the distal of M1

(M1D, primary outcome site that was not mechanically affected by the ligature or injections) and mesial and distal aspects of the second molar (M2M & M2D, respectively). The image (Figure 5) where the roots were in an even plane with their respective canal spaces widest mesio-distally was used for measurement and all measurements were performed by one examiner (AB) who was masked to the experimental groups and treatments rendered. Ten percent of the sites were then re-coded and re-measured at random to evaluate intra-examiner reliability.

Histology

All specimens were decalcified in 5% formic acid solution for approximately one month. Following decalcification, the specimens were processed and embedded in paraffin in a conventional manner. Serial sections 5-μm thick were obtained in a mesio-distal direction with the roots aligned in one plane. The sections were stained with hematoxylin and eosin (H&E) for analysis by light microscopy.

In order to characterize the connective tissue (CT) and inflammatory infiltrate, cell counts were completed at the area of interest, specifically in the CT of the papilla between M1-M2 interproximally. This was completed using direct grid-point counting of cells in 100 point intersects under 400× magnification with a light microscope (Nikon Optiphot, Tokyo, Japan). Intersections were identified as containing one of the following: uninflamed collagen, fibroblasts, polymorphonuclear leukocytes (PMNs), lymphocytes, plasma cells, macrophages, endothelial cells/blood vessel lumens, or spaces/other.

Images of the histologic sections were captured at a magnification of 40× with a digital camera (ProgRes C3, JENOPTIK). The images were saved on a

computer and then analyzed using appropriate software (ProgRes CapturePro v. 2.8.8, JENOPTIK). The images were calibrated by linear measurements and the area of inflammatory infiltrate and the total area of CT (collagen and fibroblasts) above the area of alveolar crest interproximally between M1 and M2 were measured, which allowed for a calculation of the ratio of the area of inflammatory infiltrate to the total area of CT above the alveolar bone crest (% INF). In addition, the length of CT attachment to interproximal roots was measured. All measurements and cell counts were performed by one examiner (AB) who was masked to the experimental groups and treatments rendered and 10% of the specimens were confirmed by an oral pathologist (NN).

Statistical Analysis

Eight rats per group were used in this study based on histological detection of bone gain with administration of 0.5 mg SIM injections with 5-8 rats per group (Lee et al. 2008). Each rat was used as a different experimental subject and as the unit of measurement for primary outcomes. The data obtained in the analyses were grouped and presented as means ± standard errors of the mean (SEM). To assess differences of measurements among groups for all measurements, one-way analysis of variance (ANOVA) was completed. Pearson correlations were calculated for associations between the % INF and amount of bone loss. The single measures intra-class correlation coefficient (ICC) was calculated to evaluate the intra-examiner reliability between the μ-CT measurements collected at two different measurement times. The significance level was set at $p \leq 0.05$ in all tests.

CHAPTER 4: RESULTS

Pilot Study

The mean distance from the CEJ to the alveolar crest at the distal of M1 (Figure 6) was 1.174 mm for Group 1 (ligature-alone), 1.105 mm for Group 2 (ligature + LPS), and 0.680 mm for Group 3 (LPS-only). Both of the measurements in Groups 1 and 2 were significantly greater than their contralateral controls (p = 0.03 & 0.006, respectively). The measurement for Group 3 was not significantly different than its contralateral control. Both Groups 1 and 2 caused significantly greater bone loss compared to Group 3 (p = 0.011 & 0.015, respectively). There was no difference in bone loss between Groups 1 and 2. Measurements taken using μ-CT confirmed the histologic measurements (Figure 7).

The scores for subepithelial inflammation for ligature-alone, ligature plus LPS, and LPS-alone groups were 1.75, 2.2, and 1.6, respectively, with no significant differences among groups. The ligature-alone and ligature plus LPS groups both had significantly greater inflammatory scores than their contralateral controls (p = 0.008 & 0.026, respectively), while the difference in the LPS-alone group was not statistically significant (Figure 8).

LPS-alone in the concentration used (10 μg/μL) was not an effective method to induce experimental periodontitis in rats. Both ligatures alone and ligatures plus LPS injections effectively induced experimental periodontitis as determined by interproximal bone loss; however, there was no additional benefit with the addition of LPS in the ligature model. It can be concluded from the pilot study that

experimental periodontitis can be induced effectively with ligatures alone. The hypothesis that ligatures would help create inflammation and bone loss and allow for an increased volume of LPS (and/or experimental drug in the main study) to be injected into the periodontium may be true, but the addition of LPS did not create a more destructive periodontal lesion.

Core Study

All animals' weights averaged greater than 300 grams at the conclusion of the study. The mean changes in weight from the start to the end of the study for all groups were within 10 grams of the starting weights (Figure 9), and these changes were not significantly different among groups (p = 0.1315).

Micro-computed Tomography

At the primary outcome site (M1D), Group 9 (1.5 mg SIM/SIM-mPEG) showed the least amount of bone loss compared to all other groups (Figure 10). The mean distance from the CEJ-ABC for Group 9 was 0.68 mm ± 0.05, for Group 7 (1.0 mg SIM/SIM-mPEG) was 0.75 mm ± 0.06, for Group 5 (0.5 mg SIM/SIM-mPEG) was 0.77 mm ± 0.06, for Group 3 (mPEG) was 0.85 mm ± 0.06, and for Group 1 (ligature-alone) was 1.00 mm ± 0.06. Each group showed significantly greater bone loss than their contralateral untreated controls, and all three increasing doses of SIM/SIM-mPEG showed significantly less bone loss than ligature-alone (p = 0.007, 0.005, & 0.0002, respectively). In addition, 1.5 mg SIM/SIM-mPEG showed significantly less bone loss than mPEG (p = 0.04). Bone loss at M2M and M2D was not significantly different among treatment groups (Figures 11 & 12, respectively). Finally, the

single measures ICC was 0.985 (95% confidence interval: 0.961 to 0.994), indicating a high intra-examiner reproducibility of measurements taken using the μ-CT system (Table 3).

Histology

The length of CT attachment was not significantly different among treatment groups (Figure 13). Each treatment group had a significantly greater length of CT attachment compared to their contralateral controls, except for 1.5 mg SIM/SIM-mPEG.

Each treatment group showed significantly greater areas of inflammation between M1 and M2 compared to their respective contralateral untreated control (Figure 14). Also, 1.0 mg SIM/SIM-mPEG showed a significantly greater area of inflammatory infiltrate compared to ligature-alone and mPEG groups (p = 0.002 & 0.03, respectively). No significant difference was observed among groups for % INF (p = 0.23). Also, no correlation was found between the % INF and bone loss at both M1D and M2M (Table 4).

Significantly greater percentages of lymphocytes (Figure 15) were found in the ligature-alone and mPEG groups compared to their contralateral controls (p = 0.05 & 0.003, respectively). Differences between specific dosages of SIM/SIM-mPEG compared to their contralateral untreated controls did not reach statistical significance. There were no significant differences in lymphocyte percentages for SIM/SIM-mPEG groups compared to ligature-alone and mPEG groups.

The percentage of PMNs (Figure 16) was significantly decreased in the 1.5 mg SIM/SIM-mPEG group compared to 0.5 mg SIM/SIM-mPEG and mPEG groups (p

= 0.02 & 0.03, respectively). No significant differences in PMN counts were observed between treatment groups and their contralateral controls.

A greater percentage of uninflamed CT (Figure 17) was observed for 1.0 mg SIM/SIM-mPEG compared to ligature-alone, mPEG, and 0.5 mg SIM/SIM-mPEG groups (p = 0.04, 0.0006, & 0.006, respectively), as well as 1.5 mg SIM/SIM-mPEG compared to mPEG and 0.5 mg SIM/SIM-mPEG (p = 0.001 & 0.009, respectively), and approached statistical significance when 1.5 mg SIM/SIM-mPEG was compared to ligature-alone (p = 0.07). Differences between dosages of SIM/SIM-mPEG and their contralateral untreated controls did not reach statistical significance, while ligature-alone and mPEG groups revealed significantly less uninflamed CT compared to their contralateral controls.

There were no significant differences observed between cell counts of fibroblasts, macrophages, plasma cells, endothelial cells/blood vessel lumens, or spaces/other.

CHAPTER 5: DISCUSSION

Pilot Study

The results from the pilot study confirmed that the use of a ligature alone is an effective method to create an experimental periodontitis model in a rat. This method produced a significant amount of bone loss compared to its contralateral untreated control ($p = 0.03$), as well as a significant difference compared to LPS alone ($p = 0.011$). No significant difference was found between the ligature alone and ligature plus LPS groups. In addition, the ligature-alone method produced a significantly greater amount of inflammation when compared to its contralateral untreated control. Spolidorio et al. (2014) found after placing cotton ligatures around the maxillary 2nd molars of rats that a clear evolution of the inflammatory process was evident. The authors also noted significant increases in IL-1β, TNF-α, and IL-6 following ligature-induced periodontitis and stated this method demonstrates a satisfactory outcome of the experimental periodontitis model. Furlaneto et al. (2014) also used a ligature to induce experimental periodontitis around the maxillary 2nd molar of rats. These authors observed a greater amount of alveolar bone and attachment loss and moderate-to-severe inflammatory infiltrate in experimental periodontitis rats compared to control (no treatment) at all sites analyzed (buccal, palatal, furcation, and interproximal). The authors further stated that this model allows for the successful induction of experimental periodontitis, which was in accordance with the current pilot study.

The LPS injections provided no additional benefit when used in addition to a ligature and were unable to produce a significant amount of bone loss when used alone in a series of 3 injections over one week. In addition, LPS injections-alone in a 1 μL volume did not produce a greater amount of inflammation compared to the untreated control. These results contradict those of Price et al. (2013), who reported that 10 μL of *E coli* LPS injected into the palatal/interproximal gingiva of rat molars resulted in an increase in subsculcular inflammation. Dumitrescu et al. (2004) also reported that 10 μg of LPS in a volume of 1 μL saline injected three times over one week resulted in significant gingival and periodontal inflammation with inflammatory infiltrate, apical migration of the junctional epithelium, interdental bone loss, and activation of osteoclasts at the site of injection. Ramamurthy et al. (2002) reported that 10 μL of *E coli* LPS (1 mg/mL) given through three injections over one week resulted in a loss of alveolar bone in a short time frame. This pilot study is in contrast to these reports as well. The reason the pilot study conflicted with the aforementioned reports could be explained by the difficulty in injecting the entire volume of LPS into the palatal tissue. For example, it was possible that a portion of the 1 μL volume remained in the needle lumen through capillary action, the bevel on the needle could have been too large to allow for the entire volume to enter the palatal tissue, and the taut nature of the palate could have resisted the injection pressure placed on the syringe, preventing the volume from entering the connective tissue space.

Core Study

The results of the core study showed that SIM/SIM-mPEG significantly decreased the amount of bone loss and inflamed tissue near the injection site. At the primary outcome site, 1.5 mg SIM/SIM-mPEG resulted in the least amount of bone loss compared to all other treatment groups. However, differences among individual doses of SIM/SIM-mPEG were not statistically significant. All three doses of the experimental micelle resulted in significantly less bone loss compared to the ligature-alone group, and 1.5 mg SIM/SIM-mPEG was the only dosage that resulted in significantly less bone loss than the mPEG treatment group. No differences among groups were seen at M2M and M2D, likely due to the trauma induced at the injection site (M2M) and inability of the drug to exert its effects in sites not in the close vicinity of drug delivery (M2D).

In a study (Vaziri et al. 2007) using ovariectomized (OVX) rats with ligature-induced periodontitis around mandibular molars where SIM was administered subperiosteally into the buccal mucosa, it was concluded that SIM showed protective features against the impact of periodontitis on the attachment apparatus and alveolar bone. Similarly, a study by Xu et al. (2014) found that local SIM administration in OVX rats with ligature-induced experimental periodontitis around maxillary M1 and M2 resulted in increased alveolar crest height and prevented local alveolar bone loss. Another study (Seto et al. 2008) using local SIM topically injected into periodontal defects created with ligatures around maxillary molars of rats showed that SIM recovered the ligature-induced alveolar bone resorption, showing a 46% reversal of bone height loss. Price et al. (2013) used a

SIM/alendronate-β-cyclodextrin complex that was injected prior to the induction of experimental periodontitis in rats and showed that this SIM complex has the potential to prevent episodes of periodontitis bone loss. The results of this study confirm the bone-preservation and potentially bone-regenerative effects of SIM that was observed in these reports. Because one SIM/SIM-mPEG injection was placed at the time of ligature placement and two subsequent injections were placed following experimental periodontitis induction, a combination of bone-preservation and bone-regenerative effects may have been observed. Future studies with all local applications applied following ligature removal are needed to confirm the regenerative capabilities of SIM/SIM-mPEG.

The length of connective tissue attachment was not significantly different among treatment groups. Each group, regardless of experimental protocol (injections or ligature-alone), showed significantly greater areas of inflammation above the alveolar bone crest compared to their respective contralateral controls. In addition, 1.0 mg SIM/SIM-mPEG showed a significantly greater area of inflammatory infiltrate compared to the ligature-alone and mPEG groups. The % INF also revealed no significant differences among groups, nor was there a correlation found between the % INF and bone loss near the injection site (M1D & M2M). The rationale as to why bone changes were observed at the primary outcome site without differences in % INF through SIM/SIM-mPEG injections is unknown, but could possibly be explained due to the effects the drug has on processes directly associated with bone loss, such as decreasing osteoclast differentiation by increasing the OPG/RANKL ratio, increasing osteoblast

differentiation by up-regulating BMP-2, and decreasing osteoblast apoptosis by increasing TGF-β receptors. Thus, the area of inflammation induced by experimental periodontitis may have been without consequence when treating with SIM/SIM-mPEG. This conclusion is supported by the results of Dalcico et al. (2013), who reported that orally administered SIM in rats with experimental periodontitis induced with nylon ligatures around maxillary 2nd molars resulted in a significant inhibition of bone loss as well as a reduced expression of RANKL and an increased expression of BMP-2 and OPG levels.

The percentage of PMNs was significantly decreased in the 1.5 mg SIM/SIM-mPEG group compared to 0.5 mg SIM/SIM-mPEG and mPEG groups. This dose-response trend confirms the findings of Dalcico et al. (2013), in which the authors reported a dose-dependent reduction in myeloperoxidase (MPO) activity (an indicator of the extent of neutrophil accumulation) in the gingival tissue of SIM-treated- compared to saline-treated-rats.

Lymphocytes were the predominant cell type found in the inflammatory infiltrate of the connective tissue at day 28 (termination of the study), demonstrating the chronic nature of the experimental periodontitis lesion at this time-point. This finding is in accordance with the findings of Menezes et al. (2005) and Lima et al. (2000), which demonstrated an intense inflammatory cell infiltrate of mononuclear cells by day 11 with ligature-induced periodontitis around the 2nd maxillary molar in rats. The ligature-alone- and mPEG-treated rats in the current study were found to have significantly greater percentages of lymphocytes compared to their contralateral untreated controls, indicating both the effectiveness

of the use of a ligature in the initiation of experimental periodontitis and the pro-inflammatory property of the mPEG carrier. The difference in lymphocyte count for each dose of SIM/SIM-mPEG compared to their respective contralateral untreated controls was not statistically significant, suggesting a trend toward health with SIM/SIM-mPEG use. However, there was no significant difference in this cell type for these doses compared to the ligature-alone and mPEG groups; thus, no definitive correlation regarding a decrease in lymphocytic infiltrate with the use of SIM/SIM-mPEG could be proven.

SIM/SIM-mPEG resulted in a significantly greater percentage of uninflamed CT in the 1.0 mg dose compared to ligature-alone, mPEG, and the 0.5 mg dose. Increased uninflamed CT was also noted in the 1.5 mg dose compared to mPEG and the 0.5 mg dose. The 1.5 mg dose of SIM/SIM-mPEG, when compared to ligature-alone, approached a statistically significant increase ($p = 0.07$). Thus, a dose-response trend was observed regarding SIM/SIM-mPEG and uninflamed CT fibers. Results also indicated that the inflammatory infiltrate in the connective tissue of the papilla between M1-M2 interproximally was localized to the lamina propria directly underlying the sulcular and junctional epithelium, and did not advance towards the alveolar bone crest. This also could contribute to the bone-preservation effect seen with SIM/SIM-mPEG at the primary outcome site. Furthermore, the mPEG group revealed the least amount of uninflamed CT, and as mentioned above, had significantly greater counts of PMN infiltrate compared to SIM/SIM-mPEG doses. Collectively, it can be concluded that the mPEG carrier alone increased inflammation histologically in this study.

A limited number of human studies have reported on the effects of SIM in the treatment of periodontitis. In a study of 60 patients receiving SRP plus either a locally delivered placebo or SIM, there was a greater decrease in gingival inflammation and significantly more intrabony defect fill at sites treated with scaling and root planing plus SIM (Pradeep & Thorat 2010). Another similarly designed study by the same group examining SIM's effect in treating molar Class II furcation defects in 72 patients resulted in a similar reduction in gingival inflammation and greater mean percentage of bone fill in SIM-treated subjects (Pradeep et al. 2012). The authors concluded that locally-delivered SIM provides a comfortable and flexible method to improve clinical parameters and also enhance bone formation. Also, 38 subjects with type 2 diabetes similarly treated with SIM resulted in the same inflammation reduction with significant intrabony defect fill at sites treated with SRP plus locally delivered SIM compared to SRP alone (Pradeep et al. 2013). While the human trials above are supportive of the use of statin treatment of periodontitis and other craniofacial defects, Zhang et al. (2014) states that future confirmation of these results by other investigators is crucial.

The differences in weight changes were not significantly different among groups from the start to the conclusion of the study. Further, the mean changes in weight for all groups were within 10 grams of the starting weights, or a maximum 3.3% change in weight from baseline. Thus, neither the ligature nor a combination of ligature with mPEG or any dose of SIM/SIM-mPEG had an effect on weight changes in the rats throughout the duration of the study. In the study mentioned

above, Price et al. (2013) also demonstrated no significant weight change in rats over the course of study using a SIM/alendronate-β-cyclodextrin complex.

CHAPTER 6: CONCLUSIONS

SIM/SIM-mPEG, especially 1.5 mg SIM/SIM-mPEG, significantly decreased the amount of bone loss and inflamed tissue in experimental periodontitis near the injection site. However, the mPEG carrier appears to increase inflammation and may not be well-suited for conjugation with SIM.

CHAPTER 7: FUTURE DIRECTIONS

Currently, researchers at the University of Nebraska Medical Center College of Pharmacy have developed a new SIM pro-drug in which SIM has been conjugated with pyrophosphate. This pro-drug is currently in experimental phases to test its bone anabolic and anti-inflammatory potential.

REFERENCES

Albandar JM, Brunelle JA, Kingman A. Destructive periodontal disease in adults 30 years of age and older in the United States, 1988-1994. *J Periodontol* 1999;70:13-29.

Allenspach-Petrzilka GE, Guggenheim B. Bacterial invastion of the periodontium; an important factor in the pathogenesis of periodontitis? *J Clin Periodontol* 1983;10:609-617.

Balto K, Muller R, Carrington DC, Dobeck J, Stashenko P. Quantification of periapical bone destruction in mice by micro-computed tomography. *J Dent Res* 2000;79:35-40.

Belibasakis GN, Bostanci N. The RANKL-OPG system in clinical periodontology. *J Clin Periodontol* 2012;39:239-248.

Caton JG, Ciancio SG, Blieden TM, Bradshaw M, Crout RJ, Hefti AF, Massaro JM, Polson AM, Thomas J, Walker C. Treatment with subantimicrobial dose doxycycline improves the efficacy of scaling and root planning in patients with adult periodontitis. *J Periodontol* 2000;71:521-532.

Cobb CM. Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planning. *J Clin Periodontol* 2002;29:6-16.

Coimbra LS, Rossa C Jr., Guimaraes MR, Gerlach RF, Muscara MN, Spolidorio MP, Herrera BS, Spolidorio LC. Influence of antiplatelet drugs in the pathogenesis of experimental periodontitis and periodontal repair in rats. *J Periodontol* 2011;82:767-777.

Dalcico R, de Menezes AMA, Deocleciano OB, Oria RB, Vale ML, Ribeiro RA, Brito GA. Protective mechanisms of simvastatin in experimental periodontal disease. *J Periodontol* 2013;84:1145-1157.

de Aquino SG, Guimaraes MR, Stach-Machado DR, da Silva JA, Spolidorio LC, Rossa C Jr. Differential regulation of MMP-13 expression in two models of experimentally induced periodontal disease in rats. *Archives of Oral Biology* 2009; 54:7:609-617.

Dumitrescu AL, Abd El-Aleem S, Morales-Aza B, Donaldson LF. A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation. *J Clin Periodontol* 2004; 31:596-603.

Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ. Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res* 2012;91:914-920.

Furlaneto FAC, Nunes NLT, Oliveira Filho IL, Frota NPR, Yamamoto KO, Lisboa MRP, Ervolino E, Taba M Jr., Otavio Rego R, Messora MR. Effects of locally administered tiludronic acid on experimental periodontitis in rats. *J Periodontol* 2014;85:1291-1301.

Golub LM, Lee H, Greenwald R, Ryan M, Sorsa T, Salo T, Giannobile WV. A matrix metalloproteinase inhibitor reduces bone-type collagen degradation fragments and specific collagenases in gingival crevicular fluid during adult periodontitis. *Inflamm Res* 1997;46;310-319.

Golub LM, McNamara TF, Ryan ME, Kohut B, Blieden T, Payonk G, Sipos T, Baron HJ. Adjunctive treatment with subantimicrobial doses of doxycycline: effects on gingival fluid collagenase activity and attachment loss in adult periodontitis. *J Clin Periodontol* 2001;28:146-156.

Jia Z, Zhang Y, Chen YH, Dusad A, Yuan H, Ren K, Li F, Fehringer EV, Purdue PE, Goldring SR, Daluiski A, Wong D. Simvastatin prodrug micelles target fracture and improve healing. *J Control Release* 2015;200:23-34.

Kaldahl WB, Kalkwarf KL, Patil KD, Molvar MP, Dyer JK. Long-term evaluation of periodontal therapy: I. Response to 4 therapeutic modalities. *J Periodontol* 1996;67:93-102.

Lee Y, Schmid M, marx D, Beatty M, Cullen D, Collins M, Reinhardt R. The effect of local simvastatin delivery strategies on mandibular bone formation in vivo. *Biomaterials* 2008;29:1940-1949.

Lima V, Bezerra mM, Alencar VBM, Vidal FDP, Rocha FAC, Brito GAC, Ribeiro RA. Effects of chlorpromazine on alveolar bone los in experimental periodontal disease in rats. *Eur J Oral Sci* 2000;108:123-129.

Listgarten MA. Similarity of epithelial relationships in the gingiva of rat and man. *J Periodontol* 1975;46:677-680.

McCauley LK, Nohutcu RM. Mediators of periodontal osseous destruction and remodeling: principles and implications for diagnosis and therapy. *J Periodontol* 2002;73:1377-1391.

Menezes AMA, rocha FAC, Chaves HV, Carvalho CBM, Rebeiro RA, Brito GAC. Effect of sodium alendronate on alveolar bone resorption in experimental periodontitis in rats. *J Periodontol* 2005;76:1901-1909.

Mengel R, Candir M, Shiratori K, Flores-de-Jacoby L. Digital volume tomography in the diagnosis of periodontal defects: an in vitro study on native pig and human mandibles. *J Periodontol* 2005;76:665-673.

Mogi M, Otogoto J, Ota N, Togari A. Differential expression of RANKL and osteoprotegrin in gingival crevicular fluid of patients with periodontits. *J Dent Res* 2004;83:166-169.

Mundy GR. Statins and their potential for osteoporosis. *Bone* 2001;29:495-497.

Offenbacher S. Periodontal diseases: pathogenesis. *Ann Periodontol* 1996;1:821-878.

Park CH, Abramson ZR, Taba M Jr., Jin Q, Chang J, Kreider JM, Goldstein SA, Giannobile WV. Three-dimensional micro-computed tomographic imaging of alveolar bone in experimental bone loss or repair. *J Periodontol* 2007;78:273-281.

Pellegrini G, Seol Y, Gruber R, Giannobile W. Pre-clinical models for oral and periodontal reconstructive therapies. *J Dent Res* 2009;88:1065-1076.

Powell SE. The histology of simvastatin injections in rat experimental periodontitis. 2011. [Master's thesis UNMC College of Dentistry]

Pradeep AR, Thorat MS. Clinical effect of subgingivally delivered simvastatin in the treatment of patients with chronic periodontitis: a randomized clinical trial. *J Periodontol* 2010;81:214–222.

Pradeep AR, Priyanka N, Kalra N, Naik SB, Singh SP, Martande S. Clinicalefficacy of subgingivally delivered 1.2-mg simvastatin in the treatment of individuals with class II furcation defects: a randomized controlled clinical trial. *J Periodontol* 2012;83:1472–1479.

Pradeep AR, Rao NS, Bajaj P, Kumari M. Efficacy of subingivally delivered simvastatin in the treatment of patients with type 2 diabetes and chronic periodontitis: a randomized double-masked controlled clinical trial. *J Periodontol* 2013;84:24–31.

Price U, Le H, Powell SE, Schmid MJ, Marx DB, Zhang Y, Wang D, Narayana N, Reinhardt RA. Effects of local simvastatin-alendronate conjugate in preventing periodontitis bone loss. *JPeriodontal Res* 2013;48:541–548.

Ramamurthy NS, Xu J-W, Bird J, Baxter A, Bhogal R, Wills R, Watson B, Owen D, Wolff M, Greenwald RA. Inhibition of alveolar bone loss by matrix metalloproteinase inhibitors in experimental periodontal disease. *J Periodont Res* 2002;37:1-7.

Saglie R, Elbaz JJ. Bacterial penetration into the gingival tissue in periodontal disease. *J West Soc Periodont* 1983;31:85-93.

Salvi GE, Lang NP. The effects of non-steroidal anti-inflammatory drugs (selective and non-selective) on the treatment of periodontal diseases. *Curr Pharm Des* 2005;11:1757-69.

Schroeder HE, Lindhe J. Conversion of stable established gingivitis in the dog into destructive periodontitis. *Arch Oral Biol* 1975;20:775.

Seto H, Ohba H, Tokunaga K, Hama H, Horibe M, Nagata T. Topical administration of simvastatin recovers alveolar bone loss in rats. *J Periodont Res* 2008;43:261–7.

Sgolastra F, Gatto R, Petrucci A, Monaco A. Effectiveness of systemic amoxicillin/metronidazole as adjunctive therapy to scaling and root planning in the treatment of chronic periodontitis: a systematic review and meta-analysis. *J Periodontol* 2012;83:1257-1269.

Sgolastra F, Petrucci A, Gatto R, Monaco A. Effectiveness of systemic amoxicillin/metronidazole as an adjunctive therapy to full-mouth scaling and root planning in the treatment of aggressive periodontitis: a systematic review and meta-analysis. *J Periodontol* 2012;83:731-743.

Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontol 2000* 2002;28:12-55.

Spolidorio LC, Lucas PDR, Steffens JP, da Silva HAB, Alves VTE, Spolidorio DMP, Hozhausen M. Influence of parstatin on experimental periodontal disease and repair in rats. *J Periodontol* 2014;85:1266-1274.

Stein D, Lee Y, Schmid MJ, Killpack B, Genrich MA, Narayana N, Marx DB, Cullen DM, Reinhardt RA. Local simvastatin effects on mandibular bone growth and inflammation. *J Periodontol* 2005; 76:1861-1870.

Thelaide E. The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J Clin Periodontal* 1986;13:905-911.

Triplett RG, Nevins M, Marx RE, Spagnoli DB, Oates TW, Moy PK, Boyne PJ. Pivotal, randomized, parallel evaluation of recombinant human bone morphogenetic protein-2/absorbable collagen sponge and autogenous bone graft for maxillary sinus floor augmentation. *J Oral Maxillofac Surg* 2009;67:1947-1960.

Vaziri H, Naserhojjati-Roodsari R, Tahsili-Fahadan N, Khojasteh A, Mashhadi-Abbas F, Eslami B, Dehpour AR. Effect of simvastatin administration on periodontitis-associated bone loss in ovariectomized rats. *J Periodontol* 2007;78:1561–1567.

Walker C, Thomas J, Nango S, lennon J, Wetzel j, Powala C. Long-term treatment with subantimicrobial dose doxycycline exerts no antibacterial effect on the subgingival microflora associated with adult periodontitis. *J Periodontol* 2000;71:1465-1471.

Williams RC, Jeffcoat MK, Howell TH, Rolla A, Stubbs D, Teoh KW, Reddy MS, Goldhaber P. Altering the progression of human alveolar bone loss with the non-steroidal anti-inflammatory drug flurbiprofen. *J Periodontol* 1989;60:485-490.

Xu L, Dong XW, Shen LL, Li FF, Jiang JX, Cao R, Yao HY, Shen HJ, Sun Y, Xie QM. Simvastatin delivery via inhalation attenuates airway inflammation in a murine model of asthma. *Int Immunopharmacol* 2012;12:556-64.

Xu XC, Chen H, Zhang X, Zhai ZJ, Liu XQ, Qin A, Lu EY. Simvastatin prevents alveolar bone loss in an experimental rat model of periodontitis after ovariectomy. *J Transl Med* 2014;12:284.

Zhang Y, Bradley AD, Wang D, Reinhardt RA. Statins, bone metabolism and treatment of bone catabolic diseases. *Pharmacol Res* 2014;88:53-61.

Table 1

Experimental groups of the pilot study.

Group	Number	Week 1	Week 2	Week 3	Week 4
1	5	Silk ligatures		Euthanize	
2	5	*Escherichia coli* LPS injections		Euthanize	
3	5	Silk ligatures	LPS injections		Euthanize

Table 2

Experimental groups of the core study.

Group	N	Week 1	Week 2	Week 3	Week 4
1	8	Ligatures	Remove Ligatures		Euthanize
3	8	Ligatures mPEG (10µL)	Remove Ligatures mPEG	mPEG	Euthanize
5	8	Ligatures 0.5 mg SIM/SIM-PEG (10µL)	Remove Ligatures 0.5 mg SIM/SIM-PEG	0.5 mg SIM/SIM-PEG	Euthanize
7	8	Ligatures 1.0 mg SIM/SIM-PEG (10µL)	Remove Ligatures 1.0 mg SIM/SIM-PEG	1.0 mg SIM/SIM-PEG	Euthanize
9	8	Ligatures 1.5 mg SIM/SIM-PEG (10µL)	Remove Ligatures 1.5 mg SIM/SIM-PEG	1.5 mg SIM/SIM-PEG	Euthanize
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group					

Table 3

The intra-class correlation coefficient (ICC), evaluating the intra-examiner reliability between μ-CT measurements collected at two different measurement times.

Intra-class Correlation Coefficient							
	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	0.985	0.961	0.994	133.883	17	17	.000

Table 4

Pearson correlation coefficients comparing area of inflammatory infiltrate/total area of CT above the alveolar bone crest and bone loss at M1D and M2M.

Pearson correlation coefficients			
		M1D bone loss	M2M bone loss
Percent inflammatory area	*r* value	.06850	.07373
	p value	.5847	.5563

Figure 1

Synthesis of the amphiphilic macromolecular prodrug SIM-mPEG.

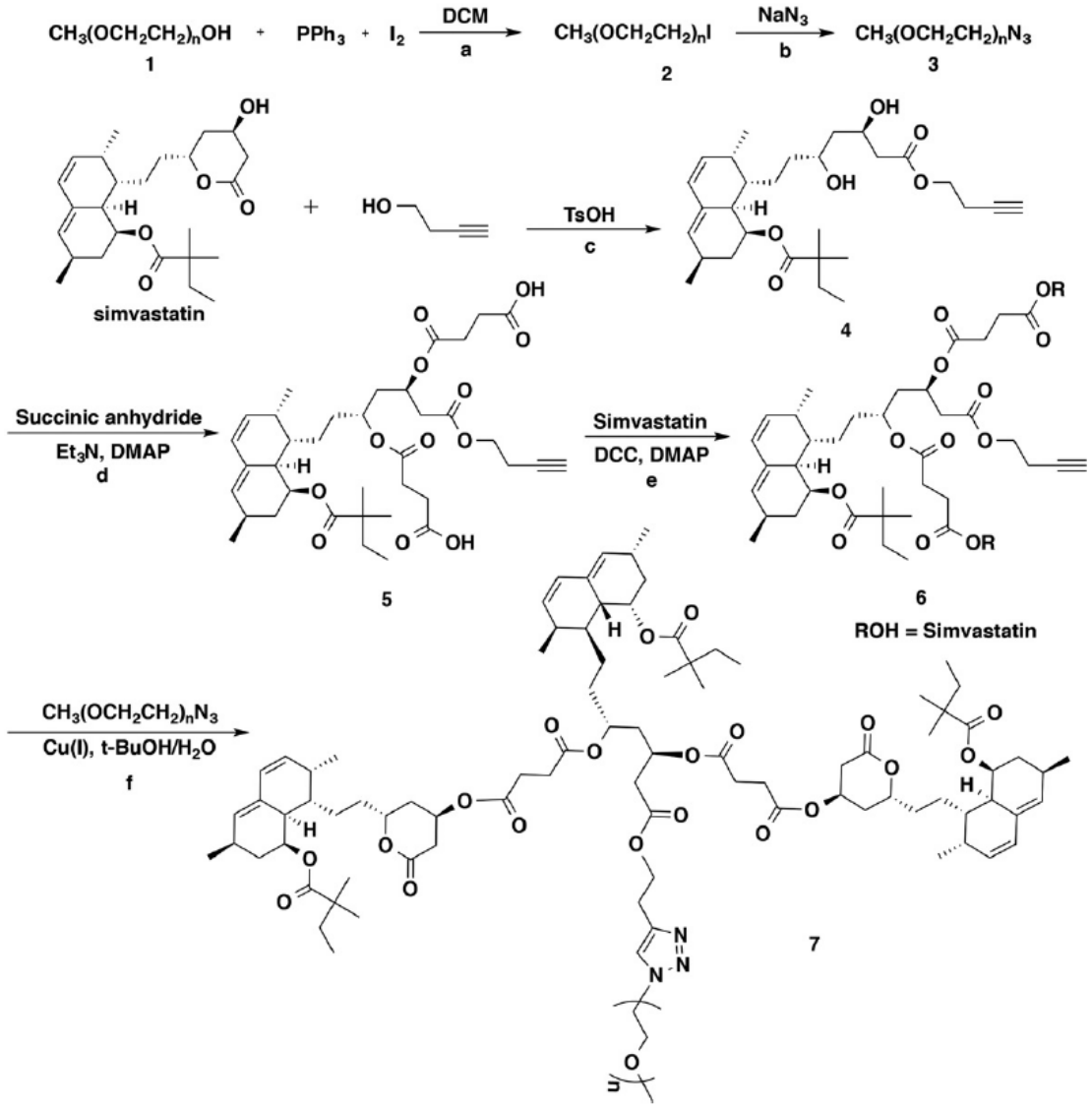


Figure 2

4-0 silk ligature in place around the maxillary right second molar.

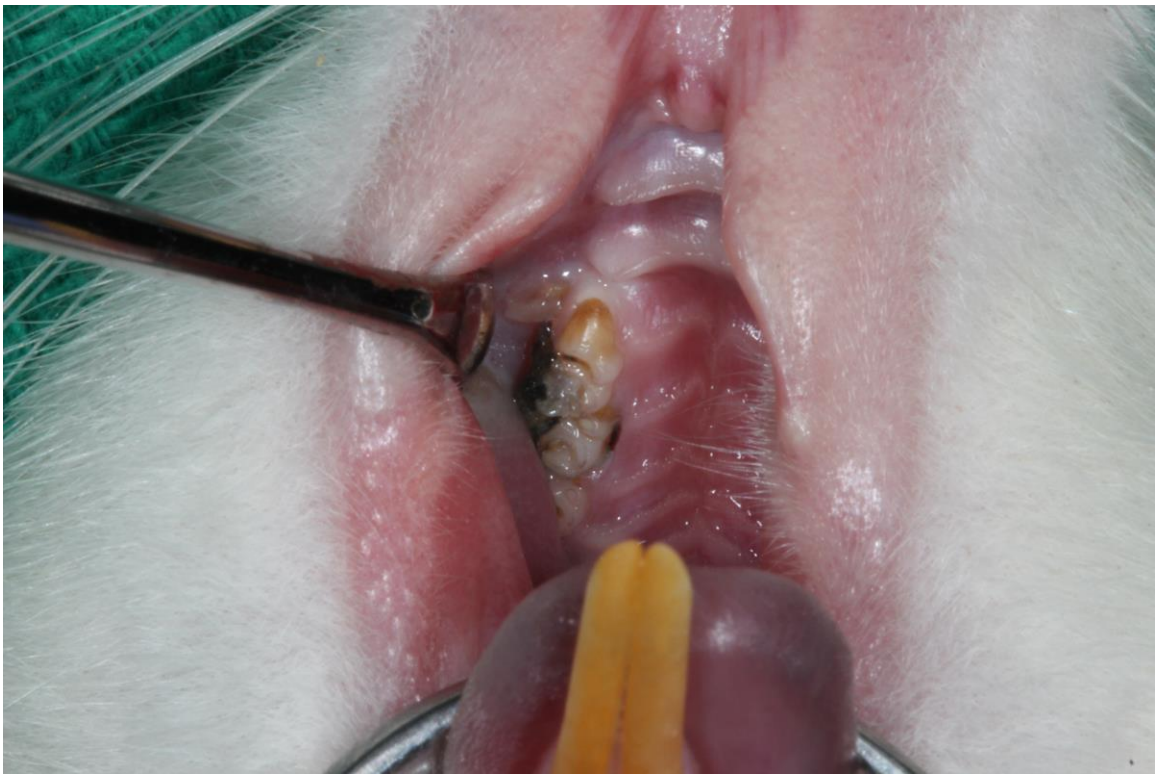


Figure 3

Local injections delivered to the palate between M1 and M2.

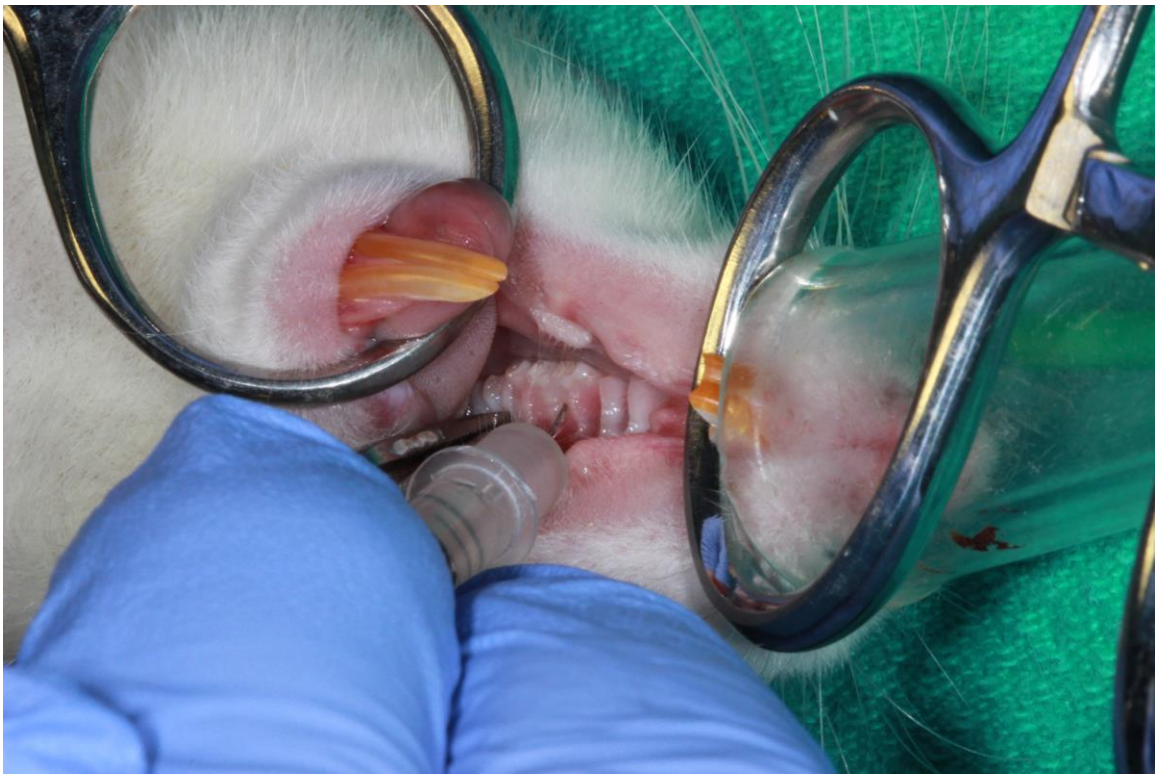


Figure 4

Alignment of specimens on three axes prior to obtaining measurements.

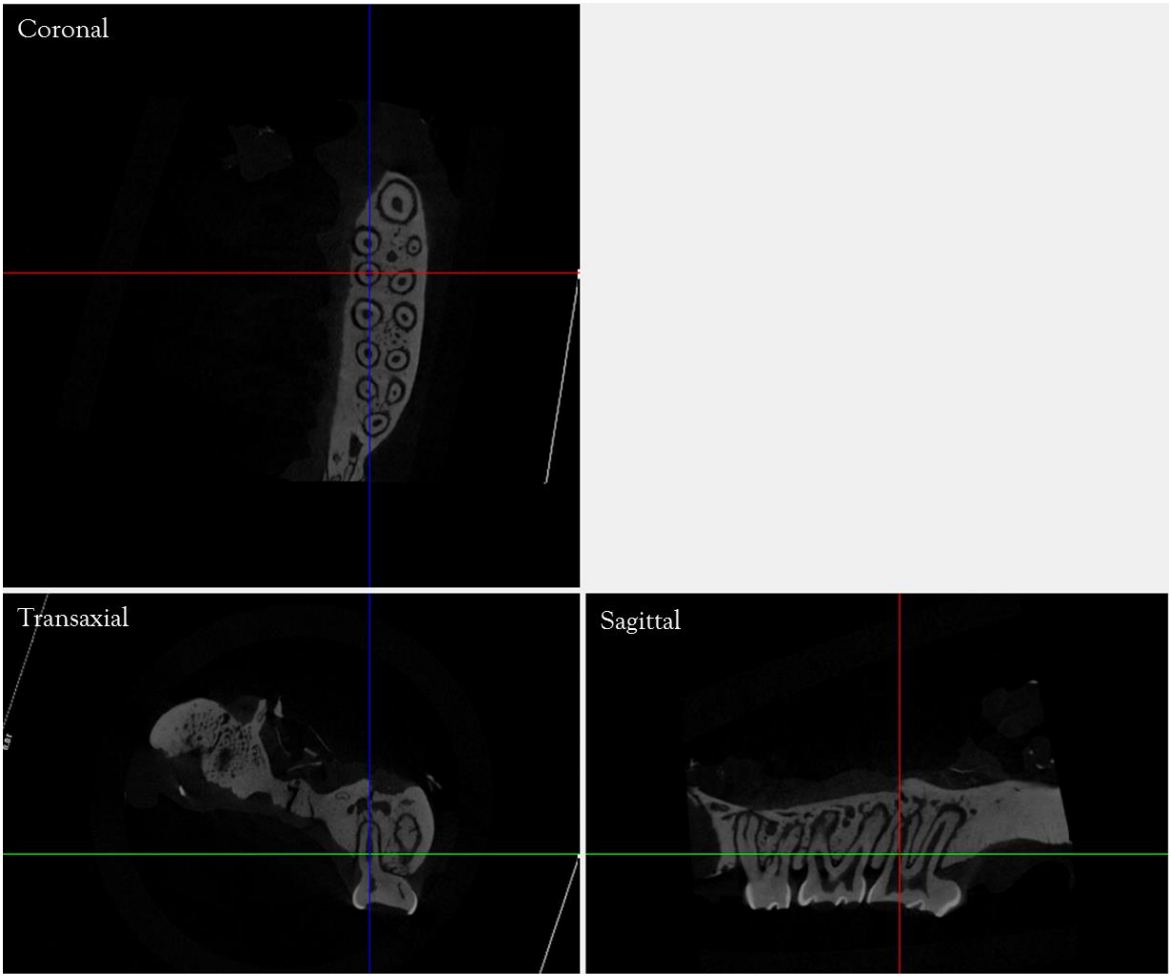
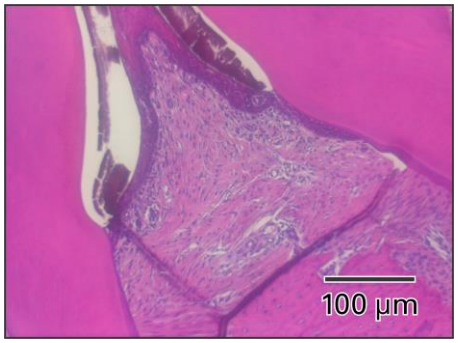
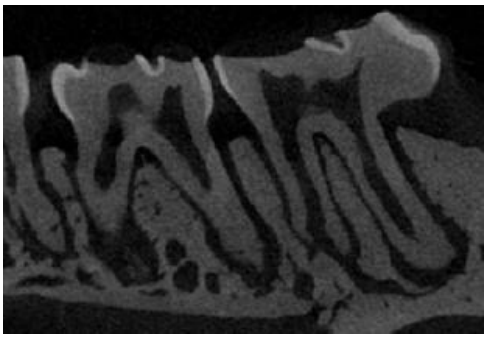


Figure 5

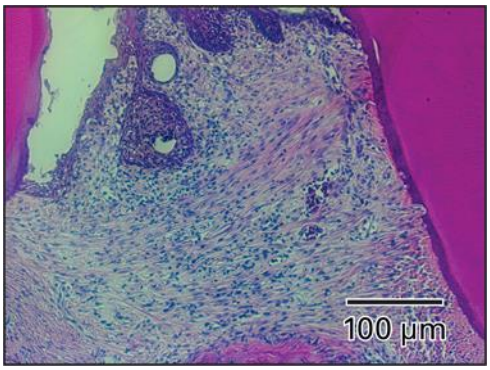
Histology shows papillary epithelium (top) and bone (bottom) and teeth M2-M1 at 100X magnification. μ-CT specimens reveal orientation of sagittal section used for measurements.



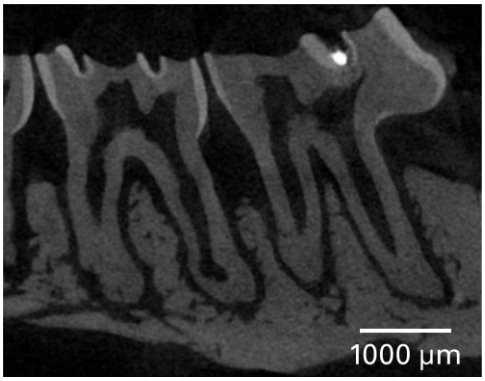
Untreated pristine control (M2-M1)



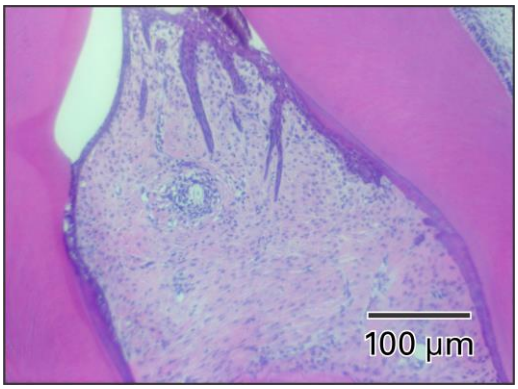
Untreated pristine control (M2-M1)



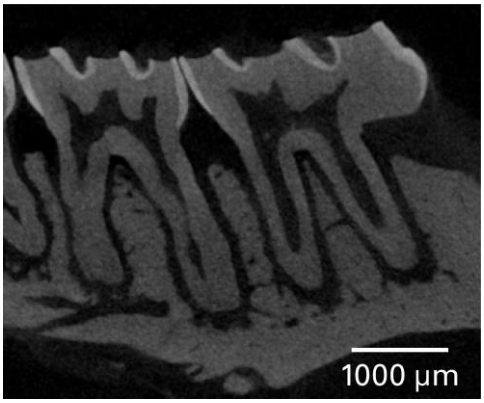
Ligature-alone (M2-M1)



Ligature-alone (M2-M1)



1.5 mg SIM/SIM-mPEG (M2-M1)



1.5 mg SIM/SIM-mPEG (M2-M1)

Figure 6

The effect of ligature-alone, ligature + LPS, and LPS-alone on the alveolar crest height at the distal of M1 as measured histologically.

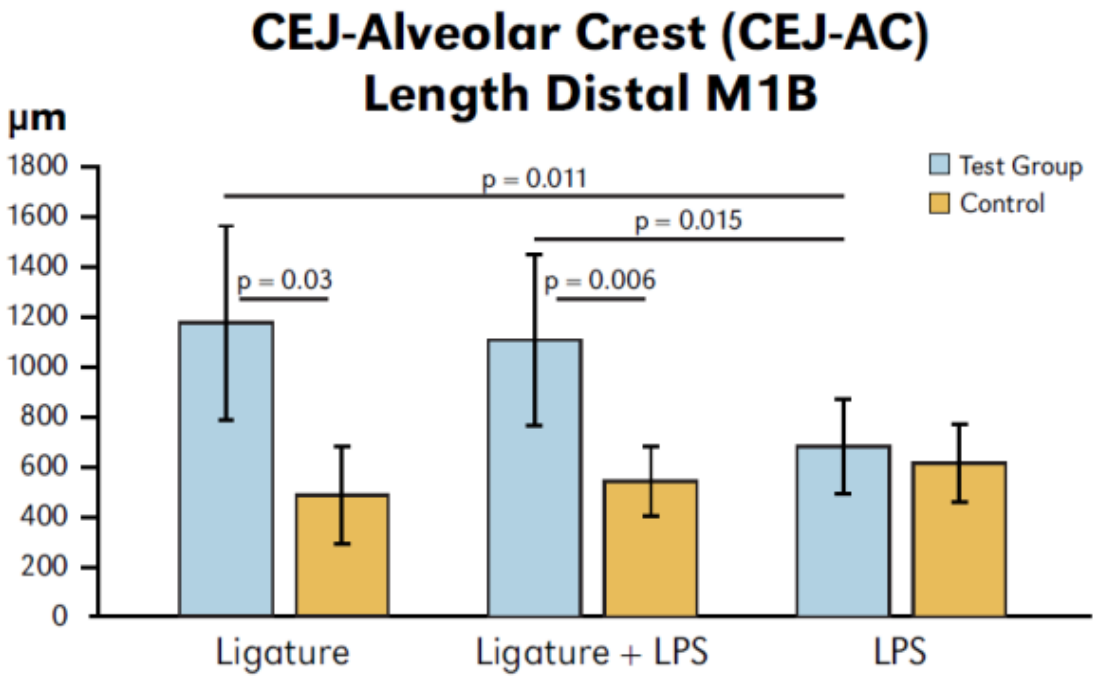


Figure 7

The effect of ligature-alone, ligature + LPS, and LPS-alone on the alveolar crest height at the distal of M1 as measured using µ-CT.

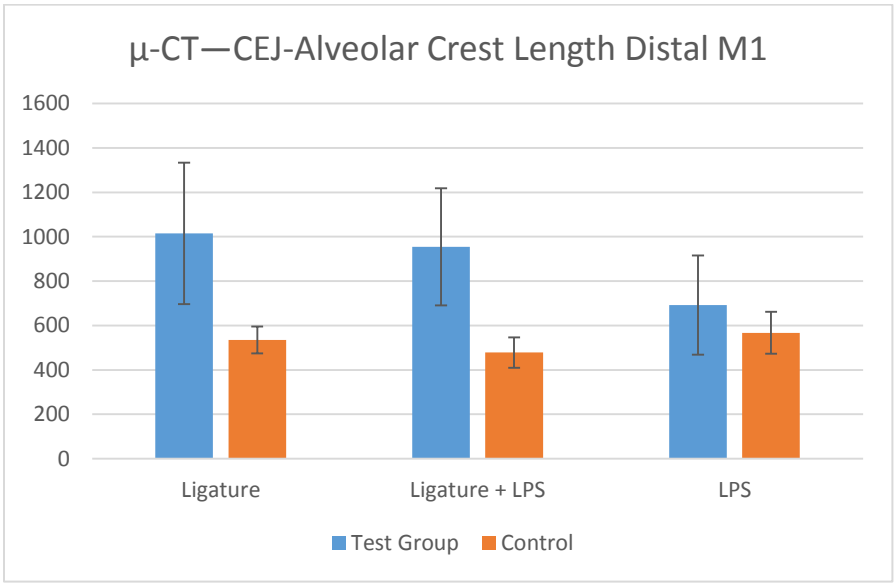


Figure 8

The effect of ligature-alone, ligature + LPS, and LPS-alone on the subepithelial inflammatory score.

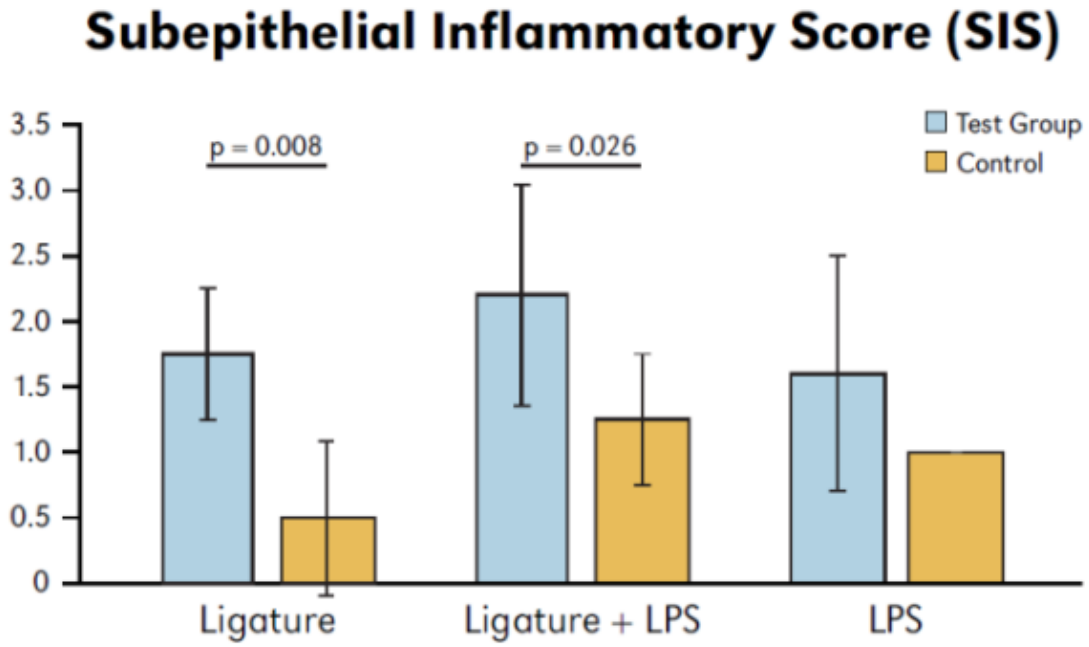
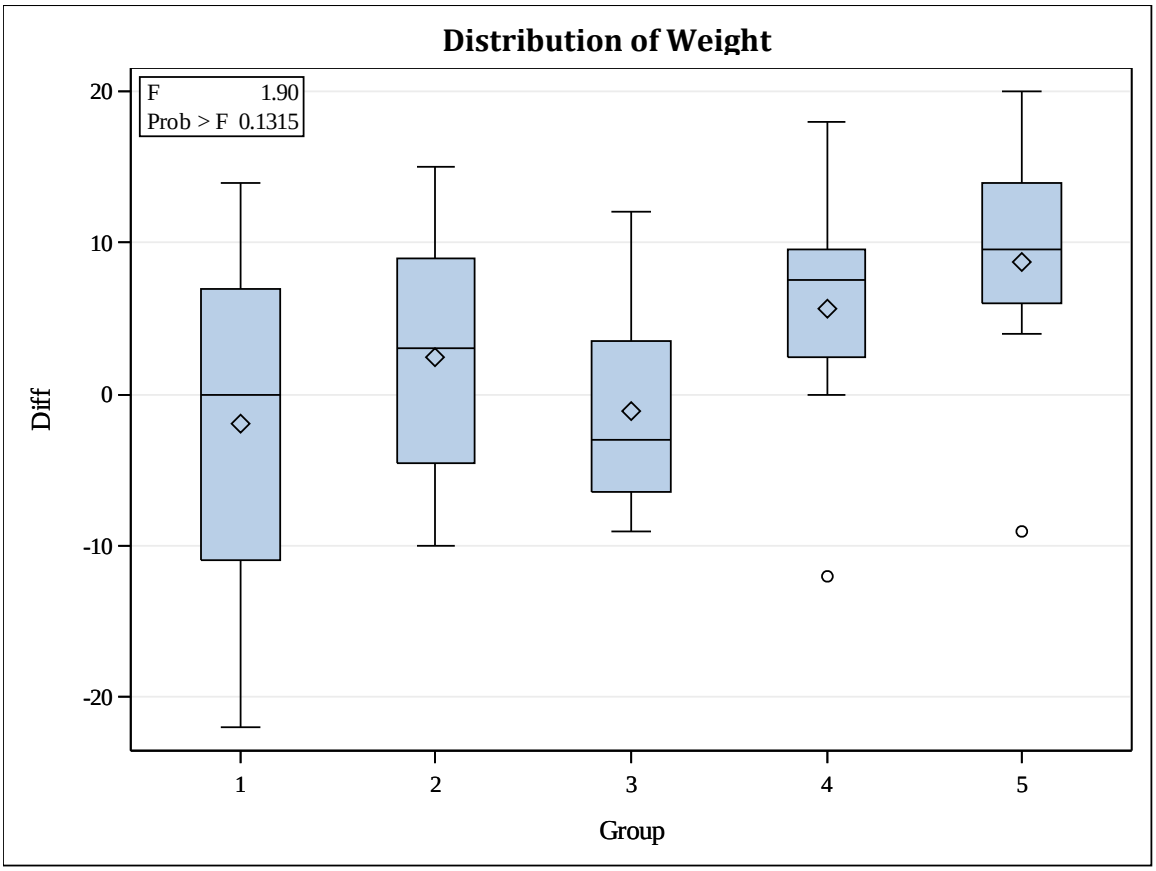


Figure 9

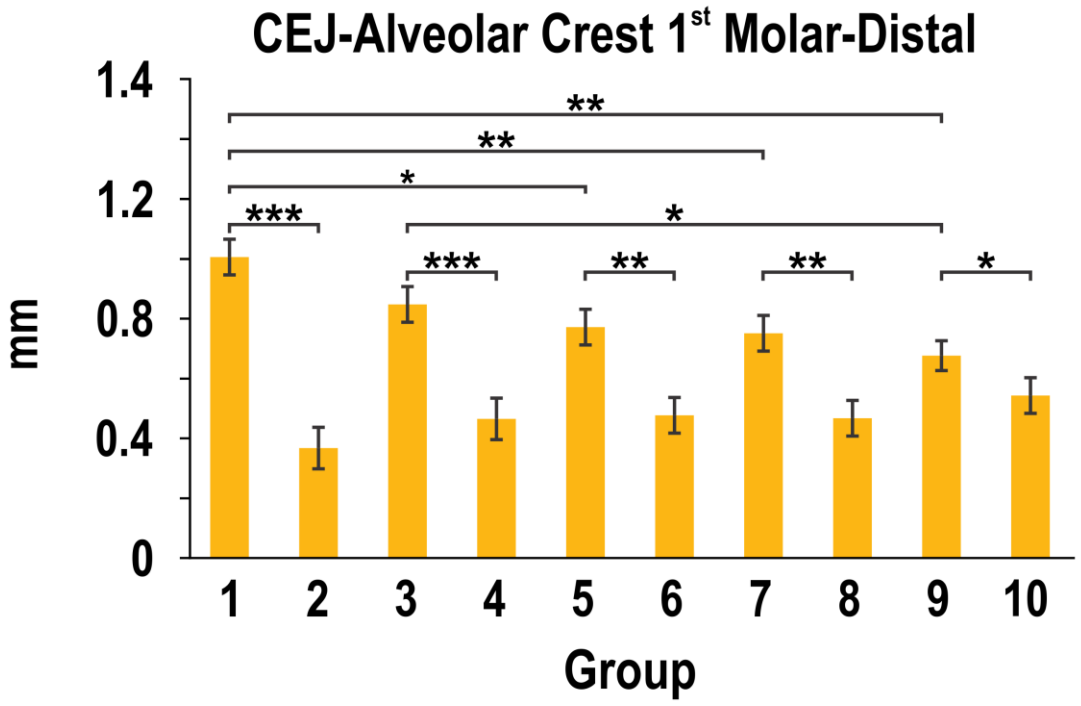
Distribution of differences in weight from start to finish of the study among groups (p = 0.1315).



Group 1 = ligature-alone
Group 2 = mPEG
Group 3 = 0.5 mg SIM/SIM-mPEG
Group 4 = 1.0 mg SIM/SIM-mPEG
Group 5 = 1.5 mg SIM/SIM-mPEG

Figure 10

CEJ-ABC at primary outcome site as measured by μ-CT.

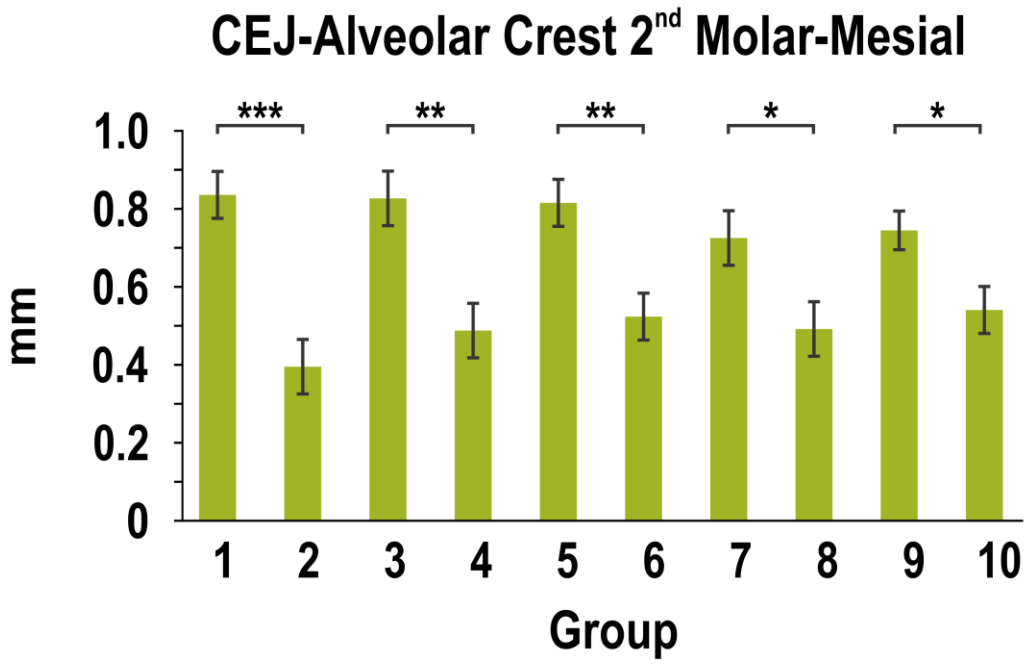


Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Figure 11

CEJ-ABC at M2M as measured by μ-CT.

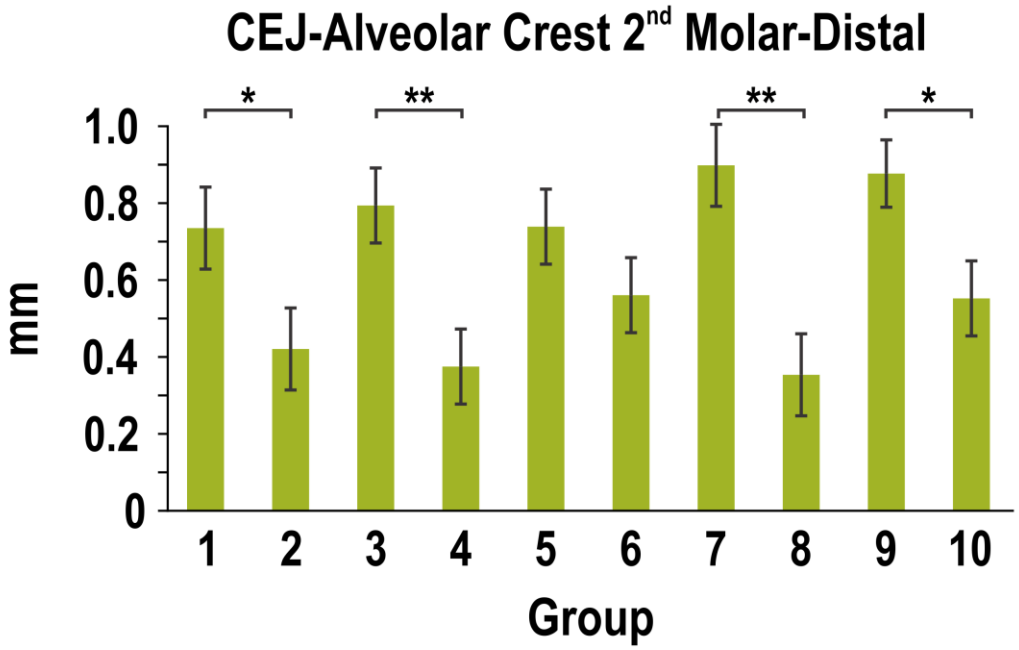


Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Figure 12

CEJ-ABC at M2D as measured by µ-CT.

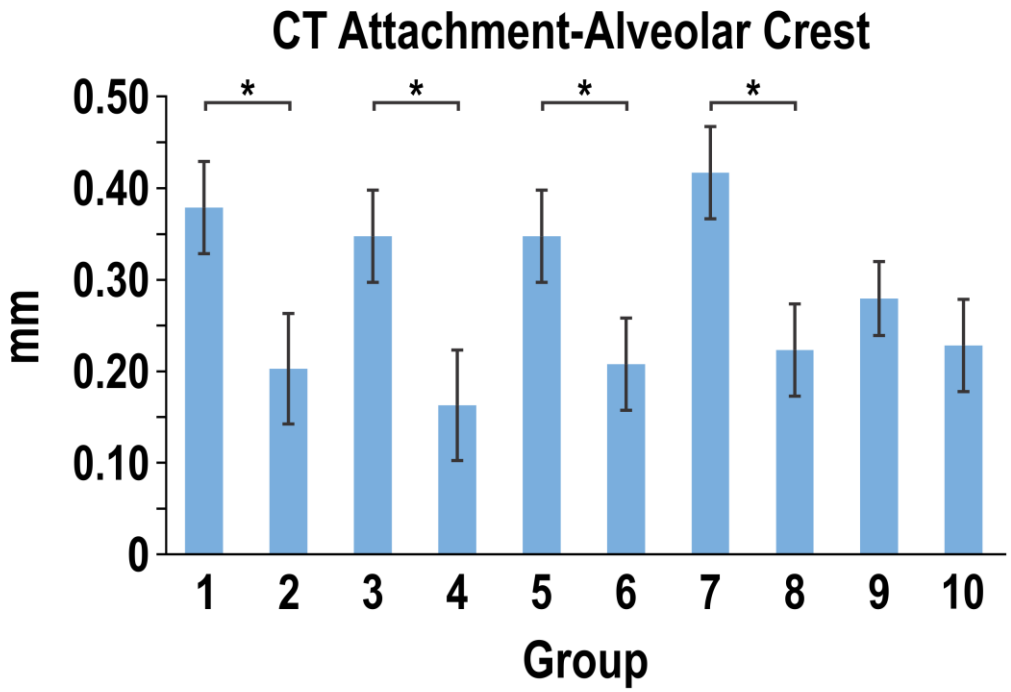


Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Figure 13

The length of CT attachment as measured histologically.

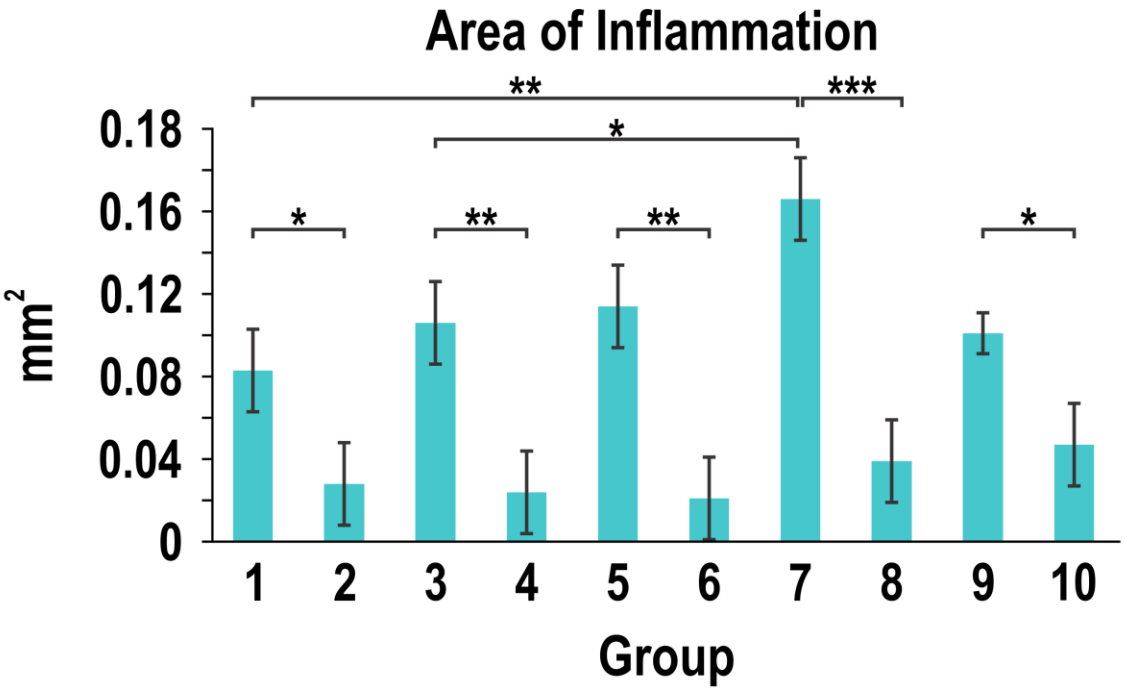


Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Figure 14

The area of inflammatory infiltrate between M1 and M2.

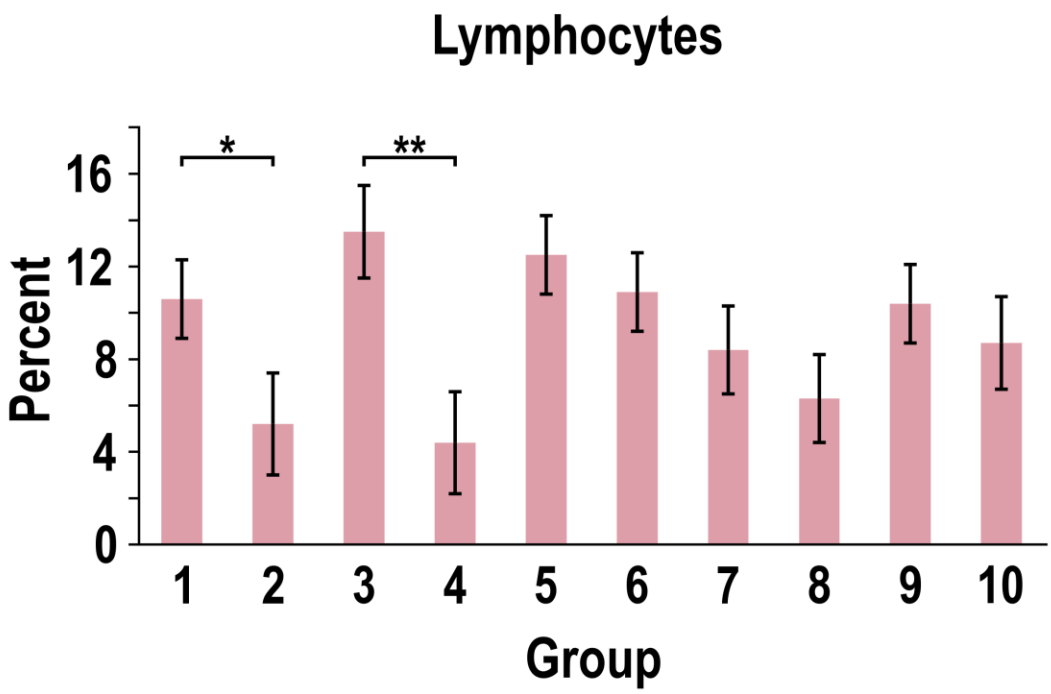


Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Figure 15

Lymphocytic infiltrate interproximally between M1 and M2.

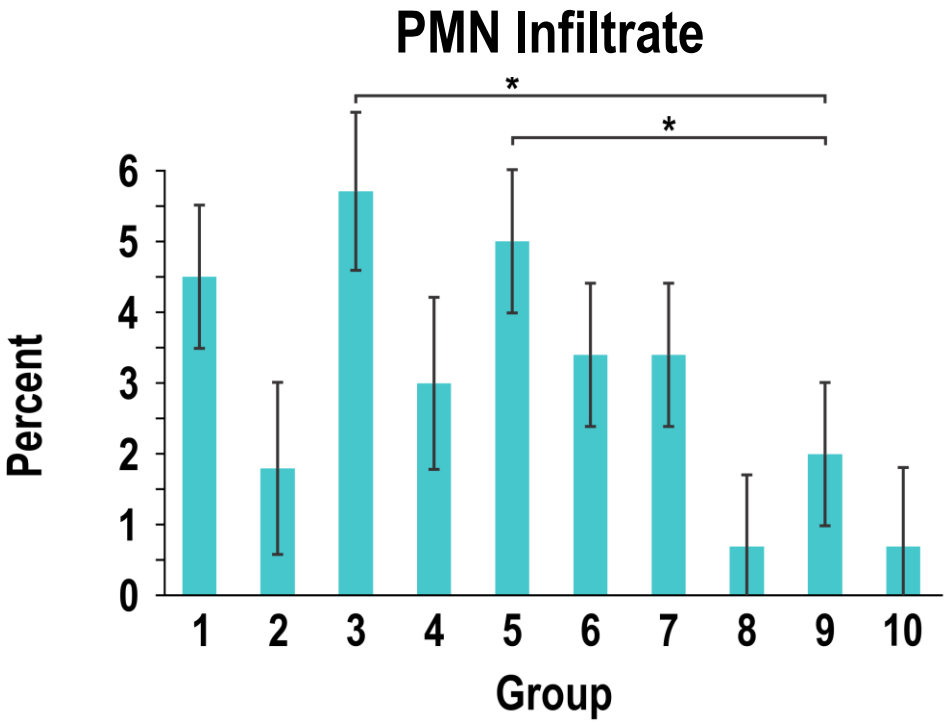


Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Figure 16

PMN infiltrate interproximally between M1 and M2.

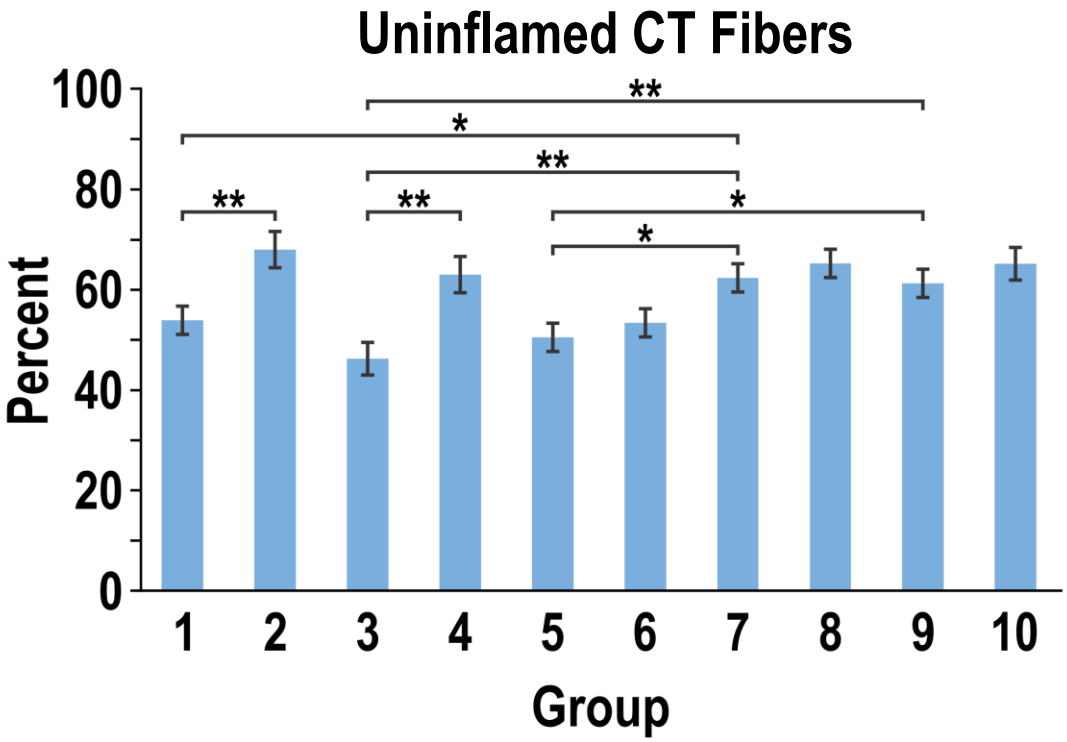


Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Figure 17

Uninflamed CT fibers interproximally between M1 and M2.



Group 1 = ligature-alone
Group 3 = mPEG
Group 5 = 0.5 mg SIM/SIM-mPEG
Group 7 = 1.0 mg SIM/SIM-mPEG
Group 9 = 1.5 mg SIM/SIM-mPEG
Groups 2, 4, 6, 8, 10 = contralateral untreated control of preceding treatment group

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

APPENDIX

Animal #	Area of inflammation	Supracrestal CT area	% INF	CEJ - apical extent of epithelium
276L	0	0	0	0
276R	0.03527	0.1146	0.307766143	0.2586
277L	0.03101	0.2531	0.28411	0.1972
277R	0.16299	0.8278	0.196895385	0.3197
278L	0.02811	0.1275	0.220470588	0.03171
278R	0.067498	0.253898	0.265846915	0.3648
279L	0	0	0	0
279R	0.14042	0.5286	0.2656451	0.1772
280L	0.03676	0.1651	0.222652938	0.1018
280R	0.05145	0.18826	0.273292255	0.2042
281L	0.0118	0.1612	0.073200993	0.05389
281R	0.04174	0.542	0.07701107	0.192
282L	0.02962	0.1842	0.160803474	0.24
282R	0.05657	0.3005	0.188252912	0.3394
283L	0	0	0	0
283R	0.1106	0.3239	0.341463415	0.3128
284L	0.01811	0.1507	0.120172528	0.1811
284R	0	0	0	0
285L	0	0	0	0

CEJ - bone	M1DB CEJ - bone µCT	M2MB CEJ - bone µCT	M2DB CEJ - bone µCT
0	0	0	0
0.4937	0	0	0
0.3646	0.405	0.468	0.51
0.9866	0.716	0.523	0.684
0.2489	0.326	0.34	0.308
0.587	0.8	0.74	0.465
0	0.456	0.395	0.421
0.8089	1.079	1.019	1.06
0.2857	0.356	0.322	0.508
0.4803	1.03	1.007	0.393
0.3159	0.288	0.402	0.218
0.6752	1.095	1.129	0.857
0.4265	0.465	0.436	0.626
0.5711	1.396	0.652	0.919
0	0.469	0.556	0.349
0.5928	0.93	0.78	0.776
0.3761	0.487	0.597	0.668
0	1.327	1.477	1.22
0	0.504	0.445	0.218

Lymphocyte	Plasma Cell	Macrophage	PMN	CT	Blood Vessel	Fibroblast	Space
0	0	0	0	0	0	0	0
15	0	0	1	59	4	21	0
6	0	0	3	68	5	18	0
11	3	0	7	46	6	22	5
2	0	0	3	70	4	19	1
10	0	0	7	59	4	220	0
0	0	0	0	0	0	0	0
7	1	0	3	55	10	24	0
8	0	0	1	67	6	15	3
10	0	0	3	61	6	14	6
5	0	0	0	63	7	25	0
9	0	0	4	59	6	22	0
5	0	0	2	72	6	15	0
16	0	0	5	58	3	17	0
0	0	0	0	0	0	0	0
7	4	0	6	34	17	21	11
5	0	0	2	67	2	22	2
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0

Animal #	Area of inflammation	Supracrestal CT area	% INF	CEJ - apical extent of epithelium
285R	0	0	0	0
286L	0	0	0	0
286R	0.12256	0.4113	0.297982008	0.2093
287L	0.012699	0.3019	0.042063597	0.3369
287R	0.0798	0.2237	0.35672776	0.4961
288L	0.007466	0.046576	0.160297149	0.1506
288R	0.1374	0.2608	0.526840491	0.3733
289L	0.03009	0.2237	0.134510505	0.02844
289R	0.0508	0.2648	0.1918429	0.3565
290L	0.0368	0.2383	0.154427193	0.2359
290R	0.077871	0.2673	0.291324355	0.3726
291L	0.01841	0.05773	0.31889832	0.3707
291R	0.04632	0.2249	0.205958204	0.275
292L	0	0	0	0
292R	0.117	0.3646	0.320899616	0.09134
293L	0.02362	0.1466	0.16111869	0.1931
293R	0.12644	0.2989	0.423017732	0.4749
294R	0.11446	0.2689	0.425660097	0.3942
295L	0.03259	0.15	0.217266667	0.04286

CEJ - bone	M1DB CEJ - bone µCT	M2MB CEJ - bone µCT	M2DB CEJ - bone µCT
0	0.951	1.014	0.61
0	0.494	0.432	0.204
0.6464	1.014	0.9	0.404
0.5137	0.464	0.56	0.305
0.6361	0.526	0.604	0.697
0.3005	0.491	0.436	0.334
0.5591	0.692	0.773	1.15
0.2993	0.399	0.456	0.265
0.6727	0.662	0.746	0.726
0.4833	0.542	0.542	0.247
0.6211	0.863	0.845	1.118
0.4912	0.516	0.509	1.034
0.7518	0.771	0.682	0.668
0	0.339	0.262	0.363
0.4393	0.829	0.728	0.61
0.3841	0.575	0.611	0.377
1.043	0.887	0.909	1.176
0.7406	0.937	0.883	0.451
0.1563	0.351	0.321	0.421

Lymphocyte	Plasma Cell	Macrophage	PMN	CT	Blood Vessel	Fibroblast	Space
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
23	0	0	16	33	16	11	1
2	0	0	1	75	8	12	0
10	2	0	6	40	3	27	0
9	0	0	3	39	0	17	32
8	0	5	5	53	10	19	0
11	0	0	2	50	9	25	1
7	0	0	1	58	6	28	0
5	0	0	2	67	6	20	0
0	0	0	0	0	0	0	0
7	0	0	2	55	2	28	6
5	0	1	9	49	6	24	6
0	0	0	0	0	0	0	0
24	2	2	3	44	2	19	5
1	0	2	4	50	10	33	0
8	0	0	3	50	7	29	3
9	0	0	6	52	11	20	2
7	0	1	2	57	15	18	0

Animal #	Area of inflammation	Supracrestal CT area	% INF	CEJ - apical extent of epithelium
295R	0.07346	0.26	0.282538462	0.4883
296L	0.02876	0.09973	0.288378622	0.08493
296R	0.09621	0.2344	0.410452218	0.4342
298L	0.01446	0.09679	0.149395599	0.1102
298R	0.2613	0.2818	0.927253371	0.4233
299L	0.014849	0.1046	0.141959847	0.2494
299R	0.1085	0.2192	0.494981752	0.3103
300L	0.021	0.1279	0.164190774	0.1003
300R	0.09871	0.1628	0.606326781	0.397
301L	0.01836	0.08773	0.209278468	0.2126
301R	0.12928	0.5449	0.237254542	0.2635
302L	0.07739	0.2496	0.31005609	0.06813
302R	0.3066	0.06737	4.550987086	0.2872
303L	0.041422	0.1382	0.299725036	0.08889
303R	0.08393	0.3003	0.279487179	0.2423
304L	0.02749	0.1226	0.224225122	0.1571
304R	0.1389	0.3803	0.36523797	0.2723
305L	0.06316	0.205	0.308097561	0.2231
305R	0.1726	0.5196	0.332178599	0.505

CEJ - bone	M1DB CEJ - bone µCT	M2MB CEJ - bone µCT	M2DB CEJ - bone µCT
0.7284	0.892	0.928	1.191
0.3492	0.506	0.611	1.416
0.7414	1.093	1.297	0.628
0.283	0.354	0.427	0.262
0.8627	0.61	0.634	0.421
0.4948	0.592	0.726	0.392
0.8558	0.901	0.944	0.552
0.2959	0.416	0.483	0.538
0.5529	0.658	0.634	0.653
0.338	0.454	0.497	0.261
0.873	0.65	0.731	0.723
0.345	0.496	0.496	0.497
0.4352	0.702	0.688	0.552
0.3942	0.487	0.579	0.438
0.6772	0.868	0.826	0.818
0.3818	0.52	0.526	0.384
0.6412	0.695	0.704	1.278
0.4365	0.717	0.599	0.569
0.6878	0.546	0.81	1.408

Lymphocyte	Plasma Cell	Macrophage	PMN	CT	Blood Vessel	Fibroblast	Space
7	1	0	0	59	11	22	0
12	2	0	0	57	0	28	0
15	1	1	1	65	0	14	3
11	0	0	2	54	3	30	0
27	0	1	13	38	12	8	1
17	0	0	8	52	0	21	4
12	0	0	2	37	13	29	7
7	0	0	6	60	6	17	5
21	1	0	7	37	10	23	1
3	0	0	1	63	5	27	0
7	0	0	1	69	7	16	0
5	0	0	2	64	10	17	2
7	1	0	2	60	1	19	0
10	1	0	0	60	2	27	0
11	0	0	2	68	5	12	2
11	0	0	0	61	3	16	9
5	1	0	4	62	6	21	0
6	0	0	1	74	5	14	0
10	1	0	5	58	5	19	2

Animal #	Area of inflammation	Supracrestal CT area	% INF	CEJ - apical extent of epithelium
306L	0.0431	0.1841	0.234111896	0.07888
306R	0.2223	0.6022	0.369146463	0.1489
307L	0.02951	0.1032	0.285949612	0.1843
307R	0	0	0	0
308L	0	0	0	0
308R	0.1129	0.3755	0.300665779	0.4524
309L	0.04346	0.203	0.21408867	0.1579
309R	0.05539	0.2403	0.230503537	0.8195
310L	0.02961	0.1432	0.206773743	0.1021
310R	0.1432	0.5387	0.265825135	0.2229
311L	0.06287	0.1427	0.440574632	0.2041

CEJ - bone	M1DB CEJ - bone µCT	M2MB CEJ - bone µCT	M2DB CEJ - bone µCT
0.3283	0.344	0.37	0.262
0.7032	0.735	0.657	0.844
0.3417	0.475	0.481	0.23
0	0	0	0
0	0	0	0
0.8342	0.851	0.744	1.203
0.4861	0.753	0.89	0.741
0.8816	1.032	0.859	1.139
0.2272	0.403	0.325	0.218
0.7432	0.649	1.13	0.399
0.4173	0.542	0.455	0.524

Lymphocyte	Plasma Cell	Macrophage	PMN	CT	Blood Vessel	Fibroblast	Space
3	0	0	1	71	5	18	1
10	1	0	4	63	7	15	0
6	0	0	0	64	8	22	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
9	3	0	6	57	2	22	1
8	0	0	2	70	8	10	2
6	0	0	2	62	3	25	3
5	0	0	0	72	5	18	0
5	0	0	1	71	3	20	0
7	0	0	1	63	8	21	0

Animal #	Area of inflammation	Supracrestal CT area	% INF	CEJ - apical extent of epithelium
311R	0.07683	0.2554	0.30082224	0.3163
312L	0.03843	0.1483	0.259136885	0.1664
312R	0.1102	0.2439	0.451824518	0.3204
313L	0.04793	0.1588	0.301826196	0.1464
313R	0.06563	0.4037	0.162571216	0.2082
314L	0.05452	0.1602	0.340324594	0.1735
314R	0.164	0.3915	0.41890166	0.23
315L	0	0	0	0
315R	0.0633	0.2273	0.278486582	0.224
316L	0	0	0	0
316R	0.05354	0.2452	0.218352365	0.1441

CEJ - bone	M1DB CEJ - bone µCT	M2MB CEJ - bone µCT	M2DB CEJ - bone µCT
0.4034	0.508	0.546	1.277
0.3171	0.423	0.437	0.416
0.456	0.451	0.52	0.668
0.4123	0.481	0.643	0.962
0.6358	0.959	0.734	1.118
0.4716	0.507	0.445	0.6
0.7567	0.69	0.771	0.704
0	0.377	0.345	0.422
0.4981	0.501	0.542	0.581
0	0	0	0
0.4403	0.753	0.793	0.613

Lymphocyte	Plasma Cell	Macrophage	PMN	CT	Blood Vessel	Fibroblast	Space
9	3	0	2	68	2	15	0
4	0	0	0	69	10	16	0
21	1	0	2	54	6	15	1
15	1	0	0	54	5	24	1
8	1	0	2	62	7	18	2
13	0	1	1	63	2	20	1
17	1	0	6	49	5	16	6
0	0	0	0	0	0	0	0
5	0	0	1	64	7	23	0
0	0	0	0	0	0	0	0
12	1	1	0	60	3	24	0

KEY:

276-283 = Ligature-alone

284-287 & 292-295 = mPEG

288-291 & 296-300 = 0.5 mg SIM/SIM-mPEG

301-308 = 1.0 mg SIM/SIM-mPEG

309-316 = 1.5 mg SIM/SIM-mPEG

Animal #	Starting Weight	Finishing Weight	Difference
276	304	318	-14
277	296	289	7
278	304	297	7
279	324	310	14
280	309	314	-5
281	323	331	-8
282	315	310	5
283	326	348	-22
284	328	324	4
285	299	309	-10
286	300	295	5
287	314	317	-3
288	292	294	-2
289	283	277	6
290	270	278	-8
291	300	309	-9
292	273	279	-6
293	297	295	2
294	330	315	15
295	326	313	13

Animal #	Starting Weight	Finishing Weight	Difference
296	310	315	-5
298	302	301	1
299	288	292	-4
300	324	312	12
301	348	340	8
302	325	317	8
303	401	394	7
304	303	298	5
305	353	342	11
306	353	335	18
307	334	346	-12
308	352	352	0
309	324	320	4
310	340	326	14
311	337	327	10
312	309	300	9
313	342	334	8
314	336	322	14
315	340	349	-9
316	328	308	20