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Evaluating the Efficacy of CXCR3-antagonist in Mediating Peri-implant Bone Loss

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Evaluating the Efficacy of CXCR3-antagonist in Mediating

Peri-implant Bone Loss

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Oral Biology

by

Ryan Lee Wong

2019

ABSTRACT OF THE THESIS

Evaluating the Efficacy of CXCR3-antagonist in Mediating

Peri-implant Bone Loss

by

Ryan Lee Wong

Master of Science in Oral Biology

University of California, Los Angeles, 2019

Professor Flavia Queiroz de Mo Pirih, Chair

BACKGROUND: Dental implants are subject to peri-implantitis, which is an inflammatory condition mediated by bacterial insult that leads to irreversible crestal peri-implant bone loss. Unfortunately, treatment modalities to arrest the condition from progressing are unpredictable. With an estimated 45% of all patients with implants that suffer from this condition, it is imperative to further study the disease to improve treatment protocols. A CXCR3-antagonist drug has been developed to inhibit immune cell migration by preventing CXCL9 and CXCL10-dependent chemotaxis. The drug, AMG-487, acts by blocking the CXCR3 receptor. Preliminary studies have shown its effectiveness in reducing periodontal bone loss in mouse models. In this study, we wish to explore its role in reducing peri-implant bone loss.

OBJECTIVE: To evaluate the efficacy of AMG-487 to reduce peri-implant bone loss.

MATERIALS AND METHODS: One-month old C57BL/6J mice had their left maxillary molars extracted. Custom-made titanium implants were placed after eight weeks of healing and were allowed to osseointegrate for one month. The mice were separated into control, ligature, and CXCR3 antagonist groups. 6-0 silk ligatures were placed around the head of the implants, and the mice were injected twice daily for two weeks with vehicle or AMG-487. The mice were then sacrificed and fixed for histology, and scanned with micro-CT for radiographic analysis.

RESULTS: The experimental groups experienced significant bone loss compared to the control group. However, there were no statistically significant differences between the ligature group and the CXCR3 antagonist group. Both experimental groups revealed soft tissue swelling and increased inflammatory infiltrates relative to the control group, but no clear differences were observed between the experimental groups. The osteoclast numbers increased significantly in the experimental groups. The CXCR3 antagonist group showed greater COX-2 staining than control, but appeared to have stained less than the ligature group.

CONCLUSION: This current study was not able to demonstrate the efficacy of the CXCR3 antagonist to sufficiently reduce ligature-induced peri-implant inflammation and bone loss.

The thesis of Ryan Lee Wong is approved.

Paulo M. Camargo

Perry Klokkevold

Sotirios Tetradis

Flavia Queiroz de Mo Pirih, Committee Chair

University of California, Los Angeles

2018

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And last but not least, I would like to thank all the laboratory members, Gregory Chan, Cameron Hankins, Kearny Chang, Dr. Rajvee Bhakta, and Dr. Makiko Ishii, and my great friend, Adam Neal, who have all really truly exemplified the meaning of great teamwork and have played a pivotal role in this project.

INTRODUCTION

Implants are a popular and highly desirable option to replace missing teeth[1]. Despite great outcomes in patient treatment, implants are subject to complications, such as peri-implantitis, which could ultimately lead to implant failures[2]. Peri-implantitis is "characterized by inflammation in the peri-implant mucosa leading to progressive loss of supportive bone[3]". It is an inflammatory condition mediated by bacterial insult that leads to soft and hard tissue destruction around osseointegrated implants in function[3,4,5]. A study performed in a Swedish population reported that peri-implantitis affects approximately 45% of all patients and 14.5% of patients present with the moderate to severe form[6].

Peri-implantitis cases are expected to increase given the increasing number of implants being placed yearly[7]. This is a growing concern because peri-implantitis does not respond well to treatment, as no protocol has proven to be predictably effective at arresting peri-implantitis from progressing[8,9]. Current treatment modalities used are derived from our therapeutic approaches to treat periodontitis[10,11]. This is because peri-implantitis and periodontitis share similar etiologies, as both diseases are related to bacterial infections and the host immune response[5,12,13]. Attempts to treat peri-implantitis non-surgically have been shown to be ineffective, whereas surgical therapy has resulted in varying and unpredictable degrees of disease resolution[9,14]. The mechanical process of subgingival calculus and biofilm removal is crucial in the treatment of periodontitis[15]. The decontamination process of implants, however, is not as

straightforward as scaling and root planing of teeth given the surface properties of implants[16]. Therefore, we propose to tackle peri-implantitis by attempting to reduce the inflammatory response pharmaceutically.

A study evaluating the genetic component of periodontitis utilized the Hybrid Mouse Diversity Panel and found a wide variation of lipopolysaccharide (LPS) - induced bone loss among different strains[17]. The parental mouse strains A/J and C57BL/6J showed the lowest and greatest susceptibility to bone loss, and further studies comparing these two strains revealed increased expression of the proinflammatory chemokines CXCL9 and CXCL10 in C57BL/6J[18]. Several cells, such as monocytes, endothelial cells, and fibroblasts secrete these chemokines to attract T-cells, macrophages, dendritic cells, and natural killer cells to sites of infection[19,20]. CXCL9 and CXCL10 are interferon (IFN)-y-inducible ligands that bind to the CXCR3 receptor[20]. CXCR3 appears to be absent on naïve T-cells, but gets upregulated during inflammation to allow these cells to infiltrate into the tissues[20,21]. Interestingly, the receptor has also been implicated in other inflammatory diseases such as arthritis and diabetes to name a few[22,23]. The periodontitis study further explored the role of these chemokines by repeating the experiment in CXCR3 knockout mice, and in wild type mice treated with an antagonist to the receptor, and found a significant decrease in the amount expected of bone loss[18] (Figure 1).

The drug developed to block the CXCR3 receptor is AMG-487[24,25]. This drug acts to inhibit immune cell migration by preventing CXCL9 and CXCL10-dependent chemotaxis. Several studies have explored the use of AMG-487 as a therapeutic agent for systemic diseases; e.g. one murine model of metastatic breast cancer showed that CXCR3 antagonism inhibits lung metastasis[26], and another one demonstrated how CXCR3 antagonism attenuated spontaneous itch in experimental allergic contact dermatitis[27]. In our study, we wish to study the role of the CXCR3 receptor in peri-implantitis by evaluating the efficacy of AMG-487 to reduce peri-implant bone loss.

MATERIALS AND METHODS

Animals:

29 C57BL/6J wild type mice from the Jackson Laboratories (Bar Harbor, ME, USA) were utilized and separated into the following groups (Fig. 2):

- 8 Control (no ligature) + vehicle

- 10 Ligature + vehicle

- 11 Ligature + CXCR3 Antagonist

Vehicle: hydroxypropyl-β-cyclodextrin (Sigma Aldrich, MO, USA)

CXCR3 Antagonist: AMG-487 (Tocris, R&D Systems, MN, USA)

Implant Surgeries:

One-month old mice had their left maxillary molars extracted followed by eight weeks of healing. Custom-made titanium implants (1mmx0.5mm) were placed and allowed to osseointregrate for one month. 6-0 silk ligatures were placed around the head of the implants for two weeks to induce peri-implant inflammation and bone loss[28].

AMG-487 and Vehicle Injections:

The mice were injected twice daily intraperitoneally for two weeks with either vehicle or AMG-487. AMG-487 was reconstituted in a 50% hydroxypropyl-β-cyclodextrin in a sonicating water bath for 2 hours with occasional vortexing. After the AMG-487 powder was completely dissolved, distilled water was added to make a final concentration of 20% hyroxypropyl-β-cyclodextrin

solution. The vehicle injection was carried out utilizing the 20% hyroxypropyl-β-cyclodextrin solution for either the control or ligature group. For the CXCR3 antaognist group, the mice were injected with AMG-487 at a concentration of 5μg/g[26].

Micro-CT Analysis:

Maxillae were scanned using a μCT scanner (Skyscan 1172; Skyscan, Aartselaar, Belgium) at 10μm resolution and X-ray energy 55 kVP and 181 μA. Both linear and volumetric measurements were performed to assess peri-implant bone loss. Linear data was converted into DICOM format and analyzed with Dolphin Software (Chatsworth, CA), and the volumetric data was analyzed using CtAN (V.1.16 Bruker, Billerica, MA, USA). For the linear bone height measurements, the implants were oriented in the sagittal and coronal planes so that the head and the body of the implant were perpendicular to each other. The distance from the junction of the head of the implant to the crest of the bone was measured on the buccal, palatal, mesial and distal surfaces, and averaged to a mean linear bone height distance per implant (n ≥ 8 mouse/group). For the volumetric analysis, the samples were oriented similarly as mentioned for the linear protocol, but using DataViewer (V.1.5.2 Bruker, Billerica, MA). The top and bottom limits for the volumetric analysis in the axial plane was determined by the lowest average of the linear data in the control group and the highest average linear data in the experimental groups. The bone loss around the body of the

implant was traced and averaged to account for the circumferential volume void of bone per group ("tissue volume (mm^3)").

Histological Analysis:

The specimens were fixed in 10% buffered formalin solution for 48 hours and were decalcified in 15% ethylenediaminetetraacetic acid (EDTA) for four weeks. Once decalcified, the ligatures were removed and the implants unscrewed counterclockwise. The samples were cut sagitally directly adjacent to the implant sockets and were embedded into paraffin. The sections were cut 5µm thick, and were stained with hematoxylin and eosin (H&E) to evaluate inflammatory infiltrate and with tartrate-resistant acid phosphatase (TRAP, Sigma Aldrich, MO, USA) to assess and quantify osteoclasts. Stained cells that were multi-nucleated and lining bone on the distal surface of the implants were counted. General inflammation was assessed qualitatively through immunohistochemistry using anti-Cox-2 (1:250, ab15191 Abcam, Cambridge, UK).

Statistics:

Linear bone height and volumetric analyses, and osteoclast quantification were represented as mean ± standard error of the mean. A two-way analysis of variance (ANOVA) was used to compare significance between groups (Prism 5; GraphPad Software, Inc. La Jolla, CA).

Study Approval

This study followed the guidelines and protocols of the Chancellor's Animal Research Committee of the University of California, Los Angeles, and that of Animal Research: Reporting In Vivo Experiments (ARRIVE). The mice were fed a soft diet ad libitum (For the duration of the study,Bio Serve; Frenchtown, NJ).

RESULTS

Clinical assessment of soft tissues:

After the maxillae were harvested, clinical images were taken immediately using a digital microscope to assess the soft tissue differences (Fig. 3). The control group without ligature showed no clinical signs of soft tissue swelling, as the gingiva was flush with the implant head and the palatal rugae were clearly defined. Both experimental groups with the ligatures showed soft tissue swelling, as the gingiva appeared edematous, hyperplastic over the implant head, and with loss of definition of the palatal rugae.

Radiographic assessment of hard tissues:

To radiographically compare the ability of the CXCR3 antagonist to decrease peri-implant bone loss in the presence of ligature-induced inflammation, the maxillae were scanned with micro-CT and analyzed for linear bone height and volumetric bone loss differences (Figure 4A-D). Both analysis protocols revealed a similar pattern in the results. Utilizing the control as the baseline bone level/volume, both experimental groups experienced statistically significant bone

loss. However, there were no statistical significant differences between the ligature group and the CXCR3 antagonist group.

Histologic assessment:

The H&E stain provided an overall qualitative assessment of the inflammatory changes on a microscopic level (Fig 5A-B). The soft tissue in the experimental groups appeared to contain more inflammatory infiltrates, suggested by polymorphonuclear leukocytes (PMNs) infiltration, as compared to the control group. Given the increased soft tissue swelling in the experimental groups, therefore increased soft tissue thickness, there was an overall greater infiltration of PMNs. The comparison between the ligature and the CXCR3 antagonist group did not reveal clear differences, as the density of infiltrated PMNs appeared to be relatively similar in both groups.

Osteoclast assessment:

Osteoclasts are the cells responsible for bone resorption, thus the bone loss in peri-implantitis. Osteoclasts were stained with TRAP (Fig. 6A-B). Multinucleated cells that lined the distal bone adjacent to implants were counted. Three sections per mouse were averaged to a total value per sample (n ≥ 3 for all groups). There was a statistical increase in the number of osteoclasts in the experimental groups relative to the control group. The ligature and the antagonist groups showed no statistical difference relative to each other.

Cyclooxygenase-2 assessment:

To qualitatively further assess general inflammation, immunohistochemistry was performed to stain for the inflammatory marker COX-2 (Fig. 7). The ligature group showed greater staining when compared to the control group. The CXCR3 antagonist group also revealed greater staining than the control group; however, it appeared that there was a decrease in the stain relative to the ligature group.

DISCUSSION

Given the increasing popularity of dental implants to replace missing or compromised teeth, there is a reduced emphasis on saving questionable teeth affected by caries, pulpal infections, or periodontal disease[29]. Dental implants, however, can develop a biologically driven complication such as peri-implantitis. Different treatment approaches have been explored to manage the disease. According to the European Federation of Periodontology, non-surgical treatment is unpredictable and has limited evidence of effectiveness. Peri-implantitis appears to respond better to surgical treatment, though the degree of resolution varies and the current evidence does not support one particular surgical treatment over another[30]. In this study, we attempted to explore a pharmaceutical approach to help decrease peri-implant inflammation and bone loss.

The overexpression of the CXCL9 and CXCL10 chemokines, found in the high bone loss phenotype strain of the LPS-induced periodontitis model[18], paved the way to study their influence in peri-implantitis. These chemokines are ligands to the CXCR3 receptor, and this ligand-receptor complex has been implicated in many inflammatory conditions both in human and mouse models, e.g. liver disease, graft rejection, cardiovascular disease, allergic reactions, and autoimmune diseases like rheumatoid arthritis, type I diabetes mellitus, and systemic lupus erythematosus[20,22,23,27,31,32].

The main function of the ligands is to act as signaling molecules to attract CXCR3+ immune cells such as T-cells, natural killer cells, and dendritic cells to

sites of infection[20]. This inflammatory pathway has an inherent self-amplification loop, which may contribute to the exacerbation of chronic inflammatory diseases and autoimmune conditions.

Herein, we aimed to study the influence of the CXCL ligands by the means of the CXCR3 antagonist, AMG-487, in the ligature-induced peri-implantitis model[28]. The mice had ligatures around the implants for two weeks and were injected twice daily with the antagonist for the duration of the insult. The purpose was to assess the efficacy of AMG-487 to sufficiently mitigate the induced peri-implant inflammation and consequently decrease the amount of expected bone loss.

Our clinical and radiographic results did not reveal differences between the ligature and the antagonist groups. Both experienced soft tissue swelling in the presence of the ligatures and the radiographic analyses revealed statistically similar linear and volumetric bone loss. The bone loss data corroborated with the TRAP analysis, as the experimental groups experienced a similar increase in the number of osteoclasts relative to the control group. Qualitatively, the histological specimens were also unable to clearly demonstrate differences between the experimental groups. In relation to controls, there was a noticeable increase in inflammatory infiltrates and COX-2 expression. COX-2 immunohistochemistry was utilized to assess general inflammation, as the enzyme is selectively induced at sites of inflammation by pro-inflammatory cytokines[33]. There appeared to be a decrease in the staining of COX-2 in the AMG-487 group as compared to the

untreated ligature group, but not enough to warrant conclusive statements. This finding does, however, suggest that the ineffectiveness of the antagonist drug to reduce inflammation could be attributed to the experimental design.

We did not have a positive control to test if the drug actually worked; for instance, it is possible that it was not dissolved properly or was mishandled. Running a LPS-induced periodontitis experiment, as published by Hiyari et al., would have helped determine if the drug functioned as intended. Next, we opted to utilize the ligature model of peri-implantitis because it allows us to bypass differences in the colonization of bacteria and the configuration of the bone loss attained resembles the defect observed in humans[34]. The model that identified the overexpression of the CXCL9 and CXCL10 ligands utilized LPS from *Porphyromonas gingivalis*, which is a potent stimulant of the immune system[35]. It has been demonstrated that these two models signal periodontal bone resorption through different inflammatory pathways, as the LPS model is Toll-like receptor 4 (TLR4) dependent, whereas the ligature model is not[36]. Interestingly, CXCL10 is also a ligand to TLR4[37]. It is possible that the ligature model may not be as effective at inducing the overexpression of the ligands, and relative to LPS, the CXCR3 antagonist may not be as effective at reducing inflammation pertaining to that specific pathway.

Another difference to consider is that periodontitis and peri-implantitis may not respond similarly to the CXCR3 antagonist, which is in line with the fact that peri-

implantitis does not respond as predictably to treatment as periodontitis. It is suggestive that these two conditions have pathophysiological differences requiring alternative treatment approaches.

CONCLUSION

In this current study, the CXCR3 antagonist treatment was not effective at reducing peri-implant inflammation and bone loss. Given the limitations of the ligature model and the lack of a positive control, further research is indicated to better evaluate the role of the CXCL9 and CXCL10 chemokines, and their respective receptor, CXCR3, in per-implantitis.
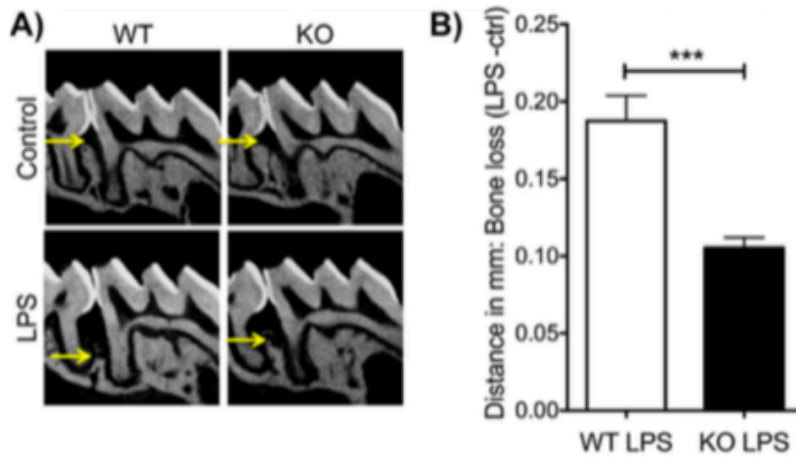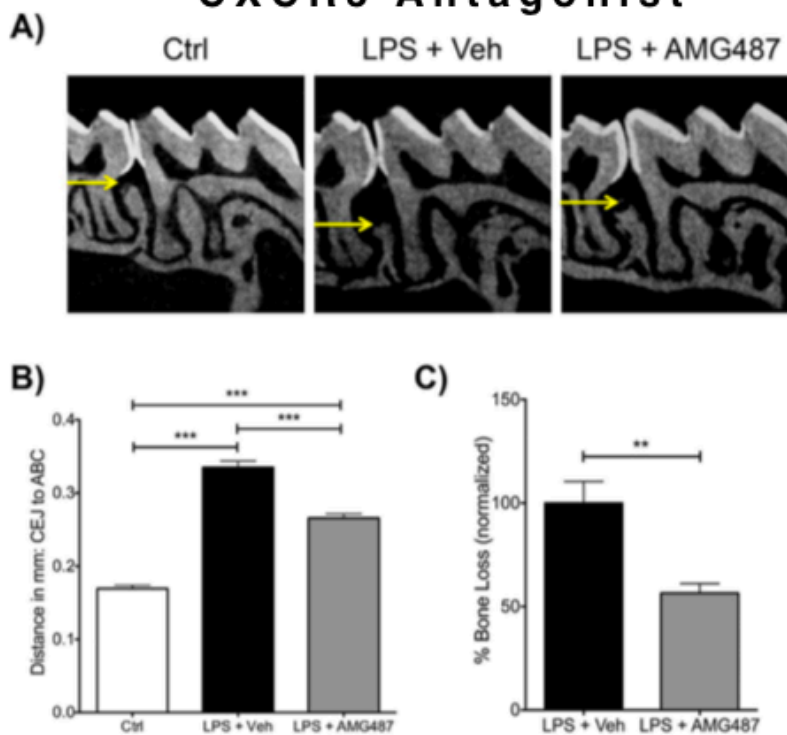
Figure 1: Preliminary data, Hiyari et al. 2018. Lipopolysaccharide-induced periodontitis using a CXCR3 knockout mouse model (n>5 mice/group) and a CXCR3 antagonist mouse model (n>5 mice/group). Manipulation of the CXCR3 receptor resulted in statistically significant decrease in bone loss in both models.
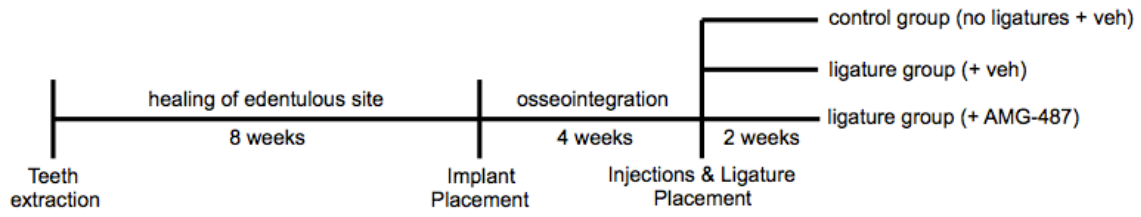
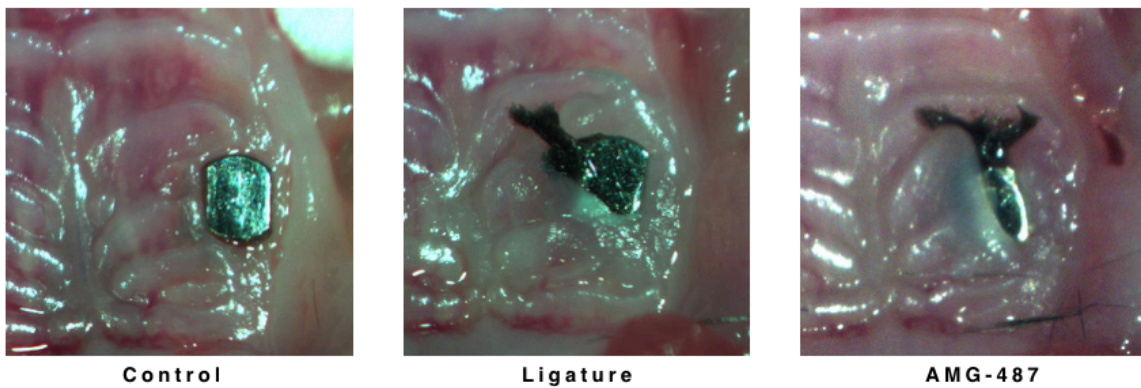Figure 2: Schematic diagram of the experimental design

Figure 3: Clinical evaluation of soft tissues. Representative clinical images of control (no ligature), ligature, and CXCR3 antagonist, AMG-487, groups. Notice increased soft tissue swelling and irregularity of the gingiva in the presence of the ligature around the implants.
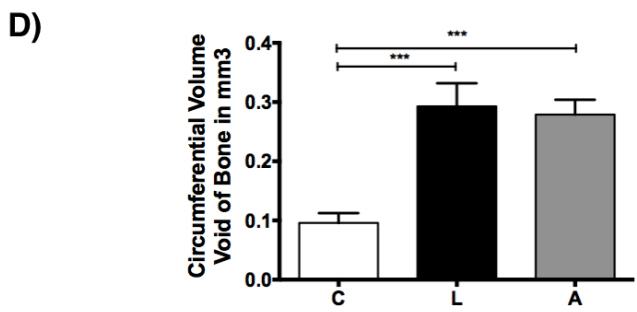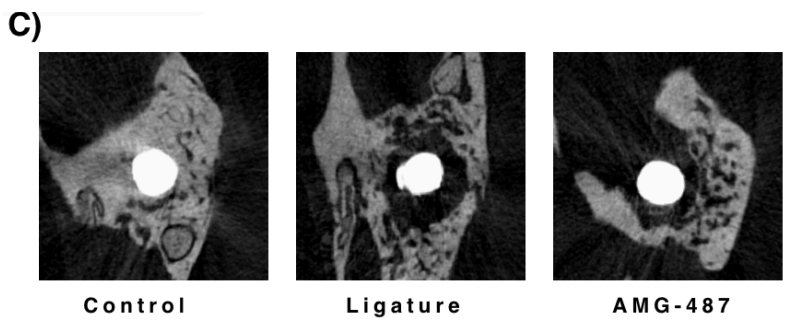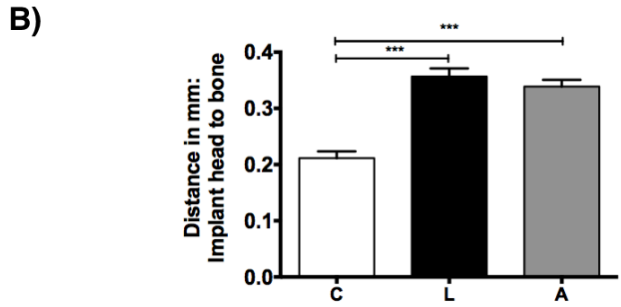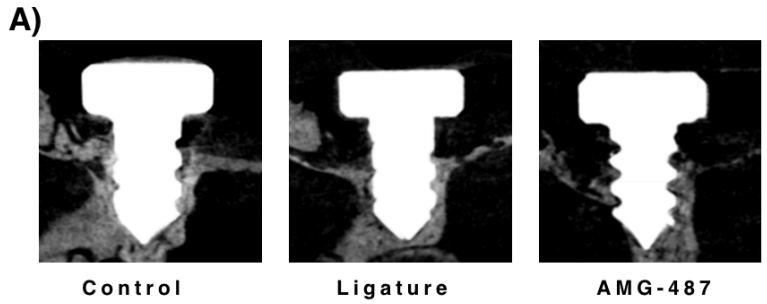
Figure 4: Radiographic analysis of bone loss. A) Representative images of the linear bone height analysis (B) in a sagittal plane. C) Representative images of the volumetric bone loss analysis (D). The control group (C) represents the expected distance from the implant head to the bone and the expected volume void bone in healthy mice. The ligature group (L) and the CXCR antagonist,

16

AMG-487, group (A) experienced statistically significant bone loss relative to the control group. There were no statistical significant bone loss differences between the ligature and the antagonist group. Data are represented as mean ± standard error of the mean. ***$p < 0.001$, ($n \geq 8$ for all groups).

A)

Control   Ligature   AMG-487

B)

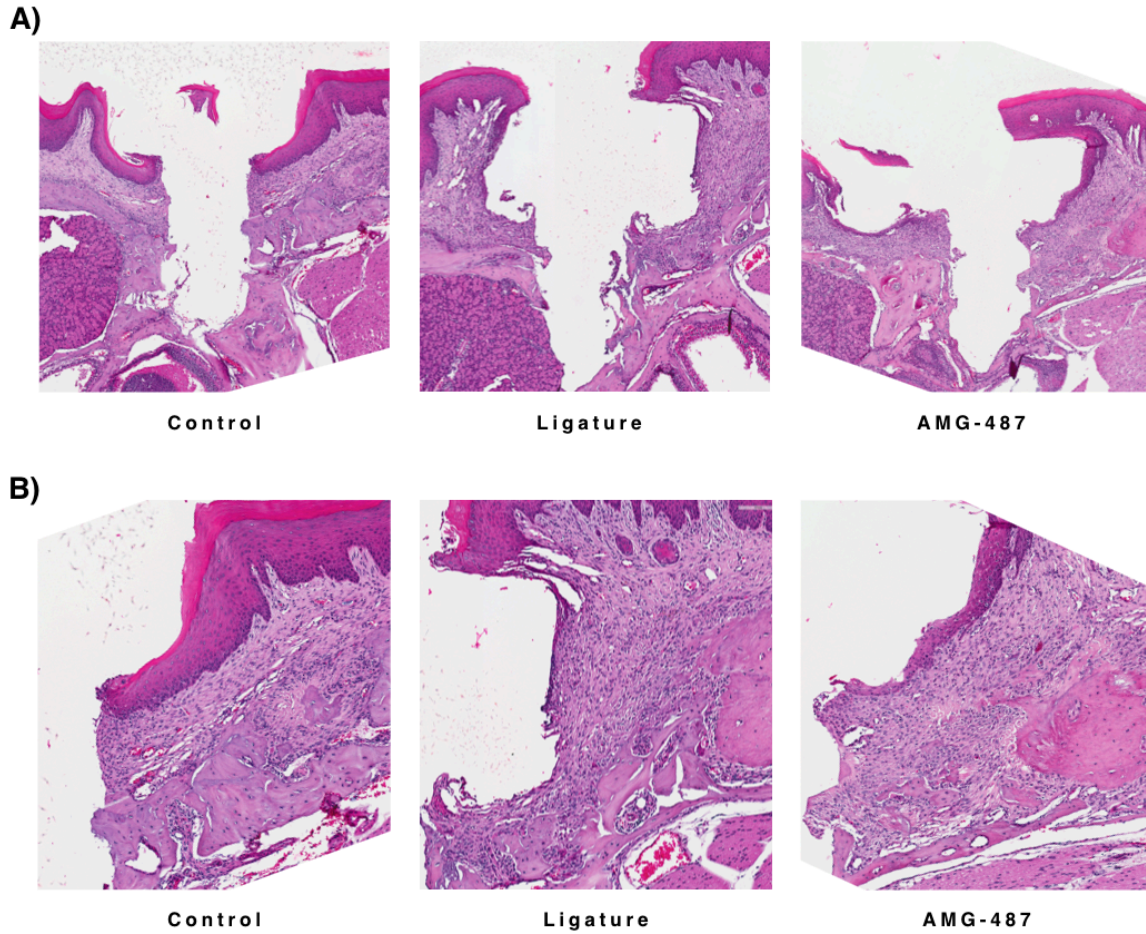Control   Ligature   AMG-487

Figure 5: Histologic assessment of assessment of inflammatory infiltrate via hematoxylin & eosin staining at 10X (A) and 20X (B) magnification. Notice the increased soft tissue thickness and inflammatory infiltrates represented by dark purple stained cells in the experimental groups.
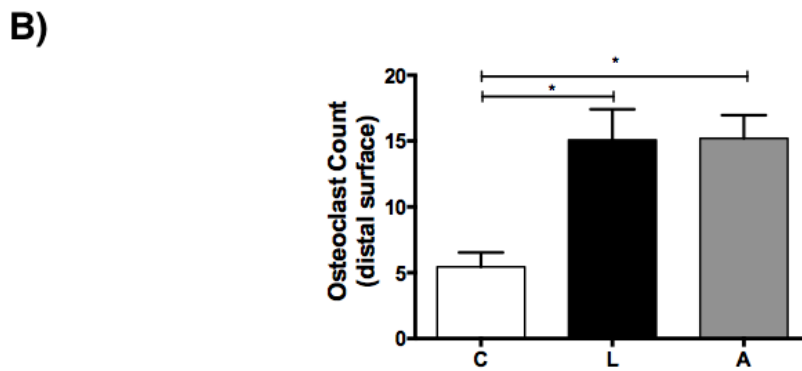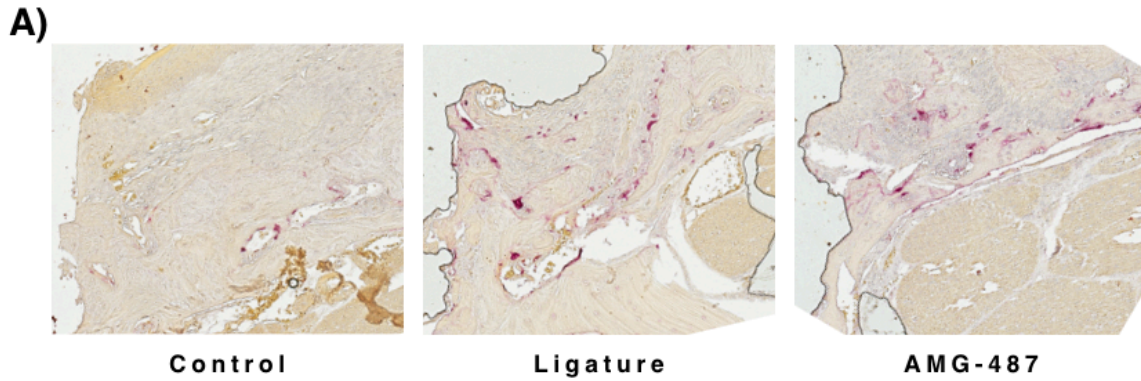
Figure 6: Histologic assessment of osteoclasts. Stained multinucleated cells that lined the distal bone adjacent to implants were counted. Three sections per mouse were averaged to a total value per sample. There was a statistical increase in the number of osteoclasts in the experimental groups relative to the control. There was no difference between the ligature and the antagonist group. *p < 0.05, (n ≥ 3 for all groups).
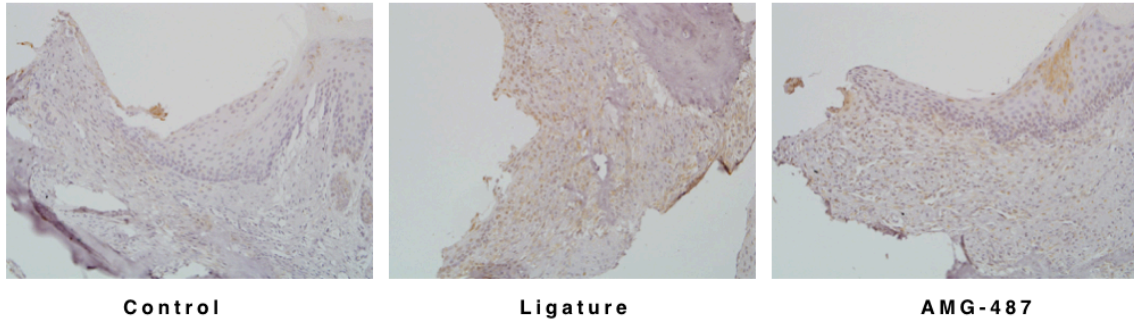
Control   Ligature   AMG-487

Figure 7: Histologic assessment of COX-2. Notice the increase in staining in the ligature group relative to the control group. The CXCR antagonist appeared to have less staining than the ligature group.

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