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The Therapeutic Potential of Wnt4 in Peri-Implantitis

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The Therapeutic Potential of Wnt4
in Peri-Implantitis

A thesis submitted in partial satisfaction of the
requirements for the degree Master of
Science in Oral Biology

by

Julia Wan Chen

2017
ABSTRACT OF THE THESIS:

The Therapeutic Potential of Wnt4 in Peri-Implantitis

By

Julia Wan Chen

Master of Science in Oral Biology

University of California, Los Angeles 2017

Professor Flavia Queiroz de Mo Pirih, Chair

Background: Peri-implantitis is an inflammatory disease process that presents with bone loss around implant fixtures. Without the proper treatment, it can lead to the eventual loss of the implant. However, controversy lies over the effectiveness of treatment modalities as the criteria for disease diagnosis and success of treatment varies in the literature. With an increasing global burden of disease comes incentive to find predictable treatment options for peri-implantitis. Wnt4 has been shown in prior studies to attenuate osteoporotic bone loss and prior studies in our lab using a murine model for peri-implantitis, found transgenic Wnt4 mice to exhibit the same pattern of attenuated bone loss in the maxilla.

Objective: To identify whether experimental recombinant Wnt4 (rWnt4) injection shows attenuated bone loss compared to a control PBS injection, in a murine model of ligature induced peri-implantitis.
Methods: Four-week-old male C57BL/6J mice had their left maxillary molars extracted. Following eight weeks of healing, titanium implants were placed in the extraction sites and allowed to integrate for four weeks. After osseointegration, evaluated by presence and lack of mobility in the implants, a 6-0 ligature was tied under the head of the implant in half of the mice to induce peri-implantitis. Half of the mice did not receive ligature placement around the implants. Each group was then divided again to either receive daily control PBS injections or experimental rWnt4 injections intraperitoneally for 30 days. Four weeks after ligature placement and daily injections, the mice were sacrificed. The maxillae were imaged using micro-computerized tomography (μCT) scanning and linear and volumetric bone loss were analyzed. Tissues from the representative samples (based on proximity to mean measurements) were stained with hematoxylin and eosin (H&E), Picrosirius Red for collagen, and Tartrate Resistant Acid Phosphatase (TRAP) for osteoclasts. Immunohistochemistry (IHC) was also performed and analyzed for NF-κB, MMP-9, and Cox-2.

Results: Clinically, four weeks after ligature placement there was increased soft tissue edema present around implants from the ligature induced peri-implantitis group compared to the no-ligature group. Radiographically, linear bone loss analysis showed statistically greater bone loss in the ligature compared to no ligature group for both the PBS and rWnt4 injection groups. The same finding was observed for the volumetric bone loss analysis around implants. There was no statistical difference found in bone loss between the rWnt4 injected groups compared to PBS injections for both the ligature and no ligature groups. TRAP staining for osteoclasts showed statistically greater numbers of osteoclasts in the PBS injection ligature group compared to PBS injection no ligature group. Picrosirius red staining showed more organized collagen fibril distribution in rWnt4 ligature vs. PBS ligature
groups. There was also a decreased amount of immunoreactivity for MMP-9, NF-κB and Cox 2 on IHC staining for the rWnt4 ligature group compared to the PBS ligature group.

Conclusion: The findings from this research study validate the ligature-induced peri-implantitis murine model and show that ligature samples have greater tissue and bone loss, making this an ideal animal model to study this disease process. Although the bone loss exhibited by rWnt4 injections was not statistically significant, it provides a preliminary framework for future studies of Wnt4 effects on bone metabolism in the maxilla. The decreased amount of immunoreactivity shown in IHC staining of MMP-9, NF-κB and Cox-2 show a positive effect of decreased inflammatory response in the soft tissues surrounding implants in the injected rWnt4 injection groups, which could be translated to the treatment of peri-implant mucositis. Given that there was a decrease in inflammatory markers, a dose response study may allow us to identify a Wnt4 dose that has a positive therapeutic effect on peri-implantitis, which could be further translated to the treatment of peri-implantitis in humans.
The thesis of Julia Wan Chen is approved.

Paulo M. Camargo

Perry Klokkevold

Flavia Queiroz de Mo Pirih, Committee Chair

University of California, Los Angeles

2017
DEDICATION:

I would like to dedicate this thesis to my loving family: my parents Stephen and Nancy, for their never-ending support and encouragement in all my life endeavors, for working so hard to provide my brother and I with endless opportunities; and to my brother Frank, for always being there for me and supporting me every step of the way.
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INTRODUCTION:

Tooth loss is a major problem that faces a majority of the population in some form or another, ranging from partial to complete edentulism. This leads to a loss in quality of life. With the advent of dental implants and its incorporation into the dental professional’s repertoire to replace missing teeth, we have been able to improve not only patients’ function, but also phonation and overall well-being (1). Dental implants have become a popular treatment option for patients who want a fixed restorative option and who do not want to sacrifice adjacent teeth for dental prostheses. With continued research and development of new biomaterials and methodology, implants have become a predictable treatment option for patients.

Although dental implants have become a well-accepted treatment option with good survival rates, they do not come without problems. The same inflammatory processes that affect the natural dentition in the form of periodontitis also affect the implant surface and supporting bone and soft tissue and are classified as peri-implant diseases. There are two forms of the disease process – peri-implant mucositis and peri-implantitis. The term peri-implantitis was first introduced into the literature by Mombelli in 1987 and was described as an infectious disease with similarities to periodontitis (2, 3). They are both initiated by an inflammatory response in the soft tissues surrounding the implant fixture and restoration (4-7). If left untreated, it can lead to bone loss around the implant fixture that can lead to eventual loss of the implant. Despite the difficulty in designing studies to investigate this disease process, with most of them being longitudinal in nature, the prevalence of peri-implant diseases has been estimated to affect 45% of the population (8-12). There is controversy over an accurate estimate of the true prevalence of peri-implant disease with inconsistencies.
stemming from both the definition and reporting of peri-implant mucositis and peri-implantitis (3). More specifically, recent studies have shown that peri-implantitis with bone loss greater than 0.5 mm is exhibited in 52% and 66% of implants at 2 and 3 years respectively (13). This pathological disease process frequently presents in patients who receive implants (14) and has a higher frequency in patients who are smokers (3). The loss of implants leads to a burden of disease, which affects the way we treat patients and the long-term prognosis of dental implants. Thus, it is imperative for research and clinical studies to be done, to find a systematic way to prevent and treat peri-implant diseases.

Because we have a better understanding of treating periodontal diseases, similarities have been drawn between peri-implantitis and periodontitis, to try to find effective treatment modalities for peri-implant diseases. Treatment of peri-implant diseases includes scaling, air abrasion, bone grafting, laser therapy, adjunctive antimicrobial therapy, and soft tissue surgery (15-18). However, there is no set methodology or proven technique for predictable treatment of this disease process (19). This is perhaps due to the inherent differences that have been found between the two disease processes.

Studies have shown that peri-implant mucositis is similar to gingivitis in humans and that it is a reversible pathologic process (20). However, the inflammatory response in peri-implant soft tissues is stronger than their gingival counterparts (21). If left untreated, the disease process progresses, and peri-implant mucositis will progress to peri-implantitis. Peri-implantitis leads to clinical features, which include increased and deepened probing pocket depth, bleeding on probing, bone loss, clinical attachment loss, and similar microbiological makeup of bacteria within deeper pocket depths (5, 22, 23). What has been found to be different, is the extent and characteristics of the bony lesions we find in peri-implant
diseases – more symmetrical and circumferential around implants compared to the surface-specific lesions found in periodontitis (5). Histologically, there are differences as well: an inflammation free zone of connective tissue separates periodontitis from the alveolar bone, whereas inflammation in peri-implantitis penetrates deep into the bone and alveolar spaces (5). An implant, unlike a tooth, lacks a periodontal ligament. Instead of a junctional epithelium, there is a hemidesmososomal attachment at the epithelium-implant interface, which is weaker and perhaps more susceptible to breakdown during the disease process (24, 25).

Recent interest has been drawn to the wingless-type MMTV integration site (Wnt), which belongs to a family of 19 proteins that are divided into canonical and non-canonical ligands, based on their dependence on β-catenin transduction (26). They are secreted cysteine-rich glycoproteins, which can act as short-range ligands to activate receptor-mediated signaling pathways (27). Wnt signaling is critical in developmental biology and has been found to be involved in early axis specification, brain patterning, intestinal development, cancer pathways, limb development, and bone and stem cell biology (28-31). In relation to dentistry, there have been studies on the role of the Wnt family of proteins and their role in bone biology (32). It has been found that canonical β-catenin dependent Wnt signaling is required for bone formation (32). Wnt ligands secreted from osteoblasts are critical for the maturation and mineralization of osteoblasts (33). Canonical Wnt pathway acts through the formation of a complex of Wnt, Frizzled, and LRP5 or LRP6. LRP5/6 are Wnt co-receptors for this canonical signaling pathway (34).

Non-canonical Wnt5a is involved in osteoblast differentiation, which results from enhanced expression of Lrp5/6 (33). The non-canonical pathway is not dependent on β-catenin and
instead exerts its effects via Ca2+ and planar cell polarity pathway, which is mediated by small G proteins such as RhoA and Rac, and c-Jun and Jnk (34). Wnt5a enhances the expression of Rank in osteoclast precursors, which then promote Rankl-induced osteoclast formation (33). More recently, studies have suggested that Wnt4 proteins can attenuate osteoporotic bone loss and inflammation via inhibition of nuclear factor-kappa B through non-canonical Wnt signaling (35). Yu et al. also showed that Wnt4 was able to inhibit osteoclast formation and bone resorption in transgenic (TG) mice expressing Wnt4 from osteoblasts. Wnt4-TG mice were protected from estrogen deficiency-induced bone loss and tumor necrosis factor alpha induced bone loss (35). A subsequent study then tested the inhibitory effects of Wnt4 on osteoclast formation in culture. Although it failed to inhibit Rankl-induced osteoclast formation, it did inhibit 1α-25(OH)2D3-induced osteoclast formation in co-cultures prepared from WT mice, but not in the mice that did not have the Opg gene. They suggested this meant that Wnt4 inhibits osteoclast formation via the expression of Opg (36).

An earlier study by Chang et al. discovered that Wnt4 enhanced in vitro osteogenic differentiation of mesenchymal stem cells (MSCs) isolated from human adult craniofacial tissues and promoted bone formation in vivo (37). They found that p38 MAPK was activated by Wnt4 in a non-canonical pathway, to lead to this effect. This was done by transfecting Wnt4 via retroviruses into the MSCs (37). Thus, expression of Wnt4 through a genomic route was effective in eliciting positive results in the craniofacial tissues, similar to our earlier data on decreased bone loss in transgenic Wnt4 mice.
PRELIMINARY DATA:

Previously, Pirih et al. developed a murine model of experimental peri-implantitis (38). By placing machined, smooth-surfaced, screw-shaped titanium implants into the maxilla of healed extraction sites and tying 6-0 silk sutures around implants, they have been able to develop a reliable method to study peri-implantitis in mice. μCT imaging and bone loss analysis showed that there was significantly greater bone loss around implants with ligatures (Fig. 1A and 1B). Histological staining with toluidine blue showed the same increased bone loss around implants and increased inflammatory infiltrates (Fig. 1C). The model serves as a platform for studying the pathogenic mechanisms of peri-implantitis, in order to evaluate potential treatment interventions and is utilized in this study.

Using transgenic Wnt4 mice and the findings from Yu et al. in this murine model of peri-implantitis, our lab has been able to study the effects of peri-implantitis in transgenic Wnt4 mice (Fig. 2). The findings are in line with the results and data from Yu et al. (35), where we show that transgenic Wnt4 mice, which overexpress the Wnt4 protein, have attenuated bone loss in cases of ligature-induced peri-implantitis in comparison to control mice. Measurements of linear bone loss averaged around the implants, normalized against their control group, show a statistically significant amount of decreased bone loss in the Wnt4 TG group vs. controls for the ligature experimental group.

Based on the findings from Yu et al. and this unpublished data from our laboratory, we have supporting evidence for the protective effects of Wnt4 in a transgenic murine model with experimentally induced conditions of peri-implantitis (38). It has been a goal in the field of periodontics and dentistry, to find a more predictable way to treat peri-implantitis and to
prevent the bone loss that we see around implants, often leading to their failure. Given the positive results we see in preventing bone loss in the transgenic Wnt4 murine model, it would be useful to find a more applicable therapeutic delivery model to potentiate the clinical effects of Wnt4.

Thus, in our study, we used the same ligature-induced peri-implantitis murine model developed by Pirih et al., to study the local delivery of injectable recombinant mouse Wnt4 protein, to investigate whether we see the same positive therapeutic results of attenuated bone loss around implant fixtures found in preliminary results, in a more clinically applicable vehicle. Research done with a murine model provides many benefits, as the mouse is small in size, easier to obtain, and has a sequenced genome (39) that offers a complete database to facilitate genetic and biological testing, to study disease processes. Taken together, we hypothesized that Wnt4 could have a therapeutic application for treatment of peri-implantitis and will elucidate our findings in the following thesis.
MATERIALS AND METHODS:

The general experimental timeline of the study is shown in Figure 3. Four-week-old male C57BL/6J mice were used for our study. Our study adhered to the Chancellor’s Animal Research Committee at the University of California, Los Angeles’ approved protocol and guidelines. The mice had their first, second, and third maxillary molars extracted as previously described (38).

Following tooth extraction, the remaining sockets healed for 8 weeks. Mice were given antibiotics diluted in their drinking water for 4 of the 8 weeks. Custom designed machine-surfaced screw type titanium implants, machined from 6AL4V titanium rods (DP Machining Inc. La Verne, CA) were placed into the healed extraction sites in the first/second maxillary molar area, one-per-mouse, and allowed to osseointegrate for 4 weeks. Osteotomy was made with a 0.03 mm carbide micro hand drill (BIG Kaiser Precision Tooling Inc. Hoffman Estates, Illinois). Implants were placed into the osteotomy site by hand, using a clock-wise screwing motion, until the head of the implant was at the crestal bone level. Mice were anesthetized using 3% isoflurane during all surgical procedures and received pain medication intraperitoneally. During the healing period, mice were given antibiotics orally in the drinking water for four weeks after implant placement.

One month after our one-stage implant placement, 6-0 silk ligatures were placed around the implant head to induce peri-implantitis. Control mice did not receive ligatures. Peri-implantitis was given the opportunity to develop for one month following ligature placement. During the entire course of peri-implantitis progression, recombinant mouse Wnt4 protein rmWnt4, CHO cell-derived (R&D Systems Minneapolis, MN) was injected intraperitoneally
(i.p.) daily for 30 days, at a dose proportional to each individual mouse's weight, based on the work of Yu et al (35). The stock recombinant Wnt4 protein (rWnt4) was reconstituted with 625 µL of phosphate buffered solution (PBS) prior to injection and stored in the appropriate temperature refrigerator. The dosage of the rWnt4 injection after this would be 8 µg/kg (35). One µL of rWnt4 per gram of mouse weight was added to 50 µL of PBS and this entire amount was injected into each mouse daily, via IP injection. To account for the changes in weight of the mice, each mouse was weighed weekly and this weight was used to formulate the dosing of the rWnt4 injections, specific for each mouse. The control mice received 80 µL of PBS injections, to serve as a vehicle injection.

There were four experimental groups as follows:

1. No ligature with i.p. PBS (control) injections
2. No ligature with recombinant i.p. Wnt4 injections at a dosage of 8 µg/kg
3. Ligature with i.p. control (PBS) injections
4. Ligature with i.p. recombinant Wnt4 injections at a dosage of 8 µg/kg

Four weeks after ligature placement and daily i.p. rWnt4 or PBS injections, the mice were sacrificed. The maxillae were harvested and fixed in 4% paraformaldehyde for 24 hours and subsequently stored in 70% ethanol for μCT scanning. Maxilla were scanned and imaged via μCT scanning (micro-CT SkyScan 1172; SkyScan, Kontich, Belgium) at 10 µm (0.010 mm) resolution and x-ray energy of 55 kVp and 181 uA. Volumetric data was converted into DICOM format and imported into Dolphin Imaging software (Chatsworth, CA) for further analysis of 3D and multi-planar reconstructed images. Bone levels were measured in this software by measuring the distance from the head of the implant to the alveolar bone crest.
Bone-level measurements in each group were averaged (mean ± standard error of the mean.

We also performed a bone volume/tissue volume (BV/TV) analysis using the μCT data to calculate a volumetric measurement of bone loss in our samples (Fig. 4). The volumetric bone loss was measured using CTAn software (V.1.16 Bruker, Billerica, MA). The maxilla sample were first oriented using DataViewer (V.1.5.2 Bruker, Billerica, MA) so that the implant head was perpendicular to the implant body in both the sagittal and coronal planes. Using CTAn, volumetric measurements were taken starting from 10 slices below the junction of the implant head and body. This represents a normal volume of soft tissue where there would be no bone in all the samples. The volumetric measurements are then taken all the way down to the first appearance of alveolar bone, which represents the volume of bone loss while excluding the implant body volume from the analysis. These circumferential volumetric measurements were all taken in the axial plane. The data from each group was averaged in order to determine the amount of circumferential bone loss in each group.

Following μCT scanning, maxillae were decalcified in 15% ethylenediaminetetraacetic acid (EDTA) for four weeks. Titanium implants were then unscrewed in a counter-clockwise fashion and embedded in paraffin. Five micron thick sections were cut in the sagittal plane. Overall tissue morphology and initial assessments were viewed on hematoxylin and eosin (H&E) stained slides and with Picrosirius Red staining using a polarized filter, which results in birefringence of the collagen fibers, which distinguishes between type I and III collagen. This allows for visualization of collagen fibrils and orientation in the tissue samples around the implants. Collagen stains red under light microscopy visualization and the yellow-orange
birefringence on polarized light microscopy (Fig. 10A) represents Type I (thick fibers) collagen and green birefringence represents Type III collagen (thin fibers). Osteoclasts were stained using Tartrate Resistant Acid Phosphatase (TRAP) staining according to standard protocols (Sigma Aldrich, MO).

After standard deparaffinization protocols were utilized, antigen retrieval was performed using 10 mM sodium citrate pH 6.0 overnight at 60C. After application of primary antibodies, the tissue samples were incubated overnight at 4C in a humidified chamber. Secondary antibodies were applied and incubated for 2.5 hours at room temperature: Anti-MMP-9 (Abcam, Cambridge MA, 1:100), Anti-Cox-2 (Abcam, 1:200), Anti-p65 (NF-κB) (Rockland, 1:250). The immunoreactions were then visualized with DAB peroxidase HRP for 15 minutes (Vector Labs, CA, USA). The slides were then digitally imaged using Aperio ImageScope Model V11.1.2.752 (Vista, CA,) for all representative slides for each experimental group.

Linear bone height measurements, volumetric bone loss measurements, and osteoclast numbers were averaged for each group (mean ± standard error of the mean) and compared to each other using a Students’ t-test (Prism 5; GraphPad Software, Inc. La Jolla, CA, USA).
RESULTS:

Clinical and Radiographic Findings of Implant Osseointegration:
At the four-week time point, initial implant osseointegration was evaluated clinically after placement of the fixture, prior to ligature placement. Implants were assessed for integration, to see if they were visually still present in the mouse maxilla. Afterwards, clinical signs of mobility and inflammation were also examined. None of the implants that were present clinically showed any signs of detectable mobility. Implant osseointegration was also assessed radiographically another 4 weeks later, after ligature placement and sacrifice of the animals via μCT scanning.

Clinical Signs of Inflammation Induced by Peri-Implantitis Development:
Four weeks after implant placement and osseointegration evaluation of the implant fixtures, 6-0 silk ligatures were tied around the neck of the implant, to induce an inflammatory response in two out of the four experimental groups. Following the four-week experimental period after ligature placement, no implants were lost from any of the experimental groups and thus were able to be subjected to further analysis.

The experimental groups with ligature placement showed visible signs of inflammation in the soft tissue surrounding the implant fixtures for both the PBS and rWnt4 groups. There was tissue edema and increased soft tissue growth around the fixtures in these samples compared to the experimental groups without ligature placement (Fig. 5).

Radiographic Assessment of Bone Level Changes in Peri-Implantitis Murine Model:
At the four-week time point after ligature placement, radiographic analysis was done on the
samples, both linearly (Fig. 6) and volumetrically (Fig. 7), to compare the differences in bone loss around the implant fixtures in all four experimental groups.

Radiographically, at four weeks, there was more bone loss in the ligature compared to no ligature groups, for both the PBS group for linear bone loss (0.385 mm ± 0.017 mm vs. 0.2185 mm ± 0.016 mm) and for volumetric bone loss (0.2903 mm³ ± 0.048 mm³ vs. 0.0585 mm³ ± 0.013 mm³) and the Wnt4 injection group for linear bone loss (0.422 mm ± 0.025 mm vs. 0.2 mm ± 0.025 mm) and for volumetric bone loss (0.3386 mm³ ± 0.072 mm³ vs. 0.0492 mm³ ± 0.019 mm³). This occurred in both the linear (Fig. 6) and volumetric bone loss (Fig. 7) analysis. The linear bone loss for both the PBS and Wnt4 groups was statistically greater in the ligature group vs. the no ligature group (p<0.001 for linear bone loss and p<0.01 for volumetric bone loss). For linear bone loss, the difference in the PBS ligature to no ligature group was -0.167 mm ± 0.024 mm and for rWnt4 ligature to no ligature group was -0.222 mm ± 0.037 mm. For volumetric bone loss, the difference in PBS ligature to no ligature group was -0.232 mm³ ± 0.055 mm³ and for rWnt4 ligature to no ligature group was -0.029 mm³ ± 0.0884 mm³.

Although, there was less bone loss measured in the Wnt4 injection group compared to PBS injection group for the no ligature placement group, this difference was not statistically significant. The Wnt4 injection, ligature placement group did not show statistical significance compared to the PBS injection ligature placement group. These findings were consistent in both the linear bone loss (Fig. 6) and the BV/TV volumetric bone loss (Fig. 7) analysis.

**Histological Changes in the Peri-Implantitis Murine Model:**

Assessment of histological changes that occurred in the different experimental groups at the
four-week time point after development of peri-implantitis via ligature placement around the neck of implants, was determined by analysis of H&E and TRAP staining (for osteoclasts).

In H&E staining at four weeks (Fig. 8), the no ligature control groups, both in PBS and Wnt4 samples, demonstrated good bone apposition and crestal bone levels against the implant indicating osseointegration of the implant (Fig. 8). There is normal epithelial and submucosal architecture that can be observed on histological staining. In contrast, for the ligature experimental groups for both the PBS and Wnt4 samples, there is more bone loss seen in the crestal bone height around the implant in comparison to the no ligature groups, along with thicker soft tissue infiltrate (Fig. 8).

On the histological slides for TRAP staining, osteoclast cells stained purple. Osteoclast numbers were counted at the four-week timepoint. An average number of osteoclast cells found on the crestal portion, within, and at the apical extent on both the mesial and distal side of the implant was used for statistical analysis (Fig. 9). There were higher numbers of osteoclast counts in the ligature peri-implantitis groups compared to no ligature groups for both the PBS (5.0 ± 0.408 cells vs. 2.33 ± 0.882 cells) and Wnt4 (5.67 ± 0.882 cells vs 3.33 ± 1.202 cells) samples. This was statistically significant for the PBS comparison groups (p<0.05). However, for the rWnt4 injection groups, there was no statistical difference found in the ligature vs. no ligature groups or in comparison to PBS injected groups (Fig. 9).

**Soft Tissue Matrix Organization:**

Assessment for soft tissue changes was assessed with Picrosirius Red (PSR) staining (Fig. 10 A), where qualitative changes in collagen I and III fiber make-up and orientation were visualized around the implants in each experimental group. There appears to be more
organized and thicker collagen fibril distribution in the no ligature vs. ligature groups for both the PBS and rWnt4 experimental groups. There is also a more organized and linear collagen fiber distribution in the soft tissue matrix of the rWnt4 experimental group for both the ligature and no ligature group in comparison to their respective PBS groups.

Immunohistochemistry staining of MMP-9 (Fig. 10 B), showed decreased amounts of immunoreactivity for the rWnt4 injection group for both the no ligature and ligature groups. However, the decrease in staining is more profound in comparing the rWnt4 ligature group to the PBS ligature group.

**Inflammatory Markers:**

Inflammation increases in both periodontal disease and peri-implant diseases and some of the first signs can be seen in the soft tissues surrounding implants, in the form of peri-mucositis. Histological samples using hematoxylin and eosin staining, and immunohistochemistry for NF-κB (Fig. 11 A) and Cox-2 (Fig. 11 B) showed increased cellular activity and inflammatory changes in both the PBS and rWnt4 ligature groups compared to their respective non-ligature groups. The rWnt4 no ligature group had less inflammation compared to the PBS no ligature group and even more significantly, the rWnt4 ligature group had decreased immunoreactivity compared to the PBS ligature group, which can be seen in both the NF-κB and Cox-2 immunohistochemistry stains.
DISCUSSION:

Peri-implantitis is an inflammatory disease process that presents with bone loss around implant fixtures. It progresses from the initial inflammatory condition of peri-implant mucositis, initiated by an inflammatory response in the soft tissues that surround an implant (4, 6, 7). Peri-implant diseases are detrimental to the long-term stability and maintenance of implants in the human jaw. Although implants are a great option to restore edentulous spaces and have been in regular use for the last 50 years (40), they are subject to the same disease process that we see around teeth. The biggest problem clinicians face is the ability to treat peri-implant diseases effectively and predictably and this has not yet been elucidated (41). A recent Cochrane systematic review concluded there is no reliable evidence for the most effective interventions to treat peri-implantitis (42). Again, this is likely due to variations in the criteria for successful treatment of peri-implant diseases.

Bone remodeling occurs via a fine balance and continuous homeostatic cycle between bone resorption and formation in order to maintain constant bone mass throughout life. It occurs through the action of osteoclasts, osteoblasts and osteocytes (33). Wnts are secreted glycoproteins that activate receptor mediated signaling pathways. The Wnt signaling pathways mediate its effects through the β-catenin dependent canonical pathway or independent non-canonical pathway (28). The β-catenin dependent canonical pathway has been studied extensively for its effects on bone biology, but recent papers have tried to characterize the non-canonical Wnts’ effect on bone metabolism as well. In studies by Yu et al., it was found that a transgenic Wnt4 murine model exhibited decreased bone loss in OVX induced estrogen deficiency mice via non-canonical signaling (35).
Based on the findings from Yu et al., preliminary studies were conducted in our laboratory using the same transgenic Wnt4 mouse model (35), which showed TG mice had statistically significant less bone loss compared to control mice in a peri-implantitis murine model (Fig. 2). Building on this initial data, we utilized the same murine model of peri-implantitis (38) to investigate the results of systemic injections of mouse rWnt4 protein on bone loss around implant fixtures. We attempted to identify a therapeutic application that would garner the same effect of attenuated bone loss for implants experiencing peri-implantitis, as seen previously by our lab.

However, after initiating our experimental study, an addendum was published by Yu et al. that modified some of the conclusions initially drawn from their study (43). They stated that TG Wnt4 mice had greater trabecular bone mass than control mice, which their initial analyses did not account for. After re-examining the data and performing analysis on the relative rate of change of bone mass, they found no statistical significance between the TG mice vs. wild-type mice (43). The conclusions drawn from the effects of injected rWnt4 protein in OVX mice are still valid. They found significantly less bone loss exhibited in OVX mice receiving rWnt4 injection immediately after ovariectomy and higher degrees of BMD and BV/TV in rWnt4 injected mice one month after bone loss was established, compared to their respective controls (43).

We did not find a statistical difference in the amount of bone loss, both linearly and volumetrically when comparing the rWnt4 ligature group vs. PBS injection ligature group as hypothesized. However, there was a statistically significant greater amount of bone loss exhibited in the ligature compared to no ligature samples for both the PBS and rWnt4 injection groups, validating the peri-implantitis murine model as shown in previous studies.
from our experimental studies.

Although human studies have been conducted to study how to treat peri-implantitis, the experiments are limited by study type, sample size, and the ability to do histological studies to verify treatment results. The treatment of peri-implantitis in humans often does not satisfy the strict criteria for a randomized controlled trial (RCT) and some studies may be underpowered. Mice share similar orofacial and genetic characteristics with humans. The mouse genome has close to 98% similarity to humans, which allows for translation back to human clinical trials (44). There are genomic, transcriptomic, and proteomic databases present for the mouse, which can be used as a basis for human studies (39, 45). Animal studies are beneficial as they offer a controlled experimental model, regulated via the environment the animals are housed in, food and water they are fed, and reproducibility of experimental methods.

We anticipated attenuated bone loss with the therapeutic injection of mouse rWnt4 protein vs. control PBS injections, based on prior studies (35) and experiments in our lab. However, there was no statistically significant difference in bone loss, both in linear and volumetric bone analyses, between the rWnt4 injection group compared to the PBS injection group, for both ligature and no ligature placement implant groups. This could be attributed to the fact that we were studying the bone loss pattern in the maxilla or craniofacial complex in our experiment and Yu et al. were studying the femur or long bones in their experimental model. Evaluating distinct sites may have resulted in different bone loss patterns based on variations in bone metabolism.

It should be noted that another limitation of this study was that we used the dosage from Yu
et al's experiment on injected rWnt4 in OVX mice (35), but did not do a dose response for our study. The most commonly used animal model for studying osteoporosis is the rodent. Rodents do not experience menopause, but the OVX procedure has been a time-honored method of producing an artificial menopause (46). The model has been studied more extensively in rats, as it exhibits most of the characteristics of human menopausal osteoporosis, but the mouse is often used for ease of manipulation of its genome (46). Most of the publications relating to mice study the short-term effects of cytokines and hormones and studies have found that the time course of bone loss is similar in mice and rats in the short term, but long term studies still need to be done (47). Because Yu et al. were studying the effects of rWnt4 on ovariectomy-induced estrogen deficient (OVX) mice, the makeup of the bony architecture is different from our control C57BL/6J mice. Thus, the dosage of the rWnt4 protein they used may not have been sufficient to see a statistically significant result in our study of mice with normal bone metabolism.

Bacteria is the underlying cause of the inflammatory disease process that causes peri-implant mucositis leading to peri-implantitis (4, 6, 7). Varying host response to inflammation and bacterial challenge can contribute to the tissue destruction seen around implants (48). Histological analysis of soft tissue changes in our samples showed destruction and disorganization of the collagen matrix via staining for MMP-9. Matrix metalloproteinases are a group of enzymes that degrade the extracellular matrix and basement membrane components and have been implicated in periodontal and peri-implant diseases (49). The major collagenase in periodontitis is MMP-8, accompanied by MMP-9 (49), which we stained for in our study. We saw that there was decreased immunoreactivity in the rWnt4 ligature group compared to the PBS ligature group. It was previously found that MMP-8 was the only collagenase found in peri-implant sites with continued bone loss (50). This
suggests that the rWnt4 injections could potentially decrease the amount of MMP-9 found in ligature-induced peri-implantitis. Correspondingly, we see less destruction and disorganization from our Picrosirius red staining of collagen in our rWnt4 ligature group compared to the PBS ligature group.

We also stained for NF-κB and Cox-2, which are indicators for pro-inflammatory reactions. NF-κB controls DNA transcription and cytokine production, and is implicated in the pathogenesis of periodontal disease and atherogenesis (51). We also know that transcripts such as TNF-alpha, IL-1B, and IL-8, which are regulated by the NF-κB pathway are up-regulated following exposure to periodontopathogens (52). We see an expected increase in the amount of immunoreactivity from no ligature to ligature group for both the PBS and rWnt4 injected experimental groups. There is also a decreased amount of staining or immunoreactivity for the rWnt4 injection groups compared to PBS, for both ligature and no-ligature groups, which implicates there is a decreased amount of inflammatory response in the injected rWnt4 groups. This also implies a decreased amount of peri-implant mucositis present in our samples. Perhaps with a future dose response analysis, we can find an appropriate dosage of rWnt4 that could have positive effects of attenuated bone loss, in addition to the decreased inflammatory response we see in our rWnt4 samples.

There are several future directions resulting from this study. First, one study could be directed at examining the amount of bone loss both linearly and volumetrically, exhibited by rWnt4 injection on the long bones (femur) vs. the maxilla, to identify any differences between the two. A calculation of the bone mineral density and linear and volumetric measurements of bone loss around the growth plate of the femur or long bones can show if there are any differences in the rWnt4 effects on long bones versus the bones in the
craniofacial complex. Another study could involve a dose response curve to see what dosage of rWnt4 protein can elicit an optimal response in the craniofacial complex and long bones. A detailed evaluation of increasing doses would help determine the ideal concentration of rWnt4 protein to characterize a particular effect.

We could also study the effects of local injections of rWnt4 protein in the maxilla to identify if local delivery of rWnt4 may elicit a more robust response on bone metabolism and decreased bone loss around implants in the maxilla, through a direct localization of the protein to the area of desired effect. A final study could involve the delivery of the rWnt4 protein via a nanoparticle vehicle, which consists of particles of sub-micron and sub-cellular size allowing for their penetration deep into tissues through capillaries and epithelial linings, to be taken up more efficiently by cells (53). This allows for efficient delivery of therapeutic agents to targeted sites in the body and time-release of the drug can be controlled based on the encapsulating polymeric characteristics (53).

In conclusion, our experiment shows a validation of the peri-implantitis murine model and provides preliminary groundwork for future studies of the effects of Wnt4 on bone metabolism in the maxilla. Due to previous work in our laboratory showing statistically significant amounts of decreased linear bone loss in a transgenic Wnt4 model around implants affected by ligature-induced peri-implantitis, this encouraged us to study whether we could see the same effects via a therapeutic application of rWnt4 protein via injection. Perhaps by using a different dose we can see the same effects of attenuated bone loss in the future. Immunohistochemistry showed a decreased amount of immunoreactivity for MMP-9, NF-κB and Cox-2 indicating that the dosage used had a positive effect on decreased inflammation in the soft tissues surrounding the implant. Picosirius red staining
also showed more organized collagen fiber orientation in injected rWnt4 mouse samples, indicating there is a positive effect on the soft tissue changes in the peri-implantitis samples. This could be translated to a positive effect on a decreased inflammatory response in the rWnt4 injected mice and be useful in the treatment of peri-implant mucositis in humans. Although the results from bone loss analysis were not statistically significant, they establish a framework for future studies of the effects of rWnt4 on bone metabolism around implants with ligature-induced peri-implantitis.
**FIGURES:**

A) Sagittal µCT image of control (left) and ligature group (right) demonstrating increased bone loss.

B) Data showing mean distance from the implant head to the crestal bone; greater bone loss observed in implant with ligature (b and c) compared to control (a).

C) Representative histological toluidine blue staining demonstrating reduced alveolar bone level and supracrestal inflammatory infiltrate in the implant with ligature placement (right).

**Figure 1. Pirih et al. 2014 preliminary data.** A) Sagittal µCT image of control (left) and ligature group (right) demonstrating increased bone loss. B) Data showing mean distance from the implant head to the crestal bone; greater bone loss observed in implant with ligature (b and c) compared to control (a). C) Representative histological toluidine blue staining demonstrating reduced alveolar bone level and supracrestal inflammatory infiltrate in the implant with ligature placement (right).
Figure 2. Preliminary data on TG Wnt4 mice from Pirih lab. Graph represents preliminary data with linear bone level measurements from the implant head to the alveolar bone crest for TG Wnt4 mice compared to WT mice. Data are mean ± SEM. ***p<.001 comparing WT control (n=3) to WT ligature (n=2). ** p<.01 comparing WT ligature (n=3) to Wnt4 ligature (n=2).
Figure 3. General schematic depicting experimental timeline of research methodology.
Figure 4. μCT image representative of linear and volumetric bone analysis of mouse samples. A) Linear measurements were taken from the head of the implant down to the crest of bone adjacent to the shaft of the implant body (red line) on the buccal, lingual, mesial, and distal surfaces of the implant on the μCT scan. B) Orientation of μCT for volumetric bone loss analysis. Red line on the sagittal and coronal section corresponds to the axial plane.
Figure 5. Clinical Images. A) Representative clinical images. Inflammation and soft tissue coverage visualized at four weeks after ligature placement. B) Graph of percentage surface area coverage over implant surface four weeks after ligature placement for each of the four experimental groups. Data are mean ± SEM, normalized to an average of implant head surface area. *p<.05 comparing PBS ligature to no ligature group (n≥5 for all groups).
Figure 6. Radiographic evaluation of linear bone loss following ligature induced Peri-Implantitis and rWnt4 injections. A) Representative sagittal μCT images of PBS and rWnt4 injected groups with both ligature and no ligature placement at the four-week time point after ligature placement. B) Graph represents the average distance calculated from implant head to alveolar bone four weeks after ligature placement. Four measurements taken on the buccal, palatal, mesial, and distal surfaces and average taken. Data are mean ± SEM. ***p<0.001 (n≥5 for all groups).
Figure 7. Radiographic evaluation of volumetric bone loss following ligature induced Peri-Implantitis and rWnt4 injections. A) Representative axial μCT images of PBS and rWnt4 injected groups with both ligature and no ligature placement at the four-week time point after ligature placement. B) Graph represents the volumetric circumferential bone loss calculated from 10 microns below the implant head to the point of bone to implant contact four weeks after ligature placement. Data are mean ± SEM. **p<0.01 (n≥5 for all groups).
Figure 8. Histological evaluation after ligature induced Peri-Implantitis and rWnt4 injections. Representative sagittal H&E images of all four experimental groups. 10X magnification.
Figure 9. Osteoclastic activity after ligature induced Peri-Implantitis and rWnt4 injections. A) TRAP staining of representative samples four weeks after ligature placement and rWnt4 or PBS injections. Staining depicts the D surface of implant. 10X magnification. B) Graph represents the averaged number of osteoclasts at the four week timepoint after ligature placement. *p<0.05 (n≥3 for all groups)
Figure 10. Histological evaluation of soft tissue changes with Picrosirius red and MMP-9. A) Picrosirius red staining, both brightfield and polarized views for all four experimental groups. Polarized view (right panel) shows birefringence of collagen fibers, distinguishing between Type I (yellow-orange) and Type III (green) collagen. There are more organized collagen fibers in the rWnt4 ligature vs. PBS ligature group. 10X magnification. B) MMP-9 immunohistochemistry shows the activity of MMP-9 in all four experimental groups, which breaks down collagen fibers. Note decreased staining in rWnt4 ligature group vs. PBS ligature group. 10X magnification.
Figure 11. Immunohistochemistry staining of NF-κB and Cox-2. A) NF-κB staining four weeks after ligature placement and injection of rWnt4 or PBS. 10X magnification. B) Cox-2 staining four weeks after ligature placement and injection of rWnt4 or PBS. Note the decreased staining visualized in rWnt4 ligature vs. PBS ligature group for both IHC stains. 10X magnification.
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