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Effects of Chemically-Modified-Curcumin (CMC2.24) on Rat Experimental Periodontitis

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Howard Hao Wang

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Abstract of the Thesis

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Periodontal disease is one of the most common chronic inflammatory diseases, and causes severe debilitation due to the loss of teeth. There are two main strategies utilized in the treatment of periodontal disease: (1) local mechanical therapy, such as scaling and root planing and/or periodontal surgery, as well as local adjunctive antimicrobial (Atridox, Arestin, and Periochip) therapy, and (2) addition of an adjunctive therapy such as host-modulation therapy (HMT). Regarding the latter, there is currently only one systemically administered FDA-approved medication for the treatment of chronic periodontitis, Periostat®, a non-antimicrobial doxycycline-based MMP-inhibitor, which was developed decades ago. The current study assessed a novel NON-tetracycline or NON-doxycycline-based host-modulation medication, which is a triketonic (rather than diketonic like doxycycline), chemically-modified curcumin 2.24 (CMC2.24), and was tested for efficacy in reducing periodontal bone loss in a rat model of experimental periodontitis. Periodontitis was induced in 50 adult male rats by repeated local

injection of phosphate buffered saline (PBS; control) or lipopolysaccharide/endotoxin (LPS; experimental) into the gingiva. Experimental groups were orally administered either curcumin or CMC2.24 (30mg/kg), whereas the untreated LPS rats and PBS rats received vehicle alone. Both the "prophylactic" and "therapeutic" models were employed. The 20 rats in Experiment 1 (the "prophylactic" protocol) were injected with either PBS or LPS, and at the same time, systemically administered daily with the vehicle, or curcumin or CMC2.24 for 2-weeks via oral gavage. The 30 rats in Experiment 2 (the "therapeutic" protocol) were sub-divided into Experiment 2A (1-week duration of CMC2.24) or 2B (2-weeks duration of CMC2.24) to assess the "therapeutic" effects of different durations of treatment AFTER periodontal disease had already been established. After sacrifice, gingival tissue, blood, and alveolar bone were collected and analyzed. The jaws were dissected, defleshed, photographed, and bone loss was measured morphometrically and radiographically. The gingival extracts and blood samples were analyzed for matrix metalloproteinases (MMP-2 and MMP-9) by gelatin zymography, and cytokines by ELISA. In general, the LPS group with no treatment exhibited increased MMP-2 and MMP-9, cytokines (IL-1β, TNF-α) and alveolar bone loss. CMC2.24 was found to be more effective than curcumin in reducing MMPs, cytokines and alveolar bone loss. CMC2.24 treatment significantly reduced the periodontal bone loss in the rats in both the "prophylactic" and "therapeutic" models of drug administration; regarding the latter model, 2-weeks of treatment was required since the shorter duration (1-week) did not show significant effects except for a reduction in MMP-9. This study found that systemic administration of CMC2.24, a novel triketonic zinc-binding chemically-modified curcumin, reduced both local and systemic inflammation by modulating the production of cytokines and MMPs and attenuated periodontal bone loss in experimental periodontitis in both the "prophylactic" and "therapeutic" models.

I. DEDICATION

This thesis is dedicated to my parents, Hui Zhen Wang and Chun Guang Wang, and my girlfriend, Simone Park, for it is their unconditional love and support that allowed me to pursue and achieve my dreams.

"Do not be desirous of having things done quickly.
Do not look at small advantages. Desire to have things
done quickly prevents their being done thoroughly.
Looking at small advantages prevents great affairs from
being accomplished."

-Confucius

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III. LIST OF ABBREVIATIONS

CMC: Chemically-modified Curcumin

HMT: Host-modulation Therapy

CAL: Clinical Attachment Level

MMP: Matrix Metalloproteinase

IL: Interleukin

TNF: Tissue Necrosis Factor

FDA: US Food and Drug Administration

RANK: Receptor Activator of Nuclear Factor KappaB

RANKL: Receptor Activator of Nuclear Factor KappaB Ligand

ELISA: Enzyme-linked Immunosorbent Assay

LPS: Lipopolysaccharide

PBS: Phosphate buffered saline

CEJ: Cemento-enamel Junction

ROI: Region-of-interest

N.S.: Not Statistically Significant

IC_{50}: The Half Maximal Inhibitory Concentration

IV. ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Ying Gu, and my co-mentor Dr. Lorne Golub, for all of their help and patience in this project. This project could not have been accomplished without their help and the help that I received from my lab members:

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Dr. Veena Raja

Dr. Deng Jie

Peggy Yang

In addition, I would like to thank my department chair and mentor, Dr. Vincent Iacono for always providing motivation for me to continue to take on more challenges and endeavors, but more importantly, to finish them.

V. CHAPTER 1: INTRODUCTION

A. Disease classification and epidemiology

Periodontal diseases have generally been classified into two main categories: gingivitis and periodontitis. Gingivitis is characterized by the presence of inflammation that is confined to the gingiva and does not induce periodontal attachment loss. Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth that results in progressive destruction of the periodontal attachment apparatus, i.e. the cementum, periodontal ligament and alveolar bone. This can lead to decreased alveolar bone density and height, which clinically translates to increased periodontal pocket depths, clinical attachment loss, recession, and mobility of the involved teeth leading to the possible loss of involved teeth if not treated [1].

Based on the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions, periodontal disease can be classified by eight major types, such as gingival diseases, chronic periodontitis, localized and generalized aggressive periodontitis, periodontitis as a manifestation of systemic diseases, necrotizing periodontal diseases, among others [2]. Based on combined data from the 2009, 2011 and 2012 National Health and Nutritional Examination Surveys conducted in the United States, it is estimated that 47.2% of the US civilian non-institutionalized population age 30 or greater has periodontitis [3]. The prevalence of periodontitis in other countries such as the United Kingdom is similar; approximately 50% of the adult population and 60% of those over the age of 65 is diagnosed with periodontitis [4]. Despite efforts in advancing the treatment modalities for periodontitis, it is still a global public health concern as both industrialized and developing countries still have similar rates of periodontal destruction in the adult population [5].

B. Etiology & pathogenesis of chronic periodontitis

Chronic periodontitis is a complex disease that has two primary etiologies: the bacterial biofilm and a destructive host response. In the pathogenesis of periodontal disease, the bacterial biofilm transitions from a Gram-positive, facultative, fermentative ecology to predominantly Gram-negative, anaerobic, chemoorganotrophic and proteolytic ecology [6]. The Forsyth group found that the "red complex," which includes *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola*, were found to be most correlated to clinical signs of periodontal destruction [7]. Aside from the virulence factors common to most bacteria, the "red complex" exhibit numerous pathogenic mechanisms such as neuraminidase activation for nutrient acquisition, evasion of host immune responses, and intracellular trafficking capabilities [8]. Their ability to induce the production of pro-inflammatory cytokines and chemokines by macrophages and other inflammatory cells that act to modify the host immune response further amplifies their pathogenicity [9].

In the presence of sustained bacterial challenge, various polymorphonuclear leukocytes, macrophages, T-cells, B-cells and other inflammatory cells are recruited to the local inflammatory sites and activated to phagocytose bacteria and release destructive pro-inflammatory mediators such as MMP-2, MMP-9, cytokines, chemokines and reactive oxygen species [10]. Cytokines such as IL-1β, IL-1α, TNF-α, IL-6 were found to be involved in the pathogenesis of periodontitis, including the activation of extracellular matrix and bone resorption pathways [11, 12]. IL-1β was found to be the most abundant in diseased tissue at 5-fold higher concentration than TNF-α and 40-fold higher concentration than IL-1α [13]. Multiple sources have found IL-1β to be one of the most important cytokines in periodontitis; IL-1β is 500-fold more potent than TNF-α and 15-fold more potent than IL-1α in stimulating bone resorption. However, synergism of IL-1β and TNF-α enhances their bone resorptive capabilities by up to 10

times [14]. Higher levels of IL-6 at diseased periodontal tissues were found to be associated with refractory periodontitis, and when the IL-6 gene was deleted in mice, there was decreased bone loss when compared to the wild-type mice [15, 16]. Injection of soluble receptor blockers to IL-1 and TNF-α was able to decrease osteoclast formation by 67% and decrease the amount of alveolar bone loss by 60% in the primate experimental periodontitis model [17].

Matrix metalloproteinases including the collagenases also play a pivotal role in the destruction of the periodontal attachment apparatus, including the loss of periodontal ligaments and alveolar bone resorption [18]. In particular, MMP-2, -8 and -9 have been found to be upregulated in inflamed periodontal sites [19, 20]. The 92-kDa pro forms of MMP-9 as well as different molecular forms of MMP-2 are gelatinases, and with the aid of collagenases, are able to degrade extracellular matrix components that are common in all tissues and organs such as elastin, Type I, II, III, IV collagen, and proteoglycan, via their zinc-dependent endopeptidase active sites [21]. Matrix metalloproteinases are secreted as pro-forms, and activation of the MMPs can occur by many different pathways. Both bacterial extracts such as that of *P. gingivalis* and host neutrophil secreted elastase have demonstrated the ability to activate latent MMP-2 significantly in a concentration and time-dependent manner [22].

C. Periodontal treatments

Current treatment modalities include non-surgical periodontal procedures, based on scaling and root planing, as well as local antimicrobial applications with minocycline microspheres (Arestin), chlorhexidine gluconate chips (Periochip) or doxycycline hyclate gels (Atridox) among others. More severe periodontal conditions may require surgical procedures, which include open flap debridement, resective osseous surgery, and/or regenerative osseous surgery [23]. In some cases, systemic antibiotic may be warranted to further enhance biofilm

control in conjunction with local periodontal procedures [24, 25]. The aforementioned periodontal treatment options only deal with one arm of the etiology, i.e. bacterial biofilm. The other arm of the etiology, the host response, requires a different type of therapy. The latter aspect can be modified by using a host-modulation medication such as Periostat® (subantimicrobial doxycycline), which has been approved by the FDA since 1998 and has proven to be efficacious in improving periodontal parameters in such patients [26-29]. Research has shown that treatment with subantimicrobial doxycycline is able to reduce the excessive MMP activity, concomitantly reduce the amount of collagen degradation, and bone resorption in patients with periodontitis [30]. Clinical improvements with scaling and root planing alone can gain a mean of 0.5 mm in clinical attachment level (CAL), while adjunctive therapy with subantimicrobial doxycycline can result in an additional 0.35 mm gain in CAL (95% CI, 0.15-0.56), representing a 70% improvement in CAL [29].

The goal of periodontal treatment is to intervene successfully in the natural progression of periodontal disease by removing both the bacterial threat and modifying the host response to decrease the pro-inflammatory cytokines, collagenases and gelatinases, while increasing anti-inflammatory cytokines, tissue inhibitors of metalloproteinases and resolvin-type molecules [23, 31, 32]. In this regard, three-pronged approaches for the treatment of periodontal disease have been recommended. The first is assessment of risk factors for periodontal disease, the second is biofilm control (i.e. antimicrobial approaches, local mechanical therapy) and the third is host-modulatory therapy [33].

D. Host-modulation therapy

In previous studies, tetracyclines such as minocycline, doxycycline and tetracycline itself were found to have unexpected non-antimicrobial properties. These previously unrecognized

properties inhibit matrix metalloproteinases by zinc ionic binding at the active site, resulting in decreased clinical severity of inflammation and less periodontal destruction in adults with chronic periodontitis [34-36]. As a result of large-scale multi-institutional, doubled-blind clinical trials, which utilized the anti-MMP activity of the calcium and zinc binding β-diketone moiety at the carbon 11 and 12 of tetracyclines, subantimicrobial doxycycline was approved by the FDA as the first in its class as a systemic drug for host-modulation as an adjunct to scaling and root planing in the treatment of periodontitis [27-29]. No other systemic drug has been approved for such use to date. Recently, research in this area has been focused on finding molecules with similar matrix metalloproteinase-inhibiting active sites as the members of the tetracycline family. Molecules such as thermorubin and the bis-aroyl methanes were earlier candidates that were not suitable for host immune response regulation due to either the antimicrobial activity (thermorubin), or their lackluster inhibitory activity towards chemokines, cytokines and matrix metalloproteinases (bis-aroyl methanes) [37-39].

In the search for such a compound, it was found that curcumin contains a calcium and zinc binding β-diketone moiety that is similar to that found in tetracyclines, and several groups have reported that it has potent anti-inflammatory effects by reducing receptor activator of nuclear factor kappaB ligand (RANKL), receptor activator of nuclear factor kappaB (RANK), osteoprotegerin, nuclear factor kappaB, tumor necrosis factor-α and interleukin-6 expressions in the rat experimental periodontitis model [40, 41]. The anti-inflammatory properties of curcumin have been demonstrated to help not only in periodontal disease but also cardiovascular diseases, cancer, arthritis and diabetes [42-45]. While curcumin has shown significant results in reduction of inflammation in these conditions, its use as an effective therapeutic drug is limited due to its low solubility and bioavailability in vivo, and its intrinsic instability towards metabolic processes. This leads to low measureable levels in the serum [46-49]. Rossa et. al (2011) have

found that while systemic administration of curcumin is capable of decreasing inflammatory markers [40], it did not show evidence of inhibition of bone loss in periodontitis to any significant degree, although other studies have shown decreased bone loss by oral administration [40, 50, 51]. These limitations of relatively low potency as an inhibitor of bone loss have lead researchers to synthesize various derivatives and analogs of curcumin to increase its bioavailability and improve its effectiveness in vivo as a therapeutic agent for anti-inflammatory diseases and to reduce bone loss in periodontal disease [39, 52-54].

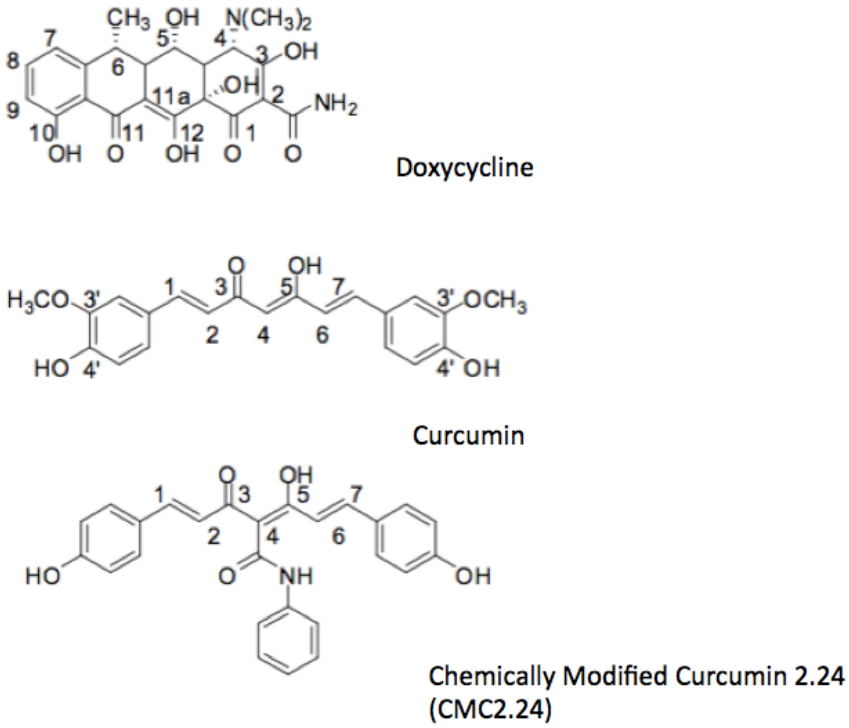


Figure 1: *Comparison of the molecular structure of doxycycline (top), curcumin (middle), CMC2.24 [1,7-bis-(4-hydroxyphenyl)-4-phenylaminocarbonyl-1E,6E-heptadien-3,5-dione] (bottom)*

Through the synthesis and experimental studies of different new derivatives of curcumin, a novel compound was identified as the most efficacious (Fig 1). CMC2.24 [1,7-bis-(4-hydroxyphenyl)-4-phenylaminocarbonyl-1E,6E-heptadien-3,5-dione] not only has increased bioavailability and stability, but its matrix metalloproteinase inhibition capabilities are also

enhanced by modifying the calcium and zinc binding β-diketone moiety to a triketone moiety that exhibits greater anti-inflammatory effects in cell culture and in vivo, as well as decreased IC_{50}, translating to a more potent MMP-inhibitor in vitro, compared to natural curcumin [46, 55] (Fig. 1).

In an in-vitro study, it was found that not only does Zn^{2+} bind more strongly to CMC2.24 compared to curcumin, but CMC2.24 exhibited increased acidity and binds more strongly to bovine serum albumin and to zinc than curcumin, which increases its biological activity [54]. Multiple in vivo experimental periodontitis studies performed on rats have confirmed the efficacy of CMC2.24 based on its ability to decrease bone loss and reduce inflammatory cytokines. In an early study, experimental periodontitis was induced in the rat model by endotoxin (LPS) injection into the gingiva at the interdental region of the maxillary molars; the contralateral side received saline injections, which served as the control. After daily oral gavage with CMC2.24 (30 mg/kg) for 2-weeks, the alveolar bone loss was calculated by way of a micro-computed tomography scan as well as morphometrically. The gingiva and blood were collected and analyzed for MMP levels as well as cytokines via ELISA. This preliminary study found that this novel triketonic CMC2.24 was able to reduce both inflammatory mediators as well as clinical alveolar bone loss [56].

The aforementioned pilot study utilized a minimum number of rats and a "split-mouth" experimental design in which half of the mouth was injected with phosphate buffered saline (PBS) and the contralateral sites were injected with lipopolysaccharide (LPS) in order to analyze both inflammatory mediators and clinical alveolar bone loss. This was a pilot study and further experiments were needed to confirm its findings. The current proposed study seeks to enhance further the evidence in the use of CMC2.24 for the treatment of experimental periodontitis by a different approach. The pilot study utilized a split-mouth design in which one side of the

maxillary molars received PBS injections (controls) and the contralateral side received LPS injections. This split-mouth design raises concerns that LPS injections in the gingiva can affect the overall systemic health of the rats, which may affect the PBS injection sites (controls). The link between oral health and systemic health has been studied for decades and periodontal conditions have been linked to diabetes, cardiovascular health and many other conditions [57, 58]. In these studies, no comparisons of the efficacy of natural curcumin compared to the novel triketonic CMC2.24 on the treatment of periodontitis were made, although more recent studies have included these comparisons. All previous studies utilizing "prophylactic" models (in which a therapeutic agent is administered either before or at the same time as the disease is initiated) with CMC2.24 have been described, as opposed to "therapeutic" models, which are more challenging since the novel drug is only administered AFTER the disease has already been established [59, 60].

E. Rationale for current research study

In our current study, we utilized individual rats in each group to eliminate possible detrimental health effects of LPS injections in the saline injected rats. A direct comparison of the effects of natural curcumin and CMC2.24 was also performed. Of importance, prior to this proposed experiment, all previous studies used a "prophylactic" model, which provides treatment at the same time that the periodontal disease is induced by local injection of LPS endotoxins. This research project envisaged two models of usage for CMC2.24: the first is a "prophylactic" model to assess the efficacy of CMC2.24 in preventing the onset of periodontal disease, similar to previous studies, and the second is the "therapeutic" model aimed at assessing the efficacy of CMC2.24 in the treatment of existing periodontal disease. Analysis of parameters such as matrix metalloproteinase levels, inflammatory mediator concentrations both locally and systemically,

and alveolar bone loss were performed to determine the in vivo efficacy of the medication in the rat model for treatment of experimentally induced periodontitis.

VI. CHAPTER 2: MATERIALS AND METHODS

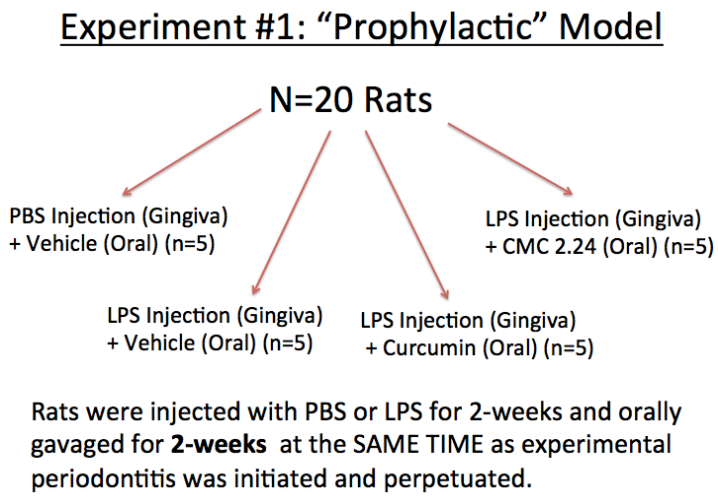
A. Animal procedures

Male adult Spargue Dawley rats, each weighing 250-300 g were distributed into the control and diseased groups (n=5 rats per group). General anesthesia was induced by inhalation of an isoflurane/oxygen mixture. Thereafter, 30 μg of lipopolysacchride (LPS) from E. coli (Strain 055:B5; Sigma Chem Co., St. Louis, MO, USA) diluted in 3 μL of phosphate buffered saline (PBS) was injected into the palatal gingiva (3 μL volume per injection) between the upper first and second molars using a Hamilton microsyringe (Agilent, Santa Clara, CA, USA), as described previously by Elburki et. al (2014) [56]. Control group rats received PBS injection at the same site as the experimental groups. PBS and LPS injections were made three times a week for 2 weeks.

The effects of CMC2.24 were assessed in both "prophylactic" and "therapeutic" models. Experiment 1 is the "prophylactic" model, in which the induction of periodontal disease with LPS injections occurred at the same time the oral administration of the drug or vehicle was initiated. The goal was to assess the ability of CMC2.24 to mitigate/prevent harmful effects of LPS induced periodontitis in comparison to natural curcumin as well as the placebo. The control group received vehicle only; i.e., carboxymethyl cellulose, a viscosity modifier or thickener. The chemically-modified curcumin was provided to us in a powder form and prior to delivery to the test subjects, it was suspended in the carboxymethyl cellulose using a polytron homogenizer. In the "prophylactic" model, 20 rats were divided into 4 groups of 5 rats each. Injections of PBS or LPS endotoxin were carried out for 2-weeks. Injections with LPS endotoxin for 2-weeks have been shown in previous studies by us and other researchers to induce experimental periodontitis in rats [56, 59, 61]. Group 1 rats received placebo injections with PBS into the gingiva bilaterally at the palatal aspect of the interdental region of the first and second molars of the

maxilla and were orally gavaged with vehicle alone; Groups 2, 3 and 4 received LPS injections bilaterally at the palatal aspect of the interdental region of the first and second molars of the maxilla as well; however, group 2 rats were oral gavaged with the vehicle alone, whereas group 3 rats received curcumin 30 mg/kg and group 4 rats received CMC2.24 30 mg/kg. PBS and LPS injections were carried out at the same time as the daily oral administration of vehicle alone, curcumin or CMC2.24 (Fig. 2a).

(a)



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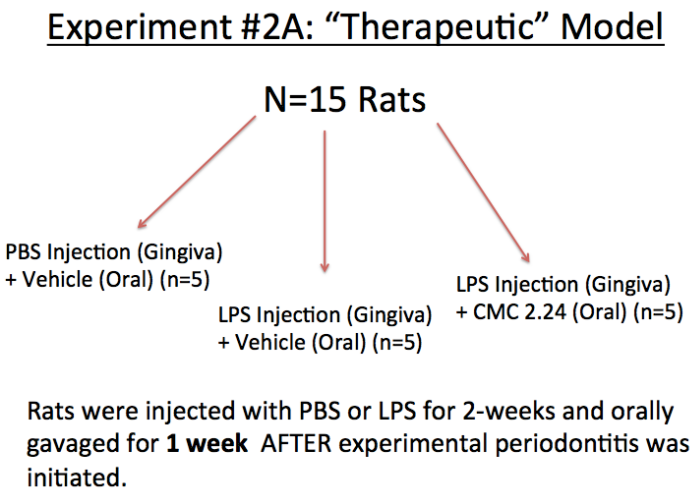

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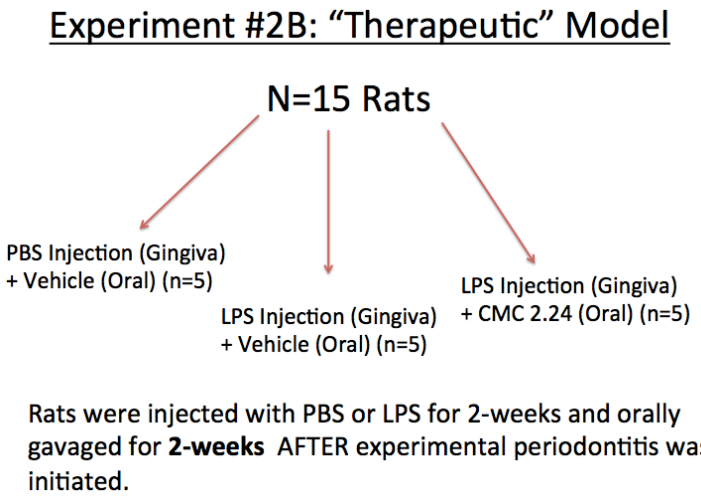


Figure 2: *Schematic diagram of Experiments 1, 2A and 2B control and experimental groups.*

A total of 30 rats were included as test subjects in experiment 2, the "therapeutic" model, to determine the efficacy of two different treatment durations, which were initiated 2-weeks after the disease had been initiated. The 30 rats were separated into groups of 15 for experiment 2A and 2B. In experiment 2A or 2B, the animals were further subdivided into groups of 5 rats, namely, the PBS + Vehicle group, LPS + Vehicle group or the LPS + CMC2.24 group (Fig 2b and 2c). Experiment 2 assessed the therapeutic potential of CMC2.24 versus the vehicle as placebo. In both experiments 2A and 2B, PBS injections in the control group (n=5) and LPS injections in the experimental groups (n=5) were carried out daily for 14 days. These injections were then discontinued to mimic the biologic condition of administering the test medication to pre-existing periodontal disease. In experiment 2A, the rats received oral gavage with vehicle alone or CMC2.24 (30 mg/kg) once per day over a 7-day period, whereas in experiment 2B, the oral gavage of either vehicle alone or CMC2.24 (30 mg/kg) was carried out for the longer duration of 14 days (Fig. 3).

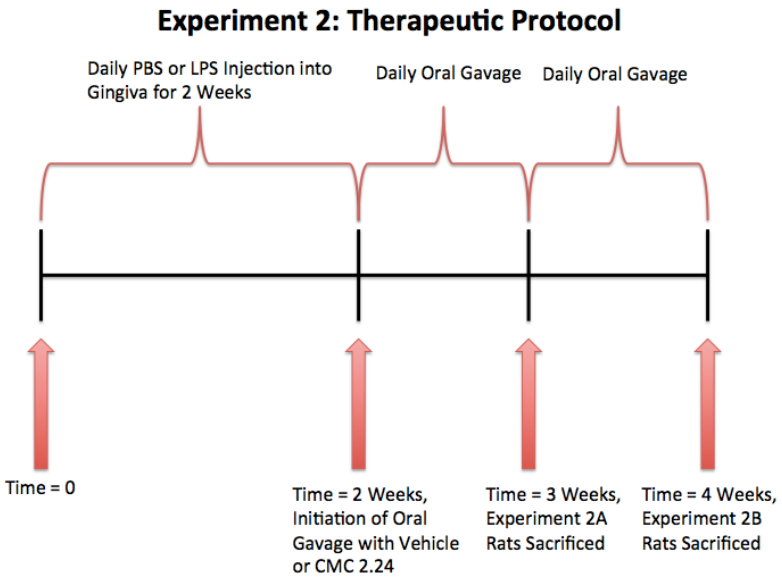


Figure 3: *Schematic diagram of Experiment 2A and 2B time line*

At the end of the experimental period, the animals were sacrificed by CO_2 inhalation and gingival tissue and maxillary alveolar processes were collected as described below. In addition, blood samples were collected and the serum and plasma were separated by standard procedure and analyzed for MMPs and cytokines as described below.

B. Gingival tissue extract

The gingival tissues were excised around maxillary 1st and 2nd molars of each rat and pooled per experimental group (5 rats per group). The gingival tissues were extracted and the MMPs were partially purified as described by us previously (Ramamurthy and Golub et. al [62, 63]). The samples were homogenized (all procedures at 4c) with a glass grinder (Kontes, Glass Co., Vineland, NJ) attached to a T-Line Lab stirrer (Model 106 Taboys Engineering Corp., NJ) in 50 mM Tris-HCl buffer (pH 7.6) containing 5 M urea, 0.2 M NaCl, and 5 mM $CaCl_2$ and then extracted overnight and centrifuged at 15,000 rpm for 1 h. The supernatants were collected and dialyzed exhaustively against 50 mM Tris buffer (pH 7.8) containing 0.2 M NaCl and 5 mM

$CaCl_2$. Ammonium sulfate was added to the dialysate to produce 60% saturation, allowed to stand overnight, and the precipitate containing the MMPs was collected by centrifugation at 15,000 rpm for 90 min. The pellets were then dissolved in the Tris buffer (pH 7.8) containing NaCl, $CaCl_2$, and 0.05% Brij solution and exhaustively dialyzed against the same buffer. Protein content of the extracts was determined by Bio-Rad Protein Assay and was used to normalize the proteins in the samples from each group (See standard curve; Fig 4).

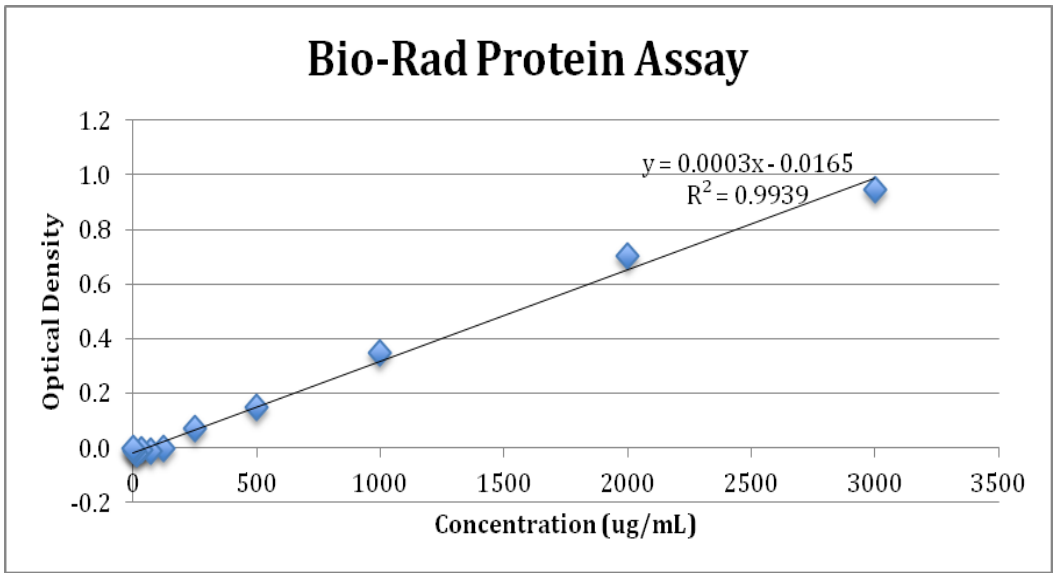


Figure 4: *Bio-Rad protein assay for normalization of protein concentrations*

C. Zymographic assay of MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B)

The relative levels of the higher molecular weight proforms and the lower molecular weight activated forms of MMP-2 and MMP-9, in the individual rat's plasma, and pooled gingival extracts from each experimental group, were determined by zymography (the gelatin zymography system was purchased from Invitrogen Corp., Carlsbad, CA). All samples were run under non-reducing denaturing conditions on the gelatin zymography system containing polyacrylamide copolymerized with gelatin at a final concentration of 1 mg/mL. After electrophoresis, the gels were washed in 2.5% Triton X-100 and incubated at 37°C overnight in

the assay buffer (40 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$; pH 7.5). After incubation, the gels were stained with Brilliant Blue Stain (Invitrogen Corp., Carlsbad, CA). Clear zones of lysis against a blue background indicated gelatinolytic activity and the molecular weight species of these MMPs were assessed using previously laboratory attained MMP-2 and MMP-9 as standards. Image J software was used to compare the density (aka intensity) of the gelatinolytic bands as previously described by Hu et. al (2010) [64].

D. Cytokines in gingiva and serum

The levels of three bone resorptive cytokines (IL-1β, IL-6, and TNF-α) were measured in serum and gingival tissue extracts by enzyme-linked immunosorbent assays (ELISAs). These assays were performed according to the manufacturer's instructions (R&D systems, Minneapolis, MN), and the results were normalized to the total concentration of protein in the samples. Blood samples from the rats in each experimental group were assayed in duplicate.

E. Morphometric analysis of alveolar bone loss

As described previously by Souza et al (2010), the soft tissues were carefully dissected to maintain the integrity of the maxillary bone specimens [65]. These were then completely defleshed by immersion in boiling water for 20 minutes and then in 2N sodium hydroxide for 10-15 minutes followed by gentle mechanical dissection of the remaining soft tissue. After washing in running water, the specimens were dried overnight in an incubator maintained at 30°C. The specimens were fixed on 3 mm thick red dental wax with their palatal or buccal surfaces facing up. Standardized orientation was achieved by positioning the specimens with the palatal cusp tip of the first and second molars superimposed on the corresponding buccal cusp tips (i.e., occlusal plane perpendicular to the ground). To validate measurement conversions, a millimeter ruler was positioned on the wax and photographed with all specimens. The specimens were

positioned under a digital single lens reflex camera mounted in tripod and digital images were obtained at 1x magnification with a maximum reproduction ratio of 1:1 utilizing a 100 mm macro lens.

A single independent examiner carried out all morphometric measurements of alveolar bone loss by measuring the distance between CEJ to alveolar bone crest at 4 positions, namely positions 6, 7, 8, 9 on the first and second molars on each surface [66] using Image J analysis software and the results were converted to millimeters using measurement of the reference millimeter grid (Fig. 5). The mean distance between CEJ and alveolar bone crest in each specimen was attained and the values for the control group (PBS injected group) were subtracted from the LPS groups as to mimic physiologic anatomy and to determine pathologic alveolar bone loss in the experimental groups. These procedures are essentially similar to the methods described previously by Chang et. al (1994) [66]. Two other independent examiners validated the reproducibility of the results after a blind study was conducted, and an inter-rater reliability score (kappa score) was calculated.

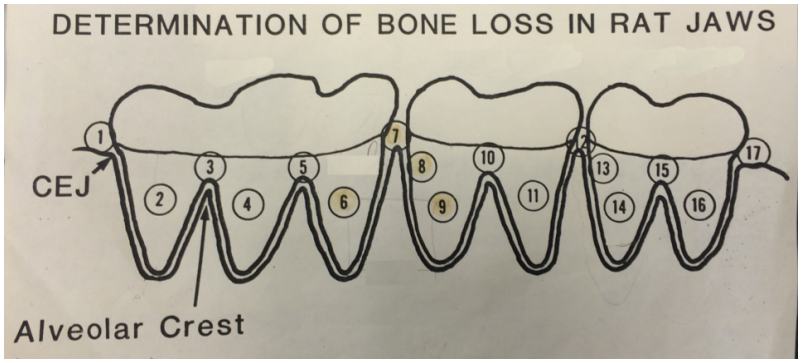


Figure 5: *Diagram indicating the positions of key study region of maxillary molar teeth in rats at positive 6, 7, 8 and 9*

F. Radiographic analysis of alveolar bone loss and density

After the soft tissue of the maxillary alveolar bone were removed, the maxillae were mounted onto dental periphery wax and radiographs are taken with a size 2 digital sensor placed parallel to the maxillae and the X-Ray collimator cone is placed perpendicular to the specimen and the digital X-ray sensor. An aluminum step wedge was placed adjacent to the specimen and the radiograph was taken similar to the procedure described by Payne et al (2007) [67]. The radiographs were taken at the same setting of exposure factors of 7 mA and 65KV at 0.10 seconds. The radiographs were processed by EMAGO dental radiography software and digitally magnified. The distance of the radiographic CEJ to the alveolar crest was measured in millimeters. Radiographic density of the alveolar bone adjacent to the first and second molar were calculated using the histogram function by the mean gray value or pixel intensity of the pixels within the examination region normalized to the gray value of the pixels in the step wedge as described by Khul et. al (2000) [68]. A similar technique of digital subtraction radiography is employed to detect the alveolar bone density in patients who are on bisphosphonate therapy [69]. In our densitometric analysis, the region-of-interest (ROI) for measuring the density of the alveolar bone was in approximately the same location (between positions 7 and 8, at the site of injection) across all samples and the same size in all of the radiographs for standardization by using the computer mouse cursor tool (cross shape) to create a square on the radiograph. The histogram measurements provide a pixel intensity value for the ROI between the first and second maxillary molars related to the value for the standard step wedge, and a ratio is calculated. This ratio represents the radiographic density of the alveolar bone of each sample, and the average radiographic density is calculated for each group and they are compared.

VII. CHAPTER 3: RESULTS

A. Experiment 1: "Prophylactic" model

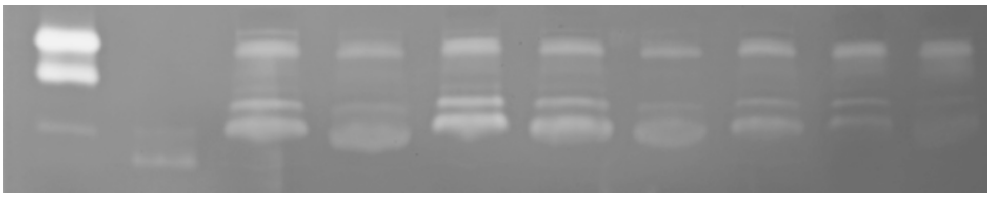
1. MMP 2 and MMP 9 in plasma (gelatin zymography)

Plasma was prepared from the blood samples collected by the standard technique of centrifuging the whole blood samples and analyzing the cell-free supernatant for MMP-2 and MMP-9 by gelatin zymography and Image J software (Figs 6 a, b, c), as described by us previously [59]. In the qualitative analysis of the gelatin zymograms, there is a general trend in which the LPS/Vehicle group shows greater band intensity compared to the normal group, the curcumin group and the CMC2.24 group. The CMC2.24 group appears to exhibit the greatest reduction in band intensity and, in general, is lower in intensity than the other LPS-treated groups (vehicle-alone or with curcumin)(Figs 6 a, b, c).

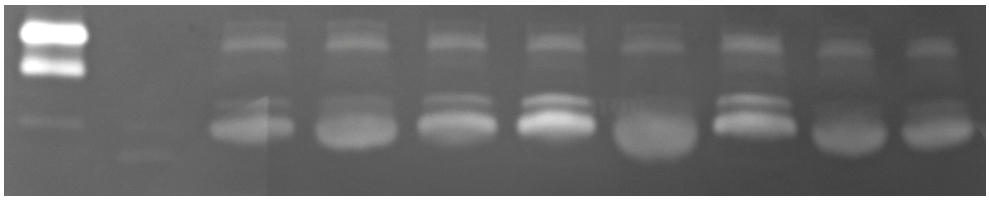
In Experiment 1, the LPS/Vehicle group shows the greatest amount of MMP-2 at 43% higher than the normal group, but the difference is not significant ($p>0.05$). In contrast, both the LPS/Curcumin and LPS/CMC2.24 groups showed a decreased level of MMP-2 compared to the LPS/Vehicle group by 14.7% and 32%, respectively, though not statistically significant (Fig 7). Similar trends are observed for activated and pro forms of MMP-9. In the pro-MMP-9 analysis, the LPS/Vehicle group has an elevation of 4.5% compared to the normal group (not statistically significant; $p>0.05$), the LPS/Curcumin group shows a decrease of 12.6% compared to the LPS/Vehicle group (not statistically significant; $p>0.05$), and the LPS/CMC2.24 group has levels that are 30% lower than the LPS/Vehicle group, which did return to normal levels (statistically significant; $p<0.05$) (Fig 8). The differences between the groups are even more pronounced in the activated-MMP-9 analysis; a significant increase of 133.6% in the LPS/Vehicle group compared to the normal group ($p<0.05$), treatment with curcumin shows a decrease of 53.2% in activated MMP-9 levels (not statistically significant; $p>0.05$), while treatment with CMC2.24 is

able to decrease activated MMP-9 levels below normal to approximately 1/3 of the level seen in the normal group and a highly significant reduction of 84.9% compared to the LPS/Vehicle group ($p<0.001$) (Fig 9).

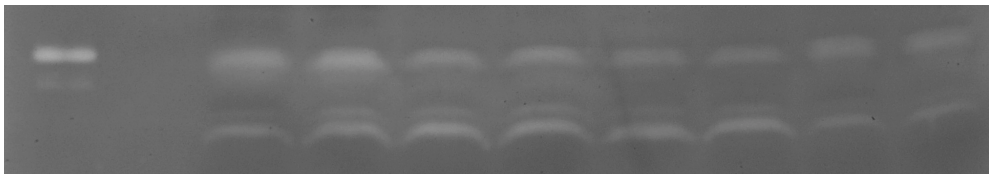
(a)



(b)



(c)



MMP 9 MMP 2 N N L L C C 2.24 2.24

Figures 6 (a), (b), (c): *Gelatin zymogram of plasma sample MMP 2 and MMP 9, zymographic plates a & b analyzes 2 rats per experimental group, (c) last sample was analyzed in duplicates of the same rat in each group because of additional un-used gel lanes*
N=normal controls, injected into the gingiva with PBS and administered vehicle, L=LPS-injected group and administered vehicle, C=LPS-injected and administered curcumin, 2.24=LPS-injected and administered CMC2.24

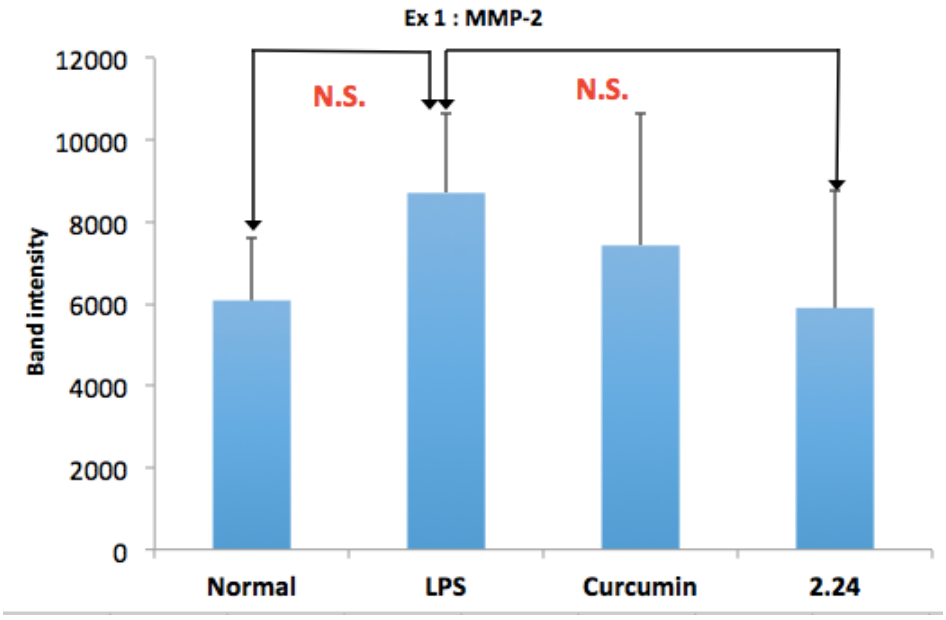


Figure 7: *Experiment 1, MMP-2 levels measured in plasma*

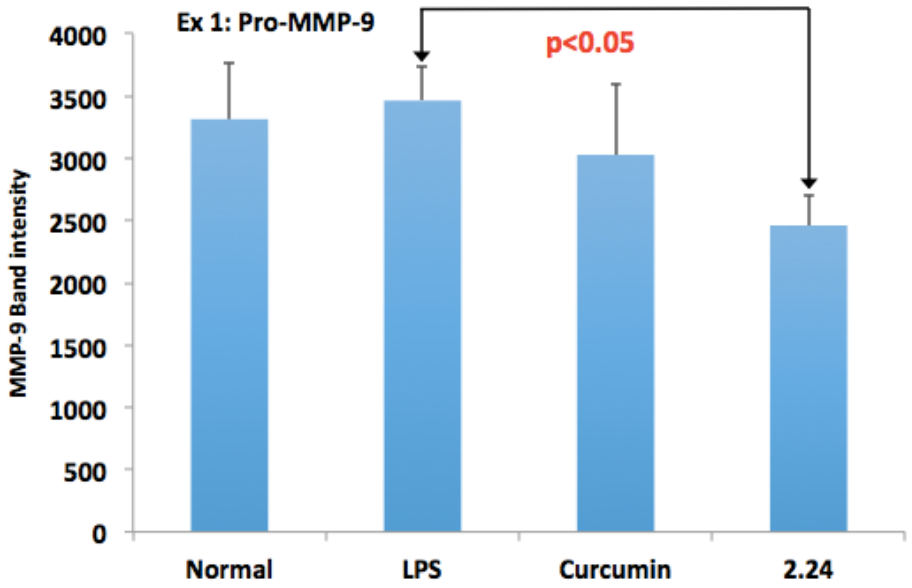


Figure 8: *Experiment 1, pro-MMP-9 levels in plasma*

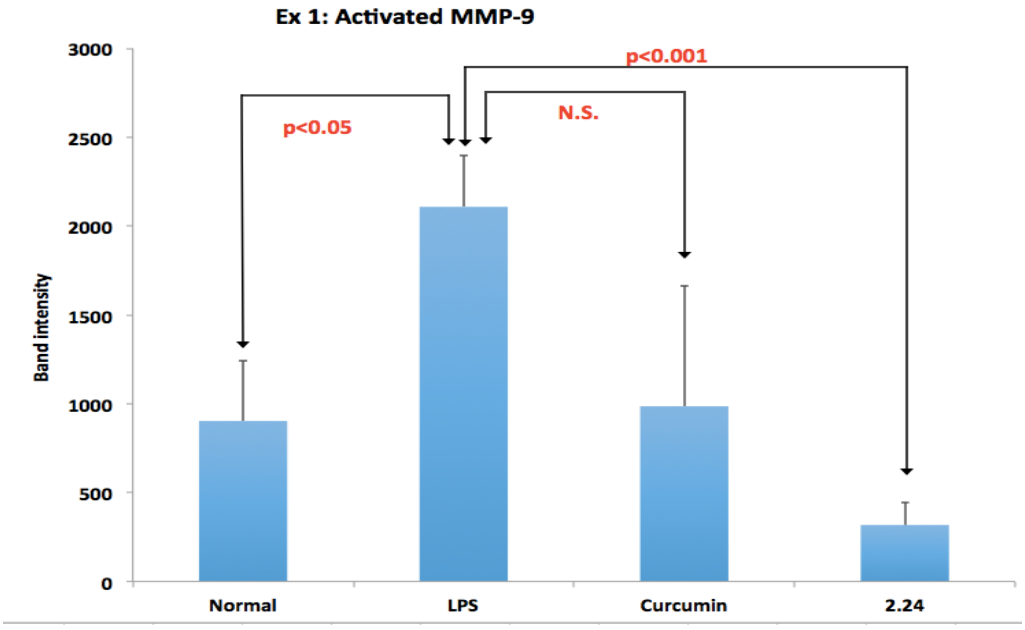


Figure 9: *Experiment 1, activated MMP-9 levels in plasma*

2. MMP 2 and MMP 9 gelatin in gingival tissue (gelatin zymography)

MMP 2 and 9 levels in the pooled gingival tissue were analyzed by gelatin zymography (Fig 10). The densitometric analysis utilizing Image J software showed that the MMP-2 levels increased by 115% in the LPS/Vehicle group when compared to the normal group. Total MMP-2 is dramatically elevated in all three LPS-groups (Fig 10), whereas, the 72 kDa pro-MMP-2 seem to be reduced by curcumin, and even more so by CMC2.24 (Fig 10). However, the most drastic change is observed in Pro-MMP-2 levels (Fig 11); the LPS/Vehicle group had an increase of 42.3% in Pro-MMP-2 compared to the normal group, treatment with curcumin decreases the Pro-MMP-2 levels by 44.9% and treatment with CMC2.24 decreases the Pro-MMP-2 levels by 59.6% when compared to the LPS/Vehicle group (Fig 11). Total MMP-9 is increased by 135% in the LPS/Vehicle group (Figs 10 & 12) when compared to the control treatment. Curcumin reduces the total MMP-9 by 16%, while treatment with CMC2.24 reduces the total MMP-9 by 48.4% when compared to the LPS/Vehicle group (Fig 12).

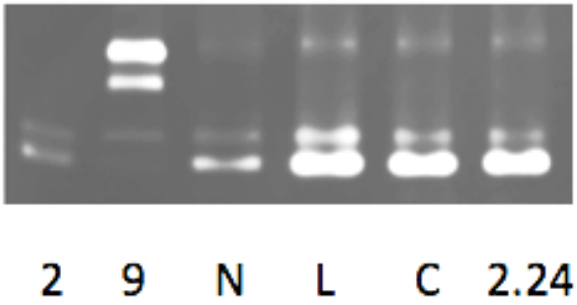


Figure 10: *Experiment 1, gel zymography of gingival tissue MMP-2 and MMP-9*

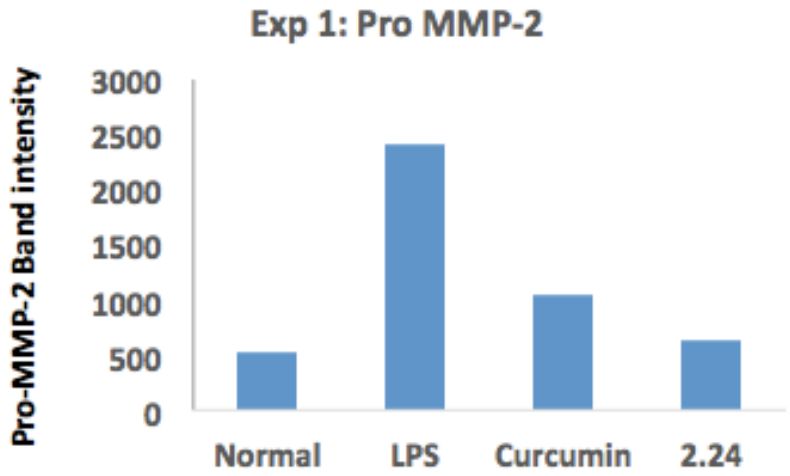


Figure 11: Experiment 1, pro-MMP-2 levels measured in gingival tissues

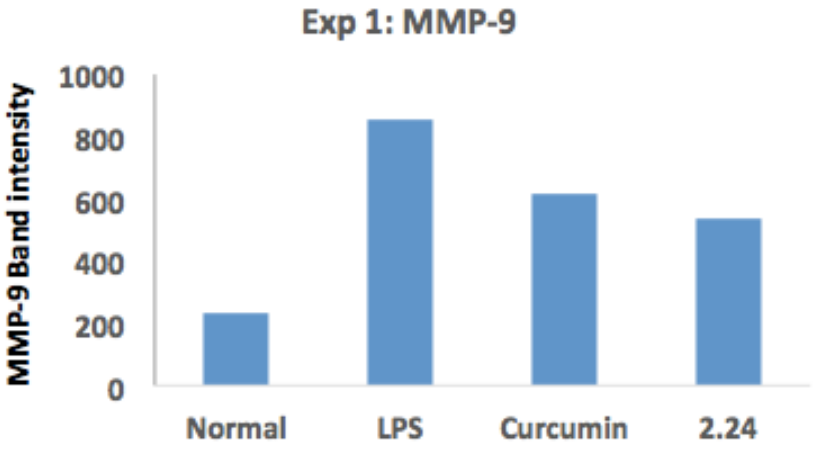


Figure 12: *Experiment 1, Total MMP-9 levels measured in gingival tissues*

3. Cytokines in gingiva

Cytokine levels in the gingiva were analyzed by ELISA techniques. The results show that compared to the normal group, there is a pronounced elevation of IL-1β levels in the LPS/vehicle group by 56.4%, whereas in the LPS/Curcumin group and LPS/CMC2.24 this is only slightly elevated by 29% and 10.3% respectively (Fig 13). Treatment with curcumin and chemically-modified curcumin 2.24 shows modest reduction of IL-6 cytokine levels when compared to the LPS/Vehicle group and the CMC2.24 treated group shows slightly greater reduction. No differences in pattern of response were seen between the groups for TNF-α (data not shown).

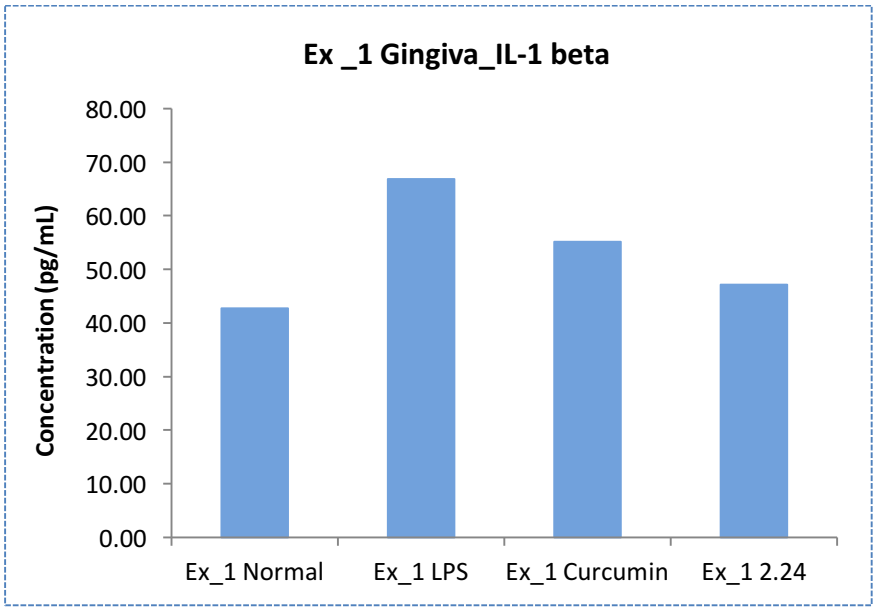


Figure 13: *Experiment 1, gingival IL-1β concentration*

4. Cytokines in serum

Serum IL-1β levels in experiment 1 demonstrates an elevation of 12% in the LPS/Vehicle group compared to the normal group, whereas in the LPS/Curcumin and LPS/CMC2.24 groups, it decreases by 11% and 6.5%, respectively, when compared to the normal group (Fig 14).

However, these mild changes, although consistent with the pattern in the pooled gingival extracts, are not statistically significant. No significant differences were observed in the serum IL-6 or TNF-α across the groups (data not shown).

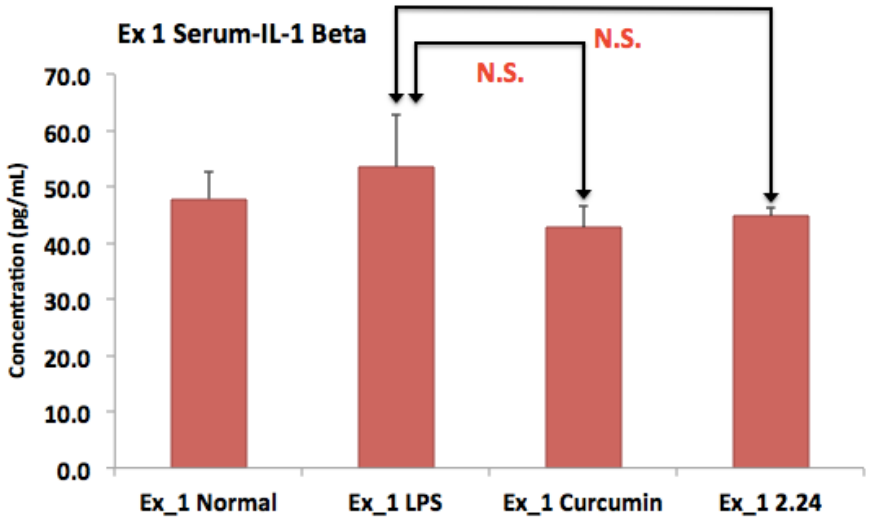


Figure 14: *Experiment 1, serum IL-1β concentration*

5. Bone morphometric and radiographic analysis

Alveolar bone loss was measured at the maxillary first and second molars, including the interproximal regions, also known as sites 6, 7, 8 and 9, at both the buccal and palatal aspects based on earlier studies by Chang et. al [66]. Bone loss was measured from the CEJ to the alveolar crest, and the bone loss in the normal group was set as the baseline for comparison to all of the other groups (Fig 15 a-d).

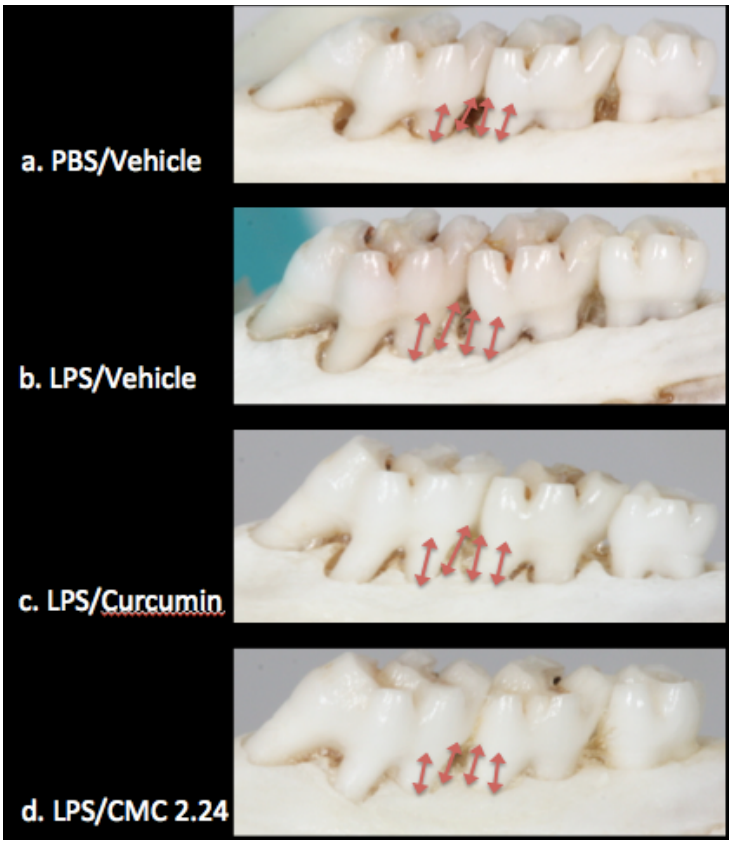


***Figure 15* (*a*)-(*d*):** *Morphometric analysis of Experiment 1 maxillary alveolar bone loss at sites #6, 7, 8, 9 on the palatal side*

The LPS/Vehicle group shows significant bone loss compared to the normal group ($p<0.01$). The LPS/Vehicle group is found to have 0.07 mm of additional alveolar bone loss compared to the normal group, and the LPS/CMC2.24 group has the least amount of bone loss compared to the normal group with only an additional loss of 0.01 mm. There was a significant reduction of 86% in pathologic bone loss in the LPS/CMC2.24 group when compared to the LPS/Vehicle group ($p<0.01$); however, interestingly, the LPS/Curcumin group has the greatest amount of alveolar bone loss, and this is statistically significant from the LPS/Vehicle group ($p<0.05$) (Fig 16). When measuring the alveolar bone loss solely on the palatal side of experiment 1 specimens where the PBS or LPS injections were given, similar profound effects are observed (Fig 17). An independent examiner and two blinded independent examiners

performed the morphometric analysis, and an inter-rater's reliability score (kappa coefficient) of 88% was achieved.

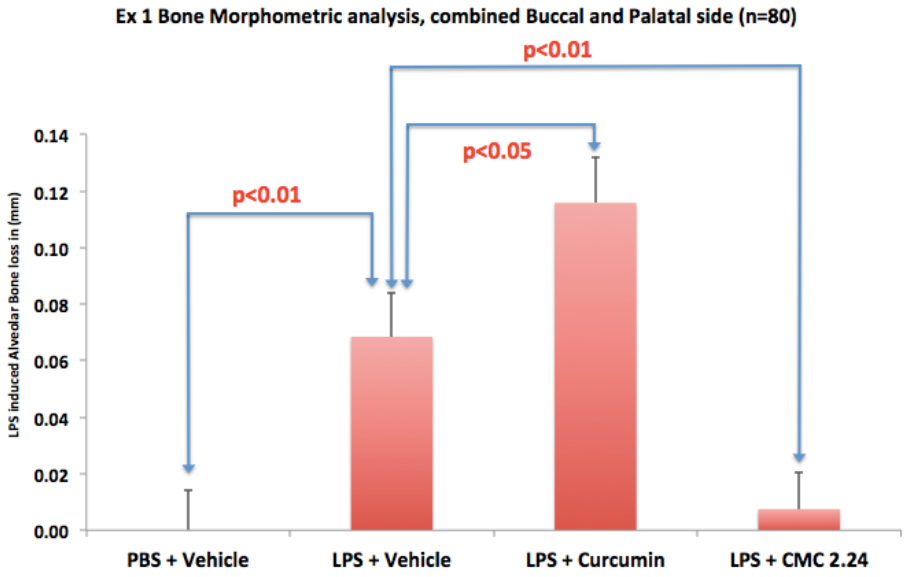


Figure 16: *Morphometric analysis of experiment 1 maxillary bone loss at positions 6, 7, 8, 9 on the buccal and palatal aspect between the 1st and 2nd molars*

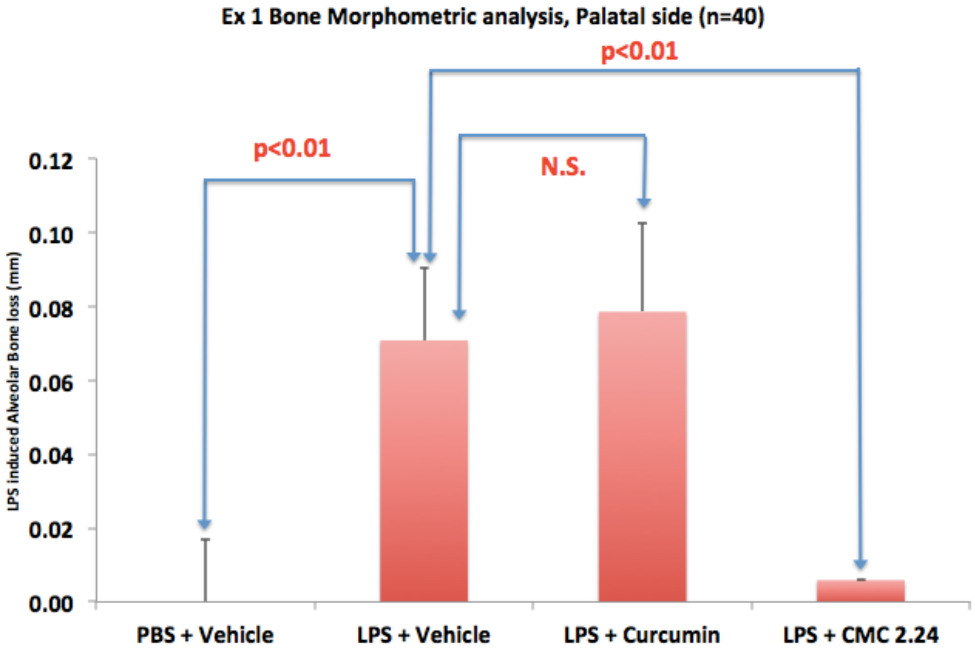
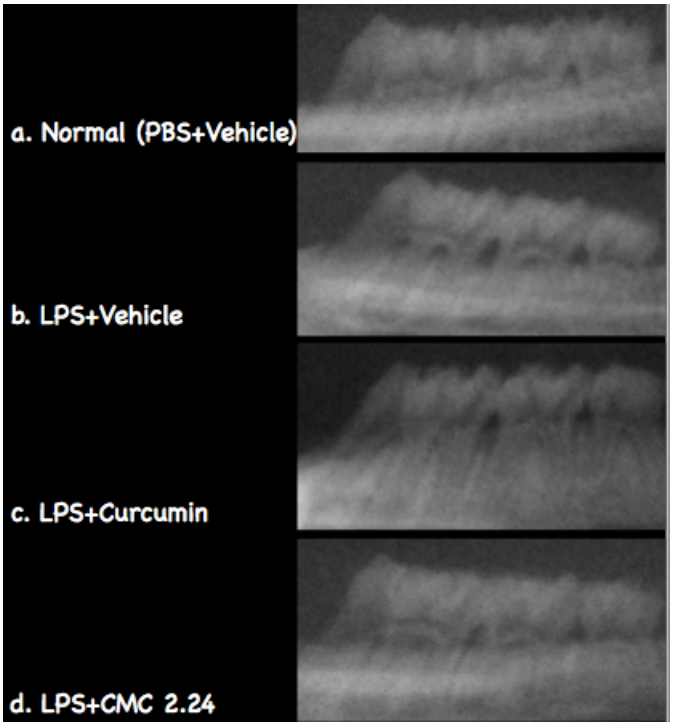


Figure 17: *Experiment 1, morphometric analysis of experiment 1 maxillary bone loss at positions 6, 7, 8, 9 only on the palatal aspect between the 1st and 2nd molars*

Radiographic analysis was performed on all of the samples and interproximal bone loss was measured at position 7 and 8 between the first and second maxillary molars by an independent examiner that was blinded to the results of the morphometric analysis. Positions 6 and 9 could not be calculated due to overlap of the teeth and the bone radiographically, meaning fewer analysis samples were included in the statistics (Fig 18 a-d). The analysis shows the same pattern of changes as the morphometric measurements. LPS/Vehicle group has significantly greater radiographic alveolar bone height loss compared to the normal group ($p<0.01$), the curcumin treated group has even greater bone loss than the LPS/Vehicle group ($p<0.05$), and the CMC2.24 treated group shows reduction of radiographic alveolar bone height loss but not statistically significant from the LPS/Vehicle group ($p>0.05$) (Fig 19).



Figures 18 (a)-(d): *Radiographic analysis of Experiment 1 maxillary alveolar bone loss at sites #6, 7, 8, 9 after 2-weeks therapy*

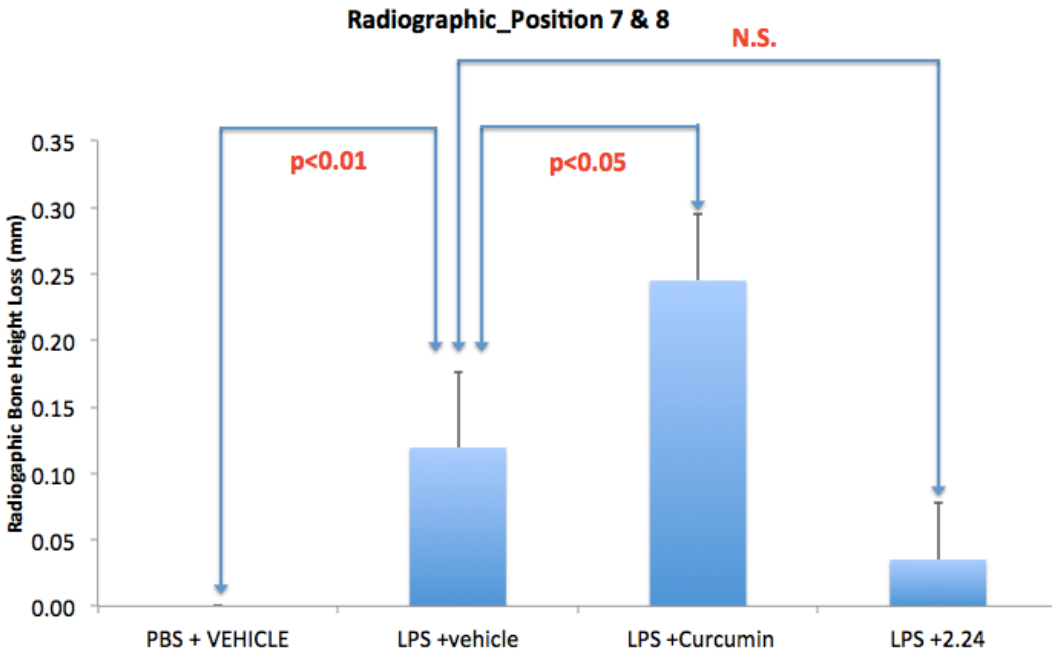


Figure 19: *Experiment 1, radiographic analysis of pathologic bone height loss at interpoximal positions 7 & 8*

The alveolar bone density compared at the interproximal regions between position 7 and 8 in the normal group was found to have the highest density ratio, and the LPS/Vehicle group had a decrease of 7% in bone density ratio in comparison. The curcumin and CMC2.24 treatments showed no significant difference in radiographic bone density from the LPS/Vehicle group and normal group (data not shown).

B. Experiment 2A: "Therapeutic" model at 1-week

1. MMP 2 and MMP 9 in plasma (gelatin zymography) at 1-week of therapy

The MMP-2 and MMP-9 concentrations in plasma and gingival tissue in experiment 2A were analyzed by the same methods as experiment 1 (Fig 20(a) & (b)). Due to the shortened duration of treatment in this experiment, no pronounced changes between the groups were observed. However, it is evident that compared to the normal and the CMC2.24 treated group,

the LPS/Vehicle group shows greater band intensity in the zymograms. This difference is most visible in the pro and activated MMP-9 bands (Figs 20a and 20b).

The MMP-2 in plasma is significantly elevated in the LPS/Vehicle group by 41.7% compared to the normal group (not statistically significant, $p>0.05$). On CMC2.24 treatment, MMP-2 in plasma is significantly reduced by 47.1%, to a level that is comparable to the normal group, though, not statistically significant ($p>0.05$) (data not shown). The pro-MMP-9 level in the plasma shows that the LPS/Vehicle group had an increase of 9% compared to the normal group and the CMC2.24 treated group has 32.3% lower MMP-9 levels compared to the LPS/Vehicle group ($p<0.05$) (Fig 21). In the activated-MMP-9, there is an increase of 92.5% in the LPS/Vehicle group compared to the normal group, and treatment with CMC2.24 decreased activated-MMP-9 levels by 79.5% ($p<0.5$) (Fig 22).

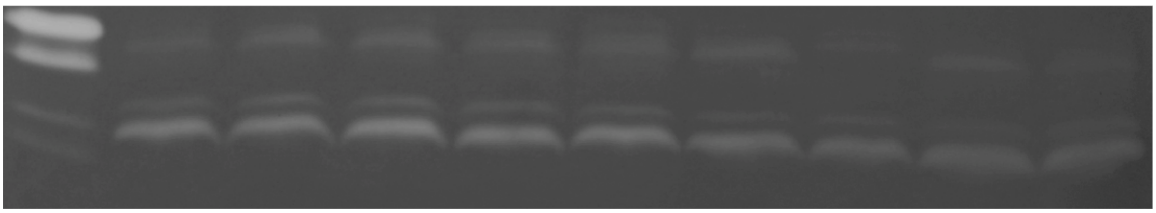


Figure 20(a): *Gel #1: Experiment 2A; gelatin zymogram of MMP-2 and MMP-9 levels in plasma at 1-week of therapy*

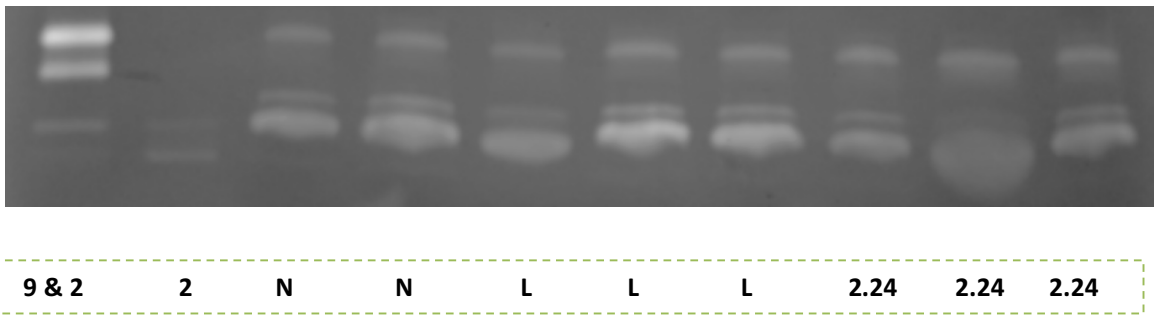


Figure 20(b): *Gel #2: Experiment 2A; gelatin zymogram of MMP-2 and MMP-9 levels in plasma at 1-week of therapy, due to additional un-used lanes, one LPS group rat, and one CMC2.24 group rat was repeatedly ran in gel #2*

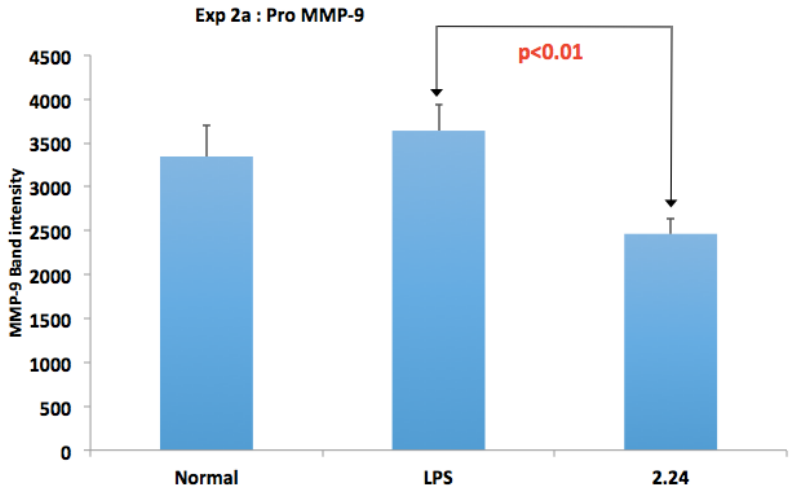


Figure 21: *Experiment 2A; pro-MMP-9 levels measured in plasma at 1-week of therapy*

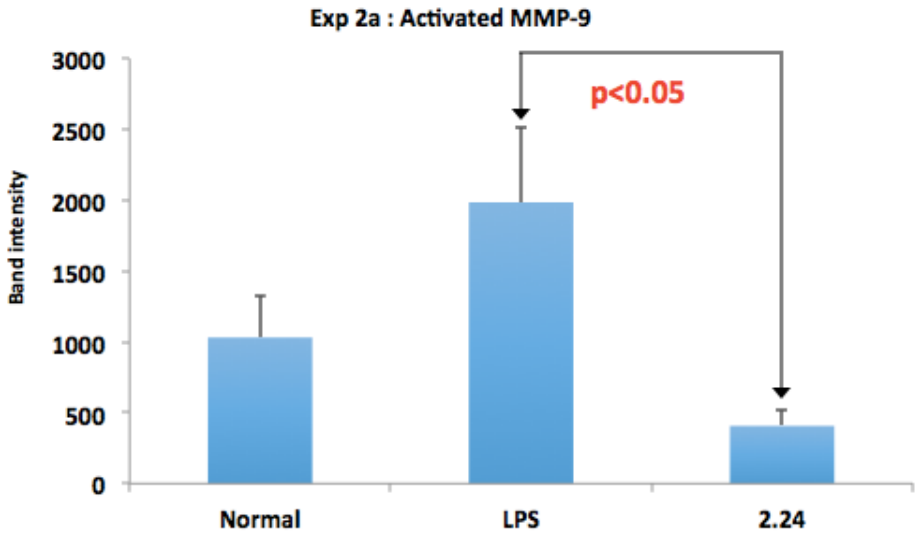


Figure 22: *Experiment 2A; activated-MMP-9 levels measured in plasma at 1-week of therapy*

2. MMP 2 and MMP 9 in gingiva (gelatin zymography) at 1-week of therapy

In gingival tissue, both MMP-2 and MMP-9 levels were analyzed by gelation zymography using ImageJ software (Fig 23a and 23b). Changes in MMP-2 amongst the three groups (control, LPS/Vehicle, and LPS/CMC2.24) were not evident at 1-week of therapy (for 2-weeks, see experiment 2B). An evaluation of MMP-9 levels in experiment 2A, the LPS/Vehicle

group induces a 65% increase in MMP-9 compared to the control group, and at this short-duration of 1-week of drug administration in this "therapeutic" protocol, CMC2.24 does not substantially reduce MMP-9 levels in the gingival tissue (data not shown).

3. Cytokines in gingiva and serum at 1-week of therapy

Cytokine concentrations were analyzed via ELISA technique, and no pattern of reduction is seen in any of the cytokines including IL-1β, IL-6, and TNF-α in both gingival tissue and serum samples at 1-week therapy with CMC2.24 treatment (data not shown).

4. Bone morphometric and radiographic analysis at 1-week of therapy

Bone morphometric analysis of Experiment 2A samples show minimal differences in the amount of alveolar bone loss and is not statistically significant. There are minimal changes in the bone density at the interproximal regions between positions 7 and 8 also (data not shown).

C. Experiment 2B: "Therapeutic" model at 2-weeks

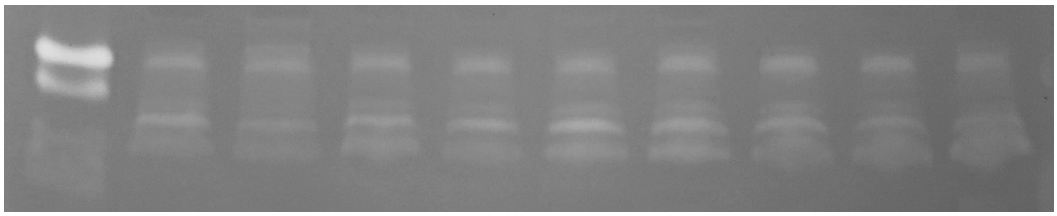
1. MMP-2 and MMP-9 in plasma (gelatin zymography) at 2-weeks of therapy

The gelatin zymograms for MMP-2 and MMP-9 indicate that the MMP-2 and MMP-9 band intensities in the normal and LPS/Vehicle group are similar; however, a noticeable decrease in band intensity is observed in the CMC2.24 treated group (Figs 23a and 23b).

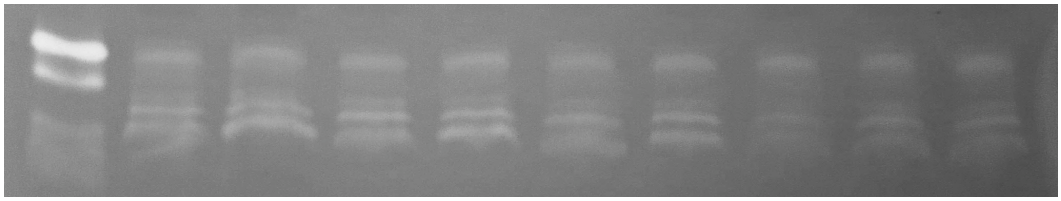
In the analytical comparison of the total MMP-2 levels, the LPS/Vehicle group had the highest band intensity with an 11% increase when compared to the normal group and oral administration with CMC2.24 for 2-weeks decreased the total MMP-2 levels by 31.8% (not statistically significant, $p>0.05$) (Fig 24). Measuring the pro-MMP-9 and activated MMP-9 levels shows a similar pattern; reductions in MMP-9 are observed with CMC2.24 treatment.

However, only the activated-MMP-9 is reduced significantly by 59.3% ($p<0.01$) compared to the LPS/Vehicle group (Fig 25).

(a)



(b)



9&2	N	N	N	L	L	L	2.24	2.24	2.24

Figure 23 (a), (b): *Experiment 2B, gel zymogram of MMP-2 and MMP-9 in plasma at 2-weeks therapy, one sample from each group was repeated in Gel 2 (b) due to unused lanes*

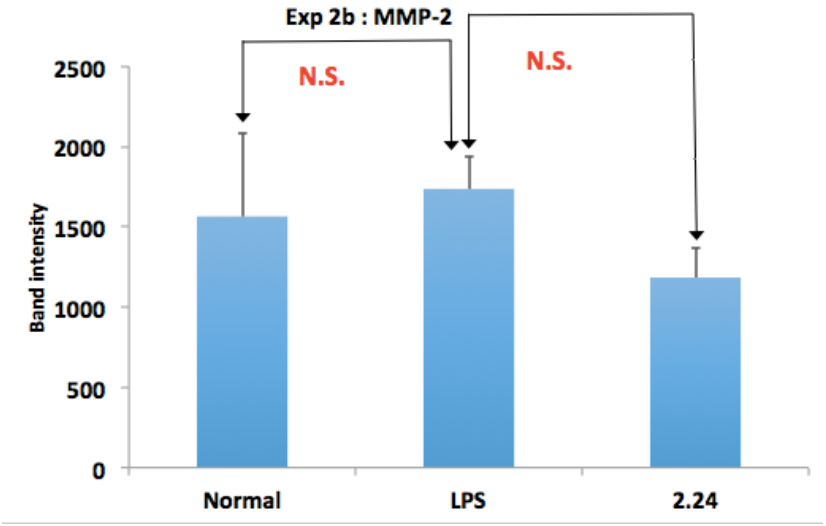


Figure 24: *Experiment 2B, MMP-2 levels in plasma at 2-weeks therapy*

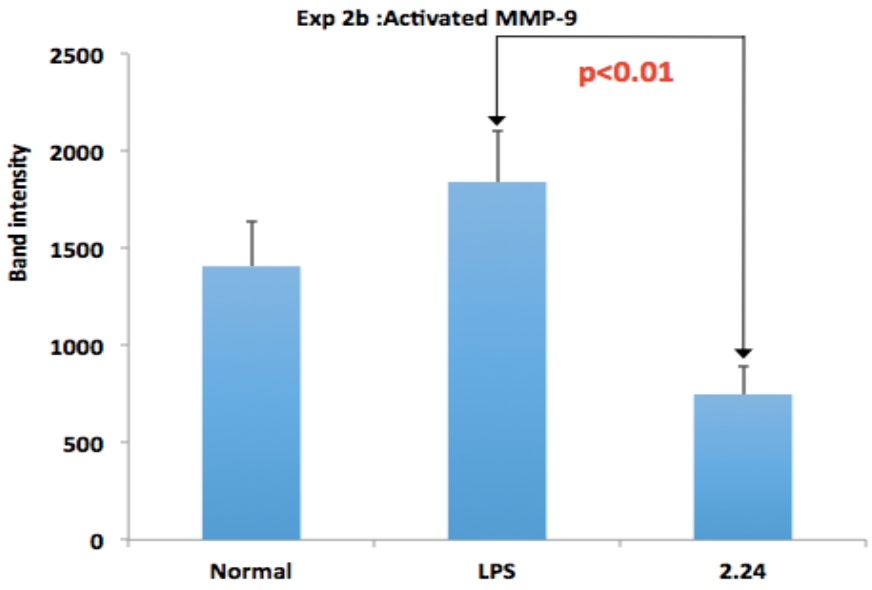


Figure 25*: Experiment 2B, activated-MMP-9 levels in plasma at 2-weeks therapy*

2. MMP-2 and MMP-9 & cytokines in gingiva at 2-weeks of therapy

The pattern of change for MMP-2, MMP-9, IL-1β, TNF-α, IL-6 were similar in that LPS tends to generate the highest level of inflammatory and collagenolytic mediators. Treatment with CMC2.24 reduces the level of pro-inflammatory mediators and MMPs to near normal levels. However, the magnitudes of these changes are modest. An example shown here for the densitometric analysis of the gelatin zymograph for gingival tissue total MMP-2 (pro- and activated MMP-2) at 2-weeks treatment demonstrates the pattern observed for MMPs (Fig 26). An example of the gingival cytokine analysis via ELISA of IL-1β is provided to show the consistent pattern observed for TNF-α as well as IL-6 (Fig 27).

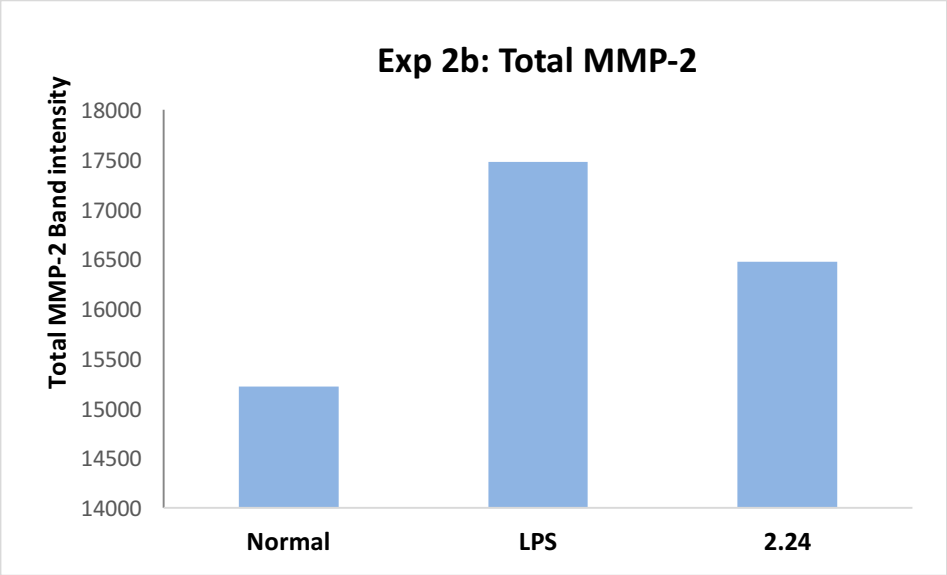


Figure 26: *Experiment 2B, total MMP-2 levels in gingiva at 2-weeks of therapy*

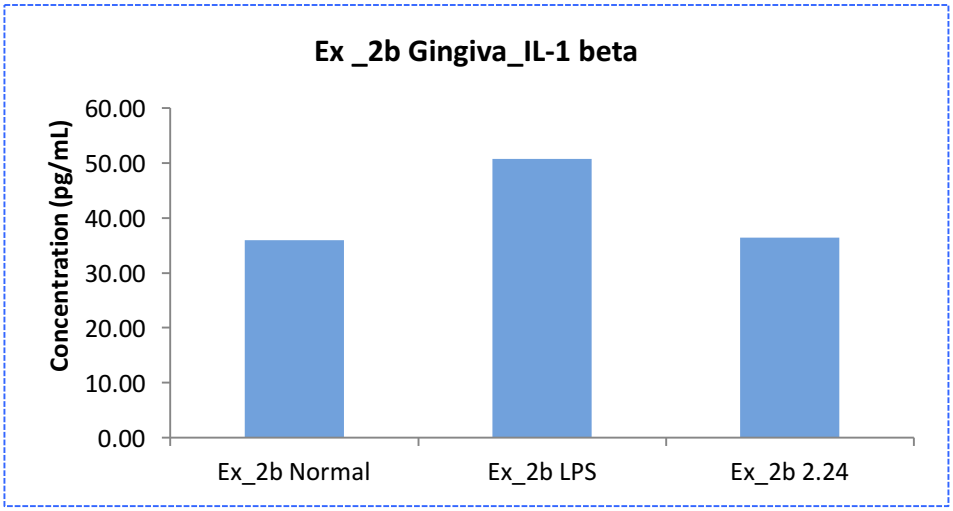


Figure 27: *Experiment 2B, IL-1β concentrations in gingiva at 2-weeks of therapy*

3. Cytokines in serum

Compared to the gingival tissue cytokine concentrations, the differences in the serum cytokine concentrations are less pronounced and lack a definitive trend. Only the IL-1β

concentration shows an elevation of 24.8% in the LPS/Vehicle group, whereas the LPS/CMC2.24 group reduces the level of IL-1β to normal levels, though it is not statistically significant ($p>0.05$) (Fig 28). The other cytokine measurements show less pronounced changes and are also not statistically significant ($p>0.05$) (data not shown).

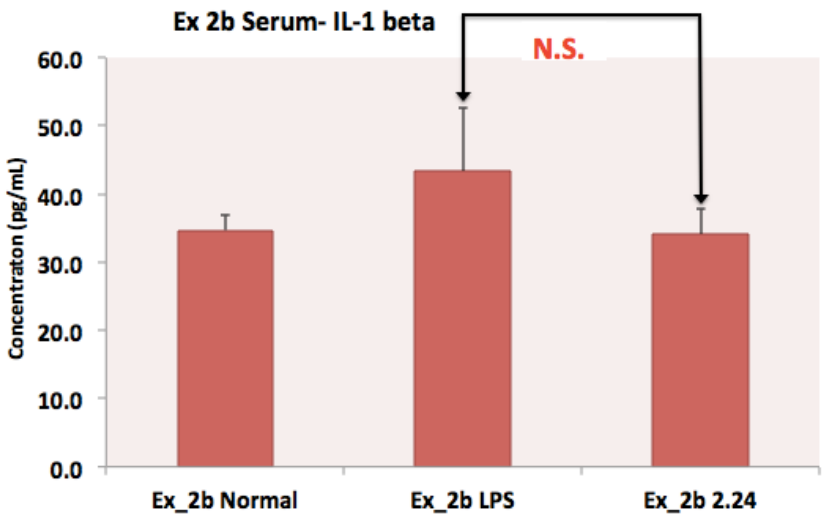


Figure 28: *Experiment 2B, IL-1β concentrations in serum at 2-weeks of therapy*

4. Bone morphometric and radiographic bone analysis at 2-weeks of therapy

As described earlier, at 1-week of treatment, bone loss changes were not statistically significant. However, as now discussed, increasing the duration of treatment does result in statistically significant results. Bone morphometric analysis results from Experiment 2B demonstrate that the LPS/Vehicle group shows an increase of 0.08 mm of bone loss compared to the normal group ($p>0.001$). However, the LPS/CMC2.24 treatment group has a 62.5% reduction in bone loss when compared to the LPS/Vehicle group ($p<0.05$) measuring all 4 positions both on the buccal and palatal aspects of the maxillae (Figs 29a-c and 30). When the bone morphometric analysis was carried out only on the palatal side of the maxillae, a similar trend is seen in the Experiment 2B group; however, due to the reduced number of sites analyzed,

these data are not statistically significant (data not shown). An independent examiner and two blinded independent examiners performed the morphometric analysis, and an inter-rater's reliability score (kappa coefficient) of 95% was achieved.

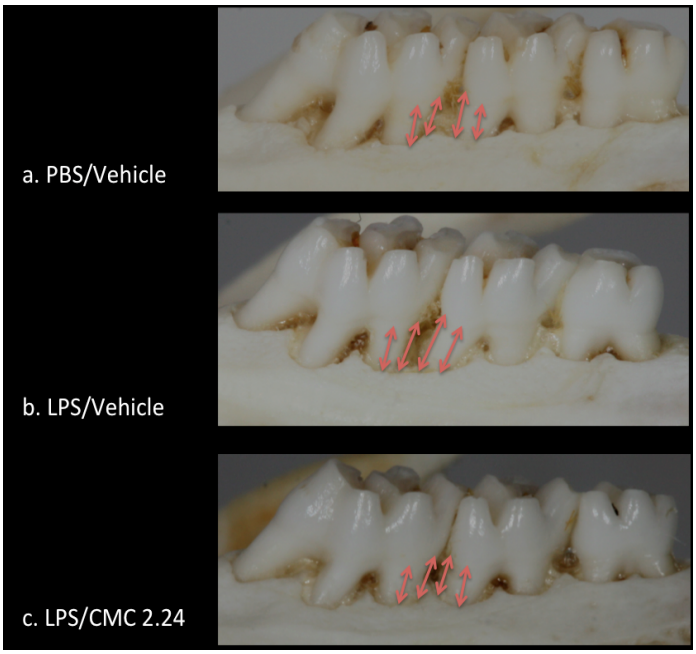


Figure 29 (a)-(c): *Morphometric analysis of Experiment 2B maxillary alveolar bone loss at sites #6, 7, 8, 9 after 2-weeks therapy*

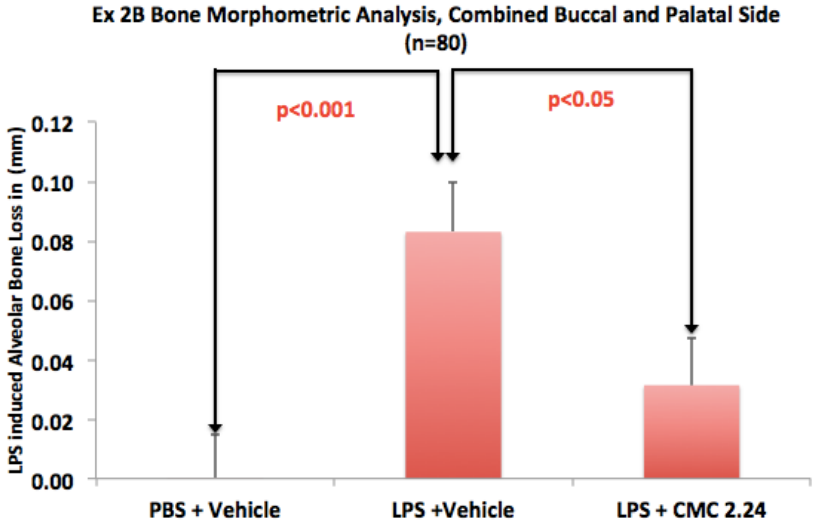


Figure 30: *Bone morphometric analysis, both buccal and palatal measurements after 2-weeks of therapy*

In experiment 2B, the radiographic analysis (performed by an independent examiner who was blinded from the results of the morphometric analysis) of bone height from CEJ to the alveolar crest at positions 7 and 8 reveals that the LPS/Vehicle group has increased radiographic bone height loss compared to the normal group by an average of 0.10 mm, whereas the LPS/CMC2.24 group was not only able to eliminate this radiographic bone height loss but actually resulted in a gain averaging 0.03 mm when compared to the radiographic bone height in the normal group, an effect that is not statistically significant ($p>0.05$) (Figs 31a-c and 32).

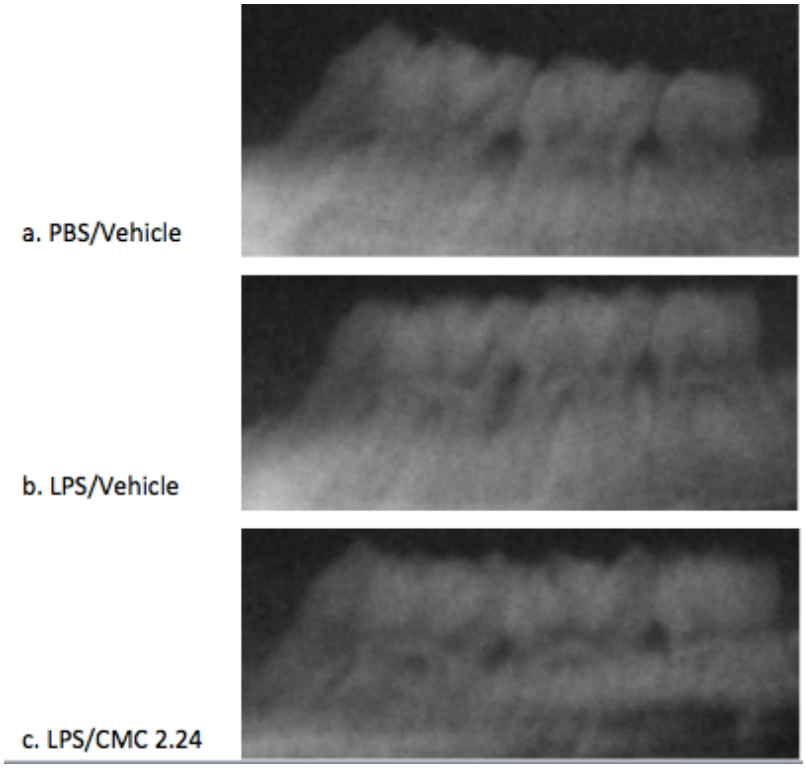


Figure 31 (a)-(c): *Radographic analysis of Experiment 2B maxillary alveolar bone loss at sites #6, 7, 8, 9 after 2-weeks therapy*

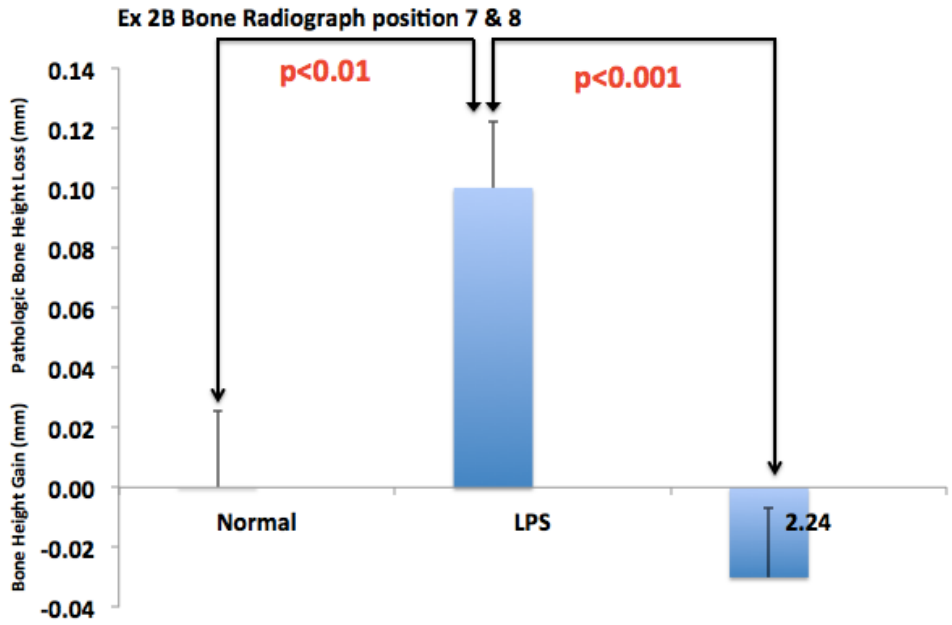


Figure 32: *Experiment 2B, radiographic analysis of bone height loss after 2-weeks therapy at positions 7 and 8, setting normal to zero radiographic bone height loss and any suggestive radiographic loss of bone height is positive and any gain of suggestive radiographic bone height is negative*

The radiographic bone density measurement, i.e., amount of pixel intensity distribution of the bone in the selected region of interest calculated in relation to a standardized step wedge, that is included in every radiograph in the interproximal bone between the first and second molars does not show significant differences between the groups ($p>0.05$) (Fig 33).

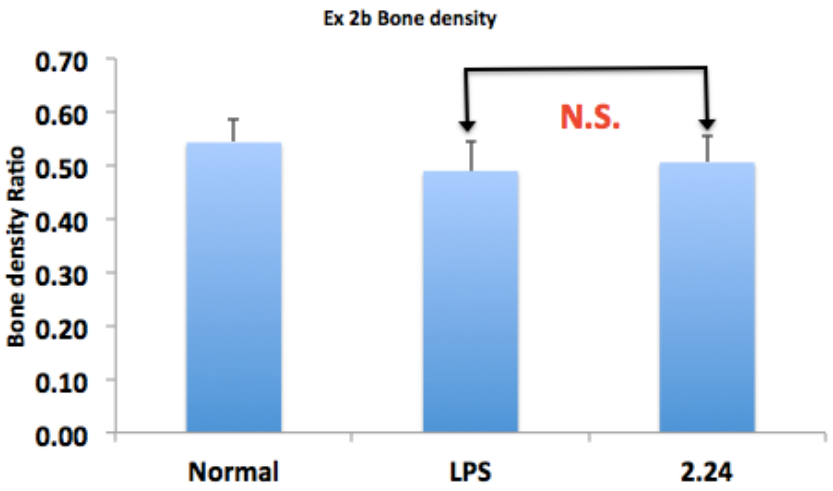


Figure 33: *Experiment 2B, density between positions 7 and 8 (site of injections) as determined by EMAGO Dental Radiology software.*
** Radiographic bone density: measure of pixel intensity of a region of interest at positions 7 and 8 using a standardized step wedge in all radiograph*

VIII: CHAPTER 4: DISCUSSION & CONCLUSIONS

Since the FDA approval and advent of Periostat® or subantimicrobial doxycycline in 1998, it still remains as the first and only FDA approved systemic medication for treating periodontal disease [28]. It is described as a host modulator, which reduces the activity of matrix metalloproteinase as an adjunctive therapy to scaling and root planing in the treatment of periodontitis. Since then (and before), subantimicrobial doxycycline has been demonstrated in a number of clinical studies, intentionally, to help in the treatment of a myriad of inflammatory diseases such as the dermatologic inflammatory disease, rosacea, as well as rheumatoid arthritis, acute coronary syndromes, post menopausal osteopenia, and acute lung disease [28, 36, 67]. However, the often misunderstood impression that Periostat® is an antibiotic at the doses used has limited its use. This led to the search for other novel and more effective drugs. Curcumin, although it is unrelated to the tetracyclines, contains the same 1,3-diketonic moiety and has been known for its anti-inflammatory property for a long period of time. A further step was taken in our lab in which the curcumin compound was chemically-modified to enhance its zinc binding active sites as well as increased stability in serum albumin. CMC2.24 was chosen as the lead compound out of many synthesized derivatives of curcumin due to its superior capabilities in vitro, in cell culture, and in vivo in the rat model. In previous studies, CMC2.24 was found to show superior potency in vitro, (i.e., the lowest IC_{50}) against a variety of matrix metalloproteinases that are associated with tissue degradation [39]. In addition, when CMC2.24 was systemically administered via oral gavage in a dosage of 30 mg/kg, it was able to reduce the secretion of pathological levels of MMP-9 with no evidence of toxicity [59]. In these researches and in previous experiments, treatment with CMC2.24 did not show any evidence of toxicity

based in part on no reduction in body weight gain with time, even in diabetic rats (data not shown).

In addition to testing the efficacy of CMC2.24 against periodontal disease and reducing inflammatory factors in diabetes, this novel experimental drug has been tested to treat many other diseases. CMC2.24 was found to enhance the activity of a new generation taxoid SBT-1214 in the suppression of prostate cancer stem cell activity and pluripotency transcription factors in vitro [70]. In another study, curcumin and different chemically-modified curcumin compounds were utilized to assess their efficacy in inhibition of the lethal factor in anthrax, and CMC2.24 was found to inhibit the lethal factor with the same potency as the parent compound, curcumin, but with improved solubility and stability. The mechanism of CMC2.24 inhibition of anthrax lethal factor can be attributed to the fact that the factor is a zinc-based metalloproteinase, and CMC2.24 is known to inhibit other zinc-metalloproteinases, namely the host-derived MMPs [56, 59, 71]. More recently, Xu et. al (2016) found that treatment with CMC2.24 is able to reduce significantly the inflammatory cell levels, NF-kB expression, and MMPs -2, -9, -12 activities in the mouse model of acute respiratory distress syndrome (ARDS) associated with pneumonia, ultimately leading to both decreased mortality and lung tissue apoptosis [72]. Impaired wound-healing in diabetics is another prolonged inflammatory condition that is characterized by abnormally high levels of proinflammatory cytokines such as IL-1β and TNF-α and also matrix metalloproteinases [73]. In a study by Zhang et. al (2016), 1% CMC2.24 topically applied to standardized skin wounds in streptozotocin-induced, severely hyperglycemic diabetic rats, was found to “normalize” wound healing in those rats over a study duration of 7 to 30 days [60].

In the current study, the efficacy of CMC2.24 was assessed in both a "prophylactic" model and a "therapeutic" model of rat experimental periodontitis. In the "prophylactic" model, the rats' gingiva were injected with PBS or LPS injections 3 times per week for 2-weeks, and at the same time during this 2-weeks period, they were orally administered either a placebo, curcumin or CMC2.24 every day. The "therapeutic" model involved injecting the rat's gingiva with PBS or LPS injections 3 times per week for 2-weeks and only providing the placebo or CMC2.24, via oral administration, after this 2-weeks period has passed. The "therapeutic" model was separated into two arms (Experiment 2A and 2B) to study the effect of the duration of treatment on periodontal disease, either a 1-week (2A) or 2-weeks (2B) administration of the placebo or CMC2.24. The "therapeutic" model is more challenging than the "prophylactic" model because the treatment starts AFTER the periodontal disease has been developed. Evidence of disease initiation and progression after 2-weeks of repeated LPS endotoxin injection was seen in the dramatic increase of MMP-2 and MMP-9, IL-1β and most importantly, the significant clinical evidence of alveolar bone loss in the LPS-injected rats.

In the "prophylactic" model, reductions of both MMP-2 and MMP-9 to levels similar to or even below the normal group were observed in both the plasma and the gingiva of these groups when treated with CMC2.24. It is of interest to note that although the objective of this study was to assess the efficacy of CMC2.24 in the treatment of local periodontal disease, the plasma levels of MMP-9 were also significantly lowered. This systemic benefit of CMC2.24 may be due to the fact that: (i) the experimental drug is systemically administered via oral gavage and (ii) that by reducing local periodontal inflammation, systemic inflammation is also decreased. This idea is supported by our past research in treating diabetic rats with CMC2.24,

which attenuated both local and systemic inflammation with a subsequent decrease of the diabetic complications [59].

MMP-2 is a gelatinase (type IV collagenase) that has been well known to be more involved in tissue turnover [74]. Research studies have also shown that MMP-9 is more associated with inflammation and the active disease state for periodontitis [75]. Both MMP-2 and MMP-9 levels are reported to be elevated in patients who have periodontitis, however, MMP-9 levels are more significantly increased in those that have active disease status [76]. CMC2.24 shows promise in treating inflammation as the data shows that there were significant reductions in pro and activated forms of MMP-9 in the "prophylactic" and in both the 1-week and 2-weeks "therapeutic" model.

In this study, cytokines such as IL-1 B, TNF-α, and IL-6 from both the gingiva and the blood were analyzed by ELISA. These cytokines were chosen because of their intimate relationship with the pathogenesis of chronic periodontitis. IL-1 B and TNF-α are both highly associated with the active disease state and inflammation of chronic periodontitis. The different secretion levels of these two cytokines in response to bacterial challenge may have an important role to play in the onset and progression of periodontitis [77]. The association between IL-1β polymorphisms and susceptibility of the host to periodontal disease has been well studied and currently there is a product on the market called PerioPredict, supplied by Interleukin Genetics that tests a patient for increased risk for periodontal disease. This highly positive association of IL-1β was mirrored in our experiment in which all of the LPS/Vehicle groups had elevated concentrations of IL-1β and treatment with CMC2.24 was able to substantially reduce IL-1β levels in gingival tissue by 29.5% when compared to the LPS/Vehicle group in the "prophylactic" model. TNF-α has been recognized as another pro-inflammatory cytokine that

plays a major role in the progression and onset of periodontitis, but its widely recognized that TNF-α is much less potent than IL-1β in key aspects of periodontal disease pathogenesis with respect to their ability to stimulate bone resorption [14]. The association between periodontitis and IL-6 is less well studied; however, polymorphisms in genes of this cytokine have contributed to increased incidences of chronic periodontitis [78]. IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine, thus the balance of IL-6 is essential in the progression and resolution of periodontal disease [79]. With this knowledge in mind, it may be beneficial for the host to reduce IL-6 levels to lower than that which is observed in the disease state but not to reduce it so much that IL-6 cannot also act as a protective cytokine with its anti-inflammatory capabilities. Similar to previous studies, both TNF-α and IL-6 cytokines did not significantly change across the groups in either the plasma or the serum in the "prophylactic" model [56].

In the "therapeutic" model, there were two aims: (i) to assess the efficacy of CMC2.24 in treatment of pre-existing LPS-induced periodontal disease, and (ii) to assess the temporal effects of CMC2.24 (1-week vs. 2-weeks regimen) in the treatment of pre-existing LPS-induced periodontal disease. In answering the first aim, which assesses the efficacy of CMC2.24 of treating pre-existing periodontal disease in the "therapeutic" model, the evidence indicates that there are significant benefits indicated by a reduction in MMPs, pro-inflammatory cytokines, and more importantly, alveolar bone loss.

In addressing the second aim of temporal efficacy, it is apparent from the results that the 1-week regimen of treatment with CMC2.24 had minimal impact amongst the three groups except that MMP-9 levels were increased in the LPS/Vehicle group, especially activated-MMP-9, which was significantly different from the normal group and CMC2.24 was able to reduce both excessive pro- and activated-MMP-9 back to normal levels ($p<0.01$, $p<0.05$, respectively).

The evidence indicates that a 1-week "therapeutic" regimen of CMC2.24 on the rat experimental periodontitis model had minimal impact on pro-inflammatory cytokines and mediators as well as on alveolar bone loss except for reducing MMP-9 levels, the MMP that is more associated with active inflammation.

Aside from the analysis of cytokines and matrix metalloproteinases, the ultimate therapeutic outcome of alveolar bone loss, at the site of LPS induced periodontitis, was assessed both morphometrically and radiographically. As previously mentioned, the location of interest is between the maxillary first and second molars, labeled sites 6, 7, 8 and 9 as described by Chang et. al [66]. In our analysis, the bone level of the normal group was set to zero in order to calculate the increased or decreased bone loss in the experimental groups. In the morphometric analysis, the CMC2.24 treated groups in both the "prophylactic" and "therapeutic" models consistently exhibited decreased alveolar bone loss compared to the LPS/Vehicle groups. There was a pronounced and significant reduction of bone loss of 86% (Kappa coefficient >88%) in experiment 1 (1-week therapy duration) compared to the LPS/Vehicle group, whereas a 62.5% reduction (Kappa coefficient >95%) was seen in experiment 2B (2-weeks therapy duration) comparing the LPS/CMC2.24 group to the LPS/Vehicle group. These reductions were also seen in the radiographic analysis that was performed independently of the morphometric analysis. In the radiographic analysis of experiment 2B, the CMC2.24 treated group actually showed some radiographic bone height gain after a 2-week therapeutic regimen, even when compared to the normal group, though not statistically significant. It can be speculated that this may be a reduction of bone height loss with systemic CMC2.24 administration or an increase in bone density because radiographic bone loss is only apparent when there is more than 30% of decalcification present. In a previously published study, Elburki et. al (2014) found similar

results in the "prophylactic" model in which treatment with CMC2.24 decreased the loss of alveolar bone height in the LPS injected rats, and returned the bone height close to their control group. These measurements were performed both morphometrically and also using micro-CT imaging [56]. In our radiographic analysis, we also measured changes in the region of interest between positions 7 and 8 at the interdental bone between the first and second molar using a step wedge and EMAGO radiographic software analysis. However, no statistically significant differences were seen in the density, but a pattern was apparent in which there was a slight reduction in bone density in the LPS/Vehicle group when compared to the CMC2.24 treated group. In the past, our laboratory has performed density analysis in the diabetic rat model, and found that they show decreased density in the skeletal long bones and alveolar bone (in preliminary studies), which was prevented by systemic administration of CMC2.24. The density changes in the current study may not be apparent due to its short duration or due to the nature of this animal model, resulting in no change in the bone density. It is also widely known that the alveolar bone (especially in the mandible) more readily undergo collagen degradation and turnover. This may play a role in the threshold for detection of bone density changes [80].

In a preliminary analysis, extrapolation of correlation (r2) was performed for MMP-2, MMP-9, also the proinflammatory cytokines IL-1β and IL-6 against alveolar bone loss. In this analysis, the values for bone loss were plotted against the various MMPs and cytokines and r2 values were calculated. There was a consistent pattern for each of the variables in experiments 1 and 2. However, it is recognized that due to the minimal number of data points, this only allows the correlations to be considered as preliminary and further studies with a greater number of data points are necessary. In experiment 1, the correlation coefficient (r2) was the highest for gingival MMP-9 levels at r2=0.54. The second strongest positive correlation with bone loss was

IL-1β concentration in the gingiva, which has a r^2 value of 0.45, followed by gingival MMP-2 levels at 0.38; the remaining factors, gingival IL-6, serum IL-1β, serum IL-6 had minimal correlation with bone loss. In experiment 2, the highest correlation coefficient (r^2) was observed in the gingival MMP-9 levels, just as it was in Experiment 1 with an r^2 value of 0.68. The next most correlated factor was IL-1β cytokine in the gingiva, which yielded an r^2 of 0.47, followed by MMP-2 with an r^2 of 0.39. The remaining factors (IL-1β, IL-6 in serum and IL-6 in gingiva) correlated only poorly with bone loss. In both experiments 1 and 2, the MMP-9 levels in gingival tissue were most positively correlated with bone loss measurements, which is consistent with what the scientific literature reports. In this regard, gingival tissue and gingival crevicular fluid levels of MMP-9 have been found to be highly elevated with increased severity of periodontal disease and these levels are substantially decreased after treatment [76].

In conclusion, this study indicates that oral administration of CMC2.24 is able to achieve inhibition of both pro-inflammatory cytokines and MMPs as well as pathologic alveolar bone loss in both "prophylactic" and "therapeutic" models of rat experimental periodontitis. It is clear that in the "therapeutic" model, which is more challenging with regard to therapy to overcome, required 2-weeks of treatment with CMC2.24 since after 1-week, there did not appear to be any significant benefits other than reduction of pro- and activated-MMP-9 levels. In future studies, it will be important to determine the therapeutic effects of CMC2.24 at longer periods of treatment (including possible systemic benefits) and with continuous LPS infection.

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