Stony Brook University

A Novel Chemically Modified Curcumin as a Pleiotropic MMP-Inhibitor: Therapeutic Potential in Locally- and Systemically-Induced Periodontal (and other) Connective Tissue Breakdown

A Dissertation Presented

by

Muna Saad Elburki

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Oral Biology and Pathology

(Periodontal Biology)

Stony Brook University

May 2015

Stony Brook University

The Graduate School

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Abstract of the Dissertation

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Periodontitis, the most common chronic inflammatory disease known to mankind, is recognized as the major cause of tooth loss in adults. Although periodontitis is initiated by anaerobic gram-negative bacteria, the destruction of the periodontium is essentially mediated by the host response.

Numerous investigators have described various pharmacologic strategies to modulate the host response during periodontal disease, however only a non-antimicrobial tetracycline formulation is FDA-approved for these patients. Of interest, this formulation of sub-antimicrobial-dose doxycycline (SDD) has shown efficacy in clinical trials in other diseases also, e.g, rheumatoid arthritis. However, a significant drawback of the approved SDD is that this novel low-dose formulation cannot be increased in order to prevent the emergence of antibiotic-

resistant bacteria. Therefore a search has been underway for new drug molecules which act on similar active-sites for matrix metalloproteinases (MMP) inhibition like the tetracyclines, but with a different phenolic superstructure. In this regard, our group recently designed new MMP-inhibitor compounds containing the same zinc-binding site as the tetracyclines, but which are bi-cyclic phenolic compounds rather than teracycles. In the current studies, we demonstrate that systemic administration of a newly developed chemically-modified-curcumin (CMC2.24) in three different rat models of periodontal disease [locally (LPS/endotoxin)-induced as well as systemically (type I diabetes)-induced models of periodontitis; and as a combination of both] significantly inhibited alveolar bone loss, and attenuated the severity of local and systemic inflammation. Moreover, this novel tri-ketonic phenylaminocarbonyl curcumin (CMC2.24) appears to reduce the pathologically-excessive levels of inducible MMPs to near normal levels, but appears to have no significant effect on the constitutive MMPs required for physiologic connective tissue turnover.

In addition to the beneficial effects on periodontal disease, induced both locally and systemically, CMC2.24 treatment also favorably affected extra-oral connective tissues, skin and skeletal bone.

Regarding molecular mechanisms, p38 MAPK and NF-κB, are both activated during the development of experimental periodontitis. Moreover, systemic treatment with CMC2.24 appeared to markedly inhibit NF-κB and p38 MAPK activation in both locally- and systemically-induced models of periodontitis.

The data in this thesis support our hypothesis that CMC2.24 is a pleiotropic MMP-inhibitor, having both intracellular and extracellular effects, which, together, reduce local and systemic inflammation and prevent hyperglycemic- and bacteria-induced tissue destruction.

Dedication

To my Mom who raised me with a love of science and supported me in all my pursuits.

"Mom, without you, there would be no me.
Your love, your attention, your guidance,
have made me who I am.
Without you, I would be lost,
wandering aimlessly,
without direction or purpose.
You showed me the way
to serve, to accomplish, to persevere.
Without you, there would be an empty space
I could never fill, no matter how I tried.
Instead, because of you,
I have joy, contentment, satisfaction and peace.
Thank you, mom.
I have always loved you
and I always will"

By Joanna Fuchs

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AEs: Adverse events

AMI: Acute myocardial infarction

ANUG: Acute necrotizing ulcerative gingivitis

ANOVA: Analysis of variance

ARDS: Acute respiratory distress syndrome

AP-1: Activator protein 1

AGEs: Advanced glycation end products

ADA: American Dental Association

APMA: Amino phenyl mercuric acetate

AOI: Area of interest

BMD: Bone mineral density

BVF: Bone volume fraction

$CaCl_{2:}$ Calcium chloride

CAL: Clinical attachment loss

CD: Cluster of differentiation

CMCs: Chemically-modified curcumins

CVD: Cardiovascular disease

CMTs: Chemically-modified tetracyclines

CRP: C-reactive protein

CP: Chronic periodontitis

CEJ: Cementoenamel junction

CO2: Carbon dioxide

DEXA: Dual-energy X-ray absorptiometry

D: Untreated diabetic

E.coli: Escerichia coli

ECM: Extracellular matrix

ELISA: Enzyme-linked immunosorbent assay

FDA: Food and drug administration

G.I.: Gingival Index

GCF: Gingival crevicular fluid

Hb A1c: Hemoglobin A1c

HNSCC: Head and neck squamous cell carcinoma

HOCl: Hypochlorous acid

HMT: Host modulatory therapy

HIV: Human immunodeficiency virus

hs-CRP: High sensitivity C-reactive protein

IL-1β: Interleukin-1beta

IL-6: Interleukin-6

IFN- γ: Interferon gamma

iNOS: Inducible nitric oxide synthase

IC_{50}: Half maximal inhibitory concentration

IBD: Inflammatory bowel disease

kDa: Kilodalton

LDL: Low-density lipoprotein

LPS: Lipopolysaccharide

MMPs: Matrix metalloproteinases

MMP-Is: MMP-inhibitors

MAMPs: Microbial associated molecular patterns

MAPKs: Mitogen activated protein kinases

NaCl: Sodium chloride

N: Non-diabetic control

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

NSAIDs: Non-steroidal anti-inflammatory drugs

NIH: National Institute of Health

NIDCR: National Institute of Dental and Craniofacial Research

OD: Optical density

OPG: Osteoprotegerin

OPG-L: Osteoprotegerin ligand

OAF: Osteoclast-activity factor

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffer saline

P. gingivalis: Porphyromonas gingivalis

PGE2: Prostaglandin E2

PISF: Peri-implant sulcular fluid

Pl. I.: Plaque Index

PMSF: Phenylmethanesulfonyl fluoride

PMNL: Polymorphonuclear leukocytes

PVDF: Polyvinylidene difluoride

RANKL: Receptor activator of nuclear factor kappa B ligand

RA: Rheumatoid arthritis

RAGEs: Receptors for AGEs

ROI: Region of interest

ROS: Reactive oxygen species

SBU: Stony Brook University

SDD: Sub-antimicrobial-dose doxycycline

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

S.E.M: Standard error of mean

SRP: Scaling and root planing

STZ: Streptozotocin

TBST: Tris-HCl Buffer SalineTween-20

TC: Tetracycline

TNF-α: Tumor necrosis factor-alpha

Th: T helper cells

TEMED: Tetramethylethylenediamine

TLR: Toll-like receptor

Tris-HCl: Tris hydrochloride

VCAM-1: Vascular cell adhesion molecule 1

WBCC: Whole blood cell cultures

µCT: Microcomputerized tomography

Acknowledgments

First and foremost I want to thank my advisor Dr. Lorne M. Golub for his guidance, persistent encouragement and support. It has been an honor to be his PhD student. He has taught me to reach for excellence and innovation in my research and the importance of translating basic science discoveries into clinical application. I appreciate his significant contribution of time, ideas, and knowledge to make my PhD experience especially productive and inspiring. The joy and enthusiasm he has for his research was contagious and motivational for me. I am also thankful for the excellent example he has provided as a successful researcher and professor.

I wish to express my gratitude to the following people who helped me to complete this dissertation:

Dr. Marcia Simon (Director of Graduate Studies) for her kindness and support.

Dr. Maria Ryan (Chair of Oral Biology and Pathology)

Dr. His-Ming Lee

Dr. Arthur Goren

Dr. Arnold Wishnia

Our collaborator in Brazil Dr. Carlos Rossa and his team

My undergraduate students whose research I supervised, Nameta Gupta, Peter Balacky, Daniel Sierra, Mark Goodenough, Dana Moore, Nicholas Terezakis, Kamil Kuflewski, Myeongkwan Kim. Your assistance in the laboratory is especially appreciated.

Mrs Marguerite T. Baldwin (Graduate Program Coordinator) and Mrs Laura Bertolotti for their kindness and support.

Particular thanks to my committee members, Dr. Francis Johnson, Dr. Mark Wolff, Dr. Sanford Simon (Sandy), and Dr. Steven Walker for their suggestions and comments on the research project.

Special thanks to the Ministry of higher education and scientific research in Libya for its financial support which enabled me to carry out this research project and the Faculty of Dentistry at Benghazi University in Libya for their support and encouragement.

Finally I would like to thank my family especially my Mom, my sister (Fatma) and my brothers (Rafa and Jamal) for their love, encouragement and support.

Vita, Publications and/or Fields of Study

Education

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Publications

1. Muna S. Elburki, Carlos Rossa, Morgana R. Guimaraes, Mark Goodenough, Hsi-Ming Lee, Fabiana A. Curylofo, Yu Zhang, Francis Johnson, and Lorne M. Golub. 2014 "A Novel Chemically Modified Curcumin Reduces Severity of Experimental Periodontal Disease in Rats: Initial Observations." Mediators of Inflammation 2014:pp.1-10. doi: 10.1155/2014/959471.

Published Abstracts

1. **M.S. Elburki**; A. Goren; H.M. Lee; Y. Zhang; N. Gupta; P. Balacky; F. Johnson; L. London; J. Grewal; and L.M. Golub. Chemically-Modified Curcumins and Alveolar Bone Loss in Diabetic Rats. J. Dent. Res., 2011, 90, Special Issue, Abstract # 2295.

2. **M.S. Elburki**; H.M. Lee; N. Gupta; P. Balacky; Y. Zhang; F. Johnson; Y. Zhang; and L.M. Golub. Chemically-Modified-Curcumins Inhibit Alveolar Bone Loss in Diabetic Rats with Peiodontitis. J. Dent. Res., 2012, 91, Special Issue, Abstract # 1528.

3. **M.S. Elburki**; C. Rossa; M. Goodenough; M.R. Guimarães; H. Lee; F. Johnson; Y. Zhang; and L.M. Golub. A Novel Chemically Modified-Curcumin Improves Experimental Periodontitis in Rats. J. Dent. Res., 2013, 92, Special Issue, Abstract # 1428.

4. **M.S. Elburki**; H.M. Lee; M. Goodenough; F. Johnson; Y. Zhang; and L.M. Golub. A novel Chemically-Modified-Curcumin "Normalizes" Dermal Collagen in Type I Diabetic Rats. Journal of Clinical and Experimental Dermatology Research. 2013, 4 (2).

5. Y. Gu; H. Lee; H. Callen; S. Rugova; **M.S. Elburki**; Y. Zhang; F. Johnson; and L.M. Golub. A Novel Chemically-Modified-Curcumin "Normalizes" Impaired Leukocyte Competence in Diabetic Rats. J. Dent. Res., 2014, 93, Special Issue, Abstract # 1025.

6. L. M. Golub; **M.S. Elburki**; H.M. Lee; F. Johnson. Novel Bi-cyclic Pleiotropic Matrix Metalloproteinases-Inhibitors ("pleio-MMPI"): Dental and Medical Therapeutic Implications. Brit. Soc. Oral Med., presented May 10th, 2014, London, UK.

7. **M.S. Elburki**; D. Moore; N. Terezakis; k. kuflewski; m. Kim; H. Lee; C. Rossa; F. Johnson; and L.M. Golub. NF-κB and MAPK Activation in Experimental-Periodontitis is Suppressed by Chemically-Modified-Curcumin. J. Dent. Res., 2015, 94, Special Issue, Abstract # 1329.

8. F.A. Curylofo Zotti; M.R. Guimaraes; **M. S. Elburki**; H.Lee; F. Johnson; C. Rossa; and L.M. Golub. A Chemically-Modified Curcumin (CMC2.24) Reduces Experimental Periodontitis Severity: Histomorphometric/Immunohistochemical Studies. J. Dent. Res., 2015, 94, Special Issue, Abstract # 1338.

9. Y. Zhang; S. A. McClain; H. M. Lee; **M.S. Elburki**; Y. Gu; M. S. Wolff; F. Johnson; and L. M. Golub A Novel Chemically-modified-curcumin (CMC) Normalizes Wound-healing in Experimental Diabetes. J. Dent. Res., 2015, 94, Special Issue, Abstract # 2256.

10. Y. Gu; H. Lee; H. Wang; Y. Yang; A. Cerciello; **M.S. Elburki**; F. Johnson; and L. M. Golub. A Novel Chemically-modified-curcumin (CMC2.24) Resolves Systemic Inflammation in Diabetic Rats. J. Dent. Res., 2015, 94, Special Issue, Abstract # 2249.

Fields of Study

Bone biology and osteoporosis; mediators of inflammation; matrix metalloproteinases; biological mechanisms in pathogenesis of periodontal disease; mechanisms linking periodontal and systemic diseases notably diabetes.

Chapter 1

Introduction

Periodontitis is a chronic inflammatory disease found worldwide. It is among the most common chronic inflammatory diseases known to mankind and is recognized as the major cause of tooth loss in adults (Katz et al. 2001). Nationwide, the prevalence of periodontal disease, in some form, is known to affect up to 50% of the adult population and is a substantial inflammatory burden which can be detrimental to over-all systemic health (Eke et al. 2012). In this regard, this common dental disease, chronic periodontitis (CP), has, over the past few decades, been increasingly linked to a variety of medical diseases such as cardiovascular disease (CVD) and stroke, increased severity of diabetes, low birth weight babies (controversial), bacterial pneumonia, rheumatoid arthritis, Crohn's disease, and pancreatic and head and neck cancer (Van Dyke et al. 1986; Beck et al. 1996; Genco 1996; Grossi et al. 1996; Offenbacher et al. 1996; Scannapieco and Mylotte 1996; Page 1998; Tezal et al. 2012; Genco and Borgnakke 2013; Mikuls et al. 2014).

For decades, periodontal disease has been known to be initiated by bacteria, organized as a plaque or microbial biofilm adherent to the teeth, particularly anaerobic gram-negative microorganisms such as *Porphyromonas gingivalis (P. gingivalis*), *Tannerella forsythia, Treponema denticola* (ie., the "red complex"; Socransky et al. 1998) and others. An important mechanism involves their microbial products, notably lipopolysaccharide (LPS) or endotoxin (a constituent of the cell wall of Gram-negative bacteria), that induces inflammation in the adjacent

gingival/periodontal tissues. However, it is now widely recognized that the breakdown of collagen and other connective tissue constituents of the gingiva and periodontal ligament, as well as osteoclast-mediated resorption of the alveolar bone, is mediated by the host response (Page 1991; Genco 1992; Golub et al. 1998a; Ryan 2002; Giannobile 2008; Gu et al. 2012; Van Dyke 2014). In this regard, it should be pointed out that, although the traditional view in the field of periodontal biology is that the anaerobic microbial biofilm initiates periodontal destruction mediated by the host response, some lines of evidence indicate that the latter can occur without an obvious microbial insult. Examples include the up-regulation of gingival MMPs in vivo due to experimental diabetes even in germ-free rats (Golub et al. 1983) and the induction of periodontal breakdown, as well as systemic osteoporosis, following ovariectomy without manipulating the periodontal microflora (Golub et al. 1999) .

In brief, LPS and other microbial products are thought to penetrate into the gingival tissues, where they initiate and propagate an immuno-inflammatory reaction, which then leads to production of elevated levels of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α). These and other inflammatory mediators such as the reactive oxygen species (ROS) (e.g., hypochlorous acid, superoxide anion), prostanoids (e.g., prostaglandin E2 or PGE2) and nitric oxide (NO) stimulate production (and, in the case of polymorphonuclear leukocytes (PMNL), degranulation and release) of various neutral proteinases including the matrix metalloproteinases (MMPs) such as (but not limited to) the collagenases MMP-1, MMP-8, and MMP-13, the gelatinases/type IV collagenases, MMP-2 and MMP-9, and serine-proteinases, notably leukocyte elastase, which degrade various collagen and non-collagenous components (e.g., proteoglycan, fibronectin, basement membranes) of the gingiva, and periodontal ligament. These host-derived mediators, such as the prostaglandins

and cytokines (e.g., IL-1β which originally was known as OAF -- osteoclast-activating factor; Jandinski 1988), promote alveolar bone resorption (Page 1998).

The research conducted in this thesis is forward-thinking because historically, and still today, treatment of chronic periodontitis has overwhelmingly focused on reducing the bacterial "burden" in the periodontal pocket and reduction of periodontopathogens e.g., *P. gingivalis.* Traditionally, this has included mechanical debridement procedures such as scaling and root planing (SRP) and surgical reduction of pocket depth to reduce the bacterial "load" in the periodontal lesion, and enhancing the effectiveness of plaque removal by optimizing oral hygiene procedures. Both topically applied (e.g., atridox; arestin; periochip) and systemic (e.g., metronidazole, azithromycin) antimicrobials/antibiotics as adjunctive therapy have also been advocated (Genco and Slots 1984) although a recent comprehensive statistical (retrospective cohort) study did not find any benefit of adjunctive systemic antibiotic therapy in the management of the periodontal patient based on the ultimate outcome/morbidity of chronic periodontitis, ie., tooth loss in adult patients (Cunha-Cruz et al. 2008). Also, of course, the excessive use of antibiotics, resulting in the emergence of antibiotic-resistant bacteria, must be considered a serious detrimental consequence. In this regard, a number of years ago (Golub et al. 1983, 1991, 1998a) our laboratory introduced and developed NON-antibiotic formulations of the common antibiotic, the tetracyclines, as host-modulating drugs (based on mechanisms of action previously unrecognized in the medical and dental fields) in the management of chronic periodontitis and various relevant medical disorders, and this has been thoroughly reviewed (Golub et al. 1991, 1998a, Gu et al. 2012). In the research documented in this thesis, a novel pleiotropic MMP-inhibitor, based on a well-known food-stuff, was studied for its potential (including the

mechanisms involved) in managing chronic periodontal disease and its link to systemic inflammation and to a common relevant systemic disease, diabetes mellitus.

For over a decade, this "food-stuff", curcumin, has received great interest as a potential medicinal compound. This is not surprising considering that curcumin, which is derived from the common spice turmeric, has long been known for its potent anti-inflammatory, anti-carcinogenic, and anti-oxidant properties (Chainani-Wu 2003). Curcumin has been shown not only to modulate periodontal disease, but also to exhibit a range of antifungal and antiviral beneficial effects in animal studies (Chainani-Wu 2003; Guimarães et al. 2011). With this background in mind, Golub laboratory in collaboration with Johnson laboratory has recently been developing a series of chemically-modified analogs of curcumin, ie., the CMCs, which have a zinc-binding site (a β-diketone moiety) similar to that in curcumin. A similar cation-binding site is found in the tetracyclines, identified earlier, and studied extensively as MMP-inhibitors (Golub et al. 1991, 1998a; Gu et al. 2012). Our newer CMCs are more soluble and more potent than our earlier bi-phenolic zinc-binding compounds, ie., the bis-aroyl methanes and earlier CMCs. These most recent CMCs are triketonic rather than diketonic, and have exhibited greater potency as MMP-inhibitors in vitro, in cell and tissue culture, and in animal models of periodontal disease based, in part, on their enhanced zinc and albumin-binding properties (Zhang et al. 2012a, 2012b; Elburki et al. 2014)

As described above, extracellular matrix (ECM) breakdown, during different periodontal diseases, is mediated by a complex cascade including host-derived proteinases (Golub et al. 1991). The significance of the host inflammatory response in periodontal disease presents an opportunity

to explore novel approaches for treating periodontitis involving the targeting of the host response (Golub et al. 1992). Host modulatory therapy (HMT), first introduced both by Williams using non-steroidal anti-inflammatory drugs or NSAIDs (Williams et al. 1984, 1987, 1988; Howell and Williams 1993) and by Golub using non-antimicrobial tetracyclines as MMP-inhibitors; (Golub et al. 1983, 1994a, 1998a); was designed to be adjunctive to the use of conventional periodontal treatments, primarily (but not only) scaling and root planing, that reduce the bacterial "load" in the periodontal pocket. HMT was developed to treat the host inflammatory and collagenolytic responses in periodontal pathogenesis, and functions by inhibiting the activity and/or down-regulating the expression of MMPs, such as the collagenases and gelatinases, as well as suppressing cytokines, such as IL-1β, IL-6, and TNF- α, and other mediators of inflammation and connective tissue destruction including the "normalization" of excessive osteoclast-mediated bone resorption (Golub et al. 1991, 1992; Gu et al. 2012). More recently, a combination of two different host-modulatory treatments (SDD and low-dose flurbiprofen) was combined in a clinical trial on periodontal patients requiring surgical treatment and did show a synergistic response in modulating host mediators (MMPs and neutrophil elastase) in the excised gingival tissues (Lee et al. 2004).

Ever since the mid-1960's-1970's, with the recognition that the host response is primarily the propagating factor in periodontitis and bone destruction (Fullmer and Gibson 1966; Goldhaber et al. 1973), the concept of HMT, and its development, has evolved progressively. Several additional approaches, beyond those described above, have also been advocated including the use of bisphosphonates, resveratrol, and, most recently, the resolvins based on omega-3 fatty acids (Preshaw 2008). However, with the earlier discovery of the unexpected ability of the tetracycline (TC) family of antibiotics (e.g., minocycline, doxycycline) to block host-derived MMPs plus other

anti-inflammatory effects of these drugs (Golub et al. 1983, 1998a), and the development of non-antimicrobial formulations of doxycycline as MMP inhibitors (Golub et al. 1994b, 1998b), only this HMT strategy has been FDA-approved and ADA-recommended as a safe and effective adjunct to non-surgical periodontal therapy. However, as discussed in detail by Elburki et al. (2014), a significant drawback of the approved sub-antimicrobial-dose doxycycline (SDD) is that the low-dose formulation cannot be increased for treating connective tissue destructive diseases such as periodontitis, since a higher level in the blood would result in the emergence of antibiotic-resistant bacteria not only that, but prolonged administration of antibiotics causes gastrointestinal problems. On the other hand, the proposed plan (see below) of prolonged therapy with the curcumin-based MMP-inhibitors (MMP-Is) for inflammatory (and other diseases) is not affected by a similar dose-restriction because this compound is not an antibiotic (Elburki et al. 2014).

Given the background described above, "a search has been underway for new drug molecules which exhibit a similar active site for MMP-inhibition as the tetracyclines" (Elburki et al. 2014) "but with a different phenolic superstructure" (Zhang et al. 2012a; Gu et al. 2013). With this strategy in mind, the therapeutic potential of tetracycline's metal-ion binding site (Golub et al. 1991, 1998a) has been expanded by the recent development of a new series of compounds with a similar zinc-binding moiety, but which are bi-cyclic rather than tetra-cyclic, ie., the chemically-modified curcumins or CMCs. The structures of these CMCs, their potency and mechanisms of action as MMP-Is, and their zinc-binding (and other) characteristics have been described recently and "lead" compounds identified (Zhang et al. 2012a, 2012b; Gu et al. 2013). This primary compound, CMC 2.24, is a phenylamino carbonyl curcumin which is tri-ketonic to enhance its zinc-binding characteristics. This differs from the di-ketonic active site on both the tetracyclines

and on traditional/natural curcumin compounds. It has shown evidence of efficacy in vitro, in cell culture, and in animal models of chronic inflammatory and other diseases (Zhang et al. 2012b, 2012b; Botchkina et al. 2013; Elburki et al. 2014).

With the background summarized above, the focus of this dissertation is the study of a new, HMT compound in animal models of periodontal disease and its potential to impact favorably the link between this most common oral-inflammatory disease and an increasingly common and debilitating systemic disease, diabetes mellitus. Therefore, the objectives of the current studies from the outset were:

1. To test the hypothesis that a newly designed chemically modified curcumin (CMC 2.24), recently found (by our laboratory) to be a potent MMP-I in vitro (Zhang et al. 2012a), can be effective in reducing the severity of experimental periodontitis in a rat model in vivo, and elucidation of the mechanisms involved; and
2. To determine whether this pleotropic MMP-I also favorably impacts: (a) systemic inflammation, and (b) the unusually severe periodontal disease associated with the hyperglycemic type I diabetic rat. The latter describes an all-too-common clinical situation in poorly-controlled diabetic patients who also exhibit severe periodontitis which can be refractory to traditional therapies.

Chapter 2

Literature Review

Periodontitis, the most common chronic inflammatory disease known world-wide, is characterized by pathologically-excessive degradation of collagen and other connective tissue constituents and accelerated resorption of the alveolar bone in the periodontal supporting structures of the teeth including the gingiva, periodontal ligament and the alveolar bone. In the USA, the prevalence of periodontal disease, in some form, is known to affect up to 48% of the adult population, distributed as 8.7% for mild, 30.0% for moderate, and 8.5% for severe periodontitis, making it a leading problem in oral healthcare, which also has systemic implications (Eke et al. 2012). As a general guide, the severity of periodontitis can be categorized "on the basis of the extent of clinical attachment loss (CAL) as follows: Mild = 1 to 2 mm CAL, Moderate = 3 to 4 mm CAL, Severe = ≥5 mm CAL" (Armitage 1999).

I. Risk factors for periodontal disease

Decades ago it was thought that all adults were essentially equally susceptible to periodontal disease. More recently, various risk factors have been identified which significantly impact the susceptibility to periodontitis and these risk factors can be divided into modifiable and non-modifiable categories that include the following:

A. Non-modifiable risk factors

1. Aging

Epidemiologic studies have revealed more periodontal disease in older individuals (65-74 years old) compared to younger individuals (Abdellatif and Burt 1987; Grossi et al. 1994, 1995). Furthermore, studies have demonstrated that elderly people exhibit greater microbial plaque accumulation and more severe periodontal disease compared to younger people, supporting the view that periodontal disease is an age-related problem, (Abdellatif and Burt 1987). However, the mechanisms explaining these observations are still unclear. Several studies indicated that periodontal disease is more severe in elderly individuals because of cumulative tissue destruction, over a longer period of time (ie aging), rather than a result of an increased rate of periodontal breakdown as humans age (Abdellatif and Burt 1987).

2. Race

Studies by Beck and coworkers showed that blacks had more advanced periodontal disease (about three times greater) compared to whites of the same age groups (Beck 1994). In studying the risk indicators for blacks and whites, they found that socioeconomic status and the Gram-negative microorganism, P. intermedia in the subgingival biofilm, were risk indicators for blacks but not for whites (Grossi et al. 1994, 1995).

3. Gender

Periodontal disease has frequently been reported to be more predominant or severe in males than in females of similar age groups. Males commonly display poorer oral hygiene than females (Grossi et al. 1994, 1995). However, even when adjusting for oral hygiene as well as

socioeconomic status and age, male gender was found to be linked with more severe periodontal disease perhaps due to hormonal differences (Grossi et al. 1994, 1995; Golub et al. 1999).

4. Genetics

An extensive review of the literature about gene polymorphisms associated with chronic periodontitis has been conducted by Laine et al. (2010). They studied polymorphisms in IL1, IL6, IL10, vitamin D receptor, and CD14 genes. They concluded that, although there is growing evidence pointing toward the association between these genes and chronic periodontitis, these associations are limited to certain populations and no gene polymorphisms can be linked to chronic periodontitis (Laine et al. 2010). The interactions between genetics and periodontal disease are complex, and studies in twins, families, and studies of genetic polymorphisms are essential before genetic effects on periodontal disease can be fully understood (Genco 1996; Genco and Borgnakke 2013).

B. Modifiable risk factors

1. Oral hygiene status and local factors

Poor oral hygiene and accumulation of dental plaque (bacterial biofilm) as well as plaque retention factors, including dental calculus, anatomical factors such as tooth morphology, developmental abnormalities of teeth, position of the teeth in the arch, amount and the quality of the surrounding gingiva, incompetent /potentially incompetent lips (mouth breathing) and iatrogenic factors such as restorations (overhanging margins; surface finish; contour), removable prostheses, orthodontic appliances, are among the many risk factors for periodontal disease which can be readily modified (Lyle 2014).

2. Smoking

The association between cigarette smoking and periodontal disease has long been recognized, and smoking is now considered one of the most important risk factors for periodontitis-associated tooth loss (Bergström and Preber 1994; Genco and Borgnakke 2013). Smoking also delays periodontal wound healing (Heasman et al. 2006). Smoking leads to vasoconstriction, perhaps due to nicotine (Genco and Borgnakke 2013). Cigar and pipe smoking appear to have similar effects on the periodontium as cigarette smoke (Albandar et al. 2000; Krall et al. 2006).

3. Alcohol

Alcohol consumption as a risk factor for periodontal disease may be associated with frequency and dose (Tezal et al. 2004), however, more studies are warranted to understand this link (Genco and Borgnakke 2013).

4. Socioeconomic status

The association between periodontal disease and socioeconomic status has been reproducible, where wide differences in socioeconomic status between different groups of people are compared. These studies comparing people from developed countries with those from developing countries suggested that periodontal disease may be linked to nutritional deficiencies seen in the latter. Another study of periodontal disease in industrialized countries, for example the United States, found that periodontal disease is more severe in people of poorer socioeconomic status (Genco 1996). Yet, in more recent studies, the link between poor socioeconomic status and more severe periodontal disease was not observed when periodontal status was adjusted for oral hygiene and smoking (Grossi et al. 1994, 1995).

5. Stress

Early studies suggested that stress and distress are associated with increased severity of periodontal disease. However, the mechanism of action has not been elucidated (Moss et al. 1996; Genco et al. 1999). Recently, several studies have examined the role of psychological stress, distress and coping skills and all of these were found to be important risk factors for periodontal disease (Peruzzo et al. 2007; Genco and Borgnakke 2013). By far the strongest correlation between stress and periodontal disease is seen in patients with acute necrotizing ulcerative gingivitis (ANUG). This condition is observed most commonly in young adults under stress (Johnson and Engel 1986).

6. Obesity and metabolic syndrome

Obesity is now recognized as an inflammatory state and is linked with numerous chronic diseases such as type II diabetes, cardiovascular disease (CVD), and cancer. Obesity is a significant risk factor for periodontal disease, and insulin resistance appears to play a role in this relationship (Lyle 2014). Scientists believe that systemic inflammation results from adipocytes in fat tissue, generating high levels of pro-inflammatory cytokines, which may provide the link to an increased risk for periodontal disease and insulin resistance (Genco et al. 2005; Genco and Borgnakke 2013).

C. Systemic diseases and conditions

Several recent studies have addressed the question of systemic diseases as risk factors for periodontal disease. Seymour et al (2007) reported, that in spite of 3000 years of a history of suspicion that oral disease can affect general health, it is only in recent years that the link between periodontal diseases and systemic conditions such as coronary heart disease and stroke, and a higher risk of preterm low birth-weight babies (Offenbacher et al. 1996; Page 1998; Craig et al.

2003; Mattila et al. 2005; Carrion et al. 2012), has been systematically studied. Likewise, recognition of the threat posed by periodontal diseases to people with chronic diseases such as, diabetes, respiratory diseases, osteoporosis, rheumatoid arthritis, and inflammatory bowel disease, is quite recent (Van Dyke et al. 1986; Grossi et al. 1996; Scannapieco and Mylotte 1996; Demmer and Desvarieux 2006; de Pablo et al. 2008; Pischon et al. 2008; Dissick et al. 2010; Martínez-Maestre et al. 2010; Genco and Borgnakke 2013; Payne et al. 2015). In spite of these epidemiological associations, the mechanisms for the different relationships are still unclear, but are increasingly being explored as described in a later section of this dissertation on the link between periodontitis and diabetes. A number of hypotheses have been proposed, such as "common susceptibility, systemic inflammation with increased circulating cytokines and mediators, direct bacterial damage to the endothelium and cross-reactivity or molecular mimicry between bacterial antigens and self-antigens" (Seymour et al. 2007). Even though not all populations or studies indicate statistically significant associations, many of the studies, which have been summarized by "meta-analyses", show significant associations, even after the traditional risk factors such as smoking, blood lipids, race, gender and obesity are adjusted. Whether these links are causally associated, or a result of underlying genetic or behavioural risk factors that are shared by both conditions, remains unclear (Bahekar et al. 2007). The following widespread systemic disorders have been investigated, most intensely in recent years, for a link to periodontal disease:

1. Diabetes

Many studies in the literature have linked periodontitis and both type I and type II diabetes (Grossi et al. 1996; Genco et al. 2005; Demmer and Desvarieux 2006; Genco and Borgnakke 2013).

Diabetes mellitus is characterized by hyperglycemia which historically has been the main diagnostic feature of the disease. Long-term hyperglycemia produces a range of cellular and molecular effects leading to oxidative stress, up-regulation of pro-inflammatory responses, and vascular changes which have been associated with numerous diabetes complications. Long-term exposure to high blood glucose levels has been linked to diabetic complications in part via the formation of advanced glycation end products (AGEs) that are produced by non-enzymatic glycation of various proteins in the host tissues (Baynes 1991; Schmidt et al. 1996). Furthermore, interaction between the AGEs with their receptors (RAGEs) has been invoked as a mechanism by which both type I and type II diabetes increases the severity of periodontitis and vice-versa (Lalla et al. 2000; Golub et al. 2006; Graves and Kayal 2008; Engebretson and Hey-Hadavi 2011). More details will be discussed in later chapters. Recently a hypothesis has been proposed linking chronic systemic inflammation with insulin resistance, which then initiates the development of type II diabetes. The initiators of inflammation are numerous and possibly include periodontal infection, which results in a cascade of events, including increased production of inflammatory mediators, stimulation of acute-phase protein synthesis, and subsequent insulin resistance that causes pathogenic changes leading to type II diabetes (Kim and Amar 2006). A more specific pathway is described in Chapter 3 (Elburki et al. 2014).

2. Cardiovascular disease

Periodontitis may be linked to systemic diseases in several ways. Lipopolysaccharide (LPS)/endotoxin in the wall of Gram-negative bacteria in the biofilms, and pro-inflammatory cytokines and other mediators from the inflamed periodontal tissues, may gain access to the circulation in pathogenic quantities (Page 1998). Furthermore, periodontitis and certain systemic

diseases, particularly cardiovascular disease (CVD), have the same risk factors such as tobacco smoking, male gender, race/ethnicity, stress, aging and, in particular, diabetes. As an example,, Carrion et al. (2012), proposed a role for blood myeloid dendritic cells in sheltering and circulating pathogens from the subgingival microbial biofilm in the periodontal pocket which are then transported to atheromatous plaques lining coronary and other arteries. This and other pathways may play a role in providing key signals for myeloid dendritic cell differentiation and atherogenic changes (Carrion et al. 2012). However, in recent years, the host response, rather than periodontopathogens, seems to be the primary focus for elucidating the link between periodontal disease and CVD. Of particular interest, when pro-inflammatory mediators such as TNF-α, IL-lβ, PGE2, IFN-γ, MMP-9 and MMP-8 reach high levels as in the tissues in periodontitis, the periodontium can, as a result, act as a renewing reservoir for release of these cytokines and MMPs into the blood stream. Cytokines and prostaglandins, which gain access to the circulation, can then stimulate and propagate systemic effects. As examples IL-lβ favors coagulation and thrombosis and delays fibrinolysis. IL-1, TNF-α, and thromboxane can cause platelet aggregation and adhesion and the formation of lipid-laden foam cells in cholesterol rich atheroma in cardiac blood vessels (Page 1998). An alternative hypothesis is that inflammatory mediators (e.g., IL-6) from the periodontal tissues (gingiva and others) enter the circulation and then, after stimulating the liver to produce acute phase proteins (e.g., C-reactive protein /CRP, fibrinogen, haptoglobin, etc), produce a systemic inflammation which can mediate CVD, e.g., atherosclerosis and disruption of the cholesterol rich plaque lining the coronary and other arteries (see below). This hypothesis links inflammatory periodontal disease to CVD, such as atherosclerosis, without necessarily invoking bacteria or their products interacting with the cardiovascular system (Mattila et al. 2005). Inflammatory mediators from the host's diseased periodontal tissues, including the cytokines, IL-

1β, TNF-α, and IL-6, may act directly (elevated in the blood, but derived from the gingiva), or indirectly (e.g., cytokines carried from the inflamed gingiva by the circulation to the liver where they induce the expression of acute phase proteins to induce cardiovascular disease; Mattila et al. 2005).

In this regard Craig et al (2003) found that the patients with severe rapidly progressing periodontitis showed a 100% higher hs-CRP level in the circulation (plus other biomarkers) than patients with mild periodontitis (Craig et al. 2003). Regarding mechanisms, CRP can form a complex with oxidized low-density lipoprotein (LDL) cholesterol which, when phagocytosed by macrophages infiltrating atherosclerosis plaques, can differentiate into foam cells (Østerud and Bjørklid 2003). The production of MMPs, for instance MMP-8 (collagenase-2) and MMP-9 (gelatinase B), by these modified inflammatory cells, can destroy the thin collagen ‘‘cap’’ that covers cholesterol- rich plaques lining the coronary arteries leading to “plaque rupture, thrombosis, and acute myocardial infarction” (Libby 1995; Lee and Libby 1997; Bench et al. 2011). Recently Salminen et al (2013), in a subset of 45 postmenopausal women from a randomized, double-blind, placebo-controlled clinical trial involving 128 postmenopausal women who exhibited both periodontal disease and systemic bone loss, demonstrated that the serum from these CVD-vulnerable women on the 2 year regimen of SDD produced a significant increase in LDL cholesterol efflux from macrophages in culture compared to the serum from the postmenopausal women treated with placebo. These findings identified one mechanism by which SDD therapy, in subjects vulnerable to CVD, can reduce the formation of cholesterol-rich atherosclerosis plaques (Salminen et al. 2013).

3. Osteoporosis

Recently a systematic review of the literature describing the relationship of osteoporosis to periodontitis, tooth loss, and mandibular atrophy has been published. Martinez-Maestre et al. (2010) found that most studies revealed that systemic osteoporosis was linked to mandibular osteoporosis, and loss of teeth (Martínez-Maestre et al. 2010). Regarding mechanisms, Golub et al. (1999), reported that experimentally inducing (by ovariectomy) postmenopausal osteoporosis in rats not only produced skeletal bone loss (as expected) but also resulted in increased alveolar bone loss measured morphometrically in the same female rats, which, in turn, was associated with pathologically-excessive MMP (collagenase) activity in extracts of the overlying gingival tissue. Of particular interest, treating these rats with a systemically-administered MMP-inhibitor, CMT-8 (chemically modified, NON-antimicrobial doxycycline), not only reduced systemic osteoporosis (femurs) but also reduced the severity of periodontal disease (ie., reduced gingival MMP activity and less alveolar bone loss) (Golub et al. 1999). Additional in vitro studies demonstrated that the MMP inhibiting properties of the non-antibiotic, tetracyclines could prevent osteoclast-mediated bone resorption (Gomes et al. 1984; Rifkin et al. 1994).

Golub et al. (2010) subsequently demonstrated, in a randomized, double-blind, placebo-controlled clinical trial involving 128 postmenopausal women who exhibited both periodontal disease and systemic bone loss, that women on a 2 year regimen of SDD showed a significant reduction in biomarkers of bone resorption in serum compared to those postmenopausal women treated with placebo (Golub et al. 2010). In the same randomized, double-blind, placebo-controlled clinical trial, Payne et al. (2011) demonstrated that postmenopausal osteopenic women, with periodontal disease treated with a 2 year regimen of SDD, showed a significant reduction in the serum biomarkers of systemic inflammation hs-CRP and MMP-9 (Payne et al. 2011).

4. Pulmonary disease

Oral bacteria have also been involved in the etiology of bacterial pneumonia, and it has been assumed that dental plaque may act as a storage site for respiratory pathogens (Garcia et al. 2001). A variety of oral bacteria have been found in infected sputum and lung abscesses, such as *A. actinomycetemcomitans*, *Actinomyces israelii*, *Capnocytophaga* species, *Eikenella corrodens* and others. As a result, it has also been postulated that people with periodontal disease may be at increased risk for bacterial pneumonia (Scannapieco and Mylotte 1996).

5. Rheumatoid arthritis

Periodontitis has repeatedly been described as a risk factor for rheumatoid arthritis (RA) (Pizzo et al. 2010) and this has recently been reviewed extensively by Payne et al. (2015). Periodontitis and RA are both chronic inflammatory diseases and share smoking as a risk factor (Mikuls et al. 2014). Even though a causal association between periodontitis and RA has not been proven, many studies have revealed a higher incidence of periodontitis in patients with RA compared to non-RA control subjects (de Pablo et al. 2008; Pischon et al. 2008; Dissick et al. 2010). Some evidence suggests that the periopathogens, *P. gingivalis*, may play a role in the etiology of RA (Rosenstein et al. 2004), although the recent review by Payne et al. (2015) stressed that the link of RA and CP is likely largely mediated by the host-response and systemic inflammation as reflected by elevated acute phase proteins in the circulation (Payne et al. 2015). Of particular relevance to the link between these two clinical inflammatory diseases, a recent-double-blind placebo controlled study carried out by O'Dell et al. (2006) demonstrated that an approved MMP-inhibitor medication, SDD, (in combination with methotrexate) reduced the severity of RA, over a 2 year time period, 3-4 times more effectively than the standard-of-care anti-inflammatory drug, methotrexate plus

placebo (O'Dell et al. 2006); note that SDD does not exhibit antibacterial/antibiotic efficacy and only modulates the patient's host response (see review by Gu et al. 2012).

6. Inflammatory bowel disease

Van Dyke and his colleagues (1986) evaluated 20 patients with inflammatory bowel disease (IBD) and proposed that the microorganism, *Wolinella*, isolated from the oral cavity of patients with IBD may play an indirect role in the pathogenesis of the IBD by modifying the host response or, more directly as an infectious agent, or both (Van Dyke et al. 1986). Lamster et al. (1978) stressed the role of an altered host response in their study showing unusually severe CP in patients with Crohn's disease (Lamster et al. 1987). More recently a case-control study by Habashneh et al. (2012) confirmed the link by observing a higher prevalence and severity of periodontitis in patients with IBD compared with controls (Habashneh et al. 2012).

7. HIV Diseases

Periodontal diseases may be the first clinical sign of HIV infection since the immunosuppression and subsequent susceptibility may alter the responses of the oral tissue as well as the composition of the oral flora. Necrotizing ulcerative periodontitis lesions are most commonly observed in individuals with systemic conditions such as HIV infection, immunosuppression and severe malnutrition (Glick et al. 1994).

8. Head and neck cancer

Patients with a history of chronic inflammatory disease in the oral cavity may be susceptible to tumor human papillomavirus (HPV) especially in patients with head and neck squamous cell

carcinoma (HNSCC). This link appears to be evident in patients with oropharyngeal cancer and less obvious in patients with oral cavity or laryngeal SCC (Tezal et al. 2012).

9. Preterm low birth weight

Studies by Offenbacher and his colleagues (1996) indicated that the incidence of preterm low birth-weight babies is significantly associated with severe periodontal disease even after adjusting for other risk factors such as smoking, alcohol drinking, drug use, urinary tract infection and malnutrition (Offenbacher et al. 1996). However, a study by Michalowicz et al. (2006) indicated that treatment of periodontal disease during pregnancy does not significantly change the incidence of preterm low birth weight (Michalowicz et al. 2006). This link remains controversial at this time (Lopez et al. 2002; McGaw 2002; Vettore et al. 2008; Saini et al. 2010).

10. Periodontitis as a manifestation of additional systemic diseases

Finally, concerning this complex and still poorly-understood topic, according to the 1999 workshop on classification of periodontal diseases and conditions (Armitage 1999), periodontitis, as a manifestation of systemic diseases, can be classified into:

1. Periodontitis associated with haematological disorders such as: Acquired neutropenia, Leukaemias and others.

2. Periodontitis associated with genetic disorders such as: Hypophosphatasia, Papillon-Lefèvre syndrome, Downs syndrome, Acatalasia, Familial and cyclic neutropenia, Leukocyte adhesion deficiency syndromes, Chediak-Higashi syndrome, Glycogen storage disease, Infantile genetic agranulocytosis, Cohen syndrome, Ehlers-Danlos syndrome (types IV and VIII) and others.

II. The etiology and pathogenesis of periodontal disease

The understanding of the etiology and pathogenesis of periodontal disease has changed significantly in the last five decades, not only with regards to the specific microorganisms associated with the onset and progression of the disease, but also the dramatically elevated emphasis on the primary role of the host response in the destruction of the periodontal tissues. In this regard, Van Dyke (2014) recently highlighted the concept that the host-response in periodontal disease may not only be induced by (or follow) the microbiological insult to the gingival/periodontal tissues (traditional view), but may also precede, or result in, the development of a recognizable pathogenic microbial biofilm; this concept addresses the recent controversy as to whether chronic periodontitis should be categorized as an "infectious" disease (traditional view) or, that chronic periodontitis is an "inflammatory" disease (Van Dyke 2014).

A. Etiology

By the mid-1960s, the concept took hold that the initiation and progression of periodontal disease is caused by the supra and subgingival dental plaque which is now called the microbial biofilm. At that time, a key series of longitudinal clinical studies were carried out by Dr. Harald Löe and his colleagues collectively called the "Experimental Gingivitis in Man." The aim of these experiments on human subjects was to determine whether or not there is a cause-effect relationship between dental plaque accumulation and the onset of gingival inflammation. The initial experiment was performed on 12 dental students who exhibited good oral hygiene and gingival health. Before beginning this study, the subjects were administered repeated dental prophylaxis and oral hygiene instruction so that their Plaque Index (Pl. I.) and Gingival Index (G.I.) were essentially zero, ie., no detectable plaque adherent to their teeth and no clinically-evident gingival inflammation. Each subject was then instructed not to brush their teeth for a period of time. Immediately, the mean Pl.

I. increased and days later the mean G.I. increased. They concluded that accumulation of dental plaque (now called the microbial biofilm) is the cause of the onset of gingivitis. Moreover, when the subjects were told to begin brushing their teeth again, the mean Pl. I. immediately decreased, and days later the mean G.I. decreased back to the normal levels seen prior to initiating this longitudinal protocol (Löe et al. 1965). Additional studies on human subjects by this group identified the shift of the plaque microflora from a predominantly aerobic Gram-positive biofilm, associated with gingival health, to a biofilm which consisted primarily of anaerobic Gram-negative microorganisms (Löe et al. 1965, 1965); the identification and characterization of these "periodontal pathogens" has been a major "driver" of periodontal research for the past 5 decades (see below)

Soon afterward Lindhe and his colleagues (1973) extended Löe's observations by showing that allowing dental plaque to accumulate in the beagle dog model was followed, in time, by a rise in the gingival crevicular fluid flow, then an increase in gingival inflammation which was assessed clinically and then months later, by increased pocket depth indicating the development of tissue breakdown and the conversion of gingivitis to periodontitis (Lindhe et al. 1973).

More recently, the focus on the etiology of periodontal disease has changed markedly with a focus on specific anaerobic microorganisms as the initiating factor. A number of specific bacteria, such as *P. gingivalis, Tannerella forsythia, Treponema denticola, Aggregatibacter actinomycetemcomitans* (previously known as *Actinobacillus actinomycetemcomitans*), *Prevotella intermedia, and others including Fusobacterium nucleatum, Wolinella recta, and spirochetes,* have been associated with severe forms of periodontal disease (Haffajee and Socransky 1994). Even today, the predominant view highlights the so-called "red complex" (which includes *P.*

gingivalis, *Tannerella forsythia, Treponema denticola)* as the primary etiologic factor in the most common periodontal disease (excluding gingivitis) chronic periodontitis (Socransky et al. 1998), although in more aggressive (and rare) forms of periodontal disease, localized and generalized aggressive periodontitis, other microorganisms have been highlighted, ie., *Aggregatibacter actinomycetemcomitans* (Richardson 2005; Haubek and Johansson 2014). Furthermore, a group of pathogens not typically present in the oral cavity has also been linked to periodontal disease, such as Enterobacteracea, Pseudomonadacea, and Acinetobacter (Slots et al. 1990). Periodontitis is a chronic inflammatory disease, in which severe forms of the disease are associated with specific bacteria that have colonized the subgingival area regardless of the host's protective mechanisms; as discussed below, the response of the host to the microbial irritants (e.g., endotoxin/LPS) is now recognized to be essentially responsible for the connective tissue breakdown and loss of alveolar bone which are the essential characteristics of this disease. Nevertheless, the vulnerability of individuals seems to vary greatly depending on which risk factors are functioning (Genco 1996). Moreover, as recently reiterated by Van Dyke (2014), this association between these specific Gram-negative microorganisms and the onset and progression of periodontitis may reflect, at least in part, the inflammation-altered-environment in the periodontal lesion (periodontal pocket) causing the normal Gram-positive microflora to shift to a more anaerobic Gram-negative microbial biofilm associated with disease (Van Dyke 2014).

B. Pathogenesis: Germ-free models and periodontitis pathogenic mechanisms--early studies

In the early 1960s, a major breakthrough was achieved by Dr. Jerome Gross at Harvard medical school when he discovered, for the first time ever, a proteolytic enzyme produced by animal tissues (the “host”) that could degrade the triple helical collagen molecule under physiologic condition of

pH and temperature; he called this enzyme, animal collagenase. Until this landmark discovery, it was widely thought that the triple helix collagen molecule, which is the major constituent of all connective tissues in the body, was not susceptible to the then-known neutral proteinases such as trypsin, plasmin, neutrophil elastase, etc. This breakthrough experiment was published in 1962 (Gross and LaPiere 1962).

Soon afterwards, another major observation regarding collagen destruction was made by a dental researcher at NIDCR/NIH, Dr. Harold Fullmer. He used the sterile J. Gross tissue-culture system, and a collagen matrix comprised of triple-helical collagen molecules, and found that human gingiva, surgically excised from a healthy site, did not produce a zone of lysis of a collagen gel in tissue culture whereas gingiva excised from a diseased site (ie., a deep pocket) produced a dramatic increase in mammalian collagenase as made evident by the zone of lysis in tissue culture, and the production of 3/4 and 1/4 split products of collagen characteristically produced by host or mammalian collagenase (Fullmer and Gibson 1966).

A few years later, Ivanyi and his colleagues in Britain (1972), described the role of the immune response in periodontal destruction independent of bacteria. Their data suggested that cell-mediated immunity plays a significant role in the pathogenesis of periodontal disease (Ivanyi et al. 1972). Subsequently, Taubman et al. (1984) determined the role of the immune response in a germ-free rat model of experimental periodontal disease. His experiments supported the idea that T-cell functions and regulation of the immune response by the thymus can have a protective and/or destructive effects in periodontal disease. He concluded that in order to control this disease, it

would be crucial to enhance the protective "arm" of the immune response, and suppress its destructive aspect (Taubman et al. 1984).

About the same time it was becoming increasingly clear that systemic diseases particularly diabetes can increase the severity of periodontal disease in both human and experimental animals (Löe 1993). In earlier experiments, Golub et al. (1973) addressed the mechanisms by which diabetes can increase periodontal breakdown. Using the tissue culture technique of Gross and LaPiere (1962) and Fullmer and Gibson (1966), he and his colleagues showed, for the first time in any tissue, that inducing experimental diabetes (alloxan or streptozotocin injection) and severe hyperglycemia dramatically increased mammalian or host derived collagenase activity; note that the tissues he cultured in the collagenolytic system were gingival. He demonstrated that the viable gingiva in tissue culture, under sterile conditions, when derived from hyperglycemic diabetic rats produced more solubilization and degradation of radioactive collagen fibers than gingiva from non-diabetic rats and that reducing blood glucose levels by treating the diabetes with insulin injections reduced the excess gingival collagenase activity. This demonstrated that diabetes increases mammalian collagenase activity as a mechanism for pathologic periodontal tissue breakdown and, assuming the same effect could be found in other tissues as well, could also provide a mechanism which mediates a variety of complications throughout the body [in fact, in recent years, diabetes has been found to increase MMPs systemically, not just locally in the gingiva; (Ramamurthy and Golub 1983; Ryan et al. 1999, 2001)].

As reviewed extensively by Golub et al. (1998a), the next question asked was: did diabetes and hyperglycemia result in pathologically excessive mammalian (host-derived) collagenase activity

in the gingiva (a) by creating an environment in the gingival crevice (e.g., elevated glucose and urea concentrations; hypoxia, etc…) favoring the overgrowth of an anaerobic Gram-negative microflora which resulted in elevated levels of endotoxin penetrating the gingival tissues, or (b) by mechanisms independent of a microbial shift and only dependent on an altered host response (a dominant pathway suggested was the long-term hyperglycemia producing AGE/RAGE interaction to up-regulate MMP production; Golub et al. 1998a; Ryan et al. 1999). In the microbially-induced pathway, diabetes was thought to alter the microbial biofilm by increasing the level of Gram-negative anaerobic bacteria in the periodontal pocket. This would result in elevated endotoxin (LPS) levels in the gingival pocket, then the endotoxin could penetrate the diabetes-damaged gingival crevicular epithelium to stimulate the host cells to produce elevated collagenase and other MMPs which would degrade collagen and the other connective tissue constituents. In the alternative pathway (host-mediated pathway), the view was that diabetes increases gingival collagenase independent of bacteria. For example, hyperglycemia would produce advanced glycation end products (AGEs) which would then react with RAGEs on the surface of host cells such as, fibroblast and macrophages which would stimulate MMP production. To identify which hypothetical pathway was correct, Golub and his group (1983) decided to suppress the microbial pathway by treating the diabetics with minocycline (a tetracycline antibiotic) to see if suppressing the bacterial pathway cancels the diabetic effect on gingival collagenase. At first glance it appeared that the excess collagenase in the diabetic gingiva was due to an overgrowth of periodontal bacteria, because this effect was inhibited by treating the diabetic rats with the antibiotic minocycline. However, to ensure that the tetracycline was not acting by some unknown non-antimicrobial mechanism, the same experiment was repeated using a germ-free rat model and, surprisingly, the effect of the drug was virtually the same. Once again diabetes, even in the germ-

free rats, dramatically increased mammalian collagenase activity, meaning that the shift in microbial effect was not related to the excess collagenase activity in the gingiva of diabetics. Even more intriguing, treating the germ-free diabetic rats with this tetracycline again reduced the excess mammalian collagenase activity to near-normal levels. This demonstrated, for the first time ever, that tetracyclines could inhibit host-derived MMPs and inhibit collagen degradation, and by mechanisms unrelated to the antibiotic activity of TCs. Ultimately, this discovery resulted in the development of several non- antibiotic formulations of tetracyclines for the treatment of a variety of collagen destructive diseases (Golub et al. 1983, 1991, 1998a; Gu et al. 2012). Moreover, this novel non-antibiotic property of tetracyclines has been confirmed by various researchers internationally (Emingil et al. 2008; Preshaw et al. 2008; Tüter et al. 2010).

C. Immunopathogenesis of Periodontal Disease--recent studies

It has been accepted traditionally that periodontal disease is induced by bacteria in dental plaque or biofilm; moreover, evidence now exists that specific microorganisms may be responsible for more aggressive forms of the disease. However, it has also been acknowledged that, some individuals can harbor these specific bacteria, and NOT show signs of disease progression (Moore and Moore 1994). Host factors related to disease susceptibility is of extreme importance to the outcome of periodontal disease, and even though periodontal pathogens are still regarded as the main initiating agents, the host's immune-inflammatory response to these pathogens plays an essential role (Seymour and Gemmell 2001). In fact, a recent essay by Van Dyke (2014) recommended that chronic periodontitis should no longer be classified as an "infectious" disease, and should now be considered (like rheumatoid arthritis, inflammatory bowel disease/Crohn's disease, psoriasis) an "immune-inflammatory" disease.

Inflammation in the periodontal tissues is regulated by the expression of mediators generated by the host that cause a number of pleiotropic events resulting in the recruitment of inflammatory cells and elaboration of biologic mediators by leukocytes and macrophages, and other cells e.g., fibroblast, epithelial cells, and bone cells. If the inflammation is temporary or acute in nature, it can protect the host by stimulating defense mechanisms combating infection and initiating wound healing. On the other hand, if the inflammation is severe and prolonged (or unresolved), it can cause substantial tissue breakdown (Graves 1999). Many cell types and mediators, including T helper 1 and T helper 2 lymphocytes, cytokines and chemokines, seem to participate in the immunopathogenesis of periodontal diseases (Garlet et al. 2003). Chronic inflammatory periodontal disease manifests itself clinically, as at least two distinct diseases. Data, based on microbiological, immunological and animal studies, have revealed that some types of periodontal disease in adults can stay "silent" or non-progressive for many years and do not jeopardize the life of the dentition (ie., gingivitis), while other types, regardless of extensive treatment, continue to progress and, eventually, can cause tooth loss (ie., periodontitis). Even though periodontal bacteria are the initiating agents in periodontitis, subsequent progression and severity of the disease are thought to be determined by the host immune response (Seymour 1987).

Similar to other chronic inflammatory diseases, a complex network of cytokines take part as vital mediators in controlling cellular interactions. As observed in many clinical and experimental studies, LPS and other products of bacteria can stimulate the host cells to release pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and others. Target cells in turn are stimulated to elaborate still other cytokines (some anti-inflammatory, e.g., IL-10), inflammatory mediators

and, in the case of tissue destructive diseases, catabolic enzymes. The cytokine network controls the inflammatory mechanisms to either intensify or restrain tissue reactions (Bickel et al. 2001).

As a result of the site-specific nature of the periodontal disease process, this network has to be securely controlled by local processes. The initial (acute) inflammatory phase includes a reactive and defensive response to the bacterial products. Increased neutrophil migration into the sulcus, increased flow of serum proteins into the tissues, proliferation of epithelial cells, and subsequent local accumulation of mononuclear cells accentuates these phenomena (Bickel et al. 2001).

The innate immune system in the periodontal microenvironment consists of multiple cell types, including epithelial cells, CD38+ Langerhans cells in oral mucosa, tissue macrophages, neutrophils, and dendritic cells (Teng 2006). Their role in the periodontium is to provide defense against the invasion of pathogens and the maintenance of tissue integrity. This innate immune system is effective in protecting the periodontium as evidenced by the limited number of bacteria actually invading the periodontal tissues and the very rare occurrence of sepsis in spite of the long-term bacterial load in the dental biofilm associated with periodontal disease. However, even though most of the microorganisms are located outside the periodontal tissues, their microbial associated molecular patterns (MAMPs) trigger innate immune responses by activating Toll-like receptor (TLR) signaling which, in turn, may initiate and modulate adaptive immune responses (Sugawara et al. 2006). These receptors are expressed by immune cells such as macrophages, neutrophils, and dendritic cells as well as by non-immune resident cells, such as fibroblasts and epithelial cells. Within the periodontal tissues, TLR2 and TLR4 expression are increased in severe periodontitis,

suggesting that these receptors have an increased capacity to signal and influence downstream cytokine expression (Ren et al. 2005; Kajita et al. 2007).

Regarding the role of the biofilm, their metabolic products, notably LPS/endotoxin component of the cell wall of Gram-negative pathogens, is one of the main microbial associated molecular patterns that can stimulate the expression and production of pro-inflammatory cytokines through activation of TLRs. These cytokines e.g., IL-1β, TNF-α, and IL-6 are considered to be the essential initiators involved in the destruction of soft and hard tissues that are the hallmarks of aggressive periodontal disease. This important role of LPS for periodontal diseases is demonstrated in the LPS-model of experimentally induced periodontal disease, where direct injection of LPS into the gingival tissues initiates a local host response that involves recruitment of inflammatory cells, generation of prostanoids and cytokines, secretion of lytic enzymes and stimulation of osteoclasts, culminating in the destruction of both soft and mineralized tissues of the periodontium (de Aquino et al. 2009). Virtually the same host response can be achieved by infecting gnotobiotic rats with a periopathogen such as *P. gingivalis* (Chang et al. 1988).

IL-1 and TNF-α can up-regulate collagenases and PGE2 synthesis which mediate breakdown of periodontal tissues. In acute gingivitis, T cells dominate, whereas in the later-occurring immune response, large numbers of B cells predominate. This has led to debates on the defensive role of cellular immune responses in gingivitis. Chronic inflammation is thought to be controlled mainly by IL-2, IFN-γ, IL-10, and IL-12, while acute inflammation is generated mainly by elevated levels of IL-1 family of cytokines, as well as TNF-α. In this regard, it should be recognized that the most common type of periodontitis, chronic periodontitis, is actually

characterized by a period of disease activity characterized by infiltration of acute inflammatory cells the PMN leukocytes which migrate into the periodontal pocket while chronic inflammatory cells remain in the periodontal connective tissue. Therefore, chronic periodontitis is characterized by “bursts” of acute inflammation and tissue destruction (exacerbation) followed by periods of remission. Moreover, changes in expression of entire cytokine families might be consistent with the concept of a change from a T helper 1 to a T helper 2 response. This change has been noticed in chronic diseases and is implicated in chronic periodontal inflammation. Furthermore, the experimental reduction of neutrophilic granulocytes in the dento-gingival area leads to enhanced subgingival microbial colonization even in the presence of mechanical elimination of supragingival plaque (Bickel et al. 2001).

Periodontal disease is a peripheral infection mediated by a variety of Gram-negative bacteria. T-lymphocytes that can be present in the dense inflammatory infiltrate in this disease. CD4+ and CD8+ T-cells are found in periodontal lesions, as memory/activated T-lymphocytes. Th1-type T-cells upregulate the production of pro-inflammatory cytokines IL-1 and TNF-α, which can cause resorption of bone by promoting the differentiation of osteoclast precursors and subsequent osteoclast activation. Such osteoclast differentiation is reliant on activation of osteoprotegerin ligand (OPG-L) production by osteoblasts. The tumor necrosis factor (TNF) family of molecules, the receptor activator for NF-kB ligand (RANKL), its receptor RANK, and the natural antagonists, osteoprotegerin (OPG), have all been found to be key modulators of bone remodeling and are directly involved in the differentiation, stimulation, and survival of osteoclasts and osteoclast precursors (Taubman and Kawai 2001). Activated CD4+ T cells express RANKL,

which can directly activate osteoclastogenesis and the loss of alveolar bone which characterizes periodontitis (Taubman and Kawai 2001).

In contrast, activated T-cells, by virtue of direct production and expression of OPG-L, can directly promote the differentiation of osteoclasts. OPG-L seems to be mostly expressed on Th1-type cells. The direct and indirect participation of T-cell in periodontal bone resorption seems to be reliant on the degree of Th 1-type T-cell recruitment into inflamed gingival tissues. This T-cell recruitment is mediated by adhesion molecules and chemokines/chemokine receptors. The T-cell characteristics in inflamed periodontal tissues can be compared with those in rheumatoid arthritis, in which bone resorption is often attributed to Th1-type T-cell involvement (Taubman and Kawai 2001).

D. Link between local chronic periodontitis and systemic circulation

Goncalves et al. (2010) reported that peripheral blood mononuclear cell (PBMC) from patients with generalized chronic periodontitis and no history of systemic diseases released increased levels of TNF-α and IL-6 in cell culture compared to healthy subjects without chronic periodontitis. The increased production of such pro-inflammatory cytokines indicates a hyper-reactivity of PBMC from patients with local periodontitis and periodontal tissue destruction plus a systemic inflammatory burden (Gonçalves et al. 2010). However, the concept that periodontal disease increases the levels of TNF-α and IL-6 in the circulation of patients remains controversial (Ide et al. 2003; D'Aiuto et al. 2004). Some studies have found higher plasma levels of TNF-α and IL-6 in subjects with periodontitis, compared to healthy controls, which are reduced after periodontal therapy, while others have not found this association (Marcaccini et al. 2009; Monteiro

et al. 2009). Another study involving PBMC also demonstrated that LPS-stimulated monocytes from periodontitis subjects released higher levels of TNF-α than those mononuclear cells from control subjects (McFarlane et al. 1990). Gustafsson et al. (2006) also showed a tendency for a higher release of TNF-α by stimulated mononuclear cells from subjects with treated periodontitis when compared to healthy ones (Gustafsson et al. 2006). On the other hand, Fokkema et al. (2002) found no differences in the release of TNF-α and IL-6 in the *E. coli* LPS-stimulated whole blood cell cultures (WBCC) comparing periodontitis and healthy subjects (Fokkema et al. 2002). These contradictory results can be explained, in part, by methodological and population differences including the inclusion of smokers, ethnic characteristics of the populations, sample size, types (chronic or aggressive) and severity of periodontitis, type of cell culture (WBCC or PBMC), duration and methods of cell stimulation and differences in assays for these inflammatory cytokines.

Similar differences in study results have been found using other types of leukocytes and other cytokines. As examples, Fokkema et al. (2002) demonstrated that the levels of IL-8 in the supernatants of LPS-stimulated WBCC from patients with periodontitis were higher than those from controls (Fokkema et al. 2002). Other studies also demonstrated that the plasma levels of IL-8 were higher in patients with periodontitis than in healthy individuals (Monteiro et al. 2009). On the other hand, Restaino et al. (2007) showed that IL-8 secretion promoted by various stimulating agents in WBCC did not differ between periodontitis and control groups (Restaíno et al. 2007). Interestingly, the authors noticed that, unlike the control group, the levels of IL-8 secreted by stimulated neutrophils from periodontitis subjects were significantly lower than those from

periodontally healthy individuals. However, methodological differences between these various studies have hindered any direct comparisons (Restaíno et al. 2007).

Goncalves et al. (2010) found no differences between periodontitis and healthy groups with regard to the levels of the anti-inflammatory cytokine, IL-10, indicating that LPS-stimulated PBMC of periodontitis and non-periodontitis subjects may present a similar ability to produce this regulatory anti-inflammatory cytokine (Gonçalves et al. 2010). These findings are supported by a previous study using the supernatants of stimulated WBCC from chronic periodontitis subjects (Fokkema et al. 2002). In relation to the plasma levels of IL-10, Monteiro et al. (2009) did not find differences in the concentration of IL-10 between chronic periodontitis and healthy subjects (Monteiro et al. 2009). On the other hand, Havemose-Poulsen et al. (2005) observed higher plasma levels of IL-10 in generalized aggressive periodontitis individuals, when compared to healthy ones (Havemose-Poulsen et al. 2005).

Increased levels of circulating monocytes have been observed in subjects with periodontitis (Fokkema et al. 2002). Gustafsson et al. (2006) demonstrated that mononuclear cells and neutrophils from subjects with treated periodontitis and slight periodontal inflammation are also hyper-reactive and susceptible to relapse of the disease when compared to periodontally healthy subjects (Gustafsson et al. 2006). In addition, data on whether periodontal therapy affects the serum levels of CRP and other systemic biomarkers are still inconclusive (Restaíno et al. 2007). Therefore, it is reasonable to suggest that subjects with periodontitis may have a constitutionally different host response in PMBCs, independent of the presence of active disease. However, it could also be argued that patients with periodontitis, as a result of this common oral inflammatory

disease, develop systemic inflammation including elevated levels of cytokines and acute phase protein, e.g., CRP. From these findings, it may also be speculated that high reactivity of immune cells to LPS may be a susceptibility factor for both periodontal tissue breakdown and systemic diseases in individuals with periodontitis.

E. Regulatory pathways in chronic periodontitis

Activation of NF-κB is known to be essential for the expression of inflammatory cytokines involved in the pathogenesis of various inflammatory diseases (DiDonato et al. 1997; Milward et al. 2007), suggesting that this cell signaling pathway could be a major target for host modulation therapies. Activation of NF-κB was also seen in oral epithelial cells exposed to the periodontal pathogens, *fusobacterium nucleatum and P. gingivalis* (Carayol et al. 2006), indicating the crucial role of NF-κB on innate immunity in the oral cavity. In this regard, stimulation of gene expression in human monocytic cell line by *P. gingivalis* LPS was abolished by inactivation of NF-κB (Nakajima et al. 2006). In another study, inhibition of NF-κB activation by endocannabinoids (derived from arachidonic acids), which are lipid mediators with immunonosuppressive and anti-inflammatory properties, decreased the production of pro-inflammatory mediators (IL-6, IL-8 and MCP-1) induced by *P. gingivalis* LPS in human gingival fibroblasts, another resident cell type with an important role in innate immune response in periodontal diseases. All of these (and other) studies indicate the role of NF-κB in the expression of fundamental mediators in periodontal disease (Xu et al. 2009). Additional details are discussed in Chapter 5.

F. Matrix Metalloproteinases (MMPs)

Matrix Metalloproteinases (MMPs), generated by animal and human tissues (the host), are neutral proteinases that physiologically/normally participate in the remodeling of the extracellular matrix (ECM) through the degradation of its various components. However, under pathologic conditions, the MMPs are expressed and activated excessively (see below). For an enzyme to be considered an MMP it must meet the following two criteria: the protein must have a zinc-binding catalytic domain and it must contain a propeptide sequence of around 80 amino acids with a conserved cysteine residue capable of coordinating with the zinc atom (Sorsa et al. 2006). These MMPs have a wide range of physiologic functions, including their ability to modify tissue during embryogenesis, tooth eruption, bone and connective tissue remodeling and removal of the endometrial lining during menstruation (Sekton 2010). In addition, MMPs also participate in wound healing, and cancer metastasis. In pathologically excessive levels/activity, these MMPs play a key role in the breakdown of tissue during various inflammatory, hormonal and neoplastic diseases (Hu et al. 2007). MMPs can be subdivided into categories such as collagenases, gelatinases, stromelysins, and membrane-type MMPs depending on their target substrates (Sorsa et al. 2006) and these are listed in Table 1 (Whittaker et al. 1999; Visse and Nagase 2003; Zhang et al. 2012a).

While a normal level of these MMPs is required for proper physiological function, overproduction of these enzymes can result in pathological conditions such as cardiovascular disease, chronic obstructive pulmonary disease, cancer, rheumatoid arthritis, osteoporosis, and periodontal disease (Payne and Golub 2011). In these illnesses, MMPs are elevated to a level where their natural inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), are incapable

of decreasing their activity back to normal (Visse and Nagase 2003). In an attempt to combat excessive collagenase activity through drug intervention, Golub group (as well as others) has been developing new drugs to "normalize" these pathologically-elevated MMPs which are associated with connective tissue (including bone) breakdown.

As background, four decades ago Golub laboratory was the first to discover that diabetes increases mammalian collagenase activity in any tissue--the tissue initially studied was the gingiva (Ramamurthy et al. 1973b), and subsequently in the skin of rats (Golub et al. 1983; Ramamurthy and Golub 1983). Their observation then led to an original discovery in therapeutics, that tetracyclines (TCs) such as doxycycline can inhibit MMPs both in vivo and in vitro, and by mechanisms that are unrelated to the antibiotic activity of these drugs (Golub et al. 1983, 1998a; Gu et al. 2012). The discovery of this unexpected property of tetracyclines has been widely acknowledged to provide a new, safe. and effective therapeutic approach to the treatment of multiple diseases which involve collagen destruction (Curci et al. 1998; O'Dell et al. 2006; Monk et al. 2011; Siller and Broadie 2012; Layton and Thiboutot 2013).

MMPs and periodontal disease

Collagen destruction is an essential component in the pathogenesis of periodontitis including bone resorption (Golub et al. 1990) and is thought to be mediated by a complex cascade involving both host-derived MMPs (and other proteinases) and their endogenous inhibitors, all initially triggered by microbial insult, e.g., LPS or endotoxin. However, host-derived MMPs play an essential physiologic role as well (Page 1991; Sorsa et al. 1992; Golub et al. 1995). Historically, Fullmer and Gibson (1966) were the first to demonstrate that human gingiva express a host-derived

collagenolytic MMP (ie., then simply called “collagenase”) and that this MMP was produced in excessive levels during periodontal disease and was a major mediator of connective tissue destruction during periodontal disease (Fullmer and Gibson 1966).

Bacteria such as *P. gingivalis* in the biofilm induce resident cells in the gingiva, such as fibroblasts and epithelial cells, or infiltrating inflammatory cells such as neutrophils, monocytes and macrophages, to synthesize and release excessive levels of MMPs which subsequently, become activated and overcome their natural inhibitors (e.g., TIMPs) (Chang et al. 1988; Sorsa et al. 1992; Golub et al. 1995).

Mechanisms involve the ability of bacterial products, such as LPS and other toxins from Gram-negative anaerobes, as well as host-derived inflammatory mediators (cytokines, prostanoids, ROS) to induce transcription of MMPs and their ultimate secretion and activation. In the case of PMN leukocytes, these mechanisms induce the degranulation of azurophilic and specific granules; the latter release their stored MMP-8 (collagenase-2) and MMP-9 (gelatinase A) into the inflamed tissues (Page 1991; Birkedal-Hansen 1993; Golub et al. 1995).

Excessive levels of MMPs have been found in inflamed gingiva, gingival crevicular fluid (GCF), peri-implant sulcular fluid (PISF), mouth-rinses and saliva of humans with periodontal diseases (Golub et al. 1995; Sorsa et al. 2006). Moreover, MMP levels and activity have been found to decrease following periodontal treatment (Golub et al. 1985, 1990, 1995; Sorsa et al. 2006; Tüter et al. 2010).

MMP-1 or collagenase-1, is constitutive and known to be expressed at low levels in periodontium. MMP-1 constitutes 1-2% in human GCF (Golub et al. 1997). MMP-8 or collagenase-2, also known as neutrophil collagenase, is stored in specific granules of the PMNL and is released during

degranulation as a result of stimuli such as the reactive oxygen species. MMP-8 can also be produced by resident cells such as gingival fibroblasts, epithelial cells, monocytes, macrophages and plasma cells during inflammation and in response to cytokines such as IL-1β (Sorsa et al. 2006).

MMP-8 is one of the essential biomarkers in periodontitis and is largely responsible for connective tissue destruction (Lee et al. 1995; Sorsa et al. 2006); it constitutes 80-90% of total collagenase in the human GCF (Golub et al. 1997) and treatment of periodontitis with scaling and root planing (SRP) and SDD dramatically decreases the levels of MMP-8 in the GCF of humans (Sorsa et al. 2010; Golub et al. 2010).

MMP-13 or collagenase-3 is also known as bone collagenase although it is also produced by other cells, e.g., epithelium (Uitto et al. 1998). During bone development and gingival wound healing MMP-13 is expressed (Sorsa et al. 2006). MMP-13 constitutes 10-20% in human GCF (Golub et al. 1997) and excessive MMP-13 levels have been detected in periodontal disease (Hernandez et al. 2006, 2009; de Aquino et al. 2009) especially in the junctional epithelium near the periodontal lesion at a histochemical level (Uitto et al. 1998).

Gelatinases include MMP-2 or gelatinase A and MMP-9 or gelatinase B. MMP-2 is constitutive and known to be expressed at low levels in the periodontium. Pro MMP-2 is activated by MMP-14, whereas proMMP-9 is activated by a serine proteinase, human trypsin-2 and oxidants such as hypochlorous acid (Sorsa et al. 2006).

MMP-9 can be secreted by different cell types such as macrophages, eosinophils, keratinocytes and osteoclasts, and, like its collagenase counterpart, MMP-8, is released during PMNL degranulation (Cuadrado et al. 2008). Elevated levels/activation of MMP-9 have been shown to be

expressed in several chronic inflammatory oral diseases including periodontitis, periimplantitis and pericoronitis (Beklen et al. 2006; Sorsa et al. 2006).

Expression and activation of MMP-9 can be triggered by a number of inflammatory mediators such as IL-1β, TNF-α, IL-2 and IFN-ϒ (Sorsa et al. 2006).

III. Traditional antimicrobial therapy

The role of bacteria in the initiation of periodontitis is well-documented. However, it is now widely accepted that the collagen and connective tissue destruction of the gingiva and periodontal ligament, and the loss of alveolar bone, is largely mediated by the host response (Ryan 2002; Gitlin and Loftin 2008).

In this regard, LPS and other substances can penetrate the gingival tissues from the subgingival biofilm, and initiate and propagate immuno-inflammation, leading to production of elevated levels of pro-inflammatory cytokines. These and other inflammatory mediators (e.g., ROS, NO) are known to stimulate production and/or release of MMPs and other neutral proteinases, eg., PMNL elastase, cathepsin G, which degrade the connective tissues of the gingiva and periodontal ligament; these and prostaglandins also help mediate alveolar bone resorption (Ryan et al. 2003).

Traditionally, non-surgical periodontal treatment has included mechanical debridement procedures such as scaling and root planing (SRP) and surgical reduction of probing depth to reduce the bacterial “load” in the periodontal pocket, plus the enhancement of the effectiveness of plaque removal by oral hygiene procedures. In addition, adjunctive antimicrobial therapy has also been advocated as follows:

(1) systemic antimicrobial therapy:

Several types of antibiotics have been studied including Tetracyline, Metronidazole, Augmentin, and Metronidazole plus Amoxycillin (Lindhe et al. 1983; Berglundh et al. 1998). Systemic antimicrobial therapy used in conjunction with root planing with or without surgery has in certain circumstances resulted in improved healing. However, the routine use of systemic antibacterial drugs in the treatment of chronic periodontal diseases cannot be recommended. Consideration may be given to the use of adjunctive systemic antimicrobials in some patients with aggressive forms of the disease, although a recent review of the literature, and statistical analysis of the reported results (retrospective cohort) by Cunha-Cruz et al. (2008) could not identify any statistically significant benefit of adjunctive systemic antibiotic therapy in the management of the periodontal patient (Cunha-Cruz et al. 2008).

(2) Local antimicrobial therapy:

Several types of topical antimicrobials are available to the practioner and include direct irrigation, and slow release materials such as Elyzol (25% Metronidazole gel), Dentomycin (2% Minocycline hydrochloride gel), Arestin (1% minocycline hydrochloride), Periochip (Chlorhexidine chip), Atridox (Doxycycline in a resorbable polymer). These local delivery systems are of limited value. They are only effective if the agent is delivered to the base of the pocket and when high levels of the antibiotic (much higher than those seen in the circulation following oral administration) persist within the pocket (Heijl et al. 1991; Williams et al. 2001; Divya and Nandakumar 2006; Tomasi et al. 2008; Pragati et al. 2009; Matesanz-Pérez et al. 2013).

IV. Systemic host modulation therapy (HMT)

In the mid 1980s, Williams and his colleagues at the Harvard School of Dental Medicine (HSDM), and Golub and his group at Stony Brook University (SBU), addressed the importance of the HMT in periodontal disease by demonstrating (1) that non-steroidal anti-inflammatory drugs or NSAIDs such as flurbiprofen can inhibit alveolar bone loss in animal studies and in human clinical trials (Williams et al. 1984, 1987, 1988; Offenbacher et al. 1992; Howell and Williams 1993), and (2) that Tetracyclines, by NON-antibacterial mechanisms, can inhibit host-derived MMPs and osteoclast activity to suppress collagenase and bone resorption, processes essential for periodontal and other (e.g., Rheumatoid arthritis) diseases (Golub et al. 1983, 1994a, 1998a).
This paradigm shift at that time, with a focus on the host response, led to the search for novel therapies for periodontitis. These host-modulating therapies included:

(1) The non-steroidal anti-inflammatory drugs (NSAIDs). These are commonly used to treat pain and inflammation and include the inhibitors of the prostaglandins and other arachidonic acid metabolites which are associated with the pathogenesis of periodontitis (Klein and Raisz 1970; Goldhaber et al. 1973; Williams et al. 1987). Therefore, it was logical to use inhibitors of arachidonic acid metabolites, such as the NSAIDs, in treatment of periodontitis (Williams et al. 1984, 1987, 1988; Offenbacher et al. 1992; Howell and Williams 1993). However, NSAIDs are frequently associated with gastrointestinal side effects, such as bleeding or perforation of gastroduodenal ulcers, (Buttgereit et al. 2001) and increased risk of CVD (Graham et al. 2005) and cannot be recommended for long-term use as a host modulating therapy (Williams et al. 1989). Also, flurbiprofen, systemically administered, did produce a "rebound" effect ie., after stopping

NSAID treatment, the disease severity rebounded and become even worse than that before this treatment was initiated (Lichtenberger et al. 1995).

(2) The Tetracyclines. These are broad-spectrum antibiotics that unexpectedly were found to inhibit host-derived MMPs (Golub et al. 1991, 1998a; Ryan et al. 2001). This property was first identified by Golub et al. in 1983 who demonstrated that a semi-synthetic tetracycline, minocycline, had the ability to reduce pathologically-excessive collagenase activity even in a germ-free diabetic rat model. This was a seminal discovery for two reasons: (1) because it identified for the first time that tetracyclines can inhibit mammalian (host-derived) collagenases and other MMPs, and (2) by mechanisms that were unrelated to their well-known antimicrobial activity (Golub et al. 1983). Additional studies soon verified and expanded the use of tetracyclines as a potential treatment for periodontal disease because of these newly-recognized characteristics and their ability to inhibit pathologically-elevated collagenase activity (Golub et al. 1984, 1985, 1991, 1998a). The success of the tetracyclines was propelled into mass-market application when several novel formulations of TCs were developed including: (1) NON- or sub-antimicrobial-doses of doxycycline (SDD), which were ultimately FDA approved for the treatment of periodontal disease (Periostat®), (see (Golub et al. 1991, 1998a, 2001; Gu et al. 2012), for reviews) and, later, a NOVEL sustained-release formulation of SDD which was approved for treating the chronic inflammatory skin condition, rosacea (Oracea®). Although a number of researchers have tried to develop MMP-Is to inhibit connective tissue degradation associated with inflammatory and other diseases, their efforts were not successful (see Hu et al. 2007, for review); the only US-FDA approved systemically administered drug as MMP-Is are those based on TCs. Doxycycline, when administered as a regular dose tetracycline, like most, if not all antibiotics, produces

complications such as gastrointestinal disturbance, increased photosensitivity, and the emergence of antibiotic- resistant microorganisms (Golub et al. 1990, 1995; Gu et al. 2012). They found, however, that lowering the dose of doxycycline to produce peak (Cmax) blood levels, <1μg/mL, could eliminate these complications while providing essentially the same MMP inhibitory properties (see Golub et al. 1991, 1998a, 2001; Gu et al. 2012, for reviews). Other benefits to SDD include its low IC_{50} value and its proven safety in clinical trials on patients with both dental and medical conditions (Ryan et al. 2001; Walker and Golub 2012). These data appear to be consistent with an earlier study by Lee et al. (2004) in which humans requiring extensive periodontal surgery, allowing analysis of the excised gingival tissues, demonstrated that a combination of a low dose NSAID (which by itself was ineffective) combined with SDD synergistically reduced MMPs (collagenase, gelatinase and even PMNL elastase) in the excised gingival tissues. With SDD as an effective treatment for periodontal disease, Golub et al. (1987) then synthesized non-antibacterial analogs of tetracyclines and the first of these chemically-modified tetracyclines (4-dedimethylamino tetracycline; CMT-1) was generated by his team in 1987 (Golub et al. 1987) by the removal of the dimethyl amino group from carbon-4 of the TC molecule which is responsible for the antibiotic activity of this class of compounds. This chemical modification did <u>not</u> impair the MMP-I activity and some of these CMTs (such as CMT-3; 6-demethyl 6 deoxy 4-dedimethyl amino tetracycline) were in fact more potent MMP-Is than the antibiotic TCs (Golub et al. 1987). A discussion of these follows.

(3) Tetracycline-derived MMP-Is. Tetracycline analogs have been developed, ie., the chemically-modified tetracyclines (or CMTs or COLs), which have lost their antibiotic activity but which retained their calcium and zinc binding sites at carbon-11 and carbon-12 (a β-diketone moiety) and

their proteinases-inhibitory properties. These have shown efficacy in experimental periodontitis and other diseases in animal studies (Ryan 2002; Roy et al. 2011) and in human clinical trials (see below). CMTs became an attractive option because, in theory, they would be able to reduce these pathologically-elevated collagenase levels at higher doses than sub-antimicrobial-dose doxycycline without creating drug-resistant bacteria. A series of CMTs were synthesized and were tested for their potency as inhibitors of MMP activity (Golub et al. 1991). Several CMTs such as CMT-1, CMT-2, CMT-3, and CMT-6 showed potential for the reduction of collagenase activity in various diseases. The lead compound, CMT-3, has shown efficacy in a pig-model of acute respiratory distress syndrome (ARDS), diabetes, arthritis and cancer (Golub et al. 1991, 1998a; Lokeshwar et al. 2001; Ryan et al. 2001; Ramamurthy et al. 2002; Gu et al. 2011a, 2011b; Roy et al. 2011) and in phase II clinical trials in humans with Kaposi's sarcoma, as an anti-angiogenesis drug. However, this compound did result in adverse events, specifically increased photosensitivity in these subjects (Dezube et al. 2006; Drucker and Rosen 2011; Richards et al. 2011) and to date, has not been developed further. However, a pilot study by Ryan et al. (2008), using a much lower oral/systemic dose of CMT-3 (ie., 10 mg/day), rather than the 50-150 mg/day in the phase I and II studies on cancer patients (Dezube et al. 2006), did appear to reduce IL-1β and MMP-8 in human periodontal pockets (Ryan et al. 2008).

(4) Bisphosphonates are widely used in the treatment of systemic metabolic bone diseases as a result of their ability to inhibit osteoclast-mediated bone resorption. Because of their ability to increase the differentiation of fibroblast into osteoblast and to inhibit the activity of osteoclasts, it was tested in clinical trials in subjects with periodontitis (Tenenbaum et al. 2002; Shinoda et al. 2008). However, within the last few years, the literature has indicated that bisphosphonate use,

particularly intravenous preparations, may be linked to osteonecrosis of the jaws, and that their efficacy in periodontitis has not been consistent (Marx et al. 2005; Woo et al. 2006).

(5) Lipid-derived mediators. Recently, new families of lipid-derived mediators, such as lipoxins, protectins, and resolvins, were found to possess potent anti-inflammatory/proresolving activity in vivo, specifically their ability to resolve acute inflammation (Serhan et al. 2008). More recently Naqvi et al. (2014) demonstrated that docosahexaenoic supplementation (an omega-6 fatty acid) combined with low-dose aspirin significantly reduced periodontitis and gingival inflammation in humans in a double-blind placebo-controlled trial of 3 months duration (Naqvi et al. 2014).
There are additional novel host response therapeutic approaches (ie., NO synthase inhibitors, p38 MAPK inhibitors, NF-κB family inhibitors and TNF antagonists) to treat periodontal diseases currently in preclinical studies in various animal models with potential host-modulation action (Kirkwood et al. 2007).

V. Curcumin

Curcumin, a hydrophobic polyphenol derived from dried rhizomes of Curcuma longa, is widely consumed, in low-dose, in food-stuffs in a number of countries (India, China) with no known ill-effects. Curcumin appears to have a wide range of activities including both biological and pharmacological activities (Bharti et al. 2004; Shishodia et al. 2005; Begum et al. 2008; Goel et al. 2008; Grynkiewicz and Slifirski 2012). Different animal models and human studies have demonstrated that curcumin is a pharmacologically safe agent, even at very high doses, at least in part due to its inefficient absorption into the systemic circulation and less-than-optimal pharmacokinetics and poor solubility. For instance, phase I clinical trials demonstrated that

curcumin, when administered even at very high doses (12 g per day), is well-tolerated with no known ill-effects (Anand et al. 2007). Curcumin currently appears to be a pharmacologically safe and efficient compound for the treatment and prevention of many diseases because of its potent anti-inflammatory, anti-carcinogenic, antimicrobial, and anti-oxidant properties (Lundvig et al. 2015; Rodrigues et al. 2015). However, while these properties are desirable, curcumin is highly insoluble, poorly bioavailable, and has to be consumed in large quantities to be (somewhat) effective (Dulbecco 2013; Rodrigues et al. 2015); Curcumin's deficient bioavailability has been stressed as a major obstacle for its approved as a medicinal agent. Curcumin administration has been reported to result in: "low serum levels, limited tissue distribution and a short half-life" that severely limits its clinical therapeutic potential (Anand et al. 2007; Dulbecco 2013).

VI. Chemically modified curcumins (CMCs)

The properties of TCs and CMTs as inhibitors of MMPs is associated, at least in part, with zinc-binding by the compounds, in the catalytic domain of these proteinases. (Zhang et al. 2012a). Curcumin (Figure 1a), which also has this enolic beta-diketone moiety was chosen as the next generation compound for MMP inhibition. However, due to curcumin's low absorption into the body and high rate of metabolism (Anand et al. 2007), it was modified in an attempt to increase its bioavailability and efficacy (Zhang et al. 2012a). In order to increase the solubility and zinc-binding of curcumin, a series of curcumin analogs were synthesized with a carbonyl substituent at the C-4 position. 4-Methoxy carbonyl curcumin (CMC 2.5) was one of the first compounds that was synthesized. Although CMC 2.5 (Figure 1b) was more soluble than curcumin, it exhibited similar potency as curcumin. The second attempt to increase the solubility and zinc-binding was accomplished by adding a phenylamino carbonyl group at the 4-position; the inhibition of MMP

activity was substantially increased as made evident from the in vitro data for CMC 2.23 and CMC 2.24 (Zhang et al. 2012a). The addition of the phenylamino carbonyl group at C4 improved the solubility, as a result of increasing the acidity of CMCs (Zhang et al. 2012a). However, in vivo studies on rats indicated that CMC 2.24 was superior to CMC 2.23 and the latter (but not the former) exhibited some signs of toxicity (Elburki et al. 2011, 2012). CMC 2.23 (Figure 1c), which is derived from curcumin 1, was the second compound tested in vivo after it showed potent inhibitory effects on MMPs in vitro. However, it was discarded because it was not as therapeutically effective as CMC 2.24 and it also showed some evidence of toxicity and was less effective in reducing diabetic complications. We can see from the chemical structure that CMC 2.23 (Figure 1c) contains the same methoxy groups on both phenolic rings as curcumin (Figure 1a), while CMC 2.24 (Figure 1d) lacks these methoxy groups and this could explain why CMC 2.23 was not effective as CMC 2.24 and more toxic (Elburki et al. 2011, 2012; Katzap et al. 2011; Zhang et al. 2012a).

The "lead" compound, CMC 2.24 (Figure 1d), differs from curcumin in that it contains a phenylamino carbonyl group at C4 (making it triketonic rather than diketonic), plus the lack of the two methoxy groups (Zhang et al. 2012a). This compound showed promising effects in vitro as it displayed IC_{50} (ie., the concentration of the compound required to inhibit 50% of the enzyme/proteinase activity in vitro) values of 2-8 μM for gelatinases (MMP-2, -9), collagenases (MMP-8, -13), and for MMPs-3, -7, -12, and -14 (Zhang et al. 2012a). Additionally, a study performed on human monocytes stimulated with endotoxin illustrated that CMC 2.24 was capable of reducing excessive levels of inflammatory mediators such as IL-1β, TNF-α, PGE2, and MCP-1 (Zhang et al. 2012a). The same monocyte study also indicated that CMC 2.24 reduced the

elevated levels of MMP-2, -9, -8, and -13 down to normal levels. It is believed that these in vitro results could be related to CMC 2.24's increased affinity to coordinate with the Zn atom (CMC 2.24 showed a dissociation constant of 765 ± 20 μM compared to 1385 ± 89 μM of curcumin). However, the main factor involved is that the enol of CMC 2.24 is largely ionized compared to curcumin, which is less ionized at physiologic pH (Zhang et al. 2012b).

Table 1- Matrix Metalloproteinases (MMPs), Class, and Substrate

Category	MMP	Enzyme	Substrate
Collagenase	1	Fibroblast Collagenase; Collagenase 1	Type I Collagen
	8	Neutrophil Collagenase; Collagenase 2	Type I, II, III Collagen
	13	Bone and epithelial Collagenase; Collagenase 3	Type I, II, III Collagen, Gelatin
Gelatinase	2	Gelatinase A	Type IV, V, VII, X Collagen, Gelatin, Elastin, Fibronectin
	9	Gelatinase B	Type I, IV, V, Gelatin, Elastin, Fibronectin
Stromelysins	3	Stromelysin-1	Types III, IV, V, IX Collagen, Laminin, Fibronectin
	10	Stromelysin-2	Types III, IV Collagen, fibronectin, laminin
	11	Stromelysin-3	Type I Collagen, Serpin
Membrane-Type	14	MT1-MMP	Pro-MMP2, Fibronectin, Casein, Elastin
	15	MT2-MMP	Pro-MMP2, Gelatin, Fibronectin, Laminin,
	16	MT3-MMP	Pro-MMP2, Type III collagen, Gelatin
	17	MT4-MMP	Pro-MMP2, Gelatin
	24	MT5-MMP	Pro-MMP2
	25	MT6-MMP	Pro-MMP2
Other	7	Matrilysin 1	Fibronectin, Gelatin Type IV, X collagen, Casein
	12	Macrophage Elastase	Elastin, Fibronectin, Proteoglycan
	19	RASI 1	Gelatin
	20	Enamelysin	Amelogenin, Aggrecan, Gelatin
	23	CA-MMP	Mca-peptide
	26	Matrilysin 2	Fribrinogen, Fibronectin, Vitronectin
	28	Epilysin	Casein

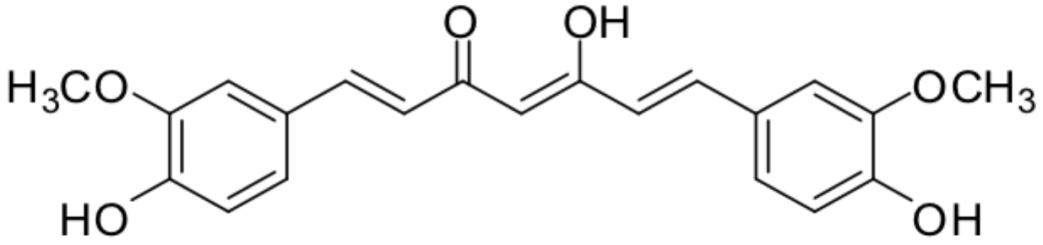
Modified from references (Whittaker et al. 1999; Visse and Nagase 2003; Zhang et al. 2012a)

Table 2- IC_{50} [µM] of several CMCs as MMP-inhibitors

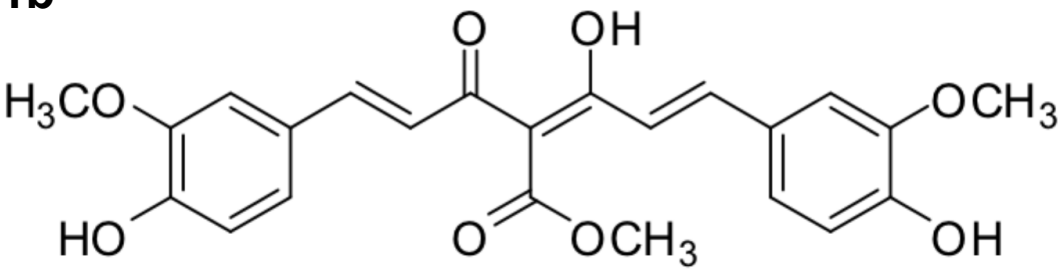
Compound / MMPs		1,10-Phenanthroline	Curcumin	CMC 2.5	CMC 2.14	CMC 2.23	CMC 2.24
Collagenase	MMP-1	42.0±1.1	85.8±1.8	74.0±3.5	76.3±6.5	68.0±3.2	69.8±2.0
	MMP-8	31.3±0.5	6.8±1.0	30.8±1.5	20.0±2.0	2.5±0.3	4.5±0.5
	MMP-13	50.0±10.4	3.7±0.3	28.3±4.4	26.7±1.7	3.3±0.3	2.7±0.7
Gelatinase	MMP-2	73.8±1.0	5.0±0.7	25.3±1.3	23.8±0.9	6.3±0.9	4.8±0.5
	MMP-9	45.0±12.6	30.0±2.9	55.0±17.3	43.3±4.4	8.7±0.7	8.0±0.6
Others	MMP-3	77.0±3.2	4.7±0.8	32.5±2.8	28.3±1.0	5.3±0.7	2.9±0.4
	MMP-7	196.8±8.8	51.8±2.5	48.8±0.5	57.5±4.6	21.5±1.0	5.0±0.7
	MMP-12	29.5±1.3	2.6±0.2	27.8±1.7	5.3±0.3	4.5±0.5	2.0±0.4
	MMP-14	43.8±4.2	29.5±3.2	48.5±4.3	40.0±8.4	41.3±4.9	15.3±3.1

1,10-Phenanthroline is the positive control, IC_{50} were measured using a synthetic fluorescent peptide substrate (Mca-Lys-Pro- Leu-Gly-Leu-Dpa-Ala-Arg-NH_2), for MMPs with cleavage site between Gly and Leu, Mean of 3-4 analyses ± S.E.M. Reproduced with permission from professor Francis Johnson, Department of Pharmacological Science and Chemistry and professor Lorne Golub, Department of Oral Biology and Pathology, Stony Brook University (Zhang et al. 2012a).

1a



1b



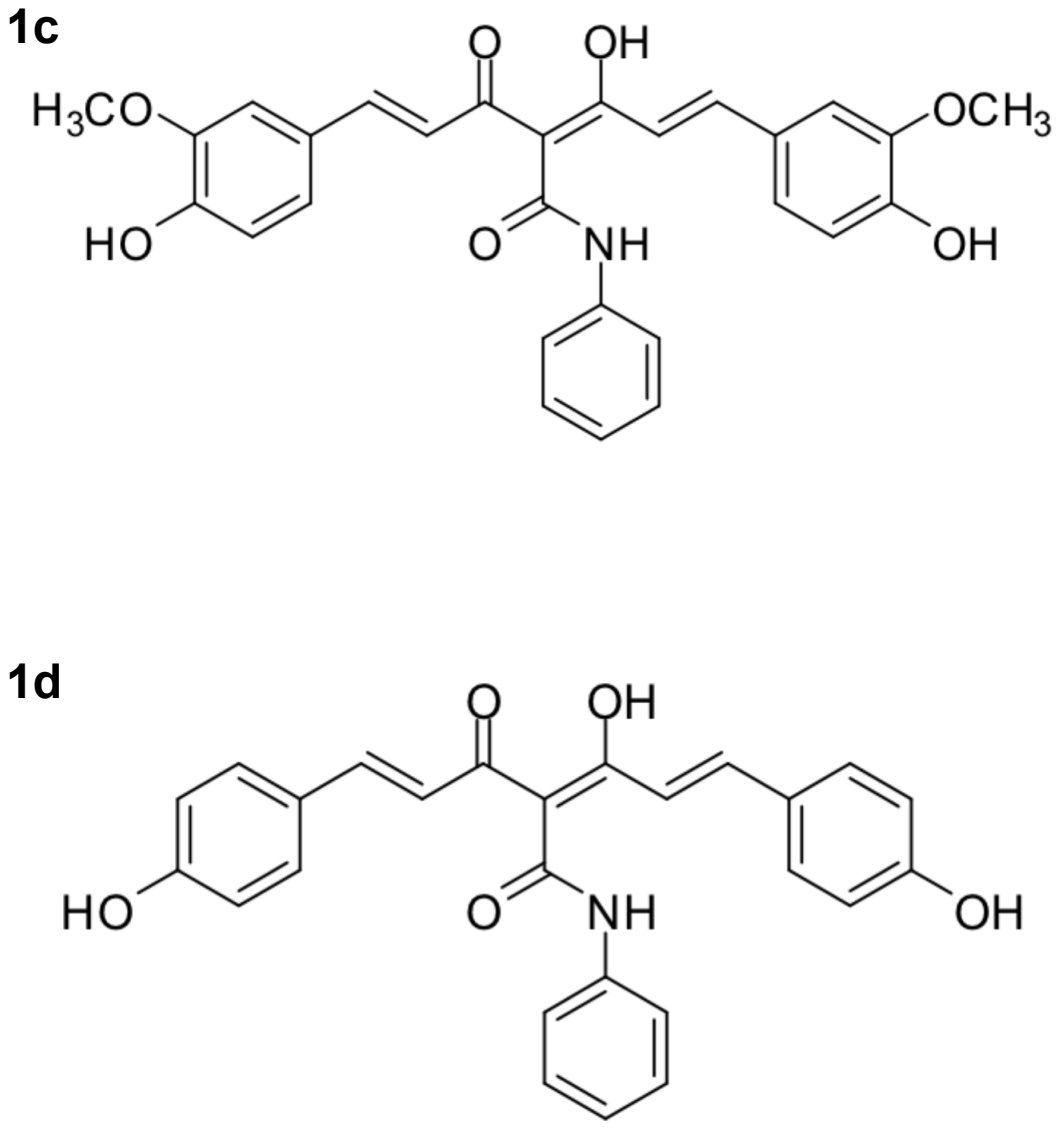


Figure 1. (a) Chemical structure of curcumin. (b) Chemical structure of CMC 2.5. (c) Chemical structure of CMC 2.23. (d) Chemical structure of CMC 2.24. Reproduced with permission from professor Francis Johnson, Department of Pharmacological Science and Chemistry at Stony Brook University.

Chapter 3

A Novel Chemically-Modified Curcumin Reduces Severity of Experimental Periodontal Disease in Rats: Initial Observations

Introduction

Over the past several decades, numerous studies have described pharmacologic strategies to utilize matrix metalloproteinase-inhibitors (MMP-Is) to prevent connective tissue breakdown associated with various inflammatory and other diseases, e.g., periodontitis, arthritis, osteoporosis, cardiovascular disease, and cancer (Overall and López-Otín 2002; Sorsa et al. 2006; Hu et al. 2007; Gu et al 2012). Recently, these have also included less obvious strategies such as (but not limited to) blocking MMP-mediated cleavage of insulin receptors in type-2 diabetics to improve insulin sensitivity (Frankwich et al. 2012) and to reduce HbA1c levels (Engebretson and Hey-Hadavi 2011). However, to date, the only orally (systemically) administered MMP-Is approved by the US-FDA and other national regulatory agencies (Europe, Canada) are those based on the surprising non-antimicrobial properties of the tetracycline antibiotics (Golub et al. 1991, 1997, 1998a; Gu et al. 2012). In this regard, studies on experimental animals and on human subjects have demonstrated the efficacy of non-antimicrobial tetracycline formulations, as pleiotropic MMP-Is, in periodontal and other diseases (Golub et al.1997, 1998a; Payne and Golub 2011; Gu et al. 2012, 2013). In addition to demonstrating that these medications, which include two-formulations of sub-antimicrobial-dose-doxycycline (both FDA-approved), can inhibit collagenolysis, connective tissue destruction, and bone resorption in the diseased periodontal tissues, other therapeutic

mechanisms have also been identified. These include suppressed expression of inflammatory mediators such as the cytokines (e.g., IL-1β, TNF-α, IL-6), prostaglandins, reactive oxygen species (e.g., HOCl) and nitric oxide, the latter reflecting the inhibition of inducible nitric oxide synthase (Amin et al. 1999; Gu et al 2013).

Given this background, a search has been underway for new drug molecules which exhibit a similar active site for MMP-inhibition as the tetracyclines " but with a different phenolic superstructure" (Gu et al. 2013). With this strategy in mind, the therapeutic potential of the tetracycline diketonic, metal-ion binding site (Golub et al. 1991, 1998a) has been expanded by the recent development of a new series of compounds with a similar zinc-binding moiety, but which are bi-cyclic rather than tetra-cyclic, ie., the chemically-modified curcumins or CMCs. The structures of these compounds, their potency and mechanisms of action as MMP-Is, and their zinc-binding (and other) characteristics have been described recently and a "lead" compound identified (Zhang et al. 2012a, 2012b; Gu et al. 2013). This compound, CMC 2.24, is a phenylamino carbonyl curcumin; is tri-ketonic (which enhances its zinc-binding characteristics) in contrast to the di-ketonic active site on both the tetracyclines and on traditional/natural curcumin compounds; and has shown evidence of efficacy in vitro, in cell and organ culture, and in animal models of chronic inflammatory and other diseases (Zhang et al. 2012a, 2012b; Botchkina et al. 2013). As additional background, recent studies have shown that natural/unmodified curcumin administered to rats with experimentally-induced periodontal disease was effective in reducing inflammatory mediators and MMPs in the gingiva and periodontal ligament, but was ineffective in reducing the excessive resorption and loss of alveolar bone (Guimarães et al. 2011). Accordingly, the current report describes the first of a series of studies which examined the efficacy of CMC 2.24 as a pleiotropic

MMP-I in several rat models of periodontitis with a particular focus on its ability to inhibit pathologic alveolar bone loss. Moreover, because of the long-standing interest in the link between the oral disease, periodontitis, and systemic inflammation (the latter associated with increased risk for various diseases notably cardiovascular disease and more severe diabetes (Payne et al. 2011; Gu et al. 2012), the effects of treatment with this novel compound on biomarkers in the circulation were also examined.

Materials and Methods

Experimental periodontal disease model:

Eleven male Holtzman rats (*Rattus norvegicus albinus*) weighing 150-250 g, were maintained under pathogen-free conditions with controlled temperature (21 ± 1^{o}C) and humidity (65–70%) and a 12 h light–dark cycle. Food and water were provided *ad libitum* throughout the experiment. General anesthesia was induced by inhalation of an isofluorane/oxygen mixture. 30 μg of lipopolysaccharide (LPS) from *Eschericia coli* (strain 055:B5 - Sigma Chem Co., St. Louis, MO, USA) diluted in phosphate buffered saline (PBS) were injected into the palatal gingiva (3 μL volume per injection) using a Hamilton micro-syringe (Agilent, Santa Clara, CA, USA) as described by us previously (Garcia de Aquino et al. 2009). These LPS injections were made into the palatal tissue between the upper 1st and 2nd molars, on the left side of the animal, three times a week for 14 days (a total of 6 injections and 180 μg of LPS in each site). The opposite side received injections of the same volume of PBS vehicle and served as the control sites ("split-mouth" protocol; see Figure 2). At the end of the experimental period, the animals were sacrificed by CO_2 inhalation and samples collected as described below. Also at the time of sacrifice, blood samples were collected and the serum and plasma were separated by standard procedure and

analyzed for MMPs and cytokines as described below. The study protocol was previously approved by the Institution's Committees (Araraquara –UNESP, SP, Brazil and Stony Brook University, NY, USA) for Experimental Animal Use.

Experimental groups:

The effects of CMC 2.24 (a phenylamino carbonyl curcumin) were assessed in a "prophylactic" model (the efficacy of this compound in a "therapeutic" model will be assessed in future studies) in which the induction of periodontal disease by LPS injections was carried out during the same period of time (14 days) as the daily oral administration of CMC 2.24 (30 mg/kg) or the vehicle-control. The test compound and the vehicle-control (a 1 mL suspension of 2% carboxymethyl cellulose) were both administered once per day over the 14 day protocol by oral intubation. The rats and their periodontal tissues were randomly distributed into the following experimental groups as illustrated in Figure 2:

Group 1-- Gingiva injected with PBS in rats systemically administered vehicle alone (n=5); Group 2-- Gingiva injected with *E.coli* LPS in the vehicle-treated rats (n=5) [note, with this "split-mouth" design, group1 and group 2 tissues involve the same 5 rats];

Group3-- Gingiva injected with PBS in rats systemically administered the test medication (CMC 2.24; n=6); and

Group 4-- Gingiva injected with *E.coli* LPS in rats systemically administered CMC 2.24 (n=6) [as above, groups 3 and 4 involve the same 6 rats]. However, for the μCT analysis, additional rats were added to each experimental group resulting in n=10 rats per group.

Gingival tissue extract and its partial purification:

The gingival tissues from the hemi-maxilla of each rat were excised and pooled per experimental group (5-6 rats per group) as described by us previously (Ramamurthy and Golub 1983; Golub et al. 1994a). The pooling of gingival tissues for each group was necessary because individual rats do not yield sufficient gingiva for enzyme analyses. The gingival tissues were extracted and the MMPs partially purified as described by us previously (Ramamurthy and Golub 1983; Golub et al. 1994a). In brief, the samples were homogenized (all procedures at 4°C) 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 $CaCl_2$, 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 0.2M 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 and exhaustively dialyzed against the same buffer. Protein content of the extracts was determined by Bio-Rad Protein Assay.

Zymographic assay of MMP-2 (gelatinase A) and MMP-9 (gelatinase B):

The relative levels of the higher molecular weight pro-forms, and the lower molecular weight activated forms of MMP-2 and MMP-9, in the pooled gingival extracts from each of the four experimental groups (Figure 3), were determined by zymography (the Gelatin zymography system was purchased from Invitrogen Corp., Carlsbad, CA). In brief, all samples were run under non-reducing denaturing conditions on the gelatin zymography system containing polyacrylamide

copolymerized with gelatin at a final concentration of 1mg/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, 10 mM $CaCl_2$, pH 7.5). After incubation, the gels were stained with SimplyBlue^{TM}SafeStain (Invitrogen Corp., Carlsbad, CA). Clear zones of lysis against a blue background indicate gelatinolytic activity, as described by us previously (Brown 2004; Lee et al. 2004; Gu et al. 2013). Densitometric analysis of the gelatinolytic bands was carried out using the Scientific Imaging system (KODAK ID 3.5, Rochester, NY).

Alveolar bone loss measurements:

Since this is a major outcome in the experimental periodontal disease model, and reducing alveolar bone loss is a key therapeutic goal in treating human inflammatory periodontal disease, two methods were used to assess the effects of CMC 2.24 on this inflammatory-driven bone loss model.

1. **Morphometric analysis of alveolar bone loss**

As described previously (Souza et al. 2011), the soft tissues were carefully dissected to maintain the integrity of the maxillary bone specimens. These were then completely defleshed by immersion in 8% sodium hypochlorite for 4 h followed by gentle mechanical scavenging of the remaining soft tissue. After washing in running water, the specimens were immediately dried with compressed air. To distinguish the cementum-enamel junction (CEJ), 1% aqueous methylene blue solution (Sigma-Aldrich, Saint Louis, MO, USA) was applied to the specimens for 1 min and then washed in running water. The specimens were fixed on 3mm thick red dental wax with their palatal surface 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 stereomicroscope (Leica MZ6, Buffalo Grove, IL, USA) and digital images were obtained at 25X magnification using a 6.1 mega pixel color digital camera coupled to the microscope. A single examiner, who was not aware of the experimental group allocation of the specimens, carried out all morphometric measurements of alveolar bone loss by delineating the area of exposed root surface of the first and second molars using an image analysis software (Leica Application Suite, v 3.8.0, Leica Microsystems, Buffalo Grove, IL. USA) and the results converted to mm^2 using measurement of the reference millimeter grid. The area of exposed root surface in each specimen was averaged according to the experimental groups. Intra-examiner calibration was performed by evaluating repeated measurements of 10 non-study images presenting alveolar bone loss similar to the present study. The intraclass correlation showed a 96.8% reproducibility.

2. Microcomputerized tomography (µCT)

Upon sacrifice, the hemi-maxillae of the rats were dissected including teeth and surrounding soft tissues, fixed for 18-24 h in 10% neutral buffered formalin at 4°C, washed in distilled water and transferred to 70% ethanol. This procedure allowed us to use these same specimens for the histological assessments used in subsequent studies (Guimaraes et al., in preparation). These samples were scanned on a microcomputer tomograph (Skyscan 1176, SkyScan, Aartselaar, Belgium) using 18 µm slices. The digital radiographic images of each sample were reconstructed into a three dimensional model (NRecon Software, SkyScan, Aartselaar, Belgium) consisting of a matrix of 18x18x18 µm and a standardized gray scale value to visualize only mineralized tissues. Using the software package Dataviewer\CTan\CTvol (Skyscan, Aartselaar, Belgica), the reconstructed tridimensional matrix of each sample was initially re-oriented in a standardized

manner on three planes: sagital, coronal and transversal. Subsequently, a cubic region of interest (ROI) of 9.72 mm^3 was defined using standardized dimensions and anatomical landmarks: cementum-enamel junction of the first molar as the coronal limit and extending vertically 1.5 mm apically; an antero-posterior dimension of 3 mm from the distal aspect of the mesial root of the first molar, and the transversal (bucco-lingual thickness) dimension of 2.16 mm (120 slices of 18 µm each). This ROI included the first molar, half of the second molar and also approximately 1 mm from the most palatal aspect of the first molar crown (including the palatal bone adjacent to the first and second molar teeth which was the site of LPS injections). We determined the relative volume of this ROI occupied by mineralized tissue in each sample. The data was averaged for each experimental group and compared by non-paired t-tests using Welch's correction for unequal variances. Significance level was set to 95%.

Immunoblotting for measurement of MMP-8 in plasma and gingival extracts:

MMP-8 levels in plasma and gingival extracts, the latter prepared as described above, were determined by Western blot analysis. In brief, samples were reduced, boiled, subjected to SDS/PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane *(*Amersham Pharmacia Biotech Inc, Piscataway, NJ). Blots were blocked with 5% non-fat dry milk for 2 h at room temperature. The membranes where then incubated with polyclonal antibodies specific for MMP-8 (Abcam PLC, Cambridge, MA) overnight at 4°C. Blots were washed and incubated with secondary antibodies purchased from Thermo Scientific for 2 h at room temperature. Detection of the bands was carried out on radiographic film by using SuperSignal West Dura Extended Duration Chemiluminescent Substrate (Thermo Fisher Scientific Inc, Waltham, MA). The band densities were quantified by scanning on a laser densitometer (Golub et al. 1995). To assess the levels of

inactive (pro-form), and smaller molecular weight active forms of the MMP-8 (collagenase-2), bands corresponding to both molecular weight forms were quantified, and the data is expressed as densitometric units and as the ratios of inactive/active forms. Recombinant rat MMP-8 (source: mouse myeloma cell line, NSO derived) from R&D Systems (Minneapolis, MN) was used as a standard for Western blot analysis of the rat plasma samples. This MMP-8 standard was incubated for 4 hours at room temperature, in the presence or absence of 1 mM amino phenyl mercuric acetate (APMA), a known activator of higher molecular weight pro-MMPs into the lower molecular weight activated forms (Golub et al. 1994a).

ELISA for measurement of MMP-13 in plasma and gingival extracts:

The level of MMP-13 was measured in the gingival tissue extracts and plasma of each rat by Enzyme-Linked Immunosorbent Assay (ELISA). This assay was performed according to the manufacturer's instructions (TSZ Scientific LLC, Framingham, MA). Blood samples from animals in each experimental group were assayed in duplicate.

Measurement of gingival tissue and serum levels of bone resorptive cytokines:

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 animals in each experimental group were assayed in duplicate.

Results:

A. <u>Local/oral measurements: gingiva and alveolar bone</u>

The levels of both MMP-2 (72 kDa pro-gelatinase) and MMP-9 (92 kDa pro-gelatinase) were assessed by gelatin zymography in pooled gingival tissue from half-jaws of rats from each experimental group (Figure 3). LPS-induced periodontal disease dramatically increased MMP-2 and MMP-9 levels in the pooled gingival tissue, while lower levels of the pro-(higher molecular weight) and activated (lower molecular weight) forms of these gelatinases were seen in the gingival tissue from all of the other experimental groups. Treatment of the rats with systemically-administered CMC 2.24 appeared to "normalize" the pathologically-excessive levels of the various molecular weight forms of these gelatinolytic MMPs in the LPS-injected gingiva assessed either visually (Figure 3a) or by densitometric analysis of the zymograms (Figure 3b). Some reduction of these MMP proteinases by CMC 2.24 administration was also seen in the gingiva from the rats without LPS injections (Figure 3a and 3b).

In a pattern reminiscent of the zymograms described above, and based on morphometric analysis of alveolar bone height loss which measured the area of exposed root relative to the cemento-enamel junction as a fixed anatomical landmark, LPS injections into the gingiva significantly ($p=0.005$) increased alveolar bone loss (Figure 4). Moreover, when the LPS-injected rats were treated by oral administration of CMC 2.24, alveolar bone loss was significantly reduced ($p=0.003$) back to the normal level seen in the rats not exposed to gingival LPS injections. Note that CMC 2.24 treatment did not affect alveolar bone loss in the control rats receiving injections of PBS vehicle rather than LPS (Figure 4).

To confirm and expand these data on alveolar bone loss in the four experimental groups (Figure 2), additional measurements using μCT were carried out. As shown in Figure 5, these data

again demonstrate that LPS increased the loss of bone in the AOI and that CMC 2.24 administration reduced this bone loss to the level seen in the control rats in which the gingiva were injected with PBS instead of LPS.

Analysis of IL-1β in extracts of the pooled gingival tissues indicated that LPS injections markedly increased the level of this pro-inflammatory cytokine since it was not detectable in the extracts of the PBS-injected gingival tissue (Figure 6a). Moreover, CMC 2.24 administration reduced the pathologically-excessive levels of IL-1β in the gingiva by 93% (Figure 6a). Similar concentrations of IL-6 were detected in the gingival tissues from the different groups of rats, however the LPS injections did not appear to affect these levels and CMC 2.24 treatment only slightly reduced the levels of this cytokine by about 15% (data not shown). TNF-α was undetectable in both gingival extracts and in serum (see below).

B. <u>Systemic measurements: Plasma and Serum</u>

In the experimental protocol used in the current study (a "split-mouth" design), MMP-8 (neutrophil-type collagenase, collagenase-2) and MMP-13 (collagenase-3) were both detected in the plasma samples from the different groups of rats but neither was detected in the gingiva (see discussion section below). Based on Western blot analysis, the plasma samples from the LPS-injected rats (half-jaw only) which were treated by oral administration of CMC 2.24 appeared to exhibit reduced levels of activated, lower molecular weight, forms of MMP-8 compared to the plasma from the LPS-treated rats administered the vehicle alone (controls) (Figure 7a). Based on the densitometric analysis of these Western blots (Figure 7a), the plasma of the CMC 2.24-treated rats with LPS-induced periodontitis exhibited a ratio of pro/active MMP-8 of 2.52 ± 0.20 (S.E.M.) which was 89.5% higher than the ratio, 1.33 ± 0.05, seen in the plasma from the vehicle-treated

LPS-periodontitis rats (Figure 7b), and this inhibition of activation of the precursor (latent) form of MMP-8 by the CMC2.24 treatment was statistically significant ($p=0.024$). Note that a 4-hour incubation of the standard recombinant rat MMP-8 with 1 mM APMA, a known activator of pro-MMPs in vitro (Golub et al. 1994a), converted the higher molecular weight proMMP-8 into the smaller molecular weight activated form of this leukocyte-type collagenase (see Figure 7a).

The plasma levels of MMP-13 assessed by ELISA were found to be about 1.1 µg/mL. Administration of CMC 2.24 to the LPS-periodontitis rats appeared to slightly reduce the levels of this collagenase in the plasma, however this effect was not statistically significant (data not shown).

Regarding the pro-inflammatory cytokines in the serum (Figure 6b), because of the "split-mouth" design (see Figure 2), there were no serum samples from rats without gingival LPS injection. However, the levels of IL-1β in the serum of these LPS-exposed rats (about 30 pg/mL) were significantly ($p=0.03$) reduced to undetectable levels by CMC 2.24 administration, a pattern similar to that seen in the gingival tissues (Figure 6a).

IL-6 was seen at higher concentrations in the serum (about 95 pg/mL) than IL-1β in the LPS-periodontitis rats and, again, CMC 2.24 appeared to reduce the level of this cytokine. However, this lesser effect (about 18% reduction) was not statistically significant (data not shown).

Discussion

This work advances a novel therapeutic strategy which uses systemically-administered medications as adjunctive therapy to modulate the host response in periodontal disease (periodontal therapy has traditionally only focused on locally suppressing the pathogenic microorganisms in the oral biofilm), with applications for other chronic inflammatory diseases as

well (see below). The clinical application of this strategy began with the discovery that tetracyclines (TCs), unexpectedly, can inhibit host-derived MMPs, inflammatory mediators (e.g., the cytokine, IL-1β), and collagen degradation including bone resorption; and by mechanisms not dependent on the anti-bacterial properties of these drugs (Golub et al.1997, 1998a; Gu et al. 2012, 2013). Soon thereafter, doxycycline was found to be a more potent MMP-inhibitor than other tetracycline antibiotics, including minocycline and tetracycline itself, and was subsequently developed and approved as a NON-antibiotic low-dose formulation for long-term administration to patients with chronic periodontitis and the dermatologic inflammatory disease, rosacea (Golub et al. 1998a; Gu et al. 2012). Based on these earlier, and the current, studies the non-tetracycline chemically-modified-curcumin (discussed below) appears to be as, or more, potent an MMP-inhibitor compound compared to doxycycline (Golub et al. 1991, 1998a; Gu et al. 2012; Zhang et al. 2012b). As one example, the IC_{50} (the concentration of the compound required to inhibit 50% of MMP activity in vitro) of doxycycline has been reported to be approximately 15μM (Golub et al. 1991, 1998a). In contrast, recent studies by our group have demonstrated IC_{50} levels of CMC2.24 at even lower μM levels (2-5 μM) when tested in vitro against MMPs such as MMP-8 (leukocyte-type collagenase), MMP-9 (leukocyte-type gelatinase), MMP-12 (macrophage metallo-elastase) and MMP-14 (membrane-type MMP) (Zhang et al. 2012b) However, a significant disadvantage of the approved sub-antimicrobial-dose formulations of doxycycline is that NO increase in the dose of this tetracycline can be prescribed to the patient [which might be desirable in order to, possibly, enhance the efficacy of this treatment in collagen-destructive diseases, e.g., periodontitis] because the low, NON-antibiotic blood levels of the drug (< 1μg/mL) produced by this formulation cannot be exceeded in order to prevent an important side-effect, namely the emergence of tetracycline-resistant or pan-antibiotic-resistant bacteria (Gu et al. 2012)

In contrast, the potential strategy of long-term administration of CMC 2.24, for inflammatory diseases, would not be undermined by this strict, low-dose, limitation because this compound is not an antibiotic in contrast to the tetracyclines.

As described earlier (see "Introduction"), natural curcumin has a similar active site (ie., the diketone zinc-binding moiety) as the tetracyclines, and can also modulate the host response including MMP-inhibition and suppression of inflammatory mediators (Banerji et al. 2004; Bharti et al. 2004; Woo et al. 2005; Kaur et al. 2006; Shakibaei et al. 2007; Begum et al. 2008; Grynkiewicz and Slifirski 2012), although it is ineffective against alveolar bone loss (see below). However, the chemically-modified curcumin, CMC 2.24, tested in the current in vivo study, has a modified active site which is triketonic as detailed by us in previous studies by Zhang et al (Zhang et al. 2012a, 2012b), and does effectively inhibit bone loss.

Recently, newer host-modulating medications have also been investigated as adjunctive treatment for periodontal disease and related medical disorders. These, in particular, have included: (1) the resolvins such as the polyunsaturated fatty acids (Serhan et al. 2008) which do not suppress the acute inflammatory response required by the host to combat infection, but which do prevent the tissue-destructive prolongation of this process, and (2) the subject of the current study, the chemically-modified curcumins (CMCs). Of importance, the latter have shown improved solubility, zinc-binding, and biological effects in comparison with natural curcumin (Zhang et al. 2012a, 2012b). Development of these CMCs is based on maintaining a similar active site for MMP-inhibition as that of the tetracyclines but with a different phenolic superstructure (Gu et al. 2013), which most recently resulted in the development of a new series of compounds with a tri-

ketonic zinc-binding moiety, but which are bisphenyl in character-cyclic rather than being tetra-cyclic, ie., the chemically-modified curcumins or CMCs. A series of these triketonic CMCs have been developed including the active CMC 2.5 (a methoxy carbonyl curcumin; (Gu et al. 2013) which, in turn, has been superseded by a more potent MMP-I compound, CMC 2.24, a phenylamino carbonyl curcumin; the latter has shown evidence of efficacy (and safety) in vitro, in cell and tissue culture, and in vivo models of several diseases including arthritis, diabetes and cancer (Katzap et al. 2011; Zhang et al. 2012a, 2012b; Botchkina et al. 2013).

The current study is the first to demonstrate efficacy of this compound, CMC 2.24, in an animal model of experimental periodontitis. Evidence of the onset and progression of this disease, induced by several injections of LPS into the gingiva of the rat, included dramatic increases in several forms (both pro-and activated) of connective tissue-destructive MMP-2 (72 kDa) and MMP-9 (92 kDa) gelatinases, elevated levels of the inflammatory cytokine often associated with periodontitis, IL-1β, and, most important in this model, a significant increase in alveolar bone loss, assessed morphometrically and by μCT, in the same jaws as the increase in gingival inflammatory mediators and MMPs (the impact of this local inflammatory disease and this experimental treatment on systemic levels of mediators is discussed below).

The potent efficacy of CMC 2.24 was demonstrated by (i) the statistically significant reduction of the LPS-induced, pathologically-elevated alveolar bone loss down to the levels seen in the healthy controls; and (ii) the essentially complete reduction of the pro- and activated, pathologically-excessive levels of MMP-2 and MMP-9, and IL-1β, in the inflamed gingival tissues back down to the un-(or barely-) detectable levels seen in the control gingiva. As a result of the

profound efficacy of this novel compound in this initial study, we now have a rationale to initiate studies using a modified animal model of experimental periodontal disease, which does not use "split mouth" design, and in a periodontitis model in which the CMC 2.24 is administered therapeutically (after the disease has been established) rather than prophylactically as in the current study. In a more recent study in which alveolar bone loss was assessed at the cellular level histomorphometrically and histochemically, a similar pattern of change was seen: namely that LPS injection increased osteoclast-mediated bone resorption, and that CMC 2.24 inhibited this mechanism of alveolar bone loss (Guimaraes et al., in preparation). The potency of the biological effects of CMC 2.24 at an oral dose of 30 mg/kg is further demonstrated by the fact that in previous experiments, we did not observe a significant decrease of inflammation-driven bone resorption with 100 mg/kg dose of natural curcumin (Guimarães et al. 2011). Interestingly, in recent experiments, we found that daily administration of 400 mg/kg of natural curcumin significantly reduced inflammatory-driven bone resorption in this model, but this dose of natural curcumin is more than 10-fold higher than the dose of CMC 2.24 administered in the current study (Guimaraes et al., in preparation).

Regarding insights into the mechanisms, plus the impact of this local disease and its treatment on the systemic condition of the host, we also observed the following : (i) the apparent reduction of IL-1β by CMC 2.24 treatment in the pooled gingival tissues was paralleled by a dramatic and significant reduction of this inflammatory mediator in the systemic circulation of the same animals, and (ii) CMC 2.24 treatment, although it did not appear to alter the total levels of MMP-8 (neutrophil-type collagenase) in the blood samples of the LPS-injected rats, it did significantly reduce the ratio of the lower molecular weight, activated, collagen-destructive forms

of this collagenase relative to the higher molecular weight, inactive, pro-forms of this MMP [note that in the current experiment MMP-8 could not be detected in the pooled gingival tissue]. Mechanisms could include the ability of CMC 2.24 to inhibit other neutral proteinases such as plasmin, elastase and MMP-1 which are known to cleave the amino-terminal pro-peptide domain of pro MMP-8, converting it into the smaller molecular weight activated forms (Golub et al. 1994a, 1998a). Of relevance to the mechanisms involving CMCs ability to inhibit pro-MMP activation, recent studies (S. Simon et al., unpublished data) indicate that 2.24 can inhibit serine neutral proteinases (ie., neutrophil elastase) which could explain the reduced conversion of the pro-proteins into smaller molecular weight activated-MMPs which was observed in the current study in the systemic circulation. Still another possible mechanism involves the potential of this compound to inhibit the production of reactive oxygen metabolites (e.g., hypochlorous acid, HOCl). These are known to mediate proteinase activation by dissociating the thiol group in the propeptide domain (Golub et al. 1994a). This mechanism is significant because MMP-8 is largely derived from the degranulation of polymorphonuclear leukocytes and, in the human periodontal pocket, MMP-8 constitutes about 80-90% of the total collagenase in this lesion; MMP-13 is the second most dominant collagenase in the periodontal pocket in humans, contributing about 10-20% of the total, and is thought to be derived from the junctional epithelium and bone cells (Golub et al.1997, 2010). However, in the rat, MMP-13 is analogous to the constitutive collagenase, MMP-1, in humans and likely plays a role in physiologic turnover of collagen rather than the pathological degradation of collagen during periodontitis. In this regard, MMP-13 also could not be detected in the inflamed gingival tissues in the rats in the current study and, although it was detected in the plasma, was not reduced by CMC 2.24 treatment suggesting a preferential effect of the test compound on pathologically-elevated, rather than on constitutive levels of these MMPs.

Additional mechanisms include the ability of natural curcumins to inhibit various signaling pathways and transcription factors involved in the expression of inflammatory mediators (AP-1, MAPK, NF-κB, STAT3) resulting in a decrease in the expression of the inactive pro-forms of the MMPs and of inflammatory cytokines and, ultimately, a marked change in the microenvironment (Bharti et al. 2004; Guimaraes et al. 2011; Souza et al. 2012).

Conclusions

The results of this initial study indicate that the oral administration of a novel, tri-ketonic phenylamino carbonyl curcumin (CMC 2.24), to rats with endotoxin (LPS)-induced periodontitis, is a significant and potent inhibitor of both pathologic alveolar bone loss and its inflammatory and collagen-destructive mediators. Moreover, this chemically-modified curcumin appears to have additional benefits by reducing the impact of this local inflammatory disease on systemic biomarkers of the host without (apparently) negatively affecting the mediators of constitutive connective tissue turnover. Studies are now underway to expand these observations in additional rat models of experimental inflammatory periodontal disease with a particular focus on CMC 2.24 effects (i) on the cellular mechanisms of alveolar bone loss; (ii) in a model in which the test medication is administered therapeutically (i.e., after the disease has been established) rather than prophylactically; and (iii) on the pharmacokinetics (such as peak blood levels; serum half-life) of this novel compound.

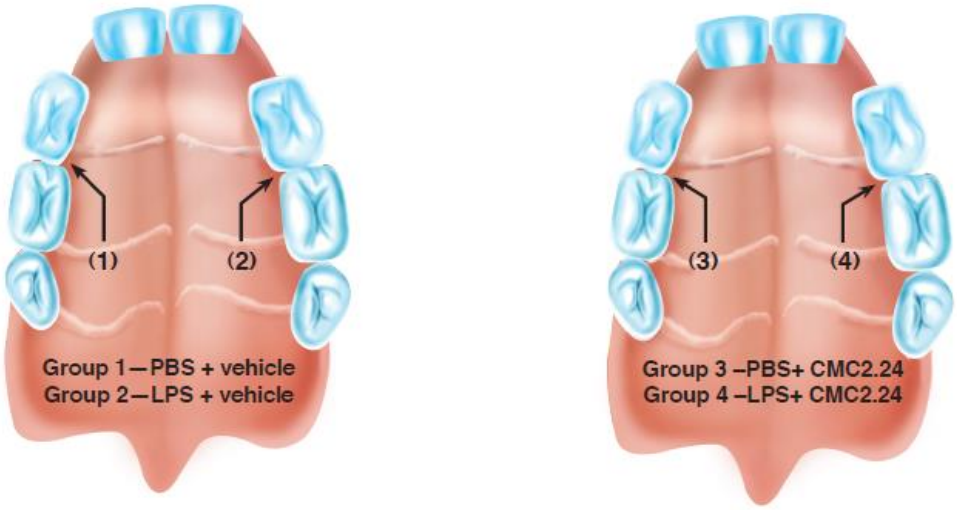


Figure 2. Diagrammatic representation of the four experimental groups using “split-mouth” protocol

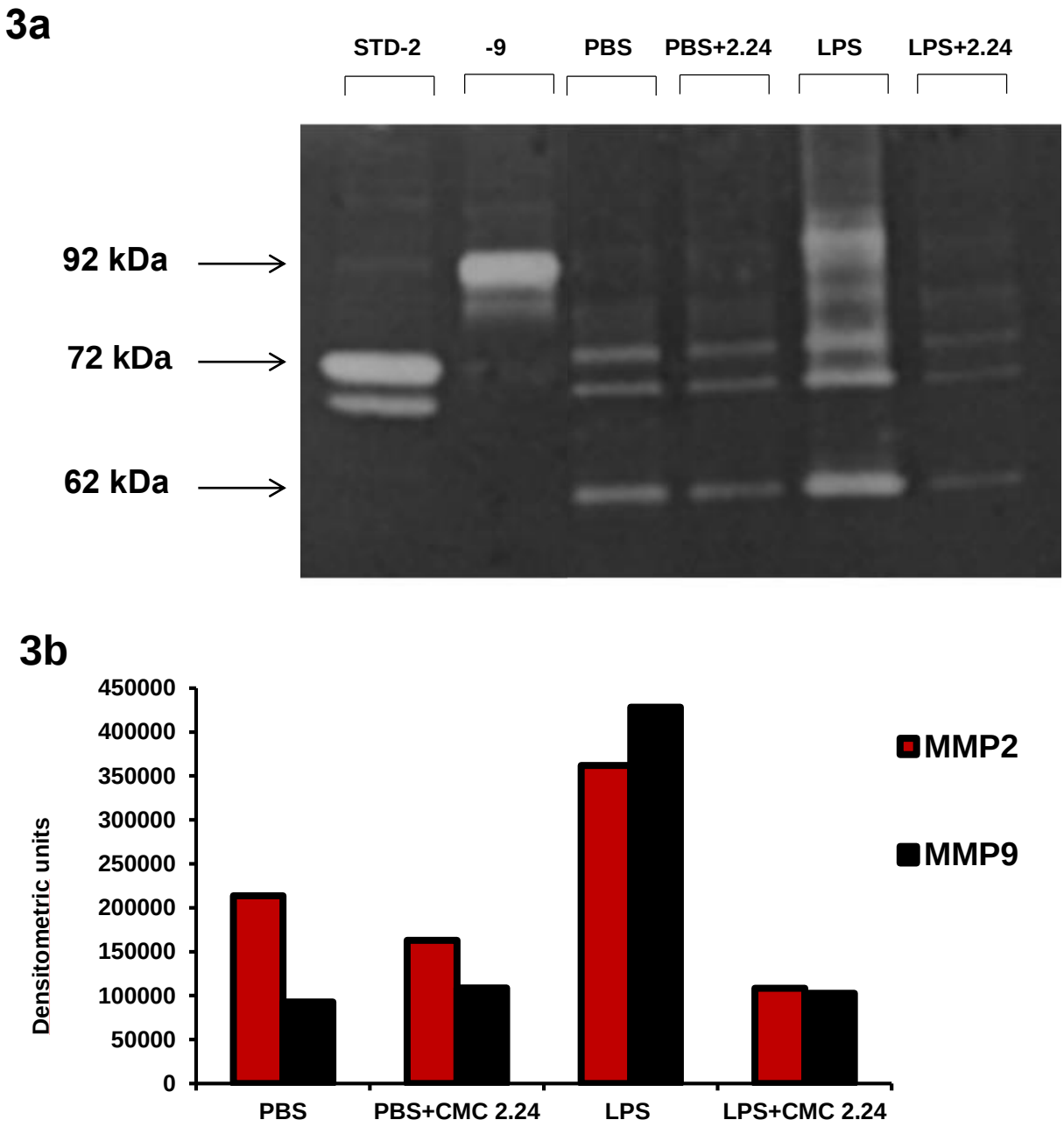


Figure 3. (a) Gelatin zymography of partially purified extract of gingiva from each experimental group showing the effect of orally-administered CMC 2.24 on gingival MMPs (-2, -9). In groups 1 and 3, all rats received PBS injections into the gingiva plus oral administration of either vehicle alone (group1) or CMC 2.24 (group3). In groups 2 and 4, all rats received LPS injections into the gingiva plus oral administration of either vehicle alone (group2) or CMC 2.24 (group4). (b) Densitometric analysis of gingival MMPs (-2, -9).

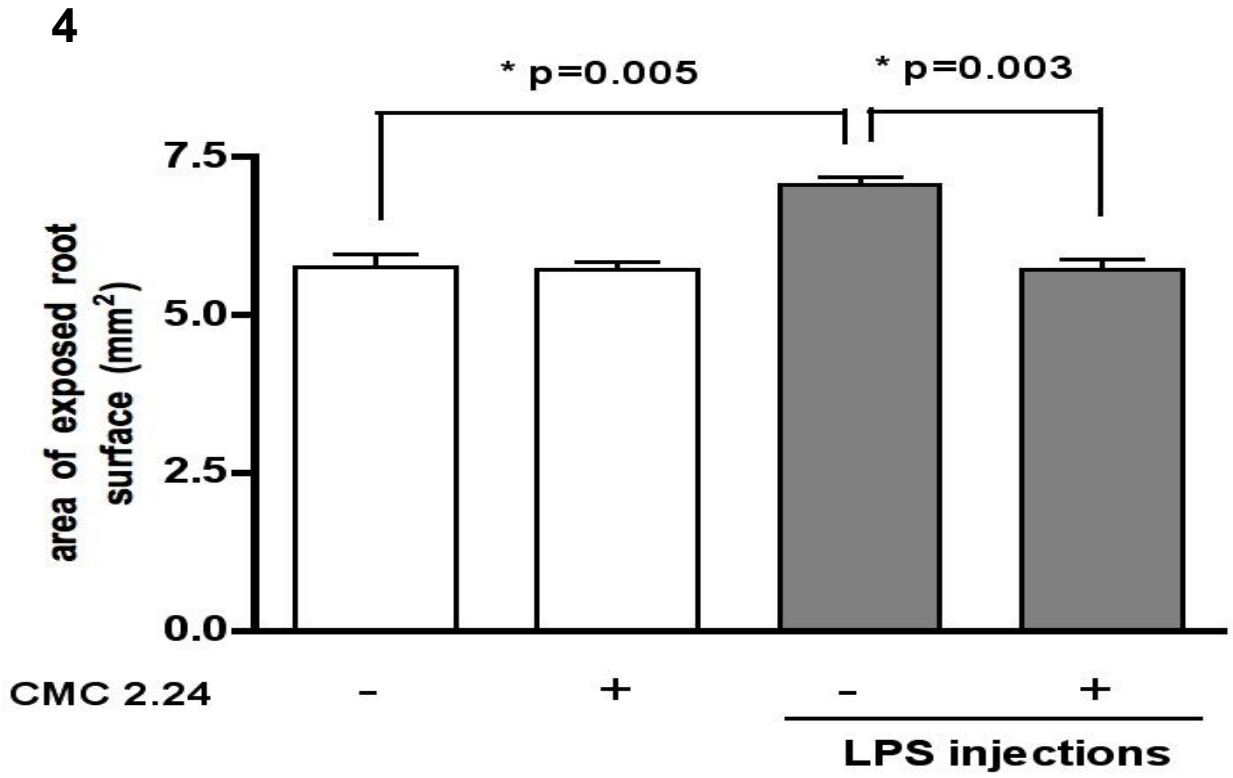


Figure 4. Direct measurements on de-fleshed hemi-maxillae demonstrate that CMC 2.24 significantly inhibits alveolar bone resorption in the in vivo model of LPS-induced periodontal disease. The bar graph presents the results of the percent of exposed root surface, which is directly proportional to the extent of bone loss, according to the experimental group. LPS caused bone loss as indicated by the significant increase of the area of exposed root surface, whereas simultaneous systemic administration of CMC 2.24 significantly reduced this area, indicating an attenuation of inflammatory-driven bone resorption. Differences between experimental conditions are indicated by the brackets and * (unpaired t-test for independent samples with Welch's correction for unequal variances).

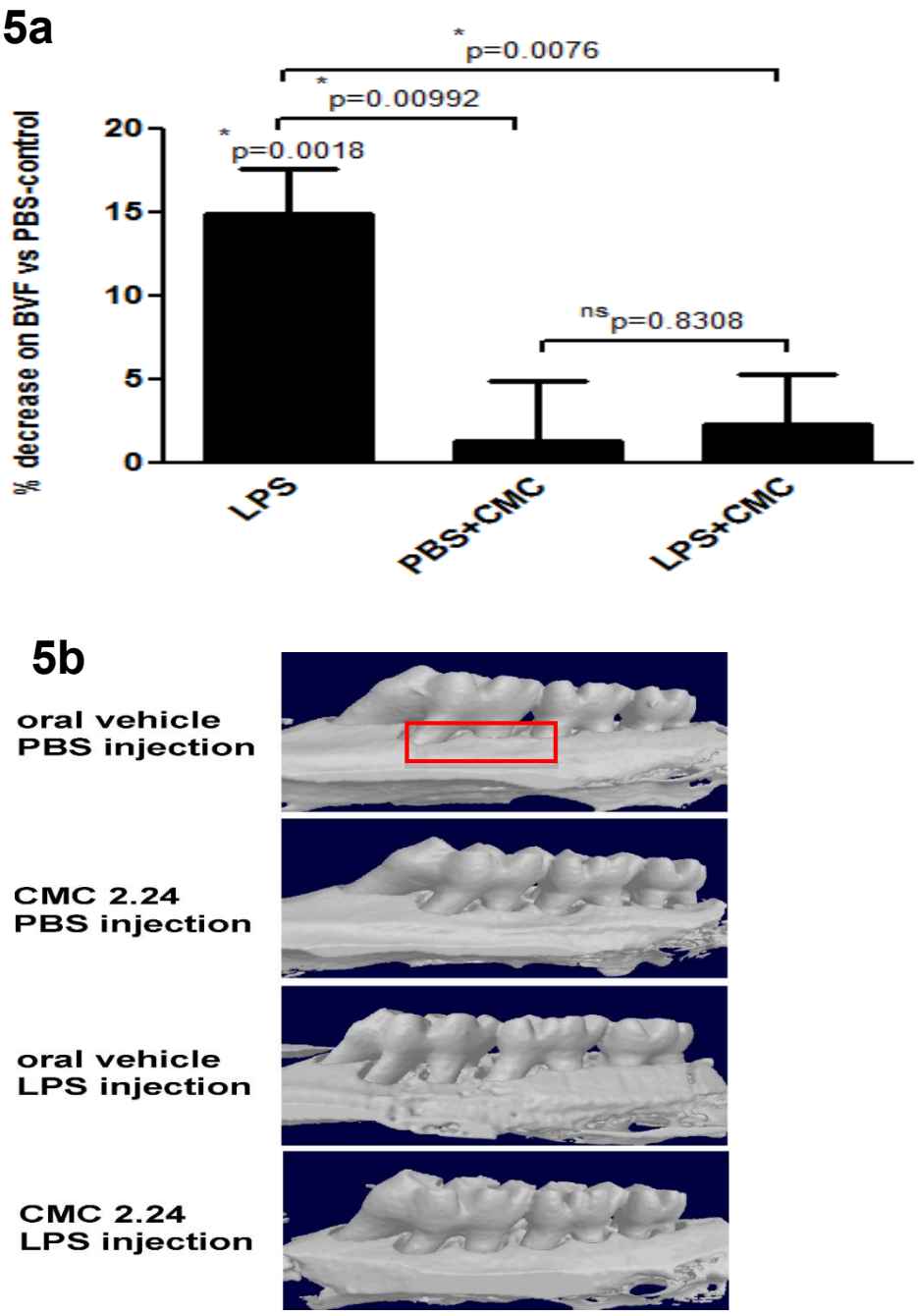


Figure 5. uCT data confirming that CMC 2.24 significantly inhibits alveolar bone resorption in the in vivo model of LPS-induced periodontal disease. Rats received either 2% carboxymethylcellulose vehicle or 30 mg/Kg of CMC 2.24 by oral intubation daily for 2 weeks. Contralateral LPS (3 µL, 30 µg) or PBS (3 µL) vehicle injections were performed 3 times/week for 14 days at the palatal aspect of first molars (see Figure 2). The bar graph presents the results of the uCT analysis as the change in the bone volume fraction (BVF) in the standardized ROI (bi-dimensionally shown as a red box in the representative image of the control) in comparison to vehicle-treated/PBS-injected samples (BVF in these samples was set to 100%, since these were assumed to present no inflammatory bone resorption). Bars indicate average and standard deviations. * indicates a significant difference in comparison to PBS-injected/vehicle-treated control. Differences between experimental conditions are indicated by the brackets and * (unpaired t-test for independent samples with Welch's correction for unequal variances). Images in Figure 5b show three dimensional rendering of the mineralized tissues in representative samples.

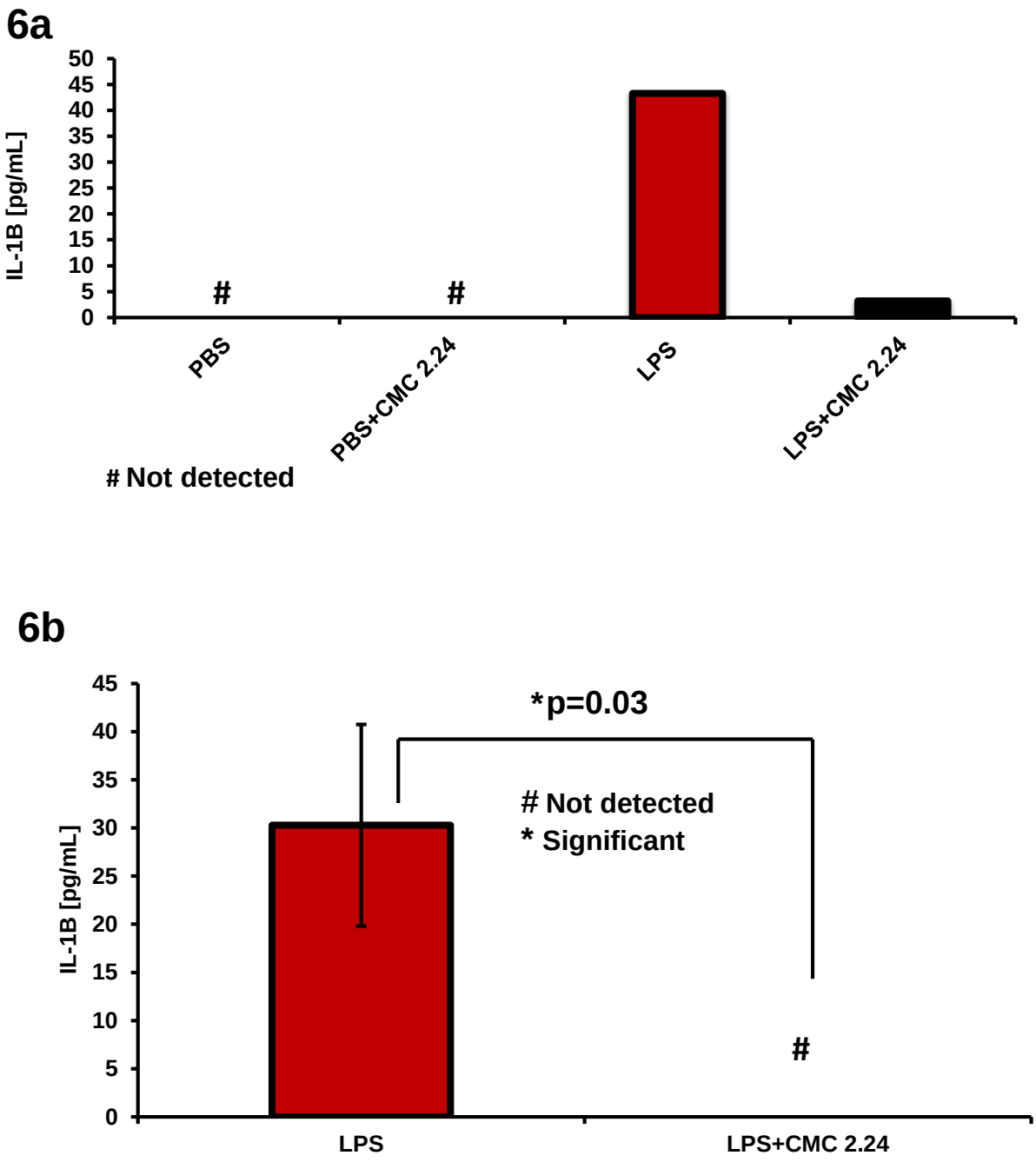


Figure 6. (a) The effect of CMC therapy on IL1-β in rat gingiva (top) and (b) serum (bottom) measured by ELISA.

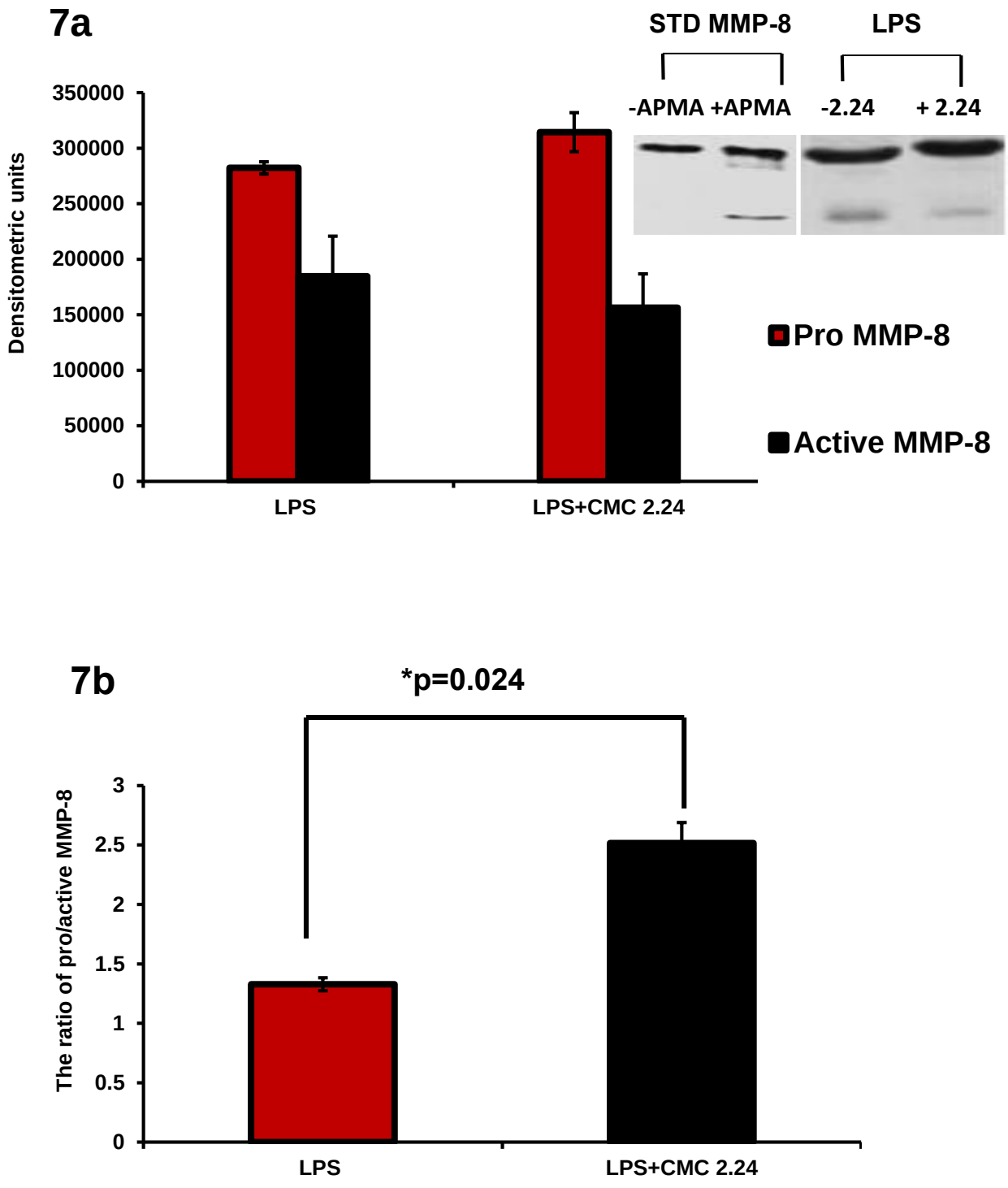


Figure 7. (a) Densitometric analysis of Western blots of MMP-8 in plasma from untreated LPS- injected rats (LPS) and LPS-injected rats treated with CMC 2.24 (LPS+CMC 2.24).Each value represents the mean of MMP-8 ± the Standard Error of the Mean (S.E.M.); representative Western blots of MMP-8 in plasma from untreated and CMC 2.24 treated rats are shown in the insert. (b) The ratio of pro/active MMP-8 calculated from densitometric analysis shown in (a) above.

Chapter 4

A Novel Chemically-Modified Curcumin Reduces the Severity of both Local and Systemic Connective Tissue Breakdown in a Rat Model of Periodontitis Exacerbated by Uncontrolled Diabetes

Introduction

As described in Chapters 1-3, periodontal disease is not only the most common of all chronic inflammatory diseases known to mankind, and the major cause of tooth loss in the adult population, it has also been linked to various medical diseases particularly diabetes (Soskolone and Klinger 2001; Taylor 2001; Ryan et al. 2003; Golub et al. 2006; Liu et al. 2006; Preshaw 2013). Also, as described previously, inflammatory periodontal disease is initiated by oral bacteria, particularly the anaerobic gram-negative microorganisms such as *Porphyromonas gingivalis (P. gingivalis*), *Prevotella intermedia, Tannerella forsythia* and others in the subgingival plaque or biofilm. Their microbial products, notably lipopolysaccharide (LPS) or endotoxin (a constituent of the cell wall of these gram negative bacteria), play an important role in inducing inflammation in the adjacent gingival and periodontal tissues. However, it is now widely accepted that the collagen and connective tissue destruction of the gingiva and periodontal ligament in the patient with periodontitis, including the loss of alveolar bone, is largely mediated by the host response, (Genco 1992; Ryan 2002; Gitlin and Loftin 2008; Gu et al. 2012; Van Dyke 2014). The latter can be amplified by systemic factors such as the diabetic/hyperglycemic state. One explanation for the link between periodontitis and diabetes, described decades ago, is the enhanced production in the

diabetic patient of host-derived MMPs in the gingival and periodontal tissues (Ramamurthy et al. 1973a; Chang et al. 1988). More recently, diabetic complications, including unusually severe periodontitis, have been linked to a hyper-inflammatory state which is reflected by the systemic inflammation, characterized in the circulation by elevated acute-phase proteins such as C-reactive protein (CRP) (Li et al. 2000). Many studies over many years have provided evidence for the links between these local and systemic diseases namely, chronic periodontitis and diabetes (Grossi et al. 1996; Genco et al. 2005; Demmer and Desvarieux 2006; Genco and Borgnakke 2013).

With regard to the mechanisms involved, it is known that advanced glycation end products (AGEs), can interact with receptors for AGEs (RAGEs) on target cells, such as macrophages and others, stimulating the production of MMPs, inflammatory cytokines (eg., TNF-α, IL-1β and IL-6) and adhesion molecules (eg., VCAM-1) as well as other mediators which promote tissue destruction (Shiau et al. 2006; Graves and Kayal 2008). Additional studies (Pankewycz et al. 1995; Purwata 2011) have also demonstrated that elevated levels of inflammatory cytokines are linked to diabetic complications. One of the most common diseases exacerbated by diabetes and by prolonged hyperglycemia is an increase in the incidence and severity of periodontitis. In this regard, periodontal disease has been considered as the sixth complication of diabetes (Löe 1993; Lalla and Papapanou 2011). A number of mechanisms have been identified to explain the link between diabetes and periodontal disease mentioned above. Additional mechanisms include increased expression not only of inflammatory mediators (Lalla et al. 2000; Santana et al. 2003), but also of excessive levels of matrix metalloproteases, and osteoclastogenic factors (Salvi et al. 1998; Lalla and Papapanou 2011). Still others (Shetty et al. 2008; Sima et al. 2010) have proposed that diabetes interferes with host defense mechanisms, including impaired leukocyte chemotaxis,

adherence, phagocytosis, and apoptosis, which then leads to prolonged (ie., unresolved) inflammation and increased susceptibility to infection, followed by enhanced connective tissue breakdown, all of which can impair wound-healing (with other serious consequences such as limb amputation and, even, death), increase severity of periodontitis, and other related complications. Diabetes also accelerates the progression of experimental periodontal disease induced by bacterial endotoxin or by silk ligatures (the latter irritate the gingival tissues both mechanically and by trapping more bacteria) (Salvi et al. 1998; Naguib et al. 2004). Additionally, previous reports have indicated that even in absence of external stimuli, such as bacterial endotoxin or silk ligatures, a chronic hyperglycemic environment in the periodontal tissues can trigger periodontal destruction as discussed later in this section (Golub et al. 2006; Claudino et al. 2007). To further complicate the explanation for the severity of diabetes-enhanced periodontal disease, the accelerated connective tissue breakdown observed both locally in the gingiva, and even that seen systemically in the skin (both mediated by pathologically- excessive, host-derived MMPs), occurs even in germ-free rats, rendered type I diabetic by streptozotocin injection (Golub et al. 1983, 1998a).

With this background in mind, Golub et al. (2006) proposed a "two-hit model" that describes how co-induction of periodontal disease initiated by the oral microflora, in conjunction with systemic inflammation, could result in intra-and extra-oral destruction of connective tissues including bone. These authors proposed that microbial products such as endotoxin generated by the subgingival microflora, provides the first "hit" in chronic destructive periodontitis. The second "hit" in this model is provided by systemic inflammation which is characterized by elevated biomarkers and mediators, such as CRP, IL-6, and MMP-9 in the circulation, which are associated with various systemic diseases such as cardiovascular disease, rheumatoid arthritis, osteoporosis,

and diabetes and which are known to be associated with destructive periodontal disease (Golub et al. 2006; Payne et al.2015). A recent example is illustrated by the studies of Frankwich et al. (2012). They demonstrated that MMPs, such as MMP-9 (which are produced in excessive levels during periodontitis, ie., the 1st "hit") can degrade insulin receptors on host cells (e.g., muscle) which would decrease insulin sensitivity and promote more severe hyperglycemia (2^{nd} "hit") which, in a similar way (e.g., through AGE-RAGE interaction and enhancement of cytokines), would promote more severe periodontitis (Frankwich et al. 2012).

As yet another example of this complex interaction between local and systemic diseases, it has been suggested that type I and type II diabetes increase the risk of periodontitis and alveolar bone loss as a result of systemic bone loss or osteoporosis. As one example of this pathway, osteoblasts incubated in tissue culture on a glycated collagen matrix (mimicking non-enzymatic glycation due to prolonged hyperglycemia) showed suppression of type I collagen synthesis, the major organic constituent of bone (Kirkwood et al. 1999). Additionally, the deregulation of inflammatory cytokines, a known effect of diabetes, is another contributing factor in the development of periodontal bone loss (Liu et al. 2006) as well as enhanced skeletal bone loss; in this regard, IL-1β, a key mediator associated with the pathogenesis of periodontitis, was historically known as "OAF" or osteoclast-activating factor (Jandinski 1988). Since this pro-inflammatory cytokine can upregulate the expression of IL-6, which in addition to being a biomarker of systemic inflammation in the circulation, it can also chemically attract osteoclasts (Lazzerini et al. 2013).

And finally, several studies have shown that inducing experimental diabetes increases MMP expression and activity (mediating excessive collagen breakdown) in tissues such as skin

and gingiva even in germ-free rats (Golub et al. 1983; Chang et al. 1988; Ramamurthy et al. 1998), and that administration of tetracyclines, by NON-antimicrobial mechanisms, can reduce this excessive proteinase activity down to constitutive/physiologic levels seen in normal non-diabetic rats (Golub et al. 1983; Ramamurthy et al. 1998).

Objectives

Based on the above introduction linking the two classes of disease in a "bidirectional manner" (Grossi and Genco 1998; Golub et al. 2006; Preshaw et al. 2012; Velea et al. 2013)**,** and the relatively, and increasingly, common clinical situation in which patients exhibit both conditions, the objective of the current experiment was to determine: (1) the effect of a model of periodontitis, complicated by diabetes, on mechanisms of tissue breakdown including bone loss, and (2) the response of the combination of this local and systemic phenotype to the pleiotropic tri-ketonic MMP-inhibitors (designed in the laboratory of Francis Johnson, PhD), CMC 2.24 and CMC 2.23. We used the type I diabetic rat model because this class of diabetes produces a severe form of periodontal disease (Deshpande et al. 2010; Meenawat et al. 2013) over a short period of time; note that the rat model of type II diabetes requires many months (Ryan et al. 1999), in contrast to the weeks required for the type I model. In addition, type I diabetes (which used to be called "brittle" or juvenile diabetes) is generally more difficult for the patient to optimally control blood glucose levels which contributes to the clinical view that this form of the disease is associated with more severe periodontal disease than type II which is primarily an adult disease, often more easily managed by the clinician.

Materials and Methods

Experimental Protocol:

24 adult male Sprague-Dawley rats (body weight 225-350g, Charles River Laboratories International, Inc., Wilmington, MA) were distributed into four groups (n=6/rats per group). The first three groups were rendered Type I diabetic via intravenous injection of streptozotocin (70 mg/kg body weight, STZ; ENZO Life Sciences, Inc., Plymouth Meeting, PA) into the tail vein after an overnight fast (Golub et al. 1978a). The rats in the fourth group were injected through the tail vein with 10 mM citrated saline buffer pH 4.5 (non-diabetic controls, N). All rats were given unlimited access to food and water. All STZ-injected rats exhibited pathologically elevated glucose levels in their urine within 24-48 hrs and the diabetic status was checked weekly by using non-enzymatic test strips (CTMI 4 LN, Cole-Tayler Marketing Inc, Redsa, Ca). One week after inducing diabetes, each rat in the first three groups was injected, to induce periodontitis, with 15 μL *E. coli* LPS (1 μg/ μL) in four different maxillary gingival sites (on the buccal and palatal surfaces on both right and left sides) every other day for one week. Beginning at the time of the first injection of LPS, two different CMCs, 2.23 and 2.24 (30 mg/kg), were administered daily via oral gavage for a period of 3 weeks to the two groups of rats treated with either CMCs 2.24 or 2.23. The untreated diabetic rats and the non-diabetic controls were administered vehicle alone control (2% carboxymethyl cellulose suspension). The dose of 30 mg/kg of these CMCs was chosen because earlier studies indicated maximal benefits at 30 mg/kg (similar to the benefits at 100 mg/kg) (Elburki et al. 2011). At the end of the three weeks of therapy, the rats in each experimental group were fasted overnight and then were sacrificed by CO_2 inhalation. Blood was retrieved from the rats by cardiac puncture for analysis of blood glucose (One Touch Ultra Glucometer; Johnson & Johnson, New Brunswick, N.J.) and HbA1c (Bayer A1CNow Self check,

Sunnyvale, CA) levels. The serum and plasma were separated by standard procedures (Thavasu et al. 1992). Gingiva, jaws, tibia and skin were collected and stored at -80ºC until used for analysis. The study protocol was previously approved by the Committee for Experimental Animal Use at Stony Brook University, NY, USA

Radiographic/morphometric measurement of alveolar bone loss:

Following sacrifice, the animals were decapitated, the jaws were defleshed and alveolar bone loss was evaluated from radiographs (see below). In brief, the maxillary jaws were removed from each rat, the gingival tissues excised and pooled per group. The jaws were then boiled in water for 10 minutes and then defleshed by dissection. The jaws were allowed to dry, large particles were brushed off and the jaws were immersed in 0.2 N NaOH at room temperature for 5 minutes to remove the remaining soft tissue debris, then rinsed with water and allowed to dry. Standardized radiographs were taken of wax-mounted jaws, images obtained with Schick size 2 CMOS sensors, and alveolar bone loss was assessed morphometrically by measuring the distance between a fixed anatomical landmark, the cementoenamel junction (CEJ), and the crest of the alveolar bone at site #7 (site #7 is located between the distal surface of 1st molar and the mesial surface of the 2nd molar of the maxillary quadrants) essentially as described by us previously (Chang et al. 1994; Ryan et al. 1999). This anatomical site was chosen to analyze alveolar bone height loss in the maxillary quadrants, because previous studies indicated that this site showed the maximum loss of alveolar bone compared to the other sites, which was confirmed in the present study (Chang et al. 1994).

Radiographic/morphometric analysis of systemic bone loss

Following sacrifice, the tibias were also collected. In brief, the right and left tibias were removed from each rat, boiled in water for 10 minutes and then defleshed by dissection. The tibias were allowed to dry, large particles were brushed off and the long bones were immersed in 0.2 N NaOH at room temperature for 5 minutes to remove the remaining soft tissue debris, then rinsed with water and allowed to dry. Cortical bone was assessed morphometrically by measuring the Pixel values at an area of interest (AOI) $1mm^2$ in the middle of the cortical plate of the diaphysis in the med- tibia (Figure 15a). Standardized radiographs were taken using an internal step-wedge with a fixed X-ray cone system, and images were obtained with Schick size 2 CMOS sensors, a technique described by us previously to assess alveolar bone density in the $1mm^2$ AOI in human dental radiographs (Payne et al. 2007; Goren et al. 2008). The internal step-wedge was made from aluminum alloy and contained 4 steps of different thickness (Figure 15d). Each radiograph was taken with the same exposure time and focal distance. Digital radiographs were imported into the software program (Schick) for analysis. The AOI chosen was in the middle of the cortical plate of the middle of the diaphysis and was readily reproducible. Using the software program described above, the radioopacity and the radiolucency of the AOI was determined by averaging pixel values using the Microsoft (Excel) program. Cortical bone density in the tibia was measured by converting Pixel values to density units. X-rays were taken on Kodak Insight film using an internal step-wedge and calculating the density of the step wedge by X-rite 361 Transmission Densitometer. The Densitometer measures density (up to 6.0 OD) and dot area. A standard curve (Figure 15b) using an aluminum step wedge of different thickness was created to convert pixels into density units.

Extraction of salt-soluble collagen:

Salt-soluble and insoluble collagen fractions were prepared from the skin by previously described techniques (Glimcher and Krane 1964; Ramamurthy et al. 1973b). In brief, the entire skin (except for that over limbs) was dissected from each rat, the adherent subcutaneous tissue was removed, the skin was weighed, skin thickness from the mid-dorsal surface was measured (in mm) with a caliper. A 6 mm circular punch biopsy from the skin (using a surgical trephine) was finely minced at 4ºC. Then the minced rat skins were extracted in cold (4ºC) neutral salt buffers (50 mM Tris-HCl buffer, pH 7.4, containing 1 M NaCl and 1 mM PMSF) for 2 days followed by centrifugation at 11,000 rpm for 20 minutes. As described by Ramamurthy et al. (1973b), the cold (4ºC) neutral-salt buffer extracts the triple-helical (undenatured) collagen molecules which are newly synthesized and not yet extensively cross-linked or polymerized. In contrast, the older collagen fibers, in which Schiff base crosslinks or even stronger inter-molecular covalent crosslinks have formed, are insoluble (4ºC) in these neutral-salt buffers. After centrifugation, the supernatants were dialyzed against 3% acetic acid. The pellet yields the insoluble (older) collagen fraction. 300 µL aliquots of the salt-soluble collagen (after thermal denaturation at 65ºC) were examined by SDS-PAGE for collagen α (monomer, 100 kDa) and β (dimer; 200 kDa) subunits (see below) (Golub et al. 1978b). The rest of the salt-soluble collagen was lyophilized using a Speed-Vac (Savant Instruments, Inc. Holbrook, NY). 700 µL of 2 N NaOH were added to both fractions of collagen and hydrolysis was accomplished by autoclaving at 120ºC for 1 hr which was repeated for another 1hr (120ºC). After neutralizing the hydrolysates, 50 µL aliquots were examined by the standard spectrophotometric (560 nm) Stegemann's reaction for hydroxyproline levels; hydroxyproline is an amino acid essentially only found in collagen and serves as a biomarker for collagen content and metabolism (Stegemann 1958; Ramamurthy et al. 1972; Golub et al. 1978b).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

Collagen subunits ie., the α and β components, were examined by polyacrylamide gel electrophoresis using a modification of the technique of Sakai and Gross (Sakai and Gross 1967) in 6% resolving gel and 3% stacking gel. The polyacrylamide gel contained 30% acrylamide mix, 10% sodium dodecyl sulfate (SDS), 1.5 M Tris (pH 8.8), 10% ammonium persulfate, 0.8% tetramethylethylenediamine (TEMED) and water. Aliquots of the salt-soluble collagen from each experimental group (0.15 g/100 mL) were denatured at 65ºC for 10 min and then mixed with an equal volume of sample buffer. A 20 µL aliquot of this mixture was loaded onto each gel [a collagen standard, collagen type I rat tail, BD Biosciences, Bedford, MA, was used as a reference to determine the molecular weight of the collagen components] and electrophoresis was performed (180 min; 100 V). The gels were stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 40% methanol, and 50% H_2O for 30 min and destained for 1 hr in 10% acetic acid, 10% ethanol, 50% methanol, and 30% H_2O with at least two changes of this solvent. Densitometric analysis of the collagen components was done as described previously (Golub et al. 1978b; Elburki et al. 2014).

Gingival tissue and skin MMPs:

The gingival tissues from the maxillary jaws were excised, weighed, and pooled per experimental group (n=6 rats per group) as described previously (Elburki et al. 2014). The gingival tissues were extracted and collagenase was partially purified as described in Chapter 3. The entire skin (except for that over limbs) was dissected from each rat, the adherent subcutaneous tissue was removed, and the skins were weighed. The skins were finely minced at 4ºC, then 100 mg of minced skin for each rat was extracted (4ºC) and collagenase in the extract was partially purified again as described in Chapter 3 (Elburki et al. 2014).

Gelatin zymography for measurement of MMP-2 and MMP-9:

Gelatin zymographic assays were used to assess the levels and molecular weight forms of MMP-2 and MMP-9 in the pooled gingival tissues (Bildt et al. 2008) and in individual blood and skin extracts from each group of rats (Ramamurthy and Golub 1983). Samples and standards were run under non-reducing denaturing conditions on gelatin zymography as described in Chapter 3 (Elburki et al. 2014).

Western blotting for measurement of MMP-8 in plasma, gingiva and skin extracts:

Western blotting techniques used to determine MMP-8 levels in plasma, gingiva and skin extracts were also described in Chapter 3 (Elburki et al. 2014).

Measurement of MMP-13 in plasma by ELISA:

The MMP-13 level in plasma was measured in duplicate by ELISA for each experimental group as described in Chapter 3 (Elburki et al. 2014).

Measurement of bone resorptive cytokines levels in gingival tissue and serum:

Three inflammatory cytokines, (IL-1β, IL-6, and TNF-α), were analyzed in duplicate in the serum samples from each rat, and in gingival tissue extracts from sites of periodontal disease, by ELISAs as described in Chapter 3 (Elburki et al. 2014).

Statistical analysis:

Each value represents the mean ± standard error of the mean (S.E.M) of 6 rats per experimental group (bone, skin, blood); however, for gingiva (because of the small amount of tissue/rat) only pooled samples were analyzed. The standard error of the mean was calculated from the standard deviation. Statistical significance of the data presented was determined by using analysis of

variance (ANOVA) or by paired students T-test. p-values of less than 0.05 were considered to be statistically significant. These analyses were carried out using Excel statistical software.

Results

Blood glucose measurements and diabetic complications:

As shown in Figures 8a and b, all three groups of diabetic rats (including vehicle-only treated diabetics plus those diabetics rats treated either with CMC 2.24 or CMC2.23) exhibited significantly elevated blood glucose and hemoglobin A1c (CMC 2.24 treatment only) levels compared to the non-diabetic control rats, and the CMC 2.24 or CMC2.23 treatments produced no detectable effect on the severity of hyperglycemia at least in the relatively short duration (3 weeks) of this treatment protocol. However, despite this apparent lack of benefit on the severity of hyperglycemia in the diabetic rats, the treatment with CMC 2.24 appeared to reduce diabetic complications even in the presence of severe hyperglycemia (Table 3). The rats were examined for the presence of adverse events (AEs) in each experimental group during the three week therapeutic protocol and at the time of sacrifice (4 weeks after inducing type I diabetes) and it was found that both the non-diabetic control (N) rats and the diabetic rats treated with CMC 2.24 showed no detectable adverse events. In contrast, the untreated diabetic group (D) suffered from tail necrosis at site of STZ injection (i.e., NON–healing tail wound), excessive tears and inflamed sclera in both eyes, and bleeding under the toe-nails. In addition to these AEs (although reduced in incidence) the diabetic rats treated with CMC 2.23 suffered mild diarrhea and showed non-healing necrosis in their tails which healed after two weeks. In contrast, half of the vehicle-treated diabetic rats showed adverse events such as bleeding under the toe-nails and tail necrosis throughout the 4-weeks experimental protocol (Table 3).

Physical measurements of gingiva and skin

As expected, four weeks after inducing diabetes by STZ injection, all diabetic rats exhibited a lack of body weight gain, polydipsia, and polyuria whereas the non-diabetic control rats progressively gained weight over the 21-day time period (Figure 9). Note that one week after inducing diabetes, the diabetic rats were treated once per day systemically, for 21 days with either CMC2.24 or CMC 2.23; the non-diabetic control rats were only treated with vehicle alone. All of the diabetic rats treated with either CMC2.24 or CMC2.23 showed no difference in body weight gain compared to vehicle-treated diabetic rats (Figure 9), even though the D+CMC 2.24 rats (but NOT the D+CMC 2.23 rats) showed reduced AEs even in the presence of severe hyperglycemia.

Gingiva

The maxillary gingiva from the each experimental group were dissected and the wet weight for each pool of tissue per group was determined. The pooled gingiva from the different groups of rats including normal, diabetic, diabetics treated with CMC 2.24, and diabetics treated with CMC 2.23 groups (n=6 rats/group), weighed 14.0 mg, 22.2 mg, 15.8 mg, and 24.2 mg, respectively (Figure 10). The normal rats showed the lowest wet weight whereas the untreated diabetic rats showed a 58.6 % increase in wet weight. The diabetic rats treated with CMC 2.24 showed 12.9% increase compared to the non-diabetic control rats and the diabetic rats treated with CMC 2.23 showed 72.9% increase in wet weight (Figure 10b). Note that only the diabetic rats treated with CMC 2.24 showed gingival weight similar to that exhibited by the gingiva from the non-diabetic controls.

Skin

Skin thickness from the mid-dorsal surface was assessed using a millimeter caliper. Skin thickness in the normal, diabetic, and diabetic rats treated with CMC 2.24, and CMC 2.23 were found to average 0.18 cm, 0.09 cm, 0.14 cm, and 0.09 cm, respectively (Figure 11). Inducing type I diabetes and severe hyperglycemia produced a statistically significant 45.7% decrease ($p=0.0004$) in skin thickness compared to the thickness of skin seen in non-diabetic control rats (thus the increased wet weight of the diabetic rat gingiva (see Figure 10) may reflect excess water accumulation, ie., inflammatory edema, the vascular component of inflammation and is not due to increased tissue (dry weight) accumulation). Systemic administration of CMC 2.24 appeared to be the most effective treatment because it significantly increased skin thickness in the diabetic rats to a level 38.1% ($p=0.004$) greater than that seen in the untreated diabetic group (Figure 11). In fact, CMC 2.24 increased skin thickness to essentially the same level as that seen in the non-diabetic control rats since there was no statistically significant difference between the two groups ($p>0.05$). Considering the two CMC treatments, orally administered CMC 2.24 normalized skin thickness whereas CMC 2.23 remained the same as the untreated diabetic rats ($p=0.9$).

These changes in the skin of the diabetics not only reflect skin atrophy (decrease in skin thickness) but also reflect decreased levels of collagen assessed biochemically by hydroxyproline analysis and by SDS/PAGE (see below).

Measurement of collagen in rat skin:

Experiments on the diabetic rats showed that daily oral administration of CMC 2.24 for 21 days increased the skin collagen as evidenced by increased content of hydroxyproline (data not shown). The hydroxyproline content, measured spectrophotometrically, was found to be 14.5, 12.0, 15.0

μg/6 mm circular punch biopsy (using a surgical trephine) in the normal, diabetic, and diabetic rats treated with CMC 2.24 groups, and the % salt- soluble collagen was found to be 30.9, 22.9, and 31.3% of the normal, diabetic, and diabetic rats treated with CMC 2.24 collagens, respectively (Figure 12). These data indicate that diabetes reduces the newly synthesized poorly-cross-linked collagen ($p=0.02$) and increases the older polymerized, insoluble, highly-cross-linked collagen. However, CMC2.24 treatment significantly increased ($p=0.03$) the newly-synthesized, salt-soluble collagen and decreased the polymerized insoluble collagen back to normal levels. Only the salt-soluble data was found to be statistically significant; the data on the % insoluble collagen was not statistically significant (Figure 12).

As shown in Figures 13a and b, the α (monomer) and β (cross-linked dimer) components of the salt-soluble fraction of collagen in skin were examined by polyacrylamide gel electrophoresis (Figure 13a). The dominant collagen in skin is type I which exhibits a 2:1 ratio of α1:α2. As shown in Figure 13, a similar ratio is seen indicating that these samples were composed mainly of type I collagen. Densitometric analysis of these monomers and their cross-linked dimers indicated that the α/β ratio was reduced by 36% (Figure 13b) in the diabetics which was significantly lower than normal ($p=0.05$). When the diabetic rats were treated with CMC 2.24 the ratio of α/β appeared to be increased by 31% which statistically was not different ($p=0.1$; NS) than the ratio for collagen in the non-diabetic control rats (Figure 13b).

Local/systemic bone measurements: alveolar bone and tibia

Based on radiographic-morphometric measurements of alveolar bone height loss using a standardized x-ray technique which measured the distance between the cementoenamel junction (CEJ; fixed anatomical land mark) and the alveolar bone crest at site #7 of the maxillary quadrants,

as described by us previously (Chang et al. 1994), the combination of systemic disease, diabetes, and LPS injections (local periodontal disease) into the gingiva in the untreated rats significantly ($p=0.02$) increased alveolar bone loss by 58%. Administration of CMC 2.24 significantly ($p=0.04$) reduced this increase by 31% (Figure 14a) essentially back to the normal levels seen in non-diabetic control rats ($p=0.25$; NS). In contrast, CMC 2.23 did not significantly reduce alveolar bone loss in the diabetic rats with experimental periodontitis (Figure 14a and b).

Systemic bone loss was measured using a radiographic-morphometric assay of cortical bone density using a standardized X-ray technique which measured the optical density at AOI ($1mm^2$) in the mid diaphysis (Figure 15a and b). As seen in Figure 15b, there is a linear positive relationship ($R^2= 0.990$) between the pixels values and O.D. based on the density of the aluminum step-wedge incorporated into each radiograph (Figure 15d). As shown in Figure 15c diabetes reduced cortical bone density slightly, however this effect was found to be statistically significant ($p=0.03$). There was a trend towards normalization of cortical bone density, using both CMC 2.24 and 2.23 therapies, but these effects were not statistically significant.

MMP-2, -9 in gingiva: gelatin zymography

The levels of both gelatinases (MMP-2 and MMP-9) were assessed by gelatin zymography in the pooled gingival tissue from the upper jaws of rats from each experimental group (Figure 16). Diabetes and LPS-induced periodontal disease appeared to increase MMP-2 levels specifically the lower molecular weight activated form of MMP-2 (however, it cannot be stated definitively that this lower molecular weight gelatinolytic enzyme is, in fact, derived from 72 kDa pro-MMP-2) in the pooled gingival tissue (note the comparison to the standard pro and active MMP-2 (std-2)) and increased the lower molecular weight activated MMP-9. However, treatment of the rats with

systemically-administered CMC 2.24 appeared to dramatically decrease the pathologically-excessive levels of both gelatinases (MMP-2; activated forms, and MMP-9 including the 92 kDa pro-form and activated forms) in the diabetic rats with LPS-injected gingiva (Figure 16a). Some reduction of MMP-9 by CMC 2.23 administration was also seen in the gingiva from the diabetic rats with LPS injections but no effect was seen on MMP-2 (Figure 16a). In addition, this lower molecular weight gelatinolytic proteinases, presumably an activated form of MMP-2, showed the same pattern ie., increased with diabetes and LPS and reduction by CMC2.24 (but not CMC 2.23) treatment, although this zone of lysis may be another type of gelatinolytic MMP, e.g., MMP-7.

MMP-2, -9 in blood: gelatin zymography

The levels of both gelatinases (MMP-2 and MMP-9) were assessed by gelatin zymography in the plasma samples from each rat but, unlike the gingiva and skin, no MMP-2 was seen in the plasma samples of any of the experimental groups (Figure 16b). Similar to the data for the diabetic rats without LPS-induced periodontitis (preliminary data not presented), in the current study the combination of these systemic and local disease significantly increase MMP-9 levels in the plasma specifically the lower molecular weight activated form ($p=0.01$). However, treatment of the rats with systemically-administered CMC 2.24 significantly decreased the pathologically-excessive levels of MMP-9 by 44% in the diabetic rats with LPS-induced periodontal disease ($p=0.02$) (Figure 16b).

MMP-2, -9 in skin: gelatin zymography

The levels of both MMP-2 (72 kDa) and MMP-9 (92 kDa) were also assessed by gelatin zymography of skin extracts but, unlike gingiva (+LPS), no MMP-9 was seen in skin of any group

(Figure 17a). Diabetes appeared to increase (a) the amount of both pro-and activated MMP-2, and (b) the ratio of lower molecular weight activated forms of MMP-2, relative to the higher molecular weight inactivated forms but this effect did not reach statistical significance. CMC 2.24 (and, to a lesser extent CMC 2.23) also appeared to reduce both molecular forms of this gelatinase (MMP 2) in skin (Figure 17a and 17b). Note that CMC 2.24 reduced the ratio of active MMP-2 to pro MMP-2 ($p=0.001$) (Figure 17c); in contrast CMC 2.23 did not reduce this ratio.

MMP-8 in gingiva, plasma and skin extracts: Western blot

Western blot was performed to measure the levels of MMP-8 in gingiva, plasma and skin extracts. MMP-8 was detected in the gingival extracts from the different groups of rats. Based on Western blot analysis, the gingival extracts from the diabetic rats with LPS-induced periodontal disease appeared to show elevated levels of both higher molecular weight pro-MMP-8 as well as lower molecular weight activated forms (Figure 18a). However, the ratio of pro/active MMP-8 in the diabetic rat gingiva appeared unaltered (Figure 18b). The evidence that the higher and lower molecular weight forms of MMP-8 represent the pro- and activated forms of this collagenase (collagenase-2), respectively, is indicated by the Western blot pattern of standard MMP-8 in the presence and absence of 1 mM APMA; this compound is traditionally used to activated the pro forms of MMPs into the lower molecular weight activated forms, as discussed by us previously (Elburki et al. 2014). As seen in Figures 18a and b, both CMC 2.24 and 2.23 appeared to reduce the relative level of the lower molecular weight, activated forms of MMP-8 (note the increased ratio of pro/active MMP-8 in gingiva from rats treated with either 2.24 or 2.23; Figure 18b). Based on the densitometric examination of these Western blots (Figure 18a), the gingiva of the CMC 2.24 treated diabetic rats with LPS-induced periodontal disease showed a ratio of pro/active MMP-

8 of 1.53 which was 78.5% higher than the ratio, 0.85, found in the gingiva from the vehicle-treated diabetic rats with LPS-induced periodontal disease (Figure 18b), and this inhibition of activation of the latent form of MMP-8 by the CMC 2.24 treatment was also noticed in the gingiva of the CMC 2.23 treated rats with diabetes and LPS-induced periodontitis which showed a ratio of pro/active MMP-8 of 1.8 which was 108.1% higher than the ratio, 0.85, found in the gingiva from the vehicle-treated diabetic rats with LPS injection (Figure 18b).

MMP-8 was also detected in the plasma (Figure 18c) and skin extracts (Figure 18d) from the different experimental groups of rats. It appeared that MMP-8 was increased in the untreated diabetic group both in plasma and skin extracts and that treatment with CMC 2.24 reduced the elevated levels of MMP-8 in plasma and skin extracts (Figure 18c and 18d). Note that the value for skin and plasma from D+2.24 rats was essentially the same as that for non-diabetic controls ($p>0.05$).

MMP-13 in plasma: ELISA

ELISA was used to measure the levels of MMP-13 in the plasma of each experimental group. In the untreated diabetic rats the levels of MMP-13 in plasma were found to be about the same as that for non-diabetic controls, ie., 1.2 ng/mL. Administration of CMC 2.24 to the diabetic rats had no effect on the levels of this collagenase in the plasma (Figure 19). Western blot of MMP-13 also showed no effect of CMC 2.24 on the levels of this collagenase (data not shown). It should be noted that MMP-13 in rodents is considered a constitutive collagenase (Mitchell et al. 1996; Elburki et al. 2014), unlike MMP-13 in humans which is inducible and has been shown to be increased in diseases such as chronic periodontitis (Rossa Jr et al. 2005).

Cytokines in gingiva, serum and skin extracts

Analysis of IL-1β in extracts of the pooled gingival tissues indicated that diabetes markedly increases the level of this pro-inflammatory cytokine since it was not measureable in the extracts of the non-diabetic control gingival tissue (Figure 20a). Moreover, CMC 2.24 (and, to a lesser extent CMC 2.23) reduces the pathologically-excessive levels of IL-1β in the gingiva back to normal level (Figure 20a). Of interest, similar effects were also seen systemically in the serum which could be analyzed statistically and were found to be significant (Figure 20b). Similar concentrations of IL-6 were seen in the gingival tissues from the normal, diabetes + LPS and diabetes + LPS+ CMC 2.23 groups of rats. However, the diabetes plus LPS and CMC2.24 treatment appeared to slightly reduce the level of this pro-inflammatory cytokine in the gingiva by 24% (Figure 21a). Nevertheless, statistically significant effects on IL-6 were seen in the serum. IL-6 exhibited higher concentrations in the serum in the diabetic rats and CMC 2.24 and CMC 2.23 did significantly reduce the level of this cytokine in the serum back to essentially normal levels (Figure 21b). TNF-α was unmeasurable in both gingival extracts and in serum. None of these cytokines was detectable in any of the skin extracts.

Discussion

It has repeatedly been demonstrated that MMPs are up-regulated in the presence of both periodontal disease and diabetes (McNamara et al. 1982; Ramamurthy and Golub 1983; Ryan et al. 1999; Claudino et al. 2012). A characteristic feature of periodontal disease is the breakdown and loss of collagen in the periodontium including the gingiva, periodontal ligament, and alveolar bone. Many inflammatory cytokines and prostanoids (e.g., PGE2), during periodontal disease, can upregulate the production of MMPs such as the collagenases MMP-1, MMP-8, and MMP-13,

which are responsible for the destruction of various constituents of extracellular matrix including collagen fibers (Wahl and Corcoran 1993; Ries and Petrides 1995; Sorsa et al. 2006). It should be noted that the effect of LPS-induced experimental periodontitis on MMPs in the gingiva and on cytokines, both locally and systemically, was discussed in the previous chapter (Elburki et al. 2014). The mechanisms for the increased activity of the MMPs in diabetes has not yet been clarified. Elevated levels of iNOS, PGE2, and pro-inflammatory cytokines have all been seen in diabetics (Bain et al. 1997) and any or all of these cell regulators could be responsible for elevated MMP expression and activity. AGEs formed during long-term diabetes can react with RAGEs to stimulate the production of various inflammatory cytokines (ie., IL-1β, IL-6, and TNF-α) which then can stimulate the production of MMPs (Shiau et al. 2006; Graves and Kayal 2008).

Ramamurthy et al. (1972) reported that alloxan-induced (type I) diabetes caused a marked reduction of both salt-soluble and acid-soluble collagens in rat gingiva compared to non-diabetic controls. A similar pattern was seen in the current study which showed a decrease in salt-soluble relative to the insoluble collagen in the skin of the STZ-diabetic rats. Ramamurthy et al. (1972) also reported that the urinary levels of hydroxyproline were elevated, which suggested that there was more collagenase activity in general in body tissues since urinary excretion of hydroxyproline is known to be associated with the breakdown products of collagen generated systemically (Ramamurthy et al. 1972). Consistent with these earlier findings, the same group (Golub et al. in 1978) demonstrated that inducing type I diabetes with STZ resulted in an augmentation of collagenase activity in the gingiva of rats (Golub et al. 1978a). Further experiments illustrated that diabetes results in a multitude of collagen abnormalities. The most relevant to periodontal disease was a study that demonstrated that diabetes caused a reduction in the density of bone in the jaws

(mandible) of rats. This osteoporotic effect in oral as well as skeletal bone was suggested to be the result, in part, of excess collagenase activity since 90% of the organic matrix of bone is type I collagen (Ramamurthy et al. 1973b).

Although, at the time, information regarding the biology of MMPs was limited (the first MMP, now called MMP-1 or collagenase-1, was originally described by Gross and LaPiere, 1962), it was still clear that these enzymes were a major factor in the adverse effects of diabetes on collagen structure and turnover and periodontal disease. This conclusion was arrived at because, in the 1960s, it was found that collagenase MMPs were the only neutral proteinases capable of degrading collagen's triple-helical structure under physiologic conditions of pH and temperature (Gross and LaPiere 1962). It is now understood that many different types of MMPs (not just collagenases) contribute to the remodeling of the extracellular matrix (Visse and Nagase 2003; Sorsa et al. 2006; Sekton 2010).

With the promising effects of CMC 2.24 as MMP and cytokine inhibitors, and with the demonstration that CMC 2.24, but not other triketonic CMC (CMC 2.23 and CMC 2.14), can significantly inhibit alveolar bone loss in diabetic rats (Elburki et al. 2011), it was of great interest to see how this compound performed in an animal model of endotoxin-induced periodontitis complicated by type I diabetes, a serious and not uncommon disease situation seen in clinical practice. Although diabetes by itself can induce periodontitis (Claudino et al. 2012), we decided in this experiment to use the diabetic rat model with endotoxin-induced periodontitis to induce a more severe and aggressive form of periodontitis because of the short duration of this experimental protocol (4 weeks) and because this particular combination of local and systemic disease is so-

often seen clinically and is particularly difficult to treat effectively. This also mimics the human condition in that diabetes increases the incidence and severity of periodontitis induced by oral bacteria. Diabetics more often than non-diabetics, show severe periodontal disease including rapid alveolar bone loss (Deshpande et al. 2010; Preshaw et al. 2012). The results of the current study are consistent with these, and other reports, in that the combination of STZ-diabetes plus LPS-periodontal disease produced the most severe and rapidly developing periodontal disease in the rat model that we have ever observed. Therefore, this severe form of periodontitis, as a complication of severe type I diabetes, with its dramatically elevated blood glucose levels (severe hyperglycemia), provides a substantial test of the potential efficacy of CMC 2.24.

The following discussion initially addresses the changes in MMPs and cytokines locally in the gingiva resulting from CMC 2.24 administration in vivo in the rat model of diabetes combined with locally (LPS)-induced periodontitis. This section is followed by a discussion of the systemic changes in blood and skin. The final section addresses changes in bone structure both locally in the periodontium, and systemically in the skeletal tissue.

Local effects

Diabetes and gingival weight

Gingival weight was examined for each experimental group. Since the dry weight of the gingiva is reduced by diabetes (Ramamurthy et al. 1974; Yu et al. 1993) while the wet weight is elevated as seen both in earlier studies and in the current experiments, the increased wet weight appears to reflect edema due to a diabetes-induced hyper-inflammatory state. Moreover, CMC 2.24 (but not CMC 2.23) appeared to reduce this accumulation of fluid in the gingiva. In addition. Ramamurthy et al. (1974) found that experimentally induced diabetes decreases collagen synthesis

in gingival tissues in vitro and in skin tissue in vivo which also indicated that the increase in wet weight was due to edema and not due to an increase in tissue (or collagen) mass (Ramamurthy et al. 1974) and that this vascular component of inflammation was reduced by CMC 2.24 treatment of the diabetic rats.

Diabetes and MMPs in gingiva

Clearly, the induction of periodontitis in the diabetic rats greatly elevated the level/activity of MMPs. These results held true for MMPs -2, -8, and -9 and agree with the data presented in the literature describing type I diabetic rat studies (Ramamurthy et al. 1972; Golub et al. 1983; Chang et al. 1996; Ryan et al. 1999, 2003) and also in our recent publication in non-diabetic rats with LPS induced periodontitis (Elburki et al. 2014). Treatment of the rats with systemically-administered CMC 2.24 appeared to dramatically decrease the pathologically-excessive levels of both gelatinases (MMP-2 and 9) in the gingiva of the diabetic rats with LPS-induced periodontal disease. A major source of MMP-9 in inflamed gingiva is derived from the specific granules in the PMN leukocyte. However, other cells in the gingiva, e.g., epithelial cells, can also produce MMP-9 as well as MMP-2. The primary effect of diabetes was an increase in the lower molecular weight activated forms of MMP-9 which were reduced by CMC 2.24; some reduction of MMP-9 by CMC 2.23 administration was also seen in the gingiva from the diabetic rats with LPS-induced periodontal disease. However, no effect of CMC 2.23 was seen on MMP-2. This agrees with previous studies that have shown that both doxycycline and curcumin are capable of reducing elevated MMP levels (Golub et al. 1990; Kundu et al. 2011). Because doxycycline, curcumin, and CMC 2.24 all have a similar pharmacophore this result is not surprising. Again these beneficial effects of CMC 2.24 occurred even though diabetic rats remained severely hyperglycemic.

MMP-8 is one of the essential biomarkers in periodontitis and is thought to be largely responsible for periodontal connective tissue destruction (Lee et al. 1995; Sorsa et al. 2006); it constitutes 80-90% of total collagenase levels in the human GCF (Golub et al. 1997). MMP-8 reduction, as a result of CMC 2.24 administration, is important because MMP-8 like MMP-9 is a leukocyte-generated collagenase known to be elevated during periodontal disease (Sorsa et al. 2011). However, fibroblasts in the gingiva may also contribute to the excessive collagenase activity since the diabetic rat skin, like the gingiva and blood, shows high levels of MMP-8 which in the skin, is likely to be of fibroblast origin, and not neutrophil, as indicated by Sorsa et al. (1994). Fibroblasts and other cells under certain conditions, such as elevated cytokines during diabetes, can be induced to secrete a neutrophil-type of collagenase, MMP-8 (Sorsa et al. 1994). This data agrees with our recent publication (Elburki et al. 2014) that illustrated that CMC 2.24 reduced excess MMP-8 activation as made evident by an increased ratio of pro-MMP-8 to active-MMP-8 after CMC 2.24 administration. Mechanisms could include the ability of CMC 2.24 to inhibit other neutral proteinases such as plasmin, elastase and MMP-1 which are known to convert pro MMP-8 into the smaller molecular weight activated forms as was discussed in Chapter 3 (Elburki et al. 2014).

Diabetes and inflammatory cytokines in gingiva

In addition to accelerated alveolar bone loss, another typical effect of diabetes-exacerbated-periodontal disease is an increased level of inflammatory cytokines (Salvi et al. 1998; Liu et al. 2006). Periodontal disease even in non-diabetics is also characterized by an increase in these cytokines in the gingival tissue and the GCF in the periodontal pocket particularly IL-1β and TNF-α (Page 1991; Wilton et al. 1992; Geivelis et al. 1993; Graves 1999). Similar results were found

in this experiment as the untreated diabetic rat group displayed abnormally high levels of inflammatory mediators in gingiva. Administration of CMC 2.24 reduced IL-6 and IL-1β down to essentially normal levels. These results agree with earlier studies demonstrating that natural curcumin reduces the same inflammatory mediators (Guimarães et al. 2011). Furthermore, this correlates well with the gingival wet weight data described earlier which showed an increase in edema in these tissues in the diabetic rats with periodontitis, and a reduction of this vascular component of inflammation due to CMC 2.24 treatment.

Systemic effects

Diabetes and MMPs in blood

The reduction by CMC 2.24 treatment of MMPs in the pooled gingival tissues was paralleled by a significant reduction in MMPs in the systemic circulation of the same severely ill animals. The induction of periodontitis in the diabetic rats greatly elevated the level of MMP-9 in the plasma. However, treating the rats with systemically-administered CMC 2.24 significantly decreased the pathologically-excessive levels of MMP-9 in the plasma of the diabetic rats with LPS-induced periodontal disease.

MMP-8 in the plasma was also increased in diabetic rats with LPS-induced periodontal disease and treatment with CMC 2.24 reduced the pathologically elevated levels of MMP-8 in plasma. Tuomainen et al. (2007) reported that there is a link between high levels of MMP-8 (neutrophil-type collagenase) in blood and cardiac events, and in addition to MMP-8, MMP-9 was also elevated in gingival crevicular fluid (GCF) in periodontal lesions in patients with chronic periodontitis (Tuomainen et al. 2007). Moreover, Blankenberg et al. (2003) reported that MMP-9, which is elevated in the periodontal pocket (see above), when elevated in the plasma of humans

with CVD, is positively correlated with the incidence of acute myocardial infarction (AMI), even fatal heart attacks. The MMP-9 levels were monitored in plasma during a long-term (4-year) clinical trial involving over 1.000 patients (Blankenberg et al. 2003).

With regards to MMP-13, its levels in plasma in the diabetic rats with LPS-induced periodontal disease were found to be about the same as that for non-diabetic controls, and the administration of CMC 2.24 to the diabetic rats had no effect on the levels of this collagenase in the plasma. MMP-13 is the second most dominant collagenase in the human periodontal pocket (Golub et al. 1997, 2010). However MMP-13 in rats is constitutive, as MMP-1 is in humans (Mitchell et al. 1996), and was found to be relatively insensitive to CMC 2.24.

Diabetes and inflammatory cytokines in blood

Chronic inflammation anywhere in the body, such as occurs in chronic periodontitis, rheumatoid arthritis, and Crohn's disease, causes an increased production of inflammatory mediators (including prostanoids, cytokines, NO, and MMPs) at the local site, which can also be detected in the systemic circulation. The pro-inflammatory cytokines in the plasma, such as IL-6, can stimulate the liver to produce acute phase proteins (particularly CRP, and other inflammatory mediators, such as fibrinogen, haptoglobin, α1 anti-chymotrypsin) which are markers of systemic inflammation and can link chronic periodontitis with other diseases such as CVD. In addition to these inflammatory biomarkers, the increased levels and activities of MMP-2, -8 and -9, both locally and systemically in the circulation, are also associated with both chronic periodontitis and CVD (Gu et al. 2011b). Of relevance to this has been the characterization of chronic periodontitis as an “inflammatory” disease rather than an “infectious” disease (Van Dyke 2014).

Despite CMC 2.24's ability to inhibit MMPs and inflammatory cytokines in the diabetic rat with periodontitis, this treatment had no effect on blood glucose levels. This is not surprising since CMC 2.24, doxycycline, and any other MMP-inhibitor mentioned in this thesis, have not shown an ability to reduce the severity of hyperglycemia (no reports in the literature) at least in short-term studies. However, a pilot clinical study by Engebretson and Hey-Hadavi indicated that the treatment of periodontitis with a three-month regimen of daily sub-antimicrobial-doses of doxycycline plus scaling and root planing, can decrease HbA1c in patients with type II diabetes who are normally taking the prescribed hypoglycemic agents (Engebretson and Hey-Hadavi 2011). These observations suggest that longer-term periodontal treatments with MMP-Is such as SDD or CMC 2.24 may, with a longer duration of treatment, decrease HbA1c. The hypothesis is that, following periodontal treatment with these agents, resolution of periodontal inflammation leads to a reduction in the levels of inflammatory mediators locally, which then results in a reduction in the levels of inflammatory mediators in the circulation. Important mediators in this process involve IL-6 and TNF-α, which are known to induce the synthesis of acute phase proteins in the liver which are then released into the circulation (e.g., CRP) which consequently impairs intracellular insulin signaling (Hotamisligil 2000; Rotter et al. 2003; Taylor et al. 2013). Thus, resolution of systemic inflammation, as a result of these periodontal therapeutic strategies could lead to better control of diabetes. Future studies could determine whether the dramatic local and systemic abnormalities seen in the current experiments, which are corrected by CMC treatment, are followed by a reduction in blood glucose and HbA1c levels when the drugs are administered for a longer period of time.

Diabetes and MMPs in skin

The MMP levels, assessed by gelatin zymography, were studied in the skin tissue from the normal, vehicle-treated diabetic, and CMC2.24-treated diabetic rats. It can be seen that inducing diabetes dramatically increased both the latent pro-forms of MMP-2, ie., 72kDa gelatinase, as well as increasing the multiple lower molecular weight activated forms of MMP-2. This experimental disease also increased the ratio of lower molecular weight activated forms of MMP-2 relative to the higher molecular weight inactivated forms indicating that diabetes, by mechanisms not yet known, can accelerate the conversion of pro-MMPs into their activated forms. When the diabetic rats were treated by oral gavage with CMC2.24, in spite of no effect on the severity of hyperglycemia, the pro-MMP-2 and the activated forms of MMP-2 in the skin were all reduced to near-normal levels. Moreover, CMC 2.24 therapy reduced the ratio of active MMP-2 to pro MMP-2. In contrast, CMC 2.23 did not reduce this ratio. These effects of diabetes and CMC 2.24 administration explain, at least in part, the loss or decrease in newly-synthesized, salt-soluble collagen in the dermis of the diabetic rats, because the MMPs preferentially degrade this poorly-cross-linked fraction rather than attacking the older insoluble, highly cross-linked collagen (Lien et al. 1984; Schneir et al. 1984b), whereas inhibition of MMPs by CMC 2.24 returned the salt-soluble collagen levels back to normal. Of interest, although diabetes appears to mimic the changes that occur in skin collagen with aging, ie., decreased collagen solubility/increased collagen crosslinking, CMC 2.24 treatment produces a younger profile of collagen in the diabetic rat skin (Figure 13) which is characterized by a higher proportion of the salt-soluble collagen and a lower percentage of cross-linked insoluble collagen. A similar pattern of change in MMPs can be seen in other collagen-rich tissues, such as the gingiva from the same groups of rats, which showed that periodontal disease in the diabetics increased MMP levels whereas CMC 2.24 treatment

dramatically reduced these matrix-degrading enzymes. One of the mechanisms by which CMC 2.24 could inhibit the activation of the 72kDa, pro-MMP-2, into the smaller molecular weight activated forms of MMP-2, is to directly block the activity of MMP-14 or MT_1-MMP which was seen previously by Zhang et al. (2012a). In fact, they demonstrated that CMC 2.24 is a much more potent inhibitor of MMP-14 in vitro than the zinc-chelator 1, 10-phenanthroline, or curcumin, or other CMCs. Also MMP-14 has been shown to activate pro-MMP-2 (Liu et al. 1999). Perhaps because CMC 2.24 can inhibit MT_1-MMP, there is a reduction in the activation of pro-MMP-2 which brings the ratio of the activated to the pro-MMP-2 back to normal (Figure 17c). Additional mechanisms such as inhibition of serine proteinase activation of MMP-2 was discussed in the previous chapter. In gingiva and skin, diabetes increased 62 kDa gelatinase but only gingiva showed MMP-9. Therefore, 62 kDa likely reflects a lower molecular weight activated form of MMP-2, not activated MMP-9, because no MMP-9 was seen in diabetic skin, only in gingiva. Increased MMP-9 in gingiva (D+LPS) but not in skin, likely reflects PMNL/macrophage MMPs due to increased inflammatory disease in gingiva (ie., increased periodontal disease in diabetic rats).

Diabetes and aging:

The effects of excessive MMP activity, together with reduced collagen synthesis and increased crosslinking, could reflect accelerated aging of the skin (and other tissues) associated with diabetes and severe hyperglycemia. These changes are normalized, in part, by CMC 2.24 which decreases MMP-mediated degradation of the newly-synthesized, soluble collagen. However, CMC 2.24 may also be affecting collagen synthesis and crosslinking to increase the ratio of new relative to old collagen in the dermis and other collagen-rich tissues, and we are currently

studying these changes as well. In addition, when the diabetic rats were treated by oral administration of our lead compound, CMC2.24, over a 3-week time period, this compound significantly increased skin thickness by 38.1%, and increased diabetic skin collagen content, counteracting the accelerated aging of the dermis induced by the diabetic state. All of these beneficial changes occurred despite the ineffectiveness of this therapy on the severity of hyperglycemia over the short-term duration (4 weeks) of these experiments.

For decades, it has been recognized that many of the complications of long-term diabetes, mimic signs of aging. These aging-like changes in diabetics, particularly in collagen-rich tissues, include decreased elasticity of arteries, increased fibrosis of lungs, decreased mobility of joints, increased cardiovascular and kidney disease, decreased skeletal bone density (osteoporosis) and enhanced oral bone loss including periodontal disease (Ramamurthy et al. 1973b; Ramamurthy and Golub 1983). The combined result of these changes in collagen-rich tissues is skin atrophy and the induction of a leathery-like texture of the skin together with impaired wound-healing (Rosenbloom et al. 1981; Ramamurthy et al. 1998). These aging-like changes in the dermis (and other tissues) of diabetic patients have been shown in the past to include a number of abnormalities in skin collagen structure and metabolism, listed below:

1. Decreased fibroblast activity and suppressed collagen secretion. Several mechanisms have been identified including (a) decreased type I procollagen mRNA transcription; (b) decreased activity of intracellular collagen biosynthetic enzymes, prolyl and lysyl hydroxylase; and (c) increased intracellular procollagen degradation. All of these mechanisms reduce collagen secretion into, and the proportion of new collagen in, the extracellular matrix (Ramamurthy et al. 1972; Schneir et al. 1981a, 1984b; Leung et al. 1986; Golub et al. 1996).

2. Increased collagen crosslinking and polymerization in the extracellular matrix. This occurs in the dermis (and other tissues, such as gingiva, bone, and tendons) during both diabetes and aging. Mechanisms involved in this accelerated aging of the connective tissue matrix include increased enzymatically-mediated (lysyl oxidase) crosslink formation and non-enzymatic glycation, the latter due to prolonged exposure of the collagen to elevated glucose levels (Garnero 2012). Diabetes appears to increase the percentage of newly synthesized collagen molecules that undergo oxidative deamination of ϵ- amino groups resulting in increased crosslink formation and decreased collagen solubility; a possible mechanism includes diabetes-enhanced activity of the enzyme, lysyl oxidase (Golub et al. 1978b; Ramamurthy et al. 1983; Schmidt et al. 1996). This could produce a skin with a leathery-like texture, as mentioned above.

3. Increased levels and activity of collagen-destructive proteolytic enzymes, the matrix metalloproteinases or MMPs, including the collagenases and gelatinases (Ramamurthy et al. 1973a; Yu et al. 1993; Ryan et al. 2001). These MMPs preferentially degrade the salt-soluble, newly-synthesized poorly-cross-linked collagen in the dermis (and other tissues) which results in a "pile-up" of insoluble older, highly-cross-linked collagen which resists turnover (Ramamurthy and Golub 1978; Schneir et al. 1981b, 1984a, 1984b; Lien et al. 1984). The net result of all of these intracellular and extracellular alterations in dermal collagen is skin atrophy, including wrinkling of the skin and bruising (defective type IV collagen of vascular basement membranes) and impaired wound healing (Ramamurthy et al. 1972). Thus, it is intriguing to note that many of these collagen abnormalities seen in the current study in diabetic rats were "normalized" by CMC 2.24 administration, again in the absence of any effect on the severe hyperglycemia.

Diabetes and bone loss

The repeatedly-discussed increase in MMP levels/activity directly correlates with alveolar bone loss, which is an essential characteristic of periodontal disease. Untreated diabetic rats exhibited a statistically significant 59.9% increase in the loss of alveolar bone height as measured from the CEJ to the alveolar crest. Treatment with CMC 2.24 significantly reduced this loss of alveolar bone. Others have reported that doxycycline, another MMP inhibitor, also inhibits alveolar bone loss in diabetic rats in vivo (Chang et al. 1994). Regarding systemic bone loss and diabetes-induced osteoporosis, and based on morphometric analysis of the cortical bone using a standardized X-ray technique which measured the optical density at a defined AOI, diabetes appeared to significantly decrease bone density in the tibia. Therefore, the effects of local (LPS) and systemic (diabetes) disease, and treatment with CMC 2.24 on alveolar bone in the periodontium, does not seem to reflect an effect on the oral microflora, since similar changes occur systemically in long bones which are not exposed to microbial insult. All of the CMCs tested appeared to improve cortical bone density. However the improvement was not statistically significant. CMC 2.24 did significantly decrease alveolar bone loss but not systemic bone loss perhaps because alveolar bone exhibits a very high turnover rate likely reflecting the fact that alveolar bone exhibits a higher trabecular/cortical ratio than long bones (Vignery and Baron 1980). The presence of osteoporosis in the tibia in diabetic rats may reflect accelerated collagen breakdown due in part to increased tissue collagenase levels (Ramamurthy et al. 1972, 1973b). In addition a deficiency of insulin appears to inhibit collagen synthesis (Brown and Liddy 1970). Therefore, a reduced rate of bone collagen synthesis may also contribute to the development of osteoporosis and periodontal bone loss. In this regard, diabetes has been shown to impair the production of bone matrix components by osteoblasts resulting in osteopenia (Sakai et al. 1990). This is consistent with our recent data that shows that diabetes decreases bone density and

osteocalcin levels which reflects suppressed osteoblastic activity (Elburki et al. 2011). We have also used quantitative radiography to assess changes in bone density due to both diabetes and treatment with CMC 2.24. An aluminum step-wedge of different thickness is widely used in such radiographic studies to measure bone optical density (OD) in terms of mm aluminum or radiographic bone aluminum equivalents (Kolbeck et al. 1999). OD is measured at areas of interest determined by the investigator. Bone OD has been shown to be a reliable method for bone mineral density (BMD) assessment when compared to Dual-energy X-ray absorptiometry (DEXA; is the "gold standard" for assessment of osteoporosis in humans), and found to be useful in measuring bone strength (Nielsen et al. 2007) as well as bone density changes that result from rheumatoid arthritis and osteoporosis in humans (Glüer et al. 1994; Nagamine et al. 2000). Although DEXA is the most common method used to measure bone mineral density (Chappard et al. 1997), it is very expensive, the equipment is not mobile and, as a result, is not often used to measure bone density in animals compared to digital radiography.

Finally, administration of CMC 2.24 demonstrated no adverse effects in these animals with both local and systemic disease. This indicates that the compound is safe in rat models at least, for this short-term time period. Because CMC 2.24 is an analog of curcumin, which is considered safe by the FDA (Grynkiewicz and Slifirski 2012), future phase I human clinical trials testing bioavailability and toxicity can be reasonably contemplated. In any case, the absence of adverse effects in the current studies, even in animals with severe illness, makes the treatment with CMC 2.24 conducive for promising future research.

Conclusion

Overall, administration of CMC 2.24 in diabetic rats with endotoxin-induced periodontitis: (1) significantly suppressed local/alveolar bone loss and also tended to reduce the severity of systemic bone loss (diabetes-associated osteoporosis), (2) attenuated the severity of local and systemic inflammation by reducing cytokine levels (IL-6, and IL-1β), and (3) reduced both pathologically-elevated levels of MMPs (MMP-2, -8, and -9) and their activation. This novel chemically-modified triketonic curcumin also increased the levels of newly-synthesized (salt-soluble) collagen and decreased the proportion of the older (insoluble) collagen in diabetic rat skin. Of interest, CMC 2.24 is the more potent inhibitory agent of MMPs in vitro and inflammatory cytokines in cell culture (Zhang et al. 2012a), compared to natural curcumin and a series of other curcumin analogs, this suggests that it has significant therapeutic potential therapeutically as an inhibitor of collagen breakdown during periodontitis and other collagen-destructive diseases. Moreover, CMC 2.24 treatment accomplished these beneficial effects in uncontrolled diabetics with severe periodontal disease even in the presence of continuing severe hyperglycemia. Although these experiments were short-term (ie., 4 weeks), it is still reasonable to speculate that in future, longer-term, studies (e.g., 3 months) CMC 2.24 administration may bring about a reduction in blood glucose and HbA1c levels, because of the reduction of the inflammatory burden in the periodontal and other tissues. Lastly, detailed pharmacokinetic and safety studies must be undertaken in two animal models before this compound can be tested in phase I human clinical trials.

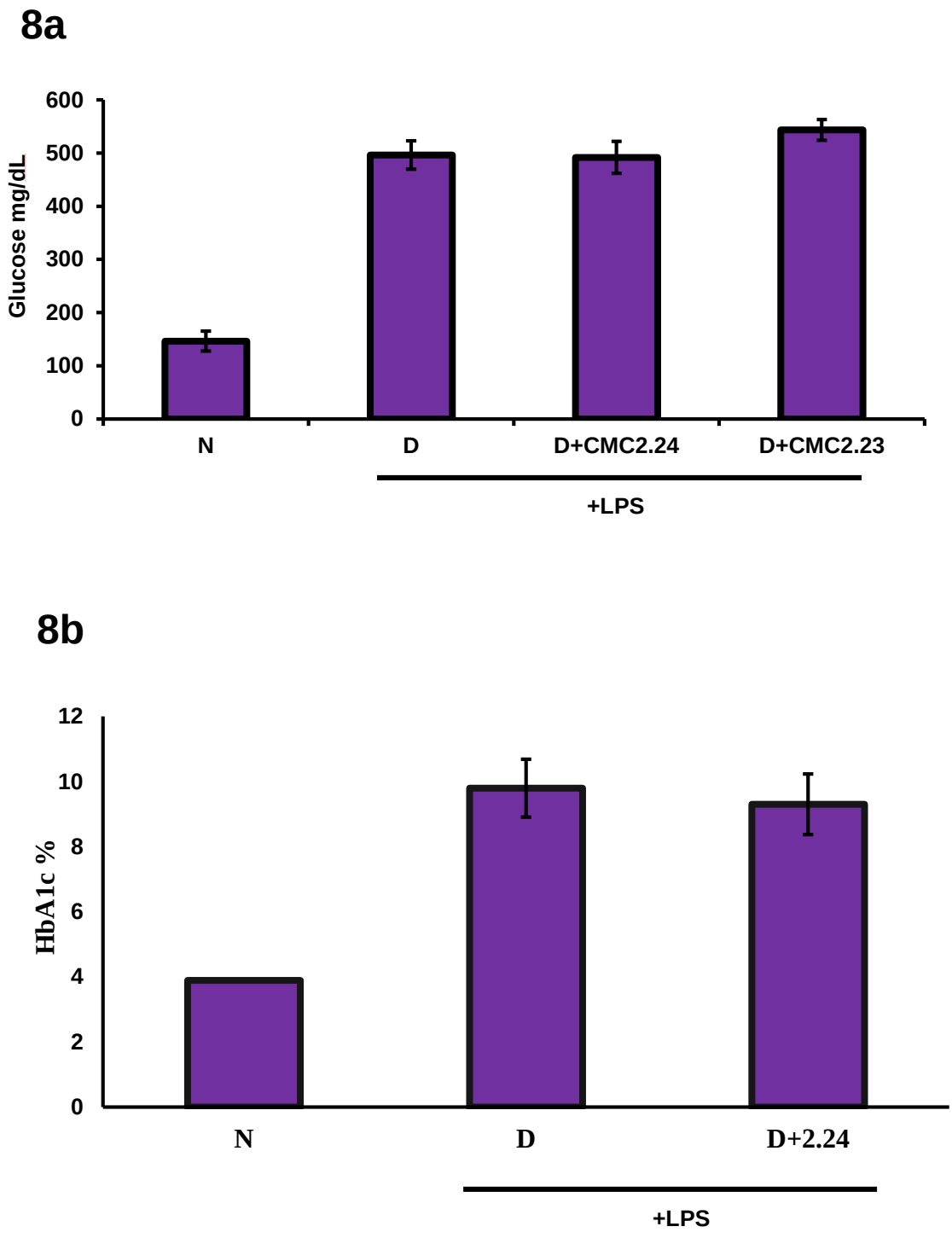


Figure 8. (a) The effect of diabetes and orally-administered CMC 2.24 and 2.23 on fasting blood glucose levels; blood samples were collected 4 weeks after STZ injection. (b) HbA1c levels in blood samples from normal, diabetic, and diabetic rats treated with CMC 2.24 four weeks after STZ injection; note, HbA1c values in the CMC 2.23 treated diabetic rats was not measured. Each value is the mean ± standard error of mean (S.E.M) of 6 rats per group.

Table 3- Diabetic Complications: Effects of CMC 2.23 and 2.24

Experimental Group (no. of rats per group)	Incidence of Adverse Events (AEs)	Description of AEs
N (n=6)	0/6	None
D+LPS (n=6)	3/6	Bleeding under toe-nails; tail necrosis; excessive tears and inflamed sclera
D+LPS plus CMC2.23 (n=6)	1/6 6/6	Tail necrosis which healed after two weeks; Mild diarrhea
D+LPS plus CMC2.24 (n=6)	0/6	None

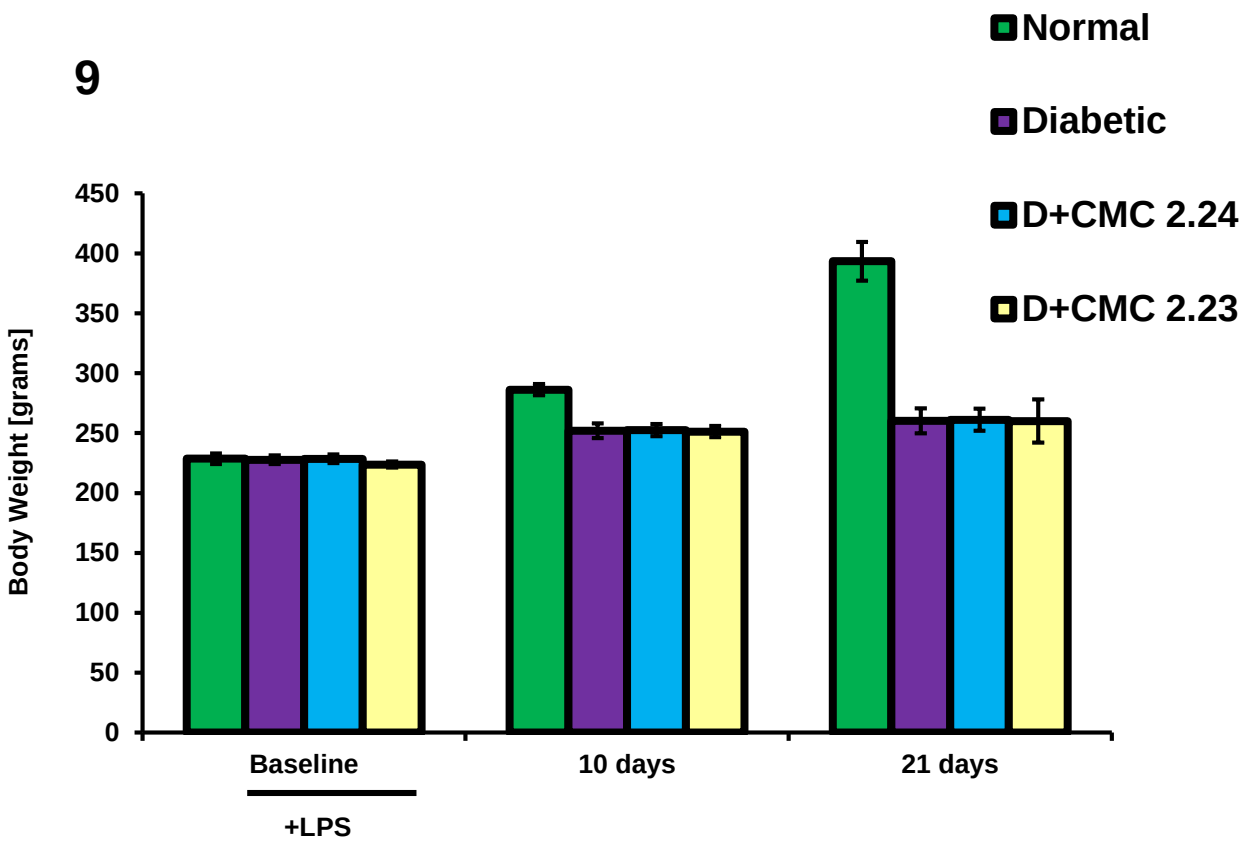


Figure 9. The effect of diabetes and orally-administered CMC 2.24 and 2.23 on body weight at base line, 10 days after starting CMC treatment, and 21 days after starting CMC treatment at the time of sacrifice. Each value is the mean ± S.E.M of 6 rats per group.

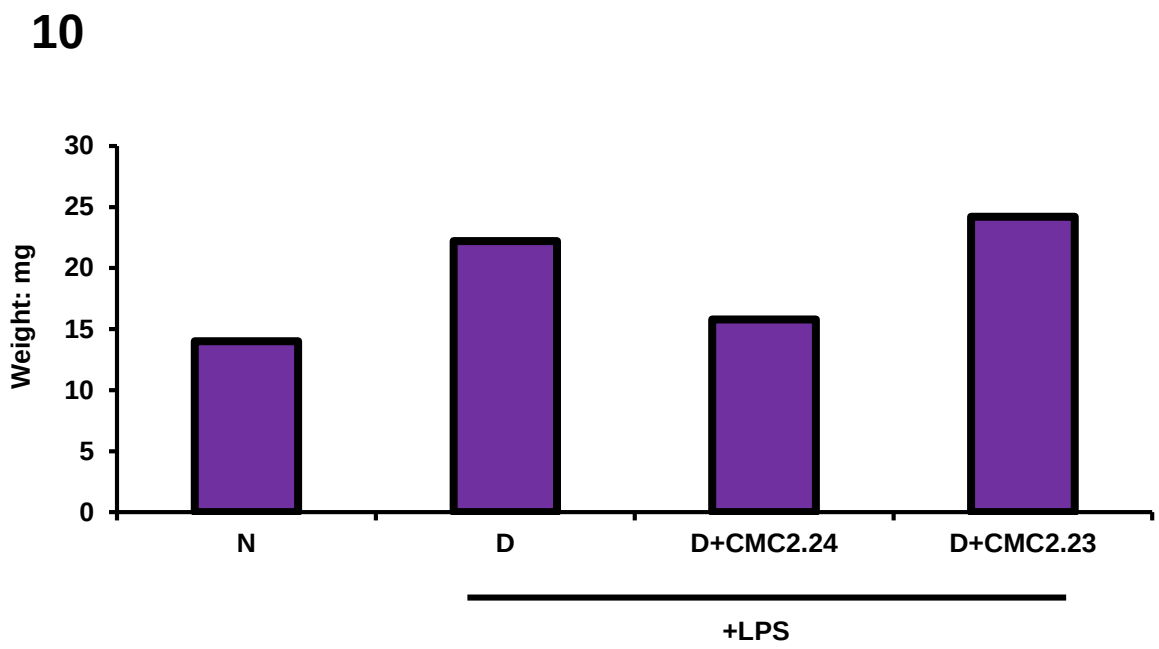


Figure 10. Total wet weight of gigiva (pool of 6 rats per group).

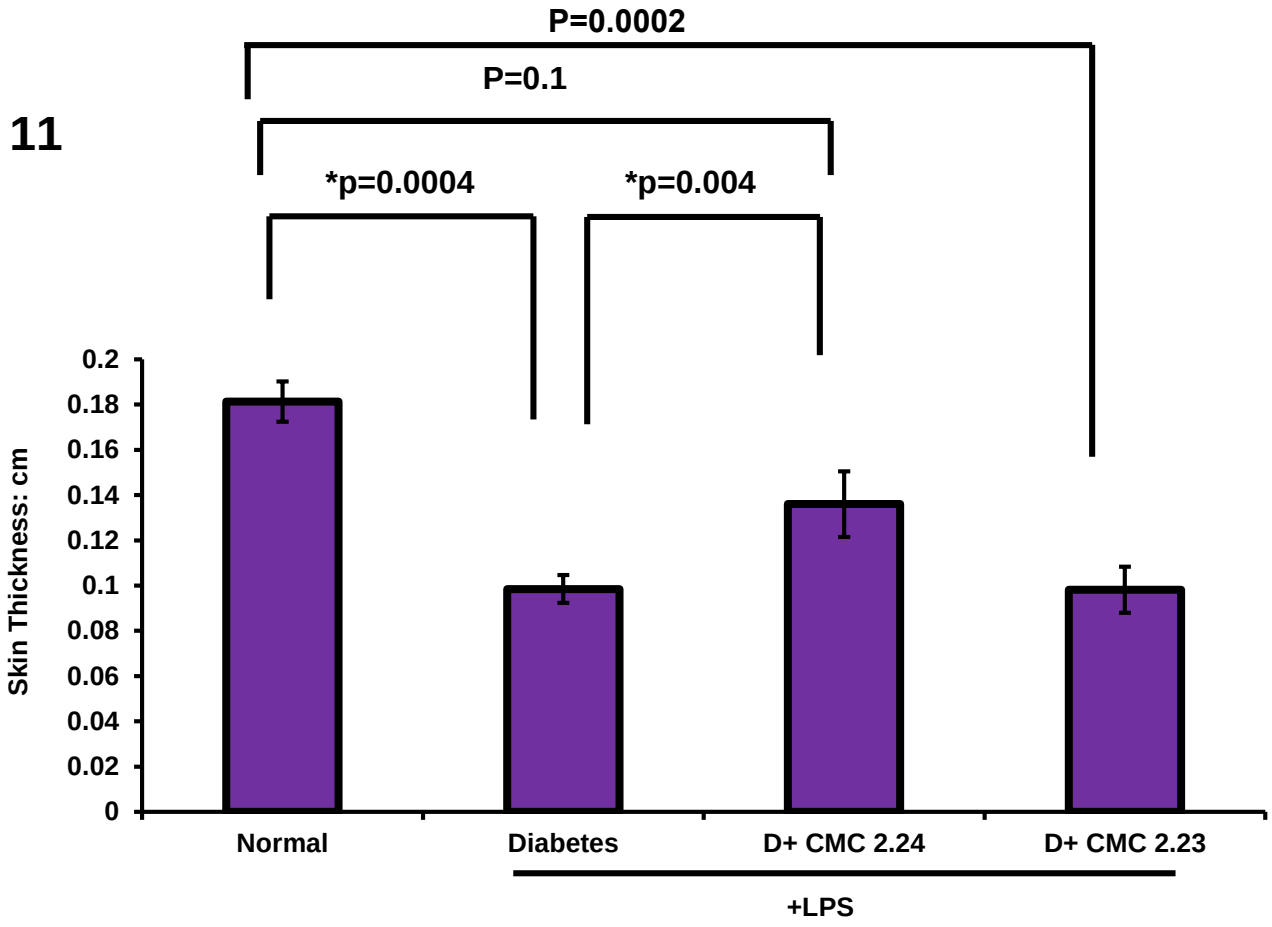


Figure 11. The effect of CMC 2.24 and CMC 2.23 administration on diabetes-induced skin atrophy (loss of skin thickness): prevention by oral administration of CMC2.24 (in spite of no effect on hyperglycemia). Each value is the mean ± S.E.M of 6 rats per group.

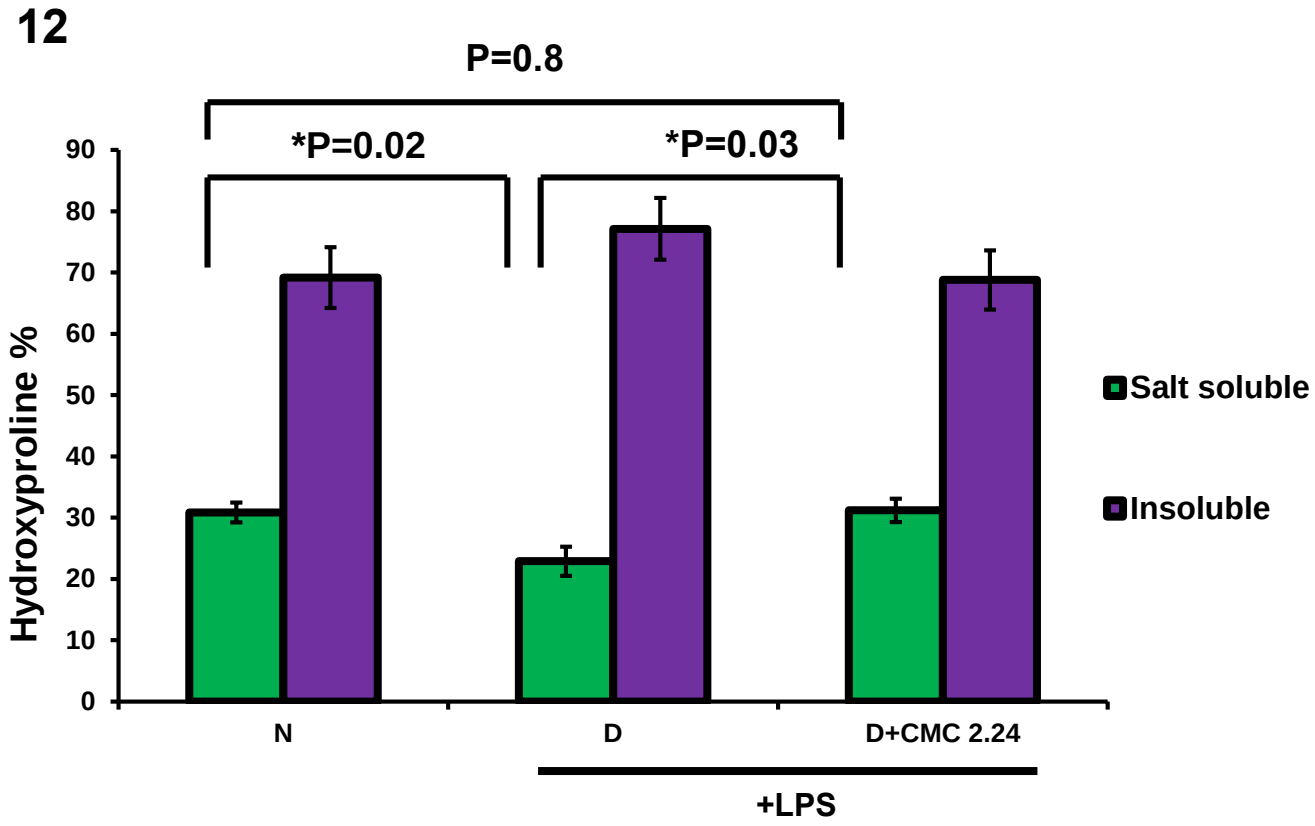


Figure 12. The effects of diabetes and CMC 2.24 treatment on the % of collagen (assessed as hydroxyproline) in skin in the salt-soluble and insoluble fractions. Each value is the mean ± S.E.M of 6 rats per group. Statistical differences between experimental conditions are indicated by the brackets and *.

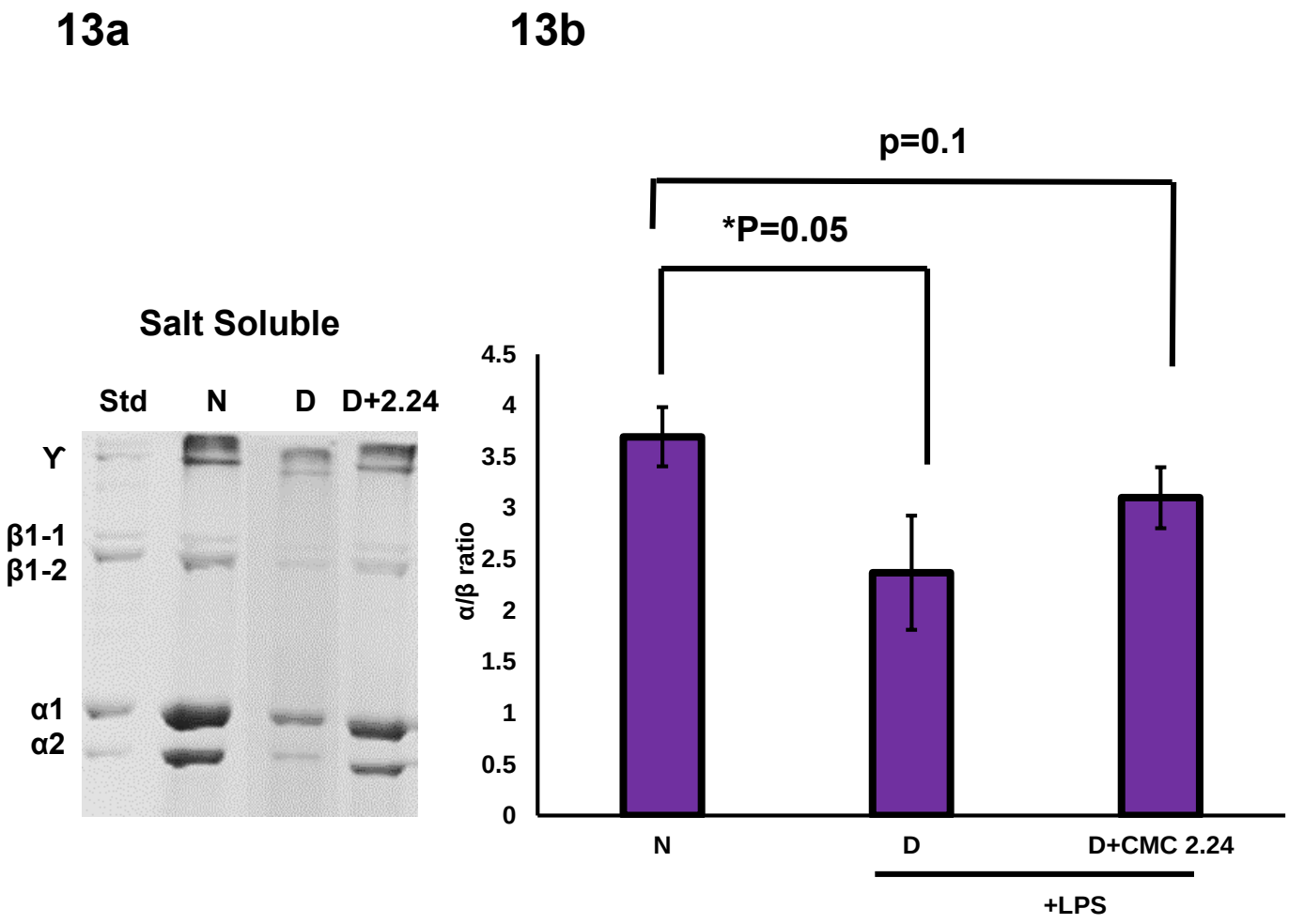
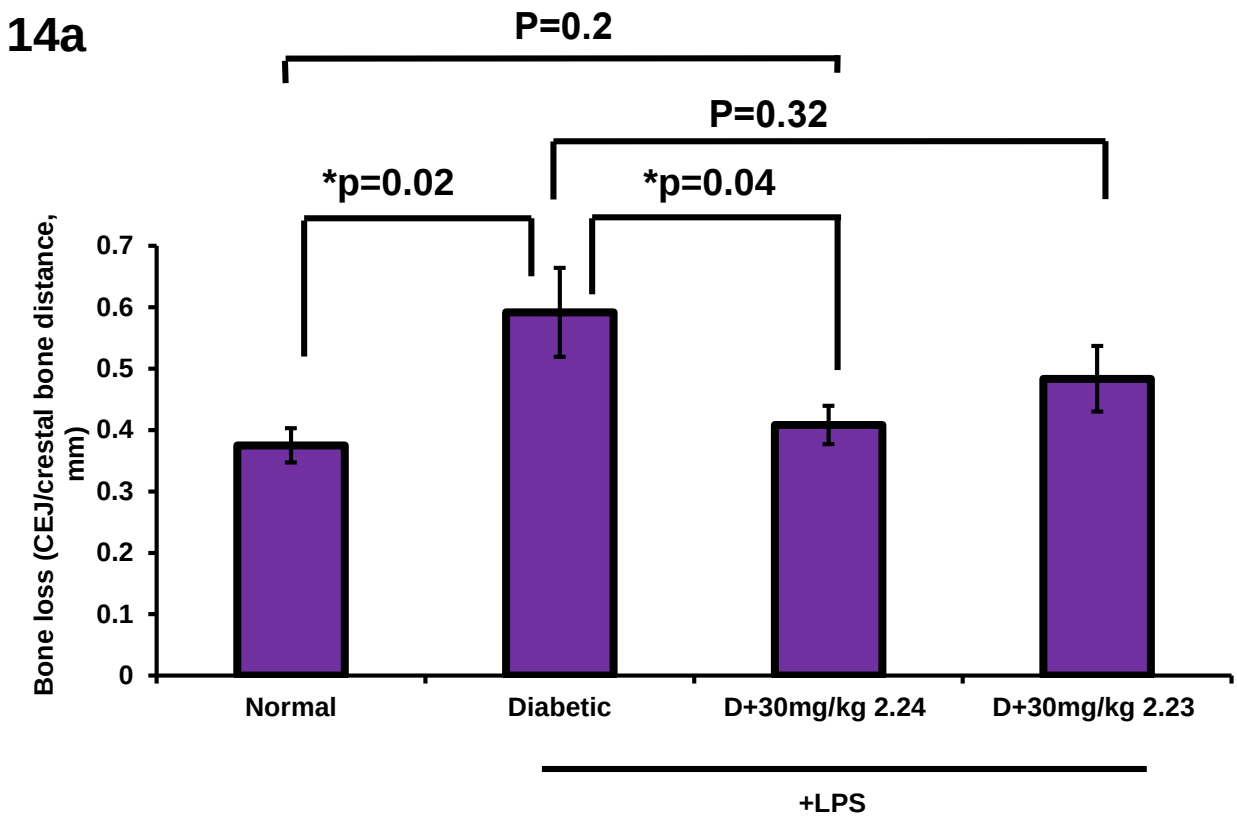


Figure 13. (a) SDS-polyacrylamide gel electrophoresis patterns of salt-soluble collagen in skin from normal, vehicle-treated diabetics, and diabetics treated with CMC 2.24. The standard type I collagen (1st lane) shows the typical α1/α2 ratios of 2:1. (b) Densitometric analysis shows that the α/β ratio in salt-soluble collagen for the three groups of rats. Each value is the mean ± S.E.M.



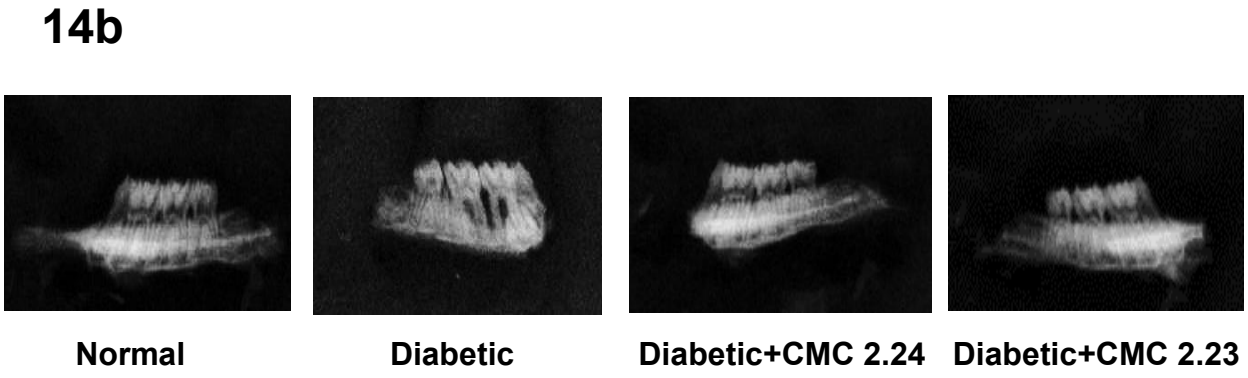
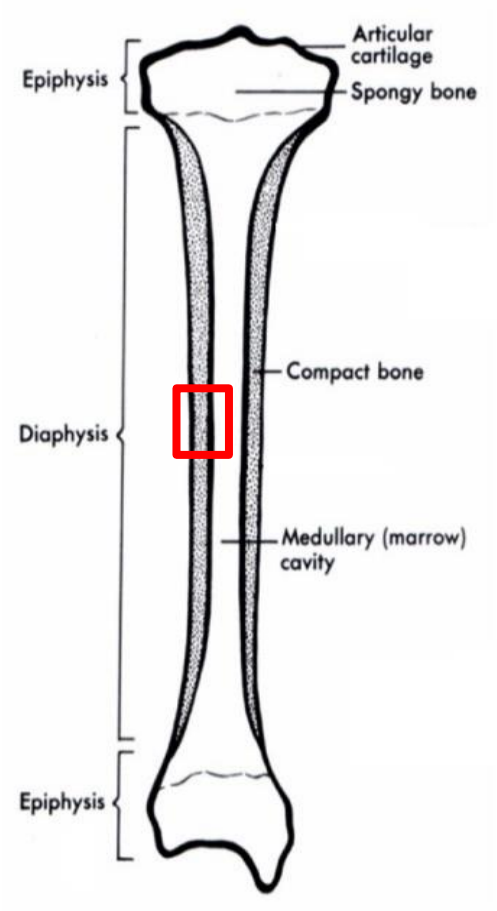
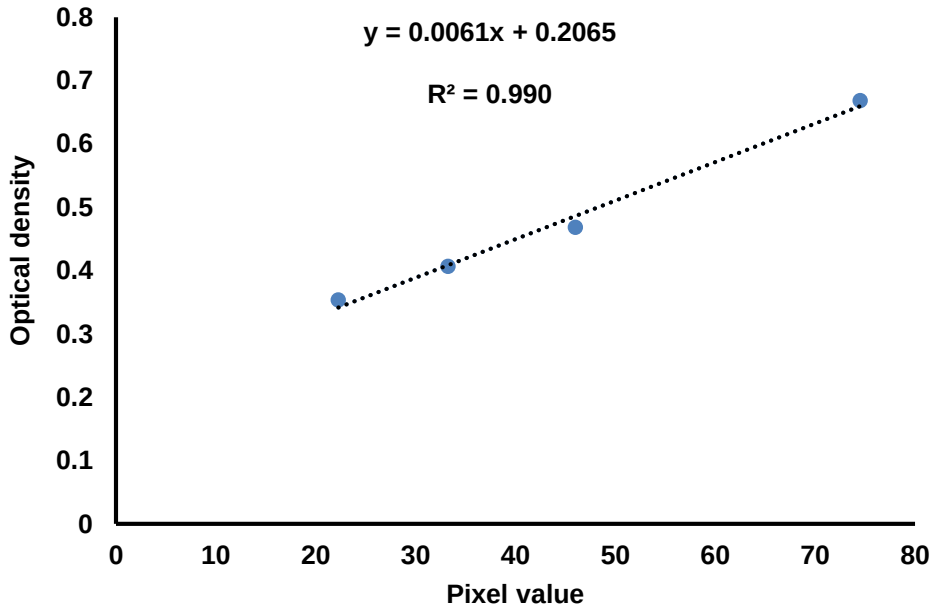


Figure 14. (a) The measurement of alveolar bone loss, from standardized radiographs of hemi maxilla (Figure 14b), from the CEJ to the crest of the alveolar bone at site # 7. Each value (mm) represents the mean of 12 jaws per experimental group ± S.E.M.

15a


Articular cartilage
Epiphysis
Spongy bone
Compact bone
Diaphysis
Medullary (marrow) cavity
Epiphysis

15b


y = 0.0061x + 0.2065
R² = 0.990
Optical density
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80
Pixel value

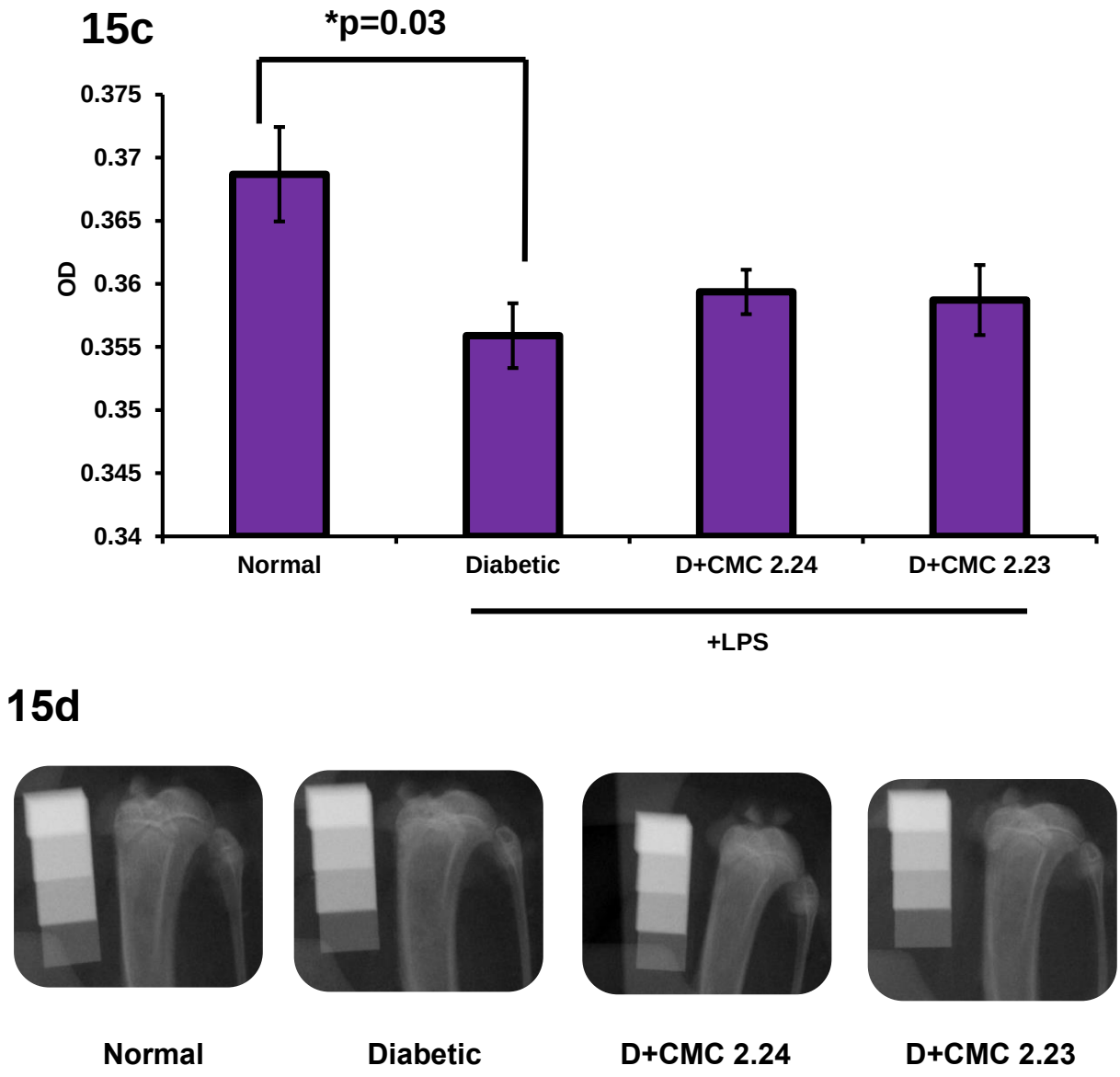


Figure 15. (a) Tibia anatomy; the red box shows area of interest (AOI) in the cortical bone. (b) Standard curve showing the positive relationship between the pixel values and the optical density of the built-in aluminum step-wedge. Each value represents the mean of 4 measurements. (c) The measurement of cortical Bone density, from standardized radiographs of tibia (Figure 15d). The bar graph presents the results of cortical bone density which was measured by converting Pixel values to density units. Each value represents the mean of 12 tibia per experimental group ± S.E.M.

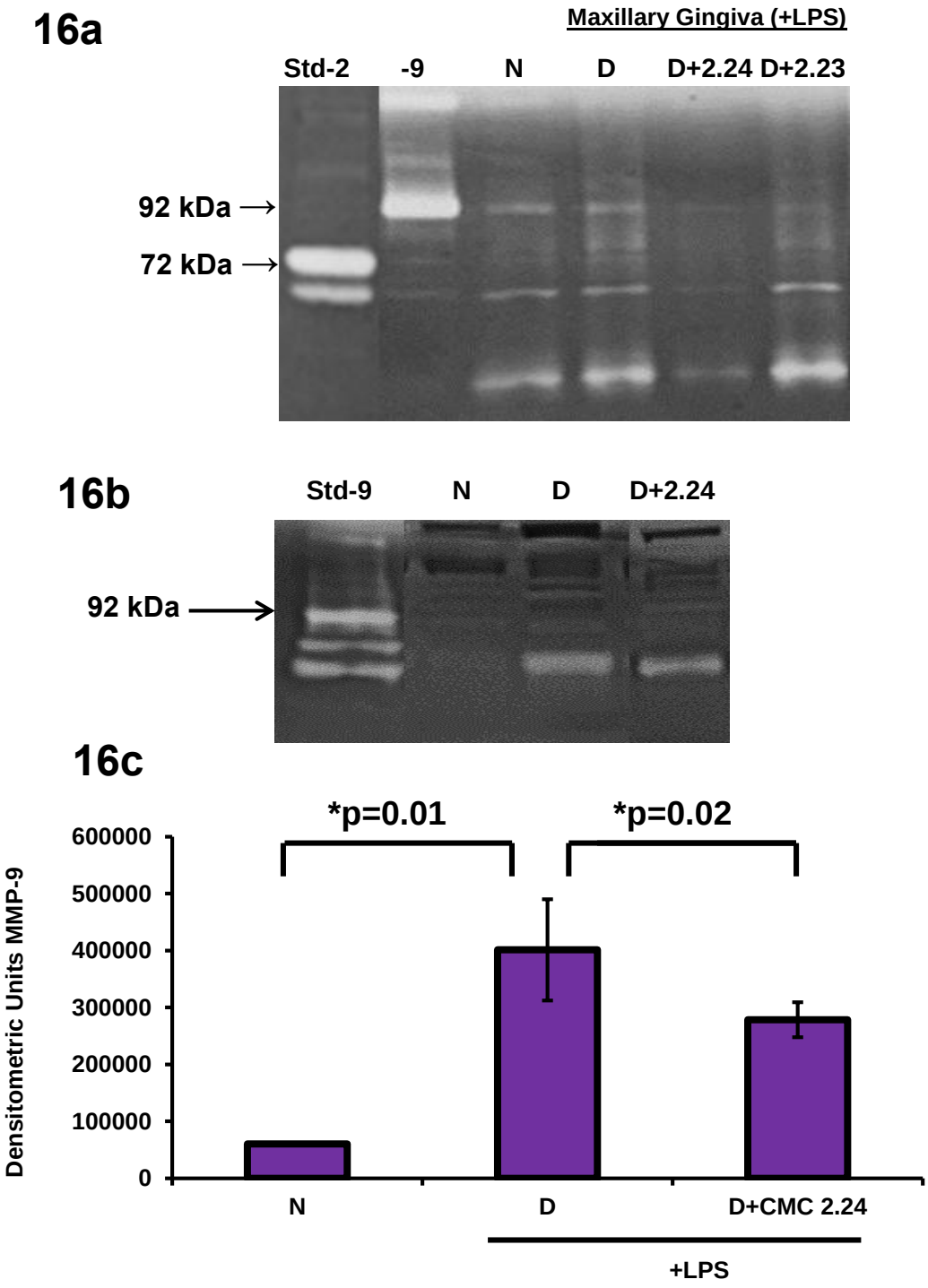
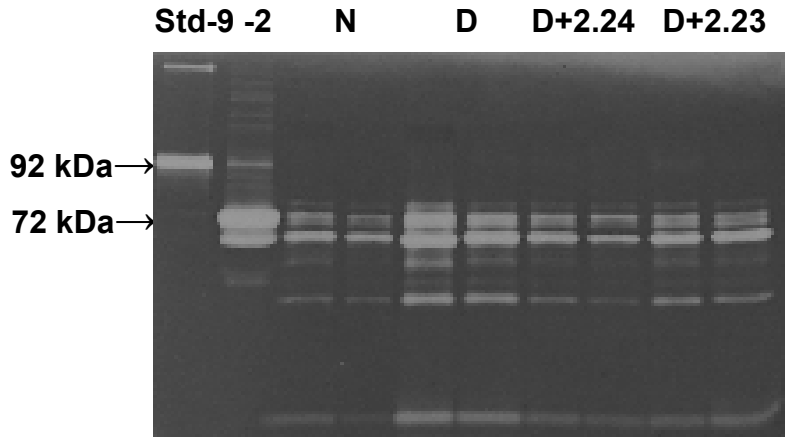
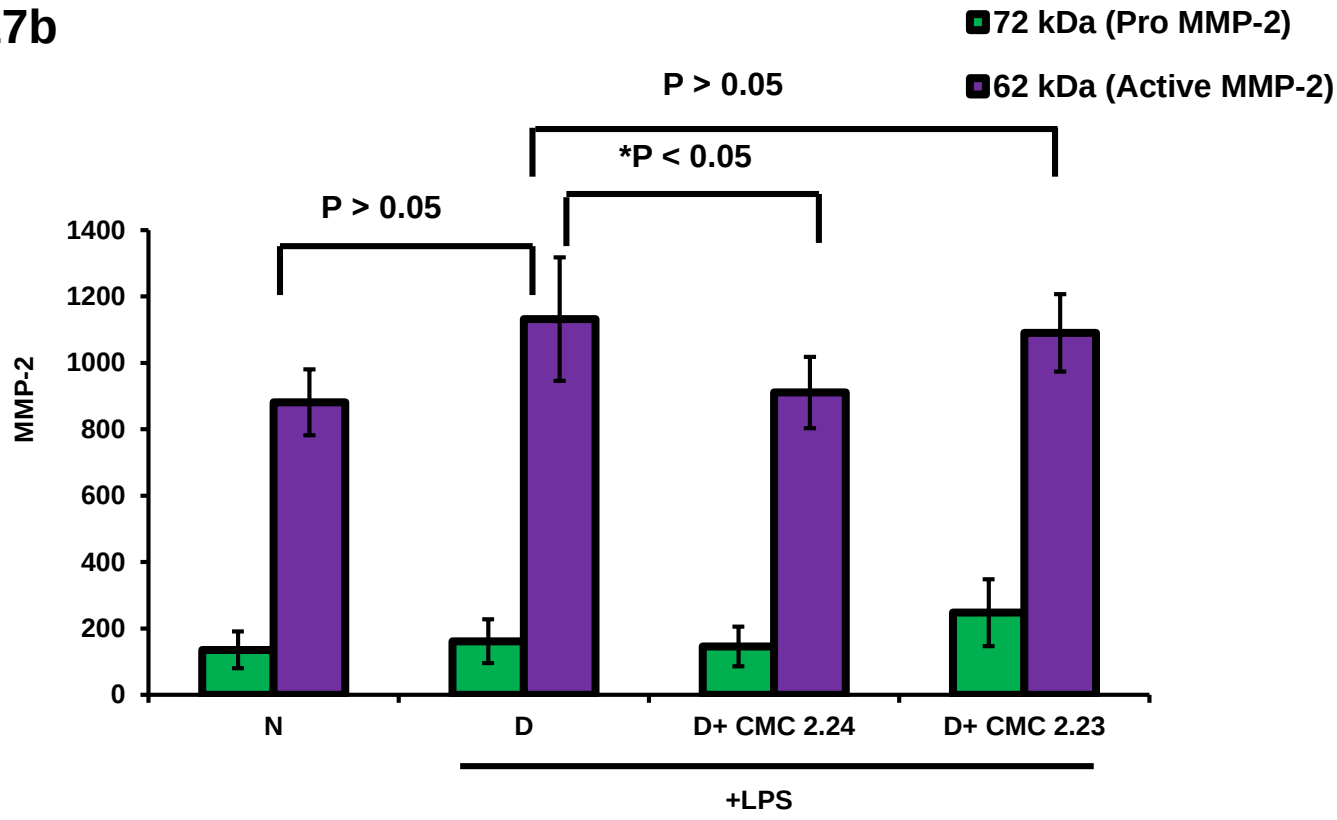


Figure 16. Gelatin zymography of partially purified extract of pooled gingiva (a) and plasma (b) from each experimental group showing the effect of orally-administered CMC 2.24 on MMPs (-2, -9). (c) Densitometric analysis of plasma MMP-9.

17a


Std-9 -2
N
D
D+2.24
D+2.23
92 kDa
72 kDa

17b


72 kDa (Pro MMP-2)
62 kDa (Active MMP-2)
P > 0.05
*P < 0.05
P > 0.05
MMP-2
1400
1200
1000
800
600
400
200
0
N
D
D+ CMC 2.24
D+ CMC 2.23
+LPS

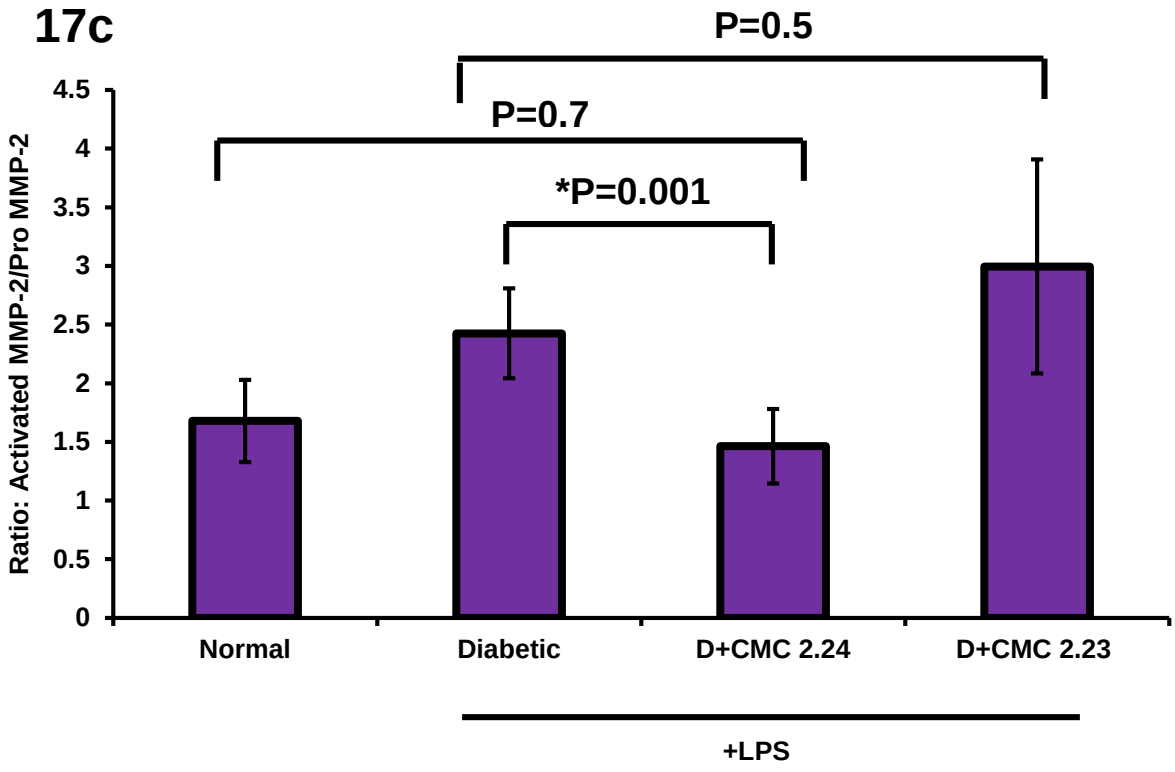
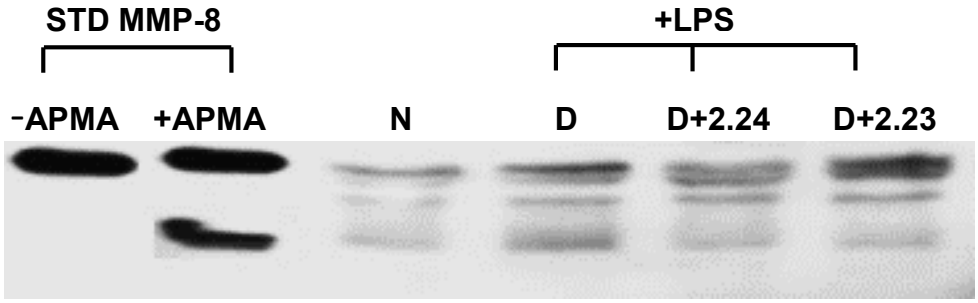
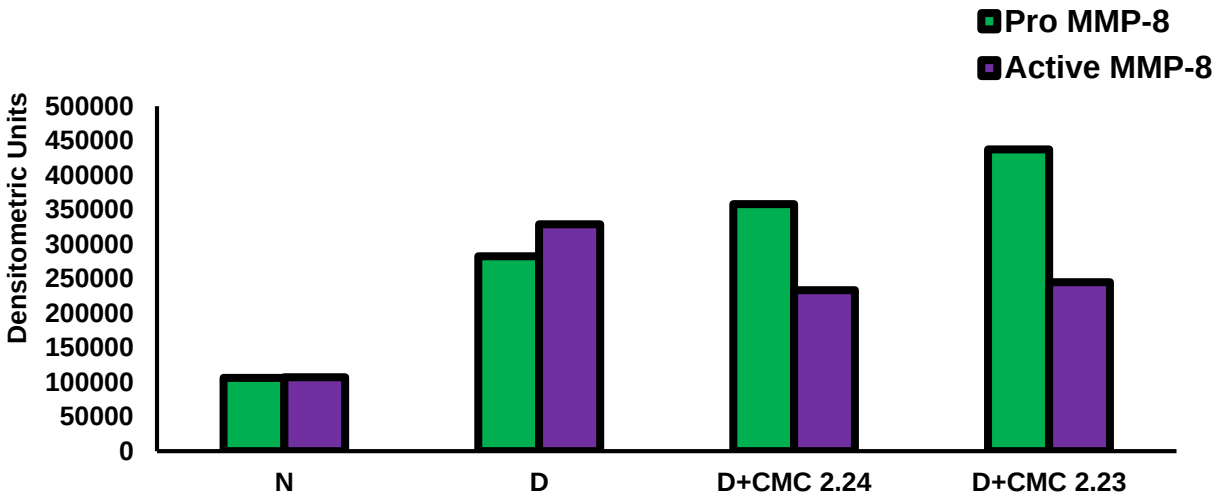


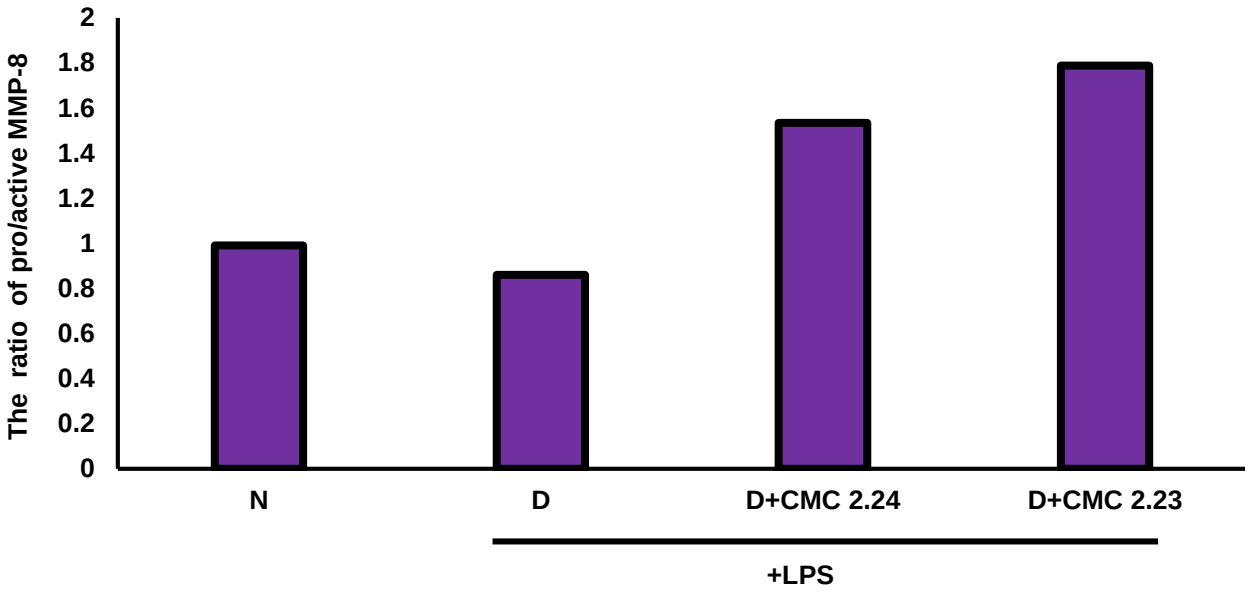
Figure 17. (a) Representative samples of gelatin zymography of partially purified extract of skin from each experimental group showing the effect of orally-administered CMC 2.24 and 2.23 on skin. Each value is the mean ± S.E.M of 6 rats per group. (b) Densitometric analysis of skin MMP-2. (c) The ratio of activated MMP2 in rat's skin.

18a


STD MMP-8
+LPS
-APMA
+APMA
N
D
D+2.24
D+2.23


Pro MMP-8
Active MMP-8
Densitometric Units
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
N
D
D+CMC 2.24
D+CMC 2.23

18b


The ratio of pro/active MMP-8
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N
D
D+CMC 2.24
D+CMC 2.23
+LPS

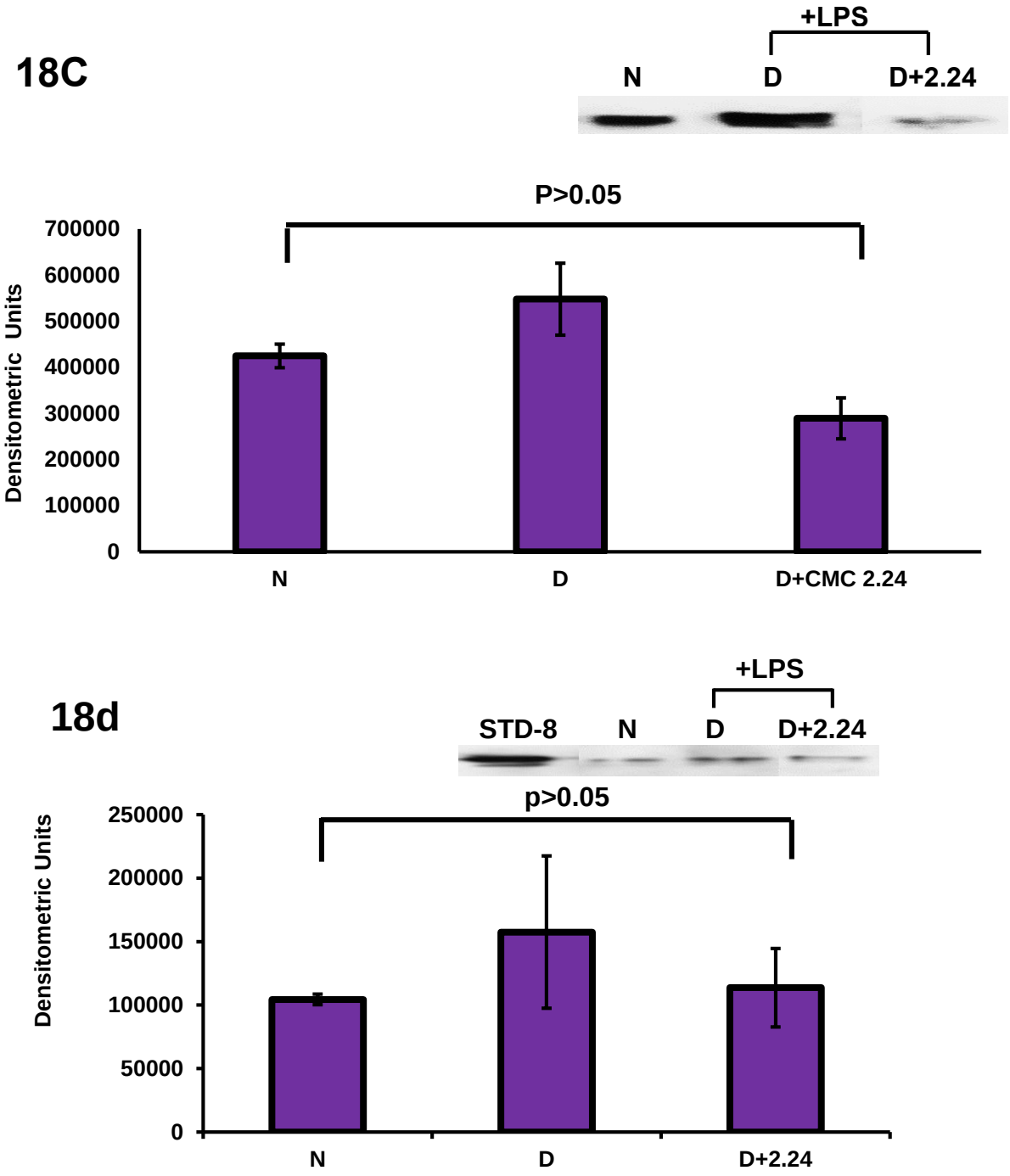


Figure 18. (a) Densitometric analysis of Western blots of MMP-8 in pooled gingiva from each experimental group; Western blots of MMP-8 in gingiva from different experimental groups and MMP-8 STD plus or minus APMA activation are shown in the insert. (b) The ratio of pro MMP-8 in rat's gingiva. (c) Densitometric analysis of Western blots of MMP-8 in plasma from each experimental group. Each value represents the mean of MMP-8 ± S.E.M; representative Western blots of MMP-8 in plasma from each experimental group are shown in the insert. (d) Densitometric analysis of Western blots of MMP-8 in skin from each experimental group. Each value represents the mean of MMP-8 ± S.E.M; representative Western blots of MMP-8 in skin from each experimental group are shown in the insert.

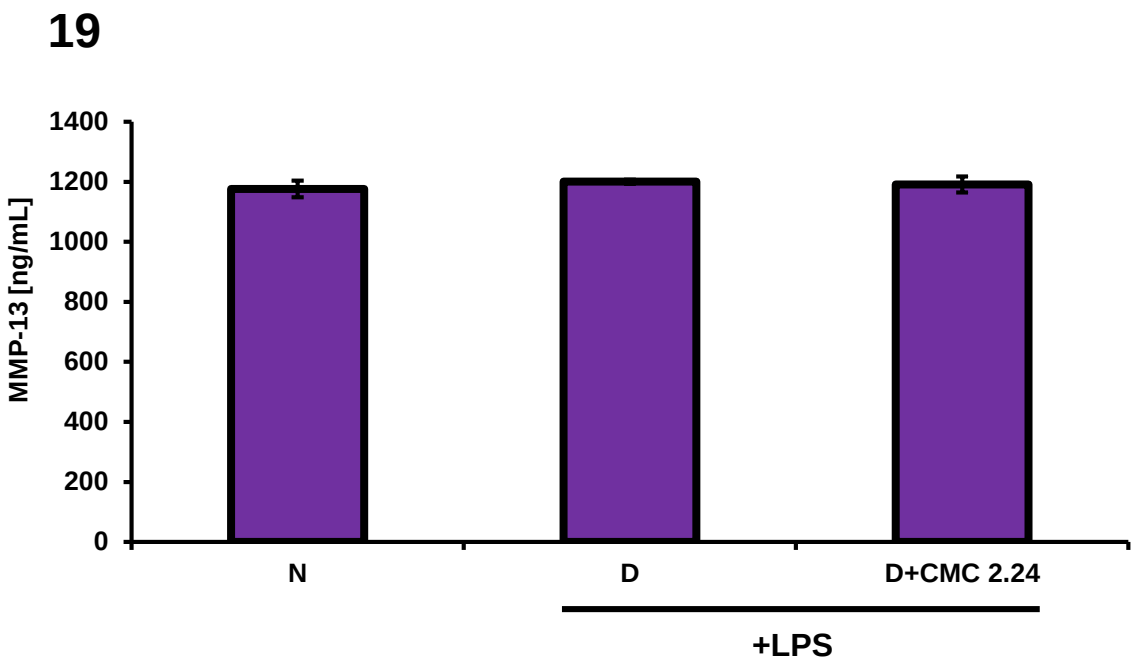


Figure 19. The effect of CMC 2.24 administration on rat MMP-13 levels in plasma from each experimental group measured by ELISA. Each value represents the mean concentration of MMP-13 ± S.E.M.

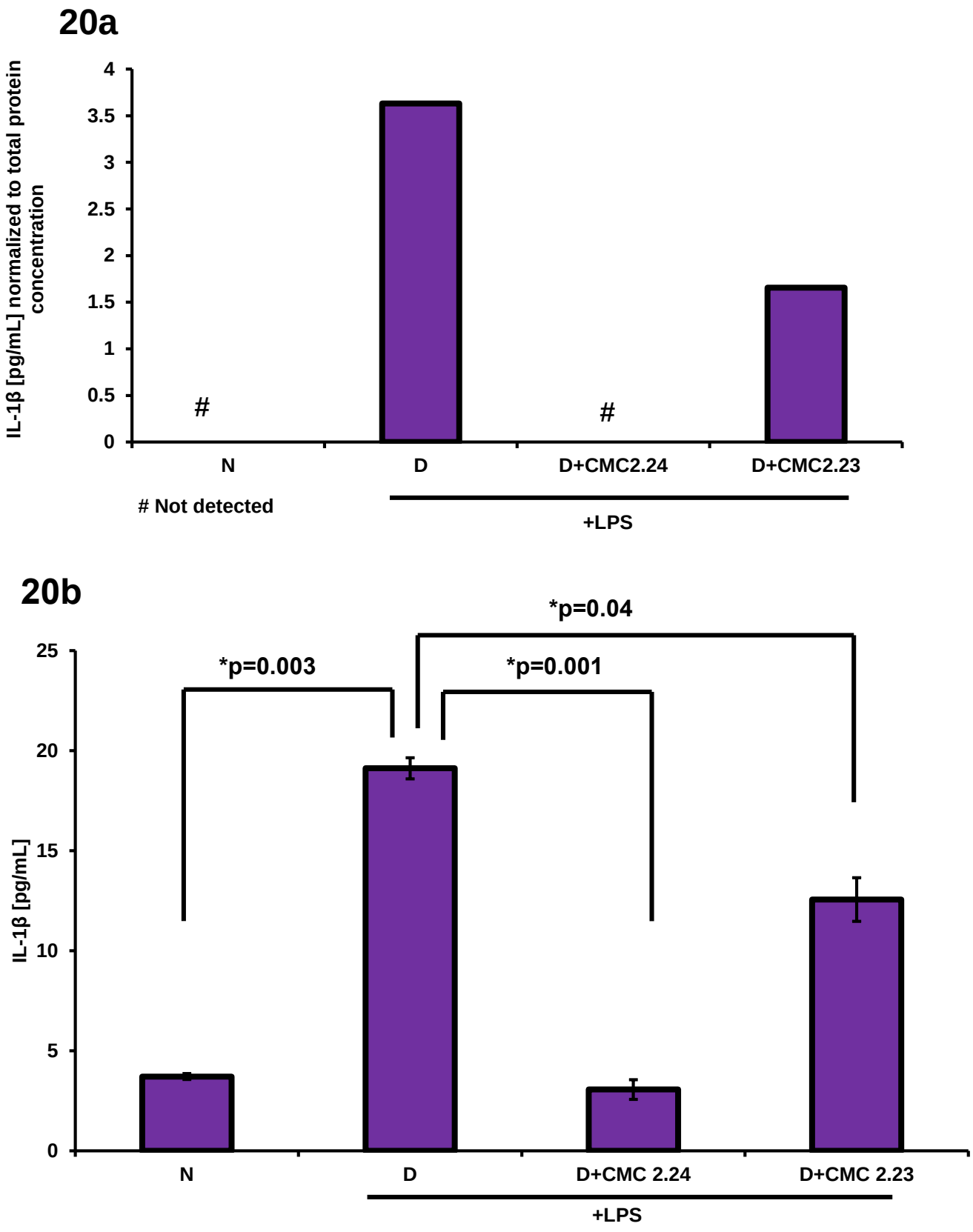


Figure 20. (a) The effect of CMC therapy on IL-1β in rat gingiva (top) and (b) serum (bottom) measured by ELISA.

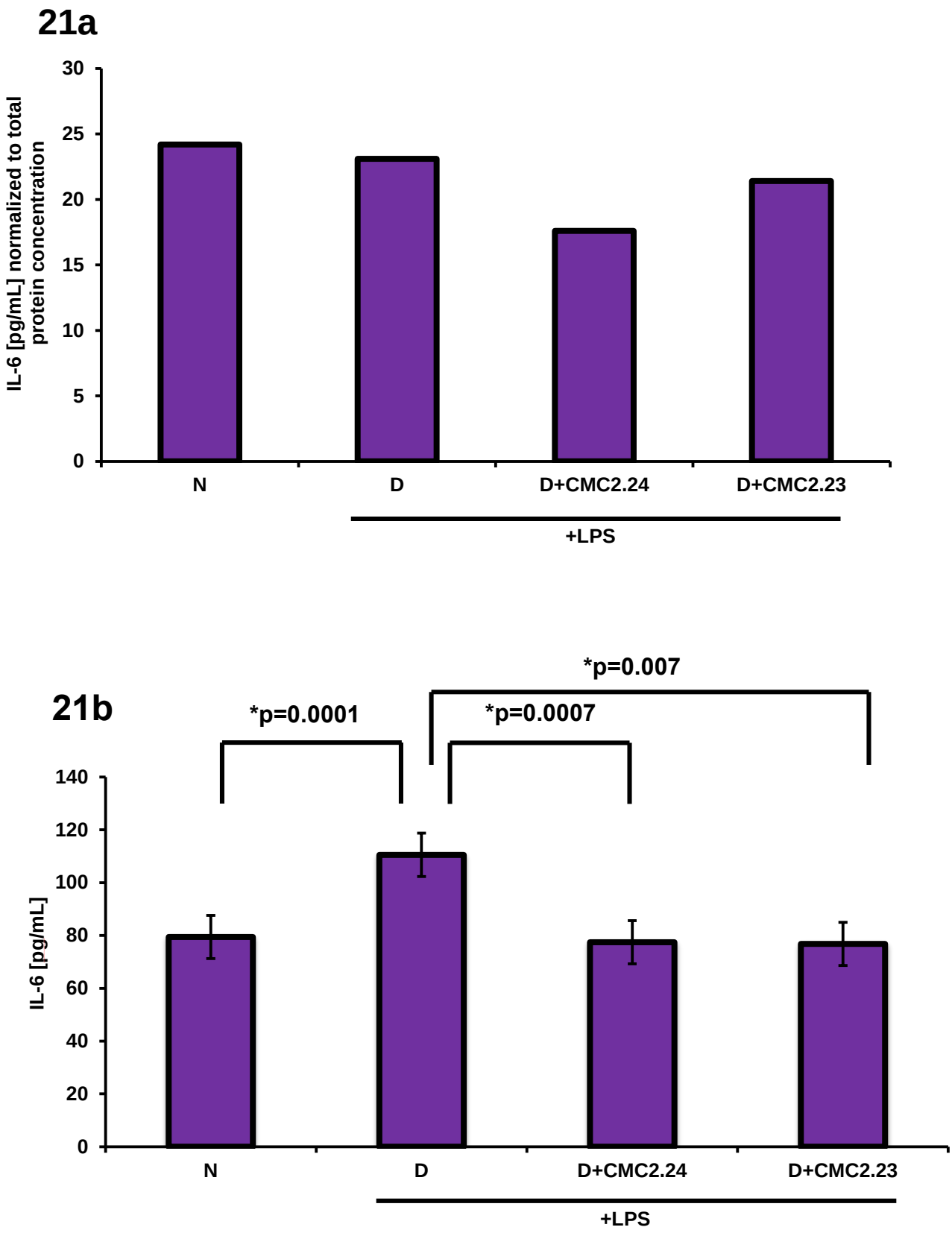


Figure 21. (a) The effect of CMC therapy on IL-6 in rat gingiva (top) and (b) serum (bottom) measured by ELISA.

Chapter 5

The Chemically Modified Curcumin (CMC 2.24) Inhibits p38 MAP Kinase and Nuclear Factor κB Activation in Locally- and Systemically-Induced Models of Experimental Periodontitis: Preliminary Results

Introduction

Periopathogenic bacteria, such as *Porphyromonas gingivalis (P. gingivalis*), generate several virulence factors, such as LPS, that can initiate the innate immune response in the host. This leads to the production of inflammatory mediators and subsequently to connective tissue breakdown including alveolar bone loss (Kirkwood et al. 2007). As a result, fibroblasts, monocytes, and macrophages within the periodontium, produce elevated levels of inflammatory mediators, such as IL-1β, IL-6, and TNF-α (Lee et al. 1995; Tsai et al. 1995; Leng and Elias 1997). These mediators upregulate the production and activation of MMPs, and prostanoids (Haffajee and Socransky 1994; Kirkwood et al. 2007) which promote osteoclast-mediated bone resorption via acid production (the proton pump which dissolves the bone mineral) and proteinase activity (cathepsin K, and MMPs) which degrade the largely type I collagen bone matrix.

The elevated blood glucose levels associated with uncontrolled diabetes is also associated with activation of the innate immune response characterized by elevated levels of inflammatory mediators including TNF-α, IL-1β and IL-6 which promote an increased severity of periodontitis

(Graves and Kayal 2008). The mechanisms by which diabetes can induce periodontal disease are not fully understood, although a number of studies have proposed that the activation of the innate immune response may be triggered through multiple pathways. For example, the long-term exposure to high glucose levels results in the non-enzymatic glycation of protein and the formation of advanced glycation end products (AGEs) (Baynes 1991; Schmidt et al. 1996). The reaction between AGEs and their receptor RAGE stimulates the production of reactive oxygen species such as superoxide anion, hypochlorous acid and others (which are believed to be the main activators of neutrophil derived pro-MMPs ie., pro-MMP-8 and pro-MMP-9) and triggers inflammatory signaling cascades, indicating the great impact of this interaction in the etiology of diabetic complications including various chronic diseases such as nephropathy, CVD and others (Agati and Schmidt 2010; Srikanth et al. 2011). In addition, AGE-RAGE reaction has been invoked as a mechanism by which diabetes increases the severity of periodontitis and vice-versa (Lalla et al. 2000; Golub et al. 2006; Graves and Kayal 2008; Engebretson and Hey-Hadavi 2011). A number of mechanisms have been proposed to explain this link between diabetes and periodontal disease which were discussed previously in Chapter 4.

The production of inflammatory mediators and cytokines is typically a rigorously controlled process which is initiated by the presence of external stimuli such as bacterial LPS and endogenous molecules such as IL-1β and TNF-α that are rapidly transported through the cytoplasm and into the nucleus where gene expression occurs (Kirkwood et al. 2007). Because the cytokine network involved in diseased periodontal tissues is very complicated and might be modified by periodontal disease activity (Bickel et al. 2001), it is important to understand the signaling pathways involved in inflammation and cytokine gene expression. This may offer another strategy

for host response modulation. The rationale for this research approach is that we focused specifically on the signaling pathways, NF-κB and p38 MAPK that are known to be important for inflammatory diseases and/or for expression of cytokines that are associated with breakdown of connective and bone resorption (Epstein et al. 1997; Patil et al. 2004; Rossa Jr et al. 2005, 2006; Mbalaviele 2006; Rogers et al. 2007; Sartori et al. 2009; Guimarães et al. 2011c; de Souza et al. 2012; Guimaraes et al. 2012).

A. Mitogen activated protein kinases

Mitogen activated protein kinases (MAPKs) are intracellular protein kinases that regulate essential biological processes and cellular functions such as proliferation, differentiation, mitosis, cell survival and apoptosis (Kyriakis and Avruch 2001). MAPK pathways play a significant role in signal transduction of cytokines, growth factors, hormones, bacterial LPS and stress and play a vital role in many phases of the inflammatory immune response (Robinson and Cobb 1997). MAPKs consist of three main subfamilies of extracellular-regulated kinases (ERK-1/-2), c-Jun N-terminal activated kinases (JNK) and p38. ERK kinases are activated by growth factors and mitogens whereas JNK and p38 are activated by pro-inflammatory cytokines or stress (Patil et al. 2004). However, the interaction and cross-activation between ERK and p38 MAPKs pathways may be encountered at different levels depending on the cell type and the nature of the stimuli. All three MAPKs are expressed in periodontal disease (Garcia de Aquino et al. 2009).

The MAPK cascade is comprised of "three-tiered" protein kinases, a MAP Kinase (MAPK), a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Activation of the MAPKs leads to phosphorylation and regulation of particular gene transcription factors (Pearson et al. 2001). These MAPKs have been associated with inflammation of the periodontal tissues

(gingiva) and alveolar bone loss via the activation of inflammatory mediators (e.g., cytokines) and MMPs (Rossa Jr et al. 2006; Garcia de Aquino et al. 2009; de Souza et al. 2012). A major pathway involves LPS from Gram-negative periopathogens binding to Toll-like receptor-4 (TLR-4), also known as CD14 receptor, on the surface of macrophages (and other cells, e.g., dendritic cells, neutrophils, monocytes, fibroblasts and epithelial cells) which then activate multiple intracellular signaling cascades resulting in phosphorylation of multiple MAPKs including p38. (Hoffmann et al. 2002; Kirkwood et al. 2003; Patil et al. 2004, 2006; Rossa Jr et al. 2006). Activation of the MAPK pathway results in activation of the transcription factor NF-κB, which is important for cytokine gene expression (Garcia de Aquino et al. 2009; de Souza et al. 2012) and subsequently, osteoclastogenesis and bone loss (Lee et al. 2000; de Souza et al. 2012). This activation of MMPK/NF-κB is recognized as fundamental for chronic inflammatory diseases such as rheumatoid arthritis and chronic periodontitis (Tak and Firestein 2001). In this regard, Rossa et al. (2006) demonstrated that p38 signaling is required for maximal expression of receptor activator of nuclear factor kappa B ligand (RANKL) in bone marrow stromal cells induced by IL-1β and TNF-α in vitro. Moreover, blocking p38 signaling in bone marrow stromal cells was found to inhibit IL-1β and TNF-α induced osteoclastogenesis in vitro (Rossa Jr et al. 2006). Therefore, suppressing p38 activation, by novel therapeutic strategies might decrease inflammatory cytokine production and its damaging effects including alveolar bone loss.

B. Nuclear factor kappa B

Nuclear factor kappa B (NF-κB) regulates genes expression involved in inflammation, innate and adaptive immunity, metastasis, and the suppression of apoptosis (Xie et al. 2012). The activation of NF-κB is an important factor in hyper-inflammatory responses and inflammation-

induced injury. LPS from Gram-negative bacteria is a main inducer of inflammatory responses via the biosynthesis and release of different inflammatory mediators, which have a role in the immuno-inflammatory response, resulting in diseases such as acute respiratory distress syndrome (ARDS) (Roy et al. 2011) and septic shock (Anrather et al. 2005). NF-κB can be activated by numerous bacteria, bacterial toxins and pro-inflammatory cytokines that are released during sepsis. NF-κB is the final destination of these septic shock stimulators (Ulevitch 2004).

NF-κB is a transcription factor present in the cytosol as an inactive heterodimer which is composed mainly of p65/p50 subunits bound to the inhibitor, IκB. This inhibitor is quickly degraded in the presence of stimuli such as bacterial LPS as well as the pro-inflammatory cytokines, IL-1β and TNF-α. This results in nuclear translocation and gene transcription following the binding of the dissociated NF-κB to a specific consensus sequence in the DNA (Shishodia et al. 2005). During activation, the inhibitory subunit IkBα is phosphorylated at Ser 32 and Ser 36 residues followed by ubiquitination-dependent degradation. The kinase which phosphorylates IκBα is known as IkB kinase (IKK) and is composed of three subunits, IKKα, IKKβ and IKKɣ (also known as NEMO) (Karin and Ben-Neriah 2000).

As described previously, curcumin is a diferuloylmethane derived from a yellow spice turmeric, isolated from the dried rhizomes of Curcuma longa, is widely consumed in food-stuffs with no known ill-effects. Curcumin is also known as a pharmacologically safe agent that has been shown to inhibit NF-κB activation and NF-κB gene expression (Singh and Aggarwal 1995; Anand et al. 2007) but, as noted earlier, natural curcumin is highly insoluble, poorly bioavailable, and has to be consumed in large quantities to show a marginal effect (Dulbecco 2013). As described earlier,

our group has developed and investigated the therapeutic potential of a novel chemically-modified curcumin; CMC 2.24, to arrest periodontitis in vivo. In this chapter, because of the, importance of p38 MAPK and NF-κB signaling pathway in inflammation, cytokine expression, and inflammatory bone loss, we assess the modulation of these pathways in an in vivo rat model of periodontal disease by systemic administration of CMC 2.24. Specifically, we determined whether this compound suppresses the activation of the signaling pathways p38 MAPK and p65 NF-κB.

Materials and Methods:

I- Locally-induced model of experimental periodontitis:

The techniques described below are modifications of those described earlier in Chapter 3. In brief, 28 male Holtzman rats (*Rattus norvegicus albinus*), weighing 150-250 g, were distributed into 4 groups each containing 7 animals. Inflammatory mediated alveolar bone loss was established in two groups of rats by repeated (every second day) local injection of LPS from *E. Coli* (30 μg) into the palatal gingiva on both sides of the maxilla, for 3 weeks; oral administration of CMC 2.24 (30 mg/kg) was initiated at the same time that LPS injections were started, however, CMC 2.24 was administered daily for 4 weeks, including 1 week after LPS injection stopped. The other LPS injected group received vehicle alone. The other two groups received injections of the same volume of PBS vehicle (no LPS) one of these groups received CMC 2.24 (30 mg/kg) suspended in 2% carboxymethyl cellulose and the other group received vehicle (1 mL of suspension of 2% carboxymethyl cellulose) alone. At the end of the experimental protocol, the animals were sacrificed, maxillary jaws were collected for alveolar bone measurement and gingiva samples were collected and analyzed for MMPs and cytokines.

II- Systemically-induced model of experimental periodontitis:

The techniques described below are also modifications of those described earlier (see Chapter 4). In brief, 12 adult male Sprague-Dawley rats (body weight 225-350 g) were divided into three groups (n=4). Two groups were rendered Type I diabetic (D) via intravenous tail injection of streptozotocin (70 mg/kg body weight). The third group was injected through the tail vein with 10mM citrated saline buffer pH4.5 (non-diabetic controls; N). One week after inducing diabetes, one of the two diabetic groups was administered CMC 2.24 (30 mg/kg) daily by oral gavage for a period of 3 weeks. The non-diabetic controls and the (other) diabetic rats were administered vehicle alone. At the end of the three weeks, the animals were sacrificed and blood was collected through cardiac puncture for blood glucose measurements. Also at the time of sacrifice, the maxillary jaws were collected for alveolar bone measurements and gingiva samples were collected and analyzed for MMPs and cytokines.

Radiographic/morphometric analysis of alveolar bone loss:

Following sacrifice, alveolar bone loss was measured morphometrically from radiographs as described previously in Chapter 4.

Gingival tissue extract and its partial purification for analyses of MMPs:

The gingival tissues were extracted and the MMPs partially purified as described previously in Chapter 3.

Gelatin zymography for measurement of MMP-2 and MMP-9:

MMP-2 and MMP-9, in the pooled gingival extracts from each of the experimental groups, were determined by gelatin zymography as described previously in Chapter 3.

ELISA for measurement of gingival tissue levels of cytokines:

Three inflammatory cytokines (IL-1β, IL-6, and TNF-α) and the anti-inflammatory cytokine, IL-10, were measured in gingival tissue extracts by standard ELISA techniques as described previously in Chapter 3.

Western blotting analysis of activated/phosphorylated NF-κB p65 and MAP kinase p38 expression levels:

Western immunoblotting was performed as described previously (see Chapter 3). Briefly, the gingival tissues from the hemi-maxilla of each rat were excised and pooled for each group (7 rats per group for experiment I and 4 rats per group for experiment II). Total protein was isolated from the gingival tissues using the detergent-based extraction buffer (Tissue Protein Extraction Reagent [T-PER]; Pierce Biotechnology; Thermo Fischer Scientific, Rockford, IL, USA) containing protease- and phosphatase-inhibitor cocktails (Complete and Phos-Stop, Roche), as per manufacturer's instructions (Pierce Biotechnology). The tissue samples were homogenized in the buffer (50 µL/mg of tissue) and centrifuged for 5 min at 16,000 g at 4°C. After centrifugation the supernatant was collected and analyzed for total protein using the Bio-Rad Protein Assay. Protein samples (60 µg) were mixed with 2x SDS sample buffer containing mercaptoethanol as a reducing agent and heat-denatured at 100°C for 5 min, then separated by gel electrophoresis using 5% to 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) and transferred to PVDF membranes (Amersham Pharmacia Biotech Inc, Piscataway, NJ) at 100 V for 2 hours. The membranes were blocked with nonfat dry milk (5%) in TBST (Tris-HCl Buffer SalineTween-20) for 2 hours at room temperature. Each membrane was then immersed three times for 5 min in TBST and incubated with the primary antibodies overnight at 4°C (1:1000 dilution in TBST; phospho-p65 and phospho-

p38; Cell Signaling Co., Danvers, USA). Detection of the primary antibodies was done with secondary antibodies conjugated to horseradish peroxidase for 2 hours at room temperature (1:5000 dilution in TBST; Cell Signaling Co., Danvers, USA). Membranes were then washed three times in TBST (Guimarães et al. 2011; Guimaraes et al. 2012). Detection of the bands was carried out as described earlier (Elburki et al. 2014, Chapter 3). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference for quantitative analysis.

Statistical analysis:

Data for bone loss were assessed radiographically and are presented as the mean ± standard error of the mean (SEM), except for the gingiva because of the need to pool the tissue (the quantities are too small to be analyzed for individual rats). The data for the gingival tissue represents pooled tissue for each group of 7 rats/group (experiment I), or groups of 4 rats/group (experiment II). We used the method described by Bildt et al. (2008), who found that, pooled tissue was necessary even in the case of humans (Bildt et al. 2008). Statistical analyses were performed with Excel statistical software. Differences amongst groups were evaluated using analysis of variance (ANOVA) or by paired students T-test, when appropriate. The unpaired t-test was used for independent samples with Welch's correction for unequal variances. p-values of less than 0.05 were considered significant.

Results

Blood glucose measurements:

As shown in Figure 22, both groups of diabetic rats (including vehicle-only treated diabetics plus those diabetics rats treated with CMC 2.24) exhibited significantly elevated blood glucose levels

compared to the non-diabetic controls rat, and CMC 2.24 treatment had no detectable effect on the severity of hyperglycemia at least in the relatively short duration (3 weeks) of the treatment. However, despite this, CMC 2.24 appeared to eliminate diabetic complications even under the condition of severe hyperglycemia (Table 4).

CMC 2.24 "normalized" locally/LPS-induced alveolar bone Loss

Based on radiographic-morphometric measurement of height loss of alveolar bone, obtained by a standardized x-ray technique, (which measured the distance between the CEJ and the alveolar bone crest at site #7 of the maxillary quadrants), LPS injections into the gingiva significantly ($p=0.0001$) increased alveolar bone loss by 53.2% (Figure 23a). Administration of CMC 2.24 significantly ($p=0.01$) reduced this increase by 22.3% back to the normal level seen in the untreated rats. It is worth noting that there was no significant difference ($p>0.05$) between bone loss measurements in the PBS group and the experimental periodontitis group (LPS) that was treated with CMC 2.24. Also the control rats, treated with CMC 2.24, did not show any bone loss in the control rats receiving injections of PBS vehicle rather than LPS (Figure 23a).

CMC 2.24 normalized systemically-induced alveolar bone Loss

In a pattern similar to that seen in the LPS-induced periodontitis rats, and which was also based on radiographic-morphometric measurement of the reduction in height of alveolar bone (measured as the distance from alveolar bone crest to the CEJ as a fixed anatomical landmark), the systemic disease, diabetes, in the untreated rats significantly ($p=0.03$) increased alveolar bone loss by 36.3%. Administration of CMC 2.24 significantly ($p<0.05$) diminished this increase by 24.4%

(Figure 23b) back to the levels seen in the non-diabetic control rats (N vs D+CMC 2.24, p=0.7; NS). (Figure 23b).

CMC 2.24 normalizes the pathologically-elevated level of MMP-9 induced by LPS

The amounts of both MMP-2 and MMP-9 were evaluated by gelatin zymography in the pooled rat gingival tissue. LPS-induced experimental periodontitis increased only the MMP-9 levels (not MMP-2) in the pooled gingival tissue. Treatment of the rats with systemically administered CMC 2.24 lowered the pathologically-elevated levels of MMP-9 both in the LPS-injected gingiva (Figure 24a) and in the gingiva from the rats lacking LPS injections. Pro- (72 kDa) and lower molecular weight- (activated) MMP-2 were reduced only in the PBS-injected gingival tissues (Figure 24a).

CMC 2.24 normalizes the pathologically-elevated level of MMP-9 induced by diabetes

The concentrations of both MMP-2 and MMP-9 were measured by gelatin zymography in pooled gingival tissue taken from the upper jaws of rats from the N and D groups (Figure 24b). Diabetes does not appear to increase MMP-2 and 9 levels in the pooled gingival tissue and may have decreased the latter in this experiment. However, treatment of the rats with systemically-administered CMC 2.24 again appeared to decrease the gelatinase, 92 kDa pro- (MMP-9), in the gingiva of diabetic rats as assessed by gelatin zymography (Figure 24b).

CMC 2.24 inhibits LPS-induced bone resorptive cytokines (IL-1β, IL-6, and TNF-α) and increases anti-inflammatory IL-10

Analysis of IL-1β in extracts of the pooled gingival tissues show that LPS injections dramatically increase the level of IL-1β since it was not measureable in the extracts of the PBS-injected gingival tissue (Figure 25a). Moreover, CMC 2.24 administration reduces the pathologically-elevated levels of IL-1β in the gingiva by 50.6% (Figure 25a). Similarly LPS injections increase the level of TNF-α and IL-6 in the gingiva and CMC 2.24 administration reduces the pathologically-elevated levels of TNF-α and IL-6 in the gingiva by 71.6% and 48.0%, respectively (Figure 26a and 27a). Analysis of IL-10 in extracts of the pooled gingival tissues indicated that LPS injections dramatically decrease the level of this anti-inflammatory cytokine since it was not measureable in the extracts of the LPS-injected gingival tissue but was detected and measured in the extracts of PBS-injected gingiva. By contrast, CMC 2.24 increased the level of IL-10 by 100% (Figure 28a).

CMC 2.24 inhibits diabetes-induced bone resorptive cytokines (IL-1β, IL-6, and TNF-α) and increases anti-inflammatory IL-10

Analysis of IL-1β in extracts of the pooled gingival tissues showed that diabetes increases the level of IL-1β by 119.2% (Figure 25b). Furthermore, CMC 2.24 reduces the pathologically-elevated levels of IL-1β in the gingiva by 54.4% back to normal level (Figure 25b). Similarly diabetes increases the level of TNF-α and IL-6 in the gingiva and CMC 2.24 administration reduces the pathologically-elevated levels of TNF-α and IL-6 in the gingiva by 14.1% and 31.2% respectively (Figure 26b and 27b). Analysis of IL-10 in extracts of the pooled gingival tissues indicated that

although diabetes decreases the level of this anti-inflammatory cytokine, CMC 2.24 did not appear to affect IL-10 levels as a result of induction of diabetes (Figure 28b).

CMC 2.24 inhibited LPS induced activation of NF-κB

To evaluate the effect of CMC 2.24 on LPS activation of NF-κB signaling pathway, gingival tissues were analyzed for phosphorylation of p65 by Western blot analysis. As shown in Figure 29, LPS increases (modestly) the activation of NF-κB. In animals which were treated with CMC 2.24 (30 mg/kg body weight), a dramatic reduction in NF-κB phosphorylation occurred in both the PBS- and the LPS-injected gingiva (Figure 29).

CMC 2.24 suppressed diabetes induced activation of NF-κB

A similar pattern was seen in the diabetic rat model of periodontal disease; diabetes alone increases activation of NF-κB (phosphorylation of p65), whereas in the animals treated with CMC 2.24 (30 mg/kg body weight), the diabetes-induced activation of NF-κB is dramatically reduced (Figure 30).

CMC 2.24 inhibited LPS induced activation of p38 MAPK

Because of the important role of p38 MAPK on gene expression of inflammatory cytokines, we next evaluated the effect of orally-administered CMC 2.24 on the activation of p38 MAPK signaling pathways by Western blot analysis. LPS injections modestly increased the activation of p38 MAPK in the gingival tissues (similar to the modest increase in NF-κB activation), which was lower in the animals treated with CMC 2.24 (30 mg/kg body weight) (Figure 31).

CMC 2.24 suppressed diabetes induced activation of p38 MAPK

A similar effect was seen in the diabetic rat model of periodontal disease. Diabetes by itself appeared to increase activation of p38 MAPK in the gingival tissues, but this was markedly suppressed in the animals treated with CMC 2.24 (30 mg/kg body weight) (Figure 32).

Discussion

Activation of NF-κB is considered to be vital for the expression of inflammatory mediators which participate in the pathogenesis of various inflammatory diseases (DiDonato et al. 1997; Mercurio 1997), a rationale which targets this pathway for host-modulation therapies. Pro-inflammatory cytokines such as IL-1β and TNF-α are produced by activated fibroblasts, monocytes, and macrophages and play a key role in the pathogenesis of periodontitis (Lee et al. 1995; Tsai et al. 1995; Leng and Elias 1997). Once these cytokines are released they stimulate the production of catabolic enzymes such as MMPs, which are largely responsible for connective tissue breakdown in chronic inflammatory diseases (Robbins et al. 2000; Largo et al. 2003). IL-1β and TNF-α also activate other mediators of inflammation such as cyclo-oxygenase-2 (Page 1998).

From our experimental work we can present data which indicate that systemically-administered CMC 2.24 effectively reduces mediators of inflammation, connective tissue breakdown, and alveolar bone loss in a locally-and systemically-induced model of experimental periodontitis. This effect on these cell-signaling pathways is seen consistently in the gingiva of rats that suffer from either locally- or systemically-induced periodontal disease it appears to be associated under various situations with a predictable reduction in inflammatory cytokines, MMPs, and bone loss. These effects can be explained, in part, by the inhibition of IL-

1β, IL-6, and TNF-α protein expression, as a result of the suppression of NF-κB activation. In addition this effect is also accompanied by inhibition of alveolar bone loss. All of these cytokines have been found to be significantly increased in diseased periodontal tissues in comparison with healthy tissues (Geivelis et al. 1993; Offenbacher et al. 1993; Lee et al. 1995; Gamonal, et al. 2000; Ejeil et al. 2003) and have also been associated with increased pocket depth and loss of attachment (Stashenko et al. 1991; Gorska et al. 2003; Kirkwood et al. 2007).

Recently studies have revealed that curcumin is able to inhibit the activation of NF-κB (Plummer et al. 1999; Gaddipati et al. 2003; Goel et al. 2008) and MAP Kinases (Woo et al. 2005; Goel et al. 2008), thereby reducing the synthesis of pro-inflammatory cytokines, such as IL-6 (Gaddipati et al. 2003), TNF-α (Banerjee et al. 2003; Fu et al. 2007) and PGE2 (Goel et al. 2008). With regard to the modulation of intracellular signaling pathways, suppression of NF-κB activation may be one of the main mechanisms by which CMC 2.24 reduces inflammation.

In the present study we have demonstrated that our "lead" compound CMC 2.24, has important beneficial therapeutic effects on the pro-inflammatory cytokines and MMPs and also can inhibit also the activation of NF-κB in a locally- or systemically-induced model of experimental periodontitis. However, it is likely that the inhibition of pro-inflammatory cytokine production by CMC 2.24 is also partially dependent on the modulation of other signaling pathways.

Another signaling pathway with p38 MAPK is involved in the expression of pro-inflammatory cytokines and bone resorption (Patil et al. 2004, 2006; Rossa Jr et al. 2005, 2006;

Mbalaviele 2006). In addition, suppression of p38 MAPK activation may provide another mechanistic reason for the anti-inflammatory effects of CMC 2.24 and the prevention of alveolar bone loss.

It is well-recognized that MMPs function as matrix-degrading enzymes that mediate extracellular matrix protein remodeling (Schmitz et al. 1996). Under normal conditions, a balance exists between MMP activity and the synthesis of new extracellular matrix. This occurs where the expression of MMPs and its tissue inhibitors (known as TIMPs) is well-controlled and balanced against new extracellular matrix proteins synthesis. Nevertheless, in periodontitis this tightly-controlled equilibrium between synthesis and degradation becomes unbalanced resulting in excessive extracellular matrix degradation as a result of unregulated MMP expression and activity and leads to bone destruction (Visse and Nagase 2003; Shakibaei et al. 2007).

The present study confirms that LPS induces up-regulation of MMP-9, as has been previously reported for this and other MMPs (Sakai et al. 2001; Esteve et al. 2002; Elburki et al. 2014). In this experiment MMP-9 levels in the gingiva of untreated diabetic rats did not increase (Figure 24b), although in other experiments this did occur (Gu et al. 2013). This inconsistent response of MMP-9 to diabetes may reflect a variable defect in chemotaxis in the leukocytes (Geerlings and Hoepelman 1999) of the diabetic rats in part due to the fact that these rats were not fasted prior to STZ injection. Here we show that CMC 2.24 inhibits MMP-9 in both models of experimental periodontitis. We propose that this may be a result of upstream inhibition of NF-κB, inhibition, because the latter regulates the expression of MMP-9 as has been shown by other investigators (Arakawa 1995; Esteve et al. 2002).

Several of the downstream effects of IL-1β and TNF-α activation, including the up-regulation of MMPs, are also controlled by activation of the transcription factor NF-κB (Mengshol et al. 2000; Sakai et al. 2001; Sylvester et al. 2001; Liacini et al. 2002; Singh et al. 2002; Largo et al. 2003). It is well-known, that phosphorylation of p65 is required for the NF-κB activation and transcriptional functions, and that this phosphorylation is mediated by IKK (Sizemore et al. 2002). In the inactive state, p65 is retained in the cytoplasm, however, when NF-κB is activated, the phosphorylated p65 subunit of NF-κB translocates to the nucleus, where it binds to NF-κB-recognition (κB) sites in the promoter regions of selected target genes, and activate their expression (Grall et al. 2003). Increased phosphorylation of p65 as a result of LPS and diabetes was clearly demonstrated by Western blot analyses of gingival tissues which also suggest the translocation of phosphorylated p65 to the nucleus. By contrast, CMC 2.24 decreased phosphorylation of p65, thereby inhibiting activation of NF-κB and consequently gene transcription.

Expression of TNF-α and IL-1β induced RANKL by p38 MAPK is required in bone marrow stromal cells in vitro. Inhibition of p38 signaling was found to inhibit IL-1β-and TNF-α-induced osteoclastogenesis (Rossa Jr et al. 2006). The upstream effector, p38 MAPK is shared by several inflammatory cytokines. Stimulated monocytes, macrophages, and fibroblasts in the periodontal tissues synthesize cytokines such as IL-1β, IL-6, and TNF-α and prostanoids such as PGE2 (Lee and Young 1996; Lee et al. 2000). These cytokines stimulate the production of many inflammatory mediators and enzymes, including MMPs, prostaglandins, and RANKL (Assuma et al. 1998; Graves et al. 1998). For example, IL-1β and TNF-α activate bone marrow stromal cells and macrophages to stimulate osteoclastogenesis through RANK–RANKL interaction (Wei et al.

2005). Inhibition of IL-1β and TNF-α decreases the loss of connective tissue attachment and alveolar bone (Graves et al. 1998; Delima et al. 2001). In the present study, Western blot analysis of gingival tissues revealed that LPS and diabetes increase phospho-p38, whereas CMC 2.24 lowers the level of phospho-p38. Furthermore, CMC 2.24 significantly protects against loss of alveolar bone height.

Conclusions

We have demonstrated that our "lead" compound CMC 2.24, has significant therapeutic effects on pro-inflammatory cytokines, MMPs, and also appears to inhibit activation of NF-κB and p38 MAPK in locally- and systemically-induced models of experimental periodontitis. Furthermore, inhibition of the NF-κB and p38 MAPK pathways appears to ameliorate periodontitis in both LPS and diabetes models of experimental periodontitis.

LPS injection and induction of diabetes both elicited the inflammatory process and increased activation of NF-κB and p38 MAPK. Systemic administration of CMC 2.24 produced a marked inhibition of NF-κB and p38 MAPK activation in periodontally diseased tissues. This treatment was accompanied by marked reductions in MMP-9, cytokines (IL-1β, IL-6 and TNF-α), and an increase in IL-10. In addition CMC 2.24 significantly protected against loss of alveolar bone height.

The findings of the present study demonstrate a potent anti-inflammatory effect of systemically-administered CMC 2.24 in both models of experimental periodontitis and suggest a potential therapeutic role for CMC 2.24 in the treatment of periodontal disease. Future studies will address the biological effects of CMC 2.24 in these in vitro and in vivo models, in relevant cells associated with periodontal disease. In addition, the inhibition of LPS signaling by CMC 2.24 could have

significant implications for the treatment of chronic inflammatory diseases caused by bacterial infections. It is now recognized that inflammation plays a crucial role in several chronic diseases such as rheumatoid arthritis, atherosclerosis and cancer (Gradisar et al. 2007). Therefore we suggest that anti-inflammatory and other beneficial effects of CMC 2.24 may be linked to the modulation of inflammation by interfering with pattern recognition receptors (e.g., TLR-4 and RAGE), activation by microbe-associated molecular patterns and endogenous molecules, thereby blocking the activation of NF-κB signaling pathway. Future studies, either (1) by using more sensitive techniques to measure activation of these cell-signaling pathways, to carry out these assays without requiring pooling tissue (gingiva) which would allow a more accurate statistical analyses, or (2) by examining other tissues, available for individual rats in greater quantity. These are needed to confirm our observations that the efficacy of 2.24 in inflammatory periodontal (and other) diseases depends, in part, on the upstream efficacy of this compound in regulating cell-signaling. However, CMC 2.24 also has pleiotropic extracellular benefits because it can decrease the conversion of pro-MMPs into active MMPs (probably by decreasing extracellular neutral proteinase activation) and can decrease the activities of the extracellular MMPs themselves.

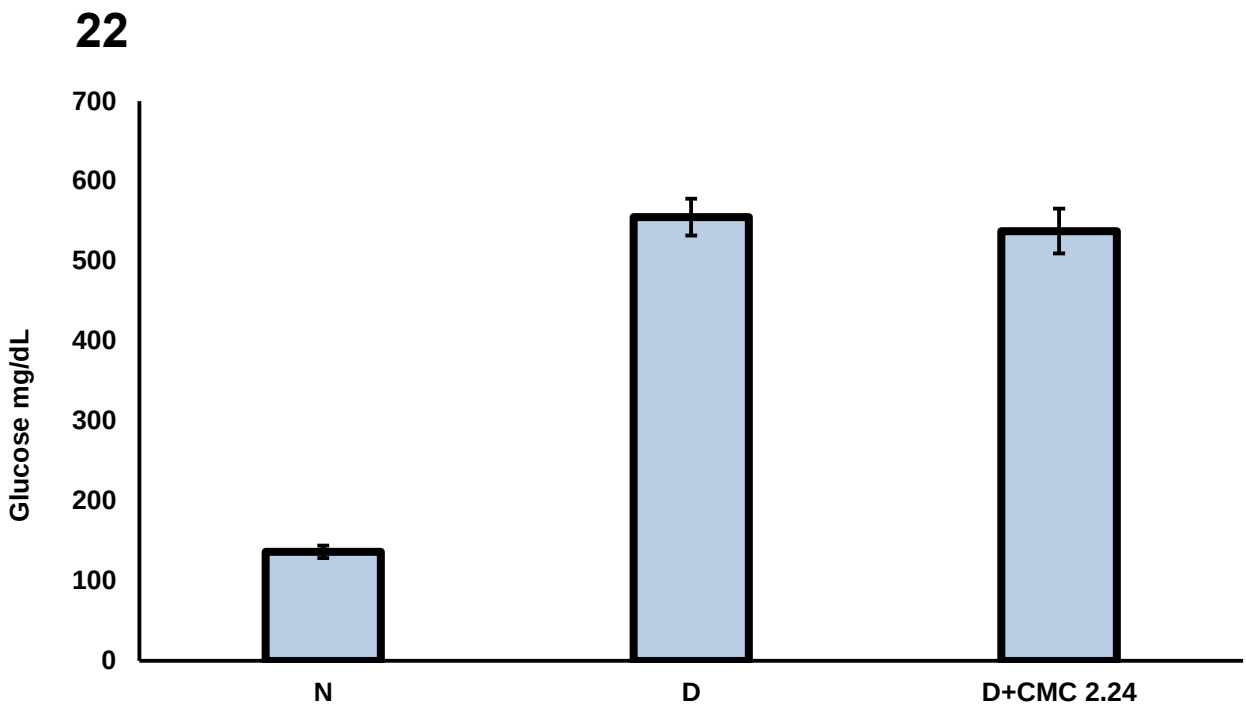
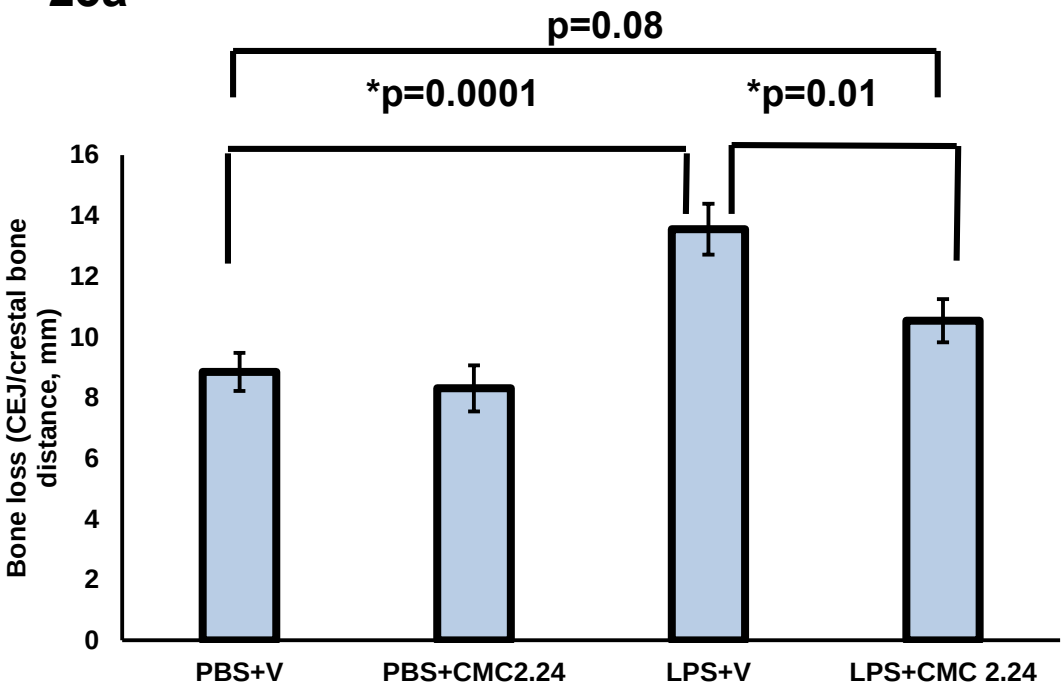


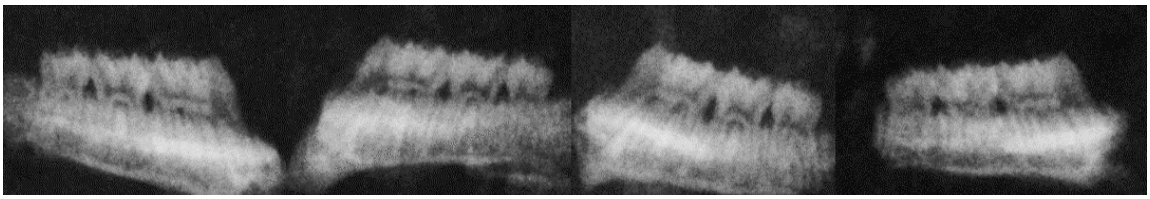
Figure 22. The effect of diabetes and orally-administered CMC 2.24 on fasting blood glucose levels; blood samples were collected 4 weeks after STZ injection. The D rats were treated either by systemic administration (oral route) of vehicle alone, or by daily administration of 30 mg/kg CMC 2.24 for 21 days beginning 7 days after inducing diabetes. Each value represents the mean ± the standard error of the mean for 4 rats per group.

Table 4- Diabetic Complications: Effects of CMC 2.24

Experimental Group (no of rats per group)	Incidence of Adverse Events (AEs)	Description of AEs
N (n=4)	0/4	None
D (n=4)	3/4	Bleeding under toe-nails; tail necrosis; excessive tears and inflamed sclera
D+CMC2.24 (n=4)	0/4	None

23a





PBS+V PBS+CMC 2.24 LPS+V LPS+CMC 2.24

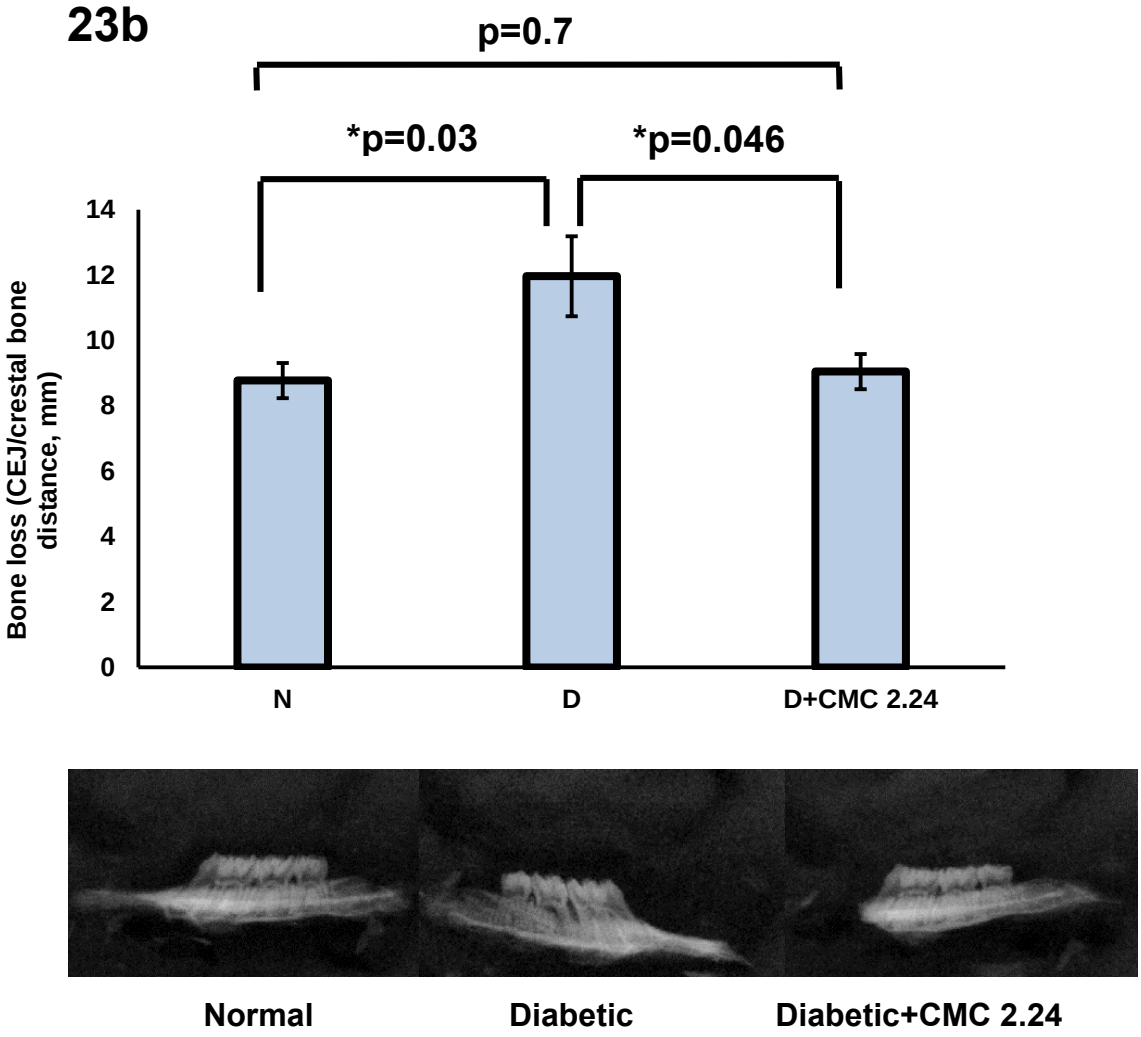


Figure 23. The measurement of alveolar bone loss, from standardized radiographs of hemi maxilla, from the CEJ to the crest of the alveolar bone at site # 7. Each value (mm) represents the mean ± S.E.M. (a) Locally-induced periodontitis. (b) Systemically-induced periodontitis.

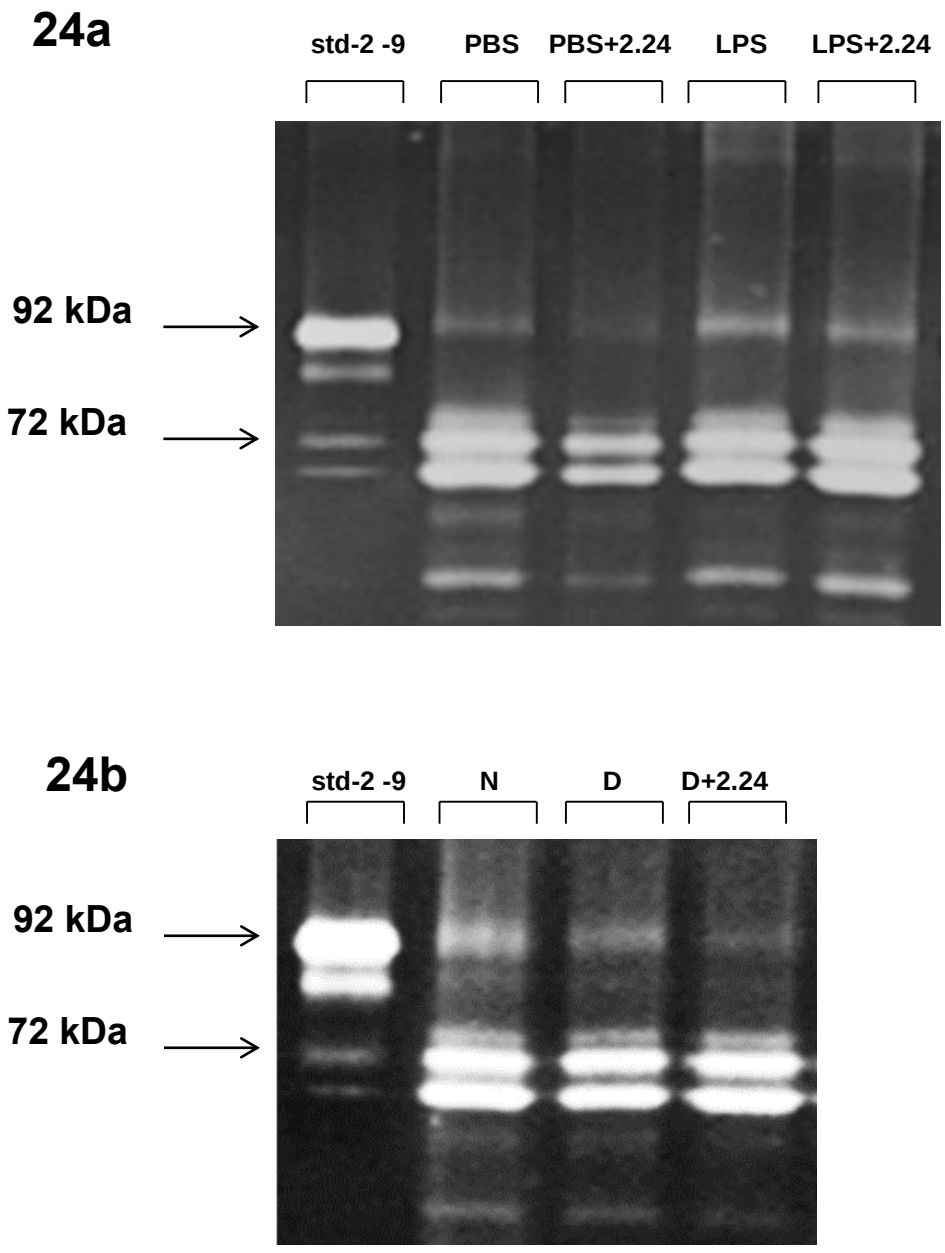
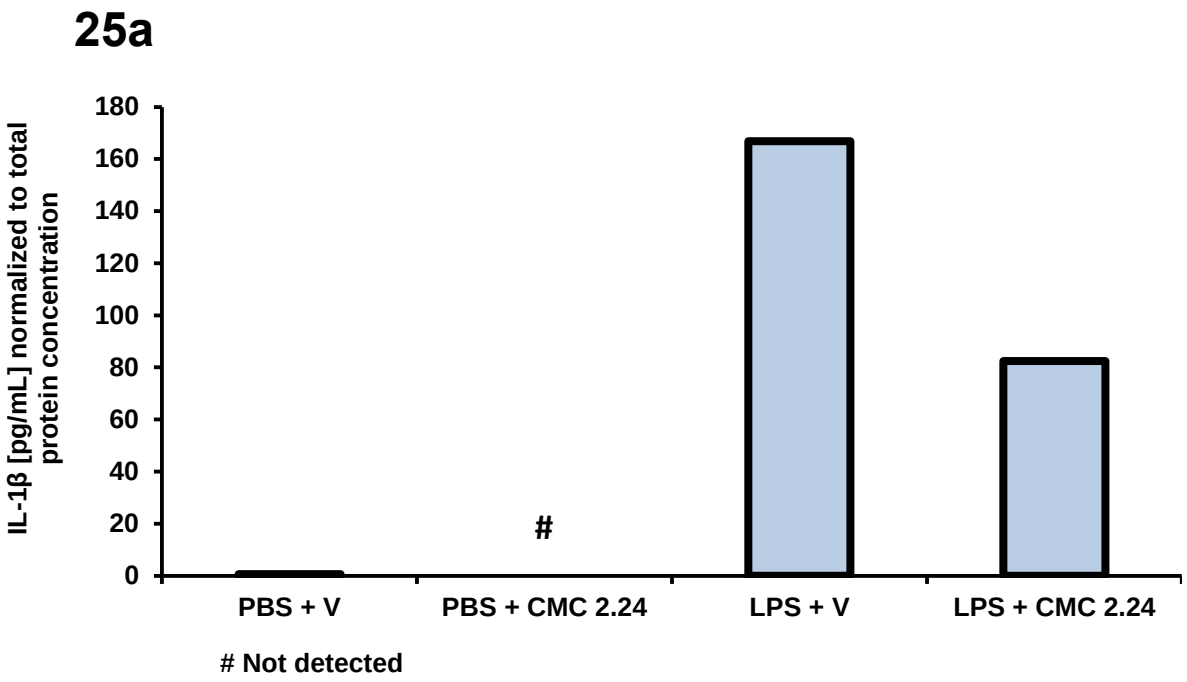


Figure 24. Gelatin zymography of partially purified extract of pooled gingiva from each experimental group showing the effect of orally-administered CMC 2.24 on gingival MMPs (-2, -9). (a) Locally induced periodontitis. (b) Systemically induced periodontitis.



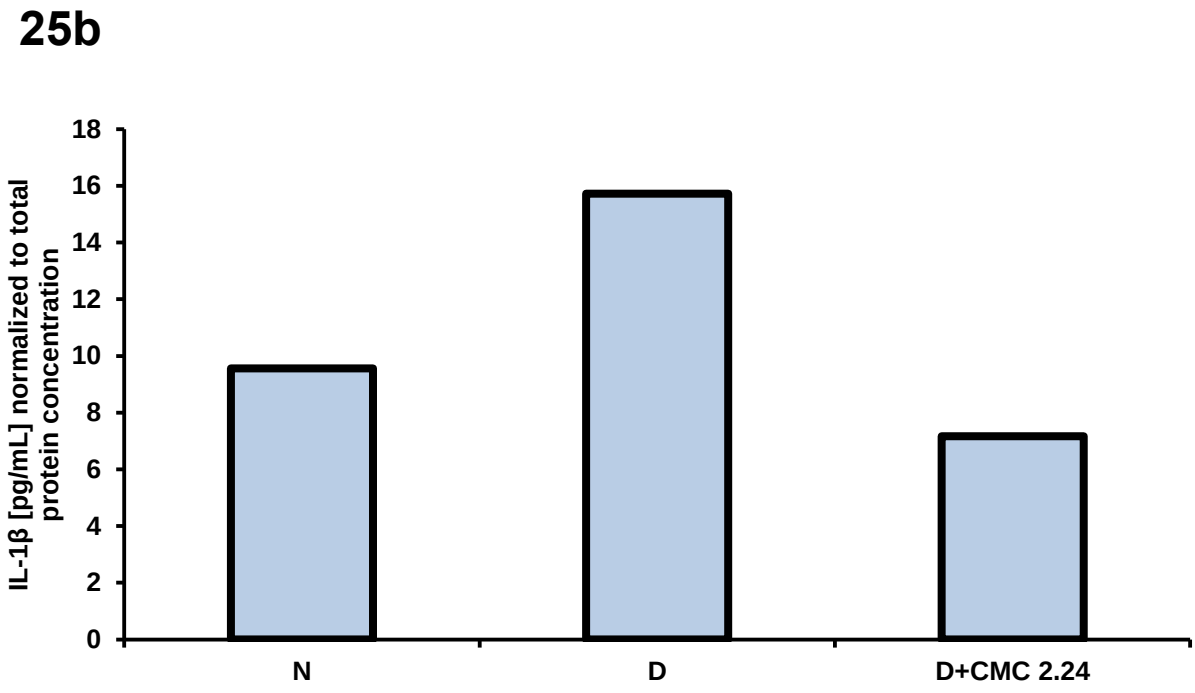


Figure 25. (a) The effect of CMC2.24 therapy on IL1-β in rat gingiva with LPS injection measured by ELISA. (b) The effect of CMC 2.24 therapy on IL1-β in diabetic rat gingiva measured by ELISA.

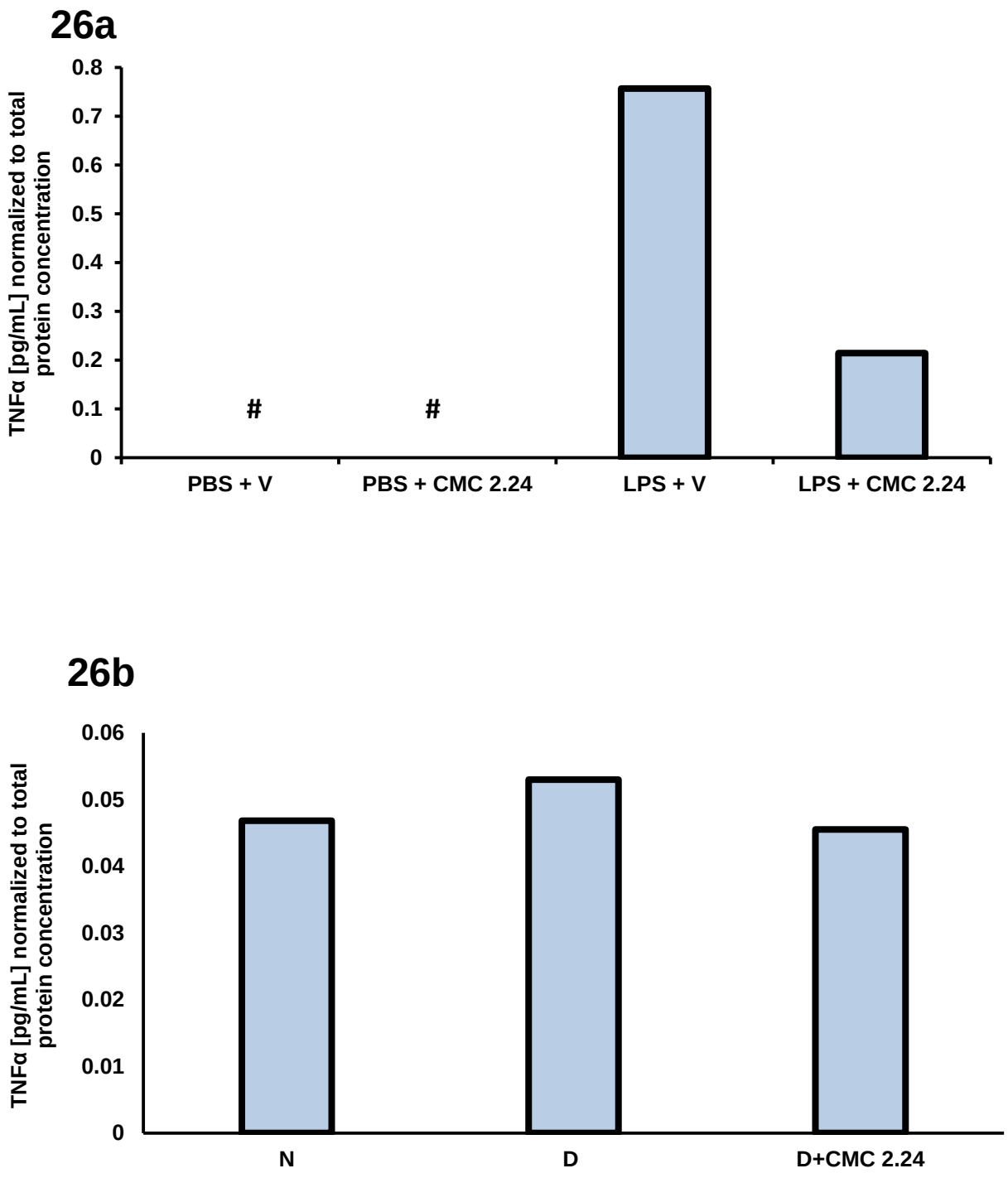
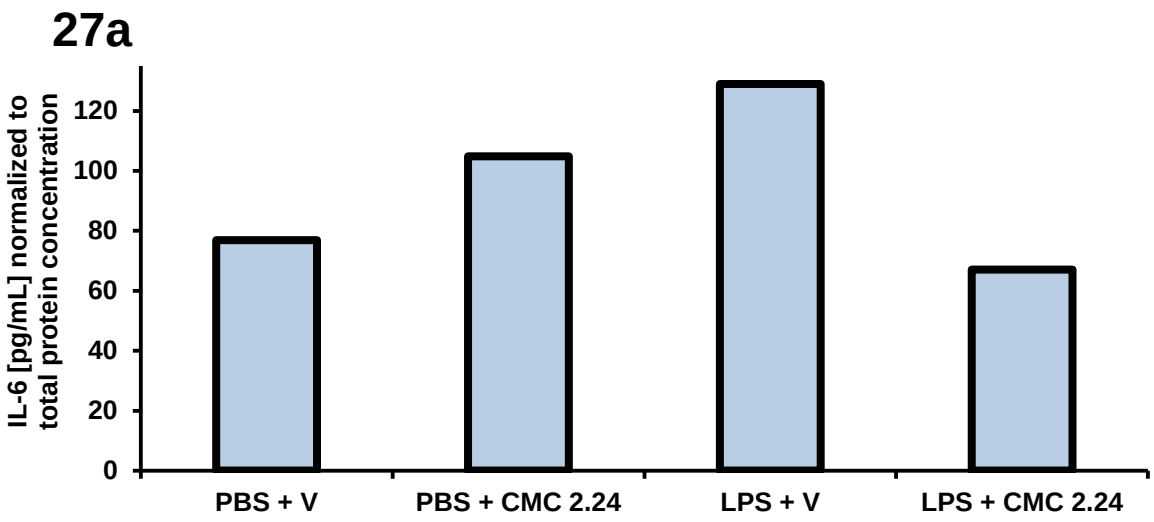


Figure 26. (a) The effect of CMC2.24 therapy on TNF-α in rat gingiva with LPS injection measured by ELISA. (b) The effect of CMC 2.24 therapy on TNF-α in diabetic rat gingiva measured by ELISA.



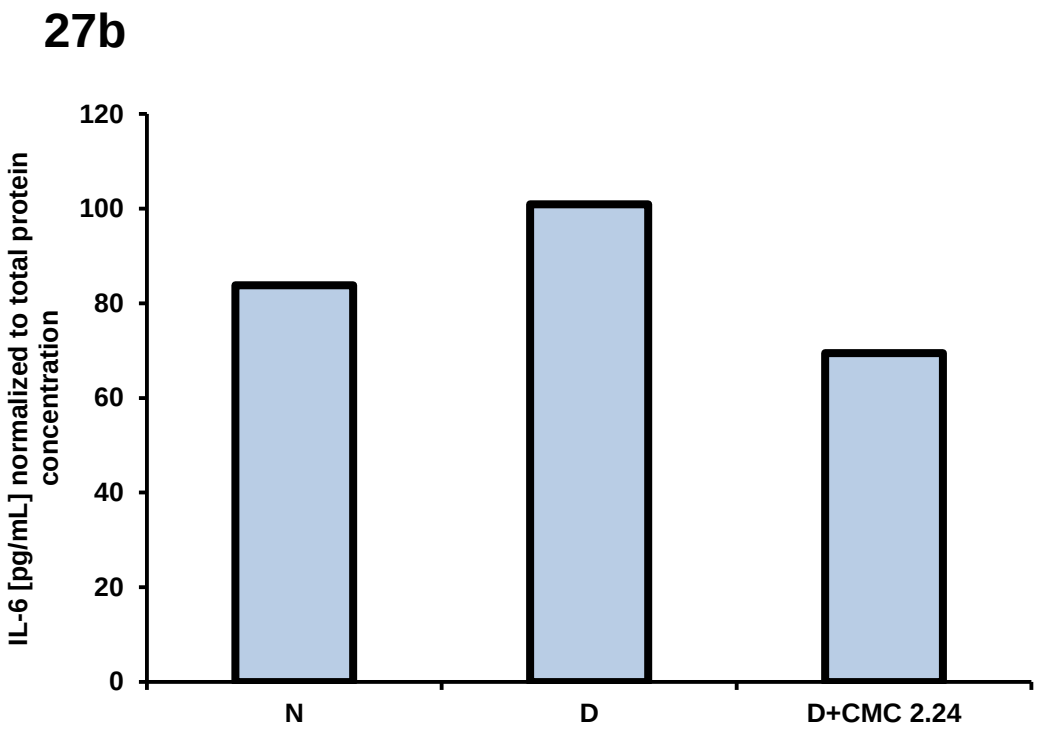


Figure 27. (a) The effect of CMC2.24 therapy on IL1-6 in rat gingiva with LPS injection measured by ELISA**.** (b) The effect of CMC 2.24 therapy on IL1-6 in diabetic rat gingiva measured by ELISA**.**

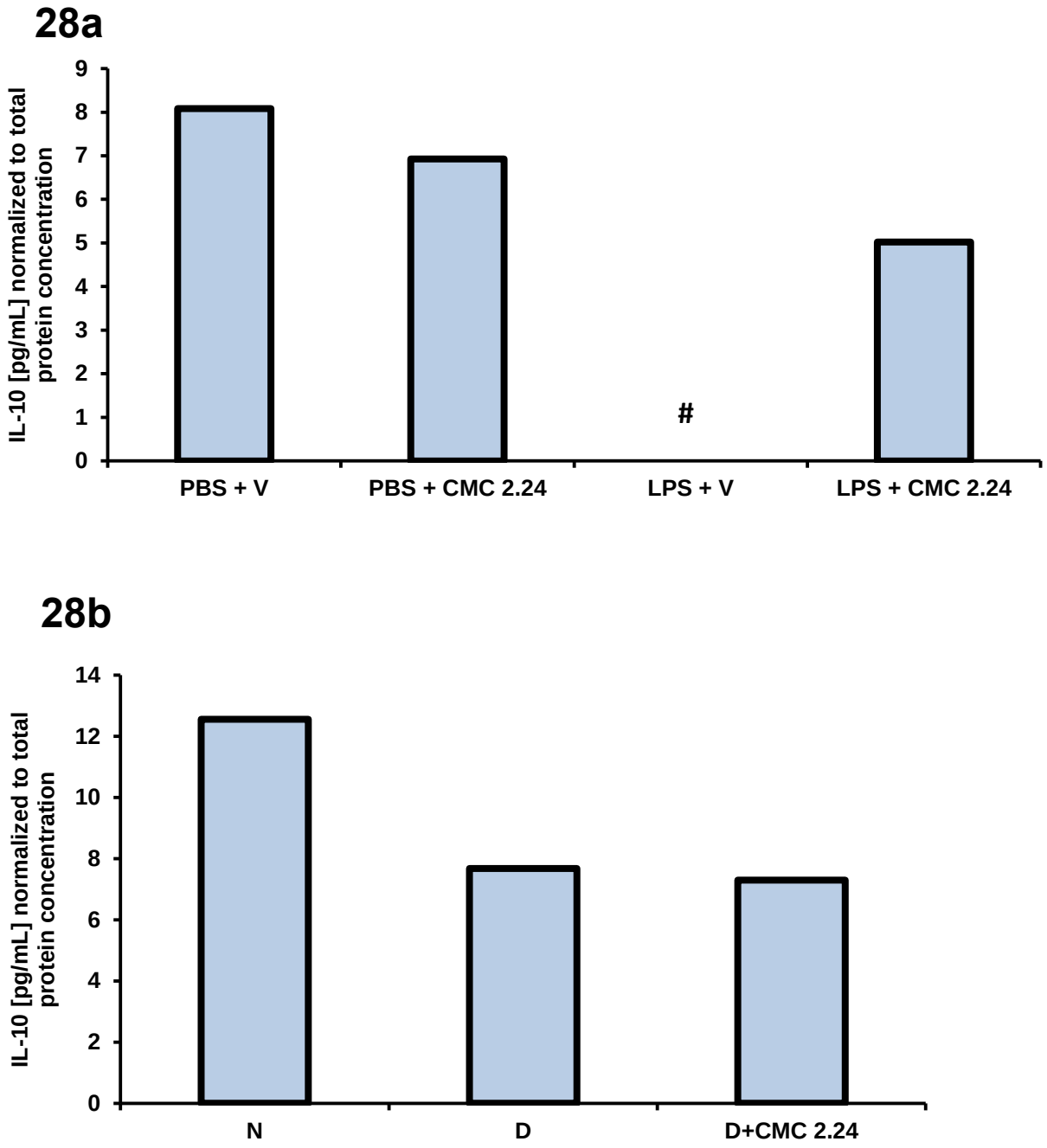


Figure 28. (a) The effect of CMC2.24 therapy on IL1-10 in rat gingiva with LPS injection measured by ELISA**.** (b) The effect of CMC 2.24 therapy on IL1-10 in diabetic rat gingiva measured by ELISA**.**

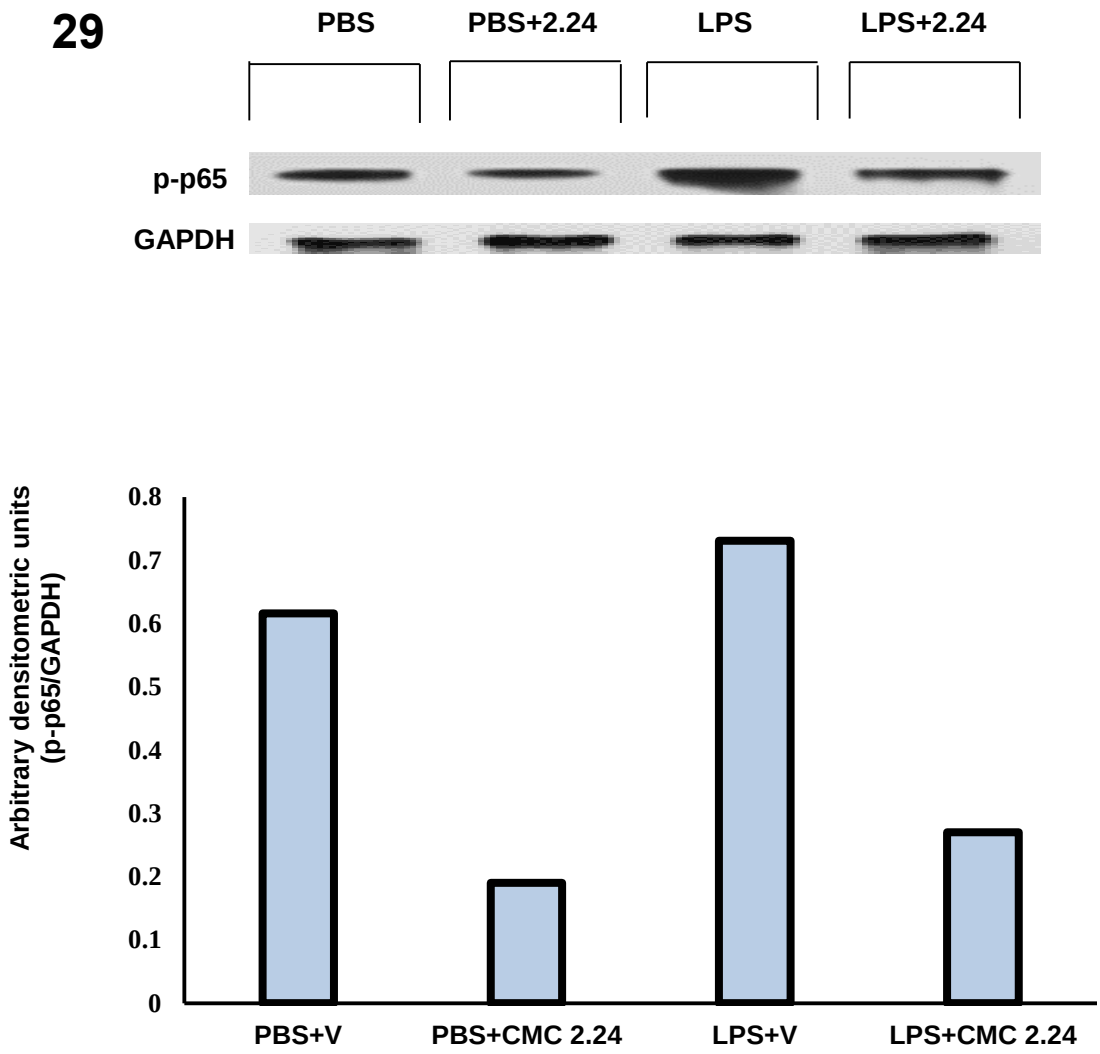


Figure 29. Modulation of nuclear factor-κB activation in gingival tissues by systemic administration of CMC 2.24. The activation of the signaling pathways was assessed by the detection of phosphorylated forms of p65. Expression levels of GAPDH are shown to confirm equal protein loading. The bar graph represents the densitometric units (p-p65/GAPDH).

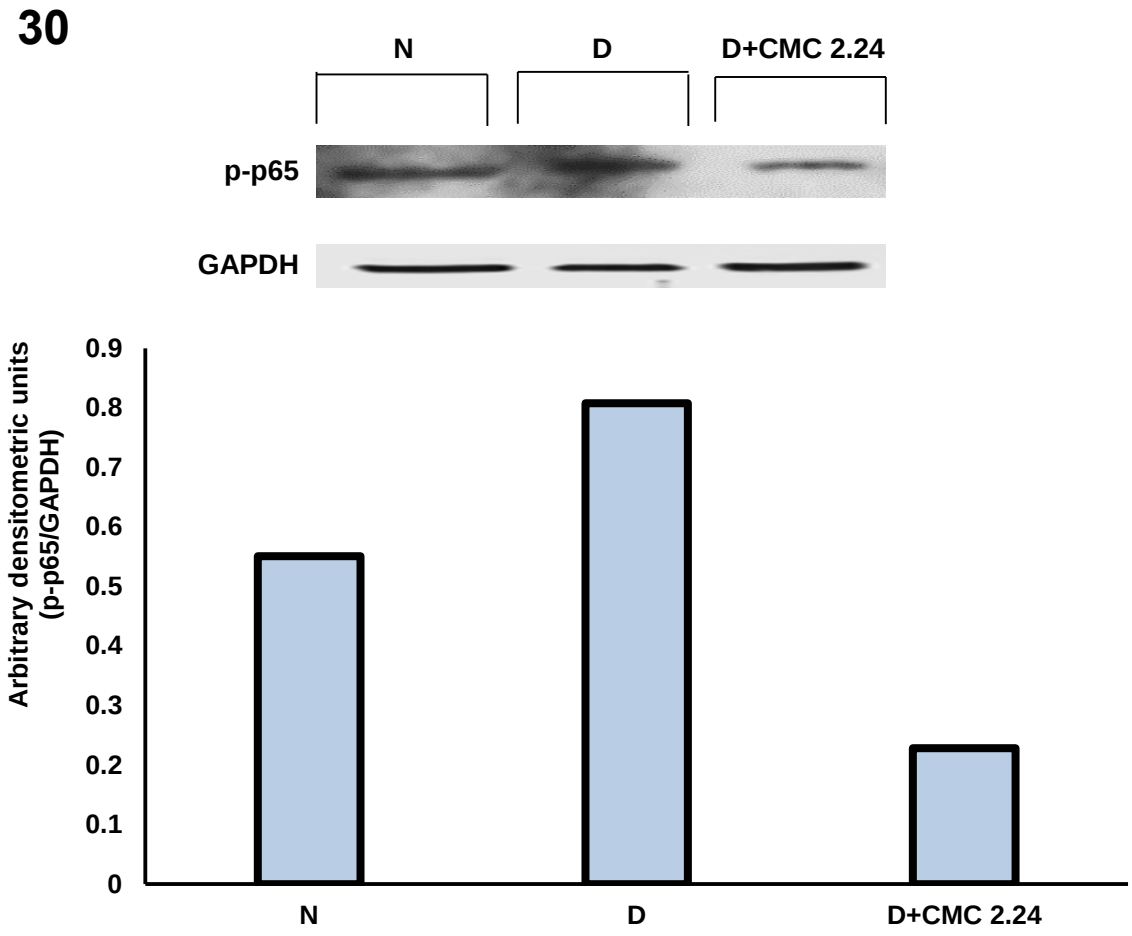


Figure 30. Modulation of nuclear factor-κB activation in gingival tissues by systemic administration of CMC 2.24. The activation of the signaling pathways was assessed by the detection of phosphorylated forms of p65 (NF-κB). Expression levels of constitutive housekeeping GAPDH are shown to confirm equal protein loading. The bar graph represents the densitometric units (p-p65/GAPDH).

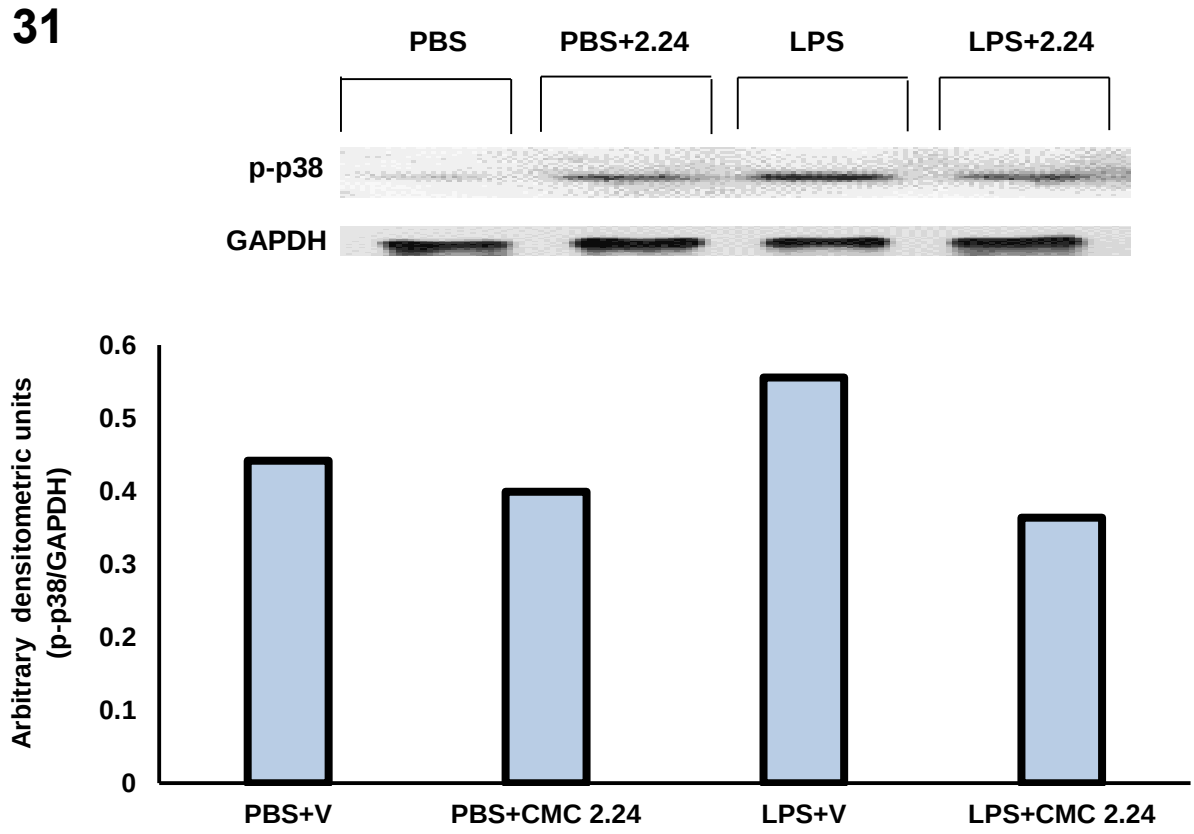


Figure 31. Modulation of p38 MAP kinase activation in gingival tissues by systemic administration of CMC 2.24. The activation of the signaling pathways was assessed by the detection of phosphorylated forms of p38 (MAPK). Expression levels of constitutive housekeeping GAPDH are shown to confirm equal loading of protein. The bar graph represents the densitometric units (p-p38/GAPDH).

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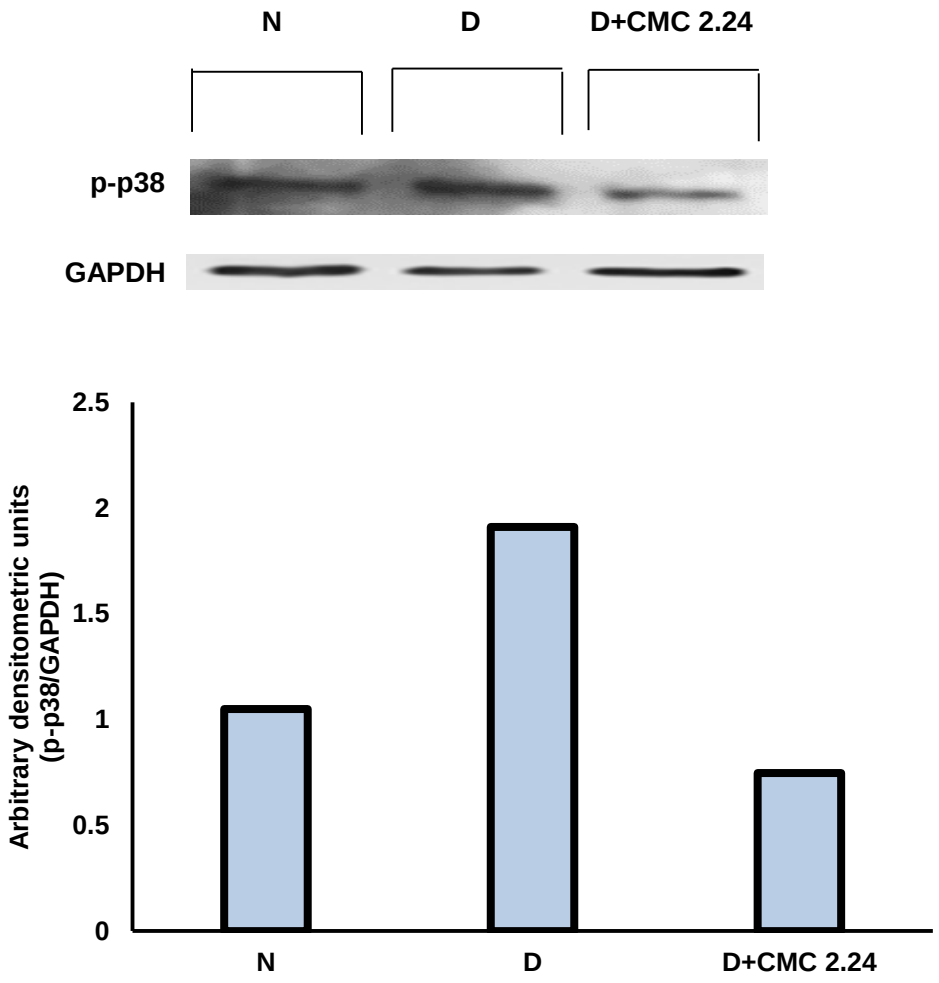

Figure 32. Modulation of p38 MAP kinase activation in gingival tissues by systemic administration of CMC 2.24. The activation of the signaling pathways was assessed by the detection of phosphorylated forms of p38 (MAPK). Expression levels of constitutive housekeeping GAPDH are shown to confirm equal protein loading. The bar graph represents the densitometric units (p-p38/GAPDH).

Chapter 6

Summary and Conclusion

I. Summary of the current studies

The destruction of both connective tissue and bone during periodontal disease is mediated, in large part, by proteinases such as the MMPs generated by the host tissues. Lipopolysaccharide (LPS; endotoxin) derived from periodontal Gram negative pathogens, induce the host tissues to produce inflammatory mediators such as the cytokines, IL-1β and TNF-α, and others, such as PGE2, ROS (e.g., HOCl), and NO (generated by inducible-nitric oxide synthase), which stimulate the expression and activation of host-derived proteinases (e.g., MMP-8 and MMP-9). Previous basic research and clinical studies have repeatedly demonstrated that our earlier- developed MMP-inhibitors, the tetracyclines (TCs), (administered at sub-antimicrobial-dose), reduce periodontal breakdown not only in the soft tissues (gingiva, periodontal ligament) but also prevent the loss of both alveolar bone density and bone height (Payne and Golub 2011). After identifying the active enolic 1,3-diketo site responsible for these host-modulating properties (Golub et al. 1991, 1998a), further research by Drs. Golub and Johnson and their colleagues resulted in the design and testing of new MMP-inhibitor compounds. These have a zinc-binding enolic site similar to the TCs, but are related to curcumin and are triketonic rather than diketonic compounds. The research, described in this thesis, demonstrates that the newer chemically-modified-curcumin (CMC 2.24) has significant inhibitory effects on pro-inflammatory cytokines and MMPs in several animal models of experimental periodontal disease. These models involve induction of experimental

periodontal disease both by local microbial factors and by a systemic disease, namely diabetes, long-known to be associated with increased severity of this common oral inflammatory condition.

In brief, oral administration of a newly-developed chemically-modified-curcumin (CMC 2.24) in three different rat models of periodontal disease [including (1) a locally (LPS/endotoxin)-induced; (2) a systemically (type I diabetes)-induced models of experimental periodontitis; and (3) a combination of both LPS and type I diabetes-induced periodontal disease] significantly inhibited local (alveolar) bone loss, attenuated the severity of local and systemic inflammation by decreasing cytokine production (IL-6 and IL-1β), and reduced pathologically-elevated levels of MMPs and their activation (MMP-2, -8, and -9). Although alveolar bone loss was measured by three different techniques including morphometric analysis of bone loss in defleshed jaws, radiographic/morphometric analysis and microcomputerized tomography (μCT), the results were consistent. They indicate that the systemic administration (by the oral route) of this novel, tri-ketonic phenylaminocarbonyl curcumin (CMC 2.24) is a potent inhibitor of pathologic alveolar bone loss and reduces inflammatory mediators in the adjacent gingival tissues. Moreover, this novel compound appears to reduce the pathologically excessive levels of inducible MMPs to near normal levels, but has little or no effect on the constitutive MMPs required for normal physiologic connective tissue turnover.

In addition to the beneficial effects on periodontal tissues, CMC 2.24 treatment also favorably affects extra-oral connective tissues including skin and skeletal bone (tibia).

Among the numerous complications of uncontrolled type I diabetes and severe hyperglycemia in humans, is skin atrophy (including impaired wound-healing; Zhang et al. 2015),

and osteoporosis which can lead to pathologic fractures (Ramamurthy et al.1973b). These abnormalities in collagen-rich tissues have been reported to result from suppressed collagen synthesis by both fibroblasts in the dermis and osteoblasts in bone (Sakai et al. 1990). An additional cause of deficiencies in these tissues is pathologically-excessive MMP activity which mediates accelerated collagenolysis (Ramamurthy et al. 1973a; Yu et al. 1993; Ryan et al. 2001). In the research described in this thesis, these additional complications of diabetes were also ameliorated by oral administration of CMC 2.24, indicating that this single daily treatment strategy, simultaneously produces both beneficial oral and systemic effects.

Also the diabetes-induced abnormalities in skin and bone, described above is consistent with previous studies in the field (Ramamurthy and Golub 1978; Schneir et al. 1981b, 1984a, 1984b). We found that inducing experimental diabetes decreased the percentage of total collagen extractable from skin by neutral salt buffers (4^{o}C). This results in an increase in the percentage of highly cross-linked, insoluble collagen. The salt-soluble collagen represents the most recently synthesized fraction in skin (and other tissues including bone matrix). However, as a result of lysyl-oxidase mediated cross-link formation during extracellular maturation of collagen, the insoluble fraction represents an older fraction of collagen. The data on collagen solubility reflects two main processes due to the diabetic condition: (1) there is suppression of collagen synthesis, and (2) MMP-mediated collagen degradation is increased. The latter can be explained by the known characteristic of MMPs to preferentially degrade the newly-synthesized non-crosslinked (soluble) collagen resulting in a greater proportion of older, highly-crosslinked collagen (Figure 33). All of the findings described in this thesis are consistent with these well-known effects on collagen metabolism in the diabetic rat model (Golub et al. 1978b; Ramamurthy et al. 1983;

Schmidt et al. 1996). Given that CMC 2.24 treatment, "normalized" the reduced solubility (at 4°C) of collagen in the skin of the diabetic rats, as well as decreasing MMP activation and activity, our new data support our view that CMC 2.24 treatment has a profound beneficial effect on collagen in skin, and on the bone matrix. Type I collagen makes up more than 90% of the organic matrix of bone and, in the current studies, CMC 2.24 administration appeared to reduce the severity of osteoporosis in the long bones (tibia) of these severely hyperglycemic rats. It should be recognized, however, that all of the experiments described in this thesis refer to a time period no longer than 4-weeks. It is possible, therefore, that in longer-term in vivo experiments, CMC 2.24 treatment might decrease significantly the severity of hyperglycemia. This also implies that reducing the severity of these complications in the presence or absence of experimental-periodontitis could result in less severe diabetes.

Regarding molecular mechanisms, p38-MAPK as well as NF-κB, were activated in the gingiva during the course of both LPS- and diabetes-induced experimental periodontitis, consistent with a hyper-inflammatory state of the several rat models of disease that were used in these studies. Moreover, systemic treatment with CMC 2.24 produces substantial inhibition of NF-κB and p38 MAPK activation in gingiva in both models of experimental periodontitis. This treatment effect was accompanied by marked reductions in MMP-9, and the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, together with an increase in the anti-inflammatory cytokine, IL-10. In addition, CMC 2.24 significantly decreased the destruction of alveolar bone which is a "hallmark" of periodontitis. Because bacterial LPS and cytokines (e.g., IL-1β and TNF-α) bind and activate Toll receptors, this initiates the MAPK pathway, and results in activation of the transcription factor NF-κB, important for cytokine gene expression. Therefore, we suggest that the anti-inflammatory and

other beneficial effects of CMC 2.24 may be linked to the down-modulation of inflammation by interfering with the activation of pattern recognition receptors such as TLR-4, RAGE or IL-1R which would consequently limit activation of NF-κB (Figure 34).

The results presented in this thesis support the hypothesis that CMC 2.24 is a pleiotropic MMP inhibitor, having both intracellular and extracellular effects. These together, reduce local and systemic inflammation and prevent hyperglycemic and bacterial-product (LPS)-associated, tissue destruction. Further safety and toxicity studies on CMC 2.24, in additional animal models will be required before clinical trials can be conducted in patients with periodontal disease.

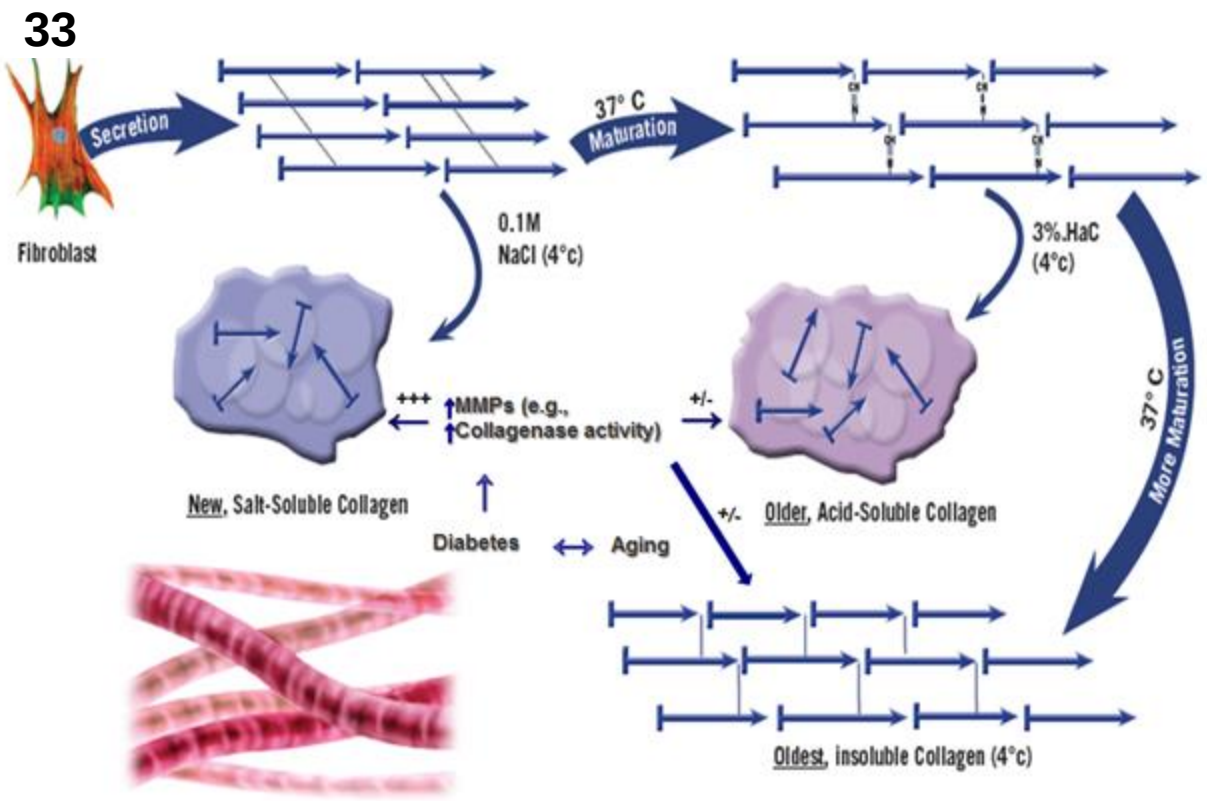

Figure 33. Extracellular maturation of collagen: Formation of inter-molecular cross links and cross-striated collagen fibrils.

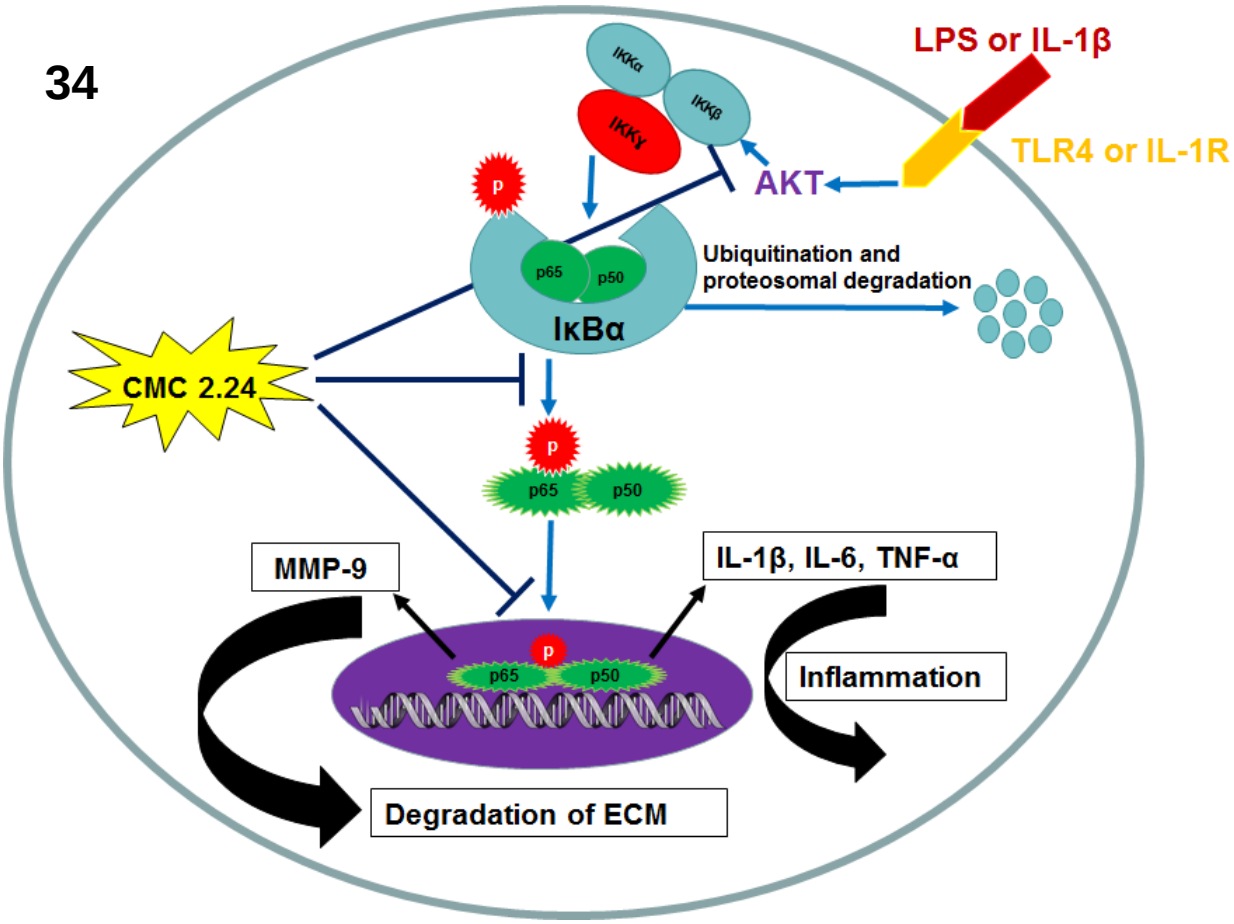

Figure 34. Working model of CMC 2.24 anti-inflammatory actions on LPS and/or cytokine- induced signal transduction in gingiva.

II. Future studies and preliminary results

Recently, a preliminary study was conducted using a more sensitive assay than are described in Chapter 5 (Western blot) to detect and quantify the activation of NF-κB (p65) in gingival samples. This assay, measures the binding of NF-κB to an oligonucleotide containing a consensus sequence in DNA by ELISA. This allows the analysis of gingival samples from individual rats without requiring the pooling of tissue. Consequently, the difference between the NF-κB activation in the gingival tissues from different experimental groups can be analyzed statistically. This was not possible when the tissues were pooled. In this recent study, the gingival tissues from the hemi-maxilla of individual rats were excised from the following groups: non-diabetic controls, diabetic rats treated with vehicle alone, and diabetic rats which were orally- administered CMC 2.24 (30 mg/kg body weight; n=6 rats per group). The extraction procedure used was that recommended by the manufacturer (Active Motif, INC, Carlsbad, CA) and total protein was isolated from the gingival tissues of each rat using a lysis buffer containing a cocktail of protease-and phosphatase-inhibitors. The tissue samples were homogenized in the lysis buffer (1 µL/mg of tissue), centrifuged for 10 min (10,000 g at 4°C), followed by collection of the supernatant and analysis for total protein using the Bio-Rad Protein Assay, and NF-κB by ELISA .

Of relevance to our previous experiments on this topic, the data on individual rat gingiva showed the same pattern of change (Figure 35) as that seen when the gingiva were pooled by group--that is, the induction of diabetes significantly increased NF-κB activation ($p=0.004$), whereas CMC 2.24 treatment reduced this parameter (D+ vehicle vs D+ CMC 2.24; $p=0.03$) essentially back to normal levels (D+CMC 2.24 vs NDC, $p>0.05$; not significant). However,

because the duration of diabetes was shorter in this study than in the earlier study (4 weeks) and the duration of treatment was also shorter, exact comparisons are not possible.

This preliminary data suggest that future studies should be carried out using both (1) the LPS-induced model of periodontitis, and (2) the diabetes-induced model of periodontitis to define more clearly, the efficacy of CMC 2.24 on the cell signaling pathways that regulate the inflammatory process and increase the expression and activation of MMPs that mediate connective tissue and bone destruction.

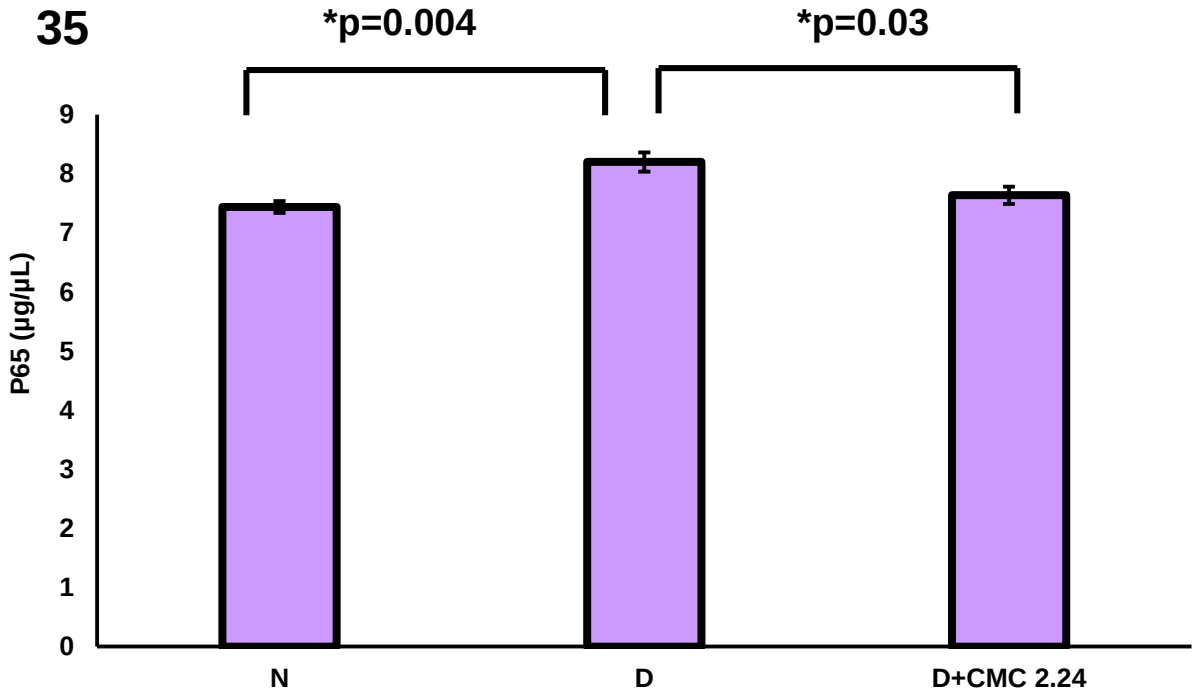

Figure 35. Modulation of nuclear factor-κB activation in gingival tissues by systemic administration of CMC 2.24. The activation of the signaling pathways was assessed by the detection of phosphorylated forms of p65 (NF-κB) by ELISA.

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