

A Novel Modified-Curcumin Promotes Resolvin-Like Activity and Reduces Bone Loss in Diabetes-Induced Experimental Periodontitis

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Purpose: Clinically, it is challenging to manage diabetic patients with periodontitis. Biochemically, both involve a wide range of inflammatory/collagenolytic conditions which exacerbate each other in a “bi-directional manner.” However, standard treatments for this type of periodontitis rely on reducing the bacterial burden and less on controlling hyperinflammation/excessive-collagenolysis. Thus, there is a crucial need for new therapeutic strategies to modulate this excessive host response and to promote enhanced resolution of inflammation. The aim of the current study is to evaluate the impact of a novel chemically-modified curcumin 2.24 (CMC2.24) on host inflammatory response in diabetic rats.

Methods: Type I diabetes was induced by streptozotocin injection; periodontal breakdown then results as a complication of uncontrolled hyperglycemia. Non-diabetic rats served as controls. CMC2.24, or the vehicle-alone, was administered by oral gavage daily for 3 weeks to the diabetics. Micro-CT was used to analyze morphometric changes and quantify bone loss. MMPs were analyzed by gelatin zymography. Cell function was examined by cell migration assay, and cytokines and resolvins were measured by ELISA.

Results: In this severe inflammatory disease model, administration of the pleiotropic CMC2.24 was found to normalize the excessive accumulation and impaired chemotactic activity of macrophages in peritoneal exudates, significantly decrease MMP-9 and pro-inflammatory cytokines to near normal levels, and markedly increase resolvin D₁ (RvD₁) levels in the thioglycolate-elicited peritoneal exudates (tPE). Similar effects on MMPs and RvD₁ were observed in the non-elicited resident peritoneal washes (rPW). Regarding clinical relevance, CMC2.24 significantly inhibited the loss of alveolar bone height, volume and mineral density (ie, diabetes-induced periodontitis and osteoporosis).

Conclusion: In conclusion, treating hyperglycemic diabetic rats with CMC2.24 (a triketonic phenylaminocarbonyl curcumin) promotes the resolution of local and systemic inflammation, reduces bone loss, in addition to suppressing collagenolytic MMPs and pro-inflammatory cytokines, suggesting a novel therapeutic strategy for treating periodontitis complicated by other chronic diseases.

Keywords: hyperglycemia, periodontitis, matrix metalloproteinases, inflammation, resolvins, host-modulatory therapy

Introduction

Interaction of periodontitis and diabetes has long been explored. Patients with both periodontitis and diabetes exhibited higher levels of oral and systemic inflammatory markers than healthy subjects, such as interleukins (IL-1 β , IL-6),^{1,2} nod-like receptor

family proteins (NLRP),¹ and tumor necrosis factor (TNF- α).³ Each condition makes the other more severe, linking the two diseases in a “bi-directional manner” as follows. Inflammatory mechanisms during periodontitis damage the tissues supporting the dentition, including gingiva, periodontal ligament, and alveolar bone, eventually leading to tooth loss, but also impact systemic health.^{4,5} The matrix metalloproteinases (MMPs), including the collagenases, MMP-1, MMP-8 and MMP-13, and gelatinases, MMP-2 and MMP-9, when produced in excessive levels (in contrast to their role, at constitutive levels, in physiologic connective tissue turnover), play a major role in the pathogenesis of numerous diseases, including periodontitis and diabetes, even COVID-19.⁶ A recent study indicates that MMP-9, as an immune-based biomarker, is associated with mortality in COVID-19 patients.⁷ Moreover, these MMPs produced locally by inflamed oral tissues, can travel through the circulation and degrade insulin receptors, which contribute to insulin resistance, promote hyperglycemia, and exacerbate the diabetic condition.^{8,9} As a result, diabetes-induced periodontitis, now called the sixth complication of diabetes,^{10,11} is very difficult to manage with traditional-mechanical therapy, ie, scaling and root planing. Therefore, new therapeutics (including MMP-inhibitors), which modulate the excessive host response and promote resolution of inflammation, are urgently needed.

To date, only two MMP-inhibitor medications have been governmentally approved (US Food & Drug Administration; also regulatory agencies in Canada & Europe), ie, the novel NON-antimicrobial formulations of the tetracycline, doxycycline, for the treatment for human periodontitis;^{12,13} and a sustained-release formulation of this medication for the treatment of a chronic inflammatory skin disease, acne-rosacea.¹⁴ The mechanism involves, at least in part, the binding of calcium and zinc, by the β -diketone moiety of this tetracycline (including minocycline and doxycycline) molecule which then inhibits the activity of these metal-dependent, host-derived, neutral proteinases.^{15,16} More recently, additional host-modulating therapy drugs (HMTs) have been developed including resolvins (polyunsaturated fatty acids) and chemically modified curcumins (CMCs). Numerous studies have supported the therapeutic potential of resolvins, such as eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), in managing chronic diseases.^{17,18} Regarding the latter, a phenylaminocarbonyl tri-ketonic chemically-modified curcumin 2.24 (CMC2.24), as a lead compound, has an enhanced/potent calcium and zinc-binding bioactivity, and has been extensively studied. This novel pleiotropic compound not only inhibits MMPs,^{19,20} but also reduces pro-

inflammatory mediators in cell and tissue culture, and in vivo small (rodent) and large (dog) animal models of various inflammatory/collagenolytic disorders (eg, periodontitis, diabetes including impaired wound healing, acute lung disease, arthritis, and cancer).^{21–26}

Hence, the rationale of this study is to describe additional novel therapeutic properties of CMC2.24: its ability to induce the production of anti-inflammatory resolvins (eg, Resolvin D₁, RvD₁), during diabetes, in addition to inhibiting excessive production of destructive MMPs and pro-inflammatory cytokines which prolong chronic inflammation, and to improve the impaired cell function. Thus, the aim was to test the therapeutic potential of CMC2.24 (a) in vivo, in severely hyperglycemic diabetic rats with/without thioglycolate stimulation in the peritoneal cavity; and (b) in ex-vivo cultured macrophages.

Materials and Methods

Animal Study

The animal study was approved by Stony Brook University’s Institutional Animal Care and Use Committee (IACUC, #230617-23), and was performed in strict accordance with the approved protocol. Animals were housed in the Division of Laboratory Animal Resources (DLAR) at Stony Brook University, with care provided by the center’s personnel. This facility follows the Animal Welfare Act (USDA enforced), the Public Health Service Act (OLAW enforced), and NY State law (DOH enforced), and is an AAALAC International accredited facility. The Three Rs (3Rs) were reviewed by SBU DLAR and approved. All procedures were conducted at the same location and were overseen by a fully-qualified veterinarian who was also available for general anesthesia, analgesia, routine, surgical care and euthanasia. Thirty-six adult male Sprague-Dawley rats (Strain code: CD001; Body Weight/Age: 276–300 grams/60–65 days; viral antibody free; Charles River Laboratories International, Inc., Wilmington, MA, USA) were used in two models: the non-elicited and elicited models. Eighteen rats in each model were randomly distributed into three groups: non-diabetic control (N), diabetes (D), and diabetes+CMC2.24 treatment (D +CMC2.24) groups (n=6 rats/group).²⁷ All rats were single housed in proper sterile-filter capped cages, and were given unlimited access to food and water. Type I diabetes was induced by I.V. injection of streptozotocin (STZ, 70mg/kg body weight; ENZO Life Sciences, Inc.,

Plymouth Meeting, PA, USA) through tail vein. The N rats were injected with 10mM citrated saline buffer, pH4.5, served as controls. Hyperglycemia were confirmed in urine by using nonenzymatic test-strips (CTMI 4 LN; Cole-Taylor Marketing Inc., Redsa, CA, USA) in all STZ-injected D and D+CMC2.24 rats. CMC2.24 was administered 30mg/kg/daily to the D +CMC2.24 group by oral gavage for 3 weeks. The N and D groups were administered vehicle-alone (1mL of a 2% carboxymethylcellulose suspension) by oral gavage for the same period of time. Each rat has an equal chance of being assigned to the treatment or vehicle-alone.

Non-Elicited Model

At the end of 3-weeks therapy, all rats (N, D, and D+CMC2.24 groups) were fasted overnight and euthanized by CO₂ inhalation. Resident peritoneal wash (rPW, non-elicited) was collected by peritoneal lavage following intraperitoneal injection of 15mL of sterile-cold PBS/3mM EDTA.

Elicited Model

Four days prior to ending of the 3-weeks therapy, all rats in three groups were injected intraperitoneally with 3% thioglycolate medium (Thermo Fisher Scientific, Waltham, MA, USA) to elicit a macrophage inflammatory response.²⁸ The thioglycolate-elicited peritoneal exudates (tPE) were collected as described previously at 3 weeks.²⁸ Jawbones were collected for micro-computerized tomography (μ CT) analysis. Blood was collected by cardiac puncture for analysis of blood glucose and glycated hemoglobin (HbA1c) levels.

Chemical Reagents

CMC2.24 was synthesized and provided by Chem-Master Intl., Inc. (99.5% pure, Stony Brook, NY, USA). Carboxymethyl cellulose as placebo was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All cell culture reagents and other chemical reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Three-Dimensional μ CT Morphometric Analysis and Quantification of Bone Loss

In the elicited model, jawbones were fixed and placed in a standardized sample holder. The maxillae were scanned using high-resolution μ CT (μ CT40; Scanco Medical, Bassersdorf, Switzerland) at 20.0 μ m voxel size, followed

by reconstruction of the μ CT images. Results were analyzed using μ CT V6.0 software on the HP open platform (OpenVMS Alpha Version 1.3–1 session manager). Two reference points (bucco-palatal embrasure) were identified on the distal surface of the 1st molar and the mesial surface of the 2nd molar at the height of contour on coronal sections. The average distance from the cemento-enamel junction to alveolar bone crest (CEJ-ABC) was measured to quantify alveolar bone loss at bucco-palatal sites of the distal surface of the 1st molar and the mesial surface of the 2nd molar.²⁹

Volume of interest (VOI) was generated by delineating continuously the contours on the axial planes between the distal root of the 1st molar and the mesial root of the 2nd molar. The relative bone VOI was calculated by excluding the root and crown volume from the total VOI, then using a bone volume fraction [bone volume/total volume (BV/TV)] to obtain the volume of mineralized bone per unit volume of the sample, based on counting voxels.²⁹ In addition, bone mineral density (BMD) was calculated by mean voxel value in units of hydroxyapatite density (mg HA/cm³).³⁰

Resident Peritoneal Washes (rPW) and Thioglycolate-Elicited Peritoneal Exudates (tPE)

The rPW and tPE were collected and centrifuged at 1000 rpm for 5 min, 4°C, to separate supernatant cell-free peritoneal fractions (CFPFs) and the mixed cells in the peritoneal cavity. The CFPFs were analyzed for MMP-2 and MMP-9 by gelatin zymography. Peritoneal monocytes/macrophages (M ϕ s) were isolated and purified from the mixed cells.

Gelatin Zymography

Assays for MMP-2 (pro-form: 72 kDa; active-form: 62 kDa) and MMP-9 (pro-form: 92 kDa; active-form: 82 kDa) in the CFPFs from rPW and tPE in non-elicited and elicited models were conducted by gelatin zymography, as described previously.²¹

Cell Culture Studies (ex-vivo)

The peritoneal mixed cells from rPW and tPE were re-suspended in PBS/3mM EDTA. The cell suspension was layered onto Lymphoprep™ (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) at a ratio of 2 to 1–1.5 (v/v) then centrifuged at 1800 rpm for 30 min at

25°C. Peritoneal M ϕ s were isolated/purified by the density gradient centrifugation and adherence as described previously.³¹ The non-elicited and elicited M ϕ s were counted and cultured in serum-free media (Thermo Fisher Scientific Inc., Boston, MA, USA) in 24-well plates for 18 hours (ex-vivo). Each well contained 10⁶ cells/mL, supplemented with 100units/mL penicillin and 100 μ g/mL streptomycin, in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Cell Migration Assay

The elicited peritoneal M ϕ s were harvested and directly counted by TC20™ Automated Cell Counter (Bio-Rad Laboratories, Inc. USA). For cell migration/chemotaxis assay, the assay kit (24-well, 5 μ m) (ab235696, Abcam plc. USA) was used with a Boyden chamber, where the cells migrate through a semi-permeable membrane under different stimuli. We used culture medium from LPS-stimulated macrophage as chemoattractant for macrophage migration in the assay. Cell migration was analyzed directly by reading fluorescence (Ex/Em = 530/590 nm) in a plate reader and was performed according to manufacturer's instructions.

ELISA Assay

The supernatants from cell culture medium (ex-vivo) of non-elicited and elicited peritoneal M ϕ s were analyzed for three pro-inflammatory cytokines: IL-1 β , IL-6, and TNF- α (R&D Systems, Inc., Minneapolis, MN, USA), and three resolvins: RvD₁ (Cayman Chemical., Ann Arbor, Michigan, USA), Resolvin E₁ (RvE₁) (MyBioSource, Inc., San Diego, CA, USA), and Lipoxin A₄ (LxA₄) (Neogen Corporation, Lexington, Kentucky, USA) by ELISA, and performed according to manufacturer's instructions.

Blood Glucose and HbA1c

Blood was collected by cardiac puncture for analysis of blood glucose (One Touch Ultra Glucometer, Johnson & Johnson, NJ, USA) and glycated hemoglobin (HbA1c) levels (Bayer A1CNow Self check, Sunnyvale, CA, USA) by standard procedures.

Statistical Analysis

SPSS19.0 (IBM) were used to analyze the data. The levels of MMPs, pro-inflammatory cytokines, resolvins, and bone evaluation for N, D, D+CMC2.24 groups were determined by analysis of variance (ANOVA), and also by Student's *t*-test (two investigators carried out the data analysis, separately), with *p* < 0.05 taken as statistically significant. Each value represented Mean (n=6/group) \pm

Standard Error (S.E.M.). All experiments were independently performed at least 3 times.

Results

Peritoneal Macrophage Cell Function Cell Accumulation and Chemotactic Activity

Diabetic rats exhibited an abnormal 77% increase (*p* < 0.05) in macrophage accumulation (Figure 1A) and a 24% reduction (*p* < 0.05) in chemotactic activity when compared to normal rats during thioglycolate-stimulation (Figure 1B). Both abnormalities were "normalized" by treatment of diabetic rats with CMC2.24. This treatment significantly reduced macrophage accumulation by 87.6%, and improved cell migration ability by 44.1% (*p* < 0.05) (Figure 1A and B).

Pro-Inflammatory Cytokines

Diabetes significantly increased the concentrations of all three pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) in the thioglycolate-elicited peritoneal M ϕ s, compared to non-diabetic controls, by 5-folds, 7.2-folds, and 1.8-folds, respectively (*p* < 0.05) (Figure 1C–E). All three cytokines were reduced to normal levels by the CMC2.24 in vivo treatment (Figure 1C–E). In addition, resident non-elicited peritoneal M ϕ s secreted overall lower levels of IL-1 β compared to thioglycolate-elicited peritoneal M ϕ s (Figure 1F), but IL-1 β was increased in D group, and treatment with systemically-administered CMC2.24 again dramatically reduced IL-1 β by 90% to normal levels (*p* < 0.005) (Figure 1F). Also, impaired M ϕ s were "normalized" by CMC2.24 therapy. No effect was observed for IL-6 or TNF- α in the resident M ϕ s.

MMPs

In the thioglycolate-elicited model, both peritoneal M ϕ s and cell-free peritoneal fractions (CFPFs), from the diabetic rats, exhibited significantly elevated MMP-9 levels. In diabetic rats, pro-MMP-9 levels were increased by 80.4% in the peritoneal M ϕ s (Figure 2A); similarly, in the CFPFs, the levels of total-MMP-9 were increased in D group by 60%, compared to non-diabetics (*p* < 0.05) (Figure 2B). Crucially, in vivo CMC2.24 treatment resulted in a significant reduction of pro-MMP-9 by 89.4% (*p* < 0.05) in the peritoneal M ϕ s, and by 33% (*p* < 0.05) of total-MMP-9 in the CFPFs, respectively, back to normal levels (Figure 2A and B).

The MMP-2 levels were not detected in the elicited peritoneal M ϕ s, while both pro- and active-MMP-2 were produced in the CFPFs. In vivo CMC2.24 treatment

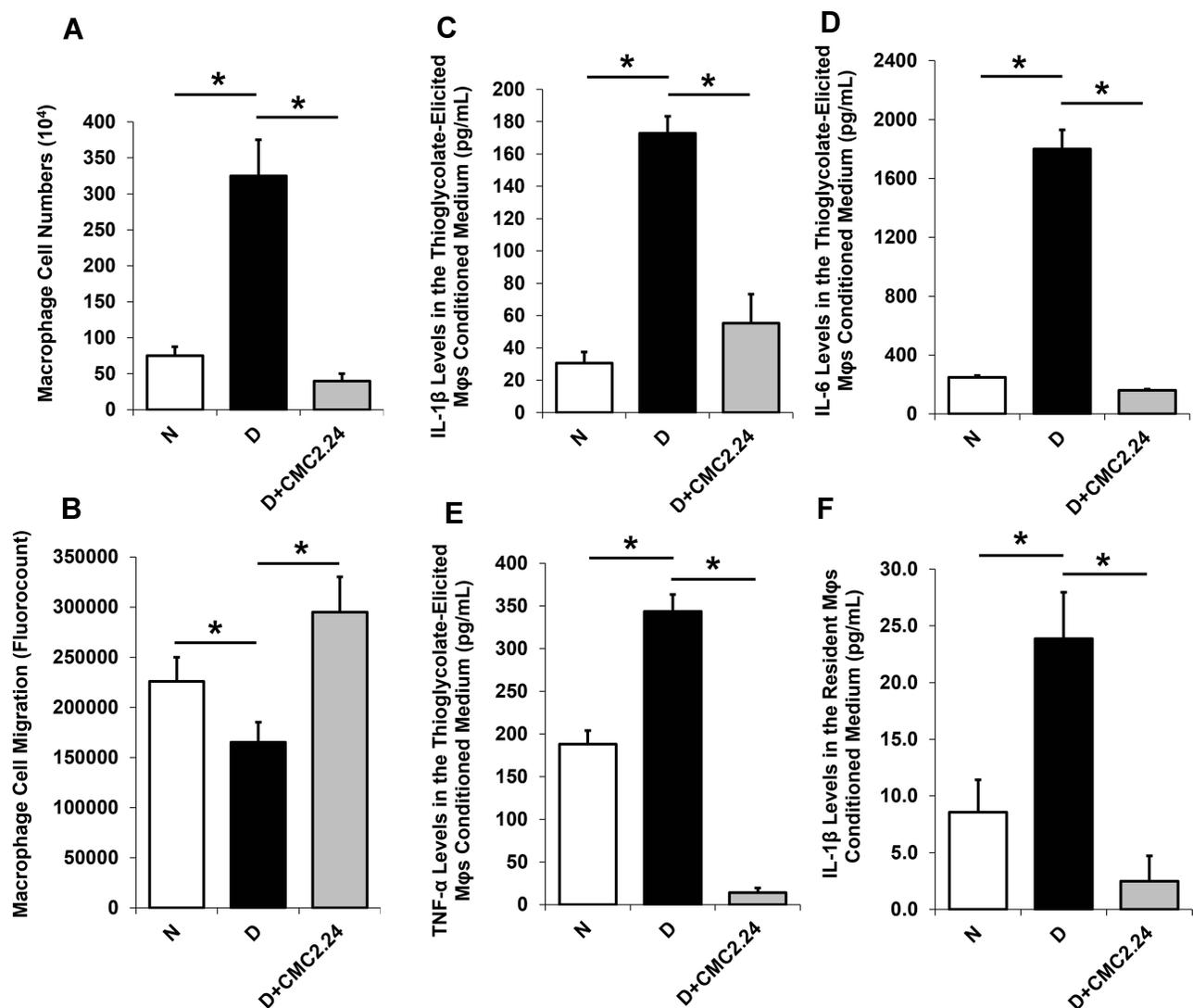


Figure 1 Effects of in vivo CMC2.24 treatment on peritoneal macrophage (Mφ) cell function and pro-inflammatory cytokines in the elicited and non-elicited models of diabetic rat. (A) Cell accumulation was measured by counting of Mφs cell numbers. Each column represents 10⁴ cells. (B) Chemotactic activity was measured by Mφs cell migration (fluorocount). (C–E) IL-1β, IL-6 and TNF-α levels in the thioglycolate-elicited Mφs conditioned media (pg/mL). (F) IL-1β levels in the resident non-elicited Mφs conditioned medium (pg/mL). Each value represents Mean (n=6/group) ± Standard Error (S.E.M.). *p < 0.05, values were compared between groups at the same time period. **Abbreviations:** N, normal group; D, diabetic group; D+CMC2.24, diabetes+CMC2.24 treatment group.

tended to reduce this gelatinase to normal levels, but the changes were not significant (data not shown).

In the non-elicited model, D group exhibited significantly increased MMP-9 levels in the CFPPFs, including the pro-form (92 kDa) ($p < 0.001$), and active-form (82 kDa) ($p < 0.05$), as well as the total-MMP-9 ($p < 0.001$), compared to N group (Figure 2C). The total-MMP-9 in D group was 81% higher than that in the non-diabetic controls. Treatment with systemically-administered CMC2.24 significantly decreased the pathologically-elevated levels of total-MMP-9 by 47% ($p < 0.05$).

Specifically, CMC2.24 treatment decreased the active-MMP-9 significantly ($p < 0.05$), compared to D group, and “normalized” this active-form of enzyme efficiently compared to N group ($p > 0.05$) (Figure 2C).

Diabetes also increased pro-MMP-2 (72 kDa) by 73% ($p < 0.001$) in the non-elicited CFPPFs, compared to normal rats, whereas CMC2.24 therapy reduced pro-MMP-2 levels by 56% ($p < 0.005$), back to normal levels (Figure 2D); the pro-MMP-2 levels in the N and D +CMC2.24 groups were not statistically different ($p > 0.05$).

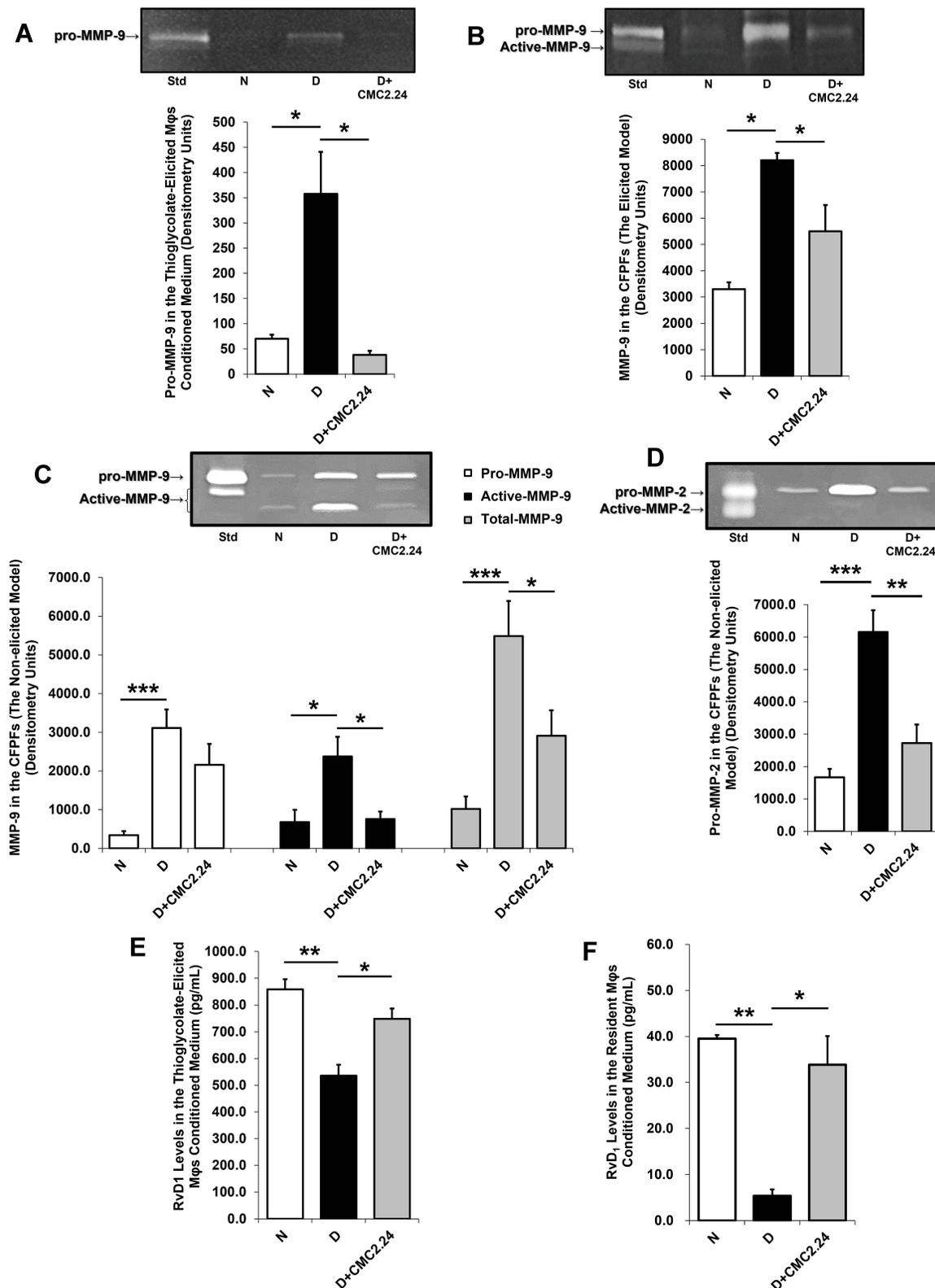


Figure 2 Effects of in vivo CMC2.24 treatment on MMPs and Resolvin D_1 in the elicited and non-elicited models of diabetic rat. (A) MMP-9 activities were analyzed by gelatin zymography and scanned by densitometer, and pro-MMP-9 levels were quantified by image J in the thioglycolate-elicited M ϕ s conditioned media. (B) MMP-9 activities were analyzed by gelatin zymography, scanned by densitometer, and quantified by image J in the elicited cell-free peritoneal fractions (CFPFs). (C) The pro-, active-, and total-MMP-9 activities were analyzed by gelatin zymography, scanned by densitometer, and quantified by image J in the non-elicited peritoneal CFPFs. (D) MMP-2 activities were analyzed by gelatin zymography and scanned by densitometer; and pro-MMP-2 levels were quantified by image J in the non-elicited peritoneal CFPFs. (E) RvD $_1$ levels (pg/mL) in thioglycolate-elicited M ϕ conditioned medium. (F) RvD $_1$ levels (pg/mL) in non-elicited resident M ϕ conditioned medium. Each value represents Mean (n=6/group) \pm Standard Error (S.E.M.). *p < 0.05; **p < 0.005; ***p < 0.001, values were compared between groups at the same time period.

Abbreviations: N, normal group; D, diabetic group; D+CMC2.24, diabetes+CMC2.24 treatment group.

Resolvins

The thioglycolate-elicited peritoneal M ϕ s produced overall much higher levels of RvD₁ (20-folds increase) than the non-elicited resident M ϕ s (Figure 2E and F). Diabetes significantly suppressed the RvD₁ levels by 36% in elicited M ϕ s (Figure 2E) and 86% in non-elicited M ϕ s (Figure 2F) ($p < 0.005$). CMC2.24 treatment of diabetics increased RvD₁ levels by 29% in elicited M ϕ s (Figure 2E) and 84% in non-elicited M ϕ s (Figure 2F) ($p < 0.05$), restoring this resolvins in the diabetics essentially back to normal levels. The other resolvins, RvE₁ and LxA₄, were undetectable in both models of rat peritoneal M ϕ s.

Diabetes-Induced Periodontitis and Osteoporosis

Alveolar Bone Loss

In the elicited model, morphometric analyses showed a pattern consistent with diabetes-induced periodontitis. The severity of alveolar bone loss, as represented by an increase of the CEJ-ABC distance bucco-palatally (Figure 3A) on the distal side of the 1st molar were significantly greater ($p < 0.05$) in D group than rats in N and D +CMC2.24 groups (Figure 3B). The lack of a significant difference in alveolar bone loss between the latter two groups ($p > 0.05$), indicated that CMC2.24 treatment was able to

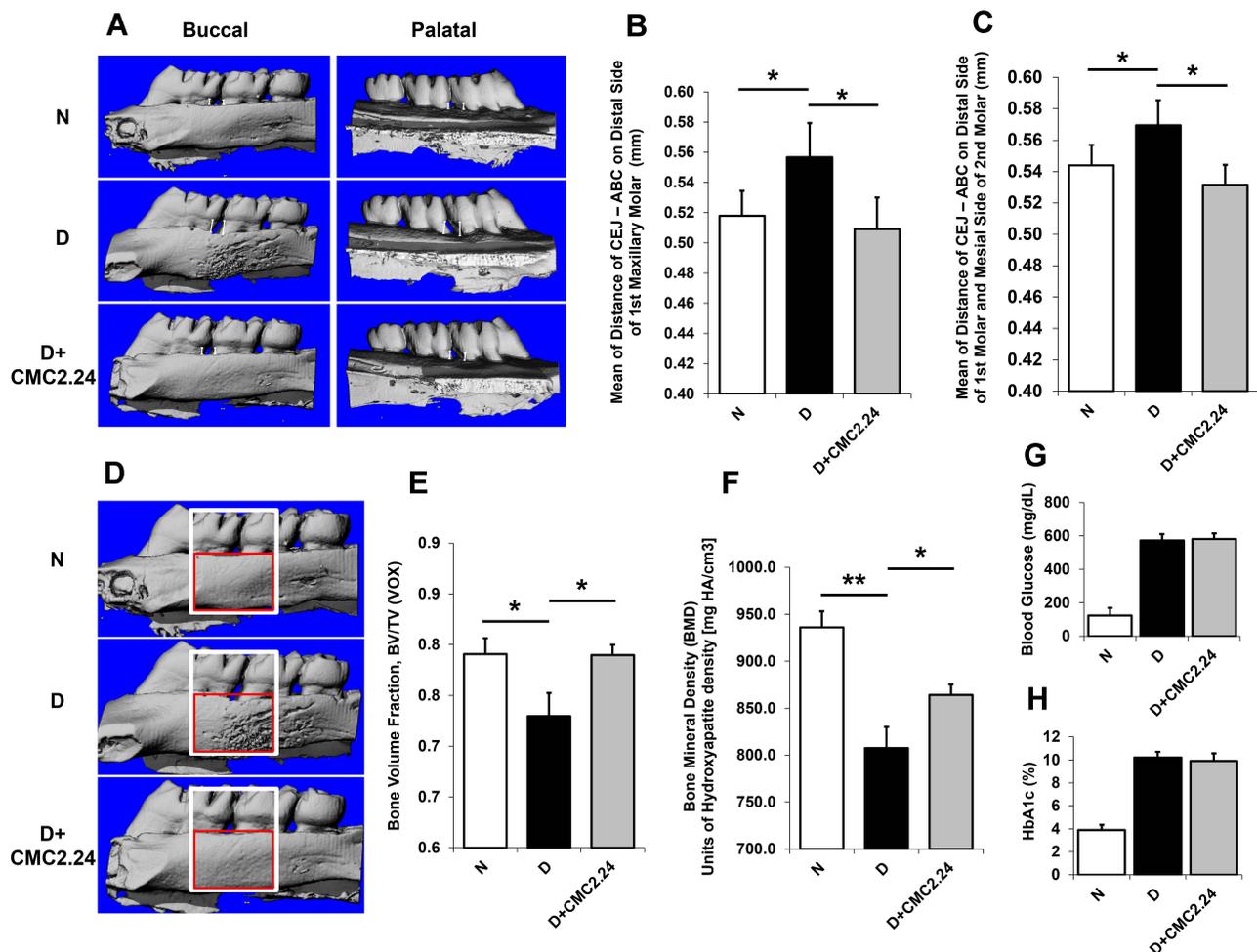


Figure 3 Morphometric analyses of diabetes-induced periodontal alveolar bone loss and osteoporosis measured by μ CT in rat with 4-day thioglycolation. (A) White "markers" (flat-end line, "I") indicates the distance from CEJ to ABC at the sites between the distal side of the 1st molar and the mesial side of the 2nd molar; bucco-palatally, representing alveolar bone loss. (B) Mean of distance (mm) of CEJ-ABC at the distal side of the 1st maxillary molar on both buccal and palatal view. (C) Mean of distance (mm) of CEJ-ABC at both the distal side of the 1st molar and the mesial side of the 2nd molar bucco-palatally. (D) Morphometric analyses of diabetes-induced osteoporosis measured by μ CT in rat with 4-day thioglycolation. The total volume of interest (VOI) area indicates in the white box. And the bone volume of interest area indicates in the red box. (E) The analysis of bone volume fraction (BV/TV) by μ CT. (F) The analysis of bone mineral density (BMD) by μ CT based on calculating the units of hydroxyapatite density (mg HA/cm³). (G) The levels of blood glucose (mg/dL) in normal/control (non-diabetic), untreated diabetic and the CMC2.24-treated diabetic rats, respectively. (H) The levels of HbA1c (%) in normal/control (non-diabetic), untreated diabetic and the CMC2.24-treated diabetic rats, respectively. Each value represents Mean ($n=6$ /group) \pm Standard Error (S.E.M.). * $p < 0.05$; ** $p < 0.005$, values were compared between groups at the same time period.

Abbreviations: VOX, based on counting voxels; TV, total volume (mm³); BV, bone volume (mm³); N, normal group; D, diabetic group; D+CMC2.24, diabetes+CMC2.24 treatment group.

“normalize” excessive alveolar bone loss. Consistent with these results, the total levels of buccal-palatal alveolar bone loss on both the distal side of the 1st molars and the mesial side of the 2nd molars were dramatically increased ($p < 0.05$) in D group, compared to the values in N and D+CMC2.24 groups (Figure 3C). Importantly, no significant difference of alveolar bone loss was detected between the latter two groups ($p > 0.05$), showing the efficacy of CMC2.24 treatment in preventing alveolar bone loss due to diabetes.

Osteoporosis of Jawbone

In the elicited model, diabetes-induced osteoporosis was observed in the jawbone. The 3D- μ CT morphometric analyses of the maxilla showed the porosity in these bones was dramatically increased in untreated diabetics, compared to N and D+CMC2.24 groups (Figure 3D). The latter two groups had no difference of porosity. This was confirmed by the analysis of bone volume fraction (BV/TV): As shown in Figure 3D, BV and TV were indicated by the red and white boxes, respectively. D rats exhibited a significantly smaller percentage of BV/TV ($p < 0.05$), indicating the lower relative BV than N and D+CMC2.24 rats (Figure 3E). CMC2.24 treatment increased ($p < 0.05$) the relative BV back to essentially normal levels (Figure 3E). Moreover, analysis of BMD was consistent with the results of relative BV, presenting a substantial reduction ($p < 0.005$) of BMD in the untreated diabetics (Figure 3F), while the reduced BMD in the maxilla of diabetic rats was “rescued” by CMC2.24 treatment ($p < 0.05$), back to normal status ($p > 0.05$) (Figure 3F).

Blood Glucose and HbA1c

The blood glucose and HbA1c levels were 600 mg/dL and 10% (Figure 3G and H), respectively, in the untreated and CMC2.24-treated diabetic rats. Note that, for all of these beneficial changes produced by CMC2.24 treatment, no improvement was seen for blood glucose and HbA1c; D and D+CMC2.24 rats were still severely hyperglycemic at the end of study, similar to our previous observation.²⁷ However, in spite of the persistent hyperglycemia, there were no adverse events in D+CMC2.24 group during the 3-week protocol, in contrast to the untreated diabetics which showed multiple complications such as tail necrosis and inflamed sclera of the eye.

Discussion

Research has shown that periodontitis is associated with other chronic systemic diseases, such as diabetes,³²

osteoporosis,³³ rheumatoid arthritis³⁴ and coronary heart disease.³⁵ For a long time, it was thought that microbial biofilm was the factor that linked periodontitis to other diseases in the body; however, more recent research³⁶ demonstrates that inflammation may be largely responsible for the association. Therefore, treating inflammation and modulating the host response may not only help manage periodontitis, but may also help with the management of other chronic inflammatory conditions.^{16,37–39}

Recently, we identified the pleiotropic properties of a novel HMT compound, CMC2.24, in three rat models of experimental periodontitis.^{21,27,40} Its ability to “normalize” the inflammation/collagenolysis was also identified in oral and extraoral tissues.^{21,27,40} In the current report, we extend these studies of CMC2.24 to include a previously unrecognized ability of this phenylaminocarbonyl curcumin to upregulate resolvin activity and “normalize” immune cell functions, in addition to extending our earlier studies on normalization of pathologically-excessive cytokines and MMPs. Thus, we found that the function of macrophages which is suppressed by diabetes, was beneficially affected by CMC2.24’s resolvin-like activity.

In brief, the impaired immune-cell function caused by hyperglycemia is associated with diabetes-induced complications,⁴¹ thus thioglycolate was used as a stimulus to induce an inflammatory response in the peritoneal cavity of diabetic rats. In the elicited model, diabetes led to an abnormally increased accumulation of macrophages in the peritoneal cavity. However, these cells exhibited impaired function characterized by: decreased chemotaxis, and increased production of both pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) and pathologically-elevated collagenolytic enzymes (MMP-2 and MMP-9). The therapeutic potential of CMC2.24’s resolvin-like activity was observed in this study by “normalizing” cell function and restoring cell activity during inflammation. And its therapeutic potential is also supported by several recent publications which indicated: (a) a distinct and early increase in circulating MMP-9 in COVID-19 patients with respiratory failure, and demonstrated that only the plasma marker, MMP-9, correlated to neutrophil count and the severity of COVID-19 related pneumonia,⁶ and (b) the potential of using HMT (NON-antibiotic doxycycline, developed and used initially for chronic inflammatory periodontitis) as a strategy for the prevention of ARDS, a deadly outcome of COVID-19.⁴²

In addition, we also evaluated the resident macrophage cell function in the non-elicited model. Similar pathologic

conditions in these cells were observed during diabetes, but at much lower levels compared to the levels under thioglycolate stimulation. Specifically, CMC2.24-treated diabetic rats showed a significant reduction of a key pro-inflammatory cytokine, IL-1 β , in the non-elicited resident macrophage. Moreover, data indicated that in the rPW, pathologically-elevated activities of two gelatinases (MMP-9 and MMP-2) were also “normalized” by CMC2.24 treatment in the diabetic rats.

Of importance, this study describes a significant increase in resolvin (RvD₁) production in both non-elicited and elicited macrophages from the diabetic rats treated with CMC2.24 *in vivo*, and is consistent with our previous observations in a macrophage cell culture study.⁴³ This important lipid mediator is derived from DHA, and exhibits a potent ability to resolve acute inflammation, thus preventing its prolongation.⁴⁴ Studies have shown that RvD₁ involves the inactivation of glycogen synthase kinase 3 beta (GSK3 β), a serine/threonine protein kinase involved in inflammation, simultaneously augmentation of GSK3 β anti-inflammatory axis, and thus enhances resolution in LPS-stimulated/TLR4-engaged primary human monocytes.⁴⁵ Although RvD₁ has usually been associated with resolution of polymorphonuclear leukocyte infiltration/migration during acute inflammation,^{46,47} our studies indicate that RvD₁ can also be produced by macrophages both in cell culture, and in animals, to resolve chronic inflammation, and was significantly increased by CMC2.24 treatment. Moreover, the enhanced activity of RvD₁ seems to be the “targeted resolvin” of CMC2.24 treatment. The other major resolvins such as RvE₁ and LxA₄ have less sensitivity to CMC2.24 treatment, compared to RvD₁. More mechanisms can be further explored in future studies.

Regarding broad therapeutic potential, we also observed a potent ability of CMC2.24 to reduce diabetes-induced bone height loss in alveolar bone, and diabetes-induced osteoporosis in rat jaws. This complication in the rats with severe hyperglycemia was characterized by increased roughness and porosity of the maxilla based on 3D-reconstructed images, and further confirmed by μ CT morphometric analysis of BV and BMD analysis. These data demonstrated no significant difference between the CMC2.24-treated diabetic rats and non-diabetics, indicating that CMC2.24 treatment prevents local and systemic bone loss (diabetes-induced periodontitis and osteoporosis). However, whether treatment with CMC2.24 can reduce the risk for pathologic fracture of skeletal bones in poorly controlled diabetics also remains to be demonstrated in future studies.

Notably, all these beneficial responses of diabetic rats to CMC2.24 occurred without any reduction in the severity of hyperglycemia. This observation suggests the potential of CMC2.24 treatment for reducing serious diabetic complications, even during uncontrolled hyperglycemia, which is supported by the lack of adverse events in the CMC2.24-treated diabetics at least during the 3-weeks protocol.

Conclusion

In conclusion, the current study indicates that *in vivo* administration of CMC2.24 to diabetic rats “normalizes” inflammatory (and other) cell functions as shown by: (a) decreasing the abnormal accumulation of macrophages in the peritoneal cavity; (b) improving inflammatory cell chemotactic activity; (c) significantly suppressing both pathologically-elevated levels of MMPs and their activation; (d) attenuating the severity of systemic inflammation as indicated by reduced pro-inflammatory cytokines in peritoneal macrophages (*ex vivo*); and (e) significantly enhancing resolvin (RvD₁) activity. These beneficial effects ultimately contributed, at least in part, to the reduction of local alveolar bone loss (diabetes-induced periodontitis), and the severity of systemic bone loss (diabetes-induced osteoporosis). These anti-collagenolytic (MMP-inhibition), anti-inflammatory, and pro-resolvin benefits occurred without any effect on severe hyperglycemia, and no adverse events were observed in the CMC2.24-treated diabetic rats, strongly indicating the therapeutic potential of this novel compound even in uncontrolled diabetics. Lastly, pharmacokinetic and safety studies⁴⁸ are being carried out in different animal models, which is necessary before this compound can be considered for testing in human clinical trials.

Data Sharing Statement

All other supporting information is available upon request from the corresponding author.

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Disclosure

LM Golub is listed as an inventor on several related patents, including CMC2.24, and these have been fully assigned to his institution, Stony Brook University, The State University of New York (SUNY). F Johnson is also listed as an inventor on several related patents, including CMC2.24, which have been fully assigned to Stony Brook University and to Chem-Master Int. Inc., on a shared basis. In addition, Traverse Biosciences Inc. has exclusively licensed patents from the Research Foundation for the State University of New York (RF/SUNY) covering the structure and use of the chemically modified curcumins for the purpose of commercialization in human and animal health. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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