COMPARISON OF THE EFFECTS OF TRADITIONAL VS. MODIFIED CALCIUM CHANNEL BLOCKERS ON MATRIX METALLOPROTEINASE PRODUCTION IN HUMAN GINGIVAL FIBROBLASTS

By

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Submitted to the Faculty of the School of Graduate Studies

of the Medical College of Georgia in partial fulfillment

of the Requirements of the Degree of Master of Science

June

2007

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This thesis is submitted by Shahin Madi, D.M.D. and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore in partial fulfillment of the requirements for the degree of Master of Science.

6/26/07

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ACKNOWLEDGMENTS

From the inception of this project, there seemed to be tremendous amount of challenges and obstacles preventing its completion. I am very grateful to many people who have aided me in overcoming them, and without whom I could not have been successful.

This thesis is dedicated to my family with love. They have provided me with unconditional support and have encouraged me to push forward.

An expression of deep gratitude is extended to the following people: Bharati Bhatt and Julie Winger for helping me with the experiments and putting my thoughts together to finish this project.

Dr. James Borke who stepped forward to take this project with the greatest insight and supportive major advisor as one could hope to find.

Dr. Carol Lapp for her expertise with tissue culture, developing the protocol for quantifying MMP, and the full support of her lab to help me complete this project..

Dr. Ahmed Khocht helped incorporate the controversy of plaque, and also encouraged me to complete this project.

Dr. Mahmood Mozaffari for helping evaluate the dose responses of my drugs and the pharmacological simulations in cell culture.

Dr. Rafik Abdelsayed for his valuable input concerning gingival overgrowth.

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I. INTRODUCTION

A. Statement of the Problem

Calcium channel blockers (CCBs) are commonly used treatments for cardiovascular disorders. They are generally associated with the undesirable side-effect of gingival overgrowth which can adversely affect esthetics, function, and increase plaque-retention. Different CCBs induce overgrowth to varying degrees. The pathogenesis of this condition has not been fully elucidated. Matrix metalloproteinases (MMPs) are important enzymes involved in tissue remodeling. Recent evidence shows that CCBs suppress gingival fibroblast production of MMPs. This suppression of MMP production may cause tissue-remodeling imbalance which leads to gingival overgrowth. Further, plaque can exacerbate the gingival overgrowth. Interleukin-1β (IL-1β) has been proven to be one of the main products of plaque in the oral cavity. We hypothesized that MMP gingival fibroblast production levels vary according to the type of CCBs used, which may explain the differences in their induction of gingival overgrowth. IL-1β was used in conjunction with the CCBs to evaluate the effects of MMP expression. The aim of this study compared the effects of two different CCBs on gingival

fibroblast MMP production *in vitro* and, using CCBs in the presence and absence of IL-1β, to account for plaque in the oral cavity.

More than 80% of patients on a regimen of ongoing traditional CCB therapy have signs of adverse oral effects such as gingival overgrowth (Fattore *et al.*, 1991). Current treatments of gingival overgrowth require cessation of the causative drug therapy and surgical excision of the affected oral tissue. Gingival overgrowth will likely reoccur with current treatment methods unless traditional CCB therapy is stopped completely. This requires the patient to weigh cardiovascular health against oral health. However, the recent advent of new or "modified" CCBs may provide an alternative to the traditional CCBs. These newer CCBs may alleviate the undesirable oral side effect of the traditional cardiovascular medication without sacrificing cardiovascular health. There is a lack of understanding of the mechanism of drug-induced gingival overgrowth and how it occurs with different CCBs. Such knowledge could lead to improved drug design or selection.

Human gingival overgrowth is believed to be a function of matrix metalloproteinase (MMP) production caused by traditional calcium channel blockers (CCBs) (Seymour, Thomason, Ellis, 1996). A new generation of calcium channel blockers may have less of an effect on MMP production and gingival overgrowth than the traditional CCBs. This research explored the differences between traditional and modified calcium channel blockers, as explained in the CCB section B-2 of this thesis, on normal human gingival fibroblast production of matrix metalloproteinases (MMPs), MMP-7 and 8, *in vitro*

using In-Cell Western Assay to monitor and quantify MMP activity. IL-1β was used to account for the plaque activity in the oral cavity.

B. Review of Related Literature

B-1. Gingival Overgrowth

Up to 83% of patients taking calcium channel blockers (CCBs) report indications of gingival overgrowth (Fattore *et al.*, 1991; Ramon *et al.*, 1984; Shaftic *et al.*, 1986). The use of CCBs such as nifedipine, verapamil, and diltiazem, for management of cardiovascular disorders including angina, arrhythmia, and hypertension, increased dramatically in recent years. While CCBs are generally well-tolerated, with only mild side effects, several CCBs are associated with a higher incidence of side effects. For example, gingival overgrowth (GO), or excessive growth of the gums, is common with diltiazem and nifedipine, but can occur with any CCB (Marshall & Bartold, 1999). Furthermore, recent studies conclude that, in patients on immunosuppressive therapy such as cyclosporine and tacrolimus, concurrent CCB therapy exacerbates gingival overgrowth (Ellis *et al.*, 2004).

In current terminology, "gingival overgrowth," has replaced the term "gingival hyperplasia" (Dongari-Bagtzoglou, 2004). After histological evaluation of tissue sections, it was determined that the gingival fibroblasts were neither increasing in size (hypertrophic), nor in number (hyperplastic) (Hassell & Hefti, 1991). The only

changes in the parameters of the biopsies were an increase in the percentage volume of vessels and the amounts of interstitial substance (Heijl & Sundin, 1987). Further histopathologies of the gingival lesions with all drugs (CCBs, anticonvulsants, and immunosuppressants) were similar, and characterized by excessive accumulation of extracelluar matrix proteins, like collagen, or amorphous ground substance (Marshall & Bartold, 1999; Dongari *et al.*, 1993; Hallmon & Rossmann, 1999). The drug-induced GO usually begins about 2-3 months after starting the initial drug therapy and reaches maximum severity in 12-18 months (Livingston, 1970). The enlarged tissue is typically pale pink, firm, dense, mulberry shaped, and fibrotic with an increased tendency to bleed. The tissue surface may appear either stippled or smooth (Carranza & Newman, 1996).

While GO is usually generalized throughout the mouth, it can be more severe in the mandibular and maxillary anterior regions where teeth are present, and is absent in edentulous areas (Carranza & Newman, 1996). The condition begins painlessly around the facial and lingual gingival tooth margins and interdental papillae. As the course of this condition progresses, the marginal and papillary enlargements unite and develop into a massive tissue covering a major portion of the crowns, and sometimes interferes with occlusion, mastication, oral hygiene, esthetics, and speech (Carranza & Newman, 1996). This enlargement is chronic in nature and increases in size with time. Surgery is usually required to eliminate the enlarged areas. However, even after surgical excision, GO usually recurs unless CCB therapy is withdrawn (Carranza & Newman, 1996).

B-2. Calcium Channel Blockers

CCBs, also called calcium antagonists, work by blocking calcium ion entry into heart muscle and blood vessels, thus causing blood vessels to dilate and heart contraction to slow. Intracellular (cytoplasmic) calcium concentrations are directly regulated through a variety of ion pumps, channels, and exchangers, providing many possible sites for therapeutic agents to act. In a cell at rest, the concentration of cytoplasmic free calcium is normally maintained at very low levels relative to the extracellular calcium. Once stimulated, the rapid elevation of cytoplasmic free calcium concentrations occurs by the opening of channels in the sarcolemma/plasmalemma and sarcoplasmic/endoplasmic reticulum (Robertson & Robertson, 1996). Upon removal of stimulation, ATP-dependent ion-pumps and Na^{+}/Ca^{++} exchangers return the cytoplasmic free calcium concentrations to resting levels.

Plasmalemmal calcium channels allow extracellular calcium to enter cells. They can be divided into three major categories of channels: voltage-dependent, receptor-operated, and stretch-operated. Receptor-operated and stretch-operated calcium-channels (e.g. $alpha_1$-adrenergic receptors) are not very sensitive to CCBs (Robertson & Robertson, 1996; Katzung & Chatterjee, 2001). Voltage-dependent calcium-channels, homologous to Na^{+}- and K^{+}-channels, consist of at least three types in the body: L, T, and N-type (Robertson & Robertson, 1996). Most CCBs work by binding to L-type channels. T and N-type channels are not very sensitive to L-type CCBs (Robertson & Robertson, 1996).

CCBs are divided into two different classes proposed by the World Heath Organization: Group A and Group B (Vanhoutte, 1987). In order to distinguish the newer from the traditional CCBs for this research we have classified them as traditional and modified CCB. Traditional CCBs (e.g. dihydropyridines, verapamil, and diltiazem) work by binding to L-type channels ("slow channels"), which are abundant in cardiac and smooth muscle. Different classes of L-type CCBs bind to various sites on the $alpha_1$-subunit, which is also the major channel-forming subunit ($alpha_2$, beta, gamma, delta subunits are also present) (Katzung & Chatterjee, 2001). Different sub-classes of L-type channels exist that may contribute to tissue selectivity. Modified CCBs with different specificities also have been developed. For example, bepridil is a drug with both Na^+ and K^+ channel blocking activity, in addition to L-type channel blocking. Nisoldipine is a dihydropyridine that inhibits calcium ion influx across cellular membranes in vascular smooth muscle, resulting in vasodilation. Nisoldipine has a higher selectivity for calcium channels in vascular smooth muscle than in cardiac muscle.

The physiology of calcium-channels and the therapeutic effects of drugs that block their activities may be related to their effects on the induction of matrix metalloproteinases (MMPs), which could lead to gingival overgrowth. MMPs play a major role in cell migration by degrading the extracellular matrix compounds (Todaro *et al.*, 2003).

B-3. Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases-mediated collagenolysis is an important pathway in the remodeling and destruction of connective tissues in gingiva affected by periodontitis (Lapp *et al.*, 2003). MMPs form a very important family of proteinases that participate in the normal turnover of periodontal tissues as well as aspects of tissue degradation during periodontal disease (Uitto *et al.*, 2003). MMPs constitute a large subfamily of zinc- and calcium-dependent endopeptidases. These enzymes should be distinguished from neutrophil elastase, cathepsin G, kinins, plasmin, and other important proteolytic enzymes. MMPs are produced by several cell types like fibroblasts, macrophages, neutrophils, synovial cells, and some epithelial cells (Kumar, Abbas, Fausto, 2005). Intracellular collagen degradation by fibroblasts is a poorly-understood pathway for the physiological remodeling of connective tissue (Arora *et al.*, 2000). MMPs can be divided into at least 5 subtypes: collagenases (MMPs-1, -8, and -13); gelatinases (MMPs-2, and -9); stromelysins (MMPs-3, -10, -11, and -19); matrilysins (MMPs-7 and -26); and membrane bound-types (MMPs-14, -15, -16, -17, -24, and -25). The MMP family of enzymes contributes to normal and pathological tissue remodeling. MMPs play a key role in the migration of normal and malignant cells throughout the body. In oral health, they are associated with tissue remodeling, angiogenesis, tooth eruption, and salivary gland morphogenesis (Lapp *et al.*, 2003). These enzymes also act as regulatory molecules by functioning in enzyme cascades and by processing matrix proteins,

cytokines (i.e. IL-1, TNF), growth factors (i.e. PDGF, FGF), and adhesion molecules, to generate fragments that enhance or reduce biological effects (Opdenakker & Van Damme, 1992).

Although the link between single MMPs and individual substrates is not direct, it is clear that, as a family, MMPs are capable of breaking down extracellular matrix components. MMPs are released from lysosomal granules of phagocytosing neutrophils (Uitto *et al.*, 2003). Bacterial plaque stimulates the release of neutrophil collagenase and activates the latent enzyme (Sorsa *et al.* 1992). In normal physiology, MMPs produced by connective tissue are thought to contribute to tissue remodeling during development, in the menstrual cycle, and as part of repair processes following tissue damage. In inflammation, when one MMP is down-regulated in a mouse model, the functions are replaced by another MMP with overlapping activity (Woessner, 1999). This result suggests that each enzyme contributes to a concerted effort that mediates changes in extracellular matrix. The obvious destructive capability of MMPs caused initial research in this area to be focused on diseases that involve breakdown of connective tissues (*e.g.*, rheumatoid arthritis, cancer, and periodontal disease) (Opdenakker & Van Damme, 1992). Leukocytes, particularly macrophages, are major sources of MMP production. MMPs released by leukocytes play vital roles in allowing these cells to extravasate and penetrate tissues, a key event in inflammatory disease.

Even though MMPs can potentially destroy gingival tissues, their activity is strictly controlled. Specific inhibition of MMPs can be mediated by the four members of the tissue inhibitor of metalloproteinase (TIMP) family, which are

proteins that regulate the extracellular activity of MMPs (Brew *et al.*, 2000). Alteration in TIMP expression is also known to occur in many disease processes and affects the extracellular matrix and growth factors to modulate cell behavior (Uitto *et al.*, 2003). MMPs are synthesized as latent zymogens and most are secreted from cells as a soluble proform, while some are membrane-type MMPs (MT-MMPs), which are not released but exert their activity on the cell surface (Uitto *et al.*, 2003). Other considerations to help explain the mechanism would be substrate recognition and cleavage, MMP inhibition, and the domain-domain interactions that occur in the activation and association of MMPs and TIMPs with the cell membrane and the extracellular matrix (Uitto *et al.*, 2003).

MMP-8 (collagenase-2) is a major MMP in neutrophils and is able to potently degrade interstitial collagen. It is able to degrade types I, II, III, VII, and X forms of collagen, gelatin, entactin, aggrecan, and tenascin (Uitto *et al.*, 2003). It is one of the major collagen-degrading enzymes in gingival crevicular fluid and saliva (Ingman *et al.* 1996). It is also believed to have a major responsibility for collagen degradation in inflamed tissue in gingivitis and adult periodontitis (Lee *et al.* 1995).

MMP-7 (matrilysin) is an epithelial MMP with a broad spectrum of substrates which can degrade fibronectin, laminin, types IV, V, IX, X, and XI forms of collagen, gelatin, elastin, entactin, tenascin, and proteoglycan core proteins. It plays an important role in normal physiology and can degrade matrix and basement membrane components and disrupt the antibacterial defense of junctional epithelium (Uitto *et al.*, 2003). MMP-7 has also been proven to

process MMP-8 to its active form, as well as MMPs in the collagenolytic pathway, suggesting that MMP-8 is an *in vivo* substrate of MMP-7 (Dozier *et al.* 2006). There have been indications that MMP-7 is associated with innate host defense in periodontal tissues (Emingil *et al.* 2006).

B-4. Interleukin-1β (IL-1β)

Cytokines are best defined as proteins (8-80 kDa molecular weight) that utilize a paracrine, autocrine, endocrine, or intracrine signaling mechanism. These potent molecules are produced in a transient and tightly regulated manner. Cytokines act by binding to specific receptors at the cell membrane that can begin a cascade that leads to induction, enhancement or inhibition of a number of cytokine-regulated genes in the nucleus.

Interleukin-1 (IL-1) is a proinflammatory cytokine that initiates many immune responses: T-cell proliferation, increased production of lymphokines by T-lymphocytes, enhancement of B-Cell antibody production, and induction of interferon synthesis (Ebersole & Taubman, 1994). This inflammation disturbs the equilibrium between production and degradation of gingival connective tissue (Ebersole & Taubman, 1994). Several studies have demonstrated the expression and production of MMPs in gingival fibroblasts when stimulated by IL-1β (Birkedal-Hansen, 1993; Lapp *et al.*, 2003; Alvares *et al.*, 1995; Nakaya *et al.*, 1997). In periodontal disease, IL-1 causes attachment loss through the stimulation of the production of proteolytic enzymes which break down the

extracellular matrix and connective tissue. Gingival fibroblasts affected by periodontitis express IL-1β as a primary form of cytokine (Honig *et al.*, 1989; Jandinski *et al.*, 1991; Tokoro *et al.*, 1996).

C. Specific Aims

1. Evaluate the influence of a dihydropyridine, nifedipine, and IL-1β on the expression of MMP-7 and 8 in normal gingival fibroblasts in culture using quantitative In-Cell Western Assay.
2. Evaluate the influence of a modified dihydropyridine, bepridil, and IL-1β on the expression of MMP-7 and 8 in normal gingival fibroblasts in culture using quantitative In-Cell Western Assay.

D. Hypothesis

The traditional CCB, nifedipine, will decrease the production of MMP-7 and 8 in normal human gingival fibroblasts, which should be further decreased under the influence of IL-1β.

The modified CCB, bepridil, will increase the production of MMP-7 and 8 by normal human gingival fibroblasts more than traditional CCBs and IL-1β should decrease MMP production.

II. MATERIALS AND METHODS

A. Human Gingival Fibroblasts and Immunocytochemistry

Cells were grown on 8-well slides for immunocytochemistry for demonstration of the presence of MMP-7 and MMP-8. Cells were processed by means of a modified avidin-biotin-peroxidase technique (Hsu *et al.*, 1981; Borke *et al.*, 1987). Briefly, cells were fixed in 10% buffered formalin. Endogenous peroxidase activity was blocked by five-minute incubation in 0.3% H_2O_2. Non-specific binding of antibodies to cells was blocked by incubation for 1 hr in 10 mg/mL casein. Rabbit anti-human MMP antibodies (Sigma Antibodies, St. Louis, MO, USA) were applied to the cells at a dilution of 1:500 for 1 hr. Control cells were processed with normal rabbit serum, substituted for the anti-MMP antibodies. After cells were washed in PBS, a 1:200 dilution of the secondary biotin-conjugated goat anti-rabbit Ig antibody (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min. After being further washed in PBS, the cells were incubated for 30 min in the avidin-peroxidase complex reagent (ABC reagent, Vector). Following additional PBS washes, the peroxidase molecule in the immobilized avidin-peroxidase complex was used to reduce H_2O_2 in the presence of diaminobenzidine tetrahydrochloride (DAB), to produce a

brown reaction product over the sites of antibody binding to MMP. After being stained, cells were dehydrated in ascending concentrations of ethanol to xylene, and coverslipped with Permount.

B. Primary Cell Cultures of Human Gingival Fibroblasts

Human gingival fibroblast (HGF) samples were isolated from healthy gingivae during routine surgical procedures. Gingival tissues were harvested and immediately placed in a sterile test tube containing Hanks buffered salt solution (HBSS) (Atlanta Biological, Norcross, GA) supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml; Life Technologies GIBCO, Rockville, MD), and fungizone (amphotericin B; 1 µg/ml; Life Technologies GIBCO, Rockville, MD), then adjusted to a pH of 7.4. After washing three times in HBSS, the gingival explants were disinfected in Tris HCL buffer containing 0.5% sodium hypochlorite for five minutes, and then rinsed three more times with HBSS. All preparations were conducted under sterile conditions in a laminar flow tissue culture hood.

The disinfected gingival tissues were minced into pieces of 1 to 2 mm^3 and transferred into 35 mm^2 culture dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). The tissue pieces were allowed to air dry slightly to enhance the attachment to the surfaces of the culture dish. After attachment, Dulbecco's modified essential medium (DMEM) (Life Technologies GIBCO, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Atlanta

Biological, Norcross, GA) plus penicillin G (100 U/ml), streptomycin (100 U/ml), and fungizone (0.5 µg/ml) was carefully added. The explant cultures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for one week at which time cell growth could usually be seen. Epithelial, cobble-stone shape and the spindle-shape fibroblasts are distinguishable using a microscope. Epithelial cells rarely establish themselves to the flask but they can be separated by splitting when they are attached to the flask (Lapp *et al.*, 2003).

For routine culture, the DMEM (minus fungizone) was replaced every three days for one week in 75 cm^2 tissue culture flasks (Lux Scientific Corporation, Newberry Park, CA). The confluent fibroblasts were detached by trypsin-EDTA treatment and transferred into five 25 cm^2 tissue culture flasks which contained 4.5 x 10^5 cells per flask. For detachment, the existing medium was aspirated and the cells were washed with sterile HBSS. The cells were counted using the protocol for the Coulter Counter provided by the manufacturer (Beckman Coulter, Inc. Fullerton, CA, USA). The cells were then incubated for one week in phenol red-free DMEM containing 4% FBS made up of 96 ml of serum-free DMEM and 4 ml of FBS. The serum-free contains 100 ml phenol red-free DMEM, 0.125 ml Insulin Transferrin Selenium (ITS), 0.5 ml Oleic Acid – Albumin, and 1 ml Bovine Serum Albumin Fraction – V solution 7.5%. The medium was changed twice. After one week of incubation, the flasks of fibroblasts were exposed to either bepridil or nifedipine for an additional 7 days. New medium and calcium channel blocker solutions were applied every 48 hours. At day five, the cells were trypsinized, counted, and transferred from the

25 cm^2 tissue culture flask to a 96-well tissue culture plate containing DMEM with 0.5% FBS plus the same drug treatment. On day six, one-half of the 96-well culture plate was exposed to IL-1β at concentration of 1 ng/mL for 24 hours. On day seven, cells were harvested using the Odyssey In-Cell Western Assay (Li-Cor Biosciences, Lincoln, Nebraska) procedure.

Crystal violet and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were used to create a dose range for nifedipine and bepridil. For the crystal violet assay, the fibroblasts were fixed with 11% glutaraldehyde (10 µL/100 µL medium) and agitated for 20 minutes. After rinsing with distilled water, plates were dried overnight. Cells were stained with 0.1% crystal violet in 0.2 M MES, pH 8.0, while shaking for 20 minutes, then were rinsed and dried. The dye was then solubilized by the addition of 100 µL / well of 10% acetic acid, and the optical density at 562 nm was determined in a microplate reader. For the MTT assay, the medium in excess of 100 µL/well was removed and 25 µL of 5 mg/mL MTT in sterile PBS was added to each well. The plates were then incubated for 3 hrs, during which time living cells converted the soluble yellow dye into an insoluble purple crystal. Cells and dye were then solubilized by the addition of 10% SDS in 25% N,N-dimethylformamide, pH 3.6. The optical density of each well was determined at 562 nm in a microplate reader.

C. In-Cell Western Assay

The medium was first removed by inversion of the plate onto sterile absorbent gauze. Using a multi-channel pipetter, 150 uL of 3.7% formaldehyde was applied to each well for 20 minutes to fix the cells. Next, the wells were washed five times with 200 µL of 1X PBS with 0.1% Triton X-100. Each wash was for a period of five minutes at room temperature on a rotary shaker at 150 rpm. After washing, 150 µL of LI-COR Odyssey blocking buffer was applied to each well for ninety minutes at room temperature with constant shaking. Next, 50 µL of MMP-7 (1:1000 dilution) or MMP-8 (1:500 dilution) primary rabbit anti-human antibody (Sigma Antibodies, St. Louis, MO, USA) was applied to the experimental wells, while 50 µL of blocking buffer was applied to the control wells for 24 hours, at room temperature with constant shaking. The wells were then washed five more times with 200 µL of 1X PBS with 0.1% Tween-20 solution for five minutes at room temperature. The secondary goat anti-rabbit (1:900 dilution, IRDye800CW conjugation) antibody (Rockland Inc., Gilbertsville, PA, USA) was applied to all the wells (50 µL) for one hour in the dark with gentle shaking at room temperature. Lastly, the cells were washed using a 1X PBS with 0.1% Tween-20 solution five times for five minutes in the dark at room temperature.

The micro plate was then placed in the Odyssey infrared imaging machine operated by Odyssey software. The plate was scanned at a medium quality with 169 resolution, 3.3 mm offset focus, and 9.0 intensity. Each well was outlined via the Odyssey protocol to measure the intensity and quantified in Excel. The non-

specific binding data was subtracted from all test wells. Each treated group (n = 6) was calculated for average intensity and standard deviation.

D. Statistical Analysis

Data was in the form of mean intensities, as calculated by the Odyssey software. These data was analyzed statistically and graphically with GraphPad Prism software. The Prism program is a powerful combination of biostatistics, curve fitting (nonlinear regression), and scientific graphing. One- and two-way ANOVA and Neuman-Keuls post-tests were used, as appropriate, to determine statistical differences. The software analyzed the data and interprets the statistical significance of the drug effects, with or without stimulation (IL-1β) using appropriate statistical tests.

In each 96-well plate, 3 wells were used for control of the primary antibody in order to make sure the primary antibody was specific to the MMP-7 or MMP-8. The control wells only had secondary antibody and any expression was subtracted from the entire data to account for non-specific responses. The data were quantified in the Odyssey software and transferred to Excel software for calculation of mean and standard deviation for the control and for each dose. Any data that were considered to be from outliers were statistically evaluated using the Grubbs' test for detecting outliers (Motulsky, 2003) and excluded from the total n value. The totals were entered in Prism software for the 2-way ANOVA analysis and P values. Statistical significance was defined as $P<0.05$.

The graphs in figures 2 – 5 represent average values plus or minus standard error of mean.

III. RESULTS

There were 6 healthy patients from which human gingival fibroblasts were obtained. Three different gingival fibroblast cultures were used for nifedipine and 3 different gingival fibroblast cultures were used for bepridil. Each gingival fibroblast culture was used for MMP-7 & MMP-8, and was incubated for a total of 7 days with IL-1β being added to half of the wells for 24 hours prior to harvesting. Two-way ANOVA was performed for the treatment and doses relative to the control.

Initially, to evaluate gingival fibroblasts' total MMP expression we used immunocytochemistry. However, after some pilot studies (figures 1a & 1b), the ability to objectively quantify total MMP expression appeared to be very challenging. Therefore, In-cell Western Assay was used since quantifying the MMPs through the fluorescent dyes was facilitated.

Each MMP-7 & -8 treatment group with either nifedipine or bepridil was measured at least 3 times to evaluate consistency and cell response. In order to make sure the gingival fibroblasts were reacting to the IL-1β, the control group had to show up-regulation of the MMP with the addition of IL-1β (figures 2-5).

MMP-7 (figures 2a & 2b) was down-regulated as the nifedipine dose increased. The addition of IL-1β usually up-regulated the MMP-7 production. There was statistically significant down-regulation of MMP-7 at each nifedipine

dose. This was most apparent at the highest dose of 10^{-6} M (figure 2a). Although figure 2a and 2b are different, figure 2b represented a more typical result.

Figure 3 is a representative of the effects of nifedipine on MMP-8 production. Basal MMP-8 (figure 3) was not significantly affected by nifedipine doses. However, IL-1β-stimulated MMP-8 production was significantly down-regulated, but not in a dose-dependent fashion.

Bepridil apparently decreased both basal and stimulated MMP-7 and MMP-8 (figures 4 & 5); however, the response was only significant at higher doses. Again, these are representative graphs of all the experiments for bepridil.

In order to compare the percentage production of the MMPs with and without drug treatment, for each experiment, the control value was set at 100%, and each treatment was compared to the control. These percentage reductions were averaged and plotted in figures 6 through 9. If control is 100%, then basal MMP-7 nifedipine down-regulation (figure 6) ranged from 10 to 34 percent of control. For basal MMP-8 (figure 7), nifedipine down-regulation ranged from 13-20 percent of control. For bepridil basal MMP-7 (figure 8) down-regulation ranged from 5 to 24 percent relative to the control. Basal MMP-8 for bepridil (figure 9) range was 11 to 38 percent of control.

For nifedipine the IL-1β-stimulated MMP-7 production (figure 6) ranges from 90 to 95% of control. Figure 7 shows that MMP-8 production is about 80 to 85% of the control for all doses. Nifedipine did not show dose dependent inhibition of IL-1β-stimulated response. For both MMP-7 and MMP-8 production

with bepridil IL-1β-stimulated response is not dramatically affected until the highest dose reached ($5x10^{-6}$ M).

Figure 1a:

Immunocytochemistry: Gingival Fibroblast (40X) ***Without*** *IL-1 beta*

Immunocytochemistry of a sample of gingival fibroblast without IL-1β stained for MMP-7 (40X magnification). Brown color is the localization of the MMP-7.

Figure 1b:

Immunocytochemistry: Gingival Fibroblast (40X) ***With*** *IL-1 beta*

Immunocytochemistry of a sample of gingival fibroblast treated with IL-1β and stained for MMP-7 (40X magnification). As above, MMP-7 staining is shown by arrows.

Figure 1a: Without IL-1β

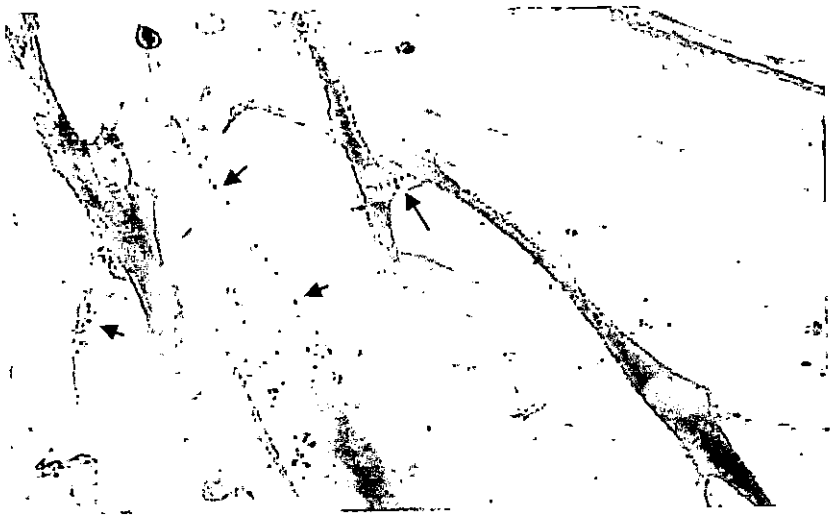


Figure 1b: With IL-1β

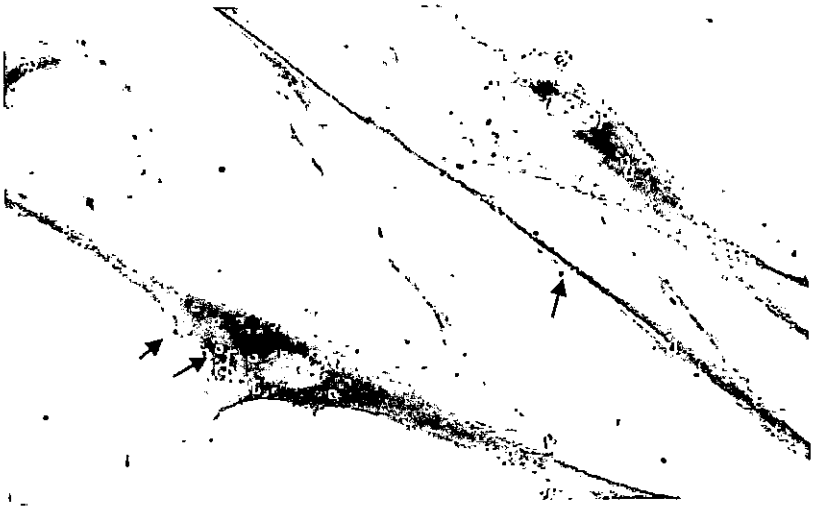


Figure 2a:

MMP-7 Nifedipine (variation)

Gingival fibroblasts were treated with nifedipine with or without IL-1β (1.0 ng/ml) were assayed for production of MMP-7 using the In-Cell Western protocol. (*) Statistical significance relative to the appropriate control P<0.05. Bars represent average values plus standard error of the mean.

Black bar: Without IL-1β

Gray bar: With IL-1β

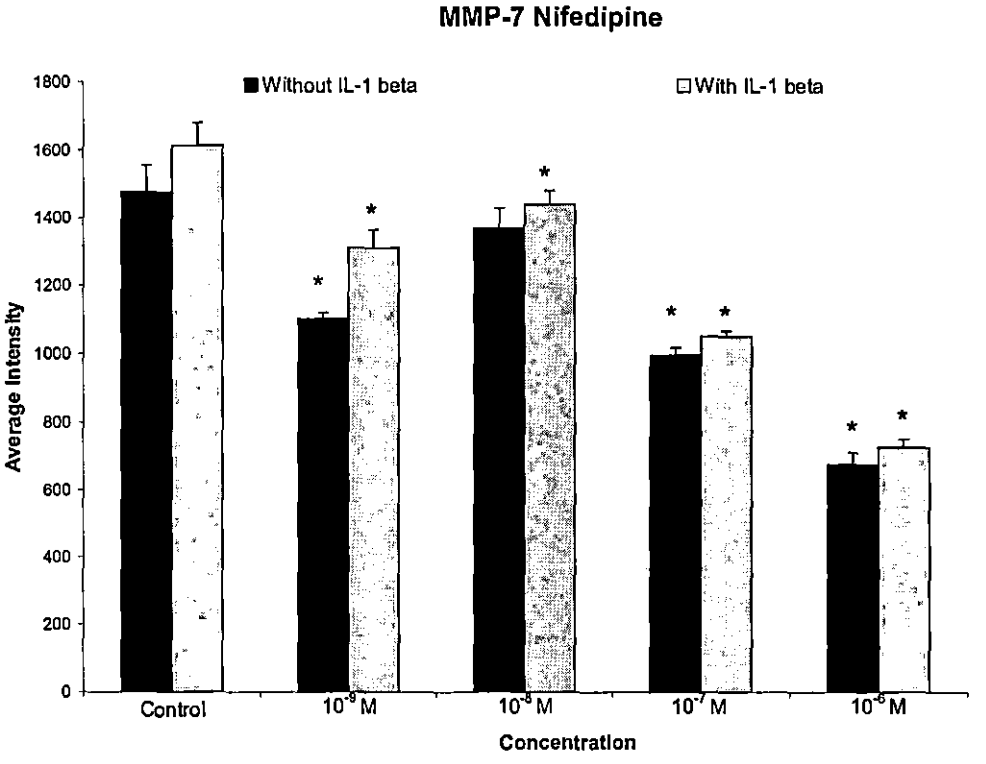

MMP-7 Nifedipine
Without IL-1 beta
With IL-1 beta
Average Intensity
1800
1600
1400
1200
1000
800
600
400
200
0
Control
10-9 M
10-8 M
10-7 M
10-6 M
Concentration

Figure 2b:

MMP-7 Nifedipine

As per figure 3a, nifedipine dose-dependently reduced basal MMP-7 production. (*) Statistical significance relative to the appropriate control P<0.05.

Black bar: Without IL-1β

Gray bar: With IL-1β

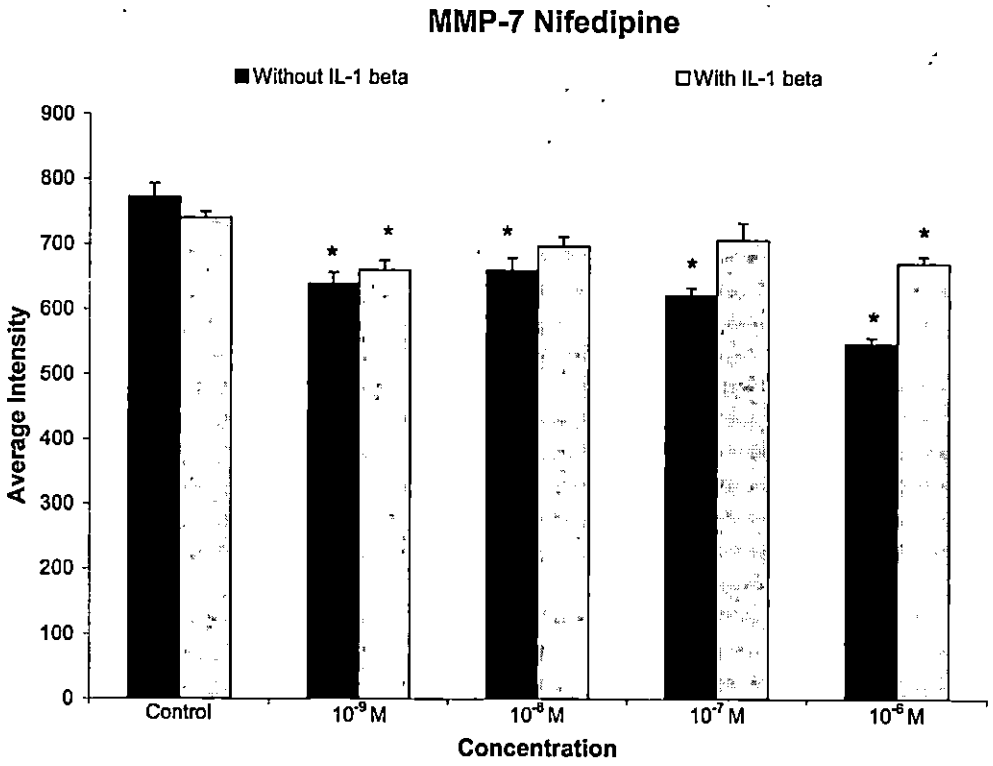

MMP-7 Nifedipine
Without IL-1 beta
With IL-1 beta
Average Intensity
900
800
700
600
500
400
300
200
100
0
Control
10-9 M
10-8 M
10-7 M
10-6 M
Concentration

Figure 3:

MMP-8 Nifedipine

Using the same protocol as for MMP-7, MMP-8 production was detected after nifedipine treatment. (*) Statistical significance relative to the appropriate control P<0.05.

Black bar: Without IL-1β

Gray bar: With IL-1β

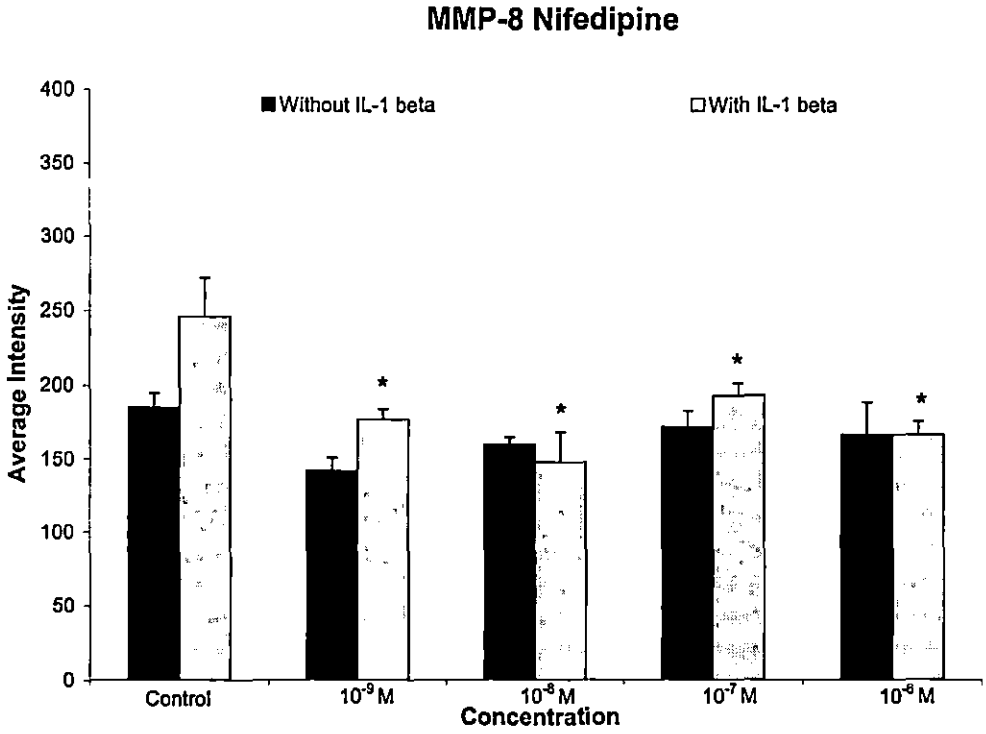

MMP-8 Nifedipine
Without IL-1 beta
With IL-1 beta
Average Intensity
400
350
300
250
200
150
100
50
0
*
*
*
*
Control
10-9 M
10-8 M
10-7 M
10-6 M
Concentration

Figure 4:

MMP-7 Bepridil

Gingival fibroblasts were treated with nifedipine with or without IL-1β (1.0 ng/ml) were assayed for production of MMP-7 using the In-Cell Western protocol. (*) Statistical significance relative to the appropriate control P<0.05. Bars represent average values plus standard error of the mean.

Black bar: Without IL-1β
Gray bar: With IL-1β

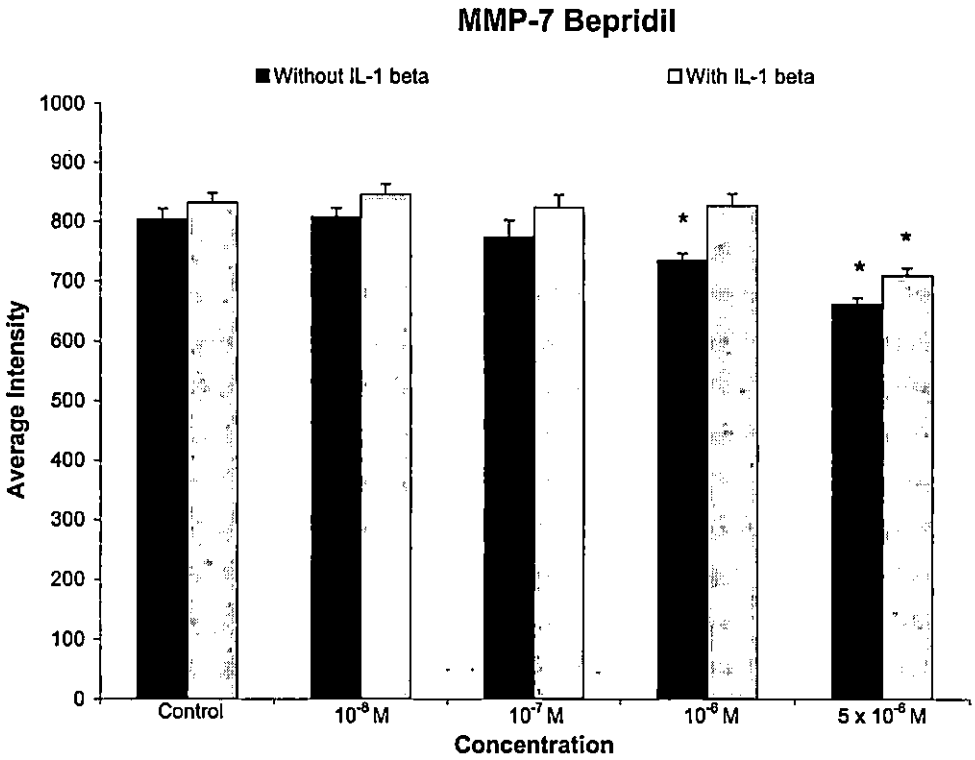

MMP-7 Bepridil
Without IL-1 beta
With IL-1 beta
Average Intensity
1000
900
800
700
600
500
400
300
200
100
0
Control
10-9 M
10-7 M
10-6 M
5 x 10-6 M
Concentration
*
*
*

Figure 5:

MMP-8 Bepridil

Using the same protocol as for MMP-7, MMP-8 production was detected after nifedipine treatment. (*) Statistical significance relative to the appropriate control P<0.05.

Black bar: Without IL-1β

Gray bar: With IL-1β

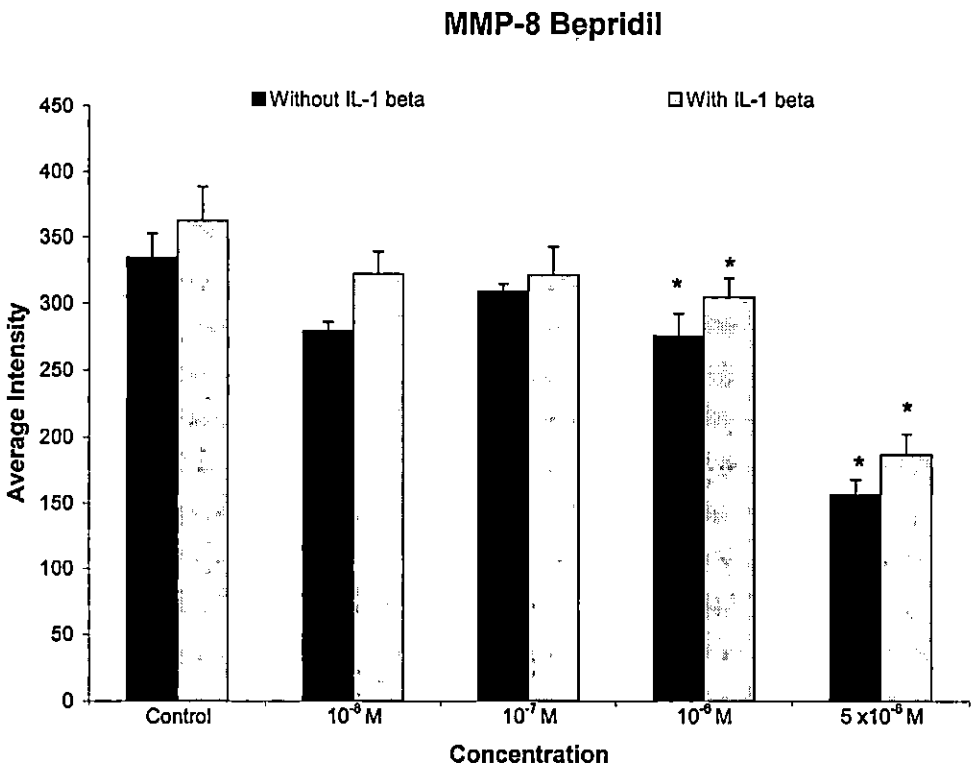

MMP-8 Bepridil
Without IL-1 beta
With IL-1 beta
Average Intensity
450
400
350
300
250
200
150
100
50
0
Control
10-8 M
10-7 M
10-6 M
5 x10-6 M
Concentration

Figure 6:

MMP-7 Nifedipine (Percent Difference)

Total raw data of MMP-7 nifedipine combined to show percentage difference relative to control. Control considered as 100%

Black bar: Without IL-1β

Gray bar: With IL-1β

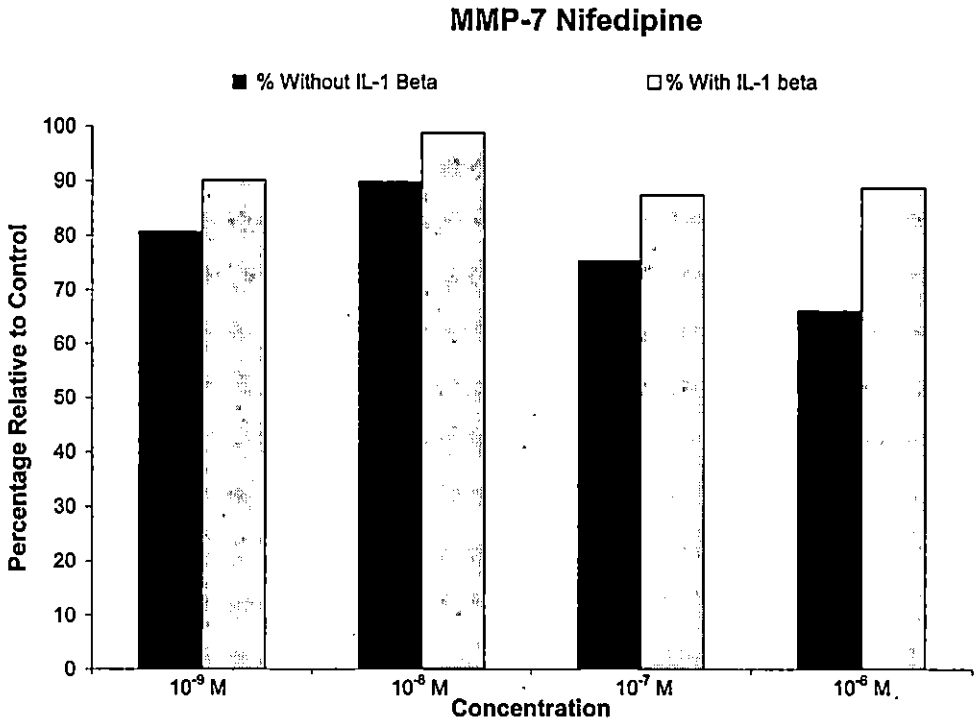

MMP-7 Nifedipine
% Without IL-1 Beta
% With IL-1 beta
Percentage Relative to Control
100
90
80
70
60
50
40
30
20
10
0
10-9 M
10-8 M
10-7 M
10-6 M
Concentration

Figure 7:

MMP-8 Nifedipine (Percent Difference)

Total raw data of MMP-8 nifedipine combined to show percentage of difference relative to control. Control considered as 100%

Black bar: Without IL-1β

Gray bar: With IL-1β

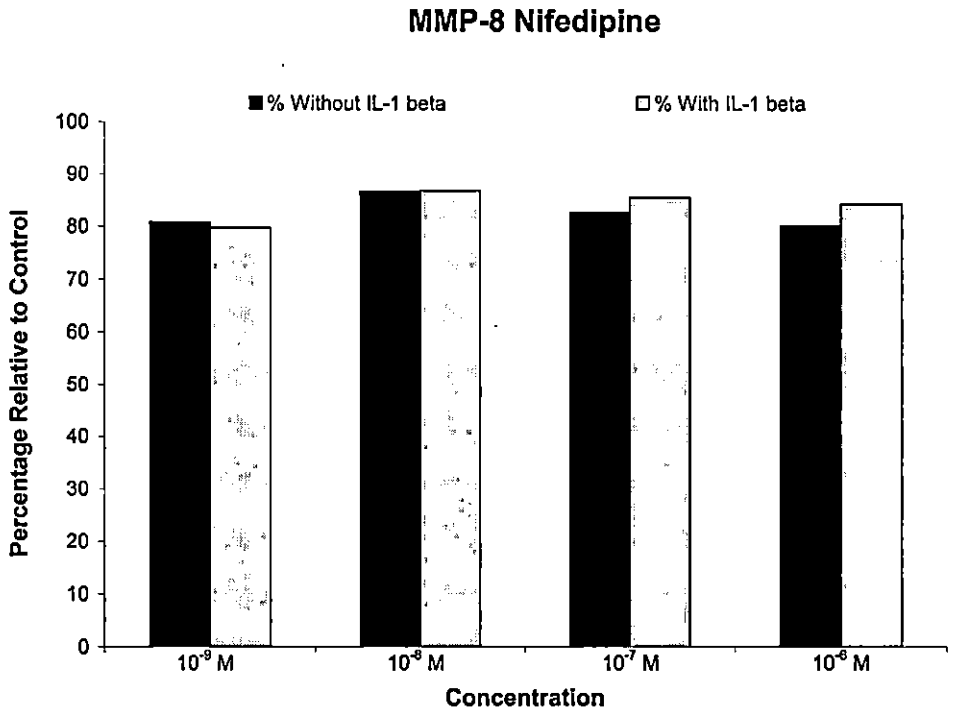

MMP-8 Nifedipine
% Without IL-1 beta
% With IL-1 beta
Percentage Relative to Control
100
90
80
70
60
50
40
30
20
10
0
10-9 M
10-8 M
10-7 M
10-6 M
Concentration

Figure 8:

MMP-7 Bepridil (Percent Difference)

Total raw data of MMP-7 bepridil combined to show percentage of difference relative to control. Control considered as 100%

Black bar: Without IL-1β

Gray bar: With IL-1β

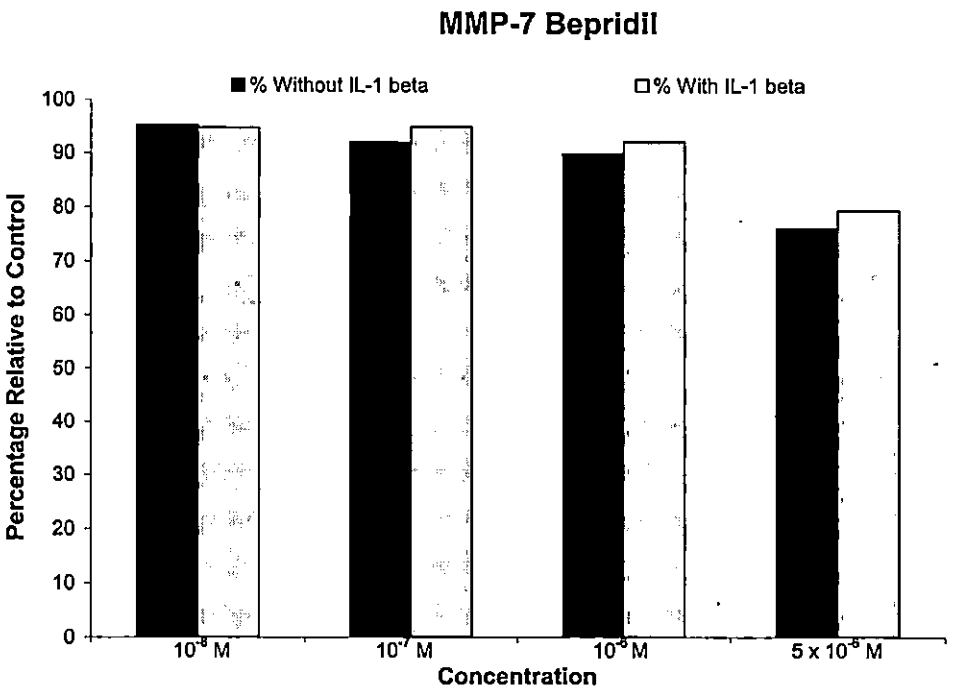

MMP-7 Bepridil
% Without IL-1 beta
% With IL-1 beta
Percentage Relative to Control
100
90
80
70
60
50
40
30
20
10
0
10⁻⁸ M
10⁻⁷ M
10⁻⁶ M
5 x 10⁻⁶ M
Concentration

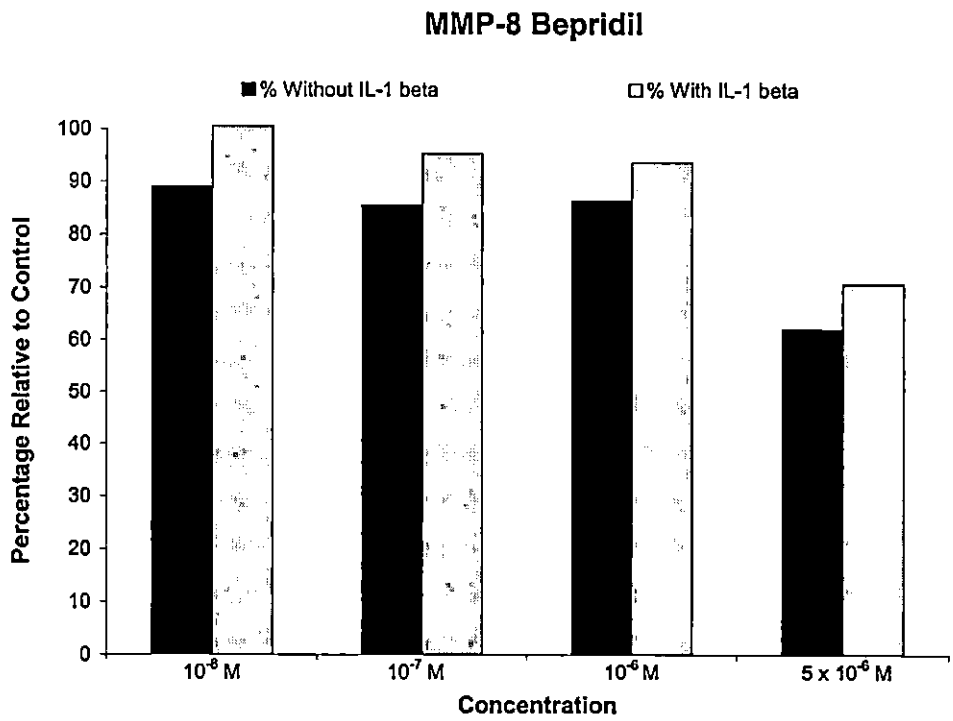
Figure 9:

MMP-8 Bepridil (Percent Difference)

Total raw data of MMP-8 bepridil combined to show percentage of difference relative to control. Control considered as 100%

Black bar: Without IL-1β

Gray bar: With IL-1β


MMP-8 Bepridil
% Without IL-1 beta
% With IL-1 beta
Percentage Relative to Control
100
90
80
70
60
50
40
30
20
10
0
10-8 M
10-7 M
10-6 M
5 x 10-6 M
Concentration

IV. DISCUSSION

Improvements in cardiovascular therapy have dramatically increased the lifespan of people in the U.S. Thus, an increasing percentage of a dentist's practice is occupied by the treatment of older persons taking chronic calcium channel blocker (CCB) drugs for disorders such as angina, arrhythmia, and hypertension. Many of these CCBs have an adverse effect in the oral cavity (i.e. gingival overgrowth) to varying degrees. Unfortunately, patients on these medications may not see physical manifestations of the adverse effects until months into therapy, which can add to the severity of the gingival overgrowth. More than 80% of patients on a regimen of ongoing traditional CCB therapy have signs of gingival overgrowth. Current treatments require cessation of the CCB drug therapy and surgical excision of the gingiva. Gingival overgrowth will likely reoccur unless the CCB therapy is stopped or switched to a non-CCB drug, which requires the patient to weigh cardiovascular health risk against oral health.

In the early 1990s, the prevailing opinion was that this class of drugs caused a true hyperplasia of the gingival tissues. Current thinking of GO as "hyperplasia" is inappropriate after histological evaluation has shown that the drug increases the extracellular tissue volume rather than increasing the number of cells. The highest number of cases of drug (CCB)-induced gingival

overgrowth in the literature has been reported with nifedipine (Adalat ®, Procardia ®). Bepridil (Vascor ®) - related cases of GO have not been cited in the literature, nor is there any listing of gingival overgrowth as a side effect in the manufacturer's product description.

Despite many investigations, the pathogenesis of the biological mechanism of gingival overgrowth is poorly understood. The drugs that are generally known to cause gingival overgrowth are the CCBs, cyclosporin A, and phenytoin (Seymour, *et al.*, 1996). There is also controversy regarding oral hygiene and plaque, as potential etiological factors (Seymour, *et al.* 1996). One study showed that when nifedipine could not be discontinued, gingival overgrowth did not reoccur after gingivectomy when extensive plaque control was maintained (Nishikawa *et al.* 1991). The change to another CCB, however, can result in continued gingival overgrowth. One study reported the disappearance of gingival overgrowth within 15 days after termination of verapamil, but overgrowth reoccurred after another CCB, diltiazem, was started (Giustiniani, Robestelli della Cuna, Marieni, 1987). Another case report of non-surgical management of a patient presenting with nifedipine-induced gingival overgrowth indicated that improving the standard of plaque control led to a complete resolution of overgrowth without recurrence (Ciantar, 1997). Evaluation of cytokines in response to the inflammation related to plaque was in another study with cyclosporine A gingival overgrowth (Atilla, Kutukculer 1998). They suggested that IL-1β levels in gingival crevicular fluid might be responsible for cyclosporine A-induced gingival overgrowth not by

itself but in combination with other factors. IL-1β levels were increased based on an *in vitro* study with an IL-1β concentration of 500 pg/mL and gingival fibroblasts exposed to nifedipine at 10^{-7} M, Johnson, Zebrowski, Dai, 2000, speculated that poor oral hygiene was a risk factor.

Our experiments exposed the gingival fibroblasts to twice the concentration of IL-1β (1.0 ng/mL) used by Johnson *et al.* study (2000). A pilot study was completed with 2.0 ng/mL and the effects of IL-1β were not altered significantly compared with the lower dose (1.0 ng/mL). Other pilot studies did not show significant effects when gingival fibroblasts were exposed to 6 h, 12h, or 16 h of IL-1β (data not shown). Maximal effects of IL-1β were seen after 24h exposure. IL-1β alone did not down-regulate the MMP-7 & MMP-8. We did see an increase of MMP expression with IL-1β for both nifedipine and bepridil. This refuted our original hypothesis that drug treatment plus "plaque" would further decrease MMPs production. Based on our study, IL-1β did not exacerbate the down regulation of MMP-7 & MMP-8. However, the interaction with TIMPs needs to be evaluated, and longer incubation than 24 hours with IL-1β might be required to show an effect. One criticism of longer incubation with IL-1β can be that total effects may be related to increased number of fibroblasts rather than cell activity.

The data in Figure 2a were different from the typical results. This illustration is provided to show the variability between the cell-lines, i.e. people. This may partially explain why certain individuals have gingival overgrowth. Pilot studies were completed to evaluate different incubation

periods of: one, three, five, and seven days (data not shown). After seven days of CCB treatment the MMP production was significantly down-regulated, and this was the time we used for the incubation period. Our results indicated that MMP-7 nifedipine was decreased by nifedipine in a dose-dependant manner (figures 2a & 2b). IL-1β-stimulated MMP-8 was reduced by nifedipine (figure 3) but not dose-dependently. Bepridil decreased both MMPs basally and IL-1β-stimulated responses only at higher doses (figure 4 & 5). The dose response for bepridil was different from that seen for nifedipine since it was seen only at higher doses. For the highest doses of both bepridil and nifedipine, there was an extremely significant down-regulation of MMP-7 & MMP-8, except for MMP-8 with nifedipine. Our results indicated that nifedipine has more direct affect on MMP-7 than MMP-8, and bepridil affected both MMP-7 & MMP-8 at high doses only. However, there appeared to be a drug-specific difference between the basal and the IL-1β-stimulated MMP production. For MMP-8, nifedipine seemed to reduce the response to IL-1β, while stimulated MMP-7 production was clearly evident even at the highest dose. The reverse seemed to be true for bepridil treatment. MMP-8 production was reduced at high dose concentrations, but the IL-1β-stimulated response was evident. For MMP-7, IL-1β failed to produce as much increase at each dose. MMP-7 was less stimulated by IL-1β with nifedipine compared to MMP-8 with nifedipine. The MMP-7 and MMP-8 response to IL-1β with bepridil was significantly affected at the highest doses. At the highest doses for both CCBs, and for both MMPs, IL-1β-stimulated MMP production was

significantly down-regulated relative to its related control. The statistical significance ($p<0.05$) in reference to the control was used to evaluate changes in MMP production. Even though at certain doses IL-1β-stimulated exposure was expressed less in reference to its control, the MMP production was equal to or higher than basal secretion indicating there was no further down regulation of the MMP.

The antibodies available for this experiment were polyclonal, which meant that we studied total MMP expression instead of evaluating the differences between the zymogen form of the MMP and the active form. Future studies with additional antibodies to delineate between the pro-form and active-form might help us to understand the pathogenesis much better.

Based on our study, while bepridil-influenced MMP expression was different from nifedipine, it is concluded that bepridil might induce gingival overgrowth in high doses. The manufacturer of bepridil reports that over 99% is bound to plasma proteins, which indicates that a small amount of bepridil is left for biological effects, like gingival overgrowth. Since we saw significant down-regulation of MMPs for bepridil at high doses, this suggested that bepridil doses needs to be high enough and/or for a longer period to see the effects clinically. Although there have been no cases of GO reported with bepridil, this study suggests that future clinical cases may occur. On the other hand, nifedipine is reported to be 92-98% bound to plasma proteins, indicating that the biological effects of the CCB might be evaluated clinically more readily (i.e. gingival overgrowth).

In summary, we concur with other studies that conclude that gingival overgrowth may be driven by the interaction between the CCB and gingival fibroblasts in a dose-dependent manner. Knowing the limitations of an *in vitro* experiment for extrapolation to clinical significance, it was still surprising to this investigator that IL-1β did not exacerbate the down-regulation of the MMPs. If plaque, represented here by IL-1β, is an etiological factor the biological mechanism may be through TIMPs, other MMPs, or other cytokines. Another study evaluating different MMP and cytokines showed similar results of up-regulation of MMP-1 expression when human gingival fibroblasts were exposed to interleukin-1α and up-regulation of TIMP-1 (Sakagami, G. *et al.* 2006). Also, there may be drug-specificity to specific MMPs that can exacerbate the down-regulation leading to overgrowth as was noticed with nifedipine to MMP-7 and bepridil to MMP-8.

V. SUMMARY

This study compared the differences between traditional (nifedipine) vs. modified (bepridil) calcium channel blockers on matrix metalloproteinase (MMP-7 and MMP-8) production in human gingival fibroblasts (HGF) *in vitro*. It also incorporated IL-1β as a stimulator representing a major exacerbating factor in plaque. HGFs were treated with the CCBs for a total of seven days, and IL-1β at 1.0 ng/mL was given the last 24 hours prior to harvesting for In-Cell Western. The total MMP-7 & -8 expressions were quantified and compared to control at different doses. Two way ANOVA ($p<0.05$) was completed for statistical significance. It was determined that the down-regulation of MMP was driven by the dose of the CCB, which could lead to gingival overgrowth. The MMP expression for each dose was up-regulated by IL-1β. This was seen that IL-1β, the stimulation similar to plaque, did not exacerbate the down-regulation of MMP-7 & MMP-8, suggesting that gingival overgrowth may be strictly driven by the CCB. If IL-1β is an etiological factor, the mechanism may be through other pathways but not directly through MMP-7 & MMP-8. Also, bepridil did down-regulated the MMP expressions at higher doses even though the pattern was different than nifedipine, suggesting bepridil may also cause gingival overgrowth in some cases.

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