Dentin Matrix Metalloprotease Inhibition by Berberine

BY

KRISTEN A. VLAGOS,
B.A., University of Illinois at Urbana-Champaign, 2006
D.D.S., University of Illinois at Chicago, 2011

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Oral Sciences
in the Graduate College of the
University of Illinois at Chicago, 2015

Chicago Illinois

Defense Committee:

Dr. Ana K. Bedran-Russo, Chair
Dr. Judy Yuan
Dr. Phimon Atsawasuwan

ACKNOWLEDGEMENTS

I would like to express my sincere gratidue to my thesis advisor, Dr. Ana K. Bedran-Russo, for her knowledge, support and guidance throughout my research project. I would also like to thank my other thesis committee members, Dr. Judy Yuan and Dr. Phimon Atsawasuwan for their support and contributions. I am especially appreciative to Dr. Cristina Vidal for her technical guidance and expertise. Her involvement was instrumental in the completion of this study.

KAV

TABLE OF CONTENTS

CHAPTER PAGE

I. INTRODUCTION **1-5**
A. BACKGROUND 1-3
B. SIGNIFICANCE OF STUDY 4
C. SPECIFIC AIMS 5
D. HYPOTHESES 6

II. LITERATURE REVIEW **7-21**
A. STRUCTURE AND REGULATION OF MMPS 7-10
B. ROLE OF MMPS IN DENTIN 11-12
C. ROLE OF MMPS IN CARIES PROGRESSION 13-15
D. ROLE OF MMPS IN HYBRID LAYER DEGRADATION 16-17
E. MMP INHIBITION IN DENTIN 18-19
F. BERBERINE AS MMP INHIBITOR 20-21

III. METHODOLOY **22-30**
A. STUDY DESIGN 22
B. RECOMBINANT MMP INHIBITION 23-25
1. Experimental design overview *23*
2. Zymographic analysis *23-24*
3. Data analysis *24*
C. ENDOGENOUS MMP INHIBITION 26-30
1.Specimen preparation *26*
2. Test groups *27*
3. Quantification of collagen degradation *28*
4. Hydroxyproline assay *29*
5. Statistical analysis *30*

IV. RESULTS **31-37**
A. GELATIN ZYMOGRAPHY 31-34
B. HYDROXYPROLINE 35-37

V. DISCUSSION **38-42**
A. STUDY FINDINGS 38-45
B. STRENGTHS AND LIMITATION OF STUDY 46-50
B. FUTURE STUDIES 51

VI. CONCLUSION **52**

CITED LITERATURE **53-60**

VITA **61-62**

LIST OF TABLES

TABLE PAGE

I. TREATMENT GROUPS 28

II. RECOMBINANT ENZYME ACTIVITY AFTER TREATMENT WITH BERBERINE 33

III. MEAN HYP RELEASE 36

LIST OF FIGURES

FIGURE PAGE

1. DOMAIN STRUCTURE OF MMPS 10
2. MMP ACTIVATION 10
3. MOLECULAR STRUCTURE OF BERBERINE 21
4. GELATIN ZYMOGRAPHY SCHEMATIC 25
5. DENTIN POWDER SPECIMEN PREPARATION FOR HYP ASSAY 27
6. TREATMENT AND INCUBATION OF DEMINERALIZED DENTIN POWDER 27
7. GELATIN ZYMOGRAM OF RMMP ACTIVITY 31
8. DENSITOMETRY PROFILES FOR GELS 32
9. PLOT OF THE PEAK AREAS AND PERCENT RMMP-2 AND -9 ACTIVITY 33
10. MEAN HYDROXYPROLINE RELEASE 37

LIST OF ABBREVIATIONS

BRB	Berberine
CHX	Chlorhexidine
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
rMMP	Recombinant matrix metalloproteinase

SUMMARY

Matrix metalloproteases (MMPs), a family of proteolytic enzymes, are known to be involved in the breakdown of collagen in dentin. While these enzymes contribute to physiological processes such as tooth morphogenesis and dentin formation, they also act in pathological ways. These enzymes participate in the progression of dental caries and failure of resin bonds. Inhibition of dentinal MMPs may aid in controlling the detrimental effects to the dental tissue. Berberine, a plant-based compound, has been demonstrated to be an effective MMP inhibitor. The aims of this study were to determine the effectiveness of berberine at inhibiting the activities of specific recombinant MMPs (types 2 and 9) and endogenous dentin MMPs.

Recombinant MMP inhibition was assessed by means of gelatin zymography. Gels containing recombinant MMP-2 and -9 were divided into the following groups according to incubation buffer: Group 1- MMPs buffer; Group 2- MMPs buffer containing 0.02% berberine; Group 3- MMP buffer containing 0.002% berberine; Group 4- MMPs buffer containing 0.0002% berberine; Group 5- MMPs buffer containing 2 mM phenantroline. Following incubation with buffer solution, enzymatic activity was quantified by measuring band intensity in the gels. All concentrations of berberine decreased both MMP-2 and -9 activities, and the effect was shown to be concentration dependent.

Endogenous MMP activity was assessed through quantification of hydroxyproline release. Samples of human demineralized dentin powder were randomly assigned into 5 groups according to incubation solution: Group 1- water;

Group 2- 0.02% berberine; Group 3- 0.2% berberine; Group 4- 0.002% chlorhexidine; Group 5- 0.02% chlorhexidine. Powder specimens were treated with the various solutions for 1 hour at 37° C under agitation. Afterwards, the specimens were washed and then incubated in artificial saliva at 37° C under agitation for a total of 7 days. The amount of hydroxyproline released in supernatant was evaluated at 24 hours, 48 hours, and 7 days. Statistical analysis was performed using two-way and one-way ANOVA and Games Howell post hoc tests ($p < 0.05$). No statistical differences in the amount of collagen degradation were observed among the groups treated with berberine as compared to the control group treated with water.

It was concluded that berberine possess inhibitory potential against recombinant MMP-2 and -9. However, inhibition of endogenous dentin proteases by berberine was not demonstrable.

I. Introduction

A. Background

Dentin is a dynamic mineralized tissue consisting of inorganic hydroxyapatite crystals entrenched within an organic extracellular matrix (ECM). Type I collagen is the main component of the ECM, representing up to ninety percent of the organic material (Linde, 1984). The remaining ten percent is made up of water and other non-collagenous proteins. The non-collagenous dentin proteins include proteoglycans, phospholipids, and enzymes. Among the enzymes, matrix metalloproteinases (MMPs) are of particular interest due to their physiological and pathological processes in dentin (Mazzoni et al., 2015).

Matrix metalloproteinases are Zn^{2+} and Ca^{2+} -dependent endopeptidases that are capable of degrading almost all components of ECM. These enzymes contribute to normal physiological processes throughout the body such as wound healing, growth and development, and cell signaling. However, the specific role of MMPs in dentin is not as well understood. It is thought that MMPs function physiologically in tooth morphogenesis and dentin formation; however, these enzymes can also participate in ECM degradation in pathological ways (Chaussain et al., 2013).

MMPs are secreted in inactive enzyme form by connective tissue cells such as fibroblasts, osteoblasts, and odontoblasts. The MMPs found within dentin are produced by odontoblasts during secretion of dentin matrix. The pro-MMPs become trapped within the dentin after calcification of the matrix. Exposure to an acidic environment can facilitate the activation of these dormant enzymes (Visse

and Nagase, 2003). The acidic exposure can occur from either carious bacterial acids or acids from bonding procedures. Once activated, the collagenolytic activity of MMPs contributes to caries progression as well as the failure of resin bonds in the hybrid layer of dentin (Chaussain et al., 2013; Mazzoni et al., 2015).

During the dental caries process, the mineral part of dentin is solubilized by acids produced by oral bacterial followed by destruction of the organic component of dentin. Bacterial proteases have long been considered the main offenders in the degradation of the dentin ECM; however, bacterial collagenolytic activity has been demonstrated to be weaker than previously thought (Tjäderhane et al., 1998; van Strijp et al., 1994, 1997; Kawasaki and Featherstone, 1997). Current evidence suggests host-derived MMPs play a more significant role than bacterial proteases in caries propagation (Chaussain-Miller et al., 2006; van Strijp et al., 2003; Mazzoni et al., 2007; Sulkala et al., 2002).

These endogenous enzymes also contribute to degradation of the hybrid layer in resin bonds. It has been reported that endogenous MMPs bound in dentin can degrade the exposed collagen fibrils within the hybrid layer if unprotected by resin (Breschi et al., 2008). The action of these enzymes is thought to be responsible for the thinning and disappearance of collagen fibrils that is observed with incompletely infiltrated dentin hybrid layers (Armstrong et al., 2004; Koshiro et al., 2005). With time, bond degradation may show up as a loss of retention and bond strength. Ultimately, this may result in marginal leakage and recurrent caries.

Inhibition of dentinal MMPs may provide therapeutic approach towards limiting the detrimental effects to the dental tissue. Preservation of collagen matrix

integrity is crucial to improve dentin-bonding durability and create stable and long lasting adhesive restorations. Hence novel treatment strategies to retard the degradation of dentinal collagen include MMP inhibitors such as berberine.

Berberine, a plant-based chemical, has been widely studied as a MMP inhibitor. Berberine is a natural compound that can be isolated from a variety of plants such as *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree turmeric), *Berberis vulgaris* (barberry), and *Hydrastis canadensis* (goldenseal) (Ikram, 1975). Berberine has long been recognized as a therapeutic agent. It has been used throughout the world for a multitude of ailments (Imanshahidi and Hosseinzadeh, 2008). Today, the medicinal properties of berberine are garnering more attention as the mechanisms of action become better understood. Berberine has been widely studied for its multiple pharmacological activities including anti-bacterial, anti-inflammatory, anti-oxidative, anti-carcinogenic, and anti-metastatic properties.

Investigation on the antineoplastic properties of berberine show that it is involved with the inhibition and down regulation of various MMPs. MMP inhibition has also been investigated within the oral environment. Berberine has been shown to inhibit MMP activity in periodontal tissue thereby reducing gingival tissue destruction (Tu et al., 2013). If berberine has a similar effect on MMPs found within dentin, then collagen degradation could be mitigated. The inhibition of MMPs by berberine has not yet been studied in dentin.

B. Significance of Study

The discovery of new therapies to inhibit the effects of endogenous MMPs in dentin is of great clinical importance. This will aid in preventing the propagation of carious lesions and help to extend the longevity of resin bonded restorations. Berberine has been shown to have an inhibitory effect on MMPs. However, the effect of berberine on specific dentinal MMP activity has not yet been studied. The data presented in the current study may offer the opportunity for further study of MMP inhibition by berberine and clinical applications.

C. Specific Aims

The purposes of this study were:

a. To determine the effect of berberine on selective recombinant MMPs.

b. To determine the effect of berberine on endogenous dentinal MMPs by examining the amount of collagen degradation.

D. Hypotheses

Berberine will inhibit dentinal MMP activity and provide a protective effect against collagen degradation. The first null hypothesis tested was that berberine has no inhibitory effect on recombinant MMPs. The second null hypothesis was that berberine has no inhibitory effect on endogenous matrix-bound proteases

II. Review of Literature

A. Structure and Regulation of MMPs

Matrix metalloproteases belong to a family of proteolytic enzymes that degrade extracellular matrix (ECM) proteins. They are classified as endopeptidases based on their cleavage of internal peptide bonds. There are 24 known vertebrate MMPs, of which 23 are found in humans (Tjäderhane et al., 2015; Visse and Nagase, 2003). Each MMP has distinct but often overlapping substrate specificities, and together they can cleave almost all ECM components. Additional targets include other proteinases, proteinase inhibitors, clotting factors, chemotactic factors, and cell-cell adhesion molecules (Yang et al., 2007).

MMPs are classified as such based on a number of common structural and functional features including the "cysteine switch" motif in the propeptide domain, and the zinc-binding motif in the catalytic domain (Visse and Nagase, 2003). Due to large stretches of sequence homology, MMPs share fairly similar three-dimensional structure.

MMPs can be differentiated into six main subgroups based on substrate specificity, which includes collagenases, gelatinases (type IV collagenases), stromelysins, matrilysins, membrane-type metalloproteinases, and other MMPs. Specific MMPs are referred to by their aforementioned common names or by a numerical nomenclature. MMPs are classically described as having two main structural areas, a pro domain and a catalytic domain (Figure 1). The pro domain functions as a regulatory structure to maintain the latency of the precursor enzyme. It is thought that the enzyme is kept inactive by virtue of a Cys-Zn bond

(referred to as the "cysteine switch" mechanism) (Figure 2). An unpaired cysteine residue in the pro domain forms a bridge with the zinc ion in the active site, displacing the water molecule which is necessary for catalysis. Disruption of the Cys-Zn bond through cleavage of the propeptide induces a conformation change that switches the enzyme into the active form.

Dissociation of the pro domain can be achieved by autolysis or by the action of other proteolytic enzymes (Hannas and Kuttan, 2013). Activation can also occur by chemical agents, heat, and low pH (Visse and Nagase, 2013). These modes of activation are thought to work through the disruption of the cysteine-zinc interaction of the cysteine switch (Visse and Nagase, 2003).

The catalytic domain controls cleavage-site specificity through its active site cleft. Pockets of the active site bind amino acids residues immediately adjacent to the scissile peptide bond. Secondary binding sites located outside the active site also contribute to substrate selectivity.

With the exception of MMP-7, -26, and -23, all MMPs have an additional regulatory hemopexin-like domain that is linked to the catalytic domain by a hinge region. When this domain is present, it regulates binding of certain substrates, membrane activation, and some proteolytic activities. It also regulates the binding of endogenous inhibitors known as TIMPs, which are involved in the local control of MMP activity in tissues.

To accomplish normal physiological functions, MMPs must be tightly regulated. Activators and inhibitors are just one form of control that occurs at the protein level. However, enzyme regulation begins at the transcriptional level via

numerous stimulatory and suppressive factors affecting gene expression (Fini et al., 1998). Post-transcriptional mechanisms can also influence MMP expression. Processing of mRNA can act to both stabilize and destabilize transcripts (Delany et al., 1995; Vincenti, 2001). Following protein synthesis, MMPs are usually directed to the cell membrane in preparation for release into the ECM. Although most MMPs are secreted constitutively, some controls exist to sequester MMPs within specific cells. For example, MMP-8 and -9 are synthesized by differentiating granulocytes in bone marrow and stored in granules of circulating neutrophils. Neutrophil activation triggered by inflammatory mediators causes the release of the enzymes into the extracellular environment (Hasty et al., 1990). Once secreted, other control mechanisms are involved in localizing MMPs to specific extracellular sites. This will often boost MMP activation, restrict the access of MMP inhibitors, concentrate MMPs within the area of their targets, and limit the extent of proteolysis to specific extracellular regions (Sternlicht and Werb, 2001).

The final regulatory mechanism of MMPs is through their own proteolytic inactivation and physical clearance. While, some protein cleavages inactivate MMPs, others, such as those that specifically remove the hemopexin domain, can produce truncated enzymes that lose their ability to cleave some substrates while maintaining their ability to cleave others (Woessner and Nagase, 2000). Such actions can also reduce their affinity for and ability to be inhibited by TIMPs (Itoh et al., 1998). The removal of the hemopexin domain can also prohibit the ability of certain MMPs to localize to the cell surface. Extracellular MMPs levels are also

controlled by the direct clearance of intact enzymes. This may occur through receptor binding, endocytosis, followed by permanent degradation.

In conclusion, MMPs are tightly regulated by several mechanisms during nearly every aspect of their existence. As knowledge in the field of MMP biology continues to grow, so will the understanding of how these enzymes regulate both physiological and pathological processes.

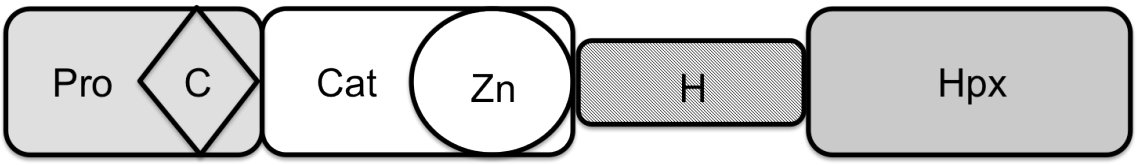


Figure 1. Domain structure of MMPs. Pro, prodomain with cysteine switch (C); Cat, catalytic domain; Zn, zinc binding site; H, hinge region; Hpx, hemopexin domain. Modified from Sternlicht and Werb, 2001, Hannas et al., 2007, and Visse and Nagase, 2003.

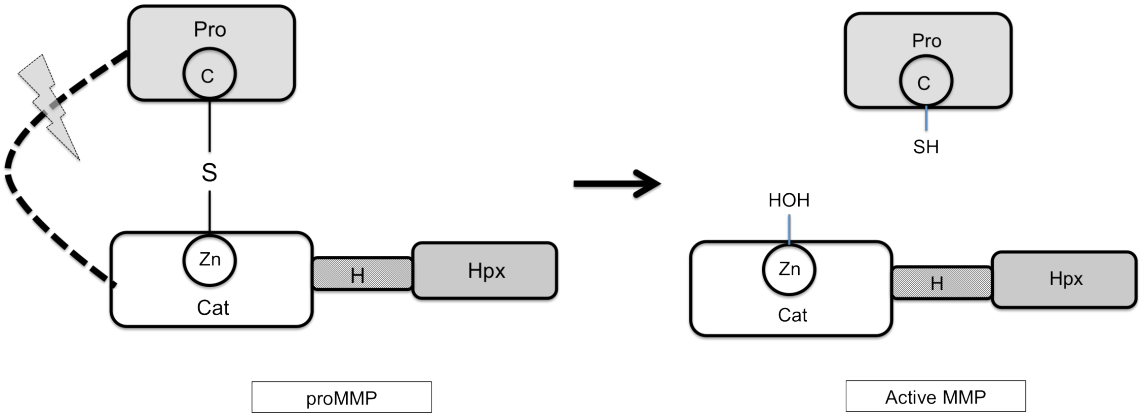


Figure 2. MMP activation. The cysteine residue (C) of the MMP prodomian (Pro) pairs with the zinc ion (Zn^{2+}) at the functional site of the catalytic domain (Cat), forming the cysteine switch (S) and hiding the active site cleft of the enzyme. Enzyme activation is achieved by removal of the prodomain which exposes the catalytic site. The hemopexin domain (Hpx) is attached to the catalytic domain by a hinge domain (H). Hpx is involved in protein-protein interactions, substrate recognition, enzyme activation and protease localization. Adapted from Visse and Nagase, 2003, Tjäderhane et al., 2013, 2015.

B. Role of matrix metalloproteases in dentin

MMPs have been suggested to participate in various physiological processes occurring in dentin. They contribute to organic matrix reorganization from initial tooth development to long term maturation and repair of dental tissues. Although the role of MMPs is to breakdown ECM components, their function extends far beyond mere deconstruction. They also tightly regulate cellular behavior through manipulation of the ECM. It has become clear that the ECM is not just a scaffold to support cells or act as a barrier. It also acts as a reservoir for biologically active molecules. Remodeling of ECM uncovers signaling molecules that affect the actions of nearby cells.

The main MMPs identified in normal dentin matrix are MMP-2, -3, -8, -9, and -20 (Mazzoni et al., 2007, 2011; Sulkala et al., 2007). During tooth development the expression of MMPs is spatially and temporally regulated in the different layers of dentin (Bourd-Boittin et al., 2005). As mineralization occurs, some of these MMPs become incorporated into dentin and remain latent until later release (Hannas, 2007; Sulkala et al., 2002). For example, MMP-2 is found to be concentrated at the dentin-enamel junction (Boushell et al., 2011). The specific localization of MMP-2 may be key to its role in the remodeling and degradation of the basement during tooth morphogenesis, which allows communication between the pre-ameloblalsts and odontoblasts (Heikinheimo and Salo, 1995). This direct contact is critical for terminal cytodifferentiation (Bourd-Boittin et al., 2005). MMP-2 has also been shown to cooperate with MMP-20 in processing the two major dental matrix

proteins, amelogenin and dentin sialoprotein, which is necessary for proper mineralization of both dentin and enamel (Bourd-Boittin et al., 2005).

The expression of MMPs begins during dentinogensis and continues as dentin matures. After tooth formation is complete, dentin deposition persists, albeit at a slower rate, in response to physiological and functional demands. Similar to primary dentinogenesis, mature odontoblasts secret dentin organic matrix, after organization in the predentin area, is mineralized. MMPs have been implicated in the degradation of predentin ECM components to facilitate its removal from the predentin as mineralization proceeds (Hall et al., 1999). While MMPs are bound within mineralized dentin, they are also secreted by odontoblasts in dentinal fluid (Sulkala et al., 2002). This suggests that MMP-20 may be involved in the subtle restructuring of dentin (Tjäderhane et al., 2001). The mechanical properties of dentin decrease significantly with age, and it has been suggested that changes in the intertubular collagen matrix contribute to these structural responses.

MMPs may also participate in the regulation of pulpal defensive reactions, such as the formation of atubular reparative dentin. MMPs may be involved in the liberation and processing of bioactive factors essential for dentin formation (Charadram et al., 2012). In turn, these dentin-derived bioactive mediators may indirectly up regulate reparative dentin formation by controlling expression and activity of MMPs (Tjäderhane et al., 2001). Thus, MMPs are involved in complex signaling cascades and have functional capacity beyond simple demolition.

C. Role of matrix metalloproteases in caries progression

Caries is a progressive disease of the calcified tissue of teeth. The pathophysiology of caries is characterized by demineralization and subsequent destruction of the organic substance of the tooth, ultimately resulting in cavitated lesions. The disease process is initiated by organic acids produced by oral bacteria (Marsh, 1994). Hydroxyapatite is solubilized by the action of these acids, causing loss of calcium and phosphate ions (Featherstone, 2008). The demineralization process is balanced by the buffering capacity of the saliva, which allows for remineralization (Featherstone, 2000). If this balance between demineralization and remineralization is disrupted, then the caries process will progress (Featherstone, 2004).

In dentin, demineralization is followed by destruction of the collagenous organic matrix. Unlike enamel, which is comprised of 0.4-0.6% organic matrix, dentin is made up of 18-20% organic material (Goldberg et al., 2011), and provides a better substrate for proteolytic degradation. Loss of mineral content in conjunction with collagen degradation synergistically contribute to the reduction in physical and mechanical dentin properties (Mazzoni et al., 2015). Once dentin is no longer able to support overlying enamel, the structure collapses and a cavity forms (Chaussain Miller et al., 2006).

Previous caries models suggested bacterial proteases were responsible for collagen degradation (Rosengren and Winblad, 1976; Jackson et al., 1997). However, this idea came into question when further studies showed that cariogenic bacteria were not capable of degrading dentin matrix after demineralization (van

Strijp et al., 1997; Tjäderhane et al., 1998). The bacteria collected from dentinal lesions created *in situ* were found not to be capable of degrading collagen in vitro, and collagenases extracted and purified from bacteria showed low activity in acidic environments (van Strijp et al., 1997). Similarly, Tjäderhane et al. (1998) demonstrated that salivary cariogenic bacteria isolated from caries-active patients showed no gelatinolytic activity.

The mounting evidence indicated that degradation of the organic matrix in dentinal caries lesions was mostly mediated by host-mediated proteases rather than bacterial action (Tjäderhane et al., 1998; Dayan et al., 1983). Dayan et al. (1983) was one of the first to demonstrate that dentin possesses collagenolytic activity. Indeed, over the next several decades, other studies confirmed the presence of intrinsic proteases in dentin (Tjäderhane et al., 1998; Mazzoni et al., 2007; Sulkala et al., 2002, 2007). Carious dentin has been reported to contain latent and active forms of MMP-2, -8, and -9 (Tjäderhane et al., 1998). In sound dentin, MMP-2 and -9 has been identified (Mazzoni et al., 2007), along with MMP-8 (Sulkala et al., 2007). Enamelsyin (MMP-20) has also been identified in in sound dentin as well as in odontoblasts and pulp tissue of both sound and carious teeth (Sulkala et al., 2002).

Our current understanding of the caries disease process is that host-derived MMPs contribute significantly to the destruction of collagen matrix. It is thought that activation of latent MMPs is facilitated by exposure to a low pH, such as the acid environment created by cariogenic bacteria (Loesche, 1986). This initial activation is followed by a period of neutralization, which potentiates MMP

proteolytic capacity (Mazzoni et al., 2006). Although activated MMPs are stable in acidic pH they are only functional at neutral pH (Mazzoni et al., 2015). Neutralization of the acid can occur by the dentinal buffering mechanism (Camps and Pashley, 2000) or through the salivary buffer system (Edgar et al., 1994), thus allowing pH-activated MMPs to cleave matrix components.

Modifications to collagen matrix by MMPs have a significant impact on the ability of dentin to repair itself. It was thought that the matrix in caries affected dentin remained mostly intact until it became heavily demineralized. However, it has been shown that dentin matrix undergoes significant restructuring even early in the demineralization process, which negatively impacts remineralization potential. For dentin to remineralize, nanometer sized apatite crystals must regrow in the gap zones between collagen fibrils (Bertassoni et al., 2009). However, in an immunohistological study by Suppa et al. (2006), it was found that intact collagen and proteoglycans are significantly reduced in caries affected dentin. Similarly, a study by Vidal et al. (2014) revealed an almost complete loss of autofluorescent signal emitted by well-structured collagen in caries affected dentin compared to normal dentin. Both studies findings indicate collagen, still believed to be remineralizable, undergoes early restructuring that affects capacity for intrafibrilllar remineralization.

D. Role of matrix metalloproteases in hybrid layer degradation

Contemporary dentistry often relies on adhesive bonding. During bonding procedures, the tooth surface is conditioned, removing mineral content either partially or completely, to open the collagen matrix (Pashley, 1992). This is performed to allow resin infiltration into the dentin forming the hybrid layer (Van Meerbeek et al., 1992). The quality of resin-collagen interface is directly related to the bond strength and its stability is crucial to the longevity of the restoration (Salvio et al., 2013). Degradation of the hybrid layer can lead to gaps between teeth and restorative materials and ultimately result in failure.

Studies have revealed that the hybrid layer created by dentin bonding systems is unstable over time (Hashimoto et al., 2000, 2003b; Breschi et al., 2008). Because adhesive monomers are not able to fully encapsulate the exposed collagen fibrils (Wang et al., 2003), they are left vulnerable to hydrolytic degradation (Hashimoto at al., 2003) as wells as attack by endogenous proteinases found within the dentin organic matrix (Pashley et al., 2004; Tjäderhane et al., 2013a). In a study by Pashley et al (2004), partially demineralized collagen beams, obtained from human dentin, were stored in artificial saliva, while control specimens were stored in artificial saliva with the addition of proteolytic enzyme inhibitors or in pure mineral oil. They found that collagen matrices were almost completed destroyed after an incubation of 250 days. The specimens incubated with enzyme inhibitors remained fully intact with significantly thicker and more organized collagen networks compared to the dentin aged in mineral oil. In this experiment, Pashley

et al. demonstrated that collagen degradation was due to host-derived enzymes rather than bacterial proteases as was previously thought.

Evidence of collagenolytic and gelatinolytic activities in dentin initially supported the potential involvement of MMPs in the disruption of collagen fibrils within the hybrid layer. Collagen matrices displayed progressive fibrillar disintegration following application of adhesive bonding systems (Garcia-Godoy et al., 2007). While MMPs are dormantly bound in dentin matrix, it has been shown that the acidic components of bonding systems cause enzyme activation (Nishitani et al., 2006; Tay et al., 2006). If the dentin matrix is not fully infiltrated with adhesive resin, activated MMPs they can begin to degrade the collagen fibrils at the bond interface (Carrilho et al., 2007). The degradation of collagen fibrils leads to destruction of the hybrid layer and loss of dentin bond strength over time (Hashimoto et al., 2003a,b).

E. Matrix metalloprotease inhibitors in dentistry

MMP activity in dentin matrices can be regulated by endogenous and exogenous inhibitors. Most of these inhibitors chelate or replace the metal ions at the active site and/or interact with the MMP propeptide fragment. Some other inhibitors prevent MMP access and inhibit activity by coating the substrate (Mazzoni et al., 2012; Tjäderhane et al., 2013b).

Exogenous inhibitors are applied as therapeutic agents that can be chemically synthesized or derived from natural sources. Several artificial MMPs inhibitors have been described, which include tetracycline and their derivatives, doxycycline and minocycline. These antibiotics have been shown both *in vitro* and *in vivo* to inhibit MMP-1, -2, -8, and -12 (Golub et al., 1995; Lauhio et al., 1995). Chemically modified tetracyclines are non-microbial but still retain the ability to inhibit the release and activity of MMPs (Golub at al., 1998; Ramamurthy et al., 1998). Zoledronate is a third generation bisphosphonate, which has the ability to inhibit MMP proteolytic activity (Teronen et al., 1997; Boissier et al., 2000).

Several other synthetic MMP inhibitors are already used in dental practice. Among them, ethylenediaminetetraacetic acid (EDTA), which is an effective zinc and calcium chelator, was recently shown to inhibit the degradation by acid-activated endogenous MMPs of dentin beams treated for 1 minute with 17% EDTA (Thompson et al., 2012). Chlorhexidine is a widely used microbial agent that has also been shown to have proteolytic inhibitory effects. Chlorhexidine has been shown to reduce the activity of MMP-2, -9 and -8 (Gendron 1999). Treatment of dentin with concentrations as low as 0.2% chlorhexidine resulted in higher bond

strength and reduced nanoleakage of resin bonds (Breschi et al 2010). Carrilho et al. (2007) showed that local application of 2% chlorhexidine for 1 minute to the etched dentin surface just before applying the dentin bonding primer was able to inhibit the degradation of the hybrid by MMPs for at least 14 months. It is thought that chlorhexidine works by chelating zinc or calcium ions at the active site (Gendron, 1999).

A different group of MMP inhibitors include those derived from natural sources. Green tea polyphenols, grape seed extracts, and cranberry proanthocyanidins have all been shown to process MMP inhibitory effects (Epasinghe et al., 2013). These compounds may have fewer side effects when compared to synthetic drugs, which makes them particularly attractive as therapeutic agents. It is thought that they can be more safely applied directly on the affected tooth or integrated in daily-used topical products (Chaussain et al., 2013).

F. Berberine as matrix metalloprotease inhibitor

Berberine is a natural isoquinoline alkaloid that can be isolated from several plants, such as Rhizoma coptidis (Huanglian), Hydrastis Canadensis (goldenseal), and Cortex phellodendri (Huangbai) (Ikram, 1975). Berberine possesses a multitude of therapeutic effects. It is known to have anti-inflammatory, anti-microbial, anti-platelet, anti-carcinogenic, and many other pharmacological activities (Imanshahidi and Hosseinzadeh, 2008).

Recently, research has focused on elucidating the pharmacological mechanisms of berberine. In particular, the inhibitory effect of berberine on matrix metalloproteinases has been shown to mitigate several disease processes.

In traditional medicine, berberine has been used for various cardiovascular conditions including hypertension and arrhythmia (Imanshahidi and Hosseinzadeh, 2008). In addition, it is thought that berberine may aid in stabilizing artherosclerotic plaques (Huang et al., 2012) and protect against neuronal damage following transient global ischemia by reducing MMP-9 activity (Hong et al., 2012).

The inhibitory action of berberine on MMP-9 has also been shown to prevent UV light induced skin inflammation and aging in human keratinocytes (Kim et al., 2008).

Several studies have demonstrated MMP inhibition by berberine may play a significant role in restricting cancer growth and progression. ECM degradation is crucial to the invasion and metastasis of cancer cells. Berberine has been widely shown to inhibit MMP activity in cancer cells, thereby inhibiting invasion and metastasis. It has been shown to inhibit the invasiveness of human tongue

squamous cell cancer (Ho et al., 2009), human breast cancer cells (Kim et al., 2012), pulmonary and melanoma cancer cells (Hamsa and Kutan, 2012) through inhibition of MMP-2 and -9. Lin et al. (2008) also demonstrated how berberine prevented invasion and expression of MMP-1, -2, and -9 in human gastric cancer cells (Lin et al., 2008). Berberine exerted an anti-invasive effect in hepatoma cells through suppression of MMP-9, however, showed negligible toxicity to normal human liver cells (Liu et al., 2011).

MMP inhibition also thought to prevent angiogenesis which is necessary for tumor growth. Berberine was shown to inhibit vascular ingrowth in tumor cells by down-regulating the expression of MMP-1 and MMP-2 (Wartenberg et al., 2003).

Within the oral environment, berberine was shown to inhibit the activity of MMP-2 and -9 and reduce the gingival degradation in periodontium (Tu et al., 2013).

There is a multitude of evidence to demonstrate that berberine is an effective MMP inhibitor, which makes berberine an attractive therapeutic agent in the treatment or prevention of diseases involving collagen degradation.

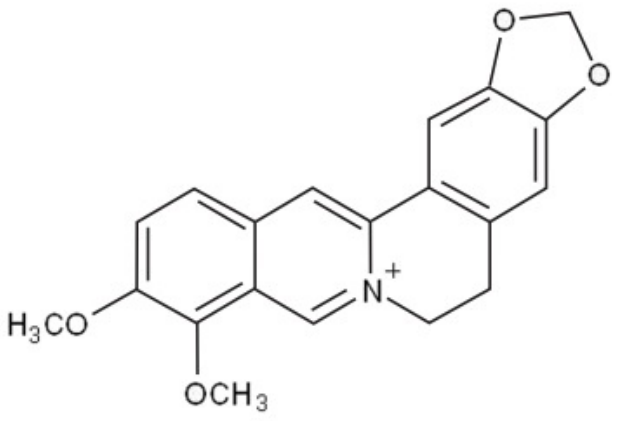


Figure 3. Molecular structure of berberine

III. Methodology

A. Study design

This study was designed as a two-part experiment to assess the inhibitory effect of berberine on MMP activity. Part one of the study was to evaluate the inhibitory effect of berberine on recombinant MMPs (rMMPs) using gelatin zymography. In this technique, proteolytic enzymes were subjected to sodium dodcylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to demonstrate their types by molecular weight and then detected by their ability to degrade a substrate (Vandooren et al., 2013). Recombinant MMP-2 and -9 were selected for this study. The enzymes were separated in gels and then treated with various concentrations of berberine (0.02, 0.002, and 0.0002%). Enzyme inhibition was assessed by ability of rMMP to degrade the collagen substrate within the gel carriers after exposure to different concentrations of berberine. The second part of the study assessed an inhibitory effect of berberine on endogenous MMPs. For this part, specimens of dentin were treated with berberine, chlorhexidine and water (control) and then assessed for collagen degradation over time. The assessment was conducted using a hydroxyproline assay to assess collagen solubilization.

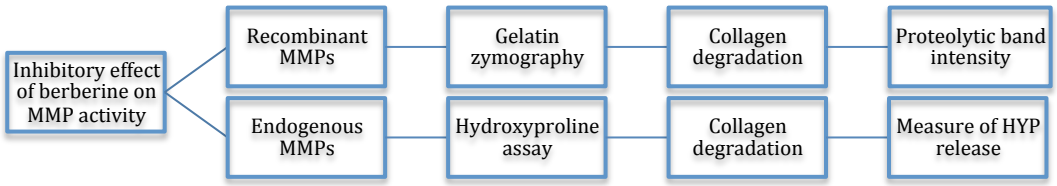


Figure 4. Flowchart of methodology

B. Recombinant matrix metalloproteinase inhibition

1. Experimental design overview

Gelatin zymography assays were conducted to assess the inhibitory effect of various concentrations of berberine on recombinant MMP-2 and -9. In gel zymography, gelatin is copolymerized in an SDS-PAGE gel as a substrate (Tjäderhane et al., 2013a). After electrophoresis, the tested protein samples are separated by their molecular weight. The gel is then incubated in buffering solution, allowing gelatinolytic enzymes in the sample to degrade the gelatin in the gel. Specific enzymes and their enzymatic activity can be determined by the location and strength of the bands in the gelatin gel (Tjäderhane et al., 2013a).

2. Zymographic analysis

Activation of enzymes was achieved by incubating samples and standards with 2 mM p-aminophenylmercuric acetate (APMA) at 37°C for 1 hour. MMP-2 and MMP-9 were electrophoresed using PowerPac Basic (Bio-Rad Laboratories, Hercules, CA, USA) under non-reducing conditions in 10% SDS-PAGE co-polymerized with 0.2% of fluorescent MDPF-labeled gelatin. Human recombinant MMP-2 and MMP-9 were obtained from AnaSpec (Fremont, CA, USA). After eletrophoresis, gels were washed in 2.5% Triton-X 100 2% for 30 min under agitation. The wash was repeated with fresh solution for an additional 30 minutes. Caution was taken to protect gels from light.

After the gels were incubated for 48 hours at 37 °C in enzyme buffer, they were divided into the following groups according to incubation buffer: Group 1-

MMPs buffer; Group 2- MMPs buffer containing 0.02% berberine; Group 3- MMP buffer containing 0.002% berberine; Group 4- MMPs buffer containing 0.0002% berberine; Group 5- MMPs buffer containing 2 mM phenantroline. Berberine was obtained from Sigma-Aldrich (St. Loius, MO, USA). Postive and negative control zymograms were included in the presence of plain buffer (Group 1) and 2 mM phenantroline (Group 5), respectvely. Images on gels were captured under UV light using Quantity One analysis software in a Gel Doc™ XR+ system (Bio-Rad Laboratories, Hercules, CA, USA).

3. Data analysis

Band intensities were quantified using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

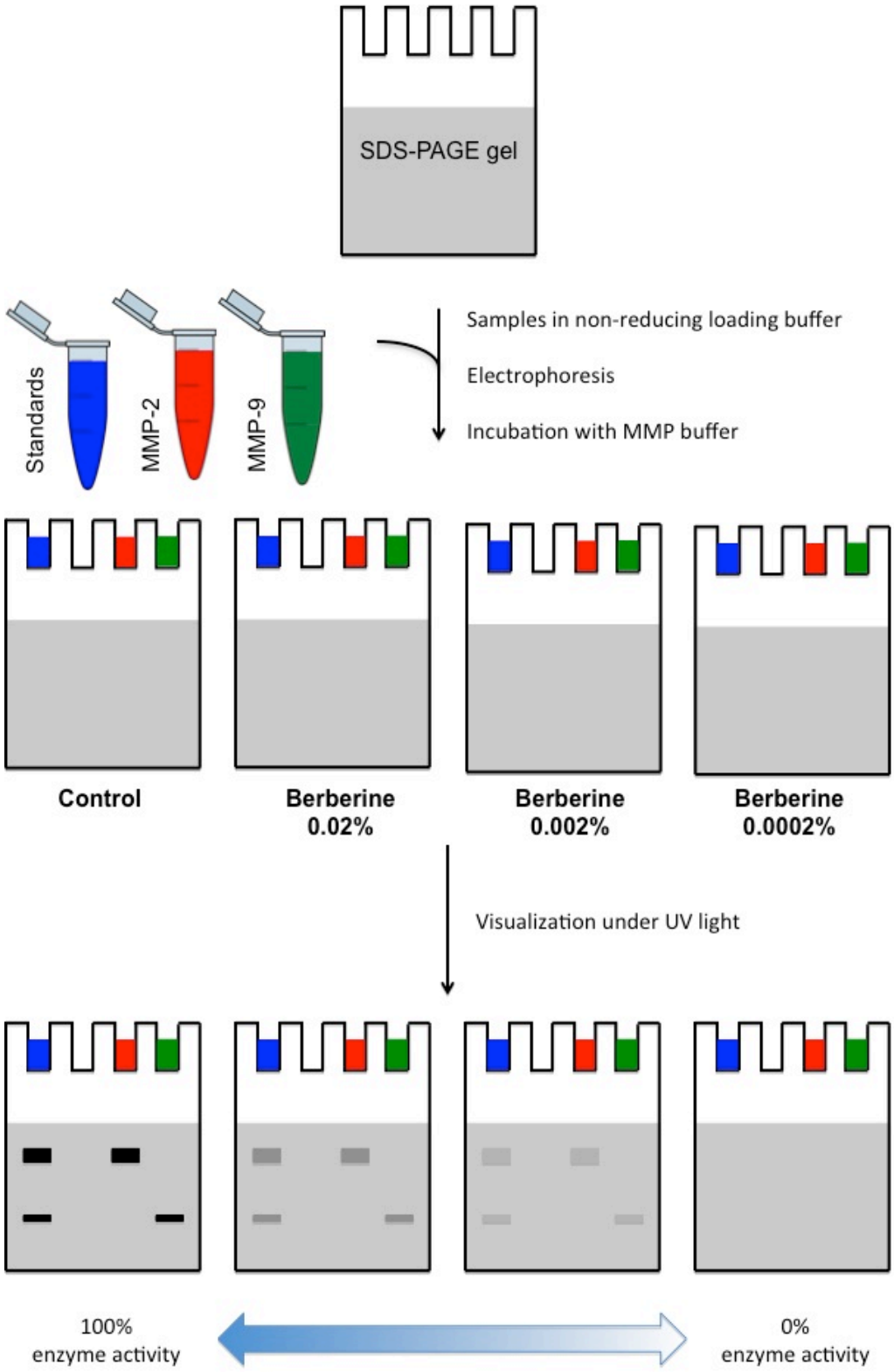


Figure 4. Gelatin Zymography Schematic

C. Endogenous MMP inhibition

1. Specimen preparation

Extracted non-carious human molars were selected. Roots were removed from the teeth and discarded (IRB/ACCC protocol: 2009-0198). Enamel was manually removed from the anatomical crown using a high speed medium grit diamond bur under copious water irrigation. The remaining dentin core was visually inspected to ensure complete removal of enamel and any residual pulp tissue. The dentin specimens were frozen with dry ice (carbon dioxide) and then manually pulverized with a steel mortar and pestle to form a homogenous dentin powder in accordance to Mazzoni et al., 2007.

The dentin powder was demineralized with 10% phosphoric acid at room temperature for 5 hours under agitation, and complete demineralization was verified radiographcially (Bedran-Russo et al., 2010) (Figure 5). Following, 4M NaOH was added to neutralize the acid and end demineralization activity. Samples were centrifuged (12,000 rpm for 10 minutes), washed twice with distilled water for 30 minutes, and thoroughly dried.

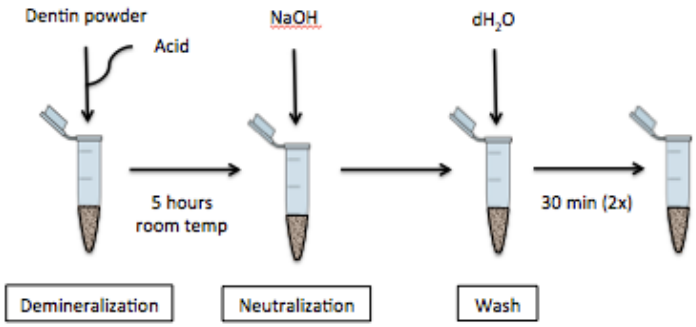


Figure 5. Dentin powder specimen preparation for HYP assay

2. Test Groups

The dentin powder was separated into 50 mg aliquots. The specimens were then divided into 5 groups (N=15) according to the treatment solution. Group 1: water; Group 2: 0.02% berberine; Group 3: 0.2% berberine; Group 4: 0.002% chlorhexidine; Group 5: 0.02% chlorhexidine. Berberine and chlorhexidine were obtained from Sigma-Aldrich (St. Loius, MO, USA). Powder specimens were incubated with the aforementioned solutions for 1 hour at 37° C under agitation and then washed three times with distilled water. Afterwards, the specimens were incubated with artificial saliva (5 mM HEPES, 2.5 mM $CaCl_2$, 0.3 mM NaN_3, 0.05 mM $ZnCl_2$ pH 7.4) at 37° C under agitation for a total of 7 days. The artificial saliva samples were collected at 24 hours, 48 hours, and 7 days. Following each collection time, specimens were returned to incubation with fresh artificial saliva. A 500 μL of artificial saliva was saved from each collected sample. The samples were lyophilized and stored at -20°C prior to hydroxyproline analysis.

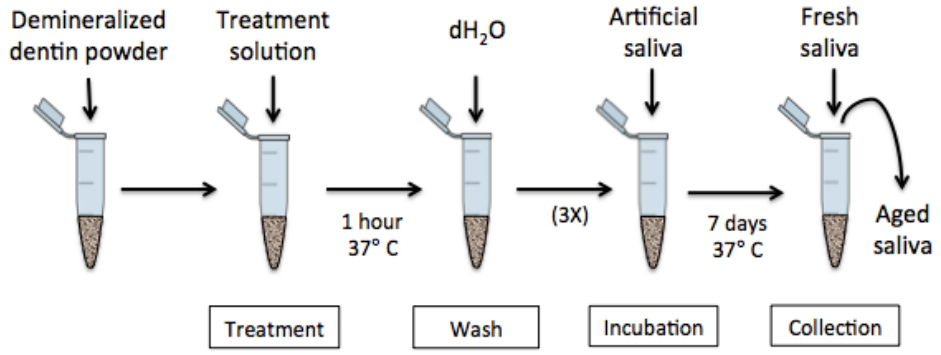


Figure 6. Treatment and incubation of demineralized dentin powder

TABLE I

TREATMENT GROUPS

Group (N=15)	Treatment solution
1	Water
2	0.02% berberine
3	0.2% berberine
4	0.02% chlorhexidine
5	0.2% chlorhexidine

3. <u>Quantification of collagen degradation</u>

Collagen degradation was assessed through an assay of hydroxyproline release as previously described by Reddy & Enwemeka (Clinical Biochemistry, 1996). Hydroxyproline is an amino acid thought to be found exclusively in collagen (Reddy and Enwemeka, 1996). Type I collagen contains about 10% hydroxyproline by mass, while most other proteins have minimal amounts or none of this amino acid (Bornstein and Sage, 1980). It is formed through the hydroxylation of proline by the enzyme prolyhydroxylase (Reddy and Enwemeka, 1996). Because hydroxyproline is quite specific to collagen, its release is a convenient measure for collagen breakdown.

In the present study, a hydroxyproline assay was utilized to directly quantify the amount of collagen degradation in extracts of dentinal tissue. The measurement is based on a colorimetric analysis of hydroxyproline. The procedure is based on alkaline hydrolysis of the tissue homogenate and subsequent determination of the free hydroxyproline in the hydrolysates (Reddy and Enwemeka 1996). Chloramine-

T is used to oxidize the free hydroxyproline for the production of a pyrrole, a heterocyclic aromatic organic compound with a chemical formula of C_4H_4NH (Loudon, 2002). The addition of Ehrlich's reagent results in formation of Ehrlich chromogen, a chromophore that can be measured quantitatively in a spectrophotometer.

4. <u>Hydroxyproline assay</u>

Aliquots of standard HYP samples were prepared by mixing a stock solution (0.5-16 μL) with 2N NaOH to reach a final volume of 50 μL. Similarly, test samples were prepared by mixing the contents of the lyophilized artificial saliva samples with 40 μL NaOH 2N to reach a final volume of 50 μL. Contents of all were vortexed and centrifuged (accuSpin Micro 17, Fisher Scientific, Waltham, Massachusetts, USA) at 1000 rpm for I minute. Afterwards, samples and standards (Sigma-Aldrich St. Loius, MO, USA) were hydrolyzed in an oven at 120°C for 60 minutes. After hydrolysis, 450 μL of chloramine T reagent was added, mixed, and incubated at room temperature for 25 minutes. Next, 500 μL of Ehrlich's reagent was added, mixed, and incubated at 65°C for 40 minutes. Absorbance of samples was read at 550 nm using a 96 well plate in a spectrophotometer (SpectraMax Plus 384, Molecular Devices; Sunnydale, CA, USA) and compared to a set of standard hydroxyproline samples.

Time points tested were 24 hours, 48 hours and 1 week. Hydroxyproline concentration was determined in μg of hydroxyproline/ml of artificial saliva solution and was normalized to the dry weight of dentin (mg).

5. Statisical analysis

Results from the hydroxyproline assay were statistically analyzed using two-way and one-way ANOVA and Games Howell post hoc tests ($p < 0.05$) using SPSS for Windows (version 19, SPSS Inc., Chicago IL USA).

IV. Results

A. Inhibition of rMMPs - gelatin zymography results

Results of the gelatin zymography are shown in Figure 2. A positive control zymogram of recombinant MMP-2 and -9 exhibited their active enzymatic activities as indicated by the intense positive bands in the gels. Zymograms of recombinant MMPs treated with berberine showed a decrease in the enzymatic activity as illustrated by the reduction in the intensity of the positive bands. All berberine concentrations decreased both MMP-2 and -9 activities. The inhibitory effect of berberine was observed to be concentration dependent. Incubation with a 0.02% berberine solution produced nearly a complete inhibition of MMP-2 and -9 activities. Activity of MMP-2 was reduced by 93% while MMP-9 was reduced by 97%. At concentrations as low as 0.0002%, berberine showed a decrease in enzymatic activity. Activities of MMP-2 and -9 were reduced by 48% and 26%, respectively. A negative control zymogram incubated with 5mM EDTA and 2mM phenanthroline showed no enzymatic activity (data not shown).

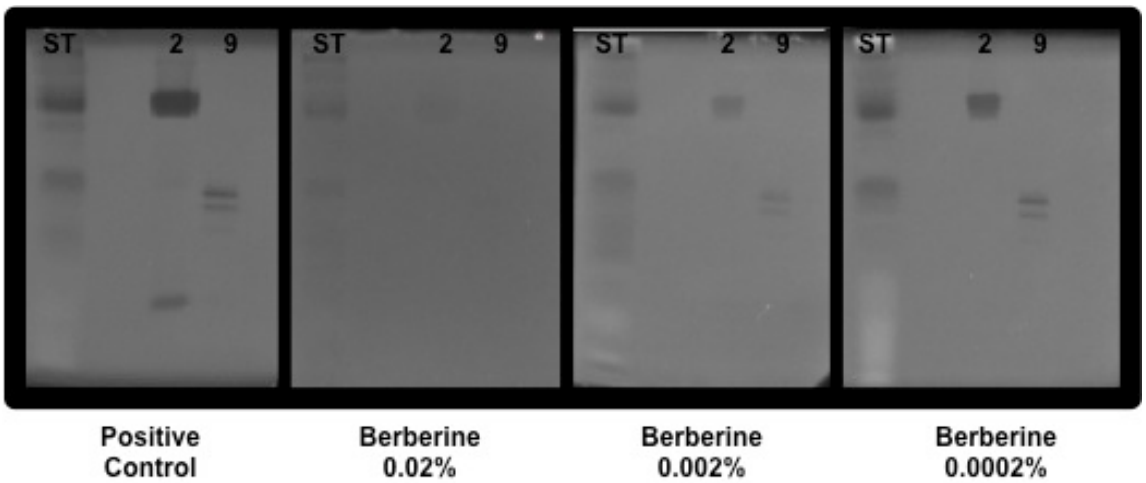


Figure 7. Gelatin zymogram of rMMP activity after treatment of various concentrations of with berberine; ST: standard molecular weight, 2: MMP-2, 9: MMP-9

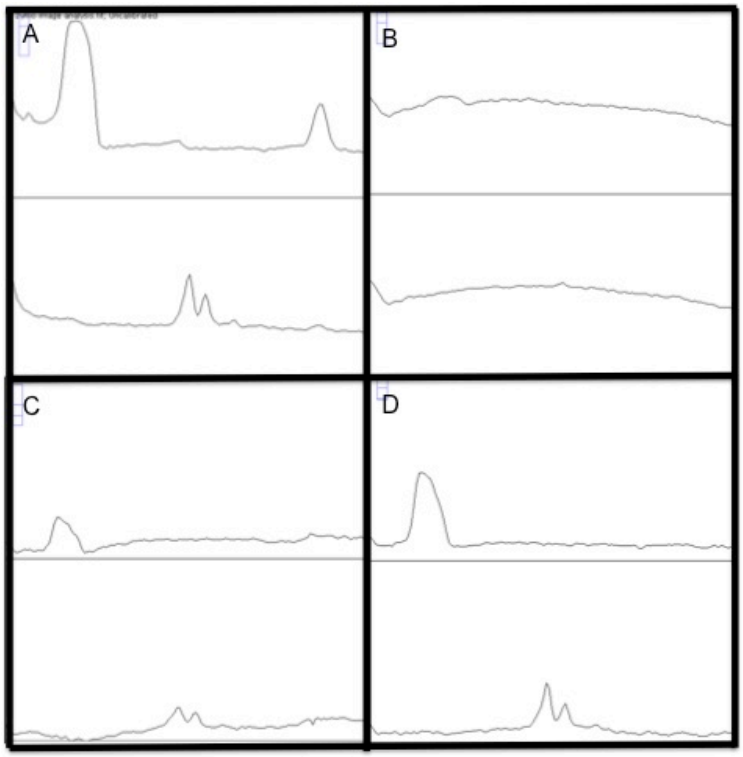


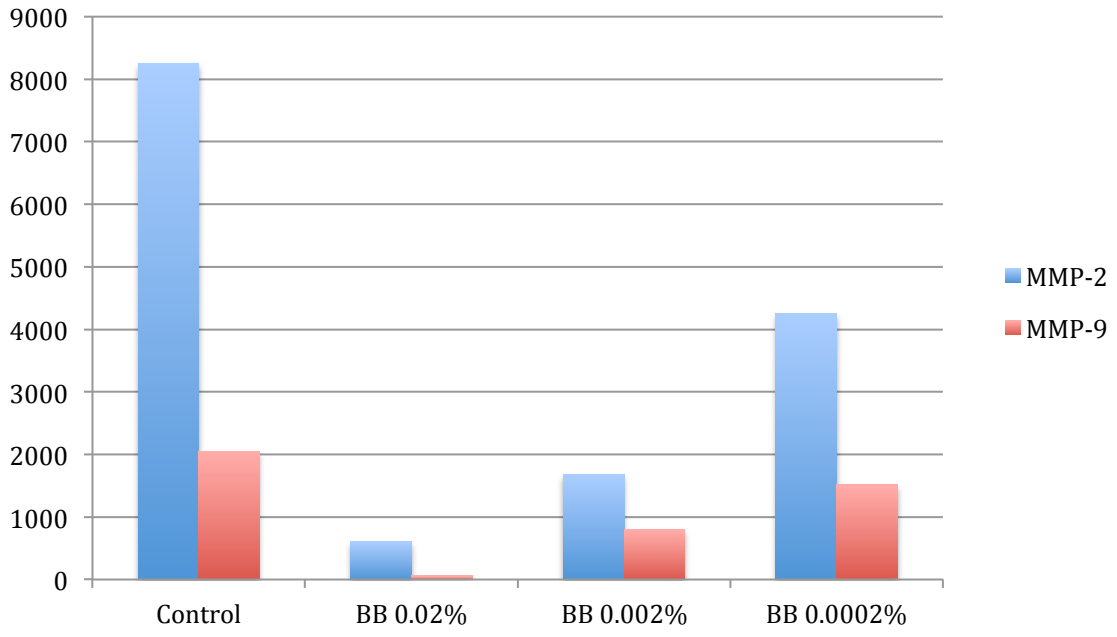
Figure 8. Densitometry profiles for gels; A. Control (MMP-2: upper graph, MMP-9: lower graph) B. Berberine 0.02% C. Berberine 0.002% D. Berberine 0.002%.

TABLE II

RECOMBINANT ENZYME ACTIVITY AFTER TREATMENT WITH BERBERINE

	rMMP-2 Peak Area	**rMMP-2 Percent Activity**	**rMMP-9 Peak Area**	**rMMP-9 Percent Activity**
Control	8250.832	100	2052.497	100
Berberine 0.02%	606.506	7.351	58.364	2.844
Berberine 0.002%	1683.012	20.398	801.841	39.067
Berberine 0.0002%	4257.033	51.595	1512.255	73.679

A.



B.

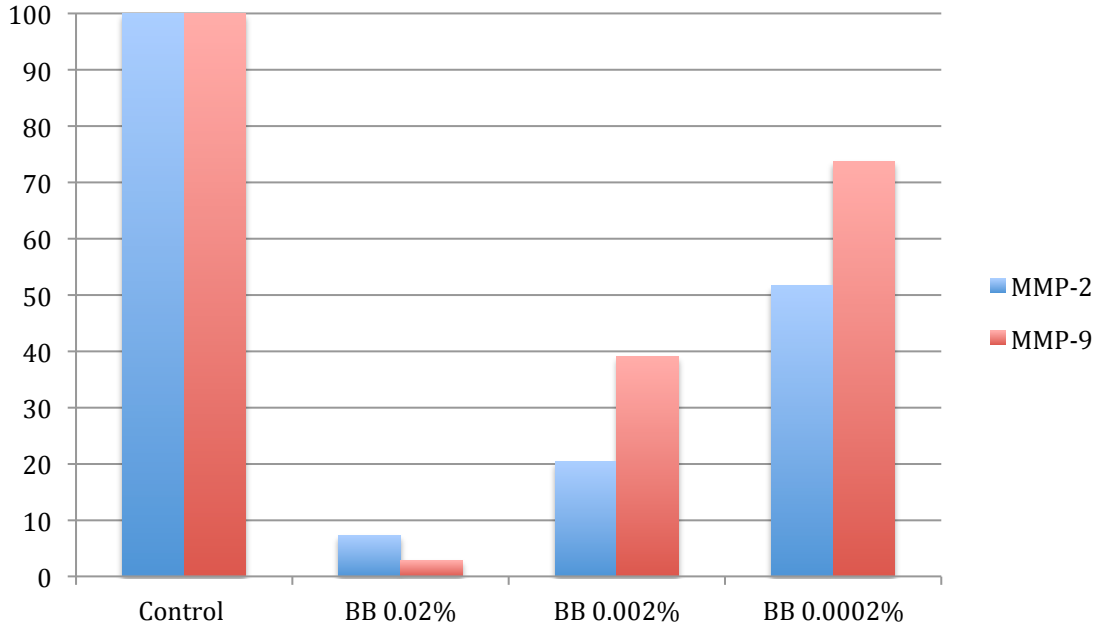


Figure 9. Plot of the peak areas (A) and percent rMMP-2 and -9 activity (B); BB: berberine

B. <u>Inhibition of endogenous MMPs – hydroxyproline assay results</u>

The findings from the hydroxyproline assay are summarized in table 1. The results were calculated as cumulative averages of hydroxyproline release (i.e, 48 hours indicates 1st and 2nd days of incubation). Statistical analysis was performed using two-way and one-way ANOVA ($p < 0.05$). Two–way ANOVA showed no significant difference among the factors ($p = 0.597$); however, a significant difference was observed within each factor ($p < 0.05$). One-way ANOVA showed a statistically significant increase in hydroxyproline release over the observation period ($p < 0.001$). Each treatment group exhibited an increase in collagen solubilization over the 1-week period of time. Post-hoc Games-Howell tests showed significant differences between some of the treatment groups. Dentin treated with 0.02% chlorhexidine had a significant reduction in collagen breakdown as compared to the negative control group (water). Treatment with 0.02% chlorhexidine had significantly less hydroxyproline release than dentin treated with 0.2% chlorhexidine. No statistical differences in the amount of collagen degradation were observed among the groups treated with berberine.

TABLE III

MEAN HYDROXYPROLINE RELEASE µg/ml/mg dentin

DW: distilled water; BB: berberine, CHX: chlorhexidine; Groups identified by different letters are significantly different.

Group	Treatment	Time			Pooled Data
		24 hours	48 hours	1 week	
1	DW	3.00 ±1.14	4.07±1.52	6.01±1.73	4.62±2.10 A
2	0.02% BB	2.79±1.69	3.47±2.08	6.22±3.45	4.16±2.87 A,B
3	0.2% BB	2.10±1.01	2.76±1.58	4.84±2.10	3.23±1.96 A,B
4	0.02% CHX	1.80±0.58	2.98±0.57	4.48±0.66	3.09±1.26 B
5	0.2% CHX	3.09±0.77	4.42±1.32	4.92±1.44	4.14±1.41 A
Pooled Data		2.58±1.22 a	3.53±1.58 b	5.43±2.23 c	

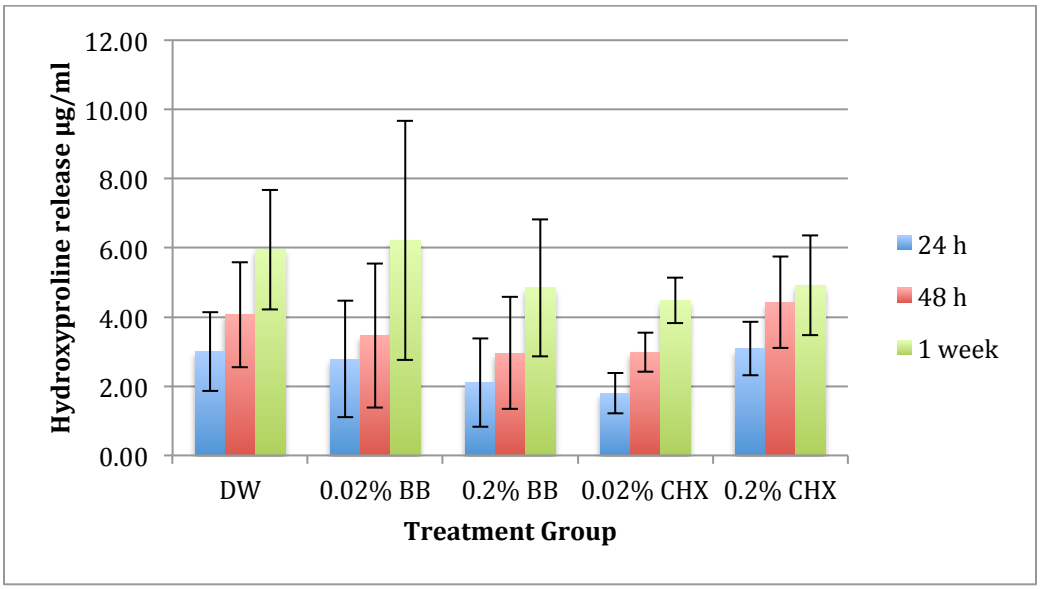


Figure 10. Mean hydroxyproline release µg/ml/mg dentin (SD); DW: distilled water; BB: berberine, CHX: chlorhexidine

V. Discussion

A. Study findings

In the present study, the inhibitory effect of berberine on recombinant and endogenous MMP activity was examined. Results of zymographic analysis showed that gelatinolytic activity of recombinant MMP-2 and -9 was inhibited by berberine. This study helps to clarify the mechanism of MMP inhibition by berberine. Berberine acts both directly and indirectly on MMPs to suppress activity. Previous studies have shown MMP inhibition by berberine is partly mediated through the suppression of upstream signaling molecules such as u-PA, AP-1, and NF-κB, which affect gene expression of MMP-2 and -9 (Ho et al., 2009; Hamsa and Kuttan, 2012, Liu et al., 2011; Kim et al., 2008,2012; Wartenberg et al., 2003; Lin et al., 2008). Direct inhibition of MMP activity by berberine has been also demonstrated (Hong et al., 2012); however, the mechanism by which this occurs is less clearly understood. Based on results of the zymography assay, in which directly applied berberine solutions suppressed recombinant MMP activity, there is further evidence to demonstrate berberine also acts directly on contact to inhibit MMP activity. While the exact mechanism of action is unknown, it is possible that berberine may bind the enzyme and induce a conformation change in the structure, which either inactivates it or prevents it from binding to collagen. Berberine could also function similar to chlorhexidine or EDTA in chelating the metal ions. Since berberine inhibited both recombinant MMPs in the experimental model, the first null hypothesis that berberbine has no effect on rMMP inhibition was rejected.

Results of the hydroxyproline assay of endogenous MMP activity showed no differences in the relative proteolytic activities in demineralized dentin among the groups treated with berberine compared to the negative control (water). The lack of endogenous MMP inhibition by berberine was a rather surprising finding given that berberine exhibited strong inhibition of recombinant MMP activity. However, studies on other potential MMPs inhibitors have demonstrated similar discrepancies between inhibition of soluble versus matrix bound MMPs (Tezvergil-Mutluay et al. 2011 a,b). Unlike recombinant enzymes, endogenous MMPs are bound within the dentin matrix and are not fully available. These enzymes (MMP-2 and -9) are bound intimately along the collagen fibrils of dentin (Mazzoni et al., 2008). When the dentin matrix mineralizes, MMPs become covered with hydroxyapatite crystals, immobilizing and inhibiting functionality of the enzymes. As long as dentin is mineralized, its proteases remain structure stable (Nishitani et al., 2006).

Because endogenous MMPs are trapped within the calcified dentin matrix, it is thought that phosphoric acid activates the pro-MMPs in part through demineralization of the dentin matrix, which allows release of complexed MMPs into the surrounding environment (Mazzoni et al., 2012). Acid demineralization also acts to remove minerals from collagen intramolecular regions, exposing cleavage sites for MMPs. Without adequate demineralization collagen fibers will be partially protected from the degradative processes of MMPs and the apparent effect of MMP inhibitors may seem diminished. TEM examination of the air-dried dentin powder after 10% phosphoric acid etching for 15 seconds showed that remnant mineralized matrices were present even in the finest dentin particles (Mazzoni et al., 2006). In

the present study, dentin powder was exposed to 10% phosphoric acid for a period of 5 hours. With such a lengthy demineralization cycle, it is likely that nearly all mineral content was removed. Thus, it was presumed that enzymes and collagen substrate were both adequately exposed.

However, it had not yet been settled whether the demineralization/activation protocol used in the current study could have had any adverse effect on endogenous MMP activity. The concern was if the pool of enzymes were not fully activated, then the apparent effect of inhibitors would be reduced. In fact, enzymatic activity varies greatly depending on the demineralization/activation treatment (Mazzoni et al., 2007). It was found that the highest MMP activities were found after citric acid treatment, whereas acetic acid and EDTA treatments yielded much lower levels of active MMPs. Other studies have actually found that acid etching dentin powder may significantly lower MMP activity (Mazzoni et al., 2006; Nishitani et al., 2006; Pashley et al., 2004). Mazzoni et al. (2006) found that acid etching mineralized dentin powder with 10% phosphoric acid for 15 seconds resulted in almost complete inactivation (98.1%) of its inherent proteolytic activity. Likewise, it was observed that treatment of mineralized dentin powder with 37% phosphoric acid gel for 15 seconds actually reduced the inherent collagenolytic activity of mineralized dentin by 65% (Pashley et al., 2004).

It is believed that etching with strong acids results in MMP inactivation because of protein denaturation. Although some MMPs are known to resist extremes in temperatures and pH (Sulkala et al., 2007), 10% and 37% phosphoric acid have a pH value of 0.4 and -0.7, respectively, which is likely to cause at least

partial enzyme denaturation upon exposure. However, others argue against the idea that strong acids can chemically denature matrix bound proteases (Tezvergil-Mutluay et al., 2013). Another theory proposed by Nishitani suggests that strong acids raise local calcium ion concentrations so that it forms an insoluble reaction product (Iwasa et al., 2011) that precipitates as fine crystals over exposed collagen and MMPs thereby blocking access of substrates. Nonetheless, it has been shown that mild acids are more effective in activating MMPs (Mazzoni et al., 2006; Nishitani et al., 2006). Tjäderhane et al. (1998) found that pH values between 2.3 and 5 were most effective in activating salivary gelatinases. Self-etching adhesives, with pH values of only 2-3, have been demonstrated to be successful MMP activators because they expose the enzymes without denaturing them (Nishitani et al., 2006).

Based on these findings, it is plausible to consider that the dentin powder may have been overly demineralized in this study resulting in less than maximal MMP activation. In spite of this, results showed hydroxyproline release in all treatment groups, signifying proteases were active and enzymatic degradation of collagen was occurring. If the demineralization procedure was ineffective in stimulating MMP activity, then we would expect an absence of hydroxyproline release in all treatment groups. Thus, it was concluded that the demineralization/activation protocol was not a likely a reason for lack of MMP inhibition by berberine.

The question remains as to why berberine lacked any sign of inhibitory effect in the dentin model. One thing important thing to consider the interplay of endogenous proteolytic enzymes with non-collagenous proteins found in dentin

matrix. There are several MMPs (MMP-1,-2,-7,-8,-9,-13) that can bind to proteoglycans (PG) and glycosaminoglycans (GAG) (Tjäderhane et al., 2013). These PG/GAG complexes may affect the binding properties and enzyme activity of MMPs. It is also thought that these complexes may facilitate MMP activation and stabilization, possibly even accelerating conversion of zymogens to active form (Tjäderhane et al., 2013). It is plausible that MMP inhibitors may not exert the same effect on the PG/GAG complexed MMPs compared to soluble or unbound enzymes. Thus, this may provide one reasonable explanation why recombinant MMPs had stronger inhibition potential than compared to dentin matrix-bound MMPs.

Furthermore, dentin is known to contain other proteolytic enzymes aside from MMP-2 and -9, including MMP-3, -8, and cystheine cathepsins (Tersariol et al., 2010). The later is a class of proteases that can also hydrolyze collagen. The inhibitory potential of berberine may not be the same for all the endogenous enzymatic components of dentin. There is evidence to support that some MMPs may be more sensitive to inhibition than others (Gendron et al., 1999). Thus, it is not known if the inhibitory effect of berberine on MMP-2 and -9 will be correlated to inhibition of other dentin proteases.

There are also inherent differences in the collagen found in gelatin (type IV) compared to dentin matrix (type I). Both collagen types are made up of three polypeptide chains, which wrap around one another to form a rope-like tight triple helical structure called tropocollagen (Linsenmayer, 2013; Traub and Piez, 1971). Type I collagen is commonly termed “fibrillar” collagen because it is capable of forming very resistant fibers due to extensive cross-linking between tropocollagen

molecules (Kalluri, 2003; Nguyen et al., 2012). Type IV collagen structure differs from type I in that it does not form fibers but rather a plane network (Kalluri, 2003). Due to these structural differences, gelatin may be more easily degraded because it is less cross-lined than dentin collagen matrix. Because gelatinases have a simpler enzymatic job, they may be more sensitive to inhibition than other collagenases. This may have contributed to the stronger inhibitory effect of berberine seen with the recombinant MMPs compared to the endogenous model.

Another possible explanation for lack of demonstrable MMP inhibition of may be related to berberine's mechanism of action. Anti-proteolytic compounds inhibit MMPs through different mechanisms such as direct inhibition of enzyme function, interference in activation of pro-MMPs, and reduced expression MMP genes and protein levels. For anti-MMP agents that work through direct inhibition, substantivity plays an important role in degree of effectiveness. Substantivity is defined as the persistence of effect of a topically applied drug, determined by the degree of physical or chemical bonding to the substrate. This prolonged association affects the duration and magnitude of enzyme suppression. The effectiveness of chlorhexidine as an anti-proteolytic agent is partly related to its substantivity with dental tissues (Carrilho et al., 2010). It is not known if berberine possesses a strong affinity to dental tissues. If berberine has a limited degree of substantivity, then it is assumed that nearly all of the compound would have been eliminated from the dentin samples during the washing steps of the experimental model. Hence, in the absence of berberine, dentinal MMPs would be free to function.

The assumption of this study was that the MMP inhibitors would have sufficient substantivity to resist the washing steps in the protocol. A study by Kim et al. (2010) found that rinsing demineralized dentin powder with water resulted in the highest release of chlorhexidine, up to 95% debonding. It is thought that water causes displacement of chlorhexidine from collagen because it has a stronger bond affinity. This is one of the reasons why studies on chlorhexidine MMP inhibition commonly examine it in conjunction with resin bonding systems (Carrilho et al., 2007; De Munck et al., 2009; Breschi et al., 2010). When dentin is treated with chlorhexidine, it becomes sealed into place when the adhesive is applied and is protected against the rinsing action of fluids. Based on these findings, it is conceivable that rinsing with water after treatment of dentin caused significant debinding of MMP inhibitors, resulting in lack of MMP inhibition in some experimental groups.

In addition, this study was carried out for a short period of time (1 week) so it is possible that a longer incubation time would have yielded different results. Although some changes in dentin are observable after 1 week of incubation following treatment with MMP inhibitors, studies suggest a time frame of about 4 weeks to quantitate the level of collagen degradation in dentin (Carrilho et al., 2009). This is deemed a reasonable time frame to evaluate the potential of MMP inhibitors. Collagen degradation by MMPs seems to be a slow process, which may take several weeks to see measurable differences (Dayan et al., 1983). At one week, inhibitors may have an insignificant impact on total proteolytic activity if the pool of

available MMPs is not in full force. Therefore, by observing collagen degradation for a longer period of time, the effect of MMP inhibitors may be more fully realized.

It was interesting to note that the lower concentration of chlorhexdine (0.02%) exhibitied a stronger anti-collagenolytic effect against compared to the higher concentration of chlorhexidine (0.2%).These results are unusual, as previous studies have clearly demonstrated that chlorhexidine is an effective MMP inhibitor. A study by Breschi et al. (2010), found that dentin gelatinolytic activity was inhibited by chlorhexidine even at concentrations as low 0.2%. However, Carrilho et al. (2010) showed that increases in chlorhexidine concentration can either increase or decrease the absorption on to dentin substrates. It is thought that due to repulsive intermolecular forces, once a molecule of chlorhexidine binds to site on the outer surface of dentin, no further uptake can take place at that site (Carrilho et al., 2010). Perhaps, application of a lower concentration of chlorhexidne resulted in the formation of a more stable layer of retained chlorhexidine on the dentin surface, whereas the higher concentration may have only oversaturated the substrate, causing a rapid release of the excess.

Berberine has been shown to be an effective inhibitor of recombinant MMP-2 and -9. The inhibitory effect on endogenous MMPs was not apparent. At this point, berberine cannot be ruled out as an effective MMP inhibitor because there may have been confounding variables in the dentin model. These factors may include issues related to inhibitor selectivity, potency, substantivity and length of study.

B. <u>Strengths and limitations of study</u>

The first part of this study tested inhibition of berberine on recombinant MMPs. This was done to initially establish evidence that berberine had potential MMP inhibitory ability. MMP-2 and -9 were selected because these MMPs would be most clinically relevant to study. The presence of host-derived MMP-2 and -9 in mature dentin have been shown to contribute to the degradation of the organic matrix (Fukae et al., 1991; Tjäderhane et al., 1998) during dental caries development (Dayan et al., 1983; Sulkala et al., 2001; Vidal et al. 2014), and throughout resin- infiltrated dentin interfaces (Nishitani et al., 2006). While preliminary zymographic data showed berberine was effective at inhibiting gelatin breakdown by recombinant MMP-2 and -9, results must be interpreted carefully. It was not known if berberine had the same inhibitory potential for endogenous MMPs. Using the same zymographic technique, this could have been further explored by running a gel using extracted human MMPs from dentin.

After establishing that berberine has potential to inhibit MMP activity, the next step was to determine if this compound could inhibit endogenous collagenolytic activity in dentin. This was necessary to establish clinical relevance of berberine as a potential dental therapeutic agent. Interestingly, the dentin model failed to show an inhibitory effect of berberine against endogenous proteases. However, it has been established that dentin is known to contain many other proteases aside from gelatinases (MMP-2 and -9). The hydroxyproline assay employed did not allow for quantifying specific proteolytic activity. This was a major drawback to the assay because could not be revealed if berberine possessed

any degree of enzyme specificity. While, the net inhibitory effect could be measured, the specific targeted proteases could not be identified using the hydroxyproline assay.

In understanding why berberine seemed to lack inhibition against endogenous MMPs, it is important to recognize that differences exist between recombinant and native MMPs, which may influence effectiveness of inhibitors. For example, recombinant MMP-9 is less glycosylated and has a lower molecular weight than natural MMP-9 (Vandooren et al., 2013). Recombinant enzymes are manufactured to ensure a purified product available in fully active form. In contrast, endogenous gelatinases are found in latent form and may be already complexed with endogenous inhibitors, PGs, and GAGs, all of which may suppress enzymatic activity. Due to possible protein complexes, endogenous MMPs may require higher concentrations of inhibitors than recombinant proteases to elicit a comparable effect. One major limiting factor faced was the upper concentration limit of berberine treatment solution that could be utilized in the dentin model. Due to the fact that purified berberine has an intense yellow hue, this restricted the upper concentration of berberine that could be used without interference of background color with spectrophotometric readings. At concentrations higher than 0.2%, berberine caused permanent staining of the dentin powder. It was found that the yellow hue would leech into the incubation solution over time. Since the hydroxyproline assay is a colorimetric assay, any aberrant background color in the samples may affect the accuracy of the results. Therefore, berberine concentrations

were kept low enough to ensure no residual staining of dentin or incubation solution.

The current study only focused on the inhibition of endogenous matrix bound MMPs. Aside from MMPs, Catehpesin were are localized and dentin and have been linked to caries diseases.

The current study also used a relatively long application time of MMP inhibitors, which may not be practical in a clinical setting. An application time of approximately 30-60 seconds should be the target time frame (Carrilho et al., 2007,2010) for achieving sufficient uptake onto the dentin surface.

C. Future studies

Further studies are needed to elucidate the efficacy of berberine against endogenous dentin MMPs, including the long-term significance on MMP inhibition. Since collagen degradation by MMPs seems to be a slow process, a longer study should be carried out. It would also be of interest to evaluate how berberine affects activity of different MMP types. Alternative assays for assessing MMP inhibition should be explored, such as Biotrak (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) that allows for quantification of specific enzyme activity. This may aid in clarifying if berberine has enzyme specificity. Future studies may explore the inhibitory effect of berberine at higher concentration levels using a non-colorimetric assay, such as high pressure liquid chromatography. In addition, other demineralization/activation and treatment protocols should be considered to ensure that dentinal MMPs reach full activation and berberine achieves maximal binding to dentin matrix. Future studies should also investigate the therapeutic role of incorporating of berberine into restorative materials, such as resin adhesives. Application of berberine in conjunction with resin bonding agent could enhance substantivity and may produce a stronger inhibitory effect. As the benefits of berberine continue to be investigated, this compound may prove to be an effective alternative to other similar chemicals with possibly fewer side effects because it is naturally- derived product (Liu et al., 2011).

VI. Conclusion

Considerable evidence supports the importance of MMPs as key host-derived mediators of pathological dentin degradation as observed in caries lesions and resin-bonded interfaces. Berberine, a plant-derived compound, has been shown to inhibit rMMP activity. Within the limits of this study, it may be concluded that berberine possess inhibitory potential against recombinant MMP-2 and -9. However, berberine seems to lack short-term inhibition against endogenous dentin proteases. Further studies are needed to assess the interactions of berberine with endogenous dentinal MMPs and methods for clinical application.

CITED LITERATURE

Armstrong SR, Vargas MA, Chung I, Pashley DH, Campbell JA, Laffoon JE, Qian F. Resin-dentin interfacial ultrastructure and microtensile dentin bond strength after five year water storage. Oper Dent 2004;29:705-712.

Bedran-Russo AKB, Vidal CMP, Dos Santos PH, Castellan CS. Long-term effect of carbodiimide on dentin matrix and resin-dentin bonds. Journal of Biomedical Materials Research B: Applied Biomaterials 2010;94B:250-255.

Bertassoni LE, Habelitz S, Kinney JH, Marshall SJ, Marshall GW, Jr. 2009. Biomechanical perspective on the remineralization of dentin. Caries Res. 43(1):70-77.

Blackburn RS, Harvey A, Kettle LL, Manian AP, Payne JD, Russell SJ. Sorption of chlorhexidine on cellulose: mechanism of binding and molecular recognition. J Phys Chem B 2007;111:8775-8784.

Bornstein, P., Sage, H. Structurally distinct collagen types. Annual Review of Biochemistry 1980;49:957-1003.

Breschi L, Martin P, Mazzoni A, Nato F, Carrilho M, Tjäderhane L, Visintini E, Ccadenaro M, Tay FR, De Stefano Dorigo E, Pashley DH. Use of a specific MMP-inhibitor (galardin) for preservation of hybrid layer. Dent Mater 2010;26:571-578.

Camps J and Pashley DH. Buffering action of human dentine in vitro. J Adhes Dent 2000;2:39-50.

Carrilho MR, Carvalho RM, Sousa EN, Nicolau J,Breschi L, Mazzoni A, Tjäderhane L, Tay FR, Agee K, Pashley DH. Substantivity of chlorhexidine to human dentin. Dent Mater. 2010;26(8):779-785.

Carrilho MR, Tay FR, Donnelly AM, Agee KA, Tjäderhane L, Mazzoni A, Breschi L, Foulger S, Pashley DH. Host-derived loss of dentin matrix stiffness associated with solubilization of collagen. Journal of Biomedical Materials Research: Part B, Applied Biomaterials 2009;90:373-380.

Carrilho MRO, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L, Reis AF, Hebling J, Mazzoni A, Breschi L, Pashley D. In vivo preservation of the hybrid layer by chlorhexidine. J Dent Res 2007;86(6):529-533.

Chaussain C, Boukpessi T, Khaddam M, Tjäderhane L, George A, Menashi S. Dentin matrix degradation by host matrix metalloproteinases: inhibition and clinical perspectives toward regeneration. Front Physiol 2013;4:308. doi:10.3389/fphys.2013.00308.

Dayan D, Binderman I, Mechanic GI. A preliminary study of activation of collagenase in carious human dentine matrix. Arch Oral Biol 1983;28:185-187.

Delany, A.M., Jeffrey, J.J., Rydziel, S. Canalis, E. 1995. Cortisol increases interstitial collagenase expression in osteoblasts by post-transcriptional mechanisms. J. *Biol. Chem*. 270:26607-12.

De Munck J, Van den Steen PE, Mine A, Van Landuyt KL, Poitevin A, Opdenakker G, Van Meerbeek B. Inhibition of enzymatic degradation of Adhesive-Dentin interfaces. J Dent Res 2009;88(12):1101-1106.

Edgar WM, Higham SM, Manning RH. Saliva stimulation and caries prevention. Adv Dent Res 1994;8(2):239-245.

Epasinghe DJ, Yiu CK, Burrow MF, Hiraishi N, Tay FR. The inhibitory effect of proanthocyanidin on soluble and collagen-bound proteases. J Dent. 2013;41(9):832-9.

Featherstone, JD. Dental caries: dynamic disease process. Aust Dent J. 2008;53(3):286-291.

Featherstone JD. The continuum of dental caries- evidence for a dynamic disease process. J Dent Res 2004;83(Spec Iss C):C39-C42.

Featherstone JD. The science and practice of caries prevention. JADA 2000;131:887-899.

Garcia-Godoy F, Tay FR, Pashley DH, Tjäderhane L, Pashley EL, King NM. In vitro degradation of resin-bonded dentin after 3 years of strorage. Am J Dent 2007;20(2):109-113.

Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinases 2,8, and 9 by chlorhexidine. Clinical and Diagnostic Laboratory Immunology 1999;6(3):437-439.

Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. Front Biosci (Elite Ed). 2011;3:711-735.

Green GD, Reagan K. Determination of hydroxyproline by high pressure liquid chromatography. Analytical Biochemistry 1992;201:265-269.

Hannas TP and Kuttan G. Berberine inhibits pulmonary metastasis through down-regulation of MMP in metastatic B16F-10 melanoma cells. Phytotherapy Research 2012;26:568-578.

Hall, R., Septier, D., Embery, G., and Goldberg, M. (1999). Stromelysin-1 (MMP-3) in forming enamel and predentine in rat incisor-coordinated distribution with proteoglycans suggests a functional role. *Histochem. J.* 31, 761-770. doi: 10.1023/A1003945902473

Hashimoto M, Ohno H, Kaga M, et al. In vivo degradation of resin-dentin bonds in humans over 1 to 3 years. J Dent Res 2000;79:1385-91.

Hashimoto M, Tay FR, Ohno H, et al. SEM and TEM analysis of water degradation of human dentinal collagen. J Biomed Mater Res B Appl Biomater 2003a;66:287-98.

Hashimoto M, Ohno H, Sano H, et al. In vitro degradation of resin-dentin bonds analyzed by microtensile bond test, scanning and transmission electron microscopy. Biomaterials 2003b;24:3795-803.

Hay, ED (Ed.). (2013). Cell biology of extracellular matrix. Springer Science & Business Media.

Ho Y-T, Yang, J-S, Li T-C, Lin J-J, Lin J-G, Lai K-C, Ma C-Y, Wood G, Chung J-G. Berberine suppresses in vitro migration and invasion of human SCC-4 tongue squamous cancer cells through the inhibitions of FAK, IKK, NF-κB, u-PA and MMP-2 and -9. Cancer Letter 2009;279:155-162.

Hong J-S, Chu Y-K, Lee H, Ahn B-H, Park J-H, Kim M-J, Lee S, Ryoo H-S, Jang J-H, Lee S-R, Park J-W. Effects of berberine on hippocampal neuronal damage and matrix metalloproteinases-9 activity following transient global cerebral ischemia. Journal of Neuroscience Research. 2012;90:489-497.

Ikram M. A review on the chemical and pharmacological aspects of genus Berberis. Planta Med 1975;53:763-768.

Imanshahidi M and Hosseinzadeh. Pharmalogical and therapeutic effects of berberis vulgaris and its active constituent, Berberine. Phytother Res 2008;22:999-1012.

Iwasa M, Tsubota K, Shimamura Y, Ando S, Miyazaki M, Platt JA. pH changes upon mixing single-step self- etching adhesives with powdered dentin. J Adhes Dent 2011;13:207-212.

Jackson RJ, Lim DV, Dao ML. Identification and analysis of a collagenolyti activity in Streptococcus mutans. Curr Microbiol 1997;34:49-54.

Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nature Reviews Cancer 2003;3(6):422–433.

Kawasaki K, Featherstoone JD. Effects of collagenase on root demineralization. J Dent Res 1997;76:588-595.

Kim S, Kim Y, Kim JE, Cho KH, Chung JH. Berberine inhibits TPA-induced MMP-9 and IL-6 expression in normal human keratinocytes. Phytomedicine 2008;15:340-347.

Kim S, Han J, Lee SK, Choi M-Y, Kim J, Lee J, Jung SP, Kim JS, Kim J-H, Choe J-H, Lee JE, Nam SJ. Berberine Supresses the TPA-induced MMP-1 and MMP-9 expressions through the inhibition of PKC-α in breast cancer cells. Journal of Surgical Research 2012;176:e21-e29.

Kim J, Uchiyama T, Carrilho M, Agee KA, Mazzoni A, Breschi L, Carvalho RM, Tjäderhane L, Looney S, Wimmer C, Tezvergil-Mutluay A, Tay FR, Pashley DH. Chlorhexidine binding to mineralized versus demineralized dentin powder. Dental Materials 2010;26:771-778.

Koshiro K, Inoue S, Sano H, De Munck J, Van Meerbeek B. In vivo degradation of resin-dentin bonds produced by a self-etch and an etch-and rinses adhesive. Eur J Oral Sci 2005;113:341-348.

Lin J-P, Yang J-S, Wu C-C, Lin S-S, Hsieh W-T, Lin M-L, Yu F-S, Yu C-S, Chen G-W, Chang Y-H, Chung J-G. Berberine induced down regulation of matrix metalloproteinase-1, -2 and -9 in humn gastic cancer celss (SNU-5) in vitro. In Vivo 2008;22:223-230.

Linde A. 1984. Noncollagenous proteins and proteoglycans in dentinogenesis. In: Anders L, editor. Dentin and dentinogensis. Vol. 2. Boca Raton (FL): CRC Press;56.

Liu B, Wang G, Yang J, Pan X, Yang Z, Zang L. Berberine inhibits human hepatoma cell invasion without cytotoxicity in healthy hepatocytes. PLoS ONE 2011;6(6):e21416. Doi:10.1371/journal.pone.0021416

Loesche WJ. Role of Streptococcus mutans in human dental decay. Microbiol Rev 1986;50(4):353-380.

Loudon, MG. 2002. Chemistry of Napthalene and the aromatic heterocycles. Organic Chemistry. Fourth ed. New York: Oxford University Press;1135-1136.

Marsh, PD. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res;8:263-271.

Mazzoni A, Mannello F, Tay FR, Tonti GAM, Papa S, Mazzotti G, Di Lenarda R, Pashley DH, Breschi L. Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin. J Dent Res 2007;86(5):436-440.

Mazzoni A, Pashley DH, Nishitani Y, Breschi L, Mannello F, Tjäderhane L, Toledano M, Pashley EL, Tay FR. Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives. Biomaterials 2006;27:4470-4476.

Mazzoni A, Tjäderhane L, Checchi V, Di Lenarda R, Salo T, Tay FR, Pashley DH, Breschi L. Role of dentin MMPs in caries progression and bond stability. J Dent Res 2015;94(2):241-251.

Mazzoni A, Papa V, Nato F, Carrilho M, Tjäderhane L, Ruggeri A Jr, Gobbi P,Mazzotti G, Tay FR, Pashley DH, Breschi L. Immunohistochemical and biochemical assay of MMP-3 in human dentine. J Dent. 2011 Mar;39(3):231-7.

Mazzoni A, Pashley DH, Tay FR, Gobbi P, Orsini G, Ruggeri A, Carrilho M, Tjäderhane, Di Lenarda R, Breschi L. Immunohistochemical identification of MMP-2 and MMP-9 in human dentin: Correlative FEI-SEM/TEM analysis. Journal of Biomedical Materials Research Part A. 2008;697-703. DOI: 10.1002/jbm.a.31920

Nguyen TT, Gobinet C, Feru J, Brassart-Pasco S, Manfait M, Piot O. Characterization of type I and IV collagens by raman microspectroscopy: Identification of spectral markers of the dermo-epidermal junction. Spectroscopy: An International Journal 2012;27(5-6):421-427.

Nishitani Y, Yoshiyama M, Wadgaonkar B, et al. Activation of gelatinolytic/collagenolytic activity in dentin by self-etching adhesives. Eur J Oral Sci 2006;114:160-6.

Niu, L. N., Zhang, L., Jiao, K., Li, F., Ding, Y. X., Wang, D. Y., et al. (2011). Localization of MMP-2, MMP-9, TIMP-1, and TIMP-2 in human coronal dentine. *J. Dent*. 39, 536-542. Doi: 10.1016/j.jdent.2011.05.004

Pashley DH. The effects of acid etching on the pulpodentin complex. Oper Dent 1992;17:229-42.

Pashley DH, Tay FR, Yiu C, et al. Collagen degradation by host-derived enzymes during aging. J Dent Res 2004;83:216-21.

Rosengren L, Winblad B. Proteolytic activity of Str:eptococcus mutans (GS-5). Oral Sugery, Oral Medicine, Oral Pathology 1976;42(6):801-809.

Salvio LA, Hipolito VD, Martins AL, de Goes MF. Hybridization quality and bond strength of adhesive systems according to interaction with dentin. Eur J Dent 2013;7:315-26.

Sulkala M, Larmas M, Sorsa T, Salo T, Tjäderhane L. The localization of matrix metalloproteinases-20 (MMP-20, enamelysin) in mature human teeth. J Dent Res 2002;81(9):603-607.

Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T, Tjäderhane L. Matrix metalloproteinases-8 (MMP-8) is the major collagenases in human dentin. Archives of Oral Biology 2007;52:121-127.

Suppa P, Ruggeri A Jr, Tay FR, Prati C, Biasotto M, Falconi M, Pashley DH, Breschi L. 2006. Reduced antigenicity of type I collagen and proteoglycans in sclerotic dentin. J Dent Res. 85(2):133-137.

Tay FR, Pashley DH, Loushine RJ, Weller RN, Monticelli F, Osorio R. Self-etching adhesives increase collagenolytic activity in radicular dentin. J Endod 2006;32:862-8.

Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Päkkönen V, Martins MT, et al. Cysteine cathepsins in human dentin-pulp complex. J Endod 2010;36:475-481.

Tezvergil-Mutluay A, Mutluay MM, Gu L, Agee KA, Carvalho RM, Manso A, Carrilho M, Tay F, Breschi L, Suh B, Pashley DH. The anti-MMP activity of benalkonium chloride. Journal of Dentistry 2011a;39(1):57-64.

Tezvergil-Mutluay A, Agee KA, Uchiyama T, Imazato S, Mutluay MM, Cadenaro M, Breschi L, Nishitani Y, Tay FR, Pashley DH. The inhibitory effect of quaternary ammonium methacrylates on soluble and matrix-bound MMPs. J Dent Res 2011b;90(4):535-540.

Tezvergil-Mutluay A, Mutluay M, Seseogullari-Dirihan R, Agee KA, Key WO, Shceffel DLS, Breschi L, Mazzoni A, Tjäderhane L, Nishitani Y, Tay FR. Effect of phosphoric acid on the degradation of human dentin matrix. J Dent Res 2013;92(1):87-91.

Tjäderhane L, Larjava H, Sorsa T, Uitto V-J, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res 1998;77:1622-9.

Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol ILS, Geraldeli S, Tezvergil-Mutluay A, Carrilho MR, Carvalho RM, Tay FR, Pashley DH. Optimizing dentin bond durabililty: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. Dental Materials 2013a;29:116-135.

Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S, Tezvergil-Mutluay A, Carrilho M, Carvalho RM, Tay FR, et al. Strategies to prevent hydrolytic degradation of the hybrid layer: a review. Dent Mater. 2013b;29(10):999-1011.

Tjäderhane L, Buzalaf MAR, Carrilho M, Chaussain C. Matrix metalloproteinases and other matrix proteinases in relation to cariology: the era of 'dentin degradomics'. Caries Res 2015;49:193-208.

Traub WW, Piez KA. The chemistry and structure of collagen. Adv Protein Chem 1971;25:243-352.

Tu HP, Fu MMJ, Kuo PJ, Chin YT, Chiang CY, Chung CL, Fu E. Berberine's effect on periodontal tissue degradation by matrix metalloproteinases: an in vitro and in vivo experiment. Phytomedicine 2013;20:1203-1210.

Van Strijp AJ, van Steenbergen TJ, ten Cate JM. Bacterial colonization of mineralized and completely demineralized dentine in situ. Caries Res. 1997; 31(5):349-355.

Van Strijp AJ, van Steenbergen TJ, de Graaff J, ten Cate JM. Bacterial colonization and degradation of demineralized dentin matrix in situ. Caries Res 1994;28:21-27.

Van Strijp AJ, Jansen DC, DeGroot J, ten Cate JM, Everts V. Host-derived proteinases and degradation of dentine collagen in situ. Caries Res 2003;37:58-65.

Vidal CM, Tjäderhane L, Scaffa PM, Persariol IL, Pashley D, Nader HB, Nascimento FD, Carrilho MR. 2014. Abundance of MMPs and cysteine cathepsins in caries-affected dentin. J Dent Res. 93(3):269-274.

Van Meerbeek B, Inokoshi S, Braem M, et al. Morphological aspects of the resin-dentin interdiffusion zone with different dentin adhesive systems. J Dent Res 1992;71:1530-40.

Yang RU, Liu H, Williams I, Chaqour B. Matrix Metalloproteinases-2 expression and apoptogenic activity in retinal pericytes. Annals of the New York Academy of Science 2007;1103:196-201.

Vincenti, M.P., 2001. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. In *Matrix Metalloproteinase Protocols*, ed. IM Clark, pp. 121-48. Totowa, NJ: Humana.

Vandooren J, Geurts N, Martens E, Van den Steen PE, Opdenakker G. Zymography methods for visualizing hydrolytic enzymes. Nature Methods 2013;10:2111-220.

Wang Y, Spencer P. Hybridization efficiency of the adhesive/dentin interface with wet bonding. J Dent Res 2003;82:141-5.

Wartenberg M, Budde P, de Mareés M, Grünheck F, Tsang SY, Huang Y, Chen Z-Y, Hescheler J, Sauer H. Inhibition of tumor-induced angiogenesis and matrix-metalloproteinases expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional chinese medicine. Laboratory Investigation 2003;83(1):87-98.

VITA

NAME: KRISTEN A. VLAGOS D.D.S.

LICENSURE: ILLINOIS STATE DENTAL BOARD LICENSE
Central Regional Board
Illinois License Registration No. 019.028730
August 2011-Current

EDUCATION: UNIVERSITY OF ILLINOIS AT CHICAGO
Advanced Certificate in Prosthodontics
Graduated May 2014

UNIVERSITY OF ILLINOIS AT CHICAGO
Doctor of Dental Surgery
Graduated May 2011

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN
Bachelor of Science in Molecular and Cellular Biology
Graduated December 2006

PROFESSIONAL EXPERIENCE:

ASSOCIATE DENTIST
March 2015 to Current
DENTAL DESIGN GROUP
Naperville, Illinois

ASSOCIATE DENTIST
July 2014 to March 2015
WESTCHESTER FAMILY DENTAL CARE
Westchester, Illinois

CLINICAL ASSOCIATE PROFESSOR
July 2014 to Current
University of Illinois at Chicago

TEACHING EXPERIENCE:

University of Illinois at Chicago

- Teaching assistant for Restorative Dentistry (REST) 322 Fixed Prosthodontics Technique I
- Teaching assistant for Restorative Dentistry (REST) 321 Complete Denture Prosthodontic Technique

- Tutor for Biochemistry and Molecular Genetics (BCMG) 411 Intro into Biochemistry

AWARDS:

Delta Dental of Illinois 2011 Outstanding Dental Student Award Recipient
Awarded in recognition for the most professionalism in patient care services

Awardee – University of Illinois at Chicago Graduate Student Research Competition- Clinic and Research Day 2014
The annual UIC Clinic and Research Day provides a forum for dental students, postgraduate residents, and research staff to present research achievements to the College and the larger research community. The research competition for this award comprised all advanced specialty programs in oral presentations.

Omicron Kappa Upsilon National Dental Honor Society
Omicron Kappa Upsilon recognizes scholarship and character in dental professionals. Founded in 1914, membership in Omicron Kappa Upsilon represents one of the highest honors attainable in the dental profession.

PROFESSIONAL ASSOCIATIONS:

American College of Prosthodontists
American Dental Association
Chicago Dental Society