EFFECTS OF DYNAMATRIX® ON ANGIOGENIC CYTOKINE AND MATRIX METALLOPROTEINASE EXPRESSION FROM HUMAN ENDOTHELIAL CELLS:
AN IN-VITRO STUDY

by

Scott Thomas Hill

Submitted to the Graduate Faculty of the School of Dentistry in partial fulfillment of the requirements for the degree of Master of Science in Dentistry, Indiana University School of Dentistry, June 2015.
Thesis accepted by the faculty of the Department of Endodontics, Indiana University School of Dentistry, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

Ygal Ehrlich

L. Jack Windsor

Josef Bringas

Ned Warner

Susan L. Zunt

Kenneth J. Spolnik
Chair of the Research Committee and Program Director

Date
ACKNOWLEDGMENTS
I would like to thank the Indiana University School of Dentistry Department of Endodontology faculty (Drs. Spolnik, Ehrlich, Bringas, and Warner). Their overwhelming support and contributions to this thesis, and my overall education, have really allowed me to have an experience that I could not have had anywhere else. I consider them not only my faculty, but also my mentors because of the examples they set as professionals. I also would like to thank the part-time endodontic faculty mentors for their contributions to my educational experiences.

I am especially thankful for Dr. L. Jack Windsor’s guidance and insight for my project. Also, I would like to thank Jadesada Palasuk for his help with the setup and explanation of the steps in the laboratory throughout the project. Without everyone’s help, I would not have been able to complete this project.

A special thanks to the Delta Dental Foundation for the grant awarded for this project. Their financial contributions allowed us to complete this project.

Thank you.
LIST OF ILLUSTRATIONS
FIGURE 1  Experimental design .......................................................... 33
FIGURE 2  DynaMatrix membrane sterilized ........................................ 34
FIGURE 3  Example of DynaMatrix trimmed to fit into the six-well plate… 35
FIGURE 4  Six–well plates with three experimental groups with culture media. Column 1: HUVECs, Column 2: HUVECs seeded on DynamMatrix; and Column 3: DynaMatrix membrane ……… 36
FIGURE 5  Array membranes placed in wells ....................................... 37
FIGURE 6  Cytokine Array: Images of scanned x-ray films for each sample group. From left to right: Cell+Membrane group (C+M); Cell-only group (C); Membrane-only group (M) ……… 38
FIGURE 7  MMP Array: Images of scanned x-ray films for each sample group. From left to right: Cell+Membrane group (C+M); Cell-only group (C); Membrane-only group (M) ……… 39
TABLE I  RayBio Human Angiogenesis Antibody Array 1 map .............. 40
TABLE II RayBio Human Matrix Metalloproteinase Antibody Array …… 41
TABLE III Data from cytokine array membranes ................................... 42
TABLE IV Data from MMP array membranes ...................................... 43
INTRODUCTION
The goal of endodontic therapy is to retain infected teeth by eliminating pulpal and periapical disease. Young, infected permanent teeth with incomplete development of the root pose significant challenges in endodontic treatment. Chemo-mechanical disinfection with conventional debridement with endodontic files and irrigation can be challenging, and so is obturating the treated immature canal.\(^1\) Regenerative endodontic (RE) procedures have become acceptable treatments for these teeth. RE procedures can be defined as “biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex.”\(^2\)

Endodontic regenerative therapy aims to establish an environment that will enable continued root development, revascularization, and revitalization of the pulp tissue. Proper disinfection of the root canal system is a prerequisite for RE.\(^3\)

Stem cells, a scaffold, and cytokines/growth factors are the three major components needed to create an environment for successful RE to occur. Currently, a blood clot created in the canal serves as an endodontic scaffold.\(^4\) Bleeding is evoked from the apical tissues leading to the formation of a blood clot in the canal. The evoked-bleeding step involves the manipulation of the periapical tissues, and stem cells are released and delivered into the root canal system.\(^5\) Soluble extracellular matrix molecules of dentin, pulp fibroblasts, and endothelial cells (EC) have also been shown to be involved in dentin-pulp regeneration.\(^6\) The blood clot scaffold is eventually replaced with pulp-like tissue and dentin pulp regeneration can be seen.\(^6\)
Failures in RE have been attributed to lack of vascularization, which could not support the blood clot scaffold. Angiogenesis is needed to increase vascularization and the creation of a vascular network. Different cell types and various cytokines/growth factors are involved in angiogenesis. These include endothelial cells, fibroblasts, neutrophils, and macrophages that produce vascular endothelial growth factor (VEGF). VEGF is a signaling protein that early in angiogenesis increases the permeability of blood vessels causing the extravasation of plasma proteins. This step leads to the creation of a temporary scaffold for migrating ECs. Macrophages produce tumor necrosis factor alpha (TNF-alpha), which also induces VEGF expression. In wound repair, VEGF expression is enhanced leading to a neovascular network that drives regeneration by increasing tissue perfusion. ECs have been shown to release the angiogenic cytokines fibroblast growth factor (FGF) and VEGF without any stimulation. VEGF increases the permeability of ECs and FGF promotes the proliferation and differentiation of ECs, smooth muscle cells, and fibroblasts.

Matrix metalloproteinases (MMPs) play a role in angiogenesis by degrading matrix molecules and by activating or liberating the growth factors, such as basic fibroblast growth factor (bFGF), VEGF, and insulin-like growth factor 1 (IGF-1) that are within the extracellular matrix. Zehng et al. showed an increase in the expression of MMP-3, MMP-9 and MMP-13 after a pulpal injury. Tissue inhibitors of matrix metalloproteinases (TIMPs) play roles in the inhibition of MMPs, and thus, in inhibiting angiogenesis. Mathieu et al. showed that ECs might play an important role in recruiting human pulp cells, as well as in forming the necessary blood vessels for angiogenesis.
It is believed that an appropriate scaffold that provides an environment that supports ECs could improve the success of RE procedures.

Alternative scaffolds to the blood clot scaffold are platelet-rich-plasma, synthetic scaffolds, and natural scaffolds.\textsuperscript{12} DynaMatrix\textregistered (Cook Biotech, West Lafayette, IN, USA) is a membrane currently used in dentistry for periodontal regeneration procedures.\textsuperscript{12,13} This acellular, three-dimensional extracellular matrix (ECM) is derived from porcine small intestine submucosa (SIS) and is composed of collagen that could be used as a framework for cell growth. The company states that it contains promoters of angiogenesis such as FGF-2, connective tissue growth factor (CTGF), and transforming growth factor beta (TGF-\textbeta).\textsuperscript{13} Research at Indiana University School of Dentistry (IUSD) has shown that DynaMatrix\textregistered has potential as an endodontic scaffold.\textsuperscript{14,15} It can support the growth of human dental pulp stem cells (HDPSCs) and human dental pulp fibroblasts (HDPFs), and positive angiogenic profiles were seen after these cells were exposed to Dynamatrix\textregistered. The effects of DynaMatrix\textregistered on ECs and MMPs have not been investigated, although both are pivotal for angiogenesis. This work will further the evidence for the potential that DynaMatrix\textregistered has in RE. It will examine the angiogenic cytokine and MMP profiles of EC exposed to Dynamatrix.

\textbf{CLINICAL SIGNIFICANCE}

DynaMatrix\textregistered membranes have been used successfully in medicine as scaffolds. Their ability to increase the angiogenic cytokine expression from endothelial cells will make them potential scaffolds for regenerative endodontics.
HYPOTHESES

1. Null: There will be no significant differences in pro-angiogenic or anti-angiogenic cytokines and in matrix metalloproteinases or tissue inhibitors of metalloproteinases expression or presence when HUVECs are seeded on the DynaMatrix® membrane as compared with the membrane or cells alone.

2. Alternative: There will be a significant difference in pro-angiogenic or anti-angiogenic cytokines and in matrix metalloproteinases or tissue inhibitors of metalloproteinases expression or presence when HUVECs are seeded on the DynaMatrix® membrane when compared with the membrane or cells alone.
REVIEW OF LITERATURE
HISTORY OF ENDODONTICS

One of the earliest descriptions of toothaches described as pain caused by cold and mastication was by Fu His in 2953 BC. For centuries descriptions of treatment for toothaches were described in Egyptian tablets, Hebrew Bibles, and medical writings of the Greeks, the Romans, and the Chinese. The Chinese theorized in the 14th century BC that dental disease was caused by tooth worms until it was disproved by the use of microscopes.

One of the first books in the English language that was considered to be exclusively devoted to dentistry was published in 1687 by Charles Allen. It described the treatment for teeth with dental transplants as “taking out the rotten teeth or stumps and putting in their places some sound ones drawn immediately out of some poor body’s head.”

The Surgeon Dentist written by Pierre Fauchard in 1728 led many to consider him “the father of modern dentistry.” In his book, he described everything from anatomy, orthodontics, pathology, and the replacement of missing teeth. It also described the treatment of various teeth with pulp cavities and root canals. He also discussed treating abscessed teeth with access cavity preparations that were left open and eventually filled with lead foil. Fauchard also treated the pulp with oils, cinnamon, and even opium in deep carious lesions, for which he used a small pin for pulp extirpation.
In 1756 Phillip Pfaff, a German dentist, described a pulp-capping procedure. He placed gold or lead over the exposed pulp. It was placed in such a way as to avoid direct contact with the pulpal tissues.\textsuperscript{18}

Bourdet described a procedure in 1757 that consisted of extracting carious teeth, filling the root canal with gold or lead, followed by replantation of the tooth.\textsuperscript{20} A similar procedure was described in the 11\textsuperscript{th} century by an Arabian physician, Avicenna.\textsuperscript{20}

The first endodontic procedure to be recorded in North America was performed by Robert Woofendale.\textsuperscript{21} He came to New York from England in 1766 and his treatment consisted of cauterizing the pulp with a hot instrument followed by cotton pellet placement into the canals.\textsuperscript{21,22} Frederick Hirsh, a German dentist, in the 18\textsuperscript{th} century, wrote about tenderness to percussion being associated with diseased teeth. His treatment suggestion was to use a red-hot probe repeatedly inserted into the cervical area of the tooth, and then to fill the tooth with lead.\textsuperscript{20}

“The Vitalistic Era” in the 19\textsuperscript{th} century began as people began to become concerned with vitality and the problems this posed for treatment.\textsuperscript{18} J.B. Gariot in 1805 suggested that obliteration of the pulp does not destroy the vitality of the tooth.\textsuperscript{18} Edward Hudson expanded on this and is often given credit for being the first to place fillings in root canals to preserve the natural dentition.\textsuperscript{21-25}

In \textit{Opinions on the Causes and Effects of Disease in the Teeth and Gums} in 1819, John Callow gives Charles Bew credit for describing blood flow into the tooth from the apical foramen and out through the dentinal wall and through the periodontal membrane.\textsuperscript{23} This thought process, the “vitalistic theory,” was in line with others of his time.\textsuperscript{23} Leonard Koecker, a German immigrant, wrote \textit{Principles of Dental Surgery}. 
This 1826 book was the standard in the field of dentistry for 50 years. He felt the tooth would become a foreign body after the pulp was destroyed, either by disease or artificially, and require the tooth to be extracted. To prevent the further extraction of teeth, Koecker popularized pulp capping similar to the manner in which Pfaff in 1756 pulp capped teeth.23,26,27

The vitalistic or double-membrane theory was formulated and presented by S. S. Fitch in 1829.18 In *Systems of Dental Surgery*, Fitch wrote about his belief that teeth were like hollow bones. He believed teeth consisted of an outer periosteum and an inner periosteum that was between the pulp and the dentin. With this double-membrane theory, he thought nourishment for the crown was exclusively from the dental pulp or lining membrane, and that the nourishment for the root was from the pulp membrane interiorly, and from the alveolar membrane exteriorly.18

There was opposition to the vitalistic theory. British surgeon and anatomist John Hunter was of the “nonvitalistic” group. Hunter believed that dentin was not like living tissue and was lacking in many of the properties of living tissue, including circulation, sensibility, and the inability to repair.18 Cuvier and Robertson from England aligned with Hunter’s views.23

The treatment of diseased pulpal tissues prior to 1836 was very unpleasant and painful. Advancements in treatment began with Shearjashub Spooner in New York. He began using arsenic trioxide to devitalize the pulp before removing it.21 The Chinese used arsenic in ancient medicine practices for the treatment of abscesses of the jaw20 and its use continued until the 1920s for devitalization of the pulp before its removal.28 In 1837
Jacob and Joseph Linderer started using essential or narcotic oil for the treatment of exposed pulps.\textsuperscript{29}

Edwin Maynard in 1838 made the first broach for the removal of the pulpal tissue by filing down a watch spring. Maynard also designed hoe-like instruments for pulpal procedures.\textsuperscript{29} The first mention of the removal of the nerve, of the cleaning of the canal, and of filling it with gold foil was by Baker in 1839.\textsuperscript{18,20}

Wooden plugs that were soaked in creosote were used in the 1850s to fill root canals.\textsuperscript{18} They used a combination of sealer made with gutta-percha, quick-lime, powdered glass, feldspar, and metal filings with the wooden plug to seal the canal.\textsuperscript{28}

Thomas Rogers presented 220 cases of pulp capping at the Odontological Society of London meeting in 1857.\textsuperscript{18} Of his 220 cases, 202 were considered to be successful.\textsuperscript{18} He outlined the conditions that when present are helpful in leading to successful pulp capping. These included: general good health, freedom from inflammatory tendencies, absence of previous considerable pain from the tooth, absence of disease in other parts of the tooth, and no use of caustics to decrease the pain.\textsuperscript{18}

S.C. Barnum introduced the rubber dam for the placement of gold foil restorations in 1864.\textsuperscript{18} The use of the rubber dam was quickly adopted in the root canal filling procedure for a more aseptic environment.\textsuperscript{20,21} Many people credit G.A. Bowman of Missouri for being the first person to use gutta-percha alone for root canal fillings.\textsuperscript{21,24,30} Bowman was also one of the inventors of the rubber dam clamp forceps in 1873.\textsuperscript{21}

Leber and Rottenstein’s work from Germany recognized that \textit{Leptothrix buccalis}, a parasite, led to a better understanding of how tooth decay could cause gangrene or necrosis of the pulp.\textsuperscript{18,31} They found its existence on tooth surfaces, carious lesions, and
within the dentinal tubules.\textsuperscript{18,31} During the same year of 1867, Magitot suggested using electric current to test the pulp.\textsuperscript{26}

In the 1870s, another theory began to gain momentum over the vitalism theory. The septic theory, backed by G.O. Rogers and Charles S. Tomes, was the thought that pathologic organisms were the biggest cause for the disease of the pulp.\textsuperscript{23} Arthur Underwood expanded on the septic theory in 1882.\textsuperscript{23} He suggested that removal of pathogens from the pulp space, via antiseptics, could prevent pulpal suppuration and alveolar abscesses.\textsuperscript{18} For the next 30 years, this idea justified the use of caustic germicides for bacterial elimination from the pulp chamber.\textsuperscript{23}

In 1895 the discovery of x-rays by Wilhelm Konrad Roentgen helped to change the accuracy of dental diagnosis.\textsuperscript{32,33} Otto Walkhoff used Roentgen’s discovery to take radiographs of his own teeth and Edmund Kells took radiographs not only for diagnosis, but for endodontic treatment as well.\textsuperscript{32,33} Also, in 1895 Dr. Bowman introduced a combination of chloroform and gutta-percha, chloropercha, which was used with gutta-percha cones to obturate the root canal. His technique was widely accepted among dentists.\textsuperscript{21}

Cocaine was used by Carl Koller in 1884 for a topical anesthetic.\textsuperscript{32,33} William Halsted performed mandibular block injections with a similar solution as Koller.\textsuperscript{32,33} Funk in 1890 introduced pressure anesthesia using crystals of cocaine for pulp removal.\textsuperscript{32,33} Novocaine (procaine) developed in 1905 by Einhorn, provided dentistry with a more effective and less toxic anesthetic.\textsuperscript{28} Initially, the use of procaine was inefficient, requiring the dissolution of a tablet in a solution, boiling, cooling and aspiration into a
syringe. It was not for nearly 25 years that the process for block anesthesia was perfected.\textsuperscript{28,29,34}

Dr. Meyer L. Rhein developed a technique using radiographs and a diagnostic wire to determine the length of the canal and degree of obturation in 1908.\textsuperscript{21,26} Also, to avoid overfilling, G.V. Black suggested measurement control to determine the length of the canal and the size of the apical foramen.\textsuperscript{18} It was not until 1913 that the first dental x-ray units were introduced.\textsuperscript{18}

Later in the 20\textsuperscript{th} century, a shift in the medical and dental community led to what is now called the Focal Infection Theory.\textsuperscript{35} This theory was not new, but was gaining momentum in part due to the work of E.C. Rosenow.\textsuperscript{35} The idea was that a large number of diseases were caused by microorganism including bacteria, fungi, and viruses that arise from within the individual to form a focus of infection.\textsuperscript{35} Rosenow demonstrated that streptococci were present in diseased organs.\textsuperscript{35} He also demonstrated that these bacteria could travel in the blood stream to establish an infection at a distant site within the organism.\textsuperscript{34} A significant boost to the focal infection theory that helped it to gain more widespread acceptance was due to William Hunter.\textsuperscript{18} Hunter, a British physician and pathologist, gave a lecture at McGill University in Montreal in 1910 called, “The Role of Sepsis and Antisepsis in Medicine.” It was later published in 1911 in \textit{Lancet}.\textsuperscript{18} This increase in the acceptance of the focal infection theory led to analysis of the validity of endodontic procedures among the dental and medical community. Because of this, many dentists and physicians recommended the extraction of all pulpless teeth. It was not until about 1930 that the pendulum began to swing to a more conservative approach.\textsuperscript{18} It was nearly a decade before this more conservative approach\textsuperscript{18} based on proper diagnosis,
aseptic techniques bacteriological culturing, and improved radiographic practices for root canal treatment\textsuperscript{36} was taught in dental schools.\textsuperscript{18}

During “the scientific era” in 1937, scientific evidence was moving toward more sound histological, biological, and pathological findings.\textsuperscript{18} Some of these scientific works by Logan were able to show that bacteria can be present in tissues without eliciting pathologic effects.\textsuperscript{22} Similarly, Tunnicliff and Hammond displayed that pulps of extracted teeth with microorganisms in them did not display any inflammatory changes.\textsuperscript{18,22} Further, work by Cecil of Cornell Medical College demonstrated little improvement of arthritis after the removal of a suspected foci of infection.\textsuperscript{18} These results led Burket to his conclusion that the improvement following the removal of foci of infection was only a causal relationship.\textsuperscript{18,37} This body of evidence played a role in discouraging the practice of indiscriminate extraction of non-vital teeth and encouraged root canal therapy.\textsuperscript{18}

The use of antibiotics for root canal therapy was led by Fred Adams and Louis Grossman in the 1940s.\textsuperscript{18,34} Penicillin was first used by Adams, who also reported the use of sulfanilamide in the treatment of periapical infections.\textsuperscript{18,34} Dr. Grossman led the way for the use of an economical and more stable form of penicillin, and he used penicillin on paper points to disinfect root canals.\textsuperscript{24}

The formation in 1943 of the formation of the American Association of Endodontists (AAE) in Chicago, Illinois, signified the beginning of organized endodontics.\textsuperscript{38} A committee formed in 1949 to discuss the possibility of forming a specialty board for endodontics. Later, the American Board of Endodontics was formed in 1956.\textsuperscript{38} The next step was to gain recognition by the American Dental Association (ADA) as a specialty. Due to the relentless efforts of many AAE members and leaders,
the ADA officially recognized endodontics as a specialty in 1963. In 1965 the first certifications of Diplomat status were given. Currently, the AAE has around 7,000 members with around one-fourth of them board certified.

THEORY OF ENDODONTICS

The goal of endodontic therapy is to maintain form and function of teeth by the elimination of a microbial insult on the pulp and periapical tissues. This is accomplished through the study of the morphology, physiology and pathology of the pulp and periapical tissues. Endodontic therapy is dependent upon the successful removal of bacteria from the root canal system to allow the conditions for the body to heal the periapical tissues and to return the tooth to a homeostatic environment.

In 1890 Miller demonstrated that the cause of apical periodontitis was due to the presence of different bacteria associated with pulpal disease. The landmark study for endodontics was in 1965 when Kakehashi et al. demonstrated that the pulps of germ-free rats survived despite being left open to the oral environment. This study showed the importance of bacterial presence in the formation of pulpal and periapical disease. Without the reduction of bacteria, apical periodontitis can occur. This highlights the importance of chemo-mechanical cleaning and shaping of the root canal system.

The importance of chemo-mechanical preparation, microbial control, and obturation were emphasized by Stewart in 1955. Of the three phases, chemo-mechanical preparation was the most significant factor. Grossman further expanded upon this concept of chemo-mechanical preparation when he described 13 principles of effective root canal therapy. The 13 principles consisted of:

1. Aseptic technique.
2. Confinement of instrumentation within the root canal system.

3. A fine, smooth instrument should be used to enter the canal and never forced apically.

4. Enlargement of the canal space from its original size with biomechanical instrumentation.

5. The root canal space should be irrigated continuously with a solution that is aseptic during instrumentation.

6. The irrigation solution should be non-irritating and remain within the canal space.

7. No special treatment is needed for a fistula.

8. Before obturation, a negative culture should be obtained.

9. A hermetic seal of the root canal system should be obtained.

10. Use of an obturation material that is not irritating to the periapical tissues.

11. Drainage must be established if an acute alveolar abscess is present.

12. Avoid injections into the area of an infection.

13. Periapical surgery may be indicated to eliminate the inflammatory or cystic tissues.

Schilder discussed how sealing the root canal system (by obturation of the root canal system in three dimensions, after chemo-mechanical preparation, to the cemento-dentinal junction or from 0.5 to 1 mm from the apex) would help protect the periapical tissues from breakdown from endodontic pathogens.\(^{48}\) Pitt Ford outlined three reasons for having a well-obturated root canal.\(^{49}\) The reasons he stated were\(^{49}\):

1. Decreased space for bacterial colonization.
2. Prevention of bacterial contamination of the apex.

3. Prevention of bacterial movement along the wall of the canal system.

Ford also stressed the importance of maintaining an aseptic environment during treatment.\textsuperscript{49}

Weine in 1996 made alterations to the principles of endodontic therapy.\textsuperscript{41} His alterations included the need for restoration of endodontically treated teeth, diagnosis, preparation, obturation, importance of debridement, use of rubber dam, maintaining instrumentation to within the canal system, post-operative observation, and case presentation to the patient during treatment planning.\textsuperscript{41} Non-surgical endodontic therapy consists of debridement of the canal chemo-mechanically with endodontic files and irrigation solutions followed by obturation of the root canal system.

**IRRIGATION SOLUTIONS**

Roughly 35 percent of the root canal system remains uninstrumented after mechanical debridement with nickel-titanium rotary instruments.\textsuperscript{50} Schilder emphasized the importance of irrigation solutions in non-surgical endodontic therapy.\textsuperscript{48} Bystrom and Sundqvist demonstrated in 1981 that bacteria still persisted even after treating canals mechanically and using saline irrigation.\textsuperscript{51} Such findings demonstrated the limitations of mechanical instrumentation without irrigation. Some teeth still had bacteria present after five treatment cycles.\textsuperscript{51} Several irrigation solutions are used to aid in the removal of more debris during chemo-mechanical preparation. Harrison outlined the properties of an ideal irrigation solution.\textsuperscript{52} They are:

1. Efficacy as an antimicrobial agent.

2. Ability to dissolve tissues.

4. Ability to aid in the debridement the root canal system.

The main irrigation solutions used today are sodium hypochlorite (NaOCl), chlorhexidine (CHX), and ethylenediaminetetraacetic acid (EDTA).

Sodium Hypochlorite

NaOCl has been shown to be an effective antimicrobial agent with greater ability over saline for elimination of microorganisms from within the root canal system. NaOCl is not available commercially around 8.0 percent and is diluted for clinical use to anywhere from 0.5 percent to 6.0 percent. At lower concentrations, NaOCl’s effect is primarily tissue-dissolving, and as the concentration increases, so does the ability to dissolve tissues, as well as to increase antimicrobial efficacy. Hand et al. found that full strength NaOCl, 5.25 percent was significantly better at dissolving pulp tissue remnants than lesser-concentrated forms. The tissue-dissolving ability is enhanced through longer duration and increases in temperature. NaOCl’s hypochlorite ion, OCl⁻, establishes an equilibrium with HOCl, hypochlorous acid, which is responsible for bacterial inactivation. NaOCl is considered the most effective irrigant for use in endodontics due to its desirable characteristics, including its antibacterial properties, its ability to dissolve necrotic tissues, its action to mechanically flush debris from within the root canal, and its lubricating ability.

Chlorhexidine

Chlorhexidine (CHX) is an effective antimicrobial against gram-positive and gram-negative bacteria, facultative anaerobes and aerobes, spores, viruses and yeast.
CHX is used in endodontics at two concentrations, 0.012 percent and 2 percent. The positive electrostatic charge of CHX binds to the negatively charged bacterial cell wall. This causes the cell wall to be disturbed, making it more permeable and diminishing the bacteria’s ability to regulate its internal environment. \(^{61,63}\) Unlike NaOCl, CHX has the ability to remain on dentin for long periods of time hindering the ability of bacteria to colonize surfaces. \(^{64}\) Rosenthal et al. in 2004 demonstrated that CHX remains within the root canal dentin and retains its antimicrobial activity for up to 12 weeks. \(^{65}\) CHX, just like NaOCl, is concentration-dependent. Two percent CHX has been shown to be more effective than 0.12-percent formulations. \(^{66}\) Due to many of these antimicrobial properties, CHX has been recommended as a final irrigant during endodontic treatment. \(^{67}\)

One reason that CHX is not used exclusively as an irrigant is due to its lack of tissue-dissolving properties. Using CHX as an adjunct to NaOCl has some drawbacks. One potential problem is when CHX and NaOCl are mixed. This can lead the precipitation of what was initially thought to be para-chloroaniline, a potential carcinogen. \(^{66}\) Later studies concluded that it was actually para-chlorophenylurea and para-chlorophenylguanidyl-1,6-diguanidyl-hexane. \(^{68}\) After irrigating with NaOCl and before a final rise with CHX, it is advised to use isopropyl alcohol \(^{69}\) to flush out NaOCl and diminish the potential for forming the precipitate.

Ethylenediaminotetraacetic Acid (EDTA)

A smear layer is formed during the mechanical debridement and shaping of the root canal. It is still not clear whether removal of this smear layer is beneficial or not. \(^{70}\) It has been suggested in a recent systematic review that the removal of the smear layer is actually beneficial in increasing the fluid-tight seal after obturation. \(^{71}\) The most effective
concentration for smear layer removal and deeper penetration of irrigation solutions has been shown to be 17 percent when used in conjunction with NaOCl.\textsuperscript{72,73} Orstavik and Haapasolo demonstrated how EDTA can remove the smear layer and improve the antimicrobial effects of other irrigating solutions deeper into the dentin.\textsuperscript{74} Due to the possibility of an improved seal after obturation,\textsuperscript{71} EDTA has become a common irrigation solution during endodontic treatment.

OBTURATION

After thorough chemo-mechanical preparation, the root canal system needs to be sealed with a filling material. Obturation with gutta percha and sealer is the most widely used method. The goal of obturation is to hermetically seal the root canal to prevent re-infection of the periapical tissues.\textsuperscript{75} Length of the obturation has been shown to affect the success of the obturation.\textsuperscript{76} The highest success rates were found in a meta-analysis when the obturation material was not found beyond the apex.\textsuperscript{76} The techniques used, and the anatomy of the tooth, play a role in controlling the length of the filling material. It has been shown that using warm gutta-percha impacts the likelihood of over-extending the obturation material past the apex.\textsuperscript{77} The last part involved in sealing the root canal system is the placement of a coronal restoration, which leads to the highest success rates.\textsuperscript{78}

REGENERATIVE ENDODONTICS

The goal of endodontic therapy is to retain infected teeth by eliminating pulpal and periapical disease. Young, infected permanent teeth with incomplete development of the root pose significant challenges in endodontic treatment. Chemo-mechanical disinfection with conventional techniques, debridement with endodontic files, and
Irrigation can be challenging, as well as obturation of the treated immature canal.\textsuperscript{1} RE procedures have become acceptable treatments for these teeth. RE procedures can be defined as “biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex.”\textsuperscript{2} Endodontic regenerative therapy aims to establish an environment that will enable continued root development, revascularization, and revitalization of the pulp tissue. Proper disinfection of the root canal system is a prerequisite for RE.\textsuperscript{3}

Stem cells, a scaffold, and cytokines/growth factors are three major components needed to create an environment for successful RE. Currently, a blood clot in the canal serves as an endodontic scaffold.\textsuperscript{4} Bleeding is evoked from the apical tissues and a blood clot is created in the canal. The evoked-bleeding step involves the manipulation of the periapical tissues; stem cells are released and delivered into the root canal system.\textsuperscript{5} Soluble extracellular matrix molecules of dentin, pulp fibroblasts, and ECs have also been shown to be involved in dentin-pulp regeneration\textsuperscript{6} and may enter with the blood clot. The blood clot scaffold is eventually replaced with pulp-like tissue; dentin and pulp regeneration can be seen, as well as continued root development.\textsuperscript{6}

RE procedures are not entirely new ideas within endodontics. The recent work by Iwaya in 2001 has increased the interest in RE within the dental community.\textsuperscript{79} The article explained through a case report the treatment of a 13-year-old female’s mandibular second premolar with a sinus tract and immature root development. The tooth was irrigated with NaOCl (5 percent) and hydrogen peroxide (3 percent). A combination of metronidazole and ciprofloxacin was used for further disinfection. Finally, calcium hydroxide was placed apically against the tissues, and the access was closed with glass-
ionomer cement and a resin composite restoration. The 30-month follow-up revealed continued root development. The case report sparked interest in development of a clinical protocol to maximize the clinical success.

Case selection is a key component for success in RE. The American Association of Endodontists (AAE) discuss in their AAE Clinical Considerations for a Regenerative Procedure the case selection and procedure for RE. Case selection involves four parts including: the tooth should be necrotic with an immature apex; a post space is not needed for the final restoration; compliance of the patient and parent, and the patient is not allergic to any of the medicaments and antibiotics needed for the procedure. Once a case is deemed appropriate, the AAE has developed a current clinical protocol.

The current protocol involves two appointments. At the first appointment, the tooth is irrigated copiously with gentle irrigation of 20 ml of 1.5 percent NaOCl. CHX is not recommended for RE procedures due to its cytotoxic effects on stem cells of the apical papilla. The canals are dried, and either calcium hydroxide or antibiotic paste is placed into the canal, and the tooth, sealed with a temporary restoration. The second visit is initiated within one to four weeks. At this visit, the use of 20 ml of 17-percent EDTA is recommended. EDTA has been shown to promote the survival of stem cells of the apical papilla (SCAP), potentially by promoting dental growth factors to be released from the dentin. A blood clot is then induced and currently serves as an endodontic scaffold for continued root development. A scaffold is very important for RE procedures. Scaffolds provide the environment necessary for “cell growth and differentiation, promoting cell adhesion, and migration.” This is induced by laceration of the apical tissues with the goal of blood clot formation to the cement-enamel junction allowing for 3
mm to 4 mm of mineral trioxide aggregate (MTA) to be placed to seal the root canal. A final restoration is then placed over the MTA.\textsuperscript{13} Failures in RE have been attributed to a lack of vascularization, which could not support the blood clot scaffold.\textsuperscript{7}

**ANGIOGENESIS**

Angiogenesis, the development of new blood vessels, is needed to increase vascularization, and the creation of a vascular network. Capillary development occurs in three different ways. They can sprout from existing capillaries, anew, and by “incorporating circulating monocytes that have transdifferentiated into endothelial cells.”\textsuperscript{9} Sprouting angiogenesis is the most studied form of angiogenesis.\textsuperscript{8} A need exists for a balance with angiogenesis to prevent a state of chronic inflammation associated with fibroproliferative disorders and metastasis tumors.\textsuperscript{85}

Different cell types and various cytokines/growth factors are involved in angiogenesis. These include endothelial cells, fibroblasts, neutrophils, and macrophages that produce vascular endothelial growth factor (VEGF). VEGF is a signaling protein that early in angiogenesis increases the permeability of blood vessels causing the extravasation of plasma proteins. This step leads to the creation of a temporary scaffold for migrating ECs.\textsuperscript{8} Macrophages produce tumor necrosis factor alpha (TNF-alpha), which also induces VEGF expression. In wound repair, VEGF expression is enhanced leading to a neovascular network that drives regeneration by increasing tissue perfusion.\textsuperscript{9} ECs have been shown to release the angiogenic cytokines fibroblast growth factor (FGF) and VEGF without any stimulation\textsuperscript{6} VEGF increases the permeability of ECs and FGF promotes the proliferation and differentiation of ECs, smooth muscle cells, and fibroblasts.
Matrix metalloproteinases (MMPs) play a role in angiogenesis by degrading extracellular matrix molecules and by activating or liberating the growth factors bFGF, VEGF, and insulin-like growth factor 1 (IGF-1) that are within the extracellular matrix.\textsuperscript{8} Zehng et al. showed an increase in the expression of MMP-3, MMP-9, and MMP-13 after a pulpal injury.\textsuperscript{10} Tissue inhibitors of matrix metalloprotienases (TIMPs) play a role in the inhibition of MMPs and therefore inhibit angiogenesis. Mathieu et al. showed that ECs might play an important role in recruiting human pulp cells, as well as in forming the necessary blood vessels for angiogenesis.\textsuperscript{11} It is believed that an appropriate scaffold that will provide an environment that will support ECs may improve the success of RE procedures.

**SCAFFOLDS**

There are multiple scaffolds used in place of a blood clot. The most commonly used alternative scaffolds are platelet-rich-plasma, synthetic and natural scaffolds.\textsuperscript{12} DynaMatrix\textsuperscript{®} (Cook Biotech, West Lafayette, IN) is a matrix currently used in dentistry for periodontal regeneration procedures.\textsuperscript{12,13} This ECM is derived from porcine small intestine submucosa (SIS) and is composed of collagen that could be used as a framework for cell growth. The intestine is processed with surfactant and ionic solutions to remove the cells and nuclear matter. The remaining structure is three-dimensional, acellular, and collagen-rich with no cross-linking. An isolation process preserves the structural components of the matrix consisting of collagens I, III, IV, and VI. The result is a framework for infiltration of cells, cytokines, and MMPs important for regeneration. The company states that it contains promoters of angiogenesis including FGF-2, connective tissue growth factor (CTGF), and transforming growth factor beta (TGF-betta).\textsuperscript{13}
Research at Indiana University School of Dentistry (IUSD) has shown that DynaMatrix® has potential as an endodontic scaffold. It can support the growth of HDPSCs and HPFs. Positive angiogenic profiles were seen after these cells were exposed to Dynamatrix®. The effects of Dynamatrix on EC, cytokines, and MMPs have not been investigated, although both are pivotal for angiogenesis. This work will further the evidence for the potential Dynamatrix has in RE.

RE procedures will continue to change and evolve as futures studies continue to enhance the understanding of the important factors needed. With this, the protocols used will continue to change, to increase the outcomes, and to enhance predictability.
METHODS AND MATERIALS
EXPERIMENTAL DESIGN

Human umbilical vein endothelial cells (HUVECs) were utilized in this *in-vitro* study. The study groups were as follows: 1) HUVECs seeded in culture only (control group 1); DynaMatrix® membranes in culture media without any cells (control group 2); and HUVECs seeded on DynaMatrix® membranes (experimental group). Conditioned media from the different groups were collected after 72 hours of incubation. The media were tested for the expression of specific cytokines and matrix metalloproteinases.

HUVECs CULTURE

The HUVECs were obtained from American Type Culture Collection (ATTC, Manassas, VA, USA) and grown in Kaighn’s Modification of Ham’s F-12 with L-Glutamine (ATTC, Manassas, VA) supplemented with 0.1 mg/ml heparin, 0.03 mg endothelial cell growth supplement (EGGS), 10-percent solution of fetal bovine serum (FBS), 100 U/ml penicillin, 50-mg/ml gentamicin, and 2.5-mg/ml of amphotericin B. HUVECs from passage 3 to passage 8 were used. HUVECs were grown, and visual inspection under the microscope confirmed the cell count.

HUVEC TREATMENT

After the HUVECs were grown, 75,000 cells were seeded per well in six well plates. Three wells were used for each group for a total of 9 wells. The experimental groups tested were as follows:

(a) Group 1: HUVECs only (C).
(b) Group 2: DynaMatrix® membrane incubated without any cells (M), and 
(c) Group 3: HUVECs seeded on DynaMatrix® membranes (C+M).

The experimental design is shown in FIGURE 1.

The cells were given 24 hours for attachment and verified with a light microscope. The serum plus media was removed and 2 mLs of serum minus media was added to each well of the 6 well plates. Then, after 72 hours of incubation at 37°C, the conditioned media from the three different experimental groups were collected and stored at -70°C until analyzed. The conditioned media from the various groups were used to test for the expression of multiple angiogenic cytokines and MMPs.

ANGIOGENIC CYTOKINE AND MMP EXPRESSION

Equal volumes, 1 mL of conditioned media, were used for analyses. The angiogenic cytokine and MMP profile from HUVECs alone or HUVECs seeded on DynaMatrix® membranes or membranes alone were evaluated utilizing RayBio Human Angiogenesis Antibody Array I (TABLE 1, RayBiotech Inc., Norcross, GA) and RayBio® Human Matrix Metalloproteinase Antibody Array (TABLE 2, RayBiotech Inc., Norcross, GA) according to the manufacturer’s protocol. Specifically, the array membranes were blocked by 1 ml of the blocking buffer, supplied by the kit, for 30 minutes. The blocking buffer was removed. Then, 1 ml of the conditioned media was added to each well and incubated overnight at 4°C. The membranes were then washed three times with 2 ml of Buffer I for five minutes, then washed twice with 2 ml of Buffer II for 5 minutes. Then, 1 ml of the Biotinylated Antibody Cocktail was then added to each well and incubated overnight at 4°C. The membranes were then washed with Buffer I and II as previously described. Then, 2 ml of Horseradish Peroxidase-Conjugated
Streptavidin was added to each well and incubated for 2 hours at 4°C. The wells were then washed with Buffer I and Buffer II again as previously described. Detection agent supplied by manufacturer was added and then the membranes were exposed to X-ray film for 5 seconds, 10 second, 20 seconds, 30 seconds and 2 minutes. The density of the cytokine expression was measured with the Quantity One 1-D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA). The experiment was repeated three times and the averages calculated.

STATISTICAL ANALYSIS

Multiple dots for each cytokine on array membranes were averaged. For each array membrane, the densities were adjusted for the background by subtracting the average value of the negative controls and then normalized by dividing by the average of the positive controls. The data were then converted back to the original scale by multiplying by the average of the positive controls for the first array membrane. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by pair-wise tests using Fisher’s Protected Least Significant Differences to control the overall significance level at 5 percent. Analyses were performed using SAS (SAS Institute Inc., Cary, NC). The distribution of the data was examined and a transformation of the data (natural logarithm, rank, etc.) was used. Based on previous studies, the study has approximately 80-percent power to detect a three-standard-deviation difference between groups, assuming two-sided tests each conducted at a 5-percent significance level.
RESULTS
The Quantity One 1-D Analysis Software (Bio-Rad) was used to measure the optical densities of the visible dots on the membranes. The background for each membrane was adjusted. The average value of the negative controls was subtracted and then normalized by dividing the average of the positives controls. There were three membranes for each group. Group comparisons were done utilizing a one-way ANOVA.

The cytokine and MMP arrays showed that the cells alone had significantly greater GRO (p = 0.0123); MCP-1 (p = 0.0123); MMP-1 (p = 0.0300); MMP-2 (p = 0.0254); MMP-10 (p = 0.0003); MMP-13 (p = 0.0186); TIMP-1 (p = 0.0254); and TIMP-2 (p = 0.0003) as compared with the membrane alone. The membrane alone had significantly less MMP-8 (p = 0.0308) when compared with cells alone.

The cells + membrane showed significantly greater values for bFGF (p = 0.0065) than the cells alone. The cells + membrane showed significantly smaller MMP-10 (p = 0.0104) and TIMP-2 (p = 0.0104) than the cells alone. The cells + membrane showed significantly smaller values than the cells alone for MMP-8 (p = 0.0065).

Cells + membrane showed significantly greater bFGF (p = 0.0308); GRO (p = 0.0300); Leptin (p = 0.0186); MCP-1 (p = 0.0300); MMP-1 (p = 0.0123); MMP-2 (p = 0.0190); MMP-10 (p = 0.0104); TIMP-1 (p = 0.0190), and TIMP-2 (p = 0.0104) compared with the membrane alone.

The cytokine array groups did not have significantly different values for ANG (p = 0.0945); EGF (p = 0.7843); ENA-78 (p = 0.2042); IFN-γ (p = 0.9670); IGF-1 (p = 0.7843); IL-6 (p = 0.0945); IL-8 (p = 0.1424); PDGF-BB (p = 0.4910); PIGF (p =
0.1424); RANTES (p = 0.7290); TGF-β1 (p = 0.7290); TIMP-1 (p = 0.1820); TIMP-2 (0.1820); Thrombopoietin (p = 0.9670); VEGF (p = 0.7843), and VEGF-D (p = 0.4910).

The MMP array groups did not have significantly different values for MMP-3 (p = 0.6262); MMP-9 (p = 0.1424), and TIMP-4 (0.7843).
FIGURES AND TABLES
FIGURE 1. Experimental design.
FIGURE 2. DynaMatrix membrane sterilized.
FIGURE 3. Example of DynaMatrix trimmed to fit into the six-well plate.
FIGURE 4. Six-well plates with three experimental groups with culture media. Column 1: HUVECs; Column 2: HUVECs seeded on DynamMatrix; and Column 3: DynaMatrix membrane.
FIGURE 5. Array membranes placed in wells.
FIGURE 6. Cytokine array: Images of scanned x-ray films for each sample group. From left to right: Cell+Membrane group (C+M); Cell-only group (C); and Membrane-only group (M).
FIGURE 7. MMP Array: Images of scanned x-ray films for each sample group. From left to right: Cell+Membrane group (C+M); Cell-only group (C); and Membrane-only group (M).
### RayBio® Human Angiogenesis Antibody Array 1 Map

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>Angiogenin</td>
<td>EGF</td>
<td>ENA-78</td>
<td>b FGF</td>
</tr>
<tr>
<td>2</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>Angiogenin</td>
<td>EGF</td>
<td>ENA-78</td>
<td>b FGF</td>
</tr>
<tr>
<td>3</td>
<td>GRO</td>
<td>IFN-γ</td>
<td>IGF-1</td>
<td>IL-6</td>
<td>IL-8</td>
<td>LEPTIN</td>
<td>MCP-1</td>
<td>PDGF-BB</td>
</tr>
<tr>
<td>4</td>
<td>GRO</td>
<td>IFN-γ</td>
<td>IGF-1</td>
<td>IL-6</td>
<td>IL-8</td>
<td>LEPTIN</td>
<td>MCP-1</td>
<td>PDGF-BB</td>
</tr>
<tr>
<td>5</td>
<td>PIGF</td>
<td>RANTES</td>
<td>TGF-β1</td>
<td>TIMP-1</td>
<td>TIMP-2</td>
<td>Thrombopoietin</td>
<td>VEGF</td>
<td>VEGF-D</td>
</tr>
<tr>
<td>6</td>
<td>PIGF</td>
<td>RANTES</td>
<td>TGF-β1</td>
<td>TIMP-1</td>
<td>TIMP-2</td>
<td>Thrombopoietin</td>
<td>VEGF</td>
<td>VEGF-D</td>
</tr>
<tr>
<td>7</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>Neg</td>
<td>POS</td>
</tr>
<tr>
<td>8</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>Neg</td>
<td>POS</td>
</tr>
</tbody>
</table>

**Note:** GRO detects CXCL1, CXCL2, CXCL3
**Note:** TGF-β1 detects only active form
**Note:** VEGF detects VEGF-165 and VEGF-121

### Key to abbreviations:

- **Angiogenin:** aka ribonuclease 5
- **EGF:** epithelial growth factor
- **ENA-78:** epithelial neutrophil-activating protein 78
- **b FGF:** basic fibroblast growth factor
- **IFN-γ:** interferon gamma
- **IGF-1:** insulin-like growth factor 1
- **IL-6, 8:** interleukin 6, 8
- **MCP-1:** monocyte chemotactic protein-1
- **PDGF-BB:** platelet-derived growth factor BB
- **PIGF:** Phosphatidylinositol-glycan biosynthesis class F protein
- **TGF-β1:** transforming growth factor beta 1
- **TIMP-1, 2:** tissue inhibitor of metalloproteinases metalloproteinase inhibitor 1, 2
- **VEGF:** vascular endothelial growth factor
- **VEGF-D:** vascular endothelial growth factor D
- **POS:** positive control
- **Neg:** negative control
TABLE II

RayBio® human matrix metalloproteinase antibody array

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>MMP-1</td>
<td>MMP-2</td>
<td>MMP-3</td>
<td>MMP-8</td>
</tr>
<tr>
<td>2</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>MMP-1</td>
<td>MMP-2</td>
<td>MMP-3</td>
<td>MMP-8</td>
</tr>
<tr>
<td>3</td>
<td>MMP-9</td>
<td>MMP-10</td>
<td>MMP-13</td>
<td>TIMP-1</td>
<td>TIMP-2</td>
<td>TIMP-4</td>
<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>4</td>
<td>MMP-9</td>
<td>MMP-10</td>
<td>MMP-13</td>
<td>TIMP-1</td>
<td>TIMP-2</td>
<td>TIMP-4</td>
<td>NEG</td>
<td>POS</td>
</tr>
</tbody>
</table>
TABLE III

Data summary of densitometry readings for cytokine array*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>C (Mean)</th>
<th>M (Mean)</th>
<th>C+M (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG</td>
<td>60012 (39064)</td>
<td>-42846 (43058)</td>
<td>129372 (46287)</td>
</tr>
<tr>
<td>bFGF</td>
<td>-45220 (44510)</td>
<td>-35128 (43536)</td>
<td>97693 (78019)</td>
</tr>
<tr>
<td>EGF</td>
<td>-43021 (43730)</td>
<td>-41951 (42880)</td>
<td>85810 (86806)</td>
</tr>
<tr>
<td>ENA-78</td>
<td>-39696 (47252)</td>
<td>-41950 (42965)</td>
<td>88730 (84731)</td>
</tr>
<tr>
<td>GRO</td>
<td>173462 (65497)</td>
<td>-40415 (44717)</td>
<td>158113 (18553)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>-40894 (45054)</td>
<td>-41232 (44346)</td>
<td>86219 (87205)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>-42763 (43699)</td>
<td>-40964 (43968)</td>
<td>85427 (87313)</td>
</tr>
<tr>
<td>IL-6</td>
<td>4144 (44794)</td>
<td>-41429 (43553)</td>
<td>108037 (63924)</td>
</tr>
<tr>
<td>IL-8</td>
<td>383171 (88302)</td>
<td>-40512 (43016)</td>
<td>310150 (157215)</td>
</tr>
<tr>
<td>LEPTIN</td>
<td>-22429 (49056)</td>
<td>-39917 (42737)</td>
<td>108537 (68966)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>271193 (81018)</td>
<td>-39463 (42372)</td>
<td>232402 (57627)</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>-39535 (44622)</td>
<td>-40165 (43670)</td>
<td>90897 (84240)</td>
</tr>
<tr>
<td>PIGF</td>
<td>-7179 (50997)</td>
<td>-41561 (45001)</td>
<td>101250 (72780)</td>
</tr>
<tr>
<td>RANTES</td>
<td>-38922 (44416)</td>
<td>-36873 (45059)</td>
<td>87397 (85444)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>-41043 (44427)</td>
<td>-39061 (43882)</td>
<td>86942 (84810)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>80033 (56341)</td>
<td>-40506 (43999)</td>
<td>141514 (27625)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>68683 (47678)</td>
<td>-40539 (43756)</td>
<td>103968 (67456)</td>
</tr>
<tr>
<td>Thrombo</td>
<td>-41778 (43985)</td>
<td>-40783 (43005)</td>
<td>86135 (85737)</td>
</tr>
<tr>
<td>VEGF</td>
<td>-41152 (44959)</td>
<td>-39576 (43038)</td>
<td>87670 (85237)</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>-43629 (44308)</td>
<td>-39307 (44125)</td>
<td>86798 (85988)</td>
</tr>
</tbody>
</table>

*Mean densitometry values for each experimental group, followed by the standard error in parenthesis. C: Cells only; M: DynaMatrix only; M+C: HUVEC seeded on DynaMatrix.
TABLE IV

Data summary of densitometry readings for the MMP array*

<table>
<thead>
<tr>
<th>MMP</th>
<th>C</th>
<th>M</th>
<th>C+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>268750 (33511)</td>
<td>26630 (2003)</td>
<td>286894 (5036)</td>
</tr>
<tr>
<td>MMP-2</td>
<td>20151 (3619)</td>
<td>12482 (786)</td>
<td>17727 (1042)</td>
</tr>
<tr>
<td>MMP-3</td>
<td>24321 (3302)</td>
<td>21833 (1137)</td>
<td>25248 (2396)</td>
</tr>
<tr>
<td>MMP-8</td>
<td>-4 (523)</td>
<td>-1983 (732)</td>
<td>-2711 (578)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>22341 (505)</td>
<td>17458 (3451)</td>
<td>15360 (553)</td>
</tr>
<tr>
<td>MMP-10</td>
<td>266741 (16450)</td>
<td>-1805 (861)</td>
<td>95033 (7677)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>21932 (1797)</td>
<td>16407 (1296)</td>
<td>19288 (1231)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>212466 (28199)</td>
<td>-432 (668)</td>
<td>223384 (8775)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>200826 (26425)</td>
<td>10493 (1460)</td>
<td>73484 (4112)</td>
</tr>
<tr>
<td>TIMP-4</td>
<td>21493 (2942)</td>
<td>23444 (455)</td>
<td>24275 (1030)</td>
</tr>
</tbody>
</table>

*Mean densitometry values for each experimental group, followed by the standard error in parenthesis. C: Cells only; M: DynaMatrix only; M+C: HUVEC seeded on DynaMatrix.
DISCUSSION
HUVECs seeded on the DynaMatrix *in vitro* grew on the membranes. This important characteristic makes DynaMatrix a possible future scaffold to be placed within the canal during regenerative endodontic procedures.

In this *in-vitro* study, the HUVECs only group (C) had statistically significant greater values for GRO and MCP-1 in the cytokine array, and MMP-1, MMP-2, MMP-10, MMP-13, TIMP-1 and TIMP-2 in the MMP array as compared with the DynaMatrix only group (M).

The experimental group (C + M) had a statistically significant increase in bFGF, GRO, Leptin, and MCP-1 in the cytokine array, and a significant increase in the MMP array of MMP-2, MMP-10, TIMP-1 and TIMP-2 compared with the DynaMatrix membrane alone. HUVECs alone also were more significant than the DynaMatrix membrane alone for GRO and MCP-1 in the cytokine array and also for MMP-2, MMP-10, MMP-13, TIMP-1 and TIMP-2 in the MMP array. This could imply an additive effect of the cells on the DynaMatrix for GRO, MCP-1, MMP-2, MMP-10, TIMP-1 and TIMP-2. The DynaMatrix membrane did not express many factors, therefore, the addition of the cells to the membrane appears to be an additive effect for many factors in the arrays.

The most significant changes were seen within the C+M group compared with the cells only group. The HUVECs seeded on the DynaMatrix membrane group (C+M) had statistically significant increases in the expression of bFGF versus both control groups in the cytokine array. This increase is not due to simple addition of bFGF through leaching of the cytokine from the membrane alone. The levels of bFGF in the experimental group
(C+M) were shown to be statistically significantly greater than those of the membrane or the cells alone. The level of bFGF in the experimental group could be due to a stimulatory effect of the DynaMatrix on the HUVECs. It appears to be more than just an additive effect due to the significant level of increase in bFGF in the experimental group. Membrane-only specimens had greater levels of bFGF than the cells alone, but were not statistically significant. The membrane-only group had no HUVECs present; therefore, the increase in bFGF in the supernatant of the membrane-only group must be a result of it leaching from the membrane.

The experimental group (C + M) has significantly less TIMP-2 and MMP-10 than the cells alone in the MMP array. The importance of TIMP-2 being statistically significantly less implies that the membrane had an inhibitory effect on the cells ability to release/express TIMP-2.

An interesting finding was that the cytokine array did show a difference between the C+M group and the C group for TIMP-2. TIMP-2 was less in the C+M group, but was not shown to be statistically significant.

The overall results of this study were an increase in pro-angiogenic cytokines and MMPs. The increase in bFGF in the experimental group was the only cytokine to increase compared with both. A pro-angiogenic cytokine, the bFGF promotes proliferation and differentiation of endothelial cells, smooth muscle cells, and fibroblast. Purified basic pituitary FGF in vitro has been shown to “induce endothelial cells to invade three-dimensional collagen matrix and to organize themselves to form characteristic tubules that resemble blood capillaries.” Studies have shown that bFGF
can stimulate characteristics of the processes for angiogenesis for inducing endothelial cell migration and invasion.\textsuperscript{86}

The other cytokines that were shown to be statistically increased in the experimental group were compared with the membrane-only group. They were GRO, Leptin, and MCP-1. GRO has been shown to be a chemotactic factor for angiogenesis, as well as MCP-1, which is recognized as an angiogenic chemokine. Leptin is an endocrine hormone that regulates adipose tissue, and it has been found to generate a growth signal to promote angiogenic processes in endothelial cells via a leptin receptor.\textsuperscript{87} It has been speculated that in addition to stimulating angiogenesis in ECs via a leptin receptor, “leptin plays a role in matrix remodeling by regulating the expression of MMPs and TIMPs.”\textsuperscript{87}

The MMPs arrays had several factors that were shown to be statistically decreased in the C+M group compared with the C group. The experimental group had a decrease in TIMP-1 compared with the cells alone. The experimental group compared with the membrane alone had an increase in TIMP-1 and TIMP-2. The membrane-only group had very small values for TIMP-1 and TIMP-2 as compared with the cells-only group and could account for the increase in the experimental group compared with the membrane-only group. The decrease in TIMP-2 demonstrated that the membrane might have an inhibitory effect on the cells’ expression of TIMP-2. The increase in TIMP-1 and TIMP-2 compared with the membrane-only group could be due to the addition of cells to the membrane having an additive effect as compared with the membrane-only, because the membrane-only group had a very small value for TIMP-1 and TIMP-2. TIMP-1 and TIMP-2 are known to inhibit all known MMPs, therefore inhibiting the degradation of
ECM and preventing angiogenesis from occurring.\textsuperscript{88} It would be a desired outcome to have an overall decrease in TIMPs to prevent inhibition of MMPs and therefore to help promote angiogenesis.

The MMP array’s decrease in MMP-10 in the experimental group compared with the cells-alone group is not a desired outcome, due to its ability to degrade the ECM and promote angiogenesis; however, the expression of many other MMPs were not negatively affected by the addition of the membrane. This decrease could be due to an inhibitory effect the membrane has on the cells’ ability to express MMP-10. The experimental group had an increase in MMP-2 and MMP-10 compared with the membrane-only group. This could be due to an additive effect of the cells to the membrane because these MMPs were not increased compared with the cell-only group. MMPs have been shown to play an important role in angiogenesis, wound healing, and inflammation.\textsuperscript{10} They also contribute to the remodeling of pulp tissues and dentin.\textsuperscript{68,90} Several inflammatory cytokines have been shown to stimulate MMP-10.\textsuperscript{69} The decrease in MMP-10 may not inhibit angiogenesis in the presence of inflammation within the canal.

It is difficult to know for sure if the preceding discussion regarding specific cytokines and MMPs observed \textit{in-vitro} would correlate to an \textit{in-vivo} setting. The individual cytokines and MMPs are affected by many different factors. They can have different effects based on their target cells, and they are dose dependent. Much of the research on the angiogenic potential of the cytokines and MMPs has been done with a focus on tumor angiogenesis. The findings in these studies may focus on different clinical conditions than those of the present study, and therefore, the outcomes may differ clinically from the regenerative endodontic procedures that we focused on.
Research at IUSD has focused on the use of DynaMatrix as a scaffold in RE regenerative endodontic procedures. The previous studies used HDPSC and HDPF. The results of these studies were that there were statistically significant differences in the expression of angiogenic cytokines in the experimental groups (HDPSC or HDPF seeded on DynaMatrix) when compared with the control groups (HDPSC or HDPF only and DynaMatrix only).\textsuperscript{14,15} The use of a matrix may allow for a more coronal advancement of the factors necessary for regeneration either by a wicking effect, or by increasing angiogenesis to the level of the scaffold. Failures in RE have been linked to the lack of vascularization within the canal that could support the blood clot scaffold,\textsuperscript{7} and using a scaffold like DynaMatrix may be a solution to this problem.

Future studies could include evaluating the angiogenic cytokine effects of HDPSC, HDPF, and HUVECs on the DynaMatrix. Eventually animal studies could be done leading to \textit{in-vivo} human studies based on the fact that DynaMatrix is currently used in dentistry and medicine as an FDA-approved resorbable membrane.
SUMMARY AND CONCLUSIONS
The null hypothesis for this study was rejected. There was a statistically significant increase in bFGF in the experimental group (HUVECs seeded on Dynamatrix) as compared with the control groups (HUVECs alone and DynaMatrix alone). In addition to bFGF, Leptin, MCP-1, GRO, MMP-2, and MMP-10 were also increased in the experimental group compared with the membrane alone. Other factors, like TIMP-2, were decreased in the experimental group as compared with the cells alone, but increased in the experimental group compared with the membrane alone. Overall, the cytokine and MMP profiles are positive for up-regulation of pro-angiogenic factors and down-regulation of some anti-angiogenic factors.

This study adds to the overall evidence that supports the use of DynaMatix for an intracanal scaffold for regenerative endodontics. DynaMatrix could increase the local angiogenic cytokines, specifically bFGF, and MMPs within the canal. The membrane may also assist in endodontic regeneration by down-regulation of TIMP-2, an inhibitor of angiogenesis, in the presence of ECs. It could also serve as a more predictable and reproducible scaffold clinically. This study demonstrated that DynaMatrix could support the growth of endothelial cells. The overall results of this study suggest that DynaMatrix could improve the vascularization within the canal by changing the microenvironment within the disinfected canal and allow for angiogenesis and tissue regeneration.
REFERENCES


57. Gulabivala K, Patel B, Evans G, Ng Y-L. Effects of mechanical and chemical procedures on root canal surfaces. Endodontic Topics


ABSTRACT
Introduction: Regenerative endodontics (RE) is a treatment alternative for the infected immature tooth to establish an environment in the canal that enables continued root development and the growth of pulp or pulp-like tissue within the canal. A scaffold created in the canal encourages the formation of vital tissue. The porcine sub-intestinal-submucosa (SIS) membrane, Dynamatrix®, has the potential to serve as an endodontic scaffold. Research at Indiana University School of Dentistry (IUSD) has shown that Dynamatrix® can support the growth of human dental pulp stem cells (HDPSC) and human pulp fibroblasts (HPF). Positive angiogenic cytokine profiles were seen after
these cells were seeded on Dynamatrix®. Endothelial cells play an important role in the formation of blood vessels and are a source of angiogenic cytokines. Exposure of these cells to DynaMatrix® may result in a positive angiogenic profile for both cytokines and matrix metalloproteinases (MMPs).

Objective: The aim of this in-vitro study was to investigate if the exposure of human endothelial cells to the DynaMatrix® membrane would result in differences in the expression of cytokines and MMPs that play roles in angiogenesis.

Materials and Methods: Human endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATTC, Manassas, VA) and used in this study. Groups were established as follows: (a) Group 1: HUVECs seeded in culture media only, (b) Group 2: DynaMatrix® membrane incubated alone in the serum-media without any cells, and (c) Group 3: HUVECs seeded on DynaMatrix® membranes. After 72 hours of incubation, the conditioned media were collected and analyzed for the expression of 20 angiogenic cytokines and MMPs utilizing cytokine and MMP protein arrays. The density of each cytokine and MMP expressed was measured, averaged, and statistically analyzed by ANOVA.

Results: Exposure of human umbilical vein endothelial cells (HUVECs) to the DynaMatrix® membrane resulted in a positive angiogenic profile for both cytokines and MMPs.

Conclusion: This work furthers the evidence for the potential of DynaMatrix® to serve as a more predictable scaffold in RE.
CURRICULUM VITAE
Scott Thomas Hill

May 2002  Northwood High School, Nappanee, IN, Diploma

May 2006  Valparaiso University, Valparaiso, IN, BS, Biology


June 2007 to May 2011  Indiana University School of Dentistry, Indianapolis, IN, DDS

July 2011 to June 2012  Richard L. Roudebush VA Medical Center, Indianapolis, IN

July 2012 to June 2013  General Practice Residency General Dentist, Private Practice, Anderson, IN

July 2013 to June 2015  Indiana University School of Dentistry, Indianapolis, IN, MSD in Endodontics

Professional Organizations

Indiana Dental Association
American Dental Association
American Association of Endodontists