EFFECTS OF DYNAMATRIX® ON ANGIOGENIC CYTOKINE EXPRESSION FROM HUMAN DENTAL PULP FIBROBLASTS: AN *IN VITRO* STUDY

by

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INTRODUCTION

For generations, the loss of, or severe damage to a body part resulted in the use of prostheses or perhaps a transplant of some kind. Materials used for transplants are categorized as follows: autograft (derived from the same individual); allograft (derived from a different individual of the same species); xenograft (derived from non-human sources), or alloplast (derived from synthetic material).[1,2]

The field of material science is evolving rapidly with technological advancements such as three-dimensional bioprinting and even gene regulation/therapy.[3,4] An increase has been observed in the use of more durable synthetic materials (e.g., Teflon, silicone) to replace or rebuild diseased tissues of the human body. Despite all advancements, many newer materials are physically and functionally different from the original human form, and they supply more of a structural rather than a functional replacement of the original tissue.[5]

The demand for original human tissue has created the field of tissue engineering from the study of cell biology.[6] At the turn of the century, this was considered a novel approach and is still being explored. Tissue engineering is the field of functional restoration of tissue structure and physiology for impaired or damaged tissues because of cancer, disease, or trauma.[7] Tissue engineering relies on living cells in various ways to maintain, enhance, or restore tissues and organs.[8] In the process, such engineering utilizes and integrates principles from various other fields such as cell transplantation, material science, and engineering.[5] An outgrowth of the increased knowledge in the field of tissue engineering has been the development of a scientific discipline termed “regenerative

medicine."[6] Regenerative therapy involves the combined interplay of three key elements: stem cells, some type of scaffold, and cytokines.[6] Regenerative therapies have become an alternate method for restoration of tissues and/or organs instead of autografts, allografts, or artificial prostheses.[9] Tissue engineering and regenerative therapies have shown promising results in the field of dentistry. The field of regenerative endodontics aims to utilize the principals of tissue engineering to replace the pulp-dentin complex in the diseased tooth.[5]

Regenerative endodontic therapy via revascularization is a form of tissue engineering that is being utilized to overcome the challenges that a necrotic immature permanent tooth presents. One of the most significant challenges apart from debridement of the canal system can be found in conventional obturation. These immature teeth have open apices, which dramatically increase the risk of over-extension of material beyond the apical foramina. Apexification utilizes calcium hydroxide (traditional approach) or more recently, mineral trioxide aggregate, to induce the formation of an apical hard tissue barrier that prevents overfill during conventional obturation.[10] Although these developments ease obturation, the tooth remains fragile with compromised periodontal support. Consequently the tooth has a less than desirable prognosis due to a high susceptibility to fracture.[1] Modern regenerative endodontic therapy aims to address these shortcomings.

Several studies have shown healing of apical periodontitis, continued development of the root apex, and an increase in root canal wall thickness[4] using regenerative endodontic techniques.[11-13] The American Association of Endodontics announced a suggested regenerative endodontic protocol that includes multiple

appointments. The canal is irrigated at the first appointment and followed by placement of a low concentration of antibiotic paste as an intracanal medicament. The patient is to be seen in three to four weeks and anesthetized without a vasoconstrictor. The medicament is then rinsed from the canal with 17-percent ethylenediaminetetraacetic acid and bleeding is evoked by careful laceration of the apical tissue. Mineral trioxide aggregate is then placed over the blood clot and the canal is sealed with a permanent coronal restoration.[14] Thus, the revascularization is accomplished by careful laceration of the apical papilla with an endodontic file to encourage hemorrhage into the empty disinfected canal space. The end result of revascularization promotes growth of vital pulp-like tissue into the disinfected canal space and continued root formation both in length and width.[11,15] The clot formed serves as a scaffold that supports mesenchymal stem cells and residential cells, including pulp fibroblasts, and provides nutrients and multiple growth factors, which are required for cell differentiation and dentin/root formation. [16]

Variations in the level of induced blood flow affect the quality of the blood clot, and the amount and quality of continued root development.[12, 17] Case reports have suggested that platelet-rich plasma can be used as a successful scaffold material.[18] However, scientists are still searching for materials that can serve as better scaffolds than the induced blood clot.[19, 20] The research on scaffold materials evolved out of early unsuccessful experiments from Nygaard Ostby and the role of blood clots in attempts at regeneration. [13] Regenerative endodontic therapy was initiated in the early 21st century. From the early 21st century until now, about 60 studies related to regenerative procedures have been conducted.[21] There are several variations in the clinical protocol for

regenerative endodontic therapy in more recent studies.[21] Despite all the technological advancements made in tissue engineering, a protocol is still needed that yields consistent results of pulp tissue regeneration inside a tooth.[21]

It is believed that an exogenous scaffold may be more predictable for pulp tissue regeneration and continued root formation than simply a blood clot. An ideal scaffold would facilitate cell organization, proliferation, differentiation, and vascularization essential for the success of pulpal regeneration.[14] DynaMatrix® is a natural membrane scaffold made of the porcine small intestine after decellularization and sterilization. It preserves the natural three-dimensional organization of extracellular matrix (ECM) molecules, especially Type I collagen, and contains functional proteins such as growth factors and cytokines.[22,23] Growth factors are any group of proteins that stimulate the growth of specific tissues, while cytokines are defined as any group of small, short-lived proteins that are released by one cell to regulate the function of another cell, thereby serving as intercellular chemical messengers.[24] The chief difference is that growth factor implies a positive effect on cell division, while cytokines are considered neutral with respect to cell proliferation. Cytokines can have positive effects (like growth factors) or negative effects. All growth factors are cytokines, yet not all cytokines are growth factors. Specifically, this study will measure the angiogenic growth factors/cytokines listed in Table I. DynaMatrix® is used as a scaffold in other areas of medical and oral surgery to promote wound healing and tissue regeneration.[23] Its application in endodontics is limited to assist with wound healing of periapical and periradicular surgeries. It should be noted that other growth factors such as dexamethasone and dental

proteins have been shown to stimulate dental pulp cells to differentiate into cells resembling odontoblasts/osteoblasts, adipocytes, or chondrocytes.[25]

The aim of this *in-vitro* study was to determine if human dental pulp fibroblasts (HDPFs) seeded on DynaMatrix® membrane would alter their production of angiogenic cytokines.

Previously, a study on DynaMatrix® membrane and dental pulp stem cells reported significant differences in angiogenic cytokine expression (Baker, unpublished data). HDPFs are the most numerous cells in the dental pulp[26] helping to form and to maintain the extracellular matrix through the production of collagen. They also have immunological properties and express many important cytokines.[15] These attributes could make the HDPFs more clinically relevant in advancing the protocol for regenerative endodontic therapy. Angiogenesis cytokine arrays have been established as a viable method for assessing the expression of angiogenic cytokine factors [27] and will be used in this study. Therefore, since cytokines can either have a negative or positive effect on cells, the hypothesis was to see if HDPFs seeded on DynaMatrix® membranes would alter the production of cytokines. Such production would be more successful if the porcine growth factors/cytokines from the Dynamatrix membrane would positively affect the HDPFs to secrete angiogenic cytokines and ultimately stimulate regeneration.

CLINICAL SIGNIFICANCE

DynaMatrix® scaffolds have been used successfully in dentistry and medicine. Their ability to support fibroblast activity will make them potential scaffolds for regenerative endodontics.

HYPOTHESES

Null: There will be no significant alterations in angiogenic cytokine expression when HDPFs are seeded on the DynaMatrix® membrane when compared with the membrane or cells alone.

Alternative: HDPFs seeded on the DynaMatrix® membrane will alter the expression of angiogenic cytokines when compared with the membrane or cells alone.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Although some authors have cited accomplishments made in endodontics from ancient times, the most remarkable advancements became much more frequent in the 17th century. The first dental book written in English was published by Charles Allen in 1687.[28] He described removing an infected tooth 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."[29] The idea of replacing an infected tooth with something else, such as another person's tooth, as Allen suggested, has proved to be a challenging task. The study of dentistry and endodontics has been an ever-changing process.

A fine piece of work, *The Surgeon Dentist,* was written by Pierre Fauchard in 1728. Fauchard, who is regarded as "the father of modern dentistry," accurately described root canal systems and pulp cavities of various teeth.[28] In even more detail, the book also described the practice of relieving abscesses and draining pus by drilling access holes in teeth. After these teeth were left open to drain for a few months, the pulp chamber was filled with lead foil.[28] Fauchard was a resourceful man and described the placement of cinnamon or the oil of cloves to the affected area of deep caries for treatment. In addition to drainage procedures, Fauchard described a pulp extirpation procedure using a small pin.[28]

Phillip Pfaff was a German dentist who cited a pulp-capping procedure in 1756, which involved cutting a piece of gold or lead to fit approximately the shape of the

opening over the pulp tissue. The metal was concavely shaped in a way to avoid direct contact with the pulp.[28]

King Louis XV of France had a personal dentist. The dentist Bourdet described in 1757 a process of extracting carious teeth, filling the root canals with gold or lead, and then replanting them, similar to the extract replant procedure used today. He also described a type of treatment involving the dislocation of a symptomatic tooth in order to sever the nerve. Then, he would immediately replace the tooth into its socket.[29] Although Bourdet was ahead of his time, he was not the first to treat symptomatic teeth in this manner. The same procedure had been described in the 11th century A.D. by the Arabian physician Avicenna.[30]

The first endodondic procedures performed in the US have been credited to Robert Woofendale. He was an English practitioner who immigrated to New York in 1766. He sought to alleviate dental pain by cauterizing the pulp with a hot instrument, followed by the placement of cotton pellets into the canals.[31,32]

By the late 1700s, a manuscript appeared on diagnosing dental disease written by a German dentist, Frederick Hirsch. His means for diagnosis were by tapping the suspected teeth, and noted that diseased teeth were tender to percussion. He recommended perforation of the tooth in the cervical area followed by insertion of a red-hot probe. The final step in his protocol was to fill the cavity with lead.[33]

In the early 19th century, there was a change in thought about infected teeth. A concept emerged regarding the importance of tooth vitality and how it related to pulp treatment. This time period was significant enough to be called, "The Vitalistic Era."[28] In 1805 J.B. Gariot became one of the first to advance the idea that obliteration of the pulp

does not destroy the vitality of the tooth.[34] In 1809 an Irishman named Edward Hudson practicing in Philadelphia built upon Gariot's theory that a pulpless tooth can remain in the mouth and began to place fillings in root canals. Hudson has been given credit for being the first dentist to do this. He designed his own instruments, which he used to pack gold foil in the canals.[31,35] Hudson was innovative for his time because he advocated the preservation of the natural dentition.[36]

In 1819 John Callow credits Charles Bew with describing the flow of blood into the pulp through the apical foramen and out through the dentinal wall and the periodontal membrane in his work, *Opinions on the Causes and Effects of Diseases in the Teeth and Gums*. Bew joined others of his time who subscribed to the vitalistic theory.[34] Leonard Koecker, who wrote *Principles of Dental Surgery* in 1826, was one of Bew's contemporaries. Koecker's book was the standard in the field for 50 years.[36] Koecker said a destroyed pulp could cause the dentinal core of the tooth to die, and that once dead, it would present itself as a foreign body, thus necessitating the extraction of the dead tooth. To avoid this fate for the tooth, Koecker advocated a pulp-capping procedure (essentially the same procedure described by Pfaff in 1756).[34,37,38]

The "vitalistic" or "double membrane" theory was formulated and presented by S.S. Fitch in his book "*System of Dental Surgery,"* in 1829. Fitch promoted the notion that teeth were like hollow bones with an outer periosteum (periodontal membrane) and an inner periosteum that lay between the pulp and the dentin. Thus Fitch believed that the crown was nourished solely by the dental pulp or its membrane, whereas the roots were nourished by the pulp membrane on the interior, and by the alveolar membrane on the exterior.[28] Support for this concept led to the practice of removing the crowns of

teeth after extirpation of the pulp. The reasoning for this practice was that the source of nourishment had been removed to the crown. However, the root was left in the socket, because it was believed to receive continued nourishment via the periodontal membrane. The root was then restored with a pivot crown.[34, 38]

John Hunter, a British surgeon and anatomist, believed that dentin possessed none of the properties of living tissue and was devoid of circulation, sensibility, and capability of repair. He was a proud supporter of an opposing philosophical position that advocated a "nonvitalistic" theory.[28] Hunter was not alone in his belief in the nonvitalistic theory; Cuvier and Robertson, also British, supported the nonvitalistic theory.[34]

In 1836 a drastic change in the approach to vital pulp extirpations was when Shearjashub Spooner of New York used a protoplasmic poison (arsenic trioxide) to devitalize the pulp before removing it.[31] Although this was a new approach in modern Western medicine, this procedure was actually invented by the ancient Chinese. In ancient Chinese medicine, the treatment for jaw abscesses was placement of arsenic trioxide.[30] Such treatment rapidly grew in popularity due to its success in eliminating the pain involved in the removal of vital pulps. Unfortunately, this also led to overuse of arsenic for devitalization of pulps and for treatment of teeth with dentin hypersensitivity. Arsenic was still being used by many dentists as recently as the 1920s to destroy the pulp before removing it.[39]

One year later in 1837, a father and son, Jacob and Joseph Linderer, advocated using essential oil as a pulpal obtundant before restoring a tooth with an exposed pulp.[40] The first root canal broach was invented by Edwin Maynard in 1838. He created it by filing a watch spring. Maynard also designed hoe-like instruments for shaping and

enlarging root canals.[41] In 1839 Baker described a treatment for an exposed nerve that included removal of the nerve, followed by cleaning of the canal and then filling it with gold foil. It is in fact Baker who has been credited with writing the first published account of pulpal extirpation, root canal cleaning, and filling.[28,30]

Throughout the decade of the 1850s, variations in root canal filling material were experimented with, including plugs of wood soaked in creosote.[28] In an attempt to create a seal with the wooden plug filling material, a liquid cement was used. This particular sealer contained gutta-percha, quick-lime, powdered glass, feldspar, and metal filings. This mixture was also used as a temporary restoration material.[39]

At a meeting of the Odontological Society of London in 1857, a man by the name of Thomas Rogers presented a series of 220 pulp-capping cases, 202 of which were considered successful. Rogers listed and described conditions that would lead to the success of a pulp-capping procedure including: general good health of the patient; freedom from inflammatory tendencies; absence of previous considerable pain from the affected tooth; absence of disease in other parts of the tooth, and no caustics to decrease the pain. Rogers' overall assessment and treatment recommendations proved to be accurate through modern times with the exception of his final protocol recommendation in the case of pulp-capping failure: three leeches and a laxative.[28]

Major innovations in endodontic treatment were seen at the close of the 19th century, when techniques began to look more like the modern techniques. Advancements in asepsis were brought about by S.C. Barnum of New York, who introduced the use of the rubber dam to isolate the tooth during the placement of gold foil restorations in 1864. It was quickly adopted for use in endodontic procedures to provide a more aseptic

environment for treatment.[30,31] In 1867 G.A. Bowman of Missouri was given credit for being the first to only use gutta-percha for filling root canals.[31,35,42]

A big year in endodontic advancements was 1867. Not only did gutta-percha make its entry, Magitot suggested using an electric current for pulp testing, and the German team of Leber and Rottenstein proved the existence of a parasite they called *Leptothrix buccalis,* which they found to exist on tooth surfaces, carious lesions, and within dentinal tubules.[37] This information led to a better understanding that tooth decay could cause gangrene (necrosis) of the pulp, and soon attempts were being made to apply Lister's principles of antiseptic treatment to the realm of pulp treatment.[28,40] Also in 1867 Joseph Lister described using carbolic acid as an antiseptic for surgical procedures.

By the late 1870s, the theory of vitalism was challenged by the septic theory. The septic theory asserted that pathogenic organisms were the most common cause of diseases of the pulp. This theory was promoted by G.O. Rogers in 1878 and Charles S. Tomes in 1879.[34] In 1882 Arthur Underwood expanded the septic theory by suggesting that if pathogens could be successfully excluded from the pulp space through the use of powerful antiseptic agents, suppuration of the pulp and resultant alveolar abscesses could be prevented.[28] This idea provided new justification for pulp therapy procedures that included caustic germicides for bacterial elimination, a practice that remained in vogue for more than 30 years.[34]

Dr. Bowman of Missouri introduced a solution of chloroform and gutta-percha, in 1895 naming it chloropercha. It was believed to have a superior seal as compared with gutta-percha alone. This technique rapidly became widely accepted. One of its chief

advocates was M.L. Rhein of New York, who further developed and described the technique several years later.[31]

One of the all time greatest inventors, Thomas Alva Edison discovered that calcium tungstate could be used to fabricate fluorescent dental mirrors in 1898. These dental mirrors aided in observing pathologic conditions and endodontic results. However, the instrument exposed clinicians and patients to excessive amounts of radiation, an appreciation of which finally led to their decline in the 1930's.[36]

Before 1905 cocaine was the anesthetic agent of choice for 20 years, yet things changed when Einhorn developed procaine (Novocaine®). This provided an alternative to the previously used cocaine that required a limited dosage due to its high toxicity. Though the invention of procaine represented a promising breakthrough in dental pain control, its wide use for mucosal injections was stifled initially by an inefficient protocol that required dissolving the tablet in solution, followed by boiling, cooling, and aspiration into a syringe. It would take another 25 years for more accessible block anesthesia techniques to be perfected.[39,41,43]

In 1908 Dr. Meyer L. Rhein, a physician-dentist from New York, developed a technique for determining canal length and degree of obturation that utilized a diagnostic wire in conjunction with radiographs.[31,37] Similarly, G.V. Black proposed measurement control to determine the length of the canal and the size of the apical foramen in an attempt to avoid overfilling.[28]

The beginning of the 20th century saw the birth of the Focal Theory of Infection (or Theory of Focal Infection) that postulates a myriad of diseases are caused by microorganisms (bacteria, fungi and viruses) that arise endogenously from a focus of

infection."[44] Hippocrates has been credited with perhaps the first report of focal infection by attributing the cure of a case of arthritis to a tooth extraction.[45] The theory experienced a resurgence due in part to E.C. Rosenow, a protégé of Frank Billings, who was a famous supporter of this theory.[44] Billings showed in 1909 that streptococci were present in many diseased organs, and that these bacteria were capable of traveling in the bloodstream to establish a separate infection at a distant site.[43]

One of the most famous supporters of the theory of focal infection was William Hunter, a British pathologist. In October of 1910, Hunter fanned the flame and propelled the theory to a state of widespread acceptance,[28] when he lectured on the topic at McGill University in Montreal. The presentation, entitled "The Role of Sepsis and Antisepsis in Medicine," was published in 1911 in the *Lancet*.[28]

The widespread popularity of the focal infection theory led to increased scrutiny of endodontic procedures. These times were dark days in the history of endodontics. Many physicians and dentists began to recommend extraction of all endodontically treated teeth, and this group of advocates was known as the "100 percenters." Others recommended removal of all non-vital teeth, and yet others even suggested that all teeth, whether diseased or not, be removed for the sake of prevention, as well as treatment, or "therapeutic edentulation" or "the clean-sweep."[44,46]

It was not until around 1930 before a more conservative approach gained popularity. Thus, the philosophy of the 100 percenters was the most influential approach in dentistry for nearly two decades.[28] The change in the dominant school of thought was driven by reexamining definitive diagnosis, aseptic techniques, bacteriological culturing, and improved radiographic practices in conjunction with root canal therapy.[47] The change

was not immediate however, as it would take close to a decade before the more conservative approach to the treatment of dental disease would become the standard.[28]

Several advancements in 1937 led to a new era, known as the "the scientific era."[28] Two landmark discoveries were made in 1937, Logan showed that bacteria can be present in normal tissues without having a pathological effect,[32] and Tunnicliff and Hammond found that microorganisms could be found in the pulps of extracted teeth "without any evidence of inflammatory tissue changes."[28,43] While there were new discoveries, reports also blatently contradicted the focal infection theory. Cecil of Cornell Medical College reported 200 cases in which arthritis had been treated by the removal of suspected foci with little evidence of positive effects. This led Burket to the conclusion that any improvement subsequent to the removal of the microbial center was more likely the result of an associative relationship[28] between the foci and the disease rather than a causative one.[48] These crucial discoveries finally brought an end to the formerly widespread practice of wholesale extraction of distressed or non-vital teeth.[28]

After the theory of focal infection slowly began to fade, the early 1940s saw a re-focusing on disinfection. Fred Adams and Louis Grossman promoted the idea of utilizing antibiotics as a supplement to root canal therapy. Adams reported used sulfanilamide, an antibiotic to treat periapical infections. He was also credited as the first to use penicillin in pulp canal therapy.[28,43] Grossman urged the use of a non-aqueous formulation of penicillin for use in endodontics. His recommendation was based on the fact that the stability of the compound decreased in cost compared with the conventional manner. Later, Dr. Grossman even used penicillin-laced paper points to disinfect root canals.[35]

With all the advancements in endodontics for centuries, there was still not a dedicated specialty or organized group of endodontists. However, this changed in 1943 when the American Association of Endodontists (AAE) was formed in Chicago, Illinois.[49] By 1949 the AAE investigated the possibility of establishing a specialty board in endodontics, and by 1956, this idea became a reality with the formation of the American Board of Endodontics.[50] Finally, due to a remarkable development and growth of endodontics during the previous 25 years and the tireless efforts of AAE leaders,[49] the American Dental Association recognized endodontics as a specialty in 1963. The first examination and certification of Diplomates occurred two years later in 1965.[28,49] The AAE currently boasts roughly 7000 members. and approximately a quarter of those members are board-certified.[51]

THEORY OF ENDODONTICS

Arguably the most foundational study in the advancement of modern endodontics was conducted in 1965 by Kakehashi, Stanley, and Fitzgerald.[52] The experiment showed that pulps of germ-free rats, when exposed and left open to food impaction, remained vital despite the trauma.[52] This highlighted the role that microbes play in endodontic infections and the supreme importance of reducing bacterial loads in order to achieve success following endodontic therapy.[53-55] Failure to achieve this goal can result in apical or periradicular periodontitis, which is defined as inflammation and destruction of the periodontium that may or may not produce symptoms.[56]

The ultimate goal of root canal therapy for both primary and secondary endodontic infections is to obtain maximal reduction in microorganisms and their toxins and to eliminate microbial insult to the pulpal and periapical tissues.[53] A 1955 study by

Stewart highlighted three phases of endodontic treatment: chemomechanical preparation, microbial control, and obturation of the root canal.[57] Of these three phases, Stewart identified chemomechanical preparation as the most important phase. This was later confirmed by Grossman,[58] who also identified 13 principles of effective root canal therapy as follows:

1. Aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic.
6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irrigating to the periapical tissues.
11. If an acute alveolar abscess is present, proper drainage must be established.
12. Injections into infectious areas should be avoided.
13. Apical surgery may be required to promote healing of the pulpless tooth.

In 1967 Schilder highlighted the importance of chemomechanical preparation of the canal to render it as aseptic as possible via instruments and antiseptics. Once there is sufficient reduction in microbial load, the next step is a three-dimensional obturation to the cementodentinal junction or 0.5 mm to 1 mm from the radiographic apex.[59] Ford

expanded on the principles listed by Schilder,[60] who cited three reasons that it is important to achieve a three-dimensional obturation. First, it leaves less space for bacterial colonization; second, it prevents apical contamination; and third, it prevents movement of bacteria along the walls of the canal. In addition to these guidelines for obturation, Ford also stressed the importance of aseptic technique including the use of rubber dam isolation, adequate coronal restoration of root-canal-treated teeth to provide another seal, and appropriate recall to monitor healing.

REGENERATIVE ENDODONTICS

Regenerative endodontic 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."[61] Though the concept of regenerative endodontic procedures is not new (Dr. B.W. Hermann reported on the use of $Ca(OH)_2$ in a case report involving a vital pulp amputation[62]), it has received increasing attention in recent years.[63,64] The typical clinical scenario in which regenerative endodontic procedures have been employed has been in cases of immature infected teeth with incompletely developed apices.[61,64,65] However, some investigators have described the potential to apply these principles to a wide array of clinical situations including mature teeth with closed apices and eventually to such endeavors as "replacement of periapical tissues, periodontal ligaments, gingiva, and even whole teeth."[61]

Some have questioned the value of applying the principles of regenerative medicine to endodontics.[61] It has been stated that the pulp plays no significant role in the fully developed tooth in terms of form, functions, or esthetics and therefore replacing it with a synthetic material as in conventional root canal treatment is the most practical

approach. Murray and colleagues refute these points,[61] citing the possibility for staining of tooth structure by endodontic filling materials and sealers.[66,67] Others, including Andreasen, have also pointed out the potential for decreased fracture resistance of root dentin caused by long-term $Ca(OH)_2$ exposure,[68] that is a part of certain treatment protocols for infected immature teeth. Others have proposed applying the principles of regenerative procedures to treat root perforations.[63]

Though minor variations exist in the precise protocols used in the regenerative endodontic procedures that have been reported in the literature, most practitioners have followed an approach that includes disinfection via irrigation of the canal space, placement of an intracanal medicament (typically $Ca(OH)_2$ or an antibiotic paste), induction of bleeding into the canal from the apical tissues, and placement of a coronal seal.[64,69] The induction of bleeding into the canal space is performed in order to provide a fibrin scaffold that can be populated by the cells involved in regeneration, as well as to provide a source for cytokines that aid in regeneration and repair.[61,64,69] The scaffold provides a biological "three-dimensional microenvironment for cell growth and differentiation, promoting cell adhesion, and migration."[70]

ANGIOGENESIS

Angiogenesis is defined as the formation of new blood vessels from preexisting capillaries, which has great importance in pulp regeneration.[71]Vasculogenesis is defined as the formation of the primary vascular plexus from preexisting vascular precursor cells in the embryo.[72] However, angiogenesis is the formation of new blood vessels from preexisting capillaries[72] and is responsible for the majority of the blood vessels formed

during physiological and pathologic conditions.[73] Thus, in the present study, the term angiogenesis will be used.

A key component to achieving success in regenerative endodontic procedures involves establishing an adequate blood supply to the tissues involved.[61, 64, 67] The establishment of such a blood supply requires the growth of existing vessels to form new branches and extensions in the periapical area and into the root canal itself.[69] This process of new blood vessel growth from previously existing blood vessels is termed "angiogenesis."[75] Other physiologic processes that involve angiogenesis include wound repair, the ovarian/menstrual cycle and embryogenesis.[76] Angiogenesis is absolutely critical to the entire process, as most cells must be less than 1 mm from a blood vessel to survive.[77]

Angiogenesis can be classified into two types: sprouting angiogenesis and intussusceptive (or splitting) angiogenesis.[75] Sprouting angiogenesis has been the most extensively studied method of vessel formation in humans, and it involves a complex series of interactions between various growth factors and cytokines that govern the development of the new vasculature.[78] Some chemical messengers induce angiogenesis (angiogenic), while others play an inhibitory role (angiostatic). The balance between the angiogenic and angiostatic factors is crucial, as aberrant angiogenesis can lead to "chronic inflammation associated with chronic fibroproliferative disorders as well as growth and metastasis of solid tumors."[76] The formation of new blood vessels through angiogenesis is mandatory to increase the survival rate of re-transplanted tissues.[71]

CYTOKINES

The definition of cytokines are "secreted proteins that regulate important cellular responses such as proliferation and differentiation."[79] Several cytokines have been identified that play important roles in the process of angiogenesis. One of the earliest steps in the process of angiogenesis involves an increase in the permeability of blood vessels in response to the presence of vascular endothelial growth factor (VEGF). This leads to extravasation of plasma proteins, which create a temporary scaffold for migrating endothelial cells[78] that are induced to migrate in part by the action of insulin like growth factor-1 (IGF-1).[80] Angiogenin and monocyte chemotactic protein-1 (MCP-1) are growth factors that aid the process by increasing new vessel growth.[81-83] Interleukin-6 and interleukin-8 (IL-6, IL-8) have also been shown to play a role in angiogenesis, sometimes promoting and sometimes inhibiting angiogenesis depending upon the interplay between multiple chemical messengers in the local microenvironment.[84-87]

Transforming growth factor-beta (TGF-β) is another angiogenesis promoting cytokine.[88] In addition to other tissues, it is found in the dentin matrix. It is anti-inflammatory, promotes wound healing, and has been used to promote mineralization of pulp tissue.[61,89] Fibroblast growth factor (FGF-2) is secreted from a wide range of cells and promotes proliferation of a variety of cell-types. It has been applied in regenerative endodontic procedures to increase stem cell numbers.[61,85] Leptin has been shown to exert atherogenic, thrombotic, and angiogenic actions on the vasculature.[90] Leptin is one of the two most-abundant, circulating adipokines that plays an important role in leading the endothelial cells to be proangiogenic.[90]

One family of enzymes that plays critical roles in multiple physiologic processes is the matrix metalloproteinase (MMP) family. MMPs aid in the development and morphogenesis of connective tissue and also function in the process of wound healing. However, when left unchecked, their activity has been implicated in multiple disease processes including tumor cell metastasis, atherosclerosis, and arthritis. Another family of molecules known as tissue inhibitors of metalloproteinases (TIMPs) play an important role in regulating the activity of MMPs.[91] There are four isotypes of the TIMPs[92] and types 1-3 have been shown to have inhibitory effects on angiogenesis.[92-94]

Interferon gamma (IFN-γ) has also been shown to inhibit angiogenesis *in vitro*. It achieves this inhibitory effect, at least in part, by opposing the action of platelet derived growth factor (PDGF).[95] PDGF, specifically heterodimer BB, has been shown to modulate endothelial proliferation and angiogenesis.[95] Another intracellular mediator, epithelial neutrophil-activating protein-78 (ENA-78), plays an important role (as its name suggests) in acute inflammation by binding to receptors on polymorphonuclear leukocytes (PMNs, neutrophils) to "mediate their spatially-localized activation."[97] However, studies have also demonstrated a strong correlation between higher ENA-78 levels and increased vascularity of certain tissues.[76, 98]

DYNAMATRIX®

Dynamatrix® is an extracellular matrix membrane derived from porcine small intestine submucosa (SIS). It is marketed to dental practitioners for use in "guided bone and guided tissue regeneration procedures."[99] According to information provided by the manufacturer (Cook Biotech, Lafayette, Indiana), "SIS is prepared by removing the tunica mucosa from the inner intestinal surface, and the serosa and tunica muscularis

from the outer intestinal surface. Further during processing with a series of surfactant and ionic solutions, the cells and nuclear matter are removed leaving behind a three-dimensional, acellular, collagen-rich extracellular matrix (ECM). The isolation and disinfection process preserves structural and functional bioactive molecules including, collagens (I, III, IV, VI) in their natural, three-dimensional states. This provides the framework for the infiltration of host cells and lasting strength during the remodeling process. Active growth factors such as fibroblast growth factor (FGF-2), connective tissue growth factor (CTGF), and transforming growth factor beta (TGF-β) promote angiogenesis, participate in vascular repair and development, and stimulate migration and proliferation of cells. Glycoproteins and other protein-carbohydrate complexes such as fibronectin, proteoglycans, and glycosaminoglycans can be found in the membranes. These serve as chemo-attractors, provide cell attachment sites in the extracellular matrix, and help to regulate the complex processes of cell migration, proliferation, and differentiation. Elastin is present in negligible amounts."[22]

With regards to its physical properties, the average pore size of the material is "~17 μm, with a range from roughly 10 μm to 50 μm. When the product is re-hydrated, the pores may be as large as 50 μm. It is extremely sensitive to the manufacturing process. Average thickness of SIS is 0.155 mm in the wet state. Dry product as provided collapses to about 0.100 mm."

The manufacturer further provides the following information concerning the safety of the product: "All of our devices are composed of porcine small intestinal submucosa (SIS). In order to ensure and maximize patient safety, Cook Biotech utilizes only healthy pigs raised at qualified and monitored producers and rendered at USDA-

certified meat packing facilities. All pigs are sourced in the United States. Further, the packing facilities must process only pigs, minimizing the potential cross-contamination with animal species known to have the potential for carrying transmissible spongiform encephalopathy. However, there are no known naturally occurring transmissible spongiform encephalopathy in pigs and cross-species transmission is highly unlikely."[99]

The aim of this *in-vitro* study was to determine if human dental pulp fibroblasts (HDPFs) seeded on DynaMatrix® membrane would alter their production of angiogenic cytokines.

HYPOTHESES

Null: There will be no significant alterations in angiogenic cytokine expression when HDPFs are seeded on the DynaMatrix® membrane when compared with the membrane or cells alone.

Alternative: HDPFs are seeded on the DynaMatrix® membrane will alter the expression of angiogenic cytokines when compared with the membrane or cells alone.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

HDPFs were used in this study. Groups were established as follows: (a) Group 1: HDPFs seeded in culture media only; (b) Group 2: DynaMatrix® membrane incubated alone in the serum-media without any cells, and (c) Experimental group: HDPFs seeded on DynaMatrix® membranes. After allowing 24 hours for cell attachment, serum minus media was added. Then, after 72 hours of incubation, the conditioned media was collected for cytokine analyses. The conditioned media were analyzed for the expression of 20 angiogenic cytokines (see Table I) utilizing cytokine arrays. The density of each cytokine expressed was measured, averaged, and statistically analyzed by ANOVA to determine the statistically significant differences among the groups with regard to the expression or presence of specific angiogenic cytokines.

HDPF CULTURES

The HDPFs had already been collected from healthy de-identified teeth extracted from patients for orthodontic treatment. These cells were then cultured and stored at 70°C. Indiana University Purdue University Indianapolis (IUPUI) Institutional Review Board (IRB) approval number 1408977286. Briefly, the teeth were transported from the clinic to the laboratory in phosphate buffered saline (PBS) solution. The extracted teeth were washed, cracked, and the pulp tissue was removed. The pulp tissue was minced into small fragments, placed in cell culture dishes, and grown in cell culture media. The cells

that grew out of the explants were sub-cultured and maintained as cell lines. HDPFs between passage 2 and passage 8 were used in the present study.

HDPF Treatment

HDPFs were fed and grown in the Dulbecco's Modified Eagle's medium (DMEM) cell culture media. Every three to four days, the cells were sub-cultured to avoid overcrowding on the plates. Cells were seeded (75,000) per well in 6-well plates. These cells were counted using a hemocytometer and a light microscope (Figure 7). The following experimental groups were tested and are shown in Figure 1:

(a) Group 1: HDPFs only (C),

(b) Group 2: DynaMatrix® membrane incubated without any cells (M).

(c) Group 3: HDPFs seeded on DynaMatrix® membranes (C+M).

Cells were given 24 hours to allow for attachment. After 24 hours, the serum plus media was removed and 2 ml of serum minus media was added to each well of the 6 well plates. After 72 hours of incubation at 37^{o}C , the conditioned media from the three different groups were collected and stored at -70^{o}C until analyzed.

Cytokine Arrays

The conditioned media from the various groups were used to test for the expression of multiple angiogenic cytokines. Equal volumes (1 ml) conditioned media were used for the cytokine analysis. The angiogenic cytokine profile from the HDPFs alone or HDPFs seeded on DynaMatrix® membranes or membrane alone were evaluated utilizing RayBio Human Angiogenesis Antibody Array I (Table I, RayBiotech Inc., Norcross, GA).[27] Briefly, the cytokine array membranes were blocked by 1.0-percent

serum for 30 minutes at room temperature and then incubated for three hours with 1 ml sample from each of the groups; two hours with biotin-conjugated antibodies provided by the kit, and then two hours with horseradish peroxidase-conjugated streptavidin. Detection agents supplied by the manufacturer were then added to each membrane for two minutes. The cytokines on the membrane were then visualized by autoradiography on x-ray film. With two dots for each type of the cytokine on each membrane, a total of four to six determinations for each cytokine were used for further semi-quantification. The density of the cytokines was measured with the Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Des Plaines, IL). The experiment was repeated three times and the averages were calculated.

Statistical Analysis

Multiple dots for each cytokine on the 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 had 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 results of the angiogenic cytokine arrays used in the study groups are presented in Table III. Membrane only (group M) was significantly higher than cells only (group C) for bFGF ($p = 0.0023$). Group C was significantly higher than group M for ANG ($p = 0.0003$), GRO ($p = 0.0104$), IL-6 ($p = 0.0104$), IL-8 ($p = 0.0104$), Leptin ($p = 0.0104$), MCP-1 ($p = 0.0003$), THPO ($p = 0.0065$), TIMP-1 ($p = 0.0254$), TIMP-2 (0.0300), and VEGF ($p = 0.0308$). Cells+Membrane (group C+M) was significantly higher than group C for bFGF (0.0249), GRO ($p = 0.0104$), IFN-γ ($p = 0.0249$), IL-6 ($p = 0.0104$), IL-8 ($p = 0.0104$), and Leptin ($p = 0.0104$). Group C was significantly higher than group C+M for ANG ($p = 0.0104$), MCP-1 ($p = 0.0104$), and THPO ($p = 0.0308$). Group C+M was significantly higher than group M for ANG ($p = 0.0104$), GRO ($p = 0.0003$), IFN-γ ($p = 0.0023$), IL-6 ($p = 0.0003$), IL-8 ($p = 0.0003$), Leptin ($p = 0.0003$), MCP-1 ($p = 0.0104$), TIMP-1 ($p = 0.0190$), TIMP-2 (0.0123), and VEGF ($p = 0.0065$).

The groups did not have significantly different p values for EGF ($p = 0.2042$), ENA-78 ($p = 0.6242$), IGF-1 ($p = 0.2817$), PDGF-BB ($p = 0.1424$), PIGF ($p = 0.2817$), RANTES ($p = 0.9670$), TGF-beta1 ($p = 0.6510$) and VEGF-D ($p = 0.1424$).

A ratio of the cytokine expression levels was calculated and is presented in Table II. The ratios of Group M and Group C+M against the control group, Group C, were determined. Overall, there was an increase in angiogenic cytokines in the significantly different cytokines when comparing group M with group C+M ratios over group C. Although there were differences in all 20 of the cytokines evaluated, the discussion and conclusions will focus only on the significantly different cytokines.

FIGURES AND TABLES

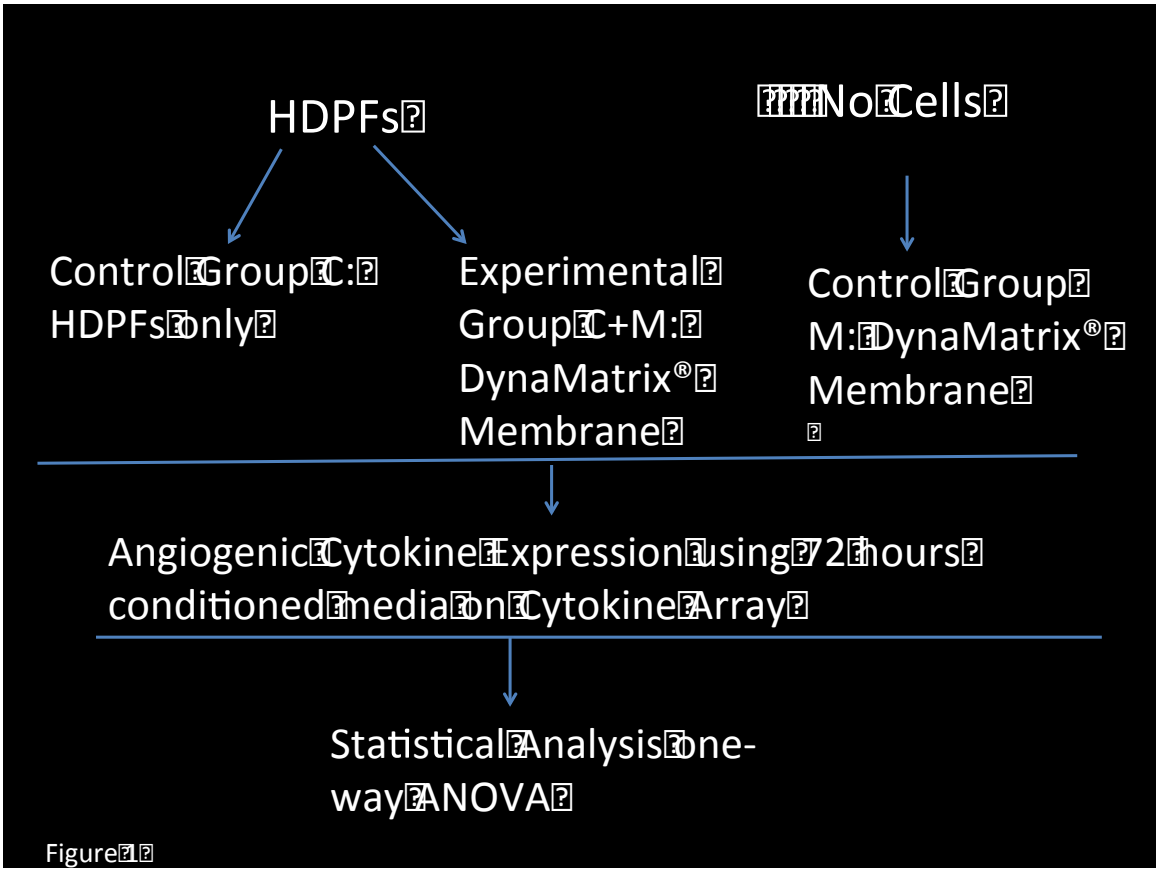


FIGURE 1. Experimental design overview.

FIGURE 2. DynaMatrix® membrane provided by manufacturer Cook Biotech Inc., West Lafayette, IN, and image of the membrane cut into circle to fit into one of the 6-well plates.

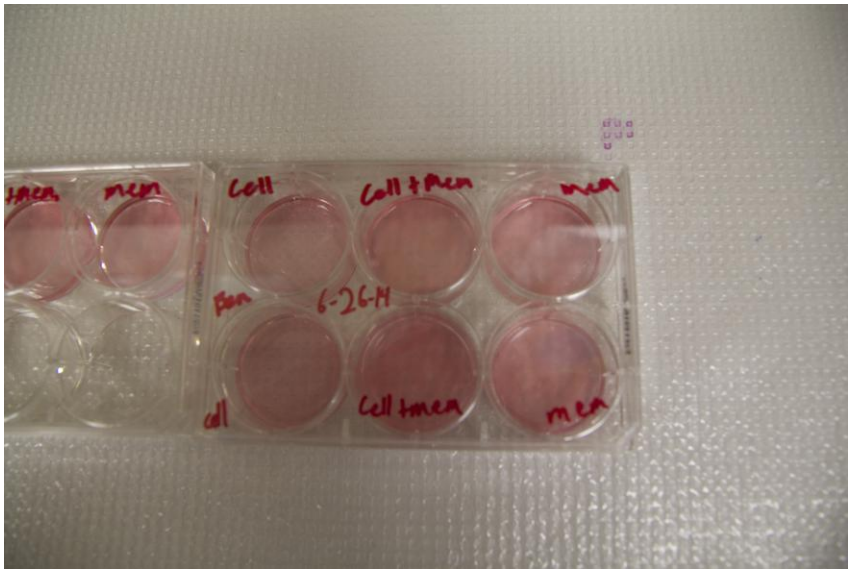


FIGURE 3. Six-well plate with the three experimental groups in all six wells.

FIGURE 4. Test tube rack with test tubes containing the supernatant collected from each of the three wells (HDPFs alone, DynaMatrix® alone, DynaMatrix® + HDPFs). The experiment was run three times resulting in 9 separate samples.

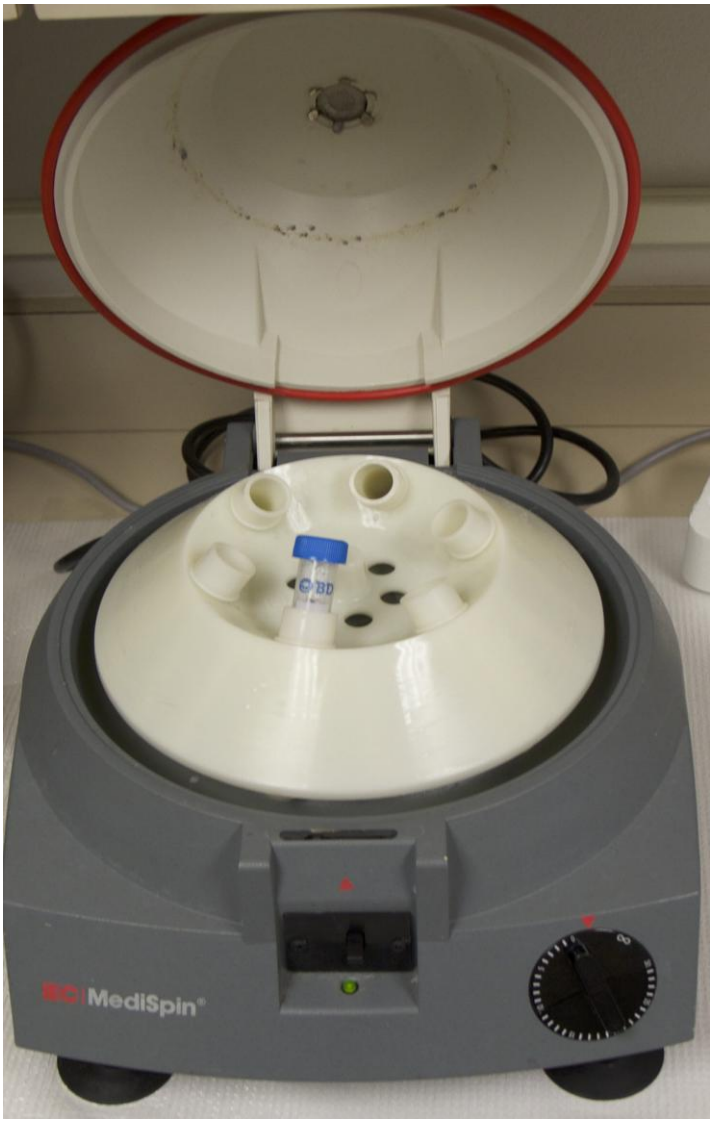


FIGURE 5. Centrifuge used to spin down cells into pellicle during cell growth and subculture phases.

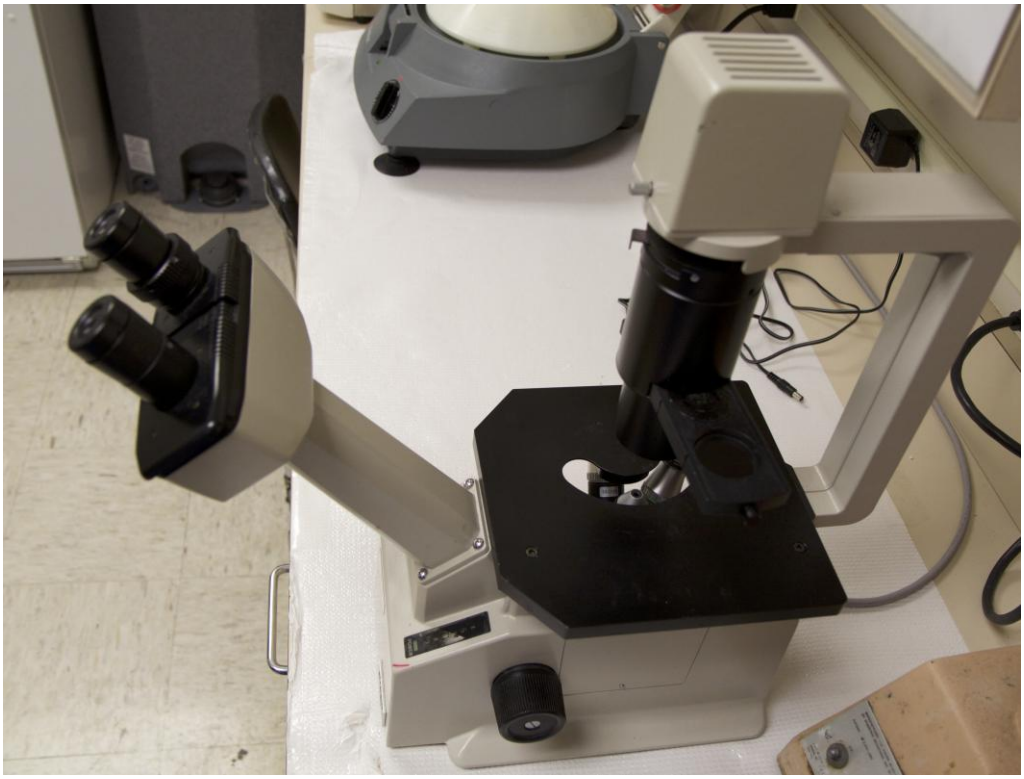


FIGURE 6. Light microscope used for counting cells.

FIGURE 7. RayBio® Human Angiogenesis Antibody Array 1 Kit. Above: closed box; below: open box.

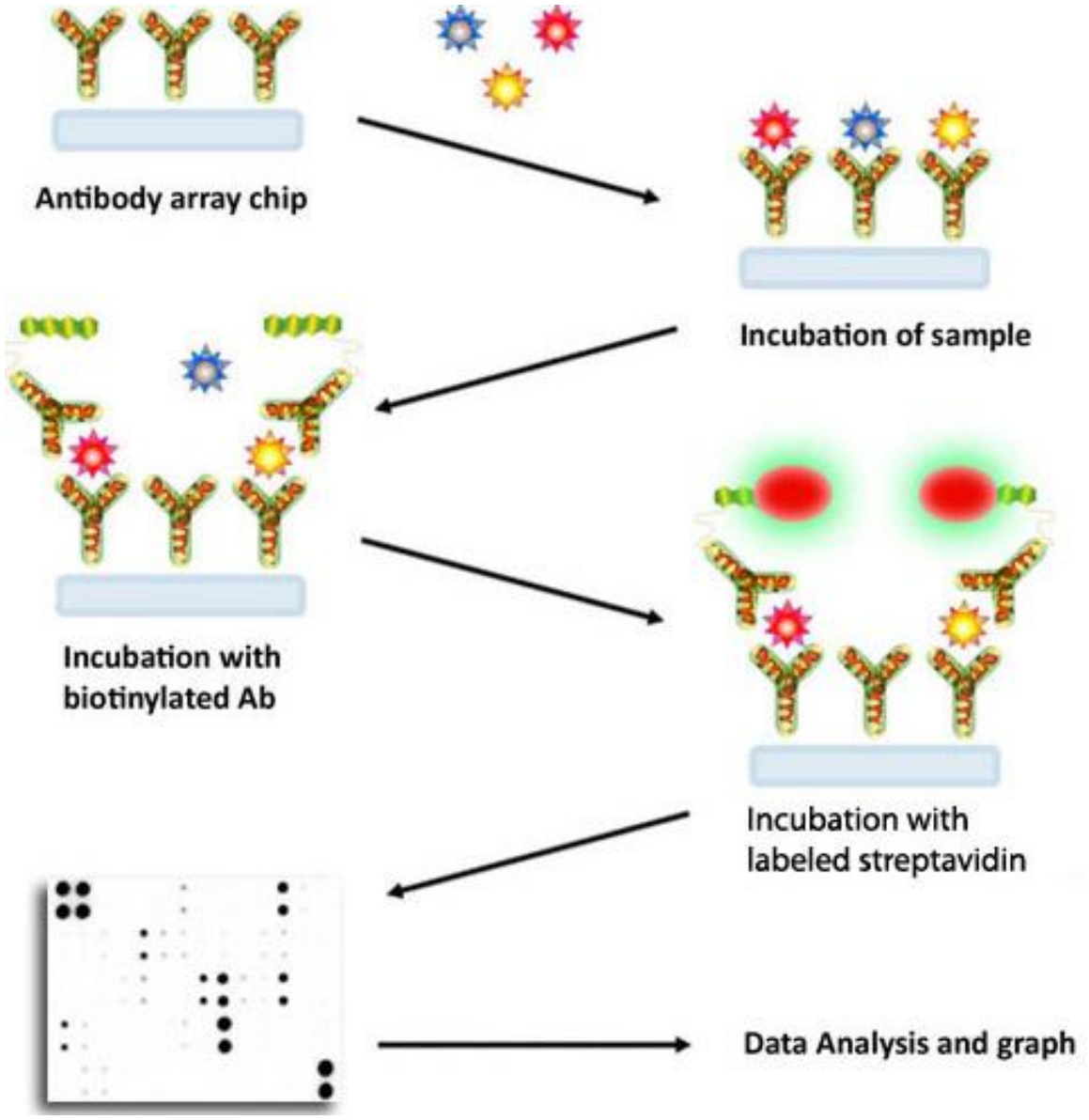
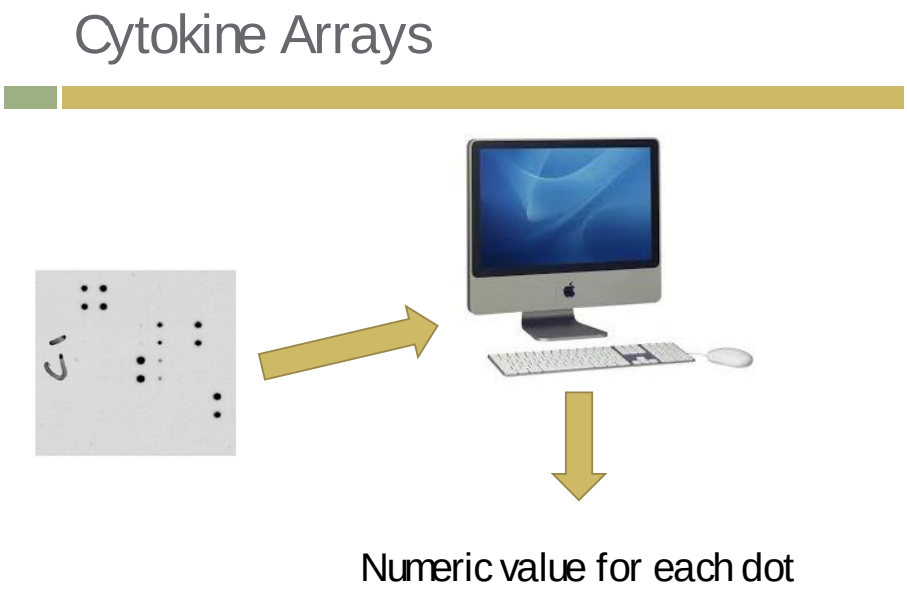
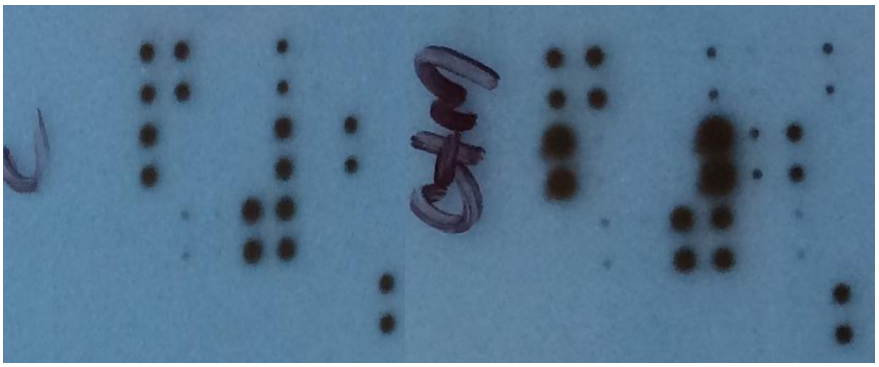


FIGURE 8. Schematic illustration of the steps involved in cytokine array analysis.





A B C

FIGURE 9. Images of autoradiography films from the various samples from this study. A: Membrane-only group; B: HDPFs-only group; C: DynaMatrix® + HDPFs group

TABLE I

RayBio® Human Angiogenesis Antibody Array 1 Map

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
2	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
3	GRO	IFN-□	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
4	GRO	IFN-□	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
5	PIGF	RANTES	TGF-□1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
6	PIGF	RANTES	TGF-□1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Neg	POS
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Neg	POS

Key to abbreviations: Angiogenin: aka ribonuclease 5, EGF: epithelial growth factor, ENA-78: epithelial neutrophil-activating protein 78, bFGF/FGF-2: 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 metallopeptidase inhibitor 1, 2, VEGF: vascular endothelial growth factor, VEGF-D: vascular endothelial growth factor D, POS: positive control, Neg: negative control.

TABLE II

Cytokine expression levels (Fold difference compared to control)

cytokine	M/C	CM/C
ANG	0.25	0.63
GRO	0.40	1.60
IFN-γ	0.50	1.71
IL-6	0.40	1.60
IL-8	0.40	1.60
LEPTIN	0.40	1.60
MCP-1	0.25	0.63
TIMP-1	0.32	1.05
TIMP-2	0.33	1.17
THPO	0.33	0.54
VEGF	0.35	1.29
bFGF	3.83	2.67

Key to abbreviations: C: Cell-only group, M: Membrane-only group, C+M: Cell + Membrane group. The highlighted numbers represent significant differences.

TABLE III

Mean for each cytokine ± Standard Deviation (s.d.) and p-values for comparisons*

Cytokine	C ± (s.d.)	M ± (s.d.)	C+M ± (s.d.)	C vs. M	C vs. C+M	M vs. C+M
ANG	3.1 ± 0.4x10^6	-0.02 ± 0.03x10^6	1.3 ± 0.1x10^6	0.0003	0.0104	0.0104
Bfgf	0.1 ± 0.08x10^6	1.3 ± 0.3x10^6	0.7 ± 0.2x10^6	0.0023	0.0249	0.0828
EGF	0.1 ± 0.4x10^6	0.06 ± 0.01x10^6	-0.001 ± 0.07x10^6	0.1435	0.1137	0.8719
ENA-78	0.1 ± 0.09x10^6	.05 ± 0.05 x10^6	-0.004 ± 0.04 x10^6	0.7895	0.3663	0.5114
GRO	6.7 ± 0.7 x10^6	0.1 ± 0.09 x10^6	13.9 ± 0.8 x10^6	0.0104	0.0104	0.0003
IFN-γ	0.1 ± 0.05 x10^6	0.003 ± 0.05x10^6	0.3 ± 0.01 x10^6	0.0828	0.0249	0.0023
IGF-1	0.08 ± 0.06x10^6	-0.04 ± 0.05x10^6	-0.01 ± 0.01 x10^6	0.1300	0.3071	0.5472
IL-6	0.1 ± 0.6 x10^6	-0.07 ± 0.03x10^6	1.4 ± 0.6 x10^6	0.0104	0.0104	0.0003
IL-8	7.1 ± 1.0 x10^6	0.5 ± 0.5 x10^6	21 ± 0.5 x10^6	0.0104	0.0104	0.0003
LEPTIN	0.1 ± 0.04 x10^6	-0.01 ± 0.02x10^6	1 ± 0.2 x10^6	0.0104	0.0104	0.0003
MCP-1	4.6 ± 0.2 x10^6	0.01 ± 0.04 x10^6	3.3 ± 0.4 x10^6	0.0003	0.0104	0.0104
PDGF-BB	0.09 ± 0.06 x10^6	-0.02 ± 0.05x10^6	-0.08 ± 0.04 x10^6	0.2029	0.0593	0.4061
PIGF	0.07 ± 0.01 x10^6	-0.019 ± 0.05x10^6	0.11 ± 0.049 x10^6	0.3071	0.5472	0.1300
RANTES	0.44 ± 0.09 x10^6	0.47 ± 0.1 x10^6	0.43 ± 0.10 x10^6	0.9009	0.9009	0.8038
TGF-ß1	0.49 ± 0.1 x10^6	0.32 ± 0.2 x10^6	0.51 ± 0.10 x10^6	0.4372	1.0000	0.4372
THPO	0.1 ± 0.02 x10^6	-0.005± 0.004x10^6	0.06 ± 0.01 x10^6	0.0065	0.0308	0.2488
TIMP-1	9.2 ± 0.8 x10^6	0.7 ± 0.7 x10^6	9.2 ± 0.5 x10^6	0.0254	0.8276	0.0190
TIMP-2	7.9 ± 0.9 x10^6	-0.01 ± 0.05x10^6	8.7 ± 0.9 x10^6	0.0300	0.5060	0.0123
VEGF	0.19 ± 0.03 x10^6	0.01 ± 0.06 x10^6	0.3 ± 0.08 x10^6	0.0308	0.2488	0.0065
VEGF-D	0.1 ± 0.04 x10^6	0.017 ± 0.04x10^6	-0.02 ± 0.02 x10^6	0.2029	0.0593	0.4061

*Highlighted numbers represent statistically significant difference $p < 0.05$.

DISCUSSION

Human dental pulp fibroblasts were capable of attaching and growing on DynaMatrix® *in vitro.* This is valuable information for possible future studies involving DynaMatrix® . This attachment characteristic of the membrane makes it a possible candidate for future use as an intra-canal scaffold for regenerative endodontic procedures.

The results of this *in-vitro* study support the claim of the manufacturer that DynaMatrix® contains "biological signals such as growth factors."[99] One of the cytokines in the membrane is fibroblast growth factor (FGF-2). The most significant increase across all of the cytokines that were evaluated was seen with basic fibroblastic growth factor (bFGF or FGF-2). bFGF was present in statistically significant higher levels in the supernatant from the DynaMatrix-only group when compared with the HDPFs-only group. The only possible conclusion for the increased bFGF levels in the supernatant of the DynaMatrix-only group, given the fact that no cells were present, is that the pro-angiogenic cytokine bFGF was released from the membrane itself. With the exception of bFGF, the levels of expression of the statistically significant other cytokines were actually higher in the HDPFs-only group as compared with the Dynamatrix-only group.

In addition to increasing the levels of bFGF from the DynaMatrix® into the supernatant, DynaMatrix® appears to have had an effect on the expression of vascular endothelial growth factor (VEGF) from the HDPFs. When the fold difference as compared with the control, or ratios of the two groups containing Dynamatrix-only and Dynamatrix® + cells were calculated, the increase in VEGF was significant (see Table

III). This implies that the increased levels of VEGF in the experimental group were not due simply to additive effects between the cytokine molecules from the DynaMatrix and those from the HDPFs, but rather that DynaMatrix actually had some sort of stimulatory effects that resulted in greater VEGF expression from the HDPFs. As Imada About stated, VEGF exerts strong chemotactic effects on human pulp cells in a dose-dependent manner.[2] Thus the increase of the cytokine VEGF would draw more pulp cells, endothelial cells, and fibroblasts to the area. Once present, they could begin to form thin cords of interconnecting cells, and exhibited elongations and branching to form a network of capillary-like structures corresponding to neoangiogensis.[100]

Another interesting finding was that the level of bFGF decreased from a ratio of 3.83 Dynamatrix-only as compared with cells only to a level of 2.67 in the experimental group (Dynamatrix+HDPFs) as compared with cells only, which is a 30-percent decrease. One explanation is that the bFGF supplied by the Dynamatrix® was metabolized by HDPFs. Another explanation is that the bFGF is binding to the cell surface. This is a positive discovery as About states "FGF-2 has been shown to enhance pulp cell proliferation."[2] This growth factor was expected to play a very important role in the current study, because fibroblasts were used. FGF-2 also plays roles in cell proliferation and differentiation important for wound healing and angiogenesis.[72]

The cytokine leptin was found to be significantly higher in the experimental group (DynaMatrix + HDPFs) as compared with both the HDPFs and DynaMatrix® groups. Leptin has been shown to exert atherogenic, thrombotic, and angiogenic actions on the vasculature.[90] Leptin is one of the two most-abundant, circulating adipokines that plays an important role in proangiogenic/proatherogenic factors in endothelial cells.[90]

Adipokines are cytokines that are secreted by adipose tissue. It is interesting to note that both *in-vivo* and *in-vitro* studies have demonstrated the activation of endothelial receptors by leptin, thus leading to capillary tube formation, a prerequisite for angiogenesis.[101]

Adipokines such as leptin have been found to up-regulate key proangiogenic molecules like the gelatinases (MMPs, MMP-2/-9).[90] Additionally, leptin has been shown to up-regulate and act synergistically with the key angiogenic mediators like FGF-2, VEGF, and its receptor VEGFR1, stimulating vascular permeability, consequently resulting in functional angiogenesis.[90] This is of particular interest, because in the present study bFGF (FGF-2) and VEGF were significantly higher in the experimental group (DynaMatrix + HDPFs), which supports the claim that leptins up-regulate other key angiogenic cytokines. These findings are conducive for new blood vessel growth.

A different mechanism appears to have altered TIMP-2 expression. TIMP-2 levels were significantly higher in both the HDPFs-only group and the experimental group (DynaMatrix + HDPFs) as compared with the DynaMatrix-only group. The expression of TIMP-2 from the HDPFs was actually not statistically different from the experimental group (DynaMatrix + HDPFs). There was no expression of TIMP-2 in the DynaMatrix-only group, as shown in Table III. Although the HDPFs express TIMP-2, the DynaMatrix did not alter this in a negative way. There are four isotypes of the TIMPs[92] and TIMPs 1-3 have been shown to have inhibitory effects on angiogenesis.[92-94] The increased levels of TIMP-2 and THPO, due to the HDPFs, both of which are inhibitors of angiogenesis, were not potentiated by the DynaMatrix®. It should be considered that angiogenesis is a complex process in a local micro-environment and that addition of other cell types might alter the environment. An unpublished study on human dental stem cells described a pro-

angiogenic environment with decreases in TIMP-1 and TIMP-2 (Baker, unpublished data).

Although the interaction between the HDPFs and DynaMatrix® resulted in some anti-angiogenic cytokine profiles, the most important angiogenic cytokines, bFGF and VEGF, for vascularization were increased in this study.[2] For example, bFGF is present in DynaMatrix® and appears to promote expression of bFGF from HDPFs or cleavage of the membrane releasing more bFGF. bFGF is secreted from a wide range of cells and promotes proliferation of a variety of cell-types. It has been applied in regenerative endodontic procedures to increase stem cell numbers, as well as interact with endothelial cells to form capillaries.[61,85,100]

IL-8 levels were found to be significantly higher in HDPFs only group and the HDPFs seeded on DynaMatrix® compared with DynaMatrix® only group. IL-8 has been shown to play a role in angiogenesis, sometimes promoting and sometimes inhibiting angiogenesis, depending upon the interplay between multiple chemical messengers in the local microenvironment.[85-87] However, IL-8 is also affiliated with inflammatory processes and has been shown to even play a "causative role in acute inflammation by recruiting and activating neutrophils."[102] A state of inflammation is generally considered detrimental to the success of regenerative endodontic procedures, especially in the case of teeth that have undergone trauma. In these teeth, damage to the predentin and precementum layers can lead to root resorption in the presence of inflammation.[103]

The measured MCP-1 levels were highest in the HDPFs only group, significantly lower in the experimental group (DyanMatrix + HDPFs), and still significantly lower in

the DynaMatrix® only group. These results exhibit a cytokine profile that would favor the goals of endodontic regenerative procedures when HDPFs are used to supplement a pro-angiogenic profile that is lacking in the DynaMatrix® membranes. MCP-1 has been shown to promote angiogenesis in multiple studies[83, 104-106]; though the exact mechanism by which it achieves this is not completely understood.[107] Furthermore, one study demonstrated novel evidence that the mechanism of MCP-1–induced angiogenesis is mediated through pathways involving VEGF and the activation of RhoA small G protein.[83] However, like IL-8, MCP-1 has been shown to be an important mediator of inflammation,[108] which may pose an obstacle to regeneration.

The preceding discussion highlights generalities concerning the roles of specific cytokines, but it is important to recognize that the effects of individual cytokines are complicated by various factors. *In vivo*, cytokines may have different effects depending upon their target cell types. Their effects can also be dose-dependent. Therefore, the findings of an *in-vitro* study have to be interpreted accordingly. In addition, many of the studies involving research into the roles of various cytokines in the process of angiogenesis use animal models and focus on tumor angiogenesis in various parts of the body other than the jaw.[105,106,109,110] The findings of these studies may involve important differences from the specific clinical situation involved in regenerative endodontic procedures.

One issue that affects the feasibility of regenerative endodontic procedures is the degradation or incorporation of the scaffold. The rate at which the scaffold resorbs should mimic as closely as possible the rate of tissue formation.[61] Collagen has been suggested as a possible intracanal scaffold for regenerative endodontic procedures.[69] A recent study

involving revascularization of rat teeth found that an injectable Type 1 collagen scaffold was replaced after eight weeks by vascularized connective tissue, thereby "confirming its biodegradability and potential use as a scaffold for pulp cell engineering."[74] Several studies have investigated techniques for treatment of bone defects in a rat model utilized SIS membranes.[111,112] The bone defects in the rat femurs were treated with bone allograft seeded with mesenchymal stem cells and growth factors. This was then wrapped with SIS that had been seeded with mesenchymal stem cells. The analysis of the SIS membrane demonstrated its complete resorption by 9 weeks.[112] As DynaMatrix® is an SIS membrane that contains collagen, it would likely exhibit a similar rate of resorption (8-10 weeks) as that found in the two previously mentioned studies, which would be appropriate for regenerative endodontic procedures.[74] As discussed above, another critical issue influencing the outcomes of regenerative endodontic procedures is the elimination of microbes from the root canal space. To this end, most suggested protocols recommend copious irrigation with NaOCl followed by placement of an intracanal medicament to maximize microbial elimination. Nevertheless, recent research indicates that even the most meticulous efforts to eradicate microbes from the root canal system are never completely successful.[113,114] In light of this reality, various authors have encouraged the development of a scaffold that contains an antimicrobial component for use in regenerative endodontic procedures.[61,64,69] DynaMatrix® has no documented antibiotic effects, so the addition of an antibiotic to the membrane or simultaneous use of the membrane with an intracanal antibiotic solution or paste would likely increase its appeal for use in regenerative endodontics.

Potential research could include a similar study design to the one utilized in this investigation, but could substitute different cells that are known to play an important role in angiogenesis, such as endothelial cells. Further clinical relevance could be achieved by studying the interaction between DynaMatrix® and a mixed population of various cell types, rather than a single cell type. The expression of different cytokines could also be studied readily as the manufacturer offers custom-made cytokine array membranes that can be designed to screen for specific cytokines as requested by the researcher.[115] Ensuing animal studies and human *in-vivo* studies could be expedited by the fact that DynaMatrix® already has a long track record of safe clinical use in humans.

SUMMARY AND CONCLUSIONS

The HDPFs utilized in this study remained viable after being seeded on DynaMatrix® in the presence of culture media *in vitro*. The null hypothesis was rejected as statistically significant differences in the expression of angiogenic cytokines were demonstrated between the experimental group (DynaMatrix® + HDPFs) when compared with the control groups (HDPFs-only or DynaMatrix-only). Specifically, bFGF was highest in the (DynaMatrix® only group), lower in the experimental group (DynaMatrix + HDPFs), and lowest in the (HDPFs-only group). In addition, tissue inhibitor of matrix metalloproteinases-2 (TIMP-2) and thrombopoietin (THPO) were significantly higher in the (HDPFs only group) than in the (DynaMatrix-only group). The levels of TIMP-2 and THPO did not significantly increase in the experimental group (DynaMatrix® + HDPFs). Both TIMP-2 and THPO are inhibitors of angiogenesis. Thus the findings support that by adding the DynaMatrix® membranes did not increase the anti-angiogenic cytokines TIMP-2 and THPO. Statistically significant differences in the levels of other cytokines were detected among the groups, but those particular cytokines were present in such small quantities that they were deemed highly unlikely to result in any biological and clinical effects. These cytokines were IL-6, IL-8, IFN-gamma and GRO.

DynaMatrix® could potentially improve the regenerative endodontic procedure by providing a predictable scaffold for cellular and vascular in-growth into the root canal system. As shown in this current study, DynaMatrix® supported HDPFs viability. Additionally, it may improve angiogenesis by increasing the quantity of pro-angiogenic cytokines present in the local microenvironment such as bFGF, VEGF and leptin. This

would be a result of the released cytokines present in DynaMatrix®, as well as possible interactions with the cells involved in regeneration that result in greater expression of angiogenic cytokines from these cells. Lastly, it may aid endodontic regenerative procedures by down-regulating the expression of potential inhibitors of angiogenesis such as THPO and TIMP-2.

Cytokines such as bFGF, ANG, VEGF and leptin promote angiogenesis or will stimulate migration and proliferation of cells. The cytokine expression profile from the cells seeded on DynaMatrix® suggests that it might be a suitable scaffold for regenerative endodontic procedures. It could improve vascularization by increasing angiogenic cytokines in the microenvironment of the disinfected root canal space, creating capillary networks and support tissue regeneration.

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ABSTRACT

EFFECTS OF DYNAMATRIX® ON ANGIOGENIC CYTOKINE EXPRESSION FROM HUMAN DENTAL PULP FIBROBLASTS: AN *IN VITRO* STUDY

by

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Introduction: An exogenous scaffold may lead to more predictable pulp tissue regeneration and continued root formation in a regenerative endodontic procedure. DynaMatrix® is a natural membrane scaffold made of porcine small intestine, currently used in periodontal regenerative surgeries.

Objective: The purpose of this study was to investigate if human dental pulp fibroblasts (HDPFs) seeded on DynaMatrix® membrane would result in an increase in the expression of angiogenic cytokines. Materials and Methods: HDPFs (75,000 per well) were seeded in 6-well plates. Three groups were tested: Group 1 (C): HDPFs in

media only; Group 2 (M): DynaMatrix® (Cook Biotech, Indianapolis, IN) alone in media; and Group 3 (C+M): HDPFs seeded on DynaMatrix® membranes. After 72 hours of incubation in serum positive, the conditioned media were collected and analyzed for the expression of 20 angiogenic cytokines utilizing RayBiotech Inc., arrays per the manufacturer's instruction. The data were analyzed by ANOVA.

Results: Group M was significantly higher than C for bFGF ($p = 0.0023$). C+M was significantly higher than M for ANG ($p = 0.0104$); GRO ($p = 0.0003$); IFN-γ ($p = 0.0023$); IL-6 ($p = 0.0003$); IL-8 ($p = 0.0003$); Leptin ($p = 0.0003$); MCP-1 ($p = 0.0104$); TIMP-1 ($p = 0.0190$); TIMP-2 (0.0123). C was significantly higher than C+M for ANG ($p = 0.0104$); MCP-1 ($p = 0.0104$); and THPO ($p = 0.0308$). Cytokines such as b-FGF, ANG, and leptin promote angiogenesis, and stimulate migration and proliferation of cells.

Conclusion: The cytokine expression profile from the cells seeded on DynaMatrix® suggests that it might be a suitable scaffold for regenerative endodontic procedures. It could improve vascularization by increasing angiogenic cytokines in the microenvironment of the treated root canal and supporting tissue regeneration.

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