EFFECTS OF DYNAMATRIX® MEMBRANE ON ANGIOGENIC CYTOKINE EXPRESSION FROM HUMAN DENTAL PULP STEM CELLS

by

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INTRODUCTION

Historically, damage to human tissues and organs has been treated with the use of prostheses or transplants. 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]

As material science has evolved, better and more durable synthetic materials (e.g., Teflon and silicone) have been used to replace or rebuild diseased tissues or parts of the human body. While these newer materials offer some superior characteristics compared with older materials, their physical and mechanical properties are still quite distinct from the original human form, and they supply more of a structural rather than a functional replacement of the original tissue.[2]

As the study of cell biology has progressed, the field of tissue engineering has evolved.[3] Tissue engineering is the field of functional restoration of tissue structure and physiology for impaired or damaged tissues because of cancer, disease, or trauma.[4] The field exploits living cells in various ways to maintain, enhance, or restore tissues and organs,[5] and in the process it utilizes and integrates principles from various other fields such as cell transplantation, material science, and engineering.[2]

An outgrowth of the increased knowledge in the field of tissue engineering has been the development of a scientific discipline termed "regenerative medicine."[3] Regenerative therapy involves the combined interplay of three key elements: stem cells, some type of scaffold, and cytokines.[2] Regenerative therapies have become an alternate

method for restoration of tissues and organs instead of autografts, allografts or artificial prostheses.[6]

Tissue engineering and regenerative therapies have shown promising results in the field of dentistry. Tissue engineering approaches can aid in either the replacement of damaged tooth structures or in the repair/regeneration of the pulp-dentin complex (regenerative endodontics).[2]

Regenerative endodontic procedures are currently performed to treat infected immature teeth utilizing the "revascularization method."[7] This method includes two steps: disinfection of the root canal followed by the laceration of apical tissues to induce blood flow into the root canal space (RCS). The blood clot formed in the canal serves as a fibrin scaffold for the accumulation of stem cells and other cells with dentin/root formation capability.[8] This protocol has resulted in continued root formation (in length and width) [9] in certain clinical cases. However, the adequacy of hemorrhage induced in the apical tissues has a significant impact on the quality of the regenerated dental structures. [10] This phenomenon indicates that the blood clot itself may play an important role in continued root formation by locally releasing molecular signals, which induce angiogenesis. Successful angiogenesis is then crucial for the vitality of the newly regenerated pulp tissues and for the initiation of continued root development. Therefore, the production of angiogenic cytokines from the local surviving cells or the effective delivery of exogenous angiogenic cytokines to the desired area is one of the key components in regenerative endodontic treatment.

It is believed that an appropriate exogenous scaffold could aid the endogenous elements (fibrin meshwork and molecular signals in blood clot) and result in more

predictable regenerative endodontic procedures.[11] Platelet-rich plasma (PRP) has been reported recently as a scaffold material whose usage has met with mixed results.[12-14] Investigators continue to search for materials that can serve as better scaffolds.[15] To accomplish the structural and functional replacement of the target tissue, the ideal scaffold must facilitate the growth of the requisite cell population (by such processes as attachment, migration, proliferation, and three-dimensional spatial organization). The two categories of materials that are most commonly used in tissue engineering are synthetic polymers such as poly(lactic) acid (PLA) and poly(glycolic) acid (PGA), and natural matrices derived from biological sources such as reconstituted collagen.[16] Extracellular matrix (ECM) membranes belong to the second category and are indeed biological membranes that are used in regenerative procedures. ECM membranes used in clinical or research studies are usually derived from porcine organs such as the urinary bladder or small intestine. After "decellularization and terminal sterilization,"[17] the ECM becomes a membrane that possesses structural and functional proteins (collagens types I, III, IV, and VI, glycosaminoglycans, glycoproteins and proteoglycans)[18] in their native three-dimensional organization, but devoid of species-specific cellular components that would trigger an immune response in the host. The preservation of the natural three-dimensional organization of the molecules in the membrane distinguishes ECM scaffolds from synthetic scaffolds and might contribute to "constructive tissue remodeling instead of scar tissue."[17]

DynaMatrix® is a biological ECM scaffold product that is marketed to dental practitioners. In addition to the structural and functional proteins mentioned above, DynaMatrix contains biological signals such as cytokines.[17] In dentistry, DynaMatrix has

been used for periodontal restorative procedures and for alveolar ridge preservation.[19] Its track record of successful use in periodontal procedures encourages investigation of the material for use in regenerative endodontic procedures. Specifically, DynaMatrix may be placed within the root canal system of the immature tooth to facilitate the continued development of the entire length of the root from the apical foramen to the level of the dentin enamel junction (DEJ). As a scaffold containing biological signals, DynaMatrix has the potential to induce angiogenesis and encourage the migration of cells that are involved in the continued development of the root canal system. This continued development of the root length and width would be particularly valuable in the cervical area of the tooth, which is prone to fracture when underdeveloped and is an area to which cells involved in continued root formation may have difficulty migrating without the aid of a scaffold.

The aim of this current study was to determine if the exposure of human dental pulp stem cells (HDPSC) to the DynaMatrix membrane will result in an increased production of angiogenic cytokines that are critical for pulp/root regeneration. Angiogenesis cytokine arrays have been established as a viable method for assessing expression of cytokines.[20] HDPSC were chosen as they are expected to be found in the apical papilla and the infected immature root canal system of teeth that current regenerative endodontic techniques are designed to treat. [21]

CLINICAL SIGNIFICANCE

If the experimental group reveals significantly different expression levels of the angiogenesis cytokines that would be conducive to pulp regeneration or root formation, further experimentation might establish the DynaMatrix membrane as a potentially beneficial scaffold for use in regenerative endodontic procedures.

HYPOTHESES

Null Hypothesis

No statistically significant differences in the expression of angiogenic cytokines will be demonstrated between the experimental group and the control groups.

Alternative Hypothesis

Statistically significant differences in the expression of angiogenic cytokines will be demonstrated between the experimental group (HDPSC seeded on DynaMatrix) when compared with the control groups (untreated cells or the DynaMatrix membrane alone without cells).

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

In 1687 Charles Allen published a book credited with being the first book in English devoted exclusively to dentistry.[22] This volume contained a description of "taking out the rotten teeth or stumps and putting in their places some sound ones drawn immediately out of some poor body's head."[23]

In 1728 Pierre Fauchard wrote *The Surgeon Dentist,* which provided accurate descriptions of root canals and pulp cavities of various teeth. The book also described the practice of relieving abscesses and draining pus by creating access holes in teeth which were left open for two to three months. Later, the pulp chamber was filled with lead foil.[22] In addition to drainage procedures, Fauchard described a pulp extirpation procedure using a small pin and a treatment for deep carious lesions, which involved application of the oil of cloves or cinnamon to the affected area for several weeks.[22]

The German dentist Phillip Pfaff (whose patient pool included Frederick the Great) mentioned a pulp-capping procedure in 1756, which involved cutting out a piece of gold or lead to fit approximately the shape of the opening over the pulp. The metal was shaped so as to be concave to avoid direct contact with the pulp.[22]

Louis XV's dentist, Bourdet, described in 1757 a process of extracting carious teeth, filling the root canals with gold or lead, and then replanting them. He also described an "intentional" endodontic treatment involving dislocation of a symptomatic tooth in order to sever the nerve, followed by immediate replacement of the tooth into its socket.[23] Bourdet was not the first to treat symptomatic teeth in this manner; the same

procedure had been described as early as the 11th century AD by the Arabian physician Avicenna.[24]

Robert Woofendale, an English practitioner who came to New York in 1766, is credited with performing the first endodontic procedures in the US. He sought to alleviate dental pain by cauterizing the pulp with a hot instrument, followed by placement of cotton pellets into the canals.[25,26] The German dentist Frederick Hirsch wrote toward the end of the 18th century about diagnosing dental disease 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.[27]

By the early 19th century, ideas were beginning to emerge concerning the importance of tooth vitality and how this concept related to pulp treatment. This time period has been dubbed "The Vitalistic Era" by some.[22] 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.[28] Building on this idea, an Irish clinician by the name of Edward Hudson, who practiced in Philadelphia, is typically given credit for being the first to place fillings in root canals in 1809. He designed his own instruments, which he used to pack gold foil in the canals.[25,29] Hudson was innovative for his time in that he advocated the preservation of natural dentition.[30]

John Callow in his work *Opinions on the Causes and Effects of Diseases in the Teeth and Gums* (1819) 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. Thus, Bew joined others of his time who subscribed to the "vitalistic theory"

of teeth.[28] One of Bew's contemporaries, a German immigrant named Leonard Koecker, wrote *Principles of Dental Surgery* in 1826. This book became a standard in the field for 50 years.[30] Koecker believed that destruction of the pulp would cause the whole dentinal core of the tooth to immediately die, thereby rendering the tooth a foreign body requiring extraction. To avoid this fate for the tooth, Koecker popularized the pulp capping procedure (essentially the same procedure described by Pfaff in 1756).[28,31,32]

In his book *System of Dental Surgery* published in 1829, SS Fitch formulated and presented the principles of the "vitalistic" or "double membrane" theory.[22] He advocated 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. With this model in mind, Fitch believed that the crown was nourished exclusively by the dental pulp or its membrane, whereas the roots were supplied by the pulp membrane on the interior and by the alveolar membrane on the exterior.[22] This concept led to the practice of removing the crown of teeth after extirpation of the pulp (since its source of sustenance had been removed), while 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.[28,32]

An opposing philosophical camp advocated a "nonvitalistic"[22] concept. The esteemed British surgeon and anatomist John Hunter believed that dentin possessed none of the properties of living tissue and was devoid of circulation, sensibility and capability of repair. Hunter was joined by Cuvier and Robertson from England, who were also proponents of the nonvitalistic theory.[28]

A significant change in the way vital pulp extirpations were performed occurred

in 1836 when Shearjashub Spooner of New York used a protoplasmic poison (arsenic trioxide) to devitalize the pulp before removing it.[25] Though novel for modern Western medicine, this approach was actually not original but actually dates back to ancient Chinese medicine when it was described as a treatment for jaw abscesses.[24] This treatment immediately became popular due to its success at practically eliminating the pain involved in the removal of vital pulps. Unfortunately, this also led to imprudent use 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.[33]

In 1837 Jacob and Joseph Linderer (father and son) advocated using essential or narcotic oil as a pulpal obtundant prior to restoring a tooth with an exposed pulp.[34] In 1838 Edwin Maynard developed a root canal broach by filing a watch spring, and also designed hoe-like instruments for shaping and enlarging root canals.[35] In 1839, Baker described a treatment for an exposed nerve that included removal of the nerve, followed by cleaning the canal and filling the canal with gold foil. He is commonly credited with writing the first published account of pulpal extirpation, canal cleaning, and root canal filling.[22,24]

Throughout the 1850s, plugs of wood soaked in creosote were used as root canal filling material.[22] Liquid cement was used in conjunction with the wooden plug to create a seal. This early sealer contained gutta-percha, quick-lime, powdered glass, feldspar, and metal filings. This concoction was also used as a temporary restoration material.[33]

In 1857, Thomas Rogers gave a presentation on pulp capping at a meeting of the Odontological Society of London. He reported on a series of 220 pulp capping cases,

202 of which were considered successful. Rogers described the conditions that would lead to the success of the 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 use of caustics to decrease the pain. Rogers' overall assessment and treatment recommendations could hold up under modern scrutiny, except for the final recommendation in his protocol in the case of pulp-capping failure: the application of three leeches and a laxative.[22]

Later innovations in the 19th century brought endodontic treatment ever closer to modern techniques. In 1864 S.C. Barnum of New York introduced the use of the rubber dam to isolate the tooth during the placement of gold foil restorations. It was quickly adopted for use in endodontic procedures to provide a more aseptic environment for treatment.[24,25] G.A. Bowman of Missouri is generally given credit for being the first to use only gutta-percha for filling root canals in 1867.[25,29,36]

Also in 1867 (the first year Joseph Lister described using carbolic acid as an antiseptic for surgical procedures), 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. 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.[22,34] In this same year, Magitot suggested using an electric current for pulp testing.[31]

By the late 1870s, the theory of vitalism was beginning to give way to the septic theory, which asserted that pathogenic organisms were the most common cause of

diseases of the pulp. This theory was advocated by G.O. Rogers in 1878 and Charles S. Tomes in 1879.[28] In 1882 Arthur Underwood further developed 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.[22] This idea provided new justification for pulp therapy procedures that included caustic germicides for bacterial elimination, a practice that remained common for more than 30 years.[28]

In 1895, Dr. Bowman of Missouri introduced a solution of chloroform and gutta-percha, which was appropriately named chloropercha. It was used with gutta-percha cones for obturation of root canals. This technique quickly gained wide acceptance, and one of its chief advocates was M.L. Rhein of New York, who further developed and described the technique several years later.[25]

Thomas Alva Edison, one of America's greatest inventors, discovered in 1898 that calcium tungstate could be used to fabricate fluorescent dental mirrors that could be used to observe pathologic conditions and endodontic results. However, the device exposed clinicians and patients to excessive amounts of radiation, an appreciation of which finally led to the declined usage of the mirrors in the 1930s.[30]

In 1905, Einhorn developed procaine (Novocaine), which provided an alternative to the previously used cocaine (which had been used as an anesthetic agent for 20 years, but only sparingly due to its high toxicity). Though it represented a promising breakthrough in dental pain control, the wide usage of procaine 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 user-friendly block anesthesia techniques to be perfected.[33,35,37]

Dr. Meyer L. Rhein, a physician-dentist from New York, developed a technique in 1908 for determining canal length and degree of obturation that utilized a diagnostic wire in conjunction with radiographs.[25,31] G.V. Black also suggested measurement control to determine the length of the canal and the size of the apical foramen to avoid overfilling.[22] In the second decade of the 20th century, ideas began percolating in the medical and dental community that would eventually be synthesized into what is now referred to as the "Focal Theory of Infection" (or Theory of Focal Infection) that postulates a myriad of diseases caused by microorganisms (bacteria, fungi, viruses) that arise endogenously from a focus of infection."[38] Though this concept was not truly new (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),[39] it experienced a resurgence due in part to E.C. Rosenow, a protégé of Frank Billings, (a name commonly affiliated with the popularization of this theory)[38] who did work in 1909 showing 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.[37]

Another major event that propelled the Theory of Focal Infection to a state of widespread acceptance[22] occurred in October of 1910, when British physician and pathologist William Hunter 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*, a respected medical journal of the time.[22]

The widespread popularity of the Theory of Focal Infection led to increased scrutiny on endodontic procedures. Many physicians and dentists began to recommend

extraction of all endodontically treated teeth (the 100 percenters). Others recommended removal of all non-vital teeth and yet others suggested that all teeth, whether diseased or not, be removed for the sake of prevention, as well as treatment ("therapeutic edentulation" or "the clean-sweep").[38,40]

The philosophy of the 100 percenters became the most popular approach to dental practice and this persisted up until approximately 1930 when the pendulum began to swing back toward more conservative approaches.[22] This change in popular sentiment included a refocusing on such things as definitive diagnosis, aseptic techniques, bacteriological culturing, and improved radiographic practices in conjunction with root canal therapy.[41] These ideas were not widely adopted immediately, however, and it would take approximately a decade before this more conservative approach to the treatment of dental disease would take hold generally.[22]

In the year 1937, multiple advances ushered in a period referred to by some as "the scientific era."[22] In that year, work by Logan showed that bacteria can be present in normal tissues without having a pathological effect,[26] and Tunnicliff and Hammond found that microorganisms could be found in the pulps of extracted teeth "without any evidence of inflammatory tissue changes."[22,37] Also in 1937, 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 information prompted Burket to conclude that any improvement following the removal of bacterial foci was more likely the result of an associative relationship"[22] between the foci and the disease rather than a causative one.[42] The work of these and other men effectively sounded the death knell for the formerly widespread practice of wholesale extraction of distressed or non-

vital teeth.[22]

In the early 1940s, Fred Adams and Louis Grossman advanced the idea of utilizing antibiotics as an adjunct to root canal therapy. Adams reported using sulfanilamide in treating periapical infections and is credited as the first to use penicillin in pulp canal therapy.[22,37] Grossman advocated use of a non-aqueous formulation of penicillin for use in endodontics citing stability of the compound decreased cost as advantages over the conventional form. Dr. Grossman would later use penicillin-laced paper points to disinfect root canals.[29]

The year 1943 marked the dawn of organized endodontics. In that year, the American Association of Endodontists (AAE) was formed in Chicago, Illinois.[43] By 1949, this group was investigating 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.[44] Finally, due to remarkable development and growth of endodontics during the previous 25 years and the tireless efforts of AAE leaders,[43] the American Dental Association recognized endodontics as a specialty in 1963. The first examination and certification of Diplomates occurred 2 years later in 1965.[22,43] The American Association of Endodontists currently boasts approximately 7000 members and approximately one-fourth of those are board certified.[45]

THEORY OF ENDODONTICS

One of the foundational studies of modern endodontics was conducted in 1965. It showed that the pulps of germ-free rats, when exposed and left open to food impaction, survived despite the trauma.[46] This highlighted the important role microbes play in endodontic infections, and the supreme importance of reducing bacterial loads in order to

achieve success following endodontic therapy.[47-49] 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.[50]

A 1955 study by Stewart highlighted three phases of endodontic treatment: chemomechanical preparation, microbial control, and obturation of the root canal.[51] Of these three phases, Stewart identified chemomechanical preparation as the most important phase. This was later confirmed by Grossman,[52] 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.

Schilder discussed the importance of chemomechanical preparation of the canal with instruments and antiseptics followed by a three-dimensional obturation to the

cementodentinal junction or 0.5 mm to 1 mm from the radiographic apex.[53] These principles were later reiterated by Ford,[54] 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. Ford also stressed the importance of aseptic technique including the use of rubber dams, adequate coronal restoration of root canal treated teeth and appropriate recall to monitor healing.

IRRIGATION

The utilization of irrigation solutions is an important part of non-surgical root canal therapy. It aids in the elimination of bacteria, bacterial by-products and debris from the root canal system. Irrigation solutions can often reach portions of the canal space that are not reached by mechanical debridement alone. Studies have shown that up to 53 percent of canal walls remain untouched following instrumentation.[55,56] Specific desirable properties of irrigation solutions were identified by Harrison[57] as follows: antimicrobial efficacy, tissue dissolution capacity, biocompatibility, and ability to adjunctively debride the root canal system. Various irrigation solution have been studied and utilized clinically.

Sodium Hypochlorite

Sodium Hypochlorite (NaOCl) is a powerful antimicrobial agent and is effective at dissolution of collagen and other components of organic tissue. It gained popularity as an antimicrobial agent during World War I and was later applied in endodontic treatment.[58] NaOCl has shown superior capacity for microbe elimination within the root

canal system when compared to sterile saline.[59] It is available in various concentrations ranging from 0.5 to 6.0 percent.[60] The concentration of the solution can affect both its tissue dissolving and antibacterial properties. At lower concentrations, NaOCl is primarily effective at dissolving necrotic tissues.[61] At higher concentrations, its tissue dissolving and antimicrobial properties are both enhanced.[62] Its tissue-dissolving efficacy can also be enhanced by increased duration of exposure, as well as increased temperature.[63,64] For every 5°-C rise in temperature, the bactericidal rates for NaOCl doubled.[65] Due to its favorable characteristics, NaOCl is used widely and often considered the single most effective endodontic irrigation solution.[66,67]

Chlorhexidine

Chlorhexidine (CHX) is a broad spectrum antimicrobial agent that is effective against yeast as well as gram-negative and gram-positive bacteria. It is available in various concentrations from 0.12 to 2 percent.[58] Its effectiveness against bacteria results from its overall positive electrostatic charge, allowing it to bind to negatively charged bacterial cell walls. This results in damage to the cell by increasing permeability of the cell wall, which diminishes the bacteria's ability to regulate its internal environment.[68,69] At low concentrations, CHX has a bacteriostatic effect primarily by interfering with cell metabolism, while at higher concentrations it acts as a detergent and is bactericidal.[58] In endodontics, CHX is useful due to its substantivity (extended microbial effect) which results from its ability to bind to hydroxyapatite.[58] This allows it to remain on dentinal walls for extended periods of time, thereby reducing bacterial colonization.[70] The antimicrobial activity of CHX is directly related to its concentration, and accordingly, 2.0- percent preparations have been shown to be more effective endodontic irrigation

solutions than 0.12-percent solutions.[71] Due to its favorable properties, CHX has been advocated as the final solution in an endodontic irrigation regimen, especially in the case of endodontic retreatment.[72]

Unlike NaOCl, CHX does not possess tissue dissolving properties, which practically necessitates that it be used in conjunction with other irrigation solutions in order to maximize chemomechanical debridement of the root canal system.[58] This presents a potential problem in that CHX has been shown to form a potentially carcinogenic precipitate when mixed with NaOCl. Originally identified as para-chloroaniline (PCA) by Basrani,[73] subsequent investigation suggested that the precipitate formed upon mixing CHX and NaOCl is actually parachlorophenylurea (PCU) and parachlorophenylguanidyl-1,6-diguanidyl-hexane (PCGH).[74]

EDTA

Endodontic instrumentation results in the formation of what has been termed a "smear layer." The smear layer consists of organic and inorganic substances such as fragments of odontoblastic processes and necrotic debris,[75] as well as fragments of hydroxyapatite. There is controversy surrounding the question of whether removal of the smear layer is advisable.[75] A relatively recently conducted systematic review and meta-analysis on the effects of the smear layer removal and its effect on sealability of the root canal system concluded that smear layer removal improves the fluid-tight seal of the obturation.[76] This finding would seem to advocate an irrigation regimen that includes irrigation solutions capable of removing the smear layer.

Ethylenediamene Tetra-Acetic Acid (EDTA) has been used as an endodontic irrigation solution. It has shown efficacy at chelation and removal of inorganic

components of the smear layer. The concentration of EDTA most commonly used in endodontics is 17 percent, which has been shown to remove the smear layer in one minute.[58] EDTA's effects are self-limiting, and its effectiveness can be decreased in the presence of organic debris.[77] However, when used in conjunction with NaOCl, a synergistic effect resulting in the removal of the entire smear layer and deeper penetration of irrigation solutions into dentinal tubules has been observed.[78,79]

OBTURATION

Chemomechanical debridement and final irrigation set the stage for the obturation phase of non-surgical root canal treatment. The objective of obturation is to prevent infection of the periapical tissues by sealing the root canal space.[80] Various materials have been utilized and advocated for use in obturation. Most techniques use a core material and a sealer to achieve a tight seal; the most commonly used core material is gutta-percha.[58] In a 1946 publication,[81] Dr. Louis Grossman identified multiple qualities that would characterize an ideal obturation material. They are that the material should be: 1) easily introduced; 2) liquid or semisolid which later solidifies; 3) capable of sealing the canal laterally and apically; 4) free of shrinkage; 5) resistant to moisture; 6) bacteriostatic; 7) devoid of a tendency to stain tooth structures; 8) biocompatible with periapical tissues; 9) easily removed; 10) sterile when introduced into the canal; and 11) radiopaque.

The quality of the obturation is related to the success of endodontic treatment. One aspect of obturation quality is the length of the fill. A recent meta-analysis investigated this issue and found that success rates are improved when the obturation material does not extend beyond the apex.[82] Peng[83] found that obturation techniques that

utilize warm gutta-percha resulted in a higher rate of overextension of obturation material, so extra care is required when using this type of technique. The highest success rates are achieved when adequate chemomechanical debridement and obturation are followed up by a high quality coronal restoration.[84]

However, some clinical situations present particular challenges to the achievement of these goals. For example, immature necrotic teeth with thin dentinal walls pose a risk of fracture open condensation of obturation materials, and the blunderbuss apical architecture make it very difficult to achieve an apical seal with traditional materials.[85] For this particular clinical situation, alternative treatment methodologies are required. These methodologies are typically referred to collectively as "regenerative endodontics."

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."[86] 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 in 1952[87] and B.N. Ostby explored the role of the blood clot in endodontics in a paper published in 1961[88]), it has received increasing attention in recent years.[89,90] The typical clinical scenario in which regenerative endodontic procedures have been employed has been in cases of immature infected teeth with incompletely developed apices.[21,86,90] 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."[86]

Some have questioned the value of applying the principles of regenerative medicine to endodontics.[86] 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[86] citing the possibility for staining of tooth structure by endodontic filling materials and sealers.[91,92] They also point out the potential for decreased fracture resistance of root dentin caused by long term $Ca(OH)_2$ exposure[93] 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.[89]

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 begins with disinfection via irrigation of the canal space.[90] Irrigation protocols for immature teeth with open apices differ from those for teeth with closed apices. For regenerative procedures, a reduced concentration of 2.5 percent NaOCl is recommended to avoid cytotoxicity to apical tissues including stem cells.[94] Additionally, the use of CHX in regeneration procedures has been discouraged due to its detrimental effects on stem cells of the apical papilla (SCAP).[94] Conversely, EDTA has been shown to promote SCAP survival. One suggested mechanism for this effect is that irrigation with EDTA promotes the release of dentinal growth factors that were embedded in the dentin during dentinogenesis.[15,86]

Irrigation is followed by placement of an intracanal medicament. Bacteria present in necrotic root canals and deep within the tubules of radicular dentin have been found to be predominantly obligate anaerobes.[95] Metronidazole has a wide spectrum of bactericidal action against oral obligate anaerobes, even within the necrotic pulp.[96,97] One study found that 99 percent of bacteria in infected root dentin were killed in the presence of metronidazole.[96] Another study found that a mixture of ciprofloxacin, metronidazole, and minocycline can sterilize root dentin.[97] These findings have led to the widespread use of triple antibiotic paste (TAP) in regenerative endodontic procedures. In addition to its effectiveness at eradicating pathogenic bacteria, this mixture has been shown to be well tolerated by apical tissues.[98] One downside of TAP, however, is that the minocycline component can cause dentinal staining.[99] Calcium hydroxide is another intracanal medicament option for regenerative endodontic procedures. It has been shown to reduce bacterial levels,[100] but it can also result in the initiation of apical hard tissue barrier formation by sterile necrosis of the apical tissues.[101] This process tends to hinder endodontic regeneration. Following placement of the intracanal medicament, the tooth undergoing regenerative treatment is sealed with a temporary restoration and the patient is recalled in three to four weeks.

At the following appointment, bleeding is induced into the canal from the apical tissues, and a permanent coronal seal is placed.[90,85] 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 of cytokines that aid in regeneration and repair.[85,86,90] The scaffold provides a biological “three dimensional microenvironment for cell growth and differentiation, promoting cell adhesion, and

migration."[102]

While little data are available concerning success rates of regenerative procedures (the majority of the literature consists of case reports with failures not being reported), a recent study did reveal that regenerative techniques were "associated with significantly greater increases in root length and thickness in comparison with calcium hydroxide apexification and MTA apexification as well as excellent overall survival rates."[103]

ANGIOGENESIS

A key component to achieving success in regenerative endodontic procedures involves establishing an adequate blood supply to the tissues involved.[11,86,90] 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.[85] This process of new blood vessel growth from previously existing blood vessels is termed "angiogenesis."[104] Other physiologic processes that involve angiogenesis include wound repair, the ovarian/menstrual cycle, and embryogenesis.[105]

Angiogenesis can be classified into two types: sprouting angiogenesis and intussusceptive (or splitting) angiogenesis.[104] 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.[106] Some of these 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."[105]

CYTOKINES

Cytokines are defined as "secreted proteins that regulate important cellular responses such as proliferation and differentiation."[107] Various cytokines that play integral roles in the process of angiogenesis have been identified. 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,[106] which are induced to migrate in part by the action of insulin like growth factor - 1 (IGF-1).[108] Angiogenin and monocyte chemotactic protein - 1 (MCP-1) are growth factors that aid the process by increasing new vessel growth.[109-111] 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.[112-115]

Transforming growth factor-beta (TGF-β) is another angiogenesis promoting cytokine.[116] 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.[86,117] Fibroblast growth factor (FGF) is secreted from a wide range of cells and promotes proliferation of a variety of cell-types. It can be applied in regenerative endodontic procedures to increase stem cell numbers.[86,113]

One family of enzymes that plays a critical role in multiple physiologic processes is the matrix metalloproteinase (MMP) family. MMPs aid in 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 (TIMP) play an important role in regulating the activity of MMPs.[118] There are four isotypes of TIMP,[119] and types 1-3 have been shown to have inhibitory effects on angiogenesis.[119-121] 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).[122] PDGF-BB has been shown to modulate endothelial proliferation and angiogenesis.[123] 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."[124] However, studies have also demonstrated a strong correlation between higher ENA-78 levels and increased vascularity of certain tissues.[105,125]

ADULT STEM CELLS

All tissues are generated from stem cell precursors.[126] Stem cells can be defined as cells that have "the ability to continuously divide and produce progeny cells that differentiate (develop) into various other types of cells or tissues."[127] Stem cells are also commonly classified as either embryonic/fetal or adult/postnatal.[128] One set of investigators expressed preference for the term *postnatal* over the term *adult* because cells that fall under this classification are present in babies, infants and children, and therefore, the term *adult* can be misleading. This same group advocated the use of the term *embryonic* rather than *fetal*, as most of the cells in this classification are indeed present in the embryonic stage rather than the fetal stage.[86]

The importance of classifying stem cells in this manner (embryonic vs. postnatal) lies in the fact that the potential for stem cells to differentiate into different cell types (i.e., plasticity) differs between the two groups.[129] Embryonic stem cells have historically shown greater plasticity than their postnatal counterparts. However, more recent studies have demonstrated that postnatal stem cells have greater potential for plasticity than previously believed.[130] Stem cells can also be described as totipotent, pluripotent, or multipotent. Totipotent cells exhibit the greatest plasticity. They are found in a new embryo (1 day to 3 days) and have the potential to differentiate into an entire individual. Pluripotent cells are also highly plastic and can differentiate into over 200 different cell types. These cells are present in the blastocyst stage (approximately 5 days to 14 days). Multipotent cells are more differentiated than the previous two types, but can still be induced to form other tissues. Sources of multipotent stem cells include fetal tissue, cord blood and postnatal stem cells such as dental pulp stem cells.[86]

HUMAN DENTAL STEM CELLS

One of the fundamental issues facing any attempt at tissue engineering is to determine the source of the cells that will be utilized.[85] For regenerative endodontic procedures, the dental pulp and tissues of the periodontium serve as a reservoir of stem cells.[86] Four different types of human dental stem cells have been identified and characterized[90]: dental pulp stem cells (DPSCs);[131] stem cells from human exfoliated deciduous teeth (SHED);[132] stem cells from apical papilla (SCAP);[133,134] and periodontal ligament stem cells (PDLSCs).[135] Of these four types, all but SHED are derived from permanent teeth.

Dental stem cells are a subclass of mesenchymal stem cells (MSCs) and they vary

in their ability to differentiate into specific tissue-forming cells. DPSCs can differentiate into odontoblast-like cells which can produce dentin-like tissue both *in vivo* and *in vitro*.[90] They can also be induced under proper conditions to differentiate into cells capable of producing such disparate tissues as nerve, muscle, cartilage, bone and even adipose.[136-140] The identification and isolation of these stem cells has led to a more complete understanding of the pulp and periodontal ligament tissues and their potential for regeneration even following tissue damage from trauma or infection. For example, the observation of severely infected immature teeth remaining capable of undergoing complete root maturation after proper disinfection procedures may be explained by the possibility that stem cells somehow evade death even in the noxious microenvironment of an infected tooth.[90,133,141]

According to one study, many adult tissues contain only 1 percent to 4 percent stem cells.[142] Therefore, these cells need to be isolated and concentrated for later re-introduction to the desired site of action or induced to migrate to the desired site via chemical messengers. There are four methods commonly utilized to identify and isolate stem cells from mixed cell populations. These include: "(a) staining the cells with specific antibody markers and using a flow cytometer in a process called fluorescent antibody cell sorting (FACS); (b) immunomagnetic bead selection; (c) immunohistochemical staining; and (d) physiological and histological criteria, including phenotype (appearance), chemotaxis, proliferation, differentiation, and mineralizing activity."[86] Of the various types of dental stem cells, dental pulp stem cells are the most studied.[86] These cells express specific surface proteins that can be utilized in the identification process. Examples include alpha-smooth muscle actin, von Willebrand

factor CD146, and 3G5.[143] Human pulp stem cells also have a fibroblast phenoptype, with specific proliferation, differentiation, and mineralizing activity patterns.[143,144]

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."[17] According to materials provided by the manufacturer (Cook Biotech, West Lafayette, IN), their SIS membranes are prepared by removing the tunica mucosa from the inner intestinal surface and the serosa and tunica muscularis from the outer intestinal surface. Next, during processing with a series of surfactant and ionic solutions, the cells and nuclear matter are removed. This results in a three-dimensional, acellular, collagen-rich extracellular matrix (ECM) that is not chemically crosslinked. 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 cytokines such as fibroblast growth factor (FGF-2), connective tissue growth factor (CTGF), and transforming growth factor beta (TGF-β) all of which 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. 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 also present in negligible amounts.

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. 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 (TSE). However, there are no known naturally occurring TSEs in pigs and cross-species transmission is highly unlikely.[145]

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

This study utilized human dental pulp stem cells (HDPSC) *in vitro.* Three different groups were tested as follows: 1) control group 1- Human Dental Pulp Stem Cell (HDPSC) culture only; 2) control group 2 – DynaMatrix membranes placed in culture media without any cells present, and 3) experimental group- HDPSC seeded on DynaMatrix membranes. The conditioned media from the various groups were collected after 72 hours and tested for the expression of specific angiogenic cytokines that may play a role in regenerative endodontic treatment.

The HDPSC used in this study were purchased form Cook Biotech (Indianapolis, IN). The protocol used by Cook Biotech to procure and treat the HDPSC has been outlined in the literature and is summarized below as published:[146]

Tissue Processing and Cell Culture

Collection and transport of extracted teeth

Extracted human third molars were obtained with informed consent through a local oral/maxillofacial surgeon from patients aged 18 years to 30 years. Once extracted, teeth were placed into chilled solution vessels containing 20 mL of sterile phosphate buffered saline and transported to the lab for processing within 24 hours.

Processing and tissue recovery

Before digestion of dental tissue, teeth were disinfected by a protocol adopted and modified from cornea banking.[147] The roots of cleaned teeth were separated from the

crown; the roots were split to reveal the dental pulp, which was then recovered with a curette for further processing or cryopreservation.

Tissue digestion

Recovered dental tissue was placed into an enzyme bath consisting of a blend of saline with Type I and Type II Collagenase to liberate the cells. Once digestion was complete, the enzyme mixture was neutralized by addition of medium and the cells were plated as previously described.[148]

Culture and expansion of fresh tissue digest

For culture of fresh digested tissue, cells were plated immediately in a T-25 flask at a density of 1 tooth digest per flask and placed into a high humidity 37º C, 5.0-percent CO2 incubator. No selection of stem cells was performed directly, and instead selective culture was used to maximize stem cell recovery. After 14 days of growth, adherent cells were dissociated from flasks and a cell count of total cells per flask was obtained via standard hemacytometer counting methods.

Cells were then sub-cultured for approximately 4 weeks, with medium changes taking place three times per week. Following trypsin dissociation, passage 3 cells were re-suspended in complete medium and were confirmed to meet minimal criteria to be called MSC by flow cytometry based on the International Society for Cell Therapy (ISCT) position paper. [149] Antibodies to human CD73, CD90, CD105, CD34, CD45, CD11b, CD19 and HLA-DR were obtained from BD Biosciences (San Jose, CA). Selected DPSC cultures were stained with the above antibodies and appropriate isotype controls per the manufacturer's instructions and were analyzed using a FACSCalibur

instrument and CellQuest software (BD Biosciences). Cells 95-percent positive for CD73, 90 and105, and 65-percent positive for CD34, 45, 11b, 19 and HLA-DR were confirmed DPSC. The cells procured from Cook Biotech were subcultured. HDPSC (50,000/well) were seeded on the plates (no membrane) or on DynaMatrix® membrane in the 6-well plates for 3 days. The conditioned media were collected and stored at -70º C before further analysis, and subsequently the concentrations of the total protein in the collected media were determined by the Bradford method using Bio-Rad protein assay.

CYTOKINE ARRAY

The release of multiple cytokines from the HDPSC or DynaMatrix alone, or from HDPSC seeded on DynaMatrix® membranes was evaluated utilizing RayBio Human Angiogenesis Antibody Array I (Table 1, RayBiotech Inc., Norcross, GA) according to the manufacturer's protocol.[20] In brief, the cytokine array membranes were blocked with 1.0-percent serum for 30 minutes and then incubated for three hours with 200 ng of total protein from each of the samples, two hours with biotin-conjugated antibodies provided by the kit, and then two hours with horseradish peroxidase–conjugated streptavidin. Detection agents supplied in the kit were mixed and applied to each membrane for two minutes. The cytokines on the membrane were then visualized by autoradiography on x-ray film. Each experiment was repeated three times. With two dots for each type of cytokine on each membrane, a total of four to six determinations for each cytokine (outliers removed) were used for further semi-quantification.

To determine the level of cytokine expression, the optical densities of the visible dots on the membrane were measured by Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA). The density of each dot was adjusted for the background using the

negative controls on each membrane and the different membranes were normalized by the positive controls on each membrane. The experiment was repeated three times and the averages were calculated. Group comparisons were performed using one-way ANOVA. Based on previous studies,[150-153] this study had approximately 80-percent power to detect a fold-change of two, assuming two-sided tests each conducted at a 5.0-percent significance level and a within-group standard deviation equal to approximately one-half fold-change.

RESULTS

To determine the levels of cytokine expression, the optical densities of the visible dots on the membrane were measured by Quantity One 1-D Analysis Software (Bio-Rad). For each membrane, the density of each dot was adjusted for the background by subtracting the average value of the negative controls on each membrane and then normalized by dividing by the average of the positive controls. The experiment was run three times resulting in three membranes for each group. This generated four to six densitometry readings (two per membrane, some statistical outliers removed) for each cytokine in each group. Group comparisons were performed using one-way ANOVA.

The cell-only group (C) had significantly lower ENA-78 ($p = 0.0166$), leptin ($p = 0.0208$), PDGF-BB ($p = 0.0034$), VEGF ($p = 0.0174$), VEGF-D ($p = 0.0230$), and bFGF ($p = 0.0047$) compared to the DynaMatrix membrane-only group (M). C had significantly higher IL-6 ($p = 0.0268$), IL-8 ($p = 0.0153$), MCP-1 ($p = 0.0021$), TIMP-1 ($p = 0.0027$), and TIMP-2 ($p = 0.0105$) compared to M.

C had significantly lower PDGF-BB ($p = 0.0182$) and bFGF ($p = 0.0009$) compared to the DynaMatrix membrane + dental pulp stem cell group (M+C). C had significantly higher IL-6 ($p = 0.0203$), IL-8 ($p = 0.0097$), MCP-1 ($p = 0.0019$), TIMP-1 ($p = 0.0006$), and TIMP-2 ($p = 0.0086$) compared to M+C. C and M+C did not have significantly different ENA-78 ($p = 0.78$), leptin ($p = 0.42$), VEGF ($p = 0.42$), or VEGF-D ($p = 0.07$).

M had significantly lower bFGF ($p = 0.0452$) compared to M+C. M had significantly higher ENA-78 ($p = 0.0178$), TIMP-1 ($p = 0.0363$), and VEGF ($p = 0.0117$)

compared to M+C. M and M+C did not have significantly different IL-6 ($p = 0.79$), IL-8 ($p = 0.63$), leptin ($p = 0.06$), MCP-1 ($p = 0.92$), PDGF-BB ($p = 0.10$), TIMP-2 ($p = 0.81$), or VEGF-D ($p = 0.34$).

The groups did not have significantly different angiogenin ($p = 0.20$), EGF ($p = 0.27$), GRO ($p = 0.60$), IFN-gamma ($p = 1.00$), IGF-I ($p = 0.79$), PIGF ($p = 0.84$), RANTES ($p = 0.74$), TGF-beta 1 ($p = 0.70$), or thrombopoietin ($p = 0.22$).

TABLES AND FIGURES

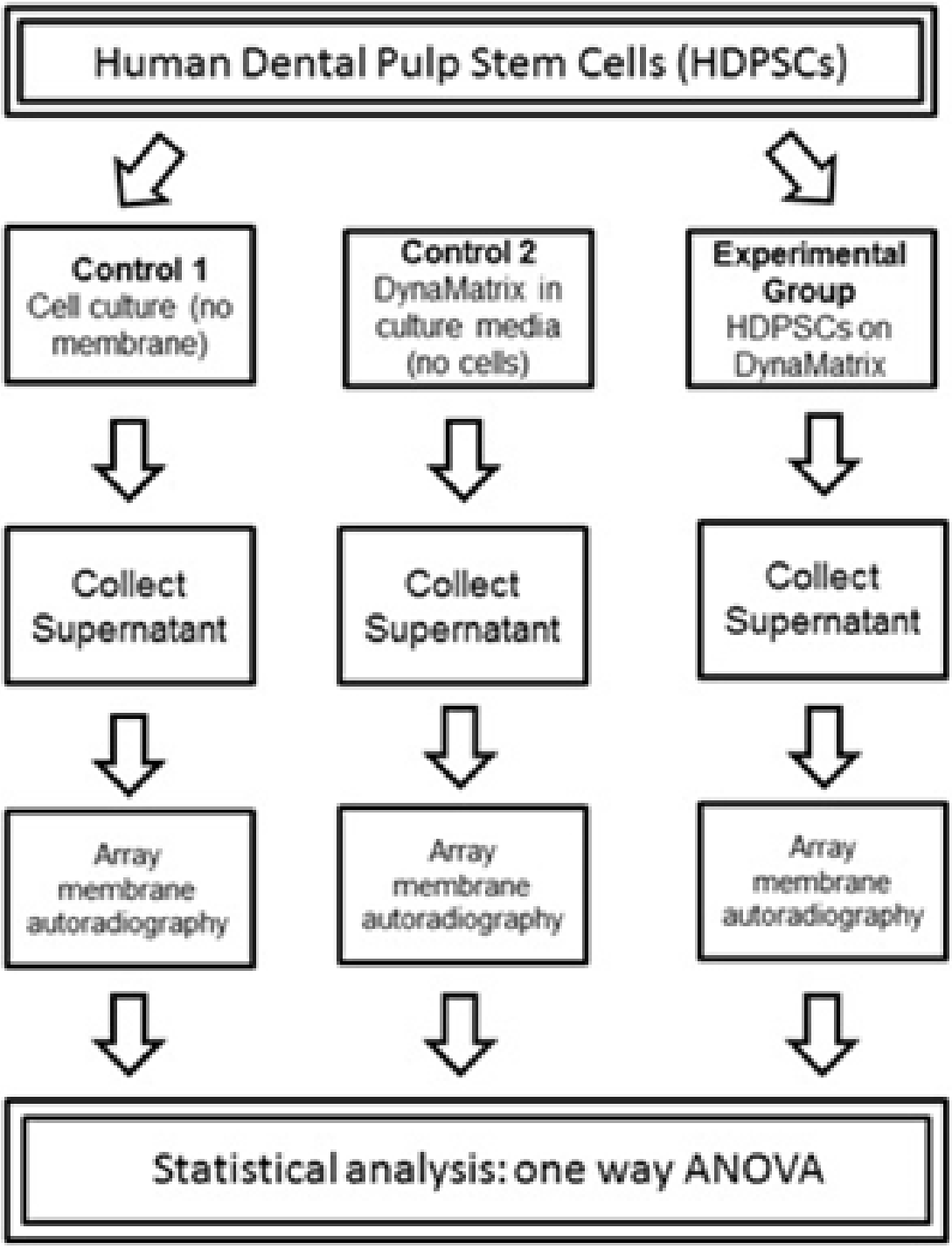

FIGURE 1. Experimental design overview.

FIGURE 2. DynaMatrix membrane provided by manufacturer in large quantity.

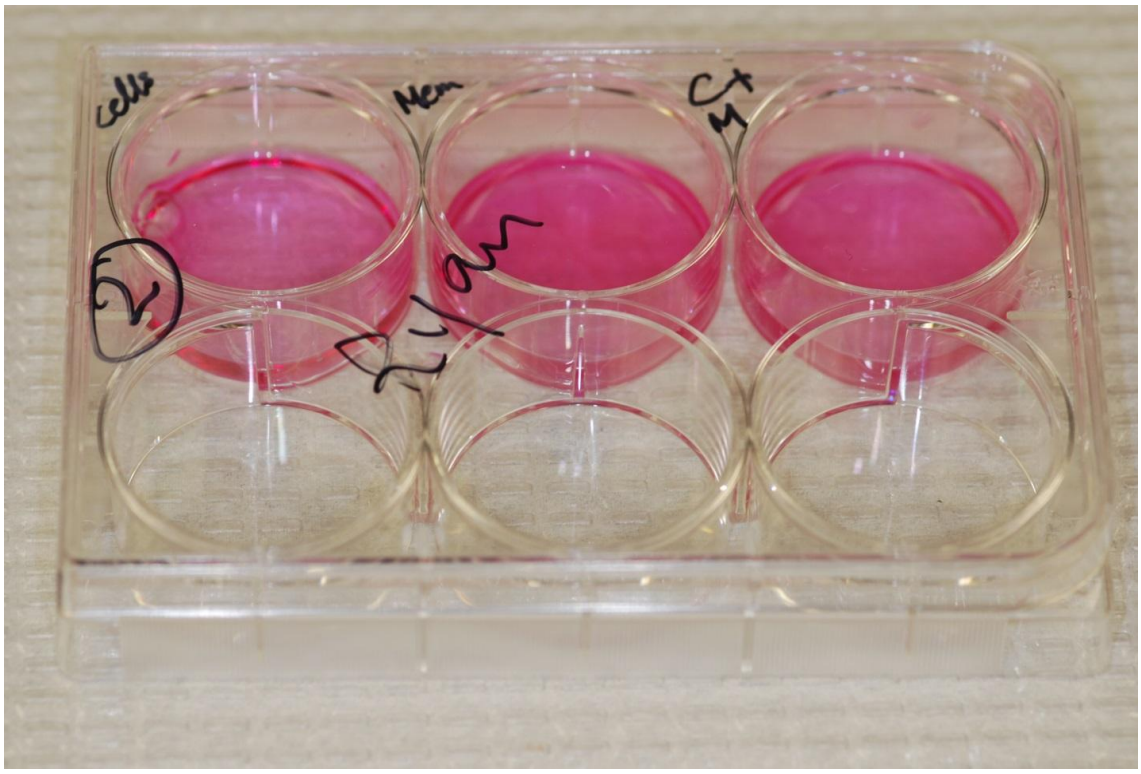

FIGURE 3. Six-well plate showing three experimental groups with culture media in top three wells. Well 1: HDPSC; Well 2: DynaMatrix membrane; and Well 3: HDPSC seeded on DynaMatrix membrane.

FIGURE 4. Test tube rack with test tubes containing the conditioned media collected from each of the three wells (HDPSC alone, DynaMatrix alone and HDPSC seeded on DynaMatrix membrane). The experiment was run three times resulting in 9 separate samples.

FIGURE 5. Bovine Serum Albumin utilized in Bradford protein assay.

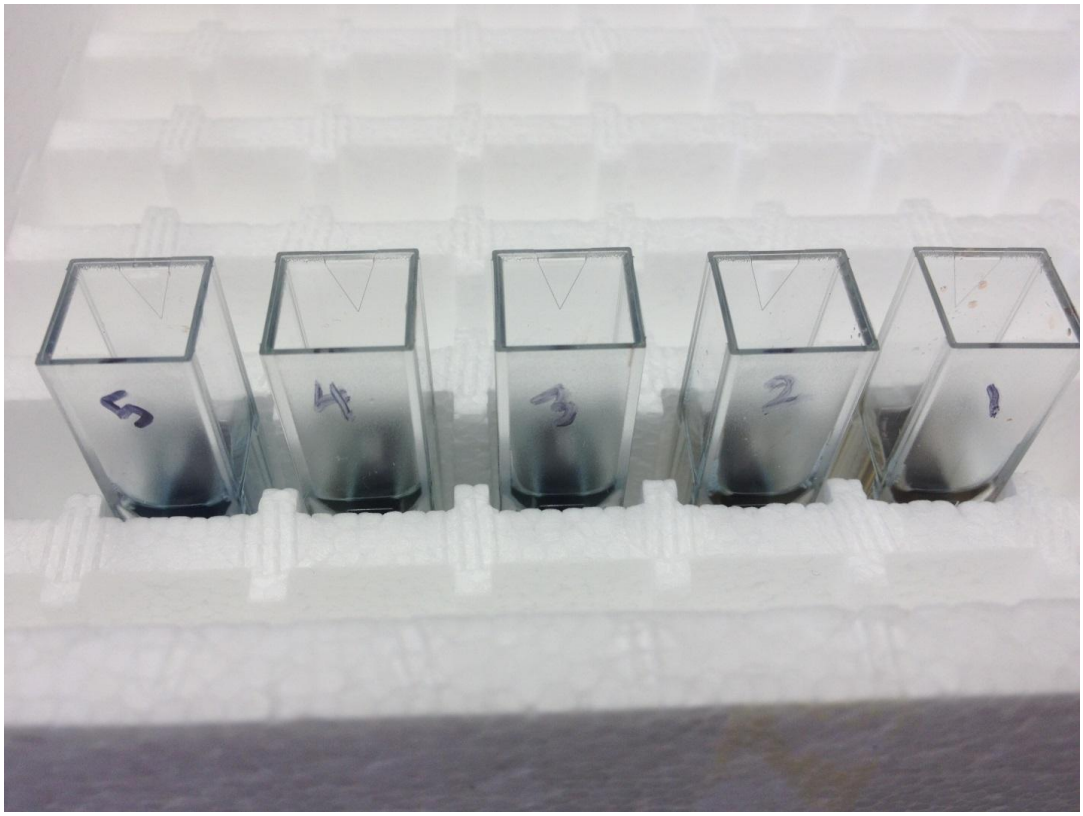

FIGURE 6. Samples were placed in vials and then into the spectrophotometer.

FIGURE 7. UV/ Visible Spectrophotometer used for assessing protein concentrations.

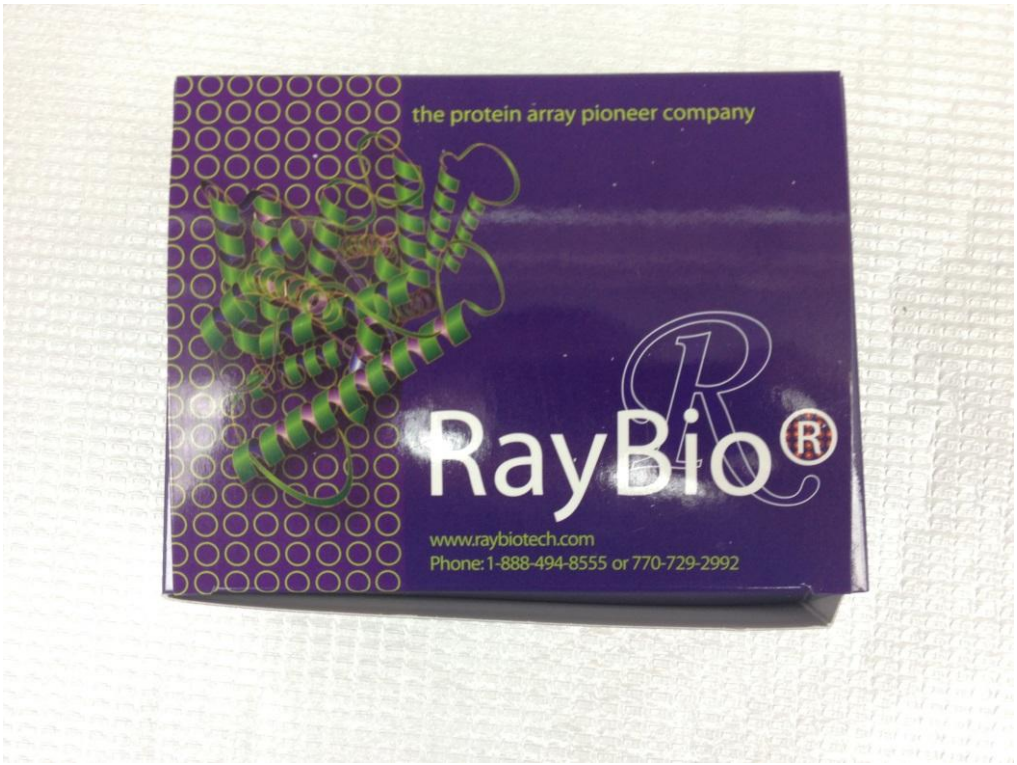

FIGURE 8. RayBio® Human Angiogenesis Antibody Array 1 Kit. Above: Closed box; Below: Open box.

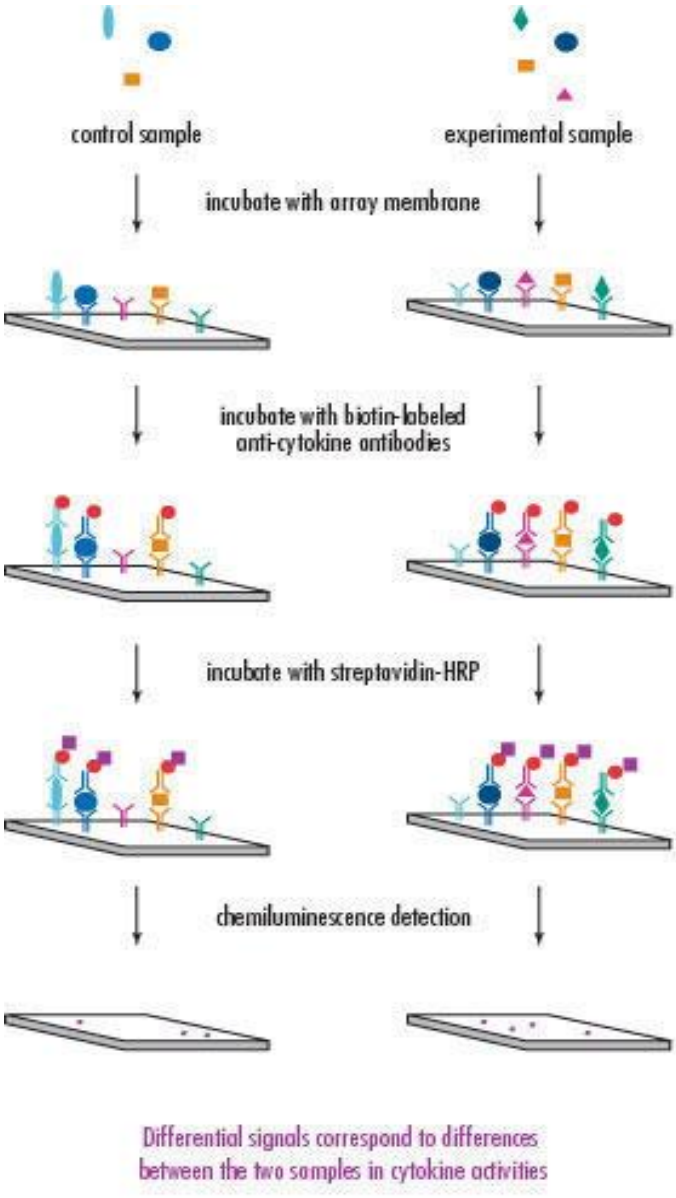

FIGURE 9. Schematic illustration of the steps involved in cytokine array analysis.

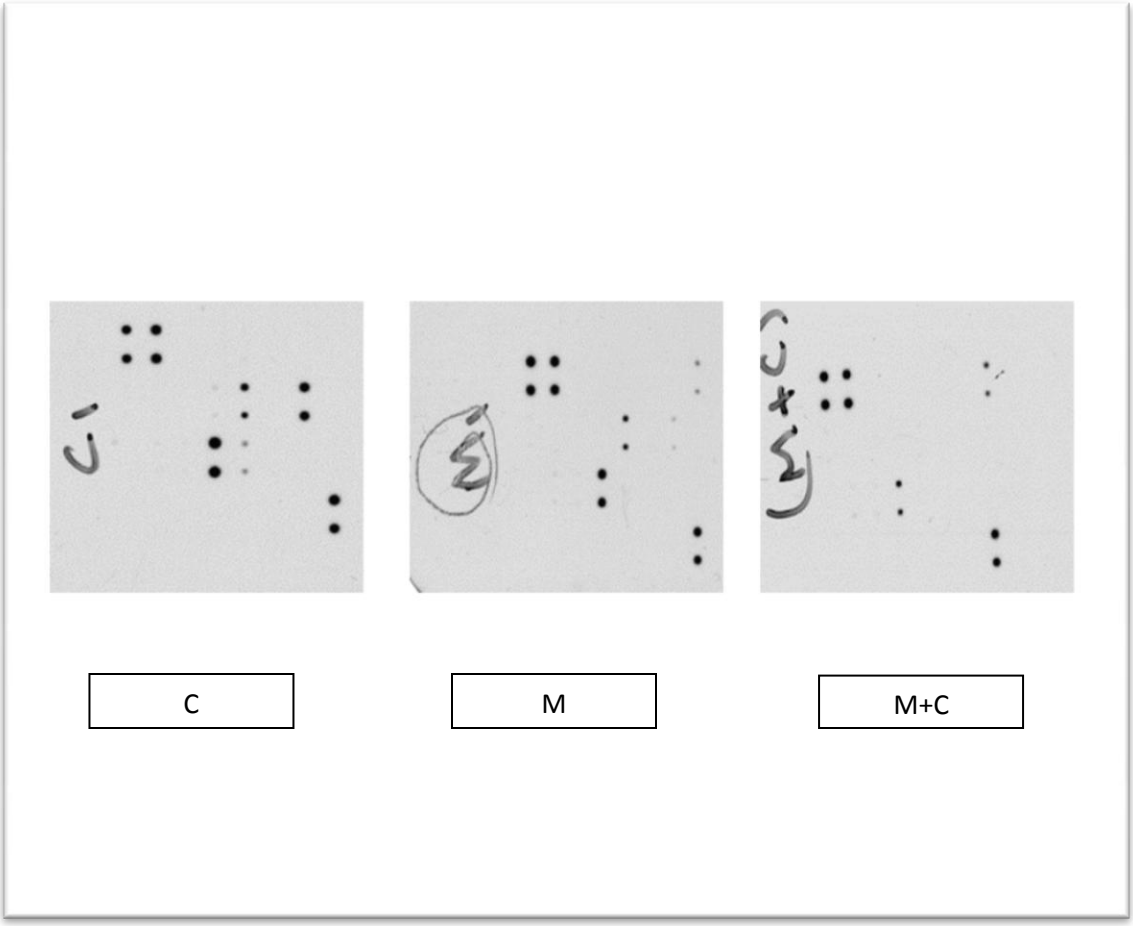

FIGURE 10. Images of autoradiography films from the various samples. From left to right: Cell-only group (C); Membrane-only group (M); HDPSC seeded on DynaMatrix group (M+C).

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-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
4	GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
5	PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
6	PIGF	RANTES	TGF-β1	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

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 metallopeptidase inhibitor 1, 2
VEGF: vascular endothelial growth factor
VEGF-D: vascular endothelial growth factor D
POS: positive control
Neg: negative control

TABLE II

Data summary of densitometry readings*

Cytokine	C	M	M+C
Angiogenin	0.0075 (0.0026)	0.0068 (0.0040)	-0.0040 (0.0062)
EGF	-0.0012 (0.0017)	0.0090 (0.0036)	-0.0011 (0.0076)
ENA-78	-0.0035 (0.0021)	0.0145 (0.0006)	-0.0048 (0.0060)
GRO	0.0141 (0.0118)	-0.0014 (0.0035)	0.0067 (0.0078)
IFN-gamma	0.0001 (0.0011)	0.0002 (0.0013)	-0.0001 (0.0063)
IGF-I	-0.0022 (0.0026)	0.0014 (0.0021)	-0.0002 (0.0062)
IL-6	0.0239 (0.0050)	0.0034 (0.0008)	0.0015 (0.0034)
IL-8	0.2541 (0.0478)	0.0351 (0.0096)	0.0042 (0.0031)
Leptin	-0.0065 (0.0014)	0.0047 (0.0020)	-0.0038 (0.0035)
MCP-1	0.5254 (0.0646)	0.0118 (0.0006)	0.0033 (0.0023)
PDGF-BB	-0.0067 (0.0009)	0.0075 (0.0010)	0.0020 (0.0030)
PIGF	-0.0049 (0.0024)	-0.0023 (0.0031)	-0.0052 (0.0060)
RANTES	0.0126 (0.0101)	0.0032 (0.0004)	0.0101 (0.0041)
TGF-beta 1	0.0067 (0.0044)	0.0025 (0.0018)	0.0015 (0.0060)
TIMP-1	0.8444 (0.0402)	0.3995 (0.0054)	0.1707 (0.0777)
TIMP-2	0.1267 (0.0242)	0.0017 (0.0082)	-0.0059 (0.0002)
Thrombopoietin	-0.0065 (0.0022)	-0.0011 (0.0001)	-0.0031 (0.0015)
VEGF	-0.0044 (0.0010)	-0.0002 (0.0002)	-0.0054 (0.0001)
VEGF-D	-0.0046 (0.0023)	0.0055 (0.0020)	0.0022 (0.0000)
bFGF	-0.0032 (0.0005)	0.1309 (0.0232)	0.2052 (0.0282)

* Mean densitometry values for each experimental group, followed by the standard error in parentheses. C: Cell-only group; M: DynaMatrix-only group; M+C: HDPSC seeded on DynaMatrix.

DISCUSSION

One important finding from this study was that human dental pulp stem cells appeared to grow on DynaMatrix *in vitro* based on the measured differences in cytokine levels between the experimental groups. This is one more characteristic of the membrane that makes it a possible candidate for future use as an intracanal scaffold for regenerative endodontic procedures.

The results of this *in vitro* study supported the claim of the manufacturer that DynaMatrix contains "biological signals such as growth factors."[17] ENA-78, leptin, PDGF-BB, VEGF, VEGF-D and bFGF were all present in higher levels in the supernatant from the DynaMatrix-only group when compared to the HDPSC-only group. Since no cells were present in the DynaMatrix-only group to secrete these cytokines, the increased levels of these cytokines in the supernatant must have resulted from their having leached out of the membrane itself. However, with the exception of bFGF, the levels of expression of the above-mentioned cytokines were so miniscule that while the differences were statistically significant, it is highly unlikely that biological or clinical significance can be ascribed to these differences. As such, the discussion that follows will highlight only cytokines that were detected in quantities deemed sufficient to reasonably anticipate some biological effect.

In addition to increasing the levels of certain cytokines by simple diffusion from the DynaMatrix into the supernatant, the DynaMatrix appears to have had an effect on the expression of bFGF by the HDPSC. The experimental group, which consisted of the HDPSC seeded on DynaMatrix, exhibited statistically significantly higher levels of bFGF

than the levels detected in the DynaMatrix-only and the HDPSC-only groups combined. This implies that the increased levels of bFGF in the experimental group were not due simply to additive effects between the cytokine molecules from the DynaMatrix and those from the HDPSC, but rather that DynaMatrix actually had some sort of stimulatory effects that resulted in greater bFGF expression from the HDPSC.

A different mechanism appears to have affected the levels of TIMP-1 and TIMP-2 expression. These levels were highest in the HDPSC-only group, significantly lower in the DynaMatrix-only group and lowest in the experimental group (DynaMatrix + cells). While the expression of TIMP-1/-2 from the HDPSC was obviously reduced in the presence of DynaMatrix, one might expect that they would still be present in at least the same levels as that those seen in the DynaMatrix-only group since the membrane is present in both groups. However, the fact that the DynaMatrix + HDPSC group exhibited the lowest TIMP-1 and TIMP-2 levels suggests that the presence of the cells reduced the diffusion of TIMP-1 and TIMP-2 from DynaMatrix into the supernatant by some mechanism. Other possible mechanisms include some chemical interactions that result in tighter binding between TIMP and DynaMatrix, or actual uptake of TIMP by HDPSC that for some reason did not occur in the HDPSC-only group.

The HDPSC-only group had significantly higher levels of IL-8, MCP-1, TIMP-1, and TIMP-2 than the other two groups. Again, this would imply some interactions between the HDPSC and DynaMatrix that results in reduced expression of these particular cytokines by the HDPSC. Otherwise, the HDPSC-only group and the experimental group (HDPSC + DynaMatrix) would have exhibited comparable levels.

It is interesting to note that in general, the interactions between the HDPSC and

DynaMatrix tended to result in an angiogenic cytokine profile that would seem to promote conditions conducive to achievement of some of the goals of regenerative endodontic procedures. For example, bFGF is present in DynaMatrix and DynaMatrix appears to promote expression of bFGF from HDPSC. FGF is secreted from a wide range of cells and promotes proliferation of a variety of cell-types. It can be applied in regenerative endodontic procedures to increase stem cell numbers.[86,113] The interaction of HDPSC and DynaMatrix also resulted in significantly lower levels of TIMP-1 and TIMP-2. There are four isotypes of TIMP[119], and types 1-3 have been shown to have an inhibitory effect on angiogenesis.[119-121] IL-8 levels were found to be significantly lower when HDPSC were seeded on DynaMatrix compared to HDPSC alone. 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.[113-115] 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."[154] 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.[155]

The decreased MCP-1 levels seem to present an exception to the general trend observed in this study that the experimental group (HDPSC + DynaMatrix) exhibited a cytokine profile conducive to the goals of endodontic regenerative procedures. MCP-1 has been shown to promote angiogenesis in multiple studies,[111,156-158] though the exact mechanism by which it achieves this is not completely understood.[159] However, like IL-

8, MCP-1 has been shown to be an important mediator of inflammation,[160] 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 effect of individual cytokines is 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 such as this may not be directly applicable to an *in-vivo* situation. 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 jaws. 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 resorbability of the scaffold. The rate at which the scaffold resorbs should mimic as closely as possible the rate of tissue formation.[86] Collagen has been suggested as a possible intracanal scaffold for regenerative endodontic procedures.[85] 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."[11] One study to investigate techniques for treatment of bone defects in a rat model utilized SIS membranes. The bone defects in the rat femurs were treated with bone allograft seeded with mesenchymal stem cells (MSC) 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.[161] 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 aforementioned studies which would be appropriate for regenerative endodontic procedures.[11]

As discussed above, another critical issue influencing the outcomes of regenerative endodontic procedures is the elimination of microbes from the root canal space (RCS). To this end, most suggested protocols recommend copious irrigation 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 RCS are never 100-percent successful.[162,163] 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.[85,86,90] DynaMatrix has no documented antibiotic effects, so 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.

Future research could include a similar study design to the one utilized in this investigation, but could substitute different dental stem cells types such as periodontal ligament stem cells (PDLSC), stem cells of the apical papilla (SCAP) or stem cells from human exfoliated deciduous teeth (SHED). 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.[164]

Eventual animal studies could follow, 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 null hypothesis was rejected because statistically significant differences in the expression of angiogenic cytokines were demonstrated between the experimental group (HDPSC seeded on DynaMatrix) when compared with the control groups (HDPSC-only or DynaMatrix-only). Specifically, bFGF was significantly highest in the DynaMatrix + HDPSC group, lower in the DynaMatrix-only group, and lowest in the HDPSC-only group. In addition, IL-8, MCP-1, TIMP-1 and TIMP-2 were highest in the HDPSC-only group, lower in the DynaMatrix-only group, and lowest in the HDPSC + DynaMatrix group. Statistically significant differences in the levels of other cytokines were detected between the groups, but those particular cytokines were present in such small quantities that they were deemed unlikely to result in any biological effects.

DynaMatrix could potentially improve the regenerative endodontic procedure by providing a predictable mechanical scaffold for cellular and vascular in-growth into the root canal system. Additionally, it may improve angiogenesis by increasing the quantity of angiogenic cytokines present in the microenvironment via release of 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 IL-8, MCP-1 and TIMP-1 and TIMP-2.

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ABSTRACT

EFFECTS OF DYNAMATRIX® MEMBRANE ON ANGIOGENIC CYTOKINE EXPRESSION FROM HUMAN DENTAL PULP STEM CELLS

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Historically, treatment protocols for infected, immature teeth involved disinfection of the root canal space (RCS) followed by creation of an apical barrier and subsequent obturation. While generally effective at removing the source of infection, these methods result in teeth with thin, underdeveloped roots that were prone to fracture over time.

An alternative to the conventional methods is now available in the form of regenerative endodontic procedures. These are currently performed to treat infected immature teeth with the "revascularization method."[7] This method includes two steps: disinfection of the root canal, and then laceration of apical tissues to induce blood flow into the root canal space. The blood clot formed in the canal serves as a fibrin scaffold for

the accumulation of stem cells and other cells with dentin/root formation capability. This protocol has resulted in continued root formation (in length and width) in certain clinical cases. However, the adequacy of the hemorrhage induced in the apical tissues has a significant impact on the quality of the regenerated dental structures. This phenomenon indicates that the blood clot itself may play an important role in continued root formation by locally releasing molecular signals, which induce angiogenesis. Successful angiogenesis is then crucial for the vitality of the newly regenerated pulp tissues and for the initiation of continued root development. Therefore, either the production of angiogenic cytokines from the local surviving cells, or the effective delivery of exogenous angiogenic cytokines to the desired area is one of the key components in regenerative endodontic treatment.

It is believed that an appropriate exogenous scaffold could aid the endogenous elements (fibrin meshwork and molecular signals in the blood clot) and result in more predictable regenerative endodontic procedures. DynaMatrix® is a biological extracellular matrix (ECM) scaffold product that is marketed to dental practitioners. In addition to structural and functional proteins (collagens types I, III, IV, and VI, glycosaminoglycans, glycoproteins, proteoglycans), DynaMatrix contains biological signals such as cytokines. Its track record of successful use in periodontal procedures encourages investigation of the material for use in regenerative endodontic procedures. Specifically, DynaMatrix may be placed within the root canal system of the immature tooth to facilitate the continued development of the entire length of the root from the apical foramen to the level of the dentin enamel junction (DEJ). As a scaffold containing biological signals, DynaMatrix has the potential to induce angiogenesis and encourage

the migration of cells that are involved in the continued development of the root canal system.

The aim of this current study was to determine if the exposure of human dental pulp stem cells (HDPSC) to the DynaMatrix membrane will result in an increased production of angiogenic growth factors that are critical for pulp/root regeneration. This study used HDPSC *in vitro.* Three different groups were tested as follows: 1) control group #1, Human Dental Pulp Stem Cell (HDPSC) culture only; 2) control group #2, DynaMatrix membranes placed in culture media without any cells present, and 3) the experimental group, HDPSC seeded on DynaMatrix® membranes. The conditioned media from the various groups were collected and tested for the expression of specific cytokines using angiogenesis cytokine arrays, which have been established as a viable method for assessing expression of angiogenic cytokines.

To determine the level of cytokine expression, the optical densities of the visible dots on the membrane were measured by Quantity One 1-D Analysis Software (Bio-Rad). For each membrane, the density of each dot was adjusted for the background by subtracting the average value of the negative controls on each membrane and then normalized by dividing by the average of the positive controls. The experiment was run three times resulting in three membranes for each group. This generated four to six densitometry readings (two per membrane, some statistical outliers removed) for each cytokine in each group. Group comparisons were performed using one-way ANOVA.

The cell-only group (C) had significantly lower ENA-78 ($p = 0.0166$); leptin ($p = 0.0208$); PDGF-BB ($p = 0.0034$); VEGF ($p = 0.0174$); VEGF-D ($p = 0.0230$), and bFGF ($p = 0.0047$) compared with the DynaMatrix membrane-only group (M). C had

significantly higher IL-6 ($p = 0.0268$); IL-8 ($p = 0.0153$); MCP-1 ($p = 0.0021$); TIMP-1 ($p = 0.0027$), and TIMP-2 ($p = 0.0105$) compared with M.

C had significantly lower PDGF-BB ($p = 0.0182$) and bFGF ($p = 0.0009$) compared with the DynaMatrix membrane + dental pulp stem cell group (M+C). Control had significantly higher IL-6 ($p = 0.0203$); IL-8 ($p = 0.0097$); MCP-1 ($p = 0.0019$); TIMP-1 ($p = 0.0006$); and TIMP-2 ($p = 0.0086$) compared with M+C. C and M+C did not have significantly different ENA-78 ($p = 0.78$); leptin ($p = 0.42$); VEGF ($p = 0.42$), or VEGF-D ($p = 0.07$).

M had significantly lower bFGF ($p = 0.0452$) compared with M+C. M had significantly higher ENA-78 ($p = 0.0178$); TIMP-1 ($p = 0.0363$); and VEGF ($p = 0.0117$) compared with M+C. M and M+C did not have significantly different IL-6 ($p = 0.79$); IL-8 ($p = 0.63$); leptin ($p = 0.06$); MCP-1 ($p = 0.92$); PDGF-BB ($p = 0.10$); TIMP-2 ($p = 0.81$), or VEGF-D ($p = 0.34$).

The results of this study indicate that that DynaMatrix may contribute to success in regenerative endodontic procedures by providing a predictable mechanical scaffold for cellular and vascular in-growth into the RCS. Additionally, it may improve angiogenesis by increasing the quantity of angiogenic growth factors present in the microenvironment via release of angiogenic cytokines present in DynaMatrix, as well as through possible interactions with the cells involved in regeneration that result in greater expression of angiogenic cytokines from these cells.

CURRICULUM VITAE

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