THE EFFECTS OF RADICULAR DENTIN TREATED WITH DOUBLE ANTIBIOTIC PASTE AND EDTA ON DENTAL PULP STEM CELL PROLIFERATION: AN *IN-VITRO* STUDY

by

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INTRODUCTION

The ultimate goal of endodontic treatment is to maintain the dentition in a physiologic functioning state by treatment of an infected root canal space. Pulpal necrosis and infection, perhaps from trauma or caries, with periapical pathosis of immature dentition present many challenges in maintaining the dentition long-term. Incomplete root formation in both length and width is typical in immature teeth and leads to difficulty in endodontic treatment.[1] These teeth will present with large canals with blunderbuss apices causing procedural challenges during cleaning, shaping, and obturation. Additionally, necrotic immature teeth with pulpal necrosis may also present with thin root walls and shorter root lengths leading to an increase in susceptibility to fracture,[2] decreasing the long-term prognosis. The traditional method of apexification addresses the issue of open apices by creating an apical hard tissue barrier in order to adequately complete the root canal procedure, but the problem of thinner root walls and shorter root lengths remains with this form of treatment.[3]

The regeneration of tissues into the canal space in immature teeth has been suggested to improve the long-term prognosis relative to the traditional method of apexification.[4,5] "The objectives of regenerative endodontic procedures are to regenerate pulp-like tissue, ideally, the pulp-dentin complex; regenerate damaged coronal dentin, such as following a carious exposure; and regenerate resorbed root, cervical or apical dentin."[6] The current recommended American Association of Endodontists (AAE) protocol[7] comprises of a two-step protocol. The first step involves canal disinfection by debridement using minimal instrumentation and copious irrigation with 1.5-percent

NaOCl solution.[8] Antibiotic paste (0.1 mg/mL) is placed in the canal for further disinfection and the access is sealed with a temporary material. One to four weeks later, the second step is initiated by re-access and the canal is irrigated with 17-percent Ethylenediamene Tetra-Acetic Acid (EDTA)[9,10] and rinsed with saline. The apical tissues are lacerated by over-instrumentation and bleeding is induced to form a blood clot into the root canal space. Mineral trioxide aggregate (MTA) is placed over the clot near the coronal third of the canal and the tooth is permanently restored.

The blood clot formed in the canal space serves as a tissue scaffold for stem cell migration, as well as other cells with hard-tissue-forming potential.[11] This migratory process into the root canal is thought to be the process resulting in hard tissue formation and further development of root length and width.[4,5,12] Numerous case reports document success and evidence of resolution of the infection and an increase in root length/thickness of the necrotic immature teeth.[13,14]

Even with successful clinical outcomes, there is limited evidence on the exact mechanisms of the process. One aspect with recent developing evidence has been the influence of intracanal solutions and their effects on the radicular dentin for cellular attachment and viability. For example, EDTA, a chelating agent, removes the smear layer, opens dentin tubules,[15] and releases growth factors from the dentin.[16-18] It has been shown that dentin pulp stem cells have an "intimate association" with dentin when pre-treated with EDTA.[19] Another study has found that EDTA promotes stem cell attachment while 2-percent chlorhexidine and 6-percent NaOCl are detrimental to stem cell viability on the root canal dentinal walls.[10] One recent study shows that antibiotic pastes affect dentin surfaces by demineralization and smear layer removal,[20] but there

exists limited evidence on stem cell attachment and viability on treated dentin walls based on this specific finding. These findings may suggest that antibiotic pastes have a benefit for stem cell attachment and proliferation similar to that of EDTA on radicular dentin. However, two recent studies suggest that antibiotic paste concentrations previously suggested in regenerative endodontic procedures have direct detrimental effects on the survival of human stem cells of the apical papilla (SCAPs) and human dental pulp stem cells (hDPSCs).[21,22] Both studies suggest the use of lower concentrations of medicaments that are not as toxic to pulp cells during endodontic regeneration. One recent study reported on indirect effects of triple antibiotic paste (TAP), double antibiotic paste (DAP), and calcium hydroxide (CaOH) showing results that high concentrations of antibiotic pastes should be avoided, but instead, low concentrations (1 mg) should be used.[23] However, there is still a lack of evidence regarding the indirect effects of medicaments in a combination of irrigants on dentin used in endodontic regeneration on stem cell toxicity and proliferation.

The aim of this in-vitro study was to evaluate the viability and cytoxicity of hDPSCs on human radicular dentin treatment with two different concentrations of DAP followed by EDTA. hDPSC were chosen as they are expected to be found in the apical papilla and are involved in the process of current regenerative endodontic techniques.[24]

CLINICAL SIGNIFICANCE

If the experimental groups reveal significantly different viability and cytotoxicity levels on dentin specimens treated with antibiotics and EDTA, the results may contribute

to the body of knowledge for further defining the current protocol, and to the work to develop a standardized protocol in regenerative endodontic procedures.

HYPOTHESES

1. Null: There is no significant difference in hDPSC viability, unattached cells, and cytotoxicity on dentin specimens among groups regardless of the type of dentin treatment or specimens left untreated.
2. Alternative: There is a significant difference in hDPSCs viability, unattached cells, and cytotoxicity among groups depending on the types of dentin treatment.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Charles Allen was credited with publishing the first book in English devoted exclusively to dentistry in 1687.[25] He described dental transplants by "taking out the rotten teeth or stumps and putting in their places some sound ones drawn immediately out of some poor body's head."[26]

In 1728 Pierre Fauchard, "the father of modern dentistry," provided descriptions of root canals and pulp cavities in his book "The Surgeon Dentist." Fauchard described pulp and canal anatomy and procedures of creating access holes in teeth for relieving abscesses. Later, these pulp chambers would be filled with foil.[25] Additionally, Fauchard described the use of a small pin for pulp extirpation and treatment for deep carious lesions with the application of different oils or cinnamon for several weeks and at times with opium.[25]

In the 18th century, the German dentist Phillip Pfaff was the first to mention a pulp-capping procedure, which involved cutting out a piece of gold or lead to fit over the pulp exposure.[25] Thomas Rogers further outlined pulp capping by presenting 202 successful cases out of 220.[25] His criteria for success included the following: good patient health, no inflammatory tendencies related to the patient, no history of previous pain, and no other disease associated with the tooth in question.

In 1757 Louis Bourdet described a process of intentional replantation by extraction of carious teeth, using gold or lead to fill the root canals, followed by replanting the tooth. He also described an "intentional" endodontic treatment involving

the dislocation of the tooth in order to sever the nerve, followed by immediate replacement back into the socket.[26] Bourdet was not the first to treat teeth in this manner as the same procedure had been described in the 11th century by Avicenna, an Arabian physician.[27]

Robert Woofendale, an English practitioner in New York, is credited in 1766 with performing the first endodontic procedures in the US by cauterizing the pulp with heat, followed by placement of cotton pellets into the canals.[28,29] Near the end of the 18th century, Frederick Hirsch, a German dentist, wrote about diagnosing dental disease by tenderness to percussion. He recommended perforation of the cervical area of the tooth and repeated insertion of a red-hot probe. Lead was used to fill the cavity.[27]

The early 19th century has been called "The Vitalistic Era" as some began to recognize the problem of vitality in connection with pulp treatment.[25] 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.[30] In 1809 Edward Hudson, an Irish practitioner in Philadelphia, is typically given credit for being the first to place fillings in root canals.[28,31] Hudson was considered innovative for his time due to his advocacy for preservation of natural dentition.[32]

John Callow from London published *Opinions on the Causes and Effects of Diseases in the Teeth and Gums* in 1819. In this work, he credited 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. Bew joined others of his time in subscribing to the "vitalistic theory" of teeth.[30]

In 1826 a German immigrant named Leonard Koecker wrote *Principles of Dental*

Surgery in 1826. This book became a standard in the field for 50 years.[32] Koecker believed when the pulp was destroyed artificially or by disease, the dentinal core immediately died. The tooth would become a foreign body requiring extraction to prevent further extraction. To avoid tooth loss, Koecker popularized the pulp capping.[30,33,34]

In 1829 SS Fitch formulated and presented the principles of the "vitalistic" or "double membrane" theory.[25] He wrote *Systems of Dental Surgery* advocating that teeth were like hollow bones with an outer periosteum (periodontal membrane) and an inner periosteum that lay between the pulp and the dentin. Furthermore, he believed that the crown was nourished exclusively by the dental pulp or lining membrane, whereas the roots were supplied by the pulp membrane on the interior and by the alveolar membrane on the exterior.[25] This led to the practice of decoronation of teeth after extirpation of the pulp and then leaving a root in the socket and placing a pivot crown on the remaining root.[30,34] It was believed the root continued receiving nourishment via the periodontal membrane.

An individual advocating a "nonvitalistic"[25] concept included British surgeon and anatomist John Hunter. He believed that dentin possessed no properties of living tissue and was lacking any circulation, sensibility, and capability of repair. Other individuals advocating the "nonvitalistic" concept included Cuvier and Robertson from England.[30]

Prior to 1836 pulp extirpation was considered a very painful procedure. This was significantly changed when Shearjashub Spooner of New York began using a protoplasmic poison (arsenic trioxide) to devitalize prior pulp prior to extirpation.[28] In actuality, Spooner was not the first to use arsenic in practice as the use of arsenic dates

back to ancient Chinese medicine for treatment for jaw abscesses.[27] The use of arsenic treatment became popular due to its success of painless pulp extirpation. Unfortunately, the imprudent use of arsenic led to devitalization of pulps, treatment of teeth with dentin hypersensitivity, and leakage through the apical foramen leading to periodontal tissue destruction.[35]

In 1837 Jacob and Joseph Linderer advocated by publication the use of essential or narcotic oil to render the pulp insensible prior to restoring a tooth with an exposed pulp.[36] In 1838 Edwin Maynard developed the first root canal broach and designed hoe-like instruments for shaping and enlarging root canals.[37] In 1839 Baker was first to describe by publication the processes of pulp extirpation, canal cleaning, and canal filling.[25,27]

Throughout the 1850s, plugs of wood soaked in creosote were used as root canal obturation material.[25] Liquid cement was used in conjunction with the wooden plug to create a seal. This was an early attempt at obturation using a solid root-filling point and cement or sealer. This early sealer was composed of gutta-percha, quick-lime, powdered glass, feldspar, and metal filings.[35]

In 1857 at the Odontological Society of London meeting, Thomas Rogers gave a presentation on pulp capping reporting on a series of 220 pulp capping cases, of which 202 cases were considered successful. The outline he provided for the conditions of success in nerve capping was as follows: general good health of the patient, freedom from inflammatory tendencies, absence of previous considerable pain in the tooth, absence of disease in other parts of the tooth, and no caustics to deaden the pain. Rogers' overall assessment and treatment recommendations were worthy of early scientific

appraisal, except for the final recommendation of application of three leeches and a laxative in pulp capping failure.[25]

In 1864, S.C. Barnum of New York introduced the use of the rubber dam during gold foil restoration placement. Rubber dams were quickly incorporated into root canal filling procedures to provide a more aseptic environment during procedures.[27,28] In 1867 G.A. Bowman of Missouri was given credit by numerous authors for being the first to use just gutta-percha for filling root canals.[28,31,38] Bowman also co-invented the rubber dam clamp forceps in 1873.[28]

Also in 1867 the German team of Leber and Rottenstein proved the existence of a parasite 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 antiseptic treatment to pulp treatment.[25,36] In this same year, Magitot suggested pulp testing using electric current.[33]

In the late 1870s, the septic theory began gaining traction while the theory of vitalism was beginning to give way. The septic theory asserted that pathogenic organisms were the most common cause of diseases of the pulp. Dr. G. O. Rogers suggested in an article published in *Dental Cosmos* in 1878 that pathogenic organisms might be the most common causes of diseases of the pulp. He concluded that successful treatment required the total destruction of these organisms. This theory was also advocated and strengthened by Charles S. Tomes in 1879 suggesting that dentin loses its vitality by septic conditions or arsenic, and that cementum also becomes infected leading to involvement of the periodontal membrane.[30] In 1882, Arthur Underwood further supported the septic theory

by suggesting that suppuration of the pulp and resultant alveolar abscesses could be prevented if pathogens could be successfully excluded from the pulp space through the use of powerful antiseptic agents.[25] This idea provided new justification for pulp therapy procedures using caustic germicides for bacterial elimination and would be widely accepted for more than 30 years.[30]

In 1895 Dr. Bowman of Missouri introduced chloropercha. Chloroform solution was used with gutta-percha cones for obturation of root canals. This technique quickly gained wide acceptance among dentists and techniques were further developed.[28]

In 1905 Einhorn developed procaine, also known as Novocaine. Procaine provided an alternative to the previously used cocaine. Cocaine had been used sparingly as an anesthetic agent for 20 years due to its high toxicity. Though it represented a promising breakthrough in dental pain control, the initial usage of procaine for mucosal injections was hindered by an inefficient protocol that required dissolving the tablet in solution, boiling, cooling, and aspiration into a syringe. Nearly 25 years later, block anesthesia techniques to be perfected.[35,37,39]

In 1908 Dr. Meyer L. Rhein, a physician and dentist from New York, developed a technique for canal length and degree of obturation that utilized a diagnostic wire in conjunction with radiographs.[28,33] G.V. Black also suggested a measurement control to determine the length of canals and the sizes of the apical foramen to keep materials within the root canal system.[25]

In the second decade of the 20th century, the “Focal Infection Theory” began permeating in the medical and dental community. This idea would eventually postulate a myriad of diseases caused by microorganisms (bacteria, fungi, or viruses) that arise

endogenously from a focus of infection.[40] Though this concept was not truly new, it experienced a resurgence due in part to the work of E.C. Rosenow.[40] In 1909 Rosenow's work showed that streptococci were present in many diseased organs, and these same bacteria were capable of traveling in the bloodstream to establish an infection at a separate distant site.[39] Another major event that accelerated the focal infection theory to widespread acceptance[25] 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 *Lancet.*[25] The popularity of the theory of focal infection led to scrutiny of endodontic procedures. Many physicians and dentists began to recommend extractions of all pulpless teeth. These clinicians were known as the 100 percenters. The popular philosophy of the 100 percenters persisted up until approximately 1930 when the philosophy began to swing back towards more conservative approaches.[25] This change included a refocusing and emphasis on definitive diagnosis, aseptic techniques, bacteriological culturing, and improved radiographic practices in conjunction with root canal therapy.[41] It would take approximately a decade before this more conservative philosophy would gain acceptance in practice and be taught in dental school.[25]

In 1937 scientific evidence based on sound histological, biological, and pathological findings paved a period referred to by some as "the scientific era."[25] Work by Logan showed that bacteria can be present in normal tissues without having pathological effects.[29] Tunnicliff and Hammond reported that microorganisms could be found in the pulps of extracted teeth "without any evidence of inflammatory tissue changes."[25,39] Also, 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 of a "mere causal relationship."[25,42] The type of work as previously mentioned helped to stop the practice of wholesale extraction of non-vital teeth and transition a new era in root canal therapy.[25]

In the early half of the 1940s, Fred Adams and Louis Grossman steer the idea of antibiotics as an adjunct to root canal therapy. Adams reported the use of sulfanilamide in treating periapical infections and is credited as the first to use penicillin in pulp canal therapy.[25,39] Grossman advocated the use of a more stable and economical non-aqueous formulation of penicillin for use in endodontics. Dr. Grossman later used penicillin-saturated paper points to disinfect root canals.[31]

The year 1943 marked the birth of organized endodontics by the formation of the American Association of Endodontists (AAE) in Chicago, Illinois. By 1949 this group had formed a committee for investigating the possibility of establishing a specialty board in endodontics. By 1956 the American Board of Endodontics was formed marking the beginning of the specialty board.[44] In 1963 the American Dental Association recognized endodontics as a specialty due to development and growth of endodontics during the previous 25 years and the relentless efforts of AAE leaders.[43] The first examination and certification of Diplomates occurred in 1965.[43] The American Association of Endodontists currently has approximately 7000 members and approximately one-fourth of those are board certified.[44]

THEORY OF ENDODONTICS

The objective of endodontic therapy is to eliminate microbial insult to the pulpal and periapical tissues to maintain natural form, function, and esthetics.[45] One foundational study in endodontics best illuminates this objective. In 1965, this study showed that exposed pulps of gnotobiotic rats survived even after being subjected to food impaction regardless of the trauma under sterile conditions.[46] This study showed the significance of bacteria involved with endodontic disease and the important role of reducing bacterial load in endodontic treatment.[47] Without this reduction, apical periodontitis, defined as destruction and inflammation of the periodontium, can occur with or without symptoms.[48]

In 1955 Stewart emphasized three phases of treatment: chemomechanical preparation, microbial control, and obturation of the root canal.[49] Furthermore, Stewart emphasized that the chemomechanical preparation was most important. Grossman further confirmed the importance of chemomechanical preparation with his 13 principles of effective root canal therapy.[50] These principles included the following: use of aseptic technique; instruments should stay within the root canal; instruments should never be forced apically; canal space must be enlarged from its original size; root canals should be continuously irrigated with an antiseptic; solutions should remain within the canal space; fistulas do not require special treatment; a negative culture should be obtained before obturation of the root canal; a hermetic seal of the root canal should be obtained; obturation material should not be irritating to the periapical tissues; and if an acute alveolar abscess is present; proper drainage must be established; injections into infectious areas should be avoided; as well as apical surgery may be required to promote healing of

the pulpless tooth.

In 1967 Schilder discussed further principles regarding chemomechanical instrumentation, antiseptics, and three-dimensional obturation to the cementodentinal junction or 0.5 mm to 1 mm from the radiographic apex.[51] Ford followed outlining three reasons why three-dimensional obturation was important.[52] The three reasons were that it leaves less space for bacterial colonization; it prevents apical contamination, and it prevents bacterial movement along the canal wall. He also emphasized aseptic technique, adequate coronal restorations, and recall appointments to monitor healing.

IRRIGATION

As Schilder mentioned,[51] one of the most critical parts to non-surgical root canal therapy is irrigation. Studies have shown that up to 53 percent of canal walls are left untouched from the limitations of instrumentation.[53,54] Irrigants can often reach these areas that are left untouched from instrumentation. Harrison outlined desirable properties of endodontic irrigants as follows: antimicrobial efficacy, tissue dissolution capacity, biocompatibility, and ability to adjunctively debride the root canal system.[55] There are numerous endodontic irrigants that are utilized clinically under these principles.

Sodium Hypochlorite

Sodium hypochlorite (NaOCl) is a potent antimicrobial and tissue dissolution agent. NaOCl has shown a superior ability for microbial elimination within the root canal system in comparison with saline.[56] NaOCl is used at various concentrations, but studies testing concentrations show enhanced tissue dissolution activity and anti-microbial activity at higher concentrations than lower concentrations.[57] Increasing

temperature and duration of exposure can increase its tissue-dissolving efficacy.[58] The increase of temperature can also increase bactericidal effects.[59] With NaOCl's numerous desirable characteristics, it is frequently considered the single most effective endodontic irrigant.[60,61]

Chlorhexidine

The broad spectrum anti-microbial chlorhexidine (CHX) is available in 0.12-percent and 2-percent concentrations. The mechanism of action involves its positive electrostatic charge that binds to the negatively charged bacterial wall resulting in permeability in the cell wall disturbing the bacteria's ability of regulating its internal environment.[62,63] One of the major advantages of CHX is its substantivity, which allows it to remain on dentinal walls for extended periods of time leading to an inhibition of bacterial colonization on surfaces.[64] The antimicrobial activity is directly related to its concentration as 2-percent formulations have been shown to be more effective endodontic irrigants when compared with 0.12 percent formulations.[65] CHX has been advocated as a final irrigant in endodontic treatment due to its favorable properties, especially in endodontic retreatment cases.[66]

The main disadvantage of CHX when compared with NaOCl is that it does not possess tissue dissolution properties. Considering this disadvantage, CHX should be used more as an adjunct endodontic irrigant to NaOCl to maximize the chemomechanical debridement of the root canal system. However, the direct mixture of CHX with NaOCl can cause the precipitation of a potential carcinogen. This carcinogenic precipitate was initially identified as para-chloroaniline,[67] but further studies have shown that the precipitate is actually para-chlorophenylurea and para-chlorophenylguanidly-1,6-

diguanidyl-hexane.[68] Studies have shown that using absolute isopropanol intermediate irrigant between NaOCl and CHX can prevent this precipitate formation.[69]

EDTA

Mechanical debridement during root canal therapy results in a smear layer over the dentin surfaces. The smear layer consists of organic and inorganic substances such as fragments of odontoblastic processes and necrotic debris,[70] and fragments of hydroxyapatite. EDTA is frequently used as an endodontic irrigant in the removal of the inorganic component of the smear layer by its chelating action. The common concentration of 17 percent used in endodontics has been shown to remove smear layer best when used in conjunction with NaOCl allowing deeper penetration of irrigants into dentinal tubules.[71,72] There exists debate as to whether the smear layer should be removed prior to obturation, but a recent systematic review and meta-analysis concluded that the fluid tight seal is improved during obturation if the smear layer is removed.[73]

OBTURATION

The final part to non-surgical root canal therapy is obturation. The goal of obturation is to hermetically seal the root canal system to prevent infection of the periapical tissues.[74] Most obturation techniques use gutta percha as core material and sealer to achieve the hermetic seal.

The quality of the obturation is a factor influencing the success of treatment. One aspect of quality is the length of the obturation or whether the obturation material stays within the root canal system. A meta-analysis addressing the length of the obturation found increased success rates when obturation material did not overextend beyond the

apex.[75] Further studies have shown that warm gutta-percha obturation techniques result in a higher rate of overextension of obturation material.[76] The last step that adds benefit to sealing the obturation material is an adequate coronal restoration. The highest rates of success are achieved by have adequate coronal restoration, which provides a coronal seal amongst other benefits.[77]

REGENERATIVE ENDODONTICS

Background

Regenerative endodontic involves "biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex."[6] Its application is specific to the treatment of immature necrotic teeth as they present with many challenges in treatment. These types of teeth present with blunderbuss apices with underdevelopment of root structure. Thin root walls are characteristic of these types of immature teeth as well. These characteristics cause difficulty with traditional methods of obturation for apical seal,[78] as well as increase the root's susceptibility to fracture.[2]

The concept of regenerative endodontics is not novel,[79,80] but recent attention has been given since a modern case report by Iwaya[81] in 2001. The report detailed that the canal was disinfected non-mechanically with 5-percent NaOCl and 3-percent H_2O_2 followed by a combination of metronidazole and ciprofloxacin for further disinfection for 5 weeks. A layer of calcium hydroxide was placed against the apical tissue and the access was sealed with glass-ionomer cement followed by adhesive composite resin. At the 30-month follow-up, the report showed results of further development of the root

structure after treatment.

Protocol

The procedure of regenerative endodontics involves two parts. First, the canal is entered and disinfected with irrigants and an intracanal medicament. The second part involves re-entry of the canal system, preparation of the canal wall surface by other irrigants, induction of a blood clot, and closure with a biocompatible material and restoration. More specifically, the current recommended American Association of Endodontists (AAE) protocol[7] comprises the previously mentioned two-step protocol. The first step involves canal disinfection by debridement using minimal instrumentation and copious irrigation with 1.5-percent NaOCl solution.[8] Antibiotic paste (0.1 mg/mL) or CaOH paste is placed in the canal for further disinfection and the access is sealed with a temporary material. One to four weeks later, the second step is initiated by re-access and the canal is irrigated with 17-percent EDTA[9,10] and rinsed with saline. The apical tissues are lacerated by over-instrumentation and bleeding is induced to form a blood clot into the root canal space. Mineral trioxide aggregate (MTA) is placed over the clot near the coronal third of the canal and the tooth is permanently restored.

The recommended protocol by the AAE appears to be an evolving guideline as research regarding endodontic regeneration continues to surface. Previous guidelines recommended using 1 g/mL concentrations,[82] while the most current guidelines recommend using antibiotic paste at 0.1 mg/mL[7] in response to direct cytotoxicity studies.[21] One recent study reported more relevant indirect effects on cell viability by showing no effects on cell viability when 1 mg/mL of double antibiotic paste (DAP) or triple antibiotic paste (TAP) on dentin disk specimens.[23] One may also argue that a

minimum of concentration of 0.14 mg/mL for DAP and 0.3 mg/mL for triple antibiotic paste (TAP) is needed based on bactericidal studies.[83] The current recommendation also states that CaOH or antibiotic paste can be used as the intracanal medicament. But based on the study by Bose,[4] better outcomes were seen with root width when antibiotics were used as an intracanal medicament as opposed to CaOH. No significant differences in resultant root length were seen between the two medicaments. As further studies in regard to protocol become published, the recommendation will become further defined on regenerative endodontics.

Scientific Principle

The regeneration of tissues into the canal space in immature teeth has been suggested to improve the long-term prognosis relative to the traditional method of apexification.[4,5] "The objectives of regenerative endodontic procedures are to regenerate pulp-like tissue, ideally, the pulp-dentin complex; regenerate damaged coronal dentin, such as following a carious exposure; and regenerate resorbed root, cervical or apical dentin."[6]

The blood clot formed into the canal space serves as a tissue scaffold for stem cell migration as well as other cells with hard-tissue-forming potential.[11] This migratory process into the root canal is thought to be the process resulting in hard tissue formation and further development of root length and width.[4,5,12] Numerous case reports show success and evidence of resolution of infection and increase in root length/thickness of necrotic immature teeth.[13,14]

The sources of stem cells involved in regenerative endodontics are from the dental pulp and tissues of the periodontium.[6] More specifically, dental pulp stem cells,[84] stem

cells of the apical papilla,[85,86] and periodontal ligament stem cells[87] have been identified and characterized. These cells can differentiate into odontoblast-like cells and produce dentin-like structures resulting in the observed root growth.[88]

Mechanisms and Recent Findings

Even with successful cases of clinical outcomes, there is evidence still being established on the exact mechanism of the process to further define a standard protocol. One aspect with recent developing evidence has been the influence of intracanal solutions and their effects on the radicular dentin for cellular attachment and viability. For an example, EDTA (a chelating agent) conditions the dentin by the removal of smear layer, opening of dentin tubules,[15] and the release of growth factors from the dentin.[16-18] It has been shown that dentin pulp stem cells have an "intimate association" with dentin when pre-treated with EDTA.[19] Another study has found that EDTA promotes stem cell attachment while 2-percent chlorhexidine and 6-percent NaOCl are detrimental to stem cell viability on root canal dentinal walls.[10] One specific study studying indirect effects established that 1.5-percent NaOCl concentration is what should be used for protocol.[8]

One recent study shows that antibiotic paste affects dentin surfaces by strong demineralization and smear layer removal,[20] but limited evidence exists about stem cell attachment and viability on treated dentin walls based on this specific finding of treated dentin. This finding may suggest that antibiotic pastes may have a similar benefit for stem cell attachment and proliferation as EDTA on radicular dentin. However, two recent studies suggest that antibiotic paste concentrations previously suggested in regenerative endodontic procedures have direct detrimental effects on the survival of SCAPs and hDPSCs.[21,22] Both studies suggest the use of lower concentrations of medicaments that

are not as toxic to pulp cells during endodontic regeneration. Another recent study reported on indirect effects of TAP, DAP, and calcium hydroxide (CaOH) and showed results that high concentrations of antibiotic pastes should be avoided and low concentrations (1 mg/mL) should be used.[23] These *in-vitro* studies do have limitations in that results do not necessarily correlate with the success of clinical cases.

There have also been suggestions from reports towards a transition away from the use of minocycline in triple antibiotic pastes[89] due to its dentin binding and staining properties.[90,91] As a substitute, DAP[92] and modified triple antibiotic pastes replacing the minocycline[93] have been suggested. The transition away from minocycline has shown to be a consideration of recent studies.[20,23,83,94]

As mechanisms of regenerative endodontics protocol are further studied and defined, further adjustments will be made in order to increase success and predictable outcomes.

MATERIALS AND METHODS

EXPERIMENT DESIGN

Groups of 4 mm x 4mm x 1 mm human radicular dentin specimens were prepared and treated with 500 mg/mL DAP (G1), 1 mg/mL DAP (G2), 500 mg/mL DAP followed by 17-percent EDTA (G3), 1 mg/mL followed by 17-percent EDTA (G4), or 17-percent EDTA (G5) and evaluated for viability, unattachment, and cytoxicity for hDPSC proliferation (Figure 1). The DAP treatment phase occurred for one week. One week of treatment with DAP was chosen based on current recommendations.[7]

INCLUSION/EXCLUSION CRITERIA

Human mandibular premolar and anterior teeth were collected and stored in 0.1-percent thymol at 4°C. Inclusion criteria were as follows: single root, complete root formation, and at least 4- mm mid-root diameter in either buccolingual or mesiodistal direction as determined with a caliper. Exclusion criteria were as follows: caries, restorations, hypocalcification, hypoplasia, cracks, or defects. Specimens were re-examined after preparation and excluded if surface defects or cracks were present.

SPECIMEN PREPARATION

Fourteen teeth that met the initial inclusion/exclusion criteria were prepared into 28 specimens (Fig. 2). Teeth were removed from 0.1-percent thymol and rinsed in deionized (DI) water for 10 seconds. Teeth were decoronated and the roots were sectioned buccolingually (Figure 3) using a high-speed saw with water irrigation (Figure 4) (Lapcraft, L'il Trimmer, Powell, OH). Each sectioned half-root were secured to a thick

acrylic plate with sticky wax and cut to a 4x4 mm dimension (Figure 5) with a double-bladed Isomet low speed saw (Buehler, Lake Bluff, IL) with water (Figure 6). Specimens were secured to a 38-mm cylindrical mounting block (Struers Inc., Cleveland, OH) with sticky wax with the cementum side facing up. Specimens were then placed into the Struer's cylinder holder and flattened with 500-grit paper at 300 rpm with water until approximately 1.6-mm specimen thickness remained using the RotoPol31/RotoForce-4 (Struers Inc.) (Figure 7). Specimens were removed from the mounting block and cleaned of sticky wax mechanically and confirmed visually. Specimens were re-mounted using sticky wax with the dentin side facing up. The cylinders with attached specimens of equal height were polished using the RotoPol 31/Rotoforce-4. The specimens were polished using 500-grit, 1200-grit, 2400-grit, and 4000-grit paper for 30 s, 40 s, 50, and 60 s, respectively, at 300 rpm with water. For each grit paper, specimens were ground until the surface of each specimen was uniformly polished. Sandpaper grit was removed from the specimens by submerging them in a flowing DI water bath for 3 minutes, ultrasonic DI water bath for 3 minutes, flowing DI water bath for 3 minutes again. The specimens were air-dried. A final polish of the specimens was accomplished on the RotoPol/31/Rotoforce-4 using a polishing pad with 1-µm diamond suspension (Struers Inc) for 3 minutes at 150 rpm without water. The diamond polishing suspension removed by placing the specimens in a flowing DI water bath for 3 minutes, followed by an ultrasonic 2-percent micro rinse solution bath for 3 minutes, and then back to a flowing DI water bath for 3 minutes again. The specimens were air-dried. Sticky wax was removed from the specimens. To confirm that the samples were entirely polished, all samples were visually inspected. The polished specimens were placed dentin side up in a

covered container lined with paper towels moistened with 0.1-percent thymol and placed into storage 4°C until sterilization with ethylene oxide had been completed.

TREATMENT

Double antibiotic consisting of a 1:1 ratio of ciprofloxacin and metronidazole (Champs Pharmacy, San Antonio, TX) (DAP) was mixed with distilled water to create two mixtures of two different concentrations (500 mg/mL and 1 mg/mL). DAP was chosen based on one of the first reports of success of pulpal regeneration[81] and based on reports of a transition away from use of minocycline in triple antibiotic pastes[89] due to its dentin staining properties.[90,91] The 500-mg/mL DAP concentration was chosen based on creating a creamy antibiotic paste based on pilot studies. The use of antibiotic pastes was the prior recommendation to the current AAE recommendation.[82,89] The 1 mg/mL DAP concentration was chosen based on a current toxicity report[23] and an antimicrobial report of minimum bactericidal DAP concentration of 0.14 mg/mL.[95] All specimens were placed in 48-well flat bottom cell culture plates (BD Falcon, San Jose, CA). Specimens in G1 and G3 were submerged with a 400 uL of 500 mg/mL DAP, and specimens in G2 and G4 were submerged with 400 uL of 1-mg/mL DAP for 1 week at 37°C with 100-percent humidity. After incubation, the specimens were removed and rinsed with 10 mL of distilled water using a micropipetter until all visible DAP has been removed. Specimens from G3, G4, and G5 were treated with 20 mL 17-percent EDTA (Champs Pharmacy, San Antonio, TX) for 10 minutes by applying and exchanging at a rate of 2 mL of 17-percent EDTA per minutes. Another group of specimens (G6) specimens did not receive any treatment and served as a negative control treatment. Each specimen was then rinsed with 500-uL phosphate buffered saline (PBS) and transferred over to a 96-well not cell

culture treated sterile plate (Thermo Scientific, Waltham, MA) with the treated side facing up for hDPSC seeding. The 96-well not cell culture treated sterile plates was designed for cells to not adhere to the bottom of the wells, and more specifically, around the dentin specimens in the experiment.

CELL CULTURE

hDPSCs (Cook General BioTechnology, Indianapolis, IN) were cultured using Dulbecco's Modified Eagle's Media (DMEM) (Hyclone, Logan, UT) supplemented with 10-percent fetal bovine serum (FBS) (Hyclone), 4 mM L-glutamine (Hyclone), 100 U/ml penicillin (Invitrogen), 50-µg/ml gentamicin (Invitrogen), and 2.5-µg/ml fungizone (Invitrogen). hDPSCs at passage 3 to 8 were utilized. 10,000 hDPSCs suspended in 100uL DMEM with FBS were seeded in each appropriate well and given 24 hours to attach to the dentin specimens. After the attachment period, the supernatant was collected from each sample and 2 uL x 50 uL DMEM with FBS was used for washing each specimen to wash out any residual unattached cells on the well surface and collected for unattached cell analysis using the lactose dehydrogenase (LDH) assay (Roche Applied Science, Indianapolis, IN). Visual inspection under the microscope of each well on the periphery of each specimen confirmed that unattached cells from the well surfaces surrounding the dentin specimens were removed. All specimens were received DMEM 2-percent FBS and given 3 days for proliferation. The water soluble tetrazolium (WST) viability (Roche Applied Science, Indianapolis, IN) and LDH cytotoxicity assays were performed at the end of the 3-day proliferation period.

CONTROL GROUPS:

G6 contained specimens that were not treated with DAP or EDTA and served as a positive control for cell seeding. Another set of wells (G7) did not contain specimens, but was subjected to cell seeding and DMEM washings as previously described to ensure cells were not attaching to the plate wells. Another set of specimens (G8) was left untreated and unseeded with cells to serve as a negative control.

LDH ASSAY

LDH cytotoxicity assay is based on the measurement of LDH activity released from the cytosol of damaged cells. The principle of this reaction involves two steps. The first step involves the conversion of lactate from the reagent added to pyruvate by the catalytic activity of LDH resulting in the reduction of NAD^+ to $NADH/H^+$. The second step involves the catalyst diaphorase mediating transfer of H^+ from $NADH/H^+$ to a tetrazolium salt to reduce into formazan. The formazan dye colorimetric results are representative of LDH activity and are quantified using a microplate spectrophotometer. LDH assays using the Cytotoxicity Detection KitPlus (Roche Applied Science, Indianapolis, IN) was performed on all samples to quantify unattached cells and cytoxicity of hDPSCs from treated and untreated dentin specimens. Then, 100 uL of unattached lysed cell supernatant from each sample 1 day after seeding of cells and supernatant 3 days after changing media was taken and transferred to another clear 96-well plate. One hundred (100) uL of fresh mixed cytoxicity reagent was added and was given 30 minutes for reaction time at room temperature. The samples were read using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 490 nm wavelength and a reference wavelength of 650 nm giving a quantified result of

cytotoxicity from each sample. Calculations were performed relative to 10,000 lysed hDPSCs.

One day after cell seeding, the supernatant from each sample was collected. A lysis buffer was added provided from the LDH assay kit and the LDH assay was performed. The unattached cells 24 hours after seeding onto specimens contained in this supernatant can undergo cell death releasing LDH, but this occurs at a variable rate. To quantify undamaged, unattached cells in addition to the LDH present from dead unattached cells, each sample was subjected to intentional lysis to sum the total LDH present from cell death and LDH released from lysis to undamaged cells. This total LDH was quantified to represent the total number of unattached cells for each sample.

WST PROLIFERATION ASSAY

WST proliferation assay is a scientific method with numerous advantages[96] to quantify cell proliferation by the reduction of WST salts to water-soluble colored formazan compounds by succinate-tetrazolim reductase, which is active in mitochondria of viable cells. With the addition of WST to the media of metabolically active cells, the degree of color changes from the formazan dye are representative of viable metabolically active cells. The colorimetric results are then quantified using a microplate spectrophotometer.

A WST proliferation assay using Cell Proliferation Reagent WST-1 (Roche Applied Science) was performed on all samples to quantify proliferation of hDPSCs on dentin specimens. Media were switched out to 100 uL of DMEM without serum for each sample at 3 days after cell seeding. Ten (10) uL of WST reagent was added to each and given 2 hours for reaction time. One hundred (100) uL of reaction mixture was taken

from each sample and transferred to a new clear 96-well plate and read using a microplate spectrophotometer at 450-nm wavelength and a reference wavelength of 650 nm giving a quantified result of viability from each sample. Background readings were subtracted from each sample and relative viability was calculated based on the untreated specimen control group (G6).

STATISTICAL METHODS

All data were checked for normality using the Shapiro-Wilk test and the normality assumptions were satisfied. The effects of the type of dentin treatment on cytotoxicity and attachment of pulp stem cells were examined using one-way ANOVA followed by Tukey-Kramer post hoc comparisons. A 5-percent level of statistical significance was applied for all analyses.

RESULTS

LDH ASSAY OF UNATTACHED CELLS

The LDH assay of all unattached lysed cells showed significantly higher levels of unattached cells from specimens applied with 500 mg/mL of DAP and 1 mg/mL DAP when compared with all other sample groups ($p < 0.05$). All other sample groups did not show any significant difference with inter-group comparison ($p < 0.05$) (Figure 8, Figure 11).

WST VIABILITY ASSAY OF CELL ON SPECIMENS AFTER 72 HOURS

The WST viability assay showed a significant decrease of viability for specimens when treated with 500 mg/mL of DAP, regardless of treatment with or without EDTA when compared with all other groups ($p < 0.05$). Specimens treated with just 500 mg/mL showed almost no viability of the cells. The highest level of viability was observed on specimens just treated with 17-percent EDTA, but was not statistically significantly different when compared with all other groups, except those treated with 500 mg/mL DAP with or without EDTA. Specimens treated with 1 mg/mL DAP showed no significant difference when compared with the group treated with 500 mg/mL DAP with 17-percent EDTA. Figure 9 and Figure 12 illustrate the results of the WST assay.

LDH CYTOTOXICITY ASSAY AFTER 72 HOURS

Cytoxicity was significantly lower in the group treated with the 500 mg/mL DAP with or without 17-percent EDTA, when compared with groups treated with 1mg/mL

DAP with 17-percent EDTA, the control, and 17-percent EDTA. Cytotoxicity was highest with the 17-percent EDTA treatment group, but not significantly higher when compared with the control and the treatment with 1mg/mL DAP with 17-percent EDTA. There was no significant difference among 1 mg/mL DAP, 1 mg/mL DAP with 17-percent EDTA, and the control. Figure 10 and Figure 13 illustrates the results of the LDH cytotoxicity assay after 72 hours.

PROLIFERATION

Data accumulated and summed from all assays resulted in quantification of total cells involved. This total number of cells from all assays showed an increase in total cells when compared with the original 10,000 cells that were seeded in certain groups representing proliferation. Proliferation trends generally followed the trend of the WST viability assay (Figure 14). Very minimal proliferation was seen in the groups treated with 500 mg/mL DAP with and without 17-percent EDTA treatment.

FIGURES

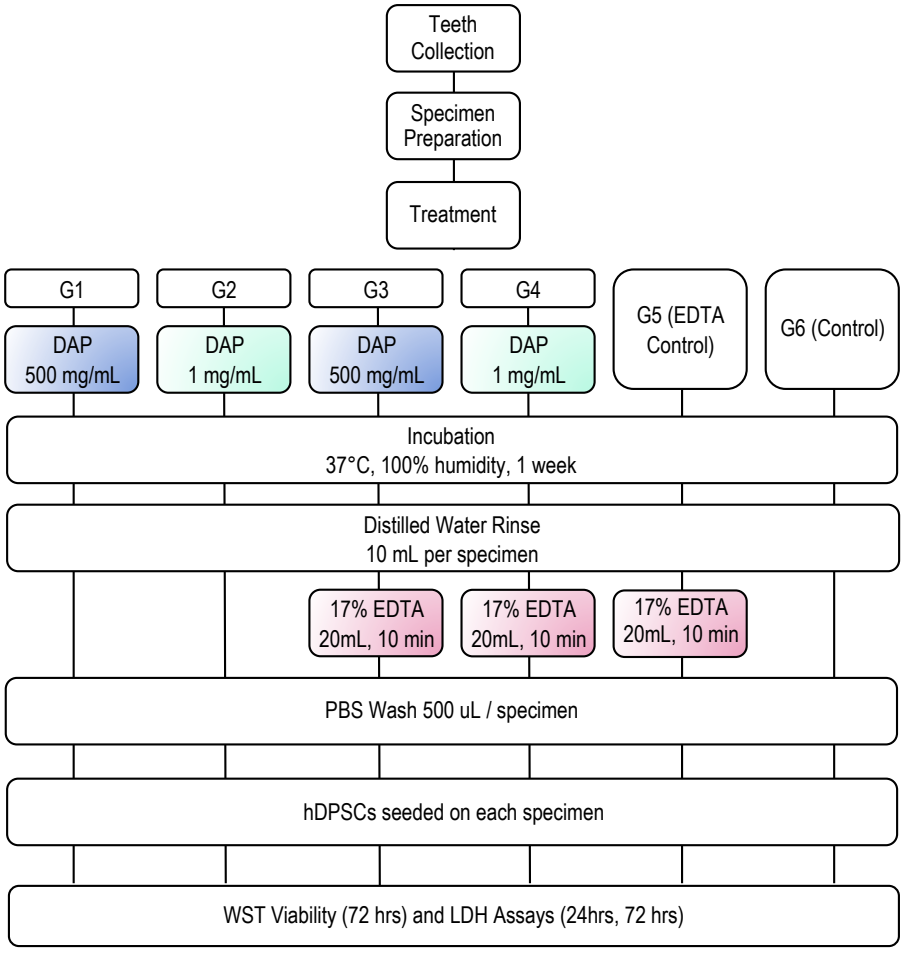


FIGURE 1. Experiment design flowchart.

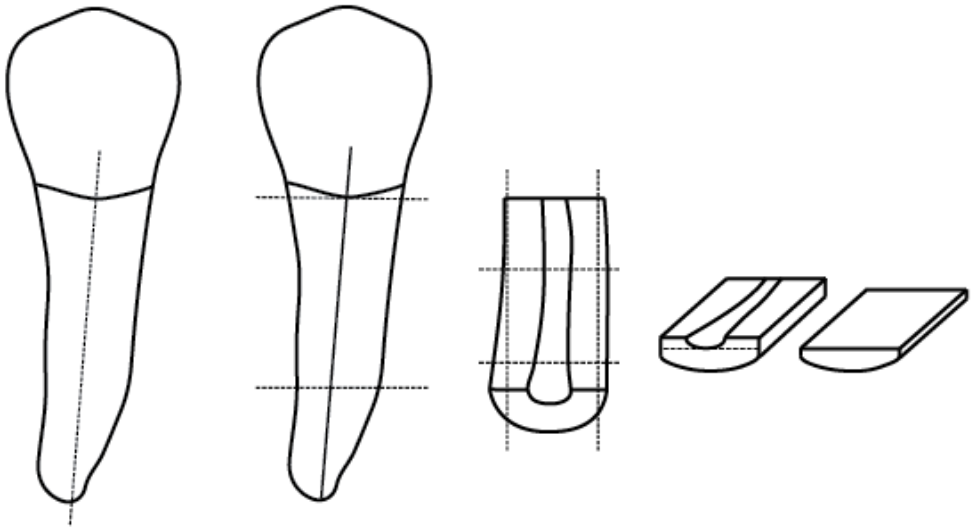


FIGURE 2. Overview of specimen preparation: Each tooth was sectioned, cut to 4x4 mm, and the inner surface was flattened.

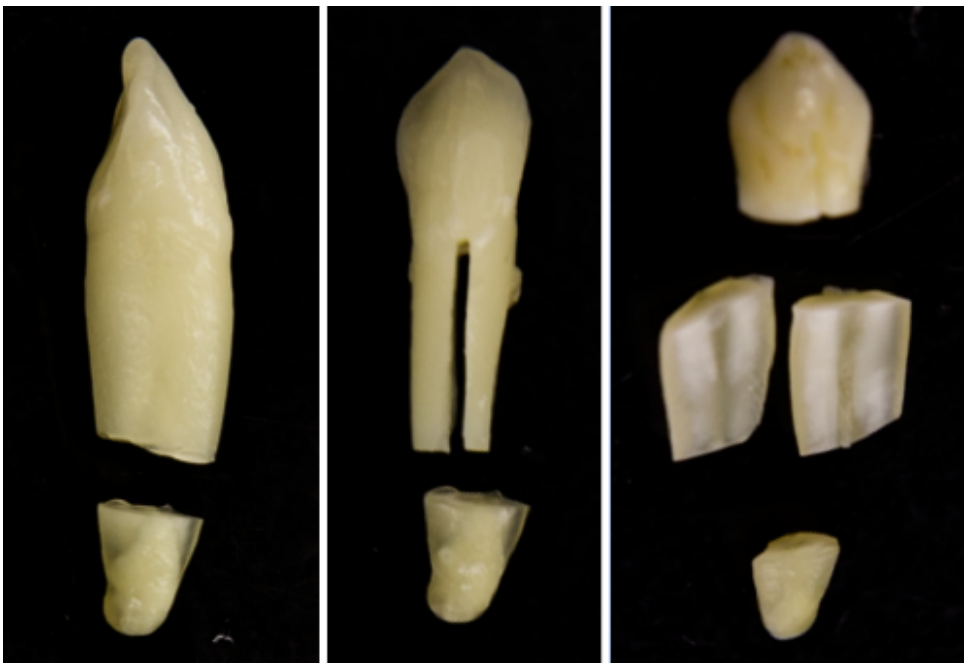


FIGURE 3. Teeth were sectioned using a high-speed saw with water irrigation.

FIGURE 4. The high-speed saw that was used with water irrigation (Lapcraft L'il Trimmer).

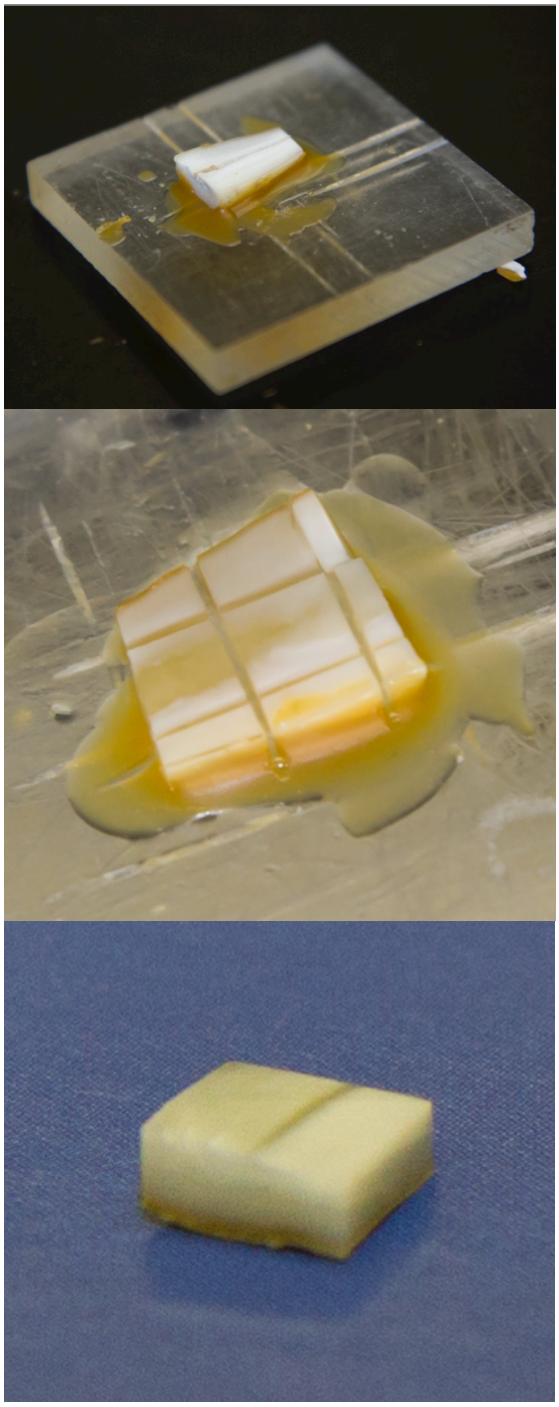


FIGURE 5. Each half-root was cut into a 4x4 mm square with a double-bladed low-speed saw with water.

FIGURE 6. The low-speed saw that was used with water irrigation (Isomet, Buhler)..

FIGURE 7. The RotoPol 31 (bottom) Rotoforce-4 (top) was used to flatten the bottom side and polish the specimen side.

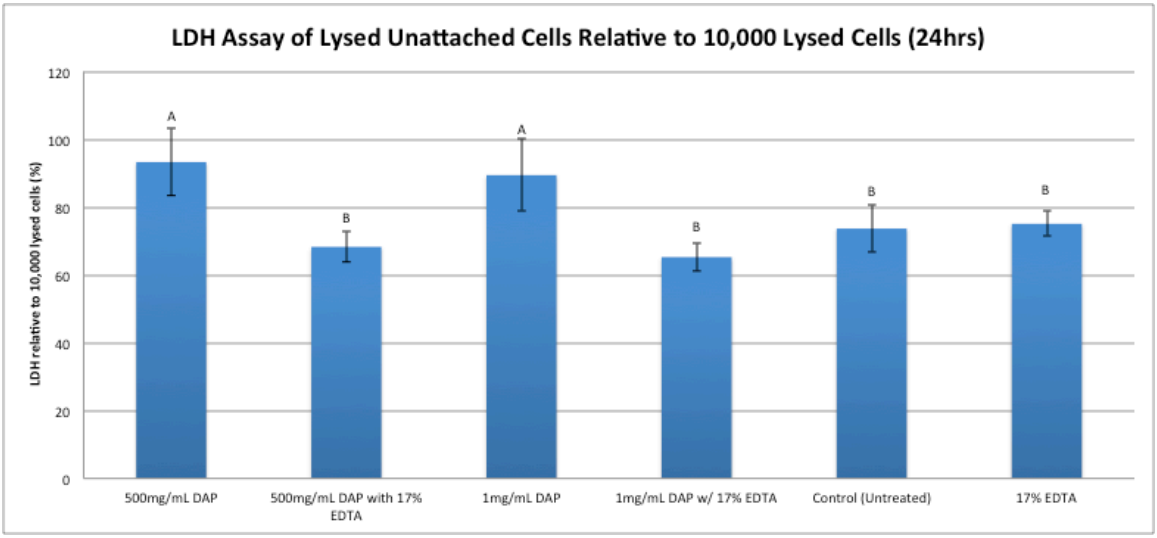


FIGURE 8. Graph showing results of LDH assay. Bars labeled "A" show statistical significant difference when compared with "B" ($p < 0.05$). Error bars represent standard deviation of each group.

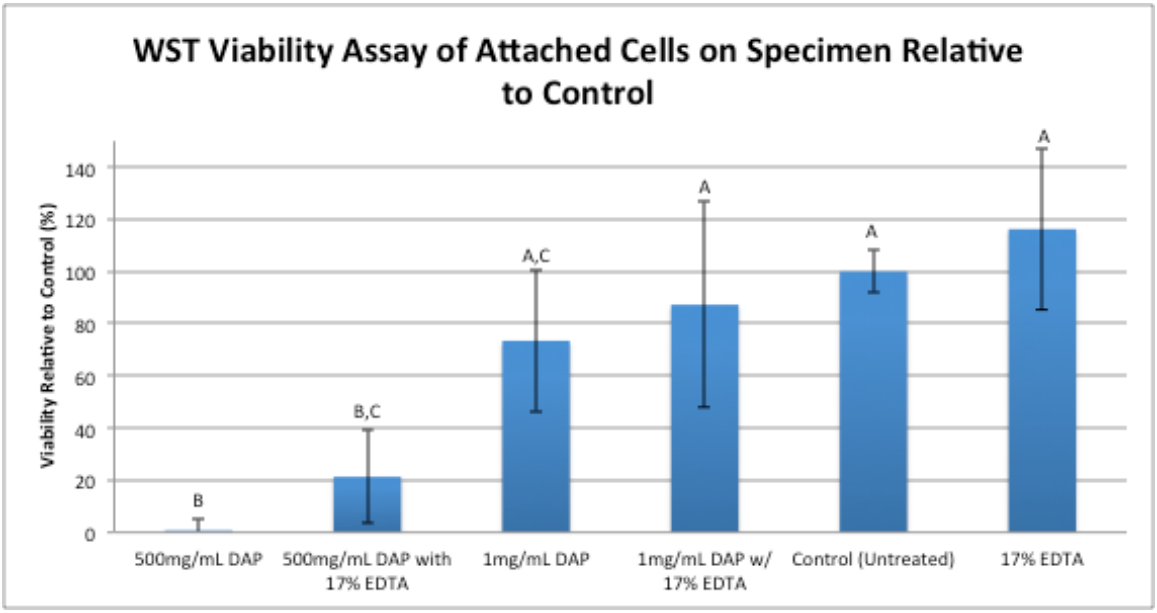


FIGURE 9. Graph showing results of WST assay. Bars labeled "A" show statistically significant difference when compared with "B" ($p < 0.05$). Bars labeled "C" show no statistically significant difference when compared with each other ($p < 0.05$).

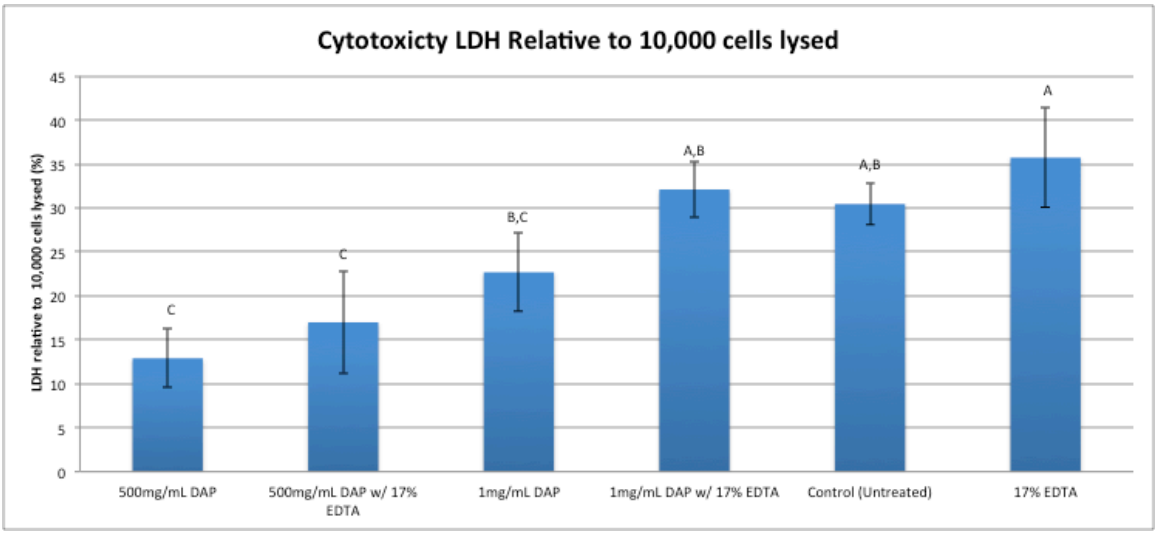
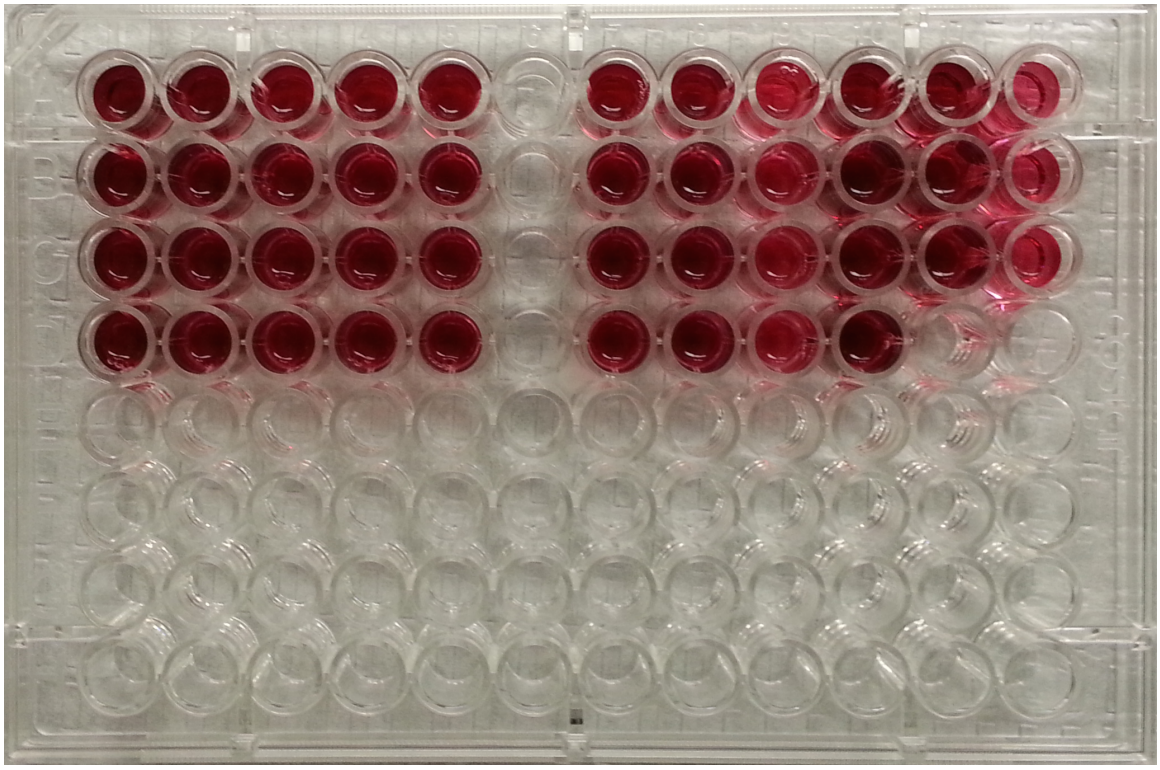
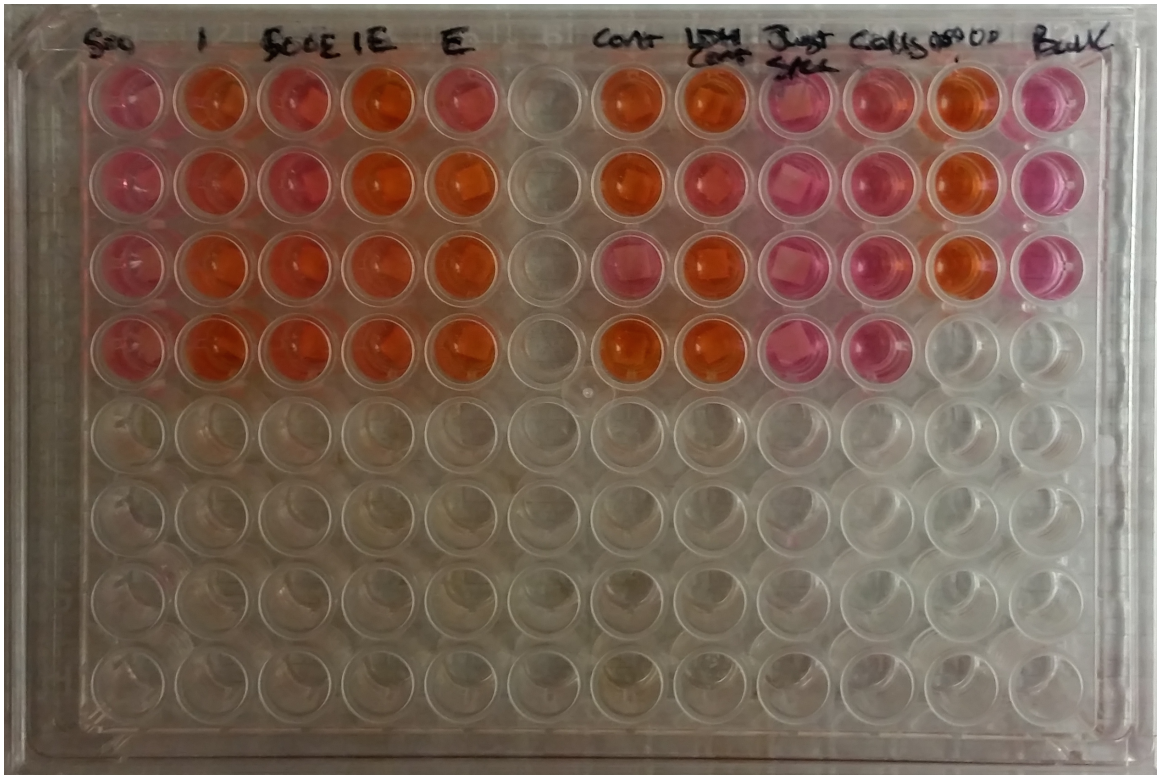


FIGURE 10. Graph showing results of LDH assay. Bars labeled "A" show statistically significant differences when compared with "C" ($p < 0.05$) Bars labeled "B" show no statistically significant differences when compared with each other ($p < 0.05$).



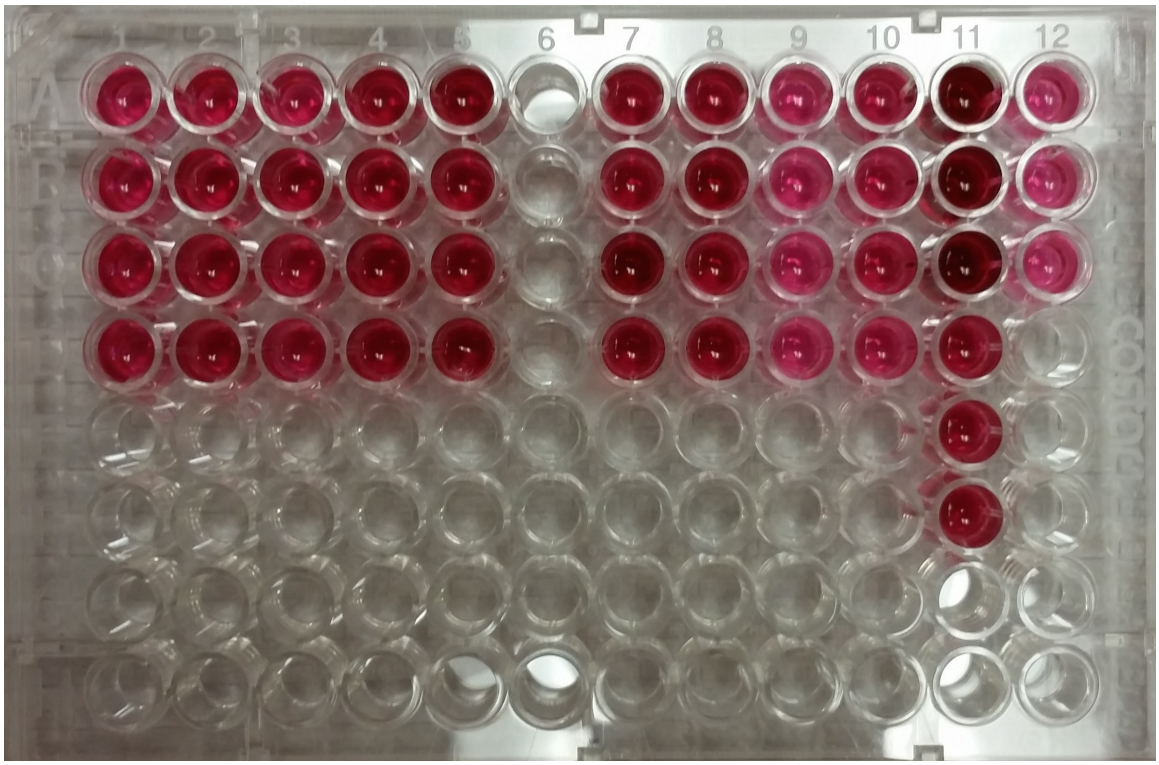
Temperature(°C)	500mg/mL DAP	500mg/mL DAP with 17% EDTA	1mg/mL DAP	1mg/mL DAP w/ 17% EDTA	Control (Untreated)	17% EDTA	Specimens only (no cells)	Cells only (no dentin specimens)	10000 cells lysed	background
490-650	2.683	2.156	2.677	1.981	2.185		1.047	2.429	2.552	0.851
	2.561	2.119	2.475	1.951	2.135	2.234	0.974	2.956	2.629	0.907
	2.281	1.992	2.319	2.049	2.314	2.117	0.982	2.381	2.665	0.96
	2.481	2.029	2.271	2.108	2.03	2.219	1.011	3.022		
avg	2.5015	2.074	2.4355	2.02225	2.166	2.19	1.0035	2.697	2.615333333	0.906
calculated	1.5955	1.168	1.5295	1.11625	1.26	1.284	0.0975	1.791	1.709333333	0
% of 10000	93.34048362	68.33073323	89.47932917	65.30323713	73.71294852	75.11700468	5.703978159	104.7776911	100	
SD	0.168842135	0.076371897	0.183043711	0.070348537	0.117929357	0.063663176	0.033070631	0.3388146	0.057726366	
SD (%)	9.877660024	4.467934698	10.70848541	4.11555404	6.899143373	3.724444784	1.93470933	19.82144697	3.377127497	
% of Cont	126.6269841	92.6984127	121.3888889	88.59126984	100	101.9047619	7.738095238	142.1428571		
SD of Cont(%)	13.40016947	6.061261675	14.52727862	5.583217227	9.359472808	5.052633029	2.624653292	26.89004764		

FIGURE 11. Top. Picture showing LDH assay of lysed unattached cells after 24 hours in a 96-well plate. Bottom. Raw and calculated data of LDH assay of lysed unattached cells after 24 hours.



Temperature(°C)	500mg/mL DAP	500mg/mL DAP with 17% EDTA	1mg/mL DAP	1mg/mL DAP w/ 17% EDTA	Control (Untreated)	17% EDTA	Specimens only (no cells)	Cells only (no dentin specimens)	back
21.6	0.138	0.237	0.701	0.957	0.801		0.135	0.26	0.167
	0.14	0.178	0.416	1.035		0.764	0.136	0.2	0.162
	0.166	0.406	0.689	0.523	0.839	0.938	0.135	0.181	0.163
	0.213	0.421	0.869	0.54	0.914	1.187	0.144	0.178	
avg.	0.16425	0.3105	0.66875	0.76375	0.851333333	0.963	0.1375	0.20475	0.164
calculated	0.00025	0.1465	0.50475	0.59975	0.687333333	0.799	-0.0265	0.04075	0
SD	0.034912987	0.121503086	0.187467997	0.270152272	0.057500725	0.212605268	0.004358899	0.03809965	0.002645751
% of Control	0.036372454	21.314258	73.43598448	87.25751697	100	116.2463628	-3.855480116	5.92870999	
SD (%)	5.079484056	17.67746165	27.27468437	39.30440425	8.36576983	30.93190127	0.634175404	5.543111063	

FIGURE 12. Top. Picture showing colorimetric WST assay of cells on specimens 72 hours after completion of seeding cells in 96-well plate. Bottom. Raw and calculated data of WST assay of cells present on specimens 72 hours after seeding.



Temperature(°C)	500mg/mL DAP	500mg/mL DAP w/ 17% EDTA	1mg/mL DAP	1mg/mL DAP w/ 17% EDTA	Control (Untreated)	17% EDTA	Specimens only (no cells)	Cells only (no dentin specimens)	10000 cells lysed	back
21.3	0.583	0.619	0.746	1.015	1.005		0.45	0.702	2.428	0.405
	0.665	0.914	0.893	1.032		1.085	0.439	0.626	2.347	0.407
	0.743	0.781	0.927	1.16	1.024	1.084	0.431	0.725	2.715	0.405
	0.711	0.73	0.954	1.1	1.098	1.289	0.447	0.556		
avg.	0.6755	0.761	0.88	1.07675	1.042333333	1.152666667	0.44175	0.65225	2.496666667	0.405666667
SD	0.069481412	0.12238464	0.092754155	0.066550106	0.049135866	0.118069189	0.008539126	0.076856468	0.193370973	0.001154701
Calculated	0.269833333	0.355333333	0.474333333	0.671083333	0.636666667	0.747	0.036083333	0.246583333	2.091	0
% of 10000 lysed	12.9045114	16.99346405	22.68452096	32.09389447	30.44795154	35.72453372	1.725649609	11.79260322	100	
SD (%)	3.322879597	5.852923939	4.435875435	3.182692799	2.349874034	5.646541786	0.40837521	3.675584312	9.247774909	

FIGURE 13. Top. Picture showing LDH cytotoxicity assay of from supernatant 72 hours after seeding cells in 96-well plate. Bottom. Raw and calculated data of LDH cytotoxicity assay from supernatant after 72 hours after seeding.

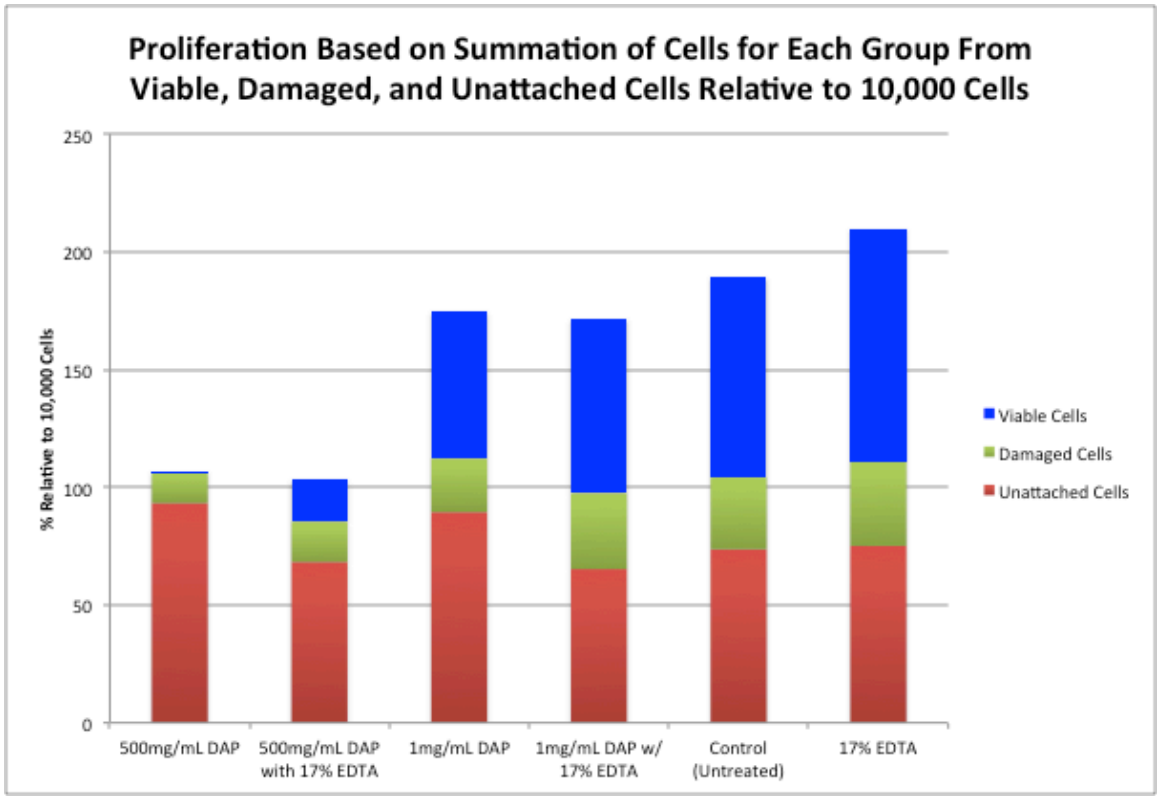


FIGURE 14. Summation of data from LDH unattached, LDH Cytotoxicity, and WST Viability assay showing trends of proliferation for all groups except when treated with 500 mg/mL DAP with and without EDTA.

DISCUSSION

The findings of the WST viability assay show that specimens treated with high concentrations of DAP (500 mg/mL) decreased hDPSC viability on those specimens. When the specimens were treated with 17-percent EDTA, the specimens did show a trend of higher levels of hDPSC viability with respect to their DAP treatment concentration, but these comparisons within their respective DAP concentration treatments were not statistically significant. The results are in agreement with previous studies that the use of high concentrations of DAP has lethal effects on the viability of dentin specimens.[21,23]

According to the statistical analysis, groups treated with 1 mg/mL DAP with and without EDTA did not show a significant difference in viability of hDPSCs compared with the controls. Again, this is in agreement with previous studies using indirect models[23] and current recommendations for using lower-concentration antibiotic formulations. The data did show a trend in the decrease of viability, which may be correlated with mild cytoxicity.

A trend of increased viability was seen when 17-percent EDTA was used for treatment within the respective antibiotic treatment groups and no-treatment group. This may be explained by the further rinsing action of the specimens by the EDTA removing remaining antibiotics that may be cytotoxic. It may also be explained by the removal of the smear layer by the EDTA,[15] which creates a surface more conducive for cell attachment.[10,19] Nonetheless, the reported benefits of EDTA irrigation appear to be consistent with trends seen in the data and current recommendations.

The data from the LDH assay of unattached cells on dentin specimens revealed that the highest levels of hDPSC that were not attach were from the 500mg/mL DAP and 1mg/mL DAP groups. High levels of unattachment seen with the group treated with DAP at 500mg/mL are consistent with data from the WST assay showing that, again, paste-like concentrations (500 mg/mL) of DAP are lethal. When data was accumulated and summed between all assay showing a trend of proliferation, very minimal to none was seen in groups treated with 500 mg/mL DAP with 17% EDTA, again confirming that high concentrations of DAP should not be used. All other groups showed a trend of proliferation.

The data obtained from this experiment re-establishes that high concentrations of DAP as an intracanal medicament during regenerative endodontic procedures may be detrimental to stem cell proliferation within the canal system. It is likely that residual DAP remained on dentin specimens despite extensive washing procedures. Recent reports have shown that triple antibiotic paste has been found to be bound to dentin despite various irrigation techniques.[97] It would not be surprising to see remaining DAP on dentin after treatment as well, given the findings of this study as well as previous reports.[23]

The results of this study suggest that treatment and conditioning of dentin with DAP and EDTA that are commonly used in regenerative endodontic procedures have an impact on the trends of stem cell viability on surfaces and proliferation. Based on these findings, it is important to choose an appropriate concentration for antibiotic intracanal medicament that has adequate antimicrobial activity while minimizing the detrimental effects on the dentin surface for proliferation and differentiation of stem cells.

Future studies include repeating experiments with increased sample sizes to statistically verify trends; changing antibiotic pastes; incorporating low-concentration sodium hypochlorite as a rinse, and switching to various dental stem cell sources. Cytotoxicity studies could be achieved by using a live/dead assay, which assesses cell viability and death within the same sample. Also, LDH assay with intentional lysis could be used instead of WST assay for the viable cells on specimens to quantify cell numbers for consistency through the experiment. The 10,000 cells that were used as a reference control on the WST assay were not unattached and could have behaved metabolically differently, which could have resulted in a skewed reading. If LDH assay had been used with intentional lysis using 10,000 cells unattached, the quantification of cells on the specimens likely would have been more accurate. Further clinical relevance could be achieved by performing in-vivo studies.

SUMMARY AND CONCLUSIONS

The null hypothesis was rejected because statistically significant differences were demonstrated in the levels of viability, unattached cells, and cytotoxicity, based on how the dentin was treated with DAP and EDTA. A significant decrease in the viability of hDPSCs was demonstrated on dentin specimens treated with high-concentration DAP in combination with and without EDTA when compared with the control groups. Dentin treated solely with 500 mg/mL DAP and 1mg/mL DAP demonstrated a significantly increased level of unattached cells when compared with all other groups. Not contributing to the overall value of the findings, the LDH cytotoxicity results showed trends similar to those of the WST assay and essentially gave background cell-death levels.

Based on the findings of this study, high concentration DAP of 500 mg/mL cannot be recommended for use as an intracanal medicament during endodontic regenerative procedures, which is in agreement with current recommendations.[7] The results are also in agreement that low-concentration DAP should be used instead. Lastly, irrigation with EDTA may have a benefit in increased stem cell viability on dentin surfaces based on the trend seen.

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ABSTRACT

THE EFFECTS OF RADICULAR DENTIN TREATED WITH DOUBLE ANTIBIOTIC PASTE AND EDTA ON DENTAL PULP STEM CELL PROLIFERATION: AN *IN-VITRO* STUDY

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Introduction: Regenerative endodontic therapy in immature teeth promotes continuation of root development and likely increases the prognosis of these teeth. The use of double antibiotic paste (DAP), equal parts of ciprofloxacin and metronidazole, followed by the dentin conditioner, ethylenediaminetetraacetic acid (EDTA), has been suggested for canal disinfection and facilitation of stem cell attachment/proliferation, respectively. However, the effect is unknown when all these agents are used on on radicular dentin surfaces to facilitate the level of stem cell proliferation.

Objectives: The aim of this in-vitro study is to compare the proliferation of human dental pulp stem cells (hDPSCs) on human radicular dentin treated with two different

concentrations of DAP followed by EDTA.

Materials and Methods: Human premolars and incisors were prepared into standardized polished 4 mm x4 mm radicular dentin specimens. Groups of specimens were treated with DAP 500 mg/mL, DAP 1 mg/mL, DAP 500 mg/mL followed by 17-percent EDTA, DAP 1 mg/mL followed by 17-percent EDTA; 17% EDTA, or no treatment. All groups treated with antibiotics were incubated with DAP at 37°C for one week. All specimens were washed with distilled water. The hDPSCs were seeded across all specimens and unattached cells were collected after 24 hours. LDH assay was completed on unattached cells for quantification. Three days after attachment, WST viability and LDH cytotoxicity assays were performed.

Hypothesis: There is no significant difference in hDPSC viability, unattachment, and cytotoxicity on dentin specimens treated with DAP and 17-percent EDTA.

Clinical Significance: These results can be used to help identify the best treatment concentrations when using DAP and/or EDTA to promote endodontic regeneration.

Results: The results demonstrated significantly less viability of hDPSCs on specimens treated with 500 mg/mL DAP with and without 17-percent EDTA. Groups treated with 1 mg/mL DAP, 1 mg/mL DAP and 17-percent EDTA, and 17-percent EDTA alone had no statistically significant difference in viability compared with control untreated dentin. The results of the unattached cells from the LDH demonstrated that cells from the specimens treated with solely 500 mg/mL and 1 mg/mL DAP had significantly higher levels of unattached cells when compared with all other groups. The LDH assays in summation with the WST assays showed a trend of a lack of proliferation on groups treated with 500 mg/mL DAP with and without 17-percent EDTA.

Conclusions: Paste-like concentrations (500 mg/mL) of DAP are detrimental to hDPSC viability, whereas the present study supports the use of low-concentration antibiotics consistent with current recommendations for intracanal medicaments used during endodontic regenerative procedures.

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