# THE EFFECTS OF NANO-HYDROXYAPATITE IN A DOUBLE ANTIBIOTIC PASTE- LOADED METHYLCELLULOSE CARRIER ON DENTAL PULP STEM CELLS

by

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# INTRODUCTION

Untreated dental caries in permanent teeth was the most prevalent health condition according to the Global Burden of Disease 2010 study (1). If caries become extensive, in children in particular, severe pain and infection can occur, affecting quality of life. Therefore, untreated dental caries creates a significant biological, psychological, social, and financial burden on the family and the healthcare system (1). Pulp necrosis of the immature permanent tooth is a disease process in children that results from infection of the dental pulp chamber before root formation has been completed, leaving the root dentin thinner and shorter, and with an open root foramen. A dental infection of this nature has potential to cause grave morbidity and even mortality. Even if the health of the child is not endangered, this anatomical underdevelopment of the root structure compromises the survivability of the tooth and is prone to fracture and carious destruction (2).

Historically, these teeth were treated through a technique known as apexification, in which the use of calcium hydroxide [$Ca(OH)_2$] is utilized (3). This technique requires long-term placement of $Ca(OH)_2$ inside the root canal with a goal of inducing the formation of an apical hard tissue barrier. The duration of placement often requires multiple visits over several years to achieve periodontal healing and bridging (2). In a retrospective study, Cvek found periapical healing of 95% and 91% of teeth immediately after treating and at a 4-year follow-up, respectively (4). Additional challenges include low patient recall, possible contamination of canal between appointments, and increased dentin brittleness, with the fracture strength of $Ca(OH)_2$-treated teeth potentially being

halved after a year (2, 5, 6). Research by Yassen et al showed that this result is likely due to the significantly higher phosphate/amide I ratios in $Ca(OH)_2$-treated dentin compared with untreated dentin (7). They proposed that the alkaline $Ca(OH)_2$ with a pH of 11.8 has a denaturation effect on the dentin organic matrix, facilitating penetration of the apatite-encapsulated collagen matrix and thus changing the nature of the internal walls of the tooth (7).

Apexification has been improved with the use of bioceramic materials such as mineral trioxide aggregate (MTA) to improve the apical plug and biological response at the apex (8-10). As an apical plug, the MTA continues the formation of an apical barrier and also creates a stop for the obturation of the canal with gutta-percha. The use of MTA could reduce the number of patient visits with an increased success rate (11), but still no increase in root-wall thickness occurs.

In light of these challenges, regenerative endodontic procedures (REPs) have gained importance. The goal of these procedures is to regenerate pulp-like tissue, ideally the pulp-dentin complex (12). The history of REPs can be dated back to the 1920s, when Dr. B.W. Hermann presented a case report on vital root amputation with $Ca(OH)_2$ (13). If successful, there is continued root formation both in length and width. Also, pulp-like tissue is hoped to be formed in the canal that responds to sensibility testing (14). Nygaard-Ostby was an early pioneer regarding revascularization of necrotic pulp canals. In *in vivo* studies he observed after inducing a blood clot within a pulpless necrotic canal, ingrowth of new tissues and after ten months a full thickness of fibrous tissues with very few lymphocytes noted (15, 16). Recent emphasis in regenerative endodontics has been

placed on certain key factors – stem cells, scaffolds, and growth factors (17) in an environment that has been properly disinfected.

## REGENERATIVE ENDODONTIC PROCEDURES

### Disinfection with TAP and DAP

Since its introduction by Hoshino et al. (18, 19), triple antibiotic paste (TAP) consisting of equal parts metronidazole, ciprofloxacin, and minocycline has been utilized as an intracanal medicament in a majority of reported regenerative cases (20). In 2004, Banchs and Trope published a case study using this combination on a necrotic immature tooth and showed that revascularization is possible (21). Since then, their results were confirmed and others managed to replicate their findings. However, concern over tooth discoloration has led to research regarding a double antibiotic paste (DAP), excluding minocycline, with equally successful anti-microbial results (22, 23). Much research has been performed on the effect of DAP against young and established biofilms in differing concentrations (24-27) with a conclusion that an effective range of 1-5 mg/ml DAP was ideally antibacterial with concentration-dependent cytotoxicity on human dental cells. In DAP and TAP concentrations greater than 0.5 and 0.25 mg/mL, respectively, cytotoxicity was found with human dental pulp stem cells (DPSC) (23, 28-30). However, other studies showed no cytotoxicity on DPSCs with DAP and TAP between 1-5 mg/mL (27, 30). The challenge has been in determining the concentration with maximal antibacterial effect and minimal cellular cytotoxicity. In their study comparing DAP and TAP to $Ca(OH)_2$, Yassen et al. noted a reduction in the phosphate/amide I ratio indicating formation of collagen-rich matrix on the surface of radicular dentin with DAP and TAP (7). This

demineralization effect might play a significant role in pulp regeneration, effectively improving the attachment and growth of host stem cells onto dentin via exposure of collagen fibers and their accompanying growth factors (7).

In recent studies within this university, water-based methylcellulose hydrogel has been utilized to deliver antibiotic paste with controlled concentrations (31, 32). Methylcellulose is a biocompatible material and has been utilized in similar carrier techniques (33, 34). In-vitro results of this technique appeared to minimize the negative effects of these medications on the immature necrotic tooth (31, 32, 35). Portions of this technique has been adopted by the American Association of Endodontists' Clinical Guidelines for regenerative endodontic procedures (36). However, the regeneration of the pulp-dentin complex has not been a consistent outcome of in vivo procedures. With this in mind, we intend to examine the potential for hydroxyapatite nanoparticles suspended into DAP/methylcellulose and its effect on DPSC activity.

## STEM CELLS

There are a number of stem cells of importance for endodontic regeneration. The stem cells in the dental region differ from embryonic stem cells which are totipotent or pluripotent in that they are multipotent, giving rise only to a select type of cells (37). The DPSC as well as stem cells from the apical papilla (SCAP) are important for regenerative endodontics (38, 39). DPSCs have the ability to form dentin and pulp-like tissues when transplanted in immunocompromised mice using hydroxyapatite/tricalcium phosphate scaffolds (40, 41). Recent studies have shown DPSCs can be harvested, preserved, and utilized in-vitro with appropriate results. For the proposed studies, we will use DPSCs as

these have the potential to generate dentin and importantly, have been used extensively in our laboratories for research with application of DAP and methylcellulose.

## HYDROXYAPATITE NANOPARTICLES

In an attempt to re-create the molecular structure of bone, collagen-hydroxyapatite scaffolds have been created using a biomimetic precipitation technique (42). Collagen comprises approximately 90% of the organic matrix of bone, known as osteoid, and is mineralized by hydroxyapatite, which is $Ca_{10}(PO_4)_6OH_2$ (42). This molar ratio varies with age, species, and type of bone, but forms the foundation for a resorbable scaffold. The ratio between calcium and phosphate, generally 1.66:1, is important in the rate of resorption within bone (42) as well as providing surface area for which dental pulp stem cells to bind (43). Hydroxyapatite is able to chemically bind to bone without inducing toxicity or inflammation, stimulating bone growth via a direct osteoinductive action on osteoblasts (44). Application and fabrication of nano-hydroxyapatite crystals for use in dentistry has been investigated with crystal size ranging from 50 to 1000 nm (44). Nano-hydroxyapatite has shown biocompatibility in multiple studies (45). Of interest, nano-hydroxyapatite in concentrations ranging from 0.10 to 0.25% in sports drinks have been shown to decrease erosion on bovine enamel in vitro (46). Phosphate and hydroxide anions released from the nano-hydroxyapatite also appeared to decrease the titratable acidity of the sports drink (46). Similar studies have shown nano-hydroxyapatite is able to occlude dentinal tubules, with significance objectively with measurement and subjectively with decreased dentin sensitivity (47-49). With this information, it is hypothesized that a secondary effect of hydroxyapatite would be to mitigate the acidity of DAP in solution and maintain a more neutral pH. Two in-vitro

studies using collagen-hydroxyapatite scaffolds found increased proliferation of mesenchymal stem cells and osteogenic differentiation, respectively(50, 51). Ning et al. showed these scaffolds have strong potential for bone tissue regeneration using differing collagen to hydroxyapatite ratios and different sized apatite crystals (50). Niemeyer et al. noted that cell infiltration and osteogenic differentiation were enhanced on collagen-hydroxyapatite scaffolds compared with a tricalcium-phosphate scaffold (51). It is for these reasons that nano-hydroxyapatite is being considered in this study, based on similar studies conducted in the laboratory of Dr. Angela Bruzzaniti, as described in the experimental approach.

It is important to note that in regenerative endodontics the DAP-loaded methylcellulose solution is not the scaffold. This solution, which would include nano-hydroxyapatite, is removed by rinsing with EDTA between 1 to 4 weeks after placement (36). The goal, which will be evaluated in future studies would be that residual hydroxyapatite remains to promote mineralization once bleeding is induced. Equally significant is clinical practicality of a material. With additional products, antibacterial efficacy and usability may decrease. Pending positive results from this study, future studies will include evaluation of antibiotic properties and, should the need arise, viscosity will be evaluated and reported upon in future studies.

## OBJECTIVE

The specific objective of this study is to investigate the effect of hydroxyapatite in a methylcellulose plus DAP paste on the growth and maturation of DPSC.

## Null Hypothesis

The addition of hydroxyapatite nanoparticles to the methylcellulose plus DAP paste will have no effect on growth and maturation of dental pulp stem cells.

## Alternative Hypothesis

The addition of hydroxyapatite nanoparticles to the methylcellulose plus DAP paste will increase the growth and maturation of dental pulp stem cells.

# REVIEW OF LITERATURE

## HISTORY OF ENDODONTICS

The first evidence of dental treatment can be dated back to approximately 5000 BC, when ancient Sumerians attributed dental caries to tooth worms (52). In 2500 BC, the death of Hesy-Re with an inscription on his tomb as "the greatest of those who deal with teeth" stands as the earliest known reference to a dentist (52). In ancient Egypt, papyri have been discovered explaining diseases of teeth and recommended cures (53). Millenia later, Hippocrates and Aristotle taught about dentistry, dental care and treatment (54).

It was not until 1684 when Anton Von Leeuenhoek observed samples of teeth under microscopic magnification that the theory of the tooth worm was disproven (55). Three years later, in 1687, the first English book devoted to dentistry was authored by Charles Allen. Though the Little Medicinal Book for All Kinds of Diseases and Infirmities of the Teeth by Artzney Buchlein, written in 1530, was the first book devoted entirely to dentistry, the book by Charles Allen, written in English, had a much larger presence. Treatment at this time included identification and removal of diseased teeth with replacement as the only option (55).

Fifty years later, the scholarly emphasis switched from removal of infected teeth to restoration and preservation of the natural dentition. In 1723, Pierre Fauchard, credited as being the Father of Modern Dentistry, authored The Surgeon Dentist and in doing so was the first to detail comprehensive dental care. Many aspects of this two-volume, sixty-four-chapter book were revolutionary presentations in literature, including dental

anatomy, restorative treatment, and denture fabrication. Importantly, he presented many endodontic ideas in his book including accessing the pulp chamber for purulence drainage and obturating teeth with lead foil (55, 56). In doing so, he became the first to discuss obturation techniques in endodontics (55). In 1756, Philipp Pfaff authored a book in German in which he described pulp capping with gold foil prior to placing a restoration (57). In the following year, Bourdet offered the technique of intentional extraction followed by replantation for endodontic therapy (55).

In the United States, Robert Woofendale was one of the earliest dentists (58). He also was an early pioneer in endodontics; he would cauterize the pulp to alleviate pulpal pain and proposed the use of cinnamon, clove, turpentine, opium, and camphor oils in alleviation of pulpal pain (54). Notably, he was a mentor of Paul Revere and John Greenwood, Sr.

For the better part of the 1800s, research emphasis was placed on the role of pulp vitality. This period is referred to as the Vitalistic era, highlighted in 1805 by J.B. Gariot declaring that destruction of the pulp does not destroy the vitality of the tooth (53). In 1802, B.T. Longbothom recommended filling the roots of teeth that for whatever reason should not be extracted. However, Edward Hudson is most often credited as having been the first to place filling material, in his case gold foil, in root canals in 1809 (55). John Callow, in 1819, crediting Charles Bew, described the flow of blood through the apical foramen (53).

Leonard Koecker wrote Principles of Dental Surgery in 1826, which was used as standard for fifty years. He, however, believed that destruction of a pulp meant the whole dentinal core of the tooth immediately died and became a foreign body and required

immediate extraction (55). His contribution, on the contrary, was to popularize the pulp capping procedure and, although he is often credited for it, utilized the same technique as Pfaff described in 1756.

The Vitalistic theory became rooted in 1829, when S. S. Fitch proposed its doctrines in System of Dental Surgery in which he theorized that the crown was nourished by the dental pulp or by its membrane, whereas the roots were supplied by the pulp membrane (53, 54). All vital pulp treatment would have been extremely painful at this time as no sedative materials had been discovered yet. In 1836, Shearjashub Spooner used arsenic trioxide to devitalize the pulp prior to removal, which became instantly successful given its painless technique (55). However, leakage of arsenic and overzealous usage lead to significant destruction of teeth and supporting tissues. Arsenic was used, however, as recently as the 1920s for pulp destruction prior to removal (59). In 1837, Jacob Linderer recommended essential oil or narcotic oil to desensitize the pulp prior to restoration (57). Then, in 1838, the first root canal broach was invented by Edwin Maynard using a watch spring, enabling treatment of teeth with smaller canals. Interestingly, Maynard became more famous for creating the Maynard Carbine rifle (55). The first recorded account of modern endodontic treatment occurred in 1839 when Baker wrote in the American Journal of Dental Science about removing nerve, cleaning and shaping the canal, and filling with gold foil (56).

Gutta-percha was introduced to dentistry in 1847 by Edwin Truman. He incorporated it as a filling base as well as denture material (60). Then, attempts were made to obturate using wood soaked in creosote and plugged with a solution of Hill's stopping (a mix of gutta-percha, lime, glass, and metal filings) and chloroform (56).

Awareness of secondary dentin was matched as the ultimate goal of pulp capping in 1850 by W. W. Codman (54). In 1857, Thomas Rogers reported a retrospective series of 220 cases of pulp capping, of which 202 he determined successful. His criteria were overall health, lack of inflammation and decrease in previous pain. His judgement was quite sound; however, in cases of failed pulp capping he prescribed three leaches and a laxative (55).

In 1864, S.C. Barnum utilized a thin sheet of rubber to isolate a tooth during gold foil operations, thus harboring in a new era of rubber dam isolation providing more aseptic conditions (56).

By the year 1865, a number of dentists were filling root canals with denture gutta-percha heated to near-molten temperatures and plugged into place. Those often credited with this technique are G.A. Bowman and E.L. Clarke, both residing in the United States (54, 55).

In 1867, Joseph Lister was practicing antiseptic techniques during surgical procedures and at the same time Leber and Rottenstein isolated a microbial organism present on tooth surface as well as inside dentinal tubules (57).

In 1870, G.V. Black looked at 10-year survival outcomes of pulp capping on 42 cases, in which only six cases the pulp survived longer than five years. In that study he promoted the use of zinc oxychloride as a capping material. Referred to as Ash's cement, it became popular as a capping medicament and root canal filler material and is often credited to N.C. Keep for its wide acceptance (54, 56).

In a pivotal article published in 1878, G.O. Rogers stated that diseased pulp may result from pathogenic organisms (61). This ushered in a new era of thinking from

vitalism to what would be referred to as septic theory (62). In the transition time between eras, Charles S. Tomes wrote that the lack of vitality of the dentin which allowed contamination of the cementum and ultimately the bone (61).

In 1882, Arthur Underwood proposed that pulpal suppuration was in fact dependent on pathogens and that the most important aspect was a sterile tooth, which led to widespread acceptance of caustic germicides used in the root canal system to eliminate bacteria for over 30 years (61).

In 1883, G.A. Mills wrote in Dental Cosmos about driving a wooden stick soaked with creosote into the root canal, followed by a "quick blow with a mallet," leaving it in place for several seconds (62). The stick was then removed with the pulp attached.

Also in 1883, Dr. Bowman introduced chloropercha, a mixture of chloroform and gutta-percha used to obturate root canals. A decade later, M.L. Rhein further developed the technique (63).

In 1885, formalin was introduced by Lepkowski and naturally became an ideal solution for fixation of residual pulp stump tissue.

In 1886, G.V. Black promoted root amputation of severely periodontally involved molars to retain teeth and maintain healthy tissues

During this time, many restorative procedures required the use of a dowel in the root canal for the crown, which indicated a greater need for endodontic therapy in more teeth. Rarely were these procedures being performed in aseptic conditions. In 1888 W.D. Miller described endodontic abscess formation as a result of pulpal infection (64). As a proponent of the septic theory, he described the human mouth as a focus of infection and advocated sealing antiseptic in a cavity preparation and protecting it from saliva for 30

minutes. He even advocated that any organ inhabited by bacteria could produce a metastatic abscess at a susceptible target (65, 66).

Chlorophenol was introduced in 1891 by Otto Walkoff, but only brought to the United States in 1899 by Hermann Prinz (62).

In 1894, J.R. Callahan suggested the use of 20 to 40% sulfuric acid to enlarge and clean root canals soaked on cotton pellet in the canal for 24 to 48 hours with no regard for potential seepage of the caustic agent into periradicular tissues (62).

In 1895, M.L. Rhein used a technique referred to as electromedication to sterilize root canals. Invented by Breuer and later known as ionization, this technique allowed less use of caustic agents to sterilize (63, 67).

Also in 1895, William C. Roentgen accidentally discovered x-rays. In the United States, C. Edmund Kells began using the x-ray in his dental practice.

Formocresol was introduced to dentistry about this time by John P. Buckley and remained popular for over 50 years and remains in use today (56, 67).

In 1905, Einhorn developed procaine (Novocaine) and H.S. Vaughn was the first to use infiltration anesthesia before pulpal extirpation (59, 67, 68).

The turn of the century ushered in a new thought process on infection within teeth and their relationship with the body. In 1904, Frank Billings noted an apparent relationship between oral sepsis and bacterial endocarditis (62). Then, in 1909, E.C. Rosenow, a student of Billings, developed the theory of focal infection by showing streptococci from diseased organs spreading through the bloodstream and establishing at a distant site (67). At the same time, Mayrhofer demonstrated a role of streptococci in pulpal infection (69). In 1910, William Hunter gave a lecture titled “The Role of Sepsis

and Antisepsis in Medicine," which was later published in the Lancet. He is quoted as calling the gold crown "a mausoleum of gold over a mass of sepsis" (66, 70). After this, despite the recent advances in dentistry, pulpless teeth became condemned to extraction. This continued for twenty-five years. Billings then replaced the term "oral sepsis" with "focal infection" (71). In 1912, Rhein countered the work of Hunter and pushed for improved techniques and aseptic protocols (71). In 1913, Rosenow developed the theory of elective affinity of organisms to tissue, where essentially an organism can choose the distant organ it wishes to infect (66).

With all this scrutiny on the biologic principles of endodontics, increased attention to biocompatible materials was considered. B.W. Hermann, in 1920, promoted the use of calcium hydroxide which he called Calxyl for root canal obturation. A decade later, he would promote calcium hydroxide for pulpal therapy as well as in treating infected canals (72).

In 1921, Rosenow and Meisser proved in animal models that the apexes of healthy teeth can be infected through external contaminants. In addition, Rosenow believed that once a tooth became infected, it remained infected (73).

In 1925, U.G. Rickert proposed the use of sealer with a pre-fitted gutta-percha cone (67). Lentulo introduced a rotary paste inserter also in 1925 made of steel wire that would carry sealer down into a canal.

In 1928, Walkoff invented an iodoformized chlorophenolcamphormenthol paste which he demonstrated was resorbable not tissue irritating (72).

In 1931, Rickert and Dixon began experimenting with principles that became what we now refer to as the hollow tube effect in which root canal space left void would, in the absence of microorganisms, initiate periapical inflammation (64).

In 1933, Dr. E.A. Jasper introduced silver points to dentistry as a means of obturation. The silver points had standard sizes and similar diameter and taper as reamer and file instruments (63).

In 1937, Logan began work that postulated microorganisms did not necessarily imply presence of infection (58). Also in 1937, Tunnicliff and Hammond demonstrated bacteria in pulps of teeth without evidence of inflammation (67). And that same year, Cecil reported arthritis cases in which the suspected foci of infection had been removed with no improvement to the patient (74).

In 1938, Zander demonstrated histologic success from vital pulp capping by showing continuous odontoblasts lined up below secondary dentin bridge (67).

In 1940, Sommer and Crowley reported that a periapical lesion demonstrated by a radiolucent area is not necessarily an infection (71).

Antibiotic application in root canals began around this time with Fred Adams in 1941 recommending the use of sulfanilamide to treat apical infection. In addition to this antibiotic, he was the first to use penicillin in endodontic therapy in 1944 (67). Although Grossman in 1944 recommended using a nonaqueous solution of penicillin to improve stability in system (67).

In 1943, the American Association of Endodontists was formed with a meeting of 20 men in Chicago (75). Harry Johnston created the term “endodontics” from two Greek words: “endon” meaning within and “ho dontas” meaning a tooth (70). The first dental

journal dedicated to the subject, The Journal of Endodontia, was published in 1946 with Orban as its first editor (71). In 1949, the American Association of Endodontists formed a committee to investigate the potential for specialty recognition. Eventually, in 1956, the American Dental Association Council on Dental Education recognized the American Board of Endodontics (70).

In 1959, Sargenti and Richter introduced N2 paste (also known as Sargenti paste) to the United States, which remained controversial due to the use of paraformaldehyde among other questionable agents as its ingredients (76). The AAE issued a position paper against its use in the 1990s (77).

By 1963, over 200 dentists in the United States were limiting their practice to endodontics (75). So, in that year, the American Dental Association recognized endodontics as a specialty of dentistry (70) and certified its first Diplomates two years later.

## THEORY OF ENDODONTICS

Endodontic therapy received a significant contribution in 1965 when Kakehashi, Stanley, and Fitzgerald performed their work on germ-free rats. In this study, exposed pulps remained vital in bacteria-free rats despite exposure to masticatory forces and exposure to the oral environment (78). The alternative group, rats that were not germ-free, experienced pulpal necrosis and periapical pathosis. The authors concluded appropriately that bacteria are responsible for pulpal and periapical disease (78). In 1974, Bergenholtz noted that traumatized teeth with periapical destruction had bacterial growth (79). In 1981, using a monkey model, Moller demonstrated that infected pulp tissue was necessary for periapical inflammation and not simply necrotic tissue (80).

These studies and many others laid the groundwork in establishing the current goal of endodontics, which is to achieve sufficient reduction in pathogens and their toxins for the body to manage healing (81, 82). Insufficient reduction of the microbial load can, therefore, lead to inflammation of the periapical tissues referred to as apical periodontitis (83). There is, therefore, a direct relationship between success of endodontic therapy and adequate reduction of bacteria.

Endodontic success was originally outlined by Dr. G.G. Stewart in three distinct phases: chemomechanical preparation, control of microbes, and sealing and obturating the root canal system (84). Most importantly, the chemomechanical preparation reduces the microbial load to a sufficient extent and establishes a space to obturate the root canal system, which Grossman was later able to corroborate and expound upon (85). He identified 13 principles for endodontic therapy:

- Clinical asepsis
- Retaining instruments within the canal system
- Never force instruments apically
- Enlarging canal space to accommodate obturation material
- Continuous irrigation throughout treatment
- Irrigation solution remains within canal space
- Fistulas do not require special attention
- Negative culture should be confirmed prior to obturation
- Obturation should result in hermetic seal to root canal system
- Obturation material should not be inflammatory to tissues
- Adequate drainage in case of acute alveolar abscess

- Avoidance of injections into infectious spaces
- Surgical approach may be necessary for adequate healing

Present-day, the understanding is that the most important objective is the elimination of bacteria and its associated contents. Schilder is credited with bringing this understanding to light in 1967 (86). Schilder also stressed the importance of obturating the root canal system in three dimensions, in which aliquots of gutta percha were placed into the canal, heated with a spreader and condensed, allowing for, what he referred to as a hermetic seal (86).

## MECHANICAL INSTRUMENTATION

Primarily, instrumentation is the process of enlarging the root canal space to adequately allow for proper delivery of irrigation and disinfection as well as ultimately obturation of the same space (85, 87). Of significant importance is the ability to maintain the original canal to avoid deviation from the canal space (88). This deviation could result in transportation or unzipping from the original canal, which may result in a perforation exiting the canal at a location other than the original apical foramen (89). Despite the importance of shaping the canals, a significant amount of the pulp canal system remains untouched after instrumentation (90-92). Knowing that bacterial penetration may reach up to 300 μm into dentinal tubules, an additional layer of disinfection is necessary (93, 94).

## CHEMICAL IRRIGATION

Incorporating antibacterial solution into instrumentation of the root canal system provides that additional benefit (95). Ideal disinfection irrigant should display the

following properties: action on endodontic bacterial biofilm, tissue dissolution, endotoxin inactivation, effect on smear layer, minimal caustic potential, and minimal allergic potential (96). Sodium hypochlorite (NaOCl) has proven itself as the solution worthy of such a challenge as it meets many of these requirements. It demonstrates an ability to dissolve organic tissue, lubricate the canal, and disinfect canal space with some effect on the smear layer and only a few reported cases of allergenicity in the literature (96, 97). Ranges of pH of NaOCl are above 11, which allows for hypochlorous acid to affect oxidative phosphorylation in cellular processes, blocking membrane activities and DNA synthesis (98-100). This process can be affected by a variety of factors, several studied include amount of exposure time, temperature upon exposure, and concentration (95, 101, 102). Regarding its minimal effect on the smear layer, it effects only the organic component; however, incorporation of one minute of ethylenediaminetetraacetic acid (EDTA) adequately removes the smear layer (103). Additionally, NaOCl demonstrates no residual effect or efficacy against endotoxin (104-106). In such cases where these requirements are desired, disinfection with 2% chlorhexidine gluconate (CHX) would be indicated (107, 108). Unfortunately, mixture of NaOCl and CHX forms a precipitate described as para-chloroaniline (PCA), which may have negative and potentially harmful effects (109, 110). This can be prevented by flushing the canal with a different irrigant between solutions.

## OBTURATION

As mentioned earlier, and described by Schilder, a hermetic seal after adequate disinfection to minimize space for bacterial penetration is necessary and defined by the AAE as obturation. Obturation should be confined to the canal system without voids and

terminate within 1 mm of the radiographic apex (111, 112). Requirements for adequate endodontic sealer were listed by Grossman in 1982 (113), which included:

1. Should create a hermetic seal
2. Tacky when mixed to “stick” to canal walls
3. Radiopaque
4. Fine powder particles to mix well with liquid
5. No shrinkage on setting
6. Should not stain tooth
7. Bacteriostatic (or at least not bacteriophilic)
8. Slow setting time
9. Insoluble in tissue fluid
10. Tissue tolerant, or non-irritating to tissue
11. Soluble in a common solvent

Endodontic sealer is used in conjunction with obturation material, primarily gutta percha, to aid in providing a hermetic seal (111).

## MICROORGANISMS

Bacteria are the challenge against which endodontics faces (78). Numerous studies look at species present within root canal systems, and comparing biofilms within necrotic pulps of mature and immature teeth, as well as primary and secondary infections where a tooth has already been endodontically treated (114-118). In the necrotic pulp of the immature tooth, as is often the case with regenerative techniques, endodontic infections most often consist of gram-negative anaerobic rods (114). The most commonly

isolated of these species is A. *naeslundii*, which has significant pathogenicity activating the innate immune response triggering an inflammatory process (115-120).

Another important pathogenic species located often in these teeth is F. *nucleatum*, which is a gram-negative obligate anaerobe (121). This species is important because of its ability to aggregate additional bacterial species, developing the biofilm (122). F. *nucleatum* and another species, P. *gingivalis* have virulence factors that lead to tissue destruction and difficulty in the host response eradication (122, 123).

Another endodontic species is E. *faecalis*, a gram-positive facultate anaerobe, which demonstrates ability to invade dentinal tubules and expresses significant virulence factors (124-126).

## MANAGING IMMATURE TEETH WITH PULPAL NECROSIS

Pulp necrosis of the immature permanent tooth results from infection of the dental pulp chamber before root formation has been completed, leaving the root dentin thinner and shorter, and with an open root foramen (4, 127). This blunderbuss apex presents a significant challenge to endodontics (22, 128). Techniques to address this include apexogenesis, apexification, and regenerative endodontics (89).

## APEXOGENESIS AND APEXIFICATION

Historically, these teeth were treated through a technique known as apexification, in which the use of calcium hydroxide [$Ca(OH)_2$] is utilized (3). This technique requires long-term placement of $Ca(OH)_2$ inside the root canal with a goal of inducing the formation of an apical hard tissue barrier. The duration of placement often requires multiple visits over several years to achieve periodontal healing and bridging (2). In a

retrospective study, Cvek found periapical healing of 95% and 91% of teeth immediately after treating and at a 4-year follow-up, respectively (4). Additional challenges include low patient recall, possible contamination of canal between appointments, and increased dentin brittleness, with the fracture strength of $Ca(OH)_2$-treated teeth potentially being halved after a year (2, 5, 6). Research by Yassen et al showed that this result is likely due to the significantly higher phosphate/amide I ratios in $Ca(OH)_2$-treated dentin compared with untreated dentin (7). They proposed that the alkaline $Ca(OH)_2$ with a pH of 11.8 has a denaturation effect on the dentin organic matrix, facilitating penetration apatite-encapsulated collagen matrix and thus changing the nature of the internal walls of the tooth (7).

Apexification has been improved with the use of bioceramic materials such as mineral trioxide aggregate (MTA) to improve the apical plug and biological response at the apex (8-10). As an apical plug, the MTA continues the formation of an apical barrier and also creates a stop for the obturation of the canal with gutta-percha. The use of MTA could reduce the number of patient visits with an increased success rate (11), but still no increase in root-wall thickness occurs.

## REGENERATIVE ENDODONTICS

Regenerative endodontics is defined as biologically-based procedures which replace damaged structures of the pulp-dentin complex (12, 129). The term "regeneration" has very specific connotation and does not truly occur within the canal chamber (130). However, AAE definition to "replace" damaged structures is quite appropriate and fitting as there is a new and unique mineralized layer that is deposited in these cases (130). When performed clinically, the AAE defines this term as a

Regenerative Endodontic Procedure (REP) (129). There are three goals of regenerative endodontics, which are: to remove the source of infection and reduce clinical symptoms, to increase root wall thickness and root length, and to have a return in sensibility response within the tooth (12). The process is based on tissue engineering and knowledge gained has benefitted from the biomedical fields. This process is accomplished via disinfection initially followed by inducing apical bleeding, which delivers a blood supply rich in mesenchymal stem cells into the canal chamber promoting tissue growth (38, 131, 132).

## HISTORY OF REGENERATIVE ENDODONTIC PROCEDURES (REPs)

Despite their recent surge in popularity, REPs are not new in the literature. Interestingly, Nygaard-Østby had performed preliminary work in the field in the 1960s and hypothesized that a blood clot formation in the canal could lead to revascularization and healing (133). He conducted research on patients with vital and non-vital pulps where root canal treatment was performed, and apices enlarged to allow bleeding into the canal system. Teeth were medicated if necrotic, restored and followed for a period of time until ultimately were extracted and sectioned. Interestingly, despite being left unfilled, all teeth had resolution of symptoms and pathosis. Some of the cases showed evidence of "regeneration" in that there was apical closure of some teeth and others developed ingrowth of connective tissue (133). Histologically, the new tissue differed from normal pulp tissue in that it included cementoblasts while lacking odontoblasts (133). Nonetheless, this was a seminal study despite the lack of attention that it received at the time.

In 1966, a study involving mixtures of antibiotic paste emerged where the investigators instrumented short of the expected vital tissue and medicated, representing

the first case where antibiotic mixtures were used in immature necrotic teeth for disinfection (134). As with Nygaard-Ostby, all teeth resolved clinical signs and symptoms of infection. However, in this study, there was a reported increased root length with all cases (134). Then, in 1971, another Nygaard-Ostby study emerged with similar results demonstrating resolution of symptoms and increased root width (15). However, the tissue within the canals remained the same, lacking the tissues expected within a normal tooth (15).

Another 30 years passed before regenerative endodontics continued its course with the first case report of successful REP. In the seminal study by Iwaya, double antibiotic paste (DAP) using the same two antibiotics (ciprofloxacin and metronidazole) as presented in this study was utilized (135). Though a case report, this paper was able to demonstrate that an appreciable amount of root width and length can develop with this procedure (136). In treatment of this infected necrotic immature tooth, Iwaya utilized 5% NaOCl and 3% hydrogen peroxide over the course of six appointments with interappointment medicament of DAP to achieve the desired results, which were demonstrable continued root length and thickness radiographically over two years later (135). Another case report by Banchs and Trope was published three years later utilizing triple antibiotic paste (TAP) consisting of ciprofloxacin, metronidazole, and minocycline (21). The necrotic pulp space of the immature permanent tooth was irrigated with 5.25% NaOCl and medicated with TAP for 28 days. Then, at the second appointment, antibiotic was removed with saline and bleeding was induced followed by a restoration to seal the chamber (21). These two studies both reported a return to sensibility testing at over two

years post-operatively (136). This protocol became the framework for treatment protocol in REPs and was utilized in multiple reported cases (137-140).

## CLINICAL ASEPSIS THROUGH DISINFECTION

We understand from Kakehashi et al. that bacteria are the cause of apical periodontitis (78). Our understanding of bacteria present within the necrotic tooth is improving with the development of Next-Generation Sequencing techniques. Currently, the identified important players within the canal are gram-negative and gram-positive species. *Fusobacterium*, *Dialister*, *Porphyromonas*, *Prevotella*, *Tanerella*, *Treponema*, Campylobacter, and *Veillonella* are the most prevalent gram-negative bacteria (141). *Parvimonas*, *Fillifactor*, *Pseudoramibacter*, *Olsenella*, *Actinomyces*, *Peptostreptococcus*, Streptococcus, Propionibacterium, and Eubacterium are the most prevalent gram-positive bacteria identified (141). In the primary endodontic infection of immature permanent teeth, as would be seen in REPs, *A. naeslundii* was identified as most prevalent (141). These species are present in complex and diverse biofilms along the canal walls as well as penetrating into dentinal tubules (142).

Bacterial species also change as the infection becomes more established and environmental conditions such as oxygen saturation and availability of nutrients changes, progressing from facultative bacteria in the presence of oxygen to eventually obligate anaerobes later in disease progression (143).

## TYPES OF IRRIGANTS

The primary goal of regenerative endodontics is resolution of clinical signs and symptoms as evidenced by radiographic healing. Chemical disinfection is critical as minimal to no mechanical instrumentation is recommended in these procedures.

### Sodium Hypochlorite (NaOCl)

Primarily, the use of NaOCl is recommended as it is antibacterial, dissolves organic tissue, and provides lubrication for instrumentation (116, 144, 145). NaOCl has been in use since its introduction in 1919 (150JJ). Concentration is recommended at 1.5% as the immature tooth presents with an open apex so as not to cause significant harm to the patient (145, 146). Equally, a greater concentration can adversely affect stem cells and growth factors located in the apical papilla (147-149).

### Calcium Hydroxide ($Ca(OH)_2$)

Introduced in the 1920s by B.W. Hermann, $Ca(OH)_2$ has been a workhorse in endodontic disinfection (13). It is effective due to its alkaline nature, with a pH above 12 and works by direct contact, inhibiting microorganism DNA replication (150). $Ca(OH)_2$ is able to inactivate lipopolysaccharide (LPS) (151, 152). $Ca(OH)_2$ demonstrates a beneficial effect for stem cell proliferation in all concentrations (20, 153, 154). However, $Ca(OH)_2$ can affect hard tissue formation by interfering with the osteoprotegrin/RANKL ratio (155) as well as negatively affecting the phosphate:amide ratio (31). Andreasen reported prolonged use of $Ca(OH)_2$ intracanal can reduce tooth fracture resistance, which was confirmed in other studies (2, 156). $Ca(OH)_2$ also has demonstrated limited effectiveness against commonly isolated endodontic microbes including *E. faecalis* and

*Porphyromonas gingivalis* (24). For these reasons, antibiotic pastes retain an influential role.

## Triple Antibiotic Paste (TAP)

To combat the multispecies nature of the bacteria present within endodontic infections, pastes consisting of multiple antibiotics have been proposed. TAP was introduced by Hoshino when examining in-vitro endodontic comparisons and found that the combination of ciprofloxacin, metronidazole, and minocycline was effective (18, 19). In-vitro studies have demonstrated that 0.3 mg/mL of TAP significantly reduces bacterial load (23, 25, 157). However, challenges exist when utilizing TAP, most notably the issue of dentin discolorization associated with minocycline (158, 159). Also, minocycline causes demineralization by binding to calcium ions with its low pH of 2.9 (7, 160). Equally and more importantly in REPs, concentrations above 1 mg/mL demonstrate detrimental effects on SCAP (153) and DPSC (23, 27, 161). Given these complications with minocycline, other antibiotics have been recommended as replacements such as cefaclor, amoxicillin, or clindamycin (136). Clindamycin is efficacious against many endodontic microbes (162, 163). When considering an alternative antimicrobial to minocycline, the term modified TAP (mTAP) has been promoted as a useful replacement (31).

## Double Antibiotic Paste (DAP)

An alternative is to remove minocycline altogether and utilize DAP, which consists of ciprofloxacin and metronidazole (164). DAP does not cause the levels of staining as found with TAP and has been found to be equally efficacious against

endodontic pathogens (24, 26, 135) while showing low cytotoxicity to dental pulp stem cells and SCAP (23, 162). This has led to growing interest in DAP as antibiotic treatment of choice.

### Ethylenediaminetetraacetic acid (EDTA)

EDTA is a viscous chelator, removing the inorganic portion of the smear layer by binding metallic ions (165). The smear layer is created mechanically and chemically and occludes the dentinal tubules causing decreased disinfection and bond strength when required (113). EDTA at a concentration of 17% removes the smear layer and releases growth factors from inside dentin (166-168). By removing the smear layer, EDTA increases surface area, possibly leading to better stem cell attachment, as evidenced by DPSC attachment to dentin pre-treated with EDTA (169, 170). EDTA can also mitigate some of the negative effects of NaOCl, again possibly increasing survival of stem cells of the apical papilla (149). If left in place too long, EDTA is able to demineralize peritubular and intertubular dentin (103).

## MAJOR DOMAINS OF REPs

The secondary goal of regenerative endodontics is increased root length and root wall thickness, followed by the tertiary goal of positive sensibility. Though the primary goal is most important, the secondary and tertiary goals are where regenerative endodontics research begins to differentiate itself from traditional endodontics. There are three major domains required to develop REPs. They include stem cells, scaffold or tissue engineering materials, and growth factors (12).

## Stem Cells

Stem cell nurturing is the tissue engineering aspect of REPs. Stem cells can be either pluripotent or multipotent, dividing into any human cell or into their identical cell, respectively (37, 131). Stem cells can be further sub-categorized according to their source. Allogenic stem cells derive from the same species. Autologous stem cells are collected from the same individual. Xenogenic stem cells originate from a different species. There exist a great number of mesenchymal stem cells (MSCs) in the periapical area of immature teeth with necrotic pulp (131). Stem cells are found in the apical papilla (SCAPs), dental pulp stem cells (DPSCs), dental follicle progenitor stem cells (DFPCs), periodontal ligament stem cells (PDLSCs), and stem cells from human exfoliated deciduous teeth (SHEDs) (38-40, 171-173). Stem cells are concentrated in the cell-rich zone of the pulp, near the odontoblastic layer (136). The above stem cell lines are essential for pulp fibroblasts, extracellular matrix, and collagen regeneration (40, 89, 174). The DPSC as well as stem cells from the apical papilla (SCAP) are important for regenerative endodontics (38, 39). DPSCs have the ability to form dentin and pulp-like tissues when transplanted in immunocompromised mice using hydroxyapatite/tricalcium phosphate scaffolds (40, 41). Based on their location in proximity to the Hertwigs epithelial root sheath (HERS), SCAP are believed to be the main source of undifferentiated cells involved in root development, whether in vital cases or with REPs (39). These SCAP have demonstrated ability to retain their vitality and stemness in an inflammatory environment as well as increased osteogenic differentiation and induction of angiogenesis (175).

## Scaffold

A scaffold is required for transport of nutrients, oxygen, and metabolites to the site of repair so that stem cells are capable of differentiating accordingly (17, 176). Nevins was the first to use a scaffold in REPs in 1976 using a collagen membrane (177). He later used a cross-linked collagen-hydroxyapatite scaffold (SynOss) in an off-label application in four cases and reported substantial hard tissue repair (178). Hutmacher identified six properties of an ideal scaffold for use in REPs (179):

1. Porous structure to allow for tissue attachment
2. Resorbable membrane
3. Cellular growth and proliferation
4. Sufficient mechanical properties
5. Biocompatible materials
6. Good handling characteristics

Often, the patient's own blood clot is indicated as the scaffold (12). However, given recent technological advancements, new scaffolds are being designed (12). Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) have been utilized as scaffold (137, 180-182). In addition, key growth factors may potentially be released by the use of these scaffolds (166, 167, 170).

## Growth Factors

Growth factors are endogenous molecules that act as a signaling molecules promoting tissue growth, maturation, healing, and repair with significant impact on the regeneration potential of stem cells (183). These growth factors are located within the dentin matrix and under certain conditions are released into surrounding environment to

influence neighboring cells and tissues (183). The use of EDTA in regeneration aids in the release of signaling molecules including transforming growth factor beta (TGF-β), which promotes stem cell differentiation into odontoblasts and signals pulp tissue mineralization while promoting wound healing (166). Additional growth factors, including bone morphogenic protein (BMP) and vascular endothelial growth factor (VEGF), promote odontoblast differentiation and vasculature formation, respectively (183).

### Nano-Hydroxyapatite (n-HA)

Nano-HA has been used in dentistry for a variety of indications ranging from desensitization by blocking of dentinal tubules (184) to remineralization of carious lesions (185). More exciting, however, has been its incorporation in the medical tissue bioengineering fields. Hydroxyapatite has a chemical formula of $Ca_{10}(PO_4)_6(OH)_2$ and is the main component of enamel, giving its bright white appearance (44). The size of n-HA ranges from 50 to 1000 nm (44). In recent bone regeneration models n-HA has been used to increase healing potential. One study in 2015 created sponge-like plugs of collagen-hydroxyapatite implanted in mice and found that an appropriate HA proportion facilitated MSC attachment, proliferation, and differentiation (50).

## CLINICAL APPLICATION

REPs address a challenge – the necrotic pulp of an immature permanent tooth – that has not been easily treated. The excitement is in the potential to regenerate tissues and re-establish vitality in a previously necrotic pulp with a potentially sizeable apical infection. However, the true benefit is in the fact that a tooth that, if treated

conventionally with apexification, would have had a questionable prognosis for the duration of its life. Now there is a probability that with increased root width and root thickness, stability will be granted for a longer time. Whether or not DAP with n-HA finds its way into the AAE Guidelines for Regenerative Endodontics, it was exciting to have researched such an interesting field.

## CURRENT RECOMMENDED GUIDELINES FOR REPs

### Case Selection:

- Tooth with necrotic pulp and an immature apex
- Pulp space not needed for post/core, final restoration
- Compliant patient/parent
- Patient not allergic to medicaments and antibiotics necessary to complete procedure

### Informed Consent

- Two or more appointments
- Use of antimicrobials
- Possible adverse effects: staining of crown/root. lack of response to treatment, pain/infection
- Alternatives: MTA apexification, no treatment, extraction
- Permission to enter information into AAE database (optional)

### First Appointment

- Local anesthesia, dental dam isolation and access
- Copious, gentle irrigation with 20-ml NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g.,

needle with closed end and side-vents, or EndoVac™), lower concentrations of NaOCl are advised 1.5-percent NaOCl (20mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues

- Dry canals with paper points
- Place $Ca(OH)_2$ or low concentration of triple antibiotic paste

If the triple antibiotic paste is used: 1) Consider sealing pulp chamber with a dentin bonding agent [to minimize risk of staining] and 2) Mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 0.1mg/ml to 1.0 mg/ml

Triple antibiotic paste has been associated with tooth discoloration

Double antibiotic paste without minocycline paste or substitution of minocycline for other antibiotic (e.g., clindamycin; amoxicillin; cefaclor) is another possible alternative as root canal disinfectant

- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining)
- Seal with 3-4 mm of a temporary restorative material such as Cavit™, IRM™, glass-ionomer, or another temporary material, dismiss patient for 1 week to 4 weeks

Second appointment (1-4 weeks after 1st visit)

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial

- Anesthesia with 3-percent mepivacaine without vasoconstrictor, dental dam isolation
- Copious, gentle irrigation with 20 ml of 17-percent EDTA
- Dry with paper points
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cemento-enamel junction), an alternative to creating a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF), or autologous fibrin matrix (AFM)
- Stop bleeding at a level that allows for 3 mm to 4 mm of restorative material
- Place a resorbable matrix such as CollaPlug™, Collacote™, CollaTape™ over the blood clot if necessary and white MTA as capping material.
- A 3-mm to 4-mm layer of glass ionomer (e.g. Fuji IX™, GC America, Alsip, IL) is flowed gently over the capping material and light- cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA (such as bioceramics or tricalcium silicate cements [e.g., Biodentine®, Septodont, Lancaster, PA]) should be considered in teeth where there is an esthetic concern
- Anterior and Premolar teeth - Consider use of Collatape/Collaplug and restoring with 3 mm of a non-staining restorative material followed by bonding a filled composite to the beveled enamel margin

Molar teeth or teeth with PFM crown - Consider use of Collatape/Collaplug and restoring with 3 mm of MTA, followed by RMGI, composite, or alloy

Follow-up

- Clinical and radiographic exam
- No pain, soft tissue swelling or sinus tract (often observed between first and second appointments)
- Resolution of apical radiolucency (often observed 6 mos. to 12 mos. after treatment)
- Increased width of root walls (generally observed before apparent increase in root length and often occurs 12 mos. to 24 mos. after treatment)
- Increased root length
- Positive pulp vitality test response
- The degree of success of REP is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
    - Primary goal: The elimination of symptoms and the evidence of bony healing
    - Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential)
    - Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue)

# MATERIALS AND METHODS

EXPERIMENTAL GROUPS

Group 1 – $Ca(OH)_2$

Group 2 – Methylcellulose

Group 3 – Methylcellulose + 1 mg/mL DAP

Group 4 – Methylcellulose + 1 mg/mL DAP + 0.25% Hydroxyapatite

Group 5 – Methylcellulose + 1 mg/mL DAP + 0.50% Hydroxyapatite

Group 6 – Methylcellulose + 1 mg/mL DAP + 1.0% Hydroxyapatite

Group 1 serves as a positive control since $Ca(OH)_2$ has known positive effects on DPSC growth (186). Group 2 serves as the negative control group. Groups 3-6 serve as the experimental groups, testing the effect of hydroxyapatite on DPSCs. No true negative control was included as this had been conducted in an earlier study within this laboratory.

MATERIALS

Materials required for this project were purchased commercially or prepared as per the methodology below. DPSC from immature third molars were previously purchased and stored in aliquots in liquid nitrogen (Cook General BioTechnology, Indianapolis, IN, USA). Cell culture 24-well plates (Alkali Scientific Inc. Cat: TP9024), Transwell chambers (Falcon Product 1.0 μm permeable support transmembrane with transparent PET membrane: #353104 ), alpha modified minimal essential media, fetal bovine serum (FBS) (Atlanta Biologicals Inc., Flowery Branch, GA, USA), penicillin-streptomycin (Life Technologies Corporation, Grand Island, NY, USA), 0.25% trypsin/EDTA (Life Technologies Corporation), DAP powder [(Double antibiotic capsules; Ciprofloxacin HCL monohydrate (LETCO: 1509220034), Metronidazole, USP (MEDISCA: 137957/B); (1:1)*400 mg/400mg; #20)], methylcellulose (Methocel 60 HG,

28-30% methoxyl basis), hydroxyapatite nanoparticles (HA nanopowder <200 nm size, Aldrich: 677418-10G), ALP assay kit (Sigma Aldrich), Alizarin red reagents and Pierce BCA Assay Kit (Thermo Scientific: 23225) were purchased commercially.

## METHODOLOGY

### Preparation of Methylcellulose containing DAP ± HA

We prepared methylcellulose containing DAP as per previously published protocols (32). Initially, 1 mg/mL concentration of DAP was made by dissolving 25 mg each of metronidazole and ciprofloxacin in 25 mL of sterile water. Antibiotics were measured using sterilized aluminum foils and DAP powder was added to the beaker containing media. Solution was mixed with magnetic stir bar at speed 6/10 for 10 minutes until DAP was fully incorporated. It should be noted that 1 mg/mL DAP fully suspended. A paste-like consistency of DAP was achieved by incorporating 2.0 g of methylcellulose powder into solution at room temperature. The solution was then separated into three divisions so that the differing concentrations of hydroxyapatite nanoparticles could be added independently. Nano-HA was added by weight; for 0.25%, 0.50%, and 1.0% (Figure 9). A homogenous mixture was created ultimately by vibrating for less than 1 minute.

### Human dental pulp stem cells (DPSC)

DPSC were used for all studies. The stem-like nature of these cells has been previously established utilizing flow cytometric analysis. The DPSCs were previously frozen in liquid nitrogen in aliquots. Cells at passages from 3 to 5 were utilized in this experiment. Sub-confluent cells were detached from the culture plate with 0.05% trypsin-

EDTA. Cells were thawed, passaged once, plated at $4x10^4$ cells/well in 24-well plates (Figure 6) and placed into 37°C/5% $CO_2$ incubator (Figure 10). For proliferation assay, cells were cultured in alpha-modified essential medium (MEM) supplemented with 10% FBS and 1 % penicillin/streptomycin. For ALP and mineralization assays, the media was also supplemented with 10 mM β–glycerolphosphate and 50 mg/ml ascorbic acid.

Proliferation

DPSCs were cultured in 24-well plates with or without the medicaments for 3 days (Figure 7). 500 μL MEM culture media were placed in the floor of the wells followed by the transwells. 750 μL media was then placed into the transwell, followed by 100 μL of each treatment, respectively, using a tuberculin syringe. This experimental plate was then incubated for 3 days at 37°C/5% $CO_2$ incubator. On day 3, a modified MTS Assay kit (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay) was then used as a measure of the cell proliferation. This assay is able to determine the ability of the cells to metabolize. The time points at which the proliferation assay was run were 0-3 hours as per previous studies in this laboratory. 200 μL of MTS reagent solution was in each culture well per time point to be read. Enough reagent solution was reserved for three extra wells of only reagent solution as a control. Transwells were removed and discarded. Media was aspirated; appropriate volume of the reagent solution added to each well in plate with the same volume of reagent solution added as control to three empty wells on the plate. The Normal ratio for the reagent is 20 mL α-MEM:4 mL MTS: 200 μL PMS. Plate reading required 1 mL of above reagent mix to each well. For reading the plate, 100 μL from each well was pipetted to 96-well plate and absorbance measured at 490 nm (Figure 14). Again, this was performed at 0, 1, 2, and 3 hours in which

timeframe the absorbance on the spectrophotometer did not saturate out (Figure 15). Caution was used throughout the procedure to minimize light exposure as reagents are light-sensitive, and plates remained in incubator when not being manipulated. In total, proliferation was performed over three trials with a total n = 10, all performed in triplicate.

Quantification of ALP Activity

DPSCs were cultured in 24-well plates with or without the medicaments for 7 days. For ALP quantification, β-GP and AA were added to the media to promote differentiation of the cells. b-GP was added to MEM media at 0.01 volume and AA was added at 0.001 volume of MEM. 500 μL MEM culture media plus β-GP/AA was placed in the floor of the well followed by the transwell. 750 μL media was then placed into the transwell, followed by 100 μL of each treatment, respectively, using a tuberculin syringe. This experimental plate was then incubated for 3 days at 37°C/5% $CO_2$ incubator. At day 3, media were changed; transwells were removed delicately with forceps and placed in new 24-well plate. Media was aspirated from wells ensuring avoidance of cells. 500 μL MEM/β-GP/AA media were added to floor of wells, transwells were returned to original well, and an additional 250 μL media were added to the transwell. Plates were returned for incubation until day 7. For cell lysis prior to ALP activity estimation, 10 mL lysis buffer were required consisting of 7.92 mL double distilled $H_2O$, 0.5 mL of 1 M Tris-Cl (pH 7.5), 0.3 mL of 5 M NaCl, and 1 mL of 10% Igepal-CA 630 (NP-40). For inhibitors, 0.25 mL of 10% sodium deoxycholate, 0.01 mL of 10 mg/mL leupeptin hydrochloride, 0.01 mL of 10 mg/mL aprotinin, and 0.01 mL of 10 mg/mL of pepstatin were used. For the substrate solution, 10 mL of double distilled $H_2O$ mixed with 10 mL of alkaline

buffer and 40 mg of 4-nitrophenyl phosphate sodium salt hexahydrate powder were made ready. And 40 μL NaOH (10 M) were mixed with 20 mL $H_2O$ for NaOH (20 mM) for 20 mL. After culture, transwell chambers containing the spent medicaments were discarded and cells in the bottom chamber were washed twice with ice cold phosphate buffered saline (PBS). Cells were lysed in 100 μL of lysis buffer and the bottom of the well was scraped with a pipette tip. Cells were carefully placed in a microcentrifuge tube and sonicated for 5 minutes (Figure 12). Microcentrifuge tubes were then spun on $13.2x10^3$ RPM for 3 minutes (Figure 13). Supernatant was then stored at -34°C until ALP assay was performed as per the laboratory protocol described below.

The colorimetric conversion hydrolyzing p-nitrophenol phosphate to p-nitrophenol was used to determine alkaline phosphatase activity according to protocols in this lab(187). A standard curve was created using serial dilution. 200 μL of standards in triplicates were added to a 96-well plate. 3 μl of the cell lysate was added to be assayed in triplicate. 100 μL of the substrate solution (40 mg of p-nitrophenyl phosphate (p-NPP), 10 mL of alkaline buffer, and 10 mL of double distilled $H_2O$) was added to each well that contained photo-sensitivity and incubated at 37°C for 1 hour. Incubation was stopped by adding 95 μl of 20 mM NaOH when the treatment groups were within the color range of the standards, determined by the colorimetric conversion of p-NPP to nitrophenol. Final volume was cooled to room temperature and optical absorbance measured at 405 nm using the plate reader (Figures 14, 17).

ALP activity was normalized by total protein in the cell lysates. For this we used the BCA kit according to the manufacturer's protocol. Briefly, a working solution of BSA 250 μg/mL in distilled $H_2O$ was used to generate the standard curve. In parallel,

aliquots of the protein lysates were added to the BCA reaction mixture. Absorbance for both the standards and samples were measured at 562 nm (Figure 14). Total protein in each lysate was determined based on the BSA standard curve (Figure 20). In total, ALP assay was evaluated in three trials with a total n = 13, all performed in triplicate.

Mineralization Assay

DPSCs were cultured in 24-well plates with or without the medicaments for 7 days. For mineralization quantification, β-GP and AA were added to the media to promote differentiation of the cells. β-GP was added to MEM media at 0.01 volume and AA was added at 0.001 volume of MEM. 500 μL MEM culture media plus β-GP/AA was placed in the floor of the well followed by the transwell. 750 μL media was then placed into the transwell, followed by 100 μL of each treatment, respectively, using a tuberculin syringe. This experimental plate was then incubated for 3 days at 37°C/5% $CO_2$ incubator. At day 3, media was changed; transwells were removed delicately with forceps and placed in new 24-well plate. Media was aspirated from wells ensuring avoidance of cells. 500 μL MEM/β-GP/AA media was added to floor of wells, transwells were returned to original well, and an additional 250 μL media was added to the transwell. Plates were returned for incubation until day 7. The mineralization plates were stopped at 7 days by fixing the cells with 500 μL of 3.7% formaldehyde in PBS added to each well and waiting 15 minutes for cell fixation. This solution was aspirated from wells and ended with fixed cells in 1 mL of PBS. Separately, Alizarin Red S solution was mixed with a stir bar. A standard curve of solution in 1.7 mL microcentrifuge tubes was then prepared. PBS was suctioned off from the fixed mineralization plates and rinsed twice with distilled $H_2O$. 500 μL per well of 40 mM Alizarin Red S were added to each well on

plate. Shaker was set to speed 20 for 10 minutes. Alizarin Red S was suctioned off and rinsed with $H_2O$ three times. Water was aspirated and washed with 1 mL of PBS to each well for 15 minutes. PBS was aspirated and 500 μL of CPC was added to each well to extract Alizarin Red S. Solution was placed on shaker at speed 20 for 15 minutes. 150 μL of standards and samples were placed into a 96-well plate (Figure 16) in triplicates and absorbance was measured at 562 nm (Figure 14). In total, mineralization assay was evaluated in three trials with a total n = 13, all performed in triplicate.

## Sample Size and Statistical Analysis

Comparisons between groups for differences in mineralization, BSA, and ALP activity were performed using analysis of variance (ANOVA), with different variances allowed for each group and a random effect included in the model to account for correlation within each of the three trials. The effects of time and group on proliferation were evaluated using repeated measures ANOVA, with different unstructured variance-covariance matrices allowed for each group and a random effect included in the model to account for correlation within each of the three trials. A simulation-based multiple comparisons procedure was used to adjust for multiple comparisons. A 5% significance level was used for all tests.

# RESULTS

# RESULTS

## Proliferation Assay

Regarding proliferation, comparisons were made at three time points; T=0, 1, 2, 3 hours. Very little statistically significant difference was noted between treatment groups at all time points. Of note, however, the MC + DAP + HA 1% group displayed significantly greater metabolic activity than that of $Ca(OH)_2$ at T= 2, 3 hours with p = 0.039 and 0.007, respectively. There was a positive trend toward greater proliferation in the three experimental groups throughout all time (Figure 18). Equally, these three groups outperformed the MC + DAP and $Ca(OH)_2$ groups; these two groups had very similar readings (Figure 19). And the MC group consistently demonstrated the lowest proliferation with the largest standard error as well.

## ALP Assay

The three experimental groups (DAP + MC + HA 0.25%, DAP + MC + HA 0.5%, DAP + MC + HA 1.0%) statistically outperformed all other groups except MC + DAP + HA 0.25% was only moderately greater than $Ca(OH)_2$, p = 0.100 (Figure 22). The other two groups, MC + DAP + HA 0.5% and MC + DAP + HA 1.0%, were significantly greater than $Ca(OH)_2$, at p = 0.002 and p = 0.010, respectively. All three experimental groups were significantly greater than the MC group and the MC + DAP group. The $Ca(OH)_2$ group was also significantly greater than the MC + DAP group, p = 0.031.

Comparing the three experimental groups, there was a trend favoring MC + DAP + HA 0.5%.

Mineralization Assay

When comparing $Ca(OH)_2$ to all three experimental HA groups (DAP + MC + HA 0.25%, DAP + MC + HA 0.5%, DAP + MC + HA 1.0%), a statistically significantly greater amount of mineralization was noted (Figure 24). MC + DAP + HA 0.25% ($p = 0.012$), MC + DAP + HA 0.5% ($p = 0.008$), and MC + DAP + HA 1.0% ($p = 0.013$) were all equally and similarly greater than the $Ca(OH)_2$ group. Interestingly, the strongest group was the MC alone group which was meant to serve as the control group demonstrating statistical significance over MC + DAP and $Ca(OH)_2$ groups with $p = 0.003$ and $p = 0.001$, respectively. One other significance occurred, which was MC + DAP + HA 0.5% over MC + DAP, at $p = 0.049$. Comparing the three experimental groups, a slight linear trend appeared matching HA concentration.

# FIGURES AND TABLES

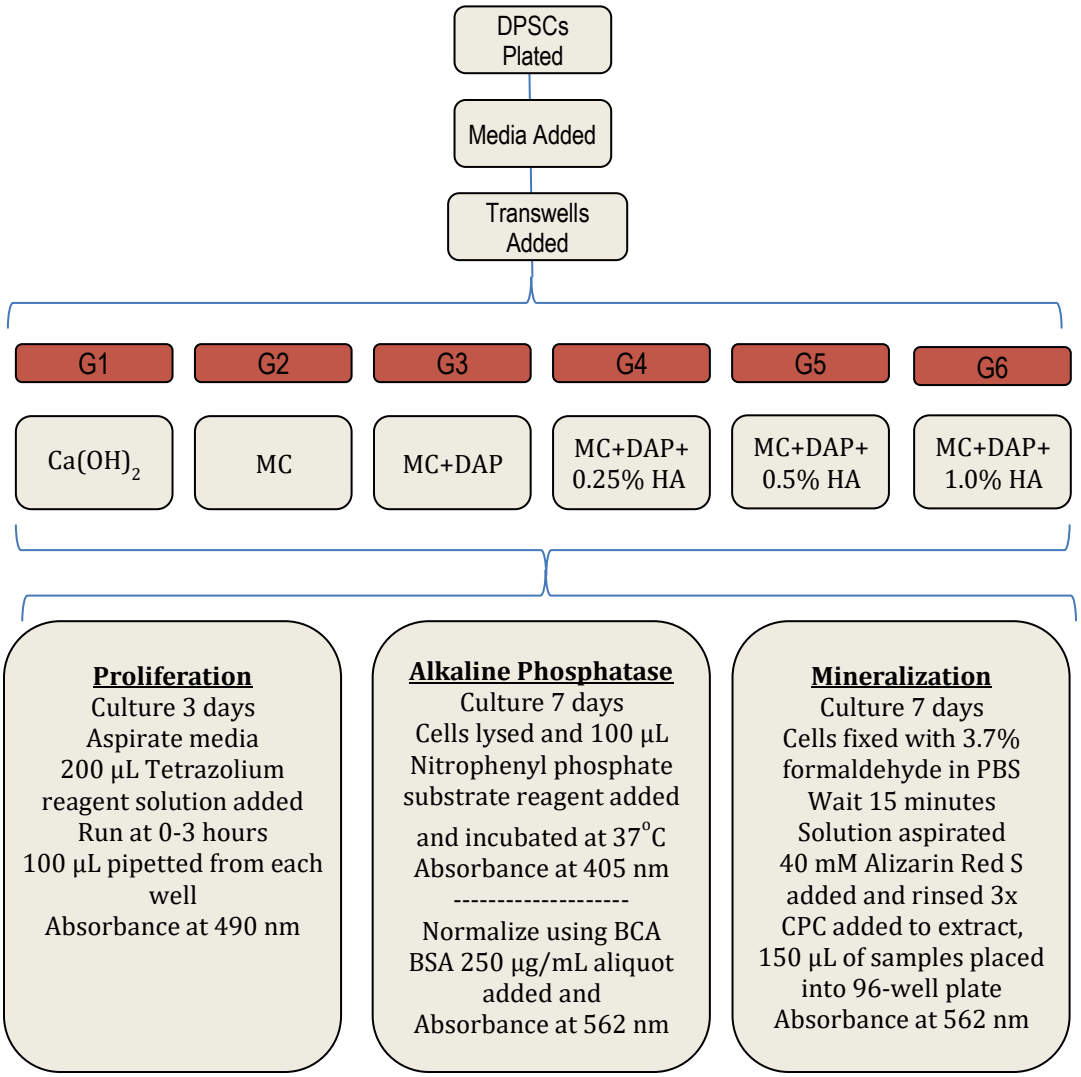


FIGURE 1. Flowchart of experimental design

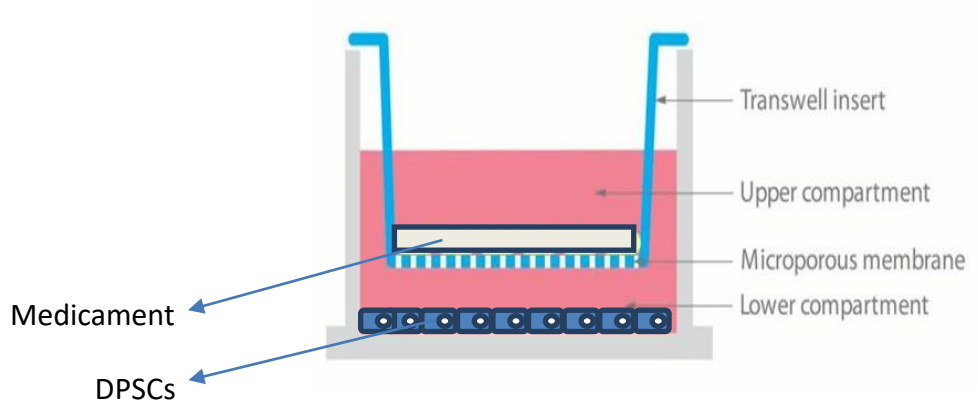


FIGURE 2. Experimental design.

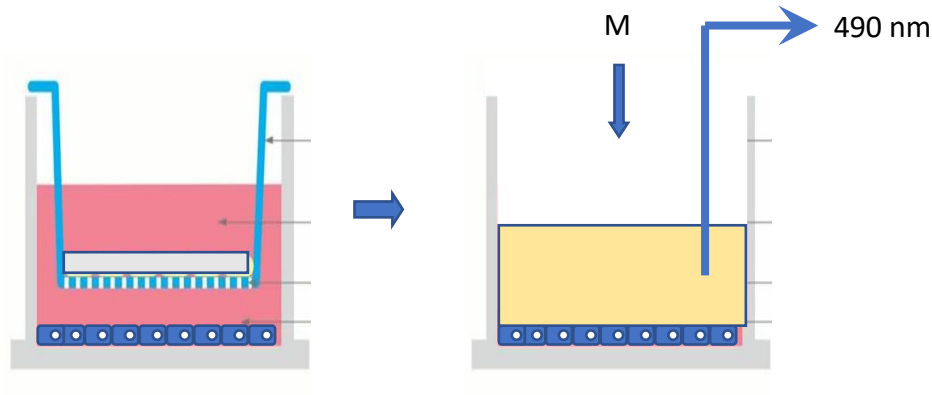


FIGURE 3. Experimental MTS assay.

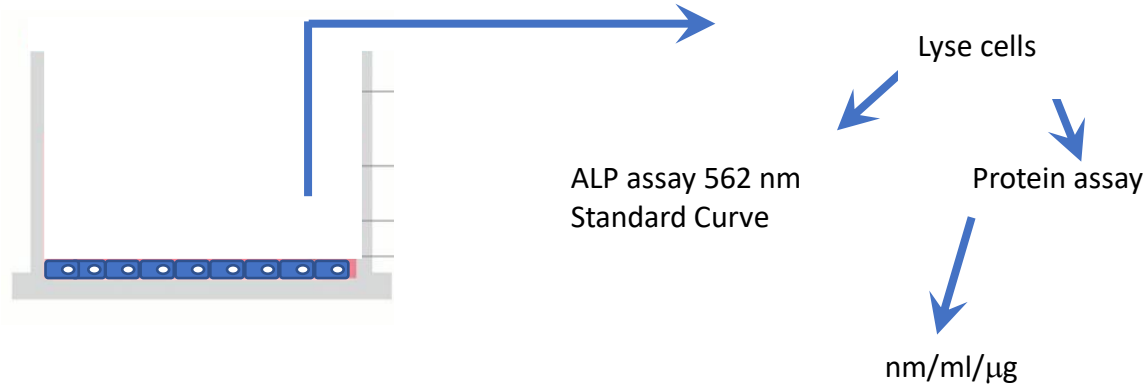


FIGURE 4. Experimental ALP assay.

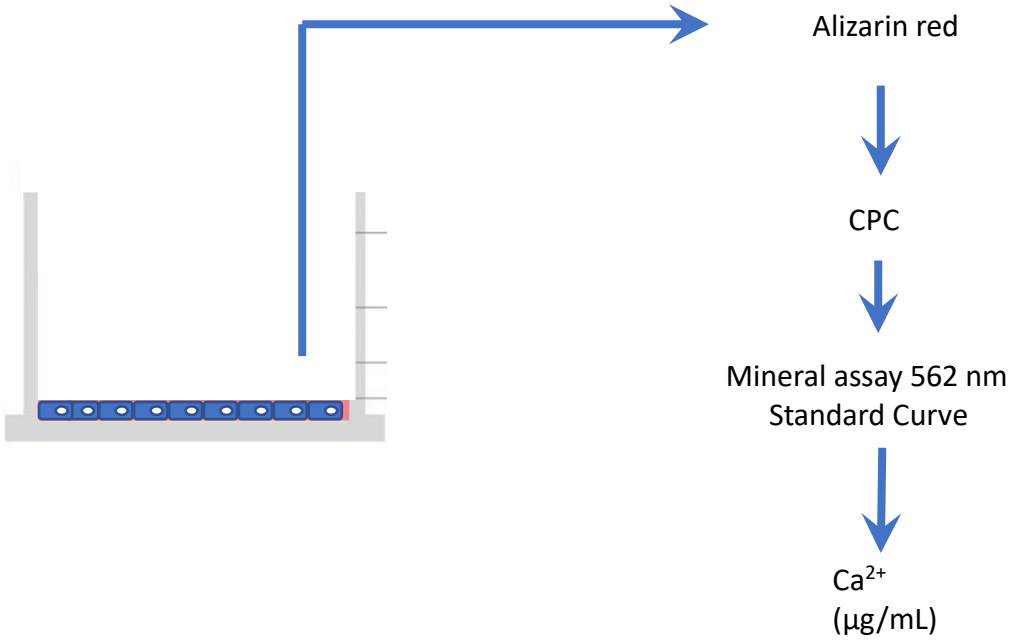


FIGURE 5. Experimental Mineralization assay.

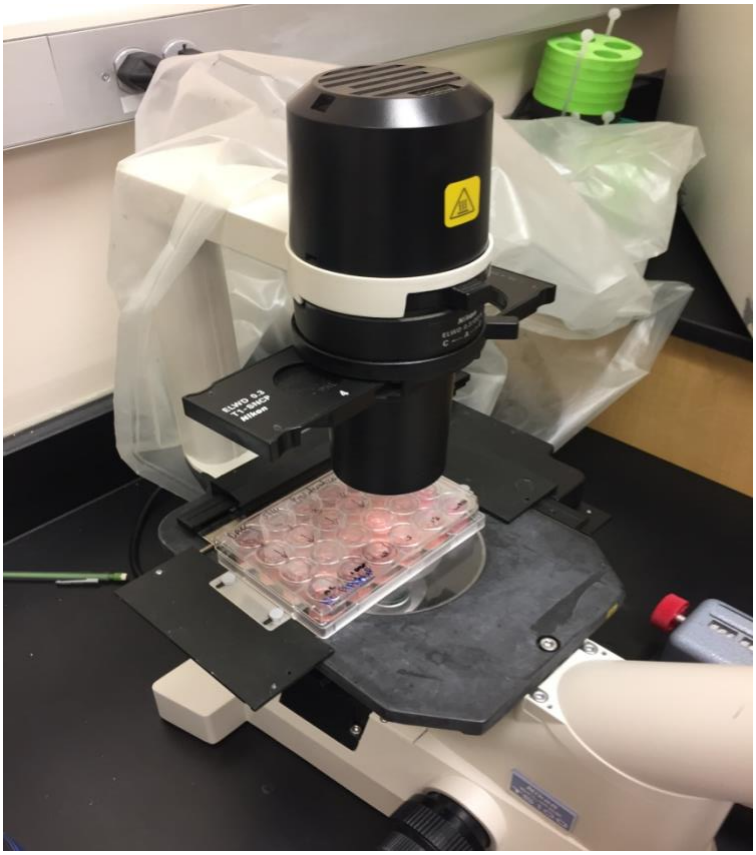

FIGURE 6. Counting DPSC after plating.

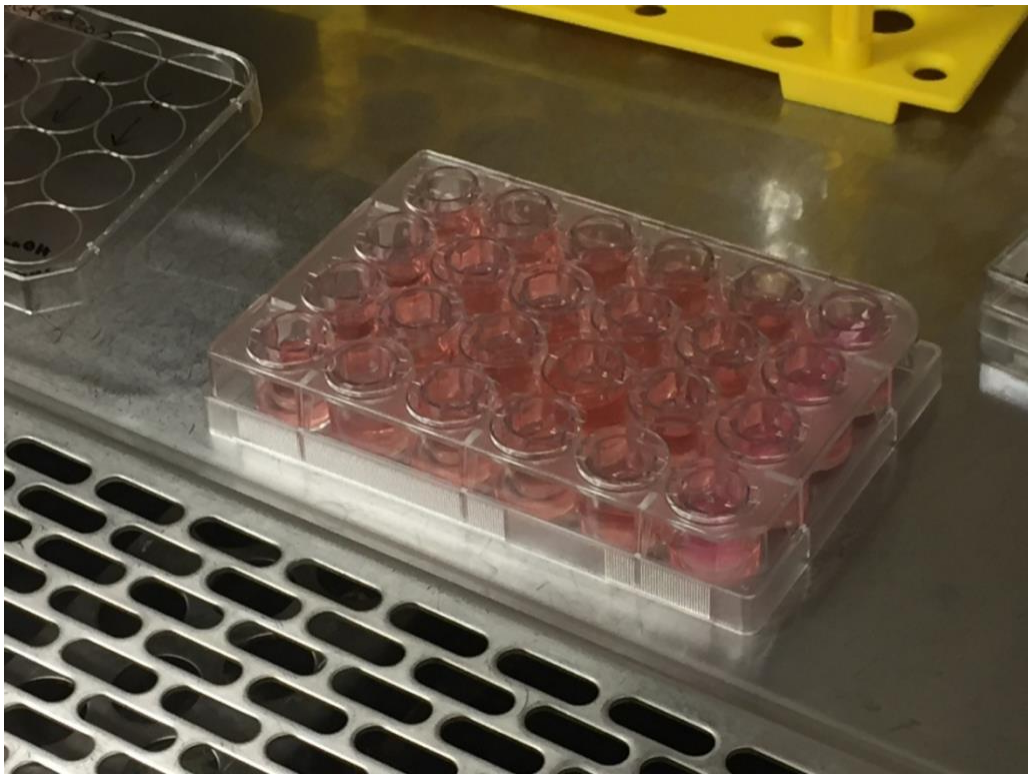

FIGURE 7. A 24-well plate with Transwells and medicaments with culture in place.

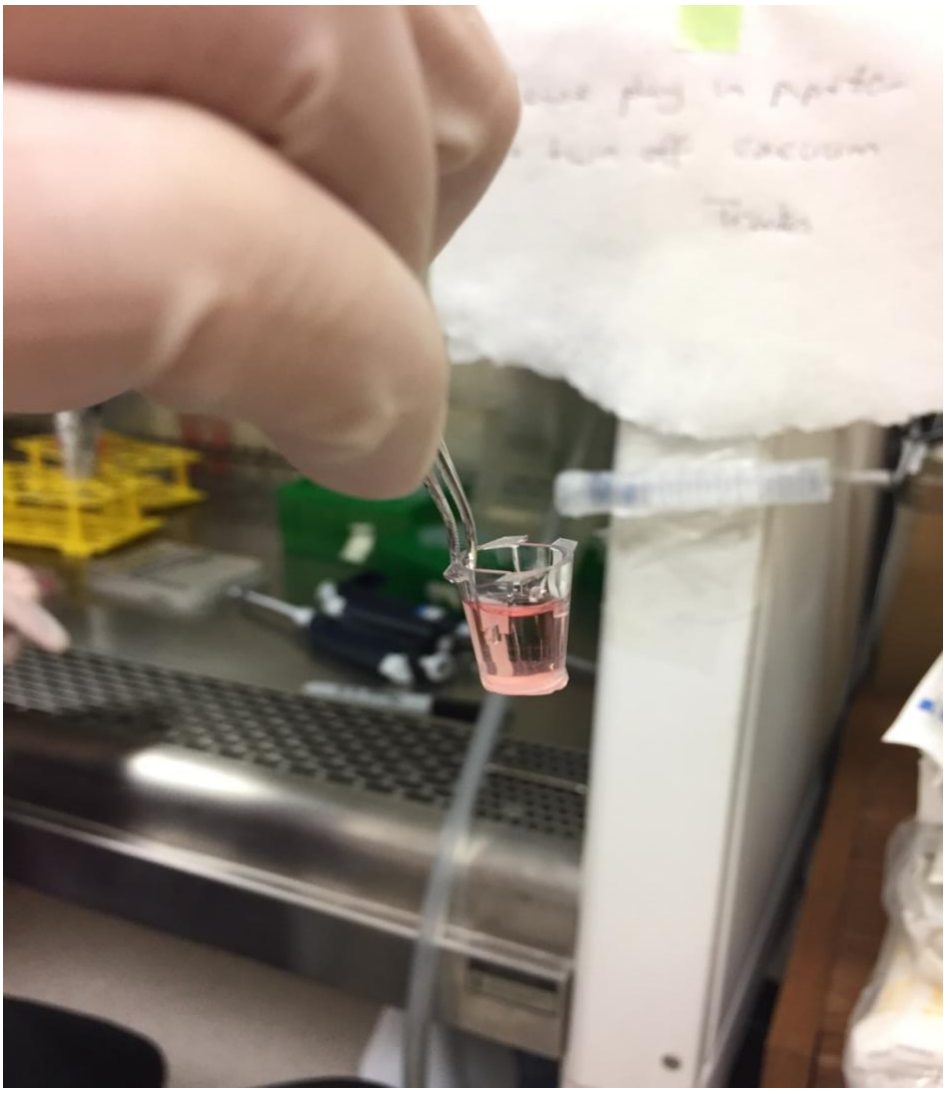

FIGURE 8. Image of 1.0-μm pore size Transwell with experimental medicament lining membrane and culture media filling well.

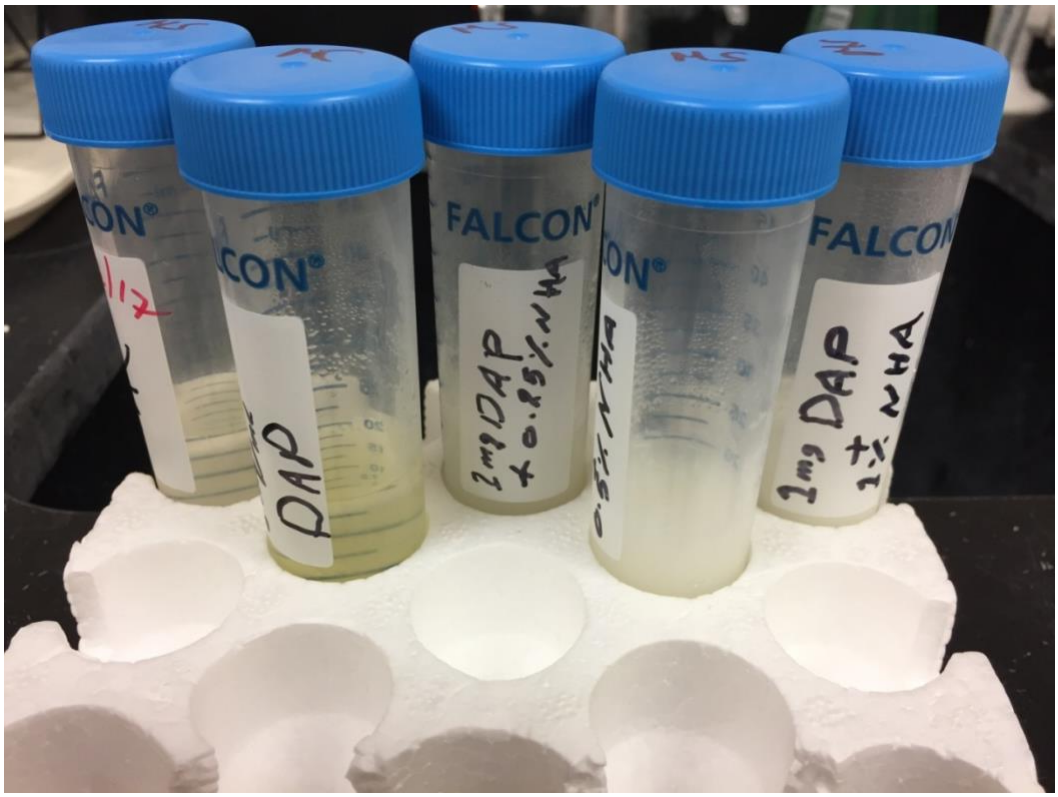


FIGURE 9. Photo of experimental groups within experimental tubes.

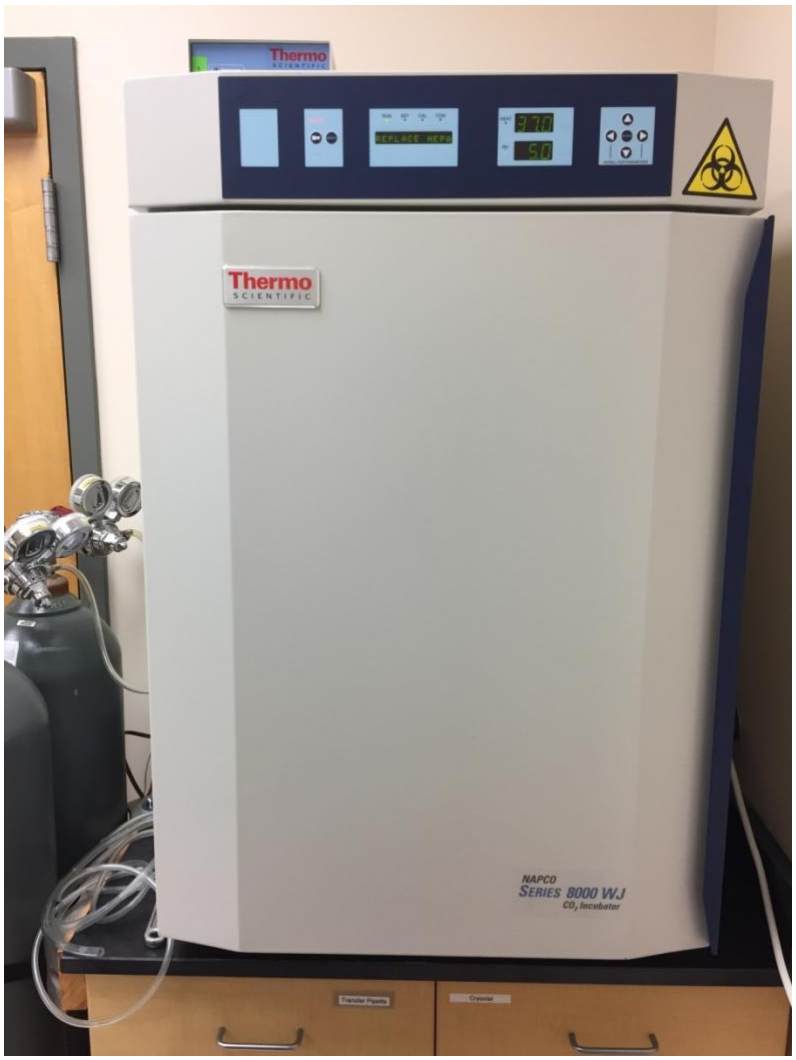


FIGURE 10. Photo of $CO_2$ incubator utilized during experimentation.

FIGURE 11. Pipetting during assay preparation.

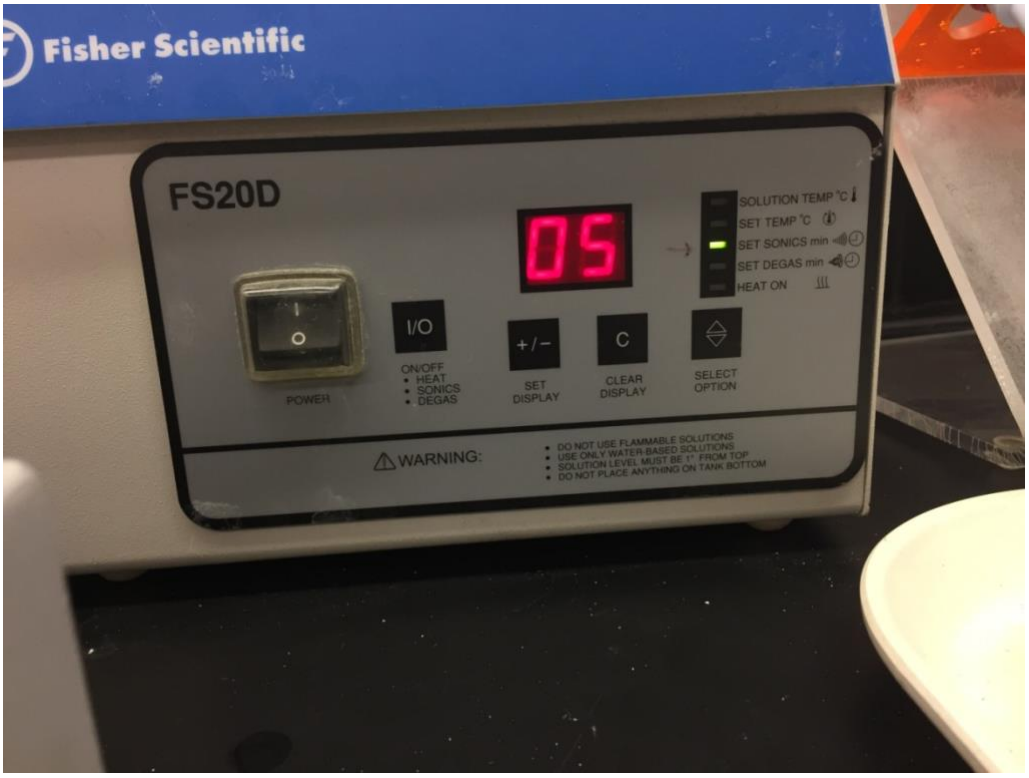


FIGURE 12. Sonication set to 5 minutes during ALP assay.


FIGURE 13. Centrifugation of tubes during assay.


FIGURE 14. Spectrophotometer utilized during experimentation for quantitative analysis of results.

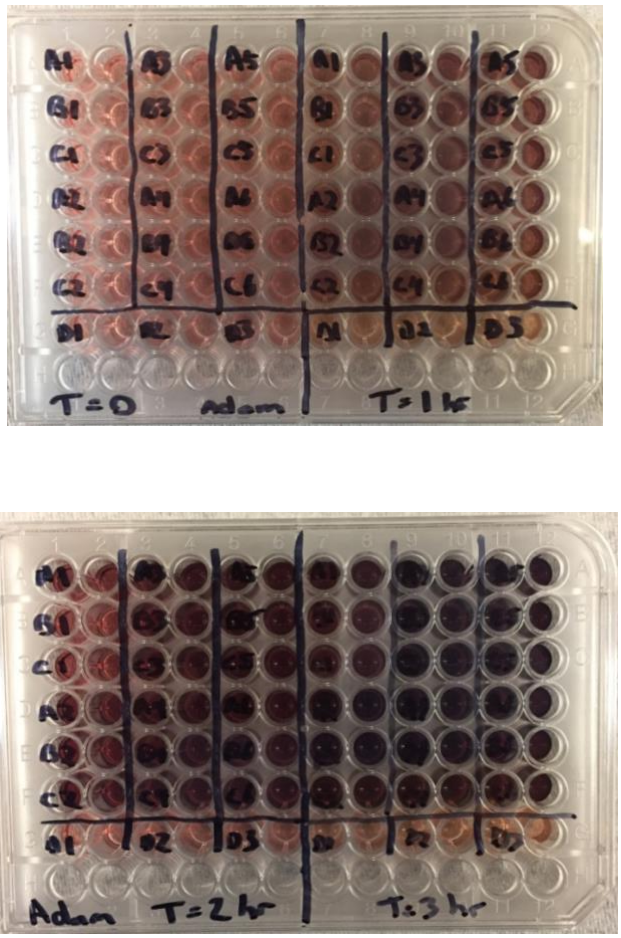


FIGURE 15. Experiment Trial 3 proliferation assay.

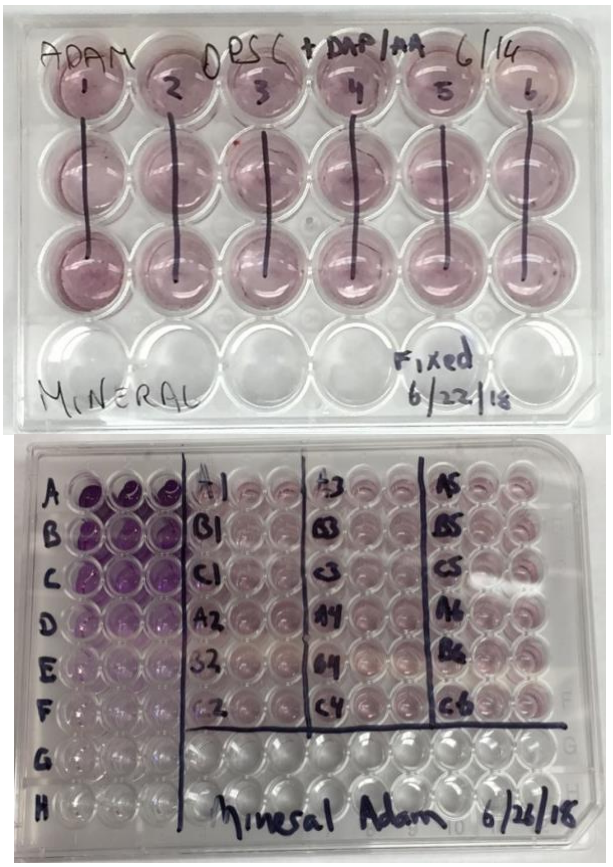


FIGURE 16. Experiment Trial 3 mineralization assay.

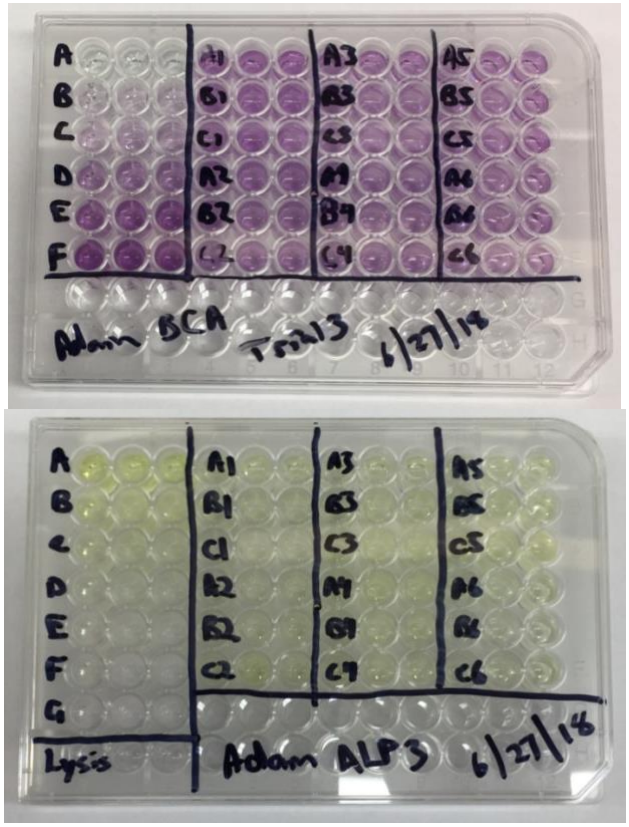


FIGURE 17. Experiment Trial 3 BSA and ALP assay.

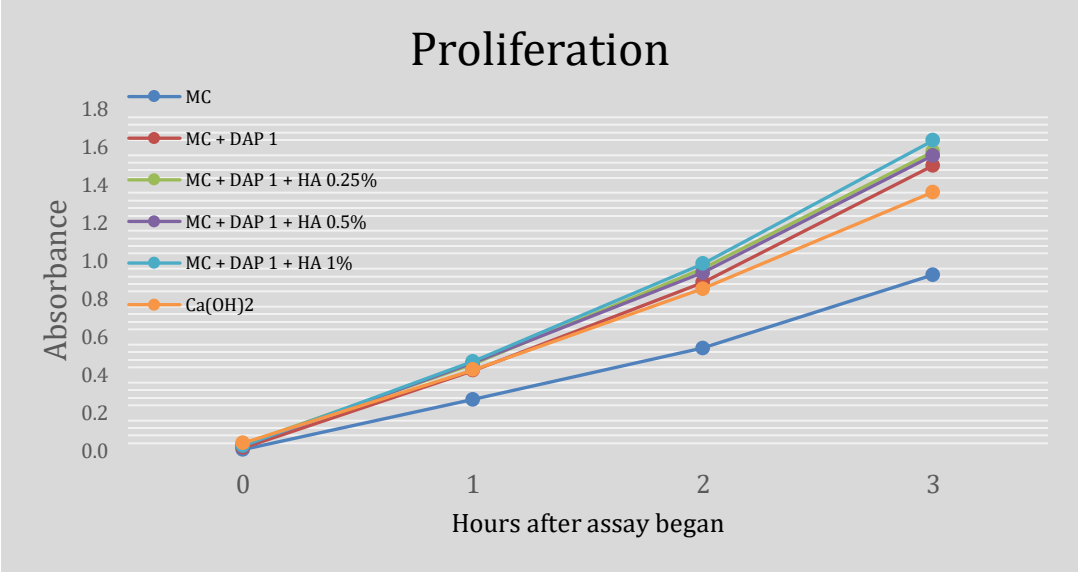


FIGURE 18. Proliferation Results: Assay quantitatively analyzed over T = 0 to 3 hours (significance demonstrated below).

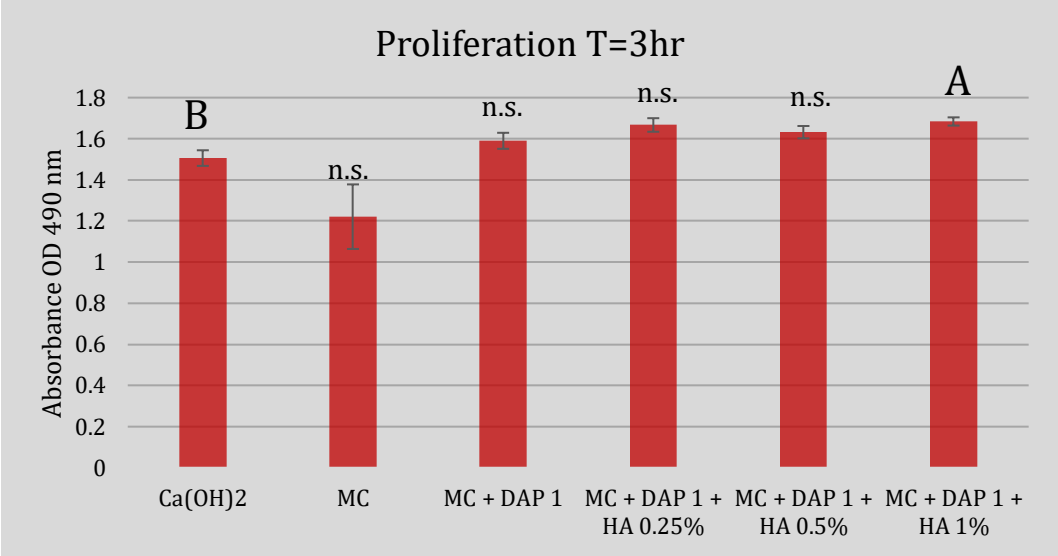


FIGURE 19. Proliferation results: Significance ($P < 0.05$) demonstrated by differing letters above respective experimental groups. Note: Graph demonstrates measurement at T = 3 hours as absorbance separated through timespan.

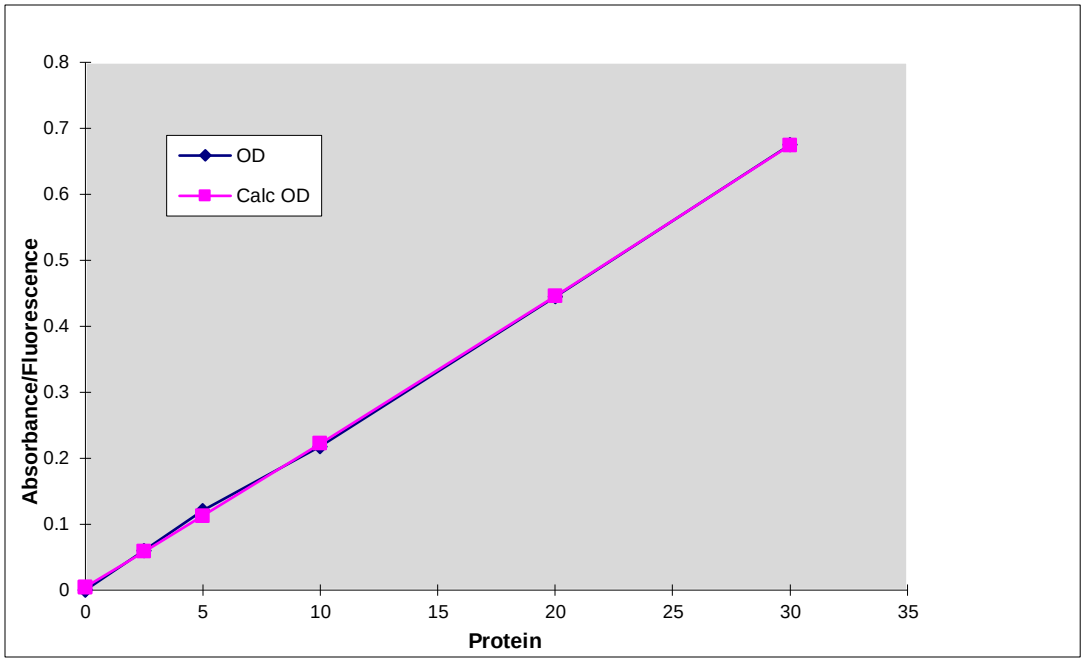


FIGURE 20. BSA standard curve for Trial 3 against which trial measurements were measured to determine ALP activity (below). Note: Standard curve was established for each of the three trials separately.

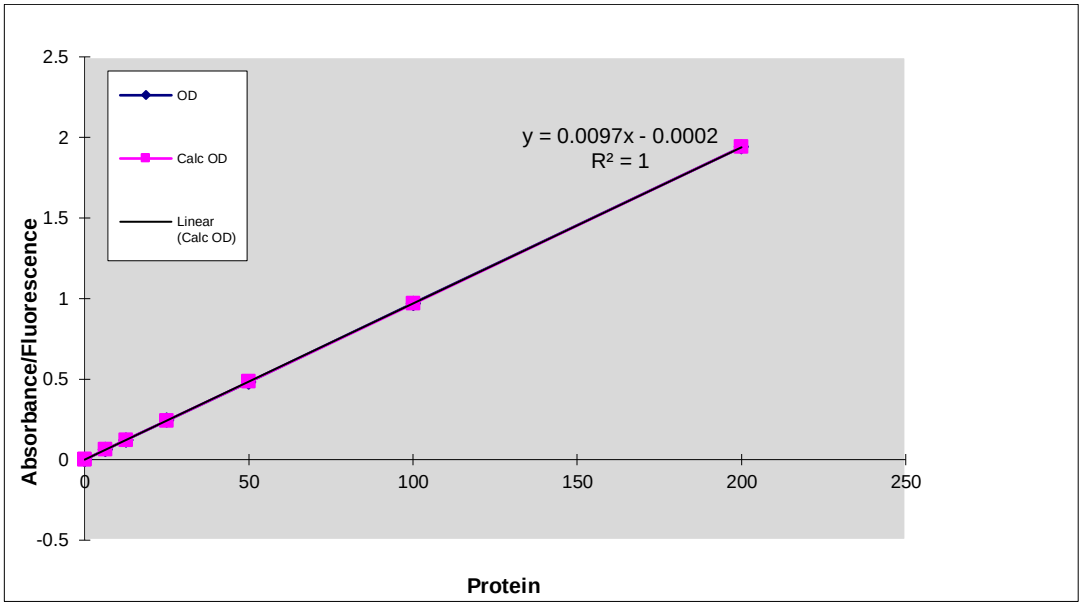


FIGURE 21. ALP standard curve for Trial 3 against which trial measurements were measured to determine ALP activity (below). Note: Standard curve was established for each of the three trials separately.

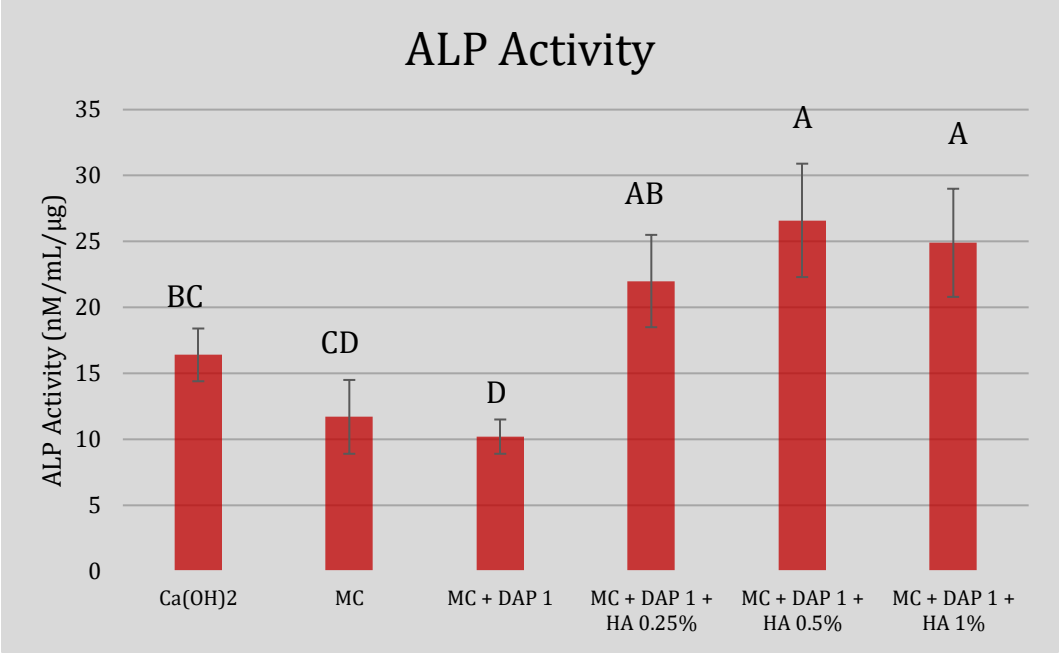


FIGURE 22. ALP Results: Significance ($P < 0.05$) demonstrated by differing letters above respective experimental groups.

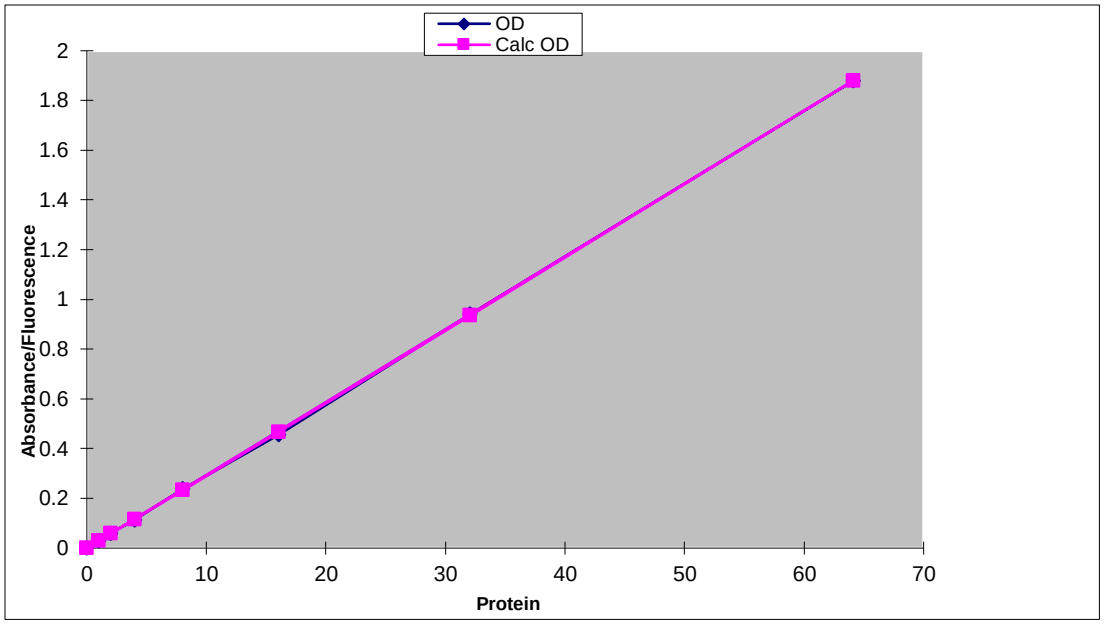


FIGURE 23. Mineralization Trial 3 standard curve against which trial measurements were measured to determine calcium deposited (below). Note: Standard curve was established for each of the three trials separately.

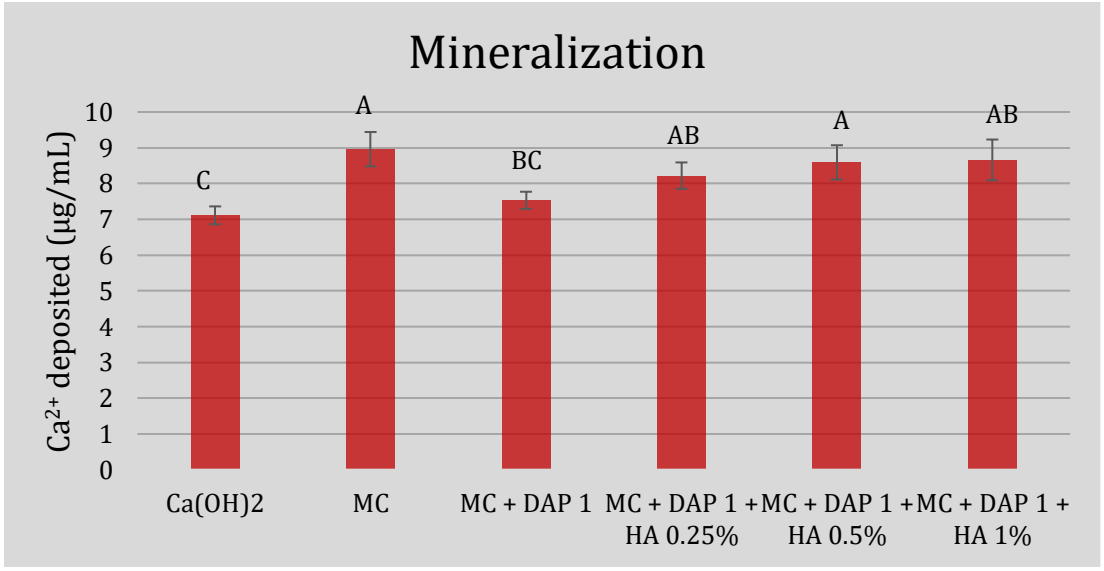


FIGURE 24. Mineralization Results: Significance ($P < 0.05$) demonstrated by differing letters above respective experimental groups.

TABLE I

Mineralization results measured in μg/mL

| Trial | Group | N | Mean | SD | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| All | Ca(OH)2 | 12 | 7.11 | 0.86 | 0.25 | 5.88 | 8.54 |
| | MC | 13 | 8.96 | 1.74 | 0.48 | 6.71 | 12.58 |
| | MC + DAP 1 | 13 | 7.53 | 0.88 | 0.24 | 5.99 | 9.05 |
| | MC + DAP 1 + HA 0.25% | 13 | 8.22 | 1.32 | 0.37 | 5.88 | 10.50 |
| | MC + DAP 1 + HA 0.5% | 13 | 8.59 | 1.74 | 0.48 | 6.17 | 12.45 |
| | MC + DAP 1 + HA 1% | 13 | 8.66 | 2.04 | 0.57 | 6.62 | 12.97 |
| 1 | Ca(OH)2 | 4 | 7.37 | 0.44 | 0.22 | 6.90 | 7.76 |
| | MC | 4 | 10.77 | 1.83 | 0.92 | 8.48 | 12.58 |
| | MC + DAP 1 | 4 | 8.19 | 0.67 | 0.34 | 7.57 | 9.05 |
| | MC + DAP 1 + HA 0.25% | 4 | 9.56 | 0.76 | 0.38 | 8.65 | 10.50 |
| | MC + DAP 1 + HA 0.5% | 4 | 10.31 | 1.89 | 0.94 | 8.43 | 12.45 |
| | MC + DAP 1 + HA 1% | 4 | 11.29 | 1.27 | 0.64 | 9.88 | 12.97 |
| 2 | Ca(OH)2 | 5 | 7.47 | 0.99 | 0.44 | 5.88 | 8.54 |
| | MC | 6 | 8.64 | 0.70 | 0.29 | 7.68 | 9.38 |
| | MC + DAP 1 | 6 | 7.63 | 0.65 | 0.26 | 6.35 | 8.14 |
| | MC + DAP 1 + HA 0.25% | 6 | 8.24 | 0.56 | 0.23 | 7.72 | 9.03 |
| | MC + DAP 1 + HA 0.5% | 6 | 8.45 | 0.48 | 0.20 | 7.66 | 8.91 |
| | MC + DAP 1 + HA 1% | 6 | 7.86 | 0.73 | 0.30 | 6.93 | 8.69 |
| 3 | Ca(OH)2 | 3 | 6.17 | 0.06 | 0.04 | 6.13 | 6.24 |
| | MC | 3 | 7.19 | 0.52 | 0.30 | 6.71 | 7.75 |
| | MC + DAP 1 | 3 | 6.44 | 0.47 | 0.27 | 5.99 | 6.92 |
| | MC + DAP 1 + HA 0.25% | 3 | 6.39 | 0.44 | 0.25 | 5.88 | 6.66 |
| | MC + DAP 1 + HA 0.5% | 3 | 6.56 | 0.41 | 0.24 | 6.17 | 6.99 |
| | MC + DAP 1 + HA 1% | 3 | 6.75 | 0.11 | 0.07 | 6.62 | 6.82 |

TABLE II

Mineralization (μg/mL) comparisons*

| Result | Difference | SE | p-value | |
|---|---|---|---|---|
| Ca(OH)2 < MC | -1.85 | 0.42 | 0.001 | * |
| Ca(OH)2 & MC + DAP 1 n.s. | -0.41 | 0.33 | 0.809 | |
| Ca(OH)2 < MC + DAP 1 + HA 0.25% | -1.10 | 0.32 | 0.012 | * |
| Ca(OH)2 < MC + DAP 1 + HA 0.5% | -1.47 | 0.41 | 0.008 | * |
| Ca(OH)2 < MC + DAP 1 + HA 1% | -1.55 | 0.45 | 0.013 | * |
| MC > MC + DAP 1 | 1.43 | 0.37 | 0.003 | * |
| MC & MC + DAP 1 + HA 0.25% n.s. | 0.75 | 0.36 | 0.297 | |
| MC & MC + DAP 1 + HA 0.5% n.s. | 0.38 | 0.44 | 0.955 | |
| MC & MC + DAP 1 + HA 1% n.s. | 0.30 | 0.48 | 0.989 | |
| MC + DAP 1 & MC + DAP 1 + HA 0.25% n.s. | -0.69 | 0.25 | 0.084 | |
| MC + DAP 1 < MC + DAP 1 + HA 0.5% | -1.06 | 0.36 | 0.049 | * |
| MC + DAP 1 & MC + DAP 1 + HA 1% n.s. | -1.13 | 0.41 | 0.074 | |
| MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 0.5% n.s. | -0.37 | 0.35 | 0.894 | |
| MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 1% n.s. | -0.44 | 0.40 | 0.871 | |
| MC + DAP 1 + HA 0.5% & MC + DAP 1 + HA 1% n.s. | -0.07 | 0.47 | 1.000 | |

*Denotes significance.

TABLE III

BSA (mg/mL) results

| Trial | Group | N | Mean | SD | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| All | Ca(OH)2 | 13 | 1.21 | 0.24 | 0.07 | 0.85 | 1.57 |
| | MC | 13 | 1.52 | 0.41 | 0.11 | 0.88 | 1.96 |
| | MC + DAP 1 | 13 | 1.26 | 0.21 | 0.06 | 0.92 | 1.57 |
| | MC + DAP 1 + HA 0.25% | 13 | 1.30 | 0.15 | 0.04 | 1.04 | 1.56 |
| | MC + DAP 1 + HA 0.5% | 13 | 1.25 | 0.20 | 0.06 | 0.89 | 1.53 |
| | MC + DAP 1 + HA 1% | 13 | 1.25 | 0.21 | 0.06 | 0.86 | 1.55 |
| 1 | Ca(OH)2 | 4 | 0.88 | 0.03 | 0.01 | 0.85 | 0.92 |
| | MC | 4 | 0.96 | 0.10 | 0.05 | 0.88 | 1.10 |
| | MC + DAP 1 | 4 | 1.00 | 0.07 | 0.04 | 0.92 | 1.09 |
| | MC + DAP 1 + HA 0.25% | 4 | 1.11 | 0.07 | 0.03 | 1.04 | 1.18 |
| | MC + DAP 1 + HA 0.5% | 4 | 0.99 | 0.07 | 0.04 | 0.89 | 1.04 |
| | MC + DAP 1 + HA 1% | 4 | 0.98 | 0.09 | 0.05 | 0.86 | 1.09 |
| 2 | Ca(OH)2 | 6 | 1.38 | 0.10 | 0.04 | 1.29 | 1.57 |
| | MC | 6 | 1.73 | 0.16 | 0.06 | 1.59 | 1.96 |
| | MC + DAP 1 | 6 | 1.30 | 0.07 | 0.03 | 1.17 | 1.38 |
| | MC + DAP 1 + HA 0.25% | 6 | 1.35 | 0.04 | 0.02 | 1.30 | 1.41 |
| | MC + DAP 1 + HA 0.5% | 6 | 1.32 | 0.07 | 0.03 | 1.23 | 1.41 |
| | MC + DAP 1 + HA 1% | 6 | 1.33 | 0.05 | 0.02 | 1.29 | 1.43 |
| 3 | Ca(OH)2 | 3 | 1.32 | 0.05 | 0.03 | 1.27 | 1.37 |
| | MC | 3 | 1.85 | 0.02 | 0.01 | 1.82 | 1.87 |
| | MC + DAP 1 | 3 | 1.53 | 0.07 | 0.04 | 1.45 | 1.57 |
| | MC + DAP 1 + HA 0.25% | 3 | 1.47 | 0.09 | 0.05 | 1.40 | 1.56 |
| | MC + DAP 1 + HA 0.5% | 3 | 1.46 | 0.07 | 0.04 | 1.39 | 1.53 |
| | MC + DAP 1 + HA 1% | 3 | 1.45 | 0.10 | 0.06 | 1.36 | 1.55 |

# TABLE IV

## ALP activity per μg protein

| Trial | Group | N | Mean | SD | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| All | Ca(OH)2 | 13 | 16.4 | 7.1 | 2.0 | 3.5 | 25.1 |
| | MC | 13 | 11.7 | 9.9 | 2.8 | 1.2 | 25.7 |
| | MC + DAP 1 | 13 | 10.2 | 4.8 | 1.3 | 4.9 | 18.6 |
| | MC + DAP 1 + HA 0.25% | 13 | 22.0 | 12.5 | 3.5 | 6.0 | 42.8 |
| | MC + DAP 1 + HA 0.5% | 13 | 26.6 | 15.4 | 4.3 | 7.2 | 49.5 |
| | MC + DAP 1 + HA 1% | 13 | 24.9 | 14.8 | 4.1 | 6.6 | 46.4 |
| 1 | Ca(OH)2 | 4 | 16.8 | 3.8 | 1.9 | 12.5 | 21.7 |
| | MC | 4 | 3.1 | 2.5 | 1.2 | 1.2 | 6.5 |
| | MC + DAP 1 | 4 | 6.6 | 1.7 | 0.8 | 5.3 | 9.0 |
| | MC + DAP 1 + HA 0.25% | 4 | 15.3 | 6.4 | 3.2 | 10.3 | 24.3 |
| | MC + DAP 1 + HA 0.5% | 4 | 18.0 | 2.9 | 1.5 | 14.7 | 21.8 |
| | MC + DAP 1 + HA 1% | 4 | 16.5 | 3.0 | 1.5 | 13.1 | 20.3 |
| 2 | Ca(OH)2 | 6 | 21.6 | 3.1 | 1.3 | 16.2 | 25.1 |
| | MC | 6 | 21.2 | 5.7 | 2.3 | 10.0 | 25.7 |
| | MC + DAP 1 | 6 | 14.7 | 2.7 | 1.1 | 10.7 | 18.6 |
| | MC + DAP 1 + HA 0.25% | 6 | 33.7 | 5.5 | 2.2 | 26.9 | 42.8 |
| | MC + DAP 1 + HA 0.5% | 6 | 41.4 | 6.7 | 2.7 | 29.1 | 49.5 |
| | MC + DAP 1 + HA 1% | 6 | 39.1 | 6.8 | 2.8 | 27.1 | 46.4 |
| 3 | Ca(OH)2 | 3 | 5.5 | 1.8 | 1.0 | 3.5 | 6.8 |
| | MC | 3 | 4.3 | 1.1 | 0.6 | 3.0 | 5.0 |
| | MC + DAP 1 | 3 | 6.1 | 1.5 | 0.8 | 4.9 | 7.7 |
| | MC + DAP 1 + HA 0.25% | 3 | 7.8 | 1.6 | 0.9 | 6.0 | 9.1 |
| | MC + DAP 1 + HA 0.5% | 3 | 8.6 | 1.4 | 0.8 | 7.2 | 10.0 |
| | MC + DAP 1 + HA 1% | 3 | 7.9 | 1.3 | 0.8 | 6.6 | 9.2 |

TABLE V

ALP activity per μg protein comparison*

| Result | Difference | SE | p-value | |
|---|---|---|---|---|
| Ca(OH)2 & MC n.s. | 4.7 | 1.9 | 0.161 | |
| Ca(OH)2 > MC + DAP 1 | 6.2 | 2.0 | 0.031 | * |
| Ca(OH)2 & MC + DAP 1 + HA 0.25% n.s. | -5.6 | 2.1 | 0.100 | |
| Ca(OH)2 < MC + DAP 1 + HA 0.5% | -10.2 | 2.6 | 0.002 | * |
| Ca(OH)2 < MC + DAP 1 + HA 1% | -8.5 | 2.5 | 0.010 | * |
| MC & MC + DAP 1 n.s. | 1.5 | 1.9 | 0.969 | |
| MC < MC + DAP 1 + HA 0.25% | -10.3 | 2.1 | <.001 | * |
| MC < MC + DAP 1 + HA 0.5% | -14.9 | 2.5 | <.001 | * |
| MC < MC + DAP 1 + HA 1% | -13.2 | 2.4 | <.001 | * |
| MC + DAP 1 < MC + DAP 1 + HA 0.25% | -11.8 | 2.1 | <.001 | * |
| MC + DAP 1 < MC + DAP 1 + HA 0.5% | -16.4 | 2.5 | <.001 | * |
| MC + DAP 1 < MC + DAP 1 + HA 1% | -14.7 | 2.4 | <.001 | * |
| MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 0.5% n.s. | -4.6 | 2.7 | 0.516 | |
| MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 1% n.s. | -2.9 | 2.5 | 0.859 | |
| MC + DAP 1 + HA 0.5% & MC + DAP 1 + HA 1% n.s. | 1.7 | 2.9 | 0.992 | |

*Denotes significance.

TABLE VI

Proliferation time = 0 hours

| Trial | Group | N | Mean | SD | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| All | Ca(OH)2 | 10 | 0.040 | 0.015 | 0.005 | 0.01 | 0.07 |
| | MC | 10 | 0.014 | 0.009 | 0.003 | 0.00 | 0.03 |
| | MC + DAP 1 | 10 | 0.013 | 0.003 | 0.001 | 0.01 | 0.02 |
| | MC + DAP 1 + HA 0.25% | 10 | 0.024 | 0.012 | 0.004 | 0.01 | 0.05 |
| | MC + DAP 1 + HA 0.5% | 9 | 0.024 | 0.008 | 0.003 | 0.01 | 0.04 |
| | MC + DAP 1 + HA 1% | 10 | 0.027 | 0.008 | 0.002 | 0.01 | 0.04 |
| 1 | Ca(OH)2 | 3 | 0.040 | 0.015 | 0.009 | 0.03 | 0.06 |
| | MC | 3 | 0.006 | 0.003 | 0.002 | 0.00 | 0.01 |
| | MC + DAP 1 | 3 | 0.015 | 0.004 | 0.002 | 0.01 | 0.02 |
| | MC + DAP 1 + HA 0.25% | 3 | 0.035 | 0.011 | 0.006 | 0.03 | 0.05 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.025 | 0.002 | 0.001 | 0.02 | 0.03 |
| | MC + DAP 1 + HA 1% | 3 | 0.026 | 0.003 | 0.002 | 0.02 | 0.03 |
| 2 | Ca(OH)2 | 4 | 0.033 | 0.015 | 0.007 | 0.01 | 0.05 |
| | MC | 4 | 0.015 | 0.001 | 0.001 | 0.01 | 0.02 |
| | MC + DAP 1 | 4 | 0.011 | 0.002 | 0.001 | 0.01 | 0.01 |
| | MC + DAP 1 + HA 0.25% | 4 | 0.014 | 0.005 | 0.002 | 0.01 | 0.02 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.016 | 0.003 | 0.002 | 0.01 | 0.02 |
| | MC + DAP 1 + HA 1% | 4 | 0.020 | 0.007 | 0.003 | 0.01 | 0.03 |
| 3 | Ca(OH)2 | 3 | 0.049 | 0.017 | 0.010 | 0.04 | 0.07 |
| | MC | 3 | 0.021 | 0.013 | 0.008 | 0.01 | 0.03 |
| | MC + DAP 1 | 3 | 0.015 | 0.002 | 0.001 | 0.01 | 0.02 |
| | MC + DAP 1 + HA 0.25% | 3 | 0.027 | 0.008 | 0.005 | 0.02 | 0.03 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.030 | 0.008 | 0.004 | 0.02 | 0.04 |
| | MC + DAP 1 + HA 1% | 3 | 0.035 | 0.001 | 0.001 | 0.03 | 0.04 |

TABLE VII

Proliferation time = 1 hours

| Trial | Group | N | Mean | SD | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| All | Ca(OH)2 | 10 | 0.431 | 0.031 | 0.010 | 0.39 | 0.49 |
| | MC | 10 | 0.323 | 0.145 | 0.046 | 0.14 | 0.49 |
| | MC + DAP 1 | 10 | 0.422 | 0.042 | 0.013 | 0.36 | 0.51 |
| | MC + DAP 1 + HA 0.25% | 10 | 0.457 | 0.041 | 0.013 | 0.38 | 0.50 |
| | MC + DAP 1 + HA 0.5% | 9 | 0.452 | 0.041 | 0.014 | 0.39 | 0.51 |
| | MC + DAP 1 + HA 1% | 10 | 0.457 | 0.030 | 0.010 | 0.42 | 0.51 |
| 1 | Ca(OH)2 | 3 | 0.427 | 0.013 | 0.008 | 0.42 | 0.44 |
| | MC | 3 | 0.270 | 0.075 | 0.044 | 0.18 | 0.32 |
| | MC + DAP 1 | 3 | 0.423 | 0.012 | 0.007 | 0.41 | 0.44 |
| | MC + DAP 1 + HA 0.25% | 3 | 0.459 | 0.015 | 0.009 | 0.44 | 0.47 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.463 | 0.018 | 0.010 | 0.45 | 0.48 |
| | MC + DAP 1 + HA 1% | 3 | 0.472 | 0.006 | 0.003 | 0.47 | 0.48 |
| 2 | Ca(OH)2 | 4 | 0.461 | 0.019 | 0.010 | 0.44 | 0.49 |
| | MC | 4 | 0.478 | 0.016 | 0.008 | 0.46 | 0.49 |
| | MC + DAP 1 | 4 | 0.454 | 0.040 | 0.020 | 0.41 | 0.51 |
| | MC + DAP 1 + HA 0.25% | 4 | 0.493 | 0.018 | 0.009 | 0.47 | 0.50 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.490 | 0.021 | 0.012 | 0.48 | 0.51 |
| | MC + DAP 1 + HA 1% | 4 | 0.474 | 0.029 | 0.015 | 0.44 | 0.51 |
| 3 | Ca(OH)2 | 3 | 0.397 | 0.012 | 0.007 | 0.39 | 0.41 |
| | MC | 3 | 0.170 | 0.032 | 0.019 | 0.14 | 0.21 |
| | MC + DAP 1 | 3 | 0.377 | 0.016 | 0.009 | 0.36 | 0.39 |
| | MC + DAP 1 + HA 0.25% | 3 | 0.406 | 0.019 | 0.011 | 0.38 | 0.42 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.404 | 0.016 | 0.009 | 0.39 | 0.42 |
| | MC + DAP 1 + HA 1% | 3 | 0.422 | 0.005 | 0.003 | 0.42 | 0.43 |

TABLE VIII

Proliferation time = 2 hours

| Trial | Group | N | Mean | SD | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| All | Ca(OH)2 | 10 | 0.917 | 0.065 | 0.021 | 0.85 | 1.00 |
| | MC | 10 | 0.719 | 0.312 | 0.099 | 0.38 | 1.10 |
| | MC + DAP 1 | 10 | 0.958 | 0.091 | 0.029 | 0.86 | 1.15 |
| | MC + DAP 1 + HA 0.25% | 10 | 0.999 | 0.055 | 0.017 | 0.91 | 1.08 |
| | MC + DAP 1 + HA 0.5% | 9 | 0.977 | 0.057 | 0.019 | 0.90 | 1.07 |
| | MC + DAP 1 + HA 1% | 10 | 0.998 | 0.027 | 0.009 | 0.97 | 1.06 |
| 1 | Ca(OH)2 | 3 | 0.855 | 0.006 | 0.003 | 0.85 | 0.86 |
| | MC | 3 | 0.540 | 0.142 | 0.082 | 0.39 | 0.67 |
| | MC + DAP 1 | 3 | 0.888 | 0.011 | 0.006 | 0.88 | 0.89 |
| | MC + DAP 1 + HA 0.25% | 3 | 0.962 | 0.022 | 0.013 | 0.94 | 0.99 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.938 | 0.026 | 0.015 | 0.92 | 0.97 |
| | MC + DAP 1 + HA 1% | 3 | 0.987 | 0.005 | 0.003 | 0.98 | 0.99 |
| 2 | Ca(OH)2 | 4 | 0.986 | 0.018 | 0.009 | 0.97 | 1.00 |
| | MC | 4 | 1.066 | 0.037 | 0.018 | 1.02 | 1.10 |
| | MC + DAP 1 | 4 | 1.040 | 0.085 | 0.042 | 0.95 | 1.15 |
| | MC + DAP 1 + HA 0.25% | 4 | 1.053 | 0.030 | 0.015 | 1.01 | 1.08 |
| | MC + DAP 1 + HA 0.5% | 3 | 1.036 | 0.040 | 0.023 | 0.99 | 1.07 |
| | MC + DAP 1 + HA 1% | 4 | 1.014 | 0.037 | 0.019 | 0.98 | 1.06 |
| 3 | Ca(OH)2 | 3 | 0.886 | 0.044 | 0.025 | 0.85 | 0.93 |
| | MC | 3 | 0.434 | 0.076 | 0.044 | 0.38 | 0.52 |
| | MC + DAP 1 | 3 | 0.918 | 0.057 | 0.033 | 0.86 | 0.98 |
| | MC + DAP 1 + HA 0.25% | 3 | 0.964 | 0.045 | 0.026 | 0.91 | 1.00 |
| | MC + DAP 1 + HA 0.5% | 3 | 0.959 | 0.053 | 0.030 | 0.90 | 1.01 |
| | MC + DAP 1 + HA 1% | 3 | 0.988 | 0.017 | 0.010 | 0.97 | 1.00 |

TABLE IX

Proliferation time = 3 hours

| Trial | Group | N | Mean | SD | SE | Min | Max |
|---|---|---|---|---|---|---|---|
| All | Ca(OH)2 | 10 | 1.506 | 0.121 | 0.038 | 1.33 | 1.66 |
| | MC | 10 | 1.221 | 0.495 | 0.157 | 0.67 | 1.80 |
| | MC + DAP 1 | 10 | 1.590 | 0.124 | 0.039 | 1.46 | 1.86 |
| | MC + DAP 1 + HA 0.25% | 10 | 1.667 | 0.104 | 0.033 | 1.54 | 1.84 |
| | MC + DAP 1 + HA 0.5% | 9 | 1.632 | 0.090 | 0.030 | 1.52 | 1.78 |
| | MC + DAP 1 + HA 1% | 10 | 1.684 | 0.064 | 0.020 | 1.63 | 1.84 |
| 1 | Ca(OH)2 | 3 | 1.365 | 0.027 | 0.016 | 1.33 | 1.38 |
| | MC | 3 | 0.928 | 0.236 | 0.136 | 0.68 | 1.15 |
| | MC + DAP 1 | 3 | 1.504 | 0.056 | 0.032 | 1.46 | 1.56 |
| | MC + DAP 1 + HA 0.25% | 3 | 1.577 | 0.041 | 0.024 | 1.54 | 1.62 |
| | MC + DAP 1 + HA 0.5% | 3 | 1.558 | 0.036 | 0.021 | 1.52 | 1.58 |
| | MC + DAP 1 + HA 1% | 3 | 1.636 | 0.005 | 0.003 | 1.63 | 1.64 |
| 2 | Ca(OH)2 | 4 | 1.625 | 0.029 | 0.015 | 1.60 | 1.66 |
| | MC | 4 | 1.771 | 0.035 | 0.017 | 1.73 | 1.80 |
| | MC + DAP 1 | 4 | 1.688 | 0.133 | 0.067 | 1.57 | 1.86 |
| | MC + DAP 1 + HA 0.25% | 4 | 1.773 | 0.059 | 0.030 | 1.70 | 1.84 |
| | MC + DAP 1 + HA 0.5% | 3 | 1.733 | 0.058 | 0.033 | 1.67 | 1.78 |
| | MC + DAP 1 + HA 1% | 4 | 1.743 | 0.065 | 0.032 | 1.70 | 1.84 |
| 3 | Ca(OH)2 | 3 | 1.490 | 0.071 | 0.041 | 1.42 | 1.56 |
| | MC | 3 | 0.781 | 0.151 | 0.087 | 0.67 | 0.95 |
| | MC + DAP 1 | 3 | 1.545 | 0.078 | 0.045 | 1.48 | 1.63 |
| | MC + DAP 1 + HA 0.25% | 3 | 1.615 | 0.052 | 0.030 | 1.56 | 1.66 |
| | MC + DAP 1 + HA 0.5% | 3 | 1.604 | 0.055 | 0.032 | 1.54 | 1.65 |
| | MC + DAP 1 + HA 1% | 3 | 1.653 | 0.011 | 0.006 | 1.64 | 1.66 |

TABLE X

Proliferation comparison

| Time | Result | Difference | SE | p-value | |
|---|---|---|---|---|---|
| All | Ca(OH)2 & MC n.s. | 0.154 | 0.078 | 0.320 | |
| | Ca(OH)2 & MC + DAP 1 n.s. | -0.022 | 0.026 | 0.955 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.25% n.s. | -0.063 | 0.023 | 0.054 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.5% n.s. | -0.047 | 0.022 | 0.257 | |
| | Ca(OH)2 < MC + DAP 1 + HA 1% | -0.068 | 0.019 | 0.005 | * |
| | MC & MC + DAP 1 n.s. | -0.176 | 0.079 | 0.195 | |
| | MC < MC + DAP 1 + HA 0.25% | -0.217 | 0.077 | 0.050 | * |
| | MC & MC + DAP 1 + HA 0.5% n.s. | -0.202 | 0.077 | 0.083 | |
| | MC < MC + DAP 1 + HA 1% | -0.222 | 0.076 | 0.039 | * |
| | MC + DAP 1 & MC + DAP 1 + HA 0.25% n.s. | -0.041 | 0.025 | 0.551 | |
| | MC + DAP 1 & MC + DAP 1 + HA 0.5% n.s. | -0.025 | 0.025 | 0.903 | |
| | MC + DAP 1 & MC + DAP 1 + HA 1% n.s. | -0.046 | 0.022 | 0.273 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 0.5% n.s. | 0.016 | 0.021 | 0.971 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 1% n.s. | -0.005 | 0.018 | 1.000 | |
| | MC + DAP 1 + HA 0.5% & MC + DAP 1 + HA 1% n.s. | -0.021 | 0.017 | 0.822 | |
| 0 | Ca(OH)2 > MC | 0.026 | 0.006 | 0.001 | * |
| | Ca(OH)2 > MC + DAP 1 | 0.027 | 0.005 | <.001 | * |
| | Ca(OH)2 & MC + DAP 1 + HA 0.25% n.s. | 0.016 | 0.005 | 0.196 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.5% n.s. | 0.017 | 0.005 | 0.057 | |
| | Ca(OH)2 & MC + DAP 1 + HA 1% n.s. | 0.013 | 0.005 | 0.308 | |
| | MC & MC + DAP 1 n.s. | 0.001 | 0.003 | 1.000 | |
| | MC & MC + DAP 1 + HA 0.25% n.s. | -0.010 | 0.004 | 0.572 | |
| | MC & MC + DAP 1 + HA 0.5% n.s. | -0.009 | 0.004 | 0.496 | |
| | MC & MC + DAP 1 + HA 1% n.s. | -0.012 | 0.004 | 0.078 | |
| | MC + DAP 1 < MC + DAP 1 + HA 0.25% | -0.011 | 0.003 | 0.022 | * |
| | MC + DAP 1 < MC + DAP 1 + HA 0.5% | -0.010 | 0.002 | <.001 | * |
| | MC + DAP 1 < MC + DAP 1 + HA 1% | -0.014 | 0.002 | <.001 | * |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 0.5% n.s. | 0.001 | 0.004 | 1.000 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 1% n.s. | -0.002 | 0.004 | 1.000 | |
| | MC + DAP 1 + HA 0.5% & MC + DAP 1 + HA 1% n.s. | -0.003 | 0.003 | 0.998 | |
| 1 | Ca(OH)2 & MC n.s. | 0.108 | 0.048 | 0.575 | |
| | Ca(OH)2 & MC + DAP 1 n.s. | 0.010 | 0.017 | 1.000 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.25% n.s. | -0.025 | 0.017 | 0.979 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.5% n.s. | -0.020 | 0.018 | 0.998 | |
| | Ca(OH)2 & MC + DAP 1 + HA 1% n.s. | -0.026 | 0.014 | 0.880 | |
| | MC & MC + DAP 1 n.s. | -0.099 | 0.049 | 0.750 | |
| | MC & MC + DAP 1 + HA 0.25% n.s. | -0.134 | 0.049 | 0.249 | |
| | MC & MC + DAP 1 + HA 0.5% n.s. | -0.129 | 0.049 | 0.311 | |
| | MC & MC + DAP 1 + HA 1% n.s. | -0.134 | 0.048 | 0.215 | |
| | MC + DAP 1 & MC + DAP 1 + HA 0.25% n.s. | -0.035 | 0.020 | 0.883 | |
| | MC + DAP 1 & MC + DAP 1 + HA 0.5% n.s. | -0.030 | 0.020 | 0.973 | |
| | MC + DAP 1 & MC + DAP 1 + HA 1% n.s. | -0.036 | 0.017 | 0.708 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 0.5% n.s. | 0.005 | 0.020 | 1.000 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 1% n.s. | -0.001 | 0.017 | 1.000 | |
| | MC + DAP 1 + HA 0.5% & MC + DAP 1 + HA 1% n.s. | -0.006 | 0.017 | 1.000 | |

*Denotes significance.

(continued)

TABLE X

Proliferation comparison
(cont.)

| | | | | | |
|---|---|---|---|---|---|
| 2 | Ca(OH)2 & MC n.s. | 0.198 | 0.102 | 0.807 | |
| | Ca(OH)2 & MC + DAP 1 n.s. | -0.041 | 0.037 | 0.999 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.25% n.s. | -0.082 | 0.028 | 0.169 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.5% n.s. | -0.060 | 0.029 | 0.730 | |
| | Ca(OH)2 < MC + DAP 1 + HA 1% | -0.081 | 0.023 | 0.039 | * |
| | MC & MC + DAP 1 n.s. | -0.239 | 0.104 | 0.553 | |
| | MC & MC + DAP 1 + HA 0.25% n.s. | -0.280 | 0.101 | 0.241 | |
| | MC & MC + DAP 1 + HA 0.5% n.s. | -0.258 | 0.102 | 0.377 | |
| | MC & MC + DAP 1 + HA 1% n.s. | -0.279 | 0.100 | 0.229 | |
| | MC + DAP 1 & MC + DAP 1 + HA 0.25% n.s. | -0.041 | 0.035 | 0.997 | |
| | MC + DAP 1 & MC + DAP 1 + HA 0.5% n.s. | -0.019 | 0.036 | 1.000 | |
| | MC + DAP 1 & MC + DAP 1 + HA 1% n.s. | -0.040 | 0.031 | 0.994 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 0.5% n.s. | 0.022 | 0.027 | 1.000 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 1% n.s. | 0.001 | 0.020 | 1.000 | |
| | MC + DAP 1 + HA 0.5% & MC + DAP 1 + HA 1% n.s. | -0.021 | 0.022 | 1.000 | |
| 3 | Ca(OH)2 & MC n.s. | 0.285 | 0.162 | 0.900 | |
| | Ca(OH)2 & MC + DAP 1 n.s. | -0.083 | 0.056 | 0.975 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.25% n.s. | -0.161 | 0.052 | 0.101 | |
| | Ca(OH)2 & MC + DAP 1 + HA 0.5% n.s. | -0.125 | 0.050 | 0.398 | |
| | Ca(OH)2 < MC + DAP 1 + HA 1% | -0.178 | 0.044 | 0.007 | * |
| | MC & MC + DAP 1 n.s. | -0.368 | 0.163 | 0.578 | |
| | MC & MC + DAP 1 + HA 0.25% n.s. | -0.446 | 0.161 | 0.241 | |
| | MC & MC + DAP 1 + HA 0.5% n.s. | -0.410 | 0.161 | 0.367 | |
| | MC & MC + DAP 1 + HA 1% n.s. | -0.463 | 0.159 | 0.173 | |
| | MC + DAP 1 & MC + DAP 1 + HA 0.25% n.s. | -0.077 | 0.052 | 0.977 | |
| | MC + DAP 1 & MC + DAP 1 + HA 0.5% n.s. | -0.042 | 0.050 | 1.000 | |
| | MC + DAP 1 & MC + DAP 1 + HA 1% n.s. | -0.094 | 0.045 | 0.708 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 0.5% n.s. | 0.036 | 0.046 | 1.000 | |
| | MC + DAP 1 + HA 0.25% & MC + DAP 1 + HA 1% n.s. | -0.017 | 0.040 | 1.000 | |
| | MC + DAP 1 + HA 0.5% & MC + DAP 1 + HA 1% n.s. | -0.053 | 0.037 | 0.985 | |

*Denotes significance.

# DISCUSSION

The field of regenerative endodontics has seen tremendous advancements in recent years. AAE Guidelines now recommend a DAP or TAP concentration between 0.1% and 1.0% antibiotic. These guidelines are based in some part on research performed at this institution. Concentrations ranging from 0.125 to 10 mg/mL of DAP and TAP demonstrated a similar antimicrobial effect on an established *E. faecalis* biofilm (23). Concentrations of 0.5 mg/mL and 0.25 mg/mL DAP and TAP showed no cytotoxicity to DPSC (23). Dentin with DAP had a significantly longer residual antibacterial effect than TAP at equal concentrations (25). A similar study looked at the residual effect of DAP on pretreated dentin at 1 and 4 weeks at varying concentrations (188). The DAP concentrations at 1 and 5 mg/mL demonstrated significant antibacterial effects against biofilm from mature teeth with necrotic pulp (188).

The introduction of methylcellulose hydrogel as a carrier for antibiotic has improved handling characteristics of the antibiotic pastes. Introduction of methylcellulose may minimize the reduction in microhardness of roots compared with equal concentration of antibiotic (31). Since this time, research performed here at this institution has been performed utilizing the same MC preparation techniques, as described in the prior section. In a 2017 study comparing concentration of DAP and its direct and residual effects on biofilms obtained from mature and immature teeth with pulp necrosis, it was concluded that 1 and 5 mg/mL DAP, and $Ca(OH)_2$ all exhibited a significant and substantial direct antibacterial effect on all biofilms (28). Additionally,

dentin pretreated with 5 mg/mL DAP had a significant residual effect against both biofilm types compared to the 1 mg/mL DAP and Ca(OH)2 groups (28).

One significant limitation of using DAP in this formulation is the inability to confirm placement of the medicament within the canal. Recent research has been conducted exploring the effects of radiopacifiers on the effect of DAP. In one study adding radiopacifiers to varying concentrations of DAP medicament, it was found that $BaSO_4$ DAP as low as 1 mg/mL showed profound direct and residual antibacterial effect on biofilms from immature teeth with pulp necrosis (189). In contraindication to these findings, recent unpublished research here at IUSD demonstrated $ZrO_2$ had a greater antibacterial effect and actually demonstrated these effects alone. Nonetheless, the effect is the same; that radiopacifiers do not negatively affect the antibacterial properties of DAP at 1 mg/mL. In recent manuscript-submitted research conducted in the same lab, the researchers performed the same experimental design to evaluate the effects of addition of $BaSO_4$ and $ZrO_2$ on DPSCs. In this research, they utilized proliferation, mineralization, and ALP assays to assess the differentiation and specialization of DPSCs and found little significant difference between the addition of $BaSO_4$ and $ZrO_2$ in regard to proliferation and slightly but not significant improved ALP activity with addition of $ZrO_2$.

The same researchers also investigated the antimicrobial properties, cytotoxicity, and differentiation potential of double antibiotic intracanal medicaments loaded into hydrogel system (161). In this study, the authors found that the direct antibacterial effects of DAP at 1, 5, and 10 mg/mL, as well as $Ca(OH)_2$ resulted in no *E. faecalis* biofilm remaining. They found less effect of 1 mg/mL DAP on a dual-species biofilm consisting of *E. faecalis* and *P. intermedia* with four of seven culture samples remaining positive for

bacteria with a statistically significant difference from the other experimental groups. The differences become even more dramatic when evaluating the residual effect of treated dentin on bacterial biofilm. Similar to previous studies, DAP at 5 and 10 mg/mL had significant residual effect on both single-species and dual-species biofilms, whereas $Ca(OH)_2$ had no residual effect on either biofilm type. The 1 mg/mL DAP group demonstrated no residual effect on either species biofilm (161). This demonstrates that DAP at lower concentrations has a diminished direct, and no residual effect, on more complex biofilms that would be present in a necrotic pulp of an immature tooth.

It would seem evident that a concentration greater than 1 mg/mL DAP would be necessary in attempt to reduce the bacterial load to allow healing. However, the study of the cellular effects of DAP offer a resounding rebuttal to that idea. When investigating the proliferation of DPSC exposed to the experimental medicaments, the authors found that compared to the positive control, 5 and 10 mg/mL DAP induced significant decreases in DPSC proliferation, whereas 1 mg/mL DAP and $Ca(OH)_2$ did not cause significant decreases in proliferation of DPSC (161). Regarding ALP activity, which demonstrates the cells ability to differentiate into an osteogenic lineage only 1 mg/mL DAP demonstrated no significant difference when compared to the positive control among the experimental groups. $Ca(OH)_2$, 5 and 10 mg/mL DAP all led to significant decreases in ALP activity. When evaluating mineralization, the MC alone and $Ca(OH)_2$ groups demonstrated significant mineral deposits when compared to the other groups. However, 5 and 10 mg/mL DAP demonstrated significant reductions in mineralization when directly compared to 1 mg/mL DAP ($P<.001$) (161).

These findings became the formative experimental design of this project. Based on the detrimental stem cell effects of DAP concentrations greater than 1 mg/mL, as well as being the current regenerative guidance of the AAE, we decided to focus on this concentration in our groups. In the previous study, the negative control did not differ statistically from other experimental groups; therefore, it was decided to not be beneficial to this research. The determination of concentration of hydroxyapatite was made experimentally. Nano-hydroxyapatite has shown an ability to chemically bind to bone without inducing toxicity or inflammation (44). In the dental literature, concentrations of nano-HA usage ranged from 0.1-1.0% (46, 47) and hydroxyapatite demonstrates an ability to chemically bind to bone without inducing toxicity or inflammation, stimulating bone growth via a direct osteoinductive action on osteoblasts (44).

The current study investigated the cytotoxicity and differentiation potential of 1 mg/mL DAP with concentrations of HA at 0.25%, 0.50%, and 1.00%, comparing these experimental groups to established groups of $Ca(OH)_2$, MC alone, and DAP 1 mg/mL. It should be noted that all DAP concentrations were formulated with MC using established protocols, the decision was made to alter the protocol for HA groups as the final step of centrifugation for 15 minutes would be expected to precipitate the HA out of suspension.

The proliferative capacity of all experimental groups containing DAP were similar, as was the $Ca(OH)_2$ group in our study. The only significance noted at T=3 hours was that of MC + DAP + HA 1% over $Ca(OH)_2$, which differs from previous findings (29, 161). However, the minimal amount of error between groups (SE = 0.038 and 0.02, respectively) demonstrates the repeatability of the trials. Interestingly, proliferation demonstrated very similar results across all experimental groups, which emphasizes that

no negative effects were imposed on DPSCs when exposed to HA which would agree with previous findings (44, 190).

Our study also demonstrated similar effects when evaluating mineral deposited in the experimental groups (161). There was, however, a difference when observing the $Ca(OH)_2$ group between experiments. No statistical analysis was performed between the two experiments, but when comparing $Ca(OH)_2$ and DAP at 1 mg/mL in the previous experiment $Ca(OH)_2$ had significantly greater mineral deposited, whereas in the current experiment $Ca(OH)_2$ had a significantly lesser mineral deposited. This is interesting, and the author is unsure of its significance, but $Ca(OH)_2$ is known to demonstrate mineral deposition in MSCs. The concern is that too much mineralization may result in excessive calcification within the root canal system *in vivo* which could lead to potential pulp canal obliteration (191). In fact, a recent study that compared $Ca(OH)_2$ to DAP/TAP medicaments in regenerative cases showed an increased frequency of intracanal calcification (77% and 46%, respectively) (192). However, as with proliferation, when comparing mineralization between DAP groups, very similar results were demonstrated among all groups. In fact, the DAP + MC + HA 0.5% group demonstrated statistically significantly greater mineralization than the DAP + MC group alone. So, a potential pitfall of HA inclusion might be canal obliteration.

However, when observing ALP activity, a clear improvement developed with the experimental HA groups. This confirms research performed in the medical literature that shows a biocompatible nature of HA, and more specifically nano-HA, to improve cellular metabolic activity (44, 190, 193). ALP activity was statistically significantly greater in the DAP + MC + HA 0.5% and DAP + MC + HA 1.0% groups than all other groups

(excluding DAP + MC + HA 0.25%) with a significant magnitude. Not only did the HA not have a detrimental effect on the differentiation potential of DPSC, it had a near-threefold greater effect than DAP + MC alone.

However, no such study of this nature has been performed prior. Though our volume of research is growing regarding DAP at concentration of 1 mg/mL, incorporating nano-HA is a novel addition. The current study investigated this growth potential at time intervals of 3-7 days based on previous designs, which can be justified based on previous studies and AAE guidelines. One week of antibiotic administration remains the minimum amount of time recommended for regenerative treatment of the immature necrotic pulp. Therefore, further investigations are recommended to examine potential growth and differentiation of DPSC at greater time points. Additionally, with the promise this study offers, further research that incorporates recently-studied radiopacifiers would be recommended.

# SUMMARY AND CONCLUSIONS

In conclusion, the null hypothesis, which stated that there would be no difference in growth and maturation of DPSCs in the presence of nano-HA, was partially rejected. The incorporation of n-HA, especially at the 0.50% and 1% levels showed no evidence of cytotoxicity on DPSCs and, in fact, demonstrated comparable metabolic activity to established medicaments and positive controls. The incorporation of n-HA showed statistically significantly increased potential to differentiate into an osteogenic or odontogenic lineage over $Ca(OH)_2$ as well as DAP. In addition, these cells showed an increased end production of calcium demonstrating their potential for incorporation in regenerative procedures.

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ABSTRACT

# THE EFFECTS OF NANO-HYDROXYAPATITE IN A DOUBLE ANTIBIOTIC PASTE- LOADED METHYLCELLULOSE CARRIER ON DENTAL PULP STEM CELLS

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Regenerative endodontic procedures (REP) require disinfection techniques to eliminate bacteria from the infected immature root canal system and promote new growth of the pulp-dentin complex. Double antibiotic paste (DAP), a mixture of ciprofloxacin and metronidazole, has shown efficacy in doing so while minimizing cytotoxicity on dental pulp stem cells (DPSC). Stem cells, scaffolding, and growth factors are necessary in the maturation, proliferation, and differentiation of mesenchymal stem cells into the root canal system. Nano-hydroxyapatite (n-HA) has a history of biocompatibility and, in addition, has shown promising effects as a tissue bioengineering material.

Objective: The aim of this in vitro study was to investigate the proliferation and mineralization of DPSC in the presence of 1% DAP and methylcellulose (MC) with varying concentrations of nano-hydroxyapatite.

Materials and Methods: DPSC were plated in 24-well plates containing culture media. The next day, semi-permeable 0.1 μm Transwell chambers were inserted into the wells to separate the reservoirs for medicaments. Treatment paste composed of methylcellulose containing 1% DAP with either 0.25%, 0.50%, or 1.0% nano-hydroxyapatite was added along with culture media. Methylcellulose alone and calcium hydroxide (Ultracal) were used as control groups. After 3 days, cells were evaluated for cytotoxic effects using an MTS proliferation assay (n = 10, in triplicate). DPSCs were also cultured with these medicaments for 7 days in osteogenic media and evaluated for alkaline phosphatase (ALP) activity and mineralization activity (n = 13, in triplicate). Comparisons between groups for differences in mineralization, BSA, and ALP activity were performed using analysis of variance (ANOVA), with different variances allowed for each group and a random effect included in the model to account for correlation within each of the three trials. A simulation-based model was used to adjust for multiple comparisons.

Results: Addition of n-HA treatment groups increased mineralization significantly greater than calcium hydroxide, with MC alone and MC+DAP+0.5% HA providing the greatest effect. Regarding ALP, all HA concentrations performed significantly greater than MC and DAP concentrations. Proliferation demonstrated similar metabolic activity in all experimental groups with few comparisons significant.

Conclusion: The challenge in REPs is to maintain survival, and preferably promote the proliferation and development of DPSCs into the pulp-dentin complex with a consistent treatment outcome. The combination of DAP with hydroxyapatite may allow for both disinfection and improved mineralization and cellular differentiation. This contribution has shown significant ability to increase stem cell differentiation into an osteogenic lineage as well as calcium deposition, indicating end goal results of regenerative procedures.

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