THE EFFECTS OF ELECTROMAGNETIC WAVE STIMULATION (EMS) ON OSTEOBLAST DIFFERENTIATION AND ACTIVITY

by

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LIST OF ABBREVIATIONS

Alkaline phosphatase……..……ALP

Apical periodontitis…….AP

Calcium hydroxide…….$Ca(OH)_2$

Calvaria derived osteoblasts……COBs

Chlorhexidine ……......CHX

Electronic apex locator…….…….EAL

Electromagnetic apical treatment…….…….EMAT

Electromagnetic stimulation…….EMS

Electromagnetic wave irradiation…….…….EMWI

Ethylenediamine tetra-acetic acid …….……EDTA

Insulin-like growth factor I and II……...IGF-I, IGF-II

Mesenchymal stem cells…….…….MSC

Mineralized trioxide aggregate……MTA

Pulsed electromagnetic field…….…….PEMF

Regenerative endodontic procedures……...……REPs

Sodium hypochlorite…….……NaOCl

Transforming growth factor beta I and II…….TGF-β1, TGF-β2

INTRODUCTION

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One goal of nonsurgical root canal therapy is to reduce the bacterial load within an infected root canal system, and the subsequent objective is to prevent or treat apical periodontitis (AP).[1] AP is the host defense response to microbial challenge emanating from the root canal system.[1] These lesions are viewed as a dynamic encounter between microbial factors and host defenses at the interface between infected radicular pulp and periodontal ligament that results in local inflammation, resorption of hard tissues, and destruction of other periapical tissues.[2] These lesions are generally identified during routine radiographic examinations.[3] 90% of periapical lesions are classified as radicular cysts, dental granulomas or abscesses,[4, 5] and of these, it was reported that 50% were granulomas, 35% were abscesses, 15% were cysts.[6] A return of the involved tooth to a healthy and functional condition without surgical intervention should be the ultimate goal in endodontic treatments[7], and ideally, all inflammatory periapical lesions should be treated with a nonsurgical root canal treatment (NSRCT) which is a conservative minimally invasive approach. Only after failure of nonsurgical approaches should surgical intervention be considered.[8]

Since nonsurgical root canal treatment is not always effective. A novel treatment, classified as electromagnetic apical treatment (EMAT), may be a promising addition to conventional nonsurgical root canal therapy for more rapid healing of periapical lesions. EMAT uses electromagnetic wave irradiation (EMWI) and the process is believed to occur via electrical and thermal energy through a mechanism known as electromagnetic stimulation (EMS). Drs Tominaga, Yumoto, and Sato from the University of Tokushima

Graduate School in Japan, completed a clinical study showing more expedient healing of periapical lesions treated with EMS as compared to a control group in which periapical lesions were not treated with EMS.[9] These findings suggest that EMS may be beneficial to bone tissue repair in apical periodontitis.

The clinical studies by Drs Tominaga, Yumoto, and Sato showed that the periapical healing times with three different patients were 3 months, 4 months, and 7 months.[9] This is a much faster rate of healing than what is currently reported for conventional nonsurgical root canal therapy, which may take 12 or more months to have complete periapical bone healing.[10] Murphy et al., found that the average radiographic rate of repair was 3.2 mm^2 per month and over 70% of periapical lesions take 12 months or longer to heal.[10] With conventional nonsurgical root canal therapy, Saatchi et al., demonstrated the 12-month periapical healing of large lesion after using calcium hydroxide as an intracanal dressing.[11] Moshari et al. showed that after a 20-month follow up, complete radiographic and clinical healing of the periapical lesion was observed.[12] Soares et al. showed the complete resolution of large periapical lesion after 2-year follow-up.[13] Valois et al. also showed complete healing of large lesions (10×15mm) after 2 years with nonsurgical root canal therapy.[14] These clinical studies did not use EMS. The use of EMS, in conjunction with routine endodontic treatment, may prove to be of benefit for faster healing rates of apical periodontitis with periapical radiolucency upon radiographic examination.[15]

EMS has been studied as a possible method of disinfection by creating solutions with electromagnetic waves that create electric and thermal energy to produce a bactericidal effect.[16] Yumoto et al. demonstrated that high-frequency electromagnetic

waves have inactivating and bactericidal activities against microbial species.[17] Previous research for EMS to stimulate bacterial killing was set at approximately 60-215 mA of current.[18] However, for EMS to stimulate osteoblasts and bone defects, low-frequency electric current at low wattage for longer periods were used.[19] In another report, a lower current for EMS of rat calvaria osteoblasts resulted in more bone deposition than the control without EMS stimulation.[19]

In the EMS technology, through the use of an electromagnetic wave irradiation device attached to an active electrode (a specially coated ISO size 10 hand file), a circuit can be created much in the same manner as an electronic apex locating device. Due to an insulating coating along the file, the electromagnetic waves are concentrated at the tip of the active electrode. Sato et al. applied this EMS technology to an in vitro model evaluating bone repair of rat calvaria defects.[19] An increase in bone healing was found, which was attributed to the stimulation of osteoblasts as well as necessary growth factors for bone formation. Stimulation with electromagnetic waves was also reported to activate an osteoblastic cell line.[19] Bone remodeling involved the coordinated actions of bone-forming osteoblasts (OB) and bone-degrading osteoclasts (OC), which are regulated by hormones, growth factors, transcription factors, and mechanical stimuli.[20] Studies using MC3T3-E1, an immortalized pre-osteoblast cell line, indicated that electromagnetic wave stimulation can promote cell proliferation and increase expression of several growth factors.[17]

Although EMS shows promise for increasing bone repair, the mechanism of action of EMS on primary osteoblastic cells is currently unclear. Additional in vitro and

ex vivo studies are required to further establish the utility of using EMS to promote osteogenesis.

OBJECTIVE

The objective of this in vitro study is to evaluate the effects of EMS on the proliferation and differentiation of primary osteoblast-lineage cells from neonatal mice, which are a more biologically relevant source of osteogenic cells than immortalized MC3T3-E1 pre-osteoblast cells previously examined by other investigators.[17] The impact of EMS on cell proliferation, alkaline phosphatase activity and mineral deposition, which are increased by mature osteoblasts, will be determined.

HYPOTHESES

- Null Hypothesis: Primary murine calvaria osteoblast-lineage cells that are treated with Electromagnetic Wave Stimulation (EMS) will have no effect on proliferation and differentiation, no effect on alkaline phosphatase activity, and no effect on mineral deposition of the primary osteoblast-lineage cells.
- Alternative Hypothesis: Primary murine calvaria osteoblast-lineage cells that are treated with Electromagnetic Wave Stimulation (EMS) will have an increased effect on the proliferation, alkaline phosphatase activity, and mineral deposition of the primary osteoblast-lineage cells.

REVIEW OF LITERATURE

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HISTORY OF ENDODONTICS

The Indus Valley Civilization (IVC), which is modern day areas of Pakistan, northeast Afghanistan, and northeastern India, has yielded evidence of dentistry being practiced as early as 7000 BC.[21] In IVC, bow drills have been found along with beads of bone, shell and turquoise, which suggests that the skilled bead craftsmen also worked on teeth.[21] The earliest dental filling is believed to be found in a 6500 year old human mandible from Slovenia, filled with beeswax.[22] The earliest theory of tooth pain can be traced back to 5000 BC from the Sumerian text that describes the belief that a "tooth worm" would cause a toothache by gnawing at the structure of the tooth, an idea that was not proven false until the 1700s.[23]

The earliest reference to a dentist is believed to be from 2500 BC, on the tomb of Hesy-Re, had the inscription "the greatest of those who dealt with teeth."[24] Around 500-300 BC in ancient Greece, Aristotle and Hippocrates wrote about dentistry, but it was not until year 1530 that the first book on dentistry was published.[25] This book was titled *The Little Medicinal Book for All Kinds of Diseases and Infirmities of the Teeth*, by Artzney Buchlein from Germany.[24] The book covers practical topics such as oral hygiene, tooth extraction, drilling teeth, and placement of gold fillings; the book was written for barbers and surgeons.[24]

In 1687, Charles Allen wrote the first English-language book solely on the subject of dentistry.[26] Modern dentistry can trace is roots back to this book and it was favored over the book by Buchlein in 1530. In 1700s, Anton von Leeuwenhoek, the " Father of

Microbiology[27]," and the "Father of Modern Microscopy[28]," observed samples of teeth under magnification and disproved the "tooth-worm" theory. He described the "animalcules" contained in plaque and tartar and produced the first drawing of bacteria.[27] Leeuwenhoek believed that fly worms nested in tooth cavities and extended to the pulp to cause pain.[28] Even though his report was incorrect, he was the first to have a theory that tooth pain arose from contamination and from "animalcules", and not from a "tooth worm."[28]

Around 30 years after Leeuwenhoek's findings, Pierre Fauchard was credited as being the "Father of Modern Dentistry." Fauchard was a French surgeon, and in 1723, he published *The Surgeon Dentist, A Treatise on Teeth*. He was credited with this title because his book was the first to describe a comprehensive system for the practice of dentistry including basic oral anatomy and function, operative and restorative techniques, and denture construction.[24, 28, 29] Fauchard could be considered as one of the most influential men in the development of endodontics because his publication presented information on accessing the pulp chamber for purulence drainage, recommendation of using a pin to extirpate the pulp, leaving the tooth open for 2-3 months, and filling the teeth with led foil.[30] Fauchard was the first to discuss obturation techniques in the beginning of endodontics.[29]

In 1756, 33 years after Fauchards publication, a German dentist named Philip Pfaff was the first to discuss his pulp capping procedure where he used a gold foil or lead over a pulp exposure.[31] In 1760, John Baker immigrated from England and set up a dental practice.[24] He is considered the earliest medically trained dentist to practice in America.[32] Shortly after this, Robert Woofendale performed one of the first recorded endodontic

procedures in the United States in 1766.[25, 28, 33] In order to reduce pulpal pain, he cauterized the pulp tissue using a heated instrument. He then used oil of cinnamon, clove oil, turpentine, opium, or camphor oil as another modality to cauterize the pulp and relieve pain.[33] Woofendale also trained a protégé, the famous Paul Revere.[28] In 1768, Paul Revere placed advertisements in a Boston newspaper offering his services as a dentist. Paul Revere verified the death of Dr. Joseph Warren in the Battle of Breed's Hill, when he identified a bridge that he constructed for Warren. This is the first known case of post-mortem dental forensics.[24]

By the end of the 18th century, Frederick Hirsch, a German practitioner, discovered that a diseased tooth elicited pain on percussion.[30] He is one of the first practitioners to document the finding of periapical pathology associated with percussion. He treated these teeth with perforation at the neck of the tooth, followed by insertion of a red-hot probe and then filled the tooth cavity with lead.[28] In 1789, hypochlorite was first produced in France. Hypochlorite solution was used as a hospital antiseptic that was sold under the trade name Esol and later called Dakin's solution.[34]

The beginning of the 1800s starts a new era called the Vitalistic era (1806-1878).[28] Vitalists believed that living organisms were different from non-living items because the living organisms had a distinctive 'energy' or 'soul'.[35] Vitalists argued that living organisms did not have a chemical basis and living organisms could not be explained by mechanistic process.[35] Vitalist chemists predicted that organic materials could not be synthesized from inorganic components.[36] However, Friedrich Wöhler synthesized urea from inorganic components in 1828.[36] Vitalism theory was progressively weakened by Wöhler's finding because the synthesis of urea was

counterevidence for the vitalist hypothesis that only organisms could make the components of living things.[36] Even though the vitalist theory was proved wrong, vitalist thought influenced dentistry by questioning pulp vitality. Many dentists disregarded Wöhler's findings and continued to follow a vitalist doctrine.[29]

Vitalist Leonard Koecker wrote *Principles of Dental Surgery in 1826,* and his beliefs dominated pulp treatment procedures for over 50 years.[28] Koecker believed that when pulp was diseased, the whole dentinal core of the tooth immediately died, and that living tissue could not remain healthy and viable near dead tissue.[37] He said that the tooth became a foreign body and recommended extraction.[28] In 1829, the publication by S.S. Fitch, *System of Dental Surgery,* solidified the vitalist theory.[28] He believed that the crown was nourished exclusively by the dental pulp, whereas the roots were supplied by the pulp membrane on the interior and by the alveolar membrane on the exterior.[38] According to this theory, the roots were still nourished by the periodontal ligament but the crown lost its vitality since the pulp was removed.[38] He the supported the procedure of removing the necrotic crown of teeth after extirpation of the pulp and then placing a replacement crown on the remaining roots.[28]

Before 1836, extirpation of vital pulp was extremely painful without the discovery of anesthesia. Hot instruments were used to cauterize the pulp and corrosive sulfuric or nitric acid was used to desiccate the pulp tissue.[28] In 1836, Shearjashub Spooner from New York, devitialized pulp by using a protoplasmic poison, arsenic trioxide.[39] Treatment with arsenic was considered a success since it was painless; however, the arsenic caused necrosis of the other vital supporting tissues and periodontium.[39] The arsenic technique was used until the 1920s. In 1837, Jacob and

Joseph Linderer recommended the use of essential oils to help with pain prior to placing a direct pulp cap and permanent filling.[38]

In 1838, inventor Edwin Maynard fabricated the first root canal broach; he filed a watch spring for others to use in canals.[40] In 1839, Baker is credited with the first publication on pulpal extirpation, cleaning and root canal filling.[30] He removed the nerve, cleaned the root canals, and he then used gold foil to fill the tooth.[29] He wrote this procedure in the *American Journal of Dental Science,* which was the world's first dental journal.[24]

Also in 1839, Charles Goodyear invented the vulcanization process for hardening rubber.[24] Vulcanite was the result, an inexpensive material easily molded to the mouth, and dentists started to use this material as a denture base.[41] In 1864, the molding process of vulcanite dentures was patented.[24] However, the dental profession fought with Goodyear on licensing fees for the next 25 years.[41] In 1847, Edwin Truman introduced the use of gutta percha to the field of endodontics and dentistry.[42] He used gutta percha for denture bases in addition to root canals.[42] For the dentists that were not introduced to gutta percha yet, creosote wood soaked plugs were used to fill root canals.[29] To seal the wooden plugs, a mixture of Hill's stopping (gutta-percha, quick-lime, powdered glass, and metal fillings), chloroform, and eucalyptus oil was used as a liquid cement.[29] This is considered one of the earliest attempts to obturate with a solid and a liquid sealer.

In 1846, William Morton used ether anesthesia for surgery during the first successful public demonstration.[24] Horace Wells had a similar demonstration one year prior, but it was considered a failure since the patient cried out in pain.[24] W.W. Codman was the first to recognize and document secondary dentin formation in 1850.[25] After pulp

exposure, Codman strived for secondary dentin formation with pulp caping.[25] Thomas Rodgers also explored the topic of pulp capping in 1857.[25] He reported 220 cases of pulp capping to the Odonatological Society of London, with 202 successful cases. His work could have been praised as an example of good scientific approach, but the latter recommendation did not have scientific evidence. He prescribed three leaches and a laxative to the cases that failed.[28]

In 1859, 26 dentists met in Niagara Falls, New York, and this was the foundation of the American Dental Association (ADA).[24] There have been many influential contributions to dentistry and endodontics during the late 1900s. A very notable contribution is the invention of the dental dam. The dental dam has been the primary accepted method of isolating the operative field since its introduction by Sanford Christie Barnum in 1864.[43] It was essential in the era before suction was available and this simple device helped solve a troublesome problem for dentists.[28]

By 1865, dentists caught on to the concept of using the denture base gutta percha as root canal filling material, the concept introduced by Truman.[40] It is often documented by many authors that G. A. Bowman was the first to have used gutta percha, and not Truman. However, Bowman is credited for the technique of hot gutta percha plugged into place.[25] Bowman was also a co-inventor in 1873 for the rubber dam clamp forceps.[40]

In 1866, Lucy Beaman Hobbs graduated from Ohio College of Dental Surgery, as the first woman to earn a dental degree.[24] In 1867, Joseph Lister invented an antiseptic rinse using carbolic acid in surgery that was later applied as an oral rinse.[31] Also in 1867, Magitot introduced the electric pulp tester (EPT) by using current to determine tooth vitality.[44] 24 years later, in 1891, J.S. Marshall advocated and popularized the electric

pulp tester.[45] The EPT still remains one of the most useful devices in diagnosing between vital and non-vital teeth.[44] In 1870, G.V. Black reviewed 42 pulp capping cases with a 10 year follow-up. G.V. Black found than only 5 cases survived longer than 5 years. He advocated the use of zinc oxychloride as a capping material, and it was referred as Ash's material. This material gained acceptance as a capping material in the United States.[25, 28, 29]

In 1878, G.O. Rogers published an article suggesting that pathogenic organisms caused disease.[37] This created a spark for a new theory, the 'septic theory', and led to the end of the vitalism era due to the recognition of the pathogenicity of bacteria.[45] In 1882, Arthur Underwood further explored the septic theory. He suggested that an alveolar abscess with suppuration of the pulp was caused by the toxic effects of pathogens. He also theorized that if powerful antiseptic agents were stronger than the pathogens, then disease would be prevented.[38] Underwood stated that if the pulp chamber and canal were sterile with antiseptics, it did not matter if the pulp was vital or non-vital.[45] This concept was influential for the specialty of endodontics and practitioners used germicides to eliminate bacteria for pulp therapy for the next 30 years.[46]

Besides the watch spring that was used as an endodontic broach,[40] there were other techniques to remove pulp tissue. In 1883, G.A. Mills wrote in *Dental Cosmos* that he used creosote dipped hickory or orangewood sticks to try to remove the pulp tissue.[45] He would put the sticks in to the canals, hit them with a mallet, and then removed them with the pulp attached. This procedure did not have much acceptance by other dentists due to the intimidating nature of the procedure.[45] Also in 1883, Bowman introduced the solution of chloroform to gutta percha, termed chloropercha.[33] He used this to obturate

the canals, an idea that was accepted by many other dentists. M.L. Rhein of New York further developed this concept during the next ten years.[45]

There were many different methods that were used to disinfect and obturate during the 1880s-1900s. In 1884, Cassius Richmond used phenol and iodine to sterilize the tooth.[45] He was also the first to use carbolized orangewood as the filling material, favoring knocking out pulps with wood and mallet.[47] Mills used the orangewood sticks to remove pulp tissue, and Richmond used the orangewood sticks as obturation material.

Arsenic was still used over amputated coronal pulps as a means of disinfection.[45] Lepkowski introduced Formalin in 1885[48], which was a better alternative to the caustic arsenic. C.T. Gramm in 1890 used copper points[39] for root canal fillings. Otto Walkoff recommended using chlorophenol to sterilize root canals in 1891.[45] In 1894, J. R. Callahan recommended the use of 20% to 40% sulfuric acid to clean root canals.[47] Cotton pellet soaked in the sulfuric acid sat in the canal for 24 to 48 hours, causing caustic damage to the periapical tissues.[47] John Buckley introduced formocresol to dentistry, and this mixture of tricresol and Formalin was widely used in 1904 and for the next 50 years.[29, 49] Most of these agents were toxic and caustic to the patient due to possible seepage through the apical foramen.[45]

It wasn't until 1895 that dentists began to steer away from the caustic sterilizing agents. Dr. Breuer was the first to use electromedication to sterilize root canals.[33, 49] This was originally called electrolization, and then was referred as ionization.[45] M.L. Rhein was an advocate of this method in the United States, and he abandoned the previous use of caustic agents.[50] Hermann Prinz then introduced the use of 1% sodium chloride solution during electrolization in 1917.[51]

During the late 1800s, there were many advances with anesthesia in dentistry. In 1884, Koller introduced cocaine as a topical anesthetic, but it did not have wide acceptance due to its extreme toxicity.[30] However, E.C. Briggs did use it as a topical to anesthetize the pulp in 1890.[30] That same year, Funk tried a method to force the anesthetic into the pulp tissue. He exposed the pulp, then he used unvulcanized rubber with a plunger-like instrument to push a solution of cocaine into the coronal pulp.[47] Although cocaine had high toxicity, many dentists sparingly used it as an esthetic for the next 20 years.[38] In 1905, Alfred Einhorn developed procaine, later marketed under the trade name Novocaine.[40] This anesthetic was difficult to use and required tedious preparation. A tablet needed to be dissolved into solution, cooled, and then aspirated into a syringe for an injection.[40, 45] H.S. Vaughn is credited as the first person to use infiltration anesthesia before pulpal removal.[45]

In 1895, Wilhelm C. Roentgen, a German physicist, accidently discovered x-rays.[52] This discovery revolutionized endodontics, dentistry, and medicine; this allowed for dentists to visualize the root canals and analyze their procedures. Shortly after Roentgen's discovery, Otto Walkhoff took the first dental x-ray in Germany.[53] The news had spread to America, and then W. J. Morton, a physician, took the first radiograph in the United States.[45] In 1896, C. Edmond Kells, a prominent New Orleans dentist, took the first dental x-ray of a living person in the United States.[24] Kells also employed the first female dental assistant to help with procedures, scheduling, and bookkeeping in 1885. Soon after this, "Lady in Attendance" signs were seen in the windows of 19th century dental offices.[24]

In 1908, dentist and physician M.L. Rhein used a wire to determine canal length and verified with radiographs.[33] This is the first recorded working length x-ray to see if the apex had been reached.[48] Also in 1908, G.V. Black, a leading reformer and educator of American dentistry, wrote, *Operative Dentistry,* and this textbook was an essential publication for 50 years.[49] He was a pioneer in the use of visual aids for teaching dentistry.[24] G.V. Black also had a recommendation to determine length and size of the apical foramen by using a measurement control, so that overfilling of the canal could be avoided.[45]

At the time when dentistry and endodontics was gaining momentum with new advancements with the discovery of x-rays, the theory of 'focal infection' was gaining attention. In 1904, Frank Billings reported an apparent correlation between oral sepsis and bacterial endocarditis.[45] This gained attention from those interested in "oral sepsis." In 1909, E.C. Rosenow, a student of Frank Billings, was interested in the bacteriologic aspect of root canal therapy and developed the theory of focal infection. He presented that streptococci could travel through the bloodstream to infect distant sites and organs.[49] Mayrhofer also presented that streptococci were in 96% of pulpal infections.[51] This information made medical doctors question the field of dentistry and endodontics.

In 1910, Dr. William Hunter, an English physician, lectured on the focal infection theory, attacking American dentistry. He condemned all pulpless teeth to extraction and he was concerned with septic conditions caused by poorly constructed prosthetic restorations. This lecture was later published in the *Lancet.*[54] Doctors and dentists began recommending extraction of all diseased teeth to prevent the disease from spreading. The focal infection theory governed medicine for the next 25 years.[55, 56]

In 1912, Rhein was one of the first to challenge Hunter and the focal infection theory. Rhein advocated for better root canal procedures, better aseptic techniques, and the use of the rubber dam.[55] Dentist such as Callahan, Johnson, Coolidge, and Prinz realized that improvement in their procedures, diagnosis, technique, and aseptic protocols will help form a better argument for saving infected teeth.[33, 57] It was during this time when Dakin recommended sodium hypochlorite as a buffered 0.5% solution for irrigation of wounds during World War I.[34] In 1918, Coolidge introduced sodium hypochlorite to endodontics as an intracanal irrigation solution, which was a monumental contribution to the aseptic protocol of endodontics.[58]

In 1937, Logan, who was also in opposition to the focal infection theory, reported that even if microorganisms were present, it did not imply infection. He stated that bacteria are often present without causing pathological damage.[49, 55] This publication helped doctors to question the focal infection theory. There were two other publications that weakened the arguments of the focal infection theory. Tunnicliff and Hammond found a lack of tissue inflammation in extracted teeth, even though microorganisms were present in the pulps of these teeth.[49, 55] And lastly, Cecil at Cornell Medical College, published that removing suspected foci from 200 patients with arthritis did not improve the conditions of the patients.[55, 59] These publications started a new "scientific era" (1940s-1960s) by providing scientific evidence with sound histological and pathological findings. Researchers were able to show with additional research that systemic disease did not start from an infected tooth. Dentists were able to promote root canal therapy and put a halt on extraction of non-vital teeth.[55, 60]

Perhaps one of the most important discoveries in medicine happened by accident. In 1928, at St. Mary's Hospital in London, Alexander Fleming accidently discovered penicillin.[61] He noticed that contaminated bacteria did not grow around the *Penicillium* genus mold spores when an uncovered Petri dish sat next to an open window. Fleming later stated, "*When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, or bacterial killer. But I suppose that was exactly what I did."*[61] This discovery transformed all of medicine and dentistry. Antibiotic application was later applied to endodontics in 1941 by Fred Adams. To treat apical infection, Adams recommended sulfanilamide. Adams was also the first to use penicillin in root canal therapy in 1944.[49, 55]

A very influential contribution to endodontics has been the development of the electronic apex locator to determine working lengths of roots. It is typically reported that the evolution and development of the electronic apex locator for use in root canal procedures was introduced from the work of Suzuki in 1942, and the device was developed by Sunada in 1962. However, the concept of using electrical means to locate the ends of root canals was brought to the dental profession as early as 1918 by Dr. Levitt Ellsworth Custer. Custer was the first to report the use of electric current to determine working length. Suzuki then revisited Custer's concepts.[62] Sunada's research and his first device became the basis for most electronic apex locators (FIGURE 1). [63]

In 1943, Harry Johnston and twenty other dentists met in Chicago as the first organized meeting of endodontics. From the meeting, the American Association of Endodontics (AAE) was founded.[64] This group of dentists also discussed and set standards for endodontic treatment, and Johnston created the term "endodontics."

Endodontics is from two Greek words, with “endon” referring to “within” and “ho dontas” meaning a “tooth.”[65] With many more dentists limiting their practice to root canal therapy, the American Association of Endodontists wanted to become recognized as a specialty.[55]

In 1945, one of the most influential mass public health contributions in history was the beginning of the community water fluoridation (CWF) movement.[66] Sodium fluoride was added to the public water systems as a preventative measure, and this began in cities of Newburgh, New York, and Grand Rapids, Michigan.[24] This public health measure created more awareness for dentistry and for dental research. In 1948, President Harry S. Truman signed the Congressional bill which established the National Institute of Dental Research; this was the beginning of federal funding for dental research.[24]

In 1949, the American Association of Endodontists formed a committee to approach the American Dental Association.[55] In 1956, the American Dental Association Dental Education Council recognized the Board of Endodontics.[65] By 1963, more than 200 American dentists limited their practice to endodontics.[64] That same year, in 1963, the American Dental Association (ADA) approved for Endodontics to be recognized as one of the dental specialties. By 1965, the first endodontic specialists achieved board certification.[65] Today, the AAE has grown tremendously and it has more than 8,000 members globally.[32]

There have been many substantial advances in endodontics in the last 60 years, ever since the foundation of AAE and since the ADA has recognized endodontics as a specialty. The use of microscopes in dentistry has become quite popular since the late 1980s. However, its origin for dental applications can be traced to the early 20th century,

when a microscope, invented by an American Biologist named Greenough, working in the Zeiss Company, was adapted for use in dentistry.[67, 68] Today, Zeiss is one of the leading companies supplying endodontic and medical microscopes.[68]

The AAE also had its own advances by publishing a position statement on dental dam use as the standard of care. While the use of a rubber dam was advocated many years prior, there was no official documentation stating that using a dental dam is the standard of care during endodontic treatment until 2010. In 2010, the AAE published a "AAE Position Statement: Dental Dams." In this position statement it says, "tooth isolation using the dental dam is the standard of care; it is integral and essential for any nonsurgical endodontic treatment."[69] The position statement was reaffirmed by the AAE in 2017.[70]

Another important contribution that forever changed the way endodontic procedures are completed was the invention of the nickel titanium rotary system. In late 1988, Harmeet Walia introduced the magical properties of a corrosion resistant alloy to the manufacturers for making the nickel–titanium (NiTi) rotary instruments.[71] Over the past two decades, the nickel–titanium (NiTi) rotary instruments have highly improved the quality of the cleaning and shaping of the root canals. The super-elasticity and shape memory of these instruments reduce the possibility of the canal transportation along with saving the time for both the patients and the clinicians. Previously, the hand endodontic files were made of carbon steel or stainless steel. Up to this date, five generations for NiTi rotary instruments have been described according to the time of introduction, properties, and method of application.[71]

Other substantial advancements in endodontics include the use of cone beam micro computed tomography (CBCT) in diagnosis and treatment planning of difficult cases, endodontic retreatments, and microsurgery.[72] Additionally, other advancements include adjunct irrigation therapies such as sonic and ultrasonic irrigation, and the Sonendo GentleWave system.[73] Regenerative endodontics has had an explosion of improvement and new advancements in the last couple years, with the latest AAE recommendations revised in 2018.[74] The importance of research and continuous advancements is paramount for the science of endodontics. These continual improvements and research developments gives patients and clinicians encouragement of continued growth for the AAE and for the endodontic specialty.

THEORY OF ENDODONTICS

A pivotal point in the field of endodontics occurred in 1965 due to research evidence by Kakehashi, Stanley and Fitzgerald.[75] This research evidence showed that microorganisms and bacteria were the cause of pulpal and periapical disease. Germ-free (gnotobiotic) rats and control (normal) rats had pulp tissue exposed, and both groups contained ordinary microflora in the oral environment. The germ-free rats did not show any signs of disease while the normal rats showed pulp necrosis with granuloma and abscess formation. This study showed that endodontic and periapical disease begins when the ordinary microflora enters into the pulp tissue and into the root canal system. The microflora can enter by means of caries, trauma, or an existing restoration.[75]

There were additional studies that followed Kakeshashi's example and looked at the impact of microorganisms on pulp tissue and pulpal disease. One study by Gunnar

Bergenholtz in 1974 showed that bacterial growth was present in traumatized teeth with periapical lesions.[76] Another study by Moller in 1981 studied the pulps of 9 monkeys, 78 teeth. 26 of the pulp chambers were kept bacteria free by sealing the chamber, while 52 were infected by the indigenous oral flora. This study showed that noninfected necrotic pulp tissue did not induce inflammatory reactions in the apical tissues, but by contract, infected pulp tissue did induce inflammatory reactions and could be seen clinically and radiographically. Facultatively anaerobic streptococci, coliform rods and obligately anaerobic bacterial strains were most frequently found.[77] These studies help develop a protocol for endodontics; the root canal system needs to be disinfected and cleaned of microorganisms to reduce the inflammatory reaction and to allow for healing.[78] Mechanical debridement and reducing the pathogens, such as lipopolysaccharides (LPS)[79], inside the root canal system, will allow for successful endodontic therapy.[80]

In 1955, prior to Kakehashi's findings, George G. Stewart stated that thorough chemomechanical cleansing of the canal is one of the most important phases of root canal therapy. He believed that the bacteria needed to be eliminated for a successful outcome, which is what Kakehashi proved ten years later. Stewart was instrumental in endodontics because he had formulated a protocol for root canal therapy. He believed that root canal therapy may be divided into three phases: chemo-mechanical preparation, microbial control, and obturation of the root canal.[81] Since chemo-mechanical cleaning of the canal was the most important step, the root canal was enlarged to reduce the number of microorganisms, to increase space inside the canal for a larger volume of medicine, and to allow for an easier obturation. In Stewart's study, he obturated the canals by lateral

condensation as described by Grossman.[81] Louis Grossman believed that there were 13 conditions that would determine the success of a root canal therapy. These included:

1. Aseptic technique
2. Instrument retention within the root canal system
3. Instruments should never be forced apically
4. Enlarging the canal space from its original size to accommodate obturation material
5. Continuous irrigation with and antiseptic throughout treatment
6. Irrigation solution should remain with the canal space
7. Fistulas do not require a special protocol
8. Negative culture should be confirmed prior to obturation of the root canal
9. Obturation should include a hermetic seal of the root canal system
10. Obturation material should not be irritating to tissues
11. Alveolar abscess should be adequately drained
12. Injections should be avoided into infectious areas
13. Surgical treatment may be required for proper healing

One of the most important aspects in endodontics is the disinfection of the root canal system and elimination of bacterial; this is still paramount in today's practice. In order to have proper aseptic technique, rubber dam isolation is necessary.[82] In 1967, Herbert Schilder developed a obturation technique, which was referred to as the "warm gutta percha vertical compaction technique," or also known as the "Schilder Technique." These concepts of using a rubber dam, proper aseptic technique, obturation with gutta

percha, and the "warm gutta percha vertical compaction technique" are still used today in modern endodontics.[82]

MECHANICAL PREPARATION/INSTRUMENTATION

The purpose of endodontic instrumentation is to remove debris[83], irrigate to the apex[84], and prepare the canal for obturation.[80] There have been many different instrumentation techniques reported in the literature. Some commonly described instrumentation techniques are the step-down by Goerig[85], the passive step-back by Torabinejad[86], the anti-curvature filing by Abou-Rass[87], the balanced forced by Roane and Sabala[87], and the crown-down technique by Morgan and Montgomery[88].

Instrumentation can be performed with a combination of hand instruments and rotary instruments. Hand instruments are usually made of stainless steel, while the rotary instruments are made of nickel titanium (NiTi) alloys.[89] NiTi alloys have two different crystalline structures, and these are the more flexible low-temperature martensite phase, and the stiffer austenite phase. There are also controlled memory (CM) files, a heat-treated NiTi material in which the austenite finish temperature is higher than the body temperature, which allows for the more flexible martensite phase of the instrument.[90] Rotary instruments have a better centering ability in the root canal system over hand instruments[83], but hand and rotary instruments have no difference in reducing the amount of bacterial inside the root canal system.[91]

Instrumentation of the coronal portion of a canal, referred as 'coronal flaring,' provides many benefits for the practitioner. Coronal flaring creates straight-line access to the apical portion of the canal[92], it can decrease the incidence of rotary instrument

separation[93], and it can allow the apical foramen to be reached more consistency with electronic apex locators.[94]

Instrumentation of the apical portion of the canal refers to the size of the master apical file (MAF) at the apex. Saini showed that when the MAF was three sizes larger than the initial apical size, outcomes were significantly improved. He also showed that further enlargement did change the outcomes.[95] Mickel found that greater bacterial reduction was noted by using the crown-down technique to gauge the apical size, then increasing by three files sizes as the final file compared to only one files size greater than the initial file selected.[96] Salzgeber and Brillant found that there was better penetration of irrigants to the apex of a root when a minimal MAF size of number 30 was used.[84]

Although instrumentation removes debris and helps shape the root canal system, it does create a smear layer. The smear layer can be found on the surfaces of the canal wall and packed inside the dentinal tubules; Mader discovered that the smear layer was located on these two areas.[97] As to removal of the smear layer, there is controversy as to whether or not this needs to take place. Sen and Taylor favor the smear layer removal. Sen stated that the smear layer blocks the disinfecting properties of sodium hypochlorite and chlorohexidine[98], and Taylor stated that there was less coronal leakage with the removal of the smear layer.[99] Clark-Holke and Drake stated that there was more bacteria inside the canals when the smear layer was removed.[100] Madison and Krell reported that leaving the smear layer did not affect the apical seal.[101]

CHEMICAL IRRIGATION

Instrumentation will remove bacteria and help shape the root canal system. However, instrumentation alone cannot fully removal all the bacteria and debris. Peters found that 35% of root canal walls were untouched after endodontic instrumentation.[102] Therefore, irrigation is required to help eradicate microorganisms and help flush out debris since instrumentation cannot removal all microbes. Zehnder reviewed the different irrigation solutions and he stated that the ideal endodontic irrigant should[103]:

- Have broad antimicrobial properties
- Be highly effective against anaerobic and facultative organisms
- inactivate lipopolysaccharides
- dissolve both vital and necrotic tissue
- either prevent formation of the smear layer or dissolve it once formed.

Zehnder stated that many irrigants have one or several of these properties, but no single one irrigant has all of these properties. Peters et al. also stated that the ideal irrigant should do the following[78]:

- Be an effective germicide and fungicide.
- Be nonirritating to the periapical tissues.
- Remain stable in solution.
- Have a prolonged antimicrobial effect.
- Be active in the presence of blood, serum, and protein derivatives of tissue.
- Have low surface tension.
- Not interfere with repair of periapical tissues.

- Not stain tooth structure.
- Be capable of inactivation in a culture medium.
- Not induce a cell-mediated immune response.
- Be able to completely remove the smear layer and be able to disinfect the underlying dentin and its tubules.
- Be nonantigenic, nontoxic, and noncarcinogenic to tissue cells surrounding the tooth.
- Have no adverse effects on the physical properties of exposed dentin.
- Have no adverse effects on the sealing ability of filling materials.
- Have a convenient application.
- Be relatively inexpensive.

Sodium hypochlorite is the most commonly used endodontic irrigant and it fulfills many of Zehnder's and Peter's criteria. Properties of sodium hypochlorite that make it an effective endodontic irrigant[104]:

- Dissolves necrotic tissue (Baumgartner and Mader)[105]
- Dissolves vital tissue (Rosenfeld)[106]
- Removes the organic component of the smear layer (Baumgartner and Mader)[105]
- Kills planktonic bacteria (Haapasalo)[107]
- Kills bacterial in established biofilms (Del Carpio-Perochena)[108]
- Kills bacteria in dentinal tubules (Wong and Cheung)[109]

As reported by AAE members, sodium hypochlorite at concentrations of 5.25% or greater are commonly used in practice.[110] Hand found that 5.25% sodium hypochlorite was the most effective at dissolving necrotic tissue.[111] Senia found that it was the most effective concentration for removing vital tissue.[112] Harrison found that this concentration was safe for clinical use and did not increase postoperative pain.[113] Morgental found that 5.25% sodium hypochlorite was more effective than either chlorhexidine or QMix irrigation solution (Dentsply) at eradicating *Enterococcus faecalis*[114]; QMix is a solution that contains chlorhexidine, EDTA, and a surfactant. Although sodium hypochlorite effectively disinfects the root canal system and dissolves tissue, it lacks the ability to dissolve the mineralized component of the smear layer.[104] Therefore, practitioners will use chelating agents as part of their irrigation protocol to dissolve the inorganic component of the smear layer.

EDTA (ethylenediaminetetraacetic acid) is a chelating agent that binds to inorganic metals via four carboxylate and two amino groups.[115] EDTA chelates calcium ions and effectively targets dental hard tissue.[116] Calt and Serper found that a 10 minute application of EDTA caused excessive dentin erosion but a 1-minute rinse of EDTA was safe and removed the smear layer completely.[116] When sodium hypochlorite and EDTA are combined, EDTA can reduce the efficacy of sodium hypochlorite. The active chlorine content of sodium hypochlorite was reduced when mixed with EDTA.[117] Practitioners must use caution when using different irrigations and should follow their protocols carefully.

Besides the popular sodium hypochlorite and EDTA irrigation solutions, chlorhexidine gluconate (CHX), is an irrigation solution also used in endodontics.

Chlorhexidine is a biguanide, and Jeansonne and White found that chlorohexidine was as effective as 5.25% sodium hypochlorite for antibacterial activity[118]. Jeansonne and White recommended chlorhexidine for cases of sodium hypochlorite allergy or on open apices cases where sodium hypochlorite extrusion may result[118]. Chlorohexidine also possesses the property of substantivity. Baca found that chlorohexidine will bind to dentin, and its antibacterial activity can persist for as many as 48 days after exposure[119]. While there are certain benefits to using chlorhexidine, there are some disadvantages. Chlorhexidine lacks the ability to dissolve organic tissue, specifically vital and necrotic pulp tissue[120]. Basrani also found that when chlorhexidine reacts with sodium hypochlorite, it forms a red-brown, carcinogenic precipitate called parachloroaniline, which can occlude dentin tubules[121]. Chlorohexidine should only be used in a dry canal, free of liquid sodium hypochlorite. Due to these disadvantages, chlorohexidine must be used with caution.

In addition to several different irrigation solutions, there are also adjunctive irrigation techniques besides needle irrigation. This includes passive ultrasonic activation, sonic activation, photodynamic therapy, EndoVac (Kerr), and Gentle Wave (Sonendo). These adjunctive irrigation technologies can provide variation and improvements in debridement, disinfection or both.[104] Whichever technique is used, it is imperative that the irrigation solutions are used with caution to prevent accident leakage or sodium hypochlorite accident.

OBTURATION

As mentioned earlier, Herbert Schilder developed an obturation technique, called the “warm gutta percha vertical compaction technique,” or also known as the “Schilder

Technique." He stated that placement of a three dimensionally adapted filling is indicated. His technique used small increments of gutta percha that were heated with a spreader, and then these increments were compacted with a plugger inside the root canal system. This created a well dense, obturated root canal filling that would create a sealed system.[122] Traditional root canal filling material is composed of gutta percha (GP). Gutta percha is closely related to rubber and is a naturally occurring polymer of isoprene.[42] Common properties of gutta percha materials are:[104]

- GP cones contain 65% of zinc oxide, 20% gutta percha, 15% waxes, resins, and metals[42].
- Trans-isoprene, chemically related to natural rubber latex[42]
- Cone form of GP is in the beta crystalline phase[123]
- Changes to alpha crystalline phase on heating 42-49°C [123]
- Careful use in patients with severe type 1 allergy to latex since similar to rubber[124]

According to the AAE Glossary of Endodontic Terms, obturation technique is defined as "the method used to fill and seal a cleaned and shaped root canal using a root canal sealer and core filling material."[125] They also define the obturation technique into seven different categories:[125]

- *carrier-based obturation*: Sealer is placed in the canal followed by a core carrier coated with gutta- percha or other materials; device is heated prior to placement.
- *continuous wave compaction technique*: A variation of warm vertical compaction in which a master point is placed in a sealer-lined canal and compacted by a

prefitted, tip-heated electrical plugger. The softened point is vertically compacted, the plugger withdrawn and canal backfilled with thermoplasticized material.

- *lateral compaction*: A sealer is placed in the canal followed by a fitted gutta-percha (or other material) master point compacted apically and laterally by a tapering spreader to make room for additional points.
- *plasticized technique:* A sealer is placed in the canal followed by a filling material that has been softened with heat or chemicals for compaction in the canals.
- *silver point (cone) technique:* A sealer is placed in the canal followed by a fitted silver point.
- *warm vertical compaction:* A sealer is placed in the canal followed by a fitted master cone. warmed and compacted vertically by a plugger to make room for additional warmed segments of filling.

From these different types of obturation techniques, carrier-based systems provide an alternative to GP but the filling quality may be inferior to the other techniques.[104] These tend to be marketed as quicker obturations and faster RCTs over the other techniques. However, Baumgardner found that carrier-based filling materials result in fills that leak significantly more than laterally or vertically condensed GP.[126] For outcomes of lateral compaction and warm vertical compaction, no differences were noted in voids between the two techniques[127], and no differences in outcomes were noted with either technique.[128] The highest success rates occur when the obturation material is 0-1mm from the radiographic apex.[129] Sealer is an essential component to obturation of a root canal and sealer should be used no matter which technique is chosen.[130] In 1982, Grossman stated that an ideal endodontic sealer should have these properties:[131, 132]

- Exhibits tackiness when mixed to provide good adhesion between it and the canal wall
- Establishes a hermetic seal
- Radiopaque
- Very fine powder, so that it can mix easily with liquid
- No shrinkage on setting
- No staining of tooth structure
- Bacteriostatic, does not encourage bacterial growth
- Exhibits a slow set
- Insoluble in tissue fluids
- Tissue tolerant; nonirritating to periradicular tissue
- Soluble in a common solvent if it is necessary to remove the root canal filling.

The most popular sealers are zinc oxide-eugenol formulations, calcium hydroide sealers, glass ionomer sealers, resin-based (epoxy resin or methacrylate resin) sealers, and calcium silicate-based sealers, which is the most recently introduced sealer. Regardless of the sealer selected, the all exhibit some level of toxicity until they have set. Therefore, extrusion of sealers into periradicular tissues should be avoided.[132]

MANAGING IMMATURE TEETH WITH PULPAL NECROSIS

Pulp necrosis in an immature permanent tooth poses treatment challenges for the practitioner.[104] Unfortunately, the pulp chamber was infected with bacteria which caused

pulp necrosis before the root apices was able to fully maturate and close.[133] The dentin walls of the tooth can be thin and weak, with a risk of possible fracture. The open apices of incompletely developed roots require additional treatment to create a proper barrier. Since there is a wide apex, endodontic materials can accidently be extruded outside of the root canal system.[134] Apexogenesis attempts to maintain a vital pulp in immature teeth, while apexification and regenerative endodontics attempts to address pulp necrosis in immature teeth.

APEXOGENESIS

According to the AAE Glossary of Endodontic terms, apexogenesis is "a vital pulp therapy procedure performed to encourage continued physiological development and formation of the root end; frequently used to describe vital pulp therapy performed to encourage the continuation of this process."[125] Apexogenesis is considered vital pulp therapy, and this includes pulp capping, partial pulpotomy, or full pulpotomy as treatment options.[104]

The goal of pulp capping is to treat the exposed vital pulp by sealing with a dental material to facilitate the formation of reparative dentin and maintenance of the vital pulp.[125] Bergenholtz found that pulp caps should be placed as soon as the exposure occurs for the best prognosis.[135] Mineral trioxide aggregate (MTA), calcium hydroxide, and Biodentine (Septodont) are materials used by practitioners for pulp capping. Calcium hydroxide has long been considered the universal standard for vital pulp therapy, ever since it was introduced by Hermann in 1920s.[136] However, clinical retrospective studies have shown that calcium hydroxide as a pulp capping material

indicated increasing failure rates over time. A study by Willershausen et al. showed 80.1% success at 1 year, 68% at 5 years, and 58.7% after 9 years.[137]

MTA was introduced by Torabinejad in the 1990s, and practitioners began to use MTA over calcium hydroxide. Torabinejad and Parirokh reported that once a MTA pulp cap is placed, coagulative necrosis of adjacent pulp tissue results, then stimulating and forming a hard tissue bridge.[138] Mente et al reported that the long-term success rates for direct pulp caps with calcium hydroxide were 58% and with MTA were 80%.[139] Biodentine, a calcium silicate, has recently been advocated as an alternative to both calcium hydroxide and MTA. It is also used in a similar manner as the CH and MTA. Nowicka et al found that Biodentine-capped pulps had a similar histological appearance to those treated with MTA[140]

Pulpotomy is the other vital pulp therapy option, and pulpotomy can be either complete or partial, depending on the extent of the pulp tissue removed.[125] Pulpotomy treatment involves removal of the coronal portion of a vital pulp to preserve the vitality of the radicular pulp Cvek found that pulpotomy treatment facilitated continued root development in immature permanent teeth. Cvek also found common postoperative complication of calcifications, internal root resorption, and pulpal necrosis.[141] If pulpal necrosis did result, then apexification or regenerative endodontic procedures were additional treatment options for pulp necrosis in an immature tooth.

APEXIFICATION

According to the AAE Glossary of Endodontic terms, apexification is “a method to include a calcific barrier in a root with an open apex or the continued apical

development of an incompletely formed root in teeth with necrotic pulps.[125] Apexification objective is to create and induce a hard tissue barrier in which endodontic filling materials can be compacted. Frank reported one of the first apexification techniques and this involved placement of calcium hydroxide into the root canal system; this would allow for the Hertwig epithelial root sheath to generate an apical barrier.[142] Jeeruphan found the survival rate for calcium hydroxide apexification to be 77%.[143] Although this was a relatively good result, Cvek reported that long term calcium hydroxide apexification has been associated with an increase in cervical root fracture.[144] He reported the incidence for cervical root fracture to be 28% to 77%.[144] Shabahang also reported that it could take up to 24 months to form the apical barrier.[145] Due to these limitations of cervical root fracture and extended treatment times, practitioners began looking at artificial apical barriers.

Practitioners began to use artificial barriers, such as MTA, for apexification procedures. Since MTA is biocompatible and able to seal in the presence of blood, it was a considered a good alternative to calcium hydroxide.[138] MTA also decreased treatment time for patients compared to CH apexification.[146] Jeeruphan reported a 95% survival of MTA apexification treated teeth, compared to only a 77% survival reported for calcium hydroxide apexification treated teeth.[143] Pace et al reported that 94% of immature teeth treated with MTA apexification were healed 10 years postoperatively.[147] Witherspoon and Ham reported a 93.5% success rate of MTA apexification in a single visit versus a 90.5% success rate in two visits.[148] One alternative to apexification for immature necrotic teeth is regenerative endodontics.

REGENERATIVE ENDODONTIC PROCEDURES (REPs)

Regenerative Endodontics is an additional treatment option for the necrotic immature tooth, besides apexification. According to the AAE Glossary of Endodontic terms, regenerative endodontics is "biologically-based procedure designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex."[125] This treatment encourages the further development of radicular dentin and attempts to decrease cervical fracture. Regenerative endodontics is a tissue engineering procedure that facilitates development of dental hard tissues, as known as Regenerative Endodontic Procedures (REPs). Nygard-Otsby is the first credited for his work with regenerative endodontics; he recognized tissue healing with a blood clot inside of the root canal system during the 1960s.[149] In 2004, Banchs and Trope presented a case report that sparked interest and attention with regenerative endodontics, and this topic is still heavily research today.[150]

According to Law in the article "Consideration for regeneration procedures" in 2013, regenerative endodontics requires a few components:

- Necrotic pulp
- An immature apex
- No need for post and core restoration
- Patient and parental compliance

The above recommendations have been expanded upon and in 2018, the AAE has published an updated "AAE Clinical Considerations for a Regenerative Procedure."[151] The current case selection recommendations include Law's contributions but also supplemented with "no antibiotic allergies for the patient and ASA 1 or ASA 2."[151] The

guidelines recommend for REPs to be completed in two visits, only if the patient is asymptomatic on the second visit.[152]

During the first visit, the tooth is disinfected with 20mL/canal of 1.5% sodium hypochlorite and 20 mL/canal of saline or 17% EDTA.[151] The reduced concentration of sodium hypochlorite from 5.25% is to prevent cell death of the apical papilla cells.[153] Then, the tooth is filled with a double antibiotic paste[152], a triple antibiotic paste[154], or calcium hydroxide, and temporized.[150] The patient then returns after 1-2 weeks for the completion of the procedure, as long as the patient is asymptomatic.[151] At second visit, anesthesia without a vasoconstrictor, such as epinephrine, is given to anesthetize the patient.[151] The tooth is again rinsed with 20 mL/canal of 17% EDTA to release growth factors from the dentin tubules.[155] The EDTA is a chelating agent that will demineralize the canal dentin layer and exposure of the collagen fibers, allowing for growth factors to be released into the RCS. Then, a 25 file is used to lacerate the apical papilla and to induce bleeding beyond the apex, releasing stem cells.[151] The blood will then fill the tooth and once at the CEJ, a collagen barrier is placed.[156] A coronal seal is placed on top of the collagen barrier, followed by BC liner, and a resin restoration.[151]

The goals of REPs include a *primary goal)* to eliminate symptoms, eliminate apical periodontitis, and to have complete bone healing; *secondary goal)* to have increased root wall thickness and/or increased root length; and *tertiary goal)* to have a positive response to vitality testing.[151] In order to achieve these goals, stem cells, growth factors, and scaffolds are necessary for tissue engineering to occur.[157] Lovelace showed that the mesenchymal stem cells that infiltrate the canal space originate in the apical papilla.[158] In one cohort study by Jeeruphan, it was reported that REPs had a 100%

survival rate.[143] In a pilot retrospective cohort study, it was reported by Alobaid that regenerative endodontics was statically equivalent to the outcome of apexification.[159]

MICROORGANISMS

There are different types of endodontic infections, and these infections can be classified into a *primary infection* or a *secondary infection*. A primary infection occurs in untreated necrotic teeth, and it appears to involve a greater number of bacterial species than a secondary infection.[104] A secondary infection is a reinfection of a previously treated tooth. Rocas and Sigueira reported that there are approximately 20 species in primary infection compared with approximately 3 species in secondary infections.[160] Figdor and Sundqvist reported that primary infections consisted of equal numbers of gram-positive and gram-negative species and there were more obligate anaerobes than aerobes present.[161] *Actinomyces naeslundii, Fusobacterium nucleatum*, and *Porphyromonas gingivalis* are examples of gram-positive species in primary infections.[162]

A. naseslundii is a gram-positive, rod shaped bacterium, and it will activate the innate immune system.[163] This activation of the innate immune system then results in activation of the inflammatory process by activating cytokines and Toll-like receptors.[164] *F. nucleatum* is a gram-negative obligate anaerobe, and this species has the ability to develop a biofilm by attaching to other bacteria.[165] *P. gingivalis* is a gram-negative anaerobe, and it has virulence factors that lead to tissue destruction of the host tissue, such as the periodontal tissues.[162] *P. gingivalis* can have LPS, fimbriae, or capsules, and

these are the main virulence factors which allows the bacterium to evade a host response.[166]

Figor and Sunqvist also reported that secondary infections contained mostly gram-positive bacteria, but there were equal numbers of anaerobes and facultative species.[161] Murad contradicts that statement on secondary infections and reported that there was a higher prevalence of gram-negative bacteria in secondary infections, especially in the present of a large periapical lesion.[167] Some of the commonly found bacterial species of secondary infections are

Actinomyces israelii, Enterococcus faecalis, and *Propionibacterium spp.*[160] *E .faecalis* is a gram-positive facultative anaerobe, commonly found as a bacteria species associated with root canal retreatment.[168] This bacterium is of particular interest due to its antimicrobial resistance. *E. faecalis* can survive starvation and has the ability to survive long periods of time in dentinal tubules without nutrients.[169] *E. faecalis* possesses a proton pump that allows for it to adapt to harsh environments.[170] Bystorm theorized that this proton pump contributes to *E. faecalis'* unique resistance to calcium hydroxide.[171] Distel found that *E. faecalis* could form biofilms, helping the bacterium increase its resistance to endodontic procedures.[172]

Some of the less frequently encountered species in endodontic infections are *Actinomyes*, spirochetes, fungi, archaea, and viruses.[104] Ferreira found that herpes simplex virus (HSV) types 1 and type 2; herpesvirus (HHV) types 6,7,8; varicella zoster virus (VZV), and human papillomavirus (HPV) can all be isolated viruses in endodontic infections.[173] Sabeti reported a presence of Epstein-Bar virus (EBV) and cytomegalovirus (CMV) in periapical lesions.[174] Von Arx reported an association between feline

herpesvirus and cased of invasive cervical root resorption in both humans and cats.[175]

USE OF ELECTRONIC APEX LOCATORS

In 1918, Custer was the first to report the use of electric current to determine working length.[63] Suzuki in 1942 found that iontophoresis in dog's teeth indicated that the electrical resistance between a root canal instrument inserted into a canal and an electrode applied to the oral mucous membrane registered consistent values. Based on Suzuki's finding, Sunada in 1962, reported that a specific value of the resistance would determine the position of the root canal terminus.[176] He reported that there is a constant value (6.5kΩ) of the electrical resistance between the mucous membrane and the periodontium, and he stated that it is possible to use this value of resistance in the estimation of the root length. Sunada showed that if an endodontic instrument is connected to an ohmmeter (Figure 1) then introduced into the canal and advanced until the ohmmeter shows the value of 40 μA, the tip of the instrument has reached the periodontal ligament at the apical foramen. [63]

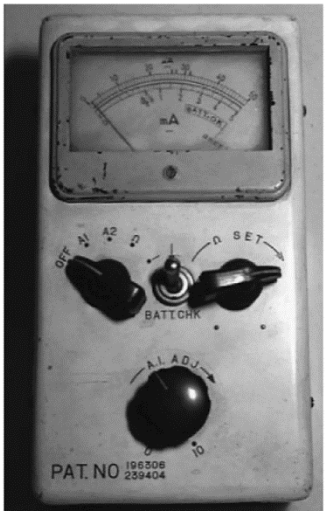


FIGURE 1. A simple d.c. ohmmeter used by Sunada 1962 for measuring the length of the root canal using direct electric current.[63]

An electronic apex locator (EAL) functions by using the human body to complete and electrical circuit. One side of the apex locator's circuitry is connected to the oral mucosa through a lip clip and the other side to a file. When the file is placed into the root canal and advanced apically, the electrical circuit is completed when the file tip touches periodontal tissue at the apex (Figure 2).[63] The electrical resistance of the electronic apex locator and the resistance between the file and oral mucosa are equal, which results in the device indicating that the apex has been reached.[63]

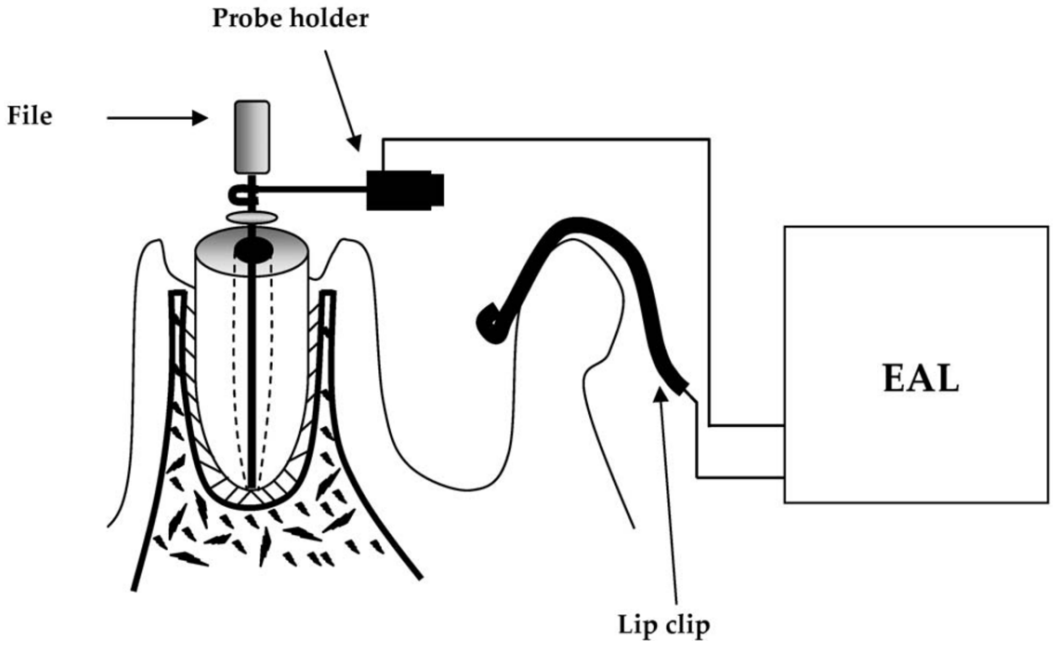


FIGURE 2. Typical circuit for electronic determination of working length in a human model with electronic apex locator (EAL).[63]

When an endodontic file penetrates inside the canal and approaches the minor apical foramen, the resistance between the endodontic file and the foramen decreases because the effective length of the resistive material (dentine, tissue, fluid) decreases. While the early and simpler devices measure resistance, other devices measure impedance using either high frequency, two frequencies, or multiple frequencies.[176] Electronic determination of working length is one of the key factors responsible for the success of endodontics. According to many articles in the literature, electronic determination is extremely accurate and predictable, therefore, it has gain tremendous popularity in root canal treatment.[177] Throughout the years, there has been many different generations of apex locators:[177]

- *First Generation Apex Locators: Measurement of electrical resistance***.** These EALs used resistance method for determining working length and measured the opposition to the flow of direct current (resistance). These were based on the principles of Suzuki and Sunada. Pain was felt by the patient due to high currents and these machines were found to not be accurate.[178]
- *Second Generation Apex Locators: Measurement of electrical impedance.* These EALs used impedance for determining working length and measured the opposition to the flow of alternating current (impedance). These units utilized the current of a single frequency and were inaccurate in the presence of irritants in the canal.[179, 180]
- *Third Generation Apex Locators: Using two different frequencies at the same time in order to measure the difference of ratio between two currents.* These EALs use two frequencies instead of one to measure the impedance to determine working length. The first apex locator in the category was Endex[181], but it required calibration for each canal. Root ZX by J. Mortia was later developed, but did not require any calibration.[182] Two different frequencies of 400 Hz and 8 kHz simultaneously were used to measure the impedance in the canal. A quotient value was then determined by dividing 8 kHz impedance value by 400 Hz impedance value. When the quotient value is 0.67, then the reading of the minor apical diameter is located.[183] Root ZX can be used in canals filled with all types of fluids because the quotient (0.67) is always the same.[183-185]
- *Fourth Generation Apex Locators: Using two or more non-simultaneous continuous frequencies in order to measure the difference or ratio between two*

currents. These EALs use multiple frequencies (2-5 frequencies) to measure the impedance in order to determine the working length.[176] Multi-frequency measurement system is used to calculate the distance from the tip of the file to the foramen by measuring impedance changes between two electrodes.[177] It is believed that the using multiple frequencies but using only one frequency at a time, and basing the measurements on the root mean square values of the signal frequency increases device accuracy and reliability.[178, 186] It was different than the third generation since it was only using one frequency at a time, not simultaneously. Root ZX II (J. Morita, Tokyo, Japan) is an example of a fourth generation EALs. Root ZX II uses the root mean square of the impedances measured at frequencies of 0.4 and 8.0 kHz.[187] The Root ZX II still uses impedance measurements to measure location within the canal, but is considered a powerful microprocessor. It is able to process the mathematical quotient and algorithm calculations required to give accurate readings.[188] Apex locators in this category were more accurate and reliable than the previous generations. [182]

- *Fifth Generation Apex Locators: Measures the capacitance and resistance of the circuit separately.* Propex II (Dentsply Maillerfer, Ballaiques, Switzerland) is an example of 5th generation. The addition of a diagnostic table including statistics of values at different positions made it easier to diagnose the position of the file.[189]

USE OF ELECTRIC CURRENT FOR DISINFECTION

Electromagnetic fields have been introduced to endodontics during the early 20th century. This use of electricity in endodontics was originally referred as "electro-sterilization." [50, 51] The purpose of electro-sterilization was to provide root canal sterilization, anesthesia by removing pulp tissue, and to help heal periodontal disease.

More research was completed on electro-sterilization and by the 1990s, Endox Endodontic system was developed. This was in the form of high frequency alternating current (HFAC) for root canal disinfection. Per the manufacturer's instructions, the system worked by thermoablation of pulp tissues and elimination of microorganisms present in the root canal. The pulp tissues contain a structure that is sensitive to the electromagnetic fields generated by a high-frequency current. The Endox emitted high-frequency electrical pulses through a probe.[190] The parameters for this system administered pulses that lasted 0.14 seconds and at a frequency of 312.5 kHz with approximately 1100 V. They found that Endox helped with disinfection, but it did not dissolve pulp tissue.[190] In 2012, Yumoto et al also reported that high-frequency electromagnetic waves eradicated bacteria.[16]

In more recent years, an electrical device has been developed and used in Japan for disinfection of the root canal system. This is the J. Morita device, and it was invented by researchers at the International Society for Electro-Magnetic Dentistry (ISEM) and J. Morita Corporation. Dr. Toshihiko Tominaga, working alongside engineer Mr. Kazu Matoba, reported a 95%-99% success rate of root canal therapy with the device in over 300 cases treated.[15, 191]

A study by Tominaga et al., used a split tooth design. This treatment was referred to as Electromagnetic Apical Treatment (EMAT) Procedure. Patients selected had bi-rooted teeth, such as a premolar or a molar, and both roots had periapical lesions. The EMAT device was used to treat one side of the tooth (treatment group), or one of the roots, while the other root (control group) did not receive treatment from the device. A pre-operative periapical radiograph and CBCT images were taken. Each lesion was measured on the periapical radiograph, and on the CBCT, the lesions were measured in 3D form. The root canal therapy was performed in two visits. Placement of intracanal medicament occurred at the first appointment, an obturation was finished at the second visit. The second root, the experimental root, was cleaned and shaped with instruments, irrigated with saline, and received one pulse from the EMAT device into the lesion and 1 pulse at the root apex. This experimental root was operated at the first visit.

Patients returned for follow-up at one, three and six months; a new periapical radiograph was taken at the follow-ups. Teeth that received treatment were divided into two groups; they were divided into the rapid healing group and the slow healing group. In the control group, the periapical lesion had reduced in size on an average of 0.38 mm per month and the volume reduction was 8.12% per month. In the experimental slow healing group, the periapical lesion had reduced in size on an average of 1.12 mm per month and volume reduction was 17.26% per month. In the experimental rapid healing group, the periapical lesion had reduced in size an average of 2.97 mm per month and the volume reduction was 33.33% per month. Overall, the results from this study showed an average time of complete healing of the periapical lesions in the experimental rapid healing group to be approximately 3 months.[15] These results suggested the EMAT procedure is

promising for periapical healing, resulting in shorter healing times after root canal therapy.

The EMAT device by the J. Morita Corporation has been used on bacterial *in-vitro* research studies, studying *E. faecalis* biofilm. The high-frequency electromagnetic impulses were emitted through an insulated K-file. Current was emitted and concentrated at the tip of the file. The settings of this device varied from 500 kHz to 1000 kHz, 70% to 80% duty cycle, and a current of 50 to 150 mA. This device was similar in appearance to the Root ZX electronic apex locator. The device was an electronic apex locator in addition to electromagnetic wave stimulation capability. The results of this study showed that electromagnetic wave stimulation did have an antibiofilm effect on two of the saline groups, without the use of sodium hypochlorite.[18]

USE OF ELECTRIC CURRENT IN THE MEDICAL FIELD

In the 1950s, Fukada and Yasuda found that when bone is stressed to the point of deformation, electrical potentials were generated.[192] Bone is electronegative in the areas of compression, and electropositive in the areas of tension. These electropositive areas produced bone, which was a significant finding, and inspired the idea that endogenous electric fields using electrical stimulation would enhance bone healing and produce bone.[193, 194] Today, there is a $500 million market for bone stimulation in the United States.[195] The use of electromagnetic stimulation to treat fractures is common, but the efficacy of this modality remains uncertain.[195] It has been suggested that the electromagnetic fields regulate growth factor production and proteoglycan and collagen synthesis. It has also been acknowledged that physical stress, such as electromagnetic

waves, supersonic waves, heat, pressure, mechanical loading and electrical stimulation promote osteogenesis and healing in bone fractures.[19, 196] It is believed that electrical stimulation will increase fracture healing rate.[197]

Pulsed electromagnetic field (PEMF) stimulation first gained approval in 1979 by the US Food and Drug Administration.[198] Since the approval of PEMF, many different research articles have studied the effects of PEMF stimulation on nonunion fractures. The success rates range from 68% to 90% for fracture healing.[199] One study looked at a system called Biomet® EBI Bone Healing System (EBI, LLC (a Zimmer Biomet company), Parsippany, NJ, USA). Biomet is a noninvasive PEMF portable device that delivers weak pulsing electrical currents at the fracture site. These currents are generated by a low-energy electromagnetic field consisting of 4.5 ms bursts of pulses, repeating at 15 Hz, with a peak magnetic field of 18 G during each 225 μs pulse.[199]

This study found a statistically significant correlation between average daily use of the Biomet® EBI Bone Healing System and a reduction in the average time to heal for non-union fractures.[199] With each additional hour of daily PEMF treatment, there was an associated 6-day reduction in average time to heal. For patients who used the device for an average of 10 hours per day when compared versus patients who used their device for an average of 1 hour per day, there was a 35%–60% reduction in the healing time.[199] Patients that used PEMF for 9 or more hours per day healed on average 76 days earlier than patients who used PEMF for 3 hours or less per day.[199] Noninvasive pulsed electromagnetic field bone growth stimulators can improve healing times for patients with impaired fracture healing.

MURINE CALVARIA OSTEOGENIC CELLS

When conducting research with cell culture, the two options are primary cells or immortalized cell lines. Cell lines have the advantage over freshly isolated cells with immediate availability of large numbers of cells, the more homogeneous nature of the cell populations in cultures, and the expected stability of the phenotype. However, over time cell lines can appear unstable and subclones of cell lines can develop in different laboratories. The resulting cell line may not be entirely representative due to colonal selection that can favor rapidly growing cells. These rapid growing cells might not express all typical features from a certain tissue. With primary cells, an obvious advantage of these cells more closely mimics the in vivo phenotype and can be isolated from humans and animal models. Therefore, in many cases, the use of primary bone cells may be preferred over the use of cell line in certain experiments.[200]

C57BL/6 mice were originally obtained from Jackson Laboratories for preparation of murine osteoblast-lineage cell culture. C57BL/6 is the most widely used inbred strain and this strain is a general-purpose strain. This mice breed are long-lived, have a low susceptibility to tumors, and was the first strain to have its genome sequenced.[201]

OSTEOBLASTS

The cells responsible for bone formation are osteoblasts. Mesenchymal stem cells (MSC) give rise to chondrocytes, muscle cells, adipocytes, and osteoblasts. Specific activation of transcription factors by morphogenetic and

developmental proteins is needed for the mesenchymal progenitor cells to commit to the osteoblastic lineage. Protein secretion and bone mineralization is the main function of osteoblasts. Once mature osteoblasts complete bone matrix mineralization and formation, osteoblasts can become embedded in the matrix it has secreted; it is then referred to as an osteocyte.[202]

High levels of alkaline phosphatase (ALP) and osteocalcin are associated with mature osteoblasts; the rate of bone formation in vivo is commonly distinguished by the circulating concentrations of these specific proteins in serum. Osteoblasts secrete specialized matrix proteins and type I collagen during the synthesis of bone matrix. Type I collagen and the other proteins forms an osteoid. Hydroxyapatite is then deposited by the osteoblast and the osteoid helps serve as a template for the matrix formation. Integrins interact between the osteoblasts and the bone matrix, and integrins play a role in osteoblast function, survival, and differentiation.[202]

Transcription factors direct osteoblast differentiation and growth, and as a result, proteins are expressed during matrix production and mineralization. The steps include proliferation, maturation, mineralization, and apoptosis; specific transcription factors regulate each stage which leads to osteoblast phenotypic markers. After completion of bone formation, 60-80% of osteoblasts die by apoptosis. Apoptosis is programed cell death and it appears at the early stages of osteoblast differentiation. It also continues throughout the life cycle of the osteoblast. Increases in osteoblasts can be achieved by either progenitor cells

increasing the rate of osteoblast production, by decreasing apoptosis rate of osteoblast death, or a combination of the two.[202]

After osteoblasts finish bone maturation and mineralization, some osteoblasts become flattened bone lining cells. These cells cover the surfaces of bones and can have a protective effect. Bone lining cells can revert back to forming bone matrix in the response to parathyroid hormone. These cells can also be in close contact with osteocytes entrapped in the bone matrix and can communicate through gap junctions. This suggests that bone lining cells can potentially exchange minerals and metabolites on the surface of bones or with bone marrow. Bone lining cells can also play a role in bone remodeling by retracting from the bone surface to create a canopy over osteoclasts and osteoblasts. When the osteocytes sense bone resorption is need, they send signals to bone lining cells to retract from the surface. This canopy creates a *bone remodeling compartment,* which contains bone marrow osteoblast precursors, blood vessels with hematopoietic osteoclast progenitors, osteocytes, and osteoblasts which is separated from the rest of the marrow. These osteoclast progenitors mature into osteoclasts by cytokines, and this initiates bone remodeling. Osteoblast precursor cells then are activated by factors released during bone matrix resorption, and they mature into bone synthesizing osteoblast cells. [202]

CYTOKINES INVOLVED IN OSTEOGENESIS

1) <u>Growth Factors Stored in Bone and Dentin:</u>

Bone contains a number of growth factors and this includes Insulin-like Growth Factors I and II (IGF-I, IGF-II), Transforming Growth Factor Beta (TGF-β1, TGF-β2),

platelet-derived growth factor (PDGF), basic and acidic fibroblast growth factor (FGF), and bone morphogenic proteins (BMPs).[203] IGF-II and TGF-β are the two most abundant growth factors present in human bone matrix, and IGF-I, PDGF, and basic fibroblast growth factor are several fold less abundant.[204] Extracts of human dentin have been shown to be mitogenic to embryonic chick bone cells.[205] This suggests that like human bone, human dentin contains growth factors.[205] Baylink et al. found that dentin extracts contain IGF-1, IGF-II, and TGF-β. These concentrations were similar to that found in human bone.[203] Dentin proteins have also exhibited osteoinductive properties.[206, 207, 208, 209] Extracts of cementum have been shown to be mitogenic to gingival fibroblasts in culture.[210] Growth factors are not only present in bone but also in dentin and cementum.[203]

2) Growth Factors Produced by Bone Cells:

Baylink et al. found that human bone cells in culture produce a number of growth factors. These are known to be stored in human bone and include IGF-I, IGF-11, TGF-β, and PDGF. IGF-II seems to be the most abundant mitogen produced by human bone cells.[203] However, in mouse and rate bone cells, IGF-I is more prevalent that IGF-II. Therefore, the amount of IGF-I and IGF-II appear to be different between rodents and humans. [203, 211]

3) Biological Actions of Bone-Derived Growth Factors in Vitro and in Vivo:

IGF-I and IGF-II have been shown to stimulate proliferation in serum-free cultures of human bone cells derived from iliac crest and calvarial bone cells derived from embryonic chick, fetal mouse, and fetal rat.[211] IGF-I1 has been shown to stimulate the synthesis of type I collagen in untransformed normal human bone cells.[211, 212] IGF-I

and IGF-I1 have been shown to increase bone collagen synthesis and decrease collagen degradation in intact rat calvaria in vitro.[213] It is hypothesized that these responses were from relatively mature osteoblast. However, TGF-β depends on cell type, culture conditions, and cell density. TGF-β has been shown to be a potent mitogen for calvaria cells derived from embryonic chick and newborn mouse osteoblasts, where it inhibited proliferation of MC3T3-EI mouse osteoblasts.[214] In humans, TGF-β appeared to be depended no cell density and dose. TGF-β stimulated cell proliferation at low doses, whereas it inhibited cell proliferation at high doses.[215] TGF-β favors osteoblast proliferation but restricts osteoblast maturation. The mechanism of restricting osteoblast maturation is from repression of gene expression involved in bone formation.[216] TGF-β has been shown to stimulate production of type I collagen in human bone cells.[217] PDGF and basic FGF have been shown to stimulate human bone cell proliferation under certain culture conditions but had no effect on collagen synthesis. The authors concluded that BMPs may act to stimulate bone formation in vivo in skeletal or ectopic sites by stimulating undifferentiated cells to proliferate and differentiate to bone cells.[217]

4) Bone Cells Secrete Other Cytokines in Osteogenesis

Mesenchymal progenitor cells give rise to preosteoblasts, osteoblasts, bone-lining cells and osteocytes.[218-221] Osteoblasts secrete extracellular matrix components such as type I collagen and fibronectin during differentiation. Osteoblasts also secrete signaling molecules, as in transforming growth factor (TGF-β), and then progress and differentiate into mature osteoblasts. Matrix mineralization process is completed by the mature osteoblasts.[218, 222]

USE OF ELECTRIC CURRENT FOR OSTEOBLAST STIMULATION

In vitro studies have reported that direct electrical current enhances extracellular matrix synthesis and proliferation of osteoblasts.[223, 224] Electromagnetic fields with pulses to osteoblasts can exert stimulating effects of proliferation and differentiation. Animal studies and case reports have shown that electrical stimulation can enhance mechanical strength in healing of artificial bone defects or bone fractures and can increase bone calcifications. Electric energies had been also used to stimulate osteoblasts in fractures, osteotomies, and spine fusions in orthopedics. [19, 225, 226]

In 2015, it was demonstrated that high-frequency electromagnetic waves increase osteoblastic cell activity and expression of growth factors via ERK1/2 and p38 MAPK pathways.[17] Electromagnetic waves can efficiently activate some regulators of growth factors, which are important for bone repair and function on osteoblasts. ERK1/2 and p38 MAPK are two types of kinases. MAPKs are serine/threonine kinases that phosphorylate transcription factors and other kinases. These kinases are responsible for proliferation and differentiation, and for other cellular processes.[227]

In 2016, Sato et al studied the effects of high-frequency electromagnetic waves on bone formation. This experiment was completed on rat calvaria defects. Bone defects were created with a diameter of 4.8 mm on the left and right sides of the calvarias of Wistar rats. The active electrode was placed in the left defect (experimental side) and high-frequency electromagnetic stimulus was applied for 1 second and repeated five times. The EMAT device by the J. Morita Corporation

had specification set at 520 kHz, 70% duty cycle, and 1.4 W electric energy. This was completed twice a week for 2 weeks. The right side was not stimulated (control group) and the after 8 weeks, hematoxylin and eosin (H&E) staining was completed on histological specimens. The rate of bone formation was measured. Histological measurement showed that the experimental group had significantly more bone formation when compared with the control group.[19]

ELECTROMAGNETIC FIELD MECHANISM OF ACTION

The exact mechanism by which the electromagnetic fields stimulate osteoblasts is not fully understood.[228] It is believed to work by possibly stimulating the cell membrane of osteoblasts,[229] and may have effects via opening of ion channels, specifically calcium channels[230], and the release of bioactive factors and membrane trafficking.[228] Something that is similar to pulsed electromagnetic fields is the technique of electroporation. Electroporation is the application of an electric field pulse that elicits a change in the cellular membrane to promote the uptake of exogenous macromolecules including DNA, RNA, and proteins.[231] Electroporation is the temporary rearrangement of the cell membrane due to a physical process in which cells are exposed to a high-voltage electric field.[232] The cells become permeable after electroporation and may take up solutes from their surrounding environment; this includes nucleic acids, proteins, carbohydrates and small molecules.[232] The mechanism of electroporation in mammalian cells is poorly understood; one theory suggests that transient leaky structures (pores) in the cellular membrane are caused by rapid administration of an electrical pulse and this allows for

other macromolecules into the cell interior.[231] One device that can be used for electroporation is called the Gene Pulser Xcell- Electroporation System (BioRad).[232, 233]

A few different studies have also shown primary cilia to play a role in transducing pulsed electromagnetic field signaling to cells; this could provide a theory to explain differences in cell responsiveness depending on their differentiation stage and the involvement of signaling pathways that could all be regulated at the receptor level.[228 234-236] Another study reported that intracellular calcium transient is necessary for pulsed electromagnetic fields to affect proliferation and differentiation of obsteoblasts.[230] A final theory on the mechanism of electromagnetic field to stimulate osteoblasts is that electrolytic generation of oxygen or oxygen radicals may increase metabolic activity of osteoblasts, which can stimulate proliferation and differentiation of the cells.[237]

Electromagnetic fields have been used to promote and expedite bone healing and to help heal apical periodontitis. The mechanism of action for electromagnetic stimulation still remains to be determined and additional research is needed to explore this topic. Additional research will help to better understand the mechanism of action of electromagnetic stimulation on osteogenesis and may allow for better clinical procedures in the future.

MATERIALS AND METHODS

MATERIALS AND METHODS

METHODOLODY BACKGROUND

MTS ASSAY

The most direct approach to quantifying cell growth is by directly counting the percentage of live and dead cells using a hemocytometer. Cell proliferation is determined by counting the number of viable osteoblasts at the end of the culture periods. The addition of 0.2% trypan blue dye to the cells can also be included. Cells undergoing apoptosis have impaired cell walls and will uptake the Trypan blue dye. Therefore, the number of live cells (excluding trypan blue) and the number of dead cells (blue) can be determined. The results are expressed as the ratio/percentage of live versus dead cells.[238]

As an alternative, less time-consuming approach to determine cell proliferation is through the use of metabolic activity assays, which is correlated with cell number. An example of such an assay is the MTS CellTiter 96® Aqueous One Solution Cell Proliferation Assay, which is colorimetric method to determine cell viability in proliferation or cytotoxicity assays. According to the Technical Bulletin by Promega, the MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product. This product is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.[239]

MTS measures metabolic and mitochondrial enzyme activity, which is correlated with cell proliferation in many mammalian cell types. Specifically, the assay measures the activity of NAD(P)H-dependent dehydrogenase enzymes which are elevated in

metabolically active cells. The enzymes will cause the reduction of MTS tetrazolium compound and generate the colored formazan product that is soluble in cell culture media. This assay produces a formazan product that has an absorbance maximum at 490 nm in phosphate-buffered saline, which will be measured using a spectrophotometer.[240]

ALKALINE PHOSPHATASE ASSAY

As detailed in the Introduction, alkaline phosphatase (ALP) is the most widely recognized biomarker for the osteoblast phenotype. ALP is an enzyme which catalyzes the hydrolysis of phosphate esters at an alkaline pH. There are three isoenzyme types of ALP and they are tissue non-specific, intestinal, and placental. Bone, liver and kidney are the three isoforms of the tissue non-specific isoenzyme of ALP. These isoforms contain the different carbohydrate moieties on the same polypeptide backbone. Bone ALP shares a common protein structure with liver ALP, but they can be differentiated by thermostability, electrophoretic mobility, chemical inactivation and lectin reactivity. In pathological bone disorder patients with osteoporosis or Paget's disease, these differences help distinguish bone ALP from liver ALP in serum and plasma. ALP was found to be associated with the plasma membrane of osteoblasts, which buds out to form the matrix vesicles seen in developing bone.[241] Changes in alkaline phosphatase level and activity are associated with various disease states in the liver and bone. Elevated levels of ALP in blood are most commonly caused by liver disease or bone disorders.[242] Alkaline phosphatase enzyme catalyzes the hydrolysis of phosphate esters in alkaline buffer and

produces organic radicals and inorganic phosphate. Alkaline phosphatase will remove phosphate groups from nucleotides, proteins, and alkaloids.[243]

Through the research of Sabokbar et al., a protocol for quantitatively measuring ALP in isolated osteoblastic cells was developed.[241] This colorimetric assay measures ALP enzymatic activity in biological samples. At alkaline pH, the ALP enzyme catalyzes the conversion of p-NPP to a yellow colored end-product, p-nitrophenol, which has a maximum absorbance at 405 nm. In the study by Sabokar et al., they found that freeze-thawing the samples twice, or freeze-thawing once or twice followed by sonication yielded the highest enzyme activities. They found that there was no significant difference between the samples that were freeze-thawed (2x) and those which were freeze-thawed (1 or 2x) and sonication, therefore they recommended freeze-thawing the samples twice before measurement of ALP activity. They also found that within 2 days of storage, the activity of the enzyme decreased by 10% from the initial activity but there was no additional loss of activity for the rest of the experimental period. They suggested that short-term storage of samples will affect the activity of the enzyme but that caution should be used when interpreting results after longer periods of storage.[241]

For the ALP assay, a standard curve is generated and is used to compare the change in absorbance with the enzyme activity of ALP.[244] To compare the amount of ALP activity to the number of cells present in each well, ALP activity must be normalized by the total protein amount. For this, a commercial bincinchoninic acid (BCA) protein assay kit[245] from ThermoFisher is used following the manufacturer's specifications. [245] [245] The ALP activity (nM/mL) for each well is divided by the total protein amount (ug) in that well.

MINERAL DEPOSITION BY OSTEOBLASTS

Mineralization assay is an in vitro assay for assessing bone cell mineral deposition. The dye can be extracted from the stained monolayer and assayed.[246] Biological mineralization is a complex process on an extracellular matrix that results in the deposition of poorly crystalline solids of calcium and phosphate, and principally bioapatites. There are two patterns of mineral deposition on an extracellular matrix. These are (i) matrix vesicle-mediated mineral initiation and (ii) heterogeneous nuclear activation of mineral crystals on collagen. This requires involvement of some noncollagenous glycoproteins that bind Ca2+ and collagen. Bone-specific glycoprotein known as bone sialoprotein (BSP) which nucleates hydroxyapatite formation in vitro has gained attention. Standford et al. were able to show that osteoblastic cell line UMR cells rapidly formed an apatitic-like, calcium-phosphate mineral associated with both the cells and their surrounding extracellular matrix.[247] Mineral formation was stimulated by phosphate. Cultures were exposed to a phosphate supplement (β-glycerolphosphate). A positive staining for calcium mineral was observed in cultures exposed to β-GP or inorganic phosphate (Pi) and increased in a linear fashion through the entire culture period. In this study, the authors found that mineral formation was not dependent on the presence of ascorbate addition to the medium.[247] After 96 hours of incubation, they found a plateau of calcium mineral associated with the cell layer. Therefore, when cultures reached a confluent state, the mineral formation had reached a maximum. The mineral deposition process is tightly controlled by phosphate supplements in a dose range (ED50=4-5 mM). Calcium appeared to function in the system by regulating the amount of mineral deposition in the cell layer prior to initiation by phosphate. During the period

of phosphate treatment, the cells formed most of its apatitic mineral as a result of a metabolic production and secretion of competent hydroxyapatite nucleators. Alizarin Red-S assay uses an Alizarin red-S (AR-S) dye which binds selectively to calcium salts. AR-S binds to approximately 2 mol of Ca2+/mol of dye in solution.[247] Alizarin Red S solution is used because it binds selectively to calcium salts (deposits), and it will stain calcium deposits with agitation in vitro.[248]

MATERIALS

Materials required for this project were purchased commercially or prepared as per the methodology below. Cell culture 24-well and 48-well plates (Alkali Scientific Inc. Fort Lauderdale, FL, USA), 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, Grand Island, NY, USA), ALP assay kit (Sigma Aldrich, St. Louis, MO, USA), Alizarin red reagents and Pierce BCA Assay Kit (Thermo Scientific, Waltham, MA, USA) were purchased commercially.

METHODOLOGY

PREPARATION OF MURINE OSTEOBLAST-LINEAGE CELLS

C57BL/6 mice were obtained from Jackson Laboratories. All mice used in this project were handled according to the guidelines of the American Association for Laboratory Animal Science using Institutional Animal Care and Use Committee

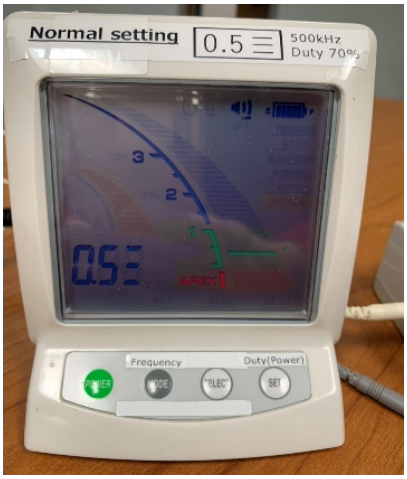
(IACUC) approved protocols (IACUC approval: DS000885R) and in according with the NIH (Guide for the Care and Use of Laboratory Animals, 1996).

Murine calvarial cells were prepared using the previously described protocol.[249, 250] Mouse calvariae are reliable source of osteoblast precursors which are easy to isolate, produce larger quantities of cells and have the capacity to proliferate and can underdo differentiation and mineralization into mature osteoblasts when cultured in osteogenic media. To prepare osteoblasts, calvaria from neonatal mice 2-3 days old were pretreated with 10 mM EDTA in PBS for 30 minutes. Male and female 2-3 day old mouse pups were combined for all preparation of calvarial osteoblasts. Next, the calvaria was subjected to sequential collagenase digestions with 0.1% collagenase type IA (Sigma, MO, USA) from Clostridium histolyticum in serum-free αMEM media (Hyclone, UT, USA) with the antibiotics, 1% (v/v) Penicillin/Streptomycin (P/S, Lonza, NJ, USA) for 30 minutes in each digestion at 37°C under shaking conditions (200 rpm). Cells were collected following incubation in collagenase from fractions 3–5, which consist of about 95% osteoblast-precursors. Calvaria-derived osteoblasts were passaged twice and expanded. Aliquots of cells were then frozen in liquid nitrogen until required, and then thawed and passaged once prior to use. For all experiments, osteoblasts were cultured in MEM plus 10% fetal bovine serum and 1% P/S.

ELECTROMAGNETIC WAVE STIMULATION (EMS)

EMS was generated using EMAT equipment from J. Morita MFG. Corp., Kyoto, Japan (Figure 3A).[16] The EMAT equipment also contained a 'Current Limiting Resistor Box' (Figure 3B). The Current Limiting Resistor Box allowed for current in mA to be changed to three different currents. These values were 10mA, 1mA, or 0.1mA. The EMS device was connected to the current limiting resistor box (Figure 3C). The EMS device set-up was used under the sterile hood during shocking of the cells (Figure 3D).

A



B

C D

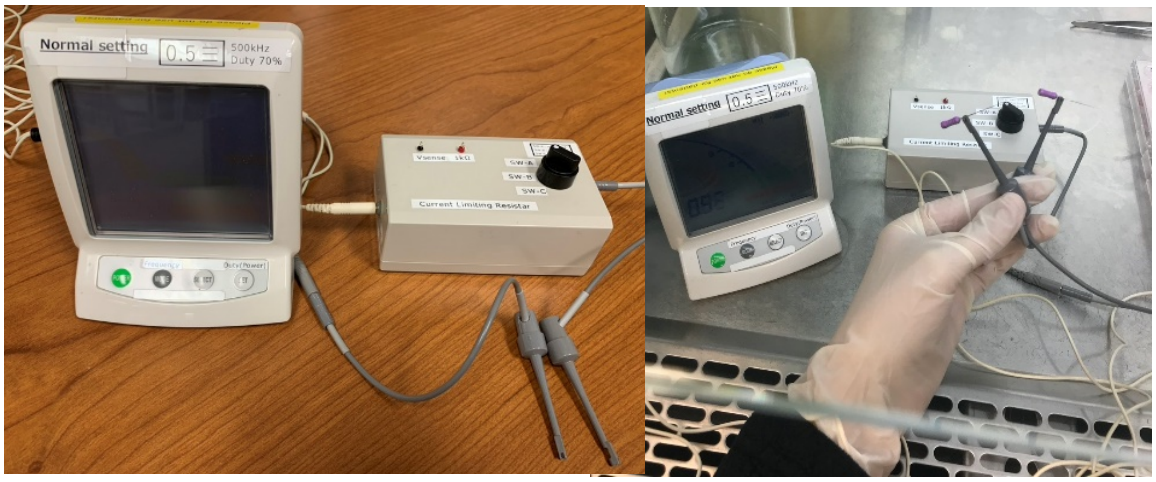


FIGURE 3. EMS Device. A. EMS device with modified J. Morita Electronic Apex Locator. B. Current Limiting Resistor Box; A = 10mA, B = 1mA, C = 0.1mA. C. EMS Device with Current Limiting Resistor Box. D. EMS Device Set-Up; Current Limiting Resistor connected to probes and to EMS device under sterile hood.

The EMS device (J. Morita; Japan) requires a complete circuit in order to generate electromagnetic wave stimulation. In a clinical scenario, a counter electrode is placed over the patient's lip in the form of a shepherd hook. The active electrode is attached to a #10 endodontics hand file (K-file No. 10; MANI, Inc., Utsunomiya, Japan) via a clip. In this *in vitro* experiment, the counter (passive) electrode was attached to an additional #10 endodontics hand file (K-file No. 10; MANI, Inc., Utsunomiya, Japan) which was attached to the current limiting resistor box (Figure 4A). The #10 endodontics hand file was used as an active electrode and was inserted into one well of the plate, into the culture media. The passive electrode was also inserted into the same well of the plate, into the cultured media (Figure 4B). This *in vitro* experiment design replicated the same

approach as in a clinical setting, with an active and passive electrode. Figure 5 is a visual representation of the osteoblast cells in cell culture with the electrodes (Figure 5).

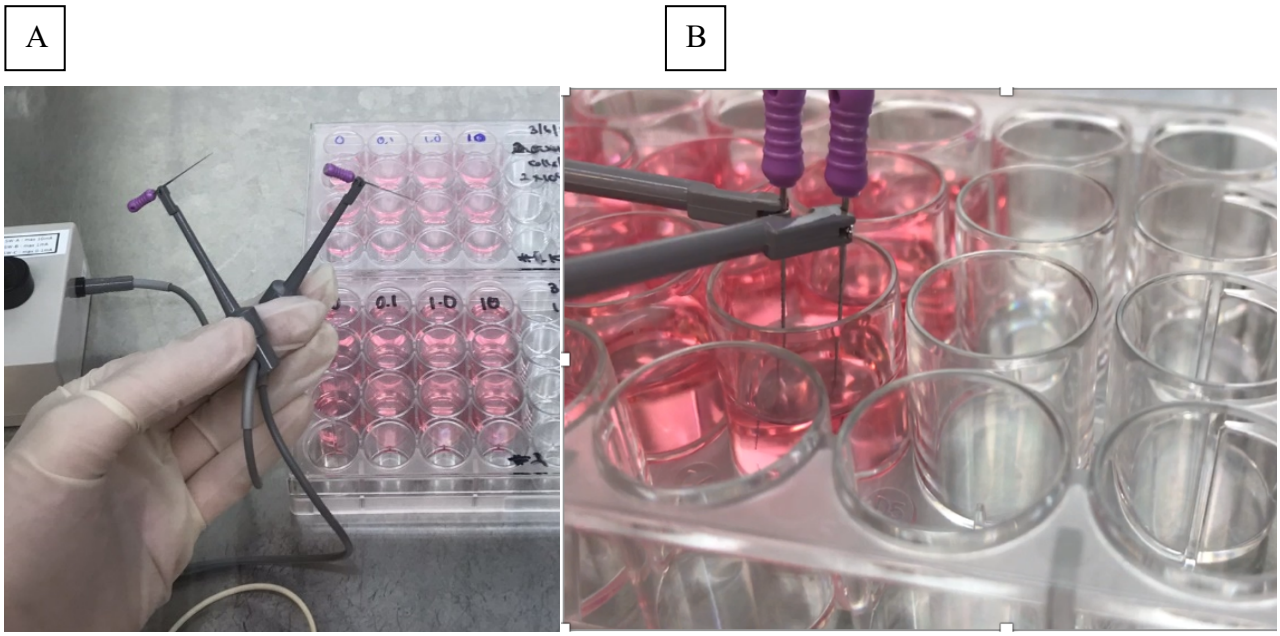


FIGURE 4. Active probe and passive probe with #10 Endodontic K-Files. A. Attached to current limiting resistor box. B. Inserted into the same well of cultured cells and media

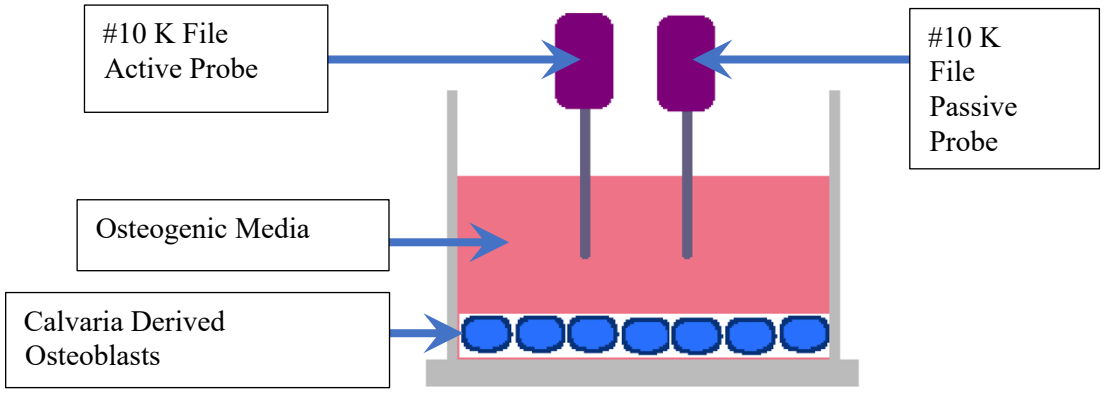


FIGURE 5. Experimental design of EMS on osteoblasts in culture.

The active electrode was a #10 coated endodontic hand file, and the parylene coating served as an insulator. The coating allowed for the electromagnetic burst to only activate the last 2-3 mm of the hand file. The current was passed intermittently at 1 sec intervals for 5 pulses, 1-second burst at the manufacturer's recommended setting of 500 kHz, 70-percent duty. The one-second bursts were controlled by a rheostat, which is pressed each time a burst is desired. The oscillation frequency of EMAT was fixed at 500 kHz by the manufacturer, set at 228 V. We tested the electrical current values of 0.1 mA, 1.0 mA and 10 mA for the EMAT device.

CONTROL AND EXPERIMENTAL GROUPS

Group 1 – no Electromagnetic Wave Stimulation (EMS)

Group 2 – 0.1 mA Electromagnetic Wave Stimulation (EMS)

Group 3 – 1.0 mA Electromagnetic Wave Stimulation (EMS)

Group 4 – 10 mA Electromagnetic Wave Stimulation (EMS)

Group 1 served as a negative control of the Calvaria derived osteoblasts with no electromagnetic wave stimulation. Groups 2-4 serve as the experimental groups, testing the effect of electromagnetic wave stimulation on calvaria derived osteoblasts. Pilot studies were performed with different shock numbers, different number of days, and different number of days of rest. Cells plated were then shocked with five, 1 second pulses, and were treated twice, two days in a row and then allowed to rest for 24, 48 or 72 hours (Figure 6).

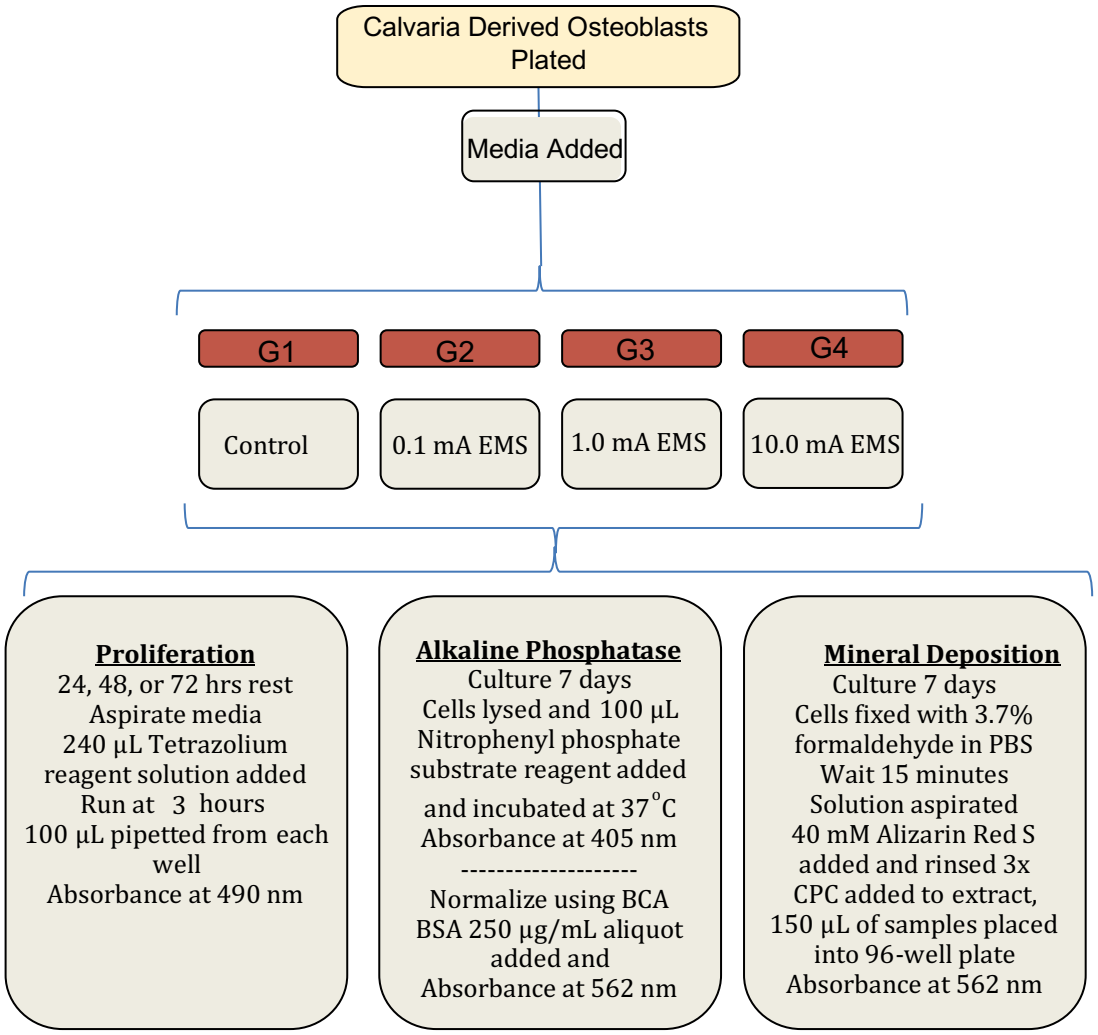


FIGURE 6. Flowchart of experimental design.

PROLIFERATION ASSAY

Murine calvarial osteoblasts, 2.5 x 10^4 cells/well or 1.5 x 10^4 cells/well were cultured in either 24-well plates or 48-well plates for 1 day (Figure 7). Cells were plated in osteogenic media consisting of 500 µL MEM plus 10% fetal bovine serum. This experimental plate was then incubated for 1 day at 37°C/5% CO_2 incubator. On day 2, the cells were shocked with the various currents per that experiment. After the cells rested for either 24, 48, or 72 hours, then the cells were ready for the proliferation assay. A modified MTS Assay kit (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay) was then used, according to the manufactures instructions (Promega), as a measure of the cell proliferation. This assay is able to determine the metabolic activity of the cells.

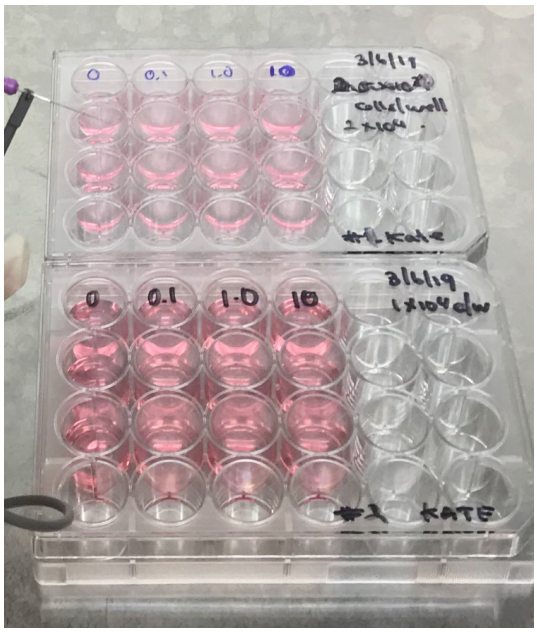


FIGURE 7. Cultured 24-well plate with calvaria derived osteoblasts and experiment set-up ready for EMS pulses.

To prepare for proliferation assay, the media was removed from each well. Proliferation solution was prepared per manufacturer's instructions. 240 µL of MTS reagent solution was then added per well. The cells were then incubated in the 37°C/5% CO_2 incubator for 3 hours. Caution was used throughout the procedure to minimize light exposure as reagents are light-sensitive, and plates remained in incubator when not being manipulated. For reading the plate, 100 µL from each well was pipetted to 96-well plate and absorbance measured at 490 nm by using the spectrophotometer. Figure 8 is a diagramed timeline of the primary osteoblasts from the start of plating the cells to the date of MTS Assay (Figure 8).

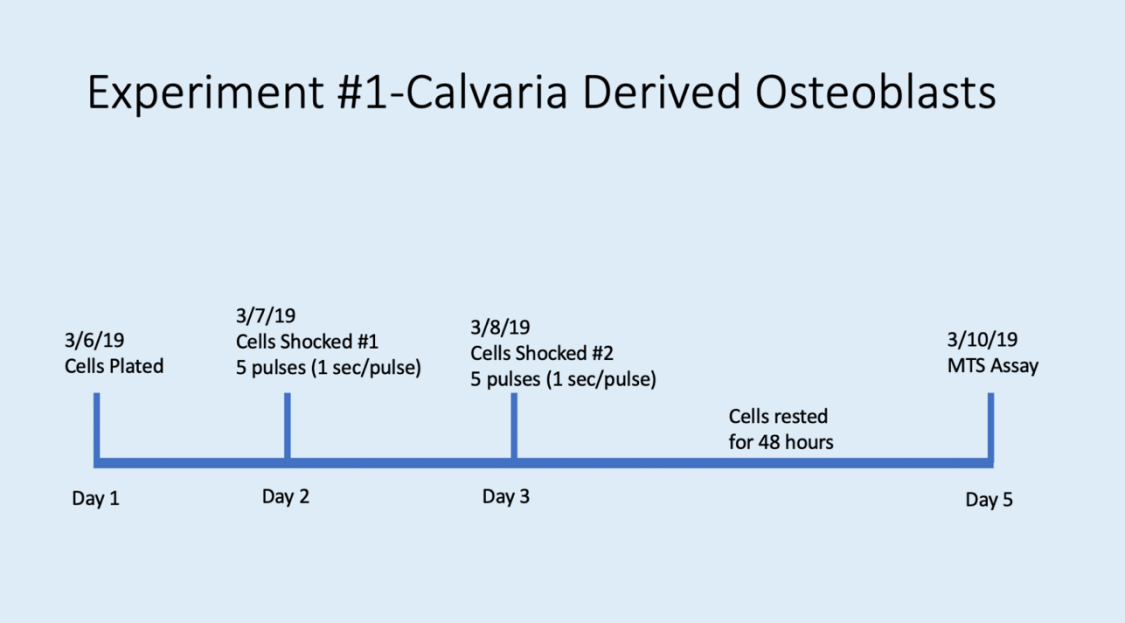


FIGURE 8. Experimental Timeline for Pilot Study Experiment #1

ALKALINE PHOSPHATASE (ALP) ACTIVITY ASSAY

Calvaria-derived osteoblasts were cultured in 24-well plates or 48-well plates at 2.5 x 10^4 or 1.5 x 10^4 cells/well in osteogenic media consisting of MEM plus 10% fetal bovine serum. After cells were treated with shocks for the various experiments, then the plates were prepared to promote differentiation of the cells. For ALP quantification, ascorbic acid (AA) and β-glycerolphosphate (β-GP) were added to the media to promote differentiation of the cells. 50 µM ascorbic acid and 5 mM β-glycerolphosphate (were added to the MEM serum, for up to 14 days to induce osteogenesis). 500 µL MEM culture media with β -GP/AA was placed into the cells on day 3 and on day 6; then lyse buffer was added to the cells. The experimental plates were incubated in the 37^{o}C/5% CO_2 incubator during this time.

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. Then, 40 µL NaOH (10 M) were mixed with 20 mL H_2O for NaOH (20 mM) for 20 mL. Cells were washed twice with ice cold phosphate buffered saline (1X PBS). Cells were lysed in 100 µL of lysis buffer for a 24 well plate and 50 µL of lysis buffer for a 48 well plate. The bottom of the well was scraped with a pipette tip. Cells were carefully placed in a microcentrifuge tube and sonicated for 5 minutes. Microcentrifuge tubes were then spun

on 13.2 x10^3 RPM for 3 minutes. Supernatant was then labeled and stored at -34^{o}C until ALP assay was performed as per the laboratory protocol described below.

According to protocols in a study by Olivos et al, the colorimetric conversion hydrolyzing *p*-nitrophenol phosphate to *p*-nitrophenol was used to determine alkaline phosphatase activity.[251] A standard curve was created using serial dilution. 200 µL of standards in duplicates 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 photosensitivity 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.

ALP activity was normalized by total protein using a Pierce™ BCA protein assay kit. The BCA kit was used 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. Total protein in each lysate was determined based on the BSA standard curve. ALP assay was evaluated and performed in duplicate.

MINERAL DEPOSITION ASSAY

Calvaria-derived osteoblasts were cultured in 24-well plates or 48-well plates at 2.5 x 10^4 or 1.5 x 10^3 cells/well in osteogenic media consisting of MEM plus 10% fetal bovine serum. After cells were treated with shocks for the various experiments, then the plates were prepared to promote mineralization of the cells. For mineralization quantification, ascorbic acid and β-glycerolphosphate were added to the media to promote differentiation of the cells. Media was aspirated from wells ensuring avoidance of cells. 50 μM ascorbic acid and 5 mM β-glycerolphosphate were added to the MEM serum, for up to 14 days to induce osteogenesis. 500 μL MEM culture media with β -GP/AA was placed into the cells on day 3 and on day 6. The experimental plates were incubated in the 37°C/5% CO_2 incubator during this time.

The mineralization plates were stopped at 7 days by fixing the cells with 500 μL of 3.7% formaldehyde in PBS (1:9 of 37% formaldehyde: 1x PBS) added to each well and waiting 15 minutes for cell fixation. The PBS helps to maintain a constant pH. When ready for the assay, the PBS solution was aspirated from wells and rinsed twice with dH_20. This ended with fixed cells in 1 mL of PBS. Separately, Alizarin Red S (40mM, pH 4.2, Sigma) solution, which binds selectively to calcium salts, was used to stain calcium deposits in each well. The Alizarin Red S solution was mixed with a stir bar before adding to the wells. PBS was suctioned off from the fixed mineralization plates and rinsed twice with dH_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 then suctioned off and rinsed with tap H_2O three times. The tap water must be used versus

distilled water because of the higher pH and additional ions and electrons in the tap water.

Tap water was aspirated and washed with 1 mL of PBS to each well for 15 minutes on the agitator. Next, the bound Alizarin Red S was extracted with 1% cetyl pyridinum chloride (Sigma) in 10mM sodium phosphate (pH 7.0). PBS was aspirated and 500 µL of CPC was added to each well to extract Alizarin Red S from the monolayer. Solution was placed on shaker at speed 20 for 15 minutes. A standard curve of solution in 1.7 mL microcentrifuge tubes was then prepared. 150 µL of standards and samples were placed into a 96-well plate in triplicates. Calcium deposition was measured by recording the absorbance of extracted Alizarin Red S at 562 nm using the spectrophotometer. A standard curve of 1-64 µg/mL Ca2+, was prepared based on the known ability of calcium binding to Alizarin Red S (2 mol of Ca2+/mol Alizarin Red S).[252]

SAMPLE SIZE AND STATISTICAL ANALYSIS

Based on previous data collected from this laboratory, a sample size of nine per group (3 replicate experiments, each performed in quadruplicate) was estimated to have 80% power to detect 25%, 50%, and 15% differences in cell proliferation, ALP, and mineralization, respectively. This is assuming two-sided tests each conducted at a 5% significance level and coefficient of variation of 0.15 for cell proliferation, 0.3 for ALP, and 0.1 for mineral deposition. However, due to unforeseen circumstances including technical breakdown, unexpected high number of optimization experiments and the university shut down due to the COVID-19 pandemic, it was not possible to perform the requisite number of individual replicate experiments to satisfy the sample size

calculations. Nevertheless, clear trends were observed, as discussed in the results and discussion. For the completed studies, all experiments were performed at least once, and in triplicate or quadruplicate samples within an experiment. Comparisons between groups for differences in proliferation, alkaline phosphate activity, and mineral deposition were performed using student's t-test. A 5% significance level was used for all tests.

RESULTS

RESULTS

EFFECT OF EMS ON THE PROLIFERATION OF OSTEOBLASTS

Regarding MTS proliferation of calvaria-derived osteoblasts, eight different experiments were performed in which we examined different cell plated number of the primary calvaria derived osteoblasts, different cell plate number and different number of days that the cells were treated with EMS (Table 1). The pulse number of 5 pulses per treatment day did not change for any of the experiments.

Experiment #	**Cell Plate**	**Well Surface Area**	**Cell Number**	**Current mA**	**Pulse Number**	**Pulse Days**
1	24-well	1.9 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	2
2	24-well	1.9 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	3
3	24-well	1.9 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	3
4	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	2
5	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	3
6	48-well	1.1 cm^2	$1.5 x10^3$ cells/well	0, 0.1, 1.0, 10	5	1
7	48-well	1.1 cm^2	$1.5 x10^3$ cells/well	0, 0.1, 1.0, 10	5	2
8	48-well	1.1 cm^2	$1.5 x10^3$ cells/well	0, 0.1, 1.0, 10	5	3

TABLE 1. Experimental Conditions for the MTS Proliferation Assay.

Using Experiment #1 as a representative data graph from the eight experiments (Figure 9), we found that there was a slight decrease in proliferation for the EMS treatment groups compared to the control but there was no significant difference in proliferation from the 0.1, 1.0, and 10 mA EMS groups compared to the control 0 mA group. These results from Experiment #1 were similar for the seven other experiments for MTS Proliferation.

Collectively, when all the data was analyzed together irrespective of plating conditions, and number of pulse days, we found that there was no statistically significant differences noted between the experiment treatment groups of EMS current of 0.1, 1.0, or 10 mA compared to the control group of 0 mA EMS current on calvaria-derived osteoblasts in any of the eight experiments (Figure 9).

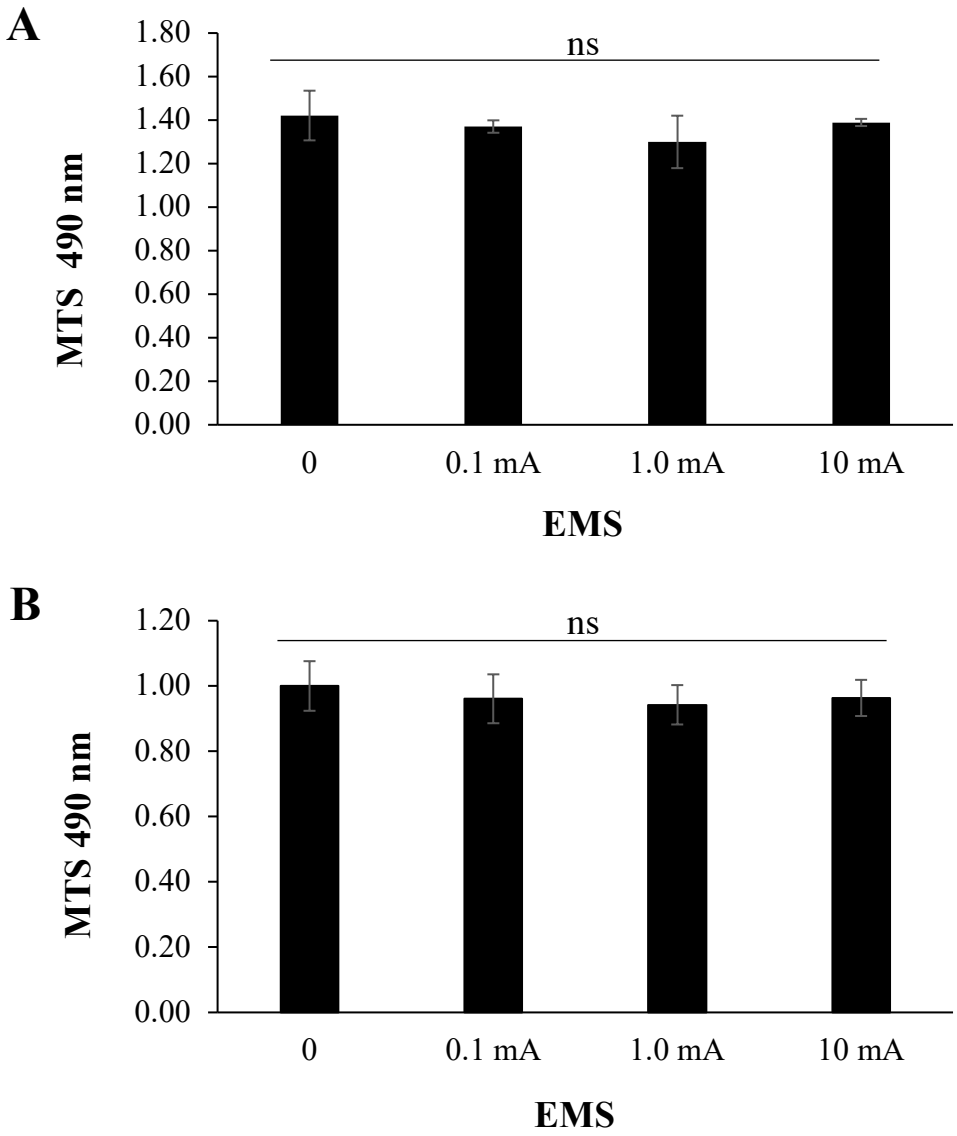


FIGURE 9. **Effect of EMS on Osteoblast Proliferation. A.** Representative experiment showing the effect of EMS (0-10 mA) on osteoblast proliferation as determined by MTS assay. Cells were treated with 5 pulses of EMS for 2 consecutive days, 2 days rest and plated at 2x10^4 cells/well. This is one representative experiment of 8 independent experiments, each performed in triplicate. The mean +/- SD is shown. (ns indicates non-significant data using student's t-test, $p>0.05$). **B.** An average of 8 independent experiments, independent of number of pulse days, days between pulses, and cell plating density. Osteoblasts were treated with EMS at the indicated milliamps followed by MTS assay. Each experiment was performed in triplicate, and the mA was normalized to the control. The data represents the mean ± standard deviation of eight independent experiments.

EFFECT OF EMS ON ALP ACTIVITY OF OSTEOBLASTS

Regarding alkaline phosphatase activity of calvaria-derived osteoblasts, three different experiments were performed in which we examined different cell plated number of the primary calvaria derived osteoblasts, different cell plate number and different number of days that the cells were treat with EMS (Table 2). The pulse number of 5 pulses per treatment day did not change for any of the experiments.

Experiment #	Cell Plate	Well Surface Area	Cell Number	Current mA	Pulse Number	Pulse Days
1	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	3
2	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	2
3	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	2

TABLE 2. Experimental Conditions for the Alkaline Phosphatase Assay.

Using Experiment #2 as a representative data graph from the three experiments (Figure 10), we found that there was a slight increase in ALP activity for the EMS treatment groups compared to the control but there was no significant difference from the 0.1, and 1.0 mA EMS groups compared to the control 0 mA group. However, there was a statistically significant difference between the treatment group of 10 mA EMS current compared to 0 mA current, favoring increased alkaline phosphatase activity.

Collectively, when all the data was analyzed together irrespective of plating conditions, and number of pulse days, we found that there was no statistically significant differences noted between the experiment treatment groups of EMS current of 0.1 and 1.0

mA compared to the control group of 0 mA current on calvaria-derived osteoblast in any of the three experiments. However, there was a statistically significant difference between the treatment group of 10 mA EMS current compared to 0 mA current, favoring increased ALP activity (Figure 10).

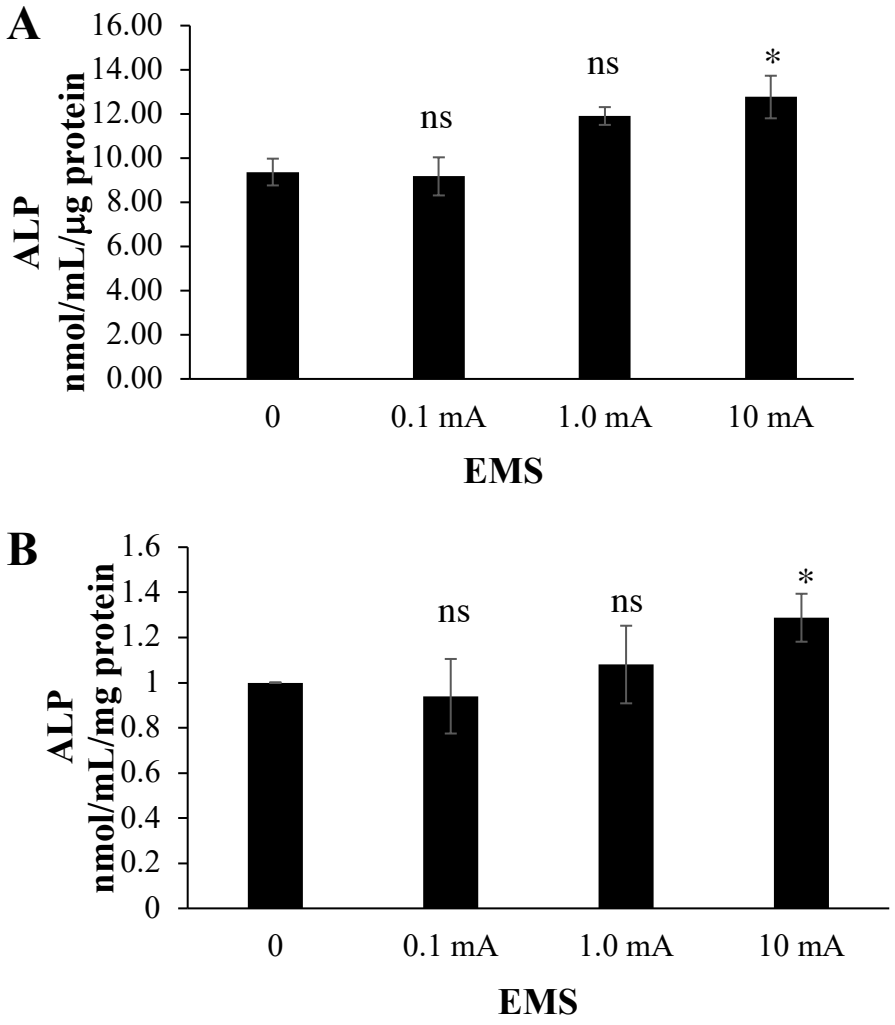


FIGURE 10. **Effect of EMS on Osteoblast Alkaline Phosphatase (ALP). A.** Representative experiment showing the effect of EMS (0-10 mA) on osteoblast ALP as determined by ALP assay. Cells were treated with 5 pulses of EMS for 2 consecutive days, 2 days rest and plated at 2x10^4 cells/well. This is one representative experiment of 3 independent experiments, each performed in triplicate. The mean +/- SD is shown (ns indicates non-significant data and * is significant using student's t-test, $p>0.05$). **B.** An average of 3 independent experiments, independent of number of pulse days, days between pulses, and cell plating density. Osteoblasts were treated with EMS at the indicated milliamps followed by ALP assay. Each experiment was performed in triplicate, and the mA was normalized to the control. The data represents the mean ± standard deviation of eight independent experiments.

EFFECT OF EMS ON MINERAL DEPOSITION BY OSTEOBLASTS

To determine the mineral deposition activity of calvaria-derived osteoblasts, three different experiments were performed in which we examined different cell plated number of the primary calvaria derived osteoblasts, different cell plate number and different number of days that the cells were treat with EMS (Table 3). The pulse number of 5 pulses per treatment day did not change for any of the experiments.

Experiment #	Cell Plate	Well Surface Area	Cell Number	Current mA	Pulse Number	Pulse Days
1	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	2
2	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	3
3	48-well	1.1 cm^2	$2x10^4$ cells/well	0, 0.1, 1.0, 10	5	2

TABLE 3. Experimental Conditions for the Mineral Deposition Assay.

Using Experiment #2 as a representative data graph from the three experiments (Figure 11), we found that there was no significant difference from the 0.1, 1.0, and 10 mA EMS groups compared to the control 0 mA group for mineral deposition activity. These results from Experiment #2 were similar for the two other experiments for mineral deposition activity.

Collectively, when all the data was analyzed together irrespective of plating conditions, and number of pulse days, we found that there was no statistically significant differences noted in mineral deposition between the experiment treatment groups of EMS current of 0.1, 1.0, or 10 mA compared to the control group of 0 mA EMS current on calvaria-derived osteoblasts in any of the three experiments (Figure 11).

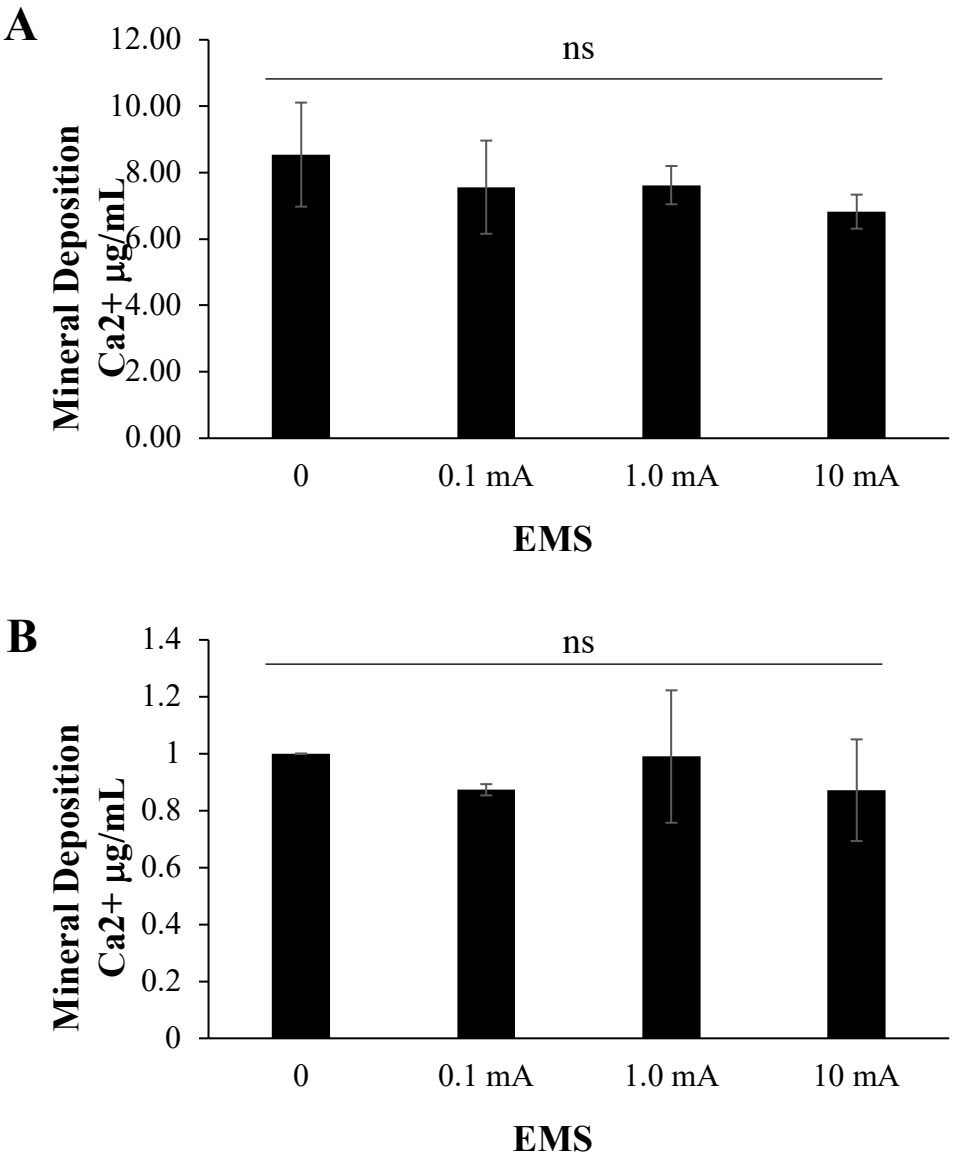


FIGURE 11. **Effect of EMS on Osteoblast Mineral Deposition. A.** Representative experiment showing the effect of EMS (0-10 mA) on osteoblast mineralization deposition as determined by Mineralization assay. Cells were treated with 5 pulses of EMS for 3 days, every other day, 1day rest and plated at 2x10^4 cells/well. This is one representative experiment of 3 independent experiments, each performed in triplicate. The mean +/- SD is shown. (ns indicates non-significant data using student's t-test, $p>0.05$) **B.** An average of 3 independent experiments, independent of number of pulse days, days between pulses, and cell plating density. Osteoblasts were treated with EMS at the indicated milliamps followed by Mineralization assay. Each experiment was performed in triplicate, and the mA was normalized to the control. The data represents the mean ± standard deviation of eight independent experiments.

DISCUSSION

DISCUSSION

The null hypothesis of this study included that primary murine calvaria osteoblast-lineage cells that were treated with EMS will have no effect on proliferation, alkaline phosphatase activity, or mineral deposition of the primary osteoblast-lineage cells. However, before the null hypothesis can be accepted or rejected, it is necessary that the sample size requirements are met. Unfortunately, a key limitation of our studies was that we were unable to replicate specific experiments to reach a sample size of nine, which was based on our sample size calculation. This was due to unexpected technical problems arose during the course of our studies, extensive optimizations experiments that were required, and to interruption of our research which was caused by the COVID-19 pandemic. Additional replicates of current experiments are required in future. Nevertheless, clear trends were observed, and in the case of ALP activity, EMS of 10 mA resulted in a statistically significant increase in ALP activity compared to the control 0 mA was observed using a sample size of N=4 (Figure 10). Therefore, the alternative hypothesis can be partially accepted, specifically for the effect of 10 mA EMS on ALP activity (Figure 10). However, with regards to MTS proliferation and mineral deposition, no statistical significance was observed (Figure 9 and Figure 10).

The diagram below (Figure 12) shows that the matrix maturation phase is characterized by maximal expression of alkaline phosphatase (indicated by AP).[253] Therefore, the increase in ALP activity we observed suggests that at 10mA EMS, the early differentiation and matrix maturation of the osteoblast lineage cells is increased. As can be seen in Figure 12, extracellular matrix mineralization occurs after ALP activity is on the decline. Given we did not observe a statistically significant increase in mineral

deposition, the data suggests that EMS-stimulated osteoblasts are still in the early phases of differentiation. Further, it is possible that extending the post treatment incubation time may be necessary to see the osteoblast become full differentiated and increase matrix mineral deposition.

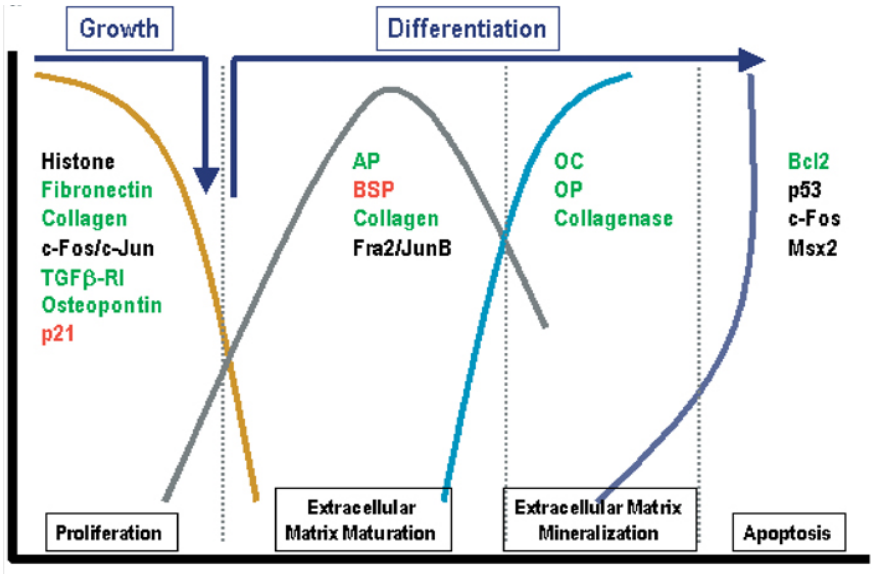


FIGURE 12. Stages of Osteoblast Growth and Differentiation. [253]

We had originally predicted that primary murine calvaria osteoblast-lineage cells treated with EMS will have an increased effect on the proliferation, alkaline phosphatase activity, and mineral deposition of the primary osteoblast-lineage cells. The results of this experiment did not show an increase in proliferation or mineral deposition and a possible reason for the absence of an EMS response could be due to too low of EMS current. The current used in the study was very low at 0.1, 1.0, and 10 mA. The manufacture of J. Morita recommended low currents in order to not cause cytotoxicity of the osteoblast cells. Previous research has shown that higher currents have antimicrobial

effects, causing death of *E. faecalis* biofilms and disinfection.[18] It is possible however, that increasing the EMS currents may result in positive cellular effects. In addition, as discussed above, optimizing the post-treatment duration, may enable matrix mineralization to occur.

We also investigated the effect of EMS on the proliferation of MC3T3-E1 pre-osteoblast cell line, and murine derived bone Marrow derived stem cells (BMSCs), which also showed no significant change in proliferation at 0.1, 1, and 10mA (data not shown). Together with the proliferation data shown using primary murine calvaria osteoblast-lineage cells, these data indicate that EMS may not have a robust effect on cell proliferation. Previous reports have shown an increase in cell proliferation using EMS but those studies were performed using an immortalized cell line, which are able to undergo unlimited proliferation.[17] Therefore, a possibility for the differences in our data, compared to the previous publications is that we used primary murine calvaria-derived osteoblasts to more closely mimic in vivo cellular responses. However, primary cells do not proliferate to the same extent as immortalized cell lines.

Previous research used an *in vivo* rat calvaria model[19] which might have a more beneficial environment compared to the current study of *in vitro* primary cells in culture. Therefore, another possibility why positive responses were not seen in our studies for proliferation and mineral deposition, compared to reports in vivo, is that lack of additional growth factors, supportive cells, or blood supply to the cells in vitro. EMS was also used as Electromagnetic Apical Treatment (EMAT)[191] in humans which showed promising results and this *in vivo* study has more favorable conditions of growth factors,

blood supply, and other favorable variables that can also influence increased healing of apical periodontitis.

The passage number of the calvarial osteoblasts also could have an effect on the findings of this research study. In addition, using mesenchymal stem cells that are not as differentiated into the osteogenic lineage may also have promising effects, especially with respect to their proliferative capacity. Additional research is needed to explore these variables.

Future studies are necessary to recognize the mechanism of the EMS technology before regenerative endodontics. Possible future studies would include examining the effect of EMS at different milliamps on proliferation, ALP activity and mineral deposition of dental pulp stem cells (DPSCs) or stem cells from apical papilla (SCAP), which are relevant for applications in the field of regenerative endodontics. Future studies would also include looking at different primary cells and earlier mesenchymal stem cells lineage which can be a more proliferative cell type.

SUMMARY AND CONCLUSIONS

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In conclusion, no significant difference was found between the experiment groups of 0.1, 1.0 and 10 mA EMS and the 0 mA control group on MTS proliferation and mineral deposition. However, the alternative hypothesis can be accepted for the effect of 10 mA EMS on ALP activity, which was statistically higher than all other groups. The data suggest that EMS has the potential to increase early differentiation of primary murine calvaria-derived osteoblasts, and consequently may have applications to bone regeneration, although additional studies are required to optimize the conditions, reproduce findings and determine the cellular mechanisms involved.

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ABSTRACT

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THE EFFECTS OF ELECTROMAGNETIC WAVE STIMULATION (EMS) ON OSTEOBLAST DIFFERENTIATION AND ACTIVITY

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Introduction: The goal of nonsurgical root canal therapy is to reduce the bacterial load within an infected root canal system, and the subsequent objective is to prevent or treat apical periodontitis. Clinical studies have shown more expedient healing of apical periodontitis treated with electromagnetic wave stimulation (EMS) as compared to apical periodontitis not treated with EMS. Stimulation of osteoblasts and growth factors has been shown when EMS was applied to rat calvaria, resulting in increased bone healing.

Objective: The purpose of this vitro study was to evaluate the effects of EMS on the proliferation and differentiation of osteoblasts. Using primary neonatal calvaria osteoblast-lineage cells, the effects of different EMS regimens on proliferation, alkaline phosphatase (ALP) activity, and mineral deposition were determined.

Materials and Methods: EMS regimen included currents of 0mA, 0.1mA, 1mA, and 10mA delivered for five consecutive 1s pulses per day for one, two, and three days. Cell proliferation was assayed after 1 or 2 days using an MTS assay. Alkaline phosphatase activity and mineral deposition were assayed after culturing the cells in osteogenic media containing ascorbic acid and β-glycerol phosphate for 7 days. Comparisons were performed using analysis of variance, with a 5% significance level.

Results: There was no statistically significant differences noted in MTS proliferation and mineral deposition between the experiment EMS treatment groups of 0.1, 1.0, and 10.0 mA compared to the control group of 0 mA current on calvaria-derived osteoblast. While there were no statistically significant

differences noted in ALP activity in the 0.1, and 1.0 mA EMS groups, compared to 0 mA control, alkaline phosphatase activity was significantly increased in the 10 mA EMS group.

Conclusion: There was no significant differences in MTS proliferation and mineral deposition of the EMS group compared to the control group. However, 10 mA EMS favored increased ALP activity suggesting EMS can promote matrix maturation by osteoblasts. Additional in vitro experimental studies, including different stem cell populations, culture duration and EMS treatment regimens are needed to understand the mechanism of action of EMS for future applications in regenerative endodontics.

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