THE EFFECT OF TRIPLE ANTIBIOTIC PASTE AND EDTA ON THE SURFACE LOSS AND SURFACE ROUGHNESS OF RADICULAR DENTIN

by

Andrew Nerness

Submitted to the Graduate Faculty of the School of Dentistry in partial fulfillment of the requirements for the degree of Master of Science in Dentistry, Indiana University School of Dentistry, June 2014.

Thesis accepted by the faculty of the Department of Endodontics, Indiana University School of Dentistry, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

Ygal Erhlich

Jeffrey Platt

Susan L. Zunt

Kenneth J. Spolnik
Chair of the Research Committee
and Program Director

Date

ACKNOWLEDGMENTS

Thank you Drs. Spolnik, Vail, Ehrlich, Legan, and Bringas for your full-time commitment to my education. Thank you, Dr. Spolnik, for offering me a position in the program, and for being an exceptional program director. Thank you to Drs. Deardorf, Adams, Sahni, Hine, Berman, and Steffel for sacrificing your time to contribute to my education. Thank you, Diane, for your administrative efforts, for keeping us organized and on track, and for your hard work preparing for accreditation. Thank you to Linda, Danielle, Jenny, Steve, and Elaine for assisting me every day in the clinic – you made my life so much easier. Thank you, Dr. Platt and Dr. Hara, for advising me in this project and for allowing me to use your labs. Thank you, Ghaeth Yassen, for all the time and effort you contributed to this research. This project would not have been as successful without your expertise and guidance. And, thank you, my classmates, for being there for me through my residency.

You have all been wonderful mentors, friends, and sources of encouragement and inspiration. Thank you for sharing your experiences and for helping me to develop my skills and prepare for my career. Thanks to you, this residency was a valuable, enjoyable and memorable experience that I will always be grateful for.

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INTRODUCTION

Tissue destruction associated with trauma or disease has been historically managed with limited options such as transplantation or restoration with artificial materials. These options have shortcomings, such as lack of function, donor shortage, host rejection, and failure of integration. In the field of endodontics, it is the diseased or necrotic pulp tissue that is removed and restored with an artificial material. Although this treatment has offered high levels of success,[1] many teeth have a reduced prognosis because of extensive structure loss, fracture, or incomplete development.

Immature teeth that develop pulpal necrosis have a compromised prognosis because the underdeveloped roots are thin and short with an increase risk for root fracture and a poor crown-to-root ratio.[2,3] Furthermore, placing the root canal filling is challenging because it is difficult to prevent material from extending beyond the open apex. Treatment strategies for these teeth have evolved over the last five decades. Apexification has helped to prevent overfill during obturation by utilizing calcium hydroxide or MTA to induce the formation of an apical hard tissue barrier.[4-6] Although these developments made it easier to place the root canal filling, it did not address the underdeveloped tooth structure. Endodontic regeneration procedures present an alternative treatment that enables continued root formation.

Regenerative endodontics can be defined as biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex.[7] In 1971, Nygaard-Ostby and Hjortdal documented the first attempted regenerative cases in necrotic teeth, but the results were unsuccessful.[8]

The current technique, although still under consideration, has had more desirable results with formation of vital tissue in the canal[9-11] and continued root development in both width and length.[12,13] All the essential elements for regeneration are present in this therapy: stem cells, a scaffold, and growth factors.[14] Blood from the apical area contains mesenchymal stem cells of the apical papilla,[15] the blood clot itself serves as a scaffold, and growth factors are released from platelets and dentin.[16] Regenerative endodontic therapy is divided into five phases: canal disinfection, dentin conditioning, induction of bleeding into the canal, coronal seal, and follow-up.[17] Several agents are used for the disinfection and conditioning phases of regenerative endodontic therapy.

Sodium hypochlorite (NaOCl) solution is an powerful alkaline germicide capable of dissolving necrotic tissue.[18] However, it is ineffective against LPS, [19,20], it can be cytotoxic to stem cells, [21] and its cytotoxicity is correlated with decreased stem cell attachment.[22] Therefore it is recommended to use a lower concentration (1.5%) at the first visit and to avoid use at the second visit when stem cells are present in the canal.[17]

Calcium hydroxide is an effective alkaline bactericide capable of LPS hydrolysis.[19,23-25] However, negative effects on dentin include superficial collagen degradation,[26] decreased tooth fracture strength when used over 30 days,[27] and reduced flexural strength of dentin.[28]

Triple antibiotic paste (Tripaste or TAP), a combination of ciprofloxacin, metronidazole and minocycline, was formulated by Hoshino et al.[29,30] and proved to be an effective topical antimicrobial on root canal dentin. However, it has several negative effects which include minocycline-induced tooth discoloration,[31,32] dentin demineralization (pH = 2.9),[26] and stem cell cytotoxicity (LC_{50} = 1mg/mL).[33] The initial

concentration of TAP that was first used was 1000 mg/mL. However, to minimize stem cell cytotoxicity, a reduced concentration of 0.1 mg/mL was recommended.[17] TAP is effective against cultivatable bacteria of endodontic lesions *in vitro* at 0.3mg/mL.[34] In a recent study, 1 mg/ml was found to be efficacious against *E. faecalis* and *P. gingivalis* biofilm.[35]

EDTA is a non-acidic (pH = 7.7) chelating agent that is used to condition the dentin after disinfection. It demineralizes dentin by removing calcium and phosphorus[36] and removes the smear layer thereby exposing dentin tubules[37] and facilitating the release of growth factors from dentin.[38-40] Furthermore, dental pulp stem cells demonstrate "intimate association" with dentin that has been pre-treated with EDTA.[16]

The AAE has provided the following protocol for regenerative therapy. At the first appointment, the canal is irrigated with 20 mL 1.5-percent NaOCl irrigation for 5 minutes, dried with paper points, filled with TAP (0.1 mg/ml) or calcium hydroxide, and temporized with Cavit and IRM for one week to four weeks. At the second appointment, the canal is irrigated with 20 mL 17-percent EDTA, irrigated with sterile saline and dried. Bleeding is induced by over-instrumentation, a collagen membrane is placed 3 mm below the CEJ; MTA is placed 3 mm to 4 mm thick, and the access is restored with glass ionomer.[17]

These agents may affect the surface properties of dentin. The surface properties of dentin can be assessed through the quantification of surface loss and surface roughness. Surface loss is a measure of the quantity of structure lost whereas surface roughness is a measure of the quality of the surface. Profilometry is frequently used to quantify these properties.[41] It has been used to study the effects of toothbrush abrasion,[42] dentifrice

erosion,[43] erosive beverages,[44] endodontic instrumentation,[45-47] bleaching agents,[48-50] the relationship of roughness to bond strengths,[51,52] and the effect of various irrigating solutions and medicaments on dentin and enamel.[36,48,53-56]

CLINICAL SIGNIFICANCE

The degree to which demineralization by EDTA and TAP affect surface loss and surface roughness is unknown. Surface loss may be correlated with reduced tooth strength and surface roughness may be correlated with stem cell attachment. Quantification of these properties will help to identify the ideal concentration and treatment duration of these agents for regenerative endodontic therapy. The ultimate goal of these agents is to adequately disinfect the canal, to achieve a surface roughness that will promote stem cell attachment, and to avoid excess surface loss that might cause tooth damage or inhibit regeneration.

OBJECTIVE

The aim of this *in-vitro* study is to quantitatively investigate the surface loss and surface roughness on human radicular dentin resulting from treatment with TAP or TAP followed by EDTA.

HYPOTHESES

1. Null: There is no significant difference in surface loss between all groups. Alternative: There is a significant difference in surface loss between at least two of the groups.

2. Null: There is no significant difference in surface roughness between all groups. Alternative: There is a significant difference in surface roughness between at least two of the groups.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Ancient Sumerian texts dated as early as 5000 BC described that worms gnawing inside a tooth cause a toothache. This was known as the "tooth worm" theory. It wasn't until 1684 when Anton von Leeuwenhoek microscopically observed microorganisms from tooth samples that this worm theory was debunked. Charles Allen published the first book about dentistry in 1687.[57] Although it did not mention endodontics, it described transplantation.

In the 1700s, endodontic treatment consisted of incomplete treatment of the pulp with various mechanical techniques, the use of a variety of chemicals and medicaments, and obturation limited to the pulp chamber. In 1728 Pierre Fauchard, "the founder of modern dentistry," wrote a book that described endodontic procedures such as mechanical debridement, the use of medicaments, and obturation of the pulp chamber with lead foil.[58] In 1756 Phillip Pfaff described pulp capping using gold or lead which were improvements from previous procedures.[59] In 1757 Bourdet described endodontic therapy by extraction and replantation in order to sever the nerve.[57] In 1766, Robert Woofendale provided the first recorded description of an endodontic procedure in this country using heat cauterization of the pulp and a filling made of cotton. He also described the use of oil of cinnamon, cloves, turpentine, opium, and camphor to relieve pain and treat the pulp.[60] Frederick Hirsch described use of "percussion" as a diagnostic test, treatment of the pulp with a red-hot probe through a cervical access, and filling with lead.[58]

During the years between 1800 and 1850, pulp and periradicular physiology became better understood; the "vitalistic theory," and pulpal anesthesia were introduced; and new instruments for debridement were designed. In 1805 J. B. Gariot introduced the concept of pulp vitality and the ability to retain a non-vital tooth.[61] In 1809 Edward Hudson placed the first fillings, which were made of gold foil, into root canals.[62] In 1819 Charles Bew described the flow of blood into the pulp. In 1807 Eleazar Parmly founded the first dental journal. In 1826 Leonard Koecker challenged the concept that a non-vital tooth could be maintained, and promoted prevention of necrosis with pulp capping procedures that had been previously described by Pfaff.[63] In 1829 SS Fitch introduced the concept of the periodontal membrane and also suggested that the coronal and root vitality were independent of each other. In 1836 Shearjashub Spooner introduced painless pulpal debridement by using arsenic to devitalize the pulp before removal. Unfortunately, this technique also caused severe damage to the periodontium.[58] In 1837 Jacob Linderer and his son, Joseph, recommended essential or narcotic oil to anesthetize the pulp.[59] In 1838 Edwin Maynard developed instruments for canal debridement including the first broach.[64] In 1839 Baker is credited with writing the first complete account of root canal therapy including pulpal debridement, cleaning, and obturation of the canals with gold foil.[58] At this time, the etiology of a toothache was speculated to be nerve exposure, fungus of the nerve (pulp polyp), pus, or periodontitis.

Between 1850 and 1900, new instruments, disinfectants, obturation materials, surgical endodontics, diagnostic tests, and prognostic factors were discovered for existing endodontic therapies. It was also during this time that the septic theory was introduced. In 1850 creosote-soaked wood plugs were used to obturate canals. The sealer used was

Hill's stopping mixed with either chloroform or eucalyptus oil. In 1847 Edwin Truman introduced gutta-percha for obturation.[65] In 1850 Codman described that the goal of pulp capping was to obtain a secondary dentin layer over the pulp.[61] In 1851 Hullihen described the first endodontic surgical procedure with flap reflection, osteotomy, and trephination of the tooth to induce pulpal hemorrhage and to relieve the congested pulp.[60] In 1857 Thomas Rogers identified prognostic factors for pulp capping. In 1858 Jonathan Taft claimed that viable dentin was more resistant to decay, identified indications for pulp capping, and recommended capping with collodion or gutta-percha dissolved in ether or chloroform followed by a gold restoration.[59] In 1864 Barnum introduced the concept of tooth isolation with rubber that evolved into the rubber dam.[66] In 1865 Clarke and Bowman introduced obturation with hot gutta-percha and Bowman invented the rubber dam retainer forceps.[66] In 1867 Magitot introduced electric current for pulpal diagnosis.[67] In 1870 G.V. Black recommend zinc oxychloride as a capping material.[58] In 1873 carbolic acid and iodoform were introduced as pulpal disinfectants.[59] In 1879 the vitalism theory began to transition into the septic theory, in which an infected tooth was the etiology of disease and disinfection was the new treatment priority.[61] In 1895 Bowman introduced chloropercha, which was used along with gutta-percha cones for obturation.

In the early 1900s, local anesthesia was applied and canal length and size determination were developed as procedures. Also, the era included the rise and fall of the focal infection theory. In 1905 Einhorn invented procaine (Novocaine) for local anesthesia.[68] In 1908 Dr. Rhein first described radiographic working length determination using a wire in the canal.[66,67] G.V. Black also recommended determination of working length and apical gauging.[69] In the 1920s, the focal infection theory was introduced,

which claimed that a focus of infection (e.g. within the tooth) would cause other diseases throughout the body.[70] In 1909 E.C. Rosenow explained that streptococci present in diseased organs could spread via the bloodstream to a new location and establish an infection.[70,71] In 1910 William Hunter gave a presentation titled "The Role of Sepsis and Antisepsis in Medicine," which propelled this theory. As a result, endodontic therapy was scrutinized and extraction was recommended for all non-vital or previously endodontically treated teeth. Shockingly, some even recommended extraction of all teeth for prevention of infection.[70,72] Thankfully, in 1930 this theory began to lose its popularity in exchange for more conservative approaches. In 1937 Logan distinguished between the presence of bacteria and infection[73] and Tunnicliff and Hammond identified microorganisms in pulps without disease.[69,71] In 1937 Burket reported 200 arthritis cases that had not resolved with removal of infection foci and concluded that the relation between the foci and arthritis was not causative but rather associative.[69] This ended the focal infection era.

In the mid 1900s, the use of antibiotics was more prevalent. Among practitioners, the American Association of Endodontists (AAE) and the American Board of Endodontics (ABE) advanced the field, and the specialty gained recognition by the ADA. The first examination for board certification was given. In 1941 Fred Adams and Louis Grossman began using antibiotics such as penicillin and sulfanilamide for the treatment of endodontic infection.[69,71] In 1943 the AAE was formed, and in 1956, the ABE was formed.[74] By 1963 endodontics had grown tremendously and was recognized by the American Dental Association as a specialty. In 2009 the AAE had 7000 members with approximately 25 percent having completed board certification.[75]

From the late 1900s and through the turn of the century, there have been many exciting developments in endodontics such as cone beam computed tomography, nickel titanium rotary files, microsurgical instruments,[76] improved magnification and lighting,[77] and better materials such as mineral trioxide aggregate.[78] These developments have permitted more accurate diagnoses,[79,80] more conservative and precise treatments,[76] and ultimately more successful long-term outcomes.[81] It was also during this time that the field of regenerative endodontics emerged. In 2006 the first regenerative endodontics conference was held in Nova Southeastern University.[82] Shortly thereafter, Murray published an article titled "Regenerative Endodontics: A Review of Current Status and a Call for Action," which outlined specific goals to help transition concepts into reality.[82] The AAE formed a standing committee dedicated to integrating regenerative procedures into practice.[82] Between 2001 and 2010, the AAE dedicated one-half million dollars to 29 regenerative projects at 13 different institutions.[83] The 2011 to 2012 ADA Current Dental Terminology included a new code (D3354) for pulpal regeneration within the endodontic section of the code, recognizing that it is an endodontic procedure.[84] In summary, in the last two decades there have been many improvements in endodontics, and the emergence of regenerative endodontics is one of the most exciting.

THEORY OF ENDODONTICS

In 1965, the crucial role of microorganisms in the pathogenesis of pulpal and periapical pathology was demonstrated in the class study by Kakehashi, Stanley, and Fitzgerald.[85] They showed that traumatized tissue in germ-free rats was able to heal, whereas in conventional rats there was inflammation, necrosis, and abscess formation. Moller et. al. in 1981 further demonstrated that infected pulp tissue, and not necrotic

tissue alone, caused periapical inflammation histologically in monkeys.[86] Therefore, the objective of endodontic therapy is to reduce the microbial load and byproducts in order to prevent and to treat pulpal and periapical pathology, and to restore the tooth to prevent microbial reinfection and permit long-term function.[87,88,89] Classic authors such as Stewart,[90] Grossman,[91] and Schilder[87,88] have described important principles for successful endodontic therapy and separated them into three phases: chemomechanical preparation, microbial control, and obturation. However, microbial control must be considered throughout all treatment phases. The phases described more commonly now are instrumentation, irrigation, and obturation.

Instrumentation

The first phase is instrumentation. The canal walls should be enlarged from the original size and enlarged apically to a minimum size 30 to permit access for disinfecting irrigants.[91-93] The original shape of the canal should be maintained.[94] Instruments should not be forced apically beyond the canal space to prevent damage to the periodontium,[91] with the exception of small patency files to enhance apical penetration of irrigants.[88,95] Although instrumentation reduces bacteria by 100 time to 1000 times,[96] between 35 percent and 53 percent of the main canal surface remains un-instrumented.[97-99] Furthermore, there are dentin tubules and extra canals of the root canal system[100] where bacteria penetrate[101] and cannot be reached by instrumentation alone. Therefore, additional disinfection is accomplished with irrigation.

Irrigation

The second phase of endodontic therapy is irrigation. The canal should be continuously irrigated with antiseptic solutions, which should remain in the canal for sufficient time.[102] Sodium hypochlorite (NaOCl) solution is recommended as the primary irrigation solution because it is an effective broad-spectrum antimicrobial and powerful dissolver of organic tissue.[103] It has a high pH of 11 and exerts its antimicrobial activity through the activity of hypochlorous acid, which disrupts oxidative phosphorylation, membrane activities, and DNA synthesis.[104-106] Temperature, exposure time, and concentration of NaOCl are important factors to consider are because they are directly proportional to the degree of tissue dissolution and penetration into dentin tubules.[102,107,108]

One limitation of NaOCl is that it is unable to dissolve the inorganic dentin particles in the "smear layer" that is generated during instrumentation.[109] It has been suggested that the smear layer may block the penetration of NaOCl into the dentin tubules.[110] Ethylenediamene Tetra-Acetic Acid (EDTA) solution addresses this problem because it is an effective chelating agent.[109] Irrigation with 17-percent EDTA for 1 min is capable of removing the smear layer.[111] When NaOCl is applied after irrigation with EDTA, it results in more debris removal than with EDTA alone.[112] A recent systematic review concluded that smear layer removal improves the fluid-tight seal after obturation of root canal system *in vitro*.[113]

Another limitation of NaOCl is that it is ineffective against endotoxin[20] and it lacks substantivity.[114,115] Chlorhexidine gluconate (CHX) is a supplemental irrigation solution that has broad-spectrum antimicrobial activity[116] with sustained antimicrobial

effects for up to 12 weeks.[117] CHX is cationic and exerts its antimicrobial effect by electrostatically binding bacteria and disrupting the cell wall.[118,119] One concern with CHX is the formation of a harmful precipitate when mixed with NaOCl. Initially, it was thought that this precipitate was a toxin called Para-chloroaniline (PCA).[120] However, more recent studies using NMR contradicted this finding instead suggesting that the precipitate was PCU and PCGH.[121] Nevertheless, PCU may be metabolized to the PCA toxin; therefore, formation of the precipitate should be prevented during treatment by flushing the canal between the two solutions.[121]

Obturation

The third phase is obturation. The root canal obturation should have a hermetic seal.[91] Obturation material must not irritate the periapical tissues.[91] Schilder concluded that the warm vertical technique was best for filling the entire root canal system.[86] A recent systematic review concluded that the success rate of obturation is significantly higher when the material is terminated 0 mm to 1mm from the radiographic apex.[122] This was confirmed with an outcome study, which identified that obturation density and length (0 mm to 2mm within the apex) are significant prognostic factors for success.[123] Finally, the use of an obturation sealer is important to provide the best seal.[122]

IMMATURE NECROTIC TEETH

Despite the 97-percent success rates achieved with conventional endodontic therapy,[1] immature teeth that develop pulpal necrosis have not had such great success. Immature necrotic teeth have a compromised prognosis because the thin and short roots increase the risk for cervical root fracture and result in a poor crown-to-root ratio.[2,3]

Obturation is challenging because there is great risk for overextension of material through the open apex.[124] Treatment strategies for these teeth have evolved over the last five decades.

Early Techniques and Apexification

Early obturation techniques involved fabrication of custom fitted gutta-percha cones. However, because the apical canal dimension was wider than mid-root dimension, a good seal could not be obtained.[125] Treatment of these cases with endodontic surgery had many pitfalls. These included fracture of fragile apical walls, compromised seal due to a large bulk of retrofill, and decreased crown-to-root ratio by apicoectomy.[125] In the 1960s, an apical barrier technique was first suggested and is now referred to as apexification.[4] This technique used calcium hydroxide to induce the formation of a calcified barrier across the apex; many successful case reports led to widespread use of this technique.[6] Calcium hydroxide has a high pH and is responsible for antimicrobial activity and causes a low-grade irritation that induces a mineralized apical barrier. However, calcium hydroxide apexification required patient compliance because of the prolonged waiting period for the apical barrier to form.

In the 1970s, others began using new materials that contained tricalcium phosphate.[125] This eventually led to the introduction of mineral trioxide aggregate (MTA) in 1995.[78] Similar to calcium hydroxide, MTA possesses a high pH and induces apical hard tissue formation, but it does so with much greater consistency.[126] MTA is hydrophilic and able to form a better apical seal in the presence of moisture.[127] MTA also serves as an apical stop, so that obturation is possible at the same visit with patient compliance less of a concern. Furthermore, newer dentin bonding techniques have been

shown to increase fracture resistance of these teeth.[128] As a result, MTA apexification has reported high success rates in the short-term. In 2007 Simon et. al. reported 81-percent success at 1 year,[129] and in 2008 Witherspoon et al. reported 93.5-percent success at 1.5 years.[130] In summary, apexification techniques have improved the prognosis for these teeth. However, the long-term prognosis may still be compromised due to the thin walls and short roots. Fortunately, the emergence of regenerative endodontics has provided potentially better outcomes for these teeth.

REGENERATIVE ENDODONTICS

Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.[131] The three requirements for tissue engineering are stem cells, scaffolds, and growth factors.[131] Regeneration refers to the use of tissue engineering principles to regenerate damaged or missing tissue. Regenerative endodontics has been defined as biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex.[7] It is important to note that the nature of the replaced tissue is what distinguishes healing by regeneration from healing by repair. Healing by true or complete regeneration occurs only when the new tissue type is identical to the tissue it is replacing and when the structure and function are completely restored.[132] In contrast, healing by repair occurs when the new tissue type is not identical or there is loss of structure or function.[132]

History

In 1971 Nygaard-Ostby documented the first attempted regenerative cases. Mature vital or necrotic teeth were mechanically debrided, chemically disinfected, and obturated.[8,133] In necrotic teeth 4-percent formaldehyde solution was used for disinfection. Although growth of fibrous connective was observed in previously vital canals, there was no growth observed in the previously necrotic teeth.[8] In 1974 Myers treated infected mature and immature teeth in monkeys. The teeth were disinfected with 5.25-percent NaOCl; the apical constrictions of the mature teeth were enlarged, and bleeding was induced into the canals. After 24 weeks tissue growth had occurred in many of the teeth; however, it was usually accompanied by periapical inflammation and root resorption, perhaps indicating inadequate disinfection or coronal seal. It is worth noting though that immature teeth responded better than the mature teeth; they demonstrated continued root growth and the largest amount of connected tissue in-growth.[134] In 1976 Nevins treated pulpless immature teeth in monkeys with biomechanical debridement followed by collagen-calcium phosphate gel for 12 weeks. Histologic evaluation revealed "revitalization" of the canal with various forms of soft and hard connective tissue including "cementum, bone, and reparative dentin."[135]

In 2001 Iwaya treated an immature necrotic tooth with periapical involvement.[136] There was a concern that mechanical instrumentation would remove potentially remaining apical vital tissue that might aid in revascularization.So the canal was disinfected non-mechanically with 5-percent NaOCl and 3-percent H_2O_2 followed by a combination of metronidazole and ciprofloxacin. After disinfection a layer of calcium hydroxide was placed against the apical tissue and the access was sealed with glass-

ionomer cement followed by adhesive composite resin. At the 30-month follow-up, radiographic exam revealed continued root growth and apical closure. In 2004 Banchs and Trope published a case report that followed a specific protocol for revascularization of immature necrotic teeth.[137] This protocol was based on the healing observed in the avulsed immature tooth that is replanted. It was suggested that if the same environment could be created for the necrotic immature tooth, revascularization should occur. First, the canal was disinfected non-mechanically with 5.25-percent NaOCl, Peridex, a mixture of ciprofloxacin, metronidazole, and minocycline for 26 days, and 5.25-percent NaOCl again. After disinfection, apical tissue was irritated with an explorer to induce bleeding into the canal to the level of the CEJ and left to clot for 15 minutes. Finally, an MTA seal was placed. After 2 years, the patient was asymptomatic, root growth was observed, and the tooth responded positively to the cold test. Subsequently, this protocol was repeated by others and led to many successful case reports with formation of vital tissue in the canal.[9-11,138]

Terminology

Different terms have been proposed to describe regenerative procedures. One of the first terms used was “revascularization.” The term “revascularization” is defined as restoration of vascularity and occurs as a natural physiologic process in all healing, whether by regeneration or repair.[139] For example, revascularization occurs during the healing of a periapical lesion.[139] Furthermore, the new tissue generated in the canal is not always vascular. Therefore, another term “revitalization” has also been proposed instead.[140] However, the term revitalization might also be misinterpreted to mean re-innervation. Most recently, the term “regenerative endodontic procedures” (REPs) has

been used to describe this treatment procedure.[141] This may be the best term to use because it describes the ideal goal of regeneration.

Recent Discoveries

In the past decade, new research has provided more insight into regenerative endodontic procedures (REPs). In 2005 Nakashima and Akamine outlined the three requirements for endodontic regeneration: stem cells, a scaffold, and growth factors.[14] In 2011 Lovelace quantified mesenchymal stem cells in the blood at the apical area and found concentrations that were 600-fold greater than levels in the systemic blood.[15] Banchs and Trope described that the blood clot that serves as a scaffold for the growth of new tissue into the pulp space.[137] Others have had success using platelet rich plasma (PRP) [9,142,143] or platelet rich fibrin (PRF)[144] as a scaffold. Several authors have also identified growth factors that are released from platelets and dentin matrix such as TGF-β and dentin sialoprotein (DSP) that are capable of inducing stem cell proliferation and differentiation.[16,38,39]

In 2009 Bose et al. quantified root development after REPs and found a 25.1-percent increase in width and 14.7-percent increase in length.[13] This development was significantly more than observed for apexification in the same study (width 0.9%, length 0%). In 2012 Jeeruphan et. al. also quantified root development within 6 months postoperatively and found a 28.2-percent increase in width and 14.9-percent increase in length.[12] This development was significantly more than observed for MTA or $Ca(OH)_2$ apexification in the same study (MTA width 0%, length 6.1%; $Ca(OH)_2$ width 1.5% length 0.4%). In 2014, Kahler et. al. reported a case series of 16 consecutive REPs and found resolution of the periapical radiolucency in 90.3 percent and complete apical

closure in 19.4 percent at 18 months.[145] These findings confirm the ability of REPs to promote root growth.

There have been a variety of findings regarding the type of tissue that is formed following REPs. In 2010 Wang et. al. treated immature dog teeth and found three types of new tissue: intracanal cementum (IC), intracanal bone (IB), and other connective tissue. The IC was located on the dentin walls and appeared similar to cellular cementum. The IB was located in the lumen and contained bone-like tissue. Surrounding the IC and IB, a connective tissue similar to periodontal ligament was also present. In 2011 Yamauchi et. al. treated immature dog teeth and found two types of new tissue: dentin-associated mineralized tissue [DAMT]) and bony islands (BI). The DAMT was located near the dentinal wall, was devoid of vasculature, and was less cellular. In contrast, the bony islands were located in the canal lumen, were vascular, contained many cells, and were similar to bone marrow. In 2013 Martin et. al. performed a REP *in vivo* and identified healing with mineralized tissue and fibrous connective tissue, but no pulp-like tissue or odontoblast-like cells were identified. Many cases reported healing without re-innervation as determined by no response to EPT or cold. Based on these findings, these cases are healing by repair rather than regeneration. However, there have also been several cases with findings more suggestive of regeneration. There have been several cases that reported presence of innervation as determined by EPT or cold.[31,136,146-148] In 2012 Shimizu et. al. identified loose pulp-like connective tissue and cells that resembled odontoblasts[149] and Torabinejad and Faras described pulp-like vital connective tissue.[142]

Indications and Outcomes for REPs

REPs so far have been largely reserved for adolescents with necrotic immature teeth with open apices. This is based on findings in trauma research that suggest apical diameters >1mm are more likely to undergo revascularization.[150] However, Laureys et. al. found in beagle dogs that an apical foramen as small as 0.32 mm permitted revascularization and concluded that the size of the apical foramen may not be as important as previously thought.[151] Assessment of outcomes for REPs in immature necrotic teeth depends on the definition of success. The AAE described three goals, in order of importance, for measuring the success of REPs: 1) elimination of symptoms and periradicular healing, 2) continued root growth, and 3) positive response to vitality testing.[17] However, the level of evidence for the outcome of REPs is low because current research is limited to case reports and case series.

Disinfection for REPs

Kakehashi et. al. demonstrated that healing occurs only in germ-free rats.[85] In 2007 Thibodeau et al. performed REPs on immature necrotic dog teeth and confirmed histologically that vital tissue only formed in teeth that were first disinfected.[152] The most common disinfection strategy combines irrigation with NaOCl followed by an intracanal medicament with either calcium hydroxide or antibiotic pastes.[138]

Although NaOCl solution is an powerful antimicrobial, it has several disadvantages in the context of REPs.[18] First, NaOCl has a concentration-dependent cytotoxicity on stem cells.[21,153] Secondly, this cytotoxicity has been correlated with decreased stem cell attachment.[22] Lastly, NaOCl has also been shown to reduce the modulus of elasticity and flexural strength of dentine at 3 percent and 5 percent

concentrations. Therefore, it is recommended to use a lower 1.5-percent concentration during the disinfection phase and to avoid use during the induction of bleeding and stem cells phase.[17]

Calcium hydroxide ($Ca(OH)_2$) is an effective alkaline bactericide capable of LPS hydrolysis.[19,23-25] Calcium hydroxide has also been found to be conducive to SCAP survival[36] and even significantly increase the proliferation of SCAPs at a concentration of 1 mg/mL.[33,154] However, $Ca(OH)_2$ also has disadvantages in the context of REPs. Andreasen et. al. found that a four-week application decreased tooth fracture strength.[27] Grigoratos et. al. found that a one-week application reduced the flexural strength of dentine. Yassen et. al. found that three-month application caused a significant increase in microhardness and reduction in root fracture resistance in extracted teeth.[155] Yassen et. al. also found that a one-week, two-week, or four-week application caused superficial collagen degradation.[26] Lastly, it was found to be less effective than triple antibiotic paste against *E. faecalis* and *P. gingivalis* biofilm.[35]

Triple antibiotic paste (Tripaste or TAP) contains a combination of ciprofloxacin, metronidazole and minocycline and was first formulated by Hoshino et. al.[29] Metronidazole is broad spectrum, bactericidal, and is effective against obligate anaerobes present in necrotic pulp. TAP was found to be significantly more effective than $Ca(OH)_2$ against *E. faecalis* and *P. gingivalis* biofilm.[35] TAP has also been shown to be effective against cultivatable bacteria of endodontic lesions *in vitro* at 0.3mg/mL.[34] However, TAP has several disadvantages in the context of REPs including discoloration, demineralization, and cytotoxicity.

Minocycline, one component of TAP, has been shown to cause discoloration in several case reports.[31,32] Minocycline binds calcium ions via chelation, forms an insoluble complex, and remains incorporated in the tooth matrix.[156] The chelating effect combined with the extremely low pH (2.9) also causes demineralization.[26] Adverse drug reactions have been observed from topical application of these antibiotics outside of dentistry.[157-159] Stem cell cytotoxicity is also a concern with TAP. The initial concentration of TAP that was first used was 1000 mg/mL. However, in 2012, Ruparel et al. found *in vitro* that direct exposure of 1 mg/mL TAP to SCAPs caused the death of 50 percent of the cells (LC_{50}).[33] Therefore, to minimize stem cell cytotoxicity, a reduced concentration of 0.1 mg/mL is now recommended.[17] Supplemental disinfection with 2-percent chlorhexidine has been shown to cause severe cytotoxicity to SCAPs and is therefore contraindicated.[153]

Dentin Conditioning for REPs

EDTA is a non-acidic (pH = 7.7) chelating agent that demineralizes dentin by removing calcium and phosphorus ions from dentin.[36,109] EDTA is thought to improve the environment for regeneration by several mechanisms. Irrigation with 17-percent EDTA is capable of removing the smear layer thereby exposing dentin tubules.[37,111] Exposure of dentin tubules has been shown to facilitate the release of growth factors from dentin.[38-40] EDTA has also been shown to increase dentin surface roughness, which may be associated with increased adherence by stem cells to dentin.[56] Dental pulp stem cells have also been shown to demonstrate “intimate association” with dentin that has been pre-treated with EDTA.[16] Finally, EDTA has been shown to partially reverse the cytotoxic effects of NaOCl thus permitting increased survival of SCAPs.[21] Although EDTA

provides several benefits for REPs, it has also been shown to cause severe peritubular and intertubular dentinal erosion when applied for 10 minutes as observed with SEM.[111]

Recommended Guidelines for REPs

A specific protocol for REPs was published by the AAE and can be divided into five phases: canal disinfection, dentin conditioning, induction of bleeding into the canal, coronal seal, and follow-up.[17] At the first appointment, the canal is irrigated with 20-mL 1.5-percent NaOCl irrigation for 5 minutes, dried with paper points, filled with TAP (0.1 mg/ml) or calcium hydroxide, and temporized with Cavit and IRM for one week to four weeks. At the second appointment, the canal is irrigated with 20-mL 17-percent EDTA, irrigated with sterile saline, and dried. Bleeding is induced by over-instrumentation of apical tissue, a collagen membrane is placed 3 mm below the CEJ, MTA is placed 3 mm to 4 mm thick, and the access is restored with glass ionomer.

SURFACE PROPERTIES AND PROFILOMETRY

Surface loss is a measure of bulk quantity of structure that is lost from a surface after being affected by some factor.[41] Surface roughness, on the other hand, is a measure of the quality of the surface and can be quantified in a variety of different ways.[41]

The most common method of quantifying roughness in dental research is with average surface roughness (Ra), which is quantified by taking the arithmetic mean of all deviations of the profile from the mean line.[41] This method gives a gross representation of how smooth or rough the surface is; however, it does not represent more detailed textural characteristics (maximums, minimums, amplitude, ratio of peaks to valleys) that

can be used to predict other factors such as wear-resistance or surface absorbance of fluids.[41]

Profilometry is commonly used to investigate the surface profile of dental hard tissues by quantifying surface loss and surface roughness.[41] It has been used to study the effects of toothbrush abrasion,[42] dentifrice erosion,[43] erosive beverages,[44] endodontic instrumentation,[45-47] bleaching agents,[48-50] the relationship of roughness to bond strengths,[51,52] and the effect of various irrigating solutions and medicaments on dentin and enamel.[36,48,53-56]

The two most common methods of profilometry are contact stylus profilometry and non-contact optical profilometry.[41] The contact type utilizes a diamond stylus that is moved laterally across the sample. It is a more established and commonly used technique and provides a high resolution. The non-contact optical type utilizes a chromatic optical sensor that does not contact the sample. One advantage of this technique is that the non-contact nature of the measurement protects the sample and makes analysis of the surface more reliable. However, this technology is somewhat new and protocols for use have yet to be standardized.

MATERIALS AND METHODS

STUDY DESIGN

In this *in-vitro* study, human radicular dentin specimens were prepared and randomly divided into six groups for treatment (Figure 1). The samples were quantitatively analyzed for surface loss and surface roughness using profilometry.

1. Group 1: No treatment (negative control).
2. Group 2: 17-percent EDTA for 5 minutes (negative and positive control).
3. Group 3: TAP 1 mg/mL for 4 weeks.
4. Group 4: TAP 1 mg/mL for 4 weeks followed by 17-percent EDTA for 5 minutes.
5. Group 5: TAP 1000 mg/mL for 4 weeks.
6. Group 6: TAP 1000 mg/mL for 4 weeks followed by 17-percent EDTA for 5 minutes.

Sample Size

Based on the results of a pilot study, a sample size of 20 specimens per group was selected because it would provide an 80% power to detect a 15μm difference in surface loss and a 0.275μm difference in surface roughness. All sample size calculations assumed two-sided tests each conducted at a 5% significance level.

Inclusion/Exclusion Criteria

Extracted human anterior teeth were collected with IRB approval (Study #: 1212010183) and stored in 0.1-percent thymol at 4°C. Teeth were included if they had a

single root, complete root formation, and minimum diameter of 4.0-mm midroot in either buccolingual or mesiodistal direction. Teeth were excluded if they had caries, restorations, hypocalcification, hypoplasia, or cracks. After preparation of the specimens, they were re-examined and excluded if any surface defects or cracks were detected.

Specimen Preparation

Teeth that met the inclusion/exclusion criteria were prepared into specimens (Figure 2). Teeth were removed from 0.1-percent thymol and rinsed in deionized (DI) water for 10 seconds. For each tooth, the root tip was removed; the root was sectioned buccolingually, and the tooth was decoronated with a saw (Lapcraft, L'il Trimmer) with water irrigation (Figure 3 and Figure 4). The inside of each half-root was flattened using a course vertical polishing wheel without water until the root canal concavity was no longer visible and the surface was smooth to an explorer. Each half-root was secured to a thick acrylic plate with sticky wax with the dentin side facing up and so that it was visually level. Each half-root was cut into a 4 mm x4 mm square with a double-bladed saw (Isomet, Buehler) with water irrigation (**Error! Reference source not found.**Figure 5, Figure 6). Specimens were removed from the acrylic plate and cleaned of sticky wax.

Specimens were embedded in acrylic resin (Buehler, VariDur) using a 12x12x8 rubber mold (Figure 7, Figure 8) A small dot of Vasoline was applied to the center of the mold floor. The specimen was placed onto the mold floor with the flattened dentin surface face down onto the Vasoline. The Vasoline was used to keep the specimen in place. Acrylic resin was prepared by mixing 2 parts Varidur powder with 1 part Varidur liquid for 20 seconds using a metal spatula; no more than 9 mL was prepared at any given time. The resin was poured into the mold at the corner to prevent air pocket formation

and left to set for 10 minutes. The acrylic specimen blocks were removed from the mold and placed in DI water for 2 minutes to dissipate heat.

First, the bottom of the specimen blocks were flattened. The specimen blocks were secured with sticky wax onto Struer's mounting cylinders, three per mounting cylinder, with the specimens face down against the cylinder (Figure 9). The back surface of the specimen blocks on the cylinder were flattened manually by hand on the RotoPol 31 using 500-grit paper at 300 rpm with water until level (Figure 10). Six cylinders of similar height, each with three specimen blocks, were placed into the cylinder holder. The back surfaces were further flatted automatically with the Rotoforce-4 on the RotoPol 31 using 500-grit paper at 300 rpm with water for 10 seconds or longer until the back of the blocks were visibly flat and smooth. The specimen blocks were removed from each cylinder, air-dried, and the sticky wax was removed. Each group of three specimen blocks was kept with its respective cylinder.

Next, the specimen side of the blocks was polished. The specimen blocks were reattached to the cylinders, but with the specimen facing up. Six cylinders with attached specimen blocks of equal height were polished automatically using the RotoPol 31/Rotoforce-4. The specimens were polished using 500-grit, 1200-grit, 2400-grit, and 4000-grit paper for 30 seconds, 40 seconds, 50 seconds, and 60 seconds respectively at 300 rpm with water. For each grit paper, specimens were ground until the surface of each specimen was uniformly polished. Sandpaper grit was removed from the specimens by submerging them in a flowing DI water bath for 3 minutes, ultrasonic DI water bath for 3 minutes, flowing DI water bath for 3 minutes again. The specimens were air-dried. A final polish of the specimens was accomplished on the RotoPol/31/Rotoforce-4 using a

polishing pad with a 1-µm diamond suspension (Struers, Inc.) for 3 minutes at 150 rpm without water. The diamond polishing suspension was removed by placing the specimens in a flowing DI water bath for 3 minutes, ultrasonic 2-percent micro rinse solution bath for 3 minutes, and a flowing DI water bath for 3 minutes again. The specimens were air-dried. Sticky wax was removed from the specimens. To confirm that the samples were adequately polished, all samples were visually inspected, and three samples were randomly selected. Roughness was measured using a stylus profilometer (Surtronic 3+, Taylor Hobson).

Additional Exclusion Criteria

Specimens were re-examined and 40 specimens were excluded because surface defects or cracks were present. This left 120 usable specimens for the study.

Taping

Two pieces of adhesive unplasticized polyvinyl chloride tape were placed over each side of the specimen leaving a central exposed area of approximately 4 mm x2 mm (Figure 11). The covered areas served as a control for the central exposed area. The tape was placed perpendicular to the long axis of the canal to control for variations between inner and outer dentin. On the side of each acrylic block, a specimen number was written from 1 to 120. The specimens were placed specimen side up in a covered container lined with paper towels moistened with 0.1-percent thymol and placed into storage for 1 week at 4°C until treatment.

Cap Fabrication

Caps were fabricated for the specimens in group 5 and group 6 to prevent condensation of the roof of the storage containers from dripping down onto the specimens and washing away the TAP (Figure 12). A dome of sticky wax was placed over acrylic block to create space for the TAP. Using a vacuum suck-down machine, soft bleaching tray material was sucked down over the block/wax and trimmed leaving a 2-mm overhang.

Random Assignment of Specimens to Groups and Blinding of Operator to Groups

Specimens were randomly assigned to each of six treatment groups using the random number generator function in Microsoft Excel. Each specimen was labeled with a random number during analysis to blind the user from the treatment groups.

Treatment

Triple antibiotic mixture consisting of a 1:1:1 ratio of ciprofloxacin, metronidazole, and minocycline, was purchased as a 498-mg capsule from Champs Pharmacy, San Antonio, TX. The triple antibiotic powder was mixed with distilled water at two different concentrations: a 1000 mg/mL paste, because this is what has been used clinically, and a 1mg/mL solution, because it has been shown to have reduced cytotoxicity to stem cells while still providing effective disinfection.

Gasket-sealed plastic containers with a small air vent were used to contain the samples during incubation to prevent dehydration. Group 1 and 2 specimens were submerged into 200 mL DI water (Figure 16). Group 3 and 4 specimens were submerged into 200 mL of 1 mg/mL TAP solution (Figure 15). Group 5 and 6 specimens were

treated with a 0.2 mL droplet of 1000 mg/mL TAP, were covered with a cap, and were placed in containers lined by paper towels saturated with 20 mL of DI water (Figure 14). All specimens were incubated for 4 weeks at 37°C with 100-percent humidity (Figure 17). A four-week duration was selected because this is within the recommended AAE guidelines and has produced successful results in many case reports. After incubation, the specimens were removed from the incubator. Specimens were rinsed with DI water for 3 minutes, submerged in an ultrasonic DI water bath for 3 minutes, and rinsed again with DI water for 3 minutes.

After incubation, specimens in group 2, 4 and 6 were submerged into 6 mL of 17-percent EDTA (Champs Pharmacy, San Antonio, TX) for 5 minutes during which a magnetic stirring rod (VWR) was activated at a speed setting of 3 (Figure 18). Group 2 specimens served as a positive control to confirm the effect of EDTA alone, and a negative control for groups 4 and 6. The tape was removed from all specimens. The specimens were placed face up in a covered container lined with paper towels moistened with distilled water and placed into storage for 1 day at 4°C until analysis.

Surface Loss Quantification

Specimens were scanned with the non-contact profilometer and S5/03 optical sensor (Proscan 2000, Scantron Industrial Products Ltd). All specimens were left uncovered to dry for 10 minutes prior to analysis to minimize potential differences associated with moisture content during scanning. A photo was taken of each specimen prior to measurement. A 2.5 mm x 1.0mm area was scanned over the center of each specimen across both the treated and untreated areas (Figure 19). The scan parameters were set to a resolution of 0.01 mm (X-axis) by 0.1 mm (Y-axis) and sample rate of

100Hz. Specimens were analyzed for surface loss using the Proscan 2000 software. Artifacts of missing data were filled using the auto-fill function. The 3-Point Step Height function was used to measure surface loss. Three regions measuring 0.6 mm x 1.0 mm were selected for analysis (Figure 20). Two regions were selected from the end of each untreated area and one was selected from the center of the treated area. The software subtracted the height of the treated area from the average of the two heights of the untreated areas (Figure 21). This result was recorded as the surface loss (μm). An image of the surface profile was captured and saved (Figure 22).

Surface Roughness Quantification

Specimens were scanned with the Taylor Hobson Surtronic 3+ contact profilometer (**Error! Reference source not found.**). All specimens were left uncovered to dry for 10 minutes prior to analysis to minimize potential differences associated with moisture content during scanning. Three 2.5-mm lines were scanned along the exposed area of each specimen. Specimens were analyzed for surface roughness using the Taylor Hobson profile analysis software. Data were leveled using the automatic leveling function and the surface roughness (Ra, μm) was obtained. The mean of the three measurements for each sample was calculated.

Statistical Methods

Summary statistics (mean, standard deviation, standard error, minimum, maximum) were calculated for each study outcome by group. One-way ANOVA was used to test the effects of TAP and EDTA on surface loss and surface roughness. Pair-

wise comparisons between groups were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5 percent.

RESULTS

Surface Loss Results

The null hypothesis that there is no significant difference in surface loss between all groups was rejected. The one-way ANOVA showed a significant effect of treatment type on surface loss ($p < 0.0001$). Mean surface loss (μm) results for all groups are listed in Figure 31 and Figure 32. Representative images of dentin surface loss for all treatment groups are shown in Figure 25 to Figure 30. TAP without EDTA caused a concentration-dependent increase in surface loss that was significant between G1, G3 and G5 ($P < 0.0001$, Figure 33). Surface loss for G1, G3, and G5 was 0.95μm ± 0.51μm, 7.55μm ± 2.72μm, and 57.95μm ± 17.33μm, respectively. TAP with EDTA caused a similar concentration-dependent increase in surface loss that was significant between G2, G4, and G6 ($P < 0.0001$, Figure 34). Surface loss for G2, G4 and G6 was 1.81μm ± 1.08μm, 6.76μm ± 2.20μm, and 67.59μm ± 17.32μm, respectively. Compared to control, addition of EDTA caused an increase in surface loss that was significant between G1 and G2 ($P = 0.002$, Figure 35). However, addition of EDTA to samples previously treated with TAP did not crease a significant difference in surface loss between G3 and G4 ($P = 0.3169$, Figure 36), or G5 and G6 ($P = 0.0712$, Figure 37).

Surface Roughness Results

The null hypothesis that there is no significant difference in surface roughness between all groups was rejected. The one-way ANOVA showed a significant effect of treatment type on surface roughness ($p < 0.0001$). Mean surface roughness (Ra) results for all groups are listed in Figure 38 and Figure 39. TAP without EDTA caused a

concentration-dependent increase in surface roughness (Figure 41). There was a significant difference between G1 and G5 ($P < 0.0001$) and between G3 and G5 ($P < 0.0001$) but not between G1 and G3 ($P = 0.1187$). Surface roughness for G1, G3, and G5 was 0.24µm ± 0.045µm, 0.29µm ± 0.134µm, and 0.68µm ± 0.204µm, respectively. TAP with EDTA caused a similar concentration-dependent increase in surface roughness (Figure 44). There was a significant difference between G2 and G6 ($P < 0.0001$) and between G4 and G6 ($P < 0.0001$) but not between G2 and G4 ($P = 0.0305$). Surface roughness for G2, G4 and G6 was 0.24µm ± 0.051µm, 0.27µm ± 0.042µm, and 0.70µm ± 0.21µm, respectively. EDTA did not create a significant difference in surface roughness between the G1 and G2 ($P = 0.6993$, Figure 42), G3 and G4 ($P = 0.4823$, Figure 43), or G5 and G6 ($P = 0.7226$, Figure 44).

FIGURES

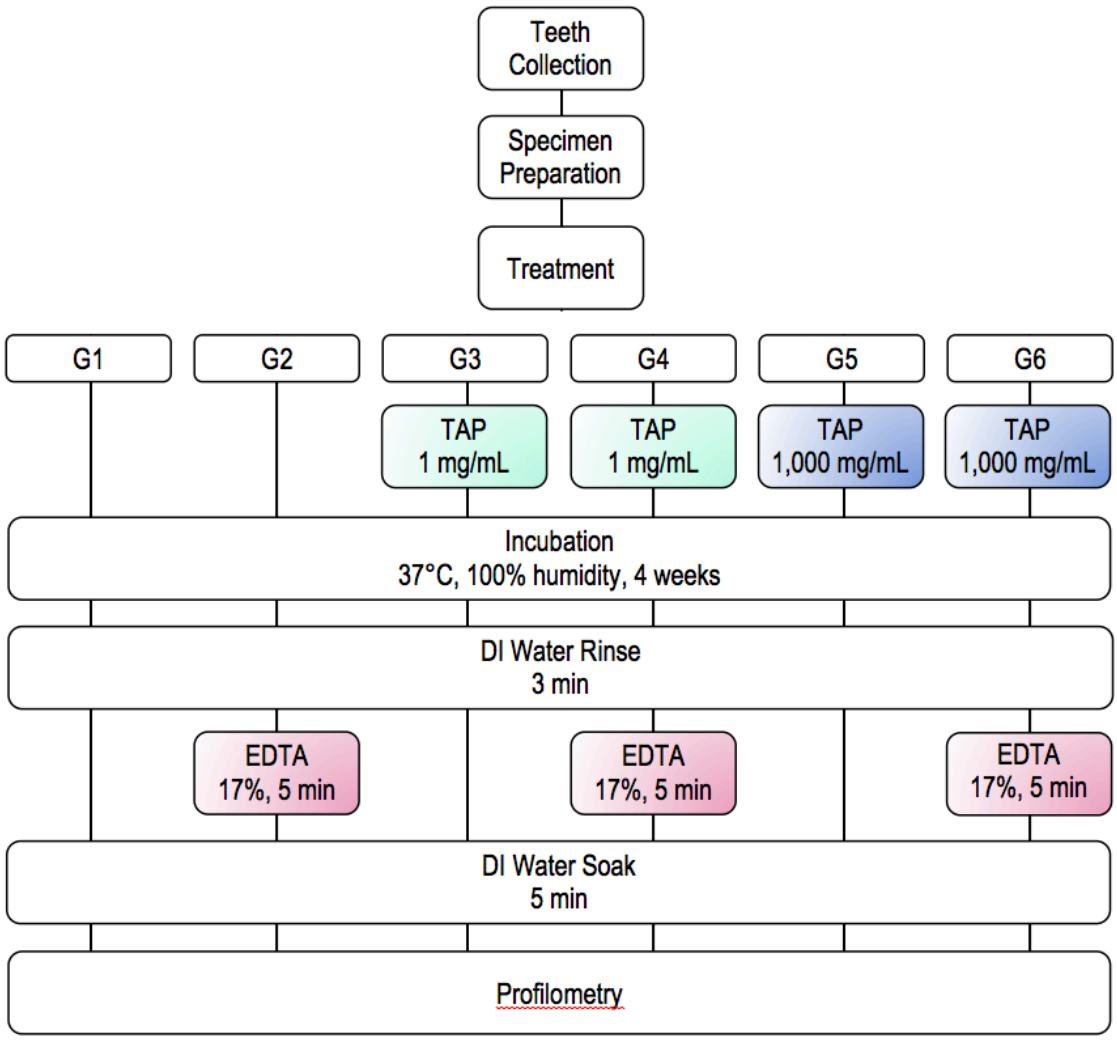

FIGURE 1. Experiment design flowchart.

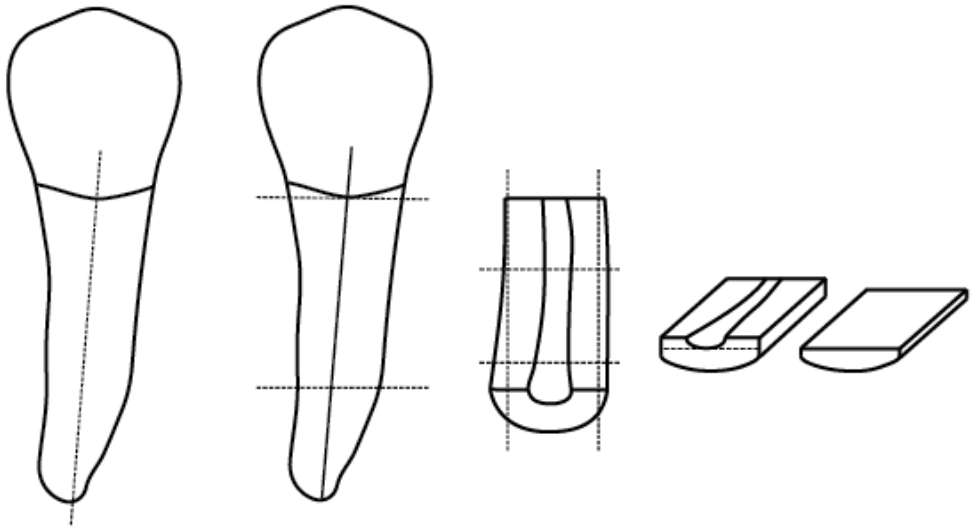

FIGURE 2. Overview of specimen preparation: Each tooth was sectioned, cut to 4x4 mm, and the inner surface was flattened.

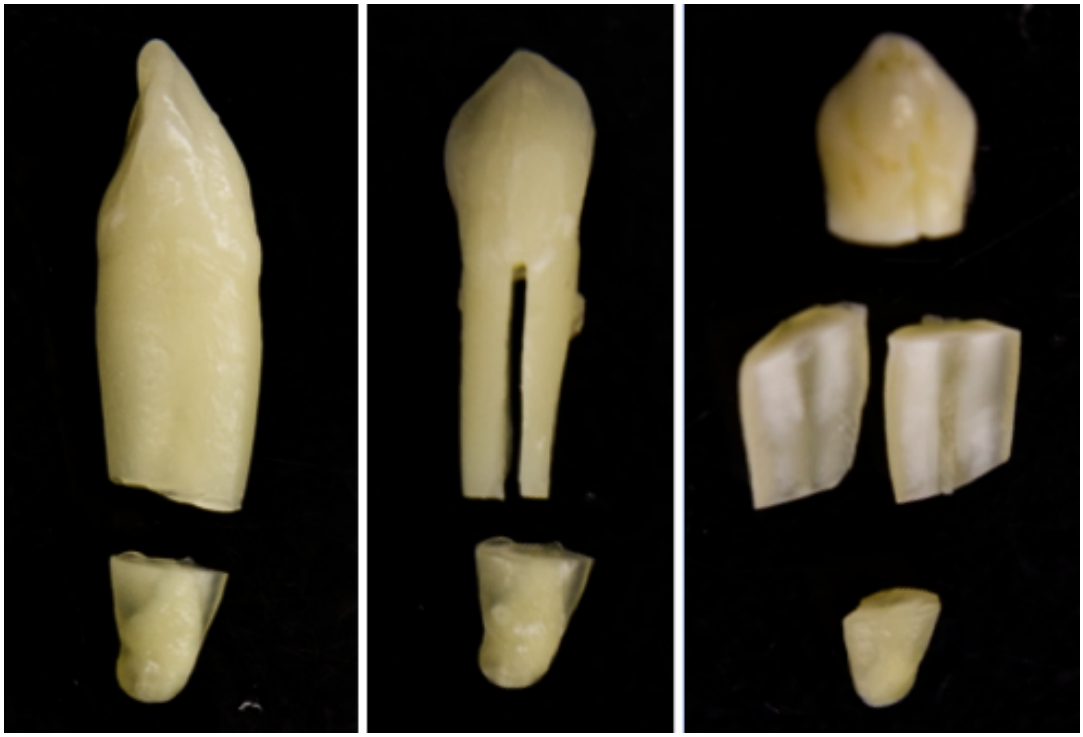

FIGURE 3. Teeth were sectioned using a saw with water irrigation.

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

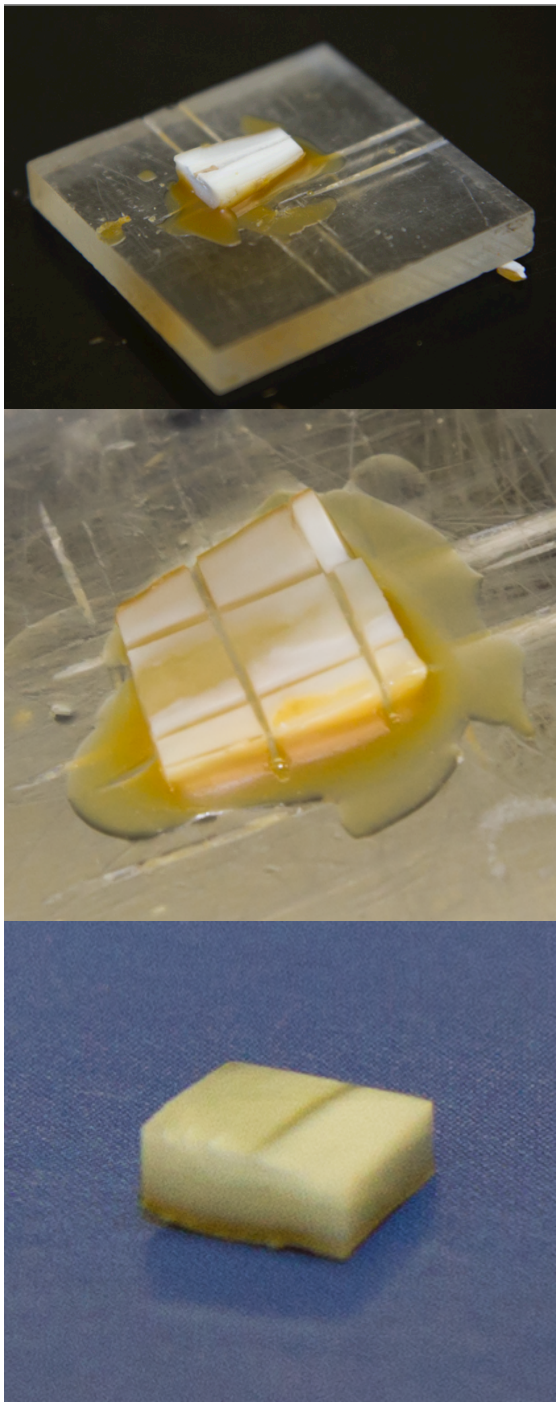

FIGURE 5. Each half-root was cut into a 4x4-mm square with a double-bladed saw with water.

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FIGURE 6. The saw used with water irrigation (Isomet, Buhler).

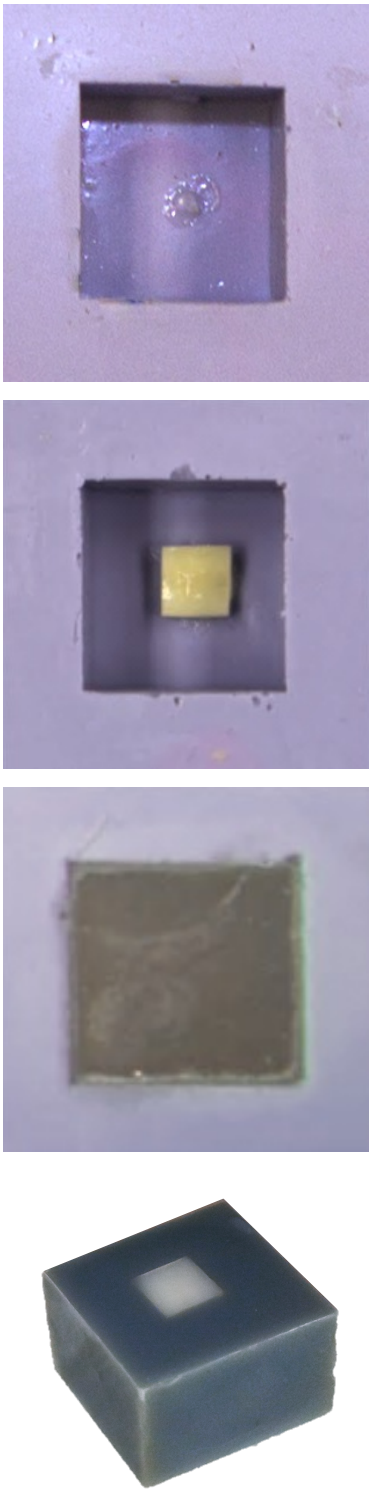

FIGURE 7. Each dentin specimen was embedded into an acrylic resin block with the dentin surface facing up.

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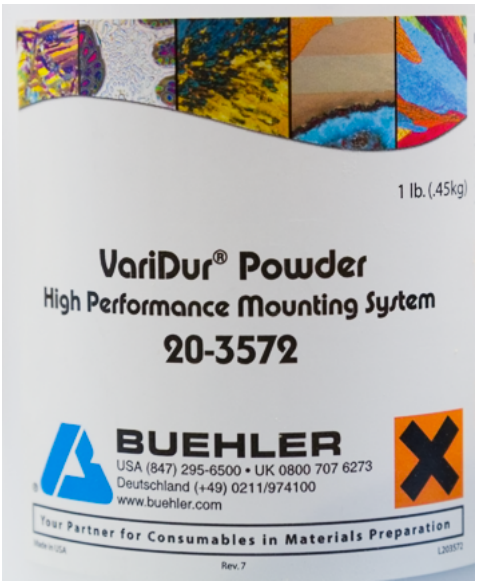

FIGURE 8. Acrylic-resin (Buehler, Varidur) powder and liquid were used to embed the specimens.

FIGURE 9. The specimen blocks were secured with the specimens face down with sticky wax onto Struer's mounting cylinder.

FIGURE 10. The RotoPol 31 (bottom) / Rotoforce-4 (top) was used to flatten the bottom side and polish the specimen side of the acrylic blocks.

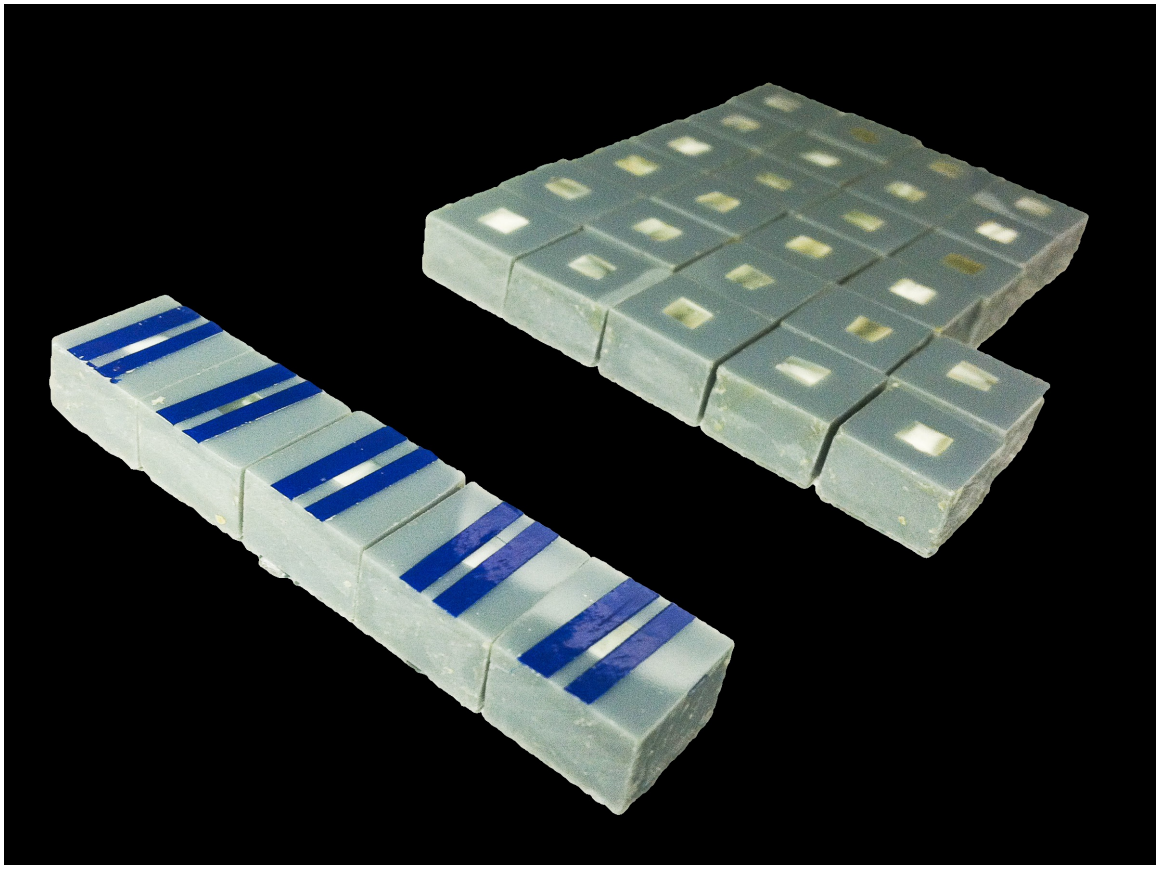

FIGURE 11. Tape was placed over each specimen perpendicular to the long axis of the canal leaving 1.0 mm to 1.25 mm exposed. The taped areas serve as a control for the exposed areas.

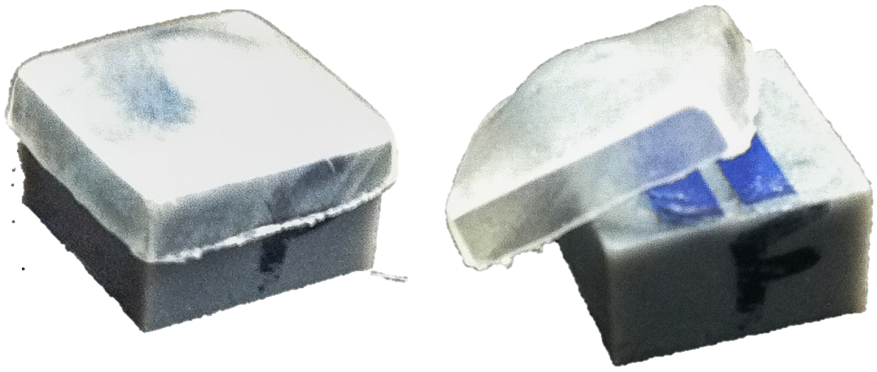

FIGURE 12. Caps were fabricated for the specimens in being treated with 1000 mg/mL TAP (groups 1 and 3) to prevent condensation on the roof of the storage containers from dripping down onto the specimens and washing away the TAP.

FIGURE 13. Gasket-sealed plastic containers with a small air vent were used to contain the samples during incubation to prevent dehydration.

FIGURE 14. Group 1 and 3 specimens were treated with a 0.2-mL droplet of 1,000 mg/mL TAP, were covered with a cap, and were placed in containers lined by paper towels saturated with 20mL of DI water.

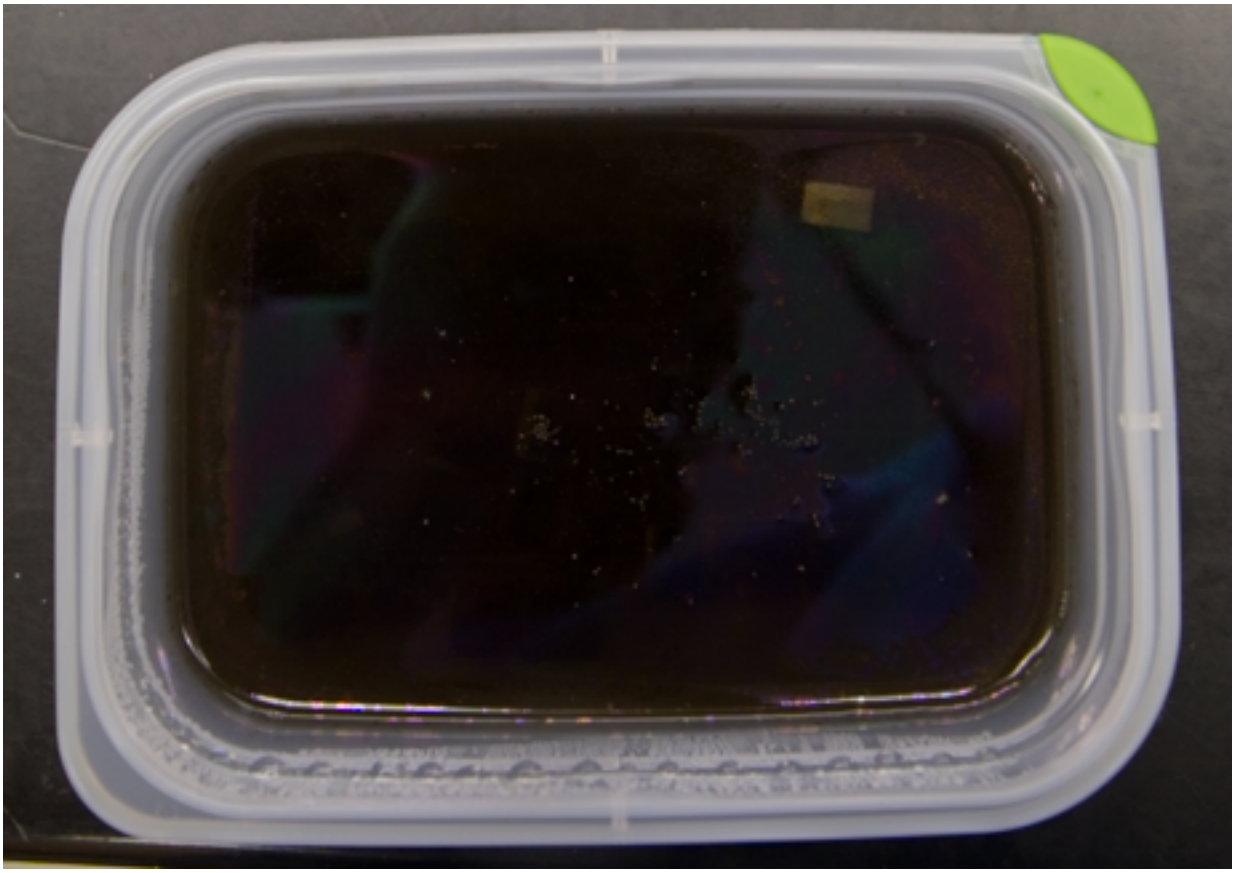

FIGURE 15. Group 2 and 4 specimens were submerged into 200 mL of 1 mg/mL TAP solution.

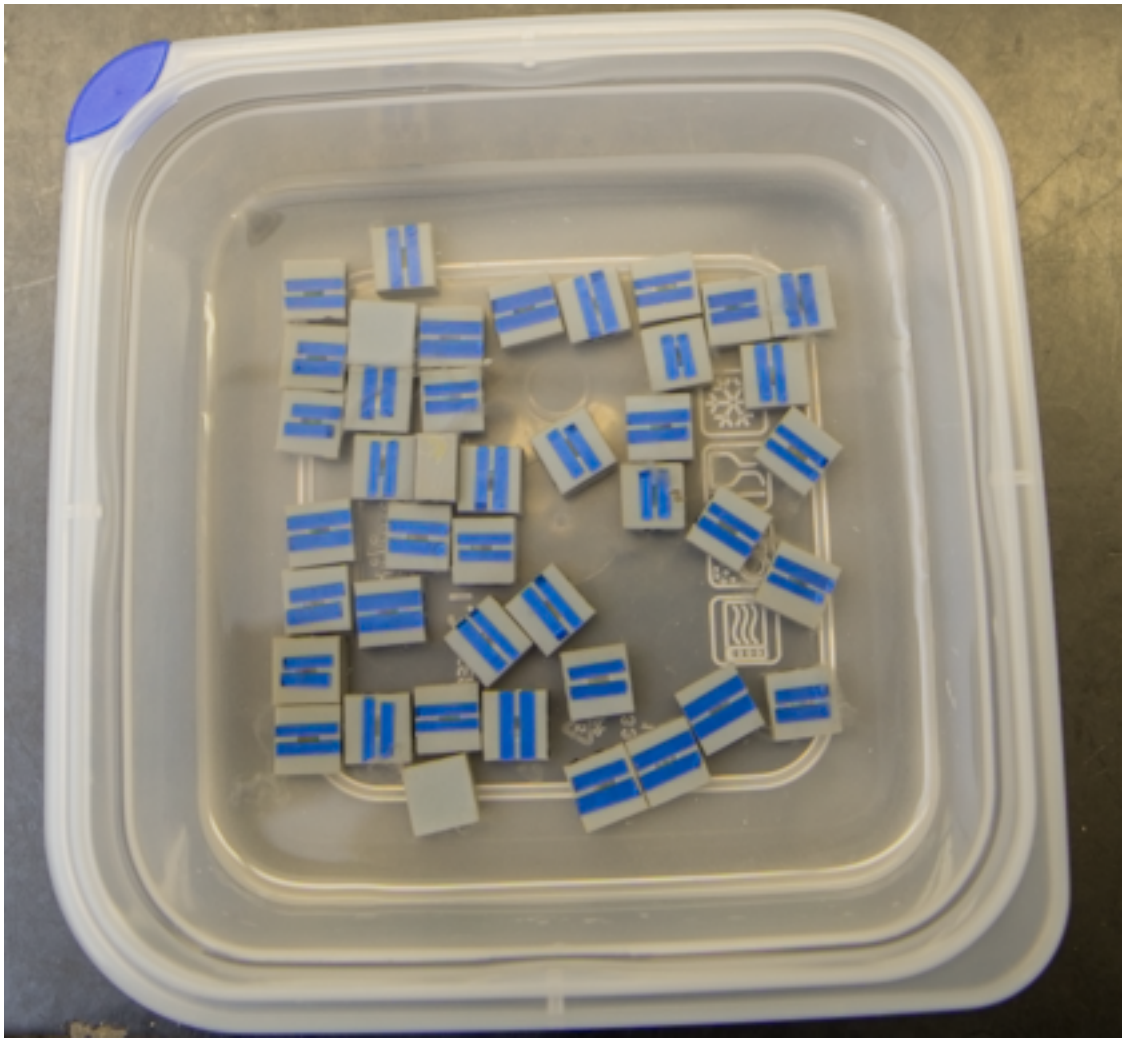

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FIGURE 16. Group 5 and 6 were submerged into 200 mL DI water.

FIGURE 17. All specimens were incubated for 4 weeks at 37°C with 100% humidity.

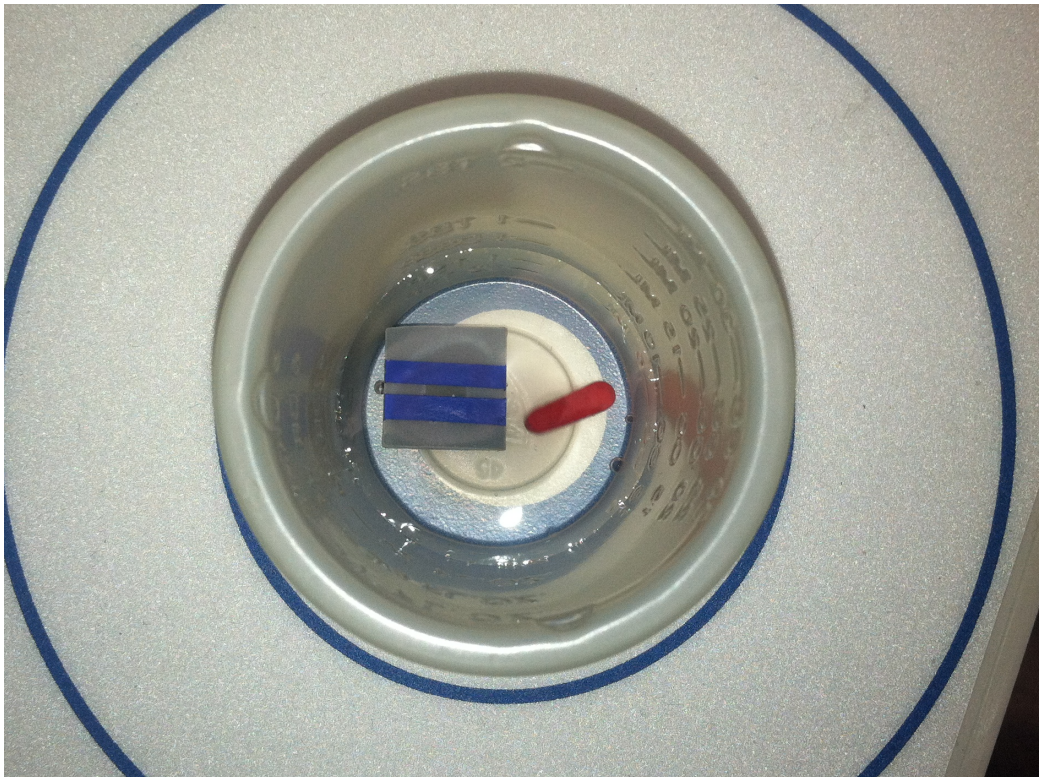

FIGURE 18. Specimens in groups 2, 4 and 6 were submerged into 6 mL of 17-percent EDTA for 5 minutes.

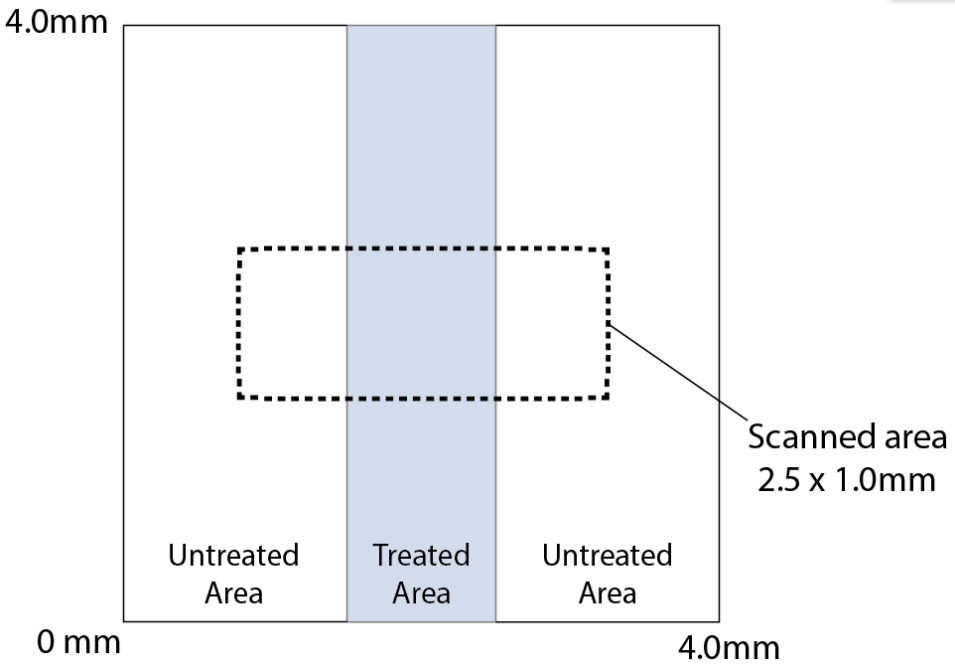

FIGURE 19. An illustration of a specimen demarcating the area scanned for surface loss analysis. The area measured 2.5mm x 1.0mm, was centered on specimen, and extended across both treated and untreated surfaces.

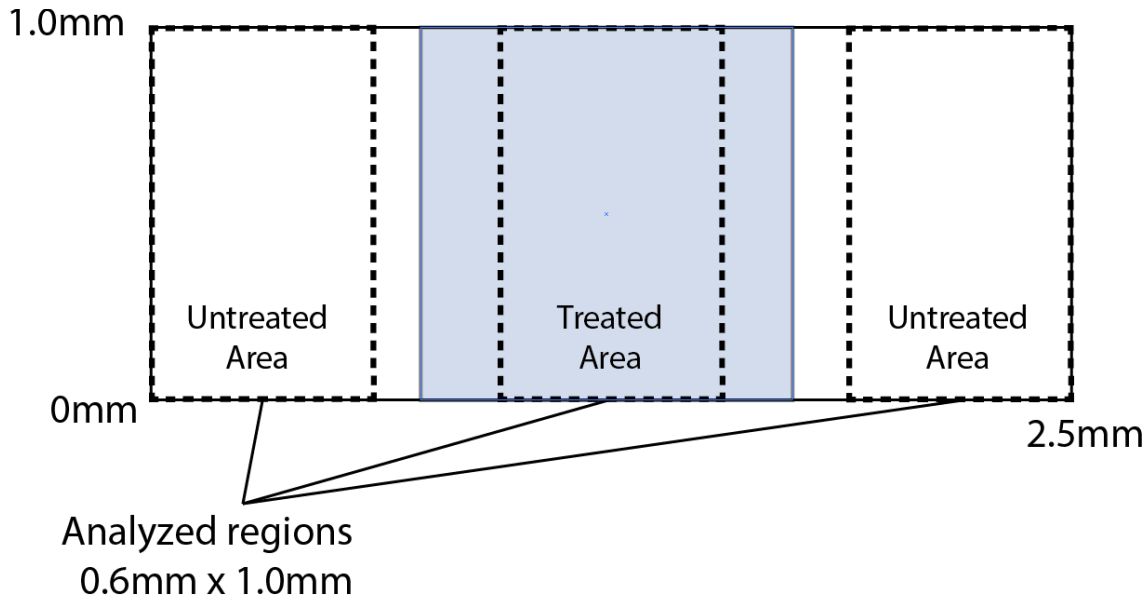

FIGURE 20. Surface loss was quantified from this 2.5 mm x 1.0 mm scanned area using the 3-Point Step Height function. Three regions measuring 0.6 mm x 1.0 mm were selected for analysis. Two regions were selected from the end of each untreated area and one was selected from the center of the treated area.

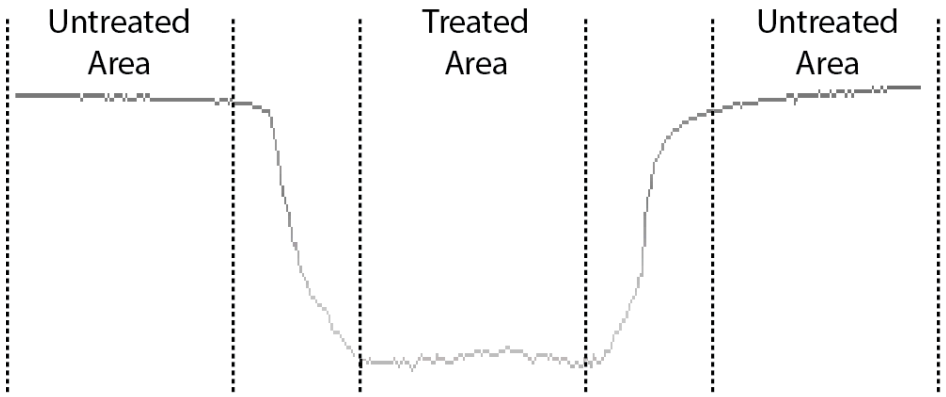

FIGURE 21. A cross-sectional view of the surface being analyzed. Surface loss was quantified using the 3-Point Step Height function, which subtracted the height of the treated area from the average height of the two untreated areas.

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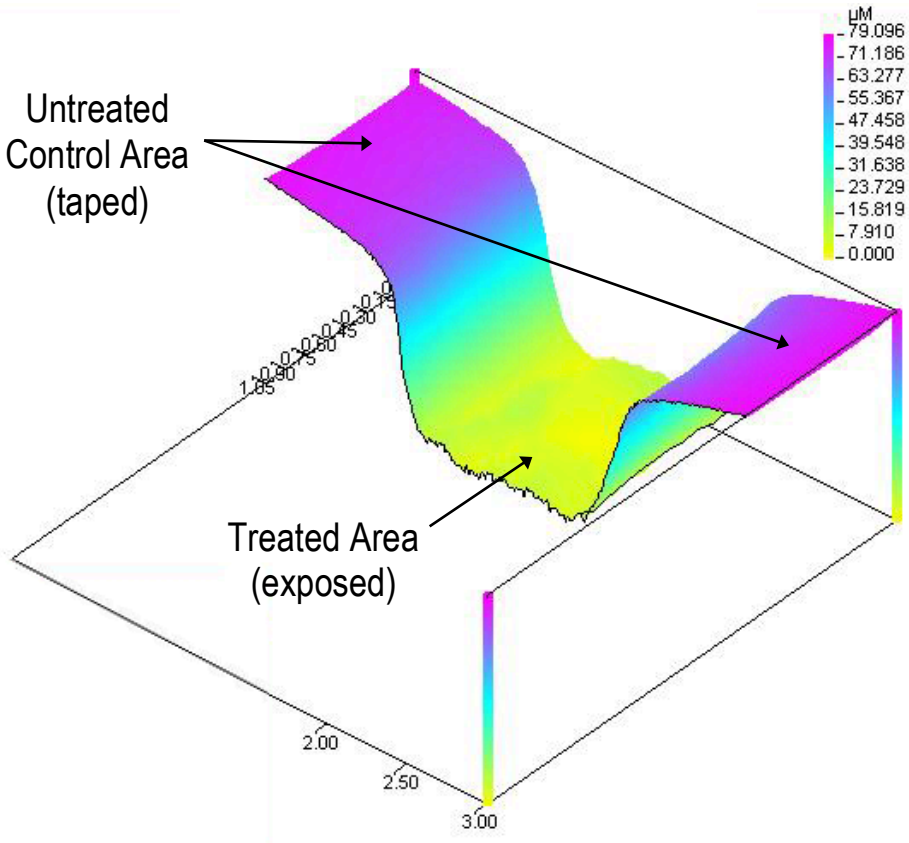

FIGURE 22. This acquired scan of a 2.5 mm x 1.0 mm area of a specimen was used for surface loss quantification. The exposed area was treated with 1000 mg/mL TAP for 4 weeks followed by 17-percent EDTA for 5 minutes.

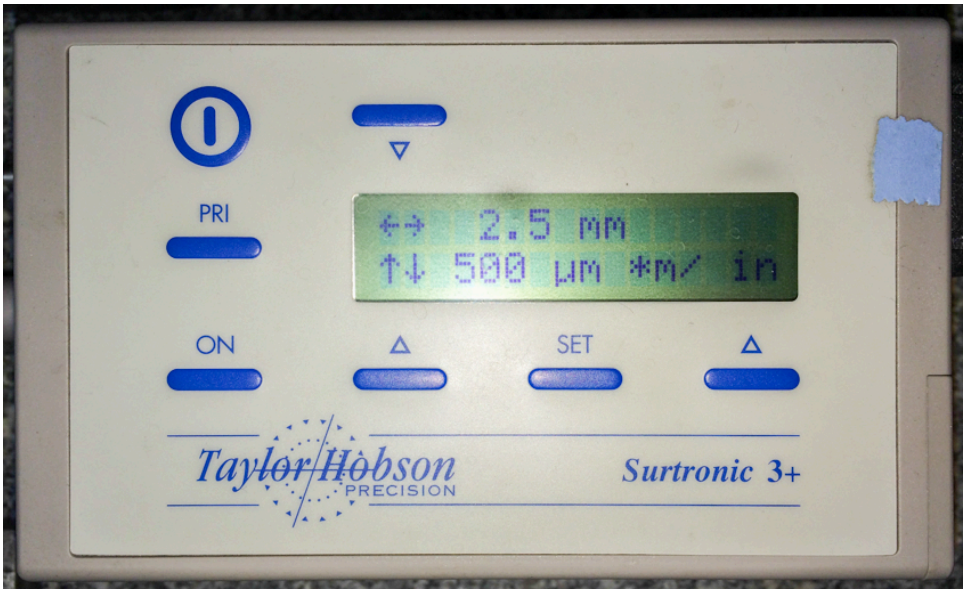

FIGURE 23. Specimens were scanned with the Taylor Hobson Surtronic 3+ contact profilometer.

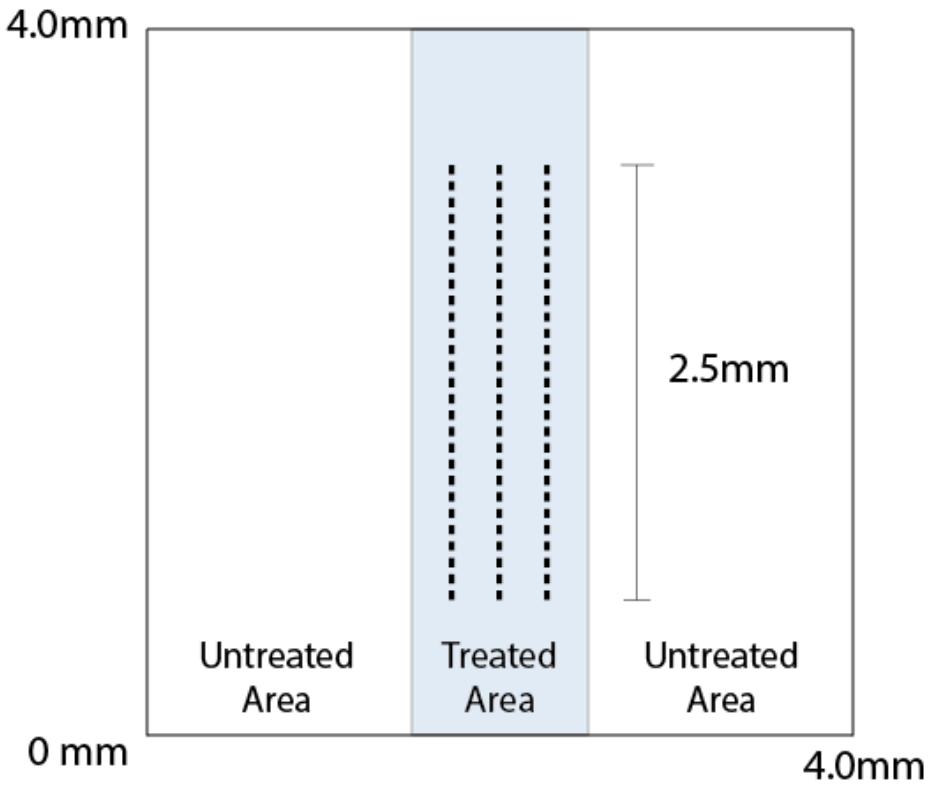

FIGURE 24. Three 2.5-mm lines were scanned across the treated area of each specimen.

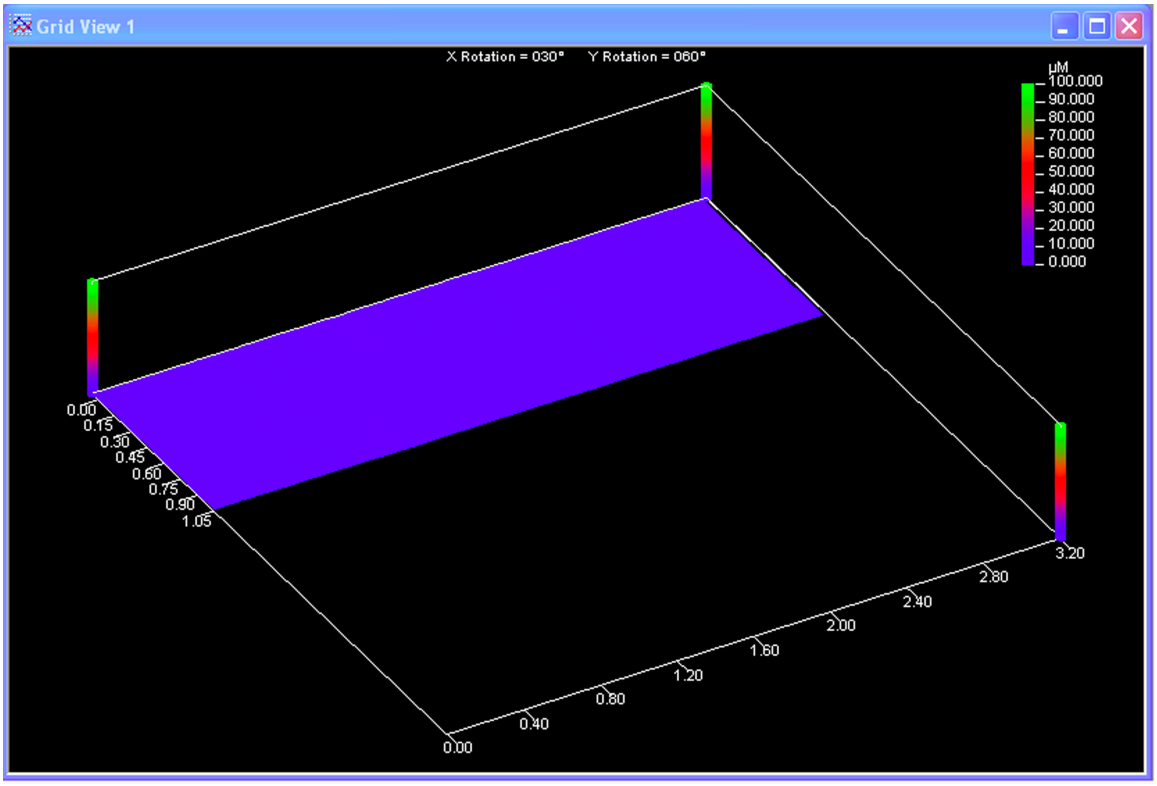

FIGURE 25. Representative image of dentin surface loss measured with optical non-contact profilometer with no treatment.

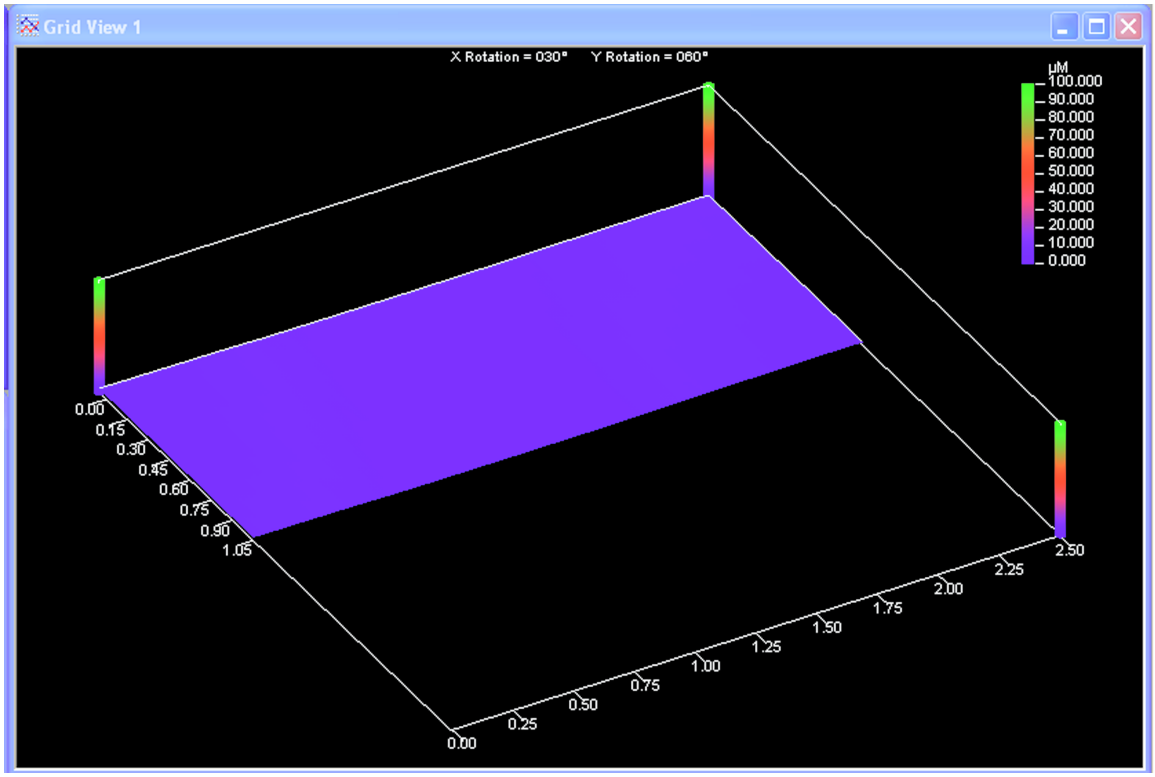

FIGURE 26. Representative image of dentin surface loss measured with optical non-contact profilometer after treatment with 17-percent EDTA.

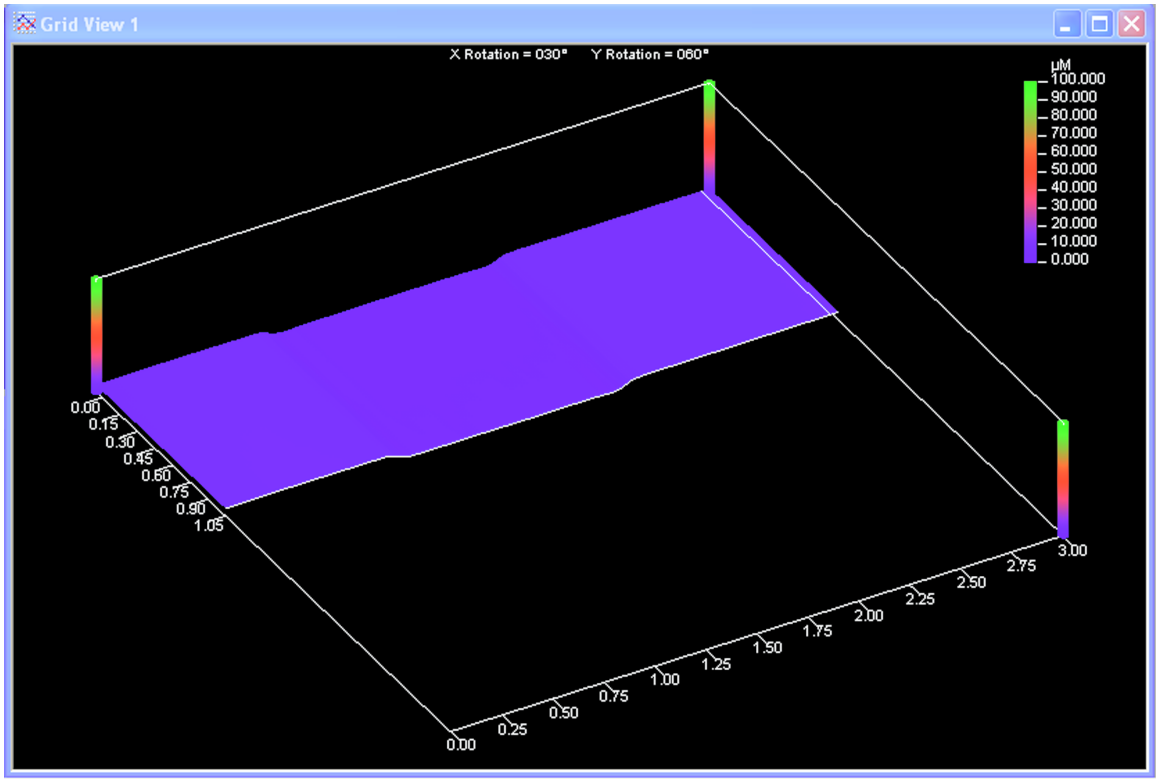

FIGURE 27. Representative image of dentin surface loss measured with optical non-contact profilometer after treatment with 1 mg/mL TAP.

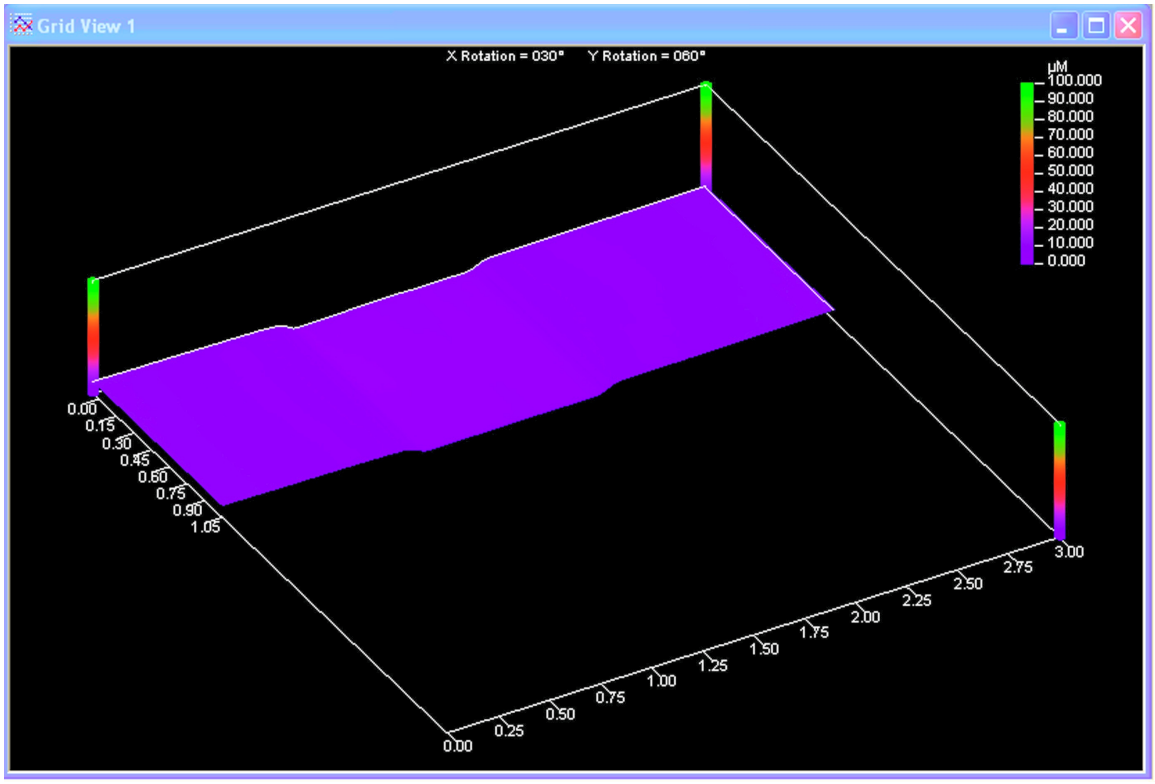

FIGURE 28. Representative image of dentin surface loss measured with optical non-contact profilometer after treatment with 1 mg/mL TAP + 17-percent EDTA.

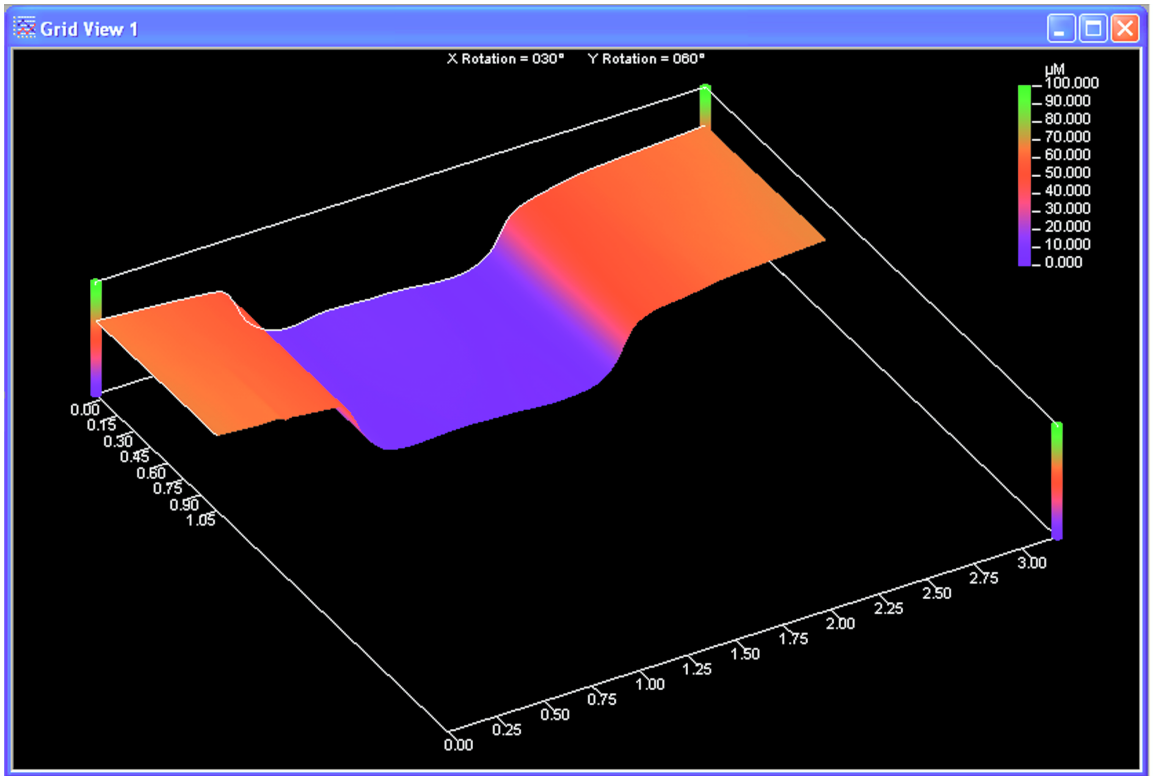

FIGURE 29. Representative image of dentin surface loss measured with optical non-contact profilometer after treatment with 1000 mg/mL TAP.

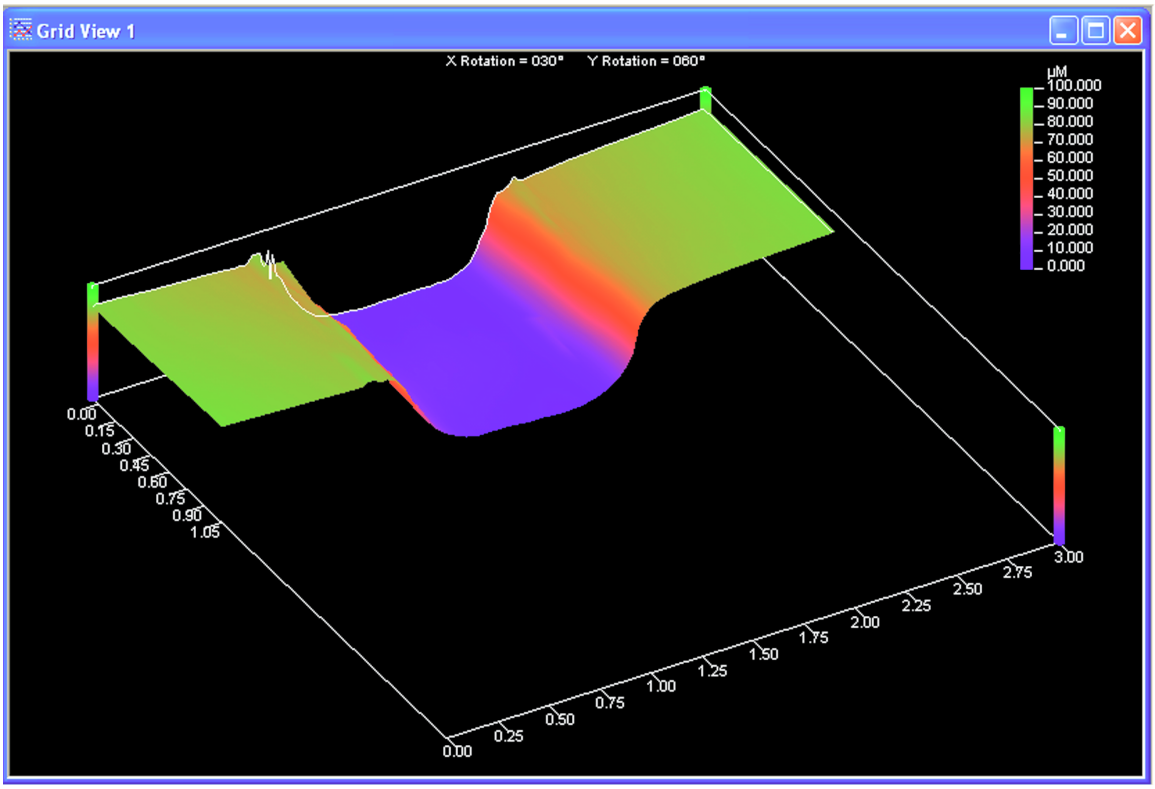

FIGURE 30. Representative image of dentin surface loss measured with optical non-contact profilometer after treatment with 1000 mg/mL TAP + 17-percent EDTA.

	Group	n	Mean (µm)	SD	Min	Max
G1	Control	17	0.95	0.51	*0.36*	*1.68*
G2	17% EDTA	20	1.81	1.08	*0.21*	*3.66*
G3	TAP 1mg/mL	20	7.55	2.62	*2.47*	*13.01*
G4	TAP 1mg/mL + 17% EDTA	18	6.76	2.20	*3.46*	*11.32*
G5	TAP 1,000mg/mL	20	57.95	15.64	*31.69*	*86.96*
G6	TAP 1,000mg/mL + 17% EDTA	19	67.59	17.33	*39.33*	*113.55*

FIGURE 31. Surface loss (µm) and summary statistics for each group.

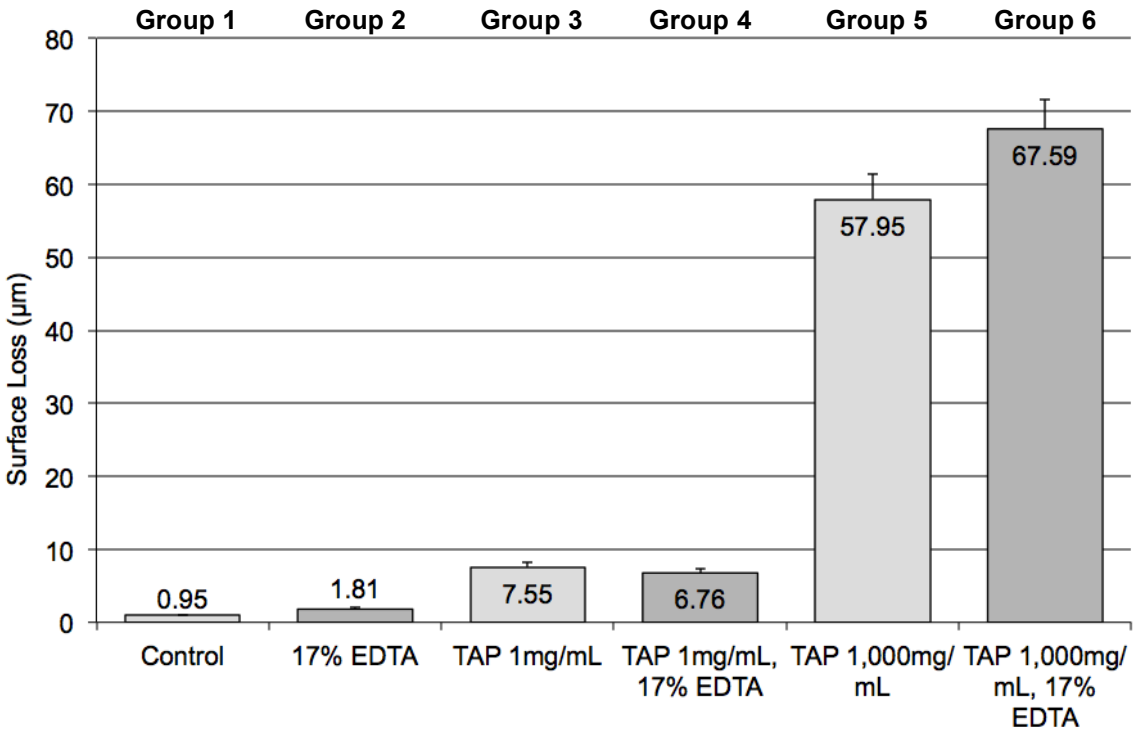

FIGURE 32. A comparison of surface loss (µm) for all treatment groups. Data are presented as the mean ± standard error of the mean.

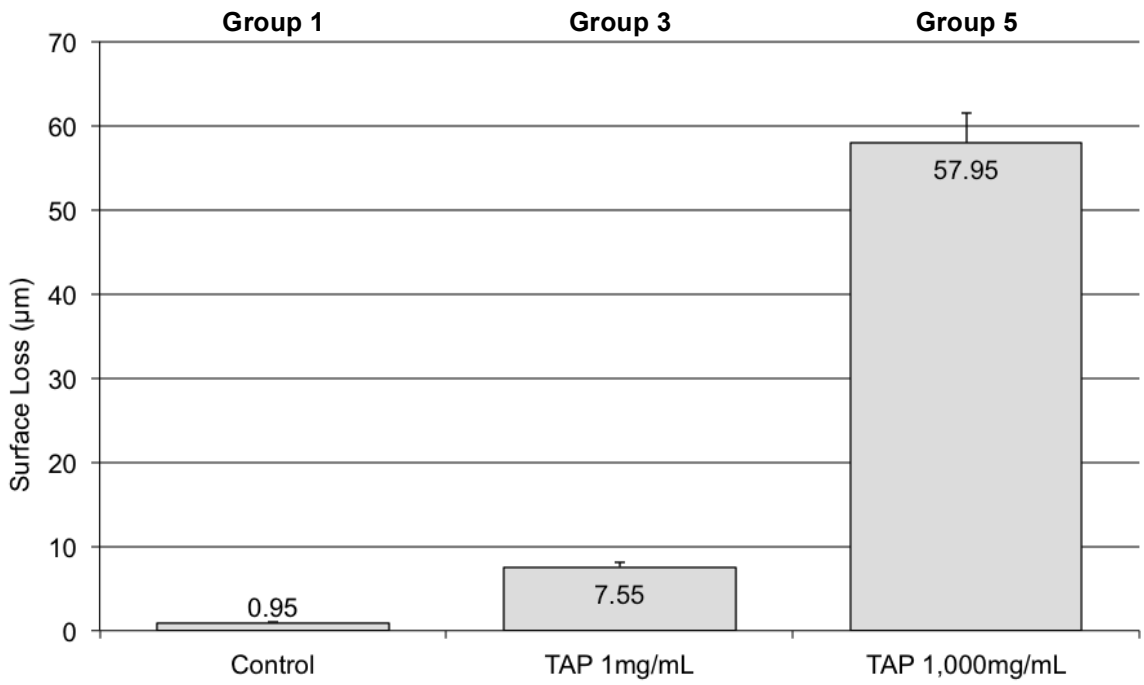

FIGURE 33. Compared to the control, TAP caused a concentration-dependent increase in surface loss that was significant between groups ($P < 0.0001$). Data are presented as the mean ± standard error of the mean.

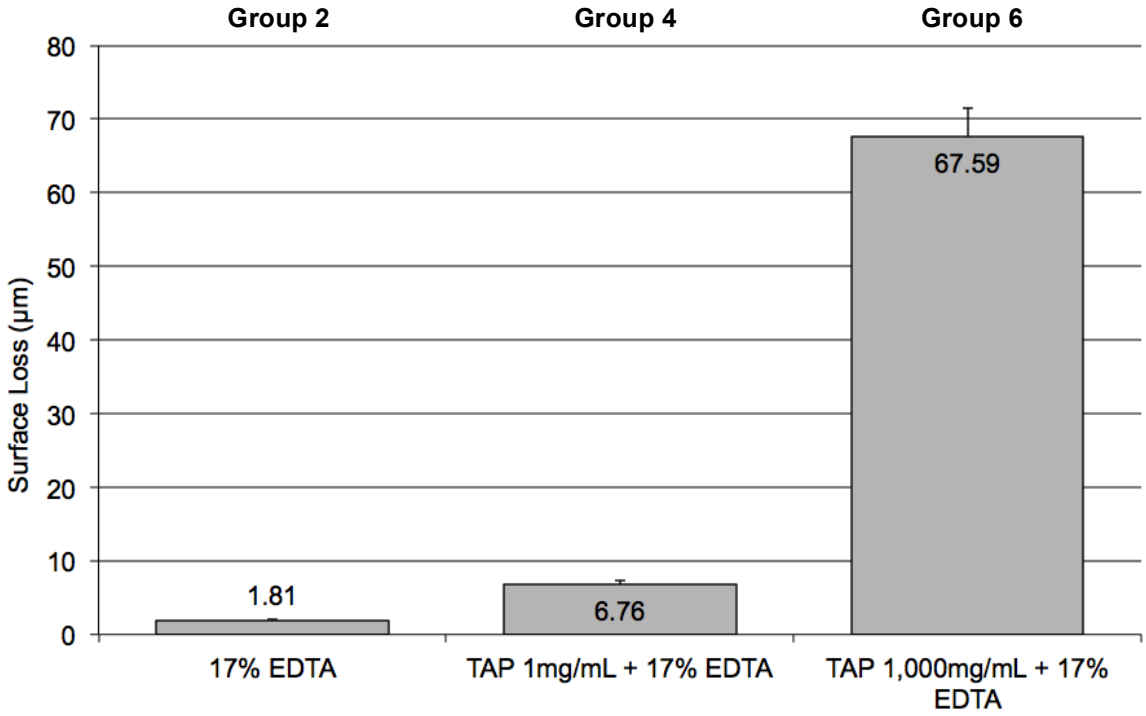

FIGURE 34. Compared to the 17-percent EDTA alone, TAP + 17-percent EDTA caused a concentration-dependent increase in surface loss that was significant ($p < 0.0001$). Data are presented as the mean ± standard error of the mean.

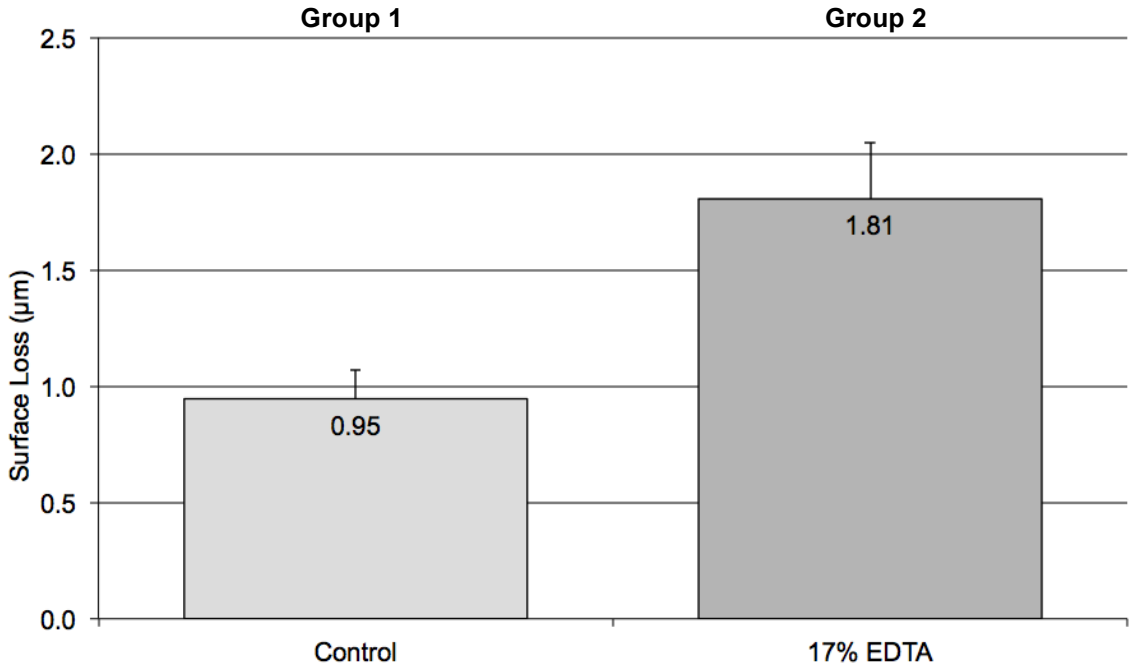

FIGURE 35. There was a significant difference in surface loss between control and the group with 17-percent EDTA ($p = 0.002$). Data are presented as the mean ± standard error of the mean.

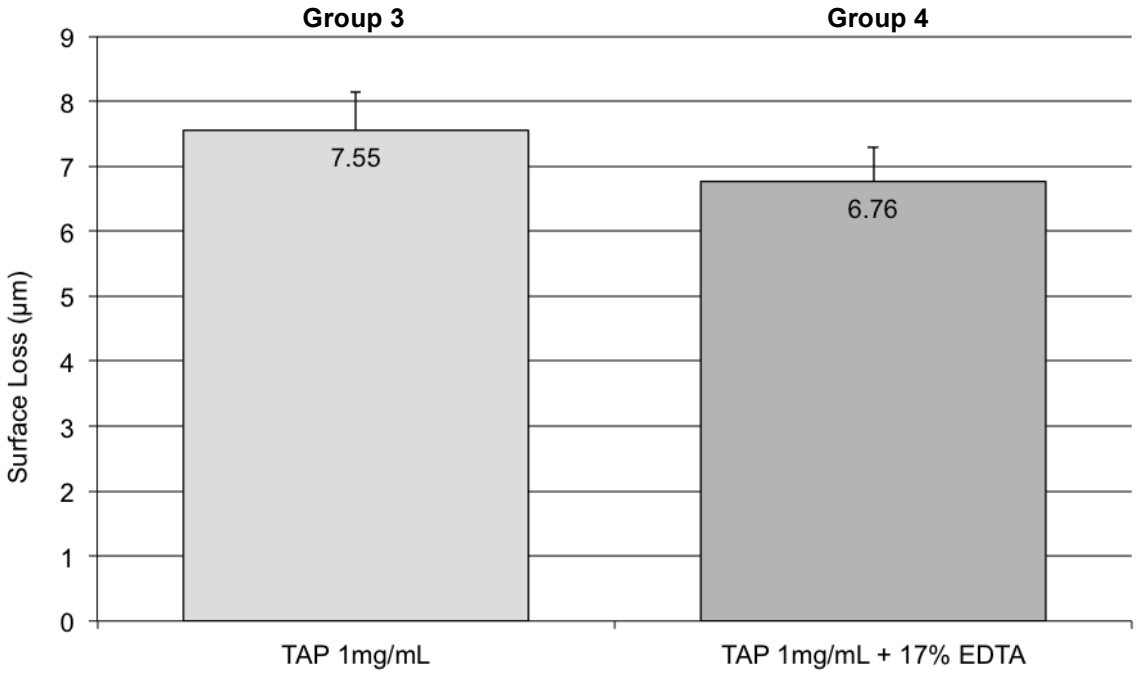

FIGURE 36. There was no significant difference in surface loss between TAP 1mg/mL without or with 17-percent EDTA ($p = 0.3169$). Data are presented as the mean ± standard error of the mean.

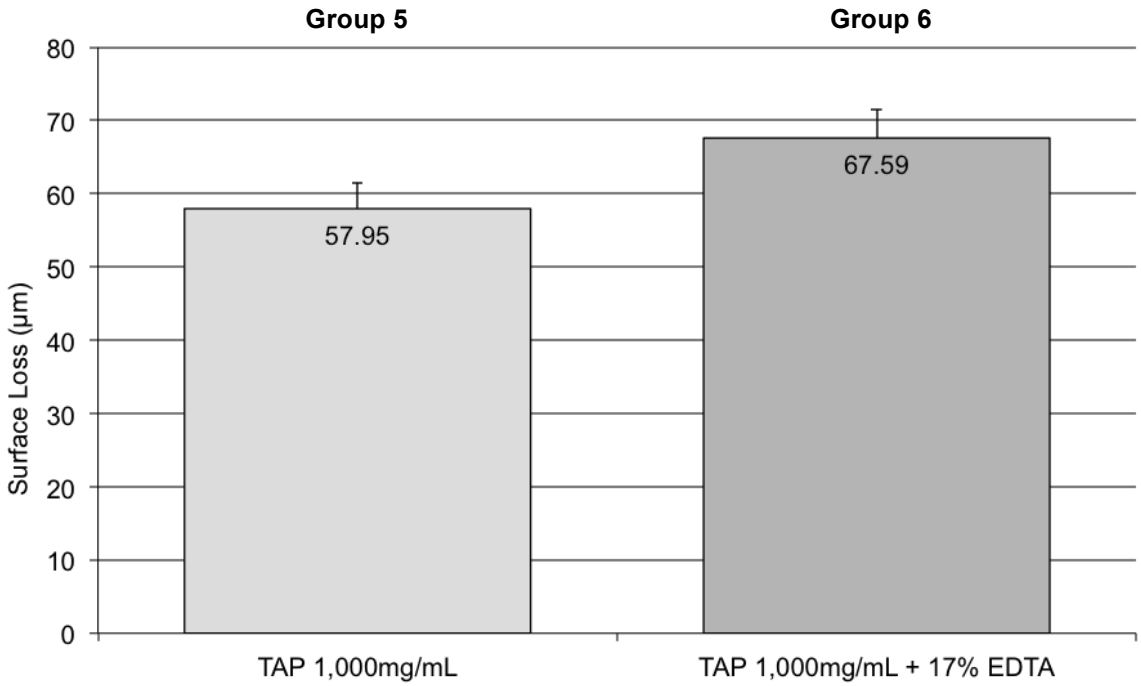

FIGURE 37. There was no significant difference in surface loss between TAP 1000 mg/mL with or without 17-percent EDTA ($p = 0.0712$). Data are presented as the mean ± standard error of the mean.

	Group	n	Mean (Ra)	SD	Min	Max
G1	Control	18	0.24	0.045	*0.17*	*0.30*
G2	17% EDTA	18	0.24	0.051	*0.16*	*0.38*
G3	TAP 1mg/mL	20	0.29	0.134	*0.14*	*0.58*
G4	TAP 1mg/mL + 17% EDTA	19	0.27	0.042	*0.21*	*0.37*
G5	TAP 1,000mg/mL	17	0.68	0.204	*0.43*	*1.06*
G6	TAP 1,000mg/mL + 17% EDTA	16	0.70	0.21	*0.51*	*1.28*

FIGURE 38. Surface roughness (Ra, µm) and summary statistics for each group.

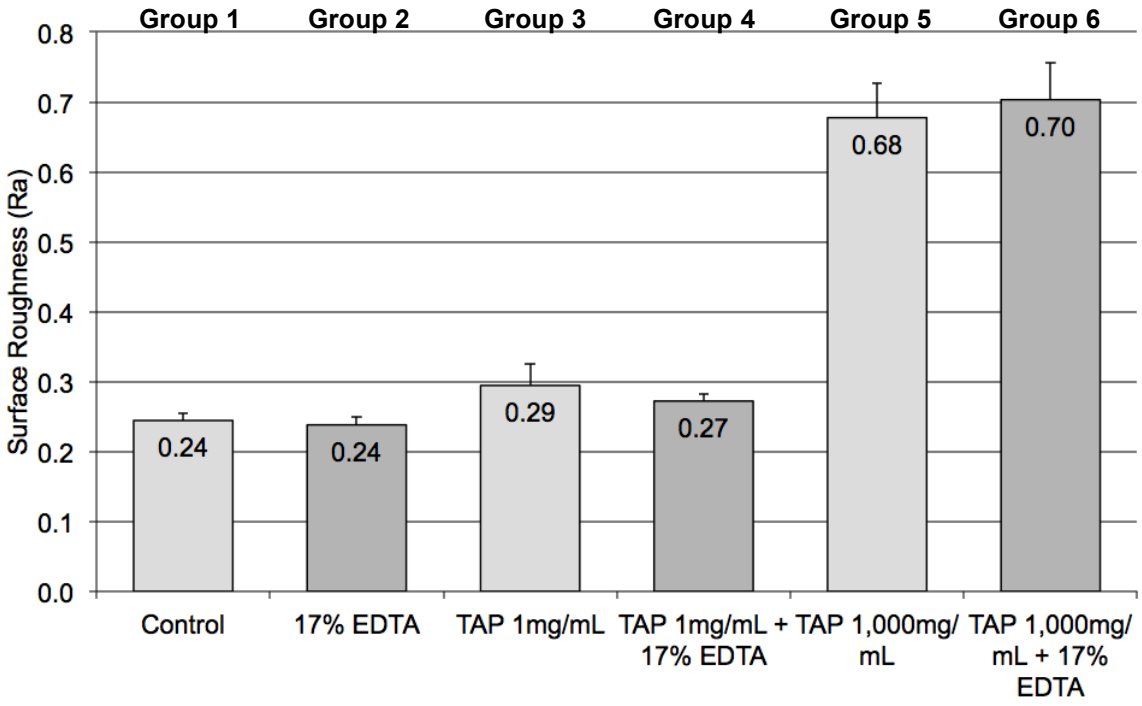

FIGURE 39. A comparison of surface roughness (Ra, μm) for all treatment groups. Data are presented as the mean ± standard error of the mean.

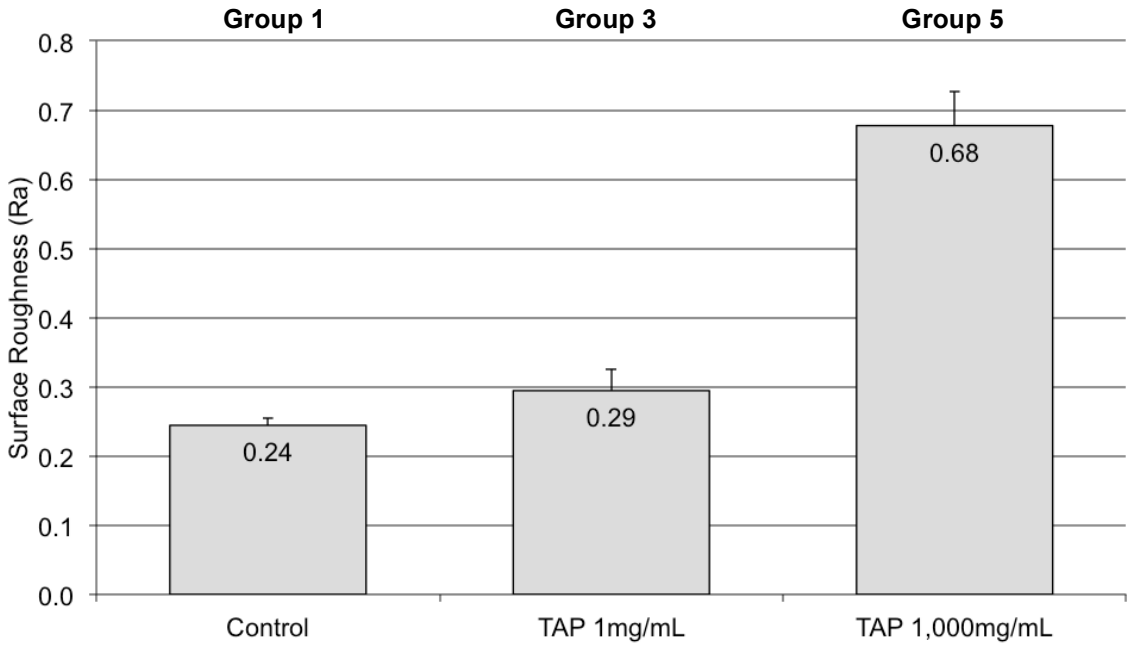

FIGURE 40. Compared with the control, TAP caused a concentration-dependent increase in surface roughness. There was a significant difference between Control and TAP 1,000mg/mL ($p < 0.0001$) and between TAP 1mg/mL and TAP 1000mg/mL ($p < 0.0001$) but not between Control and TAP 1mg/mL ($p = 0.1187$). Data are presented as the mean ± standard error of the mean.

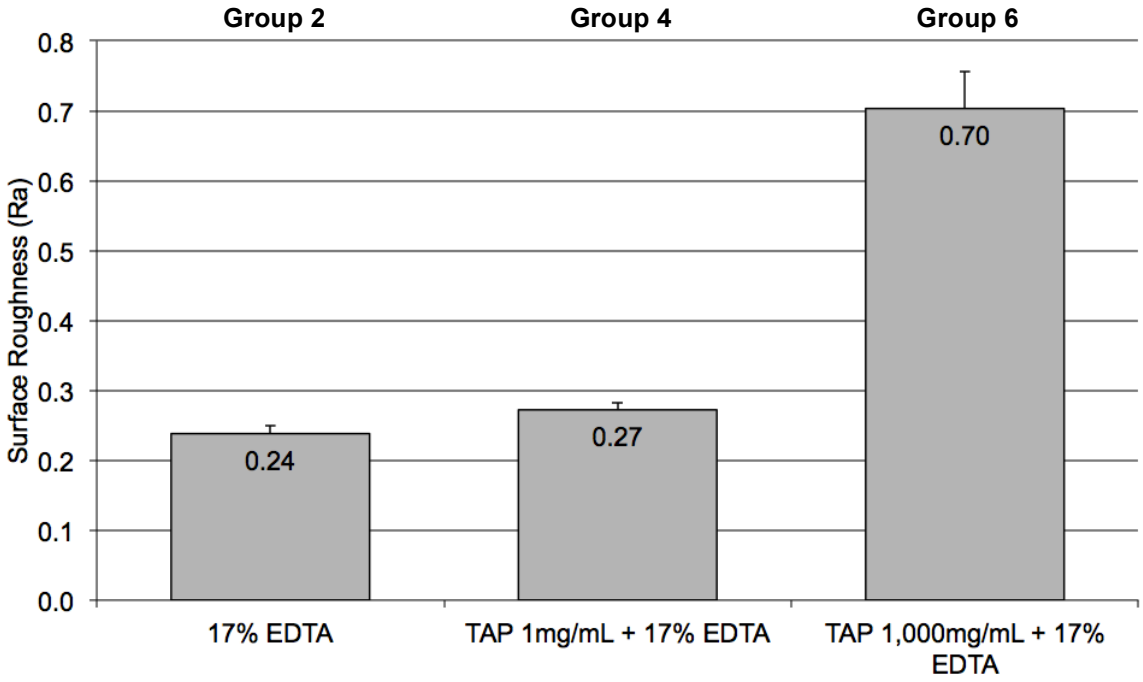

FIGURE 41. EDTA caused a similar concentration-dependent increase in surface roughness. There was a significant difference between 17-percent EDTA and TAP 1000mg/mL + 17-percent EDTA ($p < 0.0001$), and between TAP 1 mg/mL + 17-percent EDTA and TAP 1000 mg/mL + 17-percent EDTA ($p < 0.0001$); but no significant difference was between 17-percent EDTA and TAP 1 mg/mL ($p = 0.0305$). Data are presented as the mean ± standard error of the mean.

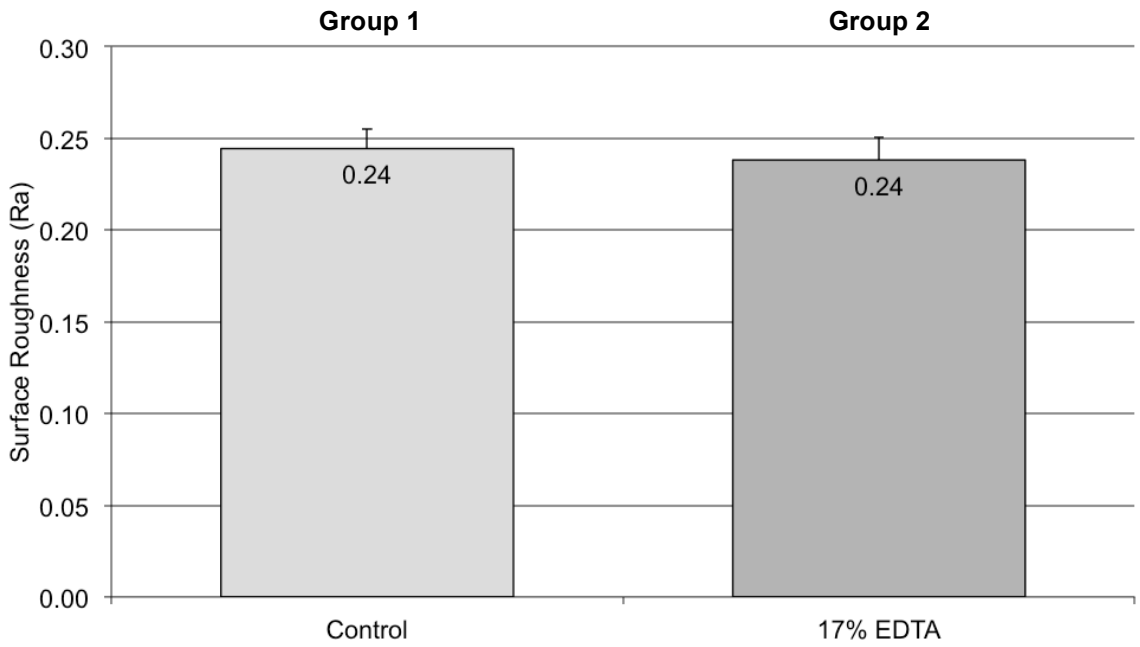

FIGURE 42. There was no significant difference in surface roughness between Control and 17% EDTA ($p = 0.6993$). Data are presented as the mean ± standard error of the mean.

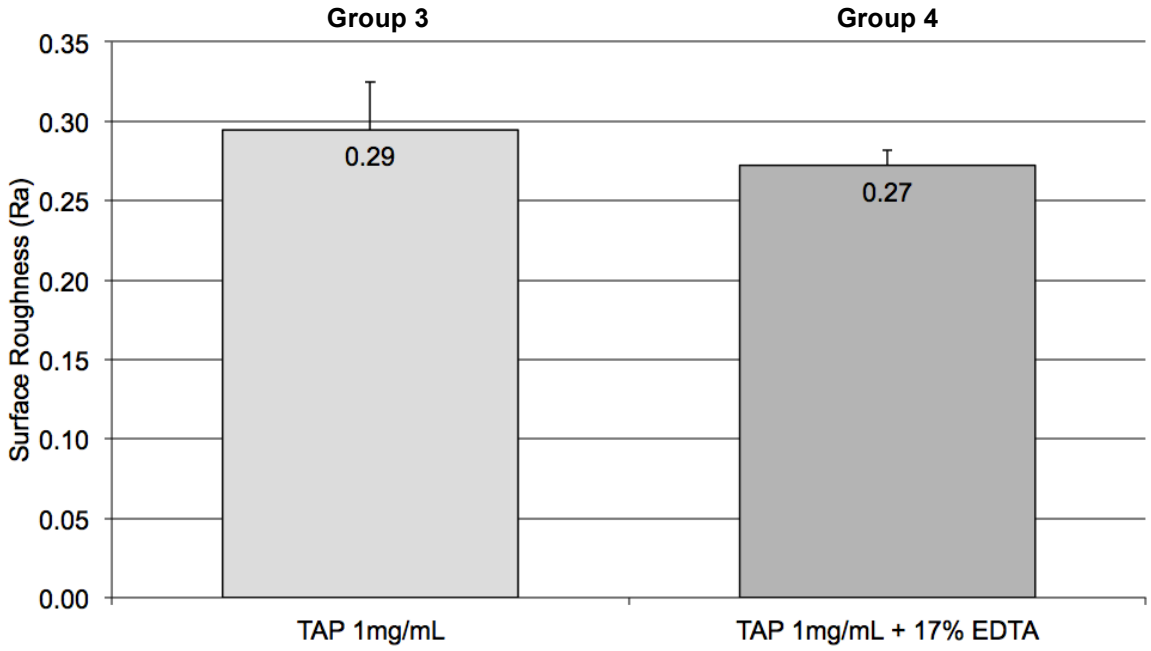

FIGURE 43. There was no significant difference in surface roughness between TAP 1mg/mL with or without 17-percent EDTA ($p = 0.4823$). Data are presented as the mean ± standard error of the mean.

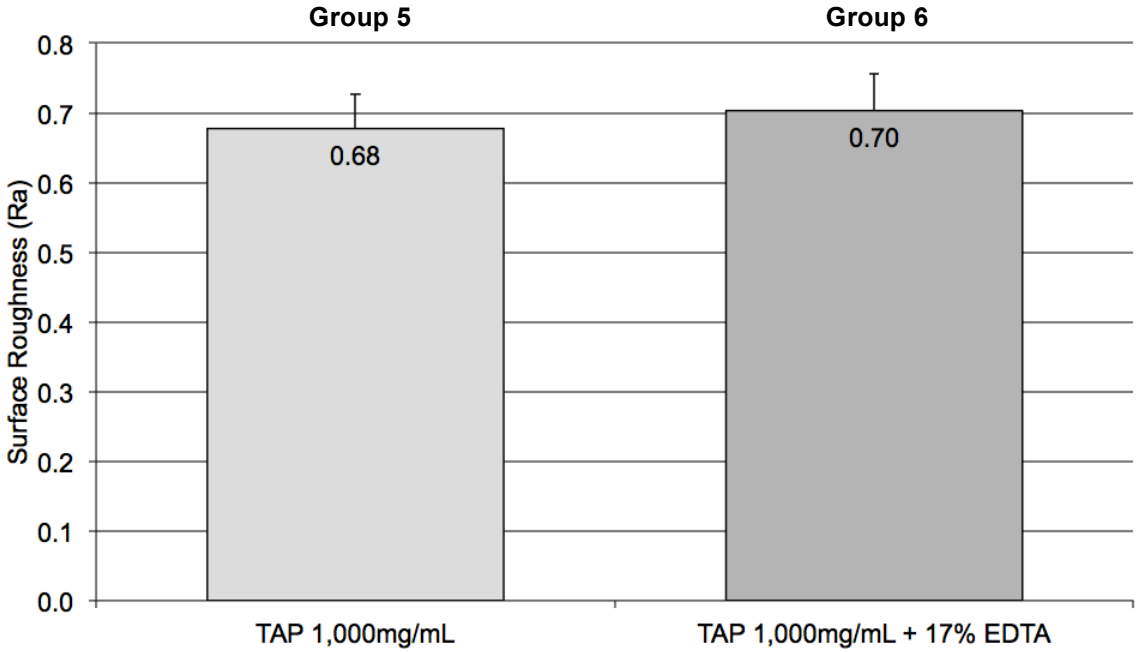

FIGURE 44. There was no significant difference in surface roughness between TAP 1000 mg/mL with or without 17-percent EDTA ($p = 0.7226$). Data are presented as the mean ± standard error of the mean.

DISCUSSION

Given minimal or no instrumentation is performed during REPs, an efficient chemical disinfection protocol is required to eradicate the endodontic pathogens and optimize the biological environment inside the root canal for regeneration. However, this chemical challenge may negatively impact the physical and mechanical properties of radicular dentin. Therefore, it is important to develop a balanced endodontic regeneration protocol that achieves effective chemical disinfection with minimal adverse effects on the physical, chemical and mechanical properties of root dentin.

Profilometry

Contact type profilometry was chosen instead of the non-contact type for surface roughness quantification for several reasons. First, the contact-type method has a more established technique and is the most commonly reported measurement in within dental studies.[41] Therefore, our results can be more accurately compared with previous studies. Secondly, the roughness values of control specimens as quantified with the contact method were consistent with values of previous studies whereas the values obtained from the non-contact method were not. The decision to use the average surface roughness (Ra) parameter for assessment of roughness was based on the fact that Ra is the most common roughness parameter used in dental research and is recognized internationally.[41]

Surface Loss

The concentration of TAP that provides a pasty consistency is about 1000 mg/mL; this concentration has been commonly used in endodontic regeneration.[33] Our study

showed that the use of 1000mg/mL TAP with or without EDTA caused significant radicular dentin erosion ranging from 57 μm to 67 μm. This strong erosive effect of 1000 mg/mL TAP could be explained by the acidic nature of TAP (pH = 2.9) and the calcium chelating property of minocycline present in TAP.[26] Irrigation solutions with calcium chelating ability such as EDTA or MTAD have been found to cause erosion of instrumented root canal dentin.[160,161] The strong erosive effect of 1000 mg/mL TAP might remove the predentin organic-rich layer adjacent to the pulp of immature teeth. This might occur indirectly through the demineralization of the supporting inorganic structures leaving the organic remains loose and susceptible to washing out. This might also occur through direct denaturation of organic proteins. Collagen denaturation has been shown to occur at pH less than 4 at 37°C.[162] The average thickness of the predentin layer was found to be 15 μm, 26 μm, and 40 μm on the cervical, middle and apical areas of immature teeth, respectively.[163] Therefore, minimizing the surface loss caused by antibiotic medications might preserve the protein-rich root canal surface layer in immature teeth and improve the outcomes of endodontic regeneration. Our study also showed that dentin treated with 1 mg/mL TAP with or without EDTA caused surface loss ranging from 6.8 μm to 7.6 μm. Although the diluted TAP treatments caused significantly higher surface loss compared to the untreated control group, this low concentration caused approximately 9 times lower dentin surface loss compared with the use of 1000 mg/mL TAP + EDTA. It is also noteworthy to mention that 1 mg/mL TAP was found to be effective against *E. faecalis* biofilm[35] and had no indirect cytotoxic effect against stem cells of the apical papillae.[154] In the current study, dentin treated with EDTA caused significantly higher surface loss than untreated dentin. However, the average surface loss

of EDTA treated dentin was limited to only 1.9 μm. Furthermore, the use of EDTA after both concentrations of TAP did not significantly increase the surface loss compared with the use of TAP alone. The EDTA minimum erosive effect reported in this study generally agrees with previous studies that reported limited root canal erosion after irrigation with EDTA.[164,165] However, the use of NaOCl followed by EDTA or the use of calcium hydroxide followed by EDTA were found to cause severe root canal erosion.[161,165,166] The combined chemical challenges of root canal irrigants and intracanal medicaments might have a synergistic erosive effect on an immature root during endodontic regeneration. This is of particular concern when in the cervical area of the root where there is no expected increase in dentin thickness after the regeneration procedure. The American Association of Endodontists has recommended the use of 0.1/mg/mL concentrations of the antibiotic medicaments during endodontic regeneration.[17] However, non-diluted TAP is still the most commonly used medicaments in recently published clinical studies[148,167] and there is no clinical evidence supporting the use of low concentrations of antibiotic in regeneration procedures.

Surface Roughness

Surface roughness measurements were also performed in the current study. A substrate with a rough surface may facilitate bacterial adhesion and biofilm formation.[168] On the other hand, optimum surface roughness may promote the adhesion and differentiation of mesenchymal stem cells in a regeneration procedure.[169] The use of 1000 mg/mL TAP with or without EDTA significantly increases roughness of radicular dentin. However, the use of 1 mg/mL TAP did not significantly increase roughness of radicular dentin. Unlike previous studies, the current investigation showed that EDTA treated

dentin did not cause significant increase in surface roughness compared to untreated control dentin. This could be explained by the relatively short EDTA treatment time used in this study (5 minutes). Previous studies that reported significant increase in dentin surface roughness after exposure to EDTA either used EDTA for 10-15 minutes[56,170] or applied EDTA after irrigation with sodium hypochlorite.[36]

Future Research

Minocycline-induced tooth discoloration has been problematic for satisfying the esthetic needs of patients.[31,32] Some have suggested the use of antimicrobial mixtures without minocycline such as modified triple antibiotic paste (mTAP)[171] or double antibiotic paste (DAP).[172] Future research could investigate the effects of these formulations on the surface properties of dentin.

Additive or synergistic effects may occur when combining the TAP and EDTA with other medicaments such as NaOCl.[161,165,166] Future research could investigate the effect TAP and EDTA when preceded by NaOCl. This combination may provide a rougher surface for stem cell attachment, but may also result in excessive surface loss. This combination would also be more clinically applicable since it more closely resembles what is currently being practiced.

In this study, EDTA was ineffective at increasing dentin surface roughness after 5 minutes. A 10-minute to 15-minute application of EDTA has been shown to cause a significant increase in surface roughness,[56,170] however, this extended duration may not be clinically practical. Future research could investigate the effect of higher concentrations of EDTA to increase roughness more quickly.

Several case reports have demonstrated successful healing with a shorter duration of antimicrobial treatment.[138] This would likely reduce surface loss but may also lead to inadequate surface roughness. Future research could investigate the effects of shorter duration treatments such as 1 week or 2 weeks on surface properties of dentin.

Despite the significant surface loss observed after 4 weeks in this study, it is unknown if this loss is clinically relevant with regard to fracture resistance. Treatment with 1000 mg/mL TAP has been shown to cause time-dependent decrease in fracture resistance that is statistically significant after 12 weeks (14-percent decrease) but not after 4 weeks. Future research should investigate both the effect of time and concentration of these solutions and medicaments on fracture resistance.

SUMMARY AND CONCLUSIONS

The null hypotheses that there is no significant difference in surface loss or surface roughness between all groups was rejected. Collectively, this study demonstrated that the 1000 mg/mL concentration of TAP used in endodontic regeneration caused a statistically significant increase in dentin surface loss and surface roughness. These effects are concentration dependent and are reduced with the 1 mg/mL concentration of TAP. The substantial amount of surface loss and surface roughness reported in this study were caused by TAP rather than EDTA. Furthermore, the use of EDTA after the two concentrations of TAP did not have significant additive effect on surface loss and surface roughness of radicular dentin. When choosing a concentration and duration of TAP and EDTA for regenerative endodontic procedures, the effect on surface loss and surface roughness should be considered. Further research is needed to determine the clinical significance of these effects on fracture resistance, stem cell attachment, and the outcome of regenerative endodontic procedures.

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ABSTRACT

THE EFFECT OF TRIPLE ANTIBIOTIC PASTE AND EDTA ON THE SURFACE LOSS AND SURFACE ROUGHNESS OF RADICULAR DENTIN

by

Andrew Nerness

Indiana University School of Dentistry
Indianapolis, Indiana

Introduction: Regenerative endodontic therapy in immature teeth with necrotic pulps triggers continued root development thereby improving the prognosis of these teeth. Several agents are under consideration for the disinfection and conditioning phases of this therapy. Triple antibiotic paste (TAP, i.e. equal parts of ciprofloxacin, metronidazole, minocycline) is used for canal disinfection and 17% EDTA solution is used for dentin conditioning. However, TAP and EDTA cause demineralization and their

effect on surface loss and surface roughness of radicular dentin during regenerative procedures has not been quantified. Surface loss may be correlated with reduced tooth strength and surface roughness may be correlated with stem cell attachment. Objectives: The aim of this in vitro study was to quantitatively investigate the surface loss and surface roughness on human radicular dentin after treatment with two concentrations of TAP followed by EDTA. Materials and Methods: Human radicular dentin specimens were prepared from extracted human anterior teeth and randomized into six experimental groups. Group 1: saline control; Group 2: 17% EDTA; Group 3: TAP 1 mg/mL; Group 4: TAP 1 mg/mL and 17% EDTA; Group 5: TAP 1,000 mg/mL; Group 6: TAP 1,000 mg/mL and 17% EDTA for 5 minutes. After TAP is applied to Groups 3-6, all groups were incubated for 4 weeks. Then, groups 2, 4, and 6 were treated with EDTA for 5 minutes. Dentin surface loss (μm) and surface roughness (Ra, μm) were quantified after various treatments using non-contact and contact profilometry, respectively. Data were analyzed by one-way analysis of variance ($\alpha = 0.05$) Hypothesis: It was hypothesized that there would be a significant difference in surface loss or surface roughness between at least two treatment groups. Results: All treatment groups showed significantly higher surface loss compared to untreated control. Dentin treated with 1g/mL TAP caused significant increase in surface loss and surface roughness compared to dentin treated with 1 mg/mL TAP. However, only 1g/mL TAP treated dentin showed significantly higher surface roughness compared to untreated control. The use of EDTA after both concentrations of TAP did not have significant additive effect on surface loss and surface roughness of dentin. Conclusion: The use of 1 mg/mL TAP can minimize surface loss

and surface roughness of radicular dentin compared to higher concentrations. The use of EDTA after TAP may not cause additional surface loss and surface roughness of dentin.

CURRICULUM VITAE

Andrew Nerness

1998 to 2002	General Education Courses Minnesota West College Worthington, MN
1998 to 2002	General Education Courses Minnesota West College Worthington, MN
2002 to 2005	BA in Biology Augustana College Sioux Falls, SD
2005 to 2006	Graduate Level Biology Courses University of Minnesota Minneapolis, MN
2007 to 2011	DDS University of Minnesota School of Dentistry Minneapolis, MN
2011 to 2012	GPR Certificate University of Minnesota/Fairview Hospital Minneapolis, MN
2012 to 2014	MSD in Endodontics, Oral Pathology Minor, Indiana University School of Dentistry Indianapolis, IN