

Effect Of Bioactive Irrigants On Intraradicular Dentin
Microstructure During Endodontic Therapy

BY

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THESIS

Submitted as partial fulfillment of the requirements

for the degree of Master of Science in Oral Sciences

in the Graduate College of the

University of Illinois at Chicago, 2012

Chicago, Illinois

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This thesis is dedicated to all the people who believed in me. My family for their love and support: my parents, Jonatas and Alzenira DeSouza, whose sacrifices and prayers have made my dreams attainable; my husband and my lovely precious daughters Isabella and Raquel. It would not have been accomplished without their encouragement at all times.

This thesis is also dedicated to the Department of Endodontics, at UIC-College of Dentistry, and especially to my professors who believed in me.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis committee, Dr. Ana Karina Bedran-Russo, Dr. Mohamed Fayad, Dr. Bradford Johnson and Dr. Christopher Wenckus for their constant support and encouragement throughout my research endeavors.

I would like to thank Dr. Bedran-Russo, my thesis advisor, for her dedication, offering her time, effort, and expertise to aid me throughout this project. I appreciate the use of her laboratory and facilities at the Restorative Dentistry Department.

I would like to acknowledge Dr. Fayad, also my thesis advisor and my mentor, who provided me with all the knowledge and support needed to help me during this process of achieving this dream in my career path.

I would also like to acknowledge Dr. Johnson and Dr. Wenckus who oversaw the development of the research project and made important contributions to the study. In addition, I would like to thank Dr. BeGole for her valuable insights and assistance with the statistical analysis of data.

A number of individuals in Dr. Bedran-Russo's lab were extremely helpful in the laboratory procedures and data collection process. These were Dr. Berdan Aydin, Dr. Lina Hassan, and my fellow residents Dr. Bahrani and Dr. Bell. Their contribution was crucial in the completion of this Master's thesis research.

I would also like to thank the AAE Foundation for the grant received toward this research project.

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LIST OF ABBREVIATIONS

TA	Tannic acid
GSE	Grape seed extract
GE	Genipin
NaOCl	Sodium hypochlorite
EDTA	Ethylenediamine tetracetic acid
CHX	Chlorhexidine
PBS	Phosphate-buffered saline
HBSS	Hank's balanced salt solution
PA	Proanthocyanidin
GD	Glutaraldehyde
FS	Flexural strength
UTS	Ultimate tensile strength
TBS	Tensile bonding strength
Td	Denaturation temperature
DSC	Differential scanning calorimetry
ANOVA	Analysis of variance
SD	Standard deviation
IRB	Institutional review board
SEM	Scanning electron microscope

SUMMARY

Sodium hypochlorite is the most recommended irrigating solution in endodontics for the removal of pulp remnants and dentin debris during instrumentation. However, it may cause changes to the organic and mechanical properties of dentin. Previous studies have shown that modification of the collagen by altering the number of cross-links enhances the mechanical properties and lowers the rate of enzymatic degradation. Plant derived natural products represent a rich source of antimicrobial compounds. Proanthocyanidin present in grape seed extract (GSE), and tannic acid have demonstrated the ability to induce inter- and intra-molecular collagen cross-links in biological tissue. Collagen cross-links affect the mechanical properties of demineralized dentin by increasing stiffness, and provides resistance against enzymatic degradation. The null hypotheses tested was that the use of crosslinking agents tannic acid (TA) and grape seed extract (GSE) as intra-canal irrigants will not have a significant effect on the flexural strength and dentin collagen biodegradation of radicular dentin.

Extracted single-rooted permanent teeth were sectioned into $7 \times 3 \times 0.3 \text{ mm}^3$ beams and randomly assigned into four groups according to the irrigation type : Group 1: Saline, Group 2: EDTA 17%, 5.25%NaOCl; Group 3:5.25% NaOCl, 20% TA , Group 4: 5.25% NaOCl, 30% GSE . Specimens were tested for flexural

SUMMARY (continued)

strength (MPa), and dentin collagen biodegradation rates by mass loss measurements , and denaturation temperature of dentin matrix using DSC. Statistical analysis were performed using one-way ANOVA test ($\alpha=0.05$) and revealed no statistically significant differences in flexural strength among groups ($p=0.595$). TA was borderline significance in reducing the collagen degradability when compared to the other irrigants, but the results were not statistically significant ($p= 0.069$).The results for the thermal stability of dentin matrix measured by DSC revealed no statistically significance ($p= 0.552$). Therefore, we concluded that the use of NaOCl, TA and GSE didn't affected the flexural strength, rate of collagen biodegradation and thermal stability of collagen thermal transition of root dentin, thus the null hypothesis was accepted.

I. INTRODUCTION

1.1 Background

Irrigating solutions flush pulpal debris, dentin chips, microorganisms, and bacterial biofilm out of uninstrumented surfaces of the root canal walls.

An ideal root canal irrigant should have a broad antimicrobial spectrum, inactivate endotoxins, low surface tension, ability to remove the smear layer, low toxicity, noncaustic to periodontal tissues, low cost, tissue solvent activity, lubricant, safe application, ability to stimulate hard tissue repair and prevent or reduce pain, effective in presence of pus and organic debris, and non-staining to the tooth and soft tissues (Penick, and Osetek, 1970; Senia et al., 1971).

The types of irrigants that have been recommended include acids, chelating agents, proteolytic enzymes, alkaline solutions, normal saline solution, and other chemical agents such as oxidizing agents.

The primary goal of an endodontic treatment is to obtain a clean root canal system free of microbiota and debris, which can then be sealed with a microbial-tight root canal filling. The chemomechanical preparation concept relates to the

use of chemically active irrigating solutions in combination with mechanical cleansing. To this end, sodium hypochlorite (NaOCl) solutions remain the most widely recommended irrigants in endodontics on the basis of their unique capacity to dissolve necrotic tissue remnants and their excellent antimicrobial potency, however, it may cause changes to the organic (mainly collagen, which acts as a matrix for the deposition of apatite crystals) and mechanical properties of dentin, such as microhardness, roughness, elastic modulus, flexural and fatigue strength, mainly due to proteolytic action of NaOCl on the collagen matrix of dentin (Sim, et al., 2001) In addition to NaOCl, the use of a chelating agent has been advocated to rid the root canal system of the smear layer consisting of dentin particles embedded in an amorphous mass of organic material that forms on the canal walls during the instrumentation procedure. It is believed that removing this layer could (a) dissolve attached microbiota and their toxins from root canal walls, (b) improve the seal of root canal fillings, and (c) reduce the potential for bacterial survival and reproduction (Torabinejad et al., 2002).

Most clinicians use NaOCl solutions in the range of 1%–5% (w/v) and EDTA at 17%. To reduce stress on endodontic instruments, it is advocated to keep the root canal system flooded with an aqueous solution during mechanical preparation (Peters et al., 2005). For that purpose, NaOCl is recommended to maximize working time on necrotic tissue remnants and microbiota. A chelating solution could then be used as the final rinse to remove the smear layer.

However, an alternating irrigating regimen with NaOCl and EDTA has also been recommended, with a final NaOCl flush after EDTA (Baumgartner et al., 1987).

Dentin is composed of approximately 22% organic material by weight. (Mareending et al., 2007), consisting primarily of Type I collagen, which determine to the mechanical properties of dentin . Sodium hypochlorite, a nonspecific oxidizing agent, is known to fragment long peptide chains and to chlorinate protein terminal groups; the resulting N-chloramines are broken down into other species.

The mineral phase in dentin protects the collagen matrix. It is therefore conceivable that demineralizing agents used for the removal of the smear layer can expedite the destructive hypochlorite effect . In other words, using a chelating agent before sodium hypochlorite exposes the organic dentin matrix, which can then be attacked by the subsequent hypochlorite rinse (Oyarzun et al., 2002). Overzealous use of irrigants might thus make root canal–treated teeth more prone to fracture. In this context, it appears important to notice that vertical root fractures are among the most common causes for the extraction of endodontically treated teeth(Mareending et al, 2007).

Cross-linkings increase the structural stability and strength of dentin collagen fibers. Previous studies had shown that modification to the collagen by

altering the number of cross-links, enhance the mechanical properties and lower rates of enzymatic degradation (Bedran-Russo, 2009).

They also contribute to making dentin collagen relatively insoluble in acid and neutral solutions as compared with collagen from most other sources. As a consequence, acid etching of dentin mostly removes the mineral phase, whereas the collagen remains almost intact (Haapasalo, 2007).

There has been a search for a natural irrigating solution with less potential for adverse effects. Plant derived natural products represent a rich source of antimicrobial compounds. Tannic acid (TA) is a condensed tannin, consisting of a complex mixture of polygalloylglucose esters, and is a naturally occurring polyphenol with weak acidity. Hydrogen bonds are formed between amide NH groups from collagen and hydroxyl groups from TA (Jastrzebska et al., 2006a).Its use on dentin affects the mechanical properties of demineralized dentin by modification of the collagen (Jastrzebska et al., 2006b). Grape seed extract (GSE), mainly composed of Proanthocyanidin (PA), is a natural cross-linker well known to readily precipitate proline rich proteins (such as collagen) due to hydrogen and covalent bonds (Bedran-Russo et al., 2007; Ku , 2007).

1.2 **Significance of the Study**

The development of a new regimen for endodontic irrigation which is not only antimicrobial but also offers low-toxicity to human tissues is of great importance. The use of biologically inspired mechanisms such as increased dentin matrix flexural strength by the presence of collagen cross-links and hydrogen bonds using plant-derived natural products and their application in endodontics is less well documented.

The data presented from the current study may open an opportunity for further studies of tannic acid and grape seed extract's efficacy and direct application in the endodontic field.

1.3 Specific Aims

The purpose of this study was:

- (a) To evaluate the effect of intracanal irrigation solutions on the mechanical properties of mineralized root dentin.
- (b) To investigate the effect of intracanal irrigation solutions on the rate of collagen biodegradation of mineralized root dentin.
- (c) To investigate the effect of intracanal irrigation solutions on the thermal stability of collagen of mineralized root dentin.

Irrigation solutions consisted of standard irrigation agents (saline; 17%EDTA> 5.25% NaOCl). Two different collagen cross linkers, 20% Tannic acid (TA) and 30% Grape seed extract (GSE) were included for comparison.

1.4 Hypothesis

The null hypothesis of the proposed study is that tannic acid (TA), grape seed extract (GSE), saline, and sodium hypochlorite (NaOCl) have no effect on the flexural strength, dentin collagen biodegradation and collagen thermal stability of intraradicular dentin after chemomechanical debridement.

II. REVIEW OF THE LITERATURE

2.1 Effect of endodontic irrigants on dentin

Chlorhexidine (CHX) is a potent antiseptic widely used for chemical plaque control in the oral cavity (Addy and Moran, 1997) at 0.1-0.2% concentration, while 2% is the recommended concentration for root canal irrigating solution (Zamany et al., 2003). The antiviral effect of CHX has also been reported (Baqui et al., 2001). Although CHX is useful as a final irrigant, it cannot be recommended as the main irrigant in endodontic cases since it is unable to dissolve organic components (Naenni et al., 2004). CHX adverse effects include tooth discoloration (Yamashita et al., 2003a), loss of taste, burning sensation of the oral mucosa, subjective dryness of the oral cavity, and tongue discoloration (Yusof and Khoo, 1988). The effectiveness of CHX to clean root canal walls is generally found to be inferior to NaOCl (Yamashita et al., 2003b).

Chelating agents act on calcified tissue by substituting sodium ions which combine with dentin to produce soluble salts for the calcium ions that are bound in a less soluble combination (Weine, 1982). EDTA, the most popular chelating agent, is a liquid solution of the sodium salt of ethylenediamine tetraacetic acid with a pH of 7.3, it was introduced into endodontics with the aim of facilitating preparation of calcified and narrow root canals by softening

root canal dentin, but it is also an irrigant with the capability to remove the smear layer, leaving the dentine tubules open.

Attempts have been made to increase antibacterial activity by adding antiseptics (EDTAC) (Nygaard, 1957) or tetracycline antibiotics (MTAD) (Torabinejad et al., 2003) to EDTA and citric acid respectively. EDTAC demonstrates similar smear-removing efficacy as EDTA, however it is more caustic (Patterson, 1963).

It's well known that sodium hypochlorite (NaOCl) is a non-specific proteolytic agent that is capable of removing organic material (through oxidation NaOCl dissolves organic tissues of predentin and pulp), as well as magnesium and carbonate ions, and have a strong antimicrobial activity.

The antimicrobial action results from the release of hypochlorous acid when it contacts organic debris, disrupting cell metabolism and leading to cell death (Siqueira et al., 1997; Baumgartner and Cuenin, 1992). Besides the wide spectrum, nonspecific killing efficacy of all microbes, hypochlorite preparations are also known to be sporicidal, virucidal and show greater tissue dissolving effects on necrotic than on vital tissues (McDonnell and Russel, 1999).

NaOCl encompasses many desirable properties of a main root canal irrigant, however, the superficial destructive effect of NaOCl on mineralized dentin is irreversible and is present irrespective of whether EDTA 17% is subsequently employed as the final active irrigant. The EDTA removes the collagen depleted apatite phase to expose the underlying dentin that is morphologically perceived as canal wall erosion (Zhang et al., 2010). NaOCl is able to remove collagen fibers and thereby to prevent the hybrid layer formation, which is considered by many authors as the fundamental qualification for a correct and adequate adhesion to dentin (Guida, 2006).

When using NaOCl over extended periods of time during treatment, NaOCl seems to have an undesired side effect on the flexural strength of dentin (Kai et al., 2010). The decline in flexural strength is probably attributed to the generation of a brittle layer of apatite crystallites that are not supported by a structurally intact collagen matrix. It's well known that the mineral component in hard connective tissues contributes to strength and elastic modulus, whereas the collagen component is responsible for toughness of the tissues (Wang et al., 2001). Destruction of the collagen matrix in mineralized tissues results in a less tough, more brittle substrate that might precipitate fatigue crack propagation during cyclic stresses (Kruzic and Ritchie, 2008). This might increase the susceptibility of root-treated teeth to post-treatment crown or root fracture. One study investigated the influence of irrigants on flexural strength of dentin bars and concluded that a 24-minute

exposure time to a 2.5% hypochlorite solution caused a significant drop in flexural strength, which might result in fracture of endodontically treated teeth (Marending et al., 2007). The relation between erosion and microhardness of root canal dentin was evaluated in one study and the author concluded that erosion was not the main factor in decreasing the dentin microhardness, whereas the amount of irrigant (2.6% NaOCl, 17%EDTA, MTAD, 2% Chlorhexidine) penetration might be the main cause (Saghiri, et al., 2009).

Armstrong et al., (2008) studied the denaturation temperature (Td) of demineralized dentin matrix as a function of infiltration with water vs. polar solvents vs. adhesive resins, and concluded that the presence of water-free organic solvents increased the thermal stability of demineralized dentin collagen matrices. Interpeptide hydrogen bonding seems to stabilize collagen to thermal challenge.

White et al., (1973) and Weadock et al., (1983-1984) studied the effect of glutaraldehyde crosslinking on physical and biological properties of collagen using collagenase derived from *Clostridium histolyticum*, which hydrolyzes the peptide bond on the amino side of Gly in -X-Gly-Pro- as its substrate specificity.

Collagen was rapidly degraded to relatively small peptides by this collagenase. Crosslinking with GD prevented the enzyme from penetrating the collagen molecule in order to reach the sites of enzymatic reaction, and

even when the enzyme dose cleaved a collagen molecule, crosslinks maintained the structure of the molecule and prevented further action of the enzyme.

According to the study by Sim et al. (2001), a 2-hour exposure of dentin to NaOCl solutions of more than 3% (w/v) significantly decreases the elastic modulus and flexure strength of human dentin compared with a control exposure to physiologic saline, thereby possibly contributing to the weakening of root canal-treated teeth.

Since many of the chemical irrigant agents exhibit adverse effects on mechanical properties of coronal and root dentin(Soares et al., 2007; Moreira et al., 2009; Pascon, 2009), the search for agents that minimize damage is increasing.

Plants produce a wide variety of secondary metabolites, which are bioactive compounds. It is widely accepted that this chemical diversity is what serves to protect plants against microbial pathogens (Dixon, 2001). Proanthocyanidin (PA), widely present in fruits, vegetables, nuts, seeds, flowers is a potent anti-oxidant cross-linking agent with vast biological activities. Recently, the use of a Grape seed extract, mainly composed of PA, has been shown to improve the mechanical properties of demineralized

dentin (Bedran-Russo et al., 2007; Bedran-Russo et al., 2008a), and reduce biodegradation rate of collagen (Macedo et al., 2009).

2.2 Collagen in Dentin

In dentin, Type I collagen acts as a scaffolding and accommodates a large portion (56%) of the mineral content in the holes and pores of the fibril (Nanci and Arnold, 2003). The mechanical properties of the collagen fibrils, such as, stability, viscoelasticity and tensile strength, depend on a highly regulated mechanism of intermolecular cross-linking between the fibrils (Sung et al., 1999).

Treating collagen with chemical collagen cross-linkers induces a greater number of cross-links between collagen molecules, leading to an increase in its tensile and other mechanical properties (Charulatha and Rajaram, 2003).

Cross-linking bonds in collagen can be induced by chemical reactions initiated by heat, pressure, radiation, change in pH or by treatment with reagents called chemical collagen cross-linkers. Chemical cross linkers can be broadly classified into naturally occurring (PA and TA) and synthetic agents (GD).

2.2 Tannic acid as a cross-linking agent

Tannic acid (TA), a commercial form of condensed tannin, is a naturally occurring polyphenol with weak acidity that has the ability to modify collagen chemically (Jastrzebska et al., 2006). It has been determined that TA functions as a collagen cross-linking agent through hydrogen-bonding mechanisms and hydrophobic effects. TA may also have anti-tumor and anti-microbial properties (Cass and Burg, 2012).

Li et al. studied tannins extracted from immature fruits of *Terminalia chebula Fructus Retz* and its application for the treatment of cutaneous wounds in rat as well as its antibacterial effects. After optimal extraction and purification, the content of tannin extracts was increased to 81% (Li et al., 2011).

Tannin extracts showed the inhibition of *Staphylococcus aureus* and *Klebsiella Pneumonia in vitro*. The results suggested that tannin extracts from dried immature fruits of *Terminalia chebula Fructus Retz* can promote cutaneous wound healing in rats, probably resulting from a powerful antibacterial and angiogenic activity of the extracts.

Hargerman et al. (1981), indicated that TA binds to proteins via hydrogen bonds between phenolic hydroxyl residues of TA and =CO of proteins.

Pankhurst showed that binding of TA and collagen is mediated by hydrogen bonds between phenolic hydroxyl residues of TA and -NH- of collagen or by forming crosslinking structures of ionic bonds between collagen side chains (Pankhurst, 1958).

In the study by (Koide and Daito, 1997), the tensile tests, imbibition test and anti-enzyme test were performed on collagen films mainly composed of Type I collagen after ultraviolet irradiation and immersion in 0.5% TA and glutaraldehyde, and demonstrated that the tensile strength increased in the GA and TA treatment groups, and the anti-enzyme activity was markedly improved by GA and TA treatments. The findings of this study showed that the crosslinking density increased with the reaction time in all crosslinking techniques used, and that mechanical properties were changed. Bedran-Russo et al. (2009a), studied the mechanical properties of tannic acid treated dentin matrix. Dentin beams specimens (0.5 x 1.7 x 6.5 mm dimensions) were made into hour-glass-shaped samples with a neck area. The samples were fully demineralized in 10% phosphoric acid solution and divided into 4 treatment groups: control (distilled water), 1% TA, 10% TA, and 20% TA. For ultimate tensile strength (UTS) evaluation, the specimens were mounted on a microtensile tester machine and tested at a crosshead speed of 1 mm/min. Analysis of this study demonstrated the ability of TA-dentin matrix complexes to increase the mechanical properties of dentin, reduce enzymatic degradation, and increase resin-dentin bond strength.

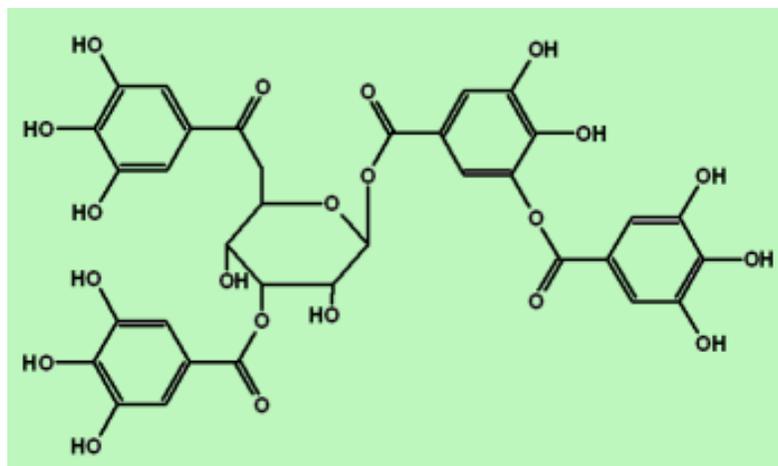


Figure1. The chemical structure of tannic acid

2.4 Grape seed extract as a cross-linking agent

Grape seed extract is composed mainly of proanthocyanidin (PA) that is naturally occurring plant metabolite bioflavonoid. PA has been proven to be safe in various clinical applications. Studies have also shown that PA increased collagen synthesis and accelerated the conversion of soluble collagen to insoluble collagen during development. PA is a potent antioxidant known to possess vasodilation, anti-inflammatory, antibacterial and immunostimulating effects.

Proanthocyanidin (PA) is widely available in fruits, vegetables, nuts, seeds, flowers and barks (Bedran-Russo et al., 2009b). As a bioflavonoid, it contains a benzene-pyran-phenolic acid molecular nucleus (referred to as

flavin) as part of its much larger molecular structure. PAs are a mixture of monomers, oligomers, and polymers of flavan-3-ols (known as catechins), which are ubiquitous in plants.

Widely used as natural antioxidants and free-radical scavengers, PAs have been proven to be safe in various clinical applications and as dietary supplements (Fujii et al., 2007; Yamakoshi et al., 2002). PAs are considered one of the most important classes of secondary metabolites in the plant kingdom (Han et al., 2003a). GSE has also been shown to improve the mechanical properties of demineralized dentin by increasing the tensile bond strength (Bedran-Russo et al., 2008b).

PA from grape seed extract (GSE) have been thought to prevent ischemia/reperfusion damage caused by reactive oxygen species such as superoxides and peroxynitrites (Aldini et al., 2003). PA from cranberry inhibited the surface-adsorbed glucosyltransferases and F-ATP activities, and the acid production by *Streptococcus mutans* (Duarte et al., 2006). Studies have also shown that PAs increased collagen synthesis and accelerated the conversion of soluble collagen to insoluble collagen during development (Rao and Steinmann, 1983). PA-treated collagen matrices were demonstrated to be nontoxic and resisted enzyme digestion in vitro and in vivo (Han et al., 2003b).

GSE interacts with proteins to induce cross-links by four different mechanisms: covalent interaction, ionic interaction, hydrogen bonding interaction or hydrophobic interactions (Han et al., 2003c; Pierpoint, 1969; Loomis, 1974). A competitive binding assay studied the relative affinity of various proteins and PA showed that proline-rich proteins like collagen have an extremely high affinity for PA based components, forming a Proline-PA complex. The stabilization of collagen fibers through hydrogen bond formation resulted in an increase in the denaturation temperature of the fixed tissue (Han et al., 2003d; Hangerman and Butler, 1981).

Al-Ammar investigated the effect of three different cross-linking agents (Glutaraldehyde [GD], Grape seed extract [GSE], and Genipin [GE]) on the tensile bond strength (TBS) of resin-dentin bonds. All solutions had the pH adjusted to 7.4 using NaOH, the dentin surface was initially etched using a 37% and 35% phosphoric acid gel for 15 seconds, then thoroughly rinsed with water for 15 seconds and kept moist, and all teeth were immersed in their respective solutions for 1 hour. The results of this study showed that the highest bond strength was observed for the GSE treated group, which was statistically higher than all the other experimental groups, demonstrating changes to the mechanical properties of dentin matrix (Al-Ammar et al, 2009).

Xie et al. used an in vitro pH-cycling model to evaluate the effect of GSE on the remineralization of artificial root caries, and concluded that GSE

positively affected the demineralization and/or remineralization processes of artificial root caries lesions, most likely through a different mechanism than that of Fluoride, demonstrating that GSE may be a promising natural agent for non-invasive root caries therapy (Xie et al, 2008).

Castellan et al. compared the effect of two different PA-based cross-linker (cocoa and grape seed extracts) on resin-dentin bonded interface using two different treatment times (10 min and 60 min), with 10 min representing a time exposure more clinical relevant, and characterized the effect of different sources of PA on the mechanical properties and resistance against enzymatic degradation of demineralized dentin matrices. Moreover, failure at the bonded interface may lead to the formation of pathways in which oral fluid, bacterial products and endogenous proteolytic enzymes can degrade the components. Deterioration of the dentin collagen fibrils has been suggested as a possible mechanism responsible for adhesive bonds degradation. The demineralized dentin treated with PA-based agents decreased collagenase degradation, increased mechanical properties and reduced water absorption. This study concluded that the quantity and types of PA may influence its interaction with collagen fibrils and consequently the resin-dentin bond strength (Castellan et al, 2011).

Bedran-Russo systematically characterized dentin matrices biomodified by proanthocyanidin-rich grape seed extract (GSE) and glutaraldehyde (GD). Changes to the biochemistry and biomechanical properties were assessed by

several assays to investigate the degree of interactions, biodegradation rates, proteoglycans interaction, and effect of collagen fibril orientation and environmental conditions on the tensile properties. The results of this study showed the highest degree of agent-dentin interaction was observed with GSE which exhibited the highest denaturation temperature, regardless of the agent concentration (Bedran-Russo et al., 2011).

Biodegradation rates remarkably decreased following biomodification of dentin matrices after 24hours of collagenase digestion (Macedo et al., 2009). A significant decrease in the proteoglycan content of GSE treated samples was observed using a micro-assay for glycosaminoglycan and histological electron microscopy, while no changes were observed for GD and control. Biomodification of dentin matrices using chemical agents not only affected the collagen biochemistry, but also involved interaction with proteoglycans.

The use of collagen cross-linking agents may induce additional formation of inter and intra-molecular cross-links, which may increase strength of dentin and may decrease the degradation rates of collagen(Macedo et al., 2009).

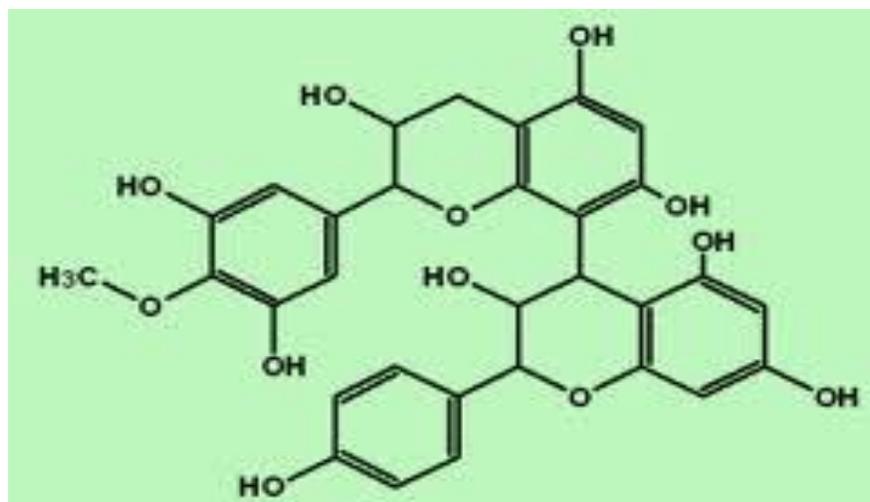


Figure2. The chemical structure of proanthocyanidin

III. METHODOLOGY

3.1 Study design

The experimental protocol was deemed exempt by the Institutional Review Board at the University of Illinois at Chicago (Appendix A, research protocol # 2011-0262).

Power analysis was performed to determine the sample size in each group and for each experimental test, based on power = 0.8 and $p=0.05$. It was determined that twenty seven dentin beams were needed for each group for the flexural strength test, with a total sample size of one hundred and eight beams. Eleven dentin beams ($n=11$) for the collagen biodegradation test, with a total sample size of forty four, and five dentin beams ($n=5$) to measure the denaturation temperature of the collagen, with a total sample size of twenty.

Tannic acid was obtained from Fisher Biotech (Fair Lawn, NJ, USA). A 20% (w/v) solution in phosphate buffer was used. Grape seed extract from MegaNatural, Polyphenolics (Madera, CA). It consisted of 97.8% PA according to data provided by the manufacturer. A 30% (w/v) solution in phosphate buffer was used in this study. Bacterial collagenase

from *Clostridium histolyticum* (type I, ≥ 125 CDU/mg solid) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Extracted permanent human anterior teeth were collected. Prior to experimentation, all teeth were stored in distilled water with 0.5% Thymol solution. All teeth were decoronated at the cemental-enamel junction, and all roots sectioned at the apical portion to a standardized length of 7mm, keeping the middle portion of the root. The prepared root samples were placed in a glass container filled with Hank's Balanced Salt Solution (HBSS) identified by tooth number. The number of root samples obtained from each tooth were assigned to four groups according to the irrigation type and also assigned to the experimental tests. Therefore each tooth had its own control.

3.2 Test groups

Specimens from each tooth were randomly divided into the following groups according to the irrigation protocol: Group 1: Control -Saline irrigation, pH 7.0; Group 2: Irrigation with 5ml of EDTA 17% (pH 7.4) for 60 seconds, followed by a final rinse with 10ml of 5.25% NaOCl (pH 12.08) for 2 minutes; Group 3: Irrigation with 10ml of 5.25% NaOCl for 2 minutes, rinse with saline, followed by 5ml of 20% TA for 60 seconds, and a final rinse with saline; Group 4: Irrigation with 10ml of 5.25% of NaOCl for 2 minutes, rinse with saline, followed by 5ml of 30% GSE for 60 seconds, and a final rinse with saline.

The pH of the 20%TA and the 30% GSE were adjusted to remain between 7.2 to 7.6, using NaOH.

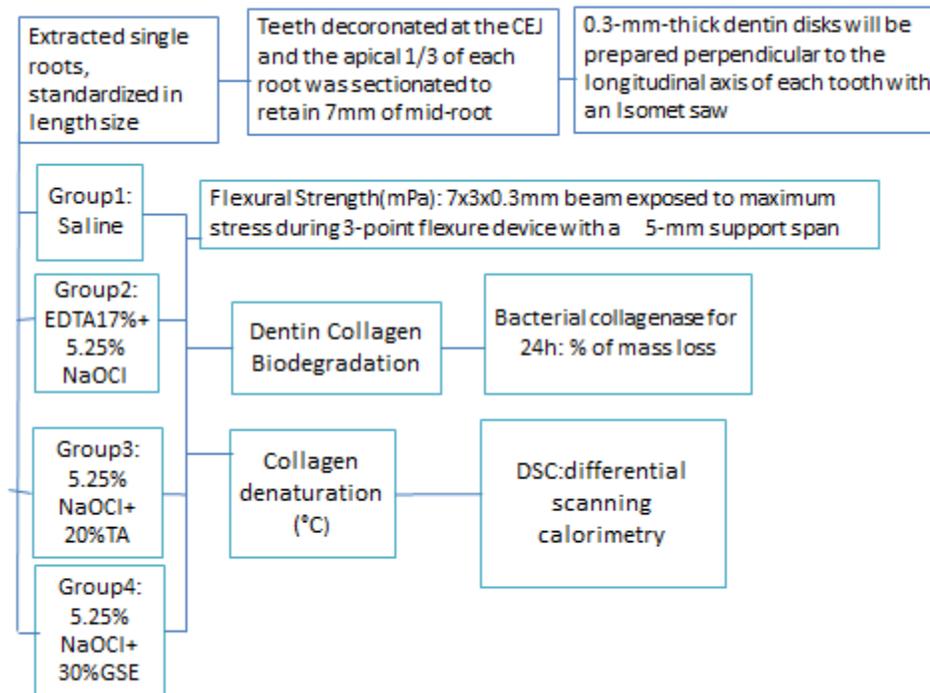


Figure3. Flowchart of the methodology used in this study.

3.3 Tooth specimen preparation

Dentin disks were obtained from mid-root dentin by sectioning in the mesio-distal direction and parallel to the longitudinal axis of each tooth with an Isomet saw(Buehler-Series 15LC Diamond, Buehler, Lake Bluff, IL, USA) under water cooling. Dentin beams were prepared from the center of each disk and further trimmed by means of a cylindrical diamond bur (#557D,

Brasseler, Savannah, GA, USA) to a final rectangular dimension of 0.3mm thickness x 3 mm width x 7 mm length.

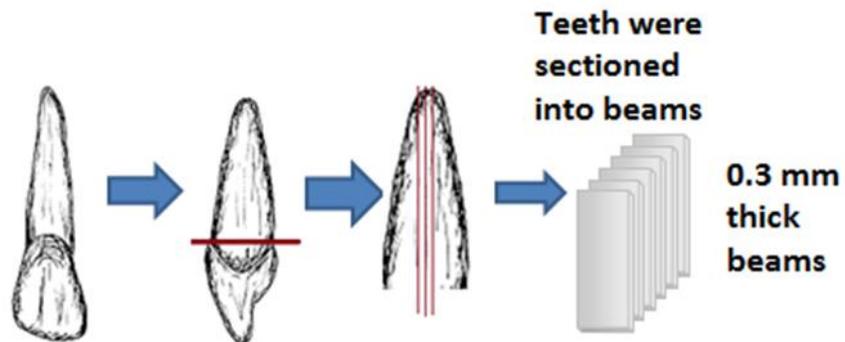


Figure4. Tooth specimen preparation procedure.

3.4 Pilot study

A pilot study was conducted prior to the actual experiment to evaluate the methodology, using a total of 12 dentin beams divided into 4 groups according to the previously mentioned methods. Data analysis was performed by one-way ANOVA test ($\alpha = 0.05$), and revealed no statistically significant differences in flexural strength among groups ($p = 0.2572$), although higher values were observed for TA ($206.90 \pm 7.55 \text{ MPa}$) in comparison to EDTA+NaOCl ($177.36 \pm 27.14 \text{ MPa}$), GSE ($182.76 \pm 29.24 \text{ MPa}$), and Saline ($168.09 \pm 19.15 \text{ MPa}$). TA significantly reduced collagen degradability ($p = 0.0324$) when compared to the other irrigants: EDTA+NaOCl (42.6%), TA (9%), GSE (29.4%), Saline (33.20%).

Therefore, the preliminary results showed that the application of chemical cross-linking agents did not enhanced the dentin strength, but TA significantly enhanced collagen stability.

3.5 Mechanical properties- Flexural Strength

Flexural strength evaluation was performed by using mid-root dentin. A 7 x 3 x 0.3 mm beam was prepared from the center of each disk to ensure that dentinal tubules were oriented parallel to the plane of maximum stress during the 3-point flexure device with a 5-mm support span. The dentin beams from each tooth were divided into 4 groups (n=27) and subjected to the irrigation protocol described above.

Each 7-mm-long beam was placed on top of the support span and loaded to fracture under water by using a universal testing machine (MTS model1125, Eden Praire, MN) at a crosshead speed of 1mm/min.

Flexural strength was calculated with the formula $\frac{3PL}{2bd^2}$, where P= load fracture(N), L= length of support span (mm), b= beam width(mm), and d= beam thickness (mm) (Figure 5).

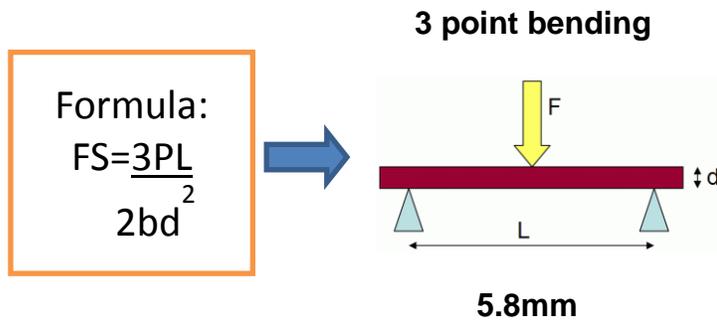


Figure 5. Beam under three point bending

3.6 Collagenase Biodegradation

Collagenase derived from *Clostridium histolyticum* hydrolyzes the peptide bond on the amino side of Gly in -X-Gly-Pro- as its substrate specificity (White et al, 1973). Collagen is rapidly degraded to relatively small peptides by this collagenase. Crosslinking is thought to prevent the enzyme from penetrating the collagen molecule in order to reach the cleavage sites. Even when the enzyme does cleave a collagen molecule, crosslinks will maintain the structure of the molecule and prevent further action of the enzyme (Weadock et al., 1983-1984).

Dentin beams described above, but with dimensions of 2mm x 2mm x 0.3mm were made, and treated according to the four groups of irrigating solutions described earlier (n=11). Specimens were then demineralized with 1.5ml 10% phosphoric acid for 5 hours at room temperature. The samples were placed in a vortex shaker positioned horizontally. The insoluble residue was washed with distilled water by repeated cycles. The samples were placed

in a dessicator under vacuum overnight to dry. The following day, samples were weighed and rehydrated with distilled water for 30 minutes. Each sample was suspended in 1.5ml of 1% w/w bacterial collagenase with ammonium bicarbonate buffer (pH=9.5) for 24 hours at 37°C. Samples were then placed in the dessicator overnight and the amount of collagen digested was calculated as a percentage of dry mass loss of the residues(initial weight/post-digestion weight x 100).

3.7 Differential Scanning Calorimetry (DSC)

Dentin disks with dimensions of 2mm x 2mm x 0.3 mm were obtained from mid-root dentin using an isomet saw described before. These specimens were submitted to the irrigation protocol mentioned above (n=5).

Measurement of denaturation temperature: Specimens sealed in high-pressure stainless steel pans and weighted were placed in a DSC (Model Pyris1 DSC, Perkin-Elmer Life and Analytical Sciences, Inc., Wellesley, MA, USA) and were scanned from 25 to 300 °C at a rate of 5 °C/min. An empty reference stainless steel pan was used as control. Subsequent cooling and reheating confirmed that the collagen denaturation was irreversible. The DSC was calibrated prior to use with iridium standards. The endothermic transition was recorded as peak denaturation temperature.

3.8 Surface Morphology (SEM) Analysis

Dentin disks with dimensions of 2mm x 2mm x 0.3 mm were obtained from mid-root dentin using an isomet saw described before. These specimens were submitted to the irrigation protocol mentioned above (n=2). After treatments, the samples were dehydrated in the following sequence: 50% alcohol for 20 minutes, 75% alcohol for 20 minutes, 95% alcohol for 30 minutes and 100% alcohol for 1 hour. The dried dentin beams were mounted on stubs, gold sputter-coated (SEM Coating Unit E5150, Polaron Equipment Ltd., PA, USA), and evaluated under SEM(S-3000 Hitachi, Tokyo, Japan).

3.9 Statistical Analysis

Data for each experiment were analyzed using one-way ANOVA (SPSS for windows, Version 19, SPSS Inc., Chicago, IL, USA). A level of significance of $\alpha = 0.05$ was used in all statistical tests (flexural strength, rate collagen biodegradation, and collagen thermal stability).

One-way ANOVA was used for comparison between groups for all treatment agents.

IV. RESULTS

4.1 Flexural Strength

The flexural strength results are summarized in Tables I and II. The mean values and standard deviation for each group are shown in Figure 6. Flexural strengths of specimens treated with TA had the highest values, followed by GSE group. One-way ANOVA showed no statistically significant differences in dentin mechanical properties evaluated among groups ($p= 0.595$).

TABLE I

MEAN VALUES WITH STANDARD DEVIATION AND STANDARD ERROR OF FLEXURAL STRENGTH(MPa) FOR DENTIN SPECIMENS.

Groups	Mean± Std. Dev.	Std. Error
Saline	194.74 ± 61.54	11.84
EDTA, NaOCl	211.02 ±49.81	9.58
NaOCl, 20%TA	214.49 ± 63.62	12.24
NaOCl,30% GSE	203.48± 52.67	10.13

TABLE II
ONE-WAY ANOVA FOR COMPARISON BETWEEN GROUPS
OF IRRIGATING AGENTS OF DENTIN SPECIMENS.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6224.432	3	2074.811	.634	.595
Within Groups	340385.529	104	3272.938		
Total	346609.961	107			

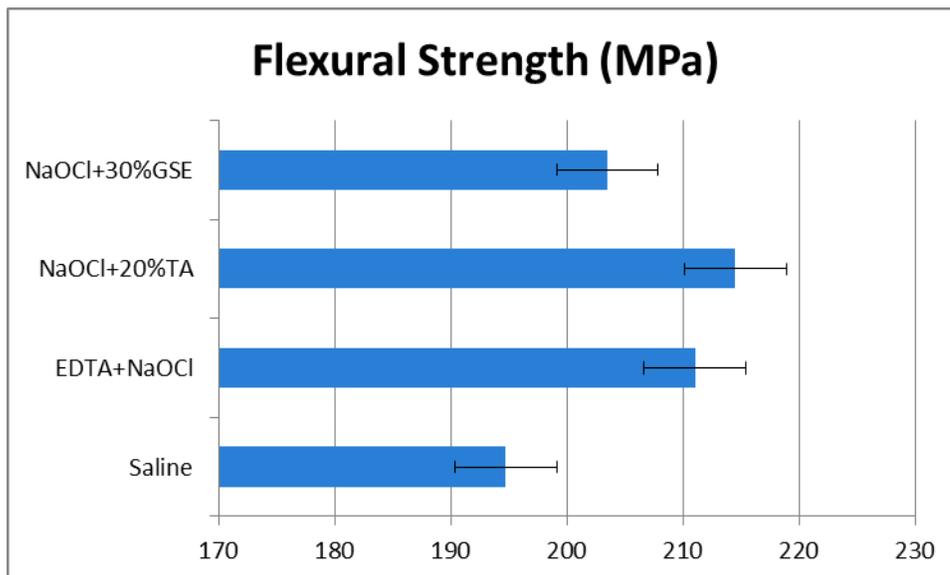


Figure6. Comparison of the mean FS among groups after treatment with irrigating solutions and maximum stress experienced within the dentin beam during 3 point bending flexural strength.

4.2 Dentin collagen biodegradation

No statistically significant differences in the rate of collagen biodegradation among groups were observed ($p=0.069$). Biodegradation rates did not significantly decrease following biomodification of dentin matrices by collagenase digestion. (Tables III and IV, Figure7).

TABLE III

MEAN VALUES WITH STANDARD DEVIATION AND STANDARD ERROR OF DENTIN COLLAGEN BIODEGRADATION (% OF MASS LOSS) FOR DENTIN SPECIMENS.

Groups	Mean± Std. Deviation	Std. Error
Saline	27.23±19.59	5.90
EDTA, NaOCl	26.67±14.27	4.30
NaOCl, 20%TA	13.77±12.14	3.66
NaOCl, 30% GSE	31.42±16.46	4.96

TABLE IV

ONE-WAY ANOVA FOR COMPARISON BETWEEN GROUPS OF % OF MASS LOSS OF DENTIN SPECIMENS AFTER 24HOURS EXPOSURE TO THE COLLAGENASE.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1924.586	3	641.529	2.550	.069
Within Groups	10061.431	40	251.536		
Total	11986.017	43			

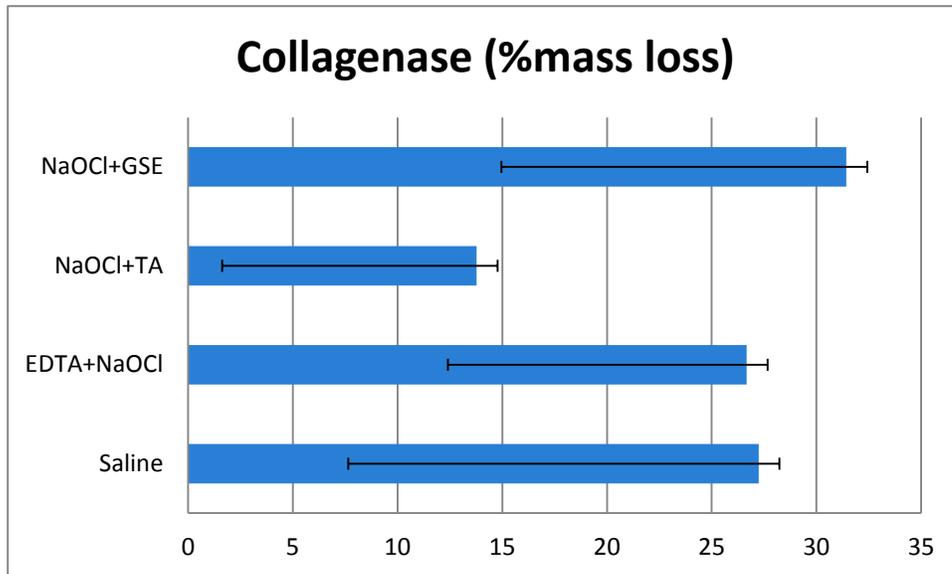


Figure 7. Comparison among groups of collagen degradation after collagenase digestion.

4.3 Differential Scanning Calorimetry (DSC)

The denaturation temperature results are shown in Tables V and VI. No statistically significant differences in the denaturation temperature of the dentin matrix among groups were observed ($p=0.55$). The collagen thermal transition of the root dentin were not affected by the collagen cross-linking treatment agents (Figures 8-12).

TABLE V

MEAN VALUES WITH STANDARD DEVIATION AND STANDARD ERROR OF DENATURATION TEMPERATURE (TD) OF DENTIN MATRIX COLLAGEN AFTER IRRIGATION TREATMENT.

Groups	Mean± Std. Deviation	Std. Error
Saline	185.18±21.72	9.71
EDTA, NaOCl	196.90±5.28	2.36
NaOCl, 20% TA	202.88±29.02	12.97
NaOCl, 30% GSE	197.36±13.65	6.10

TABLE VI

ONE-WAY ANOVA FOR COMPARISON BETWEEN GROUPS OF DENATURATION TEMPERATURE (TD) OF DENTIN MATRIX COLLAGEN SPECIMENS AFTER THE USE OF DIFFERENT IRRIGATING SOLUTIONS.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	831.388	3	277.129	.725	.552
Within Groups	6114.301	16	382.144		
Total	6945.689	19			

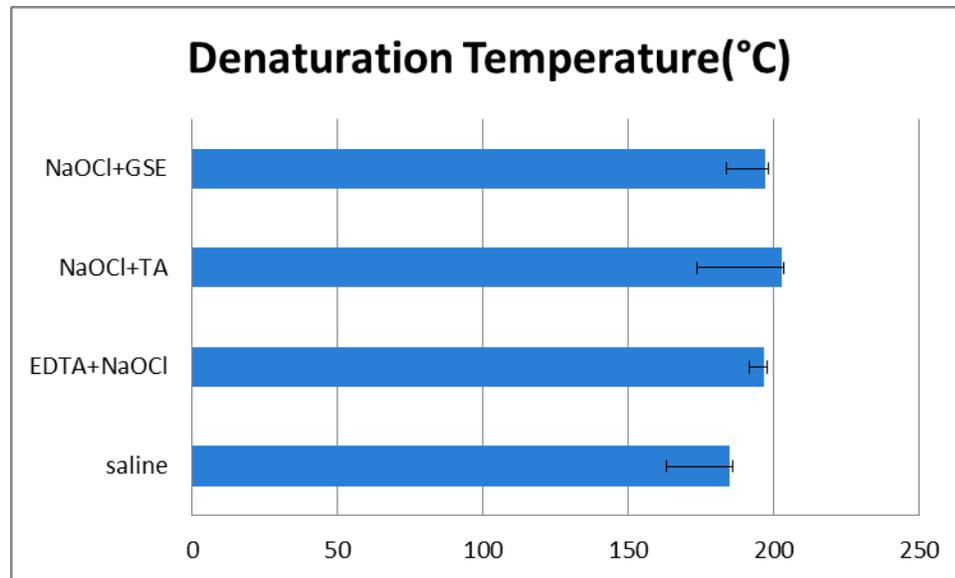


Figure 8. Comparison among groups of denaturation temperature (Td) of dentin matrix collagen

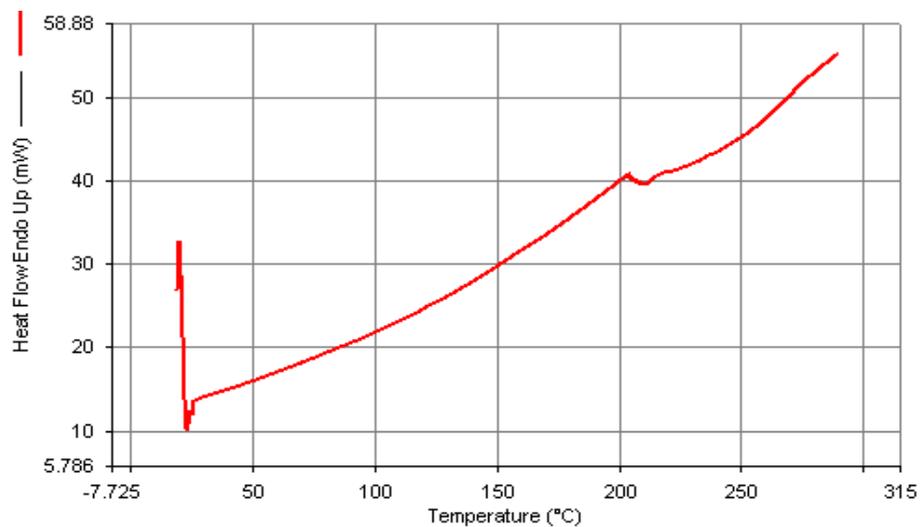


Figure 9. Saline (control group) collagen denaturation peak temperature

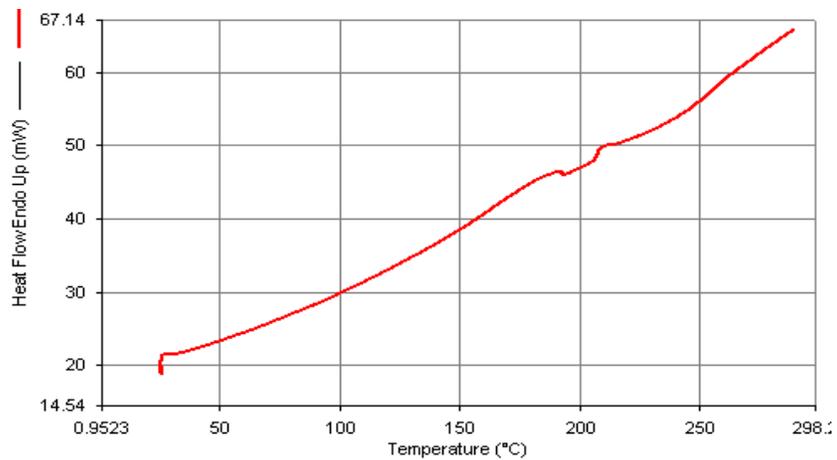


Figure 10. EDTA-5.25%NaOCl group: collagen denaturation peak temperature

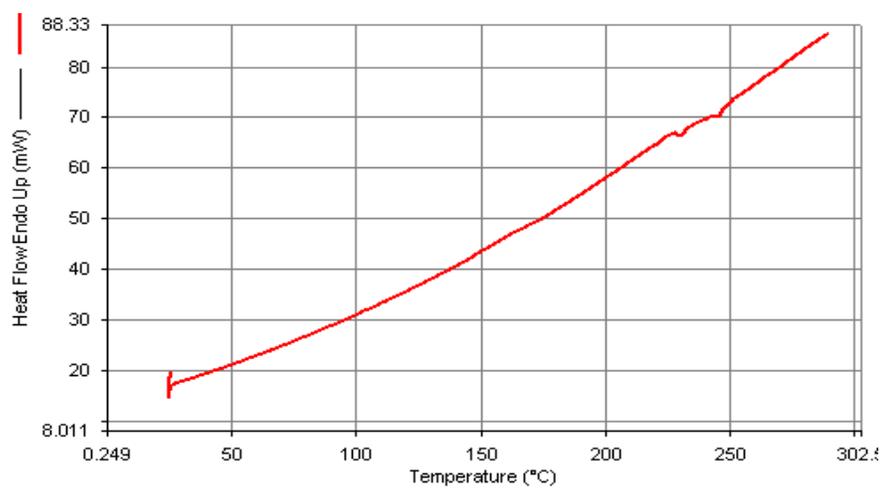


Figure 11. 20%TA group: collagen denaturation peak temperature

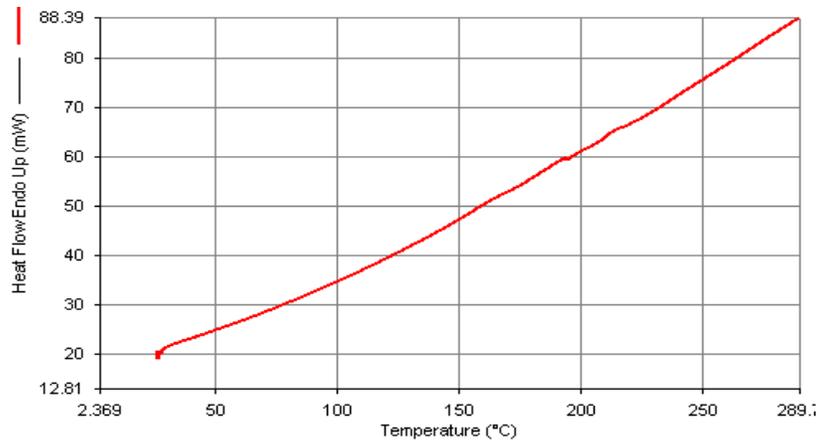


Figure 12. 30%GSE group: collagen denaturation peak temperature

4.4 SEM Analysis Results

The surface morphology was examined for each group after irrigation treatment protocol using SEM, results showed distinct pattern for EDTA-5.25%NaOCl treated group when compared to saline, TA, and GSE groups. EDTA-5.25%NaOCl group demonstrated open dentinal tubules and absence of the smear layer, most likely due to the chelating properties of the EDTA. The other groups showed similar pattern of thick layer of dentin smear covering the dentine tubules (Figures 13-16).

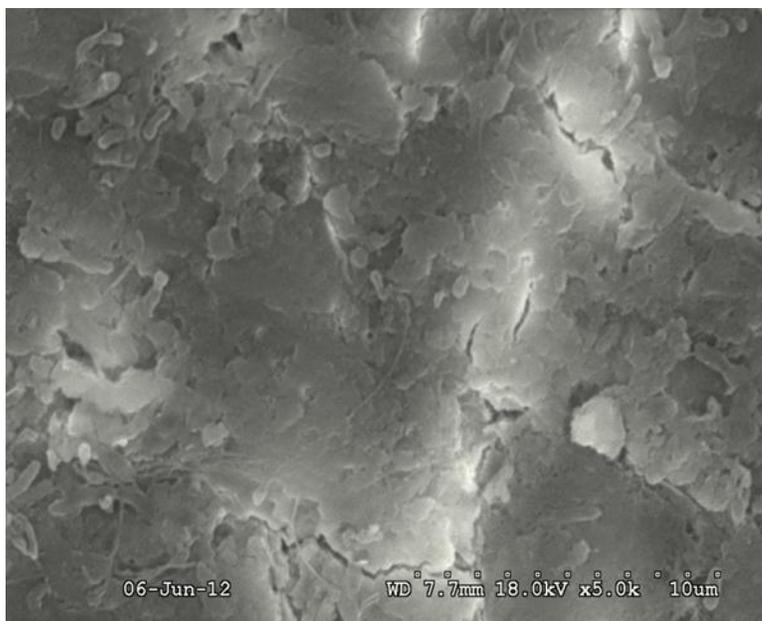


Figure 13. SEM of the saline group : surface morphology

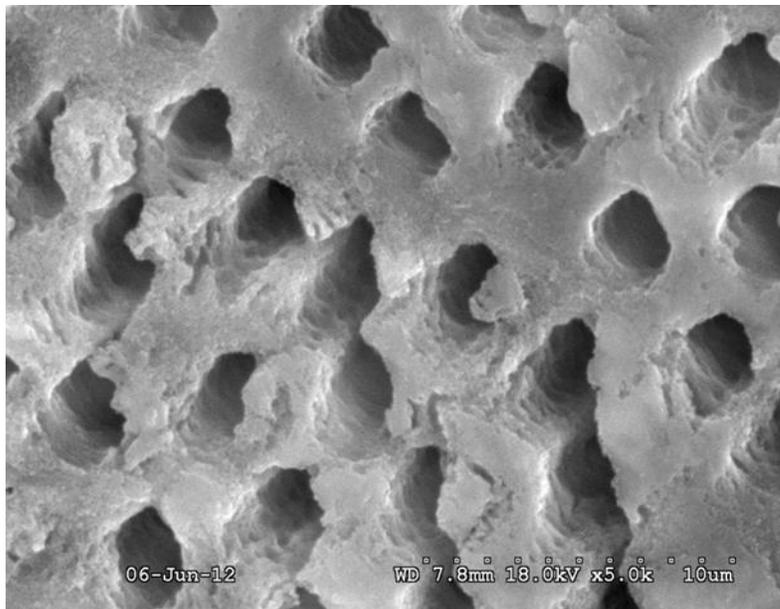


Figure 14. SEM of the EDTA-5.25%NaOCl group : surface morphology



Figure 15. SEM of the TA group: surface morphology

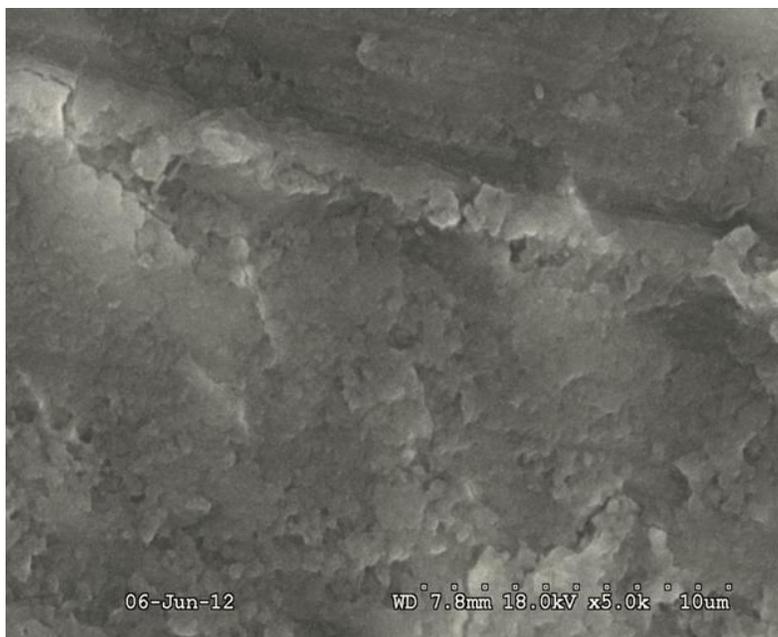


Figure 16. SEM of the GSE group: surface morphology

V. DISCUSSION

5.1 Study findings

The mechanical properties of dentin have been attributed mainly to the packing and density of mineral particles (Marshall et al., 1997; Kinney et al., 2001), the amount of intertubular dentin, and/or tubule density (Konishi et al., 2000).

The aim of this study was to evaluate the effect of intracanal irrigation solutions on the intraradicular dentin and to investigate the effects of two different collagen cross linking agents (20% Tannic acid and 30% Grape seed extract) in the intraradicular dentin. The treatment groups were compared and analysed. The analysed data and the results are discussed with respect to three aspects: comparison of the flexural strength of each cross-linking agent, the rate of collagen biodegradation, and the thermal transition of the collagen.

Storage of the teeth in 0.1% thymol for up to 3-4 months should not influence the outcome variables under investigation.

Flexural strength is a method to determine the the force applied perpendicular to the long axis of a specimen with a 0.5 mm/min crosshead speed until its moment of fracture(Vieira et al., 2012). The use of extrinsic cross-linking agents

to induce the formation of additional inter and intra-molecular cross-links in collagen and thus improving its mechanical structure and increasing the mechanical properties of dental substrates has been widely studied (Al-Ammar et al., 2009; Bedran-Russo, Castellan, Hassan, and Antunes, 2010; Bedran-Russo et al., 2007-2008-2009). They have also been shown to affect tooth demineralization and remineralization and increase resistance to caries (Walter et al., 2008; Xie et al., 2008).

In this study, TA and GSE exhibited no increase in flexural strength of partially demineralized or mineralized root dentin after treatment time (1 minute) when compared with saline or the standard irrigation (NaOCl-EDTA-NaOCl). Moreover, EDTA and NaOCl failed to demonstrate a significant effect of irrigant sequence on mechanical dentin properties. Consequently, the short-term use of EDTA (1 minute) before sodium hypochlorite did not increase the destructive effect of NaOCl on dentin and did not significantly affect the flexural strength.

The results from this study differ from Marending et al.(2007), who assessed the impact of different irrigation regimens on root dentin mechanical properties, showing that 24 minutes of NaOCl exposure significantly reduced flexure strength, and this effect was not influenced by the use of EDTA either before or after the last NaOCl exposure . Although the exposure times to NaOCl were relatively short, and irrigant volumes were low, there was still a significant effect on flexure strength on that study. On the other hand, the author stated that the

dentin specimens used in the study were exposed to the irrigants from 4 sides (immersed), and this does not correlate with the clinical environment.

Grigoratos et al. evaluated the effect of NaOCl solutions (3%, 5%) for 2 hours, and saturated calcium hydroxide ($\text{Ca}(\text{OH})_2$) solution(1 week), on the flexural strength of standardized dentin beams (Grigorato et al., 2001). The NaOCl solution was changed every 15 min to prevent saturation by reaction products and to ensure that all surfaces of the dentin beams would be exposed. The author concluded that NaOCl (3% & 5%), and saturated $\text{Ca}(\text{OH})_2$ reduced the flexural strength of dentin. The findings were in accordance with those of Sim et al.,1996, and Mareending, 2007.

Zhang evaluated the apatite/collagen ratio using dentin powder immersed in 50ml of either 5.25% or 1.3% NaOCl for up to 240 minutes and rinsed with 17%EDTA for 2 minutes, and demonstrated that collagen degradation was significantly increased and the FS of mineralized dentin significantly reduced after the use of 5.25% NaOCl for more than 1 hour, however , changes were nor significant when 1.35 NaOCl was used as irrigant for up to 4 hours (Zhang 2010).

In this study, besides the very short exposure time to the irrigants(1-2minutes), only one surface of the dentin beam (inner surface) was exposed to the irrigating solution, similar to a clinical environment, and these two factors most likely had a direct influence in the results. The current data were obtained

in a controlled laboratory environment, and direct clinical conclusions can therefore not be drawn.

The digestibility test performed in the present study demonstrated that dentin treated with cross-linkers TA and GSE was not significantly less susceptible to collagenase digestion compared to the other groups (control and EDTA-NaOCl). These findings are in disagreement with previously published studies (Macedo et al, 2009; Bedran-Russo et al, 2009; Han et al, 2003). The short exposure time of the irrigating solutions limited the cross-link formation, and might be one of the explanations for the results obtained. Another important aspect is the fact that the present study used TA in mineralized dentin tissue as opposed to Macedo' study, which did their experiment in demineralized dentin.

Thermal stability represents the resistance of the collagen molecules to unfolding as a result of heat treatment. In the present study, the increase in the denaturation temperature for TA and GSE groups may be related to certain degree of cross-linking of the treated dentin and therefore increased stability of the biomodified matrix, although not statistically significant when compared to saline and EDTA-NaOCl groups.

According to Armstrong et al, 2006 and Mogilner et al, 2002, interpeptide hydrogen bonding within collagen fibrils seems to stabilize them against thermal challenge, the authors even speculate that water breaks interpeptide hydrogen

bonds making collagen more susceptible to thermal denaturation, and these localized denaturated regions within the dentin may be more brittle and may act as sites where crack initiation could eventually arise.

Under the conditions of this experiment, it may be surmised that treatment times of 1 min, and 2 min may not be enough to induce a degree of cross-linkage of TA/GSE that might be reflected as changes in the mechanical properties of the root dentin.

5.1 Clinical relevance and limitations

In general, flexural strength is mainly affected by defects or alterations on a specimen's surface (Marending et al., 2007). Although removal of smear layer might be desirable, we have to consider which irrigant to use last. If the last irrigant is EDTA, it is reasonable to believe that the organic part of the smear layer remains on the surface of the root canal lumen. Collagen can be important for the binding of bacteria including *Enterococcus faecalis*. It might not be prudent to use EDTA as the final irrigant.

In the present study, EDTA was not used as a final irrigant, which was more clinically relevant. When used individually, EDTA and NaOCl can each affect the physical and mechanical properties of dentin adversely (Sim et al., 2001). Not surprisingly, the combined removal of the inorganic as well the organic phase gives rise to damaging effects on the peritubular and intertubular dentin(Ari et al, 2004).

However, under the conditions of this study design, this combined effect did not significantly affect the flexural strength results. This might be related to the limited time and volume of irrigating solutions.

Future research is needed to test different concentrations of crosslinking agents, different irrigation protocols, and exposure time of irrigating solutions to mimic different clinical scenarios.

VI. CONCLUSION

In conclusion, within the experimental conditions of this study, the use of NaOCl, TA and GSE did not affect the flexural strength, rate of collagen biodegradation and collagen thermal transition of root dentin. Therefore the null hypothesis was accepted.

It is important to emphasize that the results of this *in vitro* study cannot be directly extrapolated to clinical conditions where a higher volume and greater exposure time of the irrigating solutions in the root canal may result in a different outcome.

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APPENDIX

IRB for Extracted Teeth-Exempt Review

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (OVC 632)
205 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Notice of Determination of Human Subject Research

March 23, 2011

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RE: **Protocol # 2011-0262**
The Effect of Different Irrigating Solutions of Intraradicular Dentin Microstructure
During Endodontic Therapy

Dear Dr. Iampaglia:

The UIC Office for the Protection of Research Subjects received your "Determination of Whether an Activity Represents Human Subjects Research" application, and has determined that this activity **DOES NOT meet the definition of human subject research** as defined by 45 CFR 46.102(f).

You may conduct your activity without further submission to the IRB.

If this activity is used in conjunction with any other research involving human subjects or if it is modified in any way, it must be re-reviewed by OPRS staff.

The UIC Office for the Protection of Research Subjects received your "Determination of Whether an Activity Represents Human Subjects Research" application, and has determined that this activity **DOES meet the definition of human subject research** as defined by 45 CFR 46.102(f).

You must submit either a Claim of Exemption or an Initial Review Application for IRB review. Your research cannot be conducted until written notice of an exemption determination or IRB approval has been granted.

For guidance on submitting your application, please refer to the guidance at:
<http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/index.shtml>

Phone: 312-996-1711

<http://www.uic.edu/depts/ovcr/opr/>

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ABSTRACTS:

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