EFFECTS OF FLAVONOIDS ON COLLAGEN BIOSTABILITY, REMINERALIZATION AND LONGTERM DURABILITY OF NATURAL AND DENATURED DENTIN

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DOCTOR OF PHILOSOPHY

by
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EFFECTS OF FLAVONOIDS ON COLLAGEN BIOSTABILITY, REMINERALIZATION AND LONG TERM DURABILITY OF NATURAL AND DENATURED DENTIN

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ABSTRACT

Collagen biostability in the resin dentin interface determines the longevity of dental composite restorations. Maintaining this biostability becomes even more challenging in the altered and denatured substrate of caries affected dentin (CAD). In this study two strategies, the use of crosslinker and remineralizing agents were evaluated for improving collagen biostability in natural and CAD. To understand the effect of these strategies three aims were addressed: 1) characterizing the effects of natural flavonoids theaflavins (TF) and Cranberry (CR) in crosslinking natural and denatured dentin collagen; 2) evaluating the effects of remineralizing agents on natural and denatured dentin collagen in presence of a crosslinker and 3) evaluating the effect of dual- functional etchants on longevity of dental composite restorations in natural and CAD. Results of these studies were as follows. First the crosslinking interaction of natural flavonoids with dentin collagen created alterations in collagen structure that significantly improved the biostability of natural and denatured dentin collagen. Second the use of remineralizing agents with natural flavonoids crosslinker CR reduced the time required for remineralization and provided a sturdy collagen scaffold for remineralization. Third, dual-functional etchants used for a clinically feasible time of 30 seconds were able to improve the longevity of resin dentin bonds created in natural or CAD. These results showed that TF and CR are efficient crosslinkers with the ability to protect

dentin collagen against enzymatic degradation within 30 seconds treatment time. Along with improving remineralization potential, these crosslinkers are efficient dual functional etchants which can improve the longevity of resin dentin bonds.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies have examined a dissertation titled “Effects of Flavonoids on Collagen Biostability, Remineralization and Long Term Durability Natural and Denatured Dentin”, presented by Saleha Nisar, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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DEDICATION

This dissertation is dedicated to my family- specially my mom for her patience and fortitude. I would also dedicate this dissertation to all the people who have supported me throughout my education. I would not have been able to be where I am without their inspiration to drive me.

CHAPTER 1

INTRODUCTION

Despite advancements in adhesive techniques, the long term durability of tooth colored composites bonded to dentin is not reliable and require replacement after some years in use (Breschi et al. 2008; Ferracane 2013). The weak link in a dental composite restoration is the bond between the adhesive resins and dentin. This bond degrades over time under the dynamic stresses of the oral cavity hence reducing the life of a restoration and causing damage to the underlying sound dentin structure (Van Nieuwenhuysen et al. 2003; Opdam et al. 2010; Veloso et al. 2019).

The conventional bonding process involves removal of the mineral phase from the top 5-10μm of the dentin substrate by acid etching. This step is then followed by infiltration of the adhesive resin into the mineral depleted compartments of dentin collagen substrate. The infiltration creates micromechanical retention and leads to formation of a resin reinforced collagen hybrid layer. However, the adhesive resins that are currently being used are relatively hydrophobic and are not able to displace the water that fills the intrafibrillar (within the collagen fibrils) and extrafibrillar (between collagen fibrils) compartments of the collagen following acid etching. Presence of residual water in the denuded collagen matrix acts as a media for degradation of resin as well as dentin collagen (Tay and Pashley 2003). Failure of the hydrophobic resins to infiltrate the dentin collagen network leaves the collagen exposed and unprotected, thus contributing to its instability (Sauro et al. 2009; Carvalho et al. 2012). Moreover, the cavity that is prepared by a dentist has varying region of caries affected and sound dentin. Caries affected dentin has low mineral content and disorganized or denatured collagen network as compared to sound dentin (Pinna et al. 2015). Bonding to such a

substrate makes restoring dentin with long term durability, more challenging. Another factor affecting the long term durability of adhesive resins is the presence of endogenous enzymes in the dentin substrate and hybrid layer, which can be activated by acid etching. These enzymes will attack the unprotected dentin collagen that previously had mineral protection and cause degradation of the hybrid layer (Mazzoni et al. 2015b). All these factors contribute towards producing a weak bonded interface with an unstable and permeable hybrid layer. The hybrid layer formed would contain a water rich, resin and mineral sparse collagen network that will fail with aging under the dynamic stresses in the oral cavity (Sauro et al. 2009; Liu et al. 2011d).

One strategy to improve the durability of adhesive bonds is to replace the residual water in the denuded and denatured collagen matrix of the hybrid layer by apatite mineral. This process when done in the presence of non-collagenous proteins (NCP), could potentially re-mineralize intra and extrafibrillar compartments of the collagen network (Gower 2008). Another strategy is to strengthen the dentin matrix by increasing the crosslinking of collagen network, hence making it stiffer and more resistant to enzymatic attacks (Green et al. 2010; Liu et al. 2013). The stiffening effect of the collagen could reduce the risk of collagen collapse following demineralization and may pave the way for infiltration of the adhesive resin. While these strategies might be effective, a knowledge gap exists in literature regarding an explanation about the mechanisms through which these strategies work. Knowledge regarding the function of the crosslinker in improving the crosslinking density only or as an enzyme inhibitor as well has to be explored. Literature also lacks information regarding the effect of these strategies on the long term durability of restorations made in caries affected dentin. Finally, it is important to explore and comprehend the possibility of

using these two strategies together to produce long lasting resin dentin bonds in natural and denatured dentin collagen.

Hence, the main objective of this study is to determine the effect of crosslinking and remineralization on natural and denatured dentin collagen. These treatment strategies would produce structural and mechanical changes in the dentin substrates, which would provide the basis for their resistance against enzymatic degradation and contribution towards improving the long term durability of resin dentin bonds.

CHAPTER 2

BACKGROUND

Dental caries is a biofilm-mediated, sugar-driven, multifactorial, dynamic disease that results in the phasic demineralization of dental hard tissues (Pitts et al. 2017). It is one of the most prevalent health condition worldwide, affecting around 2.4 billion people (Bagramian et al. 2009; Kassebaum et al. 2015). To treat dental caries, composites have been the restorative material of choice for dentists (Correa et al. 2012). Availability of dental composites in a variety of shades allows esthetic recovery of teeth possible (Chan et al. 2010). This combined with removal of less tooth structure and their ability to adhere to teeth via adhesive resins have remarkably contributed to their popularity (Demarco et al. 2012). However, the adhesive resins that are used to bond the composite to the tooth surface form a weak resin dentin interface, which degrades over time under the dynamic stresses of the oral cavity (Hashimoto et al. 2010; Spencer et al. 2010).

Annual failure rates of around 9% have been reported in literature for dental composites (Bernardo et al. 2007; Opdam et al. 2014). A failed dental restoration is an inconvenience to both the patient and dentist. It imposes high financial cost to the patient and the health system along with an increase in the amount of clinical time spent by a dentist (Tobi et al. 1999; Forss and Widström 2004; Fernández et al. 2011). The National Institute of Dental and Craniofacial Research (NIDCR) reported that 60% of the total restorative treatments are that of replacing degraded existing fillings (Kravitz et al. 2016). An annual cost of around $4-5 billion is being spent on replacing dental fillings in the USA alone (Abt 2008; Simecek et al. 2009). Other than the burden of financial cost, inconvenience and time

spent, on replacement of dental restoration always causes irreplaceable loss of healthy tooth structure as well (Hunter et al. 1995).

The Conventional Dentin Bonding Process

The conventional dentin bonding process starts with formation of a bacteria free cavity form in the dentin substrate (fig. 1a) The dentin surface is then etched with an acid which causes removal of the mineral phase from the top 5-10μm of dentin substrate (fig. 1b). The surface is then rinsed with water and dried slightly. Dentin adhesive resin is then applied (fig. 1c) which should ideally infiltrate into the various compartments of the collagen and replace the dentinal fluid or residual water in the demineralized layer and provide protection to the acid exposed dentin collagen (Ferracane 2006). It is the infiltration of the adhesive resin which creates micromechanical retention and leads to formation of resin reinforced collagen hybrid layer. It is the stability of this hybrid layer which holds the fort for a dental composite restoration and determines its durability in the oral cavity. Failure of the hydrophobic adhesive resins to infiltrate the entire depth of acid etched demineralized dentin collagen network results in unprotected and exposed collagen network (fig. 2), which is prone to degradation by endogenous enzymes, thus contributing to its instability Final step is the application of composite (fig. 1d).

Challenging Dentin Substrates

Natural/ Sound Dentin

Dentin substrate is a hydrated composite structure composed of 50% inorganic, 30% organic material and 20% water presenting a unique challenge for bonding as compared to enamel (Spencer et al. 2006). The structure has dentinal tubules which traverse the length of the dentin. The orientation and density of these tubules vary in different locations within the

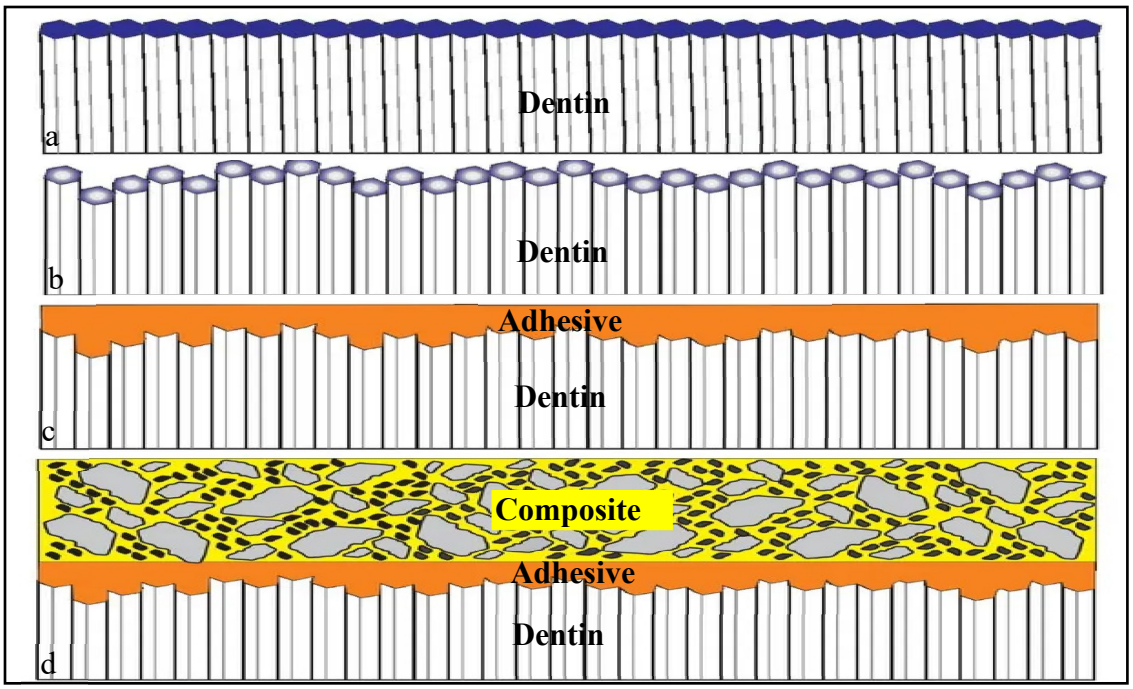

Figure 1. The conventional bonding process. Showing (a) dental cavity, (b) acid etched surface, (c) adhesive application, (d) composite application. Adapted from (Malloy et al. 2018)

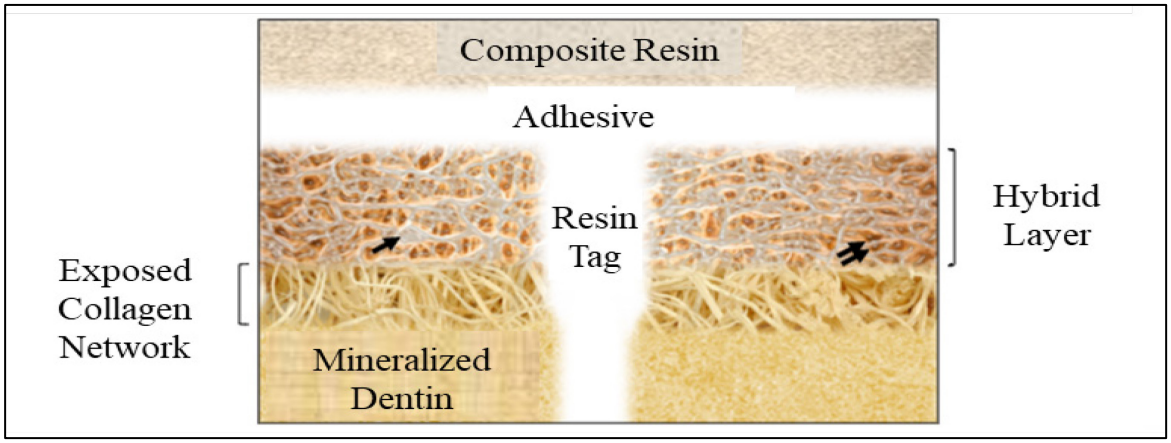

Figure 2. Adhesive reinforced collagen hybrid layer. Showing the exposed collagen network which could not be protected by the adhesive and will be vulnerable to enzymatic attack (Baldión and Cortés 2016).

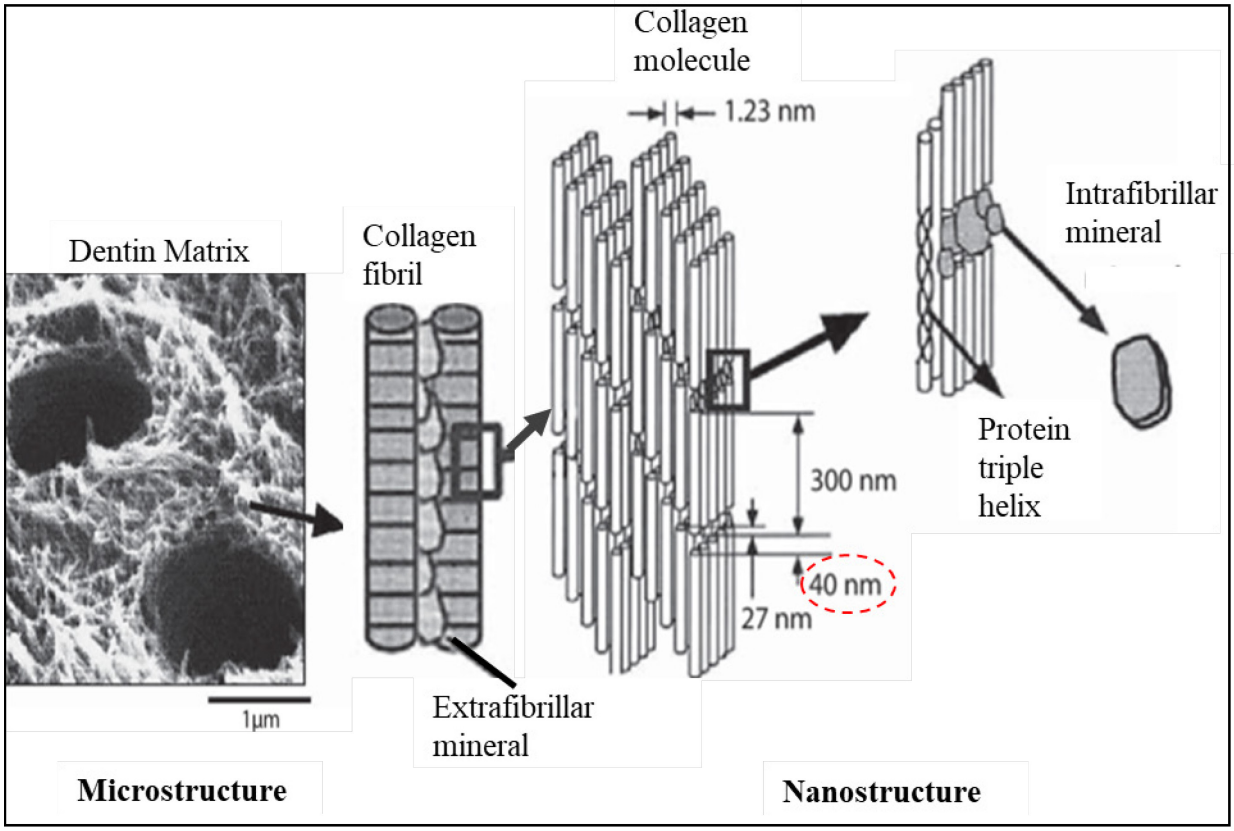

Figure 3. Structural hierarchical model of dentin matrix. From left to right: SEM image of dentin matrix showing dentin tubules surrounded by collagen fibrils. The collagen fibrils have extrafibrillar mineral between fibrils. The next illustration is of the collagen molecules that yields a 40nm gap zone into which intrafibrillar mineral is deposited (last image). Figure is not drawn to scale (Bertassoni et al. 2009).

dentin substrate with higher density and tubule diameter being close to the pulp and gingival margin (Nanci 2017). Hence a restoration which is placed in deep dentin (close to the pulp) or has a gingival margin (Class II, V) would have an added challenge of increased moisture content for the hydrophobic adhesive resin.

Dentinal matrix is made up of Type I collagen reinforced by apatite crystals. Scanning electron (SE) image of dentin matrix and its schematic hierarchical model are shown in figure 3. This collagen matrix itself is organized hierarchically into several structure levels (Wegst et al. 2015; Bertassoni 2017). The basic building block of this matrix is a triple helical tropocollagen molecule. Five tropocollagen molecules (form a microfibril) arrange themselves in a manner which leads to the creation of a gap zone 40 nanometer (nm) and an overlap zone of 27nm (Wess 2005). These zones are responsible for the 67nm (40 + 27) repetitive banding pattern seen under microscopy that is characteristic of type I collagen (Habelitz et al. 2002; Ottani et al. 2002). It is within these gap zones that intrafibrillar crystals form and grow with their c axis parallel to the long axis of the collagen fibrils (Nudelman et al. 2013). Microfibrils aggregate with each other to form collagen fibrils. Each collagen fibril has mineral deposited around them and is called extrafibrillar mineral or in between fibrils which is referred to as interfibrillar (Landis 1986). This hierarchical arrangement of the collagen fibrils and the mineral deposition plays an extremely important role in giving dentin its ideal mechanical properties (Jäger and Fratzl 2000; Kinney et al. 2003).

Within the dentin collagen matrix are present non collagenous proteins (NCP's) which are carboxylated anion's and play an important role in post translational organization and stability of collagen fibrils (Staines et al. 2012; Bertassoni and Swain 2014). These

NCP's are located in the gap zones of the collagen fibrils where they act as specific nucleators of apatite formation hence playing an important role in inducing intrafibrillar mineralization (Landis 2007).

The dentin collagen matrix also contains endogenous proteolytic enzymes like matrix metalloproteinases (MMP's) and cysteine cathepsins (Tersariol et al. 2010; Tjäderhane et al. 2013b). These enzymes are developmentally secreted as proenzymes and become trapped in the dentin substrate during tooth development and remain dormant. These enzymes become activated following acid etching or due to decrease in pH with bacterial infiltration during caries development (Mazzoni et al. 2006; Mazzoni et al. 2015a). Once activated these enzymes attack the unprotected dentin collagen fibrils within the hybrid layer causing degradation and destruction of the hybrid layer (Mazzoni et al. 2015a). The collagen fibrils in the bottom of the hybrid layer are more prone to degradation when the adhesive resin fails to penetrate the entire depth of the demineralized zone and provide protection to the exposed collagen (Hashimoto et al. 2002). The unprotected exposed collagen fibril serves as a substrate for the endogenous enzymes leading to initiation of a vicious cycle of degradation of the interfacial hybrid layer. Along with the hybrid layer, the rest of the dentin structure also gets affected, causing development of secondary caries and eventually failure of the adhesive bond (Breschi et al. 2008; Tjäderhane et al. 2013b).

Caries Affected Dentin

While the presence of endogenous enzymes and the hierarchical arrangement of natural/ sound dentin is a challenge itself, it is important to point out that while the bacteria loaded caries infected dentin is removed, the underlying caries affected dentin is preserved. Hence the final cavity prepared by a dentist has varying regions of caries affected and sound

natural dentin (Wang et al. 2007; Nakajima et al. 2011). Caries affected dentin (CAD) has its own challenges as it has an abundance of occluded dentinal tubules (Ogawa et al. 1983) with less crystalline mineral phase and low mineral content (Spencer et al. 2005; Wang et al. 2007). The mineral crystals in CAD are randomly distributed with large apatite crystallites and wider intercrystalline spaces as compared to sound dentin (Daculsi et al. 1979). Higher water content (Ito et al. 2005) and presence of disorganized or denatured collagen network in CAD have also been reported (Suppa et al. 2006; Pinna et al. 2015). As compared to sound dentin CAD is softer with lower values of elastic modulus and hardness hence has compromised mechanical properties (Marshall et al. 2001; Zheng et al. 2005).

Bonding to such a substrate makes restoring dentin with long term durability even more challenging. The altered chemical and morphological characteristics contribute towards lower bond strengths produced with CAD as compared to sound dentin irrespective of the bonding system used (Yoshiyama et al. 2002; Say et al. 2005). Following acid etching a deep, non uniformly demineralized layer is produced (Wang et al. 2007). This is due to higher susceptibility of CAD to acid etching as it is partially demineralized (Nakajima et al. 2011). Increase depth of demineralized layer produces an added challenge for the adhesive resin. The adhesive resin will have to penetrate the entire depth of demineralized zone while competing with high amounts of residual water within the CAD substrate and provide protection to the exposed collagen. High amount of residual water also interferes with polymerization of the adhesive monomers. Studies have shown lower degree of double-bond conversion of the adhesive monomers in CAD as compared to sound dentin (Spencer et al. 2005). These factors contribute towards production of a poor quality hybrid layer which would compromise the longevity of the restoration.

Potential Treatment Strategies

Crosslinking Dentin Collagen Substrate

Strengthening the dentin collagen network to stop the vicious cycle of biodegradation has been attempted by inducing exogenous crosslinks within the collagen matrix. These crosslinkers can enhance the resilience of the dentin collagen substrate hence making it more resistant to degradation. Studies have shown that the use of collagen crosslinking agents has improved the stiffness of the dentin collagen substrate (Bedran-Russo et al. 2008a; Carrilho et al. 2009; Xu and Wang 2011) and provide resistance to enzymatic degradation (Epasinghe et al. 2013; Seseogullari-Dirihan et al. 2016; Balalaie et al. 2018).

Glutaraldehyde is one of the well known synthetic chemical crosslinking agents, which is able to induce exogenous crosslinks between its aldehyde groups and amine groups of collagen (Ritter et al. 2001a; Ritter et al. 2001b). The effectiveness of glutaraldehyde treatment is time dependent (Bedran-Russo et al. 2008a; Al-Ammar et al. 2009). High concentration and exposure time of at least 10 minutes (min) is required to achieve desirable mechanical properties, which is not practical for clinical use (Bedran-Russo et al. 2008a; Pinto et al. 2015). Another drawback of glutaraldehyde is its cytotoxicity which limits its use in the oral cavity (Hass et al. 2016b). Due to its cytotoxic effects, glutaraldehyde is being replaced by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS). This chemical crosslinker works by activating the carboxylic acid groups in collagen by giving O-acylisourea groups (Bedran-Russo et al. 2010). Unlike glutaraldehyde which retains in the collagen following crosslinking, EDC/NHS crosslinking gives rise to a urea derivative. This urea byproduct produced can easily be rinsed off hence lowering the cytotoxicity concerns (Mazzoni et al. 2013).

Natural crosslinkers such as polyphenols from botanic sources have also gained popularity over the past years due to their low toxicity and availability as a sustainable resource (Yamakoshi et al. 2002; Han et al. 2003). Proanthocyanidin's (PAC) from grape seed extract (GSE) is a natural crosslinker and is considered a gold standard due to its performance in inducing exogenous crosslinks (Pierpoint 1969; Han et al. 2003; Bedran-Russo et al. 2014) as well as inhibiting endogenous enzymes (Seseogullari-Dirihan et al. 2015; Seseogullari-Dirihan et al. 2016). Unlike chemical crosslinkers GSE uses far more mechanisms to interact with collagen and induce crosslinks hence producing better results (Bedran-Russo et al. 2008a; Castellan et al. 2011). The rapidness in inducing crosslinking in short time (less than 30 seconds) makes GSE a practical choice for clinical setting (Liu et al. 2013; Liu and Wang 2013a). The building blocks of GSE are flavon-3-ol oligomer units which are linked through only one single bond between C4 to C8 or C4 to C6 (Balalaie et al. 2018). This type of linkage is also called B-type linkage (Bedran-Russo et al. 2014). The structure of GSE which is made of a mixture of dimers, trimers, tetramers and polymers, makes it a big complex molecule with high molecular weight (Gabetta et al. 2000). The large molecular size could be limiting its penetration through the entire depth of the demineralized layer of dentin collagen substrate hence reducing its effectiveness (Liu and Wang 2013a; Liu et al. 2014a). Another drawback of GSE is its radical scavenging effect which would inhibit the polymerization of the adhesive resin resulting in incomplete protection of the demineralized dentin collagen substrate (Green et al. 2010; Liu and Wang 2013b). Hence, there is a need to explore better crosslinkers which can be compatible with the adhesive resin and produce promising long term results in terms of stabilization of the hybrid layer.

The less explored PAC from cranberry (CR) extract has drawn our attention. It is also a big complex of oligomers and polymers like GSE, however unlike GSE, the monomer subunits in CR are linked through a single bond between C4 and C8 or C6 as well as through at least one intermolecular bond between O7 and C2 (fig. 4). This type of linkage (A type) plays an important role in increasing antibacterial activity (Foo et al. 2000) as well as reducing the collagen degradation by inhibiting MMP-1 and 9 (Bodet et al. 2007; La et al. 2009). Another advantage of A type PAC's is that they have better stability in solutions and lower degradation or oxidation rates as compared to B-type PAC's as in GSE (Xu et al. 2015; Aydin et al. 2019).

When the mechanical properties of the two types of PAC's were compared, CR did not perform as well as GSE. However, CR treated dentin specimens showed more stability to degradation following storage in artificial saliva (Castellan et al. 2011). This could be due to its structural ability to inhibit the endogenous enzymes hence contributing towards long term stability. Poor mechanical performance of CR in this study could also be due to the fact that the CR they used contained less than 1% PAC in it. Another study used 10% CR with PAC concentration of around 65% to evaluate degradation of demineralized dentin matrix and found no difference between the performance of the two PAC's (Boteon et al. 2017). CR with PAC concentration of 50.2% was tested as a crosslinker in our lab recently and showed good protection against enzymatic degradation (Wang et al. 2021). Based on the results of this recent study it was important to further characterize CR with A-type linkage to understand its potential role (as a crosslinker and/or MMP inhibitor) in improving the long term durability of dentin collagen. It would also be interesting to see if different types of PAC linkages have any effect on remineralization of dentin collagen.

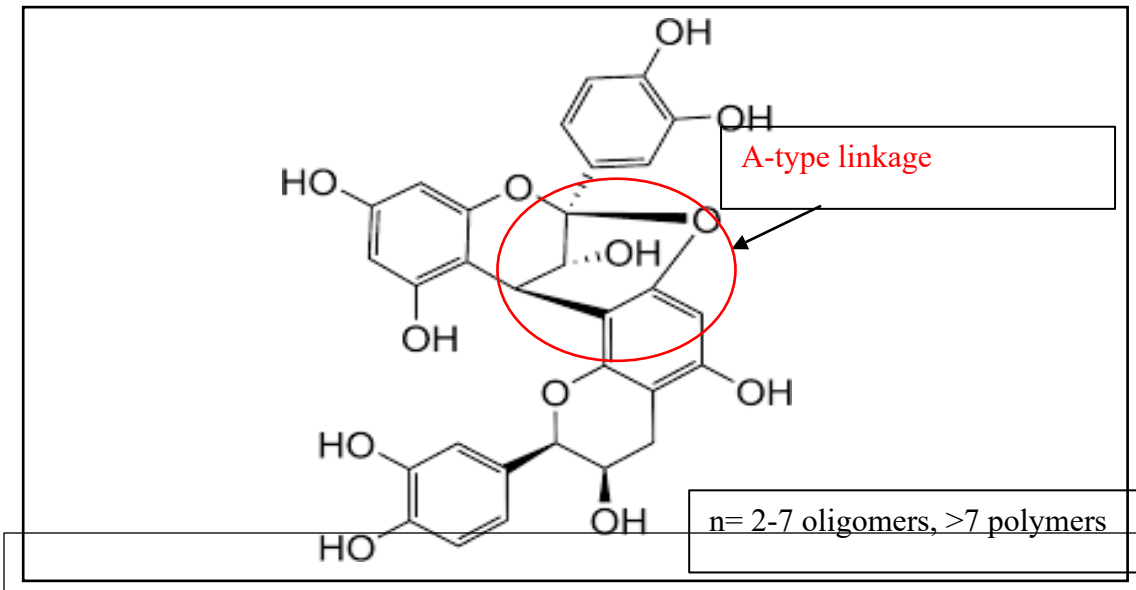

Figure 4. Schematic diagram of molecular structure of CR. Showing the A- type linkage encircled in red (Ulrey et al. 2014).

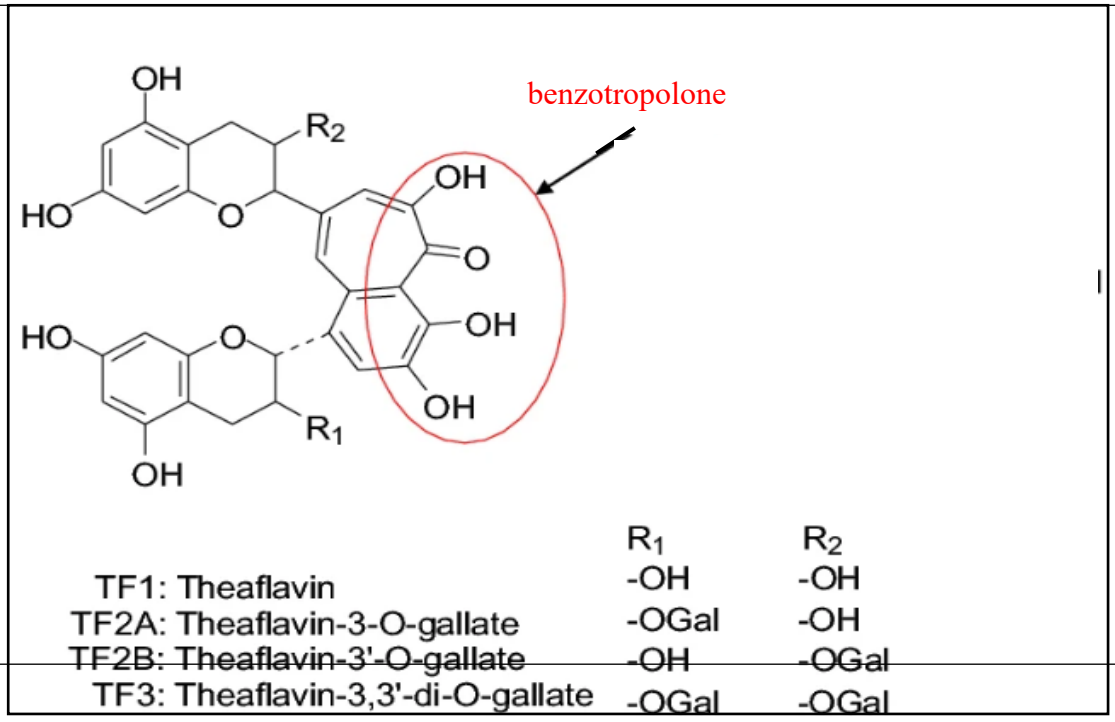

Figure 5. Schematic diagram of molecular structure of TF. Showing the benzotropolone skeleton (encircled in red). The 4 basic compounds which make TF are also mentioned below (Liu et al. 2021).

Polyphenols from black tea extract called Theaflavin's (TF) are also natural compounds with a unique and simple structure made of just 4 basic compounds as mentioned in figure 5. They are known to have good antibacterial (Xie et al. 2015), anti-cancer (Isemura et al. 2000)

and lipid lowering properties (Maron et al. 2003) similar to CR (Feghali et al. 2012). The intermolecular interaction of TF with various proteins provides the basis for its bioactivity (Sirk et al. 2011). The structure of TF has enough galloyl and phenol hydroxyl groups to promote collagen crosslinking but not so many as to inhibit adhesive resin polymerization like GSE does. Unlike PAC units which are connected by a single C-C bond, TF units are coupled through a benzotropolone structure (fig. 5). This structure has a carbonyl group next to hydroxyl group which might favor additional crosslinking. Secondly TF has a lower molecular weight as compared to GSE which would enable better penetration through the entire demineralized depth of dentin collagen substrate. These distinctive features of this compound should make it a novel crosslinker with enhanced performance on long term biostability of dentin collagen. This compound has never been used on denatured collagen to investigate its role as a potential crosslinking agent. No studies till date have been done regarding its effects on long term stability of dentin collagen or its influence on durability of resin dentin bonding.

Remineralization of Dentin Collagen Substrate

Presence of NCP's with in the collagen molecules plays an important role in mineralization of dentin, hence contribute significantly towards the mechanical properties of dentin collagen (Kinney et al. 2001; Kinney et al. 2003). Gower and colleagues used analogs of NCP's to understand and reproduce this biomimetic remineralization process in collagen

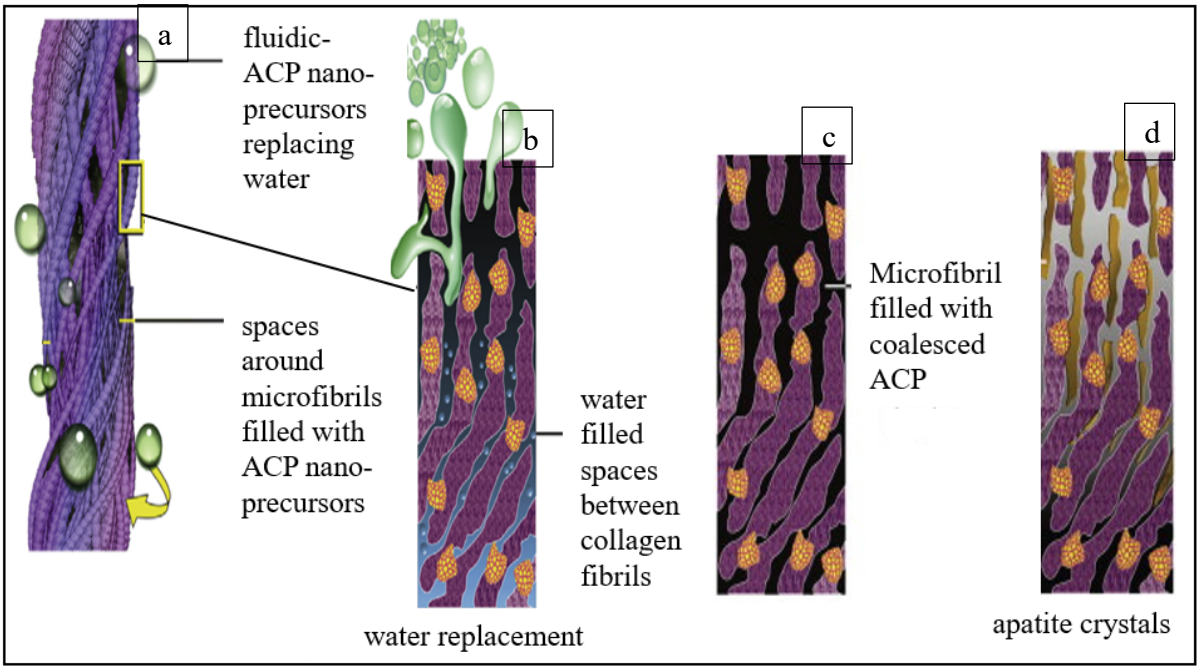

Figure 6. Schematic process of intrafibrillar mineralization. Using non collagenous proteins (NCP) in a supersaturated mineral solution. (a) Demineralized collagen fibrils surrounded by Amorphous Calcium Phosphate (ACP) nano-precursors. (b) View of microfibril at molecular level showing ACP nano-precursor flowing into the extrafibrillar and intrafibrillar compartments of collagen to replace water. (c) The ACP nano-precursors coalesce in the microfibrils and (d) grow into apatite crystals. Adapted from (Kim et al. 2010a)

matrix (Gower and Odom 2000; Dai et al. 2008). They used anionic polymers as NCP analogs in a supersaturated mineral (calcium & phosphate) solution. The highly charged anionic polymer attracted calcium and phosphate (thus inhibiting nucleation) and formed stabilized amorphous calcium phosphate (ACP) nano-precursor of the mineral phase which surround the collagen fibrils (fig. 6a) The precursor formed was termed Polymer-Induced Liquid Precursor (PILP) as it did not behave like a solid and had the ability to flow and diffuse into the intra and extrafibrillar collagen compartments where it could replace the residual water (fig. 6b), coalesce, grow and mature into apatite crystals (fig. 6c & 6d) (Olszta et al. 2007).

The PILP system has been tested on 140μm deep artificial carious lesions. The results showed 91% recovery of the elastic modulus of these lesions in 28 days (Burwell et al. 2012). However, no study has tested this system on denatured or altered dentin collagen substrate found in caries affected dentin nor has there been any report of using it with a natural crosslinker. Understanding the interaction and the resulting modification produced in collagen following the combined use of a crosslinker and remineralizing agent will enhance our comprehension regarding the possibility of the use of these strategies in the adhesive system. This understanding would also aid in developing a bifunctional compound having functional groups for both crosslinking and remineralization.

Goals of the Research

With this research we want to characterize the effects of crosslinkers CR and TF on the biostability of natural and denatured dentin collagen. We are also interested in the interaction between crosslinking and remineralization treatments on dentin collagen to enhance our

understanding for creating a stable hybrid layer and improve the long term durability of resin dentin bonds.

Specific Aims

Aim1: To characterize the effects of crosslinking treatment on the structural, mechanical properties and bio-stability against enzymatic challenges in natural and denatured dentin collagen. (Chapter 3)

Aim 2: To determine the effects of crosslinking treatments on the remineralization process of natural and denatured dentin collagen. (Chapter 4)

Aim 3: To evaluate the effects of collagen crosslinking on in vitro bonding performance and long term durability of resin dentin bonds in natural dentin and CAD. (Chapter 5)

Clinical Significance

The contribution of this project will be significant because it will characterize the structural and chemical changes in dentin (natural and denatured) bonding substrate in response to treatment with crosslinking agents. Dentin substrate and testing techniques have been designed to maintain close similarities to real clinical situations so that realistic results can be achieved. An in-depth understanding through the results achieved will ultimately provide knowledge to develop targeted strategies to improve the long term durability of resin dentin bonds.

The natural compound TF used in this study also adds novelty to the project as it has never been tested as a potential crosslinker until recently in our lab (Liu et al. 2021). While the simple structure looks attractive, TF's crosslinking ability, effect on endogenous enzymes and interaction with denatured dentin needs to be explored. Similarly, the clarity required with CR regarding its function as a crosslinker or an endogenous enzyme inhibitor, hence its

role in contributing to the long term durability of resin dentin bond will be achieved. The interaction of CR with remineralizing agents and its effect on dentin collagen biostability will also be assessed. This study will also be looking into the use of crosslinkers in etchants to produce dual-functional etchants (acid etching and collagen crosslinking at the same time), in order to reduce the number of steps a dentist will have to undergo to complete the bonding process.

Study results can reduce our knowledge gaps regarding the use of these two agents together to enhance the long term durability of resin dentin bonds. The knowledge attained can provide the basis for conducting future studies investigating the use of these agents in different components of the adhesive system. Furthermore, our findings will also contribute in providing knowledge to researchers in the field of bone remineralization, and collagen scaffold engineering to develop alternative options to bone grafts (Jee et al. 2010; Soicher et al. 2013).

CHAPTER 3

THE EFFECTS OF FLAVONOIDS ON STRUCTURAL, MECHANICAL PROPERTIES AND BIOSTABILITY OF NATURAL AND DENATURED DENTIN COLLAGEN

Introduction

Dentin collagen that forms the body of the tooth, is a structurally complex substrate which is challenging to restore. The organic matrix of dentin is composed of type I collagen which forms a 3-dimensional network stabilized through inter and intramolecular crosslinking (Light and Bailey 1980; Knott and Bailey 1998). This stable structure is disrupted during a carious attack which leads to degradation of the collagen fibers, disruption of crosslinking and alteration of the secondary collagen structure (Kuboki et al. 1977; Wang et al. 2007). Reduction in pH during caries infiltration further leads to activation of dormant endogenous enzymes in the dentin structure which starts a vicious cycle of collagen degradation in rest of the sound dentin (Mazzoni et al. 2015a).

To counteract collagen degradation, induction of exogenous crosslinks has been proposed to provide stability to the collagen structure and stop the vicious cycle of degradation (Cai et al. 2018). Naturally derived PAC rich extracts have shown to effectively provide stabilization to dentin collagen exposed to enzymatic challenges (Castellan et al. 2011; Wang et al. 2021). Type A PAC (CR) can directly inhibit endogenous enzymes present in the dentin matrix and play a crucial role in reducing collagen degradation (La et al. 2009). While some studies have rendered type CR's performance weak (Castellan et al. 2011), a recent study from our group showed that it could provide resistance to enzymatic degradation similar to the gold standard type B PAC (Wang et al. 2021). Another natural crosslinker, theaflavins (TF) which is comparatively a smaller compound than PAC was investigated in

our lab and it showed promising results when tested for dentin collagen biostability (Liu et al. 2021). Both these crosslinkers were able to crosslink dentin collagen in a clinically feasible time period of 30 seconds.

Chemical crosslinker EDC is a zero length crosslinker which unlike PAC or theaflavins, creates crosslinks in the dentin collagen without any molecule being part of the final bond created (Nakajima and Ikada 1995). This compound was able to produce inhibition of endogenous enzymes as well as improved mechanical properties of dentin when used for longer treatment time (Bedran-Russo et al. 2010; Mazzoni et al. 2014). Recent study showed high bond strengths and low activity of endogenous enzyme following 5 year storage of samples treated with EDC primer (Maravic et al. 2021). These studies used EDC for longer time (1min to 1 hour) and did not rinse off the crosslinker following application which allowed the crosslinker to continue its effect for longer than the intended treatment period.

The promising effect of these crosslinkers has been tested on sound natural dentin and information regarding their effect on the challenging substrate of denatured dentin (DD) is scarce. The caries affected dentin (CAD) substrate found in clinical setting has distinct physical, chemical, and mechanical differences from sound dentin making it a challenging substrate to work with (Angker et al. 2004; Wang et al. 2007; Ekambaram et al. 2015b). Secondly, most of the research that is carried out using CAD are related to adhesive bonding studies, which presents with a lot of discrepancies with respect to the results produced (Ferreira Nicoloso et al. 2016; Fialho et al. 2019; Hass et al. 2019). These variations are due to differences in the type of substrate used to simulate CAD. As literature does not provide a clear method to simulate the changes in CAD to achieve a homogenous and characterizable

substrate, we developed two methods (heat and acid denaturation) to carry out controlled quantitative evaluations.

The objective of this study was to evaluate the effect of 2 crosslinkers (CR and TF) on the structure, properties, and biostability against enzymatic degradation in natural and denatured dentin collagen.

Materials and Methods

Teeth Collection and Preparation

Previously extracted human third molars were collected from dental surgery clinics within the metropolitan Kansas City area. The teeth were collected without any associated patient identifiers according to a protocol approved by the University of Missouri-Kansas City adult health science institutional review board (IRB#: 12-50-NHSR). Each tooth was analyzed for visual defects or caries and sound teeth were selected. These teeth were cleaned of any remaining soft tissue and were stored in 0.9% phosphate buffered saline (PBS)[1] containing 0.002% sodium azide[2] (NaN3) at 4°C. Roots, enamel crown and surrounding enamel of the teeth were removed using a water -cooled low speed diamond saw[3] to achieve dentin blocks measuring 7mm x7mm x 2mm. A uniform smear layer was created on the surface of exposed dentin by gentle polishing on a wet 600-grit silicon carbide sandpaper[4]. The dentin blocks were further cut into two different types of samples: dentin beams with dimensions 7mm x 0.8mm x0.8mm and dentin films with dimensions 7mm x 7mm x 7µm. Dentin films were cut using a tungsten carbide knife mounted on an SM2500S microtome[5].

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[1]MDL# MFCD00131855, Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103.
[2]Cat. No. S2002, Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103.
[3]IsoMet® 1000 Precision Saw, Buehler Ltd., Lake Bluff, IL 60044.
[4]CarbiMet S,600, Item# 16100600, Buehler Ltd., Lake Bluff, IL 60044
[5]SM2500S microtome, Leica, Deerfield, IL

Preparation of Demineralized Dentin Specimens

The dentin films were demineralized with 10% phosphoric acid[6] solution for 30 min, while the dentin beams were demineralized for 5 hour (h) and the solution was changed 3 times. The specimens were then rinsed with deionized (DI) water three times for 10 min each. Complete demineralization was verified by Fourier transform infrared (FTIR) spectroscopy.

Preparation of Denatured Dentin Specimens

For heat denaturation (HD) the demineralized dentin specimens were heated in a water bath at 100˚C for 1 h. For acid denaturation (AD) the demineralized dentin specimens were put in 35% phosphoric acid for 3 h at the temperature of 37˚C. Each specimen was then rinsed with DI water three times for 30 min.

Crosslinker Treatment

Crosslinkers TF[9], CR[10], carbodiimide hydrochloride (EDC)[11] (mixed with N-Hydroxysuccinimide[12] in a ratio of 0.05M:0.02M) were tested and untreated dentin specimens (CT) were used as negative control. All crosslinkers were dissolved in DI water to achieve a final concentration of 1%. Dentin collagen films were rolled on to a cover slip with help of a small brush and each film was immersed in the respective crosslinker solution for 30 seconds (s) or 1 h. The films were then rinsed with DI water 3 times for 30 min. Dentin collagen beams were immersed in the crosslinker solutions for 1 h, followed by rinsing

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[6]Lab Chem, 300 Industry Drive, Pittsburgh, PA 15275
[9]Black Tea Extract Theaflavins 40% (PG-TF40), Lisi (Xian) Bio-Tech Inc. Baoji City, Shanxi Province, China
[10]Ocean Spray, Cranberry Extract Powder, Lakeville-Middleboro, MA 02349
[11]1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, CAS# 25952-53-8, Thermo Fisher Scientific, Waltham, MA 02451
[12]N-Hydroxysuccinimide, CAS# 6066-82-6, Sigma-Aldrich, St. Louis, MO 63178
with DI water 3 times for 30 min.

FTIR Analysis

Chemical changes associated with collagen biomodification such as formation of new bonds, alteration in band intensity and ratio were detected with FTIR spectroscopy.

The crosslinker treated and untreated dentin collagen films (n=5 per group) were spread on a cover slip[14] and were allowed to dry in vacuum for 24 h. The FTIR spectra of the films were collected at a resolution of 4 cm^{-1} with 64 scans using FTIR spectrometer[15] (with a transmission attachment) on a barium fluoride disk. The spectra were analyzed using Spectrum Software (version 5.3)[15]. Two-point baseline correction and normalization to amide I band was done and band ratios were calculated. For DD CTs the band height ratios A1455 / A1235 were measured while the band ratios (A1400 / A1450 and A 1550/1450) of spectra of all specimens were calculated. The ratio of band area of the two crosslinkers (~1145 for TF, ~1120 for CR) and Amide I band height was also quantified to evaluate the extent of interaction with dentin collagen. Amide I band height was chosen as it was not affected by heat or acid denaturation.

Weight Loss and Hydroxyproline Analysis

These tests allowed us to quantitatively analyze the resistance of dentin collagen specimens against exogenous enzymatic challenge. Resistance against an aggressive exogenous enzyme would provide evidence regarding the contribution of the crosslinker in producing stabilized dentin collagen.

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[14]Nunc® Thermanox™ coverslip TMO#174985, Sigma-Aldrich, St. Louis, MO 63178
[15]Spectrum One, Perkin-Elmer, Waltham, MA 02451
[8]Clostridium Histolyticum, EC 3.4.24.3, PIN C-0130; Sigma Chemicals, St. Louis, MO 63103

Sixty, 7 μm thick dentin collagen films were used for each treatment group. For exogenous enzymatic degradation, the treated specimens from each group were dried in vacuum and weighed. The specimens were then subjected to digestion by 0.1% collagenase[8] (exogenous enzyme) for 1 h at 37˚C in an incubator[16]. Following digestion, the digestion solution was transferred to an Eppendorf tube[17] and the digested specimen were rinsed with deionized water and centrifuged for 15 min. The rinsed water was also transferred to the same Eppendorf tube. This digestion solution was used for hydroxyproline (HYP) analysis, while the dentin collagen specimens were spread on a cover slip and dried in vacuum for 48 h. The dried collagen specimens were then weighed. Weight loss (WL) analysis was done by subtracting the weight of the specimens after digestion from their weight before digestion.

For hydroxyproline analysis, the digestion solution from each group was transferred to a glass tube[18] and hydrolyzed using 6M hydrochloric acid[19] at 110°C for 24 h. The dry residue was oxidized using copper sulfate[20], sodium hydroxide[21] and sulfuric acid[22] followed by 5% Ehrlich's reagent 23 to develop a color. The absorbance was read at 555nm with a microplate

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[16]Quincy lab, Inc.10-180 Bench Top incubator, Hogentogler, Columbia, MD 21045
[17]Eppendorf® Flex-Tubes Microcentrifuge Tubes, EP022364120, Sigma-Aldrich, St. Louis, MO 63178
[18]Aldrich® Essentials culture tube with screw cap and PTFE/rubber liner, Z740943, Sigma-Aldrich, St. Louis, MO 63178
[19]Hydrochloric acid, CAS# 7647-01-0, Sigma-Aldrich, St. Louis, MO 63178
[20]$CuSO_4$, CAS# 7758-98-7, Sigma-Aldrich, St. Louis, MO 63178
[21]NaOH pellets, CAS#1310-73-2, Sigma-Aldrich, St. Louis, MO 63178
[22]H_2SO_4, CAS#7664-93-9, Sigma-Aldrich, St. Louis, MO 63178
[23]4-(Dimethylamino) benzaldehyde, CAS#100-10-7, Sigma-Aldrich, St. Louis, MO 63178
[24]VICTOR Nivo Multimode Microplate Reader, Part# HH35000500, PerkinElmer, Waltham, MA 02451
[25]*trans*-4-Hydroxy-L-proline, CAS#7647-01-0, Sigma-Aldrich, St. Louis, MO 63178

reader[24]. The trans-4-Hydroxy-L-proline[25] was used as the standard to make the working curves for quantifying hydroxyproline released from each micro gram of dentin collagen specimens during the digestion.

In situ Zymography Analysis

This analysis allowed us to evaluate the crosslinker's ability to inhibit the activity of MMPs (endogenous enzymes), hence expanding our knowledge regarding its role in providing biostability to collagen.

Three crosslinker treated dentin collagen films per treatment group were used. The films were spread on a cover slip[14] with the help of a small brush. Self-quenched fluorescein conjugated gelatin[26] was used as MMPs substrate. An anti-fading agent[27] and the fluorescein-conjugated gelatin was diluted in a ratio of 1:1:8 in a dilution buffer (NaCl[28] 150 mM, $CaCl_2$[29] 25 mM, Tris–HCl[30] 50 mM at pH 8.0). The self -quenched fluorescein conjugated gelatin will be acted on upon by the gelatinases (MMP-2 and MMP-9) present in dentin, causing breakdown of gelatin and producing fluorescence.

Ten microliters of the fluorescent-gelatin solution were spread on top of each dentin collagen specimen. The specimens were then transferred to humidified chamber[16] and stored in dark at 37°C for 24 h. All specimens were put in a moist case before putting them in the

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[26]EnzChek™ Gelatinase/Collagenase Assay Kit, 250-2,000 assays, E-12055, Molecular Probes, Thermo Fisher Scientific, Eugene, OR 97402
[27]Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK
[28]NaCl, CAS#7647-14-5, Sigma-Aldrich, St. Louis, MO 63178
[29]$CaCl_2$, CAS#10043-52-4, Sigma-Aldrich, St. Louis, MO 63178
[30]Tris HCl, CAS#1185-53-1, Sigma-Aldrich, St. Louis, MO 63178
[31]Confocal Laser Scanning Microscope, Fluoview FV1200, Olympus, Tokyo, Japan

incubator. Following incubation, the samples were rinsed with DI water on the cover slip. Excess water was removed using Kim wipes. The amount of green fluorescence produced by the hydrolyzing effect of the conjugated gelatin by MMPs was evaluated for each specimen under a confocal laser scanning microscope (CLSM)[31] with excitation of 488 nm and emission of 530 nm wavelengths.

Mechanical Strength Testing

Creating a stable, resilient and an insoluble hybrid layer is the hallmark of a long-lasting dentin resin bond. By inducing exogenous crosslinks, the demineralized dentin collagen should be reinforced and its mechanical strength should increase. This should create a resilient collagen network that is resistant to enzymatic degradation. In this test the mechanical strength of the crosslinker treated dentin collagen (natural and HD) were measured and compared with their respective untreated CTs (CT and HD CT).

Dentin collagen beams (15 per treatment group) were used. The beams in each treatment group were stored in PBS[1] for 24 h in an incubator at 37˚C before testing. These beams were attached to the upper and lower clamps of the mechanical tester[32] using cyanoacrylate adhesive[33]. Each specimen was subjected to a 150 lb load at a speed of 0.5 mm/min. The ultimate tensile strength (UTS) and elastic modulus (EM) of each specimen was measured by dividing the maximum force at the point of failure with the cross section area of the specimen.

[32] SSTM-5000 Tensile Tester, United Calibration Corporation, CA 92649
[33] Zapit Base and accelearator, Dental Ventures of America, Inc., CA 92878

Statistical Analysis

The normality of distribution and homogeneity of variances were assesssed using Shapiro Wilk and Levene tests. WL, HYP and MMPs activity were analyzed by three-way analysis of variance (ANOVA) and Games-Howell test (α=0.05). Mechanical tensile stress results were analyzed using two-way analysis of variance.

Results

Spectral Changes in Dentin Collagen after Denaturation

Representative FTIR spectra of HD (red line), AD (blue line) and CT (black line) dentin collagen are shown in figure 7. The characteristic dentin collagen bands including amide I (~1660 cm^{-1}) associated with C=O stretching, amide II (~1555 cm^{-1}) for N-H bending and C-N stretching, amide III (~1245 cm^{-1}) for C-N, C-H and N-H deformation, C-O and C-N bending at ~1400 cm^{-1} and CH_2 scissoring at ~1450 cm^{-1} were prominent in all the spectra. A closer look revealed changes in both DD specimens, such as amide I (broadening), amide II (broadening, reduction in intensity, slight shift from ~1555 cm^{-1} to a lower wave number of ~1550 cm^{-1}, decrease in intensity at ~1400 cm^{-1} δ (C-O and C-N bending) and reduction in intensity in amide III. The HD specimen also showed a shift to lower wavenumber from ~1245 cm^{-1} to ~1242 cm^{-1}. Table 1 represents the band height ratios (A1455 cm^{-1}/ A1235 cm^{-1}) of CT, HD and AD dentin collagen. Both DD types had significantly lower band ratios compared to CT ($p < 0.001$).

The representative FTIR spectra of TF treated natural CT (top), HD (middle) and AD (bottom) dentin collagen are presented in figure 8. The black dashed line spectrum at the bottom is of the TF powder. The characteristic bands associated with dentin collagen could be assigned to all the treated specimens. A closer peek at the spectra showed pronounced

changes in all the treated specimens compared to the CT, such as amide I (broadening), amide II (broadening and reduction in intensity, except for 1 h HD, increase at ~ 1450 cm^{-1}, decrease at ~1400 cm^{-1}, increase in amide III intensity. Other prominent changes included the appearance of a band at ~1145 cm^{-1} and a bulge at ~1107 cm^{-1} which correspond to bulges seen in the TF powder at the same wave number. All these changes were more intense following 1 h TF treatment.

The FTIR spectra of CR treated dentin collagen are shown in figure 9 with the bottom spectrum (black dashed line) representing CR crosslinker. Typical bands of dentin collagen were seen in all CR treated spectra as described above. The prominent changes in all CR treated spectra include amide I (broadening), amide II (broadening and reduction in intensity, except for 1 h heat denatured, increase in intensity at ~ 1450 cm^{-1}, decrease at ~1400 cm^{-1}, increase in amide III intensity at ~1290 cm^{-1} and the emergence of shoulder at ~1120 cm^{-1} which corresponds to a bulge at the same wave number in the CR powder spectrum as well. Similarly, all these changes were more intense following 1 h CR treatment.

The FTIR spectra of EDC/NHS treated dentin collagen (solid lines) are represented in figure 10 and the bottom spectrum is of the EDC/NHS powder (black dashed line). Dentin collagen bands were seen in all the EDC/NHS treated spectra. Compared to the spectra of TF or CR treated collagen, there were much less changes in the EDC/NHS treated spectra. Only slight reduction of amides II, III and ~CH_2 bands were seen.

To further characterize the collagen crosslinking interactions, the band ratios at A1400/ A1450 (fig. 11) and A1550/ A1450 (fig. 12) were measured. Irrespective of the dentin collagen type and treatment time, TF and CR treated dentin collagen showed a statistically significant reduction in both ratios ($p < 0.001$), indicating higher crosslinker

interactions with dentin collagen. To quantify the extent of TF and CR interactions with dentin collagen, the band ratios at A1145/ Amide I for TF (fig. 13) and A1120/ Amide I for CR (fig. 14) were measured. The ratios of TF and CR treated dentin collagen were significantly higher ($p < 0.001$) when treatment time was increased from 30 s to 1 h, irrespective of dentin collagen type. The band ratio of 1 h TF treated HD dentin was highest followed by AD and natural dentin. On the other hand, 1 h CR treated natural dentin had the band ratio followed by HD and AD. Both TF and CR treated dentin collagen for 30 s had highest band ratios for AD followed by natural dentin and HD.

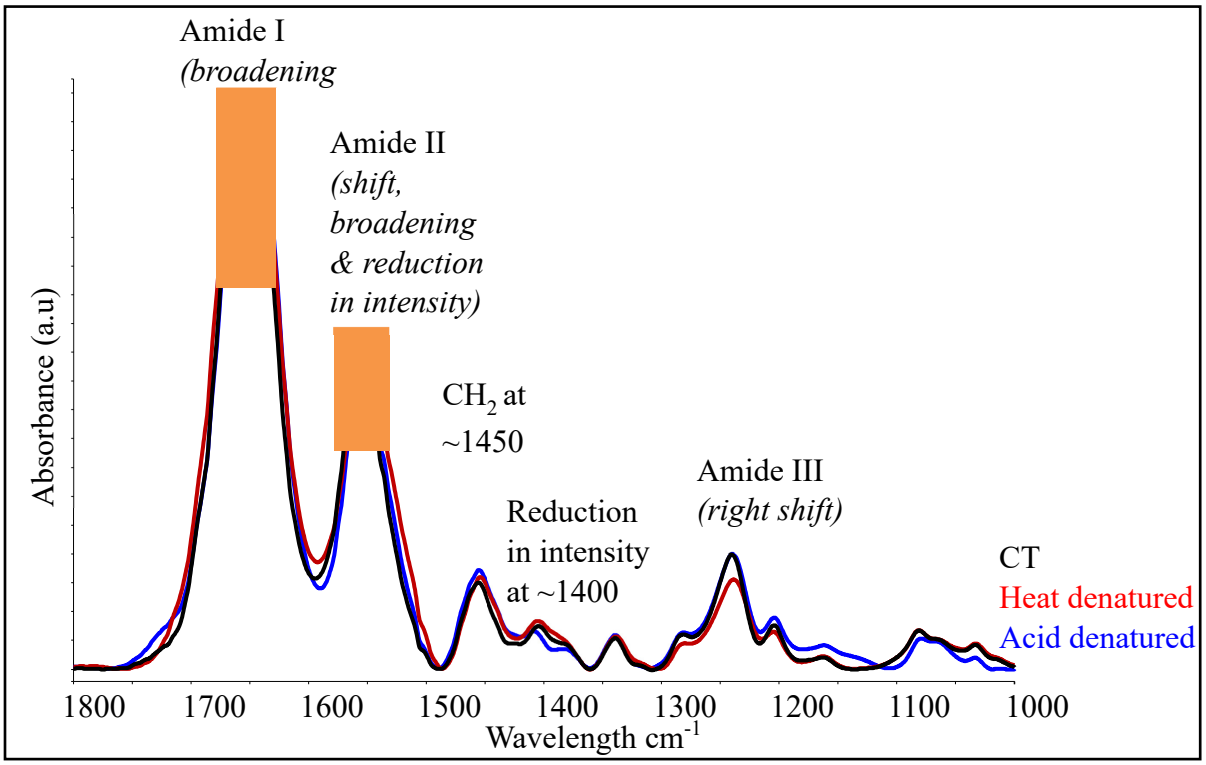

Figure 7. Representative FTIR of demineralized dentin collagen. Control (CT) in black, heat denatured (red line) and acid denatured (blue line).

TABLE 1

Means and Standard Deviations of Band Height Ratios (A1235 cm^{-1}/ A1445 cm^{-1})

Collagen type	Band height ratio (A1235 cm^{-1}/ A1445 cm^{-1})
CT	1.181 ± 0.14 A
HD	0.861 ± 0.05 B
AD	0.906 ± 0.07 B

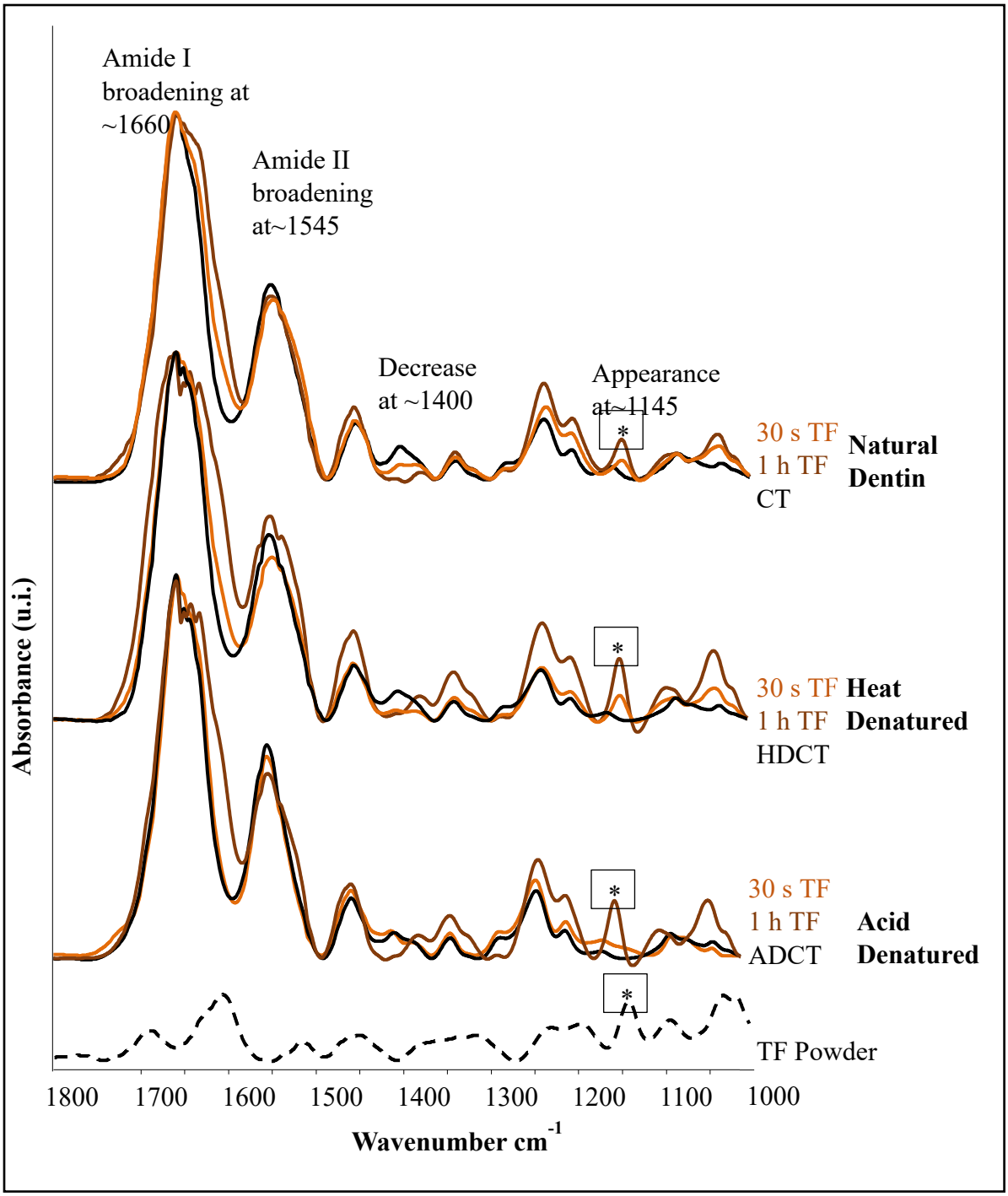

Figure 8. Representative FTIR of TF treated dentin collagen. Untreated dentin in black line (CT), 30 s (orange), 1 h (brown) TF treated dentin collagen. The top spectra are of natural dentin, middle represent heat denatured and bottom spectra are of acid denatured dentin. Dotted line (black) in the bottom represents the spectra of TF powder. Black asterisk (*) represent TF contribution.

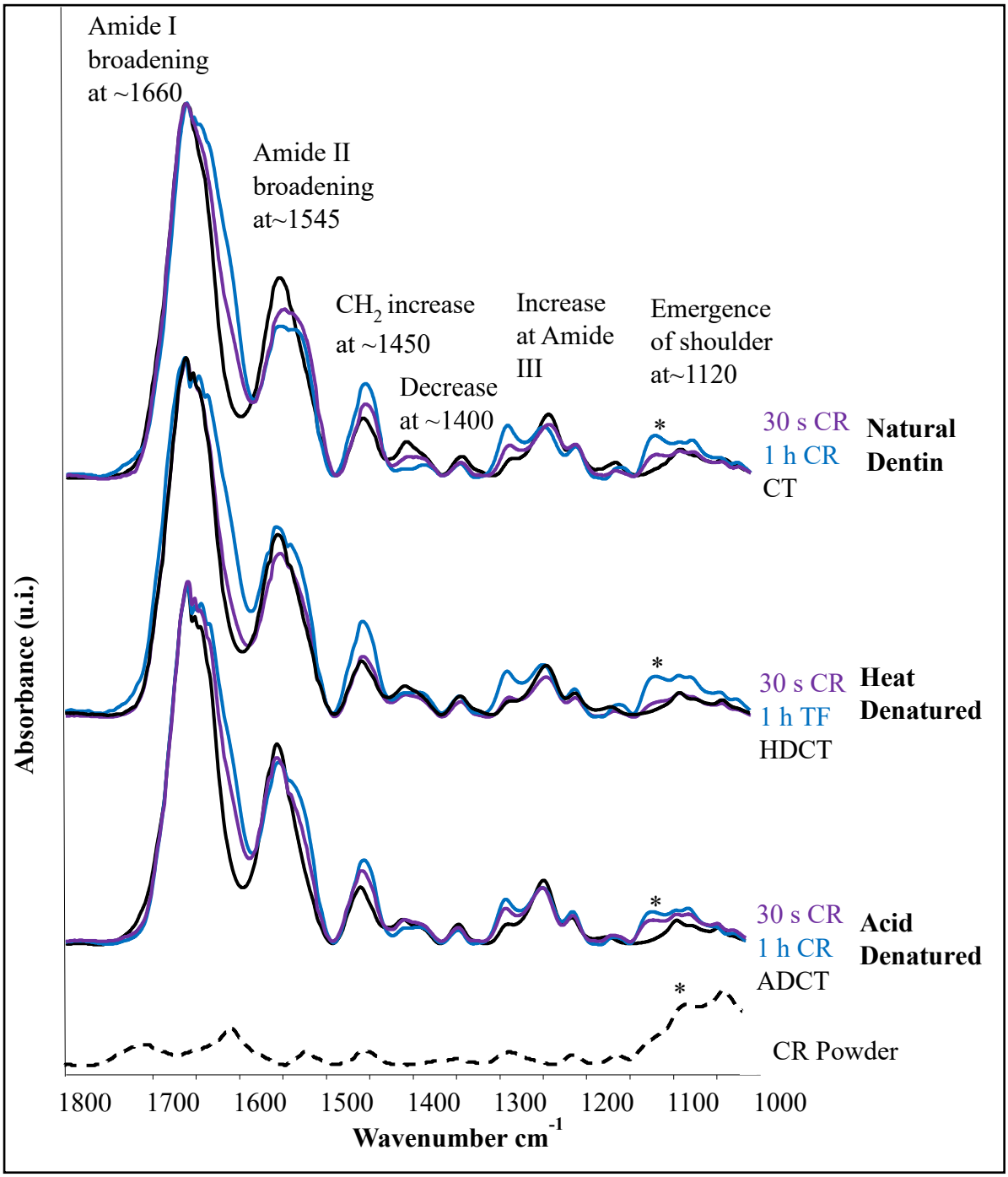

Figure 9. Representative FTIR of CR treated dentin collagen. Untreated dentin in black line (CT), 30 s (light blue), 1 h (dark blue) CR treated dentin collagen. The top spectra are of natural dentin, middle represent heat denatured and bottom spectra are of acid denatured dentin. Dotted line (black) in the bottom represents the spectra of CR powder. Black asterisk (*) represent CR contribution.

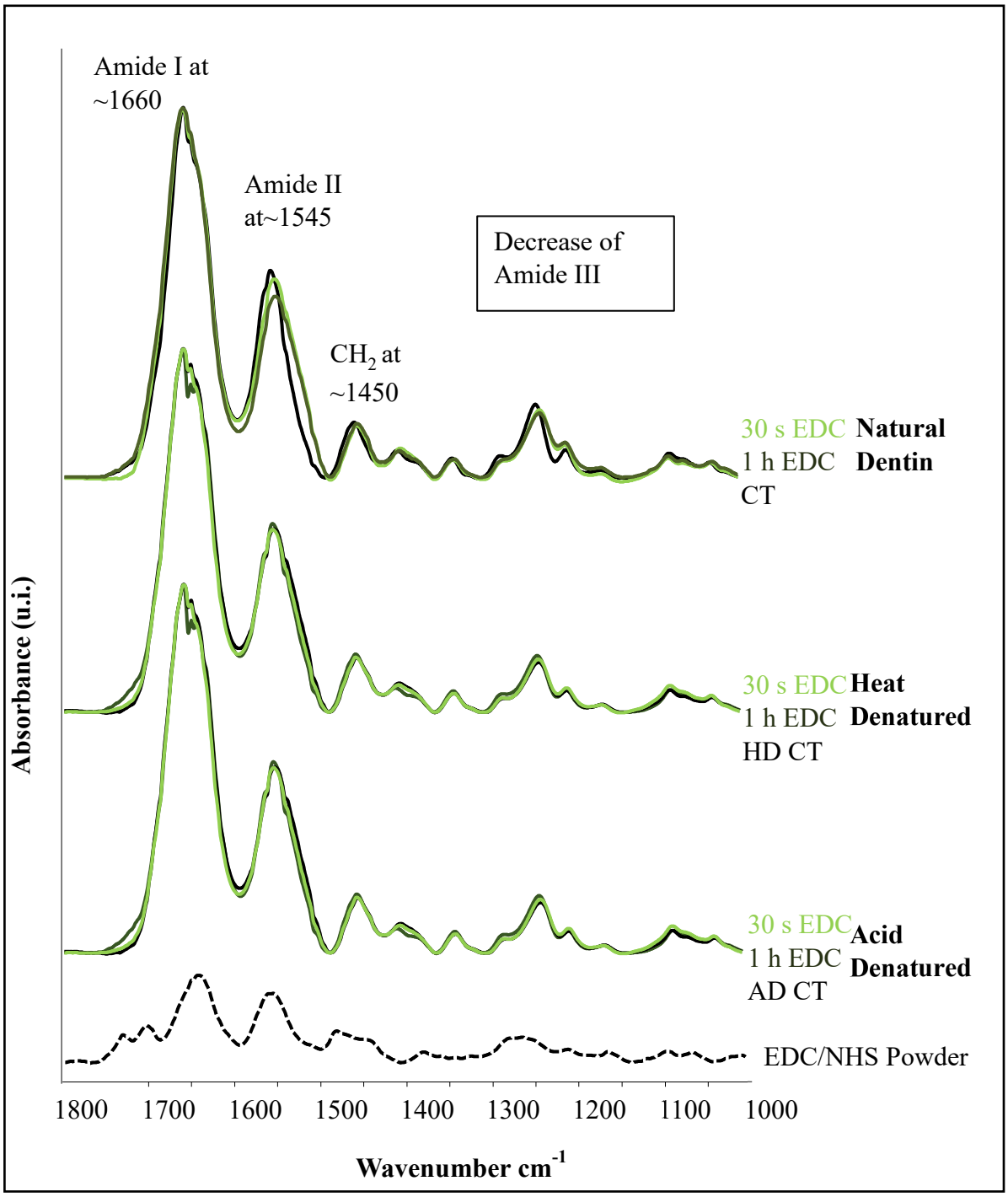

Figure 10. Representative FTIR of EDC/NHS treated dentin collagen. Untreated dentin in black line (CT), 30 s (light green), 1 h (dark green) EDC/NHS treated dentin collagen. The top spectra are of natural dentin, middle represent heat denatured and bottom spectra are of acid denatured dentin. Dotted line (black) in the bottom represents the spectra of EDC/NHS powder.

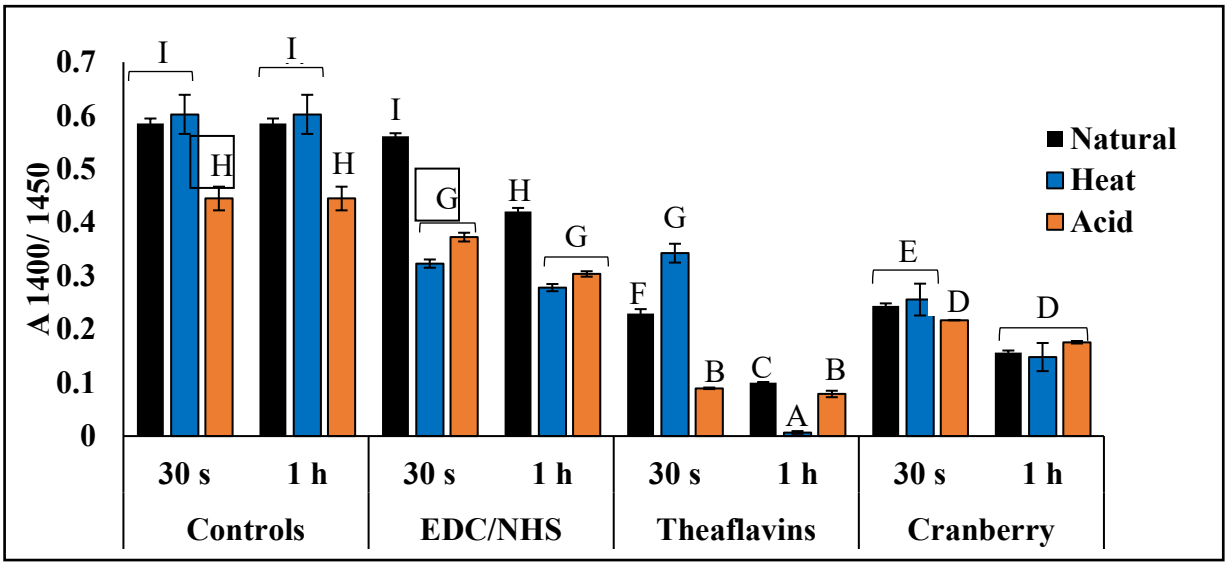

Figure 11. Band ratios of C-O and C-N bending to CH_2 (A1400/ A1450). Means with different letters are significantly different ($p < 0.05$).

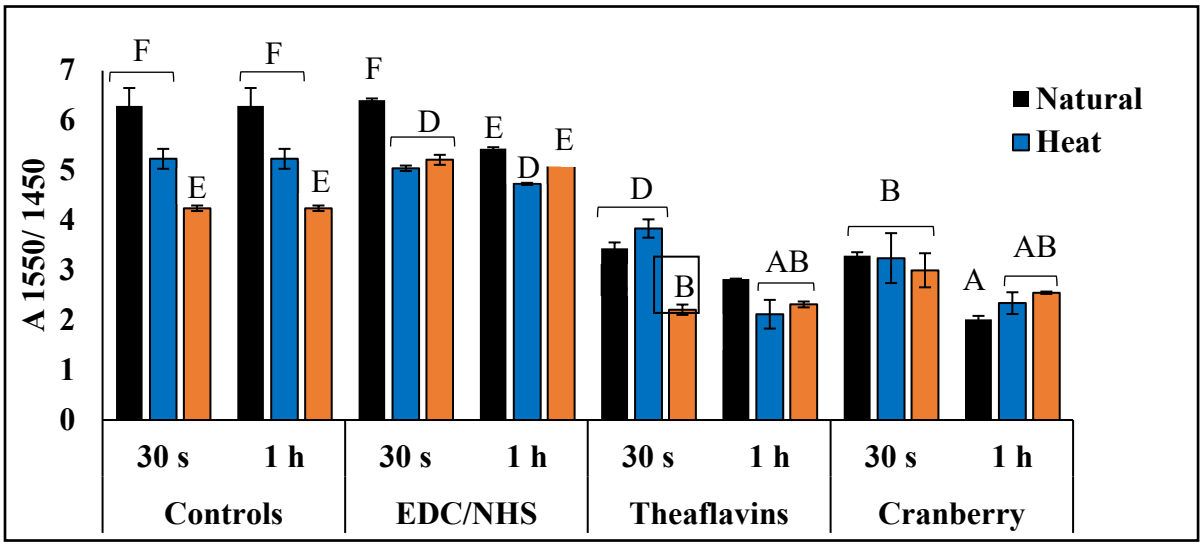

Figure 12. Band ratios of amide II to CH_2 (A1550/ A1450). Means with different letters are significantly different ($p < 0.05$).

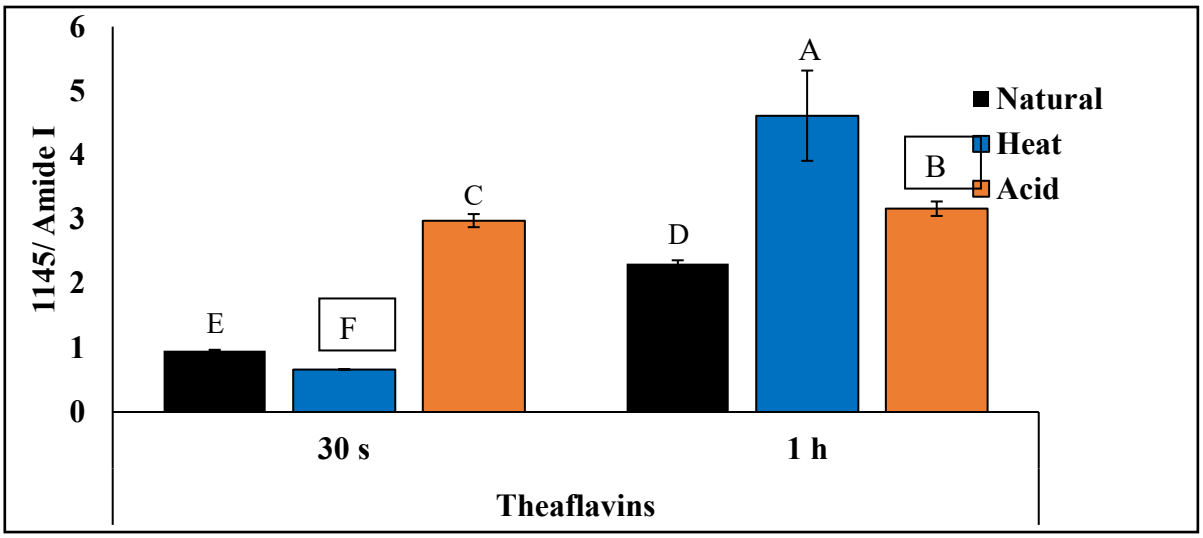

Figure 13. Band ratios of A1145/ Amide I for TF treated groups. Means with different letters are significantly different ($p < 0.05$).

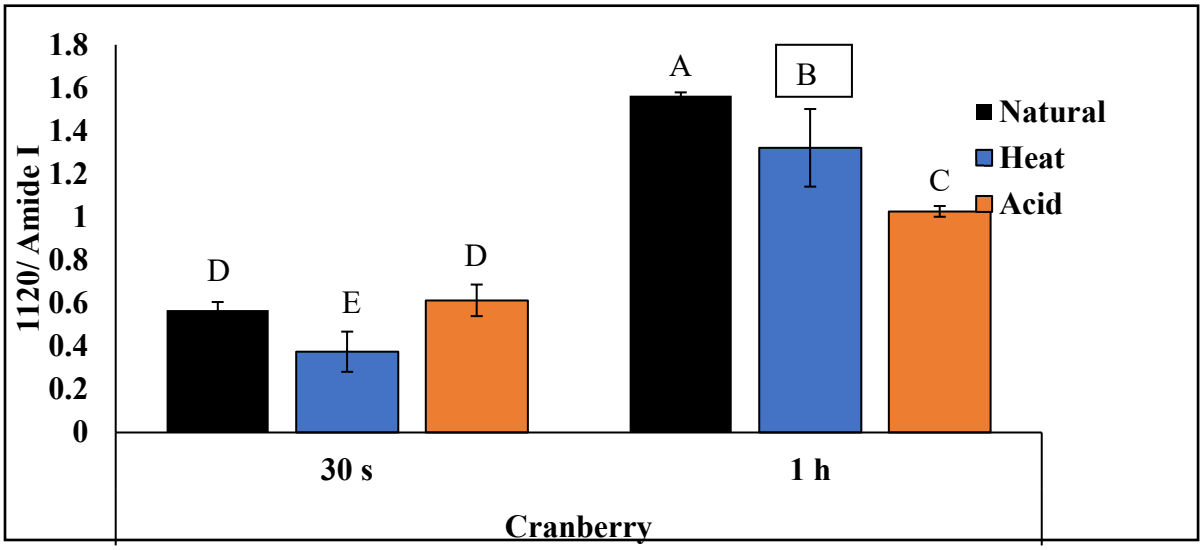

Figure 14. Band ratios of A1120/ Amide I for CR treated groups. Means with different letters are significantly different ($p < 0.05$).

Endogenous MMPs Activity in Dentin Collagen

The endogenous MMPs activity within the collagen films was assessed using confocal laser scanning microscopy and in situ zymography. The green fluorescence intensity (top) representing the activity of the endogenous MMPs and the representative images (bottom) are presented in figure 15. The untreated (CT) of natural dentin collagen showed highest overall fluorescence indicating strong MMPs activity even when compared to AD and HD ($p < 0.0001$). No statistical difference was seen in both HDCT and ADCT's and EDC/NHS treated natural and DD ($p > 0.05$) except for 1 h EDC/NHS treated natural dentin collagen which showed a dramatic reduction of ~ 90% as compared to CT and ~70% when compared to 30 s treatment time ($p < 0.0001$). Both CR and TF treated groups were able to reduce MMPs activity by ~95% when treated for 1 h irrespective of the dentin collagen type ($p > 0.05$), while 30 s treatment time had slightly lower (~90%) MMPs activity for natural dentin while CR treated AD dentin showed a reduction of ~70%.

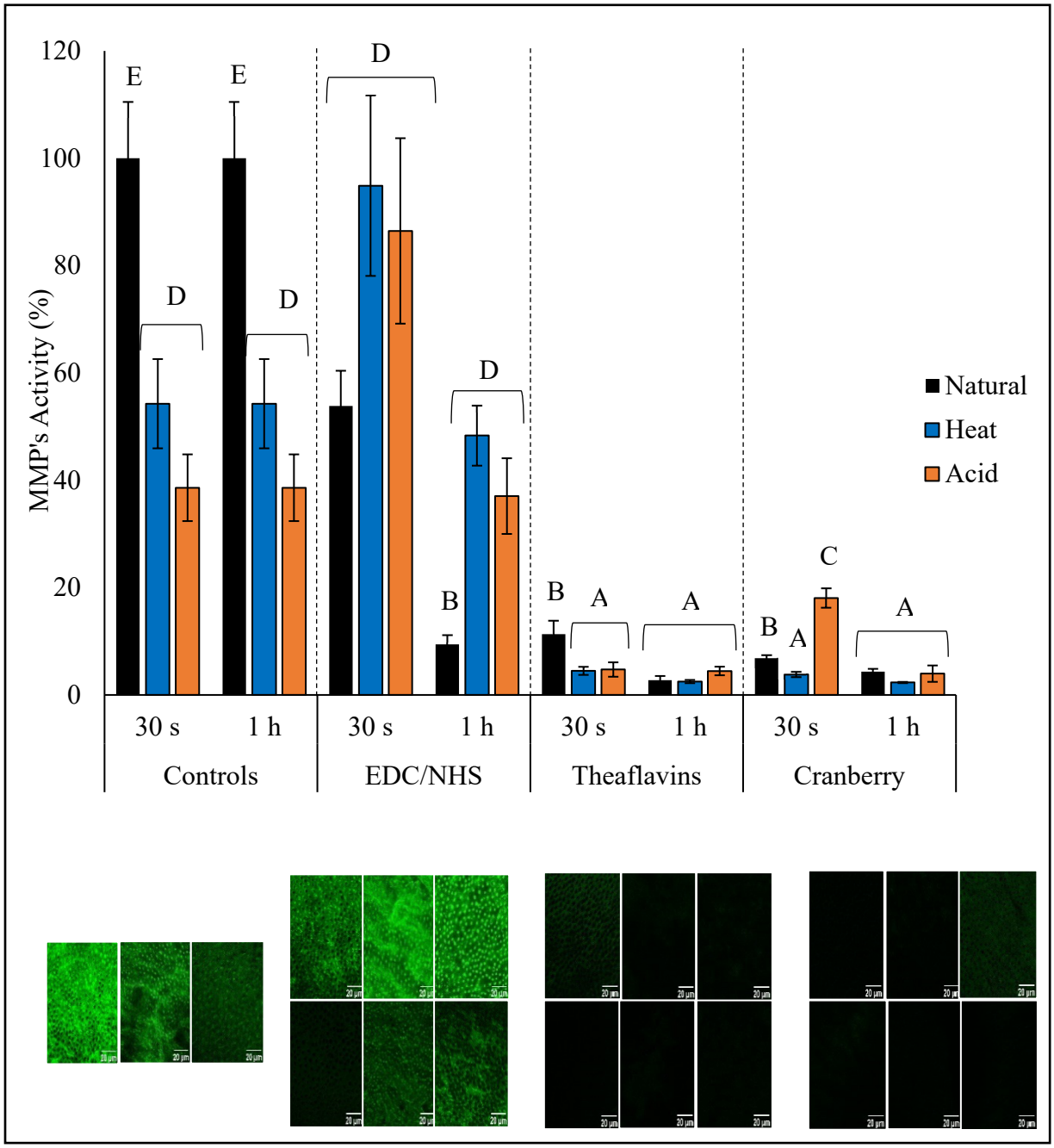

Figure 15. MMPs activity of all treatment groups. Relative green fluorescence intensity representing MMPs activity (top) and representative images (bottom) of controls and treated dentin collagen following 24h incubation in quenched fluorescein-labeled-gelatin. The images are obtained with CLSM using green channel showing fluorescence attributed to MMPs activity. Means with different letters are significantly different ($p < 0.05$).

Biostability Against Exogenous Enzymatic Degradation

Results for collagen biostability against exogenous collagenase degradation are presented in figure 16 & figure 17. Lowest WL (~10%) was seen in 1 h TF, CR treated groups irrespective of collagen substrate along with 30 s TF, CR treated natural and AD dentin collagen, indicating highest collagen biostabilization. A reduction in biostabilization was observed with EDC/NHS 1 h treatment of natural and AD dentin collagen (~45%) and for HD TF and CR (~35%) 30 s treated groups ($p < 0.0001$). Highest WL (100%) and complete collagen digestion was observed for all 30 s EDC/NHS groups and HD 1 h treatment of EDC/NHS which was comparable to all CTs ($p > 0.05$). The HYP measurements (fig. 17) showed lowest HYP release (~5µg/mg) for 1 h CR and TF treated dentin collagen except for AD which had higher HYP release of 20.4µg/mg ($p < 0.0001$), while 30 s TF and CR treated natural dentin collagen also showed low HYP release (~10µg/mg) statistically similar to 1 h ($p > 0.05$). This trend was followed by 30 s CR, TF treated AD and EDC/NHS 1 h treated natural dentin collagen (~40µg/mg). HD dentin collagen treated for 30 s with CR and TF showed a significantly higher (~75µg/mg) HYP release as compared to the other two substrates treated for the same time ($p < 0.0001$). Highest HYP release (~129 µg/mg) was observed in ADCT followed by HDCT (~111 µg/mg) which was statistically similar to HD and AD EDC/NHS 30 s treatment ($p > 0.05$).

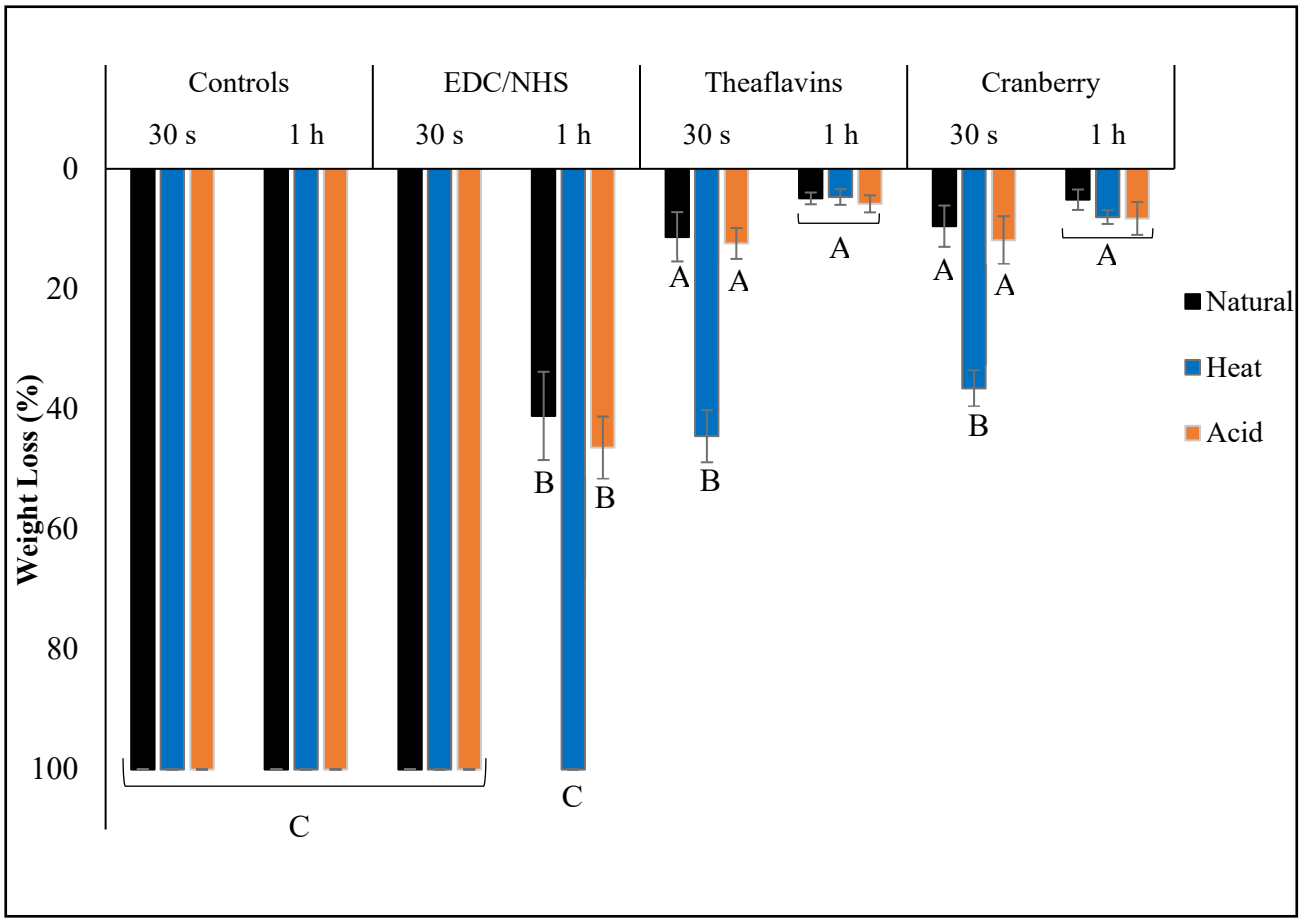

Figure 16. Percent weight loss of controls and crosslinker treated groups after digestion. Means with different letters are significantly different ($p < 0.05$).

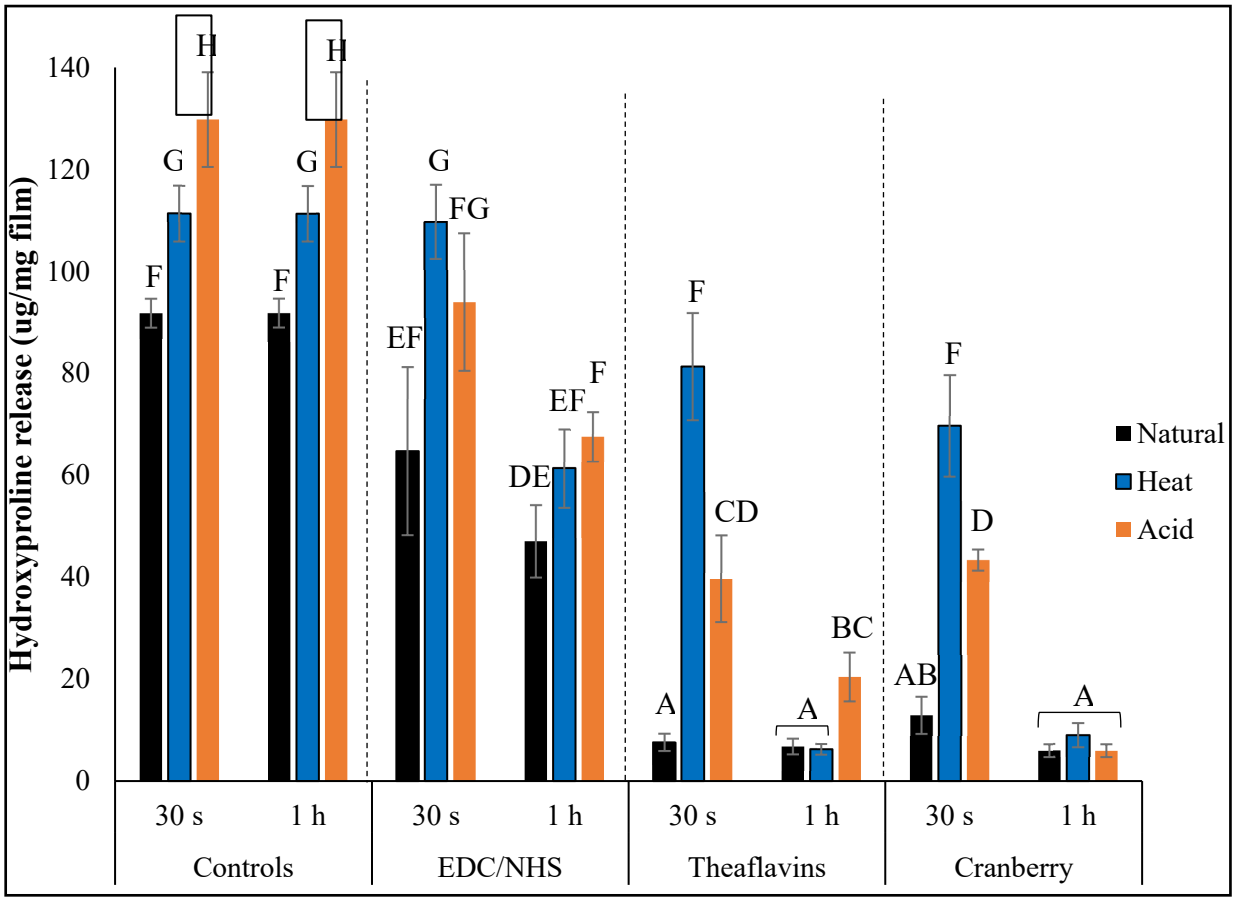

Figure 17. Hydroxyproline (HYP) release content (µg/mg film) of controls and crosslinker treated groups after digestion. Means with different letters are significantly different ($p < 0.05$).

Resistance to Mechanical Tensile Strength Testing

The ultimate tensile strength (UTS) and elastic modulus (EM) results are presented in fig. 18. Natural dentin collagen had significantly higher UTS and EM values compared to HD collagen ($p < 0.05$). Highest UTS and EM values were observed in TF and CR treated natural dentin collagen followed by TF and CR treated HD dentin collagen which had UTS values significantly similar to untreated natural dentin collagen ($p > 0.05$). EDC/NHS treatment did not show any significant effect on natural or HD dentin collagen compared to control.

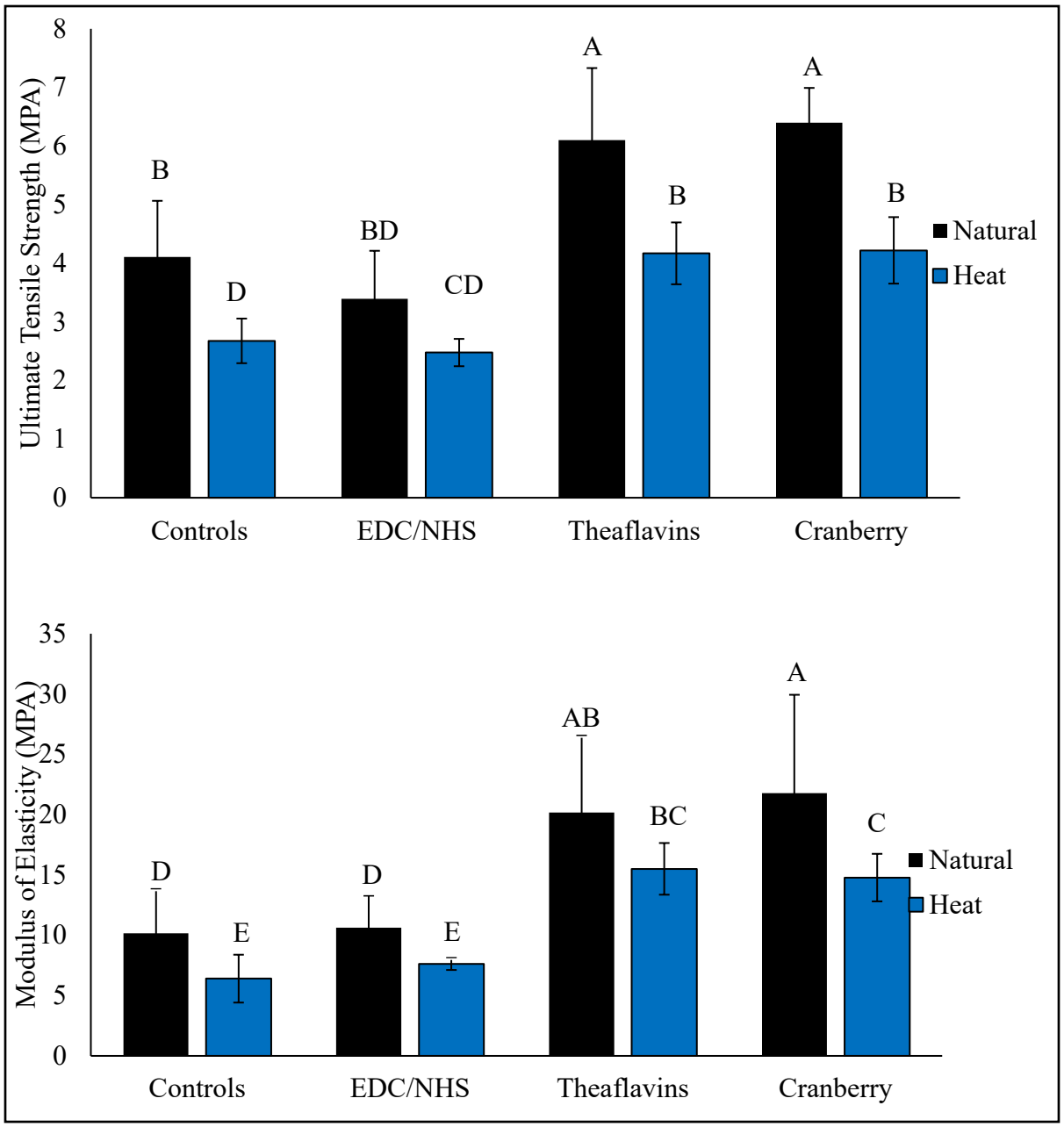

Figure 18. Ultimate tensile strength (top) and modulus of elasticity (bottom) of natural and heat denatured dentin collagen treated with crosslinkers for 1 h. Means with different letters are significantly different ($p < 0.05$).

Discussion

Dentin has a complex chemical and physical structure made of a fibrous three-dimensional network of type I collagen that is impregnated with carbonated apatite mineral. Despite having composition similar to bone, dentin is comparatively more interwoven with extensive crosslinking throughout its structure (IR 1951; Veis and Schlueter 1964). The triple helical collagen molecules are stabilized through extensive hydrogen bonds as well as inter and intramolecular crosslinks (covalent bonds) which provide stability, strength and resilience to dentin collagen (Kuboki and Mechanic 1982).

However, this resilient structure is susceptible to degradation during a carious attack which initiates a vicious cycle of endogenous enzymes activation leading to breakdown of the collagen molecules (Femiano et al. 2016). These enzymes can bind to specific sites in the collagen molecules and cause unfolding of the triple helix structure (Visse and Nagase 2003). This results in loss of crosslinks at inter and intramolecular levels causing structural alterations or denaturation within the collagen molecules which leads to compromised mechanical properties and collagen stability (Ito et al. 2005).

Simulating the carious process in dentin to achieve collagen denaturation has been attempted through acid buffers with pH cycling and microbiological caries induction which produces an artificial carious lesion of ~140 – 200 μm (Ferreira Nicoloso et al. 2016; Fialho et al. 2019). Some studies have also used dentin with an active carious lesion as a substrate for adhesive bonding (Hass et al. 2019). The drawback of using these methods is that the lesion produced is not homogenous and hence quantifying the effect of treatments would result in inaccurate interpretations. While literature does not provide clear denaturation method or technique to produce DD substrate which is quantifiable, we tested two different

methods, HD and AD to produce DD collagen. To achieve homogenous denaturation and limit the effect of diffusion of crosslinkers, we used thin dentin samples of ~7µm thickness. It has been a common practice in literature to use thick dentin beams to evaluate crosslinker induced biomodification of dentin collagen. This practice reduces the chances of homogenous diffusion of the crosslinker into the specimen and would not give an accurate representation of the crosslinker treatment. Secondly the acid etched dentin layer we are trying to biomodify through the crosslinking treatment is less than ~10µm thick. Hence to mimic clinically relevant substrate it is wise to use thin dentin collagen specimens which are (7-10 µm thick) rather than 400 to 1000 µm, wherever possible.

Characterization of the dentin collagen substrate was done using FTIR and structural alterations indicating denaturation were confirmed (fig. 7). The most prominent changes include broadening of amide I and reduction in amide II intensity, which could be due to formation of water mediated hydrogen bonds in the DD collagen (Rabotyagova et al. 2008; Stani et al. 2020). The shift of amides II and III to lower wave numbers can be assigned to structural rearrangements or depletion of the triple helix structure of dentin collagen following denaturation (Derrick 1991; Rabotyagova et al. 2008; Sanden et al. 2021). Reduction of ~1400 cm^{-1} band shows the effect of dehydration on dentin collagen fibers due to denaturation (Miles et al. 2005; Boryskina et al. 2007; Liu et al. 2013).

The band ratio of A1235/ A1450 of HD dentin collagen was 0.86 and AD was 0.9 as compared to that of natural dentin collagen (1.18). Dentin collagen with an intact triple helical structure has A1235/ A1450 value close to 1, while this value reduces to around 0.5 when the structure is completely destroyed and becomes like gelatin (Sylvester et al. 1989; Liu et al. 2014b). This reduction in the band ratio indicates that our denaturation techniques

were able to induce structural changes / damages in the collagen causing it to denature and producing a substrate similar to caries affected dentin. While WL results did not show any difference between natural and DD collagen ($p > 0.05$), HYP release (more sensitive molecular technique) was significantly higher in both DD compared to natural dentin collagen ($p < 0.001$). Structurally altered and unwound triple helix structure of DD collagen would provide more cleavage sites for collagenase to act on and be broken into smaller peptide units causing higher HYP release (Philominathan et al. 2012). Both denaturation techniques used in this study alter the collagen structure in their own unique way. Heat causes collagen structural alterations which are accompanied by gross shrinkage of the whole structure (Hoermann and Schlebusch 1971), while acid triggers hydrolysis of the collagen molecule causing it to breakdown into smaller peptides and leading to gross loss of collagen substrate (León-López et al. 2019). This is the reason why we see the highest HYP release in AD dentin.

Endogenous MMPs activity was reduced in both DD samples compared to natural dentin collagen ($p < 0.001$) which can be explained by probable denaturation of the MMP enzymes themselves hence affecting their ability to cleave collagen (Meraz-Cruz et al. 2020). Another explanation could be the alterations of the specific cleavage sites on the collagen molecule itself, such as Gly-Ile in α1 chain and Gly-leu in α2 (Messent et al. 1998) where the enzymes act on. These alterations would limit the ability of MMPs to bind to and cleave the collagen molecule.

The triple helix structure in natural dentin collagen is packed into a tight coil with extensive crosslinks making it a resilient structure (Goh et al. 2007). Denaturation would cause loss of crosslinks and unwinding of the triple helices which would affect the

mechanical integrity of dentin collagen (Wang et al. 2002; Ito et al. 2005). A significant difference was observed between the mechanical properties of natural dentin and HD collagen ($p < 0.001$) in this study. This further supports our HYP release and FTIR results, implying that structural alterations and unwinding of the triple helix structure of collagen reduces the mechanical properties of dentin collagen following denaturation. As explained earlier, the nature of AD process causes hydrolysis of collagen which leads to loss of the dentin collagen substrate. Hence only HD dentin collagen beams were used to compare mechanical properties.

Biomodification of dentin collagen using exogenous crosslinkers has shown to enhance biostability as well as mechanical properties of sound natural dentin collagen (Bedran-Russo et al. 2014). The few studies that have looked into the effect of the crosslinkers on DD have a lot of discrepancies due to the challenges of not having a homogenous substrate. FTIR results in our study showed distinct spectral changes in natural and DD collagen treated with the crosslinkers. Evidence of hydrogen bonding (between amino and amide groups of collagen and phenolic (OH) groups of TF and CR) is seen in all dentin collagen substrates treated with TF and CR, represented through spectral changes in amide I (broadening) and amide II (broadening and reduction in intensity) (He et al. 2011). Another predominant change was the reduction of the ~1400 cm^{-1} band intensity due to hydrophobic interactions causing dehydration via the replacement of bound water in collagen by TF and / or CR (Miles et al. 2005; Liu et al. 2013). The spectral changes induced by the crosslinkers were quantitively measured with A1400/ A1450 (fig. 11) and A1550/ A1450 ratios (fig. 12). Reduction in the ratio is attributed to high collagen/ crosslinker interactions. The crosslinkers TF and CR treated groups showed the lowest ratios compared to the

controls. Increased interactions would translate into induction of more crosslinks and improved protections against enzymatic degradation which can be confirmed through reduced WL and HYP release (fig. 16 & fig. 17) values of TF and CR treated groups. To further characterize the extent of collagen interactions of TF and CR, ratios of the specific contributory bands of the two crosslinkers and Amide I were measured (fig. 13 and fig. 14). An increase in the ratios meant higher collagen /crosslinker interactions. Irrespective of the dentin collagen type, both ratios significantly increased following 1 h treatment.

The changes in the FTIR spectra of EDC/NHS treated dentin collagen were less intense as compared to TF and CR, which included reduction of amide II band that could be due to a decrease in $-NH_2$ in response to increased crosslinking (Júnior et al. 2015; Oliveira et al. 2019). Unlike TF and CR which can induce crosslinking through various mechanisms, EDC/NHS is a zero length crosslinker that acts through activation of the carboxylic acid groups in collagen, which form an intermediate product (O-acylisourea) that slowly reacts with amino groups in collagen to form amide bonds (Tezvergil-Mutluay et al. 2012). Hence EDC/NHS acts by facilitating the formation of amide bonds without getting incorporated into the collagen, as is evident in the FTIR spectra which show negligible contribution of EDC/NHS powder into the treated collagen. The band ratios (fig. 11 and fig. 12) for EDC/NHS showed significant reduction ($p < 0.001$) following 1 h treatment time compared to the controls. This is due to reduction of band intensities at ~1400 cm^{-1} and Amide II (fig. 10), caused by formation of amide bonds between carboxylic acid and amines groups within the collagen molecules.

The effect of crosslinkers on the resistance to exogenous enzymatic degradation of DD collagen was also assessed. Effective crosslinking should strengthen the triple helix and

prevent unwinding of the collagen fibrils by sterically blocking the cleavage sites to bacterial collagenase and MMPs or by directly inhibiting these enzymes. For natural dentin collagen TF or CR treatment resulted in significant reduction of WL and HYP values compared to untreated dentin collagen (irrespective of treatment time). However, DD collagen showed significant resistance (with reference to HYP values) only after 1 h treatment except TF treated AD collagen which showed no difference with increasing treatment time. While treatment time of 30 s is clinically feasible, it was not enough to provide protection to DD collagen against collagenase digestion. It is important to point out that the dentin films were exposed to a challenging concentration of bacterial collagenase which is 2-6 orders of magnitude higher than the level of MMPs found in human saliva (Mäntylä et al. 2003).

Increasing the treatment time to 1 h gives the crosslinker more time to diffuse through the collagen structure and induce additional crosslinks to the unwound DD collagen, hence providing it higher resistance against collagenase degradation. This is in agreement with the A1145/ Amide I and A1120/ Amide I ratios (fig. 13 & fig. 14) which indicate higher collagen/ crosslinker interaction after 1 h treatment time. The A1145/ Amid I ratio of 30 s TF (for HD collagen) is lowest, indicating least interactions, which translated into higher WL and HYP results. While following 1 h TF treatment, HD collagen showed extensive interactions (high A1145/ Amide I ratio) with the collagen which resulted in enhanced protection against enzymatic degradation. Better performance after 1 h treatment could be due to the smaller size of TF allowing it to penetrate through the dense HD dentin collagen structure and induce crosslinks. On the other hand, increasing the treatment time did not have a significant effect on AD collagen which could be due to higher breakdown or hydrolysis of the collagen molecule resulting in less substance to crosslink compared to HD collagen.

Hence increase in treatment time would not have as dramatic effect on AD as for HD. The A1120/ Amide I ratio for natural collagen following 1 h treatment of CR showed the highest interactions, hence better protection against enzymatic degradation. Being bigger molecules, CR continues to polymerize when increasing the treatment time to 1 h, hence allowing more interactions with collagen and better protection against collagenase degradation.

Both TF and CR were able to reduce MMPs activity of denatured collagen irrespective of the treatment time, in fact they showed significantly higher reduction in MMPs activity than crosslinker treated natural dentin collagen. Both TF and CR are known to have MMP inhibition properties, hence other than introducing extensive crosslinks which could sterically block the active sites on MMPs, they can also directly inhibit MMPs and protect dentin collagen from degradation (La et al. 2009; Kong et al. 2015).

The positive control (EDC/NHS) used in this study showed no protection against collagenase degradation after 30 s, however 1 h treatment time showed significant reduction in WL and HYP values of all dentin collagen types (except WL of HD) compared to untreated controls. EDC/NHS activation results in formation of intra and inter helical crosslinks instead of intermicrofibrillar, which can be detected by sensitive techniques like HYP analysis and not by WL (Zeeman et al. 1999). This would explain why we could see significantly higher WL for EDC/NHS treated HD but similar HYP values for both DD. Being a zero length chemical crosslinker, the mechanism through which EDC/NHS works is slow and would produce better results following longer treatment time (Scheffel et al. 2014). However, some studies have also shown that crosslinking activity of EDC/NHS reaches a plateau after 1-2 h treatment time, which could be due to the limited availability of carboxyl groups on collagen for interaction or limitation of EDC/NHS to access the reaction sites

(Bedran-Russo et al. 2010). Unlike TF and CR which have more diverse mechanisms to interact with collagen, limited crosslinking potential of EDC/NHS results in comparatively poor protection against collagenase digestion.

Ability of EDC/NHS to form inter and intra helical bonds allows it to crosslink dentin collagen as well as the dentin matrix bound MMPs (Ekambaram et al. 2015a). The carboxyl and amino groups in MMPs are more accessible than those in collagen, making EDC/NHS a potent MMPs inhibitor than a collagen crosslinker (Perumal et al. 2008). This explains dramatical reduction of MMPs activity in natural dentin collagen following 1 h EDC/NHS treatment. Our studies show that the MMPs activity of EDC/NHS treated DD was same as that of untreated control indicating that EDC/NHS was not able to inhibit MMPs activity as it could for natural dentin collagen. Following denaturation, alteration of the triple helix structure as well as MMPs cleavage sites makes it challenging for EDC/NHS to effectively reduce MMPs activity.

Previous studies have shown a significant improvement in mechanical properties of natural dentin collagen treated with crosslinkers (Bedran-Russo et al. 2010; Castellan et al. 2011). As denaturation of dentin collagen causes reduction in mechanical properties, it is of vital importance that inducing exogenous crosslinking would make DD collagen stiff and provide stability against degradation. As AD caused loss of the collagen substrate, we used HD dentin collagen beams only. Our results showed significant improvement in mechanical properties of both TF and CR treated natural and HD dentin collagen compared to untreated controls. However, EDC/NHS treated natural and HD dentin collagen did not show any improvements compared to controls. These results are in disagreement with those of Bedran-Russo et al. who showed significant improvement in EDC/NHS treated natural dentin after 1

h treatment (Bedran-Russo et al. 2010). The difference in our results could be due to the lower concentration we used (0.05M EDC/ 0.02M NHS) compared to 0.3M EDC/ 0.12M NHS used in literature. Secondly, unlike the common practice in literature where samples are not rinsed following treatment, we thoroughly rinsed our samples in order to stop the continued effect of crosslinking longer than the intended time which could be another reason for lower values in our study.

Conclusions

The denaturation techniques used in this study were able to induce structural changes in dentin collagen and produce a substrate similar to CAD. Crosslinking treatment altered the collagen structure and improved the biostability of DD collagen against exogenous and endogenous enzymatic degradation compared to the controls regardless of treatment time (except for EDC/NHS 30 s treatment). It was generally more challenging to crosslink HD collagen in 30 s, however significant improvement was seen following 1 h treatment time. Both TF and CR were efficient to crosslink DD collagen, showing even better biostability than natural dentin collagen when untreated.

CHAPTER 4

THE EFFECTS OF FLAVONOIDS ON REMINERALIZATION OF NATURAL AND DENATURED DENTIN COLLAGEN.

Introduction

The body of the tooth is formed by dentin which is a mineralized composite made up of type I collagen and non collagenous proteins (NCP) (Moradian-Oldak and George 2021). The collagen fibrils are arranged in a manner that creates a 67 nm banding pattern made up of gap and overlap zones. The overlap zones formed between neighboring collagen molecules are the basis of inter-molecular crosslinking. While intra-molecular crosslinks are formed between adjacent triple helical chains through covalent bonding (Knott and Bailey 1998). The extensive crosslinking in dentin collagen contributes towards its mechanical strength and stability (Miguez et al. 2004).

The collagen in dentin matrix also provides a 3 dimensional interactive scaffold for hierarchically organized mineral deposition (Wang et al. 2012; Yao et al. 2019). The binding of NCPs in specific gap regions of the collagen fibril initiates precipitation of calcium and phosphate mineral into hydroxyapatite crystals. These crystals then coalesce and fill the intrafibrillar gap regions to form hydroxyapatite. This hierarchal organization of mineral reinforces dentin collagen and contributes to its mechanical properties (Kinney et al. 2001; Kinney et al. 2003).

Loss of mineral as well as the scaffolding properties of dentin collagen following a carious attack starts a vicious cycle of activation of endogenous enzymes and degradation of collagen. To improve the status of dentin collagen, biomimetic approaches using bioactive

agents to enhance mechanical properties have shown promising results (Balalaie et al. 2018; Saxena et al. 2022). The use of NCP analogues can stabilize the formation of amorphous calcium phosphate (ACP) particles, enabling them to diffuse into the microfibrillar spaces in dentin collagen. The stabilized ACP can replace the residual water in these spaces while undergo crystallization to form hydroxyapatite (Olszta et al. 2007). This process is termed Polymer-Induced Liquid Precursor (PILP) and it results in restoring the mineral hierarchical arrangement similar to that in dentin (Burwell et al. 2012; Saxena et al. 2022).

Another biomimetic approach is the use of crosslinkers, which have the ability to induce exogenous inter and intrafibrillar crosslinks (Cai et al. 2018). The biomodifications introduced through crosslinkers have shown to improve the mechanical properties as well as resistance against enzymatic degradation (Bedran-Russo et al. 2014). As discussed in chapter 3, the crosslinkers TF and CR showed promising results by modifying the collagen structure and improving the mechanical properties as well as resistance against degradation in natural and denatured dentin collagen in clinically feasible treatment time of 30 s.

While both these strategies have shown promising results, information regarding their combined effect is limited. To the best of our knowledge these strategies have not been used to evaluate their effect on denatured or altered dentin collagen substrate found in caries affected dentin. Nor has there been any comparative report of using PILP with a natural crosslinker. Hence the aim of our study was to evaluate the PILP technique with a natural crosslinker in natural and denatured dentin.

Materials and Methods

Collection of Teeth and Preparation

Thirty teeth were collected according to the methods explained in chapters 3. Half were kept for experiments on sound/ natural dentin and rest of them were subjected to artificial caries induction.

WL and HYP Analysis of Dentin Collagen Films

Four hundred and twenty natural and 300 heat denatured (HD) dentin collagen films (10 μm thick) were prepared following methods described in chapter 3.

Untreated natural and HD dentin collagen films were used as controls. For rest of the natural dentin collagen films, half were treated with NCP containing remineralization solution (50 mM Tris-buffer[30], 0.9% NaCl[28], 0.02% NaN_3[48], 4.5 mM $CaCl_2$[29], 2.1 mM K_2HPO_4[49], 100 μg/mL of 27 kDa pAsp[50], 39 ml DI water at pH 7.4) for 1, 2 and 7 days. The other half were treated with the crosslinker CR[10] for 30 s and rinsed with DI water for 2 min, after which 60 films were put in remineralization solution for 1 day and the other 60 films were put for 2 days. For heat denatured films, half were put in remineralization solution for 2 and 7 days while the rest of them were treated with the crosslinker out of which 60 films were put in remineralization solution for 2 days. All films subjected to remineralization solution were kept in an incubator at 37˚C for the duration of the remineralization treatment. Treated films from all the groups were subjected to WL and HYP analysis according to the methods described in chapter 3.

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[48]Sodium Azide, CAS# 26628-22-8, Sigma-Aldrich, St. Louis, MO 63178
[49]di-Potassium Hydrogen Phosphate, CAS# 7758-11-4, Sigma-Aldrich, St. Louis, MO 63178
[50]Poly aspartic acid, CAS# 25608-40-6, Alamanda Polymers Inc., Huntsville, AL 35816

Artificial Caries Induction to Achieve CAD

Occlusal enamel of 3rd molars was removed using a water-cooled low speed diamond saw[3] to expose the dentin surface. Roots of these teeth were sealed using two coats of epoxy adhesive[35] followed by two coats of nail varnish[36]. The teeth were then sterilized in an autoclave[37] at 121ºC for 20 min. Cariogenic solution (Fialho et al. 2019) containing 3.7 g of BHI broth[38], 2 g sucrose[39], 1 g glucose[40] and 0.5 g yeast extract[41] to each 100 mL of DI water. This solution was sterilized in an autoclave for 20 min at 121 °C. The cariogenic solution was then inoculated with 2% strain of *Streptococcus mutans* ATCC25175[42] (10^8CFU/mL)**.** The sealed and sterilized teeth were put in the cariogenic solution and incubated for 14 days in a humidified 5% CO_2 incubator[43] at 37˚C. The cariogenic solution (without the bacterial inoculation) was changed every 48 h. Following incubation, the teeth were sterilized again and the biofilm on the dentin surface was removed with a gauze while the epoxy and varnish layers were removed manually with a scalpel blade[44]. Presence of soft dentin was confirmed for each sample using a non-cutting instrument[45] (Carvalho et al. 2016). Soft dentin was removed with a manual curette[46] until only dark dentin that was resistant to being touched with an exploratory

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[32]SSTM-5000 Mechanical Tester, United Calibration Corporation, CA 92649
[33]Zapit, Dental Ventures of America, Corona,CA 92880
[35]Loctite 234796 super glue all-purpose adhesive, Kearneysville, WV 25430
[36]Revlon Nail Enamel #270, New York, NY
[37]Autoclave, GT14DP, Zealway, Wilmington, DE 19801
[38]Brain Heart Infusion Broth, Becton Dickinson and Company, Sparks, MD 21152
[39]Sucrose, CAS#57-50-1, Sigma-Aldrich, St. Louis, MO 63178
[40]α-D-Glucose, CAS#492-62-6, Sigma-Aldrich, St. Louis, MO 63178
[41]Yeast extract, CAS#801301-2, Sigma-Aldrich, St. Louis, MO 63178
[42]Colecao de Culturas Tropical Fundacao Andre Toselo
[43]Shel Lab SCO 5A CO2 Air Jacketed Incubator, GLOBAL INDUSTRIAL, Port Washington, NY 11050

probe (without pressure) remained. The CAD teeth were then polished with 600 grit SiC paper. Six CAD teeth were cut into dentin beams measuring 7mm x 0.8mm x0.8mm and 4 were cut into dentin blocks (7mm x 7mm x 2mm) with a water-cooled low speed diamond saw[3].

Microhardness Evaluation of CAD

The roots of 4 CAD and 2 sound teeth were removed and sectioned (perpendicular to the long axis) into two halves using a water-cooled low speed diamond saw[3]. Each section was polished with 600, 1200 and 4000 grit SiC paper. The sections were sonicated for 10 min to remove any residues. Microhardness measurements were done using Knoop indenter with a static load of 25 gram applied for 30 s. Ten indents at an interval of 100µm were made across the specimen, starting from the outermost surface of each section, and proceeding across through the depth of the specimen. Four lines of indents were made in each sectioned specimen.

Preparation of Demineralized Dentin Layer

Six teeth (3 natural and 3 CAD) were cut into dentin beams measuring 7mm x 0.8mm x0.8mm and 6 were cut into dentin blocks (7mm x 7mm x 2mm) with a water-cooled low speed diamond saw[3]. For Scanning Electron Microscopy (SEM) a notch was made in the middle of each beam from the side opposite to the treated/ abraded surface for subsequent fracturing. The surface of each beam was etched with 32% phosphoric acid[47] for 30 s followed by rinsing with DI water for 2 min.

The etched specimens were treated with the crosslinker CR[10] solutions (prepared as described in chapter 3) for 30 s followed by rinsing with DI water for 2 min. Half of the

[44]371610, Bard-Parker® Disposable Scalpels size 11, Aspen Surgical, Caledonia, MI 49316
[45]Item# 392-5236, Safco Dental Supply, Buffalo Grove, IL 60089
[46]Item# 392-5004, Safco Dental Supply, Buffalo Grove, IL 60089
[47] Scotchbond™ Universal Etchant, 3M ESPE, St. Paul, MN 55119

crosslinker treated specimens were then immersed NCP containing remineralizing solution for a period of 2 days in an incubator at 37˚C. Specimen not treated with the crosslinkers were put in the remineralization solution for 1, 2, and 7 days. Each specimen was put in 40ml of remineralization solution. For SEM, half of the specimens following their respective treatments were subjected to 0.1% collagenase digestion for 1 h at 37˚C. The specimens were rinsed with DI water for 2 min and then prepared for SEM & TEM evaluation.

Scanning Electron Microscopy (SEM) Analysis

Treated demineralized dentin (natural and CAD) when exposed to collagenase enzyme should resist degradation and collagen morphology should be preserved. The cross section of demineralized dentin with details of collagen morphology before and after collagenase degradation can be viewed with SEM. This technique provides a direct way of characterizing the structural changes in demineralized dentin collagen.

Four notched dentin beams per treatment group (2 before and 2 after collagenase digestion) were used. Each specimen was fixed in 2.5% glutaraldehyde[51] buffered with 0.1 M sodium cacodylate[52] for 1 h and dehydrated in graded solutions of ethanol starting at 33% followed by 67%, 85%, 95%, and ending with 100% for 2h. After air-drying overnight, the dentin beams were fractured and mounted on aluminum stubs with conductive tape. They were then coated with carbon and visualized at the secondary electron mode (SEM) and back-scattered electron mode (B-SEM) in SEM[52].

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[51]Gluteraldehyde solution, EMS#16220, EMS, Hatfield, PA 19440
[52]Sodium cacodylate buffer, EMS#11652, EMS, Hatfield, PA 19440
[52]FEI/Philips XL30 Field-Emission Environmental SEM, Philips, Eindhoven, The Netherlands

Transmission Electron Microscopy (TEM) Analysis

Similar to SEM, TEM will characterize structural alteration of collagen morphology. The details of the perseverance of the bending structure of collagen at nano level through TEM reaffirms the SEM results. Selected area electron diffraction (SAED) was used to identify the nature and the crystallinity of the mineral formed after remineralization treatment.

The TEM study was performed on 2 un-notched dentin beams per treatment group. Each treated specimen was fixed in the same way as above for SEM, followed by post-fixation with 1% OsO_4[53] for 1.5 h and dehydrated in graded solutions of ethanol (33%, 67%, 85%, 95%, 100%) for 2h. The specimens were then treated with 1:1 solution of ethanol and propylene oxide[54] for 30 min, followed with 100% propylene oxide for 2h, and finally with 1:1 solution of propylene oxide and epoxy resin[55] overnight. After final infiltration with pure epoxy resin, specimens were incubated in an oven at 60°C for 48h. Ultrathin (60-100 nm) sections were cut with an ultramicrotome[56]. Each section was stained with 2% uranyl acetate[58] and observed under TEM[59] at an accelerating voltage of 80kV.

Surface Topology of Demineralized Dentin Surface

Two natural dentin and 2 CAD teeth were used for this experiment. They were divided into 4 parts using nail varnish[36] as show in figure 19. In the first step varnish was applied on areas 1, 3 & 4 on all teeth. Varnish was not applied on area 2, instead it was

[53]Osmium tetroxide, CAS#20816-12-0, Sigma-Aldrich, St. Louis, MO 63178
[54]Propylene Oxide, CAS#75-56-9, Sigma-Aldrich, St. Louis, MO 63178
[55]45359, Epoxy-Embedding Kit, Sigma-Aldrich, St. Louis, MO 63178
[56]EM-UC7 ultramicrotome, Leica, Buffalo Grove, IL 60089

etched with 32% phosphoric acid[47] for 30 s followed by rinsing with DI water for 2 min. All teeth were then put for 7 days in 40ml of remineralization solution each. On day 5, varnish was removed from area 3 and area 4. These areas were etched with 32% phosphoric acid[47] for 30 s followed by rinsing with DI water for 2 min. Area 4 was then treated with 1% crosslinker (CR) for 30 s and rinsed with DI water. Each tooth was then put back in remineralization solution for 2 more days.

Following completion of treatment times, varnish was removed from area 1 and this area was etched with 32% phosphoric acid[47] for 30 s and rinsed. Each specimen was then dried in a vacuum desiccator and then coated with carbon and visualized at the secondary electron mode (SEM).

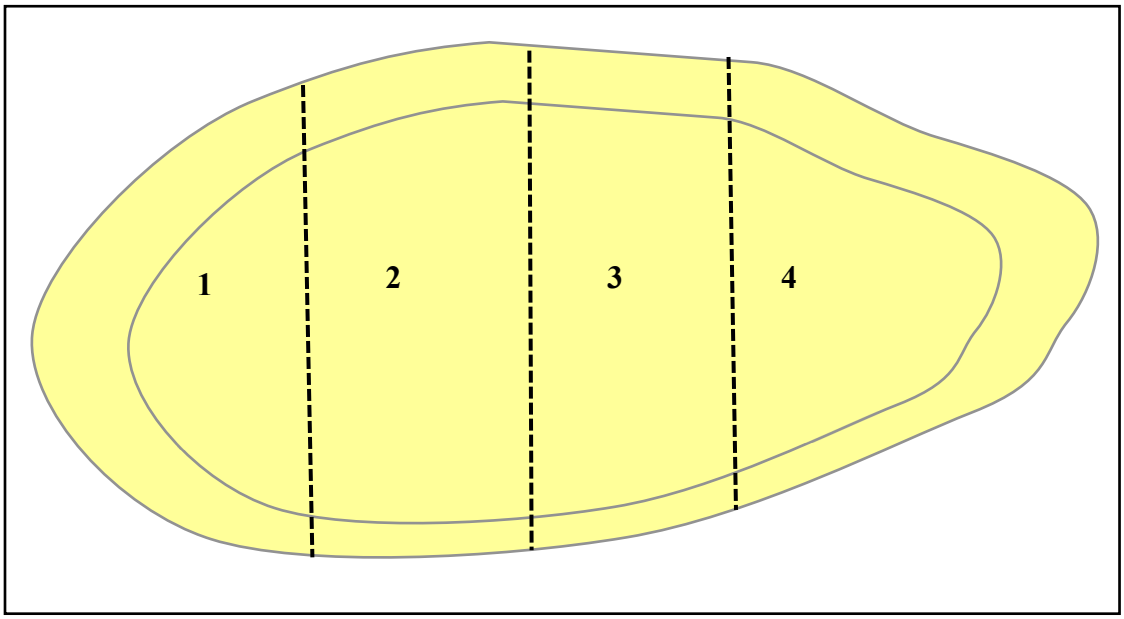

Figure 19: Representative figure explaining the division of teeth into different areas for treatment. Area 1: control (CT), Area 2: CT_7R (CT remineralized for 7 days), Area 3: CT_2R (CT remineralized for 2 days), Area 4: CR_2R (Crosslinker treated followed by 2 day remineralization).

Results

Biostability Against Exogenous Enzymatic Degradation

The collagen biostability results are presented in figure 20 & figure 21 for natural dentin collagen and in figure 22 and figure 23 for HD dentin collagen. For natural dentin collagen, highest WL was seen in CT (100%) followed by CT_1R (~67%) and CT_2R (~63%). Natural dentin collagen films treated with CR showed a WL of ~18% which was statistically similar to CT_7R ($p= 0.68$). While CR_1R and CR_2R had the lowest WL values ($p = 0.226$) which were significantly different from rest of the experimental groups ($p < 0.001$). The HYP analysis for natural dentin collagen (fig. 21) showed statistically lowest HYP release (~4 µg/mg) in CR_2R ($p < 0.001$), followed by CR_1R, CR and CT_7R experimental groups which had statistically similar HYP release values ($p > 0.05$). Experimental groups CT_1R (~67 µg/mg) and CT_2R (~62 µg/mg) had statistically similar HYP release values and were significantly lower than CT (~87 µg/mg) which had the highest HYP release ($p < 0.01$).

For HD dentin collagen the lowest WL and HYP release were seen in the experimental group CR_2R (5.5% and 3.84 µg/mg, respectively) followed by CR (17.04% and 15.74 µg/mg, respectively) which were statistically different ($p < 0.001$). Unlike natural dentin collagen, the WL and HYP release of CT_7R were significantly higher than CR ($p = 0.003$), while similar to natural dentin collagen, a significant difference was seen between CT_2R and CT_7R. The WL and HYP release values were the highest in CT (100% and 124.05 µg/mg, respectively).

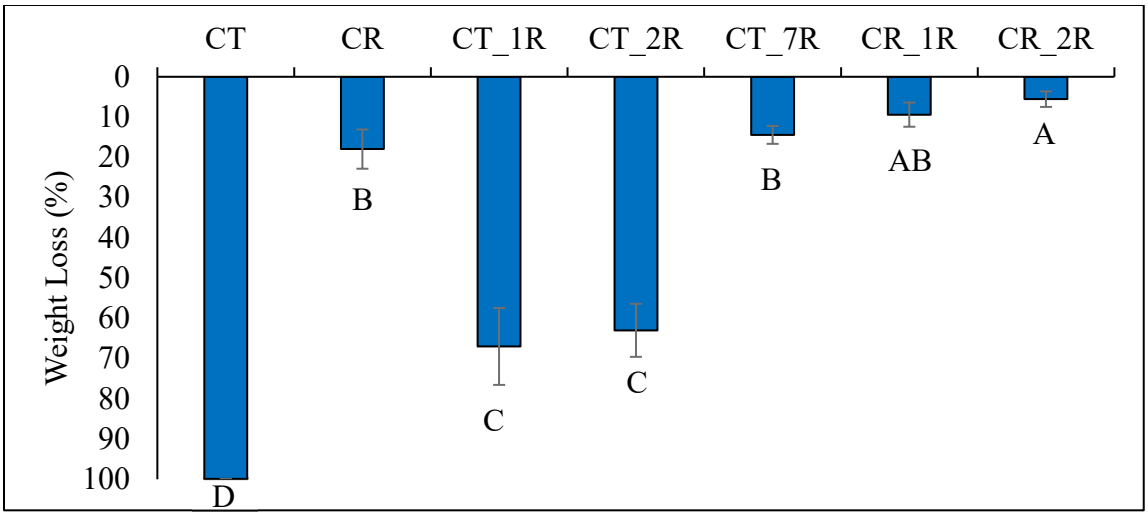

Figure 20. Percent weight loss of natural dentin controls (CT and CR) and remineralization solution treated groups after digestion. Means with different letters are significantly different ($p < 0.05$). Abbreviations: CT: Control; CR: Cranberry crosslinker; CT_2R: Control treated with remineralization solution for 2 days; CR_2R: Crosslinker treatment followed by two-day remineralization.

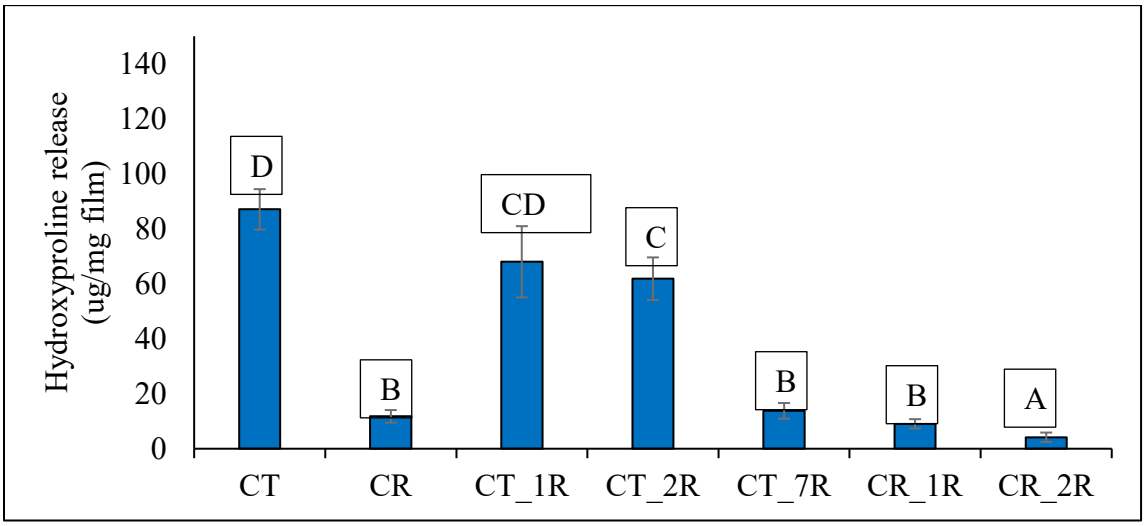

Figure 21. Hydroxyproline (HYP) release content (µg/mg film) of natural dentin collagen controls (CT and CR) and remineralization solution treated groups after digestion in 0.1% collagenase for 1 h. Means with different letters are significantly different ($p < 0.05$). Abbreviations: CT: Control; CR: Cranberry Crosslinker; CT_2R: Control treated with remineralization solution for 2 days; CR_2R: Crosslinker treatment followed by two-day remineralization.

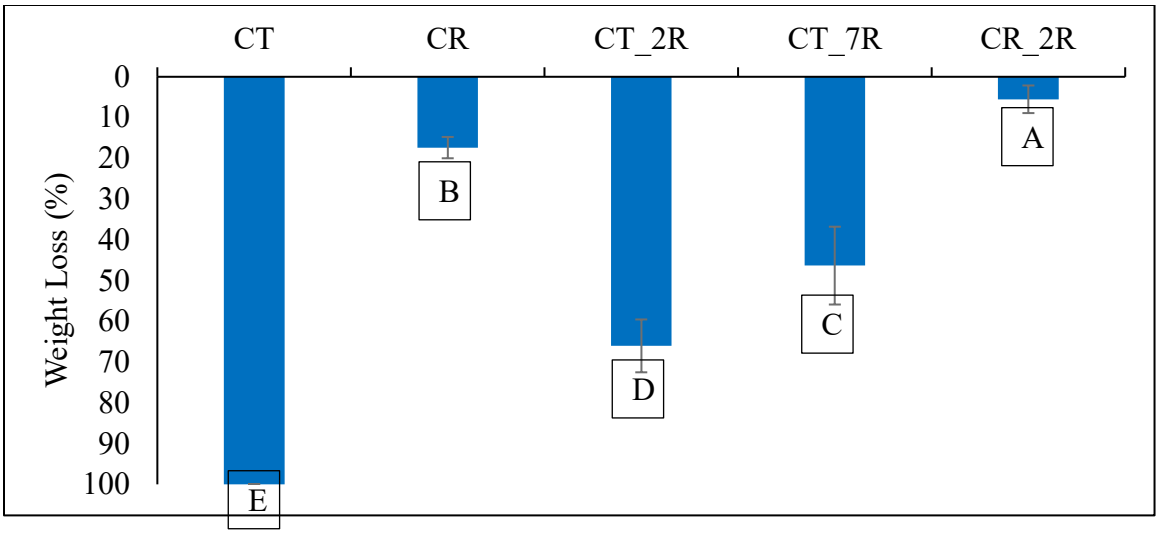

Figure 22. Percent weight loss of HD dentin collagen controls (CT and CR) and remineralization solution treated groups after digestion in 0.1% collagenase for 1 h. Means with different letters are significantly different ($p < 0.05$). Abbreviations: CT: Control; CR: Cranberry Crosslinker; CT_2R: Control treated with remineralization solution for 2 days; CR_2R: Crosslinker treatment followed by two-day remineralization.

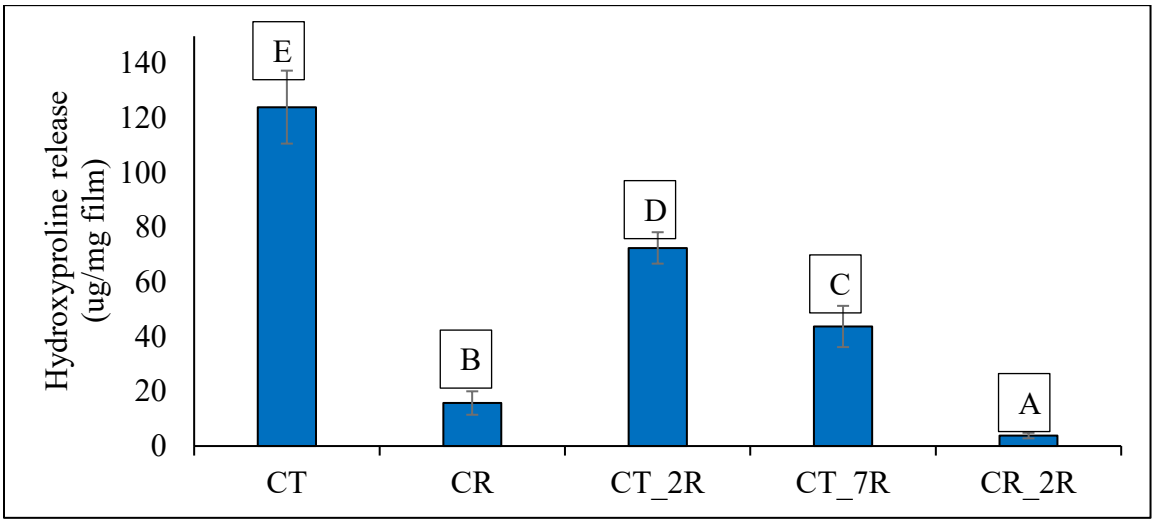

Figure 23. Hydroxyproline (HYP) release content (µg/mg film) of HD dentin collagen controls (CT and CR) and remineralization solution treated groups after digestion. Means with different letters are significantly different ($p < 0.05$). Abbreviations: CT: Control; CR: Cranberry Crosslinker; CT_2R: Control treated with remineralization solution for 2 days; CR_2R: Crosslinker treatment followed by two-day remineralization.

Characterizing CAD through Microhardness

The microhardness values from the surface and into the depth of the cavity for natural and CAD are represented in figure 24. While the microhardness values of natural dentin stayed roughly constant (~55 KHN), the microhardness values of CAD gradually increased as measurements were taken into the depth of CAD. This represents the demineralized and altered dentin collagen substrate affected by caries induction.

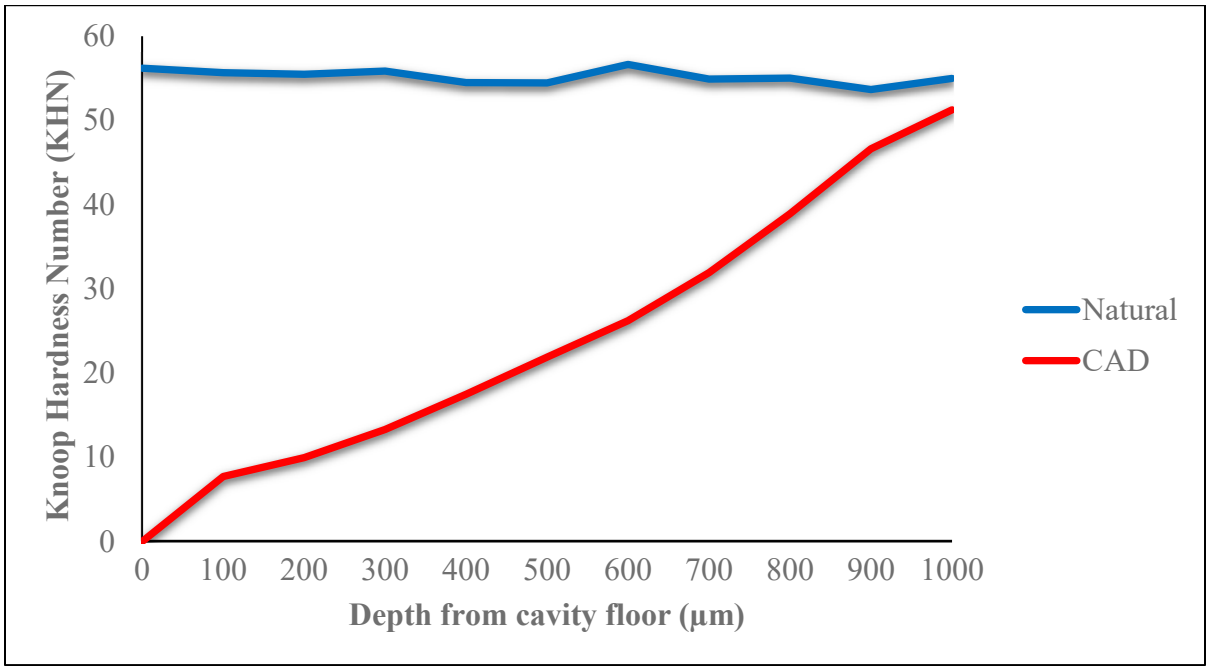

Figure 24. Microhardness values of natural and CAD from the surface and into the depth of the cavity. Abbreviations: CAD: Caries Affected Dentin; KHN: Knoop hardness number.

SEM Analysis of Demineralized Dentin Layer

The demineralized dentin layers following different treatments before and after collagenase digestion are shown in figure 25 & figure26. The collagen fibrils were visualized as demineralized dentin (DD) layer or dentin layer (DL) in secondary electron (SE) mode and a dark layer in backscattered electron (BSE) mode. This DD and DL was clearly visible in all groups of natural dentin before collagenase digestion (fig. 25). After digestion, the DD layer in control (PA) and in CT_1R was not able to survive and was completely digested in 1 h while the DL in rest of the natural dentin groups remained intact. The DD layer in CAD was not visible before or after digestion even after 14 days of remineralization. (fig. 26).

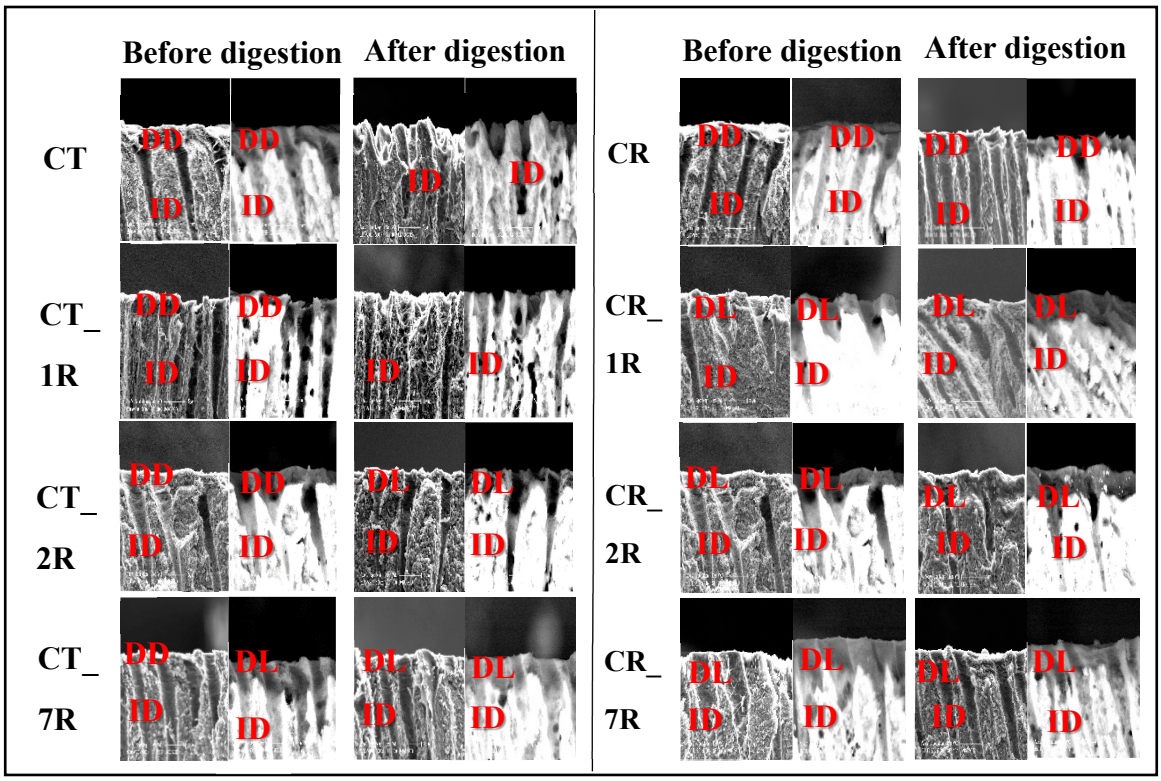

Figure 25: Representative images of demineralized dentin slabs of natural dentin for all experimental groups before and after collagenase digestion. These images were obtained from SEM in secondary-electron (SE, left) and backscattered-electron (BSE, right) modes. Abbreviations: DD: Demineralized Dentin; DL: Dentin Layer; ID: Intertubular Dentin; CT: Control; CT_1R: Control treated with one-day remineralization solution; CR: Cranberry; CR_1R: CR treatment followed by one day remineralization. All experimental groups showed an intact DD before collagenase digestion. After digestion DD did not survive in the control group (PA) and in CT_1R.

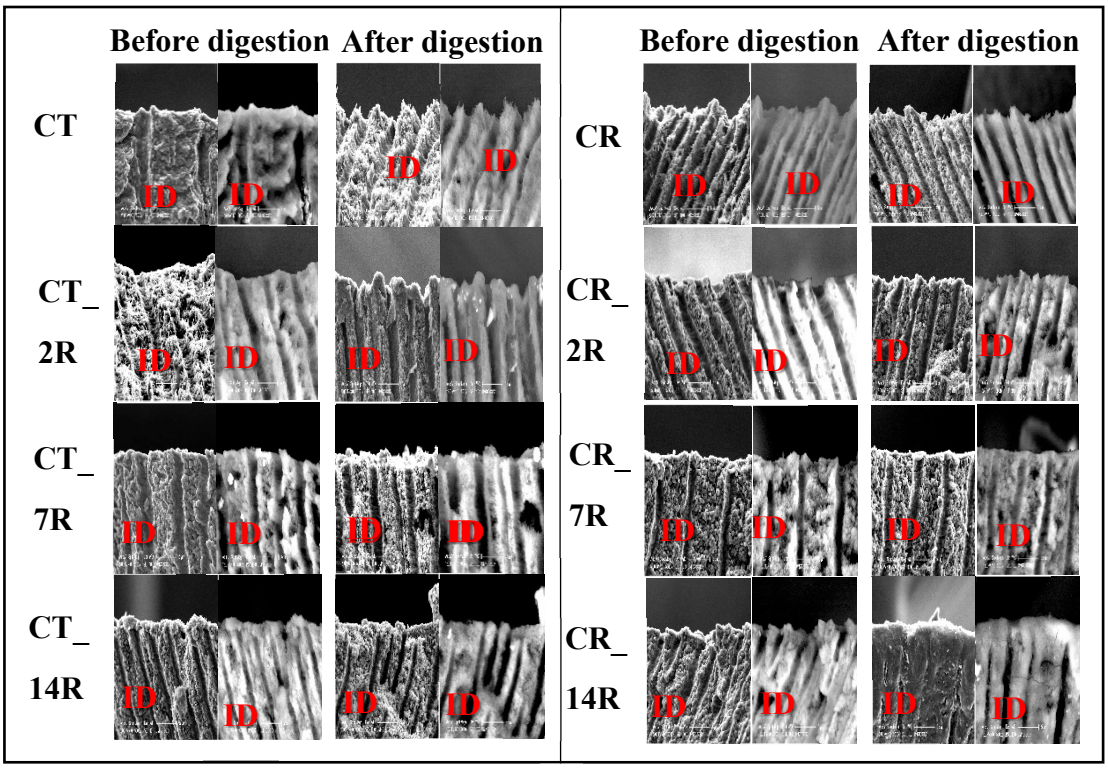

Figure 26: Representative images of CAD demineralized dentin slabs in all experimental groups before and after collagenase digestion. These images were obtained from SEM in secondary-electron (SE, left) and backscattered-electron (BSE, right) modes. Abbreviations: ID: Intertubular Dentin; CT: Control; CT_2R: Control treated with remineralization solution for 2 days; CR: Cranberry; CR_2R: Crosslinker treatment followed by two day remineralization. The DD layer was not visible in any of the experimental groups in CAD before or after digestion.

TEM analysis and SAED Pattern of Demineralized Dentin

Representative TEM images of unstained dentin specimens of treatment groups of natural dentin (left) and CAD (right) are presented in figure 27. The inset at the bottom right corner of each image represents selected area electron diffraction (SAED) patterns from the treated regions of each experimental group. The comparatively light intensity region in all images represents the treated area. In natural dentin, the treated area in CT_2R was very light and some dark areas representing mineral could be seen at the bottom of the demineralized dentin region. However, no banding pattern could be seen in the demineralized area of CT_2R. Increasing the remineralization time to 7 days as well as in CR_2R treatment group, intensified mineral deposition and banding pattern could be seen (yellow hand). The SAED patterns showed distinct rings corresponding to (002) and (211) diffractions of hydroxyapatite representing improved crystallinity in CT_7R and CR_2R treatment groups.

In CAD, the dentin collagen looked amorphous and seemed to separate after 2-day remineralization. Collagen banding pattern could not be seen in CT_2R or CT_7R treatment groups, while banding pattern could be seen in the CR_2R group with SAED pattern showing distinct diffusive rings of SAED pattern of hydroxyapatite. While the diffuse rings in SAED pattern could not be seen in the CT_2R treatment group of CAD.

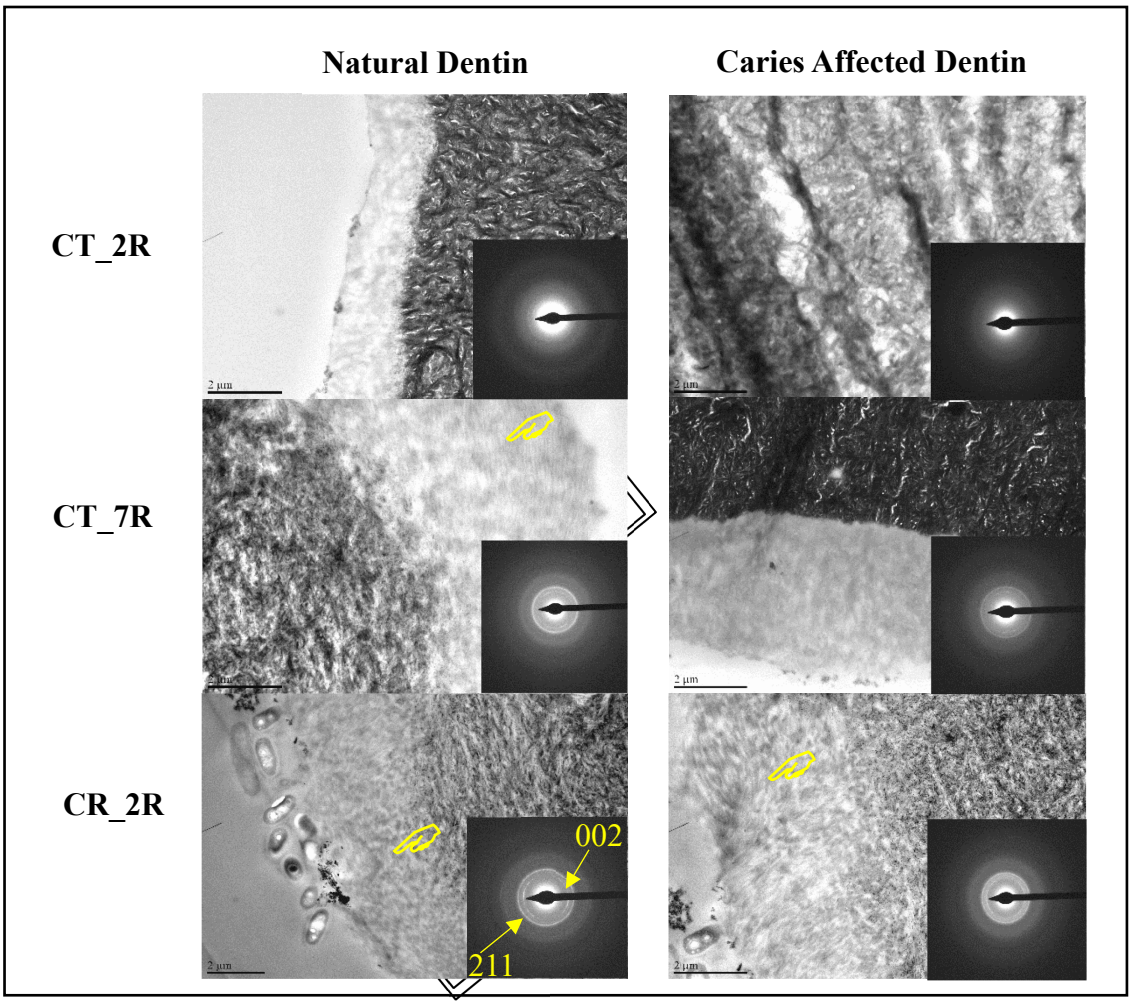

Figure 27. Representative TEM images and SAED pattern of natural dentin (left) and CAD (right) specimens of all experimental groups. The lighter areas in each image represent the treated parts of the specimens. Increase in remineralization time resulted in darker treated areas representing mineral deposition. Banding pattern of collagen (yellow hand) represents intrafibrillar mineral deposition. The SAED patterns (inset images) of the treated area for CT_7R and CR_2R are similar to diffractions of hydroxyapatite. Abbreviations: CT_2R: Control treated with remineralization solution for 2 days; CT_7R: Control treated with remineralization solution for 7 days; CR_2R: Crosslinker treatment followed by 2-day remineralization.

SEM Analysis of Demineralized Dentin Surfaces

Representative SEM images showing the surface topology of natural dentin (left) and CAD (right) are presented in figure 28. For natural dentin a significant distinction can be made among the SEM images of different treatment groups. An increase in mineral deposition (yellow hand) around the dentin tubules compared to CT could be seen after 2-day remineralization treatment. The mineral was present within and around the dentin tubules after 7-day remineralization while in the CR_2R treatment group the mineral deposition was dense and surrounded the tubules. In CAD, the mineral deposition was not as dense as that seen in natural dentin treatment groups; however, CR_2R treatment group had comparatively more mineral deposition than the rest of the groups.

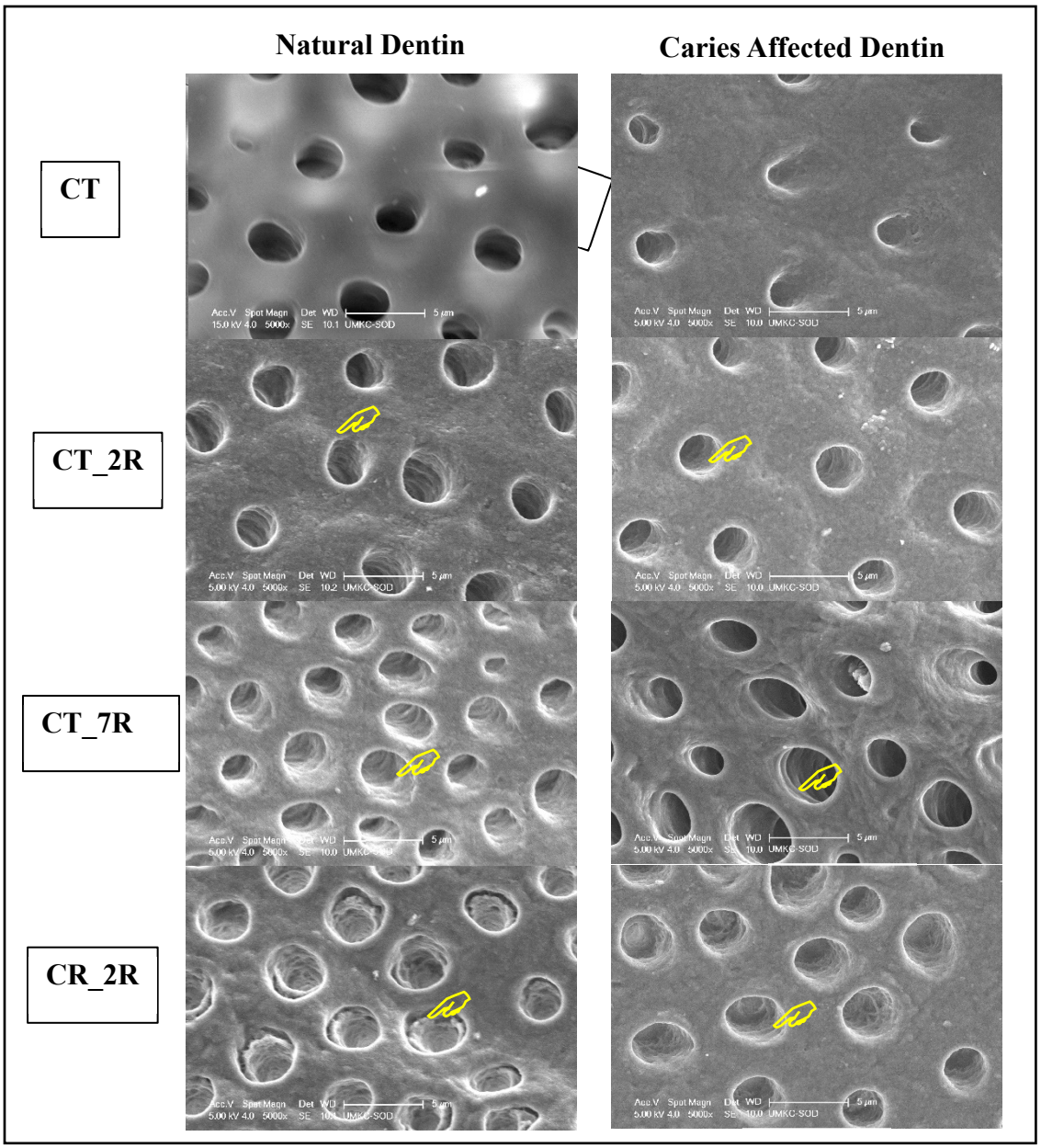

Figure 28. Representative surface topology images of natural (left) and CAD (right) dentin specimen of all experimental groups. These images were obtained through SEM in secondary-electron mode. Mineral deposition (yellow hand) around and in dental tubules can be seen after remineralization. Abbreviations: CT: Control; CR: Cranberry; CT_2R: Control treated with remineralization solution for 2 days; CT_7R: Control treated with remineralization solution for 7 days; CR_2R: Crosslinker treatment followed by two day remineralization.

Discussion

Strengthening dentin collagen and replacing mineral within the different compartments of type I collagen can reinforce the hybrid layer and contribute towards longevity of resin dentin bonds. In order to achieve remineralization, different strategies have been proposed including the use of fluoride and bioactive glass (Sauro et al. 2013). These strategies cause deposition of mineral over existing mineral apatite seed crystals which act as nuclei for regrowth (Kim et al. 2010b). However, due to absence of seed crystals in the demineralized layer following acid etching as well as in the substrate of CAD, these techniques fail to accomplish remineralization within the inter and intrafibrillar compartments of dentin collagen and contribute towards their resistance against degradation (Nancollas and Wu 2000; Liu et al. 2011c). On the other hand, the use of NCP analogues creates a complex of amorphous mineral and NCP (called PILP) which can flow into the various compartments of dentin collagen structure and reintroduce apatite mineral (Olszta et al. 2007). Mineralization occurs when PILP comes in contact with the specific gap regions in collagen fibrils (Nudelman et al. 2010; Liu et al. 2011b). The hierarchical structure of type I collagen serves as a template for remineralization and when the template is altered or compromised as in the case of CAD, the process becomes challenging. This can be seen in our WL and HYP results, where in natural dentin collagen CT_2R and CT_7R were able to resist collagenase degradation more effectively compared to the same experimental groups of HD dentin (figs. 20 – 23).

Remineralization through PILP is a time consuming process as the infiltration of the stabilized ACP into the various compartments of dentin collagen followed by crystallization both in the gap zones and on the surface of collagen takes a long time. (He et al. 2003;

Nudelman et al. 2010). This can be seen in SEM images of natural dentin, where increased exposure time to remineralization solution resulted in more mineral deposition in and around dentin tubules (fig. 28). Similarly, the demineralized dentin layer (fig. 25) in CT_2R was only partially able to resist collagenase degradation and looked weaker as compared to the groups with longer remineralization time. WL and HYP results also showed significant resistance to enzymatic degradation when increasing the remineralization treatment time from 2 to 7 days irrespective of dentin collagen substrate (fig. 20 – 23). However, enzymatic degradation of dentin collagen starts immediately following acid etching and progresses rapidly before remineralization can be achieved (Liu et al. 2011d). Hence it is imperative to protect dentin collagen while the remineralization process progresses.

Other than dentin collagen, an important component of hybrid layer is the adhesive resin, which is vulnerable to hydrolytic degradation. Presence of collagen bound water in inter and intrafibrillar compartments of dentin collagen limits infiltration as well as complete polymerization of the adhesive resin (Wang et al. 2006). This residual water serves as a functional medium for hydrolysis of the adhesive resin and contributes towards the vicious cycle of collagen degradation and weakening of the hybrid layer (Tjäderhane et al. 2013a; Betancourt et al. 2019). The efficiency of crosslinkers TF and CR to protect dentin collagen from degradation in a short clinically feasible time of 30 s has been discussed in chapter 3. The structural alterations produced by these crosslinkers resulted in reduction of endogenous enzymatic activity and increase in the resilience of dentin collagen. As both crosslinkers performed equally well, we decided to choose only one crosslinker CR for this study due to its extensive phenol groups. While using efficient crosslinkers can protect dentin collagen from degradation, the presence of residual water still presents a challenge for the adhesive

resin. In this study the strategy to use crosslinker CR prior to remineralization provided a resistant scaffold which could withstand collagenase degradation while the remineralization process continued to replace water in the collagen network, hence reducing the chances of hydrolytic degradation.

Our results showed that using natural flavonoid CR not only improved resistance to enzymatic degradation, but also resulted in reducing the time period required for remineralization. Natural dentin collagen treated for 30 s with CR resulted in similar protection against collagenase degradation as 7-day remineralization (CT_7R), while using these two strategies together (CR_2R) resulted in the lowest WL and HYP values irrespective of dentin collagen type. The SEM and TEM images (fig. 28 and fig. 27) also supported these results. In natural dentin, dense deposition of mineral in CR_2R within the dentin tubules could be seen compared to other treatment groups. Similarly, TEM images (fig. 27) showed distinct banding pattern as well as diffusive rings representing diffraction patterns for intrafibrillar mineralization. These results are in agreement with another study which used a chemical crosslinker with polyacrylic acid as NCP analogue (Chen et al. 2016). Other than providing a sturdy collagen structure for remineralization, crosslinking can reduce the nucleation energy barrier and promote phase transition, facilitating mineralization in the gap region of dentin collagen (Kong et al. 2022). Studies have also shown that crosslinkers with abundant phenolic groups accelerate mineral deposition by binding to calcium ions and promoting formation of hydroxyapatite (Xie et al. 2008; Jawale et al. 2017). Abundance of phenol groups in CR also promote hydrogen bonding and contribute towards extensive crosslinking in dentin collagen and transform passive collagen into an active template which accelerated remineralization.

In CAD the demineralized dentin layer with altered dentin collagen is weak and thick, hence would present a greater challenge for remineralization to progress. Loss of this weak demineralized dentin layer or inability of this layer to survive processing resulted in failure to produce a visible layer with SEM (fig. 26). The microhardness data (fig. 24) shows how there is a gradual increase in KHN value as we progress from the surface and into the depth of CAD. This shows how the depth of demineralized zone in CAD is greater than that of natural dentin and would explain why we could not see a demineralized dentin layer even after 14 days of remineralization treatment.

Distinct banding and SAED pattern were not visible in TEM images in the CAD demineralized layer even after 7-day remineralization treatment. However, the surface topology of CAD (fig. 28) showed some mineral deposition in and around the dentinal tubules in the CR_2R treatment group, additionally TEM images also showed a distinct banding pattern and increase in crystallinity with diffuse rings in SAED. Although the mineral is not as dense as in natural dentin, it shows that crosslinker treatment could positively impact remineralization of CAD collagen. Similarly, CR_2R treatment group showed the lowest WL and HYP values (5.5% and 3.84 μg/mg) in HD dentin collagen compared to other treatment groups. Based on our results, we can deduce that for successful remineralization in natural or caries affected dentin, the use of a crosslinker can not only accelerate remineralization, but also enhance the protection of dentin collagen against enzymatic as well as hydrolytic degradation. To the best of our knowledge this is the first study which has shown successful remineralization of CAD that is pretreated with a natural flavonoid.

Conclusions

Within the limitations of this study, we showed that remineralization of dentin collagen can produce an acid etched dentin layer that is resistant to collagenase degradation. Although remineralization is a slow process, treating dentin collagen with a crosslinker can aid in speeding up remineralization as it provides a sturdy structure and an active scaffold for mineral deposition. While protecting the altered dentin collagen of CAD is challenging with remineralizing agents alone, combining this strategy with the use of a crosslinker can aid in providing stability to altered dentin collagen substrate. For future studies a delivery method for NCP analogues needs to be designed which will ensure availability of these analogues while the process of remineralization continues to replace water bound to collagen.

CHAPTER 5

THE EFFECTS OF DUAL- FUNCTIONAL ETCHANTS ON DURABILITY OF RESIN DENTIN BONDS IN NATURAL AND CARIES AFFECTED DENTIN

Introduction

Creating an efficient and stable resin dentin bond has been a challenge since the introduction of adhesive resins in dentistry (Van Meerbeek et al. 2011). Innovations in adhesive procedures and adhesive resin formulations have been in focus compared to research on improving the state of dentin collagen itself. The hydrophilic nature, high organic content, the structural arrangement of mineral and type I collagen make dentin a challenging substrate to bond to (Pashley 1991). This substrate becomes even more challenging to restore following carious attack. In order to keep in line with the principals of minimally invasive dentistry, a dentist would remove the outer bacteria infected dentin of a carious tooth and preserve the CAD (Maltz et al. 2002). This substrate of CAD has an altered structural content, arrangement of mineral and collagen fibrils which negatively affects bonding performance (Nakajima et al. 2011; Joves et al. 2013; Isolan et al. 2018). The disorganized structure of CAD is also more susceptible to degradation by endogenous enzymes like MMPs which are highly active in this substrate.(Vidal et al. 2014; Mazzoni et al. 2015a). Degradation of collagen leads to creation of an unstable hybrid layer which would result in early failure of a restoration.

The conventional bonding process starts with etching dentin with an acid to prepare the dentin substrate for micromechanical retention of the adhesive. The most commonly used etchant in clinical practice is 30-40% phosphoric acid (PA). It is an aggressive acid that creates a thick demineralized layer which is challenging for the infiltrating resin to penetrate

completely and protect the exposed collagen fibrils. The unprotected collagen is susceptible to degradation by endogenous enzymes which cleave collagen resulting in failure of the restoration at the bonded interface (Pashley et al. 2004). The aggressive nature of PA produces structural changes in collagen (Eliades et al. 1997) and also cause removal of proteoglycans which affects the resin dentin bond effectiveness (Bedran-Russo et al. 2008b). Research on organic acids as an alternative to PA have shown promising results, specially α-hydroxy acids which are weak acids with a carboxyl group adjacent to hydroxy group in α position (Trevelin et al. 2019; Vidal et al. 2021). These water soluble, colorless α-hydroxy acids have shown to reduce the enzymatic activity in dentin and promote adequate bond strengths (Trevelin et al. 2019). However, there is no information regarding their effect on bonding performance after aging nor is there any information about their efficacy in the altered substrate of CAD.

The use of crosslinkers to strengthen the dentin collagen and reduce MMPs activity is a promising alternative to improve adhesion to dentin (Fang et al. 2012; Fialho et al. 2019). To include the crosslinkers into the adhesive system different strategies have been used. Adding the crosslinker directly into the adhesive resulted in reduced polymerization of the adhesive and negatively affected bonding performance (Epasinghe et al. 2012). Using the crosslinkers as a primer has shown promising results, however it increases the number of steps required to achieve bonding (Hass et al. 2016b). Combining the crosslinker in an acid etchant to create a dual- functional etchant is an attractive approach. It would allow etching of the dentin to remove the mineral and simultaneously protect the exposed collagen by crosslinking, hence providing a sturdy dentin matrix for adhesive infiltration (Hass et al. 2016a; de Siqueira et al. 2019). However, the solubility of crosslinkers in the etchant is

challenging and a volatile solvent such as ethanol or acetone has to be added to prevent precipitation of the crosslinkers. Moreover, when PA was combined with a hydrophobic crosslinker like proanthocyanidins (PAC), the speed at which it etched dentin was too fast for the crosslinker to catch up and crosslink the dentin collagen. Due to its hydrophobic nature and high molecular weight than PA, the diffusion of PAC would be slow which limited crosslinking of collagen through the entire demineralized depth resulting in unprotected collagen (Liu et al. 2014a). To overcome such discrepancies, testing an efficient and lower molecular weight crosslinker like TF in a dual- functional etchant is an attractive approach.

Hence our aim was to look for an alternative etchant that is less aggressive but efficiently demineralizes dentin and can provide better solubility for crosslinkers. We also wanted to compare the efficiency of two structurally dissimilar crosslinkers with different molecular weights in dual- functional etchants. The demineralized depth, bonding performance and endogenous enzyme activity of the dual- functional etchants were tested in natural dentin as well as CAD.

Materials and Methods

Preparation of Dual- functional Etchants

Two acid mixtures were used in this study. One mixture was of 35% glycolic acid[65] (GA) and 10% PA while the other mixture was that of 35% tartaric acid[75] (TA) and 10% PA. The crosslinkers (TF[9], CR[10] and EDC/NHS[11]) were added to the acid mixtures to achieve a final concentration of 1%. Controls used were 35% PA, GA+PA (GP) & TA+PA (TP) without a crosslinker as shown in table 2.

TABLE 2

Experimental etchant groups and their pH

	Groups	pH at 19.8˚C
Controls without crosslinker	35%PA (PA)	0.63
	35%TA +10%PA (TP)	0.89
	35%GA +10%PA (GP)	0.79
Treatment groups with crosslinker (Dual- functional Etchants)	35%TA +10%PA 1% theaflavin (TPTF)	0.91
	35%TA +10%PA 1% cranberry (TPCR)	0.90
	35%TA +10%PA 1% EDC/NHS(TPEDC)	0.90
	35%GA +10%PA 1% theaflavin (GPTF)	0.92
	35%GA +10%PA 1% cranberry (GPCR)	0.89
	35%GA +10%PA 1% EDC/NHS(GPEDC)	0.90

Natural and CAD Specimen Preparation for SEM

Eight dentin blocks measuring 7mm x 7mm x 2mm and 30 dentin beams measuring 1mm x 1mm x 4mm were prepared for natural dentin and CAD. The CAD specimens were prepared according to the method mentioned in Chapter 4.

SEM Analysis

The rationale for using this technique is to characterize the structural changes in etched layer of dentin. It allowed us to characterize the ability of the dual- functional etchant in preserving the collagen structure in the etched dentin following enzymatic degradation.

Notched dentin beams for natural sound dentin and CAD were prepared according to the techniques described in chapter 4. The surfaces of these beams were then etched with the dual- functional etchants as well as the control etchants. Four dentin beams per group were used. Half of the etched specimens were put in 0.1% collagenase[8] for 1 h at 37˚C. The specimens were then rinsed, followed by fixation and dehydration as explained in chapter 4. The dark areas in the back- scattered electron mode representing the demineralized depth were quantified using Image J software[73].

Bonding Procedure

A total of 126 dentin blocks (63 for natural dentin and 63 CAD) were etched for 30 s and then rinsed for 1 min. The dentin blocks were air dried for 5 s followed by application of

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[68] Adper Single Bond Plus, 3M ESPE, St. Paul, MN 55119
[69]1200mWcm^2; Radii-cal, SDI Limited, Bayswater, Australia
[65]Glycolic acid, CAS#79-14-1, Sigma-Aldrich, St. Louis, MO 63178
[75]Tartaric acid, CAS#87-69-4, Sigma-Aldrich, St. Louis, MO 63178
[66]Phosphoric acid, CAS#7664-38-2, Thermo Fisher Scientific, Waltham, MA 02451

two layers of adhesive[68] (with 5 s air drying between each layer application). The adhesive was then light cured with an LED curing light[69] for 10 s. A composite resin[33] buildup of 2mm was then done and light cured for 40 s with an LED curing light[69].

Resin Dentin Microtensile Bond Strength (μTBS) Testing

Thermocycling, and Failure Mode

Testing the bonding performance through microtensile bond test allowed us to assess the effect of the various treatment agents on tackling the *in situ* challenges to improving the long term durability of resin dentin bond. Microtensile bond test evaluates the stress a resin dentin bond could endure before failing. Results of these tests are a direct translation of the ability of these agents to reduce the degradation process at the resin dentin interface and improve the clinical efficacy of dental restorations. The restored dentin specimens were sectioned longitudinally in mesiodistal and buccolingual direction using a water- cooled low speed diamond saw[3]. The cross section of the resin dentin bonded beams (n=7 per treatment group with 20 beams per tooth) was measured with a digital caliper[75]. Half of the specimen within each group were stored in SBF[74] solution for 24 h at 37˚C before testing. The other half were put in SBF[74] solution for aging and subjected to thermocycling in a thermocycler[77] at 5˚C and 55˚C with a dwell time of 1 min each for 10,000 cycles (Gale and Darvell 1999; Soeno et al. 2004). Each beam was attached to a jig with cyanoacrylate resin and subjected to a tensile force parallel to the long axis of each specimen on a micro tensile tester[70] at a

[70]Micro tensile tester BISCO, Inc., Schaumburg, IL 60193
[63]Triboscope, Hysitron, Bruker, Minneapolis, MN 55344
[74]Kokubo T, Takadama H. How useful is SBF in predicting in vivo bone bioactivity? Biomaterials. 2006 May 1;27(15):2907-15.
[77]Eppendorf mastercycler: EP gradient S thermal cycler Thermo Fisher Scientific, Waltham, MA 02451

crosshead speed of 0.5mm/min. The μTBS values were achieved by dividing the maximum force at failure with the cross-section area of each specimen. The fractured specimens were viewed under SEM[52] for fractographic analysis to analyze the fracture patterns. The fractures identified were: adhesive/mixed (A/M); cohesive in dentin (CD); cohesive in composite (CC) and premature failure (PF).

Nanoleakage Evaluation

Presence of residual water in the hybrid layer prevents resin infiltration and contributes to the vicious cycle of resin and collagen degradation. Through this nanoleakage study, the presence of residual water at the resin dentin interface can be evaluated in response to the treatments over a period of time.

The resin dentin bonded beams (4 per tooth from each treatment group) that were not tested for μTBS were randomly selected. Half of the beams were put in a thermocycler and the other half were put in SBF for 24 h in an incubator at 37˚C. After storage these bonded beams were immersed in 50 wt% ammonical silver nitrate solution[71] and kept in total darkness for 24 h at room temperature (Tay et al. 2002). They were then rinsed with DI water for 2 min and immersed in a photo developing solution[72] for 8 h under fluorescent light. The specimens were then polished with 600,1200 and 4000 grit SiC papers followed by 1 & 0.25μm diamond pastes[61] on polishing cloth. The specimens were cleaned in an ultrasonic bath for 5 min, air dried, mounted on aluminum stubs and coated with carbon. The dentin resin interface of each sample was observed with SEM[52] in backscattered electron mode. Three images were taken of

[75]Absolute Digimatic, Mitutoyo, Tokyo, Japan
[71]Poly Scientific, NC0736121, Thermo Fisher Scientific, Waltham, MA 02451
[72]RP X-OMAT developer, Carestream, Rochester, NY 14608

each specimen, the first image was taken at the center of the bonded beam and the other two

were obtained 0.3mm to the left and right side of the first picture. The bright stained areas in the hybrid layer were quantified using Image J software[73].

In situ Zymography Analysis

Through in situ zymography the activity of endogenous enzymes within the hybrid layer was determined.

A total of 16 dentin blocks were used (8 for natural dentin and 8 for CAD). These blocks were divided longitudinally into two and randomly divided into different groups. The etching and bonding process was same as that for the μTBS test except that the adhesive was mixed with Rhodamine B[76] before applying. The bonded blocks were put in an incubator[15] at 37˚C for 24 h after which two 400μm thick dentin slices along the longitudinal axis were cut. Rest of the block was kept in SBF and subjected to thermocycling. The bonded slices were attached to a microscopic glass slide with a cyanoacrylate adhesive[32] and polished with 600,1200 and 4000 grit SiC papers to 200 μm thick. Ten microliters of the fluorescent-gelatin solution (prepared as described in chapter 3) were spread on top of each dentin specimen. The specimens were then covered by a coverslip[13] and transferred to humidified chamber[15] and stored in dark at 37ºC for 24 h. The amount of green fluorescence produced was evaluated for each specimen under a multiphoton microscope[78] in an upright configuration. The green fluorescence was imaged using a 488nm laser for excitation and emissions were captured between 495nm and 545 nm wavelengths. Rhodamine was imaged using a 552nm laser for

[73]Image J 1.53b, Wayne Rasband, NIH, USA
[76] Rhodamine B, CAS#81-88-9, Sigma-Aldrich, St. Louis, MO 63178
[78]Leica SP8 Multiphoton Microscope, Leica, Buffalo Grove, IL 60089

excitation and emissions were captured between 560nm and 620nm. For the collagen signal, second harmonic imaging was done using a Ti: Sapphire laser tuned to a wavelength of 850nm.

Back-scattered second harmonic signal was then collected through the same objective used for excitation. The amount of green fluorescence in the hybrid layer were quantified using Image J software[73].

Statistical Analysis

The normality of distribution and homogeneity of variances were assesssed using Shapiro Wilk and Levene tests. The μTBS, nanoleakage and MMPs activity were analyzed by Kruskall-Wallis test, two-way ANOVA and Tukeys test or Games-Howell (α=0.05).

Results

SEM Morphology of Demineralized Dentin Layer

The demineralization depth of dentin following etchant treatment is shown in table 3. The demineralized dentin layer (DL) was seen as collagen fibrils in secondary electron (SE) mode and a dark layer in backscattered electron (BSE) mode. This DL was clearly visible in all groups before collagenase digestion in natural dentin (fig. 29.) After digestion, the DL in all controls (PA, TA, TP, GA, GP) and in TPEDC, GPEDC was completely digested in 1 h while the DL in TF and CR containing dual- functional etchants remained intact. The DL in CAD was not visible in any group before or after digestion (fig. 30.)

TABLE 3

Demineralization Depth of Experimental Etchant Groups

	Groups	Demineralization depth (μm)
Controls without crosslinker	35%PA (PA)	6.01 (0.39)
	35%TA	0.47 (0.12)
	35%GA	0.41 (0.06)
	35%TA +10%PA (TP)	2.38 (0.30)
	35%GA +10%PA (GP)	2.28 (0.22)
Treatment groups with crosslinker (Dual -functional Etchants)	35%TA +10%PA 1% EDC/NHS(TPEDC)	2.38 (0.30)
	35%GA +10%PA 1% EDC/NHS(GPEDC)	2.28 (0.22)
	35%TA +10%PA 1% theaflavin (TPTF)	2.10 (0.27)
	35%GA +10%PA 1% theaflavin (GPTF)	2.06 (0.2)
	35%TA +10%PA 1% cranberry (TPCR)	1.9 (0.3)
	35%GA +10%PA 1% cranberry (GPCR)	1.93 (0.2)

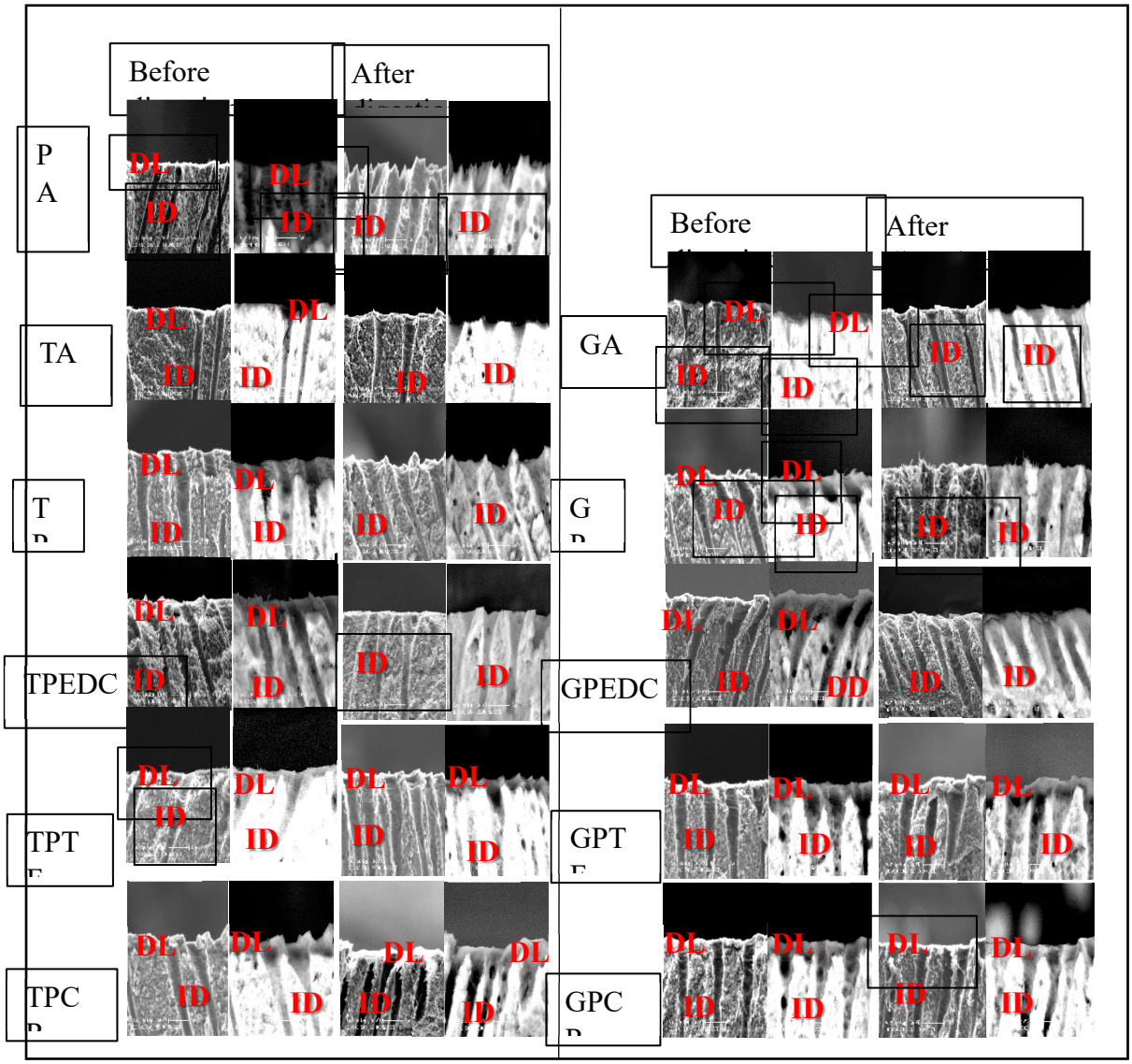

Figure 29: Representative images of etchant treated demineralized natural dentin slabs for all experimental groups before and after collagenase digestion. These images were obtained from SEM in secondary-electron (SE, left) and backscattered-electron (BSE, right) modes. Abbreviations: DL: Demineralized dentin Layer; ID: Intertubular Dentin. All experimental groups showed an intact DD before collagenase digestion. After digestion DD did not survive in the control groups (PA, TATP, GA, GP) as well as in TPEDC and GPEDC groups.

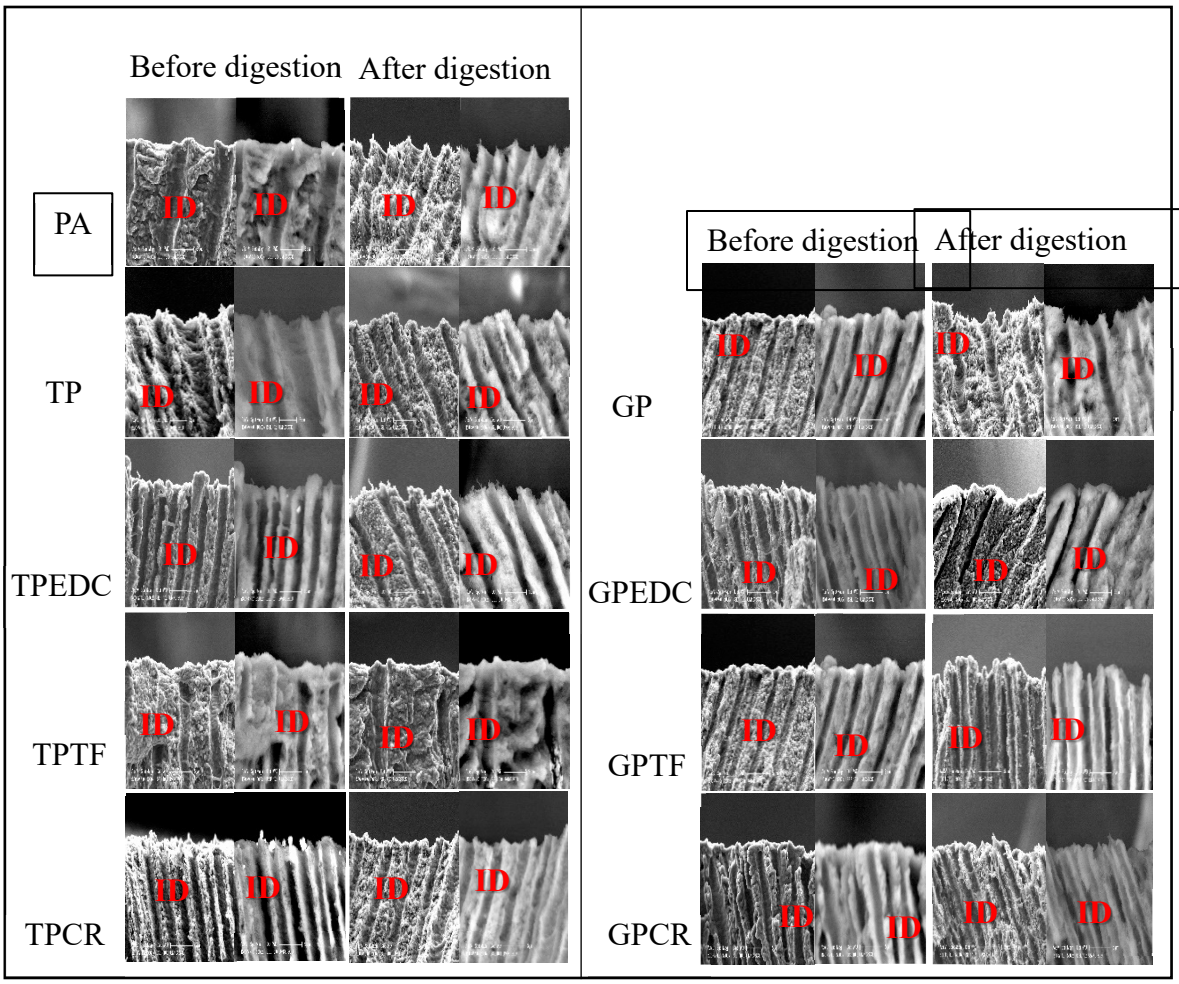

Figure 30: Representative images of etchant treated demineralized dentin slabs of CAD for all experimental groups before and after collagenase digestion. These images were obtained from SEM in secondary-electron (SE, left) and backscattered-electron (BSE, right) modes. Abbreviations: ID: Intertubular Dentin. All experimental groups failed to produce a demineralized dentin layer.

Resin Dentin Microtensile Bond Strength

The results of μTBS of natural dentin are shown in figure 31 and for CAD are shown in figure 32. The μTBS values of natural dentin are generally higher than that of CAD. A significant reduction in μTBS of all controls and TPEDC, GPEDC after thermocycling was seen ($p < 0.001$), while TF and CR containing dual- functional etchants showed no difference between 24 h and thermocycled specimens in both natural dentin and CAD ($p > 0.05$).

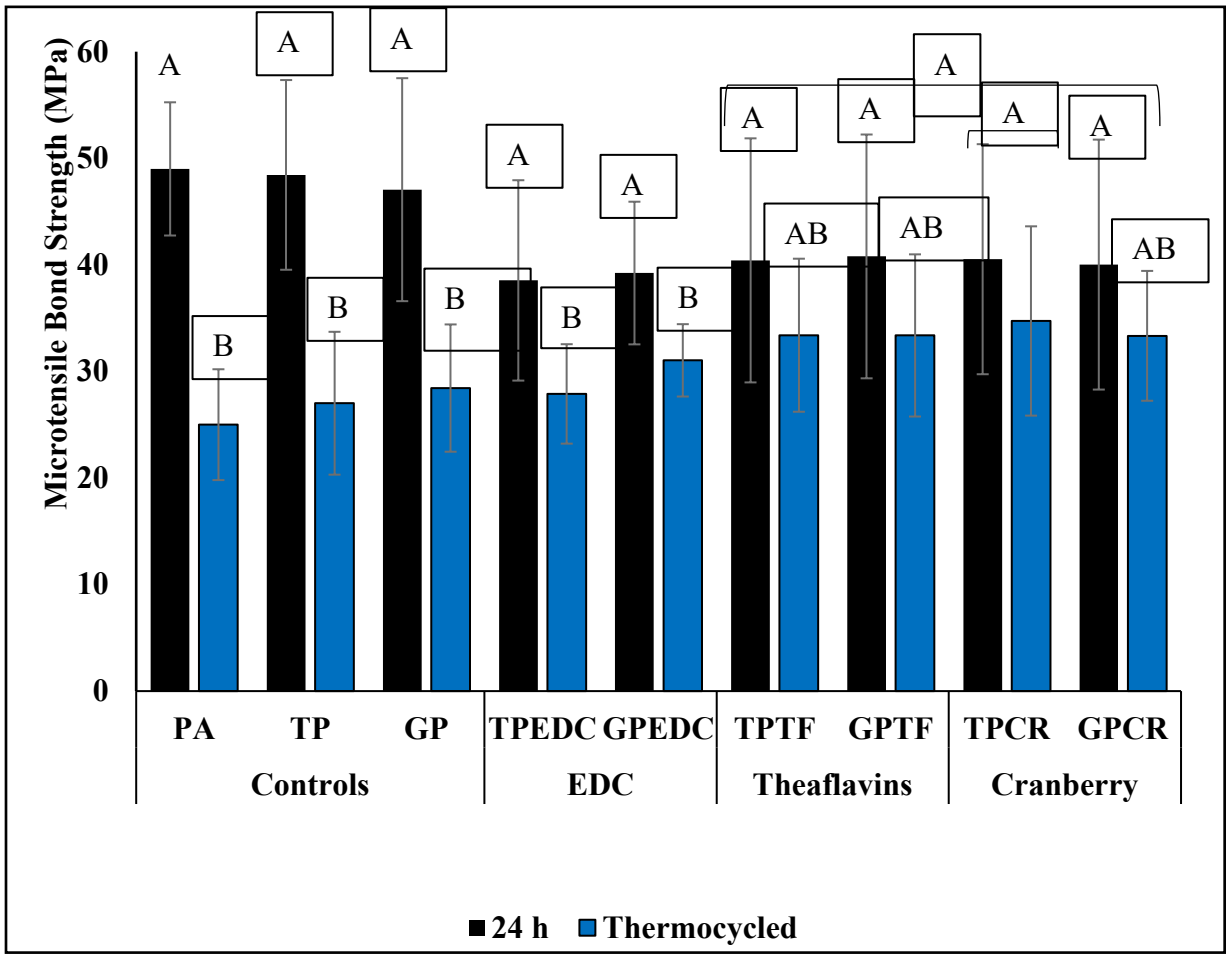

Figure 31: Means and standard deviations of microtensile bond strength of all natural dentin experimental groups. Different letters indicate statistically significant difference (Kruskal-Wallis test; $p < 0.05$).

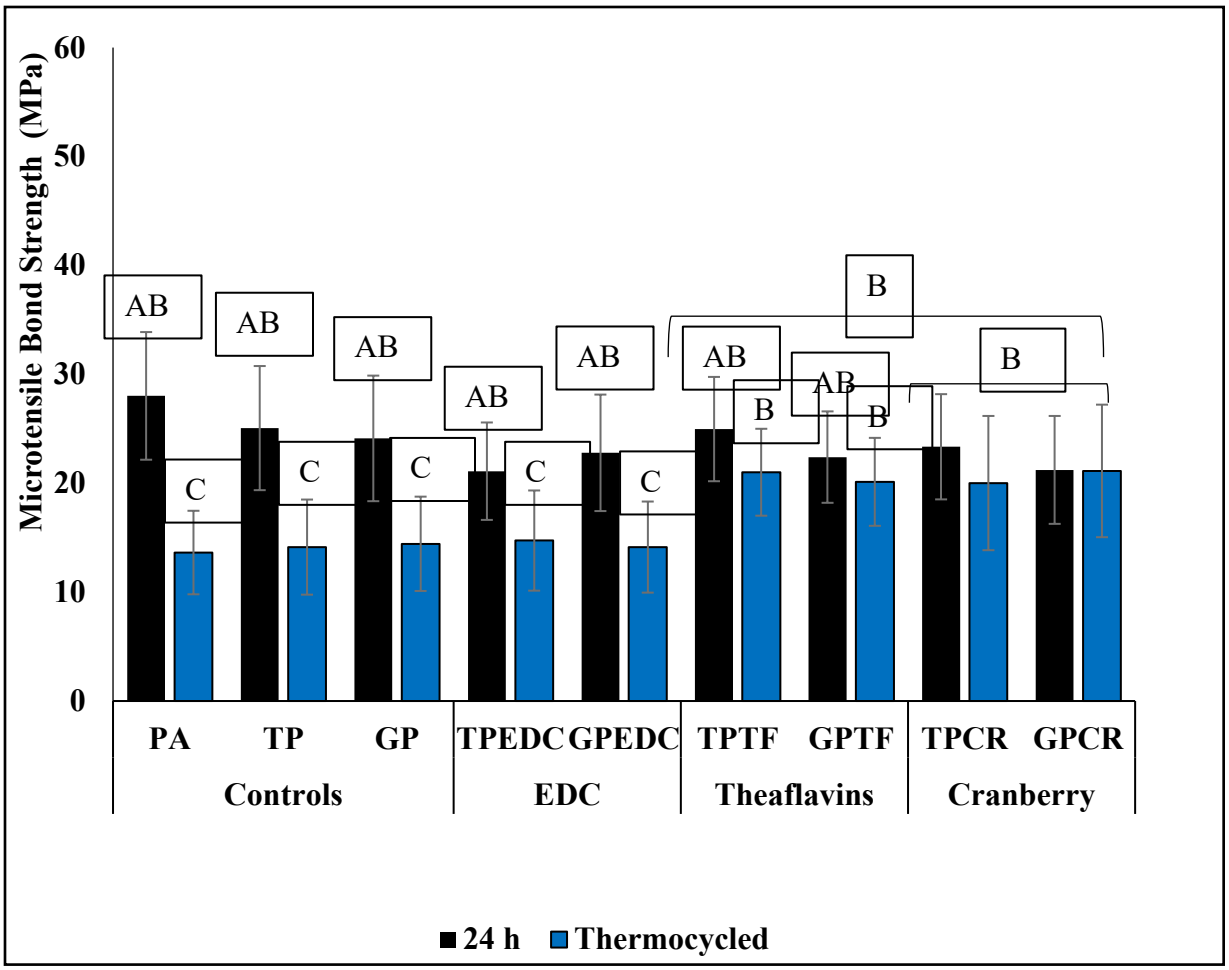

Figure 32: Means and standard deviations of microtensile bond strength of all CAD experimental groups. Different letters indicate statistically significant difference (two-way ANOVA and Tukeys test; $p < 0.05$).

Fracture Pattern Analysis

The percent distribution of fractured specimen for natural dentin are displayed in figure 33 and for CAD in figure 34. All groups had a predominance of A/M failure indicating breach in both the substrates (dentin and adhesive). For CAD, in addition to premature fracture, high prevelance of cohesive fractures were seen. A closer look at the fracture patterns revealed that the controls for natural dentin (fig. 35) had less exposed collagen and normal dentin surface morphology compared to CAD controls (fig. 36). The controls for both dentin types had fewer resin tags as compared to the dual- functional etchant groups (fig. 35 and fig. 36). The adhesive in the CAD groups generally had a very porous and amorphous morphology (fig. 36).

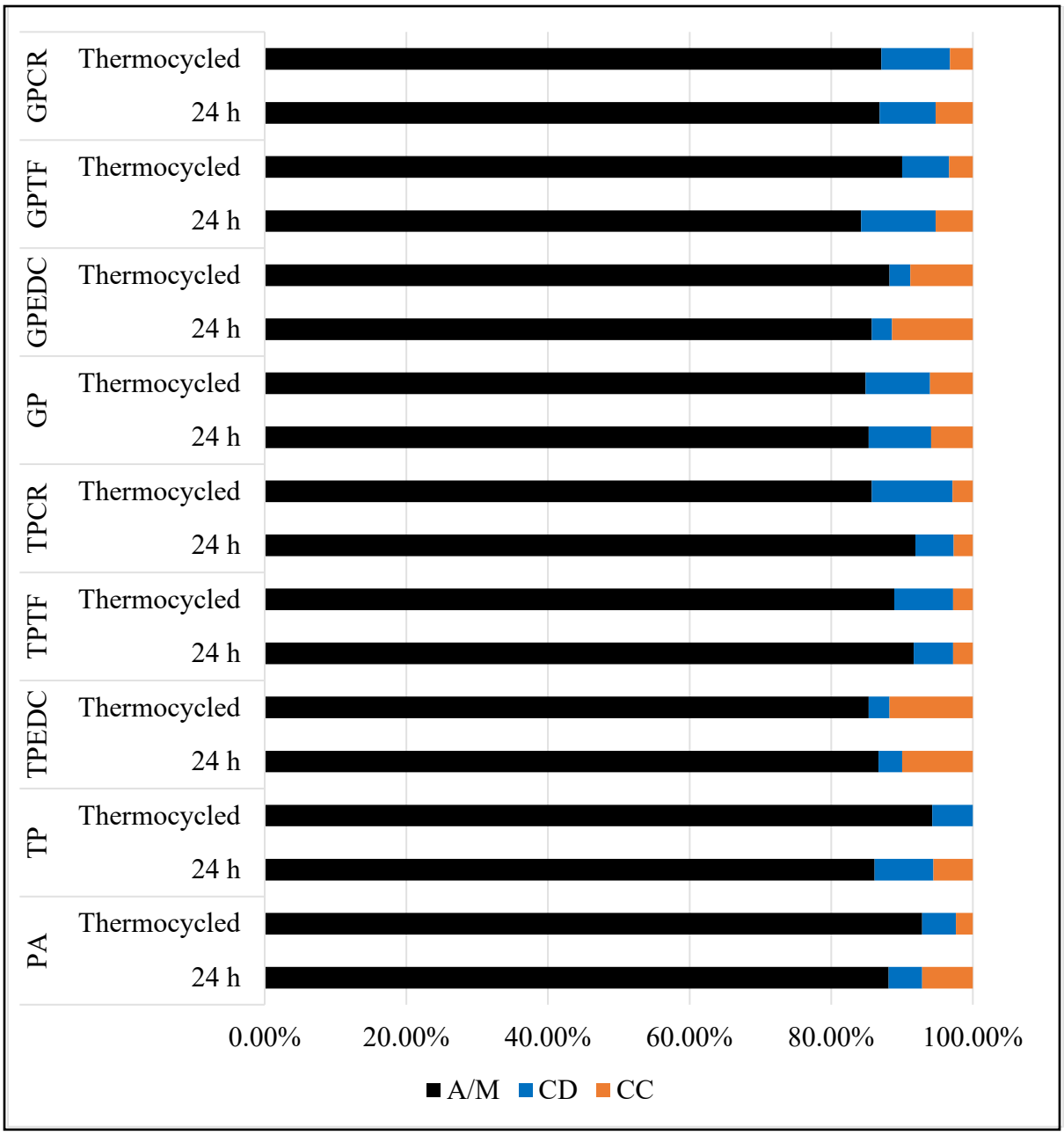

Figure 33: The percent distribution of fractured specimens according to fracture mode for all natural dentin experimental groups. Abbreviations: A/M: adhesive/mixed fracture; CD: cohesive dentin fracture; CC: cohesive composite fracture.

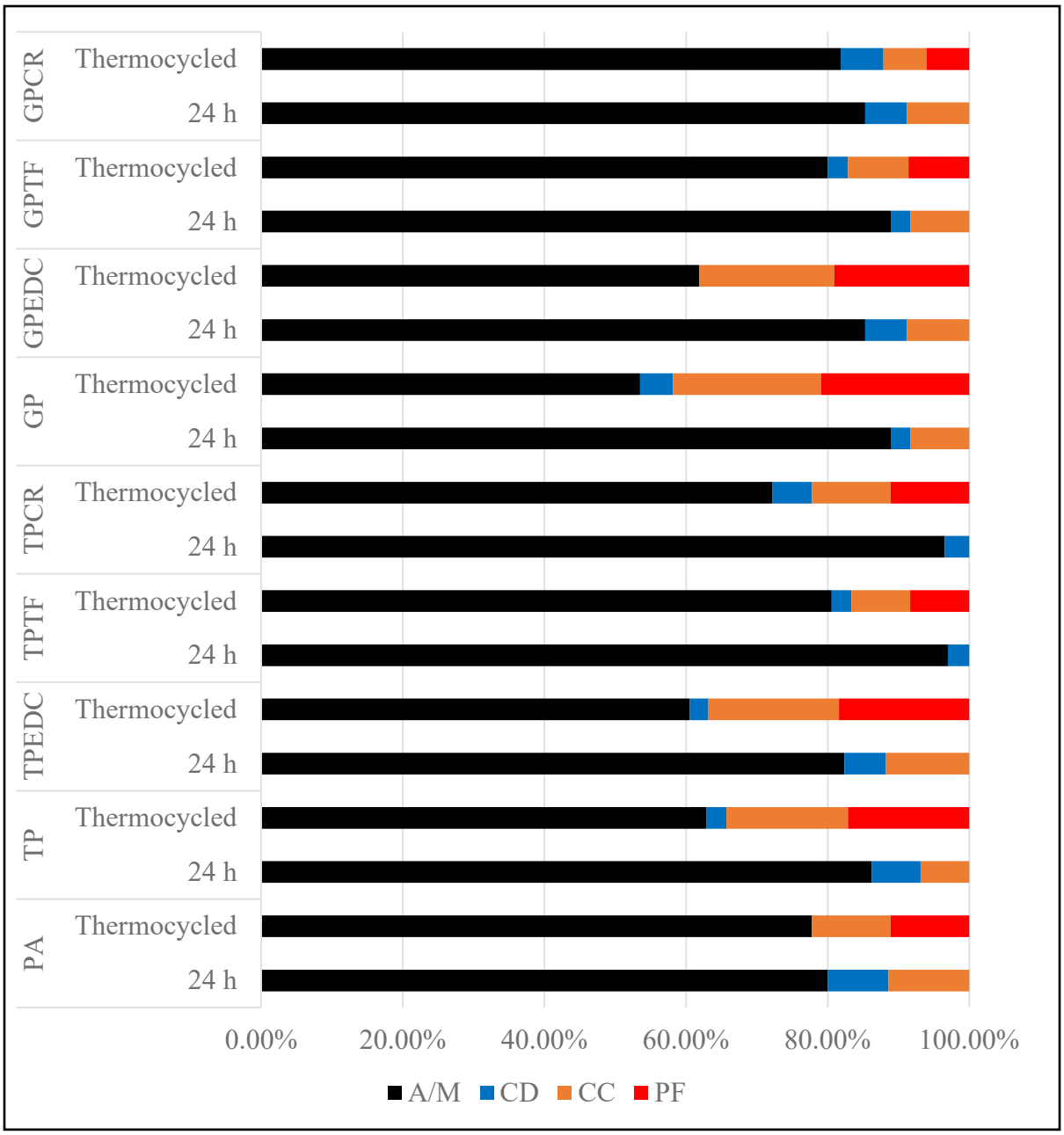

Figure 34: The percent distribution of fractured specimens according to fracture mode for all CAD experimental groups. Abbreviations: A/M: adhesive/mixed fracture; CD: cohesive dentin fracture; CC: cohesive composite fracture; PF: premature failure

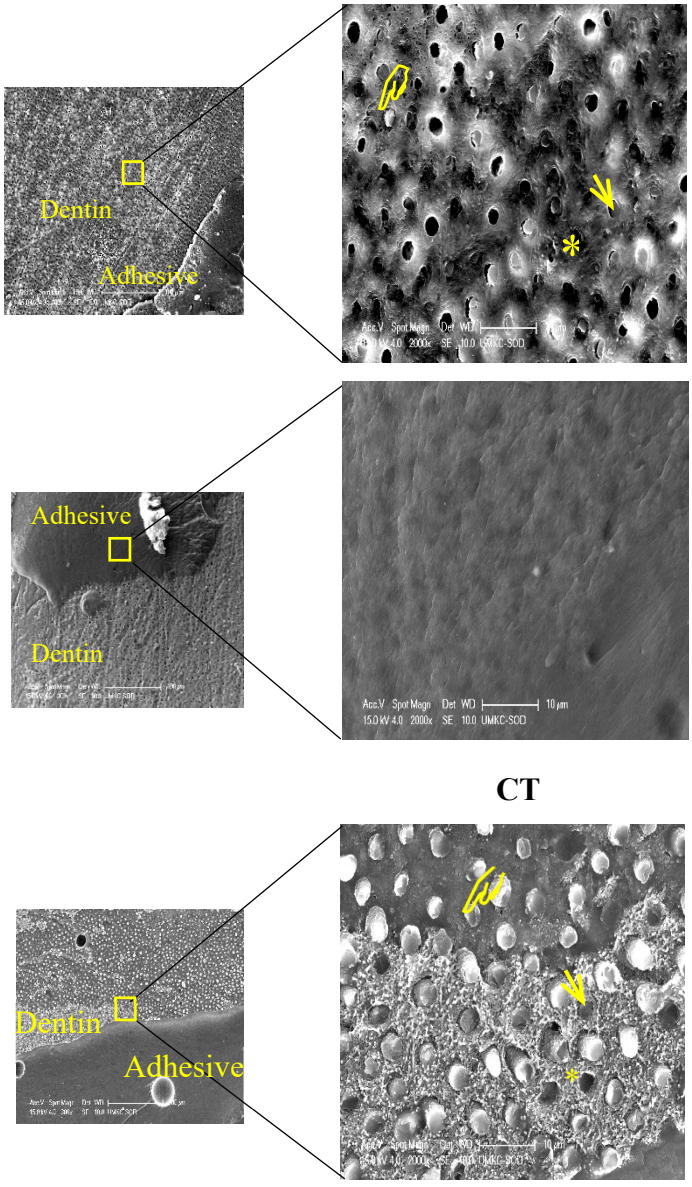

Figure 35: Representative SEM images of fractured specimens of the natural dentin CT and dual- functional etchant groups. At lower magnification a mixed fracture pattern was seen in all groups. At higher magnification control group showed few smear plugs (yellow arrow), demineralized tubules, exposed collagen (yellow asterisk) and some resin tags (yellow hands) in dentin and smooth adhesive pattern. For the dual- functional etchant group, greater number of resin tags could be seen.

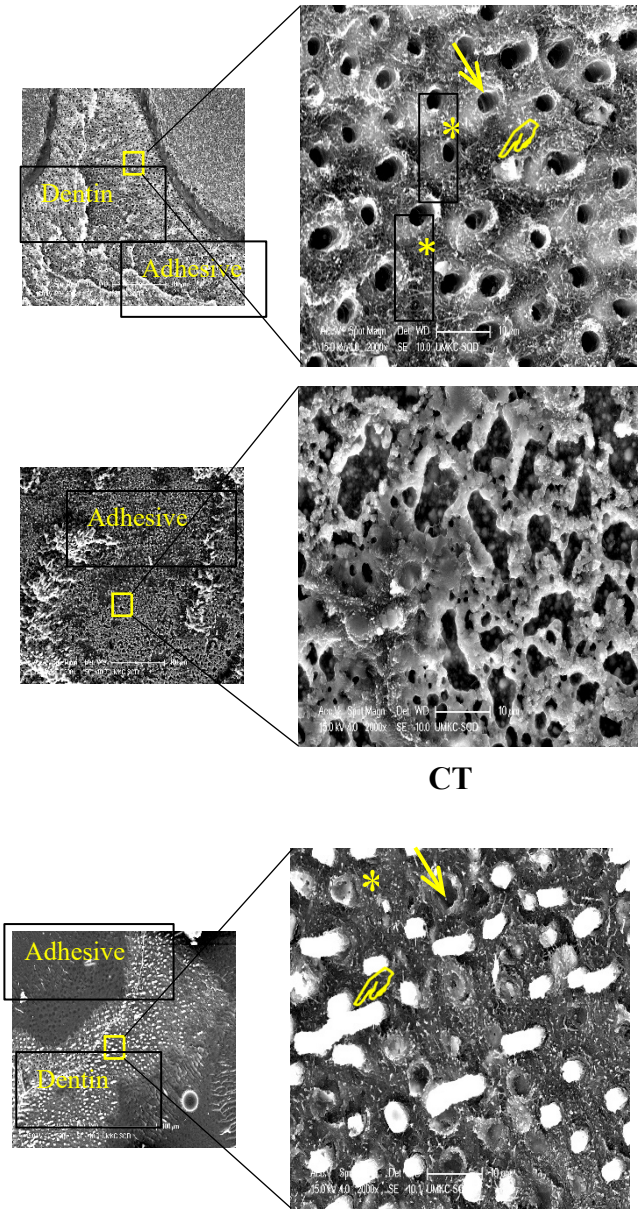

Figure 36: Representative SEM images of fractured specimens of CAD CT and dual-functional etchant groups. At lower magnification a mixed fracture pattern was seen. At higher magnification all controls showed few smear plugs (yellow arrow), demineralized tubules, exposed and disorganized collagen (yellow asterisk) and some resin tags (yellow hands). The adhesive layer in CAD showed higher porosity. For the dual- functional etchant groups, greater number and longer resin tags could be seen.

Nanoleakage Evaluation of Resin Dentin Bonds

Representative BSE images of PA, TP and TP dual- functional etchants in natural dentin are shown in figure 37 and for CAD in figure 39. The BSE images of GP and GP dual-functional etchants in natural dentin are represented in figure 38 and for CAD in figure 40. Quantitative results of silver nitrate uptake in the hybrid layer of natural dentin after 24 h and thermocycling are displayed in figure 41 and for CAD in figure 42. The nanoleakage percentage was generally higher for all CAD experimental groups compared to natural dentin. In both, natural dentin and CAD significant increase in nanoleakage percentage after thermocycling was seen between all controls and EDC containing dual- functional etchants ($p < 0.01$), while no difference was seen between TF and CR containing dual- functional etchants in both dentin types ($p > 0.05$).

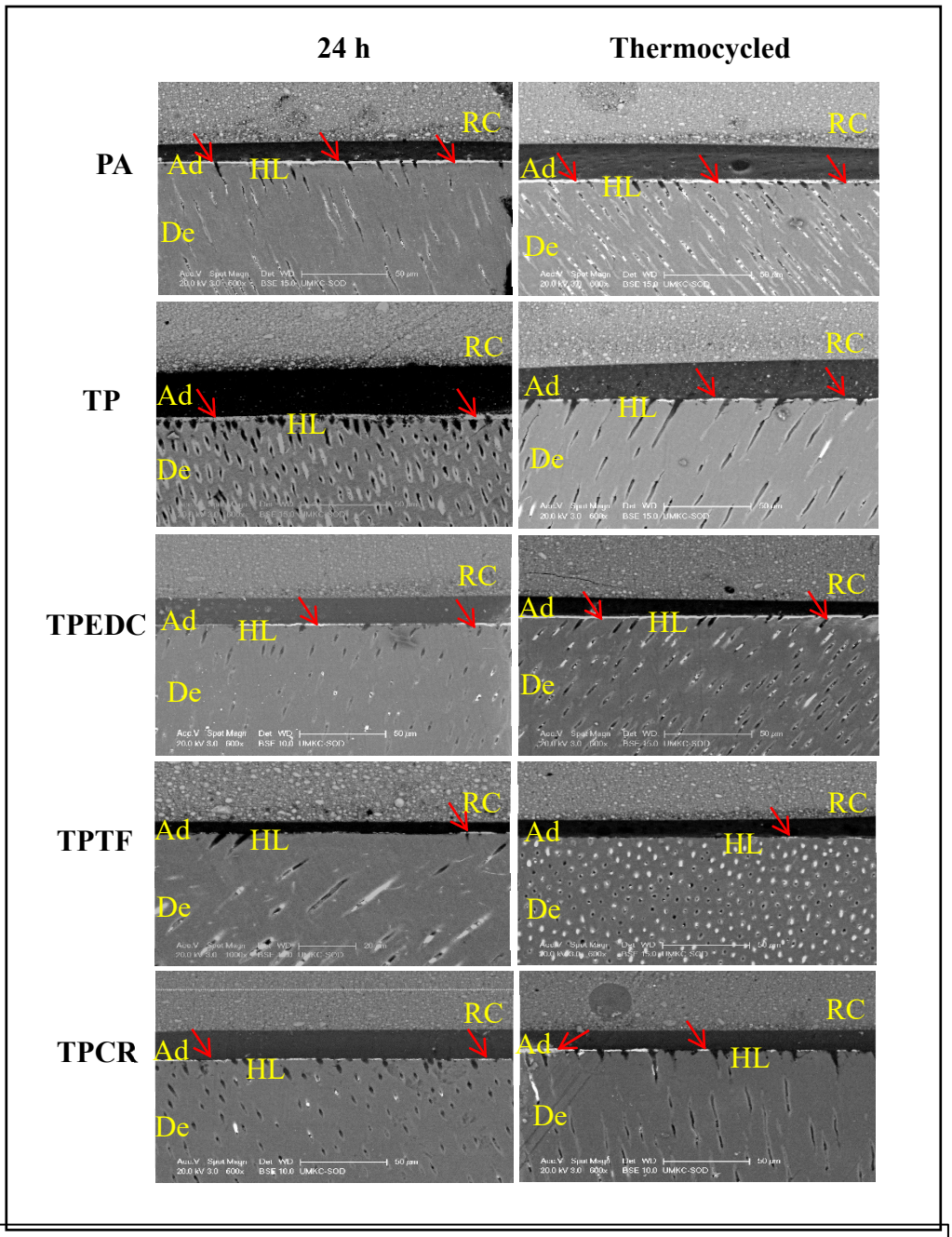

Figure 37: Representative backscattered SEM images of nanoleakage samples the resin dentin interface of PA, TP and TP dual- functional etchant groups in natural dentin. Images on left are after 24 h and on right after thermocycling in SBF solution. Silver nitrate infiltration is shown with red arrows. Abbreviations: RC: resin composite; Ad: adhesive layer; De: dentin; HL: hybrid layer.

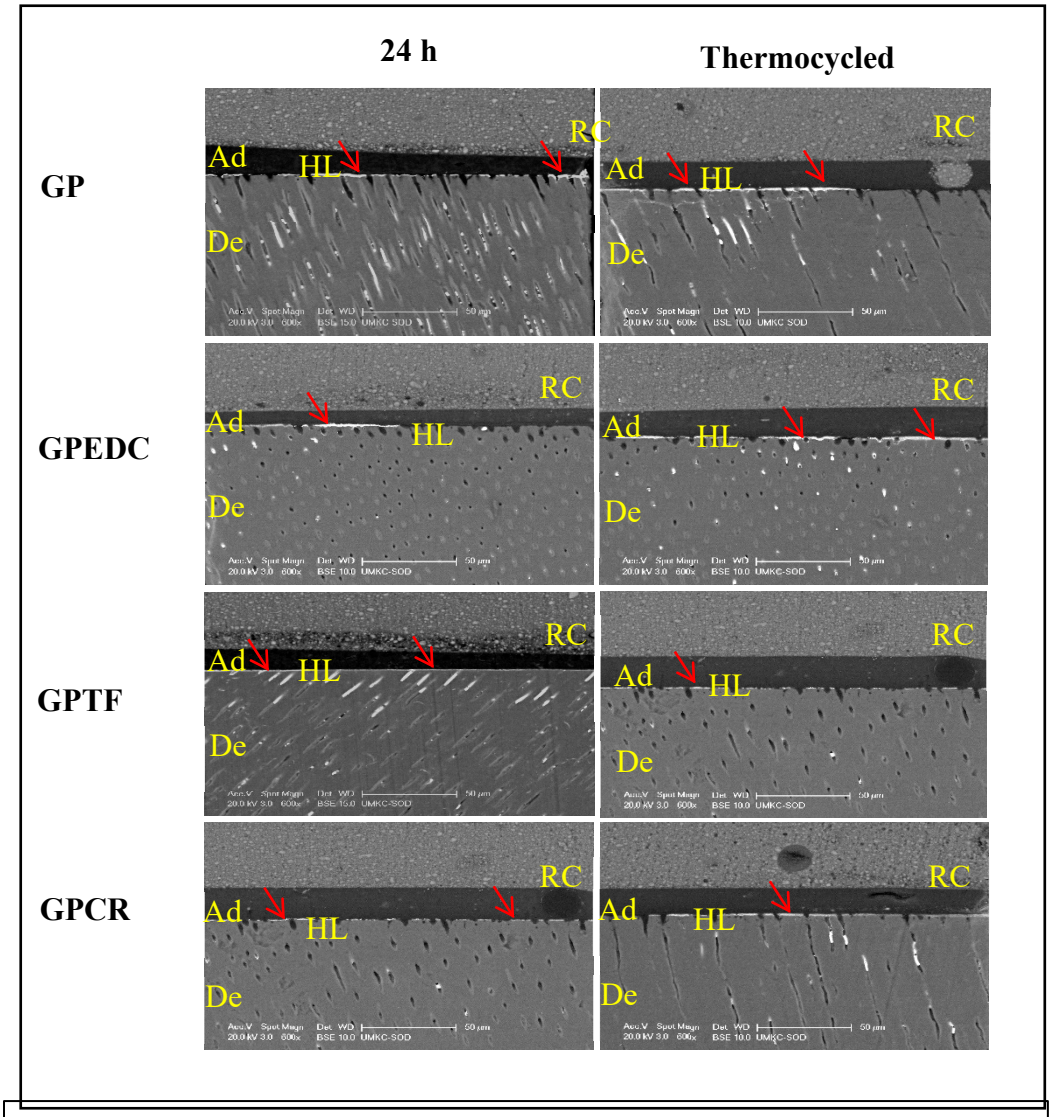

Figure 38: Representative backscattered SEM images of nanoleakage samples the resin dentin interface of GP and GP dual- functional etchant groups in natural dentin. Images on left are after 24 h and on right after thermocycling in SBF solution. Silver nitrate infiltration is shown with red arrows. Abbreviations: RC: resin composite; Ad: adhesive layer; De: dentin; HL: hybrid layer.

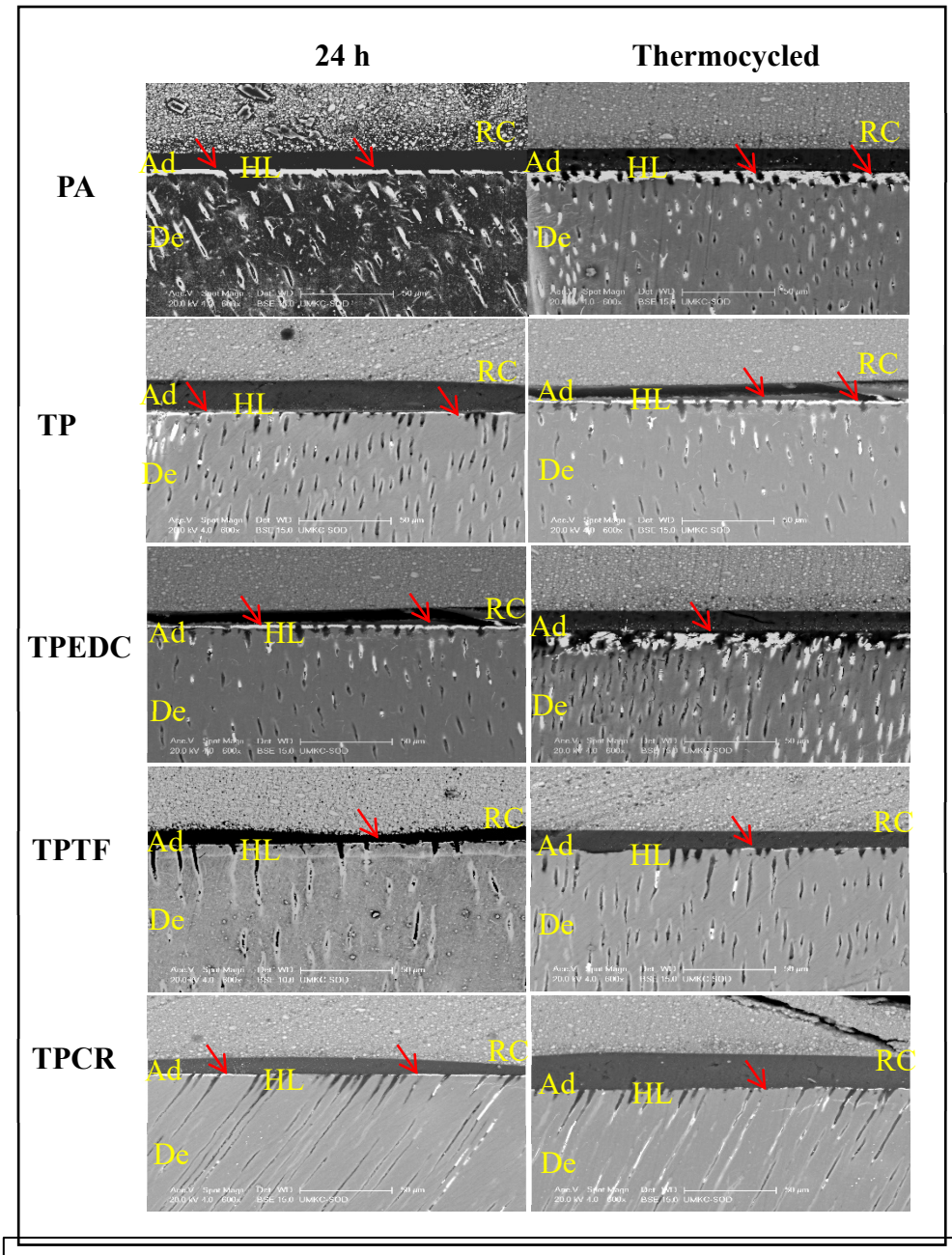

Figure 39: Representative backscattered SEM images of nanoleakage samples in the resin dentin interface of PA, TP and TP dual- functional etchant groups in CAD. The images on left are after 24 h and on right after thermocycling in SBF solution. Silver nitrate infiltration is shown with red arrows. Abbreviations: RC: resin composite; Ad: adhesive layer; De: dentin; HL: hybrid layer.

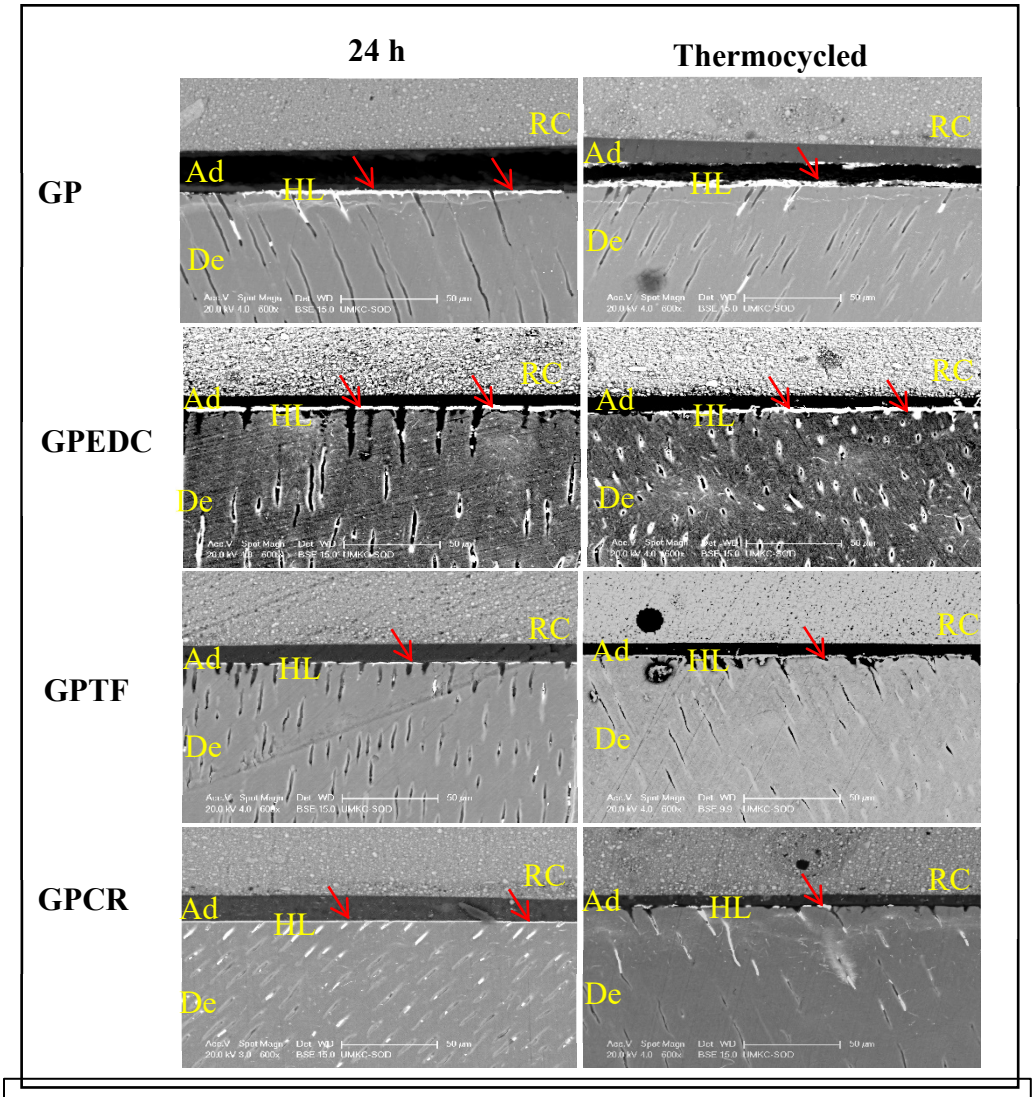

Figure 40: Representative backscattered SEM images of nanoleakage samples in the resin dentin interface of GP and GP dual- functional etchant groups in CAD. images on left are after 24 h and on right after thermocycling in SBF solution. Silver nitrate infiltration is shown with red arrows. Abbreviations: RC: resin composite; Ad: adhesive layer; De: dentin; HL: hybrid layer.

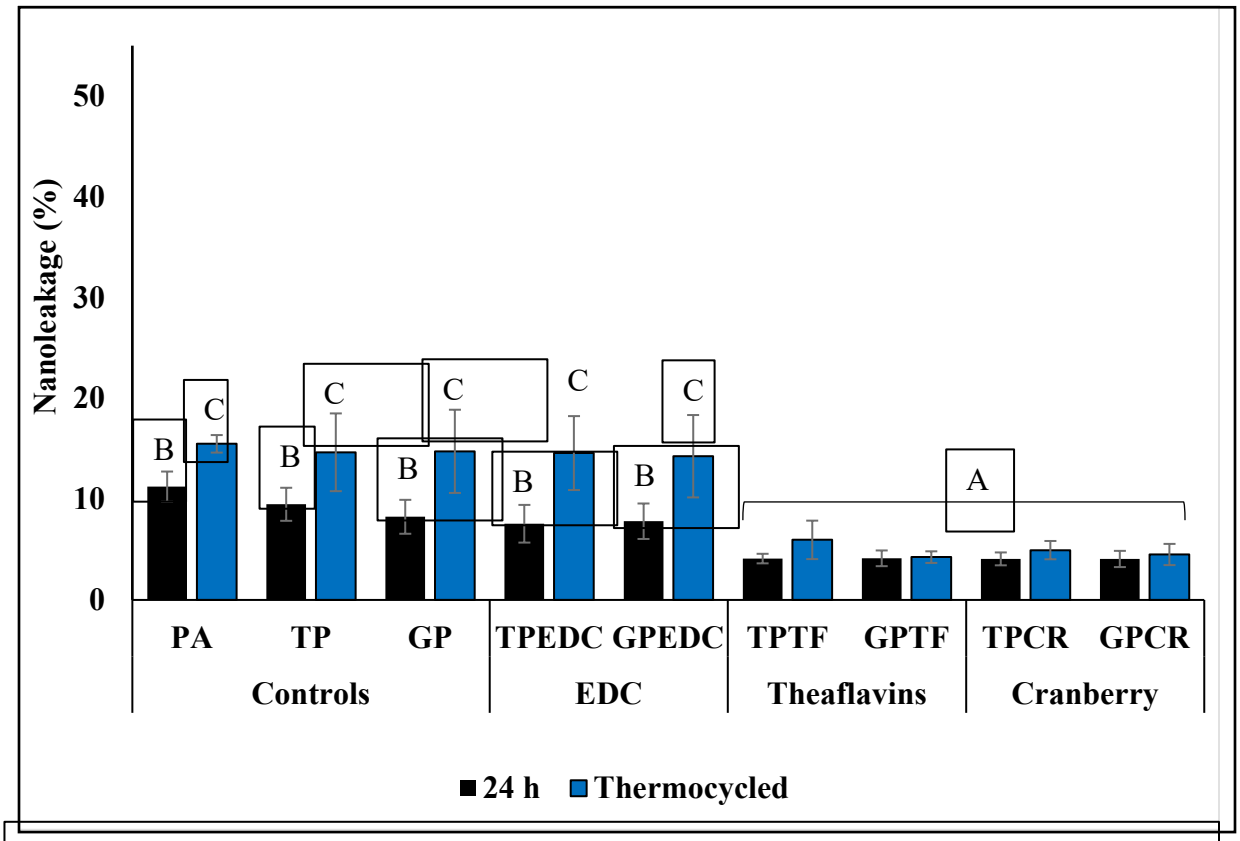

Figure 41: Means and standard deviations of nanoleakage (%) in resin dentin interface for all natural dentin experimental groups. Results following 24h and after thermocycling in SBF solution are shown. Different letters indicate statistically significant difference (two-way ANOVA and Games-Howell test; $p < 0.05$).

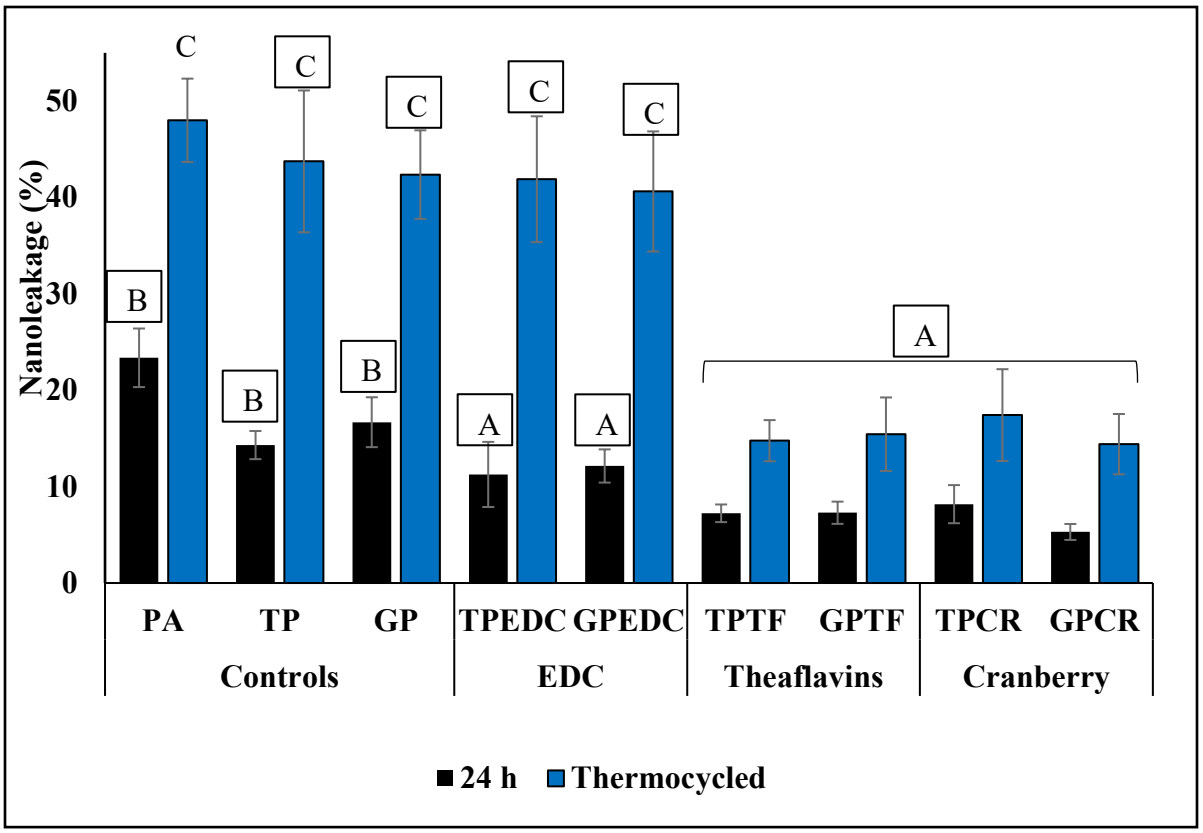

Figure 42: Means and standard deviations of nanoleakage (%) in resin dentin interface for all CAD experimental groups. The results following 24h and after thermocycling in SBF solution are shown. Different letters indicate statistically significant difference (Kruskal-Wallis test; $p < 0.05$).

In Situ Zymography

The representative multiphoton microscopy images obtained through in situ zymography of natural dentin of PA, TP and TP dual- functional etchants treated natural dentin are shown in figure 43 and for CAD in figure 45. The multiphoton images of GP and GP dual- functional etchants in natural dentin are represented in figure 44 and for CAD in figure 46. Quantified areas of green fluorescence in the hybrid layer representing MMPs activity for all experimental groups in natural dentin are shown in figure 47 and for CAD in figure 48.

The MMPs activity in CAD was significantly higher as compared to natural dentin ($p < 0.001$). The controls and EDC containing dual- functional etchant had significantly higher MMPs activity compared to TF and CR containing dual- functional etchants in natural dentin ($p < 0.001$). In natural dentin a significant increase in MMPs activity after thermocycling was seen in PA control ($p < 0.001$), while thermocycling did not affect MMPs activity in rest of the controls and the dual- functional etchant groups ($p > 0.05$). A significant difference was also seen in all CAD controls after thermocycling ($p < 0.001$). The MMPs activity for CAD was highest in the controls followed by EDC containing dual- functional etchant groups and the lowest in the TF and CR dual- functional etchants. No significant difference was seen in EDC, TF and CR containing dual- functional etchants before and after thermocycling irrespective of dentin type ($p > 0.05$).

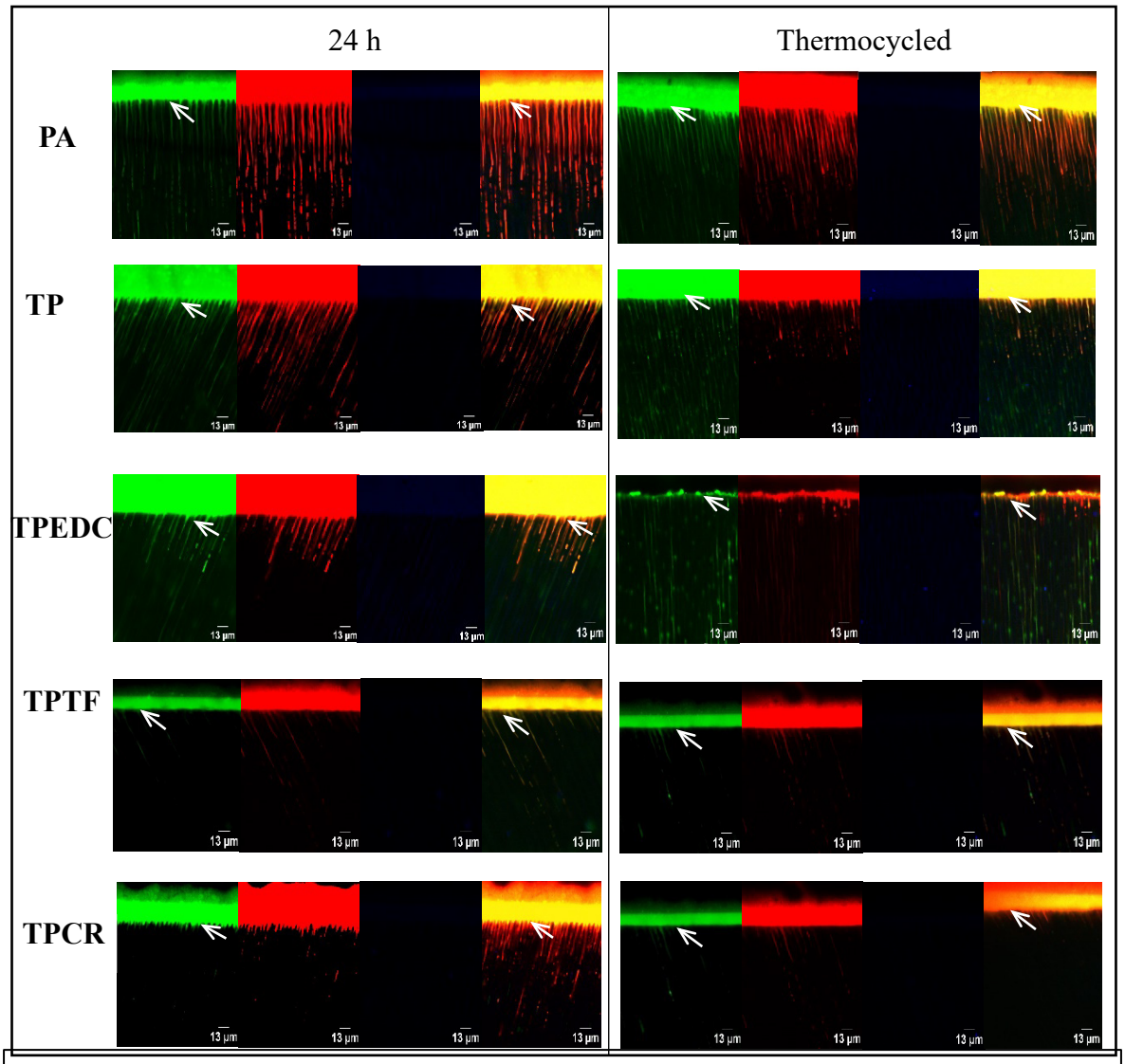

Figure 43: Representative multiphoton microscopy images of resin dentin interface of PA, TP and TP dual- functional etchant groups in natural dentin. Images on left are after 24h and after thermocycling are shown on right. For each group the first image from the left is the green fluorescence representing the MMP's, the second (red) represents the adhesive, the third image (blue) represents collagen, and the final is the merged image of the first three. White arrows represent the MMP's activity.

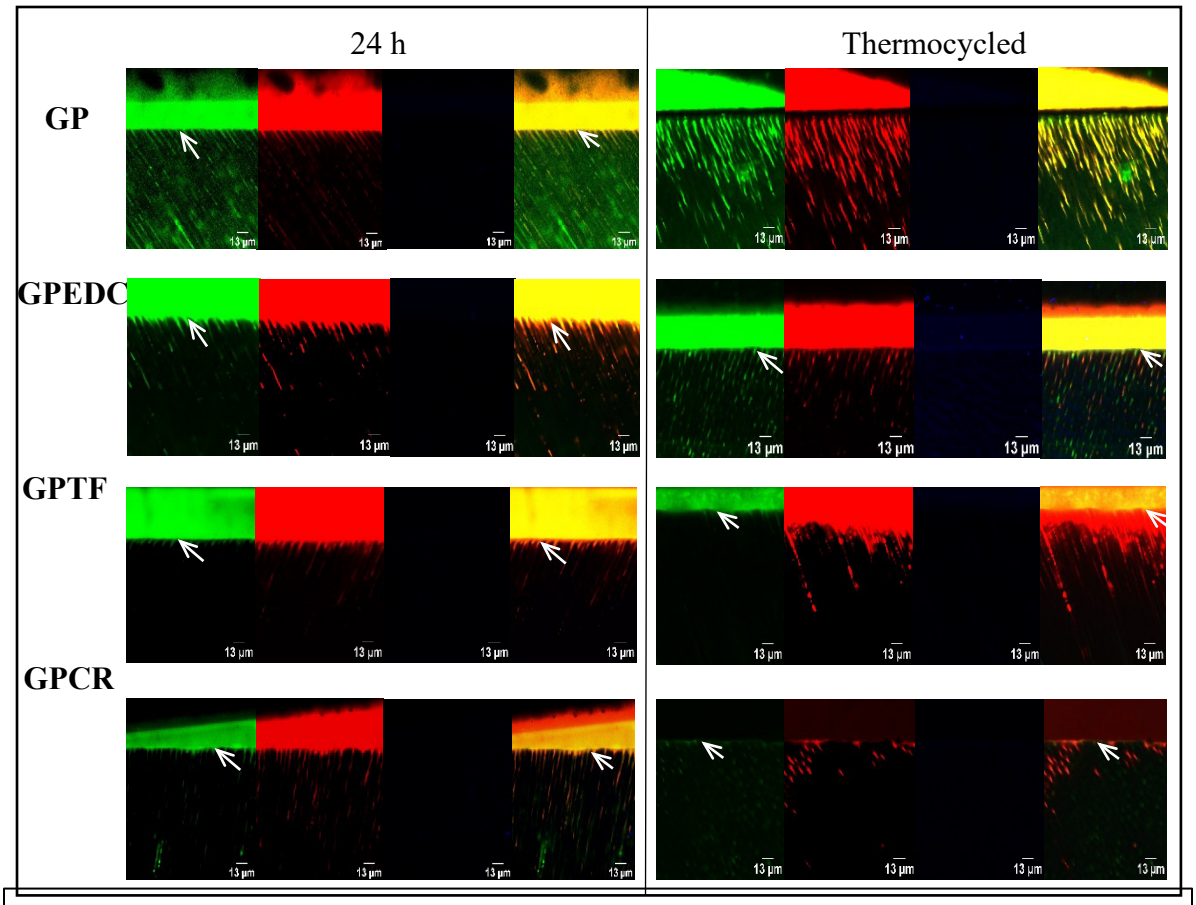

Figure 44: Representative multiphoton microscopy images of resin dentin interface of GP and GP dual- functional etchant groups in natural dentin. Images on the left are after 24h and on the right are after thermocycling. For each group the first image from the left is the green fluorescence representing the MMP's, the second (red) represents the adhesive, the third image (blue) represents collagen, and the final is the merged image of the first three. White arrows represent the MMP's activity.

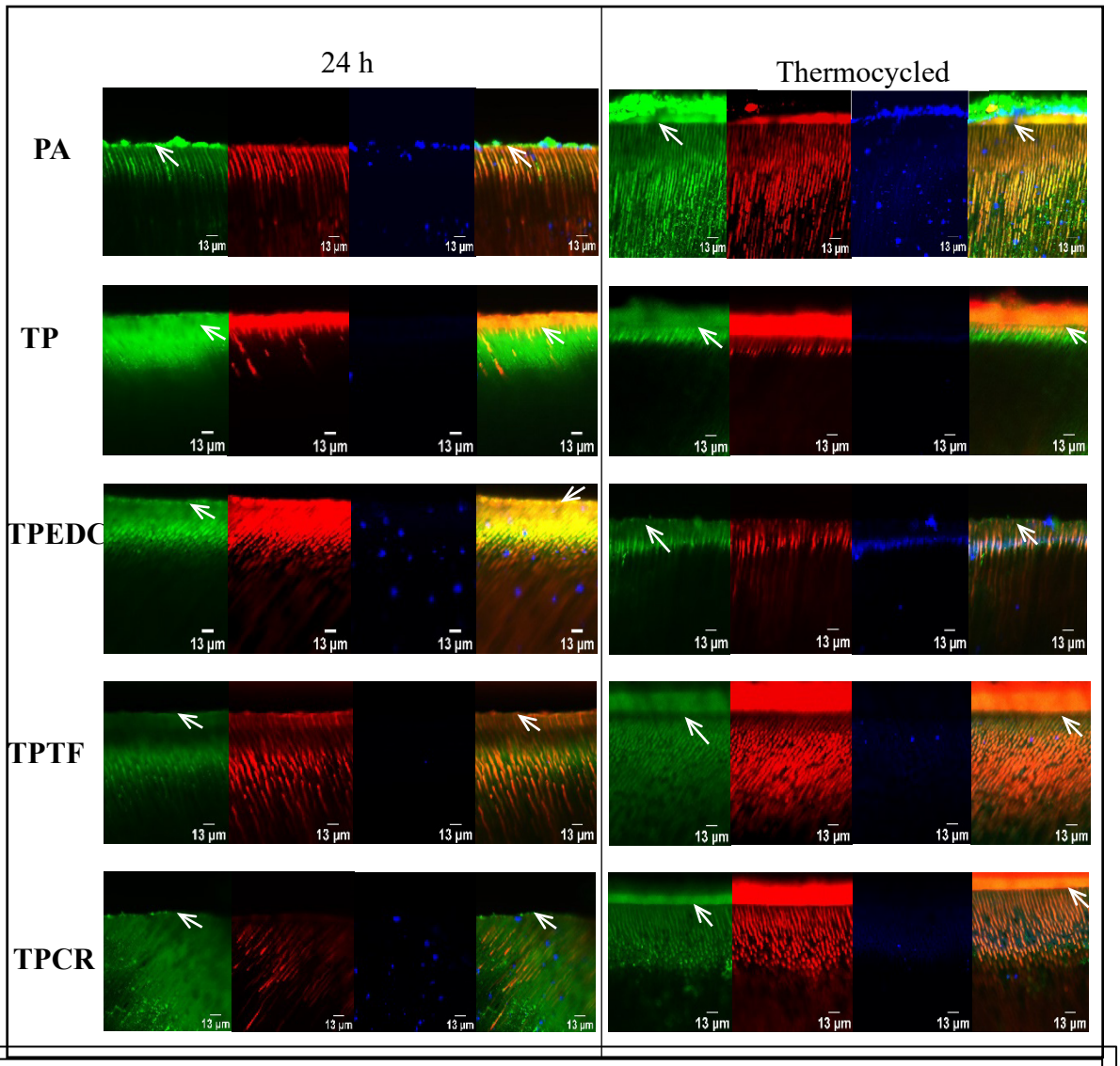

Figure 45: Representative multiphoton microscopy images of resin dentin interface of PA, TP and TP dual- functional etchants in CAD. Images on the left are after 24h and on right are after thermocycling. For each group the first image from the left is the green fluorescence representing the MMP's, the second (red) represents the adhesive, the third image (blue) represents collagen, and the final is the merged image of the first three. White arrows represent the MMP's activity.

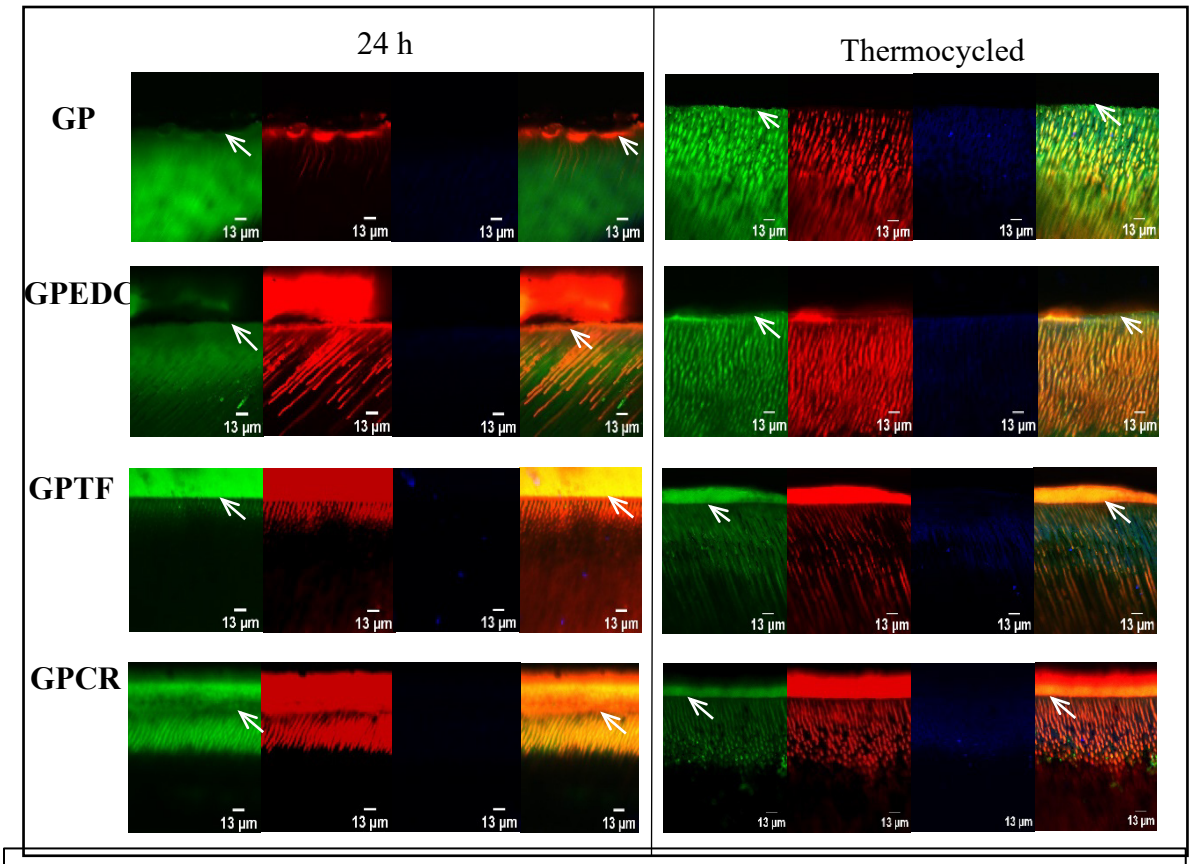

Figure 46: Representative multiphoton microscopy images of resin dentin interface of GP and GP dual- functional etchants in CAD. Images on the left are after 24h and on the right are after thermocycling. For each group the first image from the left is the green fluorescence representing the MMP's, the second (red) represents the adhesive, the third image (blue) represents collagen, and the final is the merged image of the first three. White arrows represent the MMP's activity.

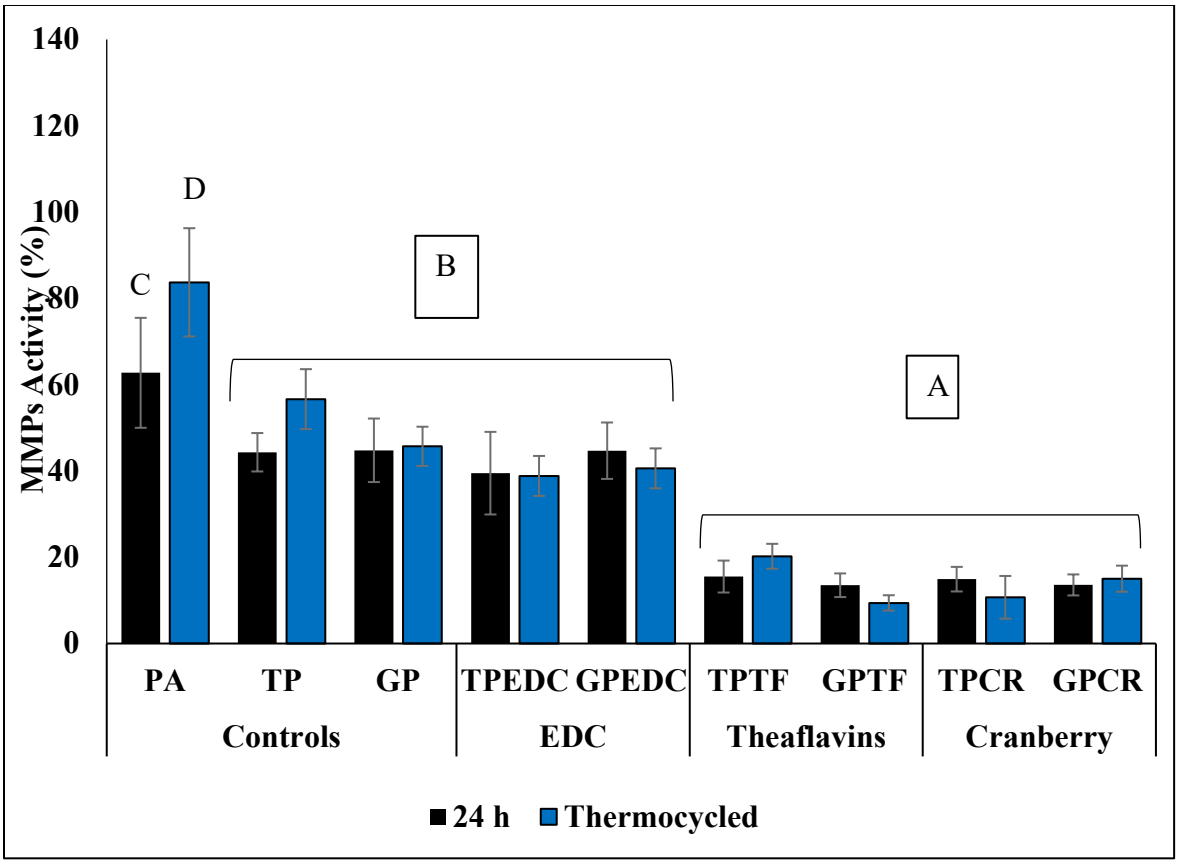

Figure 47: Representative MMP's activity in natural dentin's resin dentin interface of all experimental groups after 24h and thermocycling. Different letters indicate statistically significant difference (two-way ANOVA and Games-Howell test; $p < 0.05$).

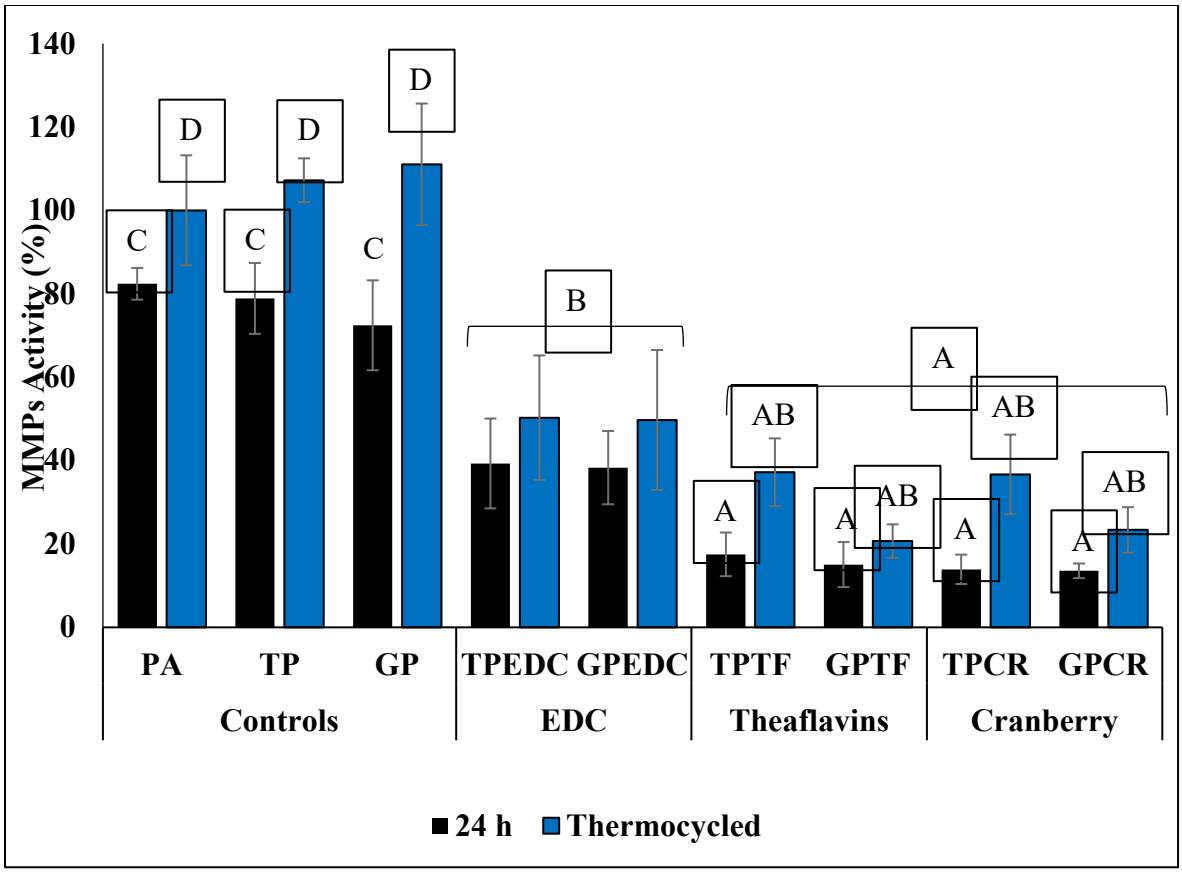

Figure 48: Representative MMP's activity in CAD's resin dentin interface of all experimental groups after 24h and thermocycling. Different letters indicate statistically significant difference (two-way ANOVA and Games-Howell test; $p < 0.05$).

Discussion

This study was designed to evaluate the effect of dual- functional etchants using a combination of organic and phosphoric acid with a crosslinker. To the best of our knowledge, this is the first study to introduce a combination of acids as dual- functional etchants. Two organic acids, TA and GA were tested to evaluate their ability to improve the solubility of crosslinkers in PA without using a volatile solvent like ethanol. The ability of the dual-functional etchants was then tested for their effect on stability of resin dentin bonds in natural and CAD.

The two organic acids used in this study (TA & GA) are weak α- hydroxy acids which have been used in dermatology for skin rejuvenation (Babilas et al. 2012). These acids when used alone as dentin etchant produced ~0.4μm demineralization depth whereas a depth of ~6μm was produced with the etchant PA (table 3). Our formulation made of a combination of organic acid with PA resulted in a depth of ~2μm. While the depth of demineralization has shown to not affect the stability of a resin dentin bond (Carvalho et al. 2012), using a dual-functional etchant creates certain challenges. For example, the crosslinkers have the ability to bind to the mineral in dentin and restrict the diffusion of etchant, hence hinder mineral removal (Xie et al. 2008). Secondly, during etching the acid attaches to the hydroxyapatite through ionic interaction and form a calcium ion complex. It is the rate at which this complex dissolves, which determines the extent of demineralization (Chien et al. 2016; Trevelin et al. 2019). Both these factors are restricted when a dual- functional etchant is used. Addition of a crosslinker reduced the demineralization efficiency and a reduction of ~45% was seen when organic acids alone were used as an etchant (Trevelin et al. 2020). The dual- functional etchant formulation used in this study caused a reduction in demineralization depth of ~20%.

As the dissociation constant of PA is higher than that of organic acids, it would effectively remove mineral or mineral ionic complexes to create a demineralized zone for adhesive infiltration. However, when used alone the aggressive nature of PA causes dissolution of intra and extrafibrillar mineral in dentin collagen, weakening and making it vulnerable to collapse during resin infiltration (El Feninat et al. 2001). By combining an acid like PA with a higher dissociation constant value which could aid in adequate mineral removal, with an organic acid that resolves the issue of crosslinker solubility while countering excessive demineralization caused by PA, is an attractive approach for etching dentin.

While the etchants were able to produce a demineralization zone in natural dentin (fig. 29), the substrate of CAD seems to have failed to produce any (fig. 30). Either the demineralized zone was too faint to be visualized using this technique or it was too weak to withstand the processing steps. The microhardness data (fig. 24) shown in chapter 4 indicates a gradual increase in KHN from surface and into the depth of CAD. This slope representing the depth of CAD could be another reason for not being able to see the demineralized layer. The substrate of CAD is exposed to bacterial acids causing loss of mineral as well as activation of endogenous enzymes resulting in a disorganized structure with altered dentin collagen (Wang et al. 2007; Mazzoni et al. 2015a). This substrate is more porous with water filled spaces which hinder infiltration and polymerization of the adhesive leading to hydrolytic degradation (Ito et al. 2005; Nakajima et al. 2011). This disorganized structure was evident in multiphoton images of CAD (fig. 45 and fig. 46) where exposed collagen shown in blue was higher compared to natural dentin (fig. 43 & fig. 44). In addition, the occurrence of occluded dentinal tubules with insoluble mineral crystals also hinders infiltration of the adhesive and formation of an effective hybrid layer. Due to these

challenges the resin dentin bonds created in CAD are less stable compared to those in natural dentin (Isolan et al. 2018).

In our study, the feeble substrate of CAD resulted in μTBS values which were around half of those in natural dentin (fig. 31 & fig. 32), which is in agreement with previous studies (Lenzi et al. 2016; Nicoloso et al. 2017; Hass et al. 2019). The altered collagen and low mineral content in CAD compromises mechanical properties of this substrate and reduces its cohesive resistance. This affects formation of a stable hybrid layer resulting in occurrence of premature failures (fig. 34) and lower bond strength values compared to sound dentin (Nakajima et al. 2011). The altered substrate of CAD is susceptible to over etching which results in formation of deep demineralization zones which are challenging for adhesive infiltration (Erhardt et al. 2008). This results in higher nanoleakage (fig. 39 & fig. 40) and MMPs activity (fig. 48) compared to natural dentin (Fialho et al. 2019; Gutierrez et al. 2022). On closer examination of the fractured CAD beams (fig. 36), higher frequency of exposed collagen fibrils which probably were poorly infiltrated by the resin were evident. A highly porous adhesive layer in fractured CAD beams was also seen, which indicates poor resin infiltration and polymerization of the adhesive (Hass et al. 2021).

Creation of high demineralization depths with aggressive etchants like PA, translate into formation of thicker hybrid layer which adversely affect the stability of resin dentin bonds (Hashimoto et al. 2000). Our study showed a significant reduction in the μTBS and increase in nanoleakage of all controls as well as EDC containing dual- functional etchant groups after thermocycling ($p < 0.001$) in natural and CAD. Thick hybrid layers formed in these experimental groups are weak and vulnerable to enzymatic degradation as the exposed collagen fibrils are not completely encapsulated and protected by the resin (Wang and

Spencer 2003; Spencer et al. 2004). However, no difference was seen in TF and CR containing dual- functional etchants ($p > 0.05$) following thermocycling, indicating improved stability and durability of resin dentin bonds in these groups. Crosslinked dentin collagen strengthens the etched organic matrix and provides a sturdy network for adhesive infiltration which would protect the exposed collagen from degradation (Liu et al. 2011a). This was reflected in extensive resin tags on the fractured specimen in the dual- functional etchants groups (fig. 35 & fig. 36). These results also show that even if the infiltrating resin is not able to encapsulate the exposed collagen completely, strengthening the exposed collagen matrix through crosslinkers can improve the stability and durability of resin dentin bonds. Hence the combination of low demineralization depth and crosslinking of the exposed collagen, makes dual- functional etchants a promising approach towards improvement of the durability of resin dentin bonds. Despite low molecular weight of TF, no difference in its performance was seen compared to CR. This could be due to the purity of TF used in this study which was ~40% compared to ~80% used in previous study (Liu et al. 2021). Using a higher analytical grade of TF with less impurities could have performed better in a dual- functional etchant and outweigh the performance of a comparatively bigger molecule of CR.

Using crosslinkers to strengthen the altered dentin collagen matrix is a promising approach, however very few studies have evaluated their effect on CAD and to best of our knowledge, none have tested the effect of dual- functional etchants on CAD. This is the first study which has evaluated the effect of crosslinkers TF and CR containing dual- functional etchants on the durability of natural and CAD. The effects and extent of interactions of TF and CR with dentin have been discussed in chapter 3. Ideally crosslinkers work better at neutral pH, however, both TF and CR crosslink dentin collagen through non covalent

interactions including hydrogen and hydrophobic bonding, which makes them efficient crosslinkers at lower pH as well (Liu et al. 2014a). Both these crosslinkers reinforce the collagen network and improve the resilience of collagen against enzymatic degradation (Liu et al. 2021; Wang et al. 2021). These crosslinkers were also able to significantly reduce activity of MMPs when used in a dual- functional etchant (fig. 47 and fig. 48). The MMPs activity of TF and CR containing dual- functional etchants was significantly lower than that of the controls and EDC containing dual- functional etchants ($p < 0.001$) and showed stability after aging ($p > 0.05$) in natural dentin and CAD. These results support the microtensile strength (fig. 31 & fig. 32) and nanoleakage (fig. 41 & fig. 42) results which showed stability in the dual- functional etchant groups after aging. Other than directly inactivating the catalytic sites on MMPs (Liu et al. 2011d), extensive crosslinks induced by TF and CR, reinforce the collagen matrix and limit the access to the collagen active sites on which MMPs act on (La et al. 2009; Kong et al. 2015). Reduction in MMPs activity would prevent collagen from degradation, provide stability to the hybrid layer and improve the longevity of a dental restoration.

Studies have shown that the activity of MMPs is pH dependent (Mazzoni et al. 2006; DeVito-Moraes et al. 2016). Our study showed significantly higher MMPs activity of PA (lowest pH) treated specimens compared to TP and GP in natural dentin ($p < 0.05$). Similarly, the MMPs activity of only PA treated specimens significantly increased after aging ($p = 0.008$) in natural dentin. Etching dentin with an aggressive etchant like PA removes more mineral (especially intrafibrillar) from dentin, exposes collagen and activates MMPs. Over time these activated MMPs start a vicious cycle of attacking unprotected collagen which leads to activation of more MMPs. This vicious cycle continued during aging and resulted in

higher MMPs activity of PA treated specimen. However, using a milder etchant like TP and/or GP would reduce the chances of over etching and activating more MMPs, hence maintaining MMP activity during aging as well. Despite of no significant difference in MMPs activity in TP and GP after aging, it did not have the same effect on microtensile bond strength and nanoleakage results, which increased significantly after aging. Although MMPs play an important role in stabilizing the hybrid layer, other factors such as hydrolytic degradation of the adhesive could have weakened the hybrid layer after aging.

Contrary to natural dentin, aging accentuated MMPs activity in all CAD controls ($p < 0.05$) which resulted in poor bond strength (fig. 32) and increased nanoleakage (fig. 42). This difference could be due to the already demineralized and febrile state of CAD, which was not affected by the etchant type. On the other hand, a significant reduction in the MMPs activity as well as no difference after aging in all dual- functional etchant groups of CAD showed the positive effect of dual- functional etchants in maintaining stability in hybrid layer of CAD as well. Unlike TF and CR containing dual- functional etchants which are able to crosslink dentin collagen through different mechanisms, EDC/NHS containing dual- functional etchants have limitations. This crosslinker acts through activation of carboxyl groups in collagen which then react with the amino group to form amide bonds (Tezvergil-Mutluay et al. 2012). In acidic conditions of an etchant, the ionization of the carboxyl group is suppressed due to low pH. (Cammarata et al. 2015). This limits the availability of activated carboxyl groups and hence formation of crosslinks, making EDC/NHS unsuitable to be used in an etchant. While EDC containing dual- functional etchants showed no significant difference in MMPs activity compared to TP & GP in natural dentin, they showed a significant reduction in CAD ($p < 0.05$). This could be due to the altered state of CAD, which

is more porous and humid, causing dilution of the acid. This change in pH would have allowed EDC to form inter and intra helical bonds in the dentin matrix bound MMPs and reduce their activity (Ekambaram et al. 2015a).

Conclusions

We propose new formulation of dual- functional etchants which can overcome the challenges of over etching and excessive mineral removal while solving the issue of crosslinker solubility, enabling creation of a strengthened demineralized zone for micromechanical retention of the adhesive. Both TF and CR containing dual- functional etchants could demineralize and crosslink dentin collagen in a clinically feasible time period of 30 s. Despite lower molecular weight of TF compared to CR, no difference between their performance was seen. Following thermocycling TF and CR containing dual- functional etchants showed biostability compared to controls irrespective of dentin type. Future studies need to be carried out to test the shelf life of these dual- functional etchants and their compatibility with other adhesives.

CHAPTER 6

CONCLUSIONS

This research focused on evaluating the role of flavonoids on the biostability and long term durability of natural and denatured dentin collagen. In addition, the effect of natural flavonoid CR on remineralizing dentin collagen was also evaluated. Within the limitations of this study the following conclusions were drawn from the following three hypothesis:

Hypothesis 1. Flavonoids affect the biostability, structural and mechanical properties of natural and denatured dentin collagen.

- Two denaturation techniques were tested which produced dentin collagen with structural alterations that were similar to CAD.
- The interactions of flavonoids (CR and TF) with natural and denatured dentin collagen altered their structure.
- The structural alterations resulted in improved biostability against enzymatic degradation as well as mechanical properties of natural and denatured dentin collagen.
- Treatment time of 30 s was challenging to protect HD dentin from enzymatic degradation however, significant improvement was seen following 1 h treatment time.

Hypothesis 2. Remineralization of natural and denatured dentin collagen can be enhanced by natural flavonoid CR.

- Remineralization of CAD is more challenging compared to natural dentin.

- Extensive phenol groups in CR produce exogenous crosslink in natural and denatured dentin collagen and provide a sturdy scaffold for remineralization.
- The phenol groups provided additional nucleation sites in dentin collagen and accelerated the remineralization process in natural and denatured dentin collagen.

Hypothesis 3. Flavonoids containing dual- functional etchants improves the long term durability of natural and CAD.

- A new formulation of dual-functional etchants using less aggressive acid was proposed.
- Natural flavonoids TF and CR containing dual- functional etchants created a demineralized layer that was resistant to enzymatic degradation in a clinically feasible treatment time for 30 s.
- The dual- functional etchant containing TF and CR showed stability after thermocycling compared to controls irrespective of dentin type.

In conclusion, this investigation has characterized the effects of two natural flavonoids (TF and CR) on natural and denatured dentin collagen. Based on our results, these flavonoids can produce structural alterations in dentin collagen and improve their resistance against enzymatic degradation, provide a sturdy and active scaffold for accelerated remineralization. When used as a dual- functional etchant they can improve the long term durability of resin dentin bonds in a clinically relevant time of 30 s.

However, future studies are required to further understand the structure activity relationship of the four TF compounds as well as the A type linkage of CR in improving dentin collagen biostability. An understanding of the number and position of phenol and

galloyl groups as well as the importance of the benzotropolone structure in resisting collagenase and esterase degradation or their interaction with collagen could provide valuable insight in creating synthetic compounds with improved function.

More research is also required for better understanding of the complexities involved in dentin collagen remineralization. Longer duration studies need to be done to evaluate the extent of mineralization. Chemical analysis to evaluate structural alteration in remineralized dentin collagen, mechanical test (nanoindentation) and the use of fluorescein labelled NCP are future studies that will help in better understanding of the process. Work also needs to be done in creating a delivery mechanism for NCP in an adhesive or in a liner or base without affecting their properties. It would also be interesting to see if the remineralization process would have similar results with pretreatment with a dual-functional etchant.

The long term goal is to create better understanding of the complex nature of denatured dentin collagen and the challenges associated with improving its resistance to degradation. This combined with an understanding of structure function relationship of crosslinker or remineralizing agents can help us design a synthetic compound with functional groups for crosslinking and remineralization that is compatible with all components of the dentin bonding system. The synthetic compound created would not only aid in improving the long term durability of resin dentin bonds but also contribute to fields of bone scaffold engineering and aid in developing a new generation of bone graft alternatives.

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VITA

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EDUCATION

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2013-2015	M.Phil in Dental Materials Riphah International University Islamabad, Pakistan
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2001-2003	Higher Secondary School Certification The City School Islamabad, Pakistan
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PROFESSIONAL EXPERIENCE

2015-2017	Assistant Professor in Department of Dental Materials, IIDC, ISD, Pakistan
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2011-2013	Demonstrator in Prosthodontics Department, IIDC, ISD, Pakistan
2010-2017	Private Practice, ISD, Pakistan

AWARDS AND ACHIEVEMENTS

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Twice volunteered a community dentistry trip to Chakkar, AJK in May and August 2009
Third Position in 1st Prof. B.D.S. Annual Exam 2005
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PRESENTATIONS AT SCIENTIFIC MEETINGS

2022 Effect of natural flavonoids on biostability of denatured dentin collagen at AADOCR/CADR Annual Meeting & Exhibition in Atlanta, GA, USA

2021 Crosslinking effect of theaflavins and type-A-proanthocyanidins on dentin collagen biostability at IADR/AADR/CADR General Session

2015 Effects of varying powder liquid ratios of properties of heat cure acrylic resin at 2nd Annual Conference of PADR, Lahore, Pakistan

PUBLICATIONS

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