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Structure-Property Relationships for Human Dentin: The Importance of Biological and Chronological Aging

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Abstract

Structure-Property Relationships for Human Dentin: The Importance of Biological and Chronological Aging

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The increase in number of senior patients has become a major challenge in the field of dentistry. Senior patients are more likely to experience tooth fracture, which often necessitates tooth extraction. Understanding the aging process and its effects on the structure-property relationships of tooth tissue is critical to the development of patient-centric oral healthcare. Previous efforts have focused on the impact of age-related mineralization, known as sclerosis, within the tooth crown. In comparison, few studies have considered age-related degradation of the organic content and its contribution to the structural behavior of dentin. This concern is of greater importance in the radicular dentin, which possesses a higher organic content. Furthermore, the tooth roots are subjected to more invasive treatments as a result of root infection, which could contribute to a unique form of age-related degradation. Therefore, the primary goal of this study is to understand the materials science of aging in the radicular dentin, including the differences in degradation that occur in vital and non-vital teeth. Chapter 1 presents a brief introduction to oral histology that supports the detailed discussions in the following chapters. Chapter 2 to Chapter 6 describe complementary studies performed to answer the overarching research questions: what are

the mechanisms of structural degradation in tooth roots with aging and how is this process affected by the loss of the vital pulp? Chapter 7 then addresses the cellular aspects of the aging process in dentin.

In Chapter 2 we start by exploring the changes in microstructure, chemical composition, and mechanical properties of radicular dentin as a function of aging. Specifically, the evolution in microstructures associated with sclerosis, mineral-to-collagen ratio and collagen cross-linking were correlated with the mechanical properties as a function of distance from the root apex. The findings indicate that the most severe degradation occurs near the root apex.

In Chapter 3 we compare the structural behavior of radicular dentin from vital and non-vital teeth of senior patients using a matched-pair approach. The results indicate that the radicular dentin of teeth with root canal treatment (RCT) followed by oral function undergoes an accelerated degradation in comparison to that in teeth with vital roots. This process appears to be most associated with the extent of collagen cross-linking.

Dehydration has been considered as a potential factor in Vertical Root Fractures (VRF) after the RCT. In Chapter 4 we characterized the spatial distribution in shrinkage of tooth roots with respect to donor age and prior RCT using digital image correlation. The RCT treated teeth of senior patients suffered the highest percentage of water loss. Dehydration-induced cracking initiates from the root surface towards the canal as a result of shrinkage.

Chapter 5 focuses on acute embrittlement of dentin due to changes in the capacity for viscous deformation in the dentin matrix. Advanced dynamic mechanical analysis was applied to the dentin matrix using scanning mode nanoindentation. The findings revealed degradation in the intertubular component of radicular dentin (i.e. the col-

lagen matrix) with aging, and an acceleration of the extent of age-related degradation near the apex and particularly within the tissue of pulpless (i.e. non-vital) teeth.

In Chapter 6, autotransplantation of teeth after cryopreservation is discussed, which is an increasingly viable method for whole tooth replacement. The objective of this effort is to determine whether cryopreservation alters the microstructure of dentin or causes a reduction of its resistance to fracture. Results revealed that hidden damage within the dentin as a result of cryopreservation decreases the fatigue resistance and could render these teeth more susceptible to fracture.

In Chapter 7, the role of odontoblasts in the progress of sclerosis and the degradation in strength of dentin with aging is discussed. The biophysical and biochemical alterations in dentin appear to result from changes in the microstructure and chemical composition of the dentinal tubules where odontoblastic extensions reside. The extent of odontoblast apoptosis is assessed along the radial direction of roots from teeth of different ages and is correlated to the chemical composition and mechanical properties of the radicular dentin.

Chapter 8 presents a summary of the overall results and conclusions. A brief discussion of future research directions is also included.

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1. Yan, W., Montoya, C., Øilo, M., Ossa,A., Paranjpe,A., Zhang,H., Arola, D.D., Reduction in Fracture Resistance of the Root with Aging. (2017), *J. Endod.*, 43 (9), 1494–1498.

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DEDICATION

to my wonderful wife Xiaojing,
who always brings out the best in me,
and to my dearest parents,
for their endless love, support and encouragement.

Chapter 1
INTRODUCTION

1.1 Motivation

During my five years PhD research in the Arola lab, I studied the aging effects on the composition, microstructure and structural integrity of oral hard tissues. Specifically, we are interested in why oral hard tissues, such as tooth dentin and enamel, undergo changes with biological and chronological aging and how that contributes to diminishing resistance to fatigue and fracture. The scope of this research is extended to the effects of treatments such as root canal therapy and cryopreservation.

The importance of this study stems from growth in the population of the aging dentate in today's world. The elderly population is much greater today than decades of the past, and it continues to rapidly increase.[1] In 2018 the number of people older than 64 years old surpassed the number of children under 5 years old (Fig. 1.1).[2] By 2030 the world is likely to have 1 billion older people, accounting for 13 percent of the total population.[3] In the United States, all baby boomers will be older than age 65 by 2030 and 1 in every 5 U.S residents will be at retirement age.[4] The same trend is observed in both developed countries and developing countries, making the aging population a global issue. In the meantime, the world also has had a steady increase in life expectancy and some nations have experienced more than a doubling of average life expectancy during the 20^{th} century.[3]

This important demographic shift has led to a decrease in the rate of edentulism(tooth loss). Dentistry is now facing new challenges for the oral health care of older adults because senior patients will no longer accept extraction and dentures

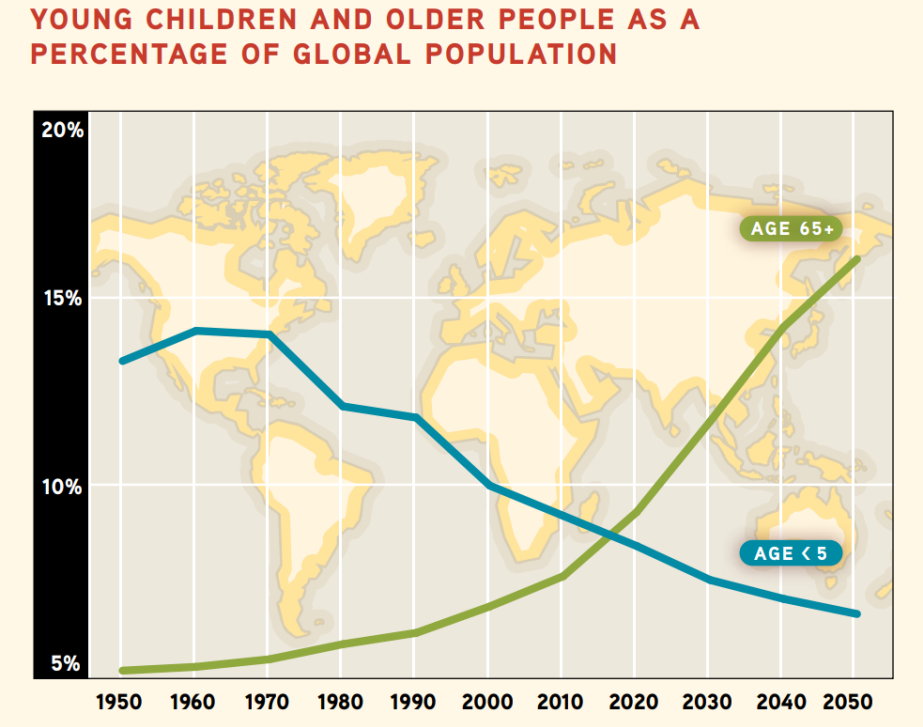

Figure 1.1: Young children and older people as a percentage of global population. Copyright 2020 United Nation.

as a standard solution to complex restorative needs. Simply put, patients are living longer and retaining their natural teeth.[5] For dental professionals, this transition is also accompanied by the growing impact of noncommunicable diseases and chronic conditions.[6] In the future, dental health care providers will be required to care for an expanded number of older adults who have retained teeth and are medically complex.[7]

One concern in the treatment of dentate seniors is that cracked teeth are much more common in this patient cohort.[8, 9] Tooth fracture most commonly occurs in patients range from 40 to 60 years old.[10] The severity and consequences of the fracture can range from minor, needing no treatment at all, to severe, resulting in

root canal therapy (RCT), or even tooth loss.[11] Thus, tooth failure due to fracture has become more prevalent with aging and the reduction of edentulism in seniors.[12, 13, 14]

The age of a human is generally defined by chronological age which refers to the actual amount of time a person has lived, whereas biological age is assessed by the actual physical and mental functions.[15] While tooth fracture correlates with chronological age for the general population, the dentition of each person has a unique biological age that is a function of their oral environment, diet, phenotype and most importantly, dental treatments. Endodontic treatment, or sometimes referred as root canal therapy (RCT), is one of the most common and well-established method to preserve natural tooth. However, researchers have observed that teeth treated by RCT are subjected to much more fractures, particularly within the root.[16, 17]

The primary goal of this study is to understand why aging and dental treatments appear to increase the risk of tooth fracture. We focus on the most abundant oral calcified tissues, dentin, because it is most often involved in clinical treatments and the diagnosis of cracks. Dentin is composed by inorganic and organic parts and is regarded as a bio-composite. The hierarchical microstructures of dentin, which is a characteristic of most hard tissues, determines its fracture properties. In order to understand the complex relationship between aging and properties of dentin, we must first understand the relationship between age and its microstructures. Therefore, the research goal can be subdivided into two components: (i) how the biological and chronological age affect microstructures of dentin, and (ii) how changes in microstructures with aging contribute to the mechanical properties of dentin.

1.2 Aging Tooth Structure

Dentin appears to be the principal tissue involved in tooth fractures as a consequence of what is called the "crack tooth syndrome".[8] The goal of this section is to set a stage for answering the first question of our research goal: how does biological aging affect the microstructures of dentin?

1.2.1 The Human Tooth

The primary function of human tooth is mastication, which involves cutting and crushing food mechanically for swallowing and digesting. Teeth must be hard and firmly attached to supporting connective tissues and the bone to fulfill the mastication function.

In humans, newborns carry 20 primary teeth, which are also called deciduous teeth. The deciduous teeth are gradually succeeded by 32 permanent teeth during childhood to accommodate the growth of the face and jaws. Both the deciduous and permanent sets of teeth are equally distributed in the maxillia (upper jaw) and mandible (lower jaw) bone and termed as the maxillary and mandibular teeth, respectively. There are 16 teeth in each arch that consist of 4 incisors, 2 canines, 4 premolars and 6 molars. They are mirrored with respect to midsagittal line(mid-line) of maxillary and mandibular dental arches (Fig. 1.2).

Tooth tissue consists of a hard, inert, acellular enamel supported by the less mineralized, more resilient, and vital hard connective tissue dentin.[18] Dentin is attached to the alveolar bones of the jaw through tooth-supporting connective tissues, consisting of the cementum and periodontal ligament. The three hard tissues of tooth are: enamel, dentin and cementum. Within the scope of crack tooth syndrome and root fracture, detailed discussion will be focused on dentin.

Each tooth consists of a crown and root (Fig. 1.3). Anatomically, the crown is

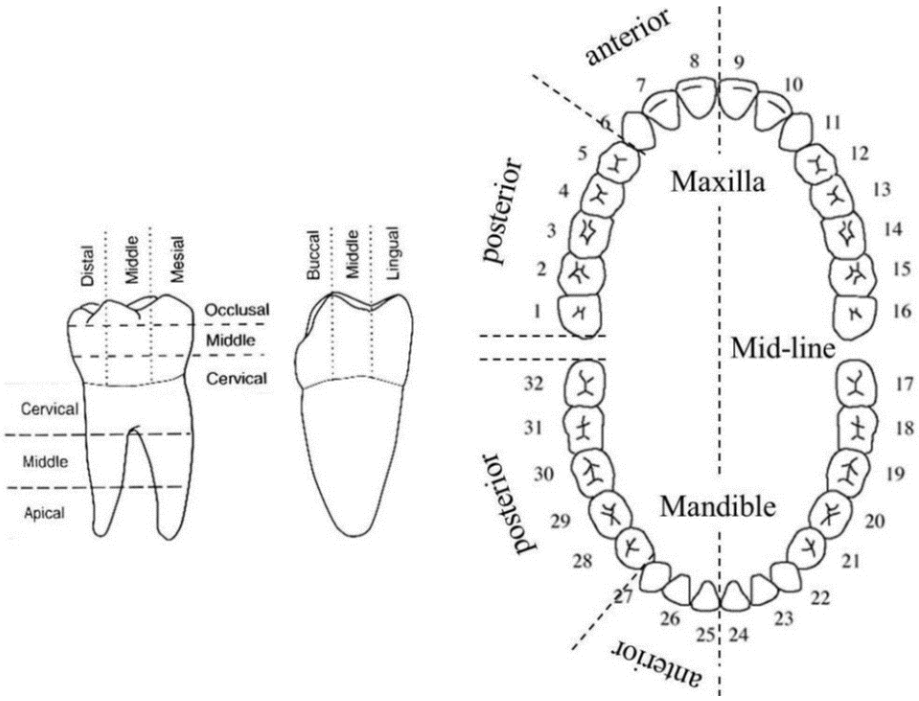

Figure 1.2: Nomenclature and numbering system of permanent human teeth. Copyright 2020 ProQuest.

defined as the portion of teeth that is covered by enamel. This term is different from the clinical crown where the crown is defined as any visible portion of the tooth in the oral cavity. The geometry of the crown can be described in terms of occlusal, mesial, distal, buccal and lingual surfaces (Fig. 1.2). The occlusal surface is the chewing surface that is furthest from the tooth root. Mesial refers to the area of the tooth facing the midline, whereas distal is the surface furthest from the midline. Similarly, the tooth surfaces of the teeth nearest to the tongue are referred to as lingual, whereas those closest to the face or buccinators muscle of the cheek are the buccal surface.[18]

The tooth root is defined as the portion below the cemento-enamel junction(CEJ) and is covered with cementum. Different from its anatomical term, the clinical root is any part of a tooth not visible in the mouth. Dentin composes most of the root, and encloses the pulp canal which is filled with soft connective tissues. Dentin from the root is usually referred to as radicular dentin. The root can be further divided into apical, middle and coronal third, each of which has different microstructural characteristics.

Dentin is a mineralized tissue of the tooth and consists of 70% mineral, 18% organic matter and 12% water by weight. Dentin forms the bulk of the tooth and provides a resilient supporting foundation for the comparatively hard and brittle enamel. The organic part is mainly fibrillar type-I collagen reinforced with apatite minerals and connected to enamel by the Dentin-Enamel Junction (DEJ). One of the most distinct features of dentin is the dentinal tubules, which is a network of microscopic channels extending radially outward from the pulp towards the DEJ and cementum. The tubule lumens are lined by a highly mineralized cuff of peritubular dentin that consists primarily of apatite crystals. Intertubular dentin occupies the region between the tubules and consists of a collagen fibril mesh oriented essentially perpendicular to the tubules and bound by apatite crystallites. In recognition of this

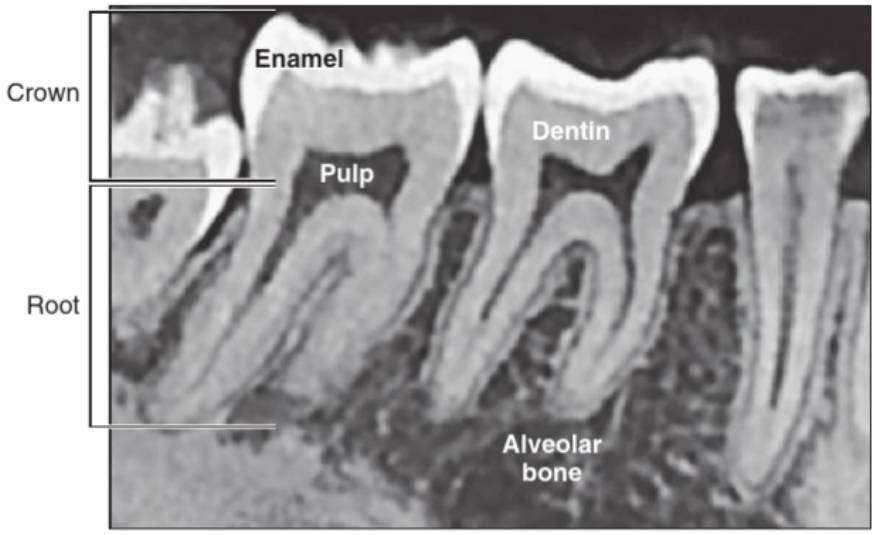

Figure 1.3: Vertical Cone Beam CT slice of mandibular molars and premolars showing the crown and root of tooth. Copyright 2020 Elsevier.

broad range of dimensional scales, dentin is often described as exhibiting a complex hierarchical structure.[19]

Tooth enamel is the most highly mineralized tissue in the human body and consists of more than 96% inorganic matter in the form of apatite crystals, 3% water and traces of organic material.[18] Enamel is an acellular tissue and does not have the ability to regenerate or repair damage that develops with oral function. Microstructurally, the enamel consists of carbonated hydroxyapatite crystals arranged in a keyhole shaped structure known as 'prisms'. The prisms, also regarded as rods, are arranged in a parallel fashion and extend roughly perpendicular in the outer enamel layer close to the tooth's surface. The middle and inner enamel layers possess a decussated pattern (also called Hunter-Schreger bands) where the rods are arranged in an alternating fashion that extends out from the DEJ at different orientations. This hierarchical ar-

rangement of rods allows enamel to dissipate energy and prevent catastrophic failure. The prism deccusation is believed to be a key factor contributing to the fracture and fatigue resistance of enamel.[20, 21]

1.2.2 Aging of Dentin

Although there are odontoblastic extensions of pulp cells within the tubules, dentin cannot be remodeled and regenerated by these cells. Therefore, changes in the structure and mechanical behavior with biological aging would be unexpected. Healthy dentin from young teeth manifests with natural white-yellow color and is opaque due to the light scattering effect within dentinal tubules (Fig. 1.4A).[22] However, dentin undergoes a gradual transition to increasing translucency with increasing age, a process regarded as sclerosis.[23] The changes causing dentin to appear more translucent is a result of occlusion of the tubule lumens by a mineral substance with a refractive index similar to that of the rest of the dentin as shown in Fig. 1.4B.[24, 25] After an adequate number of lumens have been filled by the deposition of mineral, dentin will allow light to transmit, and becomes more transparent. Sclerosis starts in the apical dentin (at the root tip) and spreads towards the crown with increasing age. Transparency begins in the root apex and becomes evident from the third decade of life. In the plane transverse to root pulp canal, sclerosis begins in the mesial and distal quarters and then spreads to the buccal and lingual quarters,[26] so the sclerotic zone forms a butterfly shape.

The exact mechanism of aging in dentin is not fully understood. Many researches have speculated that sclerosis of dentin is a physiological process stimulated either by biological factors from cells even though it cannot be regulated by cellular responses, or by external signals such as stress. Previous studies have evaluated the sclerosis of impacted teeth from senior patients.[28] Results showed that there was minimal

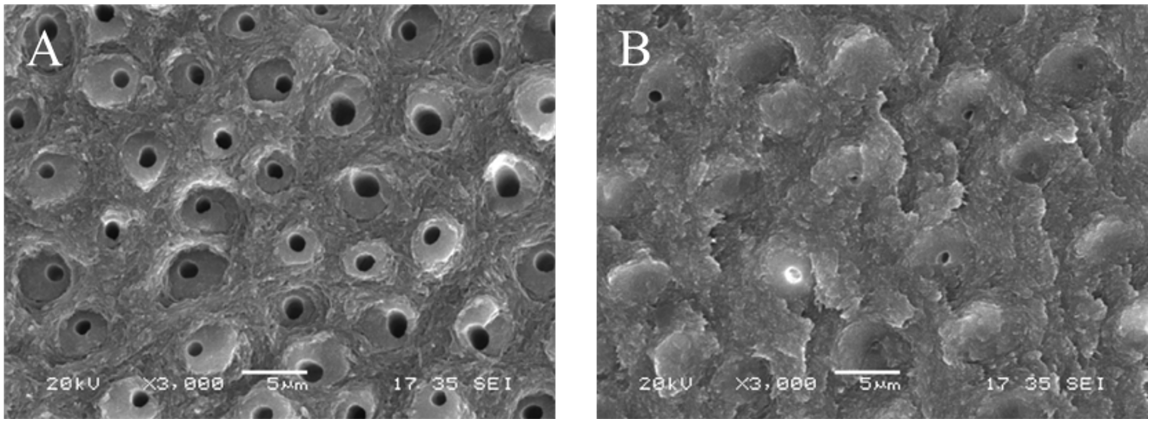

Figure 1.4: Typical micrographs from the fatigue fracture surface of young and old dentin. (A) the fatigue fracture surface from a young hydrated dentin specimen. This dentin is from a 20 years of age female patient. (B) the fatigue fracture surface from an old hydrated dentin specimen. This dentin is from a 50 years of age male patient. (Reproduced with permission from [27]. Copyright 2020 Elsevier)

evidence of filled tubules in unerupted teeth from older individuals, which suggests that the physiological sclerosis is possibly initiated by the stresses imposed by the oral environment. However, there are also contradictory reports suggesting that the amount of sclerosis increased linearly with age and that it was not markedly affected by the function of the tooth or external stimuli encountered during life.[29]

In a comparison of normal and sclerotic dentin using X-ray computed microtomography, Kinney et al. found that there is a significant increase in the degree of mineralization within the transparent dentine.[19] Further study showed that the mineral crystallites within intertubular matrix were 7–19% smaller in age-induced transparent dentin as compared to normal dentin.[30] In addition, the large mineral crystals deposited within the tubule lumens in transparent dentin were chemically similar to the intertubular mineral. They proposed that occlusion of the lumens occurs by dissolution of intertubular crystals and a re-precipitation of new mineral

crystallites within the lumens.[30]

A comprehensive understanding of the etiology of dentin sclerosis is still missing. Recently it has been purported that apoptosis of odontoblasts may be the key-factor that is responsible for the development of physiologic sclerotic dentin.[31] Increased odontoblast apoptosis has been found to have a positive relation with age.[32] The apoptotic bodies of odontoblasts become trapped within the dentinal tubules due to the continuous formation of secondary dentin and cannot be eliminated through phagocytosis. Thereafter, the cells suffer from a secondary or apoptotic necrosis leading to the release of the internal contents of pyrophosphate and hydrogen phosphate. Pyrophosphate can react with water and dehydrate the dentin. Hydrogen phosphate can react with hydroxyapatite and demineralize it, leading to the release of Ca^{2+} ions, which then contribute to the intratubular mineralization (sclerosis).

1.3 The Structure-Property Relationships in Dentin

Previous studies have investigated the relationships between dentinal mineralization(sclerosis) and fatigue strength,[33, 34] fracture toughness[35, 36] and fatigue crack growth resistance.[27, 37] The outstanding resistance to fatigue of dentin were attributed to the crack blunting, bridging and microcracking. These mechanisms were found to operate in the dentin of young donor teeth where sclerosis is not apparent and lumens are not filled by minerals. However, the intertubular dentin is more critical to resisting tooth fracture volumetrically and the collagen fibril mesh is integral to its fracture toughness. The research regarding collagen and its crosslinking has been neglected. Therefore, this section serves as a background introduction to elucidate the second research goal: what is the connection between the microstructures of dentin and its mechanical properties?

The most profound impact of age-related mechanical degradation of dentin is the

cracked tooth syndrome. Cracked teeth are routinely encountered in dental practice regardless of age, and often result in fracture.[38, 39, 40] Patients often experience discomfort associated with chewing and unexplained sensitivity to cold.[8] Teeth that are fractured are challenging to treat due to the difficulty of obtaining a clear diagnosis.[41] Macrography, transillumination, staining with dyes, diagnostic surgery, microscopy, and a cone-beam computed tomography scanning are all used to confirm the presence of cracks.[42] However, none of these techniques can reveal cracks easily or with perfect certainty.[38] Even if a crack is identified, it is hard to know how far the crack has extended. Due partly to the challenges of diagnosis, there is not a universally accepted approach for repairing cracks in teeth.[43]

The crack tooth syndrome was identified as incomplete fracture in vital teeth that involves dentin. According to the American Association of Endodontists, five types of cracked teeth are identified and ordered by the magnitude of severity, namely the craze line, fractured cusp, cracked tooth, split tooth and vertical root fractures.[44] Craze lines are tiny cracks that only affect the outer enamel surface and usually cause no concern except for aesthetic appearance. Fractured cusp refers to chipping of the tooth that does not involve the pulp. Fractured cusp usually causes minor pain and sensitivity, which can be treated with a filling or artificial crown. Cracked tooth, on the other hand, means a crack that extends from the occlusal surface vertically toward the root. The treatability of a cracked tooth via root canal therapy or artificial crown relies on whether the crack propagates over the gum line. A long-term progression of a cracked tooth can result in a split tooth. Endodontic surgeries sometimes can save a portion of the tooth but extraction is usually necessary. Last but not the least, vertical root fractures begin in the root and extend upwards. Infection of the pulp, gum or surrounding bones is usually associated with vertical root fractures and results in complete removal of the tooth.

Previous studies on aging and mechanical behavior have focused on coronal dentin. Nonetheless, vertical root fractures (VRF) are amongst the most difficult cracks to treat and involves radicular dentin (Fig. 1.5). VRF is defined as a complete or incomplete fracture from the root at any level, propagating vertically along the tooth.[45] They are usually oriented buccolingually. Root fractures present the greatest challenge to the practicing dentist due to the difficulty of identifying them visually, and the limited number of treatment options.[46, 47, 48, 49]

Radicular dentin differs from coronal dentin by the ratio of area occupied by the dentinal tubules and the collagen matrix.[50] Multiple studies report that the root has lower dentinal tubule density when compared to the crown, indicating higher percentage of intertubular dentin per unit volume of tissue, and a larger volume fraction of matrix. Mjör & Nordahl compared the tubule density and degree of branching within the crown and root of human teeth.[51] The crown exhibits significantly greater tubule density than the root, and the number of fine tubule branches (0.3 to 0.7 mm diameter) was highest in the root, particularly near the apices and in regions of low primary density. Camargo et al. divided the roots into cervical, middle, and apical thirds and concluded that the density decreased with increasing depth from the cervix to the apices.[52] The large spatial variations in microstructure could result in differences of the mechanical properties along the root length. In fact, experiments suggest that radicular dentin has larger tensile strength, fracture toughness and endurance limit than coronal dentin, which could be attributed higher fraction of organic matrix.[53] There are investigation focusing on tensile strength of root dentin involving both bovine and human teeth. Sano et al. found that the tensile strength of radicular dentin was greater than that of the crown.[54] Inoue et al. reported similar findings but noted that samples with axial and circumferential orientations of the root exhibited significantly different strengths despite having nearly identical tubule

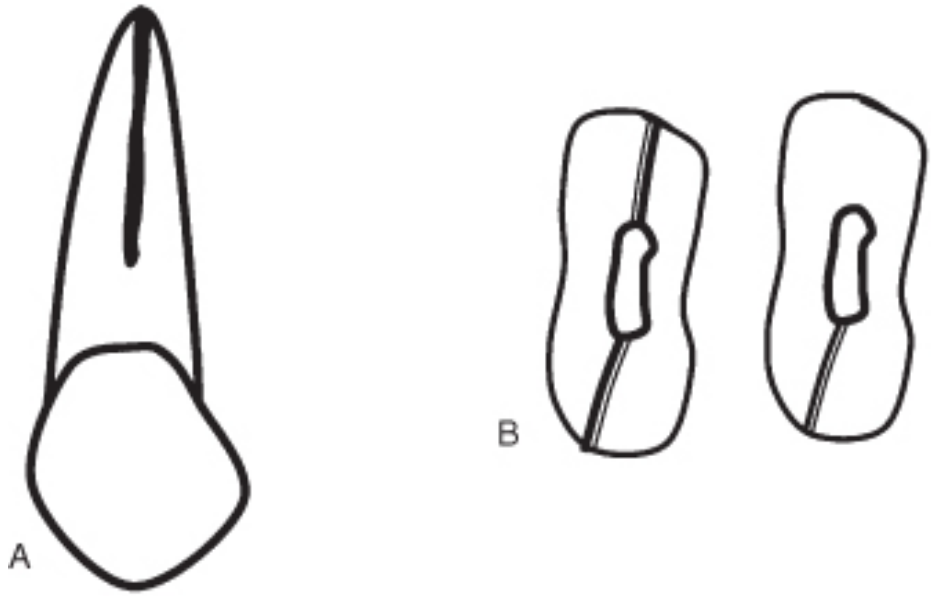

Figure 1.5: Schematic of vertical root fracture. Copyright 2020 Elsevier

orientation.[55] Their results were supported by a discussion of the dentin matrix, hypothesizing that the highest strength in the axial direction of the root (which possess a perpendicular tubule orientation) stemmed from the preferential orientation of collagen fibers in that direction.[56]

Natural physiological aging is not the only concern in root fractures. Teeth with non-vital pulp and those that have had the pulp removed during RCT are more susceptible to fracture than vital teeth.[57] In facts, VRF occurs more frequently in teeth with prior RCT.[58] There is a belief that VRFs initiate from internal dentin cracks[59] that have resulted from the RCT process. Indeed, dentin defects introduced during the endodontic procedures could be a contributing factor.[60, 61, 62] However, previous studies have also been reported that no defects could be identified within the dentin of teeth subjected to RCT and/or their contribution to VRF is unlikely.[63, 64] Clearly the cause of VRF and how potential changes in microstructure of dentin

associated with RCT contributes to the process of failure are controversial and remain unclear.[65] It is also unclear if the RCT process interferes with the natural process of aging and the corresponding rate of degradation in the resistance to fractures.

1.4 Objectives

Many factors are associated with dentin fractures. The objective of this study is to provide new fundamental knowledge concerning the change in microstructures and mechanical properties of radicular dentin as a function of chronological aging and as a result of clinical treatments. The investigation was designed with following objectives:

Objective 1

To evaluate the age-dependence in mechanical properties and microstructures of radicular dentin with respect to the organic and inorganic components of dentin.

Objective 2

To evaluate the changes of mechanical properties and microstructures related to root canal therapy and their relation to dehydration shrinkage.

Objective 3

To investigate odontoblast apoptosis and its contribution to age-related dentinal sclerosis.

Objective 4

To determine whether cryopreservation alters the microstructure of dentin and causes a reduction of its resistance to mechanical failures.

Chapter 2

REDUCTION IN FRACTURE RESISTANCE OF THE ROOT DENTIN WITH AGING

2.1 Introduction

The higher incidence of cracked teeth in senior patient has raised concerns about the diminishing fracture resistance of oral hard tissues, especially in dentin. Vertical root fractures (VRF) are amongst the most difficult cracks to treat. According to the American Association of Endodontists, a VRF is defined as a complete or incomplete fracture from the root at any level, propagating vertically along the tooth.[45] They are predominantly oriented buccolingually[66] and extended from a surface to include the root canal.[67] VRF usually extend throughout the longitudinal axis of root all the way to apex as shown in Fig. 2.1. Root fractures present the greatest challenge due to the complexity of identifying them visually, and the limited number of treatment options.[46, 47, 48, 49] Although VRF are primarily occur in endodontically treated teeth, non-endodontically treated teeth also appear to have fracture in root[68] and mostly involves mandibular molars.[69]

There is a strong positive correlation between patient age and teeth fractures, especially VRF. According to a demographic analysis on 227 teeth,[10] nearly 90% of all root fractures occurred in patients older than 40 years; the correlation with age was significant ($p < 0.0001$). The factors associated with higher VRF rate include that older patients are more likely to have had extensive operative or endodontic procedures and their teeth have had more years of physical use. Nonetheless, research also showed that a majority of VRF involved in non-endodontically treated teeth occurs in senior

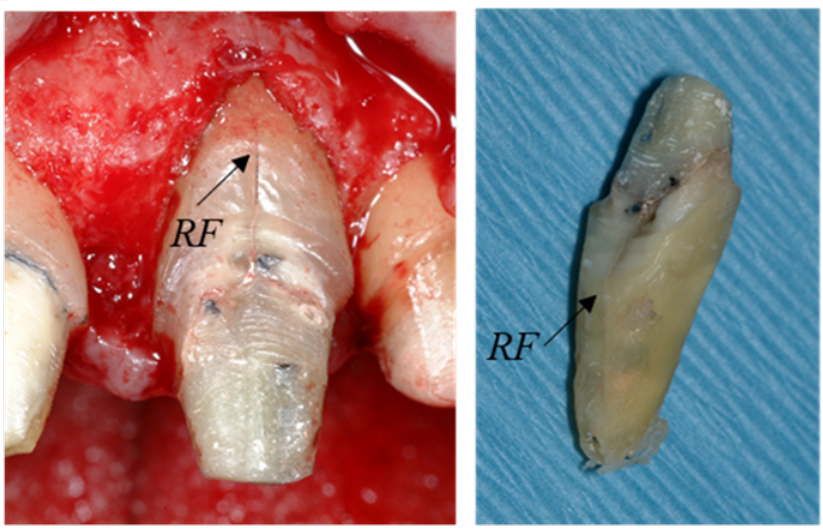

Figure 2.1: Root fracture originating from the apex in tooth #9 of a 69-year-old male patient.

patient.[68] This raises the question: Is the degradation in the mechanical properties of dentin with aging another key factor to consider in VRF?[70] It has not been considered in past discussions of VRF.

Previous studies on aging and mechanical behavior have focused on coronal dentin. Evaluation of the flexural strength of coronal dentin revealed that there is a decrease in strength of almost 20 MPa per decade of life that begins shortly after reaching adulthood.[71] With young and old groups defined as age $\leq$30 and age $\geq$55, respectively, there is a 50% reduction in strength of dentin between the young and old age groups. The ratio of the apparent endurance limit to the ultimate strength in flexure for young and old dentin was 0.5 and 0.38, respectively. The ratio of these two critical strengths distinguishes the relative fatigue resistance of a material and the smaller ratio for old dentin emphasizes its lower resistance to fatigue in comparison to young dentin. Similar investigations have shown that there is also a reduction in the fatigue

strength of dentin with age. In a study limited to coronal dentin from third molars, the old tissue exhibited significantly lower fatigue life regardless of the magnitude of cyclic stress.[33]

Dentin undergoes a gradual transition to increasing translucency with age, and is regarded as sclerosis.[23, 22] Sclerotic transparency becomes evident from the third decade of life in the root apex. However, this microstructural change is not homogeneously developed with increasing age. Sclerosis begins in the apical dentin (at the root tip) and spreads towards the crown.[18] As such, it is reasonable to speculate that dentin of the tooth root is more susceptible to the aging related degradation than coronal dentin as a result of sclerosis. Moreover, in the plane transverse to root pulp canal, sclerosis begins in the mesial and distal quarters and then spreads to the buccal and lingual quarters,[23, 24] so the sclerotic zone forms a butterfly shape.[26] The process sequentially extends to the crown.[22] The distribution and extension of sclerosis imply that occlusion of the dentinal lumens does not start simultaneously in the root and crown (Fig. 2.2). The occurrence and development of sclerotic dentin in the root and the cause of a butterfly-shaped sclerosis pattern were hypothesized to relate with the odontoblast apoptosis in the root canal.[31] A detailed discussion of the apoptotic effects on sclerotic dentin will be included in Chapter 7.

Based on the spatial distribution of dentin's microstructures introduced from Chapter 1, it appears that radicular dentin is more susceptible to aging than coronal dentin, or that it undergoes more rapid aging than in the crown. Therefore, the aim of this investigation was to evaluate the age-dependence in strength and fatigue properties of radicular dentin. The null hypothesis was that there is no decrease in strength or fatigue resistance with age.

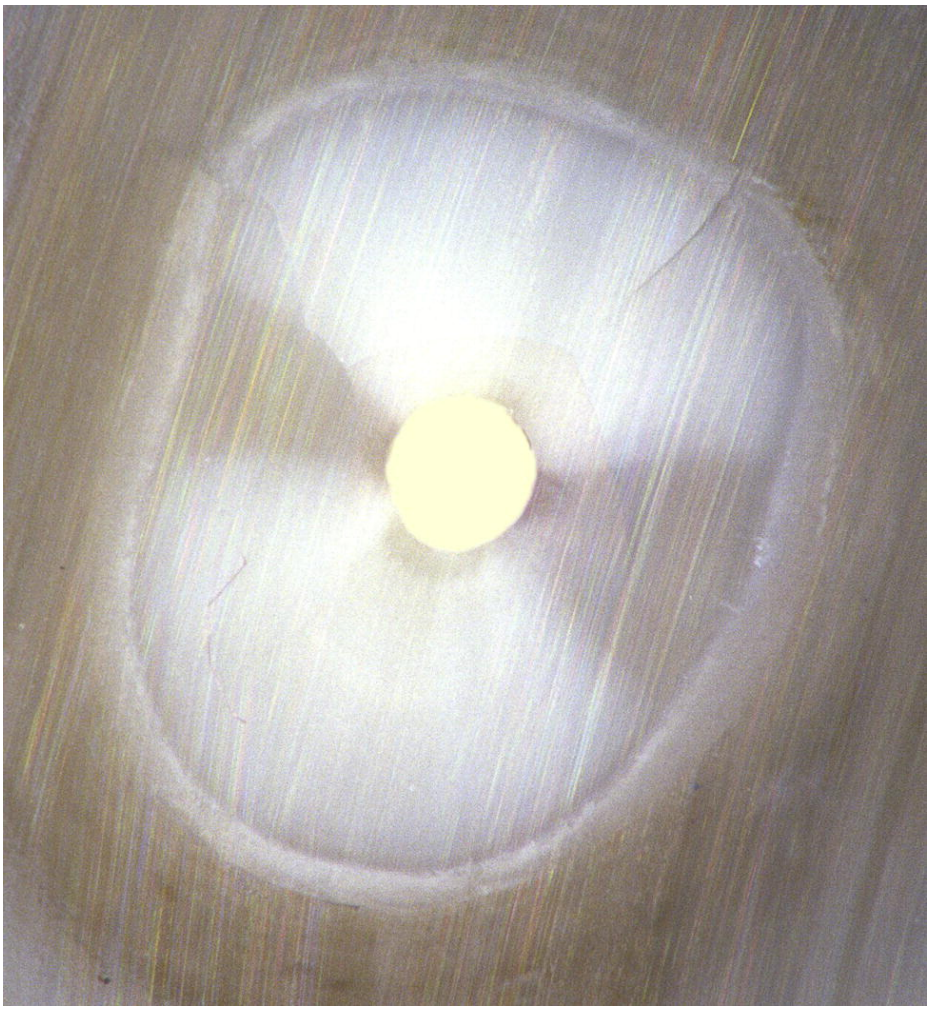

Figure 2.2: A root section under a light microscope showing the butterfly effect.[26] Copyright 2020 Elsevier.

2.2 Materials and Methods

Human single-rooted noncarious teeth were obtained from participating clinics with written consent and following a protocol approved by the Institutional Review Board of the University of Washington. Immediately after extraction, the teeth were placed in Hank's balanced salt solution and stored with record of donor age and gender. The teeth were sectioned using previously described methods[72] to obtain beams with rectangular cross-section (0.5×1.5 mm) and 8 mm length from the root in either the mesial-distal (M-D) or buccal-lingual (B-L) quarters as shown in Fig. 2.3A. Briefly, The teeth were cast in a polyester resin foundation and uniform sections of 1.5 mm thickness were obtained along the mesial–distal or bucccal–lingual axis using a numerically controlled slicer/grinder with continuous flood coolant. Primary slices were obtained equidistant from the mesial–distal and buccal–lingual aspect using diamond impregnated slicing wheels (#320 mesh abrasives). Each of the sections was mounted to a glass sheet with temporary adhesive for secondary sectioning. The dentin tubules were oriented perpendicular to the longitudinal axis of the beams. After machining, the beams were stored in Hank's balanced salt solution at 22°C for fewer than 7 days.

In evaluating flexure strength, 48 beams were obtained from 25 different donors in the B-L quarters and 54 beams were obtained from 21 different donors in the M-D quarters. According to the strength distribution, results from monotonic loading were further evaluated according to young (age ≤ 30, mean $= 23 \pm 5$ years) and old (age ≥ 55, mean $= 61 \pm 5$ years) groups. These age group definitions are also consistent with a previous study of dentin.[71] For cyclic loading, 57 beams were obtained from 41 donors in the B-L quarters, including the young (26) and old (31) groups. Similarly, 62 beams were obtained from 39 donors in the M-D quarters, including the young (33) and old (29) groups, respectively. Strengths for the two age groups were compared

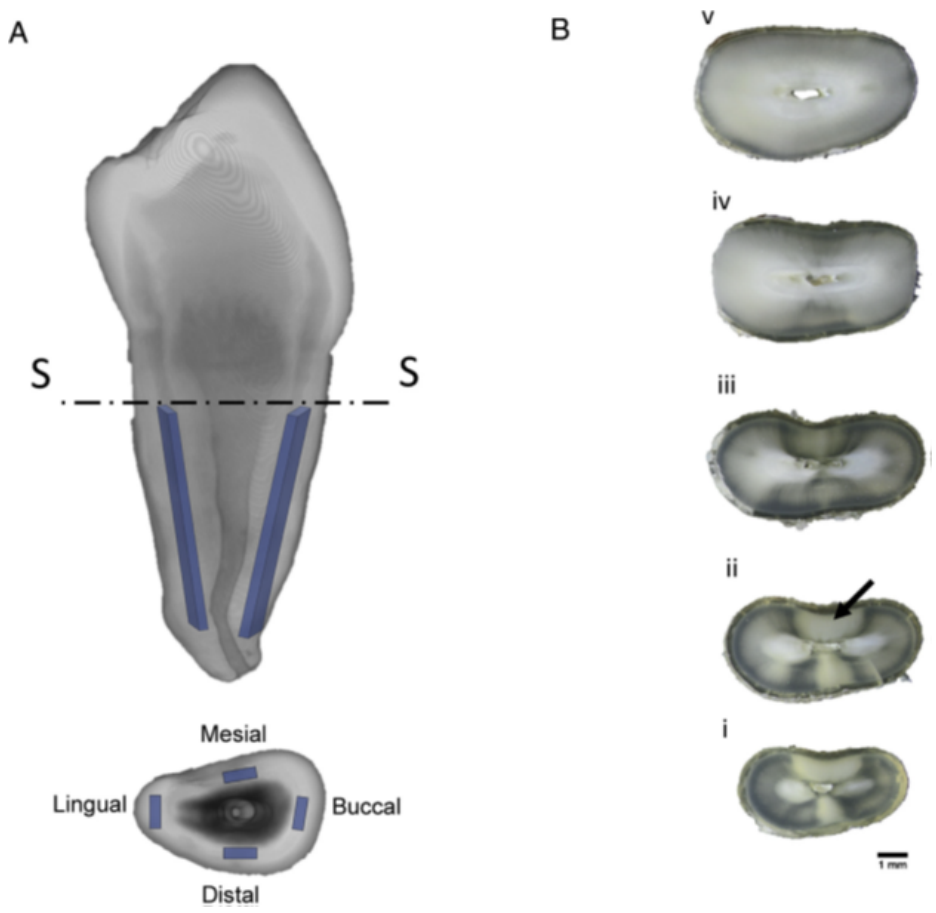

Figure 2.3: Details of specimen locations and spatial variations in sclerosis. (A) A micro–computed tomography image of a representative tooth with locations where the specimens would be obtained in the buccal-lingual quarters. Note that the root is cross-sectioned at S-S to show the positions of the specimens over the wall thickness in the buccal-lingual andmesial-distal quarters. (B) Cross sections of an incisor from a 65-year-old patient viewed under optical microscopy. Each section from i to v is obtained in increments of 2 mm from the previous and beginning at the apex. The typical "butterfly" effect is seen prominently in the first 3 sections from the apex (i–iii) and then is no longer evident after the middle third of the root. The bar length represents 1 mm and the pointer highlights the sclerotic region where evident.

using an unpaired Student t-test and the critical value (alpha) was set at 0.05.

The dentin specimens were loaded in 4-point flexure to failure using either monotonic or cyclic loading according to a scaled version of ASTM D790M for four-point flexural testing of materials with 1/3 load span arrangement.[71] All experiments were conducted using an EnduraTEC Elf Model 3200 testing system, which has a load capacity and sensitivity of 225 N and 0.001 N, respectively. All specimens are fully immersed in a HBSS bath (22°C) to maintain hydration during testing. Monotonic flexure testing was performed using displacement control loads to failure with a crosshead rate of 0.001 mm/s. The rate was established from preliminary experiments and resulted in a reasonable test duration for the range in bend displacements required for failure.[71] The load at failure and beam geometry were used to estimate the bend stress according to conventional beam theory

$$\sigma_f = \frac{FL}{bd^2}$$

where σ_f is the stress at the tensile surface at midpoint, F is the load at given point on the load-displacement curve, L is length of supporting span, b is the width of the testing beam and d is the thickness of the testing beam. Similarly, the bend strain in the dentin beams was estimated in terms of the four-point flexure arrangement and load-line displacement assuming a constant radius of curvature

$$\epsilon_f = \frac{4.76Dd}{L^2}$$

where ϵ_f is strain at the tensile surface at midpoint, D is the deflection at a given point on the load-displacement curve, L is the length of supporting span and d is the thickness of the beam. The flexural response of the dentin beams under quasi-static loading to failure was examined in terms of stress–strain diagrams and the flexure

strength was determined from the maximum bend stress.

In examining the fatigue properties of dentin, the beams were subject to load control actuation using a stress ratio (R) of 0.1. A loading frequency of 5 Hz was used to balance concerns associated with the rate of mastication and the duration of time required to complete the individual tests. Fatigue loads were selected to achieve a maximum cyclic stress between 30% and 95% of the flexure strength for that age group. The fatigue life distribution of dentin was modeled using the power law model

$$\sigma = AN^B$$

where A and B are the fatigue life coefficient and exponent, respectively, σ is the stress amplitude and N is the number of cycles to failure. The fatigue endurance limit was estimated for both young and old dentin by the cyclic stress amplitude at which the number of cycles to failure reached a horizontal asymptote. If not directly evident from the fatigue life diagram the equivalent endurance strength was estimated from the power law model at 10^7 cycles.

The microstructural characteristics of the dentin were examined quantitatively using a scanning electron microscope (model JSM- 6010PLUS/LA; JEOL, Peabody, MA) as a function of distance from the apex. Three young (mean = 18 $\pm$ 3 years) and 3 old (mean = 77 $\pm$ 8 years) single-rooted teeth were embedded, polished, and coated following established procedures.[73] Briefly, The specimens were embedded in cold-cured epoxy resin. The exposed dentin in the resin mount was polished using silicon carbide abrasive paper with successive smaller particle sizes until reaching 4000 grit. Further polishing by means of standard red felt polishing cloth wheels was then performed using diamond particle suspensions of 3 μm in size. After polishing, all samples were ultrasonically cleaned in an HBSS bath for 30 min before microscopic observation in order to eliminate particles of the diamond particle suspension or tissue

resulting from the polishing process. The polished specimens were then kept in a HBSS bath solution prior to testing. Examinations were performed 2 mm from the apex and continuing toward the crown in 2-mm increments as described in Fig. 2.3B. At each distance, 7 images were obtained at 500× in each of the 2 regions (i.e., within 1 mm of the canal and 1 mm from the cementum). In each image, the number of total tubules and occluded lumens were counted using a commercial software (ImageJ 1.8.0, freeware; National Institutes of Health, Bethesda, MD). The ratio is expressed as the occlusion ratio and average values were obtained at each location, and for the 3 teeth in each age group, to describe the spatial distributions.

An analysis of the chemical composition of the radicular dentin was performed using Raman spectroscopy (Renishaw InVia, West Dundee, IL) as a function of distance from the tooth apex. The results were used to determine the collagen cross-linking ratio. Five young (mean = 26 ± 5 years) and five old (mean = 57 ± 1 years) dentin beams obtained from freshly extracted teeth were scanned over the spectral range of 400 to 1900 cm^{-1}. A laser with 785 nm wavelength was focused onto the sample for 10 seconds through a Raman fluorescence microscope (objective 50×; Leica, Buffalo Grove, IL) with a resolution of 10×50 mm. The spectrum was acquired at distances of 2 mm away from apex and in 2 mm increments along the longitudinal direction of each tooth. The acquired Raman spectra were baseline corrected using WiRE 3.4 (Renishaw) to account for fluorescence. The cross-linking ratio was calculated from the ratio of area under pyridinoline peak at 1660 cm^{-1} and dehydrodihydroxylysinonorleucine peak at 1690 cm^{-1} according to Paschalis et al.[74] Measures of the occlusion ratio, and chemical composition from the 2 age groups were compared over the distance from the apex using a 2-way analysis of variance and the critical value (alpha) was set at 0.05.

2.3 Results

The strength distribution of the radicular dentin beams from the B-L and M-D quarters is presented in Fig. 2.4. There is a significant decrease ($p \leq 0.0001$) in strength with age, with average ($\pm$ std dev) of 199 ± 36 MPa and 122 ± 11 MPa for the young and old groups, respectively. However, no significant difference of flexure strength is observed between B-L and M-D quarters.

The fatigue strength of radicular dentin from donors within the young and old groups is shown in Fig. 2.5; the old group exhibits significantly lower fatigue strength than the young group ($p \leq 0.001$). Defined at 1×10^7 cycles, the apparent endurance limit for the young and old groups is 43.5 MPa and 29.5 MPa, respectively. According to a Rank Sum analysis, there was no difference between the B-L and M-D quarters for the young dentin. For the old group, results for the B-L and M-D quarters were significantly different ($p \leq 0.05$).

Results for the occlusion ratio are presented in Fig. 2.6. Specifically, the ratio is shown for regions close to the pulp and close to the cementum in Fig. 2.6A and 2.6B, respectively. For the young donor group, the ratio of filled lumens is almost constant throughout the longitudinal axis of tooth, with higher occlusion ratio in the region close to the cementum ($p \leq 0.05$). There is a significant increase ($p \leq 0.001$) in the number of filled lumens (and occlusion ratio) with age over the entire root length. The largest occlusion ratio was found within the apical third and closest to the pulp canal. Near the cementum, the increase in occlusion ratio was lower, but it is significant nonetheless.

The changes in the mineral-to-collagen ratio with age is shown as a function of distance from the root apex in Fig. 2.7A. There are significant increases ($p \leq 0.05$) with age and the greatest degree of increase occurs within the apical- and middle-thirds of the root. The difference occurring above 8 mm from the apex is not significant

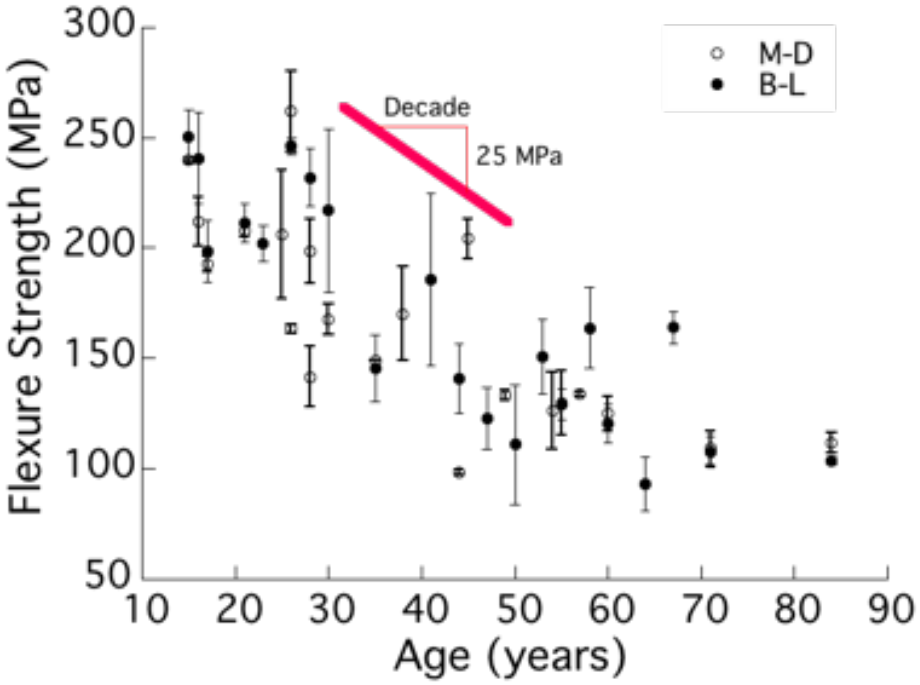

Figure 2.4: Flexure strength of beams from the mesial-distal and buccal-lingual quarters as a function of donor age. The data points with vertical bars represent the average and standard deviation in strength of beams that were obtained from multiple teeth for that donor. The overall average strength and standard deviation for the young and old age groups were estimated from the results of all flexure tests that fell within the defined age ranges.

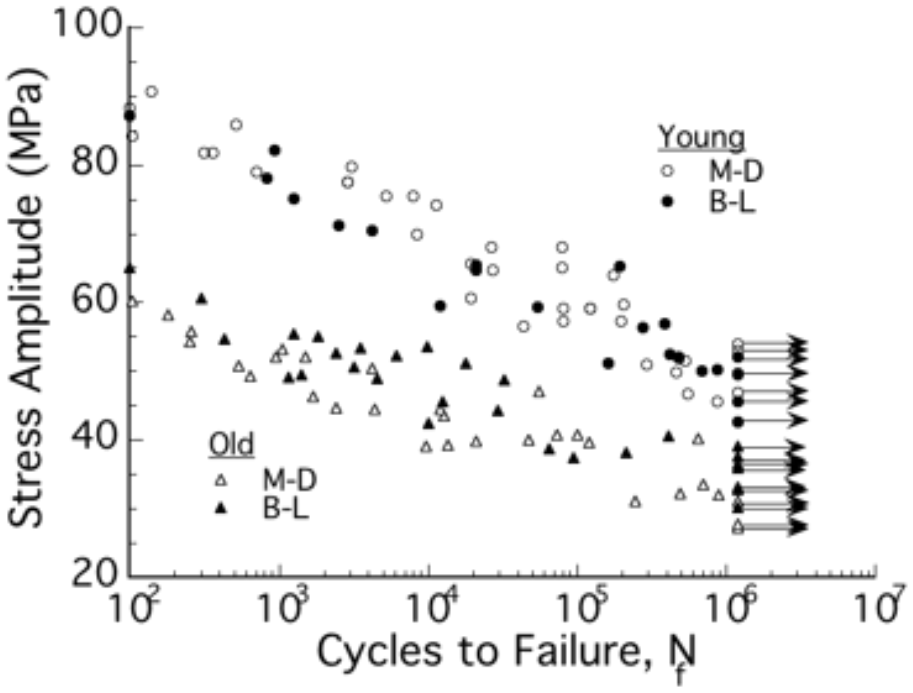

Figure 2.5: Comparison of fatigue-life distributions for the young (mean age = 22 years) and old (mean age = 58 years) mesial-distal dentin and old buccal-lingual dentin (mean age = 56). The data points with arrows at the far right of this figure represent specimens that did not fail within 1.2×10^6 cycles and the test was discontinued. B-L, buccal-lingual; M-D, mesial-distal.

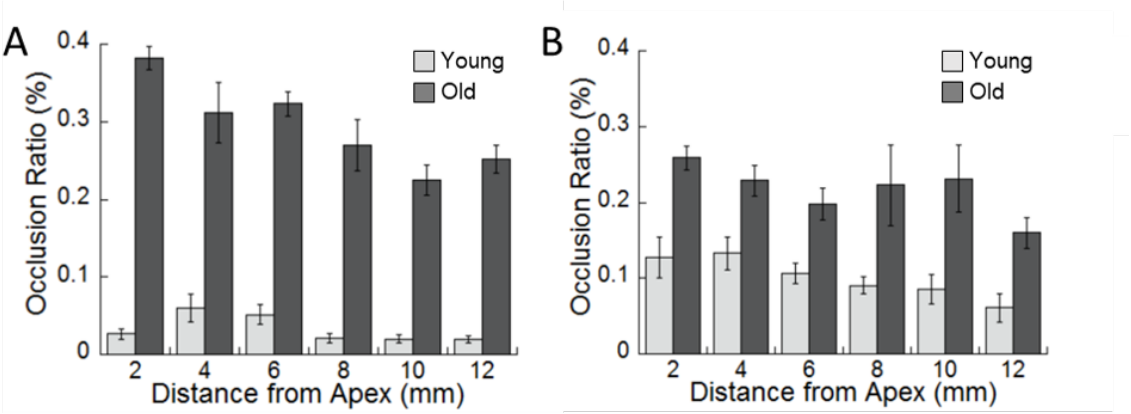

Figure 2.6: Spatial distribution in microstructure of the radicular dentin for the young and old age groups. (A) Occlusion ratio close to the pulp. This ratio represents the ratio of lumens filled with mineral relative to the total number of tubules (ie, occlusion ratio) as a function of distance from the apex for the young (mean age = 18) and old (mean age = 77) groups in the mesial-distal quarters. The occlusion ratio of the old dentin is significantly greater. (B) Occlusion ratio close to the cementum.

according to the statistical analysis. The changes in the cross-linking ratio is shown in Fig. 2.7B. The same trend is found, with significant increase as a function of age and most substantial changes close to the apex ($p \leq 0.05$).

2.4 Discussion

Results from monotonic and cyclic loading demonstrated that there were significant reductions in strength of radicular dentin with age. The average reduction in strength exceeded 25 MPa per decade and was essentially linear between 20 and 60 years of age (Fig. 2.4). There was nearly 40% reduction in average strength from the young to the old age groups. Similarly, the apparent endurance limit decreased by over 30% from the young to the old age group. These findings require rejection of the null hypothesis.

Earlier studies on coronal dentin have shown that a substantial reduction in

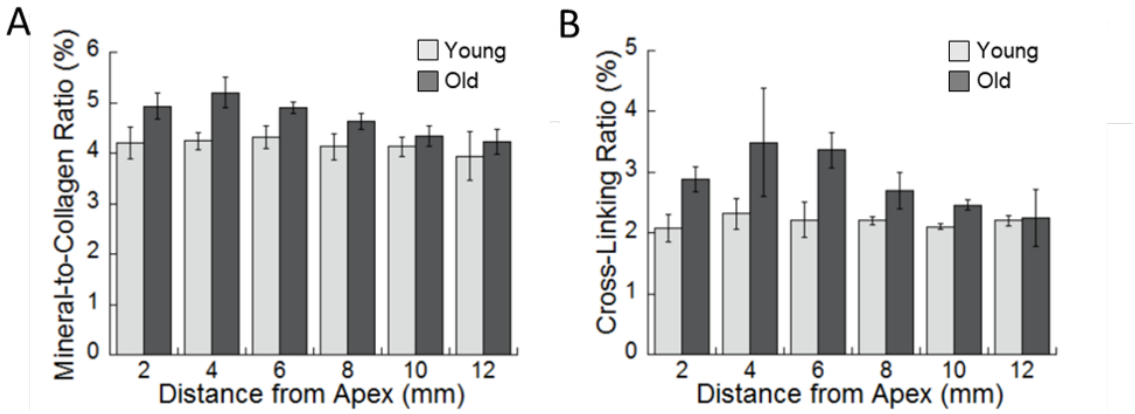

Figure 2.7: Spatial distribution in chemical composition of the radicular dentin for the young and old age groups. (A) Mineral-to-collagen ratio close to the pulp. This ratio represents the ratio of lumens filled with mineral relative to the total number of tubules (ie, occlusion ratio) as a function of distance from the apex for the young (mean age = 18) and old (mean age = 77) groups in the mesial-distal quarters. The occlusion ratio of the old dentin is significantly greater. (B) Collagen cross-linking ratio.

strength,[71] fatigue crack growth resistance,[27, 37] and fracture toughness occurs with aging.[35, 36] However, there has been limited focus on the mechanical properties of radicular dentin in comparison to the crown,[53] which is largely due to the limited tissue available and added complexity caused by the root shape. Previous work has examined the strength and fatigue response of the root, but involved bovine dentin.[55] Shinno et al. recently reported that there is a significant reduction in strength of tissue from the root and crown with increasing advanced glycation end-products, which is associated with aging.[75] However, that study did not examine the fatigue response.

In a previous evaluation of coronal dentin the flexure strength decreased from roughly 150 MPa to 80 MPa over the period of aging between the young and old groups.[71] The endurance limits of the coronal dentin reported for these two age

groups were 44 MPa and 23 MPa, respectively. These responses were obtained with exactly the same specimen size, evaluation conditions, and tubule orientation. There are two important features that become evident in the comparison. First, the apparent strength of radicular dentin under static loading is greater than that of the crown by a factor of 1.3 or greater, which agrees with earlier studies.[54, 76] Second, despite the greater flexure strength, the fatigue strength of radicular dentin is nearly equivalent to that of the crown. The endurance limit of young coronal and radicular dentin is approximately 44 MPa. Hence, the lower ratio of fatigue to static strength of radicular dentin (0.22 vs. 0.3) suggests that it is less effective at resisting the growth of damage under cyclic loading. This quality is relevant to damage that may be introduced during treatment[77, 62] as well as the contributions of compaction and mechanical cycling to the incidence of VRF.[63] Further work is necessary to understand how the microstructure of radicular dentin contributes to this behavior.

The occlusion ratio measurements in Fig. 2.6 show an interesting trend in the evolution of the root microstructure. Clearly, there is a significant increase in the number of filled tubules with aging along the entire length of the root, and the increase in occlusion ratio is greatest at the root apex. In the young donor teeth, the occlusion ratio is greatest near the cementum. However, the greatest number of filled lumens in the old group was close to the pulp, not near the cementum (Fig.2.6B). That contradicts the occlusion process in the crown where the tubules become filled first peripherally and the process continues towards the pulp.[73]

Despite the large increase in number of filled lumens with mineral along the entire root length, the increase in mineral to collagen ratio was limited primarily to the apical and middle thirds (Fig. 2.7A). The spatial distribution in this measure agrees well with the evidence of sclerosis in the sectioned roots (Fig. 2.3B), and the small degree of sclerotic dentin in the coronal third. Surprisingly, the changes in relative

mineral content were also limited. The trends in cross-linking along the root were quite different, with the largest increase occurring closer to the middle third than near the apex. Based on the distribution of the measurements in Fig. 2.7A and 2.7B, the increase in degree of cross-linking with age was more substantial than the increase in mineral to collagen ratio. That could imply that the increase in extent of collagen crosslinking is more important than the changes in mineralization to the degradation in mechanical properties with age.

In the young teeth, there were no significant differences in mineral to collagen ratio or collagen crosslinking ratio along the root length. There were spatial variations in both measures for the old age group, which implies that the rate of aging along the root length is not constant. That is a new and interesting concept. In addition, the trend in the most visible change in microstructure, namely the filling of lumens with mineral, did not correspond with the distribution in chemical composition related to either the mineral or the collagen. Apparently, the current understanding of aging in the root is rather limited. The overall design of endodontic treatments has been based on the concept that the dentin has similar properties along the entire length of the root. According to these preliminary results, that does not seem to be true in senior patients.

Although the research was motivated by a greater incidence of VRF in the teeth of seniors, the study was not performed on teeth that received root canal treatment. The calcified tissues of pulpless teeth reportedly have a lower degree of hydration than vital teeth,[78] and both the strain to fracture and fracture energy are significantly reduced for dehydrated dentin.[79] The increased brittleness of pulpless teeth has been associated with the effect of dehydration on the dentinal tubules.[80] Nevertheless, tooth age was not considered in the aforementioned studies, and an examination of root canal–treated teeth is warranted. The aging effect on root canal treated teeth is

studied and discussed in Chapter 3 and dentin dehydration with diminished fracture resistance of root is presented in Chapter 4.

Most importantly, while the age-related changes in the microstructure and chemical composition took place in the apical third of the root, the flexure strength and fatigue strength were evaluated using specimens that subjected the middle third of the root to the largest stress. According to the variations in the microstructure and chemical composition (Fig. 2.6 and 2.7), the age-related degradation in the strength and fatigue resistance could underestimate changes at the apex. An assessment of the spatial variations in degradation, and its importance to vertical root fractures will require further investigation. A detailed spatial distribution of mechanical properties in dentin was studied by scanning based nano dynamic mechanical analysis using commercial nanoindenter in Chapter 5.

2.5 Conclusion

Within the limitations of this investigation, the results showed that there are significant decreases in the strength of root dentin and its resistance to fatigue with increasing patient age. The endurance limit of radicular dentin is similar to coronal dentin. The primary period of reduction spans over the ages from 30 to 60, which agrees with results of previous study. The occlusion ratio, mineral-to-collagen ratio and collagen crosslinking ratio are significant higher in aged dentin. There is a spatial variation in the rate of lumen occlusion ratio along the root length, and particularly between the area close to the pulp and close to the cementum. The degradation in properties appears to result from a combination of changes in the microstructure and chemical composition, but additional work is required to identify the specific contributions.

Chapter 3

CONTRIBUTION OF ROOT CANAL TREATMENT TO THE FRACTURE RESISTANCE OF DENTIN

3.1 Introduction

In addition to the effects of aging,The contribution of RCT to VRF in teeth is of equal relevance.[49, 81] Teeth received RCT experience VRF more often than unrestored teeth.[16, 17] Although the survival rates can be initially high for endodontically treated permanent teeth,[82, 83] the risk of fracture increases with time are generally associated with cracks that initiate in the apical region.[58]

The higher risk of fracture in teeth after RCT has been attributed to the loss of structure and increase in stress.[57] Indeed, iatrogenic dentin removal is inevitable in RCT and can significantly lower the load to fracture in radicular dentin due to loss of effective volume.[84] The fracture behavior of the root largely depends on the remaining thickness of dentin[85, 86], and potentially on the properties of the intracanal post.[87] Lang et al. noted that all endodontic treatments involving the removal of hard tissue from the canal increased the deformability of the root and caused a reduction in the stability of the tooth.[88] In addition, for teeth requiring endodontic treatment that also receive a direct restoration, the degree of deflection and tooth strain increase significantly with the loss of axial dentin.[53] In addition, the use of irrigants and the pressure of gutta-percha condensation can also contribute to the weakening of root filled teeth.[89, 90]

The contribution of defects and flaw generated from the endodontic procedure is another factor to consider.[91] Internal pressure during lateral condensation can

introduce flaws that later undergo cyclic crack growth.[92] Rotary and reciprocating instruments also cause dentinal defects.[60] Such flaws could facilitate fatigue or fracture via cyclic crack growth, both of which would increase the rate of VRF. Shemesh et al. showed that dentinal defects (fractures, craze lines or cracks) were introduced during RCT.[77, 62] Barreto et al. found that instrumented teeth subject to apical pressure filling techniques and mechanical cycling underwent VRF.[63] In addition, rotary and reciprocating instruments cause dentinal defects[60] that undergo mechanical cycling during apical filling of the root and later under function. Many of the most popular procedures applied to RCT have shown negative impact on mechanical integrity of dentin.[93] Nonetheless, the same treatment procedures are used in RCT regardless of patient age. Given the reduction in strength and resistance to fatigue, senior patients receiving endodontic treatment are potentially far more vulnerable to VRF.

Despite the abundant evidence of dentinal crack associated with root canal preparations, there has been great controversy around whether the RCT can truly induce microcracks and whether those microcracks can actually cause any root fractures *in vivo*.[94] De-Deus et al. showed that there is a lack of casual relationship between microcracks and root canal therapy[64] because most previous studies has misleadingly identified cracks induced by post-extraction experimental phenomenon.[95] In addition, recent research has suggested that root canal preparation does not induce dentinal microcracks *in vivo*.[96] Most *ex vivo* micro–computed tomographic analysis only detect microcracks that preexisted in natural non-endodontically treated teeth that are part of the dentin microstructure.[97] Therefore, efforts have focused on the mechanical properties of dentin after RCT.

The possibility of dentin degradation in the dentin after root canal treatment is potentially of greater importance to consider in VRF after RCT.[98] Carter et al.

demonstrated a significant difference in the shear strength and toughness between vital and endodontically treated teeth.[99] Sornkul et al. reported that vital teeth have higher resistance to fracture than endodontically treated teeth under vertical and lateral forces.[100] Mireku et al. conducted fatigue testing on tooth roots to determine whether patient age contribute to the fracture resistance of teeth subjected to root canal treatment.[46] It was revealed that VRF in teeth that received RCT is more likely to occur in the teeth of older patients. However, Cheron et al. found that the elastic moduli and hardness of radicular dentin are not different before and after root canal treatment.[101] Similarly, Missau et al. reported that teeth receiving endodontic treatment and retreatment exhibit the same fatigue resistance when compared with untreated teeth.[102] Currently there is no well-established experimental analysis on the fracture behavior of dentin after RCT.

In this study the chemical composition, microstructure and strength of multiple teeth from selected donors were compared as a function of donor age and prior endodontic treatment. The null hypothesis was that there is no difference in the fracture resistance of unrestored teeth and those that have undergone endodontic treatment followed by clinical function.

3.2 Materials and Methods

Human teeth were obtained from participating oral surgeons according to an exempt protocol approved by the Institutional Review Board of the University of Washington. An inspection of the teeth was conducted at receipt for active caries or lesions, structural defects or prior restorations. Those with evidence of caries or defects were discarded. Between 6 and 13 teeth were obtained from each donor as described in Table. 3.1 and five of the donors possessed endodontically treated teeth. The teeth were immediately placed in Hank's Balanced Salt Solution (HBSS) and stored with

Table 3.1: Details concerning the sample distribution of evaluation. The investigation involved 52 total teeth obtained from 6 donors with age spanning from 46 to 74 years. A, anterior tooth; P, posterior tooth. Note that 56A and 56B represent the teeth of 2 different donors with the same age.

Age	Location		Treatment		Total
	A	P	Endo treated	Non-endo treated	
46	1	7	0	8	8
56A	5	8	5	8	13
56B	1	5	2	4	6
60	5	6	3	8	11
63	3	7	4	4	8
74	3	3	3	3	6
Total	18	36	17	35	52

record of tooth position, donor age and gender.

Those teeth selected for evaluation were cast in a polyester resin foundation and sectioned axially in the mesial-distal (M-D) direction using slow-speed diamond abrasive wheels and with continuous water irrigation. One half of each tooth was subjected to further sectioning following established methods[103] to obtain rectangular beams from the roots in the buccal-lingual (B-L) quarters as shown in Fig. 3.1A. After machining, the beams were stored in HBSS at 4°C for fewer than 2 days.

The dentin beams were subjected to four-point flexure to failure. The flexure loading apparatus consisted of a 1/3 span arrangement as shown in Fig. 3.1B. The experiments were conducted using a universal testing system (EnduraTEC Elf Model 3200; TA Instruments) with specimens immersed in a HBSS bath (22°C) to maintain

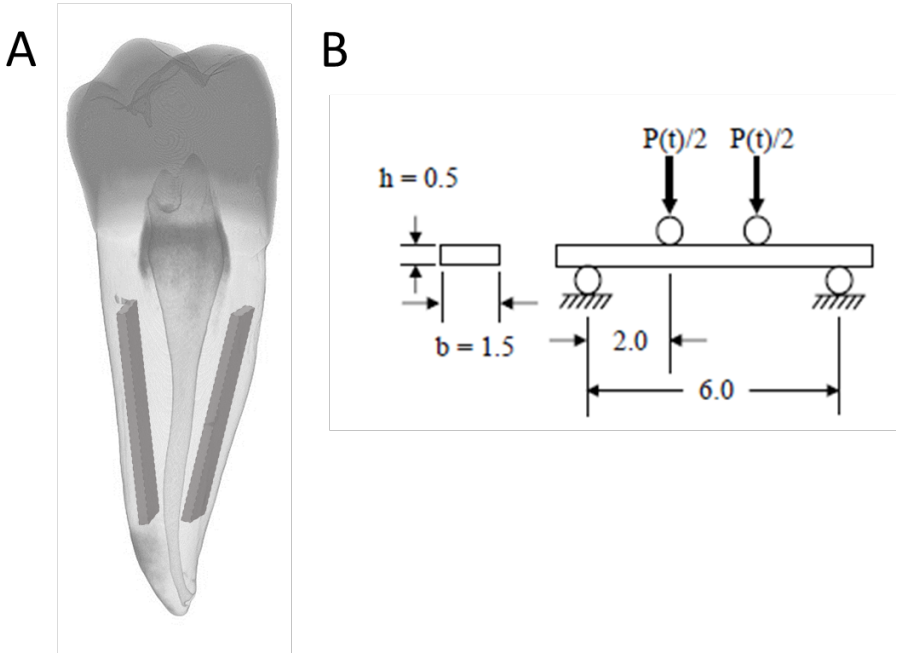

Figure 3.1: Methods of evaluating the strength and fatigue resistance in root dentin. (A) Beams were obtained from the buccal-lingual and mesial-distal regions of the teeth, and (B) the beams were subjected to 4-point flexure according to a 1/3 point loading arrangement.

hydration during testing. Quasi-static flexure was performed under displacement control loading to failure with a cross-head rate of 0.001 mm/s. The strength of each beam was determined from the maximum flexure stress to failure. The strengths of the endodontically treated and untreated dentin from the 6 donors were compared using a paired Students t-test and the critical value (alpha) was set to 0.05.

The microstructure of the radicular dentin was examined using a Scanning Electron Microscope (SEM; Model JSM- 6010PLUS/LA; JEOL, Peabody, MA) and image processing. The remaining half of each sectioned tooth was embedded in cold-cured epoxy resin exposing the root canal and longitudinal section. The exposed dentin in the resin mount was polished using silicon carbide abrasive paper from #800 to

#4000 mesh until the dentinal lumens became evident. Further polishing was performed using diamond particle suspensions to 3 μm in size. The total number of open lumens and the number of occluded lumens were counted using a commercial software (ImageJ 1.8.0, freeware; National Institutes of Health, Bethesda, MD). Results were expressed as the occlusion ratio, which is the number of occluded lumens to total number of lumens. Occlusion ratios of the endodontically treated and unrestored teeth were compared using an unpaired Students t-test and the critical value (alpha) was set to 0.05.

The chemical composition was analyzed using Raman spectroscopy (Renishaw InVia, West Dundee, IL) with scans performed over the spectral range of 400 - 1900 cm^{-1} and acquired at distances of 4 mm away from the root apex. The spectra were baseline corrected for fluorescence using WiRE 3.4. The crosslinking ratio was calculated from the ratio of area under pyridinoline (Pyr) peak at 1660 cm^{-1} and dehydrodihydroxylysinonorleucine (deH-DHLNL) peak at 1690 cm^{-1}. Mineral-to-collagen and collagen crosslinking ratios for the endodontically treated and untreated dentin were compared using an unpaired Students t-test with critical value (alpha) set to 0.05.

3.3 Results

The average flexure strength of the radicular dentin samples is presented for both the unrestored and root canal–restored teeth as a function of donor age in Fig.3.2. The average strength of radicular dentin reported by Yan et al.[103] for young donor teeth is also shown for reference (199 ± 36 MPa, age $\leq$ 30 years). All of the teeth from donors $\leq$ 55 years of age exhibited significantly lower strength ($P < 0.0001$) than that of young dentin. The average flexure strength of the unrestored dentin for the 5 donors identified as "old" is 150 ± 16 MPa, respectively. There is a general decrease

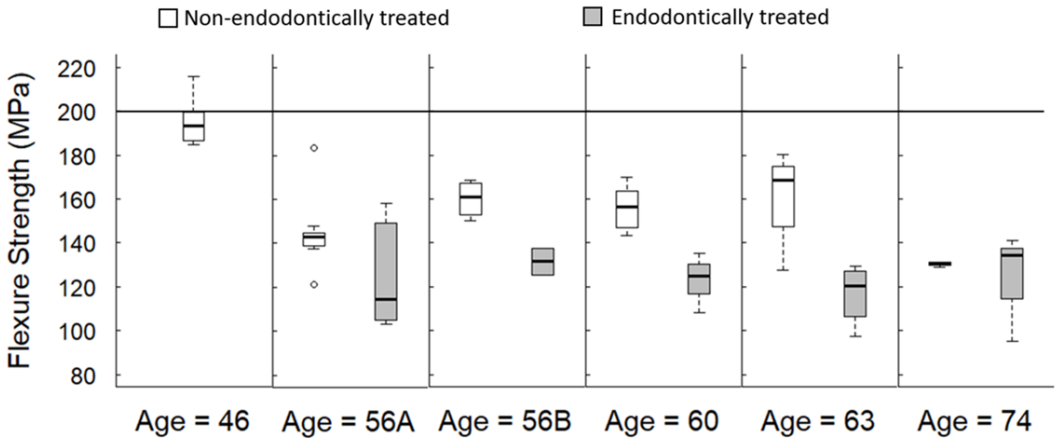

Figure 3.2: Flexure strength of root canal–treated and non–root canal–treated teeth in relationship to young radicular dentin. The average flexure strength of young radicular dentin (199.7 MPa) is shown for comparison based on Yan et al.[103]

in strength with increasing donor age for this group, and the strength for the oldest donor teeth was the lowest overall (130 ± 1.4 MPa).

For the root canal–treated teeth in Fig. 3.2, there is no trend in the strength of dentin with donor age. There was no significant difference in the strength ($P > 0.05$) of dentin from the root canal–treated teeth among the 5 donors. The overall average flexure strength of the root canal–restored dentin is 123 ± 18 MPa, which is significantly lower ($P < 0.0005$) than that of dentin from all the unrestored teeth, and also significantly lower than that of dentin from the unrestored teeth within the donor-matched dentition ($P < 0.05$).

A normalization of the flexure strength was performed for further analysis. Specifically, the flexure strength of dentin from all teeth evaluated was normalized by that from a non root canal-treated incisor (including tooth numbers 8, 9, 24, and 25) from the same donor. Results for the unrestored and the root canal-treated teeth are presented in Fig. 3.3A and 3.3B, respectively. In Fig. 3.3A, the strength of the

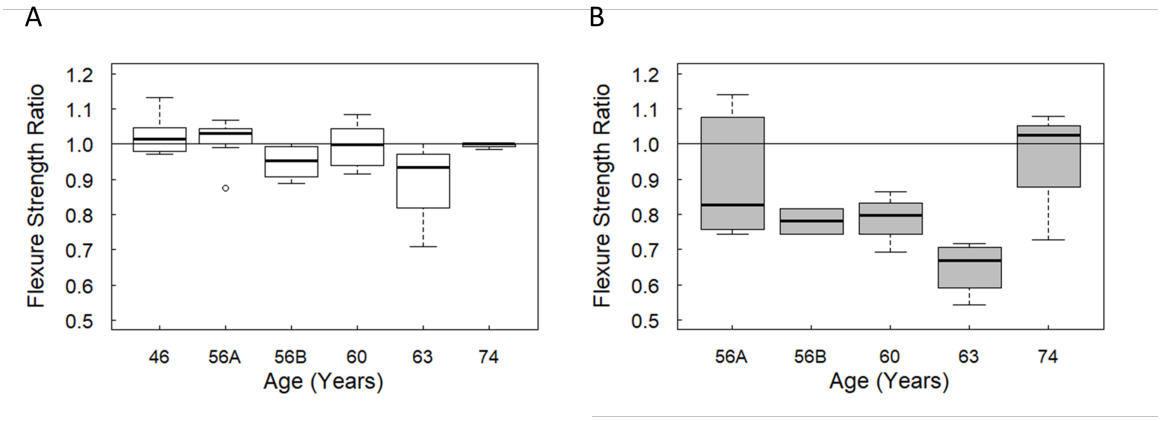

Figure 3.3: The ratio of flexure strength of dentin from the (A) unrestored and (B) root canal–restored teeth to the strength of radicular dentin from untreated incisors of the same donor.

unrestored posterior teeth is not significantly different from the incisor control. The overall average flexure strength ratio of root canal–treated teeth was 0.82 ± 0.17; the smallest ratio was 0.65 ± 0.08 and was obtained for the 63-year-old donor. For 4 of the 5 donors (56A, 56B, 60, and 63), the strength ratios of the posterior teeth were significantly lower ($P < 0.05$) in comparison with the healthy incisors. Interestingly, the root canal–treated teeth from the oldest donor did not show significant differences in strength from the unrestored incisors ($P > 0.05$) as evident in Fig. 3.3B.

Results of the chemical composition and microstructural analysis are shown in terms of the mineral-to-collagen ratio, the collagen cross-linking ratio, and the occlusion ratio in Fig. 3.4, respectively. There was no significant difference in the mineral-to-collagen ratio between the dentin of root canal– and non–root canal–treated teeth. However, dentin from the root canal–treated teeth exhibited a significantly greater collagen cross-linking ratio ($P < 0.005$) and a significantly lower occlusion ratio ($P < 0.025$).

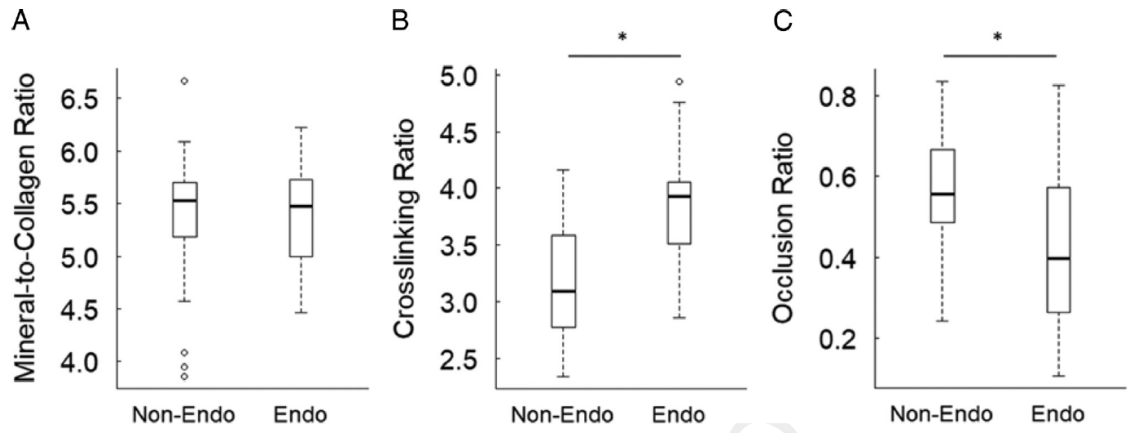

Figure 3.4: Results of Raman spectroscopy and microscopic analysis of dentin from the buccal-lingual quadrants and 4 mm from the root apex. (A) The mineral-to-collagen ratio, (B) the collagen cross-linking ratio, and (C) the occlusion ratio are presented in terms of prior root canal treatment. Endodontically treated teeth have a significantly higher cross-linking ratio ($P < .005$) and lower occlusion ratio ($P < .025$) than untreated teeth

3.4 Discussion

In this investigation, the chemical composition, microstructure, and strength of radicular dentin were evaluated from multiple teeth of randomly selected donors. A comparison of the properties performed using age- and donor-matched tooth pairs enabled an assessment of the importance of prior root canal treatment. The strength of radicular dentin from the unrestored teeth (150 ± 16 MPa) was significantly lower than that reported for young radicular dentin (199 ± 36 MPa) (Fig. 3.2) . Apart from the average strengths of dentin from teeth of the donor identified as 56A, there was also a general trend indicating a reduction in strength with increasing age, as expected. The maximum reduction in strength (35%) with regard to young dentin was exhibited by teeth from the 74-year-old donor. The variation in strength of the unrestored teeth was also the smallest for the 74-year-old donor. For radicular dentin, Yan et al.[103]

found the rate of degradation in strength is approximately 25 MPa per decade until reaching age 55 years, after which it reaches a plateau.

In contrast to the unrestored group, dentin from the restored teeth did not show age dependence (Fig. 3.2 and 3.3). Nevertheless, the average strength of the treated group was almost 20% lower than that for the unrestored group. A comparison of results in Fig. 3.2 show that, regardless of age, the strength of dentin from root canal–treated teeth is lower than that of unrestored teeth, which requires rejection of the null hypothesis. Thus, independent of the loss of tooth structure with instrumentation,[57] the strength of the root tissue decreases after root canal treatment. This is the first investigation to provide clear evidence of the changes in strength of dentin occurring after treatment and clinical function.

A major concern in previous studies on the properties of tooth tissues is the importance of patient/donor-specific oral conditions and health status. Indeed, this concern is highlighted in the strength of dentin for 2 different 56-year-old donors (Fig. 3.2). The average flexure strength for the unrestored teeth for these 2 donors is significantly different ($P < 0.05$). Microstructural characteristics such as lumen density[104] and collagen fibril diameter[76] are important. Dietary habits, use of medications, prior trauma, and even differences in masticatory habits are also plausible contributions. There is a reduction in the strength of dentin with age, but the extent of reduction is clearly patient specific. Normalizing the strength measurements with a reference from the same patient reduced the influence of patient-specific and potentially confounding factors as previously discussed. The merit of this approach is evident in Fig. 3.3A and B where all the unrestored and root canal–restored teeth were normalized to a healthy unrestored incisor. The unrestored teeth from 5 of the 6 donors have an average strength within 5% of that of the healthy incisor, indicating a very small variation in properties "within" the patient's dentition. For the root canal–restored teeth, 4 of

the 5 donors possessed strength that is 15% lower than that of the unrestored teeth, independent of the degradation related to age.

Carter et al. found that root canal–treated dentin is weaker and more brittle than vital dentin but did not establish the causes.[99] Prior studies suggest that root canal–treated teeth are more brittle because of dehydration,[105, 106] regardless of instrumentation. This is a controversial subject. For instance, Huang et al. conducted compression, indirect tensile testing, and impact testing on human dentin and showed no significant difference between the properties of dentin from treated and untreated teeth.[80] Additional evidence suggests that dehydration does not necessarily reduce the resistance to fracture[9] or fatigue crack growth[27] of dentin. Future work should focus on the changes in free and bound water in the dentin of root canal–treated teeth and their contribution to its mechanical durability.

Collagen cross-linking is considered a primary contributor to the degradation in fracture resistance of bone with age.[107] In comparing the root canal–treated teeth with their unrestored reference, there was no difference in the mineral-to-collagen ratio (Fig. 3.4A), and, although statistically significant, there was a small difference in the occlusion ratio (Fig. 3.4C). However, the root canal–treated teeth exhibited a significantly greater collagen cross-linking ratio, above that associated with aging. That signals an increase in the nonreducible hydroxypyridinium cross-links Pry and deoxypyridinoline relative to the reducible divalent cross-links deH-DHLNL and dehydrohydroxylysinonorleucine. An increase of nonreducible cross-links has been observed in dentin with aging[108] and contributes to its fragility.[109] The ratio of Pyr and deH-DHLNL characterizes collagen maturity.[110] The higher cross-linking ratio of the root canal–treated dentin (Fig. 3.4B) suggests that it is the primary contribution to the reduction in strength relative to the unrestored teeth. Because this is superposed with the effects of natural aging, root canal treatment appears to

accelerate the aging process, which causes an increase in tooth fragility

There are several limitations to this study. Root canal treatment is often applied to teeth that have been exposed to bacteria and have undergone inflammation of the pulp. Although this could be a factor in the lower strength, the mineral-to-collagen ratio did not show any differences based on treatment (Fig.3.4A). A related concern is that the period between treatment and extraction was unknown, and the treated teeth undoubtedly have unique periods of post-treatment clinical function. This topic is important and requires further exploration.

3.5 Acknowledgments

The authors thank Prof H. Allen Chen and Dr. Ying Guo for their useful comments and contributions. Supported by the Spencer Foundation (Prof. Hai Zhang).

Chapter 4

DEHYDRATION AND SHRINKAGE STRAINS IN THE DENTIN OF ENDODONTICALLY TREATED TEETH

4.1 Introduction

Dehydration has been considered a potential contributor to VRF.[111] A reduction in water content of dentin can potentially induce residual shrinkage strain on the circumferential direction.[112] It was widely accepted that dentinal dehydration that occurrs in the organic components of dentin for the weakening and reduction in the structural integrity after RCT.[79, 111, 113] About 70% of the total amount of water found in dentin manifests as bound water, which is bound by weak van der Waals forces and hydrogen bonds to the collagen and hydroxyapatite components within the dentin matrix.[114] The remaining unbound- or free-water can be found in the dentinal tubules, porosities and mineralized dentin matrix. In a normal, healthy, vital tooth, the dentinal fluid flow is under a slight positive pressure of 15 cm H_2O (1,47 kPa).[115] Approximately 85% of the free-water loss can occur within 30 minutes when the dentin is exposed to ambient condition.[79] Fully hydrated dentin shows viscoelastic properties and its fracture toughness is purported to its degree of hydration.[27, 79]

Following root canal treatment, the pulp is removed, and the root canal is shaped, cleaned, disinfected and dried before root-filling. Helfer et al. reported a 9% decrease in moisture of dentin from endodontically treated teeth.[78] It has been shown that the loss of dental pulp associated water content, as well as the loss of free-water from the dentin matrix/dentinal tubules in proximity to the root canal, can influence the mechanical integrity.[116, 113] Microcracks induced by RCT[61] or dehydration[105]

can undergo growth[27] under this shrinkage and lead to tooth fracture (Fig. 4.1). However, there is also evidence that dehydration is not the primary cause. Huang et al. conducted compression, indirect tensile testing and impact testing on human dentin to assess whether dehydration weakens dentin.[80] Their results showed no significant difference between the mechanical properties of treated and untreated dentin. Kruzic et al. also showed dehydration of dentin does not necessarily cause a reduction in the resistance to fracture.[9] There is currently no consensus whether dentinal dehydration is the primary cause of mechanical degradation of root canal treated dentin.

Digital image correlation (DIC) has been used to investigate shrinkage in dentin under free convection.[117, 116] In fact, Chen et al. used 3-D DIC to evaluate full-field deformation in dentin with dehydration.[118] However, an evaluation of dehydration-induced shrinkage in the roots of teeth with prior RCT, or specific to senior patients has not been reported. The objectives of this study were to characterize shrinkage in radicular dentin with water loss as a function of donor age and prior RCT. Moreover, the loss of bound water was analyzed spatially in radicular dentin to fully understand the relation of age and prior RCT treatment to dehydration-induced shrinkage strains. The null hypotheses of this investigation were that there is no difference in the magnitude of shrinkage strain or extent of water loss in the radicular dentin of teeth: i) regardless of patient age, and ii) whether or not the tooth received prior RCT.

4.2 Materials and Methods

Human single-rooted non-carious teeth were obtained from participating clinics with an exempt protocol approved by the Institutional Review Board of the University of Washington. The teeth were placed in Hank's balanced salt solution (HBSS) immediately after extraction. Tissue on the root surface was carefully removed and teeth

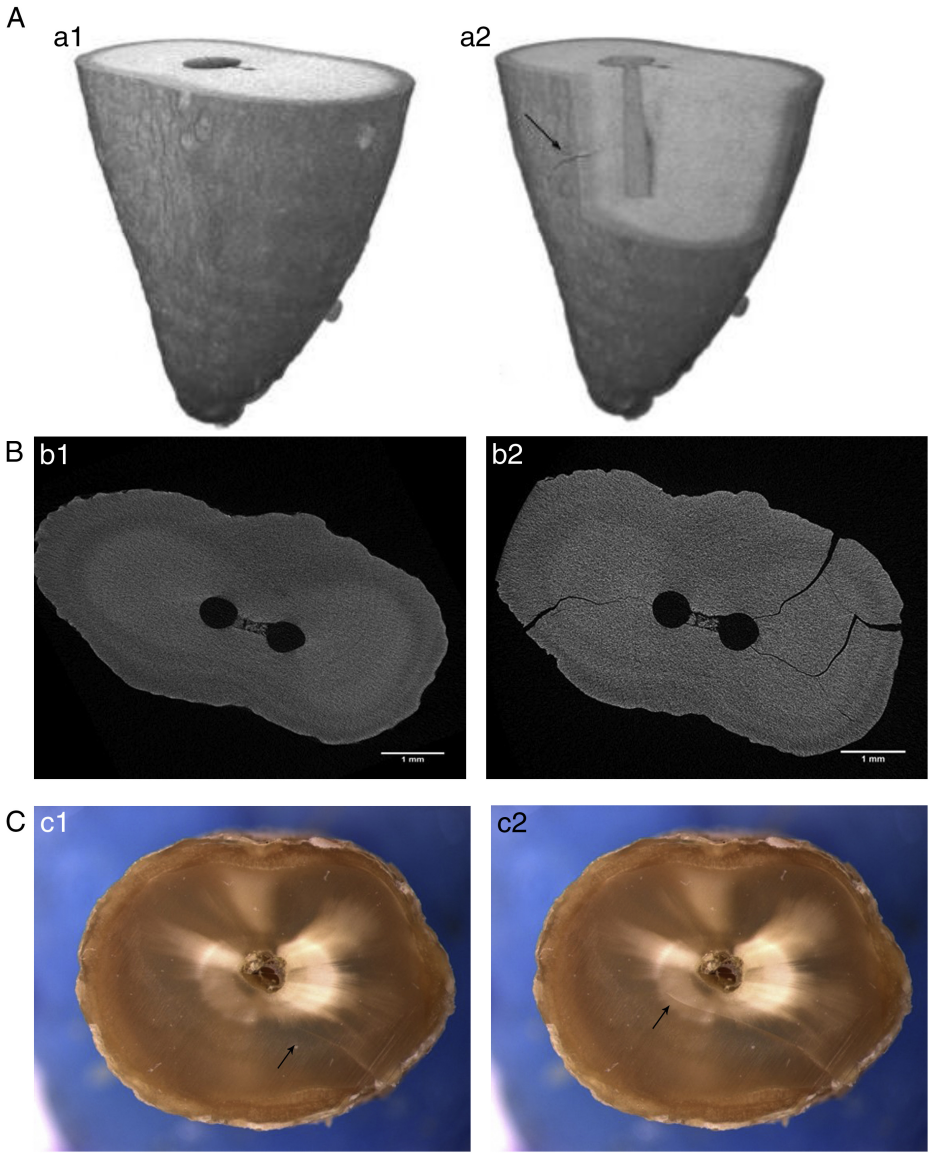

Figure 4.1: μCT and light microscope images of cracking teeth. (A) 3D renderings of a root tip before ($a1$) and after ($a2$) drying, demonstrating crack formation upon dehydration. (B) Virtual slices of identical zones in a root show comparison between wet ($b1$) and dry ($b2$) states of the sample and demonstrate appearance of cracks. (C) Crack propagation dynamics. Optical micrographs of an incipient crack observed in a slice under a light microscope immediately after cutting ($c1$) and after 5-minute air-dry at room temperature ($c2$). The arrow highlights visible crack tip, which is seen to extend significantly after drying.[105] Copyright 2020 Elsevier.

with decay or structural defects were discarded.

A total of 14 teeth were obtained from eight different patients and divided in young (n=6, age $<$ 25 yrs) and old (n=8, age $>$ 60 yrs) groups. For the old donor group, the teeth were obtained from four different donors, including one matched pair of teeth from each donor, including one untreated and one RCT tooth from mirrored locations of the mandible. For the young group, three (of the six) teeth were randomly chosen and subjected to root canal treatment *ex vivo* within the endodontic clinic at the University of Washington. Thus, seven out of the 14 teeth had RCT and four of those eight (i.e. in the old group) underwent post-treatment clinical function.

For the young RCT group, a standard protocol was adopted for treatment. The chamber was accessed with a #2 round bur and high-speed handpiece. The working length was determined utilizing 10c hand file until patent, then withdrawn 1 mm from apical foramen. The glide path was established until the 10c file was loose to 1 mm past working length. The canal was instrumented with Vortex Blue rotary files to the working length and irrigated copiously with 6% NaOCl, 17% EDTA, then 6% NaOCl again. The canal was dried with paper point to working length and obturated utilizing bioceramic sealer with Vortex Blue gutta-percha via the continuous wave obturation technique. Backfill was completed with Calamus Flow. No information was available for the RCT of the old teeth.

The teeth were cast in resin and sectioned perpendicular to the tooth length in three locations following established methods.[103] Three 1.5 mm slices were obtained between the cemento-enamel junction (CEJ) and root apex, representing apical-, middle- and coronal-third sections of the root (Fig. 4.2A). For consistency, the apical and coronal slices were sectioned 1 mm away from the apex and CEJ, respectively; the middle slice was sectioned at the midpoint. The slices were stored in HBSS at 4°C for less than 3 days. One side of the slices was prepared with a speckle coating

using air brush and oil-based ink in support of DIC.

Stereo-DIC measurement was performed using industrial monochrome CMOS cameras with 2592 × 2048 pixels and 73.0 fps (Model UI-3180CP-M-GL, IDS, Obersulm, Germany), two 50 mm fixed-focus C Mount lenses (Model V5018, Computar, Japan), a pair of extension tubes of 15 mm, and calibration plate (9 × 12, checker size: 1.5 mm) as describe in Fig. 4.2B. This configuration supported a field of view up to 25 mm × 20 mm. The two cameras were synchronized to record images simultaneously during free convection. The aperture and exposure times were adjusted to obtain the optimal brightness and contrast. The calibration plate was used to obtain the cameras' intrinsic and extrinsic parameters necessary for DIC.

To perform experiments, the prepared root sections were removed from the HBSS, gently blotted to remove excess moisture, and then placed on a polystyrene tray in air at roughly 25 ± 1°C and 60% relative humidity. Digital images of the specimens were acquired under free convection at a rate of 2 frame/s for an extended period. The first pair of images recorded at time zero served as a reference (with zero strain) and the displacement and strain were calculated using correlation methods. A commercial software (PMLAB DIC-3D, Nanjing PMLAB Sensor Tech Co., Ltd., Nanjing, China) was used to obtain the displacement and strain maps. Search parameters included a subset size of 25 × 25 pixels and step size of 3 pixels. The hoop strain was estimated from the strains along the periphery of each tooth slice. A time lapse documentation of crack initiation and growth from the outer surface of radicular dentin was also performed using optical microscopy.

Raman Spectroscopy was used to identify subtle changes in molecular structure of both the mineral and collagen components of dentin.[119] Raman Spectroscopy has been adopted to characterize the mineral- and collagen-bound water in human cortical bone,[120] and similarly in dentin.[121] Thus, it was performed using a commercial

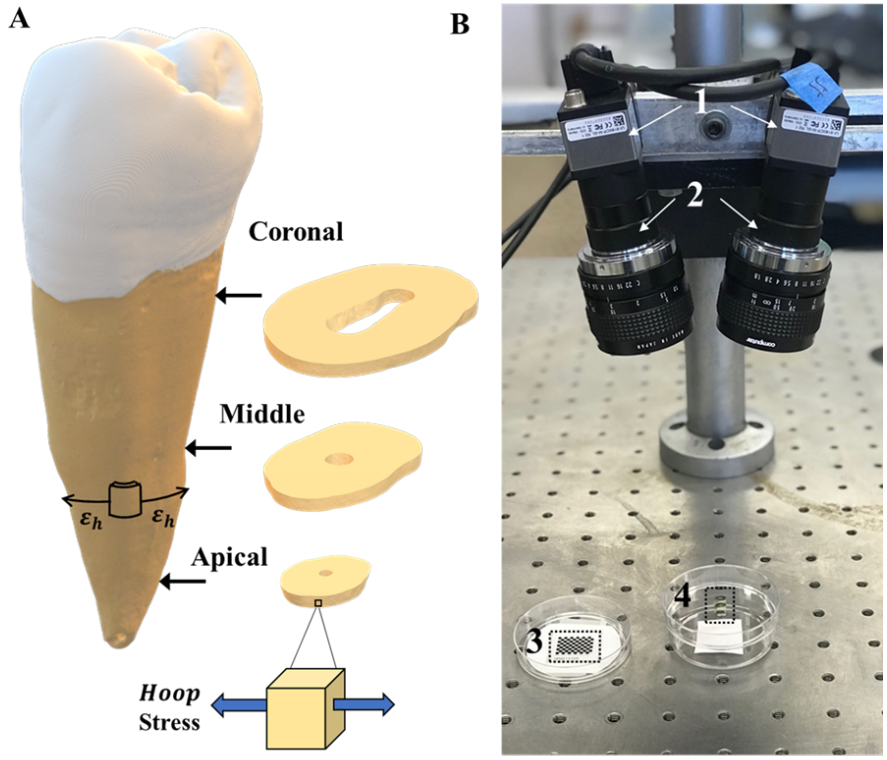

Figure 4.2: Measuring shrinkage strain with 3-D digital image correlation (DIC). A) schematic of dentin slices cut from three different locations along the root. Direction of hoop strain (ϵ_h) is indicated on the root surface. B) Experimental arrangement used for shrinkage measurement under free convection in air. (1) Camera. (2) Lens. (3) Calibration Board. (4) Dentin Sample.

unit (Renishaw InVia, West Dundee, IL, USA) over the spectral range of 2700 - 4000 cm^{-1} and at eight spots distributed around the circumference of the dentin slices. The spectra were baseline corrected for fluorescence using WiRE 3.4. The bound water ratio was calculated according to Unal et al.[120] from the ratio of area under the compounded mineral- and collagen-bound water peaks over 3100 – 3600 cm^{-1} and collagen backbone peak at 2949 cm^{-1} (Fig. 4.3).

Statistical comparisons of the shrinkage strain and bound water ratio were calculated with respect to anatomical location and age using a two-way Analysis of variance (ANOVA) and with the critical value (alpha) set at 0.05.

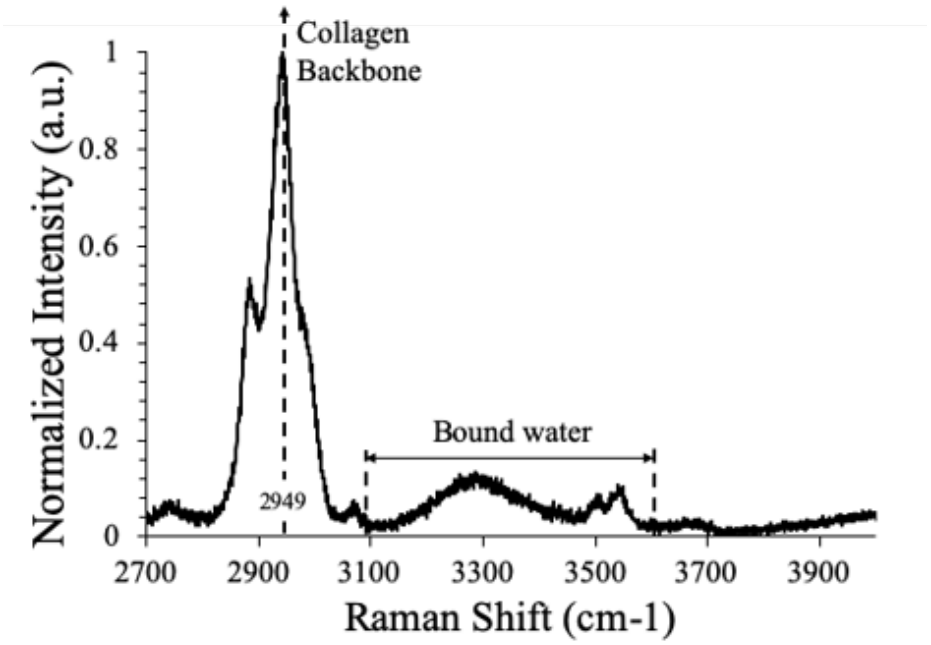

Figure 4.3: The Raman shift from 2700cm^{-1} to 4000cm^{-1} for dentin. The 2949cm^{-1} peak represents the collagen backbone while the peaks from 3100cm^{-1} to 3600cm^{-1} represent collagen and mineral related bound water. Ratio of bound water to collagen backbone by the Area-Under-Curve in radicular dentin was measured at the perimeter of dentin slices.

4.3 Results

A comparison of the average measured hoop strains that results from water loss over a 5-minute period in the apical-, middle- and coronal-thirds of teeth from each group is shown in Fig. 4.4A-D. As evident from these distributions, the hoop strain increases linearly with time, regardless of donor age and prior treatment. However, the magnitude of strain is clearly age dependent.

The average shrinkage rates in the apical-third were determined using linear regression (Fig. 4.4A-D). For the old donor teeth, the RCT group exhibited a higher rate of shrinkage than the non-RCT group ($p < 0.05$). However, for the young donor group there was no significant difference in shrinkage rate between the RCT and non-RCT groups. There was also no significant difference in the rate of shrinkage between the RCT and unrestored teeth of the middle and coronal third sections. Comparing the two unrestored groups, the apical shrinkage rate was significantly lower in the old teeth in comparison to the young teeth ($p < 0.05$).

Maps of the shrinkage strains over the entire cross-section of selected teeth from each group are shown in Fig. 4.5 after 60 min of dehydration. Regions of high strain are evident in red color and concentrate near the cementum, especially in the old RCT group. The average maximum hoop strains over time in the apical thirds of teeth from the four groups are presented in Fig. 4.6. Shrinkage strains in the old RCT group were significantly greater ($p \leq 0.05$) and nearly three time larger than the other groups. Optical microscopy showed crack initiation and growth caused by this strain (Fig. 4.7).

The bound water ratio was measured by Raman spectroscopy and calculated from the combined intensity of the mineral- and collagen-bound water peaks divided by the collagen backbone peak. The Raman spectrum for dentin from a typical non-RCT tooth is shown in Fig. 4.3. In the hydrated state, there was no difference in

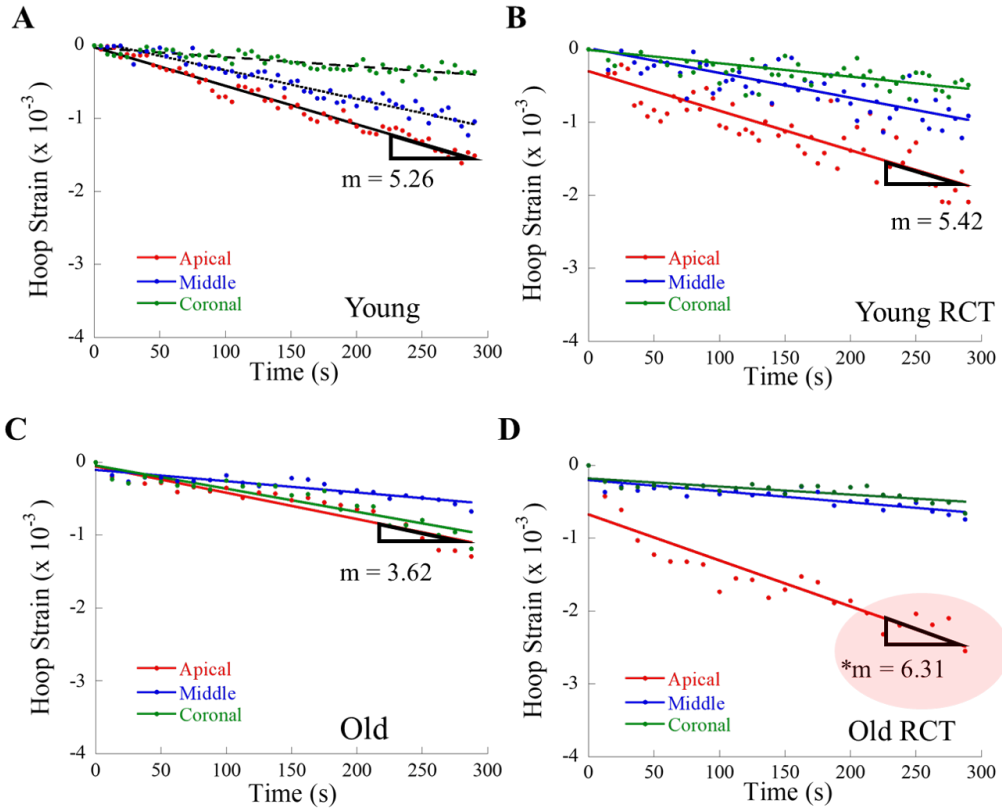

Figure 4.4: Measuring time evolution of shrinkage strain (dimensionless) in root dentin with 3D digital image correlation (DIC). (A) Young, (B) Young Root-Canal Treated, (C) Old, and (D) Old Root-Canal Treated. Shrinkage rates (m) are represented as $microstrains/sec$ $(10^6/s)$. All groups have significantly larger stain rate in the apical third of the root. $*$ indicates significant difference compared to untreated counterparts.

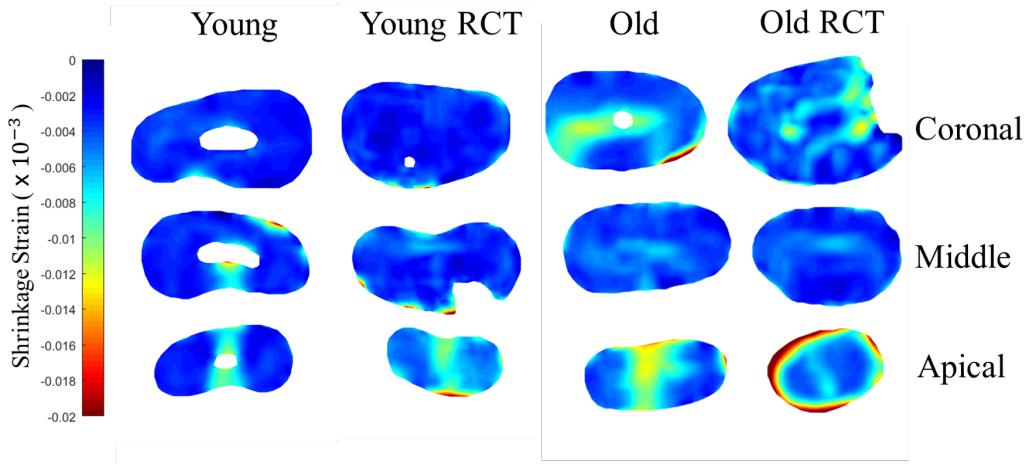

Figure 4.5: Map of shrinkage strain from in coronal, middle and apical thirds after dehydration for 60 min. Note the comparatively large strains in the Apical third of the Old RCT group.

bound water between the non-treated young and old dentin (Table 4.1). However, bound water in the old RCT group was significantly lower than that for the old non-RCT dentin. Furthermore, the decrease in bound water in the old RCT dentin after dehydration (21%) exceeded that in both the young and old non-RCT dentin (16% and 15%, respectively).

4.4 Discussion

The pulp serves as the "pump" that maintains the movement of dentinal fluid within the canal and dentin tubules.[122] Removal of the pulp during RCT disables this pump and undoubtedly interrupts the dentinal fluid distribution and the water content of dentin. Spatial variations in the water loss could result in non-uniform shrinkage, mechanical strains and the development of strain energy, particularly in regions of large strain. As such, a non-uniform distribution of water loss and consequent shrinkage in the root serve as a potential "driving force" for crack initiation and growth, the

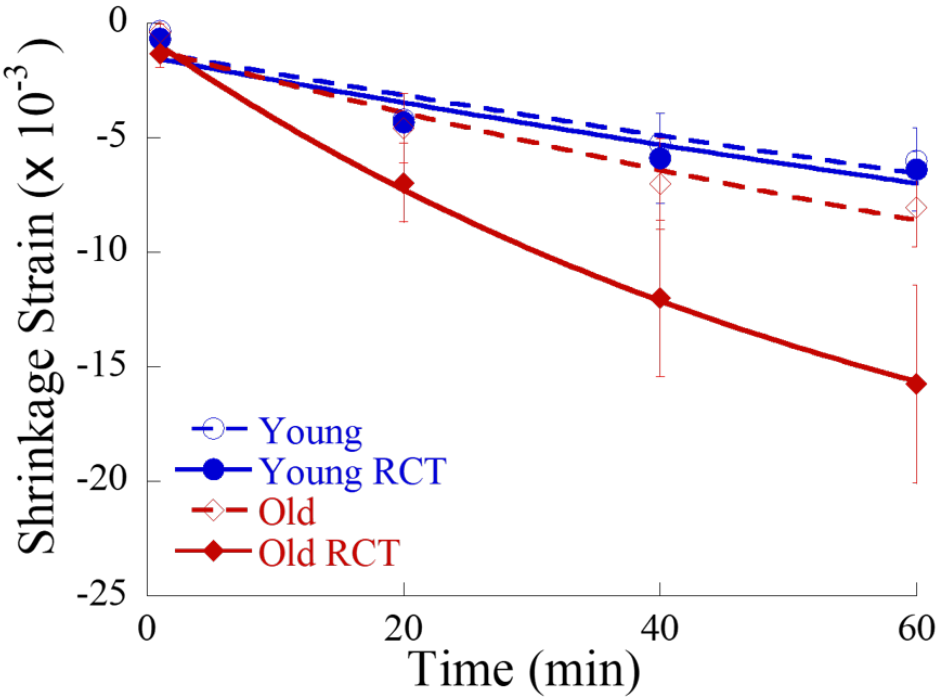

Figure 4.6: Shrinkage strain of buccal-lingual surface within the apical third over 60 minutes of dehydration. (a.u. = arbitrary units)

precursors to tooth fracture.

The measured shrinkage in roots of the four groups was a function of the donor age, which requires rejection of the first null-hypothesis. Dentin sclerosis progresses with aging, a process marked by gradual filling of the dentinal lumens with mineral that begins at the apex.[22] Sclerosis involves mineralization of the lumens as well as increased collagen cross-linking of the intertubular dentin, both of which are expected to reduce shrinkage. Indeed, the strains in the untreated old teeth were lowest overall (Fig. 4.4C). However, in comparing results for the two RCT groups, the dehydration-

induced shrinkage was substantially greater for the old group; shrinkage rate in the apical-third was nearly 75% greater than that in the untreated teeth and the young RCT group. That requires rejection of the second null-hypothesis. After 60 min of dehydration, strain in the old RCT group was 3 times higher than in the matched unrestored group. Interestingly, the disparity between the RCT and untreated teeth was not reflected in the middle- and coronal-thirds (Fig. 4.6A). The cause for this unique response requires further study.

Independent of RCT and the instrumentation process, dehydration of dentin may increase the risk of VRF. Specifically, dehydration can cause microcracking in dentin,[106] reduced structural integrity[105] and a decrease in the resistance to cyclic crack growth as shown in Table 4.1.[27] Water loss also reduces the capacity for viscous deformation, which increases the propensity for brittle behavior.[79] Furthermore, prolonged exposure to the oral environment after RCT can contribute to irreversible denaturation of the collagen fibrils in the root.,[123] which can influence the degree of shrinkage with water loss. As such, dehydration may predispose the root to fracture and work synergistically with the aforementioned driving force.

Although water loss in dentin after RCT has been a concern for decades,[124] shrinkage strain measurements and strain distribution maps from teeth receiving RCT and post-treatment function has never been reported. Previous studies have characterized the shrinkage resulting from the water loss in coronal dentin.[117, 116] However, dehydration induced shrinkage within the root is of greater importance. Regardless of age and prior restorative treatments, shrinkage rates in the apical-third of the root were significantly higher than those in the middle- and coronal-thirds (Fig. 4.4). That higher strain rate is expected from the greater volume fraction of inter-tubular matrix in the root and its contraction with water loss.[52] Furthermore, the orientation of hoop strains in the root and corresponding stress (Fig. 4.2A) can assist

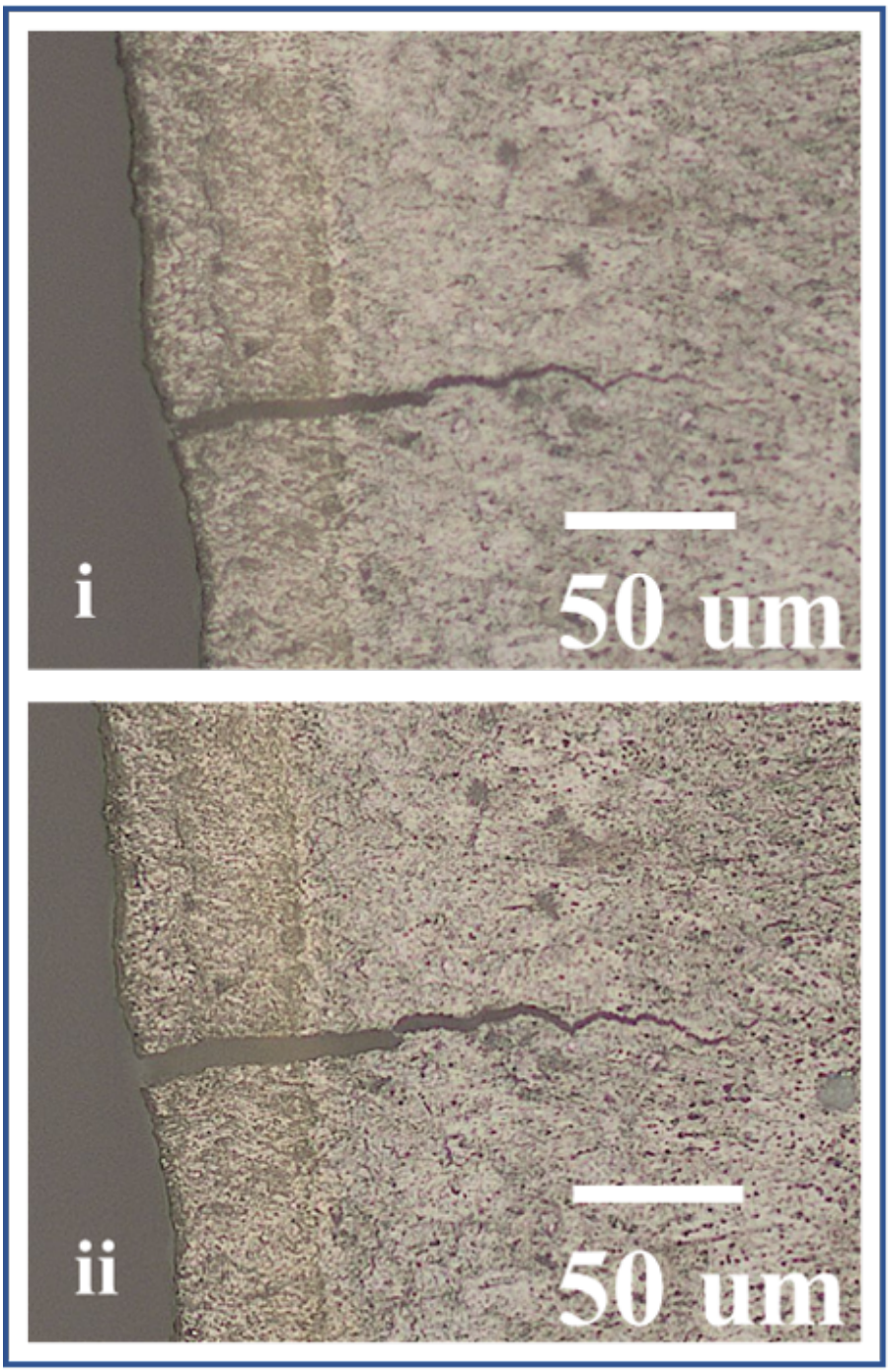

Figure 4.7: A microcrack that initiated at the outer surface of the apical third and extending inward after (i) 1 and (ii) 10 minutes of dehydration.

Table 4.1: Analysis of bound water ratio in outer dentin via Raman Spectroscopy. (a.u. = arbitrary units) Different letters in same group indicate significant difference. Different letters indicate significant difference compared to the corresponding hydrated and dehydrated tissues in the other groups.

Age/Treatment	Bound Water Ratio (a.u.)	
	Hydrated	Dehydrated
Young	0.215 ± 0.045^{a}	0.180 ± 0.028^{b}
Young-RCT	0.213 ± 0.032^{a}	0.182 ± 0.027^{b}
Old	0.207 ± 0.044^{a}	0.175 ± 0.025^{b}
Old-RCT	0.165 ± 0.041^{b}	0.131 ± 0.037^{c}

in this crack growth process. Indeed, VRF starts at the apex, where shrinkage strains were highest (Fig 4.6) and propagate coronally in the occlusal-cervical plane,[38] akin to the crack orientation in Fig. 4.7.

The collagen matrix consists of roughly 30% bound and 70% unbound water.[125] The unbound water molecules occupy the sites between adjacent collagen peptides via hydrogen bonding. Removal of that water by dehydration results in interpeptide bonding and shrinkage of the collagen.[114] Shrinkage strains in dentin are caused by the increase in interpeptide forces within the collagen fibrils with removal of water.[124] Hence, the extent of shrinkage in dentin is a function of the mineral to collagen ratio. There are fewer dentinal tubules and a higher collagen content adjacent to the cementum,[51] which exhibited more extensive shrinkage with water loss. Indeed, the largest shrinkage strains occurred close to the cementum, particularly in the RCT teeth (Fig. 4.5).

While Raman Spectroscopy showed that the extent of bound water was lowest in the RCT teeth, the percentage water loss with free convection was greatest. One limitation to this assessment is that Raman spectroscopy provides a relative measure-

ment that is unable to give absolute measures of water content. Future studies should focus on the unique response of RCT teeth after clinical function to fully understand the dehydration induced shrinkage in dentin roots.

There are several limitations to the present investigation that are important to consider. First, the oral history of the RCT teeth was not controlled and the exact period of post-treatment function is unknown. In addition, the young RCT teeth were treated *ex vivo* and did not undergo post-treatment clinical function. Lastly, it important to put this study in perspective. VRF is a multi-factorial problem and that damage related to water loss was evaluated independent of other important factors. For instance, demineralization and collagen denaturation resulting from the irrigants,[89] internal canal pressure during lateral condensation of the canal in RCT[92] and the combined effects of apex geometry and higher collagen content in this region[86] are also relevant.

4.5 Acknowledgements

The authors are grateful to Prof. H. Allen Chen and Dr. Daniel A. Estrada for their comments and contributions. This work was partially supported by the Colgate-Palmolive Company.

Chapter 5

ROOT FRACTURES IN SENIORS AND COLLAGEN EMBRITTLEMENT OF DENTIN

5.1 Introduction

As introduced in previous chapters, the microstructure of dentin undergoes a gradual transition in translucency with increasing age that results from filling of the dentinal tubules with mineral, a process regarded as sclerosis.[29] This transition in the microstructure of dentin is dynamic and progresses with aging. Dentin sclerosis occurs first and is most severe in tissue near the root apex,[22] which could be attributed to the tubule morphology in the root since the dentin tubules have a smaller lumen diameter and density in the root in relation to the crown.[8]

Although the mechanisms of dentin sclerosis are still unclear,[30, 126] the consequences are far more well known. In particular, the age-related changes in microstructure of dentin reduce the damage tolerance of the tooth.[127] Previous studies that addressed aging and the mechanical behavior of dentin have shown that the crown[71] and root[103] undergo reductions in strength. Furthermore, the fatigue strength,[33, 34] fracture toughness[35, 36] and fatigue crack growth resistance[27, 37] also undergo a significant reduction with age. However, there are two key limitations of prior studies on this topic, namely: i) most work has concentrated on the coronal dentin, and ii) investigations of the root have not considered spatial variations. Furthermore, the degradation in damage tolerance of dentin with aging has largely been attributed to what is most noticeable, i.e. the mineral that fills the dentin tubules.

In comparison, far less effort has focused on the changes to the intertubular

dentin. Changes in collagen content, or changes to inter- and intra-fibrillar collagen cross-linking, can also increase the risk of brittle fracture of mineralized tissues. For instance, investigators have concentrated on the role of collagen in the mechanical properties of bone.[128, 129, 130, 81] Results indicate that the toughness and strength of bone decreases significantly with increasing collagen denaturation. Further studies showed that strength, work to fracture, and fracture toughness of bone are all dependent on the collagen network. Results showed a significant correlation between age-related decrease in the toughness of bone and collagen matrix crosslinking. Wang et al. concentrated on the age-related changes of collagen crosslinking in bone matrix.[130] Both non-enzymatic and enzymatic crosslinking may be contributing factors causing age-related deterioration of bone quality and a degradation in resistance to facture. Consequently, it is reasonable to suspect that changes in the degree of collagen crosslinking occur with age in dentin and may be a principal factor contributing to the increasing incidence of VRF with age.

Removal of the pulp during RCT is associated with irreversible change to the dentin structure.[24] In a comparison of the strength of root dentin between teeth with age- and donor-matched controls, those with prior RCT exhibited 30% lower strength.[119] Yet, the progression of aging in non-vital teeth, specifically those that have received RCT followed by post-treatment function, and the spatial variations in degradation of the root are not understood. Root fractures in the teeth of seniors with RCT could be attributed to an acute form of degradation in mechanical properties at the apex and could manifest in the intertubular or peritubular components of dentin. Understanding this phenomenon is the first step towards the development of treatment modalities, or the development of dental materials, that account for the unique property variations.

Indentation methods have been adopted to characterize the changes in mechanical

behavior of dentin with aging and sclerosis.[131, 132] Macroscopic indentation methods are not able to discern the properties of distinct aspects of the microstructure or interfaces,[133] which is highly relevant to an evaluation of dentin. As such, nanoindentation has become a standard method for measuring the mechanical behavior of biological hard tissues at the microscopic scale and also enables an assessment of the viscous behavior. Nanoindentation or atomic-force microscopy is required to measure discrete changes in the intertubular and peritubular dentin,[134] which is relevant to the present investigation.

Nanoscopic Dynamic Mechanical Analysis (NanoDMA) is a special form of nanoindentation based structural analysis. Scanning nanoDMA, performed using scanning probe microscopy, maintains the indentation stress within the elastic range, which is preferred for highly sensitive analysis. Recent applications include evaluations of dentin bonding[135] and aging.[136] It has also been applied in investigations related to remineralization of dentin[137, 138, 139, 140] and to assess the effects of collagen crosslinking.[138, 117] However, no investigation has been reported the use of nanoDMA mapping within tooth roots to delineate the unique age-related changes of the intertubular and peritubular dentin or the differences between vital and non-vital teeth.

In the present study, scanning mode nanoDMA was used to characterize the dynamic mechanical behavior of dentin in the roots of teeth with regards to donor age, pulp vitality and histological location. Two null hypotheses were defined: i) there is no significant difference in the mechanical behavior of intertubular dentin along the length of the root with regards to donor age, and ii) there is no significant difference in the properties of intertubular dentin between vital teeth and those with prior RCT.

5.2 Materials and Methods

Human single-rooted non-carious teeth were obtained from participating clinics with an exempt protocol approved by the Institutional Review Board of the University of Washington. Single-rooted premolars were chosen for their simple physiology in comparison to molars with multiple roots. The teeth were stored in Hank's balanced salt solution (HBSS) with record of donor age and gender. Those teeth with visible caries or structural defects were discarded. A total of 12 teeth from 8 patients were selected and divided into young (n=4, age < 25), old (n=4, age > 60) and old non-vital (n=4, age > 60) groups. The four teeth in the old and old non-vital groups were matched pairs of teeth. Each pair consisted of teeth from mirrored locations of the arch from the same donor that included a vital tooth and non-vital tooth with prior RCT. Obtaining matched pairs of teeth in mirrored locations of the arch is very difficult, but very beneficial, since it reduces the variations in tooth properties across donors.

The teeth were cast in a polyester resin foundation within two weeks of receipt and sectioned axially in the mesial-distal (M-D) direction using a precision slicing/grinding machine. The resulting halves were embedded in cold-cured epoxy resin (Epofix HQ Resin and Hardener, Struers) exposing the root canal and longitudinal section. The exposed dentin sections were polished using silicon carbide abrasive paper from #800 to #4000 mesh with water irrigation until halfway through the thickness of dentin and the dentin tubules became evident. Further polishing was performed with 3 μm diamond particle suspensions and 0.04 μm colloidal alumina suspension. The specimens were subjected to 20 minutes of sonication to remove residual debris inside the dentin tubules. All of the aforementioned procedures were conducted with the tooth maintained fully hydrated in HBSS.

A dynamic mechanical analysis (DMA) of the polished sections of root dentin

was performed using Scanning Probe Microscopy (SPM) on a commercial system for nanoindentation (Hysitron Inc., Model TI980 Triboindenter, Minnesota MN). The scanning-based evaluations were performed using a Berkovich diamond indenter with 90 nm nominal tip radius, which was measured in scanning mode with quartz standard according to Ryou et al.[135] Prior to nanoDMA on teeth, a frequency sweep was performed on fused quartz from 100 Hz to 300 Hz to identify resonance components related to the machine operation. Based on the results of this process a scanning frequency of 200 Hz was used to maximize the signal to noise ratio in the evaluations of dentin. A 4 μN static indentation load and a dynamic sinusoidal load of 2 μN were applied following Ryou et al.[141] The scanning mode nanoDMA was conducted using a window of evaluation of 20 μm $\times$ 20 μm, which constitutes an area involving 5-10 dentin tubules as shown in Fig. 5.1A. The instrument performs the evaluation through 256 horizontal scans and with pixel density of 256 $\times$ 256. Properties of the intertubular and peritubular dentin were quantitatively evaluated within the apical, middle and coronal thirds (Fig. 5.1B). In each region, areas of interest within the window of evaluation were selected that corresponded to either the intertubular or peritubular dentin. Three independent nanoDMA scans were conducted in each region of each tooth to ensure that a statistical representative assessment was achieved. Then the average and standard deviation of the desired properties were determined from the scan data within that domain of relevance.

To minimize the effects of dehydration to the mechanical behavior,[142] the nanoDMA evaluations were performed in the hydrated condition with treatment of 99.4% ethylene glycol (EG).[136] Briefly, a thin layer of EG was smeared on the sample surface after removal from HBSS bath to prevent the evaporation of water during scanning. Previous results have shown that there is no influence of EG on the moduli estimated using scanning-based DMA[141] and that this approach can maintain the

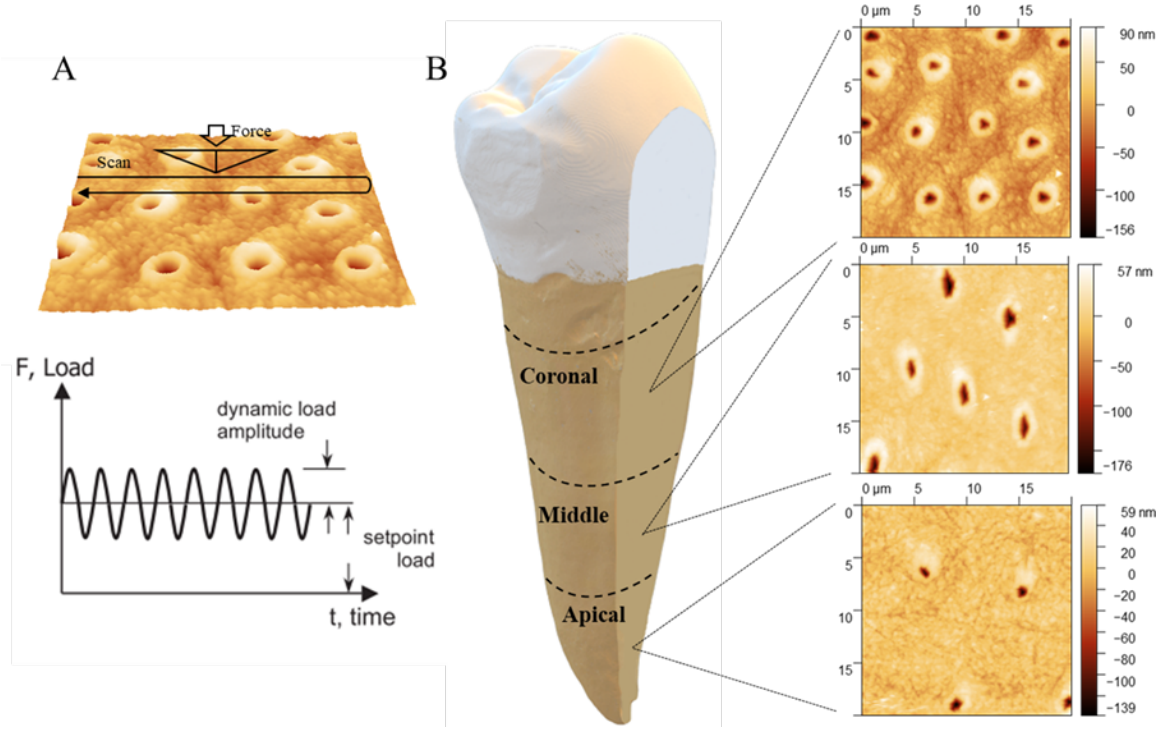

Figure 5.1: Details of the experimental evaluation. (A) nanoDMA scanning on prepared dentin surface using scanning probe microscopy (SPM) with a Berkovich indenter. Under load control, the indentation involves a static component (setpoint load) and a superposed dynamic load amplitude equivalent to 50% of the static component. (B) Demonstration of analysis within the three chosen locations along the tooth root using SPM. The root is divided into the coronal-, middle- and apical-thirds. Representative topography maps show the lumen density within each of these three regions. Note the progressively lower tubule density from the coronal to the apical third of the root in B.

hydration of the tissue for over an hour. Since the individual scans were completed with a period of less than 30 minutes, the process ensured that the properties were evaluated in the fully hydrated condition.

Within each region of evaluation, the storage (E') and loss (E'') moduli were obtained from the nanoDMA scans, which represent the elastic behavior and dampening capacity of the material, respectively. These two parameters were used in estimating the complex modulus (E^*) according to $E^* = (E'^2 + E''^2)^{1/2}$, which represents a measure of the combined influence of the storage and loss behavior. The ratio of the loss and storage moduli were used to obtain the tan delta parameter, which provides a relative measure of the viscous response of the tissue that is independent of tip geometry. A statistical analysis of the parameters was performed using a two-way Analysis of Variance (ANOVA) test with significant differences identified at $\alpha = 0.05$. The normality of the data was checked before performing the statistical analysis.

5.3 Results

Representative property maps obtained for the apical dentin of a tooth root from the young group of donors are shown in Fig. 5.2. Specifically, the surface topography and mechanical properties distributions representing the complex, storage and loss moduli, as well as the tan delta distribution are presented in this figure as denoted. These maps were obtained over a 20 μm $\times$ 20 μm window of evaluation. The dentin tubules and peritubular cuffs are clearly evident in the topography and mechanical property maps, which enabled precise identification of the regions corresponding to the intertubular and peritubular dentin. The center of the tubule lumens exhibit property values that are unrealistic due to boundary effects between the tip and open lumens. Such areas are artifacts of the indentation method and were excluded in the quantitative analysis.

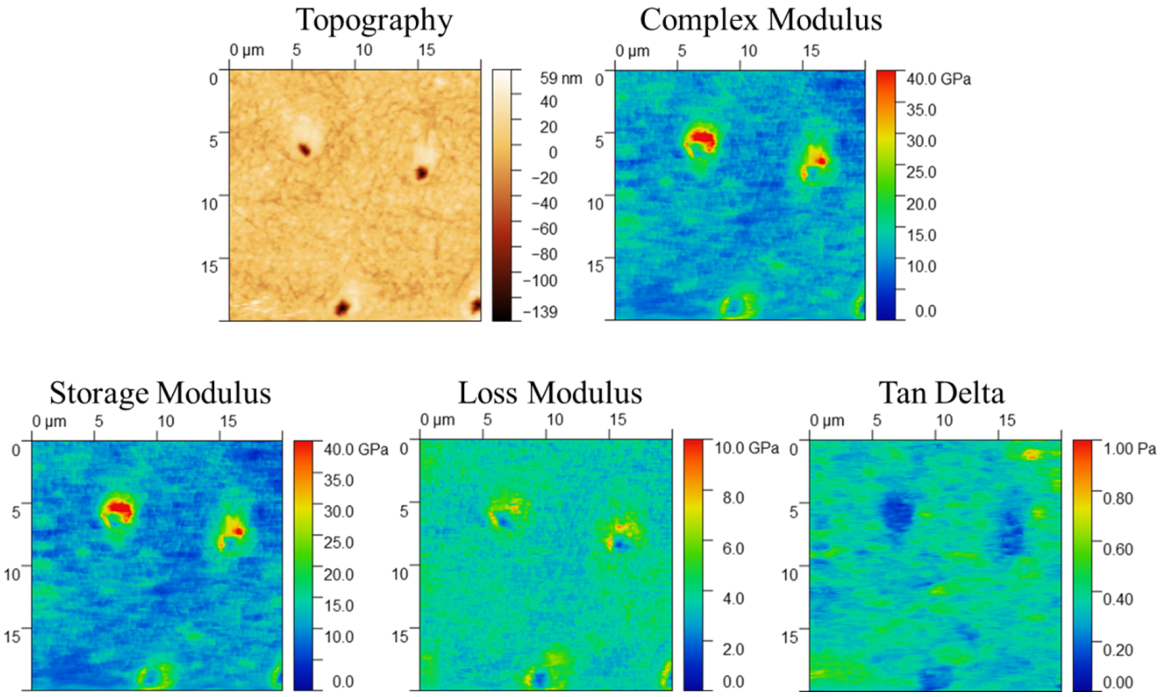

Figure 5.2: Representative property maps from the apical-third of a tooth that is part of the young vital group. Note that the maps include the surface topography, complex modulus, storage modulus, loss modulus and tan delta as labeled. The scan area involves three mostly complete dentin tubules, and partial view of a fourth, which are clearly evident in the scans.

Representative property maps for the apical dentin of roots within the vital and non-vital old groups are presented in Fig. 5.3. Shown are the property distributions for the storage and loss moduli, as well as the tan delta parameter. Note that the maps for the individual properties presented in Fig. 5.2 and Fig. 5.3 have the equivalent range for easy comparison. Differences in the complex and storage modulus between the young and old dentin are clearly evident. Specifically, the storage modulus measurements for the intertubular dentin of both old groups are substantially higher than those of the young teeth. In contrast, the tan delta values for the intertubular dentin of the old groups appear lower than those obtained for the young dentin.

A comparison of the dynamic mechanical properties of the peritubular dentin from the apical-, middle- and coronal-thirds of the roots was performed and results are shown in Fig. 5.4. Specifically, the storage and loss moduli are presented in Fig. 5.4A and 5.4B, respectively, and the tan delta parameter and complex modulus are shown in Fig. 5.4C and 5.4D, respectively. In all three locations of the root, the dynamic mechanical properties of the peritubular dentin from the old vital and non-vital teeth were not significantly different ($p > 0.05$) from those of the young teeth. However, with regards to spatial variations, the storage and complex moduli of the peritubular dentin from the old vital roots in the apical third were significantly greater ($p < 0.05$) than those values for the measures from middle- and coronal-thirds. Furthermore, there was no significant difference in the measured tan delta parameter of the peritubular dentin between the age groups and with respect to location.

Similar to the analysis of the peritubular dentin in Fig. 5.4, quantitative comparisons of the dynamic mechanical properties of the intertubular dentin are presented in Fig. 5.5. Specifically, the storage and loss moduli for tissue from the young, old and old non-vital roots are compared in Fig. 5.5A and 5.5B, respectively. Similar comparisons of the tan delta and complex modulus of the intertubular dentin are

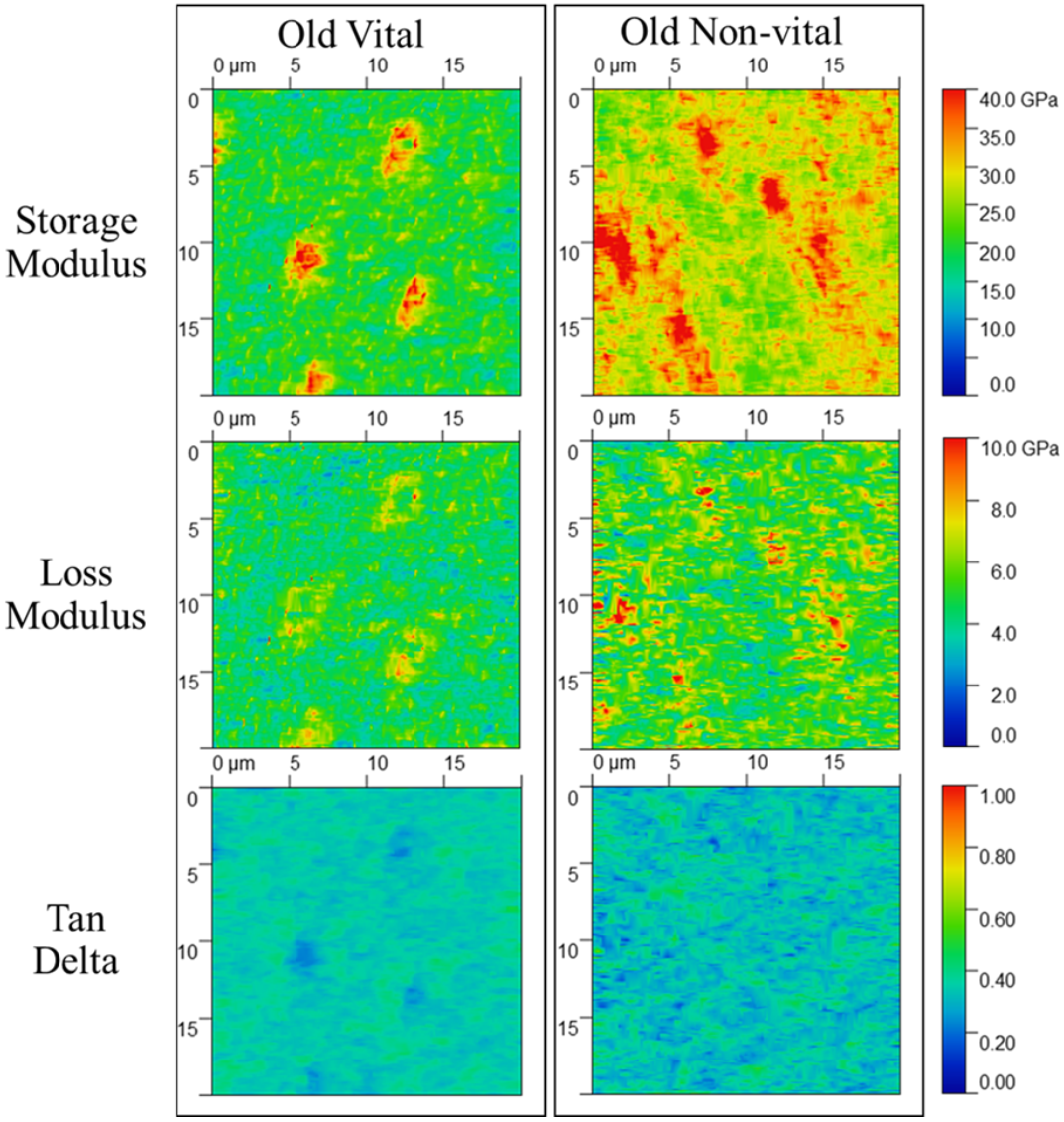

Figure 5.3: Property maps of the dynamic mechanical properties for dentin from the apical thirds of teeth representing the old vital and old non-vital groups as labeled. The nanoDMA maps for these two teeth were obtained from donor-matched tooth pairs.

shown in Fig. 5.5C and 5.5D, respectively. Regarding spatial variations, the storage and complex moduli of the intertubular dentin obtained from the old vital teeth were significantly larger ($p < 0.05$) in the apical third than in the two regions of interest located more coronally. That warrants rejection of the first null-hypothesis. In contrast, no spatial variations were identified in the properties of the young or the old non-vital teeth over the root length. Regarding differences between age groups, the intertubular dentin of the old vital teeth exhibited significantly higher ($p < 0.0005$) storage and complex moduli in the apical- and middle-thirds than the young dentin. Furthermore, the complex moduli of the intertubular dentin from roots of the old non-vital group were significantly higher in all three locations ($p < 0.0005$). That difference requires rejection of the second null hypothesis. While there was no difference in the loss modulus across age groups, the tan delta parameters of the young dentin were significantly higher than the two old groups, regardless of location ($p < 0.005$).

5.4 Discussion

According to an investigation using discrete indent mode nanoDMA, Ryou et al.[136] reported that there was no significant difference in the storage and complex moduli of peritubular dentin between young and old vital teeth. Those findings were confirmed through results of the present study in scanning mode. Nevertheless, the peritubular dentin of the old teeth in Ryou et al.[136] was reported to have significantly lower loss modulus and tan delta, implying that it has a lower capacity for viscous dampening. That trend was not observed by nanoDMA in scanning mode. The difference could be attributed to the mechanics of indentations and/or size effects associated with application of the two distinct methods. Based on its spatial relation and sensitivity, scanning nanoDMA is a more appropriate method for assessing the properties of the individual constituents. The set point load in the scanning mode used herein was 4

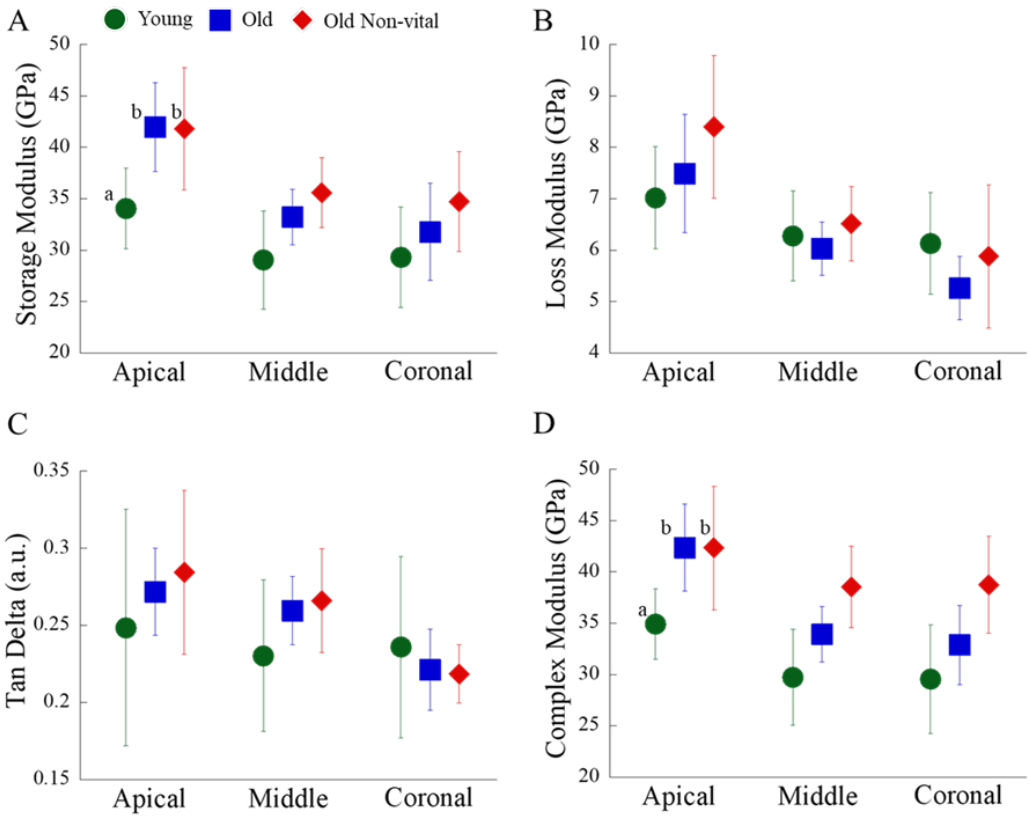

Figure 5.4: A comparison of the nanoDMA responses for young, old and old non-vital peritubular dentin. Data points within each region labeled with different letter are significantly different ($p < 0.05$). (A) Storage Modulus, (B) Loss Modulus, (C) Tan Delta, (D) Complex Modulus

μN, which is 100 times lower than in the discrete indent mode performed by Ryou et al.[136] The high sensitivity of the scanning mode analysis enabled isolating properties of the peritubular cuff from effects of its surroundings; no significant differences with age were found. Moreover, the studies by Ryou et al.[135, 136] were conducted on coronal dentin and not on tissue from the root. It is important to acknowledge the difference in the tissue from these two regions. There are no changes in the dynamic mechanical properties of the peritubular cuffs of radicular dentin with aging, except for within the apical third. Within the two old groups, there was no significant difference in the properties of the peritubular cuff between the radicular dentin of vital and RCT non-vital teeth.

To the authors' knowledge, no previous study has evaluated the spatial variation in properties of the peritubular dentin in the root. As sclerosis starts from root apex in the third decade of life,[70] it was expected that the apical-third in the old groups, where the sclerosis is most advanced, would have higher storage and complex moduli. Indeed, there was an increase in these two moduli at the root apex of the two old groups (Fig. 5.4A and 5.4D). Deposition of mineral within the dentin tubules can fundamentally change the indentation response. Occlusion of the dentin tubules changes the physical boundary conditions of the cuffs by suppressing the degree of transverse elastic deformation. Another possible mechanism is densification of the cuffs through a precipitation of crystals stimulated by the aqueous solution within the dentin tubules. The latter explanation is supported by the smaller crystal size in aged dentin with respect to that in the cuffs of young dentin.[30] Nevertheless, it only applies to the vital teeth, not those that have received RCT. The specific cause for the larger resistance to elastic deformation exhibited by the peritubular cuffs in the two old groups is unclear.

In comparison to the effort placed on the dentin tubules with aging, the evolution

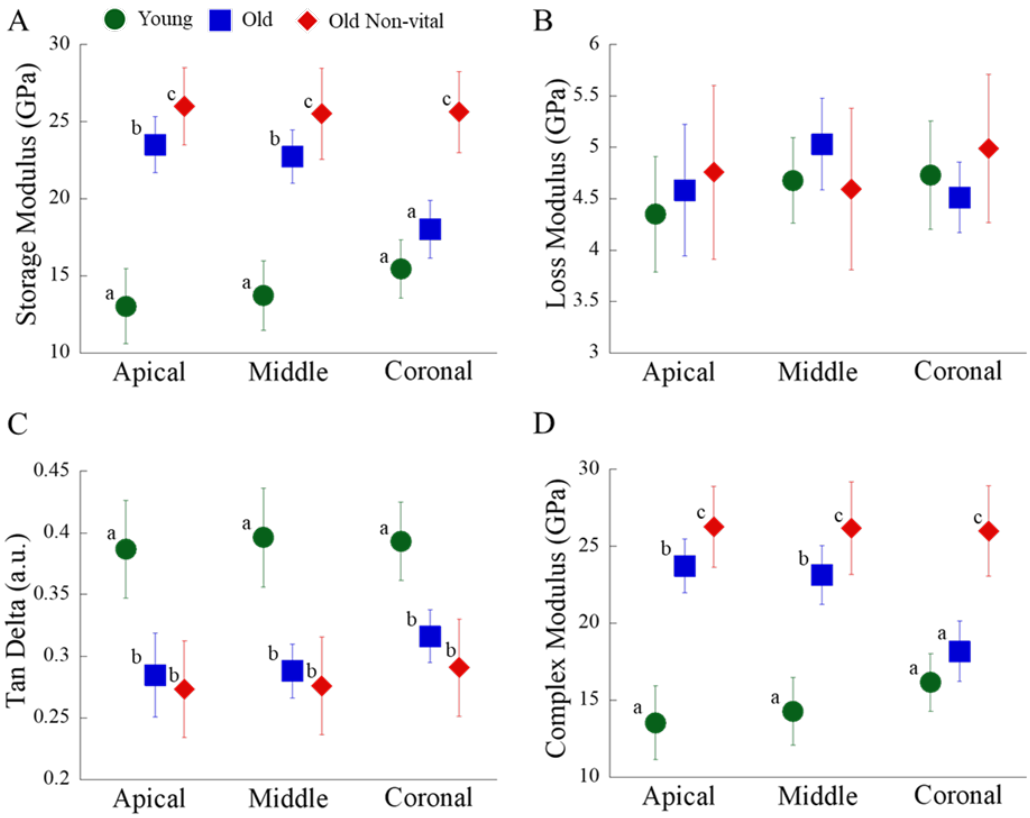

Figure 5.5: A comparison of the nanoDMA responses for young, old and old non-vital intertubular dentin. Data points within each region that are labeled with different letters are significantly different ($p < 0.05$). (A) Storage Modulus, (B) Loss Modulus, (C) Tan Delta, (D) Complex Modulus

in properties of intertubular dentin has received limited attention. In bone, the age-induced degradation in toughness is almost exclusively attributed to the collagen matrix.[143] In teeth, intertubular dentin occupies more than 90% of the volume of dentin overall and type-I collagen accounts for approximately 90% of the intertubular matrix.[18] As such, volumetrically, the intertubular dentin is more critical to resisting tooth fracture and the collagen fibril mesh is integral to its fracture toughness.[19] By virtue of its large volume fraction, property alterations of the collagen and intertubular dentin with aging are potentially more detrimental overall than the changes to the peritubular cuff and dentin tubules. This statement is most applicable to the apical third of the root where the tubule density is the lowest overall (Fig. 5.1B). As sclerosis begins in the third decade of life,[70] the apical region undergoes sclerosis for much longer time than the crown. Consequently, age-related changes in properties of dentin would be expected to be largest near the apex. Indeed, that is where the most significant changes in properties of the old vital dentin were observed (Fig. 5.5).

Kinney et al.[33] proposed a dissolution-reprecipitation theory for sclerosis where dissolution of intertubular mineral is reprecipitated within the tubules as magnesium-rich beta-tricalcium phosphate and apatite. And in support of that mechanistic theory, sclerotic dentin exhibits a reduction in crystal size in the intertubular region with respect to "normal" non-sclerotic tissue.[30] Due to the reduction in volume fraction of mineral within the intertubular space, the complex and storage modulus of intertubular dentin would be expected to decrease with age, not increase. However, both the old vital and non-vital intertubular dentin exhibited significantly higher storage and complex modulus, regardless of location (Fig. 5.5). The larger storage modulus could be caused by an increase in the mineral content or perhaps a greater extent of collage cross-linking. Yan et al.[103] recently showed that there are increases in both the mineral to collagen ratio and the extent of cross-linking in the root with

aging. Although that study was limited to vital teeth, a later study involved the roots of RCT teeth from old donors[119] and showed that non-vital teeth exhibited lower mineral to collagen ratio than that of vital teeth, but significantly larger extent of collagen cross-linking. That implies that the larger storage and complex moduli of the vital and non-vital groups could result from different mechanisms. Without more information regarding the specifics of the RCT performed, it is not possible to distinguish the specific causes to the increase in cross-linking in that study. It appears that more work is required to elucidate the aging process in non-vital dentin.

The increase in storage modulus of the intertubular dentin with age results in detrimental consequences. For the same stress applied to an arbitrary volume of intertubular dentin (Fig. 5.6A), the young dentin with comparatively low storage modulus (denoted as E for old dentin and E/2 for young dentin), can accommodate much greater strain energy to a critical stress (Fig. 5.6B). The strain energy that manifests beyond what can be stored is converted to energy for fracture. This is where viscous deformation in dentin plays a very important role. The tan delta quantifies the relative dampening capacity, which involves viscous relaxation to an applied stress as shown schematically in Fig. 5.6C. With higher loss modulus, young dentin possesses more viscous relaxation (dark arrow), and thus is capable of dissipating more strain energy. The nearly 25% decrease of tan delta in old dentin indicates diminished capacity for viscous stress relaxation in relation to young dentin. The loss of this capacity for stress relaxation through viscous behavior is expressed as an embrittlement of the tissue. Therefore, an increase in root fractures in senior patients is quite conceivable due to this embrittlement of the intertubular dentin.

There is precedence for cross-linking of the collagen to play an important role in the embrittlement with aging, especially in the non-vital teeth (Fig. 5.5D). In bone, collagen crosslinking contributes to its decline in damage tolerance with age.[130, 128]

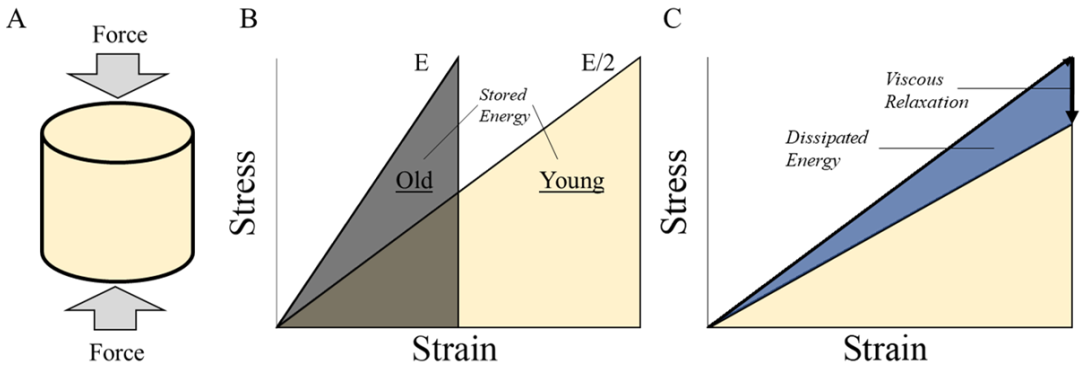

Figure 5.6: Schematic representation of the storage and loss moduli and their importance to the mechanical behavior of dentin. (A) A cylindrical volume of dentin subjected to compressive force that results in uniaxial stress and strain. (B) A comparison of the strain energy stored to failure for young and old dentin shown by the area under the stress-strain curves (assuming equivalent strength). Notice the lower strain energy storage capacity of the old dentin due to its high elastic (i.e. storage) modulus. (C) Viscous relaxation in young dentin (noted in blue) results in a reduction of the axial stress and a decrease in the strain energy within the material generated by mechanical loading. That relaxation reduces the propensity for root fracture due to the reduction in strain energy.

Dentin undergoes accelerated collagen crosslinking,[108] which contributes to a reduction in strength with age.[119] A previous study has also shown that there is a correlation between intertubular collagen crosslinking and reduction of fracture strength in donor-matched RCT and non-RCT root dentin.[65] Thus, crosslinking of the collagen matrix within the intertubular dentin is a potential contribution to the increase in storage and complex moduli overall, as well as to the changes in the apical third. This comment requires further support and assessment of the intertubular mineralization to establish that it is not a confounding factor to the changes in properties.

One of the most interesting finding is the significant differences in properties between vital and non-vital dentin of the donor matched pairs (Fig. 5.5). Structural

changes in dentin after removal of the pulp are unexpected due to absence of cellular processes as well as loss of the dentin fluid and internal pulpal pressure. Consequently, the driving forces causing the evolution of sclerosis are deactivated. However, the property maps clearly showed that non-vital dentin possessed significantly higher storage and complex moduli than the vital counterparts, and along the entire length of the tooth. This is the first report of such changes in properties of RCT teeth. Although the dynamic mechanical properties for the old dentin exhibited gradients from the apical- to coronal-thirds, indicating more advanced aging near the apex, no such spatial variations were evident in the non-vital dentin. It is reasonable to speculate that the collagen in pulpless teeth undergoes crosslinking or denaturation as a consequence of the treatment. For example, sodium hypochlorite irrigation pretreatment of dentin has been proven to effect the elastic modulus and hardness and potentially causes alteration of the collagen.[89, 144] Although plausible, this topic requires further investigation.

There are limitations to the present investigation. One of the most critical concerns is the unknown dental history of the donors. While the microstructure of dentin appears consistent among all teeth, its properties are unique from person to person.[119] This is a consequence of many factors, which may include diet, medications, parafunctional habits, etc. The patient factor was reduced by using donor-paired vital and non-vital teeth. Nevertheless, differences in the progression of aging between the donors is uncertain. Furthermore, no details were available regarding the RCT teeth, including method and time of treatment, or the period of post-treatment function. These are important factors in understanding the initiation and progression of degradation. There was also a limitation in assessing the specific changes in the microstructure and chemical composition responsible for the decrease in capacity for viscous deformation at the sub-micron level. Advanced techniques such as atom probe

tomography should be applied to examine the structural changes in dentin with aging at the atomic level.

5.5 Acknowledgement

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Chapter 6

EFFECT OF CRYOPRESERVATION OF TEETH ON THE STRUCTURAL INTEGRITY OF DENTIN

6.1 Introduction

Autogenous transplantation (i.e. autotransplantation), or the surgical movement of a tooth from one location to another in the same individual, has become an increasingly viable method for tooth restoration.[145, 146, 147, 148] According to Zachrisson et al. the three primary indications for autotransplantation are unevenly distributed multiple agenesis, agenesis of the mandibular second premolars in low-angle face types with either normal or weak facial profiles, and accidental loss or congenitally missing maxillary central and lateral incisors.[149] When performed by the proper surgeon, autotransplantation of teeth with partially formed roots compares favorably with other treatment modalities for substituting missing teeth in a long-term perspective. Nevertheless, there is a limited window of viability; the optimal time for autotransplantation of premolars to the maxillary anterior region is when the root development has reached two thirds to three fourths of the final root length.

Long-term follow-up studies have reported that the success rates for autotransplantation range from 95 to 99 percent.[146, 147, 148] Allogenic transplant (allotransplant) procedures that involve donor teeth from another human show lower success rates, which is likely due to the histo-incompatibility of donor teeth.[148] Despite the high efficacy of the procedure, there is still a problem related to the paucity of healthy donor teeth that are available for this purpose. The frequency of autotransplantation procedures is currently limited because they can only be performed when the patient

has a healthy tooth available that would already necessitate extraction.[150] This obstacle to transplantations provides motivation for the storage of healthy extracted teeth for future use in autotransplantation.[151] The ability to save premolars from routine extractions and store them for future use opens new possibilities to the field of restorative dentistry and prosthodontics.

Cryopreservation involves the long-term storage of tissues and cells by cooling them to temperatures well below the glass transition temperature of water ($-135°$C).[152] The process essentially 'suspends' biological activity and molecular movement, thereby providing the ability to save biological constructs and store them for future use. Cryopreservation treatment has been successful in the long-term preservation of cells, tissues, organs and other biological constructs.[153, 154, 155] In soft tissues, two of the most critical issues associated with the freezing process have involved direct damage caused by ice crystals forming within the cells, and secondary damage attributed to increased concentration of the remaining solutes.[156, 157, 158] Those concerns are potentially less relevant in the preservation of teeth due to the density and lower permeability of the hard tissues involved, but the prospect of damage cannot be ignored.

In general, there has been limited investigation on how cryopreservation affects the mechanical properties of enamel or dentin. Kuhl et al. explored whether cryopreservation affected the macro-scale structural integrity of teeth and reported that no cracks greater than 0.8 μm in length were detected after cryopreservation.[159] The complex anatomy and the combination of unique tissues in teeth makes them susceptible to damage as a result of cryopreservation. Indeed, cryopreservation of teeth must be approached differently than for other tissues.[160] Nevertheless, to the authors' knowledge there is no reported study that addresses whether cryopreservation causes a degradation in the microstructure of dentin or enamel if it increases the potential for structural failures.

The complex anatomy and the combination of unique tissues in teeth makes them susceptible to damage as a result of cryopreservation. Indeed, cryopreservation of teeth must be approached differently than for other tissues.[160] Overall, dentin is less mineralized than the enamel but possesses much greater water content; by volume dentin is approximately 20% water in comparison to approximately 3% for enamel.[18] Dentin is structurally anisotropic with spatial variations in composition and properties.[161, 53] The most distinctive feature of dentin is the network of microscopic tubules, which extend radially outward from the pulp to the dentino-enamel junction (DEJ). Each tubule is surrounded by a highly mineralized cuff of peritubular dentin consisting of nanoscopic apatite crystals. The intertubular dentin occupies the regions between each of the tubules and their peritubular cuff. It consists of a mesh of type I collagen fibrils that are oriented roughly in the plane perpendicular to the tubules and are reinforced by inter- and intra-fibrilar apatite crystals. The larger water content of dentin relative to enamel, as well as the sharp variations in mineral content posed by the intertubular and peritubular dentin, suggests that cryopreservation-related damage is more likely in dentin.

Cryopreservation could be detrimental to the mechanical behavior of dentin. Shrinkage stresses induced by the mismatch in contraction between enamel and dentin,[117] or during freezing of the free and bound water, could cause the formation of microcracks. Furthermore, cyclic stresses develop in teeth during mastication and as a result of temperature changes in the oral environment. If adequate in magnitude, these stresses could cause fatigue. Kinney et al. hypothesized that damage accumulation, and fatigue are contributors to tooth fracture.[19] As such, a reduction in the fatigue strength of the dentin and enamel is extremely relevant to the success of any procedure that is being contemplated in the field of restorative dentistry. Previous work has shown that microstructural damage that is not evident from microscopic

evaluations of dentin can contribute to its fatigue behavior.[162, 163, 164] Therefore, an evaluation of the mechanical behavior of cryopreserved dentin was performed that involved quasi-static and cyclic four-point flexure loading. The primary objective of this study was to determine whether cryopreservation causes a degradation of the microstructure of dentin or its resistance to fracture. While not directly associated with aging or endodontic treatment, this study does provide further understanding of the contributions from changes in the microstructure of dentin and its resistance to fracture.

6.2 Materials and Methods

A total of 73 human third molars were collected within 24 hours of extraction from participating clinics according to protocols approved by the Institutional Review Board of the University of Washington. Immediately after extraction, the teeth were placed in Hank's Balanced Salt Solution (HBSS) and stored with record of tooth number, donor age and gender. An inspection for visible decay, structural defects or prior restorations was conducted at receipt of each tooth and throughout the cryopreservation and sectioning process. Teeth with defects were discarded and replaced with additional candidates without defects.

The cryopreservation protocol used in this investigation was designed according to previous studies, which have reported evidence of high pulp-cell viability.[159, 165] Each sample was transferred from the HBSS solution to a petri dish filled with PBS and manually agitated in order to remove any contamination or soft tissue from the surface. The majority of the remaining tissue was removed using tweezers and a razor blade, with attention given not to disrupt the surface integrity, nor to apply excessive compressive forces. After washing, each tooth was transferred to a sealable tube containing 2-4 mL cryoprotectant solution with 10% (volume/weight in water) dimethyl-

sulfoxide (DMSO) and 90% fetal bovine serum after previous studies.[159, 165] The teeth were soaked in the DMSO solution for 25 min at 4°C. After temperature equilibration, each DMSO tube was placed into a larger container filled with isopropanol and stored in a −80°C freezer for an average of 15 hours. To complete the cryopreservation process, each nested sample container unit was removed from the freezer, immediately submerged in liquid nitrogen (LN) at −196°C and stored for an average of 10 days. Upon thawing the sample containers were removed from the LN tank and placed into a water bath held at 37°C, where they stayed for 25 min until the DMSO solution turned completely liquid.

After the thawing process, each tooth was removed from its container and rinsed in PBS at least three times to remove the cryoprotective agent. The teeth were stored in HBSS at 4°C for no longer than 3 days prior to sectioning. It was deemed necessary to rule out the potential effects of the cryoprotective agent on the mechanical behavior and chemical composition. Therefore, an additional three molars were collected and stored in the DMSO solution for 10 days at 5°C rather than freezing. These teeth are considered the DMSO treated group.

The teeth of all three groups (cryopreserved, non-cryopreserved and DMSO treated) were subjected to further sectioning to obtain rectangular beams of dentin from the mid coronal region (Fig. 6.1A). The beams possessed nominal cross-sections of 0.5 mm × 1.5 mm, with dentin tubules oriented perpendicular to the longitudinal axis. After machining, the beams were stored in HBSS at 22°C for fewer than 2 days.

The dentin specimens were loaded in four-point flexure to failure using either monotonic or cyclic loading based on established methods (Fig. 6.1B). The flexure loading apparatus consisted of 1/3 span arrangement as defined by ASTM D790M. The experiments were conducted using an EnduraTEC Elf Model 3200 universal testing system with specimens immersed in a bath of HBSS at room temperature

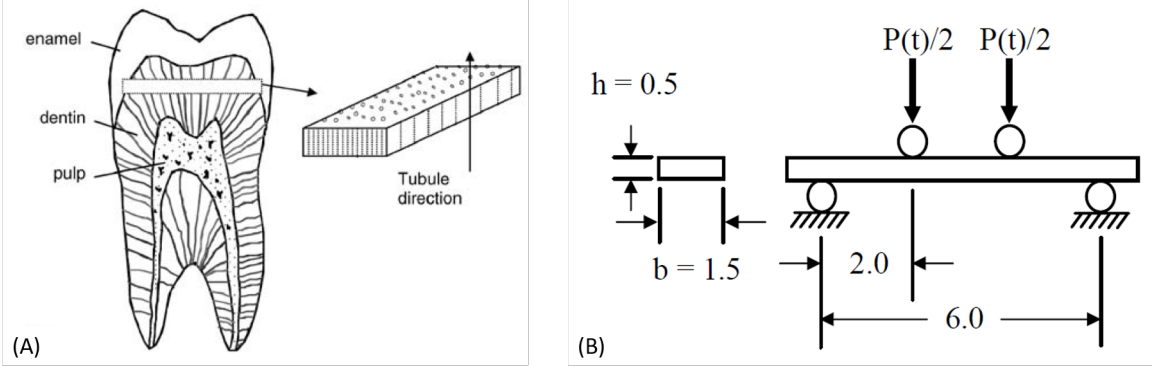

Figure 6.1: Schematic diagrams describing the experimental evaluation of the mechanical properties. (A) beams were obtained from the mid-coronal section of the donor teeth with cross-section of approximately 0.5 × 1.5 mm. All beams were obtained from a location equidistant from the DEJ and the pulpal horns. (B) the beams were subjected to quasi-static or cyclic loading in four point flexure with 1/3 load-point spacing. The coronal side of the beams was always placed on the tensile side of the flexure loading arrangement.

(22°C) to maintain hydration during testing. A detailed summary of the number of specimens prepared and tested is listed in Table 6.1. For the quasi-static investigation 52 beams were prepared from 24 molars of 11 different donors, with an average age of 23 ± 3 years. In the evaluation of fatigue properties of the cryopreserved group, 74 beams were obtained from 49 molars of 29 donors with average age of 23 ± 3 years. There is a difference in number of specimens tested for the cryopreserved and non-cryopreserved groups as evident in Table 6.1. The non-cryopreserved group is larger as results were also available from prior studies.[71] The flexure strengths for the two groups were compared using an unpaired Students t-test and the critical value (alpha) was set at 0.05. The fatigue strength distribution for the two groups were compared using a Rank Sum analysis and the critical value (alpha) was set at 0.05.

Selected specimens were inspected after flexural loading using a scanning electron microscope (model JSM-6010PLUS/LA; JEOL, Peabody, MA) to examine the

Table 6.1: Details concerning the samples evaluated in static and cyclic loading and the average age of the donors. The "control" indicates teeth that were not subjected to cryopreservation.

Group	Control			Cryopreserved		
	Teeth	Beams	Age (yrs)	Teeth	Beams	Age (yrs)
Static	16	40	24±3	8	12	22±4
Fatigue	34	52	24±3	15	22	22±3

microstructure and the fracture surface. The fracture surfaces were inspected to identify unique features that differentiated the fatigue behavior of the cryopreserved and non-cryopreserved dentin. In addition, the chemical composition of the dentin was analyzed using Raman spectroscopy (Renishaw InVia, West Dundee, IL). Three beams each were randomly selected from the cryopreserved and non-cryopreserved groups, as well as from the DMSO treated group. Raman spectra of coronal dentin representing the three groups were determined by scans over the spectral range of 400-1900 cm^{-1} following established methods.[103] For each beam evaluated, five different scans were taken randomly at the center area. The spectra were baseline corrected for fluorescence using a commercial software (WiRE 3.4, Renishaw, Gloucestershire, UK). The intensity distribution obtained from the three dentin beams of each group was normalized and averaged for comparison.

6.3 Results

A comparison of stress-strain curves resulting from flexural loading of representative cryopreserved and non-cryopreserved dentin specimens are shown in Fig. 6.2. The responses exhibited linear elastic behavior at the onset of loading followed by a region

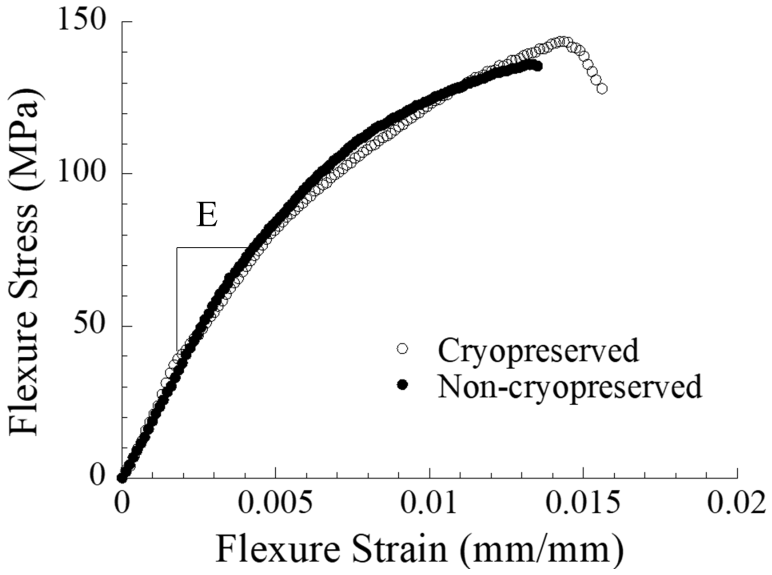

Figure 6.2: Typical flexural responses of dentin beams resulting from quasi-static four point flexure to failure.

of nonlinear deformation to failure. No differences were evidence in the constitutive behavior according to these responses. Results of the elastic modulus and strength measurements for the specimens are presented in Fig. 6.3A and 6.3B, respectively. According to the results obtained from all the dentin samples evaluated, there was no signficant difference ($p > 0.05$) in the elastic modulus between the three groups as expected. The apparent flexural strength of the non-cryopreserved (control), cryopreserved and DMSO treated dentin were 147 $\pm$ 10 MPa, 142 $\pm$ 25 MPa and 145 $\pm$ 11 MPa, respectively. There was no significant difference in the flexural strength ($p > 0.05$) between the three groups. Results for the flexure strength are in good agreement with those reported in previous studies.[71]

Fatigue life diagrams for the control and cryopreserved dentin specimens are shown in Fig. 6.4. Each data point in this figure represents the results of a single beam. The points annotated with arrows indicate specimens that did not fail within 1.2×10^6 cycles and that the cyclic loading was discontinued. They reached a level of endurance that could be used in determining the fatigue limit. According to a Wilcoxon Rank Sum analysis, the fatigue strength of the cryopreserved dentin was significantly lower than that of the non-cryopreserved group ($Z = -3.396$, $p = 0.00034$). The data representing the finite life responses were used to develop Basquin-type power law models, which are presented Fig. 6.4 and describe the mean fatigue strength distributions over the finite life history. Using these models, the apparent fatigue limits were estimated for 10^7 cycles. For the cryopreserved and non-cryopreserved dentin, the endurance limits are 37 and 44 MPa, respectively. The non-cryopreserved dentin specimens exhibited an endurance limit almost 20% greater than that of cryopreserved dentin.

No difference was found in the fracture surface features of the dentin fatigue specimens from the cryopreserved and non-cryopreserved teeth. Representative micro-

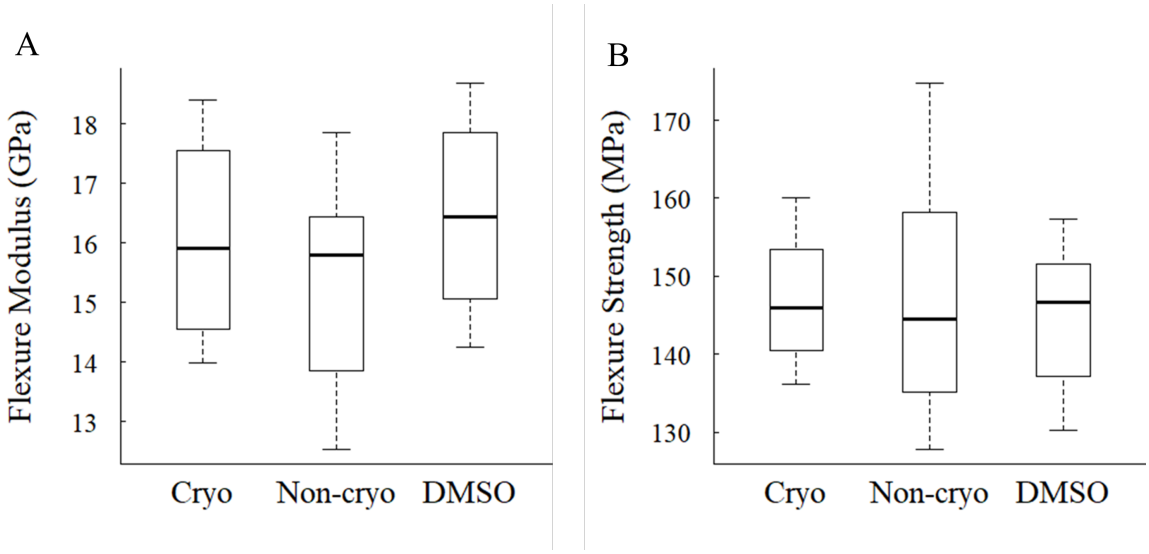

Figure 6.3: Mechanical properties of dentin beams in terms of treatment. (A) Flexure modulus. (B) Flexure Strength.

graphs of dentin specimens that were prepared for evaluation are shown in Fig. 6.5. Specifically, the surfaces of non-cryopreserved and cryopreserved specimens are shown in Fig. 6.5A and 6.5B, respectively; these represent side of the samples closest to the DEJ (i.e. the outer-dentin) and would be the surfaces of the beams to be subjected to tension in the cyclic loading experiments. No damage was evident in the microstructure of the non-cryopreserved beams. However, the surface of specimens from the cryopreserved group showed some evidence of separation between the intertubular dentin and peritubular cuffs. A micrograph from the surface of a cryopreserved beam taken at higher magnification is shown in Fig. 6.5C. Evident in this micrograph are the profuse distribution of cracks within the peritubular cuffs and the sunken nature of the peritubular cuffs with respect to the surrounding tissue. These features were not evident in the surfaces of the the non-cryopreserved samples or those that were DMSO treated but not cryopreserved (not shown).

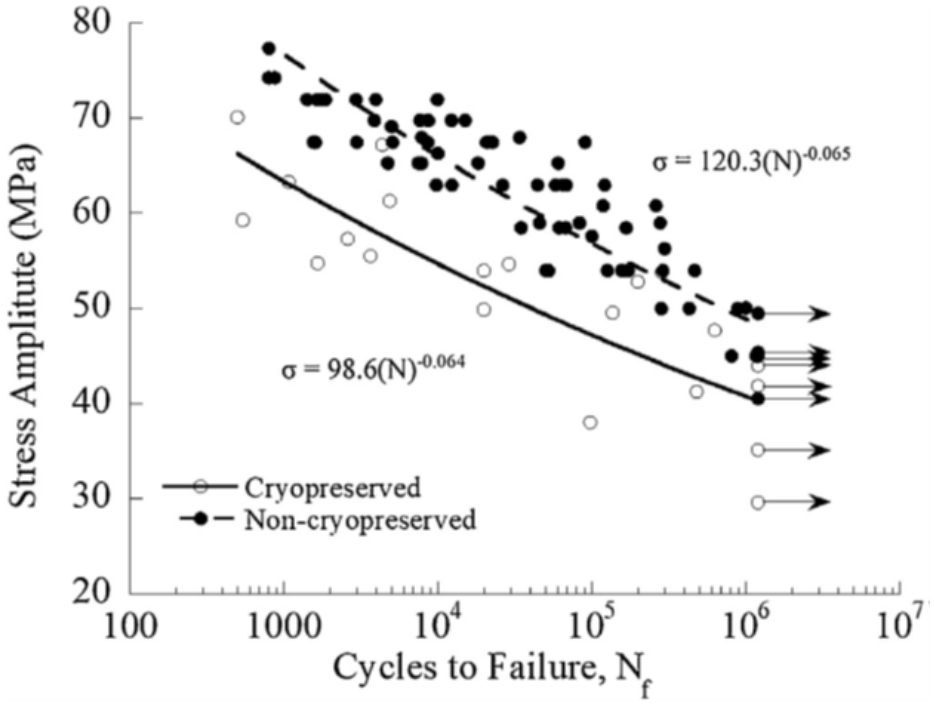

Figure 6.4: Fatigue life diagram comparing the fatigue life distributions of the cryopreserved and non-cryopreserved tissue. All data points represent results from cyclic loading of a single beam. The data points with arrows represent the responses for beams that did not fail within 1.2×10^6cycles and the cyclic loading was discontinued.

The chemical composition of the dentin beams was analyzed by Raman spectroscopy. Specifically, scans of the intensity distribution are shown over the entire spectral range for a representative beam from the control, cryopreserved and DMSO treated groups in Fig. 6.6. An inset of the intensity distribution over the range associated with the amide bands is also shown from 1000 to 1800 cm^{-1}. There is no significant difference in the intensity distributions between the three groups evaluated, which indicates that the cryopreservation process did not introduce damage to the collagen peptides.

6.4 Discussion

Previous studies concerning the cryopreservation of teeth and its potential success have focused on histological evidence and cell viability of the periodontal ligament (PDL) after transplantation.[166, 167] In comparison, the influence of cryopreservation on the residual structural integrity and mechanical properties of the tooth has received limited attention. To the authors' knowledge, no study has characterized the properties of dentin or enamel of teeth that have undergone cryopreservation. This study examined the properties of dentin after cryopreservation due to the larger water content of the tissue in comparison to enamel, and the potentially greater possibility for tissue damage due to more balanced distribution of mineral and organic content, as well as the large differences in their physical properties.

According to the monotonic tests performed on the coronal dentin samples, the apparent flexural strength of non-cryopreserved and cryopreserved dentin was 147 $\pm$ 10 and 142 $\pm$ 25 MPa, respectively. Results for the control group are consistent with those of a previous investigations using the same methods of evaluation.[71] A comparison with the flexure strength of dentin subjected to the DMSO treated condition (145 $\pm$ 11 MPa) showed that there were no significant differences in strength

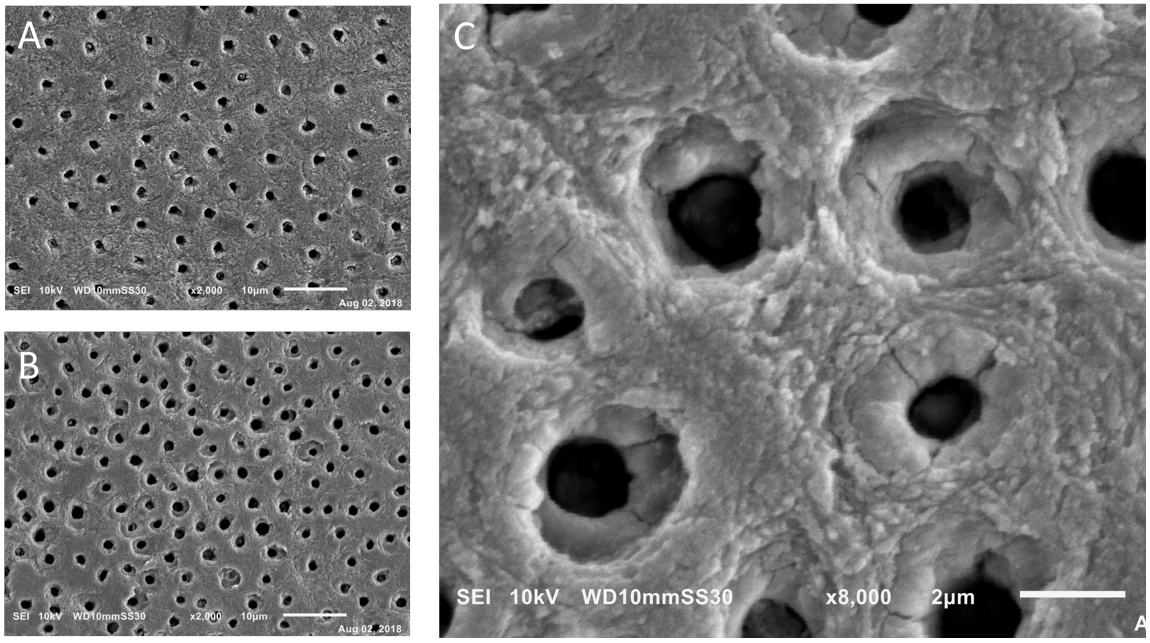

Figure 6.5: Scanning electron micrographs of the surface from representative dentin beams obtained from (A) non-cryopreserved and (B) cryopreserved teeth. A highly magnified view of the microstructure is shown for the cryopreserved sample in (C). Note the profuse microcracking of the peritubular cuffs and their sunken nature from the surrounding intertubular dentin. That observation was consistent for all the cryopreserved samples, but was not seen in the non-cryopreserved teeth.

between the three groups. Therefore, neither exposure to cryoprotectant for 10 days or the changes in temperature associated with the cryopreservation process caused damage that was sufficient enough to reduce the strength of dentin.

Although there was no significantly difference in strength, there was a significant difference observed in fatigue strength between the cryopreserved and non-cryopreserved teeth. The apparent endurance limit of dentin for the cryopreserved teeth (37 MPa) was nearly 20% lower than that of the control. It is not uncommon for treatments of dentin to have no effect on the monotonic strength, whereas cause a significant reduction of the fatigue strength.[162] The results of fatigue provide an indirect measure of the structural integrity of the tissue.[161] Clearly, the lower fatigue strength of the cryopreserved group indicates that either the extent of intrinsic damage or the rate of damage accumulation with cyclic loading is greater. Cryopreservation caused degradation to the dentin that reduced its structural integrity.

The cryopreservation procedure could cause degradation of the collagen matrix or at the interface of the collagen and mineral crystals, or even at a larger structural scale, e.g. within the peritubular cuffs. If present, degradation of the collagen should have been evident in the Raman spectra. However, there was no significant difference in the spectra between three different treatment groups in terms of collagen content (mineral to collagen ratios) and crosslinking. These results suggest that cryopreservation does not introduce microstructural alteration of the collagen matrix.

Alternatively, damage caused by the cryopreservation process could be structural in nature and induced by the formation of ice crystals or stresses borne by thermal expansion/contraction mismatch. The formation of water crystals can be dependent on multiple factors such as freezing rate, viscosity and freezing temperature.[157] A slow freezing rate has been shown to prevent crystal formation and reduce cell dam-

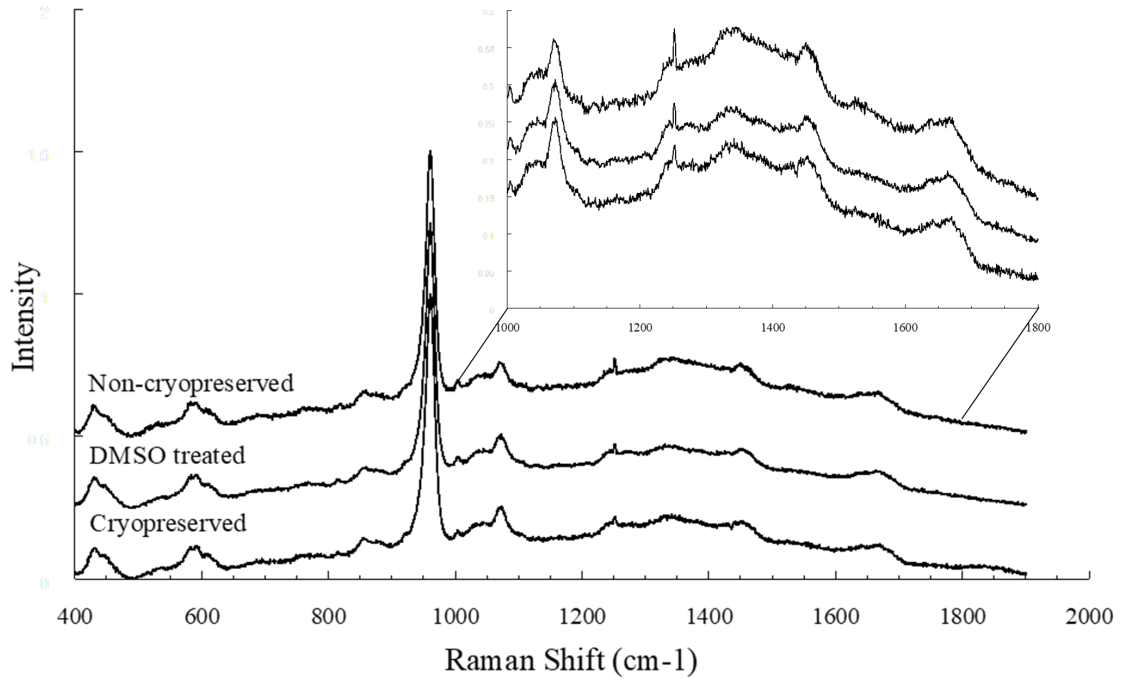

Figure 6.6: Details of results from Raman spectroscopy of the dentin samples, including the cryopreserved, non-cryopreserved and DMSO treated conditions.

age in the cryopreservation of soft tissues.[166] If a liquid has a high enough viscosity and the freezing rate is slow, it will form an amorphous solid during cooling rather than arrange into a crystal lattice. Adding solutes (i.e. cryoprotectants) aids this process by increasing the viscosity and decreasing the freezing temperature. Previous investigations involving cryopreservation of teeth showed that a cryoprotectant solution containing 10% DMSO is optimal. In fact, Schwartz et al. reported a 96% pulp viability in teeth autotransplanted after cryopreservation followed by proper DMSO treatment.[168] Hence, the lower fatigue strength of the cryopreserved dentin in the present study was not attributed to an inappropriate cryopreservation protocol.

The most plausible explanation for the lower fatigue strength of dentin from the cryopreserved teeth is that microcracks or other small defects were initiated as a result of the freeze-thaw cycle.[159] An increase in the population of intrinsic defects would accelerate the process of damage accumulation in the cryopreserved dentin during cyclic loading, and decrease the fatigue strength. Free fluid occupies about 22% of the total volume of dentin and the tubules are the major channels for diffusion within the tissue. Bound water represents up to 25% of the water in dentin and resides in interfibrillar water compartments.[125] As a result of cryopreservation, the volumetric expansion associated with the formation of ice crystals can induce mechanical stresses within and between the collagen fibrils that are detrimental. That damage is at a dimensional scale that is presently not able to be visualized.

There was microscopic evidence of cracking within the peritubular cuffs and unique disassociation between the cuffs and surrounding intertubular dentin in the cryopreserved teeth. Due to the higher mineral content of the peritubular cuff with respect to the intertubular dentin, the bound water is expected to reside in the intertubular dentin. That would cause expansion of the intertubular dentin during cryopreservation, while the peritubular cuffs undergo thermal contraction. Although this occurs on

the micro-scale, it could result in stresses that are large enough to damage the fibrils at the interface of the intertubular and peritubular dentin, and facilitate separation. Admittedly, this interpretation is highly speculative. The microstructure of dentin from the cryopreserved teeth does exhibit cracking of the cuffs, suggesting a tensile stress distribution. That damage provides supporting evidence that can be used to rationalize the significant reduction in the fatigue strength. If this damage is introduced by cryopreservation, it is unlikely that it will be repaired by remineralization cycles.

In the context of freezing to a very low temperature, differences in the thermal expansion coefficient between dentin and enamel are also relevant. A mismatch of this parameter could cause the development of microcracks that originate from restraints in the free expansion of the tissue. During the cooling process the enamel contracts first, and due to its low tensile strength may crack as a result of the induced stress. Brown et al. examined the effect of thermal cycling over temperatures ranging from 32 to 60°C on extracted human and bovine teeth.[169] Results of that investigation showed that thermal stresses caused by temperature cycling can induce microcracking and thermal fracture in both the dentin and enamel. Damage caused by temperature fluctuations of 28°C suggests that the thermal cycle associated with cryopreservation could potentially degrade the structural integrity of teeth as well, including both the dentin and enamel. If damage resulted from stresses induced by the differences in free thermal expansion/contraction of dentin and enamel, an evaluation of dentin beams cryopreserved after sectioning from the tooth could be revealing. That exercise is reserved for future study.

The primary objective of this investigation was to determine if cryopreservation caused a degradation in the structural integrity that is detrimental to the durability of dentin. Results indicate that cryopreservation of teeth reduces the fatigue strength

of dentin. Although that is an important finding to the practice of cryopreservation and its applications in dentistry, there are limitations to the investigation that are important to consider. This study was focused on dentin from the coronal third of young donor teeth. The root dentin is of equal importance to the structural behavior of teeth, and vertical root fractures initiate as a result of damage within the root. Future research should consider the spatial distributions in microstructure and properties of dentin in relation to the effects of the cryopreservation protocol. Damage resulting from cryopreservation is also possible in enamel but was not considered. A complimentary study focused on enamel is warranted. Furthermore, the degradation in fatigue resistance of dentin from the cryopreserved teeth was hypothesized to result from mechanical damage introduced by the cryopreservation treatment. Microcracks were evident in the peritubular cuffs of the cryopreserved teeth and there was evidence of weakening of the interface between the peritubular cuff and surrounding intertubular dentin. Nevertheless, microcracks were not observed within the volume of the dentin from the methods of evaluation adopted, just the surfaces of the beams. The detection of microcracks in dentin is a challenging process and could be pursued in more detail in future studies.[170] Further investigation will be required to establish the specific mechanisms responsible and if the extent of degradation can be reduced by introducing changes to the cryopreservation protocol.

6.5 Conclusion

An experimental evaluation concerning the effects of cryopreservation on the structural integrity and mechanical properties of coronal dentin from cryopreserved teeth was conducted. There was no significant difference between the flexural strength of dentin from cryopreserved teeth, DMSO treated, and non-cryopreserved (control) teeth. However, dentin from the cryopreserved teeth exhibited almost 20% reduction

in fatigue strength, which was significantly lower than the non-cryopreserved control. Therefore, within the limitations of this study, cryopreservation does cause degradation of the structural integrity of dentin, which is detrimental to its durability. Results of Raman spectroscopy showed no evidence of degradation in the collagen matrix as a result of cryopreservation or 10% DMSO cryoprotectant treatment. An analysis of the microstructure suggests that there is damage introduced in the peritubular cuffs and at the interface of the peritubular cuffs and surrounding intertubular dentin. Based on these findings, the degradation in fatigue responses appears to be attributed to mechanical damage that is introduced by the cryopreservation treatment.

Chapter 7

ODONTOBLAST APOPTOSIS AND INTRATUBULAR MINERALIZATION OF SCLEROTIC DENTIN

7.1 Introduction

As expressed in previous chapters, dentin undergoes a gradual transition in the microstructure with increasing age that causes an increase in translucency, a process regarded as sclerosis.[126] The changes causing dentin to appear more translucent is a result of occlusion of dentinal tubules by a mineral substance.[22] With the progression of sclerosis, evaluation of the flexural strength of dentin have revealed that there is a decrease in strength of almost 20 MPa per decade of life that begins shortly after reaching adulthood.[103]

A dissolution & re-precipitation theory has been proposed to explain the formation of intratubular minerals.[30] However, the exact mechanism of age-induced sclerotic dentin and its relationship with mechanical degradation is not fully understood. Previous studies have evaluated sclerosis of impacted teeth from senior patients.[28] Results showed that there was minimal evidence of filled tubules in unerupted teeth from older individuals, which implied that the physiological process responsible for sclerosis might be initiated by the stresses imposed by the oral environment. However, there are also contradictory reports suggesting that the amount of sclerosis increased linearly with age and it was not markedly affected by the tooth function or external stimuli encountered during life.[29]

Although dentin is an acellular biological hard tissue that cannot be remodeled, cells of the pulp play important roles in supporting the functionality of dentin. Odon-

toblasts are dentin-secreting cells that retain cellular activity for the whole life of a healthy tooth.[171] Once teeth are completely formed and erupted, odontoblasts transform into a mature stage that allows for secondary and reactionary dentin secretion. Odontoblasts are also critically involved in the transmission of sensory stimuli from the dentin-pulp complex.[172] While their cell bodies are attached to the pulp chamber wall, odontoblasts extend their odontoblastic processes within the dentinal tubules that are filled with extracellular fluid. This extracellular fluid is rich in calcium ions and critical to the deposition of mineral within the dentinal lumens.[173]

The odontoblast cell vitality is critical to dentin and has a direct relationship to cellular apoptosis,[32] as well as the incidence of dentin fracture. A reported study showed that non-vital teeth account for nearly 90% of VRF.[10] The age-related cellular changes, including pulp complex fibrosis, odontoblast density, cellular morphology and apoptosis, are all potential contributing factors to the age-related alteration of dentin structure.[174, 175] Recently, it has been proposed that apoptosis of odontoblasts may be the key-factor that is responsible for the development of physiologic sclerotic dentin.[31] Indeed, increased odontoblast apoptosis has been found to have positive relation with age.[32, 174] However, these studies did not consider the spatial apoptotic effects on dentin microstructure and its consequence to fracture. The apoptosis of odontoblasts requires detailed investigation in relation to the aging process and the microstructural changes that evolve in dentin.

In this study, age-related odontoblastic changes in terms of cell density and apoptosis were characterized to correlate with the changes of chemical and mechanical properties of sclerotic dentin. The aim of this study was to determine the age-related odontoblastic apoptosis and its relationship with mechanical and compositional changes of radicular dentin.

7.2 Materials and Methods

Twelve human non-carious vital teeth were obtained from participating clinics following an exempt protocol approved by the Institutional Review Board of the University of Washington. The age and gender of the donor were obtained with each tooth. The teeth were placed in 10% PBS buffered formalin immediately after extraction and then carefully inspected for the presence of flaws or decay. These teeth were further divided into young (age ≤ 25, n = 6) and old (age ≥ 60, n = 6) groups. Within 2 hours after extraction, these teeth were decalcified by Morse's solution at 4°C for 72 hours. The demineralized teeth were processed for histology using a graded ethanol series followed by xylene and embedded in paraffin. Three serial cross-sectional sections representing each thirds of the sample were cut at 5 μm thickness using a microtome and subsequently stained with hematoxylin and eosin ($H\&E$) (Fig. 7.1A). A cell density analysis was conducted with $H\&E$ staining in the apical, middle and coronal thirds of the root in both young and old group.

For immunohistochemistry staining of cleaved caspase 3, rabbit polyclonal anti mouse (CC3, 1:100 dilution, BioCare Medical, Cat.No. CP229B) were performed at the University of Washington Histology and Imaging Core. First, the slides were baked for 30 minutes at 60°C and deparaffinized on the Leica Bond Automated Immunostainer (Leica Microsystems, Buffalo Grove, IL). Antigen retrieval was performed on all slides with EDTA buffer for 10 minutes at 100°C. All subsequent steps were performed at room temperature. Blocking was performed with 10% normal Goat Serum in TBS for 20 minutes. The primary antibody diluted in Leica Primary Antibody Diluent (Leica Microsystems, Buffalo Grove, IL) was applied for 60 minutes. A secondary goat anti-rabbit horseradish peroxidase conjugated antibody (8 μg/mL) was then applied for 8 minutes. Additional blocking was adopted with Leica peroxide block for 5 minutes. Antibody complexes were visualized using Leica Bond Mixed

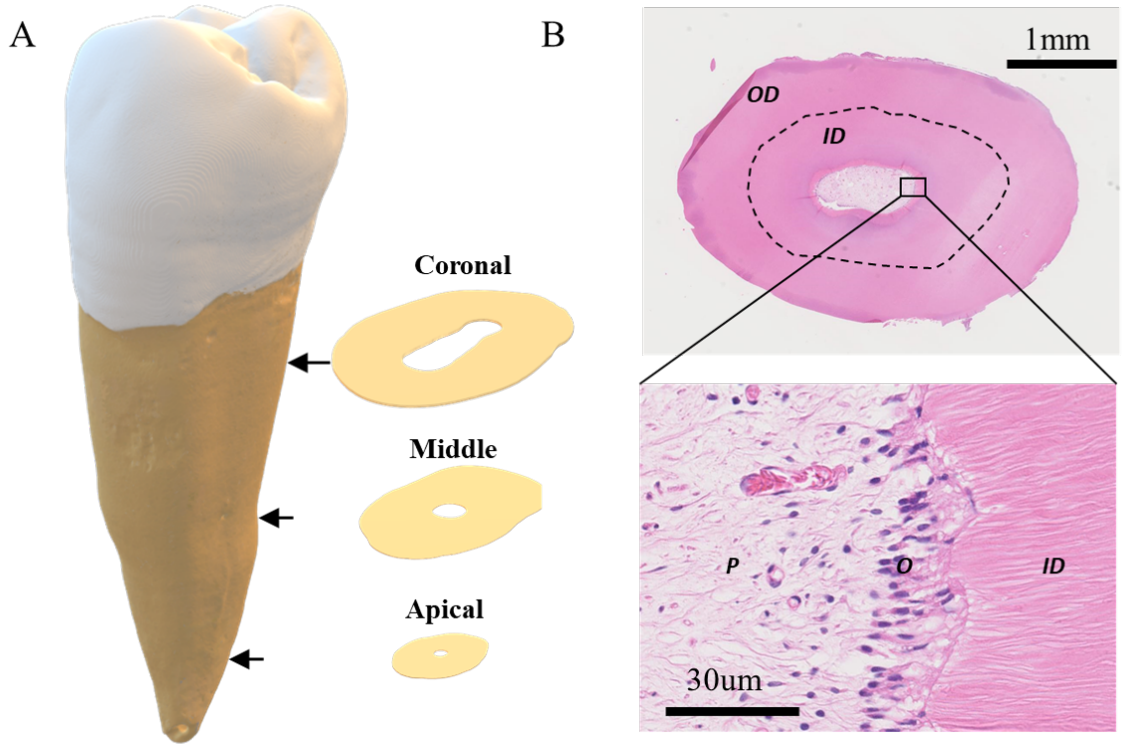

Figure 7.1: Dentin subjected to immunohistochemical staining. (A) Schematic of dentin slices cut from three different locations along the root. (B) Image of hematoxylin and eosin–stained sections showing cellular detail of areas used for cell counts and apoptosis analysis. Dentin slices were divided into four regions for Cleaved Caspase-3 staining. OD: Outer Dentin. ID: Inner Dentin. O: Odontoblast layer. P: Pulp Chamber.

Refine (DAB, 3,3'-diaminobenzidine) detection at 2× for 10 minutes. Tissues were counterstained with hematoxylin counterstain for 4 minutes followed by two rinses in water. Unless otherwise specified all reagents were obtained from Leica Microsystems.

Glass slides with dentin sections were prepared by NanoZoomer (Hamamatsu Photonics, Japan) to obtain full scanning of each section. Four regions were selected to analyze positive apoptosis staining: A: Outer dentin. B: Inner dentin. C: Odontoblast layer. D: Pulp Chamber (Fig. 7.1B). In each section, eight pictures were taken around the pulp chamber for each region. Three pictures were randomly selected from the eight pictures taken from each region at 40× magnification with picture resolution of 1920 × 1088 pixels. A total of 432 pictures were taken (6 teeth from each age groups, 3 section per tooth, 4 regions per section, and 3 pictures per region). The pictures were further assessed using IMAGEJ software (National Institutes of Health, MD) with color thresholding in the outer dentin, inner dentin, odontoblast layer and pulp chamber to obtain cleaved caspase-3 staining area fraction.

One young sample without any evidence of sclerosis and one old sample with apparent sclerotic dentin were selected for chemical composition analysis and mechanical characterization. Selected samples were sectioned along transverse axis at the middle third of the root using same slicer/grinder. The specimens were embedded in cold-cured epoxy resin. The exposed dentin in the resin mount was polished following established procedures.[73]

The polished samples were imaged by Energy-dispersive X-ray spectroscopy (EDS) using a scanning electron microscope (SEM) (Sirion XL30, FEI, Hillsboro, OR). The chemical composition of the peritubular and intertubular dentin were evaluated using single point detection. The samples were placed in a desiccator to dry for a minimum of 48 hours and then sputtered with a carbon coating. EDS spectra were normalized to 100% after the exclusion of the carbon content by specifying coating thickness. Three

hundred thousand counts were recorded per spot to achieve statistical significance for point signal acquisition. Ten points at each peritubular and intertubular site were analyzed per sample.

Scanning dynamic mechanical analysis was performed using Scanning Probe Microscopy (SPM) on a commercial system for nanoindentation (Hysitron Inc., Model TI980 Triboindenter, Minnesota, MN) to study the nanoscopic mechanical properties of the peritubular and intertubular dentin. The scanning-based evaluations were performed using a Berkovich diamond indenter with 90 nm nominal tip radius. A 4 μN static indentation load and a dynamic sinusoidal load of 2 μN were applied following Ryou et al.[136] The scanning mode nanoDMA was conducted using a window of evaluation of 20 μm $\times$ 20 μm. To minimize the effects of dehydration to the mechanical behavior,[142] the nanoDMA evaluations were performed in the hydrated condition with treatment of 99.4% ethylene glycol (EG). Previous results have shown that there is no influence of EG on the moduli estimated using scanning-based nanoindentation.[141]

All data were presented as means $\pm$ standard deviations. One-way analysis of variance (ANOVA) followed by the Tukey test was used to analyze staining area fraction of four regions between young and old group. $p < 0.05$ was regarded as statistically significant.

7.3 Results

A quantitative analysis of the cell density measurements is shown in Table 7.1. The density of odontoblasts about the pulp perimeter (per 100 μm) in both the young and old teeth decreased from the coronal to radicular dentin. The difference is significant throughout the length of the root canal in all three physiological locations. The largest decrease occurred in the apical third of the root. Surprisingly, the odontoblast cell

Table 7.1: Odontoblast density (/100μm) and percentage decrease of apical-, middle- and coronal-third of root dentin. * indicates significant difference between age groups ($p < 0.05$).

	Young	Old	Percentage Decrease
Coronal Third*	39.26±6.67	23.50±6.75	40.15%
Middle Third*	26.62±3.26	12.56±1.91	52.81%
Apical Third*	10.24±4.50	2.95±1.05	71.19%

layer in the apical third of teeth in the old group almost disappeared in 3 out of the 6 samples, with no visible cell bodies compared to young counterparts as shown in Fig. 7.2.

Fig. 7.3 shows images of CC3 staining for four areas of interest, namely the outer dentin, inner dentin, odontoblast layer and pulp chamber. Odontoblast cell extensions were partially stained by black color in old samples, as noted by the arrows. The most positive apoptosis signal was observed at the inner dentin where the odontoblast extensions are located. The odontoblast apoptosis staining fractions for regions are presented in Table 7.2. The middle third of the dentin was chosen to account for the tubule size and visibility of cells. The apoptosis staining was significantly higher in the outer dentin, inner dentin, odontoblast layer and pulp in the old teeth when compared to young teeth ($p \leq 0.05$). The largest difference occurred in the outer and inner dentin regions where the odontoblast extension processes occupy dentinal lumen.

SEM images for young and old dentin constituents are shown in Fig. 7.4A. Fig 7.4B shows the relative weight percent of phosphorous and calcium in the peritubular and intertubular dentin of the young and old teeth measured by EDS. Phosphorous content remains unchanged between young and old teeth in both the peritubular and intertubular dentin. Peritubular dentin has increased calcium from 37.5% to 43.0% whereas intertubular dentin has a decrease in calcium content from 48.8% to 39.6%. The stoichiometric ratios of calcium and phosphorous in the young and old peritubular dentin are 1.414 and 1.951, respectively. Both show statistical difference. The ratios in intertubular dentin are 1.748 and 1.682 for young and old teeth, respectively, which are not significant different.

Representative property maps of the elastics modulus obtained from scanning-based nanoindentation are shown in Fig. 7.4C. The average modulus for peritubular and intertubular dentin of young teeth were 36.2 $\pm$ 4.4 and 18.2 $\pm$ 6.2 GPa respectively. In old teeth the moduli of the peritubular and intertubular dentin were 41.9 $\pm$ 5.8 and 23.1 $\pm$ 2.3 GPa respectively. Both constituents showed significant higher modulus in old dentin. The change is most significant in the intertubular collagen matrix where the elastic moduli almost doubled from 12.7 $\pm$ 3.3 GPa to 23.9 $\pm$ 2.1 GPa.

7.4 Discussion

Dentin sclerosis is a continuous process through life. Healthy dentin from young teeth is opaque due to the light scattering that is caused by the dentinal tubules. The gradual transition of dentin transparency with aging has been attributed to the occlusion of dentinal tubules by mineral substance with a similar refractive index to that of the rest of the dentin.[22] Previous studies have shown that sclerotic dentin is subject to a detrimental mechanical degradation.[103, 71] The decrease in strength is

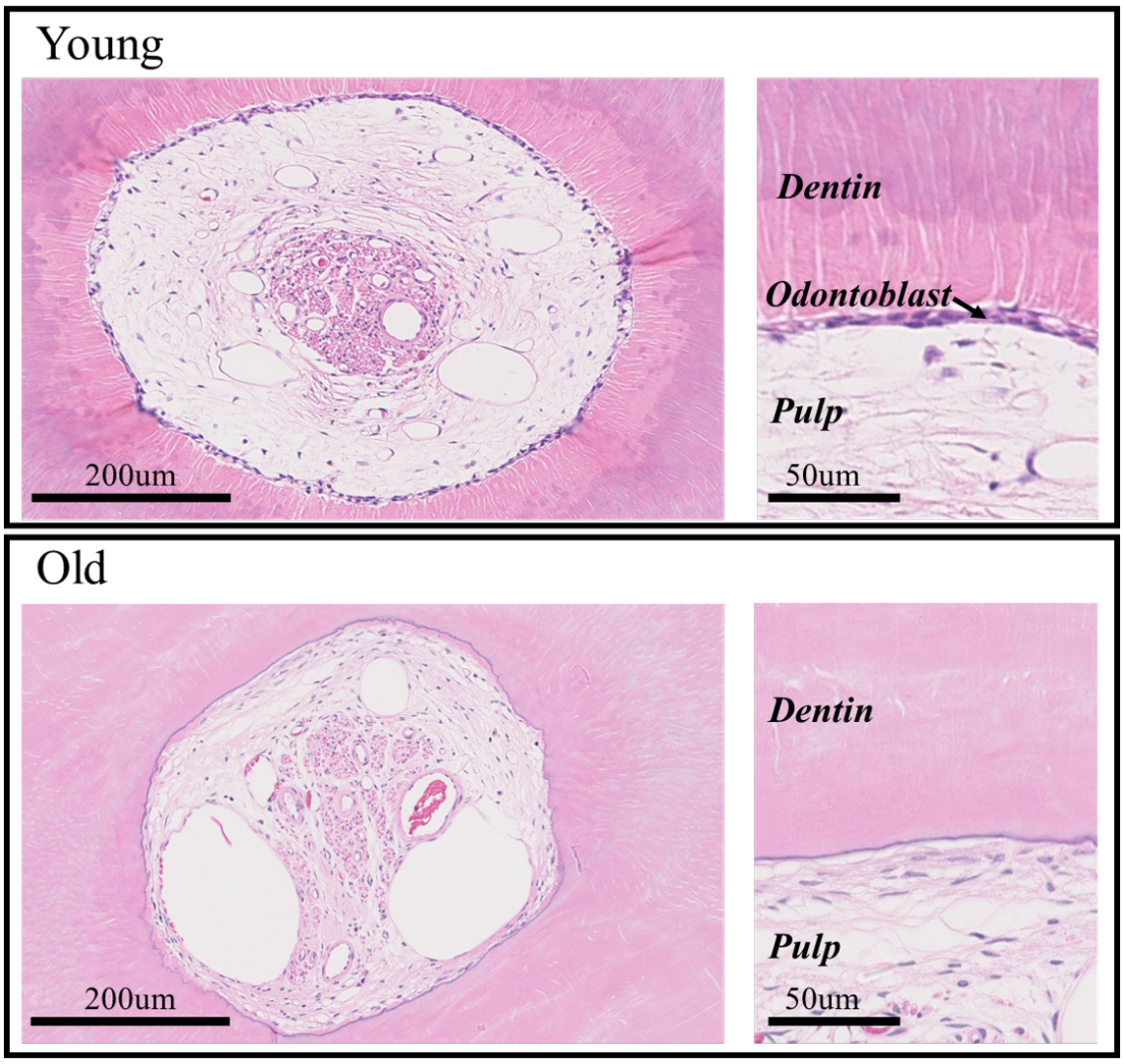

Figure 7.2: Representative images of the root canal from young and old teeth after standard H&E staining. A zoomed picture shows that the odontoblast layer in young dentin is evident whereas the old dentin has no obvious odontoblast layer.

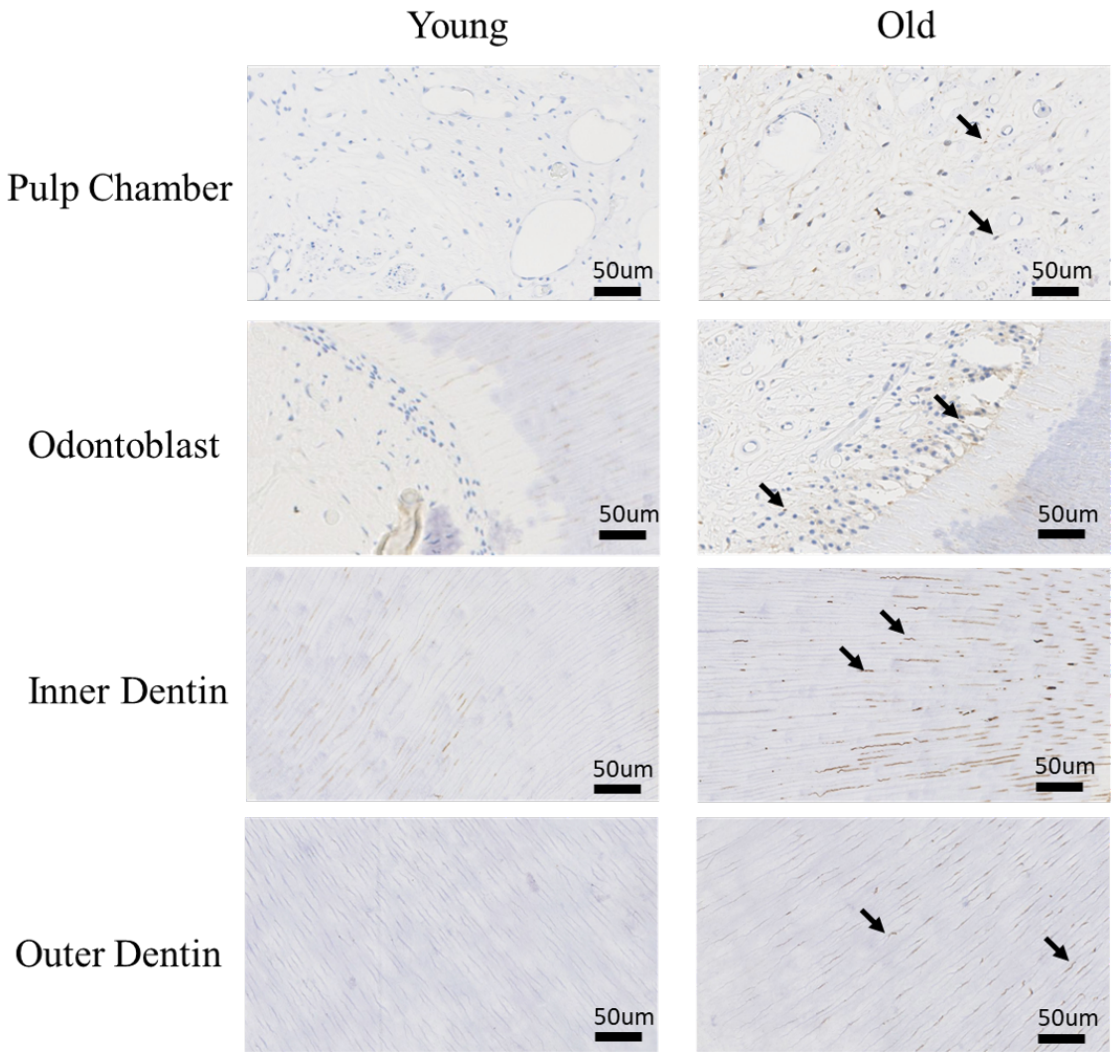

Figure 7.3: Optical images from the inner and outer dentin of young and old teeth showing Cc3 staining inside the dentinal tubules. Arrows show positive apoptosis staining from the old dentin, especially in the inner dentin area.

almost 20 MPa per decade starting from the mid-30s age. In dental practice, tooth fracture most commonly occurs in patients between 40 and 60 years old.[42, 38, 39, 40]

The number and distribution of odontoblasts change as a consequence of aging. The odontoblast cell number decreases spatially and chronologically as shown in Table 7.1. The cell density is lower in the old samples regardless of location. The largest decrease occurs in the apical third of roots, where a 71.2% decrease was observed. This finding is coincident with previous study on the overall cell density decrease in root dentin with aging.[176] Furthermore, in both young and old groups, odontoblast density is significantly lower in the radicular pulp when compared to the coronal pulp, with a 75% decrease in young teeth and nearly 90% in old teeth. The disappearance of odontoblasts resulted from aging and the narrowing pulp chamber make a concerted effect on the old radicular pulp, where odontoblast cell body is almost invisible as shown in Fig. 7.2. Since the disappearance of odontoblasts could result from programmed cell death (apoptosis), Franquin et al. performed apoptotic analysis of odontoblast and pulp cells and related the decline of cell number to apoptosis resulting pulp chamber size reduction.[32]

Odontoblast apoptosis within tooth pulps is a key factor in regulating pulp chamber remodeling.[177] However, it is unknown whether odontoblast extensions within dentinal tubules can be reached and regulated by the stimuli from pulp chambers, due to positive fluidic pressure outward[178, 179, 180] and limited tubule lumens diameter.[181, 182] In this study, the odontoblast apoptosis was characterized inside the dentinal tubules for the first time. Higher apoptosis staining was observed in all four locations of the old dentin, with the largest difference in the dentinal tubules. It is evident that caspase-3 stained signals(dark areas) are present inside dentinal tubules for the old dentin in Fig. 7.3, which indicates apoptotic odontoblast processes. The same trend has been found in both inner and outer dentin. In young dentin, the

staining is relatively low inside the dentinal tubules.

Occlusion of the dentinal lumen does not start simultaneously in the root and crown. Sclerosis begins in the apical dentin (at the root tip) and progresses towards the crown with increasing age. The differences in distribution and extension of sclerosis implies that a complex underlying mechanism is controlling the tubule occlusion.[183] It was suggested that odontoblast apoptosis could be the key contributor to the physiological sclerotic dentin formation by affecting the calcium and phosphorous ion balance in the dentinal tubule extracellular fluid.[31] In our study, the apoptotic odontoblast cell processes can be seen in the dentinal tubules and may contribute to the changes of chemical components of extracellular fluid in Table 7.2. It is known that hydroxyapatite crystallization is controlled by the ratio of the pyrophosphate (PPi) and hydrogen phosphate (Pi).[184, 185, 186, 187] The lack of phagocytosis inside the dentinal lumens can trigger the release of these contents from apoptotic cells necrosis[188] and disturb ionic balance, further inducing intratubular mineralization and occlusion.[189] Moreover, dynamic equilibrium of calcium/phosphorous ion cannot be easily regulated due to the outward physiological pressure from the pulp toward the cementum side of the roots.[190]

Our results (Fig. 7.4B) also showed that calcium from intertubular and peritubular dentin changed while the phosphorous remain unchanged. It appears that intratubular mineralization carries higher calcium ions at a cost of intertubular minerals, which support the dissolution & re-precipitation theory.[30] The elastic modulus of both the peritubular and intertubular dentin increased as shown in Fig. 7.4C. Nonetheless, the most significant change occurs in the intertubular dentin, where the elastic modulus almost doubled from young to old dentin (Fig. 7.4C). Extensive research has focused on the fracture behaviors of dentin with obturated lumens.[103, 119, 191] Counterintuitively, intertubular dentin exhibits lower elastic modulus if it is deficient

Table 7.2: Apoptotic staining area fraction (positive Cc3 signal area / Total area) of pulp chamber, odontoblasts, inner and outer dentin for young and old teeth. * indicates significant difference between age groups ($p < 0.05$). † indicates significant difference between locations($p < 0.05$).

	Young	Old
Outer Dentin*	0.011±0.002	1.547±0.399†
Inner Dentin*	0.142±0.067	2.605±1.018†
Odontoblast Layer*	4.045±1.494	7.467±2.95†
Pulp Chamber*	0.733±0.075	4.317±1.731†

in calcium.[192] Given the fact that intertubular dentin is a bio-composite with a collagen content of 30%,[193] it is plausible that the increasing stiffness stems from collagen alterations. This topic requires further investigation.

There are several limitations in the present study. First, the calcium/phosphorous ion was measured *in vitro*. Samples were dehydrated and sputter coated for SEM/EDS characterization. There are currently no methods to measure the calcium and phosphorous concentration *in vivo*. Second, samples collected from patients may deviate from each other due to factors such as oral conditions, gender, and genetic background. Animal models may be helpful in this effort since many of such factors can be controlled.

7.5 Conclusion

A decrease in the odontoblast density in the apical third of teeth is predominant with aging as very few odontoblasts were identified in the old teeth. Odontoblast apoptosis is present in the dentinal tubules, which is possibly responsible for intratubular mineralization due to imbalance of calcium and phosphorous ions. Odontoblast apoptosis appears to start at the cell extension in the tubule lumens, proceeds from outer to inner dentin, and contributes to the stoichiometric Ca/P ratio in peritubular dentin. However, the mechanical degradation seems to be unrelated to Ca/P ratio in the intertubular dentin and would require further investigation.

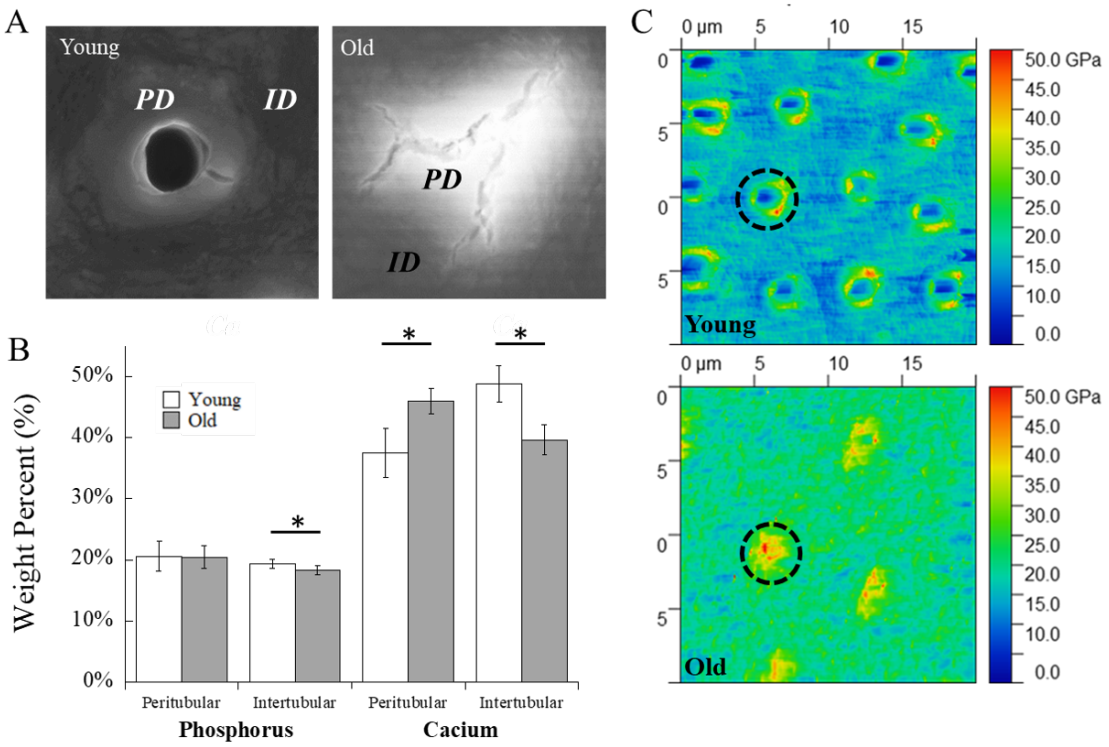

Figure 7.4: Energy-dispersive X-ray spectroscopy(EDS) analysis of peritubular dentin (PD) and intertubular dentin (ID) from young and old tooth. (A). SEM image of dentinal lumen showing peritubular and intertubular dentin. (B). Weight percentages of phosphorus and Calcium from young and old dentin by EDS. (C). Elastic moduli of young and old dentin from nanoDMA scanning. The dotted circle represents one dentinal lumen from young and old teeth. The magnitude of elastic modulus is represented by color map from red (high, 50 GPa) to blue (low, 0 GPa) as shown on the side of graph. * indicates significant difference ($p < 0.05$).

Chapter 8

CLOSING REMARKS AND CONCLUSIONS

8.1 Summary of results and Conclusions

From fish scales to human teeth, most mineralized tissues found in biological systems are considered bio-composites. The structure-property relationships play a crucial role in supporting the functions of these tissues. Many previous studies concerning aging of dentin have led to the current topic on why aging and dental treatments may cause a synergism in the degradation of dentin that results in a higher probability of tooth fractures. We have adopted and utilized conventional and cutting-edge approaches from the macro- to micro-scale and ultimately to the nano-scale to elucidate properties related to both the organic and inorganic constituents. Utilizing complementary analysis techniques such as Raman spectroscopy and scanning electron microscopy/energy-dispersive X-ray spectroscopy, the microstructures and chemical compositions can be linked to specific mechanical properties and their relationships can be better understood.

Efforts of this research from the past five years have been focused on the dentin of human teeth. The collective results showed that dentin undergoes a decrease in resistance to fatigue and fracture with chronological aging, indicating that the older age group patients have higher incidence of tooth fracture. These changes are concentrated in the root, thereby causing higher risk of root fracture. Microstructurally, dentin undergoes a mineralization process with aging that is regarded as dentinal sclerosis. This change is accompanied by collagen crosslinking which is considered more detrimental for the embrittlement of dentin because of the large proportion of

collagen and its importance to toughness. We have shown evidence from compositional measurements and nano-scale dynamic mechanical analysis, that degradation of the collagen matrix consisting of higher inter-molecular crosslinking is responsible for degradation in the resistance to fracture of the root.

The etiology of dentinal sclerosis has remained a mystery and we pursued answers from an assessment of cellular apoptosis. Within the dentinal tubules, where odontoblast apoptosis cannot be regulated by phagocytosis, the chemical contents such as pyrophosphate can be released from necrotic apoptosis bodies of odontoblast extension. Mineralization and demineralization is controlled by these crucial cytoplasms and, hence, sclerosis is apparently affiliated with apoptosis. Clear uncleaned apoptotic bodies were observed by cleaved caspase-3 staining showing old dentin is more likely to suffer from apoptotic necrosis.

We also explored why root canal therapy, one of the most common and well established methods to preserve the natural tooth that has suffered from damage of the pulp, can induce acceleration of biological aging of teeth. The dentin of teeth that have received root canal therapy has significantly lower resistance to fracture and fatigue. The structure-property relationships of teeth subjected to endodontic treatment and post-treatment clinical function were evaluated. Fracture properties alteration have been associated with lack of ability to bind water due to collagen crosslinking via digital image correlation. This change in properties can be substantial and compound with the effects of natural sclerosis and unbound water loss. The age-related degradation of teeth that have received root canal treatment is significantly higher than that of untreated teeth, even if they remain in the same patient and exposed to the same oral environment.

Finally, we have investigated effects of cryopreservation on the structural integrity and mechanical properties of dentin. Cryopreservation is a sound alternative to pre-

serve a patient's natural teeth by ultra low temperature storage. Stored teeth can be used for future restoration or stem cell bank. Our experiments showed that cryopreserved teeth had significantly lower resistance to fatigue failure after the freezing and thawing process by creating defects between mineral and organic matrix.

8.2 Future directions

Bone suffers from osteoporosis and undergoes a loss of mineral over time. Causes of osteoporosis includes lack of calcium and hormone imbalance. The aging process of the tooth is the opposite to that in bone but results in a similar outcome, i.e. a higher risk of tissue fracture in senior patients. The highly mineralized dentin undergoes a decrease resilience with aging, which is likely to cause acute brittle fractures. The first aim of future work should focus on elucidating the mechanism of mineralization in sclerotic dentin inside the dentinal tubule lumen. Current knowledge of sclerosis is not robust or complete. We hypothesized that odontoblast apoptosis and accompanying phosphate/pyrophosphate imbalance to be the originator of sclerosis but there is lack of direct evidence. Specifically, we believe the calcium and phosphate metabolism of the human body is critical to the dentin sclerosis because numerous other research studies have found that the high levels of phosphates accelerate signs of aging in many different organs. Understanding dentin sclerosis can benefit not only dentistry, but the overall aging theory of human body towards the development of anti-aging medicine.

The second future direction involves the structure-property relationships in enamel with aging. Two aspects of physical degradation dominate the aging process of enamel. Firstly, the cohesive non-collagenous protein matrix between the enamel prism crystallites undergoes continuous degradation due to age-induced imbalance of the oral pH.[18] Despite the fact that total removal of inter-prism protein accounts for over 85% reduction in fracture toughness in human enamel, [194] there is a lack of accurate experimental measurements to quantitatively determine protein loss during the aging process because of the low organic content in enamel.[195] On the other hand, chemical alteration to the mineral can also lead to an increase in crystal density and permeability.[196] The enamel mineral content is mainly composed of B-type

carbonated hydroxyapatite that has high crystallinity and low solubility compared to other mineralized tissues such as bone and dentin. The crystal properties are largely dependent on the composition of hydroxyapatite in enamel which is highly non-stoichiometric with both cation and anion impurities.[197] The mineral cation, hydroxyl group and phosphate group substitutions are potentially dependent on the patient age and large variation has been found between different age groups.[198] However, there is no systematic study and/or statistically reliable study revealing the age dependency of ionic exchanges in enamel.[199]

In the interdisciplinary fields of materials science and biology, researchers seek to understand the natural biological materials in order to design and synthesize novel materials for biomedical applications and also to take inspiration for biomimicry and biomimetics. The natural aging of teeth presently seems unstoppable. But with proper tuning of materials properties, it may be possible to avoid fractures. The ultimate goal will be slowing, stopping and even reversing the materials degradation in human teeth. However, the structure-property relationships are usually too complex to be captured by conventional methods that rely on correlation analysis. The third aim of future works should build a model on crosslinking of hard tissue collagen matrix for understanding and prediction fractures. Machine learning methods have made some exciting contributions to the study of aging. Specifically, machine learning algorithms will automatically select statistical modeling to understand the complex high dimensional concepts that are usually hard to fit by traditional models. The Arola lab has recently collaborated with the Colgate-Palmolive company to study the aging of enamel. We expect that the same methods can be applied to dentin to benefit more patients in our aging society.

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========== Appendix A

Appendix A

PUBLICATIONS

Journal Publications

1. **Yan, W.**, Renteria, C., Huang, Y., Arola D.D., A Machine Learning Approach to Investigate the Materials Science of Aging Enamel: Part I. (2020) (Submitted to Dent. Mater.)

2. **Yan, W.**, Jiang, E., Renteria, C., Paranjpe, A., Arola D.D., Zhang, H., Odontoblast Apoptosis and Intratubular Mineralization of Sclerotic Dentin with Aging. (2020) (In preparation)

3. **Yan, W.**, Jiang, H., Paranjpe, A., Zhang, H., Arola D.D., Dehydration Induced Shrinkage in Endodontically Treated and Untreated Teeth. (2020), J. Endod. (Under Review).

4. **Yan, W.**, Chen, H., Fernandez-Arteaga, J., Paranjpe,A., Zhang,H., Arola, D.D., Root Fractures in Seniors: Consequences of Acute Embrittlement of Dentin. (2020), Dent. Mater. 36 (11), 1464-1473.

5. Sun, F., Hung, H.C., **Yan, W.**, Wu, K., Shimchuk, A., Gray, S., He, W., Huang, X., Zhang, H., Inhibition of Oral Biofilm Formation by Zwitterionic Nonfouling Coating. (2020) (Submitted to J. Biomed. Mater. Res. B.)

6. **Yan, W.**, Montoya, C., Oilo, M., Ossa, A., Paranjpe, A., Zhang, H., Arola, D.D., Contribution of Root Canal Treatment to the Fracture Resistance of Dentin. (2019), J. Endod. 45 (2), 189-193.

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2. **Yan, W.**, Jiang, H., Paranjpe, A., Arola D.D., Zhang H. Odontoblast Apoptosis Contributes to Degraded Mechanical Properties in Root Dentin. 97th General Session and Exhibition of the IADR. Abstract 3185114.

3. Song, J., **Yan, W.**, Chen Y., Øilo, M., Arola, D.D. The Risk of All-Ceramic Crown Fractures due to Poisson's Effect. 47th AADR/CADR Annual Meeting. Abstract 0110.

4. Tenwalde, M., **Yan, W.**, Zhang, H., Arola, D.D. Cryopreservation of Teeth: Hidden Defects and Potential Consequences. 47th AADR/CADR Annual Meeting.

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