

Tokyo Medical and Dental University
Faculty of Dentistry
Graduate School of Medical and Dental Sciences
Oral Health Science Department
Cariology and Operative Dentistry



**Simulation of Natural Caries-affected Dentin using an Artificial
Caries Model for *in vitro* Studies**

Presented by

Gerardo José Joves Méndez

Thesis presented for the degree of Doctor of Philosophy in Dental Science

Promoter: Professor Dr. Junji Tagami
Advisors: Dr. Toru Nikaido and Dr. Go Inoue

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DEDICATION

I dedicate this work to the light shining in my life, my family, my beloved wife Dr. Andreina Martin for her endless love, understanding and support; my little angels, my dear daughters María Victoria and Dulce Maria.

This work is also dedicated to the source of my motivation, my mother Merka Méndez and father Oscar Joves.

I also dedicate this thesis to the memory of my grandfather Miguel Méndez Rubín.

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SUMMARY

Introduction

A tooth is a mineralized substrate that is always susceptible to suffer “Caries”. Caries is a multifactorial disease that in the simplest form occurs in the presence of a cariogenic biofilm attached to the tooth surface, producing acids from fermentable carbohydrates. Pathologically, dentinal caries is a multi-layered disease, in which the outer dentin is highly infected with proteolytic degradation of the collagen matrix (irreversibly demineralized) and the inner dentin has been reversibly attacked because the collagen matrix is not severely damaged, giving it potential to be repaired.

Different methods are clinically used to remove the infected dentin, ranging from pain, color, tactile hardness and dye staining to the use of self-limiting burs, chemical agents or lasers; however, there is no still gold standard method established for the caries removal. The use of dye staining solutions has been useful to differentiate the infected layer of the caries process. With the use of this approach, the dentin that is affected by bacteria, but not invaded, can be preserved. This inner affected dentin is clinically defined as the so called “caries affected-dentin”.

According to the minimal invasive (MI) concept, the caries-affected dentin should not be removed when caries is excavated; therefore, it is the main remaining substrate for interaction with the dental restorative materials. It has been well accepted that the use of direct resin composite restorations preserves sound structures and make the caries treatment more conservative, but the bonding performance of the contemporary adhesive materials has been influenced by different characteristics or properties of this tissue. Some of the main characteristics are:

mineral density, morphology and hardness.

Due to the previous characteristics, the standardization of natural caries-affected dentin for *in vitro* studies is difficult to establish and several samples are needed for a single test. Having this consideration, many authors have attempted the creation of artificial caries-like methods to test the performance of dental materials and the resulting substrates have been compared to sound dentin or to another type of methodology for the creation of it, not with the natural caries process.

The standardization of an artificial caries-affected dentin model with regard of mineral content, morphology, bond strength and hardness is being necessary to make the laboratory tests easier and comprehensible with reliable results. Therefore, the main purpose of this research project was to create a standardized caries-affected dentin model and compare it to the natural caries-affected dentin process with regard of mineral content, morphology, bond strength and hardness.

Materials and methods

A total of sixty-two human extracted teeth were used in this project. The methodology was divided into two studies:

Study 1 consisted in the use of a demineralizing method to create a standardized *in vitro* artificial caries-like lesion. A total of twenty-three carious teeth with dentin involvement were selected. The outer soft dentin was removed using a spoon excavator and then a dye solution to stain the residual infected-dentin. In order to obtain a clinically-relevant caries-affected dentin on the reduced occlusal surface, a round tungsten carbide bur attached to a low- speed hand piece without water was

used to remove the stained dentin. Thirty-four non-carious teeth were used to create artificial caries-affected dentin (ACAD) lesions. The NCAD and ACAD were compared in terms of mineral density, morphology and mechanical properties using microtensile bond strength. Transverse microradiography (TMR) was used to analyze mineral density. Scanning electron microscopy (SEM) was performed to check the morphology. The mechanical properties evaluation with regard of bonding ability, the microtensile bond strength (μ TBS) assessment was used. Two two-step self-etch adhesive systems and a resin composite to build up the teeth were used to perform the test.

Study 2 assessed the mechanical properties of intertubular dentin with regard of hardness of three substrates (n=15) including SOUND dentin, NCAD and ACAD using nanoindentation. Natural and artificial carious specimens were prepared as study 1, except sound teeth that did not need any special preparation. All specimens were prepared and subjected to the nanoindentation experiment according to previous reported protocols. A total of three hundred and twenty indentation marks were made on each sample. The nanoindentation software analyzed the data and calculated hardness from the projected area under a regime of 5 mN load.

Results

Study 1: Mineral density of NCAD and ACAD obtained by TMR was not significantly different up to a depth of 150 μ m. The morphology observation using SEM demonstrated that NCAD contains intratubular calcium-phosphate casts and differs from the ACAD where dentinal tubules remained opened. The μ TBS result was not

statistically different between NCAD and ACAD when two-step self-etch adhesive system was used.

Study 2: The intertubular dentin of NCAD revealed higher variation than ACAD and SOUND dentin by large standard deviations; meanwhile, ACAD and SOUND showed less standard deviation variation. There was found a superficial similarity of intertubular dentin hardness between ACAD and NCAD up to an indentation depth of 130 μ m. At deeper areas NCAD consistently showed lower hardness compared to both ACAD and SOUND dentin.

Discussion

It is known that the bond strength values of sound dentin in *in vitro* experiments are usually higher; on the other hand, when NCAD is being used as a testing substrate, the strength is lower. The possible reason is that the mineral density of the testing substrate may play an important role in the performance of adhesive materials. It was suggested that cavities with floor near the pulp, the bond strength is lower than superficial coronal dentin. In NCAD the speculated reason of lower bond strength is due to the process of de- and remineralization that makes the extrafibrillar minerals of the collagen matrix to be reduced and the adhesion of resin monomers with the hydroxyapatite crystals is poor.

The morphology of NCAD and ACAD differ from the intratubular calcium-phosphate casts. The enzymatic activity in NCAD is extended over a long period of time; meanwhile the ACAD model was created within 7 days of incubation. Despite this, the bond strength between NCAD and ACAD model was not affected.

The possible reason for this situation is that the resin tags created inside the intratubular dentin may not contribute to bond strength and the perfusion of the resin monomer of adhesive systems into the intertubular dentin, which play an important role in the bonding performance of the adhesive materials.

Another main significant finding of the study was the similarity in intertubular dentin hardness of NCAD and ACAD up to a depth of 130 μ m. This result is in agreement with the previous work which revealed no significant difference in mineral density in the superficial area of both substrates. It was therefore suggested that the demineralization regime could serve as an artificial caries-affected dentin model for studies involving the bond strength of resin-based dental adhesives to dentin.

Conclusions

Further studies are necessary to achieve the creation of intratubular calcium-phosphate casts in the ACAD model and artificial caries-like models simulating diversities of natural caries stages. The ACAD model created in this *in vitro* study has similar properties with regards of mineral density, bond strength and intertubular hardness when compared to the NCAD lesions.

CHAPTER 1

1.1 General background and literature review

Enamel is the hardest substance in the human body that protects the underlying dental structures; dentin and pulp. The main component of enamel is apatite derived from the hydroxyapatite $(Ca)_5(OH)(PO_4)_3$ that conforms 90% of the tissue by volume; the other component is occupied by organic material that consists primarily of proteins. On the other hand, dentin is 50% inorganic, 30% organic and 20% water by volume. Given the mineral contents of the dental hard tissues, each tooth can be damage and affected by the so-called “caries process”. Caries is a multifactorial disease that in the simplest form occurs in the presence of a cariogenic biofilm attached to the tooth surface, producing acids from fermentable carbohydrates.

When enamel is destroyed and dentin is invaded by bacteria, there is de- and remineralization of intertubular dentin that lead to the formation of intratubular calcium-phosphate crystallites. Pathologically, dentinal caries is a multi-layered disease, in which the outer dentin is highly infected with proteolytic degradation of the collagen matrix (irreversibly demineralized) and the inner dentin has been reversibly attacked because the collagen matrix is not severely damaged, giving it potential to be repaired.

Clinically, the concept of two layers of caries (infected and affected dentin) is being used (Fusayama, 1979). Caries infected dentin is mainly characterized by the presence of bacteria and denature of collagen making this layer poor in mineral density. On the other hand, caries-affected dentin is characterized by the presence of calcium-phosphate casts inside the dentinal tubules without bacteria and collagen matrix is not denatured. When caries infected dentin is being excavated, the boundary

between the infected and affected dentin is difficult to determine by visual inspection. In this regard, different methods are clinically used to remove the infected dentin, ranging from pain, color, tactile hardness and dye staining to the use of self-limiting burs, chemical agents or lasers; however, there is no still gold standard method established for the caries removal (Fusayama, 1988; Itoh et al, 2009; Pugach et al, 2009; Neves et al, 2011). Having these several criteria to remove dentin caries, the minimal invasive concept (MI) and atraumatic restorative treatment (ART) are some approaches that have been introduced to reduce the excessive removal of sound tissue while excavation (Momoi et al, 2012; Van 't et al, 2006). In the dental restorative field, the change of using “amalgam” to the “resin composite” has led to the conservation of dental tissues that previously were removed to have a good adaptation of the materials. The restoration of teeth with the use of innovative adhesive materials has allowed the practice of the minimal invasive (MI) concept that avoids the old retention form which is not needed in adhesive restorations.

In clinic, natural caries-affected dentin (NCAD) is considered as the main substrate for bonding (Nakajima et al, 2000), but the bonding performance of adhesive materials to this layer is still weak. For this reason, laboratory studies are being performed using this substrate to improve the quality of the diversity of adhesive materials but NCAD substrate is variable in terms of color, hardness, depth and extension; and many specimen preparation is required to perform a single study, making it a difficult substrate to standardize for *in vitro* research. Therefore, the rising of different methodologies to create and/or standardize caries models for laboratory studies have been developed (Marquezan et al, 2009; Zanchi et al, 2010). These methodologies are simply made by the use of demineralizing solutions (including acidified gels and/or pH cycling) or bacteria (usually *streptococcus mutans*).

The creation of laboratory caries does not result in a difficult task, but the main objective should be emphasized to establish similar characteristics and properties with the natural development of caries. Many *in vitro* studies have attempted the creation of artificial caries-like lesions with the purpose to use it as a bonding substrate, but the resulting substrates have been compared to sound dentin or to another type of method for the creation of it, not with the natural caries process. The standardization of an artificial caries-affected dentin (ACAD) with regard of mineral content, morphology, bond strength and hardness is being necessary to make the laboratory tests easier and comprehensible with reliable results. Therefore, the main purpose of this research project was to create a standardized artificial caries-affected dentin (ACAD) model and compare it to the natural caries-affected process with regard of mineral content, morphology, bond strength and hardness.

In this thesis two main studies using an *in vitro* caries-like lesion method are included:

Study 1 will discuss about an artificially caries-like model created. This substrate was used to analyze mineral density, morphology and bond strength compared to natural lesions.

Study 2 is going to explain about the mechanical properties (hardness) of sound dentin, NCAD and ACAD model created in the first study.

CHAPTER 2

STUDY 1

Mineral density, morphology and bond strength of natural *versus* artificial caries-affected dentin

Abstract

Objective: This study aimed to investigate an artificial caries-affected dentin (ACAD) model for in vitro bonding studies in comparison to natural caries-affected dentin (NCAD) of human teeth. **Material and methods:** ACAD was created over 7 days in a demineralizing solution. Mineral density (MD) at different depth levels (0 - 150 μm) was compared between NCAD and ACAD by transverse microradiography. Micro-tensile bond strengths (μTBS) of two two-step self-etch adhesives to sound dentin, NCAD and ACAD were evaluated. **Results:** Caries-affected dentin type was not a significant factor when comparing MD at different lesion levels ($p>0.05$). Under SEM, the dentinal tubules appeared occluded with crystal logs 1-2 μm in thickness in the NCAD; whereas they remained open in the ACAD. The μTBS to caries-affected dentin was lower than sound dentin, but was not affected by the type of caries ($p>0.05$). **Conclusion:** In spite of their different morphologies, the ACAD model showed similar MD and μTBS compared to NCAD.

Keywords: natural caries-affected dentin, artificial caries, TMR, mineral density, bond strength

2.1 Introduction and Objectives

According to the minimal invasive concept for restoration of cavities with dentin involvement, caries-affected dentin should be left after removal of the infected tissue (Fusayama, 1988; Momoi et al, 2012). Therefore, caries-affected dentin is predominantly the clinical substrate for bonding in many cavity preparations (Nakajima et al, 2000). Different methods are clinically used to remove the infected dentin, ranging from excavation based on pain, color, tactile hardness and dye staining to the use of self-limiting burs, chemical agents or lasers; however, there is no still gold standard method established for the caries removal (Fusayama, 1988; Itoh et al, 2009; Pugach et al, 2009; Neves et al, 2011).

Bond-strength tests are the most common laboratory methods to evaluate the bonding performance of adhesives. In order to evaluate bonding to caries-affected dentin, these tests have been usually performed on natural lesions after *in vitro* removal of the caries-infected dentin (Nakajima et al, 2005; Xuan et al, 2010; Wei et al, 2008). However, the caries-affected dentin shows a great variability which makes its use as a standardized substrate for laboratory research difficult (Marquezan et al, 2009). The bond strength of contemporary adhesive systems to caries-affected dentin is lower than that of intact dentin; it has been suggested that the lower bond strength could be due to presence of voids, collagen-rich zone at the adhesive interface and occlusion of dentinal tubules by crystal logs in the dentin (Nakajima et al, 2005). The solvents present in the adhesive materials have also influence the bond strength to this substrate (Zanchi et al, 2010). Moreover, dentin mechanical properties play a substantial role in the values of strength obtained in laboratory bond strength tests (Xuan et al, 2010), necessitating the use of a standardized substrate for comparative

adhesion studies using caries-affected dentin. The morphology of natural lesions is another limiting factor for the use of caries-affected dentin for bonding tests; in the common bond strength experiments a flat substrate is required to achieve the best interfacial loading orientation, which may be difficult considering the variability of natural lesions in shape.

Some studies have attempted to use chemical and bacterial methods to create *in vitro* caries-like lesions as substrates for bonding and testing new materials (Marquezan et al, 2009; Zanchi et al, 2010). Chemical methods using an artificial demineralizing solution to produce caries-like lesions may provide a morphological simulation and similar hardness values to natural lesions (Marquezan et al, 2009). On the other hand, while bacterial methods bear some advantages for morphological studies, they result in an excessive softness of dentin (Marquezan et al, 2009), and are technically more difficult to perform compared to chemical demineralization. In addition, bacterial creation of the lesion needs a longer period of time because the demineralization progresses slowly (Zanchi et al, 2010).

Despite the methodologies introduced for artificial caries-affected dentin, few studies have attempted to relate the properties of the resulting lesions as a bonding substrate when compared to natural lesions. Therefore, the purpose of this study was to investigate an *in vitro* model to create caries-affected dentin for bonding studies, and to compare its mineral profile, morphology and bonding properties to those of natural caries.

2.2 Materials and Methods

A total of 41 human teeth, including 27 caries-free premolars, 2 caries-free molars, 2 carious premolars and 10 carious molars, were used. The teeth were extracted as part of the treatment plan and used after the individuals' informed consent was obtained according to a protocol approved by the Human Research Ethics Committee, Tokyo Medical and Dental University (No. 725). The teeth were thoroughly cleaned from organic debris after extraction and stored in a 0.1% thymol solution until use.

2.2.1 Specimen preparation

2.2.1.1 *Natural caries-affected dentin (NCAD)*

Moderate dentin caries at the occlusal sites on the carious teeth were used. The outer soft dentin was removed using a spoon excavator (YDM, Tokyo, Japan) and then a dye solution, Caries Check (Nippon Shika Yakuhin, Yamaguchi, Japan), was used to stain the residual infected-dentin. A dental trimmer (Y-230, Yoshida, Tokyo, Japan) was then used to reduce the occlusal surface to reach close to the stained dentin at the deepest part of the carious lesion. In order to obtain a clinically-relevant caries-affected dentin on the reduced occlusal surface, a round tungsten carbide bur (No 4, ISO: 014; DENTSPLY, Tulsa, OK, USA) attached to a low-speed hand piece without water was used to remove the stained dentin (Sunago et al, 2009).

2.2.1.2 Artificial caries-affected dentin (ACAD)

A schematic representation of the experimental procedures for creation of ACAD in the study is presented in Fig. 1. The caries-free teeth were horizontally cut at the mid portion of the crown and middle-third of root using a slow-speed diamond saw (Isomet; Buehler, Lake Bluff, IL, USA) to remove cusps and root and expose the coronal dentin surface. The specimens were proximally reduced by the saw to obtain a 4×4 mm area of the dentin surface. Afterwards, an acid-resistant nail varnish (Revlon, New York, NY, USA) was applied on the proximal and bottom surfaces of each specimen with only the dentin surface remaining exposed. The specimens were immersed in 15 ml of a demineralizing solution (1.5 mM of CaCl_2 , 0.9 mM of KH_2PO_4 , 50 mM of acetic acid and 0.02% of NaN_3 with the pH value adjusted at 4.5 using NaOH) at 37 °C for 7 days. Following this, the samples were removed from the container and rinsed thoroughly with deionized water (Direct-Q UV; Millipore, Molsheim, France). Afterwards, each specimen was attached to the loading device of a polishing machine (ML-160A; Maruto, Tokyo, Japan), and the demineralized surface was ground with #600-grit SiC paper (14 cm in diameter) under 576 gr of load for 5 sec at a speed of 12 rpm under running water to create standardized dentin surface with a smear layer.

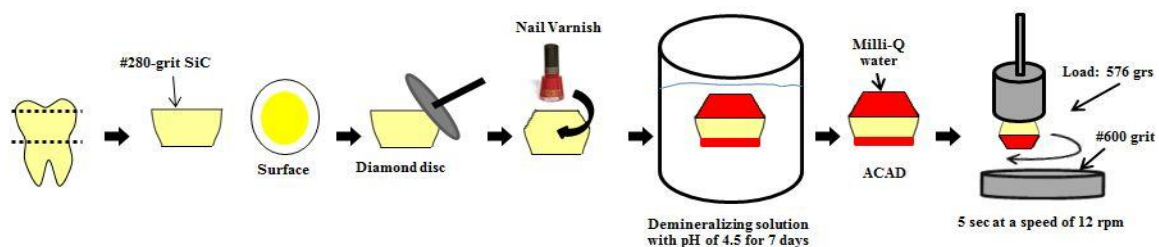


Fig 1. Schematic representation of the experimental procedures for creation of ACAD in the study. Human teeth were reduced occlusally, apically and proximally and covered by nail varnish to obtain a 4×4 mm area of the dentin surface. The specimens were immersed in demineralizing solution for 7 days and washed with deionized water. The demineralized surface was ground in a polishing device to create standardized dentin surface with a smear layer.

2.2.2 Assessment of caries-affected dentin

2.2.2.1 *Transverse Microradiography (TMR)*

TMR measurement was performed on dentin slices with $150 \pm 30 \mu\text{m}$ in thickness and approximately $3 \times 4 \text{ mm}$ in dimensions to obtain information of mineral density and mineral profile of NCAD and ACAD.

Twenty-three slices were obtained from the center of NCAD lesions, and 12 slices were obtained from the demineralized specimens in ACAD group using the low-speed diamond saw under running water. The slices were kept in a solution composed of 30 vol% of water and 70 vol% of glycerol to prevent shrinkage of collagen before densitometry measurement. The images were taken using an x-ray generator (SRO-M50; SOFRON, Tokyo, Japan) under the conditions of 25 kV, 4 mA for 20 min, with a Ni filter at 15 cm distance between the x-ray tube and the specimen. The images were captured on the x-ray glass plate film (High Precision Photo Plate PXHW; Konica Minolta Photo, Tokyo, Japan), together with 15 aluminum step-wedges and scanned as 8-bit digital images using a CCD camera (DP70; Olympus, Tokyo, Japan) attached to a microscope. Mineral density profiles were obtained using ImageJ (1.42q; NIH, Bethesda, MD, USA) and a custom visual basic application written in Microsoft Excel. The profiles were obtained over a region of interest at the center of each slice. The mineral density (vol. %) was calculated using the calibration curve, considering that the sound dentin contained 48 vol. % mineral (Natsume et al, 2011). The mean mineral density values at different depths up to $150 \mu\text{m}$ were calculated from the resulting profiles of NCAD and ACAD.

2.2.2.2 Scanning Electron Microscopic (SEM) observation

Five NCAD and six ACAD blocks (1 mm thickness) were obtained cutting each specimen at the center of the crown with the diamond saw. The samples were fixed in 2.5% glutaraldehyde for 2 h and rinsed with a 0.1 M PBS (phosphate buffer solution) before being dehydrated in graded series of ethanol (50, 60, 70, 80, 90, and 95% for 25 min each, and 100% for 60 min). The samples were finally air-dried for 24 h. Following this, a fine notch was made in the center of the bottom (pulpal) side of each specimen with a fine cylindrical diamond bur. Each slice was then gently fractured by fingers to obtain a smear-layer free axial section along the dentinal tubules. The specimens were mounted on aluminum slabs, gold-coated and then examined by SEM (S-4500, Hitachi, Ibaraki, Japan).

2.2.2.3 Microtensile Bond Strength (μ TBS) test

Ten sound premolars, 6 carious molars with NCAD and 10 premolars with ACAD were used in this part of the study. For intact dentin samples, the occlusal enamel of the sound premolars was removed using the slow-speed diamond saw to expose a flat dentin surface, which was ground with #600-grit SiC paper in the same manner as for ACAD to produce the smear layer.

Two two-step self-etching adhesive systems; Clearfil SE Bond (CSE) and Clearfil Protect Bond (CPB) (Kuraray Noritake Dental, Tokyo, Japan) were used and applied according to the manufactures instructions among three groups of dentin substrates: sound dentin, NCAD and ACAD. Each tooth was built up with a resin composite (Clearfil AP-X, shade A2, Kuraray Noritake Dental) using incremental

technique by three layers up to a height of approximately 4 mm. The bonded samples were stored in deionized water at 37 °C for 24 h to be tested. They were cut at low speed with the diamond saw under cooling water to obtain rectangular beams with cross-sectional dimensions of approximately 0.9 × 0.9 mm. Approximately 33 ± 4 dentin-composite sticks were obtained from each group of teeth (3±1 sticks for one premolar and 5±1 sticks for one molar). Pre-testing failures from cutting and attaching the sticks to the jig was not conducted. The μ TBS test was performed at a crosshead speed of 1 mm/min (EZ-test; Shimadzu, Kyoto, Japan).

2.3 Statistical analysis

All data were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA) at a significance level of $\alpha = 0.05$. Two-way ANOVA was used to compare mineral density at different levels (0, 50, 100 and 150 μ m) between NCAD and ACAD, with the “lesion level” and “type of dentin” as the two factors. The μ TBS to caries affected dentin was also analyzed by two-way ANOVA with two factors, “type of dentin” and “adhesive”. Finally, one-way ANOVA with Dunnetts T3 was used for pair comparisons that included sound dentin data as well as NCAD and ACAD of both materials.

2.4 Results

Figure 2 shows average mineral profile of the NCAD and ACAD specimens with standard deviations.

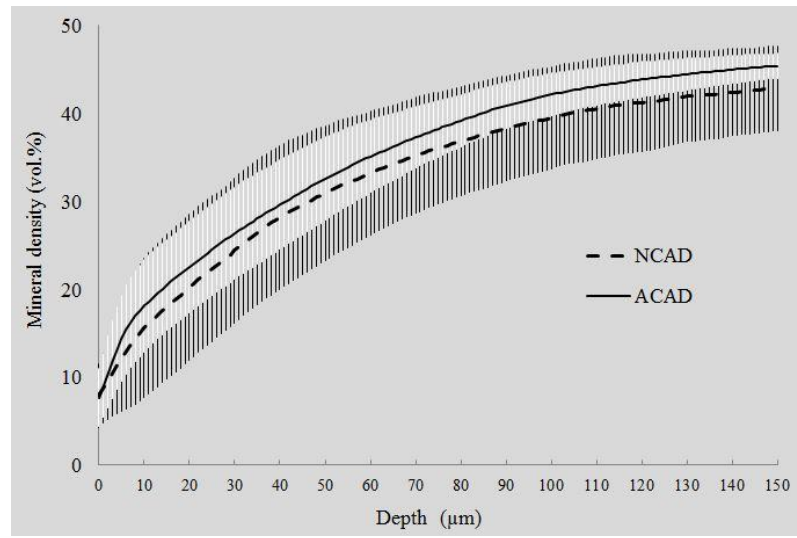


Fig 2. Average mineral profiles of all specimens in each group.

Two-way ANOVA showed that the type of dentin was not a significant factor ($p= 0.349$), while the lesion level was a significant factor affecting mineral density ($p<0.001$). The interaction of the factors was not significant ($p=0.749$). The mineral density values at each lesion level for NCAD and ACAD are presented in Table 1. Cross-sectional SEM images of the NCAD and ACAD were revealed in Fig 3. The smear layers covered the top surface in both groups. Intertubular dentin below the surface in both appeared to be porous and collagen fibrils could be observed.

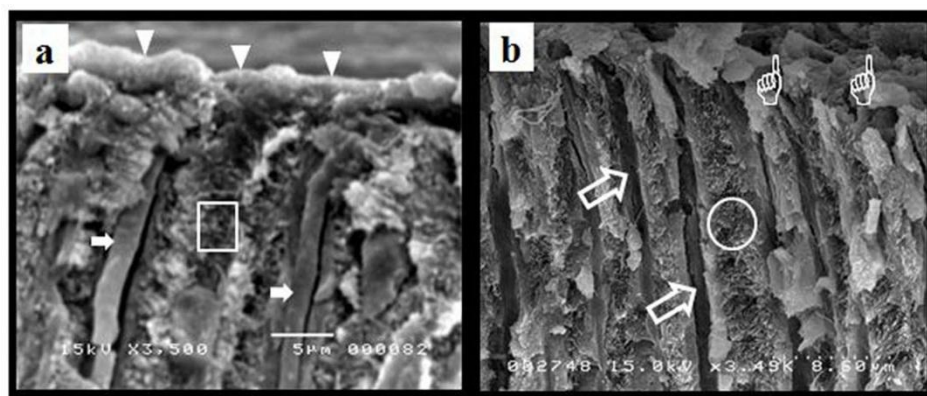


Fig 3. Cross-sectional SEM images of specimens in NCAD (a) and ACAD model (b). a) bold arrows indicate crystal logs obtruding dentinal tubules in NCAD, in the rectangle, collagen fibrils are observed within the mineral phase of the intertubular dentin; triangles indicate the presence of the smear layer on the surface; b) blank arrows show open dentinal tubules in ACAD, intertubular dentin collagen fibrils are visible in the circle; finger pointers indicate the presence of the smear layer on the surface.

However, the dentinal tubules were occluded with crystal logs 1 to 2 μm in thickness in the NCAD; whereas they remained open in the ACAD. It was difficult to detect a clear boundary between the demineralization front of the lesion and sound dentin in both NCAD and ACAD groups under SEM.

Type of lesion	vol.% at 0 μm	vol.% at 50 μm	vol.% at 100 μm	vol.% at 150 μm
NCAD	7.91 \pm 3.64	27.75 \pm 9.27	35.43 \pm 7.44	40.20 \pm 6.13
ACAD	8.30 \pm 3.60	27.37 \pm 5.61	36.44 \pm 3.84	42.32 \pm 1.80

* No significant difference between NCAD and ACAD ($p > 0.05$, two-way ANOVA).

The microtensile bond strength values are shown in Table 2. Two-way ANOVA on the results of caries-affected dentin revealed no significance for the type of caries-affected dentin ($p = 0.054$), material ($p = 0.558$) or their interaction ($p = 0.393$).

On the other hand, separate comparisons against sound dentin showed that both NCAD and ACAD substrates resulted in lower bond strengths using any adhesive ($p < 0.001$). Only for sound dentin, CSE resulted in significantly higher bond strength than CPB ($p < 0.001$).

Adhesive	Sound dentin	NCAD	ACAD
Clearfil SE Bond	80.8 \pm 18.0 (37) ^b	37.6 \pm 14.3 (35) ^a	39.8 \pm 14.2 (37) ^a
Clearfil Protect Bond	62.0 \pm 12.6 (33) ^c	34.6 \pm 9.9 (37) ^a	40.4 \pm 11.0 (34) ^a

Groups with the same letter are not significantly different ($p > 0.05$, one-way ANOVA with Dunnett T3 post-hoc comparisons). All are mean values \pm standard deviation (number of specimens).

2.5 Discussion

For treatment of caries dentin, the complete removal of caries-infected dentin is required for restoration with adhesive resin. However, diagnosis of the extent of the carious lesion to be removed is not easy in clinic. Fusayama (1979) reported that a

staining technique using a dye solution was useful to aid in the differentiation of the two layers of caries-infected and caries-affected dentin. The original dye solution, Caries Detector (Kuraray Noritake Dental) is composed of 1% acid red in propylene glycol. The carious infected dentin is stained red, while the caries-affected dentin is stained light pink and sound dentin is not stained. However, making a decision about the boundary of caries-affected dentin by the stained color is very subjective. In this study, the caries detector dye solution, Caries Check was used; it contains polypropylene glycol instead of propylene glycol in the original dye solution. Polypropylene glycol (MW 300) is a much larger molecule than propylene glycol (MW 76) and therefore lower penetration. It was reported that this dye solution stained only caries-infected dentin, which could avoid over-staining and over-excitation (Itoh et al, 2009).

The presence of a smear layer on the surface is unavoidable after cavity preparation; therefore, for *in vitro* studies where bonding tests are performed, this layer is required. Previous studies reported the use of #600-grit SiC paper to produce a thin smear layer (Lippert et al, 2011; Tay et al, 2000), but the use of a specific load or speed has not been reported before. In this study, a standardized smear layer was created using #600-grit SiC paper under controlled load and speed in ACAD. The mean mineral density values were slightly higher in ACAD than that of NCAD, while the standard deviations were greater in NCAD lesions, especially at deeper levels (Fig. 2). These findings reflect the variability of the NCAD which occur over long periods of time in contrast to the ACAD model, where the lesion was created in a short period of time under controlled conditions. Nevertheless, the ACAD profile with standard deviations always fell within the range of those in NCAD, and the type of caries-affected dentin was not a statistically significant factor when comparing mineral

density at different lesion levels. It should be noted that the NCAD lesions greatly vary in depth depending on the activity of the lesion and the time that dentin was subjected to the caries process; indeed caries may affect the whole thickness of dentin from dentin-enamel junction through to the pulp (Vidmar et al, 2012). The ACAD in the current study was compared to NCAD superficially (up to 150 μm), because in terms of the bonding interface with restorative materials, only this zone plays a role.

In accordance with the previous studies (Nakajima et al, 2005; Marshall et al, 2001) some crystal logs were found inside dentinal tubules of NCAD. NCAD is a hypomineralized tissue where the demineralization and remineralization processes have occurred during a long period of time, allowing the dissolution of the inorganic matrix which may precipitate and these create crystal logs in the dentinal tubules. However, such crystallites were not formed in the current ACAD model, because the demineralized substrate was originally sound dentin and the penetration of demineralizing agent dissolved dentin in a relatively short period of time without promoting crystal formation or a remineralizing process.

The bond strengths of the two self-etch adhesive systems to both NCAD and ACAD were significantly lower than that to normal dentin, in line with previous reports (Neves et al, 2011; Wei et al, 2008; Li et al, 2011; Yazici et al, 2004). It was suggested that the mechanical properties of caries-affected dentin which relate to the mineral content play the key role in bond strength to the substrate (Wei et al, 2008). That should help to explain why bond strength to the affected substrate was not different between CSE and CPB, while for sound dentin CSE showed the highest strength. This result was in agreement with previous study which showed higher short-term bond

strength of CSE to sound dentin in comparison to CPB (Ansani et al, 2008; Sarr et al, 2010).

The crystal logs may decrease hydraulic conductance through dentinal tubules and affect formation of resin tags (Yoshiyama et al, 2000). However, the bond strength was not different between NCAD and ACAD in the current study. It has been suggested that resin tag formation did not contribute to the bond strength, especially in the mild self-etch adhesives (Lohbauer et al, 2008). Moreover, additional etching which could remove these mineral deposits did not improve bonding of CSE to NCAD (Yazici et al, 2004). Likewise, formation of resin tags following chemomechanical removal of caries did not significantly improve bond strength of self-etch adhesives (Li et al, 2011). In the clinical situation, keeping the tubules sealed may have an advantage in decreasing pain during operation or after that. In fact, a lower incidence of post-operative sensitivity has been found in self-etch adhesives than total-etch adhesives (Unemori et al, 2004).

The interface between bonding resin and dentin is the weak link in adhesive restorations. Tsuchiya *et al.* (2004) reported the formation of a new zone beneath the hybrid layer when dentin was treated with the self-etch adhesive system. The zone was different from the conventional hybrid layer, and characterized by resistance to an acid-base challenge. Therefore, the zone named as the “acid-base resistant zone” (ABRZ), was supposed to play an important role in prevention of secondary caries, sealing of restoration margins and promotion of restoration durability. In spite of the tubule occlusion discussed above, the intertubular caries-affected dentin is a porous substrate where a thicker hybrid layer is formed by various adhesives (Yoshiyama et al, 2002). Inoue *et al.* (2006) reported that ABRZ was also observed beneath the hybrid layer in the caries-affected dentin specimens using CSE. The ABRZ of

caries-affected dentin was thicker than that of normal dentin, while its nanoindentation hardness was lower. It was also reported that the morphology of the ABRZ was influenced by the functional monomers contained in the adhesive systems. According to Yoshida *et al.* (2004), the chemical bonding potential was different among various functional monomers. CSE and CPB contain 10-Methacryloyloxydecyl dihydrogen phosphate (MDP) as a functional monomer. The capability of MDP to readily establish an intensive ionic bond with hydroxyapatite (HAp) has been demonstrated (Yoshida *et al.*, 2004). The transmission electron microscopic (TEM) observation of the adhesive-dentin interface after acid-base challenge revealed that the ABRZ contained apatite crystals and possessed a dentin-like structure, more caries-resistant than normal dentin (Nurrohman *et al.*, 2012). The ABRZ of CPB in the long-term was thicker and relatively more stable compared to CSE probably due to fluoride release (Ichikawa *et al.*, 2012; Inoue *et al.*, 2012). The attributes of ABRZ could suggest that the bonding technology could reinforce affected dentin against acid-attack, and it was proposed that such a reinforced dentin could be called as “super dentin” (Waidyasekera *et al.*, 2009; Nikaido *et al.*, 2009).

It is noteworthy that during natural caries progress, acidic conditions activate dentin-bound matrix metalloproteases (MMPs) which can degrade the organic phase (Shimada *et al.*, 2009). The intense expression of the enzymes in caries-affected dentin compared with sound dentin may induce more rapid degradation of the interface of caries-affected dentin as the bonding substrate (Toledano *et al.*, 2010).

Further studies should be performed on the morphology simulation and evaluation of the efficacy of adhesive systems to ACAD in the comparison to NCAD using various bonding systems to investigate the long-term stability of the interface.

2.6 Conclusions

The artificial-caries affected dentin showed similar mineral content and bond strength yet lower variability compared to the natural caries-affected substrate. Nevertheless, with the lack of mineral casts in dentinal tubules of artificial caries-affected dentin, their morphologies are different.

2.7 Acknowledgments

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CHAPTER 3

STUDY 2

Nanoindentation hardness of intertubular dentin in sound, demineralized and natural caries-affected dentin

Abstract

Objective: The purpose of this study was to investigate the mechanical properties of intertubular dentin in sound, natural caries-affected (NCAD) and artificial caries-affected dentin (ACAD) using nanoindentation. **Materials and methods:** Non-caries molars and caries molars with International Caries Detection and Assessment System (ICDAS II) score 5 at the occlusal site were used and caries was excavated using a spoon excavator, a round bur at low speed without water and a dye solution as guidance to detect the infected tissue. Specimens with remaining dentin thickness (RDT) >2mm were selected. ACAD teeth were created from sound teeth over 7 days in a demineralizing solution. Specimens were embedded into plastic rings with acrylic resin and then sagittal mesial-distal sectioned from crown to the long axis of the root under cooling water using a low-speed diamond blade. The surface of interest was fine polished sequentially. Hardness measurement was performed within an axial depth of 1000 μm with at least of 320 indentations on each sample. Mann-Whitney U Test was used to compare the hardness as the variable among different dentin types (SOUND, NCAD and ACAD) at each dentin depth level. **Results:** There was no significant difference in nanohardness between NCAD and ACAD up to a depth of 130 μm ($p>0.05$). NCAD consistently showed lower hardness. ACAD showed no significant difference in hardness with SOUND dentin beyond 190 μm ($p<0.05$). The lesion front in ACAD was considered to be located around the depth of 180 μm . **Conclusion:** Natural and artificial caries-affected dentin tissues were superficially comparable in intertubular nanohardness. There is a certain layer within the natural caries-affected dentin with higher hardness; however the long-term effects of caries beneath the lesion extend deeply through intertubular dentin. Sound dentin at deep areas (close to the pulp chamber) is considered to be soft.

Keywords: nanohardness, caries-affected dentin, sound dentin, artificial caries-affected dentin, remaining dentin thickness.

3.1 Introduction and Objectives

The tooth is a mineralized tissue, basically constituted by enamel, dentin and pulp. Every tooth is susceptible to be demineralized by acids from bacteria and other sources in the so called caries process. Caries is a multifactorial disease that in its simplest form occurs in the presence of a cariogenic biofilm attached to the tooth surface, producing acids from fermentable carbohydrates. Naturally, there is a dynamic balance in the oral environment where enamel can be de- and remineralized without significant loss of substance. In the stage where caries process has resulted in the breakdown of enamel surface and infection of dentin, the restoration of the tooth is necessary.

Dentin is a rigid but elastic tissue with tubules in a mineralized collagen matrix and it is considered as an elastic foundation for the enamel offering protection to the pulp (Arola and Reprogel, 2006). In between the tubules there is peritubular and intertubular dentin that are different in morphology because of crystals shape and orientation (Weiner et al, 1999).

Fusayama (1988) reported that a dye solution, which contains 1% acid red in propylene glycol, was useful to differentiate two layers of caries, the infected and caries-affected dentin (Fig. 1); natural caries-infected dentin (NCID) is the outer layer characterized by the presence of bacteria, denaturation of collagen and changes in color and appearance due to the progression time. Meanwhile, in natural caries-affected dentin (NCAD) or inner layer, there are no bacteria and the collagen is not denatured; however, the dynamic process of de- and re-mineralization of caries, lead to the formation of calcium-phosphate crystallites that block dentinal tubules and hinder the flow of dentinal fluid. Moreover, most of the teeth reveal a degree of

reduction of the pulp chamber size due to calcification; the gradual reduction of the pulp chamber size due to secondary dentin formation is considered to be a physiological change with age (Wennberg et al, 1982).

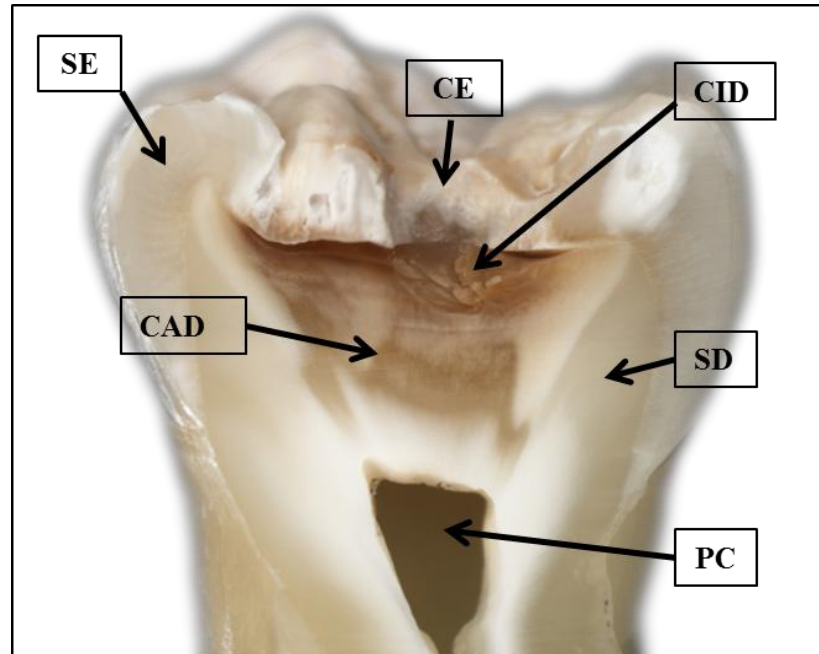


Fig 1. A light microscope cross-sectional image of a carious molar: (SE) represents sound enamel; (CE) carious enamel; (CID) caries-infected dentin; (CAD) caries-affected dentin; (SD) sound dentin; and (PC) the pulp chamber.

In clinic, according to the minimal invasive (MI) concept, the use of direct resin composite restorations has been accepted for the conservative treatment of caries, preserving sound structures of the tooth (Momoi et al, 2012). In the restoration of cavities with dentin involvement, caries-affected dentin should be preserved, being the main remaining substrate for interaction with the dental restorative materials.

Clinicians use different methods to excavate the lesion and remove the infected dentin, based on pain, color, tactile hardness, dye staining, self-limiting burs, chemical agents or lasers (Itoh et al, 2009; Pugach et al, 2009; Neves et al, 2011). Tactile hardness is one of the most common criteria used by clinicians while removing dentin caries.

Nevertheless, NCAD may be variable in terms of depth and hardness and these variables depend on the type of caries (acute, chronic and arrested), age and tooth location, caries zone in tooth and masticatory problems. In this regard, removal of the infected tissue while preserving the affected and sound structure is still a clinical challenge.

Nanoindentation test is capable of probing the mechanical properties and behavior of small volumes of dental structures (Marshall G et al, 2001; Bresciani et al, 2010; Sadr et al, 2011) and bone for in vitro studies. Thereby, different investigations have been conducted testing sound dentin and NCAD revealing that nanohardness of NCAD is lower than that of sound (Inoue et al, 2006; Ahmed et al, 2012). However, to date few studies have investigated the nanoindentation hardness of intertubular dentin of NCAD through a large axial depth from lesion surface towards the pulp.

An artificial caries-affected dentin (ACAD) model with the purpose to be used as a bonding substrate for in vitro studies was reported in the previous study; it revealed that the mineral density obtained using transverse microradiography (TMR) was comparable between NCAD and the artificial model at the superficial dentin area (Joves et al, 2013). However, it is not clear how the natural caries process is different from in vitro demineralization by acids in terms of the mechanical properties of the affected dentin.

The purpose of this study was to investigate the mechanical properties of intertubular dentin in sound, natural caries-affected and demineralized dentin using nanoindentation.

3.2 Materials and Methods

Five carious teeth with ICDAS-II score 5 (International Caries Detection and Assessment System) (Braga et al, 2009), 10 sound (n=15) were used following the guidelines approved by Tokyo Medical and Dental University Ethical Committee (No 725). They were extracted as a part of the original dental treatment plan with informed consent of the patients and thoroughly cleaned of organic debris and stored in a 0.1% thymol solution until use.

3.2.1 *Natural caries-affected dentin (NCAD)*

Five human caries molars with ICDAS II score 5 at the occlusal site were used. Two-third of the root of each tooth was trimmed (Model: Y-230, Yoshida, Tokyo, Japan) under a gentle stream of water. The outer enamel was cut with a diamond round bur (# 0876-1, ISO: 001/022, Shofu, Kyoto, Japan) at high speed using a hand piece with water spray. Following this, the outer soft caries infected dentin was carefully removed using a spoon excavator (# 1, YDM Corporation, Tokyo, Japan). A red dye solution (Caries Check, Nippon Shika Yakuhin, Yamaguchi, Japan) was used to guide while removing the residual infected-caries (red-stained) to obtain caries-affected dentin using a round stainless steel bur (# 4, ISO: 021; Morita, Tokyo, Japan) at low speed using (5,000 rpm) a hand piece without water. The remaining dentin thickness (RDT) of the coronal central area of each tooth was measured using a caliper (# 21111, YDM Corporation, Tokyo, Japan) from the occlusal surface of dentin to the bottom area in the pulp chamber. NCAD specimens with a remaining dentin thickness (RDT) more than 2 mm were included.

3.2.2 Artificial caries-affected dentin (ACAD)

Five sound molars were used to create the demineralized dentin following the ACAD model reported previously (Joves et al, 2013). Briefly, the specimens were immersed in 15 ml of a demineralizing solution (1.5 mM of CaCl₂, 0.9 mM of KH₂PO₄, 50 mM of acetic acid and 0.02% of NaN₃ with the pH value adjusted at 4.5 using NaOH) at 37 °C for 7 days. Afterwards, the demineralized surface was ground with #600-grit SiC paper (14 cm in diameter) under 576 g of load for 5 sec at a speed of 12 rpm under running water to create the smear layer.

3.2.3 Sound dentin

Five caries-free molars were used. Two-third of the root of each tooth was trimmed (Model: Y-230, Yoshida. Tokyo, Japan).

3.2.4 Sample Embedding and Polishing

NCAD and ACAD specimens were covered with a hydrocolloid dental impression material (AROMA, GC Corporation, Tokyo, Japan) on the carious surface to prevent penetration of the embedding material (UNIFAST II, GC Corporation, Tokyo, Japan). All samples were encased separately into plastic rings (2.5 cm diameter) with acrylic resin (UNIFAST II). The coronal enamel of SOUND samples protected dentin from the acrylic penetration. They were sagittal mesial-distal sectioned from crown to the long axis of the root under cooling water using a low-speed diamond blade (Isomet; Buehler, Lake Bluff, IL, USA) to obtain 1 slice (3 mm thick) from one tooth (n=15). The surface of interest was ground with an automatic lapping machine (ML-160A; Maruto, Tokyo,

Japan) from #600 up to #2000-grit SiC papers under running water. Afterwards, they were polished with diamond pastes of 6, 3, 1 and 1/4 μm particle size sequentially (Fig. 2). They were sonicated for 2 min at each step to remove any remaining diamond particles. Samples were stored in buffer solution (KCL 130mmol/L and HEPES 20mmol/L) with a pH of 7.0 adjusted using NaOH until the test was performed no later than 24 hours after polishing.

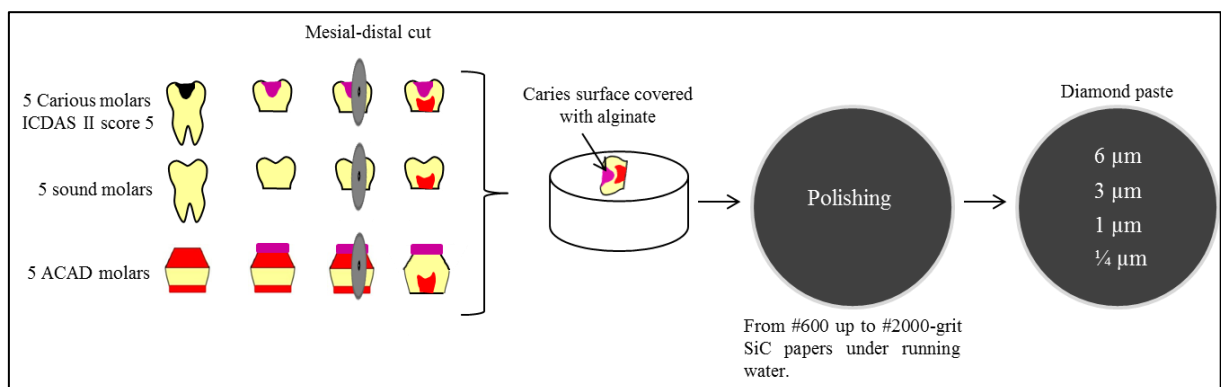


Fig 2. Schematic representation of sample preparation for nanoindentation test.

3.2.5 Nanoindentation

A nanoindentation tester (ENT-1100a, Elionix Inc., Tokyo, Japan) was used. The maximum force was 5 mN at a loading rate of 0.5 mN/s (Sadr et al., 2011) with a Berkovich diamond tip and a holding time of 1000 ms. All specimens were kept moist (room condition at 26°C, humidity 60 \pm 10%) by the application of water droplets over the sample at each 30-min interval of the nanoindentation test to prevent dehydration and shrinkage of the organic phase that affect mechanical behavior of dentin (Ryou et al, 2012). The first line of indentations was set within 5 μm from the dentin surface. The indentations were carefully programmed to be on the intertubular dentin. The first 4 rows of indentations were programmed with a 5 μm axial spacing with the first row starting around 5 μm from the surface; the spacing among the next 20 rows was 10

μm , and then 50 μm among the next 6 rows (250 μm depth), followed by 240 μm among the next 2 rows. In total, at least 320 indentations were performed on each specimen from surface to a depth of 1000 μm divided in a matrix of ten columns and thirty-two rows (Fig. 3). The nanoindentation software analyzed the data and calculated hardness from the projected area under load according to the following equation: $H = F/A_p(h)$, where F is the load and A_p is the projected area under load F , which is a function of indenter penetration depth into the substrate (h).

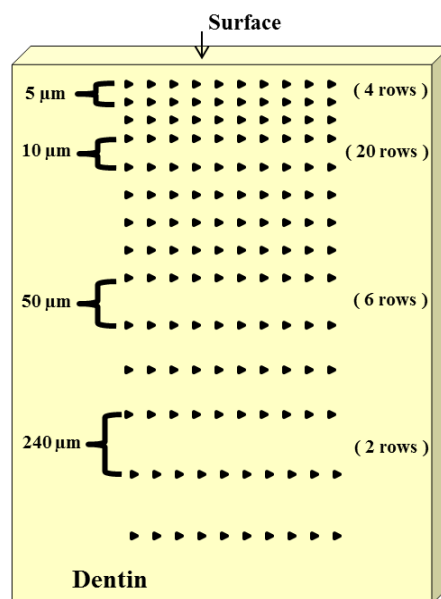


Fig 3. Schematic representation of the indentation matrix programmed for hardness assessment of dentin with axial pitch distances of 5, 10, 50 and 240 μm among rows. The total distance of the last row from the surface was 1000 μm .

3.3 Statistical analysis

Since the distribution of data in some groups was not normal (Kolmogorov-Smirnov $p < 0.05$), non-parametric Mann-Whitney U Test was used to compare the hardness as the variable among different dentin types (SOUND, NCAD and ACAD) at each dentin depth level. All data were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA) at a significance level of $\alpha = 0.05$.

3.4 Results

A CCD camera photo of a hydrated SOUND specimen with nanoindentation marks set on the intertubular dentin is presented in Fig. 4. The indentations marks (N) made using the 5 mN load are clearly visible on the intertubular dentin (I) with an approximate size of 3-5 μm in lateral dimension and 400-700 nm indentation depth.

The hardness profiles with standard deviations of NCAD, ACAD and SOUND dentin with 5 μm from the surface to the depth of dentin (1000 μm) along the axial direction of are shown in Fig 4a, b and c, respectively. Three hundred and twenty indentations were successfully performed on the intertubular dentin of the specimens (n=15). NCAD (Fig. 5a) revealed higher standard deviation of each depth, while ACAD and SOUND showed less standard deviation (Fig. 5b, c). The mean profiles of NCAD, ACAD and SOUND dentin were superimposed in Fig. 5d.

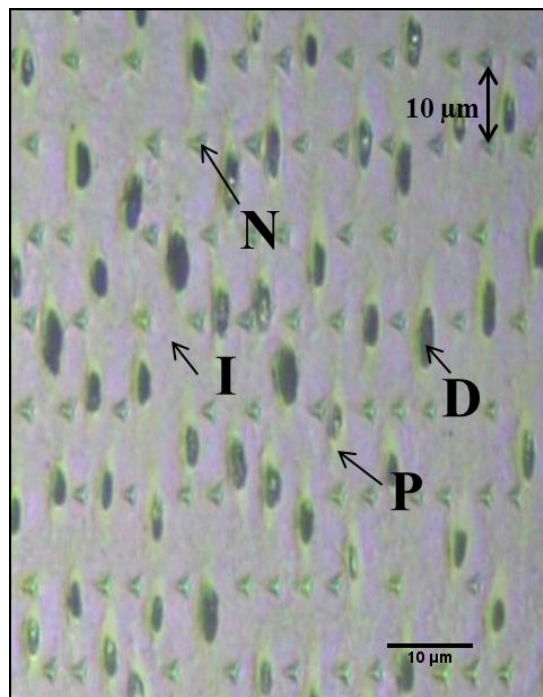


Fig 4. A real image of sound dentin showing indentations created with 5 mN loading regime and axial distance depth of 10 μm among rows: (D) shows a dentinal tubule orifice; (P) shows peritubular dentin; (N) shows an indentation mark and (I) represents intertubular dentin.

SOUND dentin from the surface to around 200 μm resulted in significantly higher hardness values than NCAD and ACAD at each dentin depth. However, hardness in NCAD and ACAD hardness have an upward tendency. ACAD reached close to the level of the sound tissue around 320 – 420 μm depth where there was no difference ($p>0.05$); while the dentin underlying NCAD did not reach the hardness level of SOUND. Moreover, all the three groups showed a similar decrement behavior from 520 μm to 1000 μm , in which NCAD consistently showed lower hardness compared to both ACAD and SOUND dentin ($p<0.05$). Since hardness of ACAD showed no significant difference with that of SOUND dentin beyond 190 μm ($p<0.05$), the lesion front in ACAD was considered to be located around the depth of 180 μm .

Table 1 shows the average hardness of SOUND, ACAD and NCAD up to a depth of 130 μm . Their hardness profiles were shown in Fig. 6. The statistical analysis revealed no significant difference in hardness between NCAD and ACAD up to 130 μm hardness depth ($p>0.05$), in which NCAD and ACAD hardness curves start within similar values (261-268 MPa) and an upward behavior towards SOUND dentin. SOUND dentin demonstrated higher hardness values with smaller standard deviations.

3.5 Discussion

Nanoindentation provides characterization of isolated components from microstructures or anatomical features of interest (Tesh et al, 2001), enabling accurate indentations in a specific selected area (Bresciani et al, 2010; Donnelly, 2011). As a complex biological tissue, nanoindentation values of dentin are

dependent on several factors including the location of the indentation (e.g. intertubular versus peritubular dentin), local composition of the tissue (i.e. mineral content, water and organic phase) as well as the nanoindentation parameters (i.e. loading regime, size of indentation, etc) (Ryou et al, 2012; Marshall et al, 2001). In this study, two structural factors were considered; caries and the depth of dentin.

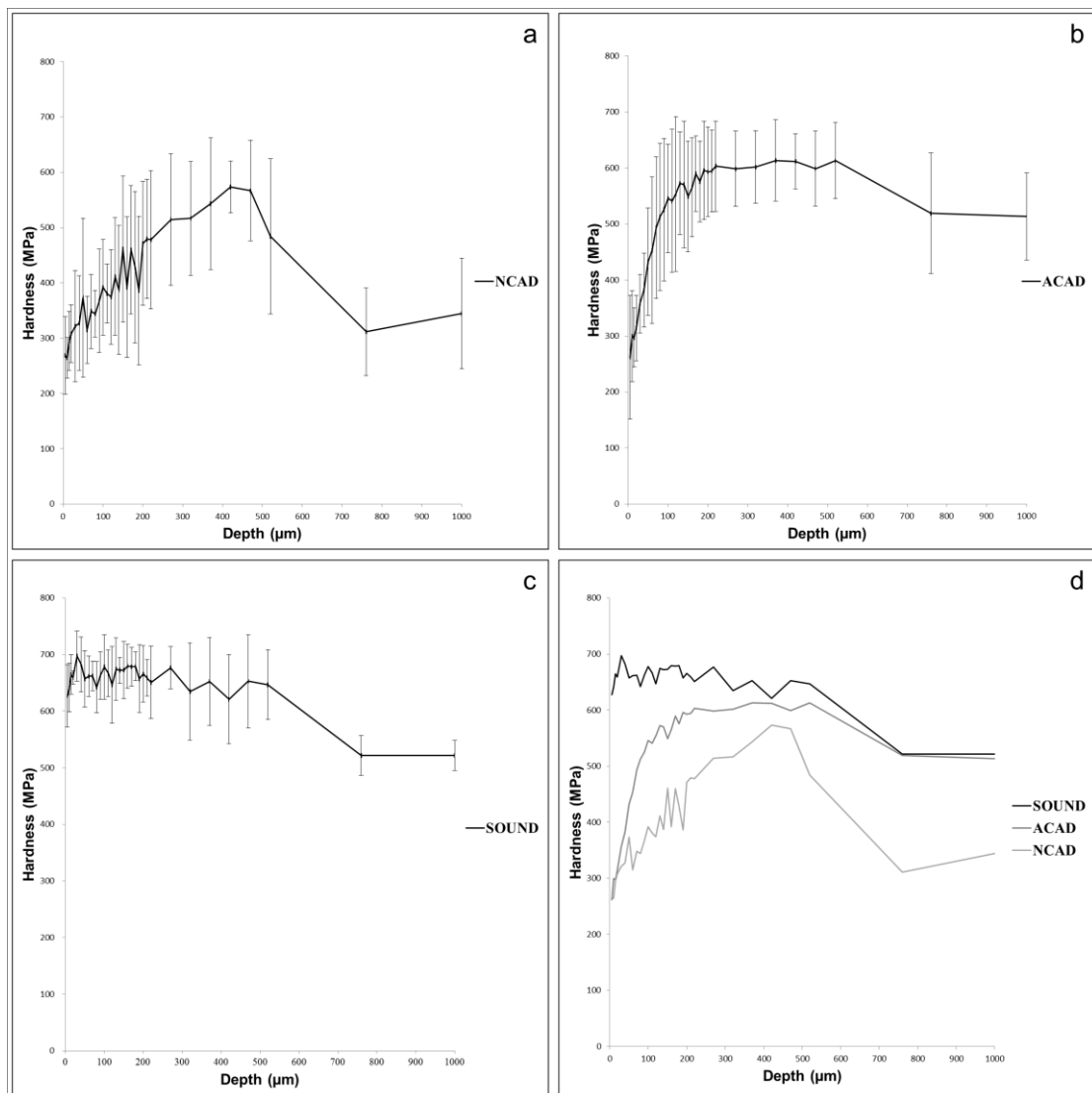


Fig 5. Representative hardness profiles with standard deviations of each type of dentin: NCAD (a), ACAD (b), SOUND (c) and; the plotted area covers 320 indentations that were made using a matrix of 32 rows of 10 indentations each; (d) shows overall hardness profiles of the three dentins.

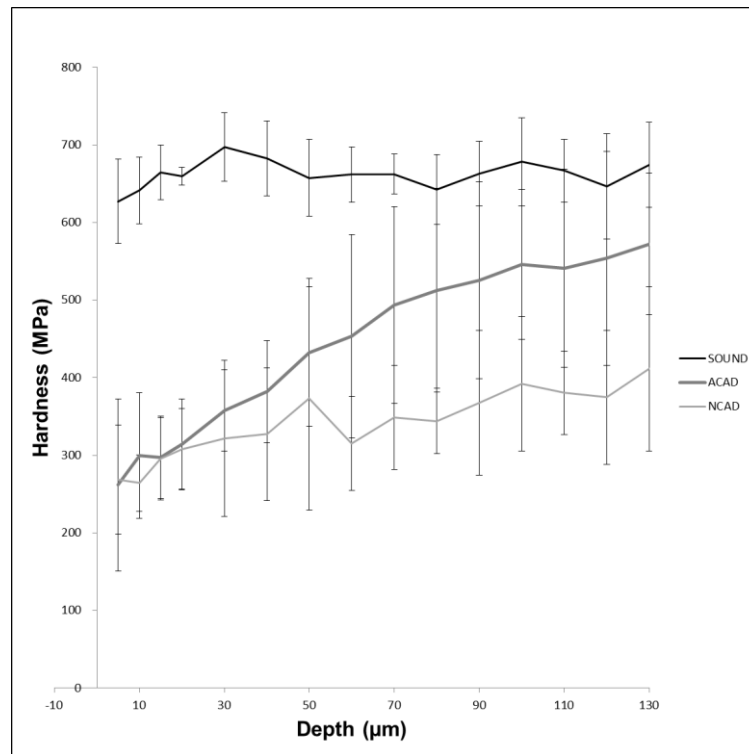


Fig 6. Representative hardness profiles with standard deviations of SOUND, ACAD and NCAD covering an axial area of 130 μm depth.

It is noted that the thickness of a thin soft substrate in nanoindentation test may affect the results; it has been suggested that the indentation depth should not surpass one-fifth of the total substrate thickness (Tsui and Pharr, 1999). The relatively small load selected for this study enabled to keep within an acceptable depth range to ensure that the local mechanical properties of intertubular dentin are measured appropriately. Nevertheless, considering the three-dimensional nature of the tissue and the constant alternating orientation of the dentinal tubules on a given cross-section, local tubule orientation (and consequently the collagen fibril arrangement in relation to the tubule orientation) and underlying presence of dentinal tubules could affect the intertubular properties (Elbaum et al, 2007).

Having different distances and location, the dentin morphology is variable and the hardness results might be affected. The RDT criterion for teeth selection was of more than 2 mm thickness in this study. ACAD and SOUND dentin were considered to have the same distance from the last row of indentations to the pulp chamber.

ACAD lesion was created in a short period of time under controlled conditions using a demineralizing solution (Joves et al, 2013). No significant difference in mineral density of superficial dentin was found between NCAD and ACAD. One finding of the current study indicated the similarity of NCAD and ACAD in hardness up to a depth of 130 μm , which is in agreement with the previous work (Joves et al, 2013). Therefore, the demineralization regime could serve as an artificial caries-affected dentin model for studies on bonding of resin-based dental adhesives.

In the present study, the only point where the intertubular hardness of NCAD reached a close value to that of SOUND dentin was seen at a depth of around 420 - 470 μm . It is important to be noted that a large number of indentations were performed over the whole NCAD lesions covering the area of 1000 μm . Previous studies using microhardness test (Lai et al, 2013; Hosoya et al, 2000) reported similar tendency of NCAD from the surface of the lesions towards the pulp chamber.

Despite similarities of NCAD and ACAD at the superficial zone, NCAD did not have similar mechanical properties to SOUND dentin or that beneath ACAD at the depth of 760 – 1000 μm . The nanohardness tendency of NCAD demonstrates that the effect of carious activity is not limited to the caries-affected area. According to the dissolution-precipitation theory, which explains the mechanism of intratubular cast formation in pathophysiological formation of transparent (sclerotic) dentin, dissolution of intertubular crystallites would result in a reduction of local mechanical properties

(Kinney et al, 2005). Over the period of time when the caries lesion progresses into dentin, the deep intertubular dentin is also prone to changes; acid from bacteria leads to activation of matrix metalloproteinases (MMPs) that affect mechanical properties by degrading the organic matrix (Tjäderhane et al, 1998), as well as accelerated calcium solubilization (Scheffel et al, 2012). Moreover, under physiological conditions of caries progression in vital teeth, reparative dentin may be formed on the ceiling and the floor of the pulp chamber as a new irregular structure (Mjör, I.A., 2009).

Table 1. Hardness average and standard deviations of an axial area of 130 μm depth from three dentins

Depth (μm) / Dentin type	SOUND	ACAD	NCAD
5	627.3 \pm 54.7 ^a	261.8 \pm 110.7 ^b	268.8 \pm 70.3 ^b
10	641.4 \pm 42.9 ^a	299.7 \pm 80.8 ^b	264.3 \pm 36.5 ^b
15	664.7 \pm 35 ^a	297.1 \pm 53 ^b	295.2 \pm 53.2 ^b
20	659.5 \pm 11.6 ^a	314.5 \pm 58.2 ^b	307.8 \pm 52.1 ^b
30	697.4 \pm 44 ^a	357.6 \pm 52.1 ^b	321.8 \pm 100.4 ^b
40	682.6 \pm 48.6 ^a	382.0 \pm 66 ^b	327.1 \pm 85.4 ^b
50	657.4 \pm 49.6 ^a	432.5 \pm 95.4 ^b	373.2 \pm 143.9 ^b
60	661.7 \pm 35.8 ^a	453.3 \pm 130.9 ^b	315.0 \pm 60.5 ^b
70	662.4 \pm 25.8 ^a	493.4 \pm 126.6 ^b	348.5 \pm 66.9 ^b
80	642.4 \pm 45 ^a	512.5 \pm 131.3 ^b	344.1 \pm 42 ^b
90	663.0 \pm 41.8 ^a	525.7 \pm 126.8 ^b	367.4 \pm 93.4 ^b
100	678.1 \pm 56.6 ^a	545.7 \pm 96.7 ^b	391.8 \pm 86.8 ^b
110	666.7 \pm 40.7 ^a	541.2 \pm 127.6 ^b	380.4 \pm 53.4 ^b
120	646.6 \pm 68 ^a	553.7 \pm 138 ^b	374.5 \pm 86.1 ^b
130	674.4 \pm 54.7 ^a	572.4 \pm 91.4 ^b	411.4 \pm 106.3 ^b

Within each row same letter means no significant difference ($p > 0.05$)

The SOUND dentin at deeper areas had been found to have lower hardness than the outer or superficial crown area (Hosoya et al, 2000). This finding was

attributed to the larger size and number of dentinal tubules (Kinney et al, 1996; Hosoya and Marshall, 2004). However, the remarkable decrease of intertubular hardness in this study at deeper areas could be attributed neither to the density of dentinal tubules nor to reparative dentin formation.

Fusayama et al (1966) had previously measured the Knoop hardness of a sclerotic layer within the NCAD, where the Knoop hardness of NCAD was even higher than that of the sound dentin. In comparison with nanoindentation test, Knoop hardness test is characterized as microhardness with larger size of indentations (in the order of 100 μm lateral dimension) covering a wider area that may not exclusively reflect the behavior of the intertubular dentin. The nanoindentation test allowed the setting of indentations performed with a small load to analyze specifically intertubular dentin as the area of interest.

Within the limitations of this study, natural caries process involves biophysiological alterations to the whole substrate that were not induced by in vitro demineralization. The ACAD model was successful in terms of mimicking mechanical properties of NCAD in the intertubular superficial zone.

3.6 Conclusions

Natural and artificial caries-affected dentin tissues were superficially comparable in nanohardness. There is a certain layer within the natural caries-affected dentin with higher hardness; however the long-term effects of caries beneath the lesion extend deeply through intertubular dentin. Sound dentin at deep areas (close to the pulp chamber) is considered to be soft.

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CHAPTER 4

GENERAL CONCLUSIONS

4.1 CONCLUSIONS

In this project, the protocol used as an artificial caries-affected dentin model was successfully compared to natural caries-affected lesions. Based on the results of the two experimental studies, it can be concluded that:

- The artificially caries dentin produced in this *in vitro* study and the natural caries-affected dentin lesions were similar in terms of mineral content and hardness at a superficial depth area. Nevertheless, the presence of mineral casts in the natural caries-affected dentin process makes the morphologies different when compared to the artificial caries-affected dentin model.
- There is a certain layer within the natural caries-affected dentin lesions with higher hardness; however the long-term effects of caries beneath the lesion extend deeply through the intertubular dentin. Sound dentin at deep areas (close to the pulp chamber) is considered to be soft.
- The use of a dye staining solution was useful to differentiate the infected layer in all of the carious specimens. Moreover, the study of innovative technologies involving caries excavation is necessary to achieve the right method and reduce the excessive sound tissue removal to practice a minimal invasive approach with patients.

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