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

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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. 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. 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). In spite of their different morphologies, the ACAD model showed similar MD and µTBS compared to NCAD.

Keywords: Natural caries-affected dentin, Artificial caries, Transverse microradiography, Mineral density, Bond strength

INTRODUCTION

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. Therefore, caries-affected dentin is predominantly the clinical substrate for bonding in many cavity preparations. 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 standardgold method established for the caries removal.

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 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. The solvents present in the adhesive materials have also influence the bond strength to this substrate. Moreover, dentin mechanical properties play a substantial role in the values of strength obtained in laboratory bond strength tests, 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. Chemical methods using an artificial demineralizing solution to produce caries-like lesions may provide a morphological simulation and similar hardness values to natural lesions. On the other hand, while bacterial methods bear some advantages for morphological studies, they result in an excessive softness of dentin, 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.

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.

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.

**Specimen preparation**

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 dentin12).

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$_2$PO$_4$, 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 s at a speed of 12 rpm under running water to create standardized dentin surface with a smear layer.

**Assessment of caries-affected dentin**

1. **Transverse Microradiography (TMR)**
   TMR measurement was performed on dentin slices with 150±30 µm in thickness and approximately 3×4 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% mineral13). The mean mineral density values at different depths up to 150 µm were calculated from the resulting profiles of NCAD and ACAD.

2. **Scanning Electron Microscopic (SEM) observation**
   Five NCAD and six ACAD blocks (1 mm thickness) were

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**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.
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).

3. Microtensile Bond Strength (µTBS) 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 µTBS test was performed at a crosshead speed of 1 mm/min (EZ-test; Shimadzu, Kyoto, Japan).

4. Statistical analysis
All data were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA) at a significance level of α=0.05. Two-way ANOVA was used to compare mineral density at different levels (0, 50, 100 and 150 µm) between NCAD and ACAD, with the “lesion level” and “type of dentin” as the two factors. The µTBS 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.

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

![Fig. 2 Average mineral profiles of all specimens in each group.](image)

Table 1  Mean values of mineral density (vol. %) of NCAD and ACAD at different depths

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>vol% at 0 µm</th>
<th>vol% at 50 µm</th>
<th>vol% at 100 µm</th>
<th>vol% at 150 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCAD</td>
<td>7.91±3.64</td>
<td>27.75±9.27</td>
<td>35.43±7.44</td>
<td>40.20±6.13</td>
</tr>
<tr>
<td>ACAD</td>
<td>8.30±3.60</td>
<td>27.37±5.61</td>
<td>36.44±3.84</td>
<td>42.32±1.80</td>
</tr>
</tbody>
</table>

*No significant difference between NCAD and ACAD (p>0.05, two-way ANOVA).
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 ($p<0.001$).

### 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 clinical practice. Fusayama\(^1\) 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-excavation\(^4\).

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\(^15-16\), 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

### Table 2 Microtensile Bond Strength (MPa) to three types of Dentin

<table>
<thead>
<tr>
<th>Adhesive</th>
<th>Sound dentin</th>
<th>NCAD</th>
<th>ACAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearfil SE Bond</td>
<td>80.8±18.0 (37)$^b$</td>
<td>37.6±14.3 (35)$^a$</td>
<td>39.8±14.2 (37)$^a$</td>
</tr>
<tr>
<td>Clearfil Protect Bond</td>
<td>62.0±12.6 (33)$^c$</td>
<td>34.6±9.9 (37)$^a$</td>
<td>40.4±11.0 (34)$^a$</td>
</tr>
</tbody>
</table>

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±standard deviation (number of specimens).
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. 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, 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. 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. That should help to explain why bond strength to affected substrate was 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.

The crystal logs may decrease hydraulic conductance through dentinal tubules and affect formation of resin tags. 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. Moreover, additional etching which could remove these mineral deposits did not improve bonding of CSE to NCAD. Likewise, formation of resin tags following chemomechanical removal of caries did not significantly improve bond strength of self-etch adhesives. 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.

The interface between bonding resin and dentin is the weak link in adhesive restorations. 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. 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 et al., 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. 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. The ABRZ of CPB in the long-term was thicker and relatively more stable compared to CSE probably due to fluoride release. 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”.

It is noteworthy that during natural caries progress, acidic conditions activate dentin-bound matrix metalloproteases (MMPs) which can degrade the organic phase. 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.

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.

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.

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