Microhardness of dentine in primary teeth after topical fluoride application

1. Introduction

Dental caries can be defined as a bacterial disease of the calcified tissues of the teeth. It is characterized by the demineralization of a tooth's inorganic content and destruction of its organic content. The development of caries in enamel and dentin is different. Unlike enamel, dentin has a relatively high organic content (20%), which consists mainly of collagen. It is a living tissue, so it can respond biologically to carious attack.

Histologically, dentinal caries is classically described as consisting of two main layers.[1] In the outer or "infected" layer, the dentin is heavily infected with bacteria, both organic matrix and mineral have been lost, and the dentin is beyond repair. In the deeper or "affected" layer, the dentin is demineralized by plaque acids, but there is no bacterial invasion. The outer part of this affected layer takes up stains and is thus called the discolored zone; underneath is the transparent zone, which consists of hypermineralized and sclerotic dentin.[1] In the past, demineralization of the enamel that had reached the dentin would have justified operative intervention. The current view, however, is that caries activity can be halted and demineralized dentin can be hardened if the cariogenic environment is altered.[2]

Arrested dentinal caries is caries in the dentin that shows no tendency to progress further. Such dentin is usually microbiologically inactive and its hardness approaches that of sound dentin.[3] Because dentin with arrested caries is exposed to the oral cavity, it has a high fluoride content and becomes hypermineralized owing to continuous remineralization from oral fluids. The lesion in arrested caries has a brown-black pigmentation and is hard on clinical probing. However, clinical diagnosis of arrested dentinal caries largely depends on the examiner's subjective assessment. Laboratory studies have thus been performed to evaluate the

remineralization and hardening of dentinal lesions during caries reversal. Measuring hardness has indeed been shown to be a reasonable method of examining the mineral content of teeth,[4] and several studies of caries or arrested caries have shown that changes in the microhardness of dentin is directly related to its mineral content.[3,5-7]

High-concentration topical fluoride agents, such as 5% sodium fluoride (NaF) varnish containing 22,600 ppm fluoride and 38% silver diamine fluoride (SDF) solution containing 44,800 ppm fluoride, have been used to arrest caries. Clinical trials have reported encouraging results after topical application of SDF solution or NaF varnish.[8-11] In those studies, treatment was considered successful if carious lesions were hard on probing. So far the anti-cariogenic effect of topical application of SDF or NaF has not been thoroughly investigated. This study therefore aimed to measure the microhardness of clinically diagnosed arrested dentinal caries of primary teeth collected from preschool children receiving regular fluoride applications for 30 months.

2. Materials and methods

Participants of this study were 4- to 5-year-old children attending the first year of 8 kindergartens in Guangzhou, China, who had caries on any primary upper anterior teeth and who had been enrolled into a clinical trial of topical fluoride for caries arrest. The main trial and this study were approved by the Ethics Committee of the Faculty of Dentistry, University of Hong Kong. The 375 participating children of the main trial were randomly assigned to receive regular topical applications of either 38% SDF solution or 5% NaF varnish on all upper anterior caries teeth. The teeth were dried and cleaned with gauze, and SDF solution or NaF varnish was then applied with a disposable dental brush. Applications were repeated every 3 months for NaF and every 12 months for SDF.[12] Treatment was considered successful (arrested caries) if the dentinal lesion was hard on probing; otherwise, treatment was considered

unsuccessful (soft active caries). The same clinical examiner conducted baseline and 18- and 30-month examinations. He was not involved in and was thus blinded to the type of fluoride applied. Clinical assessment was repeated by each examiner in about 10% of the children at each time-point to determine intra-examiner reproducibility. The Kappa statistic for duplicate examination was at least 0.95 for baseline and follow-up examinations.[9]

After 18 and 30 months, SDF varnish was effective in arresting dentin caries among this sample of children.[9,12] At the 30-month follow-up, the children were aged about 7 years and their permanent incisors were beginning to erupt. Children from either test group who had very mobile upper primary anterior teeth that were expected to exfoliate shortly were invited to take part in this study. After parental consent had been obtained, very mobile upper central incisors were extracted, fixed in 10% neutral formalin solution, and stored at 4°C until laboratory investigation of the microhardness of carious lesions.

2.1 Specimen preparation

Under streaming water, each extracted primary tooth was sectioned longitudinally along the midline of the carious lesion at a thickness of 150 μm with a copper cutting disc in a hard tissue sectioner developed by the Faculty of Dentistry, University of Hong Kong. The sectioned surface of one half was polished using a metallurgical technique (Multipol 2; Malvern Instruments, Malvern, UK). The polishing was first carried out with silicon carbide paper (Microcut P1200 PSA; Buehler, Lake Bluff, Illinois, USA) under streaming water, and then with paper discs impregnated with diamond paste (Metadi; Buehler, Lake Bluff, Illinois, USA) containing diamond grains of 15 μm, followed by 6 μm and then 1 μm. The polished specimens were checked with an optical microscope to ensure the quality of polishing.

To minimize error due to tilting and to avoid introduction of stress during microhardness testing, each section was mounted with a paralleling device and supported on a glass slide. Specimens were stored in deionized water at 23°C to keep them constantly hydrated, as drying of the dentin would cause changes in hardness and contraction.

2.2 Microhardness measurements

Microhardness, in terms of Knoop hardness number (KHN), was measured immediately after a specimen was removed from deionized water. The mounted specimen was placed under the Knoop indenter of a microhardness tester (Leitz Microhardness Tester; Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) and subjected to a load of 5 gf (49×10^{-3} N) for 10 s at each test point. Microhardness was determined at 20 successive sites below the surface of the tooth from the center of the carious lesion toward the pulp, in increments of 25 μm. The depth from the lesion surface therefore ranged from 25 to 500 μm. Three sets of KHN measurements were made on each specimen on parallel tracks approximately 150 to 200 μm apart (Figure 1). The medians of the medians of the three sets of KHN measurements at each section depth were then analyzed.

2.3 Statistical analysis

Data were analyzed with SPSS version 14.0 for Windows (SPSS Inc, Chicago, Illinois, USA). Nonparametric tests were used because, firstly, the samples in the soft caries group and arrested caries group were small, and, secondly, some data were missing as some measurements on softened areas were too soft to be measured by the microhardness tester. A nonparametric test for two independent samples (Mann-Whitney *U* test) was thus used to assess the relationship between the clinical diagnosis of caries and microhardness. The cutoff level for statistical significance was taken as 0.05.

3. Results

Nine mobile upper central primary incisors were collected. One incisor had 2 lesions, so 10 lesions were available for study. Five carious lesions had received SDF treatment; 4 were clinically diagnosed as arrested caries (Figure 2) and 1 was clinically diagnosed as soft caries. The other 5 lesions had received NaF treatment; 2 were diagnosed as arrested caries (Figure 3) and 3 as soft caries.

The microhardness of dentin, expressed as median KHN, in clinically diagnosed soft and arrested caries according to the distance from the surface lesion is shown in Figure 4. The median of the medians KHN of dentin with arrested caries was more than 40 (392 MPa) in the outer 50 μm of the carious lesion, and then ranged from 20 to 30 (196 to 294 MPa) at points between 50 and 500 μm from the surface. The median KHN of dentin with soft caries was less than 10 (98 MPa) in the outer 50 μm of the lesion, rising to slightly more than 10 in the outer 100 μm. It then gradually increased to 20 (196 MPa) at 200 μm from the surface, beyond which the median KHN ranged from about 20 to 30 (196-294 MPa).

The KHN of both test groups at depths from 225 to 500 μm were similar, at about 20 to 30 (196-294 MPa; $p>0.05$). Although the KHN in the outer 25 to 200 μm of dentin with arrested caries was higher than that of dentin with soft caries, the difference was not statistically significant ($p>0.05$).

4. Discussion

This study investigated the microhardness of dentin in arrested and soft carious lesions on primary anterior teeth that were collected from children in a 30-month clinical trial. No untreated carious teeth were available as a control group because all teeth collected in this study received regular fluoride applications. Because only children with very mobile teeth near exfoliation were invited and not all parents returned their consent form for tooth extraction, the

number of teeth collected in this study was relatively small, which may explain why the difference in microhardness between dentin in the clinically diagnosed soft and arrested carious lesions did not reach overall statistical significance.

Nevertheless, this study demonstrated a protocol that can provide useful information on microhardness of carious dentin. Although the results were intended to reflect dentinal microhardness in the hydrated state and precautions were taken to prevent drying, they cannot be taken to be true measurements of hydrated dentin because the microhardness testing was performed immediately after the specimens were removed from deionized water. Uncontrolled artifacts due to tissue contraction could thus have occurred because dehydration was inescapable during measurement. Nano-indentation has been used under fully hydrated conditions and would be the method of choice to detect incorporation of mineral into the dentin matrix.[13] Measurement of dentinal hardness is commonly performed after dehydration of the dentin specimens. Dentin contains water at a level of approximately 12% by weight and 25% by volume, and testing hydrated tissue produces more reliable and realistic results because it more closely represents the in vivo situation and avoids dehydration artifacts.[14] An ultra-microindentation system has also been recently developed and used to measure the hardness of calcified tissues in the hydrated state.[4]

Hardness testing is an indirect method of tracking changes in the mineral content of dentin, and several microhardness studies on dentin in arrested carious lesions have been published.[3,5-7] Dentinal microhardness in those studies typically ranged in KHN from 25 to 65 (245 to 638 MPa). However, microhardness results from different studies cannot be compared directly. Firstly, the method of specimen preparation can vary. Some researchers have suggested that dentin specimens be dehydrated with 100% ethanol to minimize shrinkage due to drying.[5,7] Others have suggested that dentin specimens be examined directly without ethanol

dehydration.[3,6,15,16] Not only does the hardness of carious primary dentin differ (approximately 10-fold) when it is measured under hydrated and dehydrated conditions, but it is particularly higher in more-demineralized tissue.[17] Secondly, the load used in the microhardness test varied between studies, ranging from 1.5 gf (9.8×10^{-3} N)[6] to 500 gf (4.9 N).[16] The magnitude of the load applied affects microhardness measurement, with the KHN decreasing as the applied load increases.[18] Using a load of less than 15 gf (147×10^{-3} N) to measure microhardness of carious dentin has been recommended.[5] The load used in this study was 5 gf (49×10^{-3} N), and the indentation marked on the specimens ranged from 31 μm to more than 120 μm, with 40 to 50 μm in deeper dentin. These values were similar to those in a previous study in which the mean indentation mark was 40 μm.[7]

The lack of analysis of reading error is a disadvantage in laboratory studies of microhardness. Despite this possible error, the results of microhardness measurement in this study agree with histological, chemical, and clinical observations.[3] Furthermore, any reading error in this investigation should be small because the rater became fully acquainted with the procedure.

A major factor in determining hardness of dentin is its mineral content. The collagen in dentin affects its toughness but makes no significant contribution to its overall hardness.[17] The depth of affected dentin as measured as a change in mechanical properties has been shown to be greater than that defined in clinical assessment, perhaps because dentin appears intact even when up to 50% of its mineral content has been lost.[19] The mineral content of dentin plays an important role in limiting the penetration of bacterial acid. At the macromolecular level, mineral occupies two sites within the collagen scaffold: intrafibrillar (inside the periodically spaced gaps in the collagen fibril) and extrafibrillar (in the interstices between the fibrils),[20] although the partitioning between these two sites is uncertain The mineral crystallites are

needle-like near the tooth's pulp, and their shape continuously progresses to plate-like as the enamel is approached.[20] The thickness of mineral crystallites, which is about 5 nm, stays constant at each location. Through its buffering capacity, mineral reacts and neutralizes acid as it penetrates the tooth. Investigations, however, have so far been unsuccessful in establishing a strong relationship between the reduction in hardness of carious dentin and either infection rate or discoloration.[17]

In this study, variations in hardness were observed within each group of specimens. The amount of mineral left in the teeth, which depends on the rate of caries attack and fluoride treatment, could be one of the reasons for these variations. The site of dentin indentation can also affect the hardness because the site being indented may include differently sized areas ultrastructurally.[16] In addition, dentin's mechanical properties have directionality. Dentin can be modeled as a reinforced composite of tubules in which intertubular dentin forms the matrix and tubule lumens and their associated cuffs of peritubular dentin form cylindrical fibers.[20] The tubules run continuously from the dentin-enamel junction to the pulp in the coronal dentin, and from the cementum-dentin junction to the pulp canal in the root. The regular, almost uni-axial, alignment of the tubules has led some researchers to consider that the tubules help impart dentin's dependence on orientation for its mechanical properties.[21] The intertubular dentin, which makes up the bulk of the dentin, is composed of a collagen fibril network embedded in an amorphous ground substance. Interspersed throughout the intertubular dentin are the dentinal tubules and their surrounding peritubular dentin, which has a higher inorganic content than intertubular dentin and is thus more sensitive to changes in mineralization.[22] Therefore, the depth of the carious cavity can also affect indentation behavior because the density of the dentinal tubules increases; accordingly, the hardness of dentin decreases with increased distance from the amelodentinal junction.[23]

The entire range of KHN in this study varied from less than 10 (98 MPa) to 62 (608 MPa). This range is comparable to the range of 30 to 55 (294-539 MPa) reported for dentin of primary teeth.[24] There appears to be no English-language publication on the microhardness of dentin in formalin-fixed and unfixed specimens. In this study, extracted teeth were formalin-fixed because they were collected in the field. The sectioned specimens were stored in deionized water at 4°C, which was also the method used in many other studies.[3,5-7]

Makinen et al[3] examined the microhardness of remineralized dentin and found an increase in KHN within 100 μm of the surface. This study found that the surface hardness in the outer 50 μm of dentin with arrested caries was higher than that of dentin with soft caries. There was, however, a reduction in the surface hardness beyond 75 μm for arrested carious lesions, although microhardness was consistently greater than that of the soft carious lesions in the outer 25 to 200 μm. In a study using an ultra-microindentation system to evaluate the hardness of carious dentin, microhardness increased in the outer 300 μm.[25] The increase in mechanical properties seen at the surface of carious lesions suggests an increase in mineral content and reflects the effect of remineralization to a certain depth. This study found that the KHN of dentin for both soft and arrested caries beyond a depth of 200 μm ranged from 20 to 30 (196 to 294 MPa). Similarly, Pereira and colleagues found that the effect of fluoride on microhardness beyond 400 μm from the lesion surface became insignificant.[7]

5. Conclusions

The results of this study indicate that microhardness was highest at the surface-most 50 μm of dentin with arrested caries after regular in vivo fluoride applications for 30 months. There was a difference in dentinal microhardness between samples with arrested caries and those with soft caries, but only in the outer 25 to 200 μm from the lesion surface. At distances greater than 200 μm from the lesion surface, the dentinal microhardness of both arrested soft

caries was similar. The findings suggest remineralization helps hardness recover to a certain depth.

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