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ORIGINAL ARTICLE

Confocal laser scanning microscopy study of dentinal tubules in dental caries stained with alizarin red

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Keywords

Alizarin red,
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Abstract Dentin morphology and the lesion found in dental caries have been studied for many years. It was first observed under optical microscopy, and later using electron microscopy. Confocal laser scanning microscopy (CLSM) applied with several fluorescent dyes such as alizarin red to see normal dentinal tubules. However, as far as authors aware, the CLSM studies of dentinal tubules in human caries using alizarin red is rare. The aim of this study is to examine histopathological and morphological changes in dentinal tubules of dentin caries stained with alizarin red using CLSM. Fifteen extracted carious teeth (premolar or molar) was collected and fixed in neutral formalin solution buffered with phosphate buffer, rinsed and stored in calcium free phosphate buffer saline (PBS) at 4°C. The specimens were dehydrated and embedded in resin. Longitudinal or cross sections were cut and polished and then stained with alizarin red S (100 µg/ml) in 0.5 M HCl solution for 24-48 hour at 37°C. After dehydration specimens were mounted on glass slide and examined under CLSM using epi-flourescent mode or transmission light mode with wave length of 512 nm. The images of dentinal tubules were taken serially and optimum images of three-dimensional structures were reconstructed using software of CLSM. Histopathological changes of dentinal tubules in human caries showed area of demineralized dentin, translucent zone, and normal area. The dentinal tubules were thin and had numerous branches. In conclusion, confocal microscopy revealed Study shows that confocal microscopy revealed histopathological changes in dentinal tubules affected by carious lesions.

Introduction

Dental caries remain one of the most common diseases throughout the world until today. It is caused by bacteria that colonize the tooth surface and, under certain conditions, produce sufficient acids to demineralize the enamel covering of the tooth crown or the cementum covering the root, and then the underlying dentin (Cawson and Odell, 1998).

Dentin is a mineralized connective tissue that forms the bulk of tooth, located beneath enamel and root cementum. The dentin consists of odontoblastic processes and extracellular matrix. It also consists of microscopic channels, called dentinal tubules, which radiate outward through the dentin from the pulp to the exterior cementum or enamel border (Ross *et al*., 2003).

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Dentin morphology and composition has been studied for many years. It was first characterized using optical microscopy, and later using electron microscopy with decalcified or ground section methods (Mjör and Pindborg, 1973). There is no three-dimensional (3-D) observations of caries tooth have been studied with light or electron microscopy, since thin section are difficult to prepare serially, due to the low organic content of the enamel and dentin (Nishikawa *et al*., 2003). *In vitro* study on secondary caries and tomography image of the interface between enamel and dentinal restorations using CLSM without destroying the tooth structure has been described by Al-Salihi *et al*., (2003).

Kagayama *et al*., (1999) studied dentinal tubules in human tooth that alizarin red stained vitally the dentinal tubules near the pre-dentin, but not other parts of the dentinal tubules. The study also demonstrated that the dentinal tubules surrounded by calcified extra cellular

matrix and interglobular dentin were stained with alizarin red. The coronal and radicular part of dentin showed different morphology and microscopic features.

Alizarin red is one of the fluorescent dyes that have been used for vital staining of calcified tissue. It is used in a biochemical assay to determine the presence of calcific deposition by cells of an osteogenic lineage, quantitatively by colorimetry. It is an early stage marker (days 10-16 of in vitro culture) of matrix mineralization and sometimes used to demonstrate calcium in sections (Kiernan, 2006; Lillie and Fullmer, 1976).

Study on histopathological and structural changes in dental caries using CLSM stained by modified Hematoxylene and Eosin was performed by Al-Salihi *et al*. (2003). The study demonstrates that sound enamel and dentin areas appear to be dark under CLSM, while more demineralized carious areas appear whiter. All ground sections showed different histopathological caries changes depend on the severity of lesion. Initial lesion characterized by a cone-shaped lesion with apex towards the dentin. Dentinal tubules were distorted and clefting in some teeth that showed extension of caries in dentin and lead to pulpitis.

The lesions found in dental caries have been examined by light electron microscopic method using decalcified or ground section. CLSM is a relatively new, using non-destructive technique and provide 3-D images (Al-Salihi *et al*., 2003). The used of Alizarin red in study of normal dentin was firstly reported by Kagayama *et al*., (1999). As far as we are aware, there are few publications of 3-D observation of carious teeth have been performed using fluorescent dyes such as calcein, tetracycline and rhodamin for staining.

The aim of this study was to examine histopathological findings obtained from CLSM in the lesions of human dentin caries and analyze the morphological characteristics (changes) of the dentinal tubules in lesions of human dentin caries stained with alizarin red.

Materials and Methods

Fifteen extracted carious adult permanent teeth (premolar or molar) were used in this study. The caries teeth were involved dentin, but did not exposed pulp. The teeth were then fixed a whole in 10% neutral buffered formalin for 5 days. The teeth then rinsed and stored in calcium free phosphate buffer saline (PBS) at 4° C. After that, for fixation process, specimens were dehydrated with 60%, 80%, 96% and 100% (absolute) alcohol for 24 hours of each step. Specimens then infiltrated by 10% technovit 7200 in 90% alcohol; 30% technovit in 70% alcohol; 50% technovit in 50% alcohol; 70% technovit in 30% alcohol; 90% technovit in 10% alcohol and lastly 100% technovit 7200 resin. Infiltration process with technovit resin was 24 hours of each step (Al-Salihi *et al*., 2003). All samples were then embedded and polymerized in resin fixation medium by 450 nm wavelengths. A thick section of 100-200 µm longitudinal or cross sections were performed using hard tissue cutter (EXAKT, Germany), and the dentin surface were polished with specimen polisher (EXAKT, Germany). The ground section stained with alizarin red S (100 µg/ml) in 0.5 M HCl solution for 24-48 hour at 37°C (Kagayama *et al*., 1999). The sections were polish again with polisher and rinse with water. The section were dehydrated, mounted on glass slide and examined under CLSM (Leica TCS II, Germany) using epi-fluorescence mode or transmission light mode with wavelength of 512 nm. The images of dentinal tubules were taken serially and optimum images of three-dimensional structures were reconstructed by CLSM software.

Results

Alizarin red stained dentinal tubules, interglobular dentin and inner surface of dentin (Kagayama et al., 1999). All examined teeth ground sections in this study showed histopathological changes of caries with varying degree, which revealed dentin human caries. All samples showed area of normal dentin zone (N) where dentinal tubules were condensed and thick and translucent zone (T) where dentinal tubules were thin and loose (Figure 1).

The dentinal tubules in translucent zone appear as circular tubules at cross sectional sections. However, the area of demineralized dentin appeared to be dark (Figure 2). Only few dentinal tubules stained in the translucent zone, the outline of tubules remains visible and zone appears translucent (Cawson and Odell, 1998).

The dentinal tubules appeared bamboo-like with many nodules in longitudinal sections and had numerous branches at the normal area (Figure 3a and 3b). In longitudinal sections, dentinal tubules at translucent zone were thin and smaller in size when compared to normal area (Figure 4a and 4b).

Discussion

The enamel is avascular and acellular that cannot respond to injuries, whereas the dentin is an integral part of the pulpo-dentinal organ and considered a vital tissue. Dentin and pulp have to be considered the functional unit of the endodontium, they are capable of defence reactions to carious attack (Nanci, 2008). With increasing porosity as a result of enamel demineralization, the underlying pulpo-dentinal organ therefore reacts. When the enamel lesion extends to the enamel-dentinal junction, the first sign of dentin demineralization can be seen along the junction (Fejerskov *et al*., 2003).

The result found that in area of normal dentin zone (N), dentinal tubules were condensed and

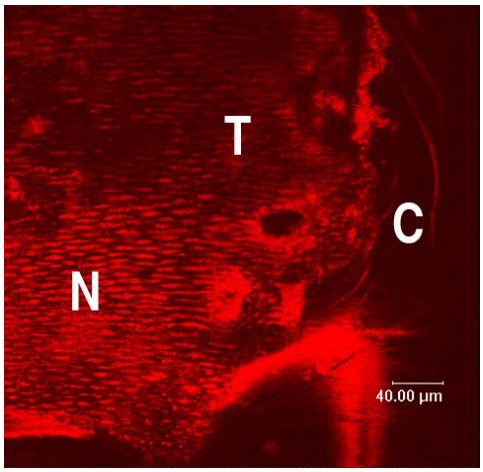


Figure 1 Normal dentin (N), translucent dentin(T) and demineralised dentin (C) zones under the CLSM in longitudinal view (100X).

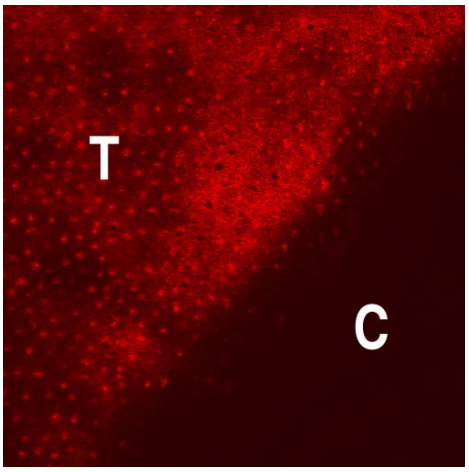
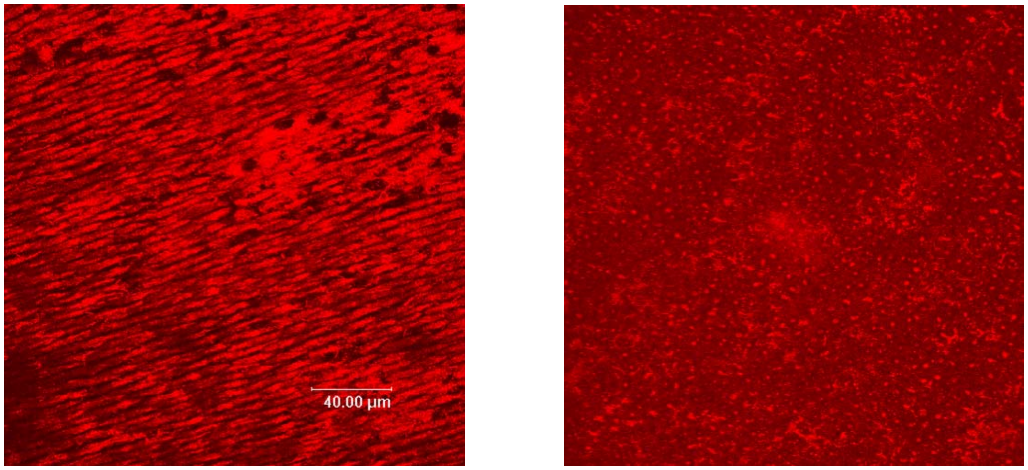
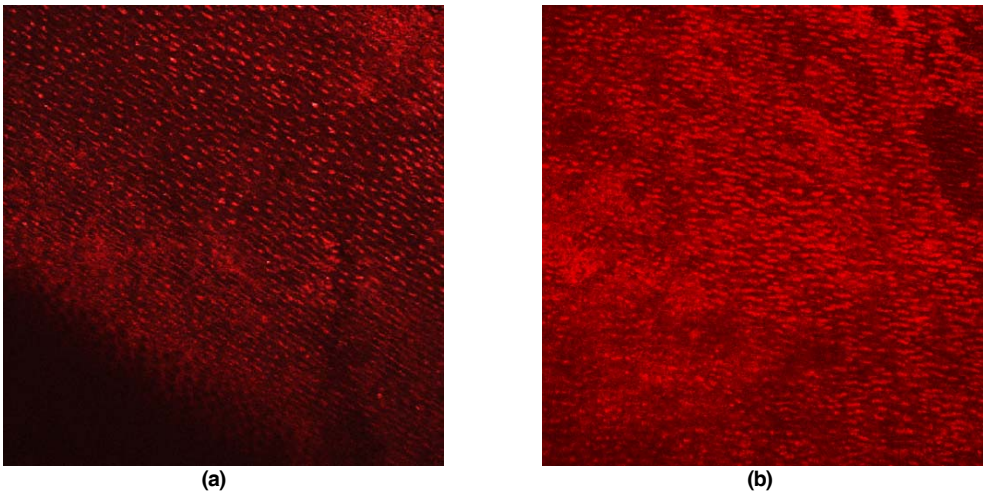


Figure 2 Demineralized dentin appears as dark area (C) followed with translucent area (T) under CLSM in cross-sectional view (63X).



(a) **(b)**

Figure 3 CLSM micrographs of dentinal tubules stained with alizarin red showing bamboo-like structure in longitudinal section. **(a)** Longitudinal view (63X) and **(b)** cross sectional view (100X).



(a) **(b)**

Figure 4 Dentinal tubules were thinner and smaller in size at translucent zone compared to normal dentin area in longitudinal section under CLSM (40X). **(a)** Translucent zone and **(b)** normal dentin area.

thick, whereas in translucent zone (T), dentinal tubules were thin and loose (Figure 1). These findings are compatible with others (Nishikawa, 1998; Arnold *et al.* 2001; Fejerskov *et al*., 2003). Nishikawa (1998) reported that in caries dentin, the lesion progressed along the dentinal tubules and lateral branches. His study also found softened dentin was observed that caused dentinal tubules were thin and loose.

Demineralization of dentin causes a pathology and morphological reaction of the dentin-pulp-complex to the carious attack. Deep active carious lesions are characterized by demineralizing dentin and the translucent zone as shown in Figure 1. The extension of demineralizing zone increases with the progression of the carious lesion. The mineral uptake in the dentin from the saliva is very limited after arrest of the disease, and therefore the demineralized dentin remain as scar in the tissue (Fejerskov *et al*., 2003).

In this study, the area of demineralized dentin in translucent zone appeared to be dark (Figure 2). Similar results also reported by Cawson and Odell (1998). The study observed that only few dentinal tubules stained in the translucent zone, the outline of tubules remains visible and zone appears translucent (Cawson and Odell, 1998). Arnold *et al.,* (2001) also found in translucent zone, dentinal tubules were less stained because of reactive process of intra tubular sclerosis. These processes are complex that result in peritubular, intratubular and reactive dentin formation within dentin tubules and the pulp chamber (Arnold *et al.* 2001). Nishikawa (1998) reported that cuboidal or rhomboid-shaped mineral crystallites were observed by scanning electron microscope (SEM) in the dentinal tubules beneath the carious lesion. Therefore, the dentinal tubules seemed to be related both to demineralisazion and remineralization (Nishikawa, 1998).

The most common defence reaction by the pulpo-dentinal organ is deposition of mineral along and within the dentinal tubules (Fejerskov *et al*., 2003). The reactive intratubular dentin sclerosis of the translucent zone is well established (Atkinson and Harcourt, 1961; Bergman and Engefeldt, 1955). The extension of the demineralizing zone increases with the progression of the carious lesion, but the extension of the translucent zone does not increase further. This may be because of limited capability of the odontoblasts to react to the carious process by producing intratubular dentin that is responsible for the sclerotic translucent zone (Arnold *et al.,* 2000).

In relatively deep carious dentin, the dentin may become hypermineralized within a limited area pulpally to the advancing demineralized zone. Depending on the size and rate of penetration of the caries, the formation of dentinal sclerosis, such dentin receives a transparent and glass-like appearance (Bergenholtz *et al*., 2003). The sclerotic and obturated dentinal tubules appear translucency because the mineral in the tubules makes dentin more homogenous, reducing scattering of light passing through the affected dentin. Sclerotic dentin is therefore often referred to as translucent (transparent) dentin or a translucent zone (Fejerskov *et al*., 2003).

Alizarin red is used in a biochemical assay to determine the presence of calcific deposition by cells of an osteogenic lineage, quantitatively by colorimetry. It is an early stage marker (days 10-16 of in vitro culture) of matrix mineralization and sometimes used to demonstrate calcium in sections (Kiernan, 2006; Lillie and Fullmer, 1976). Since dentin is composed of calcium substance, alizarin red can be use to determine the presence of calcific deposition. In contrast, alizarin cannot determine the presence of calcific deposition in area which caries activity occurs as there was demineralization of that area.

It was shown that alizarin red stained only area with mineralization; translucent zone and demineralization area appear to be dark (Figure 2). The ESEM backscattered electron mode micrograph study on dentin confirming the variation of the mineralization level of dentinal tubules (Elbaum *et al.,* 2007).

At the outer layer of coronal dentin, dentinal tubules were thin and had numerous branches as shown in Figure 3. Terminal branches are near the dentin-enamel junction. These results are comparable to the study by Kayagama that reported, in the middle layer of coronal dentin, the dentinal tubules that appear bamboo-like with many nodules in longitudinal section (Kagayama *et al*., 1999).

In the translucent zone, peritubular dentin reduces the size of dentinal tubules in order to prevent bacterial penetration and generating more mineralized sclerotic dentin (Cawson and Odell, 1998). There was a reduction of dentinal tubules size occurred in translucent zone (Ross *et al*., 2003). Similar results found in this study showed dentinal tubules were thinner and smaller in size at translucent zone (Figure 4a) as compared to the normal dentin area in longitudinal section (Figure 4b). The peritubular dentin was shown to be more mineralized than intertubular dentin, confirming the observation of Bakri and Salleh (2003) and also comparable to the harmonic generation microscopy study on dentin micro-architecture by Elbaum *et al.* (2007).

Conclusion

This study shows the ability of CLSM and alizarin red in the study of histopathological changes of dentinal tubules with carious lesions. The results obtained in this study demonstrate that confocal laser scanning microscope have the ability to focus through a caries lesion into the underlying sound dentin. Confocal microscope offers a non-destructive sensitive in demonstrating mineral changes in carious dentin.

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