Study of a Morphometric Model for Histological Evaluation of the Collagen in Dentin Carious Lesions

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Objective: *The guidelines of minimum intervention recommend the maintenance of the affected dentin; in the other hand, there are few methods able to analyze the presence of intratubular collagen. The objective of this study was to assess the collagen structure in carious dentin.* ***Study design:*** *Ten collected samples of infected and affected dentin were stained with hematoxilin-eosin (HE) and Sirius Red (SR). The areas intensively stained with SR were delimited and the percentage of these areas was measured by double-blind calibrated examiners. The mean results were subjected to the t-student test. The amount of dentinal tubules and their area (pixels) were evaluated by HE, and subjected to the Mann Whitney test.* ***Results:*** *The mean of dentinal tubules in the infected dentin was 213.22 and in the affected dentin it was 120.85 ($p<0.05$). The mean area of dentinal tubules in the infected dentin was 1175.16 and in the affected dentin was 1420.70 ($p>0.05$). The percentage of intratubular organized collagen in the infected dentin was 12.71% and in the affected dentin it was 32.37% ($p<0.05$).* ***Conclusions:*** *The histological evaluation of the collagen is a reproducible method to perform the morphometry in carious dentin and the affected dentin presents structured collagen areas where reorganization is possible.*

Keywords: *dentin, caries, histology, minimal intervention, collagen.*
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INTRODUCTION

Nowadays, in Pediatric Dentistry the atraumatic restorative technique and the minimal intervention are daily clinical activities. In both techniques, the carious infected dentin is removed while preserving the affected dentin tissue. The sound dentin is histologically divided in 80% of organic material and 20% of inorganic substance.[1] Calcium hydroxyapatite crystals constitute the inorganic mineral component. The dentin organic matrix consists of type I collagen with fractionate inclusions of glucoproteins, proteoglycans, phosphoproteins and sialoproteins impregnated in amorphous fundamental substance.[2,3,4,5,6] The collagen fibers of the intertubular sound dentin observed under electron microscopy shows gaps on the collagen of approximately 67 nm and fiber diameters between 90 and 120 nm.[7] The percentage of type I collagen, phosphoproteins and sialoproteins is greater in sound teeth than in carious ones. These three proteins are important in dentinogenesis, since they stimulate the reparative and tertiary dentin production.[6]

In the cariogenic process, toxins released from bacteria spread themselves within the enamel and initiate biochemical reactions in dentin. As the infectious process progresses, loss of minerals and matrix alteration are observed within the dentin. The pH drop activates the release of metaloproteinases that hydrolyze the organic matrix in dentin, contributing to the progression of the carious lesion.[8]

The dentinal carious lesion can be histologically divided into an outer layer (infected dentin) and inner layer (affected dentin). The outer layer presents extensive decalcification and degradation of the collagen fibers. The inner layer is characterized by intermediate decalcification of collagen fibers that can reorganize and by odontoblasts with an active recalcification process.[9,6] Currently, it is possible to observe six different layers in dentinal carious lesions: 1 – outermost zone, not capable of remineralization; 2 – translucent dentin;

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3 - sub-transluscent dentin; 4 – sclerotic dentin; 5 – dentin; 6- predentin. The layers 2, 3 and 4 correspond to areas with possible remineralization.[1]

In the minimally invasive pediatric dentistry, the total removal of infected dentin is recommended, as well as the preservation of the affected dentin. Dentin seal protects the pulp-dentin complex restricting bacterial nourishment, and inhibiting caries progression.[2,11,9,12,13,14,15] Nowadays, there are only few studies that assess the collagen behavior in decayed dentin after minimal intervention.

This research aims to study a morphometric model to evaluate the collagen in carious dentin through the measurement of reorganizable collagen areas with Sirius Red stain, and quantity and diameter of dentinal tubules with hematoxilin–eosin stain.

METHOD

This trial was approved by the Institutional Review Board of PUC-Campinas (Protocol 637/05). Ten samples of infected dentin and 10 of affected dentin were collected from 10 primary molars in the Pediatric Clinic of PUC – Campinas. The differentiation between infected dentin and affected dentin was done according to clinical criteria[15]: infected dentin: wet, highly softened tissue with no resistance on removal. Affected dentin: dried and resistant to mechanical removal with a "chip" clinical appearance.

The inclusion criteria for the samples were:

- Patients with dentin carious lesion without pulp involvement.
- Characteristic symptoms of reversible pulp inflammation.
- Patients who agree to participate in the trial and have signed the Informed Consent Form.

The exclusion criteria for the samples were:

- Carious lesion in dentin with pulp involvement, characterized by periapical radiolucent areas.
- Teeth with characteristic symptoms of non-reversible pulp inflammation;
- Teeth with clinical and radiographic signs of pulp necrosis.

After the medical history, clinical examination, X-rays, hygiene and diet education, the patient with dentinal carious lesion received local anesthesia with 2% Lidocaine, epinephrine 1:100000 (Dentisply Probem, Catanduva, Brazil), prophylaxis with Robinson brush (Microdont, São Paulo, Brazil) with slow speed and rubber dam to isolate the surgical field. The dentin carious lesion was collected with a micropunch (Ritcher, São Paulo, Brazil) and the remaining dentin was sealed with Vidrion F (SSWhite, Rio de Janeiro, Brazil) and Composite Resin Z 350 (3M ESPE, Saint Paul, USA). The biomaterials above mentioned were handled according to the manufacturer instructions.

A sound tooth selected for extraction due to severe periodontitis was used as control. Ten samples of infected and affected dentin were stained with Hematoxilin–eosin (HE), and Sirius Red (SR) and examined at (200 and 500 X). Software Tpsdig, version 1.38,was used, processing 32 images with SR-stain and 24 with HE-stain. The SR-stained areas (organized collagen) were delimited by double-blind calibrated examiners.

Formula to calculate the organized collage percentage (Figure 1). The results were analyzed with a Biostat software 4.0. To evaluate the calibration among examiners, intraclass correlation coefficient was used and the mean results were subjected to the t-student test. The golden standard for the calibration of the organized collagen areas delimitation was for SR-stained samples of sound dentin (Figure 2). The quantity and area of dentinal tubules (pixels) were evaluated in HE stain and they were subjected to the Mann Whitney test.

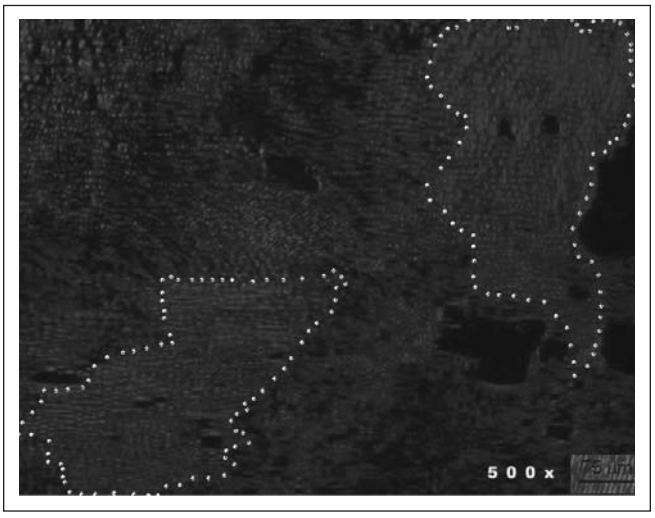

Figure 1. Measurement of Sirius Red-stained areas in the software Tpsdig. Delimited areas with dotted lines: regions with collagen similar to that of the sound dentin (control) selected by double-blinded examiners.

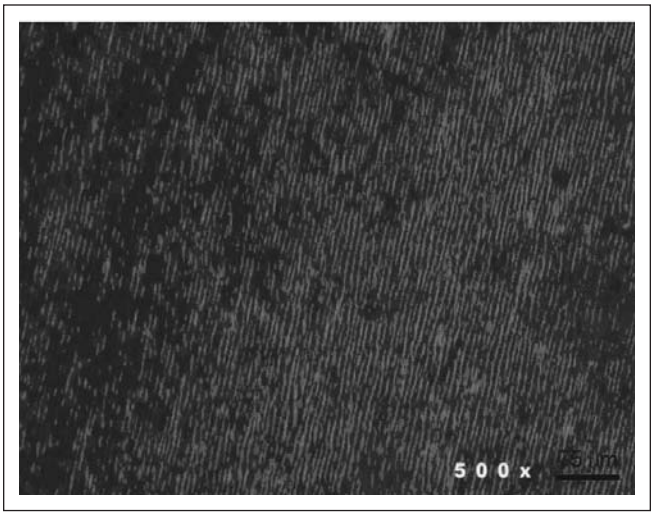

Figure 2. Golden standard: Sirius Red-stained sound dentin. Observe the absence of blackish areas that characterize the collagen organization.

RESULTS

Examiners calibration can be seen in Tables 1 and 2. The mean of dentinal tubules in the infected dentin was 213.22 and 120.85 in the affected dentin ($p<0.05$); the mean area of tubules in infected dentin was 1175.16 and in the affected dentin was 1420.79 ($p>0.05$) (Tables 3, 4) (Figures 3, 4). The mean percentage of organized collagen in the infected dentin was 12.71% and in the affected dentin it was 32.37% ($p<0.05$) (Table 5).

Table 1. Evaluation among examiners - organized collagen area – intraclass correlation coefficient - infected dentin

Variation among examiners	Experimental error	F	p-value	Intraclass correlation
4.09	0.31	13.08	p<0.0001	0.85

Table 2. Evaluation among examiners - organized collagen area – intraclass correlation coefficient – affected dentin

Variation among examiners	Experimental error	F	p-value	Intraclass correlation
0.78	0.01	41.75	p<0.0001	0.95

Table 3. Means and standard deviations of the dentinal tubules quantity (Mann Whitney test).

Infected	Affected
213.22(138.76)a	120.85(180.63)b

Different letters (a,b): statistically significant differences (p=0.0118)

Table 4. Arithmetic means and standard deviations of the dentinal tubules area – Mann Whitney test.

Infected	Affected
1175.16(729.03)a	1420.79(679.87)a

Equal letters (a,a): no statistically significant differences (p=0.4497)

Table 5. Arithmetic means and standard deviations of the organized collagen percentage – t-student test.

Infected	Affected
12.72(11.00)a	32.37(18.16)b

Different letters (a,b): statistically significant differences (p=0.0013)

DISCUSSION

The morphometric model proposed in this trial allowed the measurement of organized collagen surfaces, the amount of dentinal tubules and the quantification of carious dentin areas. This allows comparing dentin repair after cavity seal, through the quantification of the organized collagen areas and the dentinal tubules before and after restoration.

It was possible to observe 32.37% of organized collagen areas in the affected dentin, showing that this tissue presents collagen cross-links partially degraded and capable of reorganization.[2,11,9,6] Regarding to the outermost layer of carious

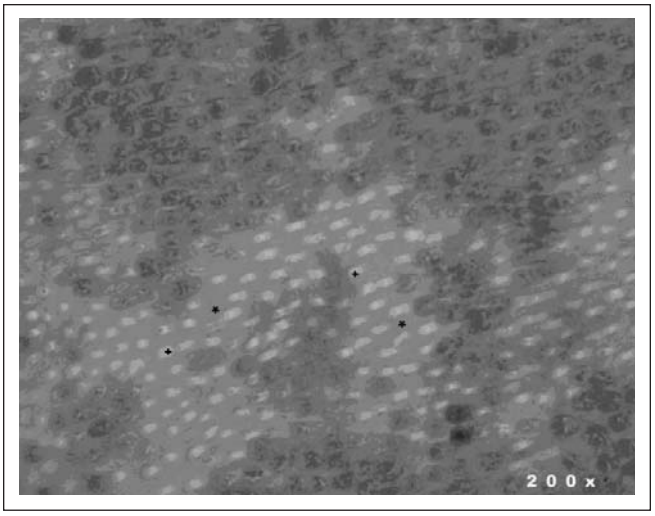

Figure 3. HE-stained infected dentin. Observe the matrix degradation in the intertubular and peritubular dentin: Intertubular dentin.* Dentinal tubules+

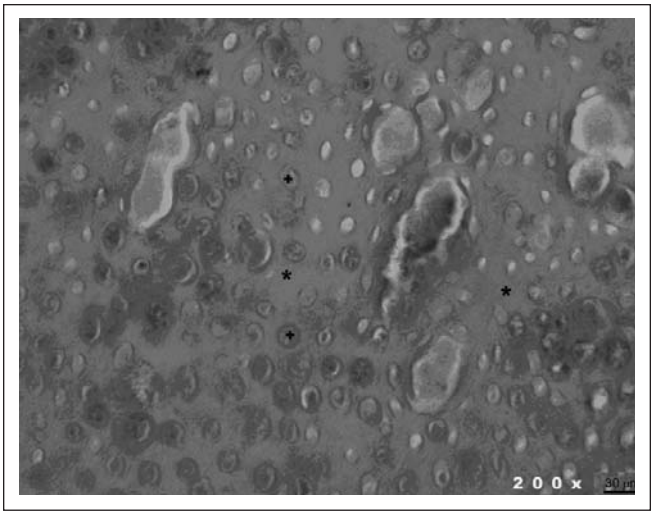

Figure 4. Affected HE-stained dentin. Observe the predominance of sound intertubular and peritubular dentinal areas. Intertubular dentin.* Dentinal tubules+.

tissue, the infected dentin presented 12.72% of organized collagen area, differing from the authors that observed irreversible degradation of the collagen matrix.[1,2, 9,16]

The quantification of the integrity of collagen fibers cross-links by measuring dihydroxylisinorleucine (DHLNL) and hydroxylisinorleucine (HLNL) and its precursors dihydroxynorleucine (DHNL) and hydroxynorleucine (HNL) has been shown in dental literature[11] as a mean to evaluate dentinal carious lesions. The sound dentin shows a marked increase of DHLNL in relation to the precursors (DHNL and HNL). The affected dentin shows increased DHNL compared with DHLNL due to the acidic pH that promotes the increase of cross-links precursors. In contrast, the infected dentin presents a decrease of DHLNL and HLNL as well as of its precursors, DHNL and HNL, exhibiting its irreversible collagen molecule alteration. Consistent results were found in this research: the affected dentin presented 32.37% of

organized collagen area and marked reduction of these areas was observed in the infected dentin, that presented 12.72% of organized regions.

In this trial, a greater number of dentinal tubules were found in the infected dentin, when compared to the affected dentin ($p<0.05$); however, when measuring those regions, greater tubular areas were found in the affected dentin comparatively to the infected dentin. Similar results were found in literature[6] showing 16% of these tubule diameters containing collagen fibrils occupying more than one fifth of the lumen. The absence of statistical difference observed in this trial among the greater tubules area in the affected dentin and the infected dentin can be supported by the marked demineralization of the infected dentin, bringing about an increase of the tubule lumen.

Reports show that the diameter of the dentinal tubules near the amelodentinal junction is approximately 0.8 μm and near the pulp is 2.5 μm.[17] Acid etching increases the tubule diameter to 35 μm.[18] This study showed that dentinal tubules had a diameter of approximately 20.4 μm in the infected dentin, and of 22.4 μm in the affected dentin. Therefore, we can see a lesser mineral loss from caries than from acid etching.

Carious teeth presented areas with no dentinal tubules or with irregular tubules in the central region, whereas in the peripheral area, the tubules were more regular. The odontoblasts located in the central region are cuboid in form, being oblong in the peripheral region. In the central region, the dentin present a decresase of type I collagen, phosphoproteins and sialoproteins when compared to the peripheral region.[6] The hematoxilin–eosin stain of the carious lesions in this study presented cuboid and oblong dentinal tubules, consistent with the literature reports.[6]

In modern restorative dentistry the minimal structural intervention is recommended, maintaining the affected dentin.[19] This study presented a model to evaluate the collagen of carious lesions in dentin and could be used to compare the sealing ability of different biomaterials and its response in affected dentinal tissue.

CONCLUSIONS

The calculation of the organized collagen percentage in SR stain associated to the quantification and characterization of the dentinal tubules area is a safe, suitable and reproducible method to perform the morphometry in dentinal carious lesions.

REFERENCES

1. Nakornchai S, Atsawasuwan P, Kitamura E, Surarit R, Yamauchi M: Partial biochemical characterisation of collagen in carious dentin of human primary teeth. Arch Oral Biol, 49: 267–273, 2004.
2. Fusayama T, Terachima S: Differentiation of two layers of carious dentin by staining. J Dent Res, 51: 866, 1972.
3. Dai X-F, Cate, ART, Limeback H: The extent and distribution of intratubular collagen fibrils in human dentine. Archs Oral Biol, 36: 775–78, 1991.
4. Chang SR, Chiego Jr D, Clarkson BH: Characterization and Identification of a Human Dentin Phosphophoryn. Calcif Tissue Int, 59: 149–153, 1996.
5. Butler WT, Brunn JC, Qin C, Mckee MD: Extracellular Matrix Proteins and the Dynamics of Dentin Formation. Connect Tissue Res, 43: 301–07, 2002.
6. Lee YL, Liu J, Clarkson BH, Lin CP, Godovikova V, Ritchie HH: Dentin – Pulp Complex Responses to Carious Lesions. Caries Res, 40: 256–264, 2006.
7. Yang B, Adelung R, Ludwig K, Böbmann K, Pashley DH, Kern M: Effect of structural change of collagen fibrils on the durability of dentin bonding. Biomaterials, 26: 5021–5031, 2005.
8. Miller CC, Fioretti F, Goldberg M, Menashi S: The Role of Matrix Metalloproteinases (MMPs) in Human Caries. J Dent Res, 85: 22–32, 2006.
9. Fusayama T: Two layers of carious dentin. Diagnosis and Treatment. Oper Dent, 4: 63–70, 1979.
10. Banerjee A, Watson TFW, Kidd EAM: Dentine carious: take it or leave it? Dent Update, 27: 272–76, 2000.
11. Kuboki Y, Ohgushi K, Fusayama T: Collagen biochemistry of the two layers of carious dentin. J Dent Res, 56: 1233–37, 1977.
12. Mertz–Fairhurst EJ, Schuster GS, Fairhust CW: Arresting caries by sealants: results of a clinical study. J Am Dent Assoc, 112: 194–7, 1986.
13. Banerjee A, Watson TFW, Kidd EAM: Dentine caries excavation: a review of current clinical techniques. Br Dent J, 188: 476–82, 2000.
14. Yoshima M, Tay FR, Toril Y, Nishitani Y, Doi J, Itou K: Resin adhesion to carious dentin. Am J Dent, 16: 47–52, 2003.
15. Pinheiro SL, Simionato MR, Imparato JCP, Oda M: Antibacterial activity of glass – ionomer cement containing antibiotic on caries lesion microorganisms. Am J Dent, 18: 261–66, 2005.
16. Hoshino E. Predominant obligate anaerobes in human carious dentin. J Dent Res, 64(2): 1195–98, 1985.
17. Pashley DH. Dentin: a dynamic substrate – a review: Scaning Microsc, 3: 161–174, 1989.
18. Pashley EL, Tão L, Mathews WG, Pashley DL: Bond strength to supeficial, intermediate and deep dentin in vivo with four dentin bonding systems. Dent Mater, 9: 19–22, 1993.
19. Türkün LS, Ates M, Türkün M, Uzer E: Antibacterial activity of two adhesive systems using various microbiological methods. J Adhes Dent, 7: 315–320, 2005.