Scientific Article

Ultrastructural and Microbiological Analysis of the Dentin Layers Affected by Caries Lesions in Primary Molars Treated by Minimal Intervention

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Abstract: *Purpose:* The purpose of this in vivo study of primary teeth was to analyze the ultrastructure and microbiology of dentin layers affected by caries lesions before and after restorations with resin-modified glass ionomer. *Methods:* Samples of carious dentin from primary teeth removed prior to restoration placement (baseline-0 day) were compared with samples taken after 30 and 60 days. Dentin from 8 primary molars was analyzed by scanning electron microscopy (SEM) and dentin from 22 primary molars was examined microbiologically to compare bacteria (total of viable counts, Streptococcus spp, Streptococcus mutans, Lactobacillus spp, and Actinomyces spp) before and after treatment (30 and 60 days). *Results:* Baseline caries samples had enlarged dentinal tubules with bacterial invasion. SEM samples after treatment suggest better tissue organization, with more compact collagen fibers arrangement and narrower dentinal tubules. The number of bacteria decreased in all samples at both 30 (98%) and 60 (96%) days, with all bacteria species showing similar trends. *Conclusions:* The minimal intervention approach is very effective to promote beneficial changes in the lesion environment and favorable conditions for the healing process in primary teeth. (Pediatr Dent 2007;29:228-34)

KEYWORDS: MINIMAL INTERVENTION, ATRAUMATIC RESTORATIVE TREATMENT, STEPWISE EXCAVATION, DENTAL CARIES

Researchers have recently turned their attention to conservative methods of treating caries lesions in dentin. Caries tissue on the cavity floor is not totally removed to maximally preserve dental structure.¹⁻⁴ These procedures are referred to as: (1) minimal intervention; (2) ultraconservative treatment; (3) atraumatic restorative treatment (ART); and (4) stepwise excavation (indirect pulp capping). The term minimal intervention in relation to dental caries includes a vast area of: (1) diagnosis; (2) prevention; and (3) control of the disease.⁵ The oral health of people from developing and developed countries can be improved by these conservative procedures.⁶

The removal of infected and damaged tissue and the use of restorative materials are fundamental requirements of modern operative dentistry.⁷⁸ Composite resin is used in ultraconservative treatments, whereas ART procedures include the use of hand instrumentation and restoration with glass ionomer cement (GIC). In studies over 3-year^{3.9.10} and 10-year⁴ intervals, conservative restorations showed

excellent retention rates and the use of glass ionomer (ART) performed comparably to conventional restorative treatment after 6 years.¹¹ Stepwise excavation had a marked reduction in bacterial growth with enhanced dentin hardness, which suggests that this management technique changed an active lesion to a slowly progressing lesion.¹²⁻¹⁵ Clearly, restoration alone will not prevent or eliminate disease. Since cavitation is a symptom of a bacterial infection, however, the first step must be to control the biofilm.¹⁶⁻¹⁸

Typical cariogenic microbiota of open active caries lesions includes, among others, the bacteria: (1) *Streptococcus mutans*; (2) *Streptococcus* spp; (3) *Lactobacillus* spp; and (4) *Actinomyces* spp.¹³ Not all bacteria in the oral cavity can ferment carbohydrates, since many species cannot withstand low pH. *Mutans streptococci* and *lactobacilli* maintain metabolic activity in low-pH environments.

Most minimal intervention studies used permanent teeth without preoperative samples.¹⁸ Therefore, whether cavity sealing changed the numbers or distribution of the microbiota is unknown. Since conservative approaches for pediatric dentistry are so important today, the impact of the intervention must be rigorously tested and clearly explained. Therefore, the authors tested the efficacy of minimal intervention by an ultrastructural and microbiological analysis of the dentin layers affected by caries lesions in primary molars.

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Methods

Research described here followed the guidelines and approval of the Human Assurance Committee (University of São Paulo, Brazil). The procedures, possible discomforts or risks, and possible benefits were fully explained to the parents of the children involved. Written consent was obtained for the participation of their children.

Healthy children of both sexes, with ages ranging from 4. to 8 years (average=5.7±1.26 SD) were selected as study subjects. All subjects had primary molars with open carious lesions in deep dentin, limited to the occlusal surface, without signs and symptoms of pulpal pathology. Periapical radiographs were taken to confirm the diagnosis and to exclude teeth with apical pathosis.

After clinical and radiographic examination, 32 teeth met the criteria for inclusion in this study (10 for scanning electron microscopy (SEM), and 22 for microbiological analysis). From each selected tooth, samples of carious dentin were removed before and after restoration (baseline and experimental samples, respectively). After 30 and 60 days, the restorations were removed for sample collection.

Common procedures for ultrastructural and microbiological analysis were used. Baseline samples were obtained after local anesthesia and isolation with rubber dam. Teeth were: (1) cleaned using a rotating brush and pumice; (2) washed thoroughly with sterile water; and (3) dried. The most superficial layer of the infected dentin was eliminated with a round bur on low-speed rotation. To make specimens available for evaluations of initial entry and re-entry, a sufficiently large layer of decayed dentin was left in each cavity. The cavity was once again washed with sterile water and dried with sterilized cotton pellets. Clinically visible residue of carious tissue on the cavity floor was divided into 2 parts (buccolingual direction) with a dentin excavator to cultivate samples at the same depth. The baseline sample was removed from the mesial portion while the experimental sample remained, so that it could be removed from the distal portion after 30 or 60 days.

Baseline samples for SEM study were collected with dentin excavators from 10 teeth, then fixed in 2% glutaraldehyde solution with a sodium phosphate buffer of 0.1M (pH 7.4) for 2 to 4 hours and postfixed in 1% osmium tetroxide in the same buffer for 1 hour. Samples were:

- 1. dehydrated in ethanol;
- 2. critical point-dried;
- ${\it 3.}\ {\it sputter-coated}\ {\it with}\ {\it gold};$ and

4. examined with a scanning electron microscope The baseline samples for microbiological study were collected from 22 teeth. Carious dentin from the mesial portion of the cavity was pulverized with a slowly rotating sterile bur. Specimens were then collected with a spoon excavator. The spoon excavator size was constant, and the volume of removed dentin was standardized (through prior practice on extracted teeth) as a level spoonful (0.43 ± 0.059 mg). One of the authors performed all the clinical procedures to help standardize data collection.

Samples were immediately transferred to flasks containing Viable Medium of Götenbörg Anaerobic¹⁹ (VMGA III), which was prepared, prereduced, and autoclaved at the microbiology laboratory (University of São Paulo, Brazil). Samples were vortexed for 60 seconds with sterile glass beads to break up aggregates of bacteria. Aliquots of 25 µL of decimal dilutions were plated in triplicate onto Brucella blood agar²⁰ to determine total viable counts (TVC). Mitis Salivarius agar (MS) and MS supplemented with sucrose and bacitracin²¹ (MSSB) were used for counting Streptococcus spp and Streptococcus mutans, respectively. Rogosa SL agar (RSL) was used to count Lactobacillus spp. Cadmium sulfate-fluoride-acridine trypticase (CFAT) agar²² was used for Actinomyces viscosus and Actinomyces naeslundii. Plates were incubated at 370°C in a candle jar for MS and MSSB and in gas-pack anaerobic jars with gas-pack anaerobic envelopes for TVC and CFAT. Plates were incubated aerobically for RSL. TVC and Actinomyces spp were counted after incubation for 5 days. Streptococci, mutans streptococci, and lactobacilli were counted at 48 hours on the basis of colony morphology.

Teeth were clinically evaluated and reopened after each experimental period. Resin-modified GIC (Vitremer, 3M, St. Paul, Minn) was used, for temporary and final restorations, including primer and gloss, following the manufacturer's instructions. SEM study samples were collected from 5 teeth after 30 days and from 3 teeth after 60 days. For the microbiological study, 10 samples were collected after 30 days and 12 after 60 days.

To collect the experimental samples, teeth were isolated with a rubber dam after local anesthesia, then polished and opened under aseptic conditions. Initially, the restorative material was removed with a high-speed diamond bur. The teeth were irrigated with sterile water and then dried with cotton wool. To complete the opening, a slow-speed round bur was used, followed by air syringe, after which the restorative material became opaque. This helped to distinguish the restorative material from the carious tissue to be collected. Samples were collected from the distal portion of the cavity (immediately beneath the restorative material) as described for baseline samples. Samples were fixed and stored (for SEM study) or transferred to the transport media (for microbiological study).

Colony-forming units (CFU) per plate were counted and transformed to CFU/sample, and the proportional reduction between the samples was calculated (baseline CFU-experimental CFU/ baseline CFU). Bacterial count was compared between treatments (Wilcoxon paired test; Statistical Package for the Social Sciences, v. 11.5.1 for Windows, SPSS Inc, Chicago, Ill).

Results

Enlargement of the dentinal tubules with bacterial invasion and an exposed collagen matrix were evident in the baseline samples (Figures 1a, 2a). SEM samples after treatment suggest better tissue organization (peritubular and intertubular dentin). This is due to: (1) more compact collagen fibers; (2) fewer bacteria; and (3) reduction or closure of dentinal tu-

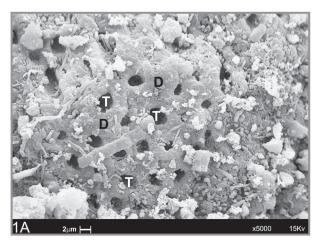


Figure 1A. Scanning electron micrograph showing the surface of a dentin sample collected before restoration placement. T=dentinal tubules; D=intertubular dentin; the bar represents $2 \mu m$.

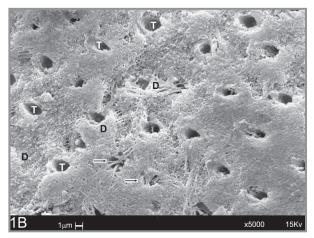


Figure 1B. Scanning electron micrograph showing the surface of a dentin sample from the same tooth in Figure 1a, collected after restoration placement (30 days). A narrowing of dentinal tubules (T) can be seen. The arrows are showing occluded tubules; the bar represents 1 µm.

bules (Figures 1b, 2b). Incidentally, baseline samples were easily excavated, while more pressure was required to excavate experimental samples. It was not possible to obtain samples from 2 teeth for the 6o-day SEM study.

Total viable counts declined from the baseline in both treatments (from 707 to 34 over 30 days and from 192 to 7 over

60 days, both P<.05, Wilcoxon test), with similar trends in the specific bacteria counts (Table 1). Average bacterial reduction was 98% over 30 days and 96% over 60 days.

Discussion

Minimal intervention treatment of caries in primary teeth resulted in an important reduction in bacterial counts. The use of dentin layers²³ in the SEM study facilitates tooth selection without the limitations inherent to dental extraction, while still permitting visualization of changes in the lesion environment (Figures 1 and 2). The affected layer beneath the infected layer comprises a zone of demineralized dentin that retains its basic dentin structure (dentin tubules, col-

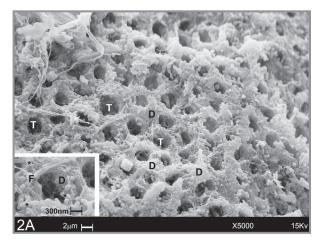


Figure 2A. Scanning electron micrograph showing the surface of a dentin sample collected before restoration placement. T=dentinal tubules; D=intertubular dentin; the bar represents 2 μ m. A higher power view shows intense demineralization. Note the exposure of collagen fibers (F) and the presence of bacteria (*); the bar represents 300 nm.

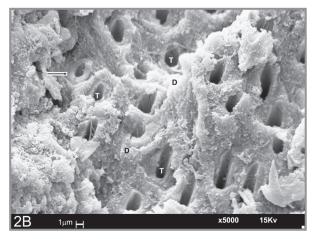


Figure 2B. Scanning electron micrograph showing the surface of a dentin sample from the same tooth in Figure 2A, collected after restoration placement (60 days). Good tubular organization is evident. The arrow highlights the peritubular dentin. T=dentinal tubules; D=intertubular dentin; the bar represents 1 μ m.

Table 1.BACTERIAL COLONY-FORMING UNITS (CFU/SAMPLE X 104) BEFORE AND AFTER RESTORATION IN
PRIMARY TEETH BY MINIMAL INTERVENTION (EXPERIMENTAL PERIOD OF 30 AND 60 DAYS)

Species		30 days			60 days	
	Before (N=10)	After(N=10)	P-value*	Before (N=12)	After (N=12)	P-value [†]
Total viable counts			.002			<.001
Mean±SD	707.30±418.00	34.00±21.41		192.10±57.50	7.22±5.26	
Median	302.50	1.33		175.00	0.79	
Range	36.0-4400.0	0.0-187.0		4.0-667.0	0.0-64.0	
Samples without growth	0	4		0	1	
Streptococcus spp			.002			<.001
Mean±SD	167.30±81.93	1.21±1.03		71.76±22.51	0.59±0.39	
Median	40.65	0.0		46.00	0.03	
Range	9.3-803.0	0.0-10.4		1.3-224.0	0.0-4.5	
Sample without growth	0	8		0	2	
Streptococcus mutans			.002			<.001
Mean±SD	33.00±7.93	0.01±0.01		53.64±19.32	0.09±0.06	
Median	25.00	0.0		16.75	0.003	
Range	2.24-79.60	0.0-0.07		0.41-200.0	0.0-0.64	
Samples without growth	0	7		0	5	
Lactobacillus spp			.004			.008
Mean±SD	27.68±7.41	1.68±1.07		41.66±34.64	0.42±0.32	
Median	32.15	0.05		0.51	0.0	
Range	0.0-57.50	0.0-10.10		0.0-419.0	0.0-3.87	
Samples without growth	1	2		4	7	
Actinomyces spp			.002			<.001
Mean±SD	563.20±331.90	1.80±1.09		91.89±37.50	0.84±0.52	
Median	266.50	0.13		46.65	0.01	
Range	6.13-3470.0	0.0-10.0		0.133-466.0	0.0-5.73	
Samples without growth	0	4		0	1	

* *P*-value for 30 days (Wilcoxon signed rank test); significance=*P*<.05. † *P*-value-for 60 days (Wilcoxon signed rank test); significance=*P*<.05.

lagen matrix) and is relatively free of bacteria.²⁴ In this layer the reversibly denatured collagen can be reorganized.² While it is difficult to differentiate clinically "infected" from "affected" dentin, eliminating the most heavily infected zone is of primary importance. Thus, only the outer layer of carious dentin must be removed—thereby permitting the preservation of the inner carious layer.² Other studies have shown that the healing process can be enhanced if the cariogenic environment is removed or altered.^{14,15,25} Deprived of nutrition, the remaining bacteria are unable to produce the acid to cause demineralization,^{1,2,24} thus inhibiting proteolytic destruction of the organic material. This study's findings support this scenario.

While this study's objectives did not include the evaluation of remineralization, the results suggest that cavity sealing contributes to remineralization. Three main points illustrate this process:

- remineralization occurs in inner carious dentin, where living odontoblasts supply calcium phosphate to the vital pulp^{2.8};
- 2. calcium concentration in dentin increases after minimal intervention treatment²⁵; and
- 3. radiographic density increases after incomplete removal and restoration of carious dentin, which suggests mineral gain.¹⁵

Therefore, minimal intervention treatment is highly recommended.

Microbiological analysis demonstrated that bacterial counts consistently declined by at least 89% and as much as 99% when compared to the baseline. In studies with different restorative materials, bacterial counts also decreased.^{1,13,15,26-28}

Weerheijm et al^{26,27} have shown that, in teeth restored with resin-modified glass ionomer the decrease in the numbers of *lactobacilli* was more pronounced than in those restored with amalgam. Both the glass ionomer and resin sealant-treated groups, however, were equally efficient at bacterial reduction. Also, Maltz et al¹⁵ have shown that, when calcium hydroxide was applied in deep caries lesions, growth did not occur in either *mutans streptococci* or *lactobacilli*.

Many organisms can respond with considerable flexibility to a changing environment.²⁹ Hence, the clinical importance of these remaining bacteria is unclear.

While fluoride may have a direct effect on caries, the effect of glass ionomer cements on bacterial counts is unknown. Remineralization of the affected dentin, even without fluoride, strongly suggests that the most important quality of a material is its ability to hermetically seal the cavity, thereby reducing or eliminating the supply of substrate for remaining microorganisms.⁷ GIC is the most conservative of the restorative materials, but requires good support from the remaining tooth structure. Resin-modified glass ionomer cements, used here, may be more durable than conventional GIC. The addition of resin components to conventional GICs improved their physical properties and bonding characteristics.^{3°-32} Adhesion with GIC is the result of an ion exchange between the cement and both enamel and dentin. This suggests that, even in the presence of demineralized tooth structure, union will still be achieved.^{17,33,34} Pulpal tolerance of the resin-modified GICs is similar to that of conventional GICs.³⁵ In this study, the authors found no postoperative symptoms.

The substantial bacterial reduction with tissue reorganization, in agreement with other studies,^{15:25} suggests that a 1-step treatment is sufficient to create favorable conditions for the healing process in primary teeth. Furthermore, the reduced number of bacteria sealed in the minimal intervention approach did not interfere with restoration survival, as also shown in long-term clinical studies.^{3:4:9-11.36:37}

The caries process can be interrupted by effectively sealing the cavity, as Mertz-Fairhurst et al⁴ have shown in ultraconservative restorations of permanent teeth. While it is possible that conservative treatments for permanent teeth are applicable to primary teeth, the biological and morphological differences of primary teeth are fundamental, and must be taken into consideration.

In this study of primary molars with occlusal restorations at 30 and 60 days post-treatment intervals, excellent tooth bonding was achieved, with no adverse reactions. It is unlikely, however, that current materials will be able to arrest caries progression completely in multiple surfaces. The success of treatment by minimal intervention depends on: (1) the correct diagnosis of the pulpal condition; (2) a hermetic seal of the cavity; and (3) an effective oral environment control.

Conclusions

Based on this study's results, the following conclusions can be made:

- 1. Favorable conditions for dentin reorganization in primary teeth were created:
 - a. upon the removal of heavily infected tissue; and b. by restoration.
- 2. Bacteria were reduced or eliminated by cavity sealing.
- 3. The minimal intervention approach is very effective in promoting:
 - a. beneficial changes in the lesion environment; and b. favorable conditions for the healing process in primary teeth.

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Abstract of Science of Literature

Caries Patterns in the Primary Dentition

This study contributed to the descriptive information of oral health status in the primary dentition, especially concerning the distribution and spatial correlation of carious lesions. Data were obtained from two surveys, the Signal-Tandmobiel® project (4,468 7-year-old schoolchildren born in 1989 from 179 schools in Flanders, Belgium) and the Tandje de Voorst - Smile for Life project (1,291 children born in 2000 and 1,315 children born in 1998 in Flanders, Belgium). Questionnaires were completed by the children's parents regarding information on oral health-related habits, and clinical examinations were completed by trained, calibrated dentist examiners. Radiographic evaluation was not included. Statistical analysis of the data was completed. Descriptive observations suggested a symmetrical distribution of caries experience at the population level. Within one subject, caries lesions tend to cluster on one side of the mouth. None of the studied variables could be shown to influence caries patterns. The authors concluded that if a subject has caries experience on one side of the mouth, lesions will tend to aggregate on the same side of the mouth, more than would be expected by chance alone.

Comments: This information may be useful to clinicians when completing a caries risk assessment for a patient, especially regarding the distribution of likely locations of cinically detectable caries lesions based on the patient's previous history of caries. **GEM**

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