In vivo Acid Etching Effect on Bacteria within Caries-Affected Dentin

F. Gu^a E. Bresciani^a T.J. Barata^c T.C. Fagundes^d M.F. Navarro^d S.H. Dickens^b
J.C. Fenno^a M.C. Peters^a

^aSchool of Dentistry, University of Michigan, Ann Arbor, Mich., and ^bAmerican Dental Association Foundation, Gaithersburg, Md., USA; ^cFederal University of Goiás, Goiânia, and ^dUniversity of São Paulo-Bauru, Bauru, Brazil

Key Words

Acid etching • Antibacterial activity • Dentin

Abstract

Acid etching procedures may disrupt residual bacteria and contribute to the success of incomplete caries removal followed by adhesive restoration. This study evaluated the in vivo effect of acid etching on cariogenic bacterial activity within affected dentin after minimally invasive treatment of caries lesions. Twenty-eight carious permanent teeth received standardized selective caries removal and random acid etch treatment (E) or not (NE) prior to adhesive restoration. Baseline and 3-month dentin biopsies were collected. The number of bacteria and activity of total bacterial cells and *Streptococcus mutans* were determined by quantitative PCR and RT-PCR. No statistically significant differences were observed in total bacterial number and activity between E and NE treatments ($p > 0.3008$). For NE, however, the residual *S. mutans* bacterial cells were reduced ($p = 0.0027$), while the activity per cell was significantly increased ($p = 0.0010$) after reentry at 3 months after restoration. This effect was not observed in group E. Although no significant differences were found between groups, this study suggests that acid

etching of affected dentin prior to adhesive restoration may directly or indirectly have an inhibitive effect on the activity of residual cariogenic bacteria. Further research is required to investigate this potential effect.

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Modern management of dental caries proposes a comprehensive approach integrating infection control and targeted prevention and remineralization of caries lesions in addition to a restorative approach. Minimally invasive dentistry adopts such a philosophy [Peters and McLean, 2001], achieving its objective of 'least invasive' surgical approach often by removal of 'infected' dentin only. Although reductions in bacterial counts and reduced activity in the remaining 'affected' dentin [Bjørndal and Larsen, 2000; Maltz et al., 2002] have frequently been reported, continued bacterial activity is often still a concern in daily clinical practice. Therefore, efforts to eliminate or reduce residual bacteria in affected dentin aiming to decrease the risk of caries progression persist, e.g. by adding antibacterial agents to dentin conditioner [Emilson and Bergenholtz, 1993; Imazato et al., 1998; Botelho, 2005], application of diamine silver fluoride

[Ag(NH₃)₂F] prior to restoration [Chu et al., 2002; Llodra et al., 2005] and employment of antibacterial liners/bases [McComb and Ericson, 1987; Palenik et al., 1992].

Acid etching is a standard procedure in adhesive dentistry to improve adhesion and retention of restorative materials and to reduce microleakage, thus providing better cavity sealing [Pioch et al., 2001]. Potential negative effects of acid treatment are its direct irritating effect on dental pulp [Fujitani et al., 1992] and widening of dentin tubule orifices. Bacteria that remain alive within the prepared cavity might manage to grow into these openings [Mejare et al., 1987]. It has been proposed that acid etching may disrupt these residual bacteria left in the cavity after caries removal [Luglié et al., 1998]. However, studies have shown that prepared cavities, even after complete caries removal, are still septic [Luglié et al., 1998] and that the total etch technique is not capable of fully removing all bacteria present [Luglié et al., 1998].

Streptococcus mutans is considered to be a major cariogenic bacterium [Marsh, 1997]. Its metabolic activity is closely related to caries initiation and progression [Marsh, 1997]. This in vivo study aimed to investigate the microbial effect of acid etching after incomplete caries removal. Cell numbers and the activity of total bacteria and *S. mutans* in affected dentin were assessed after incomplete caries removal and 3 months after restoration. We tested the hypothesis that the acid etching treatment would influence the total bacterial and *S. mutans* numbers and activity within the caries-affected dentin after a period of 3 months.

Materials and Methods

Clinical Procedures

The study was approved by the University of Michigan institutional review board (No. H03-00001883) and University of São Paulo institutional review board (No. 126/2003) and followed the CONSORT (Consolidated Standards of Reporting Trials) guidelines. Twenty subjects (14 male, 6 female) with a mean age of 42.6 (± 6.4) years presenting symptomless carious teeth were recruited for this research after informed written consent had been obtained.

In 28 teeth with caries into the middle third of dentin (15 for the nonetched group, NE, and 13 for the etched group, E), unsupported enamel was removed with a No. 330 carbide bur (Brasseler USA, Savannah, Ga., USA), followed by 'infected' dentin removal by a standardized procedure [Boston, 2003] using polymer burs (SS White Burs, Lakewood, N.J., USA). A first biopsy was taken from remaining 'affected' dentin using a No. 4 round bur (Brasseler USA) following a methodology previously described [Kidd et al., 1993]. The dentin samples were stored in 500 µl RNeasy Protect Bacteria Reagent (Qiagen, Carlsbad, Calif., USA) at -70°C until further processing. The teeth were randomly divided into 2

groups. Prior to placement of a resin-based calcium-phosphate base material [Bresciani et al., 2010; Peters et al., 2010], the cavity floor in group E (n = 13) was etched with 37% phosphoric acid (Total Etch®; Ivoclar Vivadent AG, Schaan, Liechtenstein), while group NE (n = 15) did not receive any acid etching. Randomization according to a random number table was concealed using numbered envelopes. The patients were blinded to the acid etching treatment delivered, while clinicians were not. After base placement, the enamel margins were acid etched and the cavities adhesively restored with composite resin (Epic®-TMPT Restorative; Parkell Inc., Edgewood, N.Y., USA). Three months later, the restorations were removed and a second dentin biopsy of affected dentin, at the same depth and adjacent to the first biopsy site, was taken from the cavity floor and stored using the same procedure previously described. Dental treatment was performed according to the initial treatment plan.

Preparation of DNA and cDNA Templates for Quantitative PCR

Dentin samples were thawed on ice and centrifuged at 5,000 g for 15 min to remove the RNeasy Protect reagent. The pellet was resuspended in lysis buffer consisting of 20 mM Tris (pH 8.0), 2 mM EDTA and 1.2% Triton X-100 in RNase-free water. After mild sonication (Branson Sonifier; Branson Ultrasonics, Danbury, Conn., USA; power output at 40, 3 × 10 s each), the samples were supplied with lysozyme (20 mg/ml), 0.05% methacryloyloxydecylpyridinium bromide, achromopeptidase (3.75 mg/ml), lyso-staphin (5 U/ml) and mutanolysin (250 U/ml), and incubated at 37°C for 30 min to maximize lysis, then centrifuged at 5,000 g for 15 min. The supernatant was split into 2 portions: DNA within one fourth of the lysate was isolated using the DNeasy Tissue Kit (Qiagen, Valencia, Calif., USA), and RNA within the remaining three fourths of the lysate was isolated using the RNeasy Kit (Qiagen) according to the procedures recommended by the manufacturer.

The extracted DNA was used directly for quantitative PCR (qPCR). After DNase treatment, the extracted RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (RT; Invitrogen, Carlsbad, Calif., USA) and random hexamer primers (Invitrogen) according to the manufacturer's recommendations. Absence of DNA contamination in RNA samples was confirmed by comparing the cDNA with the corresponding RNA (no-RT control) in endpoint PCR, using a 'universal' 16S rDNA primer set (5'-TTGGGTTAAGTCCCGC-3' and 5'-ATCCCC-ACCTTCCTCC-3') that amplifies a conserved region corresponding to residues 1074-1178 in *Escherichia coli* 16S rDNA. The PCR reaction mix (25 µl) contained 1 µl of template cDNA, 1 mM MgCl₂, 0.1-mM concentrations of each deoxynucleoside triphosphate, 0.4-µM concentrations of each primer, 0.5 U of *Taq* Polymerase, and *TAQ* buffer (Promega, Madison, Wisc., USA). Amplification conditions were: initial denaturation at 94°C for 4 min; 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, and final extension at 72°C for 7 min.

Quantification of Bacterial DNA and RNA Isolated from Clinical Samples

The amounts of total bacterial and *S. mutans* DNA and cDNA in each sample were determined by qPCR and quantitative RT-PCR (qRT-PCR), respectively, using a SYBR Green PCR Kit (Qia-

gen) in an iCycler (Bio-Rad, Hercules, Calif., USA) thermal cycler. The PCR conditions were as follows: denaturation at 95°C for 10 min, followed by 40 cycles at 94°C for 15 s and annealing at 55°C for 30 s and 72°C for 30 s. The 'universal' 16S rDNA primer set described above was used to detect total bacterial DNA and cDNA. An *S. mutans*-specific primer set (5'-AGAAAGGGA-CGGCTAACTA-3' and 5'-CTTCTGCACTCAAGTCAGA-3') that amplifies a 168-bp fragment of a variable region of *S. mutans* 16S rDNA corresponding to residues 486–655 in *E. coli* 16S rDNA was used to detect *S. mutans* DNA and cDNA. The specificity of this primer set was validated against available 16S rDNA sequences of oral streptococci. All analyses were performed in triplicate and included a negative control template consisting of sterile H₂O.

In order to convert the starting quantity of nucleic acids to theoretical cell numbers and to control for variation within each qPCR experiment, a standard curve was used for each experiment. Briefly, the cell number of overnight cultures of *S. mutans* (ATCC 25175) grown anaerobically in brain heart infusion medium (Difco 0037-17) was obtained by serial dilution plating on brain heart infusion plates. Colony-forming units were counted after 2-day anaerobic incubation (85% N₂, 5% CO₂ and 5% H₂) at 37°C. DNA was extracted from an *S. mutans* culture with known bacterial numbers, using the isolation procedure as described above. The qPCR reactions were carried out in duplicate using 10-fold serial dilutions of DNA templates equivalent to 3×10^{10} to 3×10^6 colony-forming units (plus a no-template control), using the same reaction conditions as described above. A standard curve was established based on the known numbers of *S. mutans* culture and corresponding cycle threshold (C_T) values of the qPCR reactions. The C_T values for successive dilutions were separated by approximately 3.3 PCR cycles, as expected for 10-fold dilutions. A linear relationship existed between the logarithm of the known number of *S. mutans* cells and corresponding C_T values generated from qPCR reactions. Levels of bacterial DNA and RNA in each dentin biopsy as reflected in C_T values for qPCR and qRT-PCR were extrapolated to cell numbers using the standard curve produced in that particular experiment. DNA data corresponded to bacterial cell numbers, while the quantity of 16S rRNA, measured by qRT-PCR, was used as an indicator of bacterial metabolic activity per cell. The term 'bacterial activity per cell' was defined as the ratio of RNA quantity to DNA quantity (RNA/DNA).

Statistical Analysis

The starting quantities of DNA and the metabolic activity per cell within each sample were considered to be continuous variables. The data were log transformed, and comparisons of bacterial cell numbers and activity per cell between groups, and between baseline and reentry within each group, were performed using the mixed model procedure in SAS (SAS version 9.12; SAS, Cary, N.C., USA). Random effects for subjects were also taken into consideration once the distribution of samples per patient was not independent.

Results

No adverse events related to the acid etching procedure were reported.

Fig. 1. DNA quantities of total bacteria and *S. mutans* at baseline and 3-month reentry. Means and SD of DNA quantities of samples within groups E and NE at baseline and reentry expressed on a logarithmic scale.

Comparative Analysis of Total Bacterial and *S. mutans* Cell Numbers

The quantity of 16S rDNA as measured by qPCR was used to estimate the number of bacterial cells in dentin biopsy samples. The mean quantities of total bacterial DNA in samples in group E at baseline and 3-month reentry corresponded to 1.26×10^8 and 9.07×10^7 cell equivalents, respectively (fig. 1). In group NE, the mean DNA quantities corresponded to 6.98×10^8 and 1.54×10^8 cell equivalents, respectively. Although minor differences between groups E and NE were observed in mean total bacterial DNA at baseline and at 3-month reentry (fig. 1), none of these were statistically significant either between groups with and without acid etching at both baseline and reentry ($p > 0.1843$) or within each group between baseline and reentry ($p > 0.4380$).

To determine whether acid etching had any effect on the amount of a major cariogenic bacterial species remaining in the restored lesion, changes in *S. mutans* DNA quantities from baseline to 3-month reentry in both groups were evaluated. The mean quantities of *S. mutans* DNA in group E biopsies at baseline and at 3-month reentry were 3.97×10^6 and 3.66×10^6 cell equivalents, respectively. The respective mean DNA quantities in group NE were 5.11×10^7 and 9.14×10^6 cell equivalents, respectively. The analysis included the effect of treatment (etch vs. no etch), time (baseline vs. 3 months) and the treatment-by-time interaction. The treatment-by-time interaction was borderline significant ($p = 0.0585$), post hoc tests showed a significant reduction in the NE group ($p = 0.0027$) and a nonsignificant reduction in the E

Table 1. Total bacterial and *S. mutans* activity per bacterial cell (RNA/DNA ratio) at baseline and reentry, and p values of comparison between baseline and reentry

Treatment of affected dentin	RNA/DNA ratio		
	baseline	reentry	p
<i>Total bacteria</i>			
E (n = 13)	0.047 ± 0.058	0.066 ± 0.065	0.3012
NE (n = 15)	0.059 ± 0.076	0.031 ± 0.020	0.4970
<i>S. mutans</i>			
E (n = 13)	0.114 ± 0.116	0.101 ± 0.107	0.7365
NE (n = 15)	0.058 ± 0.033	0.099 ± 0.041	0.0010*

Values denote means ± SD unless specified otherwise.

* Statistically significant difference.

group (p = 0.7314). There was a significant difference in the treatment groups at baseline (p = 0.0277), but not at the 3-month follow-up (p = 0.3641).

Comparative Analysis of Total Bacterial and S. mutans Relative Metabolic Activity

No statistically significant changes were found in total bacterial RNA/DNA ratio within group E or group NE at baseline and reentry (table 1), and between both groups at baseline and at reentry (p > 0.3008).

To determine whether acid etching had any effect on the metabolic activity of major cariogenic bacteria, changes in the *S. mutans* RNA/DNA ratio from baseline to 3-month reentry in both groups were evaluated (table 1).

While the total *S. mutans* cell equivalents as measured by DNA content decreased in the NE group after 3 months, the total amount of *S. mutans* 16S RNA was significantly increased (p = 0.035; data not shown), resulting in a highly significant increase in activity per cell, as measured by the RNA/DNA ratio (table 1). The increase in *S. mutans* activity in the NE group after 3 months (p = 0.0010) was the only significant difference observed for bacterial activity per cell.

Discussion

Studies on the effect of acid etching have focused on its influence on tooth structure and adhesion. Few studies have explored the potential antibacterial effect of acid etching in caries management [Torstenson et al., 1982; Mejäre et al., 1987; Luglié et al., 1998; Wicht et al., 2005].

The present study investigated the effect of acid etching on bacterial numbers and activity in 'affected' dentin 3 months after minimally invasive treatment.

This study employed qPCR methods to measure the total amount of bacterial DNA in biopsy samples in order to estimate bacterial numbers. There are more than 700 bacterial species in the oral cavity [Marsh, 1999]. Bacterial quantitation by culturing from this complex environment is rather unreliable because bacteria can only be grown if their metabolic and physiological requirements are met. More than 50% of oral bacteria are presently uncultivable, and no single culture condition supports the growth of all cultivable species. A more sensitive method for bacterial detection and quantification is PCR. This technique is very sensitive and can reliably detect fewer than 100 bacterial cells. PCR techniques have proven useful in detecting cariogenic bacteria [Igarashi et al., 2000; Kato et al., 2004; Rodis et al., 2006]. The quantification of total DNA from bacteria using qPCR offers a more accurate means of estimating bacterial cell numbers in affected dentin. By comparing results obtained using 'universal' bacterial and *S. mutans*-specific oligonucleotide primer sets, we were able to determine both the total number of bacteria and the relative proportions of *S. mutans* in the dentin biopsies.

In this study, no statistically significant difference was found in the amount of total bacterial DNA between baseline and 3-month reentry. Interestingly, the reduction in *S. mutans* DNA from baseline to reentry was statistically significant only in the NE group. The baseline *S. mutans* DNA quantities were statistically different (p = 0.0277) between groups once a higher amount of *S. mutans* DNA was detected in the NE group compared with the E group. In all cases, *S. mutans* comprised between approximately 3 and 6% of the total bacteria detected.

Our results were somewhat different than those of previously published studies using culture assays [Handelman, 1976; Bjørndal and Larsen, 2000]. One possible explanation is that qPCR is more sensitive in the detection of a much wider range of bacterial species, and that, therefore, the variation among samples may be greater. Another factor complicating the comparison with previous studies is that there are differences in sampling methodology and time frames between the studies. While our study had a larger population size than these previous studies, the statistical power was relatively low. The statistical power for comparing the mean difference in total bacterial DNA in this study was 0.1. To increase the power, a study with a larger sample size than the present robust study would be required. An obvious issue compar-

ing data from both analysis techniques is the inherently different nature of the detection mode. Traditional culture assays are only able to detect a selective group of metabolically active cells, while qPCR detects all cells, both viable and nonviable.

A major limitation of using DNA quantification to estimate total bacterial cell numbers is that this technique is not able to differentiate between viable and nonviable cells. Bacteria in nature can exist in three states: living/active, dormant/inactive and dead/nonviable. How to detect whether a bacterial cell is active or inactive has long been controversial [Choi et al., 1996; Karner and Fuhrman, 1997]. Three methods have been used to assess metabolic activity: measurement of the incorporation of radioactively labeled metabolic precursors such as thymidine or leucine [Hoch and Kirchman, 1993]; use of a redox-sensitive compound that fluoresces when reduced by an active electron transport system [Rodriguez et al., 1992], and using the amount of nucleic acids, particularly rRNA, within a cell as a proxy for metabolic activity [Lebaron et al., 2001]. As radioactively labeled precursors and redox-sensitive compounds cannot be used in vivo, detection of nucleic acids is the only option for in vivo and clinical studies.

In this study, we used qRT-PCR to determine the total amount of 16S rRNA and generated an RNA/DNA ratio as a measure of bacterial activity per cell or, more precisely, the average metabolic activity of bacterial cells (total cells or *S. mutans*) in each sample of caries-affected dentin. The RNA/DNA ratio is a well-established measure of cellular metabolic activity and has been particularly useful in metabolic studies of complex microbial environments such as marine sediments [Chícharo and Chícharo, 2008]. To our knowledge, this methodology has not previously been utilized to analyze bacterial activity in the oral environment. The RNA/DNA ratios in this study ranged between approximately 0.03 and 0.11. While rapidly growing cells would show RNA/DNA ratios of more than 1, the RNA/DNA ratios reported here are consistent with reports from other microbial communities having a low overall metabolic activity [Dell'Anno et al., 1998]. This reflects the relatively low overall metabolic activity associated with biofilms compared with cells in suspension as well as the extremely limited nutrient availability within dentin tubules.

Although acid etching treatment might be presumed to reduce bacterial activity (at least in the short term), our results showed no statistical difference in the RNA/DNA ratio of total bacterial cells between the groups with (E) and without acid etching (NE) at 3-month reentry. As not-

ed with reference to the DNA data, a potential acid etching effect on total RNA data could have been masked by the relatively large variability observed between subjects.

In contrast, omitting the etching procedure was significantly more conducive to *S. mutans* metabolic activity. The *S. mutans* RNA/DNA ratios suggest that etching had a negative effect on the subsequent metabolic activity of *S. mutans* cells remaining in the restored lesions over the 3-month study period. No significant difference was found between the E and NE groups, and additional research is needed to confirm this suggestion. While there was a nonsignificant decrease in metabolic activity in the E group as measured by RNA/DNA ratio ($p = 0.7365$), the NE group showed a significant ($p = 0.0010$) increase in metabolic activity. Several factors may have contributed to this effect. Acid etching is believed to result in improved bonding of the restoration material. Without acid etching, more nutrients may be available to the entombed bacteria due to microleakage even in the absence of a detectable loss of restoration integrity. Alternatively, the aciduric nature of *S. mutans* may have contributed to their survival potential both with and without etching, as compared to the total bacteria. To speculate further, it is not known whether there is a 'treatment effect' on bacteria that remain in carious lesions, which may be due to the exposure of the dentin to the normal atmosphere during the excavation and restoration procedure. These and other issues (including an expansion of the study to include a wider range of cariogenic bacteria) will be addressed in future studies.

Although no significant differences were found between groups, the results from this study suggest a potentially beneficial, inhibitive effect of dentin acid etching procedures on residual *S. mutans* underneath composite restorations. Further work is required to investigate this potential effect.

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