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Quantity of Remaining Bacteria and Cavity Size After Excavation with FACE, Caries Detector Dye and Conventional Excavation *In Vitro*

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Clinical Relevance

Fluorescence Aided Caries Excavation (FACE) removes heavily infected dentin more completely, without significantly increasing cavity size when compared to conventional methods.

SUMMARY

In this *in vitro* study, quantitative confocal microscopy was used to show differences in the quantity of bacteria remaining in dentin after excavation with different methods. A further parameter was the cavity volume after excavation relative to the original lesion size. Teeth with dentin caries were divided into three groups of 20 each. The caries was removed by a single operator using a slow handpiece and a round bur. In the first group, Fluorescence Aided Caries Excavation (FACE) was carried out: violet light was used to illuminate the operating field and the operator observed the cavity through a high-pass filter and removed the orange-red fluorescing areas. The second group was excavated using Caries Detector, while the third group used conventional excavation. After excavation, cavity volume was measured; samples were stained for bacteria with ethidium bromide, and they were examined using confocal microscopy under standardized conditions. The bound stain was quantified in terms of fluorescence intensity on the confocal images. Total pixel intensity was significantly lower in the FACE Group than in the Caries Detector group (p=0.046) and in the conventional excavation group (p=0.021). Differences in cavity volume relative to original lesion size were not statistically significant (p=0.86, 0.35 and 0.51). Within the limitations of

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DOI: 10.2341/06-64

this *in vitro* study, it can be concluded that FACE is more effective in removing infected dentin without significantly increasing cavity size when compared to conventional excavation and excavation with the aid of caries detector dye.

INTRODUCTION

Restorative dentistry has moved away from a "drill and fill" philosophy to a minimally invasive approach.[1] The aim of minimally invasive treatment is to conserve as much tooth substance as possible, thus extending the lifetime of a restored tooth.[2] At the same time, removal of carious dentin is necessary prior to restoring the tooth.[3] Although there still is no definitive guideline as to how much infected dentin may be allowed to remain in the cavity, it is generally accepted that heavily infected dentin needs to be removed and minimal quantities of bacteria may be left in the cavity in order to preserve dentin.[4-5]

A clinically relevant method for assessing new caries excavation methods should both quantify the infected dentin remaining in the cavity after excavation and assess the amount of dentin that has been removed. In the past, culturing methods have been used to quantify bacteria in dentin.[4,6] However, culturing methods are limited to quantifying only those bacteria that are cultivable and may have underestimated the level of infection remaining after excavation with conventional methods. It is now thought that only 50% of oral bacteria are cultivable *in vitro*.[7] More sensitive techniques are needed to quantify the total bacterial population.

A recently described caries excavation technique (Fluorescence Aided Caries Excavation [FACE]) enables the clinician to directly assess the level of bacterial infection in dentin during excavation without the need for dye.[8] This should allow selective removal of infected dentin, thus preserving non-infected dentin. While it has been shown that FACE removes bacterially infected dentin more successfully than conventional excavation,[8] it is not known whether the superior outcome of FACE, in terms of removal of the infected dentin, is accompanied by an overextension of the cavity size. This study assessed: 1) differences in the quantity of bacteria remaining after excavation and 2) the preservation of dentin, with FACE, compared to conventional excavation and Caries Detector (Kuraray, Tokyo, Japan) dye by determining cavity size after excavation.

METHODS AND MATERIALS

Sample Preparation

Sixty extracted human permanent molars with occlusal dentin caries were collected and stored for up to three months after extraction in 0.01% thymol solution at 4°C in the dark. The sample teeth were sectioned mesiodistally using a water-cooled hard tissue saw (Ultraslice 2000, Ultratec, Santa Ana, CA, USA). This allowed a stereomicroscopic assessment of lesion size (depth x width of discolored dentin and enamel) and allocation of the teeth into three groups of 20 teeth each, so that each group had the same mean lesion size mm^2 (stratified randomization) (Table 1) before excavation. Tooth halves were reassembled and embedded in acrylic resin (Sampl Kwik, Buehler, Lakebuff, IL, USA).

Vaseline was applied to the occlusal surface to allow separation of a negative record (Figure 1a) of the occlusal surface made using acrylic (Sampl Kwik).

Excavation

The operator was the same for all groups. The access cavities were made using a #557 diamond bur in a high-speed handpiece (Star Dental, Lancaster, PA, USA) under continuous water-cooling, and caries was removed using stainless steel #2 and #4 round burs in a slow-speed handpiece (Star Dental) in all groups.

In the FACE group, the cavity was excited using a 35-watt Xenon-discharge lamp and a blue band pass filter with peak transmission at 370 nm (Inspektor Research Systems Bv, Amsterdam, The Netherlands). The operator inspected the cavity through a 530 nm yellow glass filter (OG530, Schott, Mainz, Germany) and removed orange-red fluorescing dentin. The room was darkened during excavation.

In the Caries Detector group, gross caries was first removed. The teeth were dried briefly using compressed air. Caries Detector was applied to the cavity for 10 seconds. The cavity was rinsed with water for 10 seconds and dried using compressed air. Dentin, which retained stain, was selectively removed. This process

Table 1: *Mean lesion size (before excavation), cavity volume (after excavation) and relative cavity size. The differences between the groups were not statistically significant.*

Group	Mean lesion size mm^2 ± standard deviation	Mean cavity volume mm^3 ± standard deviation	Mean relative cavity size mm ± standard deviation
FACE n=20	9.2 ± 5.16	26.5 ± 19	3.0 ± 1.3
Caries Detector n=20	9.2 ± 5.16	30.2 ± 21	3.4 ± 1.4
Conventional n=20	9.2 ± 5.14	24.6 ± 12.6	3.4 ± 2.8

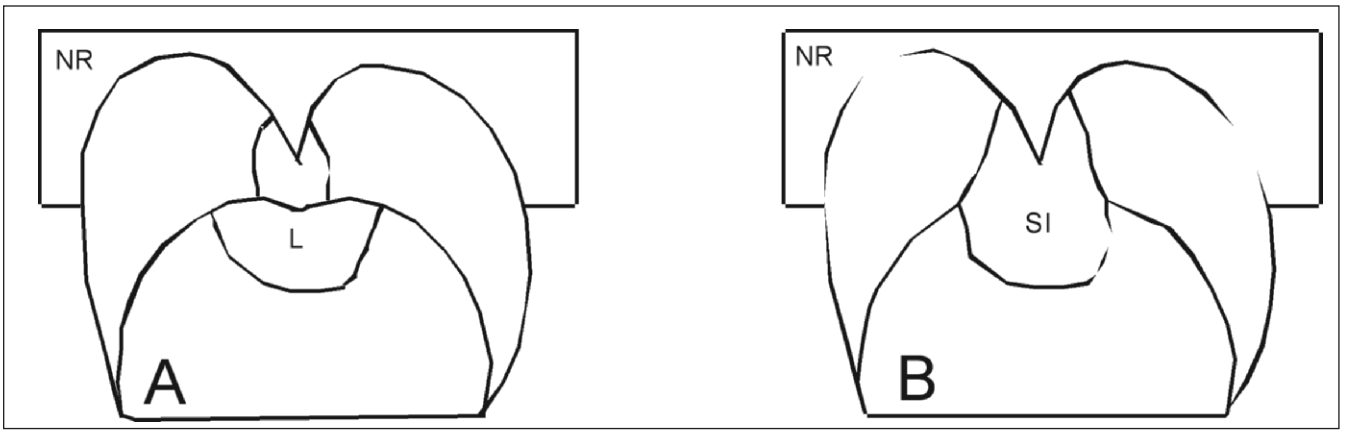

Figure 1: *A. Negative record (NR) of occlusal surface of the tooth before excavation of the lesion (L).*
B. Negative record (NR) replaced on occlusal surface after excavation to aid in making the silicone impression (SI) of the cavity.

was repeated until no Caries Detector stain remained in the cavity. The operating field was illuminated using a standard dental unit light.

In the last group, conventional excavation was carried out, and stained and softened dentin, which were detected using a sharp explorer (EX85, Hu-Friedy Inc, Chicago, IL, USA), were removed from the EDJ. In the remainder of the cavity, only soft dentin was removed. Stained hard dentin was not removed. The operating field was illuminated using a standard dental unit light.

Histology

One half of each sample tooth was used for histology. The samples were fixed in alcohol (70% ethyl alcohol), rinsed repeatedly with phosphate buffered saline, pH 7.2 (PBS) and stained with a 1:500 concentration of 10 mg/ml ethidium bromide (Molecular Probes, Eugene, OR, USA). The samples were then removed from the ethidium bromide solution, rinsed extensively in PBS and replaced in a 0.01% thymol solution in the dark.

The samples were analyzed for stain using a Biorad MRC 1024 Laser-Scanning confocal Microscope (Bio-Rad, Hemel Hempstead, UK) equipped with a Krypton-Argon laser (excitation 488 nm, detection 598 nm, 15 µm confocal slit, objective Nikon 60x water objective, NA 1.2). The CLSM imaging and analysis were carried out by a blinded examiner. All labeled specimens were scanned to determine the brightest and dimmest pixel intensities, then the black value and gain settings were adjusted so that the full dynamic range of the system was used and both over and under saturation was avoided (no zero or 255 pixels). The entire cavity outline was scanned to identify the region showing the highest fluorescence intensity. A single image of each specimen at the location with the highest fluorescence intensity was captured. In order to allow quantitative comparisons, laser power, dynamic range and confocal slit settings were kept constant for all images. Samples were scanned at a depth of 10 µm below the cut surface. The sample edge was excluded from the image to avoid inclusion of surface contaminants. The images were saved using LaserSharp software, version 3.02 (Bio-Rad).

Image Analysis

The images were analyzed using Metamorph software version 4.01 (Universal Imaging, West Chester, PA, USA). A threshold for background fluorescence was arrived at by determining the greatest pixel intensity for non-stained samples. After applying the threshold to the stained sample images to exclude background fluorescence, total pixel intensity was calculated for each image in order to quantify the amount of bound stain. The total pixel intensity for each group was calculated by adding the pixel intensity values for all 20 samples in that group.

Cavity Size

A silicone impression was made for each cavity (Dimension Garant L ESPE, Seefeld, Germany). The impression material was injected into the cavity and the acrylic record made before excavation was pressed onto the occlusal surface to limit the impression in the occlusal direction (Figure 1b). After trimming the excess impression material, the impression was weighed and the cavity volume was calculated according to the density of the impression material (1.40164 mg/mm^3).

The cavity volume for each sample was divided by its lesion size prior to excavation to give the relative cavity size.

Cavity volume mm^3/lesion size mm^2 = relative cavity size mm

Statistical Analyses

The Kolmogorov-Smirnov test showed that the pixel intensity data were not normally distributed. Comparisons among the three groups for differences in

total pixel intensity were made using the Kruskal-Wallis analysis H-test. Pairwise comparisons were made among each of the groups using the Mann-Whitney U-test. Comparisons between the groups for differences in volume of dentin removed relative to original lesion size were made using the Student's *t*-test. In all cases, the level of significance was set at $p \leq 0.05$. All statistical analyses were carried out using SPSS version 12 (SPSS, Chicago, IL, USA).

RESULTS

Histology

The level of infection remaining after excavation, represented by the amount of bound stain detected, was expressed in terms of the total pixel intensity of a CLSM image (Table 2). The total pixel intensity in the FACE group (6.76×10^3, n=20) was significantly lower ($p=0.021$) than conventional excavation (1.06×10^7, n=20) and Caries Detector (5.97×10^6, n=20, $p=0.046$). Two pulp exposures occurred during excavation in the Caries Detector group. There were no pulp exposures in either the FACE group or the Conventional group.

Cavity Size

The mean cavity volume (after excavation) for each group and the mean relative cavity size are given in Table 1. Relative cavity size was calculated for each sample individually, and the mean for each group is given in the last column. Differences between the groups were not statistically significant.

DISCUSSION

Bright surrounding light can make it difficult to identify red fluorescing dentin when using FACE outside of the mouth. This is why the room lighting was dimmed during FACE excavation in this study. This effect can be counteracted by increasing the intensity of the excitation light and does not present a problem when working inside the oral cavity. Visible violet light was used for excitation with FACE, thereby avoiding dangers for clinician and patient associated with ultraviolet light or lasers.

In this study, in order to standardize conditions for all groups, all the groups were excavated by a single operator. Future studies with several operators are needed to determine the degree of subjectivity involved in these excavation methods.

Quantitative confocal and fluorescence microscopy is widely used in conjunction with specific fluorescent probes for biological specimens. In this study, the bacteria remaining after excavation with each method were quantified by staining with ethidium bromide and analyzing the samples using confocal microscopy.

A single image was taken and analyzed for each sample. An image of the entire lesion would have been desirable, but use of a low power objective in confocal microscopy results in a thicker optical slice and loss of the confocal effect in turbid tissues. Although the entire lesion was not imaged, the entire cavity outline was scanned and the location chosen for imaging was the one that showed the greatest fluorescence intensity or the "worse case scenario" for that sample. Images were captured under standardized conditions and thresholded to eliminate background fluorescence and to allow for comparison and quantification of fluorescence intensity (total pixel intensity) of the bound stain (ethidium bromide).[9] The non-stained halves of the teeth were scanned under the same conditions to determine the level at which to set the thresholding to exclude background fluorescence from the quantitative analysis. The authors choose non-stained carious tissue rather than sound tissue, because of the strong autofluorescence of carious teeth in confocal microscopy.[10]

Table 2: *Level of Infection Remaining After Excavation*

Group	Total Pixel Intensity
FACE n=20	6.76×10^3
Caries Detector n=20	$5.97 \times 10^{6*}$
Conventional n=20	$1.06 \times 10^{7*}$

Groups marked with an asterisk did not differ significantly from each other.

In the past, culturing methods have been used to quantify bacteria in dentin.[4,6] However, culturing methods are limited to quantifying only those bacteria that are cultivable and may have underestimated the level of infection remaining after excavation with conventional methods. Current studies using Polymerase Chain Reaction (PCR) methods have detected a 40-fold greater quantity of bacteria in carious dentin than what is found using culturing methods.[11-12] In this study, the fluorescent probe (ethidium bromide) is itself DNA-specific, allowing bacteria to be detected regardless of species. Recently, PCR and Fluorescent *in situ* hybridization (FISH) techniques, using universal probes for bacteria, have been described as the gold standard for quantification of bacteria in carious dentin.[13-14]

Bacteria remaining after excavation were quantified in terms of pixel intensity of bound stain. The greatest total pixel intensity was found in the conventionally excavated group followed by the Caries Detector group, indicating that, when using these methods, a large quantity of bacteria remains after excavation. The pixel intensity for the FACE group was highly significantly lower (3 orders of magnitude, 10^3), indicating that removal of bacteria was more successful in this group.

Caries Detector dye has been shown to have a low specificity for infected dentin.[15-17] Consequently, this may result in removal of non-infected demineralized dentin, for example, circumpulpal dentin[18-20] or under-excavation of infected non-demineralized dentin. This was confirmed by the results of this study and may explain why both pulp exposures and under excavation occurred in this group.

A further aspect of this study was the assessment of cavity volume after excavation. The size of the original caries lesion was assessed using stereomicroscopy before excavation and allowed stratified randomization of the samples according to lesion size. After excavation, the smallest cavities relative to the original lesion size were in the FACE group. However, the differences between the groups were not statistically significant. This indicates greater accuracy for FACE compared to the other methods, as the residual bacteria were more completely removed without increasing the cavity size. The relatively high standard deviation for cavity size is likely due to a high variation in lesion size at the outset and might be improved upon in future studies by excluding very small and very large lesions.

This study has shown that the quantity of bacteria remaining after FACE excavation is statistically significantly lower than after conventional or caries detector excavation. The question as to whether this difference is clinically significant can only be answered by clinical studies looking at the long-term outcome after excavation with these methods. Some authors believe that biofilm at the surface of the restoration is the main factor in determining whether a lesion arrests or progresses following restoration, along with the quantity of residual bacteria beneath the restoration being irrelevant.[5,21] Still others recommend complete removal of the infected dentin before placement of the restorations.[3] It is important to remember that, when looking at the clinical outcome as a measure of whether an excavation method has been successful or not, that the excavation method is only one of numerous factors that have influenced the outcome. Therefore, it is still important for new excavation methods to be compared objectively to existing methods under *in vitro* conditions before progressing to clinical studies.

CONCLUSIONS

Within the limitations of this *in vitro* study, it can be concluded that FACE is more effective in removing heavily infected dentin without significantly increasing cavity size compared to conventional methods.

Acknowledgements

The authors thank Exing Wang, Indiana University School of Medicine, Division of Nephrology, for assistance with the image analyses; George Eckert, Indiana University School of Medicine, Division of Biostatistics, for assistance with the statistical analyses and the Indiana Center for Biological Microscopy for use of their equipment. Author ÁML gratefully acknowledges support from the Alexander von Humboldt Foundation in the form of a research fellowship.

(Received 28 April 2006)

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