

Bacteriology of deep carious lesions underneath amalgam restorations with different pulp-capping materials – an *in vivo* analysis

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ABSTRACT

Microorganisms remaining in dentin following cavity preparation may induce pulp damage, requiring the use of pulp-capping agents with antimicrobial activity underneath permanent restorations. Objective: The aims of this study were to analyze the bacteriological status of carious dentin and to assess the efficacy of different base underneath silver amalgam restorations. Material and Methods: This study was conducted on 50 patients aged 13 to 30 years. Sterile swabs were used to take samples after cavity preparation, which was assessed by microbiological culture to identify the microorganisms present. Following this, cavities were restored with silver amalgam, using one of the materials being investigated, as the base: calcium hydroxide (Group II), polyantibiotic paste (Group III), a novel light-cured fluoride-releasing hydroxyapatite-based liner (Group IV) and mineral trioxide aggregate – MTA (Group V). In Group I, the cavities were restored with silver amalgam, without any base. After 3 months, the amalgam was removed and samples taken again and analyzed for the microbial flora. Results: Lactobacilli were the most commonly isolated microorganisms in the samples of carious dentin. Groups IV and V showed negative culture in the 3-month samples. There was no statistically significant difference between Groups I, II and III. There was no significant difference between Groups IV and V ($p > 0.05$). Both Groups IV and V showed significantly better results when compared to Groups I, II and III ($p < 0.05$). Conclusions: The hydroxyapatite-based liner and MTA performed significantly better in terms of antibacterial activity than the other materials.

Key words: Dental caries. Microbiology. Calcium hydroxide. Hydroxyapatite. Mineral Trioxide Aggregate. Amalgam.

INTRODUCTION

Dental caries is one of the most prevalent diseases affecting the calcified tissues of the teeth. Although many acid producing microbes have been isolated from oral flora, *Streptococci* and *Lactobacilli* have been considered as significant ones in contributing to the initiation and progression of dental caries respectively^{4,6,8}. The infiltration of microorganisms into dentin has been studied by many authors and it has been claimed that the last traces of softened dentin is sterile, while others have opined that the softened dentin in deep cavities

may not lead to extension of decay, but maintains the acidity and thus endangers the vitality of the pulp^{3,8,20}. Furthermore, mechanical consideration that indicates removal of extensive amount of soft dentin may result in an unstable foundation for stress bearing restorations. Removal of softened dentin would further invite pulp exposure, and hence it is imperative to preserve the vitality of the pulp by meticulous conservative approach by using effective medicaments rather than resorting to endodontic therapy.

The practice of leaving uninfected, softened and stained dentin on the floor of a deep cavity is

justified³. The problem posed by secondary caries is no less cumbersome than deep caries. The dentinal tubules which contain the pioneer bacteria become distended and rupture to form liquefaction *foci*. The released toxins travel via the tubules to the pulp and cause irreversible pulpal damage⁸. Research has shown that bacteria are reduced in number and the remaining ones become calcified along with the reparative process of dentin. Many medicaments have also been tried to sterilize deep cavities. However, cavity sterilizing agents themselves may induce pulpal damage.

Calcium hydroxide is a commonly used liner in deep cavities. It has been proven to have effective antimicrobial activity and it also induces reparative dentin formation¹⁰. Considerable attention has also been given to antibiotics and antibacterial agents in operative dentistry¹³. The effectiveness of antibiotics in reducing the number of microorganisms remaining in carious dentin has not been established. Aureomycin, streptomycin and terramycin have been found to have antibacterial activity when applied on carious dentin¹⁶. Recent materials like light-cured calcium hydroxide and mineral trioxide aggregate (MTA) have also been recommended as pulp-capping agents¹⁵.

The aims of the present investigation were to study the bacteriological status of carious dentin and to assess the efficacy of calcium hydroxide, polyantibiotic paste, a novel light-cured fluoride-releasing hydroxyapatite-based liner and MTA as base underneath silver amalgam restorations.

MATERIAL AND METHODS

This study was conducted on 50 patients aged 13 to 30 years who attended the Restorative Dentistry clinic of our University. The methodology was approved by the Institutional Review Board and Ethical Committee of the University. Informed consent was obtained from all the patients. Mandibular molar teeth with deep occlusal cavities without pulp exposure were chosen for the study. The selected teeth were examined clinically for vitality by thermal testing and electric pulp testing. Percussion tests were also performed and radiographic examination was done to assess the periradicular status prior to the operative procedure.

Restorative procedures were carried out under rubber dam isolation. The tooth *in situ* was disinfected by using 70% alcohol taking precaution not to allow the disinfectant to come in contact with the carious cavity. Occlusal cavity was prepared with a sterile round bur No. 3 and 4 at low speed with intermittent cutting to avoid overheating. All softened carious material was removed with a sterile spoon excavator. Two sterile swabs were taken after rubbing on to the base of the cavity,

carefully placed into a sterile test tube and closed with a sterile cotton plug. Following this, the patients were randomly divided into five groups (n=10) as follows. The details of the patients placed in different groups were recorded:

Group I: Silver amalgam restoration was placed directly in the cavity, without any base

Group II: A liner of fast setting calcium hydroxide (Dycal, Dentsply Caulk, DE, USA) was applied on the pulpal floor of the cavity, above which a thin gold foil was placed, followed by a base of zinc phosphate above which silver amalgam (Dispersalloy, Dentsply, USA) restoration was done.

Group III: A base of polyantibiotic paste [composed of penicillin, streptomycin, achromycin, erythromycin, kanamycin, garamycin and ampicillin – 50,000 µg% as *per* recommendations of Stephan, et al.¹⁸ (1952)] was applied with a cartridge syringe above which silver amalgam restoration was done.

Group IV: A base of light-cured fluoride-releasing hydroxyapatite-based liner (LimeLite Light Cure Cavity Liner, Pulpdent Corporation, Watertown, MA, USA) was applied on the cavity floor and light cured for 30 s, above which silver amalgam restoration was done.

Group V: White MTA (ProRoot MTA, Dentsply Maillefer, Ballaigues, Switzerland) was mixed with 10% calcium chloride and taken to the cavity with a Messing Gun, above which a 1-mm-thick layer of glass ionomer cement (Riva self cure, SDI, Australia) was placed and silver amalgam restoration was done.

All patients were recalled after 3 months for a review. The teeth were isolated with rubber dam and the amalgam restorations were removed using sterile burs. Sterile swabs were used to take specimens from the floor of the cavity and transported for microbiological testing. The teeth were restored permanently with silver amalgam.

The microbiological methods were designed towards determination of the presence or absence of various cultivable microorganisms in the specimen of carious dentin and to permit general comparisons of the various organisms present in both pre-treatment and post-treatment samples. The swab specimens collected from the carious dentin from the 4 groups were inoculated into Brain Heart Infusion agar, tomato juice agar and Fastidious anaerobe agar plates (Hi Media Labs, Bangalore, India). Five percent defibrinated sheep blood was added to the media. The media were incubated at 37°C and observed daily for the presence of growth or turbidity. The culture of anaerobic microorganisms was performed in a carbon dioxide incubator (Jouan, Saint Herblain, France) in an atmosphere of 10% carbon dioxide and in the anaerobic workstation (Don Whitley Scientific, Bradford, UK) in an atmosphere of 10%

hydrogen, 10% carbon dioxide, and 80% nitrogen for 7 days. The isolated microorganisms were identified as to their genera and species, wherever possible according to their colony morphology, microscopic appearance, staining characteristics and biochemical properties.

The results were tabulated and analyzed statistically using Kruskal-Wallis test to identify the

p value and Mann Whitney U test to identify the significant groups at 5% level.

RESULTS

Figure 1 shows the bacterial flora isolated from Group I samples. Samples of carious material prior to filling, gave pure cultures of *Lactobacilli*

Individuals No.	Age/Sex	Tooth number (FDI System)	Microorganisms isolated before restoration	Microorganisms isolated 3 months after restoration
1	27 / M	46	<i>Staphylococcus aureus</i> , <i>Streptococcus viridans</i> , <i>Streptococcus mutans</i> , <i>Lactobacilli</i>	<i>Streptococcus mutans</i> , <i>Lactobacilli</i>
2	22 / M	46	<i>Staphylococcus aureus</i> , <i>Streptococcus mutans</i> , <i>Streptococcus haemolyticus</i> , <i>Peptostreptococci</i>	No growth
3	15 / F	36	<i>Streptococcus haemolyticus</i> , <i>Bacteroides</i>	No growth
4	26 / M	46	<i>Streptococcus viridans</i> , <i>Lactobacilli</i>	<i>Streptococcus viridans</i> , <i>Lactobacilli</i>
5	18 / F	46	<i>Streptococcus viridans</i> , <i>Streptococcus mutans</i> , <i>Lactobacilli</i>	Not evaluated
6	21 / F	36	<i>Staphylococcus aureus</i> , <i>Streptococcus mutans</i> , <i>Lactobacilli</i>	<i>Streptococcus mutans</i> , <i>Lactobacilli</i>
7	26 / M	46	<i>Peptostreptococci</i>	Not evaluated
8	20 / F	36	<i>Streptococcus mutans</i> , <i>Streptococcus viridans</i> , <i>Lactobacilli</i>	<i>Streptococcus mutans</i> , <i>Streptococcus viridans</i> , <i>Lactobacilli</i>
9	27 / F	36	<i>Streptococcus haemolyticus</i>	No growth
10	23 / M	46	<i>Lactobacilli</i>	No growth

Figure 1- Microorganisms present in carious dentin before and after 3 months of exposure to silver amalgam

Individuals No.	Age/Sex	Tooth number (FDI System)	Microorganisms isolated before restoration	Microorganisms isolated 3 months after restoration
1	18 / M	36	<i>Escherichia coli</i> , <i>Candida albicans</i> , <i>Streptococcus mutans</i> , <i>Streptococcus viridans</i>	<i>Escherichia coli</i>
2	22 / F	46	<i>Lactobacilli</i> , <i>Peptostreptococci</i>	<i>Peptostreptococci</i>
3	24 / F	36	<i>Escherichia coli</i> , <i>Achromobacter</i> , <i>Clostridium sporogenes</i> , <i>Peptostreptococci</i>	<i>Escherichia coli</i>
4	26 / M	36	<i>Lactobacilli</i> , <i>Staphylococcus aureus</i>	<i>Proteus rettgeri</i>
5	18 / F	46	<i>Lactobacilli</i>	No growth
6	24 / M	36	<i>Achromobacter</i> , <i>Clostridium sporogenes</i> , <i>Branhamella catarrhalis</i> , <i>Klebsiella</i>	Not evaluated
7	25 / M	46	<i>Lactobacilli</i>	No growth
8	22 / F	46	<i>Klebsiella</i> , <i>Bacteroides</i> , <i>Staphylococcus aureus</i>	Not evaluated
9	27 / M	36	<i>Lactobacilli</i>	No growth
10	23 / M	46	<i>Lactobacilli</i>	No growth

Figure 2- Microorganisms present in carious dentin before and after 3 months of exposure to a fast setting calcium hydroxide

Individuals No.	Age/Sex	Tooth number (FDI System)	Microorganisms isolated before restoration	Microorganisms isolated 3 months after restoration
1	19 / F	46	<i>Lactobacilli</i>	No growth
2	27 / F	46	<i>Lactobacilli, Fusobacterium, Klebsiella, Staphylococcus citreus</i>	<i>Staphylococcus aureus, Klebsiella</i>
3	24 / M	46	<i>Escherichia coli, Achromobacter, Clostridium sporogenes, Klebsiella, Peptostreptococci, Staphylococcus aureus, Staphylococcus citreus</i>	<i>Klebsiella, Staphylococcus aureus, Staphylococcus citreus</i>
4	22 / F	36	<i>Staphylococcus aureus, Staphylococcus citreus</i>	<i>Staphylococcus aureus, Staphylococcus citreus</i>
5	19 / F	46	<i>Lactobacilli</i>	No growth
6	24 / M	46	<i>Lactobacilli</i>	Not evaluated
7	20 / M	46	<i>Lactobacilli</i>	No growth
8	21 / M	46	<i>Klebsiella, Bacteroides</i>	<i>Klebsiella</i>
9	29 / M	36	<i>Branhamella catarrhalis, Klebsiella, Fusobacterium, Staphylococcus aureus, Staphylococcus citreus</i>	<i>Klebsiella, Staphylococcus aureus, Staphylococcus citreus</i>
10	28 / F	36	<i>Lactobacilli</i>	Not evaluated

Figure 3- Microorganisms present in carious dentin before and after 3 months of exposure to polyantibiotic paste

Individuals No.	Age/Sex	Tooth number (FDI System)	Microorganisms isolated before restoration	Microorganisms isolated 3 months after restoration
1	21 / M	36	<i>Lactobacilli</i>	No growth
2	28 / M	36	<i>Lactobacilli</i>	No growth
3	25 / M	46	<i>Fusobacterium, Streptococcus mutans</i>	No growth
4	20 / F	36	<i>Lactobacilli</i>	No growth
5	16 / F	46	<i>Lactobacilli</i>	Not evaluated
6	29 / F	36	<i>Streptococcus mutans, Staphylococcus citreus, Fusobacterium, Bacteroides</i>	No growth
7	22 / M	36	<i>Bacteroides, Streptococcus mutans</i>	No growth
8	27 / M	46	<i>Bacteroides, Streptococcus mutans, Staphylococcus aureus, Staphylococcus citreus</i>	No growth
9	26 / F	46	<i>Staphylococcus aureus, Staphylococcus citreus</i>	No growth
10	24 / M	46	<i>Fusobacterium, Bacteroides, Staphylococcus citreus, Streptococcus mutans</i>	No growth

Figure 4- Microorganisms present in carious dentin before and after 3 months of exposure to a light-cured fluoride releasing liner

in 6 cases and 4 showed a mixed flora with both aerobic and anaerobic *Streptococci* and *Bacteroides*. On re-evaluation after 3 months, only 6 patients attended the follow up. In four cases, *Streptococci* and *Lactobacilli* could be isolated.

The bacterial flora of Group II samples before and after application of calcium hydroxide is shown in Figure 2. Initial samples of 6 teeth gave pure

cultures of *Lactobacilli*. Two patients did not attend the review. In 3 cases, *Escherichia coli* and *Proteus rettgeri* were isolated. In 1 case, *Peptostreptococci* was isolated. In 4 cases, no microorganism could be isolated on re-entry.

Figure 3 shows the bacterial status of carious dentin before and after application of polyantibiotic paste. Five cases gave pure cultures of *Lactobacilli*. In

Individuals No.	Age/Sex	Tooth number (FDI System)	Microorganisms isolated before restoration	Microorganisms isolated 3 months after restoration
1	21 / M	36	<i>Staphylococcus aureus, Lactobacilli</i>	Not evaluated
2	28 / M	36	<i>Branhamella catarrhalis, Fusobacterium, Staphylococcus citreus, Streptococcus mutans</i>	No growth
3	25 / M	46	<i>Fusobacterium, Streptococcus mutans</i>	No growth
4	20 / F	36	<i>Lactobacilli</i>	No growth
5	16 / F	46	<i>Lactobacilli</i>	Not evaluated
6	29 / F	36	<i>Lactobacilli</i>	No growth
7	22 / M	36	<i>Branhamella catarrhalis, Staphylococcus aureus, Streptococcus mutans</i>	No growth
8	27 / M	46	<i>Lactobacilli</i>	Not evaluated
9	26 / F	46	<i>Streptococcus mutans, Staphylococcus citreus, Fusobacterium, Bacteroides</i>	No growth
10	24 / M	46	<i>Lactobacilli</i>	No growth

Figure 5- Microorganisms present in carious dentin before and after 3 months of exposure to mineral trioxide aggregate

Groups	Individuals who attended the review	Individuals from whom microorganisms were isolated after 3 months
I	6	4 ^a
II	8	4 ^a
III	8	5 ^a
IV	8	0 ^b
V	7	0 ^b

Different letters indicate significant results ($p < 0.05$)

Figure 6- Summary of number of patients reviewed and instances where microbes were isolated on review

1 case, *Staphylococcus aureus* and *Staphylococcus citreus* was isolated. In four cases, a mixed flora containing aerobic and anaerobic microorganisms was isolated. Only 8 patients attended the review. In three cases, no microorganism was isolated.

The bacterial flora isolated from Group IV samples in our study is shown in Figure 4. Samples of carious material prior to filling, gave pure cultures of *Lactobacilli* in 4 cases and 6 cases showed a mixed flora with both aerobic and anaerobic microorganisms. No microorganism could be isolated from the second sample in the 8 patients who attended the follow up.

The bacterial status of carious dentin before and after application of MTA and glass ionomer cement as base is given in Figure 5. Five cases of the samples of carious material prior to filling gave pure cultures of *Lactobacilli*. In 5 cases, a mixed flora containing aerobic and anaerobic microorganisms was isolated. No microorganism could be isolated in the second sample in the 7 cases who attended the review.

There was no significant difference among Groups I, II and III, or between Groups IV and V ($p > 0.05$). Both Groups IV and V showed significantly better results when compared to Groups I, II and III ($p < 0.05$) (Figure 6).

DISCUSSION

Dental caries is considered the chief cause of pulpal injury. Therefore, advancing carious lesion has been studied extensively. The pulp reactions in deep carious lesions have been attributed to bacterial toxins, not to a direct result of bacterial invasion. The removal of softened dentin has been recommended based on the fact that the acidity of remaining carious dentin in deep cavities may endanger the vitality of the pulp. It is mandatory to remove carious dentin at the dentinoenamel junction (DEJ)⁸. Simple removal of carious dentin from the cavity and placement of non bactericidal restorations are not sufficient to sterilize this environment. To eliminate the possibility of

progression of carious lesions, it is desirable to sterilize the infected dentin.

It is evident from this investigation that the deeper layers of residual caries are usually contaminated with cultivable microorganisms, which is in full agreement with the results of several studies^{4,5,11}. However, this study disagrees in part with the findings of some studies^{3,9} which reported that superficial layers of dentin are always infected, the intermediate layers occasionally infected and deeper layers of decalcified dentin of active lesions and sclerotic layers of arrested lesions are almost always sterile. The flora found at the initial opening was variable and the level at which the samples were taken could have possibly influenced this finding.

Among the bacteria isolated, *Lactobacilli* constituted 56% and this proportion is in accordance with other studies^{4,11,20}, where 80% of the same microorganism was isolated. The isolation of *Lactobacilli* is not surprising, taking into consideration the fact that this bacteria can thrive at even a low pH of 4, which is the average pH in deep carious lesions. The cariogenicity of *Streptococci* and *Peptostreptococci* in gnotobiotic rats is well established¹⁹. *Peptostreptococcus*, *Bacteroides* and *Fusobacterium* have also been isolated from carious dentin by other researchers and the findings of our study are in accordance to them^{11,10}. In one case, in Group II, *Clostridium sporogenes* was isolated. *Clostridium welchii* belongs to the same species and has been isolated in one study⁷. *Escherichia coli* and *Candida albicans* were isolated in mixed cultures containing *Achromobacter*, *Branhamella catarrhalis* and *Klebsiella*. This is in agreement with King, et al.⁹ (1965).

Only 6 patients attended the recall in Group I. In three cases, *Streptococci* and *Lactobacilli* could be isolated. King, et al.⁹ (1965) isolated microorganisms from the carious lesion during the second visit, though the number was much lesser than the initial culture. This difference is possibly due to difference in investigating procedure. It is possible that the layer of dentin sampled in this study could have been the intermediate layer of decay as classified by some researchers. Silver amalgam has been proven to be bactericidal *in vitro* by Beyth, et al.² (2007). But the results of this study were contradictory. This could have been probably because of the variations in the study conditions. Nevertheless, it is imperative to note that microorganisms were not completely eliminated under amalgam restorations. Our results are in accordance with those of Splieth, et al.¹⁷ (2003) who showed that the microbial flora underneath amalgam was similar to the one found in carious dentin and plaque, with anaerobic and facultative anaerobic gram-positive rods dominating.

In Group II, 2 patients did not attend the recall. In 4 re-entry samples, there was no growth. The antibacterial activity of calcium hydroxide has been well established in the dental literature. Reduction in the number of microorganisms could be attributed to the fact that the alkalinity of calcium hydroxide has an antimicrobial action¹² and it also neutralises the acidity of carious dentin. In 3 re-entry samples, *Escherichia coli* and *Proteus rettgeri* were isolated. However, this tooth did not show any microorganism in the initial sample. This result could have been because of contamination or sampling at different levels in the two samples. In one case, *Peptostreptococci* was isolated. This microorganism was found even in the initial sample. This is in accordance with Peters, et al.¹⁴ (2002) who found that calcium hydroxide does not eliminate *Peptostreptococci* completely.

In Group III, only 8 patients attended the recall. Five teeth presented colonies of *Staphylococcus aureus*, *Staphylococcus citreus* and *Klebsiella*, while three teeth showed no cultivable microorganisms. The reduction in microorganisms is possibly due to the placement of polyantibiotic paste, which is active against gram-positive, gram-negative microorganisms and fungi¹⁶. Based on this study, antibiotic sensitivity testing can be done for each carious lesion and the particular antibiotic can be added to the cement for treating deep carious lesions.

Group IV samples showed no evidence of cultivable microorganisms in the re-entry specimens of 8 patients who attended the review. Patients in Group IV were given a base of light-cured fluoride-releasing hydroxyapatite-based liner. The material used in this Group, LimeLite, contains hydroxyapatite in a urethane dimethacrylate resin. This is the first report in the dental literature on the use of this material in clinical settings and its efficacy on microorganisms. The manufacturer claims that this material releases calcium, hydroxyl, fluoride and phosphate ions which have cariostatic properties. This material also contains a fluoride salt that is known to have antimicrobial action. The pH of LimeLite after 7 days has been shown to be 10-11, which could also be a contributory factor for its antimicrobial action¹⁹.

MTA is one of the most promising materials to enter the realm of dentistry in some years. Its highly alkaline pH of 12.5 is probably the reason for its antimicrobial activity¹⁵. Our study showed that the results of microbiological evaluation from deep carious lesions were similar after placement of the hydroxyapatite-based liner (LimeLite) and MTA. No microorganisms could be isolated in both cases after 3 months. The only disadvantage of MTA is its prolonged setting time (approximately 2 h 45 min). However, in this study, MTA was mixed

with 10% calcium chloride because it has been shown to shorten the setting time, increase the physicomechanical properties of MTA and increase the release of calcium ions¹.

CONCLUSIONS

Several cultivable bacteria could be isolated from deep carious lesions and hence, it is important to use a liner with antimicrobial activity underneath amalgam restorations. This study showed that the hydroxyapatite-based liner had antimicrobial activity similar to MTA. Therefore, in the case of deep carious lesions, a base with broad-spectrum antimicrobial activity is preferred to preserve pulp vitality. MTA and the hydroxyapatite-based liner were superior to calcium hydroxide, which in turn was better than polyantibiotic paste, in terms of antimicrobial activity.

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