The Effect of Pulpotomy Base Material on Bacterial Penetration and Proliferation for Pulpotomized Primary Molar Teeth: A Confocal Laser Scanning Microscopy Study

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***Introduction**: the study aimed to evaluate Enterococcus Faecalis colonization in the pulp chamber in pulpotomized extracted human teeth filled by different pulpotomy base materials (PBMs), using confocal laser scanning microscopy (CLSM). **Study design**: Cavity preparations were made in 70 extracted primary molars. The pulp chambers were filled using either Intermediate restorative material (IRM), Mineral Trioxide Aggregate (MTA) or Glass ionomer (GI). Twenty-five teeth served controls. The specimens were sterilized, and coronally filled with bacterial suspension for 21 days. The specimens were cut through the furcation area, stained using LIVE/DEAD BacLight Bacterial Viability Kit and evaluated using CLSM. **Results:** The extent of fluorescent staining was larger in the GI group, compared to the IRM and MTA groups, and larger in the IRM group compared to the MTA group ($P<0.05$). The minimal and maximal bacterial penetration depths into the dentinal tubules were 55 and 695μm, respectively (mean 310μm), without differences between the materials (GI, IRM, MTA, $p>0.05$). The ratio of live bacteria to dead bacteria within the evaluated areas was higher in the GI group compared to the IRM and the MTA groups, and higher in the IRM group compared to the MTA group ($P<0.05$). There were no differences between the mesial, distal and apical parts in any of the evaluations ($p>0.05$). **Conclusions:** bacteria colonize the interface between the PBM and dentin and penetrate deeply into the dentinal tubules. The extent and the vitality of the colonized bacteria may be affected by the type of PBM.*

***Keywords**: Pulpotomy · base materials · Bacterial colonization · Enterococcus faecalis · Confocal laser scanning microscopy*

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INTRODUCTION

The preservation of the primary dentition until their natural exfoliation is highly desirable, as primary teeth are critical in maintaining arch integrity, speech, phonetics, masticatory function and esthetics [1]. Therefore, a comprehensive knowledge of the pulp pathology of the primary teeth is of great importance. Two main approaches of endodontic therapy have been described to treat pulp exposures in primary dentition: pulpotomy and root canal treatment [1].

Pulpotomy is considered as one of the most widely accepted clinical procedures for treating caries-infected pulps or iatrogenic exposure of pulps in asymptomatic primary teeth. The rationale of pulpotomy is based on the healing of the radicular pulp tissue following surgical amputation of the affected or infected coronal pulp [1]. Clinically, the technique involves amputation of the coronal pulp followed by placement of a suitable medicament, and finally restoration of the tooth in two steps: application of a pulpotomy base material (PBM) to fill the coronal pulp chamber, followed by placement of a permanent restorative material over the base [2].

Various PBMs have been advocated for use in pulpotomy procedures based on their properties such as biocompatibility, sealing ability, microleakage prevention properties, antimicrobial efficacy, and regeneration promotion potential [2].

Mineral Trioxide Aggregate (MTA), Glass ionomer (GI) and Intermediate restorative material (IRM) are widely used as PBMs filling the pulp chamber. MTA has anti-bacterial activity and a good sealing ability, as well as regenerative potential by the formation of a dentinal bridge when it contacts the pulp tissue [3,4]. Glass ionomer is a biocompatible material, which forms a chemical bond to the tooth structure, thus it has a good sealing ability and it reduces microleakage. GI is easy to handle and it has fluoride-releasing properties that are important for caries prevention in children. IRM has anti-bacterial properties and a pronounced sealing ability, thus it prevents bacterial microleakge [2,4,5].

The success rate of pulpotomy decreases over time from more than 90% during the first 6-12 months to less than 70% after 36 months or more [6]. Failure of pulpotomy in primary molars has been attributed to internal resorption, external root resorption, inter-radicular pathology, or dentigerous cyst in permanent successors of pulpotomized primary teeth. However, the success of the treatment mainly depends on the ability of the restorative materials to prevent penetration and proliferation of bacteria within the root canal system [6,7].

The main model that has been used for testing the efficacy of coronal seal and of the temporary restoration in endodontically accessed primary teeth is the traditional dye penetration model [8].

However, while using this indirect micro-leakage model, it is impossible to clarify the exact routes of bacterial penetration and colonization in the primary teeth following pulpotomy procedures [8,9].

A direct examination of bacterial penetration in the pulp chamber in pulpotomized and coronally filled teeth, using Confocal laser scanning microscopy (CLSM), overcomes these drawbacks of the traditional indirect micro-leakage models, and is capable of providing more reliable and clinically relevant results [8,9]. The aim of this study was to evaluate Enterococcus faecalis (E. faecalis) colonization in the pulp chamber in pulpotomized extracted human teeth, filled by different PBMs, using CLSM.

MATERIALS AND METHOD

After the approval of the university ethics committee, seventy freshly extracted primary first and second molars that were extracted due to orthodontic reasons were collected for this study. To qualify, the selected teeth had to have at least 3 sound walls and one half to two thirds of root length. Teeth with caries, cracks or restorations were not included in the study.

Division to study groups

The teeth were divided into following groups:

- Group 1 (n=15), IRM (Dentsply, Konstaz, Germany), followed by bacterial contamination.
- Group 2 (n=15), MTA (Angelus, Londrina, PR, Brazil), followed by bacterial contamination.
- Group 3 (n=15), GI (EQUIA-RMGIC; GC Europe, Leuven, Belgium), followed by bacterial contamination.
- Group 4 (positive control, n=5), The prepared teeth were left without base material, followed by bacterial contamination.
- Group 5 (negative control, n=5), The teeth were left without preparation and base material. The entire root chamber surface was covered with two layers of nail varnish, without further contamination.
- Group 6 (N=5) (Negative control IRM): same as group 1 but without subsequent bacterial contamination.
- Group 7 (N=5) (Negative control MTA): same as group 2 but without subsequent bacterial contamination.
- Group 8 (N=5) (Negative control GI): same as group 3 but without subsequent bacterial contamination

The PBMs were allowed to set for 24 hours at 37◦C and 100% humidity.

Tooth preparations

The teeth were cleaned with a pumice paste and a rubber prophylaxis cup [8], and then stored in distilled water for no more than 3 months. Cavity preparations were made with a no. 330 high-speed bur (Komet, Lemgo, Germany) under water coolant. Following completion of the cavity outline and the access to the pulp chamber, no. 6 carbide round bur (Komet, Lemgo, Germany) in a slow-speed hand piece was used to complete the preparation of the pulp chamber and expose the canal orifices. Then the teeth were air dried.

PBMs were prepared according to the manufacturers' recommendations, and were subsequently placed and packed to fill the pulp chamber to half of the remaining coronal height, while leaving the lateral walls clean.

The experimental model

Two coats of nail varnish were applied to the surfaces of all teeth in order to prevent bacterial leakage through lateral canals or other discontinuities in the cementum. All roots were mounted using a model as described previously [9]. In brief, each tooth was put in an Eppendorf plastic tube of 1.5 mL volume (20-mL disposable scintillation vials—Sigma-Aldrich Co., St. Louis, MO, USA) and then inserted into a glass vial (Sigma-Aldrich Co., St. Louis, MO, USA) through the opening of the rubber cap, so the plastic tube fitted tightly inside the glass vial. The junctions between the teeth, the Eppendorf, and the rubber cap were sealed with a cyanoacrylate adhesive (Krazy Glue, Columbus, OH, USA). The system was then sterilized overnight using ethylene oxide gas [10].

Simulation of Enterococcus Faecalis bacterial infection

A growth medium for Streptomycin-resistant T2-strain Enterococcus Faecalis bacteria (E.Faecalis) (ATCC® 29212™) was prepared and autoclaved. In order to prevent contamination by additional bacterial species, 0.5 mg\ml Streptomycin sulfate (Sigma-Aldrich Co. St. Louis, MO, USA) was added, as E.Faecalis is resistant to 0.5 mg\ml Streptomycin sulfate. After growth medium preparation need to describe the culturing process, and only then the filling of the teeth. Each tooth specimen was filled from the coronal part of the root canal with the freshly prepared bacterial suspension, and

then incubated at 37°C and 100% humidity. The bacterial suspension was replaced with a fresh preparation every 24 hours for a total of 21 days [9].

Preparation of samples for evaluation

After 21 days of incubation, the teeth specimens were embedded in a self-cure acrylic repair material (Triad VLC resin; Dentsply, Int., York, PA) [9], and a mesio-distal cut was performed through the pulp chamber and furcation area of each specimen with a diamond saw rotating at 500 rpm (Isomet, Buehler Ltd., Lake Bluff, IL, USA), under water cooling (Figure 1, a-b).

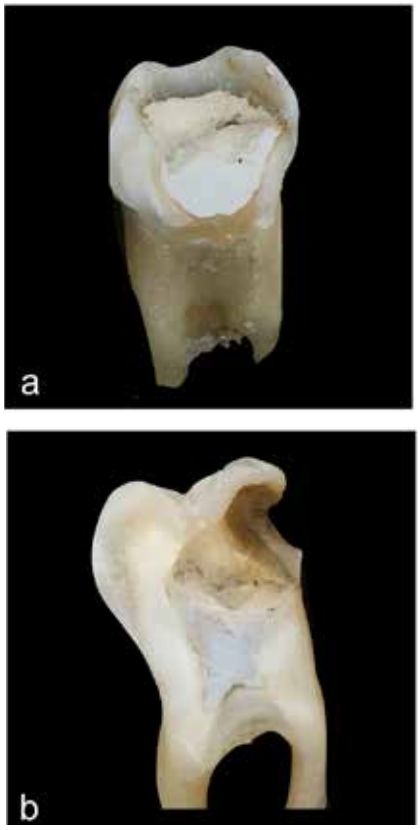

Figure 1(a,b): A mesio-distal cut was performed through the pulp chamber, pulpotomy base material and furcation area of each specimen in order to be evaluated by the Confocal laser scanning microscopy (CLSM). Prior to scanning, the cuts were stained with LIVE/DEAD BacLight Bacterial Viability Kit.

The samples were stained using LIVE/DEAD BacLight Bacterial Viability Kit L-7012 (Molecular Probes, Eugene, OR, USA), containing separate vials of the two component dyes (SYTO 9 and propidium iodide in 1:1 mixture) for staining of the biofilm. The excitation/emission maxima for these dyes are 480–500 nm for the SYTO 9 stain and 490–635 nm for propidium iodide [11].

Confocal microscopy evaluation

Immediately after the staining procedure, fluorescence from the stained bacteria was observed under a CLSM (Leica TCS SP5, Leica Microsystems CMS GmbH, Germany). Single-channel imaging and simultaneous dual-channel imaging were used to display green and red fluorescence [11,12].

The CLSM images of the bacterial biofilms were acquired at a resolution of 1024 × 1024 pixels and were analyzed by the LAS AF software (version 2.6.0.7266; Leica Microsystems CMS GmbH). The specimens were studied using a ×4 lens. The mesial, distal, and apical areas of the specimens were evaluated by the software as follows:

1. The extent of fluorescent staining within the evaluated areas was calculated.
2. The depth of bacterial colonization and penetration into the dentinal tubules was measured.
3. The vitality of the colonized bacteria was calculated as the proportion of live vs. dead bacteria.
4. The correlations between the type of the PBM material and bacterial presence and vitality were assessed.

Statistical analysis

The results were evaluated statistically using ANOVA with repeated measures to compare the proportion of live and dead bacteria with different base materials. One-way ANOVA was used to evaluate the fluorescence at the apical/mesial/distal areas, the extent of fluoresce staining within the evaluated areas, the depth of bacterial colonization, and the filling into the dentinal. $P<0.05$ was considered as statistically significant.

RESULTS

No fluorescence was observed in any of the negative control groups, and fluorescence was found in all specimens of the positive control group.

When comparing the different PBMs, the extent of fluorescent staining was significantly higher in the GI group, compared to the IRM and MTA groups ($P<0.05$). In addition, the extent of fluorescent staining was significantly higher in the IRM group compared to the MTA group ($P<0.05$). An example of the fluorescent staining in the different groups is shown in Figure 2 (a-f). Need to present the quantitative data of the extent of fluorescent staining that led to the statistical calculations.

The minimal and maximal penetration depths into the dentinal tubules were 55 and 695μm, respectively, with a mean of 310μm. No significant differences were found regarding the maximal and minimal depths of bacterial penetration into the dentinal tubules between the evaluated materials (GI, IRM, MTA) ($p>0.05$). Table 1 presents the depths of bacterial penetration into the dentinal tubules in the different groups.

When comparing the different PBMs, there were significantly more live bacteria than dead bacteria within the evaluated areas in the GI group compared to the IRM and the MTA groups ($p < 0.05$). There were also significantly more Live bacteria than dead bacteria in the IRM group compared to the MTA group ($P<0.05$) (Figures 2 (a-f) and Figure 3).

There were no significant differences between the mesial, distal and apical parts in any of the evaluations ($p>0.05$).

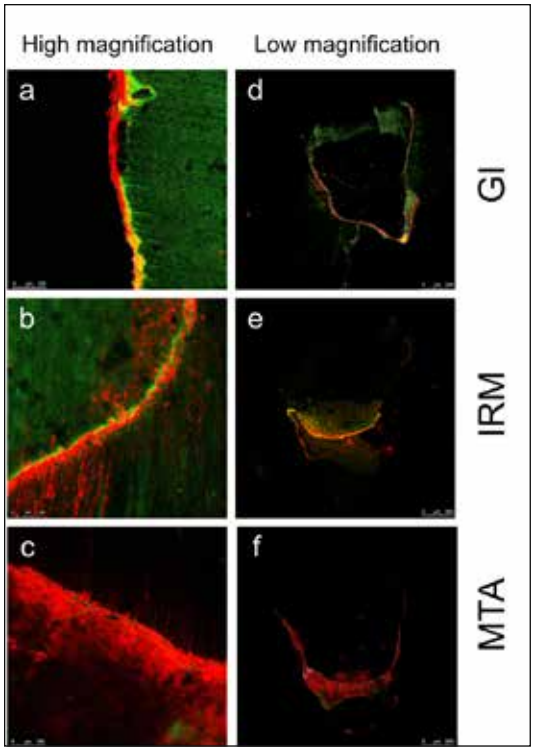

Figure 2 (a-f): Confocal laser scanning microscopy (CLSM) images of the bacterial colonization of the dentin. The infected dentin was stained with LIVE/DEAD BacLight Bacterial Viability Kit and analyzed by the LAS AF software. Vital (green) and dead (red) bacteria inside the dentinal tubules are clearly visible(a-c). A high (a-c) and low (d-f) magnification of the different materials can be seen. A higher amount of dead bacteria observed in the MTA group (c,f), compared to the IRM (b,e) and GI (a,d) groups.

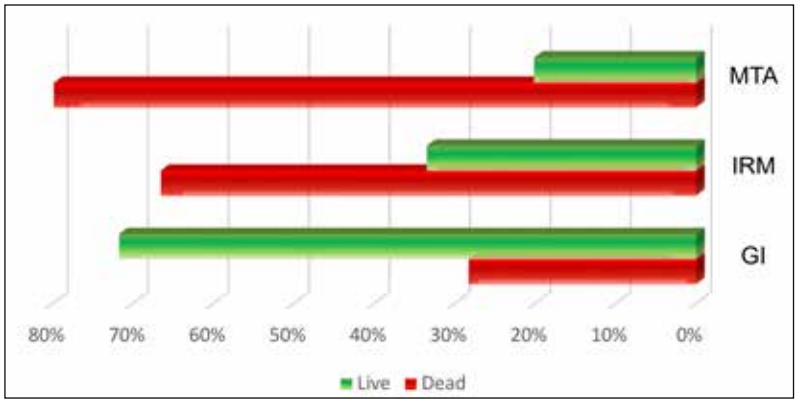

Figure 3: The average bacterial number (percentage) of live bacteria (green) and dead bacteria (red). There were significantly more live bacteria than dead bacteria within the evaluated areas in the GI group compared to the IRM and the MTA groups. There were also significantly more Live bacteria than dead bacteria in the IRM group compared to the MTA group.

Table 1 – Bacterial penetration depths into dentinal tubules (in um) for the different groups. The minimal and maximal penetration depths into the dentinal tubules were 55 and 695µm, respectively, with a mean of 310µm. No significant differences were found regarding the maximal and minimal depths of bacterial penetration into the dentinal tubules between the evaluated materials (GI, IRM, MTA).

Material	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
GI (Equia)	16	326.53	150.14	37.54	246.53	406.54	110.25	695.00
IRM	12	238.79	125.55	36.24	159.02	318.56	55.00	447.50
MTA (Angelus)	14	353.16	131.05	35.02	277.50	428.83	75.00	455.00

DISCUSSION

The main goal of pulpotomy is to maintain a symptom-free functional primary tooth until it reaches the age of its physiologic exfoliation [7]. The success of the procedure depends greatly on the ability of the coronal restoration to prevent bacterial penetration [13]. Bacteria and their byproducts are known to establish and maintain periapical inflammation [8,9,13], thus several base materials have been proposed/used to produce a hermetic seal, for preventing the penetration and the proliferation of bacteria in the root canal system [14].

Bacterial colonization of the root canal may cause an inflammatory reaction when bacterial byproducts such as endotoxins or exotoxins gain access to the periradicular tissues [15]. During the invasion of bacteria into the root canal system, bacterial biofilms colonize the dentin, and eventually the dentinal tubules may become a safe haven for bacteria [16]. Gram positive and facultative anaerobes are the most frequently isolated bacteria from root canal-treated teeth with persistent intra-radicular infections. Among them, *E. faecalis* is prevalent. In the root canal environment, *E. faecalis* bacteria play a key role in bacterial biofilm formation. Therefore, *E. faecalis* biofilms are considered as an appropriate model for evaluating root canal bacterial colonization [17-20].

Previous traditional *ex vivo* studies attempted to evaluate dye and bacterial leakage [8,21]. However, these studies were limited since they were using indirect models, which are incapable of evaluating the actual routes of bacterial penetration and colonization. Unlike the previous traditional models, in the current study, a modern model was used [9], which histologically traces the actual routes of microbial colonization *in situ*. In addition, positive and negative histological controls were used to confirm the adequacy of the experimental model [9]. No fluorescence was observed in the negative control groups, while fluorescence was found in all specimens of the positive control groups, thus confirming the reliability of the experimental model.

In the present study, the type of the base material that was used significantly affected the extent of bacterial colonization, and the viability of the penetrating bacteria. Bacteria were present more in the GI group compared to the MTA and IRM groups. There were also significantly more colonized bacteria in the IRM group compared to the MTA group. Furthermore, compared to the other evaluated PBMs (IRM and GI) MTA showed superior antimicrobial properties, as more live bacteria than dead bacteria were found in the IRM and the GI groups compared to the MTA group.

When considering the expected microleakage between MTA, IRM and GI, the results of this study are consistent with most other studies, which reported that GI leaked significantly more than MTA [22,23]. In contrast, John *et al* [24] reported no significant differences in leakage between Fuji Triage glass-ionomer and gray or white MTA. Other investigations indicated that MTA exhibited significantly less dye leakage in comparison with IRM [25].

Torabinejad *et al* and others [26-28] evaluated the clinical procedures for application of MTA, as a potential compound to seal off the pathways of communication between the root canal system and the oral cavity. MTA has been shown to have a good sealing ability due to the fact that MTA stimulates dentin bridge formation adjacent to the dental pulp [26-28].

Previous studies that assessed the antibacterial properties of MTA in various species of microorganisms reported on conflicting results, probably due to differences in the sources of the preparing material [25,29,30], as well as in the concentrations and the types of MTA used in these studies [31]. Tanomaru-Filho *et al* [30], who evaluated the antimicrobial activity of endodontic sealers, reported that MTA-based cements have an antibacterial effect on Enterococcus faecalis. In contrast, several other investigations documented that MTA had a limited antimicrobial effect against microorganisms [32]. An investigation on facultative and strict anaerobic bacteria showed that MTA had an antibacterial effect on some facultative bacteria and no effect on any species of strict anaerobes, while IRM had antibacterial effects on both types of the tested bacteria [4]. It has been shown that in aerobic conditions, MTA could generate reactive oxygen species with antimicrobial activity. Parirokh and Torabinejad [33] also found that MTA had no antibacterial effect against any of the strictly anaerobic bacteria. However, as shown by our results, it is possible that MTA's highly alkaline pH affords its antimicrobial activity even when in anaerobic condition.

Slutzky *et al* [34] have shown that IRM had antibacterial activity against E. faecalis immediately after setting, and sustained this ability for at least 1 day. Davidovich *et al* [35] evaluated the antibacterial properties of three types of GIs against Streptococcus mutans, Actinomyces viscosus and Enterococcus faecalis. No bacterial growth was reported in any of the tested bacteria. This effect lasted for at least one week for S. mutans and *A. viscosus*, but not for *E. faecalis*.

Although the bacteria penetrated deeply into the dentinal tubules to a mean depth of 310 μm (and maximal depth of 695 μm), the type of the PBM used did not affect the penetration depth. Peters et al [36] evaluated the depth of penetration of bacteria into the root dentin of permanent teeth with periapical lesions and reported that in more than half of the infected roots, bacteria were present in the deep dentin close to the cementum. They attributed their results to the fact that anaerobic culturing of dentin is a more sensitive method to detect these bacteria than histology. In that context, CLSM seems to be a favorable technique to evaluate bacterial colonization in the dentinal tubules since it allows to assess both viable and dead bacteria *in-situ* [9]; thus, it can assess the true extent of the bacterial penetration into the dentinal tubules. To the best of our knowledge this is the first assessment of bacterial colonization within the dentinal tubules of pulpotomized primary teeth.

In the current study there were no significant differences between the mesial, distal and apical parts. Further studies are needed to evaluate the bacterial colonization at different parts of the pulp chamber.

CONCLUSIONS

CLSM seems to be a reliable technique to evaluate bacterial penetration and proliferation in pulpotomized primary molar teeth, that allows to assess both viable and dead bacteria *in-situ.* Under the limitations of an *ex-vivo* model, the current study demonstrated that following pulpotomy, bacteria colonize the interface between the base material and dentin and penetrate deeply into the dentinal tubules, regardless of the type of PBM. However, the extent and the vitality of the colonized bacteria may be affected by the type of PBM.

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