THE INVASION OF BACTERIAL BIOFILMS INTO THE DENTINAL TUBULES

SHLOMO ELBAHARY

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Contents

1 General Introduction

Chapter 1

General Introduction

Endodontic infection is the infection of a tooth's root canal system and the major cause of apical periodontitis[1]. Although various chemical and physical factors can induce periradicular inflammation, scientific evidence indicates that microorganisms are essential for the progression and perpetuation of apical periodontitis[2]. Bacterial biofilms are prevalent in the apical root canals of teeth with primary and post-treatment apical periodontitis, including teeth associated with long-standing pathologic processes (e.g. large apical radiolucencies and cysts).[3] Bacterial biofilms are multicellular microbial communities that adhere to surfaces and interfaces[4]. They account for over 80% of microbial infections in the body[5,6] and can cause life-threatening infections[7] and are considered as a primary cause of apical periodontitis in teeth with infected root canal systems[8]. Intra-canal colonized bacterial communities produce constant byproducts and toxins efflux that stimulates the periapical inflammation and subsequent destruction of tissues that surround the apex of a tooth [9].

Unlike the gram-negative anaerobic flora that is found in untreated infected root canals, gram-positive and facultative anaerobes are the most frequently isolated species within treated canals in teeth with persistent intra-radicular infections, with *Enterococcus fecalis* (*E. faecalis*) being the most prevalent[10]. *E. faecalis* is not normally considered to be part of the healthy oral microbiological flora and has been identified in other common dental diseases, such as periodontal diseases, peri-implantitis, dental caries, and most commonly in secondary endodontic infections[11,12]. Therefore *E. faecalis* biofilms are considered to be an appropriate species for testing antimicrobial dental treatments[13].

The rationale for endodontic treatments is to eradicate and prevent root canal infection. This goal may be accomplished using mechanical instrumentation and chemical irrigation of the root canal between treatment sessions[14]. To prevent microorganisms from infecting or re-infecting the root and/or periradicular tissues, after completion of the root canal treatment, a root canal filling should be

placed[9], followed by adequate coronal restoration. An ideal root canal filling material should prevent bacterial colonization and ensuing leakage of bacterial by-products into the periradicular tissues. The type of the root canal filling material may affect the invasion of bacterial penetration into the dentinal tubules, and the viability of these bacteria[15]. Researchers suggested that irrigation during root canal therapy can lead to structural changes of the dentin walls of the root and changes in surface roughness[16,17]. Therefore, consideration should be given to appropriate selection of dentin preparation and irrigation protocols to match the sealer type and to allow adequate mechanical and chemical retention[18,19].

Thus, a thorough understanding of the endodontic microbiota histopathology and the actual invasion routes of bacterial infections should be the basis for the success of endodontic treatments.

Despite meticulous mechanical and chemical preparation during root canal treatment, the infection may persist[10], in most of the treated and filled root canals, and in some cases may lead to treatment failure and further complications[10,13]. Biofilms may pose a severe health threat, and in some cases, bacteria may become inaccessible to antibacterial agents and the body's immune system[20,21]. Penetration failure of antimicrobial agents may be associated with various factors, including the extracellular matrix encapsulating the biofilm cells, and multidrug resistance development of bacteria within the biofilm[20]. Many treatment regimens were evaluated to treat these persistent intra-canal infections, however, this goal remains a clinical challenge[13].

As mentioned earlier, root canal colonization of microbial biofilms as a result of either continuing bacterial contamination of the root canal-treated teeth or residual infection in the root canal system can prevent periapical healing of endodontically treated teeth. For endodontically treated teeth with apical periodontitis, surgical endodontic treatment may be indicated when non-surgical retreatment is impractical[22]. The main goal of surgical endodontic treatment is to prevent the invasion of bacteria and their by-products from the root canal system into the periradicular tissues by adequate root-end management and retrograde filling[23]. Although the success rates are impressive, in most of the cases the

bacterial biofilms remain inside the root canal system, as they were not approached during the treatment, and might lead to eventual treatment failure[24,25].

The exact mode of bacterial colonization in filled root end teeth is not fully understood. A dentin infection model has particular significance in studying apical periodontitis. Microbiological, histological, and microscopic techniques have been used to study the presence of bacteria inside root canals and within the dentinal tubules[26]. *In Vitro* studies attempted to evaluate leakage in the presence of root-end filling using different models such as the dye penetration model[27,28]. However, these studies were limited since they were using indirect laboratory models, incapable of evaluating the actual routes of bacterial penetration and colonization[26]. The most common experimental model that was used to evaluate bacterial penetration through filled or resected root canals has been the two-chamber leakage model[26]. This model presumably evaluates the penetration of bacteria from the upper chamber through the retrograde filling into the suspension located in the lower chamber, evident mainly by the appearance of turbidity in the suspension. However, inherent problems with this indirect model include the basic assumption that the leakage occurs only through the root canal space and not through additional potential routes, as well as the lack of appropriate negative controls. In addition, in most of these leakage studies, the routes of microbial leakage were not traced histologically, and thus it was difficult to quantify the bacterial colonization or to evaluate its colonization routes within the root canal space[29] . Therefore, both the reliability of these leakage models and their capability to provide a comprehensive evaluation of the root canal bacterial colonization is questionable [26].

Several microscopic techniques have been used to evaluate the bacterial colonization of dentin, including stereomicroscopy[9], scanning electron microscopy (SEM)[30], transmission electron microscopy (TEM)[31]. Thus, alternative microscopic techniques that can directly evaluate the bacterial colonization in the apical part of the resected and filled root canal are warranted.

Objectives of the thesis

This thesis aims to establish a novel and reliable experimental model that could enable to trace and quantify histologically the actual routes of bacteria and filling materials invasion into the dentinal tubules and in the total circumference of the root canal walls during various endodontic treatments, using Confocal Laser Scanning Microscopy (CLSM)-based model. CLSM was used together with live/dead bacterial staining techniques to find information regarding both the magnitude of dentin infection and vitality of the proliferating bacteria within the infected tubuli *in vitro* in order to assess their viability, even when they enter a dormant, not-culturable state.

Outline of the thesis

Chapter 2

Unlike the traditional root canal treatment where the challenge is to eliminate intra-canal bacteria, endodontic microsurgery offers an alternative to bypass the canals in teeth with apical periodontitis: the goal is to surgically block the invasion of bacteria and their by-products from the infected root canal system into the periradicular tissues with a retrograde (root end) filling[24] . The main clinical concern is that bacterial colonization and infection of the dentin and the filling-dentin interfaces following surgery may cause an inflammatory reaction when bacterial by-products such as endotoxins or exotoxins gain access to the periradicular tissues[24]. The type of root canal filling material may affect the bacterial penetration into the dentinal tubules, and the viability of these bacteria. This study aimed to evaluate *E. faecalis* colonization at the apical part of root canals in extracted human teeth following root-end resection and filling by different root-end filling materials, using CLSM.

Chapter 3

This is a subsequent study to the previous chapter. The same CLSM-based experimental model was used to assess the invasion of bacterial biofilms into dentinal tubules, this time by fluorescently labeling the different retrograde filling

materials, in three different lasers channels (Dead bacteria. Live bacteria, and filling material). The use of CLSM to assess the penetration of fluorescently labeled retrograde filling materials into the tubuli, while assessing the depth of bacterial invasion into the tubuli and their viability, may provide a comprehensive view of the microbial–pathological process following surgery and understand the influence of the filling materials and their penetration depths into the tubuli on bacterial invasion within the tubuli.

Chapter 4

Perforation in endodontic treatment can be defined as an artificial communication between the root canal or pulp chamber space and the surrounding tissues[32]. It may be pathological or iatrogenic[33]. The main goal of perforations management is sealing the defect in order to prevent bacterial contamination and create an ideal environment for tissue repair. The sealing ability and marginal adaptation of the repair material used are crucial to prevent leakage of irritants, and thus to enhance healing and treatment success[33]. Numerous materials have been introduced for furcal and root perforation repair, such as Mineral Trixode Aggregate (MTA), Amalgam, glass ionomer cement, Intermediate Restorative Material (IRM), and tricalcium phosphate. In this chapter we conducted a study to assess colonization and proliferation of *E. faecalis* in furcal perforations, repaired with different calcium-silicate based materials (MTA Angelus, Endocem, and Biodentine) by CLSM using the previously mentioned model.

Chapter 5

Pulpotomy is considered one of the most widely accepted clinical procedures for treating infected or iatrogenic exposed pulps in the primary dentition. The main goal of a pulpotomy is to maintain a symptom-free functional primary tooth until it reaches the age of its physiologic exfoliation. Several base materials have been proposed to produce a hermetic seal that prevents the penetration and the proliferation of bacteria in the root canal system. In this chapter, the different pulpotomy base materials' effects on the extent, viability, and bacterial colonization were assessed using CLSM.

Chapter 6

Internal bleaching is a non-invasive technique to treat intrinsic discoloration of teeth after root canal treatment using a potent bleaching material like sodium perborate. To improve the bleaching results dentin permeability could be increased by removing the smear layer and subsequently opening the dentinal tubules for better penetration of the bleaching material. However, this might also lead to more diffusion of bleaching agents to the outer surrounding tissues and risk post-bleaching external root resorption [34]. This study aimed to evaluate the influence of different irrigation protocols on sodium perborate penetration into dentinal tubules using CLSM

Chapter 7

An integral part of root canal treatment is the use of irrigations to disinfect the root canal system and to remove debris and tissue remnants. Currently, the most used anti-microbial irrigation protocol is a combination of sodium hypochlorite (NaOCl) and Ethylene-diamine-tetraacetic-acid (EDTA)[35]. However, the ideal irrigation sequence, volume, and application time remain disputed. Irrigation in root canal therapy can lead to structural changes of dentin and changes in surface roughness[35]. Therefore, consideration should be given to an appropriate selection of dentin preparation and irrigation protocols to match the sealer type and to allow adequate mechanical and chemical retention of the root canal filling to the root canal walls. In this chapter, we evaluated the effect of different irrigating protocols on dentin surface texture by assessing the dentin roughness using quantitative 3D surface texture analysis.

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2

Bacterial colonization in the apical part of extracted human teeth following root-end resection and filling

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Abstract

The aim of this study was to evaluate *Enterococcus faecalis* colonization at the apical part of root canals following root-end resection and filling using onfocal laser scanning microscopy (CLSM). Methods: The apical 3 mm of the root-ends of 40 extracted single rooted human teeth were resected, and retrograde cavities were prepared and filled to a depth of 3 mm using either MTA, IRM or Biodentine (n=10 each). 10 teeth served as positive and negative controls. The roots were placed in an experimental model, sterilized and coronally filled with *E. faecalis* bacterial suspension for 21 days. Then, the apical 3 mm segments were cut to get 2 slabs (coronal and apical). The slabs were stained using LIVE/DEAD BacLight Bacterial Viability kit and evaluated using CLSM. Results: The fluorescence stained areas were larger in the bucco-lingual directions compared with the mesio-distal directions ($P<0.05$) and the mean and maximal depth of bacterial colonization into the dentinal tubules were 755 and 1643 μm respectively, without differences between the root-end filling materials ($p>0.05$). However, there were more live bacteria found in the MTA group, in comparison with IRM and Biodentin groups ($p<0.05$). Conclusion: In the present study CLSM was used as a novel model to demonstrate histologically bacterial root-end colonization following root-end filling. Bacteria may colonize at the filling-dentine interfaces and deep into the dentinal tubules. This colonization was not homogenous, favoring the bucco-lingual aspects of the root, and the viability of the colonized bacteria may be affected by the type of root-end filling material.

Key words: Endodontic surgery; Root-end filling; Bacterial colonization; *Enterococcus faecalis*; Confocal laser

Introduction

The relationship between bacteria in the root canal system and periapical pathosis has been well established[1]. Root canal colonization of microbial biofilms as a result of continuing bacterial contamination of the root canal treated teeth, or residual infection in the root canal system could prevent periapical healing of endodontically treated teeth[2-6].

For teeth with apical periodontitis surgical endodontic treatment might be indicated when non-surgical retreatment is impractical [4, 5, 7, 8]. The main goal of surgical endodontic treatment is to prevent the invasion of bacteria and their by-products from the root canal system into the peri-radicular tissues by an adequate root end management and filling [4, 5, 8, 9]. Several root end-filling materials have been used in modern endodontic surgery such as mineral trioxide aggregate (MTA) [10-13], intermediate Restorative Material (IRM) [14-17], and recently also Biodentine [18-20]. A very high success rate was reported for modern surgical endodontic treatment [4, 5], however in some cases failure may occur as a result of insufficient retrograde seal and bacterial penetration and colonization [4, 5]. However, the exact mode of bacterial colonization in the apically resected root-end, filled by different types of root end filling materials, is not fully elucidated.

The most common traditional experimental model that was used to evaluate bacterial penetration through apically resected, prepared and filled root canals has been the two-chamber leakage model [21-23] that presumably evaluates the penetration of bacteria from the upper chamber and through the retrograde filling into the suspension located in the lower chamber, visible mainly by the appearance of turbidity in the suspension. However, an inherent problem with this in-direct model is the assumption that the leakage occur through the root canal space alone rather than through other potential routes, as well as the lack of appropriate negative controls [22, 23]. In addition, in most of the leakage studies, the routes of microbial leakage were not traced histologically, thus it was difficult to quantify the bacterial colonization or to evaluate its colonization routes within the root canal space [22-24]. Therefore, both the reliability of these leakage

models as well as their capability to provide a comprehensive evaluation of the bacterial colonization is questionable [22, 23], thus, alternative microscopy techniques that are capable to directly evaluate the bacterial colonization in the apical part of the resected and filled root canal is indicated.

Confocal laser scanning microscopy (CLSM) [25] has the advantage of providing a direct and quantified information about the presence and distribution of bacteria inside dentinal tubules and in the total circumference of the root canal walls [26-30]. A direct evaluation of the bacterial colonization in the apically treated and filled part of the root canal using CLSM could potentially overcome the inherent limitations of the traditional leakage models, thus, enabling more reliable and clinically relevant results.

The aim of this study was to evaluate *E. faecalis* colonization at the apical part of root canals in extracted human teeth following root-end resection and filling by different root end filling materials, using confocal laser scanning microscopy.

Materials and Methods:

Teeth selection, preparation, and allocation to groups:

Forty freshly extracted single rooted human teeth were stored in 0.05% sodium hypochlorite solution. Only fully developed teeth with one root canal with curvature not exceeding 5 degrees were included [31]. Teeth with long oval canals (when the ratio of long to short canal diameter was >2, [6]), teeth with no apical patency, teeth with an apical diameter of more than k-file #25, teeth with more than one root canal, previously endodontically treated teeth, teeth with incomplete root development or teeth with root resorption were excluded.

The crowns of the selected teeth were removed in order to obtain root specimens of 13 mm length and the working length was confirmed using a standard #10 k-file protruding from the apical foramen. The root canals were prepared to apical size #30 with stainless steel hand files (Dentsply Maillefer, Tulsa, OK, USA) using a "balanced force technique" [32]. During instrumentation copious irrigations were performed using 5% sodium hypochlorite solution. At the completion of the instrumentation a final flush of 17% EDTA followed by 5% sodium

hypochlorite solution was used to remove the smear layer [33].

The apical 3 mm of the root ends were resected without bevel using Zakaria high speed bur (Mailleffer, Ballaigues, Switzerland). Retrograde cavities were prepared to a depth of 3 mm using diamond-coated ultrasonic tips (Satelec, Paris, France) [5, 8]. The canal retrograde cavities were dried using paper points and the specimens were randomly divided into 5 groups as following:

Group 1(N=10): The 3 mm retrograde cavities were filled with mineral trioxide aggregate (MTA) (Pro Root; Dentsply-Tulsa Dental, Johnson City, TN, USA). MTA was mixed according to the manufacturer instructions, placed in the root end preparation, condensed with a micro-pluger, and stored in 100% humidity [34-36].

Group 2 (N=10): The 3 mm retrograde cavities were filled with Intermediate Restorative Material (IRM; Dentsply, Germany) mixed according to the manufacturer instructions [8, 9].

Group 3 (N=10): The 3 mm retrograde cavities were filled using Biodentine (Septodent, France) mixed according to the manufacturer instructions.

Group 4 (N=5) (Positive control): the prepared roots were left without retrograde filling.

Group 5(N=5) (Negative control): The teeth were left without retrograde preparation and filling, and the entire root surface including the apical portion was covered with two layers of nail varnish[37].

The experimental model:

Two coats of nail varnish were applied to the surfaces of all teeth excluding the resected apical portion in order to prevent bacterial leakage through lateral canals or other discontinuities in the cementum [38]. All roots were mounted using a model as described previously [39]. In brief, all roots were inserted in eppendorf plastic tubes of 1.5 mL volume (20 ml disposable scintillation vials – Sigma-Aldrich Co., St. Louis, MO) and then inserted into a glass vial (Sigma-Aldrich Co. St. Louis,

MO, USA) through the opening of the rubber cap, so it fitted tightly inside the glass vial. The junctions between the root, the eppendorf and the rubber cap were sealed with cyanoacrylate adhesive (Krazy Glue, Columbus, OH, USA).

The system was then sterilized overnight using ethylene oxide gas [24] and then placed in a 9 mL sterile glass flask containing 4 mL of Brain Heart Infusion broth (Becton Dickinson, Sparks, MD, USA), so that approximately 2 mm of the root apex was immersed in the broth [40].

Bacterial contamination of the model:

A growth medium for Streptomicin resistant T2 strain, *Enterococcus faecalis* bacteria (EF) (ATCC® 29212™) was prepared by mixing 18.5 gr of BHI with 500 ml of distilled water. The suspension was autoclaved. In order to prevent contamination by additional bacterial species, 0.5 mg\ml Streptomicin sulfate (Sigma-Aldrich Co. St. Louis, MO, USA.) was added. EF is resistant to 0.5 mg\ml Streptomicin sulfate [41].

Each specimen was filled from the coronal part of the root canal with the freshly prepared bacterial suspension and incubated at 37°C and 100% humidity. The bacterial suspension was replaced with a fresh preparation every 24 hours, and was further incubated for a total period of 21 days.

Preparation of samples for evaluation:

After 21 days of incubation the presence of turbidity in the BHI broth of each sample was recorded [42]. The specimens were embedded in a self-cure acrylic repair material (UNIFAST Trad, Gc America) and the apical 3 mm segments of each specimen containing the root end preparation and filling were cut perpendicular to the long axis of the root under water cooling with a diamond saw rotating at 500 rpm (Isomet, Buehler Ltd., Lake Bluff, IL, USA) in order to prepare 2 slabs (coronal and apical) of 1 mm thickness each [43].

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 were 480-500 nm for the SYTO 9 stain and 490-635 nm for propidium iodide [44].

Confocal microscopy evaluation:

Immediately after the staining procedure, fluorescence from the stained cells was observed under a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems CMS GmbH Germany). Single channel and simultaneous dual-channel imaging were used to display green and red fluorescence [30].

The Confocal laser scanning microscope images of the bacterial biofilm were acquired and analyzed by the LAS AF software (version 2.6.0.7266; Leica Microsystems CMS GmbH) at a resolution of 1024X1024 pixels. The specimens were observed using a X4 lens . The mesial, distal, buccal, and lingual areas of the specimens were evaluated by the software as following:

1. The size of fluoresce staining within the evaluated areas, as calculated by the software.

2. The viability of the colonized bacteria evaluated as the proportion of live and dead bacteria: the values of green fluorescence (live cells) and red fluorescence (dead cells).

3. The depth of bacterial colonization into the dentinal tubules was measured and recorded considering the canal wall as the starting point (43).

4. The stained areas of the coronal and apical slabs

Statistical evaluation:

The results were evaluated statistically as following: t-test was used to compare the proportion of live and dead bacteria with different retrograde filling materials, and to compare the stained areas at the buccal/lingual/mesial/distal areas. One-way ANOVA was used to evaluate the size of fluoresce staining within the evaluated areas and the depth of bacterial colonization into the dentinal tubules for various retrograde filling materials, and to compare the size of stained areas of the coronal and apical slabs. $P<0.05$ was considered as statistically

significant.

Results:

No fluorescence and no turbidity were observed in the negative control group, and fluorescence and turbidity were found in all the specimens of the positive control group.

The stained areas were significantly larger in the buccal and lingual directions compared with the mesial and distal directions, in all groups ($P<0.05$) (Figure 1).

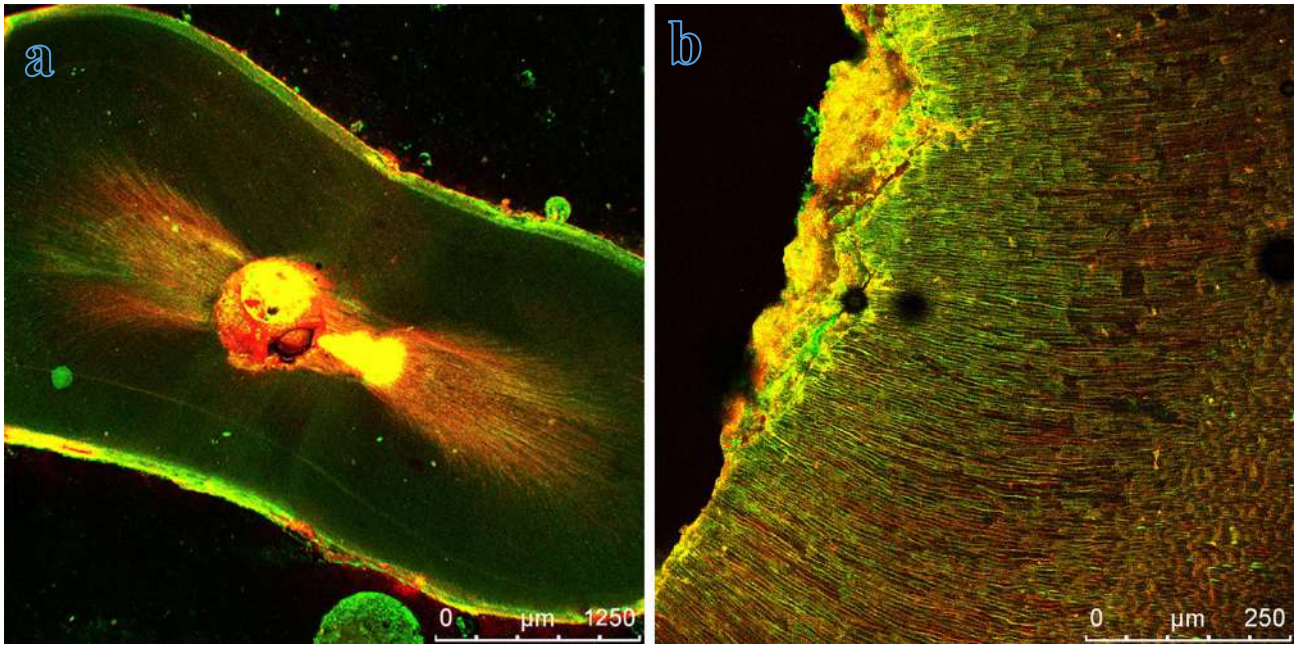

Fig. 1 Confocal laser scanning microscopy (CLSM) images of the bacterial colonization of the dentin. The infected dentin was stained with LIVE/DEAD BacLight Bacterial Viability Kit and analyzed by the LAS AF software. a, b Two different microscope fields in which vital (green) and dead (red) bacteria inside the dentinal tubules are clearly visible. The magnification of some tubules (b) shows the presence a coccoidal struc- ture in numerous branches in the radicular dentin, a butterfly-like appear- ance seen on the root cross sections (a) that occurs as a result of increased sclerosis along the tubules located on the mesial and distal sides of the canal lumen

When comparing the different retrograde filling materials (figure 2), there were no significant differences in the size of fluoresce staining within the evaluated areas (dead and live bacteria combined) ($p>0.05$). However, there were significantly more dead bacteria than live bacteria in the IRM and Biodentine groups, and there were significantly more live bacteria than dead bacteria in the MTA group ($p<0.05$).

The minimal and maximal colonization depths into the dentinal tubules were

210 and 1643 microns (μm) respectively, with mean of 755 μm. No significant differences were found regarding the depth of bacterial colonization into the dentinal tubules between the evaluated materials (MTA, IRM, Biodentine) ($p>0.05$). Table 1 presents the depth of bacterial colonization into the dentinal tubules for the different groups. There were no significant differences between the coronal and apical slabs in any of the evaluations ($p>0.05$).

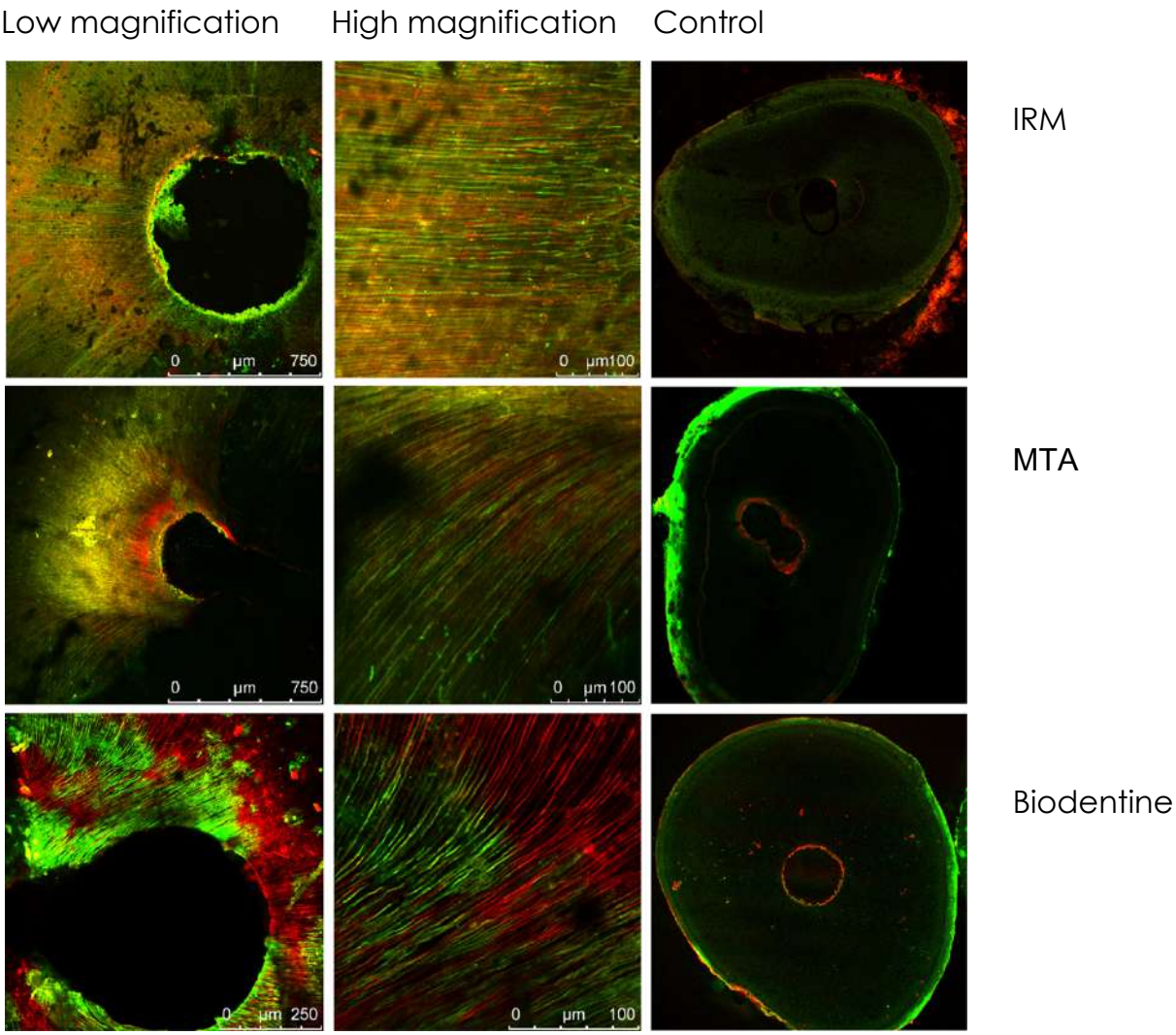

Figure.2 Confocal laser scanning microscopy (CLSM) images of experimental and control specimens after 21 days. Only the dentinal tubules of the experimental groups are infected

Table 1. Depth of bacterial colonization into the dentinal tubules (μm).

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Biodentine	7	625.35	295.01	111.50	352.51	898.19	210.50	911.18
IRM	8	673.53	308.00	108.90	416.04	931.03	456.00	1355.00
MTA	7	976.39	352.18	133.11	650.68	1302.11	591.00	1643.50
Total	22	754.56	341.17	72.74	603.30	905.83	210.50	1643.50

IRM= Intermediate Restorative Material. MTA= Mineral trioxide aggregate

Discussion:

It had been argued that the main goal of the root end management during surgical endodontic treatments is to prevent the invasion of bacteria and their by-products from the root canal system into the peri-radicular tissues [4, 5, 8, 9]. However, while the actual penetration of live bacteria into periapical tissues following the surgery may be important in some cases (e.g. extra radicular infections or periapical abscess [45, 46], the main clinical relevance is in bacterial colonization and Infection of the dentin and filling-dentine interfaces following the surgery [47, 48] since the established colonization of the root canal may cause an inflammatory reaction when bacterial byproducts such as endotoxin or exotoxins gain access to the periradicular tissues [49, 50].

Bacterial colonization of dentin is an active process mediated by cell division and availability of nutrients, and eventually the dentinal tubules may become a safe haven for bacteria [51]. Gram-positive and facultative anaerobes are the most frequently isolated species from root-canal treated teeth with persistent intra-radicular infections, and among them, *E. faecalis* is prevalent [52]. In the root canal environment, *E. faecalis* bacteria play an important role in bacterial biofilm formation, and *E. faecalis* biofilms are considered as an appropriate model for evaluating root canal bacterial colonization [53-57].

E. faecalis is a non-motile, facultative anaerobic bacteria [58], known to be a highly recalcitrant bacterium due to its ability to withstand alkaline conditions and glucose starvation, and thus it is prone to cause persistent infections [59, 60]. Peters et al. [61] argued that bacteria in dentinal tubules are entombed beneath the root canal filling and will eventually die. However, microbiological and histological studies demonstrated the growth of isolated islands of biofilms between an existing root canal filling and dentin walls [62, 63], and into the dentinal tubules [30, 51].

An ideal root-end filling material should prevent bacterial colonization and ensuing leakage of their by-products into the periradicular tissues [47]. Thus, understanding of the pathological process following endodontic surgery requires

an experimental model that is capable to assess not only the ability of the root end filling to prevent bacterial migration through the filled root end, but also to tract and quantify the exact routes of microbial colonization within the root canal space, at the filling-dentine interfaces and into the dentinal tubules [22-24].

Dentin infection model has particular significance for the study of apical periodontitis [64]. Microbiological, histologic, and microscopy techniques have been used to study the presence of bacteria inside root canals and within the dentinal tubules [65]. Previous *ex-vivo* studies attempted to evaluate leakage in the presence of root end filling using different models such as the dye penetration model. They reported that the leakage pattern may be related to different factors such as the root end-resection angel and to the exposure of dentinal tubules [66-68]. However, these studies were limited since they were using indirect models that are incapable to evaluate the actual routes of bacterial penetration and colonization. Unlike these dye penetration models or the traditional two-chamber model of bacterial leakage that suffers from significant inherent shortcomings such as uncertainty as to the real routes of the bacterial colonization in the experimental groups and the absent of proper histological controls [69], in the current study, the actual routes of microbial colonization were traced histologically and positive and negative histological controls were used to confirm the adequacy of the experimental model. In the present study no fluorescence and no turbidity were observed in the negative control group, and fluorescence and turbidity were found in all the specimens of the positive control group. Thus, in this study appropriate control groups were used and the adequacy of the experimental model to its goals was ensured.

Several microscopy techniques have been used to evaluate the bacterial colonization of dentin, including stereomicroscopy [70], scanning electron microscopy (SEM) [71, 72], transmission electron microscopy (TEM) [73], and confocal laser scanning microscopy (CLSM) (25). The use of CLSM technique is considered as useful as the traditional microbiological, histologic, standard electron microscopy and PCR- based techniques for the identification of viable bacteria in dentinal tubules [30], and has been described in previous studies [30, 43, 74]. Furthermore, the use of CLSM, along with live/dead staining method,

provides information about both the extent of the dentin infection, and also about the vitality of bacteria in the infected dentinal tubules in situ [30]. To our knowledge, this is the first study that evaluated the bacterial colonization in the apical part of extracted human teeth following root-end resection and filling using CLSM.

In the present study we found that the bacterial colonization was more extensive in the bucco-lingual direction compared with the mesio-distal direction ($P<0.05$), regardless the type of retrograde filling materials ($p>0.05$), which is in concordance with the previous study [75]. The reason may be related to a phenomenon called the ''butterfly effect'', a butterfly-like appearance seen on the root cross-sections that occurs as a result of increased sclerosis along the tubules located on the mesial and distal sides of the canal lumen. This effect is common in the single-rooted teeth of humans in a wide range of ages [76, 77].

No significant differences were found regarding the bacterial colonization area between the coronal and apical slabs ($p>0.05$). The distance between the two sections was approx. 1 mm which may explain the lack of difference. Further studies are needed to evaluate the bacterial colonization at different levels of the canal.

The depth of bacterial penetration and colonization into the dentinal tubules was not affected by the type of root end filling (MTA, IRM or Biodentine), and the maximal depth was 1643 microns (μm) with a mean of 755 μm. Peters et al [72] evaluated the depth of penetration of bacteria in root dentin of 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 more sensitive than histology to detect these bacteria. 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, thus, it is capable to assess the true extent of the bacterial penetration into the dentinal tubules.

In the present study the viability of the colonized bacteria was affected by the type of root canal filling material: there were more live bacteria found in the

MTA, in comparison with IRM and Biodentin groups. In other studies when IRM and MTA were compared, it was shown that MTA had an antibacterial effect on some of the facultative bacteria and no effect on any of the strict anaerobic bacteria, while IRM had some antibacterial effects on both types of the tested bacteria [78].

The antibacterial properties of retrograde filling materials were previously assessed [79, 80]. Slutzky et al [79] has showed that IRM was antibacterial immediately after setting for *E. faecalis* and sustained this ability for at least 1 day. Chong et al in 1994 [80] demonstrated the same in retrograde fillings. According to its manufacturer, Biodentine holds antibacterial properties due to the manifest alkalization of the environment, and also due to its high pH that exerts a clear inhibitory effect on microorganisms. In addition, the alkaline change demonstrably leads to the disinfection of adjacent hard and soft tissue structures [18-20, 81-83].

Conclusions

In the present study confocal laser scanning microscopy was used as a novel model to demonstrate histologically the actual routes of bacterial colonization in the apical part of extracted human teeth, following root-end resection and filling.

Under the limitations of an *ex-vivo* model, the current study demonstrated that following root end filling, bacteria may colonize within the root canal space, at the filling-dentine interfaces and deep into the dentinal tubules. This colonization is not homogenous, favoring the bucco-lingual aspect of the root, and the viability of the colonized bacteria may be affected by the type of root end filling material. Additional clinical studies are indicated to elucidate the exact clinical implications of the bacterial colonization characteristics of the root-end following endodontic surgery.

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The Invasion of Bacterial Biofilms into the Dentinal Tubules of Extracted Teeth Retrofilled with Fluorescently Labeled Retrograde Filling Materials

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Abstract

In this study, we evaluated the invasion of bacteria into the dentinal tubules of retrofilled extracted human teeth, and the influence of different fluorescently labeled retrograde filling materials on the bacterial invasion and viability, by means of confocal laser scanning microscopy (CLSM). The root apices of extracted teeth were cut, prepared, and filled retrogradely using either intermediate restorative material (IRM), mineral trioxide aggregate (MTA), or Biodentine. The roots were filled with *Enterococcus faecalis* bacteria from their coronal part for 21 days. Then, 3-mm-long apical segments were cut to get root axial slices, and the bacteria were fluorescently stained and evaluated by CLSM. Bacterial penetration into the dentinal tubules favored the bucco-lingual directions. The filling materials penetrated up to 957 µm into the tubuli, and the bacteria, up to 1480 µm (means: 130 and 167 µm, respectively). Biodentine fillings penetrated less and the associated bacteria penetrated deeper into the tubuli compared to MTA or IRM ($p = 0.004$). Deeper filling penetration was associated with shallower penetration of both dead and live, or live alone, bacteria ($p = 0.015$). In conclusion, the current study enables better understanding of the microbiological–pathological course after endodontic surgical procedures. It was found that even with retrograde fillings, bacteria invade deep into the dental tubules, where deeper filling penetration prevents deeper penetration of the bacteria and adversely affects the viability of the bacteria.

Keywords: Retrograde filling; *Enterococcus faecalis*; Bacterial penetration; Bacterial invasion; Root canal; Confocal microscopy; Biodentine; MTA; IRM; CLSM

Introduction

The rationale of retrograde filling material in endodontic surgery is to inhibit bacterial biofilm invasion and to prevent the invasion of bacterial toxins and byproducts into the surrounding periradicular tissues, in order to treat and prevent periapical pathology [1]. Retrograde filling is also expected to entomb any remaining bacteria in a way that would prevent bacterial invasion into the dentinal tubules, and that would eventually result in bacterial death [1]. However, studies have found that following endodontic surgery, bacterial biofilms may still colonize the root canals and also penetrate deep into the dentinal tubules [2].

As part of modern endodontic surgery protocols, a variety of retrograde filling materials are used, such as intermediate restorative material (IRM) [3], mineral trioxide aggregate (MTA) [4], and Biodentine [4].

This invasion of the bacterial biofilm manifests in the ability of viable bacteria to penetrate deep into the tubuli [5]. Thus, the depth of bacterial invasion into the dentinal tubules and their viability reflect the extent of the invasion. In a recent study, confocal laser scanning microscopy (CLSM) was used to assess the invasion of bacteria into the root apices following retrograde filling [2]. It was demonstrated that even in the presence of retrograde fillings, viable bacteria penetrated deep into the dentinal tubules [2]. However, in that study, only the bacteria were stained and evaluated using CLSM; the filling material penetration into the tubuli and its effect on the proliferating bacteria were not evaluated and remain unknown.

Recently, in another study, CLSM was used to evaluate fluorescently labeled filling material penetration into the dentinal tubules [6]. Thus, it seems beneficial to use CLSM to simultaneously evaluate both the retrograde filling material interface with the dentinal walls and its penetration into the tubuli, and its effects on the invasion of bacteria at the apically prepared and filled root canal and dentinal tubules [2,6–8]. This study enables a better understanding of the microbiological–pathological course after endodontic surgical procedures, and our null hypothesis is that the presence and type of a retrograde filling material will not affect bacterial invasion into the tubuli.

Therefore, the objectives of the study were to assess the invasion of bacterial biofilms into the tubuli of retrofilled extracted human teeth, by measuring their depth of penetration and viability, and to assess the influence of different fluorescently

labeled root-end filling materials on bacterial invasion using an established CLSM experimental model.

Materials and Methods:

Teeth collection, preparation, and distribution into groups

Based on a previously established experimental model [2], 70 single-rooted, freshly extracted human teeth were kept in 0.05% sodium hypochlorite liquid and were selected for the experiment. The experiment was approved by the Tel-Aviv University Ethics Committee on March 27, 2018, and all methods were implemented according to the relevant regulations and guidelines. All included teeth were fully developed and presented with a single straight root canal (curvature of <5 degrees) [9]. The following teeth were excluded: teeth in which the ratio of long to short canal diameters was >2 ("long oval canals") [10], root canals without apical patency or with an apical diameter of >#25 K-file, previously endodontically treated teeth, and teeth with root resorption. The crowns of the included teeth were cut, and 13-mm-long root specimens were obtained. The working length was assessed by a #15 stainless steel K-file (K-file; Dentsply Maillefer, Tulsa, OK, USA) extending beyond the apical foramen. The root canals were prepared to apical diameter #30 with standard hand K-files (K-file; Dentsply Maillefer, Tulsa, OK, USA) by the "balanced force technique" [11]. Copious irrigations were performed during the instrumentation by 5% sodium hypochlorite solution. Eventually, final irrigations were done in order to remove the smear layer (17% EDTA followed by 5% sodium hypochlorite). Then, Zakaria high-speed burs (Zakaria; Mailleffer, Ballaigues, Switzerland) were used to cut the apical 3 mm of the root apices without bevel. Three-millimeter retrograde preparations were made using diamond-coated ultrasonic retro-tips (AS3D tip; Satelec, Paris, France) [2]. Paper points were used to dry the prepared retrograde cavities. Before the retrograde filling, a standard gutta percha cone was adjusted 3 mm short of the root apex in order to control the retrograde filling depths. To avoid sealer contamination between the retrograde material and the dentin, no sealer was used.

In order to allow for analysis under CLSM, each retrograde filling material was mixed in accordance with the manufacturer's instructions and was fluorescently

labeled by adding dye (Alexa Fluor 350 dye; Life Technologies, Carlsbad, CA, USA) at an estimated ratio of 0.1% (weight) during mixing [6]. The filling was carried into the retrograde cavities by carriers (Dovgan Carriers; Quality Aspirators, Duncanville, Texas).

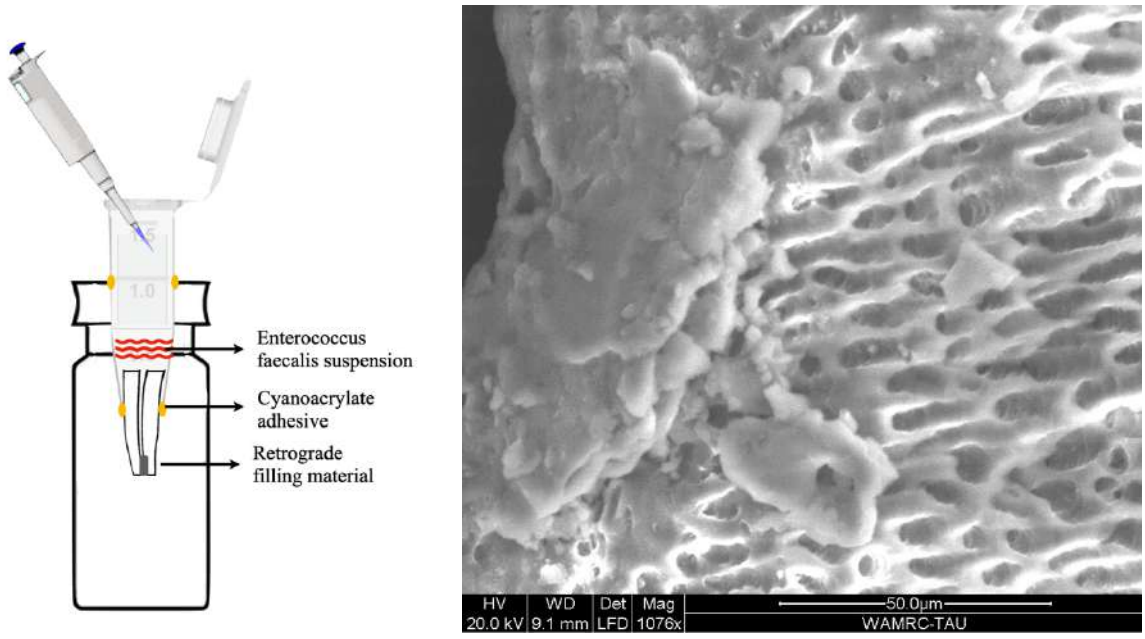
The prepared samples were then randomly allocated into eight experimental and control groups:

1. (*n* = 15): Retrograde 3 mm preparations filled with fluorescently labeled MTA (ProRoot; Dentsply Tulsa Dental, Johnson City, TN, USA).
2. (*n* = 15): Retrograde 3 mm preparations filled with fluorescently labeled IRM (IRM; Dentsply, Mannheim, Germany).
3. (*n* = 15): Retrograde 3 mm preparations filled with fluorescently labeled Biodentine (Biodentine; Septodont, Saint-Maur-des-Fossés, France).
4. (*n* = 5): Retrograde 3 mm preparations, not filled (Positive control).
5. (*n* = 5): Teeth without retrograde preparation or filling. The whole external root surface including the apical foramen was sealed with 2 layers of nail varnish (Negative control) [12,13].
6. (*n* = 5): Like Group 1 but without ensuing bacterial contamination (MTA negative control).
7. (*n* = 5): Like Group 2 but without ensuing bacterial contamination (IRM negative control).
8. (*n* = 5): Like Group 3 but without ensuing bacterial contamination (Biodentine negative control).

All specimens were then stored at 100% humidity and 37 °C for 24 h to allow for setting of the retrograde materials.

The experimental model

To avoid bacterial penetration across lateral canals or gaps in the cementum, 2 layers of nail varnish (Lilliput Nagellack; Wiesbaden, Germany) were applied to the external surfaces of the roots, without covering the prepared apical areas [13]. Then, the roots were placed in a previously described experimental model [2] (Figure 1a).



(**a**) (**b**)

Figure 1. (**a**): Illustration of the tested model. *E.faecalis=Enterococcus faecalis*. (**b**): Scanning electron microscopy (SEM) images demonstrating bacterial penetration into the dentinal tubules.

The roots were placed in 1.5 mL plastic Eppendorf tubes (disposable scintillation vials; Sigma-Aldrich, St. Louis, MO) and then placed in a glass vial (clear glass vial; Sigma-Aldrich, St. Louis, MO, USA) tightly through the rubber cap. The interfaces between the Eppendorf, the root, and the rubber cap were sealed using cyanoacrylate adhesive (Krazy Glue; Columbus, OH, USA).

Simulation of Enterococcus faecalis bacterial infection

Ethylene oxide gas was used to sterilize the roots [14]. Then, a growth medium for streptomycin-resistant T2 strain *E. faecalis* bacteria (ATCC 29212) was autoclaved. Since *E. faecalis* is resistant to streptomycin sulfate, 0.5 mg/ml streptomycin sulfate (Sigma-Aldrich; St. Louis, MO, USA) was then added to avoid possible contamination by other bacterial species [15].

The roots were then coronally filled with *E. faecalis* bacterial suspension using a pipette and incubated at 100% humidity and 37 °C. The bacterial suspension was freshly prepared and replaced every day up to 21 days [2] (Figure 1b).

Preparing the roots for the assessments

Following 21 days of incubation, the roots were fixed in a self-cure acrylic repair material (UNIFAST Trad; GC, St. Alsip, IL, USA). Then, in order to prepare samples of

root dentin axial slices for the assessments, 3 mm sections of the root ends were cut vertically to the long axis of the root, using water cooling and a 500 rpm diamond saw (Isomet; Buehler, Lake Bluff, IL, USA). [2]. In order to validate the model, one slice from each tooth was scanned in Environmental SEM (ESEM). The ESEM slices were viewed in environmental "wet" mode using a Philips XL30 ESEM-Feg (FEI/Philpips Electron Otpics, Eindhoven, The Netherlands) (working conditions: 5 °C, 2.9–5.9 torr gas pressure, 80% relative humidity, 6–9 kV). Five interesting spots on each sample were selected.

The prepared samples were stained using a Live and Dead Bacterial Viability kit (L-7012 Molecular Probes; Eugene, OR, USA) [2], which contains two distinct vials of the two component dyes (propidium iodide and SYTO 9 in a 1-to-1 mixture) for the staining of the evaluated bacteria. The excitation/emission maxima of the dyes were 480–500 nm for the SYTO 9 (staining live bacteria in green), 490–635 nm for propidium iodide (staining dead bacteria in red) [7], and 365–440 nm for the Alexa Fluor 350 (retrograde filling material stained in blue) [16].

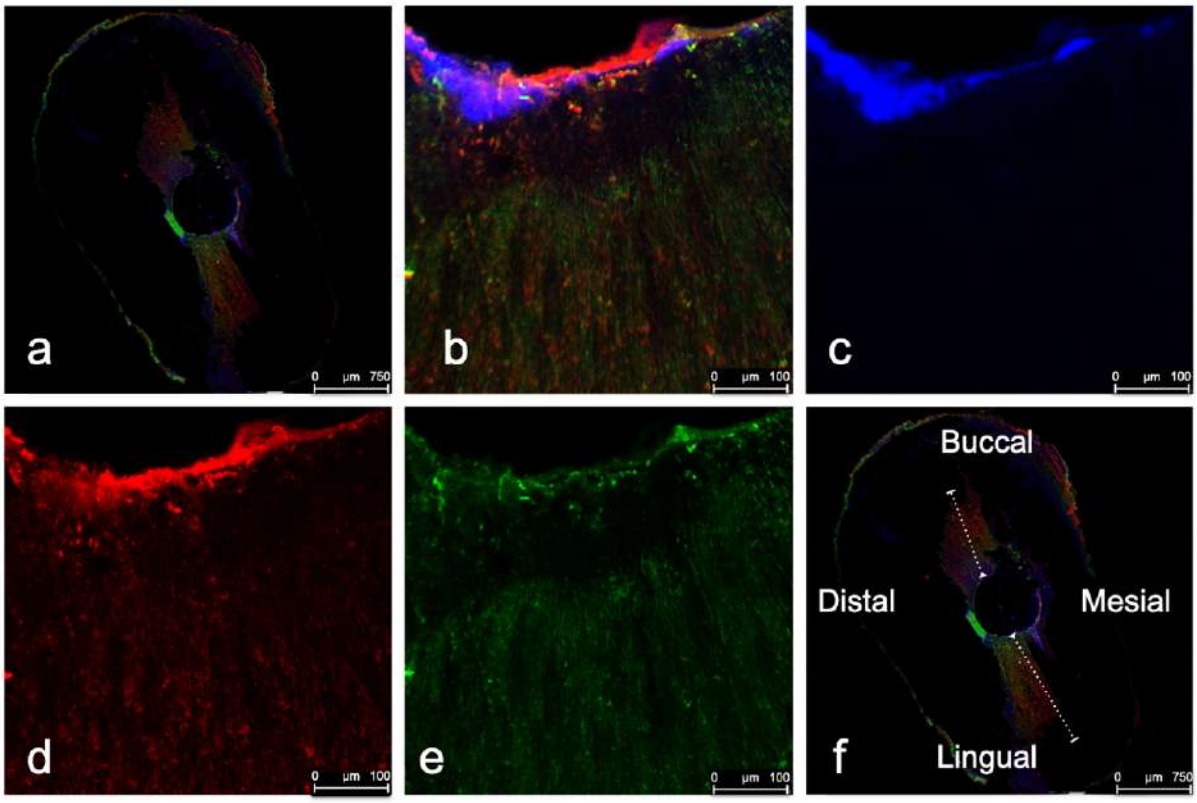
Confocal laser microscopy assessment

Following the staining of the bacteria and the filling materials, their fluorescence was assessed by CLSM (Leica TCS SP5; Leica Microsystems CMS, Wetzlar, Germany). The evaluations were performed at the mesial, distal, buccal, and lingual areas of the root dentin axial slices. The red, green, and blue fluorescence was displayed using single-channel and simultaneous triple-channel imaging [2,16], producing images of the bacteria and of the fillings at a resolution of 1024 × 1024 pixels.

The images were then evaluated using dedicated software (LAS AF, version 2.6.0.7266; Leica Microsystems CMS, Wetzlar, Germany). The slices were assessed by a × 4 lens, and the extent of fluorescence staining within the buccal, lingual, mesial, and distal areas of the slices was assessed [2,6]. The following measurements were performed to assess the invasion of the bacteria into the dental tubuli (penetration depth and viability), and to assess the influence of the retrograde filling on the bacterial invasion (Figure 2):

1. The depths of bacterial invasion and filling penetrations within the tubuli were measured at the buccal, lingual, mesial, and distal areas of the root dentin axial slices, defining the root canal wall as the beginning point (Figure 2f).

2. The bacterial viability was calculated as the proportions of live and dead bacteria.
3. The correlation between the filling material type and penetration depth, and



that between the bacterial invasion depth and viability, was determined.

Figure 2. (**a–f**): Demonstrating confocal laser scanning microscopy (CLSM) images (magnification scale; a: 0–750 µm, b–f: 0–100 µm), from the positive control group) of the *E. faecalis* bacterial invasion into the dentinal tubules. Live bacteria are presented in green (**e**), dead bacteria are presented in red (**d**), and the retrograde filling is presented in blue (**c**). The bacteria and the filling materials can be seen at high (**b–e**) and low (**a**,**f**) magnification. The minimal, maximal, and mean depths of bacterial and filling penetration within the tubuli were assessed by defining the canal wall as the beginning point (**f**).

Statistical evaluation

The results were statistically analyzed using SPSS software (SPSS version 22; SPSS Inc, Chicago, IL). One-way ANOVA tests were performed to assess the fluorescence at the buccal, lingual, mesial, and distal areas and to evaluate the level of fluorescence staining within each area (in the same group and between the different groups), the depth of bacterial penetration into the dentinal tubules, and the depth of filling penetration into the tubules. Pearson's Chi-squared test was performed to check for dependency between the bacterial viability and the filling material used. Chi-squared tests with Yates' continuity correction were performed

to evaluate the bacterial viability effect difference between the materials. $p < 0.05$ was considered statistically significant.

Results

Bacterial fluorescence was not detected in the negative control specimens, and fluorescence was detected in all the specimens of the positive control group.

The extents of the stained areas (bacteria and filling) were larger in the lingual and buccal areas compared to the distal and mesial areas. However, the differences between these areas was not statistically significant between the different retrograde filling material groups ($p = 0.094$) (Figure 2a,f).

Looking at all groups, the filling material and bacterial penetration depths within the tubuli were variable, with minimal and maximal filling penetration depths of 11 and 957 µm, respectively (mean of 130 ± 158 µm), and minimal and maximal bacterial penetration depths of 9 and 1480 µm, respectively (mean of 167 ± 317 µm) (Table 1). In addition, a negative correlation was found between the depth of filling material penetration and the bacterial penetration, where greater penetration depth of the filling material into the tubuli was associated with significantly shallower bacterial penetration depth (live and dead bacteria combined) ($p = 0.015$).

When comparing the filling and bacterial penetration depths between the filling groups, in the Biodentine group, bacteria penetrated deeper within the tubuli, while the filling penetration depths were shallower in comparison to the other materials (IRM, MTA) ($p = 0.004$) (Table 1).

Filling Material Penetration				**Bacterial Penetration**				
SD	median	max	Min	SD	median	max	Min	**Group**
198	183	644	58	158	146	500	33	MTA
158	311	957	11	342	129	996	9	IRM
155	93[a]	535[a]	32	451	663[b]	1480	160	Biodentine

-	-	-	-	189	1200[c]	1610	890[c]	Control

Table 1. Presents the penetration depths (in μm) of the retrograde filling materials and bacteria within the tubuli. The minimal and maximal bacterial penetration depths into the dentinal tubules were 1 and 1480 μm, respectively, with a mean of 167 μm. The minimal and maximal filling penetration depths into the dentinal tubules were 0 and 957 μm, respectively, with a mean of 130 μm. In the Biodentine group, in comparison to the other materials (mineral trioxide aggregate (MTA), intermediate restorative material (IRM)), bacteria penetrated deeper into the dentin (b: one-way ANOVA, $p = 0.0021$), while the filling depth was lower (a: one-way ANOVA, $p = 0.04$). In the control group, bacteria penetrated deeper into the dentin in comparison to other groups (c: one-way ANOVA, $p = 0.00018$).

Generally, more dead bacteria were detected compared to live bacteria in all the experimental groups ($p = 0.003$), without significant differences between the filling materials ($p = 0.087$) (Figure 3).

However, a significant negative correlation was found between the depth of filling material penetration and the penetration depth of live bacteria, where deeper filling penetration was associated with shallower penetration depths of live bacteria ($p = 0.024$) (Figures 4 and 5).

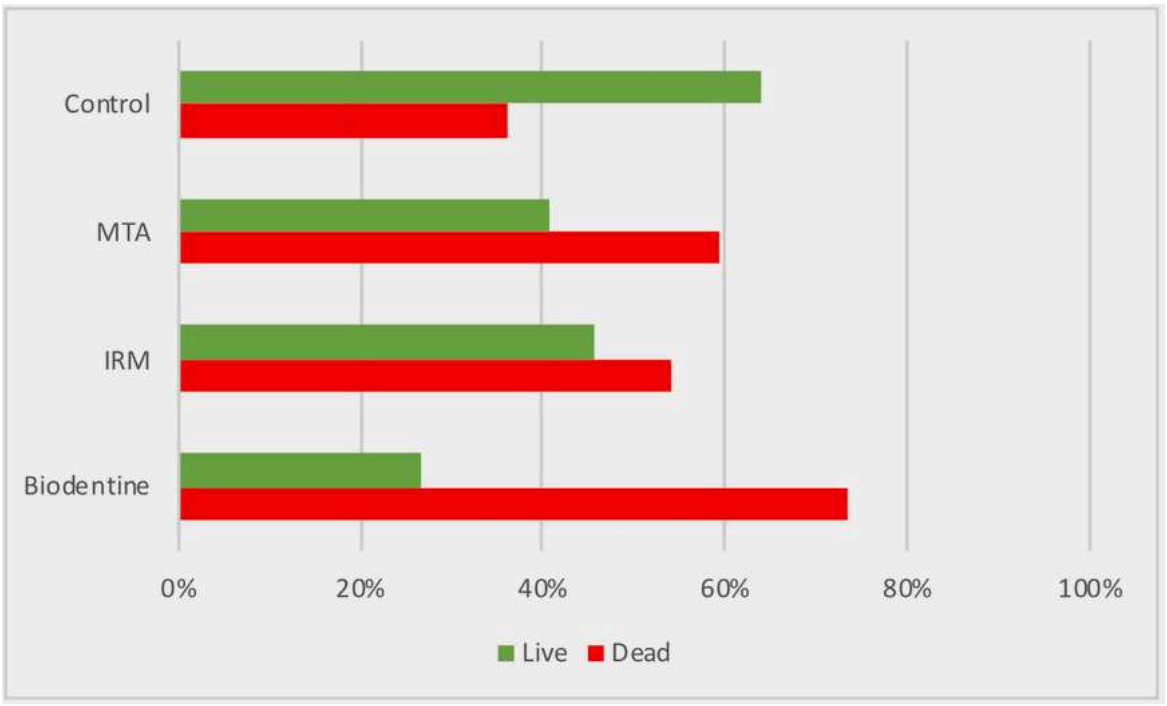


Figure 3. Average percentages of detected dead bacteria (red) and live bacteria (green). There were significantly more dead bacteria than live bacteria in all the experimental groups (Chi-squared, $p = 0.003$).

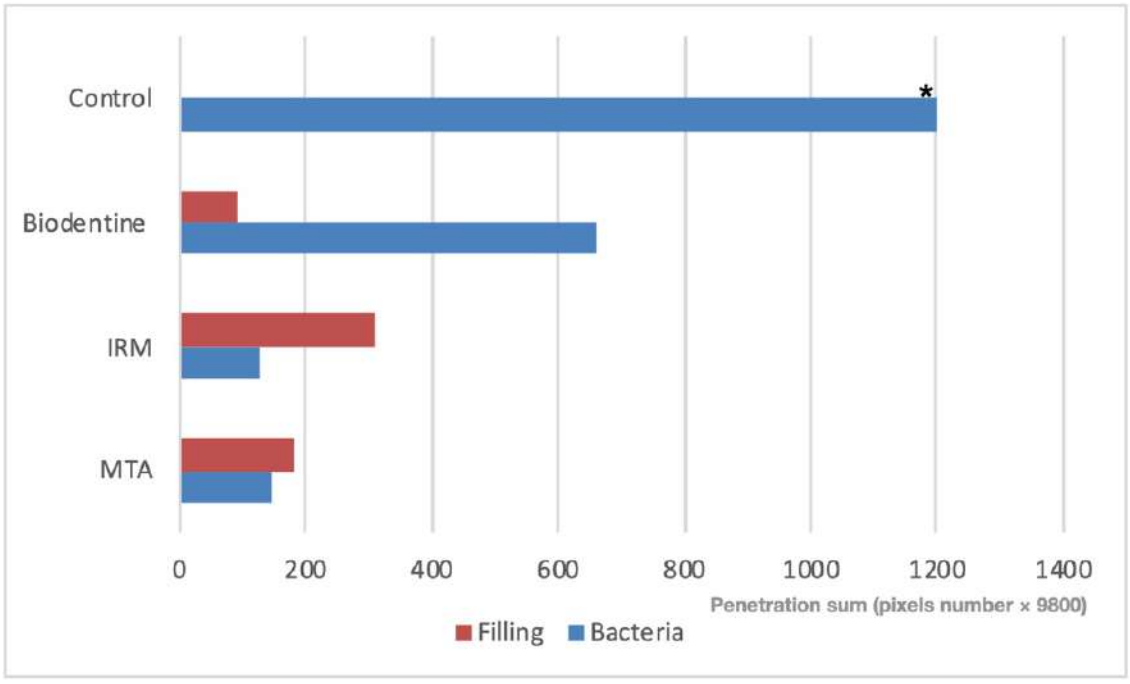


Figure 4. Fluorescence penetration amounts for the different groups (presented as the number of pixels × 9800) (one-way ANOVA).

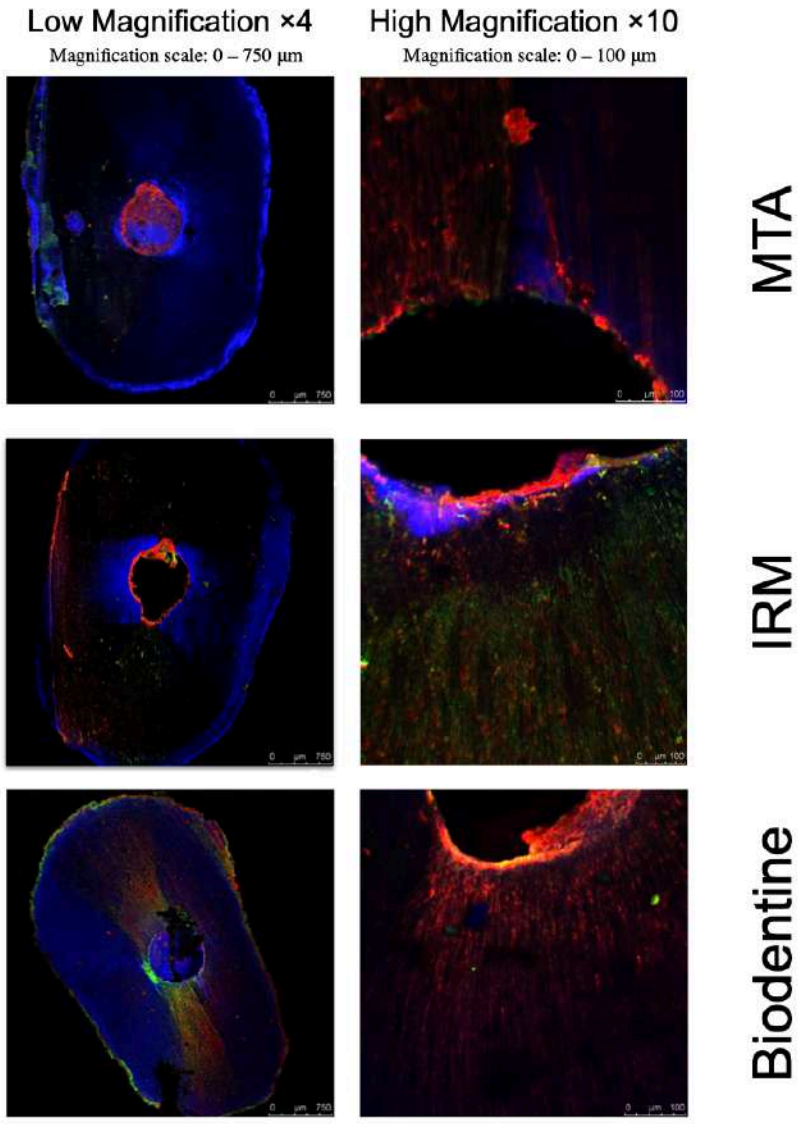


Figure 5. CLSM images presenting the detected fluorescence of the different experimental groups following 21 days of bacterial contamination. Dead (red) and live (green) bacteria within the tubuli, and the filling material (blue) are demonstrated.

Discussion

Following endodontic surgery, bacteria may still invade a retrofilled root canal and deep into the dentinal tubules [1,17], potentially leading to an inflammatory reaction when bacterial by-products invade the surrounding periradicular tissues [18]. These bacteria form biofilms, which are complex ecological communities that use diverse mechanisms to secure themselves against a harsh environment, including the immune system and antibacterial agents [19].

Gram-positive facultative anaerobes are the most commonly detected bacteria in endodontically treated teeth presenting with persistent endodontic infections [2]. Among them, *E. faecalis* plays a major role in bacterial biofilm invasion and is considered a suitable model for assessing root canal bacterial invasion [2,8].

Many microscopic, microbiological, and histological experimental models have been used to assess bacterial invasion within endodontically treated root canals [20,21]. The most common traditional experimental model is the two-chamber bacterial leakage model, which is used to assess the diffusion of bacteria from an upper chamber and over the retrograde filling to a suspension placed in the lower chamber. However, it has been reported that this traditional model suffers from many inherent limitations, such as a lack of proper control groups and histological evaluation, and that its reliability is compromised [22,23,24].

Additional *ex vivo* models to simulate and assess bacterial invasion, such as the traditional dye-penetration model [25] or the high-pressure replica technique used to assess the pore morphologies and apical leakage of sealers in retrofilled teeth [26], are restricted because in these traditional models indirect evaluations are used, incapable of demonstrating the real routes of bacterial invasion, and because of the absence of proper histological controls in these models [22]. Furthermore, under the adverse conditions of a filled root canal, bacteria can increase their stress tolerance by entering a dormant state where they are viable but not culturable [5,27], thus challenging the efficacy of traditional culture-based studies [5,19,27].

The authors of a recent study established a novel and reliable experimental model that enabled them to trace and quantify biofilm invasion into the dentinal tubules [2]. With this CLSM-based model, the actual routes of bacterial invasion are tracked histologically, and negative and positive histological controls are used to verify the suitability of the experimental model. Furthermore, the true viability of the proliferating bacteria, even when they enter a dormant, not-culturable state, can be assessed [5,19,27]. As a result, using CLSM together with live/dead bacterial staining techniques offers information regarding both the magnitude of the dentin infection and the vitality of the proliferating bacteria within the infected tubuli in situ [2,8], thus enabling a better understanding of the microbiological–pathological course after endodontic surgical procedures [2].

In the current study, we used this CLSM-based experimental model to assess the invasion of bacterial biofilms into the dentinal tubules. Furthermore, bacterial invasion was assessed in the presence of fluorescently labeled retrograde filling materials. The use of CLSM to assess the penetration of the retrograde filling materials into the tubuli [6,28], while assessing the depth of the bacterial invasion into the

tubuli and their viability, provides a comprehensive view of the microbial–pathological process following surgery. Bacterial fluorescence was not detected in the negative control group, while fluorescence was detected in the positive control group specimens, confirming the reliability of the experimental model.

The rationale of a retrograde filling material is to inhibit bacterial biofilm growth and the outflow of bacterial byproducts and toxins into the surrounding periradicular tissues [29]. In addition, the root-end filling is expected to entomb any remaining bacteria beneath the filling in a way that would eventually result in bacterial death [30]. However, studies found bacterial biofilm at the interfaces between the filling and the canals' dentin walls, and also deep in the dentinal tubules [7,15]. In order to provide a comprehensive view and analysis of the relationships between the proliferating biofilm and the filling material, in the current study, different filling materials were fluorescently labeled in order to evaluate and understand the influence of the filling materials and their penetration depths into the tubuli on bacterial invasion within the tubuli.

The same as in a previous study [2], in the current study, we found a pattern of bacterial invasion and filling penetration in which the bucco-lingual direction was preferred compared to the mesio-distal direction, regardless of the type of filling material. This finding was also supported by previous investigations [2,23,24,31]. The fact that both the fillings and the bacteria penetrated deeper in the bucco-lingual direction may be associated to an anatomical–physiological phenomenon, "the butterfly effect", which means a butterfly-like appearance observed on the cross sections of roots that are associated with higher sclerosis down the tubuli at the distal and mesial sides of the root canal [24]. This phenomenon is commonly detected in single-rooted human teeth in a broad range of ages [32,33]. Similarly, in a study by Rechenberg et al. [24], histological observations revealed that the penetration of microorganisms might predominantly happen through tubular aspects of the dentin, whereas atubular or sclerotic dentin and the interfaces between dentin and sealer remained bacteria-tight [24]. Thus, it seems that while a two-chamber model is not suitable for the evaluation of bacterial leakage, it reliably reflects the invasion of bacteria into the tubuli [2,23,24].

The bacterial invasion depths into the dentinal tubules were variable, reaching as deep as 1480 µm (with a mean of 167 µm), which corroborates the findings of a

previous study [2]. Peters et al. [34] evaluated the viable endodontic bacteria in the root dentin of infected teeth presenting with apical periodontitis using culturing methods. They found that in the majority of the roots, bacteria were identified deep within the dentin near the cementum. They also found that an anaerobic culturing method is more sensitive than histology in identifying these bacteria in the dentin. Within that scope, confocal microscopy seems to be a promising technique to assess bacterial penetration within the tubuli because it enables us to evaluate live and dead bacteria, as well as bacteria in dormant, not-culturable states [5,19,27]. Thus, CLSM is capable of assessing the actual level of bacterial invasion within the tubuli in situ [2].

In the current study, bacterial penetration into the dentinal tubules was significantly influenced by the type of filling material and its penetration depth into the dentinal tubules. For all materials, it was found that deeper filling penetration within the tubuli was significantly related with shallower bacterial penetration. This finding confirms that an appropriate retrograde filling material may inhibit bacterial penetration across the dentin into the periradicular tissues of teeth following root end resection and filling [35]. The fact that the Biodentine fillings penetrated less, and their associated bacterial penetration depths were deeper compared to the MTA and IRM groups ($p < 0.05$), may suggest that the type of root end filling material and its ability to penetrate into the tubuli may play a significant part in the risk of bacterial invasion following endodontic surgery (Figure 4).

Biodentine is a material based on calcium silicate that has several applications, including as a root-end filling material used in endodontic surgical procedures [36]. The material is prepared by the silicate-based cement technique with some adjustments aimed to improve its physical properties and handling. It had been claimed that Biodentine has a fast setting time due to its increased particle size, the addition of calcium chloride to its liquid constituent, and its decreased liquid content [35,37]. However, its bigger particle size may also explain its shallower penetration depth into the dentinal tubules compared with the other materials and its lesser ability to prevent bacterial invasion deep into the dentinal tubules as compared to the other evaluated materials.

Generally, in the presence of a filling material, significantly more dead bacteria than live bacteria were detected within the dental tubules ($p = 0.003$, Figure 3),

without significant differences between the filling materials ($p = 0.087$). Nevertheless, deeper filling penetration was associated with shallower penetration depths of live bacteria ($p = 0.015$) (Figure 4). The fact that in the present investigation the viability of the proliferating bacteria was influenced by the filling material penetration depth, rather than by the filling material type, may seemingly contradict the results of other studies that focused on the direct antimicrobial properties of different retrograde filling materials by assessing their spectra of affected bacteria, effectiveness periods, and antimicrobial mechanisms [37–41]. Torabinejad et al. [38] reported that MTA has no effect on any of the strict anaerobic bacteria and some antibacterial effect on several of the facultative bacteria, whereas other materials such as IRM possess antibacterial properties on both groups of bacteria [38]. Additional studies have reported that immediately after its setting, IRM has antibacterial effects on *E. faecalis* and sustains these capabilities for at least one day [39], while others [40] reported identical effects in root-end fillings. Others claimed that Biodentine possesses antibacterial capabilities because of its relatively high pH that has an inhibitory effect on bacteria, and because of alkalization of the ecosystem that leads to the decontamination of neighboring soft and hard tissues [37,41].

However, in addition to the direct antibacterial effect of the retrograde filling material, its capability to penetrate and to entomb the bacteria is responsible for the prevention of bacterial invasion [1]. Our findings suggest that the antibacterial properties of the retrograde filling material are related to its penetration capacity deep into the tubuli.

In the current study we used an *ex vivo* model to simulate an *in vivo* clinical scenario. It should be noted that the experimental methods used to assess biofilm formation do not accurately simulate true *in vivo* conditions; therefore, the methods' ability to provide clinically relevant information is limited. However, in the current study, we used representative surfaces to grow the biofilm—*ex vivo* dentin tissue samples—which are more likely to provide information that is relevant to true *in vivo* endodontic infections [42].

5. Conclusions

Given the limitations of this *ex vivo* model, this study enables better understanding of the microbiological–pathological course after endodontic

surgical procedures. It was found that even with retrograde fillings, bacteria invade deep into the dental tubules (up to 1480 µm). This invasion is variable and is affected by the root tubular anatomy and by the root-end filling material and its actual penetration depth into the dentinal tubules. The ability of a filling material to penetrate deep into the dental tubules is associated with lesser penetration of the bacteria and reduced viability of the invading bacteria. Additional clinical studies are indicated to elucidate the clinical implications of bacterial invasion into retrofilled root ends following endodontic surgery. In addition, clinical and experimental assessments of retrograde filling materials should take into consideration not only the direct antimicrobial effect of the filling material but also its chemical and physical properties that affect its penetration capacity deep into the tubuli.

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4

Bacterial Colonization and Proliferation in Furcal Perforation Repaired by Different Materials

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Abstract

Background: Following furcal perforations, bacteria may colonize the defect and cause inflammation and periodontal destruction. This study evaluates, using confocal laser scanning microscopy (CLSM), *E.Faecalis* colonization and proliferation in furcal perforations repaired with different materials. (2) Methods: Furcal perforations were created in 55 extracted human mandibular molars and repaired using either MTA-Angelus, Endocem or Biodentine. The specimens were coronally subjected to *E.Faecalis* suspension for 21 days. The specimens were then stained using LIVE/DEAD Viability Kit and evaluated using CLSM. (3) Results: No significant difference was obtained in the size of the stained areas between the different materials (P=0.083). However, there were significantly more dead bacteria than live bacteria at the circumference of the perforation defect (P=0.0041). There was significantly higher live:dead bacteira ratio in the MTA Angelus group (P=0.001). The minimum and maximum penetration depths of bacteria into the dentinal tubules were 159 and 1790 µm, respectively, with a mean of 713 µm. There was significantly more live bacteria than dead bacteria inside the dentinal tubules (P=0.0023) in all groups. (4) Conclusions: Following perforation repair using different materials, bacteria may colonize the interface between the repair material and dentin, and penetrate through the dentinal tubules. The type of repair material may affect the vitality of the colonized bacteria

Keywords: Bacterial colonization; Confocal laser scanning microscopy; *Enterococcus faecalis*; Perforation; Repair materials

Introduction

Perforation can be defined as an artificial communication between root canal space and the surrounding tissues of tooth (periodontium) or oral environment [1]. Perforations can be pathological: caused by root resorption or caries; or iatrogenic: as a result of dental procedures during access cavity preparation, canal negotiation or post space preparation [1, 2] . Various factors may affect the treatment outcome, such as time elapsed to the perforation repair, location of the perforation, its size, repair material and the experience of the operator [1, 3–5].

The main goal of perforations management is sealing the defect in order to prevent bacterial contamination, inflammation and loss of periodontal attachment, and creating an ideal environment for tissue repair. Furthermore, the sealing ability and marginal adaptation of the repair material used is also crucial to prevent leakage of irritants, and thus to enhance the chance of success[6-8]. Numerous materials have been introduced for furcal and root perforation repair, such as MTA, Amalgam, glass ionomer cement, Intermediate Restorative Material and tricalcium phosphate [4,7–11]. Use of biocompatible materials in perforation repair can be associated with reduced inflammatory response in the surrounding tissues [3,9].

The ability of the different restorative materials to repair the perforation defect has been assessed by different in-vitro experimental settings, including bacterial leakage model [12], radioisotopes [13], dye penetration [11] and fluid filtration method [14]. However, the reliability of those techniques has been questioned due to the fact that the leakage may occur not only through the interface between the material and the dentine wall, but also through additional potential routes when the appropriate negative controls are not included. Additionally, they do not give information about the ability of the bacteria to penetrate into the dentin. In this field, histological sections allow to assess the presence and the distribution of bacteria in the dentin tubules, but fail to determine the vitality of the bacteria.

Use of alternative microscopic techniques are necessary to analyze bacterial leakage and penetration depth into the dentinal tubules. Confocal laser scanning microscopy (CLSM) has been considered as an alternative microscopic technique

providing quantitative and clinically relevant data about the presence or absence and extension of bacteria colonized through dentinal tubules along with root canal walls circumferencially[15–17]. Thus, CLSM can be preferred for the assessment of the bacterial colonization in the perforation defects repaired with the various materials in order to overcome the mentioned limitations of the conventional in-vitro experimental settings [18]

We conducted this study to assess colonization and proliferation of *Enterococcus faecalis* in furcal perforations, repaired with MTA Angelus, Endocem and Biodentine, by using confocal laser scanning microscopy. The null hypothesis was that bacterial penetration does not depend on the type of repair material.

Materials and Methods

Preparation of specimens

The study approval was obtained through Ethics Committee of Tel-Aviv University (No: 230.17, January 23, 2018) and all methods were carried out in accordance with the relevant regulations and guidelines.

Fifty-five freshly extracted human permanent molars for periodontal reasons were used in this study. Teeth with previous endodontic treatment, visible sign of root resorption, caries, root fractures, or immature apices were excluded from the study. Teeth with fused roots or with a single root were excluded as well. Once the teeth were cleaned of debris and soft tissue remnants and then the teeth were kept in phosphate-buffered saline until use.

The teeth were decoronated to expose the pulp chamber cavities, and 5 mm of the apical segment of the roots were amputated using high speed diamond disc (IPR Diamond Disc; Dentsply Int./Maillefer, Ballaigues, Switzerland) under water cooling. Pulpal remnants were removed from pulp chamber and root canals using Barbed Broache (Barbed Broache; Dentsply Int./Maillefer, Ballaigues, Switzerland). The canal orifices and apical foramen of each root were filled with resin composite (TE; Ivoclar Vivadent AG, Schaan, Liechtenstein, German). The perforations were created in the center of the pulpal floor using a round #14 diamond bur under

optical microscope (OPMI pico Dental Surgical Microscope, Carl Zeiss Meditec, Dublin, CA, United States) at 6× magnification [19].

The specimens were randomly divided into the following experimental groups:

Group 1 (n = 10): The perforation defects were repaired using MTA Angelus (Angelus, Londrina, PR, Brazil), mixed according to the manufacturer's recommendations: a metal spatula was used, on sterilized glass slab, to mix 1:1 ratio of MTA Angelus with distilled water. The mixture was homogeneous and had a similar consistency to wet sand.

Group 2 (n = 10): The perforation defects were repaired using Endocem MTA (Maruchi, Wonju, Korea) cement, mixed according to the manufacturer's recommendations: a metal spatula was used, on sterilized glass slab, to mix 300mg Endocem MTA powder to 0.12cc liquid ratio.

Group 3 (n = 10): The perforation defects were repaired using Biodentine (Septodont, Saint-Maur-des-Fossés, France), mixed according to the manufacturer's recommendations. First closed capsule gently tapped on a hard surface to dispense the powder. Five drops of liquid was added into the capsule and then it was subjected to a triturator for 30 seconds.

Group 4 (n = 5) (positive control): The created perforations were left without repair material.

Group 5 (n = 5) (negative control): The teeth were left without perforation and repair material, and two layers of nail varnish covered the external tooth surface

Group 6 (n = 5) (negative control MTA Angelus): same as group 1 without bacterial contamination.

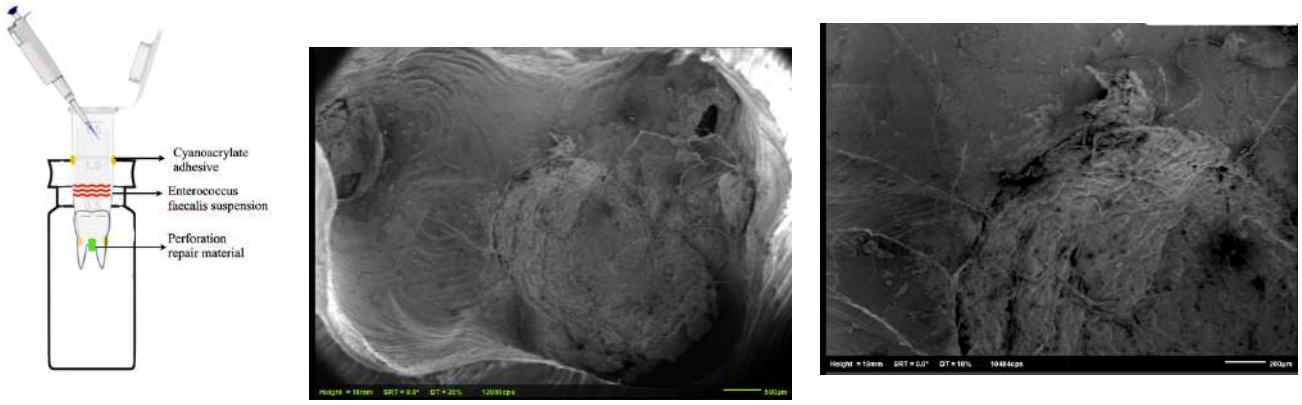
Group 7 (n = 5) (negative control Endocem MTA): same as group 2 without bacterial contamination.

Group 8 (n = 5) (negative control Biodentine): same as group 3 without bacterial contamination.

All teeth were left at 37°C and 100% humidity for 24 hours.

The experimental setting:

To avoid any bacterial leakage through accessory canals or other discontinuities in the cementum, two layers of nail varnish were covered the external surfaces of all teeth, except for the perforation site. According to a model described previously, all specimens were placed in the glass vials (Sigma-Aldrich, St. Louis, MO, USA) through the rubber cap. Cyanoacrylate adhesive was used to seal the interfaces between the specimens and the rubber cap (Krazy Glue; Krazy Glue, Columbus, OH, USA) (Figure 1a) [20,21].



(a) (b) (c)

Figure 1. (a): Illustration of the experimental model. (b-c): SEM images displaying bacterial penetration in the perforation site

Simulation of E. faecalis contamination:

The specimens were sterilized overnight with ethylene oxide gas. A growth medium for *E. faecalis* (ATCC® 29212™) was prepared and then autoclaved. 0.5 mg\ml Streptomicin sulfate (Streptomicin sulfate; Sigma-Aldrich, St. Louis, MO, USA) was mixed to the suspension to prevent contamination by additional bacterial species. Freshly prepared bacterial suspension was added to coronal part of the each specimen pulp chamber and incubated at 37◦C and 100% humidity and replaced every 24 h for a total of 21 days.

Confocal laser scanning microscopy analysis:

The specimens were embedded in a self-cure acrylic (Unifast Trad, Alsip, IL) after incubation period. The specimens were cut through the perforation site containing the repair materials, perpendicularly to the long axis of the root, using a diamond

saw at 500 rpm (Isomet; Buehler, Lake Bluff, IL, USA) under continuous water irrigation. The specimens were stained using LIVE/DEAD BacLight Bacterial Viability kit L-7012 (LIVE/DEAD BacLight Bacterial Viability Kit *for microscopy and quantitative assays; Molecular Probes, Eugene, OR, USA), including separate vials of the two component dyes (SYTO 9 and propidium iodide in 1:1 mixture). The excitation/emission maxima for these dyes were 480-500 nm for the SYTO 9 stain (live bacteria stained in green) and 490-635 nm for propidium iodide (dead bacteria stained in red) [22]. Environmental SEM (ESEM) was acquired in environmental "wet" mode by using Philips XL30 ESEM-Feg (FEI/Philpips Electron Otpics, Eindhoven, The Netherlands) (operating conditions: 5°C, 2.9-5.9 torr gas pressure, 80% relative humidity, 6-9 kV) to scan one slice from each tooth in order to validate the bacterial leakage model. Five interesting spots on each specimen were chosen(Figure 1b-c).

Following the staining the specimens, fluorescence from the stained areas were observed immediately under CLSM (Leica TCS SP5; Leica Microsystems CMS, Germany). Simultaneous and single channel imaging were utilized to show green and red fluorescence.

The CLSM images of materials and dentinal tubules were taken at a resolution of 1024×1024 pixels. All extensions of contaminated dentinal tubules of the specimens were analyzed using the software (LAS AF software, version 2.6.0.7266; Leica Microsystems CMS, Germany) [18,23,24]. First, the size of fluorescent staining area in the related region and then, penetration of bacteria into the dentinal tubules considering the canal wall as the starting point were measured. The ratio of green and red stained areas was interpreted as the vitality of the bacteria and it was evaluated whether the material used had an effect on this ratio.

Statistical analysis

Mead's resource equation was used in order to estimate the sample size. One way ANOVA was performed in order to assess the size of fluorescent stained areas and the penetration depth of bacteria into the dentinal tubules with the various perforation repair materials. Pearson's Chi-squared test was also used to analyze dependency between bacteria being alive or dead and the material used for perforation repair. The significance level was set at $p<0.05$. SPSS program (IBM Corp., 2011, Version 21.0. Armonk, NY: USA) was used for the statistical analysis.

Results

No fluorescence was detected in the negative control groups (groups 5-8), while positive control group was showed fluorescence (group 4). There were no significant differences (P=0.15) between the stained areas in buccal, lingual, mesial and distal directions, for all groups (Figure 2). When evaluating the stained areas (buccal, lingual, mesial and distal), it was found that there were no significant differences between the repair materials (both in total and in live:dead ratio) (P=0.083).

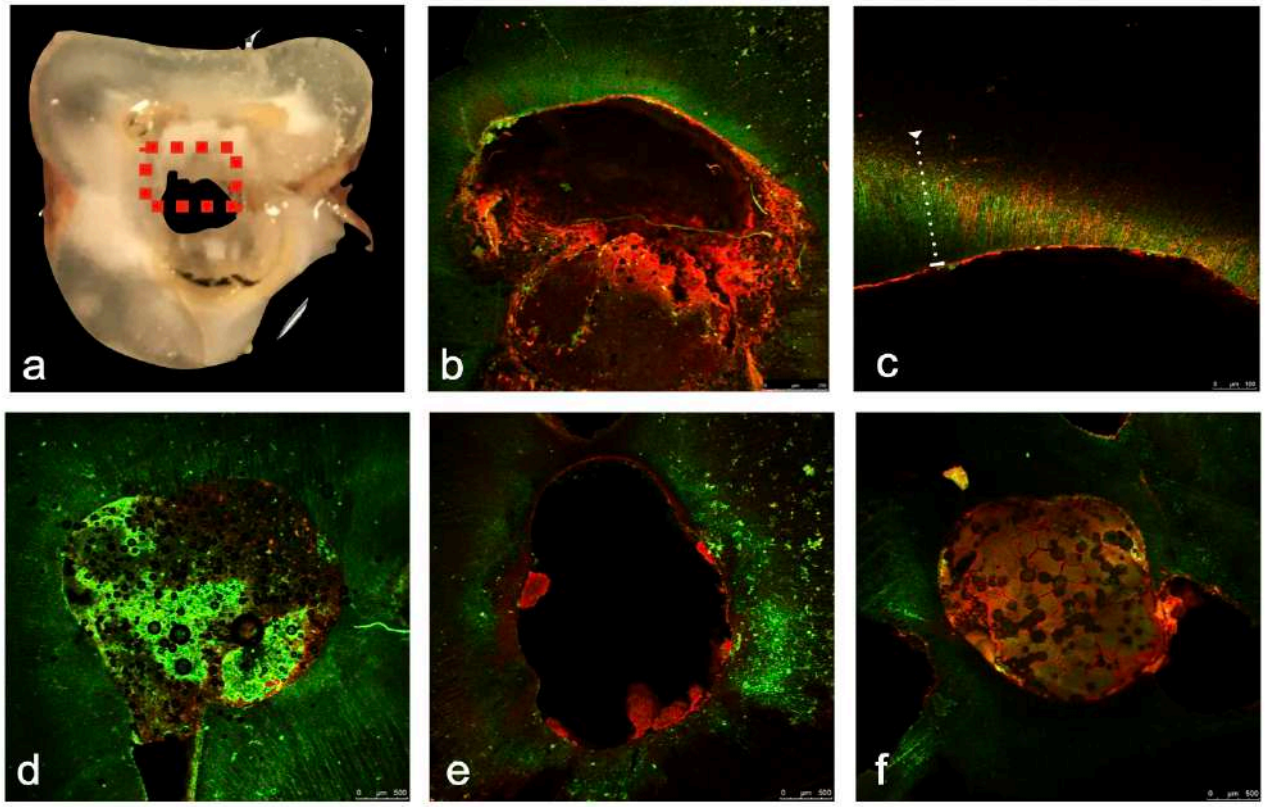


Figure 2 (a-f). CLSM images. The specimens were cut through the perforation site containing the repair materials perpendicularly to the long axis of the root in order to be evaluated by the CLSM (a). Prior to scanning, LIVE/DEAD Kit stained the infected dentin. Vital (green) and dead (red) bacteria inside the dentinal tubules in the evaluated areas are clearly visible (b-f). Positive control group showed fluorescence (b,c), MTA Angelus group (d), MTA CEM group (e) and Biodentine group (f). Significantly more dead bacteria than live bacteria was detected on the dentinal surface at the circumference of the perforation defect (d-f) and significantly more live bacteria than dead bacteria at the depth of the dentin inside the dentinal tubules (b-f) in all groups.

There was significantly more dead bacteria than live bacteria on the dentinal surface at the circumference of the perforation defect (P=0.0041) and significantly more live bacteria than dead bacteria at the depth of the dentin inside the dentinal tubules for all directions (P=0.0023) in all groups. (Figure 3). There was significantly higher live:dead bacteria ratio in the MTA Angelus group when compared to other groups (P=0.001).

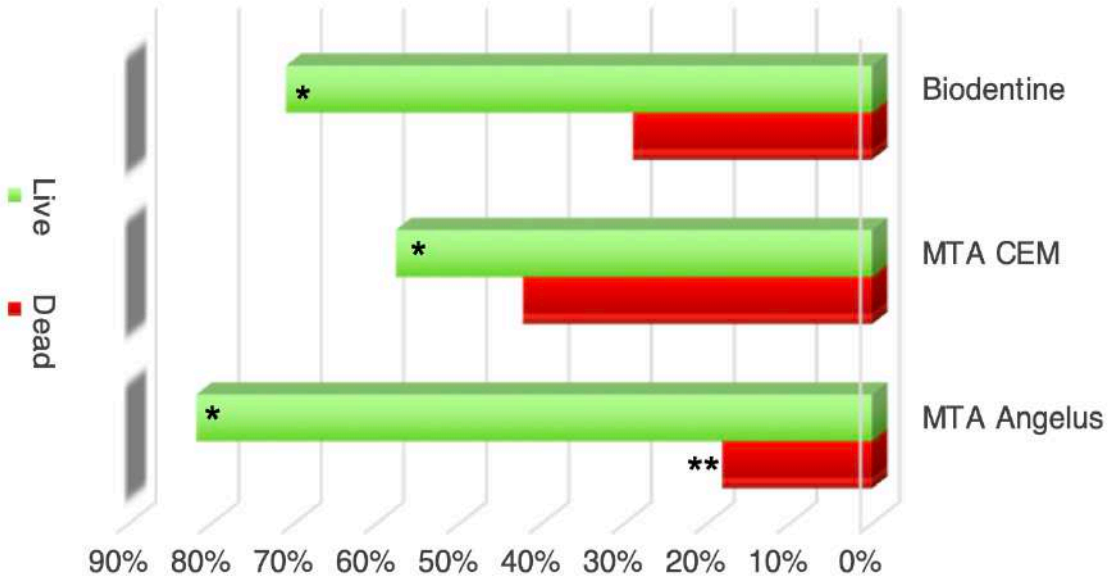


Figure 3: The average percentages of live (green) and dead bacteria (red).

No significant differences were observed between the tested repair materials in terms of bacterial penetration depth through the dentinal tubules (P=0.277). The minimum and maximum penetration depths were 159 and 1790 µm, respectively, with a mean of 713 µm (Table 1).

Table 1. Bacterial Penetration depth (in um) in the different groups

	Min	Max	Median	Stdv	P-value
MTA Angelus	159	1790	987	455	P=0.092
MTA CEM	211	782	832	176	P=0.229
Biodentine	224	1641	720	289	P=0.079

Discussion

Understanding the sealing ability of materials used in the repair of perforations and predicting the amount and direction in the presence of bacterial leakage have paramount importance in revealing the pathologies related to perforations. [25,26]. CLSM could be considered as a useful modality of choice such as traditionally standard electron microscopy along with PCR-based techniques to identify viable bacteria in dentinal tubules [16,17,24,27]. Additionally, using CLSM with the live/dead staining method allows to assess dentinal tubules in terms of extent of contamination and vitality of bacteria in those tubules [16,17,24]. To our knowledge, there is no study that assessed the bacterial colonization in perforation sites of extracted human molar teeth repaired with three different materials using CLSM. In the current study, unlike previous bacterial leakage models, the actual routes of bacterial colonization were showed histologically. Positive and four negative histological controls were used to confirm the validity of the experimental model.

After perforation occurs, the success of the treatment can associated with a well-placed repair material that should prevent communication between the root canal space, peri-radicular tissues and oral environment[28–32]. MTA is a hydrophilic calcium-silicate based material and compatible with moist conditions such as perforation sites [33]. It has various favorable properties like biocompatibility, non-cytotoxicity, radiopacity, availability and tissue regeneration [8,34–37] and it can also induce cementogenesis and osteogenesis [11,12]. However, there are several drawbacks such as long setting time, difficulties in manipulation and tooth discoloration potential [38–40]. Recently, novel calcium-silicate based materials have been introduced aiming to overcome those MTA shortcomings [41,42].

Biodentine is widely used bioactive material consist of tricalcium silicate, calcium carbonate, zirconium oxide and calcium chloride. Biodentine has been reported to not only have improved sealing ability, short setting time [42,43], but also show bioactivity, and biomineralization properties [44-48].

Another recently introduced fast-setting calcium silicate based cement is Endocem MTA which composed of calcium oxide, silicate oxide, aluminum oxide, bismuth trioxide and fine particles of pozzolan. Its fast-setting property depends on presence of pozzolan which is a siliceous and/or aluminous material. Endocem MTA

exhibited acceptable biocompatibility, reasonable mineralization effect and less discoloration potential when compared to traditional MTA [41].

Alkaline pH is not suitable for most bacteria to survive. Growth of Enterococcus faecalis could be suppressed at pH of 10.5–11.0 whereas no bacteria could survive at pH higher than 11.5 [47]. Calcium-silicate based materials forms silicate gel at their surface when mixed with water. Calcium hydroxide in silicate gel releases hydroxyl ions into the environment resulting in increased pH [48, 49]. Antibacterial effect of calcium-silicate based materials has generally been related to their high pH levels, although this assumption is not totally approved in clinical situations. In the present study, higher dead bacteria was detected compared to live bacteria on the inner dentinal surface of the perforation defect. However, more live bacteria was detected compared to dead bacteria at the depth of the dentin inside the dentinal tubules. This can be attributed to the decrease of pH through dentine tubules from the perforation defect outwards - due to the buffering capacity of dentine.

Up to now, no study has compared colonization depth *of E. faecalis* when using MTA-Ang, Biodentine and Endocem as perforation repair materials. Although no significant differences were found among tested materials in terms of stained areas, lower values were calculated for Endocem group. All tested materials in the present study were calcium-slicate based. Unlike MTA-Angelus and Biodentine, Endocem contains fluoride *E. faecalis* [50]. Similar to our results, a previous study by Tsesis et al (2018) compared bacterial colonization depth of different materials for retrograde filling, found that MTA-Angelus and Biodentine showed similar performance [18]. In the present study, the vitality of the colonized bacteria was affected by the type of repair material. Significantly more live bacteria were detected in the MTA Angelus group when compared to the Endocem and Biodentine groups. On the contrary, a previous study by Jardine et al (2019) evaluated the viability of multispecies microcosm in vicinity of bioceramic cements using a confocal laser microscopy. They found that MTA-Angelus was found to be as effective as Biodentine in terms of antimicrobial activity [51]. Use of different microorganisms may explain the different findings. A limitation of this study is the use of an in vitro model that does not mimic

the exact clinical situation but resembles one. Further *in vivo* studies are needed to assess the antibacterial activity of the various calcium slicate-based materials.

Conclusions

Considering the limitations of an *ex-vivo* setting, the present study demonstrated that bacteria may colonize at the interface between the repair material and the dentin walls, and may penetrate into the dentinal tubules. The type of repair material may affect the vitality of the colonized bacteria.

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5

The Effect of Pulpotomy Base Material on Bacterial Penetration and Proliferation for Pulpotomized Primary Molar Teeth

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Abstract

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). Materials and Methods: 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), withought 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

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* colonization in the pulp chamber in pulpotomized extracted human teeth, filled by different PBMs, using CLSM.

Materials and methods

Teeth selection

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.

Teeth preparation

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.

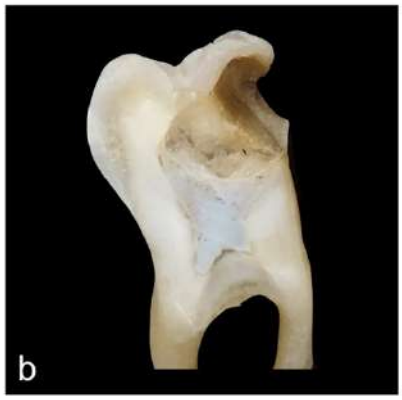

Figure 1(a,b): A mesio-distal cut was performed through the pulp chamber, pulpotomy base material and furcation area of each specimen in order to be evaluated by the Confocal laser scanning microscopy (CLSM). Prior to scanning, the cuts were stained with LIVE/DEAD BacLight Bacterial Viability Kit.

The 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 E. faecalis bacterial infection:

A growth medium for Streptomycin-resistant T2-strain *E. faecalis* bacteria (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).

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 ($p>0.05$).

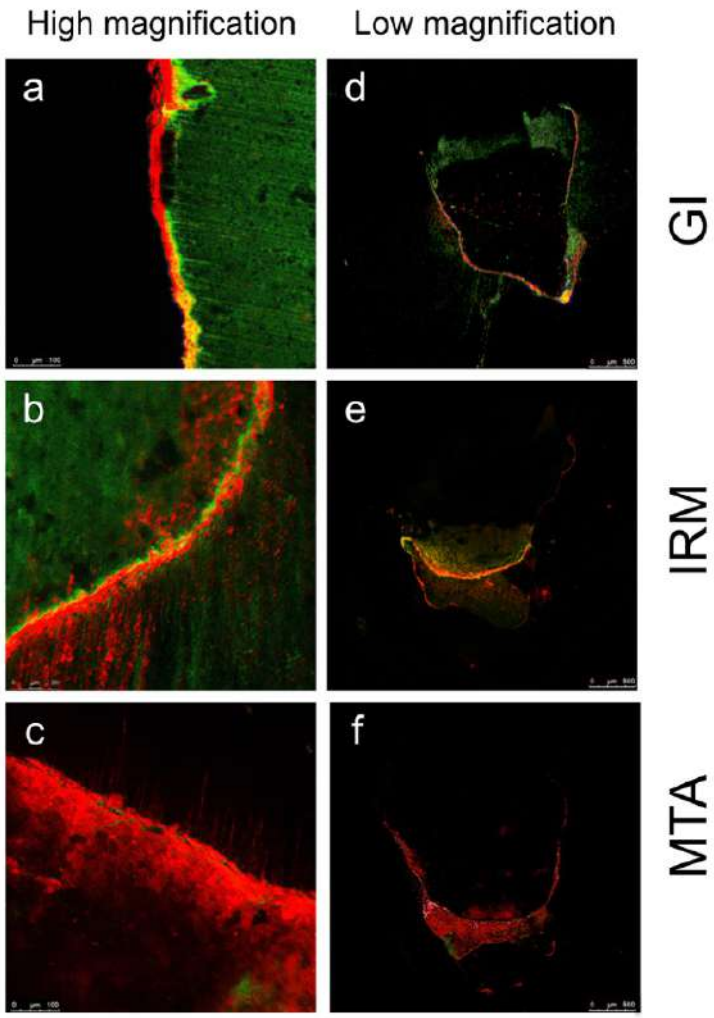

Figure 2 (a-f): Confocal laser scanning microscopy (CLSM) images of the bacterial colonization of the dentin. The infected dentin was stained with LIVE/DEAD BacLight Bacterial Viability Kit and analyzed by the LAS AF software. Vital (green) and dead (red) bacteria inside the dentinal tubules are clearly 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.

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

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).

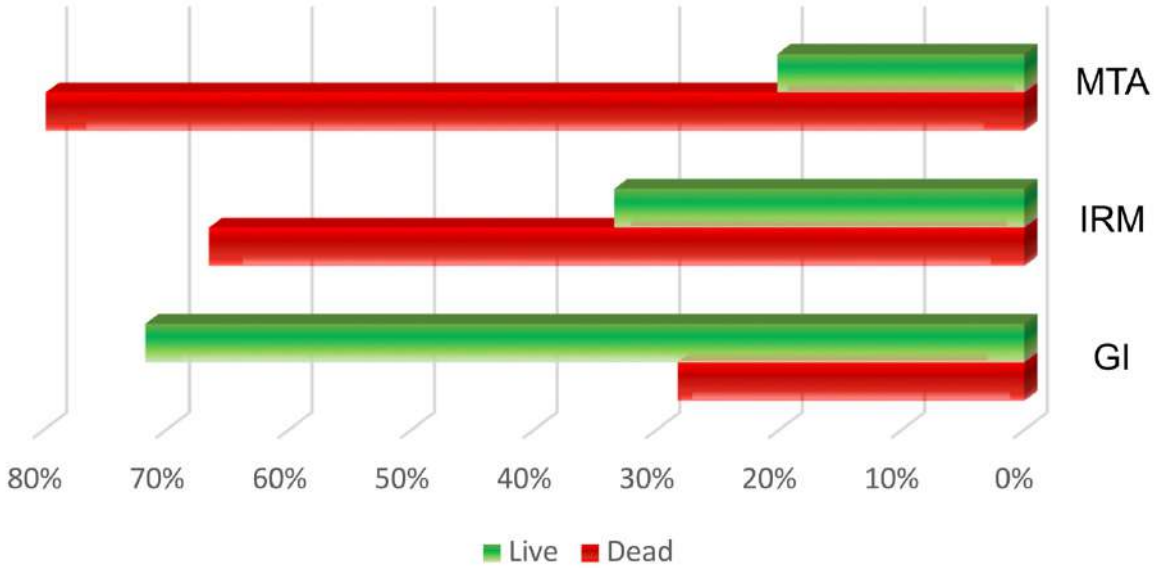

Figure 3: The average bacterial number (percentage) of live bacteria (green) and dead bacteria (red). There were significantly more live bacteria than dead bacteria within the evaluated areas in the GI group compared to the IRM and the MTA groups. There were also significantly more Live bacteria than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

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 *E. 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 *E. 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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6

Influence of Irrigation Protocol on Peroxide Penetration into Dentinal Tubules Following Internal Bleaching

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Abstract

Introduction: Discoloration of anterior teeth can result in cosmetic impairment in young children. The walking bleach technique stands out because of its esthetic results with minor side effects. Little information is available regarding the influence of various irrigation solutions on peroxide penetration. The aim of this study is to evaluate the influence of different irrigation protocols on peroxide penetration into dentinal tubules using confocal laser scanning microscopy (CLSM). Materials and methods: Cavity preparations were made in 50 extracted permanent premolars. The teeth went through different irrigation sequences: A. control B. saline C. EDTA, NaOCl D. phosphoric acid E. EDTA, NaOCl, phosphoric acid. Then, mixture of fluorescent dyed sodium perborate paste was placed along the pulp chamber and the coronal access cavity, and was refilled at days 7, 14 and 21. Results: The minimal and maximal penetration depths were 324 and 3045 µm, respectively, with a mean of 1607µm. The stained areas were significantly larger in the buccal and lingual directions ($P<0.05$). Groups B and C showed significantly larger penetration in weeks 2 and 3 compared to week 1 ($P<0.05$). Group D and E showed significantly larger penetration compared to groups B and C at all times ($P<0.05$).Conclusion: Bleaching agents penetrate to the extra-radicular region of teeth; however, the level of peroxide penetration is significantly higher when the irrigation sequence consists of phosphoric acid prior the bleaching agent placement.

Key Words: Discoloration, Bleaching, Sodium perborate, Peroxide, CLSM

Introduction

Dental trauma and pulpal infections are part of the routine pediatric dentistry. Common consequences in these cases are alterations in dental color[1–3], compromising patients' esthetics and their interactions in social environment [4–6]. Bleaching intends to preserve dental structure already weakened and to show immediate esthetic results.

Materials used for bleaching pulpless teeth are evaluated for the speed and efficacy in re-establishing the natural color of the teeth and for their potential (or lack thereof) of causing damage to surrounding structures [7–9]. An adverse effect that has been reported following internal tooth bleaching is cervical root resorption (an inflammatory mediated external resorption of the root)[10].

One of the most important properties of a bleaching material is its ability to allow penetration of the bleaching agent through dentinal tubules permeability [3,8]. The deeper the penetration, the more pigment that causes chromatic alteration of the dental tissues can be reversed by the oxidation reaction[3,8].

Several procedures have been reported to increase dentinal tubules permeability for the bleaching agents, including phosphoric acid etching of tooth structure before placing bleaching agents [11], smear layer removal [12] and heat application [13],[14]. However, thermocatalytic bleaching techniques have recently been questioned due to the deleterious effects that may be produced on dentinal structures [15],[16]. As to the agents used for bleaching of non-vital teeth, sodium perborate paste has shown improved esthetic results when prepared with either hydrogen peroxide or distilled water as the liquid vehicle. The 10% carbamide peroxide has provided similar performance to sodium perborate for internal bleaching [17],[18]. The water-based sodium perborate paste has been reported to have less potential to harm dental tissues [19]-[20]. Different types of sodium perborate (mono-, tri- or tetra-hydrated) may be used with similar results [21],[22]. Sodium perborate is an oxidizing agent available as a powder. It is stable when dry; however, in the presence of acid, warm air or water, it breaks down to form sodium metaborate, hydrogen peroxide, and nascent oxygen. H_2O_2 is released during the decomposition of perborate[23].

Sodium perborate is easier to control and safer than concentrated hydrogen peroxide solutions[1]. The first description of the walking bleach technique with a mixture of sodium perborate and distilled water was mentioned in a congress report by Marsh and published by Salvas[24]. In this procedure, the mixture was left in the pulp cavity for a few days, and the access cavity was sealed with provisional cement.

The penetration of peroxide into dentin has been previously presented [8,25–28]. However, little information is available regarding the influence of various irrigation solutions on sodium perborate penetration. Therefore, the aim of this study is to evaluate the influence of different irrigation protocols on sodium perborate penetration into dentinal tubules using confocal laser scanning microscopy (CLSM).

Materials and Methods

50 fully developed human permanent premolar teeth were used in this study, following the approval of the Tel Aviv University ethics committee. All teeth were freshly extracted for periodontal reasons from male and female patients (age range 30–45 years). Teeth with previous root canal treatment, root resorption, or caries and teeth with root fractures were excluded from the study. Once the tooth was extracted, the debris and soft tissue remnants were removed from the root with a sharp scalpel. Then, the teeth were stored in phosphate-buffered saline until used for the study.

Occlusal access openings were prepared using Endo-Access bur (Dentsply-Maillefer, Ballaigues, Switzerland). The root canals were prepared to a standard shape using Gates Glidden burs (Kerr Dental, Orange, CA, USA) to the apical foramen. The teeth were then randomly divided into five experimental groups of 10 teeth, each group went through a different irrigation sequence:

Group A (10 teeth): negative control, saline irrigation without a following bleaching agent.
Group B (10 teeth): saline irrigation
Group C (10 teeth): 17% EDTA followed by 3% NaOCl and final saline irrigation

Group D (10 teeth): 37% phosphoric acid followed by saline irrigation
Group E (10 teeth): 17% EDTA followed by 3% NaOCl, 37% phosphoric acid and final saline irrigation.
After the irrigation sequence, the bleaching agent - sodium perborate (Merck KGaA, Darmstadt, Germany) mixed with distilled water in a ratio of 2 g of powder to 1 mL of liquid - was used. In order to analyze fluorescence under confocal laser microscopy, Rhodamine B dye (Sigma-Aldrich, St. Louis, MO, USA) isothiyocyanate fluorescent was added (maximum absorption = 570 nm, maximum emission = 720 nm) to an approximate concentration of 0.1%. The mixture of fluorescent dye and sodium perborate paste (Merck KGaA, Darmstadt, Germany) was placed along the pulp chamber and the coronal access cavity.

A dry cotton pellet was placed inside the pulp chamber in all groups, and the access cavities were sealed with glass ionomer cement (GC Fuji IX GP; GC America Inc, Alsip, IL). The teeth were then stored at 100% humidity and 37°C. During the experiment, the teeth were re-rinsed and the pulp chamber was refilled with fresh bleaching paste at days 7, 14 and 21.

Preparation of Specimen for Microscopy

The specimens were embedded in a self-cure acrylic repair material (UNIFAST Trad, GC America) and cut perpendicularly to the long axis of the root under water cooling with a diamond saw rotating at 500 rpm (Isomet, Buehler Ltd., Lake Bluff, IL, USA) in order to obtain five slabs for each specimen of 1 mm thickness.

Confocal Microscopy Evaluation

Fluorescence from the stained bleaching agents was observed under a confocal laser scanning microscope (CLSM) (Leica TCS SP5, Leica Microsystems CMS GmbH, Germany). Single-channel imaging was used to display red fluorescence. The CLSM images were acquired at a resolution of 1024×1024 pixels and analyzed by the LAS AF software (version 2.6.0.7266; Leica Microsystems CMS GmbH). The specimens observed using a ×4 lens. The mesial, distal, buccal, and lingual areas of the specimens were evaluated by the software as follows:
1. The size of fluorescent staining within the evaluated areas, as calculated by the software.

2. The depth of penetration into the dentinal tubules was measured and recorded considering the canal wall as the starting point (figure 1f).

Statistical Methods

The results were evaluated statistically using Mann Whitney U test to compare the proportion between the buccal/lingual/mesial/distal areas. Kruskal-Wallis test was used to evaluate the fluorescence staining within the evaluated areas. $P<0.05$ was considered as statistically significant.

Results

No fluorescence was observed in the control group, and fluorescence was found in all specimens of the other groups (figure 1 a-f). The minimal and maximal penetration depths into the dentinal tubules were 324 and 3045 µm, respectively, with a mean of 1607µm. Table 1 presents the fluorescence stained sodium perborate penetration depths into the dentinal tubules in the different groups. The extent and amount of the stained areas were significantly larger in the buccal and lingual directions compared to the mesial and distal directions in all groups (figure 1d, Table 1, $P<0.05$).

Groups B and C showed significantly larger penetration in weeks 2 and 3 compared to week 1 ($P<0.05$). Group D and E showed significantly larger penetration compared to groups B and C at all times ($P<0.05$) (Figure 2, 1a,d,e).

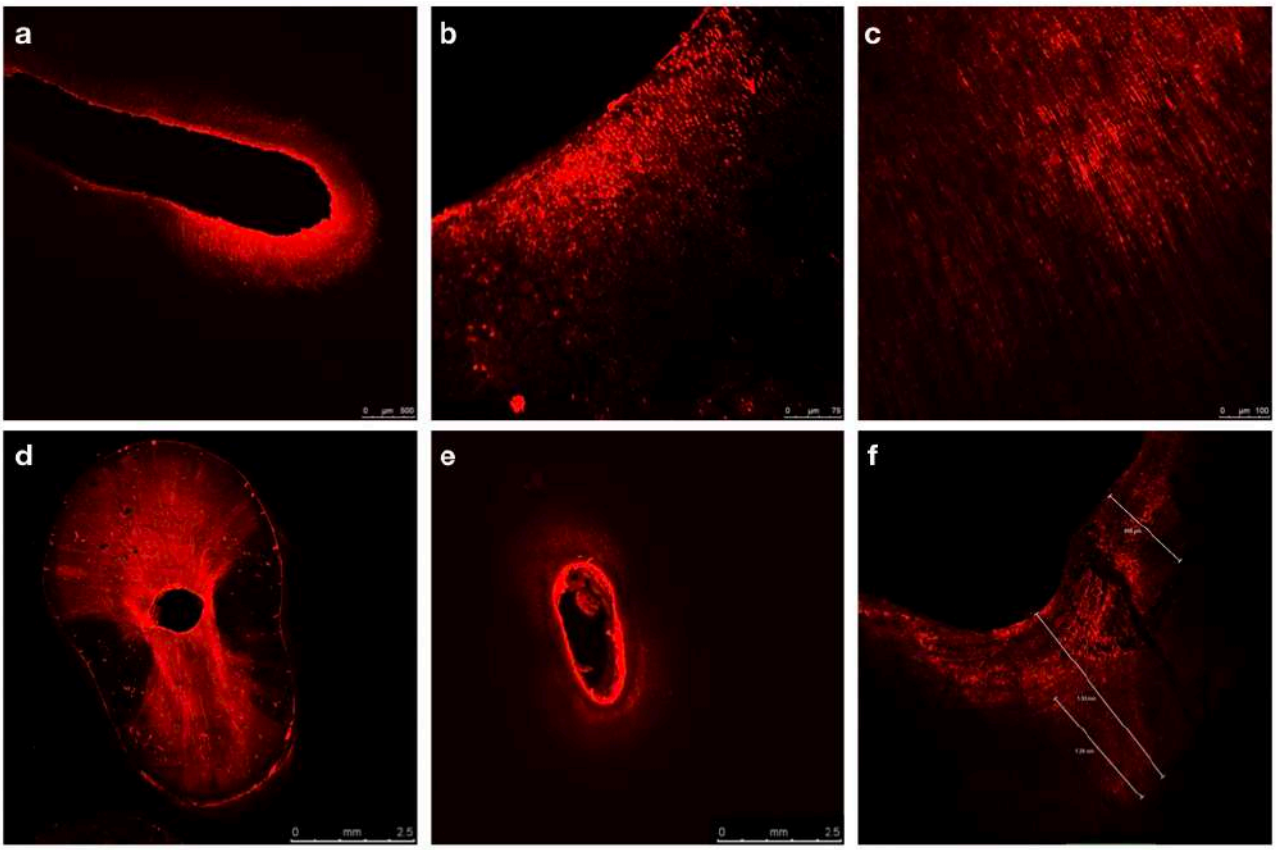

Figure 1: Confocal laser scanning microscopy (CLSM) images of the different groups. Penetration of peroxide (red) inside the dentinal tubules is clearly visible (a-f), in lower (a) and Higher (b,c) magnifcations. A butterfly-like appearance is seen on the root cross section (d) that occurs as a result of increased sclerosis along the tubules located on the mesial and distal sides of the canal lumen. Penetration depth was measured and recorded considering the canal wall as the starting point (f). The peroxide penetration was significantly higher when the irrigation sequence consists of phosphoric acid prior the bleaching agent placement (d) when comparing the other groups (a,e).

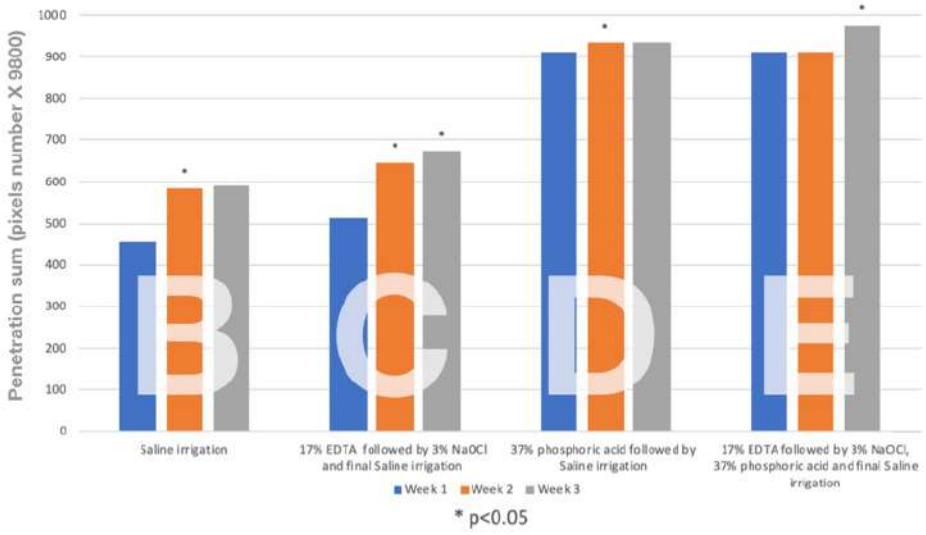

Figure 2: Fluorescence Penetration amount for the different groups (presents as the number of pixel X 9800). Groups B and C showed significantly larger penetration in weeks 2 and 3 compared to week 1 ($P<0.05$). Group D and E showed significantly larger penetration compared to groups B and C at all times ($P<0.05$).

	Minimun Penetration	Maximum Penetration	Mean	Std. Deviation	P-value
Group B: Saline					
Week 1	433	2660	1492	481.9	
Week 2	655	2709	1503	789.6	0.003
Week 3	655	2709	1503	789.6	0.04
Group C: 17% EDTA followed by 3% NaOCl and final Saline irrigation					
Week 1	324	2700	1366.4	841	
Week 2	450	2700	1427	844	0.022
Week 3	380	2700	1332	860	0.169
Group D: 37% phosphoric acid followed by Saline irrigation					
Week 1	567	3000	1682	1015.6	
Week 2	789	3045	1964	836	0.027
Week 3	789	3045	1964	836	
Group E: 17% EDTA followed by 3% NaOCl, 37% phosphoric acid					
Week 1	567	3000	1684	1013	
Week 2	567	3000	1684	1013	0.00
Week 3	567	3000	1684	1013	0.00

Table 1: Presents peroxide penetration depths in um into the dentinal tubules for the different groups. The minimal and maximal penetration depths into the dentinal tubules were 324 and 3045 µm, respectively, with a mean of 1607µm. The extent areas were significantly larger in the buccal and lingual directions compared to the mesial and distal directions in all groups.

Discussion

This study compared dentinal peroxide penetration levels after different irrigation sequences. Confocal laser scanning microscopy was used in the present study to analyze the flow of peroxide penetration because it is a technology which combines optical microscopy, physical-chemical principles and computing resources for acquisition and processing of images[29–31]. The system uses a laser source to promote excitation of fluorophores. The laser beams may diffuse through the dentin, enamel and biofilms, thus detecting their inner structures and forming several two-dimensional images[32].

Confocal laser scanning microscopy has some advantages compared to scanning electron microscopy, including histological evaluation and other methodologies for assessing penetration of endodontic materials[30,32,33]. This

technology may also be used in microbiological studies for quantifying bacteria within dentinal tubules[34,35]. Previous *ex-vivo* studies attempted to evaluate peroxide penetration [8,25–27]. However, these studies were limited since they were using indirect models, which are incapable of evaluating the actual routes of peroxide penetration. Unlike the previous traditional models, in the current study a novel model was used, which histologically traces the actual routes of penetration *in-situ* [36,37]. In addition, negative histological controls were used to confirm the adequacy of the experimental model. No fluorescence was observed in the negative control groups, confirming the reliability of the experimental model.

Maximal penetration depth into the dentinal tubules was 3045 µm. Intracoronal bleaching requires healthy periodontal tissues and a root canal that is properly obturated to prevent the bleaching agent from reaching the periapical tissues[38]. Previous studies have shown peroxide from bleaching agents to penetrate from the pulp chamber to the cervical region[39–41] during bleaching procedures. Radicular peroxide penetration should be as limited as possible, because the biological threshold of peroxide compounds causing irreversible damage to dental hard and soft tissue is unknown[21]. An adverse effect that has been reported following internal tooth bleaching is cervical root resorption[10]. It is important to note that there are a large number of cases of cervical root resorption after NVB in teeth that previously experienced dental trauma[42]. The underlying mechanism for this effect is unclear, but it has been suggested that the bleaching agent reaches the periodontal tissues through the dentinal tubules and initiates an inflammatory reaction[43]. In vitro studies using extracted teeth showed that hydrogen peroxide placed in the pulp chamber penetrated the dentine [8,25,26,44,45], and that the penetration is greater in teeth with cervical cemental defects[39].

The fact that the fluorescence staining penetrated deeper in the bucco-lingual direction may be related to an anatomical-physiological phenomenon known as the "Butterfly Effect", a butterfly-like appearance seen on root cross sections, that results from increased sclerosis along the dentinal tubules located on the mesial and distal sides of the canal lumen[46]. This effect is common in the single-rooted teeth of humans in a wide range of ages[47].

Prior to the application of the bleaching agent, preparation of the pulp cavity is required in order to remove remnants of restorative materials, root-filling materials, and necrotic pulp tissue[1]. Additional cleaning of the pulp cavity with sodium hypochlorite is also recommended[48]. In some reports, conditioning of the dentin surface of the access cavity with 37% phosphoric acid is suggested in order to remove the smear layer and in order to open the dentinal tubules. This promotes the penetration of the bleaching agent deep into the tubules and increases its effectiveness[49]. Dentin permeability and cementum integrity play a key role in determining radicular penetration. The goal of the clinician is to increase the dentin permeability in order to facilitate the bleaching procedure and in order to achieve better esthetic results. However, this may lead to more diffusion of bleaching agents to the outer surrounding tissues [23]. In our study etching treated groups (D and E) showed significantly higher penetration of peroxide. In recent years it has been recommended to add the acid etching during the preparation of the pulp chamber prior to bleaching procedures [23,50]. This step was introduced after the recognition that dentinal surfaces prepared by rotary instruments are covered by a so-called "smear layer" consisting of enamel and dentin particles, mineralized collagen matrix, blood products and bacteria (6-8). Smear layer removal has been recommended in dentistry before composite restoration and prior to root canal canal filling[51–54]. Walton et al.[55] advocated a 50% solution of phosphoric acid application for 60 seconds for smear layer removal[55]. The removal of the smear layer by acid etching has been shown to increase significantly the permeability of dentin in both vital and endodontically treated teeth, by opening up the orifices of the dentinal tubules[56,57]. One might then expect improved penetration of bleaching agents into the stained dentin, thus enhancing their effectiveness[58,59]. Although an improvement in bleaching is hypothesized, the technique has not been universally accepted [3-5,9]. In addition, whether or not this extra etching step significantly improves the quality of an endodontic bleaching procedure has not been established [60,61]. Casey et al.[49] were unable to distinguish a significant difference between the effectiveness of the bleaching procedures using etching. Group E showed lesser penetration compared to group D, even though both groups included etching. A possible explanation for the differences in penetration may be attributed to the

prior preparation of dentin using EDTA and NaOCl solutions that were incompletely rinsed off and were left inside the dentinal tubules, resulting in dilution of phosphoric acid concentration.

Bleaching success seems largely dependent on the application duration of the bleaching agent [62]. In our study, in groups B and C, where no etching had been used, a significant higher peroxide penetration was observed on the second week compared to the first week. However, in groups D and E, where etching conditioning was involved, penetration did not increase over time. Other studies [1,23] have stated that bleaching agents should be changed every 3-7 days and up to 4 times, while some showed preferable results after 3 weeks[63].

Conclusions

bleaching agents penetrate to the extra-radicular region of teeth; however, the level of peroxide penetration is significantly higher when the irrigation sequence consists of phosphoric acid prior the bleaching agent placement. This fact may carry more risk of post-bleaching external root resorption, especially in traumatized dentition.

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7

Effects of different irrigation protocols on dentin surfaces

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Abstract

Combination of EDTA and sodium hypochlorite (NaOCl) has been advocated as an effective irrigation methodology to remove organic and inorganic matter in root canal therapy. It was suggested that both might lead to structural changes of the dentinal wall surface, depending on the order of application. This study aims to evaluate the effect of different irrigating protocols on dentin surfaces using quantitative 3D surface texture analysis. Data stems from 120 human root dentin sections, divided into four groups. Each was prepared according to one of the following protocols: (1) 17% EDTA; (2) 17% EDTA followed by 5.25% NaOCl; (3) 5.25% NaOCl; and (4) 5.25% NaOCl followed by 17% EDTA. Each dentin sample was examined for its three-dimensional surface texture using a high-resolution confocal disc-scanning measuring system. EDTA 17% and the combined EDTA 17% & NaOCl 5.25% showed significantly higher roughness properties compared to NaOCl 5.25% alone. However, the order of the irrigation did not affect the dentin roughness properties. The results indicate that although irrigating the root canal using NaOCl and EDTA affects the dentin surface texture by increasing its roughness, the exact sequence of irrigation using these materials has no significant effect.

Keywords: Dentin; Erosion; Irrigation; Roughness; Surface texture analysis

Introduction

An integral part of root canal treatment is the use of irrigations to disinfect the root canal system and to remove debris and tissue remnants[1,2].The current methods of root canal preparation might produce a smear layer that covers the instrumented areas of the canal walls[3]. The smear layer contains inorganic and organic substances such as fragments of odontoblastic processes and necrotic debris[1], and may contain bacteria. It may act as a physical barrier and affect the sealing efficiency of the root canal filling. Moreover, irrigation is used for the removal of the smear layer[2,4]. Therefore, it requires the use of a chelating agent and a soft-tissue solvent[5]. The combination of Ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite (NaOCl) was suggested as an effective irrigation procedure to disinfect the root canal and to eliminate the organic and inorganic materials[1]. However, no agreement exists in the literature regarding the ideal irrigation sequence, volume, and application time[6–8]. In most cases, NaOCl is used during instrumentation, and EDTA is used preferably at the end of instrumentation to complete the removal of the smear layer[6,7]. The usage of NaOCl ensures a high disinfecting efficacy and enables the material to penetrate into the dentin. In contrast, a final flush of NaOCl has also been advocated, to allow better penetration of the NaOCl to areas that were earlier covered with the smear layer[8].

It was suggested that the usage of irrigation in root canal therapy can lead to structural changes of the dentin walls of the root, and thus might cause reduction of dentin strength, microhardness, and changes in surface roughness[9–11]. Surface roughness is a component of surface texture, which is evaluated by the deviation of an ideal surface from the real surface. Specifically,, if these deviations are large, the surface is rough; if they are small, the surface is smooth[12].
Structural and surface changes in the dentin may contribute to the development of root fractures; hence, they might have significant clinical implications[13–15]. Baumgartner and Mader[16] reported that applying EDTA and NaOCl solutions to the root canal resulted in an eroded appearance of the dentin and enlarged tubular orifice diameters.

New technologies based on Scale-Sensitive Fractal Analysis of high-resolution, three-dimensional surface reconstructions advanced the ability to produce accurate, measurable analysis for surface texture[17]. Similar to Scale-Sensitive Fractal Analysis[18], quantitative 3D surface texture analysis (3DST) has become an established method for dietary reconstruction, by analyzing dental surfaces in ungulates[19,20], primates[21], and rodents[22]. Surface texture analysis was used to analyze tooth surfaces for various purposes in evolutionary biology and has opened up opportunities for various applications in clinical practice[17,23]. The 3DST analysis uses standardized parameters from engineering applications (ISO 25178-2[24]), allowing reliable characterization of tooth attrition in a micrometer scale. The 3DST analysis of the wear and the change in surface texture can reveal the mechanical process[17,21,23] and the physical properties of the contacting materials[25]. As such, the aim of the current study was to evaluate the effect of different irrigating protocols on dentin surface texture by assessing the dentin roughness using quantitative 3D surface texture analysis.

Materials and Methods

Preparation of Dentin Specimens

The study was approved by the Tel-Aviv University Ethics Committee, and all methods were performed in accordance with the relevant guidelines and regulations. 50 fully developed human permanent anterior teeth were used in this study. All teeth were freshly extracted for periodontal reasons from male and female patients (age range 30–45 years). Teeth with previous root canal treatment, root resorption, or caries, as well as teeth with root fractures were excluded from the study.

Once the tooth was extracted, the debris and soft tissue remnants were removed from the root with a sharp scalpel. Then the teeth were stored in phosphate-buffered saline until used for the study.

For the preparation of the specimens, the crowns were removed by a cut at the cemento-enamel junction, using a high-speed tungsten 28 mm zekrya bur (De-Trey, Dentsply, Konstanz, Germany) under water coolant. The remaining roots were

sectioned perpendicular to the long axis of the root under water cooling with a diamond saw rotating at 500 rpm (Isomet, Buehler Ltd., Lake Bluff, IL, USA). Each root was cut to produce three slices of 1 mm thickness each (150 dentin slices in total, Fig. 1a). The slices were ground to achieve a smooth surface with 400, 600, 800 and 1,200 grit polishing papers consecutively under distilled water, to remove any surface irregularities. After each grinding step, dentin samples were rinsed and ultrasonicated in purified water.

The 120 dentin slices were randomly divided into four groups (containing 30 slices in each group), and each was subjected to one of the following irrigation protocols (Fig. 1b):

(1) The first group (E) was immersed in 17% EDTA (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 10 minutes; (2) The second group (EN) was immersed in 17% EDTA for 10 minutes followed by 5.25% NaOCl (Sigma-Aldrich, Inc.) for 10 minutes; (3) The third group (N) was immersed in 5.25% NaOCl for 10 minutes; (4) The forth group (NE) was immersed in 5.25% NaOCl for 10 minutes followed by 17% EDTA for 10 minutes.

After the slices were removed from the irrigation solution, the samples were rinsed copiously and kept in deionized and distilled water for no longer than 20 min prior to further analysis.

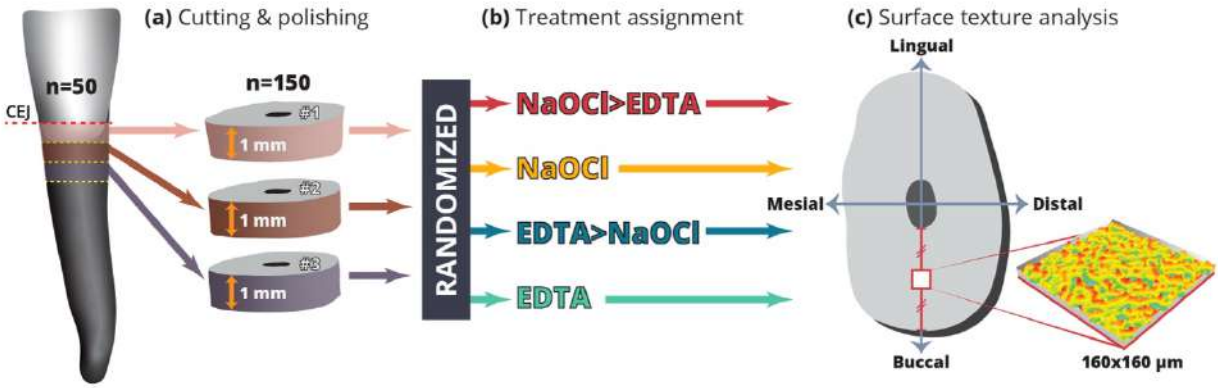

Figure 1: Scheme of the experimental protocol. (a) For each tooth (n=50), three slices of 1 mm thickness each were cut using a low-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) and ground to achieve a smooth surface with 400-, 600-, 800-, and 1,200-grit polishing papers. **(b)** A total of 120 dentin slices were randomly assigned to 4 treatment groups (containing 30 slices in each group). **(c)** For each slice, the midpoint between the pulp chamber and the buccal-most point was identified along the bucco-lingual axis to obtain a 160x160 µm surface measurement using a high-resolution confocal disc-scanning measuring system (100x long distance lens, µsurf explorer, NanoFocus AG, Germany). Subsequently, quantitative 3D surface texture analysis per spot of 160x160 µm was conducted in Mountains Map Premium software (v 7.3.7, DigitalSurf, France).

Surface Texture Analysis

Surface texture analysis of the dentin slices was conducted using a high-resolution confocal disc-scanning measuring system (100x long distance lens, μsurf explorer, NanoFocus AG, Germany). For each slice, the midpoint between the pulp chamber and the buccal-most point was identified along the bucco-lingual axis (Fig. 1c), and quantitative 3D surface texture analysis per spot of 160x160 μm was conducted[26]. Surface roughness was measured using Mountains Map Premium software (v 7.3.7, DigitalSurf, France). The parameters used for evaluation of the surface roughness were based on standardized ISO parameters (ISO 25178-2) (Table 1), each representing distinctive characteristics of the surface texture[27].

Statistical analysis was carried out using SPSS (v. 21.0). Significance was set to $p<0.05$. The outliers were removed from the analysis using the ROUT method (Q =1%)[28]. Kolmogorov-Smirnov tests were carried out to verify the normality of the measurement distributions. For parametric measurements (with normal distribution), one-way analysis of variance (ANOVA) with post hoc (Tukey) tests was carried out. For non-normally distributed parameters, Kruskal-Wallis analysis with post hoc (Bonferroni) was used. Between-group principal component analysis (PCA) of the measurements that significantly differed between the studied zones was carried out using the PAST software (v.3.16,[29]).

Ethical approval

The study was approved by the Tel-Aviv University Ethics Committee (approval number 230.17) and all methods were performed in accordance with the relevant guidelines and regulations.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Results

The means and standard deviations of roughness values of dentin surfaces for the treatment groups are listed in Table **1**.

The protocols of EDTA 17% and the combined EDTA 17% & NaOCl 5.25% showed significantly higher roughness properties compared to NaOCl 5.25% alone (Table **1**, Figs. **2-3**); 10 out of 30 parameters (Sy, Sal, Sda, Sdr, Spd, Spc, S10z, S5v, Sda, Sha) showed significant differences. Moreover, the order of the irrigation for NE and EN groups did not affect the roughness properties. In other words, the protocol of addition NaOCl 5.25% following 10 minutes EDTA 17% did not change significantly the surface texture compared to the protocol of adding EDTA 17% following 10 minutes of NaOCl 5.25% ($p>0.05$). Nineteen out of 30 parameters (Ssk, Sku, Sp, Sy, Sz, Sal, Str, Vm, Vy, Vmp, Vmc. Vvc, Vyy, Spd, Spc, S10z, Sda, Sha, Shy) showed no significant differences in this regard.

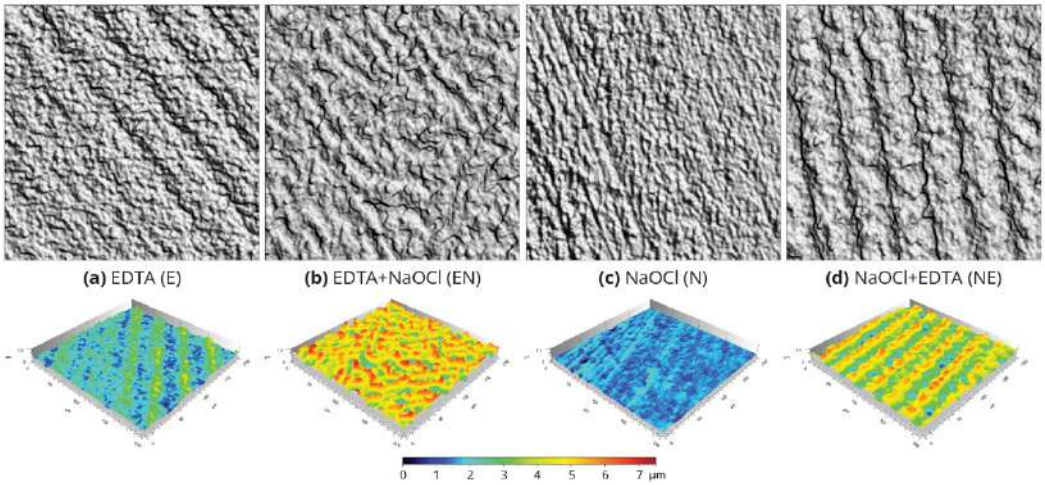

Figure 2: Surface image (top) and surface topography (bottom) of the dentin surfaces following the irrigation protocols. A 160 × 160 µm image was acquired using a high-resolution confocal disc-scanning measuring system (100× long distance lens, µsurf explorer; NanoFocus AG). (**a**) EDTA 17%; (**b**) EDTA 17% followed by NaOCl 5.25%; (**c**) NaOCl 5.25%; (**d**) NaOCl 5.25% followed by EDTA 17%. Note the reduced roughness of the NaOCl 5.25% protocol (**c**) compared to the others (**a-b, d**). The color map indicates the measured height in micrometers above the lowest point for each surface.

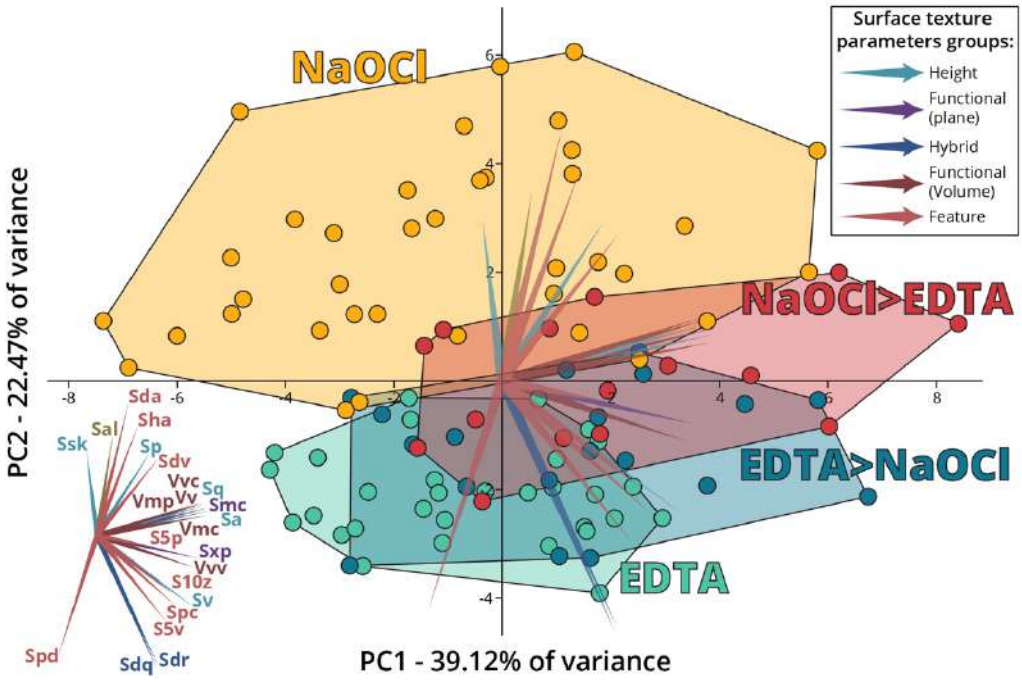

Figure 3: Between-group principal component analysis (PCA) of the dentin surface roughness parameters. Graphic presentation of the overall difference between the 4 experimental groups (E, EN, N, NE). The first two principal components explain 61.59% of the variance of the measured surface texture parameters (ISO 25178-2[30]). Each parameter is marked as a vector that indicates the contribution of the parameter to the difference between the regions.

Discussion

In the current study we used a new methodology, the 3DST, to evaluate the effect of different irrigating protocols used in root canal treatments on the dentin surfaces by assessing changes in dentin roughness, which can reflect the surface properties and characteristics. Surface roughness is one of the most frequently used test methods to evaluate the effects of different irrigations on dental tissues[9,23,31,32].

This approach has several advantages as compared to the common methods in the literature[33], profilometry was used as a quantitative method to determine surface hardness (enamel) and microradiography (dentine). Scanning electron microscopy (SEM) was the most commonly used method for qualitative study of the dentin erosion. However, according to the previous studies[34], the traditional SEM analysis method entails the following important distortions: the high degree of magnification establishes visualization of a location that is too small and does not represent the total canal area; it does not allow a longitudinal study because preparation of the specimen does not allow reuse; there is no observation of wet

areas; there is anatomic variation between specimens; and there is a possibility of researcher bias in choosing the area to be evaluated. Several different methods have been used to score the erosion the dentin layer remaining after biomechanical preparation[13,31,35–37] for instance, computerized systems for quantitative analysis of dental tissue[34,38]. The 3DST was previously suggested as a reliable method to characterize wear and changes in surfaces texture in human enamel[17,23]. Yet, this is the first study to analyze the dentin surface though this prism. The advantage of the 3DST method is that it allows reliable characterization of tooth surfaces in high resolution, both qualitatively and quantitatively. The data attained can shed light on the mechanical properties and function of the studied surface[21,25]. In contrast, the gold standard methodology for dentin roughness analysis is the scanning electron microscopy (SEM), which mostly induces qualitative rather than quantitative results for the dentin surface characterization[34].

Several different methods were used to evaluate the effect of biomechanical preparation on the dentin surface texture[13,31,36,37], including computerized systems for quantitative analysis of dental tissue[34,38]. This method is based on a set of images acquired from a large number of X-Y locations of a sample at different experimental times. Such data enable analysis of modifications, such as chemical etching, over time.

Although, these computerized systems represent a development over the traditional qualitative dentin surface SEM analysis, the main limitation they present involves the restricted depth of focus in optical microscopy, requiring a practically flat sample surface. Therefore, the accuracy in measuring the changes in roughness through the depth of the dentin is not ideal. The 3DST system overcomes this disadvantage, as 30 different parameters can be used to examine the changes in the dentin surface in 3D[34].

In this study we chose to use the dentin disc model, that was used past studies[39,40]. The model was found applicable to demonstrate the tubule due to its complex system of canaliculi that branch off from the main tubules at a variety

of angles[41]. That being said, this is an in vitro study and does not mimic the exact clinical situation but resembles one.

Using the 3DST method, we were able to quantitatively analyze the dentin surface directly, before and after several irrigation procedures. This is the first study to utilize all 30 standardized ISO parameters for dentin roughness evaluation. Thus, the effect of each irrigation solution could be analyzed and the dentin roughness could be described. The use of NaOCl and EDTA have been recommended during chemomechanical preparation[31]. We found that when in contact with these materials, the dentin may change its physical, chemical, and structural properties. They may decrease the dentin microhardness[42], change its flexural strength[43] and modulus of elasticity[37,43], cause irreversible damage of the dentin microstructure[44,45], and oxidize the organic matrix denaturing the collagen components of the dentin surface[46]. Relating these facts to the possibility of clinical occurrences, degradation of collagen matrix in mineralized tissues results in a less resistant and more brittle substrate, which can make the endodontically treated teeth more susceptible to crown or root fracture[47].

Dentin is composed of an organic core-dense collagen network, covered by an inorganic outer sheath-hydroxyl apatite coating[13]. NaOCl, which is a strong base and nonspecific oxidizer, causes degradation of the amino acids by neutralization and chloramination reactions [37]. Type I collagen and glycosaminoglycan have been found to lose their immunoreactivity after NaOCl treatment[11]. EDTA is a chelating agent and, therefore, responsible for removing the smear layer[2]. Moreover, EDTA was reported to have an antibacterial effect[48,49], probably due to the chemical chelation involving the external bacterial membrane[49,50].

Both EDTA and NaOCl are necessary for the preparation of the root canal; yet the sequence of the irrigation usage remains debated. It was suggested that when NaOCl is used prior to EDTA, the hydroxyapatite coating appears to shield the collagen fibers from the dissolving effect of NaOCl. However, when NaOCl is used after EDTA, the collagen that was already exposed to demineralizing agents is more intensively subjected to the dissolving effect of NaOCl [50].

The 10 minutes time that was chosen in this study was based on past studies. A clinical trial by Byström and Sunvqvist[51] proved that the cleansing of NaOCl and EDTA were efficient at 10 minutes. Another study[52] showed that the EDTA demineralization process of the dentin is a time dependent process, A critical time was shown to be 10 minutes. Other authors[53] proved that the removal of the smear layer by EDTA and NaOCl was efficient at 10 minutes exposure time.
The sequence of irrigation – NaOCl first followed by EDTA or the opposite – did not have a significant impact on the dentin surface roughness; yet some tendency could be noted, as more roughness appeared when NaOCl was used as the final irrigation. This finding does not corroborate findings from a previous study by Qian et al.[45] that showed higher dentin roughness when NaOCl was used following EDTA. Moreover, in that study, erosion was not detected when EDTA was used following NaOCl[45], the different result can be explained for the different methodology. Wang et al[54] used energy-dispersive X-ray spectroscopy to examine erosion level by the calcium and phosphorus release in the samples, and found that NaOCl followed by final EDTA irrigation caused minimal dentin erosion. In the present study there was a significant increase of the roughness levels when EDTA 17% was used alone or in combinations with NaOCl, regardless the order of use. It is conceivable to assume that the EDTA is the main cause for the increase in roughness parameters, while the addition of NaOCl (regardless of the sequence) did not show a significant influence. In order to attain optimal conditions for root canal therapy, the irrigation protocol should result in a disinfected root canal, free of all organic debris, microorganisms, and smear layer[31,44,47,55], whereas dentin biomechanical properties should not be harmed[31,44,47,55].
Currently, there is insufficient data to conclude whether the change in surface roughness is harmful for the root dentin and the tooth and determine how it might influence the outcome of the endodontic treatment. As is commonly known, the mineral component in hard connective tissues contributes to strength and toughness of the tooth, whereas collagen is responsible for elastic modulus[47,50,56]. Therefore, any change in dentin structure or configuration (e.g., roughness, thickness of the root, and the amount of sclerotic dentin) might serve as a contributing factor in the formation of vertical root fracture[13–15].

However, for clinical purposes, the softening effect of chemical solutions on root canal surface roughness might be an advantage, as it permits improved preparation and negotiation of tight root canals[57]. Moreover, dentin tubules become more patent and the surface roughness increases, thereby allowing improved micromechanical bonding of endodontic sealers and other materials that require surface irregularities for the adhesive to penetrate and adhere [9,58,59].

Further research is needed in order to reveal how dentin hardness and its biomechanical properties are affected by the usage of different irrigation protocols.

Conclusions

The results of the current study indicate that although irrigating the root canal using NaOCl and EDTA affects the dentin surface texture by increasing its roughness, the exact sequence of irrigation using these materials has no significant effect. Our results imply that these irrigations may adversely affect the mechanical properties of the dentin and the risk for complications following root canal treatments.

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8 General Discussion

Chapter 8

General discussion

The rationale for endodontic treatment is to eradicate the infection and to prevent microorganisms from infecting or re-infecting the root and/or peri radicular tissues [1]. Microorganisms are essential for the progression and perpetuation of different forms of apical periodontitis[2–4].

The exact model of bacterial colonization root-filled teeth is not fully elucidated and previous *in vitro* studies attempted to evaluate leakage in the presence of fillings using different models, however, these studies were limited since they were using indirect models, incapable of evaluating the actual routes of bacterial penetration and colonization[5]. Thus, the first aim of this study was to establish a proper dentin infection model. In **chapter 1** we established a novel technique based on CLSM and live & Dead staining that could overcome the difficulties of the past models. In **chapters 1 and 2** we presented the CLSM-based model, and the actual routes of bacterial invasion were traced histologically, and negative and positive histological controls were used to verify the suitability of the experimental model, enabling a better understanding of the microbiological–pathological course after endodontic and surgical procedures. In **chapter 3** We also used this CLSM-based model to assess the invasion of bacterial biofilms into the dentinal tubules in the presence of fluorescently labeled retrograde filling materials. Bacterial fluorescence was not detected in all the negative control groups, while fluorescence was detected in the positive control group specimens, confirming the reliability of the experimental model.

In all **chapters** of this thesis, we used *Eenterococcus faecalis. E. faecalis* is frequently recovered from persistent infections of endodontically treated teeth. These infections can manifest as chronic or acute inflammations followed by the destruction of the surrounding tissues around the tip of the tooth root with subsequent development of abscesses. Thus *E. faecalis* biofilms are considered as an appropriate model for evaluating root canal bacterial colonization[6,7].

Many irrigation solutions and medicaments were suggested for the elimination of *E. faecalis* infections, including mixtures of an antibiotic and a detergent[8]. Sodium hypochlorite (NaOCl), the commonly used anti-bacterial irrigation solution for the treatment of root-canal was found efficient in reducing the replicative properties of the biofilm's cells [9]. The combination of Ethylene-diamine-tetraacetic-acid (EDTA) and NaOCl was suggested as an effective irrigation procedure to disinfect the root canal and to eliminate organic and inorganic materials. In **chapter 7**, we used a new methodology to analyze the 3D surface area roughness of dentin after applying different irrigation solutions. The results indicated that irrigating the root canal using NaOCl and EDTA affects the dentin surface texture by increasing its roughness, the exact sequence of irrigation using these materials showed no significant difference. Canal sealer adhesive strength depends on chemical and mechanical retention. A smooth surface is more efficient in the case of chemical bonding, providing better adhesion.

While the penetration of live bacteria into periapical tissues following apical surgery may be important in some cases, the main clinically relevant concern is that of bacterial colonization and infection of the dentin and the filling-dentin interfaces following apical surgery. Several root-end filling materials have been used in modern endodontic surgery, such as IRM[10] and other bioceramic materials (e.g. MTA[11]and Biodentine[12]). The antibacterial properties of retrograde root canal filling materials were previously assessed[13]. Slutzky et al[14] has shown that IRM has antibacterial properties against *E. faecalis* after setting and sustaining this ability for at least 1 day. Chong et al[15] demonstrated the same effects in retrograde fillings. According to its manufacturer, Biodentine possesses antibacterial properties due to the alkalization of the environment and its high pH that exerts a clear inhibitory effect on microorganisms[16].

Peters et al.[17] argued that bacteria in the dentinal tubules are entombed beneath the root canal filling and will eventually die. However, microbiological and histological studies demonstrated the growth of isolated islands of biofilms between an existing root canal filling and dentin walls, and into the dentinal tubules[17]. In **chapters 2 and 3**, we reported that bacterial colonization and proliferation into the dentinal tubules may be influenced by the type of filling material and its penetration

depth into the dentinal tubules. In **chapters 2-5** we found that the viability of the colonized bacteria was also affected by the material type. Those findings resurfaced in **chapter 4**, dealing with perforations management and choosing sealing materials to prevent bacterial contamination, inflammation, and loss of periodontal attachment, and creating an ideal environment for tissue repair. **Chapter 4** assessed the colonization and proliferation of bacteria in furcal perforations, repaired with different materials by using CLSM, and found similar results and we concluded that the clinician's choice of repair material may be crucial for tooth survival.

Besides the benefits mentioned of the use of bioceramic materials in **chapters 2-5**, it might have an adverse effect and may cause severe discoloration of the dentin over time[18]. anterior teeth, particularly the upper ones, have a key impact on facial and oral aesthetics. These can severely affect the quality of life, causing physical, social, and psychological impairment[19,20]. When discoloration occurs following root canal treatment, non-vital bleaching might be indicated. In this technique, a mixture of sodium perborate and distilled water is usually mixed and placed in the pulp cavity[21,22]. One of the most important properties of a bleaching material is its ability to allow penetration of the bleaching agent through dentinal tubules' permeability[23–25]. In **chapter 6**, we used the CLSM-based model to evaluate the influence of different irrigation protocols on sodium perborate penetration into dentinal tubules. A mean of 1607µm penetration depth was found which demonstrates that bleaching agents penetrate to the extra-radicular region of teeth, it was also found that the level of peroxide penetration is higher when the irrigation sequence consists of phosphoric acid before the bleaching agent placement. **Chapter 6** states that this fact may carry more risk of post-bleaching external root resorption, especially in the traumatized dentition.

The findings of our studies in **chapters 2-5** indicate that even with the presence of filling materials, bacteria may invade deep into the dental tubules, all the way to the extra-radicular region. This corroborates with other studies. Peters et al[17] evaluated the depth of penetration of bacteria into the root dentin of 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.

In **chapters 2 and 3**[26,27] we found a pattern of bacterial invasion and filling penetration in which the buccolingual direction was preferred compared to the mesiodistal direction , regardless of the type of filling material. This finding was also supported by previous investigations[28–31]. The fact that both the fillings and the bacteria penetrated deeper in the buccolingual direction may be associated with an anatomical–physiological phenomenon, "the butterfly effect", which means a butterfly-like appearance observed on the cross-sections of roots that are associated with higher sclerosis down the tubuli at the distal and mesial sides of the root canal[31–33]. This phenomenon is commonly detected in single-rooted human teeth in a wide range of ages[32,33]. Similarly, in a study by Rechenberg et al.[31], histological observations revealed that the penetration of microorganisms might predominantly happen through tubular aspects of the dentin, whereas a-tubular or sclerotic dentin and the interfaces between dentin and sealer remained bacteria-tight[31]. According to these results, it can be assumed that peri-apical lesions will first develop in the buccal or lingual areas before the mesial and distal, though further investigation should be made.

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9 Summary and conclusions

Chapter 9

Summary and Conclusions

This study aimed to establish a novel and reliable experimental model for tracing and quantifying histologically the routes of bacteria and filling materials invasion into the dentinal tubules during different stages of root canal treatments. Furthermore, this study aimed to use Confocal Laser Scanning Microscopy (CLSM) together with live/dead bacterial staining techniques to find information regarding both the magnitude of dentin infection and the vitality of the proliferating bacteria within the infected tubuli *in vitro*.

Chapter 2 – In this chapter, we established the CLSM model which was later used throughout the thesis. To our knowledge, this was the first study that evaluated bacterial colonization in the apical part of extracted human teeth following root-end resection and filling, using CLSM. The current study demonstrated that following root-end filling, bacteria may colonize within the root canal space at the filling-dentine interfaces and penetrate deep into the dentinal tubules. This colonization is not homogenous, favoring the buccolingual aspect of the root. The viability of the colonized bacteria was affected by the type of root-end filling material.

Chapter 3 – This subsequent study enabled a better understanding of the microbiological–pathological course after endodontic surgical procedures since the retrograde filling was fluorescence-labeled. It was found that even with retrograde fillings, bacteria invade deep into the dentinal tubules. Furthermore, deeper filling penetration prevents deeper penetration of the bacteria and adversely affects their viability.

Chapter 4 - The ability of different restorative materials to repair perforation defects has been assessed by various *in vitro* experimental methods and reagents before. In this chapter, we used CLSM to evaluate *Enterococcus faecalis* colonization and

proliferation in furcal perforations repaired with different materials. This chapter demonstrates that bacteria may colonize the interface between the repair material and the dentin walls and may penetrate the dentinal tubules. We concluded that clinicians should choose perforation repair material with care since it has implications for the viability of the colonizing bacterial.

Chapter 5 – According to this study, CLSM seems to be a reliable technique to evaluate bacterial penetration and proliferation in pulpotomized primary molar teeth. The current study demonstrates that following pulpotomy, regardless of the type of pulpotomy base material we chose, bacteria may penetrate and colonize between base material and dentin. However, the extent and the vitality of the colonized bacteria were affected by the type of the pulpotomy base material.

Chapter 6 – Discoloration of anterior teeth after endodontic treatment can result in cosmetic impairment. The intra-coronal application of bleaching agents (e.g. carbamide peroxide, sodium perborate, hydrogen peroxide) has been used successfully for whitening root-filled teeth. Little information is available regarding the influence of various irrigation solutions on peroxide penetration. In this chapter, we evaluated the influence of different irrigation protocols on the bleaching agent into dentinal tubules using our CLSM based model. We found that peroxide penetrates as far as the extra-radicular region of teeth. The level of peroxide penetration was significantly higher when the irrigation sequence consists of phosphoric acid before the bleaching agent placement.

Chapter 7 –In this chapter, we introduced a new methodology to analyze the 3D surface area roughness of dentin after applying different irrigation solutions. The results indicated that although irrigating the root canal using Sodium hypochlorite (NaOCl) and Ethylene-diamine-tetraacetic-acid (EDTA) affects the dentin surface texture by increasing its roughness and the exact sequence of irrigation using these materials has no significant effect. Further studies using CLSM based model are required to determine the effect of irrigation sequence on bacteria and filling penetration.

Conclusions

1. CLSM seems to be a reliable technique to evaluate bacterial penetration and proliferation into dentinal tubules, that allows assessing both viable and dead bacteria *in vitro*.
2. Bacteria invade deep into the dental tubules. This invasion is variable and is affected by the root tubular anatomy and by the root-end filling material and its actual penetration depth into the dentinal tubules.
3. Bleaching agents penetrate the extra-radicular region of teeth; however, the level of peroxide penetration is significantly higher when the irrigation sequence consists of phosphoric acid before the bleaching agent placement.
4. Irrigating the root canal using NaOCl and EDTA increases its roughness, but the exact sequence of irrigation has no significant effect. Further study is required to determine the effect of irrigation sequence on chemical retention.

Hoofdstuk 9

Samenvatting en conclusies

Het doel van dit onderzoek was het tot stand brengen van een nieuw en betrouwbaar experimenteel model voor het histologisch traceren en kwantificeren van de invasieve paden van bacteriën en vulmaterialen in de dentinale tubuli tijdens verschillende fasen van wortelkanaalbehandelingen. Verder had dit onderzoek tot doel confocale laserscanningmicroscopie (CLSM) te gebruiken in combinatie met kleuringstechnieken van levende/dode bacteriën om informatie te vinden over zowel de omvang van de dentine-infectie als de levensvatbaarheid van de prolifererende bacteriën binnenin de geïnfecteerde tubuli *in vitro*.

Hoofdstuk 2 – In dit hoofdstuk hebben we het CLSM-model opgesteld dat later in de thesis werd gebruikt. Voor zover wij weten, was dit het eerste onderzoek dat bacteriële kolonisatie heeft beoordeeld in het apicale deel van geëxtraheerde humane tanden na apexresectie en retrograde vulling, met behulp van CLSM. Het huidige onderzoek toonde aan dat na retrograde vulling van de apex, kolonisatie van bacteriën kan ontstaan binnen het wortelkanaal op de grensvlakken van de vulling en het dentine, en dat deze diep kan doordringen in de dentinale tubuli. De kolonisatie is niet homogeen en vindt vooral plaats aan de buccolinguale zijde van de wortel. De levensvatbaarheid van de gekoloniseerde bacteriën werd beïnvloed door het soort retrograde vulmateriaal.

Hoofdstuk 3 – Dit vervolgonderzoek gaf meer inzicht in het microbiologisch-pathologisch beloop na endodontische chirurgische ingrepen, omdat de retrograde vulling was voorzien van fluorescentiemarkering. Het bleek dat zelfs met retrograde vullingen, bacteriën tot diep in de dentinale tubuli doordringen. Verder bleek een diepere penetratie van vulmateriaal een diepere penetratie van bacteriën te voorkomen en een negatief effect te hebben op de levensvatbaarheid van de bacteriën.

Hoofdstuk 4 – Het vermogen van verschillende restauratieve materialen om perforatiedefecten af te sluiten werd eerder onderzocht met verschillende *in vitro* experimentele methoden en materialen. In dit hoofdstuk hebben we CLSM gebruikt voor het beoordelen van de kolonisatie en proliferatie van *Enterococcus faecalis* in furcale perforaties die zijn behandeld met verschillende materialen. Dit hoofdstuk toont aan dat kolonisatie van bacteriën kan plaatsvinden op het grensvlak van het restauratiemateriaal en de dentinewanden, en kan doordringen in de dentinale tubuli. We hebben geconcludeerd dat tandartsen het restauratiemateriaal voor de perforatie zorgvuldig moeten kiezen, omdat dit materiaal gevolgen heeft voor de levensvatbaarheid van de koloniserende bacteriën.

Hoofdstuk 5 – Volgens dit onderzoek lijkt CLSM een betrouwbare techniek te zijn voor het beoordelen van bacteriële penetratie en proliferatie in primaire molaren na pulpotomie. Het huidige onderzoek toont aan dat bacteriën na pulpotomie kunnen doordringen tussen het basismateriaal en dentine en dit kunnen koloniseren, onafhankelijk van het soort basismateriaal dat voor de pulpotomie werd gekozen. Desondanks werd de omvang en de vitaliteit van de bacteriële kolonisatie beïnvloed door het soort basismateriaal voor de pulpotomie.

Hoofdstuk 6 – Verkleuring van voortanden na endodontische behandeling kan leiden tot verminderde esthetiek. De intracoronale toepassing van bleekmiddelen (bijv. carbamideperoxide, natriumperboraat, waterstofperoxide) is met succes toegepast voor het bleken van wortelgevulde tanden. Er is weinig informatie beschikbaar over de invloed van verschillende irrigatieoplossingen op de penetratie van peroxide. In dit hoofdstuk hebben we de invloed beoordeeld van verschillende irrigatieprotocollen op het bleekmiddel in de dentinale tubuli met behulp van ons op CLSM gebaseerde model. We hebben gezien dat peroxide doordringt tot in het extraradiculaire gebied van de tanden. De mate van peroxidepenetratie was significant hoger wanneer de irrigatiesequentie bestond uit fosforzuur voorafgaand aan plaatsing van het bleekmiddel.

Hoofdstuk 7 – In dit hoofdstuk hebben we een nieuwe methodiek geïntroduceerd voor het analyseren van de oppervlakteruwheid van het 3D-oppervlak van

dentine na het toepassen van verschillende irrigatieoplossingen. Uit de resultaten bleek dat het irrigeren van het wortelkanaal met natriumhypochloriet (NaOCl) en ethyleendiaminetetra-azijnzuur (EDTA) weliswaar invloed heeft op de structuur van het dentineoppervlak door de oppervlakteruwheid ervan te vergroten, maar dat de exacte irrigatiesequentie van deze materialen geen significant effect had. Voor het bepalen van het effect van de irrigatiesequentie op de penetratie van bacteriën en vulmateriaal, is nader onderzoek met op CLSM gebaseerde modellen nodig.

Conclusies

1. CLSM lijkt een betrouwbare techniek te zijn voor het beoordelen van de penetratie en proliferatie van bacteriën in dentinale tubuli, waarmee zowel levende als dode bacteriën *in vitro* beoordeeld kunnen worden.
2. Bacteriën dringen diep door in de dentinale tubuli. Deze invasie is variabel en wordt beïnvloed door de tubulaire structuur van de wortel en door het gebruikte retrograde vulmateriaal en de daadwerkelijke penetratiediepte ervan in de dentinale tubuli.
3. Bleekmiddelen dringen door in het extraradiculaire gebied van tanden. De mate van peroxidepenetratie is echter significant hoger wanneer de irrigatiesequentie bestond uit fosforzuur voorafgaand aan plaatsing van het bleekmiddel.
4. Wortelkanaalirrigatie met NaOCl en EDTA verhoogt de oppervlakteruwheid, maar de exacte irrigatiesequentie heeft geen significant effect. Nader onderzoek is nodig voor het bepalen van het effect van de irrigatiesequentie op chemische retentie.

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