The use of confocal laser scanning microscopy for the study of dentin infection

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Bacteria and their products in the root canal system of necrotic teeth are the main etiological factor of apical periodontitis. One of the major advantages of confocal microscopy is the ability to analyze by thin optical sections the subsurface structure of biological samples. We describe the ability of the confocal microscopy to investigate the pattern of dentin infection provided by different infection models used in endodontic research that includes laboratory and in situ infected dentin. Confocal pictures of in vivo infected dentin from necrotic root canals are also presented. The ability to allow 3D observation of bacterial colonization may have the potential to significantly contribute to in situ morphological studies of endodontic infections as well as the ex vivo antibacterial effects of endodontic substances and procedures.

Keywords: bacteria; dentin; confocal microscopy; pulp necrosis; biofilms; oral microbiology.

1. Introduction

Bacteria and their products in the root canal system of necrotic teeth are the main etiological factor of apical periodontitis [1, 2]. The significance of root canal decontamination to achieve healing of apical periodontitis has been addressed by several studies [3-6]. These studies provide the basis for the study of root canal disinfections protocols that include the testing of instrumentation techniques, antibacterial compounds and root canal dressings.

Ideally, the evaluation of different antibacterial protocols should be performed under a clinical environment [7]. However, in vivo studies are time consuming and difficult to standardized. In addition, microscopic analyses and clinical protocols to test new devices as, instruments or antibacterial compounds could be difficult to perform in humans without preliminary laboratory studies or because ethical considerations. By these reasons, alternative models are commonly used. These models include: the bovine or human infection dentin model using extracted teeth [8], in situ models [9, 10], animal models [11] and ex vivo models [12]. Validation of dentin infection models includes microbiological and microscopic analyses [13, 14].

The development of confocal microscope (CLSM) and the improvement of direct viable staining methods have stimulated the study of bacteria’s viability in the last years, especially in the biofilm area. The use of CLSM for the study of dentin infection and oral biofilms has been reported in some early studies [15-17]. Potential advantages of confocal microscopy for the study of dentin infection includes: observation of live bacteria in dentinal tubules and root canal walls by the use of vital staining techniques [15, 16, 18], identification of labeled bacteria by in situ hybridization [19] and 3D visualization of structural organization in biofilms [20]. Optical sectioning and the ability to study the subsurface of hydrated specimens could be useful to analyze samples that are performed by sectioning undecalcified dentin avoiding contaminants or artifacts situated at the surface layer.

To date, there is only two published data [19, 21] about distribution of bacteria and biofilms in necrotic human root canals using the confocal microscope, maybe, because of laboratory procedures to study clinical samples are limited or are not completely developed. The aims of this work are: 1. To describe the ability of a confocal microscopy technique to investigate the pattern of dentin infection provided by different models used in endodontic research that includes laboratory and in situ infected dentin; animal and human infected dentin from necrotic root canals were also evaluated.

2. Materials and Methods

The sample of this study were: five bovine teeth infected with the bacteria Enterococcus faecalis under laboratory conditions, six bovine sterile dentin blocks used in an intraoral device by a volunteer to induce dentin infection of the specimens and two distal roots of mandibular molars and one extracted root of a maxillary pre-molar extracted by non-restorable carious lesions and vertical fracture.

2.1 In vitro dentin infection

Five bovine teeth with a length of about 7-8 mm were prepared by sectioning the root tip, and after the crowns were removed at below the cemento-enamel junction. Each root canal was enlarged to a size of a gates glidden bur # 5...
To create the bacterial inoculums, isolated colonies (24 h) of pure cultures of *Enterococcus faecalis* grown aerobically on BHI agar plates were suspended in 3.0 ml of BHI. For dentin infection, under laminar flow five blocks were transferred individually into 3 ml of BHI inoculated with 200µl of an overnight *E. faecalis* suspension for 21 days. BHI was changed every 48 hours, and the purity of the broth was verified to avoid contamination. A sterile sample was used as negative control.

2.2 In situ dentin infection

This model includes the use of an intraoral appliance according to Zaura [22]. Six Dentin sections (3mmx3mmx2mm) were obtained from sterile bovine radicular dentin. The samples were treated with 2.5% sodium hypochlorite for 15 minutes and 17% EDTA for 3 minutes. The dentin samples were fixed in an intraoral orthodontic device to allow the biofilm development. In order to standardize the microbiota and rate of biofilm development, one healthy single volunteer used the intraoral device for 36 hours. The ethical committee involving human research approved the use of the intraoral device (064/2009). Informed consent was also requested. At the end of the experiment the dentin specimens were carefully washed with 100µl of sterile distilled water prior to the staining procedure. Two sterile samples that were not in contact with the oral environment were used as negative control.

2.3 Human infected dentin

Two non-vital distal roots of two mandibular first molars extracted by non-restorable carious lesions and one maxillary pre-molar extracted by a vertical fracture, were included. The mandibular first molars were part of a study that involves the evaluation of different root canal filling techniques. After the extraction a round bur was used to expose the pulp chamber to facilitate the fixation procedure. Then, the teeth were fixed in 10% formalin buffer for 72 hours. Prior to the sectioning and staining procedures the external surface of the teeth was cleaned and all the organic tissue attached to the root was eliminated carefully using a razor blade and alcohol.

2.4 General sectioning, staining and CLSM procedures

The samples of the in vitro and the extracted human teeth were sectioned using a 0.3mm Isomet saw under constant cooling using sterile distilled water. The roots were fixed on an Isomet platform using a low fusion impression compound (Kerr, MI, USA). Two 1mm sections were taken form each root corresponding to the apical and middle third of each root. The dentin segments from the in vitro experiment were not sectioned because they were flat before the infection being unnecessary the section for confocal analysis. The dentin sections corresponding to the in vitro and in situ experiments and the human sections were washed carefully with 100µl of sterile distilled water and stained with 100µl of 0.01% acridine orange in a dark environment for 30 minutes and then rinsed with 100µl of distilled water; such dye has the ability to bind with bacterial RNA emitting red fluorescence and to bind with bacterial DNA emitting green fluorescence. After the staining procedure, the corresponding specimens were immediately analyzed by the CLSM technique. The negative controls of the in situ and in vitro experiments were stained with the same protocol. For the human infected dentin study, an extracted vital tooth was used as negative control. All dentin samples were examined on an inverted Leica TCS-SPE confocal microscope (Leica Microsystems GmbH, Mannheim, Germany) using a modified chamber device. For the acridine orange staining the excitation and emission for RNA was 460 and 650 nm; the excitation and emission for DNA was 500 and 526 nm respectively. The sequential frame scan mode was used in order to prevent crosstalk. The in vitro, human and animal infected dentin specimens were scanned at the subsurface level, 5-10µm inside the dentin structure. The dentin from the in situ experiment was scanned from the top of the biofilm to the dentin surface. All the samples were observed using the 5X lens, 10X lens, 40X oil lens, 63X oil lens and 63X oil lens with an additional zoom of 3X. The 40X pictures were obtained by using 20-40 sections of 1 µm step size in a format of 1024 X 1024 pixels. The 63X zoom 3 pictures were taken by using a 0.3µm step size. The images were acquired using the Leica Application Suite-Advanced Fluorescence software (LAS AF, Leica Mannheim, Germany). Representative 3D reconstructions were performed from the confocal stacks using the open-source software OsiriX 3.6.1 available at: http://www.osiris-viewer.com/Downloads.html, running under Leopard Mac OS X 10.6.3 software (Apple computer Inc., Cupertino CA, USA).

3. Results and discussion

3.1 In vitro dentin infection

At 100X magnification all the root canals studied appear empty with no evidence of organic structures in root canal walls (Fig 2A). 40 X oil lens examination showed fluorescence in dentinal tubules that represented a clear dentin
infection in all the studied samples (Fig 1C). The more common finding was the presence of dentin infection with discrete colonization of the root canal walls, usually between 2-4 layers of cells (Fig 2B, D). Very sparse isolated bacterial colonies were also occasionally observed, bacteria in this structures appears to be coaggregated and were only visible using the 63X oil lens with zoom 3 (Fig 1E). Bacterial penetration in dentinal tubules was variable, usually 1 or 2 operative fields using the 40x oil lens (100-400µm).

![Image](image_url)

**Fig. 1** (A) 100X picture of the root canal wall (*) of bovine dentin infected under laboratory conditions, despite the presence of dentin infection (detail) there is absence of visible organic structures in root canal walls using this magnification (B) root canal wall of in vitro infected specimen showed weak colonization of root canal walls (*), infection of dentinal tubules is present (detail). (C-D) infected dentinal tubules in almost absence of canal wall colonization, a thin layer of cells can be seen (arrow) (E) a *Enterococcus faecalis* colony can be seen attached to the dentin, bacteria appears to be coaggregated.

### 3.2 In situ model

The experimental samples appeared intensely stained (Fig.2A). Confocal scanning was performed at this magnification; at least 50-100µm of scanning was required to acquire all the information in the Z-projection. This information showed that a high volume of labeled material (Biomass) was present on the dentin (Fig 2A-E). Carefully examination of these structures reveals the presence of complex biofilms with many morphological variations usually as heterogeneous forms, mushroom-like structures, or firmly compacted to the dentin structure. In very isolated areas, the process of initial colonization of the dentin was evident (Figure 2B). Initial stages of dentin infection in this model begin with a layer of bacteria of some micrometers (2-5µm) firmly adhered to the dentin. Initial colonization of dentinal tubules at this stage was visible using the 63X oil lens (Fig. 2B-C). Bacteria appear to be coaggregated in localized points forming a mass that was limited to some micrometers. These isolated biofilms spread forming a firm layer that has intense fluorescence and usually do not exceed from 10µm in the Z-plane (Fig 2C). More complex higher structures in the Z-plane could also be found; these structures seem to be in relation with biomass growth usually seen as mushroom-like structures that exceeded 50µm in height. (Figure 2D).
3.2 Human infected root canals

The presence of organic structures in root canal walls discernible at relative low magnifications (100X) was the common characteristic (Figure 3B). In spite of the presence of a firmly attached layer of bacteria in form of biofilm could be seen in some samples (Figure 3B, D), another sections showed the presence of amorphous organic structures attached to the canal walls. Dentin infection appears to be highly correlated with the presence of these amorphous structures (Figure 4B-C). In limited area bacteria appears to be enclosed inside these structures (Figure. 3D). Observation of the 3D pictures showed irregular and mushroom-like surfaces morphologies on root canal walls (Fig. 3D-E, 4B). Negative controls evidenced stained peritubular dentin without bacteria (Fig. 5A-B). Dentinal tubules of vital teeth appeared stained, maybe by the presence of odontoblastic prolongations. No bacterial profiles were evident. Clear differences between the infected and non-infected dentin were evident (Fig. 5C).

Sawing undecalcified dentin and pulp tissue for microscopically purposes have been performed by previous studies. Lan et al. [23] used an Isomet saw machine to slice tooth specimens for histological purposes, he describe that this method has a low risk of laceration of the pulp. Gonçalves et al. [24] sliced extracted vital mandibular molars using an Isomet saw for implantation purposes in mices to study pulp angiogenesis; the pulp vitality appeared not be affected by the cutting process and the histological sections not revealed artifacts product of the cutting process. In this context, we verified that an interesting confocal microscopy property is the ability to perform scanning at the sub-surface level avoiding the smear layer and contamination created by the sectioning procedure on in vitro and in vivo samples. This procedure appears to be important to future studies that include fresh extracted teeth to study metabolic activity of microorganisms in root canals, since the inclusion of the teeth in paraffin or resin for sectioning can eliminate the bacteria’s viability. Advantages of the sawing technique are that specimen processing and analyzes of the samples can be performed in a reasonable time. However, one disadvantage of this technique is that is impossible to get thin slices. The use of thin slices (2-5µm) is critical to show bacterial contamination at the apical third level for example. Another limitation is that bacteria suspended in a fluid phase in root canal walls are lost during the section procedure, limiting the analyzes only to the bacteria and organic structures attached to root canal walls and bacteria situated in dentinal tubules. We think that many problems can be solved combining other microscopical techniques in the same sample as correlative optical and transmission electron microscopy where thin sections can be performed and compared with the confocal images or using a hard-tissue microtome [25, 26].
Fig. 3  (A) Detail of carious infected dentinal tubules of the cervical third of a necrotic root canal. The lumen of dentinal tubules appears enlarged and infected. (B) 400X 3D reconstruction of middle third section of the same specimen shows infected dentinal tubules (square), organic structures attached to root canal walls (*) and tertiary dentin (#). A detail of the square is shown in (C). Dentinal tubules appear stained but no bacterial profiles are seen. A section from the confocal stack shows the presence of coccoidal cells in the dentinal tubules (arrow in detail). (D) Apical section shows less tubular density in the dentin and bacterial profiles are clearly seen attached to the root canal walls (area in the square is shown in detail in E). A 3D projection of the attached structure (detail in D) shows an evident compact biofilm with visible bacterial profiles and smooth surface.
Fig. 4  Maxillary pre-molar extracted by non-restaurable fracture is shown in (A). A 100X transversal apical section can be seen in the detail in (B). Intense colonization of the root canal wall by diverse attached mushroom-like biofilms structures is evident. 3D reconstruction showing clearly the source of bacterial cells in dentinal tubules, that is the biofilm in root canal wall (B). A 630X magnification with zoom 3 shows intense dentinal tubule infection. The bacterial profiles are restricted to dentinal tubules and not in the intertubular dentin (C).

Fig. 5  Negative controls from in vitro and in situ experiments are shown in (A-B). Dentin structure of a non-infected teeth is shown in (C). Dentinal tubules appear stained maybe by the presence of odontoblastic prolongations, no bacterial profiles are evident in any sample.

In summary, this work showed that high-resolution images of necrotic root canals and biofilms developed on dentin can be achieved using confocal microscopy, the fact that the slicing technique described in this study allows the capture of high-resolution images showed that research about biofilms and distribution of bacterial DNA in root canals in situ is promissory. Future refinements of this technique including the use of the fluorescence in situ hybridization and the use
of other staining protocols as the Live/Dead technique will allow to show the description of the tridimensional distribution of bacteria in endodontic biofilms and most important, the establishment of the metabolic activity and vitality of bacteria in infected root canals from fresh non-dehydrated samples. Discrepancies between the different infections models used in endodontic research should be taken into account for a more reliable interpretation of microbiological laboratory studies and their application to the clinical practice. This detail needs to be addressed in future studies.

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References