Photodiagnosis and Photodynamic Therapy 22 (2018) 19–25

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

An *in vivo* evaluation of microbial diversity before and after the photo-activated disinfection in primary endodontic infections: Traditional phenotypic and molecular approaches

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ARTICLE INFO

Keywords:
Anaerobic bacteria
Antimicrobial photodynamic therapy
Diode laser
PCR
Primary endodontic infection
Toluidine blue O

ABSTRACT

Background: It is essential to identify the root canal microbial diversity and count. This is due to the polymicrobial nature of the primary endodontic infection that is associated with the microbial diversity and increased resistance to the antimicrobial agents. Photo-activated disinfection (PAD), also known as antimicrobial photodynamic therapy, is a new promising non-antibiotic approach, studied to prevent microbial resistance and treatment failure.

Materials and methods: In this study, we investigated the effect of PAD on reduction of microbial diversity and count, related with primary endodontic infections. Microbial specimens were collected before PAD from patients infected with the primary endodontic infection. PAD with toluidine blue O (TBO), in combination with diode laser, was performed on infected root canals. Resampling was carried out on the root canal after PAD, and microorganisms were identified by classical microbiological tests using biochemical and analytical profile index (API ® 20A) assays and nucleic acid approaches.

Results: From the 36 subjects studied before TBO-PAD, 187 cultivable isolates from 14 different genera and 19 various microbial species were retrieved. Of the bacterial isolates, 45.4% were strict anaerobes including *Veillonella parvula*, *Porphyromonas gingivalis, Propionibacterium acnes*, *Lactobacillus acidophilus, Campylobacter rectus*, and *Slackia exigua*, in order of their frequency; 45.4% were facultative anaerobes; and 9.2% were microaerophilic bacteria (*Aggregatibacter actinomycetemcomitans*). This *in vivo* study revealed significant decrease in the microbial diversity and count of the infected root canal after TBO-PAD ($P < 0.05$); whereas *P. gingivalis* strains, the most resistance microorganisms, were recovered in 34% of the samples after TBO-mediated PAD ($P > 0.05$).

Conclusions: TBO-mediated PAD is an effective in exhibiting efficient antimicrobial activity due to the substantial reduction of the microbial diversity and count in the primary endodontic infections.

1. Introduction

Primary endodontic infection is characterized by presence of wide range of microbial diversity dominated by anaerobes that initially invade the dental pulp [1]; this includes the large microbial diversity and count, which is constantly updated, and the microbial resistance to antimicrobial agents, used as intracanal medication in root canal treatment [2,3].

Root canal treatment is generally carried out to control or eliminate the pulp inflammation and periapical infection by eradicating the pathogens [2,4]. Accordingly, treatment of endodontic infections involves chemomechanical procedures using antimicrobial agents such as chlorhexidine, sodium hypochlorite, and calcium hydroxide [5]. Although the intracanal microbial communities are significantly reduced by the chemomechanical procedures, successful treatment is based on the effective decontamination of the root canal system [4]. Alternatively, the resistance of the intracanal microbial communities to antimicrobial agents has increased [6]. Although decontamination with antimicrobial agents seems an appropriate approach, it is practically difficult to eliminate the intracanal microbiota completely [7]. Literature suggests that development of new technologies to eliminate the persistent microorganisms remains a challenge [8–10]; however, photo-activated disinfection (PAD), also known as antimicrobial photodynamic therapy or photochemotherapy, is known to be an effective adjunctive therapy, along with the traditional root canal irrigates and antimicrobial agents, in eliminating the intracanal microbiota [5,11].

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https://doi.org/10.1016/j.pdpdt.2018.02.016
Received 20 September 2017; Received in revised form 1 February 2018; Accepted 20 February 2018
Available online 24 February 2018
1572-1000/

PAD acts through a non-toxic photosensitizer and a low dose irradiation with a suitable wavelength of visible light. The photosensitizer is excited in the presence of oxygen, thus producing high reactive oxygen species, which are toxic for the microbial cells [12].

The intracanal microbiota is found to be highly diverse [1]. A thorough examination of the microbial load and content in the primary endodontic infections could advance the treatment strategies and outcome of endodontic therapy. To the best of our knowledge, no data exists on the microbial load combined with microbial composition analysis, before and after the PAD treatment, *in vivo*. Therefore, this clinical study aimed to determine the composition and load of the root canal microbial community in the primary endodontic infection by using both microbiological culture and molecular technique, before and after the PAD treatment.

2. Material and methods

2.1. Subjects and case selection

Out of 128 subjects who attended the private dental clinic, Tehran, Iran, for their endodontic treatment, 36 (28%) patients with apical periodontitis were selected for this study based on stringent criteria and 92 (72%) subjects were excluded. Inclusion criteria involved teeth with closed apex, one single-root, intact pulp chamber walls, intact root, and primary infected pulps, which were confirmed by common endodontic pulp vitality tests and radiography. Exclusion criteria involved patients who received antibiotic treatment in the previous month, cases with severe systemic disease such as diabetes mellitus, cardiovascular diseases, primary immunodeficiency diseases, and pregnancy or lactation, as well as teeth that could not be suitably isolated using a rubber dam, and teeth with a periodontal pocket probing depth greater than 4 mm. The Ethics Committee of the Iranian Registry of Clinical Trials (IRCT ID: IRCT2015031321455N1) approved the protocol describing the specimen collection for this investigation.

2.2. Root canal sampling procedure

Sampling was done from the root canals in strict aseptic conditions, as described previously [13]. Briefly, the supragingival calculus was removed by scaling and cleansing with pumice before the rubber dam application, and existing restorations and caries were removed by sterile high- and low-speed burs. After the application of previously disinfected rubber-dam, the tooth and the surrounding field were cleansed with 30% hydrogen peroxide and were then decontaminated with 2.5% sodium hypochlorite solution (Sigma-Aldrich, USA) for 1 min. Subsequently, 5% sodium thiosulfate (Sigma-Aldrich, USA) was used for 2 min to neutralize the disinfectants. Before entering the pulp chamber using new sterile bur without the use of water spray, the pulp chamber floor and walls were disinfected according to the same protocol of disinfection and neutralization of disinfectant procedures as described for rubber-dam and tooth. The sterility of the pulp chamber was checked using a microbiological sample by sterile paper points No. 30 (Meta Biomed, Korea), which was immediately transferred aseptically to tubes containing 2 mL of brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) for 24 h. Samples with positive culture results were excluded from the study. Patency of the root canal to the apical region was established using endodontic k-files (#15–30) with minimal instrumentation, where possible, until the first microbiological sample was taken without any irrigation with sterile normal saline and/or chemical solutions [13,14]. Root canal sampling was thus appropriately coincidental with digital X-ray radiography of canal length. Furthermore, the canal length was determined by an electronic apex locator (Raypex 5; VDW, Munich, Germany) and confirmed by digital X-ray.

Samples were initially collected with one sterile paper point No. 30 that were introduced to a level approximately 1 mm less than the tooth apex. The paper point was retained at one place for 1 min and was then transferred with sterile forceps to croute containing Viability Medium Göteborg Agar (VMGA) III transport medium. Samples were instantly carried to the microbiology laboratory within 15 min.

2.3. Photosensitizer and light source for endodontic therapy

After root canal sampling, the root canals were treated using PAD technique. Based on a previous study, a stock solution of toluidine blue O (TBO) (Sigma-Aldrich, Steinheim, Germany) with higher purity as a photosensitizer at a final concentration of 100 μg/mL was prepared in sterile 0.9% (wt/vol) sodium chloride, and was filtered with a 0.22 μm pore size membrane filter [15]. TBO was kept under dark conditions prior to use. Furthermore, the root canal was filled with TBO solution using a sterile insulin syringe for 5 min.

The canal was then irradiated by a diode laser (DX82, Konftec, Taiwan) at 635 nm wavelength with a fiber optic coin delivery system (diameter 0.25 mm; WF-354A, Konftec, Taiwan) which ends in an optic cap (diameter 7.5 mm) for DX82 diode laser device. Diode energy was emitted in continuous wave mode for 60 s per treatment. The root sampling and microbiological analysis was performed after the PAD procedure, as aforementioned (Section 2.2). At the end of sampling after PAD, the root canal treatment was carried out by an endodontist based on the usual treatment procedures [16]. Polymicrobial feature of two samples (i and ii) of primary endodontic infections on enriched blood agar medium before and after the PAD was shown in Fig. 1.

2.4. Samples processing for cultivation

Samples in VMGA III vials were dispersed with a vortex for 30–45 s, and 10-fold serial dilutions were made in 90 μL BHI broth. Eventually, 10 μL from each dilution was plated on the BHI agar plates (Merck, Darmstadt, Germany) supplemented with 5% defibrinated sheep blood, 0.6% (wt/vol) yeast extract (Merck, Darmstadt, Germany), 5 mg/L hemin, and 1 mg/L menadione (both purchased from Sigma-Aldrich, Steinheim, Germany). The plates were incubated at 37 °C anaerobically (80% N_2, 10% CO_2, and 10% H_2) using the Anoxomat System™ (MART Microbiology BV, The Netherlands) for up to 7 days.

Moreover, similar dilutions were plated on the BHI agar containing 5% defibrinated sheep blood (sBHI agar) for the growth of the aerobic, microaerophilic, and facultative bacteria, and in Sabouraud dextrose agar (SD agar; Oxoid, Basingstoke, UK) containing 100 μg/mL chloramphenicol for detection of *Candida* spp.; the sBHI agar and SD agar were incubated at 37 °C for 24–48 h in aerobic and microaerophilic (in 10% CO_2) conditions, depending on the type of microorganisms. All the samples were cultivated in triplicate, and after incubation, colony forming units were counted in each medium using Miles and Misra method for isolating various phenotypes [17].

2.5. Traditional culture identification

All microorganisms involved in the primary endodontic infection in this study were detectable by cultivation. Gram staining was performed on the pure isolates of root canal, and the microbial species were preliminarily characterized based on their colony features (*i.e.*, size, color, shape, surface, hemolysis consistency, and brightness). Conventional biochemical tests were performed to identification of the aerobic, microaerophilic, and facultative microorganisms. The most obligate anaerobic bacteria were identified by the analytical profile index (API ® 20A; BioMerieux, Marcy l'Etoile, France) assay. The isolates that could not be traced by API ® 20A method were identified by 16S ribosomal RNA (*16S rRNA*) gene sequencing, according to a previous study [2].