National and Kapodistrian University of Athens

School of Dentistry

RESEARCH PROTOCOL

TITLE OF PROPOSED PhD THESIS:

Clinical antibacterial efficacy and treatment outcome after implementing various root canal irrigating procedures

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DATE OF SUBMISSION:

Athens, 12/12/2022

Introduction-Scientific background

One of the main objectives of endodontic treatment is to eliminate the bacterial load located inside the root canal system. Endodontic cases with pulp necrosis and apical periodontitis are mainly characterized by an initial high bacterial load irrespective of presence or absence of symptoms (1,2). Chemomechanical preparation is the most important stage of endodontic treatment which is mainly correlated with the establishment of a favorable treatment outcome. Although the irrigation solutions used during root canal instrumentation play a significant role on root canal system disinfection, they cannot completely eliminate the intracanal bacteria population. The main reason of this issue is that the biofilms created by the bacteria due to chronic endodontic disease seem to be extremely resistant to the chemical agents used (3-5).

Endodontic treatment failure usually occurs when an intraradicular infection is not properly controlled by treatment procedures (6). Infection is present virtually in all cases of post-treatment apical periodontitis (7-8). Studies have reported that the treatment outcome is negatively affected by bacterial persistence in the root canal at the time of filling (6-7). For this reason, final irrigation with sodium hypochlorite is considered as one of the most important stages of root canal disinfection. At this point, the mechanical instrumentation has been completed and final irrigation is anticipated to decrease further the microbial load under a level in which periapical disease can be controlled and the healing process of the periapical tissues may begin. However, even after appropriate conditions a residual bacterial load may be present inside the root canal system affecting possibly the outcome of endodontic treatment (9).

Intending to solve this issue, a number of supplemental procedures have been proposed to increase effectiveness of irrigating solutions and to enhance canal disinfection, including agitation with hand files or plastic instruments or the use of sonic and ultrasonic devices (10-11). Recently, a new file, the XP-EndoFinisher (#25/.00 [XPF; FKG Dentaire, LaChaux de Fonds, Switzerland]), was introduced as a complementary universal instrument that can be used after root canal instrumentation with any file system with a diameter of ISO 25 or more for cleaning highly complex morphologies and difficult-to-reach canal areas, such as oval canals with isthmuses, apical deltas or ramifications (12-14). When working inside the root canal, XPF can be enlarged up to a 6- mm diameter and has a minimal effect on the dentin tissue on the root canal

walls. So far, only a clinical trial has utilized XP EndoFinisher to study the effects of the instrument on the outcome of root canal treatment concluding that the use of this specific file as a supplementary tool did not affect the success rate of the treatment in posterior teeth with apical periodontitis (15). However, this study did not evaluate the influence of the instrument on the reduction of bacterial load of the canals. That was performed by two other similar clinical studies which showed a remarkable bacterial reduction after the use of the XP- EndoFinisher instrument (16, 17). Until now, no study has been performed which investigates simultaneously the influence of various supplementing irrigation procedures on the intracanal bacterial load and the outcome rate of the treatment.

Unlike previous similar studies that have used culture-dependent approaches with certain disadvantages such as low sensitivity, difficulty or inability of many species to grow or be detected and time consuming which have mainly led to the underestimation of intracanal microbiota, the present study will use a culture-independent approach to identify and quantify intracanal bacteria after different stages of root canal disinfection. More specifically, Real-Time PCR, also mentioned as Quantitative (qPCR) will be used to quantify not only the total bacterial load but also the quantity amount of two specific bacterial species. The technique is very sensitive and highly efficient with the ability to detect both cultivable and uncultivable species without need to control anaerobic conditions. However, it does not come without limitations; the main one is that the viability and pathogenicity of the detected microorganisms cannot be specified (18,19,20).

So, as already mentioned, besides the effort to quantify the reduction of the total microbial load during chemomechanical preparation, the present study, have selected four bacterial species namely, *Pseudoramibacter alactolyticus* ,*Treponema denticola, Streptococcus anginosus and Porphyromonas endodontalis* to be studied regarding their quantitative changes throughout chemical disinfection. The rationale behind the selection of those specific species was to include both Gram-positive (*Pseudoramibacter alactolyticus, Streptococcus anginosus, Porpyromonas endodontalis*) and Gram-negative species (*Treponema Denticola*), as most abundant and resistant in asymptomatic or symptomatic cases of apical periodontitis (21-24). In addition, all species has been shown that may survive during endodontic procedures and may be encountered

in secondary infections. The latter as a result would be of outmost importance to be correlated with the clinical results of healing process after one year recall examination.

As previously mentioned and so far, limited data have been published investigating the XP finisher as supplementary method of effectively reducing the total amount of bacteria in the root canal system during the root canal treatment procedure. On the other hand, Passive Ultrasonic Irrigation (PUI) is considered as a gold standard technique which is widely used in order to activate the irrigating solutions inside the root canal system and it will be used as comparator to XP Endo finisher activation technique.

Aims of the study

The main aim of the study is to evaluate and compare the antibacterial efficacy of three final root canal chemomechanical preparation strategies, namely final irrigation of NaOCl 2.5%, final irrigation of NaOCl 2.5% plus PUI and final irrigation of NaOCl 2.5% plus XP EndoFinisher. Total bacterial load that will remain after chemomechanical preparation after final irrigation of NaOCl 2.5% will be compared to the number of bacteria calculated after the first and the second part of the disinfection with the supplementary means.

The second aim is to evaluate the antibacterial efficacy of irrigants activated by ultrasonic irrigation and of mechanical supplement preparation of XP EndoFinisher on the amount of decrease in the number of four different bacterial species *Pseudoramibacter alactolyticus*, *Treponema Denticola, Streptococcus anginosus* and *Porphyromonas endodontalis* which have been shown, as already mentioned, that are abundant in asymptomatic and symptomatic primary and secondary infections respectively located inside.

Finally, the antibacterial efficacy of the above three different root canal preparation strategies will be compared in terms of treatment outcome. All patients will be recalled for one-year follow-up examination where the outcome of endodontic treatment will be evaluated through clinical and radiographic examination. This will be done by evaluating the possible decrease of the size periapical lesion or the complete healing of the periapical tissues and the possible existence or not of clinical pathologic signs and symptoms.

Materials & Methods

Study design

The study has been designed as two-arm, parallel, randomized clinical. The randomization of the teeth for sampling will be performed through the use of a special software (www.randomizer.org). All the treatment and sampling procedures will be carried out by the same investigator (PhD candidate Dr. Ch. Papadopoulou).

Sample size calculation (Power analysis)

According to published articles by Amaral et al (16) and Ballal et al (17) use of final with NaOCl (concentration 2.5%), may induce a reduction of total bacterial load of 55% (100% initially- 45% after chemomechanical preparation), in terms of percentage difference of Quantitative Polymerase Chain Reaction–positive samples. If we assume an expected reduction in bacterial load positive samples of 95%, for the novel intervention (NaOCl 2,5%), which accounts for a percentage difference in reduction between the interventions of 40%, with an alpha value of 0.05 and an assumed power of 80%, the total sample required is 36 patients (18 per group). To account for any losses to follow- up, the final recruited number of patients will be raised to 22 per group, that is 66 patients in total.

Study population

The study population will be comprised of patients who will be referred for endodontic treatment at the Postgraduate program of the Department of Endodontics of School of Dentistry (National and Kapodistrian University of Athens). Based on the estimated sample size, a total of 66 patients will participate in the study. Sixty-six single rooted will be selected and randomly allocated to each experimental group.

A number of inclusion and exclusion criteria will be set for the participation of patients and eligibility of teeth in the study as follows:

Inclusion criteria

- Informed consent by the patients who wish to participate in the study.
- Single-rooted teeth with pulp necrosis confirmed by pulp sensibility tests, negative response to both cold and electric pulp testing and radiographic evidence of apical periodontitis.
- Teeth with relatively straight canals, complete root development and no radiographic evidence of pulp canal obliteration.

Exclusion criteria

- Patients who have received antibiotic treatment the last 3 months or need chemoprophylaxis for dental treatment.
- Teeth with previous endodontic treatment.
- Teeth with cracks or incomplete vertical root fracture which disturbs the integrity of the pulp chamber walls or teeth with the pulp chamber exposed to oral environment.
- Teeth with periodontal pocket more than 4mm.

Initial clinical and radiographic examination

All teeth will be examined clinically for the possible presence of periodontal pocket, percussion and palpation tests will be performed as well as pulp sensibility tests (cold and electric pulp testing). A negative response to pulp sensibility tests; absence of pulpal bleeding during access cavity preparation; and the presence of periapical radiolucency, possible presence of sinus tract, purulent drainage or swelling will be the foremost clinical parameters considered for diagnosis of pulpal necrosis. Preoperative radiographic evaluation will include two periapical radiographs with different horizontal angles by using the parallel cone technique.

Only teeth with intact pulp chamber walls, necrotic pulps as confirmed by negative response to pulp sensibility tests, and radiographic evidence of apical periodontitis will be included.

Treatment and sampling procedures

The compliance to aseptic techniques is essential throughout the entire experimental procedures (25). Before the application of the rubber dam, supragingival scaling will be carried

out at the area of interest. Caries and defective restorations will be removed. Then, the specific tooth will be isolated. An established disinfecting procedure will follow at the operating field, the tooth and the clamp. Firstly, 3% hydrogen peroxide will be applied and then 2,5% NaOCl at all the above-mentioned surfaces. A sterile high-speed bur under constant sterile saline irrigation will be used for access cavity preparation. When the access is completed, the field will be disinfected again including the access cavity. Inactivation of NaOCl, will be carried out through the use of 5% sodium thiosulphate and then a paper point will be scrubbed at the access cavity walls in order to take a sterility control sample. Only teeth of which their sterility samples are negative will be included in the study.

The first sample (S1) will then be taken. A saline irrigation will be performed within the root canal, a sterile K file # 15 will be inserted 1 mm less than the radiographic apex, following a estimation obtained from the initial radiograph and subsequent accurate calculation by using an apex locator (Root ZX mini, Morita, USA). Then, mild mechanical instrumentation will be performed. Three sterile paper cones will be positioned sequentially at the same length to absorb the liquid of the canal, each cone remaining in place for at least 1 minute. The paper cones will be aseptically transferred to Tris-EDTA buffer (10 mmol / L Tris-HCl, 1 mmol / L EDTA, pH = 7.6) and immediately frozen at _20_C. Subsequently, complete chemomechanical root canal preparation will be performed. Root canal irrigation will be performed each time from one instrument to the next with a luer lock syringe and a 27G needle (Endo Eze, Ultradent).

The cervical and mid enlargement will be enlarged with mechanical Gates - Glidden burs. The apical third quarter will be mechanically instrumented with NiTi RaCe (FKG) cones of .02 to 40 / 04 where the second sample (S2) will be taken. After the chemomechanical preparation, the teeth will be divided in three groups. The group A will be supplementally irrigated with a final irrigation of NaOCl 2,5%. After that, a sample will be taken (S3). The other group, group B will be additional prepared with PUI. A sample will be taken (S3). The group C will be supplementally prepared with the XP Endo Finisher. A sample will be taken (S3). Samples S2 and S3 will be taken according to the procedure described for sampling S1. Clinical samples will be thawed to room temperature, and DNA will be extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the protocol recommended by the manufacturer. To quantify the total bacterial load

and levels of the four bacterial specise before and after treatment procedures, 16S ribosomal RNA gene–targeted qPCR will be performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 Real-time PCR instrument (Applied Biosystems) in a total reaction volume of 20 mL. Primers, qPCR conditions, and data analyses will follow.

DNA extraction & Quantitative PCR analysis

DNA from clinical samples will be isolated by commercially available Genomic purification kit according to the manufacturer's instructions. Yield and purity of isolated DNA will be determined spectrophoto-metrically and then diluted so that concentrations between samples are equal. Quantitative PCR (qPCR) also referred to as real-time PCR will be used for studying total microbial load and relative abundance of specific phylogenetic groups of microorganisms. qPCR is based on the real-time detection of a reporter molecule whose fluorescence increases as PCR product accumulates during each amplification cycle and allows for a relatively rapid yet quantitative assessment. Reactions will be performed on 96-well plates, using the Mx3005P Real-Time PCR System (Agilent) and the SYBR Select Master Mix (Applied Biosystems). Each reaction will consist of 2x SYBR Green buffer MIX with Taq polymerase, 0.3-0.5 µM of each primer, 5 µl of DNA template and water to 20µl. All reactions shall be prepared in duplicate and will be subjected to an initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 3-15 seconds, annealing at primer-specific temperatures indicated below (Table 2) for 30-60 seconds and amplicon extension at 72 °C for 60 seconds. Product specificity will be assessed by melting curve analysis and selected samples will be run on 2% agarose gels for size assessment.

Statistical analysis

Descriptive statistics will be performed for the pre-defined variables. Normality of distribution of residuals and homoskedacity will be checked first for the continuous analyzed data. A fisher's exact test will be performed to assess any differences in the qPCR positive samples between the three interventions in all stages in terms of final irrigation with NaOCl and the two supplemental procedures. Univariable and multivariable (median) regression analysis will be performed to assess the effect of different supplemental procedures on the reduction of bacterial load in teeth with apical periodontitis. Interaction effects will be checked though the likelihood ratio test.

Effect sizes and 95% CIs will also be presented. The level of statistical significance will be set at 95%. All analyses will be conducted with Stata version 15.1 (Stata Corporation, College Station, Texas, USA).

Follow up examination

Patients of all groups will be followed-up for 12 months to assess the outcome of the treatment:

- Two recall examinations will be performed at 6 and 12 months, respectively.
- As already mentioned, a standardized radiographic examination will be performed taking a periapical radiograph using the parallel cone technique. Possible reduction of periapical lesions in follow up radiographs will be evaluated compared to initial radiographs using the periapical PAI index. The PAI index score is used in endodontic outcome studies to define the absence or the presence of periapical lesions or the reduction of the lesion over time as a result of the healing procedure. This method is considered a reproducible and unbiased method for validating radiographic healing. The periapical index (PAI) provides an ordinal scale of 5 scores ranging from 1 (healthy) to 5 (severe apical periodontitis with exacerbating features). Its validity is based on the use of reference radiographs of teeth with verified histological diagnoses (26-29).
- Clinical examination will be also performed for any pathologic signs and symptoms and to assess if the tooth is or remains functional.

Clinical significance

- This is a randomized clinical study which evaluates simultaneously the antibacterial effect of three different root canal preparation strategies on intracanal bacterial load.
- In addition, a correlation between these canal preparation strategies and the treatment outcome will be investigated. This will be done for first time.
- The results of this study will be of utmost clinical significance and may be directly applied to clinical practice of both endodontists and general practitioners.

Expected outcomes

- A significant reduction in the total microbial load after chemomechanical procedure is anticipated. Final irrigation with 2.5% sodium hypochlorite is expected to lead further to significant better results. No further bacterial reduction is anticipated with the other two preparation strategies.
- In terms of treatment outcome, no significant associations are expected to be found among the root canal preparation strategies in the XP Endo Finisher compared to the PUI group.
- All four bacterial species are expected in a significantly lower number in S2 samples and even smaller although not significantly in S3 samples.

Schedule (phases) of the study

1st YEAR

1st semester: Literature Review

2nd semester: Treatment of patients- sampling procedures

3d semester: Treatment of patients - sampling procedures

4th semester: Treatment of patients - sampling procedures

2nd YEAR

1st semester: Learning laboratory procedures

2nd semester: Performing laboratory procedures

3rd semester: Performing laboratory procedures (qPCR)

4th semester: Performing laboratory procedures (qPCR)

3d YEAR

1st semester: Statistical analysis

2nd semester: Thesis Writing

3rd semester: Thesis Writing

4th semester: Thesis Writing

Places where the research will be held

1. Postgraduate clinic of endodontics, Dental School, National & Kapodistrian University of Athens

2. Laboratory of basic sciences and oral biology - basic sciences. Dental School, National & Kapodistrian University of Athens

3. Laboratory of oral biology – Periodontology. Dental School, National & Kapodistrian University of Athens.

4. Laboratory of Biology, Biochemistry and Physiology of Human and Microorganisms. Harokopio University School of Health Sciences and Management Department of Dietetics and Nutrition.

Budget of the study

Self-subsidized. The cost is estimated up to 5000 Euros.

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