National and Kapodistrian University of Athens

School of Dentistry

RESEARCH PROTOCOL FOR APPROVAL

TITLE OF PROPOSED PhD THESIS:

Clinical efficacy of various irrigation parameters in the reduction of intracanal microbiota

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Introduction

Microbial infection is the main cause of pulp necrosis leading to subsequent establishment of apical periodontitis. The primary role of microorganisms on the development and maintenance of periapical disease has been long documented through classic and historic studies. A favorable endodontic treatment outcome is directly related to the eradication or the sufficient reduction of microbial load located inside the root canal system. Chemomechanical preparation provides a significant reduction of the intracanal microbial load. However, sterile root canals cannot be obtained, and sometimes the remaining bacteria may compromise the outcome *(Barbosa-Ribeiro et al. 2021).* Thus, the study and development of additional clinical approaches for further root canal disinfection is essential.

Root canal irrigation is a fundamental part of chemomechanical procedure *(Haapasalo et al. 2014).* It complements mechanical preparation by the removal of residual bacteria, debris and necrotic pulp tissue, especially in areas that remain untouched by mechanical instrumentation procedure. Despite the great number of irrigants that have been proposed for use during clinical practice, sodium hypochlorite (NaOCl) remains the most commonly used irrigant due its tissue dissolution properties and its high antimicrobial capacity. Nevertheless, the optimal concentration of NaOCl (0,5-5%) for use in clinical practice is a matter of argument in endodontic research. Higher concentrations may exhibit better antimicrobial properties but the concomitant possible toxic effects on periradicular tissues should always be taken into consideration. So, an appropriate balance needs to be found between an adequate disinfection of the root canal system through the use of NaOCl and the minimal toxic effects of the irrigant on periapical tissues.

Traditionally, culture-dependent approaches have been used to identify and quantify intracanal bacteria after different stages of root canal disinfection. However, these methods have major limitations such as low sensitivity, difficulty or inability of many species to grow and time consuming. The first two limitations have led to the underestimation of intracanal microbiota which may cause endodontic infection or persist after therapeutic procedures. Molecular methods have been applied to overcome the aforementioned disadvantages of culture-dependent approaches. They are characterized by high sensitivity and specificity,

ability to detect both cultivable and uncultivable species without need to control anaerobic conditions. More specifically, Real-time PCR, also mentioned as Quantitative (qPCR) has been used to quantify single bacterial species or the total bacterial load of an infected root canal system. It is a very sensitive and highly efficient technique but it does not come without limitations; the main one is that the viability and pathogenicity of the detected microorganisms cannot be specified *(Siqueira and Rôças 2005).*

Final irrigation with sodium hypolclorite is considered as one of the most important stages of root canal disinfection. The mechanical root canal preparation has been completed and final irrigation is anticipated to decrease further the microbial load under a level in which periapical disease can be eliminated and the healing process of the periapical tissues will begin. Final irrigation can be categorized in three main parameters; the concentration and the volume of the main irrigant and the irrigation time. So far, few studies have investigated the effect of final irrigation time with NaOCl, whereas no study has quantified the volume and concentration of NaOCl that may lead to a sufficient decrease of the intracanal microbial load. Although some authors suggest that application time and volume of irrigant compensate the impact of concentration *(Gazzaneo et al. 2019)*, the antimicrobial effect of various parameters of final irrigation has not been studied extensively yet. This highlights the need to investigate independently the parameters of final irrigation stage, namely the concentration, the application time and the volume of NaOCL and the contribution of each of them to the reduction of intracanal microbial load.

The reduction of total microbial load during chemomichanical preparation will be estimated using Real-Time PCR based on 16S ribosomal RNA (rRNA). To expand the aims of the study, 2 bacterial species namely, *Pseudoramibacter alactolyticus* and *Treponema Denticola* were selected to be studied regarding their initial numbers and their quantitative changes throughout chemical disinfection. The rationale behind the selection of those specific species was to include both a Gram-positive and a Gram-negative species, the first (*Pseudoramibacter alactolyticus*) as most abundant in asymptomatic cases of apical periodontitis (Siqueira and Rôcas 2003) and the second (*Treponema Denticola*) as most abundant in symptomatic cases of apical periodontitis (Cavrini et al. 2008). In additions, both 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.

Finally, the selected microorganisms have not been extensively studied in endodontic literature and yet in endodontic infections in Greek-living population.

Aims of the Study

The aim of the study is two-fold, first, to evaluate the clinical antibacterial efficacy of two different NaOCl concentrations (2,5% and 5%) under a predefined irrigant flow rate in teeth with pulp necrosis and apical periodontitis by using Real-time PCR. This will be examined through the calculation of the total bacterial load before any treatment procedure and the possible bacteria reduction after each treatment procedure (chemomechanical preparation, and final irrigation treatments). In addition, the antimicrobial efficacy of the above two different concentrations of NaOCl will be compared in terms of treatment outcome. All patients will be asked for one-year recall examination where the outcome of endodontic treatment will be evaluated through clinical and radiographic examination.

Second, to evaluate the efficacy of final irrigation by assessing, if possible, a numerical definition for that "so called" as "copious irrigation". For this purpose, the total amount of final irrigation will be divided in two equal parts of volume/ time (15ml for 5 minutes each). Total bacterial load that will remail after chemomechanical preparation will be compared to the number of bacteria calculated after the first and the second part of irrigation. It will be investigated if final irrigation provides to further microbial reduction and whether prolonged irrigation augments the antibacterial effect. The null hypothesis of this study is that increased volume/ time of irrigation will lead to similar bacteria reduction of shorter volume/ time but higher concentration, viz that higher NaOCl concentration compensates the antimicrobial effect of a more "copious" irrigation.

Besides the total microbial load, the antibacterial efficacy of final irrigation procedure against two different bacterial species (namely *Pseudoramibacter alactolyticus* and *Treponema denticola)* will also be examined. First, the prevalence of these specific species will be investigated in primary endodontic infections in Greek-living population. In addition, the effect of chemomechanical preparation and final irrigation procedure on the number of these species will be relatively examined though the reduction curves that will be obtained by qPCR.

Materials and Methods:

Study design: The present research will be designed as two-arm, parallel, randomized clinical study. 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. Agapi Zervaki).

Sample size calculation (Power analysis)

According to Rocas et al. (2016) and Rodriguez et al. (2017), use of 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 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 44 patients in total. Sample size calculation is appeared below in detail.

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Estimated sample sizes for a two-sample proportions test
Ho: p2 = p1 versus Ha: p2 != p1

Study parameters:

        alpha =    0.0500
        power =    0.8000
        delta =    0.4000  (difference)
           p1 =    0.5500
           p2 =    0.9500

Estimated sample sizes:

            N =        36
  N per group =        18
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Study population: The study population will be comprised of patients who will 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 44 patients will participate in the study. Forty-four 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 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.
- Teeth with periodontal pocket more than 4mm. *(Paiva et al. 2012).*

Initial clinical and radiographic examination

In the first appointment the whole process of treatment and sampling procedure will be explained to each patient, and they will sign an informed consent form. Firstly, the medical history of each patient will be recorded. Clinical examination will take place to a control tooth and the tooth of interest, and sensibility (cold and electric pulp test), palpation, percussion tests will be followed by periodontal examination. Finally, a periapical radiograph using parallel cone technique will complete the initial clinical and radiographic examination. If a patient is likely to possess a recent CBCT taken for other reasons, it will be recorded.

Treatment and sampling procedures: The compliance to aseptic techniques is essential throughout the entire experimental procedures (*Rôças and Siqueira 2011a, b*). Before the application of 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. A double 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 including the access cavity will be disinfected again. Inactivation of NaOCl, will be carried out through the 5% sodium thiosulphate will be used 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.

Immediately after access cavity preparation and the aforementioned decontamination procedure, the first microbiological sample will be taken (**Sample S1**). The root canal and the pulp chamber will be irrigated with sterile saline, a sterile K file #15 will be inserted in the canal to advance the irrigant and then the canal walls will be smoothly filed to the working length determined by using an apex locator. Three sterile paper points will be successively inserted in the same length for at least 1 minute, to absorb the fluid of the canal. The cutting part of the file and the paper points will aseptically be transferred to cyotubes containing Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH = 7.6) and immediately be frozen at -20^{o}C.

Patients will be randomly divided into 2 experimental groups, each of 22 teeth, where different NaOCl concentrations 2,5% (Group A) and 5% (Group B) will be used. Working length (WL) will be established according to apex locator (Root ZX mini, Morita). Chemomechanical preparation will be completed in the same appointment. In the including teeth, Sx Protaper gold (Dentsply Maillefer, Ballaigues, Switzerland)) will be used for coronal flaring at 2/3 of the WL, Smarttrack (Nikinc, Eindhoven, Netherlands) 17/.04-45/.04 and Hyflex (Coltene/Whaledent, Altstätten, Switzerland.) 50/04 will be used for the instrumentation of the root canal, all at WL. Master apical file will range from 40/.04 to 50/.04, depending on the initial instrument of root canal. Six rotary files, apart from the orifice opener will be used in each case. Root canal mechanical instrumentation will be the same for both groups A and B. The volume of irrigant and the time of irrigation between files will be 2ml for 30 seconds for both groups leading therefore to a total volume of irrigant of 18ml for 270 seconds for the chemical preparation. A disposable luer lock syringe with a 27G needle (Endo Eze, Ultradent, South Jordan, UT) will deliver the irrigant throughout the procedure. The needle will be inserted up to 4 mm from working length. After the completion of preparation, root canals will be dried with paper points and flushed with 5ml of 5% sodium thiosulphate, for NaOCl

deactivation. A small file will be inserted and instrumented the canal and a second sample (**Sample S2**) will be taken as described for Sample 1. After the chemomechanical preparation, an extra rinse will follow. The volume of irrigant used will be *15 ml* and the time of irrigation *5 minutes* for both groups. Canals will be dried with paper points and **Sample S3** will be taken with the exact procedure outlined above. Finally, another irrigation of *15ml in 5minutes* will follow for each group and **Sample S4** will be taken as described.

All teeth will be obturated with warm vertical compaction with System/Obtura at the same appointment. Access cavities will be filled with temporary cement Cavit G and the patients will be referred for permanent restoration

DNA extraction & Quantitative PCR analysis

The samples will be immediately transferred to laboratory. DNA from clinical samples will be extracted by using the QIAGEN DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Quantification of total bacteria before and after treatment procedures will be performed using 16S ribosomal RNA (rRNA) gene-targeted quantitative real-time PCR (qPCR) using the Mx3005P Real-Time PCR System (Agilent) and the SYBR Select Master Mix (Applied Biosystems, Foster City, CA) in a total reaction volume of 20 mL, as described in literature *(Rôças and Siqueira 2012, Antunes et al. 2015)*. Except for the universal primers, species-specific primers for *P. alactolyticus* and *T. denticola* will be used.

Table 1. Primers used for qPCR assays, annealing temperatures and size (base pairs, bp) of the amplicons

Target group	Forward primer	Reverse primer	Annealing Temp	Amplicon size
total bacteria 1	ACT CCT ACG GGA GGC AGC AG	ATT ACC GCG GCT GCT GG	53 °C	200 bp
Pseudoramibacter alactolyticus 2	CGAATAAGTCAG TGCCGG	CTTCGCTTCCCTTTG TTCAG	55°C	421 pb
Treponema denticola 3	TAATACCGAATG TGCTCATTTACA T-3'	TCAAAGAAGCAT TCCCTCTTCTTCTTA	60	316 bp

Each reaction will consist of 2x SYBR Green buffer MIX with Taq polymerase, 0.3-0.5 µM of each primer, 5µl of DNA template and water to 20µl. All reactions shall be prepared in duplicate for samples and standards. An initial denaturation at 95 °C for 3 minutes will be followed by 40 cycles of denaturation at 95 °C for 3-15 seconds, annealing at primer-specific temperatures indicated above (Table 1) for 30-60 seconds and amplicon extension at 72 °C for 60 seconds. Quantification of bacteria load will be performed based on the standard curves constructed using a DNA standard (ZymoBIOMICS Microbial Community DNA Standard).

Recall examination

The outcome of endodontic treatment in all examined cases will be assessed. Clinical and radiographic evaluation will be performed as described in the initial examination. Using periapical index (PAI) *(Orstavick et al. 1986*), the score of each tooth at one year after treatment will be recorded. For scores ≤2 will be addressed as healed, whereas those showing a score ≥3 will be addressed as not healed. All the follow up examinations will be performed by the same clinician. However, the evaluation of follow-up radiographs will be performed by two independent and experienced endodontists who will be blinded to the treatment groups. The evaluators will be calibrated before the final radiographic evaluation and a Kappa coefficient will be provided for interexaminer agreement.

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 analyzed continuous data. A fisher's exact test will be performed to assess any differences in the qPCR positive samples between the two interventions in terms of concentration and in terms of volume/ time. Univariable and multivariable (median) regression analysis will be performed to assess the effect of volume/ time and concentration of NaOCl parameters 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).

Clinical significance

- To the best of our knowledge, this is the first randomized clinical study which evaluates the effect of different NaOCl concentrations on intracanal bacterial load.
- In this study, there is the high expectation to define the ambiguous term "copious irrigation". The separation of final irrigation in two time/ volume interval aims to find if there is a cutoff point where no further bacteria reduction will be performed.
- The use of two different NaOCl concentrations in these time/ volume intervals intends to answer if higher concentrations compensate the effect of more "copious" irrigations.

- The effect of NaOCl concentration on treatment outcome will be examined.
- Bacteria load that will remain inside the root canal system after chemomechanical procedure will be correlated with treatment outcome, in order to set a threshold for bacteria reduction and success of endodontic treatment.
- 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

According to the preexisting literature and to the study design a significant reduction in the total microbial load after chemomicanical procedure is anticipated. Irrigation with 5% sodium hypoclorite is expected to give better antimicrobial results. Also, final irrigation may lead to higher antimicrobial reduction until a cutoff point where no further antibacterial efficacy of irrigation could be detected. The antimicrobial effect of higher concentration of NaOCl may be compensated to higher volume/ time of irrigation, and this is also the null hypothesis of the study.

In terms of treatment outcome, a tendency for higher success rate is expected in the higher NaOCl concentration group compared to lower NaOCl concentration group.

Pseudoramibacter alactolyticus and *Treponema Denticola* are expected to be found in less than 50% of samples. Their counts are expected to reduce significantly but not eradication is expected in the after-treatment samples.

Time schedule of the study

1st YEAR

1st semester: Literature Review

2nd semester: Writing the Protocol

3d semester: Protocol Presentation, Ethics Committee Approval

4th semester: Treatment of patients, Writing general part of thesis

2nd YEAR

1st semester: Treatment of patients, Writing general part of thesis

2nd semester: Treatment of patients, DNA extraction

3d semester: Treatment of patients, DNA extraction

4th semester: Performing laboratory procedures (qPCR)

3d YEAR

1st semester: Performing laboratory procedures (qPCR)

2nd semester: Statistical analysis, Thesis Writing, Paper writing

3d semester: Thesis Writing

4th semester: Thesis Presentation

Place of Research

The current PhD study will be performed at the Dental School of Athens UOA. Specifically, patients who proceed at the Department of Endodontics in the Undergraduate or Postgraduate Clinic for root canal treatment will be included. Endodontic treatments will be performed in the Postgraduate Endodontic Clinic. The storage of samples and the elaboration of laboratory procedures will take place at the Laboratory of Basic Science and Oral Biology. Real-time PCR will be performed at the Laboratory of Microbiology in the Dental School of Athens.

Budget

It is an entirely self-financed study. The estimated cost is 5000 euros.

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