Assessment of Antimicrobial Efficacy of Nano Chitosan, Chlorhexidine, Chlorhexidine/ Nano Chitosan Combination versus Sodium Hypochlorite Irrigation in Patients with Necrotic Mandibular Premolars: A Randomized Clinical Trial

تقييم الفعالية المضادة للميكروبات للنانو شيتوزان، الكلورهيكسيدين، و المزيج بين النانوشيتوزان والكلورهيكسيدين مقارنة بالكلور في ري قنوات اللب عند المعالجة اللبية للمرضي المصابين بتنخر اللب في الضواحك السفلية: تجربة إكلينيكية بالإنتقاء العشوائي.

Submitted for partial fulfillment of the **Ph. D** requirements in Faculty of Dentistry
Cairo University

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Administrative information

1. **Title:** Assessment of Antimicrobial Efficacy of Nano Chitosan, Chlorhexidine, Chlorhexidine/ Nano Chitosan Combination versus Sodium Hypochlorite Irrigation in Patients with Necrotic Mandibular Premolars: A Randomized Clinical Trial

2. **Trial registration:** This study was registered on **Protocol Registration and Results System (PRS).**

   - Website: www.clinicaltrails.gov
   - ClinicalTrail.gov ID NCT03668899.
   - Release Date: September 15, 2018.

3. **Protocol version:** version 1.
4. **Funding:** Totally Self-funding
   Sponsor: Cairo University, Faculty of Dentistry**.**

5. **Trial roles and responsibilities:**

Maha Nasr Morsy Abo shanab

Assistant lecturer of Endodontics, Faculty of Dentistry, ERU University. The main investigator; will be responsible for funding the study, clinical part of the study, screening and recruitment of sample, follow up the patients, writing the thesis, interpretation of results and drawing conclusions.

Prof. Dr. Alaa Diab

Professor of Endodontics, Faculty of Dentistry, Cairo University. Main Supervisor, Guidance throughout the clinical part, responsible for reviewing the thesis and drawing conclusions.

Dr. Nehal Nabil

Lecturer of Endodontics, Faculty of Dentistry, Cairo University. Co-supervisor, she will guide throughout the clinical part, analysis of the collected data, proof reading of the thesis and drawing conclusions.

Dr. Amira Farouk Hussein

Lecturer of Clinical Pathology, Faculty of medicine, Kasr Al Ainy, Cairo University. Co-supervisor, she will be responsible for the microbiology part.

6. Introduction

a. Description of the research question:

PICOS

P - Adult patients with necrotic mandibular premolars.
I- Interventions

I_1: Irrigation with nano chitosan

I_2: Irrigation with chlorhexidine

I_3: Irrigation with Chlorhexidine/Chitosan combination

C- Irrigation with Sodium Hypochlorite.
O- Primary Outcome: Antimicrobial efficacy of the different irrigants.
Secondary Outcome: Postoperative pain.
S- Randomized controlled trial.

Formulated question:

In necrotic mandibular posterior teeth, would irrigating the canals using either Nano chitosan, Chlorhexidine or a combination of Nano chitosan and Chlorhexidine versus Sodium Hypochlorite have different effects on canal disinfection and postoperative pain?

Statement of the problem:

Bacteria play a major role in the pathogenesis of apical periodontitis; therefore, success of endodontic treatment is dependent on its complete eradication before obturation[1].

Sodium hypochlorite (NaOCl); the gold standard for irrigation has many disadvantages including irritation of the periapical tissues and burning of surrounding tissues[2,3]. Therefore, the present study aims to explore new irrigating solutions probably more effective and less irritating to the periapical tissues than NaOCl.

Rationale for carrying out the trial:

Many studies have been conducted to assess the antimicrobial efficacy of sodium hypochlorite, (as a gold standard) compared to other suggested alternatives such as Chlorhexidine (CHX), both in vitro[4,5] and in-vivo[6,7].

Zandi et al 2016, evaluated the antimicrobial efficacy of 2% Chlorhexidine compared to 1% Sodium hypochlorite irrigation in treating teeth with apical periodontitis and found that bth solutions are similarly effective in reducing bacterial cell counts[6]. The results of this study agreed with the results of a previous study that was conducted by Ercan et al on 2004 where the antimicrobial efficacy of 2% CHX was compared to that of 5.25% NaOCl in treating necrotic teeth with periapical pathosis and concluded that both solutions were significantly effective to reduce the microorganisms in the teeth with necrotic pulp, periapical pathologies, or both, and could be used successfully as an irrigant solution[7].

Regarding postoperative pain, Almeida et al 2012, evaluated postoperative pain after single visit root canal treatment using either 2% CHX or 5.25% NaOCl as irrigating solutions

and found a low incidence of postoperative pain in both groups with no statistically significant difference between them[8].

A recent study by Farzaneh et al evaluated the effects of two different concentrations of NaOCl ,2.5% and 5.25%, on postoperative pain after single visit endodontic treatment of molars with symptomatic irreversible pulpitis and found that 5.25% NaOCl irrigation resulted in significantly less postoperative pain in the first 72 hours postoperatively[9].

The current available evidence has inconclusive results when comparing the antimicrobial efficacy of NaOCl to CHX as suggested by a recent systematic review conducted by Goncalves et al 2016, they included only 5 clinical studies out of 172 reviewed articles and found that the results of the studies were inconsistent and recommended further clinical trials to reach conclusive results[10].

Recent studies has shed the light on a wide range of natural substances like Chitosan, Propolis, and other herbal solutions that are claimed to be as effective against bacteria as NaOCl, less toxic and less irritant[2,3,11 ,12,13,14] and also introduced nanoparticles with their known antimicrobial capabilities as endodontic irrigants[15,16].

An in vitro study in 2017 claimed that the combination of 2% Chlorhexidine and 2% Chitosan is as effective as NaOCl against *E. Faecalis* in biofilms[3].

Since within the scope of our search no study evaluated the antimicrobial effects of Nano chitosan, Chlorhexidine/Chitosan combination as possible natural irrigants to those of NaOCl for irrigation in-vivo.

The **Aim** of the current study is to compare the antimicrobial effects of Nano chitosan, chlorhexidine, and their combination to NaOCl in patients with necrotic mandibular Premolars.

Review of literature:

Chemo-mechanical preparation:

It is well established that the most probable cause failure of endodontic treatment is the presence of a persisting infection. Therefore, it is important that chemo-mechanical canal preparation be directed towards complete eradication of all bacteria from the root canal system so that the best possible prognosis can be achieved in endodontic treatment[1].

"Only a few studies have evaluated the effect of infection at the time of root filling on the prognosis of treatment. These studies have shown that the success rate of endodontic treatment is approximately 10-15% lower for teeth which yield a positive culture before obturation than for teeth which yield a negative culture"[1].

Dunavant et al 2006[4], compared the efficacy of sodium hypochlorite to ,Smear Clear, Chlorhexidine, REDTA and BioPure MTAD against *E. Faecalis.* They submerged an in vitro grown *E. Faecalis* biofilm in wells containing either 1% NaOCl, 6% NaOCl, Smear Clear, 2% CHX, REDTA or BioPure MTAD for one and five minutes. Phosphate buffered saline was used as a negative control while 6% NaOCl was used as a positive control. After that the biofilms were re-cultured on THB agar plates and the number of colony-forming units (CFUs) was count. There was no significant difference between 1% NaOCl and 6% NaOCl yet, there was a significant difference between 1% and 6% NaOCl and all other tested irrigants with BioPure MTAD being the least effective solution.

Perochena et al 2015[16]**,** studied the chelating and antibacterial effects of chitosan nanoparticles (CNPs) on bovine dentin. Five groups of bovine dentin sections (20 per group) were used in the study. They used 2.5% sodium hypochlorite (NaOCl) for 20 min, 17% ethylenediaminetetraacetic acid (EDTA) for 3 min and 1.29 mg/mL CNPs for 3 min as irrigating solutions. The groups were irrigated with distilled water as acontrol, NaOCl alone, NaOCl then EDTA, NaOCl then EDTA then CNPs or NaOCl then CNPs. After irrigation, half of the samples were used to assess the chelating effect of the solutions using scanning electronic microscopy, while the other half were infected intra-orally to examine the post-treatment bacterial biofilm forming capacity under confocal laser scanning microscopy. The results of this study showed that smear layer removal was significant in all of the groups except the control and NaOCl groups and that the samples treated with CNPs were resistant to biofilm formation significantly more than other treatment groups. Therefore, they concluded that CNPs irrigation was superior to NaOCl in that as well as inhibiting bacterial growth it also can remove the smear layer effectively.

Rocas et al 2016[17]**,** evaluated the antibacterial effects of 2% chlorhexidine and 2.5 % NaOCl irrigation during rotary preparation of contaminated root canals. A total of 50 single rooted teeth with infected necrotic pulps were included in the study (two groups n=25). All root canals were prepared using BioRace rotary file system. Irrigation was done using either 2. NaOCl in one group or 2% CHX in the other group. Two samples were collected from

each canal S1 before chemomecanical preparation and S2 after chemomechanical preparation. In this study, the number of viable bacteria was determined by a quantitative polymerase chain reaction all initial samples (S1) were contaminated while in NaOCl group 44% of the treated canals were still contaminated and in CHX group 40% of the treated canals were contaminated. This difference was not statistically significant.

Zandi et al 2016[6]**,** evaluated the antimicrobial efficacy of 2% chlorhexidine compared to 1% sodium hypochlorite used as irrigants in the retreatment of endodontically treated teeth with apical periodontitis. A total of 67 patients were randomly assigned to either 1% NaOCl group (n=29) or 2% CHX group (n=38). Three samples were taken from each tooth; S1 before preparation, S2 after preparation and irrigation with either of the tested irrigants and S3 after application of calcium hydroxide intracanal medication. The amount of bacterial reduction was evaluated using 16s ribosomal RNA gene-based polymerase chain reaction. The results of this study suggested that both solutions are similarly effective in reducing bacterial cell count.

Jaiswal et al 2017[3]**,** evaluated antibacterial efficacy of Chitosan, Chlorhexidine, Propolis and Sodium hypochlorite on *E. Faecalis* biofilm invitro, Ninety single rooted mandibular premolars were included in the study. The root canals were then instrumented with rotary ProTaper instruments to an apical size of F3. 2 ml of 5% NaOCl was used as an irrigant during preparation. Teeth then vertically sectioned into two halves and divided into nine experimental groups with 20 samples each. irrigated with 3 ml of each irrigant for 10 minutes. Group 1: 5% NaOCl, group 2: 2% Chlorhexidine, group 3: 1% Acetic acid, group 4: Propolis, group 5: 0.2% Chitosan, group 6: 0.2%Chitosan+2%Chlorhexidine, group 7: 1% Chitosan+1%Chlorhexidine, group 8: 2%Chitosan+2%Chlorhexidine and group 9: Saline (negative control). The results of this study showed that Chitosan/ Chlorhexidine combination, Chlorhexidine and Propolis were as effective as sodium hypochlorite, so they concluded that their use as natural alternatives for NaOCl could be advantageous to overcome the disadvantages of NaOCl.

Yadav et al 2017[12], evaluated antibacterial effects and cytotoxicity of 0.25% Chitosan, 0.5% Chitosan, 2% chlorhexidine and 3% sodium hypochlorite against *E. Faecalis* and *C. Albicans*. *C. albicans* and *E. faecalis* cultures were prepared in vitro, then the antimicrobial activity of the tested solutions (0.25% Chitosan, 0.5% Chitosan, 2% CHX and 3% NaOCl) against them was evaluated using agar diffusion, microdilution and biofilm susceptibility tests. Saline was used as a negative control. For the cytotoxicity evaluation fresh blood was centrifuged then plasma was abstracted and packed cell volume of red blood corpuscles was obtained. Then 1 ml of packed RBCs was added to 4 ml of saline. 100µl of this diluted RBCs was distributed to 18 test tubes to obtain 3 groups (Chitosan, Chlorhexidine and hypochlorite), 6 test tubes each. For all groups, the first test tube was kept as a control in which no irrigant was added. In the second test tube 10 µl of the irrigant was integrated. 20 µl was integrated to the third test tube, 30 µl to the fourth test tube, 40 µl to the fifth test tube and 50 µl to the test tube. Tubes were incubated for 3 minutes then hemoglobin percentage after hemolysis was detected utilizing an automated hemoanalyzer. The results of this study

suggested that chitosan could be used as a non-toxic natural alternative to NaOCl since the antibacterial activity of the all chitosan groups was comparable to 3% NaOCl and 2% Chlorhexidine and that the chitosan showed no cytotoxicity at 3mg/ml.

6. b. Choice of comparators:

Sodium hypochlorite (NaOCl) was considered the most efficient of all available solutions for its superior antimicrobial effects and tissue dissolving abilities[16]. Therefore, as a gold standard for irrigation; it was chosen as a comparator in the current study.

7. **Objectives:**

- **Research hypothesis:**

There is no difference in the antimicrobial efficacy among Nano chitosan, Chlorhexidine, Chlorhexidine/Nano Chitosan and Sodium hypochlorite in root canal treatment.

- **Primary objective:**

The primary objective of this study is to assess the antimicrobial efficacy of Nano-chitosan, chlorhexidine and Chlorhexidine/Nano Chitosan combination versus NaOCl.

- **Secondary objective:**

The secondary objective is to assess the incidence and severity of postoperative pain after root canal treatment using either Nano-chitosan, CHX, Chitosan/CHX combination or NaOCl as a final flush before obturation.

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C- Irrigation with 5.25% sodium hypochlorite.
O_1- Antimicrobial efficacy of the different irrigants.
O_2- postoperative pain.
S- Randomized controlled trial.

8. Trial design:

- A Randomized controlled clinical trial.
- Unicenter: A trial will be carried out by one person in one hospital.
- Randomization double blinded: Laboratory assessor and participant will not know the method of treatment.
- Equal randomization: participants with equal probabilities for intervention.
- Positive controlled: All groups receiving treatment.
- "Parallel group study: Each group of patients receives a single treatment simultaneously".
- Two assessors will assess the results.

III. Methods

A) Participants, interventions & outcomes

9. Study setting:

- This study will be carried out on patients attending outpatient clinics in the Department of Endodontics, Faculty of Dentistry; Postgraduate Endodontics clinic 7th floor new section. Cairo University, Egypt. Adec 200 U.S.A dental units.

10. Eligibility criteria:

10. a. Inclusion criteria

1. Patient's age between 22-42 years.
2. Both male and female.
3. Patient who are medically free and with good health
4. Necrotic Mandibular Premolars.

10. b. Exclusion criteria:

1. Pregnant females.
2. Teeth with vital inflamed pulps, symptomatic periapical abscess and facial cellulitis, periodontally diseased and hopeless teeth.
3. Teeth with previous fillings and/ or previous endodontic treatment.

11. Interventions:

11. a. General operative procedures:

- Eligible patients will be randomly divided into equal groups (Nano Chitosan group), (Chlorhexidine group), (Chlorhexidine/Chitosan combination group) and the control group (Sodium Hypochlorite group).
- **Preoperative measures (for all groups):**
  - Medical and dental history will be obtained from all patients participating in this research. Clinical and radiographic evaluation for each tooth included in this study will be recorded.
  - Pain scale chart will be given to each patient to rate his /her pain level before endodontic treatment as preoperative reading on an NRS scale.
  - Tooth will be anaesthetized using 1.8 ml Mepivacaine HCl 2% - Levonordefrin 1:20000 (Carpule Mepecaine-L, Alexandria Company for Pharmaceuticals and Chemical Industries, Egypt).
  - The tooth will be isolated with rubber dam to maintain aseptic field, after isolation the tooth and surrounding field will be disinfected by a protocol using 3% hydrogen peroxide 2.5% sodium Hypochlorite before and after coronal access cavity preparation[17].
  - The initial sample S1 will be collected from root canals before preparation using sterile # 15 paper points.

- Sterile saline solution will be injected in the canals followed by the insertion of a #15 K-file (Mani, Japan) to at least 1 mm short of the apex as confirmed by apex locator (Root ZX, J.Morita USA, Irvine, CA)[17] to facilitate the insertion of the paper points to collect the samples.
- Each paper point will remain in place for at least 1 minute then immediately placed in test tubes containing reduced transport fluid and sent to the Microbiology Laboratory, Faculty of Medicine, Cairo University for microbiologic processing[7].
- Root canals will be mechanically prepared in a crown-down approach using the rotary files (M-Pro file system) in an endodontic motor (X-Smart, Dentsply Maillefer, USA) according to the manufacture instructions.

The following sequence will be used for all groups:

  - # 18 file taper (orifice opener) 0.09 will be used to prepare two thirds of estimated working length.
  - # 20 file taper 0.04 will used to full working length.
  - #25 file taper 0.06 will used to full working length.
  - #35file taper 0.04 will used to full working length.

- The canals will be thoroughly irrigated for 1 minute between every two subsequent files using 2 ml of 2.5% NaOCl.
- After complete preparation all canals will be flushed with EDTA followed by sterile saline.

- **Intervention for test group A**
  - After complete preparation, the canals will be flushed using 5 ml of Nano chitosan (Nano tech, Egypt) in a plastic syringe using Navitip needles (Ultradent, South Jordan,UT).
- **Intervention for test group B**
  - After complete preparation, the canals will be flushed using 5 ml of Chlorhexidine (CHX-Plus, Cosmopack, Egypt) in a plastic syringe using Navitip needles (Ultradent, South Jordan,UT).
- **Intervention for test group C**
  - After complete preparation, the canals will be flushed using 5 ml of a combination of Chlorhexidine and nano chitosan in a plastic syringe using Navitip needles (Ultradent, South Jordan,UT).
- **Intervention for control group**
  - After complete preparation, the canals will be flushed using 5 ml of 5.25% sodium hypochlorite (Clorex, Egypt Ltd, Egypt) in a plastic syringe using Navitip needles (Ultradent, South Jordan,UT).

- After flushing the canals with each irrigating solution all canals are to be flushed with 10 ml sterile saline and the final sample S2 is to be collected in the same manner as S1.
- After dryness, root canals will be obturated using lateral compaction technique by selection of master cone corresponding to the same size as the master apical file, and then a finger spreader will be used to allow space for auxiliaries. All canals will be sealed with a resin sealer (ADSEAL, META Biomed CO., LTD Chungbuk. Korea).

- After obturation, a cotton pellet will be placed in the pulp chamber and the access cavity will be closed with a temporary filling.

Post-operative care:

- All patients will receive postoperative instructions, in case of moderate and severe pain, patients will be allowed to take Ibuprofen (400mg), and instructed to record number of Ibuprofen tablets.

11. b. Strategies to improve adherence to intervention protocol:

- Face to face adherence reminder session, phone calls and text message reminders will tack place to stress on the post-operative instructions to record postoperative pain levels.

12. Outcomes:

Primary outcome:

- **Antibacterial efficacy:** will be assessed by counting the number of colony forming units (CFU) before and after irrigation (S1 and S2) for each patient[18]. Collected samples will be cultured in serial dilution on blood agar plates, incubated for 24 hours, and then the number of colony forming units (CFUs) will be counted per ml.

Outcome	Tool	Unit	Time
antibacterial efficacy	Number of CFUs[17]	Numerical data	Samples collected before and after preparation and transferred to the laboratory for immediate cultivation.

Secondary outcome:

- **Postoperative pain:** will be recorded on a Numerical Rating scale (NRS), pain intensity will be recorded preoperatively and postoperatively after 6, 12, 24, 48, 72 hours and 7 days. The NRS consists of a10-cm line anchored by two extremes "No pain" and "pain as bad as could be", patient will be asked to choose the mark that represent their level of pain. Pain level will be assigned to one of 4 categorical scores: **1**, None(0); **2**,Mild(1-3); **3**,Moderate(4-6); **4**,Severe (7-10)[19].

Outcome	Tool	Unit	Time
Postoperative pain	Numerical rating scale NRS[18]	Categorial data	Immediately after treatment and Up to 7 days after endodontic treatment

13. Participants time line

STUDY PERIOD									
Time point	T0	T1	F1	F2	F3	F4	F5	F6	F7
Activity									
Enrollment									
Eligibility criteria	x								
Informed consent		x							
Allocation		x							
Intervention									
Access		x							
Initial sample		x							
Cleaning and shaping		x							
Final sample		x							
Obturation		x							
Assessment									
Intracanal bacterial count		x							
Postoperative pain			x	x	x	x	x	x	x

T0: Enrollment of the patient after diagnosis and radiographic evaluation.
T1: Signing the informed consent and allocation of the patient to either intervention or control group. Starting the procedure, taking the first root canal sample before cleaning and shaping, second root canal sample after cleaning and shaping, obturation.
F1: Patients will be asked to evaluate their postoperative pain levels using a numerical rating scale (NRS)
F2: similarly, at 6 hours.
F3: similarly, at 12 hours.
F4: similarly, at 24hours.
F5: similarly, at 48 hours.
F6: similarly, at 72 hours.
F7: similarly, at 7 days.

14. Sample Size:

- The aim of the current study is to compare the antimicrobial effects of Nano chitosan, Chlorhexidine, Chlorhexidine/ Nano Chitosan combination to NaOCl in patients with necrotic mandibular posterior teeth. Based on a previous study by Shingare & Chaugule 2011[2] the difference in bacterial count between at least 2 groups is 75±75.

Using power 80% and 5% significance level we will need to study 17 in each group. This number is to be increased to a sample size of 20 to compensate for losses during follow up. Sample size calculation was achieved using PS: Power and Sample Size Calculation Software Version 3.1.2 (Vanderbilt University, Nashville, Tennessee, USA).

15. Recruitment strategy:

- M.N. will select patients from the outpatient clinic of the Department of Endodontics, Faculty of Dentistry, Cairo University, Egypt.
- "Screening of patients will be carried on until the target population is achieved"[20].
- "Identifying and recruiting potential subjects is achieved through patient database"[20].

16. Allocation

16.a. Randomization:

Sequence generation: using computer-generated random numbers.

- List will be created on (https://www.random.org/), the patients will be randomly classified into four groups:
  - "Group A: Irrigation with Nano chitosan".
  - "Group B: Irrigation with Chlorhexidine
  - "Group C: Irrigation with Chlorhexidine/Nano Chitosan combination".
  - "Group D: Irrigation with 5.25% Sodium Hypochlorite".
- Allocation ratio will be 1:1.
- This method will be done by the co-supervisor.

16.b. Allocation – concealment mechanism:

- According to the allocation sequence obtained from the computer software, the numbers that generate randomly from the software will be written in small folded opaque papers then insert into Envelope. (Opaque sealed envelope and sign across sea**).**
- All those papers will be ready before conducting any procedure. This method will be done by the co-supervisor. That Envelops will be placed in a container (box), each participant will grasp one envelope blindly before operation and will be assigned accordingly. The number of papers will decrease as each patient picks his number and so on.

16. c. Allocation - Implementation:

- Principle investigator (M.N) will referee all participants' number to the co-supervisor who will generate the random sequence, and assign the patients for intervention or control group. Neither the assessor nor the patients know the criteria of allocation.

17. Blinding:

- "Blinded participants: The patient will be informed of the steps of the treatment (as mentioned in the consent form) without getting into details".

- "Blinded Assessors: Blinding to the laboratory assessors is done by not involving them in sequence generation or allocation concealment or treatment options".

C) Data collection, management, and analysis:

18. Data collection methods:

- Base line data will be recorded on a personal history chart prior to clinical examination.
- Antimicrobial efficacy will be measured by counting the number of colony forming units CFU of bacteria after cultivating the collected samples[18].
- Paper based pain scale (VAS) assessments will be filled by the patient preoperatively, and postoperatively after 6, 12, 24, 48,72 hours and after7 days[15].

19. Data management:

- Patient files will be stored in numerical order and stored in secure and accessible place. M.N will assignee each patient a folder containing all their data including primary and secondary outcomes, patient's data recorded as a hard copy with the signed consent. "Also, a soft copy of the data will be kept on operators' laptops and will be backed up on an external hard disk".
- All data will be maintained in storage for 1 year following completion of the study.

20. Statistical methods:

- Data will be analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 21 (SPSS Inc., Chicago, IL). Numerical data will be described as mean and standard deviation or median and range. Data will be explored for normality using Kolmogrov-Smirnov test and Shapiro-Wilk test. Comparisons between 4 groups for normally distributed numeric variables will be done using the ANOVA while for nonnormally distributed numeric variables will be done by Kruskal Wallis test. A p-value less than or equal to 0.05 will be considered statistically significant. All tests will be two tailed.
- Categorical data will be described as numbers and percentages and comparisons will be done by chi square test or fisher exact as appropriate

D) Data monitoring:

- The data will be monitored by the main supervisor (A.D) for any modification of the trial if needed

21. Harms:

- Any temporary or permanent adverse effect will be recorded and documented and treated.

22. Auditing:

- The study supervisors: Prof Dr. Alaa Diab and, dr Nehal Nabil will regularly assess the trial process and documents. "Adherence to trial interventions and policies to protect participants, including reporting of complications and their treatments. "[20,21]. Auditing of the study design will be done by the evidence based committee.

IV. Ethics and dissemination

23. Research ethics approval:

- "This protocol and the template informed consent form will be reviewed by the Ethics Committee of Scientific Research - Faculty of Dentistry, Cairo University".

24. Protocol amendments:

- "Any amendment to the protocol which may affect the conduct of the research, potential benefit of the patient or may have impact on patient safety, inclusive changes of study objectives, study design, sample sizes, study procedures, or significant administrative aspects will demand a formal modification to the protocol"[16, 17]. Such modifications will be agreed upon by the Council of the Department of Endodontics.

25. Consent:

- The treatment plan will be fully explained to the patient and after educating the patient with all the needed data and complications that could occur, an Arabic consent form will be signed by patients who are willing to participate.

26. Confidentiality:

- "All study- associated information will be maintained securely. All participant data will be stored in locked lockers in areas with restricted entrance. All experimental specimens, reports, data gathering process, and administrative charts will be recognized by a coded ID number only to keep participant exclusiveness. All documentation that comprise personal identifiers including name, age, sex, identification number, address, phone number, work address will be secured disparately from study records identified by code number. All available databases will be secured with countersign- preserved entry systems"[16, 17]**.**

27. Declaration of interests:

- This study is a part of a PhD degree in Endodontics, Faculty of Dentistry; Cairo University. No financial conflict of interests is confirmed. The study is self-funded by the principal investigator.

28. Access to data:

- "All Principal investigators will be given entrance to the data sets. All data sets will be kept secure. To ensure confidentiality, data dispersed to project team members will be blinded of any recognizing participant data"[16, 17].

29. Dissemination policy:

- Study results will be published as partial fulfillment of the requirements for PHD degree in Endodontics.
- Topics proposed for presentation or publication will be expanded to the authors.

V. Appendices:

VI: Statement of originality:

- Within the scope of our search no study evaluated the antimicrobial effects of Nano chitosan, Chlorhexidine, Chlorhexidine/ Nano Chitosan combination to those of NaOCl for irrigation in endodontic treatment of necrotic teeth in-vivo; therefore, this study is an original study**.**

References

1. SJÖGREN U, FIGDOR D, PERSSON S, SUNDQVIST G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *Int Endod J*. 1997;30(5):297-306.
2. Shingare P, Chaugule V. Comparative evaluation of antimicrobial activity of miswak, propolis, sodium hypochlorite and saline as root canal irrigants by microbial culturing and quantification in chronically exposed primary teeth. *Germs*. 2011;1(1):12-21.
3. Jaiswal N, Sinha DJ, Singh UP, Singh K, Jandial UA, Goel S. Evaluation of antibacterial efficacy of Chitosan, Chlorhexidine, Propolis and Sodium hypochlorite on Enterococcus faecalis biofilm: An in vitro study. *J Clin Exp Dent*. 2017;9(9):e1066-e1074.
4. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL. Comparative Evaluation of Endodontic Irrigants against Enterococcus faecalis Biofilms. *J Endod*. 2006;32(6):527-531.
5. Sen BH, Safavi KE, Spångberg LS. Antifungal effects of sodium hypochlorite and chlorhexidine in root canals. *J Endod*. 1999;25(4):235-238.
6. Zandi H, Rodrigues RCV, Kristoffersen AK, et al. Antibacterial Effectiveness of 2 Root Canal Irrigants in Root-filled Teeth with Infection: A Randomized Clinical Trial. *J Endod*. 2016;42(9):1307-1313.
7. Ercan E, Ozekinci T, Atakul F, Gül K. Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: in vivo study. *J Endod*. 2004;30(2):84-87.
8. Almeida G, Marques E, De Martin AS, da Silveira Bueno CE, Nowakowski A, Cunha RS. Influence of irrigating solution on postoperative pain following single-visit endodontic treatment: randomized clinical trial. *J Can Dent Assoc*. 2012;78:c84.
9. Farzaneh S, Parirokh M, Nakhaee N, Abbott P V. Effect of two different concentrations of sodium hypochlorite on postoperative pain following single-visit root canal treatment : a triple-blind randomized clinical trial. 2018.
10. Gonçalves LS, Rodrigues RCV, Andrade Junior CV, Soares RG, Vettore MV. The effect of sodium hypochlorite and chlorhexidine as irrigant solutions for root canal disinfection: A systematic review of clinical trials. *J Endod*. 2016;42(4):527-532.
11. Silva P V., Guedes DFC, Nakadi F V., Pécora JD, Cruz-Filho AM. Chitosan: A new solution for removal of smear layer after root canal instrumentation. *Int Endod J*. 2013;46(4):332-338.
12. Yadav P, Chaudhary S, Saxena RK, Talwar S, Yadav S. Evaluation of antimicrobial and antifungal efficacy of chitosan as endodontic irrigant against Enterococcus Faecalis and Candida albicans biofilm formed on tooth substrate. *J Clin Exp Dent*. 2017;9(3):e361-e367.
13. Ballal N V., Kundabala M, Bhat KS, et al. Susceptibility of Candida albicans and Enterococcus faecalis to Chitosan, Chlorhexidine gluconate and their combination in vitro. *Aust Endod J*. 2009;35(1):29-33.
14. Skoskiewicz-Malinowska K, Kaczmarek U, Malicka B, Walczak K, Zietek M. Application of Chitosan and Propolis in Endodontic Treatment: A Review. *Mini-Reviews Med Chem*. 2017;17(5):410-434. doi:10.2174/1389557516666160418122510
15. Shrestha A, Kishen A. Antibacterial Nanoparticles in Endodontics: A Review. *J*

Endod. 2016;42(10):1417-1426.
16. Del Carpio-Perochena A, Bramante CM, Duarte MA, de Moura MR, Aouada FA, Kishen A. Chelating and antibacterial properties of chitosan nanoparticles on dentin. *Restor Dent Endod*. 2015;40(3):195-201.
17. Rôças IN, Provenzano JC, Neves MAS, Siqueira JF. Disinfecting Effects of Rotary Instrumentation with Either 2.5% Sodium Hypochlorite or 2% Chlorhexidine as the Main Irrigant: A Randomized Clinical Study. *J Endod*. 2016;42(6):943-947.
18. Siqueira JF, Rôças IN, Favieri A, Lima KC. Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite. *J Endod*. 2000;26(6):331-334.
19. Pak JG, White SN. Pain Prevalence and Severity before, during, and after Root Canal Treatment: A Systematic Review. *J Endod*. 2011;37(4):429-438.
20. Chan AW, Tetzlaff JM, Gutzsche PC, et al. SPIRIT 2013 explanation and elaboration: guidance for protocols of clinical trials. *BMJ*. 2013;346:1-42.
21. Chan A-W, Tetzlaff J, Altman D, Laupacis A, Gøtzsche P, Krleža-Jerić K. Research and Reporting Methods Annals of Internal Medicine SPIRIT 2013 Statement : Defining Standard Protocol Items for Clinical Trials. *Ann Intern Med*. 2013;158(3):200-207.