doi:10.1111/iej.13532

Effect of Neem (Azadirachta indica) versus 2.5% sodium hypochlorite as root canal irrigants on the intensity of post-operative pain and the amount of endotoxins in mandibular molars with necrotic pulps: a randomized controlled trial

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Abstract

Hosny NS, El Khodary SA, El Boghdadi RM, Shaker OG. Effect of Neem (Azadirachta indica) versus 2.5% sodium hypochlorite as root canal irrigants on the intensity of post-operative pain and the amount of endotoxins in mandibular molars with necrotic pulps: a randomized controlled trial. *International Endodontic Journal*, **54**, 1434–1447, 2021.

Aim To assess the effect of Neem versus 2.5% NaOCl as root canal irrigants on the intensity of post-operative pain and amount of endotoxins following root canal treatment of mandibular molars with necrotic pulps.

Methodology This parallel, prospective, double-blinded, randomized controlled trial with allocation ratio 1:1 was conducted in the out-patient clinic of the Endodontic Department, Faculty of Dentistry, Cairo University, Egypt. Fifty healthy patients with mandibular molars with necrotic pulps were randomly assigned into two equal groups using computer software. In the intervention group, root canals were irrigated using Neem; whilst 2.5% NaOCl was used in the control group. A standard root canal treatment was performed in two visits using ProTaper Next rotary files, with no intracanal medication. Pain intensity was assessed using a numerical rating scale (NRS) 6, 12, 24 and 48 h following instrumentation and canal filling. Endotoxin samples were collected using three paper points before and after canal instrumentation and a sandwich ELISA method was used to quantify the level of endotoxins. Demographic, baseline, and outcome data were collected and analysed using chi-square tests (for the comparisons of categorical variables), Mann–Whitney tests (for non-normally distributed variables) and Student's *t* tests (for normally distributed variables), A P-value < 0.05 was considered to be statistically significant.

Results The mean pain scores within the two groups decreased continually over time. The mean pain scores in the Neem group were lower than those in the 2.5% NaOCl group at 6, 12, 24 and 48 h following instrumentation and canal filling with no significant difference between them except at 24 h following instrumentation ($P = 0.012$). Both irrigants significantly reduced endotoxin levels compared to the pre-instrumentation samples ($P < 0.001$) by 8% for the NaOCL group and 18% for the Neem group.

Conclusion Neem and 2.5% NaOCl were not significantly different in terms of reducing the intensity of post-operative pain during all follow-up periods except at 24 h following instrumentation where Neem was associated with lower pain intensity. Both irrigants significantly reduced endotoxin levels but were not effective in eliminating endotoxins completely from root canals of mandibular molars with necrotic pulps.

Keywords: endotoxins, necrotic pulp, Neem (Azadirachta indica), post-operative pain, root canal irrigants, sodium hypochlorite (NaOCl).

accepted 16 April 2021

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Introduction

Post-operative pain following root canal treatment is a significant problem for both patients and clinicians (Kherlakian *et al.* 2016, Farzaneh *et al.* 2018). Some patients report moderate to severe pain that may alter their quality of life in terms of eating, relaxing, sleeping, and their general function (Farzaneh *et al.* 2018). The incidence of post-operative pain has been reported to range from 1.7 to 70% of patients, but could reach up to 80% within the first 24 h (Polycarpou *et al.* 2005, Sathorn *et al.* 2008, Pak & White 2011, Alamassi 2017). Post-operative pain is multifactorial and is a consequence of acute periapical inflammation in response to mechanical, chemical and/or microbial insult of periapical tissues during, and/or after root canal treatment (Siqueira 2003).

Microorganisms and their by-products play an important role in the induction and development of periapical disease. Gram-negative bacteria have unique virulence factors such as endotoxins, also known as lipopolysaccharide (LPS), which are anchored in the outer cell membrane and released during cell division/multiplication or death causing a biological reaction that leads to the development and maintenance of periapical inflammation and bone resorption (Valera *et al.* 2015). Clinical investigations have revealed the presence of endotoxins in 100% of root canal samples in primary and secondary infected root canals with apical periodontitis (Martinho & Gomes 2008, Gomes *et al.* 2012). Its neutralization/removal during root canal treatment is important for the healing of periapical tissues (Gomes *et al.* 2012).

Irrigants play a crucial role in root canal disinfection and to overcome the shortcomings of instruments and instrumentation techniques (Almeida *et al.* 2012). The gold standard and the most commonly used root canal irrigant is sodium hypochlorite (NaOCl) due to its high antimicrobial activity, antibiofilm activity and soft tissue dissolving capabilities (da Silva *et al.* 2015). However, it possesses undesirable characteristics such as being irritant to the periapical tissues even at low concentration, especially when extruded beyond the apex in case of teeth with open apex and with nonvital pulps (Boutsioukis *et al.* 2010, Mostafa *et al.* 2020). It can induce an inflammatory reaction and might cause acute immediate symptoms (Hypochlorite accident) (Guivarc'h *et al.* 2017). Moreover, its unpleasant taste, odour, inability to remove smear layer, and its proteolytic effect has negative impacts on the dentine causing unwanted physical and mechanical changes (Podar *et al.* 2015, Souza *et al.* 2019). Because of these events, there is a need to search for a biocompatible and dentine-friendly irrigant with better patient acceptance in taste and odour to overcome NaOCl limitations and its side effects.

Several studies have assessed the effect of NaOCl root canal irrigant on post-operative pain (Zarei & Bidar 2006, Bashetty & Hedge, 2010, Almeida *et al.* 2012, da Silva *et al.* 2015, Farzaneh *et al.* 2018, Khateeb *et al.* 2019, Verma *et al.* 2019, Mostafa *et al.* 2020); yet, there are a limited number of evidence-based investigations that compare NaOCl with other herbal irrigants.

Herbal and natural products have gained research interest worldwide in recent years due to their medicinal properties such as biocompatibility, antimicrobial activity, anti-inflammatory and antioxidant properties which have favoured their use in endodontics as root canal irrigants (Ravishankar *et al.* 2011). One of these natural products is Neem (Azadirachta indica) which is also known as the margosa tree. Neem is an evergreen tree belonging to Meliaceae family. The US National Academy of Sciences stated that Neem is 'A tree for solving global problems' due to its numerous biological activities (Ghonmode *et al.* 2013) and rich source of valuable active components such as nimbidine, nimbin and nimbolide which are responsible for uncoupling mitochondrial oxidative phosphorylation by inhibiting the respiratory chain and resulting in anti-adherence activity which in turn affects the microbial adhesion, colonization and reduces the number of microorganisms in root canals (Gupta *et al.* 2019, Islas *et al.* 2020). Neem plays a role as an anti-inflammatory agent by regulating pro-inflammatory enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) (Alzohairy 2016, Gupta *et al.* 2019). Neem is also biocompatible to human periodontal ligament cells and does not cause severe irritation to periapical tissues (Alzohairy 2016).

The results of available studies reported that the use of herbal irrigants is promising; however, there is a lack in clinical studies assessing the effect of Neem on patient-related outcomes to support evidence-based decisions. Therefore, the research question of this study was to test whether root canal irrigation using Neem (Azadirachta indica) in comparison with 2.5% sodium hypochlorite would result in a difference in

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post-operative pain and endotoxin levels in mandibular molars with necrotic pulps treated in two visits.

The null hypothesis of this study was that there was no difference in post-operative pain and endotoxin levels after root canal irrigation using Neem (Azadirachta indica) in comparison with 2.5% NaOCl.

Materials and methods

The study was designed as a prospective, parallel, double-blinded (where the patients and the statistician were blinded to the intervention), single-centre and randomized clinical trial with an allocation ratio 1:1.

The trial was reported according to the Preferred Reporting Items for RAndomized Trials in Endodontics (PRIRATE) 2020 guidelines (Nagendrababu *et al.* 2020). The protocol was registered at the ClinicalTrials.gov Identifier: NCT03312153.

The protocol of this study and the informed consent format were reviewed with the respect to scientific content and compliance with applicable research and human subjects' regulation, and approved by the IRB/ECs (approval number 51117) (institutional review boards/ethical committee) in the Faculty of Dentistry, Cairo University, Egypt.

Patients were asked to follow the general instructions and to sign a printed informed consent that explained the aim of the study, its procedures, benefits and possible harms, and to fill the outcomes data charts using a numerical rating scale (NRS) from 0 to 10 by choosing a mark so that their pain levels were categorized into 4 categories as follows: 0 = no pain, 1–3 = mild pain, 4–6 = moderate pain and 7–10 = severe pain at 6, 12, 24 and 48 h after canal instrumentation and canal filling accurately and honestly, and to return them to the principal investigator (N.S.). Endotoxin samples were collected by three paper points immediately following access cavity preparation (pre-instrumentation samples) (S1) and completion of canal instrumentation (post-instrumentation samples) (S2). A sandwich ELISA method was used for the quantification of endotoxins.

Eligibility criteria

The inclusion criteria for patients were healthy patients (Category: American Society of Anesthesiologists class 1) (ASA House of Delegates 2014) aged between 16 and 65 years with mandibular first and second molar teeth diagnosed with pulp necrosis with or without apical periodontitis. The final diagnosis was confirmed through the history of chief complaint reporting no pain with hot and/or cold stimuli, negative response to both thermal test using a hot instrument and electric pulp tester (Denjoy DY310 Dental Pulp Tester; Denjoy, Henan, China), and radiographic examination using Digora intraoral periapical sensor plate and software (Digora; Soredex, Helsinki, Finland) showing mandibular molars with (smaller than 3 mm (0–2 mm)) or without periapical radiolucency.

The exclusion criteria were teeth with a periapical radiolucency of more than 3 mm, immature roots, previously accessed or endodontically treated, deep periodontal pockets more than 4 mm, or teeth associated with vertical root fractures, coronal perforation, calcification, and external or internal root resorptions. Patients who could not interpret the NRS, if antibiotics had been consumed during the last 3 months before the study or any medication (e.g. analgesics) that could alter their perception of pain, patients with diabetes, immune-compromising, and immune-suppression disease and pregnant women were, also, excluded.

Sample size calculation

Sample size was calculated by considering data from the previous study of Bashetty & Hedge (2010) on the effect of different irrigants on post-operative pain. The difference in pain score between the two groups was 1 ± 1. Using power 80% and 5% significance level, so 17 patients in each group were needed. This number was increased to 20 to adjust for using a non-parametric test. The number was increased again to a total sample size of 25 in each group to compensate for losses during follow-up (25% more than the calculated). Sample size calculation was achieved using PS: Power and Sample Size Calculation software version 3.1.2 (Vanderbilt University, Nashville, TN, USA).

Seventy patients were recruited from the outpatient clinic of the Endodontic Department, Faculty of Dentistry, Cairo University, from June 2019 to December 2019. Ten patients were excluded as they had consumed antibiotics and/or anti-inflammatory drugs before root canal treatment, five previously accessed teeth, and five patients with immune-compromising and immune-suppression diseases. Fifty patients fulfilled the inclusion criteria and agreed to participate in the trial. All endodontic procedures were performed by a single operator and principal investigator (N.S.) at the outpatient clinic of the Endodontic Department, Faculty of Dentistry, Cairo University, Egypt.

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Randomization and blinding

Fifty numbers (1-50) were generated by computer software (http://www.random.org/) and randomly allocated to either intervention (Neem) or control (2.5% NaOCl) group in Microsoft Excel sheet (Microsoft Corporation, Redmond, WA, USA) using block randomization of 2 blocks with 25 patients in each block and printed on a table. The table was kept and only accessed by a co-investigator (S.K.) who was contacted to reveal the assigned group of each patient. Allocation concealment was performed using eight folded numbered papers in opaque, sealed envelopes by the operator (N.S.) to be selected by the patients. After diagnosis and acceptance of the patient who satisfied the eligibility criteria for the study, the patients selected an envelope with a written number. Based on this number the patient was allocated to either Neem or 2.5% NaOCl group after contacting the co-investigator.

Neem leaf extract preparation

An ethanolic Neem extract was prepared in accordance with Prasad *et al.* (2016). Mature fresh Neem (Azadirachta indica) leaves were collected from the medicinal garden of the Faculty of Pharmacy, Cairo University, and washed in sterilized distilled water and dried. Ethanolic Neem extract was prepared by macerating 150 g of dry weight powder of Neem leaves with 300 mL of 95% (w/v) ethyl alcohol for a week in a round bottom flask with occasional shaking. The flask was kept in a dark environment to avoid the effect of light on the active ingredients of Neem. The extract was then filtered through a muslin cloth and finally through Whatman No.1 filter paper. Alcohol was removed using Rota-vapor under reduced pressure and kept in the desiccators overnight. The residue was then dissolved in 300 mL of 9.99% Dimethyl Sulfoxide (DMSO) to make the final 50% concentration and was kept in the refrigerator.

Root canal treatment procedures and sampling

Root canal treatment was carried out in two visits. At the first visit, a NRS was given to the patient to record the level of pre-operative pain before root canal treatment. The tooth was anaesthetized using an inferior alveolar nerve block (1.8 mL of 2% Mepivacaine HCl with Levonordefrin 1:20 000) (Mepivacaine, Alexandria Co. for pharmaceuticals & chemical industries, Alexandria, Egypt).

The method used for endotoxin sampling procedures has been described in several studies (Jacinto *et al.* 2005, Martinho & Gomes, 2008, Gomes *et al.* 2009). The tooth was isolated using a rubber dam, and the operative field including the tooth, the clamp and rubber dam sheet was disinfected using a sterile swab moistened with 3% H2O2 (v/v) for 30 s followed by 5.25% NaOCl (Egyptian Company for household products under license of Clorox Co. Cairo, Egypt) for 30 s.

Access cavity preparation was performed in two stages: The first stage was performed to allow complete removal of carious lesions and restoration using a high-speed sterile round bur without exposure of the pulp chamber. In the second stage, before entering the pulp chamber, the access cavity was disinfected following the protocol previously mentioned. Another sterile round and Endo-Z burs (Dentsply Sirona, Ballaigues, Switzerland) were used to access the pulp chamber and 5% sodium thiosulphate was used to inactivate the disinfecting agents. The sterility of the internal and external tooth surfaces was checked by taking a swab sample and streaking on blood agar plates with subsequent incubation at 37 °C in both aerobic and anaerobic conditions. If any positive culture was detected, the sample was then excluded from the study.

The first endotoxin sample (**S1**) was taken by introducing three sterile paper points (size 15) (Meta Biomed Co. Ltd, Chungbuk, Korea) individually to the approximate working length of the wider / largest canal determined radiographically from diagnostic radiographs. Each paper point was left in the canal for 1 min. The three samples were placed in a sterile empty Eppendorf tube for further Enzyme-linked immunosorbent assay (ELISA) and stored at −20 °C.

After the first sampling, the pulp chamber was flushed with a sterile saline solution. Glide path of root canals was established by introducing a size 10 K-file (Mani Inc., Utsunomiya, Tochigi, Japan) to check patency followed by using a Proglider rotary file (Dentsply Sirona). The working length was determined using an electronic apex locator (Root ZX; J. Morita, Irvine, CA, USA) and radiographically confirmed to be 0.5–1 mm short of the radiographic apex. Root canals were mechanically prepared in the intervention (Neem) and the control (2.5% NaOCl) groups using a crown-down technique using a ProTaper Next rotary system (PTN) (Dentsply Sirona) in a torque-controlled endodontic motor (X-SMART® Plus; Dentsply Sirona) with torque 2 Ncm and speed

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300 rpm using a gentle in and out brushing motion to the full working length, in a sequence of X1, X2, X3 (mesial and distal root with two canals) or X4 (distal root with one canal).

Before the use of PTN and between each two consecutive instruments. The root canals were irrigated with the allocated irrigant (Neem in the intervention group and 2.5% NaOCl in the control group) (3 mL for 1 min) using a 30-gauge side-vented needle (KerrHawe SA, Bioggio, Switzerland). The needle was inserted 2 mm short of the working length, using a rubber stop as a guide, and the root canal was irrigated, whilst the needle was moved up and down. The prepared canals up to X3 file received a total of 12 mL of the irrigants for 4 min, whilst the prepared canals up to X4 file received a total of 15 mL of irrigants for 5 min. After completion of mechanical preparation and during the final irrigation process in both Neem and 2.5% NaOCl groups, a manual dynamic irrigation was performed with the push-pull motion of a well-fitted gutta-percha cone corresponding to the last PTN file used for 30s.

The effect of NaOCl was neutralized in the control group using 3 mL of sodium thiosulfate for 1 min. Then, the root canals in both Neem and NaOCl groups were flushed using 5 mL of sterile saline solution for 90 s, followed by 3 mL of 17% EDTA (Prevest DenPro Limited Company, Jammu, India) solution for 1 min (Verma *et al.* 2019) to remove the smear layer and the final rinse was 3 mL of sterile saline solution for 1 min. The second sample (S2) was taken as previously described for the first sample (S1). The samples were transferred to the laboratory in the Biochemistry Department, Cairo University and stored at −20 °C so that all the samples were processed at the same time by ELISA.

At the end of the first visit, a dry cotton pellet was placed in the pulp chamber, and the access cavity was restored using a temporary restoration (Cavit; 3M ESPE, Seefeld, Germany) without intracanal medication.

In the second visit 7 days later, the tooth was isolated with a rubber dam and the temporary restoration was removed. A paper point was inserted to working length to confirm the absence of exudate or foul odour (Emara *et al.* 2019). Each root canal was irrigated with 3 mL of sterile saline solution for 1 min. PTN master cone (Dentsply Sirona) was selected corresponding to the size of the last PTN file used, and a radiograph was taken to ensure the proper length of the master cones. After master cones selection, each canal was irrigated with 3 mL of either 2.5% NaOCl or Neem depending on the group for 1 min followed by 3 mL 17% EDTA for 1 min and a final rinse with 3 mL of sterile saline for 1 min. Then, the canals were dried using sterile matched PTN paper points (Dentsply Sirona) and were filled using a cold lateral condensation technique with AdSeal resin-based root canal sealer (AdSeal; Meta Biomed CO. LTD, Chungbuk, Korea). The access cavity was restored using a temporary restoration (Cavit; 3M ESPE).

After each visit, the patients were given Ibuprofen (400 mg) (Novartis Pharma, Cairo, Egypt) and were instructed to take it only in case of moderate or severe pain and to record the number and time of analgesic tablets taken.

Three days later following root filling, all patients returned their NRS charts and were referred to the Fixed Prosthodontics Department, Faculty of Dentistry, Cairo University for the final coronal restoration.

Outcome assessment

The patients were asked to rate the severity of pain using a numerical rating scale (NRS). The patients were instructed to fill the pain chart at the observation periods (6, 12, 24 and 48 h) following instrumentation and canal filling. The operator kept in contact with the patients by phone, to remind them to fill the NRS chart and to ensure accurate adherence to instructions. In case of severe pain and/or swelling occurred and the patient had to be evaluated, an emergency visit was scheduled, root canals were irrigated with the same protocol followed in the first visit by (N.S.). Systemic antibiotic (Augmentin 1 g/12 h/5 days) (GlaxoSmithKline, Cairo, Egypt) and analgesics (Ibuprofen 600 mg/8 h/5 days) (Novartis Pharma, Cairo, Egypt) were prescribed in the presence of swelling.

The secondary outcome was the quantification of endotoxins using the sandwich ELISA method after access cavity preparation and after completion of instrumentation. The method from a previous study was followed (Alarbeed *et al.* 2019). The Micro- Elisa strip plate provided in the ELISA kit (Human endotoxin (ET) ELISA Kit) provided by SunLong Biotech Co. Ltd, (Catalogue Number: SL0652Hu; Zhejiang, China) was pre-coated with an antibody specific to human endotoxin (ET).

The endotoxins were extracted from the paper points by the addition of 50 uL of phosphate-buffered

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saline (PBS) using a micropipette then vortexed for 15 s followed by centrifugation for 5 min at 3000 × *g*. Samples were added to the appropriate Micro-ELISA strip plate wells and incubated at 37 °C for 30 min. A well was left empty as a blank control.

The washing procedure was done by using the wash solution after resting for 30 s and this procedure was repeated 5 times. Then, a horseradish peroxidase (HRP)-conjugated antibody specific for ET was added to each well and incubated at 37 °C for 30 min. For the colouring procedure, 50 μL of TMB substrate solution was added to each well and incubated at 37 °C for 15 min. The wells that contained ET and HRP conjugated ET antibody appeared blue in colour and then turned yellow after adding 50 μL of the stop solution to terminate the reaction.

The optical density (OD) was measured spectrophotometrically using a Microtiter plate reader (STAT FAX 2100; Awareness Technology Inc., Palm City, FL, USA) to read the absorbance at 450 nm. The optical density (OD) value of the blank control well was set as zero, and the assay was carried out within 15 min. Calculation of the results was done as the OD value was proportional to the concentration of ET. The concentration of ET in the samples was calculated by comparing the OD of the samples to the standard curve (concentrations of Human ET Standard on (*x*-axis) and its corresponding reading of optical density (OD) (*y*-axis).

Statistical analysis

All data were coded to allow statistician blinding and entered using the statistical package SPSS version 22 (SPSS Inc., Chicago, IL, USA). Pain score data described as mean and standard deviation. The data were explored for normality using Kolmogorov–Smirnov test and Shapiro–Wilk test. Comparisons between the two groups for normally distributed numerical variables were done using Student's *t*-tests (T) whilst Mann–Whitney tests (*U*) was used for non-normally distributed variables. Comparisons between categorical variables were performed using chi-square tests (X^2). A *P*-value less than or equal to 0.05 was considered statistically significant.

Results

Of 70 patients, 50 patients met the inclusion criteria and were included in the statistical analysis without dropouts or exclusions. The flow of patients through each phase of the trial is shown in Figure 1. The baseline demographic data and clinical features of the patients are presented in Table 1. There was no significant difference between the two groups regarding the baseline data of mean age values ($P = 0.76$), gender distribution ($P = 0.18$), tooth type distribution ($P = 1$), presence of periapical lesions ($P = 0.37$), pain on percussion ($P = 0.7$) and the mean pre-operative pain scores ($P = 0.64$).

The severity of post-operative pain is presented in Table 2 and Figure 2 and was significantly lower in the intervention group compared with the control group only at 24 h following canal instrumentation ($P = 0.046$).

The intensity of post-operative pain within the two groups is presented in Figure 3 and was associated with a continuous decrease over time. The mean pain scores in the intervention group were lower than those in the control group at 6, 12, 24 and 48 h following instrumentation and canal filling with no significant difference between them except at 24 h following canal instrumentation ($P = 0.012$).

After root canal instrumentation, 6 patients in the control group (3 patients took 1 tablet, 2 patients took 2 tablets and 1 patient took 3 tablets) and 8 patients in the intervention group (3 patients took 1 tablet, 2 patients took 2 tablets and 3 patients took 3 tablets) due to moderate pain. There was no significant difference between the two groups regarding the incidence of analgesic intake (32% in the intervention group versus 24% in the control group ($P = 0.52$).

Sterility samples were negative for microbial growth, so all the samples were included in the analysis. The mean values of endotoxin samples in the two groups are represented in Table 3. Both groups were associated with a significant reduction in the amount of endotoxins compared to the pre-instrumentation sample with a significant difference between them in which the intervention group recorded the lowest mean value compared to the control group ($P = 0.02$). Both irrigants were able to reduce endotoxins amount compared with the pre-instrumentation sample by 8% for the control group and 18% for the intervention group.

Discussion

The use of herbal and natural products continues to gain popularity due to several advantages including easy availability, relatively low cost, low toxicity, increased shelf life, lack of microbial resistance and

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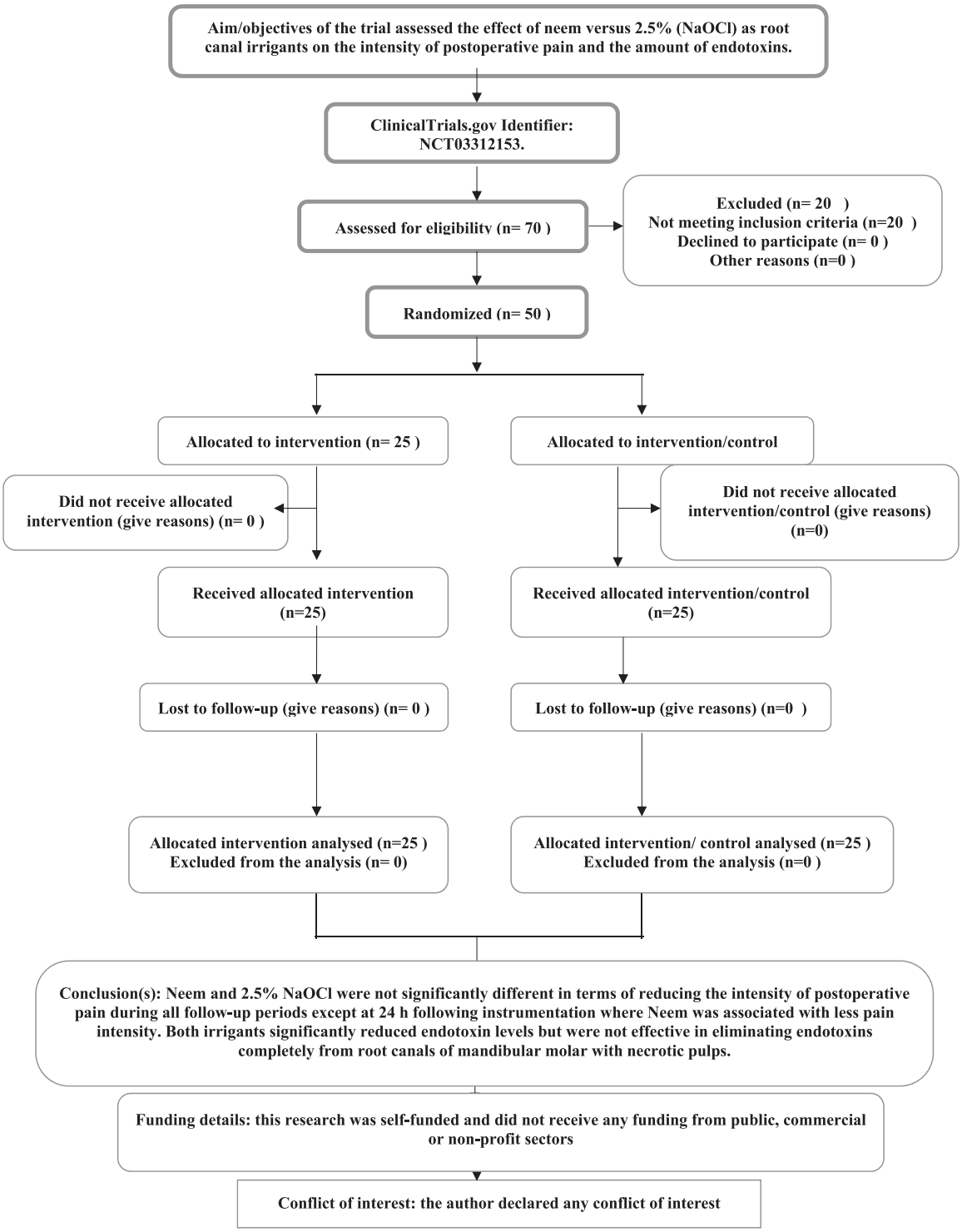

Figure 1 PRIRATE 2020 flowchart.

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Table 1 Demographic data and clinical features of the patients in the two groups

	Control group $n = 25$	Intervention group $n = 25$	*P*-value
Gender [*n* (%)]			
Female	17 (68%)	16 (64%)	0.76
Male	8 (32%)	9 (36%)	
Age (Mean ± SD)	29 ± 10.9	32.96 ± 9.8	0.18
Tooth type [*n* (%)]			
Lower 6	18 (72%)	18 (72%)	1
Lower 7	7 (28%)	7 (28%)	
Presence of periapical lesion			
No	15 (60%)	17 (68%)	0.37
Yes	10 (40%)	8 (32%)	
Pain on percussion			
No	12 (48%)	14 (56%)	0.7
Yes	13 (52%)	11 (44%)	
Pre-operative pain (Mean± SD)	1.6 ± 1.7	1.8 ± 2.08	0.64

SD, standard deviation.

Table 2 Severity and incidence [n (%)] of pain at the different pain categories for the 2.5% NaOCl and the Neem groups using chi-square test

Period	Pain + category	Control group (2.5%NaOCl) n = 25	Intervention group (Neem) n = 25	*P*-value
PreOP	No pain	12 (48%)	11 (44%)	0.62
	Mild pain	10 (40%)	8 (32%)	
	Moderate	3 (12%)	5 (20%)	
	Severe	0 (0%)	1 (4%)	
POP6 h	No pain	2 (8%)	8 (32%)	0.14
	Mild	18 (72%)	13 (52%)	
	Moderate	5 (20%)	4 (16%)	
POP12 h	No pain	6 (24%)	14 (56%)	0.053
	Mild	16 (64%)	8 (32%)	
	Moderate	3 (12%)	3 (12%)	
POP24 h	No pain	13 (52%)	21 (84%)	0.046*
	Mild	11 (44%)	4 (16%)	
	Moderate	1 (4%)	0 (0%)	
POP48 h	No pain	23 (92%)	22 (88%)	0.8
	Mild	1 (4%)	2 (8%)	
	Moderate	1 (4%)	1 (4%)	
Preob	No pain	24 (96%)	25 (100%)	0.5
	Mild	1 (4%)	0 (0%)	
	Moderate	0 (0%)	0 (0%)	
	Severe	0 (0%)	0 (0%)	
POPob 6 h	No pain	11 (44%)	12 (48%)	0.7
	Mild	14 (56%)	13 (52%)	
	Moderate	0 (0%)	0 (0%)	
POPob12 h	No pain	15 (60%)	18 (72%)	0.37
	Mild	10 (40%)	7 (28%)	
	Moderate	0 (0%)	0 (0%)	
POPob24 h	No pain	23 (92%)	23 (92%)	1
	Mild	2(8%)	2 (8%)	
	Moderate	0 (0%)	0 (0%)	
POPob48 h	No pain	25 (100%)	25 (100%)	1
	Mild	0 (0%)	0 (0%)	
	Moderate	0 (0%)	0 (0%)	

PreOP, pre-operative pain; POP6 h, pain 6 h after instrumentation, POP12 h, pain 12 h after instrumentation; POP24 h, pain 24 h after instrumentation; POP48 h, pain 48 h after instrumentation; Preob, pre-obturation pain; POPob 6 h, pain 6 h after obturation; POPob12 h, pain 12 h after obturation; POPob 24 h, pain 24 after obturation; POPob 48 h, pain 48 after obturation

*Statistical significance ($P < 0.05$).

relatively safe active constituents that have a significant physiologic/ biological effect (Maroli *et al.* 2017). Global health seeks to merge the use of herbal and natural products with evidence-based research for better understanding of their properties and effects on humans (Islas *et al.* 2020).

Neem (Azadirachta indica) has gained research interest in endodontics worldwide due to its wide range of medicinal properties as antibacterial, antifungal, antipyretic, analgesic, antioxidant and anti-inflammatory (Singh *et al.* 2017, Islas *et al.* 2020). Laboratory studies have revealed encouraging results including the use of Neem as a root canal irrigant (Sundaram *et al.* 2016, Ambhore *et al.* 2017, Esmail *et al.* 2020). Yet, there is a lack of clinical studies on this irrigant to support its ability to reduce post-operative endodontic pain and remove bacteria and bacterial byproducts from infected root canals. Therefore, this double-blinded randomized controlled trial was conducted to assess and confirm the clinical validity of using Neem (Azadirachta indica) as a herbal root canal irrigant versus 2.5% sodium hypochlorite on the intensity of post-operative pain and endotoxin levels in mandibular molars with necrotic pulps.

Fifty patients with mandibular molars with necrotic pulps without or with periapical radiolucency smaller than 3 mm were included. It has been reported that mandibular molars have the highest incidence of post-operative pain and so represent the worst-case scenario (Sadaf & Ahmad 2014, Shibu 2015, Mostafa *et al.* 2020). Teeth with necrotic pulps have a higher incidence of post-operative pain and flare-up than teeth with vital pulps (Siqueira 2003, El Mubarak *et al.* 2010, AlRahabi 2017, Sun *et al.* 2018). Moreover, they are more likely to extrude irrigants to periapical tissues than teeth with vital pulps (Farzaneh *et al.* 2018, Mostafa *et al.* 2020). Teeth with periapical radiolucencies of more than 3 mm were excluded

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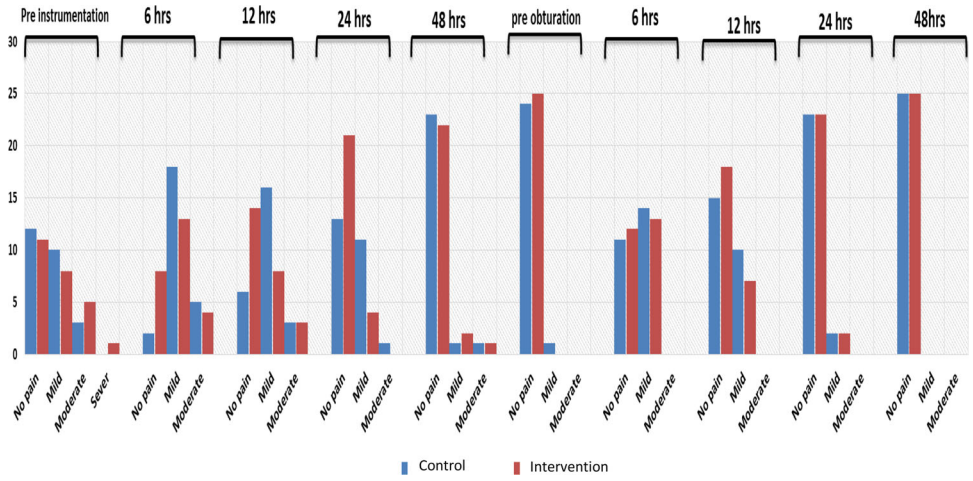

Figure 2 Bar chart representing the severity of pain following canal instrumentation and canal filling at different follow-up periods for each group.

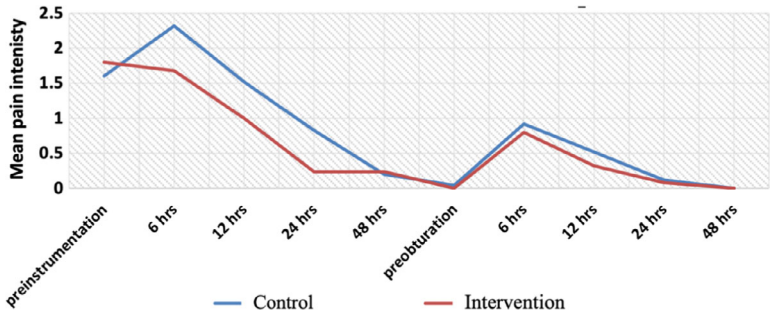

Figure 3 Line chart representing the changes in the intensity of pain at different follow-up periods for each group.

Table 3 Mean and standard deviation values of endotoxin levels

		S1	S2	*P*-value	Percentage of reduction
Control group	Mean± SD	245.7 ± 43.7	227.8 ± 45.8	<0.001*	8%
Intervention group	Mean± SD	243.4 ± 49	199.3 ± 39	<0.001*	18%
P-value		0.8	0.02*		

*Statistical significance ($P < 0.05$).

as they could represent a long-standing infection and may represent a cystic transformation or extra-radicular infections, which have a negative influence on the success rate (Nair 2006, Ng *et al.* 2011).

Root canal treatment was completed in two visits with no intracanal medication to reduce the numbers of confounding factors that could influence pain by avoiding its potential effect on post-operative pain especially when extruded periapically (Bashetty & Hegde 2010, Mostafa *et al.* 2020). A two-visit approach was adopted as previous studies have reported that patients treated in one-visit experienced more post-operative pain within the first week and required more painkiller intake (Figini *et al.* 2008, Schwendicke & Gostemeyer 2017). Furthermore, producing comparable results with previous studies that assessed post-operative pain after using NaOCl in patients with necrotic pulps (Bashetty & Hedge, 2010, Mostafa *et al.* 2020).

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NaOCl 2.5% was used in this study because it is less cytotoxic than 5.25% NaOCl. Previous randomized clinical trials reported that using lower concentrations of NaOCl was associated with less intense and frequent post-endodontic pain than 5.25% NaOCl in treating mandibular molars with nonvital pulps (Verma *et al.* 2019, Mostafa *et al.* 2020), with no effect on its antibacterial efficacy (Frough-reyhani *et al.* 2016, Zand *et al.* 2016).

Ethanol and Dimethyl sulfoxide (DMSO) was used as solvents in the present study. Ethanol 95% has better dissolving capabilities. The active components present in Neem are made of phenol groups that dissolve in an organic solvent but not in water. So, the ethanolic extracts have higher concentrations/amounts of bioactive compounds when compared with acetone, water and methanol (Altemimi *et al.* 2017). After 7 days, alcohol was evaporated using Rota-vapor to reduce the number of confounding factors that may affect the level of endotoxins by eliminating the antibacterial effect of alcohol. Thus, the real active ingredients of the extract are Neem components. Previous studies compared the antibacterial activity of ethanolic and aqueous Neem extracts and revealed that the ethanolic extract was better and more efficient than the aqueous extract (Aarati *et al.* 2011, Sinha *et al.* 2015). DMSO 9.99% is a non-toxic clear, colourless, hygroscopic organic solvent that is safe in humans with no antimicrobial activity (Singaravelu *et al.* 2019). DMSO has a unique capability to penetrate through living tissues due to its relative polar nature, without affecting the active components of Neem extract (Divya Kumari *et al.* 2019).

In the present study, root canal preparation was completed using a ProTaper Next (PTN) rotary system, as it has reported to be associated with less debris extrusion apically (Koçak *et al.* 2015) due to the progressive and regressive percentage taper that helps in removing more debris in the coronal direction, which has a clinical implication in reducing the incidence of post-operative pain (Arora & Joshi 2017).

An 11 point numerical rating scale (NRS) was used to evaluate pain intensity. It is a standardized assessment measure easier to use than a visual analogue scale, and more sensitive than a verbal rating scale (Hjermstad *et al.* 2011). It has been commonly used as an outcome measure in several studies (Najm *et al.* 2018, Ahmed *et al.* 2019, Mostafa *et al.* 2020).

Pain was assessed at 6, 12, 24 and 48 h following instrumentation and canal filling. The first 6 h was selected to determine pain level after the effect of the local anaesthesia has completely disappeared (Singh & Garg 2012). A maximum of 48 h was selected because the incidence and severity of post-operative pain are highest in the first 24 h and significantly decreases to minimal levels after 48 h post-operatively (Pak & White 2011).

Statistical analysis revealed that both groups had similar baseline characteristics in terms of age, gender, presence of periapical lesion, pain on percussion and pre-operative pain scores indicating successful randomization. The mean pain scores in the two groups decreased dramatically through the follow-up periods. Both group reported the highest mean pain score was at 6 h which decreased to reach the lowest value at 48 h following instrumentation and canal filling, this is in agreement with the findings of other clinical trials (Bashetty & Hedge, 2010, Pak & White 2011, Mostafa *et al.* 2020).

Comparison between the two groups revealed that there was a continuous decrease in the pain intensity over time with no significant difference between them at all follow-up periods except at 24 h following canal instrumentation where the intervention group recorded a lower pain level than the control group.

The rationale for decreasing of pain following the use of Neem could be associated with its high antibacterial activity, anti-inflammatory, antipyretic, analgesic and biocompatibility. Neem also has an anti-adherence activity by altering bacterial adhesion and their ability to colonize (Rosaline *et al.* 2013). Khetarpal *et al.* (2014) stated that the presence of nimbidin and nimbolide, which are the active constituents of Neem can cause bacterial cell wall lysis. Neem also plays a role as an anti-inflammatory agent by regulating the pro-inflammatory enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) (Alzohairy 2016).

On the contrary, Maroli *et al.* (2017) evaluated post-operative pain after using Neem, calcium hydroxide and 0.1% CHX as intracanal medicaments. Their results revealed that Neem was less efficacious than calcium hydroxide and CHX in reducing interappointment pain. The variability of the results may be attributed to the difference in the sample size where 5 patients were included in each group and the difference in methodology where Neem was used as intracanal medicament in form of a powder mixed with saline.

An ELISA method has limitations as it is time-consuming and tedious/laborious assay procedure as

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well as it provides a low level of sensitivity in the detection of biological molecules as microRNAs (Hosseini *et al.* 2018). However, a sandwich ELISA technique is highly sensitive (2-5 times more than direct or indirect ELISAs), specific (two antibodies were used to detect the antigen) and reproducible that provides valuable data of being able to detect and quantify substances such as peptides, proteins and antibodies that make it an efficient biotechnological tool in scientific research (Crowther 2000, Alarbeed *et al.* 2019).

Both irrigants were associated with a significant reduction in endotoxin levels compared to the pre-instrumentation samples by 8% for the NaOCl group and 18% for the Neem group. The presence of a significant difference could be attributed to the high antibacterial effect of Neem and NaOCl when compared with other irrigants as reported in previous investigations (Ambhore *et al.* 2017, Esmail *et al.* 2020).

The high level of remaining endotoxins in the two groups confirmed that instruments and irrigants failed to penetrate the confined areas of the complex root canal system. This is similar to the findings of previous studies as the reduction of endotoxins after chemo-mechanical preparation was reported to be 47% by Gomes *et al.* (2009), this variability in the results may be attributed to the difference in the methodology where 2.5% NaOCl was compared with CHX gel and Limulus amebocyte lysate assay was used to quantify endotoxins. Martinho & Gomes (2008) reported the reduction of endotoxins was 59.99%, the contrast of the studies may be attributed to the difference in the sample size as only 24 canals were included and the difference in the methodology where Limulus amebocyte lysate assay was used to quantify endotoxin levels.

It is important to highlight that the polymicrobial nature of root canal infections has been reported and a sandwich ELISA method succeeded in quantifying the amount of endotoxins that were released/liberated from dead gram-negative bacteria, but it does not give an indication of the effect of the irrigants on the reduction of live bacterial count and on the removal of biofilm from the root canal wall. Further clinical investigations are recommended to evaluate the clinical implication of residual infections after root canal treatment and how they affect the outcome of the treatment.

The limitations of the study may be the use of manual irrigant activation technique. Although it is safe, convenient, cost-effective and has a little chance to extrude irrigants apically. However, it has limited ability to allow irrigant to penetrate deeper into dentinal tubules. Another limitation of the trial may be the inclusion of both teeth with and without apical periodontitis, thus including a broader spectrum of patients to increase the generalizability of the study findings. Further clinical studies are recommended to include only teeth associated with apical periodontitis or without apical periodontitis, not both to reduce the numbers of confounding factors as the intracanal microbiota of these teeth are different.

The results of this study revealed that using Neem and NaOCl alone is not effective in eliminating endotoxin from infected root canals. Further clinical studies are recommended to assess the effect of different concentrations of Neem and NaOCl with active irrigation protocols and intracanal medicaments on bacteria and/or biofilm, and endotoxins reduction, and to determine if Neem can be used as an alternative irrigant to NaOCl in endodontics.

Conclusion

Neem and 2.5% NaOCl were not significantly different in terms of reducing the intensity of post-operative pain during all follow-up periods except at 24 h following canal instrumentation where Neem was associated with less pain intensity. Both irrigants significantly reduced endotoxin levels but were not effective in eliminating endotoxins completely from root canals of mandibular molar with necrotic pulps.

Funding information

This research was self-funded and did not receive funding from public, commercial or non-profit sectors.

Conflict of interest

The authors have stated explicitly that there is no conflict of interests in connection with this article.

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