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EVALUATION OF ANTIMICROBIAL ACTIVITY OF MANUALLY AGITATE (NANO- CHITOSAN AND NANO- PROPOLIS) AGAINST *ENTEROCOCCUS FAECALIS* IN COMPARISON WITH SODIUM HYPOCHLORITE: AN IN-VITRO STUDY

Dalia Ali Ahmed Moukarab*

ABSTRACT

Aim: The purpose of this study was to evaluate in an in-vitro model the antibacterial efficacy of manually agitated nano- chitosan and nano-propolis as root canal irrigation protocols in comparison to manually agitated sodium hypochlorite against *Enterococcus Faecalis*.

Material and Methods: The root canals of 50 single rooted human premolars were flattened to a standard length of 18mm cleaned and shaped to a size F5 Protaper, apices closed with composite, fixed into an Eppendorf vial with silicon impression material, autoclaved, and randomly assigned to the test groups (n=10) . Negative control was autoclaved and microbial analyzed. The other 40 specimens were contaminated with freshly prepared suspension of *E. Faecalis* and incubated for 4 weeks and equally divided into 4 groups positive control group (n=10) was not subjected to any further treatments, group A final irrigation with 5.25% NaOCl, group B final irrigation with nano-chitosan and group C final irrigation with nano- propolis ; all irrigation protocols were combined with manual agitation with well fitted gutta perch cone for 2 minutes under a laminar flow hood. Microbial samples were collected from all the root specimens and colony forming units were counted and transformed into log CFU. The collected data were statistically analyzed.

Results: There was no statistical significant difference in log CFU count between group A (treated with NaOCl) and group B, C (treated with CNP and PNP) while groups A, B, C showed statistically significant difference from positive control group with ($p < 0.05$).

Conclusion: With in the limitations of the present study it can be concluded that all irrigation protocols had efficient antibacterial effect against *E. faecalis*.

Manually agitated (CNP or PNP) can be considered as a more safe, efficient, and simple alternative instead of sodium hypochlorite against *E. faecalis*.

KEY WORDS: Nano-chitosan, Nano- propolis, Enterococcus faecalis, Manual agitation, Root canal irrigants.

* Lecturer of Endodontics, Department of Endodontics, Faculty of Dentistry, Minia University

INTRODUCTION

Along term successful endodontic treatment primarily depends on elimination of microorganisms from the complex root canal system; while this statement seems very simple it isn't. Mechanical debridement and shaping of the root canal falls short of totally eradicating all microorganisms from the root canal hence ,different irrigating solutions are used not only to help physically in removing debris from the root canal but in dissolving soft tissues and killing bacterial especially from areas where instruments are inaccessible.[1-5]

Enterococcus Faecalis is one of the most commonly identified microorganisms in failed endodontic treatments. [6,7]

It is a facultative anaerobic gram positive cocci known for its resistance among root canal flora, and has been frequently isolated in both pulpal and periapical lesions. **Stuart et al** [8] attributed this to its many virulence factors including lipoteichoic acid, cytolysin, pheromones, lytic enzymes and aggregation substances , its ability to adhere to tooth substance and its high ability to suppress lymphatic activity which potentially contributes to endodontic treatment failure.

Love [9] further studied *E. faecalis* role in endodontic failure and attributed it to the ability of ***E. faecalis*** to invade the dentinal tubules and its high affinity to bind to dentin collagen where it can hid from instrumentation and irrigation solutions along with the ability to with stand long periods of starvation and resist high concentrations of intracanal medicaments..

Sodium hypochlorite has been considered for decades as the gold standard endodontic irrigant with soft tissue dissolving characteristic, wide range antimicrobial activity and lubrication.[10,11,12]. Though proven to be effective as an irrigant many side effects have been observed over the years as toxicity to the periapical tissue, allergic reactions, reduction of flexure strength of dentin leading to its weakness and susceptibility to deformation and fracture. [13]

Furthermore, when sodium hypochlorite is extruded into the periapical tissue serious side effects arise as sever inflammation, ecchymosis, hematoma, necrosis and Parathesia [14]

These inherent drawbacks of chemical irrigants and constant increase in antibiotic resistance create an ongoing urge to discover and explore new natural alternative medicaments.

In the pursuit of natural alternatives for chemicals; Chitosan emerged; a natural polysaccharide which is the principle component of crustacean exoskeleton which has been recently introduced in to the field of dentistry. [15] It is described as a nontoxic cationic biopolymer which is biocompatible, biodegradable and has the ability to improve dentin surface properties and elevates dentin resistance to collagenase degradation. [16] Moreover, chitosan possesses prolonged antibacterial activity against a broad range of microorganisms.[17-20] Furthermore; it significantly improves bond strength to dentin. [21]

Kishen et al [22] tested nano-chitosan for its efficacy in eliminating bacterial biofilm and found it to be significantly efficient in removing it. **Del Carpio- Perochena etal** [23] showed that nano-chitosan was significantly efficient in removing smear layer and bacterial biofilm moreover it prevented recolonization of bacteria on the root dentin. Authors recommended nano- chitosan to be used as an alternative for EDTA.

Shi et al [24] explained the powerful antibacterial property to the high surface area and charged density which allow these unique particles not only to contact more surface area of bacterial cell but also to be attracted to the negatively charged cell membrane eventually causing its death. **Abraham et al** [25] studied the effect of different activation methods with chitosan irrigation in root canals to remove smear layer from the apical third and found that combining diode laser or endoActivator with

chitosan established significantly more smear layer removal.

Another natural substance is propolis; a brownish resinous substance collected by bees from plants which is used for comb reinforcement in hives and keeps the hive environment aseptic. This substance is known for its potent antioxidant, anti-inflammatory and antimicrobial activity.[26]

This encouraged its use in dentistry for many treatments as direct and indirect pulp capping, caries prevention, root canal disinfection, accelerating surgical wound healing and as a storage media for avulsed teeth. [27, 28]

Kilic etal [29] showed that propolis can break the resistance produced by certain bacterial strains as S aureus and E faecalis which are significant in endodontic treatment. Authors recommended its use in disinfecting root canals. **Parolia etal** [30] confirmed that ethanolic propolis extract induced bone regeneration in addition to dentin bridge formation which was demonstrated when propolis comes in contact with exposed pulpal tissues.

Al-Qathami etal [26] tested antimicrobial activity of propolis and found it to be equal to that of sodium hypochlorite. **Kousedghi etal** [31] compared propolis to calcium hydroxide against *E. faecalis* and *lactobacillus spp. and pepto-streptococcus spp*. Results showed propolis to be more efficient than calcium hydroxide. **Khurshidetal etal** [32] found that antibacterial efficacy of ethanolic based propolis against *E. faecalis* was between Chlorohexidine and calcium hydroxide and recommended its use as an endodontic irrigation solution.

Saha etal [33] attributed the antioxidant, anti-inflammatory and antibacterial properties characteristic of propolis to the flavonoids content with in it. **Elgendy and Fayyad** [34] tested propolis and propolis nanoparticles for their biocompatibility via testing cytotoxicity and apoptotic changes on dental pulp stem cells. They found both substances to be biocompatible and recommended them to be used in endodontic regenerative purposes. Recently nano propolis particles have been incorporated in to orthodontic composite which bonds to enamel. **Sodagar etal** [35] tested it for antimicrobial activity and found it significantly efficient.

For an irrigant to preform efficient disinfection it must come in to direct contact the microorganisms, unfortunately syringe irrigation falls short of reaching all internal surface irregularities of the canal, anastomosis between canals, fins and apical portion of the canal.[36] Therefore many studies advocate to actively moving irrigation solutions to amplify their effect and improve irrigation dynamics. Gently moving well- fitted gutta percha master cone inside an instrumented canal- manual dynamic irrigation- filled with irrigant in an up \ down movement significantly improves cleansing ability and antimicrobial activity.[37] **MeGill et al** [38] compared manual gutta perch agitation to automated dynamic irrigation; results showed that manual dynamic irrigation was more efficient that Rins- Endo system.

Nanotechnology has revolutionized in many aspects of the medical field especially in dentistry. It is the technology which provides particle size of 0.1nm to 100nm. This unique size range allows the material to amplify its physical, chemical and biological properties which increases their efficacy, accuracy and speed of action. [39-41]

There recently growing interest in using endodontic materials in their nano form which has shown exceptional efficiency in fulfilling their objectives. This justifies the ongoing research and comparison with the conventional ways to reach optimum treatment.

The null hypothesis of this study is that manually agitated (CNP or PNP) is not as efficient antibacterial protocols as manually agitated sodium hypochlorite against *E. Faecalis*.

AIM OF THE STUDY

The aim of this study is to evaluate antibacterial efficacy of manually agitated nano-chitosan (CNP) and nano-propolis (PNP) against *E. faecalis* in comparison with manually agitated sodium hypochlorite in an in-vitro model.

MATERIALS AND METHODS

Selection and preparation of teeth

Fifty recently extracted human mandibular premolars with fully formed apices were collected for this study. Teeth were extracted for orthodontic or periodontal reasons. Only intact teeth with single root canal were included in this study. Teeth were thoroughly cleaned of external surface debris, soft tissue remnants and calculus; then stored in saline till use.

All teeth samples were flattened to establish a standardized tooth length of 18mm using a diamond stone (Diatech, Coltene, Switzerland) to establish uniform specimens. Then root canals were instrumented using Protaper system to size F5 (Dentsply, Maillefer, Switzerland); in between instruments 5.25% NaOCl was used as irrigation, for final irrigation 3 ml of 17% EDTA for 3 min was used followed by 3ml of 5% NaOCl for 3 min and final flush with 5ml of distilled water.

Root canals were dried with paper points and the apices were sealed with composite resin, two layers of nail varnish were applied to cover the external root surface to avoid bacterial leakage. Each specimen was fixed with silicon impression material in an Eppendorf vial (this was to facilitate handling and identification); then placed into a carrier box which was the inserted in an autoclave sachet and autoclaved for 30 min at 121°C. Ten specimens were then subjected to microbial analysis and served as negative control group.

Contamination of the specimens

E. Faecalis preparation:

A pure E Faecalis culture (ATCC 29212) was grown over night in brain heart infusion (BHI) broth at 37°C. The turbidity was adjusted to 0.5 Mac Farland standard and the obtained cell density was 1.5×10^{8} cell\ml.

Specimen contamination:

The remaining 40 specimens were filled with *E faecalis* suspension using sterile micropipettes and a sterile K- file # 15 was used to ensure bacterial suspension penetration in to the working length. All contaminated specimens were incubated at 37°C for 4 weeks and the root canal contents were refreshed every 3 days.

Preparation of the irrigation solutions:

- Sodium hypochlorite NaOCl (Egyptian company for household bleach-Egypt); the concentration was adjusted at Faculty of Pharmacy, Minia University (5.25% w\v NaOCl solution)
- Nano-chitosan CNP (Nano Tech, dream land, Egypt); Chitosan was milled in a multidimensional swipe nano-ball-milling machine in a process based on inotropic gelation of CS. Then 2 grams were diluted in 100 ml of 1 % acetic acid which was then stirred for 2 hours using a magnetic stirring machine till a crystalline homogenous solution was produces. [42]
- Nano-propolis PNP (Emtenan Company, Cairo, Egypt); Propolis was prepared by milling in a ball-milling machine for 24 hours then dissolved in ethanol (20 mg/ml) and stirred using a magnetic stirring machine till a homogenous solution was produced then filtered.

Classification of the groups:

Ten specimens (n= 10) were assigned to each group

1. Negative control group: specimens were autoclaved and no further treatment was done

(This group ensured that no contamination of specimens occurred).

2. Positive control group: specimens were infected after autoclaving and no further treatment was carried out. (This group ensured that proper infection took place for all the infected specimens)
3. Group A: specimens were autoclaved and infected; then 5.25% NaOCl irrigation was delivered into the root canal and agitated manually with a well fitted gutta percha point.
4. Group B: specimens were autoclaved and infected; then 2% nano-chitosan (CNP) irrigation was delivered into the root canal and agitated manually with a well fitted gutta percha point.
5. Group C: specimens were autoclaved and infected; then nano-propolis (PNP) irrigation was delivered into the root canal and agitated manually with a well fitted gutta percha point.

Irrigation protocol:

- All irrigation procedures were performed in laminar flow hood under a septic condition, with sterile gloves and sterile syringe for each specimen.
- For irrigation protocol; the same standardized procedure was carried out for all specimens.
- A Disposable 27 gauge conventional syringe was used to deliver the irrigation solution; and the needle tip was placed in the root canal 1mm from the working length (at 17mm).
- A total of 5ml irrigation solution was used for each specimen over a total of 5 minutes divided as following:
  - Initially 3 ml irrigation solution was applied to the root canal for 3 minutes in conventional syringe irrigation.
  - Followed by 2ml of the irrigation solution applied to the root canal and manually agitated with a well fitted gutta percha point in a gentle push\ pull movement for 2 minutes.
- All specimens were manually agitated with gutta percha point size F5.
- One investigator was assigned to perform all the irrigation protocols.
- At the end of the tested irrigation protocols all specimens were irrigated with 5ml sterile saline.

Microbial analysis:

After irrigation of the specimens 3 sterile paper points F5 (Dentsply, Maillefer, Switzerland) were introduced into the root canals to the full working length for 60 seconds each; then transferred to a labeled Eppendorf vials containing 1 ml of sterile PSB (Phosphate- Saline Buffer). All vials were vortexed for 1 minute. Tenfold standard sequential dilution of each vial was performed and the bacterial count in colony forming units for each ml was calculated.

Statistical analysis:

The log transformation of each CFU\ ml count was performed, the collected data were coded, tabulated, and statistically analyzed using SPSS program (Statistical Package for Social Sciences) software version 25. Distribution of the data was done by Shapiro Wilk test. Descriptive statistics were done for non-parametric quantitative data by median and interquartile range (IQR). Analyses were done for non-parametric quantitative data between the five groups using Kruskal Wallis test followed by Mann Whitney test between each two groups. The level of significance was set at (P value < 0.05).

RESULTS

The median log and IQR of bacterial count in (log CFU \ml $^{-1}$) for all study groups and statistical analysis (Table 1, Figure 1).

All specimens of the negative control group showed zero bacterial count while the specimens of positive control group recorded the highest bacterial count.

Regarding the tested irrigation protocols; group A recorded the lowest median bacterial count at 0.4 followed by group B and C at (0.7, 1) respectively. The results showed a statistically significant difference in comparison with positive control group $p < 0.001$ indicating the strong antibacterial activity of the three tested irrigation protocols. On the other hand no statistically significant difference was found among the 3 test groups (A, B, C) $p > 0.05$ indicating that antibacterial activity of group B (treated with CNP)and group C(treated with PNP) were comparable to that of group A (treated with NaOCl).

TABLE (1) Median and IQR of bacterial count in (log CFU\ml $^{-1}$) for each group and Kruskal Wallis test for non-parametric quantitative data between the five groups followed by Mann Whitney test between each two groups

		Control -Ve	Control +Ve	Group A (Sodium hypochlorite)	Group B (Nano-chitosan)	Group C (Nano-propolis)	P value
		N=10	N=10	N=10	N=10	N=10	
Bacterial count	*Median (IQR)*	0 (0-0)	6.1 (6-6.5)	0.4 (0-1.6)	0.7 (0.2-1.4)	1 (0.3-1.1)	*<0.001**
P value (between each two groups)							
Control –Ve			*<0.001**	*0.002**	*0.001**	*<0.001**	
Control +Ve				*<0.001**	*<0.001**	*<0.001**	
Group A					*0.423*	*0.325*	
Group B						*0.820*	

IQR: interquartile range ****: Significant level at P value < 0.05***

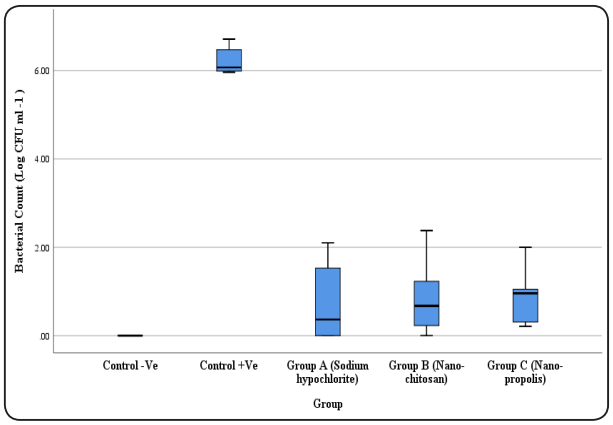

Fig. (1) Boxplot of Median and interquartile range (IQR) of bacterial count in (log CFU\ml -1) for all groups

DISCUSSION

The goal of endodontic treatment is complete debridement and disinfection of the root canal system; this is established not only by instrumentation but in combination with variable irrigation protocols. This justifies the continuous search for new irrigation solutions and techniques.

In the present study *E. faecalis* was chosen as bacterial infection maker which is the microorganism usually isolated from failed endodontic treatment due to its ability to survive irrigation solution, intracanal

medicaments, harsh root canal environmental stresses and its ability to form protective biofilms all of which is well documented. Moreover; *E. faecalis* possesses many virulence factors including lipoteichoic acid, cytolysin, pheromones, lytic enzymes and aggregation substances, its ability to adhere to tooth substance and its high ability to suppress lymphatic activity which potentially contributes to endodontic treatment failure. [43-48]

Human intact single rooted teeth were prepared and contaminated with *E Faecalis* and incubated for 4 weeks to ensure formation of a biofilm, this is to create a model that was clinically more relevant and replicate the usual endodontic significance. Many studies have confirmed that microorganisms grow in biofilms and that they reach 1000 times more resistance to antimicrobial agents than the planktonically grown bacteria. [49, 50]

All teeth included in this study were standardized to the same length and preparation procedures. The tested irrigation protocols were also standardized to have the same solution (volume, temperature, and irrigation duration and agitation duration)

Regarding the present study natural irrigations was used in nano form to amplify their antimicrobial effect. Nano-chitosan was suggested as an alternative for NaOCl due to its ability to break down bacterial cell membrane and interference with protein synthesis along with its biocompatibility which has been confirmed in previous studies. [51] Nano- propolis was also chosen for this study due to its low toxicity and high antimicrobial activity which allows it to be a safer alternative for NaOCl[26, 27, 28]. Irrigation protocol utilized manual dynamic irrigation which has found may advocates in literature confirming its superiority to syringe irrigation and a number of automated dynamic irrigation. [36-38]

Regarding the present study 10 specimens served as negative control and were autoclaved only and yield no bacterial count ensuring that specimens were completely sterile, in addition to 10 more specimens served as positive control which was contaminated and did not undergo further treatment and showed the highest bacterial count ensuring the uniform contamination and microbial loading levels of all specimens.

In the present study group A (which was treated with NaOCl) showed lowest bacterial count with a median of 0.4 log CFU\ml which is significantly lower than that of the positive control indicating its strong antibacterial efficacy. The results came in accordance with many previous studies.[8-13] **Gianrdino et al** [52] showed that 5.25% NaOCl was able to eradicate *E. faecalis* biofilm in 30 seconds, in addition to **Dunavant etal** [53] whom demonstrated that NaOCl killed all bacterial colonies within an organized biofilm.

This is mainly attributed to the chlorine release which affects a broad range microbe along with its ability to dissolve organic debris due to its proteolytic effect together with release of oxygen that eradicates anaerobic bacteria.[54]

Furthermore the tested irrigation protocol group B (treated with CNP) showed significantly low median 0.7 log CFU\ml; indicating the powerful antimicrobial efficacy of CNP against E faecalis. This comes in accordance with previous literature [16-20] Which is attributed to interaction between the positively charged CNP and the negatively charged bacterial cell membrane which impairs cellular exchange with medium; leading to leakage of the intracellular components and bacterial death.[23, 24]

In the current study group C (treated with PNP) recorded median log CFU\ml of 1 which is significantly lower than that of the initial bacterial loading of the positive control group with ($P < 0.001$); demonstrating the antibacterial efficacy against *E faecalis* which is consistent with previous studies. [27- 32]

Saha etal [33] referred the antibacterial activity of propolis to the flavonoid content which causes structural and functional damage; interfere with cell membrane integrity, inhibit the bacterial mobility, enzyme activity, cell- division and alters cell membrane permeability. Moreover; **Horvath etal** [55] attributed antibacterial activity to decrease the mRNA synthesis which leads to stopping of protein production killing the microorganism from within. While **Xie et al** [56] explained antibacterial mechanisms of flavonoids due to inhibition of biofilm formation and break down the bacterial and biofilm attachment to dentin, energy metabolism inhibition and attenuation of the pathogenicity.

In the present study chitosan and propolis irrigation solution were used in the nanoparticles form which also may have attributed to the powerful antibacterial effect against *E faecalis*; this is well documented in literature that nanoparticle size of a substance allows materials to acquire higher accuracy, efficiency and amplifies the antibacterial effect of many irrigants. [39-41]

Furthermore; the combination of these antibacterial agents with manual gutta percha agitation may explain the significantly low bacterial count. Manual dynamic irrigation has proven to be very valuable due to the physical pushing irrigant into the hard to reach canal complexities, providing more contact with bacterial colonies and breaking bacterial biofilms off the dentinal walls [37, 38]

No statistical significance difference was found between Groups (A, B, C) indicating that antimicrobial efficacy of manual agitated CNP and PNP is as effective as that of NaOCl which is considered the most potent endodontic irrigant which is in agreement with previous study by **Jaiswal et al** [57] and hence; the null hypothesis was refused.

CONCLUSION

Within the limitations of the present study it can be concluded that:

- All irrigation protocols had efficient antibacterial effect against *E. faecalis*.
- Manually agitated (nano- chitosan or nano-propolis) can be considered as a more safe, efficient, and simple alternative for sodium hypochlorite against *E. faecalis*.

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