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Evaluation of Antimicrobial Efficacy of Nano Chitosan and Chlorohexidine versus Sodium Hypochlorite as Final Rinse against *Enterococcus Faecalis*

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ABSTRACT

Purpose: This study aimed to evaluate the antimicrobial efficacy of nano chitosan and chlorohexidine versus sodium hypochlorite as final rinse against *Enterococcus faecalis*. **Methods:** Forty single rooted teeth were selected. All root canals were prepared manually to #50 file. Finally, the teeth were sterilized by gamma rays. Each root canal was inoculated with 0.5 ml of *E. faecalis*. Specimens were divided randomly into 4 main groups (10 specimens each) according to the irrigating solutions. Group 1: nano chitosan 0.2%, group 2: Chlorhexidine 2%, group 3: alternating solution of nano chitosan 0.2% & Chlorhexidine 2%, and group 4: Sodium hypochlorite 3%. The mean of bacterial count before and after final irrigation by the tested solutions were measured. **Results:** The highest mean difference value of bacterial count was found in 3% sodium hypochlorite, followed by alternating solution of 0.2% nano chitosan & 2% chlorhexidine, then 2% chlorhexidine, and 0.2% nano chitosan came lastly. However, there was no statistically significant difference among the tested groups. **Conclusions:** alternating solution of 0.2% nano chitosan & 2% chlorhexidine when used as antibacterial irrigant gives promising results and can be used as an alternative to NaOCl solution.

KEYWORDS

Nano chitosan, Chlorohexidine, Enterococcus Faecalis, Final rinse.

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INTRODUCTION

The most common etiological factors of pulpal diseases are microorganisms and their metabolic products [1]. The elimination of germs from the diseased root canal system is critical to the effectiveness of endodontic treatment [2]. Despite the fact that bacteria are the most common microorganisms discovered in primary endodontic infections, several investigations have found fungi in infected root canals [3].

It was shown that in post-treatment endodontic flare-ups besides Streptococci, Lactobacilli, Actinomyces and fungi, the Gram-positive bacterial species *Enterococcus faecalis (E. faecalis)* is often detected. Investigations have shown that in cases of secondary root canal infections the prevalence of *E. faecalis* can reach values up to 70%. *E. faecalis* is also known to be rather unsusceptible towards many disinfectants' material applied during antiseptic root canal treatment [4]. It is found in 22-77 percent of root canal failure cases and plays an essential role in the etiology of periradicular lesions after root canal treatment. Lytic enzymes, cytolysin, aggregation material, pheromones, and lipoteichoic acid are all virulence factors found in *E. faecalis*. It has been demonstrated to stick to host cells, express proteins that help it to compete with other bacteria, and change host reactions. *E. faecalis* can inhibit lymphocyte activity, which could lead to endodontic failure [5].

Because of its capacity to dissolve organic debris and great antibacterial potential, sodium hypochlorite has long been the gold standard for irrigation. However, there are several significant disadvantages to using sodium hypochlorite, including irritation to periapical tissues, unpleasant taste, high toxicity, corrosion of instruments, inability to remove smear layer, burning of surrounding tissues, and a decrease in elastic modulus and flexural strength of dentin[6,7].

Chlorhexidine (CHX) is a broad-spectrum antibacterial drug with significant antimicrobial action and minimal toxicity[8]. CHX has been proven in vitro to have long-lasting antibacterial activity in the root canal after being administered as an endodontic irrigant. As a result of its particular ability to bind to dentin, its efficiency as an antibacterial agent, and its substantivity in the root canal system, CHX has been proposed as a root canal irrigant [9].

Because of the disadvantages of the antimicrobial irrigants that are present, the search for new alternatives such as chitosan is essential. Chitosan is a non-synthetic polysaccharide, which consists of conjugated polymers of glucosamine and N-acetyl glucosamine. Partial deacetylation of chitin results in chitosan production. It is biocompatible, biodegradable, bio adhesive and has no documented toxicity. In addition, it is a good disinfectant agent. Its decreased price has increased its usage for many applications in the fields of medicine and pharmaceuticals. Also due to the high capability of chitosan to chelate different metal ions in acidic environment, it can successfully remove the inorganic component of smear layer from the root canals after mechanical preparation [10,11].

Nanomaterials are natural or synthetic materials that contain particles in unbound states, aggregates, or agglomerates, with exterior dimensions of 1 nm to 100 nm for 50% or more of the particles. [12]. It has been discovered that the size of nanoparticles affects their antibacterial activity, where the smaller particles the more the antibacterial activity [13-15]. Therefore, this study was directed to evaluate the antimicrobial efficacy of nano chitosan and chlorohexidine versus sodium hypochlorite as final rinse against *E. faecalis*.

MATERIAL AND METHODS

Teeth selection

This study used a total of 40 anonymous single-rooted teeth that were extracted for orthodontic purposes. The teeth were taken from Al-Azhar University's Oral and Maxillofacial Surgery

Department's outpatient clinic (Girls). Ethical consent for the use of excised human teeth was obtained in compliance with rules from the Faculty of Dental Medicine Al-Azhar University's Research Ethics Committee (Code: REC-EN-21-07). Teeth were obtained from patients who have given their consent for describing their approval of using their biological samples. Mandibular premolars with single root canals and mature apices were selected. Teeth were soaked in 5.25% sodium hypochlorite solution (Egyptian Detergent Company, Egypt) to remove any soft tissue covering the root surface and any calculus by the aid of ultrasonic scaler (wood packer, china). The roots of all teeth were sectioned at CEJ using a diamond tapered stone mounted on high speed hand piece (W&H Burmoos, Austria) with water spray. Flattening of the coronal portion of the roots was done with wheel stone.

K- File No.10 (DENTSPLY, Maillefer, Tulsa, OK) was introduced into the canal till the tip of the file was seen from the apex to ensured patency. The teeth were stored in normal saline solution 0.9% at room temperature till time of use.

Root canal preparation

Root canals were instrumented manually from initial file till size 50 with copious irrigation of 5 mL of freshly prepared solution of 2.5% NaOCl to remove biofilm, and 17% EDTA for smear layer removal, followed by 2.5% NaOCl respectively in all the samples. The 5 ml of NaOCl were divided as 1 ml after each file. Final irrigation was done with saline [10].

Grouping of samples

According to the final irrigation, samples were sorted into four groups, each with ten samples:

Group 1: Nano chitosan 0.2%.

Group 2: Chlorhexidine 2%.

Group 3: Alternating solution of nano chitosan 0.2% and Chlorhexidine 2%.

Group 4: Sodium hypochlorite 3%.

5 ml of each tested solution was used as a final irrigation.

Preparation of Nano Chitosan 0.2% (Nano Tech, Dream Land, Egypt).

2 gm of chitosan was diluted in 100 ml of 1% acetic acid, then was stirred for 2 hours using a magnetic stirring machine till a crystalline homogenous solution. After that, chitosan was milled in a multidimensional swipe nano-ball-milling machine in a process based on inotropic gelation of chitosan [16].

Samples' Sterilization (Egyptian Atomic Energy Authority):

Samples were inserted into sterilization bags and sterilized using gamma radiation (Cobalt 60 irradiator with dose rate of 1.774 KGY with total dose of 25 KGY) [17].

Inoculation of Enterococcus faecalis into prepared sample:

E. faecalis used in this study were obtained from patients randomly selected for research purposes at Microbiology Department, Faculty of Medicine for Girls, Al Azhar University. *E. faecalis* were prepared by growing single strain on a bile-esculin plate for 24 hours at 37°c, the purity of the culture were confirmed by culture characteristics (Fig.1a,b), microscopic examination (Fig.2) and biochemical reactions (Fig.3)

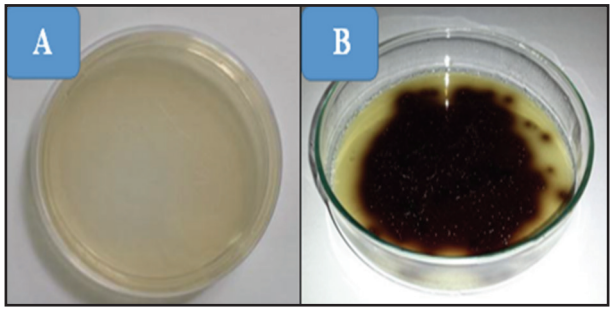

Figure (1) A photograph showing bile-esculin agar plate (A) uninoculated (B)inoculated.

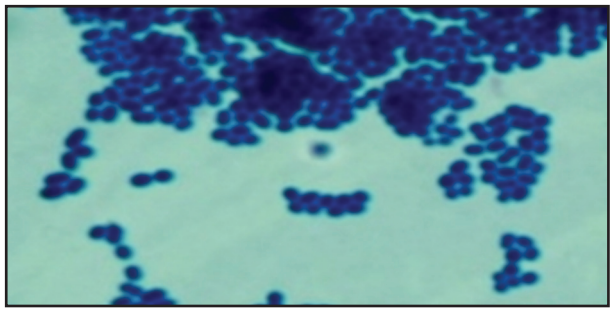

Figure (2) A photograph showing a microscopic examination of *E. faecalis*.

Figure (3) A photograph showing a test tube with negative result of catalase test.

Following sterilization of the samples, the root canal of each sample was flooded with a suspension of the isolated *E. faecalis* using sterile 1ml insulin syringe. For maximum penetration of the bacterial suspension into the entire root canal system, sterile size #15 K files was used in an up and down movement. Infused samples were placed individually inside eppendorfs with 2 ml of Brain Heart Infusion Broth (BHI), closed then were inserted inside a rack and placed in the incubator at 37°c for 48 hours, for allowing bacteria to multiply and proliferate.

Bacterial counting was done two times for each root throughout the study as follows: after incubation of root canals and after irrigation of root canal with the tested irrigation of each group.

The bacterial sample was taken from each root canal by insertion of three sterile absorbent paper point size #20 successively inside each root canal to be saturated for 1 min for each point. The paper point was removed from the canal using sterile tweezer and placed in a sterile plastic tube containing 1ml saline. The falcon tube was then sealed tightly with its screw and agitated by hand for 30 sec.

After agitation, the three-paper points were removed from the plastic tube and the cultivation process was done using serial dilution as follows: four sterile plastic tubes (each tube contain 0.9 ml of sterile saline) in a test-tube rack were labeled 1:10, $1:10^2$, $1:10^3$ and $1:10^4$ representing different levels of dilution. Two hundred μl of solutions from the original tube were placed in the first tube (1:10) of saline using a sterile automated pipettes and suitable different sterile tips used in between the different dilutions to avoid carry-over of bacteria.

The solution was thoroughly mixed in the tube by sucking up and then expelling the content three times gently. This process was repeated three more times using solution from 1:10 to dilute 1:102 and from 1:102 to dilute 1:103 and so on. Then culture every dilution on a separate bile oscaline plate, then the plates with cultivated bacteria were incubated for 24 hours at 37°C and 100% humidity.

The number of colonies was counted at the end of the incubation time, and all plates with less than 300 colonies were chosen, because more than 300 colonies on the bile esculin plate leads to a high degree of inaccuracy. Counting too many small colonies or counting overlapping colonies can cause a high count to be skewed. Then the number of bacterial colonies forming units (CFUs) per milliliter was counted and calculated according to this equation:

Number of colonies (CFUs) = bacteria X **ml dilution** X **amount plated.**

The data was collected, tabulated, and then statistically analyzed.

Statistical analysis:

For each group, the mean and standard deviation were computed. Using the Kolmogorov-Smirnov and Shapiro-Wilk tests, the data was shown to have a parametric (normal) distribution. The difference between the groups in non-related samples was compared using an independent-sample t test. The significance level was set at P≤0.05. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

RESULTS

The results of this study were listed in Table (1) and illustrated in Figure (4).

Table (1) *Mean difference values and standard deviation of bacterial count before and after the final irrigation by the tested solutions (1.5x10^8 bacteria/ml)*

Variables		Difference of bacterial count	
		Mean	SD
Group 1	Nano Chitosan 0.2%	- 0.245 x10x10	0.5234 x10x10
Group 2	Chlorhexidine 2%.	- 0.214 x10x10	0.22231 x10x10
Group 3	Alternating solution of Nano Chitosan 0.2% and Chlorhexidine 2%.	- 0.195 x10x10	0.30354 x10x10
Group 4	Sodium hypochlorite 3%.	- 0.158 x10x10	0.3024 x10x10
P-value		**0.051**^{ns}	

ns; non-significant (P > 0.05).

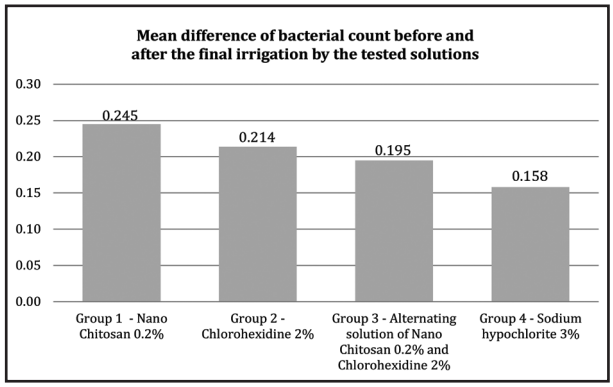

Figure (3) A bar chart showing the mean difference of bacterial count before and after final irrigation by the tested solutions.

There was no statistically significant difference among the tested groups where (P=0.051). The highest mean difference value of bacterial count was found in sodium hypochlorite 3% (- 0.158 x10x10 ± 0.3024x10x10), followed by alternating solution of nano chitosan 0.2% and chlorhexidine 2% (-0.195 x10x10±0.30354x10x10), then chlorhexidine 2% (-0.214 x10x10 ± 0.22231x10x10), and nano chitosan 0.2% (- 0.245 x10x10 ± 0.5234x10x10) came lastly.

DISCUSSION

The experimental design of the present study aimed to evaluate the antibacterial efficacy of nano chitosan 0.2%, Chlorhexidine 2%, alternating solution of nano chitosan 0.2% & Chlorhexidine 2%, and sodium hypochlorite 3% against *E. faecalis*. Natural teeth were employed in this investigation to imitate clinical conditions because artificial teeth do not simulate natural dentin. Teeth were sectioned at the cemento-enamel junction to standardize the cutting site [18].

Canals were prepared with #50 K-file and irrigated with 5 ml 2.6% sodium hypochlorite irrigation between each file, allowing better penetration of the irrigating solutions into the canals[10].

Teeth were undergoing sterilization; to completely eradicate all forms of bacteria that may be found in the root canal system; by gamma rays which is effective and introduce no detectable changes in dentin while other methods (ethylene oxide, dry heat, and autoclaving) of sterilization alter the structure of the dentin [19]. Bacterial sampling for checking of sterilization were done before inoculation of bacteria and this is served as negative control [20].

This study used *E. faecalis*, a Gram-positive facultative anaerobe that is typically detected in the root canals of failing endodontically treated cases[21].

In this study each root canal was inoculated with 0.5ml of *E. faecalis*, with density adjusted to the turbidity of 0.5 McFarland standard

($1.5x10^8$ bacteria/ml)[22], this was followed by incubation period 48 hrs to allow deeper penetration of *E. Faecalis* into dentinal tubules [23].

The bacterial sampling counting process was done two times throughout the study; after incubation of root canals for 48 hrs to count the number of bacteria that already inoculated into the canal and to compare it with that found after irrigation of root canal with the tested irrigations.

The highest mean difference value of bacterial count was found in 3% sodium hypochlorite, followed by alternating solution of 0.2% nano chitosan & 2% chlorhexidine, then 2% chlorhexidine, and 0.2% nano chitosan came lastly.

The antibacterial effect of chitosan might be due to the interaction of positively charged chitosan with a negatively charged bacterial cell that changed the permeability of the bacterial cell and might result in bacterial death [24]. The catatonically charged amino group in chitosan is thought to combine with anionic components on the cell surface, such as N-acetyl muramic acid, sialic acid, and neurotic acid, to suppress bacterial growth by interfering with medium exchanges, chelating transition metal ions, and inhibiting enzymes.

The use of natural irrigant in nano form could 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 a previous study [25].

The combination of chitosan and chlorohexidine was found to be effective as sodium hypochlorite[18]. Ballal et al. [11] found that a 2% chlorhexidine (CHX) gel mixed with chitosan had the best antibacterial activity against Candida albicans and E. faecalis. The findings of this investigation are in accordance with those of Yadav et al. [16], who found that that the antibacterial activity of the chitosan groups was comparable to that of 3% NaOCl and 2% Chlorhexidine.

CONCLUSION

Alternating solution of 0.2% nano chitosan & 2% chlorhexidine when used as antibacterial irrigant gives promising results and can be used as an alternative to NaOCl solution.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest

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