Comparative Evaluation of the Antibacterial Effect of Different Combinations of Etidronate, Nanochitosan and NaOCl on E. Faecalis Biofilm

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Abstract

The use of irrigating solutions and chelating agents during root canal treatment is an indispensable step; attempts to develop an irrigating solution with effective chelating and antibacterial properties are ongoing.

This study aimed to compare the antibacterial efficacy of etidronate mixed with 5.25% NaOCl or with normal saline, 1% nano-chitosan (NCP), to an experimental combination of etidronate, NaOCl, and NCP against E.faecalis biofilm.

After the bacteria were incubated for 48 hours, the minimal inhibitory concentration (MIC) was determined on E.faecalis ATCC 4083 strain. E. faecalis biofilm model was created, and irrigated with different solutions as follows: Group (1): 5.25% NaOCl, Group (2): 1% NCP, Group (3): etidronate /5.25% NaOCl, Group (4): etidronate /saline, Group (5): etidronate/1% NCP/ 5.25%NaOCl, Group (6): 1% NCP / 5.25% NaOCl. Quantification of cell biomass and the percentage of live and dead cells in the biomass was assessed for each group.

The highest mean value of bacteria was for the NCP (0.61±0.12) followed by etidronate/saline (0.15±0.00). The NCP and etidronate /saline groups showed significant differences from each other and are significantly higher bacteria than other groups. Etidronate / NaOCl, Etidronate /NaOCl/NCP, and NaOCl /NCP showed significantly higher values than the negative control group (NaOCl) which showed the lowest values.

Mixing the Etidronate (Dual Rinse® HEDP) with 5.25% NaOCl showed a significantly higher bactericidal effect compared to mixing with saline. 1% nano chitosan was ineffective against E.faecalis biofilm. Mixing 1% nano-chitosan with Etidronate and NaOCl does not improve the anti-biofilm activity.

Experimental article (J Int Dent Med Res 2022; 15(4): 1429-1433)

Keywords: Endodontic irrigant, Etidronate, Dual Rinse HEDP, Nano Chitosan, E. faecalis, antibacterial, Chelating agent.

Received date: 16 July 2022 **Accept date:** 09 August 2022

Introduction

Successful root canal treatment relies heavily on thorough eradication of the polymicrobial nature of endodontic infection; which otherwise leads to treatment failure [1]. Current instrumentation techniques have fallen short of reaching nearly 35% of the total surface area of the root canal; substantially relying on irrigants and their hydrodynamics to take on this mission [2]. Enterococcus faecalis is the most recognized facultative anaerobe species persistent in treated root canals, taking shelter in the extracellular polymeric matrix of biofilm which protects against nutritional deprivation, and traditional antimicrobial agents [1, 3]. Sodium hypochlorite (NaOCl) is the most widely used irrigant in the endodontic field, owing to its antimicrobial potential, and ability to break down organic tissues. Accordingly, it will remain our primary irrigant and will not be entirely[4].

Chelating vehicles such as ethylenediaminetetraacetic acid (EDTA) and citric acid have been used to dissolve the inorganic element of the smear layer on the instrumented canal walls [5]. Chelators supplement the action of

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NaOCl either as a final flush (potent chelator) or blended with NaOCl throughout instrumentation (weak chelator). Therefore, the continuous chelation technique using a variety of weak chelates solutions that do not interfere with the NaOCl effect as Etidronate or Chitosan takes a great deal of interest in the ongoing research in an attempt to simplify the irrigation protocol [6, 7]. It has been proven that these agents can be mixed with NaOCl without consuming its free available chlorine in the short term [8].

Etidronate is a salt of etidronic acid, a nitrogen-free bisphosphonate or (1-hydroxyethane-1,1-diyl) bis (phosphonic acid) which is abbreviated as HEDP [9]. Etidronic acid is a biocompatible chelator that is used in conjunction with NaOCl as a ‘‘soft’’ less aggressive chelator on dentin than EDTA [10]. Recently, Etidronate capsules intended for root canal irrigation as Dual Rinse® HEDP (Medcem, Weinfelden, Switzerland) were introduced, each capsule consisting of 0.90 g powder of etidronic acid, to be added to 10 mL of NaOCl between 1 and 5% immediately before treatment and remains active for 1 hour [11]. This decalcifying agent has been promoted to simplify the current irrigation protocol to a single-step mixture. It is also designed to be implemented in the continuous chelating protocol [12].

Chitosan, as a natural linear polysaccharide of N-acetyl-D-glucosamine, is obtained from the deacetylation of chitin in crustacean shells. Recently, chitosan has gained significant attention amongst endodontic researchers, not only because it imparts a comprehensive role as a broad-spectrum antimicrobial, but also owing to its biocompatibility, biodegradability, lack of toxicity, and its chelating ability of its acidic pH [13]. However, some properties and challenges such as low water solubility, low specific molecular weight, and purity still obstruct the widespread use of chitosan [14]. Moreover, nano-based irrigants have been increasingly utilized in recent years. In this regard, better root canal space disinfecting strategies involving chitosan nanoparticles have been the scope of many recent studies [15].

Thus in this study, we aimed to compare the antibacterial efficacy of Etidronate powder mixed with either NaOCl or normal saline, 1% nano-chitosan (NCP), and an experimental combination of Etidronate, NCP, and NaOCl against E. faecalis biofilm.

Materials and methods

This study was approved by the RAKMHSU Research & Ethics Committee Ras Al Khaimah, UAE (RAKMHSU-REC-156-2021/22-F-D).

Irrigant preparation

The NCP solution (NanoGate, Egypt) was prepared according to the ionotropic gelation process [16], blank nanoparticles were obtained upon the addition of a tripolyphosphate (TPP) aqueous solution to a Chitosan solution, then 1% NCP suspension was obtained by a predetermined dilution process. The Etidronate solution was prepared by mixing 0.9 gm of Etidronate (Dual Rinse® HEDP powder, Medcem, Weinfelden, Switzerland) either with 9.1 gm of 5.25% NaOCl solution (Egyptian company for household detergents, Egypt) or with 9.1 gm of normal saline solution (ADWIK pharmaceuticals, Egypt). The experimental solution was made by mixing 0.9 gm of Etidronate powder with 9.1 gm of 5.25% NaOCl and 9.1 gm of 1% NCP suspension.

Minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of the tested irrigants that had the potential to inhibit bacterial growth, it was determined by the broth microdilution method according to Weigand et al [17]. Four sets of 9 sterile 10ml glass test tubes were prepared to contain 1ml of broth (brain heart infusion). The previously prepared endodontic irrigants with different concentrations were added to the first tube of each set to achieve a dilution of 1000 mg/ml. Eight serial dilutions of the irrigants were executed up to the dilution of 3.9 mg/ml in the eighth tube in each set. The bacterial suspension was then prepared by suspending colonies of tested control strain in sterile saline to adjust turbidity to 0.1 McFarland. One millimeter of the bacterial suspension was added to each test tube in the 5 sets, and then the sets were incubated at 37ºC for 24h. The MIC value of each irrigant was determined by the highest dilution in each set with no visible turbidity.

Development of E. faecalis biofilm

Under strict aseptic conditions, 5mL of Enterococcus faecalis ATCC 19433 (American Type Cell Culture Collection) was grown aerobically from frozen stock cultures that were

stored at -80°C in Tryptone Soya Broth TSB (Oxoid) supplemented with 15% glycerol. The bacteria were grown for 18 - 20 hours in TSB, (Oxoid) at 37ºC, a 5% CO2 supplemented atmosphere in an incubator. The bacterial strain development is confirmed with gram stain.

Antibiofilm activity

Dilutions of 1:100 were prepared, of which 100 um in sets of 8 wells were plated in a 96-well plate for each of the tested irrigant groups as follows: Group (1): 5.25% NaOCl, Group (2): 1% NCP, Group (3): Etidronate /5.25% NaOCl, Group (4): Etidronate /saline, Group (5): Etidronate /1% NCP / 5.25%NaOCl, Group (6): 1% NCP / 5.25% NaOCl, in addition to positive and negative control groups, were all incubated for 48 hours. The wells were stained with 125 uL of 0.1% crystal violet for 10 minutes and then rinsed to remove excess stain, leaving only the stain bound to biofilm at the well bottom. The plate was then laid down on a paper towel for complete dryness; after mixing with acetic acid, it was read with a plate-reader at the OD595nm of each well. The optical density values of the sample-containing wells were subtracted from the average of the blank wells OD. The optical density average was normalized to the averages of the control wells. The higher mean value for spectrophotometric color intensity indicates increased bacterial growth and a lack of antibiofilm activities.

Statistical analysis

Numerical data were represented as mean and standard deviation (SD) values. Shapiro-Wilk's test was used to test for normality. Homogeneity of variances was tested using Levene's test. Data were parametric and showed variance homogeneity so they were analyzed using a one-way ANOVA test followed by Tukey's post hoc test. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.1.2 for Windows.

Results

The MIC was 3.1% for all groups except for Etidronate/saline which was 12%, while the used 3% suspension of NCP showed limited antibacterial effect at its full concentration in the MIC plates. Table (1) shows the means and standard deviation for the spectrophotometric color intensity results of the antibiofilm activity for the different irrigant solutions and displayed significant differences between the tested groups ($p<0.05$). The maximum antimicrobial activity was in group 1 (NaOCl) which revealed no statistically significant difference from the negative control; in contrast group 2 (NCP) showed the least antimicrobial activity with a significant difference from the positive control, followed by group 4 (Etidronate/saline). The combination in group 5 (Etidronate/NCP/ NaOCl) was found to significantly enhance the antibiofilm activity similar to group 3 (Etidronate/NaOCl) and group 6 (NCP/NaOCl) but still significantly lower than Group 1 (NaOCl) or the positive control (Fig 1).

Color intensity (Mean±SD)								*p-value*
NaOCl	*NCP*	*Etidronate /NaOCl*	*Etidronate /Saline*	*Etidronate /NCP/ NaOCl*	*NCP/ NaOCl*	*Positive control*	*Negative control*	
0.07 ± 0.01^{E}	0.61 ± 0.12^{B}	0.09 ± 0.01^{D}	0.15 ± 0.01^{C}	0.09 ± 0.01^{D}	0.09 ± 0.004^{D}	1.27 ± 0.02^{A}	0.07 ± 0.01^{E}	<0.001 *
Means with different superscript letters are significantly different (p<0.05).								

Table 1. Means and standard deviation for the spectrophotometric color intensity results of the antibiofilm activity for the tested irrigant solutions.

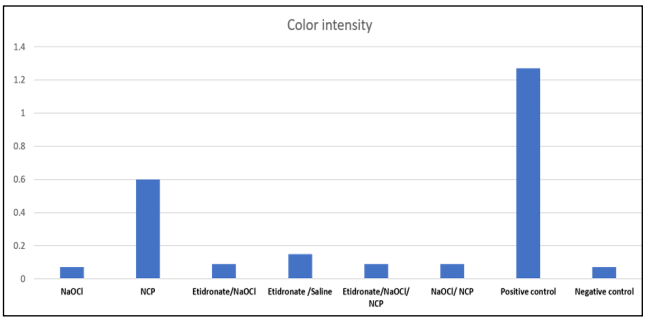

Figure 1. Bar chart showing mean and standard deviation values of color intensity values in different groups.

Discussion

Eradication of biofilm-based infection is fundamental to augmenting endodontic treatment outcomes [18] .E. faecalis is a facultative microorganism that has a non-fastidious nature, forms a monospecies biofilm, and exhibits resistance to chemo-mechanical preparation of the root canal, consequently being the main cause of failure in treated cases [19, 20]. E. faecalis biofilm was selected in the present study for its significance in endodontic infection. One of the most commonly used and readily available culture media for E.faecalis is Tryptone Soya

broth, accordingly it was used in the current study [21]. Chelators primarily employed for endodontic use were claimed to show weak antibiofilm potential, especially against Gram-positive bacteria such as E.faecalis [5]. Therefore attempts to develop new formulations with inherent chelation and antibacterial potential are an ongoing process. This study aimed to compare the antibacterial potential of Etidronate mixed with either 5.25% NaOCl or saline, 1% nano-chitosan (NCP), to different combinations of Etidronate, NaOCl, and NCP against E.faecalis biofilm.

In the present study, Dual Rinse® HEDP mixture was prepared by mixing a 1:1 ratio of 5% NaOCl aqueous solution and 18% HEDP powder, resulting in a formulation of 2.5% NaOCl/ 9% HEDP solution. This mixture maintains 77% of the initially available chlorine molecules long after 60 min, thus maintaining a prolonged proteolytic and antibacterial effect of NaOCl [22].

A limited number of studies have evaluated the antibacterial effect of Dual Rinse® HEDP, one randomized clinical trial [23] showed the antibacterial effect of 2.5 % NaOCl after mixing with Dual Rinse HEDP was not aggravated. On the contrary, the Dual Rinse® HEDP was found to promote the disinfection capacity of NaOCl in other studies[3, 24], and this comes in agreement with our results. In comparison to the classic alternating irrigation with NaOCl and EDTA, the NaOCl/Dual Rinse HEDP mixture showed higher antimicrobial efficacy [11]. In our study, mixing Etidronate with normal saline showed limited antibacterial results, which comes inconsistent with previous studies that proved the ineffectiveness of the sole use of chelators as an antibiofilm agent when compared to NaOCl alone [25, 26].

Recently, chitosan was introduced as an alternative irrigant with claims of having an antimicrobial potential. Chitosan is a polycationic molecule, which interacts with the negatively charged outer cell wall of bacteria to detriment its permeability causing leakage of intercellular components followed by binding to bacterial DNA inhibiting its replication, therefore, the death of bacterial cells is inevitable [27]. Another possible mechanism is that chitosan acts as a chelating agent that selectively binds to trace mineral elements that cause the production of toxins and inhibit microbial growth. In addition, chitosan showed antibacterial ability by interfering with bacterial adhesion to dentin and thus impairing biofilm formation [28]. Chitosan is only dissolved in an acidic condition as it is a basic polysaccharide with a pKa of 6.5. Such acidic conditions may affect the chitosan chemical properties and cytotoxicity which is a time-dependent effect [27-30].

NCP has been used as an endodontic irrigation material, with several variants noted in the literature, thus in an attempt to boost its antibacterial effect, the nano form (CNP) was used in our study. Nanoparticles provide higher surface area, charge density, and a greater degree of interaction with the bacterial cell wall [31]. However, this comes in disagreement with our results, as utilizing 1% NCP alone resulted in a weak antimicrobial efficacy against the E.faecalis biofilm. This contradiction may be attributed to variations in its concentration, molecular weight, positive charge density, polycationic structure, chelating capacity, media pH, ionic strength, and application time [32, 33], even the oxidation reaction after mixing with NaOCl may alter chitosan properties, structure, and molecular weight [34].

The NCP preparation method is one of the major factors that affect its outcome properties. Different methods have been used to prepare NCP such as emulsion cross-linking, spray drying, reverse micellar method, template polymerization, polyelectrolyte complex, and precipitation. In the current study, NCP was prepared by ionotropic gelation which might cause a limitation of amino groups in the NCP backbone leading to relatively weak positive charge centers [35, 36]. Other possible factors such as solution pH and degree of deacetylation could directly influence the performance of chitosan [15, 37]. However, in harmony with our results, using NCP in combinations with NaOCl and Etidronate enhances its antibiofilm action which can be attributed to its ongoing slow antibacterial release [28, 38].

Conclusions

Within the limitations of the current study, mixing the Etidronate powder (Dual Rinse® HEDP) with saline showed a significantly less bactericidal effect compared to mixing with 5.25%NaOCl. 1% nano chitosan solution alone was ineffective against E. faecalis biofilm, and the viable bacteria were significantly more than other tested solutions. Mixing the nano-chitosan

with Dual Rinse® HEDP powder and NaOCl did not enhance the antibacterial effect.

Declaration of Interest

The authors deny any conflicts of interest related to this study.

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