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ORIGINAL ARTICLE

Antibacterial efficacy of copper-added chitosan nanoparticles: a confocal laser scanning microscopy analysis

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Abstract

The aim of this study was to evaluate the antibacterial efficacy of copper added chitosan nanoparticles (CU-CNPs) as an irrigation solution with different irrigants in terms of eliminating *Enterococcus Faecalis* (*E. faecalis)* from the root canals. Fifty mandibular premolar teeth were prepared and infected with *E. faecalis* for 21 days. After the incubation period, samples were randomly divided into a control group irrigated with distilled water and 4 experimental groups ($n = 10$) irrigated with as follows, %6 NaOCl, %6 NaOCl + %9 editronate (HEBP), Chitosan nanoparticles (CNPs), and CU-CNPs. To calculate the proportion of dead *E. faecalis* cell volume, stained using LIVE/DEAD BacLight Bacterial Viability Kit and were scanned using confocal laser scanning microscope (CLSM). All the irrigation solutions significantly *(*$P < .05$*)* killed the bacteria in the canal, except for the control group. CU-CNPs solution killed the highest ($P < .05$*)* number of bacteria compared with the other experimental groups. No significant difference was found between CNPs, NaOCl + HEBP, and NaOCl in terms of antibacterial activity. CU-CNPs solution was exhibited higher antibacterial efficacy against *E. faecalis.*

Keywords Chitosan nanoparticules · *Enterococcus faecalis* · Irrigation · Root canal disinfection · Sodium hypochlorite

Introduction

The main objective of root canal treatment is to prevent the development of apical periodontitis or to cure the existing disease. The microorganisms in the root canal system are held responsible as the main etiological factor for the development of pulpal and periapical diseases [1]. In endodontic treatment, in order to eliminate the microorganisms from the root canal system, the antimicrobial agents are recommended to use together with mechanical preparation [2]. However, these microorganisms may exhibit resistance to changing environmental conditions [3] and antimicrobial agents [4] by creating a biofilm structure. Among these microorganisms, the most widely discussed one is *Enterococcus faecalis* (*E. faecalis*), which is one of the members of the *gram*-positive cocci group and known to be capable of creating biofilm structure and playing role in the development of periradicular lesions in persistent root canal infections [5]. *E. faecalis* can also exhibit resistance against the disinfection protocols [6].

In root canal treatment, various antimicrobial agents are widely used together with mechanical preparation for disinfection purposes. Sodium hypochlorite (NaOCl) is the most frequently used irrigation solution because of its ability to dissolve the organic tissues and neutralize the toxic products, as well as having high antimicrobial activity [7]. Despite its high antibacterial properties, 6% NaOCl has not been found to kill all bacteria even at different time intervals [8]. In addition, the high surface tension of NaOCl limits its penetration into dentinal tubules, isthmus, and lateral canals and causes it to remain insufficient for removing the smear layer

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[9]. Due to these disadvantages of NaOCl, new irrigation solutions with strong antibacterial efficacy have been sought.

Nowadays, various nanoparticles have been proposed to be used as an antibacterial agent in root canals for disinfection [10, 11] due to their wide spectrum of activity and biocompatibility [12]. Since these nanoparticles have high surface area and charge density, they interact with the negatively charged surface of the bacterial cells and show high antibacterial activity [13]. One of these nanoparticles is chitosan nanoparticles (CNPs) which improves the mechanical and chemical properties of chitosan solution, the additions can be made to the free hydroxyl and amino groups on the polymer chains [14]. The most important modification among them is CNPs which are manufactured using sodium tripolyphosphate. Since CNPs have physicochemical features such as sensitivity, antibacterial activity (higher affinity Gram-negative and Gram-positive bacteria for a quantum-sized effect), and bioavailability, they are widely used in biomechanical practice and drug administration systems [15, 16]. To increase the antibacterial activity of CNPs, elements such as silver [17], manganese, zinc, and copper may be added [18].

In literature, there is no information on the antimicrobial activity of copper-added CNPs (CU-CNPs) against the dentin contaminated with *E. Feacalis*. The aim of the present study is to evaluate the antibacterial efficacy of NaOCl, HEBP, CNPs, and copper-added CNPs (CU-CNPs) final irrigation solution on root canal dentin using confocal laser scanning microscope (CLSM). The null hypothesis is that there would be no difference between different final irrigation solutions in terms of antibacterial efficacy against *E. Faecalis*.

Materials and methods

Specimen selection and preparation

Upon the approval of the ethics committee (2017/205), 50 single-root mandibular premolar teeth that have been diagnosed with extraction due to the reasons independent from this study were involved in the present study. The radiographs of teeth were taken from different angles and it was confirmed that there was no pathological condition such as calcification and resorption and that they had a uniform canal structure. The soft and hard tissue residuals around the teeth were mechanically removed by using a periodontal curette. Root dentin blocks were prepared according to Ma et al. [19]. The root dentin blocks were enlarged to the size of Gates Glidden drill #6 (1.5 mm in diameter) (Dentsply-Sirona, Ballaigues, Switzerland) at 300 rpm under water cooling. A thin groove was made in the middle of the cylindrical specimen by a low-speed handpiece with a small round bur, and each dentin block was split with a blade and a hammer into two semi-cylindrical halves. Finally, samples were prepared approximately 4 × 4 × 2 mm in size [19]. The selected teeth were kept in 0.1% thymol solution at 4 °C until the experiments. To remove the smear layer, all specimens were rinsed with 6% NaOCl and 1 ml 17% EDTA solution (CanalPro, Coltène-Whaledent, Altstätten, Switzerland) for 4 min in an ultrasonic bath (Sankei Giken Industry Co Ltd, MIE, Tokyo, Japan), and finally immersed in distilled water for 1 min. Each semi-cylindrical half was placed in a filter tube with the canal side up. Composite resin was used to seal any gap between the specimen and the inner wall of the tube and light-cured for 20 s. To achieve the dentin contamination only within the root canals, the outer surfaces of specimens were coated with 2 layers of red nail polish (Flormar, Istanbul, Turkey). All the specimens were sterilized at 121 °C for 30 min using autoclave (Sercon, Modeo HG, Mogi das Cruzes, SP, Brazil).

Dentin contamination with *Enterococcus faecalis*

24-h pure culture suspension of *E. faecalis* (ATCC 29,212) was obtained with incubation in Brain Heart Infusion Broth (BHI) agar at 37 °C. The resultant suspension was centrifuged at 1000 rpm for 10 min to achieve an appropriate amount of bacterial culture. After the inoculation of 1×10^8 (CFU)/mL bacteria to the teeth by diluting with BHI agar, the incubation was achieved at 37 °C and 95% moisture for 21 days. After the incubation, the teeth were aseptically removed from the tubes. In order to determine the bacteria attaching to the samples, the surrounding culture environment and planktonic bacteria population were removed by rinsing with sterile 1 × phosphate-buffered saline (PBS). Then, each specimen was added with 15 µl physiological saline solution (0.9% NaCI). This procedure was repeated for twice for each specimen.

Achieving chitosan nanoparticles and copper-added chitosan nanoparticles

Chitosan nanoparticles were synthesized by the ionotropic gelation process [20]. Briefly, chitosan (0.3 g) was dissolved in 1% v/v acetic acid. The solution was stirred continuously on a magnetic stirrer at ambient temperature (25 ± 3 °C) and 1 mL of sodium tripolyphosphate (1% TPP, v/v) was added dropwise to 25 mL chitosan solution to form chitosan nanoparticles. For hardening, stirring was further continued for 30 min. End of the reaction, nanoparticles were purified by using centrifugation at 9000 g for 30 min. These particles have been referred to as CNP.

Copper-loaded chitosan nanoparticles were obtained by adding a solution of 100 µg/mL copper ions (Merck KgaA, Darmstadt, Germany) to the chitosan nanoparticle

suspension (0.375%, w/v) before purification and the purification was then carried out as for the chitosan nanoparticles described above [18].

Irrigation protocols

After a 21-day inoculation period, the specimens were placed in a sterile metallic device. Then, in order to evaluate the antimicrobial efficacies of irrigation agents, the teeth were randomly divided into 5 groups ($n=10$) and then the disinfecting solution was placed on the root canal wall of the specimens as follows,

Group 1 (Control): 5 ml distilled water for 3 min,
Group 2: 5 ml 6% NaOCl for 3 min,
Group 3: 5 ml 6% NaOCl + 9% HEBP'nin (Cublen K8514 GR; Zschimmer & Schwarz, Mohsdorf, Germany) for 3 min,
Group 4: 5 ml CNPs for 3 min,
Group 5: 5 ml CU-CNPs were irrigated for 3 min.

Confocal laser scanning microscopic analysis

Two halves of each tooth specimen were stained for 20 min at room temperature in a dark environment by using a 30 µl LIVE/DEAD®BackLight bacterial viability kit (Invitrogen Molecular Probes, Eugene, OR, USA). This kit can stain the alive bacteria, which have green pigment, with the SYTO9® that is a green, fluorescent nucleic acid dye, and the dead bacteria, which have red pigment, with propidium iodine dye that is a red fluorescent dye. Following a 20-min staining period, each specimen was rinsed with PBS to remove the residual fluorescent dye.

Twenty cross-sections were obtained in each group and the specimens were placed on the lamina. The specimens were examined using CLSM (Carl Zeiss LSM 510, Carl Zeiss Microscopy, Jena, Germany) with helium laser light sources. The scanning of the samples in CLSM was made according to the study of Ma et al. [19]. Digital images were imported to the Image J program (ImageJ software, National Institutes of Health) to measure the total dentinal surface penetration area. Killed bacteria was determined by calculating the ratio of red fluorescence to green and red fluorescence [21].

Statistical analysis

The proportions of dead cell volume after exposure to different solutions at 3 weeks were subjected to univariate analysis of variance using SPSS 16.0 (SPSS Inc, Chicago, IL). The normal distribution of the data was determined using the Shapiro–Wilk test. The statistical significance was analyzed by one-way analysis of variance and the Tukey post hoc test. The statistical significance was set at a 95% confidence level.

Results

After 21 days of incubation, the penetration of the root canal surfaces of *E. faecalis* specimens in the positive control group was confirmed using CLSM (Fig. 1). All the irrigation solutions tested here significantly decreased the number of *E. faecalis* living in the root canal surfaces when compared to the beginning ($P<0.05$). When compared to the distilled water, all the irrigation solutions showed a significantly higher level of antimicrobial activity against *E. faecalis* ($P<0.05$). No statistically significant difference was found between CNPs, NaOCl + HEBP, and NaOCl in terms of antimicrobial efficacy ($P > 0.05$). When compared to the other solutions, CU-CNPs showed a higher level of antibacterial efficacy ($P<0.05$) (Table 1).

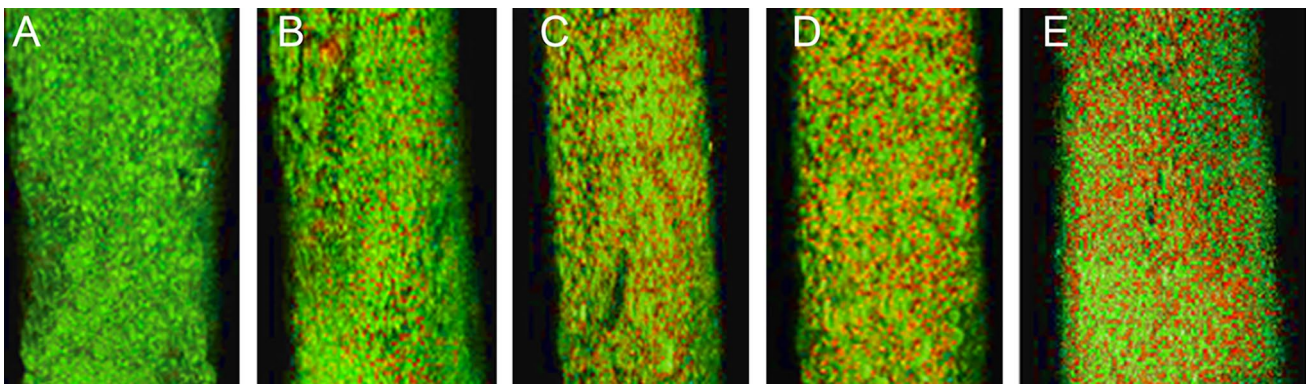

Fig. 1 CLSM of 3 weeks old *E. Faecalis* infected root canal surface after exposure to different irrigation solutions and viability staining. **a** Distilled Water **b** %6 NaOCl **c** %6 NaOCl/%9 HEBP **d** CNPs **e** CU-CNPs

Table 1 Proportion of dead *E.faecalis* cell volume in the root canal surface exposed to different disinfecting solutions after 3-week incubation

	Distilled Water	%6 NaOCl	%6NaOCl/ %9 HEBP	CNPs	CU-CNPs
Proportion of Dead Cell Volume	0.04 ± 0.01^{a}	0.52 ± 0.06^{b}	0.54 ± 0.06^{b}	0.50 ± 0.06^{b}	0.70 ± 0.11^{c}

*Different superscript letters indicate statistically significant differences between groups ($P < 0.05$) (^{a,b,c} for columns)

Discussion

In the present study, it was aimed to examine the antimicrobial efficacies of NaOCl/HEBP, CU-CNPs, and CNPs, which are produced as an alternative to NaOCl solution, against *E. faecalis* using CLSM. According to the results obtained here, it was determined that there were differences between final irrigation solutions' antibacterial efficacies against *E. faecalis* and, thus, the null hypothesis was rejected.

In this study, *E. faecalis*, which is one of the most frequently seen microorganisms in persistent endodontic infections and capable of creating biofilm and showing resistance against antimicrobial agents, was used to contaminate root canals [22]. An experimental design that is suitable for ensuring the homogeneous contamination of *E. faecalis* on the root canal surfaces, confirming the efficacies of irrigation solutions, and comparing them was chosen [23]. In accordance with the chosen experimental model, the bacteria were centrifuged [19] and it was aimed to have them penetrate the root canal surface and to produce dentin specimens infected with similar numbers of bacteria [8, 19]. To better mimic the clinical conditions ex-vivo, the outer surfaces of specimens were covered with two layers of nail polish and it was aimed to have the bacteria reach only to the root canal lumen. Thus, an appropriate environment was obtained for the growth of bacteria by making use of the dentin infection model utilized here.

Although similar effects can be obtained with larger volumes and lower concentrations of NaOCl, higher concentration of NaOCl is considered to be used in endodontics, as the usage time of solution is reduced with nickel-titanium single-file systems in root canal system [24]. The preferred sodium hypochlorite solution was 6% in the present study, as it is known that higher concentrations of NaOCl solution have a bacterial killing activity and intracanal pulpal dissolution in a shorter time than observed in lower concentrations [25]. In the present study, the duration of irrigation solutions' exposure to dentin was set to be 3 min in order to prevent HEBP from influencing the antibacterial efficacy of NaOCl in the short term and to prevent NaOCl from exhibiting toxic effect [8, 19, 26, 27]. Moreover, since the standard solutions are sufficiently effective for 3-min contact with dentin, no longer time for exposure was necessary [8].

In the traditional fluorescent microscope, it was only possible to obtain a blurred image because of the light being out of the focus plane when used with staining tests. This blurriness makes it impossible to distinguish spotted dentine and structure and to select cells individually. Moreover, in the traditional fluorescent microscope, it is necessary to demineralize the dentin specimens by creating an artificial situation [28]. For these reasons, CLSM is used for obtaining more clear images of objects. As well as providing a chance to have a deep view into the dentinal tubules, CLSM also is capable of individually showing the bacteria in dentinal tubules [29]. In the present study, CLSM and bacterial viability staining tests were used and, after exposing the specimens to different irrigation solutions, a platform was achieved for quantitatively measuring the bacterial destruction [19].

According to the results obtained here, it was observed that the bacteria on the mature biofilms (21-day) on the root canal surfaces were more resistant to the irrigation solutions. The results obtained in previous studies also corroborate this finding [8, 26]. Since many bacteria are delivered to the dentinal tubules by centrifuging, the cells at the depths of tubules may have limited access to nutrients during the maturation of biofilm and they might thus be more resistant to the irrigation solution [26]. In their study, Shen et al. [30] reported that the bacteria on the biofilms having limited nutrients are more resistant to the disinfectants.

Except for the control group, the other groups in the present study showed a high level of antibacterial effect on *E. feacalis*. In previous studies, it was reported that 6% NaOCl is more effective on *E. feacalis* when compared to lower concentration NaOCl solution [8, 19, 26, 31]. However, the sought for alternative irrigation solutions having an antibacterial effect and low level of cytotoxicity was initiated because NaOCl has a toxic effect and damages the dentin as the concentration [32] and time-in-the-canal [33] of NaOCl increase. CNPs were preferred in the present study for their antibacterial properties and efficacy against *E. faecalis* [34]. Since it has cationic properties and a large surface area, chitosan gets absorbed by the negatively loaded biofilm and thus causes destruction of the bacterial cell membrane [34].

Then, it penetrates into the intracellular components and death of bacterial cells is achieved. It was reported that chitosan showed higher level of antibacterial activity against gram-positive bacteria when compared to the gram-negative ones [18]. In a study, in which CNPs were used as medicament together with Ca $(OH)_2$ for 7 and 14 days [35], it was reported that *E. faecalis* was eliminated by 69.27% and 87.77%. CNP incorporated root canal sealers were reported to retain their antibacterial efficacy even after 4 weeks [36].

According to the results obtained here, CU-CNPs showed a significantly higher level of antibacterial activity than the other solutions did ($P < 0.05$). CU-CNPs have high antibacterial efficacy since they have a high level of surface load increasing the affinity to the membrane of negatively loaded bacteria. Moreover, CNPs create antibacterial activity by means of controlled release of copper ions dissociated in small nuclei [18]. Thus, CU-CNPs may exhibit continuous antibacterial activity.

In the present study, no difference was found between CNPs, NaOCl, and NaOCl/HEBP groups in terms of antibacterial efficacy. Given the previous studies in literature, it was reported that no difference was found between NaOCl and NaOCl/HEBP in terms of antibacterial efficacy and this result corroborates the current findings [25, 37]. It can be thought that, since it is manufactured as a chelation agent for removing the smear layer, HEBP may not increase the efficacy of NaOCl when used as the final irrigation agent. However, in the present study, none of the protocols could completely remove *E. feacalis* from the root canal surfaces.

The fact that there is not only one bacterium causing root canal infection is a limitation of the present study. One should be careful while adapting the results to the clinical conditions because of the polymicrobial nature of canal. In further studies, different irrigation solutions and endodontic procedures might be more comprehensively discussed by making use of advanced experimental models investigating pathogen microorganisms together. More study is needed to investigate on this CU-CNPs solution's effects on the vital tissues and the practical usability of this solution in the clinical environment should be investigated. It is anticipated that CU-CNPs solution could be applied broadly as an irrigation solution in endodontics for their high antibacterial activity and acceptable biocompatibilities.

Within the limitations of the current study, none of the irrigation solutions examined here could completely eliminate *E. faecalis* that has penetrated the dentin surface. Among the tested irrigation solutions, the higher antibacterial efficacy was exhibited by the CU-CNPs solution.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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