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Minimum Inhibitory Concentration of Iron Oxide Nanoparticles with Hydrogen Peroxide against Endodontic *Enterococcus faecalis*

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الخلاصة

الأهداف: تهدف الدراسة الى تقييم التأثير الأساسي المضاد للبكتيريا لجسيمات أكسيد الحديد النانوية بالاشتراك مع بيروكسيد الهيدروجين من خلال تقييم تأثيرها ضد البكتيريا المكورة البرازية في نموذج العوالق ومقارنتها مع الصوديوم هايبوكلورايت، بيروكسيد الهيدروجين، جسيمات اكسيد الحديد النانوية عن طريق تحديد الحد الأدنى لتركيز المثبط والحد الأدنى المميت. **المواد وطرائق العمل**: تم تخفيف المحاليل التالية (صوديوم هايبوكلورايت، جسيمات اكسيد الحديد النانوية، بيروكسيد الهيدروجين،، جسيمات اكسيد الحديد النانوية بالاشتراك مع بيروكسيد الهيدروجين) بمستنبت نقيع الدماغ والقلب بشكل متسلسل ثم إضافة ١٠٠ مايكرولتر من المحاليل المايكروبية. تم الحصول على النتائج من خلال ملاحظة عكرة المحاليل ونمو الجراثيم على صفائح الاكار. **النتائج**: أظهرت جسيمات اكسيد الحديد النانوية بالاشتراك مع بيروكسيد الهيدروجين أنها أكثر فعالية ضد البكتيريا المكورة البرازية من الصوديوم هايبوكلورايت، جسيمات اكسيد الحديد النانوية، بيروكسيد الهيدروجين ، حيث كانت هناك حاجة لتخفيف أقل لمنع نمو البكتيريا. أظهر صوديوم هايبوكلورايت تأثيرًا مضادًا للبكتيريا مكافئًا لتأثير بيروكسيد الهيدروجين ، بينما أظهرت جسيمات اكسيد الحديد النانوية نموًا محفزًا عند التركيز العالي الذي تم اختباره. **الاستنتاجات**: تظهر جسيمات اكسيد الحديد النانوية بالاشتراك مع بيروكسيد الهيدروجين خصائص واعدة مضادة للبكتيريا كمحلول ري داخل القناة للتخلص من البكتيريا المكورة البرازية داخل قناة الجذر المصابة.

ABSTRACT

Aims: This study aimed to assess the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) effect of iron oxide nanoparticles (IONPs) in combination with hydrogen peroxide (H_2O_2) by evaluating its effect against endodontic Enterococcus Faecalis (E. faecalis) in the planktonic model. **Materials and Methods**: the tested irrigants groups (IONPs, 3% H_2O_2, 5.25% NaOCl, IONPs+H_2O_2) were serially diluted in brain heart infusion broth, and 100 µl of standard microbial suspensions of E. faecalis were added. The results were obtained based on turbidity and growth on selective enterococcus agar plates. **Result**: IONPs+H_2O_2 showed to be more effective against E. faecalis than IONPs, 3% H_2O_2 and 5.25% NaOCl as lower dilutions were required to inhibit the growth of the bacteria. NaOCl exhibited an antibacterial effect equivalent to that of H_2O_2, while IONPs exhibited a stimulatory growth at the high tested concentration. **Conclusion**s: IONPs+H_2O_2 showing promissory antibacterial properties as an intracanal irrigating solution to control E. faecalis from the infected root canal.

Keywords: Iron Oxide, Nanoparticles, *Enterococcus faecalis*, Sodium Hypochlorite

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INTRODUCTION

The Periapical disease is triggered by a bacterial infection that invades the root canal system and causes an immunoinflammatory host reaction that damages apical tissues [(1)]. Endodontic treatment is intended to control intracanal infection and avoid re-infection to achieve periapical healing. Cleaning and mechanical instrumentation eliminate bacterial contamination; however, it is not sufficient to remove it entirely [(2)]. Also, more resistant bacteria to endodontic treatments, such as *E. faecalis* are usually present and decrease the success rate of the therapy. *E. faecalis*, gram-positive facultative anaerobes, has been found in primary infections and is localized in one-third of the teeth with the chronic periapical disease[3]. So, the eradication of *E. faecalis* from the root canal system is important to enhance the prognosis of endodontic treatment.

Several antimicrobial solutions are used in endodontic therapy, like sodium hypochlorite (NaOCl), chlorhexidine gluconate (CHX), hydrogen peroxide (H_2O_2), and Biopure MTAD. NaOCl eradicates *E. faecalis* almost instantly after contact. However, some researchers have shown that higher concentrations and more contact time of NaOCl are required to eradicate *E. faecalis*[4,5].

Recently, the use of nanoparticles as antimicrobial agents has attracted considerable interest, especially in the biomedical field, due to their specific antibacterial properties. These properties include their ability to use several pathways to eradicate microbial cells and their low propensity for developing microbial resistance [6,7].

IONPs have been commonly used clinically as contrast agents for magnetic resonance imaging due to their high biocompatibility and capacity to penetrate biological tissues such as those found in tumors and atherosclerotic plaques[8]. IONPs have been shown to demonstrate enzyme mimetic behavior comparable to natural peroxidase, which can activate H_2O_2 in *vitro* [9] and thus has been referred to as nanozymes[10]. H_2O_2 has antibacterial activity with free radicals' generation, so it is widely used for general cleaning and disinfection purposes (at concentrations as high as 10%)[11]. However, the antibacterial effects of H_2O_2 are modest when used alone[12]. Hence, this research aimed to assess the minimum inhibitory concentration and minimum bactericidal concentration effect of IONPs in combination with H_2O_2 by evaluating its effect against endodontic *E. faecalis* in the planktonic model.

MATERIALS AND METHODS

Preparation of Nanoparticle Suspensions

IONPs (purity more than 99.7%) with a size 25-50 nm were purchased from US NANO (US Research Nanomaterials Inc., Houston, Texas, USA). To prepare a stock solution with 8mg/ml IONPs, 160mg of the nanoparticles were turned into 20ml of deionized water. For the optimal dispensing of the particles, the vortex system was used for 15 minutes. The prepa-

ration of nanoparticle suspension and the microbial experiments were carried out concurrently to avoid errors.

Preparation of the Microbial Suspensions

The bacterial suspension was prepared by adding 50µL of cultured *E. faecalis* ATCC 29212 into 5ml of brain heart infusion and incubated at 37ºC for 24hrs. Then, the bacteria were diluted to reach 0.5 McFarland turbidity that is equivalent to (1-1.5×10^8) Colony-forming unit, confirmed the accuracy by spectrophotometer (EMC-11D-V; Germany) at (620nm) wavelength and the absorbance (0.1nm)[13].

Minimum Inhibitory Concentration (MIC) Test

Using the standard method suggested by the national committee of the clinical laboratory standards (CLSI), MIC was calculated for the samples in contact with the nanoparticle suspension [14].

To determine MIC of the tested irrigant solutions (5.25% NaOCl, 3% H_2O_2, IONPs, IONPs+ H_2O_2), serial tube dilution was used. Forty test tubes were prepared, and two milliliters of BHI broth was poured into each test tube using a sterile pipette, capped tightly, and then autoclaved. The tubes were then divided into four groups of 10 test tubes each.

For the IONPs group, 4 mL of the IONPs was applied to the first tube and mixed. Then 2 ml of this mixture was transferred to the second tube and then applied to the next tube. This method was carried out up to the tenth tube (the resultant concentrations decreased from the first to the tenth tube, respectively, from 8, 4, 2, 1, 0,5, 0,25, 0,125, 0,062, 0,031 to the end of 0,015 mg/ml). The same procedure was repeated for NaOCl, H_2O_2, IONPs+H_2O_2 samples. The irrigant was gradually diluted from 1:2 to 1:1024 in the last test tube. Finally, 100 µl of standard microbial suspensions of *E. faecalis* were added to test tubes 1 to 10. The test tubes were incubated at 37°C for 24 hrs[15].

Then, the microbial growth was determined by the presence or absence of turbidity and compared to the control. The positive control tube was filled with BHI medium and 0.1ml of a standardized *E. faecalis* suspension with 0.5 McFarland (10^8 CFU/mL). The negative control tube was filled with BHI media only. The highest dilution that had no turbidity represents the minimum inhibitory concentration [16].

Minimum Bactericidal Concentration (MBC)

MBC was determined by subculturing MIC tubes of the previous test which had no turbidity on plates of enterococcus selective media to examine bacterial growth for *E. faecalis*. The plates were incubated at 37°C for 48 hrs. Those plates whose MIC concentration revealed no bacterial growth were referred to as Minimum Bactericidal Concentration[16].

RESULTS

MIC and MBC for the four irrigants (5.25% NaOCl, 3% H_2O_2, IONPs, IONPs+ H_2O_2) against *E. faecalis* is shown in Table (1).

Table (1): MIC and MBC of All tested irrigant against *E. faecalis.*

Treatment group	MIC	MBC
IONPs+H_2O_2	1:1024 (tenth dilution)	1:1024 (tenth dilution)
IONPs	1:1024 (tenth dilution)	1:512 (ninth dilution)
H_2O_2	1:64 (sixth dilution)	1:16 (fourth dilution)
NaOCl	1:64 (sixth dilution)	1:64 (sixth dilution)

Results of the (MIC) test revealed that all irrigant solutions used had an inhibitory effect on *E. faecalis* after 24 hrs of incubation. However, IONPs combination with H_2O_2 was the most effective among irrigants against *E. faecalis*. It required the lowest concentration, where its tenth dilution showed no turbidity after 24 hours of incubation. 5.25% NaOCl and 3% H_2O_2 having shown their inhibitory effect against *E. faecalis* down to their sixth dilution. For IONPs suspension, the tubes from one to eighth dilution was shown turbidity, then the turbidity was absent on the ninth and tenth dilution.

The Minimum Bactericidal Concentration (MBC) is the minimum concentration of irrigants that showed no growth of *E. faecalis* on selective Enterococci media. Results revealed that all irrigating solutions were bactericidal after 24 hours of incubation. IONPs combination with H_2O_2 was the most effective among all tested irrigants where the lowest concentration (tenth dilution) was needed to kill all bacteria. H_2O_2 was able to kill bacteria at their fourth dilution, whereas, NaOCl require the same inhibitory concentration to kill *E. faecalis.*

DISCUSSION

MIC "is the lowest concentration of a material that will inhibit the visible growth of a microorganism after overnight incubation"[17]. A lower MIC is an indication of a better antimicrobial agent[17].

NaOCl inhibited the growth of *E. faecalis* in as high as a sixth dilution. This result comes in agreement with joy Sinha *et al*., (2017) who studied the MIC of NaOCl against *E. faecalis* and showed that the MIC was 0.16 %[18]. However, these results are in contrast with those reported by Neelakantan *et al*., (2003) who showed in their study that the MIC of 3% NaOCl was 0.4%[19]. This discrepancy might be related to the different strains of *E. faecalis* that were examined in the different studies.

Our study revealed that IONPs alone enhanced the growth of *E. faecalis* in suspension from the highest tested concentrations of 8mg/ml to 0.031 mg/ml. However, a bacteriostatic effect has been observed at low IONPs concentrations, that is, from 0.031 to 0.01 mg/ml. This finding comes into agreement with Torres-Gómez *et al*., (2019) who evaluated the MIC of different concentrations of IONPs alone against *E. faecalis* and showed that All of the concentrations above 0.07 mg/ml showed tur-

bidity, which results in a low antibacterial activity[20].

Previous studies have also documented the stimulatory effect of IONPs alone on microbial development, that is, on *E. coli, P. aeruginosa,* and *E. faecalis*[21, 22]. These findings may be explained by the ability of the microbial strains to use the iron oxide in high concentrations as a metabolic supply of iron, which is known to positively control the rate of the microbial growth and other physiological processes [23].

Our result showed that IONPs + H_2O_2 proved to have an inhibitory effect against *E. faecalis* at all the tested concentrations. The irrigant combination results in the Fenton-like reaction. Fenton reaction is a catalytic process that converts H_2O_2 into a highly toxic hydroxyl free radical. Reactive oxygen species generated by this reaction can easily diffuse into the cell cytoplasm, triggering ROS-induced release in the mitochondria and result in cell death[24]. Until the time of our study, there was no study conducted to evaluate the MIC of the IONPs in combination with H_2O_2 on planktonic *E. faecalis*.

CONCLUSIONS

According to the results of this study, IONPs exhibited a stimulatory effect directly correlated with high concentrations of the tested nanoparticles, but when it is combined with H_2O_2, it has a superior antibacterial effect at all tested concentrations comparable to other conventional solution used in this analysis. Therefore, considering this investigation, a therapeutic window can be provided to use IONPs+H_2O_2 as a novel intracanal irrigant. However, further investigations are required to consider the use of this combining solution as an irrigant in primary polymicrobial endodontic infections.

Conflict of Interest

Authors declare no conflict of interest.

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