



Experimental Research

Antibacterial effects and cellular mechanisms of iron oxide magnetic nanoparticles coated by piroctone olamine against some cariogenic bacteria



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ABSTRACT

Background: The present study aims to study antibacterial effects and cellular mechanisms of iron oxide magnetic nanoparticles loaded with piroctone olamine (Fe₃O₄@PO NPs) against some cariogenic bacteria (*Streptococcus mutans* and *Actinomyces viscosus*).

Methods: Nanoparticles was synthesized by the coprecipitation method. Antibacterial effects of Fe₃O₄@PO NPs were performed by calculating the minimum inhibitory concentration (MIC). We also evaluated the level of reactive oxygen species (ROS) and protein leakage to assess whether antibacterial effects may be dependent on these mechanisms.

Results: The results demonstrated that PO showed the lowest antibacterial effect compared to other drugs tested with MICs values of 53.33 and 64 µg/ml for *S. mutans* and *A. viscosus*, respectively. In contrast, the highest antibacterial effect was related to Fe₃O₄@PONPs with MICs values of 2.66 and 3.33 µg/ml for *S. mutans* and *A. viscosus*, respectively. Fe₃O₄@PONPs, Fe₃O₄MNP, and PO markedly increased ($p < 0.001$) ROS production and protein leakage of tested bacteria at $\geq 1/4$ MIC, $\geq 1/3$ MIC, and $1/2$ MIC, respectively.

Conclusion: The findings of the present survey revealed the promising antibacterial effects of Fe₃O₄@PONP against some cariogenic bacteria; whereas it triggered the ROS production and protein leakage as the possible antibacterial mode of action of anti-infective agents. However, additional surveys are necessary to elucidate the accurate mechanisms of these nanoparticles.

1. Introduction

Tooth decay as a stable harm in the enamel or hard portion of teeth is considered the most common infectious disease of oral cavity [1]. A number of cariogenic bacteria, e.g. *Actinomyces* spp., *Nocardia* spp., and *Streptococcus* spp., that are able to generate acid and consequently produce dental caries [2]. Today, a number of synthetic agents, e.g. chlorhexidine, quaternary ammonium salts, and fluoride have been generally applied to eliminate bacteria that live in the mouth [3]. In recent decades, nanomaterials and nanoparticles have revolutionized the diagnosis and treatment various infectious diseases [4]. Antimicrobial nanoparticles are strongly promising due to having several benefits

including large surface-area-to-mass ratio, ultra-small sizes, and unique physical and chemical possessions [5,6]. Nowadays, it has been proven that NPs play a potent antibacterial role through controlling the formation of oral biofilms through their biocidal, anti-adhesion and delivery capability [7–9]. Piroctone olamine is an ethanolamine salt derived from hydroxamic acid piroctone, which has been shown to have antibacterial and antifungal activity in recent years [10]. The present study aims to study antibacterial effects and cellular mechanisms of iron oxide magnetic nanoparticles loaded with piroctone olamine (Fe₃O₄@PO NPs) against some cariogenic bacteria (*Streptococcus mutans* and *Actinomyces viscosus*).

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2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulphate (SDS, 0.1%), tryptic soy broth (TSB), blood agar (BA), and mitis salivarius agar (MSA), müller-hinton broth culture medium were purchased from Merck, Germany. 2', 7'-dichloro-fluorescein diacetate (DCFH-DA) was prepared from Sigma-Aldrich, Germany.

2.2. Synthesis of Fe₃O₄ MNP coated with pyroctone olamine (PO)

At first, Fe₃O₄ MNP was synthesized based on the alkaline technique of aqueous solution containing Fe²⁺ + and Fe³⁺ ions in the absence of oxygen and by normalizing the temperature and pH conditions [11]. Then, Fe₃O₄@PO NPs was synthesized using the precipitation method according to the method described elsewhere.

2.3. Physicochemical properties of synthesized nanoparticles

The fabrication of nanoparticles was confirmed by the UV-Vis absorption spectrum and in the range of 4000–400 and with a resolution of 4 cm⁻¹. The size and morphology of the synthesized nanoparticles will be examined using a 15 kv electron microscope (SEM) with a magnification of ×10 and a resolution of 1 nm. XRD device is used to determine the properties of crystalline nanoparticles. FT-IR analysis is performed to identify potential functional groups and biomolecules responsible for the synthesis and coating agents of nanoparticles.

2.4. Antibacterial effect of NPs

2.4.1. Bacteria

A. viscosus (PTCC 1202) and *S. mutans* (ATCC 35668) were cultured in TSB, BA, and MSA at 37 °C with 5% CO₂. Lastly, growing bacteria were approved using some tests, e.g. Gram staining, optochin, catalase, and bacitracin.

2.4.2. Preparation of Standard McFarland 0.5 solution

Standard McFarland 0.5 solution, was prepared based on the method described by Panpaliya et al. [12]. To prepare of 0.5 McFarland Solution of tested Bacteria, a number of bacterial colonies were mixed with physiological serum and then turbidity of mixture was compared with the dilution of standard 0.5 McFarland [12].

2.4.3. Micro broth dilution

Micro broth dilution technique based on the Clinical and Laboratory Standards Institute (CLSI) guidelines was applied to determine the minimum inhibitory concentration (MIC) of Fe₃O₄@PO NPs against on *A. viscosus* and *S. mutans* [13]. In this method, wells with no color are considered as MICs. Chlorhexidine and normal saline were used as the positive and negative control, respectively. In the next step, the lowest concentration of the Fe₃O₄@PO NPs with no bacteria lived was described as the minimum bactericidal concentrations (MBC).

2.5. Effect of the reactive oxygen species (ROS) generation

To assess whether antibacterial effects may be dependent on ROS, we used DCFH-DA to study the ROS level provoked by Fe₃O₄MNP, PO, and Fe₃O₄@PO NP. To do this, *S. mutans* and *A. viscosus* cells were treated with DCFH-DA (10 µM) at 37 °C for 30 min. In the next step, bacteria were tested with Fe₃O₄MNP, PO, and Fe₃O₄@PONP at 1/4 MIC, 1/3 MIC, and 1/2 MIC for 180 min. In the last step, the fluorescence intensity of tested drugs was read at excitation/emission wavelength of 488/525 nm [14].

2.6. Effects on the protein leakage

Effect of tested nanoparticles on the protein leakage was determined according to the technique defined by Du et al. [15]. To perform this test, after incubating the with Fe₃O₄MNP, PO, and Fe₃O₄@PONP at 1/4 MIC, 1/3 MIC, and 1/2 MIC at 37 °C for 2 h, the mixture was centrifuged at 4000 rpm 5 min. Then, Bradford reagent (950 µL) was added to the participate and the protein content was evaluated based on the Bradford's method [15]. The positive and negative control were SDS and normal saline, respectively. The absorbance of the solution was detected at 590 nm a microplate reader spectrophotometer (BioTek, USA).

2.7. Statistical analysis

All statistical tests are accomplished using SPSS software version 25.0 (SPSS Inc., Chicago, Ill., USA). The independent *t*-test, ANOVA, Tukey and *post hoc* test are applied to compare the results between groups. P < 0.05 will be reflected as a significant level.

3. Results and discussion

Today, some microbial pathogens have been recognized in plaque which is acidogenic and aciduric nature and answerable beginning and also development of dental caries in humans [1,2]. Therefore, targeting such bacteria to prevent plaque creation and development is considered as the first choice of therapy [16]. Because of the insufficiency of synthetic antimicrobial agents as well as emergence of multi drug resistant bacteria, it seems necessary to find a new reliable, biologically safe and naturally available antibacterial agents. Today, magnetic nanoparticles are used as new materials for various purposes such as biomedicine, diagnostic and therapeutic purposes [17]. The present study aims to study antibacterial effects and cellular mechanisms of Fe₃O₄@PO NPs against *S. mutans* and *A. viscosus*.

The size of Fe₃O₄ NP and Fe₃O₄ @ PO NPs were in the range of 1–40 and 5–55 nm, respectively. While Fe₃O₄ NP and Fe₃O₄ @ PO NP particles were mostly 10–15 and 15–20 nm in size, respectively. As shown in Suppl 1C1, the XRD arrangement for Fe₃O₄ @ PO NP with peaks 2θ, 30.1°, 35.6°, 43.3°, 53.5°, 57°, 63° and 74° displayed that nanoparticles Correctly synthesized. Supplementary 1C2 shows that Fe₃O₄ MNP and Fe₃O₄ @ PO NP have perfect ferromagnetic properties. Fe₃O₄@PO NP and Fe₃O₄ and displayed saturation magnetization with values of 28.2 and 43.7 EMU/G. In this study, FTIR measurements were used to assess the functional groups located on the surface of nanoparticles (Suppl 1D). Fe₃O₄ NP displayed a typical Fe–O bond adsorption at 632 cm⁻¹. Whereas, Fe₃O₄ @ PO NP the main peaks at 3000, 1,625, 1,500, 1,366, 1190 and 627 wave numbers (cm⁻¹) for O–H or N–H, C = O, C = C, C–N indicate the functional groups CO or CN and Fe–O, respectively. It shows similar long peaks in the FTIR PO spectrum (2,962, 1,622, 1,497, 1366 and 1187 cm⁻¹). Which indicates that the coating process is done correctly.

Considering the antibacterial effect of NPs, the MIC after 3 replications for Fe₃O₄ @ PO NPs, Fe₃O₄ NPs, PO as well as chlorhexidine as a control drug on both bacterial species is listed in Table 1. The results

Table 1

Antibacterial effects of Fe₃O₄ @ PO NPs, Fe₃O₄ NPs, and PO against some cariogenic bacteria by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentrations (MBC).

Drug	MIC (µg/ml)			
	<i>A. viscosus</i>		<i>S. mutans</i>	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Fe ₃ O ₄ NPs	10.66 ± 4.6	13.33 ± 4.6	10.66 ± 1.15	10.66 ± 1.15
Fe ₃ O ₄ @PO NPs	3.33 ± 1.15*	3.33 ± 1.15*	2.66 ± 1.15*	3.33 ± 1.15*
PO	64.0 ± 0.0	64.0 ± 0.0	53.33 ± 18.4	53.33 ± 18.4
Chlorhexidine	5.33 ± 2.4	5.33 ± 2.4	4.66 ± 1.15	4.66 ± 1.15

demonstrated that PO showed the lowest antibacterial effect compared to other drugs tested with MICs values of 53.33 and 64 $\mu\text{g}/\text{ml}$ for *S. mutans* and *A. viscosus*, respectively. In contrast, the highest antibacterial effect was related to Fe3O4@PONPs with MICs values of 2.66 and 3.33 $\mu\text{g}/\text{ml}$ for *S. mutans* and *A. viscosus*, respectively. The obtained results showed that Fe3O4@PONPs had a significant ($P < 0.05$) antibacterial effect compared to chlorhexidine. The results also showed that although the levels of Fe3O4 NPs and PO significantly inhibited the growth of both bacteria, this antibacterial effect was not significant compared to the control drug.

Since the production of ROS and oxidative stress is well-known as one of the key mechanisms of bactericidal effects of anti-infective agents, we evaluated that whether antibacterial effects of Fe3O4MNP, PO, and Fe3O4@PONP can be related to ROS by DCFH-DA assay. Our results demonstrated that a significant increase ($p < 0.001$) of fluorescence intensity was observed with the increase of Fe3O4@PONP concentrations ($p < 0.001$), representing that the Fe3O4@PONP facilitated ROS creation compared with the control group. On the other hand, Fe3O4MNP and PO markedly increased ($p < 0.001$) ROS production at $\geq 1/3$ MIC and $1/2$ MIC, respectively. Effects of Fe3O4MNP, PO, and Fe3O4@PONP on the protein leakage of *S. mutans* and *A. viscosus* were shown in Fig. 1. The findings revealed exhibited that the Fe3O4MNP and PO showed considerable ($p < 0.001$) protein leakage at $2/4$ MIC and $\geq 1/3$ MIC, respectively; whereas Fe3O4@PONP displayed the highest effect

protein leakage of *S. mutans* and *A. viscosus* in all tested concentrations ($p < 0.001$) (Fig. 2).

Thukkaram et al. evaluated the *in vitro* antibacterial activity of Fe3O4NPs at concentrations of 0.01, 0.05, 0.1, and 0.15 mg/ml by determining the zone of inhibition and bacterial adhesion to surfaces. They reported that Fe₃O₄ NPs significantly inhibited the biofilm growth bacterial adhesion in *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* on different biomaterial surfaces after 30 min of incubation [18]. Gabrielyan et al. have reported the *in vitro* antibacterial effects and action mechanisms (e.g., evaluation of H⁺ -fluxes through bacterial membrane, ATPase activity assay, medium pH, redox potential and H₂ yield determinations) of silver (Ag) and citric acid coated Fe₃O₄NPs against *E. coli* wild type and kanamycin-resistant strains, as well as on *Salmonella typhimurium* MDC1759. They results exhibited the potent antibacterial effect on tested bacteria in dose-dependent response through altering in membrane permeability and membrane-bound enzyme activity [19]. Armijo et al., have also revealed the antimicrobial effects of iron oxide nanoparticles at concentrations of 1.78–17.35 mg/ml on the biofilms of *P. aeruginosa* as the main Gram-negative causative bacteria for nosocomial infections by the disk diffusion method and biofilm inhibition [20].

The amount of protein leakage of *S. mutans* and *A. viscosus* after exposure with Fe3O4MNP, PO, and Fe3O4@PONP at $1/4$ MIC, $1/3$ MIC, and $1/2$ MIC. The findings revealed that the green synthesized Fe3O4MNP, PO, and Fe3O4@PONP at $\geq 1/3$ MIC, $1/2$ MIC, and $\geq 1/4$

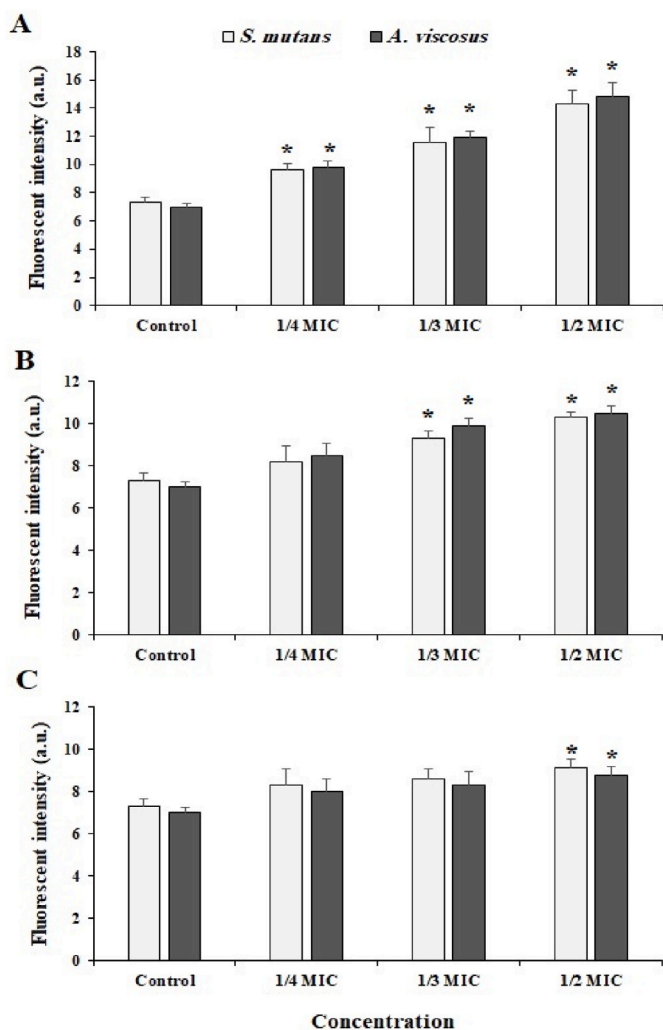


Fig. 1. The effect of Fe3O4 @ PO NPs (A), Fe3O4 NPs (A), and PO (C) on the production of reactive oxygen species (ROS) in the tested bacteria. Mean \pm SD ($n = 3$). * $p < 0.001$ significant change compared with control group.

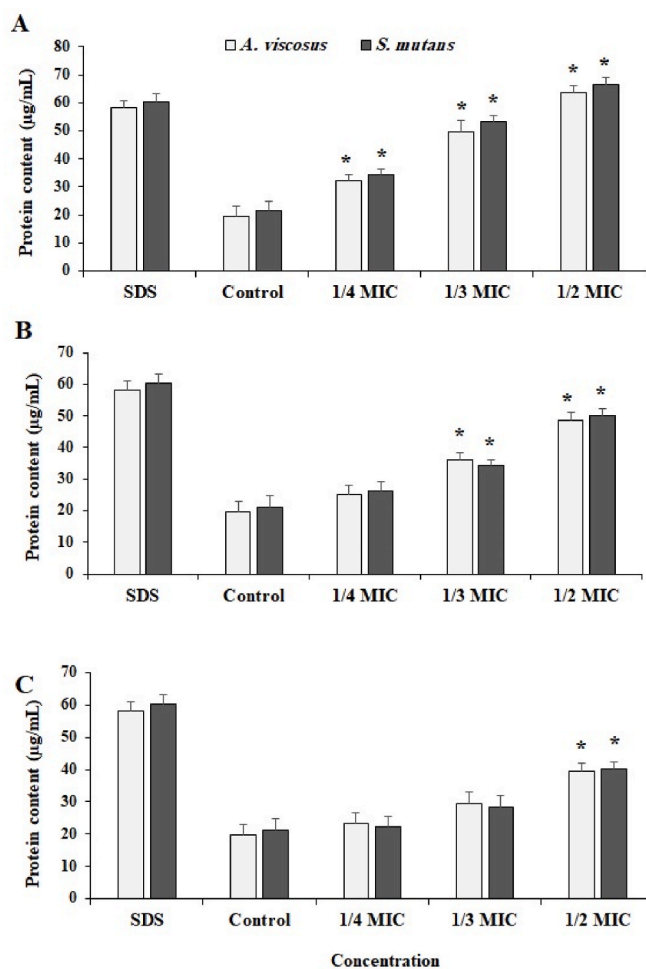


Fig. 2. Effects of Fe3O4@PONP (A), Fe3O4MNP (B), and PO (C) on the protein leakage of *S. mutans* and *A. viscosus* on protein leakage of the tested bacteria at $1/4$ MIC, $1/3$ MIC, and $1/2$ MIC. Mean \pm SD ($n = 3$). * $p < 0.001$ significant change compared with control group.

MIC markedly ($p < 0.001$) increased the protein leakage, respectively. With respect to the antimicrobial mode action of Fe NPs, previous studies demonstrated that these NPs through interaction with bacterial wall and their diffusion into the bacterial cell, triggering membrane destruction and destroy bacteria [21–23]. Regarding the cytotoxicity, Albalawi et al. have reported that Fe₃O₄ NPs and Fe₃O₄@PO NPs displayed that in MTT assay, no considerable cytotoxicity in murine macrophage J774 cells with the CC₅₀ value of 645.25 and 358.3 µg/mL for Fe₃O₄ NPs and Fe₃O₄@PO NPs, respectively [24]. Shakibaei et al., also have exhibited that Fe₃O₄@PO NPs and Fe₃O₄ NPs at 120 µg/mL showed no considerable cytotoxicity against Hs68 normal cell line; where they caused just 25% and 11% cytotoxicity, respectively [25].

The limitations of this research are the failure to conduct additional studies to determine the exact cellular mechanisms of these nanoparticles against pathogenic bacteria, failure to investigate cytotoxicity on normal human cells, and finally to investigate its antibacterial effects on other pathogenic bacteria. As the future implications of this work, we can point out that if additional studies are carried out, especially to determine the exact mechanisms of the effect of these nanoparticles and their side effects, and in the next step to confirm their effectiveness in the clinical phase, they can be used in different forms, e.g., mouthwash, toothpaste, to prevent tooth decay.

4. Conclusion

The results of this experimental survey demonstrated that Fe₃O₄@PO NPs exhibited better antibacterial effect in comparison with chlorhexidine; whereas had no cytotoxicity on some normal cell lines. However, further studies to elucidate the accurate mechanism as well as systemic toxicity especially in clinical settings are required.

Ethical approval

Not applicable.

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Author statement

Sajad Ghorbanzadeh: study concept or design.

Arshak Razlansari: data analysis or interpretation.

Somayeh Delfani, Fatemeh Karami, and Mojtaba Shakibaei: data collection.

Faranak Rezaei: writing the paper.

Registration of research studies

1. Name of the registry:
2. Unique Identifying number or registration ID:
3. Hyperlink to your specific registration (must be publicly accessible and will be checked):

Guarantor

Faranak Rezaei.

Consent

NA.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amsu.2022.104291>.

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