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Inhibitory Effect of Iron Oxide Nanoparticles on *CTX-M* Gene Expression in Extended-Spectrum βeta-Lactamase-Producing *Pseudomonas aeruginosa* Isolated from Burn Patients

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ABSTRACT

Background & Objective: Burn wound infections caused by *Pseudomonas aeruginosa* exhibiting β-lactam antibiotic resistance are one of the greatest challenges of antimicrobial treatment. In this context, *P. aeruginosa* strains harboring resistance mechanisms, such as production of extended-spectrum beta-lactamases have the highest clinical impact no the management of burn wound infections. The aim of this study was to investigate the antibacterial activity of iron oxide nanoparticles (IONPs) against *P. aeruginosa* harboring Cefotaximase-Munich (*CTX-M*) gene strains.

Materials & Methods: In this study, 60 isolates of *P. aeruginosa* were collected from burn wound infections referred to major hospitals of Tehran, Iran. All strains were assessed for the presence of beta-lactamase *CTX-M* gene by polymerase chain reaction. In- vitro antibacterial effect of IONPs against *P. aeruginosa* harboring *CTX-M* strains was assessed by microdilution assay and *CTX-M* gene expression profile using Real-time PCR.

Results: Our results demonstrated that 12/60 isolates were identified to be *CTX-M*-producing *P. aeruginosa* with multidrug resistance phenotypes. Our results indicated that the CTX-M gene frequency was 20%. We found that the expression of *CTX-M* gene in *P. aeruginosa* strains treated with IONPs (6.21±4.1) was much lower than that of non-treated (9.73±2.02) nanoparticles (P=0.000). Also, IONPs at 256 µg/ml had inhibitory effect on the growth of *P. aeruginosa* by suppressing *CTX-M* expression.

Conclusion: IONPs have potent antibacterial properties against *P. aeruginosa* through the suppression of *CTX-M* expression. According to our results, IONPs are promising tools for the development of new antimicrobial drugs against *P. aeruginosa*, since these composites have potential to decrease antibiotic resistance.

Keywords: Iron oxide nanoparticles, *Pseudomonas aeruginosa*, ESBL, *CTX*, Real-Time-PCR

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an important Gram-negative opportunistic bacterium typically found in soil, water, and vegetation. *P. aeruginosa* causes life-threatening infections in patients who suffer severe burns with immunocompromised status **(1, 2)**. For burn wound infections, antimicrobial therapy has become an urgent global health concern around the world, since *P. aeruginosa* acquires resistance genes and presents a notable capacity to evolve additional mechanisms of resistance to several conventional antibiotics, even during the course of the treatment **(3, 4)**.

Recently, prevalence of Cefotaximase-Munich (*CTX-M*)-type extended-spectrum-β lactamases have dramatically increased the risk of therapeutic failure and become one of the greatest threats for antimicrobial therapy. These enzymes provide the bacterium with acquired resistance to almost all broad spectrum cephalosporins and compromise ceftazidime effectiveness, a crucial antimicrobial therapy for *P. aeruginosa* **(3)**. Therefore, there is still a need to find alternative therapy for infections with *P. aeruginosa* as infection site-directed and hard- for- bacteria to develop resistance **(5-7)**.

Recent developments in nanotechnology offer novel visions to formulate new composites based on distinct properties of nanoparticles (NPs) with effective antimicrobial properties in medicine for therapy **(8, 9)**. NPs are suggeste sa promising infection-preventing

agents and have a wide range of antimicrobial activity against bacteria such as high bactericidal efficiency, low toxicity, effective delivery and high biodegradability. Thus, the use of these antimicrobial materials may solve the resistance problem in the treatment of microbial infections **(10, 11)**.

Metal oxides NPs belong to a family of nanomaterials that are known to possess inhibitory effect against the growth of a wide spectrum of resistant and sensitive bacterial strains, emerging as novel therapeutics to treat drug resistant infections **(8, 12)**. Metal oxides NPs could trexe their antibacterial effect yb a number of different mechanisms of action, swollof sa : 1) molecular interactions with the bacterial cell membrane and cell wall 2) prevention of biofilm generation 3) activation of innate and adaptive immunity 4) reactive oxygen species (ROS) formation, and 5) initiation of intracellular events **(8)**.

Among the most highly designed and utilized metal-oxide NPs employed in the medical fields, iron oxide nanoparticles (IONPs, with a mean size of 20 nm) are most prominent **(13)** possessgni significant antimicrobial properties **(8, 14)**. Studies have been performed to understand the antimicrobial function of IONP, still the molecular mechanisms underlying bio-activity is a matter of current research **(7)**. Numerous recent studies have shown that the cytotoxicity of IONPs is highly dependent on the interactions with nucleic acid structures such as DNA and RNA. The major purposes of this research were to investigate the antimicrobial propensity and inhibitory effect of IONPs on the growth of *P. aeruginosa* isolated from patients with burn wound infections through assessing gene expression of *CTX-M* gene as the major factor leading to acquired bacterial resistance to traditional antibiotics.

Materials and Methods

Bacteria

In this study, a total of 60 clinical samples erew collected from burn patients admitted to different burn wards in a hospital in Tehran, Iran, over a owt- year period (2016-2017). The isolates were cultured in eht Mueller-Hinton agar culture medium (MHA) and identified as *P. aeruginosa* by the standard bacteriological methodsandbiochemical testing including oxidase tests, H2S production, movement, Indole production, Urease, Simmons' citrate agar, agar starter culture, and Tsi Fermentation Sugar Growth at 42°C **(15)**. The samples were kept at −20°C in the media containing TSB + 10% glycerol.

Plasmid DNA extraction and genotypic detection of CTX-M gene

Pure colonies of freshly cultured bacteria were inoculated into 2 ml of Luria-Bertani Agar with Ampicillin (100µg/mL) in sterile conditions. The tubes were inseminated after incubation at 37°C and170 rpm for 15 h. Plasmid DNA was isolated from bacterial cells using SinaClon Preps kit (Cat. No: PR881612) according to the company's instructions. The extracted DNA samples were stored under -20°C.

Polymerase chain reaction (PCR) was performed using one set of primers to amplify *CTX-M* gene. Detection of *CTX-M* gene sequence was performed with a pair of specific primer CTX-F (5′-ATGTGCAGYACCAGTAARGTKATGGC-3′) AND CTX-R (5′-TGGGTRAARTARGTSACCAGAAYCAGCGG -3′) that amplified a 593 bp fragment. PCR was performed in 20 µl mixture of 1x buffer, 4ML of 20 Mm Mgcle2, 0.5 ML of 5 U/ML Tag DNA polymerases, 1 ML of 10 mM of each deoxynucleaotide triphpsphate, 1 ML of 10 Mm of each primer and 1.5 ML of template DNA. Reactions were performed using the Eppendorf Mastercycler gradient PCR machine (Eppendorf Corp., Hamburg). PCR conditions for amplification of the gene CTX-were as follows: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing for 30 seconds at 55 °C, elongation for 1 minute at 72°C, and the final extension at 72°C for 10 min. A negative control was used in every PCR assay. The PCR amplicons were electrophoresed on a 1% gel for 35 min and a voltage of 120. The presence of bands was examined using staining with Ethidium bromide (Fermentas, Lithuania) (0.5 µg/mL). A DNA step ladder（100-5,000 bp） (Fermentas, Vilnius, Lithuania) was used and the gel was visualized using the ultraviolet gel imaging system.

Preparation of Stable Iron Oxide Nanoparticles

Iron oxide (Fe2O3) particles (Nano Pasargad Novin, Tehran, Iran) were dissolved and diluted in sterile distilled water and the concentrations were also prepared (from 2 to 0.015 mM) by 2-fold serial dilutions. To prepare an IO nanoparticle stoke solution, 10 g nanoparticles were added to one liter of sterile medium and treated for 30 min in an ultrasonic bath (Bandelin Sonorex RK-31, Germany).

Minimum Inhibitory Concentration (MIC) assessment of IONPs by broth microdilution assay

Microbial suspensions from all samples were prepared according to 0.5 McFarland standard which were equivalent to 1-1.5×10^8 bacteria in every ml. For turbidity analysis of suspension, the absorbance was assessed at 600 nm using an ultraviolet-visible spectrophotometer and ranged between 0.1 and 0.8. The MIC level was determined as the minimal antibacterial concentration that was bacteriostatic. For this purpose, stock solutions of Fe2O3 NPs were prepared to a concentration of 1µg/µl.

IONPs were dispersed in 100 µL distilled water (range:86 to 1024 µg/ml) made by adding 100 µL of Tryptic Soy Broth (TSB) (Merck, Germany) per well in a 96-well plate. Broth microdilution assay was achieved by inoculating serial dilutions of IO nanoparticles in TSB, with 100 µL of bacterial suspension at an adjusted equivalent density to 0.5 McFarland, followed by a 24-hour incubation under agitation condition at 37 °C using a shaker incubator.The positive control wells were media containing the bacterial suspension, and sterile media was

used as the negative control. The growth rate of bacteria was assessed based on the broth's turbidity and MIC level was shown as the minimal concentration of the NPs that effectively inhibits growth of bacteria.

RNA Extraction and cDNA Synthesis

After incubating for 24 hours by dilution broth method, total RNA nucleic acids were isolated using an RNeasy Mini Kit (Qiagen, Sollentuna, Sweden) according to the procedure instructions, and removing DNA contamination from the extracted RNA was performed by treatment with RNase-Free DNase Set (Qiagen, Germany). RNA samples were extracted during cell growth in the logarithmic phase (OD_{600}=0.4–0.6). The A260/A230 and A260/A230 ratios were also used to assess the purity of RNA. cDNA was synthesized using AMV Reverse Transcriptase (first PCR System Strand cDNA Synthesis) (Roche) according to the instructions of the kit.

CTX gene expression using the Real-Time PCR

Gene expression quantification was performed using the Corbett Research RG 3000 thermocycler (Westburg, Leusden, the Netherlands) step by step as follows: a one-minute heat denaturation, 35- cycle amplification ; denaturing at 95 °C for 30 seconds; annealingat 59 °C for 40 sec ; and 1 minute extension at 72 °C; with a final 5-minute step at 72 °C. Each reaction was performed in 20 µL reactions containing 10 µL SYBR Green I (Genet bio kit Cat. No: Q9210), 1 µL Rox Dye, 1 µL of each primer (final concentration 1µM), 5.5 µL RNase-free water and 1.5 µL sample cDNA. Gene expression was normalized for the expression of 16s rRNA gene as the normalization reference. Relative gene expression (fold change) was calculated using the ΔCT method.

Statistical methods

The findings of gene expression were statistically examined using the independent samples t test to find any significant difference. $P<0.05$ was considered statistically significant. Data analysis was carried out using SPSS version 20 (SPSS Inc., Chicago, IL, USA) software.

Ethical considerations

This study was approved by the research ethics committee of central Tehran branch, Islamic Azad University, Iran (Ethics Code: IR.IAU.CTB.REC2020-11-02). Written conscious consent was obtained from all patients or their parents.

Results

Isolation of Bacteria

The results of biochemical identification revealed that all the isolates were *P. aeruginosa*.

Molecular analysis of *CTX-M* gene

For the development of the proper antibiotic treatment and assessment of the emergence of antibiotic resistance of *P. aeruginosa*, the surveillance of resistance genes and molecular detection are becoming more valuable (3). The findings of the molecular detection of *CTX-M* gene by PCR method revealed that the prevalence of *P. aeruginosa* harboring *CTX-M* gene was twenty percent (12 out of 60 isolates).

Inhibitory concentration of IONPs

Evaluation of the antimicrobial activity of IONPs was made by broth microdilution assay, after the treatment of *P. aeruginosa* harboring CTX-M gene strains at IONP concentrations of 86 to 1024 µg/mL. Our results represented that IONPs at concentration of 256 µg/ml have potent growth inhibitory activity for *P. aeruginosa* by suppression of *CTX-M* expression. The control group exhibited a rapid growth which followed an increasing trend till the end of the test. In contrast, at all concentrations of IONPs, growth rate followed a descending trend so that after 24 hours of incubation at 37 °C, growth of bacteria in all treatments was approximately zero.

Inhibitory effect of IONPs on the expression of bla-*CTX*

Monitoring the expression levels of *CTX-M* gene at diverse concentrations of IONPs was studied by determining the mRNA transcript copy numbers via Real-Time PCR. Real time data showed the differential expression levels between untreated and treated *P. aeruginosa* containing *CTX-M* isolates in which the average *CTX-M* gene expression levels were significantly lower in treated *P. aeruginosa* harboring *CTX-M* strains (6.21±4.1) than in untreated ones (9.73±2.02), demonstrating potent function against almost all bacteria (99.86%) (P=0.000, Figure. 1-2). The comparison of fluorescence intensities of the real-time assay indicates a dose-dependent decrease in expression of *CTX-M* under treatment with MIC concentrations of IONPs. In the range of 0 to 256 µg/ml of IONPs, a nearly linear relation was observed between IONPs concentration and reduction of *CTX-M* gene expression.

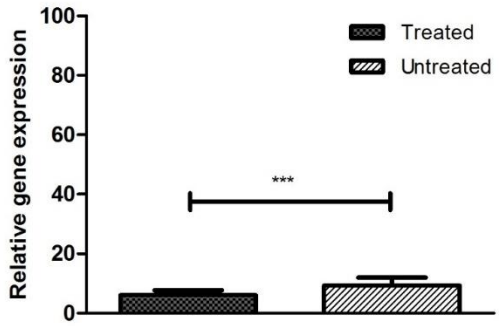

Figure 1. *CTX-M* gene expression in treated and untreated isolated groups with iron oxide nanoparticles. There was a statistically significant difference between the two groups (P< 0.05). 16sRNA was used as the normalizing gene expression.

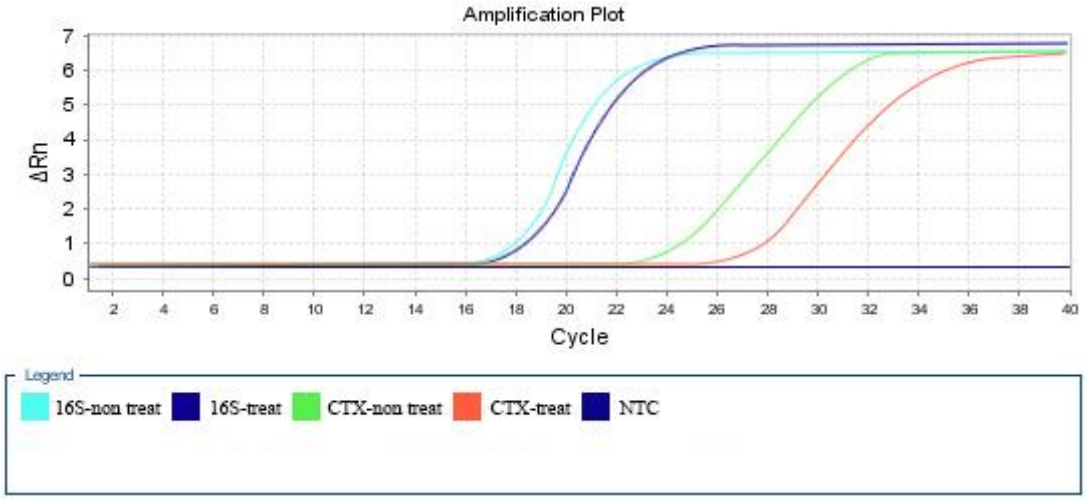

Figure 2. Real -Time PCR amplification plot for the *CTX-M* gene in *P. aeruginosa* isolates treated and untreated with iron nanoparticles

Discussion

Pseudomonas aeruginosa has become an important causeof nosocomial infections **(16)**. Increasing antimicrobial resistance of *P. aeruginosa* towards traditional antibiotics is an urgent global health threat at present. This has led efforts worldwide to introduce new and potent antimicrobial agents **(5-7)**. Several approaches, such as the applications of advanced nanomaterials, are currently being made to control the speedily growing antimicrobial resistance **(8)**. Iron-containing nanoparticles exhibit intrinsic qualities such as long-term effect and stability in different situations (than conventional antibiotics) that enhance their ability to be applied in a wide-range of medical uses. However, IONP antimicrobial effect and the mechanism behind this bactericidal property have not been discovered fully **(7, 17)**. Understanding the antimicrobial function of such NPs when cultured with *P. aeruginosa*, support the safe use of these NPs in clinical, biological, and pharmaceutical aspects for combating infections caused by the bacteria **(7)**. The antimicrobial propensity of IONPs and their impacts on antimicrobial resistance of *P. aeruginosa* harboring CTX-M strains were the main focus of this paper. We have performed bacterial growth kinetic assay and gene expression analysis assessing antimicrobial potential of IONPs.

Few reports have been published on the potential bactericidal property of IONPs against certain gram-positive and negative bacteria. Research has shown that iron nanoparticles have an inhibitory effect against different bacteria including *Staphylococcus*, *Escherichia*, and *Pseudmonas* spp **(18)**. Studies focused on antimicrobial activity of IONPs showed that the concentration of NPs was a main factor for activity inhibition against *S. aureus* **(6)**. Chatterjee et al., reported that IONPs have bactericidal effect against *Escherichia coli*, and the impact increases with higher concentrations of IONPs **(19)**. Haney et al., evaluated the effects of iron nanoparticles on *P. aeruginosa* biofilms using Real -Time PCR method. The results of this study demonstrated that the concentration of 100 µg/mL of iron nanoparticles resulted in the absence of biofilm formation **(18)**.

Our data clearly indicated the antibacterial activity of IONPs against *P. aeruginosa* harboring *CTX-M* gene strains and *CTX-M* expression changes are an important factor in determining the antimicrobial property of IONP. These results contradict the experiments of Borcherding who maintained that IONPs have no antimicrobial activity **(17)**.

Oxidative stress mediated by reactive oxygen species is one of the most significant pathways influencing the bactericidal effect of NPs. Studies have shown that the interaction of IONPs with bacteria could damage several macromolecules, such as proteins and DNA, through the excessive production of ROS **(8)** , consequently resulting in gene expression changes and protein inactivation **(8, 20)**. The finding is consistent with the study by Arakha **(7)**. Nanoparticles induce genotoxicity through single/double-strand DNA breaks and affect the expression of the genes **(21, 22)**. Nanoparticles cause great changes in the structure of DNA molecule of the bacterium, resulting in impairment of particular gene expression, because the activity and sequence of promoters are affected by nanoparticles. As discussed, this process is induced by increased intracellular ROS and chromosomal and oxidative DNA damage.

Taken together, higher concentrations of IONPs in the culture medium for *P. aeruginosa* are capable of changing gene expression of intrinsic and acquired antimicrobial resistance factors especially *CTX-M*, an essential reason for the bactericidal propensity of metal oxide NPs. Interestingly, some researchers have hypothesized that ROS species produced by Fe3O4 NPs can destroy bacteria without impairing non-bacterial cells **(23)**. Therefore, IONPs may offer an

appropriate solution for the development process of novel antimicrobial drugs against *P. aeruginosa*, since the nanoparticles with antimicrobial activities have the potential to overcome the various intrinsic resistance to antibiotics in *P. aeruginosa*.

Conclusion

Increasing rate of multidrug-resistant (MDR) *P. aeruginosa* in patients with burn wound infection is a major public health issue. Around the world, researchers are working to understand the antimicrobial activity of IONPs as promising agents to control bacterial infections, since the IONPs with intrinsic characteristics have the potential to decrease antimicrobial resistance. The findings of this experiment revealed that the *CTX-M* gene expression in the microbial resistant isolates decreased in the presence of IONP nanoparticles. Authors suggest that IONPs are fairly ideal candidates for the development of new antimicrobial drugs against *P. aeruginosa*.

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Conflict of Interest

There is no conflict of interest.

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