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Original Article

Zinc oxide nanoparticles as a new approach to eradicate the biofilm of *Pseudomonas aeruginosa* isolated from clinical samples

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Abstract

Background: Zinc oxide (ZnO) nanoparticles are particularly notable in the biomedical field, especially for antimicrobial applications, due to their beneficial properties. This study aimed to investigate the antimicrobial activity of ZnO nanoparticles against *Pseudomonas aeruginosa* (*P. aeruginosa*) isolated from clinical samples.

Methods: A microdilution test was conducted to assess the effectiveness of the synthesized against *P. aeruginosa*. Additionally, the impact of the zinc oxide nanocomposite on the expression of biofilm-related genes, including *algD*, *lecA*, and *lecB*, was measured using the Real-Time PCR method.

Results: The minimum inhibitory concentration (MIC) of the ZnO nanocomposite against *P. aeruginosa* was found to be 625 µg/ml. At half the MIC concentration, ZnO nanoparticles demonstrated a 54% inhibition of biofilm formation. The expression levels of the *algD*, *lecA*, and *lecB* genes were reduced by 3.3 ($p < 0.001$), 1.8 ($p < 0.05$), and 3.1 folds ($p < 0.05$), respectively.

Conclusion: Overall, ZnO nanoparticles, by reducing the expression levels of the *algD*, *LecA*, and *LecB* genes in *P. aeruginosa*, can be utilized as a novel therapeutic approach to improve and treat infection-related biofilms.

Keywords: Antibacterial, ZnO nanoparticles, Biofilm, Pseudomonas aeruginosa.

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There is a significant issue with the spread of multidrug-resistant (MDR) microorganisms that requires further focus. MDR isolates are linked to an estimated 1.27 million fatalities (1). To prevent further deaths, the World Health Organization published a list of priority infections. *Acinetobacter baumannii*, *Enterobacteriaceae*, and *P. aeruginosa* are classified as members of the critical group. Nosocomial infections are mostly caused by these bacteria. Among these isolates, P. *aeruginosa* isolates are the leading cause of nosocomial infections, particularly in patients (2) Only two or three antibiotics are effective against the named pathogens (3). Antibiotic resistance in bacteria have many mechanisms. Biofilm formation is an important main factor that can lead to resistance (4). Some infectious immunocompromised diseases such as periodontitis, endocarditis and chronic lung disease in cystic fibrosis patients have been related with biofilm formation (5). Alginate (α-L-guluronic acid and β-D-mannuronic acid) has a vital role in the biofilm formation of *P. aeruginosa*. It also helps protect against bacterial infections by binding to antibiotics in biofilms. and encoded by *alg*D gene (5). It is an important enzyme to make alginate polymers. Pseudomonas aeruginosa can produce LecA and LecB, which are associated with the bacterial virulence. These lectins had a role in biofilm formation on the surfaces, but its mechanisms remain unknown (6). Today, biomaterials are considered an alternative to developing novel antibacterial drugs (7).

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Nanomaterials have attracted widespread attention due to their uniquely high surface area. Metals such as zinc, copper, silver, and gold routinely is used as nanoparticle (NP). Nanoparticles can damage the integrity of the microbial membrane (8). Reactive oxygen species (ROS) produced by NPs released into the cytoplasm have the ability to damage bacterial cell membranes, causing cell lysis and shrinkage (9). Zinc (Zn) and its oxide (ZnO) are well-studied metals that affect living organisms. Zinc is a reactive element and is highly reducible (10). It is easily oxidized to form. It can act based on the following mechanisms: it disrupts cell membrane, binds to proteins and DNA, increases ROS, disrupts DNA amplification processes, and alters the expression (more down-regulate) of many genes (11, 12). Several studies have demonstrated that nanoparticles ZnO directly inactive some Gram-negative and Gram-positive bacteria (13, 14). The present research aimed to investigate the antibacterial activity of ZnO nanoparticles against *P. aeruginosa* isolated from clinical samples.

Methods

Bacterial samples: *P. aeruginosa* isolates were collected from blood samples at the Shahid Beheshti hospital of Kashan, Iran between March 25, 2022 and February 30, 2023. The following tests was performed to identify the *P. aeruginosa* bacteria: growth on MacConkey agar, growth at 42 °C, Gram staining, the oxidase test, the motility test, the catalase test, the urease test, the indole test and citrate utilization. First, all isolates were collected from clinical samples, and then *P. aeruginosa* isolates producing moderate and strong biofilms were included in the study. Isolates lacking biofilm production, as well as isolates with weak biofilms, were excluded from the study. The present study was approved by the Research Ethics Committee of Kashan University of Medical Sciences (IR.KAUMS.MEDNT.REC.1400.215).

Preparation of nanoparticles: First, 1 mmol of sodium nitrate was added to 30 mL of water, and was placed on a magnetic stirrer at a temperature of 50°C for one hour until it became completely homogeneous. Then 0.1 mmol of sodium hydroxide was added to the solution under ultrasonication. The solution was kept at 80°C for one hour. Then the precipitate was washed three times with water and methanol. To calcine the product, it was placed in the oven at 500°C for 1 hour. The observation of brown powder indicated the formation of nanoparticles. Then the physical and chemical structure of zinc oxide nanoparticles was evaluated. Semi-quantitative Energy Dispersive X-Ray Spectroscopy (EDX) was performed to determine constituent elements and crystal size of nanoparticles, and scanning electron microscope (SEM) was performed to estimate particle morphology and size. 25 mg of ZnO NPs were added to 5 mL of distilled water to create the ZnO NPs stock solution. The NPs were then sonicated at room temperature for 30 minutes at a power of 100 w and 40 kHz. NPs were stored at 4 °C.

MIC determination: To evaluate activity antibacterial of the ZnO NPs, we used the broth microdilution technique. In short, we filled the 96-well plates with 100 µL of Mueller Hinton Broth (MHB) medium. The 96-well plates were then filled with 100 µL of each concentration of ZnO NPs. Subsequently, 10 µL of the diluted 1.5×10^6 CFU solution (final concertation: 5×10^5 CFU/mL) was introduced into every well. For 18-20 hours, the plates were incubated at 37 °C. To find the MIC value, the optical density was measured following incubation. The MIC denotes the lowest concentration at which the bacteria could not grow visibly. We used MHB as the negative control and MHB plus bacteria as the positive control. The antibiotic used as a control was imipenem. Every experiment was conducted thrice, in triplicate (15).

Antibiofilm activity: Crystal violet staining protocol was used to determine activity of ZnO-NPs against biofilm formation. First, 100 µL of bacterial suspension (10^8 CFU/mL) was added into 96-well microplates. Then, 100µL of different concentrations of ZnO NPs (sub-MIC, MIC, and 2MIC) added into each well of micro plates. After incubation at 37 °C for 24 h, for removing of non-adhered cells, each well was aspirated and was washed three times with Phosphate-buffered saline (PBS). The micro plate was dried in an atmosphere of air. The methanol was added to wells (20 min) for fixing the adherent cells. Crystal violet in concentration of 0.2% (10 min) was used to stain the cells. Microplate was washed with PBS. Finally, an ELISA reader system (Awareness Technologies, USA) was used to determine the optical density of wells at 570 nm (16).

Isolation of RNA and performing real-time PCR: In this study, real-time PCR was conducted to assess the expression levels of the *algD*, *lecA*, and *lecB* genes of P. *aeruginosa* after treatment of clinical samples with ZnO-NPs at concentration of 1/2 MIC. Initially, TRIzol reagent was used to extract total RNA from bacterial cells treated with ZnO NPs at their minimum inhibition concentrations. A Nanodrop spectrophotometer (Thermo Scientific, USA) was used to detect the absorbance of RNA at 260 nm to verify the amount of extracted RNA. Following the manufacturer's instructions, the complementary DNA (cDNA) was created using the Favorgen cDNA synthesis

kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan). The IQ5 real-time PCR cycler (Bio-Rad, Germany) was used to carry out the relative real-time PCR. 10 µL of 2X SYBR green master mix (Amplicon, Denmark), 0.5 µL of each primer, 5 µL of cDNA, and 4 µL of deionized water were added to the reaction mixture. The thermal cycling schedule was as follows: 15 minutes at 94 °C, followed by 40 cycles of 20 seconds at 94 °C and 60 seconds at 60 °C. Table 1 lists the primers used in this study. The housekeeping genes employed were gyrA and oprL. We computed fold changes using the 2^{-ddCt} method (17).

Statistical analysis: The results are presented as mean ± standard deviation. Data analysis was performed using GraphPad Prism 8 statistical software, and a p-value of < 0.05 was considered significant. The Kolmogorov–Smirnov test and Mann-whitney test were used to compare the effect of ZnO-NPs concentrations (1/2 MIC, MIC, 2MIC) on the bacterial biofilm.

Table 1. Primers used for Real-Time PCR

Target gene	Sequence primer (5'➔3')	Annealing temperature
algD	Fw: TGATCTGCCAGGACCACAAGC Rv: CGCATCAACGAACCGAGCAT	60°C
lecA	Fw: GCCAGTTACGGACCTACCCAG Rv: GTGCAACCCAACGGAACAACC	60°C
lecB	Fw: CGTCACCGCCTTCGCCAAC Rv: ACCTGTGCCGAGACCAGATCC	60°C
oprL	Fw: CCGCGAGTACAATATGGCTCT Rv: CCGGACGCTCTTTACCATAGGAA	60°C

Fw, forward primer and Rv, reverse primer

Results

Antibacterial activity of zinc oxide nanoparticles: In the current study, the inhibitory activity of ZnO-NPs on *P. aeruginosa* isolates was determined by microdilution method and compared with the control group. The average MIC of ZnO nanoparticles was 625 µg/ml and the average minimum bactericidal concentration (MBC) was 1250 µg/ml for *Pseudomonas* isolates ($p < 0.05$). These findings show that the nanoparticles can be effective in preventing and inhibiting the growth of *P. aeruginosa* bacteria strains. The results also showed that *P. aeruginosa* isolates are very sensitive to nanoparticles. The findings confirmed that the higher concentration of ZnO nanoparticles had higher antibacterial activity.

Biofilm inhibition test: Fifty-five percent of the isolates had a strong biofilm that were used for measuring of effect of ZnO-NPs. The effectiveness of ZnO nanoparticles to prevent the formation of *P. aeruginosa* biofilm was assessed at dilutions of MIC, 1/2 MIC, and 2 MIC. The findings showed that ZnO-NPs significantly prevent the biofilm formation of *P. aeruginosa* isolates and this inhibitory effect is dependent on nanoparticles concentration. Based on this, ZnO-NPs at a concentration of 1/2 MIC after 24 hours inhibited the biofilm formation by an average of 54% (figure 1) ($p < 0.001$).

Measuring the expression level of biofilm related genes: Isolates of *P. aeruginosa* with strong biofilms were treated with ZnO-NPs at concentration of 1/2 MIC to evaluate *algD, lecA and lecB* genes expression by Real Time PCR. In this study, the expression level of biofilm genes was compared between untreated and treated isolates. As shown in figure 2, the expression ratio of *algD* decreased after ZnO-NPs treatment. Therefore, bacterial cells treated with nanoparticles at a concentration of 1/2 MIC showed 3.3-fold decrease in the expression levels of *algD* gene ($p < 0.001$). As shown in figure 3, the changes in LecA gene expression of *P. aeruginosa* after treatment with ZnO-NPs showed a decrease of 1.81- fold compared to the control group ($P < 0.05$). Also, it can be shown that the effect of zinc oxide on the expression of the *LecA* gene was less than that of the *algD* gene. Moreover, as shown in figure 4, the expression of *LecB* gene decreased after treatment with nanoparticles compared to the control group. So that, the bacteria that were exposed to nanoparticles showed a 3.1-fold decrease in gene expression ($p < 0.05$). ZnO-NPs had the most impact on lowering *algD* gene expression out of the three genes under study.

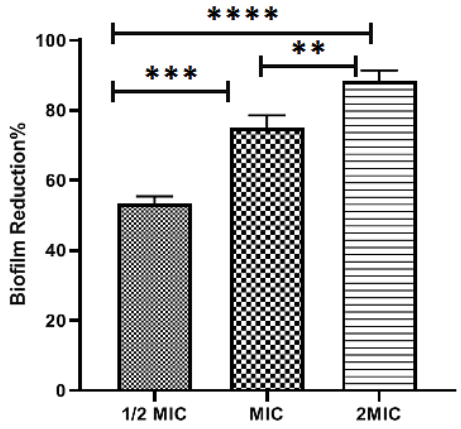

Figure 1. The activity of ZnO nanoparticles (concentration of ½ MIC, MIC and 2 MIC) against 24-hour preformed *Pseudomonas aeruginosa* biofilms

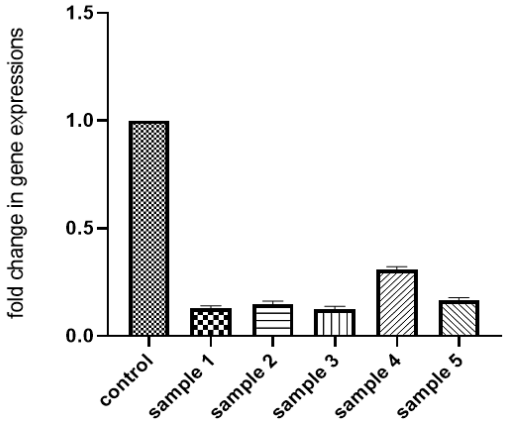

Figure 2. The effect of ZnO nanoparticles (concentration of ½ MIC) on the biofilm-related gene *algD*

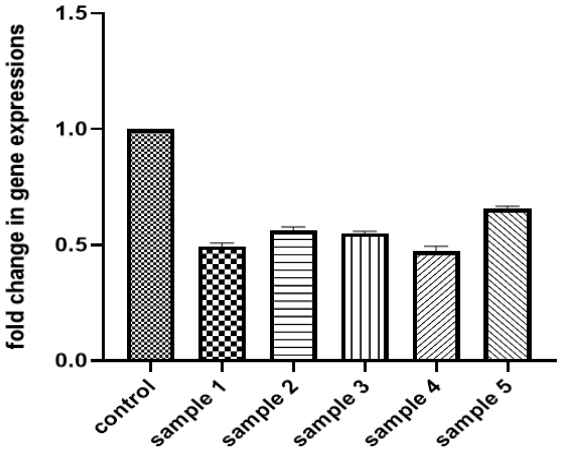

Figure 3. The effect of ZnO nanoparticles (concentration of ½ MIC) on the biofilm-related gene *lecA*

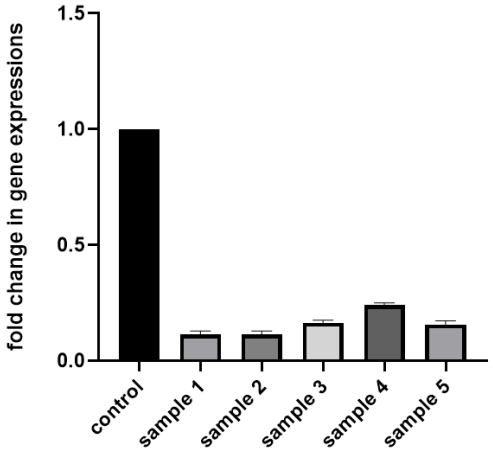

Figure 4. The effect of ZnO nanoparticles (concentration of ½ MIC) on the biofilm-related gene *lecB*

Discussion

Biofilm development is a distinctive feature of *P. aeruginosa*, a major opportunistic bacterial pathogen that has the ability to colonize surfaces. From a clinical perspective, biofilms significantly contribute to persistent and chronic infections by diminishing the immune response and the effectiveness of antibacterial treatments (18, 19). Exploring new drugs that specifically target biofilms can lead to finding the new innovate strategies to manage infections (20). The rate of biofilm formation among *P. aeruginosa* isolates from patients across various sites in Iran ranged from 43.5% to 99.5%, with an overall average of 86.5%. In recent years, several methods have been developed to eliminate biofilms. Certain nanoparticles, including ~~zinc~~ Zn and ZnO-NPs, exhibit antibactericidal properties and significant therapeutic effectiveness in targeting biofilm-producing bacteria. (21). In the current study, we present that the MIC of this compound against *P. aeruginosa* strains is 625 µg/ml. The precise mechanism of its antibacterial activity is not completely understood, and various other factors may also play a role in its antibacterial effects. Numerous studies have demonstrated the antibacterial activity of ZnO-NPs. Pati et al. demonstrated that ZnO-NPs can destroy the integrity of bacterial cell membranes, decrease cell surface hydrophobicity, and down-regulate the expression of genes associated with oxidative stress resistance in bacteria (22). In a study conducted by Valadebigi in 2023, the effects of ~~zinc~~ Zn nanoparticles on *Pseudomonas* biofilms were examined. The results indicated that zinc oxide nanoparticles at concentrations below 800 µg/ml were effective in preventing the formation of biofilms in *Pseudomonas* strains (17). Hassani et al. investigated the antibacterial and anti-biofilm properties of ZnO-NPs against clinical isolates

of *P. aeruginosa*. They found that the MIC50 and MIC90 for the isolates they studied were 150 µg/ml and 175 µg/ml, respectively. They noted ZnO-NPs exhibited an anti-biofilm effect at concentrations ranging from 50 to 350 µg/ml (23). In a study by Saadat et al., the effectiveness of ZnO-NPs (size ranging from 30 to 90 nm) against *P. aeruginosa* was reported at a concentration of 300 µg/ml (24). In Abdelraheem's study, ZnO-NPs were shown to effectively inhibit the growth of *P. aeruginosa* and limit biofilm formation. The antibacterial and anti-biofilm effects increased progressively with higher concentrations of ZnO-NPs. The MIC50 and MIC90 for the studied isolates were 64 µg/ml and 128 µg/ml, respectively (25). In this study, we investigated the ability of *P. aeruginosa* isolated from hospitalized patients in Kashan hospitals to form biofilms and assessed the impact of ZnO-NPs on biofilm formation by *P. aeruginosa*. In the present study sub-MIC levels of ZnO-NPs demonstrated antibiofilm activity against biofilm-producing *P. aeruginosa*. We compared the expression of biofilm-related genes between untreated and treated samples using RT-PCR. The results indicated that ZnO-NPs, by reducing *alg*D expression, significantly inhibit biofilm formation in *P. aeruginosa* isolates. According to the findings of this study, we identified a correlation between the degrees of biofilm formation, the expression of the *alg*D gene, and the levels of ZnO-NPs. Reduced expression of *alg*D was associated with a decrease in biofilm production. Thus, ZnO-NPs with reduced *alg*D expression can significantly inhibit the biofilm formation of *P. aeruginosa*. In another study, *P. aeruginosa* strains treated with ZnO@Glu-TSC, thymol, and a combination of ZnO@Glu-TSC and thymol exhibited reductions in *alg*D expression by 2.5, 4.3, and 2.2 folds, respectively (26). The two bacterial lectins, LecA and LecB, are virulence factors potentially associated with biofilm formation in *P. aeruginosa (27).* The present study indicates that ZnO-NPs reduce biofilm formation in *P. aeruginosa* and decrease the expression of the lectins LecA and LecB, as well as AlgD, in these bacteria. In Abdelraheem's study, ZnO-NPs significantly down-regulated the expression of the *lec*A gene in biofilm-forming clinical isolates of *P. aeruginosa*, showing a 4.7-fold change ($p < 0.0004$). According to our results, the *lec*A gene expression of *P. aeruginos*a after treatment with ZnO-NPs showed an average decrease of 1.8-fold. The strength of the present study was the evaluation of the effect of nanoparticles on clinical isolates. A limitation of the study was the small number of clinical isolates. It is better to evaluate the effect of ZnO-NPs on a larger number of clinical isolates.

In the current study, we reported that the ZnO-NPs can act as a suitable tool for treatment of infection-related biofilms. This nanoplatform, by reducing the expression levels of the *algD*, *lecA*, and *lec*B genes in *P. aeruginosa*, can be utilized as a novel therapeutic approach to improve and treat infection-related biofilms. Overall, the excellent physicochemical properties and remarkable biological activities of ZnO-NPs underscore the significance of our designs in developing new antibacterial agents to eradicate infection-related biofilms.

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Conflict of interests: The authors declared that they have no competing interest.

Authors' contribution: Ali Nazari-Alam and Ali Sobhani Nasab designed the study. Reyhaneh Rezaei Ghamsari acquired the data. Mohammad Shayestehpour analyzed and interpreted the data.

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