

Novel Combinations of Synthesized ZnO NPs and Ceftazidime: Evaluation of their Activity against Standards and New Clinically Isolated *Pseudomonas aeruginosa*

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Abstract

Background: Antibiotic resistant bacteria can be considered as a main problem in infection management. Zinc oxide nanoparticles (ZnO NPs), individually or in combination with antibiotics, can be considered as good candidates for struggling against drug resistant bacteria.

Methods: In this study, Zinc oxide nanoparticles were synthesized using sol-gel method in low temperature as a cost effective procedure and characterized by X-ray diffraction and Scanning Electron Microscopy. Antibacterial activity of 9 new combinations of Zinc oxide nanoparticles and ceftazidime was assessed against standards and new clinically isolated multi drug resistant *Pseudomonas aeruginosa* (*P. aeruginosa*), in order to evaluate enhancement effect of synthesized Zinc oxide nanoparticles on antibacterial activity of ceftazidime.

Results: The results indicated that desirable effects can be seen at 6 and 7 mM of Zinc oxide nanoparticles (60 to 100% inhibition). Moreover, after evaluation of 9 new combinations with various concentrations of both components, it was demonstrated that Zinc oxide nanoparticles can enhance the antibacterial activity of ceftazidime, against some bacterial strains of *P. aeruginosa*. The highest activity was observed with the concentration of 20 µg/ml ceftazidime in the presence of 5, 6 or 7 mM of Zinc oxide nanoparticles.

Conclusion: Zinc oxide nanoparticles in appropriate concentrations can be proposed as new and promising candidates for overcoming bacterial resistance.

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Key words: Antibiotic resistance, Ceftazidime, *Pseudomonas aeruginosa*, ZnO NPs

Introduction

Due to the rapid development in generation of antibiotic resistant bacteria, providing new antibacterial drugs or combination of different agents is urgently needed. In this field, metal nanoparticles (such as Ag, Au, Zn and etc.) have attracted the attention of several researchers because of their desirable antibacterial effects^{1,2}. Different characteristics of Zinc oxide nanoparticles (ZnO NPs) such as antibacterial and antiviral activity, photocatalytic activity, high chemical constancy, wide range of radiation absorption, low toxicity and biocompatibility make them good candidates for their use in food, cosmetics and pharmaceutical products³⁻⁵. In addition to distinct antimicrobial activity of ZnO particles, they can accelerate wound healing and prevent inflammation and itching, so their use in der-

matological products like wound dressings is very useful⁶.

Different methods such as sol-gel processing⁷, homogeneous precipitation⁸, mechanical milling⁹, organometallic synthesis¹⁰, microwave method¹¹, spray pyrolysis¹², thermal evaporation¹³ and mechanochemical synthesis¹⁴ can be used for synthesis of ZnO nanoparticles. Among these methods, sol-gel is more practical because it is a simple and cost effective process which leads to pure nano ZnO synthesis¹⁵.

Ceftazidime is one of the third-generation of cephalosporins, which can lead to development of Extended-Spectrum Beta-Lactamases (ESBLs) in gram negative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*). Production of ESBL generates resistant bac-

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teria and so causes a lot of complications in infection management¹⁶.

Previously, antimicrobial activity of commercial zinc and titanium dioxide nanoparticles were tested against two metallo beta-lactamase and biofilm producing isolates of *P. aeruginosa*. Both nanoparticles showed appreciable activity at all tested concentrations⁵. Recently, TiO₂ nanoparticles in combination with ceftazidime and cefotaxime were used in another study against multi-drug-resistant nosocomial *P. aeruginosa*. Obtained results showed that these metal nanoparticles can significantly enhance antimicrobial activity of ceftazidime¹⁷. In this study, antibacterial activities of ZnO nanoparticles (which were synthesized using sol-gel method) were tested individually or in combination with ceftazidime against different standards and MDR isolates of *P. aeruginosa* to evaluate the possible enhancement of antibacterial effect of ceftazidime. Considering that all *P. aeruginosa* isolates used in this study were ceftazidime resistant, an attempt was made to use and optimize the simply synthesized ZnO nanoparticles for overcoming their resistance for the first time.

Materials and Methods

Materials

Bacterial strains: Standard strains of *P. aeruginosa* ATCC27853, PAO1 and MH873 (which is a mucoid strain isolated from a patient with cystic fibrosis in Copenhagen Denmark as a kind gift from Prof. Nail Hobej, three isolates from clinical samples exhibiting MDR phenotype resistant to three classes of antibiotics aminoglycosides, cephalosporins and quinolones (Res 1, Res2 and Res3) were used in this study.

Chemicals

Zinc acetate dihydrate Zn (CH₃COO)₂·2H₂O, ammonia (NH₃, M=17) and tritonx-100 were obtained from Sigma Chemical Co, USA. Tryptic soy broth and agar (casein soya bean digest broth and agar from OXOID, Germany) were used as bacterial growth media.

Methods

Preparation of ZnO nanoparticles: A solution containing 0.2 M zinc acetate was made in deionized water. 25 ml of this solution was poured into a flask and placed on a magnetic stirrer (Staffordshire, ST15 OSA, UK). Then, 4.5 ml triton x-100 was added and incubated at room temperature overnight in order to form a stable suspension at neutral pH. In the next step, ammonia solution (2.5% v/v) was added dropwise, until reaching the pH of solution up to 9 and then, the solution was stirred overnight, to form the complete sediment. The sediment was separated by centrifuge (Eppendorf AG 22331 Hamburg, Germany) at 18514×g for 15 min. The sediment was washed twice with deionised water and absolute ethanol, respectively, and then was placed on a 60°C oven to dry. Dried sediment was calcined in a furnace at 400 °C for 1 hr¹⁵.

Characterization of ZnO nanoparticles

Morphology evaluation: The structure and surface characteristics of nanoparticles were visualized by Scanning Electron Microscopy (SEM Hitachi, S4160, Tokyo, Japan). The nanoparticles were sputter-coated with gold for 10 min at 6 mA and 6 kV (DC) under argon gas and were observed for morphology at an acceleration voltage of 15 kV. Particle size diameter was determined using CLEMEX® particle image analysis software package 4.7.

X-ray diffraction analysis: For identification of crystallization process, phase characterization and determination of nanoparticle size, X-ray diffraction (XRD) (D₄-BRUKER and Cu- α radiation at 30 kV and 20 mA) of ZnO samples was carried out.

Antimicrobial activity evaluations

Different concentrations of ZnO (5, 6 and 7 mM) were subjected to antimicrobial activity tests using colony counting method. For testing the bacterial growth behavior after exposure to different concentrations of ZnO nanoparticles, fresh colonies of each bacterium were grown in 3 ml Tryptic Soy Broth (TSB) in shaker incubator at 37 °C overnight. Then, bacterial cells were collected by centrifugation at 1157×g for 10 min at 4 °C and the bacterial pellet was washed three times with 3 ml of sterile Phosphate Buffered Saline (PBS) pH=7.4 and resuspended in 3 ml of PBS. Turbidity of suspension was adjusted to 0.5 McFarland standard (OD₆₀₀=0.08-0.13), and the required cell density corresponding to ~10⁸ Colony Forming Unit per milliliter (CFU/ml) was prepared. Bacterial suspension was further diluted and 100 μ l of 10⁻³ dilution of bacterial suspension was added to vials containing different concentrations of ZnO nanoparticles. Broth media without bacterial inoculation containing various concentrations (5-7 mM) of ZnO NPs were used as controls. All vials were incubated at 35 °C and then aliquots of 10 μ l bacterial broth were sampled from each vial after 18 hr¹⁷. The colony forming the unit was counted and compared with controls. Inhibitory rate of nanoparticles was calculated using equation (1):

Eq (1):

Inhibitory rate of bacterial growth=100*(CFU of control-CFU of sample)/(CFU of control)

Enhancement effect evaluation

Enhancement effect of ZnO nanoparticles on ceftazidime activity was studied by turbidity measurement using ELISA reader (Authos 2020-Austria). For evaluation of antimicrobial activity of nanoparticles, and their enhancement with ceftazidime, 40 μ l of different concentrations of ceftazidime (8, 20 and 32 μ g/ml) and ZnO nanoparticles (5,6 and 7 mM) and their combinations (Table 1) were added to each well using sterile 96 well plates. Then, 200 μ l of broth media was added to each well and 40 μ l of bacterial suspension (standard or isolated MDR *P. aeurogenosa*) was cultured in wells. Optical density of each well was measured be-

Table 1. Different combinations of ceftazidime and ZnO nanoparticles

Combination	Final concentration of each compound in the combination	
	ZnO NPs (mM)	Ceftazidime (µg/ml)
A	5	-
B	6	-
C	7	-
D	-	8
E	-	20
F	-	32
G	5	32
H	5	20
I	5	8
J	6	32
K	6	20
L	6	8
M	7	32
N	7	20
O	7	8

Table 2. X-ray analysis of synthesized ZnO NPs

2θ(°C)	D (Å)	Height
36.252	2.575	100.00
31.769	2.814	47.63
34.421	2.603	58.80

"d": The distance between crystalline surfaces
 "θ": diffraction angle of the peak

fore and after treatment. Wells without nanoparticle and/or drug were considered as controls. Plate was incubated at 37 °C for 18 hr. Optical density of each well was measured by ELISA reader at 630 nm⁵.

Statistical analysis

Data were expressed as mean±standard error. Statistical analysis was performed using Student t-test and the significance level was defined as p<0.05. Data were analyzed by GraphPad Prism statistical software (version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Morphology evaluation

The ZnO nanoparticles were gold-covered and characterized by SEM to investigate their structures and surface characteristics. It was revealed that particles were spherical and nanoparticles were homogeneous with the size of 30 nm approximately (Figure 1A).

XRD analysis

ZnO nanoparticles revealed the hexagonal wurtzite structure and the peaks were indexed according to JCPDS card No.36-1451/19-1458, peaks were observ-

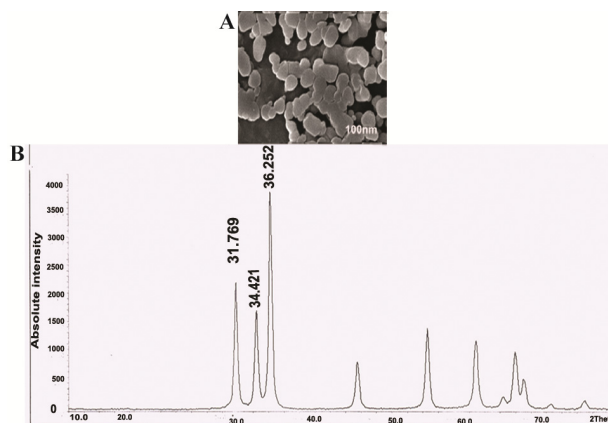


Figure 1. A) SEM image of ZnO nanoparticles (acceleration voltage of 15 kv, CLEMEX® 391 particle image analysis software package 4.7.). B) XRD pattern of ZnO nanoparticles synthesized 392 by sol-gel method (D4-BRUKER and Cu-κ radiation at 30 kv and 20 A.

ed at 2θ of 36.252°, 31.769 and 34.421 which were indexed as (1 0 1),(1 0 0) and (0 0 2) diffraction planes, respectively (Figure 1B and Table 2). The average crystallite size from XRD was calculated from X-ray line broadening of the (1 0 1) diffraction line using the Scherrer equation (2), where d is the grain size, λ is the wavelength of the X-ray (Cu Kα, 0.15418 nm), β is the full-width at the half-height of the peak, and θ is the diffraction angle of the peak. Our results clearly indicated the main peak caused by ZnO NPs with high purity. The size of calculated nanoparticles was 30-40 nm at 400 °C.

Antimicrobial activity evaluations

The bactericidal activity of ZnO NPs was tested against standard and isolated bacteria by turbidity measurement. The results of our study showed that all concentrations of ZnO nanoparticles in size of 30-40 nm can inhibit the bacterial growth (standards and isolated MDR *P. aeruginosa*) between approximately 30-100% (Figure 2 B).

Enhancement effect evaluation

Different combinations of ZnO nanoparticles and ceftazidime (as indicated in Table1) were tested on PAO1 and Res1 *P. aeruginosa*. Based on synthesis and evaluation of three concentrations of ZnO nanoparticles as described above, in this step, three concentrations of ceftazidim with ZnO NPs were combined sequentially. Combination of the highest concentration of ZnO Np (7 mM) and 32 µg/ml ceftazidime (combination M), enhanced inhibition effect in comparison to individual ceftazidime on resistant bacteria (Res1) significantly (approximately 30%) (Figure 3). In combinations J and G in which the concentration of nanoparticles was 6 and 5 mM, respectively, the inhibition effect significantly decreased as well.

$$Eq(2): \left[d = \frac{0.9\lambda}{\beta \cos\theta} \right]$$

ZnO Nanoparticles and its Enhancement Effect on Ceftazidime Activity

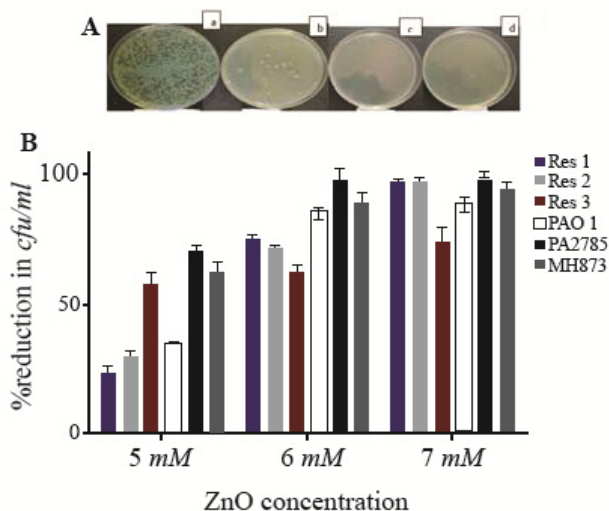


Figure 2. A) Growth of different isolated of *P. aeruginosa* after 18 hr exposure to 7 mM of 399 ZnO NPs. a: Control (without NPs), MDR isolated strains b: Res 1, c: Res 2 and d: Res3. B) 400 Inhibitory rate (%) of different concentrations of ZnO NPs against standards (PAO1, 401 ATCC27853, MH873) and 3 MDR isolates of *P. aeruginosa* indicated in text after 18 hr 402 exposure to ZnO NPs measured by colony count method (B).

In the next step, the evaluations using the lower concentration of ceftazidime (20 $\mu\text{g/ml}$) were continued on resistant bacteria. In all combinations including H, K and N (ZnO NPs=5, 6 and 7 mM respectively), the inhibitory effects significantly increased compared to pure antibiotics (20 $\mu\text{g/ml}$) (Figure 2). However, there was no increase in antibacterial activity of combinations when the concentration of ceftazidime was 8 $\mu\text{g/ml}$.

Discussion

ZnO NPs were synthesized by sol-gel as an affordable high performance method with the size of approximately 100 nm. Obtained results indicated that these nanoparticles can be effective on some standards and MDR *P. aeruginosa* used in this study. Moreover, some combinations of ceftazidime and ZnO NPs could incredibly inhibit the growth of ceftazidime resistant bacteria.

There was a direct relationship between the inhibitory effect and concentration of ZnO NPs against both standard and resistant bacteria. Maximum inhibition (near to 100%) was demonstrated at concentration of 7 mM of ZnO NPs against resistant strains (Res1, Res2) (Figure 2A). Nanoparticles with concentration of 6 mM, caused growth inhibition about 60% in all of the three isolated resistant bacteria. Moreover, the growth of standard bacteria in the presence of 6 and 7 mM ZnO NPs was apparently inhibited more than 80%. These results are in good agreement with the results obtained by other researchers^{6,17}.

Other studies showed that ZnO NPs with the size of 30 nm and the concentration of 6 mM can inhibit the growth of methicillin resistance *Staphylococcus aureus*

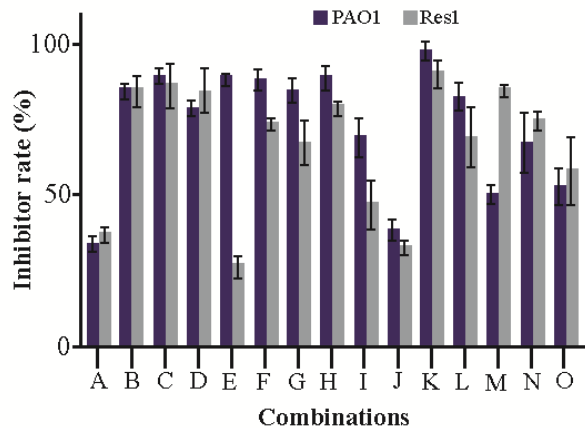
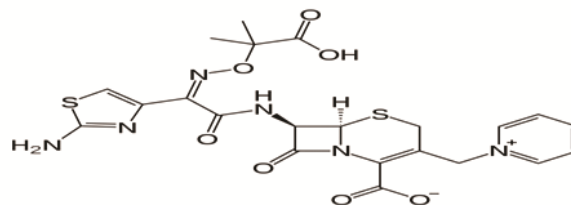


Figure 3. Inhibitory rate (%) of different combinations (according to Table 1) against PAO1 and 406 resistance isolated *P. aeruginosa* (Res1) after 18 hr exposure.



Scheme 1, Ceftazidime structure.

(MRSA), *Escherichia coli*, and many other microorganisms¹⁷⁻¹⁹.

Our results express the effectiveness of ZnO nanoparticles on both standard and isolated MDR *P. aeruginosa* at 6 and 7 mM concentrations. Different mechanisms of antibacterial effect of nano ZnO has been evaluated by some investigators²⁰. Some results showed that nanoparticles are more effective on gram positive bacteria than gram negative ones^{21,22}. However, our results indicated that ZnO nanoparticles have desirable effects on *P. aeruginosa* as a gram negative bacterium.

Generally, two different mechanisms have been proposed for this effect:

1- Producing Radical Oxygen Species (ROS) by ZnO as a semiconductor with a wide band gap²³⁻²⁶. If ZnO radiates with photon energies higher than its band gap, electrons move from the valence band to the conduction band of the particle. So, a positive hole in the valence band and a free electron in the conduction band are produced. These positive holes at the surface of nanoparticles react with hydroxyl groups and create hydroxyl radicals by absorbing the water. Moreover, free electron in the conduction band creates a superoxide ion in the presence of oxygen, which can become a hydroxyl radical as well. Therefore, the generation of all derivatives of active oxygen is considered important which can destroy the bacterial cell^{25,27}.

This hypothesis is confirmed by Xie *et al* who showed that the level of oxidative stress genes in-

creased dramatically after bacterial exposure to ZnO nanoparticles. Their results indicated that the expression of KatA in bacteria, a single catalase enzyme whose expression level increases in exposure to H₂O₂, is about 52-fold higher than normal level after treating with ZnO NPs²⁸. Moreover, this ROS can obliterate bacterial cell by membrane lipid molecules peroxidation as well²⁹.

2- Another possible mechanism is the interaction of ZnO nanoparticles with thiol group of bacterial cell membrane proteins which ultimately leads to cell death³⁰.

As mentioned before, some combinations of ZnO NPs and ceftazidime could inhibit growth of ceftazidime resistant bacteria. Antimicrobial activity of different combinations compared to their related pure groups (ZnO NPs and ceftazidime individually) against resistance isolated *P. aeruginosa* revealed that four combinations (H, K, M, N) have significantly more inhibitory rate in comparison to ceftazidime alone ($p < 0.005$). Two combinations (G, H) have higher inhibitory rate in comparison to pure ZnO NPs ($p < 0.006$). The difference between combinations M or N and the ZnO NPs alone were not significant ($p > 0.05$).

Ceftazidime, a betalactam-antibacterial agent from third generation of cephalosporins with bactericidal activity, has three active moieties in its structure (Scheme 1). Aminothiazole ring and propyl carboxy group at position 7 are responsible for increasing affinity to penicillin binding proteins of gram negative bacilli. Another active part of ceftazidime molecule is a pyridine group at position 3 which leads to rapid intrabacterial penetration. Similar to fluoroquinolones which were demonstrated earlier³⁰, it seems that hydroxylated surface of ZnO nanoparticles can interact with positively charged pyridinium ring nitrogen at position 3 and make a network between two molecules. This ionic network can stabilize ceftazidime molecule and enhance its antimicrobial effect. Another possible mechanism is the interaction of Zn, as a metal chelating ion, with the propylcarboxy group (at position 7) of ceftazidime and making a stable Zn-antibiotic nanoparticle complex^{31,32}. Moreover, interference of ZnO NPs with the bacterial cell membrane can facilitate the entrance of ceftazidime into the bacterial cell and increase its effect³³. These three mechanisms may be responsible for the antibacterial activity enhancement of ceftazidime in presence of ZnO nanoparticles in some concentrations.

Antibacterial evaluations of different combinations on standard strain, PAO1, showed that only two combinations (K and L) have more inhibitory effects compared to ceftazidime alone (Figure 3). Considering that the standard bacteria are susceptible to ceftazidime and this antibiotic has high inhibitory effect on them, it is expected that most of the combinations have no highlighted effects compared to ceftazidime. The effectiveness of combinations H and I was not significantly dif-

ferent from ceftazidime, and this was also true for the combinations L and N in comparison with ZnO NPs. However, four combinations (G, H, I and K) had higher inhibitory effects compared to ZnO NPs against PAO1 ($p < 0.001$). This result could be due to the great antibacterial activity of ceftazidime on PAO1 which leads to overall high effects in combinations.

Conclusion

ZnO nanoparticles with size of 30-40 nm were successfully synthesized using sol-gel as a simple and cost effective method. These nanoparticles were highly effective against both standard and isolated MDR *P. aeruginosa* in concentrations of 6 and 7 mM. Moreover, combination of ZnO nanoparticles with ceftazidime increased anti bacterial activity of ceftazidime in different concentrations. Even though the increased inhibition effects were seen when the ceftazidime was 20 µg/ml in presence of 5, 6 and 7 mM of ZnO NPs (compared to antibiotic only), the best and more effective combination was formula "H" which included ZnO 5 mM and ceftazidime 20 µg/ml. This combination showed significant higher inhibitory effect compared to both nanoparticles and ceftazidime alone against MDR *P. aeruginosa*. Taken together, it can be concluded that the use of ZnO nanoparticles can open a novel vision for inhibition of microbial resistance even alone or in combination with antibiotics. However, this finding requires more investigations on various resistant bacteria, *in vitro* and *in vivo*.

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