

Antibacterial Efficacy of Linezolid Alone and in Combination with Zinc Oxide Nanoparticles against Methicillin-Resistant *S. Aureus* Clinical Isolates

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Abstract

Skin and soft tissue infections caused by methicillin-resistant *S. aureus* (MRSA) are among the most common bacterial infections. Linezolid is a cortisone drug for the treatment of infections caused by MRSA. However, developing resistance to linezolid creates a hurdle in the treatment of these infections. The present study aimed to determine the activity of linezolid alone and in combination with zinc oxide nanoparticles (ZnO-NPs) for the purpose of reducing resistance and enhancing its efficacy. For this study, MRSA isolates were taken and confirmed by using the antibiotic susceptibility testing method. The minimum inhibitory concentration (MIC) of both antibiotics and nanoparticles against MRSA clinical isolates was done by using the broth microdilution method. A checkerboard assay has used the determination of the combined activity of linezolid and ZnO-NPs. ZnO-NPs displayed a spherical shape with smooth surface morphology and had a mean size of 10 nm to 20 nm, with a zeta potential of 3.57 mV. The activity of ZnO-NPs against MRSA clinical isolates was 200 µg/ml. Almost 81% of isolates were found sensitive to linezolid with MIC lower than 4 µg/ml, and 19% were resistant, having MIC greater than 4 µg/ml. The combination of an antibiotic and nanoparticles reduced the activity of each of them twofold. The current study revealed that both linezolid and ZnO-NPs have antimicrobial activity against MRSA when used alone. The combination of both medications reduces each other's MIC twofold and has an antagonistic impact. Further research is needed to determine the mechanism through which these medications inhibit each other's activity. (International Journal of Biomedicine. 2022;12(3):454-458.).

Keywords: nanoparticles • linezolid • zinc oxide • *S. aureus* • tissue infections

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Abbreviations

BMDM, broth microdilution method; **CLSL**, Clinical Laboratory Standard Institute; **FIC**, fractional inhibitory concentration; **LRSA**, linezolid-resistant *S. aureus*; **MIC**, minimum inhibitory concentration; **MRSA**, methicillin-resistant *S. aureus*; **MSSA**, methicillin-sensitive *S. aureus*; **SSTIs**, skin and soft tissue infections; **ZnO-NPs**, zinc oxide nanoparticles.

Introduction

Staphylococcus aureus can cause severe life-threatening infections that affect millions of people every year.⁽¹⁾ Among the infections, skin and soft tissue infections (SSTIs) are commonly caused by community-associated MRSA (CA-MRSA), less commonly by healthcare-associated MRSA (HA-MRSA), and rarely by methicillin-sensitive *S. aureus* (MSSA).⁽²⁾ MRSA is currently the most commonly known antibiotic-resistant pathogen around the globe, including East Asia, North Africa, the Middle East, Europe, and the Americas.⁽³⁾

Linezolid belongs to the oxazolidinone class of antibiotics approved by the FDA in 2000.⁽⁴⁾ Linezolid is used to treat infections, including SSTIs, pneumonia, and osteomyelitis, caused by MRSA.⁽⁵⁾ The drug binds to the larger 50S subunit of the bacterial ribosome and inhibits its attachment with the 30S subunit. This results from preventing the initiation complex and inhibiting the translation of mRNA.⁽⁶⁾ According to global studies reports and data from the USA, 1% of *Staphylococcus aureus* and 2% of coagulase-negative *S. aureus* produce resistance to linezolid.⁽⁷⁾ Until now multifocal outbreaks of linezolid-resistant *S. aureus* (LRSA) have been reported, determining that linezolid resistance may occur both by the vertical and horizontal transmission of genes.⁽⁸⁾

With the limited options available for therapeutic remedies due to the increase in resistance, the focus has now been directed toward alternative treatment options against such antibiotic-resistant pathogens.⁽⁹⁾ At present, inorganic metal oxides, like calcium oxide (CaO), magnesium oxide (MgO), titanium oxide (TiO₂), and zinc oxide (ZnO), have gathered attention due to their potential as antimicrobial, anticancer, and antiprotzoal agents, owing to their stability and safety.⁽¹⁰⁾

ZnO has been referred to as “generally recognized as safe” by the FDA in the US.⁽¹¹⁾ The ZnO-NPs shape, size, surface state, crystal structure, and dispensability contribute to their medicinal applications.⁽¹²⁾ The mechanism of ZnO-NP antimicrobial activity involves the generation of hydrogen peroxide and the release of Zn²⁺ ions.⁽¹³⁾ Hydrogen peroxide generated on the surface of ZnO can penetrate the bacterial cells and effectively inhibit cell growth.⁽¹⁴⁾ A previous study revealed that antibiotics when used with nanoparticles will further increase the antimicrobial activity of the antibiotics manifold.⁽¹⁵⁾ However, few studies have addressed the use of a combination of metal nanoparticles and, especially, ZnO with antibiotics against bacteria.⁽¹⁶⁾ Currently, extensive studies are becoming available on using silver nanoparticles combined with different antibiotics against both gram-positive and gram-negative bacteria.^(17,18)

Thus, the present study was designed to evaluate the anti-staphylococcal activity of nanoparticles alone and in combination with antibiotics toward the development of a new biocidal combination against pathogens.

Materials and Methods

Study design and sample collection

The study was carried out in the Department of Microbiology at the Institute of Basic Medical Sciences,

Khyber Medical University Peshawar, Pakistan. A total of 100 clinical isolates of MRSA were collected from August to October 2021 from Combined Military Hospital, Hayat Abad Medical Complex, and North West General Hospital.

Culturing and biochemical testing

The clinical isolates were inoculated on nutrient agar, mannitol salt agar, and blood agar, and the isolates were identified by colony morphology, gram staining, coagulase, and DNase test. MRSA was confirmed by the agar diffusion method (Kirby-Bauer) using a 30 µg cefoxitin disc recommended by Clinical Laboratory Standard Institute (CLSI).

Zinc oxide nanoparticle synthesis

ZnO-NPs were synthesized by the wet chemical method; zinc acetate dehydrates (C₄H₆O₄Zn. H₂O₂) and sodium hydroxide (NaOH) (Sigma-Aldrich, St Louis, MO, USA) were used. In this experiment, a large beaker of water was heated to 65°C; meanwhile, we dissolved 0.10g Zn in 25 mL isopropanol by heating in a fume hood for about 15 minutes. To chill 125 ml isopropanol the flask was placed in an ice bath. After complete dissolution of zinc acetate, 125 ml isopropanol was added to it. Chilled 15 ml of 0.050 M NaOH was added to the solution slowly. The flask with the mixed solution was placed in the 65°C water bath for 5-10 minutes.

Characterization of ZnO-NPs

To confirm NP synthesis and to study size and surface morphology, samples were taken for microscopy in screw-cap tubes containing PBS buffer and were transported to the department of physics at Peshawar university. The accelerating voltage used was 20 kV with a magnification of ×20,000 to ×45,000. The surface charge of the zinc oxide nanoparticle was determined by using the Malvern zeta sizer machine. ZnO-NPs were dissolved in deionized water, and the zeta potential charge was analyzed by PSS Nicomp380 ZLS particles sizer.

Determination of the activity of linezolid and nanoparticles

MRSA isolates were sub-cultured on blood agar, and after overnight incubation at 37°C, three to five morphologically similar colonies were emulsified in sterile isotonic saline. The suspension was adjusted to 0.5 McFarland standards (106 CFU/ml). To determine the activity of linezolid and ZnO-NPs, BMDM was used. Linezolid powder (Sigma-Aldrich Co) was purchased and a stock solution was made. Different concentrations of linezolid (0.6125-128 µg/ml) and ZnO-NPs (40-1000 µg/ml) were incorporated into a 96-well micro titer plate containing MHB media and inoculum. The plates were then placed in a shaking incubator for 24h at 37°C and 200 rpm.

Checkboard assay

To detect the activity of linezolid alone and in combination with ZnO-NPs, a broth microdilution checkboard assay was used. The first antibiotic of the combination was serially diluted along the ordinate, while the second drug was diluted along the abscissa.

MTT assay

The MIC of the clinical isolates was read by using an MTT assay. The MTT reagent at a concentration of 10 µl was added to each well after 24 hours of incubation at 37°C. The plate was wrapped in aluminum foil after adding MTT dye as this dye is

light sensitive, and the plate was further incubated for 4 hours at 35°C. After 4 hours of incubation, the plate was centrifuged at 3000 rpm for 3 minutes. The supernatant was removed from all wells and was diluted with 100 µl of DMSO for the purpose of stopping further enzymatic reactions. The plate was incubated again for 1 hour in a shaking incubator at 37°C. The absorbance was checked at an optical density of 570 nm by a microplate reader. As a positive control, media containing bacteria was added to wells without any drug. Negative control had only media and drugs but no bacteria were added. The result of MIC was interpreted according to the breakpoints given by CLSI 2017, where $MIC \leq 4$ mg/mL was taken as susceptible. An *S. aureus* ATCC 29213 reference strain was used as a control. Results were interpreted by using SPSS, and the quantities' variables, like MIC50 and MIC90, were calculated.

Results

Out of 100 clinical isolates, only 90 were found to be coagulase positive while 10 were found to be coagulase-negative *S. aureus*. All 90 clinical isolates were methicillin-resistant by using the cefoxitin 30 µg disc recommended by CLSI (Fig.1).



Fig. 1. *S. aureus* resistance to cefoxitin.

The efficacy of linezolid against MRSA was checked by BMDM, wherein out of a total of 90 isolates, 16.6% of MRSA isolates had MIC up to 0.5 µg/ml, 21% up to 1 µg/ml, 26.6% had 2 µg/ml, 14.4% had 4 µg/ml, 13.3% had 8 µg/ml, and 7.78% had MIC 16 µg/ml. According to the CLSI guidelines, the breakpoint of linezolid is 4 µg/ml. In this study, 81% of MRSA isolates were sensitive to linezolid and 19% of isolates were found linezolid resistant. ZnO-NPs were prepared by a wet chemical method. The size and morphology of ZnO-NPs were evaluated by scanning electron microscope (SEM). The nanoparticle displays a spherical shape with size ranging from 10 nm to 20 nm in diameter (Fig.2).

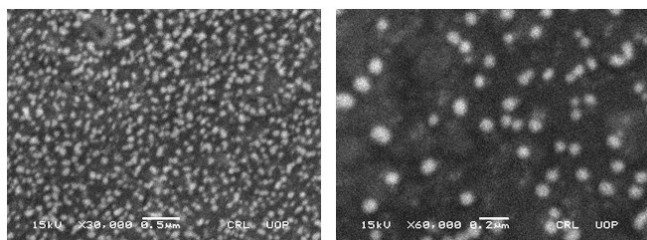


Fig. 2. SEM image of ZnO-NPs with size of 10 nm to 20 nm.

To analyze the zeta potential of ZnO-NPs, zeta sizers were used. Zeta potential plays important role in nanoparticle stability and coupling of the nanoparticle to biological surfaces. The zeta potential value of ZnO-NPs is 1.04 mV within an average size of 10 nm to 20 nm (Fig.3).

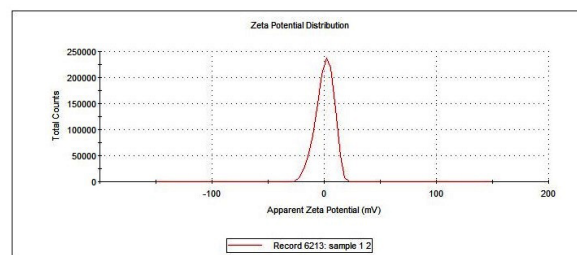


Fig. 3. Zeta potential value of ZnO-NPs (1.04 mV)

The activity of nanoparticles was performed on 90 MRSA clinical isolates (Fig.4). Results showed the minimum concentration of nanoparticles that inhibit the growth of MRSA was 200 µg/ml. To evaluate the effect of both antibiotics and nanoparticles, the FIC index was calculated.

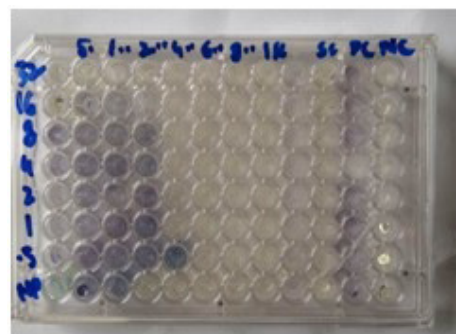


Fig. 4. Broth microdilution checkerboard assay: wells with color show growth while colorless show no growth.

$$FIC = \frac{MIC \text{ of Drug A in combination}}{MIC \text{ of Drug A alone}} + \frac{MIC \text{ of Drug B in combination}}{MIC \text{ of Drug B alone}}$$

$$FIC = \frac{32}{16} + \frac{600}{200}$$

$$\Sigma FIC = 5$$

The FIC index showed a strong antagonistic effect when nanoparticles and antibiotics were used in combination.

Discussion

MRSA is an important human pathogen responsible for causing both hospital- and community-associated infections.⁽¹⁹⁾ SSTIs caused by MRSA have a high frequency and can affect deep tissues, resulting in morbidity and mortality.⁽²⁰⁾ *S. aureus* is considered one of the major “ESKAPE pathogens” as it can acquire immediate resistance to new therapies.^(21,22) In the current study, 81% of MRSA isolates were found to be sensitive to linezolid and 19% of isolates were found to be linezolid

resistant. A similar study that was conducted at Fukuoka University Chikushi Hospital, Japan, from the year 2008 to 2012 found all MRSA isolates were sensitive to linezolid. According to that study, MRSA isolates with an MIC of 1 µg/ml decreased from 76.3% in 2008 to 35.4% in 2012. Those with a MIC >1 increased from 23.7% to 64.6% over time.⁽²³⁾ Another researcher used BMDM for 100 MRSA and 50 MSSA isolates and reported that all MRSAs were susceptible to linezolid.⁽²⁴⁾ Furthermore, another study conducted in the Peshawar region by Ahmad et al.⁽²⁵⁾ in 2014 reported 17% resistance to linezolid by using the same BMDM. The activity of nanoparticles was performed on 90 MRSA clinical isolates. Results showed the minimum concentration of nanoparticles that inhibit the growth of MRSA was 200 µg/ml.⁽²⁶⁾ Jesline et al.⁽²⁷⁾ used the disc diffusion method and reported the activity of ZnO-NPs against MRSA isolates at a concentration of 200 µg/ml and high activity of 500 µg/ml. Moreover, Aleaghi et al.⁽²⁸⁾ reported a minimum inhibitory concentration of ZnO-NPs at 625 µg/ml and the highest activity at 1250 µg/ml. The difference in the result is due to the size of the nanoparticles, which influences their activity, even when used in low concentrations.⁽²⁹⁾

The combined activity of both compounds was analyzed by using their fractional inhibitory concentration (FIC). Both antibiotics and ZnO-NPs were physically mixed at various concentrations. The FIC value indicates a strong antagonist effect when both compounds are used in combination. Jelinkova et al.⁽³⁰⁾ used the disc diffusion method for ZnO-NPs along with different antibiotics and reported no improvement in the activity of penicillin and cloxacillin when used with ZnO-NPs against *E. coli*. Moreover, Mohler et al.⁽³¹⁾ used a physical combination of ZnO-NPs and antibiotics using the same BMDM and reported a fourfold increase in the MICs of antibiotics. The difference in the result is that they used different antibiotics but not linezolid. Smekalova et al.⁽³²⁾ used BMDM to detect antimicrobial activity of antibiotics and silver nanoparticles alone and in combination against antibiotic-resistant pathogens, and reported the highest activity of gentamicin and penicillin G when used in combination with nanoparticles against *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*. No one has used linezolid in combination with ZnO-NPs. In conclusion, using nanoparticles and linezolid in combination was found to have no improved antibacterial activity.

Competing Interests

The authors declare that they have no competing interests.

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