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Topical application of zinc oxide nanoparticles reduces bacterial skin infection in mice and exhibits antibacterial activity by inducing oxidative stress response and cell membrane disintegration in macrophages

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Abstract

Here we studied immunological and antibacterial mechanisms of zinc oxide nanoparticles (ZnO-NPs) against human pathogens. ZnO-NPs showed more activity against *Staphylococcus aureus* and least against *Mycobacterium bovis*-BCG. However, BCG killing was significantly increased in synergy with antituberculous-drug rifampicin. Antibacterial mechanistic studies showed that ZnO-NPs disrupt bacterial cell membrane integrity, reduce cell surface hydrophobicity and down-regulate the transcription of oxidative stress-resistance genes in bacteria. ZnO-NP treatment also augmented the intracellular bacterial killing by inducing reactive oxygen species production and co-localization with *Mycobacterium smegmatis*-GFP in macrophages. Moreover, ZnO-NPs disrupted biofilm formation and inhibited hemolysis by hemolysin toxin producing *S. aureus*. Intradermal administration of ZnO-NPs significantly reduced the skin infection, bacterial load and inflammation in mice, and also improved infected skin architecture. We envision that this study offers novel insights into antimicrobial actions of ZnO-NPs and also demonstrates ZnO-NPs as a novel class of topical anti-infective agent for the treatment of skin infections.

Key words: Zinc oxide nanoparticles; Antibacterial; Biofilm; Pathogens; Cytotoxicity; Inflammation; Skin infection; Mice

Background

Treatment of bacterial infections has become a major challenge in the medical field. For many years the conventional antibiotic therapy has been critical in the fight against microbial infections. However, the disease-causing microbes that have become resistant to antibiotics are an increasing health problem.[1] It is mainly due to microbes that cause infections became remarkably resilient and has developed several ways to resist antibiotics. This obliges the scientific community to constantly design better therapeutic strategies, including new drugs. Recently applications of nanoparticles (NPs) have expanded considerably. NPs have been successfully used for the delivery of therapeutic agents,[2] in disease diagnostics,[3] to reduce bacterial infections in skin and burn wounds[4,5] and to prevent bacterial colonization on medical devices.[6] Because of their unique mode of action and potent antimicrobial activities against a spectrum of bacteria, the prospectus of development of new generation antibiotics makes NPs as an attractive alternative to antibiotics to overcome the drug resistance problem.

Many reports have been published on other biomedical applications; however, very limited information is available on the *in-vivo* antibacterial efficacy of metal oxide NPs, their ability to kill intracellular pathogens and mechanisms of action. Among the

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NPs, silver (Ag), gold (Au) and zinc oxide (ZnO) have been demonstrated with pronounced antibacterial activities. Out of them, the use of Ag and Au on industrial scale is limited due to their high cost. Therefore, current research focuses on ZnO as an antibacterial and immunomodulatory agent. In addition to their direct bactericidal activity, NPs are also known to disrupt biofilm formation, which augments resistance to drugs and aids pathogen to establish chronic infections[7] and modulate the secretion of cytokines.[8,9] Previously, we have shown that starch and chitosan-capped silver nanoparticles exert antibacterial activity against pathogens and also inhibit the biofilm formation without causing any cytotoxic and genotoxic effects on macrophages.[10]

ZnO is listed safe by the U.S. Food and Drug administration (21CFR182.8991). ZnO nanomaterials are used in various biological applications including drug delivery, bioimaging probes, and cancer treatment.[11-13] ZnO nano-size particles show more pronounced antimicrobial activities than large particles.[14] Although, ZnO nanoparticles (ZnO-NPs) have been shown with antibacterial activities, there is no comprehensive study on their antibacterial effect against Gram-positive, Gram-negative, mycobacteria and clinical drug resistant strains, mechanism of action and *in vivo* efficacy of ZnO-NPs to treat the bacterial infections in mice model. *Mycobacteria* and *Pseudomonas* are a leading cause of microbial airborne illness that can develop into as life-threatening disease called tuberculosis and chronic lung infection, respectively. *Staphylococcus* species are mainly responsible for skin infections.[15]

In this study, we synthesized ZnO-NPs using biopolymer starch as capping agent and investigated their immunological and antimicrobial properties against a panel of human pathogens and drug-resistant clinical isolates representing Gram-positive(*Staphylococcus aureus,* methicillin resistant *Staphylococcus aureus*), Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa)* and acid fast (*Mycobacterium smegmatis*, *Mycobacterium bovis*-BCG) bacteria. We also investigated the mechanism of antibacterial activity and feasibility of ZnO-NPs to treat skin infection caused by *S. aureus* in murine model. Gram-positive bacteria were found to be more susceptible to ZnO-NPs as compared to Gram-negative and acid fast bacteria. Among mycobacterial strains, *M. bovis*-BCG resisted the killing effect. However, ZnO-NPs exhibited effective killing of BCG in synergy with an anti-tuberculous drug rifampicin. Importantly, ZnO-NPs also killed clinical methicillin resistant *Staphylococcus aureus* (MRSA) strain quite efficiently, inhibited the biofilm formation and also reduced the lysis of red blood cells (RBCs) caused by hemolysin toxin producing *S. aureus*. ZnO-NPs killed bacteria by disrupting the cell membrane and by down-regulating the expression of oxidative-stress resistance genes thereby making bacteria prone to oxidative stress. Moreover, we have shown that ZnO-NPs significantly reduced the bacterial burden after inducing skin infection with *S. aureus* in mice model and also inhibited intracellular survival of *M. smegmatis* in infected macrophages. The intracellular killing of *M. smegmatis* was attributed to increase in the production of reactive oxygen species (ROS) in response to ZnO-NP treatment. Confocal microscopy results showed co-localization of labelled ZnO-NPs with *M. smegmatis*-GFP (green fluorescent protein) bacteria. The combined data support the biomedical application of ZnO NPs as an antibacterial therapeutic agent.

Methods

Bacterial strains and cell culture conditions

S. aureus ATCC-25923, *E. coli*, *P. aeruginosa* PAO1 and MRSA ATCC-43300 strains were grown in Luria Bertani (LB) medium at 37 °C and 180 r.p.m. *M. smegmatis* mc2155 and *M. bovis*-BCG were grown in Middlebrook's 7H9 broth medium supplemented with 1% OADC (*Oleic Albumin Dextrose Catalase*) and 0.05% Tween 80 (Merck) at 120 r.p.m. To stabilize GFP, medium was supplied with hygromycin (50 μg/ml). The human monocyte THP-1 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS, 2 mM L-glutamine and 2.5 mM HEPES. The mouse macrophage RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HiMedia) supplemented with 10% FBS, 1% penicillin-streptomycin solution, and 1% L-glutamine.

Synthesis of ZnO nanoparticles

The ZnO-NPs were synthesized by wet chemical method using zinc nitrate and sodium hydroxide (NaOH) as precursors and soluble starch as stabilizing agent.[16] 0.1 M of zinc nitrate was dissolved in 100 ml of 0.5% starch solution. After complete dissolution of zinc nitrate, equal volume of 0.2 M of NaOH solution was added slowly under constant stirring for 2 h. The solution was allowed to settle overnight, centrifuged at 10,000 g for 10 min and washed thrice with distilled water to remove the byproducts and the excessive starch. After washing, the ZnO-NPs were sonicated for 10 min in sterile water.

Nanoparticle characterization

Synthesized ZnO-NPs were characterized by UV-Visible spectroscopy (Epoch, BioTek, Germany) at a resolution of 1 nm from 200 to 900 nm. For TEM, a drop of aqueous solution of ZnO-NPs was placed on the carbon-coated copper grids. The samples were dried and kept overnight under a desiccator before loading them onto a specimen holder. The TEM measurements were performed on JEM-2100, HRTEM, JEOL, JAPAN operating at 200 kV. The size distribution and zeta potential of the ZnO-NPs were determined by DLS (Zeta sizer Nano ZS Malvern Instruments, UK) at room temperature.

In vitro killing assay

To determine the antibacterial activity of ZnO-NPs, various concentrations of ZnO-NPs were incubated with 4-5 × 10^5 bacteria in LB or 7H9 medium in 96-well round bottom plates in triplicates. Bacteria were harvested at the indicated time points and the number of colony forming units (CFUs) was assayed by plating suitably diluted cultures on LB plates. All samples were plated in triplicate and values were averaged from three independent trials.

Biofilm assay

Overnight grown cultures were washed with PBS, resuspended in Muller Hinton broth (MHB) and optical density (OD)