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

Rashmirekha Pati, MSc\textsuperscript{a}, Ranjit Kumar Mehta, MSc\textsuperscript{a}, Soumitra Mohanty, MSc\textsuperscript{a}, Avinash Padhi, MTech\textsuperscript{a}, Mitali Sengupta, MSc\textsuperscript{a}, Baskarlingam Vaseeharan, PhD\textsuperscript{b}, Chandan Goswami, PhD\textsuperscript{c}, Avinash Sonawane, PhD\textsuperscript{a,*}

\textsuperscript{a}School of Biotechnology, KIIT University, Bhubaneswar, Orissa, India
\textsuperscript{b}Department of Animal Health and Management, Alagappa University, Karaikudi, Tamil Nadu, India
\textsuperscript{c}School of Biology, NISER, Bhubaneswar, Orissa, India

Received 24 September 2013; accepted 22 February 2014

Abstract

Here we studied immunological and antibacterial mechanisms of zinc oxide nanoparticles (ZnO-NPs) against human pathogens. ZnO-NPs showed more activity against \textit{Staphylococcus aureus} and least against \textit{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 \textit{Mycobacterium smegmatis}-GFP in macrophages. Moreover, ZnO-NPs disrupted biofilm formation and inhibited hemolysis by hemolysin toxin producing \textit{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.

From the Clinical Editor: This in-depth study demonstrates properties of ZnO nanoparticles in infection prevention and treatment in several skin infection models, dissecting the potential mechanisms of action of these nanoparticles and paving the way to human applications. © 2014 Elsevier Inc. All rights reserved.

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

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.\textsuperscript{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,\textsuperscript{2} in disease diagnostics,\textsuperscript{3} to reduce bacterial infections in skin and burn wounds,\textsuperscript{4,5} and to prevent bacterial colonization on medical devices.\textsuperscript{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 \textit{in-vivo} antibacterial efficacy of metal oxide NPs, their ability to kill intracellular pathogens and mechanisms of action. Among the

\textsuperscript{*}Corresponding author at: School of Biotechnology, Campus-11, KIIT University, Bhubaneswar, Orissa-751024, India.
\textit{E-mail address: aasonawane@kiitbiotech.ac.in} (A. Sonawane).

http://dx.doi.org/10.1016/j.nano.2014.02.012
1549-9634/© 2014 Elsevier Inc. All rights reserved.
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 and modulate the secretion of cytokines. Preceding that ZnO-NPs exert antibacterial activity against pathogens and also inhibit the biofilm formation without causing any cytotoxic and genotoxic effects on macrophages.

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. ZnO nano-size particles show more pronounced antimicrobial activities than large particles. 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.

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 antibiotic 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. 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 × 10^7 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)
at 600 nm was adjusted to 1.0. Bacterial biofilms were developed on a glass slide after incubating the bacterial cells with different concentrations of ZnO-NPs for 24 h. Confocal laser scanning microscopy (CLSM, Zeiss LSM710 meta, Germany) was used to determine the bacterial biofilm architecture, thickness and morphology. The mature biofilms formed on glass slides were transferred to a 24 well plate containing 2 ml staining solution (PBS with 0.2% acridine orange), incubated for 15 min at 37 °C and visualized under CLSM. The images were acquired from control and nanoparticles treated biofilms and processed using Zen 2009 image software.

Hydrophobicity index of bacterial biofilm

Overnight grown bacterial cells (control and nanoparticles treated) were resuspended in MHB, optical density at 595 nm was adjusted to 1.0 ± 0.01, and toluene (1 ml) was added to the cell suspension in a test tube and was vortexed for 1 min. The mixture was then allowed to settle for 30 min and the OD of the aqueous phase was measured. Hydrophobicity index (HI) of microbial cells was calculated by the formula \[ (A_0 - A) / A_0 \times 100, \] where \( A_0 \) and \( A \) are the initial and final optical densities of the aqueous phase. The results were expressed as the proportion of the cells which were excluded from the aqueous phase, determined using above equation.17

Scanning electron microscopy

*S. aureus* (2 × 10^7 CFU/ml) and *M. bovis* (BCG) 1 × 10^8 CFU/ml were grown in presence (300 µg/ml) and absence of ZnO-NPs for 24 h at 37 °C. Then the cells were fixed with 3.7% formaldehyde for 30 min. The samples were detected using LFDS detector at low vacuum pressure and analyzed by scanning electron microscope (FEI Quanta 250, Czech Republic).

Cytotoxicity assay

Peripheral blood monocyte cells (PBMCs) were isolated from human blood using 2.5% dextran T-500 as described previously.18 THP-1 and PBMCs (1 × 10^5 cells/well) were grown in 24-well plates at 37 °C, 5% CO_2 for 24 h followed by treatment of cells with different concentrations of ZnO-NPs for another 24 h. Cell viability was determined by MTT assay as described previously.19

Effect of ZnO-NPs on intracellular killing of *M. smegmatis*

THP-1 (2 × 10^5) cells were treated with different concentration of ZnO-NPs 1 h before and after *M. smegmatis* infection, referred as “pretreated” and “post-treated” conditions, respectively. These cells were exposed to *M. smegmatis* at an MOI 10 for 24 h. Extracellular bacteria were killed by addition of 20 µg/ml gentamycin. Cells infected with bacteria alone were used as control. After the incubation period, cells were washed, lysed with 0.5% Triton X-100 and intracellular survival was determined by plating serially diluted samples on LB plates and the colonies were enumerated after 3 days.

Endocytosis of nanoparticles by THP-1 cells

ZnO-NPs were labeled by addition of 5 µg/ml of fluorescein isothiocyanate (FITC) and the mixture was stirred continuously for 8 h in dark. Phorbol myristate acetate (PMA) (20 nM) differentiated THP-1 cells (1 × 10^5 cells/ml) were grown on glass cover slips in 24-well plate. FITC labeled ZnO-NPs were added to the cells. RPMI media with FITC only was taken as control. Then the plate was incubated for 1 h, cells were washed with 1X PBS and Flow cytometry was performed by analyzing 10,000 gated cells using an FACS Caliber flow cytometer and Cell Quest software (BD, USA). For qualitative analysis, FITC-NP treated cells were fixed with 3.7% paraformaldehyde for 30 min at 37 °C. After incubation, cells were washed with 1X PBS and mounted on glass slides. The images were visualized by using fluorescence microscope (Nikon, Japan).

Quantification of ROS production

ROS production was determined by staining THP-1 cells with Dichloro-dihydro-fluorescein diacetate (DCFH-DA), which is a lipid-permeable non-fluorescent compound. The fluorescent intensity is proportional to the amount of ROS produced by the cells. 2 × 10^5 THP-1 cells were treated with 100 and 500 µg/ml concentrations of ZnO-NPs. Untreated and DMSO treated treated cells were taken as control. Then 20 µM DCFH-DA was added to the cells at different time points and the cells were incubated for 30 min at 37 °C. Then the cells were harvested, washed with ice-cold PBS and the fluorescence emission of DCF was measured by FACS Calibur flow cytometer (BD, USA).

Co-localization studies by confocal microscopy

ZnO-NPs were labeled with propidium iodide (1 µg/ml). 1 × 10^5 RAW264.7 mouse macrophage cells were grown on glass coverslips and then treated with PI-labeled ZnO NPs following infection with *M. smegmatis*-GFP for 2 h. The extracellular bacteria were killed by addition of 20 µg/ml gentamycin. After 12 h of incubation period, cells were washed with 1X PBS and fixed with 3.7% formaldehyde for 30 min, mounted by a DAPI containing mounting solution (Invitrogen). The samples were excited at 535 nm and 395 nm. The emission was collected at 617 nm and 509 nm. The samples were analyzed by confocal microscopy equipped with argon laser (LSM 780, Zeiss, Germany).

Hemolysis assay

All experiments with the human blood were approved by the institutional review board of KIIT University. To determine the effect of ZnO-NPs on RBC lysis, RBCs were isolated from human blood, incubated with *S. aureus* followed by treatment with different concentrations of ZnO-NPs for 24 h. Pyrogen free saline and Triton X-100 were used as negative and positive controls, respectively. After incubation, an aliquot of reaction mixture was taken and centrifuged at 8000 g for 2 minutes at room temperature. The absorbance of supernatants was measured at 405 nm using ELISA reader (Biotek, Germany) in a 96 well microtiter plate. The percentage of hemolysis was
calculated by comparing the absorbance with positive and negative controls.

**Real-time RT-PCR**

*S. aureus* ATCC-25923 was cultured with (300 μg/ml) and without ZnO-NPs for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen) as per the manufacturer’s protocol. cDNA synthesis was performed using cDNA synthesis kit (Thermo). The synthesized cDNA was used as a template for RT-PCR amplification using gene specific primers. The primer pairs used for the real time RT-PCR are listed in Table 1. All reactions were performed in a total reaction volume of 10 μl using SYBR® Green PCR mastermix (Applied Biosystems, USA), and carried out in Real Plex master cycler (Eppendorf, Germany) with initial denaturation at 95 °C for 10 min, final denaturation at 95 °C for 30 sec, annealing at 52 °C for 30 sec and extension at 72 °C for 20 sec to generate 200 bp amplicons. The mRNA levels were normalized to the transcript levels of 16s rRNA and the relative fold changes were calculated.

**Mice experiments**

Six to eight week old female BALB/c mice were used for all experiments. All mice were kept in our animal facilities, in high efficiency particulate air (HEPA)-filter bearing cages under 12 h light cycles, and were given sterile chow and autoclaved water *ad libitum*. All animal experiments were performed in accordance with national guidelines for the care and handling of laboratory animals and have been approved by the Institutional Animal Ethical Committee. Mice (5 mice in each group) were shaved on the back and injected intradermally with *S. aureus* (1 × 10^7 CFUs) in 150 μl of sterile 1X PBS using 30 gauge insulin syringe. Following experimental conditions were used: mice infected with bacteria only, mice given bacteria and ZnO-NPs (1 g/kg body weight) simultaneously, mice treated with ZnO-NPs one day after infection, mice treated with PBS and ZnO-NPs. Seven days after infection, mice were anesthetized and sacrificed. Lesional skin specimens were homogenized by a tissue homogenizer (Tissue Lyser II, Qiagen, Germany) and *S. aureus* survival was determined by plating serially diluted homogenized samples on LB plates after 24 h incubation.

For histopathological studies, the skin biopsy specimens were taken immediately after mice were sacrificed and fixed in phosphate buffered (pH 7.4) containing 4% formalin. The formalin fixed biopsy specimens were embedded in paraffin and the sections obtained and stained with hematoxylin and eosine.

**Statistical analysis**

Significant differences between the groups were determined by one-way and two-way ANOVA. All the statistical calculations were performed with the help of GraphPad prism version 5.0. Significance was indicated as *** for *P* < 0.001; ** for *P* < 0.01 and * for *P* < 0.05.

**Results**

**Characterization of ZnO nanoparticles**

The synthesized starch-capped ZnO-NPs showed an ultraviolet-absorption spectrum at 360 nm, which is a typical absorption band of ZnO-NPs (Figure 1, A). Shape and size of the NPs were determined by TEM (Figure 1, B). The TEM micrograph showed the spherical shape NPs. The average particle size of ZnO-NPs was about 500 nm, while surface zeta potential was measured as −0.56 mV (Figure 1, C and D).

**Antibacterial activity of ZnO nanoparticles**

We tested the *in vitro* antibacterial activity of ZnO-NPs against a panel of human pathogens representing Gram-positive, Gram-negative, acid fast and clinical drug resistant MRSA strain of *S. aureus* by a CFU assay. As shown in Figure 2, ZnO-NPs significantly killed bacteria in a dose-dependent manner. After 6 h of incubation approximately 78% and 91% (*P* ≤ 0.01 and *P* ≤ 0.001) of the *M. smegmatis* population was killed at 500 and 1000 μg/ml concentrations, respectively, whereas after 24 h of incubation about 93% (*P* ≤ 0.001) of bacteria were killed at 500 μg/ml and no viable bacteria were found at 1000 μg/ml concentration (Figure 2, A). In comparison to *M. smegmatis*, *M. bovis*-BCG, which resembles to the pathogenic *M. tuberculosis* strain, was found to be resistant to ZnO-NPs action. However, combination of ZnO-NPs and rifampicin (0.7 μg/ml), which is the potent first line anti-tuberculosis drug, significantly reduced the survival of *M. bovis* BCG (Figure 2, C). This dose of rifampicin (0.7 μg/ml) was determined as MIC for *M. smegmatis* and BCG (data not shown). In case of Gram-negative bacteria, about 50% (*P* ≤ 0.001) of *E. coli* and *P. aeruginosa* population were killed at 500 μg/ml concentration, where no colonies were observed at 1000 μg/ml concentrations after 24 h of incubation (Figures 2, D and E). Among all the tested bacteria, *S. aureus* was found to be more susceptible to ZnO-NP such that more than 92% and 98% (*P* ≤ 0.001) bacterial population was killed at 500 and 1000 μg/ml concentrations, respectively, whereas after 24 h of incubation no viable bacterial colonies were found at the same doses (Figure 2, F). We also investigated the antibacterial activity of ZnO-NPs against a clinical drug-resistant MRSA strain of *S. aureus*. As shown in Figure 2, G, approximately 92% and 71% (*P* ≤ 0.001) killing of MRSA strain was observed at 500 μg/ml after 6 and 24 h, respectively, and no viable colonies were found at 750 μg/ml concentration after 6 h of
incubation, indicating that ZnO-NPs are also active against drug-resistant bacteria. To check whether the antibacterial activity of ZnO-NPs is size dependent, we studied antibacterial activity of smaller size ZnO-NPs (<50 nm) procured from Sigma (Cat No. 677450). Similar killing pattern was observed against _M. smegmatis_, _P. aeruginosa_, _S. aureus_ and _S. aureus_ (MRSA) strains, although all four strains exhibited slightly more resistance to ZnO-NP action (Supplementary Figure S1). Therefore, subsequent experiments were performed with larger size (500 nm) ZnO-NPs.

**ZnO-NPs treatment disintegrate bacterial cell membrane**

To find out the mechanism of killing, scanning electron microscopy was performed to observe the bacterial cell morphology after treatment with ZnO-NPs. As shown in Figure 2, _H_, the cell membrane of _S. aureus_ was completely disrupted after treatment with minimal effective dose (300 μg/ml) (Figure 2, _F_) of ZnO-NPs for 24 h, whereas untreated _S. aureus_ showed intact coccoid chain like structures. In case of _M. bovis-BCG_, no changes in cell morphology were observed after the treatment with same dose of ZnO-NPs (Figure 2, _J_).

**ZnO-NP treatment downregulates the transcription of oxidative stress related genes in _S. aureus_**

Induction of oxidative stress is one of the potential bacterial killing mechanisms. To further understand the mechanism of bacterial killing by ZnO-NPs, we determined the transcription levels of oxidative stress resistant genes in _S. aureus_ after treatment with 300 μg/ml of ZnO-NPs. For this peroxide stress regulon perR, fur and katA genes were selected. Several studies have shown that these genes induce oxidative stress resistance mechanisms in bacteria. Moreover, catalase encoded by _katA_ is also known as virulence determinants of _S. aureus_. The expression values were normalized to 16s rRNA transcript levels. As shown in Figure 2, _J_, treatment with ZnO-NPs markedly decreased the transcription of _perR_ and _katA_ by 10 and 3.1 fold, respectively, whereas no change in the transcription level of _fur_ was observed when compared to untreated bacteria. The above data indicate that ZnO-NPs kill bacteria by disintegrating the cell membrane and by downregulating the transcription of oxidative stress resistance genes thereby making them susceptible to oxidative stress.

**Effect on biofilm formation and cell surface hydrophobicity**

Among the bacteria tested here, _P. aeruginosa_ and _S. aureus_ are known to form biofilms. We evaluated the effect of ZnO-NPs on biofilm formation, which is a major virulence factor of bacteria. The microscopic studies showed well developed biofilm formation by _P. aeruginosa_ and _S. aureus_, whereas treatment with ZnO-NPs inhibited biofilm formation in a dose dependent manner (Figure 3, _A_). CLSM images showed development of dense biofilm formation on glass slides by both strains, while treatment with ZnO-NPs showed disintegrated and recalcitrant biofilm architecture. The crystal violet staining also showed dose dependent inhibition of biofilm formation (data not shown). The percentage of hydrophobicity index was also decreased after treatment with ZnO-NPs such that 63% and 43% hydrophobicity inhibition of _P. aeruginosa_ and _S. aureus_, respectively was observed as compared to untreated bacteria (Figure 3, _B_).

**Cytotoxic effect of ZnO-NPs on macrophages and monocytes**

Despite their wide biological applications, the use of metallic nanoparticles as an antibacterial agent is limited due to their cytotoxic effect on mammalian cells. Previously we and several other studies have shown that capping of metallic
Figure 2. Dose dependent killing of bacteria by nanoparticles. (A) *Mycobacterium smegmatis*, (B) *M. bovis*-BCG, (C) ZnO-NPs with rifampicin against *M. bovis*-BCG, (D) *Escherichia coli*, (E) *Pseudomonas aeruginosa*, (F) *Staphylococcus aureus*, and (G) MRSA *Staphylococcus aureus*. Bacteria were incubated with different concentrations of ZnO NPs and their survival was determined at the indicated time points by CFU assay. Media containing bacteria alone was used as control. ND means no bacterial growth was detected. Scanning electron microscopy images of *Staphylococcus aureus* (H) and *M. bovis*-BCG (I) after incubation with (300 μg/ml) and without ZnO-NPs for 24 hours. (J) Relative gene expression levels of *perR*, *fur* and *katA* in *S. aureus* following exposure to ZnO-NPs (300 μg/ml). Results are represented as normalized to the untreated control levels. Experiments were performed in triplicates, results are shown mean ± SD; **P ≤ 0.01; ***P ≤ 0.001.
Figure 3. Effect of zinc oxide nanoparticles on biofilm formation. Antibiofilm activity of ZnO-NPs was checked by incubating (A) *P. aeruginosa* and *S. aureus* with different concentrations of ZnO-NPs for 24 h. Biofilm formation was checked by confocal laser scanning microscopy. Determination of hydrophobicity index of *P. aeruginosa* (B) and *S. aureus* (C) after treatment with 500 μg/ml ZnO-NPs for 24 h. Experiments were performed in triplicates, results are shown mean ± SD; **P ≤ 0.01; ***P ≤ 0.001.
nanoparticles with biological matrix can preserve the antibacterial activity without exerting cytotoxic effect on mammalian cells due to slow release of metallic ions from the gel matrix. In this study, we determined the cytotoxic effect of ZnO-NPs against peripheral blood mononuclear cells (PBMCs) and human monocytic THP-1 cells by the MTT assay. ZnO-NPs exerted significantly less (15% reduction in cell viability) cytotoxic effect on PBMCs at 1000 μg/ml concentration (Figure 4, A). In case of THP-1 cells, no cytotoxic effect was observed up to 500 μg/ml concentration (Figure 4, B), which is an effective bactericidal dose against the pathogens tested in this study. The intensity of dye is directly proportional to the number of viable cells. ZnO-NPs induced generation of ROS in THP-1 cells. Cells were treated with 100 and 500 μg/ml concentrations. ROS production was determined at 6 and 24 h after ZnO-NP treatment by DCFH-DA staining method using 10,000 gated cells by flow cytometry. Experiments were performed in triplicates, results are shown mean ± SD; ***P ≤ 0.001.

Figure 4. Cytotoxic activity of ZnO-NPs on (A) PBMCs and (B) THP-1 cells. Cells were treated with different concentrations of ZnO-NPs for 24 h, cell viability was determined by MTT assay. ZnO-NPs exhibit intracellular killing activity against Mycobacterium smegmatis. THP-1 cells were incubated with different concentrations of ZnO-NPs 1 h before (pre-treatment), and 1 h after (post treatment) M. smegmatis infection. Macrophages infected with bacteria alone were used as control. The cells were lysed at 6 h (C) and 24 h (D) post infection and the intracellular bacterial survival was determined by CFU assay. Endocytosis of FITC-labelled ZnO NPs in THP-1 cells was studied using (E) fluorescence microscopy, and (F) flow cytometry analysis. (G) Localization of PI labelled ZnO-NPs and M. smegmatis-GFP in RAW 264.7 mouse macrophage cells. RAW 264.7 cells were infected with M. smegmatis-GFP (green) and then treated with 50 μg/ml PI labelled ZnO-NPs (red) for 12 h. The localization of ZnO-NPs and M. smegmatis was analyzed using confocal microscopy. (H) ZnO-NPs induced generation of ROS in THP-1 cells. Cells were treated with 100 and 500 μg/ml concentrations. ROS production was determined at 6 and 24 h after ZnO-NP treatment by DCFH-DA staining method using 10,000 gated cells by flow cytometry. Experiments were performed in triplicates, results are shown mean ± SD; ***P ≤ 0.001.
Figure 5. (A) ZnO-NPs inhibited RBC lysis induced by *Staphylococcus aureus*. RBCs were incubated with *S. aureus* alone, or *S. aureus* with different concentrations of ZnO-NPs. Triton X-100 (1%) and PBS were used as positive and negative control, respectively. (B, C) Survival of *S. aureus* in ZnO-NP treated mice. Mice were infected intradermally with *S. aureus* and treated with ZnO-NPs simultaneously (*S. aureus + ZnO NP*) or 1 day after infection (*S. aureus + 1d ZnO-NP*). Mice injected with PBS and ZnO-NP alone were used as control. Seven days after infection, skin lesions were cut, homogenized and bacterial count was determined by CFU assay. (D) Histological appearance of mice skin biopsy; PBS treated, *S. aureus* infected, *S. aureus* infected and ZnO-NP treated and only ZnO-NP treated. On day 6, biopsy specimens were taken immediately after the termination of the experiment, fixed in 4% neutral buffered formalin and embedded in paraffin. The biopsy specimens were stained with hematoxylin and eosin. Numbered arrows indicate the following: 1, epidermis; 2, dermis; 3, sebaceous gland; 4, bacteria; 5, disrupted epidermal layer; 6, polymorphous infiltrate, consisting of mononuclear cells including lymphocytes and neutrophils. Experiments were performed in triplicates, results are shown mean ± SD. ***P ≤ 0.001.
study (Figure 2). However, a significant reduction in cell viability (85%) was observed at higher doses (Figure 5, B). These data indicated that the starch-capped ZnO-NPs show antibacterial activity without being harmful to macrophages at the bactericidal dose (500 μg/ml).

ZnO-NP treatment reduces intracellular bacterial burden in macrophages

Macrophages can engulf any kind of particles of a certain size. We assessed whether macrophages can also endocytose ZnO-NPs, which will lead to intracellular killing of bacteria. Since mycobacteria are an intracellular pathogen and reside inside the macrophages, we tested the intracellular burden of M. smegmatis in macrophages before and after ZnO-NP treatment. For this, THP-1 differentiated macrophage cells were treated with 50, 100 and 400 μg/ml of ZnO-NPs 1 h before (pretreatment) and 1 h after (post-treatment) M. smegmatis infection. As shown above ZnO-NP up to 500 μg/ml concentration is not toxic to THP-1 cells (Figure 4, B). Dose dependent intracellular killing of M. smegmatis was observed when compared with untreated cells (Figure 4, C and D). After 24 h of infection approximately 30% and 60% (P ≤ 0.001) reduction in intracellular survival was observed at 100 μg/ml doses, whereas at 400 μg/ml concentration about 75% and 89% (P ≤ 0.001) killing was observed in pre- and post-treated THP-1 cells, respectively.

Endocytosis of ZnO-NPs by macrophages

As described above, we observed an intracellular killing of M. smegmatis in THP-1 cells after treatment with ZnO-NPs. We hypothesized that the killing effect could be either ZnO-NPs are internalized and targeted to phagosomes where mycobacteria reside, thereby causing direct bacterial killing, or by activating the THP-1 cells. We performed fluorescence microscopic and flow cytometry studies to evaluate internalization of exogenously added FITC-conjugated ZnO-NPs in THP-1 cells. Our microscopic studies showed active endocytosis of FITC-ZnO-NPs (Figure 4, E). Flow cytometry analysis also showed significant amount of FITC-ZnO-NP is taken up by THP-1 cells (Figure 4, F).

Propidium iodide labeled ZnO-NPs co-localize with M. smegmatis-GFP in macrophages

To determine whether the ZnO-NPs kill intracellular mycobacteria by direct interaction with bacteria, THP-1 macrophages were infected with M. smegmatis-GFP, treated with PI-labelled ZnO-NPs and the intracellular localization of ZnO-NPs and M. smegmatis-GFP was studied by confocal microscopy. As shown in Figure 4, G, labelled ZnO-NPs co-localized with M. smegmatis-GFP.

ZnO-NPs induce ROS production in THP-1 cells

Another mechanism by which macrophages kill intracellular mycobacteria is induction of ROS and nitric oxide (NO) production. Therefore to further investigate the mechanism of bacterial killing, we measured the level of ROS and NO as a result of ZnO-NP treatment in THP-1 cells. Cells were exposed to 100 and 500 μg/ml of ZnO-NPs and the level of ROS production was determined by flow cytometry using DCFH staining. As shown in Figure 4, H, ROS production was significantly increased after 6 h treatment with 100 and 500 μg/ml concentrations of ZnO-NPs, while the level of ROS decreased at the same doses after 24 h of treatment. No ROS production was observed in untreated and DMSO treated control cells. We observed no significant changes in NO production following exposure of THP-1 cells to the same doses of ZnO-NPs (data not shown). The above results indicate that ZnO-NPs kill intracellular bacteria by direct interaction and by inducing the production of ROS in macrophages.

ZnO nanoparticles reduce hemolysis caused by S. aureus

Staphylococcal α-hemolysin, a major virulence factor, lyse the host cells by forming pores in the cell membrane. We showed that addition of ZnO-NPs inhibited the RBC lysis caused by S. aureus. As shown in Figure 5, A, about 60% cell lysis was observed after incubation with S. aureus, whereas in the presence of 100 and 750 μg/ml ZnO-NPs less than 10% and 15% (P ≤ 0.001) cell lysis was observed. Addition of ZnO-NPs alone caused 6% and 45% cell lysis at 100 μg/ml and 750 μg/ml concentrations, respectively.

ZnO-NPs reduce S. aureus burden in mouse skin infection

To check the in vivo antibacterial efficacy of ZnO-NPs, BALB/C mice were infected intradermally with S. aureus. Seven days after infection bacterial burden was determined by homogenizing the infected skin lesion and plating the serially diluted samples on LB plates. 25 mice were divided into 5 groups: mice injected with PBS only (group A), mice infected with S. aureus (group B), mice received S. aureus and ZnO-NPs simultaneously (group C), mice infected with S. aureus first and then treated with ZnO-NPs 24 h after infection (group D), and mice treated with ZnO-NPs only (group E). All the mice in group B developed skin infection which can be seen by the presence of black patch at the site of injection after 72 h of infection (Figure 5, B), whereas no skin infection developed in other group of mice. The body weight and movement of all mice were also monitored. The mice remained active with normal movement and body weight except group B, where the body weight slightly decreased during the course of infection. The CFU assay showed that the bacterial burden reduced significantly (118 fold) in group C mice as compared to group B mice (P ≤ 0.001; Figure 5, C). On the other hand the bacterial burden was slightly higher in group D mice but still significantly less (29 fold) compared with group B mice. These results indicate that the treatment with ZnO-NPs reduces bacterial load under in vivo conditions. We also compared in vivo antibacterial efficacy of smaller size (<50 nm) ZnO-NPs in S. aureus infected mice. Treatment with ZnO-NPs (<50 nm) showed significant decrease in S. aureus burden in group C (165 fold) and group D (2 fold) mice, respectively (Supplementary Figure S2).

Histopathological analysis

We next analysed the skin architecture, bacterial burden and the inflammatory changes in ZnO-NP treated and S. aureus.
infected mice skin lesions 7 days post infection by hematoxylin and eosin (HE) staining. Remarkable changes were observed in the subcutaneous tissue of *S. aureus* infected mice skin lesions (group B), whereas PBS (group A), *S. aureus* with ZnO-NP (group C), and ZnO-NP treated (group E) skin biopsy sections showed normal morphological features. The epidermal and dermal layers and circular sebaceous glands appeared normal and intact in all the tissue sections prepared from group A, C and E. In contrast, skin sections from group B mice showed completely disrupted epidermal layer, degenerated sebaceous glands and nuclear bodies in the dermis. Moreover, significant infiltration of polymorphous cells and large burden of *S. aureus* was seen in the epidermal layer of group B mice (Figure 5, D).

**Discussion**

In recent years treatment of bacterial infections has become a serious concern due to the increased problem of resistance against conventional antibiotics. It remains a major challenge to treat intracellular infections because many antimicrobials are not able to diffuse effectively through the cell membrane, thus have low activity inside the cells. Therefore, it is important to develop alternative therapeutic strategies including new class of antibiotics, which can kill the drug resistant strains without harming the host cells.

The *in vitro* antibacterial assay showed distinct differences in the susceptibility to ZnO-NPs. *S. aureus* was found to be more susceptible whereas the mycobacterial vaccine strain *M. bovis*-BCG showed resistance to ZnO-NPs. This may be due to the differences in the cell wall composition of Gram-positive and Gram-negative bacteria. Cell wall teichoic acids appear to be the binding sites for some molecules that cleave the bacterial peptidoglycan. So, increased susceptibility of *S. aureus* to ZnO-NPs could be due to interaction between cationic starch and anionic teichoic acid. In comparison, mycobacterial cell wall structure is more complex and rich in lipids, which limits the diffusion of drugs inside the bacterial cell. Therefore, the resistance of BCG to ZnO-NPs could be due to lack of diffusion of ZnO-NPs through hydrophobic cell wall. This is evident from scanning electron microscopic studies, which showed intact cell morphology of BCG after treatment with bactericidal dose of ZnO-NPs. In contrast, the morphology of more susceptible *S. aureus* was found to be irreversibly damaged at the same dose. The cell damage could be due to inactivation of some membrane enzyme functions by ZnO-NPs. Previous studies reported that treatment with silver nanoparticles damages the structure of *Escherichia coli* cell membrane and depresses the activity of some membraneous enzymes, which cause the bacterial death eventually. However, treatment with a combination of rifampicin and ZnO-NPs led to increased killing of BCG. It has been reported that treatment with ZnO-NPs causes changes in the membrane permeability. Rifampicin kills mycobacteria by binding to the β subunit of the RNA polymerase thus interferes in the RNA synthesis. So treatment with ZnO-NPs may have facilitated the transport of rifampicin inside the mycobacterial cell by altering cell membrane permeability and thereby killing of mycobacteria by inhibiting RNA synthesis. MRSA strains are a major cause of nosocomial infections worldwide. ZnO-NPs were also found to be effective against MRSA strain of *S. aureus*, which is resistant to beta-lactam antibiotics. Our results also implicate that ZnO-NPs can be used for the treatment of drug-resistant bacteria. High activity of ZnO-NPs could be attributed to induction of oxidative stress by inactivating enzymes, generating hydrogen peroxide and causing bacterial cell death. Indeed, we found that treatment with ZnO-NPs resulted in down-regulation of peroxide stress regulon **perR** and **katA** transcripts, which have the major role in oxidative stress resistance pathway in *S. aureus*. Many bacteria control oxidative stress pathway through the transcriptional regulation of different oxidative stress resistance gene.

*S. aureus* and *P. aeruginosa* are known to synthesize exopolysaccharides that play a crucial role in the bacterial initial adhesion to host cells and the development of a complex biofilm structure, which is then difficult to combat with host defenses and antibiotics. The anti-biofilm activity could be due to inhibition of exopolysaccharide synthesis because it has been shown that metallic nanoparticles impair exopolysaccharide synthesis, which limits biofilm formation. Rough lipopolysaccharide synthesized by pathogenic *P. aeruginosa* strains, prevents the binding of drug molecules. In case of *S. aureus*, glucosamine based polysaccharide intercellular adhesion molecules are responsible for cell-cell adhesion and binding of external molecules. These deviations in anti-biofilm activity may be the results of different capacities of ZnO-NP binding to bacterial compounds. It has been shown that coatings of ZnO-NPs inhibit biofilm formation by *Streptococcus mutans*. Targeting the hydrophobicity index is a novel way of inhibiting biofilm formation. Exopolysaccharide and cell surface hydrophobicity play an important role in bacteria-host cell interactions and biofilm architectures in microbes. Previous reports confirm that cell surface hydrophobicity helps in the reduction of biofilm production in different micro organisms including *Candida* sp. We report that treatment with ZnO-NPs reduces hydrophobicity index of *P. aeruginosa* and *S. aureus* that leads to the inhibition of biofilm formation.

An important aspect for any therapeutic molecule is that the molecule should affect the function of target molecule without harming the mammalian cells. Here we have shown that ZnO-NPs have no cytotoxic effects on PBMCs and THP-1 cells at the bactericidal dose. However, treatment with higher doses of ZnO-NPs reduced the cell viability. These results indicate the suitability of ZnO-NPs as an antibacterial molecule.

Treatment of intracellular pathogens is a major issue in medical field; therefore, it is important to deliver the therapeutic molecules to the target sites that would otherwise be inaccessible due to presence of physical barriers. Our microscopic and FACS studies also showed active endocytosis of FITC-labelled ZnO-NPs by THP-1 cells. Exogenous addition of ZnO-NPs found to kill intracellular *M. smegmatis*. The killing of *M. smegmatis* could be due to delivery of endocytosed ZnO-NPs to macrophage phagosomes where *M. smegmatis* resides or due to the activation of macrophages by inducing the synthesis of pro-inflammatory cytokines or superoxide radicals. Indeed, we observed more production of ROS in THP-1 cells after treatment with ZnO-NPs. Production of superoxide radicals such as ROS is...
known to play a critical role in antimycobacterial effector function of macrophages. Confocal microscopic studies showed co-localization of bacteria and ZnO-NPs indicating that the observed bacterial killing is due to production of ROS and direct interaction between nanoparticles and the bacteria inside the macrophages. However, further study is required to elucidate whether nanoparticles are targeted to endocytic compartments where mycobacteria resides.

S. aureus secretes α-hemolysin monomers that bind to the outer membrane of host cells. This results in irreversible osmotic swelling and lysis of host cell. S. aureus causes lysis of erythrocytes and neutrophils by secreting some cytolytic peptides. In this study we observed that ZnO-NPs inhibited the lysis of RBCs induced by S. aureus. It remains to elucidate whether ZnO-NPs affect the synthesis of hemolysin or bind to hemolysin thereby make it unavailable for binding to RBCs. However, at higher concentration (750 μg/mL) ZnO-NPs alone caused lysis of RBCs, whereas in the presence of bacteria same dose of NPs showed less cell lysis. It is possible that ZnO-NPs bind to bacteria and mask the ligands, and therefore make them less accessible for binding to RBCs.

Further we showed that administration of ZnO-NPs significantly reduced the skin infection caused by S. aureus. The differences can be confirmed by observing the improved skin architecture, reduced bacterial burden, less inflammation in histopathology images of S. aureus infected and ZnO-NP treated mice indicating that these NPs can be used as topical anti-infective agent. In conclusion, our preclinical studies demonstrate potential antibacterial application and also elucidate the mechanisms by which ZnO-NPs exhibit antibacterial activity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.02.012.

References


