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A STUDY ON THE EFFECT OF ZINC OXIDE NANOPARTICLES IN STAPHYLOCOCCUS AUREUS

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ABSTRACT

Research concerning the impact of inorganic nanoparticles on cellular health will enable new developments in nanobiotechnology to reach their fullest potential. An improved understanding of nanoparticles and biological cell interactions can lead to the development of new sensing, diagnostic, and treatment capabilities, such as improved targeted drug delivery, gene therapy, magnetic resonance imaging (MRI) contrast agents, and biological warfare agent detection etc. The objective of the present study is to observe the growth-inhibitory effect of Zinc Oxide nanoparticles on the micro organism, *Staphylococcus aureus. S. aureus* is a gram positive micro organism and is consistently one of the four causes of nosocomial infections. Antibacterial activity, study of growth curve, protein estimation and SDS PAGE analysis were performed on *S. aureus* treated with different concentrations of 20, 40, 60, 80 and 100 µg/mL of ZnO nanoparticles. These studies have shown positive results for the antibacterial activity of ZnO nanoparticles against *S. aureus* and an indication that there occurs an optimal concentration for the application of ZnO nanoparticles which shows maximum antibacterial activity. Thus, it is useful to understand the nanopartices and its interaction with the microorganisms and its application in treatment. The results obtained from the protein estimation reveal that the proteins are important biological molecules that are fundamental to the proper functioning of cells and the organism and the nanoparticles bring about changes in the protein composition and levels.

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KEY WORDS

Zinc Oxide, Nanoparticles, Staphylococcus aureus, antibacterial effect, protein expression.

INTRODUCTION

One of the major threats in the health care industry is the emergence of microbial organisms resistant to various antibiotics, and other treatment methods. Researchers have tried to develop new, effective antimicrobial reagents that are free of resistance and cost. Such problems and needs have led to the resurgence in the use of Nanoparticle-based treatments that may be linked to broad-spectrum activity and far lower propensity to induce microbial resistance than antibiotics. Inorganic nanocrystalline metal oxides such as ZnO are particularly interesting because they can be prepared with extremely high surface areas, and are more suitable for biological applications. The inorganic antibacterial materials have advantages over organic antibacterial material that the former shows superior durability, less toxicity and greater selectivity and heat resistance[1].The present study reports that ZnO nanoparticles can be applied effectively for the control of micro-organisms and the prevention of infections caused by *Staphylococcus aureus*. *Staphylococcus aureus*, a gram positive micro organism, is consistently one of the four causes of nosocomial infections. The Staphylococci are quintessential pyrogenic cocci. Infection with staphylococci is almost always accompanied by accumulation of large amounts of pus. In fact, staphylococci are responsible for more than 80% of all suppurative infections and are capable of causing diverse conditions such as boils, folliculitis, pneumonia, acute enteritis, burn infections with bacteremia, scaled skin syndrome and toxic shock syndrome[2].

Results of various studies have shown that the antibacterial activity is greater at nano scale than micro scale. The exact mechanism is not clearly understood but the probable mechanisms include, role of reactive oxygen species (ROS) generated on the surface of particles, zinc ion release membrane dysfunction, membrane internalisation[3], intracellular biotransformation of the nanoparticles or that the nanoparticles have dissolved to Zn^{2+} to enact toxicity[4]. Various studies in *E.coli* have confirmed the antibacterial behaviour of suspensions of zinc oxide nanoparticles [5, 6, 7]. The results of the antibacterial studies revealed that the use of ZnO in Nanohydroxyapatite (n-HA)/zinc oxide (ZnO) complex possessed strong antibacterial capability; the antibacterial rate was 99.45% to *S. aureus*[8]. Studies [9, 10, 11] show that ZnO nanoparticles were most effective for S. aureus and it was suggested to have a strong affinity to the cells of *S. aureus*.

MATERIALS AND METHODS

Zinc Oxide (ZnO) Nanoparticle Preparation

Commercially obtained ZnO nanoparticles (Himedia) with a size approximately <100 nm were procured. Stock suspensions with concentrations of 1 mg/mL were prepared by suspending in distilled water and sonication is carried out for about 5 minutes to allow better dispersion of nanoparticles and obtain homogenised suspension.

Preparation of Staphylococcus aureus Culture

Mannitol salt agar (MSA) is a selective medium used for the isolation of pathogenic staphylococci. On MSA, pathogenic *Staphylococcus aureus* produces small

colonies surrounded by yellow zones. The reason for this change in colour is that *S. aureus* ferments the mannitol, producing an acid, which, in turn, changes the indicator from red to yellow. The growth of other types of bacteria is inhibited.

S. aureus was grown in MSA medium at room temperature. The lysogeny broth was prepared in six different conical flasks. The flasks were treated with different concentrations of Zinc Oxide nanoparticles (20 µg, 40 µg, 60 µg,80 µg 100µg). Each of the conical flasks was inoculated with *S. aureus* strain and kept it in shaker for incubation at room temperature at 150 rpm.

Antibacterial Test - The Agar Diffusion Test or Bauer-Kirby Test

Staphylococcus aureus is inoculated over the dried surface of Muller-Hinton agar plate by streaking using L rod or swab with 12 hours incubated organism over the entire sterile agar surface. This procedure was repeated two more times, and the plate was rotated to ensure an even distribution of inoculum. The appropriate number of wells is made on the surface of agar plate and load with different concentrations of ZnO nanoparticles with 20µg/20µL to 100µg/100µL. After 16-18 hours incubation, each plate was examined and measured for the diameters of the zones of complete inhibition including the diameter of the wells.

Colony-Forming Unit

The serial dilutions of the bacterial suspensions were required. The plates are divided into numbered sectors. The inoculum suspension is deposited as drops of 0.02ml from a height of 2.5cm on to the medium where it spreads over an area of 1.5 – 2.0cm diameter. Each of the 6 plates receives one drop of each dilution in separate numbered sectors. The plates are incubated for 18 – 24 hours and observed for growth. Sectors where more than 20 colonies are present without any confluence are utilized to make the viable counts. Viable count per 0.02ml for a dilution is obtained by taking the average of counts for that dilution in all the six plates.

Growth* rate of *Staphylococcus aureus

Freshly grown bacterial inoculum is incubated in the presence of 20,40,60,80, 100µl/ml of Zinc Oxide nanoparticles are added in different flasks to observe the bacterial cell growth pattern at room temperature and 150 rpm. Total solution used in each flask is 50 mL of broth and the growth rate is indexed by measuring optical density (OD) at 600nm. The readings obtained were plotted and comparative studies are performed for different concentrations of ZnO nanoparticles.

Estimation of Protein

The *Staphylococcus aureus* culture samples have been taken in six different conical flasks. These samples were treated with different concentrations of zinc oxide nanoparticles ranging from 0, 20, 40, 60, 80 and 100 µg/mL. The cultures were then kept for incubation overnight at room temperature at 150 rpm in shaker. The samples are then taken after 24 hours for protein estimation by Lowry's method[12]. However for estimation of protein by Sodium dodecyl Sulphate Poly-Acryl amide Gel Electrophoresis (SDS-PAGE - 12%) samples have to be prepared separately *Staphylococcus aureu*s strain, grown without agitation for 24 h at 37°C in lysogeny broth. Cells from 20 ml of culture were harvested by centrifugation at 10000 rpm, washed twice with phosphate-buffered saline without Mg^{2+} and $Ca^{2+,}$ and recentrifuged. The cells were suspended in 10 ml of ice-cold acetone (analytical grade), allowed to stand on ice for 5 min, and collected by centrifugation 10000 rpm. Samples treated with acetone for 5 to 30 min or the use of more acetone did not change the efficiency of protein extraction. The proteins extracted by incubating with 1.0 ml of 1% sodium dodecyl sulphate

SDS) for 2 min the supernatants were used for protein estimation by SDS PAGE.

SEM analysis of Zinc Oxide Nanoparticles

The scanning electron microscope works by bouncing electrons off of the surface and forming an image from the reflected electrons. SEM allows a good deal of analytical data to be collected, in addition to the formed image**.** The zinc oxide nanoparticles are taken in its powdered form and SEM analysis is performed using Scanning Electron Microscope. The SEM analysis is carried out at different magnifications ranging from 15000 X to 35000 X**.** It gives the approximate size of the zinc oxide nanoparticles.

RESULTS

Study on the effect of Zinc Oxide (ZnO) nanoparticles against *Staphylococcus aureus* which is a pathogenic organism has been done. The antibacterial activity and the effect on growth and on protein level due to the interaction of nanoparticles were observed from 20, 40, 60, 80 and 100 µg/mL concentrations. The results of the present study are given below.

Antibacterial activity of ZnO Nanoparticles against S. aureus

From the results obtained due to the antimicrobial activity of ZnO nanoparticles on *S. aureus* it was interesting to note that as the concentration of nanoparticles increases, the zone of inhibition also increases i.e. a minimum for control (almost none) to a maximum in 100 µg/mL (Figure 1, Table 1).

Table 1
Antibacterial activity of ZnO Nanoparticles against S. aureus

Zone Of Inhibition of *S. aureus* (mm) at various concentrations					
Control	**20 µg/mL**	**40 µg/mL**	**60 µg/mL**	**80 µg/mL**	**100 µg/mL**
0	11.26±1.23	12.12±1.45	13.41±1.67	13.46±1.53	14.52±2.01

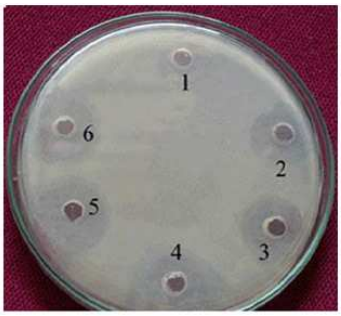

Fig 1
Antibacterial activity of ZnO nanoparticles against S. aureus (Kirby-Bauer test)

Colony Forming Units (CFU) in S. aureus when treated with ZnO Nanoparticles

The number of CFU of *S. aureus* has reduced significantly with increasing ZnO nanoparticles. Minimum number of CFU observed in the control. Virtually no CFU were observed in the samples containing ZnO at the highest concentration i.e. 100 µg/mL. The bacterial growth inhibition trend found in CFU data has matched well with the results of optical density (Figure 2, Table 2)

Table 2
Colony forming units in S. aureus treated with ZnO nanoparticles.

Colony forming units in *S. aureus* for different concentrations in ZnO nanoparticles					
Control	**20 µg/mL**	**40 µg/mL**	**60 µg/mL**	**80 µg/mL**	**100 µg/mL**
43±4	24±3	12±2	7±2	6±2	2±1

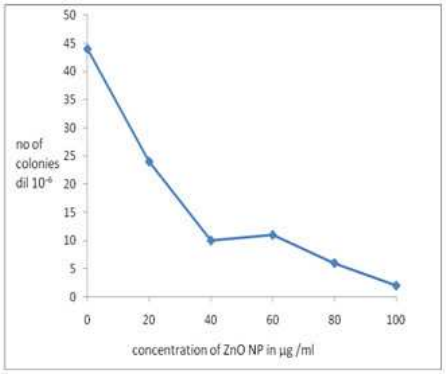

Fig 2
Graph - Colony Forming Units
Concentration of NP vs. Number of colonies

1. Control
2. 20 µg/ml
3. 40 µg/ml
4. 60 µg/ml
5. 80 µg/ml
6. 100 µg/ml

Growth rate of Staphylococcus aureus

The effect of different concentrations of ZnO on the growth of *Staphylococcus aureus* was studied at 25˚ C and 30˚ C at a pH of 7. Figures 3, 4 represent the optical absorption in the growth medium in comparison to the control to increasing concentration of ZnO nanoparticle. It has been found that at a lower concentration of nanoparticle i.e. at 20 µg/mL and 40 µg/mL the optical absorption is lesser than that at higher concentration.

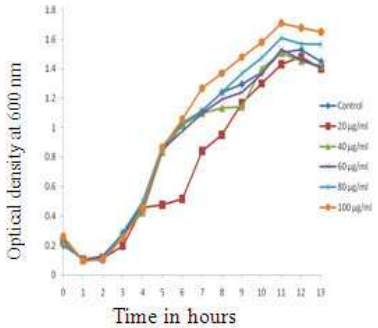

Fig 3
Bacterial Growth curve at 25˚C

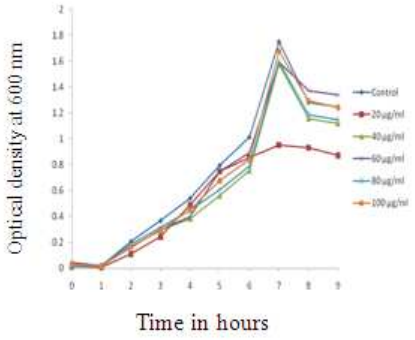

Fig 4
Bacterial Growth curve at 30□ C

Estimation of Protein

Figure 5 reports the results of the changes in the level of protein in *S. aureus* due to Zinc oxide nanoparticles obtained by estimation of total protein by Lowry's method[12]. Maximum level of protein was estimated in control as there is a decrease in protein level as concentration decreases up to 60 µg/mL.

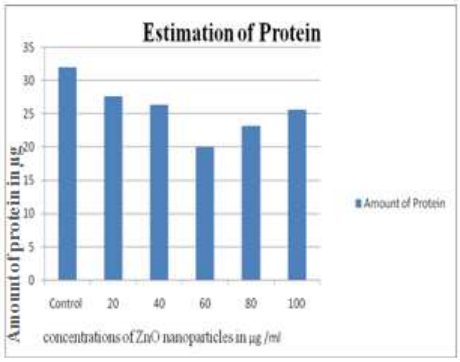

Fig 5
Estimation of Protein

Figure 6 represents the protein profile of *S.aureus* treated with 0, 20, 40, 60 and 80 µg/mL of ZnO nanoparticles. The proteins are separated from 20 kD to 80 kD. The preparations contain no proteins having masses greater than 100 kD. The separations of proteins were well observed in 12% SDS PAGE. The amount of protein separated was observed to be lowest at 20 µg/mL.

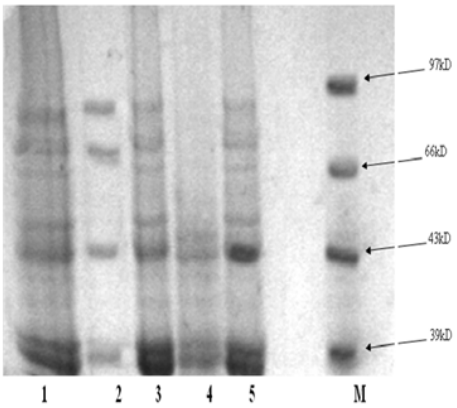

Fig 6
12% SDS PAGE analysis for protein sample.

SEM Analysis of Nanoparticles

ZnO nanoparticles obtained commercially from sigma Aldrich were characterized by SEM analysis and the size of the particles were recorded to be of less than 100nm, which is confirmed in Figure 7

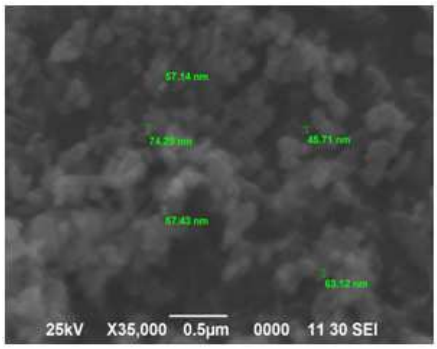

Fig 7
SEM analysis of ZnO nanoparticles

(For bacterial culture treated with different concentrations of ZnO nanoparticles)

1. **Control**
2. **20 µg/mL ZnO Nanoparticle**
3. **40 µg/mL ZnO Nanoparticle**
4. **60 µg/mL ZnO Nanoparticle**
5. **80 µg/mL ZnO Nanoparticle**

M. Protein Marker

DISCUSSION

Antibacterial activity of ZnO Nanoparticles against S. aureus

As the concentration of nanoparticles increases, the zone of inhibition also increases. This maybe due to the destructive effect of ZnO nanoparticles with the cells and increased production of active oxygen such as H_2O_2, leads to the cell death. ZnO nanoparticles, after its adherence to the surface of the cell membrane, results in disturbance in its respiration as it interact with enzymes of the respiration chains of bacteria. These results were in accordance with the result obtained by others[9].

Colony Forming Units (CFU) in S. aureus when treated with ZnO Nanoparticles

CFU has reduced significantly with increasing ZnO nanoparticle loadings. Thus the present observation indicated that the growth rate of *S. aureus* is much affected by the ZnO nanoparticles. The growth rate of the bacteria is affected due to the interaction of the nanoparticles in the cells. Nanoparticles have larger surface area available for interactions which enhances the bactericidal effect than the large sized particles and hence they impart cytotoxicity to the micro organisms.Our results was in accordance with[13] They reported the enhancing effect of ZnO nanoparticles on antibacterial activity[13].

Growth* rate of *Staphylococcus aureus

At a lower concentration of nanoparticle i.e. at 20 µg/mL and 40 µg/mL the optical absorption is lesser than that at higher concentration. This has been attributed to the reduced growth of the bacterial cells. Higher optical density means greater growth. Hence the concentrations of 20 µg/mL and 40 µg/mL of ZnO nanoparticles have been found to be more effective bactericides than higher concentrations when compared to the control this was due to the higher optical absorption. This suggests that the effect of ZnO nanoparticles is not entirely dependent on increasing concentration of nanoparticles. There is an optimum concentration which has higher effect compared to those concentrations that are more or less than the optimum.

Estimation of Protein

ZnO nanoparticles, which have a good bacteriostatic effect causes membrane disorganisation in Gram Positive organism. The surface modification of ZnO nanoparticles causes an increase in membrane permeability and the cellular internalization of these nanoparticles. This causes changes in the level of Proteins[14]. The level of protein decreased as the concentration of the nanoparticle increases. This may be due to Zinc oxide nanoparticle which is toxic and reactive towards proteins. A possible explanation is that the antibacterial effect of ZnO is based on the abrasive surface texture of ZnO. ZnO nanoparticles have been found to be abrasive due to surface defects[6] and they bind to protein molecules and as a result cellular metabolism is inhibited causing death of micro organism. It is believed that nanoparticles after penetration into bacteria inactivate their enzymes, generate Hydrogen peroxide and cause bacterial cell death[15].

SEM Analysis of Nanoparticles

ZnO nanoparticles were obtained commercially from sigma Aldrich and were characterized by SEM analysis and the sizes of the particles were found to be of less than 100nm.

CONCLUSION

In prokaryotic systems, cell death due to interactions between reactive oxygen species (ROS) and proteins, DNA, or membrane structures can be induced by oxidative stress. There occurs a concern in toxicity and safety issues regarding the expanding growth of nanotechnology and nano biotechnology, and related industrial products. Because of their wide range of practical applications including their use in sunscreens and cosmetics, and these recent indications of their toxic nature, nano scale metal oxides such as ZnO is a current focus of the Nanotechnology Safety Initiative under National Institute of Environmental Health and Safety.

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Experimental observations have explained significantly the antibacterial behaviour of Zinc Oxide (ZnO) nanoparticles. Previous studies with silver (Ag) nanoparticles, which is widely used as a biocide, showed that it is effective only with Gram negative bacteria. In the present study, it is well observed that the zinc oxide nanoparticles can be used as an effective biocide for Gram positive bacteria *Staphylococcus aureus.* From the results obtained in our study it is well understood that the proteins are the important biological molecules which are fundamental to the proper functioning of cells in the micro organisms.

REFERENCES

[1] Nagarajan P, Rajagopalan V, Enhanced bioactivity of ZnO nanoparticles- an antimicrobial study, Scientific Technol Adv Materiol, 7(4): 354-358, (2008)

[2] Edmond MB, Vancomycin-resistant Staphylococcus aureus Perspectives on Measures needed for control, Annual Int Medica, 124: 329-334, (1996)

[3] Shantikumar N, Abhilash S, VV Divya R, Deepthy M, Seema N, K Manzoor Satish R, Antibacterial activity of zinc oxide nanoparticles, J Medica Microbiol, 24: 465-471, (2008)

[4] Ma H, Bertsch PM, Glenn TC, Kabengi NJ, Williams PL, Toxicity of manufactured zinc oxide nanoparticles in the nematode Caenorhabditis elegans, Environmental Toxicol Chem, 28(6): 1324-30, (2009)

[5] Andrew LN, What can be inferred from bacterium–nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles? Ecotoxicol 17: 362–371, (2008)

[6] Darryl NW, Sheryl HE, Tracey RPH, Evaluation of the microbial growth response to inorganic Nanoparticles, J Nanobiotechnol 4(3): 1477-1480, (2006)

[7] Lingling Z, Yunhong J, Yulong D, Malcolm P and David Y, Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids), J Nanoparticle Research, 9: 479–489, (2007)

[8] Li Y, Xiao W, Zhang L, Zuo Y, Xue J, Jansen JA, Synthesis, characterization, and antibacterial activities of a novel nanohydroxyapatite/zinc oxide complex, J Biomedical Materia Research Articles Jun 15, 85(4): 929-37

[9] Jones N, Ray B, Ranjit KT, Manna AC, Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms, FEMS Microbiol Letters, 279(1): 71-6, (2008)

[10] Ohira T, Yamamoto O, Iida Y, Nakagawa ZE, Antibacterial activity of ZnO powder with crystallographic orientation, Appl

Environmental Microbiol, 74(7): 2171-8, (2008)
[11] Sawai J, Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay, J Microbiol Methods, 54(2):177-82, (2003)
[12] Hartree EE, Total Protein Estimation by Lowry's Method, Anal Biochem, 48: 422, (1972)
[13] Banoee M, Seif S, Nazari ZE, Jafari-Fesharaki P, Shahverdi HR, Moballegh A, Moghaddam KM, Shahverdi AR, ZnO nanoparticles enhanced antibacterial activity of ciprofloxacin against *Staphylococcus aureus* and *Escherichia coli*, E publication, 11(3): 77-79, (2010)
[14] Huang Z, Zheng X, Yan D, Yin G, Liao X, Kang Y, Yao Y, Huang D, Hao B, Toxicological effect of ZnO nanoparticles based on bacteria, Langmuir, 24(8):4140-4, (2008)
[15] Sierra J, Ruiz F, Pena D, Gutierrez FM, The Antimicrobial sensitivity of Streptococcus mutants to Nanoparticles of silver, zinc oxide and Gold, Int J Nanoparticles 2(1-6): 402-415, (2008)