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RESEARCH PAPER

The effect of ZnO nanoparticles on bacterial load of experimental infectious wounds contaminated with Staphylococcus aureus in mice

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ABSTRACT

Objective (s): Bacterial infection is an important cause of delayed wound healing. Staphylococcus aureus (S. aureus) is the main agent causing these infections. Zinc Oxide (ZnO) nanoparticles have antibacterial activity and also accelerate the wound healing process. The aim of the present study is to evaluate the effect of ZnO nanoparticles on bacterial load reduction of the wound infection.

Materials and Methods: Broth dilution method was used to determine MIC. The MIC of ZnO nanoparticles was determined 125 µg/ml. ZnO nanoparticles had a bacteriostatic effect against S. aureus and inhibited bacterial growth in in vitro. Thirty six mice were prepared and divided into three groups. Skin wound created on the back of all of them, the bacterial suspension (^{106} CFU of S. aureus) inoculated to each wound site and finally, three groups were treated with 40 µl of ZnO nanoparticles, tetracycline, and normal saline respectively.

Results: Superficial and depth bacterial load were determined on days 7, 14, 21. The results showed that bacterial load reduction of ZnO nanoparticles group was significantly different with the negative control group ($p<0.05$). Significant reduction of the deep bacterial load was observed in the ZnO nanoparticles group comparing to control group on day 21 ($p< 0.05$).

Conclusion: The present results showed that the topical application of ZnO nanoparticles is very effective in the bacterial load reduction. Based on our findings the ZnO nanoparticles may reduce the bacterial load of wound infection so will accelerate the wound healing.

Key words: *Bacterial load, Staphylococcus aureus, Wounds infection, Zinc oxide nanoparticls*

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INTRODUCTION

Bacterial infection causes the postponement in the wound healing process [1, 2]. It is reported that approximately 75% of deaths subsequent burn injury, caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [3]. *S. aureus* is frequently isolated from human skin and mucous membranes. This organism leads to a broad spectrum of skin and acute infections [4]. Also is the most common pathogen in the surgical area infections [5]. One to two percent of people suffer from chronic wounds that lead to increase mortality and cost of treatment. *S. aureus* and *P. aeruginosa* are the most common bacteria detected from chronic leg ulcers [6]. On the other hand emergence of resistant strains to antibiotics are strongly growing such as Methicillin, Penicillin, Vancomycin, and Quinolone resistance that is leading to failure antibiotic therapy [7]. Therefore, alternative treatment programs are a great urgent need today.

Several studies indicated that some inorganic metal oxides have great antibacterial properties. Inorganic metal oxides show more constancy, robustness and long shelf life than organic antimicrobials that it's the major benefits of application inorganic metal oxides [8].

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The local use of anti-infective is the most effective method for wound treatment [9]. The local use of Zinc Oxide (ZnO) improves acute and chronic wounds healing due to antibacterial, anti-inflammatory and increasing re-epithelialization properties [10-13]. The antibacterial properties of ZnO nano-sizes are much more than large particles [14]. Antibacterial effects of ZnO nanoparticles have been indicated toward a wide spectrum of organisms such as *Escherichia coli* and *S. aureus* [8]. Also, the biofilm formation inhibited by *S. aureus* and *P. aeruginosa* [15, 16].

Although, ZnO nanoparticles have been frequently reported with antibacterial properties but the very limited studies are available to treat the bacterial infections on the *in vivo*. The antibacterial activity of ZnO nanoparticles is affected by particle sizes and concentration. ZnO nanoparticles antibacterial activity directly correlates with their sizes. This dependency is also influenced by the concentration of NPs. ZnO nanoparticles with a smaller size (higher specific surface areas) showed highest antibacterial activity [8]. So in the present study, the particle of ZnO nanoparticles with sizes 10-30 nm was used.

In the present study, the effect of ZnO nanoparticles investigated on bacterial load of experimental infectious wounds contaminated with *S. aureus* in mice.

MATERIALS AND METHODS

Bacterium and Zinc Oxide nanoparticles

S. aureus (ATCC 25923 - MAST Company, UK) was provided by the Faculty of Veterinary Medicine, Urmia University. ZnO nanoparticles with an average size of 10 – 30 nm were purchased from US Reasearch Nanomaterials, Inc. USA.

Bacterial suspensions

To prepare a bacterial suspension, bacteria were cultured in Mueller Hinton Broth that was incubated for 18 h at 37° C. The bacteria were centrifuged at the 10000 g for 10 min at 4° C. The supernatant was discarded and bacteria were washed twice with phosphate-buffered saline (PBS) solution and finally were dissolved in PBS solution. The bacterial suspension was measured in OD 600nm (10^8 CFU/ml) based on the turbidity of 0.5 McFarland [17].

MIC determination

Broth dilution method was used to determine MIC (minimum inhibitory concentration). Generally, serial doubling dilutions of ZnO nanoparticles were prepared using Mueller-Hinton broth and finally, fresh culture bacteria was added to each test tube then incubated using shaking incubator for 24 hours at 37° C. Inhibition of cell growth was defined by counting the amount of CFUs on the plates or by the turbidities of the cell cultures. The first test tube that showed no change in turbidity were further proved for bacterial culturability by spreading 100-µl of the broth cultures onto Mueller-Hinton agar plates to determine the bactericidal or bacteriostatic effect of ZnO nanoparticles [18].

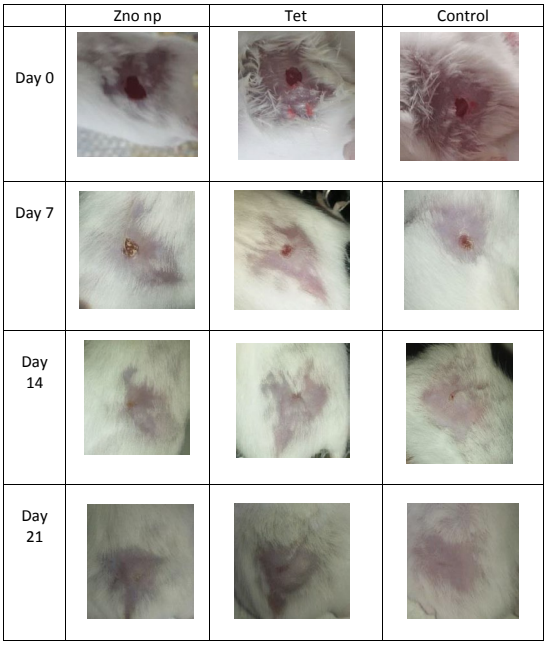

Fig 1 .Photographs of wounds in ZnO nanoparticles,Tet and negative control on day 0, 7, 14 and 21

Mice wound infection and treatment

Thirty-six male mice (20-30 g) were prepared and kept up with standard pellet diet and water ad libitum for 14 days to be adapted before the examination and their health was evaluated. Also during the study were maintained under pathogen free conditions. Mice were randomly divided into three groups (n=12) including ZnO nanoparticles, tetracycline (positive control) and normal saline (negative control). Mice were kept in accordance with the international guidelines principles of laboratory animal use and care [19]. All Mice were anesthetized with ketamine (100 mg/kg, Woerden, Netherland) and xylazine (5 mg/kg,

Table 1. Superficial bacterial load average in wounds area of experimental groups post treatment*

Day	Bacterial count of ZnO nanoparticles group	Bacterial count of positive control group	Bacterial count of negative control group
7	3.2×10 CFU/100µl	3.3×10 CFU/100µl	3.6×10^3 CFU/100µl
14	4.6×10 CFU/100µl	1.2×10^2 CFU/100µl	2.2×10^3 CFU/100µl
21	CFU/100µl	30 CFU/100µl	69 CFU/100µl

*Result of Tukey,s analysis indicate significant different (p< 0.05) in the between groups of ZnO nanoparticles and positive control groups with negative control on day 7, 14 and 21

Woerden, Netherland). The back of the mice were disinfected with 70% ethanol and hair shaved [20] and full thickness skin wounds (3 mm in diameter) were created by a sterile punch biopsy [21]. The immediately wound area were inoculated using 10 µl (10^6 CFU) of bacterial suspensions to each wound site. After the infection, the treatment was performed in all groups [19, 22]; ZnO nanoparticles group: 40 µl of ZnO nanoparticles, Tetracycline group: 40 µl of tetracycline (8 mg/kg) [22], control group: 40 µl of normal saline.

Two tests were performed for assessment of bacterial load. Swab test: This test is to determine the bacterial load of the wound surface. From the wound surface was sampled with a sterile swab. Samples were transferred to a suitable the transmission medium. Serial dilutions of the suspension ($1:10^3$ to $1:10^{12}$) were made with sterile broth media and were cultured on Mueller-Hinton agar for numbering the bacterial load.

The second test was performed to determine the bacterial load in the deep tissue. For this purpose, the wound with adjacent normal tissue (2.5 × 2.5 cm2) was cut out. Tissue samples were weighed and homogenized with a pestle then were solved in 2 ml sterile PBS. Finally, the samples were serially diluted with sterile broth media and cultured in Mueller-Hinton agar for numbering the bacterial load ($1:10^3$ to $1:10^{12}$) [23]. The bacterial load of tissue was estimated by:

CFU/Gram= Plate Count × (1/dilution) × 10/ Wt. of Homogenized Tissue

Table 2. deep bacterial load average in wounds area of experimental groups post treatment*

Treatment	Day	Bacterial count
ZnO nanoparticles	21	1×10^2 CFU/g
Positive control	21	1×10^3 CFU/g
Negative control	21	1.4×10^4 CFU/g

*Result of Tukey,s analysis indicate significant different (p< 0.05) in the between groups of ZnO nanoparticles and positive control groups with negative control on day 7, 14 and 21

Statistical analysis

All Statistical data were analyzed by a one-way ANOVA with Tukey-Kramer post-test using SPSS 16.0 (Chicago, IL). Values of p< 0.05 were considered statistically significant.

RESULTS

Minimum Inhibitory Concentration (MIC)

Bacterial growth was prevented at 125 µg/ml of ZnO nanoparticles. The results showed that ZnO nanoparticles had a bacteriostatic effect towards *S. aureus* and inhibited bacterial growth.

Superficial bacterial load

Counting the bacterial load of the wound surface showed reducing the bacterial amount in the treated groups (Table 1). Bacterial load reduction was observed in ZnO nanoparticles and tetracycline groups on day 7, 14, 21. The primary inoculation of bacteria was nearly 10^6 that it reaches to 0 CFU/100µl (Table 1) and 1×10^2 CFU/g (Table 2) on day 21. The ZnO nanoparticles group showed the quick decline at day 0 to day 21. The greatest amounts of bacteria were determined in the negative control group. Reduction of the bacterial load was significant in ZnO nanoparticles on days 7, 14 and 21 compared with negative control group (p< 0.05) So that no bacterial growth was observed on day 21 (Table 1). The ZnO nanoparticles group showed a reduction bacterial load compared with tetracycline group on days 7, 14 and 21, but the difference was not statiscally significant.

However, results of bacterial load were significant reduce in the ZnO nanoparticles and Tet groups comparing to control group particularly on day 21 (p< 0.05).

Deep bacterial load

The reduction of bacterial growth in ZnO nanoparticles group was observed so that in some samples did not grow any bacteria (Table 2). By

day 21, the treated group showed decreases in deep skin bacterial concentration (1 ×10^2 CFU/g) compared with negative control group (1.4 ×10^4) (Table 2).

However, significant decrease ($p< 0.05$) in deep skin bacterial load in the ZnO nanoparticles and tetracycline group were found comparing with control group. The ZnO nanoparticles group (1 ×10^2 CFU/g) showed a reduction in deep bacterial load compared with tetracycline group (1 ×10^3 CFU/g), but the difference was not statiscally significant.

DISCUSSION

The emergence of antibiotic resistance threaten public health [24]. *S. aureus* has recognized as a common agent of infection and is responsible for a board spectrum of superficial and acute skin infections [4]. In the United States, 11 million people admit to the hospital due to these infections and 464 thousand people hospitalize annually [4]. Bacterial contamination of wounds led to the delayed wound healing process [25] and due to the abundance of antibiotic resistance [7], antibiotic therapy is not efficient. Today researchers are presented new alternative antibacterials such as metal nanoparticles. Among the metal nanoparticles, ZnO nanoparticles are highly regarded. Many studies have shown that ZnO nanoparticles have a potential antibacterial effect [8, 26]. Antibacterial properties of Zinc Oxide related to reactive oxygen species (ROS) production which destroys bacterial cell wall and thereby causes the death of the organism [27]. Local application of zinc oxide accelerate wound healing according to the anti-bacterial, anti-inflammatory, increase reepithelization and activation of metalloproteinase enzymes properties researchers have suggested [11, 28].

In this study, we demonstrated that ZnO nanoparticles have a bacteriostatic effect against *S. aureus* and it inhibited the growth of bacteria at a concentration of 125 µg/ml. Jones *et al* also demonstrated that nanoparticles of zinc oxide have many uses as a bacteriostatic agent [26]. Also, Zhang *et al* [29] showed the bacteriostatic effect of ZnO nanoparticles against *E. coli*, but Xie et al [18] demonstrated that the action of ZnO nanoparticles against *C. jejuni* was bacteriocidal. Raghupathi *et al* [8] showed that antibacterial effect of ZnO nanoparticles is inversely related to its size. So in the peresent study ZnO nanoparticles with sizes, 10-30 nm were used to raise the antibacterial effect of ZnO nanoparticles in wound infection treatment. Several studies have shown that topical application of ZnO nanoparticles is increased angiogenesis [30] and wound healing [31].

In the present study, the effects of ZnO nanoparticles and tetracycline in reducing the bacterial load of the wound infection investigated. In this study, the hypothesis that the ZnO nanoparticles along enhance bacterial clearance during wound healing contaminated with *S. aureus*. Tetracycline was considered as a positive control or standard. According to previous studies, infection rates directly related to the amount of inoculated bacteria. Inoculation of 10^6 microorganisms can cause the infections without mortality [32].

Our results showed that the use of zinc oxide nanoparticles is quite effective in reducing the surface and deep bacterial load on days 7, 14, 21. The surface infection level was significantly improved at later time points in treatment groups.

The reduction of the bacterial load was more significant different compare with control group especially on day 21. However, results of bacterial load were not statistically significant among ZnO nanoparticles and positive groups. Paty *et al* in 2015 reported that local application of ZnO nanoparticles reduced the bacterial load in skin infection created by *S. aureus* [16]. These results are consistent with our findings and suggest that ZnO nanoparticles alone is effective in reducing the bacterial load of the wound and can be used to prevent of wound infection.

One of the main criterions on drug therapy is to be non-toxic to cells. ZnO nanoparticles toxicity depends on their concentrations and sizes. It is proposed low concentrations of ZnO nanoparticles are nontoxic to eukaryotic cells. Paty et al 2015 showed that ZnO nanoparticles at the bactericidal dose have no detrimental effects on PBMCs and THP-1 cells and prevented the lysis of RBCs by *S. aureus* and also significantly decreased the skin infection, bacterial load, and inflammation in mice [33].

CONCLUSION

The present study has shown the antibacterial effect of ZnO nanoparticles on *S. aureus* so reduced the bacterial load of wounds. Based on present findings, local application of ZnO nanoparticles may help wound healing processing due to the reduction of bacterial load.

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CONFLICT OF INTEREST

Author has no received research grants. The author declares that he has no conflict of interest.

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