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Zinc oxide nanoparticles accelerate the healing of methicillin-resistant *Staphylococcus aureus* (MRSA)-infected wounds in rabbits

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ABSTRACT

Objective: To synthesize zinc oxide nanoparticles (ZnONPs) and evaluate their antibacterial and wound healing effects against wounds infected with methicillin-resistant *Staphylococcus aureus* (MRSA).

Methods: ZnONPs were prepared by sol-gel method and characterized by X-ray diffraction (XRD) analysis and scanning electron microscopy (SEM). A total of 18 rabbits were divided into three groups: the ZnONPs group, the gentamicin group and the control group. A wound of 3 cm^2 was inflicted on each rabbit and contaminated with MRSA inoculum. Treatment was started from the fourth day post-surgery. Wound healing, microbiological analysis, and histopathological analysis were performed to assess the efficacy of ZnONPs ointment.

Results: XRD analysis confirmed the hexagonal wurtzite structure of the ZnONPs with an average crystallite size of 29.23 nm. SEM revealed discoid-shaped ZnONPs with a rough surface and an average size of 48.36 nm. Energy-dispersive X-ray analysis confirmed the purity of ZnONPs. Moreover, the particle size ranged from 100-700 nm with a high agglomeration trend. Treatment with ZnONPs promoted MRSA-infected wound healing. In addition, ZnONPs showed a good antibacterial effect as evidenced by a dose-dependent increase in the zone of inhibition.

Conclusions: ZnONPs accelerate the healing of MRSA-infected wounds. Therefore, it can be explored for the treatment of MRSA infection.

KEYWORDS: MRSA; Wound healing; ZnO; Nanoparticles; Ointment

1. Introduction

Wound healing is a highly regulated and complicated process that can be affected by various diseases and factors[1,2]. The healing process has been disoriented as a result of wound because of the interrupted skin barrier. Hemostasis, inflammation, proliferation and tissue remodeling are the four stages of wound healing[3]. These phases cannot progress through the proper sequence in acute and chronic wounds which leads to pathological inflammation due to an uncoordinated healing process. A recent study found that medicare costs between $28.1 to $96.8 billion annually in the USA[4]. The factors that affect wound healing are categorized as local factors

Significance

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is a serious problem that needs to be addressed urgently. The present study shows that zinc oxide nanoparticles have the potential to treat MRSA infection by accelerating wound healing and inhibiting bacterial growth. Further studies are needed to investigate the optimal dosage and formulation of the zinc oxide nanoparticles for the treatment of MRSA-infected wounds.

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(oxygenation and infections) and systemic factors (age, sex, and hormones). Microorganisms present normally in the skin get access to the injured skin. Wound contamination refers to the presence of bacteria on the surface of a wound without multiplying, while wound colonization refers to the replication of bacteria on the wound surface without invading the wound tissue or triggering a host immune response. Both contamination and colonization are typical in wound healing by secondary intention. Furthermore, the mere existence of organisms in nonviable tissue without invading viable tissue does not qualify as a wound infection[5].

Wound infections can be mild and self-healing or severe and life-threatening. The most frequent species of microorganisms that cause wound infections are *Acinetobacter* spp., *Pseudomonas* spp., *Escherichia coli*, *Klebsiella*, *Proteus*, *Enterobacter*, *Citrobacter*, and anaerobes such as *Clostridium* and *Peptostreptococcus* spp.[6]. It has become more challenging to treat wound infections as a result of the presence of fungi, polymicrobial flora, and methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA). Antimicrobial resistance, which has become a great threat to public health in the world, is also a major problem in all clinical settings[7]. MRSA is one of the most common strains of wound infections, affecting large areas of the skin that are deeper into the soft tissues, including abscesses, burns, cellulitis, or deep ulcers with infections.

Applications of nanoparticles (NPs) have significantly increased recently[8]. NPs have been utilized successfully in the administration of medicinal substances, in the diagnosis of diseases, to lessen bacterial infections in burn wounds and skin, and to stop bacterial colonization on medical devices. To combat the issue of drug resistance, NPs are a desirable substitute for antibiotics due to the possibility of producing next-generation antibiotics and The United States Food and Drug Administration has classified ZnO as safe (21CFR182.8991). They also have a unique mode of action and powerful antimicrobial activities against a spectrum of bacteria[9]. Numerous matrix metalloproteinases contribute to the healing process and are activated by zinc, an essential cofactor. Zinc-dependent matrix metalloproteinase encourages keratinocyte migration, endothelial cell proliferation, and re-epithelialization, all of which increase angiogenesis[10]. Due to their numerous uses in both human and veterinary medicine, nanostructures made of ZnO are quite significant. They are advantageous due to their antibacterial, antineoplastic, antigenic, and wound-healing properties. Due to their small size, they have strong macromolecular healing properties and can fight foreign diseases by acting as an antimicrobial agent[11] and enhance wound healing by acting as antimicrobial adhesive for tissues[12]. Considering the abovementioned benefits of ZnO nanoparticles (ZnONPs), the current study was designed to assess the antibacterial and wound healing effects of ZnONPs on MRSA-infected wounds.

2. Materials and methods

2.1. Ethical approval

The University of Veterinary and Animal Sciences' Ethics Committee, located in Lahore, Punjab, Pakistan, provided its clearance, Vide no: 370/(13-09-2021).

2.2. Bacterial culture

MRSA stock culture was taken from the Institute of Microbiology, University of Veterinary and Animals Science, Lahore, Punjab, Pakistan.

2.3. Synthesis of ZnONPs

In order to synthesize powdered ZnO, 200 mL precursor solutions of 0.25 M $Zn(CH_3COO)_2 \cdot 2H_2O$ and 0.5 M NaOH were prepared in deionized water. The solution was slowly combined for 10 min with constant stirring. The precipitated form of ZnO was stirred for 6 h using the sol-gel technique to create a suspension. After filtering and many washes in deionized water, the colorless residue was recovered. The gel created was dried for 5 h at 80℃ in a vacuum oven and milled into a fine powder[11].

2.4. ZnONPs characterization

ZnONPs were characterized by scanning electron microscopy (SEM, Quanta 250. FEG, USA), energy dispersive X-ray analysis (EDX, INCA Oxford Instruments, UK), X-ray diffractometry (XRD, Jeol JDX- 3532, Japan) and DLS (Zeta sizer Nano ZS, Melvern, USA)[13].

2.5. Antibacterial effect of ZnONPs

The antibacterial effect of ZnONPs was assessed by agar well diffusion method. ZnONPs at 3 mg/mL were dissolved in propylene glycol[14,15]. The pure culture of MRSA was grown to attain 0.5 McFarland turbidity diluted to 1×10^8 CFU and spread uniformly on Muller Hinton agar plates, a sterile cork borer of 6 mm diameter was used to make two wells in each plate. After swabbing or spreading of MRSA culture, wells were filled with 50 µL (150 µg) and 100 µL (300 µg) of ZnONPs solution respectively. Plates were then incubated at 37℃ overnight and zone of inhibition was measured.

2.6. Assessment of wound healing effect of ZnONPs

2.6.1. Preparation of ointment

Ointment (1% ZnONPs ointment) was prepared from the synthesized ZnONPs by mixing 1 g of ZnONPs in 99 g of petroleum jelly[11].

2.6.2. Experimental model

For the experimental trial, a total of 18 disease-free rabbits of both sexes (6 and 8 weeks old), weighing between 1 500 and 2 000 g were chosen. These rabbits were kept in the animal housing facility at the Department of Veterinary Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan. All the rabbits were acclimatized for 2 weeks. During the trial, clean bedding and adequate ventilation were provided along with a light duration of 8 to 10 h and indoor temperature of 25 ℃ to 30 ℃. Seven days before the start of the trial, 40 mg/kg bodyweight of ivermectin was subcutaneously injected for deworming. Wheat straw was used for the bedding, which was replaced every day. Fresh food was given to the rabbits twice daily. Rabbits were randomly assigned to three treatment groups, six rabbits in each group. After shaving the experimental area, wounds of 3 cm^2 diameter were created on the lateral side using a surgical blade. MRSA inoculum was added to the wound[16]. Ten microliter (10 μL) of the bacterial suspension (10^8 CFU/mL) was added in each wound[17]. Treatment of wounds was started three days after wound contamination to allow the establishment of infection. Wounds were treated topically, twice daily until day 36. Group 1 was treated with ZnONPs, group 2 was treated with gentamicin cream, and group 3 was kept as a control and was treated with normal saline.

2.6.3. Infection confirmation

Skin and soft tissue infections are clinical conditions that involve microbial invasion of the skin's layers and the underlying soft tissues. Skin and soft tissue infections can vary in etiology, severity, and presentation. Pyoderma is a minor infection, while necrotizing fasciitis is a serious, potentially fatal condition[18].

After three days of wound contamination, infection was confirmed by observing the pus formation at the wound site. Later, the wound specimen was taken in a sterile tube and sent to the laboratory for MRSA identification. The MRSA was identified by colony characteristics, microscopy, and phenotypic confirmation by disk diffusion test. Briefly, the specimen was cultured in Tryptic Soya Broth (TSB) at 37 ℃ for 12-16 h for the enrichment, and later, streaking was done on blood agar to check hemolytic pattern following the same incubation conditions. The isolated colony was picked and streaked on Mannitol Salt Agar (MSA), which acts as differential media and differentiates between mannitol fermenters and non-fermenters. The appearance of yellow colonies[19] on MSA indicates mannitol fermenter, and *S. aureus* is positive for MSA. The final confirmation was done by various biochemical tests such as tube coagulase test[20,21], catalase test, *etc*. After confirmation of *S. aureus*, the single isolated colony was picked and subcultured in nutrient broth supplemented with salt to restrict the contamination of other *Staphylococci*. The disk diffusion assay was done for phenotypic confirmation of MRSA. Briefly, the inoculum turbidity was adjusted to 0.5 McFarland Standard and swabbed on Mueller-Hinton agar, and 1 μg oxacillin discs were placed aseptically, and plates were incubated at 37 ℃ for 18-20 h. After incubation, the zone of inhibition around the disks was compared with standard zones in Clinical Laboratory and Standards Institute guidelines[22] to declare it MRSA.

2.6.4. Wound size and contraction rate

Wound size was measured at regular intervals on days 4, 7, 14, 21, and 28 by using a Vernier caliper. Wound contraction rate was also evaluated by using the formula[23].

Wound contraction rate=(Wound contraction at specific day−Wound size at day 0)/Wound size at day 0×100

2.6.5. Histopathological examination

All the rabbits were anesthetized first by using a combination of xylazine and ketamine (4.3 mg/kg and 29.1 mg/kg, *i.m.*), then a lethal dose of ketamine (600 mg, *i.v.*) was injected to euthanize the rabbits[24]. Histopathological examination was performed to evaluate wound healing in different treatment groups using a microscope (LABOMED® USA). A skin tissue sample of healed skin was taken at the end of the study, fixed in 10% neutral buffered formalin, and stained with hematoxylin and eosin[25]. Histopathological examination of the treated groups was performed with the software LABOMED PixelPro. Measurements of the epidermal, upper, and lower dermal regions of tissues were performed under the light microscope (LABOMED® USA) at 10×.

2.7. Statistical analysis

All the experimental results were expressed as mean ± standard deviation. Statistical data was analyzed by one-way analysis of variance (ANOVA) through SPSS version 25 statistical computer software and $P<0.05$ was considered significantly different[25].

3. Results

3.1. ZnONPs characterization

3.1.1. Structural properties of ZnONPs

Since ZnO was synthesized by reacting NaOH with $ZnSO_4$, the as-synthesized ZnO was structurally characterized using XRD. The diffractogram showed all sharp and standard peaks for hexagonal wurtzite ZnO crystal (JCPDS card no. 36–1451). The peak (002) at 34.135° (2θ) facet appeared slightly longer as compared to that obtained at (100) 31.326° (2θ) facet. As indicated in diffractogram of synthesized ZnONPs, the main peak appeared at 36.275° (2θ) (Figure 1A). Crystallite size calculated using FWHM 0.393 of highest peak was 29.23 nm. Peaks at 100 and 002 confirmed the

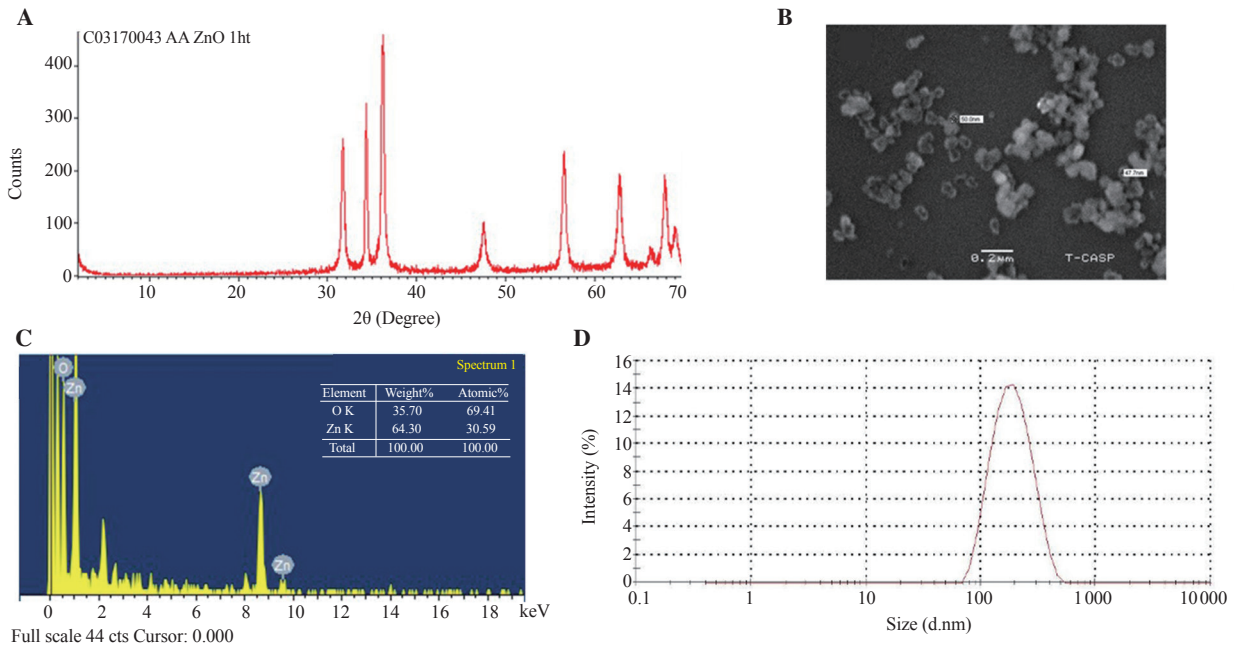


Figure 1. (A) X-ray diffractometry, (B) scanning electron microscopy, and (C) energy dispersive X-ray analyses as well as (D) size of the synthesized zinc oxide nanoparticles (ZnONPs).

unequal trend of growth towards a-axis and c-axis of the unit cell of hexagonal crystal. The absence of any extra peak confirmed the purity of the synthesized sample. Lattice constants found were a =3.248 0 Å and c=5.212 4 Å giving the aspect ratio 1.601.

3.1.2. Morphology and elemental composition of ZnONPs

The surface and morphology of ZnONPs were determined by SEM (Figure 1B). Discoid NPs of low thickness were prepared by sol-gel method. The dimensions of oval-shaped nanodiscs were measured by Image J software. The average particle was 48.36 nm. The discs had a rough surface to capture bacteria. The surface was supposed to be positively charged to attract negatively charged bacteria.

The elemental composition of ZnO was determined by EDX (Figure 1C), which provided the spectrum with peaks of Zn and O. The absence of any other peak confirmed the purity of the sample prepared. The weight % of Zn was higher but atomic % of O was much higher than Zn. This fact was favored for reactive oxidative stress (ROS) production, as the concentration of surface-adhered oxygen was very high. The high surface charge could also be justified by a higher concentration of oxygen detected at the surface.

3.1.3. Particle size of ZnONPs

The particle size was determined by diffused light scattering technique by dispersing powdered ZnO in DI water. The results indicated that particle size ranged from 100-700 nm and particles increased in size due to high agglomeration trend of strongly charged particles (Figure 1D).

3.2. Confirmation of MRSA infection

Pus was observed as a sign of infection and a sample was taken by swab for further evaluation. Growth of *S. aureus* colonies was confirmed on MSA (Supplementary Figure 1A). Catalase and coagulase positive test was confirmed (Supplementary Figure 1C). In response to the phenotypic confirmation of MRSA, the infection was confirmed as a contaminated wound due to the absence of a zone of inhibition against the 1 μg oxacillin disk. Furthermore, the morphological examination showed that *S. aureus* appeared round and formed grape-like clusters (Supplementary Figure 1B), indicating rabbit wounds were infected with *S. aureus*.

3.3. Antibacterial effect of ZnONPs

The average mean of the zone of inhibition around the well having 50 μL of (150 μg) ZnONPs was 11.9 mm while around the well having 100 μL (300 μg) of ZnONPs showed a 14 mm zone of inhibition. However, no zone of inhibition was observed around the disc having 1 μg oxacillin. Therefore, the antibacterial effect of 300 μg ZnONPs was higher than that of 150 μg (Figure 2).

3.4. Wound healing time

ZnONP ointment treatment significantly promoted wound healing as compared to the gentamicin-treated group and the control group ($P < 0.05$). The average time of would healing was (23.33 ± 1.49) days for the ZnONPs-treated group and (27.66 ± 0.69) days for the

gentamicin-treated group. In contrast, the wound in the control group was not healed till (34.00 ± 1.88) days (Figure 3).

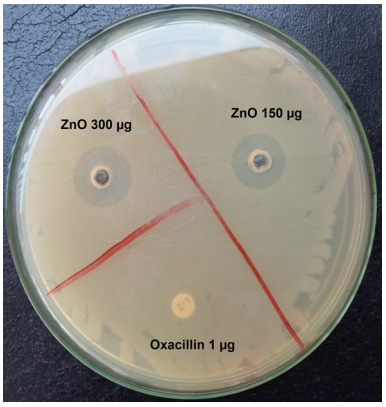


Figure 2. Zones of inhibition of ZnONPs against MRSA.

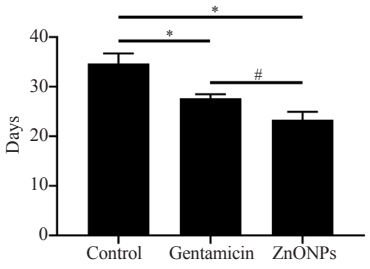


Figure 3. Wound healing time of rabbits treated with ZnONPs. Data are analyzed by one-way ANOVA followed by Dunnett's test. $^{*}P<0.001$ compared to the control. $^{\#}P<0.001$ compared to the gentamicin group.

3.5. Wound contraction rate

Wounds were contaminated with MRSA and kept untreated for initial three days, therefore, pus was evident and wounds did not contract. As the treatment started, wounds started healing and decreased in size at different rates in different groups. Wounds were completely healed in the ZnONPs-treated group as compared to the gentamicin-treated group and the control group. The ZnONPs-treated group showed faster wound healing in terms of wound contraction rate and all the glandular structure of the skin was effectively recovered compared with other groups (Figures 4 and 5).

3.6. Histopathological analysis

Histopathological examination revealed the complete absence of keratin layer and fused dermal layers, there was no complete distinction between upper and lower dermal layers in the control group. Newly formed blood vessels, less amount of collagen fiber content, and skin glandular structures were observed in the gentamycin-treated group. In addition, in this group, epidermal thickness and the number of epidermal keratinocytes were not fully recovered. Disruption in stratum cornium (keratinized layer) and re-epithelization was also observed. In the ZnONPs-treated group, epidermal thickness, ample amount of collagen fibers, angiogenesis, and recovered keratin layer were found. The epidermal layer of the ZnONPs-treated group was thicker as compared to other groups (Figure 6).

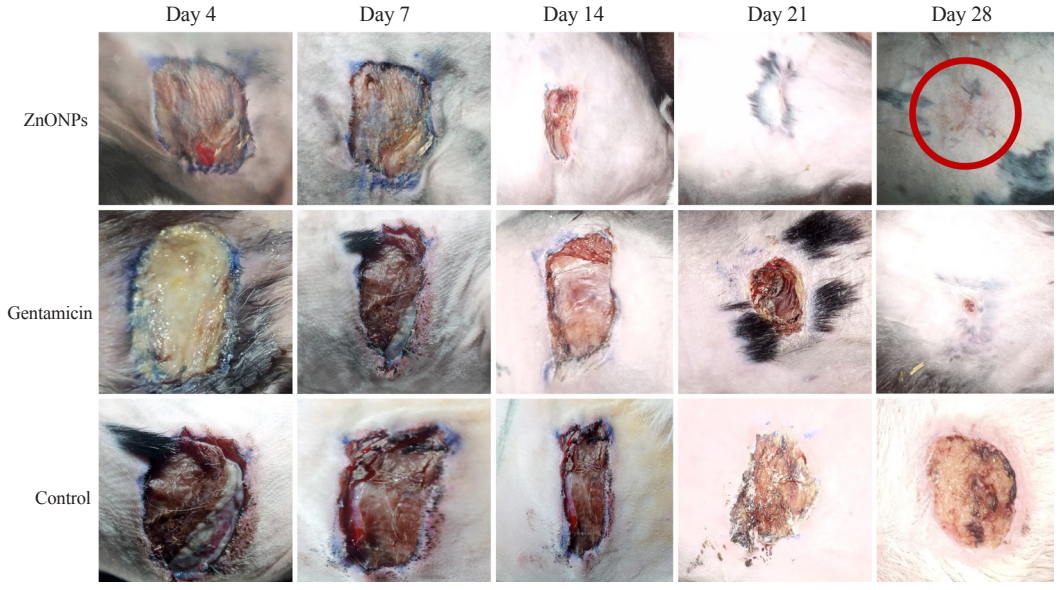


Figure 4. Morphological appearance of wound healing on different days in rabbits.

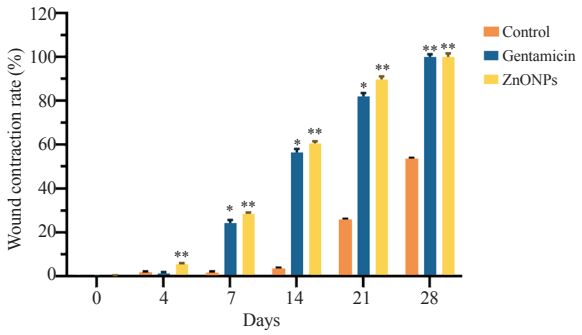


Figure 5. Wound contraction rate after ZnONPs treatment. Data are analyzed by two-way ANOVA followed by Dunnett's test. The symbol denotes that the values significantly differ between the control and treated groups ($^{*}P<0.05$, $^{**}P<0.01$).

4. Discussion

Due to the growing issue of resistance against traditional antibiotics, treating bacterial infections has recently become a critical concern. Since many antibiotics cannot efficiently diffuse across cell membranes and have minimal effectiveness inside the cells, treating intracellular infections continues to be very difficult. Therefore, it is crucial to find alternate healing methods, such as a new class of antibiotics that can eradicate drug-resistant bacteria without endangering the health of the host cells.

Infection from mono- or polymicrobial aerobic and anaerobic microorganisms that are resistant to biocides and can form dense biofilms frequently makes wound healing more difficult. Because of their heightened phenotypic resistance to biocides and host defense mechanisms, these specialized multicellular microbial aggregates significantly diminish the efficacy of antimicrobial therapies[26]. The most difficult aetiologies in the treatment of chronic wounds are *S. aureus* and a few enterobacterial species because of their ability to build biofilms that are difficult to remove[27]. Exopolysaccharides produced by *S. aureus* and *Pseudomonas aeruginosa* are known to be essential for the establishment of a complex biofilm structure as well as the initial attachment of bacteria to host cells[28], which makes it challenging to treat with antibiotics and host defenses. Metallic NPs prevent the formation of the biofilm by hindering exopolysaccharide synthesis which is the main characteristic to exert anti-biofilm activity[29]. For cell-cell attachment and the binding of outside molecules in *S. aureus*, glucosamine-based polysaccharide intercellular adhesion molecules are in charge[30]. These variations in anti-biofilm action could be caused by the varying strengths with which ZnONP binds to the various components of bacteria. *Streptococcus mutans* cannot form a biofilm when coated with ZnONPs, according to previous research[31]. Every therapeutic chemical must alter the functions of the target molecule without endangering mammalian cells. In a previous study, we demonstrated that ZnONPs, when administered at bactericidal doses, had no deleterious effects on peripheral blood mononuclear cells or THP-1 cells. The viability of the cells was decreased by treatment with greater dosages of ZnONPs. These findings demonstrate ZnONPs' applicability as an antibacterial chemical[9]. Confocal microscopy investigations revealed co-localization of bacteria and ZnONPs, indicating that the bacterial death was brought about by the direct contact between the NPs and the bacteria inside the macrophages as well as ROS generation[9].

Normal redox signals are harmed while infected wounds heal, which causes ROS levels to increase[32], followed by a compromised antioxidant defense function[33]. Additionally, oxidative stress occurs when ROS are produced excessively, impairing the functionality of skin fibroblasts and keratinocytes[33]. ROS is intimately associated with inflammation, according to numerous studies[34], and can hinder the healing of wounds in a number of ways[35]. In order to increase skin wound healing, an efficient technique must be investigated

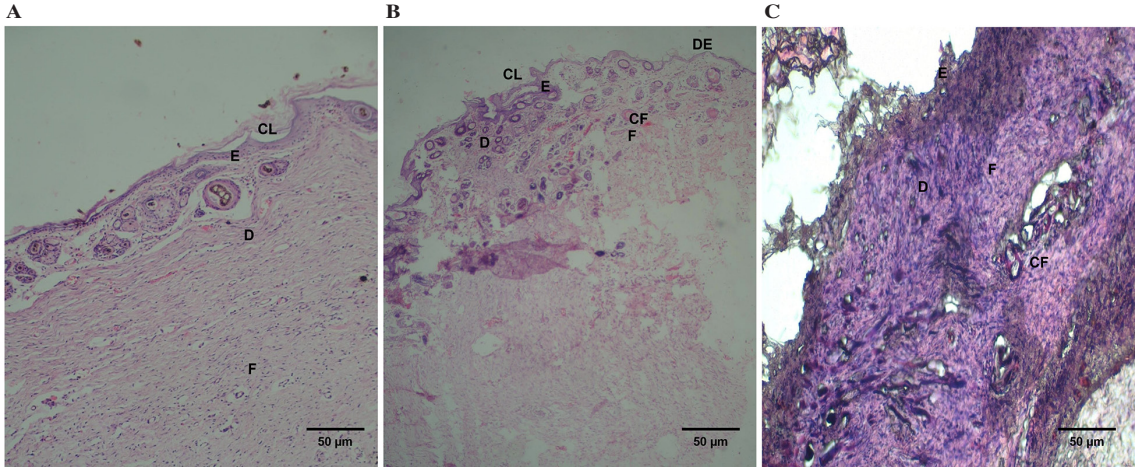


Figure 6. Histopathology of different treatment groups. (A) ZnO-NPs; (B) Gentamycin; (C) Control. A lack of the keratin layer is observed in the control group, while gentamycin treatment results in only partial regeneration of skin, blood vessels, and collagen. Thicker epidermis, collagen, angiogenesis, and keratin recovery are all observed after ZnONPs treatment, demonstrating improved results. E=epidermis, D=dermis, DE=disruption of the epidermis, CL=cornified (keratinized) cell layer, F=fibroblasts, CF=collagen fibers.

to simultaneously boost the antibacterial, ROS scavenging, and anti-inflammatory capabilities[36]. The intrinsic antibacterial characteristics of NPs or their use as drug carriers in nanotechnology offer intriguing new avenues for the development of effective antimicrobial wound dressings. Metal NPs are currently being used more frequently in the creation of nanostructured skin-care dressings and coatings (such as silver, gold, zinc oxide, and magnetite). Despite being metallic, these NPs are most frequently used because of their special abilities to exert antibacterial activity and penetrate deeply into the skin[37].

ZnONPs' antibacterial properties have drawn substantial interest on a global scale, especially since nanotechnology was used to create particles with a diameter in the nanometer range. ZnONPs have appealing antibacterial properties that promote their usage as a therapeutic antimicrobial agent. Through many methods, ZnO can cause bacterial inhibition or death. ZnO can directly destroy cell integrity of bacteria, cause the release of Zn^{+2} ions as antimicrobial, and produce ROS that can penetrate bacterial membranes and cause bacterial damage and death[38]. The strongest indicator of MRSA and methicillin-resistant *Staphylococcus epidermidis*'s vulnerability to ZnONPs may be related to their cell wall plasmolysis, or the detachment of their cytoplasm from their cell wall, as the harmful effect of ZnONPs on bacterial surface was visualized by the examination using electron microscopy[39].

This study was planned to combat the problem of antibacterial resistance affecting the normal wound healing cascade. For this purpose, ZnONPs were used to evaluate their wound healing potential against MRSA-contaminated wounds in an *in vivo* model along with *in vitro* experiments. The finding of this study revealed that ZnONPs have an antibacterial effect against MRSA and promote the healing in MRSA-contaminated wound. The average size of ZnONPs was measured as 100-700 nm which was almost similar to the study conducted to evaluate the antibacterial effect of ZnONPs against MRSA in mouse skin healing[9]. ZnONPs showed significant antibacterial effects compared with its original large size particles[40]. Different parameters including the wound healing time and wound contraction rate, histopathological changes and reconstruction of damaged cells along with *in vitro* antibacterial activity of the ZnONPs were evaluated. All the parameters showed positive changes in the group treated with ZnONPs as compared to the gentamicin and control groups. Regarding wound healing time, our result was also in agreement with the study in which topical application of ZnONPs hydrogel was applied, and wound was completely healed in two weeks[41]. *In vitro* antibacterial results showed that ZnONPs induced different zones of inhibitions against MRSA. The antibacterial effect of ZnONPs is also supported by another research[14]. The slightly larger zone of inhibition reported previously is due to the small size of NPs (21.56 nm) as compared to the present study[42]. These results are similar to the study that reported enhanced or better wound healing by ZnONPs in bacterial-contaminated wounds in mice than gentamicin[43]. Our results are also similar to the study in which MRSA which has been reported to be resistant to a variety of broad-spectrum antibiotics was successfully inhibited by synthetic ZnONPs[39]. Gram-positive bacteria were more susceptible to ZnONPs in comparison to Gram-negative bacteria[44]. In the future, compositions using ZnONPs may be used for exterior uses as antibacterial agents in ointments and lotions because numerous studies have shown that families of unrelated hydrophobic groups are equally effective in killing germs[45].

In conclusion, this study highlights the promising antibacterial properties of ZnONPs, demonstrating their effectiveness against MRSA and their potential to enhance wound healing. ZnONPs exhibited superior antibacterial activity compared to larger particles, with positive effects on wound closure time, contraction rate, histopathological changes, and cell regeneration. These findings suggest a valuable role of ZnONPs in combating antibiotic resistance and improving wound care, particularly for Gram-positive bacteria, opening avenues for their use in topical formulations like ointments and lotions. However, further investigation is required to verify the effect of NPs against other Gram-positive and Gram-negative bacteria along with the identification of wound healing and antibaceterial mechanism of action of the ZnONPs.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

MA, ASC and AA designed the work and contributed to the concept. MA, HBR, MHS, HBA and AA performed experiments and data validation. MA wrote original draft, and ASC and AA performed the critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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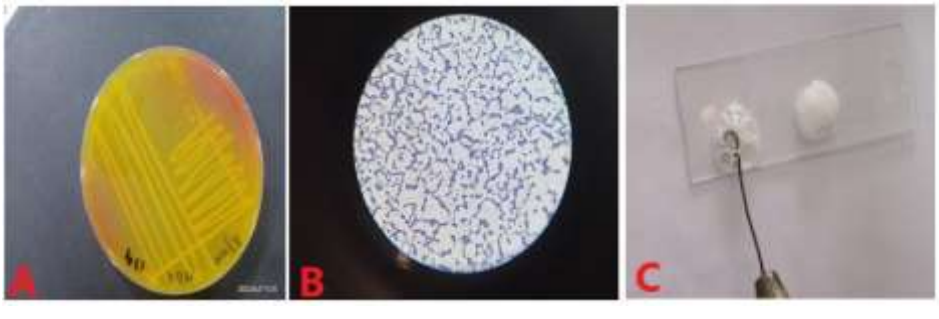
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Zinc oxide nanoparticles accelerate the healing of methicillin-resistant *Staphylococcus aureus* (MRSA)-infected wounds in rabbits

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Supplementary Figure. Morphological confirmation of *Staphylococcus aureus.* (A) Golden yellow color colonies of *Staphylococcus aureus*; (B) Gram staining and grape like cluster appearance under a microscope (magnification: 10×); (C) Catalase +ve test.