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Histopathological Evaluation of the Application of Zinc Nanoparticles in Conjunction With Chitosan Dressing on the Wound Healing of Methicillin-Resistant *Staphylococcus aureus* Infected Skin Wounds in Rats

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Abstract

Objectives: Wound healing and tissue regeneration have raised serious challenges for healthcare systems worldwide, affecting both aesthetic and health-related aspects of the community well-being. Wound healing is a complex sequence of physical, cellular, and molecular events involving cell recruitment, proliferation, as well as the synthesis and accumulation of the new extracellular matrix components. The present study aimed to histopathologically evaluate the application of zinc nanoparticles in conjunction with chitosan dressing for the wound healing of methicillin-resistant Staphylococcus aureus (MRSA) infected skin wounds in rats.

Materials and Methods: In this experimental investigation, 28 healthy male rats were randomly divided into 4 groups, each of which including 7 rats. The wound healing process was evaluated based on histopathological studies and biochemical findings on days 7, 14, and 21.

Results: According to the results from the two-way ANOVA analysis, the means of wound area in the study groups were statistically and significantly different on all evaluated days ($P<0.001$). The highest wound area was associated with group III (i.e., wound with zinc oxide) on days 6, 18, 15, 12, and 9, while the lowest wound area was observed in group IV (i.e., wound with chitosan-nanoparticles-zinc oxide). A statistically significant difference was also found among the study groups in terms of the mean values of hydroxyproline ($P<0.001$). According to the post hoc Tukey test for pairwise comparisons, a significant difference was observed among all groups regarding the hydroxyproline values ($P<0.001$). Additionally, the highest collagen density was detected in the chitosan-nanoparticles-zinc oxide group.

Conclusions: The application of biodegradable chitosan-nanoparticles-zinc oxide dressing significantly improved the histopathological and biochemical indices of the full-thickness skin wound healing. This study highlighted the effectiveness of chitosan-nanoparticles-zinc oxide dressing in wound healing enhancement, indicating its potential application in wound healing.

Keywords: Wound healing, Full-thickness wound, Chitosan-nanoparticles-zinc oxide, Sprague-Dawley rats

Introduction

Wound healing and its processes are among the most serious concerns to the healthcare systems worldwide, affecting both aesthetic and public health perspectives. The skin serves as one of the body's primary defensive barriers against the pathogenic agents (1). Wound healing is a complex sequence of physical, cellular, and molecular events, involving cell recruitment to the wound site, cellular proliferation, and synthesis, leading to the accumulation of new extracellular matrix components. While this process is naturally initiated and ended in wounds, its outcomes are not always satisfactory in terms of the healing speed and the tissue repair quality. Therefore, numerous studies have been conducted to investigate the factors affecting both pace and quality of the wound healing. A wound signifies a disturbance in the natural continuity of anatomical or functional structures of the body, such as organs, tissues, or cells. Skin damage increases the susceptibility to foreign agents and results in fluid loss and alteration of the natural form of the skin (2).

Autografting (i.e., self-transplantation of skin from a healthy area) is considered a basic technique for treating the patients with extensive skin injuries. However, the invasive nature of this method, coupled with a limited availability of the donor sites and the potential formation of conspicuous scar tissue, is problematic to the patients (3). Another emerging approach involves the application of autologous skin (i.e., implanting a small section of epidermis under controlled laboratory conditions) to establish a broad graftable surface on the damaged area for dealing with, particularly, deep burns. However, this new method suffers from the drawbacks including its invasive nature and the formation of scar tissue in the graft region (4). Given the above discussion, the adoption

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Key Messages

- Healing of infected wounds is the main challenge in treatment of patients suffering from this condition. Application of zinc nanoparticles in combination with chitosan may be useful.

of novel non-invasive therapeutic approaches is deemed a promising substitute for the traditional methods.

Various strategies have been employed for dressing different types of wounds. An appropriate covering should establish a moist and clean environment, mitigate mechanical pressure and trauma, reduce edema, stimulate regeneration, and be cost-effective (5). Chitosan (i.e., poly-D-glucosamine) is one of the derivatives of deacetylated chitin. Chitosan can dissolve in organic acids (e.g., acetic acid) and assume forms like sponges, foams, or thin films. The biological attributes of chitosan and its oligomers are well-established. Enzymes gradually hydrolyze the chitosan film, resulting in the formation of chito-oligomers. These oligomers facilitate the proper deposition and orientation of collagen fibrils within the intracellular matrix (6). Moreover, tissue-based findings have indicated that the chitosan film promotes the migration of inflammatory cells and enhances the cellular organization. Application of various doses of zinc oxide in numerous studies has been found to enhance wound healing by stimulating angiogenesis (7).

Zinc deficiency reduces the levels of immunoglobulins IgA, IgM, and IgG. Additionally, low zinc levels below the standard level result in a decreased mitogenic stimulation and lymphocyte proliferation (8). Extensive and multidimensional research is currently underway, as researchers aim to control infections by combining those techniques that sensitize bacteria to antibiotics normally ineffective against the resistant bacteria. One of the naturally occurring pathogenic bacteria of the skin flora is Staphylococcus aureus which, given the right conditions, can cause skin wound infections. Current estimates indicate that millions of people worldwide suffer from the chronic wounds (9). Poor hygienic conditions in some developing countries are the main cause of this issue. It is believed that the application of zinc nanoparticles in combination with chitosan dressings has the potential to promote the healing of infected wounds, as demonstrated in a rat model.

Materials and Methods

To conduct the present experimental study, 28 adult male Wistar rats weighing 190 ± 10 g were obtained from the School of Medicine, Lorestan University of Medical Sciences. The animals were transferred to the Razi Research Center and kept in standard cages under the natural light-dark conditions and consistent room temperature for the rats. Then they underwent a two-week acclimatization period before initiation of the study in order to minimize the negative effects of the stress caused by an unfamiliar environment on the study outcomes. The rats were fed with standard pellets and had free access to water and food pellets. The rats were randomly divided into four groups, each of which including seven rats. these four groups were as follows: group 1: control (untreated open wounds); group 2: creation of a infected wound along with regular chitosan application; group 3: creation of an infected wound along with zinc nanoparticles; and group 4: creation of an infected wound with chitosan-zinc oxide nanoparicles. Wounds in different groups were treated on a daily basis for three days.

Anesthesia and Wound Creation

Anesthesia was induced by intraperitoneally (IP) administering a combination of 2% xylazine HCl (5 mg/kg) and 10% ketamine HCl (50 mg/kg). After anesthesia, the Wistar rats were placed in a supine position on a surgical table. The dorsal thoracic region of the rats was prepared and scrubbed and, then, two circular wounds with a diameter of ten millimeters were created using a biopsy punch and scalpel. The wounds were positioned symmetrically on each side of the vertebral column. Cyanoacrylate adhesive was used to place circular donut-shaped dressings over the wounds, ensuring that the wounds were centered in the dressings. The dressings were secured to the surrounding skin using four simple interrupted nylon 3-0 sutures. By creating these wounds using the excision model, the epidermal, dermal, and hypodermal layers were completely excised.

Wound Creation and Care during the Study

After creating the wounds and excising the underlying tissue, the wound surface was contaminated by applying bacterial inoculum. The wounds were left open, and the corresponding treatment was applied to each group. The animals were housed in the designated cages according to their respective treatment groups and provided with daily care until the end of the sampling period. It should be noted that the treatment application on the wounds without anesthesia was performed for one hour and continued for three days.

After immobilizing the rats, a mixture of ketamine and xylazine was injected intraperitoneally at a ratio of 3:1 (for larger rats) or 2:1 (for smaller rats) until they were anesthetized. After inducing anesthesia, the backs of the rats were shaved using a hair trimmer, and the sterilized skin was swabbed with alcohol-soaked cotton. Circular areas with a diameter of 1 square centimeter and full thickness were excised using sterile surgical blades. Finally, the excised areas were inoculated with a sterile suspension of *Staphylococcus aureus* equivalent to a McFarland tube of 0.5. The infected wounds in each group were then dressed with the appropriate dressings and covered with sterile adhesive strips.

Preparing Dressing Rings

Dressing rings were prepared using laser cutting in circular dimensions with an outer diameter of 30 mm and a central circular area with a diameter of 20 mm. The discs were thoroughly washed with a cleaning agent and then rinsed with distilled water. The discs were immersed in a 20 000 ppm sodium hypochlorite solution for 30 minutes, followed by rinsing with distilled water, and then placed in 70% ethanol for 30 minutes. After this period, the discs were placed under a hood on a sterile gas stream to dry. The dried discs were stored in sterile containers until use, ensuring the complete packaging.

Preparation of Standard Turbidity 0.5 McFarland

For preparing the 0.5 McFarland standard solution, 5 mL of a 1.75% (w/v) solution of barium chloride dihydrate (BaCl2.2H2O) was added to 99.5 mL of a 1% (v/v) solution of sulfuric acid (1 mL of sulfuric acid in 99 mL of distilled water). The resulting standard solution was poured into a covered container and stored in a dark place at a temperature of 22 to 25 °C for up to six months. Prior to use, the solution should be thoroughly mixed. The optical density (OD) of this solution at a wavelength of 625 nm should be between 0.08 and 1.0. This turbidity corresponds to a bacterial concentration of $1–2 \times 10^8$ CFU/mL.

Preparing Bacterial Inoculum and Infecting the Wound

First, methicillin-resistant *Staphylococcus aureus* (MRSA) purchased from the Pasteur Institute was streaked onto a non-selective agar medium (e.g., brain heart infusion) to obtain the isolated or single colonies. The plates were then incubated overnight at 37 °C. Next, 1 or 2 isolated colonies were transferred to a test tube containing 5 mL of physiological saline solution and were thoroughly mixed using a shaker. In another test tube, 5 mL of the 0.5 McFarland standard solution was added. The turbidity of the saline content of the isolated colonies was visually compared to the turbidity of the 0.5 McFarland tube under a light source in front of a white background with black lines. If the saline turbidity was lower than the 0.5 McFarland standards, more colonies were added and mixed. Conversely, if the saline turbidity was higher, the saline solution was added to adjust the turbidity. Subsequently, the turbidity of the saline solution was controlled using a spectrophotometer at a wavelength of 625 nm, and the OD was between 0.08 and 1.0. This turbidity corresponds to a bacterial concentration of $1–2 \times 10^8$ CFU/mL. For infecting the wound, 100 µL of the prepared bacterial inoculum were added to the standardized wound area.

Construction of Biodegradable Biomembrane Layer

The construction of the biodegradable biomembrane layer of chitosan-nano zinc was carried out according to the method described by Bai et al (12). In summary, 10 mL of an aqueous chitosan polysaccharide solution (0.5% w/v) was mixed with 7.5 mL of a 0.23M ascorbic acid solution and 5 mL of a 0.2M acetic acid solution. Then, 0.25 mL of a 1.0M zinc solution was slowly added to this mixture. It should be noted that the color of the mixture changes from colorless to red during the reaction as evidence of chitosan-zinc nanoparticle production. Finally, the mixture was diluted with distilled water to a volume of 50 mL to reach a final concentration of 200 mg of chitosan-zinc nanoparticles per liter. After synthesis, 10 mL of this mixture was diafiltrated against 2 L of distilled water using permeable membranes, capable of passing molecules with a molecular weight of 12 kDa, for 2 hours at room temperature and was prepared for application.

Determination of Hydroxyproline Levels

Hydroxyproline is an essential amino acid that constitutes collagen, and its determination provides an accurate insight into the level of collagen tissue during wound healing. The proposed method (13) was employed to determine hydroxyproline levels. On day 21 after wound creation, tissue samples were obtained from the wound center with equal weights from different groups. One gram of each sample was dried at 60 °C and hydrolyzed in 6 N hydrochloric acid at 130 °C in the test tubes. Then, the reaction was neutralized with a pH 7 buffer solution and exposed to chloramine-T for 20 minutes for oxidation. The reaction was terminated by adding 4M perchloric acid, and the resulting color was evaluated at 557 nm wavelength using an Erlich's reagent in a spectrophotometer (UV-visible).

Tissue Sampling Method for Histopathological Examination

For histopathological studies and evaluation of the pathological parameters, wounds created on the right side from the six different groups were considered. On days 7, 14, and 21 post-operation, four rats were selected from each group, and the tissue samples including the wound margin along with 2 mm of the healthy skin were obtained. The samples were immediately placed in 10% buffered formalin and used to prepare histopathological slides stained with hematoxylin-eosin and Masson's trichrome. The percentage of collagen fiber density (150 pixels per square centimeter) in the study groups was evaluated using the Image Color Summarizer 0.76 software. A uniform magnification of 40 was used for all histopathological sections in this study.

Planimetric Evaluation

After creating all infected wounds, accompanied by a measuring scale marked on the skin for software measurement, digital photographs were taken using a digital camera, considering day zero as the starting point. This process was repeated on days 3, 6, 9, 12, 15, 18, and 21. The required scale was defined and the wound area was calculated using the measurement tool in Adobe Acrobat.

Data Analysis

After collecting the required information, the descriptive statistics were used to describe the data by calculating the central and dispersion indices for quantitative variables and frequency and percentage for qualitative variables. The normality of the data was measured using the Shapiro-Wilk test. Then the independent t-tests, paired t-test, and repeated-measures analysis of variance (ANOVA) were performed when the data distribution was normal, but the non-parametric equivalents of these tests were used for data analysis if the data distribution was not normal. All statistical tests were bilaterally performed using SPSS version 22 software. A $P<0.05$ was considered as a significant level.

Results

Figure 1 shows SEM and TEM images of the zinc oxide nanoparticles.

According to the results from the two-way ANOVA, the difference among the study groups regarding the mean wound area on all days (21, 18, 15, 12, 9, 6) was statistically significant ($P<0.001$). On day 6 of the study, the highest wound area was observed in group 1 (i.e., group with non-infected open wounds), and the lowest wound area was observed in group 4 (i.e., group with non-infected wounds along with chitosan and zinc nanoparticles).

On days 18, 15, 12, and 9, the highest wound area was associated with group 3 (i.e., group with infected wounds receiving silver oxide), and the lowest wound area was observed in group 4 (i.e., group with infected wounds along with chitosan and zinc nanoparticles) (Figure 2).

On day 21 of the study, the highest wound area was observed in the group with infected wounds receiving silver oxide, and the lowest wound area was observed in groups with non-infected wounds (group 1), non-infected wounds with chitosan dressing (group 2), and infected wounds with chitosan and zinc nanoparticles dressing (group 4).

In the Tukey post hoc test, the differences between each group and other groups were statistically significant in terms of the wound area values ($P<0.001$). All pairwise comparisons among the study groups in the Tukey post hoc test were statistically significant ($P<0.001$).

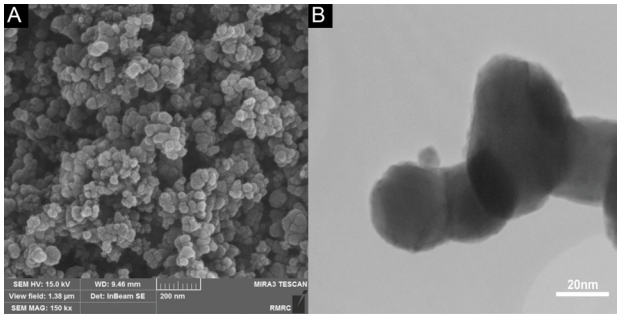

Figure 1. A: SEM Image of Zinc Oxide Nanoparticles, Scale Bar: 100 µm; **B:** TEM Image of Zinc Oxide Nanoparticles.

Figure 3 presents the number of polymorphonuclear cells, Figure 4 shows the number of mononuclear cells, and Figure 5 depicts the number of fibroblasts in experimental groups 1 to 4. A significant difference was seen among the groups regarding the number of the given cells.

The given data on the number of polymorphonuclear cells, mononuclear cells, and fibroblasts in experimental groups 1 to 4 is presented in Figures 3, 4, and 5 respectively.

A significant difference was seen among the control and other groups regarding the number of polymorphonuclear, mononuclear and fibroblast cells. Histological results showed that there was a significant difference between group 4 and other groups in terms of, especially, the cell infiltration and neovascularization. The migration process of the inflammatory cells and fibroblasts for collagen formation was evident in chitosan and nano zinc treatment groups.

Based on Figure 6, the first group showed an increase in the replacement of the epidermis by fibrinoleukocyte and acute inflammatory cells (neutrophils), resulting in

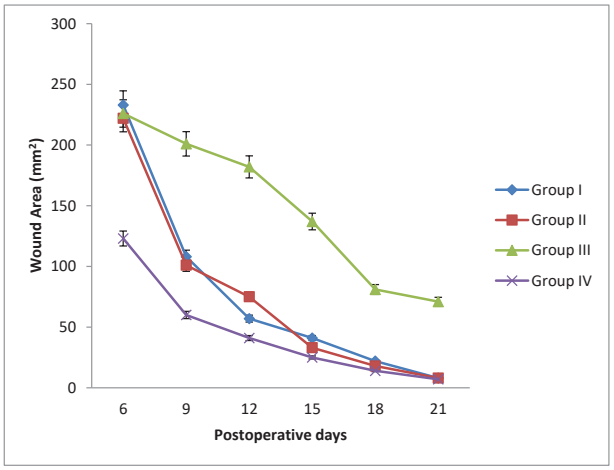

Figure 2. Planimetric Evaluation of Wound Area in Different Groups. The third group had the highest wound area, while the fourth group had the lowest. No significant changes were observed among the other groups.

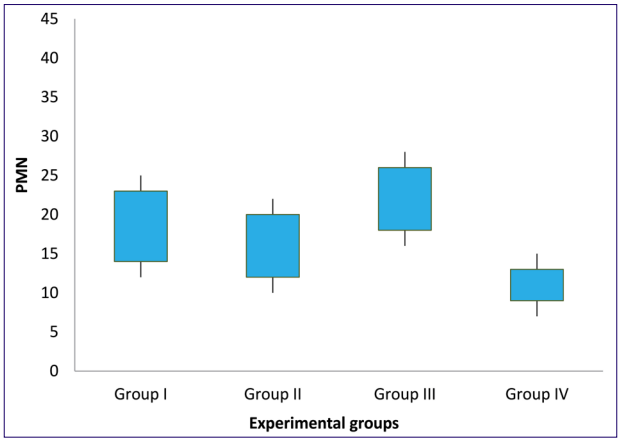

Figure 3. Box-and-Whisker Plots of the Polymorph Nuclear Cells Number in Excisional Model of the Rat's Skin in Experimental Groups. Results were expressed as mean ± SEM.

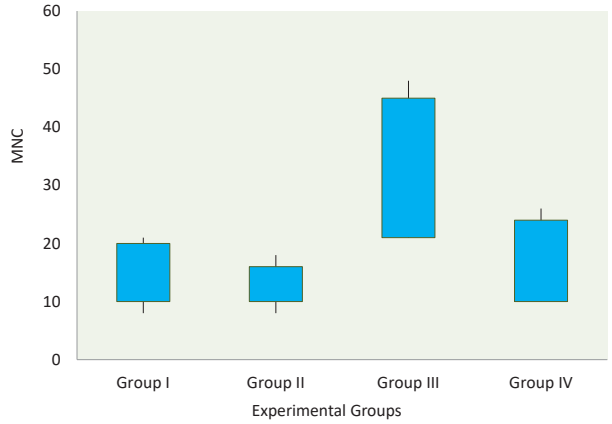

Figure 4. Box-and-Whisker Plots of the Mononuclear Cells Number in Excisional Model of the Rat's Skin in Experimental Groups. MNC, Mononuclear cell.

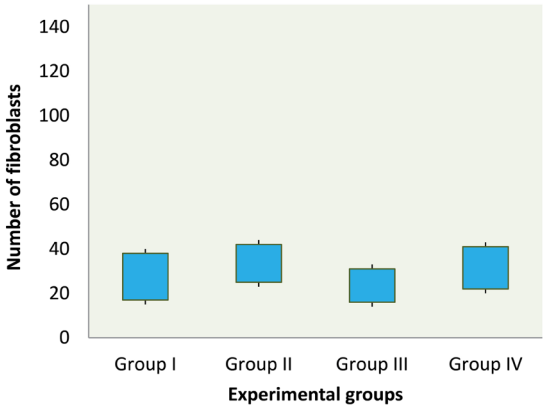

Figure 5. Box-and-whisker Plots of the Fibroblasts Number in Excisional Model of the Rat's Skin in Experimental Groups.

loss of the epidermis. Group 3 exhibited signs of repair and inflammation in the epidermis, while group 4 showed regeneration of the epidermis and replacement of the dermis with fibroblast cells (fibrotic tissue) and a small number of chronic inflammatory cells.

According to the results of the one-way ANOVA test, the difference among the study groups in terms of the mean values of hydroxyproline was statistically significant ($P<0.001$). The hydroxyproline values in groups 1 to 4 were 49.55 ± 2.34, 75.65 ± 3.43, 77.38 ± 2.48, and 99.13 ± 3.87 mg/g, respectively.

The study groups were evaluated on days 7, 14, and 21 in terms of vascularity, epithelialization, and collagen content and, according to the obtained results, epithelialization reached a moderate level on day 7, a severe level on day 14, and a very severe level on day 21.

According to the observations, the values of collagen fiber density were average, severe, and very severe on days 7, 14, and 2, respectively. The positive and significant effect of the fourth group's treatment was confirmed by evaluating the collagen content. The percentage of collagen fiber density in the treatment group with a biodegradable chitosan-zinc nanofilm layer was statistically and significantly different from those of other groups (Table 1).

Discussion

The first line of defense breached during surgeries and invasive procedures is the skin, which serves as the entry point for pathogenic agents into the body. Thus, the quicker the wound inflicted on this defensive barrier is closed, the more effectively the entry of pathogenic organisms into the body is prevented (1). Metal and metal oxide nanoparticles have received significant attention recently due to their unique properties such as lower toxicity, biocompatibility, enhanced penetration,

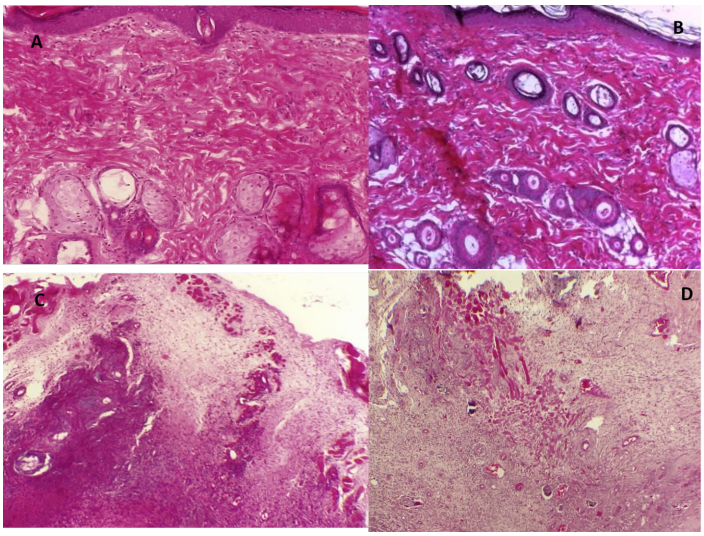

Figure 6. Histological Characteristics of Rat Skin after Wound Creation in Experimental Groups in Excisional Wound Model. A: control group; B: chitosan group; C: zinc oxide nanoparicles; D: chitosan-zinc oxide nanoparticles. Wounds with surrounding skin were prepared for histological microscopic evaluation by H&E staining (×400).

Table 1. Evaluation of Intensity of Histological Parameters in Experimental Groups

Groups	Days	Histological Parameters				
		Acute Hemorrhage	Congestion	Vascularization	Epithelialization	Collagen
Group I	7	+++	+++	+	-	+
	14	++	+	+	+	++
	21	-	-	++	++	++
Group II	7	+++	+++	-	-	-
	14	+++	+++	+	+	+
	21	++	++	+	+	+
Group III	7	+++	+++	-	-	-
	14	+++	++	+	+	+
	21	+++	++	+	+	+
Group IV	7	+*	+*	+++*	++*	++*
	14	-	-	+++*	++*	++*
	21	-	-	+++*	+++*	+++*

Classification of histological parameters based on the intensity of occurrence: - absence; + discrete; ++ moderate; +++ intense; and ++++ very intense. Histopathological damages were assessed on days, 7, 14, and 21 of lesion, as explained in material and methods section.*P<0.05 vs. groups II and III.

and greater dispersion within various biological tissues compared to their larger molecular counterparts (14).

Chitosan was first discovered in 1811 by Henri Braconnot, a French chemist and pharmacist (15). By interacting with red blood cells, chitosan promotes the rapid and effective formation of a blood clot to prevent hemorrhage. Chitosan has been recently used in bandages and other hemostatic materials in the United States. It prevents dehydration and contamination of wounds, and improves conditions for wound healing. It has been reported that the gradual release of nanoparticles inhibits the potential toxicity resulting from the rapid absorption of certain drugs within the living organisms (16). In the present study, therefore, chitosan was employed as a carrier for nanoparticles.

A study by Bano et al investigating the application of chitosan and its derivatives for wound dressings highlighted the various applications of chitosan for wound and burn treatment. Chitosan, due to its molecular weight and degree of deacetylation, was explored for its antimicrobial and wound healing properties. Histological examinations demonstrated that chitosan effectively induced the structural regeneration and granulation tissue formation in wound treatment. Our study also found a faster wound healing trend and a reduction in wound area in the chitosan-nanozinc oxide group compared to other groups, which may have been attributed to the formation of granulation tissue epithelium. This finding was in line with the result of the study by Bano et al (17).

Comparative studies have recently attracted the attention of scientists and researchers worldwide. These studies offer new insights into current knowledge and provide valuable information about the pharmacological properties of the nanoparticles compared to their larger molecular counterparts (18).

In a study by Mirastschijski et al on 33 male participants, a mixture of copper, zinc, and selenium was prescribed for wounds resulting from furuncles. Tissue sampling and the observation of the healing process revealed that this mixture stimulated the host's defense mechanism and accelerated the wound healing mechanisms, such as matrix metalloproteinase activity. These findings were consistent with our study results, suggesting that the faster wound healing in the chitosan-nanozinc oxide group may have been due to an enhanced host defense and immune system against the microorganisms (19).

Kaushik et al investigated the wound infections as a primary factor delaying the wound healing. This study indicated that the fibroblast cell growth with ZnO nanoparticles was greater for the larger particle sizes. Electron microscopy and wound area analysis confirmed the antimicrobial activity of ZnO nanoparticles, highlighting the greater antimicrobial efficacy for smaller-sized ZnO particles (20).

In a study conducted by Jayasuriya et al, adhesive strength of cells and toxicity of films prepared on the second and fifth day were measured using osteoblasts. It was observed that the stability of osteoblast decreased in films containing ZnO NPs in levels higher than 5%. The results of this study indicated that ZnO nanoparticles may have enhanced the mechanical properties of the pure chitosan films, but their application was limited to the biomedical and bioengineering applications due to their toxic effects; therefore, they may have been only used in low percentages (21).

Vingsbo Lundberg et al revealed that when treating the root canal, a local treatment with retapamulin and mupirocin was significantly more effective than a systemic treatment with linezolid and vancomycin in dealing with MRSA skin infections. Retapamulin and mupirocin may have been used as alternatives to fusidic acid for treating MRSA skin infections when resistance was suspected (22).

In another study by Cahú et al, some layers with and without ZnO particles were produced based on chitosan containing gelatin and chondroitin 4-sulfate (C4S) and tested in vitro to assess their potential wound healing properties. According to their study results, chitosans had different molecular weights based on their degree of

deacetylation (0.5%±80%). Layer solutions (i.e., chitosan, C4S, and gelatin) plus ZnO suspension showed no toxicity to fibroblasts and keratinocytes. Chitosan may have agglutinated the red blood cells, but the layer solutions caused no hemolysis. Their results also indicated that a stable chitosan-based layer with low toxicity and the ability to release components may have created an environment compatible with the cell growth. Chitosan-based layers, compared to control samples (51%), increased wound improvement (65% to 86% wound contraction) in the skin after 6 days. Furthermore, the histological analysis showed an increased tissue granulation in chitosan layers and chitosan/gelatin/C4S/ZnO. Their study concluded that the improved biopolymer composites based on chitosan may have been used for enhanced medical applications (e.g., wound healing) (23).

Reshmi et al found that the nanofibrous layers of nanochitosan (NC) enriched with poly (ε-caprolactone) (PCL) were produced for wound coverage and controlled curcumin release. Their study results demonstrated that the PCL/15NC nanofiber layer served as a promising material for wound coverage and transdermal patches with adjustable drug release under pH stimulation (24).

Golbui Daghdari et al investigated the impact of ZnO nanoparticles on the bacterial load reduction in wounds infected with *S. aureus* bacteria. According to their study results, ZnO nanoparticles exhibited antibacterial effects against *S. aureus* and reduced the bacterial growth in in vitro experiments. Moreover, ZnO nanoparticles were capable of reducing the bacterial load in infected wounds and accelerating the wound healing process (25).

Antioxidant enzymes collaborate as antioxidant shields in the body to eliminate the reactive oxygen species and prevent oxidative damage to cells and tissues. Even a slight imbalance in the physiological concentrations of these enzymes can lead to a deficiency in the body's antioxidant system and an increased susceptibility to the oxidative damage. In the present study, the balance of the antioxidant system in the group receiving chitosan-nanozinc accelerated the wound healing process. Our results were consistent with the results from a study by Goel et al (26).

Zinc primarily exerts its inhibitory and limiting effects on oxidative stress through the activity of the enzyme glutathione peroxidase. Glutathione peroxidase is arguably the most important antioxidant enzyme that prevents oxidative damage to cells, including endothelial cells (27). This mechanism could be the main factor in epithelialization and prevention of the damage to endothelial vessels, resulting in an improved blood circulation and an accelerated wound healing process.

The migration of epithelial cells toward the center of the wound requires a tissue bed rich in granulation tissue. Granulation tissue is also a vital source of myofibroblasts necessary for the process of contraction and wound closure. The contraction process mainly occurs independently of the epithelialization, meaning that while the newly-formed covering tissue moves toward the center of the wound, the existing skin also exhibits independent movement. Although these two processes are independent, their ultimate outcome is the coverage of an area of the wound lacking covering tissue. In wounds where the edges are tightly sutured, epithelialization begins when there is no defect (24 to 48 hours later) because there is no gap for the tissue to fill. In open wounds, however, the epithelialization does not occur until an appropriate granulation tissue bed is formed to cover the wound (28). Our study examined the collagen density and demonstrated that positive effects of chitosan-zinc nanoparticles could be considered as a treatment protocol in an infected wound. Furthermore, the results indicated that the percentage of collagen fiber density in the treatment group with biodegradable chitosan-nanozinc film was significantly different from those of other groups. These findings were consistent with the results of a study by Abbaszadeh et al (29). In the given study, which aimed to assess collagen density through histopathological evaluation using chitosan, it was shown that the application of chitosan and selenium nanoparticles increased the collagen levels at the wound site and accelerated the wound healing process. Their findings were in line with the results of our study, suggesting that the rapid wound healing and improvement may have been due to the increased hydroxyproline content at the wound site.

According to our study findings, the biodegradable chitosan-nanozinc film group, compared to other groups, contributed to the formation of a more suitable granulation tissue. Given the above-mentioned importance of granulation tissue, it was implied that chitosan served as a carrier or binder for zinc nanoparticles and, therefore, enhanced the efficiency of these nanoparticles. This enhancement potentially prevents the microbial infections through epithelialization and formation of covering tissue and, consequently, accelerates the wound healing process.

Limitations of the Study

The omission of molecular tests to assess the cellular events within the wound represents a notable limitation of our study.

Conclusions

The increase in hydroxyproline content was suggestive of the enhancement of collagen synthesis. Higher levels of hydroxyproline (i.e., milligrams per gram of tissue) during the wound healing process in various treatment groups indicated that chitosan-nanozinc was effective in improving and accelerating the collagen synthesis as well as in organization and arrangement of the collagen fibrils, ultimately increasing the wound tensile strength. Taking into account the results from the planimetric evaluations, it was implied that the chitosan-nanozinc combination

facilitated the wound closure.

In sum, it was found that chitosan-supported zinc nanoparticles had greater potential for healing the full-thickness infected wounds in rats than zinc nanoparticles alone. It was also determined that the application of biodegradable chitosan-nanozinc films facilitated the formation of a granulation tissue with a more appropriate thickness, improved angiogenesis, and increased collagen deposition in the wound as well as a faster wound healing process.

Recommendations

Taking into account our study findings, it was recommended that the biodegradable chitosan-nanozinc films should be used to accelerate the healing of infected wounds. As for clinical applications, however, it was suggested that specific regulations should be adopted regarding their application. Since wound healing and those processes accelerating this pathway were of a great significance during the studied period, the dressings with these characteristics may have been extensively used in the coming years.

Authors' Contribution

Conceptualization: Leila Zarei.
Data curation: Asghar Rajabzadeh.
Formal analysis: Leila Zarei.
Funding acquisition: Abolfazl Abbaszadeh.
Investigation: Hesammadin Hosseini.
Methodology: Hesammadin Hosseini.
Project administration: Asghar Rajabzadeh.
Resources: Abolfazl Abbaszadeh.
Software: Hesammadin Hosseini.
Supervision: Leila Zarei.
Validation: Asghar Rajabzadeh.
Visualization: Abolfazl Abbaszadeh.
Writing–original draft: Leila Zarei.
Writing–review & editing: Asghar Rajabzadeh.

Conflict of Interests

Authors have no conflict of interest.

Ethical Issues

The ethical aspects of this study were approved in accordance with the guidelines of the Ethics Committee of the Ministry of Health with code IR.LUMS.REC.1399.094. After euthanizing the animals, moreover, their bodies were disposed of following sanitary and ethical principles.

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References

1. Wysocki AB. Skin anatomy, physiology, and pathophysiology. Nurs Clin North Am. 1999;34(4):777-797.
2. Hansbrough JF. Current status of skin replacements for coverage of extensive burn wounds. J Trauma. 1990;30(12 Suppl):S155-160. doi:10.1097/00005373-199012001-00030
3. Ben-Bassat H, Chaouat M, Segal N, Zumai E, Wexler MR, Eldad A. How long can cryopreserved skin be stored to maintain adequate graft performance? Burns. 2001;27(5):425-431. doi:10.1016/s0305-4179(00)00162-5
4. Gajiwala K, Gajiwala AL. Evaluation of lyophilised, gamma-irradiated amnion as a biological dressing. Cell Tissue Bank. 2004;5(2):73-80. doi:10.1023/B:CATB.0000034076.16744.4b
5. Lorenz PH, Longaker MT. Wounds: biology, pathology, and management. In: Norton JA, Barie PS, Bollinger RR, Chang AE, Lowry SF, Mulvihill SJ, eds. Surgery: basic science and clinical evidence. 2nd ed. New York: Springer; 2008. pp. 191–208. doi: 10.1007/0-387-22744-X_7
6. Wang H, Zhang J, Yu H. Elemental selenium at nano size possesses lower toxicity without compromising the fundamental effect on selenoenzymes: comparison with selenomethionine in mice. Free Radic Biol Med. 2007;42(10):1524-1533. doi:10.1016/j.freeradbiomed.2007.02.013
7. Bajpai S, Mishra M, Kumar H, Tripathi K, Kumar Singh S, Prasad Pandey H. Effect of selenium on connexin expression, angiogenesis, and antioxidant status in diabetic wound healing. Biological Trace Element. 2011;144(1-3):327-38. doi: 10.1007/s12011-011-9097-7.
8. Maywald M, Rink L. Zinc in Human Health and Infectious Diseases. Biomulecules. 2022;12(12):1748. doi: 10.3390/biom12121748
9. Horkan L, Stansfield G, Miller M. An analysis of systematic reviews undertaken on standard advanced wound dressings in the last 10 years. J Wound Care. 2009;18(7):298-304. doi:10.12968/jowc.2009.18.7.43113
10. Almekinders LC, Gilbert JA. Healing of experimental muscle strains and the effects of nonsteroidal antiinflammatory medication. Am J Sports Med. 1986;14(4):303-308. doi:10.1177/036354658601400411
11. Bucknall TE. The effect of local infection upon wound healing: an experimental study. Br J Surg. 1980;67(12):851-855. doi:10.1002/bjs.1800671205
12. Bai Y, Wang Y, Zhou Y, Li W, Zheng W. Modification and modulation of saccharides on elemental selenium nanoparticles in liquid phase. Mater Lett. 2008;62(15):2311-2314. doi:10.1016/j.matlet.2007.11.098
13. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem. 1996;29(3):225-229. doi:10.1016/0009-9120(96)00003-6
14. Jain PK, Huang X, El-Sayed IH, El-Sayed MA. Noble metals on the nanoscale: optical and photothermal properties and some applications in imaging, sensing, biology, and medicine. Acc Chem Res. 2008;41(12):1578-1586. doi:10.1021/ar7002804
15. Labrude P, Becq C. [Pharmacist and chemist Henri Braconnot]. Rev Hist Pharm (Paris). 2003;51(337):61-78. [French].
16. Miller C, McMullin B, Ghaffari A, et al. Gaseous nitric oxide bactericidal activity retained during intermittent high-dose short duration exposure. Nitric Oxide. 2009;20(1):16-23. doi:10.1016/j.niox.2008.08.002
17. Bano I, Arshad M, Yasin T, Ghauri MA, Younus M. Chitosan: a potential biopolymer for wound management. Int J Biol Macromol. 2017;102:380-383. doi:10.1016/j.ijbiomac.2017.04.047
18. Asri-Rezaei S, Dalir-Naghadeh B, Nazarizadeh A, Noori-Sabzikar Z. Comparative study of cardio-protective effects of zinc oxide nanoparticles and zinc sulfate in streptozotocin-induced diabetic rats. J Trace Elem Med Biol. 2017;42:129-141. doi:10.1016/j.jtemb.2017.04.013
19. Mirastschijski U, Martin A, Jorgensen LN, Sampson B, Ågren MS. Zinc, copper, and selenium tissue levels and their relation to subcutaneous abscess, minor surgery, and wound healing in humans. Biol Trace Elem Res. 2013;153(1-3):76-83. doi:10.1007/s12011-013-9658-z
20. Kaushik M, Niranjan R, Thangam R, et al. Investigations on the antimicrobial activity and wound healing potential of ZnO nanoparticles. Appl Surf Sci. 2019;479:1169-1177. doi:10.1016/j.apsusc.2019.02.189
21. Jayasuriya AC, Aryaei A, Jayatissa AH. ZnO nanoparticles induced effects on nanomechanical behavior and cell viability of chitosan films. Mater Sci Eng C Mater Biol Appl.

2013;33(7):3688-3696. doi:10.1016/j.msec.2013.04.057
22. Vingsbo Lundberg C, Frimodt-Møller N. Efficacy of topical and systemic antibiotic treatment of meticillin-resistant *Staphylococcus aureus* in a murine superficial skin wound infection model. Int J Antimicrob Agents. 2013;42(3):272-275. doi:10.1016/j.ijantimicag.2013.05.008
23. Cahú TB, Silva RA, Silva RPF, et al. Evaluation of chitosan-based films containing gelatin, chondroitin 4-sulfate and ZnO for wound healing. Appl Biochem Biotechnol. 2017;183(3):765-777. doi:10.1007/s12010-017-2462-z
24. Reshmi CR, Suja PS, Manaf O, Sanu PP, Sujith A. Nanochitosan enriched poly ε-caprolactone electrospun wound dressing membranes: a fine tuning of physicochemical properties, hemocompatibility and curcumin release profile. Int J Biol Macromol. 2018;108:1261-1272. doi:10.1016/j.ijbiomac.2017.11.035
25. Golbui Daghdari S, Ahmadi M, Dastmalchi Saei H, Tehrani AA. The effect of ZnO nanoparticles on bacterial load of experimental infectious wounds contaminated with *Staphylococcus aureus* in mice. Nanomed J. 2017;4(4):232-236. doi:10.22038/nmj.2017.04.005
26. Goel A, Dani V, Dhawan DK. Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. Chem Biol Interact. 2005;156(2-3):131-140. doi:10.1016/j.cbi.2005.08.004
27. Narayan AR, Deepthi SK. Role of selenium supplementation in thermal burn patients. World J Pharm Pharm Sci. 2015;4(7):970-975.
28. MacPhail CM. Surgery of the integumentary system. In: Fossum TW, ed. Small Animal Surgery. 4th ed. Philadelphia, USA: Elsevier; 2013:190-218.
29. Abbaszadeh A, Tehmasebi-Foolad A, Rajabzadeh A, Beigi-Brojeni N, Zarei L. Effects of chitosan/nano selenium biofilm on infected wound healing in rats; an experimental study. Bull Emerg Trauma. 2019;7(3):284-291. doi:10.29252/beat-0703012