



Effects of Chitosan/Nano Selenium Biofilm on Infected Wound Healing in Rats; An Experimental Study

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ABSTRACT

Objective: The present study was aimed at assessment of effect of application of Chitosan/Nano Selenium biofilm on infected wound healing in rats

Methods: Sixty-eight male Wistar rats were randomized into four groups of 17 animals each. In group I (Normal) the wounds were created with no infection. In group II (MRSA), the wounds were infected with methicillin resistant *Staphylococcus aureus* (MRSA). In group III (MRSA/CHIT), animals with infected wounds were dressed with chitosan biofilm only. In group IV (MRSA/CHIT/NS), animals with infected wounds were dressed with Chitosan/Nano Selenium biofilm.

Results: There were significant differences in comparisons of group IV and other groups, particularly in terms of cellular infiltration and neovascularization. During the study period, scores for neovascularization was significantly higher in group IV rats than other groups ($p < 0.05$). Polymorphonuclear (PMN) and mononuclear (MNC) cell count and fibroblast cell proliferation in group IV were significantly higher than those of other experimental groups ($p < 0.05$)

Conclusion: Chitosan/Nano Selenium biofilm resulted in significant improvement in histopathological indices in full thickness infected wound healing.

Keywords: Infected wound; Chitosan/Nano Selenium; Biofilm; Rat.

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Introduction

Open wounds are particularly prone to infection, especially by bacteria, and also provide an entry point for systemic infections. Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins that will be produced

with concomitant killing of regenerating cells. Consequently, there is a need to stimulate healing and restore the normal functions of the affected part of the body to ease the discomfort and pain associated with wounds, preventing infection, and activating tissue repair processes [1]. *Staphylococcus aureus* (*S. aureus*) is an important cause of nosocomial

infections in most health centers [2].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most widespread bacterial pathogen causing various infections ranging from skin and soft tissue infections to serious invasive infections, such as pneumonia, endocarditis, bacteremia and sepsis [3, 4]. It is estimated that Multi-drug resistant *Staphylococcus aureus* infections leads to high mortality with an associated annual health care costs [5, 6]. Despite this high mortality rate, there are relatively few new antibacterial agents in the pharmaceutical pipeline [7]. Instead, the majority of antibiotics developed in the last decade are molecules re-engineered from existing antibiotic classes for which underlying resistance mechanisms are already present [8]. Therefore, effective new therapeutic options for treatment of infections caused by multidrug resistant *S. aureus* are urgently needed.

Well-designed scaffold with a suitable porous structure can support cell migration and guide vascular infiltration, making it an ideal dermal substitute for wound regeneration. Several *in vitro* studies that focused on determining the optimal mean pore size of collagen-based scaffolds indicated that large pores ($\geq 250 \mu\text{m}$) favor cell attachment, proliferation and migration [9]. The basic strategy of engineered tissue regeneration is the construction of a biocompatible scaffold that, in combination with living cells and/or bioactive molecules, replaces, regenerates, or repairs damaged tissues. The scaffold should possess suitable properties, like biocompatibility, controlled porosity and permeability, and, additionally, support for cell attachment and proliferation. This artificial “dermal layer” needs to adhere to and integrate with the wound, which is not always successful for the current artificial dermal analogues available [10].

Chitosan is a high-molecular weight natural polymer. It is nonpoisonous; it can accelerate wound healing, reduce blood cholesterol levels, stimulate the immune response and can be biologically decomposed. It has a stronger antimicrobial property compared to chitin in avoiding fungi because it has an active group that will bind to microbes, so it can inhibit microbial growth. Chitosan has a good chemical reactivity because it has a number of hydroxyl (OH) and amine groups (NH₂) attaching to its chain. One of its important characteristics is that it has a positive charge in acidic solution. The substance is a stronger antifungal factor compared to chitin. In addition, chitosan is polycationic, so it can be used as a clotting agent [11-13]. An increasing number of products emerging from the application of nanotechnology to the science of wound healing is currently under clinical investigation. The nanoscale strategies, both carrier, drug related and scaffold target the main phases of wound repair [14].

In recent years, nanoparticles have emerged as important platforms to treat skin wounds. Silver, gold, and copper nanoparticles, as well as titanium and zinc oxide nanoparticles, have shown

potential therapeutic effects on wound healing. Due to their specific characteristics, nanoparticles such as nanocapsules, polymersomes, solid lipid nanoparticles, and polymeric nanocomplexes are ideal vehicles to improve the effect of drugs (antibiotics, growth factors, etc.) aimed at wound healing. On the other hand, if active excipients are added during the formulation, such as hyaluronate or chitosan, the nanomedicine could significantly improve its potential. In addition, the inclusion of nanoparticles in different pharmaceutical materials may enhance the beneficial effects of the formulations, and allow achieving a better dose control [15].

Selenium is one of the essential trace elements for humans. The bioavailability of Se is related to its different chemical species. Recently, elemental selenium nanoparticles are attracting more and more attention due to their excellent high biological activity and lower toxicity [16]. Elemental selenium nanoparticles in liquid phase can be used as the materials for medical purposes [17]. For these applications, it is important to have good stability of elemental selenium nanoparticles in liquid phase. One of the effective methods for stability of nanoparticles in liquid phase is to add modifiers. Others used the chitosan as modifiers for the fabrication of elemental selenium nanoparticles [18]. The objective of the present study was to assess effect of Chitosan/Nano Selenium biofilm on infected wound healing in rats

Materials and Methods

Animals

Sixty-eight adult healthy male Wistar rats weighting approximately 240g were used and housed in individual cages under room temperature (22±3 °C), and humidity (60±5%) with natural light/dark cycle, and had *ad libitum* access to standard pellet diet and water throughout the study.

Ethical Considerations

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health followed in works of others [15]. The research project has received the confirmation of the Institution Ethics Committee with the number of IR.LUMS.REC.1397.114.

Preparation of Chitosan/Nano Selenium Biofilm

Water-soluble chitosan solution was prepared using a method described by others [19]. Briefly, Medium molecular weight crab shell chitosan was dissolved (~400 kDa, 85% deacetylated, Sigma- Aldrich St. Louis, MO, USA) into an aqueous solution (1% v/v) of glacial acetic acid (Merck, Germany) to a concentration of 2% (w/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (50°C) for 3 hours. The resultant chitosan solution was filtered through Whatman

filter paper after vacuum filtration to remove any undissolved particles. For the preparation of elemental selenium nanoparticle sol, 1.5 mL 0.227 mol/L Vc was mixed with 1.0 mL 2.40 mol/L acetum, then the appropriate amounts of 5.36 mmol/L Se(IV) solution was added into the mixtures, the mixed solution was diluted to 10 mL. For the preparation of selenium nanoparticle-chitosan solution, appropriate amounts of chitosan solution were mixed with 1.5 mL 0.227 mol/L Vc and 1.0 mL 2.40 mol/L acetum, respectively. The appropriate amounts of 5.36 mmol/L Se(IV) solution was added into the mixtures, then the mixed solution was all diluted to 10 mL. Philips diffractometer was used to obtain X-ray diffraction pattern. Field emission scanning electron microscopy FESEM studies (Philips ES 30 KWO) were used to determine morphology of the scaffold (Figure 1).

Design of Study

Sixty-eight rats were randomized into four groups of 17 animals each. Nine animals in each group were served for histological, five for planimetric studies and three rats for microbiological assessments. In group I (Normal) the wounds were created with no infection. In group II (MRSA), the wounds were infected with MRSA. In group III (MRSA/CHIT), animals with infected wounds were dressed with chitosan biofilm only. In group IV (MRSA/CHIT/NS), animals with infected wounds were dressed with Chitosan/Nano Selenium biofilm.

Wound Creation and Infection Procedures

Rats were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg of b. w.) (Alfasan, Netherlands) and xylazine (5mg/kg of b.w.) (Alfasan, Netherlands), the hair on their back was shaved and the skin cleansed with 70% alcohol solution. Following shaving and aseptic preparation, a circular

excision wound was made by cutting away a full thickness of 10 mm in diameter excision circle wounds extending through the panniculus carnosus using a 10 mm dermal biopsy with homemade sterile punch. Small gauze was placed over each wound and then inoculated with 5×10^7 CFU of *Staphylococcus aureus* ATCC 43300. The methicillin-resistant *S. aureus* ATCC 43300 strain was commercially available. The pocket was closed by means of 4-0 nylon sutures and this procedure resulted in a local abscess after 24 h. The rats were returned to individual cages and they were examined daily. After 24 h, the wounds were opened, the gauze removed for quantitative bacterial cultures and treatment started. In Normal group, 0.1 mL sterile saline 0.9% solution was added to the wounds with no infection. In groups with infected wounds, the wounds were infected with MRSA and only treated with 0.1 mL the sterile saline 0.9% solution. The rats were placed in their individual cages warmed by a heater and allowed to recover fully from anesthesia.

Microbiological Assessments

Briefly, for total bacterial count on days 7 and 14 of treatment after wound creation the granulated tissues were excised aseptically. Then, 0.1 g of sample was crushed and homogenized in sterile mortar containing 10 ml of sterile saline. The homogenized sample was serially diluted in tube containing 9 ml of sterile saline to 10^{-5} . The diluted samples were cultured on plate count agar (Merck KGaA, Darmstadt, Germany) superficially and duplicated. The cultured plates were incubated at 37 °C for 24 to 48 hours. After incubation, all colonies were counted and results described as CFU/g of granulation tissue [20].

Planimetric Studies

Wound-healing property was evaluated by wound contraction percentage and wound closure time.

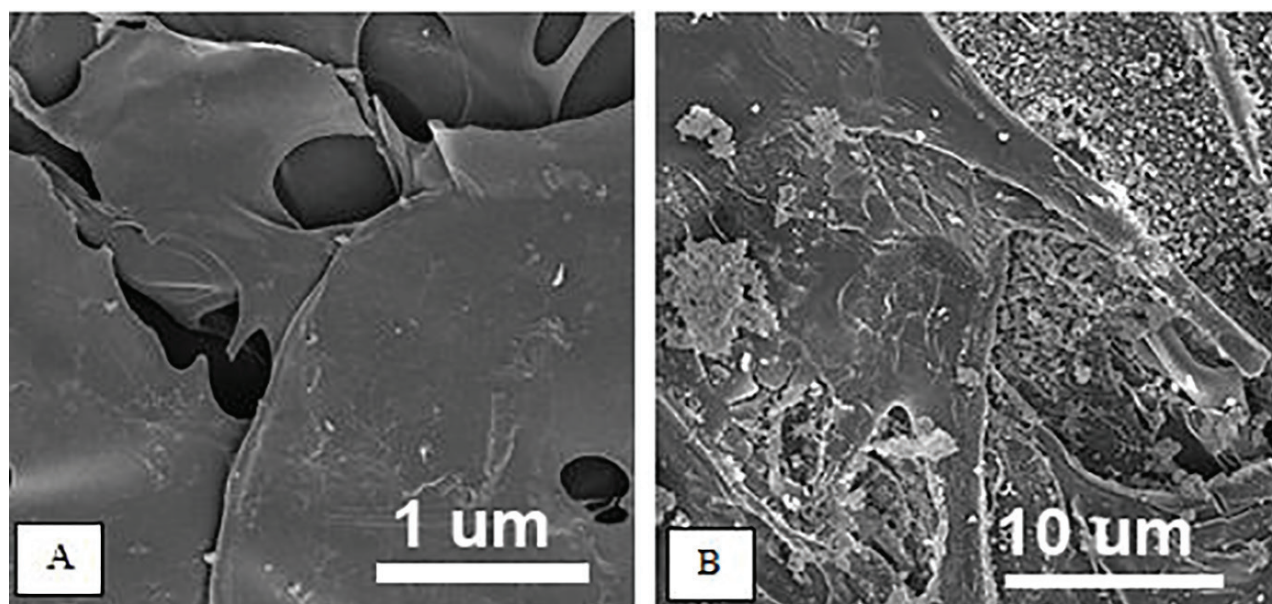


Fig. 1. Micrograph of scanning electron microscope to evaluate (A) ultra-structure of porosity of chitosan scaffold and (B) morphology of Chitosan/Nano Selenium biofilm.

Photographs were taken immediately after wounding and on days 6, 9, 12, 15, 18 and 21 post-wounding by a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc, San Jose, CA, USA) and wound contraction percentage was calculated using the following formula: Percentage of wound contraction = $(A_0 - A_t) / A_0 \times 100$

Where A_0 is the original wound area and A_t is the wound area at the time of imaging. All rats were closely observed for any infection and if they showed signs of infection were separated, excluded from the study and replaced.

Histological Studies

The tissue samples were taken on 7, 14, 21 days after surgery from periphery of the wound along with normal skin and fixed in 10% buffered formalin, dehydrated and embedded in paraffin wax, sectioned at 5 μ m and stained with hematoxylin and eosin (H&E) stains. Photomicrographs were obtained under light microscope to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Cellular infiltration including the number of mononuclear cells, polymorphonuclear cells and fibroblastic aggregation were quantitatively evaluated.

Determination of Hydroxyproline Levels

On the day 21 after surgery, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content. As a major part of collagen, hydroxyproline has an essential role in collagen stability. The collagen is the major component of extracellular tissue, which gives support and strength. Tissues were dried in a hot air oven at 60–70 °C to constant weight and were hydrolyzed in 6N HCl at 130 °C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by addition of

0.4M perchloric acid and color was developed with the help of Ehrlich reagent at 60 °C and measured at 557 nm using UV-visible spectrophotometer.

Statistical Analysis

Differences among groups were evaluated by Kruskal–Wallis variance analysis. When the P-value from the Kruskal–Wallis test statistics was statistically significant, multiple comparison tests were used to know differences. Student's t-test was used for evaluation of test results. SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A p -value was set at 0.05.

Results

Microbiology

In animals of group IV whose infected wounds were treated with nano selenium, the counts of *S. aureus* cultured in the wound tissues were significantly lower than in the infected wounds in groups II and III ($p=0.001$). No animals died due to infection or anesthetics. The uninfected wounds had no CFU/g of *S. aureus* count. Local application of nano selenium in combination with chitosan biofilm significantly reduced the rate of total bacterial count on 7 and 14 days post-wounding compared in groups II and III ($p=0.001$).

Planimetric Findings

Wound contraction percentage in different groups within the study period is shown in Figure 2. The healing rate of wounds in group IV was significantly different compared to groups II and III ($p=0.001$).

Histological Findings

There were significant differences in comparisons of group IV and other groups, particularly in terms of cellular infiltration and neovascularization. During the study period, scores for neovascularization was significantly higher in group IV rats than other groups ($p<0.05$). Polymorphonuclear (PMN) and

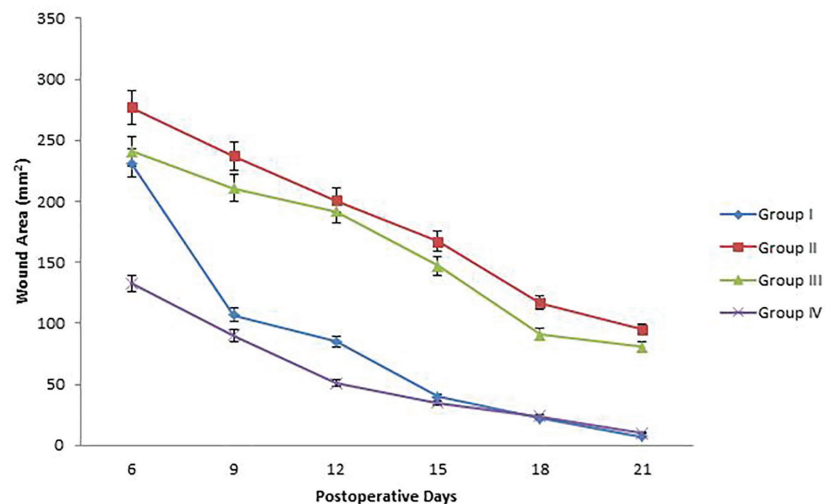


Fig. 2. Line graph indicating reduction in wound area in experimental groups. Results were expressed as mean \pm SEM. * $P<0.05$ vs. other experimental groups.

mononuclear (MNC) cell count and fibroblast cell proliferation in group IV were significantly higher than those of other experimental groups ($p < 0.05$) (Figures 3-6).

Findings of Hydroxyproline Levels

Proline is hydroxylated to form hydroxyproline after protein synthesis. Hydroxyproline contents in groups I to V were found to be 49.62 ± 2.54 , 75.19 ± 3.88 , 77.12 ± 2.73 and 97.12 ± 3.57 mg/g, respectively. Hydroxyproline contents were significantly increased in the group IV which implies more collagen deposition compared to groups II and III ($p = 0.001$).

Discussion

Although the wound healing process occurs by itself, spontaneously, and does not require much help, there are various risk factors such as infection, supply of blood, nutritional status and other factors that influence the resolution of this process [21]. It is well known that attack by microbes, which invade the skin barrier, delays the natural wound healing process [22]. MRSA is increasing in infections and is a serious threat to patients in health care facilities and the community. There are many reports in the literature that researchers have been working on various scaffolds and agents to combat MRSA related infections [23-33]. Resistance to common antibiotics makes treating MRSA costly and difficult. The main end point observed in this study, wound contraction and reduction in wound area, was accelerated by treating the wounds with chitosan and nano selenium. All the parameters observed (presence of necrotic tissue, clotting and crust, re-epithelialization and granulation tissue growth, bacterial count) were affected; suggesting that chitosan and nano selenium was effective against MRSA. Local application of chitosan and nano selenium biofilm at the wound site produced significant wound healing activity.

In excisional wound model there was a significant decrease in wound area. This indicated improved collagen maturation by increased cross linking. The balance between synthesis and breakdown and so deposition of collagen is important in wound healing and development of wound strength [34]. Hydroxyproline is a major component of the collagen that permits the sharp twisting of the collagen helix. It helps on providing stability to the triple-helical structure of collagen by forming hydrogen bonds. Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen content [35]. Increase in hydroxyproline content in group V indicated increased collagen content, since hydroxyproline is the direct estimate of collagen synthesis.

Nanoparticles (NPs) have become significant in the regenerative medicine field in the last two decades

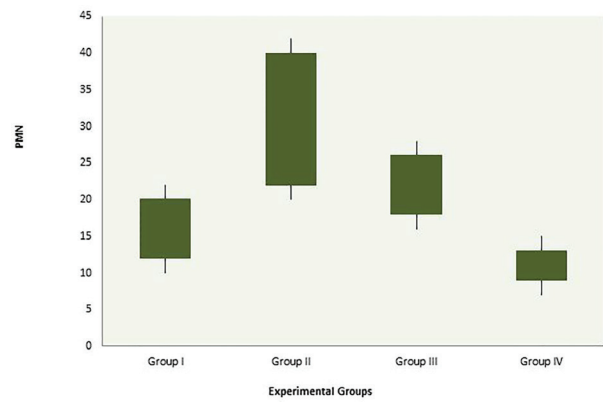


Fig. 3. Box-and-whisker plots of number of polymorph nuclear cells in excisional model of the rat's skin in experimental groups. Results were expressed as mean \pm SEM.

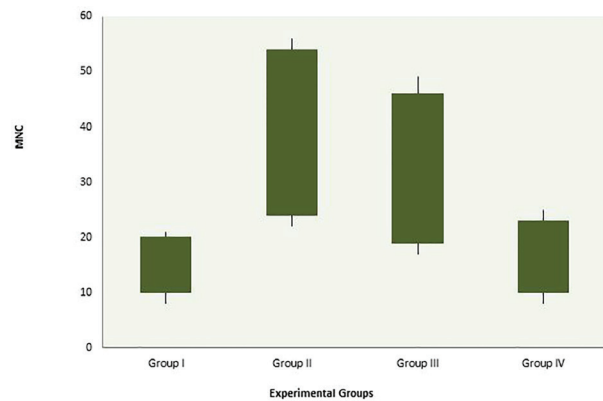


Fig. 4. Line graph indicating number of mononuclear cells in excisional model of the rat's skin in experimental groups. Results were expressed as mean \pm SEM. * $p < 0.05$ vs other experimental groups.

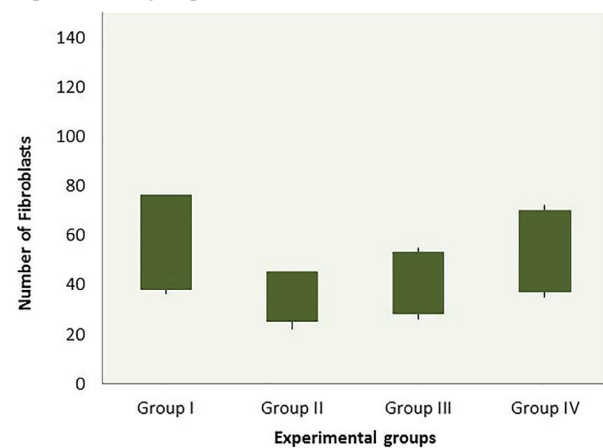


Fig. 5. Box-and-whisker plots of number of fibroblasts in excisional model of the rat's skin in experimental groups. Results were expressed as mean \pm SEM.

[36]. Many biological processes happen at through mechanisms that fundamentally act at the nanometer scale. Thus, materials such as NPs can be used as unique tools for drug delivery, imaging, sensing, and probing biological processes [37]. In the context of wound healing, the special properties of NPs like electric conductivity, antimicrobial activity, and high surface to volume ratio, swelling, and contraction make NPs versatile resources.

Several reports have demonstrated that there

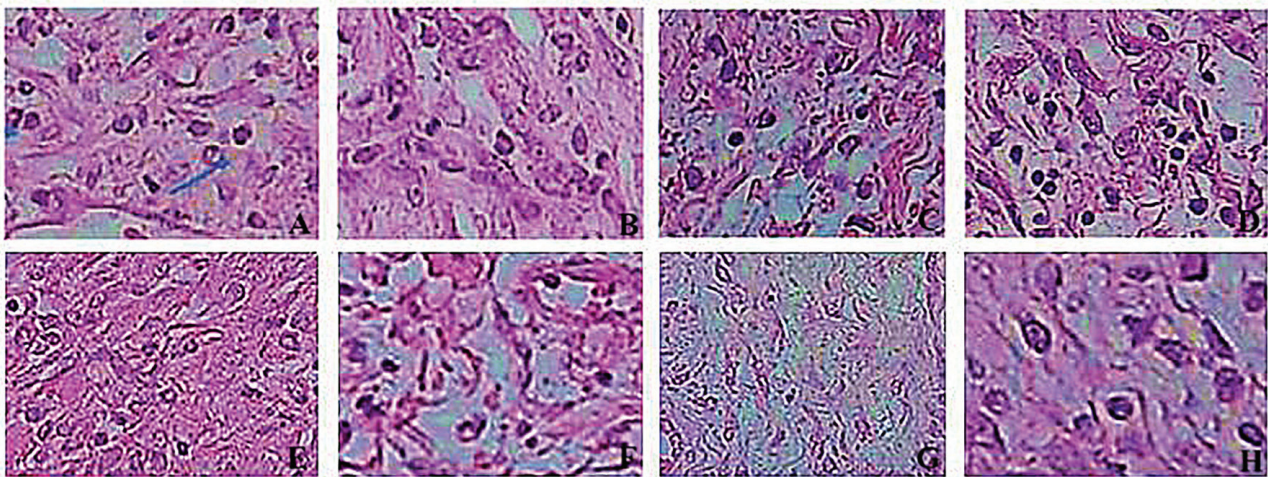


Fig. 6. Histological characteristics of rat skin on the days 7 (First row) and 14 (Second row) after wound creation in excisional wound model in experimental groups. A to D groups I to IV, respectively, and E to H groups I to IV. Wounds with surrounding skin were prepared for histological microscopic evaluation by H&E staining ($\times 400$).

is a beneficial effect of chitosan as a biologically active dressing in wound management. It has been reported that the application of chitosan to the open wounds in dogs induced exudate, which has a high growth factor activity, and induced infiltration by inflammatory cells and granulation tissue formation accompanied by angiogenesis [38, 39]. Chitosan-membrane-based wound products have been investigated both in laboratory animals and humans, however, are still at the early stages of development. Since 1980, chitosan and its derivatives have been used in skin and wound management products in Japan. Beschitin W, an artificial skin prepared from chitin threads, has been developed for human use and is on the market [40, 41].

We selected chitosan as a dressing material due to its biocompatibility, biodegradability, haemostatic activity, anti-inflammatory activity and property to accelerate wound healing [42]. The N-acetyl glucosamine (NAG) present in chitin and chitosan is a major component of dermal tissue which is essential for repair of scar tissue. Its positive surface charge enables it to effectively support cell growth and promotes surface induced thrombosis and blood coagulation. Free amino groups which are present on the chitosan membrane surface may form polyelectrolyte complexes with acidic groups of the cellular elements of blood [42]. It has several advantages over other type of disinfectants because it possesses a higher antimicrobial activity, a broader spectrum of activity, a higher killing rate and a lower toxicity toward mammalian cells. However, synthetic polymers are available at a lower price than biopolymer chitosan, substitution of chitosan by these synthetic polymers could reduce the price of chitosan-based films with safe effect on their functionality [42]. The use of nanocomposites containing metals and metal oxides allows the therapeutic use of both unique properties of nanoparticles and polymer matrix properties. In this case, often the use of nanoparticles in nanocomposite

structure allows not only to increase the stability of nanoparticles but also to reduce their potential cytotoxicity [43].

Selenium is a nonmetal that has some metal properties. The use of selenium in nanoform is promising for regenerative medicine. It is well known that nanoselenium is a highly effective long-acting antioxidant. Its local introduction in the area of injury can lead to violation of redox signaling. Selenium nanoparticles have been investigated for various medical applications and as a potential material for orthopedic implants [44]. Currently, studies which indicate precisely the ability of the selenium compounds to inhibit bacterial growth and formation of bacterial biofilms are also available [44]. Selenium has reported to show significant antiproliferative activity against HeLa and HepG2 cell lines [45]. The wound healing activity of selenium nanoparticles have revealed that 5% selenium ointment heals the excision wound of Wistar rats up to 85% within 18 days compared to the standard ointment [45].

Biomaterials derived from natural products can provide materials with greater complexity and composition. In order to mimic the extracellular matrix (ECM) conditions of the wound and to provide a scaffold for the fibroblasts for collagen deposition, ECM-based therapies have gained popularity [46]. A phase I clinical trial using fibroin to enhance wound healing is currently underway. Finally, there have been numerous marine polysaccharide hydrogels like marine collagen from *Stomolophus nomurai meleagris*, *Oncorhynchus keta*, *Lates calcarifer*, *Stichopus japonicas*, and *Salmo salar*, alginate from *Macrocystis pyrifera*, chitosan from crabs and shrimps, which are bioactive and increase wound healing rates in mice [25]. In the present study, Chitosan/Nano Selenium biofilm film revealed that there was a significant difference by means of histopathological examinations in group IV compared to other experimental groups and showed significant effect on inflammatory infiltration and

number of fibroblasts in time-dependent activity. This showed promising effect of Chitosan/Nano Selenium biofilm on wound healing.

In conclusion, Chitosan/Nano Selenium biofilm resulted in significant improvement in planimetric and histopathological indices in full thickness wound healing. Thus, from this study it could be concluded that Chitosan/Nano Selenium biofilm have a reproducible wound healing potential and hereby justifies its use in practice.

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Conflicts of Interest: None declared.

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