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RESEARCH ARTICLE

Antibacterial Activity of Nisin-Silver Nanoparticles on *Staphylococcus epidermidis* and Therapeutic Effects in Experimental Skin Infection in Mice

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Abstract

Today technology using nanoparticle when treatment pathogentic microorganism and we focused on this here .It was found that the species of *Staphylococcus epidermidis* used in present study were sensitive to Levofloxacin .The aim of report effect (Nisin-Silver Nanoparticles) on ability of complete healing injury comparing using Nisin only. In vivo study revealed that silver nanopasrticles treatment of *S. epidermidis* contaminated injured skin showed good healing process contain complete regeneration of the epithelial cells of the epidermis and good prognosis and increase of cellulartiy of the dermal content compared with untreated group. In conclusion, treatment of skin infected with *S.epidermidis* using silver nanoparticles at different concentration may limit the skin damage, localized the lesion to the incision site and enhance the healing process.

Key words: *Staphylococcus epidermidis, Nisin A, silver nano particles.*

Introduction

Staphylococcus epidermidis is the most public species of coagulase-negative staphylococci (CoNS) and. *S. epidermidis* is a Gram-positive bacterium and able to form biofilms. Is the most common types of normal human skin microbiota A frequent skin coloniser, *S. epidermidis* usually contaminates clinical microbiology samples, but is also a common cause of healthcare-associated infection [1]. Due to the tendency to form biofilms, *S. epidermidis* is a leading reason of infections related to medical devices, such as prosthetic valve endocarditis, central venous line-associated bloodstream infections, and surgical position infections (e.g. hip and knee prosthetic joint infections).

CoNS were the most commonly identified cause of central line-associated bloodstream infections in observation of healthcare-associated infections acquired in severe care units in 11 European Union (EU) Member States in 2015 and the second-most common bacterium isolated from hip and knee prosthetic joint infections in surveillance of surgical position infections in the European Union/European Economic Area (EU/EEA) [2]. Antimicrobial agents are administered concomitantly with replacement or removal of the device. Healthcare-associated strains of *S. epidermidis* tend to be multidrug-resistant, with resistance to meticillin ranging from 75% to 90% [3].Healthcare-associated strains of *S. epidermidis* produce extracellular biofilms that hinder the action of most antimicrobial agents and host immune response, thus creating treatment of medical device infections challenging and often needing the replacement or deletion of the contaminated device for effective treatment of the infection.

Silver nanoparticles (AgNPs), have strong permeability and effective broad-spectrum antibacterial properties, and it used to produce a range of antibacterial therapeutic products, such as, toothpaste, gynecologic suppository and wound dressing [4]. Thus, there is a need for “green chemistry” that

comprises a clean, nontoxic, and environmentally-friendly manner of nanoparticles synthesis, as an alternative to conventional techniques; biological methods are considered ecologically sound for the nanomaterials fabrication and safe [5]. On the other hand, nanoparticles accumulated in the organs, and they may have a toxic effect in persistence. Could take a long period to clear, however, the data of AgNPs toxicities based on *in vivo* studies is very inadequate and often controversial [6]. Bacteriocins are antimicrobial peptides produce by certain bacterial strains, which are immune to them, to compete by preventing the growth of other bacteria existing in their environment.

Nisin is a 35-mer bacteriocin of the lantibiotics group, whose use as a food preservative was approved by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO),and European Union (food additive number E234, EEC, 1983) and it is currently the most studied and characterized of all the bacteriocins produced by lactic acid bacteria (LAB) [7]. Bacteriocins structure are peptides ribosoma-synthesized many microorganism and can exhibit not wide spectra of activity (targeting members of the same species), whereas others display broader activity spectra [8].Many reports 1 antibiotics extensively subclass of bacteriocins, which includes (staphylococcin C55 and NisinA) amongst others, several type highly activity anti-clinically relevant and food-borne pathogens [9].

Material and Methods

Staphylococcus Epidermidis Isolate

The strain of *Staphylococcus epidermidis* from human skin was obtained from Department of Biology-College of Science, University of Baghdad, it was confirmed by using Api-Staph identification System and vitek 2 systems, further confirmation was made by PCR.

Antibiotic Sensitivity Test

For inoculums, standard homogenized *Staphylococcus epidermidis* was prepared in normal saline and the suspending was diluted to 0.5× 10^8 CFU ml compared with McFarland tubes [10]. Antibiotic sensitivity test for *S. epidermidis* was done by Kirby-Bauer disk diffusion method against Oxacillin OX (1), Erythromycin E (15), Levofloxacin LEV (5), Cloxacillin, CX (1), Cefepime, and FEP (30). The zones of inhibition were measured (mm) and compared with pretive chart a documented standard, the zone of inhibition (in mm) Clinical and Laboratory Standards Institute [11].

DNA Extraction

Genomic DNA was extracted from the detected bacterial isolates according to the protocol of Wizard Genomic DNA Purification Kit, Promega. Quantus Florometer was used to detect the concentration of extracted DNA

Primers Selection

The set of primers 27F (AG AG TT TG AT CT TGGCTCAG) and 1492R (TA CG GT TA CC TT GT TA CG AC TT) was used for amplification of 16s rRNA for identification of bacteria at gene level [12].

Preparation of Silver Nitrate

Silver nitrate was brought from Lobachemie. Weigh 0.0169 gm of silver nitrate and dissolve in 100 ml of distilled water to obtain three concentrations (25, 50 and 75 mM) in amber colored bottle.

Synthesis of Silver Nanoparticles

Three ml of prepared solution was added to 40 ml of silver nitrate solution in 100 ml conical flask. At room temperature Incubate for 2- 3 hours .Control is made containing only 40 ml of silver nitrate solution.

Characterization of Silver Nanoparticles

Then synthesis of silver nanoparticles was checked in UV-Visible spectroscopy at the wavelength of 300 - 700nm

Show Figure (1).

Figure1: preparation of nisin-SNPs

Synthesis and Optimization of Nisin-Silver nanoparticles Compounds

Fifty mM of AgNO3 solution was prepared and different concentrations of Nisin Making double serial dilutions (16, 32 and 64 µg/ml) in collection beaker (10 ml) to 50 mM silver nitrate solution after that was mixed with continuous stirring slowly.

Synthesis of AgNO3 at Room Temperature

The solution above mentioned are incubated at 37C (Room temperature) for 27hrs and observed the change color and checking every one day. UV-V is spectra using for the solution to monitor and measured then the flask was incubation at room temperature for another two days until the completion of the reaction.

Bacteriocin Nisin A:

Bacterocin was preparation from a commercial (Nisin A obtained from Cayman Chemical Company) shown Figure (2) as according to [13] at concentration (31.25, 62.50 and125 µg\ ml).

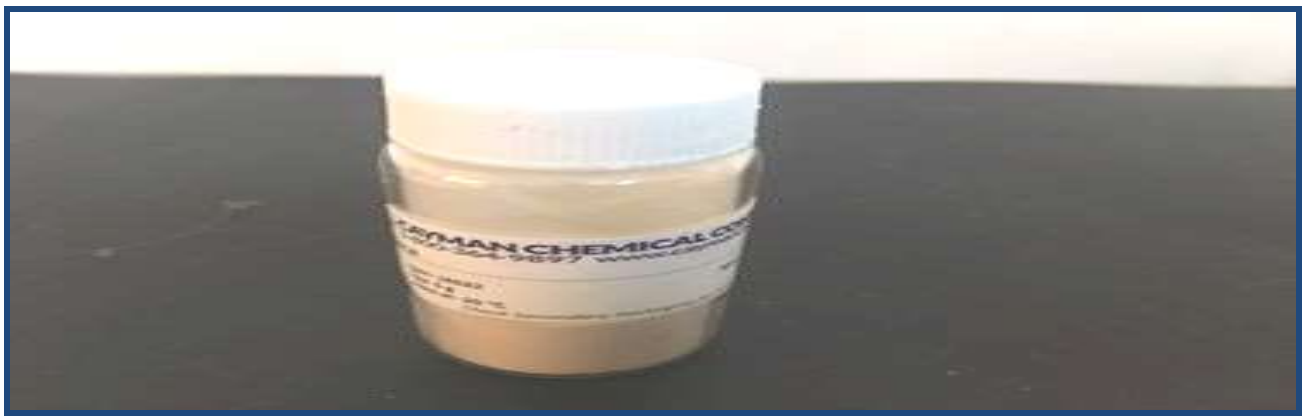

Figure 2: Nisin A obtained from Cayman Chemical Company

Agar Well Diffusion Assay (AWD):

Efficiency different concentration of Nisin A measurement activity was carried out by serial two-fold dilutions method by AWD [14].

Cream Preparation using Nisin-Silver nanoparticles

Cream formulation with AgNPs was prepared with Croda Base CR2 (Al-Maha Cosmetics factory). Briefly was prepared at a concentration of containing 10 ml AgNO3 at 50 mM added to 90 ml of Nisin A at conctration 64 µg/ ml (Figure 3).

Figure 3: Cream Nisin-Silver nanoparticles

Mice

The mice (male) were obtained from weight gm; mice were kept in disinfected cages and fed pellets and water *ad libitum*.

Experimental Design

Fifty mice were divided into 3 groups, the mice in the 1st, 2nd and 3rd (n=15 for each group) were anesthetized with an intraperitoneal injection of a mixture of xylazine (5 mg/kg) and ketamine (75 mg/kg), then the hair of the right flank was shaved (3×2 cm) using electrical shaver and the remaining hair was shaved using disposable hand shaver. The shaved area was cleaned by soap and sterile D.W., after drying skin wound was induced according to method of

(15) briefly, 3 parallel wounds line were made using sterile lancet. The 1st group considered as control positive group (injury only). The injured skin in the 2nd group were contaminated with *S. epidermidis* 0.5×10^8 cfu/ml and did not receive any treatment, while the injured skin of mice in the 3nd group was contaminated by *S. epidermidis* using one drop containing 0.5×10^8 cfu/ml and treated with Nisin-Silver Nanoparticles (conc. 64µg\ ml) after 2 hrs of infection and the treatment repeated every 12 hr. [16].

Result and Discussion

Isolation and Identification *S epidermidis*

After culture on Mannitol Salt Agar have an not ability to ferment mannitol and turn the color of medium from red to yellow were classified as a presumptive *S epidermidis*isolate .Thus, this medium was considered as selective and a differentiated medium to *Staphylococcus spp*.Whilethe isolates on Blood agar showed yellow-gray colonies are (4-3) mm in diameter on the zones of ß-hemolysis.This description is mentioned by [17] (Figure 4).

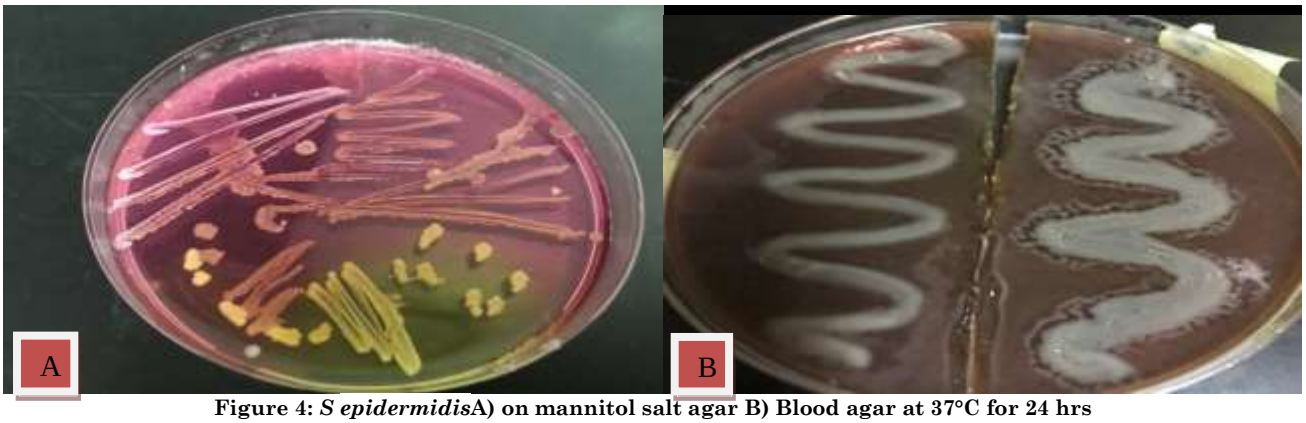

Figure 4: *S epidermidis*A) on mannitol salt agar B) Blood agar at 37°C for 24 hrs

(AST) of *Sepidermidis*

Figure (5) show various levels susceptibilities to different antibiotics among isolates that were observed by Disk diffusion method. Isolates was multi-resistance for antibiotics with a high level against, Oxacillin, Cefoxitin, and Erythromycin. But sensitivity to Levofloxacinthis result was similar to that acquired by [18].

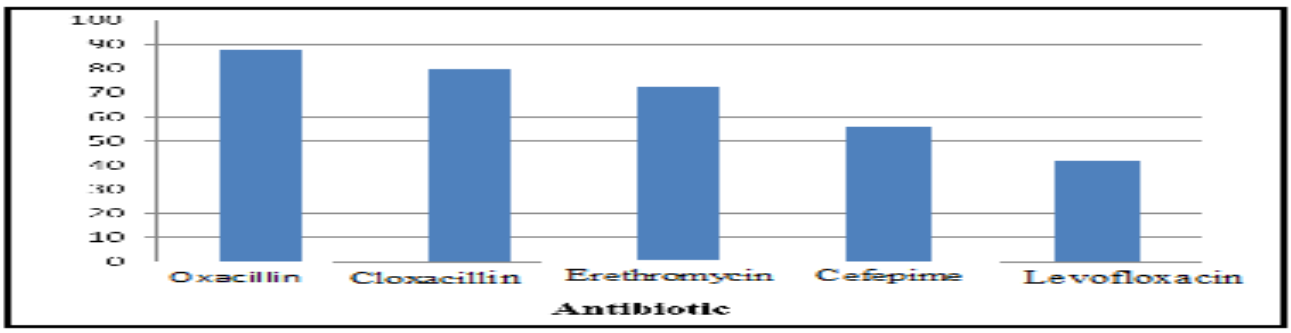

Figure 5: Antibiotic susceptibility test of S *epidermidis*

Molecular Diagnosis of *S Epidermidis*

The results shown that multiplex PCR analysis for both strain were confirmed by AST and Vitek 2 system gave positive results for multiplex Polymerase chain reaction (PCR).

Figure 6: Agarose gel (1%) electrophoresis (100v/mAmp for 90min) of amplified *16s rRNA* (1500pb) from bacterial DNA stained with ethidium bromide. Lane M. 100 bp DNA ladder, Lane 1. Unknown bacterial isolates

Study of Nisin-Silver Nanoparticles compounds characterization:

Spectral Properties of the Nisin-Silver Nanoparticles

Figure (6) revealed a strong surface plasmon placed around 400 nm. This indicates the creation of nisin-silver nanoparticles. It is not likely to detect nisin at 280 nm since it does not comprise any aromatic amino acids, the 215 nm was selection.

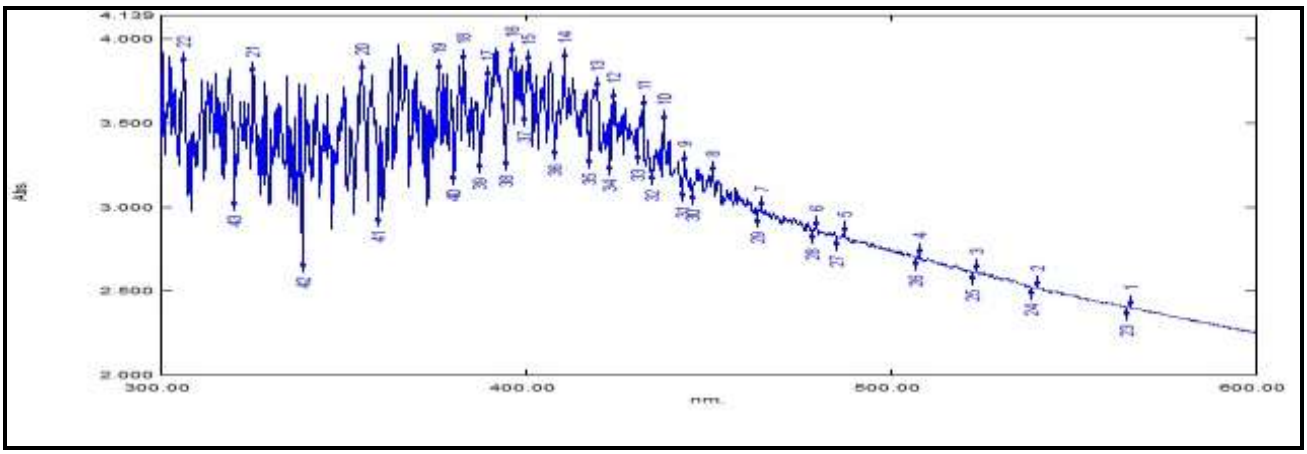

Figure7: Absorption spectra of Nisin-silver nanoparticles

Atomic Force Electron Microscopy (AFM)

The AFM micrograph acquired for the Nisin-silver nanoparticles Figure (7) shows the surface roughness alterations and the surface roughness change [root mean square (Rp)] values were recognized. For the sample the roughness value was 56 nm and the section analysis of the sample's grain size value was 42 nm.

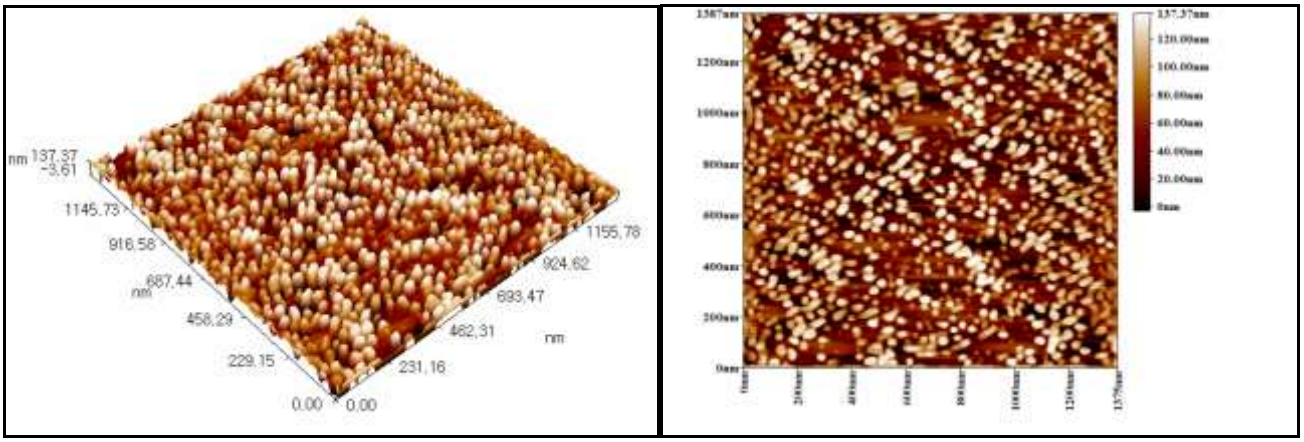

Figure 8: AFM for nisin-silver nanoparticles

Transmission Electron Microscopy Analysis (TEM) of Silver Nanoparticles

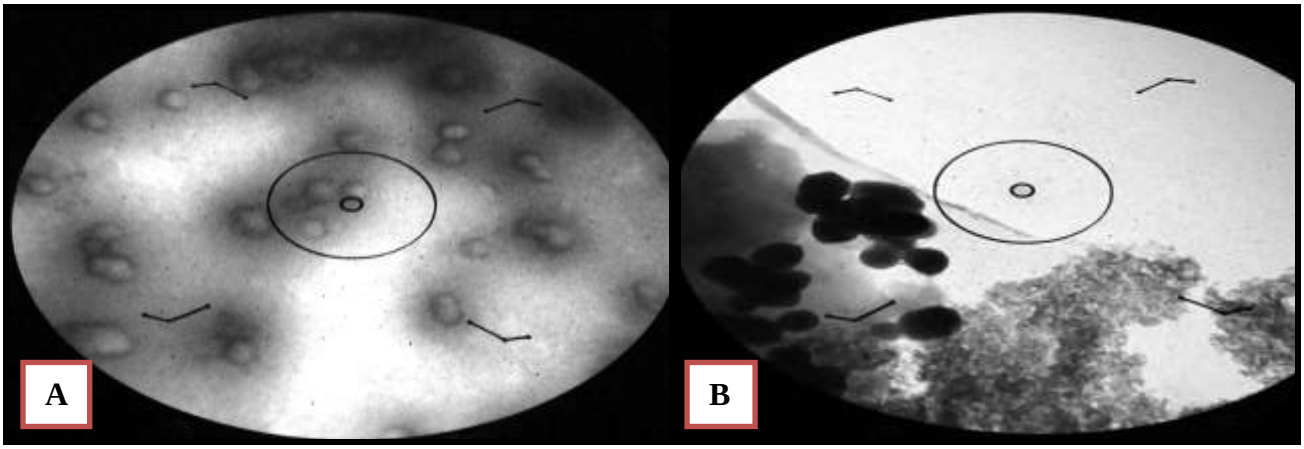

Figure 9: TEM of A) *S. epidermidis*before treatment Nisin-Silver Nanoparticles B) *S. epidermidis* after treatment Nisin-Silver Nanoparticles

Nisin-Silver Nanoparticles:

The well diffusion agar method (WDA) was used to detection *S epidermidis*sensitivity to word Nisin-Silver Nanoparticles.

The result was recorded below in Figure (9) the concentration (31, 62 and 124) µg\ mlwas appeared the inhibition zone size is (11and 9) mm recpectively while at125µg\ mlnot any inhibition, the result agree with [19].

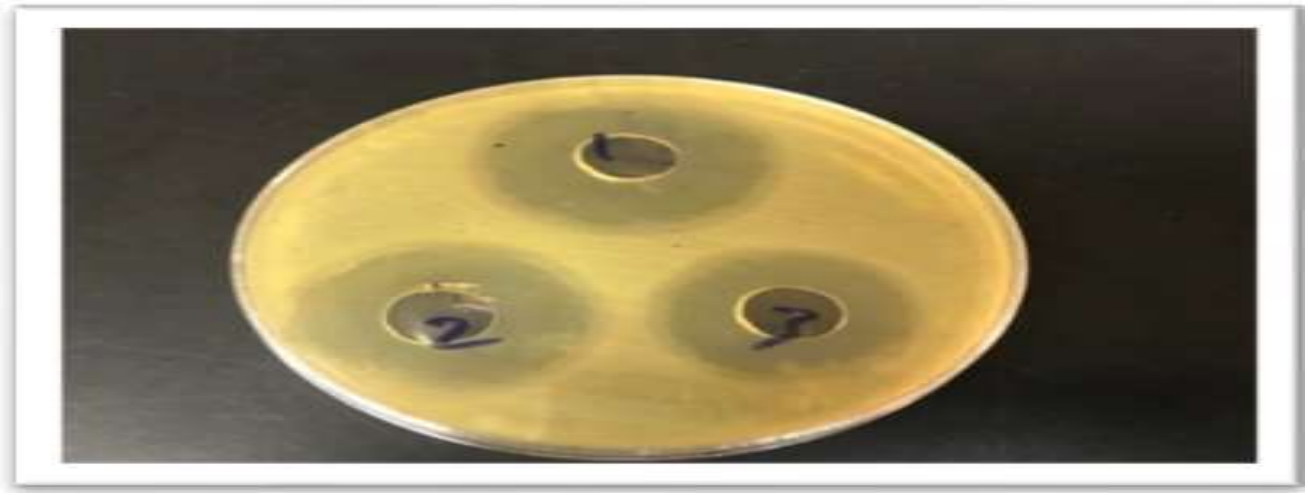

Figure 10: MIC for Nisin-Silver Nanoparticles at different concentration on (MHA) at 37°C for 24 hr

The previous study by [18] showed that Nisin A was active against MRSAand *Staphylococcusepidermidis*less than other strain in MIC range 2mg/l at 24 hrs. Many studies have regarded the activity of Nisin for use as antimicrobial therapeutic [20].

Histopathology

The lesion in the skin of mice in the control positive group, 48 hr post injury, characterized by complete loss of the epidermal layer which replaced by necrotic tissue and inflammatory cells mainly neutrophils, in addition severe infiltration of neutrophils in the dermal layer (Figure 10a), also there is severe congestion of blood vessels and mild hemorrhage in the dermal layer. At day 6 post injury, the lesion was less severe and signs of healing represented by regeneration of the epithelia of the epidermis which extended under the necrotic tissue, the epithelial cells cytoplasm were vaculated, while the collagen fiber in the dermis appear loos and irregular (Figure 10b) At 12 days post injury there is almost complete regeneration of the epithelial cells of the epidermis with mild infiltration of neutrophils in the dermal layer (Figure 11c).

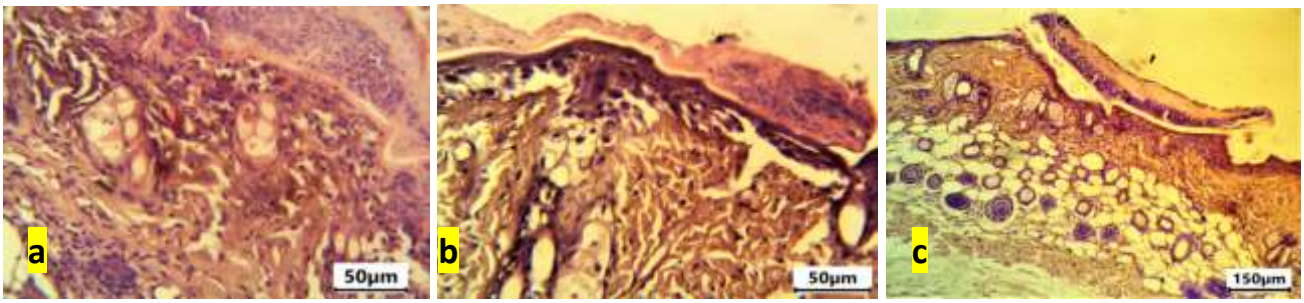

Figure 10: Sections of skin in the control positive group showed: (a) 48 hr post injury, cellular debris replaced the epithelial layer and infiltration of neutrophils in the dermis. (b) 6 days post injury, thin layer of regenerated epithelia with severe vaculation of their cytoplasm, while dermis showed loss and irregular collagen fibers. (c) 12 days post injury, whole regeneration of the epidermal layer with mild infiltration of neutrophils in the dermal layer

In the second (infected) group, 2 day post injury (24 hr post infection) there is severe destruction of the infected skin represented by severe necrosis and complete loss of the epidermal layer with severe infiltration of neutrophils in demise and congestion of blood vessels (Figure 11a). At 6 day post infection the lesion become more severe and small abscess was seen, in addition there is severe hemorrhage in the dermal layer (Figure 11b). At 12 day post infection the lesion become more severe the epithelial layer failed to regenerate and 3 out of five samples showed large abscess formation and lesion extend to the muscular layer in which neutrophils and fibrous tissue presented between muscle fibers (Figure 11c). Other section showed severe hemorrhage in the dermal layer.

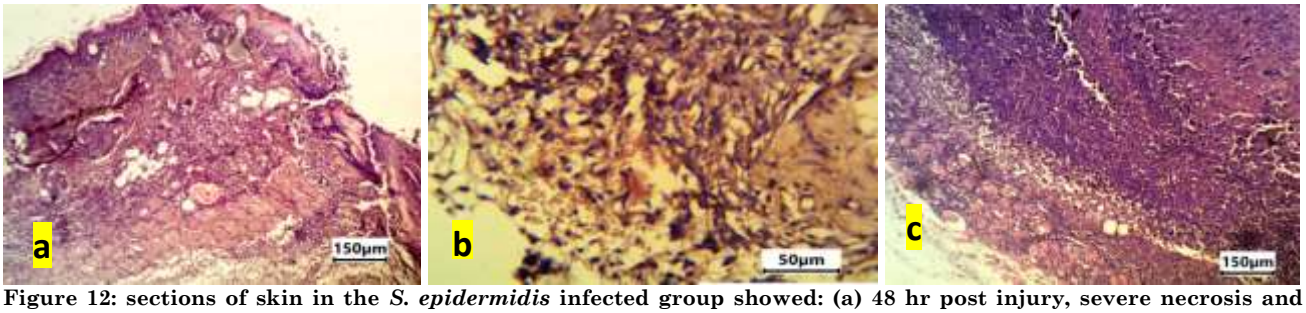

Figure 12: sections of skin in the *S. epidermidis* infected group showed: (a) 48 hr post injury, severe necrosis and complete loss of the epidermal layer with severe infiltration of neutrophils in demise and congestion of blood vessels. (b) six days post injury, severe hemorrhage in the dermal layer. (c) 12 days post injury; showed large abscess formation and lesion extend to the muscular layer in which neutrophils and fibrous tissue presented between muscle fibers

In the 3rd group (treated with nisin-silver nanoparticles, at 2 day post infection, the lesion in 3 section were less severe from untreated group, but 2 section showed necrosis and complete loss of the epidermal layer and infiltration of neutrophils in the dermis (Figure 12a) At 6 day post treatment there is complete regeneration of epithelial cells under necrotic tissue with proliferation of collagen fiber in the dermis, while one section showed healed ulcer which characterized by regeneration of epithelial layer under the necrotic tissue with few neutrophils and monocytes infiltration in the dermal layer in addition to proliferation of collagenous fiber (Figure 12b), At 12 day post treatment, 3 sections out 5 showed complete regeneration of the epithelial layer and dermis restore its normal architecture, while 2 sections showed mild hyperplasia of the epithelial cells of the epidermis and proliferation of dense fibrous tissue in the incision site in the dermis with few mononuclear cells infiltration between the fibrous tissue (Figure 12c).

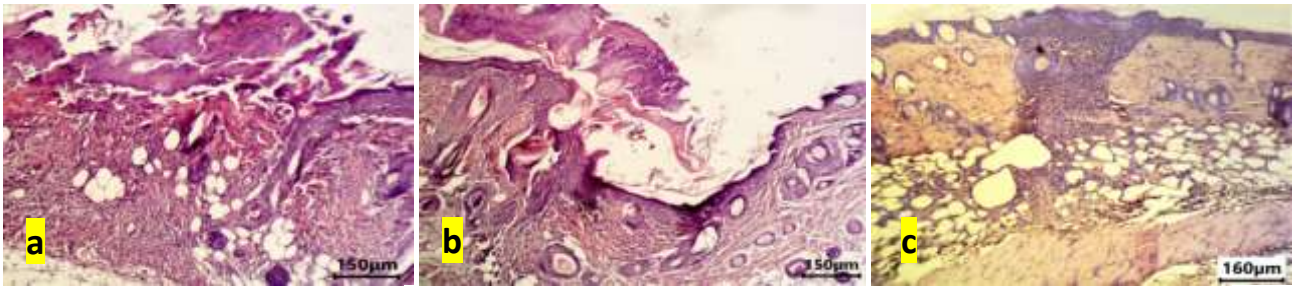

Figure 13: sections of skin in the nisin-silver nanoparticles treated group showed: (a) 48 hr post injury, severe necrosis and complete loss of the epidermal layer and infiltration of neutrophils in the dermis. (b) six days post injury, healed ulcer characterized by regeneration of epithelial layer under the necrotic tissue with few neutrophils and monocytes infiltration. (c) 12 days post injury, mild hyperplasia of the epidermal epithelia and proliferation of dense fibrous tissue in the incision site in the dermis with few MNCs infiltration between the fibrous tissues

In vivo study in the 1st group revealed normal process of wound healing which characterized by influx of inflammatory cells especially PMNs espically during the first 72 hr, and after 72 hr there is decrease in neutrophils with infiltration of few macrophages and this indicate a normal process of wound healing [15, 21].

In the second group the lesion may contributed to the virulence factors such as biofilm formation which is one of the most important virulence factor of S. epidermidis. The adaptation to environmental factors and the metabolic shift contributes to *S. epidermidis* success in colonization of host tissue and medical devices, and protects the bacteria against the hosts immune system [22] and attempts of antibiotic treatments [23, 24]. The detachment phase involves the detachment of single cells or cell cluster by several mechanisms and is thought to be crucial for the dissemination of the bacteria. Biofilms protect the bacteria and modulate the innate immune system of the host and enhance the pathogenesis of *S. epidermidis*.

Polysaccharide intercellular adhesion (PIA) block the effects of both cationic and anionic AMPs [25] and reducing opsonization of C3b and IgG binding on the bacterial surface and protects *S. epidermidis* from phagocytosis [25, 6, 27, 28]. In addition, the absence of macrophage in this group may be due to lack of contact between the bacteria and PRRs on the leukocytes, and the induction of a poor NF-κB mediated macrophage inflammatory response [29, 30].

The interaction of S. epidermidis biofilms and macrophages may reduce macrophages response to strong pro-inflammatory compounds such as LPS [31]. The histopathological changes in the 3rd group (treated group) characterized by the localization of the lesion to injured area with less damage in most sections compare with section of the untreated group.

Healing progression at 6 and 12 day post treatment may contribute to the combination of the antibacterial effects of nicin and the effect of silver nanoparticles on skin healing. Nisin bind the cell membrane using the ionic interactions of the C-terminus forming pores in the cell membrane by the penetration of the hydrophobic N-terminus leading to leakage of cellular materials through disruption of the proton motive force [32, 33, 34]. SNPs have a fast and broad spectrum antibacterial activity against both Gram-positive and -negative bacteria [35, 36].The current results of skin healing agreed with who demonstrated that silver nanoparticle dressings application has shown to enhance reepithelization, and proved to be effective in the prevention and treatment of contaminated areas.

Conclusion

In conclusion, we suggests the effects of Nisin Aagainst *S.epidermidis*at different concentrationtreatment of *S.epidermidis* infected skin with Nisin-Silver Nanoparticles limit the skin damage and localized the lesion to the incision site with good healing process.

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