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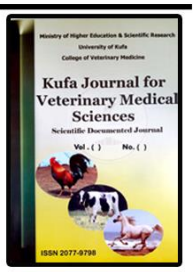

# Efficacy of Silver Nanoparticles, Antimicrobial peptides and Blue Light Combination on Healing Infected Wound by Methicillin Resistance *Staphylococcus aureus* in Rats

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**Abstract**

This study was carried out to evaluate the effectiveness of the triple combination of biologically synthesized silver nanoparticles, anti-microbial peptides and visible blue light against pathogenic clinical methicillin resistance *Staphylococcus aureus* (MRSA) *in vivo* after inducing full thickness skin injury in the initially induced diabetic rats. This experiment of *in vivo* study was for evaluation the role of the combinations in promoting wound healing after wound inducing infection using pathogenic MRSA (0.25ml from 1.5 $\times 10^8$ cfu/ml) in diabetic rats. 108 diabetic rats (aged 8-10 weeks weighing 200-250g) were divided equally into nine groups, (treatment started after the appearance of infection signs). Group A: Negative control group, group B: Positive control group, Group C: infected treated with AgNPs 8µg/ml, group D: infected treated with Gramicidin 16µg/ml, group E: infected treated with blue light for 1 hour, group F: infected treated with combination of AgNPs 0.25µg/ml with blue light for 1 hour, group G: infected treated with combination of Gramicidin 8 µg/ml and blue light for 1 hour, group H: infected treated with combination of AgNPs and Gramicidin 0.25µg/ml, 0.5 µg/ml respectively, group I : infected treated with triple combination of AgNPs ,Gramicidin 0.25µg/ml, 0.5µg/ml respectively and blue light for 1 hour. All groups treated topically daily for three days. Wound healing was assessed by measuring wound contraction at 7, 14 and 21 days post wound induction. Viable bacterial count at 21 day post wound induction. Results were according to data analysis, healing rate of treated rat with the triple combination was significantly ($p < 0.001$) faster (within 7 days) than treated rat with other double combination and each agent alone. All symptoms returned to near normal values at the end of treatment. In conclusion the topical therapy of the triple combination of AgNPs, visible blue light and antimicrobial peptides promote wound healing process in diabetic infected wound in a short period of time. A new approach can be used to combat serious infections caused by MRSA by combining AgNPs, blue light, and antibiotics.

**Key words: Silver nanoparticles, Antimicrobial peptides, blue light, MRSA, Wound Healing**

## فاعلية جسيمات الفضة النانوية والببتيدات المضادة للجراثيم والضوء الأزرق المرئي على الجروح المصابة بالمكورات العنقودية الذهبية المقاومة للميثيسيلين في الجرذان.

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** باحث

**الخلاصة:**
أجريت هذه الدراسة لتقييم فعالية التركيبة الدوائية الثلاثية المكونة من دقائق الفضة النانوية المصنعة حيويا والبيتيدات المضادة للجراثيم والضوء الأزرق المرئي ضد عزلة المكورات العنقودية الذهبية المقاومة للمثيسيلين الممرضة (MRSA) داخل الجسم الحي بعد إحداث جرح لجميع طبقات الجلد في الجرذان المصابة مسبقا بداء السكري. اجريت هذه التجربة داخل الجسم الحي لتقييم دور التركيبات في تعزيز التئام الجروح بعد احداث الجرح المصاب باستخدام (MRSA) الممرضة ( 0.25 مليلتر من $1.5 \times 10^8$ cfu/ml) في الجرذان المصابة بداء السكري. تم اخذ 108 من الجرذان المصابة بداء السكري قسمت بالتساوي إلى تسع مجموعات ( تم البدء بالعلاج بعد ظهور علامات الإصابة).مجموعة أ : مجموعة السيطرة السالبة , مجموعة ب :مجموعة السيطرة الموجبة, مجموعة ج: مصابة ومعالجة بدقائق الفضة النانوية بتركيز 8µg/ml, مجموعة د: مصابة معالجة بعقار الگرامسيدين بتركيز 16µg/ml , مجموعة  : مصابة معالجة بالضوء الأزرق المرئي لمدة ساعة ,مجموعة و: مصابة معالجة بتركيبة دقائق الفضة النانوية 0.25µg/ml مع الضوء الأزرق لمدة ساعة, مجموعة ز: مصابة معالجة بتركيبة الگرامسيدين 8 µg/ml مع الضوء الازرق المرئي لمدة ساعة واحدة, مجموعة ح: مصابة معالجة بالتركيبة المزدوجة لدقائق الفضة النانوية و عقارالگرامسيدين بتركيز 0.25µg/ml, 0.5 µg/m ,على التوالي, مجموعة ط : مصابة معالجة بالتركيبة الثلاثية المكونة من دقائق الفضة النانوية وعقارالگرامسيدين بتركيز 0.25µg/ml, 0.5 µg/ml على التوالي مع الضوء الأزرق المرئي لمدة ساعة. تم علاج كل المجموعات موضعيا ويوميا لمدة ثلاثة أيام. و تم تقييم شفاء الجروح بقياس قطر الجرح في الأيام 21,14,7 بعد إحداث الجرح. أيضا تم تقدير العد البكتيري الحي في يوم 21 بعد احداث الجرح. سجلت نتائج الدراسة في الجسم الحي وفقا لتحليل المعطيات , كان معدل شفاء الجروح في الجرذان المعالجة بالعلاج الثلاثي أسرع بصورة واضحة إحصائيا($p < 0.001$) خلال 7 أيام مقارنة بالجرذان المعالجة بالعلاجات المزدوجة والعلاجات المستخدمة على حده. عادت جميع الأعراض بما في ذلك العلامات السريرية إلى القيم القريبة من الطبيعية في نهاية العلاج. وبالتالي نستنتج إن العلاج الموضعي للتركيبة الثلاثية المكونة من دقائق الفضة النانوية والضوء الازرق المرئي والبيتيدات المضادة للجراثيم تعززعملية التئام الجروح في الجروح المصابة في فترة قصيرة من الوقت. وبالتالي يمكن استخدام نهج جديد في مكافحة الإصابات الخطيرة التي تسببها المكورات العنقودية الذهبية المقاومة للمثيسيلين من خلال الجمع بين دقائق الفضة النانوية AgNPs , الضوء الأزرق المرئي والمضادات الحيوية.

## Introduction

Diabetes prevalence is increasing in the world, and the appropriate management of patients with diabetes has become increasingly important for the prevention of hospital-acquired infections (1). Surgical wound infections are the second most common cause of nosocomial infections (2). The high rate of surgical wound infections is associated with higher morbidity, mortality and increased medical expenses (3). In spite of the new antibiotics available today, surgical wound infection still remains a threat due to secondary bacterial contamination and widespread use of prophylactic antibiotics that lead to emergence of multi-drug resistant bacteria (4). *S. aureus* is the most important pathogen in diabetic wound infections; even when it is not the only isolate, it is usually a component of a mixed infection (5). Treatment of infections caused by *Staphylococcus aureus* has become more difficult because of the emergence of multidrug-resistant isolates (6). Methicillin-resistant *S. aureus (*MRSA) presents problems for patients and healthcare facility-staff whose immune system is compromised, or who have open access to their bodies via wounds, catheters or drips (7). Failure of antibiotics to manage infections caused by multidrug-resistant (MDR) pathogens, especially MRSA, has triggered much research effort for finding alternative antimicrobial approaches with higher efficiency and less resistance developed by the microorganisms. Silver nanoparticles (AgNPs) have been reconsidered as a potential alternative to conventional antimicrobial agents (8). The use of AgNPs alone or in combination with other antimicrobial agents has been suggested as a potential alternative for traditional treatment of

infections caused by MDR pathogens (9). AgNPs were found to exhibit antibacterial activity against MRSA in vitro when tested alone or in combination with other antimicrobial agents (10,11). Blue light is recently attracting increasing attention as a novel phototherapy-based antimicrobial agent that has significant antimicrobial activity against a broad range of bacterial and fungal pathogens with less chance to resistance development compared to antibiotics (12,13). Furthermore, blue light was bactericidal, their expanded in responding to the occurrence of pathogens through the span of treatment, they are in the same way active against antimicrobial resistant pathogens and was fit for harming bacteria that produce biofilm, and have low intrinsic toxicities (14,15). therefore the aim of this study to evaluate the combination activity in promoting wounds healing in experimentally induced hyperglycemic rats.

**Materials and methods**

**- Preparation of bacteria**

The *S. aureus (*MRSA) was obtained from Al-Hilla Surgical Hospital, Iraq. The bacteria were cultured in Muller-Hinton broth and the suspension was centrifuged at 1000 rpm for 15 min. The supernatant was discarded and the bacteria were diluted to $10^8$ CFU $mL^{-1}$ in sterile phosphate-buffered saline. Ten µL of the bacterial suspension (1.5 $\times 10^8$ cfu/ml) were added to each wound after induction of full thickness skin incision, immediately (16).

**- Experimental Animals**

One hundred eight rats aged 8-10 weeks weighing 200-250g supplied from animal house of college of veterinary medicine/Baghdad university were used to perform the experiment of the present study from (October 2017 to March 2018). They were housed and maintained in a conventional animal facility, with controlled conditions of temperature ($20\pm5C^{o}$)The animals were fed commercial pellet ad libitum. Fresh water was also provided ad libitum. Each group of rat were housed in a plastic cage containing hard wood chip as bedding, and the bedding was changed every three days to ensure a clean environment.

**- Induction of Diabetes in rats**

Diabetes was induced by the administration of alloxan monohydrate in a dose 150 mg/ kg body weight intraperitoneally (I.P) for two successive days (17). A group of 12 rats with blood glucose levels above 250 mg/dL was selected for the experiment.

**- Creation of full thickness skin defect**

All rat were anesthetized with I.M. injection of (90 mg/kg.BW ketamine), in combination with 40 mg/kg.BW xylazine. The skin area of the back of rat were prepared aseptically for surgery and full thickness skin wounds (4 mm in diameter) were made on dorsal midline using sterile biopsy punch equipment. The wounds were left open without any dressing during the experiment (18). the rat were randomly divided into nine groups of 12 animals.

**- Induction of infection**

After creation of the full thickness skin incision, 10 µl of the prepared bacterial isolate (MRSA) at concentration of $1.5\times10^8$ CFU/mL was spread along the created wound and let to interfere within the wound and left the wound opened. Then leave the animals until appearance of wound inflammatory and infection signs then started with treatment with therapeutic preparations according to each group:

**- Group (A):** infected wound left without any treatment.

- **Group (B):** infected wound treated with glycerine topically daily with AgNPs for three days.
- **Group (C):** infected wound treated with AgNPs alone topically at conc.8µg/ml daily and for three days.
- **Group (D):** infected wound treated with Gramicidin alone topically at conc. 16µg/ml daily and for three days.
- **Group (E):** infected wound treated with blue light exposure alone for 1 hour daily and for three days
- **Group (F):** infected wound treated with combination of AgNPs topically at conc. 0.25µg/ml with exposure of blue light for 1 hour daily and for three days.
- **Group (G):** infected wound treated with combination of Gramicidin topically at conc. 8 µg/ml and blue light exposure for 1 hour daily and for three days.
- **Group (H):** infected wound treated with combination of AgNPs and Gramicidin topically at conc. 0.25µg/ml, 0.5 µg/ml respectively, daily and for three days.
- **Group (I):** infected wound treated with triple combination of AgNPs and Gramicidin topically at conc. 0.25µg/ml, 0.5µg/ml respectively with blue light exposure for 1 hour daily and for three days.

**- Parameters**

**Wound size measurement**

All induced wounds were photographed at consecutive time intervals (all images were taken from a distance of 25 cm of wound sites and without zooming). The photos which taken immediately after the induction of wound represent Day 0.As well as Digital photographs were captured on days 0, 7, 14 and 21 of the experiment for wound healing inspection. in respect of evaluate healing efficacy, the induced wounds scar sizes were measured. Wound size (provides the quantity of the wound area) was determined every post-treating day and compared with the initial wound area using the following formula: (19).

$$\text{wound contraction } (\%) = \frac{A_o - A_t}{A_o} x100$$

where, *A0* is the initial wound area while *At* represent the area of wound at the time of photo capturing and bacterial counting (on days 0, 7, 14 and 21, respectively). Image analysis software was used for evaluation of the wound area by taking the images and calibration in same days.

**Wound infection measurement**

Two methods were applied for evaluation of bacterial load at the wound site on day 21. First method was carried out by taking a swab from the wound surface for analyzing surface bacteria. Then the sample was carried to a proper diluents. After that to estimate the bacterial concentration, the suspensions were serially diluted from $1{:}10^3$ to $1{:}10^{12}$ with sterile Muller-Hinton Broth media and each dilution was spread on Muller-Hinton-agar plates.

The second method estimate infection of tissue depth on the 21st day. The entire wound with the nearby normal skin was excised, then tissue biopsies were homogenized in a tissue grinder after weighing and then homogenized in 1.50 mL of normal saline (20). After that the homogeneous tissue 1:10 was placed in the dilution blanks using normal saline in order to serial dilutions preparation, and each dilution was placed on Mannitol Salt Agar plates. The CFU per gram of tissue was calculated by the following equation (21)

$$CFU/g = \frac{Plate\ count\ \times\ (1\ per\ dilution)\ \times\ 10}{Weight\ of\ homogenized\ tissue}$$

**Statistical Analysis**

Statistical analysis was applied by two ways ANOVA with least significant differences (LSD) to compare groups means .Probability level $P<0.05$ and to test the significant differences between the averages of the traits and considered statistically significant applying the SAS method (22).

**Results**

**- Evaluation the activity of Silver Nanoparticles in Promoting Healing of Surgical Wound in Experimentally Induced Diabetic Rats**

**- Clinical Symptoms of the Rats and Evidence of Diabetes**

The clinical signs appeared on the animals soon after intraperitoneal injection of alloxan monohydrate for two consecutive days was general weakness, depression, loss of appetite, and change in the color of hair. The evidence of diabetes was proven by checking blood sugar of rat after two days Fig (1). Furthermore, follow up the blood glucose elevation along the period of the experiment and checked at different time.

**Fig. (1) Clinical signs of Depression and General Weakness after the incidence Diabetes**

**- Evaluation of Wound Healing Process**

**- Morphologically:**

Wounds created by 14.5mm$^2$ biopsy punch were consistent in size and shape without significant bleeding. All wound surfaces post-wounding at the time of MRSA challenge with obvious signs of infection. Signs of inflammation pain, swelling, induced slight erythema, exudate, was noted over each wound, the wounds accumulated approximately around the wounds 0.1mL of exudate fig.(2). Exudate is ‘‘material, such as fluid, cells or cellular debris, which has escaped from blood vessels or is produced by cells locally, and has been deposited in tissue or tissue surfaces. Larger amounts of exudate were observed on the wounds of control group, and were yellow in color and thicker in consistency than those from the treated group

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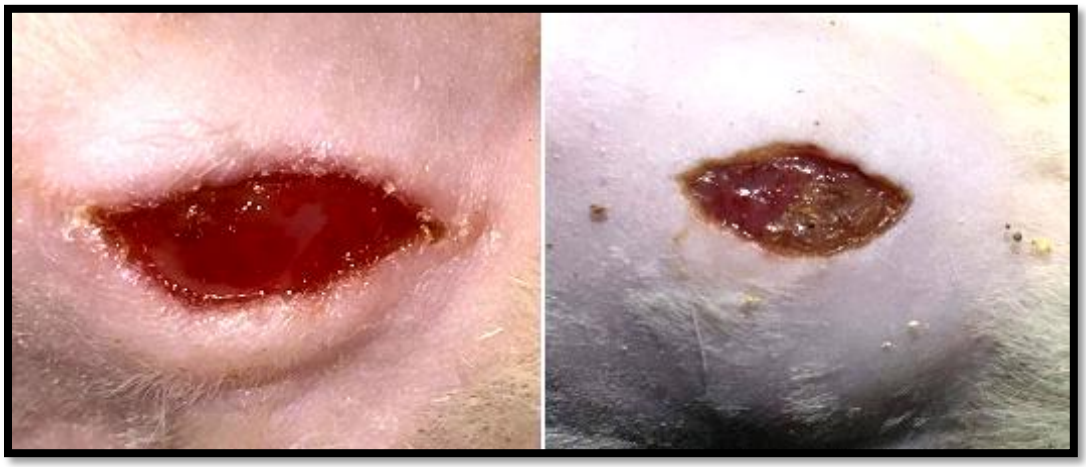

**Fig. (2) gross signs of infection (redness, swelling, pus) of wound**

On day 7, the wounds in triple combination treated looked complete healing by gross examination and showed within 14 day, regenerated hair encircling the wound fig. (1, 2, 3, 4, 5, 6, 7, 8, 9). On day 14, exudates dried and formed scabs on all the wounds treated with double combination and single agent, except control group. We define scab as a crust of dried blood, serum, and exudate over the wound during healing. At day 21, the wounds in the control group were still covered by scabs and the wounds in another treated groups looked healed by gross examination fig. (1, 2, 3, 4, 5, 6, 7, 8, 9).

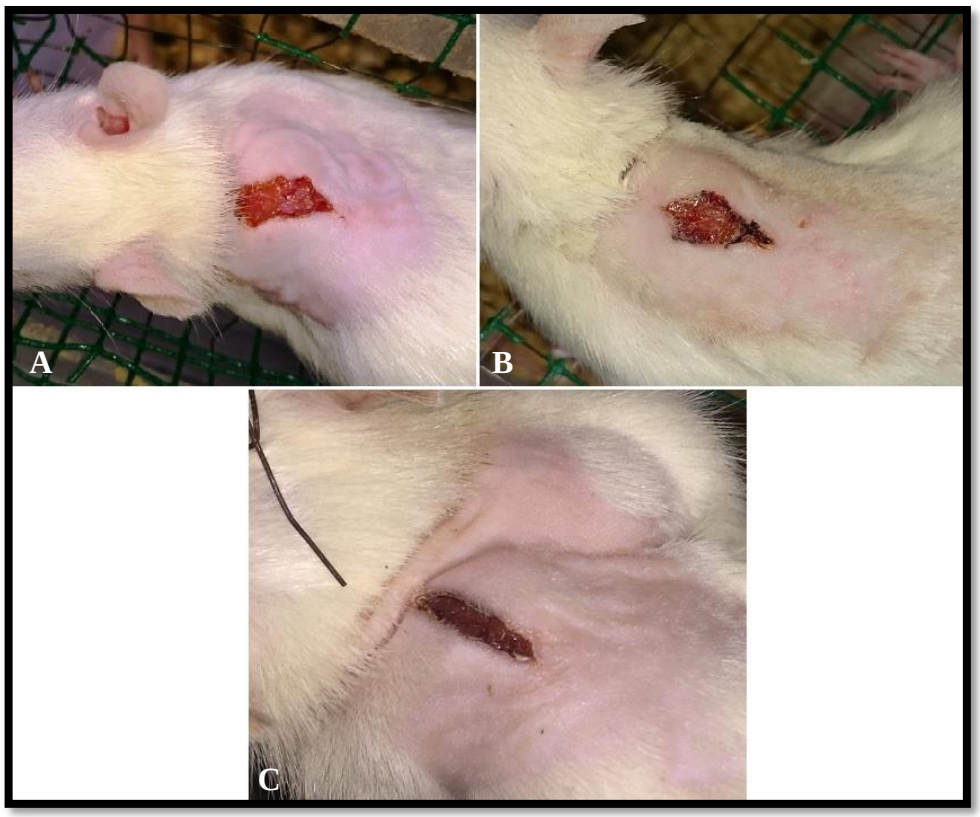


**Fig.(1) Imaged showed wound healing process of group not treated A-7 days, B-14 days, C- 21 days**

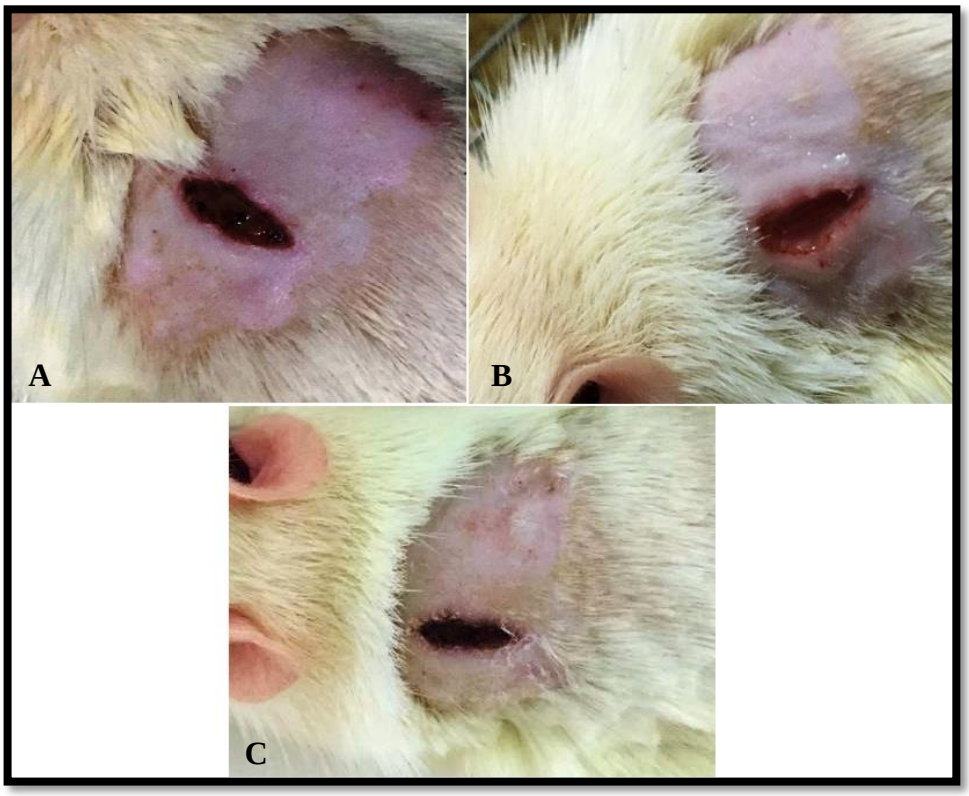


**Fig.(2) Imaged showed wound healing process of group treated with glycerin onlyA-7 days, B-14 days, C- 21 days**

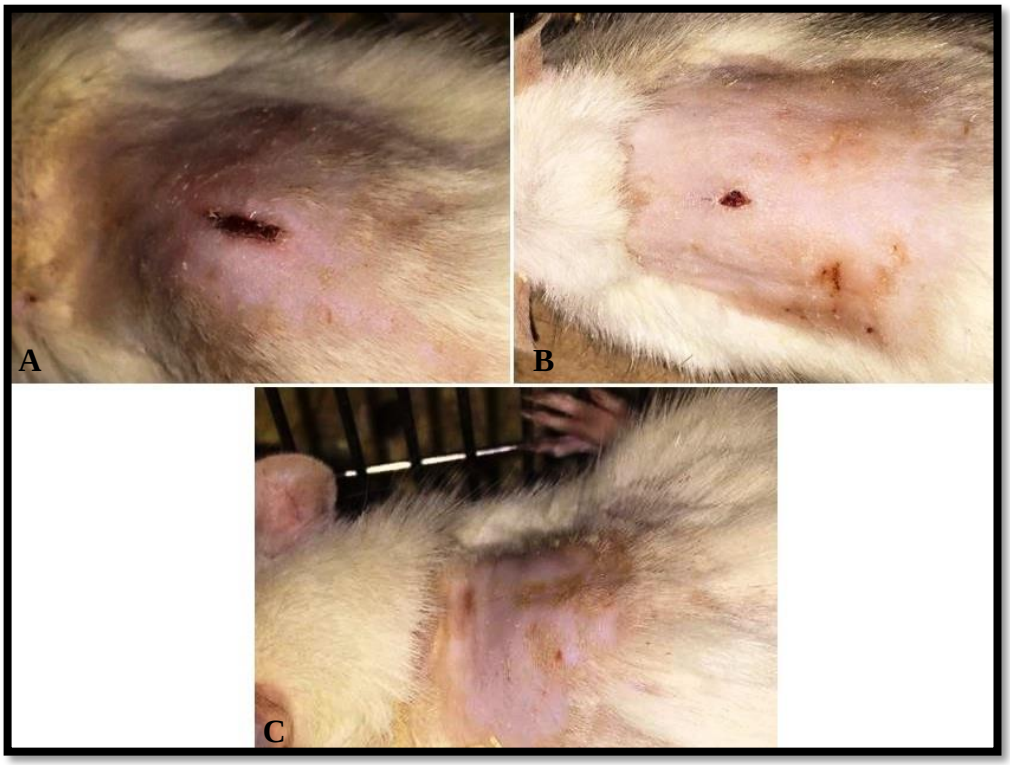


**Fig.(3) Imaged showed wound healing process of group treated with AgNPs only, A-7 days, B-14 days, C- 21 days**

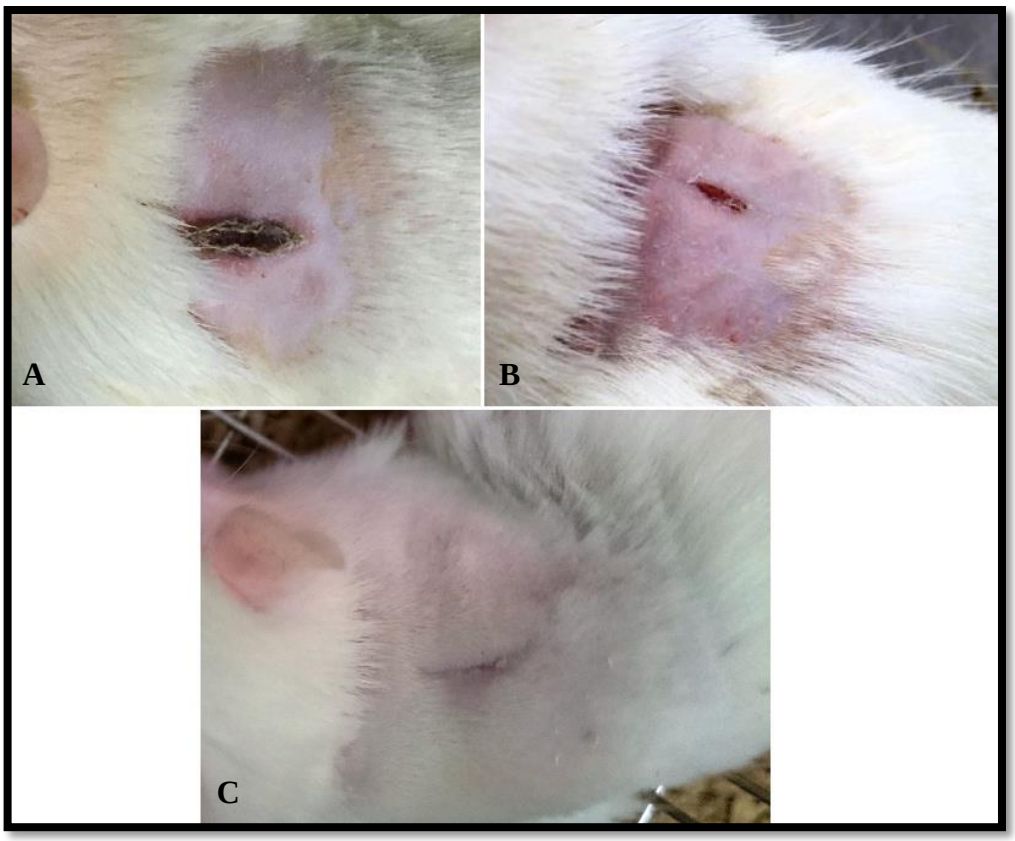


**Fig.(4) Imaged showed wound healing process of group treated with Gramicidin only, A-7 days, B-14 days, C- 21 days**

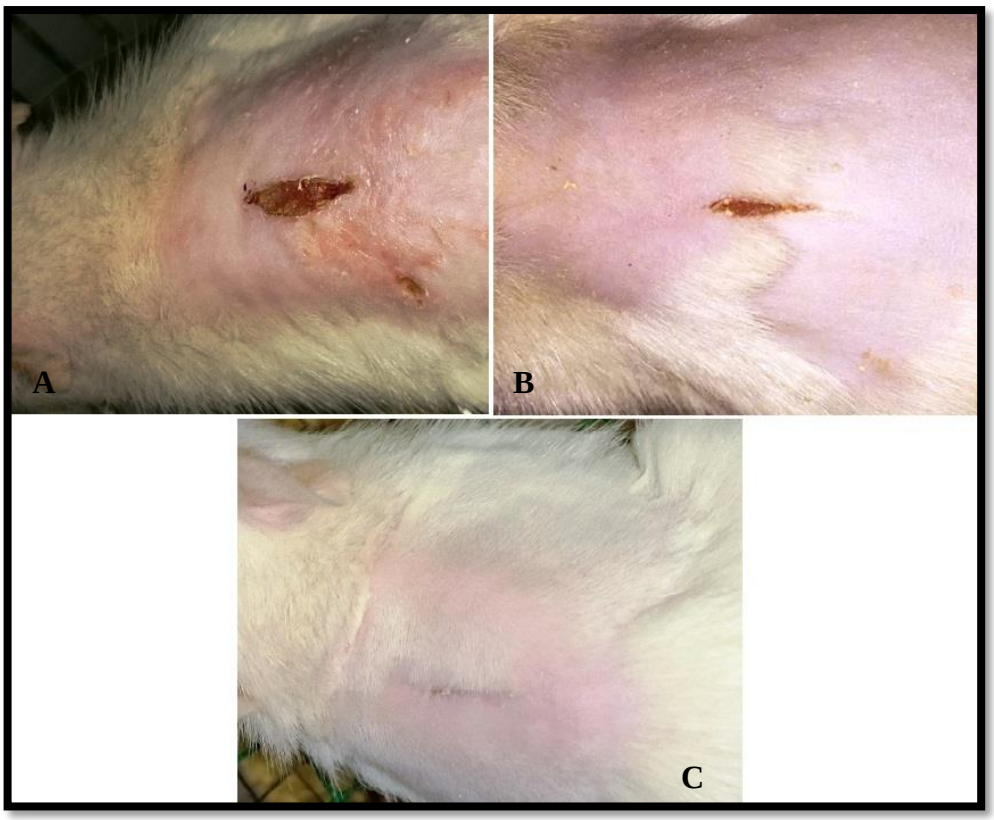


**Fig.(5) Imaged showed wound healing process of group treated with Blue light exposure only, A-7 days, B-14 days, C- 21 days**

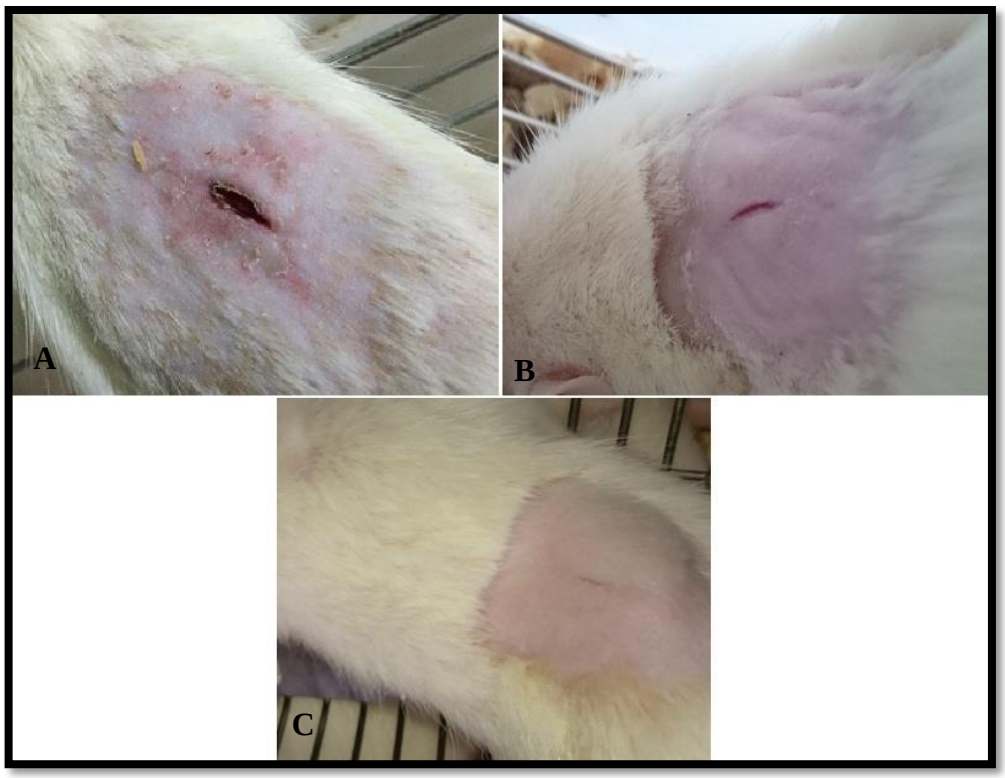


**Fig.(6) Imaged showed wound healing process of group treated with AgNPs+Gramicidin, A-7 days, B-14 days, C- 21 days**

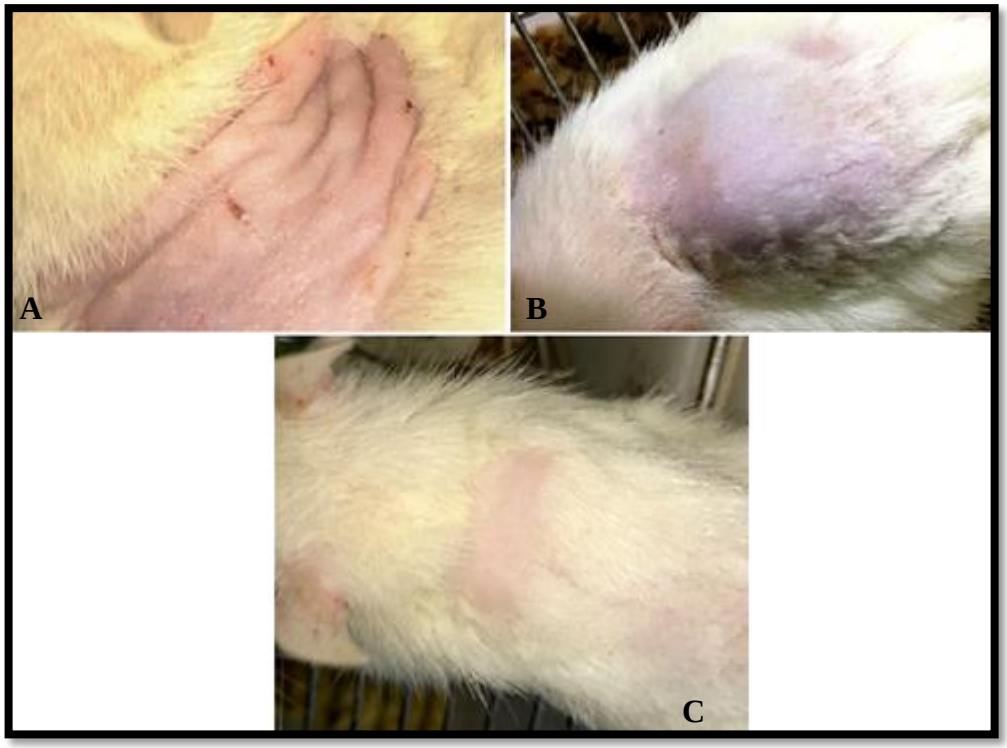


**Fig.(7) Imaged showed wound healing process of group treated with AgNPs + Gramicidin + Blue light, A-7 days, B-14 days, C- 21 days**

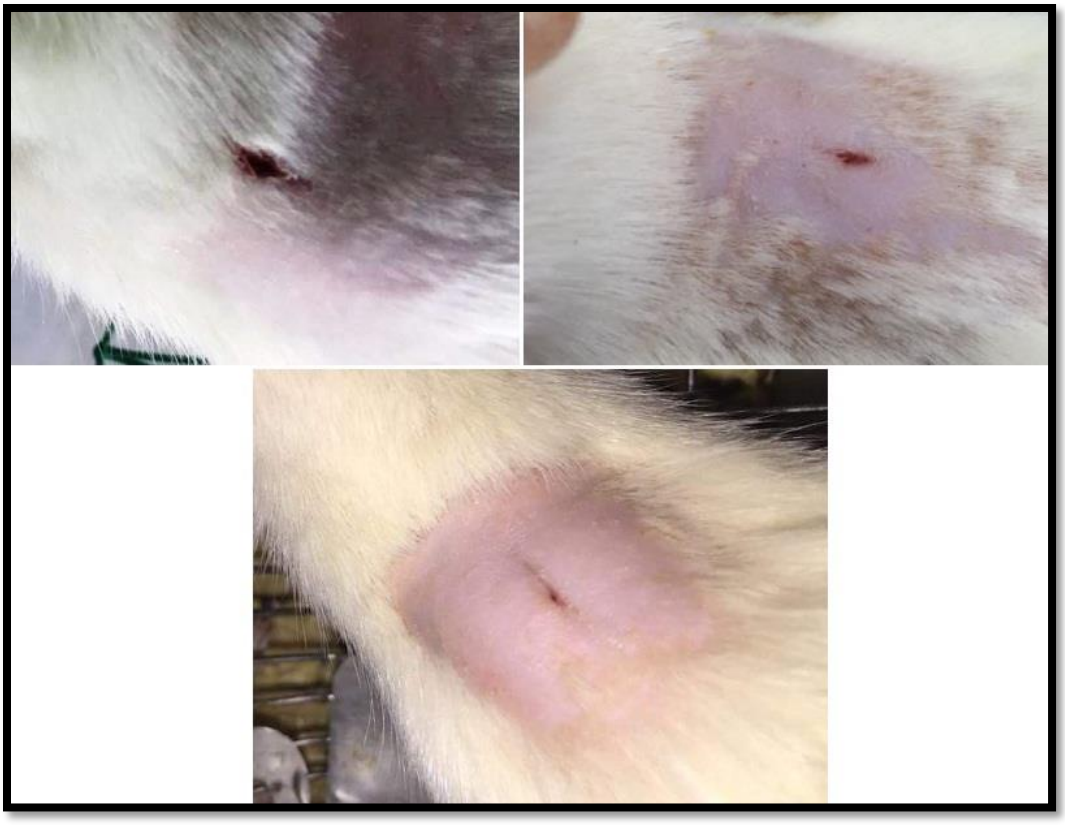

**Fig.(8) Imaged showed wound healing process of group treated with AgNPs + Blue light exposure, A-7 days, B-14 days, C- 21 days**

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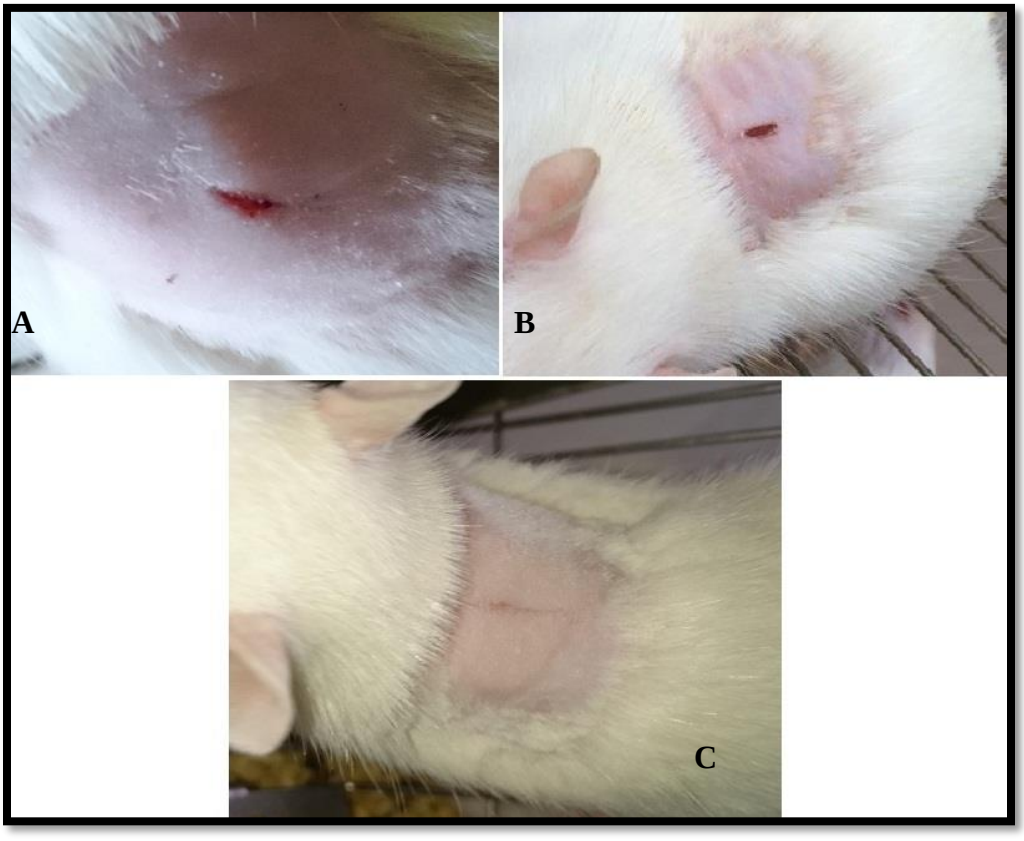


**Fig.(9) Imaged showed wound healing process of group treated with Gramicidin + Blue light exposure, A-7 days, B-14 days, C- 21 days**

**Measurement of wound area**

The result in the table (1) illustrated the effect of triple combination AgNPs, gramicidin and blue light and other treatment on wound bed area. Increased wound closure was observed in group treating with triple combination which record at 7 days 3.35±0.12 (76.81%) and reached 100% closure wound at day 21 compared with another double treated group (F, G and H) which recorded, 56.05, 51.28 and 38.06 % respectively at 7 days, and reached 91.14, 85.32 and 98.26 respectively closer at 21 days. In another hand, higher significant closure wound in treated group with AgNPs 93.07% at 21 days compared with gramicidin and blue light treated group78.75 and 77.92% respectively, in compared with control groups which showed no observed improvement in wound contraction 66.36 and 66.92 in negative and positive control respectively.

**Table (1): Effect of topical application of AgNPs, Gramicidin, blue light and combination on wound area ($mm^2$). Data are presented as mean ± SE.**

| Days / Groups | | Wound area (% wound contraction) | | | |
|---|---|---|---|---|---|
| | day O | Day 7 | Day 14 | Day 21 | |
| **Negative Control (G A)** | 14.45 | 12.25±0.12 (15.22) A a | 9.85±0.15 (31.83) A a | 4.78±0.25 (66.92) A b | **LSD= 2.957** |
| **Positive Control (G B)** | 14.45 | 12.32±0.27 (14.74) A a | 8.88±0.05 (38.54) A a | 4.86±0.11 (66.36) A b | **LSD= 3.548** |
| **AgNPs (G C)** | 14.45 | 8.25±0.15 (42.90) C a | 3.05±0.14 (78.89) C b | 1.00±0.10 (93.07) D b | **LSD=4.639** |
| **Gramicidin (G D)** | 14.45 | 10.05±0.17 (30.44) B a | 6.05±0.12 (58.13) B b | 3.07±0.18 (78.75) B b | **LSD= 4.852** |
| **blue light (G E)** | 14.45 | 10.85±0.12 (24.91) A a | 7.24±0.05 (49.89) A b | 3.19±0.02 (77.92) B c | **LSD= 3.389** |
| **AgNPs+ blue (G F)** | 14.45 | 7.04±0.12 (51.28) C a | 3.16±0.15 (78.13) C b | 1.28±0.19 (91.14) C b | **LSD= 3.645** |
| **Gramicidin+blue (G G)** | 14.45 | 8.95±0.11(38.06) B a | 5.05±0.13 (65.05) B b | 2.12±0.10 (85.32) C b | **LSD= 3.874** |
| **AgNPs+ Gram. (G H)** | 14.45 | 6.35±0.15 (56.05) D a | 3.03±0.12 (79.03) C b | 0.25±0.10 (98.26) D c | **LSD= 2.598** |
| **AgNPs+ Gram.+ blue (G I)** | 14.45 | 3.35±0.12 (76.81) E a | 0.05±0.12 (99.65) D b | 0.00±0.10 (100) D c | **LSD=** 2.942 |
| | | **LSD= 1.594** | **LSD=2.274** | **LSD=0.957** | |

**N=12** Different capital letters denote significant differences (p□0.05) between group.
Different small letters denote significant differences(p□ 0.05) within groups.
**Group A**: Negative control group (infected non treated).
**Group B**: Positive control group (infected with MRSA and treated glycerin).

**- Viable bacterial count**

The result in table (2) illustrated the total and methicillin resistance *Staph.aureus* bacterial count of wound bed in treated and untreated group. Data analysis showed that good inhibition of total bacteria and MRSA growth was gained in all groups treated with AgNPs compared with other treated groups. MRSA count in control group was significantly higher than treated rat was more than 500 colonies on medium after 7 days of infection. At 21 day after treatment significant reduction in wound total and MRSA bacterial count was obtained in all treated groups in compared with control groups.

**Table (2) Bacterial count of wound bed in treated and untreated groups**

| Groups | Bacteria | Days | Bacterial count |
|---|---|---|---|
| **Negative Control** | Total | 21 | $56.32\times10^{6}$ |
| | *S. aureus* | 21 | 22.00 cfu/gm |
| | | | |
| **Positive Control** | Total | 21 | $55.42\times10^{6}$ |
| | *S. aureus* | 21 | 27.00 cfu/gm |
| | | | |
| **AgNPs** | Total | 21 | $1.02\times10^{3}$ |
| | *S. aureus* | 21 | 00.00 cfu/gm |
| | | | |
| **Gramicidin** | Total | 21 | $5.42\times10^{6}$ |
| | *S. aureus* | 21 | 1.05 cfu/gm |
| | | | |
| **Blue light** | Total | 21 | $6.12\times10^{4}$ |
| | *S. aureus* | 21 | 2.00 cfu/gm |
| | | | |
| **AgNPs + Gramicidin** | Total | 21 | $1.02\times10^{4}$ |
| | *S. aureus* | 21 | 0.00 cfu/gm |
| | | | |
| **AgNPs + blue light** | Total | 21 | $2.14\times10^{4}$ |
| | *S. aureus* | 21 | 0.00 cfu/gm |
| | | | |
| **Gramicidin + blue light** | Total | 21 | $6.42\times10^{4}$ |
| | *S. aureus* | 21 | 2.55 cfu/gm |
| | | | |
| **AgNPs+ Gram.+ blue** | Total | 21 | 0.00 |
| | *S. aureus* | 21 | 00.00 cfu/gm |

## Discussion

Nanomaterials with antimicrobial activity that elevate the effectiveness and safety of antimicrobial administration are called nano-antibiotics (23). Their capability in control of infection has been explored and demonstrated *in vitro* and *in vivo* (24). Due to prompt prevalence of multidrug-resistant pathogens and insufficient research regarding antibiotic production; the AgNPs could be useful alternatives of routine antibiotic therapy.

Attention to the wound tissue remodeling and its infection is critical

for quick repair with no side effect. Wound healing is a treatment priority especially for diabetic or infected wounds suffering patients (25). Silver is a broad-spectrum antimicrobial that inhibits growth of microbes (26). It has been previously shown that AgNPs have *in vitro* antibacterial activities against *S. aureus* (27). In this study, we report *in vivo* capabilities of AgNPs that appear to accelerate healing of wounds inoculated with *S. aureus* in a rat model of skin wound. The results of this study indicated that AgNPs had the ability in relieve pain significantly when its applied topically on the infected surgical wounds in experimentally induced diabetic rats. This reduction in pain may be due to the analgesic effect of AgNPs.

Many researchers proved the analgesic action of AgNPs by exerting thermal stimulus on rat tail, and they noticed maximum delay in tail flick after application of AgNPs suggested that it might have some analgesic properties (28,29). Also the findings of this experiment showed that AgNPs able to accelerate infected surgical wounds healing significantly in comparison with the other groups of diabetic rats. The result of this study was in agreement with other studies showed that the application of AgNPs enhanced wound healing (30-34). AgNPs could improve the healing of wound initially on the basis of the known antimicrobial property, as well as the anti-inflammatory effect. A great problem in the infected surgical wounds healing is the bacterial contamination. The presence of bacteria, mainly multi-resistant organisms and bacterial MRSA in the wound delays the wound-healing process because of competing with host cells for nutrients and oxygen. Furthermore, their waste products are also toxic to host cells (35).

Wound healing was potentially slowed or arrested by a number of different events and conditions. One event that hinders wound healing is colonization of the wound bed by microorganisms (36). In addition to the production of a variety of toxins and proteases, the presence of microorganisms in a wound may also lead to a prolonged inflammatory response. The host inflammatory response is remarkably effective at eliminating the invading microbial population, but that same process over time may also damage the surrounding tissues (37). The presence of a bacterial burden in a wound stimulates a pro-inflammatory environment, the presence of bacteria induces also migration of monocytes, macrophages, and leukocytes, all of which initially act in an appropriate fashion but later produce a response that is exaggerated and deleterious. This is evidenced by the fact that wounds associated with a heavy bacterial load often show healing failure. In addition, bacteria and bacterial products, such as endotoxins and metalloproteinases, can cause disturbances in all phases of wound healing (38,39).

Increased bacterial burden in a wound also affects tissue oxygen availability. Leukocytes are needed in the burn to kill phagocytic bacteria-by mechanisms that involve consumption of significant amounts of molecular oxygen. In severely under perfused wounds, increased oxygen consumption by inflammatory cells can act as a sump, ‘‘stealing’’ oxygen required for basic wound metabolism. In addition, the white blood cells’ inflammatory response needed to kill bacteria increases the release of damaging oxygen free radicals. Antioxidant properties of AgNPs may be accelerate wounds healing by scavenging these free radicals, without proper antioxidant activities, wound healing might be delayed, or severe tissue damage can occur. The increased production of

enzymes and the release of toxins can also facilitate an induced cellular failure (38). Simultaneously these reasons might be explain why the group infected with MRSA and didn't receive any treatment showed poor signs of healing with progressed inflammation along the period of study.

Besides its antimicrobial activity, silver was proven to have other beneficial effects on the wound bed. A number of the biochemical effects of silver on the wound have been documented, there was important relationship between tissue destruction by a group of collagenase enzymes known as MMP and tissue synthesis which is stimulated by growth factors. It is well recognized that MMP were needed to heal a wound, but excess levels of MMP degrade fibronectin and peptide growth factors. This effect is exacerbated further by diminished levels of tissue inhibitors of metalloproteinase (TIMPs) (40). Silver-based technologies in particular provide added benefits by down-regulating MMPs to levels that facilitate wound healing (38).

Wright *et al* noted reduced levels of MMP and a higher frequency of apoptosis in a porcine model of contaminated wounds treated with nanocrystalline silver confirming that silver alters the inflammatory events in the wound (41). The present study showed that AgNPs accelerate wound healing and this acceleration is more noticeable in all groups contain AgNPs especially in triple combination (AgNPs, gramicidin and blue light). Reportedly, AgNPs are responsible for reducing the time required for hyperactive cells (myofibroblasts) involved in generation of contractile force in the wound and reverse the inflammatory processes more quickly compared to antibiotic application (42,43).

Topical application of AgNPs effectively enhanced the remodeling of wounds area and diameter and skin macroscopic appearance in rat. In this study, it was observed that 7 days after treatment, in triple combination groups treated, total and *S. aureus* bacterial loads reduced significantly. AgNPs, gramicidin and blue light were more effective than other treated groups AgNPs in reducing bacterial loads, so total and *S. aureus* amounts were 0 CFU/gm in day 21 after wound induction. In control group, 21 days after wound induction, the amounts of total bacteria and 7 days after wound induction, the amounts of *S. aureus* increased, so they were uncountable in plate. This difference in total and *S. aureus* amounts may be due to antibacterial activities of AgNPs against gram-negative and gram-positive bacteria, and this result was in agreement with Ibrahim *et al* results who reported the antibacterial activity of the ecofreindly synthesized AgNPs against pathogenic *Staph.aureus* in dose dependent manner. There is no clinical study about the effects of AgNPs on bacterial load, however, the *in vitro* antibacterial effects of AgNPs are well documented (44).

**Conclusion**

From the results we can conclude topical therapy of the triple combination of AgNPs, visible blue light and antimicrobial peptides promote wound healing process in diabetic infected wound in a short period of time. A new approach can be used to combat serious infections caused by MRSA by combining AgNPs, blue light, and antibiotics.

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