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Silver Nanoparticles Preparation and their Effect on Full-thickness Skin Wound Healing in Rabbit Model

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ABSTRACT

With the increased prevalence of antibiotic-resistant bacteria infections, there is a pressed need for innovative antimicrobial agent. Metal nanoparticle, especially those made of noble metals such as silver, show excellent properties such as signaling the regeneration of collagen, low toxicity in vivo, bacteriostatic and bactericidal activities, and inhibiting the targeting of bacteria in wound. Therefore, this study aims to prepare silver nanoparticles then investigate its antimicrobial effect in wound healing in animal model. Silver nanoparticles were successfully prepared by chemical reduction of silver nitrate, then characterized by UV-Visible absorption spectroscopy. The in-vivo study was done by application of colloidal solution of silver nanoparticles on excisional full- thickness skin defect on the dorsum of New Zeeland rabbit as an animal model. The results indicated significant reduction of the sepsis in two infected cases after the 1st week followed by complete recovery after the 2nd dose of (SNPs drops). While the other cases showed wound closure without any sepsis which demonstrated the high efficiency of silver nanoparticles as antimicrobial agent when compared with the control group which showed persistent signs of sepsis in the all cases. The histological investigation in the control groupproved that the quality of the healing process was very poor. The lengths of the newly formed epithelium, collagen deposition, and the granulation thickness are considered the positive signs of the wound healing process which obviously observed with the animal treated with SNPs.it could be concluded that silver nanoparticles can be used as an effective antimicrobial agent as promising step instead of using systemic the antibiotics.

1. INTRODUCTION.

Nanotechnology can be defined as the science of fabrication, characterization and application of materials with nanometer scale (one billion of meter), which have very interesting physical, chemical, optical and biological characteristics (Sahoo and Labhasetwar, 2003). Wound healing remains a challenging clinical problem for which efficient wound management is necessary. In response to the injury or as a recovery or healing process, the major priority is to stop hemorrhage, to avoid excessive blood loss, and prevent microbial infection by infiltration of immune cells, such as neutrophils or macrophages. More importantly, it is critical to restore the function of the damaged tissue or cell through rapid healing (Shevchenko et al., 2010). For centuries silver ions and silver based compounds has been used widely due to their antimicrobial effect. In 1700, silver nitrate was used for the treatment of venereal diseases, fistulae from salivary glands, and bone and perianal abscesses (Klasen, 2000), and silver nitrate was also used with different concentrations for fresh burn treatment (Castellano et al., 2007), while in the 1940s, after penicillin was introduced, the use of

silver for the treatment of bacterial infections minimized (Hugo, 1992). Recently due to emergence of multi-drug resistant microorganisms, silver again came in the picture and introducing of silver ions in form of nanoparticles gives greater antimicrobial efficacy, Not only against pathogenic bacteria but also against other viruses and eukaryotic microorganisms (Rizzello and Pompa, 2014 ; Paladini et al., 2015). The exact mechanism of antimicrobial effect of silver nanoparticles is not clearly known. However, there are different theories about their action against microorganisms. Silver nanoparticles have the ability to anchor and penetrate the cell membrane then change their permeability lead to cell death (Sondi and Salopek-Sondi, 2004). Danilczuk et al. (2006), suggested that the formation of free radicals by silver nanoparticles increase the porosity of bacterial cell membrane and hence cell death, in which investigated by electron spin resonance spectroscopy studies. It has also been proposed that there can be release of silver ions by the nanoparticles (Feng et al., 2000) ,and these ions can interact with the thiol groups of many vital enzymes and inactivate them (Matsumura et al., 2003). Silver nanoparticle synthesis could be done by three major ways, chemical, physical and recently by biological methods (Prabhu and Poulose, 2012). Silver nanomaterials can be obtained by both the so called 'top-down' and 'bottom-up' methods. The top-down method involves the mechanical grinding of bulk metals and subsequent stabilization of the resulting nanosized metal particles by the addition of colloidal protecting agents (Gaffet et al., 1996). The bottom-up methods, on the other hand, include reduction of metals, electrochemical methods, and sonodecomposition (Prabhu and Poulose, 2012). Studies over the years have proven that it is difficult to remove silver completely if deposited in the body. Animal studies have indicated that silver can be excreted through the hair, urine, and feces majorly (DiVincenzo et al., 1985). The silver particles can enter the body through the skin from where they enter the blood stream and are taken to various organs and are finally excreted out through urine or feces (Prabhu and Poulose, 2012). Rabbits possess characteristics desirable in a laboratory animal model including convenient size, longer life span, strain specific, have good temperaments, are easily handled and are relatively inexpensive (Mir and Darzi, 2009). However, It is important to remember that all models have limitations (Greenhalgh and Warden, 2001). Rabbit models have been criticized because the major mechanism of wound closure is contraction, whereas re-epithelialization and granulation tissue (GT) formation are the major forces involved in human and other animals wound healing (Davidson et al., 2013). Contraction is the process in which the surrounding skin is pulled circumferentially toward an open wound, which reduces the wound without forming new tissues, speeds up the wound healing, and minimizes scar formation (Ehrlich and Needle, 1983). Rabbits contain a panniculus carnosus, a thin sheet of striated muscle lying between the subcutaneous fat and dermal layer as well as a loosely organized subdermal fascial plane. Upon injury, this anatomy permits wound contraction that is often misrepresented as ''wound closure'' and therefore as ''healing'' (Davidson et al., 2013). Wound fixation is the mechanism in which minimize the healing by contraction, suturing of the wound corner is one of different methods that have been done to control wound closure by contraction.

2. MATERIALS AND METHODS:

The present investigation was divided into In vitro and In vivo studies.

2.1. In vitro study:

2.1.1.Silver Nanoparticles preparation:

Silver nitrate (AgNO3,99% meets analytical specification of Ph. Eur., BP, USP,
(Sigma-Aldrich) and tri-sodium citrate (C6H5O7Na3.2H2O, 99.99%) were used for the preparation of silver nanoparticles. Silver colloid was prepared by chemical reduction according to (Van Dong et al., 2012). All solutions of reacting materials were prepared in deionized water. In typical experiment, 50 ml of 0.002 M AgNO3 was heated to boil. To this solution 5 mL of 1 % trisodium citrate was added drop by drop. During the process, solutions were mixed vigorously and heated until change of color was evident (grayish yellow). Then it was removed from the heating device and stirred until cooled to room temperature.

2.1.2.Characterization:

Silver nanoparticles formation was characterized by UV-Visible absorption spectroscopy. Absorption spectra were recorded using a double beam UV–Vis spectrophotometer (Thermo Fisher Spectronic, faculty of pharmacy). The absorption spectra of diluted solutions of the prepared AgNPs in aqueous medium were recorded within the appropriate scan range (300–750 nm). The spectra of pure solvent were taken as a calibrating reference. Measurements were performed at room temperature.

2.2 In vivo study:

2.2.1. Animals and Study design:

All experimental animal procedures were carried out in institution of graduate studies and research lab, according to the institutional ethical guidelines for the care and use of laboratory animals (Council, 2010), the protocol was approved by Faculty of veterinary medicine, Alexandria University. Five New Zealand white rabbits (males, 3 months, 2-2.5 kg) were used in the study, before any experimental manipulations were initiated the rabbits were allowed to acclimate for at least 7 days. Two full thickness wound excision were made in the dorsum of each animal. The left defect used as control (n = 5 wounds) left without any treatment. While the right defect (n = 5 wounds) treated with silver nanoparticles drops (100 Microliter) for three successive days after surgical procedure.

2.3. Surgical procedure:

Pre-operative preparation: The animals were anesthetized by intramuscular administration of xylazine in dose (5mg\kg) followed by ketamine in dose (35mg\kg). The dorsum of each animal was prepared aseptically for the surgical procedure. By using marker and plastic template model (3x3 cm), mark the borders of the excisional wound. Cover the whole back by sterile drape with large square window around the marked site as showed (fig. 1/A).

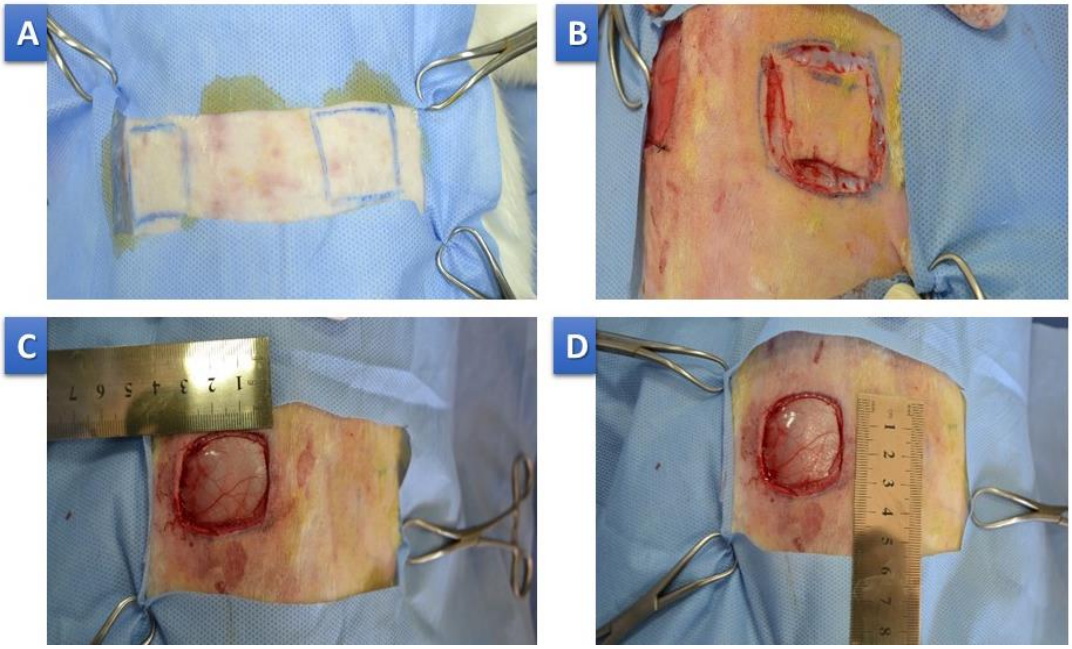

Fig. (1): Surgical procedure, (A): mark the excisional defect area, (B): regular incision includes the full thickness layers, (C & D): measurements of the defect area (3x3cm^2).

Surgical operation: Using the scalpel for sharp regular incision using the marked borders as a guide, in which the incision was deep enough to remove all the skin layers combined with the panniculus carnosus as showed (fig. 1/B). Dissection of the skin flap by using toothed forceps and scissor to make sure for removing the whole skin segment with s/c layer. Two excisional full thickness wound square in shape with diameters (3x3cm) were created on dorsum sides (right and left), with 3cm separating area between them and away from the neck. Four stiches were performed in the wound corner for the control and treated groups, in which fix the skin deeply with the muscle. Left wound (control) was washed with saline. Right wound (SNPs) was treated by drops from the freshly prepared solution was applied on the wound area daily at the same time for three successive days. Post-operative care: The wound was covered by Adhesive nonwoven sterile wound dressing. Loose wrapping of the whole back with long sterile gauze bandage as showed in (Fig. 2). Injection of sterile saline s/c which prepared to be at body temperature. The animal was kept under observation until recovery of the anesthesia, then transfer to their cages.

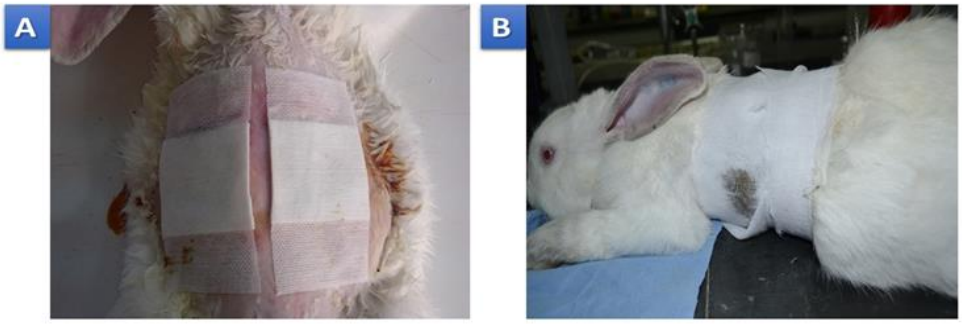

Fig. (2): post-operative care, (A): cover the wound area with adhesive dressing, (B): losse wrapping with sterile gauze.

observation until recovery of the anesthesia, then transfer to their cages.

2.4.Wound healing assessment:

Follow up of the wound closure process was done visually on the 7th, 14th and 21st day of the wound induction for signs of sepsis and inflammation.

•The wound areas were photographed by means of a digital camera (Nikon D3100, Japan with DX SWM VR Aspherical, Thailand) and measured by image J software then wound healing was calculated by the following equations:

Wound area % = (Wt /Wi) x 100. Zarandi et al., 2015).

Wound Healing Rate % = (Wi – unhealed area)/ Wi x 100 (Khafaga et al., 2018).

Where Wt is the wound area at certain time point, Wi is the initial wound area.

2.5.Histopathological Assessment:

The surrounding skin and muscle including the wound areas were carefully dissected after wound closure (after 3rd week), fixed using 10% neutral buffered formalin. Hematoxylin and eosin (H&E) staining was used to evaluate presence of necrosis, inflammatory cells, hemorrhage, granulation tissue (GT) extent, re-epithelization, and thick epidermis formation.

3.RESULTS:

3.1 Silver nanoparticles Preparation

- The color of the solution slowly turned into grayish yellow during adding the sodium tri citrate drops in the boiled silver nitrate solution, indicating formation of silver nanoparticles, showed Fig. (3/1).
- Spectrophotometer measurements showed sharp absorption peak at 430 nm showed in Fig (3/2).

Fig (3): Characterization of silver nanoparticles, (3/1): The color change from (A) transparent (silver nitrate salt) into (B) pale yellow (silver nanoparticles), (3/2): The arrow refers to the absorbance peak at 430 nm.

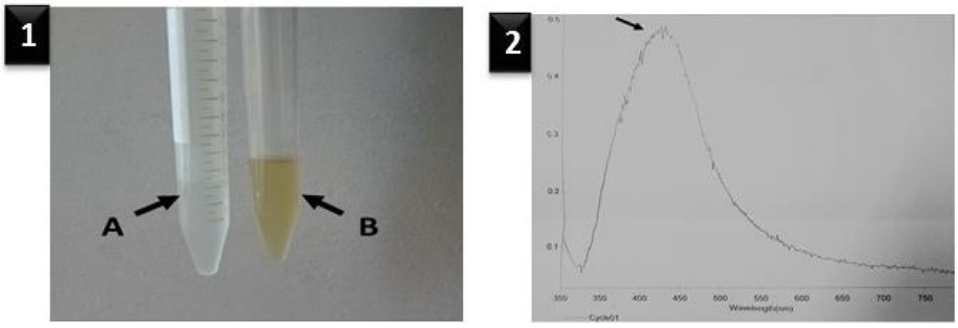

Fig (3): Characterization of silver nanoparticles, (3/1): **The color change** from (A) transparent (silver nitrate salt) into (B) pale yellow (silver nanoparticles), (3/2): The arrow refers to the absorbance peak at 430 nm.

3.2 Macroscopic investigation:

Wound area was observed after 7 days of the wound induction, in (control group) signs of sepsis (inflammation associated with pus formation) in the wound corner was observed in the all cases, and then from the 7th day treatment with warm water wash, evacuation of the pus, and disinfected with povidone iodine were made through the next three days, in which there was no signs of progress but also increase in the

abscess area, so decision was made to begin treatment with systemic antibiotic (ciprofloxacin 250mg) for 3 days. While in the group that treated with silver nanoparticles, signs of infection were observed with two cases only, after the 7th day, they were treated by the same procedures done with the control group, but systemic antibiotic was replace by repeated doses of silver nanoparticles drops (50-100 Microliter) for two days, day after day.

Wound area was measured on the 7th day, showed that there was no significant difference between control and SNPs groups and confirmed by calculating the percent of the healing rate SNPs (42.11%) and control (42.13%)

•Second week results showed that the sepsis with the control group increased and spread to the whole wound bed even after systemic antibiotic administration, while in SNPs group after the second dose of silver nanoparticles drops, complete recovery from infection and replaced by healthy tissue.

On the 14th day, the wound area showed that the control group was significantly higher than SNPs group ($P < 0.05$) Also, the healing rate percent confirmed the results as the healing rate in the SNPs (92.44%) was higher than the control group (87.55%).

•Third week results showed persistence of the pus accumulation in the wound corner even after systemic antibiotic administration, while absence of any signs of sepsis in the treated group (SNPs) after second dose of silver nanoparticles as showed Fig. (5).

On the 21st day, wound area measurements showed no significant difference between two groups ($P > 0.05$). The healing rate percent in control (98.8%).

•Both control and SNPs groups were observed for 3 days after the wound induction, there were no signs of inflammation, edema and erythema around the wound edges.

3.3 Histopathological assessment:

The histological samples were taken after wound closure in control and SNPs group, showed improvement in the healing quality in the treated group (SNPs), as complete covering epidermal layer formed in control and treated group but significant increase of the epidermal layer thickness in the treated when compared with the control group. Regarding the SNPs-treated group, resolution of

Inflammation and few blood vessels were noticed (Fig. 7).

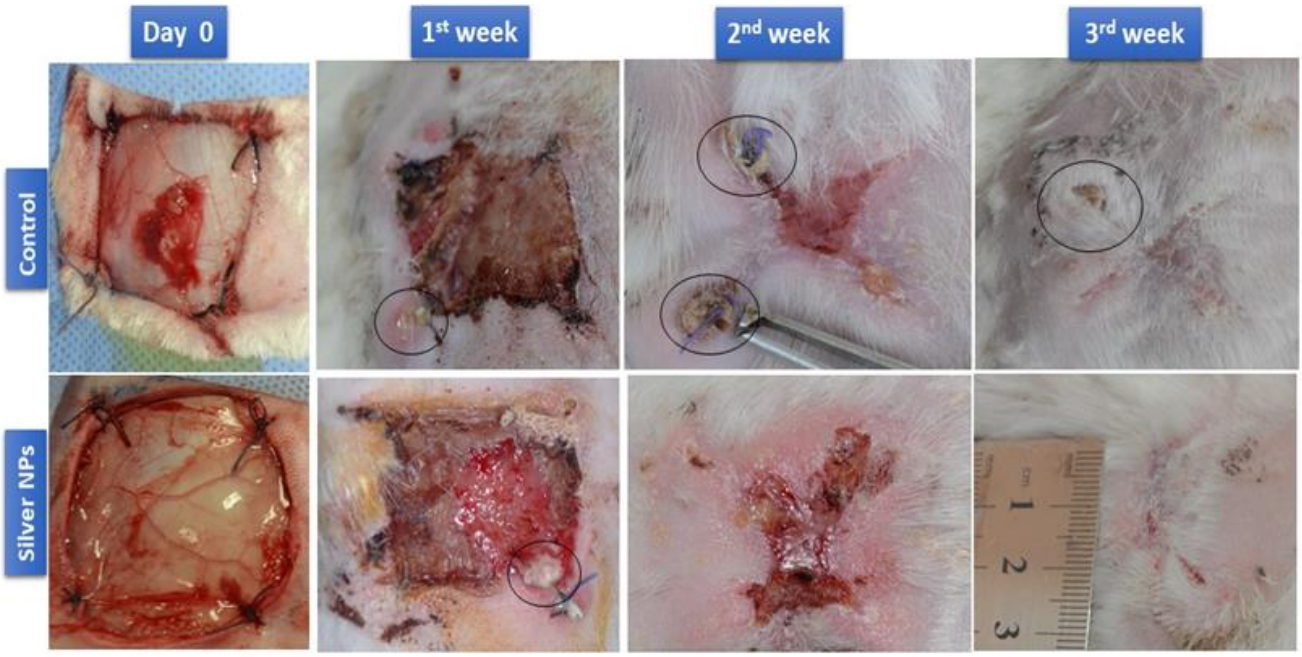

Fig. (5): Macroscopic investigation of the wound area through three weeks from day 0 until wound closure in control and treated groups.

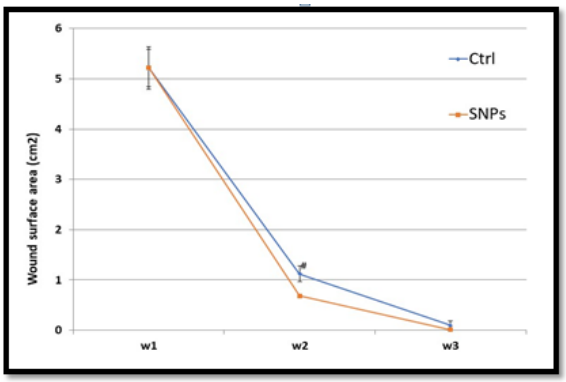

Fig. (6): Macroscopic evaluation of wound healing. Wounds' surface area (in cm^2) was measured for all animals once a week till week 3 (starting from the day of wound induction). Data were shown as mean ± SD. Different symbols indicate statistical significance ($p < 0.05$) compared to the other groups within the same time interval.

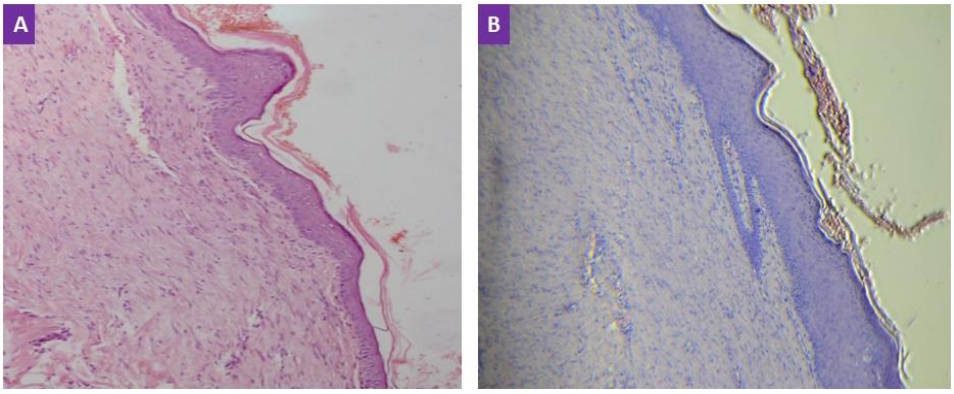

Fig. (7): Microscopic histological investigation after wound closure by H&E stain (A) control group 100x, (B): treated (SNPs) group 100x.

4. DISSCUSION

Nanoparticles are clusters of atoms in the range 1-100 nm. Nanoscale materials have emerged up as novel antimicrobial agents due to their large surface area to volume ratio (Kim et al., 2007).The prepared silver nitrate solution showed change in the color from transparent to pale yellow after heating with trisodium citrate dropping, indicated the reduction of the Ag+ ions and formation of silver nanoparticles which agreed with this previous study (Zhou and Wang, 2012). These colors arise due to excitation of surface plasmon vibrations in the metal nanoparticles as explained with (Mulvaney, 1996).

The characterization absorption spectra were the important properties of the Ag NPs, and the UV-Vis spectra was a good method for characterization of the formation and growth of Ag NPs, in which silver nitrate and sodium citrate had no peaks in the 300 ~ 600 nm wavelength range.Many strategies were performed to alleviate skin contraction in rabbit model. Full thickness skin defect down to the panniculus muscle in which this method was suggested by (Karypidis et al., 2011). And also, the defect (3x3 cm2) was made to be large enough to remained unhealed for longer time which give the chance for the wound to be healed by reepithelization more than contraction.

The elasticity of the dorsum of rabbit skin reduced the tension around the wound in the healing process, thus activate wound contraction which may lead to false outcomes. So, skin fixation strategy by suturing the wound corners was performed. Wound localization is an important point, in which we should have sufficient space of unwounded area between the right and left wound sides, and the wound area should not be near to the neck to allow easily handling of the animal during the experiment from its neck without wound disruption. So, two excisional wounds were performed on the dorsum.

The method of silver nanoparticles application on the wound area is critical point, in which the application of SNPs as solution not allow complete antimicrobial coverage to the wound bed and required another repeated doses this observation agree with the findings in this study (Li et al., 2013a).

Macroscopically there was no sign of dermal toxicity, i.e., gross signs of erythema or edema, in any of the groups in our study following application of silver nanoparticles on the wound which agree with (Chowdhury et al., 2014). Although the macroscopic investigation of the wound area of the negative control group

showed that it has been apparently healed, the microscopic investigation proved that the quality of the healing process was very poor. The lengths of the newly formed epithelium, collagen deposition, and the granulation thickness are considered the positive signs of the wound healing process which obviously observed with the animal treated with SNPs. The antimicrobial effect and very rapid bactericidal action of SNPs instead of using systemic antibiotic which agree with the findings in (Ip et al., 2006) who investigate the effect of the SNPs in vitro comparing with other bactericidal agents.

5. CONCLUSION

This study reports that Silver nanoparticle application on wound healing not only effective as antimicrobial agent but also has a role in improving the quality of the healed skin, which clearly observed by the histopathological evaluation.

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