Original Research

**Sliver nanoparticles accelerate skin wound healing in mice (Mus musculus) through suppression of innate immune system**

Mohammad Saeed Heydarnejad¹*, Samira Rahnama², Mohsen Mobini-Dehkordi³, Parisa Yarmohammadi², Hoori Ashnai⁴

¹Research Institute of Biotechnology, Shahrekord University, Iran
²Department of Biology, Shahrekord University, Iran
³Department of Genetics, Shahrekord University, Iran
⁴Department of Biology, Islamic Azad University of Shahrekord, Iran

**Abstract**

**Objective(s):** This study aimed to find the effects of silver nanoparticles (Ag-NPs) (40 nm) on skin wound healing in mice Mus musculus when innate immune system has been suppressed.

**Materials and Methods:** A group of 50 BALB/c mice of about 8 weeks (weighting 24.2 ± 3.0 g) were randomly divided into two groups: Ag-NPs and control group, each with 25 mice. Once a day at the same time, a volume of 50 microliters from the nanosilver solution (10ppm) was applied to the wound bed in the Ag-NPs group while in the untreated (control) group no nanosilver solution was used but the wound area was washed by a physiological solution. The experiment lasted for 14 days. Transforming growth factor beta (TGF-β), complement component C3, and two other immune system factors involving in inflammation, namely C-reactive protein (CRP) and rheumatoid factor (RF) in sera of both groups were assessed and then confirmed by complement CH50 level of the blood.

**Results:** The results show that wound healing is a complex process involving coordinated interactions between diverse immunological and biological systems and that Ag-NPs significantly accelerated wound healing and reduce scar appearance through suppression of immune system as indicated by decreasing levels of all inflammatory factors measured in this study.

**Conclusion:** Exposure of mice to Ag-NPs can result in significant changes in innate immune function at the molecular levels. The study improves our understanding of nanoparticle interaction with components of the immune system and suggests that Ag-NPs have strong anti-inflammatory effects on skin wound healing and reduce scarring.

**Keywords:** Innate immune system, Mus musculus, Silver nanoparticles (Ag-NPs), Skin wound

*Corresponding author: Mohammad Saeed Heydarnejad, Research Institute of Biotechnology, Shahrekord University, P.O. Box 115, Shahrekord 88186, Iran.
Tel & Fax: +983814424419, Email: msh@utas.edu.au or m_heydarnejad@yahoo.com
Acceleration of wound healing by silver nanoparticles

Introduction
Silver nanoparticles (Ag-NPs) are clusters of silver atoms that range in diameter from 1 to 100 nm and are attracting interest as antibacterial and antimicrobial agents for applications in medicine. They have also increasingly been used for coatings on various textiles and certain implants, for the treatment of wounds and burns, as a water disinfectant, and in air-freshener sprays (1, 2). Recent evidence suggests that Ag-NPs have potent anti-inflammatory effects (3, 4, 5) and accelerate wound healing (6, 7). Skin wound healing proceeds through an overlapping pattern of events including coagulation, inflammation, proliferation, matrix and tissue remodeling. The ultimate goal for wound healing is a speedy recovery with minimal scarring and maximal function (8). For this efficient and highly controlled repair process to take place, numerous cell-signaling events are required (3). The use of antimicrobial prophylaxis is important in reducing the wound’s microbial load (9). Indeed, when associated with a heavy bacterial burden, the rate of wound healing is reduced. A cascade of reactions including an essential innate immune system of host is initiated following skin injury which finally leads to restoration of tissue integrity and function (10). The repair process of skin wound starts immediately during which various growth factors such as transforming growth factor beta (TGF-β) will release (11). TGF-β is the growth factor affecting all cell types that are involved in all stages of wound healing (12). TGF-β is released by macrophages and platelets. It acts as a potent chemo-attractant for monocytes, macrophages, neutrophils, lymphocytes, and fibroblasts. TGF-β stimulates release of other growth factors and induces its own autoexpression. In addition, TGF-β plays an important role in tissue fibrosis and post-injury scarring (3). The complement system is composed of a set of blood proteins that circulate and work with each other to boost immunity and to promote inflammatory responses and host defense. They primary perform the function of destroying viruses and bacteria. The primary components of the complement system are nine in number and they are designated from C1 to C9. This system is known to have a profound inflammatory response. The molecules of the complement system interact in two different enzymatic cascades: The classical and the alternative pathways. The classical pathway is activated by an antibody bound to a foreign particle. The first component is the complement 1 (C1), which can also be activated by IgM and IgG immunoglobulin. C4, then C2 are cleaved to activate C3 and C5 (Bonaparte, et al, 2008). The alternative pathway of the complement system is an innate component of the immune system's natural defense against infections. The alternative pathway is one of three complement pathways that opsonize and kill pathogens. The pathway is triggered when the C3b protein directly binds the microbe (13).
Two other factors involve in inflammation are rheumatoid factor (RF) and C-reactive protein (CRP). Rheumatoid factor (RF) is the autoantibody (antibody directed against an organism's own tissues) that is most relevant in rheumatoid arthritis (14). It is defined as an antibody against the Fc portion of IgG. RF and IgG join to form immune complexes that contribute to the disease process. In fact, RF is most relevant in rheumatoid arthritis. However, the presence of RF in serum can also indicate the occurrence of suspected autoimmune activity unrelated to rheumatoid arthritis, such as that associated with tissue or organ rejection. In such instances, It is hypothesized that RF may serve as one of several serological markers for autoimmunity. Skin wound healing is a complex biological process that requires cellular interactions between varieties of cells, including immune cells. C-reactive protein...
CRP is a protein found in the blood, the levels of which rise in response to inflammation. Its physiological role is to bind to phosphocholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system. CRP binds to phosphocholine on microbes and damaged cells and enhances phagocytosis by macrophages. Thus, CRP participates in the clearance of necrotic and apoptotic cells. In other words, CRP is used mainly as a marker of inflammation (15). Measuring and charting CRP values can prove useful in determining disease progress or the effectiveness of treatments.

CH50 is a blood test called total complement activity. It is performed to check the level of the compliment system. The compliment system helps the ability of phagocytic cells and antibodies to clear any pathogens from an organism. Indeed, CH50 is a blood test that helps us determine whether protein abnormalities and deficiencies in the complement system are responsible for any increase in autoimmune activity. The test basically helps to closely monitor autoimmune disease activity. The titer of the complement classical pathway is expressed by CH50 (16). The study aims to address the effects of Ag-NPs on skin wound healing in mice Mus musculus when innate immune system has been suppressed.

**Materials and Methods**

**Mice holding**

Animal test was performed with compliance of the local ethics committee. A group of 50 BALB/c mice of about 8 weeks (weighting 24.2±3.0 g) were purchased from Medical Faculty of Shahrekord University and then transferred to the laboratory. The animals were in a single group and maintained on commercial pellet diet, given deionized water ad libitum and kept in plastic cages in a 20±2 °C, 50–70% relative humidity room with a 12-h light/dark cycle. The photoperiod was provided by fluorescent tubes (Thorn, 36 W, white light), and all lighting was excluded during the scotophase. A timer was used to turn the lights on and off.

After 2 weeks acclimation, the mice were randomly divided into two groups: Ag-NPs and the control group, each with 25 mice.

The animals were kept fasting over night before treatment. The mice were examined daily for infections.

**Preparation of Ag-NPs**

Silver nanoparticles (Ag-NPs) were purchased from Nano Pars Co., Iran with a purity of 95%.

The final concentration of solution was 10 ppm. The mean diameter of Ag-NPs averaged 40 nm (and ranged from 35 to 45 nm), according to the manufacturer.

**Excisional wound model & experiment**

Anesthesia for experimentation was achieved with an intramuscular injection of 10 ml ketamine, 0.5 ml acepromazine, 2 ml Diazepam and about 0.5 ml Xylazine solution at a dose of 50 mg/Kg. A 2.0 × 2.0 cm2 full-thickness excisional wound was created after anesthesia.

The dorsal area of each mouse was carefully shaved and the skin was disinfected with iodine.

Then injury was made with some up to 10 mm in diameter, following anesthesia. Once a day at the same time, a volume of 50 microliters from the nanosilver solution (10 ppm) was applied to the wound bed in the Ag-NPs group daily at a given time. In the untreated (control) group no nanosilver solution was used but the wound area was washed by a physiological solution. Each group of mouse was housed separately. The experiment lasted for 14 days. Samplings (n= 8) were conducted on days 2, 7 and 14, during which the animals were sacrificed. The blood was obtained.
Acceleration of wound healing by silver nanoparticles

directly from heart. The serum was obtained by centrifugation of the whole blood at 3000 rpm for 15 min.
Enzyme-linked immunesorbent assay (ELISA) were used to measure serum concentrations of all complement components. Level of TGF-β was also measured using commercial ELISA kit (Boster mouse TGF-β ELISA) from R&D Systems, China.
To determine wound surface area the Image Analysis tool was used. Statistical analyses were performed using Student's paired t-test, one-way ANOVA and Tukey post-hoc test. A p value of 0.05 was considered significant.
The results showed the average value ± standard deviation.

Results
Serum level of the TGF-β (important in promoting angiogenesis and attracting macrophages) were measured. At day 2 after injury, control group of mice displayed significantly greater concentrations of TGF-β than that of mice treated with Ag-NPs (P < 0.05) (Figure 1).
At days 7 and 14, the level of TGF-β clearly decreased in both groups. The greater concentration of TGF-β on day 2 in the control group suggests that Ag-NPs improved TGF-β production, which correlates with an increase in angiogenesis and enhanced wound healing.
The activity of C3 as a marker for complement activation was also assessed. After 2 days of the experiment, level of C3 significantly decreased in mice treated with Ag-NPs as compared with control (untreated) group, indicating there is a serious inflammation process in the wound area of the control.
This may show that when Ag-NPs are present on the surface of the wound area, these particles could more completely reduce C3 activity but accelerate skin wound healing, as compared with the control group.
Total serum level of RF activity in both groups of the experiment showed significant difference at day 2 so that the RF level in the control group was 42.0 ± 2.4 while it was 11.3 ± 1.3 IU/ml in the treatment one. As inflammation is a normal part of the wound healing process, the results suggest that Ag-NPs could reduce inflammatory process and enhance skin wound healing in the mice treated with Ag-NPs as compared with the control group. At day 7, the level of RF however, diminished in both groups, significantly in the control one, indicating the reduction of inflammation process particularly in untreated mice.
The CRP value difference was statistically significant between the two groups at day 2 (p<0.05). At day 2 after injury, the average of CRP in the control group was 27.76 ± 4.154 mg/dL while it was 2.59± .251 in the treatment.
This clearly shows that measurement of CRP can be used to monitor inflammatory state of the skin wound so that Ag-NPs play an important role to reduce CRP production in the wound area.
Figure 1 shows the changes in CRP with time. Afterwards, the level of CRP decreased in both groups. Finally, the levels of CH50 activity in both groups were compared. These tended to increase at days 2 and 7 after injury.
However, a significant difference was found between two groups at day 14, maybe due to the effect of Ag-NPs on the injury causing this complement component remained active.
This shows that there were no deficiencies in the complement system of all mice models and all the results associated with the complement system probably were correct. Furthermore, as scar formation is essential in wound healing (17), scar surface area was determined. There was marked difference in the macroscopic appearance of healed wounds 14 days after wounding (Figure 3).
Figure 1. Top left; serum TGF-β. Wound tissues from control group displayed significantly greater concentrations of TGF-β than that of mice treated with Ag-NPs, particularly at day 2. Top right; serum level of C3. There was a serious inflammation process in the wound area as indicated by reduced activity of C3 in the treatment group. Down left; RF activity. A significant decrease of RF in treatment group shows that Ag-NPs could reduce inflammatory process and enhance skin wound healing. Down right; CRP value. This graph shows that the Ag-NPs play an important role to reduce CRP production in the wound area as indicated by sharp reduction of CRP in the treatment group. Data are given as the average values and error bars denote SDs.

The sites of the wounds treated with Ag-NPs had little scar.
By contrast, the scar had not disappeared in the control group within this period. The results show that Ag-NPs have strong effect on skin wound and accelerate healing.

Discussion
The results of the current study show that silver nanoparticles (Ag-NPs) can improve skin wound healing and reduce scar appearance through suppression of innate immune system, as induction of immune tolerance by nanoparticles can be considered a form of desirable immunosuppression (18).
Silver nanoparticles (Ag-NPs) appear to possess potent antimicrobial activity to reduce infections. In fact, Ag-NPs with strong biocidal effects are toxic to microorganisms. They can kill bacteria that cause diseases through the contamination of food, water, and wounds (19). The mechanism of Ag-NPs on microorganisms is not completely clear, however, Ag-NPs interact with a wide range of molecular processes within microorganisms from inhibition of growth to loss of infectivity to cell death (20). For instance, free radicals derived from the surface of Ag-NPs had been suggested for the antimicrobial activity (21). Skin wound healing is a dynamic process involving the coordinated interaction of blood cells, proteins, proteases, growth factors, and extracellular matrix components.

Wound healing is an essential physiological process mediated by a variety of factors responsible for the regeneration and reorganization of damaged tissue toward its normal architecture (22). The wound healing response can be subdivided into a sequence of specific events, made up of three stages: the inflammatory stage, the proliferative stage, and the maturation stage.

The immune system is primarily active during the inflammatory stage. Typically, the wound healing involves steps that include inflammation around the site of injury, angiogenesis and the development of granulation tissue, repair of the connective tissue and epithelium, and ultimately remodeling that leads to a healed wound (9). The decreased activity of inflammatory factors measured in the current study, i.e., TGF-β, Complement system, CRP, RF is agreement with other studies in which Ag-NPs have anti-inflammatory effects and improve wound healing e.g. Chaloupka et al. (23). In addition, the results of this study show that Ag-NPs could suppress complement activity.

In fact, activation of the complement cascade can be harmful if particles enter the systemic circulation because this may lead to hypersensitivity reactions and anaphylaxis (24, 25, 26). TGF-β plays an important role in wound healing and tissue remodeling. It is the growth factor affecting all cell types that are involved in all stages of wound healing (12). Because level of TGF-β in the control group of the present study was found to be increase, thus this factor served as a chemo-attractant for neutrophils, macrophages and fibroblasts.

In addition, scar appearance and the extent of inflammation at the wound site were decreased in the mice treated with Ag-NPs so that the wound area was little and transient compared with the control group. Similarly, Tian et al. (3) in investigating the wound-healing properties of Ag-NPs found rapid healing and improved cosmetic appearance in an animal model. This is because TGF-β plays an important role in tissue fibrosis and post-injury scarring.

In addition, Wright et al. (27) have proved that Ag-NPs inflammatory effects on skin wound healing significantly suppress inflammatory cytokines and induced apoptosis of inflammatory cells. Complement system is the first line of the innate immunity (28, 29).

It is comprised a group of proteins that functions primarily to fight infection. The central results of activation of pathways are to deposit the opsonin C3 on bacterial to promote inflammation, phagocytosis and to lyse bacteria. As the most common complement protein in serum is C3 (30), the reduced level of C3 at day 2 after injury in the current study confirm that this complement component with a chemotactic response to neutrophils releases inflammatory agents into the wound area (31). Similarly, a reduction in detectable levels of two other therapeutic modalities namely RF and CRP in mice treated with Ag-NPs, when
compared with the control, was interpreted as evidence of intense anti-inflammatory reaction in the former. The results support the view that Ag-NPs indeed have efficient anti-inflammatory effect on wound healing (3, 8, 9).

In addition, the titer of the complement classical pathway is expressed by CH50 (16), the normal level of complement CH50 of the current study showed that there were no protein abnormalities and/or deficiencies in the complement system.

Figure 3. Ag-NPs accelerate skin wound healing and achieve superior cosmetic outcome. Time taken for skin wounds to heal in mice treated with Ag-NPs (right pictures) as compared with no treatment (left pictures).
Acceleration of wound healing by silver nanoparticles

Conclusion
Exposure of mice to Ag-NPs can result in significant changes in innate immune function at the molecular levels. Furthermore, the immunosuppression effects of Ag-NPs have been observed with decreased serum levels of TGF-β, C3, RF, CRP following confirmed by CH50 in the blood. The study improves our understanding of nanoparticle interaction with components of the immune system and suggests that Ag-NPs have strong anti-inflammatory effects on skin and reduce scarring.

Acknowledgments
This work was funded by the Research Institute of Biotechnology, Shahrekord University, Iran.

References
14. RAS; Rheumatoid Arthritis Symptoms”. Retrieved 2011.
Heydarnejad MS, et al