Silver Nanoparticles Can Relieve Progressive Necrosis by Regulating Macrophage Activation in Burn Wounds

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Research

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Abstract

**Purpose:** To investigate the positive influence of AgNP on preventing early burn-wound progression.

**Patients and methods:** After a deep burn, progression of burns may occur in the initial or surrounding area even after thermal factors have been removed. Due to the influence of many factors such as residual heat, the risk of burn stagnant area deterioration is very high which is considered to be salvable. Silver nanoparticles (AgNP), which has been wide used in burns, is a strong antibacterial agent that has been reported to regulate inflammation. Hence, to investigate the positive influence of AgNP on avoid early burn wound progressive deterioration, a isomorphic “comb” burn animal model was made in this study which were treated with silver nanoparticles. The wound tissues were taken for molecular biological and histological evaluation, which revealed that AgNP alleviated histological deterioration in burn stagnant area.

**Results:** Furthermore, AgNP can alleviate the early progressive necrosis and inflammatory response of burn wound, which was associated with excitation in M2 macrophage and a suppression of M1 macrophage.

**Conclusion:** In conclusion, we demonstrate that AgNP has a protective effect on acute burn wound deterioration in a rat model. It might be regulated by the macrophage activation-induced inflammation and apoptosis.

Introduction

Silver nanoparticles, a new type of antibacterial material, has characteristics of large surface area, small particle size, low melting point, and high activity. Its antibacterial activity is far greater than that of traditional ionic antibacterial agents\(^1\)\(^{-5}\). Therefore, most experts believe that AgNPs promote wound healing mainly through its highly effective antibacterial effect\(^6\)\(^{-10}\). In the treatment of infectious wounds, AgNPs rely on these unique effects to kill pathogenic bacteria and promote wound healing. However, it is difficult to explain its effectiveness in many aseptic wounds by this antibacterial mechanism. Our previous studies show that the use of AgNPs for aseptic wounds can also effectively promote wound healing, indicating that AgNPs promote wound healing per se. AgNPs can improve the inflammatory response of wounds, accelerate wound healing, and improve the prognosis of wounds, and its anti-inflammatory effect may play a pivotal role. The anti-inflammatory function of AgNPs is related to their regulation of inflammatory cytokine release. The burn wound includes three zones: the center is the coagulation and necrosis zone, the outermost is the hyperemia zone, and the area between them is the stasis zone. The skin damage in the stasis zone is reversible\(^11\)\(^{-13}\). The major changes in the tissues of the hyperemia and stasis zones include increased permeability of capillaries and their expansion, which cause abnormal exchange between normal blood vessels and the interstitial compartment, a loss of semipermeable membrane function of the capillaries, and quick accumulation of plasma-like fluid from blood vessels in the interstitial compartment or exudation from the wound surface. Even after removal of
thermal factors, edema of the cells and tissue will occur, leading to delayed or progressive tissue necrosis. This suggests there are transition obstacles from the inflammatory phase in early burn wounds to later phases. In most cases, if there is no intervention or erroneous intervention, secondary skin necrosis and coagulation will occur in the damaged part of the skin. Most studies about AgNPs focus on their antibacterial function, but there are fewer studies on improvement of the progressive necrosis of burn wound edges based on the inflammatory regulation.

This study first investigated whether AgNPs can prevent the progressive necrosis of wound margins. It then explored the related mechanism by studying the effect of AgNPs on alleviating the excessive inflammatory response in wounds. We hypothesized that after entering the wound surface, macrophage phagocytosis of AgNPs inhibits the activation of M1 macrophages through different pathways, shortens the inflammatory response period (exudation and infection period), and protects the coagulating and hyperthermic areas at the edge of the wound from further necrosis. Generally speaking, macrophages tend to be activated as M2 macrophages. This process is associated with transition of the wound from the early inflammatory phase to the repair phase and the promotion of wound healing. In this study, aiming to translate basic research to clinical application, we investigated the infiltration and activation of macrophages in the three burn zones (necrosis zone, hyperemia zone, and stasis zone) at different stages after scalding in a comb burn model. AgNPs were applied to the scald wounds. Changes in infiltration and activation of macrophages in burn wounds in different zones after different periods of time were studied. Based on the reversibility of tissue damage in the stasis zone and the repair of wounds in other zones, we investigated the mechanism by which AgNPs mediate macrophage activation to promote wound healing.

Results

Macroscopic and pathological studies of AgNPs for comb burn wounds

The results showed that the wound gradually shrank in both the control group and the experimental group. However, the redness and swelling at the edges of the control group were more obvious than in the experimental group. At later stages, wound healing depended on the shrinkage of the surrounding skin because of wound deepening and decrustation. In the experimental group, the wound did not progressively deepen and healed as a second-degree burn, with a smaller extent of wound shrinkage (Fig. 1). Hematoxylin and eosin (H&E) staining showed that with collagen degeneration and repeated infection of wounds, typical features were present in the burn wound, including thinning of the epidermis, elongation of epithelial nuclei, and dermal swelling. We observed that the administration of AgNPs alleviated these changes. In contrast, the tissue damage observed in the control group was similar to that found in untreated burns, and three days after the burn, the progressive necrosis significantly expanded in three dimensions (Fig. 2). Seven days after surgery, the inflammation and hyperemia caused by this progressive injury persisted, while the experimental group showed orderly epithelial growth and slow
recovery in some areas of incomplete necrosis. Nine days after surgery, early decrustation of necrotic tissues in the wounds of the experimental group promoted significant epithelization of the wounds. However, the wounds of the control group did not heal obviously, with crust adhesion.

**AgNPs can improve the inflammatory response of wound marginal tissue**

AgNPs significantly reduce the inflammatory response in the hyperemia zone in burn wounds in rats. The level of CD68 is an indicator of the distribution and migration of macrophages and reflects the degree of inflammatory response in the wound. Within 3 days after the burn, the level of macrophage infiltration in the tissue around the burn increased significantly (Fig. 2). The levels of macrophage infiltration in both groups decreased 7 days after the burn, but there was a significant difference between groups (Fig. 2). In addition, the burn induced a significant downregulation of tumor necrosis factor alpha (TNF-α) at all three time points. To further verify the inflammatory response at the wound margin, we performed molecular biology tests on CD68 and TNF-α in the wound marginal tissue.

**Expression of inflammatory factors in wounds (real-time quantitative PCR)**

Several inflammatory factors, such as CD-68 and TNF-α, were detected using real-time quantitative PCR to characterize the degree of inflammatory reaction at the gene expression level. In general, the various factors in the two groups of wounds showed different expression patterns (Fig. 2b). The CD68 level in the control group continued to climb after intervention. In contrast, it was lower in the AgNP group and reached a peak 7 days after intervention, while it was particularly higher in the control group (p < 0.05). Nine days after intervention, the CD68 level became similar between the two groups. TNF-α mRNA expression had the same pattern as CD68, being consistently higher in the control group than the experimental group.

**AgNPs can improve macrophage activation in wound**

The expression of inducible nitric oxide synthase (iNOS), arginase-I, MHC-II, and FIZZ-1 were detected by Western blot, which are closely related to the inflammatory response, to characterize macrophage activation at the protein level. The results are shown in Fig. 3. Overall, the bands of iNOS in the control group were denser than those in the AgNP group at each time point, while the bands of the M2 macrophage-related proteins arginase-I, MHC-II, and FIZZ-1 were lighter.

**Real-time quantitative PCR**

The extent of inflammation at the gene level was characterized by real-time quantitative PCR of several inflammatory active factors, such as iNOS, arginase-I, MHC-II, and FIZZ-1. In general, the expression of each gene in the two groups fluctuated over time (Fig. 3). iNOS in the control group increased at 3 days after intervention and reached its peak at the 7th day. The expression of iNOS in the AgNP group was lower. In contrast, the mRNA expression of arginase-1 continued to increase in both groups, but with
relatively lower expression in the control group. The expression of MHC-II mRNA was also relatively high in the experimental group, reaching a peak 7 days after intervention, and it gradually decreased, while its expression in the control group was relatively stable and low. Although the expression of FIZZ-1 mRNA was not significantly different between the two groups at day 3, it was relatively high in the AgNP group at 7 days.

Discussion

When used in burns or trauma, AgNPs can effectively promote wound healing, not only because of their bacteriostatic effect but also their inhibition of inflammation. This indicates that AgNPs can improve healing of the wound partly based on their antibacterial effect. In vivo and in vitro studies have shown that the mechanism by which AgNPs promote wound repair should be their anti-inflammatory effect. Inflammation and microbial growth are two important factors affecting wound healing. In most cases, the two factors arise concurrently and affect each other. Wound infection inevitably cause inflammation. Continuous inflammation and repeated infections are associated with progressive necrosis of burns wound.

The normal wound healing process includes a bleeding phase, inflammation phase, repair phase, and reconstruction phase. This seems to go along with the zoning of the wound, namely, the coagulation necrosis zone, the stasis zone, and the hyperemia zone. The hyperemia zone is the marginal area of a burn wound, with the most robust chemotactic accumulation of inflammatory cells, and the damage in this zone is reversible. In the inflammatory response process, in which many monocytes chemotactically aggregate at a site, macrophages play an irreplaceable role because they can engulf necrotic cells and infected microorganisms and can also secrete many chemokines and cytokines to regulate collagen secretion, cell proliferation, growth, and vascularization granulation [16, 17]. The two major pathways of macrophage activation include classical activation pathway (M1) and selective activation pathway (M2) [18, 19]. Scientists have studied the gene expression of the two types of activated macrophages at a wound using gene chip technology, and they found that 11 M1 marker genes and seven M2 marker genes were expressed 2 days after a burn. However, between 4 and 8 days after burn, the gene expression shifted to one M1 gene and nine M2 genes expressed. This indicates that the number of each type of activated macrophage may change during the wound healing process. In the middle and late stages of healing, M2 macrophages predominate [20]. These findings show that different types of macrophage activation during the wound healing process fluctuate in an orderly way. When the regular macrophage activation process is disrupted, normal wound healing may stagnate. This can lead to repeated vicious cycles of inflammation and infection.

The hyperemia and stasis zones in the burn wound are in the edge of the wound but the center of the inflammatory storm. How to effectively and correctly induce the inflammatory response in these zones for wound healing is the core of this study. In this study, we employed AgNPs to treat burn wounds and investigated their regulation of the inflammatory response in the region, in an attempt to reduce the occurrence of progressive necrosis of the wound. The mechanism is related to the activation of
macrophages in the wound: AgNPs can specifically activate M2 macrophages to effectively reduce progressive necrosis at the wound margin.

The mechanism by which they promote healing has always been attributed wholly to their effective bacteriostatic effect. In this study, we hypothesized that the mechanism by which they effectively improve wound inflammation and relieve progressive necrosis of the wound is related to activation of macrophages. The AgNPs in the wound may exert their antibacterial effects while being engulfed by macrophages to affect the activation of these cells. Therefore, we hypothesize that AgNPs can affect the activation of M2 macrophages within a specific time frame to repair and rebuild damaged tissue.

Through proteomics research, scientists have discovered that AgNPs can inhibit the expression of pro-inflammatory factors. The mechanism may be due to the antimicrobial activity of AgNPs and their effect on MMP activity [13, 21]. AgNPs can inhibit the secretion of M1-type macrophage-related factors TNF-α and iNOS [22, 23]. After AgNPs enter the circulatory system, they can be engulfed by blood cells to cause the apoptosis of blood cells and lower the expression of TNF-α and IL-5 [24, 25]. In contrast, the expression of TGF-β1 is very low in the nonscarring healing site of fetal skin. Indeed, TGF-β antagonism can significantly reduce inflammation in healed wounds and scars compared with control treatments. All these data indicate that TGF-β plays a vital role in tissue fibrosis and scar formation [26–27].

M2 macrophage-related protein Arginase-1, MHC-II, and FIZZ-1, play an important role in wound tissue reconstruction. It reduces collagen shrinkage by secreting collagenase to reduce collagen synthesis and deposition [28]. They can reduce the infiltration of mononuclear cells and M1 macrophages in the wound, inhibit the secretion of pro-inflammatory factors, and effectively regulate the inflammation of the wound [29–30].

The activation of macrophages and the occurrence and development of inflammation have a profound effect on the wound healing and. Under normal circumstances, a reasonable and orderly inflammatory response can ensure normal and rapid healing. In this study, immunohistochemistry, RT-PCR, and Western blot were used to study the expression of inflammatory factors. A reliable surface marker of macrophages, CD68 can be expressed in cells such as macrophages, monocytes, and Kupffer cells and is a direct indicator of inflammation. M1 macrophage-associated TNF-α is potential promoter of fibroblast proliferation and are usually expressed low in fetal wounds [25]. Overexpression of these factors at a wound is associated with delayed wound healing. The low expression of TGF-β, CD68, and IL-6, which indicated downregulation of the M1 macrophage response, is the basis for the improvement of inflammation and can be used as an index to predict wound healing and scarring. Western blot and RT-PCR results showed that the expression of proteins, such as CD68 and TNF-α, associated with M1 macrophages was suppressed.

The activation of M2 macrophages play an important role in the process of wound repair and reconstruction. They inhibit collagen synthesis and cross-linking through the synthesis of collagenase and thereby reduce collagen fibrosis and wound shrinkage [31]. The expression of iNOS is directly related to the activation of M1 macrophages. Arginase-1, MHC-II, and FIZZ-1 are important inducers of M2
macrophage activation. RT-PCR and Western blot showed that the expression of iNOS was significantly reduced, while the expression of arginase-1, MHC-II, and FIZZ-1 was significantly increased. These findings indirectly indicate that the activation of M2 macrophages was relatively increased.

In the experimental group, the regulation of these factors by AgNPs indirectly regulated inflammation. These findings have increased our confidence in the efficacy of AgNPs. However, they also indicate that AgNPs will have many unknown reactions with the immune system in the body. The molecular mechanisms of their activity need to be further investigated.

Conclusion

AgNPs can improve the progressive necrosis of the hyperemia and stasis zones of the burn wound edge and improve the inflammatory response in these zones. AgNPs can temporarily protect wounds with their anti-infection ability and stimulation of the immune response to promote orderly wound repair and regeneration. In this study, the application of AgNPs to the wound significantly reduced the inflammatory response of the wound bed, thereby slowing the progressive necrosis. This indicates that the inflammatory response is essential in the wound healing process. Immunohistochemistry and molecular biology experiments have demonstrated that AgNPs improved wound inflammation and progressive necrosis of wounds by inducing the differentiation of M2 macrophages.

Material And Methods

Experimental animals burn model

This study strictly abides by the rules and regulations stipulated by the Animal care and use Committee of the Second Affiliated Hospital of Zhejiang University Medical College (2016 – 144), and the guidelines for the care and use of laboratory animals issued by the National Institutes of health. In particular, a custom-made rectangular copper billet (20 mm × 10mm) was scalded in boiling water for 5 minutes and then applied to the back of SD rat (adult male) for 15s rafter induction of skin surface anesthesia. 4 bands whole thickness burn (20 mm × 10 mm) with 3 normal skin gaps (20 mm × 5 mm) represented the blood stasis area were made on the back of rats. The area of burn wound is about 4% of TBSA. All rat models were placed in separate cages. In experimental group, PVP-I with 10ppm silver nanoparticles was used on born wound compared with only PVP-I on control group immediately after burn and every 24 hours.

Animal grouping and research design

The animals were randomly assigned to two groups, PVP-I group and AgNP group (n = 8 per group). The animals in AgNp groups received wet packing with PVP-I with silver nanoparticles. The rats in the PVP-I groups were given (2ml) of 5% PVP-I wet packing. The rats in the two groups were euthanized using an overdose of pentobarbital sodium at 0, 3, 7, 9 days post burn. Skin Tissues with 2 mm between burned wound on each side was harvested from each burned rat. They were stored in 4% paraformaldehyde at
4°C or −80 °C refrigerator for histological, immunofluorescence analyses, Polymerase Chain Reaction and Western blot assays. The corresponding normal skin strips were collected as control groups.

After being fixed by 4% paraformaldehyde, dehydrated by alcohol and transparent by xylene, the wound tissues were immersed in paraffin solution and placed in cooling table for cooling. The prepared tissue wax block was fixed on the specimen rack at the selection to the largest surface of the specimen. Each specimen was cut at least 3 pieces with appropriate thickness.

**Immunohistochemical analysis**

For the immunohistochemical analysis, Paraffin sections were dewaxed to water and washed with PBS for 10 min × After 3 times (standing or shaking table), the antibody to be tested was marked with pencil on the slices. Add 3% H2O2 to seal the endogenous catalase, and then put it in the wet box at room temperature for 20 minutes. Heat 0.01M sodium citrate buffer solution (pH6.0) to about 95 °C in water bath, heat tissue slices for 45 minutes, and then slowly cool for 5-10 min. after drying or wiping with absorbent paper, the area of the specimen was circled with histochemical pen. 5% BSA was added dropwise and sealed at room temperature for 1 h after washing. Then, the slides were treated with rabbit at 4°C overnight. The first antibody, anti-CD68 primary antibody (1:100; Santa Cruz, USA) and rabbit anti-TNF-α primary antibody (1:100; Abcam, UK) were incubated at 4 °C overnight. Wash them with PBS for 5 min × Three times (standing or shaking, HRP labeled). DAB was used to color the substrate under microscopic observation to control reaction time (5-30min), then terminate the reaction by washing. Dye the slides with hematoxylin for 1 minute (appropriately extended according to the dyeing situation), then wash it with distilled water and 1% hydrochloric acid alcohol to remove the floating color and return to blue. The film was sealed and photographed under a microscope.

**RNA isolation and RT-qPCR analysis**

Samples were collected 3, 7 and 9 days after burns to isolate RNA for RT qPCR analysis. Each sample was dissolved with 1 ml Trizol reagent (Invitrogen, USA) to isolate RNA. After RNA was dissolved in depc-H2O solution, the content and purity of RNA were determined by UV spectrophotometer. cDNA library was generated from RNA template by using m-mlv reverse transcriptase cDNA synthesis Kit (Promega, USA). The iNOS, arginase-1, MHC-II and α-1 genes of GAPDH were amplified by RT qPCR. The iQTM5 multiplex real-time PCR detector (bio rad, USA) was used for 40 cycles. The expression level of each target gene was normalized to GAPDH and calculated by bio rad iCycler software (version 2.0). Primer express 2.0 (ABI, USA) and beacon Designer (bio RAD) software were used for fluorescent primer design (Shanghai Bioengineering Co., Ltd).

**Western blot analysis**

Western blot analysis was performed as previously described. Briefly, lysates extracted from frozen tissue specimens in RIPA lysis buffer (50 mM of Tris, 150 mM of sodium chloride, 0.1% SDS, 0.5% sodium deoxycholate, and 0.5% Triton X-100) were centrifuged at 12,000 rpm for 15 minutes at 4°C, separated by SDS-PAGE, and transferred onto PVDF membranes (Millipore, USA). After incubation with antibodies
overnight at room temperature, proteins were detected using an ECL Western Blotting Substrate (Pierce, USA) following treatment with 5% skim milk for one hour at room temperature. The antibodies used included rabbit anti-INOS primary antibody (Abcam), rabbit anti-Arginase-1 primary antibody (Abcam), mouse anti-MHC-II primary antibody (Abcam), and mouse anti-FIZZ-1 primary antibody (Abcam).

**Statistical analysis**

All experiments were repeated at least 3 times, and the data were statistically analyzed by SPSS version 16.0 software. The experimental data are expressed as mean ± standard deviation. After the data were tested by normality and variance homogeneity, the student's t test (bilateral) and single factor analysis were used to test whether there was statistical difference. P < 0.05 was statistically significant.

**Declarations**

**Ethics approval and consent to participate**

This animal study was approved by the Ethical Committee in Second Affiliated Hospital of Medical College, Zhejiang University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

CH and XW guided the experimental procedures. CY, SW and SG participated in establishing the experimental animal model. CY, AK and QL performed the immunostaining, Histology. CY and SW participated in the design of the study and the writing of the manuscript. QL and XG performed the improvement of the language presentation. All authors read and approved the final manuscript.
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Availability of data and material

Not applicable.

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Figures

Figure 1

General observations and HE staining of burn models after burn injury or AgNP administration. Representative general images show progressive merging in the interspaces between two burn zones A-D PVP-I group and E-H AgNP group. Representative HE-stained slices show the typical histological changes in the different groups. The progression boundary is marked with a dotted line, and the initial burn regions are labelled with asterisks, presenting obvious hyalinization. Scale bars=200μm, n=8 per group. ATX, astaxanthin.
Figure 2

(a) Immunostaining of CD68 and TGF-β of sections of AgNP and PVP-I at 3, 6 and 9d post burns. (b) The relevant Real-time quantitative analysis of AgNP and PVP-I. *Indicates a statistically significant difference, P<0.05. The bar indicates 50μm.
Figure 3

AgNP downregulated inflammatory mediators and upregulated macrophage activation associated factors in wounds at day 3, 7 and 9 post burns. Real-time quantitative analysis of iNOS, arginase-I, MHC-II, and FIZZ-1 mRNA expression. Western blotting analysis of iNOS, arginase-I, MHC-II, and FIZZ-1 at day 3, 7, 9 post burn, respectively.