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RESEARCH PAPER

Histological and Immunohistochemical Evaluation of Silver Nanoparticle-Mediated Wound Healing in Rabbits

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ABSTRACT

Silver nanoparticles (AgNPs) have been explored for their antimicrobial properties and potential in wound healing applications. This study investigates the effect of AgNPs on wound healing in a rabbit model. AgNPs were synthesized using a green synthesis approach and characterized by TEM and UV-Vis spectroscopy. Excisional wounds penetrating through the full depth of the skin were surgically induced on the ear of 15 New Zealand White rabbits. The specimens were categorized into 3 groups: control (saline), AgNP-low (0.1 mg/mL), and AgNP-high (1 mg/mL). Wound dressings containing the respective treatments were applied daily. The dimensions of the wounds were assessed at four time points: immediately after wound creation (day 0) and on the 3rd, 7th, and 14th days post-injury. Histological analysis and immunohistochemical staining for collagen I and III were performed on wound tissue samples. AgNPs were successfully synthesized, with an average size of 15 nm. Wound closure in the groups treated with AgNP was noticeably quicker when contrasted with the control group. On day 14, the AgNP-high group showed the highest percentage of wound closure (95.2±2.1%), followed by the AgNP-low group (88.7±3.5%) and control (76.4±4.2%). Histological analysis revealed enhanced re-epithelialization, collagen deposition, and neovascularization in the AgNP-treated groups. Immunohistochemical staining demonstrated increased manifestation of collagen I and III in the AgNP groups. The use of AgNPs in a rabbit wound healing model demonstrated significant improvements across multiple parameters, including expedited wound closure, more efficient re-epithelialization, and a notable increase in collagen synthesis and deposition. This evidence implies that AgNPs have the potential to be used as a medicinal aid for enhanced wound healing. Additional research is required to clarify the fundamental processes and optimize the AgNP formulation for clinical applications.

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INTRODUCTION

Wound recovery is a sophisticated and multifaceted process that progresses through several interlinked phases, starting with the control of bleeding (hemostasis), followed by inflammation, cell proliferation and tissue development, and ultimately, the restructuring and maturation of the repaired tissue [1]. The main objective of the wound healing process is to repair the injured tissue and restore its structural integrity and functional capabilities. However, impaired wound healing remains a significant challenge in clinical practice, leading to chronic wounds, which are associated with substantial morbidity, diminished life quality, coupled with escalated expenses in healthcare [2,3]. Consequently, it is imperative to explore and develop innovative treatment approaches that can improve wound healing outcomes and address the shortcomings of existing therapies.

The efficacy of silver in wound treatment has been extensively documented, building upon its centuries-long reputation as a potent antimicrobial substance [4–7]. With the emergence of nanotechnology, silver nanoparticles (AgNPs) have emerged as a promising alternative to conventional silver-based treatments for wound healing. AgNPs exhibit distinctive physicochemical characteristics, such as an elevated ratio of surface area to volume which enhances their biological activity [8,9]. Moreover, AgNPs have been shown to possess potent antimicrobial activity against a wide range of bacteria, fungi, and viruses, making them an interesting option for preventing and treating wound infections [10].

The antimicrobial mechanisms of AgNPs involve multiple pathways, such as disruption of bacterial cell membranes, interference with DNA replication, and induction of oxidative stress [11–13]. In addition to their antimicrobial properties, AgNPs have been reported to modulate the inflammatory response, promote angiogenesis, and stimulate the expansion and migration of keratinocytes and fibroblasts, which are crucial for wound healing [14–16]. The observations from these studies indicate that AgNPs have the potential to accelerate wound healing by creating a favorable environment for tissue regeneration.

A variety of strategies, including biological, chemical, and physical approaches, have been employed in the synthesis of AgNPs [17]. Among these, the green synthesis approach, which utilizes plant extracts, has garnered considerable interest due to its eco-friendliness, cost-effectiveness, and biocompatibility [18]. Aloe vera, a medicinal plant known for its wound healing properties, has been successfully used as a reducing catalyst in the eco-friendly production of AgNPs [19,20]. Aloe vera leaf extract contains numerous bioactive compounds, like polysaccharides, anthraquinones, and flavonoids, which can reduce silver ions to AgNPs and stabilize them [21].

Numerous investigations have explored the wound healing capabilities of AgNPs in both laboratory and living organism settings. One in vitro experiment demonstrated that AgNPs stimulated the growth and movement of human skin cells, namely dermal fibroblasts and keratinocytes, indicating their potential to accelerate the wound repair process [22–24]. Another study demonstrated that AgNP-coated dressings accelerated wound closure and improved collagen deposition in a rat model of excisional wounds [25]. In a comparable manner, when AgNPs were integrated into hydrogels based on chitosan and applied to mice with wounds penetrating the entire depth of their skin, the treatment was observed to facilitate the wound healing process [26].

Despite the promising results, the exact mechanisms underlying the wound healing properties of AgNPs remain to be fully elucidated. Moreover, the optimal concentration and formulation of AgNPs for wound healing applications have not been well established. Therefore, more research is required to investigate the dose-dependent effects of AgNPs on wound healing and to characterize the cellular and molecular mechanisms involved in their therapeutic action.

The use of animal subjects is indispensable for evaluating the effectiveness and potential side effects of new wound healing approaches before their translation into clinical practice. The rabbit model of excisional wounds has been widely used to assess the efficacy of various wound healing agents, including AgNPs [27–29]. Rabbits are an attractive model for wound healing studies due to their ease of handling, relatively large wound size, and similarity to human skin in terms of structure and healing process [28]. Additionally, rabbits have been shown to develop chronic wounds under certain conditions, making them suitable for investigating impaired wound healing [30].

Histological and immunohistochemical techniques are valuable tools for evaluating the progression of wound healing at the tissue level. Hematoxylin and eosin (H&E) staining serves as a powerful tool for analyzing the progression of wound healing, as it allows investigators to assess vital components of the process, including the restoration of the epithelial barrier, the growth of new granulation tissue, and the synthesis and incorporation of collagen into the healing wound [21]. Immunohistochemical staining for specific markers, like collagen types I and III, can provide insights into the quality and maturity of the extracellular matrix in the healing wound [12].

This research is designed to examine the impact of AgNPs on wound healing in a rabbit model using histological and immunohistochemical techniques. We hypothesize that AgNPs synthesized using Aloe vera leaf extract will accelerate wound closure, improve re-epithelialization, enhance collagen deposition, and promote neovascularization in a dose-dependent manner. The results of this research will add to the development of AgNP-based wound healing therapies and provide a basis for future clinical trials.

MATERIALS AND METHODS

Creation and analysis of AgNPs

AgNPs were synthesized using a green synthesis approach with Aloe vera leaf extract as a reducing agent. Fresh Aloe vera leaves were collected, rinsed comprehensively with deionized water, and segmented into petite fragments. The leaves were then blended to obtain a homogeneous extract, which was filtered using Whatman #1 filter paper. The filtered substance was stored at 4°C for subsequent utilization.

In the process of synthesizing AgNPs, 10 mL of the extract from Aloe vera leaves was combined with 90 mL of a 1 mM solution of silver nitrate and stirred continuously at room temperature for 30 min. The creation of AgNPs was signaled by a shift in color from transparent to a brown hue. The synthesized AgNPs were subjected to centrifugation at a speed of 10,000 rpm for a duration of 20 minutes, and the pellet was washed three times with distilled water to remove any impurities. The purified AgNPs were then resuspended in distilled water and preserved at 4°C for further characterization and use.

The synthesized AgNPs were analyzed using UV-Vis spectroscopy and transmission electron microscopy (TEM). In preparation for TEM imaging, a small aliquot of the dispersion of AgNPs was placed on a copper grid that was layered with a thin coating of carbon, and it was left to dry naturally in the surrounding environment. The specimens were subsequently examined under a JEOL JEM-2100 TEM operating at a 200 kV accelerating voltage. ImageJ software was employed to analyze the TEM images and determine the size and morphology of the AgNPs. UV-Vis spectroscopy was performed utilizing a Shimadzu UV-1800 spectrophotometer, measurements were taken within the wavelength spectrum of 300-800 nm to confirm the formation of AgNPs.

Animal model and experimental design

The experimental protocol involving animals was inspected and authorized by the university's Institutional Animal Care and Use Committee (IACUC) and executed in adherence with the established standards for the humane treatment and utilization of animals in research settings 15 New Zealand White rabbits (weighing 2.5-3.0 kg) were acquired from a licensed animal supplier and housed in individual cages under regulated ambient conditions (temperature: 22±2°C, humidity: 50±10%, 12-hour light/dark cycle). During a span of seven days prior to the initiation of the research, the rabbits were given unrestricted access to a standard diet formulated for their species and a continuous supply of fresh water, allowing them to acclimate to their new environment.

The study population of rabbits was divided into three randomly assigned groups, each allocated with five creatures: a saline-treated control group, a low-dose AgNP treatment group (0.1 mg/mL), and a high-dose AgNP treatment group (1 mg/mL). Before the infliction of wounds, the rabbits were sedated using an intramuscular administration of ketamine (35 mg/kg) and xylazine (5 mg/kg). The skin on the ear was trimmed and sterilized with a solution of 70% ethanol. Six excisional wounds of full thickness, each measuring 1 cm by 1 cm, were inflicted on the ear skin of each rabbit using a sterile surgical blade. The wounds were spaced at least 2 cm apart to prevent interference.

Wound treatment and monitoring

Immediately after wound creation, the wounds were treated with the respective treatments: saline (control), AgNP-low (0.1 mg/mL), or AgNP-

high (1 mg/mL). Sterile gauze dressings soaked in the treatment solutions were applied to the wounds and secured with adhesive bandages. The dressings were changed daily, and the wounds were cleaned with sterile saline before applying fresh dressings.

The dimensions of the wound were assessed at four time points: the day of wound induction (day 0) and on the 3rd, 7th, and 14th days post-injury using digital photography and image analysis software (ImageJ). Having determined the wound area, the researchers employed the subsequent formula to calculate the percentage of wound closure (Eq. 1).

Histological and immunohistochemical analysis

On the 14th day, the rabbits were euthanized using an intravenous injection of pentobarbital sodium (100 mg/kg). The wound tissue samples were excised, stabilized using 8% solution of neutral buffered formalin, followed by embedding in paraffin wax. Slices that were 5 µm in thickness were prepared and dyed with hematoxylin and eosin (H&E) for the purpose of histological examination. The stained sections were evaluated for re-epithelialization, collagen deposition, and neovascularization using a light microscope (Olympus BX51, Japan).

Immunohistochemical staining for collagen I and III was performed on the paraffin-embedded sections. The slices underwent a process of deparaffinization and rehydration, followed by antigen retrieval facilitated by the use of a citrate buffer with a pH value of 6.0. The slices were subsequently treated with primary antibodies against collagen I (ab34710, Abcam) and collagen

$$\text{Wound closure (\%)} = \left[\frac{\text{Initial wound area} - \text{Wound area at day } X}{\text{Initial wound area}}\right] \times 100 \quad (1)$$

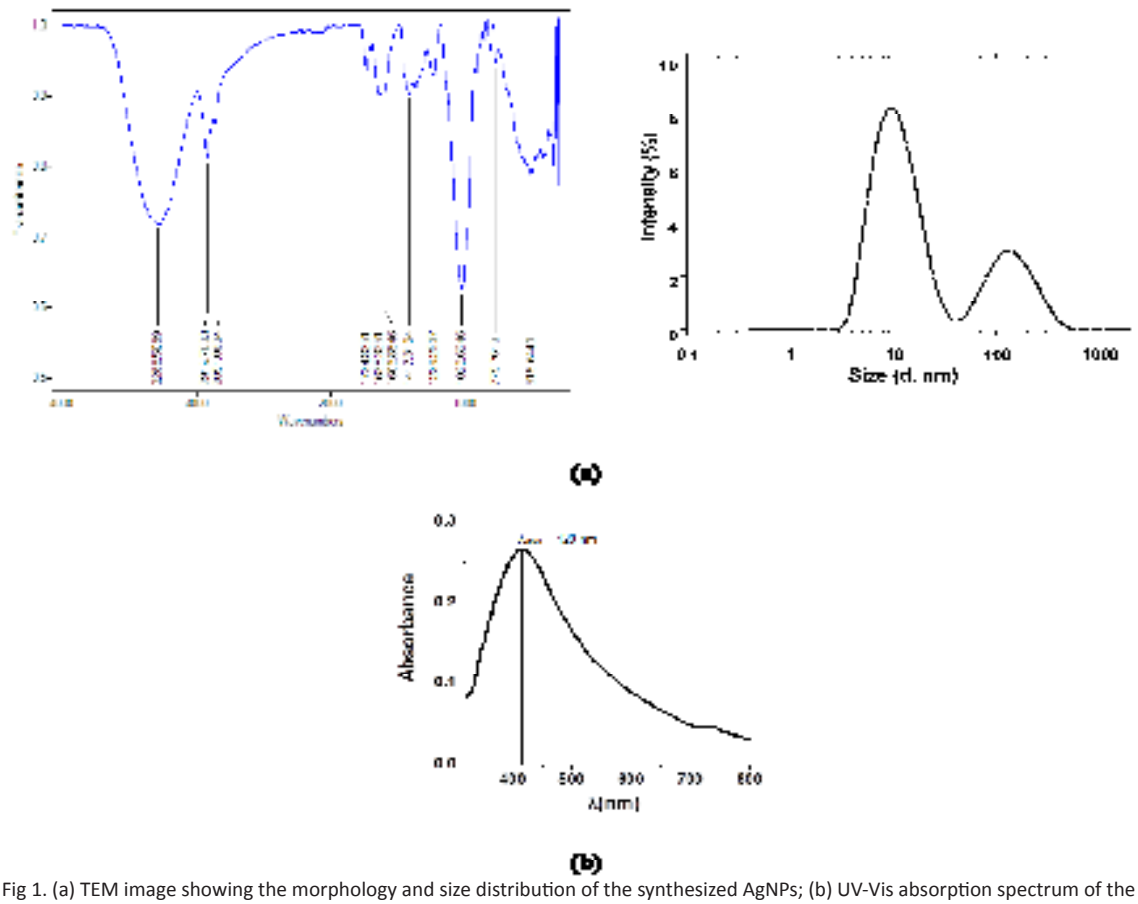

Fig 1. (a) TEM image showing the morphology and size distribution of the synthesized AgNPs; (b) UV-Vis absorption spectrum of the AgNP suspension.

III (ab7778, Abcam) overnight at 4°C. After the washing procedure, the slices were subjected to a one-hour incubation at room temperature with secondary antibodies linked to horseradish peroxidase (HRP). The next steps involved staining the sections with DAB (3,3'-diaminobenzidine) and applying a hematoxylin counterstain. The immunostained slices were examined using a light microscope, and the intensity of staining was evaluated employing a semi-quantitative grading scheme where 0 signifies no staining, 1 indicates weak staining, 2 represents moderate staining, and 3 denotes strong staining.

Statistical analysis

Data were presented as the mean ± standard deviation (SD) for all experimental groups. GraphPad Prism 8 software was employed to conduct statistical analyses. The group means were evaluated using a one-way ANOVA, supplemented with Tukey's post hoc test for detailed comparison. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Characterization of AgNPs

TEM analysis revealed that the synthesized AgNPs had a spherical shape and a narrow size distribution, with an average diameter of 15 ± 3 nm (Fig. 1a). The UV-Vis spectrum of the AgNP suspension exhibited a characteristic surface plasmon resonance (SPR) peak at 420 nm, which verified the successful synthesis of AgNPs (Fig. 1b).

Wound closure

The rate of wound closure displayed a consistent upward trajectory in all experimental groups throughout the 14-day monitoring period (Table 1). On day 3, the AgNP-high group showed a significantly higher wound closure percentage (28.5 ± 2.8%) compared to the control group (17.2 ± 3.1%, $p < 0.05$). On day 7, both AgNP-treated groups exhibited significantly faster wound closure (AgNP-low: 63.8 ± 4.2%, AgNP-high: 75.1 ± 3.6%) compared to the control group (48.9 ± 3.9%, $p < 0.01$). At day 14, the AgNP-high group achieved the highest wound closure percentage (95.2 ± 2.1%), followed by the AgNP-low group (88.7 ± 3.5%) and the control group (76.4 ± 4.2%). The statistical significance of the disparities across all groups was confirmed ($p < 0.001$).

Immunohistochemical analysis

Immunohistochemical staining indicated an elevated expression of both collagen I and III in the groups treated with AgNPs, as opposed to the control group (Table 2). The AgNP-high group showed the highest intensity of staining for both collagen I (2.8 ± 0.4) and collagen III (2.6 ± 0.5), followed by the AgNP-low group (collagen I: 2.2 ± 0.4, collagen III: 2.0 ± 0.7) and the control group (collagen I: 1.4 ± 0.5, collagen III: 1.2 ± 0.4). The differences in collagen I and III expression among the groups were statistically significant ($p < 0.01$).

Histological analysis

The sections of wound tissue which underwent H&E staining on day 14 revealed distinct differences in the healing process among the groups (Fig. 2). Relative to the control group, the groups administered with AgNPs presented a more advanced re-epithelialization, characterized by an epithelial layer with greater thickness and better organization. The AgNP-high group exhibited the most advanced re-epithelialization, with a nearly complete epithelial coverage of the

Table 1. Wound closure percentage (mean ± SD) in different groups over 14 days.

Group	Day 0	Day 3	Day 7	Day 14
Control	0	17.2 ± 3.1	48.9 ± 3.9	76.4 ± 4.2
AgNP-low	0	23.6 ± 2.9	63.8 ± 4.2*	88.7 ± 3.5**
AgNP-high	0	28.5 ± 2.8*	75.1 ± 3.6**	95.2 ± 2.1***

Note: *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

Table 2. Immunohistochemical staining intensity (mean ± SD) for collagen I and III in different groups on day 14.

Group	Collagen I	Collagen III
Control	1.4 ± 0.5	1.2 ± 0.4
AgNP-low	2.2 ± 0.4*	2.0 ± 0.7*
AgNP-high	2.8 ± 0.4**	2.6 ± 0.5**

Note: *p < 0.05, **p < 0.01 compared to the control group.

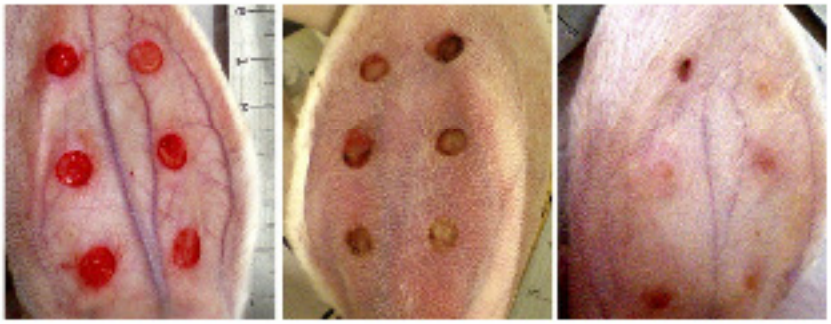

Fig. 2. Representative histological images (H&E stained) of wound tissue sections from each group on day 14, highlighting differences in re-epithelialization, collagen deposition, and neovascularization.

wound area. Moreover, the AgNP-treated groups demonstrated increased collagen deposition and a more organized arrangement of collagen fibers in the dermis compared to the control group. The AgNP-high group displayed the most abundant and mature collagen fibers. Neovascularization was also more prominent in the AgNP-treated groups, with a higher density of blood vessels in the granulation tissue in relation to the control group.

Histological scoring of re-epithelialization, collagen deposition, and neovascularization on day 14 (Table 3) further confirmed the dose-dependent effects of AgNPs on wound healing. The AgNP-high group showed the highest scores for re-epithelialization (2.8 ± 0.4), collagen deposition (2.6 ± 0.5), and neovascularization (2.4 ± 0.5), accompanied by the AgNP-low group and the control group. The differences in histological scores the groups treated with AgNPs and the control group were statistically significant ($p < 0.05$ for AgNP-low, $p < 0.01$ for AgNP-high).

The results of this study demonstrate that AgNPs synthesized using Aloe vera leaf extract accelerate wound healing in a rabbit model of full-thickness excisional wounds. The AgNP-treated groups exhibited increased collagen deposition, enhanced re-epithelialization, faster wound closure, and improved neovascularization compared to the control group. These findings suggest that AgNPs have the potential to be used as a therapeutic agent for promoting wound healing.

The faster wound closure observed in the AgNP-treated groups can be accredit to the antimicrobial and anti-inflammatory properties of AgNPs. AgNPs have been shown to inhibit the growth of a wide range of bacteria, encompassing those typically identified in wound contaminations, such as Staphylococcus aureus and Pseudomonas aeruginosa [19]. By reducing the bacterial load in the wound bed, AgNPs create a favorable environment for tissue regeneration and prevent the prolongation of the inflammatory phase, which can impede wound healing [15].

The enhanced re-epithelialization observed in the AgNP-treated groups suggests that AgNPs stimulate the proliferation and migration of keratinocytes, which are essential for the formation of a new epithelial layer over the wound surface. This finding is consistent with previous in vitro studies that have demonstrated the ability of AgNPs to promote keratinocyte proliferation and migration [22]. The increased collagen deposition and organized arrangement of collagen fibers in the AgNP-treated groups indicate that AgNPs stimulate the activity of fibroblasts, which are responsible for synthesizing and remodeling the extracellular matrix. It has been shown that AgNPs can promote the upregulation of transforming

Table 3. Histological scoring (mean ± SD) of re-epithelialization, collagen deposition, and neovascularization in different groups on day 14.

Group	Re-epithelialization	Collagen deposition	Neovascularization
Control	1.8 ± 0.4	1.6 ± 0.5	1.4 ± 0.5
AgNP-low	2.4 ± 0.5*	2.2 ± 0.4*	2.0 ± 0.7*
AgNP-high	2.8 ± 0.4**	2.6 ± 0.5**	2.4 ± 0.5**

Note: Scoring system: 0: none, 1: minimal, 2: moderate, 3: complete. *$p < 0.05$, **$p < 0.01$ compared to the control group.

growth factor-beta (TGF-β) and other growth factors that contribute to collagen production [24].

The improved neovascularization in the AgNP-treated groups suggests that AgNPs promote angiogenesis, which is crucial for supplying nutrients and oxygen to the healing tissue. Vascular endothelial growth factor (VEGF), along with other growth factors, is responsible for regulating the formation of new blood vessels in the body, which has been shown to be upregulated by AgNPs [20]. The dose-dependent effects of AgNPs observed in this study, with the higher concentration (1 mg/mL) yielding better wound healing outcomes, highlight the importance of optimizing the AgNP dose for maximum therapeutic benefit.

Immunohistochemical staining for collagen I and III provides insights into the quality and maturity of the extracellular matrix in the healing wounds. Collagen I is the predominant collagen type in normal skin, while collagen III is more abundant in early wound healing and gradually replaced by collagen I as the wound matures [8]. The increased expression of both collagen types in the AgNP-treated groups suggests that AgNPs promote collagen synthesis and maturation, leading to improved wound strength and reduced scarring.

The application of Aloe vera leaf concentrate for the green synthesis of AgNPs offers several advantages over conventional chemical synthesis methods. Aloe vera contains various bioactive compounds, such as polysaccharides, anthraquinones, and flavonoids, which act as reducing and stabilizing agents during the synthesis process [13]. These compounds not only facilitate the formation of AgNPs but also enhance their biocompatibility and wound healing properties. Moreover, green synthesis using plant extracts is an eco-friendly and cost-effective perspective that minimizes the use of toxic chemicals and reduces the environmental impact of nanoparticle production.

The rabbit model of full-thickness excisional wounds used in this study is a well-established model for evaluating wound healing interventions. Rabbits have been widely used in wound healing studies due to their similarity to human skin in terms of structure and healing process [30]. The creation of multiple wounds on each rabbit allows for the comparison of different treatments within the same animal, reducing inter-individual variability and the number of animals required. However, it is important to acknowledge the limitations of animal models and the need for further studies to assess the safety and efficacy of AgNPs in human subjects.

CONCLUSION

In conclusion, this study demonstrates that AgNPs synthesized using Aloe vera leaf extract speed up wound healing in a rabbit model of full-thickness excisional wounds. In comparison to the control group, the groups treated with AgNPs demonstrated more rapid wound closure, improved re-epithelialization, greater collagen deposition, and better neovascularization. The dose-dependent effects of AgNPs highlight the importance of optimizing the AgNP concentration for maximum therapeutic benefit. The results of this study suggest that AgNPs could be a promising new therapeutic option for enhancing the wound healing process. However, further research is required to determine the underlying mechanisms of action, assess the long-term safety and efficacy, and optimize the formulation and delivery methods of AgNPs for clinical applications.

CONFLICTS OF INTEREST

The authors affirm that no conflict of interest exists concerning the publication of this manuscript.

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