Synergetic Potential of Green Synthesized Nano-Antibiotic Combinational Therapy For Wound Healing

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Abstract

The increasing evidences of chronic surgical wounds and its associated complications in association with bacterial resistance to conventional antibiotics. Therefore, current antibiotic resistance crisis ultimately calls for the need of alternative antibacterial options and nanotechnology could be a solution. Furthermore, nano-antibiotics combinations will provide synergy with reducing the dosage of both agents, which can enhance biocompatibility and may aid in limiting the global crisis of emerging multidrug resistance. Specifically, in the context of cytotoxicity of nanoparticles, in the present study, green synthesized nanoparticles (Ag NPs, ZnO NPs) are used for the combination with antibiotic neomycin to investigate *in-vivo* wound healing activity to facilitate the tissue repair with minimized toxicity. Our results showed that, *in-vivo* potent synergetic wound healing effectiveness and faster wound contraction of prepared gel formulations from the green synthesized nanoparticles combinations with neomycin compared with neomycin or nanoparticles alone. These results point to the opportunity provided by this approach to realize the unmet needs and future directions with lustrous prospects in combinational herbal nanomedicine to combat the multi drug resistant bacteria.

1. Introduction

Wound healing is a highly coordinated process with a cascade of overlapping biological processes such as hemostasis, inflammation, proliferation and maturation with ultimate healing through minimal scarring.[1,2] The associated complications of surgical wound infections due to multidrug resistant (MDR) pathogens are more problematic than the prime cause of surgery. These MDR pathogens with their associated biofilms are capable of inducing apoptosis, and release of inflammatory cytokines that cause chronic inflammation preventing re-epithelialization with broader complications, thus have been an incessant global problem with high mortality rate.[3] Therefore, the rapid emergence of drug resistance and scarcity for novel classes of antibiotics, nanotechnological strategies to prevent MDR infections and to target the pathophysiology and complexity of wound healing process.

Under this scope, Metallic nanoparticles for instance, silver (Ag), zinc oxide (ZnO) are being extensively used to treat wound infections, but often suffer from the concentration dependent toxicity, which has remained as a matter of concern.[4] In recent past, phyto-nanotechnology could be a solution for not only minimizing cytotoxicity but also economical and effective route. In addition, plant extracts are rich in bioactive compounds with the aid of biocompatibility and further contribute to reduce and stabilize for the synthesis of nanomaterials.[5,6] Further, plant metabolites may impart additional activity in wound healing process due to their wide range of medicinal properties.[7] Recently, Ag and ZnO nanoparticles have proven their outstanding antimicrobial and wound healing properties *in vitro* and *in vivo*.[3, 8–11] Among them, green synthesized nanoparticles have proved to exhibit enhanced activity with optimum biocompatibility.[5,12,13]

Considering these perspectives, green nano-antibiotic combinatorial therapy with decreased dose of antibiotics and nanomaterials to circumvent antibiotic resistance and toxicity issues. This combinatorial

action prevents resistance; as bacteria cannot develop multiple gene mutations simultaneously by effectively killing bacteria without attaining resistance to antibiotics and also prevents toxicity through imparting synergetic activity at lower concentrations of nanoparticles and drug.[5] Although there are few reports on combinational formulations for *in vitro* antibacterial activity of nanoparticles with antibiotics, there is no report till date on *in-vivo* combinatorial wound healing activity of green synthesized nanoparticles with antibiotics.[5]

Towards this end, the present study aimed to evaluate the wound healing activity of *Annona squamosa* (AS) mediated Ag and ZnO nanoparticles and the Ag and ZnO nanoparticles in combination with antibiotic neomycin in comparison with commercial antibiotic. Here in, to perform the activity, nanoparticles and their combinations with neomycin were formulated in to gels and evaluated for optimum characteristics of a gel before assessing the wound healing activity. The schematic representation of the experimentation is shown in Figure 1.

2. Experimental Section

2.1 Preparation, characterization, antimicrobial activity, biocompatibility of Ag NP and ZnO NPs

The current study utilizes the Ag and ZnO nanoparticles synthesized via a green method using *Annona squamosa* leaf extract as a reducing agent. Method of synthesis, characterization *in vitro* antibacterial activity of prepared Ag and ZnO nanoparticles, nanoparticles in combination with neomycin and the biocompatibility of synthesized nanoparticles were reported in our previous study.[12,13]

2.2. Assessment of combinational drug of neomycin and Ag and ZnO nanoparticles

The combination of Ag and ZnO with neomycin that was prepared for antibacterial activity assessment *in vitro* in our previous study was assessed for interaction between nanoparticle and neomycin using UV-Vis spectroscopy.

In brief, 10 mL of nano particle dispersion (25 µg/mL-Ag nanoparticles, 50 µg/mL-ZnO nanoparticle) and 10 mL of neomycin solution (50 µg/mL) were taken in a 50 mL beaker and kept under continuous stirring at 800 rpm at room temperature (~27°C) for 24 h to make a composite dispersion (without aggregation). The prepared combinational drug was assessed for interaction between nanoparticle and neomycin using UV-Vis spectroscopy in the wavelength range of 200 nm to 800 nm. The dispersion of the combinational drug was centrifuged at 5000 rpm for 15 min to remove the unconjugated drug and the sediments were dried to form a powder, % yield was calculated and stored for further use

$$\%\text{Yield} = \frac{\text{Obtainednanoparticlesweight}}{\text{Totalweighttaken(NPs + antibiotic)}} \times 100$$

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The loading efficiency of the combinational drug was estimated by collecting 5 mL of supernatant solution and diluting with 95 mL of distilled water in a 100 mL volumetric flask. Absorbance of the diluted combinational drug suspension was evaluated using UV Spectrophotometer placing distilled water as blank. The % entrapment was determined by calculating the loading efficiency by using the formulae;

$$\mathrm{Loadingefficiency} = \frac{\mathrm{Totalamountofdrug} - \mathrm{Amountofunbounddrug}}{\mathrm{WeightofobtainedNPs}}$$

2.3. Formulation combination gels

Carbopol 934 (Sigma Aldrich) was passed through US Standard 60 mesh (250 μm) and weighed accurately for formulating 1% w/w gel with a batch size of 40 g. Half of the required quantity of water was added to the pre weighed beaker containing Carbopol and kept for continuous stirring until Carbopol attains sufficient gel consistency. Desired quantity of Ag and ZnO nanoparticle and Ag NP-neomycin (SNNP)/ZnO NP-neomycin (ZNNP) required for the formulation was added slowly to the gel base under continuous stirring for about 60 min to avoid agglomeration of nanoparticles. Water was added to the beaker to adjust the weight of the gel to approximately 39.5 g and added with 2-4 drops of tri ethanolamine to attain desired consistency and the final weight of the gel was adjusted to 40 g. The gel was stored in an airtight container. The concentrations of Ag and ZnO nanoparticles, and SNNP and ZNNP were selected based on antibacterial and cytotoxicity efficacy from our previous study.[12,13] Briefly, 25 μg/mL of Ag NP, 50 μg/mL of ZnO NP showed similar zone of inhibition which is identical to zone of inhibition of 50 μg/mL of neomycin. Therefore, the concentrations of Ag NP (0.25% w/w), ZnO NP (0.5% w/w), and their combinations with similar dose, i.e; 0.375% w/w SNNP (0.125% w/w Ag NP+0.25% w/w neomycin), 0.5% w/w ZNNP (0.25% w/w ZnO NP+0.25% w/w neomycin) were prepared and evaluated for antibacterial activity which resulted in synergetic activity.[12,13] With the same concentrations of nanoparticles and nanoparticle combinations, the gels were prepared and evaluated for different parameters.

2.3.1. Appearance and homogeneity

Physical appearance and homogeneity of the prepared nanoparticle gels were examined by visual observation.

2.3.2. pH measurement

pH measurement of the gel was carried out using a digital pH meter (ELICO LI 120) by dipping the combination electrode in to gel system to cover the electrode.

2.3.3. Viscosity

Viscosity of gel was determined by Brookfield viscometer (S-16, model LVDV-E, cup and bob) using small volume adaptor at 25°C. The viscosity was noted where the maximum torque was attained.

2.3.4. Spreadability

Two glass slides (25 X 75 mm) were taken for each of the formulation. 1 g of gel was placed along the width (25 mm) of the 1st glass slide and it was fixed in a position. Another slide was gently moved with firm pressure on the 1st slide instantly from the gel side of the 1st slide. The length of the smear formed by the gel on the 1st slide was measured and indicated as a measure of the spreadability for that particular gel formulation.[14]

2.3.5. *Extrudability of the gel*

The gel formulation under study was filled in clean, lacquered aluminum collapsible tube of 5 g capacity with 3 mm orifice. The ointment tube was kept between thumb and pointing finger and sufficient pressure was applied from the bottom side of the tube. The quantity of gel coming out of the tube with single press and shape of the gel extruded was examined.[14]

2.4. Wound healing activity

Albino Wistar rats were purchased from Mahavir Enterprises, Hyderabad. Wistar albino healthy male rats weighing 300-400 g were selected for the wound healing studies. They were housed in polypropylene cages under standard environmental conditions of temperature (25±2°C), humidity (45±5% RH) and 11-12 h light and dark cycles. The animals were housed individually with sterile paddy husk bedding and free access to food and water *ad libitum*. The experiments were performed in accordance with ethical norms as approved by CPSCEA guidelines and Institutional Animal Ethical Committee Ref. no. IAEC No.439/PO/01/a/CPCSEA.

Group numbers (I-VI) were allotted to the animals with six animals in each group and are represented in Table 1. Group II was treated with 0.5% w/w commercial neomycin sulphate cream.

Table 1
Treatments for wound healing activity (n=3)

Group number	Average weight of animals (g) mean±S.D	Treatment
I	335±12	Untreated
II	349±8	Neomycin cream commercial formulation (0.5% w/w)
III	354±23	Ag NP (0.25% w/w)
IV	365±18	ZnO NP (0.5% w/w)
V	389±12	Ag NP+Neomycin (SNNP-0.375% w/w)
VI	381.5±15	ZnO NP+Neomycin (ZNNP-0.5% w/w)

Prior to 24 h of the test (dose application), hair on the dorsal side was shaved cleanly for each rat, exposing 6 cm^2 area of skin approximately. Excision wound model was selected in order to evaluate the wound healing activity in adult male albino Wistar rats. The animals were anaesthetized by xylazine HCl (50mg/Kg) and ketamine hydrochloride (10mg/Kg). An excision wound of area around 500 mm^2 was made by using surgical knife on the dorsal side for all the animals. 0.5 g of each treatment was evenly applied to 500 mm^2 area of the wounded skin of each rat twice a day for a period of 14 days. The consequent changes in the wound area was monitored at regular time intervals for skin irritation, percentage of wound contraction and finally subjected the histopathological studies on the 14th day.

2.4.1. Skin irritation study

Skin reaction at the site of application was subjectively assessed at different time intervals starting with 1 h and subsequently at every application for 14 days.

2.4.2. Percentage of wound contraction

Wound area was measured by measuring the wound length and breadth at different periods of treatment. Further, the progressive changes in the area of the wound, percentage reduction in the original wound size were expressed in terms of wound contraction.

$$\%\mathrm{Woundcontraction} = 1 - \frac{A_d}{A_0} * 100$$

Where, A_0 = wound area on day zero, A_d = wound area on corresponding days

2.4.3. Histopathological studies

The skin tissue samples were collected on 14th day at the site of the wounds from different groups (I-VI) for evaluation of the extent of wound healing by histopathological studies. Biopsy specimens were

collected with utmost care, preserved using 10% buffered formalin for 24 h at room temperature and pathology studies were performed by staining with haemotoxylin and eosin and histophotographs were captured using microscope OLYMPUS BX 51 under 10 x magnifications.

2.5. Results

2.5.1. Assessment of combinational drug of neomycin and Ag and ZnO nanoparticles

The prepared combinational drug (SNNP and ZNNP) was subjected to scanning for absorbance peaks using UV-Visible spectrophotometer. A comparative UV spectral analysis of nanoparticle (Ag and ZnO), neomycin and their combination is shown in Figure 2. The absorbance peak of neomycin appeared as a band near 250 nm with low intensity. The Ag, ZnO nanoparticles showed absorbance bands around 440 nm, 375 nm respectively. The combination of SNNP obtained with enhanced absorbance at Ag nanoparticle peak area with a small shift and broadened absorption band, shown in Fig. 2 **(a).** The absorption spectrum for ZNNP displayed increased intensity with a slight shift in peak position as shown in Fig. 2 **(b).** The % yield of the combinational drug and loading efficiency was calculated to be 82.67±0.24% and 84.12± 0.32, 81.13±0.26% and 82.67±0.13 for SNNP, ZNNP respectively.

2.5.2. Preliminary analysis of the formulated combination gels

Four gel formulations of Ag NPs alone, ZnO alone and in combinations with neomycin (SNNP and ZNNP) were prepared using Carbopol 934 (1%) and were evaluated for physical appearance, pH, viscosity, spreadability, extrudability. Results of the study are given in Table 2. All the preparations were with desired consistency and optimal pH (6-7) with good spreadability. It was observed that the quantity of gel extruded with a single press was approximately 0.5 g with spherical viscous drop of diameter 0.3-0.4 mm.

Table 2
Evaluation parameters of nanoparticle gels

Gel formulation	Physical appearance	pH*	Viscosity* (centi poise)	Spreadability* (mm^2)	Extrudability (g)
Ag NP	Dark grey, smooth, homogenously dispersed, translucent	6.23	1892	1366.5	0.5
ZnO NP	White, smooth, homogenously dispersed, translucent	6.38	1972	1322	0.4
SNNP	Grey, smooth, homogenously dispersed, translucent	6.66	2080	1061	0.5
ZNNP	White, smooth, homogenously dispersed, translucent	6.99	2724	1363.5	0.45

*NP-nanoparticle, SNNP- Silver neomycin nanoparticles gel treatment, ZNNP-Zinc neomycin nanoparticle gel treatment

2.5.3. Wound healing activity

The protocol was initiated immediately after injury. No skin irritation or reddening was observed at site of wounds after application of the gel formulations during the course of 14 day study. There was slight infection in two of the 6 animals in control group (I) by day 4. Erythema/edema/infection to the skin was not observed in any of the groups (II-VI) on application of neomycin and nanoparticle/combination gels during the 14 days of study. The areas of wound contraction in control group and on application of neomycin and gel formulations on different days are given in Table 3 and are represented in Figure 3.

Table 3
Wound healing activity of commercial neomycin cream and nanoparticle gels

Treatment (Groups)	% Wound contraction				
	3rd day	5th day	10th day	12th day	14th day
Control (I)	11.42±0.13	21.2±1.18	43.1±0.26	61.34±2.24	72.8±0.46
Neomycin (II)	20.46±0.18	32.62±2.42	60.21±0.12	71.31±3.24	84.62±0.29
Ag NP (III)	22.76±2.96	33.76±0.45	61.15±1.02	73.38±0.86	86.34±2.75
ZnO NP (IV)	20.32±0.34	31.76±0.21	61.42±0.88	70.65±1.41	82.86±0.62
SNNP (V)	24.52±1.21	36.83±1.52	67.37±0.17	80.78±0.34	92.17±0.62
ZNNP (VI)	23.78±2.21	38.78±0.76	68.56±1.22	80.24±0.33	90.61±1.23

*NP-nanoparticle, SNNP- Silver neomycin nanoparticles gel treatment, ZNNP-Zinc neomycin nanoparticle gel treatment

The % wound contraction with all the formulations (84-92.17%) was higher than that of control group I ($p<0.05$, statistitically significant). Compared to % wound contraction in standard (commercial neomycin) group II (84.62%), the % wound contraction was higher in group III (86%), whereas ZnO nanoparticle treated group IV showed less % wound contraction (82.86%) than that commercial neomycin group II. The % wound contraction in combinational groups V, VI, (90-92%) were relatively higher than that of groups II, III, IV which might owe to synergetic action of Ag and ZnO nanoparticles and neomycin, and are statistically evident with $p<0.05$ when analyzed suing Graph pad prism software. The photographs of wounds from different groups were taken at specific intervals for visual comparison, and presented to assist the results of rate of wound healing. The photographs captured on 1st, 5th, 10th and 14th day of the study from all groups are shown in Figure 4, which clearly percepts that wound closure on treatment with nanoparticle combination gels are efficient than that of commercial neomycin and nanoparticles gels alone.

The histopathological photographs are shown in Figure 5. Histophotograph of group I specimens showed ulcerated epidermis and dermis with fibro collagenous stroma with scattered lymphoplasmacytic infiltrates and edema. Whereas, normal skin epidermis was noticed in histophotograph of group II specimen along with fibro collagenous stroma, mild lymphoplasmacytic infiltrates and mild edema in dermis. Specimens from group III and IV showed normal skin epidermis and dermis with fibro collagenous stroma with mild lymphoplasmacytic infiltrates without any edema. The specimens from Group V, VI showed normal skin epidermis with fibro collagenous stroma, high lymphoplasmacytic infiltrates and thin blood vessels. These results from combinations indicated that wound healing activity was high in combinations than that of individual treatments.

2.6. Discussion

This study utilizes the Ag and ZnO nanoparticles synthesized via a green method using *Annona squamosa* leaf extract as a reducing agent. The results of the Ag and ZnO nanoparticle synthesis, characterization, *in vitro* antibacterial activity and biocompatibility were described in our previous study[12,13]. In spite of enormous efficiency of nanoparticles in antibacterial treatment, they have not entered the antibiotic pipeline till date, due to certain lack of *in vivo* research and clinical trials. To bridge up the void, the research was further focused on assessing the wound healing activity on Wistar albino rats by formulating the nanoparticles and their combinations with neomycin in to gel form.

An earlier report of Aqeel Y *et al*. investigated the effect of chemically synthesized Au nanoparticle conjugation with chlorhexidine and Ag nanoparticle conjugation with neomycin by using UV-Vis analysis for estimation of combinational drug formation.[8] Based on the earlier report, the prepared combinational drug was subjected to scanning for absorbance peaks using UV-Visible spectrophotometer, which showed deviation of characteristic peak of Ag and ZnO with enhanced absorption and slight shift in peak position in presence of other drug leads to apparent formation of combinational drug.

Gel formulation was preferred for the study, as among topical semisolid preparations, gel holds longer residence time on the skin due to high viscosity and moisturizing effect on flaky skin because of their occlusive properties, bio-adhesiveness, minimal irritability and easy to apply with better release of the active constituent.[16–18] The concentrations were selected based on our previous study to establish a *in vitro-in vivo* correlation.

Wound healing response begins the moment when the tissue is injured. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase collagen production, which subsequently restores the epithelial tissue.[19] To enhance healing process without causing infections by MDR bacteria, much research is focused on resistance free antibiotics. In such a perspective, nanoparticle combination gels have proved to be effective with enhanced wound healing potential without causing infections. Moreover, the prepared nanoparticles are biocompatible and hence no toxicity issue can be expected.[12,13]

There are studies on wound healing activity of Ag nanoparticles on wound healing activities of nanoparticles alone and their alloys mediated with plant derivatives, such as *Euphorbia milli* plant extract mediated silver nanoparticles by Gong CP et al. revealing effective wound healing potential by formulating 10% Ag nanoparticle gel[16]. Madhurima Paul et al.[17] shown wound healing potential of *Pongamia pinnata* seed extract mediated silver nanoparticles on Wistar albino rats by formulating in to a gel and have shown rapid reepithelialisation when compared to the standard drug. Thangavel S et al.[18] have prepared actinobacterial mediated Ag, Au nanoparticles and their alloy and examined the wound healing activity on Wistar albino rats by preparing 10% nanoparticle formulation. The results revealed the early reepithelialisation in case of Ag nanoparticles and Ag/Au alloy nanoparticles with excellent anti-inflammatory activity via inhibition of IL-6 and TNF-α than standard drug. In addition, there are few reports on wound healing activities of nanoparticles (chemically synthesized) in combination with antibiotics. [20,21] However compared to these reports of chemically synthesized Ag nanoparticles, we have

used 1% gel for study and have shown effective wound healing with combinations of green synthesized nanoparticles as evident from histopathological studies.

With respect to ZnO, there were few reports on chemically synthesized nano ZnO for wound healing applications demonstrating enhancement of collagen synthesis and reepithelialisation of skin. [22,23] However, there are no reports on wound healing activity of green synthesized ZnO nanoparticles till date.

On focus, there are no reports noted till date on wound healing study with green synthesized nanoparticles in combination with any antibiotic which is unique for the study except for a report on CuO and neomycin for wound healing, investigated by our team.[24]

2.7. Conclusion

The preliminary investigation of wound healing indicated the suitability of these nanoparticles in prevention of infection at the site of wound. Nanoparticles contributed for wound healing on par with commercial neomycin cream in a more effective manner than that of untreated wound. Nanoparticles in combinations with neomycin (SNNP, ZNNP) showed an improved wound healing than that of individual treatments. This was clearly evident from the results of group I where mild infection was observed to the untreated wound. Besides, in groups II-VI, no infection was found when treated with neomycin or nanoparticles alone or in combinations. Moreover, earlier researchers used 10% nano gel formulations of silver for wound healing activity. Whereas in the present study, 0.25% or 0.5% concentration of nanoparticles/combination gels were used with prominent wound healing activity without any infections, which might be due to the synergetic wound healing effect of *Annona squamosa* associated with nanoparticles. Hence, the present *in vivo* study correlates with the results of our previous *in vitro* study and these combinations can be an assured paradigm to be an alternative to conventional antibiotics without toxicity. However, further research need to be explored to give more conclusions related to wound healing capacity and other possibilities of these nanoparticles for effective treatment of infected wounds caused by resistant bacteria.

Abbreviations

MDR, Multi drug resistance; AS, *Annona squamosa*; NP, nanoparticles; SNNP; Silver neomycin combibination; ZNNP, Zinc Oxide neomycin combination

Declarations

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Ethical Approval

All procedures performed in animal study were in accordance with the ethical standards of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) institution or through practice where studies were carried out.

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Figures

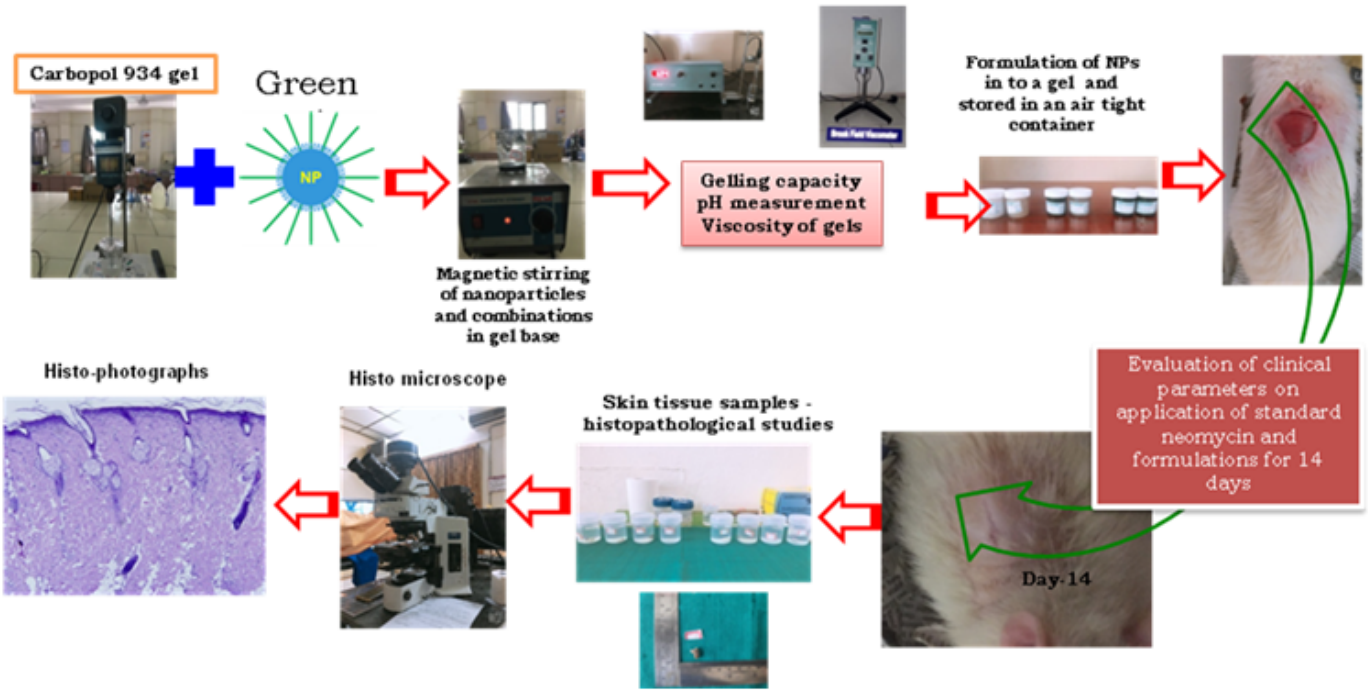

Figure 1

Schematic representation of wound healing experiment on treating with nanoparticle gel.

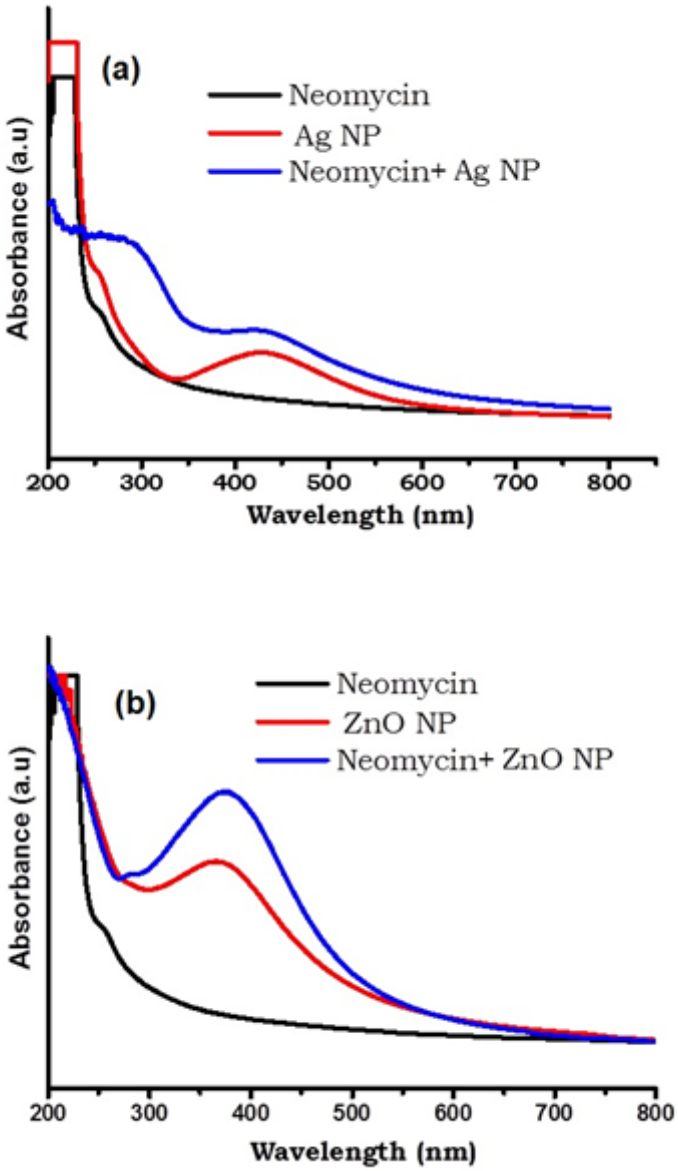

Figure 2

Assessment of nanoparticle (NP)-neomycin combinational drug.

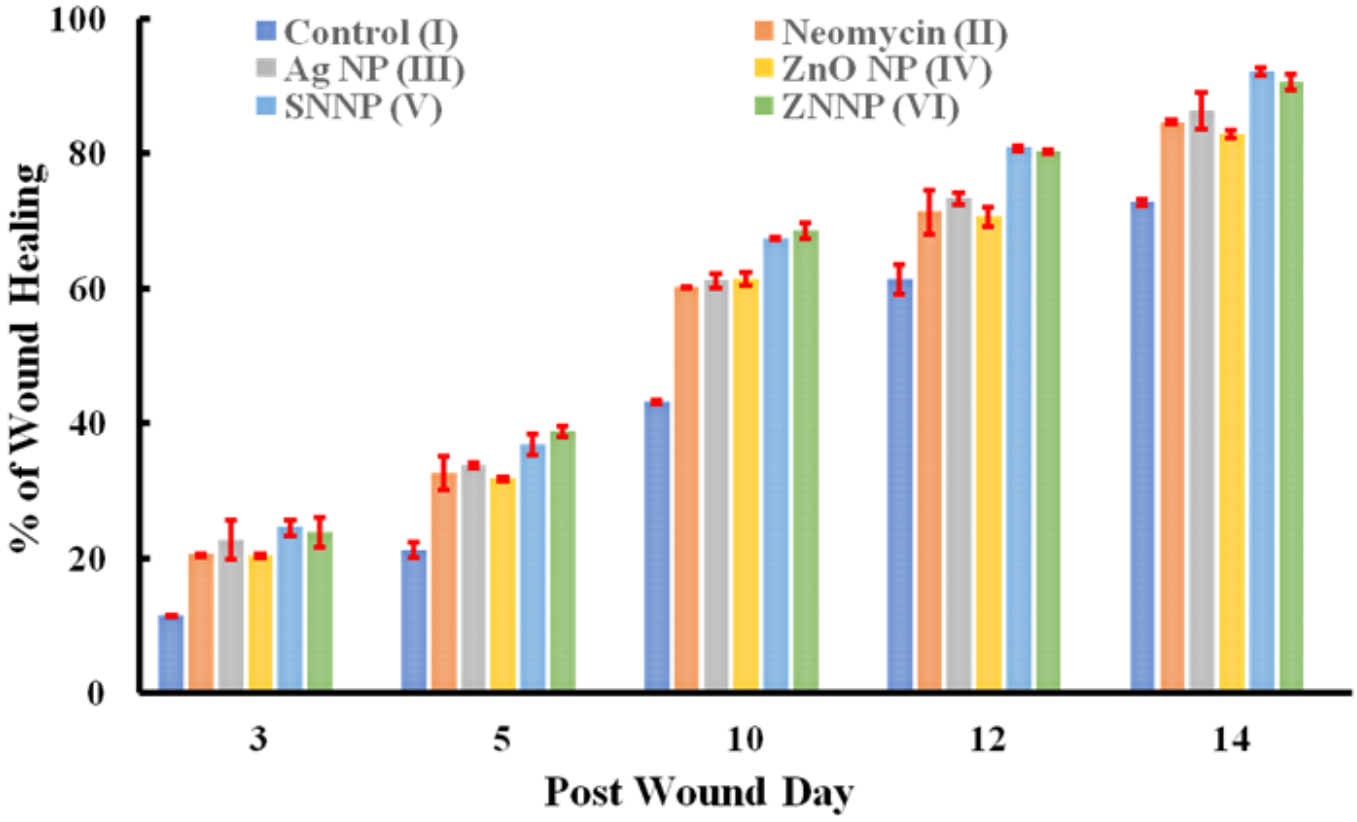

Figure 3

Percentage of Wound healing on treatment with commercial neomycin, nanoparticle (NP) and nanoparticle combination (SNNP, ZNNP) gels. ($P<0.05$)

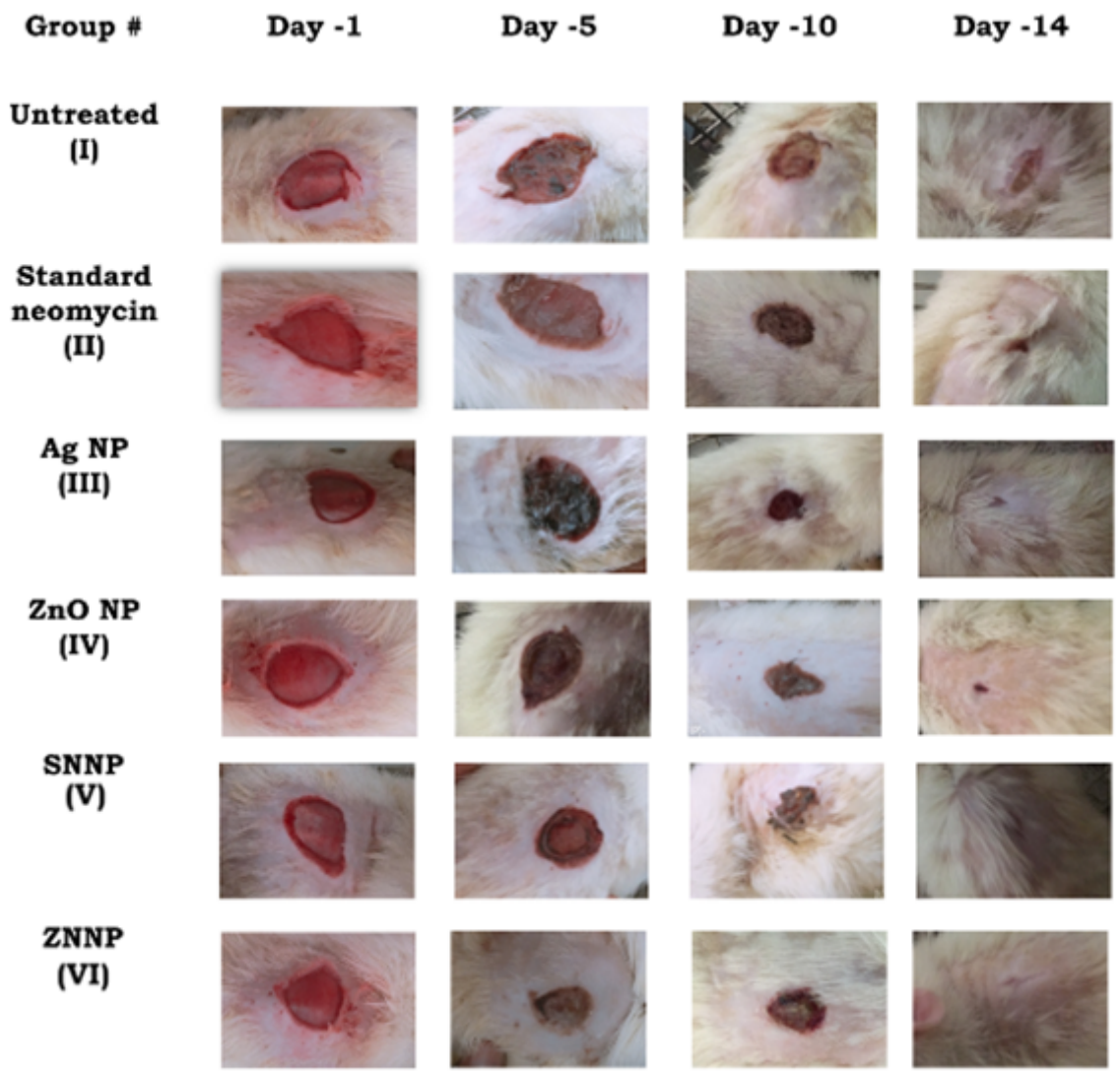

Figure 4

Photographs of wounds on different days with different nanoparticle (Ag NP, ZnO NP, SNNP, ZNNP) gel treatments.

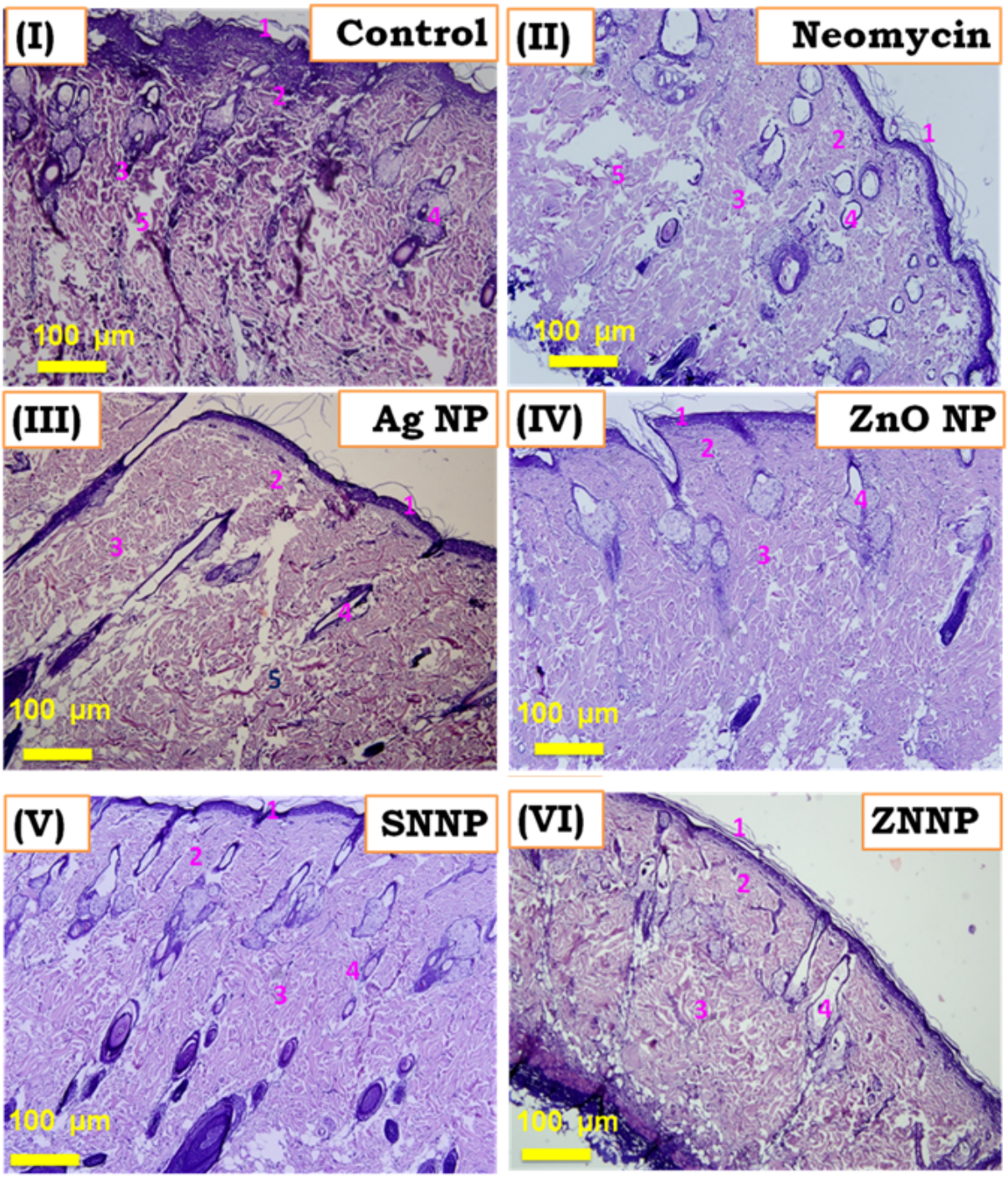

Figure 5

Histopathological images of wounds from animals on 14th day by undergoing treatment with different nanoparticle (Ag NP, ZnO NP, SNNP, ZNNP) gels. Here 1,2,3,4,5 denotes epidermis, dermis, fibroblast, blood vessels and edema respectively.