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Significant *in-Vitro* and *in-Vivo* Antimicrobial and Antibiofilm Activity of Colloidal Silver Nanoparticles (cAgNPs) in Chronic Diabetic Foot Ulcers

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Abstract

Infection is a foremost challenge in the cases of wound care, especially in cases of chronic wounds. The present study was conducted to determine the antimicrobial and antibiofilm activity of the colloidal silver nanoparticles (cAgNPs) on Gram positive organisms and to evaluate the *in-vivo* response of cAgNPs on patients of chronic diabetic foot ulcers (DFUs). cAgNPs were tested against selected Gram-positive organisms like methicillin-sensitive and resistant *Staphylococcus aureus* (MSSA, MRSA), *Enterococcus faecalis* and vancomycin resistant enterococci (VRE) using microbroth dilution assay to estimate minimum inhibitory/bactericidal concentration (MIC/MBC). Biofilm inhibition capacity and time kill assay was performed. Further, the *in-vivo* response of topical application of cAgNPs was evaluated on patients of DFUs. The susceptibility testing demonstrated the MIC and MBC values of the cAgNPs ranging from 0.5μg/ml to 1.0 μg/ml and 1.0 μg/ml to 8 μg/ml against the tested organisms respectively. The cAgNPs showed inhibition of biofilm formation in the low, medium and high biofilm producers by 91%, 83% and 75% respectively at the highest concentration (52ppm). The time kill kinetics showed significant reduction in the number of viable cells ($p < 0.0001$). Significant reduction in microbial load ($p = 0.0062$) and in the number of moderate to strong biofilm producing organisms ($p = 0.0069$) after treatment with cAgNPs was seen. cAgNPs exhibited significant *in-vitro* bactericidal and bacteriostatic activity against MRSA, MSSA and VRE respectively along with anti-biofilm activity. Additionally, cAgNPs showed significant reduction in microbial load of the chronic DFUs.

Keywords

gram-positive, MIC, MBC, time kill kinetics, wound healing

Introduction

Infection in chronic wounds is one of the foremost challenges with respect to wound care and management.[1] Delayed healing is accompanied with exudate and false collagen formation often leading to impaired blood circulation.[2] This further complicates the wound milieu thus preventing drug delivery at the desired site. Additionally, the burden of biofilm producing bacteria in these wounds further complicate drug delivery.[3] In this regard, use of silver (Ag) in wound care in various forms has gained importance.

Silver (Ag), had been previously used as antimicrobials until the mid of 20th century, when it was replaced by antibiotics.[4] Silver and its compounds have been found to be highly toxic to several classes of bacterial species[5,6] including *Staphylococcus aureus*, *Pseudomonas* and *Escherichia coli*.[7–9] Nanostructured materials have displayed better potential in clinical diagnostics because of their large

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Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the colloidal silver nanoparticles against the bacterial strains.

S.No.	Strains	MIC (µg/ml)	MBC (µg/ml)
1.	*Enterococcus faecalis* (ATCC 29212)	0.5	4.0
2.	Vancomycin resistant enterococci (VRE)	0.5	8.0
3.	Methicillin sensitive *Staphylococcus aureus* MSSA (ATCC 25923)	0.5	1.0
4.	Methicillin resistant *Staphylococcus aureus* MRSA (ATCC 33591)	1.0	2.0
5.	Methicillin resistant *Staphylococcus aureus* MRSA (Clinical)	1.0	2.0

Table 2. Percentage Reduction of Biofilm in low, medium and high Biofilm Producers.

Conc Strain	2µg/mL	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL	40 µg/mL	52 µg/mL
Low Biofilm producer	No appreciable reduction			45%	47%	72%	91%
Medium Biofilm producer	24%	28%	38%	40%	47%	71%	83%
High Biofilm producer	43%	45%	46%	47%	44%	62%	75%

surface area, better stability and practical applications.[10] The antibacterial property of the silver nanoparticles (AgNPs) has been attributed to different mechanisms such as its interaction with the thiol group present in the proteins and enzymes leading to the reactive oxygen species (ROS) production which is lethal to cells.[11] AgNPs have also been known to interfere with the process of replication leading to cell death.[12] Colloidal silver produces ROS in Gram negative organisms and to a lesser amount in Gram positive organisms.[13,14] This difference could be attributed to the differences in the thickness, composition and structure of cell wall.

Due to the scenario of increasing bacterial resistance, research on silver and its compounds have recaptured interest. With this background, the overall objective of the study was to screen the colloidal silver nanoparticles (cAgNPs) against selected Gram-positive organisms like methicillin-sensitive and resistant *Staphylococcus aureus*, *Enterococcus faecalis* and vancomycin resistant enterococci (VRE) using microbroth dilution assay and to estimate their minimum inhibitory/bactericidal concentration (MIC/MBC). Additionally, the cAgNPs were tested for biofilm inhibition capacity and time kill kinetics against the tested micro-organisms. The *in-vitro* assay was compounded by studying the *in-vivo* response of this compound on patients of chronic diabetic foot ulcers.

Materials and Methods

Ethical Statement

The study was ethically approved by Institute of medical sciences ethical committee. Informed written consent was obtained from all the patients included in the clinical study.

Colloidal Silver Nanoparticles

The cAgNPs was provided by AB NYLON TECH SDN. BHD (Company Registration no. 1209931H). The stock solution was used to prepare different concentration of the cAgNPs ranging from 0.125 µg/ml to 52 µg/ml in different assays.

Microbes and Media

Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591) and methicillin-sensitive *Staphylococcus aureus* (MSSA, ATCC 25923), MRSA (Clinical isolate), *Enterococcus faecalis* (ATCC 29212) and vancomycin resistant enterococci (VRE, clinical isolate) were included in this study. Clinical isolates included in the study had been characterized using the standard methods[15,16] and had been published elsewhere as a reference. All the clinical isolates were collected from the repository in the Infection control section, Department of Microbiology, [[REDACTED FOR PEER REVIEW]]. The bacterial isolates were grown in blood agar medium for overnight incubation.

Dimethyl sulfoxide, Resazurin dye (Sigma-Aldrich Chemicals Pvt. Ltd, India), Tryptic soy agar (TSA) and Cation adjusted Muller Hinton broth (HiMedia laboratories Pvt. Ltd, India) were used in this study.

Determination of Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of the cAgNPs was evaluated by broth microdilution method using resazurin microtitre assay against all the test organisms.[17] MICs were determined using a concentration range from 32 to 0.125 µg/ml. Suspensions of bacteria at log growth phase were incubated with serial dilution of cAgNPs in 96 well microplate at 37° C for overnight. The plates were also evaluated for visible turbidity prior to addition of resazurin. Standard antibiotic solutions of ciprofloxacin (Stock solution 10mg/mL) were used as control at the same concentration range. Wells containing only sterile media was used as media control to validate the experiment. All experiments were performed in duplicates.

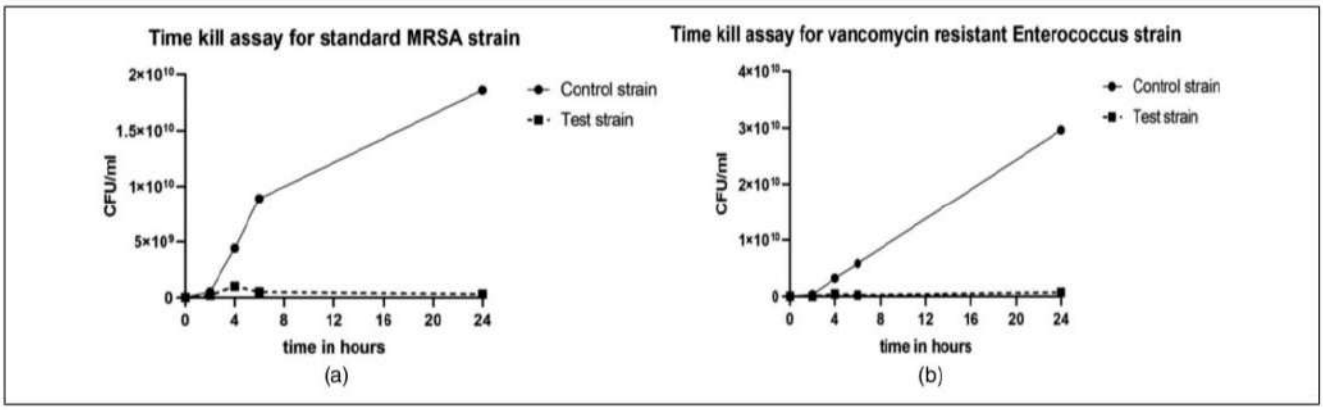

Figure 1. Graphical depiction of the time kill assay of (a) standard MRSA isolate and (b) VRE isolate.

Table 3. The Demographic and Clinical Profile of the Chronic DFU Patients and Wound parameters.

Variables	Initial	Final	p-value
Mean age	56.40 ± 10.05 years		NA
Male: Female	1.5: 1		
Mean duration of ulcers	10.90 ± 6.43 weeks		
Average time of healing	8.90 ± 2.91 weeks		
Mean size of wounds	27.83 cm^2	3.30 cm^2	
Gram negative: Gram positive isolates	2.7: 1	2.2: 1	0.7144
Microbial load (>10^6 cfu/mL)	20	8	0.0062*
High to moderate biofilm producers	27/34 (73.52%)	11/26 (42.3%)	0.0069*

Determination of Microtiter Plate Based minimum Bactericidal Concentration (MBC)

The MBC determination was done using an amended version of the method described in BSAC Guide to Sensitivity Testing.[18] MBC were determined at the end of the incubation period by plating an aliquot of 10 µl from each well demonstrating no visible growth on TSA plates not supplemented with cAgNPs. Resultant colonies were counted after incubation at 37°C for overnight. The MBC endpoint was defined as the lowest concentration of the antimicrobial agent giving at least a 99.9% killing of the initial inoculums where no visible growth of the bacteria was observed.

Evaluation of Antibiofilm Activity

The activity of the cAgNPs on biofilm formation was examined on standardized low, moderate and high biofilm producing strains of *Staphylococcus aureus*.[19] Briefly, the bacterial suspensions were prepared by diluting the overnight cultures to get 1.5×10^8 colony forming units (CFU)/mL. In 96-well microtiter plate, 10 µL aliquot of bacterial suspension, the cAgNPs concentration ranging from 2 µg/mL to 52 µg/mL and freshly prepared Luria Bertani broth media containing 0.25% glucose was taken, maintaining the final volume at 200 µL. The cAgNPs free medium containing bacterial suspension was taken as positive control while wells containing only broth medium served as negative controls. After 48 hours of incubation, each well of the plate was washed thrice with sterile phosphate buffer saline, air dried and stained with 200µL of 0.1% crystal violet. Subsequently incubated at room temperature for 20 min and the remaining excess stain was solubilized with 95% ethanol. The plate was again kept for short 20 min incubation at room temperature. The optical density at 578 nm (OD578) of each well was then measured using micro-titer plate reader (LisaScan® EM Elisa plate reader, Transasia Bio-Medicals Ltd, India).

The antibiofilm activity of the cAgNPs was determined using the formula:

Percentage reduction in biofilm formation = (O.D. of control well – O.D. of test well/ O.D. of control well) × 100

Time-Kill Kinetics Assay

The time-kill kinetics of the cAgNPs was carried out against the MRSA (ATCC 33591) and VRE strains.[20] Briefly, each strain was grown overnight in LB broth medium at 37 °C in shaker incubator with a constant shaking at 150 rpm. Further 1ml aliquots of the cultures in stationary phase were inoculated into Mueller-Hinton media to get an initial optical density of 0.01 at 578 nm (OD578). These cultures were then incubated at 37 °C and 180 rpm agitation. When bacterial growth reached an OD578 of 0.35 ie early log phase (measured through LisaScan® EM Elisa plate reader), the cultures were supplemented with 1 × MIC of cAgNP solution. Cultures were then incubated at 37 °C under constant shaking. The growth was monitored at the time intervals

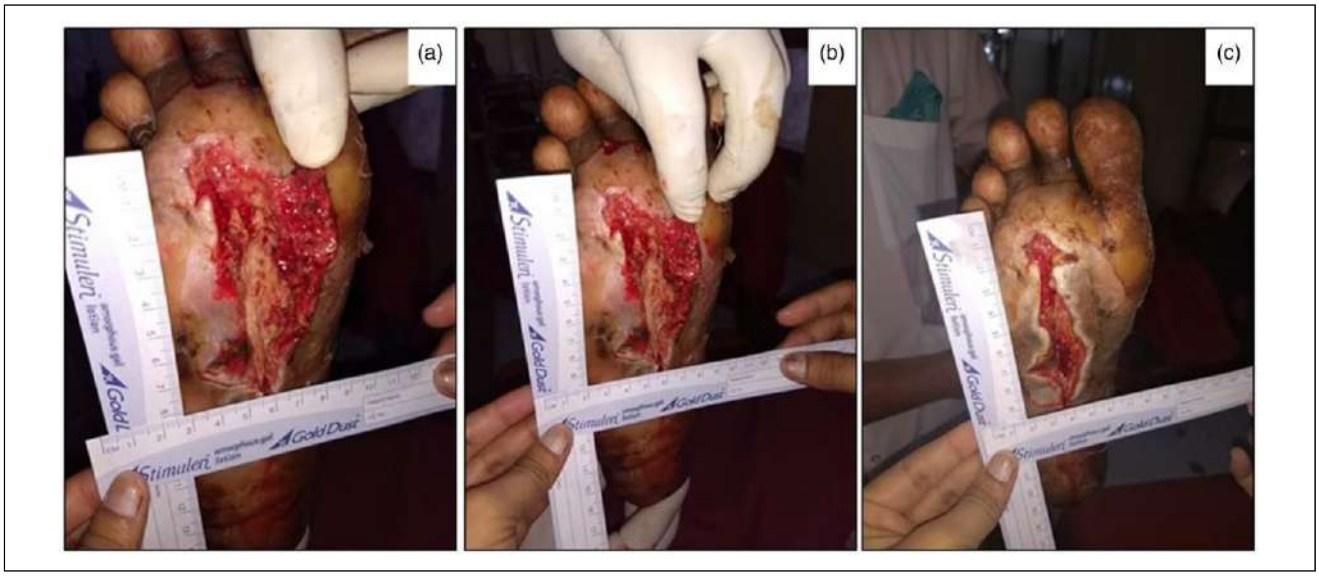

Figure 2. Chronological evolution of the DFU (a) initial appearance (b) after 8 days of treatment (c) after 21 days of treatment with cAgNPs.

Figure 3. Chronological evolution of the DFU (a) initial appearance (b) after 3 weeks of treatment (c) after 8 weeks of treatment with cAgNPs.

of 0, 2, 4, 6 and 24 hours. An aliquot of 100 µL was serially diluted and plated on TSA media for enumeration of CFU. A control test was included for the test organisms without the use of cAgNPs. The experiments were performed in duplicates and a graph of log10 CFU/mL was plotted against time.

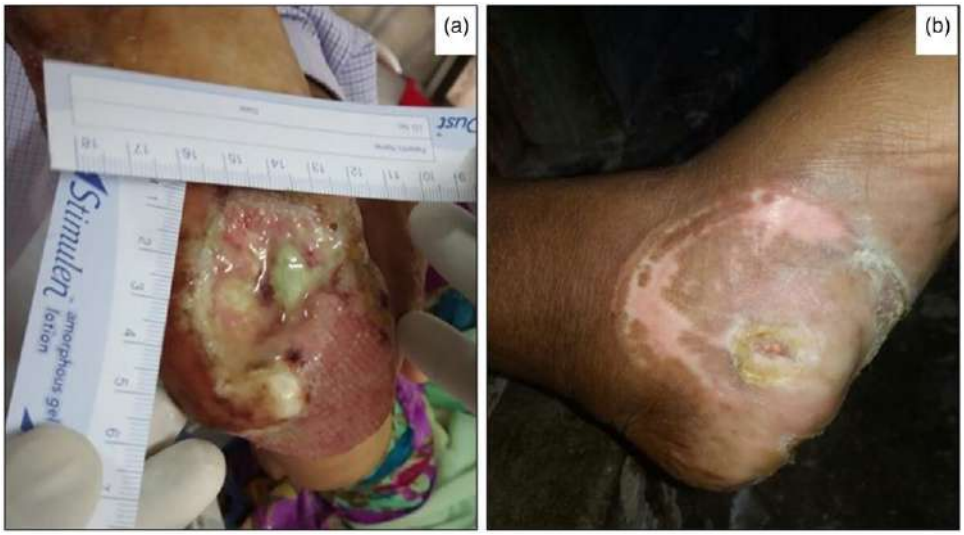

Figure 4. Chronological evolution of the DFU (a) initial appearance (b) after 10 weeks of treatment with cAgNPs.

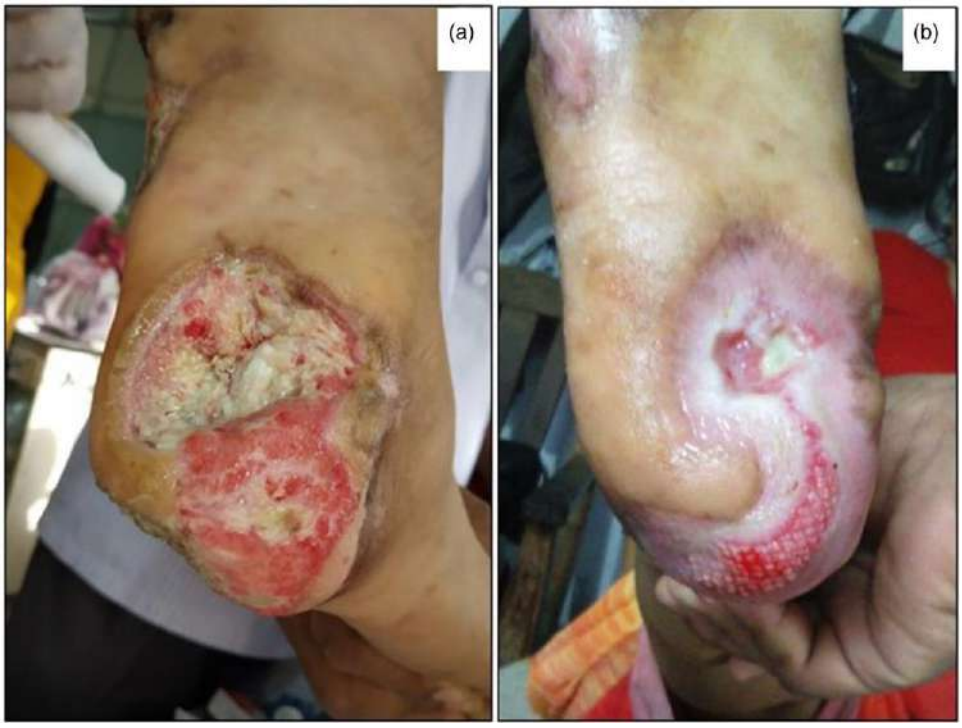

Figure 5. Chronological evolution of the DFU (a) initial appearance (b) after 8 weeks of treatment with cAgNPs.

Clinical Study

This was conducted in the Wound care unit of Department of General Surgery. A total of 20 patients were enrolled for this study based on the selection criteria. The inclusion criteria included patients with chronic infected DFU (≥1 month) without any vascular or other large wounds, aged 18-65 years, attending the wound clinic for wound care and who provided an informed consent to participate in the study. All the DFUs included in the present study were Grade 2 ulcers

based on the Wagner-Meggitt classification of DFU. Regular wound care with dressing and debridement of the callus and necrotic tissue was performed in all the cases. Along with it the use of appropriate footwear providing protection to the foot and sufficiently alleviating the pressure were recommended. All the patients were empirically given broad spectrum antibiotics (third generation cephalosporins) which was modified based on the culture reports of the wound. The definition of infection in the wounds was considered as per standard.[21] Those cases with serious comorbidities, malignancy, pregnant women, osteomyelitis, ulcer involving gangrenous area, systemic sepsis, acquired immunodeficiency syndrome (AIDS), peripheral arterial disease, concurrent venous disease, < 2 visits in the wound clinic were excluded. The standard care provided to the subjects every week included cleaning of wounds with distilled water, generous application of gauze soaked in 24-30 ppm cAgNPs solution followed by closure of the wound by a secondary dressing. Microbial load estimation[22] and biofilm formation[19] of isolated organisms from pus swabs was performed at every change of dressing based on standard method. Response to treatment was measured by change in wound size and microbial burden.

Statistical Analysis

The reduction in colony forming units of bacterial cells in time-kill assay were compared using statistical tool t-test with the help of GraphPad prism version 5 Software (La Jolla California USA). Further, several wound parameters of the studied subjects in the initial phase and after 8 weeks of treatment were compared by Chi-square test.

Table 4. The Bacterial Combinations Detected in the Initial and Final DFUs.

	Bacterial combinations	Initial N (%)	Final N (%)
Monomicrobial	*Escherichia coli*	4 (20)	3 (15)
	Staphylococcus aureus	4 (20)	3 (15)
	Pseudomonas aeruginosa	2 (10)	3 (15)
	Citrobacter sp.	2 (10)	2 (10)
	Klebsiella pneumoniae	0	3 (15)
	Total	12	14
Polymicrobial	*Klebsiella pneumoniae* + *Proteus mirabilis*	2 (10)	0
	Escherichia coli + *Klebsiella pneumoniae* + *Pseudomonas aeruginosa*	1 (5)	0
	Escherichia coli + *Klebsiella pneumoniae* + *Staphylococcus aureus*	1 (5)	2 (10)
	Escherichia coli + *Citrobacter* sp. + *Staphylococcus aureus*	2 (10)	0
	Escherichia coli + *Klebsiella pneumoniae* + *Staphylococcus aureus*	2 (10)	0
	Escherichia coli + *Staphylococcus aureus*	0	3 (15)
	Total	8	5
No growth	-	0	1 (5)

Results

Antimicrobial Activity

The susceptibility testing demonstrated the MIC values and MBC values of the cAgNPs ranging from 0.5µg/mL to 1.0 µg/mL and 1.0 µg/mL to 8 µg/mL against the different test organisms respectively. MBC/MIC ratio of the cAgNPs against the *Enterococcus faecalis* (ATCC 29212) and VRE was 8 and 16 respectively. In the case of MSSA (ATCC 25923), MRSA (ATCC 33591) and MRSA (Clinical) the MBC/MIC ratio was found to be 2 for each. The complete data on MIC and MBC values has been shown in Table 1.

Antibiofilm Activity

The cAgNPs showed inhibition of biofilm formation in the low biofilm producers and medium biofilm producers by 91% and 83% respectively at the highest concentration of 52µg/mL. Whereas in high biofilm producers, biofilm formation was inhibited by 75% at the highest concentration. The percentage reduction of biofilm in low, medium and high biofilm producers at different concentration ranges has been shown in Table 2.

Time-Kill Kinetics

The time kill kinetics of cAgNPs obtained against the MRSA (ATCC 33591) and VRE strains showed reduction in the number of viable cells within 4-6 hours of exposure as compared to the control (organisms without cAgNPs). The reduction in colony forming units of MRSA and VRE (t-test; $P < 0.0001$) were significant in presence of cAgNPs. The time kill activity against MRSA (ATCC 33591) and VRE has been graphically shown in Figure 1.

Effect on Wound Parameters

Among the 20 cases with infected DFU, mean age was 56.4 ± 10.056 years and mean duration of DFU was 10.9 ± 6.433 weeks. As compared to the pre and post treatment (8weeks) with cAgNPs, there was significant reduction in microbial load and in the number of biofilms producing organisms ($p < 0.05$). The wound parameters have been shown in Table 3. Figures 2–5 show the images of changes in parameters of wounds in few representative cases following treatment. The different bacterial combinations detected in the initial and final DFUs has been included in Table 4.

The number of cases with polymicrobial growth reduced at final assessment by 15% (3 cases). The statistical comparison between monomicrobial and polymicrobial bacterial flora in the initial and final DFU was not found to be significant (Odd's Ratio: 1.86, 95% Confidence interval: 0.48-7.25, p-value: 0.367)

Discussion

Research into silver and its compound especially antimicrobial activity has attracted attention lately. The present study reported significant antimicrobial activity of the cAgNPs against the Gram-positive micro-organisms as well as on chronic infected DFUs. To decrease the variability in wound aetiology, we limited our inclusion criteria to those with only DFU without any vascular or other large wounds. Our study is in concordance with the previous literature which demonstrates significant antimicrobial activity of the AgNPs prepared using different approaches such as sol-gel method, photo assisted reduction of silver ions and chemical reduction method.[23–26] However, the MIC and MBC values obtained in the present study with the tested product was much lower than in these previous studies. Also, *Enterococcus faecalis* and VRE showed higher MBC values as compared to the *Staphylococcus aureus* strains. *Enterococcus faecalis* has been known for its better tolerance to the antimicrobial agents. The exact phenomenon behind such an acceptance is not known, however superoxide dismutase (SOD) activity in *Enterococci* has been found to be linked with higher tolerance against the antimicrobial agents.[27]

The antimicrobial agents are classified as bactericidal if MBC/MIC ratio is ≤ 4 and bacteriostatic if MBC/MIC is > 4.[28] The ratios obtained for the test organisms in the present study reports that the cAgNPs were bactericidal for the MRSA (ATCC 33591), MSSA (ATCC 25923) and MRSA (Clinical isolate). However, with the MBC/MIC ratio > 4, the cAgNPs were bacteriostatic for *Enterococcus faecalis* (ATCC 29212) and Vancomycin resistant *Enterococcus* (VRE). The bacteriostatic agents only inhibit the multiplication of the pathogens and require host immunity for complete elimination of the pathogenic micro-organisms.[29]

Biofilms provide protection to the pathogenic micro-organisms against the adverse environment conditions and the antimicrobial agents.[30] They can be an initial source of the disease outbreaks associated with the medical devices.[12] This study showed that anti-biofilm activity of the cAgNPs against the biofilm producers which is in concordance with the previous study which demonstrates that the AgNPs can effectively prevent formation of biofilms as well as inhibit bacteria in established biofilms.[30] Biofilms increase the tolerance of the pathogenic micro-organisms against the antimicrobial agents making it hard to control the infection. However, the present study demonstrates that the cAgNPs are lethal to the micro-organisms associated with the biofilm. The mechanism responsible for the antibiofilm activity of the cAgNPs can be the interference or inhibition of the exopolysaccharides caused by silver ions.[30] In the present study, low biofilm producers showed no appreciable reduction at low concentration of the cAgNPs, which can be due to negative impact of some other virulence factors on cAgNPs contributing in the survival of the organism.[31]

There are a limited number of recent reports on the time kill kinetics of the cAgNPs against the MRSA and VRE. A recent study showed that silver nanoparticle was able to produce bactericidal effect against MRSA and VRE at 24 hours at 2 × MIC of cAgNPs.[14] However, in the present study the bactericidal effect was obtained at a lower concentration and within less timespan. These differences can be due to the better activity of the cAgNPs used in the present study or due to the variance in the strains included in the study.

The present study reported that the cAgNPs have potential activity on the healing of the chronic DFUs. Our findings are in-line with recent studies that demonstrate that AgNPs can cause rapid wound healing in the case of DFUs.[32,33] The AgNPs encourage wound healing by proliferation and migration of the keratinocytes and they also promote the fibroblast differentiation to myofibroblasts. The AgNPs have been reported to induce differentiation of cell lineages and decrease of edema resulting in re-epithelialisation of wounds and faster closure. Though the diversity of the bacterial flora in the DFUs in terms of the ratio of Gram negative to Gram positive isolates remained unchanged, the number of cases with polymicro-bial flora reduced at the final assessment of the DFU.

The study was not without the limitation as a small population size was available for the clinical study and the detection of anaerobes could not be done due to the unavailability of resources and facilities. However, this study provides valuable information which can form a base-line data for further research in the field of silver nanomedicine. Further, the study of wound healing in the murine models to evaluate the cellular and immunological mechanisms involved during the healing of DFUs through cAgNPs could be done to provide a clear perspective.

Conclusion

This study provides evidence cAgNPs exhibit significant *in-vitro* bactericidal and bacteriostatic activity against MRSA, MSSA and VRE respectively. Further, this study also shows that these cAgNPs possess anti-biofilm activity against the low, medium as well as high biofilm producers. In patients with chronic DFU significant reduction in microbial load and moderate to high biofilm producers was seen

after application of cAgNPs. Development and use of such compounds should be done in order to meet the problem of developing drug resistance.

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Declaration of Conflicting Interests

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Ethical Approval

The study was approved by the Institute ethical committee.

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References

1. Negut I, Grumezescu V, Grumezescu AM. Treatment strategies for infected wounds. *Molecules*. 2018;23(9):2392. doi:10.3390/molecules23092392
2. Hess CT. Checklist for factors affecting wound healing. *Adv Skin Wound Care.* 2011;24(4):192. doi:10.1097/01.ASW.0000396300.04173.ec
3. Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. *Virulence*. 2018;9(1):522-554. doi:10.1080/21505594.2017.1313372
4. Sim W, Barnard RT, Blaskovich MA, Ziora ZM. Antimicrobial silver in medicinal and consumer applications: a patent review of the past decade (2007–2017). *Antibiotics*. 2018;7(4):93. doi:10.3390/antibiotics7040093
5. Slawson RM, Trevors JT, Lee H. Silver accumulation and resistance in Pseudomonas stutzeri. *Arch Microbiol.* 1992;158(6):398-404. doi:10.1007/BF00276299
6. Zhao G, Stevens SE. Multiple parameters for the comprehensive evaluation of the susceptibility of Escherichia coli to the silver ion. *Biometals*. 1998;11(1):27-32. doi:10.1023/A:1009253223055
7. Sacco P, Travan A, Borgogna M, Paoletti S, Marsich E. Silver-containing antimicrobial membrane based on chitosan-TPP hydrogel for the treatment of wounds. *J Mater Sci: Mater Med.* 2015;26(3):128. doi:10.1007/s10856-015-5474-7
8. Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. *J Colloid Interface Sci.* 2004;275-(1):177-182. doi:10.1016/j.jcis.2004.02.012
9. Ramalingam B, Parandhaman T, Das SK. Antibacterial effects of biosynthesized silver nanoparticles on surface ultrastructure and nanomechanical properties of gram-negative bacteria viz. Escherichia coli and Pseudomonas aeruginosa. *ACS Appl Mater Interfaces.* 2016;8(7):4963-4976. doi:10.1021/acsami.6b00161
10. Wang L, Hu C, Shao L. The antimicrobial activity of nanoparticles: present situation and prospects for the future. *Int J Nanomed.* 2017;2017(12):1227. doi:10.2147/IJN.S121956
11. Gordon O, Slenters TV, Brunetto PS, et al. Silver coordination polymers for prevention of implant infection: thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. *Antimicrob Agents Chemother*. 2010;54(10):4208-4218. doi:10.1128/AAC.01830-09
12. Prabhu S, Poulose EK. Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. *Int Nano Lett.* 2012;2(1):1. doi:10.1186/2228-5326-2-32
13. Zou L, Wang J, Gao Y, et al. Synergistic antibacterial activity of silver with antibiotics correlating with the upregulation of the ROS production. *Sci. Rep*. 2018;8(1):1-1.29311619
14. Domínguez AV, Algaba RA, Canturri AM, Villodres ÁR, Smani Y. Antibacterial activity of colloidal silver against gram-negative and gram-positive bacteria. *Antibiotics*. 2020;9(1):36. doi:10.3390/antibiotics9010036
15. Ross P W. *Streptococcus and Enterococcus*. In: Collee JG, Fraser AG, Marmion BP, Simmons A, eds. *Mackie & McCartney Practical Medical Microbiology*. 14th. Churchill Livingstone; 1996:263-274.
16. Crichton PB. Enterobacteriaceae: Escherichia, Klebsiella, Proteus and other genera. *Mackie and McCartney Practical Medical Microbiology*. 1996:361-364.
17. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods*. 2007;42(4):321-324. doi:10.1016/j.ymeth.2007.01.006
18. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother*. 2001; 48(suppl_1):5-16. doi:10.1093/jac/48.suppl_1.5
19. Sharma S, Das A, Banerjee T, Barman H, Yadav G, Kumar A. Adaptations of carbapenem resistant Acinetobacter baumannii (CRAB) in the hospital environment causing sustained outbreak. *J Med Microbiol.* 2021;70(3):001345. doi:10.1099/jmm.0.001345
20. Kara EM, Yılmaz M, Çelik BÖ. In vitro activities of ceftazidime/avibactam alone or in combination with antibiotics against multidrug-resistant Acinetobacter baumannii isolates. *J Glob Antimicrob Resist.* 2019;17:137-141. doi:10.1016/j.jgar.2018.12.004
21. Mavrogenis AF, Megaloikonomos PD, Antoniadou T, et al. Current concepts for the evaluation and management of diabetic foot ulcers. *EFORT Open Reviews*. 2018;3(9):513-525. doi:10.1302/2058-5241.3.180010
22. Banerjee T, Das A, Singh A, Bansal R, Basu S. The microflora of chronic diabetic foot ulcers based on culture and molecular examination: a descriptive study. *Wound Management & Prevention*. 2019;65(5):16-23. doi:10.25270/wmp.2019.5.1623

23. Ansari MA, Khan HM, Khan AA, et al. Antibacterial activity of silver nanoparticles dispersion against MSSA and MRSA isolated from wounds in a tertiary care hospital of north India. *Int J Appl Biol Pharm*. 2011;2(4):34-42.
24. Lkhagvajav N, Yasa I, Celik E, Koizhaiganova M, Sari O. Antimicrobial activity of colloidal silver nanoparticles prepared by sol-gel method. *Dig J Nanomater Biostructures*. 2011;6(1):149-154.
25. Jain J, Arora S, Rajwade JM, Omray P, Khandelwal S, Paknikar KM. Silver nanoparticles in therapeutics: development of an antimicrobial gel formulation for topical use. *Mol Pharm.* 2009;6(5):1388-1401. doi:10.1021/mp900056g
26. Samadi N, Hosseini SV, Fazeli A, Fazeli MR. Synthesis and antimicrobial effects of silver nanoparticles produced by chemical reduction method. *DARU J Pharm Sci*. 2010;18(3):168.
27. Ladjouzi R, Bizzini A, Lebreton F, et al. Analysis of the tolerance of pathogenic enterococci and Staphylococcus aureus to cell wall active antibiotics. *J Antimicrob Chemother.* 2013;68-(9):2083-2091. doi:10.1093/jac/dkt157
28. Keepers TR, Gomez M, Celeri C, Nichols WW, Krause KM. Bactericidal activity, absence of serum effect, and time-kill kinetics of ceftazidime-avibactam against β-lactamase-producing Enterobacteriaceae and Pseudomonas aeruginosa. *Antimicrob Agents Chemother.* 2014;58(9):5297-5305. doi:10.1128/AAC.02894-14
29. Pankey GA, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis.* 2004;38-(6):864-870. doi:10.1086/381972
30. Martinez-Gutierrez F, Boegli L, Agostinho A, et al. Anti-biofilm activity of silver nanoparticles against different microorganisms. *Biofouling*. 2013;29(6):651-660. doi:10.1080/08927014.2013.794225
31. Oogai Y, Matsuo M, Hashimoto M, Kato F, Sugai M, Komatsuzawa H. Expression of virulence factors by Staphylococcus aureus grown in serum. *Appl Environ Microbiol.* 2011; 77(22):8097-8105. doi:10.1128/AEM.05316-11
32. Almonaci Hernández CA, Juarez-Moreno K, Castañeda-Juarez ME, Almanza-Reyes H, Pestryakov A, Bogdanchikova N. Silver nanoparticles for the rapid healing of diabetic foot ulcers. *Int. J. Med. Nano Res*. 2017;4-(01910.23937):2378-3664.
33. Thirumurugan G, Veni VS, Ramachandran S, Seshagiri Rao JV, Dhanaraju MD. Superior wound healing effect of topically delivered silver nanoparticle formulation using eco-friendly potato plant pathogenic fungus: synthesis and characterization. *J Biomed Nanotechnol.* 2011;7(5):659-666. doi:10.1166/jbn.2011.1336

Managing infection in chronic wounds with nano colloidal silver

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Wound infections present a significant clinical challenge that impacts on patient morbidity and treatment cost. This case study evaluated patients receiving standard wound management coupled with a nano colloidal silver (NCS) spray of 30 ppm. The evaluation included 17 patients (12 male, 5 female) aged between 27 to 77 years who presented with chronic non-healing wounds and treated using NCS. The patients were assessed using the hospital's standard protocol and received a minimum of two NCS applications per week, which was sprayed directly onto the wound prior to a secondary dressing application. This was continued until the symptoms of infection had cleared fully and wound healing was observed. The criteria used for wound healing was based on clinical observations which included reduction in signs of infection, such as inflammation, redness, exudate, odour, suspected biofilm and pain, and the observation of proper epithelialization the wound. The majority of non-healing wounds were venous ulcers in origin and the average wound healing time was around 5 weeks. Swabs were not taken for this case study but will be needed for a more robust study.

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Wound infections present a significant clinical challenge, impacting on patient morbidity and treatment cost (Wilkinson et al, 2011). A chronic wound is a wound that does not heal in an orderly set of stages and in a predictable amount of time. Wounds that do not heal within three months can also be considered chronic (Iqbal et al, 2017). These commonly include diabetic foot ulcers (DFUs), venous ulcers (VUs) and other non-healing wounds, such as infected wounds, traumatic wounds and others. Management of these chronic non-healing wounds poses a great challenge because they are associated with underlying disorders and are prone to complications, including secondary infections and risk of amputation. Any wound that does not heal within a few weeks should be examined by a healthcare professional because it might be infected, or might reflect an underlying disease (Iqbal et al, 2017).

Recently, NCS received considerable interest for treating chronic non-healing wounds where it has shown its effectiveness by reducing bioburden and inflammation (Wilkinson et al, 2011). This may be due to the ability of NCS to release bigger Ag+ ions at a greater rate than any other silver-based treatments due to the form and size of the nanoparticles (Wang et al, 2017). The Ag+ ion is an active antimicrobial entity as it is capable to interfere with thiol (–SH) groups and provokes the generation of reactive oxygen species (ROS) — a major contributor to its antibacterial efficacy (Wilkinson et al, 2011). NCS can release Ag+ ions at a greater rate than bulk silver by virtue of their large surface area, so if released via a dressing, these also have the potential to cross biological compartments. This review aims to consolidate recent findings with regards to the efficacy and safety of different formulations of silver used as an antiseptic agent in dressings, summarising the features

Table 1. Wound type and time to reduction in the bacterial bioburden (weeks)

No	Wound Type	Age	Gender	Initial size (cm^2)	Final size (cm^2)	Slough (initial) present	Slough (final) present	Biofilm (initial) present	Biofilm (final) present	No of weeks
1	VU	27	M	Multiple	N/A	No	No	Yes	No	7
2	VU	39	M	Multiple	Fully closed	No	No	Yes	No	9
3	VU	43	F	13.5	1.25	No	No	Yes	No	5
4	VU	63	F	37.5	12.5	No	No	Yes	Yes	6
5	VU	63	F	2.25	Fully closed	Yes	Yes	Yes	Yes	1
6	VU	36	M	36.0	26	No	No	Yes	No	7
7	VU	64	M	18.0	16.5	No	No	Yes	No	3
8	VU	51	F	10.0	6	No	No	Yes	No	3
9	VU	69	M	2.25	Fully closed	No	No	Yes	No	5
10	VU	37	M	4.0	3.28	No	No	Yes	No	5
11	DFU	65	M	11.25	10.5	No	No	Yes	No	3
12	DFU	59	M	42.9	22.65	No	No	Yes	No	7
13	DFU	67	M	5.0	1.0	Yes	No	Yes	No	7
14	DFU	65	M	35.0	13.0	No	No	Yes	No	5
15	TW	77	M	22.5	19.5	No	No	Yes	No	4
16	TW	77	M	25.5	241	No	No	Yes	No	7
17	IW	52	F	8.75	8.25	No	No	Yes	No	6

of NCS, with particular attention to the dose-dependencies for biological effects, highlighting the need for information on their uptake and potential biological effects (Wilkinson et al, 2011).

In the past, silver has been employed as an anti-microbial agent to prevent the transmission of bacteria. Medical applications are documented in the literature throughout the 17th and 18th centuries. The bactericidal activity of silver is well established. Silver nitrate was used topically throughout the 1800s for the treatment of burns, ulcerations, and infected wounds, and although its use declined after World War II (Roberts et al, 2017)and the advent of antibiotics, Fox revitalized its use in the form of silver sulfadiazine in 1968 (Politano et al, 2013).

This article reports on wound management using NCS as primary treatment in a Wound Care Clinic at the Department of Internal Medicine, Hospital Kuala Lumpur.

Material and methods

This clinical study was conducted at the Wound Care Unit, Department of Internal Medicine, Kuala Lumpur Hospital. The following materials and methods were used during the treatment:

- The wounds were cleansed with distilled water only
- NCS (30 ppm) was applied generously in two layers from a spraying distance of 10 cm
- After spraying the wound bed, the wound was closed with a secondary dressing (such as melolin, or polyurethane foam)
- For patients with VUs additional compression system bandages were used.

Study participants

A total of 17 outpatients (12 male and 5 female) between the age of 27 and 77 years with non-healing wounds were included for this observational case report. These patients had different types of chronic wounds, which included 2 Trauma Wounds (TWs), 1 Infected Wound (IW), 4 DFUs and 10 VUs.

Results

The patient distributions based on wound type and the results are displayed in *Table 1*. Out of the 17 patients, 10 (59%) had VUs with wound sizes ranging from 2.25 cm^2 to 25 cm^2, 4 (23%) had DFUs with wound size ranging from 5 cm^2 to 42.9 cm^2 and 3 (18%) had other non-healing wounds with wound size ranging from 8.75 cm^2 to 25.5 cm^2.

Out of the ten patients with VUs, 9 (90%) patients had exudating wounds with suspected biofilm, slough, heavy odour with signs of maceration *[Table 2]*. The average time to completely clear the infection in patients with VUs was observed to be around

5 weeks. Meanwhile, there were 4 (100%) patients with DFUs and 2 (75%) patients with TWs, all their wounds were suspected of have biofilm.

The infection signs in the DFUs and other non-healing wounds subsided in an average of 5 weeks. A limitation is that the swabs were not taken as this is a pilot study observing the usage of the new NCS.

The longest suspected infection eradication time, up to 9 weeks, was observed in patients with VUs. Out of all the patients, 3 experienced full wound closure.

Discussion

All of the 17 patients underwent a wound assessment, using the hospital's standard protocol, where trained wound care nurses look at the tissue visually for slough and suspected biofilm. On assessment, there were signs of infection and inflammation such as redness, suspected biofilm, slough and increased exudates in those patients with macerated wounds. The clinicians used NCS spray to clear infections. The products contained in the spray are active against a broad range of bacteria, fungi and viruses, including antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE). Furthermore, there may be significant topical anti-inflammatory and analgesic effects that encourages blood vessel formation (neovascularization).

NCS has been shown to be active against bacteria because of its ability to be absorbed into the wound environment and the bacteria wall due to its nano form and size (Tian et al, 2007).

All the 17 patients showed good improvement in terms of wound size and reduction of signs of infection without any allergic reactions. It has been noted that all 17 cases had a 30% improvement. Depending on the wound size, the treatment was continued for around 4 to 5 weeks until the signs of infection was completely cleared. However, in patients with large wounds it took 7 to 8 weeks. NCS application was stopped once the infection symptoms had been cleared while dressings, such as foam dressings, continued to be applied until the full closure of the wounds.

Figures 1 to 7 show pictures of wounds of seven patients taken before NCS was applied and at the time the infection had fully cleared.

It appears that treating chronic wounds has become more of a challenge recently. Electron microscopy of biopsies from chronic wounds found that 60% of the specimens contained biofilm structures in comparison with only 6% of biopsies from acute wounds (James et al, 2008). These wounds are mainly those

Table 2. Wound type and infection signs

No	Type	Age	Gender	Biofilms suspected	Maceration	Exudate	Swelling	Pain	Odour
1	VU	27	M	√	√	√	0	0	√
2	VU	39	M	√	√	√	0	0	√
3	VU	43	F	√	√	√	√	√	√
4	VU	63	F	√	√	√	0	0	√
5	VU	63	F	√	√	√	√	√	√
6	VU	36	M	√	√	√	0	0	√
7	VU	64	M	√	√	√	0	0	√
8	VU	51	F	√	√	√	√	√	√
9	VU	69	M	√	√	0	0	0	√
10	VU	37	M	√	√	√	0	0	√
11	DFU	65	M	√	√	√	0	0	√
12	DFU	59	M	√	√	√	√	√	√
13	DFU	67	M	√	√	√	0	0	√
14	DFU	65	M	√	√	√	0	0	√
15	TW	77	M	√	√	√	√	√	√
16	TW	77	M	√	√	√	√	√	√
17	IW	52	F	0	√	0	√	√	0

Figures 1 to 7. Images of wounds taken before starting NCS application and at the time the infection had fully cleared.

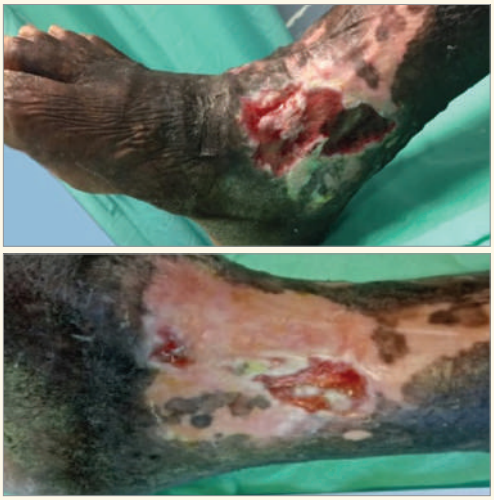

Figure 1. A 59-year-old male with a DFU improved within 7 weeks

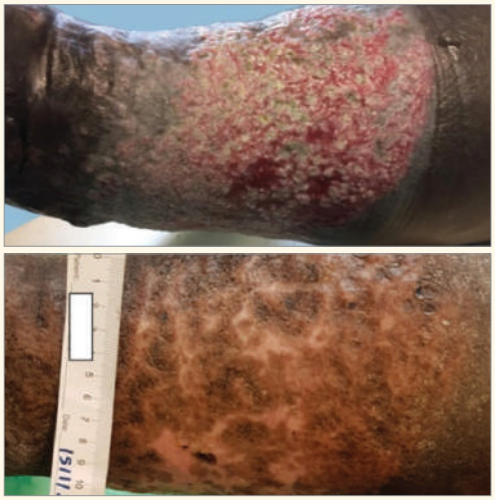

Figure 2. A 39-year-old male with a VU completely healed within 9 weeks

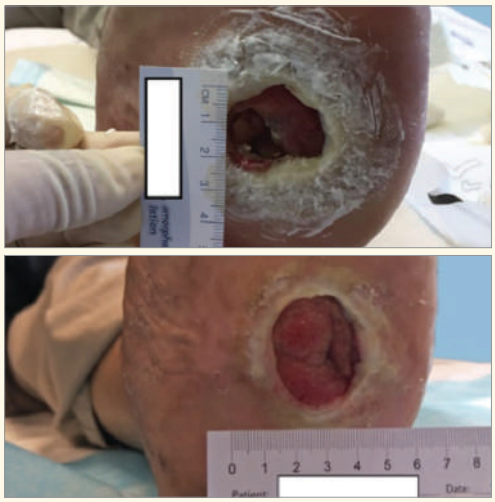

Figure 3. A 67-year-old male with a DFU improved within 7 weeks

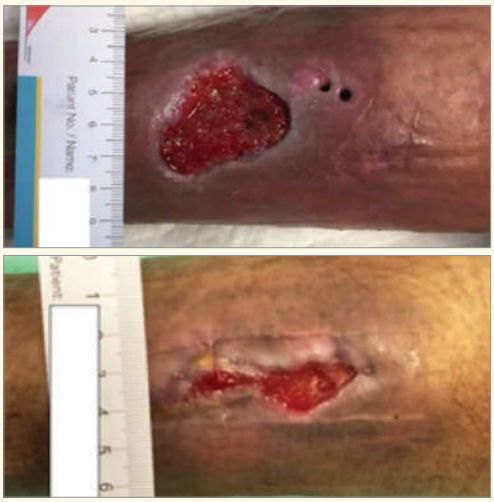

Figure 4. A 77-year-old male with a TW improved within 4 weeks

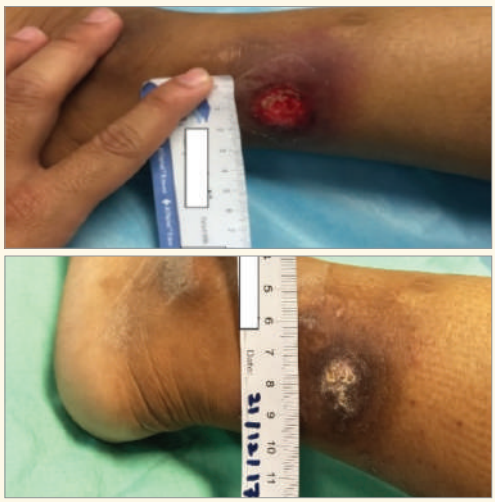

Figure 5. A 52-year-old female with a IW, which closed fully after 6 weeks

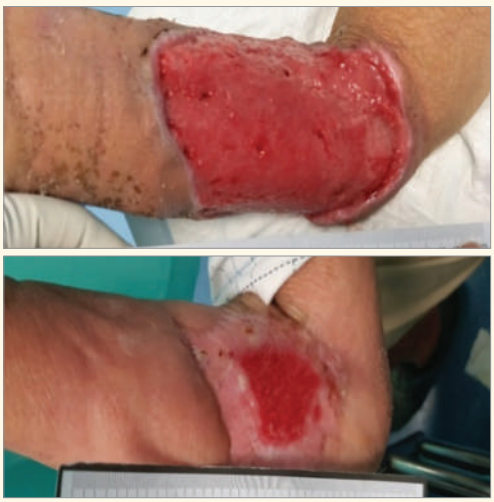

Figure 6. A 77-year-old male with a TW, which showed improvements within 7 weeks

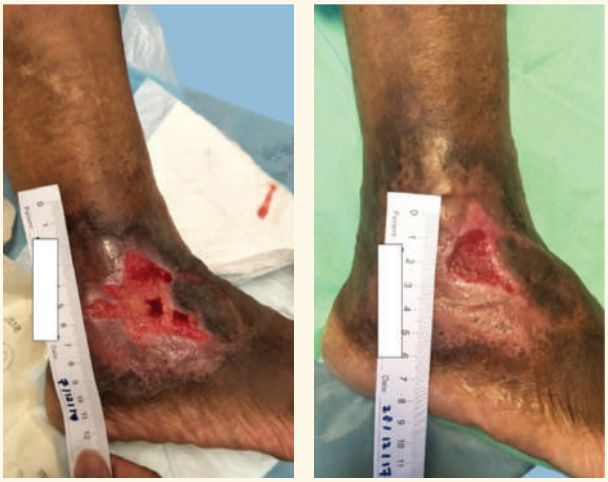

Figure 7. A 51-year-old female with a VU, which improved within 3 weeks

associated with antibiotic-resistant bacteria which form biofilms that can resist the action of antibiotics and the human immune system (Phillips et al, 2010). It is known that both Gram-negative and Gram-positive bacteria can form biofilms, while the most common biofilm-forming bacteria associated with human disease are *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Markowska et al, 2013).

Nevertheless, it has been known that biofilms are not resistant to nano silver particles (Richter et al, 2017) and the author observed in this clinical evaluation that NSC had been effective in reducing infection and managing the bio-burden, even though none of these patients were on any antibiotics.

Conclusion

In summary, it is safe to mention that NCS exerted a positive effect resulting in faster healing time and reduction in the bacterial bioburden, which has been proven to be an excellent option to achieve accelerated wound healing in managing wounds at primary health care centers. WAS

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References

Iqbal A, Jan A, Wajid MA1, Tariq S (2017) Management of chronic non-healing wounds by hirudotherapy. *World J Plast Surg* 6(1): 9–17 James GA, Swogger E, Wolcott R et al (2008) Biofilms in chronic wounds. *Wound Repair Regen* 16(1): 37-44

Markowska K, Grudniak Anna M, Wolska KI (2013) *Silver Nanoparticles as An Alternative Strategy Against Bacterial Biofilms*. Available at: https://pdfs.semanticscholar.org/2538/047689879aa67f02c2ea0b5d28a8feac596f.pdf (accessed 20 June 2019)

Phillips PL, Wolcott RD, Fletcher J, Schultz GS (2010) Biofilms Made Easy. *Wounds International* 1(3): 1–6

Politano A, Campbell KT, Rosenberger LH et al (2013) Use of silver in the prevention and treatment of infections: silver review. *Surg Infect (Larchmt)* 14(1): 8–20

Richter K, Facal T, Vandecandelaere I et al (2017) Taking the silver bullet colloidal silver particles for the topical treatment of biofilm-related infections. *ACS Appl Mater Interfaces* 9(26): 21631–8

Roberts CD, Leaper DJ, Assadian O (2017) The role of topical antiseptic agents within antimicrobial stewardship strategies for prevention and treatment of surgical site and chronic open wound infection. *Adv Wound Care (New Rochelle)* 6(2): 63–71

Wang L, Hu C, Shao (2017) The antimicrobial activity of nanoparticles: present situation and prospects for the future. *Int J Nanomedicine* 12: 1227–49

Wilkinson LJ, White RJ, Chipman JK (2011) Silver and nanoparticles of silver in wound dressings: a review of efficacy and safety. *J Wound Care* 20(11): 543–9