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ORIGINAL ARTICLE

Efficacy of Hyaluronic Acid-capped Silver Nanoparticles Against the Top Five Clinical Bacterial Isolates from Open Fracture Wounds

Hansel Gould B. Cocjin, MD, FPOA,[1] Shaun A. Porras, MD, FPOA,[1] John Paolo J. Sanchez, MD, FPOA,[1] Rene P. Magalona, MD,[1] Ma. Ella Muriel F. Valdevieso, MD, DPBO,[1] Alain C. Juayang, RMT, PhD,[2] David S. Hinolan, MSc, RPh,[3] Hazel Joan T. Balomit, RN[4]

[1]*Department of Orthopaedics and Traumatology, Corazon Locsin Montelibano Memorial Regional Hospital, Bacolod City, Negros Occidental, Philippines*
[2]*Department of Pathology and Molecular Laboratory, Corazon Locsin Montelibano Memorial Regional Hospital, Bacolod City, Negros Occidental, Philippines*
[3]*Pharmacy Program, Colegio San Agustin-Bacolod, Bacolod City, Philippines*
[4]*HeaRTnovation Hub, Corazon Locsin Montelibano Memorial Regional Hospital, Bacolod City, Negros Occidental, Philippines*

ABSTRACT

Introduction. Orthopaedic infections from open fracture wounds remain a significant clinical problem. While the use of silver nanoparticles has become popular because of promising antibacterial properties, clinical application is limited due to unstable particle size during synthesis and storage. Hyaluronic acid, a sugar molecule used by the body for tissue repair, can potentially stabilize silver nanoparticles, but this capability has not yet been proven.

Methodology. The silver nanoparticle (AgNP) was synthesized through a redox reaction using hydrogen peroxide. The hyaluronic acid (HA) was used to modify the surface of the silver nanoparticles by acting as a capping agent. The antibacterial properties were tested against the top five clinical bacterial isolates from Orthopaedic wounds reported by the institution's Infection Control Committee using the MTT[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay.

Results. The hyaluronic acid-capped (HA-AgNP) and uncapped (AgNP) silver nanoparticles exhibited antibacterial properties, and the capped silver nanoparticle exhibited stability. The HA-AgNP were observed to have dose-dependent antibacterial activity. Specifically, at 100 mcg/mL, HA-AgNP exterminated 60% of the clinical isolates including *S. aureus*, *P. aeruginosa*, *K. pneumonae*, *E. coli*, and MRSA with half-maximal inhibitory concentrations (IC50) of 42.11, 102, 58.5, 63.2 and 65.6 mcg/mL, respectively.

Conclusion. The synthesized HA-AgNP showed promising antibacterial activity against the top five clinical bacterial isolates from orthopaedic wound infections, and stable dose-dependent activity as compared to the uncapped AgNP.

Keywords. silver nanoparticles, hyaluronic acid capped silver nanoparticles, orthopaedic wound bacterial isolates, open fracture infections

INTRODUCTION

Orthopaedic infections remain a major health issue. Open fractures require antibiotics, adequate debridement, and appropriate stabilization.[1-4] Infection rates range from 0-2%, 2-10%, and 10-50%, for Gustilo Type 1, Type II, and Type III fractures, respectively.[5-9]

Emerging drug-resistant bacteria further exacerbate the problem, necessitating investigation and development of new antimicrobial agents, which can be costly. Silver-based antimicrobial agents and antiseptics have broad-spectrum antibacterial activity and are less likely to induce antimicrobial resistance.[10-13] In addition, silver ion-based agents were highly toxic to as many as 12 species of bacteria.[14] Their efficiency,

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Corresponding author: Hansel Gould B. Cocjin, MD, FPOA
Medical Specialist III, Department of Orthopaedics and Traumatology, Corazon Locsin Montelibano Memorial Regional Hospital, Lacson St., Bacolod City, Negros Occidental, 6100, Philippines
Tel. No.: (034) 703-1350
E-mail: hanselgouldcocjin@gmail.com
ORCiD: https://orcid.org/0000-0001-6010-431X

reliability, and biocompatibility are further enhanced when reduced in size to nanoparticles, and coated with amphiphilic hyperbranched macromolecules.[15,16] Silver-based antimicrobial compounds are continuously being evaluated but their use is limited because resistance and toxicity have not yet been extensively studied.[17]

One example is the wound healing agent silver-chitosan compound. The inclusion of silver nanoparticles (AgNPs) improved antimicrobial activity against drug-sensitive and drug-resistant microorganisms.[18,19] Another example is AgNP synthesized from Lansium domesticum fruit peel extract which was histocompatible and reduced time to wound closure.[20] In vitro toxicity studies show that AgNPs at 1.56–6.25 g/mL concentrations are safe for use.[21]

Commercially, silver-containing antimicrobial compounds, such as silver nitrate, silver sulphadiazine, silver sulphadiazine/chlorhexidine, silver sulphadiazine with cerium nitrate, and silver sulphadiazine impregnated lipid-colloid wound dressing are available. Innovations such as Acticoat™ and Silverlon ensure a controlled release of silver nanoparticles to the wound surfaces.[22] Use of these dressings for seven days resulted in bacterial death.[23] Wound dressings manufactured through green methods, such as bacterially-synthesized cellulose, have also been incorporated with AgNPs.[24]

The antimicrobial action of the silver nanoparticles is initiated upon contact with a peptidoglycan cell wall of the microorganism, penetration into the plasma membrane, and interaction with cytoplasmic DNA and proteins.[25] Ionic interaction with organic compounds can help explain the antibacterial properties. Clinical studies showed that AgNPs reduced bacterial colonization while producing lesser discomfort in the healing process.[26]

This study evaluated the use of hyaluronic acid as a coating mechanism for silver nanoparticles and the antibacterial efficacy of hyaluronic acid-capped silver nanoparticles against the top five clinical orthopaedic wound isolates based on the institution's antibiogram. The study intended to provide foundational evidence on the minimum inhibitory concentration of hyaluronic acid-capped silver nanoparticles.

METHODOLOGY

This experimental laboratory study was performed in Corazon Locsin Montelibano Memorial Regional Hospital, Bacolod City, in collaboration with the University of San Agustin, Bacolod City. The study was approved by the institution's PHREB accredited ethics review committee. The hyaluronic acid-capped silver nanoparticles (HA-AgNP) were synthesized at Colegio San Agustin-Bacolod's Pharmacy Laboratory. The top five clinical bacterial isolates from orthopaedic wounds were identified based on the hospital Infection Control Committee antibiogram annual reports for the previous five years. The bacterial isolates were retrieved from the Microbiology section of the Department of Pathology of Corazon Locsin Montelibano Memorial Regional Hospital. The ESKAPE isolates tested were *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli,* and Multidrug Resistant *Staphylococcus aureus (MRSA).* Antimicrobial properties were tested using the MTT[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay in three replicates in the institution's laboratory department.

Synthesis of Hyaluronic Acid-capped Silver Nanoparticles (HA-AgNP) and Non-capped Nanoparticles (AgNP)

The loading capacity of AgNP was calculated based on the elemental ratio of silver to carbon ratio where silver represented AgNPs and carbon represented HA. Theoretically, 1 mole of HA contained 7 moles of carbon, while 1 mole of AgNP contained 1 mole of silver.

The Hyaluronic Acid-capped Silver Nanoparticles (HA-AgNP) were synthesized with a few modifications (Figure 1).[27]

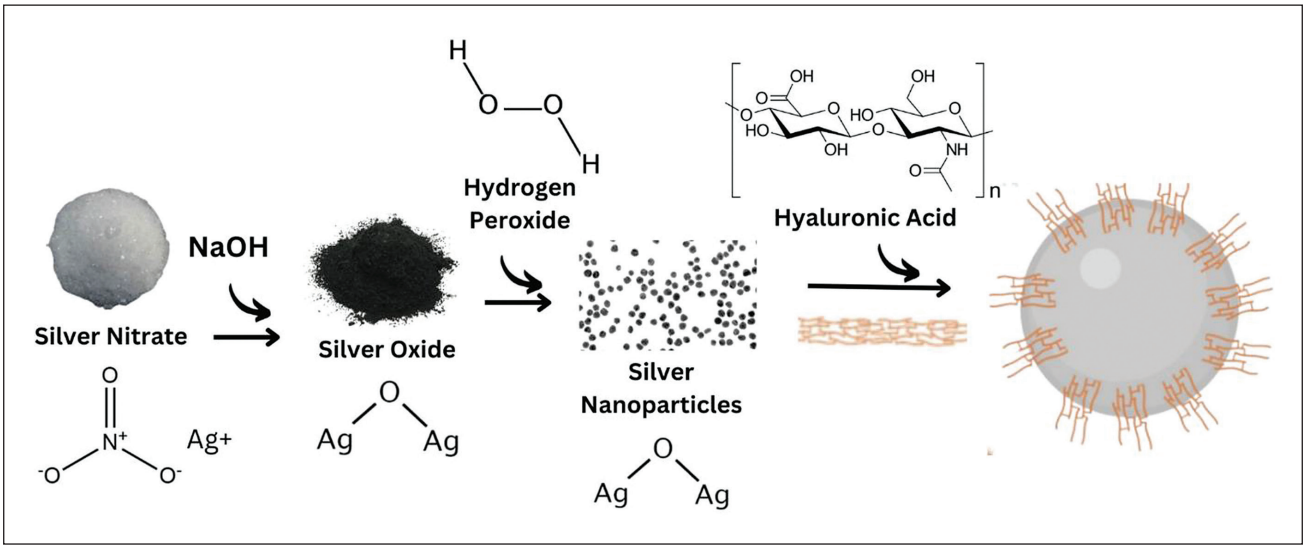

Figure 1. Synthesis of Hyaluronic Acid-capped Silver Nanoparticles (HA-AgNP).

- Hyaluronic acid (HA) and silver nitrate (AgN) solutions were each prepared into 5 mM solutions.
- In a beaker, 10 mL of silver nitrate was added. Sodium hydroxide (50 mcl, 1 M) was added dropwise while stirring at 25° C.
- On this solution, 10 mL of 30% hydrogen peroxide was added dropwise while stirring at 25° C.
- The solution was then stirred continuously for 1 hour in the dark.
- The gray solution was centrifuged at 1150 rpm at 8° C via ultracentrifugation and washed thrice with deionized water to obtain silver nanoparticles (AgNP).
- Then, 1.4 mg of freshly harvested AgNP was mixed with 100 PPM hyaluronic acid in 10 mL deionized water.
- The pH was then adjusted to 4, then the solution was stirred for 1 hour at 25° C in the dark to obtain hyaluronic acid (HA)-capped AgNP.
- HA-AgNP was then washed thrice with deionized water.

MTT assay

Bacterial isolates, with a density of 10^4 per well were seeded on a 96-well plate for 24 hours in Tryptic Soy (TS) broth (Figure 2).

- After 24 hours, the growth medium was pipetted out and the bacterial colonies were gently washed once with Phosphate Buffered Saline (PBS).
- The growth media containing varying concentrations of AgNP, HA-AgNP (100 mcg/mL, 50 mcg/mL, and 25 mcg/mL) and gentamicin (2.5 mg/mL) were seeded, and left for another 24 hours.
- After 24 hours, the growth media containing the AgNP, HA-AgNP and gentamicin was pipetted out, and gently washed thrice with PBS. Afterwards, MTT reagent diluted at a ratio of 1:9 was added.
- After 4 hours, the MTT reagent was pipetted out of the growth media, the crystals were dissolved in Dimethyl Sulfoxide (DMSO) (pH adjusted to 8 using Sodium Hydroxide) and their optical density was read by the microplate reader set at 590 nm.
- An online calculator was used to determine the Inhibition Concentration at 50% (IC50) of the material to each bacterium (https://www.aatbio.com/tools/ic50-calculator).
- The MTT assay was done in three replicates.

Statistical analysis

The percent viability for each dose of the material (AgNP vs. HA-AgNP) was analyzed using one-way ANOVA followed by Tukey's post-hoc test via an online software tool (https://astatsa.com/OneWay_Anova_with_TukeyHSD/). Statistical significance was determined based on these tests. In addition, the R^2 value was used to assess the correlation between dose and percent cell viability, indicating the antimicrobial effect. An R^2 value closer to 1 suggests a stronger correlation, with values above 0.8 generally considered indicative of a strong positive correlation.

RESULTS

The antibacterial efficacy of hyaluronic acid-capped silver nanoparticles (HA-AgNPs) was compared to uncapped silver nanoparticles (AgNPs) against five clinical ESKAPE pathogens: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and methicillin-resistant *S. aureus* (MRSA). As shown in Figures 3 and 4, both nanoparticle types exhibited concentration-dependent antibacterial activity, with increasing doses (25, 50, and 100 μg/mL) resulting in decreased bacterial cell viability. Notably, HA-AgNPs consistently demonstrated enhanced bactericidal effects compared to uncapped AgNPs across all tested isolates. This enhancement was particularly evident at lower concentrations. For instance, HA-AgNPs at 50 μg/mL frequently outperformed AgNPs at 100 μg/mL. Among the pathogens tested, *K. pneumoniae*, *E. coli*, and MRSA exhibited the highest susceptibility to HA-AgNPs, with bacterial cell viability significantly reduced relative to both AgNP-treated and gentamicin-treated controls (p <0.05). Furthermore, higher R^2 values in the HA-AgNP-treated groups indicate

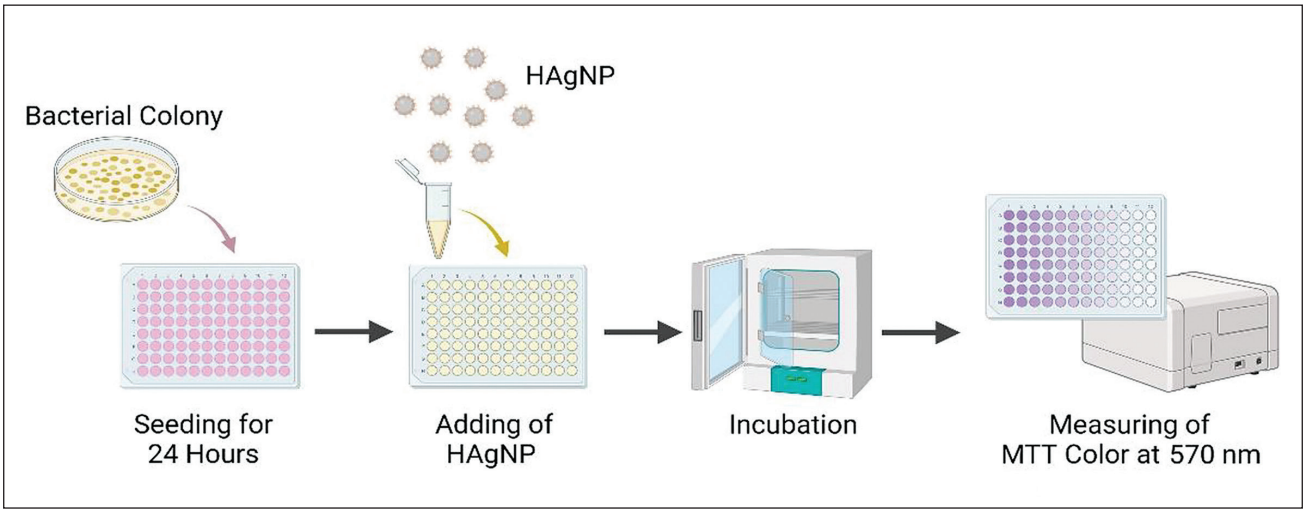

Figure 2. MTT Assay of HA-AgNP.

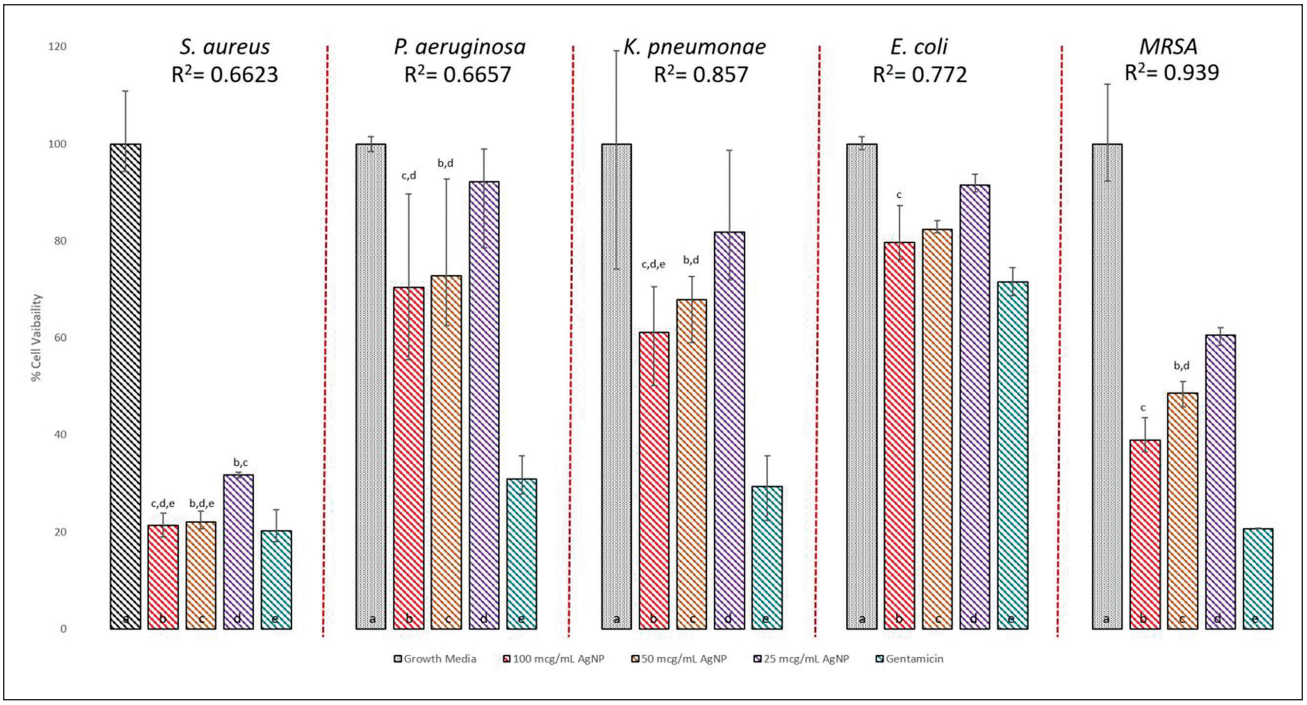

Figure 3. Antibacterial activity of AgNP against ESKAPE isolates.

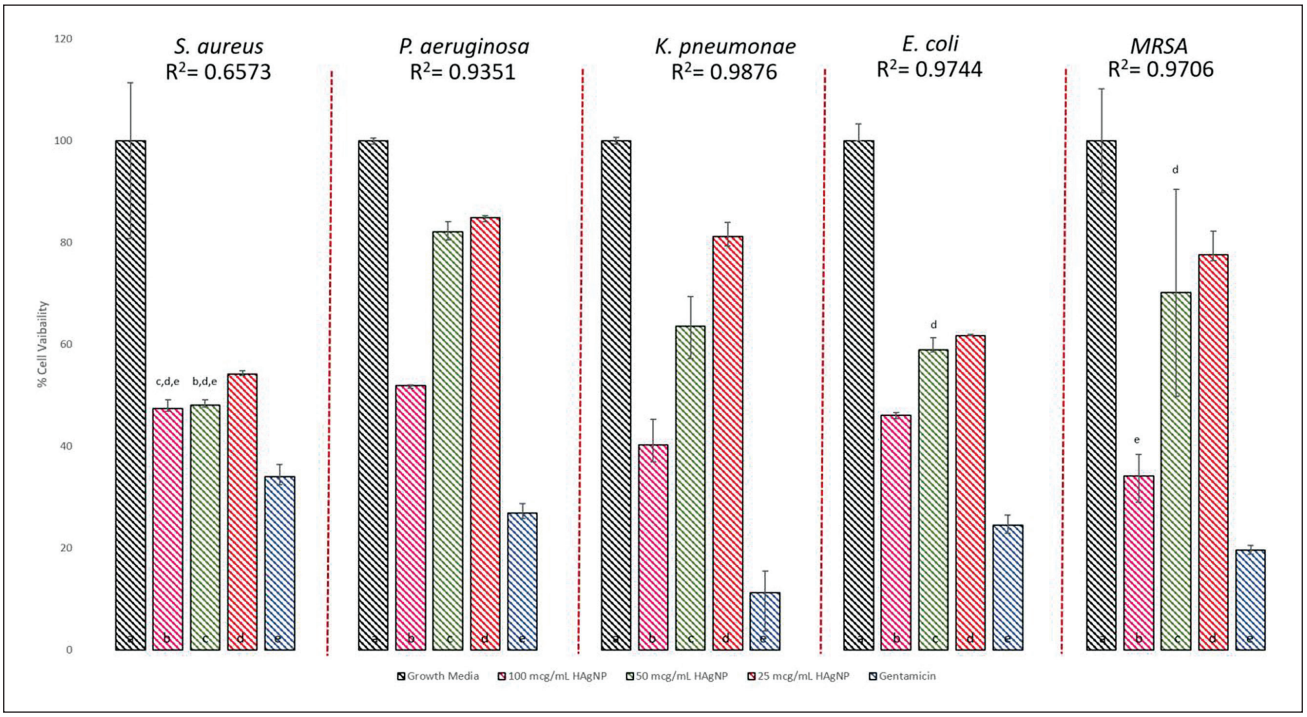

Figure 4. Antibacterial activity of HA-AgNP against ESKAPE isolates.

greater consistency in the antibacterial response. These findings suggest that HA functionalization improved the overall antimicrobial efficacy against multidrug-resistant pathogens.

The AgNP at 100 mcg/mL was able to reduce the bacterial loads of *Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa), Klebsiella pneumonae (K. pneumonae), Escherichia coli (E. coli),* and *Multidrug Resistant Staphylococcus aureus* (MRSA), by approximately 80%, 30%, 40%, 21%, and 60%, respectively. In addition, the AgNP significantly eliminated *S. aureus* at concentrations of 100 mcg/mL and 50 mcg/mL. Lastly, the AgNP showed dose-dependent antibacterial effects on *K. pneumonae, E. coli and MRSA* as shown in Figure 3. It was noted that there was no significant difference between the doses.

HA-AgNP showed moderate antibacterial activity in killing the said clinical isolates, in additional to being more reliably dose-dependent.

On average, 100 mcg/mL of HA-AgNP was able to exterminate 60% of the top five clinical isolates, and, showed dose-dependent antibacterial activity when compared to AgNP alone in all clinical isolates except *S. aureus.* The IC50 values of HA-AgNP were 42.11, 102, 58.5, 63.2 and 65.6 mcg/mL on *Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa), Klebsiella pneumonae (K. pneumonae), Escherichia coli (E. coli),* and *Multidrug Resistant Staphylococcus aureus* (MRSA), respectively, as shown in Figure 4. The stable, dose-dependent antibacterial activity of HA-AgNP can be mainly attributed to hyaluronic acid's ability in stabilizing the particle size of AgNP.

DISCUSSION

In recent years, AgNP have gained much attention as potential antibacterial agents,[28] especially on traumatic wounds and skin lesions which pose a significant cause of infections.[29,30] Silver nanoparticles can help innovate wound care, especially among surgical patients.[31,32] However, particle size is difficult to control during and after synthesis.[33] Silver nanoparticles are unstable after reduction, forming colloidal systems bigger than the expected size, reducing efficacy.[34-36]

Various methods such as mechanical reduction, laser ablation, lithography, and ball milling have been tried, but these reduce the AgNP yield and don't guarantee size uniformity.[37-39] Organic synthesis through enzymatic reduction of fungi, bacteria, and plants can produce AgNPs, but is difficult to control.[40-42] The conventional method of reduction through citric acid, tannic acids, and polysaccharides poses the same problems.[43,44]

Particle stability has been largely improved with the addition of capping agents[45-47] such as tannic acid, luteolin, chitosan, and collagen.[48-51] One study synthesized HA-AgNP as a ligand on radioactive tagging agents. However, its potential as an antibacterial has not yet been explored. Among the various polysaccharide capping agents, HA shows much promise as it also has an innate ability to kill bacteria and acts as a biological tissue cement which aids in wound healing.[52] Hyaluronic acid is composed of N-acetyl-glucosamine, and glucuronic acid, a monosaccharide containing carboxylic acid moiety.[53] Silver derivatives can easily attach to the carboxylate functional group, which can further be reduced to silver alone.[54,55] Collectively, this silver will form a colloid, but the presence of an HA tail enables control of the colloidal formation, which results in AgNPs with excellent physico-chemical and pharmacological properties.[56] In addition, hyaluronic acid acts as a bacteriostatic barrier with a proven long-term safety profile.[57-59]

While many studies have tested AgNP against gram-positive and gram-negative bacteria,[60-68] Pseudomonas and Cronobacter species,[69,70] yeast, and *E. coli*,[71-73] none have tested HA-capped AgNP.

The study tested HA-AgNP and AgNPs antibacterial activity against *S. aureus, P. aeruginosa, K. pneumoniae, E. coli* and Multidrug Resistant *Staphylococcus aureus* (MRSA). Both *S. aureus* and MRSA were highly susceptible to the AgNP at 100 mcg/mL with a bacterial reduction of 80% and 62%, respectively. This is no surprise as AgNP strongly interacts with the abundant peptidoglycan in gram positive bacteria cell walls.[74-77]

CONCLUSION

The HA-AgNP resulted in effective and dose-dependent antibacterial activity in all bacteria except for S. aureus. This enhanced activity is due to the more consistent particle size as hyaluronic acid encapsulated the silver nanoparticles, inhibiting their apparent particle aggregation. The stability was assessed through its dose-dependent antibacterial activity and, theoretically, the particle capping stabilizing properties. However, the study recommends further examination of the particle stability through scanning electron microscopy.

This capped AgNP resulted in better antibacterial activity against *E. coli* and *S. aureus*,[78,79] likely due to the uniform and smaller particle sizes.[80,81] The bactericidal effect of HA-AgNP at 100 mcg/mL on S. aureus and MRSA was statistically comparable with the positive control gentamicin at 2 mcg/mL. The synthesis and antibacterial activity of HA-AgNP showed its potential as a tool in destroying bacteria, enhancing wound healing, and combating antibiotic resistance.

STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

CREDIT AUTHOR STATEMENT

HGC: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition; **SP:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition; **JPS:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition; **RM:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition; **MEMV:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition; **AJ:** Conceptualization,

Methodology, Software, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Project administration; **DH:** Conceptualization, Methodology, Software, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Project administration; **JHB:** Conceptualization, Methodology, Software, Validation, Formal analysis, Resources, Writing – original draft preparation, Writing – review and editing, Visualization, Project administration

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed in this study are included in the published article.

FUNDING SOURCE

None.

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