The antibacterial effect of nanosilver fluoride in relation to caries activity in primary teeth: a protocol for a randomized controlled clinical trial

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Abstract
Background: Minimally invasive dentistry is a highly convenient and efficient method of managing caries in pediatric patients. Silver diamine fluoride (SDF) is commonly used to arrest active caries lesions. However, the associated black stain, possibility of soft tissue injury, and unpleasant taste often limit its use. Recently, nanosilver fluoride (NSF) emerged as a promising topical fluoride agent with potent cariostatic and antibacterial potentials. This novel anticaries agent has gained attention as an alternative to overcome the drawbacks of SDF in caries arrest.

Objectives: To assess the antibacterial effect of NSF in relation to caries activity in dentin caries lesions, as well as to investigate the change in saliva bacterial levels in primary teeth in comparison to SDF after 1 and 3 months.

Materials and methods: Fifty children aged 4 to 6 years old with active dentin caries lesions (score 5 according to International Detection and Assessment System (ICDAS II) criteria) will be enrolled in the study. They will be equally and randomly allocated into 2 groups: a group receiving NSF and a control group receiving SDF treatment. Microbiological samples will be collected from the carious lesions and from unstimulated saliva at the baseline and at the 1 and 3 months' follow‑up appointments. Bacterial counts will be assessed using Mitis Salivarius agar (selective culture media for S. mutans) and Rogosa agar (selective culture media for lactobacilli), and the results will be expressed in colony-forming units. Data regarding the children's oral health will be collected and their dmf index will be scored. The arrest of active carious lesions will be measured at the follow‑up appointments according to ICDAS II criteria.

Results: The relation between bacterial colony counts and lesion activity for both groups will be assessed, as well as the change in salivary bacterial counts. The collected data will be statistically evaluated and tabulated. This clinical trial has been registered on ClinicalTrials.gov in January 2022 (original version) with ID: NCT05221749.

Keywords: Nanosilver fluoride, Silver diamine fluoride, Antibacterial, Caries, Microbiology, Saliva, Primary teeth, Pediatric dentistry, Clinical trial

Introduction
Dental caries is a chronic disease, ranking among the most highly prevalent diseases in children [1]. Minimally invasive approaches to prevent and arrest caries lesions provide methods to address the caries problem that are affordable and convenient and do not require sophisticated equipment [2]. Silver diamine fluoride (SDF)

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has a proven antibacterial action [3] and assists enamel remineralization [4], and is available for arresting caries as a simple, non-invasive medication [5, 6]. Its major drawback is the black staining of carious tissue and that it may induce reversible slightly painful lesions in the oral mucosa that usually disappear within 48 h [7]. Moreover, it has a reported unpleasant taste, and there are concerns about toxicity [7, 8].

The use of nanotechnology in dentistry has recently attracted significant attention [9], specifically, formulations of silver nanoparticles (AgNPs). Nanosilver fluoride (NSF) is a yellow solution containing uniformly dispersed AgNPs and fluoride. This new agent is safe to be used in humans [10, 11] and has several desirable properties. Multiple in vitro researches have investigated the antimicrobial effects of AgNPs on carious tooth structures and have confirmed the substantial antibacterial effect of NSF on Streptococcus mutans and Lactobacilli [12–16]. Additionally, several studies [10, 17, 18] have reported that NSF is effective in arresting active dentine caries without any distinctive tissue pigmentation.

To our knowledge, no publication has reported the in vivo antimicrobial efficiency of NSF on the primary dentition in comparison to SDF. Therefore, this study will be conducted to evaluate and compare the antibacterial and caries activity of NSF versus SDF in dentin caries lesions and saliva in primary teeth. Specifically, in active lesions have a distinct cavity with visibly exposed dentine, where the cavity feels soft or leathery on gentle probing (known as active score 5 ICDAS II lesions). The null hypothesis of this study is that no statistically significant difference will be detected between NSF and SDF by comparing their antibacterial effect in relation to caries activity in dentin caries lesions, as well as that there will be no statistically significant difference in the change in salivary bacterial levels after 1 and 3 months of follow-up.

Materials and methods

Study design

This will be a parallel, two-arm, equivalence randomized controlled clinical trial, with a 1:1 allocation ratio. It will be set up and reported according to the CONSORT guidelines [19]. It has been approved by the ethics committee of the Faculty of Dentistry, Alexandria University IRB NO: 00010556 – IORG 0008839 and been registered on ClinicalTrials.gov in January 2022 with ID: NCT05221749, https://clinicaltrials.gov/ct2/show/NCT05221749. The detailed procedures that shall be taken in ethics consideration are mentioned in the supplementary material. The study plan is demonstrated in Fig. 1, and the study timeline is demonstrated in Fig. 2.

The PICOT question is: will children (4–6 years old) with an active carious lesion (ICDAS score 5) (population; P) using nanosilver fluoride varnish (Intervention; I) in comparison to silver diamine fluoride (comparison; C) show a greater percentage decrease in bacterial counts (outcome; O) after 3-month follow-up (time; T)?

Study setting and location

The study will be conducted in the Department of Pediatric Dentistry and Dental Public Health of the Faculty of Dentistry and the Department of Medical Microbiology and Immunology of the Faculty of Medicine. The nanosilver fluoride will be prepared and characterized at the Center of Excellence for Research in Regenerative Medicine and Applications (CERRMA), Faculty of Medicine, Alexandria University, Alexandria, Egypt.

Sample size estimation

Sample size was estimated assuming 5% alpha error and 80% study power. According to Dos Santos et al. [3], after 5 months, the NSF group had 72.7% arrested decay. Therefore, it was estimated that 14.54% of lesions treated with NSF will be arrested after 1 month. According to Milgrom et al. [3], 51.7% of lesions treated with 38% silver diamine fluoride (SDF) have been arrested after 21 days. Based on difference between two independent proportions, the minimum sample size was calculated to be 24 patients per group, increased to 25 teeth to make up for lost to follow-up cases. Total sample size = number per group × number of groups = 25 × 2 = 50 patients. The sample size estimation was based on Rosner’s method [20] and calculated using G*Power 3.1.9.7 [21].

Eligibility criteria

The participants to be enrolled in this study will be recruited after fulfilling the following criteria:

**Inclusion criteria**

- Children 4–6 years old.
- The presence of at least one active carious lesion on a primary tooth, with a score of 5 according to the International Detection and Assessment System (ICDAS II), detected by visual-tactile inspection to assess lesion severity and activity [22] (Appendix 2, Tables 1 and 2).
- Completion of an informed consent to participate in the study (Appendix 1).

**Exclusion criteria**

- Children reporting spontaneous or elicited pain from caries or showing any signs of pulpal infec-
tion, swelling, abscess, obvious discoloration of the tooth, or premature mobility [7].

- Reported usage of local or systemic antibiotics, chlorhexidine, or fluoride mouthwashes within the last 2 weeks [23].
- Children who have special health care needs or who are undergoing medical treatment for chronic or acute diseases that affect salivary flow.
- Allergy or sensitivity to silver or any of the materials included in the study [21].
- Child weight less than 10 Kg (to avoid concerns for toxicity) [24].

**Randomization and allocation**

Subjects complying with the inclusion criteria will be randomly assigned using a computer-generated list of random numbers to either the NSF or the SDF group [25]. Allocation will be performed by a trial independent individual and the allocation ratio is intended to be equal (1:1). Participants will be randomly and equally allocated to one of the two treatment groups:

- Intervention group (NSF): Twenty-five children meeting the eligibility criteria will receive nanosilver fluoride.
• Control group (SDF): Twenty-five children meeting the eligibility criteria will receive 38% silver diamine fluoride.

Allocation concealment
An assistant will be responsible for giving each participant a serial number that will be used for his/her allocation. A duplicate of this number will be kept in an opaque envelope indicating to which group the patient belongs. This envelope will be kept by a trial independent individual who will be assigned the role of opening it only at the time of intervention; so that the group to which the child is allocated is concealed from the investigator [26]. The investigator will not be blinded to the treatment type as the SDF solution has a bluish tint and will stain the active caries lesions black, while the NSF solution has a yellowish tint and is not expected to stain the lesions. However, the participants, statistician, and microbiologist will be blinded to the treatment group.

Training, calibration, and reliability
The principal investigator will be trained and calibrated by the study supervisors. Training and calibration will be regarding the use of ICDAS II criteria in the diagnosis of the teeth to be included in the study [27], application of both test materials [28], and evaluation of outcomes. The examination of 10 participants meeting the eligibility criteria will be carried out followed by re-examination after 7 days for the determination of intra-examiner agreement measured by Cohen’s kappa (κ). Obtaining a kappa score $\geq 0.8$ is considered excellent reliability. These participants will not be included in the study sample.

Nanosilver fluoride preparation

**Synthesis and characterization of silver nanoparticles**
In a 250-ml beaker, 8 mg of silver nitrate (Sigma-Aldrich, US) will be added to 50 mL of deionized water containing 0.5% (v/v) polyethylene glycol (PEG-400, Sigma-Aldrich, US). The solution will be heated until the boiling point, then 1 mL containing 50.5 mg of trisodium citrate (Alpha Chemika, India) will be added. Change in the color of the solution to yellow will indicate the formation of AgNPs [29].

The preliminary detection of synthesized AgNPs will be carried out using a UV–visible spectrophotometer (Nanodrop, DeNovix, DS-11 FX+, US) by scanning the absorbance spectra in the range of 200–800 nm wavelength. The average particle size, polydispersity index (PDI), and particle charge of AgNPs will be performed by the dynamic light scattering technique using Zeta-seizer (Nano ZS, Malvern Instruments, Worcestershire, UK), with a dilution ratio of 1:6 [30], in addition to transmission electron microscopy (TEM) (JEOL JEM-1400 series 120kV TEM).

**Cytotoxicity assay**
The safety profile of the synthesized PEG-nanosilver will be investigated on human gingival fibroblasts to ensure its safety. All cultivating materials [Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and antibiotics (penicillin and streptomycin)] will be obtained from
Gibco, US. Reagents of cytotoxicity assay as MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide will be purchased from SERVA-Electrophoresis GmbH, Germany, while dimethyl sulfoxide (DMSO) will be obtained from Fisher Chemical (Fisher Scientific), UK.

The cytotoxic effect of PEG-coated AgNPs on the viability of the gingival fibroblasts will be evaluated using MTT assay at 24 h incubation period. The cells from the primary culture will be seeded onto a 96-well culture plate containing 100 μL of DMEM/well at a density of 7000 cells/well. The cells will be incubated for 24 h at 37 °C in 5% CO₂. The gingival fibroblasts will then be treated with serial concentrations ranging from 10 to 1000 μM of PEG-AgNPs. Untreated cells will be used as control.

After 24 h incubation, the cells will be washed twice with PBS and then 100 μL of MTT (0.5 mg/mL DMEM) will be added to each well and incubated at 37 °C for 3–4h. Thereafter, the medium containing MTT will be removed, and the cells will be lysed by adding 100 μL /well DMSO in darkness. Then, an ELISA reader will quantify the optical density (OD) of the dissolved formazan crystals at 570 nm using a micro plate scanning spectrophotometer (ELISA reader, Infinite F15 TECAN, Switzerland). The percentage of cell viability will be expressed as mean ± SD according to the following formula: Viable cell % = (OD treated / OD untreated) × 100, where “OD treated” represents the absorbance value of the treated sample and “OD untreated” represents the absorbance value of the corresponding untreated sample [31]. The cytotoxicity assay will be performed in triplicates for 3 independent experiments.

**Determination of MIC of silver nanoparticles**

The MIC (mean inhibitory concentration) of PEG-AgNPs will be determined by visual comparison of the differences in turbidity in a 96-well plate [32]. *Streptococcus mutans* (proficiency testing strain identified by the College of American Pathologists as *S. mutans*) will be prepared in brain heart infusion with 2% sucrose for a concentration of 0.5 on the McFarland scale, and this will be verified using colorimetric measurement (Vitek, bioMérieux, Mexico City, Mexico). This concentration is equivalent to 1.5 × 10⁶ CFU/mL. The MIC of PEG-AgNPs solution will be determined with ranges of 0.25–1024 mg/L in double fold dilutions prepared according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method [33].

In microdilution plates, 100 μL of the dispersion form of AgNPs will be placed in wells with predetermined serial dilutions, and then 100 μL brain–heart infusion with 2% sucrose will be inoculated onto the wells with *S. mutans* at 1 × 10⁶ CFU/mL. The first and last columns of the plates will be used as positive and negative controls, respectively. Finally, the plates will be incubated at 37 °C for 24 h. Determination of the MIC will be done based on turbidity.

**Functionalization of silver nanoparticles with sodium fluoride**

For fluoridation of the formulated AgNPs, 22,600 ppm of sodium fluoride will be dissolved in 10 ml of PEG-AgNPs at the determined MIC. The fluoridated nanosuspension will then be left under stirring, in a light proof brown bottle, overnight, at room temperature [17].

**Treatment protocol**

**Effect modification/confounders**

The socioeconomic level, dietary, and oral health practices of children (as reported by their caregivers) will be measured at baseline using the World Health Organization’s questionnaire for assessment of children’s oral hygiene habits (Appendix 3) [34]. The participants will be provided the needed dental treatments before the beginning of the study period and after its completion. This is to avoid having any confounding factors (new restorations, extractions, topical fluoride treatments) that may cause an unaccounted change in the microbiological levels before and after the interventions. This is to ensure that the change in bacterial level is mainly brought about by the intervention groups and to provide an outcome measurement that is as accurate as possible.

**Baseline examination**

After obtaining the informed consent from the care giver/parent, the researcher (NA) will provide oral hygiene instructions to each of the study participants and will educate them about the importance of maintaining good oral hygiene and a proper diet [35]. The patients and caregivers will be informed about the therapeutic intervention to be applied, the possible outcomes, and side effects in detail. All the examinations and interventions will be done by one calibrated examiner. All of the lesions in the children’s oral cavity indicated for treatment with NSF or SDF will be treated and sampled for microbiological analysis [28]. No more than one drop of SDF or NSF shall be used in one appointment to avoid concerns for toxicity [24, 28].

On the day of the intervention, the children will be asked to refrain from tooth brushing in the morning, as well as eating and drinking (except water) for at least 2 h before the appointment. The dmf index will be recorded for each patient. For caries treatment in all groups, no caries or unsupported enamel will be removed. Unstimulated whole saliva samples will be first collected by asking the patient to drool in a collection cup until at least 1 mL of
saliva is collected [36]. Then any gross debris surrounding the teeth to be sampled will be gently removed and dried with cotton gauze. The teeth will then be partially isolated using cotton rolls and a saliva ejector. Additionally, petroleum jelly will be applied to the gingiva for additional protection. A 2-mm-wide microbrush will be rubbed on the carious lesion for 4 s to collect the baseline microbiological sample [3]. The microbrush will then be inserted in a sterile test tube containing 1 mL of saline and transported to the microbiology laboratory within 1–2 h [37]. After collection of the baseline samples, the intervention treatments will be applied as follows:

i. Intervention group (NSF) [17]
   - A drop of NSF will be dispensed into a sterile plastic dappen dish.
   - Affected tooth surfaces will be dried with a gentle flow of air.
   - The solution will be applied to the carious lesions using a microbrush.
   - The NSF solution will be left in contact with the tooth surface for 1 min.
   - Any excess will be removed with a cotton pellet to minimize systemic absorption.

ii. Control group (SDF) [38]
   - A drop of 38% SDF will be dispensed into a sterile plastic dappen dish.
   - Affected tooth surfaces will be dried with a gentle flow of air.
   - A microbrush will be dipped into the SDF and dabbed on the side of the dappen dish to remove excess liquid before applying the solution to only the affected tooth surface.
   - The SDF will be allowed to dry on the lesion for 1 min.
   - Any excess will be removed with a cotton pellet to minimize systemic absorption.

Follow-up examination All patients will be recalled for follow-ups after 1 and 3 months. On the day of the recall appointment, patient preparation will be done as mentioned before and microbiological assessment will be performed and CFU counts will be recorded. Lesion activity will be assessed according to the ICDAS II criteria. Using a blunt ended probe, if the cavity is found to be shiny and feels hard on gentle probing of the dentin, it will be recorded as an inactive lesion. While if it remains soft or leathery on gentle probing, it will be recorded as an active lesion (Appendix 2, Table 2).

Microbiological procedure

Sample dilution
All samples will be dispersed by vortex for 30 s then 10-fold serially diluted using sterile saline. A measure of the dilution will then be used for traditional plate culturing methods [8, 39].

Culture
Aliquots of 10 μl of each dilution will be inoculated into freshly prepared Mitis Salivarius agar—a selective culture media for *S. mutans* prepared according to the manufacturer’s instructions (Difco Laboratories Inc, NJ, USA)—and Rogosa agar—a selective culture media for lactobacilli prepared according to the manufacturer’s instructions (Himedia Laboratories, Mumbai, India)—respectively, using a micropipette.

- Mitis Salivarius agar plates will be incubated anaerobically in a CO₂ incubator (Binder redLINE incubator model RI 115-U) with an atmosphere containing 10% CO₂ at 37 °C for 72 h to detect *Streptococcus mutans* counts.
- Rogosa agar plates will be incubated aerobically at 37 °C for 48 h to detect *Lactobacilli* count.

Isolation and enumeration
Following the predetermined incubation period, colonies grown on the specified media will be counted and
represented as colony-forming unit (CFU)/mL. *Streptococcus mutans* will be identified based on their characteristic morphology on Mitis Salivarius agar plates. Similarly, *Lactobacilli* will be identified biochemically and microscopically based on their morphology.

The number of colonies will be determined and expressed as colony-forming units using the following equation: \[ \text{CFU/mL} = \text{no of colonies} \times \text{dilution factor/volume taken in mL}. \]

### Discussion

The use of SDF has been greatly popularized in the last years after its FDA approval in 2014 [42]. However, due to the permanent unesthetic black stain and possibility of soft tissue injury, it is sometimes unacceptable to patients especially for the treatment of anterior teeth [43]. Additionally, soft tissue burns have been reported after the use of SDF [3]. The incorporation of nanotechnology into modern pediatric dentistry may pave the way for a more efficient minimally invasive caries treatment. NSF is a promising cariostatic agent with a potent antibacterial activity and does not produce a black stain [14, 44]. Nanosilver particles have been widely used in a range of medical procedures ranging from treatment [45] and drug delivery [46], to medical device coating [47] and wound dressings [48]. The use of nanosilver products is continually increasing and their incorporation into the dental field holds a promising future. Especially, with the rise of the COVID-19 pandemic, the need for effective minimally invasive dental treatments is of paramount importance. Many dental institutions are not adequately equipped to face the widely spreading virus [49], and the implementation of non-aerosol generating dental procedures is of vital to limit the spread of the virus.

It is important to ensure that the nanosilver product in use is safe and poses no health risks as there have been some concerns related to the cytotoxicity of the nanosilver used [50, 51]. Previous clinical trials using NSF intraorally have synthesized AgNPs using sodium borohydride as a reducing agent [10, 52]. Sodium borohydride is known to be a highly toxic and corrosive chemical that is commonly used in bleaching wood, and its fumes are known to be odorless and cause severe irritation to the lungs and skin. Its use poses a recognized health hazard [53, 54]. For the obvious safety concerns, an alternative method of nanosilver synthesis for clinical investigations would be more appropriate. Therefore, trisodium citrate—an FDA-approved salt widely used in personal and dental care products— [55, 56] was used as a reducing agent instead of sodium borohydride. Additionally, the synthesized AgNPs were PEGylated to increase the stability of the nanosuspension and to prevent particle aggregation. Polyethylene glycol is a popular FDA-approved polymer used extensively as a pharmaceutical stabilizing agent [57, 58]. Characterization tests and cytotoxicity assays are important to ensure the safety of the synthesized formulation for clinical use.

The determination of the suitability of nanosilver fluoride as an anticaries agent and as a valid alternative to SDF is a multifaceted feat. While previous studies have
investigated its effect on caries activity [17, 18] and antibacterial potential [12–14], there still remains a lack in the knowledge surrounding its clinical performance as an antibacterial agent. This clinical trial aims to bridge the gap in the available literature on the clinical antibacterial effect of nanosilver fluoride in caries of the primary dentition.

Ethics approval and consent to participate
This study protocol received approval from the Ethics Committee of the Faculty of Dentistry, Alexandria University (IORG 0008839 - IRB No. 0359-12/2021). A signed informed consent will be obtained by the principal investigator (NA) from every parent. The informed consent that will be signed by the parents will encompass the data to be collected and its intended analysis and use for the study purpose, and it will thoroughly explain the trial procedures, benefits, possible harms/risks, and outline the measures taken to ensure the privacy and confidentiality of the participants. Additionally, the informed consent will include a picture of the black stain caused by SDF treatment. Carious teeth not treated with SDF or NSF, or those showing signs of failure after treatment, or new carious lesions that will appear during the follow-up period, will be appropriately treated. The voluntary nature of the study will be emphasized, and parents will be informed of their right to withdraw from the trial at any time without incurring any penalties.

Data monitoring
The participants will be contacted by phone after 48 h of the intervention to monitor any reported adverse events or any other unintended effects; if more than half of the participants report adverse events, such as soft tissue injury or pain, the study will be terminated. Furthermore, an interim analysis of the change in microbiological count and lesion activity of the sampled lesions will be conducted at the 1-month follow-up appointment. The study will be terminated if the majority of the treated teeth show signs of continued caries lesion activity as defined by the International Detection and Assessment System (ICDAS II). Active caries lesions will be detected by visual-tactile inspection to assess lesion severity and activity, where if the cavity is found to be shiny and feels hard on gentle probing of the dentin, it is considered an active lesion. Additionally, if the majority of the lesions show signs of caries progression and/or pulpal pathology, the study will be immediately terminated, and the participants will receive the needed restorative treatment. Emphasis will be made on the fact that participants are free to withdraw from the study at any time. No data monitoring committee (DMC) is available at the researcher’s institution and therefore no external body outside of the study team will monitor the data or have access to it.

Harms
Participants will be contacted by phone after 48 h of the intervention and then monthly for the duration of the trial to assess reported adverse events or any other unintended effects. This will also be assessed at the 1- and 3-month follow-up appointments.

Biological samples
All collected biological samples (lesion and saliva samples) and associated participant-related data will be coded to protect participant confidentiality. Each participant will be provided with a serial number that will only be accessible to the principal investigator. The biological samples will be cultivated within 1–2 h of collection; upon determining the CFU counts for each sample, the sample will be safely and permanently disposed of. No samples will be stored in any way for future use or for use in any ancillary research.

Trial status
This is the original version of the protocol, issued in January 2022. The recruitment phase is planned to start in March 2022 and end approximately by May 2022. Any changes or protocol amendments will be accounted for in the public study record available on clinical trials.gov ID: NCT05221749.

Confidentiality
Data entry will be completed by the principal investigator (NA). The principal investigator cannot be blinded to the interventions as the SDF will inevitably stain the caries lesion black while the NSF is not expected to cause any color change. The principal investigator will also be coding the participants, where each participant will be given a unique serial number that will be stored in a secured file only accessible to the principal investigator. This file will not be shared with anyone and will be stored in a password-protected online drive. Identification numbers will ensure participant confidentiality during data collection and analysis. Only this code/identifier number will be used to identify participants’ samples during microbiological laboratory procedures and during statistical analysis of the results. No biological samples of any participant will be stored for further use after the completion of the microbiological assessment.

Ancillary and post-trial care
Carious teeth not treated with SDF or NSF, or those showing signs of failure after treatment, or new carious lesions that will appear during the follow-up period, will be appropriately treated.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13063-022-06477-5.

Additional file 1: Appendix 1. Informed Consent Form to Participate in a Research Study. Appendix 2. Table 1. ICDAS II severity criteria [59]. Table 2. ICDAS II caries activity criteria [59]. Appendix 3. WHO questionnaire for oral health assessment of children [34].

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Dissemination policy
The results of the study will be disseminated through international peer-reviewed journals and conferences. There is no intention to use the services of professional writers. All authors of the final manuscript will fulfill the authorship criteria as recommended by the International Committee of Medical Journal Editors. Participant-level dataset and statistical code will be made available on reasonable request from the principal investigator.

Authors’ contributions
NA is the trial’s principal investigator. NA, MME, and DMT conceptualized the study, designed its methods, and drafted the manuscript. MME and SNE will design and optimize the functionalized nanosilver particles and conduct any related characterization and in vitro cell studies and analysis. NA will recruit participants, perform clinical procedures, and collect biological samples. SE will perform microbiological procedures and assessment. NA and MME will analyze the data. NA, MME, and DMT will interpret the study results. All authors revised and reviewed the manuscript and contributed to the final approval of the manuscript. All authors will fulfill authorship eligibility guidelines according to the International Committee of Medical Journal Editors (ICMJE).

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Availability of data and materials
The final data set for the proposed study will be available upon reasonable request from the corresponding author.

Declarations

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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