Research article

Effects of silver nanoparticles on multiple drug-resistant strains of *Staphylococcus aureus* from periodontal infection: An alternative approach for antimicrobial therapy

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ABSTRACT

Introduction and Aim: Most cases of periodontitis are associated with microorganisms. The Gram-positive bacteria *Staphylococcus aureus* is considered as one of the important organisms associated with periodontal infections. This study investigated the effect of silver nanoparticles as well as the antiseptic agent chlorhexidine on multi-drug resistant *S. aureus* isolated from periodontal infections.

Materials and Methods: In this study, with help from dentists, 266 clinical samples were collected from dental patients who had periodontal infection. *S. aureus* isolated from samples was tested for their antibiotic susceptibility profiles. Silver nanoparticles and chlorhexidine were evaluated for their antibacterial activity against these *S. aureus* isolates.

Results: *S. aureus* strains isolated from periodontal infection patients in this study were found to be multidrug resistant. AgNPs obtained using *E. coli* showed high inhibition of *S. aureus* growth when used in different concentrations (5, 10, 15, 20, 25mM). Chlorhexidine also exhibited antibacterial activity against *S. aureus*. Combination of AgNPs with penicillin and ciprofloxacin had an increasing significant effect on the sensitivity of *S. aureus*. Similarly, chlorhexidine in combination with penicillin and ciprofloxacin also showed an inhibitory effect on the growth of *S. aureus*.

Conclusion: AgNPs and chlorhexidine combined with antibiotics used in treatment of *S. aureus* isolated from periodontal disease showed a good antibacterial effect which suggests its use as an antibacterial agent against periodontitis associated bacteria.

Keywords: Periodontal infection; silver nanoparticles; chlorhexidine; *Staphylococcus aureus*; multiple drug resistance.

INTRODUCTION

Periodontal disease is an infectious chronic inflammatory illness that affects the periodontal tissues that protect and/or support the teeth. As with other infections, the interactions between bacteria and the host determine the nature of the resulting disease (1). Some bacterial species have been linked to the emergence of periodontal diseases including *Staphylococci* spp, specifically *Staphylococcus aureus* and *S. epidermidis* (2, 3). Oral *S. aureus* has been associated with dentoalveolar infections and oral mucosal lesions, and shown to colonize the tongue, saliva, mucosal surfaces, supra gingival tooth surfaces, and the periodontal pockets (4). Antibiotics are the first line of treatment. However, antibiotic usage, frequently fails to eliminate these infections due to multi-drug resistant *Staphylococcus* spp. (5).

Recent breakthroughs in nanotechnology have resulted in the development of nanoparticles with novel physicochemical properties and functions that can outperform standard antibacterial agents (6, 7). The use of nanoparticles, particularly AgNPs, as antibiotic supplements has lately gained attention in academia, business, and the field of nanomedicine (8, 9). AgNPs have been demonstrated to be effective antibacterial agents against both gram-negative and gram-positive bacteria, particularly at very low concentrations (10). These nanoparticles are known to exert their effect by acting on bacterial cell walls resulting in increased permeability as well as by respiratory chain inactivation (10). AgNPs have been studied for their antimicrobial effect against a wide variety of bacteria spp. including *Pseudomonas aeruginosa, S. aureus, Proteus mirabilis, Escherichia coli,* and other (11,12).

This study aimed to isolate *S. aureus* from periodontal disease patients and investigate the effects of various antibiotics, biologically produced AgNPs and chlorhexidine on these bacterial spp. We also aimed to find if there was any synergy between AgNPs and antibiotics on *S. aureus*, as well as between chlorhexidine and antibiotics. Finally, we investigated the effects of AgNPs, antibiotics, the synergy of AgNPs and antibiotics, and chlorhexidine on *S. aureus* gene expression.

MATERIALS AND METHODS

Samples

266 clinical dental samples were collected from patients with periodontal disease visiting the Dental Specialization Centre in Fallujah city, Al-Fallujah Teaching Hospital, and outpatient dental clinics in Fallujah between the beginning of October 2020 and the end of January 2021. After measuring the depth of the pocket (greater than 4 mm), the samples were collected using cotton swabs with the assistance of dentists. *S. aureus* in these samples were identified by first growing them on blood agar and Mannitol salt agar media. Typical colonies that developed were subjected to Gram staining and typical *S. aureus* biochemical tests which included mannitol fermentation, coagulase test, catalase test, beta-hemolysis production. The confirmatory diagnosis for *S. aureus* was made by using the VITEK 2 System (BioMerieux, France).

Antibiotic sensitivity test

Disk diffusion method that described by Kirby was carried out to detect antibiotic resistance pattern of *S. aureus* to ten antibiotics (Penicillin, Ciprofloxacin, Tetracycline, Erythromycin, Clindamycin, Augmentin, Ceftriaxone, Vancomycin, Chloramphenicol, and Gentamicin). The inhibition zone diameter was measured compared with CLSI 2012 (13).

The nanotechnology studies

Biosynthesis of silver nanoparticles (AgNPs) produced by using *E. coli*

E. coli will grow in Luria broth medium and incubated on an orbital shaker at 37 C° and 200 rpm. After 24 hr of growth, the biomass was harvested and centrifuged at 10000 rpm for 10 mins. To 200 ml of the bacterial supernatant taken separately in five tubes, silver nitrate solution (100ml for all concentration) was added at a concentration of 5, 10, 15, 20 and 25 mM. The last tube (control) consisted of 200 ml of supernatant with no AgNO3 added. The mixture was mixed and incubated at 30C° for 24 h in the dark. After 24 hours, the color change from yellow to brown was noted (14).

Characterization of nanoparticles

X-ray diffraction (XRD)

XRD was used to characterize the silver nanoparticles.

Ultraviolet visible spectroscopy

The reduction of pure AgNPs was measured using (UV) visible spectroscopy between 300 nm and 700 nm (15).

Scanning Electron Microscope (SEM)

SEM was used to determine the morphology and size of the nanoparticles. The composition of the material was assessed using EDS analysis (connected to a SEM) for point and mapping analysis.

Atomic Force Electron Microscopy (AFM)

AFM images were captured on the Nano structured film of AgNPs collected for 5 minutes by vertical adsorption from the colloid solution on glass plates and dried in the air. Simultaneously, spatial, and chemical spectral information of the nanoparticles were obtained as described previously (15).

Transmission Electron Microscopy (TEM)

TEM reveals the shape and crystal structure (if any) of the particles, as well as their size. A drop of the nanoparticle suspension was placed on a carbon-coated copper grid and the water allowed to evaporate inside a vacuum dryer to create the grid for TEM analysis. TEM was used to scan the grid containing AgNPs.

Anti-bacterial activity of AgNPs and chlorhexidine

The antibacterial activity of AgNPs and chlorhexidine was tested by the disk diffusion method, with three replications for *S. aureus*. Discs (6 mm dia.) prepared from Whatman No. 3 filter paper, sterilized in an autoclave and saturated with AgNPs and chlorhexidine (each separately) for one hr, and left to dry. *S. aureus* bacterial suspension was spread plated onto Mueller-Hinton agar medium, followed by placing discs saturated with AgNPs and chlorhexidine discs using sterile forceps on the plates (each separately), and incubating the plates at 37°C. After 24 h, the zones of inhibition (in diameter) around AgNPs and chlorhexidine discs were measured.

MIC determination for AgNPs and chlorhexidine

To determine the MIC of AgNPs and chlorhexidine against *S. aureus*, several dilutions of AgNPs (25 mM) and chlorhexidine solution were prepared, Whatman No. 3 filter paper discs (diameter of 6 mm), sterilized and saturated with different concentrations of AgNPs and chlorhexidine (each separately) for one hour and left to dry. Each disc was placed on bacteria spread plated onto Muller Hunton agar plates, followed by incubation at 37 °C for 18-24 hrs. The diameter of the inhibition zone (mm) around the AgNPs and chlorhexidine discs were measured and the MIC which is the lowest concentration inhibiting bacterial growth was recorded.

Synergy test between antibiotics with AgNPs and antibiotics with chlorhexidine

The synergy was tested between (Penicillins and Ciprofloxacin) with AgNPs and between (Penicillins and Ciprofloxacin) and chlorhexidine (each separately) against *S. aureus*, by three replications. Briefly, pure colonies of *S. aureus* were picked and transferred to tubes containing 5 ml of physiological salt solution to obtain a suspension of McFarlan tube no. 0.5 turbidity. The suspension was plated onto Muller Hinton medium followed by placing Penicillin and Ciprofloxacin discs saturated with AgNPs and

chlorhexidine (each separately). The plates were incubated at 37 °C for 18-24 hours, and the diameter of the inhibition zone was measured and compared with the results of using the antibiotics, AgNPs, and chlorhexidine alone.

Gene expression studies

RNA was isolated using the Trizol method described previously (16). Expression of the *ica*A gene was studied using the One-Step qRT- PCR Kit (Promega Corporation, USA) based on the manufacturer's instruction. Briefly, the reaction mixture (10 µl) consisted of 0.5 µl each of forward and reverse *icaA* gene primers 0.5 µl of the Probe, 0.25 µl of the reverse transcriptase RT enzyme, 1.5 µl of RNA template and the final volume made up with 1.75 of nuclease free water. qRT-PCR was carried out in a qRT-PCR machine (MIC Bio Molecular System, Australia) and the RNA concentration quantified using Quantus fluorometer.

The *ica*A gene expression studies were carried out on 16 *S. aureus* isolates which included isolates treated with AgNPs and Penicillins, isolates treated with Ciprofloxacin, isolates treated with AgNPs mixed with Penicillins, isolates treated with mixed AgNPs and Ciprofloxacin, isolates treated with mixed AgNPs, Penicillins, and Ciprofloxacin, and isolates treated with mixed AgNPs, penicillins, and ciprofloxacin, and isolates that treated with chlorhexidine.

The Livak equation was used to calculate the Cycle Threshold (Ct) values that appear in the form of Dissociation Curve at which the fluorescence is at its peak and these values represent the amount of gene expression of the target gene and the titration gene in the isolates.

Statistical analysis

Data on *S. aureus* antibiotic susceptibility was subjected to statistical analysis using GraphPad Prism 5.0 software.

RESULTS

Staphylococcus aureus could be isolated from 62 out of the 266 periodontal infection samples in this study. The antibiotic resistance profile of *S. aureus* in this study is shown in Fig.1. As seen, most of the values were lower than the control values, the highest resistance of this bacteria was towards Penicillin, while the least resistance was by Ciprofloxacin (12 mm). The inhibition zones of antibiotics chloramphenicol, gentamicin, clindamycin, erythro-mycin and tetracycline were 10, 10, 23, 26 and 21 mm respectively

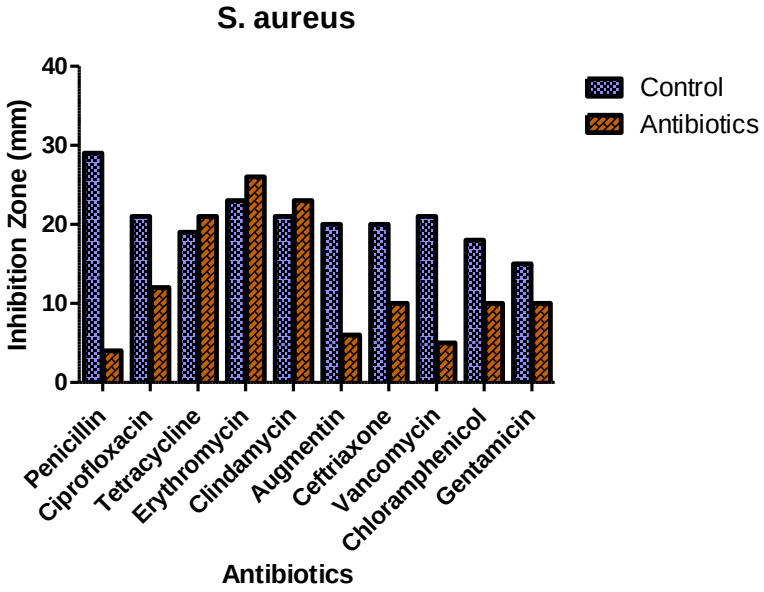

Fig. 1 Antibiotic Susceptibility of *S. aureus*

AgNP biosynthesis by *Escherichia coli*

E. coli showed the capacity to synthesize AgNPs extracellularly. The synthesis of AgNPs was confirmed by a colour change from pale yellow to dark brown (Fig. 2).

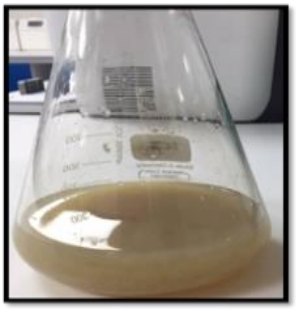

Fig. 2: Dark brown solution indicating the AgNP synthesis by *E. coli*

Characterization of biogenic silver nanoparticles

X-ray diffraction (XRD)

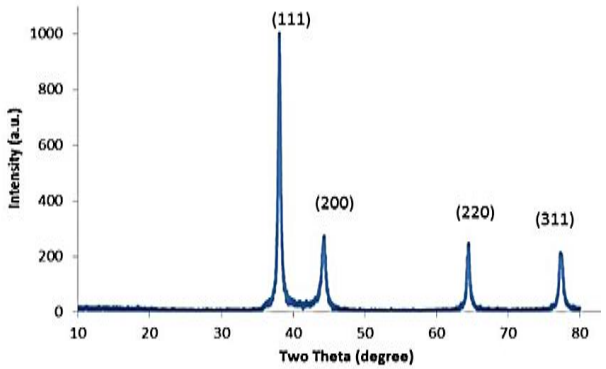

Fig. 3: XRD analysis of Biosynthesized nanoparticles from *E. coli*

The XRD pattern of AgNPs is shown in Fig. 3. The primary peaks are at 38.80, 44.340, 64.50, and 77.480, which correspond to the planes (111), (200), (220), and (311), respectively.

The ultraviolet (UV)-visible spectroscopy

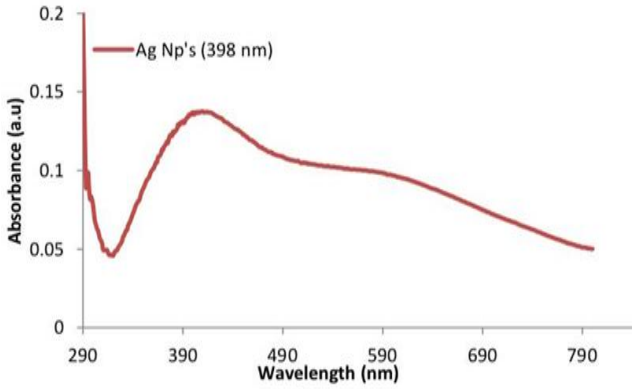

Fig. 4: The UV-spectroscopy shows a significant absorbance peak at 398 nm.

The absorption peak at 398 nm indicates the presence of surface plasmon resonance (SPR) of nanoparticles (Fig. 4).

Scanning electron microscope (SEM) analysis

The results demonstrated well dispersed and homogeneous nanoparticles with a diameter of 24.57 nm for AgNPs with varying forms, the majority of which were spherically shaped (Fig. 5).

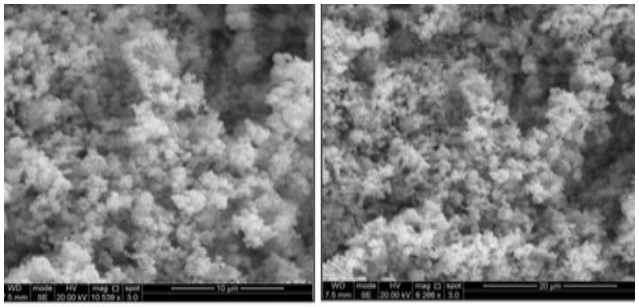

Fig. 5: SEM micrograph of a silver nanoparticle

Atomic force microscope (AFM)

The AFM image revealed the shape of AgNPs, as well as the average diameter and roughness; the average diameter of silver nanoparticles was 33.53 nm (Figs. 6A and 6B).

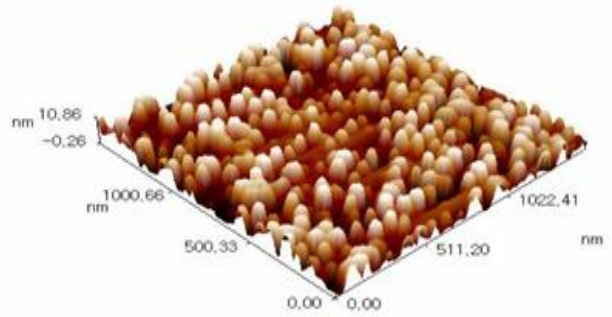

Fig. 6A: 3-D AFM image of biogenic AgNPs

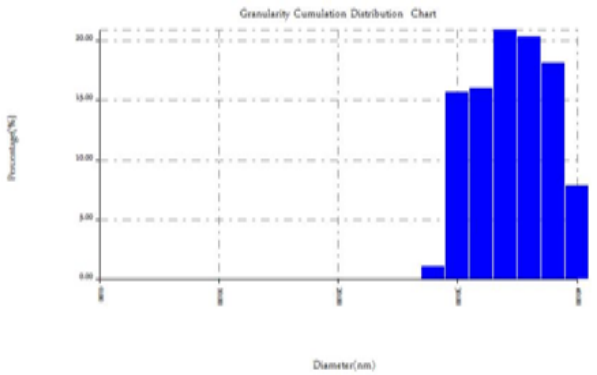

Fig. 6B: Granularity cumulating distribution map of biogenic AgNPs

Transmission electron microscopy

The outcome demonstrated the diverse forms and sizes of AgNPs (Fig.7). AgNPs were primarily in the 19-21 nm size range in the current investigation.

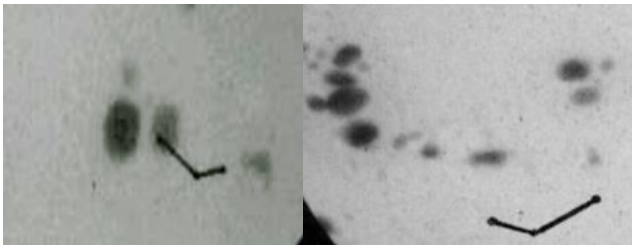

Fig. 7: TEM images of AgNPs (19-21 nm)

Antibacterial activity of AgNPs and Chlorhexidine in-*vitro*

The antibacterial activity of AgNPs and Chlorhexidine was tested using the disks diffusion method, our result showed that there is significant inhibition effect of AgNPs on *S. aureus* (Table 1).

Table 1: The effect of AgNPs with different concentrations on *S. aureus*

No	AgNPs mM concentration	Averaged diameters of inhibition zone (mm)
38	25	1
26	20	2
18	15	3
9	10	4
0	5	5

Furthermore, examination of the antibacterial activity of chlorhexidine revealed that the width of the inhibition zone for *S. aureus* was 13 mm.

Synergistic effect of silver nanoparticles with antibiotics and Chlorhexidine with antibiotics against *S. aureus*

The current study's findings revealed that the interaction of AgNPs (25 mM) with antibiotics increased the *S. aureus* sensitivity to antibiotics (Table 5). For e.g., while the zone of inhibition for ciprofloxacin alone was 12mm, this increased to 23.2 mm when ciprofloxacin was used in combination with AgNPs. Similarly, the antibacterial effect of chlorhexidine was observed to be enhanced when used in combination with the antibiotic's ciprofloxacin as well as procaine (Table 2).

Table 2: The inhibition zone diameter (mm) of treatments against *S. aureus*

Treatments	Inhibition zone (mm)
Ciprofloxacin	12
Procaine	0
Ciprofloxacin+AgNPs	23.2
Procaine+AgNPs	28.5
Chlorhexidine	13
Ciprofloxacin+ Chlorhexidine	20
Procaine+Chlorhexidine	15

Quantification of gene expression using qRT-PCR technology

In this study the RNA concentration was extracted from *S. aureus* that treated with AgNPs (25mM), penicillin, ciprofloxacin, mixed AgNPs with the penicillin, mixed AgNPs with ciprofloxacin, mixed AgNPs with penicillin and ciprofloxacin, and also RNA was extracted from *S. aureus* that treated with Chlorhexidine in sub-MIC concentrations and by two replicates for each treatment, RNA was obtained with high concentrations, ranging from 290-97.8 ng/µl (Table 6).

Table 3: *S. aureus* RNA concentration and *ica*4 gene expression values obtained for different treatments

No.	Treatment	RNA concentration (ng/µl)	*icaA* gene expression values $2^{-\Delta\Delta CT}$ (folding)
1	Control	250	1
2	AgNPs	280	0.057
3	Procaine	213	2.07
4	Ciprofloxacin	273	1.3
5	AgNPs- Procaine	112	0.0308
6	AgNPs- Ciprofloxacin	290	0.0115
7	AgNPs-Ciprofloxacin-Procaine	97.8	0.0002
8	Chlorohexidine	280	0.098

The Ct result for each treatment was compared with Ct for gene expression of the housekeeping *icaA* gene. The *S.aureus ica*4 gene expression values calculated based on Livak equation for each treatment is presented in Table 3. The results showed that the gene expression was considerably reduced when treated with AgNPs as well as AgNPs in combination with either procaine, ciprofloxacin or both when compared to control (Table 3). The lowest expression was seen in treatment with AgNps combined with ciprofloxacin and procaine (0.0002) (Table 3). The *ica*4 gene expression in *S. aureus* was also seen to substantially decrease (0.098) when treated with Chlorhexidine when compared to control (Table 3).

DISCUSSION

S. aureus is regarded as a transitory bacterium in the oral cavity. Patients with periodontal disease may be repositories of these opportunistic microorganisms in the oral cavity, together with other microorganisms (2,17). An earlier study showed *S. aureus* isolated from periodontal infection to be resistant to the commonly used antibiotics ampicillin (92.6%), penicillin (90.7%), oxacillin (11.1%) and erythromycin (5.6%) (18). *S. aureus* in this study showed complete resistance to the antibiotic procaine, while it was moderately resistant to ciprofloxacin. However, when used with AgNPs the efficacy of these antibiotics was found to be higher in this study. These findings agree with a similar work carried out earlier (18), wherein antibiotics such as vancomycin, gentamicin, streptomycin, ampicillin, and kanamycin when combined with AgNPs showed enhanced efficacy against *P. aeruginosa, S. aureus*, and *E. coli*. Antibacterial nanoparticles have been used in endodontic treatments (19) as these nanoparticles have the capacity to adhere to bacterial cell walls leading to the disruption of the bacteria. Thus, the enhanced sensitivity observed in the presence of AgNPs could be attributed to these nanoparticles and their capacity to be bactericidal. Our studies also revealed the antibacterial effect of AgNPs to increase with increasing concentration which is in line with a previous study which also showed the concentration of AgNPs to have an effect on its antibacterial action (20).

Similarly studies with the antiseptic agent chlorhexidine in this study also showed that although it could control the growth of *S. aureus*, the inhibition being much higher when used with AgNPs. Chlorhexidine a cationic bisbiguanide antiseptic, is a positively charged hydrophilic and lipophilic molecule

that interacts with phospholipids and lipopolysaccharides of cell membranes resulting in disruption of cell membranes, enabling it to enter the cells and cause cytoplasmic coagulation (21). Chlorhexidine has been popularly used in dressings and obturating pastes for teeth with peri radicular pathosis, to successfully destroy most bacteria within dentinal tubules of teeth.

Synergy studies for chlorhexidine with antibiotics also showed an increasing effect to the sensitivity of *S. aureus* to antibiotics in this study, which is in line with an earlier work which showed that catheters impregnated with chlorhexidine together with minocycline and rifampicin prevented biofilm formation by gram negative bacteria (22) resulting in decreased incidence of catheter-related infections (23). There are many studies related to find alternative of antibiotics to inhibition bacterial growth or some of virulence factors (24-26).

To understand how exactly the combination of AgNP with antibiotics or AgNP with chlorohexidine enhances the antibacterial effect, we also investigated the gene expression of the *S. aureus* housekeeping gene ica4. Our studies showed that AgNPs when combined either with antibiotics or antiseptics greatly reduced the ica4 gene expression levels, which shows that AgNPs could be acting at a molecular level to exert its antibacterial effect. Study on mechanism of CuO and ZnO nanoparticles have revealed these nanoparticles to generate DNA single-strand breaks and affect gene expression (27). Hence, further molecular studies on the synergistic effect of AgNPs with antibiotics and antiseptics are needed to find the exact mechanism of their antibacterial activity of this gram-positive bacterium.

CONCLUSION

The current study's findings indicate that biologically produced AgNPs were efficient against the bacterium *S. aureus.* The use of biologically produced AgNPs is a viable and alternative technique for treating MDR bacterial infections.

CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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