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Synergistic antibacterial activity of Silver, Zinc oxide and Copper oxide nanoaprticles against *Camphylobacter jejuni* isolates from humans with diarrohea and detection of virulence factor genes.

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Abstract

Campylobacter jejuni (*C. jejuni*) is the most common cause of foodborne gastroenteritis worldwide. The bacteria induce diarrhea and inflammation by invading the intestinal epithelium. Nanotechnology is currently contributing substantially to the development of a broad range of innovative technologies in the medical field. To date, very little work has been done to investigate the efficacy of antimicrobial nanoparticles against the gastrointestinal pathogens. The present study characterized the nanoparticles by SEM, UV spectroscopic and XRD analysis. Also, this study is to report on the virulence factors and synergistic effectiveness of CuO, Ag and ZnO nanoparticles against Campylobacter through invitro condition. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by a broth microdilution method in 96 well plates. The MIC for all Campylobacter strains was in the order of Ag _ CuO _ ZnO nanoparticles. Ag nanoparticles were the most effective against *Camphylobacter jejuni*, Which was determined as bacteriostatic. Meanwhile in synergism, with the help of three different mechanism action of nanoaprticles determined complete bactericidal activity. On the other hand, a low prevalence of the virB11, wlaN, and iam putative pathogenic genes were detected in only 3 strains, respectively.

Key words: Nanotechnology, Camphylobacter, minimum bactericidal concentration, pathogens and silver.

INTRODUCTION:

Campylobacter jejuni is the most prevalent pathogenic bacterium of zoonotic gastroenteritis [1]. Typical symptoms provoked by C. jejuni are watery to bloody diarrhea, abdominal pain, fever, and nausea [2]. This human pathogen is present in the intestinal microbiota of farm animals, especially poultry, which is the main source of infection for humans by ingestion of contaminated or undercooked food [1].

The bacteria adhere to the mucus and the surface of intestinal epithelial cells, invade the intestinal epithelium, while they pass the cells via the transcellular or paracellular route [3]. Consequently, direct epithelial barrier defects such as dysregulation of tight junction (TJ) proteins, induction of epithelial lesions or also indirect effects by inflammatory responses of epithelial or immune cells occur [4].

The pathogenesis of Campylobacter infection is complex and still poorly understood. However, it is believed that the expression of genes involved in motility, colonization, epithelial cell invasion, and toxin production play an important role in the disease development [5]. Mobility of the bacterial cells, involving the coordination of several genes (i.e., flaA and flhA), is essential for passage through the stomach and gut environment where Campylobacter produces several cell-surface proteins (encoded by the cadF, docA, racR, virB11, ciaB, and iam genes) that promote to adhere to and invade intestinal epithelial cells [6]. The bacteria can also excrete several cytotoxins (encoded by the cdtA, cdtB, cdtC, wlaN genes) that contribute to the development of the disease. Furthermore, C. jejuni is able to produce superoxide dismutase enzyme (encoded by the sodB marker), which catalyzes the breakdown of superoxide radicals and it is one of the bacterial major defense mechanisms against oxidative damage [7]. There has been an increasing trend of antimicrobial resistance in Campylobacter isolated within the food chain and humans in recent years.

Macrolides (i.e., erythromycin) and fluoroquinolones (i.e., ciprofloxacin) are considered as the first- and secondchoice of antimicrobials, respectively for the treatment of human Campylobacter infections. [8] Most campylobacteriosis cases are usually self-limiting and do not require hospitalization and antimicrobial treatment. However, the therapy is required in children with fever, increasing bloody diarrhea, and in eldery or immunocompromised patients with severe and prolonged systemic disorders nanotechnology has been used to enhance food safety in production machinery through the use of machine coatings and nano-sieves (e.g., to filter out bacteria).

Silver (Ag) nanoparticles (NP) and nanocomposites are the most widely used antimicrobial nanomaterials in the food industry. Ag NPs display biocidal activity against a broad range of Gram-positive and Gram-negative microorganisms, yeast, moulds and viruses. The antimicrobial activity of Ag nanomaterials is mainly based on the following mechanisms: (a) release of Ag ions which

bind to electron donor groups in molecules containing sulphur, oxygen or nitrogen, (b) disruption of DNA replication and, (c) oxidative stress through the catalysis of reactive oxygen species (ROS) formation [9]. A number of metal and metal oxide nanomaterials have also long been suggested to be effective as antimicrobials. Their intrinsic physicochemical properties allow excessive formation of reactive oxygen species (ROS), leading to oxidative stress and subsequent cell damage. In addition, the release of metal ions outside the cell, at the cell surface, or within the cell can alter cellular structure or function. Zinc oxide and copper oxide nanomaterials have been utilized in food packaging and coatings because of their antimicrobial and/or antifungal properties. Like Ag NPs, zinc oxide and copper oxide NPs have been shown to have a wide range of antibacterial activities against both Gram-positive and Gram-negative bacteria, including major foodborne pathogens like Escherichia coli O157:H7, Salmonella, Listeria monocytogenes, Campylobacter jejuni and Staphylococcus aureus Zinc oxide (bulk, > 100 nm) has also been granted Generally. [10] Copper oxide nanoparticles have been shown to demonstrate antibacterial activity against a number of Gram-positive and Gram-negative bacteria.

Several factors can have an impact on the effect of NPs against microorganisms, including the size, shape, stability and concentration of the NP. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values are popular and broadly useful determinants of the relative antimicrobial activity of many synthetic nanomaterials [11].

Therefore, the objective of this study was to investigate the efficacy of antimicrobial NPs against *Campylobacter jejuni*. To achieve this, the *in vitro* Synergistic activity of silver, zinc oxide (ZnO) and copper oxide (CuO) NPs was assessed against 10 isolates of *Campylobacter jejuni*.

MATERIALS AND METHODS

Bacterial culture conditions

The ten strains used in this study, all isolated from Human samples, *Campylobacter jejuni* isolates were grown on Mueller Hinton (MH) agar at 42^0C for 48 h in a microaerophilic atmosphere created with Campygen satchets (Thermo Fisher Scientific, Australia). Colonies were suspended in MH broth to an OD 595 of 0.2 (approximately 107 cfu/mL). This suspension was further diluted in MH broth to obtain a culture of 105 cfu/mL.

Identification of *Campylobacter jejuni*

Microscopic stool examination by Gram stain is useful for rapid diagnosis and treatment of Campylobacter infection in patients with acute onset diarrhoea.

Nanoparticle preparation:

ZnO NP powder (<50 nm) was purchased from Sigma (Chennai). The CuO (50 nm) suspension in Milli-Q water and Ag (20 nm) NP suspension in aqueous polyvinyl pyrrolidone (PVP), were purchased from Nanocomposites (Sisco research laboratory, Chennai) at a concentration of 1 mg/mL. Bulk forms of CuO (size <7 mm). AgNO3 were used for comparison and were purchased from Sigma. All powdered material (ZnO NPs and AgNO3) was mixed with LB/MH broth at 22^0C (between 1 mg/ mL and 10 mg/mL) and vortexed for 30 s. The ZnO NPs were sonicated in a bath sonicator (Sigma, chennai) operating at 48Wfor 30 min and used within 3 h of preparation. NP suspensions were vortexed for 30 s before each experiment and for 10 s before each use.

Characterization of silver nanoparticles

The NPs mixed LB broth was centrifuged twice to isolate the AgNPs, CuONPs, ZnO NPs and to eliminate the unwanted surplus. It was dried in a hot air oven for 30 minutes at a temperature of 100^0C.

Synthesized silver nanoparticles, Copper oxide nanoparticles and Zinc oxide nanoparticles were confirmed by UV–Vis spectroscopy and it was carried out using UV-Vis spectrophotometer in the 200–1100 nm range. Detailed analysis of the morphology, size and distribution of the nanoparticles was documented by Scanning Electron Microscopy (SEM) machine and the presence of reflection by XRD patterns.

Presence of Virulence Factor Genes:

Campylobacter isolates were tested for the presence of the following virulence genes: flaA and flhA (involved in motility), cadF, docA, racR, virB11 (responsible for adhesion and

colonization) cdtA, cdtB, cdtC, wlaN (cytotoxin production). Additionally, the gene markers such as ciaB and iam responsible for the invasiveness of Campylobacter, and sodB (stress response) were also amplified.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC):

The MIC was determined by a broth microdilution method using LB or MH (for Campylobacter) broth in 96 well plates. Twelve concentrations of the selected NPs were prepared by 2-fold dilution in LB or MH broth. The twelve concentrations for CuO and Ag NPs were 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.098 and 0.049 mg CuO NP or Ag NP/mL (to a maximum of three decimal places). The ZnO NP and bulk control concentrations were 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.063, 19.531, 9.766, 4.883 and 2.441 mg ZnO/mL (to a maximum of three decimal places). Addition to each 96 well plate was always in the order of broth, NP and then bacteria. Row A was a negative control containing 100 mL LB broth and 100 mL of the relevant NP. NPs were added to the control to allow for any effect of light scattering due to the NPs. Rows B to G contained 100 mL of NP, 50mL of LB broth and 50 mL of 105 CFU/mL bacterial suspension. Row H was the positive control, containing 150 mL of LB broth and 50 mL of the bacterial suspension. For Campylobacter, the OD595was read at 0 h and again at 48 h after incubation at 42 ^{0}C in a microaerophilic atmosphere.

The MIC of each isolate was determined in triplicate on two plates, resulting in a total of six replicates for each

strain/NP combination. The MBC was calculated from the same 96 well plates used for the MIC by counting the bacteria from wells with no or very little visible growth on either LB or MH agar. For the purposes of this study the MIC is the lowest concentration resulting in no visible growth. The MBC is the lowest concentration that kills 99.9% of the initial population.

RESULTS:

The present cross sectional study was conducted at Clinical Microbiology Laboratory of Saveetha Medical College and Hospital during the period of Nov 2018 to Feb 2019. Ethical clearance was obtained.

Out of 10 samples yielding *Camphylobacter jejuni*, 6 (60%) were from female patients and 4 (40%) were from male patients.

Among 10 samples of *Camphylobacter jejuni*, all the samples were from Gastroenterology (100%).

Cultural characteristics:

Colonies were suspended in LB broth to an OD595 between 0.03 and 0.04, approximately 107 CFU/mL (Pharmacia Novaspec II). This was further diluted in LB broth to obtain a culture of 105 CFU/mL for use in MIC and 104 for growth studies. Campylobacter jejuni isolates were grown on Mueller Hinton (MH) agar at 42^0C for 48 h in a microaerophilic atmosphere created with Campygen satchets (Thermo Fisher Scientific, Australia). Colonies were suspended in MH broth to an OD595 of 0.2 (approximately 107 cfu/mL). This suspension was further diluted in MH broth to obtain a culture of 105 cfu/mL. Gram-negative spiral rods were noted on Gram stain of stool sample [figure: 1].

Figure: 1 Result for gram staining

Presence of Virulence genes:

All of them were positive for 9 out of 10 putative virulence genemarkers tested, i.e., flaA, cadF, docA, racR, cdtA, cdtB, cdtC, ciaB, and sodB. On the other hand, a low prevalence of the putative pathogenic marker genes was associated with the virB11 which was detected in only 1 of the total number of strains. Furthermore, the wlaN and iam genes were found in 2 nd and 3 rd of all strains tested, respectively.

Characterization of nanoparticles

UV-visible spectroscopy

The synthesis of silver, Zinc oxide and Copper oxide nanoparticles had been confirmed by UV-visible spectroscopy. The UV-visible spectrum showed distinct absorption beak at (279nm, 212nm and 429nm respectively). Thus the reduction of all the 3 nanoparticles were confirmed by the UV- visible spectra. [Figure: 2]

Figure:2 Graphical representation of UV- visible spectra

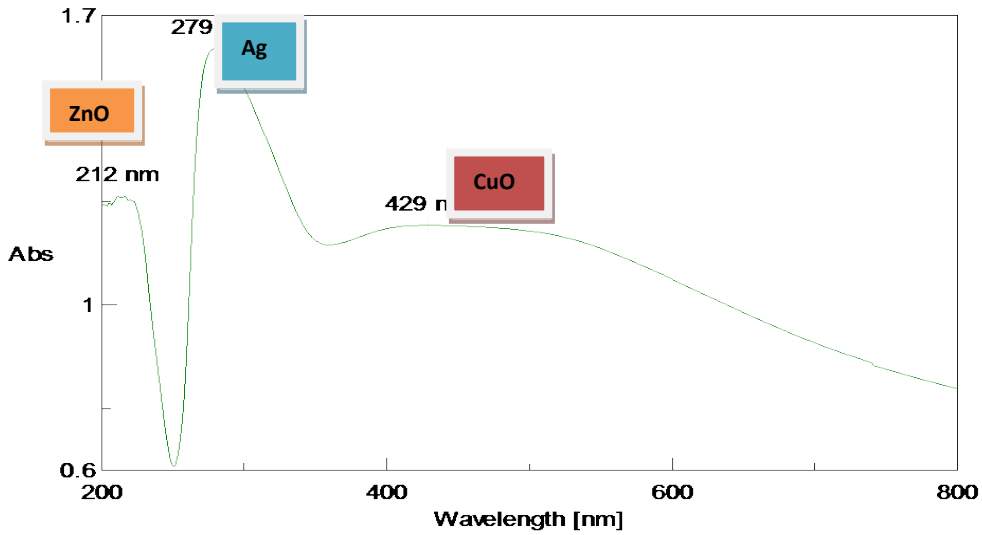
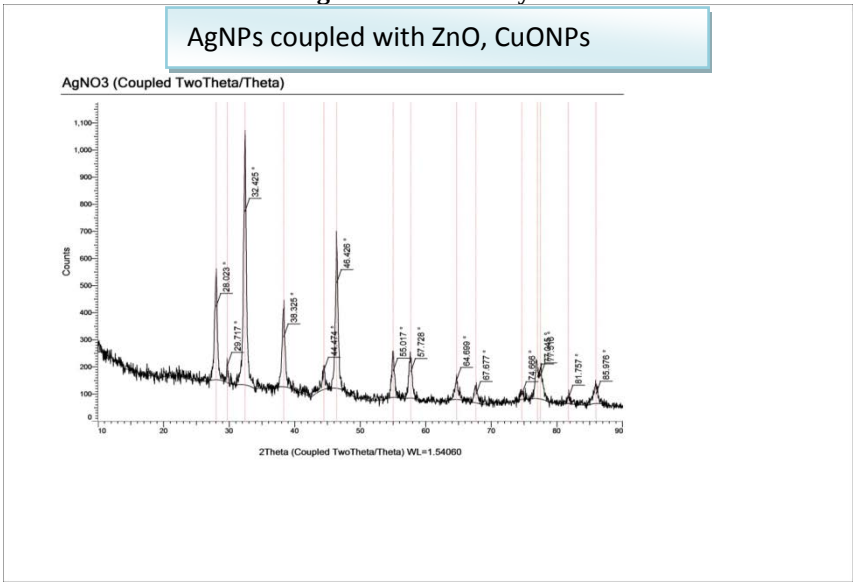


Figure: 3 XRD analysis



XRD patterns:

All the 3 nanoparticles were characterized according to bragg reflection. [Figure: 3]

SEM analysis:

Morphological character and size details of the silver, zinc oxide and copper oxide nanoparticles were presented by SEM images. All the size of the nanoparticles was investigational from the SEM image between the ranges of 40-90 nm (Fig 4).

Figure: 4 SEM results

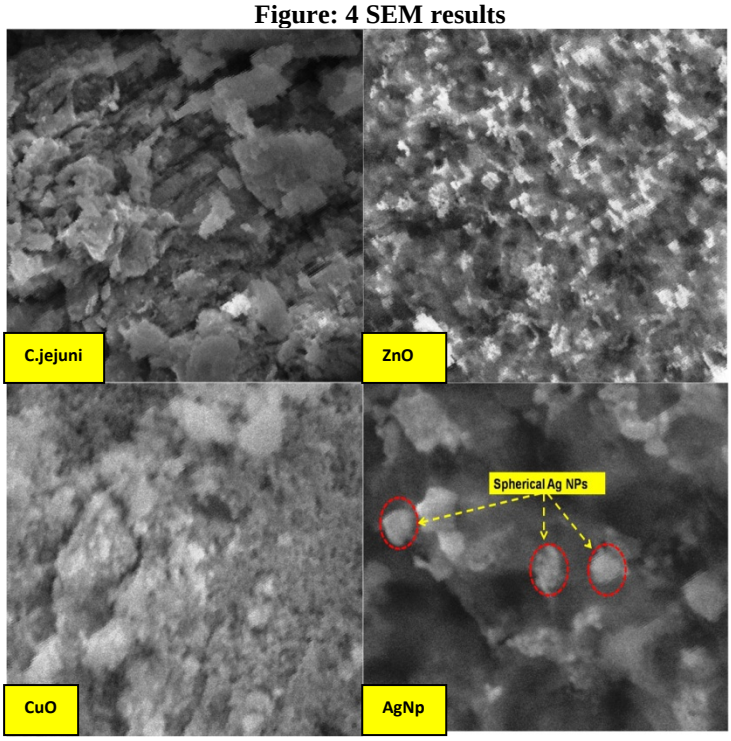


Table: 1 MIC and MBC values of silver, zinc oxide and copper oxide nanoparticles against Campylobacter.

Camphylobacter strains	MIC [µg/ml]			MBC [µg/ml]		
	Nanoparticles					
	Ag	**CuO**	**ZnO**	**Ag**	**CuO**	**ZnO**
1st strain	**3.125**	**6.25**	**25**	**6.25**	**12.5**	**3.125**
2nd strain	**6.25**	**12.5**	**25**	**12.5**	**12.5**	**12.5**
3rd strain	**3.125**	**12.5**	**50**	**12.5**	**6.25**	**6.25**

MIC and MBC

The MIC for each strain against the tested NPs is listed in table:1. For all strains Ag NPs had the lowest MIC followed by CuO and ZNO NPs. The MIC of CuO against *Camphylobacter jejuni* was not determined as the maximum concentration it was possible to test was not inhibitory. All Campylobacter strains had a lower MIC tested, across all NPs. The MBC for each strain is also displayed in Table 1. The concentrations used were not high enough to determine the MBC of CuO (>100 mg/mL) and ZNO (>5000 mg/mL) NPs against *Camphylobacter jejuni*. The MBC of all NPs against Campylobacter was found to be either the same as the MIC or no more than one dilution different.

DISCUSSION:

In this study the in vitro activity of NPs of Ag, ZnO and CuO were assessed against ten relevant isolates of Campylobacter. The antibacterial activity of the 3 NPs was compared to each other, by determining the MIC and MBC in liquid media. As Campylobacter is an obligate microaerophile and therefore unlikely to grow in the anaerobic environment, growth experiments were not performed on any of the ten Campylobacter strains.

The results of this study indicated that Campylobacter was more sensitive to each NP and its dissolved form with MIC values ranging from 2 to 50 times lower. In the only other published study to compare the level of resistance of Campylobacter to NPs, Xie et al. (2011) reported that the MIC of ZnO NPs for C. jejuni was 8- to 16-fold lower than that for Salmonella enterica serovar Enteritidis. Xie et al. (2011) concluded that the ZnO NPs displayed extremely strong activity against C. Jejuni compared to Salmonella due to the different tolerances of these organisms to oxidative stress induced by NPs.

The MIC and MBC readings for Campylobacter were taken 24 h. Also, the growth media, temperature and atmosphere during incubation varied between cultures of Campylobacter (MH broth, 42 oC, microaerophilic). For metal and metal oxide materials, depending on the detailed nature of the surfaces of the metal NPs and the manufacture's preparation, these NPs can dissolve, aggregate, or remain suspended as single particles in aqueous solutions (Stebounova, Guio, & Grassian, 2011).

The state of the NPs, i.e., whether they are present as isolated particles, aggregate, or dissolve will also vary depending on the environmental conditions (pH, ionic strength, presence of organic matter, etc.) (Stebounova et al., 2011). The size of the NP's in LB broth as characterized by UV spectroscopy, is comparable to the size stated by the manufacturers. However the size of the NP's as measured by the SEM analysis that some aggregation may occur in LB broth. The preparation of the material for the SEM analysis was conducted by staff in a different laboratory to all other experiments including preparation of NP's for XRD. Thus, an awareness of the characterisation of physical and chemical properties of nanomaterials under different conditions is essential for understanding their behaviour and potential effect on microorganisms.

All ten Campylobacter strains were sensitive to Ag NPs. While direct comparison between our results and those in the published literature is hampered by variation in the methods used, our results are consistent with those of Zarei, Jamnejad, and Khajehali (2014). Zarei et al. (2014) reported that Ag NPs displayed antimicrobial activity against a single strain of Camphylobater grown in tryptone soya broth at 35 oC, reporting an MIC of 3.12 mg/mL and an MBC of 6.25 mg/mL. While the MIC and MBC reported by Zarei et al. (2014) are lower than those reported in the present study, this may in part be due to the smaller size of the Ag NPs used in their study (10 nm) compared to the average size in this study (20 nm) or differences in NP dissolution behaviour.

To our knowledge, the present study is the first to examine the synergistic antimicrobial effect of Ag NPs, Zn O, CuO NPs against Campylobacter. As the leading cause of bacterial gastroenteritis in India, furthering our knowledge of potential control measures for Campylobacter is an important step towards lowering the incidence of food borne disease. The Campylobacter isolates exhibited an MIC ranging between 3.125 and 6.25 mg/mL and an MBC between 3.12 and 6.25 mg/mL. In a report by Hastings, Colles, McCarthy, Maiden, and Sheppard (2011), inclusion of a Ag ion biocide in poultry transportation crates was shown to be effective in reducing the level of Campylobacter during normal crate use compared to standard crates (Hastings et al., 2011). Ag NPs might exhibit additional antimicrobial capabilities not exerted by bulk or ionic silver (Marambio-Jones & Hoek, 2010). In terms of the comparatively lower level of resistance exhibited by Campylobacter to Ag NPs,

However, it is important to note that limited evidence has been reported of the resistance shown by Ag-resistant strains to Ag NPs (Marambio-Jones & Hoek, 2010). All five Campylobacter strains displayed a higher level of inhibition to the dissolved form of Ag compared to the nano-form as measured by MIC. While the AgNO3 solution was prepared by

allowing for the weight of Ag only, when comparing the number of Ag molecules in the AgNO3 solution/mL against the number of Ag particles/mL in the NP solution there are approximately five times the number of molecules in AgNO3 solution.

The ineffectiveness of CuO NPs against the five Salmonella strains may be explained by the arsenal of Cu detoxification strategies that Camphylobacter contains (Pontel et al., 2014).

Pontel et al. (2014) also identified a number of novel copper upregulated genes coding for putative detoxification factors. The MIC for the ten strains (312.5e625 mg ZnO/mL) is lower than that reported by Tayel et al. (2011). The relative sensitivity of Campylobacter (MIC 25e50 mg/mL) to ZnO NPs is in keeping with the findings of Xie et al. (2011). Xie et al. (2011) reported that ZnO NPs exhibited remarkable antibacterial activity against C. jejuni, even at low concentrations. ZnO NPs induced significant morphological changes, measurable membrane leakage, and substantial increases (up to 52-fold) in oxidative stress

gene expression in C. jejuni (Xie et al., 2011). Xie et al. (2011) investigated the mechanism of action of ZnO NPs against C. Jejuni by examining cell morphology, membrane permeability and gene expression and proposed that the primary antibacterial mechanism of ZnO NPs is likely due to oxidative stress in C. jejuni cells. Earlier studies have also proposed possible mechanisms of cell inactivation by metal oxide NPs, including membrane damage caused by direct or electrostatic interaction between the NPs and cell surfaces, cellular internalization of the NPs, and the production of active oxygen species such as H2O2 in cells due to metal oxides (G. Fu, Vary, & Lin, 2005).

CONCLUSION:

Nanomaterials exhibit outstanding antibacterial properties thatv give rise to many otentially beneficial applications in the food industry. The effectiveness of Ag and CuO NPs against the leading cause of foodborne bacterial gastroenteritis, Campylobacter, was demonstrated for the first time and this study also confirms the effectiveness of ZnO against poultry sourced Campylobacter strains.

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Conflict of interest: The authors declare no conflict of interest.

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