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Original Article

Antibacterial activity of microwave synthesized hydroxyapatite against cariogenic bacteria: A preliminary study

Ahmad Zharif Ibrahim [a,b], Alaa Sabah Hussein [a,*], Hasnah Begum Said Gulam Khan [c], Norzalina Ghazali [b]

[a] Centre of Paediatric Dentistry and Orthodontic Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh, Selangor, Malaysia
[b] Department of Paediatric Dentistry & Orthodontics, Faculty of Dentistry, Universiti Sains Islam Malaysia, Kuala Lumpur, Malaysia
[c] Centre of Preclinical Science Studies, Faculty of Dentistry, Universiti Teknologi MARA, Sungai Buloh, Selangor, Malaysia

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ABSTRACT

Introduction: The effects of hydroxyapatite (HA) on oral bacteria and biofilm remains inconclusive, with conflicting results. Studies assessing its effect against caries-causing bacteria are limited.
Objective: This study aimed to explore the antibacterial activity of HA synthesized using microwave against two of the most common cariogenic bacteria, *Streptococcus mutans (S. mutans)* and *Streptococcus sobrinus (S. sobrinus)*.
Methods: HA was chemically synthesized using a microwave. To verify the existence of the crystalline phase and the calcium and phosphate content, X-ray diffraction (XRD) and energy-dispersive X-ray (EDX) analysis were employed, respectively. Reduction in bacterial growth was used to assess the antibacterial effects of 10 %, 20 %, and 30 % HA against the tested bacteria.
Results: The presence of the hydroxyapatite crystallite phase was verified using XRD, while EDX revealed the Calcium to Phosphorus (Ca/P) ratio to be 1.6. In response to the 10 %, 20 %, and 30 % HA, *S. mutans* were reduced by 14.5 %, 15.6 %, and 23.4 %, whereas *S. sobrinus* decreased by 17.1 %, 60.8 %, and 98.6 %, respectively.
Conclusion: Microwave-synthesized HA could have antibacterial properties against caries-causing bacteria with different potencies depending on concentration and bacteria.

1. Introduction

Hydroxyapatite (HA), a calcium phosphate compound, is structurally and chemically similar to natural bone, tooth enamel, and dentin (Oberbek et al., 2018). Due to its superior biocompatibility and excellent bioactivity properties, it has been used to treat and repair regeneration conditions in soft and bone tissues across various medical fields (Ding et al., 2017).

There are mixed findings regarding the action of HA towards oral bacteria and biofilm. HA was reported to not affect biofilm attachment (Schestakow et al., 2022) and may instead intensify *Streptococcus mutans (S. mutans)* growth and biofilm formation, especially in the presence of fermentable sugar (Park et al., 2019). Contrastingly, HA was found to aggregate temporarily on the tooth surface, attach around salivary oral bacteria, and reduce the bacterial adhesion that prevents biofilm formation (Kensche et al., 2017). An in-vitro study reported that coating the dentin surface with nano-sized HA could prevent *S. mutans* growth after 24 h (Erdem et al., 2020). It can also impair bacterial biofilm's metabolic capability in producing lactic acid and extracellular polysaccharides (Luo et al., 2020).

Streptococcus species are the most common cariogenic bacteria (Meyer and Enax, 2018), especially *S. mutans* and *Streptococcus sobrinus (S. sobrinus)*. Both bacteria are believed to be the primary aetiological agent for deep carious lesion (Conrads et al., 2014). Young children with both bacteria have a higher risk of having early childhood caries (Saraithong et al., 2015). In addition, more advanced caries was frequently found when both bacteria were present (Holbrook and Magnúsdóttir, 2012). Caries was observed to be more prevalent among children presenting with both bacteria than those only with *S. mutans* (Okada et al., 2005; Sánchez-Acedo et al., 2013).

A study has previously introduced a novel method of synthesizing antibacterial HA using microwave-assisted combustion (Lamkhao et al., 2019). However, the antibacterial HA produced by this method has never been tested against cariogenic bacteria or biofilm. Antibacterial

* Corresponding author.
E-mail address: dr_alaasabah@yahoo.com (A.S. Hussein).

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activity against cariogenic bacteria or biofilm could enhance the potential of HA in caries management. Hence, this study aims to investigate the antibacterial activity of microwave-synthesized HA against cariogenic bacteria, namely, *S. mutans* and *S. sobrinus*.

2. Materials and methods

2.1. Synthesis of HA through microwave-assisted combustion

The preparation of the HA followed the protocol recommended by Lamkhao et al. (2019). The procedure is summarised and illustrated in Fig. 1.

2.2. Characterisation of the synthesized HA powder

HA powder formation was confirmed by X-ray diffraction or XRD (Rigaku Ultima IV, Japan) and energy dispersive X-ray spectroscopy or EDX under FESEM (Fei Quanta 450 Feg, Netherlands). The XRD analysis of the powder was performed using an X-ray diffractometer (Cooper K-alpha, CuKα radiation with wavelength, $\lambda = 0.15418$ nm). Patterns of diffraction were observed in a continuous mode at room temperature over the 2θ (angle between transmitted and diffracted beam) range of 10–80° at 3° per minute. Next, the powder's XRD patterns were compared with the International Centre for Diffraction Data's standard data for HA (Powder Diffraction File, PDF, Number: 01-086-0740). The powder's Ca/P ratio was targeted at around HA's theoretical (stoichiometry) value of 1.67. An elemental mapping of oxygen, phosphorus, and calcium was recorded for this purpose.

2.3. Preparation of bacterial suspension

S. mutans (ATCC 25175) and *S. sobrinus* (ATCC 33478) in glycerol stock were thawed and inoculated in 30 mL of Brain Heart Infusion (BHI) broth and BHI agar media (Oxoid, Hampshire, England). The inoculated medium was then incubated at 37 °C for 18–24 h, and the colony morphology of the culture was observed the next day. Gram staining was conducted to observe the cell morphology of the selected bacteria. A single colony of each bacterial culture was taken, inoculated in a fresh BHI medium, and incubated overnight at 37 °C. On the following day, the bacterial suspension was prepared. The number of bacterial cells in the suspension was standardized to an OD625nm of 0.08–0.13, equivalent to 10^8 colony-forming units per ml (CFU/ml) or 0.5 McFarland. Then, 50 µl of the bacterial suspension was transferred to 10 ml of BHI broth to produce an inoculum of 5×10^5 CFU/ml (European Committee for Antibacterial Sensitivity Testing (EUCAST) of The European Society of Clinical Microbiology and Infectious Disease (ESCMID), 2003).

2.4. Antibacterial activity test

The antimicrobial susceptibility test of the synthesized HA powder against *S. mutans* (ATCC 25175) or *S. sobrinus* (ATCC 33478) was conducted by measuring the percentage of bacterial growth reduction against control. The HA powder was premeasured and added to the 24-well plate. The test wells contained 50 mg, 100 mg, and 150 mg of HA. Then, 500 µl of bacterial suspension prepared above was added to each well to give a concentration of 50 mg/500 µl, 100 mg/500 µl, and 150 mg/500 µl of HA, which is equivalent to 10 %, 20 %, and 30 % HA, respectively. As the control, another 500 µl of bacterial suspension was added to another well without any material. The mixtures were incubated anaerobically at 37 °C for 24 h. After an overnight incubation, serial dilution was made and plated on BHI agar in triplicates as the technical replicate. The agar plates were incubated anaerobically at 37 °C, and the colonies were counted the next day.

The above procedures were repeated using bacterial suspension kept at 4 °C for 3 and 7 days (biologically distinct bacteria samples) to produce biological replicates. The technical replicate (similar sample, repeated measurements) ensures a precise and reproducible experimental protocol, while the biological replicates (biologically distinct samples within the same species and similar procedures) capture any biological variation.

2.5. Statistical analysis

Statistical analysis was done using IBM Statistical Package for Social Sciences version 28.0 (SPSS Inc., Chicago, IL, USA). The data distribution for log CFU/µl failed to meet conditions for normality due to its small sample size. The Kruskal-Wallis test was used to evaluate the difference in colony counts between the groups. Post-hoc pairwise analysis was performed using the Dunn's test. The software automatically adjusted the P-values according to Bonferroni correction for multiple tests. The confidence interval was 95 %, and results with a p-value less than 0.05 were considered statistically significant.

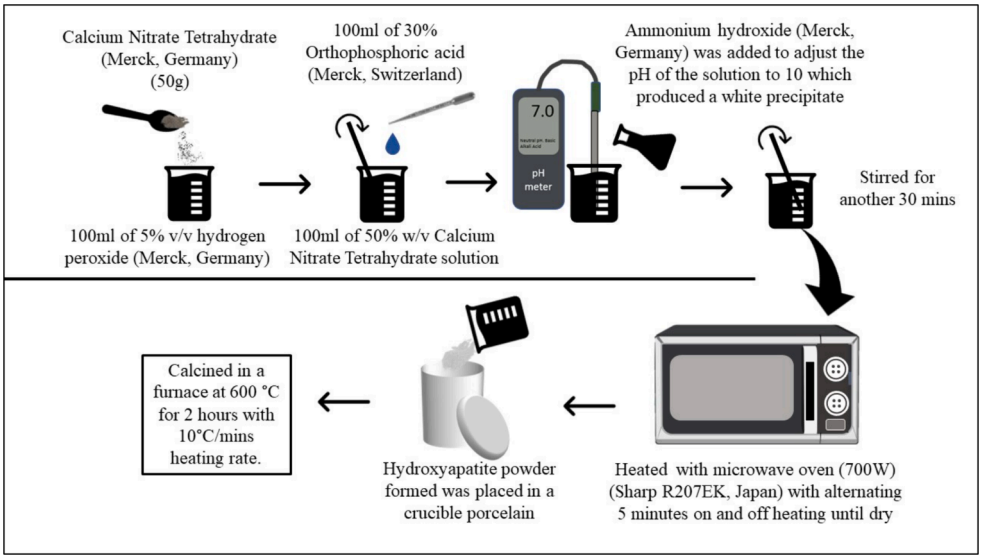

Fig. 1. Synthesis process of HA.

3. Results

3.1. Characterization of the synthesised HA

The X-ray diffraction pattern indicates present of an HA phase in the synthesized powder. The Miller indices of 002, 211, 112, and 300, which correspond to the standard HA diffraction peaks were found in the synthesized powder (Fig. 2). The synthesized HA had a crystallinity percentage of 75.42 %. The crystallinity percentage was calculated from the ratio between the areas of the crystalline peaks and the total area. Based on the Scherrer equation, the average crystallite size was 29.59 nm. The sample's Ca/P ratio and elemental mapping were obtained by an area scan using semiquantitative EDX analysis. The Ca/P ratio of the synthesized HA was 1.57 (the theoretical value of HA is 1.67). The weightage of the available elements found in the powder is listed in Table 1.

3.2. Antibacterial activity of the synthesized HA

Standard colony counting of *S. mutans* and *S. sobrinus* was performed to measure the antibacterial activity of different concentrations of HA. The reduction percentage increased as the concentration increased for both bacteria. The *S. mutans* growth was reduced by 14.5 %, 15.6 %, and 23.4 % in reaction to 10 %, 20 %, and 30 % HA, respectively (Fig. 3). In addition, the *S. sobrinus* growth was reduced by 17.1 %, 60.8 %, and 98.6 % in reaction to 10 %, 20 %, and 30 % HA, respectively (Fig. 3). The reduction percentage demonstrates a decreasing trend as the concentration of HA decreases.

The Kruskal Wallis test demonstrated that the colony counts (log CFU) between the groups for each replicate are statistically significant ($p < 0.05$) for both bacteria. A pairwise comparison using Dunn's test indicated that 30 % HA has a significantly lower colony count (Log CFU/µl) of *S. mutans* (5.24, IQR 0.0) compared to the control (6.84, IQR, 0.0) ($p = 0.027$). Similarly, there was a significantly lower colony count (Log CFU/µl) of *S. sobrinus* growth (0.10, IQR 0.0) compared to control (7.07, IQR 0.0) in response to 30 % HA ($p = 0.019$). The comparison of the other group revealed no significant differences in colony counts, as presented in Tables 2 and 3.

4. Discussion

The present study aimed to replicate the synthesis of HA using a microwave and assess its antibacterial properties against *S. mutans* and *S. sobrinus*, two of the most common cariogenic streptococcal species (Meyer and Enax, 2018). The results indicate that the HA possesses

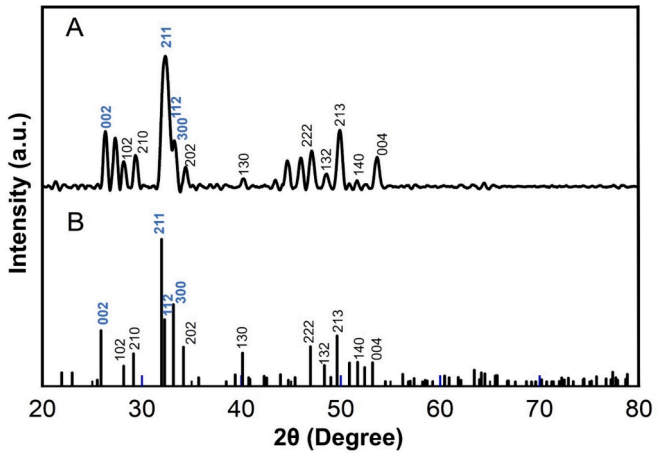

Fig. 2. XRD pattern showing peaks of the synthesized HA powder (A) compared to standard HA pattern from PDF: 01-086-0740 (B).

Table 1
Elemental composition of the powder from EDX analysis.

Element	Weightage %
O	51.04
P	19.04
Ca	29.92

antibacterial properties against both bacteria, albeit at varying effectiveness levels depending on the concentration. The higher the concentration, the more effective the HA reduces bacterial growth; this finding is further supported by a previous study (Tin-Oo et al., 2007). The 30 % HA displayed the highest reductions of bacterial growth for both *S. mutans* (23.4 %) and *S. sobrinus* (98.6 %).

A study by Tin-Oo et al. (2007) reported that viable *S. mutans* growth *was* 100 % inhibited by HA concentrations of 20 % and more. However, the present study reported a lower reduction in viable *S. mutans* at 20 % (15.6 %) and 30 % (23.4 %) HA. This discrepancy could be due to the nature of the solution preparation and bacterial growth. In the present study, the 500 µl bacterial suspension contributes 100 % of the solvent, making up the solution's concentration. Conversely, in the previous study, only 25 or 50 µl of the bacterial suspension was added to the solution made up of BHI broth. Besides, the solutions in the present study were incubated anaerobically, which further enhanced bacterial growth (Ahn et al., 2009). Nevertheless, both findings agree with another study that reported a significant reduction in the number of bacteria that adhere to enamel slabs after treatment with 5 % HA compared to the control (Kensche et al., 2017).

The present study could be the first study that reports on the antibacterial activity of HA against *S. sobrinus,* as no previous studies have been found to investigate HA against this bacterium. Based on our findings, *S. sobrinus* is more susceptible to HA compared to *S. mutans.* The reduction percentage in the viability of *S. sobrinus* at 10 % HA (17.1 %) is comparable to 30 % HA activity against *S. mutans* (23.4 %). There are no earlier results to compare this against as no prior studies have assessed HA against both *S. mutans* and *S. sobrinus.*

However, a comparison regarding the antibacterial action against both bacteria can be made from other materials. In contrast to the current findings, *S. mutans* were reported to be more susceptible when tested or exposed to other materials compared to *S. sobrinus* (Järvinen et al., 1995; Song et al., 2006). In the study by Jarvinen et al. (1995), a higher minimum inhibitory concentration (MIC) of chlorhexidine was needed for *S. sobrinus* (≤2 µg/ ml) compared to *S. mutans* (≤1 µg/ml) (Järvinen et al., 1995). Another study also corroborates this finding, whereby higher resistance of *S. sobrinus* was indicated by higher MIC of the test materials for *S. sobrinus* (4 mg/ml) compared to *S. mutans* (2 mg/ml) (Song et al., 2006).

Other studies have also reported comparable findings that indicate higher susceptibility or lower resistance of *S. sobrinus* compared to *S. mutans*. In addition, *S. sobrinus* was more susceptible to vitamin D_3, with MIC and minimum bactericidal concentration (MBC) almost four times lower than *S. mutans* (Almoudi et al., 2021). This finding is in line with another study that found lower MIC and MBC values of the test material for *S. sobrinus* than for *S. mutans* (Pandit et al., 2013).

While the precise reason for these differences in susceptibility between *S. sobrinus* and *S. mutans* is yet to be clearly understood, it could be due to different actions of the same material on both bacteria. For example, in the material tested by Pandit et al. (2013), although it impaired the acid tolerance of both bacteria, leading to death, the mechanism involved or the action exerted building up to this effect differed for each bacteria (Pandit et al., 2013). The acid tolerance mechanism of *S. mutans* was dependent on an increase in F-ATPase activity, while *S. sobrinus* was dependent on phosphoenolpyruvate phosphotransferase system (PTS) activity (Nascimento et al., 2004). The other possible reason is that *S. sobrinus* might have different proteins

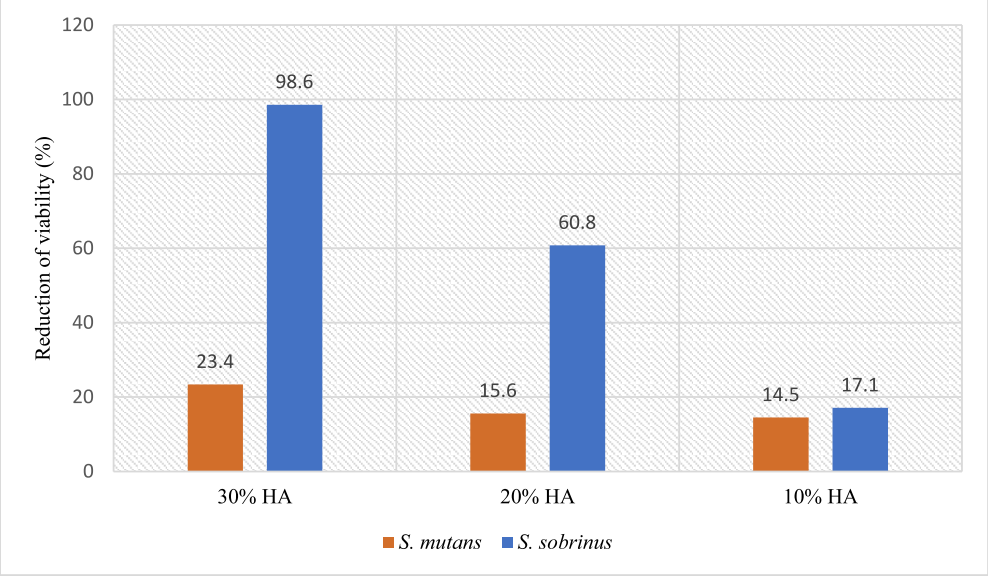

Fig. 3. Reduction percentage of *S. mutans* and *S. sobrinus* growth compared to the control.

Table 2
Comparing the colony counts of *S. mutans* between the groups.

Intervention	Log CFU/µl (n = 3) Median (IQR)	X^2 Statistic (df)[a]	P-value	Post-hoc pairwise comparisons[b]			
				Control	30 % HA	20 % HA	10 % HA
Control	6.84 (0)	9.186 (3)	0.027		0.019	0.323	1.000
30 % HA	5.24 (0)			0.019		1.000	0.533
20 % HA	5.77 (0)			0.323	1.000		1.000
10 % HA	5.85 (0)			1.000	0.533	1.000	

[a] Kruskal Wallis test.
[b] Dunn's test (P-values adjusted by Bonferroni correction for multiple tests).

Table 3
Comparing the colony counts of *S. sobrinus* between the groups.

Intervention	Log CFU/µl (n = 3) Median (IQR)	X^2 Statistic (df)[a]	P-value	Post-hoc pairwise comparisons[b]			
				Control	30 % HA	20 % HA	10 % HA
Control	7.07 (0)	9.974 (3)	0.019		0.013	0.325	1.000
30 % HA	0.10 (0)			0.013		1.000	0.325
20 % HA	2.77 (0)			0.325	1.000		1.000
10 % HA	5.86 (0)			1.000	0.325	1.000	

[a] Kruskal Wallis test.
[b] Dunn's test (P-values adjusted by Bonferroni correction for multiple tests).

whose functions are yet to be discovered, contributing to its resistance. Alternatively, it might have lesser or weaker genes, contributing to its increased susceptibility than *S. mutans* (Conrads et al., 2014).

The exact mechanism of HA action on the bacteria tested in the present study, *S. mutans* and *S. sobrinus*, is not clearly understood because of limited information. HA might hinder the metabolism and acid production of oral bacteria, especially *S. mutans* (Luo et al., 2020). In addition, the antibacterial properties of HA synthesized using a microwave could be attributed to the development of free radicals during the heating reaction (Lamkhao et al., 2019) or the formation of nanoparticles (Wang et al., 2017). The nano-sized particles may possibly penetrate cell causing toxicity to the bacteria (Xu et al., 2007).

In the present study, HA was successfully synthesized using a microwave. Microwave-assisted synthesis is a simple yet fast and effective way of synthesizing HA. The microwave's electromagnetic field increases the kinetic energy of molecules, causing them to collide, rotate, and rub; this generates heat that is transmitted swiftly and uniformly throughout the solution (Hu et al., 2021). This method produces high purity, small-sized HA with narrowly distributed particles (Corrales et al., 2014; Eliaz and Metoki, 2017).

The limitation of the present study is that it is designed as a preliminary study for the preparation and application of an HA paste in arresting dentin caries. The sample size is acknowledged to be rather small despite the utilisation of non-parametric test. In addition, the antibacterial activity testing does not indicate the value of MIC or MBC, as the concentration tested is limited to 10 %, 20 %, and 30 %. These concentrations were chosen based on the commercially available and most commonly investigated HA concentration, which is 10 % (Anil et al., 2022). A limitation to the 30 % concentration was made because another preliminary trial optimizing HA paste preparation revealed that at higher concentrations, the paste consistency was not suitable to be applied for the investigation.

Nevertheless, the antibacterial activity of microwave-synthesized HA discovered by this study should be considered as it still contributes to the

existing body of knowledge regarding the material. Further studies with more established and standardized protocols are warranted to confirm its antibacterial properties and elucidate its mechanism of action against these bacteria.

5. Conclusion

This study evaluated the antibacterial activity of microwave-synthesized HA against two of the most common cariogenic *mutans* streptococci, namely, *S. mutans* and *S. sobrinus.* Microwave-synthesized HA can be easily replicated and rapidly produced. The findings demonstrate that HA may possess antibacterial properties against both bacteria with different efficacies depending on concentration and the bacterial strains. Further research is recommended to corroborate its antibacterial sensitivity, especially against cariogenic bacteria, and unravel its mechanism of antibacterial activity.

6. Ethical approval

Ethical approval for the research study was obtained from Universiti Teknologi MARA, UiTM's Research Ethics Committee, to be run at the laboratories of the Faculty of Dentistry, UiTM Sungai Buloh under reference number REC/08/2021 (MR/662).

7. Informed consent

Given that the study did not involve human participants, the requirement for obtaining informed consent was not applicable. However, the ethical principles governing the responsible conduct of research were consistently followed, ensuring the privacy and confidentiality of any related data and results.

8. Animal welfare

The in vitro nature of this study precluded the use of live animals. No animal subjects were involved or harmed in any aspect of the research. The commitment to the ethical treatment of animals extends to using alternative methods and techniques that prevent the need for animal experimentation.

CRediT authorship contribution statement

Ahmad Zharif Ibrahim: Conceptualization, Methodology, Writing – original draft, Visualization, Resources. **Alaa Sabah Hussein:** Conceptualization, Supervision, Methodology, Writing – review & editing. **Hasnah Begum Said Gulam Khan:** Supervision, Methodology, Writing – review & editing. **Norzalina Ghazali:** Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This original article (preliminary study) is part of a research study funded by the Universiti Sains Islam Malaysia's Research Grant (Grant Number: PPPI/FPG/0122/USIM/13122) with co-author Dr. Norzalina Ghazali as the principal investigator while Dr. Ahmad Zharif Ibrahim and Dr. Alaa Sabah Hussein as the members. The funding body has no interest in the research findings, does not intervene in the way research is conducted, and does not benefit financially from the publication of this article and the study's outcomes.

All the authors have had no personal conflict or financial gain throughout the study's commencement until this article's submission for publication. Hence, to our knowledge, no conflicts of interest are to be declared.

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