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Comparative Study of the Synergistic Antimicrobial Efficacy of Chitosan with Chlorhexidine, Silver, and Propolis in Human Saliva

Alaa S. Nasef[1*], Wael E. Jamil[2], Asmaa A. Mosleh[3], Einas H. Elshatory[4]

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azhardentj@azhar.edu.eg

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Restorative Dentistry
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ABSTRACT

Purpose: The aim of the study was to evaluate and compare the synergistic antimicrobial effects of chitosan with chlorhexidine, silver, and propolis in human saliva. **Materials and method:** High and low concentrations of chitosan solution were prepared and added to the chlorhexidine solution, silver nitrate, and propolis extract. Antimicrobial testing was performed using colony-forming units (CFU) and disc diffusion tests with chitosan solution in both concentrations as a positive control. Biofilms containing *Streptococcus mutans (S. mutans)* were grown on 56 enamel blocks and subjected to the treatment solutions then diluted and grown on specific S. mutans media for colony counting. Inhibition zones were measured by immersing filter paper discs in the treatment solutions and incubating them on agar plates inoculated with S. mutans bacteria. **Results:** Statistical analysis showed that 5% chitosan/ nanosilver mixture (Chit/AgNps) had the highest synergistic antimicrobial effect in both tests with a mean value of (8.571 CFU/mL) for CFU and (44 mm) for disc diffusion. **Conclusion:** 5% Chit/AgNps showed the most promising antimicrobial efficacy against *S.mutans* among all mixtures.

KEYWORDS

Chitosan, Ssynergistic, Antimicrobial

INTRODUCTION

Dental caries is a biofilm-mediated, diet modulated, multifactorial, non-communicable, dynamic disease resulting in net mineral loss of dental hard tissues[1,2]. It is determined by biological, behavioral, psychosocial, and environmental factors. As a consequence of this process, a caries lesion develops. The bacterium *Streptococcus mutans* (*S. mutans*) is considered the main etiological agent of dental caries[3].

1. Police Officer-Dentist at the New Cairo Medical Complex for Police Officers, Cairo, Egypt.
2. Professor of Operative Dentistry Department, Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt.
3. Lecturer of Operative Dentistry Department, Faculty of Dental Medicine for Girls, Azhar University, Cairo, Egypt.
4. Professor of Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt.

* Corresponding author email:

Oral cariogenic biofilms start as an acquired pellicle on the tooth surface. This pellicle grows into a biofilm that contains receptors for the attachment of *S. mutans* bacteria[4]. In theory, caries incidence can be reduced by decreasing the load of *S. mutans* in the oral cavity[5].

The use of natural antimicrobial agents has gained great interest in the past few years. Chitosan is the second most abundant polysaccharide in nature following cellulose[6]. It shows high levels of biocompatibility, biodegradability with no toxicity[7]. It's a polycation in acidic pH giving it distinctive characteristics of being mucoadhesive, antimicrobial, and enhancing permeability[8]. It is used widely in different fields as a drug carrier and has been combined with different materials such as antibiotics, chemicals, metals, and other natural components to produce a synergistic antimicrobial effect while decreasing the undesirable effects of these materials[9].

Chlorohexidine is considered a gold standard antimicrobial agent[10]. It comes after fluoride as the most effective agent used for caries prevention[11]. Yet, chlorhexidine has several drawbacks such as tooth discoloration, alteration of taste, burning sensation, and desquamative lesions in some individuals[12].

Silver is a noble metal that has been used in medicine over the decades owing to its strong antimicrobial properties. Limitations in the use of silver ions include the deposition of silver precipitates in the skin and eyes[13]. Substituting silver ions with silver nanoparticles increased the antimicrobial effect and biocompatibility of silver to be used in various studies[14].

Propolis is a resinous substance collected by honeybees from buds and exudates of certain trees and stored inside their hives. It has antimicrobial, anti-inflammatory, antioxidant, and healing properties[15]. Flavonoids, aromatic acids, and esters are responsible for the antimicrobial properties of propolis[16].

It was found that on coupling chitosan with chlorhexidine, nanosilver, and propolis, a synergistic antimicrobial effect on *S. mutans* was observed and the undesirable effects were reduced.

This study aimed to compare and evaluate the synergistic antimicrobial effect of three chitosan mixtures combined with each of chlorhexidine, silver nanoparticles and propolis. Chitosan solution at the concentration of 1% and 5% was used to prepare high and low chitosan concentrations of chitosan/chlorohexidine, chitosan/nanosilver, and chitosan/propolis to recommend the use of the mixture with the highest antimicrobial properties for caries prevention.

MATERIAL AND METHODS

Preparation of chitosan solutions (Chit)

This study involved the preparation of two concentrations of chitosan, 1% (weight/volume) to represent low concentration and 5% to represent high concentration. Low molecular weight (LMW) chitosan powder (50-190 KDa-ChitoClear-Primex) was dissolved in acetic acid (1 gm Chit/ 100 ml acid for 1% conc and 5gm Chit/ 100 ml acid for 5% conc.). The solutions were placed on a magnetic stirrer for 24 hours to dissolve the chitosan in the acid.

Preparation of Chitosan/Chlorhexidine (Chit/CHX) mixture

2ml of chlorhexidine were added to 50 ml of each of the chitosan solutions, followed by shaking for 10 minutes to get a product of low and high concentrations of chitosan in the Chit/CHX mixtures[17].

Preparation of Chitosan/Nanosilver (Chit/AgNps) mixture

1-5 mL of an aqueous solution of silver nitrate $AgNO_3$ (0.1 mol/L) was mixed with 50 ml of the 1% chitosan solution and the resulting solution was

stirred for 30 minutes at 30ºC until a pink solution was formed. A freshly prepared aqueous solution of sodium borohydride $NaBH_4$ was quickly added to the previous mixture and stirred for another 90 minutes. The amount of $NaBH_4$ was 1-5 times the $AgNO_3$. Then, silver chitosan colloids were then precipitated out with acetone, filtered, and washed then dried in a vacuum at 60ºC for 48 hours then crosslinked with glutaraldehyde gas for 24 hours at 37ºC to obtain the yellow chitosan/nanosilver composites.

The procedure was then repeated with the same steps but substituting 1% chitosan solution with the 5% chitosan solution[18,19,20,21].

Preparation of Chitosan/Propolis (Chit/Prop) mixture

Grounded propolis was added to 80% ethanol and kept in dark for 7 days with periodical mixing using a homogenizer. The resulting extract was filtered and concentrated at 50ºC and then the resulting resin was dissolved in 95% ethanol to a final concentration of 200 mg/mL of ethanolic propolis extract (EPE).

The chitosan solution was prepared by dissolving chitosan in an aqueous solution of acetic acid. Glycerol was then added to the solution to act as a plasticizer at the concentration of 2% (w/v). Tween 20 was added to the chitosan solution at a concentration of 0.05% (v/v) to improve its adhesion and wettability. Ethanolic propolis extract (EPE) was then incorporated in the chitosan solution to reach a final concentration of EPE resin/chitosan in the solution at 10% by weight and 2% by weight[22].

Saliva collection

Saliva was collected from two Egyptian adults with normal salivary flow and with a history of previous caries but no current active caries, with no gingivitis and no history of antibiotic administration over the past 6 months. Donors were asked to chew on a sterile rubber material and saliva was collected in sterile falcon tubes after obtaining ethical approval from the Research and Ethics committee of the Faculty of Dental Medicine of Al-Azhar University (Girls Branch), Cairo – Egypt, code: P-OP-22-02. Donors were informed of the study. A verbal and signed consent was obtained[23].

Antimicrobial efficacy assessment

Colony Forming Units (CFUs)

56 enamel blocks with dimensions (4×7×1 mm) were cut from the coronal portion of bovine incisors and sterilized. They were immersed in filtered human saliva to create a salivary pellicle then placed in ultrafiltered tryptone-yeast extract broth (UTYEB) containing S. mutans and incubated for 7 days to allow biofilm formation[24].

Enamel blocks were then removed from the UTYEB medium and divided to 8 groups with 7 blocks each to receive the following treatment solutions: 1% Chit, 5% Chit, 1% Chit/CHX, 5% Chit/CHX, 1% Chit/AgNps, 5% Chit/AgNps, 1% Chit/Prop, 5% Chit/Prop. Each enamel bock was submerged in 3 ml of one of the treatment solutions for 24 hours. Enamel blocks were then removed, washed, and placed in a fresh UTYEB with S. mutans and incubated for 48 hours.

The blocks were removed from the inoculum and washed with 0.9% NaCl, placed in sterile glass tubes, and sonicated to detach the biofilm. The suspension was then diluted and inoculated on plates containing Brain Heart Infusion (BHI) agar to determine the number of viable organisms via colony count[24].

Disc Diffusion (DD)

S. mutans was cultured in Petri dishes containing blood agar. Filter paper discs of 6 mm diameter were sterilized and then impregnated with 20μL of each treatment solution. They were placed immediately on the agar plates and incubated for 24 hours. A frosted glass appearance indicated the growth of S. mutans bacteria and clear inhibition zones around the discs were measured[25] (Fig. 1).

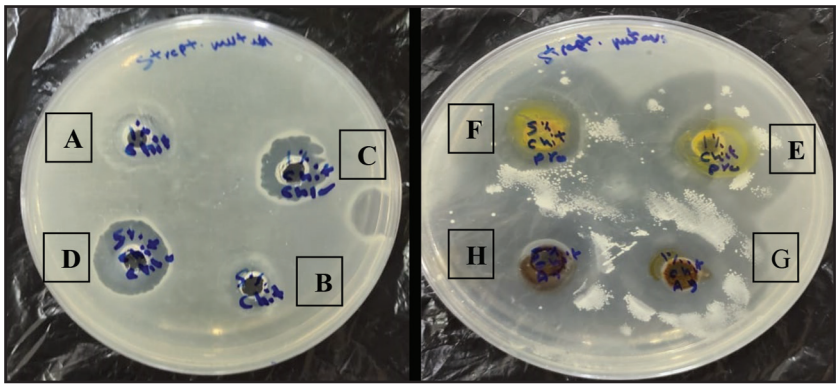

Figure (1) Inhibition zones around filter paper discs immersed in treatment solutions on agar plates inoculated with S. mutans. A) 1% Chit, B) 5% Chit, C) 1%Chit/CHX, D) 5% Chit/CHX, E) 1% Chit/Prop, F) 5% Chit/Prop, G) 1%Chit/AgNps, H) 5%Chit/AgNps

RESULTS

Regarding the inhibition of *S. mutans* ATCC 25175, there was a statistically significant difference between 1% Chit and 5% Chit. Also, 1% Chit/CHX and 5% Chit/CHX showed a statistically significant difference between. Furthermore, a statistically significant difference was found between 1% Chit/Prop and 5%Chit/Prop. However, there was no significant difference between 1% Chit/AgNps and 5% Chit/AgNps as shown in table (1) figure (2), (3).

Table (1) *Comparison between the means±SD of low and high chitosan concentrations within the mixtures using CFU*

	Chitosan	Chit/ CHX	Chit/ AgNps	Chit/ Prop
Low 1%	514.3 ±288.9	81.29 ±16.22	1.29 ±1.38	23.29 ±13.02
High 5%	30.43 ±27.81	2.14 ±1.21	0.86 ±1.57	0.86 ±1.21
Test value	-4.411μ	-12.875μ	-0.544μ	-4.538μ
P-value	0.008*	0.000*	0.596	0.007*
Significance level	HS	HS	NS	HS

**: statistically significant at $p \leq 0.05$, HS: statistically highly significant at $p \leq 0.01$,*

NS: statistically non-significant at $p > 0.05$.

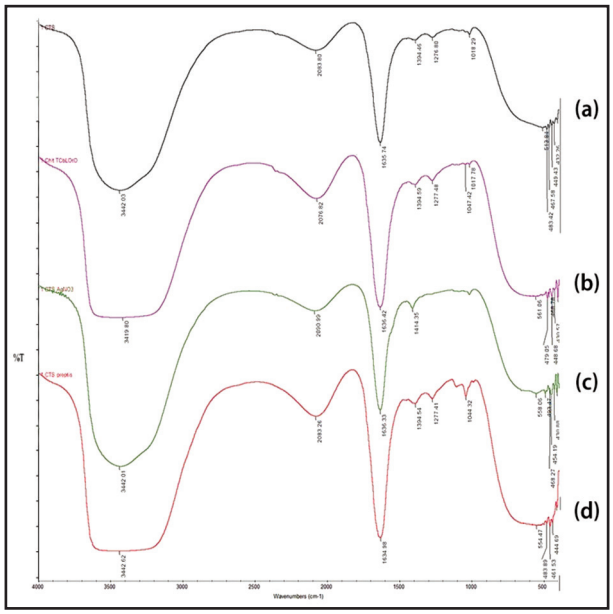

Figure (2) FTIR analysis for (a) chitosan, (b) Chit/CHX, (c) Chit/AgNPs, (d) Chit/Prop at 5% concentration

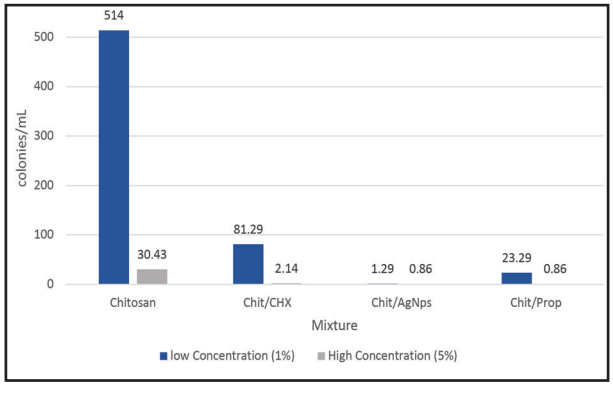

Figure (3) A graph showing the comparison of the mean colony counts (measured in colonies/mL) of mixtures using low and high chitosan concentration.

Regarding the inhibition of *S. mutans* ATCC 25175, there was a statistically significant difference between 1% Chit and 5% Chit. Also, a statistically significant difference was found between 1% Chit/CHX and 5% Chit/CHX. Furthermore, 1% Chit/Prop and 5%Chit/Prop showed a statistically significant difference between them. Finally, there was a statistically significant difference between 1% Chit/AgNps and 5% Chit/AgNps as shown in table (2) figure (4).

Table (2) *Comparison between the means of low and high chitosan concentrations within the mixtures using Disc Diffusion Method*

	Chitosan	Chit/ CHX	Chit/ AgNps	Chit/ Prop
Low (1%)	12.00 ± 0.65 SD	15.00 ± 0.65 SD	39.00 ± 1.58 SD	34.14 ± 1.31 SD
High (5%)	13.86 ±0.63 SD	20.00 ± 1.68 SD	44.00 ±1.29 SD	40.00 ±0.82 SD
Test Value	5.436	7.344	6.486	10.032
P-value	0.000*	0.000*	0.000*	0.000*
Significance level	HS	HS	HS	HS

**: statistically significant at $p \leq 0.05$, HS: statistically highly significant at $p \leq 0.01$,*

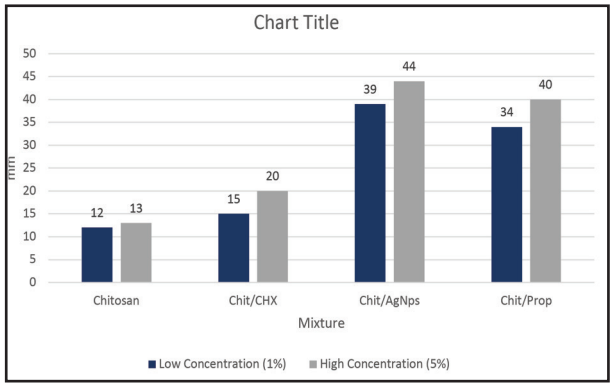

Figure (4) A graph showing a comparison of the mean inhibition zone diameters (measured in mm) of the mixtures with both low and high chitosan concentrations.

A comparison between the mean results of both tests using high and low chitosan concentrations is shown in figure (5). High CFU results indicate low antimicrobial effects while high Disc Diffusion results indicate high antimicrobial effects.

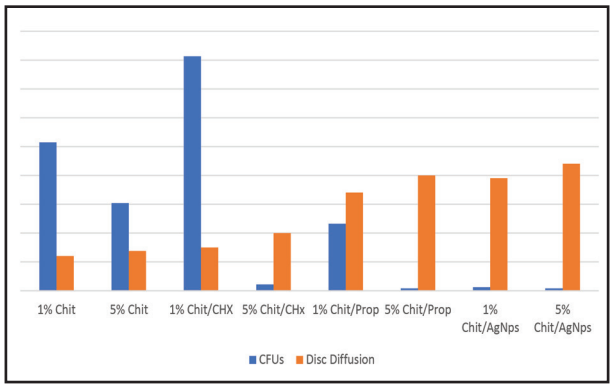

Figure (5) A graph showing the mean results of both antimicrobial tests.

DISCUSSION

Dental caries is considered one of the oldest and most common diseases of mankind. It results from tooth-adherent specific bacteria, primarily *Streptococcus mutans (S. mutans)*. This bacterium attaches to the tooth surface and grows within the dental biofilm (commonly known as dental plaque) [1]. In theory, reducing the total S. mutans load within the oral cavity will aid in caries prevention[26]. The medical model of caries management has gained interest rather than the surgical model over the past years. The medical model includes preventing the occurrence of dental caries, stopping its progression, and reversing the damage caused by it. For the medical model to succeed, it needs to include four essential components which are: 1. control of the bacterial infection, 2. reduction of the risk levels, 3. remineralization of the teeth, 4. long term follow up[27]. In this study, we focused on the first component, the control of bacterial infection.

Natural antimicrobials have been recently used to decrease the undesirable effects of standard ones. Chitosan is a naturally occurring polymer found in the exoskeleton of crustaceans, insects, and some fungi[28]. It is present in different molecular weights and degrees of deacetylation with the lowest

molecular weight chitosan showing the highest water solubility and highest antimicrobial properties when compared to the high molecular weight chitosan[29]. Chitosan was found to exhibit great synergistic antimicrobial effects when combined with different materials. In this study, we combined chitosan at two different concentrations with chlorhexidine, nanosilver, and propolis. Chlorhexidine, silver, and propolis are known for their antimicrobial properties and when combined with chitosan a synergistic antimicrobial effect was observed. This study aimed to compare the synergistic antimicrobial effect between these two materials.

Low molecular weight (LMW) chitosan was used in this study since it possesses' higher antimicrobial effects and higher solubility compared to high molecular weight (HMW) chitosan[29]. Chlorhexidine digluconate is a commonly used antimicrobial irrigant used in endodontic practice. It is considered a gold standard antimicrobial agent[10]. Decreasing the size of the silver ions to a nanoscale increased the antimicrobial capabilities of silver ions by decreasing its undesirable effects[14]. $AgNO_3$ was used to form the Chitosan/Nanosilver (Chit/AgNPs), silver nitrate ($AgNO_3$) was reduced in the presence of chitosan solutions with sodium borohydride ($NaBH_4$)[18]. Ethanolic propolis extract (EPE) was first prepared. EPE was used in this study because it showed higher flavonoid content than water extract of propolis[30]. EPE was then added to the chitosan solutions. FTIR image analysis was performed to show if any chemical interactions occurred between chitosan and the selected materials as shown in figure (2).

Two tests were chosen for the assessment of the antimicrobial effects. Colony forming units (CFU) represents the number of viable bacteria present within the dental biofilm present on tooth surface while Disc Diffusion measures the inhibition zone diameters on an agar plate inoculated with S. mutans containing filter paper discs impregnated with the tested mixtures.

This study used chitosan (Chit) with both high and low concentrations as a positive control, with 1% Chit representing the low concentration and 5% Chit representing the high concentration. Both control groups showed an acceptable antimicrobial effect with the 5% Chit showing a higher antimicrobial effect than 1% Chit. This agrees with a study that shows increasing the concentration of chitosan in a solution increases its antimicrobial effect[31].

5% Chit/AgNps showed the highest antimicrobial effect in both tests with a mean of (0.86 ± 1.57 CFU/L) and a mean inhibition zone diameter (44± 1.29 mm). This may be due to the combination of several factors. First, higher chitosan concentration increases the antimicrobial effect of the overall mixture[31]. Second, decreasing the particle size of silver within the nanoscale allows for better antimicrobial effects through increasing the surface area available for interaction with the bacteria[32]. Third, FTIR image results show the presence of an electrostatic bond between the chitosan and silver nanoparticles which comes in agreement with a previous study[18]. This electrostatic bond may have enhanced the synergistic antimicrobial effect between the two materials giving it the highest effect in both tests compared to the other mixtures.

5% Chitosan/Propolis (Chit/Prop) showed high antimicrobial effects. The mean CFU results were (0.86 ±1.21 CFU/L) and (40± 0.82 mm) for disc diffusion. This high result may be attributed to the high concentration of chitosan. This high result comes in agreement with a study that showed high antimicrobial effects on S. mutans after using a Chit/Prop mixture. This study showed that increasing the propolis concentration enhances the antimicrobial effect[33]. FTIR image results showed hydrogen bond formation between the chitosan and propolis which may have played a role in the strong antimicrobial effect observed.

1% Chit/AgNps showed a high antimicrobial effect that is almost similar to that of 5% Chit/Prop. It showed a mean value of (1.29 ± 1.38 CFU/ml)

which shows a higher antimicrobial effect than 5% Chit/Prop. Yet disc diffusion results showed a mean of (39± 1.58 mm) which is less than that of the latter. The high and almost similar results may be attributed to the high antimicrobial effect of nanosilver particles. A study showed that nanosilver fluoride was capable of decreasing the growth of bacteria in both a single species biofilm containing S. mutans and Enterococcus Faecalis while propolis fluoride was capable of decreasing bacterial growth in a single species biofilm only when compared to silver diamine fluoride control[34]. This comes in agreement with this study that silver nanoparticles show better antimicrobial effects within the dental biofilm. This may have compensated for the decrease in the chitosan concentration which enhances the antimicrobial effect.

High antimicrobial effects were observed with 1% Chit/Prop. It showed a mean of (23.29 ±13.02 CFU/ml) and a mean disc diffusion diameter of (34.143± 1.31 mm). 5% Chitosan/Chlorhexidine (Chit/CHX) showed a mean of (2.14 ±1.21 CFU/ml) and (20± 1.68 mm). This conflict in the results may indicate the efficacy of the antimicrobial mixtures inside the human mouth since the CFU results were an indication of the strength of the antimicrobial agents within the biofilm formed on the tooth surface. This differs from a study that indicates that both propolis and chlorhexidine show the same antibacterial effects on S. mutans[35]. Accordingly, 5% Chit/CHX should have shown higher results in both tests. Yet the difference in the results in our study may be attributed to the presence of chitosan. FTIR image analysis showed the presence of hydrogen bonds between both chitosan and propolis, yet no bonds were shown between chitosan and chlorhexidine. This may have affected the inhibition zone diameter of 1% Chit/Prop to be higher than 5% Chit/CHX.

On the other hand, substantivity is an important characteristic of CHX allowing it to remain attached to the tissues and constantly released to the surrounding tissues[36]. This property may have decreased the colony counts in 5% Chit/CHX to overcome the effect of the hydrogen bonds in 1% Chit/CHX. In addition, higher chitosan concentrations prevent bacterial agglutination thus may have decreased the CFU result[37].

1% Chit/CHX, showed the least antimicrobial properties with a mean of (81.29 ±16.22 CFU/ml) for CFUs and a mean of (15 ± 0.65 mm) for disc diffusion. This may be attributed to the lower concentration of chitosan. FTIR image of Chit/CHX mixture showed no interactions between the two compounds especially in the 1% Chit/CHX mixture, unlike the electrostatic bond and hydrogen bond found between chitosan and silver and propolis respectively.

CONCLUSION

Within the limitations of the current study and for the tested material, the following could be concluded:

1. 5% Chit/AgNps show the highest antimicrobial effect against S. mutans bacteria.
2. Increasing the chitosan concentration has a big influence on the antimicrobial effect.
3. Presence of bonds between chitosan and other materials increases the synergistic antimicrobial effect, the stronger the bond, the stronger the synergistic antimicrobial effect.

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DECLARATION OF INTEREST

It's been made clear by the authors that there isn't any conflict of interest that may skew the findings.

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