



RESEARCH ARTICLE

Efficacy of a silver colloidal gel against selected oral bacteria *in vitro* [version 1; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: It is necessary to develop new strategies to protect against bacteria such as *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius*, which contribute to tooth decay and plaque formation. Our current study investigated the efficacy of a colloidal silver gel in inhibiting biofilm formation by these principal oral bacteria, *in vitro*. The aim of this study was to assess the efficacy of a colloidal silver gel formulation for inhibiting bacterial biofilm formation (Ag-gel) by the principal bacteria that cause plaque formation and tooth decay.

Methods: The effect of Ag-gel on viability of *S. mutans*, *S. sanguis*, and *S. salivarius* was assessed by quantifying their colony forming units (CFU) in presence or absence of the test gel. The effect of this formulation on biofilm-forming ability of these bacteria was studied through scanning electron microscopy.

Results: Using the CFU assays, over 6 logs of inhibition (100%) were found for *S. mutans*, *S. sanguis*, and *S. salivarius* for the Ag-gel-treated bacteria when compared with the control gel. In addition, the Ag-gel also inhibited biofilm formation by these three bacteria mixed together. These results were confirmed by scanning electron microscopy.

Conclusions: The Ag-gel was effective in preventing biofilm formation by *S. mutans*, *S. sanguis*, and *S. salivarius*. This Ag-gel should be tested for the ability to block plaque formation in the mouth, through its use as a tooth paste.

Keywords

Silver, biofilm, dental plaque, dental caries

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Introduction

Problems associated with maintenance of oral health are faced by many people throughout the world, irrespective of their age and gender. The most common oral problems amongst all are dental caries, bleeding gums (periodontal diseases) and oral cancers¹. Over few decades, the severity and prevalence of dental caries, and oral cancer, which can be a fatal condition, have increased^{2,3}.

In the US, caries were estimated to be five times as common as asthma and seven times as common as allergic rhinitis³. According to the World Health Organization (WHO), dental caries are caused due to high sugar consumption, which is also linked with being overweight and obesity⁴. The incidence of periodontal diseases is estimated to be about 20–50% of the global population⁵. There are two approaches for the management of caries: extraction and preventions. The primary treatment modality for these caries, though very painful, is extraction of carious teeth^{2,3}. The routine prevention measures for dental caries are to maintain oral hygiene, involving the use of fluoride toothpaste and/or xylitol^{6,7}. Looking at the currently prevailing painful treatment, there is a need for new products to be developed for the prevention of oral cavities. The pathological organisms responsible for these caries/ periodontal diseases are *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus salivarius*.

Since mouth washes and different tinctures have been found to be ineffective against dental biofilm formation⁸, finding novel products effective against cariogenic microbes like *S. mutans* is important. While Listerine[®], has some antimicrobial activity, toothpastes such as Toss-K and Senquel-AD have no activity against four important dental caries pathogens⁹. Thus, the search continues for more effective agent(s)¹⁰. A novel product that is effective against biofilm formation would be an important contribution to chewing sticks, toothpastes or other dental products.

S. mutans has the ability to adhere the enamel surface, produce acid metabolites, build glycogen reserves and to synthesize extracellular polysaccharides. Mutans streptococci create acidic environment creating a risk for formation of cavity. During the formation of dental plaque, *S. mutans* adhere to primary colonizers by cell to cell interaction which forms biofilm on the teeth which induces bacterial growth¹¹.

Streptococcus sanguis is normally found in the human oral cavity. Due to low cariogenicity, it forms a colony on tooth surface which gets aggregated by other oral bacteria and leads to maturation of dental plaque¹². Another organism, *Streptococcus salivarius* belonging to the *salivarius* subspecies is found in oral cavity in humans a few hours after birth and remain there as the predominant inhabitant. All these organisms enhance caries formation and thus the progression of periodontal disease. The aforementioned treatment modalities are unsuccessful in controlling or killing these bacteria and hence in prevention of caries.

Silver has been used since ancient times as antibacterial agent for various pathological elements. During the last century, the antimicrobial action of silver has been investigated¹³. Colloidal silver is observed to be less toxic than ionic silver and has good

compatibility with human cells. Silver was found to be effective in dentine desensitizer and is used as root canal disinfectant¹⁴. Silver nanoparticles are also used in dental material depending on the type of material being used. For example, titanium samples are mainly soaked in AgNO₃ solution for dental implants to avoid bacterial contamination¹⁵. The mechanism of action of silver compounds on carious tooth is to inhibit demineralization process and anti-bacterial effect by interfering with bacterial cell membrane, cytoplasmic enzyme and inhibition of DNA replication of bacteria¹⁴.

Oral health being a global concern, it is essential to develop strategies to prevent dental caries and plaque formation. This study aimed at investigating the efficacy of a colloidal silver gel in inhibiting biofilm formation *in vitro* by the principal oral bacteria, *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus salivarius*.

Methods

Bacterial strains, media, and growth conditions

Streptococcus salivarius strain ATCC[®] 13419[™], *Streptococcus sanguis* ATCC[®] 10556[™], and *Streptococcus mutans* strain ATCC[®] 35668[™] were obtained from Remel (Lenexa, KS, USA). *S. salivarius*, *S. sanguis*, and *S. mutans* were routinely grown in Brain Heart Infusion (BHI, #53286, Sigma-Aldrich, St. Louis, MO), at 37°C for 24 h.

Colloidal silver materials

Colloidal silver in a gel form was obtained from Viridis BioPharma Pvt., Ltd. (Mumbai, India). It was tested by evenly spreading 0.5 g on a 6-mm blank paper disc (BD Diagnostic System, Sparks, US) inoculated with the bacteria listed in the above paragraph. We assessed the bacteria remaining on the disc by the CFU assays below.

In vitro colony forming unit (CFU) assays

A total of three blank sterile (6 mm diameter) cellulose paper discs were placed onto individual LB agar plates. Approximately 1x10³ CFUs of the test bacteria were inoculated onto each disc. In the biofilm mixture study, approximately 4x10² of each bacterium were combined together. Either no gel (untreated), Ag-gel, or placebo gel (Viridis gel without Ag), were placed over the discs inoculated with bacteria. The plates were incubated under micro-aerobic conditions, which were generated by placing the plates inside a gas jar containing an EZ GasPak (Catalog no. 260678, BD, Franklin Lakes, NJ, USA) at 37°C for 24 h. Following incubation, each cellulose disc was analyzed for the remaining viable bacteria by the CFU assay as previously described¹⁶. Each piece was carefully removed from the well, rinsed gently with sterile distilled H₂O, and placed in a microcentrifuge tube containing 1 ml PBS. The tubes were placed in a water bath sonicator for 5 min to loosen the cells within the biofilm and then vigorously vortexed 3 times for 1 min to detach the cells. Suspended cells were serially diluted 10-fold in PBS, and 10-μl aliquots of each dilution were spotted onto BHI plates. The plates were incubated at 37°C for 24 h. In experiments where no bacteria were detected, the remaining 900 μl of undiluted samples were tested. Thus, the equation for back-calculating the bacterial concentration

was CFU x dilution factor x 100, with the exception of the 100- μ l sample which was calculated as CFU x 10. This means that the smallest number of bacteria that we could detect would be approximately 1 bacterium. All experiments were performed in triplicate.

Scanning electron microscopy (SEM)

Biofilms formed on discs were prepared for SEM by standard techniques and the experiment was performed as previously described¹⁶⁻¹⁸. *S. salivarius*, *S. sanguis*, *S. mutans* or a mixture of *S. salivarius*, *S. sanguis*, and *S. mutans* biofilms were established on cellulose discs (with or without silver gel, as described above). After 24 h of incubation, each cellulose disc and any adherent bacteria were fixed with 2% (wt/vol) glutaraldehyde in filter-sterilized 0.05 M PBS (pH 7.4) at room temperature for 16 h and then rinsed three times for 15 min each in 0.05 M PBS. The fixed cellulose discs were then dehydrated in successive ethanol-water mixtures with increasing ethanol concentrations (20%, 40%, 60%, 80%, and 95% [vol/vol]) for 15 min each and then twice in absolute ethanol for 15 min. The ethanol-dehydrated samples were then placed in an absolute ethanol bath, which was placed in an EMS 850 critical point drier (Electron Microscopy Sciences, Hatfield, PA). The ethanol was replaced by successive additions of liquid carbon dioxide. Once the liquid CO₂ had replaced the ethanol, the chamber was heated under pressure to reach the critical evaporation point of carbon dioxide. The chamber was then slowly vented of gaseous CO₂ and the dry samples removed. The dried samples were affixed to

aluminum mounts with double-sided carbon adhesive tape and sputter-coated with platinum and palladium to a thickness of 18 nm. Observations were performed at 5 to 7 kV with a scanning electron microscope (Hitachi S-570; Japan). Five fields of view at 5,000X-10,000X magnification were taken at randomly chosen areas from the optic surface of each sample. A biofilm-positive field was defined as being occupied by biofilm over at least half of the visible area.

Statistical analysis

The results of the CFU assays were analyzed with Prism[®] version 4.03 (GraphPad Software, San Diego, US) with 95% confidence intervals (CIs) of the difference. Comparisons of the *in vitro* biofilms formed on the cellulose discs with either Ag-gel dressings or Ag-free ones were analyzed by a two-tailed unpaired t-test to determine significant differences. All experiments were done in triplicate. The significance limit was $P < 0.05$.

Results

Effect of Ag-gel on bacterial biofilm formation for *in vitro* CFU studies

The results for the 24-h micro-aerobic *in vitro* studies using *S. salivarius*, *S. sanguis*, and *S. mutans* isolates, as well as the mixture of all three, are illustrated in Figure 1. As seen in Figure 1A-D, the cellulose discs that had no treatment, and those that were treated with the placebo gel, showed over 6 logs of bacterial growth in each case. However, the silver

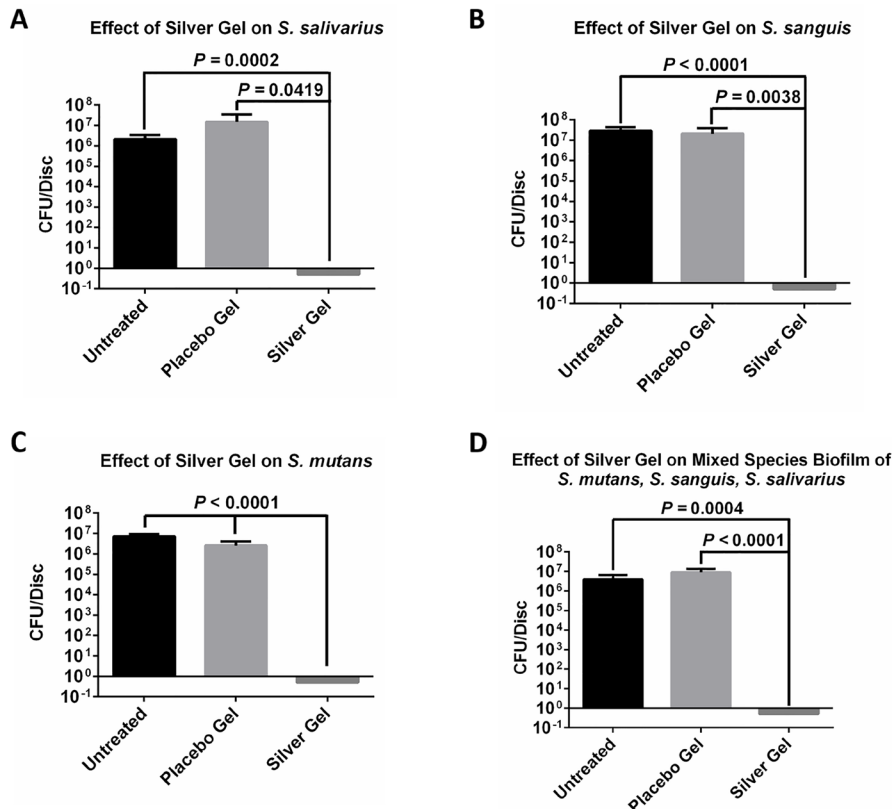


Figure 1. Colony forming unit analysis of biofilm formation. (A) *S. salivarius*, (B) *S. sanguis*, (C) *S. mutans* or (D) all three on untreated discs, discs treated with placebo gel or discs treated with colloidal silver gel.

containing gel showed 100% inhibition (over 6 log of killing) in all cases with *S. salivarius*, *S. sanguis*, *S. mutans*, or the combination of all three strains, when compared with the control, as showed in **Figure 1A–D**. Raw data are available on OSF¹⁹.

Effect of Ag-gel dressing on bacterial biofilm formation for *in vitro* SEM studies

To confirm results of CFU assay described in previous section, the biofilm formation of *S. salivarius*, *S. sanguis*, *S. mutans* or

the mixture of all three strains was studied on cellulose discs by SEM. *S. salivarius*, *S. sanguis*, *S. mutans* and the mixture of all three strains, were inoculated onto the discs in the same manner as for the CFU biofilm assay. Untreated discs were coated with placebo gel only. As above the discs were incubated for 24 h under micro-aerobic conditions at 37°C. As seen in **Figure 2**, *S. salivarius*, *S. sanguis*, *S. mutans* and the mixture of all three strains, formed typical biofilms characterized by the presence of micro-colonies on the cellulose

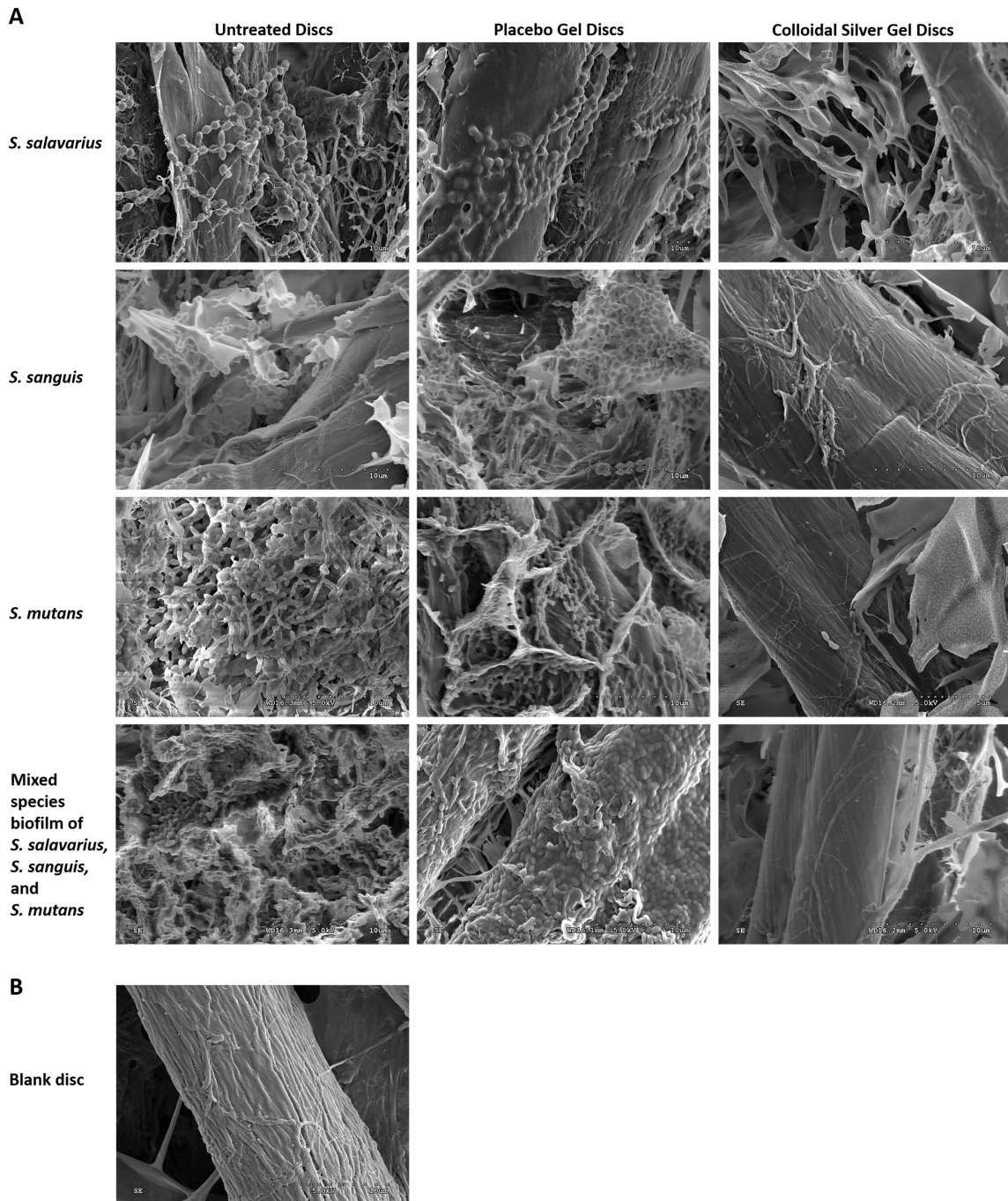


Figure 2. Scanning electron microscopy (SEM) imaging. (A) SEM analysis of *S. salivarius*, *S. sanguis* or *S. mutans* biofilm formation on untreated discs, discs treated with placebo gel or discs treated with colloidal silver gel. **(B)** SEM of a blank disc.

discs receiving no treatment, or treated with the placebo gel. However, no bacteria were seen on the cellulose discs treated with colloidal silver gel. These results confirm those obtained with the CFU assay. Raw SEM images are available on OSF¹⁹.

Discussion

There are over 700 different species that contribute to the formation of dental biofilm (plaque)^{9–11}. Among these species, *Streptococcus mutans*, *Streptococcus salivarius*, and *Streptococcus sanguinis* are the main members of this plaque^{20–22}. These bacteria are also considered to be the primary etiologic agents of human dental caries^{23–26}. It is the interaction of *S. mutans* with other streptococci that is thought to be important in dental plaque formation.²⁷ *S. mutans* can cause cariogenicity by the production of glucosyltransferase enzymes that allows glucose from sucrose to be used for the synthesis of glucan, and have also been implicated in heart problems^{28,29}. *S. sanguinis* is another common organism in dental plaque, which can colonize dental cavities. An additional problem is that this organism is also often found in the bloodstream. This allows it to attach to heart valves, causing bacterial endocarditis²². Thus, *S. sanguinis* is a key agent in infective endocarditis²¹.

Since *S. salivarius* is also part of the normal human flora, it can contaminate sterile body fluid. Thus, therapeutic interventions that disrupt the cells protecting the blood vessels can allow it to enter the blood stream and cause problems in areas such as the meninges and the cerebrospinal fluid.^{30–33} This results in a variety of infections such as meningitis and bacteraemia along with many other bacterial problems.^{27,30,34–39} Thus, these three bacterial species can not only work together to form plaque on teeth, but can play a major role in other medical problems in the body.

The objective of the current study was to evaluate the test Ag-gel for its efficacy to either control or annihilate the growth of these 3 organisms. The results present quantitative data of the antimicrobial effect on *S. salivarius*, *S. sanguis*, or *S. mutans* bacteria, of Ag-gel, layered on a cellulose disc which was inoculated with these bacteria or a combination of all three bacterial strains. The CFU assay results of *in vitro* studies using Ag-gel treated dressings showed over 6 log of killing (100%) for *S. salivarius*, *S. sanguis*, or *S. mutans* as compared with a control

gel dressing containing no Ag-gel. Since biofilms adhere strongly to surfaces, the experiments were also studied by SEM. These SEM studies confirmed the CFU results with *S. salivarius*, *S. sanguis*, or *S. mutans* biofilms, in the presence of Ag-gel or placebo gel. As seen in Figure 2, mature biofilms formed in the presence of the placebo gel, but none in the presence of the Ag-gel.

The mixture of these three bacteria was also studied, since it is proposed that the combination of bacteria is more resistant to growth inhibition than the individual bacteria. Similar results to the individual bacteria, (>6-log kill rate, 100%) were obtained with the combination of the three bacteria by both the CFU assay and by SEM studies.

Conclusion

An Ag-gel was found to be capable of over 6 log (100%) inhibition of *S. salivarius*, *S. sanguis*, or *S. mutans* bacteria, or a mixture of all three bacteria forming biofilms on cellulose discs by CFU studies. These results were confirmed by SEM studies of biofilm formation by *S. salivarius*, *S. sanguis*, or *S. mutans* or a mixture of all three bacteria, where the Ag-gel dressing showed total inhibition of biofilm formation on cellulose discs. These results indicate that use of a colloidal silver gel is an effective way to inhibit the formation of biofilms by the most common bacteria implicated in oral plaque formation, and this gel stands good potential to be developed into an effective commercial dentifrice product.

Data availability

Raw data for this study are available on OSF. DOI: <https://doi.org/10.17605/OSF.IO/AJNYU>¹⁹.

Data are available under the terms of the [Creative Commons Zero](#) “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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This study by Tran *et al.*, is important, but preliminary report that shows colloidal silver to be effective against three Streptococcus species. Following are some of the concerns I have regarding this manuscript.

1. The authors state that “the Ag-gel should be tested for the ability to block plaque formation in the mouth, through its use as a toothpaste”. Let’s consider the following: (a) Oral microbiome is very rich and its composition is important for health status of an individual and (b) Literature says colloidal silver is toxic to the host cells.

Considering (a) and (b), using the Ag-gel as a toothpaste may not be a good idea as it is not specific towards the pathogens. Thus, I recommend the authors to measure the cost-benefit ratio and mention about the conditions or stages of oral disease at which it could possibly be used as a toothpaste.

1. Why only one concentration of 0.5g of colloidal silver used for the studies?
2. Please provide the details for the figures in the legends, like (i) what is the sample size, (ii) what do the error bars indicate, and (iii) which statistical test was performed on the data.
3. I fail to understand the biological relevance of negative data of CFU in Fig. 1.
4. What happens to biofilm formation at lower concentrations of silver?
5. As the previous reviewers commented, eradication of biofilms should also be tested. Therefore, this manuscript should accompany the Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) of colloidal silver.
6. Determination of interaction of colloidal silver with antibiotics is also an important test that has to be performed.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biofilms.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 16 April 2019

<https://doi.org/10.5256/f1000research.19360.r45453>

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Mohan Kulkarni

Department of Chemistry, Biochemistry Division, Savitribai Phule Pune University, Pune, India

Caries has been the one of the most prevalent dental conditions which needs effective treatment. Products currently in use such as mouthwashes recommended for oral hygiene are not very effective against dental biofilm formation.

This study evaluates the efficacy of colloidal silver gel formulations in inhibiting bacterial biofilm formation by *S. mutans*, *S. sanguis*, and *S. salivarius* which cause plaque formation. The choice of colloidal silver over ionic silver in view of its lower toxicity is well justified and so also the choice of bacteria for evaluating the efficacy of colloidal silver.

The effectiveness of colloidal silver has been demonstrated by quantifying colony forming units (CFU). Appropriate controls and statistical analyses have been provided to support the results. The results are further supported by scanning electron microscopy. The results show that no biofilms are formed in the presence of colloidal silver gel but do form in the control experiments carried out with placebo.

Since the objective of the work is to develop a treatment for the prevention of caries, it would be desirable to optimize the concentration of colloidal silver in the gel in future. In addition to prevention of biofilm formation if colloidal silver is shown to be useful in disrupting biofilms already formed, it would enhance the scope of treatment.

I recommend the indexing of the paper "Efficacy of a silver colloidal gel against selected oral bacteria in vitro".

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Polymers, nanoparticles, drug delivery, patents.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 11 March 2019

<https://doi.org/10.5256/f1000research.19360.r45449>

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Vijay Kothari 

Institute of Science, Nirma University, Ahmedabad, India

This study has evaluated antibacterial efficacy of a colloidal silver gel against three important oral bacteria. In context of the currently available dentifrice products not being full effective in preventing the widespread problem of dental caries, need and research for new more effective dentifrices is justified. Their idea of assaying the test gel not only against individual bacteria, but also against the mixed-species is a logical one, as that represents real-life situations a bit more closely. In the recent past, maintenance of good oral health has been shown to be important for good cardiovascular health, healthy pregnancy, etc.

They may consider re-framing the last sentence of the third paragraph in 'Introduction'. They may also

check for correct placement of all references; For e.g. references 2-3 have been cited in first paragraph in an oral health context, but their titles seem to be more relevant to wound management.

While, I do not have any reservations in recommending this article in its present form for indexing, I suggest for following additional experiments for future studies by these authors:

1. Effect of this gel on pre-formed biofilms of oral bacteria should be checked on two different parameters i.e. whether this gel can kill bacteria in biofilm, and whether it can eradicate the pre-formed biofilm with/without killing the bacteria in it.
2. Results of current study indicate their gel to possess a bactericidal action. They may consider extending the incubation till 48-72 hours after transferring the gel-exposed bacteria onto fresh gel-free media, to confirm the true bactericidal effect, and absence of post-antibiotic effect (PAE).
3. They may consider determination of MIC, MBC, and time required to kill. The latter is important as typical dentifrice products like mouthwashes/toothpaste get few seconds/minutes to exert their effect in the mouth.
4. It will be interesting to investigate whether at sub-inhibitory concentrations their product can reduce lactic acid production by the Streptococci, which is a major virulence factor involved in demineralization of teeth. Whether sub-inhibitory concentrations can inhibit quorum-sensing in these bacteria?
5. In the current study, cellulose discs were used as surface for biofilm formation. In future studies, they may consider some material similar to teeth-substance (e.g. calcium) as the surface for biofilm formation.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antimicrobial Resistance (AMR); Bacterial biofilms; Antimicrobials; Traditional Medicine; Bioacoustics; Plant extracts; Quorum Sensing

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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