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ORIGINAL ARTICLE

Antibacterial Efficacy of Silver Diamine Fluoride Compared to Casein Phosphopeptide-Amorphous Calcium Phosphate Against *Streptococcus mutans* in a Biofilm Caries Model

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ABSTRACT

Objective: To compare the antibacterial efficacy of silver diamine fluoride (SDF) with a product containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) against *Streptococcus mutans* using a biofilm caries model. **Material and Methods:** Twenty-seven saliva-coated dentine blocks obtained from extracted human teeth were inoculated with *Streptococcus mutans* monospecies biofilm in this *in vitro* study. The biofilms were then exposed to 10% sucrose in brain heart infusion broth eight times daily for seven days. After the biofilm growth period, the dentine blocks (n=9 per group) were treated with one of the following substances: 1) sterile saline (control), 2) 38% SDF, and 3) a product containing CPP-ACP. Then, the samples were incubated at 37°C for 48 hours, and the numbers of viable microorganisms in the biofilms were counted and compared. ANOVA and Tukey's HSD tests were used to analyze the data ($p<0.05$). **Results:** The number of viable bacteria, as determined by the number of colony-forming units (CFU mL^{-1}) of *Streptococcus mutans*, was significantly reduced following treatment with SDF and the CPP-ACP product ($p<0.05$). However, SDF showed superior antibacterial activity compared to the CPP-ACP product (mean CFU mL^{-1} =zero compared to 96 x 10^6) ($p<0.05$). **Conclusion:** SDF has higher antibacterial activity against cariogenic *Streptococcus mutans* biofilm than the CPP-ACP product. The CPP-ACP product showed antibacterial activity, but it was limited.

Keywords: Biofilms; Dental Caries; Fluoride Treatment; Diamines; Streptococcus mutans.

Introduction

Dental caries continues to be one of the most prevalent chronic diseases in the world, affecting people across all age groups and countries [1,2]. Carious lesions can be prevented using fluoride-based materials such as professionally applied varnishes [3]. Among the fluoride agents, silver diamine fluoride (SDF) has drawn much attention in the past decade [4]. SDF is a clear liquid that combines silver's antibacterial effects and fluoride's remineralizing effects [5]. Previous studies have tested several SDF concentrations [6-8]. However, the highest concentration of SDF (38%) has shown the best results in arresting caries among children [5,6].

The mechanism of action of SDF remains unclear until now. The suggested mechanism has been that fluoride strengthens the tooth structure under attack by the acid byproducts of bacterial metabolism, decreasing its solubility. Still, SDF may also interfere with the biofilm, killing bacteria that cause the local environmental imbalance that demineralizes dental tissues [5]. Few *in vitro* studies have reported that SDF prevents the formation of *Streptococcus mutans* or *Actinomyces naeslundii* mono-species biofilms [9,10]. At the same time, others have documented its remineralizing potential on enamel and dentine [9,11]. However, a significant drawback of SDF is that as the caries lesions become arrested, the precipitation of silver byproducts in the dental tissues stains the lesions black, which can deter its use in visible areas [12]. The high concentration of silver and fluoride has also raised some concern, especially with repeated applications on very young children [5]. Also, SDF is unavailable in certain countries, including the UK [11].

Another well-recognized remineralizing agent is Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), a natural protein extracted from milk casein [13]. CPP-ACP functions by providing calcium and phosphate ions, which suppress demineralization and induce remineralization of early carious lesions [14]. Few studies have indicated that CPP-ACP exhibited a controlling potential on plaque and could reduce the growth and adherence of cariogenic bacteria [14–17]. Mao et al. [14] observed a 39% reduction in the bacterial biofilm with CPP-ACP-modified glass ionomer cement (5%), with this ability being primarily manifested in the inhibition of *S. mutans*. Similarly, Jafari et al. [18] detected growth inhibition zones in *S. mutans* treated with CPP-ACP varnishes. On the contrary, Hajiahmadi et al. [19] failed to find any antibacterial effect of CPP-ACP against *S. mutans* at any concentration; of note, however, is that Jafari et al. [18] and Hajiahmadi et al. [19] did not incorporate a caries model in their studies.

To our knowledge, none of the published studies compared the antibacterial efficacy of SDF with that of CPP-ACP using a biofilm caries model. Biofilm models are essential to create standardized procedures for comparing the effectiveness of different materials and simulating highly cariogenic conditions on the tooth surface. Control of biofilm formation and accumulation is crucial in reducing the incidence of dental caries [20]. Therefore, the purpose of this *in vitro* study was to compare the antibacterial efficacy of SDF with that of CPP-ACP against *S. mutans* using a biofilm caries model. The tested null hypothesis was that no statistically significant differences would exist among the agents tested.

Material and Methods

Ethical Clearance

This study has been approved by the ethical committee of the College of Dentistry/Qassim University (reference number: DEC_I/7002/2018).

Sample Preparation

A total of 20 sound-extracted third molars for orthodontic purposes were used in this study. A water-cooled diamond disk (Super Diamond Disk No. 800.104.355.524.190; NTI-Kahla GmbH, Germany) was used to

section the roots of sample teeth to provide 27 dentine blocks (2 ×2 × 4mm). Dentine blocks were selected as they have been considered suitable as a substrate for biofilm formation [21]. No power analysis was used to determine sample size; however, we based our sample size on the work of Chu et al. [9], where a sample size of at least eight blocks per group was sufficient to have a power of 0.8 and α=0.05. The blocks were sonicated in distilled water for 10 minutes to remove cutting debris and then etched with 37% phosphoric acid for 15 seconds to eliminate the smear layer. Then, the blocks were sterilized by immersion in 0.5% NaOCl for 10 minutes [22] and rinsed with sterile saline. Afterward, they were immersed in filtered, pooled human saliva and agitated (60 rpm for 30 min at 37ºC) to simulate pellicle formation.

The pooled human saliva used in the present study was obtained from caries active otherwise healthy donors who abstained from oral hygiene for 24 hours and from food ingestion for two hours before saliva collection.

Biofilm Growth

We used a modified method to that adopted by Savas et al. [20], *S. mutans* UA159 (American Type Culture Collection 700610, Rockville, MD, USA) colonies were transferred to brain heart infusion broth (Millipore Sigma, St. Louis, MO, USA) supplemented with sucrose and incubated at 37ºC under 10% CO_2. S. mutans UA159 was inoculated into brain heart infusion broth and incubated until an optical density of 1.5 at 600 nm/mL was obtained for biofilm formation.

The dentine blocks with human salivary pellicles were positioned in 24-well plates, each well containing 2.0 mL of the inoculum and incubated at 37ºC under 10% CO_2 to allow bacterial adhesion. Then, dentine blocks with biofilms were transferred to fresh brain heart infusion broth and exposed for 1 minute, eight times per day, to 10% sucrose for seven days to allow the bacteria to grow and mature in the biofilm as a period of 7 days was previously reported to be sufficient for a cariogenic and mature biofilm to be obtained [9,23]. After each sucrose exposure, the blocks were washed three times in 0.01% NaCl. The culture medium was changed daily after the first and last sucrose exposures.

Exposure to Tested Substances

A stock solution of the CPP-ACP product (GC tooth mousse) was initially prepared by suspending 3g in 10 mL sterile water. Then, for treatment, the dentine blocks were removed and divided into two experimental groups (n=18 blocks) and a control group (n=9 blocks). The biofilms of the experimental groups were treated with either 38% SDF (n=9 blocks) or CPP-ACP (stock solution) (n=9 blocks), while those of the control (n=9 blocks) were subjected to sterile saline. A micro brush was used to apply each material to the surfaces of the dentine blocks for 3 minutes (Table 1).

Table 1. The tested materials and their chemical composition.

Tested Substance	Trade Name	Chemical Composition	Manufacturer
38% SDF	FAgamin	$AgF(NH_3)_2$	Tedequim, Argentina
CPP-ACP	GC tooth mousse	Pure water, Glycerol, CPP-ACP, D-Sorbitol, Silicon Dioxide, CMC-Na, Propylene glycol, Titanium dioxide, Xylitol, Phosphoric acid, Guar gum, Zinc Oxide, Sodium Saccharin, Ethyl p-hydroxybenzoate, magnesium oxide, Butyl p-hydroxybenzoate, Propyl p-hydroxybenzoate	GC Australia, Australia
Sterile Saline (control)	-	0.9% sodium chloride	-

Biofilm Assessment and Estimating the Total Bacterial Colony Forming Units (CFU mL^{-1})

After topical application of the tested materials, the dentine blocks were washed with sterile water to avoid residual materials for 1 min. Then, the dentine blocks were returned to the 24-well plates and incubated for 48 hours, after which they were washed three times in 0.9% NaCl and transferred individually to sterile glass tubes containing 9 mL of 0.9 NaCl. The tubes were sonicated for 30 seconds at 20 w to detach the biofilms that had formed on the blocks.

To determine the total CFU mL^{-1}, an aliquot (1 mL) of the homogenized suspension was serially diluted in 0.9 NaCl (10^0–10^8), and a 1-mL suspension from each dilution was inoculated onto agar plates which contained brain-heart infusion broth. The plates were incubated in 10% CO2 at 37°C for 48 hours to determine the number of viable microorganisms. Then, the number of CFU mL^{-1} was counted by dividing each agar plate into four quadrants and counting the colonies on one quadrant. Then, the number gained was multiplied by four to get the whole number of colonies on the agar plate (Figure 1).

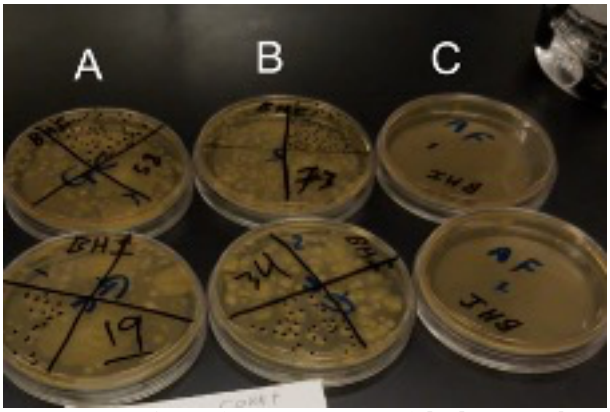

Figure 1. A sample of the agar plates following treatment with the tested materials. A: CPP-ACP (GC tooth mousse), B: sterile saline, C: Silver diamine fluoride.

Statistical Analysis

The SPSS computer software version 22.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Descriptive statistics, including means, standard deviations, and frequency distribution, were calculated for each group. The results of CFU mL^{-1} from all groups were submitted to one-way ANOVA and the posthoc Tukey test to demonstrate differences between pairs of groups. Probability p-value < 0.05 was set as the reference for statistically significant results.

Results

The means of CFU mL^{-1} for dentine blocks treated with 38% SDF, CPP-ACP, and sterile saline (control) are shown in Table 2. Only in the group treated with 38% SDF the mean of CFU mL^{-1} of *S. mutans* was zero. The group treated with CPP-ACP showed lower mean CFU mL^{-1} than the control group. The difference between the three treatment methods was significant ($p<0.05$). Post-hoc Tukey test was performed to determine the differences within treatment groups. A significant difference was found between the SDF group and CPP-ACP and saline groups. Likewise, a significant difference was found between CPP-ACP and saline groups ($p<0.05$).

Table 2. The total viable microorganisms in the biofilm after treatment procedures.

		S. mutans in the Biofilm (CFU mL^{-1})			
Tested Medium	**N**	**Mean**	**SD**	**p-value**	**Pairwise Comparisons (p-value)**
38% SDF [a]	9	0.00	0.00		0.003* (a *vs.* b)
GC tooth mousse [b]	9	96x10^6	34.64x10^6	<0.0001*	<0.0001*(a *vs.* c)
Sterile saline (control) [c]	9	301.33x10^6	16.16x10^6		<0.0001* (b *vs.* c)

SDF: Silver Diamine Fluoride; SD: Standard Deviation; *Statistically Significant Difference.

Discussion

SDF and CPP-ACP are two agents reported to have remineralizing abilities. Bearing antimicrobial potential is an essential property for remineralizing agents. The antibacterial efficacy of SDF and CPP-ACP were assessed individually in a few studies with variable results, especially CPP-ACP [15,17-19]. However, this study is the first to compare the antibacterial efficacy of both using an *S. mutans* biofilm model. *S. mutans* has been repeatedly considered the most cariogenic microorganism in dental biofilm with the ability to form mono-species biofilms [9,20]. The *S. mutans* biofilm model has shown sufficient sensitivity to biofilm changes in the presence of antimicrobial substances [20]. Most previous studies employed *S. mutans* to test the antibacterial efficacy of SDF and CPP-ACP. To form a biofilm, *S. mutans* needs to adhere to a substrate. Saliva components and pellicle layer must be present in the environment to achieve bacterial colonization and adhesion [17]. In the present study, human saliva was used to facilitate the adhesion of bacteria as well as form the pellicle layer and start the biofilm formation step. Further, to stimulate the enzymatic synthesis of adhesive glucans (bacterial cell wall polysaccharides) from sucrose, the blocks were exposed to 10% sucrose- for one minute, eight times a day, then glucans could mediate firm adherence to the surfaces of the dentine blocks [17].

In the present study, the tested agents were compared to sterile saline, previously used as a control for antibacterial studies [21,24]. All materials were applied with a micro brush to the surface of the blocks, which is consistent with previous studies that mentioned using a micro brush [9,11,23], a brush [20], or a cotton-tipped applicator [17] to apply SDF and/or CPP-ACP to the surface of the specimens. Additionally, the evaluation of CFU mL^{-1} count is an expression of the number of viable bacteria in the biofilm, and this has been deemed a sufficiently accurate measure to compare the efficacy of the tested agents [24]. However, mono-species biofilms in microplate systems differ significantly from complex in vivo multispecies plaque biofilms in both survival and pathogenic potential [9]. The human mouth involves 30 genera representing at least 500 species interacting [25]. Therefore, the results cannot be extrapolated to the in vivo situation, and caution should be exercised in their interpretation [9].

Based on the results obtained in the current study, the tested null hypothesis was rejected since differences were observed in the antibacterial activities of the agents tested. Significant differences were observed between the control group and both treatment groups. However, the mean CFU mL-1 of S. mutans was only zero in the SDF group. This finding is consistent with previous studies [9,10,20,24]. CPP-ACP showed intermediate results, with significantly lower mean CFU mL^{-1} of *S. mutans* than the control group, which had confluent growth of live *S. mutans* and high mean CFU mL^{-1} count but a significantly higher mean value than the SDF group. This implies that SDF had a prominent bactericidal effect compared to CPP-ACP. It has been reported that different brands of SDF products may vary in their composition as manufacturers have yet to disclose all of the ingredients in their SDF products [4]. However, regardless of the brand, 38% SDF should contain high silver and fluoride ion concentrations. Silver ions have a bactericidal nature against biofilm formation by primarily inactivating the enzymes responsible for synthesizing glucan, which contributes to the bulk of biofilms. Also, they play an essential role in the sucrose-dependent adhesion of organisms to tooth

surfaces. On the contrary, fluoride in high concentration binds to bacterial cell constituents and influences responsible enzymes of carbohydrate metabolism of acidogenic oral bacteria and their sugar uptake [23].

Our findings concerning CPP-ACP are in agreement with those of Erdem et al. [15] and Sahin and Oznurhan [17], who found that after using CPP-ACP, the survival of *S. mutans* in biofilm decreased as compared to the untreated control group, but insignificantly. However, the current study observed a significantly lower mean CFU mL-1 count of S. mutans after CPP-ACP treatment. These results may be because the casein fractions in the milk have some inhibitory effect on the adhesion of *S. mutans* to the tooth surface and can alter the microbial content of plaque biofilm [17]. However, this effect is perhaps limited and insufficient on the biofilm compared to a similar treatment duration with a concentrated form of SDF. Consequently, it warrants further studies to assess the effect of more concentrated forms of CPP-ACP and perhaps longer and repeated treatment durations on the dental biofilm. SDF application times in the literature ranged from ten seconds to three minutes, with various degrees of success that were not time-dependent [5]. The current study adopted a 3-minute application period for both SDF and CPP-ACP for standardization and following previous protocols using 38% SDF [20].

Furthermore, a CPP-ACP solution of a 1:3 ratio in sterile water was prepared and investigated following what was done by Kumar et al. [13]. Of note is that Sahin and Oznurhan [17] found lower absorbance values and consequently reduced survival of *S. mutans* in biofilm receiving a single application of CPP-ACP for 1 minute than the control, but the difference was insignificant. Furthermore, Kumar et al. [13] found that CPP-ACP had better remineralizing potential when applied as a topical coating without dilution. Consequently, adopting a more extended treatment duration for CPP-ACP and perhaps applying it directly may give different results on the *S. mutans* dental biofilm. Furthermore, assessing the effect of repeated application of CPP-ACP is important to simulate the frequent, at times daily, application of CPP-ACP in clinical conditions.

The present study provided valuable insights into the antibacterial effect of both SDF and CPP-ACP against *S. mutans* biofilm. However, certain limitations should be addressed; as mentioned earlier, mono-species biofilm models differ from the complex in vivo multispecies plaque biofilms. Consequently, the results should be carefully interpreted. Furthermore, the tested CPP-ACP product also contained Xylitol, among other constituents. Xylitol is a non-cariogenic sugar substitute known for its caries inhibitory effect. The presence of Xylitol in the CPP-ACP product could be a confounding factor that might have contributed to the reduced levels of *S. mutans* following treatment with the CPP-ACP product in the present study. However, it is worth mentioning that the American Academy of Pediatric Dentistry recently supported the lack of consistent evidence concerning the caries-inhibitory effect of Xylitol in children and the unlikelihood that Xylitol causes significant reductions in *S. mutans* levels [26]. Consequently, it is unlikely that Xylitol provided a synergistic effect to the antibacterial effect observed with the CPP-ACP product in the present study. Other limitations would be that we did not assess the acidogenicity of the biofilm to verify the pH level. Furthermore, no attempt to analyze the changes in mineral status in the dentine blocks (demineralization assessment) was performed. Consequently, further *in vitro* studies are needed to assess the antibacterial efficacy of CPP-ACP, in particular on cariogenic biofilms in concentrated forms as well as with extended treatment periods and repeated treatments while considering the acidogenicity of the biofilm and the demineralization status of the teeth specimens.

Conclusion

Silver Diamine Fluoride is a bactericidal agent with superior antibacterial activity compared to CPP-ACP on the *S. mutans* dental biofilm. CPP-ACP had antibacterial activity but was limited compared to silver diamine fluoride.

Authors' Contributions

SNAHA	https://orcid.org/0000-0001-5210-5128	Conceptualization, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing, Review and Editing, Visualization and Supervision.
SAO	----	Methodology, Resources, Project Administration and Funding Acquisition.
RIF	https://orcid.org/0000-0003-3542-2775	Validation and Formal Analysis.

All authors declare that they contributed to a critical review of intellectual content and approval of the final version to be published.

Financial Support

None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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