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A comparison of the antimicrobial efficacy of silver diamine fluoride and silver nitrate: an ex vivo study

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A comparison of the antimicrobial efficacy of silver diamine fluoride and silver nitrate on various cariogenic bacteria: an *ex vivo* study

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

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Abstract

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Purpose: The use of silver-based antimicrobials is an emerging method for the treatment of dental caries. In this study, the authors compare the efficacy of the two most prominent silver-based therapeutics, silver diamine fluoride (SDF) and silver nitrate ($AgNO_3$), on cariogenic and non-cariogenic multispecies biofilms. Currently there is a lack of studies comparing the efficacy of SDF to $AgNO_3$.

Methods: Plaque samples from anterior and posterior tooth sites from children presenting both with early childhood caries and caries-free children were collected, pooled, and utilized to create four *ex vivo* biofilm systems in artificial saliva. SDF and $AgNO_3$ were administered to these biofilms and bacterial survival was quantified and compared to untreated controls.

Results: Each of the four pooled sample types was applied to plates coated in artificial saliva + 1% sucrose. Both SDF and $AgNO_3$ were very effective against plaque derived biofilms when compared to untreated biofilms ($P<0.001$). There was no significant difference ($P>0.05$) in the potency of each compound.

Conclusions: SDF and $AgNO_3$ significantly inhibit *ex vivo* cariogenic and non-cariogenic biofilms at similar levels.

Introduction

Dental caries represents a significant source of morbidity among pediatric patients[1] and a global economic burden.[2] The Centers for Disease Control and Prevention reports that dental caries is the most prevalent chronic disease in our nation's children.[3] More than 40 percent of children have caries by the time they reach kindergarten.[4] The prevalence of caries within the two to five year old age group among higher income families is 18 percent, while that of children from low-income families was 42 percent.[5,6] The burden of this chronic disease experience falls on children of low socioeconomic status, and those with medical and behavioral challenges, limited access to dental care and those who need multiple dental visits. If Early Childhood Caries (ECC) remains untreated, oral health–related quality of life, body weight, growth, school attendance, and school performance can be affected.[7,8] Preventive management and cost-effective strategies can effectively arrest and even completely reverse the caries process, and so the interest in the use of silver medicaments renewed and has been growing.

Silver has been adopted as an antimicrobial material for thousands of years. It has long been a common antimicrobial agent for medical use because of its broad spectrum of antibacterial activity, lack of bacterial resistance, and low toxicity [9] A Roman pharmacopoeia written in 69 B.C. mentioned the use of silver as a disinfectant.[10] In 1917, Howe's solution was reported to disinfect caries lesions and continued to be used for nearly one-half of a century as a sterilizing and disclosing agent for bacterial invasion of dentin.[11,12] However, the use of silver products became subsidiary when penicillin and other antibiotics were introduced in the 1950s.

Additionally, the introduction of injectable local anesthesia also led to a shift from medical to surgical management of caries.

When applied by a professional with appropriate care, Silver Diamine Fluoride (SDF) is likely safe. Two components of the medicament may be of potential concern for the health and safety of patients, Fluoride and Silver. Serum concentration of fluoride following SDF application per manufacturer recommendations posed little toxicity risk and was below EPA oral reference for fluoride dose in adults.[13] In female and male rat and mouse studies conducted to determine the lethal dose (LD50) of silver diamine fluoride by oral and subcutaneous administration. Average LD50 by oral administration was 520 mg/kg, and by subcutaneous administration was 380 mg/kg. In one drop (25 microliters) of 38% SDF, there is 9.5 mg of silver. One drop of SDF is sufficient to treat at least five carious teeth. Thus the relative safety margin of using an entire drop on a 10kg child is: 380 mg/kg LD50 / 0.95 mg / kg dose = 400-fold safety margin[14] In one drop (25 microliters) of 25% Silver Nitrate (SN or $AgNO_3$), there is 6.25 mg of silver. The amount at which a dose could be lethal is 2 grams for a 70kg child. A dose of 6.25 mg of SN would then be 320 times less than the lethal dose.[15] Temporary staining to skin which resolves in 2-14 days, and mildly painful white lesion in the mucosa, from inadvertent contact with 38% SDF has been noted in the literature.[16,17] Although there has been a favorable pulpal response noted in the literature, including the presence of abundant reparative dentin and a wide odontoblast layer found in a histological assessment of the dental pulps of carious primary teeth treated with 40% silver fluoride, the toxicity and biocompatibility of silver compounds require further evaluation and should not be placed on exposed pulps as this agent can penetrate deep into dentin, and if the caries extends to the pulp, the agent can, as well which could cause a

pulpal irritation[17–19]; thus, teeth with deep caries lesions should be closely monitored clinically and radiographically.[17]

The *in vivo* mechanism(s) of action of SDF and $AgNO_3$ are a subject of ongoing research. It is thought they arrest lesions via the silver ions lysing bacteria. The silver ions interact with the bacterial cell envelope, the molecules inside the cell (e.g., nucleic acids and enzymes), and inhibit the main respiratory chain proteins causing an increase in the production of reactive oxygen species (ROS), which contributes to the death of the bacteria.[20] Fluoride has antimicrobial action at high concentrations[21], and *ex vivo* studies show more resistance to demineralization following treatment with SDF than with matching fluoride solutions without silver.[22,23] Thus, both silver and fluoride may contribute to both bactericidal effects and lesion strengthening.

It has been reported that SDF ($Ag(NH_3)_2F$) reacts with the tooth mineral hydroxyapatite (HA)($Ca_{10}(PO_4)_6(OH)_2$) to release calcium fluoride (CaF_2) and silver phosphate (Ag_3PO_4), which are responsible for the prevention and hardening of dental caries. The CaF_2 formed provides a reservoir of fluoride for the formation of fluorapatite ($Ca_{10}(PO_4)_6F_2$), which is more resistant to acid attack than HA [23]

In one study, Klein et al compared the progression of carious lesions after treating them with four chemotherapeutic regimens: silver nitrate ($AgNO_3$); silver fluoride/stannous fluoride (AgF/SnF_2); silver diamine fluoride (SDF); and chlorhexidine (CHX). Their study demonstrated 29% and 19% less lesion progression in those treated with a single AgF/SnF_2 or $AgNO_3$ application, respectively, than did the control group treated with isotonic solution. [24]

Zhao et al performed an *in vitro* study assessing the caries arresting ability of silver nitrate solution, followed by sodium fluoride varnish. They evaluated the dentin surface morphology, crystal characteristics, carious lesion depth and collagen matrix degradation of the specimens by scanning electron microscopy, X-ray diffraction, X-ray microtomography and spectrophotometry with a hydroxyproline assay. Scanning electron microscopy showed that dentin collagen was exposed in the control group, but not in groups SF (25% Silver Nitrate/5% Sodium Fluoride) and SDF, while clusters of granular spherical grains were formed in the SDF and SF groups. The results of this in vitro study indicate that the use of silver nitrate solution and sodium fluoride varnish is effective in inhibiting dentin demineralization and dentin collagen degradation.[25]

Hill et al treated freshly extracted teeth, with no caries, with different dilutions of nitric acid for varying lengths of time. A test was then made to compare the solubility of treated and untreated enamel by the weight-loss of each substance when subjected to acid demineralization. They concluded that enamel, after being treated with silver nitrate, becomes more resistant to the action of weak organic acids.

Mei et al investigated the effects of silver diamine fluoride (SDF) on dentin carious lesions with a *S. mutans* and *L. acidophilus* dual-species cariogenic biofilm. The dead-to-live ratios from confocal laser scanning microscopy (CLSM) images, which indicate strength of anti-microbial effect, were significantly higher after topical SDF application. They also found the log of colony forming units (CFU) counts for both *L. acidophilus* and *S. mutans* in the SDF group to be significantly lower than that in control groups ($p<0.01$), yet higher than those in mono-species biofilm, which indicates an increased antimicrobial tolerance of the species in dual-species.[26]

The mechanisms of increased antimicrobial tolerance in dual-species biofilms are not clear so far, but it was suggested that cell to cell communication may play a role.[27]

Mei et al also investigated the antimicrobial effects of SDF on multi-species cariogenic biofilms composed of five common cariogenic bacteria (*Streptococcus mutans, Streptococcus sobrinus, Lactobacillus acidophilus, Lactobacillus rhamnosus* and *Actinomyces naeslundii*). The colony forming units (CFU) results revealed fewer CFU's in the test group compared with the control group ($p < 0.01$). Scanning electron microscopy and confocal microscopy showed less bacterial growth in the test group, and confluent cariogenic biofilm in the control group ($p < 0.01$). The microhardness and weight percentages of calcium and phosphorus in the test group from the outermost 50 μm were higher than in the control group ($p < 0.05$). The energy dispersive spectroscopy (EDS) showed that calcium and phosphorus were higher in outer 50 mum in test groups than in the control. The Fourier transform infra-red spectroscopy (FTIR) revealed less exposed collagen I in the test lesions compared with the control group ($p < 0.01$). 38% SDF was found to inhibit the growth of multi-species cariogenic biofilms.[28]

Recent reviews of the literature have identified multiple *in vitro,* clinical and animal studies, and other types of articles such as several review articles or short communications. A few *ex vivo* and *in vitro* studies have examined the antimicrobial efficacy of SDF on multi-species cariogenic biofilms.[29–31] The bacteria included in most of these studies were *Streptococcus mutans, Streptococcus sobrinus, Lactobacillus acidophilus, Lactobacillus rhamnosus,* and *Actinomyces.naeslundii*.[29,32] Caries, however, is a polymicrobial infection process, caused by more than 700 species. Most SDF and SN studies have not studied the effects the medicaments have on the complex biofilm. A discussion on the use of $AgNO_3$ and NaF by the Oregon Dental Association concluded that more research demonstrating efficacy and safety

is required to meet the standard for evidence-based dentistry.[33] In 2017, the SDF Panel of the American Association of Pediatric Dentistry (AAPD) issued a guideline that supports the use of 38 percent SDF for the arrest of cavitated caries lesions in primary teeth as part of a comprehensive caries management program. This recommendation was found to be a conditional one based on low quality evidence due to major limitations of the supporting literature including lack of calibration and/or evidence of agreement for examiners assessing clinical outcomes and unclear definitions or inconsistent criteria for caries lesion activity[17].

There are many implications that would result from the proposed study. First, it would be the first time an *in vitro/ex vivo* study has studied the effects of SDF and SN/NaF in the same study. This would not only contribute to filling the gap in the current understanding of both products, but it could indicate whether more discussion is necessary regarding which product to use, based on antimicrobial efficacy and other factors. Secondly, it would be one of the few studies to examine the effects each product has on complex biofilms. This is clinically relevant as clinical caries is not the result of one organism, but an entire oral microbiome. Currently, there is a greater understanding of the efficacy of SDF than SN. More research on SN/NaF is needed and necessary. This study could be useful in guiding further research in this field and help the dental community design and implement further *in vitro/ex vivo/in vivo* studies to fill the gap in knowledge and contribute to our use of evidence based dentistry.

There were three specific aims of this research study:

Specific Aim 1: To develop a reliable model to replicate an *ex vivo* biofilm

Specific Aim 2: To study the antimicrobial efficacy of SDF and SN/NaF on multi-species complex microbiomes

Specific Aim 3: To compare the antimicrobial efficacy of SN/NaF and SDF.

There were two working hypotheses in this research study:

SN/NaF will demonstrate a comparable antimicrobial efficacy to SDF.

SN/NaF will demonstrate a greater antimicrobial efficacy than SN alone.

Methods

This study was approved by the Virginia Commonwealth University Institutional Review Board (IRB HM20011845). Our study sample included twenty children who presented to the VCU Graduate Pediatric Dentistry clinic for routine dental care. Inclusion criteria was medically healthy children in the primary dentition. Participants were divided into two groups:

- Group of children with Early Childhood Caries (ECC, n=20)
- Group of caries free children (n=17)

Guardians of children gave verbal consent and waiver of assent for participation of their children.

Sample collection and bacterial cultures

Two plaque samples (one anterior, one posterior) were collected from each participant. All sites were air dried, and cotton roll isolation was used. Supra-gingival plaque was gently removed from the tooth via a sterile curette and stored in 1 mL of Brain Heart Infusion (BHI) medium + 10% filtered artificial saliva[34] in a microcentrifuge tube on ice. The sample was immediately transported into an anaerobic chamber where the sample was split into two 250 µl aliquots. The remaining undiluted sample (approximately 500 µl) was stored at -80 ºC in 10% glycerol. The aliquots were either diluted with another 250 µl of BHI medium or 250 µl Shi medium to lower the oxygen level of the sample. Samples were incubated overnight in an artificial atmosphere (composed of 85% N_2, 5% H_2, and 10% CO_2) at 37 ºC using a Coy anaerobic chamber (Ann Arbor, MI). After overnight incubation the aliquots were stored at -80

ºC with 10% of glycerol. Sample aliquots from ten patients were pooled together and aliquoted to 50 µl of each for the following study.

Formation of Biofilms:

Each *ex vivo* biofilm was seeded in a saliva coated plate and grown in artificial saliva + 1% sucrose for 48 hours. Artificial saliva was refreshed after 24 hours. To measure survival, ex vivo biofilms were plated on a mixture of supplemented BHI and L-MRS agar. This creates a rich media that supports the growth of multiple cariogenic strains.

After growing each sample individually, samples of each category were combined and grown in biofilm media consisting of 1-part BHI broth, 1-part L-MRS broth, and 1% sucrose, and an *ex vivo* multi species model was created. To form biofilms for treatment, a 24-well plate was coated with artificial saliva allowing the bacteria to attach to the salivary proteins and form a biofilm in each well. The combined bacterial samples were grown in biofilm media and added to each well. Several different conditions were tested to create a suitable biofilm including biofilms grown in media or artificial saliva (Fig. 2A-B). Samples were incubated for 24 hours under anaerobic conditions at 37°C, after which old media was aspirated off and fresh media added and grown for 24 additional hours.

Antimicrobial Treatment:

After 48 hours of growth, the biofilms were treated with the following treatment groups:

1. SDF (38% Advantage Arrest™)
2. 25% SN (25% - Gordon Laboratories)
3. Saline (negative control)

For treatment, excess media was aspirated off and the biofilms washed with saline. Approximately 250 μL of each treatment chemical, enough to cover the bottom of the well, was added directly to the biofilm for 1 minute. After 1 minute of exposure, treatment was removed, and the biofilm was washed 3 times with saline. After treatment, artificial saliva + 1% sucrose was added to the biofilms and grown for 24 hours at 37 *C.

After post-treatment incubation, biofilms were re-suspended in artificial saliva using cell scraper and aspiration. The *ex vivo* biofilms were diluted and plated on 50/50 BHI and L-MRS agar plates, to support the growth of a wide variety of bacterial species. The plates were incubated for 2-3 days under anaerobic conditions. Colonies were counted on the plate to quantify bacterial survival.

Statistical analysis:

After post-treatment incubation, colonies were counted and recorded. All data in the paper was representative of four biological replicates. To determine statistical significance, colony counts of treated and untreated samples were compared by a paired, two-way T-test. Error in graphs and tables is representative of the standard deviation of all biological replicates.

Results

To gain further insight into the therapeutic potential of both $AgNO_3$ and SDF we developed an ex vivo system to compare the two treatments. In this model, plaque biofilm samples were collected from anterior and posterior sites from both healthy children (HA and HP) and children presenting with early childhood caries (CA and CP). Each sample type was pooled and grown, and these pooled samples served as seeds for the inoculation of biofilms. Several different conditions were tested to create a suitable biofilm including biofilms grown in media or artificial saliva (Figure 2). Successful biofilms required both saliva (saliva coating the plate or grown in artificial saliva) and sucrose. A mixture of fructose and glucose was not a suitable substitute for sucrose (Figure 2 A). A system using an artificial saliva-1% sucrose mixture was chosen for treatment studies.

In the untreated samples, there was no significant difference ($P>0.05$) in the bacteria recovered with each yielding approximately 8.4 Log(CFU/mL) (Table 1, Figure 2). Each *ex vivo* biofilm type was treated with 38% SDF or 25% $AgNO_3$ for 1 minute. The bacteria recovered from the healthy biofilms, anterior and posterior, treated with $AgNO_3$ was (0.70±0.39) CFUs, and (0.22±0.27), (0.69±0.39) CFUs was the amount of bacteria recovered from the healthy anterior and healthy posterior biofilms treated with 38% SDF. Very few live bacteria were detected in the treatment groups. For the caries biofilms, the bacteria recovered from the $AgNO_3$ treatment group was (1.22±0.87) and (0.82±0.37) CFUs from the anterior and posterior samples, respectively. The CFUs from the caries anterior samples treated with 38% SDF was higher than that recovered from the caries posterior samples (1.2±1.06) CFUs. This difference was

statistically insignificant ($P<0.001$). This decrease in the bacteria recovered from the treated samples when compared to the untreated biofilm of the same type is statistically significant ($P<0.001$). In both treatments, the bacteria recovered was ~1 log CFU or below, indicating that most of the biofilm was not viable. Furthermore, there was no significant difference in bacteria recovered between the $AgNO_3$ and SDF treated samples (Figure 2,Table 1).

Discussion

Thirty-eight percent silver diamine fluoride (SDF) is an effective anti-cariogenic agent with high fluoride release and capacity to both remineralize the tooth surface and increase its surface hardness[35]. In the summer of 2014, SDF was approved by the U.S. Food and Drug as a desensitizing agent and it became commercially available the spring of 2015. It has been used off-label as a caries arresting medicament since 38% SDF promises to outperform fluoride varnish for caries arrest and to become an invaluable tool for caries prevention and management [36,37] with caries lesion arrest rates upwards of 70%.[17]

Many human infections are directly caused by or exacerbated by biofilms, and they present a unique challenge to the treatment of these infectious diseases.[38] Dental caries is a prime example of a biofilm mediated disease. The bacteria responsible for caries produce a complex extra-cellular matrix that can complicate treatment.[39] Thus, all potential therapeutics or treatments for caries must be tested against biofilms to ensure they can penetrate it to eradicate the bacteria within. Our literature currently lacks studies comparing the efficacy of SDF and $AgNO_3$ on *ex vivo* biofilms resembling the complex multispecies microbiomes of the oral cavity.

The purpose of this study was to develop an *ex vivo* model to replicate the complex *in vivo* bacterial community specific to the human teeth surfaces. This model, in conjunction with technical advances in 16S rRNA gene analysis, will help investigate bacterial community profiles associated with the early childhood caries in the young primary dentition, and compare them to bacterial communities found on healthy teeth and in dentally healthy children. It will

also help us to better understand the microbial interactions in caries pathogenesis and test current and potential therapeutics for dental caries control and prevention. The model will help us to better understand the efficacy of these medicaments on microbiomes excluding all other factors of variability in the morphology of cavities. These factors include ease of isolation and application of medicaments on anterior teeth versus posterior teeth in general and on uncooperative patients in particular. This may help us to understand the reported higher caries arrest rate on the maxillary anterior teeth and buccal/lingual smooth surfaces.[40,41] To the best of our knowledge and based on a PubMed® search conducted by the author using the keywords silver diamine fluoride, silver nitrate, silver compounds, *ex vivo* studies, posterior and anterior carious lesions, is will be one of the first studies to compare the efficacy of both silver medicaments on plaque samples from anterior and posterior teeth. A protocol based on the University of California, San Francisco (UCSF) protocol for the treatment of carious lesions with SDF was designed. This included a one minute long, direct exposure of the compound to the lesion or biofilm followed by a wash using saline.[14] To equally judge the effectiveness of both $AgNO_3$ and SDF under similar conditions, we opted to treat each biofilm with each silver compound using a similar protocol. Plaque samples taken from healthy and carious teeth surfaces were grown in an artificial saliva-sucrose mixture on saliva coated plates to best mimic *in vivo* conditions. Our studies reveal that $AgNO_3$ and 38% SDF are both equally potent on the *ex vivo* biofilms when compared to untreated samples ($P<0.001$). Furthermore, there was no significant difference ($P<0.05$) in the potency between $AgNO_3$ and 38% SDF.

Taking into consideration the remarkable degree of variation in individual sites within the oral cavity[42], and the significantly lower success rate in the treatment of carious lesions on posterior teeth when compared to anterior teeth reported in recent clinical trials, plaque samples

were collected from anterior and posterior sites and were compared to determine if bacterial composition plays a role in the efficacy of the silver medicaments. There was no significant difference in the effectiveness of 38% SDF or $AgNO_3$ when comparing the anterior to posterior *ex vivo* biofilms ($P>0.05$) of the same type. This is clinically significant for clinicians that treat patients in the pre-cooperative, uncooperative groups and medically challenged groups with working time limited to a few minutes and possibly only a few seconds. In such cases, clinicians may prefer to use $AgNO_3$ over 38% SDF which is found to have a faster onset of action. Other clinicians may prefer to use 38% SDF over $AgNO_3$ as the application of fluoride varnish after treatment with 38% SDF is optional. The CDT code D1354, Interim Caries Arresting Medicament Application, used when treating teeth with either 38% SDF or $AgNO_3$ does not include the cost of using any type of fluoride varnish.

Future studies should compare the efficacy of SDF and SN treatments on microbiomes from healthy and diseased sites. This *ex vivo* model, in conjunction with technical advances in 16S rRNA gene analysis, will help in sequencing bacterial species that survived the silver treatments which will help us investigate any possible antimicrobial tolerance to SDF and SN/NaF. Both silver medicaments can be used as a tool in high caries patients as a step in their Oral Disease Control Therapy phase (ODCT). Plaque samples before and after treatment can be collected, CFUs counted and resistant species sequenced to assess patient's readiness for definitive restorative and prosthetic treatment.

Conclusion

Consistent with previous studies employing silver compounds as anti-caries agents, both $AgNO_3$ and SDF are potent bactericidal agents. The *in vitro* and *ex vivo* studies support the effectiveness of $AgNO_3$ and SDF as a caries management strategy. Furthermore, there was no significant difference in the potency each compound had on multispecies, cariogenic biofilms.

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Table 1: Colony counts of treated ex vivo biofilms

Sample	Untreated	$AgNO_3$	$AgNO_3$ Vs SDF	SDF
Healthy Anterior	8.30 ± 0.29	0.70 ± 0.39 P<0.001	P>0.05	0.22 ± 0.27 P<0.001
Healthy Posterior	8.19 ± 0.36	0.70 ± 0.39 P<0.001	P>0.05	0.69 ± 0.39 P<0.001
Caries Anterior	8.45 ± 0.11	1.22 ± 0.87 P<0.001	P>0.05	1.985 ± 1.65 P<0.001
Caries Posterior	8.62 ± 0.11	0.82 ± 0.37 P<0.001	P>0.05	1.2 ± 1.06 P<0.001

*All non-P values shown as Log CFUs

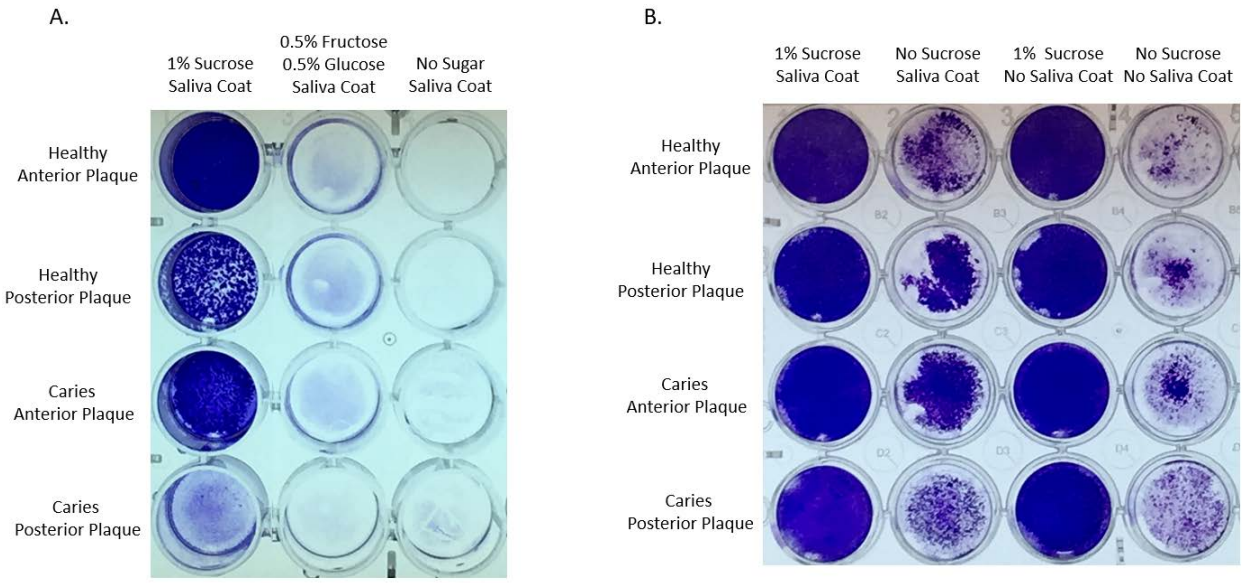

Figure 1: Creation of ex vivo Biofilms. Biomass of each biofilm was observed via crystal violet staining. Each plaque sample was grown in media or artificial saliva plus/minus sugar to determine biofilm formation in vitro. ***A.*** *Ex vivo biofilms created using a mixture of L-MRS/supplemented BHI media.* ***B.*** *Ex vivo biofilms created using artificial saliva. Biofilms derived from 4 different sites: HA – Healthy Anterior, HP – Healthy Posterior, CA – Caries Anterior, CP – Caries Posterior.*

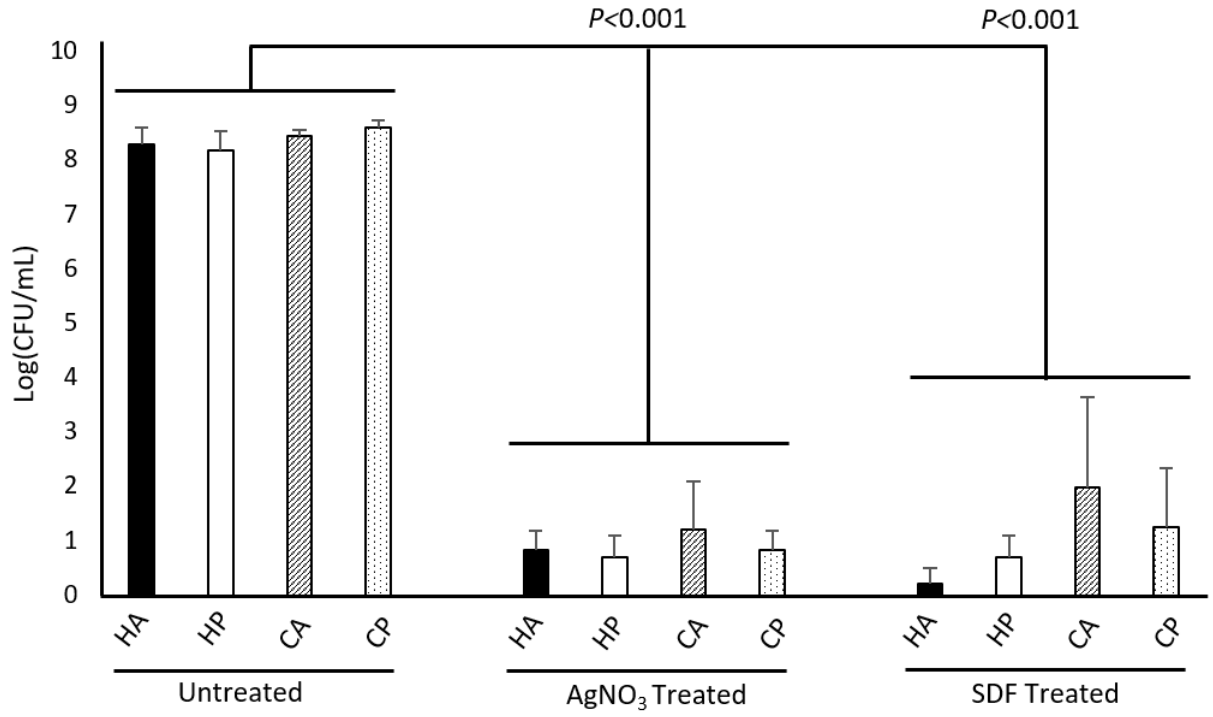

Figure 2: Treatment of ex vivo biofilms using AgNO3 and SDF

Each biofilm was treated with $AgNO_3$ or SDF for 1 minute and subsequently washed with saline. Bacteria in biofilms were re-suspended and plated on a mixture of L-MRS/supplemented BHI to quantify survival. Statistical significance was tested using paired t-test on biofilms of same type (n=4). There was no significant difference between treatment types. HA – Healthy Anterior, HP – Healthy Posterior, CA – caries Posterior