

ANTIMICROBIAL EFFECTS IN VITRO OF SILVER DIAMINE FLUORIDE
AGAINST SELECTED HUMAN RED AND ORANGE COMPLEX
PERIODONTAL PATHOGENS.

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ABSTRACT

Objectives: Silver diamine fluoride is approved by the United States Food and Drug Administration for intraoral human treatment of tooth hypersensitivity, and it has also been employed world-wide as an emerging method to arrest tooth decay. A 38% silver diamine fluoride formulation, comprised of 25% silver, 5% fluoride, and 8% ammonia as a solvent, is commercially available in the United States. One of the main mechanisms underlying the dental caries arrest potential of silver diamine fluoride is the silver component, which exerts pronounced antimicrobial activity against cariogenic bacteria.

Interestingly, studies initiated in the late 1990s demonstrated marked susceptibility of periodontal bacterial pathogens to silver nitrate. However, efforts to develop silver-based, slow-release biodegradable wafers for subgingival placement into periodontal pockets were not commercially successful. At present, no commercial products are available which employ silver ions to combat periodontal bacterial pathogens in periodontal disease treatment. It is not known whether the 38% silver diamine fluoride product commercially available in the United States possesses antimicrobial activity against periodontal bacterial pathogens, and potentially, have application in periodontal therapeutic regimens.

As a result, the objective of this study was to test the *in vitro* antimicrobial effects of silver diamine fluoride on freshly-isolated red and orange complex periodontal pathogens from severe human periodontitis lesions.

Methods: Paper point subgingival biofilm samples from 24 adults with severe periodontitis that were to be discarded after microbiological analysis at the Temple

University School of Dentistry Oral Microbiology Testing Service Laboratory were secondarily employed in this study. Dilution aliquots from each subgingival specimen were mixed with either 38% or 19% silver diamine fluoride, inoculated onto enriched Brucella blood agar plates, and incubated anaerobically for 7 days at 37°C. Bacterial species growing subsequent to the silver diamine fluoride exposure were considered to be resistant to that concentration of silver diamine fluoride. Total viable counts in silver diamine fluoride-exposed subgingival specimens were quantitated, and established phenotypic criteria employed to identify the following red and orange complex periodontal pathogens: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, *Campylobacter rectus*, *Fusobacterium nucleatum* group species, and *Streptococcus constellatus*. Other cultivable isolates recovered from silver diamine fluoride-exposed subgingival specimens were identified using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry and Bruker MALDI Biotyper analytic software. Subgingival sample dilution aliquots not exposed to silver diamine fluoride were similarly processed as controls for comparison with silver diamine fluoride-exposed specimens

Paired t-tests compared mean total subgingival viable counts, and mean total subgingival proportions of the evaluated anaerobic red and orange complex periodontal pathogens per patient, between subgingival biofilm samples exposed and not exposed in vitro to 38% or 19% silver diamine fluoride, with a *P*-value of ≤ 0.05 required for statistical significance.

Results: Subgingival specimens exposed in vitro to either 38% or 19% silver diamine fluoride yielded significantly lower total subgingival viable counts per patient

than those not exposed to silver diamine fluoride ($P < 0.001$, paired t-test), with no statistically significant differences found between 38% and 19% silver diamine fluoride exposures ($P = 0.370$, paired t-test).

All evaluated red and orange complex periodontal pathogens were suppressed below detection levels in 21 (87.5%) of subgingival samples after in vitro exposure to 38% silver diamine fluoride. Three other patient specimens treated with 38% silver diamine fluoride each had persistence of *P. micra*. Similarly, 21 (87.5%) of subgingival specimens also were culture-negative for red and orange complex periodontal pathogens after 19% silver diamine fluoride exposure, with two other patient samples showing persistence of *P. micra*, and a third sample persistence of *S. constellatus*. Total subgingival proportions of red and orange complex periodontal pathogens averaged 0.6% per patient in subgingival specimens exposed in vitro to 38% silver diamine fluoride, and 0.5% per patient in those exposed to 19% silver diamine fluoride, which were both significantly lower than 25.9% mean proportions detected in subgingival biofilms not exposed to silver diamine fluoride ($P < 0.0001$, paired t-test). No statistically significant differences were found between 38% and 19% silver diamine fluoride relative to suppression of total red and orange complex periodontal pathogen proportions ($P = 0.345$, paired t-test).

Various *Streptococcus* species, particularly *Streptococcus oralis*, were the most frequently recovered microorganisms in subgingival biofilm specimens after exposure to both 38% and 19% silver diamine fluoride, indicative of their in vitro resistance to silver diamine fluoride.

Conclusions: Silver diamine fluoride demonstrated substantial antimicrobial activity against fresh clinical isolates of red and orange complex periodontal pathogens, and total viable counts, in subgingival biofilm specimens from severe periodontitis patients, with no statistically significant differences found between silver diamine fluoride concentrations of 38% and 19%.

The dramatic in vitro suppression of red and orange complex periodontal pathogens in subgingival biofilm specimens by silver diamine fluoride, along with its selection of silver diamine fluoride-resistant species of *Streptococcus* that are associated with periodontal health, suggests a new therapeutic use for silver diamine fluoride in the management of human periodontal infections.

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CHAPTER 1

INTRODUCTION

Silver diamine fluoride was approved for marketing in the United States in August, 2014 by the United States Food and Drug Administration for intraoral human treatment of dentin tooth hypersensitivity (Horst et al. 2016). Silver ions in silver diamine fluoride solutions form a squamous layer over the surface of exposed dentin, and help plug dentinal tubules, in mitigating dentin hypersensitivity (Mei et al. 2016). The commercially available 38% silver diamine fluoride product in the United States is comprised of 25% silver, 5% fluoride, and 8% ammonia as a stabilizing agent (Horst, 2018).

Silver diamine fluoride also has also employed world-wide to arrest human tooth decay (Horst 2018). The underlying mechanisms for arresting dental caries with silver diamine fluoride are purported to stem from the antimicrobial effects of silver against cariogenic bacteria, and promotion of tooth remineralization by fluoride (Horst et al. 2016).

Interestingly, studies initiated in the late 1990s demonstrated marked susceptibility of periodontal bacterial pathogens to silver nitrate (mean minimal bactericidal concentrations = 0.5 mg/ml), including many red and orange complex periodontal pathogens associated with severe human periodontitis (Socransky et al. 1998), such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Campylobacter rectus*, but not periodontal health-associated streptococci (Spacciapoli et al. 2001). Related to this, Straub et al. (2001)

conducted a phase 1 clinical study of a bioresorbable wafer loaded with 12% silver nitrate for slow release into periodontal pockets. Silver concentrations in gingival crevicular fluid after subgingival placement of wafer averaged $> 10 \mu\text{g/ml}$ in each study patient over a 14-day period, and significantly decreased total subgingival anaerobic counts without inducing any adverse local or systemic side-effects (Straub et al. 2001).

However, further research on silver-loaded periodontal wafers has not been reported since 2001, and no commercial products are presently available in the United States which employs silver ions to combat bacterial pathogens in periodontal disease treatment. In regard to this, it is not known whether the 38% silver diamine fluoride product commercially available in the United States possesses antimicrobial activity against periodontal bacterial pathogens, and whether the product has usefulness in periodontal therapeutic regimens.

As a result, the objective of this study was to test the in vitro antimicrobial effects of silver diamine fluoride on freshly-isolated red and orange complex periodontal pathogens from severe human periodontitis lesions.

CHAPTER 2

MATERIALS AND METHODS

Laboratory Facilities

This study was performed in the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia, Pennsylvania. The study data was obtained through secondary laboratory use of human subgingival biofilm samples without intervention or interactions with any living individuals, and not involving any identifiable private patient information. Thus, this study did not involve human subjects, as defined by United States Department of Health and Human Services regulations at 45 CFR part 46.116(f), and did not require, as determined in writing, review and approval from the Temple University Institutional Review Board.

Subgingival Biofilm Samples

Subgingival biofilm samples from 24 adults with severe periodontitis were used in this study. The subgingival samples were submitted to the OMTS Laboratory for microbiological analysis by subscribing private practicing periodontists extramural to Temple University. The samples were normally discarded by the OMTS Laboratory after completion of the requested microbiological testing, but were secondarily used in this study after removal of all unique patient identifiers. As a result, no data is available for patient age, gender, systemic health history, medications, smoking status, or nature of patient clinical/radiographic periodontal parameters, beyond information that they were 35 years of age or older, and were identified by their treating periodontist as having severe periodontitis.

The subgingival biofilm samples were obtained from the patients by their treating periodontists following standardized sampling instructions from the OMTS Laboratory. Supragingival dental plaque was first removed from 3-5 periodontal sites in the patients which exhibited moderate (5-6 mm) to deep periodontal probing depths (≥ 7 mm) and gingival inflammation, after which they were isolated with cotton rolls and air drying to exclude saliva contamination into the microbial samples. Following these steps, one to two sterile paper points were then advanced with sterile forceps into each isolated periodontal site for approximately 10 seconds in order to collect subgingival biofilm specimens for microbial culture. The paper points were then placed together into a single glass vial containing 6-8 glass beads of 3 mm in diameter, and 2.0 ml of pre-reduced, anaerobically sterilized and stored Möller's VMGA III transport media (Möller 1966), which possesses a high preservation capability for oral microorganisms after sampling during transit to the laboratory (Möller 1966, Dahlén et al. 1989, Dahlén et al. 1993).

The pooled subgingival biofilm samples were then transported by overnight delivery services to the OMTS Laboratory in Philadelphia, Pennsylvania, which has been in continuous operation since its founding in 1991. The OMTS Laboratory is state-licensed for high-complexity bacteriological analysis by the Pennsylvania Department of Health (Clinical Laboratory Permit No. 021872) as an oral microbiology reference laboratory. The OMTS Laboratory is also federally certified by the US Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments (CLIA) regulations (CLIA Certificate No. 39D0707385), and fulfills all

proficiency testing, quality control, patient test management, personnel requirements, and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the United States (Rauch & Nichols, 2007).

Subgingival Biofilm Culture

When initially received at the OMTS Laboratory, the VMGA III vials were warmed to 37°C for 10 minutes prior to processing in order to liquefy gelatin in the VMGA III transport medium. The sampled microorganisms were then mechanically dispersed in the medium with a Vortex mixer at the maximal setting for 45 seconds. Serial 10-fold dilutions of the dispersed bacteria were carried out in Möller's VMG I anaerobic dispersion solution, comprised of pre-reduced, anaerobically sterilized 0.25% tryptose-0.25% thiotone E peptone-0.5% NaCl (Möller 1966). Using a sterile bent glass rod, 0.1 ml aliquots of various serial dilutions were plated onto pre-reduced, enriched Brucella blood agar (EBBA) medium, comprised of 4.3% Brucella agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione. The EBBA inoculated plates were incubated at 37°C for 7 days in a upright heated incubator (Caron, Marietta, OH, USA) in jars containing an 85% N₂-10% H₂-5% CO₂ anaerobic atmosphere introduced by an Anoxomat™ Mark II automatic jar evacuation-replacement system (Advanced Instruments, Inc., Norwood, MA, USA) (Brazier & Smith 1989), and used to quantitate total anaerobic viable counts, and determine the presence and levels of the following red and orange complex periodontal pathogens: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella*

intermedia/nigrescens, *Parvimonas micra*, *Campylobacter rectus*, *Fusobacterium nucleatum* group species, and *Streptococcus constellatus*.

Subgingival Biofilm Eradication Assay with Silver Diamine Fluoride

A subgingival biofilm species eradication assay was employed to test the antimicrobial effects of in vitro exposure of 19% and 38% silver diamine fluoride solutions on subgingival biofilm specimens from severe periodontitis patients, similar to the in vitro method described by Pozhitkov et al. (2015) for testing the effects of diluted bleach on subgingival microorganisms.

One ml aliquots of 10^{-6} subgingival biofilm dilutions from each study patient were mixed with either 0.05 ml of a 38% silver diamine fluoride commercial solution (Advantage Arrest[®], Elevate Oral Care, West Palm Beach, FL, USA), or 0.05 ml of a 19% silver diamine fluoride solution, which was created by mixing 0.025 ml of the 38% silver diamine fluoride commercial solution with 0.025 ml of sterile water (Figure 1).



Figure 1. Packaging for commercial silver diamine fluoride product approved for human intraoral use by the United States Food and Drug Administration.

The 0.15 ml total mixtures of subgingival biofilm bacteria and silver diamine fluoride were then inoculated onto EBBA plates, and incubated anaerobically for 7 days at 37°C. Bacterial species growing on EBBA plates subsequent to in vitro mixture with either 38% or 19% silver diamine fluoride were considered to be resistant to that concentration of silver diamine fluoride. Aliquots of 10⁻⁶ specimen dilutions inoculated onto EBBA plates without silver diamine fluoride exposure served as controls for comparison with silver diamine fluoride-exposed subgingival biofilm specimens.

Bacterial Species Identification

On all EBBA plates, *P. gingivalis* identification was based on colony morphology and brown-black pigmentation, lack of autofluorescence with long-wave ultraviolet light (Slots & Reynolds 1982), and a positive CAAM test for trypsin-like activity (Slots 1987). *T. forsythia* isolates were identified as gram-negative, non-motile, anaerobic rods exhibiting grey-pink speckled, convex, pinpoint colonies seen with a stereomicroscope, lack of long-wave ultraviolet light autofluorescence, and positive for trypsin-like enzyme activity (Rams & van Winkelhoff 2005). *P. intermedia/nigrescens*, which represented clinical isolates of either *Prevotella intermedia* and/or *Prevotella nigrescens* due to the inability of phenotypic identification methods to reliably differentiate between them (Rams et al. 2018), was recognized as autofluorescent red-positive, black-pigmented colonies exhibiting lactose MUG-test negative (Alcoforado et al. 1987) and trypsin CAAM test-negative reactions. *P. micra* was identified as small (minute to 1.0 mm in diameter), shiny, non-hemolytic, mainly opaque white, circular, convex surface colonies on anaerobically incubated EBBA (Rams et al. 1992). Presumptive *C. rectus* species

isolates were identified as previously described (Rams et al. 1993) on the basis of motility, colonial and cellular morphology from other organisms as short, straight, motile rods forming small (< 1.5 mm in diameter), glistening black, convex colonies with entire edges. *F. nucleatum* group isolates were presumptively identified, as specified by Jousimies-Somer et al. (2002), as gram-negative, non-motile, anaerobic, slender, fusiform rods with pointed cell ends (needle-shaped morphology), exhibiting circular, entire, raised, catalase-negative, non-pigmented, non-agar pitting, “bread crumb”-like or speckled colonies under a magnification loupe on anaerobically incubated EBBA, and demonstrating an autofluorescent chartreuse (pale yellow-green) colony color when exposed in a dark room to long-wave ultraviolet light (Brazier 1986). *S. constellatus* was defined as gram-positive, lactose MUG-test negative, non-motile, facultative cocci demonstrating small white, opaque, circular, beta-hemolytic, surface colonies with irregular edges (Rams et al. 2011).

Proportional subject recovery of test bacterial species was calculated as the percent recovery of each test species colony forming units (CFU) among the total cultivable subgingival anaerobic viable count as determined on EBBA plates where the inoculated subgingival specimens were not exposed in vitro to either 19% or 38% silver diamine fluoride.

Other bacterial species recovered on EBBA plates where the subgingival specimen was exposed to one of the silver diamine fluoride concentrations were identified using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry and Bruker MALDI Biotyper analytic software (Bruker Daltonics,

Billerica, MA, USA), following procedures previously described (Rams et al. 2016, 2018). In brief, a single colony of the bacterial isolate was picked with a sterile wooden toothpick from the surface of EBBA medium plates, direct-spotted as a thin film into individual circles on the surface of a polished steel MALDI-TOF mass spectrometry, and allowed to dry at room temperature. A 1.0 µl overlay of a 98-100% formic acid solution was placed and allowed to air dry over the colony smears to facilitate on-plate extraction of bacterial cell proteins. Each spot was then subjected to a second overlay solution with 1.0 µl of a matrix mixture, comprised of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid, and allowed to dry at room temperature. A bacterial test standard, containing *Escherichia coli* DH 5 alpha strain ribosomal proteins, was prepared according to manufacturer instructions, and processed similar to the other bacterial isolates. Negative control spots on the target plate were left blank or with the dried matrix solution alone.

Mass spectra for each isolate was acquired using a Microflex LT benchtop mass spectrometer equipped with a 20-Hz nitrogen laser (Bruker Daltonics). The instrument was operated with Bruker Daltonics FlexControl software (version 3.4) in a linear positive mode within a 2-20 kDa mass range, with voltage settings of 20 kV for ion source one, 18.05 kV for ion source two, 6.0 kV for the lens, and 2,560 V for the linear detector. Mass spectra were automatically obtained using an average 500 laser shots, and analyzed with Bruker Daltonics MALDI Biotyper 3.1 software and the MBT 6903 MSP Library, to determine the most likely microbial species identification. A MALDI Biotyper score, generated as a level of probability by the software, of ≥ 1.7 was utilized

as a threshold for reliable species identification, as recommended for assessment of anaerobic bacteria (Hsu & Burnham 2014). Scores of < 1.7 were considered to provide less reliable bacterial identification. A MALDI Biotyper log score ≥ 2.0 was considered to represent definitive species identification.

Figure 2 summarizes how the subgingival biofilm species eradication assay was carried out in the present study.

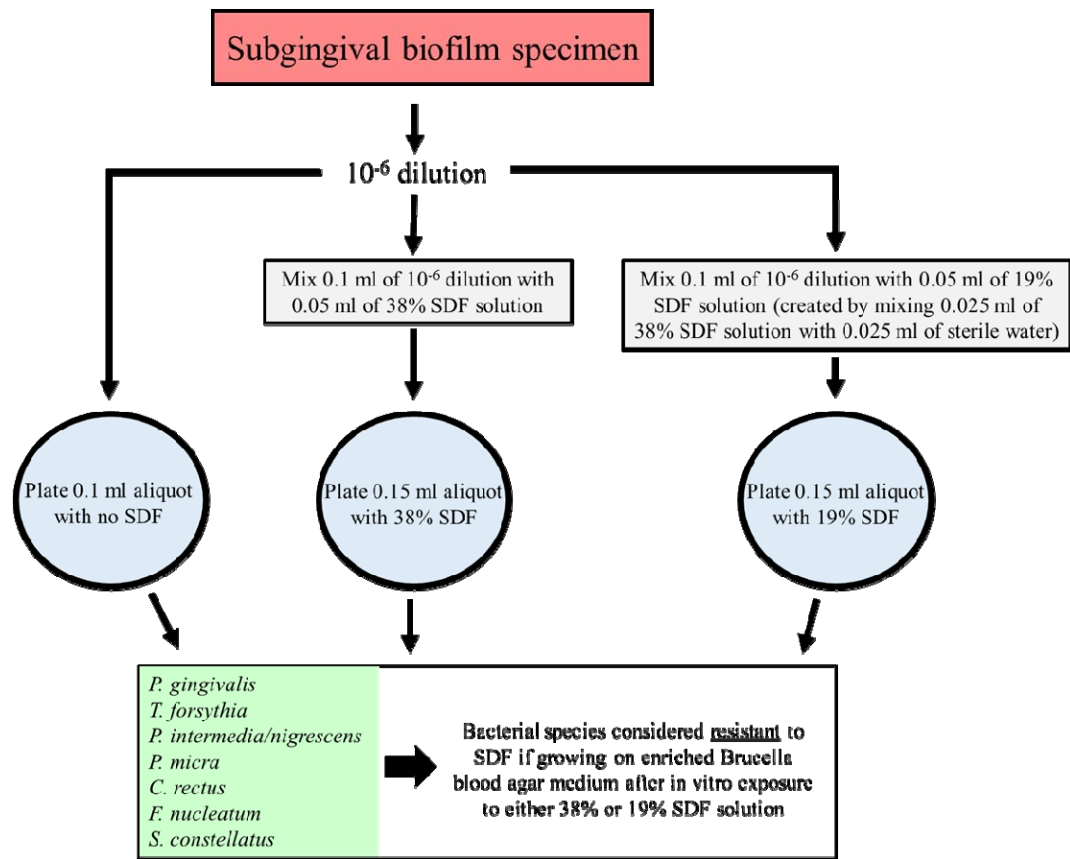


Figure 2. Flow chart of subgingival biofilm species eradication assay for 38% and 19% silver diamine fluoride (SDF).

All laboratory culture media preparation, specimen inoculation, and culture analysis were carried out in a standardized fashion by a single oral microbiology laboratory technician (Jackie Sautter), with oversight and review by the laboratory director (Dr. Thomas E. Rams). Dr. Rams carried out all MALDI-TOF mass spectrometry laboratory procedures.

Data Analysis

Mean total subgingival viable counts for each subgingival sample exposed and not exposed in vitro to the silver diamine fluoride solutions were calculated and transformed to \log_{10} values. For each of the test bacterial species, the number and proportion of organism-positive patients was determined, along with the organism's mean subgingival proportional recovery and standard deviation (SD) or standard error (SE), as well as the number and proportion of patients with bacterial species resistant to either 38% or 19% silver diamine fluoride in vitro exposure. Total subgingival proportions of red and orange complex periodontal pathogens (Carvalho et al. 2005, Page & Rams 2013, McCawley et al. 2018) were determined by summing together individual species data for each patient, and then calculating total mean values across all patients.

A paired t-test compared mean total subgingival viable counts, and mean total subgingival proportions of red and orange complex periodontal pathogens per patient, between patient subgingival samples exposed and not exposed in vitro to either 38% or 19% silver diamine fluoride, with a P -value of ≤ 0.05 required for statistical significance. The PC-based STATA/SE 14.2 for Windows (StataCorp PL, College Station, TX, USA) 64-bit statistical software package was used in the data analysis.

CHAPTER 3

RESULTS

Subgingival Biofilm Species

Table 1 lists subgingival bacterial species recovered in the 24 study patient specimens not exposed to silver diamine fluoride.

Table 1. Presence and Proportional Recovery of Selected Periodontal Pathogens in Subgingival Biofilm Specimens Without Exposure to Silver Diamine Fluoride

Species	No. (%) of positive patients	% recovery in species-positive patients \pm SD	Range %
<u>Red complex species:</u>			
<i>P. gingivalis</i>	3 (12.5)	7.0 \pm 5.2	1.0-10.0
<i>T. forsythia</i>	12 (50.0)	1.1 \pm 0.6	0.3-2.0
<u>Orange complex species:</u>			
<i>P. intermedia/nigrescens</i>	23 (95.8)	11.6 \pm 13.3	0.1-46.7
<i>P. micra</i>	24 (100)	6.4 \pm 4.1	0.7-13.8
<i>C. rectus</i>	4 (16.7)	0.2 \pm 0.1	0.1-0.3
<i>F. nucleatum</i>	21 (87.5)	7.6 \pm 4.7	1.9-18.0
<i>S. constellatus</i>	1 (4.2)	0.1 \pm 0.0	0.1

All (100%) of the study patients harbored *P. micra*, 95.8% *P. intermedia/nigrescens*, and 87.5% *F. nucleatum*, with subgingival proportions of these three orange

complex periodontal pathogens averaging 6.4%, 11.6%, and 7.6% in species-positive patient, respectively.

Among red complex periodontal pathogens, *T. forsythia* was present in 50% of the study patients at a mean subgingival level of 1.1% per species-positive patient. *P. gingivalis* was recovered in only 3 (12.5%) study patients, averaging 7.0% of subgingival isolates in species-positive patients.

Total subgingival proportions of red and orange complex periodontal pathogens averaged 25.9 ± 3.7 (SE) % per patient in subgingival biofilm patient specimens not exposed in vitro to silver diamine fluoride.

Silver Diamine Fluoride In Vitro Effects on Subgingival Biofilm Species

Total subgingival viable counts in subgingival biofilm patient specimens not exposed to silver diamine fluoride averaged 1.96 ± 0.09 (SE) $\log_{10} \times 10^6$ CFU per patient. In comparison, total subgingival viable counts in specimens exposed in vitro to 38% silver diamine fluoride averaged 0.61 ± 0.66 (SE) $\log_{10} \times 10^6$ CFU per patient, while those exposed in vitro to 19% silver diamine fluoride averaged 0.80 ± 0.16 (SE) $\log_{10} \times 10^6$ CFU per patient. In paired test-test analysis, subgingival specimens exposed in vitro to either 38% or 19% silver diamine fluoride yielded significantly lower total subgingival viable counts per patient than those not exposed to silver diamine fluoride ($P < 0.001$). However, no statistically significant differences in mean total subgingival viable counts were found for samples exposed to 38% silver diamine fluoride as compared to 19% silver diamine fluoride ($P = 0.370$, paired t-test) (Figure 3).

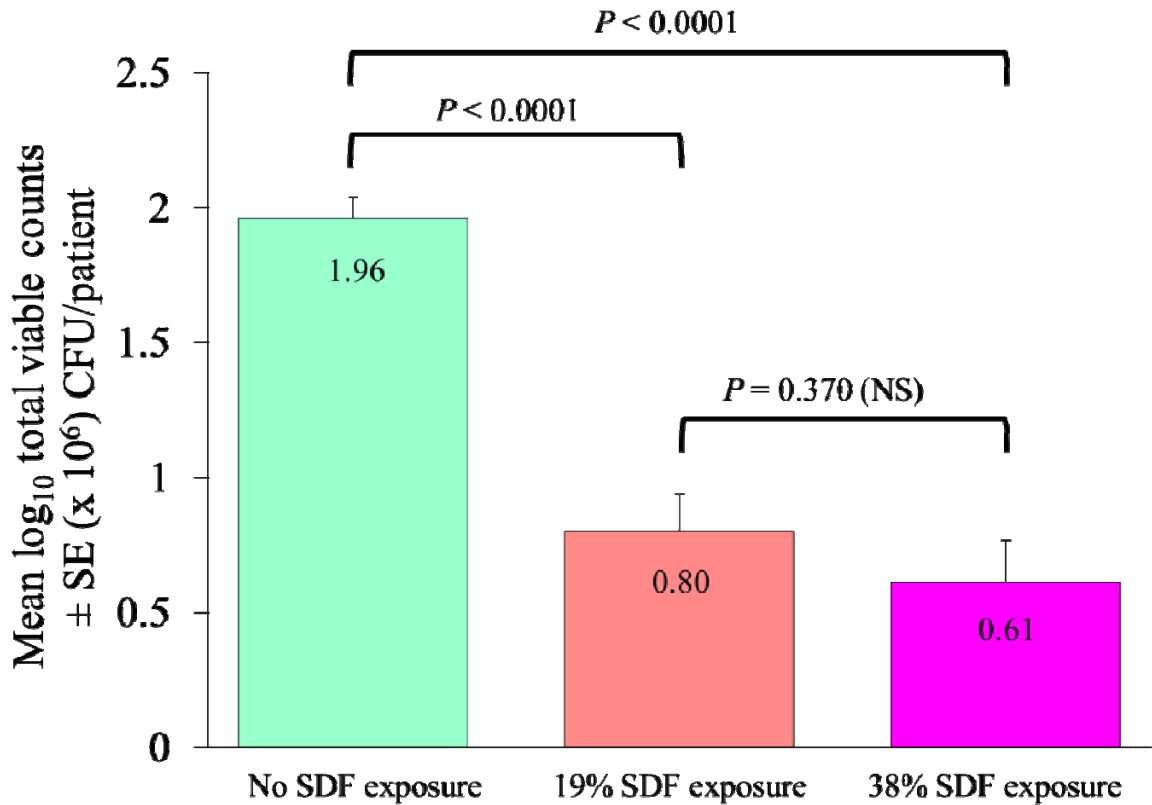


Figure 3. Average total viable counts in subgingival biofilm specimens exposed and not exposed in vitro to silver diamine fluoride (SDF).

All evaluated red and orange complex periodontal pathogens were suppressed below detection levels in 21 (87.5%) of the 24 subgingival samples after in vitro exposure to 38% silver diamine fluoride. Three other patient specimens treated with 38% silver diamine fluoride each had persistence of *P. micra* clinical isolates. Similarly, 21 (87.5%) of 24 subgingival specimens also were culture-negative for red and orange complex periodontal pathogens after 19% silver diamine fluoride exposure. Two patient samples treated with 19% silver diamine fluoride each showed persistence of *P. micra*, with persistence of *S. constellatus* in a third patient sample.

Total subgingival proportions of red and orange complex periodontal pathogens in subgingival specimens exposed in vitro to 38% silver diamine fluoride averaged 0.6 ± 1.9 (SE) % per patient, and to 19% silver diamine fluoride 0.5 ± 1.8 (SE) % per patient. Both of these proportions were significantly lower than the mean 25.9% levels per patient found for red and orange complex periodontal pathogens in subgingival biofilms not exposed to silver diamine fluoride ($P < 0.0001$, paired t-test). However, no statistically significant differences in mean total subgingival proportions of red and orange complex periodontal pathogens were found between samples exposed to 38% silver diamine fluoride as compared to 19% silver diamine fluoride ($P = 0.345$, paired t-test) (Figure 4).

Figures 5 and 6 provide representative views of EBBA culture plates illustrating the marked antimicrobial effects exerted in vitro by 38% and 19% silver diamine fluoride on total viable microbial counts and red and orange complex periodontal pathogens in subgingival biofilm specimens from severe human periodontitis lesions.

Other Subgingival Biofilm Species Resistant In Vitro to Silver Diamine Fluoride

MALDI-TOF mass spectrometry identified various *Streptococcus* species, most often *Streptococcus oralis*, as the most frequent bacterial species resistant in vitro to 38% and 19% silver diamine fluoride (Table 2).

Among the 24 patient subgingival biofilm specimens exposed in vitro to 19% silver diamine fluoride, 8 (33.3%) exhibited no growth in the 10^{-6} sample dilution aliquot, and 14 (58.3%) yielded only various *Streptococcus* species (listed in Table 3). In subgingival samples exposed in vitro to 38% silver diamine fluoride, 6 (25.0%) exhibited no growth in the 10^{-6} sample dilution aliquot, and 13 (54.2%) yielded only various

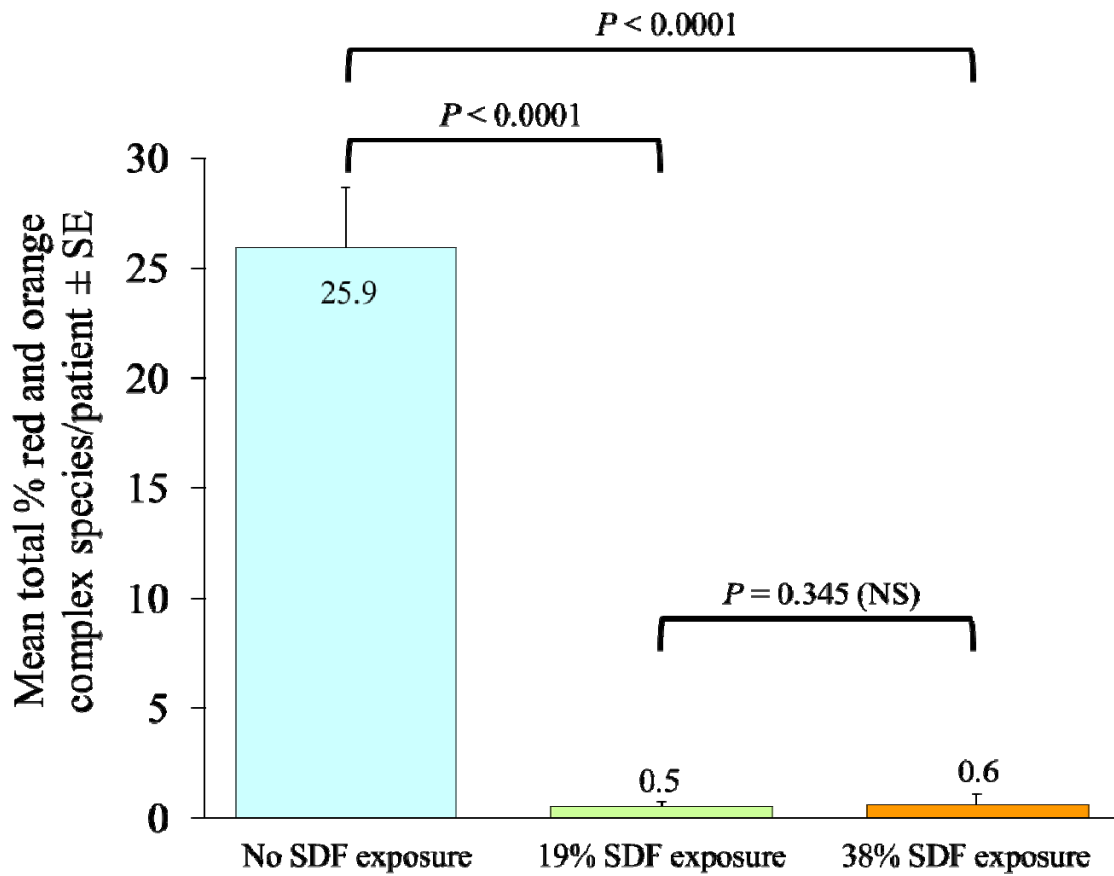


Figure 4. Average total proportions of red and orange complex periodontal pathogens in subgingival biofilm specimens exposed and not exposed in vitro to silver diamine fluoride (SDF). Paired t-tests assessed mean differences between subgingival specimen types. NS = not statistically significant.

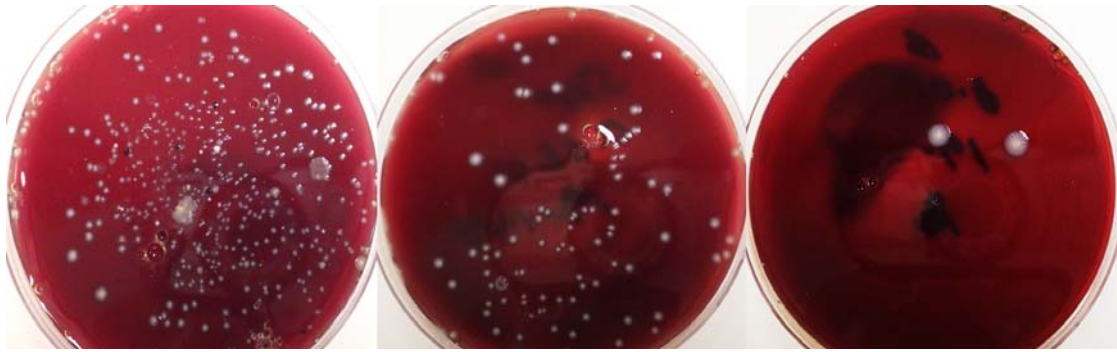


Figure 5. Example #1 of a subgingival biofilm species exposed and not exposed in vitro to silver diamine fluoride. Non-exposed specimen aliquot (left) yielded 0.5% *P. intermedia/nigrescens*, 5.6% *P. micra*, and 4.7% *F. nucleatum*. Specimen aliquot exposed to 19% silver diamine fluoride revealed only *Streptococcus oralis* (center), whereas aliquot exposed to 38% silver diamine fluoride had only one CFU each of *S. oralis* and *Streptococcus mitis* (right).

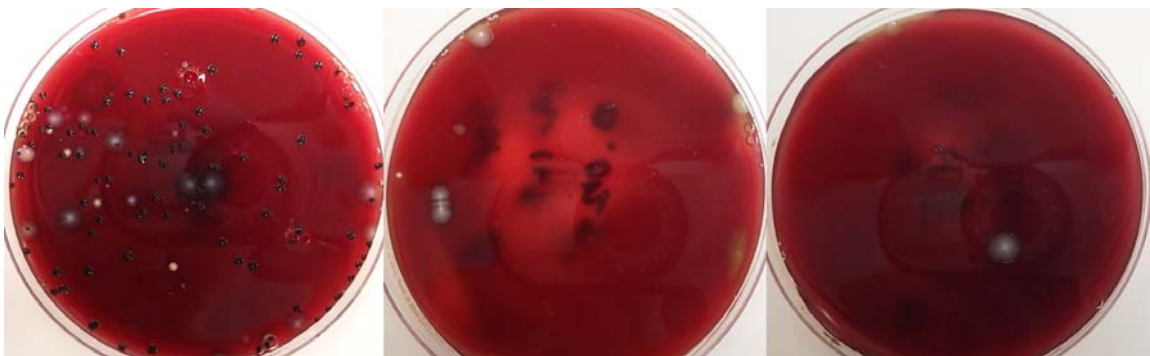


Figure 6. Example #2 of a subgingival biofilm specimen exposed and not exposed in vitro to silver diamine fluoride. Non-exposed specimen aliquot (left) grew 1.4% *T. forsythia*, 46.7% *P. intermedia/nigrescens*, and 0.7% *P. micra*. Specimen aliquots exposed to 19% silver diamine fluoride (center) and 38% silver diamine fluoride (right) both yielded only *Streptococcus cristatus*.

Table 2. Other Subgingival Biofilm Species Resistant In Vitro to Silver Diamine Fluoride

Species:	<u>No. of samples with in vitro resistant species</u>	
	19% silver diamine fluoride	38% silver diamine fluoride
<i>Streptococcus</i> species	2	2
<i>Streptococcus oralis</i>	7	9
<i>Streptococcus mitis</i>	3	3
<i>Streptococcus parasanguinis</i>	2	2
<i>Streptococcus sanguinis</i>	0	1
<i>Streptococcus cristatus</i>	2	2
<i>Streptococcus pneumoniae</i>	0	1
<i>Streptococcus anginosus</i>	1	4
<i>Streptococcus intermedius</i>	1	0
<i>Staphylococcus epidermidis</i>	0	1
<i>Actinomyces odontolyticus</i>	0	1
<i>Actinomyces oris</i>	0	1

Streptococcus species (listed in Table 3). Other organisms resistant in vitro to silver diamine fluoride included *Staphylococcus epidermidis*, *Actinomyces oris*, and *Actinomyces odontolyticus* (Table 3).

An example of MALDI-TOF mass spectrometry identification of a *Streptococcus oralis* clinical isolate resistant to silver diamine fluoride is provided in Figure 7.

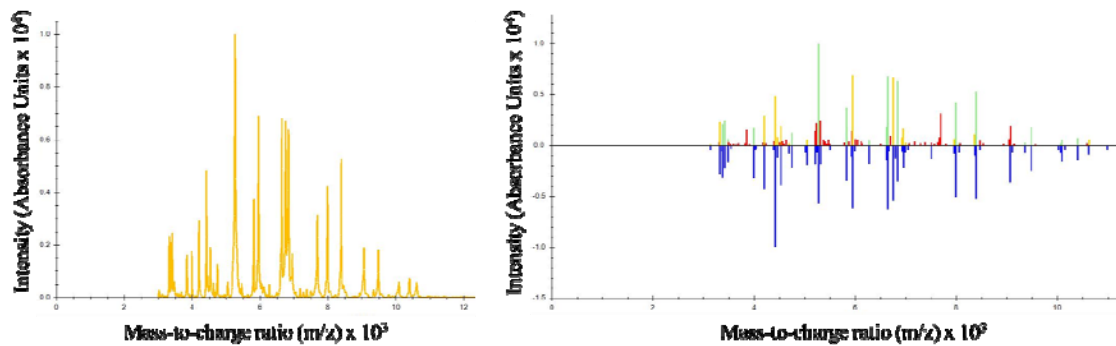


Figure 7. Example of *Streptococcus oralis* identification by MALDI-TOF mass spectrometry. Mass spectra of cultivable clinical isolate resistant to silver diamine fluoride (left) is compared to MALDI Biotyper reference library, with a definitive mass spectra peak match (mass spectrometry log score = 2.170) obtained for *S. oralis* (right). On right, blue peaks in bottom half with inverted intensity scale correspond to a known protein profile for *S. oralis*. Color of peaks in upper half reflects degree of matching of the clinical isolate with the lower half reference library *S. oralis* protein profile, with green = full match, yellow = partial match, and red = no match, for the mass spectra peaks.

CHAPTER 4

DISCUSSION

The major findings from this study are that both 38% and 19% silver diamine fluoride demonstrated substantial antimicrobial effects in vitro that significantly reduced total viable counts, and significantly reduced proportions of red and orange complex periodontal pathogens, in subgingival biofilm specimens from severe periodontitis patients. Interestingly, no statistically significant differences were found between 38% and 19% silver diamine fluoride in their antimicrobial effects on subgingival microorganisms. Among the 24 severe periodontitis subgingival specimens tested, 21 (87.5%) were culture-negative for red and orange complex periodontal pathogens after 38% silver diamine fluoride in vitro exposure, and an identical number after 19% silver diamine fluoride in vitro exposure.

Silver diamine fluoride in vitro exposure reduced total subgingival proportions of red and orange complex periodontal pathogens in subgingival specimens from a mean 25.9% levels per patient in unexposed samples to an average 0.6 ± 1.9 (SE) % per patient in samples exposed to 38% silver diamine fluoride, and to an average 0.5 ± 1.8 (SE) % per patient in those exposed to 19% silver diamine fluoride. Importantly from a cost aspect, no statistically significant differences in mean total subgingival proportions of red and orange complex periodontal pathogens were found between samples exposed to 19% silver diamine fluoride as compared to 38% silver diamine fluoride. Similarly, total viable counts in subgingival biofilm specimens were significantly reduced by both concentrations of silver diamine fluoride, with no statistically significant differences in

mean total subgingival viable counts associated with samples exposed to 19% silver diamine fluoride as compared to those exposed to 38% silver diamine fluoride. Thus, relative to a potential application of silver diamine fluoride in periodontal therapy, there appears to be a similar antimicrobial effect against periodontal pathogens with a 50% diluted concentration of the commercial silver diamine fluoride product (providing a 19% concentration) as is attained with a full-strength 38% concentration. This may make clinical application of the silver diamine fluoride commercial product less costly to implement, and more acceptable for clinicians and patients in dental practice settings.

The antimicrobial effects of 38% and 19% silver diamine fluoride on red and orange complex periodontal pathogens are in agreement with prior research with silver nitrate, where various red and orange complex periodontal pathogens were similarly susceptible in vitro to silver nitrate (Spacciapoli et al. 2001). However, the present study findings expand this area of knowledge to include silver diamine fluoride testing on fresh subgingival clinical isolates from severe periodontal pockets, as compared to laboratory stock strains of periodontal microorganisms subjected to repeated subculturing.

Additionally, the finding of primarily *Streptococcus* species as the predominant bacteria surviving in cultivable subgingival biofilms after silver diamine fluoride in vitro exposure is also potentially important relative to its application in periodontal therapy, and is consistent with prior silver nitrate research findings showing little in vitro antimicrobial activity of silver against oral streptococci (Spacciapoli et al. 2001). It is also consistent with a recent clinical study of 38% silver diamine fluoride placed onto carious lesions, where RNA sequencing analysis found no consistent changes in the

relative abundance of dental caries-associated microorganisms, including *Streptococcus mutans*, after silver diamine fluoride applications. As a result, the introduction of silver diamine fluoride into periodontal pockets may selectively reduce red and orange complex periodontal pathogens and spare periodontal health-associated *Streptococcus* species, providing streptococci of low or negligible periodontal pathogenicity with an ecologic advantage to preferentially recolonize subgingival sites and dominate in newly-developed microbial biofilms that would be inhibitory to periodontal pathogens and favor clinical periodontal stability (Hillman et al. 1985, Hillman & Socransky 1987).

Limitations of the present study need to be appreciated. Only laboratory-based testing of silver diamine fluoride against selected periodontal pathogens was conducted. It is not known if the in vitro findings in this study will similarly occur in an in vivo clinical situation. No detailed patient information was available to include in the data analysis on patient gender, age, systemic health history, medications, smoking status, or their clinical and radiographic periodontal parameters. Only selected anaerobic red and orange complex periodontal pathogens were evaluated in the subgingival plaque biofilm specimens prior to silver diamine fluoride exposure, without assessment of additional periodontal pathogens that were likely present. Only phenotypic criteria were employed to identify the targeted anaerobic red and orange complex periodontal pathogens, instead of potentially more precise molecular or biochemical methods. However, recent validation studies confirmed that the phenotypic methods used in the present study to identify *P. gingivalis* and *P. intermedia/nigrescens* clinical isolates highly correlate to species identification obtained via proteomic spectral fingerprinting of bacterial

ribosomal protein profiles by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Rams et al. 2016, 2018). The in vitro antimicrobial effects of additional dilutions of silver diamine fluoride beyond the 50% dilution employed in the present study were not examined.

However, based on the favorable in vitro antimicrobial effects of silver diamine fluoride against major periodontal bacterial pathogens, additional research, particularly clinical studies, are indicated to further establish the potential value of silver diamine fluoride in periodontal disease therapy and prevention.

CHAPTER 5

CONCLUSIONS

Silver diamine fluoride demonstrated substantial antimicrobial activity against fresh clinical isolates of red and orange complex periodontal pathogens, and total viable counts, in subgingival biofilm specimens from severe periodontitis patients, with no statistically significant differences found between silver diamine fluoride concentrations of 38% and 19%.

The dramatic in vitro suppression of red and orange complex periodontal pathogens in subgingival biofilm specimens by silver diamine fluoride, along with its selection of silver diamine fluoride-resistant species of *Streptococcus* that are associated with periodontal health, suggests a new therapeutic use for silver diamine fluoride in the management of human periodontal infections.

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