An In Vitro Pilot Study on the Effects of Silver Diamine Fluoride on Periodontal Pathogens and Three-Dimensional Scaffolds of Fibroblasts and Epithelium

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ABSTRACT

**Background:** Silver diamine fluoride (SDF) has been recently approved by the US Food and Drug Administration for caries control because of its bactericidal effects and remineralization properties.

**Aim:** In this study, we evaluated the bactericidal effects of SDF on periodontal pathogens and its effect on epithelium and fibroblasts using human skin equivalents.

**Materials and Methods:** *S. mutans, P. gingivalis, and A. actinomycetemcomitans* were cultured in mono-species biofilms and exposed to increasing concentrations of SDF, using chlorhexidine as a positive control. Cultures were then inoculated on agar plates to assess viability. Human gingival fibroblasts in 2D cultures were exposed to 1 µL of 0.394% of SDF and observed using real-time imaging. Finally, SDF was applied to human, 3D tissue scaffolds of fibroblasts and keratinocytes, termed human skin equivalents (HSE). A clinical dose of 38% SDF was applied and HSE were cultured for 12hrs, 1, 3, 5 and 10 days. The tissue was observed clinically and histologically with hematoxylin and eosin staining as well as TUNEL staining to check for apoptosis.

**Results:** *S. mutans* and *A. actinomycetemcomitans* growth was completely inhibited using all dilutions of SDF, whereas *P. gingivalis* was still viable when treated with 0.197% and 0.098% of SDF but not at higher SDF concentrations. Real-time imaging showed immediate necrosis of single-layer fibroblasts upon contact with 1µL of 0.394% of SDF. Application of SDF to HSE showed maturation of a whitish lesion from 12-24 hours to a pigmented, crusted tissue after 3 to 10 days. Histological evaluation showed a disruption of the complete layer of surface epithelium extending to the first few cell layers of fibroblasts. In treated tissues, the epithelium as well as the upper half of the connective tissue contained several apoptotic cells.
**Conclusion:** Our data show that SDF have bactericidal properties against two periodontal pathogens: *P. gingivalis, and A. actinomycetemcomitans*. SDF caused immediate necrosis of mono-layer fibroblasts. Additionally, it caused apoptosis of epithelial cells in an HSE model, but these effects did not extend to the full extent of layered fibroblasts.
DEDICATION

I would like to dedicate this thesis to Eunice—the best partner anyone could hope for—one I can always count on to shoulder any trials together and makes each day a pure delight to be shared.

I would also like to dedicate my thesis to my parents, whose incredible sacrifices laid the bedrock for my education and whose continuous encouragement allows me to continue reaching for the next rung in the educational ladder. Now that I have come to the end of this journey, I can only look back and accredit each step to you both.

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TABLE OF CONTENTS

DEDICATION..............................................................................................................v
ACKNOWLEDGEMENTS............................................................................................vi
TABLE OF CONTENTS..............................................................................................vii
LIST OF TABLES......................................................................................................ix
LIST OF FIGURES..................................................................................................x
LIST OF ABBREVIATIONS.......................................................................................xi

Introduction..............................................................................................................2
  1. Silver Diamine Fluoride....................................................................................2
  2. SDF and Caries..................................................................................................2
  3. Silver: Mechanism of Action..........................................................................3
  4. Fluoride: Mechanism of Action.......................................................................4
  5. Periodontal Disease..........................................................................................5
  6. Exposure of Soft Tissue to SDF......................................................................7
  7. Human Skin Equivalents.................................................................................8

Aim and Hypothesis................................................................................................9

Materials and Methods .......................................................................................10

Results...................................................................................................................14

Discussion..............................................................................................................19

Conclusion.............................................................................................................23

References.............................................................................................................24

APPENDICES.........................................................................................................27

Appendix A: Tables................................................................................................27

Appendix B: Figures...............................................................................................28
LIST OF TABLES

Table 1: Bacterial Growth Conditions
LIST OF ABBREVIATIONS

Chlorhexidine (CHX)
Dubocco’s Modified Eagle’s Medium (DMEM)
Fetal bovine serum (FBS)
Hematoxylin and Eosin (H&E)
Human Gingival Fibroblasts (HGF)
Human Skin Equivalents (HSE)
Penicillin-Streptomycin (P/S)
LIST OF FIGURES

Figure 1: *S. mutans* inoculation on 96 well plate with all exposures………………28
Figure 2: Schematic of quadrant streaking of agar plates…………………………29
Figure 3: HSE constructs………………………………………………………………30
Figure 4: *S. mutans* results on agar plates…………………………………………31
Figure 5A: *P. gingivalis* results on agar plates……………………………………32
Figure 5B: Enlarged view of *P. gingivalis* on agar plates…………………………33
Figure 6: *A. actinomycetemcomitans* results on agar plates………………………34
Figure 7: HGF-1 2D effects with SDF……………………………………………………35
Figure 8: HSE after immediate application of SDF, …………………………………36
Figure 9: HSE prior to embedding…………………………………………………..37
Figure 10: HSE with H&E staining…………………………………………………38
Figure 11: HSE, H&E staining with samples from all time points…………………39
Figure 12: HSE, TUNEL staining with samples from all time points………………40
An In Vitro Pilot Study on the Effects of Silver Diamine Fluoride on Periodontal Pathogens and Three-Dimensional Scaffolds of Fibroblasts and Epithelium
Introduction

1. SILVER DIAMINE FLUORIDE (SDF):
SDF (Ag(NH3)2F) was approved by the U.S. Food and Drug Administration (FDA) in 2014 as a treatment for dentinal sensitivity. Shortly after, in 2016, the FDA modified its labeled use to include interim caries arresting medicament. The fast turnover in FDA approval may be in part due its qualification in meeting the World Health Organization (WHO) “Millennium Developmental Goals for Oral Health Initiative” [1]. The WHO 2020 initiative prioritizes the development of an oral health tool that may be used in an emergency, be treated preventatively, be widely accessible, and remain cost effective. SDF is a colorless, odorless solution that was first shown to treat dentinal hypersensitivity by plugging dentin tubules with a protective layer of a high concentration of aqueous silver, thus alleviating sensitivity [2]. The application of SDF is simple and efficacious—SDF is applied with a microbrush using cotton roll isolation; following a one-week period, the carious lesion is hardened with a black pigmentation. [3]. The immediate clinical efficacy of SDF lies in the synergistic effects of silver and fluoride, in which both dissimilar ions result in a greater total effect in conjunction, rather than in its individual capacity. Silver and fluoride ions each have a mechanism of action involving the bacterial and dentinal components of a carious lesion [4].

2. SDF AND CARIES:
Dental caries is a multispecies, bacterial biofilm-related etiologic disease resulting in the breakdown of enamel and dentin. SDF (38%) has been shown to inhibit developing biofilms of S. mutans and A. naeslundii monospecies cultured on dentinal sections for 7 days [5]. Further investigations also indicated the inhibitory effects of 38% SDF on a multispecies cariogenic
biofilm in an artificial mouth model consisting of \textit{S. mutans}, \textit{S. sobrinus}, \textit{L. acidophilus}, \textit{L. rhamnosus}, and \textit{A. naeslundii} on dentin surface cultured for 14 days [6]. To date, there is only one published report on the effects of SDF in vivo on \textit{S. mutans}, as compared to fluoride varnish and acidulated phosphate fluoride gel. Bacterial quantification was performed on saliva samples taken from buccal and lingual surfaces of all teeth 2 hours after brushing. There was an overall reduction in \textit{S. mutans} counts from all three interventions, but SDF statistically significantly outperformed its counterparts [7].

3. **SILVER: MECHANISM OF ACTION:**

Antimicrobial effects of silver have long been reported from 1000 BC in its use in potable water, to 1700 AD with the use of silver nitrate in treatment of venereal diseases or salivary gland fistulae, and even now to its current use in medicine for eye infections or wound dressings [8]. When dissolved in water, silver ions have three oxidative states in which only its monovalent form produces an antibiotic effect [9]. Silver I ion is a soft lewis acid with an empty electron shell in its outer orbit and it harnesses this property to absorb electrons from anionic components of bacteria [10]. Silver compounds are attracted to negatively charged bacterial enzymes [8, 11], cell walls and membranes [12], leading to bacterial cell death. The charged silver particles also target intracellular components such as bacterial DNA [8], and amino acids, resulting in the formation of organometallic complexes that further anabolize resulting in saturation of silver ions that further exacerbate DNA alterations [9]. With the disruption of bacterial DNA and protein sulfhydryl groups, there is DNA unwinding, inhibition of respiratory processes, inhibition of cell-wall synthesis, and a finite stop in cell division. This chain reaction leads to inhibition of cell wall formation and bacterial cell death [13].
Silver plays an indirect role in the protection of dentin. Dentin is made up of 20% organic components, 90% of which is collagen type I [14]. In its physiologic form, collagen is formed by an alpha-triple helical structure, and its catabolic domain is embedded and inaccessible to proteolytic enzymes [15]. In disease states, there is a lowering of pH that results in initial activation of matrix metalloproteinases (MMPs) and cathepsins, the enzymes responsible for collagen degradation and their activators, respectively [16]. Through various host or bacterial MMPs unwinding and cleaving the collagen, breakdown occurs. Silver inhibits the process of collagenolysis with the obstruction of MMPs. Specifically, within the dentinal pulpal complex, collagen is cleaved by MMP-8, MMP-2, MMP-9, MMP-3, MMP-14, and MMP-20 [17]. SDF at a 38% concentration was shown to have 79% inhibition on MMP-2, 94% of MMP-8, and 82% of MMP-9 [18].

4. FLUORIDE: MECHANISM OF ACTION:

Much like its silver counterpart, fluoride has also been shown to inhibit MMP-2, MMP-9, [19, 20] and cathepsins B and K within minutes of incubation [18]. A proposed mechanism of action is anionic fluoride’s electronegative attraction to the zinc portion of MMPs, rendering them inactive [19]. Fluoride ions also transiently affect the proton permeability of bacterial cell membranes, but the crux of its effect lies in its ability to firmly bind to dentinal minerals. Dentin consists of 70% inorganic minerals, mainly consisting of hydroxyapatite (HA). Lowered pH lead to dissolution and partial demineralization of HA crystals. With the application of fluoride ions, there is an exchange with hydroxyl ions, leading to partial, to complete substitution ranging from fluorohydroxyapatite (FHA) and fluorapatite, respectively [4]. The fluorine embedded within the apatite crystal leads to improved isotropic distribution of anions throughout the lattice network.
than the replaced hydroxyl group [21]. The chemical stability of FHA lends itself to more stability in acidic environments and reversal in remineralization [4]. Displaced silver ions precipitate out to form a silver-protein squamous layer, effectively plugging exposed dentinal tubules [3].

To summarize, SDF exhibits bactericidal effects that prevent further bacterial destruction of dentin and remineralizing properties that reinforce and strengthen compromised dentinal structures. However, SDF is not known for its collagenolytic inhibitory effects, but this is a key feature as collagen is a central building block of the dentition and the periodontium.

5. PERIODONTAL DISEASE:

The periodontium is the structure anchoring the dentition within the alveolar process and is made up of the gingiva, cementum, periodontal ligament, and alveolar bone. Periodontal disease is an inflammatory process in which there is an imbalance between the host response and bacterial equilibrium. While there are viruses, fungi, among other factors that contribute to the pathogenicity of periodontal disease, the main contributor are bacteria. Thus, the primary pathogenic etiology in periodontology mirrors cariology in that it is related to multi-species bacterial biofilms. Cariogenic bacteria such as gram-positive cocci first populate the supragingival plaque, followed by gram negative cocci and rods, then followed by filamentous forms [22]. As described by Page and Schroeder, the host response will react to these bacterial pathogens in the initial and early forms of gingivitis involving recruitment of neutrophils and lymphocytes, whereas an established lesion exhibits plasma cells [23]. However, the distinguishing feature of the advanced lesion (periodontitis), comes with loss of attachment. Concurrently, as the topography changes, opportunistic pathogens may invade the subgingival
region. As such, there is a shift towards anaerobic, mobile, gram-negative bacteria, leading to a transition into a dysbiotic biofilm. The shift towards dysbiosis is facilitated with certain keystone pathogens such as *Porphyromonas gingivalis* [24]. *P. gingivalis* is capable of appropriating the recruitment of Toll Like Receptor-2 (TLR2), a receptor responsible for identifying pathogen-associated molecular patterns (PAMPs) and signaling the innate immune response of the adaptive immune system, along with components of the complement system with C5a [25]. By utilizing these mechanisms, they do not act as prototypical pro-inflammatory bacteria by nature, and a relatively small number can disproportionately affect the host [24].

The traditional regiment for the treatment of periodontal disease is to eliminate the bacteria and their associated biofilm through implementation of proper oral hygiene, mechanical professional debridement (i.e., nonsurgical and surgical), and therapeutic means (e.g., local or systemic antibiotics or antiseptics). While the majority of patients respond well to conventional mechanical therapy, there are a subset of patients (0.5-4%) that experience persistent, destructive periodontal disease [26]. The 1989 World Workshop in Clinical Periodontics introduced the term refractory periodontitis (RP) to describe these patients that do not respond well to conventional therapy. Although the term is outdated and over-generalizes a multifactorial disease entity, it is a helpful reference to describe patients who demonstrate additional attachment loss over longitudinal, strict recall schedules [27]. Antibiotics are currently the adjunctive standard for treatment of patients with RP [28]. However, due to the nature of its heterogeneous microbiological profile, there is no standard antibiotic regiment [29]. The heterogeneity of RP poses a problem to antibiotic therapy, as different pathogens respond differently to various antibiotics—an improper pairing may lead to ineffective or potentially harmful effects. Attempts
have been made to qualify pathogens associated with RP. Walker and Gordon showed a predominance of *P. intermedia* and *P. gingivalis* [30]. The timing of periodontal breakdown also incorporates another difficulty in the treatment of periodontal disease. Typically, periodontal disease follows the random burst theory, as hypothesized by Socransky in which the disease process follows periods of quiescence and remission [31]. Studies have been made regarding progressing lesions in periodontal disease, among these of which there are *P. gingivalis* and *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) [32]. *A. actinomycetemcomitans* is another bacteria of interest as it is implicated in previously termed “localized juvenile periodontitis” which also has its own timeline of asynchronous multiple burst theory, centered around puberty [33].

While these disease profiles may exhibit differences in temporal profile, the mechanism of action of the surrounding collagen breakdown is largely the same. The periodontal extracellular matrix largely consists of type I collagen, dysbiosis will result in an influx of endogenous MMPs produced by host fibroblasts, epithelial cells and macrophages [16]. Specifically, in periodontal disease, the MMPs most involved are MMP-8, MMP-9; MMP-14 is also involved but to a lesser extent [17]. Currently, MMP-8 is considered the main collagenase in periodontitis and is the only bio-marker on the market available for chairside periodontal testing [34].

6. **EXPOSURE OF SOFT TISSUE TO SDF:**

Silver nitrate’s inhibition of fibroblast proliferation, and at higher concentrations, lethality has been demonstrated [35]. A very recent study has also shown the cytotoxic effects of SDF on human gingival fibroblasts [36]. In the primary SDF application protocol from the University of
California in San Francisco (the pilot school for SDF studies in the U.S.), there is mention of a transient, white lesion developing on the gingiva exposed to SDF [3]. Others have substantiated this clinical observation of a mildly painful, white lesion forming and dissipating within 48 hours [37]. A researcher in Peru took intraoral photos (before application of SDF, immediately after, 24 hours after, and 1 week after), and assessments were written about the gingiva, quantifying it with an “erythema score” and Loe’s gingival index. Reports showed an initial increase in inflammation at the 24-hour mark, only to be quickly resolved or even improved [38].

7. Human Skin Equivalent (HSE):
A recent, novel development in cell culture comes with the fabrication of human, three-dimensional models that can imitate in vivo similar effects of epidermal reactions, such as differentiation or signaling, otherwise lost in a two-dimensional system [39]. Human dermal fibroblasts are seeded onto an acellular dermal matrix and allowed to mature; keratinocytes are then seeded on top and allowed to differentiate per normal physiology with an air-liquid interface. Epidermal cells are able to differentiate into all necessary cell layers and is not considered mature until the development of the stratum corneum [40]. Although both fibroblast and keratinocytes used in our study originated from neonatal foreskin, this model exhibited stratified squamous epithelium and fibroblast-rich dermal layer which recapitulates gingiva morphology [39].
Aims and Hypotheses:

1. Aims

The objectives of this study were to study the effects of SDF on periodontal pathogens (compared to cariogenic bacteria) and on epithelial cells and fibroblasts.

Specifically, our research questions were:

a. Will SDF have bactericidal effects on select periodontal pathogens?

b. Will SDF negatively impact fibroblasts and epithelial cells health?

2. Hypotheses:

a. Application of SDF will yield the same level of bactericidal effects on established cariogenic \textit{S. mutans}, compared to periodontal pathogens \textit{P. gingivalis}, and \textit{A. actinomycetemcomitans} in mono-species biofilms.

b. Application of SDF will impact viability of fibroblasts and epithelial cells in 2D-culture as well as in HSE model.
Materials and Methods

**Bacterial strain and inoculum preparation:**

Bacterial strains were purchased from American Type Culture Collection (ATCC), (Manassas, VA). Three bacterial strains were used: *S. mutans* (ATCC 25175), *A. actinomycetemcomitans* (ATCC 33384), and *P. gingivalis* (ATCC 33277). *S. mutans* and *A. actinomycetemcomitans* were grown on Brain Heart Infusion agar, while *P. gingivalis* was grown on Tryptic soy agar with 5% defibrinated sheep blood. All bacteria were grown at 37°C with *S. mutans* grown in aerobic conditions, *A. actinomycetemcomitans* in microaerophilic condition with 5% CO₂, and *P. gingivalis* grown in anaerobic conditions as described in Table 1. Discrete colonies were isolated using the quadrant streak plate method and incubated for 24 hours for *S. mutans* and 72 hours for *A. actinomycetemcomitans* and *P. gingivalis*. Plates were streaked in triplicates. Single colonies were then selected from each plate and a final inoculum was prepared using a mass spectrophotometer to an optical density of 600 nm (OD₆₀₀).

**Silver Diamine Fluoride preparation and application:**

Advantage Arrest Silver Diamine Fluoride 38% was obtained from Elevate Oral Care (West Palm Beach, FL). Serial dilutions were prepared of SDF in distilled water; initial concentration reflects clinical dose of 38%, followed by 19%, 9.5%, 4.75%, 2.37%, 1.18%, 0.593%, and 0.296%. Dilutions were prepared fresh with each use, and the stock solution was kept in a sterile, opaque container at room temperature. A 96-well plate (Fisherbrand 96 well plates sterile, Fisher Scientific, Leicestershire, UK) was used for each bacterium. Each of the three previously isolated bacterial inoculums were plated in duplicate, as shown in Figure 1. Each well received 10 μL of bacterial inoculum, 140 μL of broth, and 50 μL of SDF with each respective dilution.
As a result, final concentrations of SDF in each well used were: 12.6%, 6.3%, 3.15%, 1.57%, 0.788%, 0.394%, 0.197%, 0.098%. For each colony, two positive controls were used with 50 µL of CHX in place of SDF and two negative controls were used with 50 µL of additional broth in place of SDF. Plates were then incubated in respective conditions at 37°C.

**Bacterial culture:**

Plates were covered with breathable plate seals and incubated at 37°C for 48 hours. Following the incubation period, bacteria were streaked onto agar plates with four delineated quadrants, as depicted in Figure 2. Each plate included a positive control (CHX), a negative control (broth), and one of two of the dilution series. Each condition was performed 3 times using different colonies. *S. mutans* and *A. actinomycetemcomitans* were grown on Brain Heart Infusion agar plates and *P. gingivalis* was grown on Tryptic soy agar with 5% defibrinated sheep blood. Each dilution of SDF along with positive and negative controls were plated on individual agar plates for *A. actinomycetemcomitans*.

**Cell Culture and SDF application:**

HGF-1 cells (CRL-2014, ATCC, Manassas, VA) were cultured in 75 cm² tissue culture flasks in growth media using Dubocco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5% Penicillin-Streptomycin (P/S). Cells were incubated at 37°C in microaerophilic conditions with change of media every 3 days.

HGF-1 cells, from passage 2, were seeded in 6 well plates at 300,000 cells/well in growth media. 1µL of 0.394 was applied to HGF-1 cells. Cells were then washed with 1xPBS and exposed to 10 µL of Trypan blue (T6146, Sigma Aldrich) to visualize cell death.
Human Skin Equivalent Culture:

Three-dimension models of HSE obtained from Garlick Lab (Tufts School of Dental Medicine, Boston, MA) consisting of a basal acellular collagen layer, a collagen matrix with human dermal fibroblasts, and human, epidermal keratinocytes cultured from human neonatal foreskin keratinocytes [39]. Skin constructs are engineered to have a liquid air interface which allows for full differentiation of epithelial layers with multiple cell types. The HSE used was matured for 10 days and exhibited basement membrane, normal basal, spinous, granular, and keratinized layers [40]. Cornification medium was changed every two to three days. Three drops of 3µL of 38% SDF were applied directly onto the 3D tissues and harvested at 12 hours, 1, 3, 5, and 10 days post-treatment. Plates were photographed after applying SDF and followed for ten days (Figure 3); photographs were also taken prior to dissection using NI-150 Nikon High Intensity Illuminator, Nikon SMz645 Dissecting Stereomicroscope, with a Nikon CoolPix4500 4X zoom with 28mm threaded eyepiece.

After incubation, samples were dissected away from the inserts using fresh 12 blade scalpels and tissue forceps and divided into three and placed in tissue processing cassettes (Fisher Scientific Electron Microscopy Sciences CellSafe Biopsy Insert). Samples were fixed overnight in 4% formalin made in phosphate buffer saline (PBS). Samples were then washed with PBS and dehydrated through increasing gradients of EtOH and Xylene, using standard sequential dehydration techniques. Tissues were then embedded, with tissue standing on edge, in hot paraffin wax. Blocs were then sectioned to 6 µm, floated in water bath, and mounted onto positively charged slides (Thermo Scientific™ Shandon™ ColorFrost™ Plus Slides).
**Histology H&E and TUNEL Stain:**

Slides were first incubated for 15 minutes at 60°C in IsoTemp Oven then processed for H&E staining per standard protocol. Slides were dehydrated with xylene, and rehydrated with decreasing concentrations of ethanol to distilled water prior. After rehydration, slides were placed in Mayer’s Hematoxylin solution for two minutes. Slides were then rinsed with distilled water and acid alcohol prior to placement into 1% Lithium Carbonate, in which slides turn a purple-bluish hue. Slides were then quickly dipped in 80% ethanol prior to exposure to Eosin for 3 minutes. Finally, slides were dehydrated back through increasing concentrations of ethanol to xylene. Slides were then mounted with VectaMount mounting media. Additional slides were processed for apoptosis staining following the manufacturer protocol (Thermofisher Scientific) using Click-it™ TUNEL Colorimetric IHC Detection Kit. Due to extensive details involved along with number of steps, it is advised to review manufacturer’s protocol. Histological images were taken in brightfield using a Nikon Eclipse E600 Fluorescence Microscope and SPOT Idea 3.0 Mp Color Digital Camera.
Results

Effect of SDF on bacterial growth:

Initially, we planned to monitor bacterial growth by measuring the optical density using a microplate reader. Unfortunately, immediately upon addition of SDF solution, a precipitate formed between silver ions and bacterial components, resulting in an initial foaming reaction that matured into a dark precipitate as can be seen in the first 8 columns of Figure 1. Therefore, bacterial viability was assessed by inoculation of respective cultures on agar plates. Positive and negative controls of chlorhexidine and broth reflected expected outcomes of growth and inhibition for each bacterial colony, ensuring sound protocol.

As shown in Figure 4 and in agreement with previous reports, S. mutans growth was completely inhibited by SDF. Of note, this study shows bacterial inhibitory effects of SDF even at the lowest dilution tested of 0.098%. As shown in Figure 5, similar findings of bacterial inhibition were found for P. gingivalis at higher concentrations of SDF (Fig. 5A), however bacterial colonies were noted at SDF concentrations of 0.197% and 0.098% (Fig. 5B). Please note that the dark staining in the lower right region of the first plate at 12.6% SDF is an artifact from the silver precipitate reacting with the sheep blood present in the agar. Due to the need for longer incubation periods for A. actinomycetemcomitans and expected spread, incubation design was modified from quadrant streaking to individual inoculations on separate petri dishes. Viability patterns of A. actinomycetemcomitans are depicted in Figure 6, showing a similar pattern, as seen with S. mutans, exhibiting complete bacterial inhibition even with the lowest concentration of 0.098% SDF.
Effect of SDF on fibroblasts viability:

As the previous portion of the study identified reproducible bactericidal effects of SDF at 0.394% on all three bacterial biofilms in question, this was the concentration selected to compare to the undiluted concentration of SDF at 12.6%. HGF-1 cells were to be incubated and observed over a 3-day period. However, upon observation under an inverted microscope, addition of 1µL of 0.394% SDF caused a rapid change in cell morphology indicating cell death. Figure 7 shows HGF-1 cells prior to inoculation (Fig. 7A), the SDF front between affected and virgin cells (Fig. 7B), the affected region (Fig. 7C), application of Trypan blue to check for cell viability (Fig. 7D), and dense infiltrate of dead cells (appearing as blue, round cells) in affected region versus sparsely infiltrated region in the periphery to SDF exposure (Fig. 7E). As reaction was so immediate and drastic, it was determined that no further concentrations nor longer incubation times were required. In addition, we also concluded that if keratinocytes were to be seeded onto two-dimensional cultures, SDF would most likely cause the same rapid cell death observed with fibroblasts. Therefore, 3D epidermal matrices, HSE, were used, and for optimized clinical generalizability, the full concentration dosage of 38% SDF was used.

Effect of SDF on HSE:

Gross clinical examination. HSE were matured for 10 days and either left untreated or received drops of a 38% SDF solution. Figure 8 illustrates the reaction of HSE to SDF application, recorded immediately, at 30 minutes, and at 60 minutes. By 30 minutes, the silver ions begun to precipitate out of solution, and by 60 minutes, the droplet has spread and infiltrated the superficial keratinized layer, resulting in a larger, grayish lesion with indistinct boundaries. Figure 9 compares visual appearance of untreated HSE (control) with constructs exposed to three
drops of 38% SDF and followed for 12 hours, 1, 3, 5, and 10 days. Within the first day, the overall appearance of the lesions was similar to initial observations at 60 minutes; lesions are white-grayish opaque with indistinct borders. However, there are some fine details of differentiation: at 12 hours, lesions surfaces appear reticulated and surrounding tissues are smooth, whereas at 24 hours, there appears to be more retraction of surface lesion and surrounding tissues. At 3 days, the signature dark pigmentation starts to develop with an induration of the affected keratinized layer of epithelium showing an eschar, leathery appearance. From this time point on, the lesions have a defined boundary with erythematous margins outlining a portion of the lesion, showing inflammation extending into contacted epithelium. Day 5 shows no significant changes, aside from continued darkening of pigmentation and continued appearance of inflammation in neighboring epithelial regions. At day 10, the lesion has formed a blackened crust that has begun to peel away from borders due to extent of contraction within the lesion.

**Histological examination.** HSE were fixed, embedded in paraffin and sections stained with H&E. Figure 10 shows the histology of untreated HSE using different magnifications. Figure 10C, depicts in ascending order the different layers of HSE constructs: acellular collagen (AC), cellular collagen (CC), connective tissue (CT), and differentiated layers of the epithelium. Figure 10D, taken under a higher magnification, depicts the different cell layers of the fully differentiated epithelium. The stratum basale lies superficial to the underlying connective tissue and can be identified by their contiguous, nucleated cuboidal cells. Stratum spinosum is the next layer and contains less tightly organized cuboidal cells with smaller nuclei. Stratum granulosum follows, identifiable with purple keratohyaline granules within a flatter squamous epithelium.
Finally, the stratum corneum forms the topmost layer, made up of completely dead cells adapted in a flattened keratinized cell layer. These human, neonatal foreskin fibroblasts and keratinocytes contracts adopt a morphology very similar to that of the fully differentiated gingiva. However, an important difference of this HSE model from human oral epithelium is that the epithelial layer in this model does not form invaginations into the underlying lamina propria and instead lies in a flat, linear topography. Nevertheless, we believe that this model is suitable to study the effect of SDF on fibroblasts and epithelial cells.

Figure 11 shows the juxtaposition of all H&E stained HSE samples from all studied time points. Unfortunately, during processing, the epithelial layer of the 24-hour sample was lost, leaving only the connective tissue and underlying collagen portion. All samples spanning all time points have some commonalities: fibroblasts embedded within the deeper portions of the connective tissue exhibit a flat morphology, indicating viability whereas fibroblasts towards the surface exhibit a rounder appearance, suggesting an apoptotic state. It is important to note that this is also observed in the control sample as well. The 12-hour specimen exhibits an intact epithelium, with the exception of the superficial layers of the stratum corneum. However, beginning at day 3, continuing onto day 10, the epithelial layer is completely lifted, being adhered to the contracting stratum corneum.

**Staining for apoptotic cells.** Apoptosis of keratinocytes and fibroblasts in HSE was assessed with TUNEL staining following exposure to SDF. The non-treated HSE was also stained to provide a negative control, and only showed minimal apoptosis occurring among fibroblasts in the most superficial region of the connective tissue; no epithelial cells stained for apoptosis (Fig. 12A).
Tissues exposed to SDF (Fig. 12B-F) show apoptotic cells throughout all nucleated layers of the epithelium and within the underlying connective tissue. These results indicate that application of SDF can induce apoptosis throughout multiple layers of the HSE model. Treated tissues harvested earlier show apoptotic cells extending throughout the upper half of the connective tissue portions (Fig. 12B-D). However, samples taken at later time points such as Day 5 and Day 10 show fewer apoptotic cells that are limited to the first few cell layers of connective tissues which may indicate a potential regenerative capacity within HSE models.
Discussion

The use of SDF as a caries arresting agent is valued for its many clinical benefits such as the bactericidal effects on cariogenic bacteria, ability to harden softened dentin, and relief of dentinal hypersensitivity [3]. Each of these functions, relate back to SDF’s synergistic effects of silver and fluoride ions [4]. The effect of SDF may also hold promising implications within the periodontal discipline. Similar to caries, gingivitis and periodontitis are also borne of a disease process involving bacterial dysbiosis [6]. Much like a carious lesion, with the softening of the enamel and dentin, a periodontal lesion also involves loss of structural integrity of the surrounding gingiva, cementum, PDL, and bone—both disease processes involve the breakdown of collagen [15, 41]. Lastly, with exposure of dentin, odontoblastic processes may experience fluid movement with the change in hydrodynamics resulting in dentinal hypersensitivity—a sensation common with carious lesions with close proximity to the pulp as well as areas of recession with loss of attachment in periodontitis [42, 43].

*In-vitro* studies have shown bactericidal effects of SDF on gram-positive, cariogenic bacteria such as *S. mutans*, *S. sobrinus*, *L. acidophilus*, *L. rhamnosus*, and *A. naeslundii* [6]. The bactericidal efficacy of the drug is attributed to the silver ion’s cationic effect in disturbing negatively charged bacterial components of gram-positive bacteria. The findings of the current study are in agreement with previous reports: *S. mutans*, used as a positive control, showed complete inhibition by SDF. This study shows that the inhibitory effects of SDF hold even to the lowest dilution of 0.098% used in our study.

While no current evidence exists regarding the effect of SDF on gram-negative bacterial structures, a hypothesis can be drawn regarding silver’s cationic properties. The key
differentiating characteristic of gram-negative bacteria is their negatively charged outer membrane [44]. Studies using cationic agents such as magnesium or chlorhexidine have shown competitive ionic binding onto negatively charged lipopolysaccharides, affecting ion-pairing resulting in an electrostatic charge, affecting the lateral packing and permeability of the outer membrane [45, 46]. Specifically regarding silver particle effect of gram negative outer cell membranes, advances have been made in the field of Silver nanoparticles (AgNP). AgNP were initially created to combat the undesired staining produced by SDF precipitate—as silver particles decrease in size, it becomes more antimicrobial and more transparent, causing minimal staining [44]. AgNP are <10 nm in diameter, and are essentially micelle aggregate structures of silver ions that may readily attach to bacterial cell walls of both gram-positive and gram-negative bacteria, causing membrane permeability [47].

The antimicrobial effects of silver on gram-negative periodontal pathogens have been shown previously, with the use of silver nitrate on *P. gingivalis* and *A. actinomycetemcomitans*, among others [48]. It was postulated that the silver may interfere with sulphydryl bonds of essential bacterial enzymes, much like SDF [8]. While the specific nature of remains unknown, the bactericidal effects of SDF on gram-negative bacteria has been demonstrated in this pilot study. Bacterial inhibition was observed in *P. gingivalis* at higher concentrations of SDF ranging from 12.6% to 0.394%. However, bacterial growth was observed at the lower concentrations of 0.197% and 0.098%. Previous findings using silver nitrate report a $3\log_{10}$ reduction in *P. gingivalis* at a concentration of 0.5 µg/mL [48]—a much lower concentration than the most dilute concentration of SDF used in this study (1.97 µg/mL), perhaps suggesting higher potency of silver nitrate. However, *A. actinomycetemcomitans* exhibited complete bacterial inhibition.
even at lower concentrations of SDF. One limitation of this study was that the design was merely to answer whether bactericidal effects would be noted for periodontal pathogens at varied concentrations with a very binary outcome. Future studies should involve quantification of data using viable count assays to determine CFUs for each bacterium.

Dentinal-pulpal collagen is cleaved by MMP-8, MMP-2, MMP-9, MMP-3, MMP-14, and MMP-20 [17]. Meanwhile, MMPs most involved in periodontal lesions are MMP-8, MMP-9—and MMP-14 to a lesser extent [17]. MMP-8 is currently considered the gold standard and is the only bio-marker on the market available for chairside periodontal testing [34]. Interestingly, SDF has the ability to inhibit MMP-2, MMP-8, MMP-9, cathepsins B and K [18]. If the clinical results are so dramatic for the instantaneous halt of carious lesions, it would be interesting to investigate similar effects within periodontal lesions. A next step in the study would be to collect media of the HSEs throughout the incubation process with SDF to assess the signaling pathways involving cytokines changes with MMPs and cathepsins.

In an in-vitro study with cultured fibroblasts, silver has been shown to be cytotoxic in silver nitrate at 14x10^{-5} % with a contact time of 2 hours [35]. Likewise, SDF incubated in hydroxyapatite discs were shown to be cytotoxic to human gingival fibroblasts at 0.01% and it further retained its cytotoxic effects even after 9 weeks of rinsing with artificial saliva [36]. Our results concur with previous 2D in-vitro studies of human gingival fibroblasts: the toxicity is instantaneous and severe. Upon application of 1µL of 0.394% SDF, human gingival fibroblasts showed an immediate disruption of the attachment process. Fibroblasts changed from a well-attached cell with thin cytoplasmic extensions and smooth surfaces to one with broad
cytoplasmic extensions filled with blebs and micro-villi, and staining blue with Trypan blue, indicating cell death. However, 2D matrices reflect little of the anatomy found intraorally in which fibroblasts would be safely embedded within a connective tissue structure. As such, one of the novelties of this study was the use of a 3D HSE structure. One unexpected finding was regarding the limited reach of SDF within the connective tissue layers. All treated regions of the epithelium formed a scab-like layer with apoptosis noted in the supra-basal and basal layers of the epithelium, along with the superficial layer of connective tissue, but it appears that the destruction is not felt by the deepest layers of connective tissues. Apoptotic cells were noted deep into the middle third of the connective tissue at 12 hours and 24 hours and were noted even deeper within the bottom third of the connective tissue at the 3-day mark. However, a shift is noted at day 5 and day 10 in which the apoptotic cells are only noted in the most superficial layers of the connective tissue. This shift in apoptotic cells may indicate a shedding or regenerative capacity of the HSE in which connective tissue layers continue to develop at the deepest layers and shed the superficial layers, resulting in an upward trend of apoptotic cells. This finding corresponds to the untreated tissue samples exhibiting very minimal apoptotic markers at the most superficial layers of the connective tissue.

Another added benefit of using a 3D matrix such as HSE was to observe the clinical changes to the surface of the epithelium immediately, daily, and weekly after application of SDF. As reported in previous findings, a transient, white lesion will initially develop as soon as SDF contacts epithelium [3, 37]. However, in our study the lesion did not shed—obviously due to the static nature of this epithelial growth—but instead darkened, and contracted forming a scab-like process. The surrounding tissue around the scab at day 5 and day 10 have increased erythema,
which corresponds to a study in which inflammation was noted to increase at the 24-hour mark [38].

Conclusion

In summary, our study shows the bactericidal effect of SDF on gram negative bacteria. This finding, coupled with the concept of SDF’s inhibition of MMP-8, 9, and 14 has promising implications for the inhibition of actively progressing periodontal lesions. This study also shows that the maximal damage that SDF may cause to the epithelium and connective tissue within a 3-D HSE construct was limited to the epithelium and top half of connective tissue, suggesting that it might be safe to use on actively progressing periodontal lesions.
References


Appendix I: Tables

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Media</th>
<th>Incubation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td>Brain Heart Infusion</td>
<td>Aerobic, 24 hours, 37°C</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>Tryptic soy with 5% Defibrinated Sheep Blood</td>
<td>Anaerobic, 48 hours, 37°C</td>
</tr>
<tr>
<td><em>A. actinomyctemcomitans</em></td>
<td>Brain Heart Infusion</td>
<td>Microaerophilic (5% CO₂), 72 hours, 37°C</td>
</tr>
</tbody>
</table>

N= 6 for all groups
Appendix II: Images

**Figure 1:** *S. mutans* inoculated in Brain Heart Infusion broth in a 96 well plate with different conditions applied across columns: ordered in decreasing serial dilution for SDF from 12.6% to 0.098%, chlorhexidine (CHX, positive control), and broth (negative control). Each bacterial colony was used in duplicate with 3 colonies total, shown in the six rows. This process was duplicated for each bacterial type. The same format was used for *P. gingivalis* and *A. actinomycetemcomitans*. Note the significant dark pigmentation from columns 1-6 (SDF 12.6%-0.394%) resulting from the precipitation of SDF and bacteria.
**Figure 2:** Diagram illustrating the template used for quadrant streaking on agar plates. Top two quadrants reserved for positive (CHX) and negative (broth) control with the bottom two quadrants receiving various dilutions of SDF.

*S. Mutans*  

<table>
<thead>
<tr>
<th>Dilution</th>
<th>CHX (+)</th>
<th>Broth (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF: 12.6%</td>
<td>6.3%</td>
<td>3.15%, 1.57%, 0.788%, 0.394%, 0.197%, 0.098%</td>
</tr>
</tbody>
</table>

*Quadrant division on agar plate*
Figure 3: HSE constructs are engineered to have liquid, air interface with cornification medium. Three µL of a 38% SDF solution were applied directly onto the 3D tissues and incubated for 12 hours, 1, 3, 5, or 10 days post-treatment.
**Figure 4: Effect of SDF on *S. mutans* growth.** *S. mutans* was streaked on brain heart infusion agar plates. Results from one set of experiment with the first rows showing complete inhibition of growth across all dilutions of SDF (annotated in bottom two quadrants of agar plates). Following rows, labeled Colony II, Colony III confirm findings in triplicate study. This process was repeated twice.
Figure 5: Effect of SDF on *P. gingivalis* growth. (A). *P. gingivalis* was streaked on tryptic soy with 5% defibrinated sheep blood agar plates. Note the SDF precipitate artifact in 12.6% concentration lower left quadrant. Colonies growth was noted in quadrants with 0.197% and 0.098% SDF. (B). Shows enlarged images of the plates for better visualization of *P. gingivalis* growth at in the presence of 0.197% and 0.098% SDF. Process repeated twice.
B.

(Broth) (CHX)
12.6% 6.3%
3.15% 1.57%

(Broth) (CHX)
0.788% 0.394%
0.197% 0.098%
Figure 6: Effect of SDF on *A. actinomycetemcomitans* growth. *A. actinomycetemcomitans* streaked onto brain heart infusion agar plates. Due to the need of longer incubation periods and fast growth and spread of *A. actinomycetemcomitans* on agar plates, colonies could not be cultured by quadrant as with the other bacteria. Colonies were instead inoculated on separate petri dishes as indicated below. No bacterial growth was noted with any concentration of SDF. Process repeated twice.
Figure 7: Effect of SDF on 2D cultures of human gingival fibroblasts (HGF). (A) HGF-1 cells prior to addition of SDF. (B) SDF front showing zone of affected cells (white asterisk) upon application of 1 µL of 0.394% SDF and peripheral untreated cells (black asterisk). (C) Treated region with SDF showing complete cell detachment. (D) Application of Trypan blue for detection of cell viability. (E) Dense infiltrate of dead cells (appearing as blue, round cells) of treated region. (F) Sparsely infiltrated region of dead cells in peripheral untreated region. Time lapsed indicated in lower right corner.
Figure 8: Effect of SDF on HSE constructs. Photos were taken immediately, 30 minutes, and 60 minutes after application of 3 μL of 38% SDF using a NI-Nikon High Intensity Illuminator, Nikon SMz645 Dissecting Stereomicroscope, and a Nikon CoolPix4500 4X zoom with 28mm threaded eyepiece.
Figure 9: Effect of SDF on HSE constructs. (A) Negative control, untreated construct. B through F are treated constructs that received 3 µL of 38% SDF and incubated for: (B) 12 hours: center of lesion is white-gray and reticulated with smooth surrounding tissues; (C) 24 hours: color of lesion shifts to a more grayish hue with slight contraction noted across surface of lesion and surrounding tissues; (D) 3 days: dark pigmentation starts to develop with an induration of the affected keratinized layer of epithelium; (E) 5 days: Continued darkening of pigmentation with inflammation in surrounding epithelium; and (F) 10 days: Complete induration noted with blackened crust and contraction of eschar from underlying tissue.
Figure 10: Histological examination of H&E stained HSE construct. (A) HSE at 4x magnification. (B) 20x magnification and (C) 40x magnification showing: acellular collagen (AC), cellular collagen (CC), connective tissue with fibroblasts (CT), and further enhanced in (D) 60x magnification image showing the epithelium consisting of the basale layer (B), stratum spinosum (S), stratum granulosum (G), and stratum corneum keratinized layer (K). Scale bar 50 µm.
Figure 11: Histological examination of H&E stained HSE constructs. (A): Untreated construct. (B) 12 hours: intact epithelium noted, with the exception of the superficial layers of the stratum corneum. (C) 24 hours: Epithelial layer of the 24-hour sample was lost during processing leaving only the connective tissue and underlying collagen portion. (D) 3 days: Epithelium has lifted with contracted stratum corneum with largely unaffected connective tissue. (E) 5 days: Stratum granulosum is very spread out with intact stratum basale. Connective tissue also looks unaffected. (F) 10 days: Epithelium has also lifted away from connective tissue and appears very deformed and nearly indistinguishable between various epithelial layers with change in morphology. Connective tissue appears unaffected.
Figure 12: TUNEL staining for apoptotic cells. Apoptosis of keratinocytes and fibroblasts in HSE was assessed with TUNEL staining—apoptotic cells will stain with a dark pigmentation, indicated with red arrows. (A) Negative control, untreated construct shows minimal apoptosis occurring among fibroblasts in the most superficial region of the connective tissue; no epithelial cells stained for apoptosis. (B) 12 hours: Apoptotic cells noted throughout all nucleated layers of the epithelium and within the underlying connective tissue. (C) 24 hours: Missing epithelial layer lost during processing, but fibroblasts staining for apoptosis are limited to first few layers of connective tissue. (D) 3 days: Basal and suprabasal layers of epithelium are adherent to keratinized layer above and all stain dark for apoptosis along with superficial layer of connective tissue. (E) 5 days: Fibroblasts stain for apoptosis throughout all regions of connective tissue (F) 10 days: Fewer apoptotic cells that are limited to the first few cell layers of connective tissues.