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Title Page

1) Title: An In-vitro Comparison of Four Antibacterial Agents with and without Nicotine and their Effects on Human Gingival Fibroblasts

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VEGA is bacteriostatic at 1/4 dilution and demonstrates no detrimental effects on HGF proliferation or cytotoxicity up to 1/10,000 dilution and thus, may enhance healing outcomes.

Title: An In-vitro Comparison of Four Antibacterial Agents with and without Nicotine and their Effects on Human Gingival Fibroblasts

Abstract

Background: To compare anti-bacterial activity of 0.12% Chlorhexidine (CHX), 10% Povidone Iodine (PVD), Vega Oral Care Gel (VEGA) and Antioxidant Gel (AO) on *Streptococcus mutans*, *Streptococcus sanguis, Fusobacterium nucleatum* and *Porphyromonas gingivalis* with and without nicotine and to evaluate their effects on human gingival fibroblasts (HGFs).

Methods: *S. mutans*, *S. sanguis*, *P. gingivalis* and *F. nucleatum* were incubated with serial dilutions (1/4, 1/8, 1/16, 1/32 and 1/64) of anti-bacterial agents in media (with and without nicotine). Minimum inhibitory and minimum bactericidal concentrations (MIC/MBC) were measured, and confocal microscopy was performed. HGFs were exposed to serial dilutions (1/10, 1/100, 1/1000 and 1/10,000) of antibacterial agents with media. Water-soluble

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tetrazolium-1 (WST-1) assay and lactate dehydrogenase (LDH) assay were used to assess proliferation and cytotoxicity towards HGFs.

Results: CHX and PVD significantly inhibited growth of all bacterial species ($p<0.0001$) at all dilutions. AO and VEGA inhibited growth of all bacterial species up to only the 1/4 dilution. CHX and PVD decreased HGF proliferation at 1/10 and 1/100 dilution, while AO at all dilutions ($p<0.05$). CHX and AO were cytotoxic at all dilutions ($p<0.05$). VEGA was not cytotoxic to HGFs and did not affect HGF proliferation at any dilution ($p>0.05$). An increased bacterial growth was seen for all species except *P. gingivalis* with addition of nicotine.

Conclusion: CHX and PVD demonstrate superior antibacterial properties, but significantly reduce HGF proliferation. AO is bacteriostatic at lower dilutions but is highly toxic to HGFs. VEGA was bacteriostatic and demonstrated no detrimental effects on HGF’s.

Key Words:

Fibroblast(s), Antimicrobial(s), Antioxidant(s), wound healing, nicotine

1. Introduction

Surgical therapy is routinely performed for treatment of periodontal disease to allow resolution of periodontitis. It is also indicated for pre-prosthetic dental augmentation, dental implant placement, periodontal and peri implant soft/ hard tissue deficiency correction. However, surgical therapy may involve complications, amongst which post-surgical infection is of prime significance. In a retrospective study, an overall prevalence of 2.09% for post-surgical infections was reported.[1] Prophylactic antibiotics are often used for prevention of post-surgical infections in medically compromised patients and to prevent bacterial endocarditis.[1] However, use of antibiotics to prevent infections after periodontal surgery is

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primarily based on empirical evidence, remains controversial and indiscriminate overuse of antibiotics has contributed to the emergence of antibiotic resistance.

Smoking is considered a significant risk factor for development and progression of periodontal disease.[2, 3] Nicotine, due to its vasoconstrictive actions, decreases gingival blood flow and impairs revascularization of soft and hard tissues, inhibiting fibroblast proliferation and collagen production thus, negatively affecting wound healing.[2,4] Additionally, the presence of nicotine alters the subgingival plaque microbiota favoring binding of periodontopathic bacterial species thus, increasing biofilm formation and altering the MIC.[2, 5]

Chlorhexidine (CHX) mouth rinse has proven to be beneficial to reduce post-surgical infection due to its bactericidal acitvity.[1] The cationic structure of the CHX molecule has great affinity towards the negatively charged bacterial cell wall causing loss of osmotic equilibrium, cytoplasmic membrane extrusion, and bacterial cell wall lysis.[4] A systematic review concluded that CHX is a valuable post-surgical antibacterial agent when oral hygiene is compromised as it aids in healing, reduces biofilm formation and bleeding.[5] Despite these benefits, CHX leads to yellow-brown staining of teeth, calculus formation on prolonged use and taste alteration. [6] A dose-dependent reduction in proliferation of human gingival fibroblasts (HGFs) along with a reduction in collagen and non-collagen protein formation was observed in an in-vitro study emphasizing that CHX might pose toxic effects on HGFs thus, negatively impacting wound healing.[9]

Povidone iodine (PVD) is another antimicrobial agent commonly used for wound healing and prevention of post-surgical infection. Release of free iodine leads to inhibition of vital bacterial cellular mechanisms and oxidation of essential components of the bacterial cell membranes exerting a strong bactericidal effect.[10] PVD has been extensively used for pre-surgical site preparation, intraoperative flushing, surgical and burn wound healing.[10] However, cytotoxicity analysis have revealed that even dilute concentrations of 10% PVD is

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toxic to human skin fibroblasts.[9] PVD must also be used with caution in patients allergic to iodine, with thyroid disorders and those undergoing radioiodine therapy.[11]

Recently, a novel, herb-based formulation, Vega Oral Care Gel (VEGA) was developed that aids in healing and post-operative pain management while reducing opioid intake.[12] VEGA contains aconitum, arnica, calendula, chamomilla, echinacea, gelsemium, hepar, hypericum, ignatia, mercurius and ruta as its active ingredients. The listed inactive ingredients include allantoin, hydroxyethyl cellulose, peppermint oil, sodium benzoate and xylitol. The cumulative effect of each ingredient contributes to overall post-operative pain reduction, accelerated healing with reduced bruising, antimicrobial, and anti-inflammatory properties thus, rendering VEGA as a potentially useful alternative for post-surgical procedures. Additionally, one recent study has demonstrated superior biocompatibility to HGFs with VEGA.[13] However, in-vitro evidence supporting VEGA as an effective antibacterial agent is still sparse.

Antioxidant Gel (AO) is also used for post-surgical care.[14] Its key ingredients are phloretin and ferulic acid, both working synergistically to produce a powerful antioxidant effect. Apart from the antioxidant effect, ferulic acid plays a role in angiogenesis by affecting the activity of various growth factors such as VEGF, PDGF, etc. It also accelerates regeneration and wound healing due to faster epithelialization and granuloma formation.[15] A recent in-vitro study concluded that AO stimulates HGF proliferation thus, enhancing wound healing.[16] AO also contains menthol, peppermint oil, sage oil, clove flower oil, thyme and xylitol which have soothing, antioxidant and anti-inflammatory properties.

Reduction of post-surgical infection and stimulation of wound healing are important properties to be considered when selecting a post-operative care agent.

There have been many studies demonstrating the superior efficacy of 0.12% CHX and 10% PVD as effective antibacterial agents for prevention of post-surgical procedures.[7, 10]

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However, no research to date has examined the antibacterial efficacy and effects on HGFs of VEGA and AO.

Hence, the aim of this study was to compare the anti-bacterial efficacy of 0.12% CHX, 10% PVD, VEGA and AO on *S. mutans, S. sanguis, F. nucleatum* and *P. gingivalis* with and without the addition of nicotine. A secondary objective was to evaluate the effects of these antibacterial agents on HGFs in regard to toxicity and effect on cell proliferation.

2. Materials and Methods

Serial dilutions of all the antibacterial agents, (0.12% CHX[¶], 10% PVD[#], VEGA Oral Care Gel[**] and AO gel[††]) were prepared as 1/4, 1/8, 1/16, 1/32 and 1/64 dilutions by addition of the bacterial media (Tryptic Soy Broth or Brain Heart Infusion broth with yeast extract and vitamin K and hemin) based on a previous study.[17] Nicotine (2 mg/ml) was added to the nicotine groups for each bacterial species.

2.1 Bacterial Cultures and Culture Plate

Overnight cultures of *S. mutans* (UA159 strain, ATCC 700610) and *S. sanguis* (ATCC 10556) were grown in tryptic soy broth (TSB) at 37°C with 5% CO_2. *P. gingivalis* (ATCC 33277) and *F. nucleatum* (ATCC 10953) were grown in brain heart infusion broth with 5 gm/L of yeast extract (BHI-YE) and 5% v/v of vitamin K and hemin (BHI-YE) at 37°C anaerobically in a Gas Pak chamber. Ten µL aliquots of this overnight culture of each bacteria were placed into wells of a 96-well flat bottom microtiter plate with 190 µL of tryptic soy broth sucrose (TSBS) for the streptococcal species or BHI-YE for *P. gingivalis*

[¶] Peridex™ 3M ESPE
[#] Betadine Solution, Purdue Products L.P. Stamford, CT, USA
[**] StellaLife® Vega Oral Care Gel, Glenview, IL, USA
[††] PerioSciences® AO ProVantage Gel, Inc. Dallas, TX, USA

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and *F. nucleatum* and the serial dilutions of the antibacterial agents. A control of 10 µL of bacteria with 190 µL of media and a sterility control of 200 µL of only media were included.

2.2 Minimal Inhibitory Concentration and Minimal Bactericidal Concentration

Absorbance of the broth culture was measured after 24-hour incubation using a spectrophotometer[‡‡] at 595 nm to assess the MIC of each agent. Ten µL of the remaining culture supernatant was taken from each sample and pipetted onto blood agar plates for a 24-hour incubation to assess the MBC. Additionally, 50 µL of the supernatant was diluted with 2 µL of the BacLight Live/Dead dye[§§] in 8-well plates for confocal microscopy to assess the viability (live/dead) of bacteria using a confocal microscope at 20x magnification[‖].

2.3 Human Gingival Fibroblast Cell Culture

HGFs[¶¶] were seeded (25,000 cells/well) in 24-well plates with 1 ml Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). The plates were incubated for 24 hours to allow the cells to attach. The media were then removed, and the cells were exposed to 1 ml of serum-free DMEM with serial dilutions (1/10, 1/100, 1/1000 and 1/10,000) of the antibacterial agents for 72 hours based on a previously conducted pilot study.

2.4 Water-soluble Tetrazolium-1 (WST-1) Assay

WST-1 assay[***] was used to measure the mitochondrial dehydrogenase activity of the HGFs thus, reflecting the overall viability of the cells and their proliferation. After 72 hours, the media in the 24-well plates were removed and cell proliferation reagent WST-1 (100 µl

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[‡‡] Molecular Devices, LLC, San Jose, CA, USA
[§§] Live/ Dead™ BacLight™ Bacterial Viability Kit, Thermo Fisher Scientific, Life Technologies Corporation, Oregon, USA
[‖] Olympus FV1000 MPE, Olympus America Inc., Center Valley, PA, USA
[¶¶] ScienCell Research Laboratories, Carlsbad, San Diego, CA, USA
[***] Roche Applied Science, Indianapolis, IN, USA

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WST-1 and 900 µl serum-free DMEM) was added and the plate was incubated for 1 hour at 37°C and 5% CO_2. A 100 µl sample from each of the 24-well plates were transferred into a 96-well plate and the absorbance values of the sample were measured against the background control (media without cells) using a microplate reader[##] at 450 nm. The absorbance value of each sample was then compared with the negative control (untreated cells).

2.5 Lactate Dehydrogenase (LDH) Assay

LDH assay[***] was used to assess the cytotoxicity of the agents towards HGFs. HGFs were treated with serial dilutions of antibacterial agents as described above for WST-1 assay. The high control (total cell death-maximum LDH release) was generated by adding 5% lysis solution in serum-free DMEM to the control cells, while the low control (LDH activity from untreated cells) consisted of serum-free DMEM from the untreated control cells. Serum-free DMEM without HGFs was utilized as the background control (LDH activity in medium). After 72 hours of incubation, 100 µl media from each of the wells was transferred to a 96-well plate and 100 µl of the assay reagent mix as per the manufacturer, was added to each well. The plates were incubated for 15 minutes at room temperature. The absorbance was measured at 490 nm using a microplate reader[##]. The percentage release of LDH from the treated cells was calculated by comparing to the maximum release of LDH. To determine the cytotoxicity, the absorbance values of the background was subtracted from that of the experimental samples and the cytotoxicity was calculated.

2.6 Statistical Analysis

The experiments were repeated three times and the mean values were calculated. Two-way ANOVA was used to compare the effects of the antibacterial agents, nicotine exposure and

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their interaction. Based on prior studies, the coefficient of variation was estimated to be 0.5. With four wells of each dilution in each of three replicates of the study, the study had an 80% power to detect a ratio of means of 1.8 (80% increase in means) between any two antibacterial agents, in the presence or absence of nicotine with $p<0.0001$.

One-way ANOVA and the post-hoc Tukey's test was used to compare the difference between the groups for the HGF cell cultures. The level of significance was set at $p<0.05$. Analyses were performed using SAS version 9.4[***].

3. Results

3.1 Minimal Inhibitory Concentrations

The growth of *S. mutans* was significantly suppressed by addition of all the antibacterial agents at a 1/4 dilution when compared to the control ($p<0.0001$) (Figure. 1A). With the addition of nicotine, only PVD and VEGA were found to be significant. At the 1/64 dilution, only CHX was found to significantly suppress *S. mutans* growth ($p<0.0001$) (Figure 1B). An increase in *S. mutans* growth was observed with the addition of nicotine however, no significant differences were noted between the nicotine and no nicotine group for any of the antibacterial agents.

The growth of *S. sanguis* was significantly suppressed by the addition of all antibacterial agents at a 1/4 dilution when compared to the control ($p<0.0001$) (Figure. 1C). With the addition of nicotine, all agents except VEGA were found to be significant. As the dilution of the agents were increased only CHX up to the 1/64 dilution was found to significantly suppress *S. sanguis* growth ($p<0.0001$) (Figure 1D). An increase in *S. sanguis* growth was observed on addition of nicotine however, no significant differences were noted between the nicotine and no nicotine group for any of the antibacterial agents at same dilution.

[***] SAS Version 9.4, SAS Institute Inc., Cary, NC, USA

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The growth of *F. nucleatum* was significantly suppressed by all antibacterial agents at a 1/4 dilution when compared to the control ($p<0.0001$) except VEGA without nicotine ($p=0.05$) and AO without nicotine ($p= 0.01$) (Figure. 2A). At a 1/64 dilution, no significant differences were noted between any groups when compared to the control except AO which significantly suppressed the growth of *F. nucleatum* in the presence of nicotine ($p<0.0001$) (Figure. 2B). An increase in *F. nucleatum* growth was observed with addition of nicotine however, no significant differences were noted between the nicotine and no nicotine group for any of the antibacterial agents except PVD at 1/32 and 1/64 dilution ($p<0.0001$).

The growth of *P. gingivalis* was significantly suppressed by PVD without nicotine, AO, and VEGA at the 1/4 dilution when compared to the control ($p<0.0001$) (Figure. 2C). No significant differences were noted between any of the antibacterial agents at the 1/64 dilution and the control (Figure 2D). A decrease in *P. gingivalis* growth was observed with addition of nicotine, however no significant differences were noted between the nicotine and the no nicotine group for any of the antibacterial agents at the same dilution.

The MIC values for each bacterial species with and without the presence of nicotine have been summarized in Table 1.

The data for other dilutions of the four agents for each bacterial specie is included in supplementary files (See Tables S1-S4 in Online Journal of Periodontology)

3.2 Minimal Bactericidal Concentrations

Bacterial growth was seen on blood agar plates for VEGA and AO group for all bacterial species at all tested dilutions (data not included). CHX and PVD inhibited the bacterial growth for all bacterial species at all dilutions. The MBC values for each bacterial species with and without the presence of nicotine have been summarized in Table 1.

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3.3 Confocal Microscopy

CHX and PVD completely lysed all the bacterial species at the 1/4 dilution and intense red staining (indicating bacterial cell death) was observed at 1/64 dilution (Figure. 3). VEGA and AO demonstrated a mixture of red (dead bacterial cells) and green stained bacteria (live bacterial cells) at a 1/4 dilution for *S. mutans* (Figure. 3). However, a more intense red stain was observed for *S. sanguis* for both VEGA and AO at the 1/4 dilution (Figure. 3). Lysis of *F. nucleatum* was noted for the 1/4 dilution of AO, whereas VEGA demonstrated a partial lysis at the same dilution (Figure. 3). Less red staining was observed for both VEGA and AO at 1/4 dilutions for the *P. gingivalis* species (Figure. 3).

3.4 Water-soluble Tetrazolium-1 Assay for HGF Proliferation

Exposure to CHX at 1/10 and 1/100 dilutions negatively affected HGF proliferation when compared to the control ($p<0.05$). HGF proliferation was significantly decreased at 1/10, 1/100 and 1/1000 dilutions of PVD when compared to the control ($p<0.05$). (Table 2)

VEGA did not significantly ($p>0.05$) affect HGF proliferation at any tested dilution, when compared to the control. In contrast, AO negatively affected HGF proliferation when compared to the control at all tested dilutions ($p<0.05$). (Table 2)

3.5 Lactate Dehydrogenase Assay for HGF Cytotoxicity

Significant cytotoxicity towards HGFs was seen at all tested dilutions of CHX ($p<0.05$). PVD was also found to be significantly cytotoxic towards HGFs at 1/10 and 1/100 dilution ($p<0.05$). VEGA did not demonstrate any cytotoxicity towards HGFs at any tested dilution ($p>0.05$), whereas AO was found to be cytotoxic towards HGFs at all tested dilutions ($p<0.05$) when compared to the control. (Table 2)

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When these antibacterial agent-treated and control HGFs were observed under a 20x microscope, VEGA was found to resemble the control group most closely, with minimum negative effect on HGF proliferation. CHX and AO treatment led to lysis of HGFs at all tested dilutions, whereas PVD demonstrated lysis of HGFs up to the 1/100 dilution. (See Figure S1 in Online Journal of Periodontology)

4. Discussion

This study was undertaken to compare the antibacterial effects of 0.12% CHX, 10% PVD, VEGA and AO against *S. mutans*, *S. sanguis*, *F. nucleatum* and *P. gingivalis* with and without the presence of nicotine and to investigate their impact on the proliferation of and cytotoxicity towards HGFs. It was observed that although CHX and PVD significantly inhibited the growth of all bacterial species up to a 1/64 dilution (based on MBC), they had a significant negative impact on HGF proliferation and were cytotoxic towards HGFs at dilutions of 1/10, 1/100 and 1/1000. VEGA was found to be bacteriostatic at a 1/4 dilution when compared to the control and inferior to CHX and PVD. However, it was favorable towards HGF proliferation and was not cytotoxic at any tested dilution. AO was bacteriostatic at a dilution of 1/4, inferior to CHX and PVD and comparable to VEGA, however, it negatively affected HGF proliferation and was cytotoxic at all tested dilutions.

The superior antibacterial properties of CHX have been widely investigated in the past, thus rendering this agent as a gold standard for post-surgical use.[7] In a 6-week randomized control study, use of 0.12% CHX in patients after periodontal surgery exhibited improved wound healing and post-surgical patient management when compared to a placebo rinse.[18] Periodontal soft tissue healing along with patients' self-reported questionnaire on pain, taste and compliance were noted which demonstrated superior results for CHX over placebo rinse.[18] Similar results were found in our study where CHX inhibited the growth of all

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bacterial species up to the 1/64 dilution. The higher absorbance values noted for *F. nucleatum* and *P. gingivalis* with CHX (Fig. 2) could be due the protein precipitation properties of CHX that induced more turbidity in the culture media. However, results from blood agar plates and confocal microscopy clearly reflect the superior bactericidal property of CHX. It was also found that CHX reduced HGF proliferation and induced cytotoxic effects at all tested dilutions. In a previous study investigating the cellular effects of CHX, the authors concluded that the use of commercially available concentrations (0.12%) or diluted concentrations (as low as 0.00009%) of CHX to surgical sites for short periods of time prior to wound closure can conceivably have serious cytotoxic effects on HGFs and may negatively affect wound healing.[9]

PVD has a bactericidal effect similar to pure iodine, being highly effective against most bacteria including putative periodontal pathogens.[19] Similar results were obtained in the current study, with PVD significantly inhibiting all bacterial species up to the 1/64 dilution. The confocal images also illustrated, bacterial lysis thus, reflecting the bactericidal action of PVD. However, PVD was found to negatively affect HGF proliferation and demonstrated a high degree of cytotoxicity up to the 1/1000 dilution. Contradictory results have been found in a systematic review which compared PVD with silver sulfadiazine, rifamycin and other non-antiseptic dressings and the main parameters evaluated were wound healing, bacterial counts, and adverse effects. This review concluded that PVD does not impair wound healing.[20] However, this was a review article which included many clinical trials and evaluation of wound healing was based on duration of closure, epithelialization which could be subjective.[20] Another study concluded that even dilute concentrations of 10% PVP-iodine was toxic to human skin fibroblasts where their growth was retarded at 0.01%, 0.025% and totally inhibited by 0.1% and 1% of PVD, similar to the current study.[11]

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The present study is the first report to evaluate the antibacterial efficacy of the AO gel. The antibacterial efficacy of AO was comparable to CHX and PVD at 1/4 dilution, however, bacterial growth was observed on blood agar plates along with the confocal images depicting both dead and live bacteria. This establishes that AO is bacteriostatic at lower dilutions but has a minimal bactericidal effect. Also, it was observed that AO significantly affected HGF proliferation and demonstrated high cytotoxicity towards HGFs at all tested dilutions. This is in contrast to a previous study by Miguel that demonstrated that combinations of pure bioactive mixtures of antioxidants (resveratrol, ferulic acid, phloretin and tetrahydro curcuminoids) were not toxic to HGF and periodontal ligament cells.[16] However, the use of various mixtures, could have impacted these results and different assays were used for analysis.

VEGA was recognized recently as an effective antimicrobial agent with accelerated healing properties.[12] The sixteen key ingredients that are a part of the Homeopathic Pharmacopeia of United States (HPUC) are used to relieve pain, have anti-inflammatory properties and currently are in use for a treatment of various illnesses. They prevent bruising, swelling and demonstrate antiseptic and antioxidant properties.[21-24] Lee and Suzuki evaluated the efficacy of VEGA as a pre-emptive analgesic following mandibular block bone graft surgery and found that post-operative pain intensity was significantly reduced in the patients who had received the VEGA recovery kit when compared to the patients that only received opioid analgesics.[12] Literature regarding antibacterial efficacy of VEGA is sparse, however some case reports published with the company have found reduced bacterial loads and faster wound healing. Our study was the first to evaluate the antibacterial efficacy of VEGA. It was seen that at the 1/4 dilution, VEGA is bacteriostatic, however, it does not exert a bactericidal effect. An interesting finding reported by our study was that VEGA did not negatively affect HGF proliferation and was not cytotoxic at any tested dilution. These results are similar to a

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recent study that compared CHX and VEGA for their effect on HGF viability, proliferation, survival, and expression of growth factors.[13] It was concluded that VEGA was superior to CHX in terms of cellular biocompatibility, HGF migration and proliferation, gene expression and collagen synthesis.[13] Thus, VEGA may be a useful alternative to the other antibacterial agents, reducing the incidence of post-surgical infections and post-operative pain while promoting wound healing.

An increased bacterial growth was seen for all species except *P. gingivalis* with the addition of nicotine which is in accordance with previous studies.[25] A scanning electron microscopy study revealed that biofilm formation and metabolic activity of *S. mutans* increases in a nicotine-dependent manner up to 16.0 mg/ml of nicotine with a thicker biofilm and more spherical bacterial cells and the lowest dose which had significant results was 2 mg/ml which is the nicotine dose used in the present study.[26] A previous unpublished study conducted at Indiana University has also demonstrated that nicotine enhances the binding of periodontal pathogens to *F. nucleatum,* thus establishing that nicotine has an important role in the progression and resolution of subgingival infections. Due to a significant antibacterial activity of all agents at a 1/4 dilution with nicotine, it can be inferred that these antibacterial agents might be potentially advantageous for periodontal wound healing under a nicotine environment.

S. mutans, *S. sanguis*, *F. nucleatum* and *P. gingivalis* were selected as the primary bacterial species for our study because, the initial subgingival biofilm formation begins with the adhesion of early colonizers such as *Streptococci* species followed by colonization of the later inhabitants comprised of *F. nucleatum*, *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola* that are more pathogenic and are associated with periodontal destruction and post-surgical infections. [27-29]

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Some of the limitations of the present study could be the discrepancy in the consistencies of the antibacterial agents used. While CHX and PVD were used as mouth rinses, AO and VEGA were used as gel formulations and thus, the results may be interpreted with caution. The exact concentrations of the active agents in AO and VEGA are unknown and were used as packaged by the manufacturers, however, the protocol for preparation of serial dilutions were standardized for all agents. Effects of the agents on a biofilm need to be analyzed and undertaken in future studies. Although the in-vitro design was conceptualized to mimic an in-vivo environment, further studies are needed to evaluate the effects of these antibacterial agents in a clinical setting.

5. Conclusion

Within the limitations of the study, and from the data obtained it can be concluded that CHX and PVD demonstrate superior antibacterial properties, but significantly reduce HGF proliferation. AO is bacteriostatic at lower dilutions but is highly toxic to HGFs. VEGA is bacteriostatic at lower dilutions and demonstrates no detrimental effects on HGF proliferation or cytotoxicity. This favorable property of VEGA towards fibroblasts may enhance healing outcomes especially for surgical procedures prior to complete wound closure. The results of this study may be extrapolated for developing a post-surgical protocol for periodontal or peri-implant mucogingival and regenerative procedures. Future clinical studies to investigate the effects of these agents and patient reported outcomes need to be undertaken.

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Conflict of Interest

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Figure legends:

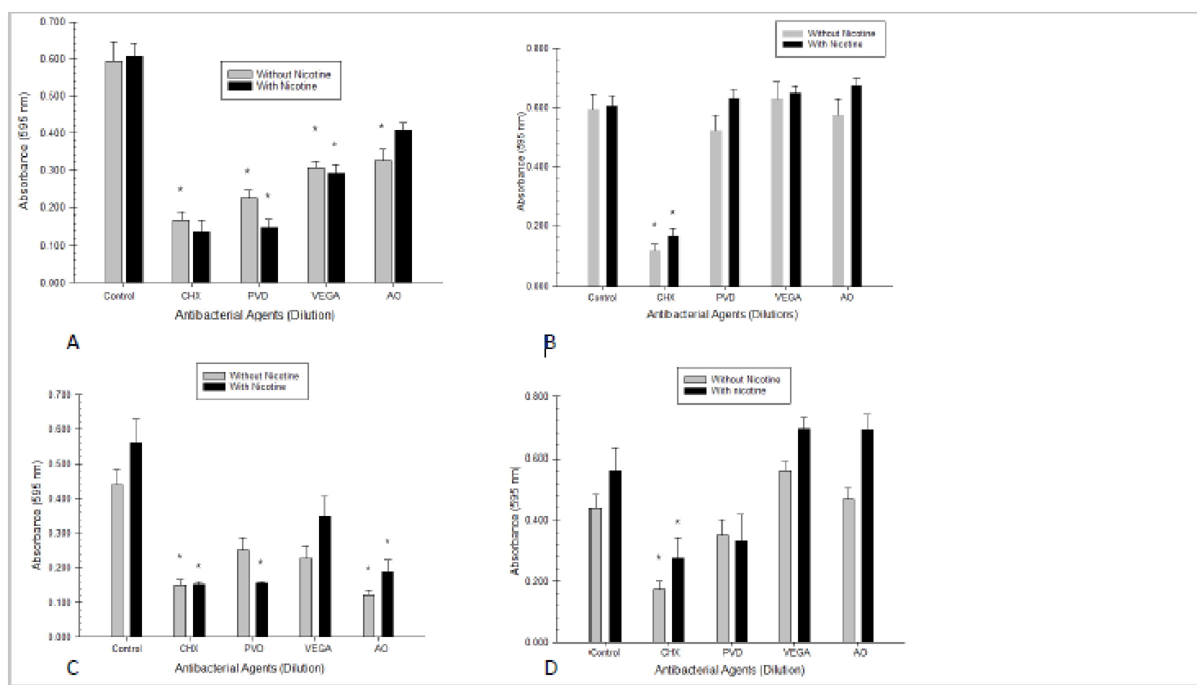

Figure 1 A-B: Effect of antibacterial agents on *S. mutans* growth at 1/4 and 1/64 dilutions. All agents, significantly inhibited *S. mutans* growth at the 1/4 dilution ($p<0.0001$). CHX demonstrated lower absorbance values, illustrating significant *S. mutans* inhibition up to the 1/64 dilution ($p<0.0001$). No significant differences were noted with the addition of nicotine.

Figure 1 C-D: Effect of antibacterial agents on *S. sanguis* growth at 1/4 and 1/64 dilutions. All agents significantly inhibited *S. sanguis* growth ($p<0.0001$). CHX demonstrated lower absorbance values, illustrating significant *S. sanguis* inhibition up to the 1/64 dilution ($p<0.0001$). No significant differences were noted with the addition of nicotine.

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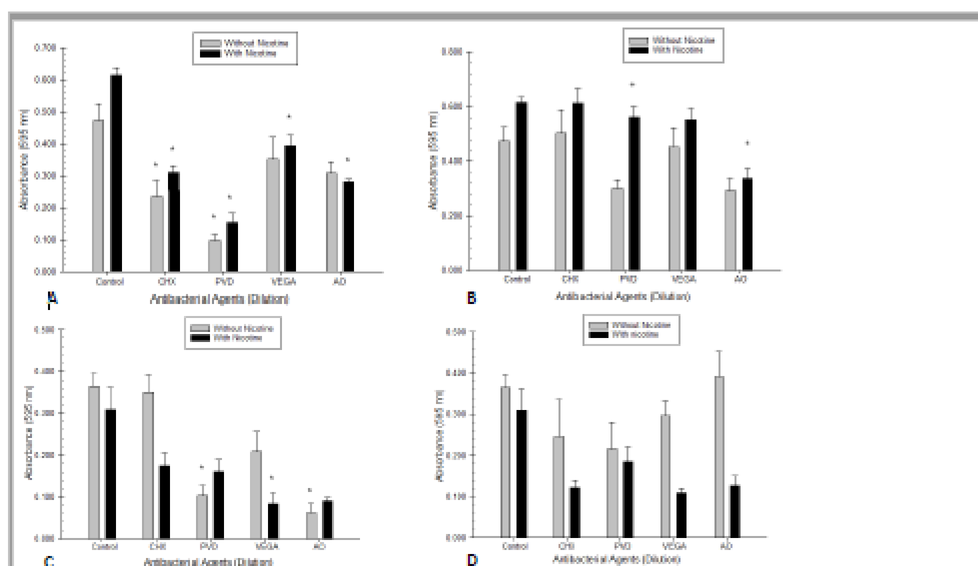

Figure 2 A-B: Effect of antibacterial agents on *F. nucleatum* growth at 1/4 and 1/64 dilutions. All agents significantly inhibited *F. nucleatum* growth at lower dilutions ($p<0.0001$) except AO and VEGA without the addition of nicotine.

Figure 2 C-D: Effect of antibacterial agents on *P. gingivalis* growth at 1/4 and 1/64 dilutions. PVD and AO without addition of nicotine and VEGA with addition of nicotine demonstrated significant *P. gingivalis* growth at lower dilutions ($p<0.0001$). No significant differences were noted for any agent at higher dilutions when compared to the control.

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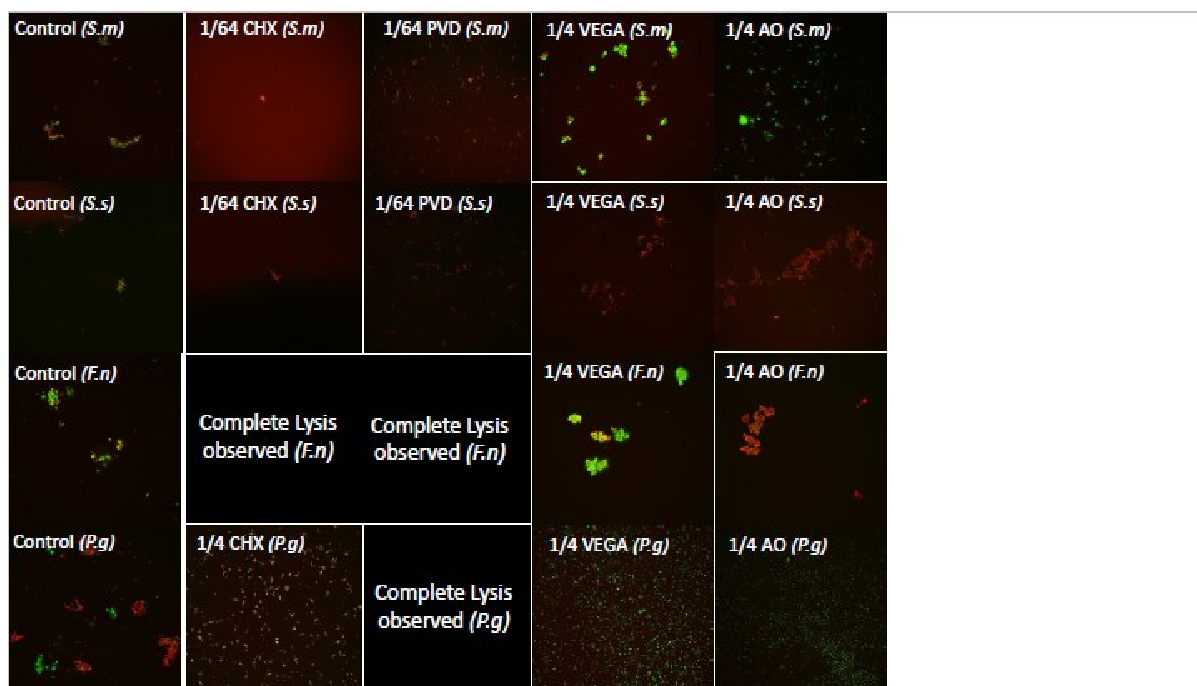

Figure 3: Confocal images demonstrating bacterial species vitality on exposure to the antibacterial agents. Live/dead staining was done with viable cells appearing green and red cells representing non-viable cells. Complete lysis made it difficult to obtain images for CHX at 1/4 and 1/64 dilution for *F. nucleatum* and 1/4 and 1/64 dilution of PVD for *F. nucleatum* and *P. gingivalis.*

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Table 1: MIC and MBC for *S. mutans*, *S. sanguis*, *F. nucleatum*, *P. gingivalis* with and without nicotine for all antibacterial agents.

		With Nicotine		Without Nicotine	
Bacterial Specie	**Antibacterial Agent**	**MIC**	**MBC**	**MIC**	**MBC**
S. mutans	CHX	1/64	1/64	1/64	1/64
	PVD	1/16	1/64	1/16	1/64
	VEGA	1/4	<1/4	1/4	<1/4
	AO	1/4	<1/4	1/4	<1/4
S. sanguis	CHX	1/64	1/64	1/64	1/64
	PVD	1/32	1/64	1/16	1/64
	VEGA	1/4	<1/4	1/4	<1/4
	AO	1/4	<1/4	1/4	<1/4
F. nucleatum	CHX	1/32	1/64	1/32	1/64
	PVD	1/4	1/64	1/4	1/64
	VEGA	1/4	<1/4	<1/4	<1/4
	AO	1/4	<1/4	<1/4	<1/4
P. gingivalis	CHX	1/32	1/64	1/64	1/64
	PVD	1/32	1/64	1/64	1/64
	VEGA	1/4	<1/4	1/4	<1/4
	AO	1/4	<1/4	1/4	<1/4

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Table 2: Effect of antibacterial agents on HGFs proliferation as measured by WST-1 Assay and cytotoxicity towards HGFs as measured by LDH Assay. CHX and PVD significantly affected HGF proliferation at 1/10 and 1/100 dilutions ($p<0.05$). AO affected HGF proliferation at all dilutions ($p<0.05$). VEGA did not affect HGF proliferation at any dilution ($p>0.05$). CHX and AO were cytotoxic at all dilutions while PVD was cytotoxic at 1/10 and 1/100 dilution ($p<0.05$). VEGA did not exhibit cytotoxicity at any dilution ($p>0.05$).

Agent	Dilution	HGF Proliferation (WST-1 Assay)			Cytotoxicity towards HGF (LDH Assay)		
		Mean ± S.D	Standard Error	Significance	Mean ± S.D	Standard Error	Significance
Control		100	.00		0	.00	
CHX	1/10	-1.104 ± 0.97	0.561	.000*	111.07 ± 7.29	4.210	.000*
	1/100	1.48 ± 0.56	0.325	.000*	105.56 ± 8.29	4.787	.000*
	1/1000	79.04 ± 16.3	9.442	.092	19.71 ± 1.37	0.791	.009*

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	1/10,000	85.35 ± 11.1	6.435	.322	19.35 ± 5.31	3.069	.011*
PVD	1/10	1.880 ± 0.62	0.360	.000*	-24.74 ± 8.02	4.63	.018*
	1/100	2.94 ± 0.87	0.507	.000	37.16 ± 14.9	8.65	.001
	1/1000	74.85 ± 10.2	5.920	.001	6.63 ± 1.11	0.642	.824
	1/10,000	111.51 ± 3.84	2.221	.096	2.80 ± 2.38	1.377	.990
VEGA	1/10	93.98 ± 4.51	2.607	.990	5.77 ± 7.88	4.551	.850
	1/100	99.25 ± 13.1	7.615	1.000	0.46 ± 7.40	4.273	1.000
	1/1000	97.83 ± 31.7	18.306	1.000	-1.50 ± 6.98	4.035	.999*
	1/10,000	103.26 ± 10.9	6.312	.999	-0.71 ± 9.18	5.301	1.000

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AO	1/10	12.04 ± 0.24	0.143	.000	51.77 ± 6.88	3.976	.000
	1/00	3.59 ± 0.51	0.295	.000	51.35 ± 7.79	4.499	.000
	1/1000	48.42 ± 2.70	1.561	.000*	46.66 ± 5.92	3.420	.000*
	1/10,000	55.57 ± 10.5	6.083	.000*	25.97 ± 8.08	4.665	.004*

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Supplementary Table 1: Antibacterial efficacy of antibacterial agents towards *S. mutans* at all dilutions (1/4-1/64) with and without nicotine.

Supplementary Table 2: Antibacterial efficacy of antibacterial agents towards *S. sanguis* at all dilutions (1/4-1/64) with and without nicotine.

Supplementary Table 3: Antibacterial efficacy of antibacterial agents towards *F. nucleatum* at all dilutions (1/4-1/64) with and without nicotine.

Supplementary Table 4: Antibacterial efficacy of antibacterial agents towards *P. gingivalis* at all dilutions (1/4-1/64) with and without nicotine.

Supplementary Figure 1: Control and antibacterial agent treated HGFs under a 20x confocal microscope demonstrating no cellular lysis with 1/10 and 1/10,000 VEGA. CHX, PVD, AO demonstrate lysis of HGFs at all tested dilutions.

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