Phase I Clinical Trial of a Chlorhexidine Diacetate Intraoral Delivery System in Medically Healthy Gingivitis Subjects

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ABSTRACT

Steven M. Van Scoyoc: Phase I Clinical Trial of a Chlorhexidine Diacetate Intraoral Delivery System in Medically Healthy Gingivitis Subjects.

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Chlorhexidine diacetate (CDA) was incorporated into an ethylene vinyl acetate (EVA) mouthguard. This single center, 5-week, open-label trial, evaluated six medically healthy subjects with moderate gingivitis. Subjects wore the mouthguard 12 hrs/day for 21 days.

Adverse events were recorded and the pharmacokinetic profiles of CDA in serum and saliva were evaluated. Plaque samples were evaluated for changes in microbial susceptibility to CDA, bacterial counts characterized under aerobic and anaerobic conditions, and specific subgingival microorganisms.

Results show limited adverse events, minimal systemic exposure to CDA, prolonged intraoral delivery, and no evidence of microbial CDA resistance. A reduction in the total bacterial counts of aerobic and anaerobic microbes, and an improvement in clinical signs of periodontal inflammation occurred.

Data suggest that the CDA-EVA mouthguard is safe for human use with minimal systemic exposure. Additionally, CDA treatment does not alter the CDA-susceptibility of the oral flora and may reduce total bacterial counts.

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LIST OF ABBREVIATIONS & SYMBOLS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
AE(s)	Adverse event(s)
AIDS	Acquired Immunodeficiency Syndrome
ANCOVA	Analysis of Co-Variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BBE	Bacteroides bile esculin
BEA	Bile esculin azide
B. fragilis	*Bacteroides fragilis*
BOP	Bleeding on probing
ºC	Centigrade
CBA	Columbia blood agar
CD4+	Cluster of differentiation 4
CDA	Candida diagnostic agar

CFU	Colony forming units
C. gracilis	*Campylobacter gracilis*
CHX	Chlorhexidine
Cmax	Maximum concentration
C. rectus	*Campylobacter rectus*
CSSI	Calculus surface severity index
CVE	Crystal violet-erythromycin
D	Day
DI	Discoloration index
E. corrodens	*Eikenella corrodens*
EVA	Ethylene vinyl acetate
FDA	Food and Drug Administration
F. nucleatum	*Fusobacterium nucleatum*
HPLC	High performance liquid chromatography
HIV	Human immunodeficiency virus
hr(s)	hour(s)

ID	Identification
IND	Investigational new drug
IRB	Institutional Review Board
IS	International standard
l	Liter
LKV	Laked blood with kanamycin and vancomycin
M	Molar
mEq	Milliequivalent
mg	Milligram
µg	Microgram
µm	Micrometer
MIC	Minimum inhibitory concentration
ml	Milliliter
mm	Millimeter
MTBE	Tert-butyl methyl ether
nm	Nanometer

NUP	Necrotizing ulcerative periodontitis
p	p-value
P. anaerobius	*Peptostreptococcus anaerobius*
P. aeruginosa	*Pseudomonas aeruginosa*
PD	Pocket depth
P. micros	*Peptostreptococcus micros*
PEA	Phenylethyl alcohol agar
P. gingivalis	*Porphyromonas gingivalis*
PHN	Phenacetin
PI	Plaque index
P. intermedia	*Prevotella intermedia*
QC	Quality control
RDW	Red blood cell distribution width
RPM	Rotations per minute
S. aureus	*Staphylococcus aureus*
SD	Standard deviation

SE	Standard error
sp.	species
T. forsythia	*Tanerella forsythia*
Tmax	Time to maximum concentration
TSA	Trypticase soy agar
TSA-NAM	Trypticase soy agar with sheep blood, heme and N-acetyl muramic acid
TSBV	TSA serum bacitracin vancomycin
UV	Ultraviolet
WC	Wilkins Chalgren anaerobic broth

Pharmacokinetic, Safety and Clinical Assessment of a Chlorhexidine Diacetate Intraoral Delivery System

Abstract

Our research team has incorporated 2.5% (by weight) chlorhexidine diacetate (CDA) into a novel intraoral delivery system. *In vitro* testing with the ethylene vinyl acetate (EVA) delivery system indicates sustained concentrations of chlorhexidine for up to 14 days. The proposed eventual use of this delivery system will include treatment of HIV-associated periodontitis patients. **Objectives**: 1) to evaluate the safety of a 2.5% CDA-loaded delivery system in medically healthy subjects with generalized moderate plaque-induced gingivitis, and 2) to document the pharmacokinetic profiles of CDA in serum following intraoral placement of the delivery system. **Methods**: This single center, 5-week, open-label trial, evaluated 6 medically healthy subjects with generalized moderate plaque-induced gingivitis. Subjects wore the EVA delivery system 12 hrs/day for 21 days in the maxillary arch. Examination of oral soft tissues and pharmacokinetic monitoring occurred at Baseline, Days 1, 2, 3, 7, 14, 21 and 35. Blood chemistries, hematology and urinalysis were performed at screening and Day 21. All adverse events (AEs) were documented. Serum and saliva samples were analyzed using high performance liquid chromatography (HPLC). Periodontal probing, gingival index, plaque score, dental calculus and discoloration index assessments occurred at Baseline, Day 21 and Day 35. **Results**: All patients tolerated the delivery system, and 18 non-severe adverse events were recorded. Calculus and discoloration indices were not increased with the use of the delivery system. No significant aberrations in blood chemistries,

hematology or urinalysis occurred during the course of the study. Ninety-four percent of serum samples had levels of CDA below the detectable limit of 0.010 μg/ml, and all samples were ≤ 0.012μg/ml. Over the 21-day course of treatment, salivary CDA levels increased two hours post-dosing at all time points. Most strikingly, analysis of covariance revealed a significant decrease in mean pocket depth in the maxillary arch over 21 days ($p<0.05$) when controlling for changes in the mandibular arch. **Conclusions:** The data suggest that the drug delivery system is safe for human use with minimal systemic exposure, and CDA is released from the EVA polymer over 21 days of dosing. Trends in the data also suggest that local chlorhexidine diacetate may reduce the clinical signs of inflammation associated with periodontal disease. [Supported by DE15267-01, RR00046]

Introduction

Chlorhexidine (CHX) is a broad spectrum antimicrobial, which has activity against both Gram-negative and Gram-positive bacteria, yeast, fungi, and viruses.[1-4] The oral rinse, chlorhexidine digluconate (0.2%) has been shown to be effective in reducing clinical signs of gingival inflammation[5]. However, CHX's ability to maintain substantivity within saliva is reported to be limited to 5 hours following rinse use.[6] Delivery of CHX at therapeutic concentrations at a constant rate over an extended period of time is desirable for the treatment of chronic periodontal conditions. Our group has developed a biocompatible copolymer (ethylene vinyl acetate or EVA) to release chlorhexidine diacetate (CDA) at a near constant rate over several weeks *in vitro*.[7-9] The use of this CDA-containing polymer was submitted to the US Food and Drug Administration (FDA) as an Investigational New Drug (IND #73,126).

Individuals who are immunocompromised are at risk for oral opportunistic infections. These conditions can lead to significant morbidity for patients. In particular, patients with deficient immune responses are susceptible to destructive and necrotizing forms of periodontal diseases.[10, 11] Our long term goal for the CDA-EVA mouthguard is the development of a sustained release intraoral delivery system for the treatment of human immunodeficiency virus (HIV)-associated oral infections.

The aims of this phase I, single center, 35-day, open-label, clinical trial were: 1) to evaluate the safety of a 2.5% CDA-loaded delivery system in medically healthy subjects with generalized moderate plaque-induced gingivitis, and 2) to document the pharmacokinetic profiles of CDA in serum and saliva following intraoral placement of the delivery system.

Materials and Methods

Study Design

Following protocol approval by the University of North Carolina Biomedical Institutional Review Board (IRB) at the University of North Carolina at Chapel Hill, six medically healthy adult subjects were recruited to participate. This population size was derived as a convenience sample based on logistical and not power considerations.

For inclusion in this study, subjects had to be 18 years of age or older, have a functional complement of ≥ 20 teeth with four teeth having probing depths of 4-5 mm and a minimum percent bleeding on probing score of at least 30%. Eligible subjects had to be able and willing to perform study procedures, and they had to provide written informed consent. Participants denied any periodontal or endodontic therapy other than prophylaxis within six months prior to enrollment. They also denied any history of a heart murmur, valvular disease, prosthetic joint replacement, chronic or active infectious disease and use of any medication

known to affect inflammation such as aspirin, nonsteroidal anti-inflammatory drugs, steroids, statins, phenytoin, calcium antagonists, cyclosporin and coumadin within one month of the screening exam. Subjects reported not using antibiotics or chlorhexidine mouthrinse within three months of screening. Subjects were excluded if they exhibited gross oral pathology, severe unrestored dental caries or clinically significant laboratory abnormalities. Subjects of both sexes were included; however, female subjects were excluded if they were pregnant, lactating or not using adequate contraceptive methods.

The study design for this clinical trial included screening, treatment and post-treatment phases (Figure 1). During the screening phase subjects received an intraoral examination. Within 14 days, subjects were recalled for laboratory testing including blood chemistries, complete blood counts, urinalysis, and urine pregnancy testing for females. Additionally, maxillary impressions were taken to fabricate the study mouthguards.

Experimental Treatment

Mouthguards containing 2.5% CDA in an EVA copolymer were custom fabricated for each subject for use over the 21-day treatment period. Accordingly, EVA sheets containing 2.5% CDA were vacuum-formed to fit the subject's maxillary arch. Individual mouthguards were trimmed and smoothed to the dentition's height of contour for a firm fit. Prior to dispensing the study mouthguard at visit 2, each mouthguard was weighed. Experimental mouthguards were dispensed in a dental appliance box labeled with the investigator's name and address, patient ID number, IND statement and instructions for use and storage. These instructions included rinsing the mouthguard with water before placement and after removal, wearing the mouthguard 12 hours per day, avoiding prolonged washing of mouthguard, not wearing the mouthguard while eating, and leaving it out for 30 minutes after

brushing teeth. No mechanical intervention or oral hygiene instructions were given to subjects. Study mouthguards were stored at room temperature (20-25°C) at the test center or the subject's home.

Safety and Clinical Evaluation

On Day 0 (visit 2), subjects entered the testing facility for a one-night inpatient stay. Five milliliters of whole blood and 2ml of unstimulated whole saliva were collected within 15 minutes prior to insertion of the delivery system. Blood draws and saliva collection occurred at 15 and 30 minutes post-dosing and at 1, 2, 3, 6, 9, 12, and 18 hours post-dosing. At hour 12, subjects removed the CDA-EVA delivery system. On Day 1 (visit 2 continued), blood and saliva samples were collected at 24 hours (following initial dosing on Day 0). The delivery system was reinserted and blood and saliva samples were collected two hours post-dosing on Day 1. The delivery system was worn by the subjects for 12 hours per day for a total of 21 days. Subject compliance and adverse events (AEs) were assessed via the review of subject diaries.

Subjects were evaluated clinically for safety and clinical outcomes on days 2, 3, 7, 14, and 21 of the treatment period. Safety assessments included measurement of vital signs and examination of the subjects' extra- and intraoral tissues for signs of pathology. Assessment of tooth discoloration (DI)[12] and calculus surface severity index (CSSI)[13]occurred at Baseline, Day 7 and Day 21. Any abnormal tissue findings were described with respect to onset, location, size (severity) and diagnosis. In addition, data were collected regarding adverse events. Clinical efficacy parameters included probing depth (PD), clinical attachment level (CAL), plaque index (PI),[14] gingival index (GI),[1515] and percent of sites bleeding to probing

(BOP) were recorded at screening and on Day 21 using a manual University of North Carolina (UNC-15) periodontal probe (Hu-Friedy Inc., Chicago, Illinois, USA).

Two clinical examiners (HH and SV) were calibrated on all study parameters prior to study procedures. Reliability was confirmed with a greater than 90% intra-examiner agreement and greater than 85% inter-examiner agreement on clinical parameters (e.g., PD agreement within 1mm). One of the two examiners performed all clinical assessments for each subject throughout the treatment period

Following the Day 21 visit, subjects entered a post-treatment period lasting two-weeks. On Day 21, subjects discontinued use of the mouthguard and returned it to the examiners. Subjects were recalled on Day 35 for a final study visit (post-treatment visit) for safety assessments including an oral examination, saliva and serum collection and laboratory re-testing.

Pharmacokinetic Evaluations

Five milliliters (± 2ml) of whole blood were collected for each pharmacokinetic sample. Blood samples were allowed to coagulate for 30 minutes and then centrifuged at 4,400 RPM for 20 minutes and stored immediately stored at -20°C until analysis. Whole, unstimulated saliva was collected via expectoration until approximately 2ml was obtained. Saliva samples were frozen -20°C until analysis. CDA drug concentrations in blood serum and saliva were measured using a novel developed and validated high performance liquid chromatography (HPLC)/Ultraviolet (UV) method. Briefly, extraction of the analyte from a serum and saliva matrix was performed by liquid-liquid extraction using tert-butyl methyl ether (MTBE). Accurately, 200ml of standards, quality controls (QCs), blanks, and unknowns were placed into a 2.0ml conical (eppendorf) tube. Next, 50µl of a 2µg/ml of the

internal standard (IS) phenacetin (PHN), and 50ml of 1.5M sodium hydroxide were added. Then 1.5ml of organic extraction solution MTBE was added to the tube. All tubes were vortex-mixed for 25 minutes followed by centrifugation for 3 minutes at 12,000 RPM. The extract was quickly frozen in an acetone dry ice bath. This acetone dry ice bath was prepared to hold 24 samples. The supernatant was poured into a clean 1.5ml conical tube and evaporated to dryness in a water bath (45°C) under a low stream of N_2 gas for approximately 15 minutes. Dried samples were reconstituted in 50µl of mobile phase (1:1 mixture of 25 M sodium phosphate pH6.0: mobile phase B). The reconstituted solution tubes were vortex-mixed for one minute and centrifuged at approximately 12,000 RPM for 5 minutes. Afterwards, the samples were transferred into HPLC 2-ml vials inside a polypropylene insert and placed in the instrument autosampler. Forty µl of the sample were injected. Sample analysis was conducted by reverse phase chromatography. The analyte and IS were eluted by a gradient mode of mobile phase from a RESTEK Ultra Aqueous C-18 (3.2 X 100 mm, 3.0µm particle size) analytical column and RESTEK Ultra Aquous C18 (2.1 X 10mm, 3.0µm) guard column. The two mobile phases were: *Mobile Phase A* (25mM sodium phosphate pH 3.0) and *Mobile Phase B* (500ml acetonitrile, 500ml methanol, 500µl heptafluorobutyric acid and 250µl triethylamine). The analyte peak was detected by an Agilent UV detector at 259nm. Concentration data in µg/ml were collected using Agilent Chemstation software.

Statistical Analysis

Baseline and demographic data were summarized with descriptive statistics (mean, median, standard deviation, range) for continuous data and with frequency tables for discrete data. Changes in clinical indices and periodontal probing parameters (from screening to Day 21)

were summarized with descriptive statistics. T-tests and an analysis of co-variance (ANCOVA). Adverse event data was summarized by frequency, severity and possible relationship to the study medication. Adverse events and other safety parameters were presented using the "intent-to-treat principle." The concentration of CDA in serum and saliva for each time point was expressed as the mean, standard deviation and range. Standard pharmacokinetic parameters were estimated for saliva analyses. These parameters included area under the curve (AUC), maximum concentration (C_{max}) and time to maximum concentration (T_{max}), which was summarized with descriptive statistics.

Results

Study Subjects

Of the seven subjects screened, all met the inclusion criteria, and six subjects were enrolled. All six subjects completed the study and were included in safety, clinical and pharmacokinetic analyses. The subjects had a mean age of 41±14 years (range, 27-58). Five (83.3%) were female, five (83.3%) were Caucasian, and one (16.6%) was African American. All subjects were non-smokers. The mean number of teeth per subject was 27.2 ±1.

Safety results

Safety tests performed at screening and Day 21 yielded blood chemistry (12 tests), hematology (10 tests) and urinalysis (17 tests). Out of the 468 tests, four were outside the normal range. One subject had a low post-treatment urine specific gravity (1.000, normal range: 1.003-1.030), one subject had high red blood cell distribution width (RDW) both pre- and post-treatment (16.8% and 16.5%, respectively; normal range: 12.0-15.0%), and one

subject had a high pre-treatment chloride level (110mEq/l, normal range: 98-107). All laboratory test aberrations were determined to be not clinically significant.

All patients tolerated the CDA delivery system, and no severe AEs were reported. However, 18 non-severe AEs were reported over the course of the trial. All AEs were categorized as Grade I according to the CTCAE v3.0 scale.[16] The most frequently reported AEs were oral lesions. There were nine lesions in three subjects. Six of the lesions were categorized as "unlikely related" or "not related" due to patients reporting a history of recent non-specific oral trauma. Three lesions were categorized as "likely related" to the study mouthguard, and all three ulcerations were reported in the same subject over a 1 month time period. No treatment was required for any of the oral lesions, and all lesions resolved upon completion of the study. The second most reported AE was headache (7 reports in 3 subjects). All AEs were graded as either "not related" or "unlikely related" to the study mouthguard The remaining two AEs were hematomas attributed to venipuncture, and no treatment was required.

Initial CSSI and DI were low, and no significant changes in CSSI or DI occurred throughout the study (Table 1). Indeed, these indices decreased on average from Baseline to Day 21.

All enrolled subjects completed the study and reported compliance with wearing the mouthguard for 12 hours/day throughout the duration of the study.

Clinical indices

At baseline, the mean pocket depth was 2.46±0.06mm, the mean CAL was 2.19±0.22mm, the mean BOP was 48.45±10.49%, the mean GI was 1.00±0.42 and the mean PI was 0.45± 0.17 (Table 1).

At visit 7 (Day 21), the mean pocket depth was 2.26±0.08mm, the mean CAL was 1.98±0.23mm, the mean BOP was 30.76±9.22%, the mean GI was 0.54±0.18, and the mean PI was 0.38±0.17. Changes in PD, GI and %BOP (Baseline to Day 21) were statistically significant ($p<0.05$) (Table 2). Analysis of covariance revealed a significant decrease [Baseline: 2.38mm (SE=0.04), Day 21: 2.21mm (SE=0.04)] in mean pocket depth in the maxillary arch over 21 days ($p<0.01$) when controlling for changes in the mandibular arch (Table 3). Mean PI, CAL and BOP decreased while GI increased, although the changes were not statistically significant (Table 3).

Pharmacokinetic Results

The CDA concentrations in saliva over the first 24 hours for each subject are shown in Figure 2. The mean AUC for all patients (n=6) was 42.87µg·hr/ml ±42.68 (9.98-128.15µg·hr/ml) (Figure 3). The concentration peaked (C_{max}=5.69µg/ml±4.37, 1.70-13.85µg/ml) at 2.54 hours±4.64 (0.25-12 hours) post mouthguard insertion. The concentration of CDA then decreased over 24 hours reaching 0.11±0.17µg/ml at 24 hours after the insertion of the mouthguard. All subjects showed a progressive decrease of CDA concentration following the removal of the mouthguard at 12 hours. Mean CDA levels both pre and post-dosing on days 1, 2, 3, 7, 14 and 21 are shown in Figure 4. At 2 hours post-dosing, the mean concentration of CDA in the saliva increased at all timepoints. The change was statistically significant ($p<0.05$) on days 1, 3 and 21. Ninety-four percent (94%) of serum samples had CDA levels below the detectable limit of 0.010 µg/ml and all samples were ≤ 0.012µg/ml.

Discussion

Immunocompromised patients are at risk for oral opportunistic infections. There is significant morbidity for patients with deficient immune responses who have destructive or necrotic forms of periodontal disease. Patients with HIV and deficient T helper (CD4+) lymphocytes are at risk for a destructive periodontal condition called "necrotizing ulcerative periodontitis" (NUP).[11] The condition is marked by gingival papillary necrosis, gingival bleeding, fetid oral malodor, clinical attachment loss and alveolar bone resorption. Although a minority of HIV-infected patients present with NUP (approximately 5%), the condition is a source of morbidity (oral pain) for these individuals.[17] In addition, the occurrence of NUP is predictive of a decrease in CD4+ lymphocyte counts below 200 in patients and an increased tendency to progress to AIDS[18, 19] Current treatment protocols for NUP include thorough debridement (scaling and root planing) of periodontal tissues and adjunctive systemic (peroral) antibiotics.[20] Selective antibiotics such as metronidazole are most often used to limit the effects on the commensal flora of the gastrointestinal and other body systems; however, all peroral antibiotics pose a risk for overgrowth of opportunistic pathogens like *Candida* species. For these reasons, there is a great public health need to develop and test new, safe and efficacious interventions for NUP and other oral conditions in HIV-infected patients. The long term goal for our investigative group is the development of a sustained release intraoral delivery system for the treatment of HIV-associated oral infections. For this study, medically healthy gingivitis subjects were selected in order to evaluate the safety of the CDA-EVA intraoral delivery system. The use of healthy subjects or volunteers is standard practice for phase I trials on safety of novel drugs prior to evaluation in study

subjects with systemic disease or more advanced oral disease who my experience a higher risk for toxicity and /or adverse events.

The results of this study substantiate the claim for *in vivo* sustained long-term CDA release from the EVA mouthguard[8] while demonstrating safety of this drug delivery system in medically healthy gingivitis subjects. The only reported AE in the oral environment was ulceration or tissue sloughing. It is unlikely that the CDA was responsible for ulcerations or desquamation as CHX gluconate mouthrinses have shown no effect on the keratinized oral epithelia following two years of daily or bi-daily rinses with 0.2% chlorhexidine gluconate.[21] In the present study, the three ulcerations that were attributed to the delivery system occurred in one subject. These ulcerations were likely a result of mechanical irritation from the mouthguard. The most commonly reported side effects associated with CHX digluconate oral rinses are: (1) an increase in staining of teeth and other oral surfaces, (2) an increase in calculus formation, and (3) an alteration in taste perception.[22] Our results show no significant increase in calculus formation or discoloration with the use of the CDA-EVA system. Although taste perception was not measured directly, no self reports of taste alteration were noted. The lack of side effects from the CDA-EVA system indicates its potential to safely deliver chlorhexidine *in vivo*.

As expected, no significant aberrations in blood chemistry or urinalysis occurred in this study. This result is consistent with a report of no altered white cell differential counts, liver function or kidney function in humans following two years daily rinsing with 0.2% chlorhexidine digluconate.[23]

The pharmacokinetic results of this study show that subjects received a minimal systemic exposure to CDA as evidenced by mostly (94%) non-detectable serum levels throughout the

study. When detected, levels were below 0.012µg/ml. These results are consistent with a variety of studies demonstrating no detectable systemic exposure of subjects exposed to either an ingested CHX gluconate or a sustained-release 2.5mg CHX gluconate intrasulcular chip.[24] It should be noted that although 6% of our samples did have detectable levels, our detection methods were 20 times more sensitive than the previously used methods.[24] Chlorhexidine has been shown to be poorly absorbed in human and animal tissues and is primarily metabolized in the liver and kidney.[25] Soskolne *et al.*[24] report no detectable levels of chlorhexidine in serum from 1 hour up to five days post-treatment with a sustained-release 2.5mg chlorhexidine gluconate intrasulcular chip and another study [22]shows the mean plasma level of chlorhexidine gluconate reached a peak of 0.206 µg/ml in humans 30 minutes after ingesting a 300 mg dose of the drug. Detectable levels of CHX gluconate were not present in the plasma of subjects ingesting CHX gluconate 12 hours after administration.[22] Regarding systemic exposure, our results confirm that the 2.5% CDA-EVA delivery system is as safe as comparable low system exposures of chlorhexidine.

The mean peak concentration of CDA in saliva for all subjects was 5.69µg/ml with a range of 1.70-13.85 µg/ml. The subjects in this study have a lower maximum salivary concentration of chlorhexdine than the reported concentration for subjects in other studies who rinsed with 0.2% CHX gluconate (C_{max}= 153±27µg/ml).[26] Lower levels of detectable chlorhexidine may indicate a limited ability for the 2.5% CDA-EVA delivery system to provide a dose capable of inhibiting the growth of oral pathogens at the current concentration. Stanley[27] reports that the minimum inhibitory concentration (MIC) of many oral pathogens ranges from 31-250µg/ml. However, Arnold *et al.* report the MIC of *P. gingivalis, S. mutans, and F. nucleatum* to be less than 1µg/ml. [28] Additionally, chlorhexidine

can alter microbial activity even at sub-MIC levels.[29] Sub-MIC levels of chlorhexidine have been shown to alter adenosine triphosphate (ATP) synthase activity and membrane ion gradients in streptococci[30] as well as inhibit the trypsin-like protease of *P. gingivalis*.[31, 32] Additionally, Marsh *et al.*[33] showed that the inhibition of acid production by oral streptococci was due to chlorhexidine's ability to inactivate the phosphoenolpyruvate-phosphotransferase sugar transport system. These effects of chlorhexidine at sub-MIC levels may significantly contribute to their effectiveness after the initial release of drug at higher concentrations. Most importantly, comparison of saliva concentrations when using the EVA mouthguard to rinsing with chlorhexidine digluconate may be inappropriate. Chlorhexidine rinses potentially deliver the drug throughout the entire mouth whereas the CDA-EVA mouthguard may target CDA delivery to the treated arch. There is a potential that the concentration of CDA is higher at the interface between the mouthguard and the maxillary tissue in the study while whole saliva collections may be significantly diluted.

An interesting observation regarding the concentration of CDA in saliva is that five of the six subjects (83.3%) demonstrated an increase in CDA concentration from 9 to 12 hours. All subjects entered the test facility in the late afternoon, and thus most subjects were sleeping between hours 9 and 12. Because salivary flow is decreased during sleep,[34] it is possible that lower salivary flow contributed to a build up of CDA in the saliva and thus a higher concentration of CDA in the saliva at 12 hours. Further investigation into the increase of CDA concentrations after twelve hours should be evaluated in the phase II trial.

It should be noted that salivary levels of CDA show great variability among subjects on days 1-21, both pre- and post-dosing. This may be attributed to variation in salivary flow and mouthguard surface area, which depends on a patients' oral anatomy. Nonetheless, CDA

was released in measurable quantities from the delivery system in all subjects throughout the entire 21-day study. Even when CDA levels were below quantifiable limits pre-dosing, all subjects had detectable salivary levels of CDA two hours post-dosing at all time points. This difference was statistically significant on Day 1, 3 and 14. The small study sample size may account for the lack of significance at the other time points. These results substantiate *in vitro* studies demonstrating CDA release for several weeks from CDA-EVA films.[288]

Trends toward improvement of clinical parameters used as surrogate measures of periodontal disease were observed with treatment. Most strikingly, a significant improvement in pocket depth was noted in the treated arch despite the small sample size of the study and despite the absences of any professional mechanical therapy. Although the results may be influenced by the Hawthorne effect or examiner bias since this was an open-label trial, the observed clinical improvement of periodontal parameters with CDA treatment in gingivitis subjects is encouraging.

Conclusions

The data suggest that the use of the CDA-EVA intraoral drug delivery system is safe for human use with minimal systemic exposure. Trends in the data suggest CDA released from an EVA delivery system may reduce the clinical signs of inflammatory periodontal disease. Interpretation of the results of this phase I clinical trial is limited due to the small sample size, lack of control group and open label design. A randomized, double blind, clinical trial in HIV subjects with periodontitis to confirm the safety and to elucidate the efficacy of this novel delivery system is underway.

Microbial Assessment of Chlorhexidine Diacetate Mouthguard: Phase I Clinical Trial

Abstract

Chlorhexidine diacetate (CDA) has been incorporated (2.5% by weight) into an ethylene vinyl acetate (EVA) intraoral delivery system for the proposed treatment indication of HIV-associated periodontitis. *In vitro* testing indicates sustained CDA delivery for at least 14 days. **Objectives:** 1) to evaluate changes in the oral biofilm composition with CDA treatment in medically healthy subjects with gingivitis, and 2) to evaluate CDA-susceptibility patterns of plaque samples before, during and after CDA-EVA treatment. **Methods:** Six adult subjects with moderate plaque-induced gingivitis were recruited for this open-label, phase I trial. CDA-EVA mouthguards were fabricated to fit the maxillary arches of subjects and worn 12 hours/day for 21 days. Microbiological samples were obtained at Days 0 (pre-dosing), 7 and 35, and quantitatively cultured using selective and differential media under appropriate atmospheres. In addition, the CDA-susceptibilities were determined by measuring the zones of growth inhibition (total and partial) of bacterial lawns that developed around standardized 2.5% CDA-EVA disks (6mm in diameter) placed on inoculated agar surfaces spread-plated with the individual subgingival plaque samples. **Results:** CDA-EVA treatment significantly decreased both aerobic (Day 0 vs. Day 7, p=0.05; Day 0 vs. Day 35, p=0.01) and anaerobic (Day 0 vs. Day 35, p=0.01) bacteria. In general, CDA-EVA treatment resulted in mean reductions of *Porphyromonus gingivalis, Prevotella intermedia* and *Candida* species. All plaque samples were susceptible to total inhibition by CDA. No

significant changes were observed for mean zones of total or partial inhibition in the individual plaque samples before, during and after the treatment period. **Conclusion:** CDA-EVA treatment does not alter the CDA-susceptibility profile of the oral flora and may reduce aerobic and anaerobic bacteria in healthy subjects with gingivitis. A phase II trial in HIV infected subjects may proceed. [Supported by DE15267-01, RR00046]

Introduction

One of the determining factors of periodontal health versus disease is the microbial composition found in the oral cavity. Accordingly the oral flora is very complex and the microbial population that forms in gingival sulci and on the surfaces of teeth of a patient with healthy gingiva differs from the bacterial populations found in moderate gingivitis and periodontitis.[35] Microscopic examination of plaque from chronic periodontitis patients show a highly anaerobic (90%) and gram-negative (75%) flora.[36] The diversity of microorganisms appears higher in the subgingival plaque of periodontitis patients than in health. Paster *et al.* estimated that there are approximately 415 species in subgingival plaque.[37] Socransky *et al.* [38] analyzed subgingival plaque of 185 subjects with or without periodontitis and determined the presence and levels of 40 subgingival taxa. They showed that species cluster into five different complexes. Orange complex (*Prevotella* species, *Fusobacterium* species and *Campylobacter* species) and red complex (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*) species were found to have an association in that the bacteria from the red complex were rarely seen without the presence of bacteria from the orange complex. Additionally, the presence of red complex bacteria was strongly related to destructive periodontitis characterized by increased probing depth and bleeding on probing. Tanner *et*

al. .[39] studied the bacterial species associated with the initial development of a periodontal lesion. This longitudinal study attempted to identify the organisms associated with the shift from health to disease. Their data suggest that *Tannerella forsythia, Campylobacter rectus* and *Selenomonas noxia* were the major species that characterized sites converting from health to disease. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were detected infrequently in the study population, which suggests that these pathogens might arrive later during the disease process. This study demonstrates that bacteria that cause periodontal disease are not isolated organisms but are part of a developing bacterial biofilm.

Individuals who are immunocompromised are at risk for oral opportunistic infections. In particular, patients with deficient immune responses are susceptible to destructive and necrotizing forms of periodontal diseases known as necrotizing ulcerative periodontitis (NUP).[10, 11] Although the clinical presentation of HIV-associated oral conditions may differ from chronic periodontitis, the pathogenic bacteria associated with periodontal disease in HIV patients resembles that of normally healthy subjects suffering from periodontal disease.[40] Microbiologic studies indicate high levels of *P. intermedia* and spirochetes are often seen in NUP patients.[41]

Current non-surgical periodontal therapies targeted at reducing pathogenic bacteria include mechanical debridement and chemotherapeutics, as well as systemic and locally delivered antibiotics. The use of systemic antibiotics can lead to allergic reactions, digestive disturbances and overgrowth of opportunistic pathogens. Thus, topical and locally delivered antimicrobials may provide benefits in the treatment of a variety of periodontal conditions while reducing risks for negative systemic side effects.

Our investigative group has incorporated chlorhexidine diacetate (CDA) into an ethylene vinyl acetate (EVA) copolymer for the treatment of periodontitis. The present study was conducted to evaluate changes in the oral biofilm with CDA-EVA treatment in medically healthy subjects with gingivitis and to evaluate susceptibility patterns of plaque samples before, during and after CDA-EVA treatment.

Materials and Methods

Study Design

Subject recruitment and experimental treatment for this investigation are described elsewhere.[42] Briefly, following a protocol approval by the Biomedical IRB of the University of North Carolina at Chapel Hill, six medically healthy consenting adult subjects were recruited to participate. This sample size was derived as a convenience sample and was chosen based on logistical and not power considerations.

This was a single center, five-week, open label, phase I clinical trial. Subjects had moderate-plaque induced gingivitis defined as four or more teeth with 4-5mm pocket depths. Subjects were treated with a CDA-EVA mouthguard 12 hours per day for 21 days followed by a 14-day post-treatment period. Clinical indices were measured at Baseline (Day 0) and Day 21. Plaque samples were collected at Baseline, Day 7 and Day 35 (Figure 1).

Plaque collection

Four subgingival plaque samples were obtained from mesial surfaces of the maxillary first and second molars (four sites total) prior to mouthguard insertion at Baseline and on Days 7 and 35. For each designated site, the area was air dried and isolated with cotton rolls. Plaque samples were collected from the subgingival sites using medium-size sterile paper

points. Samples were placed into separate screw-cap tubes containing buffered mineral salts with sodium thioglycolate and L-cysteine (Liquid Dental Transport Medium, Anaerobe Systems, Morang Hill, CA, USA) (Figure 1).

Culture technique and identification

All samples were transported to the laboratory, maintained at room temperature and processed in less than two hours. Samples were transferred to 4ml of pre-reduced Wilkins Chalgren (WC) anaerobic broth (Oxoid.Ltd., Basingstoke, Hampshire, England) capped securely and vortexed. Ten-fold serial dilutions in WC broth were performed.

All sample dilutions were spiral plated (Spiral Plater Model D from Microbiology International, Frederick, MD, USA) for quantitative culture using enriched non-selective, selective and differential media. Total recoverable colony forming units (CFU)/ml were determined for each sample. Total counts for aerobic growth were made on either chocolate agar in 5% CO_2 at 37ºC or on sheep blood agar in ambient air at 37ºC, whichever yielded the higher count. Total counts for anaerobic growth were made on trypticase soy agar supplemented with sheep blood, heme, menadione and N-acetyl muramic acid (TSA-NAM from Anaerobe Systems, Morgan Hill, CA, USA) grown at 35ºC in a flexible film chamber (Coy Laboratories Inc., Ann Arbor, MI, USA) with an atmosphere of 10% H_2-5% CO_2-85% N_2.

In order to identify specific bacteria in the subjects' dental plaque, the samples were grown on a variety of selective and non-selective media. These media (Anaerobe Systems, Morgan Hill, CA, USA or from Difco Laboratories, Detroit, MI, USA) are listed in Table 4. Total counts and subcounts based on distinct colony types were determined on each of these media. These aerobic and anaerobic media along with biochemical and morphology analysis

permitted the quantitative identification of the following microorganisms*: P. gingivalis, T. forsythia, Prevotella. intermedia, Capnocytophaga* species*, Peptostreptococcus anaerobius, P. micros, P. nigrescens, Fusobacterium nucleatum, Fusobacterium periodonticum, F.* species*, Eikenella corrodens, C. rectus, C. gracilis, Bacteroides fragilis, Actinomyces* species*, A. actinomycetemcomitans, Candida albicans,* non-albicans *Candida* species*, Enterococcus faecalis, Staphylococcus aureus, Streptococcus pyogenes, S. mutans, Lactobacillus acidophilus, Haemophilus* species*, S. pneumonia,* all enteric bacteria*,* group B and group D *Streptococci.*

Sensitivity Analysis

Each of the four subgingival plaque samples from subjects on Days 0, 7 and 35 were analyzed for sensitivity to CDA. Aliquots of 1ml of undiluted samples in WC were spread plated manually on TSA-NAM plates. If 1ml was not available, a 1:10 dilution was used. Disks (6mm in diameter) cut from standard 2.5% CDA-EVA films were evenly spaced on the freshly inoculated agar surface. These plaque bacteria were allowed to grow to a confluent lawn in an atmosphere of 10%H_2-5%CO_2-85%N_2 at 35ºC. Zones of total inhibition were measured as diameter using calipers and recorded in millimeters. Additionally, zones of partial inhibition, defined as clear demarcations where there was discernible inhibition of some, but not all of the mixture of bacteria, were recorded.

Results

Study subjects and treatment

Six subjects, met the inclusion criteria, signed consent forms and were enrolled in the study. All subjects who entered the study completed the study and were included in the

microbial analyses. The subjects had a mean age of 41±14 years (range: 27-58), and the mean number of teeth per subject was 27.2 ±1. Five (83.3%) subjects were Caucasian, and one (16.6%) was African American. Five (83.3%) of the subjects were female, and all subjects were non-smokers. In general, study treatments were well-tolerated, and the safety and clinical effects are detailed elsewhere[42]

Resistance evaluation

Mean zones of total and partial inhibition are shown in Figure 5. The mean zone of total inhibition on Day 0 was 12.15±1.97mm. On Day 7 and 35, the mean zones of total inhibition were 12.23±1.69mm and 12.27±1.28mm, respectively. There were no statistically significant differences in the zones of inhibition between Days 0 and 7 (p=0.81) or between Days 0 and 35 (p=0.46). The partial zones of inhibition also showed no statistically significant changes from Day 0 to Day 7 (p=0.78) and Day 0 to Day 35 (p=0.66).

Microbial Load

Total recoverable CFUs/ml from plaque samples are shown in Figure 6. On Days 0, 7, and 35 the total recoverable CFU for aerobic species was 4.92±0.22 (SE) $\log_{10}$ CFU/ml. On days 7 and 35 the levels were 4.57±0.14 (SE) $\log_{10}$ CFU/ml and 4.17±0.20 (SE) $\log_{10}$ CFU/ml, respectively. Total recoverable anaerobic CFUs on days 0, 7 and 35 were 5.15±0.22 (SE) $\log_{10}$ CFU/ml, 5.36±0.33 (SE) $\log_{10d}$ CFU/ml, 4.72±0.25 (SE) $\log_{10}$ CFU/ml, respectively. Overall, there was a statistically significant decrease of both aerobic (Day 0 vs. Day 7, p=0.05; Day 0 vs. Day 35, p=0.01) and anaerobic (Day 0 vs. Day 35, p=0.01) bacteria with CDA treatment.

Plaque Composition

Specific bacteria identified in the four subgingival sites from the six subjects at Baseline (Day 0), Day 7 and Day 35 are summarized in Figure 7. Over the 35-day study, the percent of sites harboring *P. gingivalis*, *P. intermedia, Capnocytophaga* species*, P. anaerobius* and *E. corrodens* decreased. In contrast from Baseline to Day 35, there was an increase in the percentage of sites harboring *P. nigrescens*, *F. nucleatum* and *C. gracilis*. *P. gingivalis* was found in all subjects during at least one timepoint throughout the study. *T. forsythus, P. micros, Wolinella* species*,* and *C. rectus* were not recovered in any of the samples. *Candida* species were present at baseline in nine samples from three of the patients. The number of positive sites decreased to eight on Day 7, and to one site on Day 35 (Figure 8).

Discussion

Chlorhexidine (CHX) is a commonly used active chemotherapeutic for managing periodontal diseases. Chlorhexidine is marketed worldwide as a 0.12% or 0.2% mouthrinse,[5, 22] but it is also available in a variety of other forms including gels,[43] toothpastes,[44] and a biodegradable local delivery chip[45] that is placed subgingivally. In general, these formulations are based on the diacetate salt of CHX.

CHX is a broad spectrum antimicrobial agent with activity against a wide range of Gram-positive and Gram-negative bacteria found in the supragingival and subgingival plaque.[46, 47] Its mode of action varies but includes lysing cell membranes, disrupting structural organization and congealing of cytoplasm at high concentrations.[48] Additionally, CHX has a dicationic charge, which allows it to bind to oral tissues, and which enhances

substantivity in the oral cavity. A clinical study in gingivitis patients showed that rinsing with a 0.12% CHX mouthrinse reduced the number of black pigmented *Bacteroides* species after 6 months.[49] Another treatment group from this same trial had subgingival irrigation with a 0.06% CHX and demonstrated a significant reduction in the number of Gram-negative anaerobic rods and black-pigmented *Bacteroides* species.[49] After 3 months, both CHX regimens resulted in an increase in Gram-positive facultative anaerobic species, which are often associated with periodontal health.

Previous reports by both Daneshmand *et al.*[50] and Grisi *et al*.[51] failed to show that the adjunctive use of a subgingival chlorhexidine chip (PerioChip, DexcelPharma, Jerusalem, Israel) improved microbial levels of periodontal pathogens over scaling and root planing alone. However, a recent multicenter randomized controlled clinical trial by Paolantonio and coworkers demonstrated that following the adjunctive use of a chlorhexidine chip in experimental sites with probing depths ≥ 5mm and bleeding on probing, total bacterial counts at 15 days and 1 month were significantly lower than in sites treated with scaling and root planing alone.[52] Additionally, sites treated with the chlorhexidine chip had significantly greater decreases at 15 days and 3 months for *C. rectus,* and at 15 days, 1 month and 3 months for *T. forsythia* over scaling and root planing alone. In the current phase I clinical trial, gingivitis subjects did not harbor *C. rectus* or *T. forsythia* either pre- or post-treatment. We did however observe a reduction in the number of sites culturing positive for other periodontal pathogens such as *P. gingivalis* and *P. intermedia* with CDA treatment.

During this phase I clinical trial, it was important to determine if treatment would lead to the formation of bacterial resistance to chlorhexidine. Stickler and colleagues have reported the existence of CHX resistant strains of *Pseudomona, Proteus* and *Providencia*.[53]

Additionally, *S. aureus* has been shown to be resistant to CHX. *S.aureus* also has the ability to transfer resistance to *E. coli* via recombinant plasmids.[54] According to one report, *Serratia marcescans* had increased resistance to CHX following repeated exposure to various contact lens solutions containing 0.001 to 0.006% chlorhexidine.[55] Additionally, *Pseudomonas aeruginosa* showed a greater than seven-fold increase in its minimum inhibitory concentration (MIC) to CHX digluconate following six days of repeated exposure to 5mg/l of CHX.[56] Another study by Brooks and coworkers demonstrated that some isolates of *P. aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* could multiply in 1:2 dilution of 2% chlorhexidine liquid soap.[57]

In contrast, Russell and Day argue that laboratory tests are inconclusive as to whether organisms can be pressured to become highly resistant to CHX.[58] Cookson *et al.*[59] subjected methicillin-resistant *Staphylococcus aureus* (MRSA) and methecillin-sensitive *Staphylococcus aureus* (MSSA) to repeated exposures of chlorhexidine *in vivo* and *in vitro*, but failed to document resistance formation. In dental studies, investigators who monitored CHX oral continuous rinsing twice a day for 2 years in humans, reported no reduction of plaque inhibition and only minor reduction of salivary bacterial sensitivity.[5, 23] Walker reviewed the microbiological effects of mouthrinses and concurs that resistance formation is not seen with extended use of CHX.[60]

The present study supports the use of chlorhexidine as a safe topical chemotherapeutic for the treatment of periodontal disease. Throughout the study, CDA produced a consistent zone of total inhibition of bacterial growth when tested on the subjects' plaque samples. In addition, there were differential sensitivities of the various bacterial populations in the plaque samples as evidenced by partial zones of inhibition. Baseline data

show that all plaque samples collected from all subjects were sensitive to CDA as demonstrated by zones of total and partial inhibition when exposed to CDA. Additionally, similar zones of total inhibition and partial inhibition were measured at all subsequent time point. These data indicate that over the 21-day treatment and the 14 day post-treatment periods, the oral flora did not develop or select for resistance to CDA. Because the zones of partial inhibition remained consistent throughout the study, it can be concluded that CDA treatment over 21 days did not result in resistance formation or preferential selection of strains with low sensitivity to CDA.

Another objective of this study was to evaluate the changes in oral bacteria throughout a 21-day dosing with the 2.5% CDA-EVA mouthguard. Accordingly, subjects had a statistically significant decrease in the recoverable CFU from baseline to day 35 for both aerobic and anaerobic species. These results indicate that the level of CDA delivered to the sites of plaque sampling may have been sufficient to suppress the overall oral flora in the absence of any professional mechanical treatment. Alternatively, decreased bacterial levels may have been influenced by the Hawthorne effect. Maximum CDA levels measured in the saliva of each subject ranged from 1.7µg/ml to 13.8µg/ml (mean C_{max} = 5.69 ±4.37 µg/ml)[42] throughout the course of the study and were greater than the MIC of *S. mutans* (aerobic) (0.39 µg/ml), *P. gingivalis* A7436 (1.56 µg/ml), *P. gingivalis* HG405 (1.56ug/ml µg/ml) and *F. nucleatum* (0.78 µg/ml) as determined in our preliminary studies.[28]

In our population of patients with minimal clinical signs of periodontal disease, a plethora of putative periodontal pathogens were identified in multiple sites. This is consistent with Socransky and coworkers who reported that even in healthy sites with 3-5mm pocket depths, periodontal pathogens (e.g., *P. gingivalis* and *P. intermedia*) could be

recovered[61]. In the majority of healthy sites, the levels of these bacteria were typically less than 10^5. This finding is consistent with those of the present report where samples containing red and orange[38] complex bacteria were detected at levels less than 10^5 in all but three sites. At the site level, no consistent pattern of microbial change could be identified; however, pooled data for all subjects showed a decrease in the percentage of plaque samples colonized with *P .gingivalis* and *P. intermedia,* which have been associated with periodontal pathology and progressive attachment loss.[62] The lack of consistent microbial change at the site level may be due to the small sample size of this investigation as well as the minimal baseline clinical disease present in these subjects.

Three subjects had sites positive for *Candida* species at baseline. In two of these subjects, there were no *Candida* species by day 35, and in the third subject *Candida* species were detected in only one of the two originally infected sites on Day 35. CHX is well documented as an anti-fungal agent. Hiom and coworkers have shown that CHX diacetate can have lethal activity against *Candida albicans* and *Candida glabrata*[2]. Additionally, Ferretti and coworkers[63] showed in a double blind clinical trial that CHX oral rinse can control oral candidiasis in bone marrow transplantation patients. The trend for a reduction in *Candida* species following treatment with the CDA-EVA mouthguard may be important as we initiate the phase II trial in HIV-infected subjects who particularly susceptible to opportunistic infections with *Candida* species.[64]

Conclusions

The data suggest that the use of the 2.5% CDA-EVA drug delivery system is safe for use in medically healthy gingivitis subjects without the development of microbial resistance to

CDA or selection of putative/opportunistic pathogens. Additionally, the overall level of both aerobic and anaerobic microorganisms may be reduced with the use of the CDA-EVA mouthguard. While interpretation of the results of this small, unblinded clinical trial is limited, testing in HIV subjects with periodontal disease may proceed.

Table 1.
Mean ±SD Baseline Clinical Parameters (N=6 subjects)

	PD	CAL	%BOP	PI	GI
Subject 1	2.39	1.90	42.9	0.23	0.69
Subject 2	2.37	2.26	30.9	0.40	0.52
Subject 3	2.49	2.24	52.0	0.46	1.02
Subject 4	2.47	5.53	48.2	0.35	0.75
Subject 5	2.53	2.06	57.7	0.66	1.49
Subject 6	2.49	2.11	58.9	0.63	1.52
All Subjects	**2.46±0.06**	**2.19±0.22**	**48.45±10.49**	**0.45±0.17**	**1.00±0.42**

Table 2.
Mean ± SD for Clinical Parameters at Baseline and Day 21 (N=6 subjects)

Parameter	Baseline	Day 21	*p**
Mean PD (mm)	2.46±0.06	2.26±0.08	0.001
Mean CAL (mm)	2.19±0.22	1.98±0.23	0.14
Mean GI	1.00±0.42	0.54±0.18	0.03
Mean PI	0.45±0.17	0.38±0.17	0.46
Mean BOP (%)	48.45±10.49	30.76±9.22	0.002
Mean CSSI	0.06±0.06	0.04±0.03	0.46
Mean DI	0.07±0.04	0.05±0.05	0.47

*Student *t* test

Table 3.
Mean ± SE for Clinical Parameters for the Maxillary Arch
Adjusting for Mandibular Arch

Parameter	Baseline	Day 21	*p**
Mean PD (mm)	2.38 (0.04)	2.21 (0.04)	0.04
Mean CAL (mm)	1.99 (0.09)	1.90 (0.09)	0.54
Mean GI	0.74 (0.11)	0.75 (0.11)	0.96
Mean PI	0.42 (0.07)	0.34 (0.07)	0.42
% BOP	44.6 (5.40)	34.4 (5.40)	0.26
Mean CSSI (x100)	0.34 (0.50)	0.83 (0.50)	0.51
Mean DI	0.09 (0.02)	0.06 (0.02)	0.41

*ANCOVA

Table 4.
Selective and non-selective media utilized for flora identification in sub-gingival plaque samples

Aerobic	Anaerobic
BEA	CBA
Chocolate	CVE
MacConkey's	BBE
Mycobiotic	LKV
Mannitol salt	TSBV
Saboraud's Dextrose	TSA-NAM
Sheep blood agar	Mitis-salivarius-bacitracin
	Campylobacter-Wolinella
	Lactobacillus selective
	Mitis-salivarius

Figure 1: Collection schedule for serum, saliva, plaque samples and clinical indicies Day 0 to Day 35

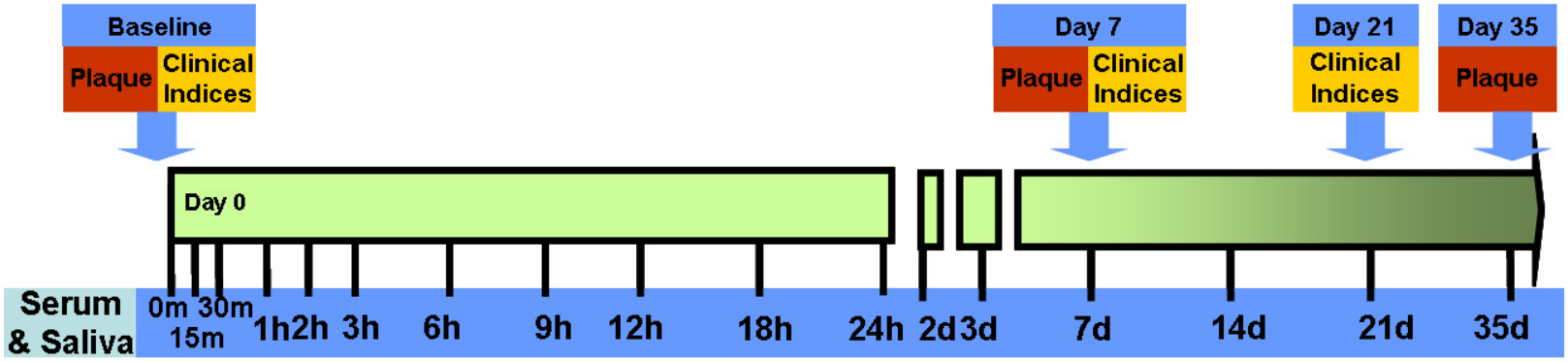


Figure 2. Concentration of CDA in saliva from Baseline to 24 hours for each subject (N=6)

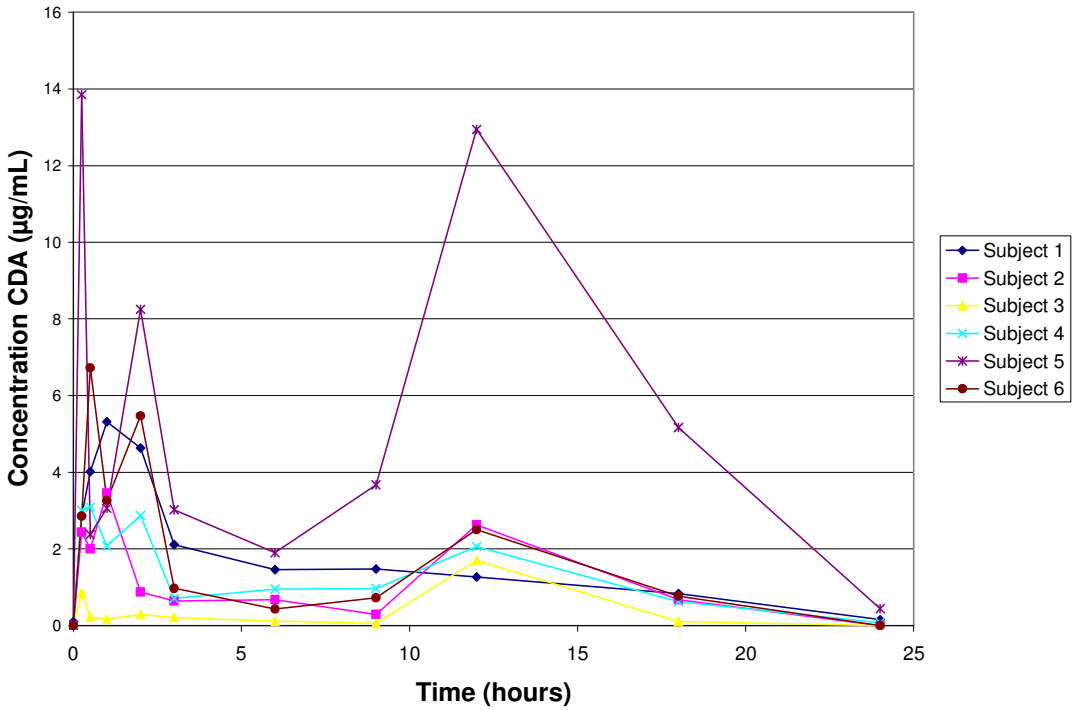


Figure 3. Mean (SD) concentration of CDA in saliva from baseline to 24 hours

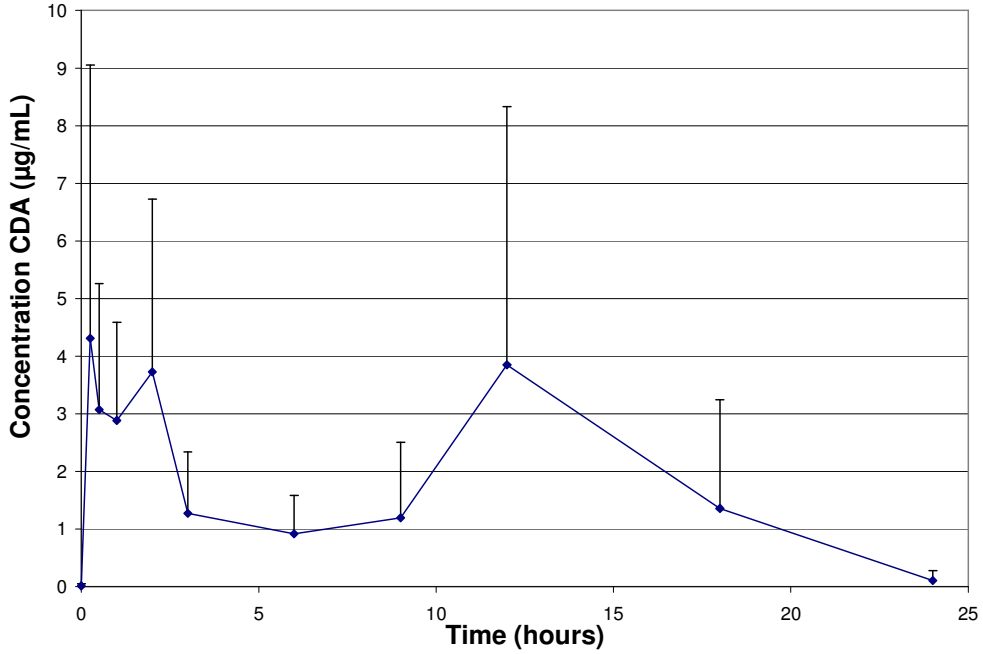


Figure 4. CDA concentration (µg/ml) in saliva day 1, 2, 3, 7, 14 and 21 prior to dosing and two hours post-dosing. (*p<0.05)

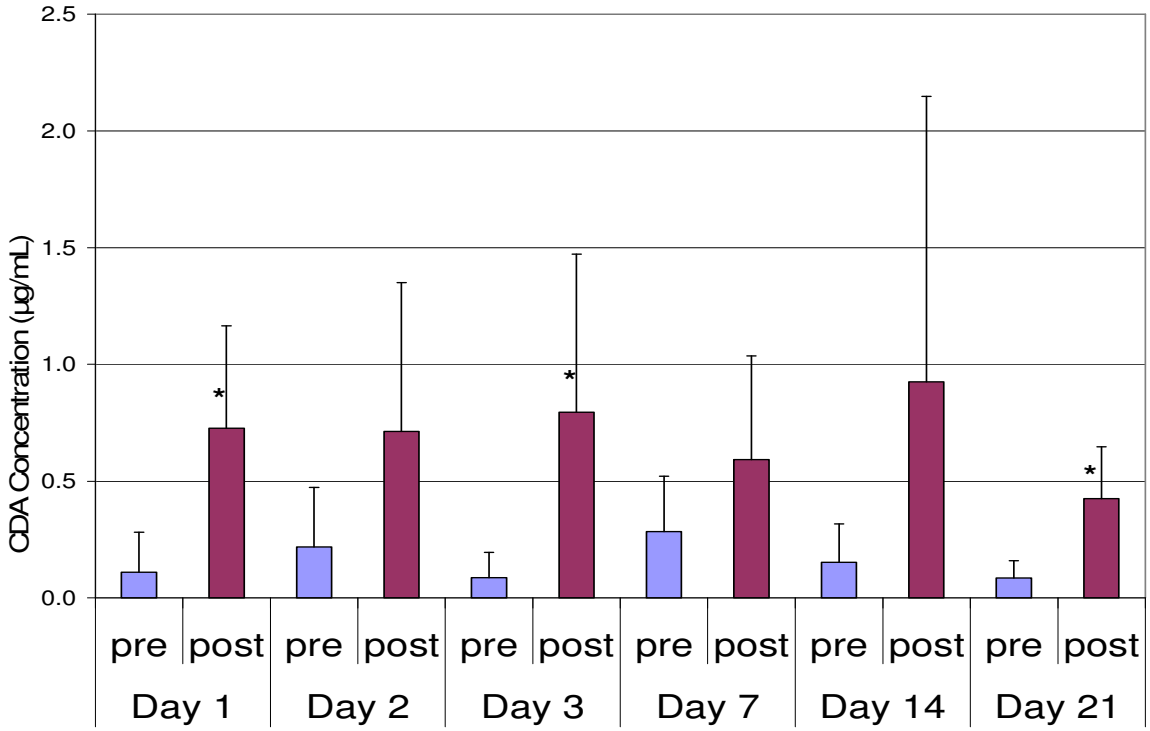


Figure 5. Mean (SD) Zones of Total and Partial Zones of Inhibition

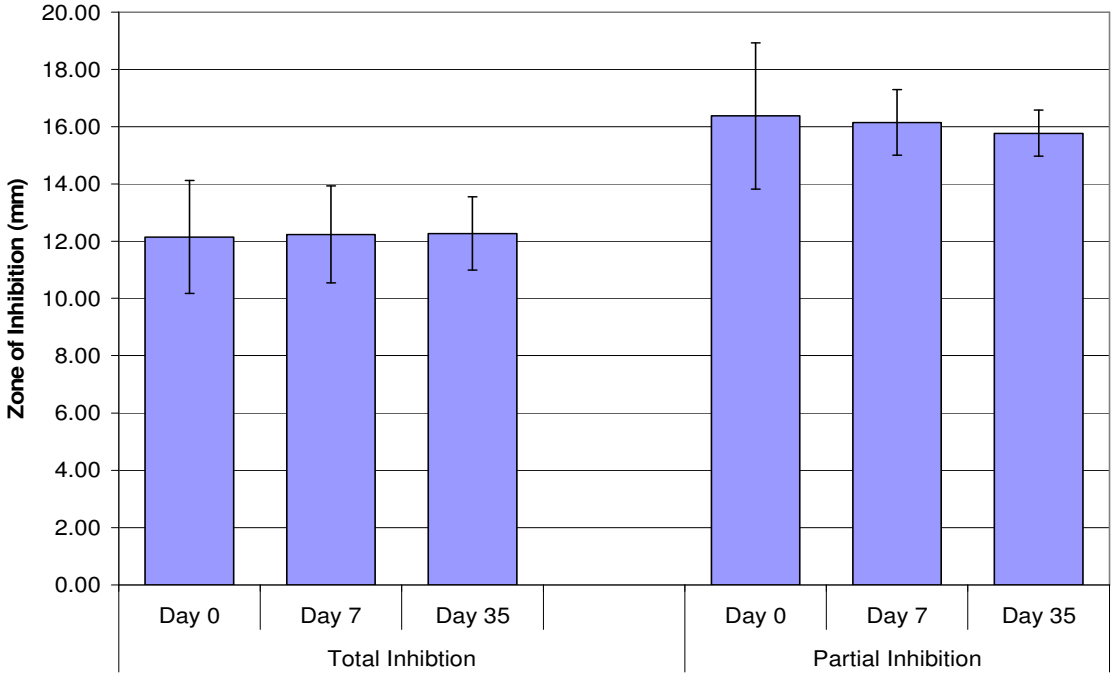


Figure 6. Mean (SD) Total Recoverable Colony Forming Units (*p<0.05)

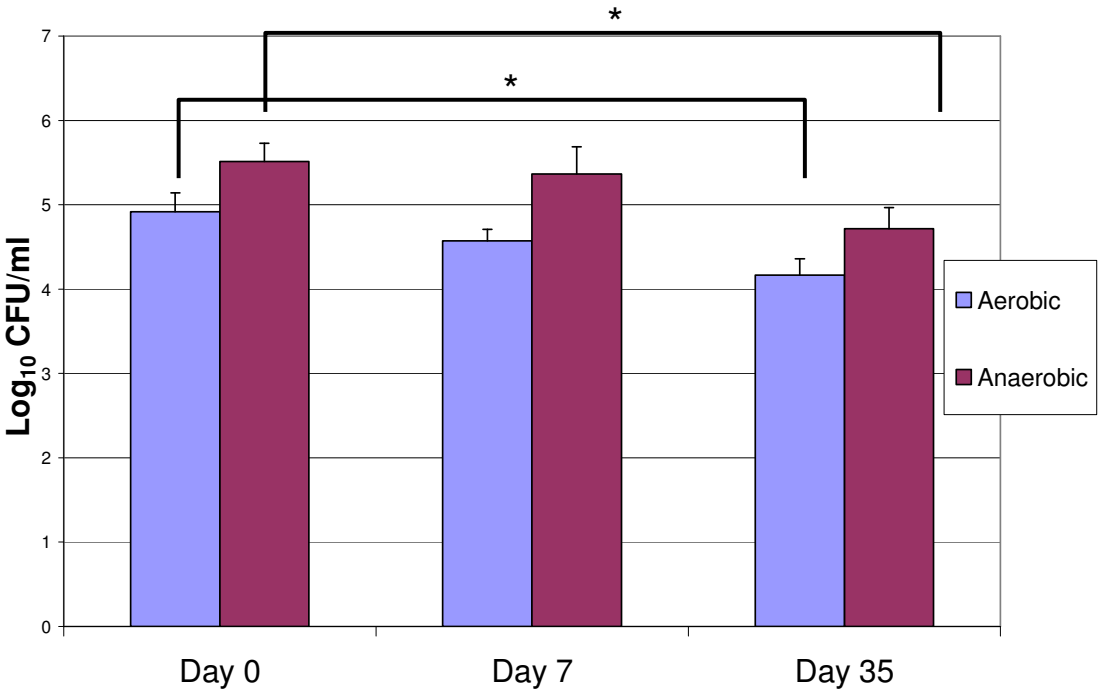


Figure 7. Percent of sites colonized with various bacteria on Day 0, Day 7 and Day 35

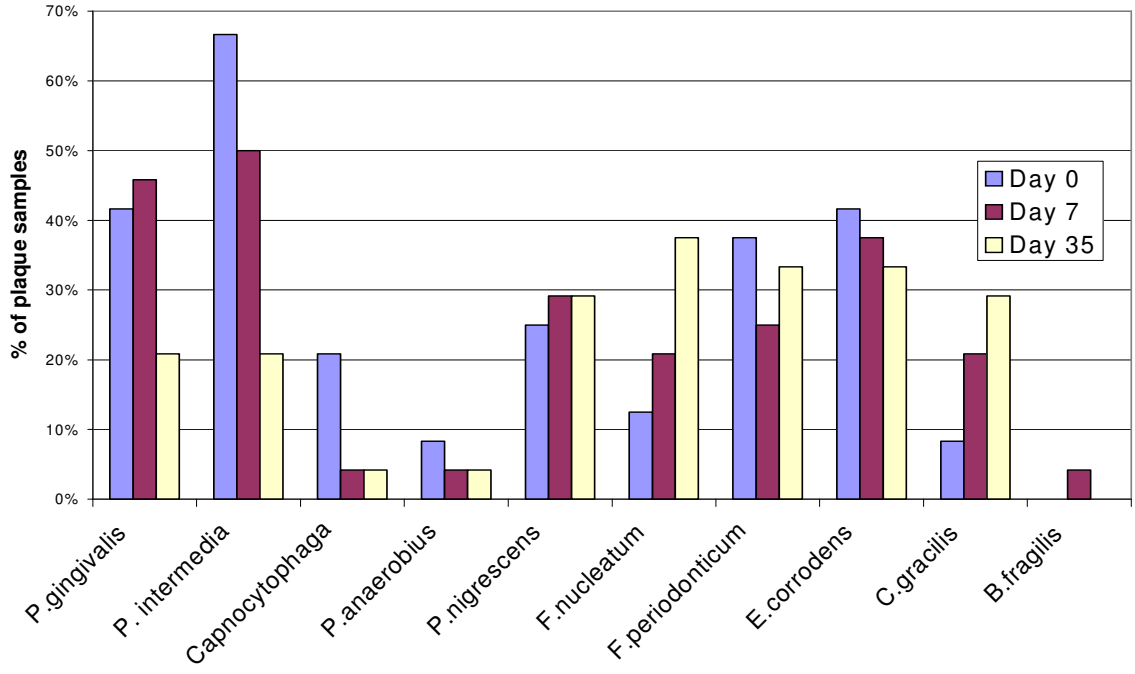
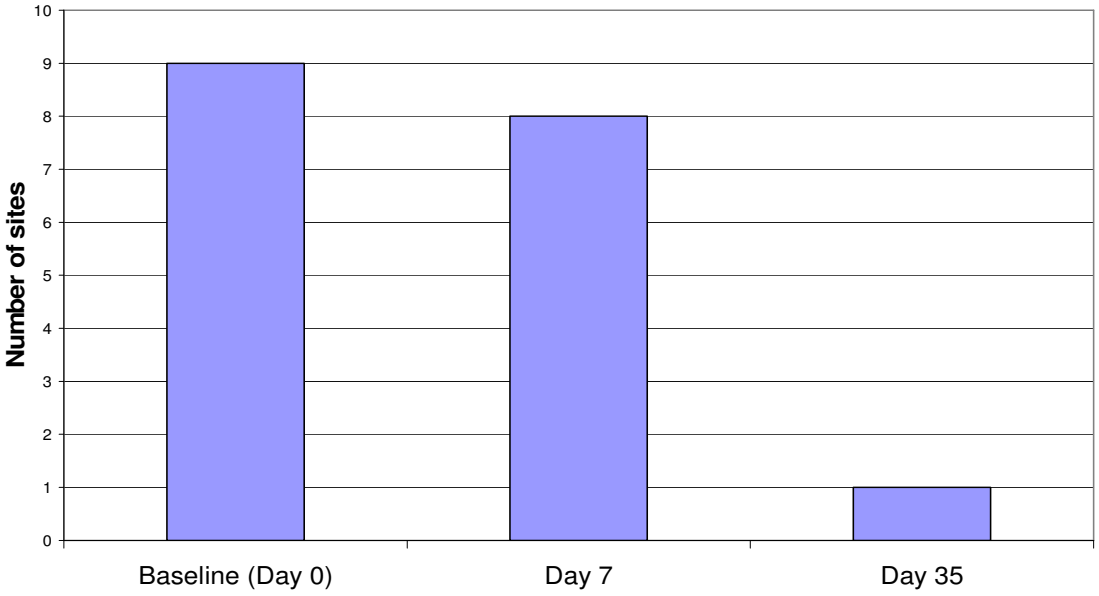


Figure 8. Number of sites colonized with *Candida species* on Day 0, Day 7 and Day 35



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