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Subantimicrobial Dose Doxycycline Effects on Osteopenic Bone Loss: Microbiologic Results

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Abstract

Background: Based on microbiologic concerns, the safety of a 24-month regimen of subantimicrobial dose doxycycline (SDD; 20 mg twice a day) was evaluated in postmenopausal osteopenic women with periodontitis in a double-blind, placebo-controlled, randomized clinical trial.

Methods: Subgingival samples were collected from two sites (probing depth ≥ 5 mm) in each of 128 subjects at baseline, with the same sites resampled at the conclusion of the 2-year period. The samples were enumerated on selective and non-selective media and on doxycycline (4 μ g/ml) medium. Up to five different colonial morphologies were subcultured from the doxycycline medium, identified to species, and susceptibilities determined to doxycycline and five other antibiotics. Data were analyzed for microbial differences in total colony forming units (CFU), periodontal and opportunistic pathogens, and changes in species and in susceptibilities of isolates recovered on doxycycline medium.

Results: There was no significant evidence that changes in total anaerobic counts over the treatment period ($P = 0.96$) differed between treatment groups. Likewise, periodontal pathogens, opportunistic pathogens, or normal flora did not differ descriptively between groups. Although there was a significant increase ($P < 0.001$) in the total CFU recovered from the 4 μ g/ml doxycycline plates at 24 months for SDD versus placebo, the percentage that was clinically resistant to doxycycline (minimal inhibitory concentration [MIC] ≥ 16 μ g/ml) decreased over the 24-month period in both groups and did not differ between the treatment groups (SDD: 79% to 76%; placebo: 83% to 70%; $P = 0.2$). There were no significant differences ($P > 0.28$ for each) in the change in cross-resistance between the groups for doxycycline and the other five antibiotics.

Conclusions: No antimicrobial effect on the subgingival flora was detected following treatment with SDD for 24 months, relative to baseline or to placebo. The increase in initial resistance (at 4 μ g/ml) did not translate into a significant increase in the percent resistant to doxycycline (MIC ≥ 16 μ g/ml) for patients in the SDD group.

Keywords

Antibiotic; clinical trials; doxycycline

A substantial body of evidence has demonstrated that the adjunctive use of subantimicrobial dose doxycycline (SDD), consisting of doxycycline hyclate, 20 mg twice a day, provides a significant additional benefit to scaling and root planing in the treatment of periodontitis.¹⁻⁷ At this dosage, the benefits obtained are derived from the anticollagenase and anti-inflammatory properties of doxycycline, rather than its anti-microbial properties.⁸⁻¹⁰ Microbial studies have documented the lack of any antimicrobial effect on the normal flora, periodontal and/or opportunistic pathogens, or change in antibiotic susceptibilities following the use of SDD for up to 9 months in double-blinded, placebo controlled, multicentered studies. These studies examined the effect, or lack of effect, of SDD on the subgingival flora¹¹⁻¹³ and on antibiotic resistances within this flora¹¹ in a periodontitis population. Likewise, there was no detectable effect of a 9-month regimen of SDD on the intestinal flora of a periodontitis population cross-sectionally, relative to placebo control, or longitudinally within the SDD treatment group.¹⁴

The effect of SDD on the vaginal flora also was examined in this study. Although the number of subjects that participated was limited, no differences were detected in the vaginal flora. In a completely different population, the effect of SDD relative to placebo was determined on the skin flora. No differences were found in the bacterial composition or in antibiotic resistances following a 6-month regimen of SDD relative to placebo.¹⁵ In all of the above studies, it was concluded that SDD did not result in changes to the normal flora, did not lead to the overgrowth or the colonization of the flora by periodontal or opportunistic pathogens, and did not result in increased resistances to doxycycline or to multiantibiotic regimens.

The above studies were conducted in generalized periodontitis populations, generally for periods ≤ 9 months. However, to the best of our knowledge, the efficacy of SDD in treating periodontitis in potentially vulnerable populations, such as postmenopausal estrogen-deficient women, has not been examined.^{16,17} The objectives of our clinical trial were to examine the efficacy of SDD in reducing progressive alveolar bone loss and attachment loss in this patient population. The microbiologic study was designed to address the safety of SDD in this patient population by determining if SDD, given twice a day for 24 months, had any detectable effect on the subgingival microbiota as determined by changes or shifts in the normal flora, emergence of periodontal and/or opportunistic pathogens, changes in resistance to doxycycline, and an increase in multiantibiotic resistance.

MATERIALS AND METHODS

Subject Population

To be eligible for participation in this clinical trial, patients could not have had active periodontal therapy, including quadrant root planing or periodontal surgery, within the past year. In addition, subjects had to have visited their dentist regularly (e.g., at least twice per year) for teeth cleanings. Subjects were entered at two clinical sites: the University of Nebraska Medical Center College of Dentistry (UNMC) and The State University of New York at Stony Brook School of Dental Medicine (Stony Brook). Twenty-five percent of all posterior interproximal sites examined at the baseline visit had a probing depth (PD) ≥ 5 mm.

Inclusion/Exclusion Criteria

Inclusion criteria included being 45 to 70 years of age at telephone screening; postmenopausal for ≥ 6 months and not receiving hormone replacement therapy; osteopenia of the lumbar spine or femoral neck (T-score: -1.0 to -2.5 inclusive); history of generalized moderate to advanced periodontitis and undergoing periodontal maintenance; and having at least nine posterior teeth and at least two sites with PD ≥ 5 mm together with bleeding on probing, clinical attachment level loss ≥ 5 mm, and radiographic evidence of alveolar bone height loss. Subjects also had to

be in good general health and willing to sign UNMC and Stony Brook Institutional Review Board–approved consent forms.

Exclusion criteria included allergy or hypersensitivity to tetracyclines; diseases or regular drug therapy that would affect the inflammatory or immune response (e.g., chronic use of non-steroidal anti-inflammatory drugs) or bone remodeling (e.g., prescription estrogens, bisphosphonates, calcitonin, or steroids); requirement for antibiotic premedication; diabetes; and normal bone mineral density at the lumbar spine and femoral neck (T-score above -1.0) or osteoporosis of the lumbar spine or femoral neck (T-score: <-2.5).

Study Design

This study was a two-center, double-blind, placebo-controlled, randomized, 2-year clinical trial; each subject was instructed to take all study medications daily for 2 years. Subjects were recruited and randomized on a rolling-admission basis beginning in June 2002 and ending October 2003. The last subject completed the clinical trial in October 2005. Study participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. The study had two treatment arms: doxycycline, 20 mg twice a day (SDD), and a placebo look-alike, twice a day. Sixty-four eligible subjects were randomized into each treatment arm. All subjects received daily calcium (1,200 mg) and vitamin D (400 IU) supplements. All subjects received periodontal maintenance every 3 to 4 months throughout the study that was delivered by the subjects' own dental care providers, not by the study clinicians. Few of the microbial sites received active periodontal therapy during the study (this active therapy was needed in a few cases because of the presence of progressive periodontitis that required “rescue therapy”) or were adjacent to sites with extractions performed.

At each visit, starting at baseline, a drug accountability form was completed and the subjects were counseled on the importance of taking the study medications in the prescribed manner. At each postbaseline visit, the expected number of pills consumed was calculated. The actual number of pills consumed was assumed to be the number dispensed minus the number returned. Percent adherence was calculated as the actual/expected $\times 100$. Observations from subjects taking $<80\%$ of the study drug, based on pill counts, were excluded in the per-protocol analyses. The analysis results were similar between intent-to-treat (ITT) and per-protocol subsets.

Microbiologic Sampling

After receiving written informed consent from the subject, subgingival samples were collected from two sites with PD ≥ 5 mm at baseline and from the same sites 24 months later. The sites selected for microbiology sampling were separate from those used for the collection of gingival crevicular fluid (described elsewhere) so that the collection of one type of sample would not have an effect on the collection of the other type of sample. If two sites with PD ≥ 5 mm, which had not been sampled for gingival crevicular fluid, were not available, sites with shallower pockets were chosen. Across the study patients at baseline, 52% had two sites sampled with PD ≥ 5 mm, 34% had one site with PD ≥ 5 mm, and 14% had shallower (<5 mm) pockets sampled. Following the removal of any visible supragingival plaque from the site, subgingival samples were collected by inserting a single sterile endodontic paper point apically into the pocket until resistance was felt; then, the paper point was moved across the horizontal axis of the tooth and left in place for ~ 10 seconds. The paper point was removed and placed into a 2-ml snap-top tube that contained 1 ml sterile Amies transport medium¹⁸ supplemented with 1% gelatin to provide a semisolid medium and 0.1% sodium thioglycolate as a reducing agent. Both samples collected from the subject were pooled together in the same tube because the objectives were to determine the effect on the subject rather than the site. Following the sample collection, the tube was labeled with the subject number, placed in an approved shipping container, and shipped for next-day delivery by an overnight courier. At the time of shipment,

the microbiology laboratories were notified of the number of samples and the subject numbers being shipped.

Microbiologic Processing

Once received in the microbiology laboratories, the samples were logged in and processed immediately. The sample was dispersed from the paper points by anaerobic sonication in a water-containing cup horn^{||} for 10 seconds at a setting of 60% power output. Following sonication, a series of 10-fold serial dilutions were made in prereduced, anaerobically sterilized Ringer solution¹⁹ using an anaerobic transfer unit to maintain anaerobiosis. Aliquots (0.1 ml) of the appropriate dilutions were spread on the surface of agar media and incubated as shown in Table 1. Duplicate series of plates were used for each sample.

Colony Counts

Following the prescribed incubation period, the plates were examined, confirmatory tests were performed as appropriate (Table 1), and the colony forming units (CFU) present at a countable dilution were enumerated. Anaerobic counts were determined from the plate dilution that gave rise to 30 to 300 CFU. For all other media, colony counts were taken from plates with 30 to 300 CFU if available. If <30 colonies were present on the most diluted plate, the colony number present was counted, provided that more than a single colony was detected. A single colony on a plate was considered a “0” count. Each set of duplicate sets of plates was counted individually, and the data were recorded.

Doxycycline Resistance

To determine the proportion of the recovered flora with resistance to doxycycline, the sample was plated on trypticase-soy blood agar supplemented with hemin and menadione (TSBA-HK) containing 4 µg doxycycline per milliliter, as described previously.^{13,27} The number of CFU present on the doxycycline-containing plates was determined and expressed as a proportion of the total anaerobic counts obtained on TSBA-HK without doxycycline. For identification of the microorganisms with resistance to doxycycline, a representative of each of the five most numerous resistant colony types, when present, was subcultured and identified to genus and species level by capillary gas-liquid chromatography as described by Moore et al.²⁸

Antibiotic Susceptibilities/Resistances

Antibiotic susceptibilities and resistances were determined for each isolate that survived the identification process by agar dilution methodology, as described previously.²⁹ The antibiotics tested were doxycycline, tetracycline, minocycline, erythromycin, clindamycin, and amoxicillin. Each antibiotic was tested in increasing two-fold concentrations from 0.25 to 128 µg/ml.

Statistical Analyses

To account for the correlation among measures within a patient over time, generalized estimating equation (GEE) analysis³⁰ was used to fit linear regression models for the log₁₀ transformed anaerobic count and logistic regression models for the odds of doxycycline resistance (minimal inhibitory concentration [MIC] ≥16 µg/ml), the odds of cross-resistance between two antibiotics, and the odds of detection of opportunistic pathogens. The log₁₀ transformation was successful in normalizing the residuals from the regression models and stabilizing the variance. Covariates in the regression models included time, treatment, and their interaction, as well as randomization stratification factors, baseline smoking status, and study

^{||}Model W-225R, Heat Systems-Ultrasonics, Farmingdale, NY.

center. An exchangeable working correlation structure was used. For the percentage of bacteria resistant to doxycycline at 4 µg/ml endpoint, the average percentage resistant was calculated across the duplicate plates for each patient and each time point and was transformed using a natural logarithm transformation. The difference in the natural logarithm of the average between 24 months and baseline was used as the outcome measure in a linear regression model, adjusted for baseline smoking status and study center, to examine differences between study drugs. A similar approach was used for actinomyces and streptococci counts.

Analysis was done on the ITT sample and on a per-protocol sample, including those subjects who did not use steroids or bisphosphonates or antibiotics, those who were at least 80% adherent in taking the study drug based on pill counts, and those microbial collection sites that did not have extractions performed on adjacent teeth within 6 months. Observed data were analyzed and multiple imputation algorithms also were used to impute missing data values and analyses were rerun.³¹ Results based on the imputed data were very similar to the observed data results and are therefore not presented. Results for the per-protocol analyses were similar to the ITT results and are therefore not presented. Subgroup analyses were performed as defined by baseline smoking status, study center, and patient adherence to study medication. Tests of interactions were used to identify significant subgroup effects. Only those interactions significant at the 0.05 α level are reported.

RESULTS

One hundred twenty-eight eligible women, aged 45 to 70 years, underwent randomization. Regarding race, one hundred twenty-three women (96%) were white, three (2%) were Asian, and two (2%) were African American. Regarding ethnicity, 123 subjects were not Hispanic or Latino (96%), while five subjects were Hispanic or Latino (4%). The SDD and placebo groups were comparable and well-matched with respect to all baseline characteristics. Subjects were ~9 years postmenopausal on average; 63% of subjects were >5 years postmenopausal, and 37% were within 5 years of menopause. Twenty percent of subjects were current smokers, and 30% of subjects were former smokers.

One baseline sample was lost in transit and was not discovered in time for a replacement sample to be collected. Eighteen subjects were without 24-month samples because of a need for antibiotic premedication ($N = 3$) or subject dropout prior to the 24-month visit ($N = 15$). Across both treatment groups, $\geq 90\%$ of the subjects had at least one periodontal maintenance visit in each 6-month study period. No subject was missing microbiologic data at both sampled sites because of extractions or active periodontal therapy. Also, no sampled site was adjacent to teeth extracted within 6 months of the 24-month visit, and no sampled site had active treatment within 6 months of the 24-month visit. No more than 8% of the subjects in either treatment group were taking hormone replacement therapy, bisphosphonates, or steroids during each 6-month study period, and no more than 5% had taken more than two courses of antibiotics during each 6-month study period. Five placebo and two SDD patients took hormonal or steroid medications between the 12- and 18-month visits, and three placebo and two SDD patients took one of these between the 18- and 24-month visits. Observations from these subjects were excluded in the per-protocol analyses.

Based on ITT analysis, the odds of periodontitis progression for relative clinical attachment level, measured using an electronic probe,[¶] over 2 years were 19% lower in the SDD group than in the placebo group (odds ratio [OR] = 0.81; 95% confidence interval [CI]: 0.67 to 0.97; $P = 0.03$).

[¶]Florida Disk Probe, Florida Probe, Gainesville, FL.

Treatment Effect on Total Anaerobic Counts

Total anaerobic counts recovered were transformed by the $\log_{10}$ function and are summarized for each treatment at baseline and 24 months in Table 2.

In the SDD group, there was essentially no change from baseline to 24 months, and the slight reduction in the placebo group over 24 months was not statistically significant ($P > 0.4$ for each group). There was no statistically significant difference between placebo and SDD treatment groups in the change over time in the mean $\log_{10}$ anaerobic counts ($P = 0.96$). Furthermore, based on regression modeling, the mean change in $\log_{10}$ anaerobic counts over the 24-month period was estimated to be a 0.04-unit decrease for each treatment group, resulting in an estimated difference of 0 between the treatment group mean change values (95% CI: -0.16 to 0.16).

Smoking Effect

GEE regression analysis suggested that the effect of SDD relative to placebo over the 24-month period differed by subject smoking status ($P = 0.02$). Among smokers on placebo, the median $\log_{10}$ anaerobic counts increased over time from 6.90 to 6.99; however, for smokers on SDD, the median counts decreased over time from 7.06 to 6.88. For the non-smokers, there was essentially no change in the placebo group (from 6.95 to 6.91) or in the SDD group (from 7.15 to 7.18). These data seemed to be skewed slightly by the low anaerobic counts obtained for a few subjects. If these low counts are excluded, the interaction was no longer significant ($P = 0.14$).

Effect on Normal Flora

The effects of treatment on the actinomyces and streptococci, both of which are considered to be part of the normal subgingival flora, were determined by transforming the counts recovered to $\log_{10}$ function, with any 0 values set to 1 prior to transformation. Descriptive summaries of these measures are given in Table 3.

For the actinomyces and the streptococci, the measures changed only slightly over time, and there were no significant differences in the median changes over time between the treatment groups ($P = 0.5$ for actinomyces, $P = 0.3$ for streptococci).

Effect on Periodontal and Opportunistic Pathogens

The prevalence of each periodontal pathogen (*Porphyromonas gingivalis*, *Tannerella forsythensis*, and *Actinobacillus actinomycetemcomitans*) was $<1\%$ across time points and treatment groups. The opportunistic pathogens, *Candida*, *Staphylococcus aureus*, and enterics, also were not common, with prevalences no greater than 26%, 15%, and 35%, respectively, at each time point for each treatment group. There were no significant differences between the treatment groups for the change in the odds of detection over time for the more common pathogens ($P = 0.4$ for *Candida* and enterics). For *Prevotella intermedia*, *Eikenella corrodens*, and *Fusobacterium nucleatum*, there was no evidence of a change within either treatment group over time (data not shown).

Effect on Antibiotic Resistance

The effect of treatment on antibiotic resistance over time was analyzed to determine 1) whether an increase in resistance to doxycycline occurred as measured by a change in the MICs of doxycycline-resistant strains recovered and a change in proportion of strains considered to be clinically resistant; 2) the change or increase in specific species demonstrating resistance to doxycycline; and 3) the change in multiantibiotic resistance.

Percentage of bacteria recovered on doxycycline (4 µg/ml) medium.—The CFU recovered on the doxycycline-containing medium were converted to percentage of total recovered anaerobic flora by dividing the total CFU on the doxycycline medium by the total anaerobic counts recovered on the same basal medium but without doxycycline. A descriptive summary of these data is presented in Table 4.

Based on median values, a higher percentage of the total recovered flora was resistant to 4 µg doxycycline per milliliter at 24 months (43.19%) compared to baseline (4.99%) in the SDD treatment group, whereas there was little change in the placebo group over time (from 4.89% at baseline to 5.44% at 24 months). This difference in change over time between the two treatment groups was statistically significant ($P < 0.001$).

Change in MICs for strains resistant to doxycycline at 4 µg/ml.—Although a higher proportion of the recovered flora from SDD subjects was resistant to 4 µg/ml of doxycycline at the 24-month time point, this does not infer clinical or therapeutic resistance. The National Committee for Clinical Laboratory Standards (NCCLS) has established the following breakpoints for doxycycline: susceptible (≤ 4 µg/ml), intermediate (>4 to <16 µg/ml), and resistant (≥ 16 µg/ml). The doxycycline data were examined to determine the MIC required to inhibit 50% (MIC₅₀) and 90% (MIC₉₀) of the strains tested. The data also were examined to determine the proportions that fell into the classifications of susceptible, intermediate, and resistant. These data are summarized in Table 5.

There was no increase in the concentration of doxycycline required to inhibit 50% and 90% of the isolated strains at 24 months relative to baseline. Fifty percent of strains in the SDD group were inhibited at 24 months by 16 µg/ml of doxycycline, and 90% were inhibited by 64 µg/ml. This is the same or less than the placebo group at 24 months and the SDD group at baseline.

The examination of the proportion of these strains considered susceptible, intermediate, or resistant to doxycycline revealed that a small decrease (2.5%) occurred in the percentage of strains considered resistant at 24 months in the SDD group relative to baseline. This decrease was accompanied by a slight increase in the intermediate group (0.8%) and in the susceptible group (1.7%). Based on logistic regression modeling, the odds of resistance decreased over the treatment period by 51% (relative change) for the placebo group and by 14% (relative change) for the SDD group. These changes in resistance over the treatment period did not differ significantly between the groups ($P = 0.2$). These data indicated that treatment with SDD for 24 months did not result in a significant increase in susceptibilities to doxycycline (as determined by change in MICs) or a significant increase in the proportion of the isolates recovered that were characterized as resistant to therapeutic levels.

There was significant evidence that the effect of treatment over time differed by study center ($P = 0.01$, study center by treatment by time interaction). The change in resistance, as determined by MIC values ≥ 16 µg/ml, did not differ significantly between the SDD group (change from 84% of the isolates at baseline with MICs ≥ 16 µg/ml to 77% at 24 months) and the placebo group (from 77% at baseline to 71% at 24 months) among UNMC subjects ($P = 0.7$), but it did differ among Stony Brook subjects ($P = 0.004$). At Stony Brook, within the placebo group, the percentage of the isolates with MICs ≥ 16 µg/ml decreased from 92% at baseline to 68% at 24 months; within the SDD group, the percentage increased from 67% at baseline to 75% at 24 months. At the 24-month time point, there was no significant difference in the odds of resistance between the SDD and placebo groups at Stony Brook (OR = 1.62 [SDD relative to placebo]; 95% CI: 0.52 to 4.98, $P = 0.4$). The significant difference between the Stony Brook treatment groups in the change in the percentage of isolates with MICs ≥ 16 µg/ml likely was due to the high percentage at baseline among the placebo subjects.

The MIC₅₀ and MIC₉₀ of the other five antibiotics tested are given in Table 6. The only increases were noted with amoxicillin and clindamycin. In both treatment groups, there was a similar increase in the amount of amoxicillin and clindamycin required for inhibition of the bacterial isolates.

The percentage of strains that were resistant to doxycycline and each of the other five antibiotics tested was determined to investigate cross-resistance between antibiotics (Table 7). The values obtained at 24 months were similar for both treatment groups; furthermore, based on regression modeling, there was no significant evidence that changes in cross-resistance over time differed between the treatment groups for any of the five antibiotics ($P > 0.28$ for each).

The subcultures isolated from the doxycycline-containing medium that survived the identification process were examined for the proportion that each taxonomic group contributed to the total number isolated to determine whether changes had occurred in those species with resistance to 4 µg doxycycline per milliliter during the treatment period. The proportions and identity of the major bacterial taxa recovered at each sample period demonstrated no notable microbial changes between treatments over time. These data are summarized in Table 8.

DISCUSSION

Previous studies¹¹⁻¹³ of the subgingival flora reported that treatment with SDD for up to 9 months exerted no detectable antimicrobial effect on the bacteria recovered from periodontal pockets. A comprehensive study¹² involving 60 periodontally diseased adult subjects and conducted independently at two separate institutions over a 9-month treatment period followed by a 3-month no-treatment period failed to detect any antimicrobial effect on the subgingival microbial flora. There was no indication of a shift in the normal flora; no overgrowth or colonization by exogenous or endogenous pathogens; and no acquisition of, nor an increase in, antibiotic resistance.¹¹⁻¹³ A separate study¹⁴ of the same basic design found no antimicrobial effects of a 9-month regimen of SDD on the fecal or vaginal flora of an adult non-aggressive periodontitis population. The current study, although of longer duration and consisting of a larger number of subjects, yielded the same conclusions. Essentially, there were no differences in the total viable anaerobic counts recovered between treatments or within treatments over time. Likewise, there were no statistically or microbially significant changes in the actinomyces and/or streptococci associated with the normal subgingival flora. There were no increases in the periodontal pathogens or the opportunistic pathogens present over time or between treatments. However, there was a statistically significant increase in the proportion of the flora that was recovered on 4 µg/ml of doxycycline in the SDD treatment group at 24 months relative to baseline that was greater than the change seen among placebo patients at 24 months relative to baseline. The question is whether this increase in the number or proportion of the bacteria recovered on 4 µg/ml of doxycycline is clinically significant. The breakpoints as established by the NCCLS for determining susceptibilities/resistances for doxycycline are ≤4 µg/ml (susceptible), >4 to <16 µg/ml (intermediate), and ≥16 µg/ml (resistant). Based on these breakpoints, it would have been logical to use 16 µg doxycycline per milliliter to select for isolates that would be considered resistant to the drug. Historically, 16 µg/ml proved to be too high to yield a sufficient number of isolates for testing because samples from a number of periodontitis subjects failed to yield any recoverable isolates at this concentration (Walker, unpublished data). Conversely, the use of a very low concentration of doxycycline (e.g., 1 µg/ml) yielded too many isolates to make identification and susceptible testing economically feasible. Thus, 4 µg/ml was selected arbitrarily because it routinely yields a manageable number of bacterial isolates for testing.

Examination of the MICs obtained for the isolates recovered on 4 µg/ml of doxycycline did not demonstrate an increase in the proportions that were considered resistant (MICs ≥16 µg/

ml). The actual proportion of the strains resistant to 16 µg/ml decreased slightly from baseline to 24 months in the SDD group: 78.8% and 76.3%, respectively. A decrease of 13.1% was seen in the placebo group. The MIC₅₀ values for placebo and SDD showed a two-fold decrease, from 32 µg/ml at baseline to 16 µg/ml at 24 months; the MIC₉₀ value remained the same for placebo (128 µg/ml) and SDD (64 µg/ml) at baseline and 24 months. A statistically significant difference was detected between the two centers in the percentage of the isolates with resistance to ≥16 µg doxycycline per milliliter. Stony Brook had an unusually high percentage (92%) of the isolates in the placebo group at baseline with MICs ≥16 µg/ml, whereas the SDD group was considerably lower (67%). At 24 months, the percentages of isolates with MICs ≥16 µg/ml were similar, 68% and 75%, respectively, for the placebo and SDD groups. The latter percentages were similar to those associated with the UNMC subjects. The decline in the percentage considered resistant to therapeutic levels of doxycycline in the Stony Brook placebo group was considered to be due to the abnormally high proportion present at baseline. It is unclear why this group had such a high initial percentage of isolates with resistance to 16 µg doxycycline per milliliter.

Another parameter for evaluating changes in resistance is the major bacterial groups at each sample period that were recovered on the doxycycline medium. Although minor differences existed, as would be expected, there is no evidence that SDD selected for, or resulted in, the increase of a particular species or bacterial group over time. The composition of the bacterial strains recovered on medium containing 4 µg/ml of doxycycline was relatively similar in the bacterial species present and their relative compositions. There was no appearance of new resistant species in the SDD group at 24 months relative to baseline. The conclusions obtained from these different analyses of resistance markers indicated that there were no increases in clinical or therapeutic doxycycline resistance levels during a 24-month treatment period relative to baseline and placebo values.

The question still remains as to why an increase was seen in the proportion recovered on 4 µg/ml of doxycycline at 24 months. One possibility may have been compliance with the twice a day dosage. In retrospect, 2 years is a long time to expect subjects to adhere to a twice a day dosing schedule. A comprehensive review of the literature has confirmed the inverse relationship in compliance for the frequency of dosage and the length of time the dosage is given.³² Although the remaining tablets for placebo and doxycycline were counted at each clinical visit, this does not ensure that the prescribed dosage regimen was followed rigidly. Blood was collected from the subjects over the course of this study and was subjected to various analyses, including doxycycline blood levels. In some instances, the doxycycline levels were higher than the mean steady state of 0.4 µg/ml established for SDD, 20 mg twice a day.³³ This could have resulted if the subjects took the daily dose, 2 × 20 mg, simultaneously before the blood draw. There was no intermediate microbial sample point, so it is not known when the increased resistance to 4 µg/ml of doxycycline occurred or how long it may have been maintained. Regardless of the fact that an increase was found in the percentage of the flora recovered on 4 µg/ml of doxycycline, this did not translate into an increase in clinical resistance as determined by doxycycline concentrations required to inhibit 50% and 90% of the isolates, the proportion of the isolates considered clinically resistant to the drug, or the acquisition of resistance by previously susceptible bacterial species.

Examination of the relationship between doxycycline resistance (MIC) and resistances to each of the five other antibiotics failed to detect significant differences between the treatment groups. One would expect, if cross-resistance resulted from a common mechanism in response to doxycycline exposure, that the probability of cross-resistance would increase significantly over the treatment period in the SDD group relative to placebo. This was not the case, even for tetracycline and minocycline, which share similar resistant mechanisms with doxycycline. GEE regression analysis found that the odds of cross-resistance, based on MIC values, for

doxycycline and tetracycline decreased by 32% (relative change) and that for doxycycline and minocycline decreased by 40% over the 2-year treatment period for SDD subjects. There were no significant differences in the odds of resistance between doxycycline and tetracycline or minocycline between the two treatment groups over the treatment period ($P > 0.28$ for each).

The total sample size ($N = 128$ randomized patients) was justified based on the primary study endpoint, radiographic evidence of a clinically significant decrease in alveolar bone density from baseline. The study was not powered to establish equivalence between SDD and placebo in terms of the microbiologic endpoints. Although no significant microbiologic effects were observed, beyond the greater proportion of the flora that was recovered on medium containing 4 µg doxycycline per milliliter in the SDD group at 24 months, conclusions of equivalence cannot be drawn.

CONCLUSIONS

No evidence was present that the use of SDD for a period of 24 months in a population of periodontally diseased osteopenic women exerted any detectable effect on the microbial flora as determined by total anaerobic counts and total counts for actinomyces and streptococci. There was no evidence that SDD resulted in the colonization or overgrowth by periodontal and/or opportunistic pathogens. Although a greater proportion of the flora was recovered on medium containing 4 µg doxycycline per milliliter in the SDD group at 24 months, this did not translate into an increase in resistance to doxycycline as measured by the concentration of drug required to inhibit 50% and 90% of bacterial isolates or an increase in the proportion of the isolates considered to be therapeutically resistant. There was no evidence of emergence of resistance in species previously found to be susceptible in either treatment group. There also was no evidence that exposure to doxycycline, 20 mg twice a day, resulted in cross- or multiantibiotic resistance.

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Table 1

Target Microorganisms, Media, Incubation Conditions, and Confirmatory Tests for the Recovery and Enumeration of Various Bacterial Taxa Associated With Human Periodontal Pockets

Microbial Taxa or Species	Medium	Incubation Conditions	Confirmatory Tests
Total anaerobic counts	TSBA-HK ¹⁹	Anaerobic, 37°C, 5 to 7 days	None
Total facultative counts	TSBA-HK	10% CO ₂ , 37°C, 5 to 7 days	None
Total doxycycline-resistant counts	TSBA-HK with doxycycline (4 µg/ml) ¹³	Anaerobic, 37°C, 5 to 7 days	None
<i>Actinomyces</i>	CFAT ²⁰	Anaerobic, 37°C, 5 to 7 days	Colony morphology, Gram stain
<i>Streptococcus</i>	Mitis salivarius agar + tellurite ²¹	Anaerobic, 37°C, 5 to 7 days	Colony morphology, Gram stain
<i>Actinobacillus actinomycetemcomitans</i>	TSBV agar ²²	10% CO ₂ , 37°C, 5 to 7 days	Colony morphology, Gram stain, catalase reaction
<i>Eikenella corrodens</i>	Clindamycin agar [*]	Anaerobic, 37°C, 5 to 7 days	Colony morphology, oxidase test
<i>Porphyromonas gingivalis</i>	TSBA + HK	Anaerobic, 37°C, 7 to 10 days	Colony morphology, trypsin positive, UV negative
<i>Prevotella intermedia</i>	TSBA + HK	Anaerobic, 37°C, 7 to 10 days	Colony morphology, trypsin negative, UV positive
<i>Fusobacterium nucleatum</i>	CVE agar ²³	Anaerobic, 37°C, 5 to 7 days	Colony morphology, indol positive
Enterics	McConkey's agar ²⁴	Aerobic, 37°C, 2 to 3 days	Colonial and cellular morphologies
<i>Staphylococcus aureus</i>	Mannitol salt agar ²⁵	Aerobic, 37°C, 2 to 3 days	Yellow-pigmented colony
<i>Candida</i>	Mycosel agar ²⁶	Aerobic, 37°C, 2 to 3 days	Colonial and cellular morphologies

UV = short-wave ultraviolet light.

* Clindamycin agar consisted of TSBA supplemented with 4 µg clindamycin-HCl per milliliter of medium.

Table 2
Descriptive Summary of Log₁₀ Anaerobic Count Values for Placebo and SDD Treatment Groups at Baseline and 24 Months

Measurement	N*	Minimum	25th Percentile	Median	75th Percentile	Maximum
Placebo-baseline	126	5.86	6.79	6.94	7.08	7.68
Placebo-24 months	116	4.43	6.74	6.91	7.15	7.73
Placebo change [†] from baseline	57	-1.96	-0.25	-0.08	+0.25	+1.50
SDD-baseline	128	6.34	6.82	7.00	7.15	7.48
SDD-24 months	102	6.34	6.78	6.99	7.15	7.45
SDD change [†] from baseline	51	-0.93	-0.24	-0.03	+0.24	+0.82

* Duplicate counts obtained for each sample at baseline and 24 months or the number of patients for the change values.

[†] Change calculated as the mean 24-month measure minus the mean baseline measure.

Table 3

Descriptive Summary of Log₁₀ Actinomyces and Streptococci Counts for Placebo and SDD Treatment Groups at Baseline and 24 Months

Measurement	N*	Minimum	25th Percentile	Median	75th Percentile	Maximum
Actinomyces						
Placebo-baseline	124	0.00	6.31	6.60	6.83	7.20
Placebo-24 months	114	0.00	6.32	6.63	6.82	7.86
Placebo change [†] from baseline	55	-6.85	-0.44	-0.005	+0.35	+6.60
SDD-baseline	128	0.00	6.32	6.61	6.79	7.28
SDD-24 months	102	0.00	6.36	6.60	6.86	7.30
SDD change [†] from baseline	51	-6.67	-0.33	+0.087	+0.43	+6.34
Streptococci						
Placebo-baseline	124	0.00	5.51	6.53	6.86	7.34
Placebo-24 months	116	0.00	5.62	6.59	6.82	7.51
Placebo change [†] from baseline	56	-6.90	-0.45	+0.065	+1.13	+5.39
SDD-baseline	128	0.00	5.42	6.56	6.86	7.45
SDD-24 months	102	0.00	6.40	6.60	6.75	7.72
SDD change [†] from baseline	51	-2.97	-0.33	+0.019	+0.47	+6.51

* Duplicate counts obtained for each sample at baseline and 24 months or the number of patients for the change values.

[†] Change calculated as the mean 24-month measure minus the mean baseline measure.

Descriptive Statistics for the Percentage of Doxycycline-Resistant (4 µg/ml) Bacteria for Placebo and SDD Treatment Groups at Baseline and 24 Months

Measurement	N*	Minimum	25th Percentile	Median	75th Percentile	Maximum
Placebo-baseline	62	0.005	1.41	4.89	8.87	53.69
Placebo-24 months	58	0.00	1.80	5.44	19.14	77.11
Placebo change [†] from baseline	56	-42.17	-6.30	-0.18	+12.64	+73.41
SDD-baseline	63	0.005	1.38	4.99	23.39	72.50
SDD-24 months	49	0.18	13.81	43.19	58.00	97.37
SDD change [†] from baseline	48	-41.60	+0.26	+28.57	+47.94	+97.31

* Number of subjects; the duplicate measurements for each subject at each time point were averaged.

[†] Change calculated as the mean 24-month measure minus the mean baseline measure.

MIC₅₀ and MIC₉₀ of Bacterial Strains and the Percentage of Strains Considered to be Susceptible, Intermediate, or Resistant to Doxycycline Based on NCCLS Breakpoint

Table 5

	N*	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Susceptible (%) (≤4 µg/ml)	Intermediate (%) (>4 to <16 µg/ml)	Resistant (%) (≥16 µg/ml)
Placebo-baseline	129	32	128	10.9	6.2	83.0
Placebo-24 months	103	16	128	25.2	4.9	69.9
SDD-baseline	151	32	64	16.6	4.6	78.8
SDD-24 months	93	16	64	18.3	5.4	76.3

* Number of strains tested.

Table 6
MIC₅₀ and MIC₉₀ of Five Other Antibiotics for the Bacterial Strains Isolated on Doxycycline (4 µg/ml) Medium

	Amoxicillin		Erythromycin		Clindamycin		Minocycline		Tetracycline	
	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)
Placebo-baseline	2	>128	8	>128	1	>128	32	64	64	128
Placebo-24 months	8	>128	8	>128	4	>128	16	128	16	128
SDD-baseline	1	64	2	>128	1	>128	32	64	32	128
SDD-24 months	8	>128	2	>128	2	>128	16	64	16	128

Table 7

Percentage of Isolates Resistant to Doxycycline and Another Antibiotic for SDD and Placebo Treatment Groups at Baseline and 24 Months

	Placebo-Baseline	Placebo-24 Months	SDD-Baseline	SDD-24 Months
Minocycline	72.4	52.4	70.7	59.1
Tetracycline	74.4	56.3	66.2	57.0
Amoxicillin	30.0	41.8	20.1	38.7
Erythromycin	50.8	40.8	38.0	35.5
Clindamycin	29.8	41.8	24.8	31.2

Table 8

Percentage Present for Each Treatment and Period for the Predominant Bacterial Groups Isolated on Medium Containing 4 µg Doxycycline per Milliliter

Bacterial Taxa	Placebo-Baseline (n = 140) [*]	SDD-Baseline (n = 161) [*]	Placebo-24 Months (n = 128) [*]	SDD-24 Months (n = 139) [*]
<i>Actinomyces</i>	1.4	3.1	0.8	2.2
<i>Bacteroides coagulans</i>	2.9	1.2	13.3	4.3
Other <i>Bacteroides</i>	1.4	2.5	0.8	1.4
<i>Bifidobacterium</i>	1.4	1.2	1.6	1.4
<i>Clostridium clostridioforme</i>	6.4	6.2	9.4	10.1
<i>Campylobacter concisus</i>	14.3	9.9	7.0	5.0
Other <i>Campylobacter</i>	2.9	3.1	3.1	2.2
<i>Eubacterium</i>	1.4	1.9	1.6	0.7
<i>Fusobacterium</i>	10.7	10.6	4.7	5.0
<i>Lactobacillus</i>	2.9	1.9	0.8	3.6
<i>Neisseria</i>	2.1	3.1	0.8	1.4
<i>Prevotella</i>	1.4	1.9	1.6	1.4
<i>Streptococcus</i>	14.3	13.7	15.6	11.5
<i>Veillonella</i>	1.4	0.6	0.8	1.4
Facultative Gram-negative rod D27	3.6	4.3	3.1	3.6
Unidentified Gram-positive rods lost on subculture [†]	9.3	5.0	5.5	7.9
Unidentified Gram-negative rods lost on subculture [†]	8.6	13.7	9.4	7.9
Other [‡]	13.6	16.1	20.3	28.8

^{*} Total number of isolates recovered.

[†] Did not survive the identification process.

[‡] Represents a number of different species that were seen only once at each treatment period.