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Long-Term Treatment With Subantimicrobial Dose Doxycycline Exerts No Antibacterial Effect on the Subgingival Microflora Associated With Adult Periodontitis

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Background: The purpose of this study was to determine whether treatment with subantimicrobial dose doxycycline (SDD), 20 mg bid, exerted an antimicrobial effect on the microflora associated with adult periodontitis.

Methods: Following the approval of the protocol and informed consent forms by the respective IRBs at the (University of Florida and West Virginia University, 76 subjects with adult periodontitis were entered and randomly assigned to receive SDD or placebo. A split-mouth design was utilized, with each subject receiving subgingival scaling and root planing (SRP) in two quadrants immediately following baseline data collection, while the remaining two quadrants were left unscaled (non-SRP). Microbial samples were collected prior to treatment, after 3, 6, and 9 months of treatment, and after 3 months of no treatment. The samples were examined by microscopy and by enumeration on selective and non-selective media.

Results: All treatments resulted in statistically significant decreases in the proportions of spirochetes and motile rods ($P < 0.05$) and in an increase in the proportion of coccoid forms ($P < 0.0001$) relative to baseline. No between-treatment differences were detected between the SDD and placebo treatments in either the SRP or non-SRP design, with the exception of the small and large spirochetal groups. The spirochetal proportions present in the SDD group were significantly lower ($P < 0.05$) than the paired placebo group during the 9-month treatment and was preceded by a significant decrease ($P < 0.01$) in the proportion of microbiologic sample sites that bled on probing. No between-treatment differences were detected in any of the other microbial parameters.

Conclusion: The microbial differences observed were attributed to the anticollagenase and anti-inflammatory properties of SDD and not to an antimicrobial effect. *J Periodontol* 2000;71: 1465-1471.

KEY WORDS

Periodontitis/microbiology; doxycycline/therapeutic use; clinical trials, controlled.

Subantimicrobial dose doxycycline (SDD) consisting of 20 mg doxycycline hyclate[§] bid has been approved as an adjunct to periodontal scaling and root planing (SRP) for the treatment of adult periodontitis. Doxycycline, like tetracycline and minocycline, in addition to being a broad-spectrum antimicrobial agent, also has inhibitory activity on host-derived collagenases and other matrix metalloproteinases by mechanisms independent of its antimicrobial properties. Specifically, tetracyclines inhibit the activity of mammalian neutrophil and osteoblast collagenases that appear crucial in the destruction of Type I and II collagen found in the periodontal ligament.^{1,2} Apart from their anticollagenase activity, tetracyclines are also reported to have anti-inflammatory properties and to be potent inhibitors of osteoclast function.³ Doxycycline is the most potent anticollagenase inhibitor of the commercially available tetracyclines with IC50 values of 16 to 20 μM for collagenases from PMNs, dental plaque, and gingival tissue.^{4,5} Several short-term clinical studies have reported that SDD resulted in a decrease in collagenase activity which was accompanied by a beneficial and significant improvement in attachment

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levels and probing depths.^{6,7} More recently, a long-term, multi-centered clinical study compared the efficacy of a 9-month regimen of SDD following SRP to a placebo control and found that the use of SDD/SRP showed statistically significant improvements in attachment level and probing depth relative to SRP with a placebo.⁸

Substantial evidence indicates that the adjunctive use of SDD provides a significant benefit to SRP due to its anticollagenase and anti-inflammatory activities rather than to its antimicrobial activity. However, serious concern has been expressed that even subantimicrobial levels of doxycycline may exert a detrimental effect on the subgingival flora. Such an effect could result in the disruption or suppression of the normal flora and lead to its colonization or overgrowth by periodontal or opportunistic pathogens. The purpose of this study was to stringently evaluate the effects of a 9-month regimen of 20 mg doxycycline bid relative to a placebo control on the subgingival flora.

MATERIALS AND METHODS

Study Design

Clinical and microbial data were collected at the University of Florida and West Virginia University from subjects with adult periodontitis during a 9-month treatment period followed by a 3-month no-treatment period. Microbiological samples of subgingival plaque were collected prior to the initiation of treatment (baseline), after 3, 6, and 9 months of treatment, and at 3 months post-treatment. A total of 76 subjects (38 at each study site) with adult periodontitis who met the inclusion and exclusion criteria set forth in the experimental protocol were entered into the placebo-controlled, double-blind treatment phase.

The design of the study was as follows: A split-mouth design was used where two quadrants in each subject received scaling and root planing (SRP) while the opposite two quadrants did not (non-SRP). The quadrants selected to receive SRP were required to have a minimum of two sites with probing depth (PD) and loss of attachment level (AL) of ≥ 5 but ≤ 9 mm and that bled on probing. The non-SRP quadrants may or may not have met this criteria. Each subject was then randomly assigned to receive either SDD or placebo treatment. Thus, in effect, there were four treatment groups: SRP-SDD, non-SRP-SDD, SRP-placebo, and non-SRP-placebo. SRP-placebo was considered as a positive control, while non-SRP placebo was a true negative control. Thus, the study was considered to consist of two parallel experiments. SRP-SDD and non-

SRP-SDD were paired as were non-SRP-SDD and non-SRP-placebo so that the SDD was the variable tested.

All subjects who completed the 9-month treatment phase were invited to continue in a 3-month no-treatment phase. Of the 67 subjects who completed the

9-month treatment phase, 27 of 36 and 26 of 29 subjects at the University of Florida and West Virginia University, respectively, returned for sampling at the end of the 3-month no-treatment period.

A total of 4 sites, distributed in a minimum of 3 quadrants (4 quadrants were selected where possible), with PD ≥ 5 mm but ≤ 8 mm were selected in each subject for microbial sampling; two sites were from the SRP quadrants and two from the non-SRP quadrants. These sample sites were retained throughout the study. Plaque samples were collected using sterile endodontic paper points as previously described.⁹ The two microbial samples collected from the SRP sites were pooled by subject and then processed, as were the two samples from the non-SRP sites.

Microbial Enumeration

Immediately following collection, samples were transported to the microbiology laboratories. The samples were gently sonicated to dispense adherent plaque and then processed. Each sample was examined by direct microscopy and by culture on selective and non-selective media.

Microscopy. A 10 μ l aliquot of the sample was removed under anaerobic conditions and placed on a clean slide for examination at 1,000 \times by dark-field microscopy. Eight distinct cellular morphotypes were distinguished and enumerated as previously described.¹⁰

Selective and non-selective media. Following a series of 10-fold dilutions in pre-reduced, anaerobic-sterilized Ringers solution, performed under strict anaerobic conditions, 0.1 ml aliquots were dispensed onto agar plates and spread with sterile glass rods. The following taxa were enumerated on selective and non-selective media: total anaerobic counts, total facultative counts, total *Streptococcus*, total *Actinomyces*, *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, enteric bacteria, *Staphylococcus aureus*, and *Candida*. Estimates of obligate anaerobic bacteria were determined by subtracting the total facultative count from the total anaerobic count. If the facultative count was greater than the anaerobic count, a zero value was entered for the obligate anaerobes. Bacteria tentatively identified as *P. intermedia* are, in reality, *P. intermedia sensu lato* since *P. intermedia* was not differentiated from *P. nigrescens*.

Statistical Analyses

The study was considered to consist of two parallel experiments, each of which was designed to test for differences between doxycycline treatment and a placebo control. One design sought for differences following conventional periodontal treatment consisting of mechanical scaling and root planing (SRP), and the second sought for differences without the initial peri-

odontal therapy of scaling and root planing (non-SRP). With this in mind, the resulting data sets were analyzed with the subject as the statistical unit to detect if differences existed at any sample period between doxycycline-treated and placebo-treated subjects.

The factorial ANOVA and Fisher's PLSD test were utilized to determine if statistically significant differences were present between the paired treatment groups at each sample period. The repeated measures ANOVA was used for longitudinal analyses to test for differences within a treatment. If differences were detected longitudinally, the paired *t* test was used to detect the location of the differences. In cases where outliers were suspected, e.g., microbial culture counts that could influence parametric analyses, the Wilcoxon signed rank, a non-parametric version of the paired *t* test, was used to verify statistical significance. Since the paired *t* test and Wilcoxon signed rank require matched samples from the same subject and the 3-month post-treatment data were derived from fewer subjects than the 9-month data set, it was necessary to construct a new data set limited to those subjects who consented to participate in the 3-month no-treatment phase for analyses seeking differences in the latter.

A total of 78 subjects were entered at the two study sites with the expectation that a minimum of 65 subjects would complete the 9-month treatment phase of the study. This sample size, if equally split, had a 90% power of detecting a difference of 1 log₁₀ in microbial counts between SDD and the paired treatment. All statistical comparisons were based on *P* ≤ 0.05.

RESULTS

Microscopic Enumeration

Differences between and within treatment groups were analyzed for each of the following morphological groups: small, intermediate, and large spirochetes; motile rods; coccoid forms; non-motile rods; fusiforms; and filamentous rods.

Between-treatment differences. No between-treatment differences were detected for any morphological group other than the spirochetes. In the SRP design, the proportion of small spirochetes (Table 1) present at the 3- and 6-month sample periods and the proportion of large spirochetes (Table 2) present at the 6-month sample were significantly lower in the SDD group than in the placebo group (*P* < 0.05). In the non-SRP design, the proportions of both the small and large spirochetes present at the 9-month sample were significantly lower in the SDD group than in the placebo group (*P* < 0.05).

Within-treatment differences. Differences within a treatment were analyzed using the paired *t* test to detect if the treatment had any significant effect on a particular morphologic group. Both the SDD and

Table 1.

Mean Percentage of Small Spirochetes Relative to Total Microscopic Flora for SDD and Placebo Treatment Groups in SRP and Non-SRP Design

Treatment Group	Baseline	3 Months Post	
		SRP	Non-SRP
SDD	10.35	3.28†	4.95†
Placebo	10.36	10.40*	5.89†
SDD	9.98	7.36	6.58*
Placebo	11.42	9.51	9.79*

* Statistically significant differences (*P* ≤ 0.05) between SDD and placebo treatment groups

† Statistically significant within-treatment differences (*P* ≤ 0.05) relative to baseline

Table 2.

Mean Percentage of Large Spirochetes Relative to Total Microscopic Flora for SDD and Placebo Treatment Groups in SRP and Non-SRP Design

Treatment Group	Baseline	3 Months Post	
		SRP	Non-SRP
SDD	3.34	0.13†	0.58†
Placebo	4.29	1.13*	1.74†
SDD	3.22	0.56†	0.56*†
Placebo	3.13	1.25†	1.79*†

* Statistically significant differences (*P* ≤ 0.05) between SDD and placebo treatment groups.

† Statistically significant within-treatment differences (*P* ≤ 0.05) relative to baseline.

placebo treatments, regardless of SRP or non-SRP design, produced statistically significant reductions in both the intermediate and large spirochetes (Tables 2 and 3). In the SRP design, the SDD treatment yielded significant reductions in small spirochetes, relative to baseline, for all 9 months of treatment, while the placebo treatment demonstrated only significant reductions at the 9-month sample period (Table 1). Significant reductions in the proportion of motile rods were detected for all treatments at all sample periods relative to baseline (Table 4). Significant increases (*P* < 0.0001) were found in the proportion of coccoid forms, relative to baseline, for all sample periods (Table 5). No significant changes were noted during any treat-

Table 3.

Mean Percentage of Intermediate Spirochetes Relative to Total Microscopic Flora for SDD and Placebo Treatment Groups in SRP and Non-SRP Design

Design	Treatment Group	Baseline	Treatment Period			3 Months Post
			3 Months	6 Months	9 Months	
SRP	SDD	15.57	19.06*	1.62*	1.88*	
	Placebo	13.43	13.05	3.77*	5.63*	
Non-SRP	SDD	13.56	13.05	2.85*	5.86*	
	Placebo	13.94	13.05	4.00*	6.33*	

* Statistically significant within-treatment differences ($P \leq 0.001$) relative to baseline.

Table 4.

Mean Percentage of Motile Rods Relative to Total Microscopic Flora for SDD and Placebo Treatment Groups in SRP and Non-SRP Design

Design	Treatment Group	Baseline	Treatment Period			3 Months Post
			3 Months	6 Months	9 Months	
SRP	SDD	6.93	6.93	2.29*	1.55*	
	Placebo	6.63	6.63	1.23*	1.93*	
Non-SRP	SDD	8.64	8.64	1.79*	2.64*	
	Placebo	6.53	6.53	1.66*	3.46*	

* Statistically significant within-treatment differences ($P \leq 0.05$) relative to baseline.

ment in the proportion of non-motile rods, fusiforms, or filamentous rods present at any sample period.

Culture Enumeration

As with the microscopic parameters, data analyses were conducted to detect statistically significant differences both between and within the treatment groups.

Between-treatment differences. With one single exception, no statistically significant differences ($P > 0.300$) were detected between SDD and placebo treatments in either the SRP or non-SRP design at any sample period for the total cultivable bacterial mass (total anaerobic counts, total facultative counts, or obligate anaerobes), normal flora (total streptococci, total actinomyces), putative periodontal pathogens (*P. gingivalis*, *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans*, or *E. corrodens*), or opportunistic pathogens (*Candida*,

enteric bacteria, or *S. aureus*). The only exception was that the total facultative counts were significantly higher ($P = 0.0146$) in the placebo treatment compared to the SDD treatment group in the SRP design at the 6-month sample period. No differences were detected between treatments at any other sample period ($P > 0.3000$).

Within-treatment differences. The means of the colony forming units (CFUs) for total anaerobic counts, facultative counts, and obligate anaerobes obtained at each sample period for each treatment are given in Figures 1 through 3. Statistically significant increases were detected with the paired *t* test in both the total anaerobic counts and the obligate anaerobes present at 3 months relative to baseline for the SDD and placebo treatments in both designs. Significant increases were also detected at 6 months, relative to baseline, for both the SDD and placebo treatments in the non-SRP design. However, when these data were reanalyzed using the Wilcoxon signed rank test to minimize the effects of extreme outliers, statistically significant increases were detected only in the placebo treatment in the non-SRP design for the total anaerobic counts and the obligate anaerobes at 3 and 6 months relative to baseline ($P < 0.02$). Significant increases were noted in the number of facultative counts present at 6 months relative to baseline for the placebo treatment in both the SRP and non-SRP designs, but these increases were not statistically significant when reanalyzed using the Wilcoxon signed rank test. No statistically significant differences were detected within the SDD or placebo treatment groups in the SRP and non-SRP design by either the paired *t* test or Wilcoxon signed rank test in any of the following microbial groups: streptococci, *Actinomyces*, *P. gingivalis*, *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans*, *E. corrodens*, *Candida*, enterics, or *S. aureus*.

Clinical Parameters Associated With Microbial Sample Sites

Since statistically significant microbial decreases, either between or within treatments, during the 9-month treatment period were associated with motile groups (spirochetes and motile rods) that have been used as indicators of disease activity, the clinical indices obtained for the microbiology sample sites at each sample period were analyzed.

Between-treatment differences. No statistically significant differences were detected between the SDD and placebo treatments in the SRP design for either AL or PD at any sample period. However, in the SRP design, the percentage of BOP sites (Fig. 4) in the SDD group was significantly lower ($P < 0.01$) than the placebo group at all sample periods following baseline. In the non-SRP design, significant gains ($P < 0.01$) in AL were present in the SDD group at 3, 6, and 9

Table 5.
Mean Percentage of Coccoid Forms Relative to Total Microscopic Flora for SDD and Placebo Treatment Groups in SRP and Non-SRP Design

Treatment Group	Baseline	3 months	6 months	9 months
SDD	14.87	37.04*	41.36*	
Placebo	13.11	31.31*	37.20*	
SDD	14.23	39.05*	39.32*	
Placebo	13.20	30.54*	30.92*	

* Statistically significant within-treatment differences ($P < 0.0001$) relative to baseline.

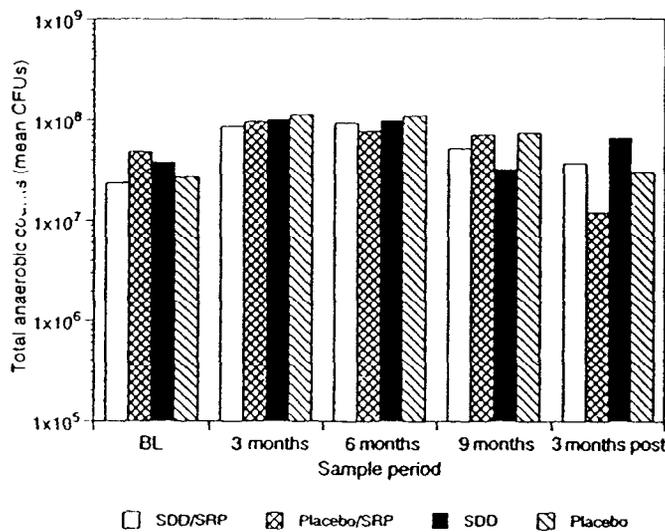


Figure 1.
 Total cultivable anaerobic counts (means) obtained for each treatment at each sample period.

months and fewer sites bled on probing ($P < 0.005$) at 6 and 9 months. No differences were detected in PD at any sample period.

Within-treatment differences. Statistically significant ($P < 0.0001$) increases in AL and decreases in PD were detected at 3, 6, and 9 months, relative to baseline, regardless of treatment or design. No significant differences were detected between either the 3-, 6-, or 9-month measurements relative to each other. Significant decreases in proportion of BOP sites (Fig. 1) were noted at 3, 6, and 9 months, relative to baseline, for the SDD group in the SRP design ($P < 0.0005$) and in the non-SRP design ($P < 0.01$). Significant decreases

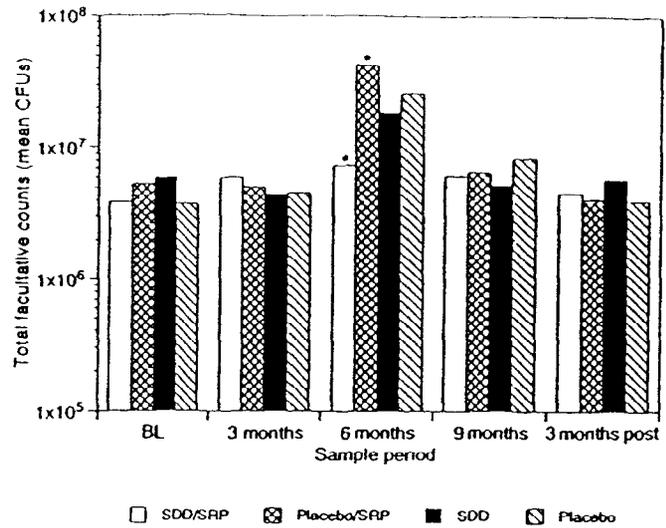


Figure 2.
 Total cultivable facultative counts (means) obtained for each treatment at each sample period (*statistical significance between treatments, $P < 0.02$).

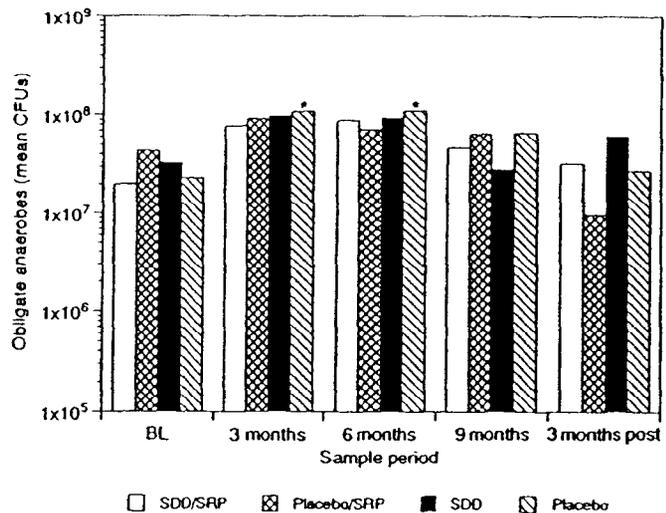


Figure 3.
 Obligate anaerobic counts (means) obtained for each treatment at each sample period (*statistical significance relative to baseline, $P < 0.02$).

in BOP sites were noted in the placebo group in the SRP design at 3 and 6 months ($P < 0.001$) relative to baseline but not at 9 months, and in the placebo group in the non-SRP design at 3 months ($P < 0.005$) but not at 6 or 9 months.

DISCUSSION

The principal objective of this investigation was to determine whether SDD exerted any detectable effect on the subgingival flora that could be attributed to

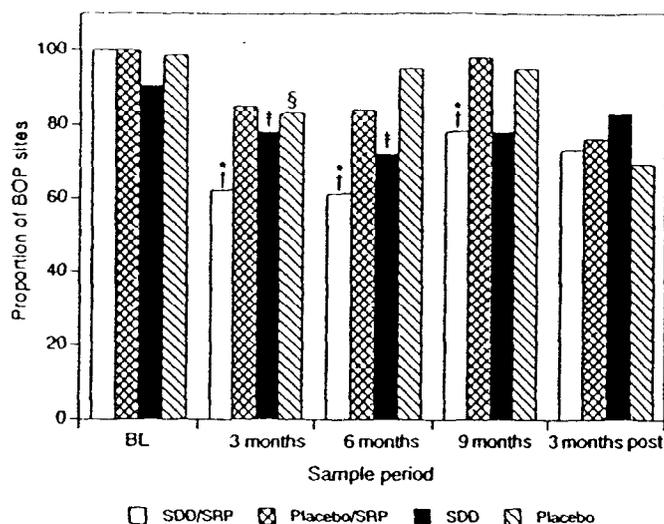


Figure 4.

Percentage of microbial sample sites bleeding on probing for each treatment at each sample period (*statistical significance between treatments, $P < 0.01$; †relative to baseline, $P < 0.0005$; ‡relative to baseline, $P < 0.01$; §relative to baseline, $P < 0.005$)

antimicrobial activity. Doxycycline is normally given at a daily dose of 100 mg, following a loading dose of 200 mg, which yields biologically active levels of 8 to 16 $\mu\text{g/ml}$ in the gingival crevicular fluid and around 4 $\mu\text{g/ml}$ in the blood.¹¹ Studies in human volunteers have demonstrated that 20 mg doxycycline bid yields steady-state serum concentrations of 0.6 to 0.8 $\mu\text{g/ml}$ (unpublished data). This level is considerably below the minimal inhibitory concentration (MIC) determined in vitro for the vast majority of the bacteria isolated from the subgingival flora.^{12,13} Since subgingival plaque exists as a biofilm rather than in a planktonic state,¹⁴ even higher drug concentrations are probably necessary for in vivo inhibition. Even so, the possibility exists that levels obtained with SDD might be inhibitory for certain bacteria that are exquisitely sensitive to the tetracyclines. Therefore, in this study, a comprehensive microbial examination of the subgingival flora was conducted by microscopy and culture enumeration in an attempt to detect differences between and within treatments that could be contributed to an antimicrobial effect.

No statistical or microbiological differences in any of the microbial parameters enumerated were detected between SDD and placebo treatments in either the SRP or non-SRP design, with the exception of the spirochetes. In both designs, the small and large spirochetal groups were found to be significantly lower at certain periods in the SDD treatment than in the corresponding placebo group. There are several possible explanations for the suppression of the spirochetes in the SDD groups. One is that the levels of doxycycline obtained in the periodontal pocket are inhibitory for

these organisms. Although the large spirochetes have not been cultivated and their sensitivity to the tetracyclines is unknown, it is generally thought that the small spirochetes are relatively sensitive to the tetracyclines, although resistance has been reported.¹⁵ Therefore, it might be argued that the suppression of the spirochetes was due to the antimicrobial activity of doxycycline. However, other bacterial groups are equally sensitive, if not more so. Almost all isolates of *P. gingivalis* are inhibited in vitro by $\leq 0.25 \mu\text{g/ml}$ of the tetracyclines.^{12,13,16} Neither we nor a number of other investigators have been successful in isolating wild-type strains of this organism with naturally occurring resistance to the tetracyclines. In the study reported here, there were no differences between treatments at either West Virginia University or the University of Florida in the numbers of *P. gingivalis* recovered at any sample period. This tends to argue against the possibility that the decrease in the relative proportion of the spirochetes was due to antimicrobial activity, since corresponding decreases in the numbers or proportions of *P. gingivalis* were not found.

Another possibility that has been advanced is that the decrease in spirochetes was due to the periodontal pocket becoming more aerobic. Since the spirochetes are thought to have a relatively low redox (Eh) requirement for growth,¹⁷ an increase in the Eh of the pocket might favor the growth of more oxygen-sensitive species at the expense of the spirochetes. However, this would most likely occur following mechanical disruption of the structure of the plaque biofilm. If this were the case, one would not expect to find treatment differences between SDD and placebo treatments in the SRP design, since both groups received periodontal scaling prior to the adjunctive treatment regimen.

The most likely explanation for the observed spirochetal differences between treatments is probably related to an improvement in the health of the periodontal pocket. There was significantly less inflammation as determined by the proportion of sites bleeding on probing in both SDD groups relative to placebo. The proportion of bleeding sites was significantly lower in the SDD/SRP group than the placebo group at 3, 6, and 9 months ($P < 0.005$) and in the SDD/non-SRP group at 6 and 9 months ($P < 0.005$). Within-treatment analyses revealed statistically significant improvements for all treatments in AL, PD, and BOP. Concurrently with these improvements in clinical indices, within-treatment analyses detected statistically significant decreases in spirochetes and motile rods with corresponding increases in coccoid forms. Since microscopic motility and bleeding on probing are often useful as indicators of disease activity, it seems reasonable to expect some relationship between the two. Therefore, we think the most logical explanation for the

between-treatment differences in spirochetes is that the microbial sample sites improved in health due to the anti-inflammatory properties of the drug so that fewer nutrients were available to support the growth of spirochetes. It could be argued that the decrease in the spirochetal population was responsible for the improvement in health, with the decrease being due to the antimicrobial activity of the drug. We do not think that this is likely due to the fact that between-treatment differences were not detected in any of the other microbial parameters. If the decrease in the number of sites bleeding on probing was due to an antimicrobial effect, between-treatment differences in microbial parameters should occur prior to the detection of improvements in clinical indices. In this study, the proportion of sites bleeding on probing had decreased prior to the detection of significant between-treatment differences in the proportions of small and large spirochetes. Since the clinical effect was observed before the microbial effect, we think this supports the hypothesis that the between-treatment differences were due to the drug's anti-inflammatory effect rather than to its antimicrobial effect.

In conclusion, no antimicrobial effect could be detected during or following a 9-month treatment regimen with 20 mg doxycycline bid, relative to placebo control, on total bacterial counts, the normal flora, or on either periodontal or opportunistic pathogens. Doxycycline had no detectable antimicrobial effect on 21 different microbial parameters commonly used to evaluate changes in the subgingival microflora.

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