Minocycline HCl Microspheres Reduce Red-Complex Bacteria in Periodontal Disease Therapy

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Background: The objective of this trial was to measure the antimicrobial effects of a minocycline HCl microsphere (MM) local drug-delivery system when used as an adjunct to scaling and root planing (SRP). DNA probe analysis for 40 bacteria was used to evaluate the oral bacteria of 127 subjects with moderate to advanced chronic periodontitis.

Methods: Subjects were randomly assigned to either SRP alone (N = 65) or MM + SRP (N = 62). The primary endpoints of this study were changes in numbers and proportions of the red-complex bacteria (RCB) and the sum of *Porphyromonas gingivalis*, *Tannerella forsythia* (formally *T. forsythensis*), and *Treponema denticola* relative to 40 oral bacteria at each test site from baseline to day 30. Numbers of RCB from the five test sites were averaged to provide a value for each subject.

Results: MM + SRP reduced the proportion of RCB by 6.49% and the numbers by 9.4×10^5. The reduction in RCB proportions and numbers by SRP alone (5.03% and 5.1×10^5, respectively) was significantly less. In addition, MM + SRP reduced probing depth by 1.38 mm (compared to 1.01 mm by SRP alone), bleeding on probing was reduced by 25.2% (compared to 13.8% by SRP alone), and a clinical attachment level gain of 1.16 mm (compared to 0.80 mm by SRP alone) was achieved.

Conclusion: These observations support the hypothesis that RCBs are responsible for periodontal disease and that local antimicrobial therapy using MM + SRP effectively reduces numbers of RCBs and their proportions to a greater extent than SRP alone. *J Periodontol 2007;78:1568-1579.*

KEY WORDS

Bacteria; dental scaling; drug delivery systems; microbiology; minocycline; periodontitis.

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Studies[1,2] of the microbial environment of periodontal disease have led to the recognition of the importance of red-complex bacteria (RCB; i.e., *Porphyromonas gingivalis*, *Tannerella forsythia* (formally *T. forsythensis*), and *Treponema denticola*) as a climax chronic periodontal disease–associated ecology. Many consider microbiologic response to be a prime requisite for effective periodontal therapy.[2] However, because the microbiologic effects after intrapocket delivery of antimicrobials are not well understood, many important questions have not yet been addressed. By focusing on the therapeutic outcome of reducing the proportion of RCB, we seek to evaluate periodontal treatments in their ability to shift microbial ecology from a pathogenic state to a condition of relative health.

The RCBs are all Gram negative, anaerobic, and asacharolytic, with virulence factors that include an array of proteolytic enzymes.[3] *P. gingivalis* and *T. forsythia* are rod-shaped bacteria, whereas *T. denticola* is a spirochete. Together, these three species have been strongly associated with chronic periodontal disease[3] and recognized as periodontal pathogens. The purpose of this study was to measure the antimicrobial effectiveness of minocycline HCl microspheres (MM)** using DNA probe technology adjunctively with

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scaling and root planing (SRP) in patients with moderate-to-advanced periodontal disease.

MATERIALS AND METHODS

This was a phase IV, five-center, single-blind, randomized, parallel-group study of 127 subjects with moderate-to-advanced chronic periodontitis who had at least five teeth with probing depths (PDs) ≥5 mm (test sites). Subjects were randomly assigned to receive either SRP alone (N = 65) or MM + SRP (N = 62), using a precomposed, pairwise, random-assignment strategy balanced for smoking, which allowed for smoking or non-smoking pairs. All subjects received an informed consent that had been reviewed and approved by the Institutional Review Board (IRB) of each center. In addition, the total project protocol was reviewed and approved by the Forsyth IRB. All examiners were calibrated for probing technique and clinical attachment level (CAL) evaluation before the study, and each of the five centers were calibrated on plaque sampling and processing by a single gold standard study coordinator.

Subjects

Subjects were recruited voluntarily from five periodontology clinics in the United States. A total of 130 subjects were enrolled in this trial between January 21, 2004 and August 12, 2004. Three subjects were excluded: one with incomplete data and two with periodontal abscess formation. Hence, 127 subjects were available for complete clinical and microbiologic analysis. Subjects were included if they were willing and able to read, understand, and sign the informed consent form; had good general health as evidenced by their medical history; were between 30 and 65 years of age; were available for the duration of the study; were willing to discontinue use of any mouthrinse for the duration of the study; had ≥16 teeth (excluding third molars and implants); and had five sites with PDs ≥5 mm in five non-adjacent interproximal spaces, excluding the distal of terminal teeth. Subjects were excluded from the study if they were pregnant, lactating, or were women of child-bearing age not using acceptable methods of birth control (e.g., hormonal, barriers, or abstinence). Subjects were also excluded if they had any periodontal therapy within the previous 3 months (excluding maintenance therapy); had received systemic or local antibiotic therapy within 3 months of enrollment; had a condition requiring prophylactic antibiotics; had systemic conditions that could influence the course of periodontal disease (i.e., diabetes or autoimmune disease); had aggressive periodontitis, acute necrotizing ulcerative gingivitis, or gross dental decay; or were allergic to any of the tetracyclines.

The population was composed entirely of outpatients of unrestricted gender or race with no serious systemic diseases. Patients were balanced by smoking (yes or no); a smoker was defined as an individual who had smoked ≥10 cigarettes on a daily basis within the past 12 months.

Study Conduct

At each center, single-blind conditions were maintained by assuring that the examiner was not aware of the subject treatment, and all treatments were administered by a separate clinician. A single gold standard coordinator calibrated examiners at each of the five sites for probing technique and CAL evaluation. The technique for plaque sampling was in accordance with the Forsyth standard method, and the five centers were calibrated on plaque sampling and processing according to this method. All subjects received a full-mouth examination to measure baseline PD, bleeding on probing (BOP), and CAL. At the screening visit, five non-adjacent test sites were selected with PD ≥5 mm. Subgingival plaque samples were collected for microbiologic (DNA probe) analysis from the test sites at baseline. All subjects were treated with full-mouth SRP. Subjects were assigned by pairwise randomization to either SRP alone or MM + SRP. Full-mouth measurements of PD, BOP, and CAL were repeated on day 30, and subgingival plaque samples were taken again from the same five designated test sites.

Treatments

All subjects received baseline full-mouth SRP at a maximum of two visits ≤10 days apart. Use of hand curets, ultrasonic instruments, and local anesthesia was permitted. This procedure was performed by a periodontist, dentist, or registered dental hygienist affiliated with the study center.

For those subjects randomized to the local antibiotic treatment arm of the study, a single-unit dose of MM containing 1 mg minocycline and ~3 mg polyglycolic acid in a microsphere formulation was administered to all periodontal pockets ≥5 mm. The dispenser tip was inserted subgingivally at the treatment site. The clinician depressed the plunger until resistance was felt and slowly withdrew the dispenser coronally while continuing to dispense the unit dose of MM. Patients were instructed to postpone brushing their teeth for 12 hours and to abstain from using interdental cleaning devices for 10 days after the local antimicrobial treatment.

Sample Collection

Five non-adjacent test sites with pockets ≥5 mm were selected in advance for sampling. Plaque samples from each were collected using sterile Gracey curets. A single scaler sample was taken by placing the curet at the base of the pocket and scraping along the full length of the root surface. The plaque sample removed by this procedure was transferred to a labeled

snap-top vial containing 150 μl of Tris buffer (TE, Tris-EDTA [10 mM Tris, 1 mM EDTA]). On completion of the sample-taking procedure, samples were made alkaline by addition of 100 μl freshly made 0.5 M sodium hydroxide solution. All microbiologic assessments were conducted at The Forsyth Institute. A previous study[4] indicated that the DNA of bacterial plaque samples prepared in this manner are stable at an ambient temperature for ≥3 months, and all samples were assayed within that time interval.

Microbiologic Enumeration of Organisms

The methods for enumeration of bacteria used in this study have been described in detail elsewhere.[4,5] On receipt, each plaque sample was boiled for 5 minutes and neutralized using 0.8 ml 5 M ammonium acetate. Samples were placed into the extended slots of a minislot,[††] concentrated onto a nylon membrane[‡‡] by a vacuum, and fixed to the membrane by exposure to ultraviolet (UV) light followed by baking at 120°C for 20 minutes.

Digoxigenin-labeled, whole-genomic DNA probes were prepared using a random primer technique. This involves growing bacteria on agar or in broth media, harvesting the cells, and extracting, purifying, and labeling the bacterial DNA from each of the test species. Each harvested cell growth was transferred to a microcentrifuge tube containing 1 ml TE buffer. After washing the cells, either proteinase K (for Gram-negative strains) or lysozyme plus achromopeptidase (for Gram-positive strains) was added, and the mixture was incubated at 37°C for 1 hour to disrupt the cell walls. DNA was isolated and purified, and the concentration was determined by UV absorption at 260 nm. The DNA was labeled with digoxigenin using a random-primer technique.

Whole cell DNA standards were prepared by adjusting the optical density (600 nm) of cell suspensions harvested from agar plates or broth media to an optical density of 1.0 ($\sim 10^9$ cells). Subsequent dilutions were based on microscopic counts in a defined volume. Routine standards (10^5 and 10^6) used on each checkerboard run were prepared from a DNA cocktail of all 40 bacterial species, each adjusted to provide the same signal as the whole cell standard. The DNA probe method was adjusted to detect $\sim 10^4$ bacteria (sensitivity), with 93.5% of cross-reactions exhibiting <5% of the homologous probe signal (specificity).[4]

Microbiologic data were recorded by computerized techniques as $100 \times \log_{10} N$, with N being the number of bacteria in each of the 40 species monitored for each of the five teeth in the study participants.

Analyses

The primary endpoint of this study was changes in the proportion of the RCBs. Numbers of RCB from the five test sites were averaged to provide a single-value estimate for each subject. The RCB prevalence (proportion) was computed as the sum of the numbers of *P. gingivalis*, *T. forsythia*, and *T. denticola* divided by the sum of the numbers of all 40 bacteria measured. Secondarily, the numbers of RCB themselves were evaluated.

Secondary outcome variables included mean numbers of *P. gingivalis*, *T. forsythia*, and *T. denticola* taken individually, prevalence of *P. gingivalis*, *T. forsythia*, and *T. denticola*, change in PD, change in CAL, and change in BOP at all sites with PD ≥5 mm at baseline. Discrete baseline demographic differences between treatment groups were evaluated using Pearson χ^2. Differences in continuous variables (i.e., age, microbiologic measures, and clinical measures) were evaluated using a one-way analysis of variance (ANOVA). Outcome measures were assessed for underlying distributional characteristics, and $\log_{10}$ transformations were performed as necessary. ANOVA and analysis of covariance (ANCOVA) were used to evaluate mean differences in measurements from baseline to 30 days between treatment groups, as well as for possible differences between centers and interactions between the two effects. No interim analyses were performed. Statistical significance was determined by $P < 0.05$ between treatment groups.

Microbiologic analysis for changes in RCB was evaluated by considering bacterial numbers and proportions in both their native and $\log_{10}$-transformed formats. The most commonly used analytical procedure was ANCOVA at 30 days, which was measured by using the baseline values as the covariate and the 30-day values as the outcome. When normality conditions were improved, analysis of $\log_{10}$-transformed values was selected; when they seemed to be violated by all distributions, non-parametric Kruskal-Wallis analysis was used. The Kruskal-Wallis analysis was also used when testing all 40 bacteria for significant change. In this case, $P < 0.001$ was used as a significance criteria to compensate for multiple testing. The distributional characteristics of the proportion, number, and $\log_{10}$ transformations of baseline RCBs measured with a DNA probe are shown in Figure 1. The $\log_{10}$ transformation of bacterial proportion and numbers were reasonably symmetrical (more closely reflecting a normal distribution), whereas the distributions of RCB proportions and numbers were skewed. Thus, statistics based on normality assumptions could reasonably be computed for the $\log_{10}$-transformed bacterial proportions and the $\log_{10}$-transformed number of bacteria. Statistical analysis of variables that were neither normal alone nor made normal by

log$_{10}$ transformation was accomplished by non-parametric analysis using the Mann-Whitney U test.

Power analysis based on data reported in a previous study[6] revealed that 64 subjects per treatment group would achieve 80% power to detect a difference of 0.4 between the null hypothesis that both group means are 0.8 for mean total sum of primary pathogens and the alternative hypothesis that the mean of the SRP-alone group is 0.4. In addition, the estimated group SDs are 0.8 with a type I error rate of 5%.

Figure 1.
Distribution of DNA probe measurements and log$_{10}$ transformations of RCB values at baseline.

RESULTS

A total of 127 subjects were included in the efficacy analysis: 62 subjects in the MM + SRP group and 65 subjects in the SRP-alone group. The demographic distribution of subjects included in the MM + SRP group and the SRP-alone group was well matched at baseline (Table 1): mean age was 50.9 and 48.9 years, 39% and 54% were women, 63% and 60% were white, and 19 and 18 subjects were current smokers, respectively. Of the clinical measures, no significant differences in baseline PD, numbers of pockets, BOP, or CAL between treatment groups were detected. The mean minocycline dose exposure of the 62 subjects treated with MM was 24.0 ± 12.7 mg (range, 5 to 72 mg) based on a dose of 1 mg/site.

Table 1.

Demographic Characteristics of Study Population by Treatment Group

Category	Characteristic	MM + SRP (n = 62)	SRP Alone (n = 65)	P Value*
Age[†]	Years	50.9 ± 7.9	48.9 ± 8.1	0.15
Gender	Male	38 (61.3%)	30 (46.2%)	0.09
	Female	24 (38.7%)	35 (53.8%)	
Smoking status	Never	24	22	0.65
	Current	19	18	
	Former	19	25	
Race	White	39	39	0.93
	African American	18	21	
	Hispanic	2	1	
	Asian	1	2	
	Other	2	2	
Center	Forsyth Institute, Boston, MA	6	5	0.98
	University of Maryland, Baltimore, MD	17	19	
	Private practice, Los Angeles, CA	7	9	
	University of Tennessee, Memphis, TN	8	8	
	State University of New York at Buffalo, Buffalo, NY	24	24	
Clinical[†]	Number of pockets ≥5 mm	24.0 ± 12.7	28.4 ± 16.8	0.10
	PD (mm) for full mouth	3.87 ± 0.45	3.92 ± 0.51	0.61
	BOP (%) for full mouth	60.9 ± 24.7	61.4 ± 25.5	0.91
	CAL (mm) for full mouth[‡]	4.16 ± 1.0919	4.14 ± 1.09	0.91
	PD (mm) for microsites only	5.57 ± 0.57	5.60 ± 0.60	0.71
	BOP (%) for microsites only	78.6 ± 21.9	78.1 ± 22.0	0.90
	CAL (mm) for microsites only[‡]	5.59 ± 1.24	5.68 ± 1.20	0.67

* Significance level between treatment groups.
† Mean ± SD.
‡ MM + SRP (n = 60); SRP alone (n = 64).

The total number of possible bacterial analyses performed in this study was 1,270 (127 subjects × five teeth × two visits). Single samples were lost from three subjects on the second visit (four teeth were sampled instead of five). The actual number of bacterial plaque samples analyzed was 1,267. The maximum total number of bacterial determinations was 50,800 (1,270 bacterial analyses × 40 species). Because of the extraction of test site teeth and visible DNA contamination from extraneous sources, only 49,776 (98%) determinations (of 50,800) were available for analysis.

A general survey of all changes in bacterial proportions (Table 2) in response to MM + SRP treatment revealed that the largest statistically significant reductions occurred within the RCB, especially *P. gingivalis* and *T. forsythia*. Significant reductions in proportions were also seen in two orange complex bacteria (*Campylobacter gracilis* and *Prevotella nigrescens*) and one unclassified spirochete (*Treponema socranskii*). Significant increases in proportions were seen in three bacteria: one actinomycete (*Actinomyces naeslundii 2*), one streptococcus (*Streptococcus sanguis*), and one member of the purple complex (*Veillonella parvula*). In comparison, SRP alone produced only small changes in proportions, none of which were statistically significant.

A general survey of changes in bacterial numbers (Table 3) revealed that numbers of bacteria generally changed in the same directions as the proportions. Three bacteria were significantly reduced by treatment with MM + SRP (*P. gingivalis*, *Eubacterium nodatum*, and *P. nigrescens*). The numbers of bacteria were not significantly altered by SRP treatment.

Primary Endpoints

Subjects treated with MM + SRP achieved a significantly greater mean reduction in the proportion of RCBs at 30 days compared to those treated with SRP alone (6.5% versus 5.0%, respectively; $P = 0.0005$; Fig. 2). In fact, for each category of percent reduction in RCB proportions from baseline, subjects treated with MM + SRP achieved a reduction of ~5% greater than that with SRP alone. However, this difference was twice as large in subjects achieving the highest reduction in RCB number (≥50%; Table 4).

A density distribution of the median RBC proportion is shown in Figure 3. The change in distribution between baseline and 30 days indicates that periodontal samples contained lower proportions of RCB after both treatments. However, a comparison of distances between median values at baseline and 30 days indicates that the median percent RCB reduction after treatment with MM + SRP was significantly greater than that with SRP alone (5.9% versus 3.8%, respectively; $P = 0.0003$).

The distribution characteristics of $\log_{10}$-transformed RCB proportions are shown in Figure 4. The $\log_{10}$ transformation greatly improved the normality of the distribution. Distances between mean values of the normal approximations of the distribution at baseline and 30 days indicate that the effect of MM + SRP in reducing $\log_{10}$ RCBs was greater than that of SRP alone. Baseline geometric mean values of $\log_{10}$ RCB proportions were 1.06 (11.5%) for MM + SRP and 1.11 (12.9%) for SRP alone. One month later, geometric mean values were 0.77 (5.9%) and 0.93 (8.5%), respectively. There was no significant difference in reduction of $\log_{10}$ RCB proportion based on study center, gender, age, race, or smoking status (Table 5).

MM + SRP also reduced the number of RCBs at day 30 to a significantly greater extent than SRP alone. Mean RCB numbers were reduced from 18.9×10^5 to 9.50×10^5 (50%) by MM + SRP and from 19.3×10^5 to 14.2×10^5 (26%) by SRP alone ($P = 0.002$; Fig. 5). For each category of percent reduction in RCB numbers from baseline, subjects treated with MM + SRP achieved a 20% greater reduction than those treated with SRP alone (Table 4). This difference is considerably greater than the 5% between-group difference identified for reduction of RCB proportions. More than twice as many subjects in the MM + SRP group achieved the two highest levels of RCB number reduction (≥40% and ≥50%) compared to SRP alone. Unlike the reduction in $\log_{10}$ RCB proportions with response rates not affected by covariables, study center and smoking status significantly contributed to reduction in $\log_{10}$ RCB numbers (Table 5). The fact that the analysis by proportions did not exhibit any significant covariant contributions but that differences between centers in RCB numbers occurred attests to the difficulty in standardizing the plaque sampling methodology. Thus, this effect was normalized by focusing primarily on bacterial proportions.

Secondary Endpoints

Microbiologic endpoints. MM + SRP reduced the proportions and numbers of each of the RCB individually, relative to the 40 bacteria measured to a greater extent than SRP alone (Table 6). These reductions were all statistically significant ($P < 0.05$), with the exception of the reduction in the number of *T. forsythensis*, which was borderline significant ($P = 0.07$). Considering that the difference in reduction in *T. forsythensis* proportions was highly significant ($P = 0.0009$), it may be concluded that the lower significance level observed in reduction in *T. forsythia* numbers was the result of the increased variability associated with the analysis of bacterial numbers.

Clinical endpoints. MM + SRP significantly improved clinical outcome measures compared to SRP

Table 2.

Mean Proportions (%) of Bacteria at Periodontal Sites at Baseline and 30-Day Change After Treatment by SRP With or Without MM

| | | MM + SRP | | | | SRP Alone | | | |
| | | Baseline | | Change | | Baseline | | Change | |
Group	Bacteria	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Pathogens	Aggregatibacter actinomycetemcomitans (serotypes a and b)	0.66	0.07	−0.02	0.09	0.64	0.05	0.03	0.05
	Porphyromonas gingivalis	4.21	0.65	**2.51**	0.58	4.44	0.47	1.68	0.34
	Tannerella forsythia	5.72	0.40	**2.65**	0.38	6.83	0.58	2.48	0.46
	Treponema denticola	3.77	0.33	1.40	0.31	4.01	0.32	0.77	0.26
Orange complex	Campylobacter gracilis	1.86	0.16	**0.22**	0.13	1.89	0.14	−0.45	0.12
	Campylobacter rectus	1.83	0.13	0.17	0.11	1.82	0.10	−0.11	0.12
	Campylobacter showae	0.81	0.07	−0.05	0.08	0.71	0.05	−0.15	0.06
	Eubacterium nodatum	1.37	0.16	0.61	0.13	1.39	0.14	0.29	0.10
	Fusobacterium nucleatum ss. nucleatum	3.85	0.19	0.46	0.20	4.55	0.28	0.03	0.23
	Fusobacterium nucleatum ss. polymorphum	1.87	0.12	0.20	0.13	1.64	0.11	−0.40	0.11
	Fusobacterium nucleatum ss. vincentii	5.81	0.29	0.79	0.32	6.00	0.27	−0.25	0.35
	Fusobacterium periodonticum	2.88	0.18	0.27	0.14	3.35	0.20	0.03	0.13
	Parvimonas micra (formerly Peptostreptococcus micros or Micromonas micros)	2.03	0.16	0.50	0.16	2.11	0.16	0.34	0.17
	Prevotella intermedia	3.34	0.41	0.81	0.23	3.02	0.26	−0.10	0.19
	Prevotella nigrescens	3.76	0.27	**1.23**	0.28	3.39	0.23	−0.55	0.25
	Streptococcus constellatus	1.50	0.11	0.19	0.08	1.53	0.10	0.32	0.10
Actinomycetes	Actinomyces gerencseriae	3.08	0.27	−1.53	0.30	2.72	0.19	−0.57	0.17
	Actinomyces israelii	2.76	0.19	−1.05	0.33	2.77	0.19	0.06	0.12
	Actinomyces naeslundii 1	2.75	0.33	−0.82	0.22	2.52	0.22	−0.36	0.13
	Actinomyces naeslundii 2 (A. viscosus)	4.75	0.34	**−4.44**	0.55	4.73	0.31	−1.66	0.38
Streptococci	Streptococcus anginosus	1.88	0.18	0.19	0.12	2.02	0.16	0.29	0.11
	Streptococcus gordonii	2.28	0.14	0.31	0.13	2.28	0.14	0.61	0.14
	Streptococcus intermedius	0.88	0.23	0.28	0.22	0.72	0.07	0.07	0.12
	Streptococcus mitis	1.24	0.11	0.12	0.11	1.25	0.09	0.27	0.08
	Streptococcus oralis	1.85	0.17	0.42	0.17	1.58	0.14	0.42	0.18
	Streptococcus sanguis	1.35	0.09	**−0.26**	0.19	1.26	0.09	0.22	0.11
Purple complex	Actinomyces odontolyticus (serotype I)	1.95	0.15	−0.75	0.18	1.78	0.15	−0.14	0.08
	Veillonella parvula	6.18	0.31	**−2.96**	0.62	6.06	0.37	−0.30	0.33
Green complex	Capnocytophaga gingivalis	1.31	0.14	−1.84	0.31	1.54	0.18	−0.57	0.21
	Capnocytophaga ochracea	2.47	0.16	−0.03	0.16	2.17	0.12	−0.38	0.13
	Capnocytophaga sputigena	1.56	0.14	−0.83	0.17	1.67	0.18	−0.50	0.17
	Eikenella corrodens	0.79	0.08	−0.37	0.08	0.91	0.09	−0.27	0.09
Unknown	Eubacterium saburreum	1.45	0.09	0.03	0.13	1.30	0.08	−0.29	0.10
	Gemella morbillorum	1.71	0.12	0.08	0.12	1.59	0.12	0.05	0.11
	Leptotrichia buccalis	2.00	0.11	−0.17	0.15	2.22	0.18	−0.30	0.14
	Neisseria mucosa	4.49	0.24	−0.57	0.32	5.10	0.42	0.13	0.28
	Prevotella melaninogenica	1.70	0.14	0.00	0.14	1.86	0.16	−0.20	0.16
	Propionibacterium acnes (serotypes I and II)	0.93	0.08	−0.13	0.06	0.88	0.10	−0.04	0.05
	Selenomonas noxia	4.27	0.28	0.93	0.24	3.58	0.21	−0.20	0.15
	Treponema socranskii	2.85	0.25	**1.01**	0.14	2.80	0.30	−0.15	0.35

Positive changes are a reduction in proportions. **Bold** values represent within-group changes that were statistically significant ($P<0.001$) by Kruskal-Wallis non-parametric analysis (adjusted for multiple comparisons; overall $P<0.05$).

Table 3.

Mean Numbers ($\times 10^5$) of Bacteria at Periodontal Sites at Baseline and 30-Day Change After Treatment by SRP With or Without MM

Group	Bacteria	MM + SRP				SRP Alone			
		Baseline		Change		Baseline		Change	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Pathogens	Aggregatibacter actinomycetemcomitans (serotypes a and b)	1.04	0.18	0.17	0.14	0.85	0.11	−0.13	0.11
	Porphyromonas gingivalis	5.61	1.17	**3.71**	1.03	5.06	0.60	1.46	0.50
	Tannerella forsythia	9.16	1.93	4.32	1.29	10.62	2.53	1.96	1.16
	Treponema denticola	4.35	0.43	1.40	0.38	3.84	0.32	0.00	0.33
Orange complex	Campylobacter gracilis	2.66	0.32	−0.03	0.27	2.70	0.45	−1.10	0.23
	Campylobacter rectus	2.86	0.36	0.50	0.22	2.64	0.38	−0.31	0.25
	Campylobacter showae	1.42	0.18	0.00	0.13	1.12	0.21	−0.58	0.19
	Eubacterium nodatum	1.60	0.20	**0.70**	0.18	1.56	0.19	0.12	0.15
	Fusobacterium nucleatum ss. nucleatum	8.04	2.37	0.48	1.25	9.37	2.77	−1.37	1.92
	Fusobacterium nucleatum ss. polymorphum	2.33	0.23	0.09	0.24	1.94	0.22	−1.05	0.23
	Fusobacterium nucleatum ss. vincentii	7.83	1.03	0.57	0.53	8.27	1.27	−0.64	0.54
	Fusobacterium periodonticum	6.09	1.67	0.48	0.42	7.68	2.37	−0.60	1.29
	Parvimonas micra (formerly Peptostreptococcus micros or Micromonas micros)	3.56	0.75	1.11	0.57	3.37	0.68	0.11	0.34
	Prevotella intermedia	7.26	2.92	1.82	1.57	7.32	3.11	−0.92	0.77
	Prevotella nigrescens	6.02	1.03	**1.52**	0.52	5.45	1.01	−1.67	0.65
	Streptococcus constellatus	2.23	0.31	0.36	0.17	2.45	0.43	0.60	0.27
Actinomycetes	Actinomyces gerencseriae	3.19	0.24	−1.94	0.37	3.13	0.29	−1.26	0.25
	Actinomyces israelii	3.31	0.30	−0.97	0.33	3.49	0.45	−0.40	0.28
	Actinomyces naeslundii 1	3.07	0.28	−0.90	0.33	3.33	0.45	−0.43	0.20
	Actinomyces naeslundii 2 (A. viscosus)	5.07	0.38	−4.38	0.67	5.36	0.49	−1.86	0.36
Streptococci	Streptococcus anginosus	2.82	0.37	0.36	0.19	2.98	0.44	0.37	0.25
	Streptococcus gordonii	3.40	0.42	0.39	0.25	3.27	0.56	0.72	0.38
	Streptococcus intermedius	2.91	1.40	0.93	0.68	1.83	0.63	−0.21	0.97
	Streptococcus mitis	1.86	0.26	0.29	0.19	1.85	0.31	0.26	0.21
	Streptococcus oralis	2.54	0.28	0.72	0.23	2.28	0.32	0.51	0.34
	Streptococcus sanguis	2.46	0.58	−0.03	0.22	2.44	0.75	0.36	0.47
Purple complex	Actinomyces odontolyticus (serotype I)	2.72	0.32	−0.62	0.20	2.34	0.31	−0.54	0.26
	Veillonella parvula	7.89	0.65	−2.58	0.72	7.36	0.73	−1.41	0.64
Green complex	Capnocytophaga gingivalis	2.00	0.30	−2.83	0.60	3.65	1.41	−0.86	0.46
	Capnocytophaga ochracea	3.02	0.29	0.13	0.23	2.42	0.24	−0.99	0.24
	Capnocytophaga sputigena	2.19	0.26	−1.03	0.24	1.98	0.30	−0.98	0.27
	Eikenella corrodens	1.44	0.29	−0.62	0.15	1.75	0.40	−0.55	0.30
Not classified	Eubacterium saburreum	2.90	0.62	−0.01	0.29	2.89	0.84	−0.51	0.38
	Gemella morbillorum	3.22	0.62	0.42	0.21	2.79	0.55	0.20	0.34
	Leptotrichia buccalis	3.46	0.58	−0.06	0.33	4.51	1.45	−0.56	0.75
	Neisseria mucosa	6.35	0.80	−0.30	0.39	7.74	1.33	0.01	0.87
	Prevotella melaninogenica	3.45	0.69	−0.08	0.42	3.14	0.63	−0.64	0.36
	Propionibacterium acnes (serotypes I and II)	3.14	1.45	0.20	0.59	3.11	1.48	−0.13	0.81
	Selenomonas noxia	5.19	0.38	0.82	0.28	4.45	0.38	−0.97	0.33
	Treponema socranskii	9.18	3.44	2.08	0.85	9.96	3.97	0.85	1.68

Positive changes are a reduction in proportions. **Bold** values represent within-group changes that were statistically significant ($P < 0.001$) by Kruskal-Wallis non-parametric analysis (adjusted for multiple comparisons; overall $P < 0.05$).

Figure 2.
Mean percent reduction in the proportion of RCB (sum of P. gingivalis, *T. forsythia, and* T. denticola*) from baseline to day 30. Brackets represent SE (0.88% and 1.00% for the MM + SRP and SRP alone groups, respectively).*

Table 4.

Number and Percentage of Subjects With Each Category of Percent Reduction From Baseline in RCB Proportion or Number

Category of Reduction	RCB Proportion		RCB Number	
	MM + SRP (n = 62)	SRP Alone (n = 65)	MM + SRP (n = 62)	SRP Alone (n = 65)
≥15%	41 (66.1%)	39 (60.0%)	41 (66.1%)	29 (44.6%)
≥25%	36 (58.1%)	34 (52.3%)	34 (54.8%)	20 (30.8%)
≥30%	33 (53.2%)	32 (49.2%)	33 (53.2%)	19 (29.2%)
≥35%	29 (46.8%)	29 (44.6%)	31 (50.0%)	18 (27.7%)
≥40%	27 (43.5%)	26 (40.0%)	30 (48.4%)	15 (23.1%)
≥50%	25 (40.3%)	20 (30.8%)	22 (35.5%)	11 (16.9%)

Figure 3.
Density distribution of the RCB proportions. The difference between groups was highly significant (P = 0.0003 by Mann-Whitney analysis). Each dot represents 1 of the 127 subjects in the study. The box plot provides the median and the first- and third-quartile boundaries of each group at each visit. Illustrated differences are between median values at baseline and 1 month.

alone (Table 6). The mean reduction in PD was 1.38 mm in the MM + SRP group compared to 1.01 mm in the SRP-alone group ($P = 0.00004$). The mean gain in CAL between groups was also statistically significant (1.16 and 0.80 mm in the MM + SRP and SRP-alone groups, respectively; $P = .0004$). The mean percent reduction of BOP for sites that had at least one pocket ≥5 mm at baseline was nearly twice as high in the MM + SRP group as it was in the SRP-alone group (25.2% versus 13.8%, respectively; $P = 0.009$).

Effects on Other Bacteria and Bacterial Parameters

Baseline RCB numbers were $\sim 19 \times 10^5$ per tooth. The mean reduction in numbers from baseline was 49% (9.4×10^5) by MM + SRP and 27% (5.1×10^5) by SRP alone ($P = 0.002$). Baseline RCB proportions were $\sim 15\%$. The mean reduction in proportions was 6.5% by MM + SRP and 5.0% by SRP alone ($P = 0.0005$).

Bacterial load, defined as the total number of bacteria on a tooth surface, was estimated by considering the total number of bacteria in each sample. The average total number of bacteria per tooth for each subject was calculated as the sum of all 40 bacteria averaged over the five sites measured. There was a trend toward greater reduction in total average number of bacteria at 30 days in the MM + SRP group compared to the SRP-alone group. The average total number of bacteria per site was reduced from 156×10^5 at baseline to 149×10^5 at 30 days in the MM + SRP group, whereas in the SRP-alone group, there was a slight increase from 159×10^5 at baseline to 169×10^5 at 30 days (not significant).

Changes were also observed in the proportions for some of the bacteria not belonging to the RCBs. Significant reductions in *P. nigrescens*, *T. socranskii*, *E. nodatum*, and *P. micra* were seen in the group treated by MM + SRP. No statistically significant effects in these groups of bacteria were observed in the group treated with SRP alone. Proportions of *A. naeslundii 2* and *Actinomyces gerencseriae* significantly increased in both treatment groups. *V. parvula* and *Capnocytophaga gingivalis* also significantly increased after treatment with MM + SRP but not for the SRP-alone group.

DISCUSSION

It is now recognized that chronic periodontitis is an infectious disease[2,7] in which the bacteria

Figure 4.
Density distribution of log_{10}-transformed RCB proportions. The normal curve represents the computed geometric mean and SD of each group at each visit. The difference between treatment groups was highly significant ($P = 0.0005$ by ANCOVA).

P. gingivalis, *T. forsythia*, and *T. denticola* play a primary role.[2] In addition, research has shown that locally delivered adjunctive antimicrobial therapy is an effective means to enhance therapeutic outcomes.[6-16] This study showed that the adjunctive antimicrobial-effect of MM + SRP on *P. gingivalis*, *T. forsythia*, and *T. denticola* exceeds the antibacterial effect of conventional therapy (i.e., SRP).

Microbiologic outcome as a primary endpoint tests a periodontal therapy in its ability to affect bacteria responsible for the therapeutic response. Only now, with the advent of molecular microbiology, does this objective become practical. Two fundamental problems that have been addressed in this clinical study design are consideration of sufficient power to show differences between two treatments (both of which lower numbers and proportions of bacteria) and controlling variability inherent in microbiologic data. Although many studies[17-19] have included microbiologic measurements to help understand clinical changes, these have largely been evaluated as differences with respect to baseline values and are inadequately pow-

ered to compare microbiologic effects between treatments. To our knowledge, this is the first randomized controlled trial designed with sufficient power to compare treatments of periodontal infection through microbiologic analysis.

A review of the analysis leads to the conclusion that a statistically significant effect on RCBs occurred and that the numbers and proportions of these bacteria were reduced both as a group and individually. Both test (MM + SRP) and control (SRP alone) treatments reduced RCBs, but the inclusion of MM significantly increased this effect. The reduction in RCB proportions indicated that the therapy significantly changed the composition of the periodontal biofilm. Typically, 14% of the periodontal bacteria were in the RCB at baseline. Thirty days after treatment, the periodontal biofilm from subjects treated with conventional therapy had 10% RCB compared to 7% for those who received MM therapy. In a recent review of microbiologic objectives of periodontal therapy,[20] it was suggested that the microbiologic goal of periodontal therapy is to lower periodontal pathogens to levels seen in healthy patients (~7% RCBs).

The differences in PD that accompanied these microbiologic changes were average reductions of 1.01 mm by SRP alone and 1.38 mm by MM + SRP. This difference (0.37 mm) was statistically significant ($P = 0.00004$), indicating that MM + SRP was more effective than SRP alone in reducing PD. From a microbiologic standpoint, reduction in PD is associated with habitat modification for those bacteria that grow to a greater density in deeper pockets (RCB and orange complex bacteria).[2] BOP occurred in ~78% of sites at baseline and was reduced to 64% by SRP and to 53% by MM + SRP, a highly significant difference ($P = 0.009$). The effect of MM was to decrease bleeding by 78% – 53% = 25% compared to 78% – 64% = 14% by SRP alone. By this measure, MM + SRP had almost twice the effect of SRP alone. From a microbiologic standpoint, reduction in pocket bleeding is a measure of reduced inflammation and nutrient flow to support growth of asaccharolytic species that depend on proteolysis for energy (such as RCBs).

The microbiologic response after SRP has been reported previously[21] as a reduction of RCBs. These observations are in agreement with the findings of this study. In addition, the reduction in RCBs was shown to be statistically greater with the adjunctive inclusion

Table 5.

Analysis of Covariance: Log_{10}-Transformed RCB Proportions* and Numbers[†]

Source	Sum of Squares	df	Mean Square	F Ratio	P Value
RCB proportions					
Treatment	0.636	1	0.636	12.800	**0.00051**
Center	0.061	4	0.015	0.306	0.87376
Gender	0.028	1	0.028	0.572	0.45099
Age	0.002	1	0.002	0.038	0.84645
Race	0.154	4	0.038	0.772	0.54536
Smoking	0.066	2	0.033	0.666	0.51601
Baseline	0.864	1	0.864	17.392	**0.00006**
Error	5.566	112	0.050		
RCB numbers					
Treatment	1.04382	1	1.04382	10.10552	**0.00191**
Center	1.58883	4	0.39721	3.84546	**0.00576**
Gender	0.00001	1	0.00001	0.00008	0.99267
Age	0.22253	1	0.22253	2.15441	0.14496
Race	0.91336	4	0.22834	2.21062	0.07232
Smoking	0.86705	2	0.43353	4.19706	**0.01747**
Baseline	4.15281	1	4.15281	40.20438	**0.00000**

Bold values represent statistically significant ($P < 0.05$) contributions from factors tested by ANCOVA.
* N = 127. Squared multiple R = 0.31.
† N = 127. Squared multiple R = 0.50.

Figure 5.
Mean reduction in the number of RCB (sum of P. gingivalis, T. forsythia, and T. denticola) from baseline to day 30. Brackets represent SE (both 2.43×10^5).

of MM. Systemic administration of metronidazole and amoxicillin has also been reported to lower RCB counts.[19] In a recent review,[22] the authors concluded that adjunctive antimicrobial therapy generally improved the clinical response of conventional periodontal therapy. It should also be noted that lower levels of RCBs associated with significant clinical improvement are consistent with the behavior of pathogenic organisms.

In this study, MM produced an increase in *Actinomyces* species. In contrast, metronidazole has been shown to have little effect and amoxicillin decreases proportions and numbers of *Actinomyces* species.[19]

Considering that with the 40 bacteria monitored, the only significant decreases in proportions occurred in the RCBs and four other bacteria (*P. micra*, *E. nodatum*, *T. socranskii*, and *P. nigrescens*), it could be concluded that treatment with MM was remarkably specific. This is in contrast to the generally held concept that tetracyclines are broad-spectrum antibiotics. This apparent contradiction likely results from the fact that treatment is directed toward a biofilm that is intrinsically stable; therefore, only a small number of susceptible species are affected even by a broad-spectrum antimicrobial.

As a general trend, the data in Table 2 indicate that treatment by MM + SRP significantly changed plaque composition, whereas SRP alone had little effect. Reductions were seen in red and orange complex bacteria, those species for which a pathogenic association is strongest. In contrast, increases in actinomycetes, streptococci, purple complex, and green complex bacteria after treatment by MM + SRP suggest that they could be considered beneficial or at least indicators of health-associated biofilm modification. A comparison of Tables 2 and 3 indicates that changes in composition (proportions) were statistically more sensitive than changes in numbers. This is likely because of variability in sample taking that is decreased by the normalization to compute proportions.

This study was designed and powered to reveal the difference in microbial effect that can be achieved by SRP alone and MM + SRP. It used the power of objectively measured bacterial presence by DNA probe analysis. Using this technology, the analysis of hundreds of samples required for statistical power could be accomplished. The primary endpoint of changes in bacterial proportions was selected because it minimized differences in microbiologic sampling techniques between centers by the normalization procedure. Normalization was achieved by adding the numbers of all bacteria evaluated as an estimate of the total bacterial load. Estimates based on microscopic count suggest that the DNA probe method accounts for 55% to 60% of the bacteria in subgingival biofilms.[2] Hence, the use of this sum is a reasonable value for normalization. A second feature is to select the combination of three bacteria thought to be pathogens (i.e., *P. gingivalis*, *T. forsythia*, and *T. denticola*) as a primary outcome variable. Using the combined presence of

Table 6.

Mean Reduction of Secondary Outcome Variables Including Clinical Measures and the Proportion and Number of *P. gingivalis, T. forsythia,* and *T. denticola* in Subjects Treated With MM Plus SRP or SRP Alone

Parameter	MM + SRP	SRP Alone	*P* Value
PD reduction (mm)*	1.38	1.01	**0.00004**
CAL reduction (mm)*	1.16	0.80	**0.0004**
BOP reduction (%)*	25.2%	13.8%	**0.009**
P. gingivalis proportions	2.5%	1.7%	**0.00007**
T. forsythia proportions	2.7%	2.5%	**0.0009**
T. denticola proportions	1.4%	0.8%	**0.004**
P. gingivalis numbers	3.71×10^5	1.54×10^5	**0.0001**
T. forsythia numbers	4.32×10^5	3.57×10^5	0.07
T. denticola numbers	1.40×10^5	$-0.003 \times 10^{5\dagger}$	**0.01**

Bold values represent statistically significant (*P*<0.05) differences between groups treated by MM + SRP and SRP alone based on variables tested by ANCOVA.
* Computed at teeth with at least one baseline PD >5 mm.
† Negative values indicate an increase from baseline to 30 days.

these periodontal pathogens increases the mean difference between test (MM + SRP) and control (SRP alone). These methods of treating microbiologic data minimized differences in the numbers of bacteria in a sample and maximized differences between test and control to overcome the characteristic variability of microbiologic measurement. Variability in periodontal probe measurements was controlled in this study by initial calibration of clinicians against a single gold standard coordinator. This procedure was considered key to obtaining reproducible clinical measurements.

The agreement between DNA probe measurement and culture procedures, although not perfect, is acceptable.[23,24] It is generally recognized that DNA probe methods produce higher bacterial counts. This could be the result of bacterial clumping that occurs in culture. On the other hand, different bacteria have different numbers of DNA copies, which could elevate DNA probe counts. Despite the fact that both conventional culture and DNA probe techniques have deficiencies, diagnostic accuracy of 0.5 to 0.8 based on culture as the gold standard have been reported.[24]

DNA probe measurement provides new tools for the clinical study of bacterial changes associated with antimicrobial therapies. The ability to control methodology to a greater extent than possible using conventional microbiology and reduce the cost per sample evaluated creates the opportunity to apply statistical models for testing differences between treatments. In

the design phase of this study, it was recognized that bacterial measures are notoriously variable because samples are taken from five investigational study sites by five different clinicians. These measures can be improved by averaging across periodontal treatment sites within each subject and by dividing by the estimated total number of bacteria to obtain proportions. This normalization procedure reduced the variability in numbers of bacteria in a sample. The study could not have been done without the development of molecular microbiology. All of these experimental features were found to be essential, and they will likely serve as a guide to meaningful clinical studies that measure changes in periodontal bacteria in the future.

CONCLUSIONS

The antimicrobial effect of locally delivered MM exhibited surprising specificity of action in being directed almost entirely toward inhibition of bacteria generally considered periodontal pathogens with little inhibitory effect on other species. In addition, adjunctive MM significantly reduced RCB, PD, CAL, and BOP to a greater extent than treatment by SRP alone. Based on risk/benefit analysis it would seem that regular use of MM would be preferred because there is little or no risk, and it provides a clear benefit.

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