Long-term evaluation of the antimicrobial susceptibility and microbial profile of subgingival biofilms in individuals with aggressive periodontitis

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Abstract

This study evaluates the antimicrobial susceptibility and composition of subgingival biofilms in generalized aggressive periodontitis (GAP) patients treated using mechanical/antimicrobial therapies, including chlorhexidine (CHX), amoxicillin (AMX) and metronidazole (MET). GAP patients allocated to the placebo (C, n = 15) or test group (T, n = 16) received full-mouth disinfection with CHX, scaling and root planning, and systemic AMX (500 mg)/MET (250 mg) or placebos. Subgingival plaque samples were obtained at baseline, 3, 6, 9 and 12 months post-therapy from 3-4 periodontal pockets, and the samples were pooled and cultivated under anaerobic conditions. The minimum inhibitory concentrations (MICs) of AMX, MET and CHX were assessed using the microdilution method. Bacterial species present in the cultivated biofilm were identified by checkerboard DNA-DNA hybridization. At baseline, no differences in the MICs between groups were observed for the 3 antimicrobials. In the T group, significant increases in the MICs of CHX (p < 0.05) and AMX (p < 0.01) were detected during the first 3 months; however, the MIC of MET decreased at 12 months (p < 0.05). For several species, the MICs significantly changed over time in both groups, i.e., Streptococci MICs tended to increase, while for several periodontal pathogens, the MICs diminished. A transitory increase in the MIC of the subgingival biofilm to AMX and CHX was observed in GAP patients treated using enhanced mechanical therapy with topical CHX and systemic AMX/MET. Both protocols presented limited effects on the cultivable subgingival microbiota.

Key words: aggressive periodontitis, biofilms, microbial sensitivity tests, DNA probes.

Introduction

Generalized aggressive periodontitis (GAP) is a severe form of periodontal disease characterized by the widespread destruction of the periodontium at a high progression rate in young subjects (Armitage, 1999). The adjunctive use of antimicrobials combined with the mechanical removal of the subgingival biofilm has been demonstrated as an effective therapeutic strategy for treating GAP (Herrera et al., 2002, 2008; Haftajee et al., 2003). Specifically, the administration of amoxicillin (AMX) and metronidazole (MET), combined or not with the topical use of chlorhexidine (CHX), provides significant clinical and microbiological benefits for GAP patients post-therapy (Guerrero et al., 2005). However, some patients with severe periodontal destruction do not respond favorably to mechanical/antimicrobial therapy (Colombo et al., 1998, 2009). Treatment failure might have several causes, including the existence of subgingival microbiota resistant to the drugs of choice (Listgarten et al., 1993, Mejia et al., 1995). Antimicrobial resistance has become a serious problem for the treatment of a large number of infections worldwide. The inappropriate and irrational use of antimicrobials leads
to the emergence, spread and persistence of resistant microorganisms, resulting in prolonged illness and greater risk of death (Gootz, 2010). Thus, an effective antimicrobial protocol for treating periodontitis should consider the severity of the disease, the general health of the host, the target microorganisms, and the pharmacokinetics, adverse effects and costs of the drug (Seymour and Hogg, 2008, Heasman et al., 2011). Moreover, periodontal diseases are polymicrobial, and biofilm-related infections widely vary in microbial composition and diversity among sites and individuals with similar clinical manifestations (Socransky and Haffajee, 2002). Bacterial species growing in biofilms are less susceptible to antimicrobial action (Costerton et al., 1999). Nevertheless, few studies have directly examined the in vitro antimicrobial susceptibility of subgingival plaques in biofilms or mixed cultures (Wright et al., 1997, Eick et al., 2004, Sedlacek and Walker, 2007). This assessment could provide additional information on the susceptibility of periodontal microbiota in GAP prior to the use of antimicrobials. Furthermore, subsequent evaluation of the drug administration might reveal potential changes in the resistance profile of this microbiota. Thus, the aims of the current study were to determine the bacterial composition and antimicrobial susceptibility profile of the subgingival biofilm in GAP patients before and up to 12 months after treatment with CHX, AMX, MET or placebo.

Material and Methods

Subject population

This study was conducted as a randomized, double-blinded, placebo-controlled, single-center, 12-month clinical trial as previously described (Heller et al., 2011, Varela et al., 2011, Silva-Senem et al., 2013). The study protocol was approved through the Ethics in Human Research Committee of the Institute for Community Health Studies at the Federal University of Rio de Janeiro, Brazil (EHRC/ICHRS-FURJ, protocol #45/2007). The subjects were selected between March 2008 and June 2009 from a pool of first-time patients referred to the Division of Graduate Periodontics of the School of Dentistry at the Federal University of Rio de Janeiro (UFRJ), Brazil. Included patients were diagnosed with GAP according to criteria of the American Academy of Periodontology (Armitage, 1999). In addition, the patients were between 18-39 years of age and had at least 16 teeth and 4 sites on different teeth (3 sites other than central incisors or first molars), with a probing pocket depth (PPD) ≥ 6 mm and clinical attachment level (CAL) ≥ 5 mm and bleeding on probing (BOP). The exclusion criteria were allergy to penicillin, MET or CHX; diabetes; immunodeficiency; required antibiotic coverage for periodontal procedures; long-term use of anti-inflammatory medication; periodontal treatment and/or use of antibiotics in the last 6 months; and pregnancy and nursing

(Heller et al., 2011, Varela et al., 2011, Silva-Senem et al., 2013).

Clinical examination and treatment protocols

A trained and calibrated examiner (D. H.) performed clinical exams at baseline, 3, 6, 9 and 12 months post-therapy. The full-mouth clinical measurements included PPD, CAL, presence or absence of BOP, supragingival visible plaque and gingival marginal bleeding. An experienced periodontist (V.M.C.) administered periodontal treatment. The patients received full-mouth debridement with ultrasonics, complemented by the irrigation of all pockets with a 0.2% CHX gel within 24 h. Additionally, patients were instructed to rinse and gargle twice a day with a 0.12% CHX solution and brush the tongue with the same CHX gel for the next 45 days. The patients were subsequently assigned either to the test (T, systemic administration of AMX 500 mg + MET 250 mg) or the control group (C, placebo tablets). Antimicrobials or placebos were prescribed 3 times a day for 10 days, starting at the moment of assignment. In the following week, the patients were treated with staged quadrant manual scaling and root planning, followed by pocket irrigation with 0.2% CHX gel within 4-6 weeks. The patients returned at 3, 6, 9 and 12 months for clinical re-evaluation, microbiological sampling, oral hygiene evaluation, and supragingival plaque and calculus removal. Furthermore, sites with PPD > 4 mm and BOP were re-instrumented under local anesthesia (Heller et al., 2011, Varela et al., 2011, Silva-Senem et al., 2013).

Subgingival biofilm sampling

Subgingival biofilm samples were collected from 3-4 of the deepest sites (PPD) using individual sterile Gracey curettes (Hu-Friedy, Chicago, IL, USA). The material was pooled, placed into cryogenic tubes containing 1 mL of mycoplasmal broth with 10% DMSO and stored at -20 °C.

Determination of the MIC

Susceptibility testing was performed using the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, formerly NCCLS, 2004), with modifications. The pooled samples were anaerobically cultured in pre-reduced supplemented BHI broth (BBL) for 48 h at 37 °C. The mixed culture was centrifuged, and the bacterial suspension was subsequently adjusted to ~1.5 x 10⁶ colony forming units (cfu)/mL in saline solution (0.9%). A 10-μL aliquot of the suspension was dispensed into the wells of 96-well, round-bottom microtiter plates (TPP), containing 100 μL of two-fold serial dilutions of the AMX and MET antimicrobials (Sigma-Aldrich Co.). The antimicrobials were administered at final concentrations ranging from 128 to 0.25 μg/mL for AMX and MET. For CHX, 22 μL of the bacterial suspensions were placed into wells containing 88 μL of the antimicro-
bials diluted in PRAS-supplemented BHI broth to final concentrations ranging from 2% to 0.02%. Each microplate included positive (bacterial suspension without antimicrobial treatment) and negative controls (medium only), and all experiments were performed in duplicate. The microplates were incubated under anaerobic conditions for 48 h at 37 °C. One examiner obtained visual readings. The MIC was defined as the lowest antimicrobial concentration yielding no visual bacterial growth.

Determination of the Composition of the Subgingival Biofilm through Checkerboard DNA-DNA Hybridization

The composition of the subgingival biofilm samples cultivated in the microplates without antimicrobials (positive controls) at baseline, 3, 6, 9 and 12 months after treatment was determined using the checkerboard method (Socransky et al., 1994), with modifications (Heller et al., 2011).

Statistical analysis

A statistical program (SPSS, Statistical Package for the Social Sciences, version 19.0, IBM) was used for all analyses. The clinical and demographic features of the groups were compared using the Mann-Whitney and Chi-square tests. The MICs of each antimicrobial for each patient was averaged within the groups at all time points. Significant differences between groups and over time were examined using the Mann-Whitney, Friedman and Wilcoxon signed rank tests. For the checkerboard data, the levels of each species were computed for each sample and patient and averaged within each group. For graphic presentation, the levels (scores 0 to 5) of each species in a sample were converted to absolute numbers and log10 transformed. Comparisons between groups over time were evaluated using the Mann-Whitney and Friedman tests, whereas the differences between two time points were assessed using the Wilcoxon signed rank test. For the checkerboard analysis, adjustments for multiple comparisons were made according to Socransky et al. (1991). Briefly, an overall p of 0.05 = 1 - (1 - k)⁻¹ was computed, where k was the desired individual p value. Thus, a p value < 0.00095 was considered to be statistically significant at p < 0.05. The level of significance for all the other analyses was 5%.

Results

Information on adverse events, adherence to the local and systemic antimicrobial regimen, and the demographic and full-mouth periodontal clinical features of the subjects in both therapeutic groups has been published elsewhere (Heller et al., 2011, Varela et al., 2011, Silva-Senem et al., 2013). The MICs for the three antimicrobials in subgingival biofilm samples obtained from GAP patients before and up to 1 year after both treatment protocols are shown in Figures 1A-C. At baseline, no significant differences between groups were observed for the MICs of all tested antimicrobials (p > 0.05, Mann-Whitney test). However, significant increases in the MICs of CHX (p < 0.05, Figure 1A) and AMX (p < 0.01, Figure 1B) were detected in the T group at 3 months compared with all other time points (Friedman and Wilcoxon tests). Significant differences over time were also observed for the MIC of MET (p < 0.05, Friedman test), which decreased at 12 months post-therapy in the T group (Figure 1C). In the C group, no significant changes in the MICs of any antimicrobial were observed over time post-therapy (p > 0.05, Friedman test). Moreover, no significant differences in the MICs of CHX, AMX or MET between groups were detected at 3, 6, 9 and 12 months post-therapy (p > 0.05, Mann-Whitney test).

Figure 1 - Mean (± SD) of the MICs of chlorhexidine (A), amoxicillin (B) and metronidazole (C) in the two therapeutic groups at baseline, 3, 6, 9 and 12 months after periodontal therapy. No differences between groups were observed at any time point (p > 0.05, Mann-Whitney test). *p < 0.05 and †p < 0.01 refer to significant differences between the 3-month visit and the other time points in the test groups (Wilcoxon sign rank test).
The composition of the subgingival biofilm cultivated in vitro from patients of the two clinical groups is presented in Figure 2. The species were ordered into different microbial complexes according to Socransky et al. (1998). The mean levels of the tested species (Table 1) were computed for both groups at each time point. At baseline, high mean levels of bacteria ($4.4 \times 10^5$ cells) were detected in both groups, including several periodontal pathogens. No significant differences between groups regarding bacterial mean levels were observed for any species at any time point (adjusted $p < 0.00095$, Mann-Whitney test). When mean counts of these species were evaluated within each group over time, few significant changes were observed (Figure 2). The numbers of Streptococcus spp. increased, while the number of periodontal pathogens, such as Aggregatibacter actinomycetemcomitans, Tannerella forsythia, Parvimonas micra and Treponema socranskii, diminished in both groups. However, only Streptococcus gordonii and Streptococcus oralis increased, whereas Neisseria gonorrhoeae significantly decreased at 12 months after treatment in the control group (Friedman test, $p < 0.00095$). In the test group, Actinomyces israelii,

Table 1 - Bacterial taxa used for development of whole genomic DNA probes tested against subgingival biofilm samples.

| Species                          | Strain   | Species                          | Strain   |
|---------------------------------|----------|---------------------------------|----------|
| Aggregatibacter actinomycetemcomitans a | 43718 | Neisseria polysaccharea          | 43768 |
| Aggregatibacter actinomycetemcomitans b | 29523 | Neisseria sicca                 | 29256 |
| Aggregatibacter actinomycetemcomitans c | 625a | Neisseria subflava               | 49275 |
| Actinomyces gerenserica         | 23860 | Neisseria meningitidis          | 13077 |
| Actinomyces israelii            | 12102 | Neisseria lactamica             | 23970 |
| Actinomyces odontolyticus       | 17929 | Neisseria gonorrhoeae           | 21824 |
| Actinomyces naeslundii          | 12104 | Neisseria mucosa                | 19696 |
| Actinomyces oris                | 43146 | Pantoea agglomerans             | 27155 |
| Actinomyces meyer               | 35568 | Parvimonas micra                | 33270 |
| Actinobacter baumannii          | 19606 | Prevotella melaninogenica       | 25845 |
| Bacteroides fragilis            | 25285 | Porphyromonas gingivalis        | 33277 |
| Capnocytophaga gingivalis       | 33624 | Prevotella intermedia           | 25611 |
| Capnocytophaga ochracea         | 33596 | Prevotella nigrescens           | 33563 |
| Campylobacter rectus            | 33238 | Propionibacterium acnes I       | 11827 |
| Campylobacter showae            | 51146 | Propionibacterium acnes II      | 43541 |
| Clostridium difficile           | 98689 | Peptostreptococcus anaerobius   | 27337 |
| Dialister pneumosintes          | GBA27b | Prevotella tannerae             | 51259 |
| Eubacterium nodatum             | 33099 | Pseudomonas aeruginosa          | 10145 |
| Eubacterium saburreum           | 33271 | Rothia dentocariosa             | 17931 |
| Eikenella corrodens             | 23834 | Selenomonas noxia               | 33359 |
| Enterococcus faecalis           | 10100 | Streptococcus anginosus         | 33397 |
| Escherichia coli                | 10799 | Streptococcus constellatus      | 27823 |
| Enterobacter cloacae            | 10699 | Streptococcus mitis             | 49456 |
| Enterobacter sakazakii          | 12868 | Streptococcus oralis            | 35037 |
| Enterobacter aerogenes          | 13048 | Streptococcus sanguinis         | 10556 |
| Enterobacter gergoviae          | 33028 | Streptococcus gordonii          | 10558 |
| Filifactor alocis               | 35896 | Streptococcus intermedius       | 27335 |
| Fusobacterium necrophorum       | 25286 | Salmonella enterica sorv. typhi | 6539 |
| Fusobacterium periodonticum     | 33693 | Staphylococcus aureus           | 33591 |
| Fusobacterium nucleatum ss. vincentii | 49256 | Streptococcus pneumoniae        | 49619 |
| Haemophilus aphrophilus         | 33389 | Tannerella forsythia            | 43037 |
| Helicobacter pylori             | 43504 | Treponema denticola             | BI1 |
| Klebsiella pneumoniae           | 10031 | Treponema socranskii            | S11 |
| Klebsiella oxytoca              | 12833 | Veillonella parvula             | 10790 |

ATCC (American Type Culture Collection, Rockville, MD), The Forsyth Institute, (Boston, MA).
Bacteroides fragilis, N. gonorrhoeae and Neisseria mucosa were reduced, and Acinetobacter baumannii, Campylobacter rectus, Filifactor alocis, Salmonella enterica and Streptococcus pneumoniae significantly increased over time post-therapy (Friedman test, \( p < 0.00095 \)).

Discussion

The use of systemic antimicrobials as adjunct treatments to mechanical therapy in GAP is controversial. There are no specific antimicrobial therapy protocols for treating different forms of periodontitis, and among the currently employed protocols, none of these therapies completely eliminated the need for retreatment (Herrera et al., 2002, 2008; Haffajee et al., 2003). The systemic administration of antimicrobials should always consider the risk-benefits for the patients, particularly the costs and adverse effects of additional drugs (Seymour and Hogg, 2008, Heasman et al., 2011). In general, the restricted use of systemic antimicrobials is the best strategy to avoid the increase in resistance worldwide (Enne, 2010). Thus, alternative approaches of intensive mechanical debridement combined with topical antimicrobials, such as CHX, have been attempted for treating severe forms of periodontitis (Quirynen et al., 1995; Sigusch et al., 2005). In previous studies (Heller et al., 2004; Haffajee et al., 2003; Enne, 2010).
After systemic treatment with AMX and MET, a significant but transitory increase in the MICs of AMX and CHX was observed in the T group. Although a similar pattern was detected in the placebo group, the changes in the MICs of all antimicrobials over time were not significant for this group. Interestingly, topical CHX was extensively used in both groups, but the increase in the MIC of this antimicrobial was significant only in the T group. Conceivably, the systemic administration of AMX and/or MET might have a synergistic impact on the susceptibility of the microbiota to CHX, reflecting ecological shifts in the periodontal microbiota. Other studies have also reported the selective and transient pressure of systemic antimicrobials on the susceptibility of the subgingival microbiota on the mixed culture of subgingival plaques.

The composition of the cultivable periodontal microbiota was evaluated before and after treatment in both groups. At baseline, high levels of many of the tested bacterial species, including periodontal pathogens, were detected in the periodontitis-related biofilm in both groups, consistent with previous studies (Socransky et al., 1998, Socransky and Haffajee, 2002, 2005). Nevertheless, genes associated with MET resistance have been determined in Bacteroides spp. (Trinnet et al., 1996). In addition, periodontal pathogens cultivated in biofilms are 100 times more resistant to MET compared with planktonic cultures (Wright et al., 1997; Eick et al., 2004; Sedlacek and Walker, 2007).
implications, as pathogenic species colonizing the periodontal biofilm might be more resistant to antimicrobials. Previous studies have suggested that major clinical and microbiological changes after mechanical therapy with or without antimicrobials are typically more pronounced in the first 3 months after therapy (Xajigeorgiou et al., 2006, Mestnik et al., 2010, Yek et al., 2010). However, as shown in figure 2, a few species continued to diminish after 9 and 12 months in both groups. For example, A. actinomycetemcomitans and P. nigrescens were not detected in the cultivated biofilm at 9 and 12 months after both treatment protocols. The reinforcement in oral hygiene and re-instrumentation during the monitoring visits might have contributed to the continuous reduction of certain pathogenic species.

Thus, these data indicate that enhanced mechanical periodontal therapy associated with the extensive topical use of CHX and systemic administration of AMX and MET leads to a transitory increase in the MICs of the subgingival biofilm to AMX and CHX. Notably, resistance was not evaluated in the present study because there are no breakpoints to assess susceptibility or resistance when MICs are obtained upon biofilm analysis. Both therapeutic protocols presented similar and limited effects on the composition of the cultivable subgingival microbiota over time. Given the similar clinical benefits of both approaches (Heller et al., 2011, Varela et al., 2011, Silva-Senem et al., 2013), the enhanced mechanical periodontal therapy associated with the topical use of CHX may be suggested as a potential and effective alternative for the treatment of individuals with GAP, without major implications on the susceptibility profile of the periodontal microbiota.

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