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SUMMARY

PHD THESIS

OXIDATIVE STRESS EVALUATION IN PATIENTS WITH PERIODONTITIS TREATED WITH ANTIBIOTIC THERAPY

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INTRODUCTION

Homeostasis of tissues affected by periodontal disease is achieved by the mechanical removal of bacterial deposits on the surface of teeth by scaling and root planing (SRP). However, this procedure cannot remove residual bacteria in root locations that are inaccessible to mechanical instrumentation; therefore, subgingival biofilm cannot be completely eliminated. The insufficient reduction of bacteria is associated with therapeutic failures, and the persistence of bacterial species after mechanical debridement has been associated with additional tissue destruction. Under these conditions, the use of systemic antibiotics as an adjunct to initial periodontal therapy may be helpful, especially because the disruption of biofilm by mechanical instrumentation makes bacteria more susceptible to antibiotics.

Until recently, the antibiotic combination of amoxicillin (AMX) plus metronidazole (MTZ) administered for 7-8 days has been the protocol most widely used to achieve clinical and microbiological efficacy. However, despite the impressive amount of research that has demonstrated the beneficial effects of the AMX + MTZ combination as an adjuvant to SRP, there is still no consensus in the literature concerning the optimal duration and antibiotic dosing. Various adjunctive regimens of AMX + MTZ have been proposed. Moreover, the different species of periodontal pathogens often have different susceptibilities to particular antibiotics. Few authors have investigated the resistance of perio-pathogenic bacteria to antibiotics both before and after periodontal treatment. However, the existing studies show that despite the initial antibiotic resistance of some species, most perio-pathogens are sensitive to antibiotics during their administration, and systemic antibiotics reduce the number of resistant strains at the end of non-surgical periodontal therapy, when compared with the initial situation. In periodontal disease, pathogens such as *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* interact with the host organism and produce a systemic inflam- mation, which affects the tissue balance through the action of a large number of cytokines and chemokines released by normal connective tissue residents such as mast cells, fibroblasts or by acquired connective tissue participants such as activated macrophages. The production of reactive oxygen species is a component of the bone resorption process that occurs during periodontal disease, and contributes to its aggravation. However, the source of reactive oxygen species and their role in the pathogenesis of periodontitis remain unclear. Studies in human subjects have revealed that periodontal disease is associated with systemic oxidative stress that induces some minor localized inflammation.

RESEARCH MAIN OBJECTIVES

1. Oxidative stress evaluation in patients with chronic periodontitis who have been treated with non-surgical periodontal therapy alone, compared with patients treated with non-surgical periodontal therapy followed by adjuvant systemic antibiotic therapy.
2. Comparison of changes in periodontal clinical parameters, microbiological changes and systemic oxidative stress as a result of adjuvant systemic administration of amoxicillin (AMX) and metronidazole (MTZ) to non-surgical periodontal therapy, for short (3 days) or long (7 days) days) administration, in patients with chronic periodontitis.
3. Evaluation of changes in resistance of subgingival pathogens to the mentioned antibiotics, before and after periodontal treatment.

STUDY I
Oxidative stress evaluation in patients with chronic periodontitis treated with non-surgical periodontal therapy with or without adjuvant systemic antibiotic therapy- a non-randomized controlled clinical pilot study

INTRODUCTION

Oxidative stress is incriminated in the pathophysiology of both systemic diseases with a high prevalence, such as hypertension, atherosclerosis, diabetes, and periodontitis. Markers of oxidative stress were highlighted in saliva, which confers it pathogenicity in oral disorders.
Oxidative stress is characterized by an imbalance between the production of reactive oxygen species, and the ability of biological systems to fight these destructive molecules and to induce repairing processes. Antioxidants are defined as substances, which, in low concentrations, when compared to an oxidizable substrate, delay or postpone the oxidation of that substrate.

Oxidative stress occurs when there is an imbalance between oxidants and antioxidants, the reactive oxygen species gaining ground, and generating the destruction of tissues.
Studies have shown that there is a significant decrease in the concentration of antioxidants in saliva of periodontal patients, when compared to healthy individuals, while oxygen-derived free radicals and the products of their reactions play an important role in the pathogenesis of chronic inflammatory disorders, like periodontitis. Glutathione is considered to be an important antioxidant that limit cell injury induced by reactive oxygen species and has an essential role in the control of the inflammatory processes and the redox reactions. Patients with periodontitis display a reduced total antioxidant capacity of the saliva, and lower concentrations of reduced glutathione (GSH) both in serum and in gingival crevicular fluid. The primary goal of non-surgical periodontal therapy is to control microbial periodontal infection by removing bacterial biofilm, calculus and toxins from periodontally involved root surfaces. This goal is reached by mechanical root instrumentation using ultrasound and hand instruments, alone or in conjunction with various local antimicrobials. The benefits of systemic antibiotic therapy as an adjunct to periodontal non-surgical treatment have been consistently debated in the literature, and a wide variety of antibiotics has been investigated as an adjunct to mechanical debridement of the periodontal pockets. The combination of amoxicillin and metronidazole administered after non-surgical periodontal therapy has been shown to be one of the most promising antibiotic protocols in the treatment of periodontitis.

MATERIAL AND METHODS

Sixteen patients, were investigated clinically and radiographically at baseline (before therapy). The following clinical parameters were assessed: periodontal pocket depth (PPD), clinical attachment level (CAL), bleeding on probing (BOP), and full-mouth plaque score (FMPS). After the measurements, full-mouth scaling and root planing under local anesthesia was performed. In the test group, additional systemic antimicrobial agents were distributed after SRP sessions. Subjects were clinically and biochemically monitored at baseline and were reevaluated in the same manner at the three months periodontal recall.

At the end of the non-surgical therapy session, the clinician allocated the patients to one of the two treatment groups, and gave their medications along with instructions for intake:

- Control group: SRP alone + placebo (N=8);
- Test group: SRP followed by systemic Amoxicillin and Metronidazole (SRP + AMX + MTZ) (both 500 mg, three times daily, seven days, N=8).

In order to evaluate the level of oxidative stress, blood samples were taken and the d-ROM test was used to measure the level of reactive oxygen metabolites and the BAP test was used for the analysis of the biological antioxidant potential (Diacron International®, Grosseto, Italia).

Unstimulated whole saliva samples were collected for the evaluation of C-reactive protein (CRP) using CRP cards (Analyticon Biotechnologies® AG, Germania) and GSH level, trough spectrophotometric method.

RESULTS

Mean age of the patients in control group was 50.62± 6.39 years old and in test group 37.62±5.31 years old.

Both PPD and CAL changes presented a statistical significance (p=0.01), showing reductions at the three months reevaluation meaning that the primary outcome of the periodontal therapy was achieved.

The FMPS decreased in both groups, but statistically significantly only in control group (p=0.02). The BOP in control group decreased from 23.5±11.35% to 18.5±13.29% and in test group from 29.75±13.38% to 12.75±10.2%, fact that reveals that antibiotics have a greater impact in the inflammation control.

Detectable CRP levels remained in more patients in the control group (seven out of eight) than in the test group (four out of eight).

After three months, GSH mean values decreased in the control group from 68.68±75.37 µmol/L to 65.14± 66.71 µmol/L and in test group from 48.73±33.89 µmol/L to 46.46±21.59 µmol/L.

d-ROMs and BAP values have changed in the following manner:

- Control group: both d-ROMs and BAP increased (d-ROMs from 448.94±128.42 U CARR to 458.91± 137.11 U CARR and BAP from 1783.3±510.04 µmol/L to 2319.9 µmol/L);
- Test group: both d-ROMs and BAP decreased (d-ROMs from 491.83±134.85 U CARR to 375.58± 126.06 U CARR, p=0.01, and BAP from 2246.18± 918.35 µmol/L to 1890.16±582.71 µmol/L)

DISSCUTIONS

Similar results were obtained by Savita et al. (2015) at the three months reevaluation of salivary GHS and by Öngöz et al. (2016) at one month. Its decrease may be due its consumption in the mechanisms of the neutralization of free radicals as a scavenger. The GSH reduction tendency in our findings may be the result of its use in the local antioxidant systems.

Related to our research, the studies of Tsai et al. (2005), Akalin et al. (2007), Konopka et al. (2007) and Chapple et al. (2007) carried on human subjects have highlighted the fact that periodontitis is associated with a systemic state of oxidative stress level by inducing a minor local inflammatory status. In association with a low total antioxidant capacity, a direct correlation among the features of periodontal disease, the systemic inflammatory status and oxidative stress may be stated. The fact that periodontal disease generates oxidative stress formation, or, conversely, that it may be a result of oxidative stress is an aspect that remains insufficiently proven and further research is needed. Another finding in our study that is supported by authors like Ehmke et al. (2005) and Feres et al. (2012) is that better clinical results were found measuring mean full-mouth PPD or CAL as endpoint, in the evaluation of the outcome provided by the use of adjuvant antibiotic medication, compared to the treatment with SRP alone.

CONCLUSIONS

1. The detectable CRP values remained present in more patients in the *placebo* group than in the antibiotic group.
2. The mean values of GSH decreased in both investigated groups.
3. The bleeding index had a more pronounced decrease in the test group, suggesting that adjuvant antibiotic therapy to SRP has a greater impact in controlling inflammation.
4. In patients receiving periodontal therapy combined with adjuvant antibiotic therapy, the oxidative stress status decreased from a very high level to a medium level after 3 months from the time of treatment.
5. The reduction of reactive oxygen metabolites levels may also be attributed to the complementary use of antibiotics to non-surgical periodontal therapy.

STUDY II

The effect of two regimens of systemic adjuvant antibiotic therapy to non-surgical periodontal treatment on the clinical, microbiological, bacterial resistance and oxidative stress values in patients with chronic periodontitis- a randomized triple-blind *placebo*-controlled clinical trial

INTRODUCTION

Homeostasis of tissues affected by periodontal disease is achieved by the mechanical removal of bacterial deposits on the surface of teeth by scaling and root planing. However, this procedure cannot remove residual bacteria in root locations that are inaccessible to mechanical instrumentation; therefore, subgingival biofilm cannot be completely eliminated. The insufficient reduction of bacteria is associated with therapeutic failures, and the persistence of bacterial species after mechanical debridement has been associated with additional tissue destruction.

Under these conditions, the use of systemic antibiotics as an adjunct to initial periodontal therapy may be helpful, especially because the disruption of biofilm by mechanical

instrumentation makes bacteria more susceptible to antibiotics. Until recently, the antibiotic combination of amoxicillin plus metronidazole administered for 7-8 days has been the protocol most widely used to achieve clinical and microbiological efficacy. However, despite the impressive amount of research that has demonstrated the beneficial effects of the AMX + MTZ combination as an adjuvant to SRP, there is still no consensus in the literature concerning the optimal duration and antibiotic dosing. Various adjunctive regimens of AMX + MTZ have been proposed. In the literature, the durations of systemic treatment with MTZ, AMX or their combination range from 7-8 days to 10-14 days. When both the duration of administration and the doses are considered, the variation in study design is even greater. The only exception appears to be the dose of AMX, which in the vast majority of studies is 500 mg per day, three times daily (TID). On the other hand, the recommended ideal dose of MTZ has been cited as 200, 250, 400 or 500 mg. Recently, substantial changes have been proposed to the adjuvant systemic antibiotic administration protocol used to treat patients with chronic periodontitis. In studies evaluating exclusively the change in clinical parameters in patients with chronic periodontitis, the proposed changes have reduced the duration of antibiotic intake from 7 to 3 days, and increase the dose from 3 x 250-375 mg/day to 3 x 500 mg/day.

These modifications are controversial due to the possibility that perio-pathogenic bacteria might develop antibiotic resistance. However, regard- less of the dose and duration, it is important that antibiotics be taken at their minimum bactericidal concentration to limit the risk for side effects and the development of microbial antibiotic resistance. The causative factors for this resistance remain uncontrolled, and national strategies to address the problem are lacking.

Currently, the 3-day systemic antibiotic adjunctive regimen used in non-surgical periodontal therapy has been compared with a 7-day regimen in only two studies conducted by the same investigators who only evaluated changes in clinical periodontal parameters.

MATERIAL AND METHODS

This prospective, *placebo*-controlled, triple-blinded, randomized clinical trial was registered in the ISRCTN Registry of Clinical Trials (ISRCTN12816166), and follows the guidelines described in the CONSORT 2010 statement on clinical trials.

The study population consisted of 46 patients (males and females, mean age, 46.24±12.81 years; range, 27-80 years) who had clinical and radiographic signs of generalized chronic periodontitis as described by Armitage in 1999. All patients underwent a clinical and radiographic baseline (before therapy) examination that assessed the following parameters: periodontal pocket depth (PPD), clinical attachment level (CAL), full mouth bleeding score (FMBS), and full-mouth plaque score (FMPS). After completing the measurements, all pockets with PPD ≥4 mm were scaled and root planed under local anesthesia with Gracey curettes and ultrasonic instruments by the same clinician (who followed the protocol used for One-Stage Full-Mouth Disinfection-OSFMD.

At the end of the non-surgical therapy session, one investigator (the randomizer) used a number generator (www.random.org) to assign each patient to one of three treatment groups. Each position on the randomization list was associated with a medication package number that corresponded to a pre-packed medication bag that contained instructions for medication intake. One bag was handed to each patient. The patients in group A (control group, n=14) received non-surgical periodontal treatment plus treatment with *placebos* for 7 days. Patients in group B (n=16) received non-surgical periodontal treatment combined with the systemic administration of AMX and MTZ (SRP + AMX + MTZ; 500 mg, TID) for 3 days, followed by treatment with *placebos* for 4 days. Patients in group C (test group, n=16) received non-surgical periodontal treatment combined with SRP + AMX + MTZ (500 mg TID) for 7 days. Each medication bag contained four identical vials, and each vial contained tasteless and identical types of capsules. Each vial was numbered, and the patient was instructed to take one capsule from each vial

every 8 h as follows: from vials no. 1 and 2 during the first 3 days after SRP, and from vials no. 3 and 4 during the following 4 days. The *placebo* group (group A) had only *placebo* capsules in all the vials.

Group B had AMX and MTZ in vials no. 1 and 2, and *placebo* in vials no. 3 and 4. Group C had AMX and MTZ in all four vials. The clinician, the randomizer and the patients were blinded against the antibiotic regimen.

During the initial evaluation, samples of subgingival plaque were collected from the deepest periodontal pockets in each quadrant and used to identify the existing bacterial strains and their resistance to systemic antimicrobial agents prior to treatment. This protocol was repeated at the three-month re-evaluation to assess post-treatment bacterial suppression and identify strain resistance after long- or short-term antibiotic intake periods. Eight paper points were inserted in each patient, and four of those paper points were inserted into sterile sealed Eppendorf tubes and sent for polymerase chain reaction (PCR) testing that was performed with a commercial Micro-Ident® Kit. The samples were tested for the following bacterial strains: *Aggregatibacter actinomycetem- comitans (Aa), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Tanererella forsythia (Tf), and Treponema denticola (Td)*.

The other four paper points were placed in vials that contained thioglycolate and resazurin. The homogenized samples were seeded into Columbia-agar plates and Schaedler-agar plates and for *Aa,* TSBV (Triptic Soy- Serum-Bacitracin-Vancomycin-Agar) plates were used. The different strains of bacteria were identified by using ViteK2 ANC Kits and Rapid ID 32A Kits. Bacterial resistance to antibiotics was evaluated by comparing the identified bacterial strains, at baseline and at three months. The minimum inhibitory concentrations (MICs) for AMX and MTZ were determined by using the Epsilometer technique (E-test®; AB Biodisk), on *Brucella* Blood Agar plates (bioMérieux®). In order to evaluate the level of oxidative stress, blood samples were taken and the d-ROM test was used to measure the level of reactive oxygen metabolites and the BAP test was used for the analysis of the biological antioxidant potential (Diacron International®, Grosseto, Italia).

RESULTS

The PPD, CAL, and FMBS values, the incidence of deep pockets (PPD ≥6 mm) and the corresponding CAL, and the number of sites with a PPD ≥6 mm, all showed significant changes between the initial and final evaluation in all three treatment groups. We also found significant decreases in FMPS in groups A and B, but not in group C. While more pronounced changes in PPD, CAL and FMBS, and greater decreases in PPD and CAL in initial deep sites were associated with a longer course of antibiotic intake, those differences were not statistically significant. There were, however, significant differences between groups with regard to the decrease in the number of sites with a PPD ≥6 mm. Post-hoc tests showed these differences were due to a greater decrease in group C than in group A (p=0.023); however, the differences between groups A and B, and between groups B and C, were not statistically significant (p>0.05 in both cases).

The detection scores for *Aa, Pg, Pi, Tf* and *Td* at baseline were not significantly different among the different treatment groups (p>0.05 for all species).

In general, the detection scores for those pathogens remained stationary or decreased over time, with only a few exceptions, namely in the case of *Aa* (1 patient in group A and 1 patient in group B) and *Pi* (1 patient in group A and 1 patient in group B). The decreases in detection scores for *Pg, Tf* and *Td* in all three groups, and for *Pi* in groups B and C were statistically significant. Significant differences among groups were found regarding changes that occurred in the detection frequency scores for *Aa* and *Tf* between baseline and the 3-month re-evaluation. For *Aa*, Mann-Whitney tests showed differences between groups A and C (p=0.048) and between groups B and C (p=0.048), but not between groups A and B; whereas

for *Tf,* groups A and B were found to be different from group C (p<0.001 in both cases), but not from each other (p=0.920).

A total of 69 bacterial isolates were identified in the bacterial cultures (average of 1.5 isolates per patient) before treatment. At the three-month reassessment, the total number of isolates was considerably reduced; however, 27 species could still be identified.

There was no resistance to AMX prior to treatment; however, after treatment, one strain of *Actinomyces israelii* (group A) and one strain of *Anareroccocus prevotii* (group C) were found to be resistant. The latter strain was identified in the same patient in group C, and was classified as being antibiotic sensitive prior to treatment, but later became resistant to both AMX and MTZ.

Resistance to MTZ prior to treatment was identified in a total of 13 strains.Resistance to MTZ after treatment was found in one strain of *Pi* (group A) and one strain *Anareroccocus prevotii* (group C).

There was a statistically significant decrease in the mean d-ROM values in group C, when compared with the mean baseline values. There were also significant differences between groups with respect to changes in d-ROM values. The decreases in mean d-ROM values in group C were significantly greater than those in groups A (p=0.012) and B (p=0.025); however, there was no difference between groups A and B (p=0.525).

DISSCUTIONS

To the best of our knowledge, this is the first study to investigate the clinical and microbiological effects of two different regimens of systemic antibiotic therapy given adjunctive to non-surgical periodontal therapy in patients with periodontitis. Moreover, this study also investigated changes that occurred in systemic oxidative stress markers and the resistance of main perio-pathogens. Unlike recent studies that only focused on clinical changes in moderate and deep pockets, our study also considered the mean full-mouth PPD as an endpoint.

Data from the literature attest that periodontitis patients are more susceptible to an imbalance in oxidative-antioxidant processes when compared with healthy subjectsand periodontal therapy can have a beneficial effect on both clinical and biochemical parameters. High levels of oxidative species are usually found in subjects with risk factors such as cigarette smoking, alcohol abuse, an unbalanced diet, or who have diseases associated with changes in oxidative balance, such as cardiovascular diseases, metabolic diseases, neurodegenerative diseases, autoimmune rheumatic diseases (rheumatoid arthritis, systemic lupus erythematosus), skin diseases like psoriasis, and periodontitis. The fact that the three groups were not homogeneous with respect to PPD and CAL at baseline could represent a potential limitation. However, in our analysis of changes that occurred between appointments, this deficiency was handled by adjusting for the baseline values of the parameters, while testing for group effects in the analysis of covariance. Furthermore, the fact that one strain of *Anaeroccocus prevotii* was identified in the same patient as being antibiotic sensitive prior to treatment, and then became resistant to both AMX and MTZ after 7 days, suggests that certain strains can acquire bacterial resistance during systemic antibiotic therapy.

From a clinical point of view, our microbiologic and oxidative stress data suggest that a 7-day systemic antibiotic regimen remains the regimen of choice as an adjuvant to SRP.

CONCLUSIONS

1. With all the limitations of this study, it can be concluded that non-surgical periodontal therapy administered in combination with a 7-day antibiotic regimen has been shown to be more effective in improving the clinical parameters, compared with a 3-day antibiotic regimen, in patients with severe chronic. Periodontitis.

2. In patients with chronic periodontitis, in terms of identifying the main periodontal-pathogenic bacteria (*Aa* and *Td*), a superior improvement with the 7-day antibiotic regimen has been demonstrated.
3. The 7-day systemic adjuvant antibiotic regimen improves the values of systemic oxidative stress markers (decreased d-ROMs) in patients with chronic periodontitis.
4. Bacterial resistance was identified in fewer bacterial strains after antibiotic treatment administered adjuvant to SRP, than before treatment.

GENERAL CONCLUSIONS

1. The detectable CRP values remained present in more patients in the *placebo* group than in the antibiotic group.

2. The mean values of GSH decreased in both investigated groups.

3. The bleeding index had a more pronounced decrease in the test group, suggesting that adjuvant antibiotic therapy to SRP has a greater impact in controlling inflammation.

4. In patients receiving periodontal therapy combined with adjuvant antibiotic therapy, the oxidative stress status decreased from a very high level to a medium level after 3 months from the time of treatment.

5. The reduction of reactive oxygen metabolites levels may also be attributed to the complementary use of antibiotics to non-surgical periodontal therapy.

6. With all the limitations of this study, it can be concluded that non-surgical periodontal therapy administered in combination with a 7-day antibiotic regimen has been shown to be more effective in improving the clinical parameters, compared with a 3-day antibiotic regimen, in patients with severe chronic periodontitis.

7. In patients with chronic periodontitis, in terms of identifying the main periodontal-pathogenic bacteria (*Aa* and *Td*), a superior improvement with the 7-day antibiotic regimen has been demonstrated.

8. The 7-day systemic adjuvant antibiotic regimen improves the values of systemic oxidative stress markers (decreased d-ROMs) in patients with chronic periodontitis.

9. Bacterial resistance was identified in fewer bacterial strains after antibiotic treatment administered adjuvant to SRP, than before treatment.