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DETECTION AND QUANTIFICATION OF PATHOGENIC MICROORGANISMS IN PATIENTS WITH CHRONIC PERIODONTITIS BEFORE AND AFTER TREATMENT WITH CHLORHEXIDINE DIGLUCONATE

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The aim of this study was to evaluate the efficiency of chlorhexidine digluconate solution (CHX) as an adjunct to nonsurgical therapy (NPT) of patients with chronic periodontitis (CP), by analyzing the presence and quantity of periopathogenic microorganisms in subgingival biofilm. DNA was extracted from the subgingival biofilm obtained from 40 patients with CP divided into two groups (NPT+CHX and NPT alone as control) at baseline and 2 months after the therapy. The presence of selected periodontal pathogens (*A. actinomycetemcomitans*, *P. gingivalis*, *E. corrodens*, *T. denticola*, and *T. forsythia*) was determined by polymerase chain reaction (PCR), while total bacterial load was assessed by quantitative PCR. The incidence of microorganisms decreased following treatment, both with NPT+CHX and NPT alone, but without reaching statistically significant difference in the NPT group. In the NPT+CHX group, a significant reduction of prevalence of two species: *T. denticola* (P = 0.008) and *T. forsythia* (P = 0.016), as well as of total microorganism count (P = 0.002) was observed two months after treatment. In conclusion, the present findings support the use of CHX as adjunctive therapy in CP.

Keywords: Chronic periodontitis, Periopathogenic microorganisms, PCR, qPCR, Chlorhexidine digluconate

INTRODUCTION

Periodontitis, with its polymicrobial etiology, is among the most frequent oral inflammatory diseases affecting more than 50% of the world's adult population (ZHANG *et al*.,

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2014). Species strongly associated with periodontitis development include, among others, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingiva*, *Treponema denticola*, and *Tannerella forsythia* (SLOTS and TING, 1999). Nonsurgical periodontal treatment (NPT) leads to ecological changes in the subgingival environment, i.e. in the composition of the microbiota (SOCRANSKY *et al*., 2013). Yet, it has been proven by several studies that periopathogenic flora from all oral niches sometimes cannot be efficiently removed only by mechanical disruption (CUGINI *et al*., 2000). Indeed, risk factors such as deep pockets, furcations and vertical defects may affect NPT outcomes in patients with chronic periodontitis (CP) if only mechanical debridement is applied (DRISKO, 2001). Consequently, dentists often use antimicrobial pharmacological agents, either antiseptics, or local/systematic antibiotics. Although novel chemical antiplaque agents are being continuously developed (SANZ *et al*., 2012), chlorhexidine digluconate (CHX) is still one of the most commonly applied adjunctive products in CP therapy, due to its well established local antibacterial efficiency and lack of systemic toxicity (PRIETTO *et al*., 2020). On the other hand, some studies point to possible side effects of CHX such as tooth pigmentation, peeling of the mucosa and taste changes (DA COSTA *et al*., 2017), prompting further need of weighing the pros and cons of its implementation in the treatment of CP.

The aim of the present study was to evaluate the efficacy of CHX as an adjunct to NPT, by analyzing crucial representatives of periopathogenic microorganisms' incidence before and after therapy, as well as the total bacterial load.

Patient selection

This case-control study was approved by the Ethics Committee of the School of Dental Medicine, University of Belgrade (Protocol n° 36/4 on February, 25th 2020) and was conducted in full accordance with the requirements of the 1964 Helsinki Declaration and its amendments. All participants have signed an informed consent to participate in the study.

The study included 40 patients with CP, both genders, aged 25 to 55 years, with good general health. Participants were randomly assigned to two equal groups (n = 20). The first group of CP patients was named 'NPT+CHX' group and they underwent NPT combined with CHX. The second group of CP patients, who underwent only nonsurgical periodontal treatment, was the control group named 'NPT'.

All patients had evident signs of acute inflammation (radiographic evidence of generalized alveolar bone loss >30%, presence of at least one pocket with probing pocket depth >5mm per quadrant and positive bleeding on probing). Exclusion criteria were: a history of systemic conditions such as heart disease, diabetes and other types of disorders that could affect the periodontal tissues or could impact the metabolic profile; pregnant and lactating women; presence of less than 15 teeth in the oral cavity; use of food supplements such as vitamin C and E in the last three months; restrictive diets in the last three months; patients taking any form of antioxidant supplements in the last three months; patients who have undergone periodontal therapy during the last three months and those who have been using antibiotics during the last three months.

Clinical examination and periodontal treatment

All patients were evaluated clinically and radiographically during their first visit, when they also received oral hygiene instructions and a medical history was taken. After that, subgingival biofilm samples were collected. NPT was performed in both patient groups during the next two visits, at week one and week two (two quadrants per visit), using hand instruments (Gracey, Hu-Friedy, Chicago, USA) and ultrasonic scalers (Cavitron Select, Dentsply, York, PA, USA) under local anesthesia (2% lidocaine with adrenaline, 1: 100 000).

During each NPT appointment oral hygiene instructions were repeated. NPT+CHX patients were further advised to rinse their mouth for 60s with 15ml of 0,12% CHX solution (Hibidex DAP, Galenika, Belgrade, Serbia), twice a day, after brushing teeth during these two weeks, and they were also asked to restrain from eating or drinking minimum 30 minutes after rinsing.

Two months after therapy completion, patients from both groups were called in for a clinical reassessment and microorganisms sampling.

Microorganisms' sampling

After saliva sampling and clinical measurements, in both analyzed groups, following the careful removal of supragingival biofilm, areas were washed with water spray, isolated with cotton rolls, and gently dried. Subgingival biofilm samples were collected by sterile endodontic paper points (No. 35), which were inserted in the site with deepest periodontal pockets in each quarter and kept there for 30 s. After that, paper points were transferred to tubes containing 1 ml of reduced transfer fluid (RTF). Two paper points were used for every sampling site and were pooled in the same tube and stored at -72°C until performing DNA extraction. Following the protocol, total bacterial DNA was isolated from the samples, using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Microorganisms' detection by PCR

The presence of 16S ribosomal DNA of *Porphyromonas gingivalis*, *Treponema denticola*, *Eikenella corrodens*, *Tannarella forsythia* and *Aggregatibacter actinomycetemcomitans* was detected using the standard PCR method, 0.2 μM of specific primers (Table 1), DreamTaq DNA polymerase (Thermo Fisher Scientific™; Waltham, MA, USA) and 5 μL of bacterial DNA isolate.

PCR was performed under the following conditions: initial denaturation (95°C for 3 minutes), cycling (35 rounds of: denaturation (94°C for 45 seconds), hybridization for 60 seconds (temperature given in Table 1), and elongation (72°C for 60 seconds), and final elongation (72°C for 5 minutes). Strains obtained from American Type Culture Collection (ATCC): *P. gingivalis* (ATCC 33277), *T. denticola* (ATCC 35405), *E. corrodens* (ATCC 23834), *T. forsythia* (ATCC 43037) and *A. actinomycetemcomitans* (ATCC 33384) were used as positive controls, and PCR reactions with water instead of bacterial DNA were used as negative controls. Products were separated by polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized under UV light. A band of expected length (Table 1) confirmed microorganism presence.

Table 1. Primers used for PCR, their hybridization temperatures and product sizes.

Species	Sequence (5' – 3')	Hybridization temperature (°C)	Product size (bp)
Porphyromonas gingivalis	Fwd AGGCAGCTTGCCATACTGCG Rv ACTGTTAGCAACTACCGATGT	55	400
Treponema denticola	Fwd TAATACCGAATGTGCTCATTTACAT Rv TCAAAGAAGCATTCCCTCTTCTTCTTA	60	316
Eikenella corrodens	Fwd CTAATACCGCATACGTCCTAAG Rv CTACTAAGCAATCAAGTTGCCC	55	688
Tannerella forsythia	Fwd GCGTATGTAACCTGCCCGCA Rv TGCTTCAGTGTCAGTTATACCT	55	600
Aggregatibacter actinomycetemcomitans	Fwd GCTAATACCGCGTAGAGTCGG Rv ATTTCACACCTCACTTAAAGGT	55	500

Quantification of microorganisms by real-time PCR

Total bacteria quantity was estimated by quantitative PCR (qPCR) as previously described by BRAJOVIC *et al.* (2016) The amplification of highly conserved target regions of 16S rRNA was performed using SensiFAST SYBR® Hi-ROX Kit (Bioline Reagents Ltd, London, UK) and the following primers: Fw 5′-TCCTACGGGAGCACAGT′-3 and Rv 5′GGACTACCAGGGTATCTAATCCTGTT-3′. The reference strain used for standard curve analysis was *Prevotella melaninogenica* (ATCC 25845), and the results were expressed as total gene copy number.

Statistical analysis

Statistical data analysis was performed using Statistical Package for Social Science (SPSS software package, version 20.0; SPSS Inc., Chicago, IL, USA). Categorical variables were presented as frequency of a certain category. The significance of differences between categorical variables was analyzed by using McNemar Test (for variables before and after intervention) or Pearson's Chi-square test (for variables in two independent samples). According to the normality test results, comparisons were performed using non-parametric Wilcoxon Signed Ranks Test (for variables before and after intervention) or nonparametric Mann-Whitney U test (for variables in two independent samples). All the analyses were considered statistically significant at $P<0.05$.

RESULTS

Microorganisms' detection The incidence of microorganisms decreased following both types of treatment, but without reaching statistically significant difference in the NPT group. However, in the NPT+CHX group, out of the five analyzed bacterial species, two showed a statistically significant decrease of incidence after the treatment: *T. denticola* ($P = 0.008$) and *T. forsythia* ($P = 0.016$) (Table 2).

Table 2. Distribution of periopathogenic bacteria in subgingival plaque at baseline and two months after applied therapy in NPT and in NPT+CHX group

Species	Treatment group	Baseline n* (%)	Two months after therapy n (%)	P value**
Porphyromonas gingivalis	NPT	19 (100%)	16 (84.2%)	0.250
	NPT+CHX	18 (94.7%)	17 (89.5%)	1.000
Treponema denticola	NPT	8 (42.1%)	4 (21.1%)	0.289
	NPT+CHX	12 (63.2%)	3 (15.8%)	0.008
Eikenella corrodens	NPT	16 (84.2%)	13 (68.4%)	0.250
	NPT+CHX	18 (94.7%)	16 (84.2%)	1.000
Tannerella forsythia	NPT	16 (84.2%)	9 (47.4%)	0.650
	NPT+CHX	19 (100%)	11 (57.9%)	0.016
Aggregatibacter actinomycetemcomitans	NPT	18 (94.7%)	15 (78.9%)	0.375
	NPT+CHX	15 (78.9%)	13 (68.4%)	1.000

*n – number of positive samples.

**P values calculated using McNemar Test, significant at P<0.05 (bold).

Microorganisms' quantification

In the NPT+CHX group, a significant reduction in total microorganism count was observed after two months (from $5.30\times10^6\pm6.06\times10^6$ to $1.28\times10^6\pm2.18\times10^6$, P=0.002), while this was not the case in the NPT group (from $3.02\times10^6\pm3.94\times10^6$ to $1.07\times10^6\pm1.74\times10^6$, P=0.094) (Figure 1).

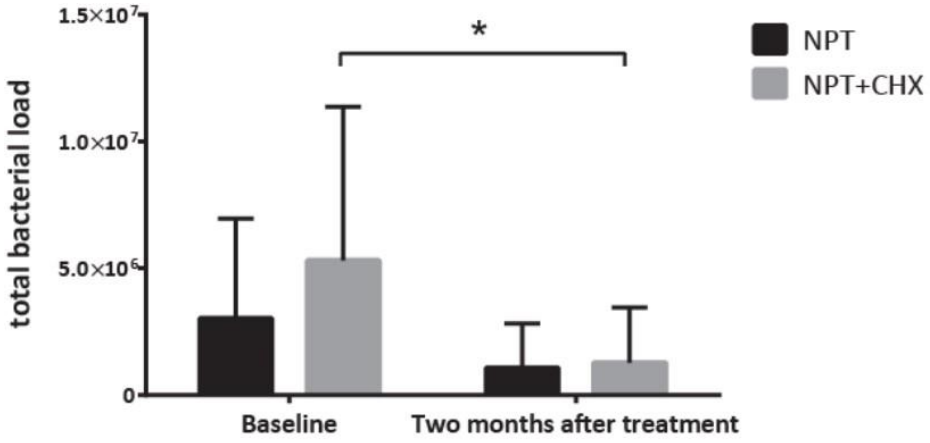

Figure 1. Prevalence of microorganisms presented as total bacterial load, at baseline and two months after therapy completion in NPT and NPT+CHX groups.

DISCUSSION

Monitoring different biomarkers, including pathogenic microorganisms, in the course of periodontal treatment is highly recommended in order to correctly evaluate the results of the applied therapy and, if necessary, modify it. Our study showed that in terms of microorganisms' incidence, there was evidence of a general decrease of all examined bacterial species both after NPT and after NPT+CHX, but without reaching statistical significance in the NPT group. On the other hand, in the NPT+CHX group *T. denticola* and *T. forsythia* showed a statistically significant decrease, pointing to the fact that NPT combined with CHX achieved greater effects in microorganisms' eradication. Our results are fully in agreement with those obtained by Calderini et al., who used two CHX formulations (CHX digluconate and CHX dihydochloride) and found a decrease of all analyzed bacterial species with both of the CHX formulations (CALDERINI *et al*., 2013). It also supports earlier findings regarding the remarkable efficiency of CHX as a local antiseptic in different clinical/laboratory settings (HAFFAJEE *et al*., 2008; NELSON-FILHO *et al*., 2012). In the study of Preus et al. it was also shown that NPT combined with adjunctive metronidazole treatment, resulted in more pronounced *T. forsythia* decrease than NPT alone, which suggests that and adjunct to the basic periodontal therapy is needed (PREUS *et al*., 2015). Krück et al. in their study of different adjunctive treatment of CP showed that, regarding microbiological findings, povidone-iodine seemed to give better results than chlorhexidine or sodium chloride (KRÜCK *et al*., 2012).

Also, reduction of total bacteria count was present after two months in both NPT and NPT+CHX groups, but the difference was significant only when CHX was applied. This confirms the recently published findings of Lauritano et al., although in their study CHX was in the form of gel, not a mouth rinse (DORINA *et al*., 2019) . Namely, they showed a statistically significant reduction in the total bacterial load in the test group, while in the control group the decrease was not significant. This indicates that despite of some possible side effects, CHX is worth considering as an adjunct to NPT in periodontitis treatment. The present study had some limitations associated with the impossibility of strict control of CHX therapy which patients performed independently at home and the short-term follow-up of two months that could have interfered with the results. So, it is necessary to monitor patients in a longer period after conservative therapy with control examinations every month. Further research involving a larger number of patients is also mandatory.

In conclusion**,** the obtained results indicate a significant reduction of bacteria incidence pointing to positive effects of adjunctive CHX therapy in patients with chronic periodontitis.

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REFERENCES

BRAJOVIĆ, G., B. POPOVIC, M. PULETIĆ, M. KOSTIĆ, J. MILAŠIN (2016): Estimation of total bacteria by real-time PCR in patients with periodontal disease. Srp. Arh. Celok. Lek., *144*(1–2):10–4.

CALDERINI, A., G. PANTALEO, A, ROSSI, D. GAZZOLO, E. POLIZZI (2013): Adjunctive effect of chlorhexidine antiseptics in mechanical periodontal treatment: first results of a preliminary case series. Int. J. Dent. Hyg., *11*(3):180-5.

CUGINI, M.A., A.D. HAFFAJEE, C. SMITH, R.L.JR, KENT, S.S. SOCRANSKY (2000): The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results. J. Clin. Periodontol., *27*(1):30–6.

DA COSTA, L.F.N.P., C. DA S.F., AMARAL, D. DA S. A.T.T., BARBIRATO LEAO., M.F. FOGACCI (2017): Chlorhexidine mouthwash as an adjunct to mechanical therapy in chronic periodontitis: A meta-analysis. J. Am. Dent Assoc., *148*(5):308–18.

DRISKO, C.H. (2001): Nonsurgical periodontal therapy. Periodontol, 2000, *25*(1):77–88.

HAFFAJEE, A.D., T. YASKELL, S.S. SOCRANSKY (2008): Antimicrobial effectiveness of an herbal mouth rinse compared with an essential oil and a chlorhexidine mouth rinse. J. Am. Dent. Assoc., *139*(5):606-11.

KRÜCK, C., S. EICK, G.U, KNÖFLER, R.E, PURSCHWITZ, H.F.R. JENTSCH (2012): Clinical and Microbiologic Results 12 Months After Scaling and Root Planing With Different Irrigation Solutions in Patients With Moderate Chronic Periodontitis: A Pilot Randomized Trial. J. Periodontol., *83*(3):312–20.

LAURITANO, D., A. PALMIERI, O. D'ORTO, A. BOLZONI, L. OTTRIA, M. GARGARI, M, DI GIROLAMO, V. CANDOTTO (2019): The use of a new chemical device based on silver and cationic surfactants as a new approach for daily oral hygiene: A preliminary study on a group of periodontal patients. Int. J. Immunopathol. Pharmacol., *33*:2058738419868101.

NELSON-FILHO, P., K.O. CARPIO-HORTA, M.C.D. DAMIAO ANDRUCIOLI, M. FERES, R.A .BEZERRA DA SILVA, F.W.,GARCIA PAULA-SILVA, F.L. ROMANO (2012): Molecular detection of Aggregatibacter actinomycetemcomitans on metallic brackets by the checkerboard DNA-DNA hybridization technique; Am J Orthod Dentofacial Orthop; *142*(4):481-6.

PREUS, H.R., G. DAHLEN, P. GJERMO, V. BAELUM (2015): Microbiologic Observations After Four Treatment Strategies Among Patients With Periodontitis Maintaining a High Standard of Oral Hygiene: Secondary Analysis of a Randomized Controlled Clinical Trial. J Periodontol.; *86*(7):856–65.

PRIETTO, N.R., T.M. MARTINS, C.D.S. SANTINONI, N.M. POLA, E. ERVOLINO, A.M. BIELEMANN, F.R.M. LEITE (2020): Treatment of experimental periodontitis with chlorhexidine as adjuvant to scaling and root planing. Arch. Oral Biol., :*110*.

SANZ, I., B., ALONSO, M. CARASOL, D. HERRERA, M. SANZ (2012): Nonsurgical treatment of periodontitis. J. Evid. Based Dent. Pract., 2012;*12*(3 Suppl):76–86.

SLOTS, J. and M, TING (1999): Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in human periodontal disease: occurrence and treatment. Periodontol., 2000, *20*: 82–121.

SOCRANSKY, S.S., A.D, HAFFAJEE, R. TELES, J.L. WENNSTROM, J, LINDHE, A. BOGREN, H, HASTURK, T. VAN DYKE, X. WANG, J.M, GOODSON (2013): Effect of periodontal therapy on the subgingival microbiota over a 2-year monitoring period. I. Overall effect and kinetics of change. J. Clin. Periodontol., *40*(8):771–80.

ZHANG, Q., Z. LI, C, WANG, T, SHEN, Y. YANG, S. CHOTIVICHIEN, L. WANG (2014): Prevalence and predictors for periodontitis among adults in China, 2010.; Glob Health Action; *1*:1–7.

DETEKCIJA I KVANTIFIKACIJA PATOGENIH MIKROORGANIZAMA KOD PACIJENATA SA HRONIČNIM PARODONTITISOM PRE I NAKON TRETMANA HLORHEKSIDIN-DIGLUKONATOM

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Izvod

Cilj ovog istraživanja bio je procena efikasnosti rastvora hlorheksidin-diglukonata kao dopune nehirurškoj terapiji (NHT) hroničnog parodontitisa (HP), analizom prisustva i količine parodontopatogenih mikroorganizama u subgingivalnom biofilmu. DNK je izolovana iz uzoraka subgingivalnog biofilma dobijenih od 40 pacijenata sa HP podeljenih u dve grupe (NHT+hlorheksidin i samo NHT kao kontrola) na početku i dva meseca nakon završetka terapije. Prisustvo određenih parodontalnih patogena (*A. actinomycetemcomitans*, *P. gingivalis*, *E. corrodens*, *T. denticola*, and *T. forsythia*) detektovano je lančanom reakcijom polimeraze (PCR), a ukupna količina bakterija lančanom reakcijom polimeraze u realnom vremenu (kvantitativni PCR). Incidenca mikroorganizma se smanjila nakon tretmana i u NHT+hlorheksidin i u NHT grupi, ali nije bila statistički značajna u NHT grupi. Statistički značajna redukcija dve bakterijske vrste: *T. denticola* (P = 0.008) and *T. forsythia* (P = 0.016), kao i smanjenje ukupne količine bakterija (P = 0.002) bili su prisutni u NHT+hlorheksidin grupi dva meseca nakon završetka terapije. Rezultati ove studije govore u prilog upotrebi hlorheksidin-diglukonata kao dopunskog sredstva u terapiji hroničnog parodontitisa.

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