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Effects of therapy with two combinations of antibiotics on the imbalance of MMP-2/TIMP-2 in chronic periodontitis

MARILENA BĂTĂIOSU[1)], CITTO IULIAN TAISESCU[2)], CĂTĂLINA GABRIELA PISOSCHI[3)], EUGEN IONUŢ PASCU[4)], MIHAELA JANA ŢUCULINĂ[5)], LUMINIŢA DĂGUCI[6)], CONSTANTIN DĂGUCI[6)], ILEANA MONICA BANIŢĂ[7)]

[1)]*Department of Pedodontics, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*
[2)]*Department of Functional Sciences, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, Romania*
[3)]*Department of Biochemistry, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania*
[4)]*Department of Oro-Maxillo-Facial Surgery, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*
[5)]*Department of Endodontics, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*
[6)]*Department of Oral Health, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*
[7)]*Department of Morphological Sciences, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*

Abstract

Periodontitis represents a chronic bacterial infection that induces immuno-inflammatory conditions affecting gingiva and tooth-supporting tissues. The role of some biological mediators in periodontal disease was widely investigated, especially that of MMP-8 and MMP-9. Recently, MMP-2 was also considered to be an appropriate therapeutic target for prevention of periodontal disease progression. However, effects of the combination of metronidazole with amoxicillin or spiramycin on the release and activation of MMP-2 and the balance MMP-2/TIMP-2 were rarely studied. This study was designed to assess the influence of two combinations of antibiotics used for treatment of periodontitis on the balance MMP-2/TIMP-2. Gingival samples obtained from patients with no pharmacological treated chronic periodontitis and those receiving either the association between amoxicillin–metronidazole and spyramicin–metronidazole were processed for paraffin embedding and then used to perform immunohistochemical reactions in order to detect MMP-2 and TIMP-2. All subjects were evaluated clinically and radiographic at the first visit and after treatment completed, the Löe & Silnees gingival index at six sites per tooth for the whole mouth being recorded. Statistical analysis was performed using non-parametrical techniques. Gingiva samples from untreated chronic periodontitis patients revealed a diffuse positive reaction for MMP-2 in the epithelium and also in fibroblasts and macrophages from the lamina propria. For gingiva samples from patients treated with antibiotics, MMP-2 positive reaction was restricted to deep epithelial layers and few cells of the connective tissue. No significant difference was observed for TIMP-2 expression. The clinical indexes were in accordance with immunohistochemical results. After treatment, gingival index values were significantly lower then before (p<0.001) in both groups treated with antibiotics. *Conclusions*: The two combinations of antibiotics tested in our study seem to have a dual ability to reduce inflammation as well as to inhibit MMP-2 activity.

Keywords: chronic periodontitis, MMP-2, TIMP-2, periodontal therapy, antibiotic treatment.

Introduction

Periodontitis is a chronic inflammatory condition caused by pathogenic microorganisms originating in the dental plaque. Although microbial flora is essential for disease initiation, environmental and genetic factors contribute to the progression and severity of the periodontal disease [1]. Periodontal disease is associated with anaerobic and microaerophilic parodontopathogens that release enzymes able to damage the periodontal tissues [2]. Inflammatory and immune mediators released in response to bacteria and bacterial products have significant effects on the periodontal connective tissues with subsequent loss of supporting structures of the periodontium. Because of the interaction between the bacterial infection and host response, irreversible destruction of periodontal connective tissue and alveolar bone occurs [3]. Therefore, the inflammatory cells like polymorphonuclears (PMN), which normally provide protection as microphages, can themselves be the major contributor to degradation of the periodontium [4]. In the presence of bacterial infection, large amounts of matrix metalloproteinases (MMP) are released by PMN [5]. MMP are a family of at least 12 different zinc-dependent enzymes produced by various cells [6] that can act together and in association with others biological factors to degrade the extracellular matrix (ECM) from the human body. In chronic periodontitis, collagen, which constitutes the dominant structural framework of ECM in periodontal tissue, is the main target of MMP and so is degraded by their net overproduction [7]. MMP have an important role in connective tissues remodeling and therefore they exists in high concentrations in inflamed gingiva. Among MMP, MMP-2, MMP-8 and MMP-9 are the most involved in periodontitis [8].

MMP-2 (gelatinase A) is able to cleave type IV collagen and also native type I collagen, which is the abundant component of gingival connective tissue matrix and alveolar bone. Collagen degradation is one of the main factors for uncontrolled destructive lesions in chronic periodontitis. Gelatinase A was found in the inflammatory infiltrate from lamina propria. Kubota *et al.* reported increased MMP-2 expression in inflamed gingival samples from patients with periodontal disease [9]. Normally, an inflammatory response is self-resolving and an imbalance

between MMP and their endogenous inhibitors – tissue inhibitors of matrix metalloproteinases (TIMP) – that ensure uncontrolled MMP-mediated tissue damage does not occur [10]. MMP-2 activity is controlled by TIMP-2. For periodontitis progression, MMP release and activity increase and concentration of TIMP decreases [10].

The role of inflammatory mediators (interleukins, tumor necrosis factor-α, prostaglandin E2) in periodontal disease (bone resorption and connective tissue destruction by overproduction of MMP) was widely investigated [11]. Therefore, inhibition of MMPs is considered an appropriate therapeutic target for prevention of periodontal disease progression. Indeed, subantimicrobial doses of tetracycline family antibiotics, known as MMP inhibitors, can be effective therapeutic adjuncts for the treatment of periodontitis, but the studies about the effects of systemic antibiotics treatment on MMP/TIMP balance were rarely [12]. In a previous study, we reported the general effect of these antibiotics on matrix remodeling in periodontal disease [13].

In this paper, we report the correlations between the clinical aspects assessed by the Löe & Silness gingival index (GI) and the histological changes of the gingival tissues in two different combinations of antibiotic treatment (amoxicillin with metronidazole and spiramycin with metronidazole) of chronic periodontitis.

Materials and Methods

A total of 19 subjects, both males and females, aged from 35 to 67 years, diagnosed with chronic periodontitis in the Department of Periodontology, were included in this study. Medical and dental histories were assessed. None of them had a history or current manifestation of systemic diseases. Patients with severe medical disorders (diabetes mellitus, immunological disorders, hepatitis, and HIV infection) and pregnant woman were excluded from the study.

The selection was made according to the clinical and radiographic criteria proposed by the *International World Workshop for a Classification of Periodontal Diseases and Conditions* in 1999 [14]. The study was performed with the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania. A written informed consent was obtained from each participant.

Three different groups of subjects were involved in the study:

- Control Group (n=5) – included patients treated by scaling and root planning without antibiotics or other antimicrobial agents;
- Ist Group (n=7) – patients treated by scaling and root planning followed immediately by adjunctive oral administration of a combination between amoxicillin (3×500 mg/day) and metronidazole (3×250 mg/day) for seven days;
- IInd Group (n=7) – patients treated by scaling and root planning followed immediately by adjunctive oral administration of a combination between spiramycin (3×500 mg/day) and metronidazole (3×250 mg/day) for seven days.

All subjects were evaluated clinically and radiographic at the first visit and the Löe & Silnees GI at six sites per tooth for whole mouth was assessed.

For histological examination and immunohistochemical tests, gingival samples including pocket epithelium and adjacent connective tissues were collected during extraction and surgery. After fixation in 4% buffered paraformaldehyde, gingival tissues were processed for paraffin embedding. Sections of 3 μm were cut and stained for usual examination in light microscopy (Hematoxylin and Eosin – HE, and trichromic Goldner–Szekely – GS) and for immunohistochemical reactions.

The primary antibodies used in the present study are described in Table 1.

Table 1 – ***The panel of antibodies used for the immunohistochemical study***

Antibodies	Vendor Code	Dilution
Mouse monoclonal antibody anti-MMP-2	Santa Cruz Biotechnology, sc 13595	1:100
Mouse monoclonal antibody anti-TIMP-2	Santa Cruz Biotechnology, sc 21735	1:200

Detection of MMP-2 and TIMP-2 was performed using Avidin–Biotin–Peroxidase method (Vectastain, Vector Laboratories, USA). 3,3'-Diaminobenzidine tetrahydrochloride and hydrogen peroxide were used for color development and Mayer's Hematoxylin for nuclear counterstaining. The sections were then observed using an inverted Nikon Eclipse E600 microscope equipped with a digital camera and Lucia 5 software. For each antibody tested was performed a negative control by replacing the primary antibody with phosphate buffer saline solution pH 7.4–7.6.

Statistical analysis was performed using non-parametrical techniques. Kruskal–Wallis test was used for intergroup comparisons before and after therapy. Wilcoxon test was used to compare all study groups before and after treatment with a significance level 0.001. Clinical index was also compared with immunohistochemical changes for a better understanding of progression of inflammation under antibiotic action. All data analysis was performed using a statistical package (SPSS 10.0, Abacus Concepts, USA).

Results

Histological aspects of cases included in our study varied dependent the presence or not of the periodontal therapy. Therefore, HE and trichromic GS staining revealed an increased inflammatory response in gingival tissues before treatment (Figure 1, a and b), characterized by an important presence of proinflammatory cells, especially lymphocytes and mast cells. We remarked the presence of hyperkeratosis in the superficial epithelial layer and also many high and ramified papillae (rete pegs).

The images obtained for samples from patients with untreated chronic periodontitis showed a highly positive reaction for MMP-2 in epithelial layers and also in lamina propria (Figure 2). In the Ist Group, patients with periodontitis treated by oral administration of amoxicillin and metronidazole, the positivity for MMP-2 was also present in lamina propria but in a more localized manner, and in the epithelium was restricted to deep layers (Figure 3).

Samples obtained from the patients treated with the combination between spiramycin and metronidazole displayed a positive reaction for MMP-2 in cells of the connective tissue, especially in phagocytes and also in few fibroblasts (Figure 4).

Immunohistochemical reactions for TIMP-2 displayed an obviously different pattern between the groups studied. Figure 5 shows the results of the immunoreaction for TIMP-2 for a gingival sample obtained from a patient with untreated chronic periodontitis. The immune response revealed a marked positivity in keratinocytes from all the epithelial layers, while non-keratinocyte cells were negative. In the chorion, the immune response was diffuse and scarce. For the patients treated with the combination between amoxicillin and metronidazole, TIMP-2 was present in keratinocytes from basal and spinous layers and mainly in the loose connective tissue of the papillae and at epithelial–connective tissue interface (Figure 6).

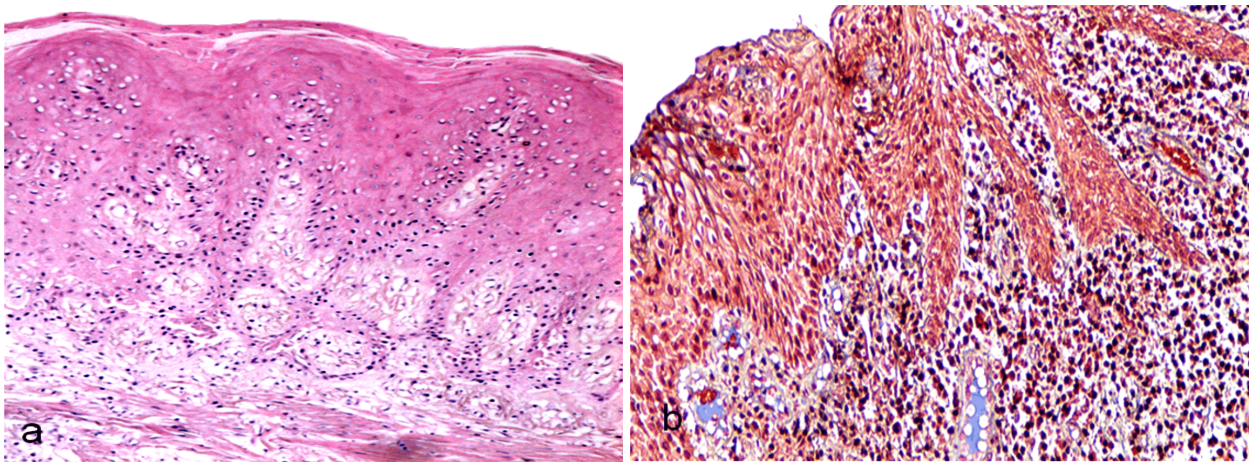

Figure 1 – ***Untreated chronic periodontitis: (a) Gingival mucosa – inflammatory infiltrate in epithelial basal layer (HE staining, ×100); (b) Sulcular mucosa – great incidence of proinflammatory cells in the superficial layer of connective tissue (trichromic GS, ×200).***

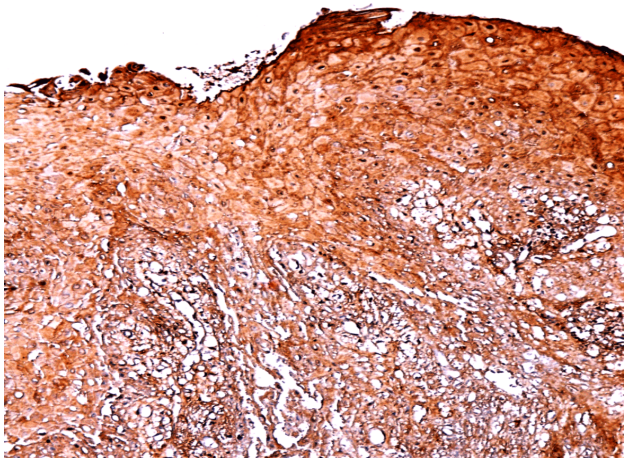

Figure 2 – ***Untreated chronic periodontitis. Intense positive reaction for MMP-2 (IHC, ×200).***

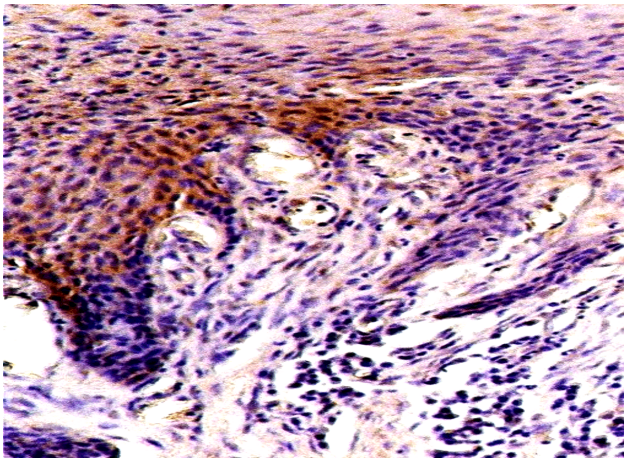

Figure 3 – ***Chronic periodontitis treated with amoxicillin and metronidazole. Diffuse positive reaction for MMP-2 in deep epithelial layers (IHC, ×400).***

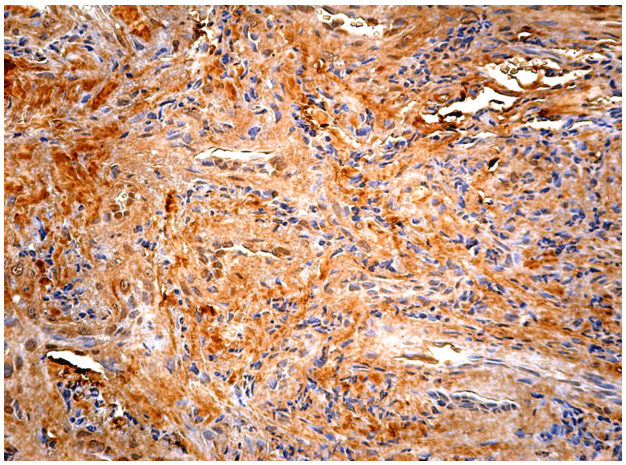

Figure 4 – ***Chronic periodontitis treated with spiramicyn and metronidazole. Positive reaction for MMP-2 in pro-inflammatory cells and in fibroblasts (IHC, ×200).***

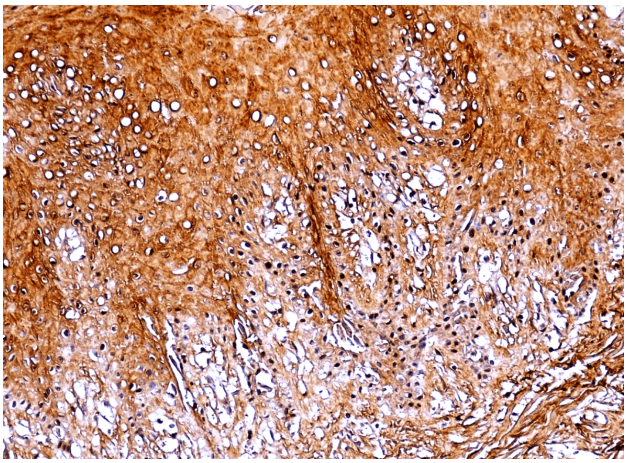

Figure 5 – ***Untreated chronic periodontitis. TIMP-2 immunostaining; marked positivity in epithelial keratinocytes and in extracellular matrix of lamina propria (IHC, ×200).***

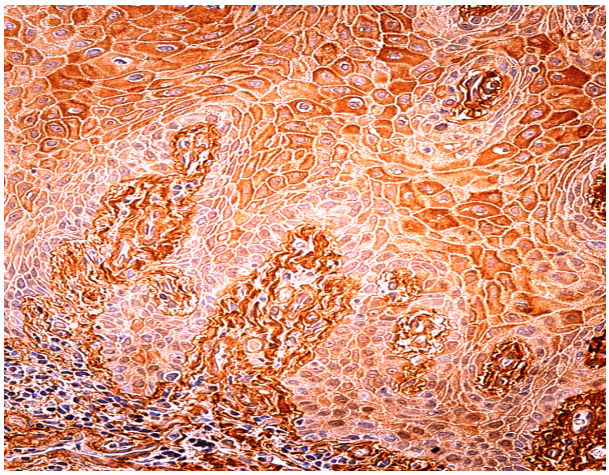

Figure 6 – ***Chronic periodontitis treated with amoxicillin and metronidazole. TIMP-2 positive reaction in keratinocytes from basal and spinous layers (IHC, ×400).***

Gingival samples from the IInd Group, patients treated with spiramycin and metronidazole, revealed a positive reaction for TIMP-2 in the same epithelial and connective structures as in the Ist Group, but the positivity was dimmed (data not shown).

After clinical examination, we compared the baseline values of gingival index with the results obtained after one week of treatment with the two combinations of antibiotics.

Regarding the gingival index at baseline, no significant difference was noticed between the study groups before treatment (p>0.05) (Table 2).

Table 2 – ***Mean of the gingival index before treatment***

Group	Gingival index before treatment (mean)	Standard deviation	*P*-value (Kruskal–Wallis test)
Control Group	2.135	0.534	0.804
Ist Group (A+M)	2.011	0.536	0.804
IInd Group (S+M)	1.966	0.463	0.804

Because the results have not a Gaussian distribution, for the comparison of data obtained before and after treatment, we used the Wilcoxon test (Table 3). Comparing GI values at baseline and after treatment, we obtained significant differences (p<0.001).

Table 3 – ***Gingival index values before and after treatment***

Group	Gingival index before treatment (mean)	Gingival index after treatment (mean)	*P*-value (Wilcoxon test)
Control Group	2.135	0.520	0.006
Ist Group (A+M)	2.011	0.266	0.00001
IInd Group (S+M)	1.966	0.253	0.00001

The means of gingival index for all groups after treatment were significantly different (p<0.01) (Table 4).

Table 4 – ***Mean of the gingival index after treatment***

Group	Gingival index after treatment (mean)	Standard deviation	*P*-value (Kruskal–Wallis test)
Control Group	0.520	0.189	0.006
Ist Group (A+M)	0.266	0.219	0.006
IInd Group (S+M)	0.253	0.227	0.006

Discussion

The conventional treatment of periodontal disease consists on the reduction of bacterial infection by mechanical removal of pathogen agents using scaling and root planning [15]. Many studies indicated that the level of microbial flora is decreased and the clinical signs are also improved at patients treated by this method. In our study, we noticed the same results, so, post-therapeutic, the values of clinical indexes were decreased compared to the control group.

Although scaling and root planning eliminate a great part of the deposits from the root surface and promote tissue healing, researches demonstrated a "smear layer" containing residual calculus, bacteria and bacterial matrix remnants on cementum. Therefore, however the biofilm seems to be totally removed, the patients present a progression of periodontal disease with peculiar periodontal attachment and bone loss [16].

A variety of antimicrobial agents have been successfully used to support mechanical biofilm removal, which has been evaluated by recent studies [17, 18]. The subgingival biofilm has been analyzed in culture and by specific molecular techniques such as PCR or DNA hybridization before and after administration of antibiotics [19, 20]. A highly satisfying clinical response was observed. This finding was confirmed by various studies that demonstrated that adjunctive systemic administration of a combination between metronidazole and amoxicillin [21] was beneficial in periodontitis treatment. Another combination, between spiramycin and metronidazole [22] was also used, being successful especially at patients hypersensitive to amoxicillin or other β-lactam antibiotics. The results reported in such anterior studies motivated us to choose for our research these two combinations of antibiotics.

While microbial dental plaque is essential for disease initiation, it is well recognized that in periodontitis the major component of soft and hard tissue destruction occurs because of the activation of the host immune defense mechanisms in response tzzthe presence of bacterial biofilm. Inflammatory mediators such as interleukins (IL-1, IL-6), tumor necrosis factor-α (TNF-α) and prostaglandin E2 (PGE2) have an important role in activation and stimulation of bone resorption and periodontal attachment loss by an overproduction of MMP [23]. MMP is an important family of up to 20 molecules represented by proteolytic enzymes that are involved in ECM degradation and remodeling. Among MMP, MMP-2, MMP-8 and MMP-9 are the most discussed in relation to chronic periodontitis [24, 25].

The inhibition of MMP activity is ensured by a wide

range of biomolecules such as cytokines and growth factors [26] *via* their natural tissue inhibitors, TIMPs. Of the four types of TIMPs, TIMP-2 can regulate the local expression of interstitial collagenase during development, remodeling and repair processes [27]. Consequently, the progression of the periodontal disease could be the result of an imbalance MMPs/TIMPs.

Paradoxically, the immuno-inflammatory host response, which have especially defensive role, is responsible for much of the breakdown of the periodontal tissues. Because of these observations, Preshaw *et al.* have proposed a new concept for treatment of chronic periodontitis, which consists in a reduction of bacterial activity combined with host modulation therapy [28]. The principle of such experiment is very simple: if chronic periodontitis is characterized by high levels of cytokines, prostaglandins and MMPs in periodontal tissues, periodontium homeostasis in health tissue is characterized by the opposite. Therefore, the objective of the host modulatory therapy is to restore the balance between proinflammatory (destructive) and anti-inflammatory (protective) mediators.

Immunohistochemical reactions made on samples from considered groups allow us to assess the effects of antibiotics on the balance between the factors who determine connective tissue turnover. In the Ist Group (patients treated with amoxicillin and metronidazole), MMP-2 had very week positivity in the epithelium and in the lamina propria, especially in residents cells. This pattern of expression, considered together with the high positivity, mainly epithelial, observed in the control group, can be interpreted like a return to a normal MMP-2 expression in the mucosa after antibiotic treatment.

Also, the immunohistochemical reactions for TIMP-2 displayed an obviously different pattern between the groups studied. For the samples obtained from patients treated with the combination metronidazole–amoxicillin, TIMP-2 was present in keratinocytes from basal and spinous layers and mainly in the loose connective tissue of the papillae and at the interface epithelium–connective tissue.

In the IInd Group, patients treated with the combination between spiramycin and metronidazole, TIMP-2 positive reaction was present in the same epithelial and connective structures as in the Ist Group, but the positivity was dimmed. Therefore, because of antibiotic treatment, MMP release and activity decrease and TIMP level increases.

The clinical index was in accordance with immunohistochemical results. In both groups treated with antibiotics, gingival index was significantly lower than before treatment (p<0.001).

The values for the IInd Group were lower compared to the Ist Group, but the difference between the two groups was not significantly (p>0.05). The clinical indexes in groups treated with antibiotics were reduced compared to control group (p<0.001).

Among antibiotics, tetracyclines and recently metronidazole were investigated like host modulator agents [11]. Studies related to conventional periodontal treatment combined with subantimicrobial doses of doxycicline or topical application of minocycline showed that clinical indexes have been improved [12].

These agents produce host modulation effects by different molecular mechanisms, including inhibition of osteoclasts and, more important, inhibition of MMPs [29].

Tetracyclines (especially minocycline and doxycycline) have been widely used in periodontal treatment for their antimicrobial effects, but their systemic administration may cause side effects. For this reason, tetracyclines were studied in subantimicrobial doses, which attenuate antimicrobial actions and also can inhibit tissue collagenase from periodontium [30]. Golub *et al.* discovered that tetracyclines worked well as host modulatory agents because they have the abilities to inhibit MMPs. In addition, a subantimicrobial dose of tetracycline will not cause the development of antibiotic resistance in the normal periodontal microorganisms [31]. These clinical researches emphasize that these medications can be used as host modulatory treatment, which control and suppress the progression of chronic periodontitis.

Metronidazole is considered an efficient antimicrobial agent against a wide range of microorganisms, especially anaerobic germs. In chronic periodontitis, it is prescribed in support to conventional periodontal treatment, single or in association with amoxicillin, ciprofloxacin, spiramycin or other antibiotics. Many studies have demonstrated the effectiveness of metronidazole on several anaerobic periodontal pathogens [32]. The potential therapeutic effects of this antimicrobial agent based on the interaction with periodontal tissues have not been investigated extensively. However, there are some studies about the immunomodulatory effects of metronidazole. Therefore, Rizzo *et al.* suggest the absence of a cytotoxic effect of metronidazole on human periodontal ligament cells and the inhibition of proinflammatory cytokines, especially TNF-α and IL-1β induced by *Porphyromonas gingivalis* [33]. Other studies showed that metronidazole therapy reduces the number of immune cells and IL-8 level in infiltrates, suggesting that is a correlation between chemokine and periodontal status [34]. Horwood & Yamada demonstrated that the presence of metronidazole may help prevent periodontal tissue destruction by altering the ability of inflammatory cells to produce other cytokines, IL-12 and IL-18 [35, 36].

The conventional periodontal treatment (scaling and root planning) and systemic antimicrobial therapy target different aspects of the pathogenesis of chronic periodontitis. Therefore, the aims of mechanical treatment are to remove subgingival plaque and calculus with reduced local external inflammation. Systemic antibiotic administration is absorbed and can be distributed to the whole body, including all supportive periodontal tissues; so, it had an antimicrobial effect but in the same time, modulated also the host responses, such inhibiting host derived proteinases [37].

Further investigations will be necessary for better understanding the rapports between systemic antibiotic therapy and host modulatory response in accordance with individual variation of synthesis and release of matrix modulators.

Conclusions

The two combinations of antibiotics used in our study (association amoxicillin and metronidazole or spyramicin

and metronidazole) exert the same influence on clinical aspects and appear to have a dual ability to reduce inflammation as well as to inhibit MMP-2 activity. These antimicrobial agents decrease the inflammation by antimicrobial and also by immunomodulatory effects. The ability of these combinations of antibiotics to determine immunomodulatory effects and the clinical implications must be better clarified in future studies. Will be necessary to investigate the molecular mechanisms through which these antibiotics exert anti-inflammatory effects and to identify the cellular pathways involved.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

All authors have equal contributions to the study and the publication.

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Corresponding author
Citto Iulian Taisescu, Lecturer, MD, PhD, Department of Functional Sciences, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone +40722–520 531, e-mail: taisescu@yahoo.com

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