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Assessment of aprotinin influence on periodontal clinical status and matrix metalloproteinases 1, 2 and their tissue inhibitors saliva concentrations in patients with chronic periodontitis

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ABSTRACT

Purpose: Assessment of the effect of treatment with aprotinin-containing drug on the clinical status of the periodontal tissue and on the concentrations of metalloproteinases released in the course of periodontitis (MMP-1, MMP-2) as well as their tissue inhibitors (TIMP-1 and TIMP-2) in the saliva of patients with chronic periodontitis (CP).

Material/Methods: The study involved 25 subjects with CP (39-68 years), including 16 women and 9 men. The patients were prescribed aprotinin preparation to be taken for 2 weeks. The control group (C) involved 14 healthy subjects (41-65 years), including 10 women and 4 men. Two periodontal indices were assessed: the approximal plaque index (API) and bleeding on probing index (BOP). Periodontal pocket depth and clinical attachment level were also evaluated. The concentrations of MMP-1 and MMP-2 as well as TIMP-1 and TIMP-2 were determined by the ELISA method.

Results: The mean salivary MMP-1 concentration in patients with CP was significantly higher before and after treatment, as compared to healthy subjects. The mean salivary MMP-2 concentration in CP patients at baseline was also higher as compared to the C group and increased after treatment. The mean salivary TIMP-1 and TIMP-2 concentration in CP patients was higher as compared to C group and increased after treatment.

Conclusions: Since the mean MMPs levels were found to be growing it can be assumed that aprotinin has no significant effect on the regulation of MMPs in the saliva of CP patients. It thus seems that aprotinin application after scaling has no additional therapeutic effect.

Key words: aprotinin, matrix metalloproteinases, chronic periodontitis

INTRODUCTION

Periodontitis is a progressive disease of the tooth supporting apparatus affecting the gingiva, periodontal ligament, root cement and alveolar bone [1]. Its pathophysiology is substantially complex. Bacterial plaque is the primary etiologic factor associated with periodontitis, with *Aggregatibacter actinomycetemcomitans* and the red complex bacteria being the most virulent [2]. Microorganisms that initiate the inflammatory process activate inflammatory cells, including neutrophils [3]. Disintegration of neutrophilic granulocytes and release of enzymes are known to induce damage to matrix proteoglycans, which in consequence leads to periodontal tissue destruction [4]. Although the primary cause of the disease is accumulation of dental plaque, some people show individual vulnerability to the development and rapid and severe course of the disease. The character of the local and general response of the body to the pathogenic bacteria may affect the clinical

course of periodontitis. Risk factors predisposing to this pathology have been the subject of numerous studies. It is believed that young people with periodontitis occurring with rapid bone loss have general immune dysfunctions, among which impairment of neutrophilic granulocytes and monocytes (adhesion, chemotaxis, phagocytosis), and excessive release of proinflammatory cytokines are the most characteristic. In such cases, periodontal destruction occurs due to the spreading of an inflammation which was primarily initiated as the host defense mechanism and was not suppressed at the right time [5]. Periopathogenic bacteria can induce tissue destruction using three pathways:

- by a direct release of proteolytic enzymes that degrade periodontal structures without intervention of the host cells,
- through the production of toxins and enzymes that may stimulate cells to release the degrading enzymes,
- by stimulating the immune response manifested as a release of cytokines from lymphocytes and macrophages, which may activate one or more pathways of degradation [6].

The relationship between microorganisms and periodontitis was discovered a long time ago and since that time treatment has been oriented at elimination of pathogens that cause the disease. Studies conducted during the last 20-year-period have found the host-response associated factors to be the key to the understanding of the pathological process. One of these factors is the family of enzymes called matrix metalloproteinases (MMPs) [7]. These proteolytic enzymes are considered to be capable of degrading extracellular matrix proteins of the connective tissue. Damage to periodontal collagen is caused mainly by MMP-8 and MMP-9 from neutrophils, MMP-2 from fibroblasts, MMP-13 from gingival epithelial cells and MMP-1 that can be found in fibroblasts, keratinocytes, osteoblasts, chondrocytes, monocytes, macrophages and epithelial cells [8-12]. According to the criteria of substrate specificity and the biochemical structure of enzymes, MMP-1 belongs to the group of collagenases and is known to cause degradation of collagen type I, II, III, VI and X. On the other hand, MMP-2 is a gelatinase-A and collagen type IV degrading gelatin [13]. In healthy tissues, these metalloproteinases modulate normal protein transformations. Many researchers have undertaken an attempt to explain the mechanisms of excessive metalloproteinase secretion and to determine endo- and exogenic factors that modify this secretion. During inflammation, proinflammatory cytokines (IL-1, TNFα) in periodontal tissue stimulate cells of inflammatory infiltrate to secrete greater amounts of metalloproteinases, including MMP-1 and MMP-2, which leads to an excessive degradation of the connective tissue [14]. In physiological conditions, the concentration and activity of metalloproteinases are regulated by their tissue inhibitors (TIMPs) secreted by most cells of the connective tissue and inflammatory infiltrate. The imbalance between MMPs and TIMPs plays an essentials role in the connective tissue degradation. TIMP-1, -2, -3 and -4 have been identified, with TIMP-1 playing the major role in MMP-1 regulation and TIMP-2 in MMP-2 regulation [12,15]. There has been a long search for extrasystemic metalloproteinase inhibitors that could support periodontal therapy. At the moment, doxycycline administered in small doses and non-antibacterial tetracycline derivatives – CMTs seem to have such properties [16].

As widely demonstrated, oral hygiene condition, and thus the amount of dental plaque, directly correlates with the occurrence and advancement of chronic periodontitis. The oral hygiene condition and dental plaque microflora are potentially modifiable factors in the etiology of periodontitis. However, complete elimination of oral microorganisms is not possible. Therefore, periodontal therapy mainly focuses on the reduction in the number of pathogenic microorganisms and aims to achieve a balance between microflora and the host [5,17].

Mechanotherapy performed to remove dental plaque and root planning is the major therapeutic procedure. In the case of shallow periodontal pockets, the elimination of dental plaque by means of professional hygienic procedures usually relieves the inflammatory symptoms and can be frequently considered a sufficient therapy. Deeper pockets are less accessible and proper scaling can be difficult. Therefore, additional pharmacotherapy, both local and general, may increase the efficacy of periodontal treatment and is undoubtedly less invasive than surgical procedure. However, being additional, it cannot be an alternative to subgingival and supragingival plaque removal. The exclusive administration of antibacterial drugs shows only limited effects as the structure of dental plaque hinders its complete penetration. Moreover, the bacteria present in the pockets to a large extent neutralize the action of drugs. Thus, the professional cleaning of hard dental tissue should be supplemented by the application of a drug potent to sufficiently penetrate the bacterial biofilm. A new aprotinin - containing drug designed to treat inflammatory periodontal lesions has appeared recently on the Polish market. According to the producer, aprotinin reduces the pathogenic effect of the enzymes, serine proteases, on the periodontal tissue.

Therefore, we decided to assess the effect of treatment with aprotinin-containing drug on the clinical status of the periodontal tissue and on the concentrations of metalloproteinases released in the course of periodontitis (MMP-1, MMP-2) as well as their tissue inhibitors (TIMP-1 and TIMP-2) in the saliva of patients with chronic periodontitis (CP).

MATERIAL AND METHODS

The study involved 25 subjects with chronic periodontitis aged 39-68 years, including 16 women and 9 men. All subjects were non-smokers, and none of them had a history or current manifestation of systemic disease. The diagnosis of chronic periodontitis was made on the basis of past dental history, clinical parameters and radiographic patterns of alveolar bone loss. The patients with periodontitis received non-surgical

periodontal treatment, which comprised instructions about oral hygiene and supra- and subgingival debridement (scaling and root planning). The treatment took one session of 1 hour. The scaling and root planning was performed with ultrasonic instruments and by a single trained operator. Measurements of probing depth and attachment level were recorded at six points around each tooth. The patients were prescribed aprotinin preparation (Trascodent, Jelfa, Poland) to be taken for 2 weeks. Trascodent was applied onto the gingival margin twice a day after toothbrushing. The patients were advised not to have meals, not to drink or rinse the mouth for two hours after drug application. The periodontal tissue was examined three times: prior to pharmacotherapy (baseline), 2 weeks and 2 months later. Two periodontal indices were assessed: the approximal plaque index (API) and bleeding on probing index (BOP) [18,19]. Periodontal pocket depth (PPD, in mm) and clinical attachment level (CAL, in mm) were also evaluated. A periodontal probe PCPUNC 15 (Hu-Friedy, USA) was used for examination.

The control group involved 14 healthy subjects aged 41-65 years, including 10 women and 4 men. Controls exhibited no signs of periodontal disease as determined by the absence of CAL and no sites with probing depth >3mm. Full-mouth periodontal examinations were performed on the control subjects.

No-stimulated saliva (10 ml) was collected three times on the days when clinical examination was performed. The saliva was collected on empty stomach. Before collecting the subjects rinsed their mouths with water. All samples were frozen and kept at -20°C units used for enzyme assays.

The concentrations of MMP-1 and MMP-2 as well as TIMP-1 and TIMP-2 were determined by the immunoenzymatic method ELISA using Quantikine Kits (R&D Systems, USA) with specific human monoclonal antibodies. Salivary MMPs and TIMPs concentrations were expressed in ng/ml.

Statistical analysis was performed using the computer program Statistica 8.0 PL. The U Mann-Whitney test was used to compare between the groups. The t-Student test for pairs was applied to assess changes in parameters at time intervals. Differences were considered statistically significant at $p \leq 0.05$.

Table 1. **Clinical parameters (mean ± standard deviation) in CP group before and after treatment.**

Parameter	Examination I	Examination II	Examination III
API	68.17±20.86	51.08±27.72*	52.28±24.92**
BOP	29.28±15.34	20.04±15.17*	26.64±20.05
PPD	3.26±0.67	2.97±0.70*	2.99±0.69**
CAL	5.23±1.04	4.98±1.17*	5.03±1.15

*- statistically significant difference between I and II examination
**- statistically significant difference between I and III examination

RESULTS

The clinical examination conducted after treatment with aprotinin revealed a reduction in the periodontal parameters studied. After two weeks, the mean values of API and BOP were found to decrease statistically significantly ($p=0.0001$, $p=0.0002$), but increased after 2 months as compared to the findings at examination II. The mean PPD was also significantly reduced after two weeks of treatment ($p=0.0000001$) and the value remained at a similar level after 2 months. CAL showed a significant improvement ($p=0.0014$) after 2 weeks, but worsened insignificantly after two months of the therapy (*Tab. 1).*

The mean salivary MMP-1 concentration in patients with CP was significantly higher both before ($p=0.00006$) and after treatment, as compared to healthy subjects. The mean levels of this enzyme before and after aprotinin treatment were comparable (*Tab. 2, Fig.1*)

The mean salivary MMP-2 concentration in CP patients at baseline was also significantly higher as compared to the control group ($p=0.0198$) and increased after treatment. Following treatment, the level of this enzyme in CP patients remained significantly higher in comparison with healthy subjects ($p=0.0364$) (*Tab. 2, Fig. 3*).

The mean salivary TIMP-1 concentration in CP patients was higher as compared to group C and increased after treatment with aprotinin, the differences being statistically insignificant (*Tab. 2, Fig. 2*).

The mean TIMP-2 level was significantly higher in CP patients as compared to the control group ($p=0.0127$). It

Table 2. **Mean concentrations of MMP-1, MMP-2, TIMP-1and TIMP-2 (± standard deviation) in CP group before and after treatment and in C group.**

Group	Examination	MMP-1	MMP-2	TIMP-1	TIMP-2
C		0.002±0.01	0.359±0.20	68.220±51.38	15.242±4.13
CP	I	0.154±0.11*	0.736±0.57*	94.929±100.89	23.907±12.29*
	II	0.170±0.15#	1.105±0.89**#	106.947±118.84	29.074±13.96#
	III	0.197±0.19##	1.308±1.16***##	145.395±150.50	29.891±15.41##

*- statistically significant difference between C and CP groups in I examination
**- statistically significant difference between I and II examination in CP group
***- statistically significant difference between I and III examination in CP group
- statistically significant difference between C and CP groups in II examination
#- statistically significant difference between C and CP groups in III examination

Figure 1. **Mean concentrations of MMP-1 in CP group before and after treatment and in C group.**

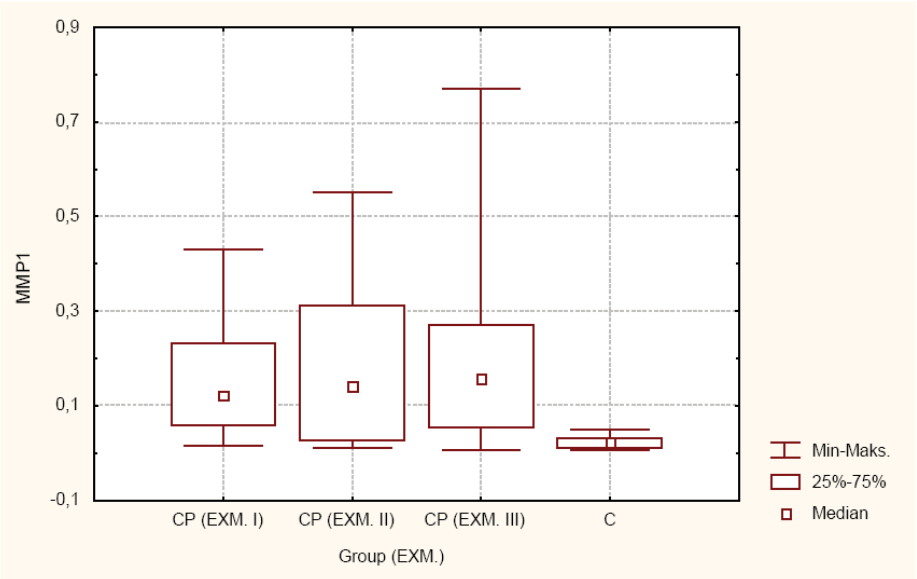
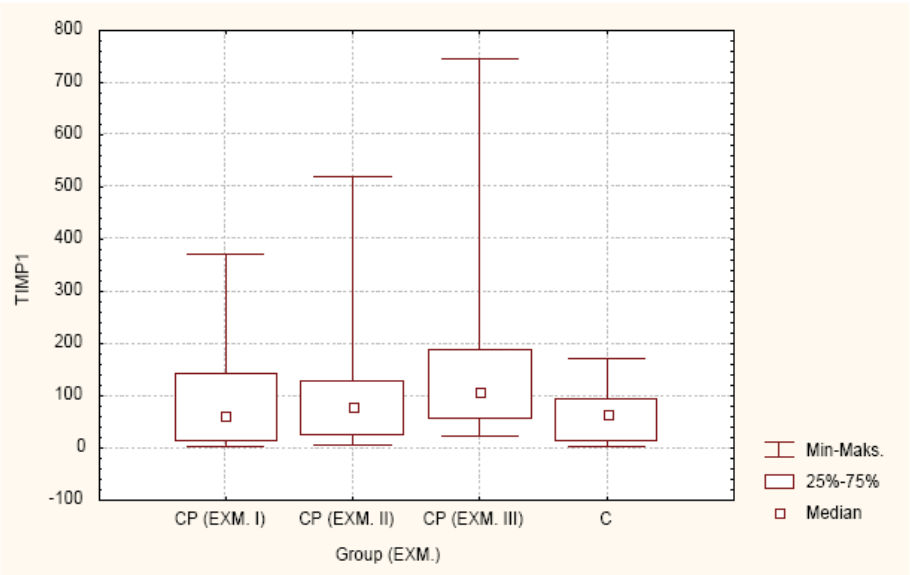


Figure 2. **Mean concentrations of TIMP-1 in CP group before and after treatment and in C group.**



increased after a two-week treatment and was still at the same level after 2 months, although the increase was statistically insignificant (*Tab. 2, Fig. 4*).

DISCUSSION

Periodontal treatment aims to achieve the disease remission by bacterial reduction in periodontal pockets and in consequence to limit their proteolytic action on the periodontal tissue [20]. Currently, the role of topical application of various chemotherapeutics and their application mode as an additional therapy in patients with periodontitis is widely discussed [21,22]. The preparation used in the current study was gel Trascodent, containing aprotinin and other substances, such as propylene glycol, hypromelose (Hydroxypropylmethylcellulose), disodium versenian (Disodium EDTA), propyl hydrobenzoate (Methylparaben, Propylparaben) and water [23]. Aprotinin, a polypeptide with molecular weight of approximately 6500, is an active substance and a specific inhibitor of serine proteases: trypsin, plasmin, kallikrein and chymotrypsin. It is known to neutralize fibrinolytic action of plasmin and inhibit some of the plasminogen activators that exert a fibrinolytic effect. Forming a complex with these enzymes, aprotinin occupies the place of active serines. Its mechanism consists in the

Figure 3. **Mean concentrations of MMP-2 in CP group before and after treatment and in C group.**

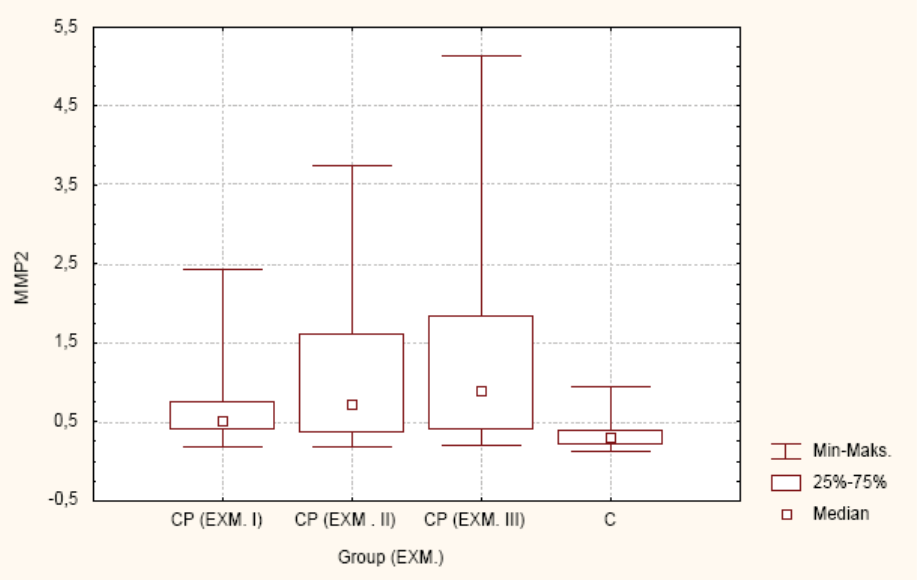
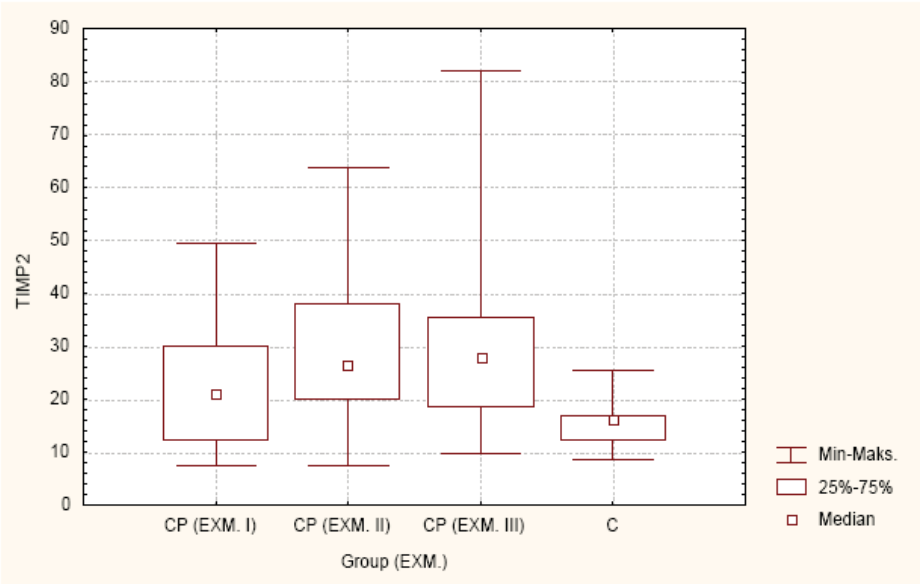


Figure 4. **Mean concentrations of TIMP-2 in CP group before and after treatment and in C group.**



inhibition of the fibrinolytic and kininopoietic systems. The inhibition of the kininopoietic system is associated with the inactivation of kallikrein, i.e. the enzyme releasing kinin from inactive precursors, whereas the inhibition of the fibrinolytic system is caused by inactivation of plasmin [24-28]. Aprotinin inhibits the action of enzymes released from lysosomes of a damaged tissue, exerting its effect in various periods of coagulation and fibrinolysis. Through its proteolytic action and inhibition of plasminogen activators, aprotinin alleviates and relieves pain, suppresses bleeding and reduces clinical symptoms of inflammatory states – reddening and swelling [23,25]. Aprotinin as a serine protease inhibitor regulates concentrations and activities of MMPs. This regulation may occur on various levels: transcription by cytokines (IL-1, TNF-α), hormones (parathormon-PTH), bacterial products (lipopolysaccharides-LPS), sequestration of enzymes to intercellular follicles, proenzyme activation (metal ions, oxidants, detergents, other proteolytic enzymes, plasmin), and on level of substrate specificity, through environmental pH and tissue proteinase inhibitors TIMPs [13]. TIMPs are produced by the same cells that generate metalloproteinases. They form a family of four structurally affiliated proteins that double monitor MMPs. TIMPs have been found to inhibit the process of transformation of pro-MMPs to MMPs and their active forms. Tissue inhibitors of proteinases are present in intercellular spaces, in blood plasma and in other body fluids

[29]. Two of them, i.e. TIMP-1 and TIMP-2, are produced in a soluble form and detected in blood serum. The major inhibitor of MMPs is a glycoprotein, 28 kDa TIMP-1, produced by most cells. Another inhibitor is a non-glycosylated protein, 21 kDa TIMP-2, produced by fibroblasts and endothelial cells, which is able to bind pro-gelatin A and control its activation. TIMP-1 expression is also regulated by cytokines, mainly by IL-1, TNF-α, as well as tumour growth factor (TGF-β), IL-6, IL-10. IL-1 induces synthesis of metalloproteinases and serin proteinases, but also inhibits TIMP-1 synthesis. The effect of TNF-α is similar to the action of IL-1, and both cytokines act synergistically, which potentiates their catabolic effect. However, TIMP-2 susceptibility to the action of cytokines has not been demonstrated [30]. Tissue inhibitors of metalloproteinases regulate degradation of the extracellular matrix both via elimination of proteinases and inhibition of MMP activation, by forming non-covalent bonds with active or latent forms of metalloproteinases at the mole ratio of 1:1. The inhibitory mechanism of metalloproteinase activation involves TIMP-induced blocking of the possibility of N-terminal fragment detachment [13].

In our study, statistically significant reductions in BOP and API indices were noted after a two-week therapy with the aprotinin-containing drug, although these findings may not have been connected with pharmacotherapy. Our patients, apart from pharmacotherapy, underwent a routine hygienic procedure accompanied by oral hygiene instruction, which allowed bacterial reduction. However, a follow-up examination conducted 2 months later showed an increase in BOP and API as compared to examination II (after 2 weeks). These results seem to confirm the hypothesis that not all patients are able to comply with the standard periodontal treatment involving mechanotherapy and hygienic regime. Improvement was also noted in another clinical parameter - PPD, whose values dropped statistically significantly after 2 weeks of treatment and remained practically the same at examination III (after 2 months). The reduction in clinical parameters obtained in our study is comparable to those reported by other authors [31-34].

Additionally, we made an attempt to assess the concentrations of MMP-1, MMP-2, TIMP-1 and TIMP-2 in the saliva of patients with chronic periodontitis. The salivary levels of all these enzymes were substantially increased in CP patients as compared to the control group. Elevated levels of MMP-1 and MMP-2 in the saliva and in the gingival crevicular fluid in periodontitis patients have also been observed by other authors [8]. We found a further increase in the concentrations of MMP-1, MMP-2, TIMP-1 and TIMP-2 after treatment with aprotinin preparation, with statistical significance noted only for MMP-2. Our findings cannot be compared to the results of other authors as data are lacking on the effect of aprotinin-containing preparations on the periodontium.

Lack of correlations of the clinical parameters BOP, API and PPD with the levels of metalloproteinases and their tissue inhibitors may suggest that the latter are not only associated with oral hygiene condition at the time of examination. MMPs regulation occurs on many levels and the inhibitor of serine proteases is only one of the many inhibitory factors. Figueredo et al [34] found that some sites continued to show collagenolytic activity 30 days after treatment and observed no correlation between the clinical outcome and protease levels at baseline or after treatment. Similar findings have also been reported by other authors [35,36]. Palcanis et al [37] demonstrated that GI and PI were not significantly altered along with changes in elastase; however, the level of elastase in the gingival crevicular fluid increased significantly at the sites with a considerable loss of connective-tissue attachment and bone resorption. The process of proliferation and activation of cells secreting MMPs and TIMPs is varied and may depend on the disease duration, previous treatment or patient's general health condition. Besides, metalloproteinases play a major role in such physiological processes as embryogenesis, angiogenesis, ovulation or healing [38]. Therefore, their elevated levels may also suggest healing processes in the periodontal tissue after scaling.

CONCLUSIONS

Since the mean MMPs levels were found to be growing it can be assumed that aprotinin has no significant effect on the regulation of metalloproteinases in the saliva of CP patients. This condition can also be due to poor pocket penetration by the drug applied onto the gingiva. It thus seems that aprotinin application after scaling has no additional therapeutic effect especially that the improvement in the clinical parameters assessed is typical of the mechanotherapeutic procedure.

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