Quantification of Proinflammatory Molecules (IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α) in Crevicular Fluid and Serum in Patients with Endo-Periodontal Lesions

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The present research proposes an assessment of the localized inflammatory burden but also at the systemic level by quantitating the pro-inflammatory molecules (IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α) with endo-periodontal lesions. The study was performed on a group of 146 subjects who, following clinical and radiological examinations, were divided into five groups: healthy endo-periodontal patients, patients with periodontitis, patients with moderate periodontitis, patients with severe periodontitis and patients presenting combined endo-periodontal lesions. IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α were analysed in crevicular fluid by ELISA and serum by fluorescence flowcytometry. IL1-α and IL1-β showed serum and crevicular fluid values significantly higher than the healthy subjects. Values for IL2, IL12, TNF-α and IFN-γ measured in crevicular fluid were higher for groups II, III, IV, and V compared to the group of healthy endo-periodontal subjects. IL2 showed significantly higher serum values for groups III, IV and V than group I. Serum IL12 values were significantly higher than healthy periodontal subjects only for patients with severe periodontitis and endo-periodontal syndrome. Following serum determinations of proinflammatory molecules, patients with endo-periodontal lesions demonstrated significantly higher values even in subjects with severe periodontitis; these data indicate a much higher risk for these patients to develop and maintain systemic maladies, as is the role local inflammation can play over the general inflammatory status of the patient.

Keywords: proinflammatory molecules, periodontal disease, endo-periodontal lesions

Inflammation and resorption of the alveolar bone in most cases is a consequence of the interaction between the microbial infection and the response of the host [1]. The critical role of bacteria in the development of periapical lesions was demonstrated by mechanical exposure of dental pulp to the oral cavity of germ-free animals. In these animals, the pulp exposure is healed with an initial or transient inflammatory response in pulp tissue, followed by a reparative response from the pulp cells and leading to the formation of a new dentin-like matrix linking to the exposed site. In contrast, mechanical pulp exposure in animals with normal oral bacteria causes an infection of the dental pulp, pulp tissue necrosis and chronic infection that prevents the repair process. The infection persists because necrotic tissue of dental pulp is inaccessible to leukocytes and therefore constitutes a bacteria-protected reservoir [2]. Chronic inflammation stimulated by bacteria and their products in the periapical area of the tooth leads to localized bone resorption, which is *decoupled*, so there is no bone repair without treatment. The result consists in the formation and expansion of granulomas or cysts in apical tissues [3].

Periodontium consists in a set of tissues in the immediate vicinity of the tooth, with a complex biofilm that includes various bacterial species [4]. Although the consensus is that periodontal diseases are stimulated by bacterial adhesion to the surface of the teeth, there is controversy over which bacteria stimulate the irreversible degradation of periodontal tissues in periodontitis [5]. Evidence from studies that are not based on bacterial culture techniques suggest that there are approximately 700 bacterial species in the oral cavity [6].

The presence of microbial pathogens in periodontal and periapical environments triggers an initial production of proinflammatory cytokines such as TNF-α and IL1-β that stimulate the expression and activation of matrix metalloproteinases (MMPs) that degrade the extracellular connective tissue matrix. Cytokines such as TNF-α can stimulate osteoclastogenesis independently, while other cytokines stimulate RANKL expression which leads to the formation and activity of osteoclasts. Innate and acquired combined immune responses can lead to high levels of inflammation and bone resorption. These proinflammatory cytokines are believed to generate an amplification loop that contributes to the progression of periodontal and periapical lesions. On the contrary, cytokines produced by Th2 and Treg cells, such as IL-4 and IL-10, have the opposite effect in part by stimulating the production of matrix metalloproteinase and OPG inhibitors, as well as by limiting cytokine production inflammatory.

The present research proposes an assessment of the localized inflammatory burden but also at the systemic level by quantitating the pro-inflammatory molecules (IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α) in subjects with endo-periodontal lesions.

Experimental part

The study was performed on a group of 146 subjects who, following clinical and radiological examinations, were divided into five groups: healthy endo-periodontal patients (n = 24) (group I), patients with superficial periodontitis (probing depth less than 4mm) (n = 36) (group II), patients with moderate periodontitis (probing depth of 4-6mm) (n

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= 32) (group III), patients with severe periodontitis (probing depth greater than 6mm) (n = 25) (group IV) and patients presenting endo-periodontal combined lesions (n = 29) (group V). Patients in groups II, III and IV did not have active endodontic lesions. Also, all subjects included in the study were systemically healthy.

We excluded patients with anti-inflammatory medication over the past 6 months, patients who had endodontic or periodontal treatment in the last 12 months and smokers.

Each subject included in the study was informed about the purpose and methodology of the study, with written agreement obtained from each subject.

The patients underwent a complex endodontic and periodontal clinical examination. Clinical examinations were completed with retro-dental-alveolar radiographs.

For crevicular fluid analysis of IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α, the teeth were isolated with cotton rolls before harvesting crevicular fluid samples. The supra-gingival plaque was also carefully removed and the sites were gently dried with the air spray. A sterile paper cone was inserted into each selected periodontal site, left for 30 seconds, and then immediately inserted into sterile Eppendorf tubes which were stored at -20°C. In the case of visible contamination with blood, the paper cone has been removed and a new site has been selected. For the determination of inflammatory markers, the paper cones were thawed, cut at 1 cm in length and thawed with 50 µL 1X [13 mM Na2HPO4, 7 mM NaHPO4, 100 mM NaCl (*p*H 7.0)] phosphate buffer at 4°C. Further, the paper cones were centrifuged at 13000 xg for 10 min at 4°C [7].

For serum assay of IL1-α, IL1-β, IL2, IL12, IFN-γ, TNF-α, a 15 mL blood sample was obtained by venipuncture from each participant. The serum was collected by centrifugation, aliquoted, stored and processed at the end of the study. Inflammatory markers were identified simultaneously using flow cytometer multiplex assays (BD™ Cytometric Bead Array (CBA, BD Bioscience, San Jose, CA, USA). CBA is a series of discrete spectral particles that can be used to capture soluble analytes. Analyses are then measured by fluorescence emission and flow cytometry detection. The method uses a series of different particles that are stably labelled with a fluorescent dye whose emission wavelength is read at ~ 650 nm. Each different group is labelled with a discrete level of fluorescent dye so that it can be distinguished by mean fluorescence intensity. The spheres in each group are covalently coupled to antibodies that can specifically capture a particular type of molecule present in biological fluids. This type of test was chosen because it allows a full analysis of the biomarkers involved in periodontitis and systemic diseases.

The data were recorded and analysed statistically. For statistical analysis, the SPSS 20.0 (IBM) program and $p < 0.05$ was considered to indicate a statistically significant difference. Continuous variables with a normal distribution are expressed as mean ± standard deviations of the mean and were analysed using parametric tests (the T test for pairs or independent samples). Fisher and McNemar tests were used to compare frequencies between affiliated or independent samples.

Results and discussions

This clinical and paraclinical study was performed on a group of 146 subjects; the mean age was 46.28 ± 11.37 years. The group was comprised of 67 female subjects (45.89%) and 79 male subjects (54.11%). As far as the environment of origin is concerned, 53 subjects came from rural areas (36.30%) and 93 from urban areas (63.70%). Demographic data by study groups are shown in table 1.

IL1-α showed serum and crevicular fluid values significantly higher than the healthy subjects; also the values recorded in the group with endo-periodontal syndrome patients were significantly higher than the group with severe periodontitis. Similar differences were also observed for serum and GCF values of IL1-β.

Values for IL2, IL12, TNF-α and IFN-γ measured in crevicular fluid were higher for groups II, III, IV and V than

Table 1

DEMOGRAPHIC DATA OF SUBJECTS BY STUDY GROUPS

		I (n=24)	II (n=36)	III (n=32)	IV (n=25)	V (n=29)	Total (n=146)
Mean age ± SD		37.25 ± 9.64	39.86 ± 8.47	43.78 ± 13.98	59.87 ± 13.67	50.64 ± 14.21	46.28±11.37
Gender	**Male**	11 (45.83%)	23 (63.89%)	15 (46.87%)	17 (68.00%)	13 (44.83%)	79 (54.11%)
	Female	13 (54.17%)	13 (36.11%)	17 (53.13%)	8 (32.00%)	16 (55.17%)	67 (45.89%)
Environment of origin	**Urban**	16 (66.67%)	21 (58.33%)	17 (53.13%)	18 (72.00%)	21 (72.41%)	93 (63.70%)
	Rural	8 (33.33%)	15 (41.67%)	15 (46.87%)	7 (28.00%)	8 (27.59%)	53 (36.30%)
SD: Standard Deviation							

Table 2

VALUES OBTAINED FROM CREVICULAR FLUID DETERMINATION OF PROINFLAMMATORY MOLECULES

GCF (pg/ml)	I (n=24)	II (n=36)	III (n=32)	IV (n=25)	V (n=29)	*P* Value group V/ group IV
IL1-α	13.24±3.23	22.02±4.23	27.81±11.49*	33.45±8.68*	41.74±12.45*	*P* <0.05
IL1-β	12.32±5.81	19.21±7.91	25.76±10.66*	31.92±9.42*	43.19±12.97*	*P* <0.05
IL2	0.33±0.07	8.39±3.44*	29.97±11.84*	42.73±9.75*	56.59±21.32*	*P* <0.001
IL12	0.21±0.02	17.23±8.78*	21.48±7.32*	30.04±7.44*	46.94±22.64*	*P*<0.001
TNF-α	3.14±2.1	19.47±7.43*	28.77±7.37*	53.19±12.54*	79.83±27.97*	*P*<0.001
IFN-γ	2.88±1.14	11.29±5.22*	15.42±6.22*	24.32±10.03*	38.42±12.45*	*P* <0.05
Values are expressed as the mean value ± Standard Deviation; * indicates a p value of <0.05						

the group of healthy endo-periodontal subjects; following comparison of the values obtained for groups IV and V, we noticed significant differences (P <0.001) for the group with patients with endo-periodontal lesions. Values of proinflammatory molecules determined in crevicular fluid are shown in table 2.

IL2 showed significantly higher serum values †for groups III, IV and V than group I; although the values †recorded for the group with superficial periodontitis were higher, the differences did not reach a level of statistical significance (P = 0.843); it is noteworthy that the mean serum level of IL12 was significantly higher for the endo-periodontal syndrome group compared to subjects with severe periodontitis (table 3).

Serum IL12 values were significantly higher than healthy periodontal subjects only for patients with severe periodontitis and endo-periodontal syndrome, significantly higher for group V vs. group IV (P <0.001).

TNF-α and IFN- γ demonstrated significantly higher values for all pathological groups compared to the group of healthy endo-periodontal subjects; in addition, we noted for both proinflammatory molecules significantly higher serum values for the endo-periodontal syndrome subjects versus subjects with severe periodontitis. Data on serum determinations are shown in table 3.

The initiation of an inflammatory cascade in lesions of endodontic origin includes the complex interaction of several cell types involving the activation of endothelial cells, PMNs, macrophages, lymphocytes and osteoclasts that lead to rapid bone destruction. The complex host response involves both innate and aquired immune response cells. Rapid bone destruction from endodontic lesions is initiated by multiple bacteria or their products, including lipopolysaccharides (LPS) [8]. It is believed that bacteria stimulate resorption by inducing proinflammatory cytokines, such as IL1-β, IL-1α, RANKL or TNF-α [9]. Initial activation of the host response occurs by stimulating Toll-like (TLR) receptors and nucleotide-binding oligomerase (NOD) receptors [10]. Both TLR and NOD are highly expressed on multiple cell types associated with endodontic lesions, including monocytes / macrophages, granulocytes, pulp fibroblasts, osteoclast precursors, and mesenchymal cells [11].

Activation of these receptors leads to stimulation of multiple proinflammatory cytokines, including IL-1, TNF-α and IL-6, and was associated with increased production of RANKL, osteoclastogenesis and bone resorption [8]. Multiple studies have reinforced the concept that the development of bone resorption in endodontic lesions involves the adaptive immune response. Types of cells predominant of endodontic lesions in a rat model have been shown to be T cells, followed by B cells and monocytes / macrophages. Multiple T cell responses have been associated with endodontic lesions including Th1 (IL-2 and IFN-γ), Th2 (IL-4 and IL-5) and T lymphocytes (Tregs, IL-10 and TGF-β) Th17 (IL-17A). In fact, key transcription factors essential for the differentiation of Th1, Th2 and Tregs, T-beta, GATA-3 and FOXβ3, respectively, were found in periapical lesions as well as IL-17A, the prototypic cytokine produced by Th17 cells [12].

Endodontic lesions have been associated with multiple proinflammatory cytokines and chemokines. Interleukins, especially IL1-α and IL1-β, are produced in periapical lesions by several cell types, including macrophages, osteoclasts, PMNs and fibroblasts. The role of IL-1 in stimulating periapical bone destruction has been demonstrated using interleukine-1 receptor antagonists to demonstrate a 60% reduction in lesion development [13]. It appears that much of the osteoclastogenic activity induced in periapical lesions is specifically related to interleukin-1a formation [8]. However, when IL-1 receptor signalling is completely eliminated, there is an increase in the size of the lesion and systemic morbidity. In the present study, IL1-α showed significantly higher serum and crevicular fluid values than healthy subjects; also the values recorded in the group with patients with endo-periodontal syndrome were significantly higher than the lot with severe periodontitis, suggesting a much higher systemic load for these patients.

The IL2, IL12, TNF-α and IFN-γ levels measured in crevicular fluid were higher for groups II, III, IV and V vs. the group of healthy endo-periodontal subjects; After comparison of the values obtained for the groups IV and V we noticed significant differences (for the group with patients with endo-periodontal lesions.) TNF-α expression was identified in endodontic lesions by cells such as PMN, monocytes / macrophages and fibroblasts and may contribute to lesion formation [14]. In the present study, TNF-α demonstrated significantly higher values for all groups with pathology than the group with healthy endo-periodontal subjects; In addition, we noticed significantly higher serum values for the endo-periodontal syndrome versus subjects with severe periodontitis. Based on similar studies, local production of TNF-α can be assumed to play a role in increasing the regulation of host response to bacteria and stimulating bone resorption during periodontitis. It is also possible that oral bacteria, in addition to the cause of local pathology, may contribute to systemic conditions by increasing the production of cytokines following bacteraemia. Interestingly, *P. gingivalis* LPS stimulates a strong local inflammatory response, but a weak systemic inflammatory response [15].

IFN-γ is a lymphokine produced by lymphocytes and natural killer cells that has been implicated in periodontal

Table 3
VALUES OBTAINED FROM SERUM DETERMINATION OF PROINFLAMMATORY MOLECULES

Serum (pg/ml)	I (n=24)	II (n=36)	III (n=32)	IV (n=25)	V (n=29)	*P* Valu group V/ group IV
IL1-α	0.14±0.02	1.76±0.14	3.12±1.44*	4.33±2.38*	7.23±3.43*	*P* <0.05
IL1-β	0.31±0.12	1.44±1.26	4.22±2.37*	5.54±2.45*	8.41±3.59*	*P* <0.05
IL2	4.42±10.27	5.01±2.43	10.28±3.23*	19.21±7.33*	32.63±7.33*	*P*<0.001
IL12	1.21±0.09	1.97±1.28	2.76±1.49	5.29±2.98*	10.55±3.79*	*P*<0.001
TNF-α	0.65±0.10	3.02±1.12*	3.28±1.95*	6.84±2.55*	8.97±4.21*	*P*<0.05
IFN-γ	12.36±3.75	17.57±4.23*	19.24±6.44*	21.10±7.47*	38.94±9.79*	*P*<0.001

Values are expressed as the mean value ± Standard Deviation; * indicates a p value of <0.05

bone loss. Its expression is associated with Th1 lymphocytes. Mice with a genetic ablation of IFN-γ have a *P. gingivalis*-induced bone loss compared to wild-type controls. T cells are an important source of IFN-γ in periodontitis and have been linked to the increase in RANKL expression [16]. We obtained significantly higher serum levels of IFN-γ for all pathological groups compared to the group with healthy endo-periodontal subjects; moreover, the values were significantly higher for the endo-periodontal syndrome group versus subjects with severe periodontitis.

It is well known that, in its unsteady state, the spread of infection and the inflammatory process in the nearby tissue compartments is possible and can cause severe inflammatory conditions, but fortunately rare. Moreover, in view of increasing the awareness of a potential relationship between persistent, inflammatory oral cavity disorders and diseases of other organs of the body, acute and chronic manifestations of apical periodontitis can also be involved [17]. Siqueira and Rocas [18] cite how primordial apical periodontitis and post-treatment can influence the general health of the individual and remain a question that requires response in endodontic microbiology.

The possible link between chronic inflammatory processes of infectious origin and periodontal disease with systemic health is today one of the most interesting issues faced by the medical and dental scientific community [19]. A question arises as to whether cell-to-cell direct interactions between periodontal or endodontic bacteria and host cells as well as between different human cells or autocrine and paracrine stimulation loops can influence the function of the distal tissues and organs resulting in pathogenesis or which contributes to the pathological mechanism of systemic diseases.

Conclusions

The values of inflammatory molecules in crevicular fluid of patients with periodontal pathology reflect the higher degree of local inflammation in endo-periodontal patients. Although patients with severe periodontitis and those with endo-periodontal syndrome exhibited the highest values, the levels were significantly higher for the latter, providing a much more severe molecular picture than the other patient categories.

Following serum determinations of proinflammatory molecules, patients with endo-periodontal lesions demonstrated significantly higher values †even than subjects with severe periodontitis; these data indicate a much higher risk for these patients to develop and maintain systemic maladies, as is the role local inflammation can play over the general inflammatory status of the patient. Therefore, treatment at all stages of these patients acquires an increased level of complexity, higher even than cases of severe periodontitis.

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