

CHANGES IN SALIVA PROTEIN COMPOSITION IN PATIENTS WITH PERIODONTAL DISEASE

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ABSTRACT

Periodontitis is a chronic inflammatory disease characterized by tissue destruction which is usually diagnosed through clinical and radiographic signs. The detection of changes in the chemical composition of saliva could be used to reflect gingivo-periodontal alterations. The aim of this study was to identify salivary parameters that could identify different stages of the periodontal disease.

The study group included 118 adults, 89 of them with mild, moderate or severe chronic periodontitis. The remaining participants comprised the control group. Total saliva was analyzed for physical and chemical properties. Dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein detection and zymography for type IV collagenase identification.

Salivary flow rate, pH and buffer capacity showed similar values in all groups. Proteins were augmented in severe periodontitis, as also shown by SDS-PAGE. Hydroxyproline rose significantly in all periodontal groups as secretory Immunoglobulin A significantly diminished compared with the control group. An increase in peroxidase was detected in moderate and severe periodontitis. All salivary samples contained 200-116-92 kDa gelatinases; minor bands at 66-31 kDa were also present in all periodontitis groups. Calcium levels showed significant differences between all periodontitis groups compared with the control group.

Quantitative changes in the chemical composition of the saliva of patients with periodontal disease could be of significance in the diagnosis and progression of periodontal disease.

Key words: whole saliva, periodontal disease.

CAMBIOS EN LA COMPOSICIÓN PROTEICA DE LA SALIVA EN PACIENTES CON ENFERMEDAD PERIODONTAL

RESUMEN

La Periodontitis es una enfermedad inflamatoria crónica caracterizada por destrucción tisular que se diagnostica generalmente a través de signos clínicos y radiográficos. Sin embargo la detección de cambios en la composición química de la saliva podrían ser empleados para reflejar alteraciones gingivo-periodontales. El objetivo de este estudio fue encontrar parámetros salivales que pudieran identificar diferentes estadios de la enfermedad periodontal.

El grupo de estudio incluyó 118 adultos, 89 de los cuales con enfermedad periodontal crónica leve, moderada o severa. El resto constituyó el grupo control. Se analizaron las propiedades físicas y químicas de la saliva total. La separación de proteínas fue realizada empleando la electroforesis en geles de poli-acrilamida conteniendo dodecil sulfato de sodio y la identificación de colagenasa tipo IV se realizó empleando la zimografía.

Flujo salival, pH y capacidad bufferante mostraron valores similares en todos los grupos. Las proteínas estuvieron aumentadas

en la periodontitis grave lo cual también se mostró por medio de la electroforesis. Hidroxiprolina aumentó significativamente en todos los grupos de pacientes con enfermedad periodontal, mientras IgA secretoria se encontró significativamente disminuida respecto al grupo control. Un incremento en los valores de peroxidasa se detectó en las periodontitis moderada y grave. Todas las muestras contenían gelatinasas de 200-116-92 kDa; sin embargo todos los grupos de pacientes con enfermedad periodontal también presentaron bandas de peso molecular menor (66-31 kDa). Los niveles de calcio mostraron diferencias significativas entre todos los grupos de pacientes con periodontitis cuando se los comparó con el grupo control.

Cambios cuantitativos en la composición química de la saliva de pacientes con enfermedad periodontal podrían tener significancia en el diagnóstico y progresión de la enfermedad periodontal.

Palabras clave: saliva total, enfermedad periodontal.

INTRODUCTION

Saliva is described as a heterogeneous fluid constituted by proteins, glycoproteins, and electrolytes. It has been suggested that individual variations in salivary composition may influence oral health¹.

Periodontal diseases and caries are plaque-associated dental diseases². Periodontal diseases are chronic inflammatory entities characterized by infiltration of leukocytes, loss of connective tissue and alveolar bone resorption. Proteolytic enzymes released by the host cells are associated to tissue destruction. In this way,

Table 1: Characteristics Associated to Control Subjects and to the Clinical and Radiographic Diagnoses of Mild, Moderate and Severe Chronic Periodontitis Groups

Diagnoses	Clinical and Radiographic Characteristics
Control	Gingival index =0. Plaque Index 20%. Probing depth 3mm. No attachment loss on the basis of clinical criteria. Characteristics compatible with health: light pink gums with a rough appearance. Absence of bleeding on probing, furcation, mobility and bony defects.
Mild Periodontitis	Gingival index >1. Plaque Index >20%. Probing depth 4mm. Pocket depth and attachment level (measurement and radiographic analysis) not exceeding one third of the length of the root. Bleeding on probing. Absence of furcation, mobility and bony defects.
Moderate Periodontitis	Gingival index >1. Plaque Index >20%. Probing depth >5mm. Pocket depth and attachment level (measurement and radiographic analysis) between one third and half the length of the root. Bleeding on probing. First degree furcation. Eventual first degree mobility.
Severe Periodontitis	Gingival index >1. Plaque Index >20%. Probing depth >7mm. Pocket depth and attachment level (measurement and radiographic analysis) over half the length of the root. Bleeding on probing. Second or third degree furcation. Eventual second or third degree mobility. Angular bony defects.

matrix metalloproteinases (MMPs) can degrade most of the extracellular matrix components³. The main source of type IV collagenase would be polymorphonuclear cells (PMN) that enter the oral cavity through the gingival sulcus^{4,5}. Collagen breakdown in connective tissue can be measured by hydroxyproline (Hyp) determination⁶. However, no chemical determinations have been reported in total saliva of periodontal patients. Total proteins in saliva are approximately 3% of those present in plasma. When analyzed by Dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), visual inspection revealed more than 40 protein bands⁷. The major immunoglobulin type in saliva is secretory Immunoglobulin A (sIgA). Previous investigations determined that it increases in periodontal disease and suggested that sIgA plays an important role in oral mucosa defence⁸. Many biochemical systems such as salivary peroxidase are known to be involved in soft-tissue repair and bacterial attack⁹. The major components of the peroxidase system complex include different forms of lactoperoxidase secreted by the salivary glands, and mieloperoxidase produced by PMN

in gingival crevicular fluid¹⁰. Mucins are also glycoproteins that can be isolated from total saliva and have shown to have antimicrobial activity. In this way, their alteration could also contribute to develop periodontal disease.² Glucose has not been described in periodontitis.

The buffering action of saliva may help to prevent demineralization. Some investigations showed a positive correlation between high salivary calcium content and periodontitis¹¹. Salivary flow rate, pH and buffer capacity were analyzed in relation to calculus formation. When total salivary flow increases, calcium concentration also increases¹². Therefore, the currently accepted concept is that periodontitis-affected subjects have a high intraoral mineralization potential¹³.

Although salivary parameters in patients with periodontal disease have been described; no associations with the different states of periodontitis have been demonstrated yet.

The aim of the present study was to identify a combination of salivary markers that exhibit statistically significant differences between patients with varying degrees of chronic periodontitis and periodontitis-free subjects. Having identified these parameters, they could be used as a tool for diagnosis and disease progression even in the early stages, given that chemical alterations should occur before macroscopic tissue changes are evident.

MATERIALS AND METHODS

Patient Selection

The study was performed on a group of patients with chronic periodontitis and a control group of periodontitis-free subjects. The control (C) group was composed of 29 subjects (14 males and 15 females) aged 45.9 ± 10.6 years. Patients were classified according to their clinical diagnosis, according to Lindhe (1998)¹⁴ in mild periodontitis (MiP) (n=29) (17 males and 12 females) aged 42.6 ± 0.6 years, moderate periodontitis (MoP) (n=34) (18 males and 16 females) aged 40.8 ± 10.5 years and severe periodontitis (SP) (n=26) (12 males and 14 females) aged 47.2 ± 11.2 years, as shown in Table 1.

Patient selection was based on preliminary screening. Inclusion criteria were as follows: 1) at least 2 sites with > 4 mm of attachment loss in each quadrant; 2) a minimum of 20 natural teeth excluding third molars; 3) no systemic medical affections that might impact directly on periodontal status; 4) no history of antibiotic use or periodontal treatment. Inclusion criteria 2-4 applied to the control group. Subjects were selected only according to the inclusion and the exclusion criteria from those who attended the Faculty of Dentistry, National University of Tucumán, for periodontal consultation. Written informed consents were obtained from all individuals prior to their participation.

Clinical Parameters

Clinical diagnoses were determined by a calibrated investigator and the following parameters at the sampled sites were considered: Gingival Index (GI)¹⁵, Plaque Index (PI)¹⁶, probing pocket depth (PD), bleeding on probing and attachment loss level. Bone resorption in chronic adult periodontitis was determined on the basis of clinical and radiographic criteria; periapical radiographs were taken using a standardized long-cone paralleling technique.

Saliva Collection

Patients were instructed not to eat or drink for 2 h prior to sample collection. Unstimulated whole saliva was collected at the initial visit, previous to the periodontal treatment, between 8-10 am for 5 min. Saliva accumulated in the antero-vestibular and sublingual region of the mouth was aspirated avoiding contact with the mucosa¹⁷. Then saliva was placed in a glass tube on ice. Volume, pH, and buffer capacity were determined. Saliva was centrifuged at 10,000 rpm for 10 min at 4°C and immediately frozen at -20°C until chemical determinations were performed.

Physical and Chemical Determinations

pH was measured with a digital pHmeter (Broadley-James Corp. Irvine, California, USA) and buffer capacity was determined by the method described by Ericsson¹⁸ modified for small volumes. Determinations included total proteins by the colorimetric method of Lowry et al. (1951)¹⁹ as determined by other authors²⁰, mucins by the method

Table 2: Means ± SE of pH, Buffer Capacity and Volumen Total / minute in Total Saliva of Control and Chronic Periodontitis Groups

Clinical Diagnoses	pH	Buffer Capacity	Total Volume /min
C (n=29)	6.66 ± 0.08	1.85 ± 0.13	0.25 ± 0.02
MiP (n=29)	6.81 ± 0.20	2.13 ± 0.26	0.20 ± 0.03
MoP (n=34)	6.55 ± 0.08	1.98 ± 0.15	0.23 ± 0.04
SP (n=26)	6.34 ± 0.11	2.33 ± 0.48	0.19 ± 0.02

C: control, MiP: mild periodontitis; MoP: moderate periodontitis; SP: severe periodontitis

described by Mehl et al. (1949)²¹, peroxidase as described by Mansson-Rahemtulla et al. (1986)²² and Hyp as described by Jamall et al. (1981)²³. sIgA was determined by radial immunodiffusion (Diffu Platte Lab, Argentina). Salivary calcium and phosphorus were determined by colorimetric methods (Wiener Lab, Argentina), and glucose was assessed by the glucose-oxidase method (Wiener Lab, Argentina).

SDS-PAGE was performed according to the method described by Schwartz et al. (1995)⁷ for salivary samples using a 12.5% resolving gel and a 4.0% stacking gel. Gels were fixed and stained in a solution of 0.1% Coomassie Brilliant Blue R-250.

Collagenases were analyzed by zymography using the method described by Ingman et al. (1994)²⁴. 10 µl samples of saliva were loaded in each lane of a 12% SDS-PAGE containing 1mg/ml gelatin as the substrate. Collagenase activity was identified on the gel as light bands on a dark background. SDS-PAGE and zymographies were performed with at least 10 individual samples.

Statistical Analysis

The data were analyzed with a SPSS system. Differences among groups were analyzed by ANOVA One Way test. When the differences were significant, the Tukey-test was used.

RESULTS

None of the determinations showed statistical differences between sexes. Volume, pH and buffer capacity did not evidence statistically significant variations between periodontally-affected and periodontitis-free subjects (Table 2).

Results of the chemical determinations are shown in Fig 1. Total proteins were significantly higher in

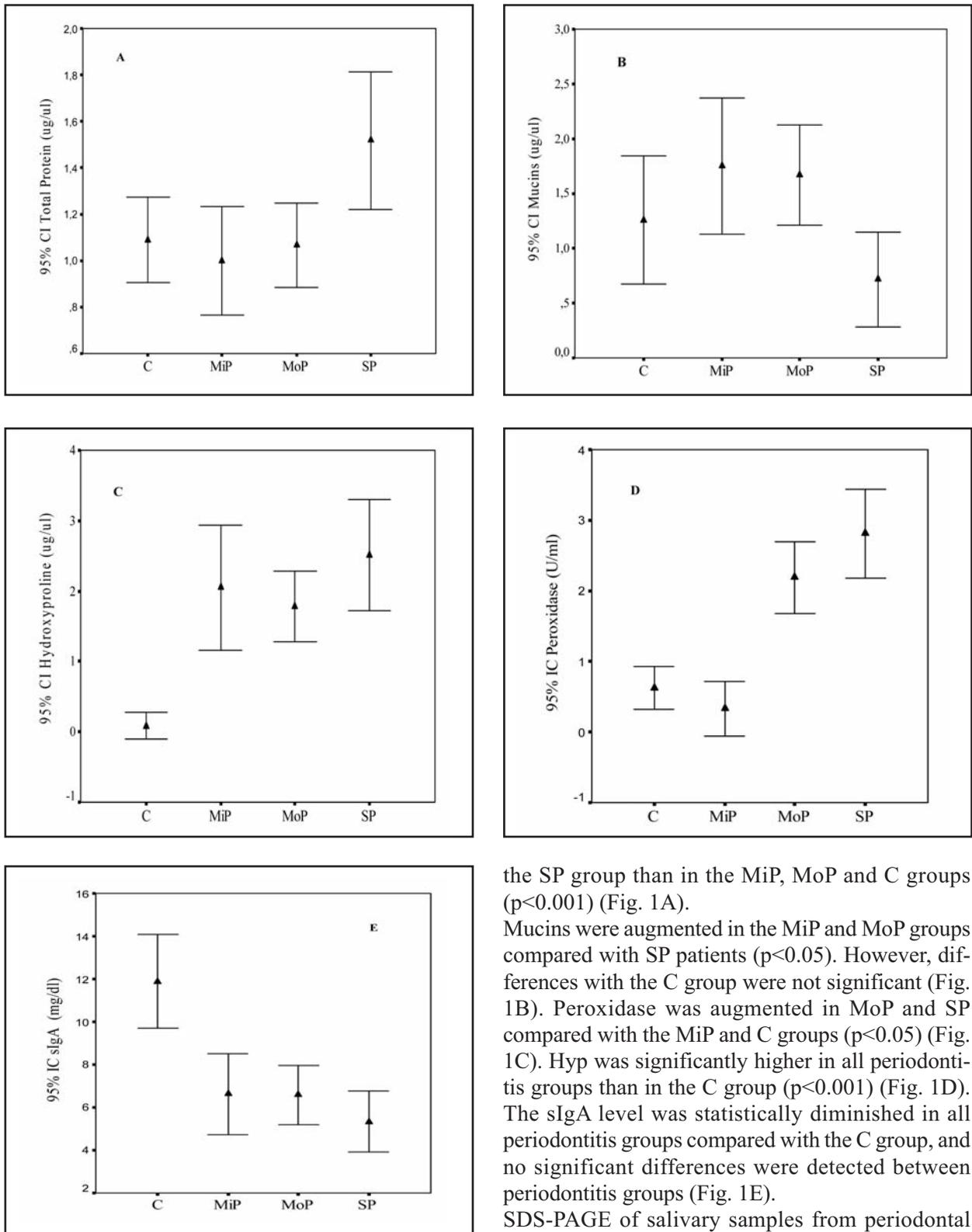


Fig. 1: Means and standard error of salivary chemical determinations of the control (C) and mild (MiP), moderate (MoP) and severe (SP) chronic periodontitis groups. A. Total proteins (mg/ml); B. Mucins (mg/ml); C. Peroxidase (U/ml); D. Hydroxyproline (mg/ml); E. sIgA (mg/dl).

the SP group than in the MiP, MoP and C groups ($p < 0.001$) (Fig. 1A).

Mucins were augmented in the MiP and MoP groups compared with SP patients ($p < 0.05$). However, differences with the C group were not significant (Fig. 1B). Peroxidase was augmented in MoP and SP compared with the MiP and C groups ($p < 0.05$) (Fig. 1C). Hyp was significantly higher in all periodontitis groups than in the C group ($p < 0.001$) (Fig. 1D). The sIgA level was statistically diminished in all periodontitis groups compared with the C group, and no significant differences were detected between periodontitis groups (Fig. 1E).

SDS-PAGE of salivary samples from periodontal patients showed a number of protein bands that increased with the degree of the periodontal disease compared to C subjects (Fig. 2 A-B). These proteins were especially localized in the upper third of the gel. Saliva of all groups, including C, contained 200-116-

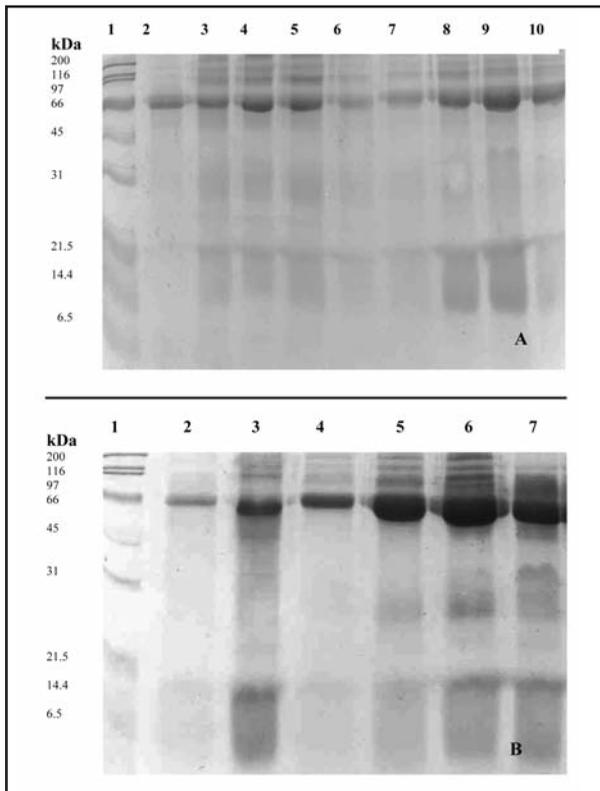


Fig. 2: SDS-PAGE of saliva A. Control group (C) (lines 2-10) and B mild (MiP) (lines 2-4), moderate (MoP) (lines 5-6) and severe (SP) (lines 7) chronic periodontitis groups. Molecular size markers (lines 1 A and B) are included.

92 kDa gelatinases. Other gelatinolytic bands were also observed in the region of 66-31 kDa in all periodontitis groups (Fig. 3).

Calcium levels showed significant differences between all periodontitis groups compared with C ($p < 0.001$) (Fig. 4A); differences were also detected between SP and MiP and MoP ($p < 0.001$). Phosphorus levels were significantly higher in the SP and MoP groups compared with the C group ($p < 0.05$) (Fig. 4B). Glucose showed no differences between all periodontitis and C groups (data not shown).

Table 3 expresses the range of values of the salivary chemical parameters that were statistically altered in relation to the different stages of the periodontal disease. The healthy state could be distinguished from the different states of the disease by hydroxiprolin, sIgA, calcium, and the high molecular weight bands of collagenases. The determination of the enzyme peroxidase, calcium and phosphorus could distinguish MiP from MoP and SP, while SP could be distinguished from MoP by the high values of total proteins, calcium and the low levels of sIgA.

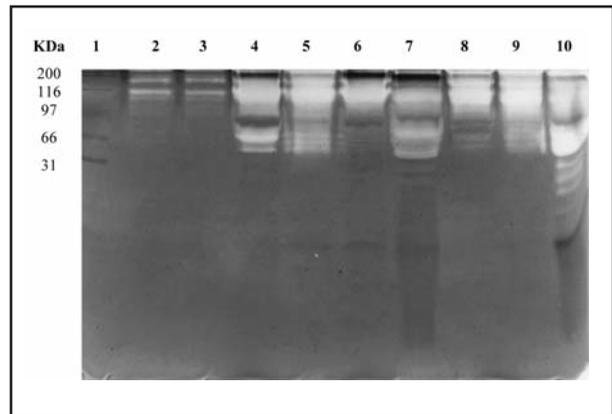


Fig. 3: SDS-PAGE of the gelatinolytic activity of saliva of the control (C) (lines 2-3) and mild (MiP) (lines 4-5), moderate (MoP) (lines 6-8) and severe (SP) (lines 9-10) chronic periodontitis groups. Molecular size markers (line 1) are included.

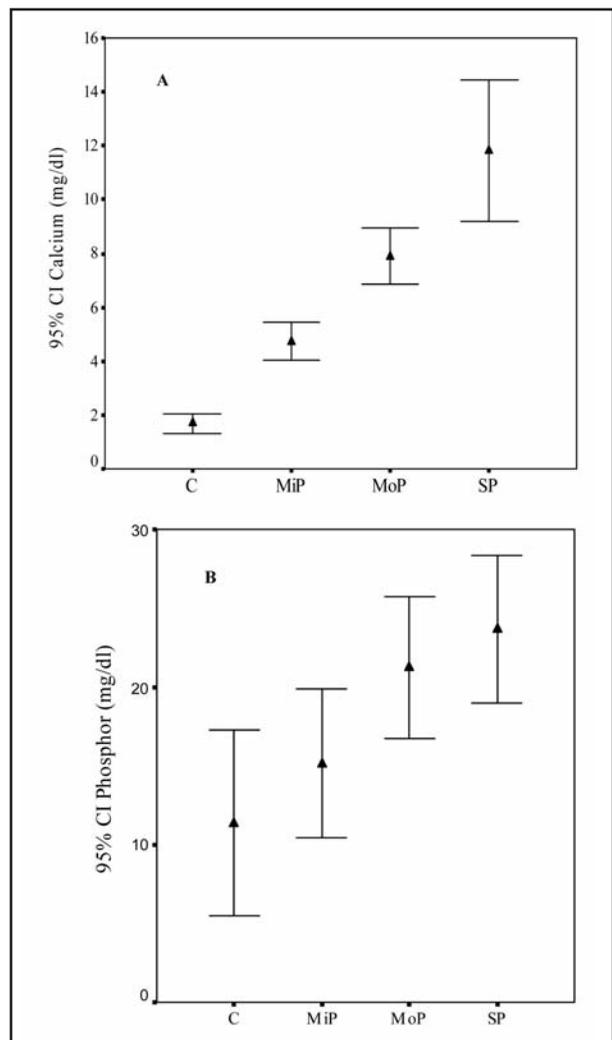


Fig. 4: Means and standard error of salivary ions of the control (C) and mild (MiP), moderate (MoP) and severe (SP) chronic periodontitis groups. A. Calcium (mg/dl) B. Phosphorus (mg/dl).

Table 3: Concentration Ranges for Total Proteins, Hidroxyproline, Peroxidase, Calcium and Phosphorus in Total Saliva of Control and Chronic Periodontitis Groups

Clinical Diagnosis	C	MiP	MoP	SP
Total Proteins (mg/ml)	0.7-1.2	0.7-1.2	0.7-1.2	> 1.2
Hidroxyproline (g/l)	< 1	2-3	2-3	> 3
Peroxidase (U/ml)	< 1	< 1	1.5 - 2	> 2
Calcium (mg/dl)	< 4	4-6	7-9	> 9
Phosphorus (mg/dl)	5-17	10-20	17-27	> 20
Collagenase (kDa)	200-97	200-97	200-31	200-31
SDS PAGE (200- 6.5 kDa)	Light bands	Light bands	Intense bands	Intense bands

C: control, MiP: mild periodontitis; MoP: moderate periodontitis; SP: severe periodontitis

DISCUSSION

For many years, assessment of salivary flow rate and composition has been used to understand variations in oral diseases. Saliva has become a topic of increasing interest as compared with other biological fluids such as blood and urine. Within this context saliva analysis would constitute a contributory aid in clinical diagnosis of active periodontitis, assessment of risk factors and evaluation of disease progression. In this study the chemical composition of total saliva of patients with chronic periodontal disease was analyzed. Patients were classified according to their clinical symptoms, according to Lindhe (1998)¹⁴ in three groups: MiP, MoP and SP. In this study, gelatinase activity increased in all periodontal patients, as evidenced by the corresponding bands of type IV collagenase in the 31-66 kDa region in keeping with Ingman et al. (1994)²⁴. Bands were also detected in the area of high molecular weight, both in healthy individuals and in periodontal patients. They could correspond to polymerized or complex enzymes bonded by disulfide bridges present in saliva²⁵. The 92-109 kDa gelatinase bands could be derived from neutrophils, which are the main cellular source of a 92 kDa gelatinase or MMP-9, that has also been shown to be synthesized by epithelial and bone cells²⁶, and gingival granula-

tion-tissue fibroblasts²⁷. The bands of 69-80 kDa are forms represented by gelatinase or MMP-2 originated from fibroblasts, macrophages or bone cells²⁶. The low molecular weight forms could result from the action of proteolytic enzymes or of multiple stages of an autocatalytic cascade²⁸.

In the present study, Hyp values significantly differed between the periodontitis and the C group. Considering that the ages of all groups were similar, tissue destruction and our results of Hyp and collagenase determination evidence that collagen would be the main protein that increases with periodontal damage. However, these results on total basal saliva contrast with data from other studies on stimulated saliva, which revealed no differences in the protein content of patients with periodontitis compared to healthy subjects²⁹. Akalin et al. (1992)⁶ found that Hyp levels in gingival tissue and in GCF from periodontally healthy subjects in different age groups were higher in the younger group than in the older one. However, there are no references in the literature that salivary Hyp variations were detected in different stages of periodontitis.

Total proteins level in saliva of patients with severe forms of periodontitis was significantly higher than in the C group. Values for controls, around 1.0 mg/ml, were similar to those reported by other authors^{20,30}, and those around 1.6 mg/ml in subjects with periodontal disease²⁹ were similar to those in this study for the SP group. This rise is partly due to the contribution of proteins from GCF, since its increase in whole saliva varies according to the degree of gingival inflammation¹⁵. In previous studies we demonstrated that GCF of patients with periodontitis has a high protein content³¹. It was also demonstrated that plasmatic protein concentration in dental pellicle samples increased 2-10 times from healthy to inflamed conditions³. The rise of proteins was also evident through SDS-PAGE of salivary samples from periodontal patients. SDS-PAGE for C was coincident with those reported by Schwartz et al. (1995)⁷. Regarding salivary proteins with antimicrobial activity, significant differences compared to the C group were found in our study for peroxidase of patients with MoP and SP, but not for sIgA. One of the most important functions of salivary peroxidase is the control of oral bacteria that form dental plaque and of the imbalance in the buccal ecology¹⁰. A high level of peroxidase activity in saliva is described for periodontitis patients concomitantly with an

increase in the gingival index²². As for the other determinations, no associations with the different stages of periodontitis were demonstrated before. Periopathogenic bacteria give rise to the degranulation of leucocytes resulting in the release of myeloperoxidase³². Its activity is increased in gingival fluid during inflammation and this increase may be reflected in whole saliva³³. These authors reported similar values of peroxidase activity (0.65 ± 0.43 U/ml) for subjects with periodontitis to those in our study for MiP. The authors recommended caution in the interpretation, since these values represent a single-point measurement and they do not necessarily reflect the status of the disease process. We also found even higher salivary values of peroxidase activity in MoP and SP.

In the present study, sIgA levels evidenced the loss of the healthy state; other authors^{33,34} reported variations (8 ± 4 mg/dl) that are similar to those in this study for subjects with MiP, MoP and SP. From our results it seems that in the SP the immune control could be much less efficient than in the MiP and MoP. The increase in mucins was not particular to a specific stage of periodontitis. In relation to the antibacterial function of saliva it is suggested that specifically MG2 is likely to be an important component of the innate immune system. The increase of MG2 could be due to the immune imbalance that characterizes the periodontal disease³⁵. These proteins can form homo and heterotropic complexes with other proteins such as sIgA³⁶, which favor bacteria agglutination². High calcium concentration of saliva seems to be a characteristic feature of periodontitis affected subjects, since people with a high content of intraoral calcium could be susceptible to develop calculus and periodontal disease, regardless of the periodontal treatment³⁷. Our results and those of other groups^{29,38} are similar for the C (4.96 ± 1.04 mg/dl) and for the MiP and MoP groups (7.17 ± 1.55 mg/dl). However, higher values were recorded for the SP saliva.

In this study, significant differences in phosphorus levels between the SP and the C groups were obtained, unlike other authors^{29,38} who found no significant differences between subjects with (10.55 ± 2.16 mg/dl)

and without (9.24 ± 1.05 mg/dl) periodontitis. Our results would evidence that in the SP, as occurred with plasmatic proteins, calcium and phosphorus could originate in blood as a consequence of advanced tissue destruction.

Regarding pH, salivary volume per minute and buffer capacity, no differences were observed between groups. Results obtained in our study for salivary flow rate and pH in C subjects and periodontal subjects were in agreement with other authors^{13,33}. However, the SP group showed less salivary flow rate than the C group, although the difference was not significant^{29,37}. Regarding the buffer capacity in healthy subjects and in subjects with periodontitis we found lower values than those reported by Grahn et al. (1998)³³ and Sewón et al. (1990)²⁹, perhaps due to the use of a different method of determination.

Although many studies analyze the composition of saliva in relation to the periodontal health state, the issue of the different degrees of the periodontal affection has not been addressed to date. The present study provides evidence for the association between some chemical parameters and the presence of periodontal disease, regardless of the status of the disease. The present data reveal that certain parameters vary with the progression of the affection. The periodontal disease state could be distinguished from the healthy state through Hyp, sIgA determinations and the light molecular weight bands of collagenases. The determination of the enzyme peroxidase, total proteins, calcium and phosphorus could be used as progression markers of the disease since their values rose from the MiP to the SP.

Chemical components may not only be identified but also quantified in order to understand the biological properties of saliva in the pathobiology of oral diseases. The same factors should be investigated after treatment in further studies. Although this is not an epidemiological study, and the daily dental practice is not usually associated to the biochemical investigation, there are many reasons to think that Dentistry and Periodontology could take advantage of this knowledge and apply it to follow the patients with periodontal disease.

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