Original Article

sIgA, peroxidase and collagenase in saliva of smokers aggressive periodontal patients

Myriam A. Koss^{a,*}, Cecilia E. Castro^b, Agustina M. Gramajo^a, María E. López^a^a Department of Biochemistry, School of Dentistry, National University of Tucumán, Argentina^b Department of Periodontology, School of Dentistry, National University of Tucumán, Argentina

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ABSTRACT

Background: Tobacco smoking is strongly associated with destructive periodontal disease, alveolar bone loss and poor response to periodontal therapy. The aim of this study was to investigate the effect of smoking on antimicrobials and destructive proteins in the saliva of patients with generalized aggressive periodontal disease.

Material and methods: The study group consisted of 55 adult subjects. They were classified according to their clinical diagnoses either as having generalized aggressive periodontitis ($n = 27$; 13 smokers and 14 non-smokers) or as periodontitis-free control group ($n = 28$; 14 smokers and 14 non-smokers). Total proteins, sIgA, peroxidase and collagenase were quantified in whole saliva. Data were analyzed using one-way ANOVA and Tukey tests.

Results: Statistical differences were found between smokers with periodontal disease and healthy control subjects for sIgA, and between non-smokers with periodontal disease and healthy control subjects for peroxidase. Collagenase activity was higher in smokers and non-smokers with periodontal disease, and tobacco use was found to increase collagenase activity in healthy subjects.

Conclusion: Whole saliva from all patients with periodontal disease had higher sIgA and lower peroxidase content. Smokers with periodontitis had higher sIgA than smokers without periodontitis. Smokers with and without periodontitis had higher collagenase activity than non-smokers (with and without periodontitis).

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1. Introduction

Periodontal diseases are chronic inflammatory entities characterized by infiltration of leukocytes, loss of connective tissue and alveolar bone resorption.¹ Aggressive periodontitis (AgP) was redefined as a complex disease exhibiting microbial alteration and cellular dysfunction, which differs from chronic periodontal disease in the underlying molecular mechanisms of its pathogenesis.² It comprises a heterogeneous group of periodontal diseases that affect adolescents and young adults. It is characterized by very rapid loss of periodontal tissue in otherwise clinically healthy subjects, and can be classified into localized (LAgP) and generalized forms (GAgP). Previous studies have investigated some factors that may increase host susceptibility to tissue destruction, including

genetic factors, functional defects of polymorphonuclear (PMN) leukocytes and monocytes, and environmental risk factors. Cigarette smoking may be an important contributor to the development of periodontal disease. Evidence suggests that periodontitis is more prevalent in smokers than in non-smokers. Tobacco smoking is strongly associated with destructive periodontal disease, alveolar bone loss and poor response to periodontal therapy.³ This suggests that smoking might interfere with the host's immune response. Tobacco metabolites suppress neutrophil function and inhibit the immune response. Nicotine has adverse effects on the role of PMN, altering the function of chemotaxis and phagocytosis and interfering with the production of immunoglobulins (Igs). Cigarette smoking can alter T-cell immune regulation and B-cell differentiation, generating a decrease in the production of Igs, which protect the oral mucosa against periodontal pathogenic bacteria.⁴

PMNs appear to play a pivotal role in aggressive periodontitis. Neutrophil granules are responsible for the neutrophil function and can be divided into three main types, according to their azurophil granule constituents. Myeloperoxidase is the most

* Corresponding author at: Cátedra de Química Biológica – Facultad de Odontología – UNT, Av. Benjamín Aráoz 800, 4000 San Miguel de Tucumán, Argentina.

E-mail address: myriam.koss@odontologia.unt.edu.ar (M.A. Koss).

effective microbial and cytotoxic mechanism of leucocytes. Oral peroxidase is composed of two enzymes: salivary peroxidase, which contributes 80% of oral peroxidase, and myeloperoxidase which contributes the remaining 20%.⁵

Damage to periodontal tissue is usually detected by means of periodontal probing, which shows loss of attachment of the tooth, or by radiographs that detect alveolar bone loss. These methods also evaluate the damage caused by previous destruction episodes, resulting in a retrospective diagnosis. Increasing attention is being given to saliva testing due to the need for a simple, non-invasive method for assessing drug intoxicated patients. Collecting saliva is easy, involves neither stress nor risk of injuries from needles, and patients can collect it themselves after instruction. Saliva contains a wide array of components that are very sensitive to toxic substances, and reflects a real-time level of biomarkers.⁶

Some inflammatory mediators and other molecules originated from tissue destruction have been detected in gingivo-crevicular fluid (GCF) and saliva of patients with chronic periodontitis (ChP) or adult periodontitis.^{7,8} Results of our previous studies have shown significant statistical differences in certain GCF markers and whole saliva between patients with ChP and periodontally healthy subjects^{9,10} and between patients with AgP and periodontally healthy individuals.¹¹

The aim of this study was to investigate the effect of smoking on total salivary slgA, peroxidase and collagenase in patients with generalized aggressive periodontal disease and to investigate the relationships with their clinical parameters.

2. Material and methods

2.1. Clinical parameters

Clinical considerations for patients with periodontitis have been published previously,⁹ taking into consideration an adaptation of Lindhe.¹²

Clinical parameters were assessed at six sites on each tooth (mesio-buccal, medio-buccal, disto-buccal, mesio-lingual, medio-lingual, disto-lingual) using a manual periodontal probe (Hu-Friedy, NC, USA). The following parameters were included: gingival index (GI),¹³ plaque index (PI),¹⁴ bleeding on probing (BOP) up to 15 s after gentle testing, probing depth (PD) (distance between the gingival margin and the bottom of the sulcus/pocket) and clinical attachment level (CAL) (distance between cement-enamel junction and the bottom of the sulcus/pocket). Bone resorption in GAgP was established on the basis of clinical and radiographic criteria. Periapical radiographs were taken using a standardized long-cone paralleling technique. Patients with GAgP included in this study had moderate (M) to advanced (A) forms of the disease and exhibited at least one site with the clinical features M: GI > 1, PI > 20%, BOP, and PD and CAL between 5 and 6 mm; A: GI > 1, PI > 20%, BOP, and PD and CAL ≥ 7 mm; C subjects: GI < 1, PI < 20%, no BOP, PD ≤ 3 mm and no CAL.

2.2. Study population

The study group consisted of 55 adults. They were classified according to their clinical diagnoses as having generalized aggressive periodontitis (GAgP) ($n = 27$) aged 26.9 ± 4.3 yrs, of whom 13 were smokers (sGAgP) (6 females, and 7 males) and 14 were non-smokers (nsGAgP) (6 females and 8 males). The control group (C) consisted of 28 periodontitis-free adult subjects aged 25.8 ± 2.9 yrs, of whom 14 were smokers (sC) (7 females and 7 males) and 14 were non-smokers (nC) (6 females and 8 males). All subjects attended the School of Dentistry at the National University of Tucumán for periodontal consultation. Written informed consent was obtained from all patients prior to participation.

Inclusion criteria for all subjects were a minimum of 20 natural teeth excluding third molars GI > 1, PI > 1, BOP, PD and CAL ≥ 4.5 mm for patients and GI < 1, PI < 1, no BOP, PD and CAL < 3 mm for the C group.

Exclusion criteria for all subjects diagnosed with GAgP included systemic diseases, periodontal therapy prior to saliva collection, and use of antibiotics, steroidal or non-steroidal anti-inflammatory agents in the 6 months prior to the study.

Individuals who had smoked more than ten cigarettes per day for at least 5 years were considered smokers.¹⁵

2.3. 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, before periodontal treatment, between 8 and 10 a.m. for 10 min. The smoking volunteers were asked not to smoke for 1 h prior to the experiment. While in a sitting position, the participants were asked to swallow saliva, then remain motionless and allow the saliva to drain passively for 10 min over the lower lip into a sterile plastic vial on ice. Saliva accumulated in the antero-vestibular and sublingual regions of the mouth was collected with a saliva ejector, avoiding contact with the mucosa, and placed in a test tube on ice. Whole saliva was centrifuged at 10,000 rpm for 10 min at 4 °C and immediately frozen at –20 °C until chemical determinations were performed.

2.4. Chemical determinations

20 µl of saliva were used to quantify total proteins following Lowry (1951).¹⁶ slgA was determined by the radial immune diffusion method by placing 5 µl of total saliva twice (Diffu Platte Lab, Argentina). Peroxidase was quantified following Mansson-Rahemtulla et al. (1986).¹⁷ 50 µM 5,5-dithionitrobenzoic acid in 100 mM phosphate buffer pH 5.6 were placed in a 2 ml spectrophotometer cuvette and 2-mercaptoethanol was added in order to obtain an absorbance between 0.8 and 1.0 at 412 nm. Then 100 µl of 90 mM potassium sulfocyanide, 100 µl of centrifuged total saliva and 100 µl of 2 mM H₂O₂ were mixed. Readings were performed at 10 s and 20 s. Collagenases were analyzed by zymography using the method described by Ingman et al. (1994).¹⁸ 10 µl salivary samples were loaded in each lane of a 12% SDS-PAGE containing 1 mg/ml gelatin as 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.

2.5. Statistical analysis

Data were analyzed by the SPSS system (11.0). Differences among groups were studied using one-way ANOVA. When differences were significant, the Tukey test was applied. Variables of the subjects were correlated by means of Spearman rank correlation analysis.

3. Results

Total proteins showed no significant difference ($p > 0.05$) between GAgP and C groups (Fig. 1a). Considering smoking, total proteins were lower in sC than in nS, although the difference was not significant ($p > 0.05$) (Fig. 1b).

Peroxidase was statistically lower in GAgP than in C ($p < 0.05$) (Fig. 1c). Considering smoking, we found statistical differences ($p < 0.05$) for peroxidase between nsGAgP and nS but not between C and GAgP smoking and non-smoking subgroups (Fig. 1d).

Fig. 1. (a) Mean and standard error of salivary total proteins in control (C) and generalized aggressive periodontitis (GAgP) groups. (b) Mean and standard error of salivary total proteins in control (C) smokers and non-smokers, and in generalized aggressive periodontitis (GAgP) smoking and non-smoking groups. (c) Mean and standard error of salivary peroxidase in control (C) and generalized aggressive periodontitis (GAgP) groups. (d) Mean and standard error of salivary peroxidase in control (C) smoking and non-smoking, and in generalized aggressive periodontitis (GAgP) smoking and non-smoking groups. (e) Mean and standard error of salivary secretory IgA in control (C) and generalized aggressive periodontitis (GAgP) groups. (f). Mean and standard error of salivary peroxidase in control (C) smoking and non-smoking, and in generalized aggressive periodontitis (GAgP) smoking and non-smoking groups.

Fig. 2. (a) Zymography of the gelatinolytic activity on sodium dodecyl sulphate polyacrylamide gel electrophoresis of saliva of control non-smoking subjects (lanes 2–3), and of aggressive periodontitis non-smoking patients (lanes 4–8). Molecular size markers (lane 1) are included. (b) Zymography of the gelatinolytic activity on sodium dodecyl sulphate polyacrylamide gel electrophoresis of saliva of control smoking subjects (lanes 2–3), and of aggressive periodontitis smoking patients (lanes 4–8). Molecular size markers (lane 1) are included.

slgA was higher ($p < 0.05$) in GAgP than in C (Fig. 1e). Considering smoking, we found statistical differences ($p < 0.05$) between sAgP and sC groups, and between nsGAgP and sC, but not between C and GAgP smoking and non-smoking subgroups (Fig. 1f).

The saliva of all subjects contained 200–116–92 kDa gelatinases (Fig. 2a and b). Control smokers show two gelatinolytic bands in the region of 31 kDa and lower molecular weights (Fig. 2b) than nsC. In smokers and non-smokers with periodontal disease, higher gelatinase activity in regions between 66 and 31 kDa was also observed; especially for sAgP.

Peroxidase correlated negatively with CAL, BOP ($p < 0.05$) and GI ($p < 0.01$). BOP, GI ($p < 0.05$) and PI ($p < 0.01$) were found to correlate positively with smoking (Table 1).

4. Discussion

Smoking is a major risk factor contributing to the pathogenesis of oral diseases. It was hypothesized that the increased susceptibility of cigarette smokers to infections reflects multifunctional

alteration of their local and systemic inflammatory and adaptive immune responses.¹⁹ Reduced phagocyte activity of neutrophils was also reported in smokers, which could be responsible for a decreased defense of the gingiva against bacterial attack.²⁰ Our study found significant differences in slgA, peroxidase and collagenase in the saliva of smoking and non-smoking healthy subjects and patients with aggressive periodontitis.

Patients with AgP had higher slgA level and collagenolytic activity and lower salivary peroxidase activity, which is negatively correlated with CAL, BOP and GI.

It has been observed that cigarette smoking can alter the salivary system by increasing salivary fluid, and reducing certain salivary enzymes and the immune system function. Hence a reduction of the immunoglobulins contained in saliva may be an increased risk factor for the host mucosa with regard to periodontal pathogens.⁴ Olayanju et al. (2012)²⁰ found that patients with periodontitis had higher slgA and concluded that slgA could therefore be used as a screening tool for periodontitis. Another study evaluated the change of slgA level in response to periodontal treatment in patients with AgP,²¹ finding a decrease in salivary slgA in resting and stimulated saliva between baseline and after 4 weeks treatment.

Our study found higher slgA in periodontal patients, and no correlation with clinical parameters. In the smoker control group, slgA level was lower than in the aggressive periodontitis smoking and non-smoking groups. The smoker control group had non-significantly lower slgA than the non-smoker group, in agreement with Gio et al. (2007),²² who demonstrated that slgA concentration was greater in non-smokers than in smokers. A very different profile of responses was observed when patients were stratified by smoking habits. Antibody to the periodontal pathogens were significantly elevated in smoker with periodontitis and significantly decreased in periodontally healthy smokers.¹⁹

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and key enzymes in extracellular collagen matrix degradation, derived predominantly from leukocytes during the acute stage of the periodontal disease. Among these MMPs, neutrophil collagenase (MMP-8) and fibroblast-type collagenase (MMP1) are the key initiators of collagen degradation. Cleavage products denature spontaneously to gelatine at body temperature, and can be further degraded by gelatinases/type IV collagenase.¹⁸ Miller et al. (2006),⁸ observed elevated mean levels of MMP-8 in saliva of subjects with periodontitis, which were highly correlated with BP, CAL and PD measures. This suggests that the collagen degradation phase of periodontitis may be measurable in saliva and may thus be useful for monitoring the activity of the disease. Previous studies conducted by our group found that saliva from healthy subjects and patients with chronic periodontal disease contained gelatinase bands around 200–116–92 kDa.⁹ The 92–109 kDa gelatinase/type IV collagenase bands could be derived from neutrophils, which are the main cellular source of a 92 kDa gelatinase/type IV collagenase or MMP-9. Type IV collagenase has also been shown to be synthesized by epithelial and bone cells, and gingival granulation-tissue fibroblasts. The 69–80 kDa bands are forms represented by gelatinase or MMP-2 originated from fibroblasts, macrophages or bone cells. The low molecular weight

Table 1
Correlations between slgA, peroxidase and smoking habits and clinical parameters.

	Probing depth	Clinical attachment level	Bleeding on probing	Plaque index	Gingival index
slgA	0.229	0.206	0.237	0.119	0.160
Peroxidase	–0.272	–0.352*	–0.340*	–0.191	–0.380**
Smoking habits	0.136	0.207	0.315*	0.454**	0.321*

* $p < 0.05$ Spearman rank correlation.

** $p < 0.01$.

forms may result from the action of proteolytic enzymes or from multiple stages of an autocatalytic cascade.²³

In this study, we found collagenolytic bands in the area of high molecular weight, both in healthy subjects and in patients with aggressive periodontitis. When the influence of smoking was studied, it was found that collagenolytic activity was higher in healthy smokers than in healthy non-smokers in the zone of 31 and 14.5 kDa zymography bands. Collagenolytic bands in the zone between 66 and 6.5 kDa were also higher in smokers with periodontal disease than in non-smokers with periodontal disease. Liu et al. (2006)²⁴ have reported that smoking modulates the expression of MMP-8 in periodontal tissues, being significantly higher in the periodontal tissue of smokers than in non-smokers.

A peroxidase level in non-smokers with periodontitis was lower than in the non-smoker control group. This difference was not observed between smokers with and without periodontitis. Saxén et al. (1990)²⁵ also found that salivary peroxidase activity was significantly lower in whole saliva samples from patients with juvenile periodontitis and that leukocyte-derived myeloperoxidase was present in significantly lower amounts in whole saliva of these patients. They suggest that because both glandular (salivary peroxidase) and polymorphonuclear-cell-derived (myeloperoxidase) enzyme activities were low in aggressive periodontitis patients, suppressed peroxidase-mediated host defence mechanisms may be characteristic of these patients. When smoking was considered, there were no significant differences in levels of peroxidase in the control group, however, a slight decrease was observed in smokers, as also reported by other research, Reznick et al. (2003),⁵ showed a sharp drop of oral peroxidase activity in smokers and no smoker after smoking a single cigarette. However, in the smokers periodontal patients an increase in peroxidase level was found. Rudolf et al. (2008),²⁶ have shown elevated myeloperoxidase in smokers, apparently resulting from nicotine stimulation of neutrophils, which results in higher levels of oxidative stress to various tissues.

In our study a positive correlation with smoking was found for BOP, PI and GI. This finding confirm previous researches who has provided evidence that the periodontal destruction observed in smokers can be connected not only to the presence of bacterial plaque and of periodontal pathogenic species but also to a impaired neutrophil chemotaxis and migration in the periodontium, contributing to increase the periodontal disease susceptibility in smokers. Cigarette components generate a thicker layer of bacterial plaque, which appears to be less easily removed by patients, and maintain a chronic level of inflammation of gingival tissues.^{4–27}

Total proteins did not differ among the different groups considered in this study. However, when each protein was considered separately, sIgA, peroxidase and collagenase differed according to periodontal status and/or smoking status. Differences in concentration of certain components of total saliva therefore reflect differences in periodontal health and tobacco use. The small sample is an important limitation of this study. Therefore, further research with a larger sample is necessary to confirm the results observed in the study.

5. Conclusion

In comparison to healthy subjects, all periodontal patients had a higher level of sIgA and a lower level of peroxidase in whole saliva. In smokers, periodontal patients had a higher level of sIgA than controls, and in non-smokers, periodontal patients had a lower peroxidase level than controls.

Smoker control subjects and smoker periodontal patients had higher salivary collagenase activity than both groups of non-smokers.

Smokers and periodontal patients had greater bacterial plaque, gingival inflammation and BOP than non-smokers.

Conflicts of interest

The authors have none to declare.

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References

1. Savage A, Eaton K, Moles D, Needleman I. A systematic review of definitions of periodontitis and methods that have been used to identify this disease. *J Clin Periodontol*. 2009;36:458–467.
2. Armitage GC. Development of a classification system for periodontal disease and conditions. *Ann Periodontol*. 1999;4:1–6.
3. Giannopoulou C, Kamma JJ, Mombelli A. Effect of inflammation, smoking and stress on gingival crevicular fluid cytokine level. *J Clin Periodontol*. 2003;30:145–153.
4. Giuca MR, Pasini M, Tecco S, Giuca G, Marzo G. Levels of salivary immunoglobulins and periodontal evaluation in smoking patients. *BMC Immunol*. 2014;15:25.
5. Reznick AZ, Klein I, Eiserich JP, Cross CE, Nagler RM. Inhibition of oral peroxidase activity by cigarette smoke: *in vivo* and *in vitro* studies. *Free Radic Biol Med*. 2003;34:377–384.
6. Schätzle M, Loe H, Ramseier C, et al. Clinical course of chronic periodontitis: effect of lifelong light smoking (20 years) on loss of attachment and teeth. *J Invest Clin Dent*. 2010;1:8–15.
7. Budunelli N, Kinane DF. Host derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *J Clin Periodontol*. 2011;38:85–105.
8. Miller CS, King Jr CP, Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing periodontal disease: a cross-sectional study. *J Am Dent Assoc*. 2006;137:322–329.
9. Koss MA, Castro CE, Salúm KM, López ME. Changes in saliva protein composition of patients with periodontal disease. *Acta Odontol Latinoam*. 2009;22(2):105–115.
10. Koss MA, Castro CE, Salúm KM, Kishimoto E, Takagi S, López ME. Gingival crevicular fluid biomarkers in patients with gingivitis and chronic periodontitis. *J Hard Tissue Biol*. 2010;19(2):111–116.
11. Castro CE, Koss MA, López ME. Intracytoplasmic enzymes in gingival crevicular fluid of patients with aggressive periodontitis. *J Periodontol Res*. 2011;46:522–527.
12. Lindhe J. Examination of patients with periodontal disease. In: Harring, Lang, eds. *Clinical Periodontology and Implant Dentistry*. Copenhagen: Munksgaard; 1998:383–395.
13. Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand*. 1963;21:533–551.
14. Silness J, Loe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal conditions. *Acta Odontol Scand*. 1964;22:121–135.
15. Castro CE, Manlla AM, López ME. Periodontal disease: association with smoking habit and systemic diseases. *Clin Int J Braz Dent*. 2013;9:100–105.
16. Lowry OH. Protein measurement with the folin phenol reagent. *J Biol Chem*. 1951;193:265–275.
17. Mansson-Rahemtulla B, Baldone DC, Pruitt KM, Rahemtulla F. Specific assays for peroxidase in human saliva. *Arch Oral Biol*. 1986;31:661–668.
18. Ingman T, Sorsa T, Lindy O, Koski H, Konttinen YT. Multiple forms of gelatinases/type IV collagenases in saliva and gingival fluid of periodontitis patients. *J Clin Periodontol*. 1994;21:26–31.
19. Ebersole JL, Steffen MJ, Thomas MV, Al Sabbagh M. Smoking-related cotinine levels and host responses in chronic periodontitis. *J Periodontol Res*. 2014;49:642–651.
20. Olayanju OA, Rahamon SK, Joseph IO, Arinola OG. Salivary immunoglobulin classes in Nigerians smokers with periodontitis. *World J Biol Chem*. 2012;26:180–183.
21. Hägwald SJ, Fishel DL, Christan CE, Bernimoulin JP, Kage A. Salivary IgA in response to periodontal treatment. *Eur J Oral Sci*. 2003;111:203–208.
22. Gio N, Hirai Y, Harada H, Ikari A, Ono T, Kinane N. Comparison of peroxidase response to mental arithmetic stress in saliva of smokers and non-smokers. *J Toxicol Sci*. 2007;32:121–127.
23. Mäkelä M, Salo T, Uitto VJ, Larjava H. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. *J Dent Res*. 1994;73:1397–1406.
24. Liu KZ, Hynes A, Man A, Alsagheer A, Singer DL, Scott DL. Increased local matrix metalloproteinase-8 expression in the periodontal connective tissues of smokers with periodontal disease. *Biochim Biophys Acta*. 2006;1762:775–780.
25. Saxén L, Tenovu J, Vilja P. Salivary defence mechanisms in juvenile periodontitis. *Acta Odontol Scand*. 1990;48:399–407.
26. Rudolf TK, Rudolf V, Baldus S. Contribution of myeloperoxidase to smoking-dependent vascular inflammation. *Proc Am Thorac Soc*. 2008;5:820–823.
27. Tymkiw KD, Thunell DH, Johnson GK, et al. Influence of smoking on gingival crevicular fluid cytokines in severe chronic periodontitis. *J Clin Periodontol*. 2011;38:219–228.