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Increased micronuclei and nuclear abnormalities in buccal mucosa and oxidative damage in saliva from patients with chronic and aggressive periodontal diseases

A. L. Zamora-Perez[1], Y. M. Ortiz-García[1], B. P. Lazalde-Ramos[2], C. Guerrero-Velázquez[1], B. C. Gómez-Meda[3], M. Á. Ramírez-Aguilar[4], G. M. Zúñiga-González[5]

[1]Instituto de Investigación en Odontología, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Jalisco, México, [2]Unidad Académica de Ciencias Químicas, Universidad Autónoma de Zacatecas, Zacatecas, México, [3]Instituto de Biología Molecular en Medicina y Terapia Génica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Jalisco, México, [4]Especialidad en Periodoncia, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Jalisco, México and [5]Laboratorio de Mutagénesis, Centro de Investigación Biomédica de Occidente, Instituto Mexicano del Seguro Social, Guadalajara, Jalisco, México

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Background and Objective: Periodontal disease is a chronic bacterial infection characterized by connective tissue breakdown and alveolar bone destruction because of inflammatory and immune response caused by periodontopathogens and long-term release of reactive oxygen species. A high number of reactive oxygen species result in periodontal tissue damage through multiple mechanisms such as lipid peroxidation, protein denaturation and DNA damage. The aim of this study was to evaluate DNA and oxidative damage in subjects with chronic or aggressive periodontitis and healthy controls.

Material and Methods: Buccal mucosa cells and whole saliva were collected from 160 subjects, who were divided into three groups: subjects with chronic periodontitis (CP) (n = 58), subjects with aggressive periodontitis (AgP) (n = 42) and a control group (n = 60). DNA damage was determined by counting micronuclei (MN) and nuclear abnormalities (NAs) in exfoliated cells, including binucleated cells, cells with nuclear buds and karyolitic, karyorrhectic, condensed chromatin and pyknotic cells. The degree of oxidative stress was determined by quantifying 8-hydroxy-2′-deoxyguanosine (8-OHdG) in whole saliva.

Results: Subjects with CP or AgP presented significantly more ($p < 0.05$) MN and NAs and higher levels of 8-OHdG ($p < 0.05$) compared with the control group.

Conclusion: Our results indicate that subjects with periodontitis (CP or AgP) exhibited an increase in the frequency of MN, NAs and 8-OHdG, which is directly related to DNA damage. In addition, a positive correlation exists between oxidative stress produced by periodontitis disease and MN.

Dra en C. Ana Lourdes Zamora-Perez, PhD, José María Echauri y Juan Díaz Covarrubias s/n, Col. Independencia, Guadalajara, Jalisco C.P. 44340, México
Tel: 01 (33) 10585200, ext. 33747
Fax: 01 (33) 36171019
e-mail: anazamora@gmail.com

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Introduction

Periodontal diseases is one of the most widespread oral diseases, which includes the inflammatory disorder of periodontitis characterized by gingival bleeding, periodontal pocket formation, destruction of connective tissue attachment, and alveolar bone resorption eventually leading to tooth loss (1). Periodontitis is initiated by the complex interaction between the presence of microorganisms, such as *Porphyromonas gingivalis*, *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans*, in the dental plaque that forms adjacent to the teeth, and the host (2–4). These pathogens are associated with the progressive form of the disease (4–7).

Two main forms have been identified, chronic (CP) and aggressive (AgP) periodontitis, and are characterized by gingival bleeding, periodontal pocket formation, destruction of connective tissue attachment and alveolar bone resorption eventually leading to tooth loss. However, destruction of connective tissue attachment is faster in AgP than in chronic periodontitis (2,4,8,9).

Increasing evidence indicates that, in periodontitis, reactive oxygen species (ROS) derived predominantly from neutrophils are implicated in the destruction of periodontal tissue; as a result, oxidative stress is increased during periodontitis (10–13). ROS such as superoxide anion, hydroxyl radical, nitrous oxide and hydrogen peroxides (14) are produced through the bacteria–host interaction-mediated pathway, stimulating polymorphonuclear leukocytes to produce superoxide radicals through respiratory burst (11). This action results in an increased ROS concentration, leading to oxidative damage to the periodontal tissues with an impaired circulating antioxidant/oxidant balance (15). Oxidative stress can result in DNA damage, including the oxidation of nucleosides, which could cause DNA strand breaks (16,17); this type of damage could have teratogenic or carcinogenic consequences (18). One method for determining DNA damage is the micronuclei (MN) assay (19–21).

MN originate from chromosome fragments or whole chromosomes that lag behind in anaphase during nuclear division (22). The evaluation of MN detects both clastogenic and aneugenic events (23). The presence of MN is an indicator of the effect of mutagenic or genotoxic agents, especially micronucleogenic compounds (19–21,23,24). The MN can be easily assessed in exfoliated epithelial cells such as human oral mucosa cells (19,21,24). The MN and nuclear abnormalities (NAs) observed in exfoliated cells of the oral mucosa reflect chromosomal aberrations that are formed in the proliferating basal layer of the epithelium and subsequently migrate to the surface of the epithelium (26). The buccal exfoliated epithelial cells constitute biomarkers of genotoxicity, allowing the use of a simple diagnostic assay and the response of target tissue to the action of carcinogens to avoid the need for cell culture (25,26). Therefore, the buccal MN cytome assay is a minimally invasive, useful *in vivo* method for monitoring genetic damage in human subjects (19–21,25,28).

Several studies have indicated that 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in body fluids are biomarkers of oxidative stress (29–32); 8-OHdG is the most common stable product of oxidative DNA damage caused by ROS (32) and has been shown to be increased in body fluids and tissues in many diseases, including diabetes mellitus (33), cancer (34), rheumatoid arthritis (35) and, recently, periodontitis (11,12). Oral health is an important aspect of the overall health status of an individual, and several reports associate oral health and dental factors with DNA damage (36–38) because of the increased oxidative stress (12). The aim of this study was to determine the amount of DNA damage by means of a buccal MN cytome assay and the amount of oxidative DNA damage by quantifying 8-OHdG in whole saliva from patients with CP and AgP and to determine whether there is a correlation between DNA damage and oxidative stress levels.

Material and methods

Study population

The study was carried out in 160 subjects who were selected from individuals who attended the Periodontal Clinic of the Dentistry School of the University of Guadalajara because of periodontal problems or for routine dental checkups. The study groups were divided as follows: clinically healthy periodontia ($n = 60$), CP ($n = 58$) and AgP ($n = 42$).

All participants had similar socioeconomic backgrounds. Those who agreed to participate in the study signed a letter of informed consent and answered a questionnaire about personal information concerning smoking habits, alcohol consumption, illness, gender, age, alimentary habits and consumption of drugs or antioxidants. All participants were in generally good health and had not received therapy for periodontal disease. Pregnant women, alcohol consumers, smokers, individuals with any systemic disease, or those who were on long-term medications that could be confounding factors were excluded from the study. Exclusion criteria also included subjects with severe diseases, such as rheumatic diseases, diabetes, chronic liver diseases and cancer, as well as those who had recently undergone radiological procedures (< 1 mo). This study was approved by the medical ethics review committee at the Universidad de Guadalajara, Guadalajara, Jalisco, México (Register number CI-7908), and all subjects provided their written approval before participating, according to the Mexican General Health Law and the NOM-008-SSA2-1993 norm.

Clinical periodontal measurements

Clinical examinations were performed on all existing teeth of the participants, and periodontal status was assessed by measuring the sites with plaque (SP) (4,39), bleeding on probing (BOP), probing depth and clinical attachment level. The examination was performed using a periodontal

probe (Hu-friedy, Chicago, IL, USA), and the mean sulcular depth was calculated (4). Clinical parameters were obtained on six sites per tooth with the exception of clinical attachment level, for which four sites were examined, and the results were expressed as the percentage of sites (SP and BOP) and as means ± SD (for probing depth and clinical attachment level) (4). All clinical examinations were carried out by a single investigator from the periodontology department.

Study groups

Dental and medical records were obtained for all participants and a periodontal examination was performed. Based on this information and according to the clinical and radiographic classification of the American Academy of Periodontology (2) the periodontal diagnosis was made.

Healthy periodontia or control group— Special care was taken to select the controls among the subjects who had healthy periodontal status and oral hygiene. This group included 38 women and 22 men (mean age 40.13) with no history of any periodontal disease, no clinical signs of gingival inflammation, good oral health and healthy dental status, no evidence of clinical attachment loss, sulcular bleeding or radiographic evidence of bone loss. The gingival tissue of this group was defined as clinically healthy when the mean sulcular depth was ≤ 3 mm and there was no evidence of BOP at any surface (4,40,41).

Chronic periodontitis group— This group included 38 women and 20 men (mean age 42.21) with inflammation in the gingival, dental plaque formation and a probing depth of ≥ 6 mm in at least six sites and a clinical attachment level of ≥ 5 mm.

Aggressive periodontitis group— This group included 26 women and 16 men (mean age 35.85). Subjects with AgP presented severe damage and a clinical attachment level of ≥ 5 mm on eight or more teeth, at least three of which were not central incisors or first molars, and presented at least two sites showing a probing depth of ≥ 5 mm and familial aggregation; i.e. at least one other family member either presenting or with a history of periodontal disease.

In both periodontitis groups, gingiva with redness or marked redness, edema, glazing, BOP or spontaneous bleeding was considered inflamed (4,40).

Sample preparation and analysis

Samples of oral mucosa cells and saliva were taken from all participants.

Buccal micronuclei cytome assay

Subjects were asked to rinse their mouths with water, and a slide was used to collect cells from the oral mucosa of the right and left cheeks. Samples were spread directly on to two separate pre-cleaned and pre-coded slides (28). Smears were air-dried and fixed with 80% methanol for 48 h and then stained with acridine orange (CAS no. 10127023; Sigma-Aldrich, St. Louis, MO, USA). Pre-coded slides were examined by one reader, who blindly counted the MN and NAs, including binucleated cells, cells with nuclear buds and karyolitic, karyorrhectic, condensed chromatin and pyknotic cells. The criteria used for scoring MN and NAs were according to those described by Thomas *et al.* (27), and the number of cells with MN and NAs were evaluated among 2000 cells using an Olympus CX31 microscope equipped with epifluorescence and oil immersion objectives (×60 and ×100; Olympus, Tokyo, Japan). The results are presented as the number of cells with MN or NAs per 1000 cells.

Salivary 8-hydroxy-2′-deoxyguanosine determination

Whole, non-stimulated saliva samples were collected in the morning under resting conditions in a quiet room. The saliva collection was performed at least 30 min after food or liquid ingestion. Participants' mouths were rinsed with water and 2 mL of whole saliva was collected in disposable tubes and the collection time was approximately 5 min. Samples were stored at −80°C until analysis. A single freeze process was performed.

A competitive ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA) was used for determination of 8-OHdG in the saliva samples. Saliva samples were centrifuged at 10,000 ***g*** for 10 min, and determination of 8-OHdG levels was conducted following the kit manufacturer's protocol: DNA damage ELISA uses an 8-OHdG monoclonal antibody to competitively bind 8-OHdG in the sample, standard or 8-OHdG pre-bound to the wells of a 96-well immunoassay plate. Anti-8-OHdG bound to 8-OHdG in the sample or standard were washed away, whereas those captured by the immobilized 8-OHdG were detected using a horseradish peroxidase-conjugated secondary antibody. The assay was developed with a tetramethylbenzidine substrate, and the absorbance was measured in a microplate reader at 450 nm. The intensity of the yellow color is inversely proportional to the concentration of 8-OHdG.

Statistical analysis

The results are expressed as means ± SD. The chi-squared test was used to compare the SP and BOP of the study groups. All data were tested for normality using the Kolmogorov–Smirnov test. Differences in MN and NA values were evaluated using Mann–Whitney's *U* test for intergroup comparison. One-way ANOVA followed by a *post hoc* Bonferroni test was used to compare 8-OHdG, probing depth and clinical attachment level within groups. A Spearman correlation was performed to test the relationship between MN and 8-OHdG. All tests were performed using the Statistical Program for Social Sciences (SPSS v11.0) for Windows (SPSS, Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Variables such as age, gender and clinical data for all participants are

described in Table 1. The ages of the participants in the different groups were similar. Neither participant age nor gender was used as a stratification criterion because there were no differences among the three groups.

In terms of clinical data, participants with CP and AgP showed a high percentage of SP and BOP compared with the control group. The probing depth values were significantly higher in the CP ($p = 0.001$) and AgP groups ($p = 0.001$) compared with the control group, and the AgP group showed significantly higher probing depth values ($p = 0.02$) than the CP. The clinical attachment level values were higher in the CP ($p = 0.001$) and AgP groups ($p = 0.001$) compared with the control group, but no difference was observed between the CP and AgP groups (Table 1).

DNA damage was evaluated by studying the MN and NAs in oral mucosa cells. DNA damage markers (MN, nuclear bud and binucleated cells) are shown in Fig. 1 and cytotoxic or cell death markers (karyolitic, karyorrhectic, condensed chromatin and pyknotic cells taken from participants in the study) are shown in Fig. 2.

The frequencies of cells with MN, cells with nuclear buds and binucleated cells (DNA damage markers), karyolitic, karyorrhectic and condensed chromatin cells (cytotoxicity/cell death markers) and the statistical evaluation of their frequencies in the different groups are all shown in Table 2.

Intergroup comparisons show that the frequencies of MN ($p = 0.001$), cells with nuclear buds ($p = 0.001$), binucleated cells (chronic periodontitis: $p = 0.001$; AgP: $p = 0.02$), condensed chromatin ($p = 0.001$) and karyorrhectic cells ($p = 0.001$) were significantly higher in the CP and AgP groups compared with the control group (Table 2). In addition, the numbers of MN ($p = 0.001$), cells with nuclear buds ($p = 0.001$), binucleated cells ($p = 0.001$) and condensed chromatin cells ($p = 0.03$) were significantly higher in the AgP group compared with the CP group (Table 2).

Oxidative DNA damage was determined using the 8-OHdG enzyme in whole saliva samples from subjects with and without periodontitis. In the overall periodontitis group (without taking into consideration the severity of the clinical features), subjects presented significantly increased ($p = 0.001$) 8-OHdG levels compared with the control group (Table 2). Moreover, when we compared 8-OHdG levels according to the severity of periodontal disease, CP ($p = 0.001$) and AgP ($p = 0.001$) groups presented significantly increased 8-OH-dG levels compared with the control group, and in the AgP group, this value was significantly ($p = 0.001$) higher than in the CP group (Table 2).

We searched for a correlation between MN and 8-OHdG enzyme levels in whole saliva from subjects with CP and AgP. Statistically significant positive correlations ($p = 0.001$) were observed between MN and 8-OHdG enzyme levels in the CP group with a confidence level of 95%. The fitted model explains 23.52% of the variability in MN, with a correlation coefficient of 0.48 (Fig. 3A). Furthermore, a positive correlation ($p = 0.003$) was found between the MN and 8-OHdG levels in the AgP group with a confidence level of 95%. The fitted model explains 27.99% of the variability in MN, with a correlation coefficient of 0.52 (Fig. 3B).

Discussion

DNA damage can produce a wide variety of effects on human health (18). Several of these effects could appear immediately, whereas others do not become evident until much later. Several chronic diseases have been studied to understand their mechanisms of perpetuation of clinical complications and the development of secondary diseases such as cancer (31). Oxidative stress and, therefore, DNA damage has an important impact on the pathogenesis of chronic disease (18).

Periodontal diseases are inflammatory disorders that are the result of the interactions between periodontopathogens and the host defense immune

Table 1. General profile and clinical measurements of subjects from the study

	Groups					
		Periodontitis		*p*-value		
Variables	Control	CP	AgP	Control vs. CP	Control vs. AgP	CP vs. AgP
n	60	58	42			
Age (years)	40.13 ± 8.41	42.21 ± 10.09	35.85 ± 6.89	NS	NS	NS
Gender						
Female (%)	38 (63)	38 (66)	26 (62)	NS	NS	NS
Male (%)	22 (37)	20 (20)	16 (38)			
SP (%)	0	100	100	$p = 0.001$	$p = 0.001$	NS
BOP (%)	0	100	100	$p = 0.001$	$p = 0.001$	NS
PD (mm)	1.89 ± 0.60	4.23 ± 0.90	5.78 ± 0.70	$p = 0.001$	$p = 0.001$	$p = 0.02$
CAL (mm)	0.50 ± 0.40	5.12 ± 1.50	5.45 ± 1.90	$p = 0.001$	$p = 0.001$	NS

AgP, aggressive periodontitis; BOP, bleeding on probing; CAL, clinical attachment level; CP, chronic periodontitis; *n*, sample size; PD, probing depth; SP, sites with plaque. Gender, SP and BOP data are expressed as percentages. In the case of periodontitis groups, all sites in all teeth were bleeding. Age, PD and CAL values are expressed as mean ± SD. Chi-squared test was used to compare gender, SP and BOP. One-way ANOVA followed by *post hoc* Bonferroni test was used to compare age, PD and CAL.

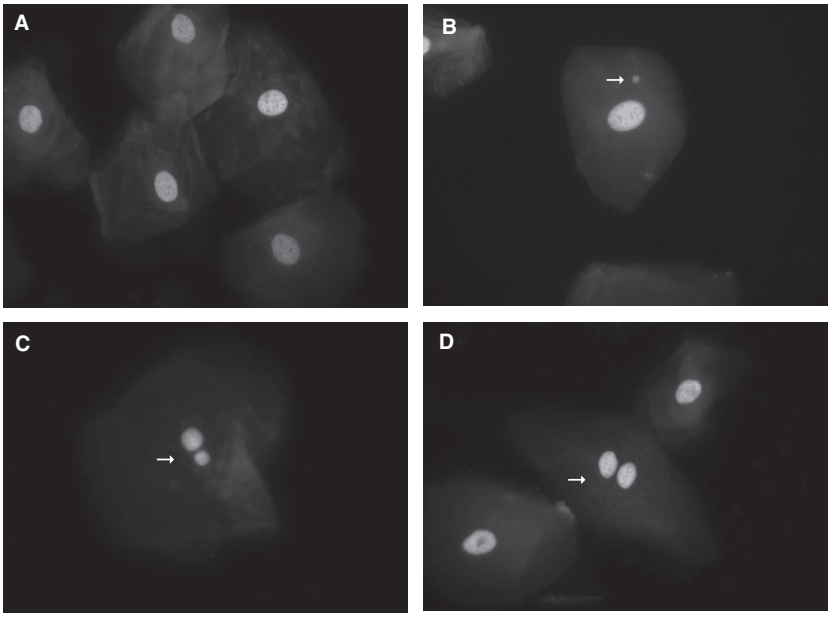

Fig. 1. DNA damage or genotoxicity marker from buccal mucosa cells evaluated in the present study: (A) normal cells; (B) micronuclei; (C) nuclear bud; and (D) binucleated cells (oil-immersion objective 60x, acridine orange stain).

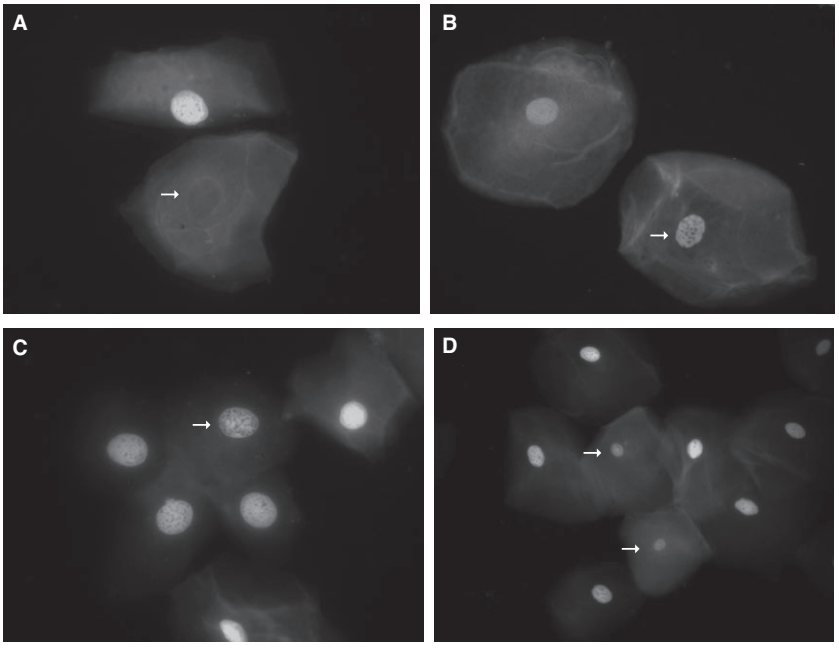

Fig. 2. Cell death or cytotoxicity markers from buccal mucosa cells evaluated in the present study: (A) karyolytic; (B) karyorrhectic; (C) condensed chromatin; and (D) pyknotic (oil-immersion objective 60x, acridine orange stain).

system and environmental factors, which results in uncontrolled immune response (1,42). Activation of the immune system, and the production of oxygen radicals and their related metabolites that occur in periodontitis, contributes to increased ROS production and oxidative stress, which is reported to be involved in many diseases (11). Among the ROS formed, H_2O_2 and OH radicals are potent oxidants that can affect nucleic acids and modify bases in the DNA, contributing to an increase in DNA damage (32) and therefore MN formation.

This study was designed to evaluate DNA damage by measuring the increase in MN and NAs in buccal mucosa cells and oxidative damage by quantifying the level of 8-OHdG in whole saliva from subjects with CP or AgP or from a control group. We also determined whether there is a correlation between DNA damage and oxidative stress levels.

Our results indicate that individuals with periodontal disease (CP or AgP) presented greater DNA damage than control individuals. In the CP group, the MN frequency and the number of cells with nuclear buds were 2.4-fold higher than in the control group, and the frequency of binucleated cells was 2.6-fold higher (Table 2). In the AgP group, the MN frequency was 3.8-fold higher, cells with nuclear buds were 2.9-fold higher, and the frequency of binucleated cells was 2.8-fold higher compared with the control group (Table 2), indicating that individuals with periodontal disease had a greater amount of DNA damage than the spontaneous levels of damage that occur in individuals without periodontitis. Here we show that periodontal disease could be a risk factor for DNA damage, and it will be a modifiable risk factor as periodontitis can be prevented and treated.

The cytotoxicity parameters were also significantly higher in the CP or AgP groups compared with those observed in the control group. In the CP group, condensed chromatin cells increased 2.3-fold and karyorrhectic cells increased 2.04-fold (Table 2), and in the AgP group condensed chromatin cells increased 3.1-fold and karyorrhectic cells increased 2.2-fold (Table 2). According to our study, the DNA damage markers and cytotoxicity parameters were also significantly higher in the AgP group compared with those observed in the CP group, the MN frequency increased 1.5-fold, cells with nuclear buds increased 1.2-fold, the frequency of binucleated cells increased 1.4-fold and condensed chromatin cells increased 1.3-fold (Table 2). Under our work conditions,

Table 2. Periodontal disease effect on frequencies of micronuclei and other nuclear abnormalities and OHdG salivary levels

	Groups			
Nuclear abnormalities	Control (n = 60)	CP (n = 58)	AgP (n = 42)	p-value
Micronuclei	0.63 ± 0.44	1.54 ± 0.80	2.40 ± 0.71	0.001[a,b,c]
Cells with nuclear buds	2.79 ± 0.82	6.73 ± 1.95	8.35 ± 2.04	0.001[a,b,c]
Binucleated cells	1.05 ± 0.71	2.08 ± 0.88	2.98 ± 0.91	0.001[a,c]/0.02[b]
Condensed chromatin cells	1.68 ± 1.56	3.96 ± 0.98	5.36 ± 1.49	0.001[a,b]/0.03[c]
Karyolitic cells	0.09 ± 0.23	0.13 ± 0.26	0.27 ± 0.53	NS
Karyorrhectic cells	0.73 ± 0.66	1.49 ± 0.92	1.63 ± 0.73	0.001[a,b]
Pyknotic cells	0.17 ± 0.33	0.22 ± 0.36	0.13 ± 0.27	NS

	Groups			
	Control (n = 60)	Periodontitis (n = 100)		p-value
8-OHdG (ng/mL)	5.10 ± 1.44	11.13 ± 1.91		p = 0.001[d]
	Control (n = 60	CP (n = 58)	AgP (n = 42)	p-value
8-OHdG (ng/mL)	5.10 ± 1.44	10.31 ± 1.51	12.13 ± 1.26	p = 0.001[e,f] p = 0.02[g]

8-OHdG, 8-hydroxy-2′-deoxyguanosine; AgP, aggressive periodontitis; CP, chronic periodontitis; n, sample size; NS, not significant. Data are expressed as mean ± SD. Results are presented as the number of cells with micronuclei or nuclear abnormalities per 1000 cells. Differences in MN and NA values were evaluated using Mann-Whitney's U test for intergroup comparison (control vs. CP[a], control vs. AgP[b] and CP vs. AP[c]). Student's t-test was used to compare 8-OHdG between the control and periodontitis[d] groupand one-way ANOVA followed by Bonferroni *post hoc* was used to compare 8-OHdG levels between control group vs. CP[e] and AgP[f] groups and AgP vs. CP[g] groups.

these findings could suggest that periodontitis in the aggressive form could be related to the increase in both DNA damage and cytotoxicity. Our results agree with those reported by Bastos-Aires *et al*. (38) who performed a preliminary study of MN levels in oral exfoliated cells from 15 patients with periodontitis using 15 control subjects. Their results showed a significant increase in MN frequency in patients with periodontitis (2.3-fold) relative to the control group, and the researchers concluded that the periodontal status might affect the MN reference level. Furthermore, Bloching *et al*. (36), suggested a possible association between periodontal status and increase in MN number in the buccal mucosa. However, D'Agostini *et al*. evaluate the induction of MN and binucleated cells in the gingival epithelium, as related to smoking habits and the occurrence of periodontal disease (43). Their results did not show any significant association between cytogenetic damage in gingival cells, smoking habits and gingivitis or periodontal disease. However, in this study, the lack of induction of MN and binucleated cells might be explained by taking in to account the sampled size was small compared with our study and another concern could be that cells from the gingival epithelial were collected from the gingival sulcus, which is a part of the epithelia that could be less exposed compared with cheek, as it is known that levels of MN and NAs can differ in oral cavity epithelium (44). Therefore, scoring the full spectrum of cytological and NAs is important in addition to MN for the diagnosis of diseases or pathological changes that may lead to the development of cancer.

Salivary 8-OHdG levels have been intensively studied in several oral pathologies, including patients with periodontitis (10,45–48) and oral cancer (34). Oxidative stress due to the formation of ROS, which is stimulated by neutrophils, causes periodontal tissue damage (11,48). Increased ROS formation, reduction of antioxidant enzyme levels and defects in the DNA restoration mechanism led to increased oxidative DNA damage (49,50). The results obtained for oxidative DNA damage determined by 8-OHdG enzyme levels in whole saliva samples from subjects with and without periodontitis indicate that individuals with periodontitis presented 2.18-fold higher levels of 8-OHdG enzyme compared with the control group. When individuals with periodontitis were divided according to disease severity into CP and AgP groups, the AgP group presented a significant increase of 8-OHdG enzyme levels compared with the CP and control groups. Our results are consistent with those reported in numerous studies that have demonstrated an increase in salivary 8-OHdG levels in patients with periodontal disease compared with healthy periodontal subjects (10,45–48). Furthermore, several authors have suggested that elevated levels of 8–OHdG may be a marker for disease activity and may indirectly reflect disease severity parameters such as clinical attachment level (11,13,51).

In the present work, we report that individuals manifesting the most advanced periodontal disease (AgP group) exhibit the highest levels of salivary 8-OHdG and MN compared with control groups. These data are consistent with some works who report that in aggressive forms of periodontitis, polymorphonuclear leukocytes appear to be functionally activated and exhibit increased production of ROS that could destroy pathogens and damage host tissues in the surrounding area (11,52,53).

Moreover, a correlation between the results obtained for DNA damage and oxidative stress was performed to test whether the increase in free radicals produced in periodontal disease was the cause of DNA damage in these patients (33).

We found a significant positive correlation between MN and 8-OHdG enzyme levels in the CP and AgP groups, indicating that these variables are associated and leave open the possibility that they could be linked to the progression of periodontal diseases (21,32,33). Canakçi *et al*. (47) reported that the increased 8-OHdG levels might signify premature

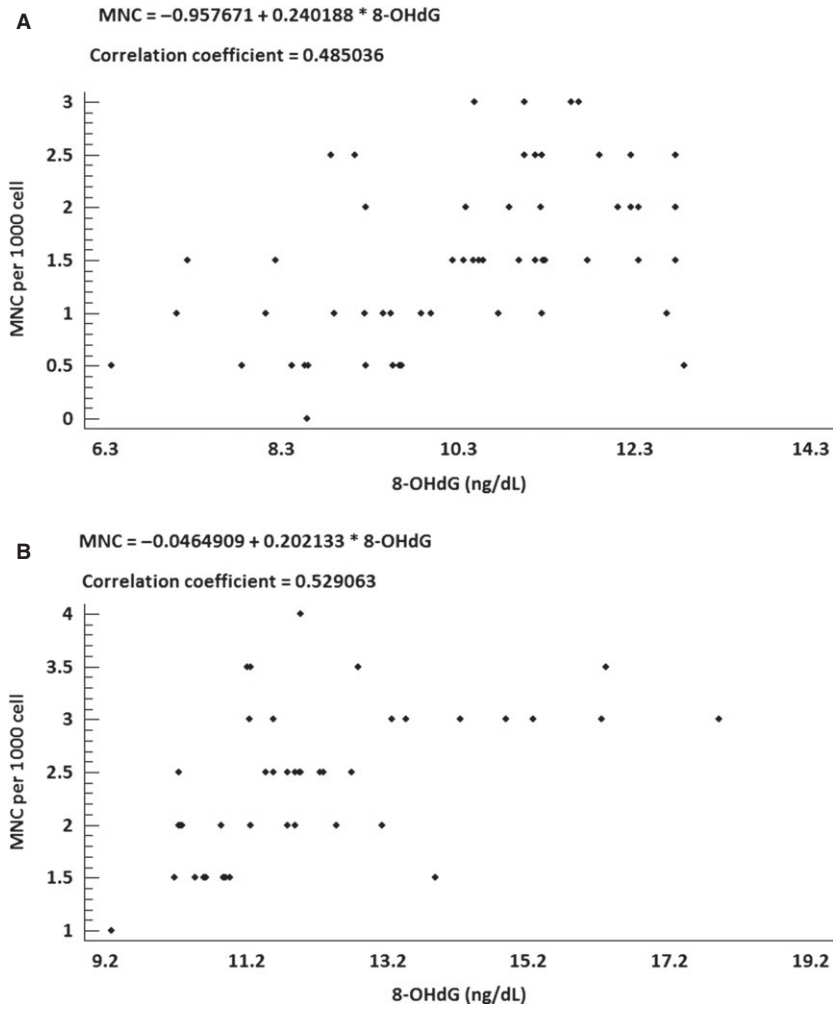

Fig. 3. Spearman's correlation coefficient between 8-OHdG and MN in the chronic (A) and aggressive periodontitis (B) groups. The scatter plot of the correlations between 8-OHdG and MN shows the relationships between of DNA oxidative damage determined by quantifying for the 8-OHdG and the number of MN from oral mucosa cells in: (A) the chronic periodontitis group (Spearman's correlation coefficient $r = 0.048$, $p = 0.001$), and (B) the aggressive periodontitis group (Spearman's correlation coefficient $r = 0.529063$, $p = 0.52$). 8-OHdG, 8-hydroxy-2′-deoxyguanosine; MNC, micronucleated cells.

oxidative mitochondrial DNA damage. However, the results of Nomoto *et al*. (54) indicate that mitochondrial DNA undergoes oxidative stress earlier than nuclear DNA.

The significant positive correlation between MN (DNA damage biomarker related to nuclear damage) and the 8-OHdG enzyme levels observed here in the CP and AgP groups are related to more intense DNA and oxidative damage that occurs during the development of periodontitis and could be used as a parameter of disease severity. In addition, 8-OHdG enzyme levels obtained in the present study from subjects with periodontitis are higher compared with those reported previously in the literature (13,45–47). This may tentatively be attributed to the: use of whole nonstimulated saliva in comparison to the analysis of stimulated saliva; exclusion of smoking subjects, as some studies report that 8-OHdG levels in urine and lymphocyte are affected by smoking and age (48); and classification of periodontal status and our population was larger than those reported (13,45–47).

Oxidative damage to genetic material is the result of the interaction of DNA with ROS, in particular radical $OH^{\cdot}$ (32). This oxidative damage leads to increasing MN and NAs number caused by DNA strand breaks and increased 8-OH-dG levels, which is a product of DNA oxidation (33).

Periodontal diseases are widespread in human populations and represent a significant public health problem (36). Periodontitis includes chronic and aggressive forms, often resulting in severe alteration of the periodontal tissues and tooth loss (1–3) and oxidative damage plays a fundamental pathogenic role (11,45–48). Here, we identified an increase in MN and NAs numbers in buccal mucosa cells and 8-OHdG in whole saliva from patients with periodontal disease (CP or AgP). DNA damage is a critical event not only in the initiation phase but also in the promotion and progression phases, which could be related to carcinogenesis events. Moreover, recent studies have associated periodontitis with head and neck cancer (55), pancreatic cancer (56), colon cancer (57) and orodigestive cancers (59), which are relevant to the control of this disease and to promote the importance of good oral health.

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