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Expression of IL-1β, IL-6, TNF-α, and iNOS in pregnant women with periodontal disease

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ABSTRACT. Periodontal disease is one of the most prevalent oral diseases. An association between this disease and pregnancy has been suggested, but available findings are controversial. We evaluated the expression levels of interleukins (IL-1β and IL-6), tumor necrosis factor-alpha (TNF-α), and inducible nitric oxide synthase (iNOS) in pregnant women with and without periodontal disease in comparison with non-pregnant women with and without periodontal disease since studies have suggested a relationship between periodontitis and the expression levels of these genes. The women in the sample were distributed into four groups: pregnant and non-pregnant women, with

or without periodontal disease, a total of 32 women. The periodontal condition was evaluated according to the probing depth, clinical attachment level and bleeding on probing. Analysis of gene expression was performed by real-time PCR. Comparisons were made of the level of gene expression among the four groups. Expression of IL-1β in the non-pregnant women with periodontal disease was 12.6 times higher than in the non-pregnant women without periodontal disease ($P < 0.01$), while expression of TNF-α in the non-pregnant women without periodontal disease was 3.5 times higher than in the pregnant women with periodontal disease ($P < 0.05$). Despite these differences, our overall findings indicate no differences in the expression levels of the cytokines *IL-1β*, *IL-6*, *TNF-α*, and *iNOS* in pregnant women with and without periodontal disease in comparison with expression of the same genes in non-pregnant women with and without periodontal disease, suggesting that periodontal disease is not influenced by pregnancy.

Key words: Cytokines; Gingiva; Real-time polymerase chain reaction; Periodontitis

INTRODUCTION

Periodontal disease is the second most common oral disease in the world and is an important public health problem. Depending on the diagnostic criteria, its prevalence in adults varies from 10 to 60% (Albandar and Rams, 2002). The disease affects the periodontal tissue and starts with colonization of the gingival tissue by bacteria from the accumulation of dental biofilm, or plaque (López et al., 2002). The activation of the immune system by periodontal pathogens results in the excessive production of pro-inflammatory cytokines, which play an important role in the onset and progression of the disease (Teng, 2006).

The periodontal health of pregnant women has been extensively studied during the last four decades, but the findings are controversial (Gürsoy et al., 2008). Previous studies of various ethnic populations have shown that oral tissues can be affected by pregnancy (Kornman and Loesche, 1980; Gürsoy et al., 2008). According to Laine (2002), pregnancy does not cause periodontal disease but can aggravate pre-existing conditions. The usual classification of periodontal disease states that gingivitis during pregnancy is induced by plaque and is affected by systemic factors (Armitage, 1999), with onset related to immunosuppression in the second trimester (Lopatin et al., 1980).

Various studies have suggested a relationship between the progression of periodontitis and the expression levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α), and nitric oxide synthase (iNOS) in gingival crevicular fluid, gingival tissue, and blood with regard to both the protein and messenger RNA (mRNA) levels (Kendall et al., 2000; Lappin et al., 2000; Bickel et al., 2001; Hirose et al., 2001; Ejeil et al., 2003; Faizuddin et al., 2003; Figuero et al., 2010; Carrillo-de-Albornoz et al., 2012). However, only a few human studies have investigated the association between increased gingival inflammation during pregnancy and local immune system changes (Figuero et al., 2010; Carrillo-de-Albornoz et al., 2012).

Therefore, the objective of this study was to identify and evaluate the expression levels

of the *IL-1β*, *IL-6*, *TNF-α*, and *iNOS* genes in pregnant women with and without periodontal disease compared with those in non-pregnant women with and without periodontal disease through real-time polymerase chain reaction (PCR) analysis of gingival tissue.

MATERIAL AND METHODS

This cross-sectional study was approved by the Human Research Ethics Committee of Juiz de Fora Federal University (process No. 263/2008), located in Juiz de Fora, Minas Gerais, Brazil. All participants signed an informed consent form. Data were collected between November 2009 and November 2010. Pregnant women were recruited during routine prenatal care visits from the Department of Women's Health of the Municipal Public Health Service of Juiz de Fora, and non-pregnant women were recruited from the Reception Department of the School of Dentistry of Juiz de Fora Federal University. Age, ethnicity, occupation, education, and economic situation were recorded for all participants. Economic classification relied on various household characteristics, such as type of dwelling, belongings (such as appliances, televisions, DVD players, and cars), and educational level of household members. This information was used to stratify and classify the economic situation into good, regular, and poor (Associação Brasileira de Empresas de Pesquisa - ABEP, 2003).

Sample population

Of 120 women initially examined, 32 were included in the study according to the inclusion/exclusion criteria. The women were distributed into four groups (N = 8) as follows: pregnant women with periodontal disease (PrD), pregnant women without periodontal disease (PrND), non-pregnant women with periodontal disease (N-PrD), and non-pregnant women without periodontal disease (N-PrND).

All subjects were in good general health, aged 18 to 40 years (Agueda et al., 2008), and had 15 or more teeth (Vettore et al., 2008) per the inclusion criteria. Among the pregnant women, only those in the second trimester (13-24 weeks of gestation) as confirmed by ultrasound examination were included.

Exclusion criteria were 1) diagnosis of diabetes mellitus, high blood pressure, or immunosuppression owing to systemic impairment; 2) receipt of orthodontic treatment, dental prophylaxis, or periodontal dental treatment within the previous six months; 3) smoking habit, alcohol or illegal drug intake, and 4) use of systemic anticonvulsants, anxiolytics, or antibiotics within the past 3 months. In addition, pregnant women with multiple gestations, diagnosis of malformed fetus, or history of miscarriage were excluded (Agueda et al., 2008; Vettore et al., 2008).

Periodontal examination

Periodontal condition was evaluated according to the following clinical parameters: probing depth (PD), clinical attachment level (CAL), presence of calculus, and bleeding on probing. The clinical periodontal measures were recorded at six sites for each tooth (mesiobuccal, mediobuccal, distobuccal, distolingual, mediolingual, and mesiolingual), with the exception of the third molars. A North Carolina periodontal probe (PCP-UNC 15; Hu-Friedy Manufacturing

Inc., Chicago, IL, USA) was used to make the periodontal measurements. Clinical parameters were assessed by a single-trained and -calibrated examiner (C.C.M.O.). Intra-examiner agreement showed Cohen's kappa coefficients of 1 for PD and 0.75 for CAL. Subjects in whom four or more teeth had one or more sites with a PD greater than or equal to 4 mm and a CAL greater than or equal to 3 mm at the same site were diagnosed with periodontal disease (López et al., 2002).

Collection of gingival tissue

Gingival tissue samples were collected from patients under local anesthesia using 2% lidocaine with adrenaline. An inverse bevel incision was made to obtain tissue from the lower part of the interdental papilla. The tissue samples included part of the epithelial pocket, conjunctive tissue, and granulation tissue (Pan et al., 2010). The samples were immediately immersed in 1.5 mL RNAlater® (Ambion, Austin, TX, USA), according to manufacturer instructions, for subsequent RNA extraction.

RNA extraction and gene expression analysis with real-time PCR

Total RNA from the gingival tissue samples was extracted using an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer instructions. To remove genomic DNA contamination, we carried out digestion with DNase I (RNase-free DNase Set; Qiagen). The quality of the RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA), and the concentrations were determined through spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA). The mean RNA integrity number of the samples was 7.04.

First-strand cDNA was synthesized using a SuperScript™ III First-Strand Synthesis System SuperMix (Invitrogen, Carlsbad, CA, USA). The mean concentrations of the samples were estimated with spectrophotometry and stored at -20°C until being subjected to real-time PCR, which was performed using SYBR Green® PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer instructions. The primers used to evaluate the gene expression are described in Table 1. Four target genes were analyzed (*IL-1β*, *IL-6*, *TNF-α*, and *iNOS*), and two endogenous controls [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and *β-actin* genes] were used to normalize the expression data.

Table 1. Sequences of the primers used in the real-time PCR.

Gene	Sequence (5'-3')	Amplicon (bp)	Reference or ID*
IL-1β	F: GCACGATGCACCTGTACGAT R: AGACATCACCAAGCTTTTTTGCT	69	BC008678.1
IL-6	F: CCAGGAGCCCAGCTATGAAC R: GAGCAGCCCCAGGGAGAA	70	AK301141.1
TNF-α	F: CAGAGGGAAGAGTTCCCCAG R: CCTTGGTCTGGTAGGAGACG	325	Hirose et al., 2001
iNOS	F: CCTCGGCTCCAGCATGTAC R: TGGGACAGCTTCTGATCAATG	66	NM_002046
GAPDH	F: GGTGGTCTCCTCTGACTTCAACA R: GTTGCTGTAGCCAAATTCGTTGT	127	Douglas et al., 2008
β-actin	F: TGACGGGGTCACCCACACTGTGCCCATCTA R: CTAGAAGCATTTGCGGTGGACGATGGAGGG	661	Hirose et al., 2001

*ID in the GenBank (http://www.ncbi.nlm.nih.gov/).

Before real-time quantification, the reactions for all the genes were optimized. To do so, we tested three concentrations of cDNA (100, 200, and 400 ng/reaction) along with three primer dilutions (50, 100, and 200 nM). After determining the best conditions, we constructed a standard curve for each gene in which the serial cDNA dilutions were plotted against their respective cycle thresholds to obtain the efficiency of each reaction.

The amplification efficiency for each gene is shown in Table 2. No peaks were observed that referred to the primer dimers or unspecific products in the dissociation curve (data not shown). The coefficient of variation of the duplicates of the cycle thresholds did not exceed 5% (data not shown).

Table 2. Primer and cDNA concentration, reaction efficiency and melting temperature (Tm) of the amplified fragment for each gene.

Gene	Primer (nM)	cDNA (ng/reaction)	Efficiency	Tm (°C)
IL-1β	50	400	0.8	78.5
IL-6	100	400	0.9	81.4
TNF-α	50	100	0.9	86.2
iNOS	100	400	0.9	79.0
GAPDH	200	100	0.8	81.7
β-actin	50	100	0.9	86.5

Each sample was tested in duplicate on 96-well optical reaction plates, sealed with optical adhesive film, and amplified in a 7300 Real-Time PCR System (Applied Biosystems), with each target amplified separately. The reaction conditions were 95°C for 5 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Statistical analysis

Statistical analysis was carried out using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) for clinical data. The nominal data are reported as absolute and relative frequencies, and the numerical data are reported as means ± standard deviation (SD). The cycle threshold data obtained during real-time PCR were analyzed using the REST© 2009 program developed by M. Pfaffl (Munich Technical University) and Qiagen (available at http://www.gene-quantification.de/rest-2009.html) to compare the difference in expression between the treatments by means of bootstrapping and randomization techniques. A P value of < 0.05 was considered to be statistically significant.

RESULTS

Table 3 describes the socio-demographic characteristics of the study groups. The mean ages and SDs of the groups were PrD: 28.50 ± 4.66 years; PrND: 22.13 ± 3.98 years; N-PrD: 30.00 ± 5.71 years, and N-PrND: 26.13 ± 5.36 years. The corresponding figures for gestational age were PrD: 20.71 ± 3.19 weeks and PrND: 19.38 ± 4.13 weeks.

The means and SDs of the clinical periodontal parameters are shown in Table 4. The women in the PrND and N-PrND groups displayed no pockets and had no attachment loss. The women in the PrD and N-PrD groups presented moderate or severe disease according to the clinical parameters (López et al., 2002).

Table 3. Socio-demographic characteristics of the groups studied.

Socio-demographic variables	N-PrD group	N-PrND group	PrD group	PrND group
Age (years)				
18-29	4 (50.0)	5 (62.5)	5 (62.5)	7 (87.5)
≥30	4 (50.0)	3 (37.5)	3 (37.5)	1 (12.5)
Ethnicity				
Caucasian	2 (25.0)	3 (37.5)	3 (37.5)	2 (25.0)
Non-Caucasian	6 (75.0)	5 (62.5)	5 (62.5)	6 (75.0)
Occupation				
Housewife	4 (50.0)	4 (50.0)	5 (62.5)	4 (50.0)
Employee	4 (50.0)	4 (50.0)	3 (37.5)	4 (50.0)
Level of education (years)				
0-8	5 (62.5)	5 (62.5)	5 (62.5)	5 (62.5)
≥8	3 (37.5)	3 (37.5)	3 (37.5)	3 (37.5)
Economic status				
Regular economic situation	3 (37.5)	2 (25.0)	3 (37.5)	1 (12.5)
Poor economic situation	5 (62.5)	6 (75.0)	5 (62.5)	7 (87.5)

Data are reported as number with percent in parentheses. N-PrD = non-pregnant women with periodontal disease; N-PrND = non-pregnant women without periodontal disease; PrD = pregnant women with periodontal disease; PrND = pregnant women without periodontal disease.

Table 4. Clinical periodontal parameters (mean ± standard deviation) according to the groups studied.

Variable	N-PrD group	N-PrND group	PrD group	PrND group
Number of teeth	24.25 ± 3.15	25.38 ± 2.39	25.38 ± 2.88	27.75 ± 0.46
Teeth with BOP (%)	73.15 ± 26.78	58.62 ± 34.70	77.95 ± 21.79	37.86 ± 23.87
Teeth with PC (%)	26.72 ± 22.78	20.16 ± 17.14	44.03 ± 19.80	14.81 ± 18.27
Teeth with PD = 4-5 mm (%)	30.80 ± 13.67	NA	15.70 ± 7.25	NA
Teeth with PD ≥6 mm (%)	2.21 ± 3.26	NA	14.29 ± 20.00	NA
Teeth with CAL = 3-5 mm (%)	23.43 ± 11.90	NA	17.43 ± 8.26	NA

Data are reported as means ± SD. BOP = bleeding on probe; PC = presence of calculus; PD = probing depth; CAL = clinical attachment lost; NA = not applicable. For other abbreviations, see legend to Table 3.

Table 5 presents a comparison of gene expression levels in the four groups. The expression of *IL-1β* in the N-PrD group was 12.6 times that in the N-PrND group ($P < 0.01$), whereas the expression of *TNF-α* in the N-PrND group was 3.5 times that in the N-PrD group ($P < 0.05$). No significant difference in expression of *IL-6* and *iNOS* ($P > 0.05$) was found between the groups.

Table 5. Relative expression of the *IL-1β*, *IL-6*, *iNOS*, and *TNF-α* genes in pregnant women with and without periodontal disease in relation to non-pregnant women with and without periodontal disease.

	Gene	Relative expression	Standard error	Probability
N-PrD x N-PrND	*IL-1β***	12.597	1.092-155.822	0.009
	IL-6	0.503	0.036-5.677	0.482
	iNOS	1.213	0.147-3.698	0.919
	*TNF-α**	0.285	0.075-1.142	0.02
PrND x N-PrND	*IL-1β*	1.122	0.045-34.317	0.895
	IL-6	0.555	0.101-3.281	0.33
	iNOS	1.596	0.302-8.618	0.384
	TNF-α	0.466	0.161-1.353	0.061
N-PrD x PrD	*IL-1β*	1.451	0.254-8.422	0.547
	IL-6	1.471	0.097-33.73	0.706
	iNOS	1.593	0.071-16.644	0.714
	TNF-α	0.964	0.338-2.877	0.932
PrND x PrD	*IL-1β*	0.432	0.101-1.929	0.16
	IL-6	2.185	0.34-14.462	0.236
	iNOS	2.046	0.169-25.652	0.485
	TNF-α	1.575	0.595-3.769	0.217

For abbreviations, see legends to Table 3. Numbers greater than 1 = greater expression in first group; numbers less than 1 = lower expression in first group (*$P < 0.05$; **$P < 0.01$).

A tendency was found for the PrND group to present the same expression profile as the N-PrD group compared with the N-PrND group, with an increase in the expression of *IL-1β* and *iNOS* and a lower relative expression of *IL-6* and *TNF-α* but without statistical significance (see Table 5; $P > 0.05$). The expression profile of the genes also appeared to follow the same pattern in PrND and N-PrD groups in relation to the PrD group, except for *IL-1β* and *TNF-α*, although the difference was, again, not statistically significant ($P > 0.05$).

DISCUSSION

Periodontitis is an inflammatory disease associated with infection of the periodontal tissues that results in local increases in pro-inflammatory cytokine levels. Cytokines play a central role in the inflammatory process associated with the destruction of the periodontium. Many studies have found that immune response varies greatly among individuals (Teng, 2006; Reher et al., 2007).

The heterogeneous diagnostic criteria and definitions of periodontal disease in previous studies have led to discrepancies in their findings (Carrillo-de-Albornoz et al., 2012). A clear strength of the design of our present study is the application of all the inclusion and exclusion criteria from previous studies to the selection of subjects (Agueda et al., 2008; Vettore et al., 2008). In addition, the definition of periodontal disease used by López et al. (2002), which includes PD and CAL, was selected. These criteria were chosen to implement an unequivocal clinical definition of patients with periodontal disease.

The clinical examination was performed in all subjects by a single researcher to eliminate subjective variability among examiners and assure the reliability of the data. In addition, the socio-demographic data were similar among the groups, avoiding any influence of these variables on the periodontal condition.

To our knowledge, this study is the first to examine the expression of *IL-1β*, *IL-6*, *TNF-α*, and *iNOS* in gingival tissue in pregnant and non-pregnant women with and without periodontal disease. Although gingival inflammation in pregnant women is clinically and histologically well documented, its etiology has not yet been established and the predilection of only some pregnant women for acute gingival inflammation is unexplained. Changes in the maternal immune system and level of biochemical mediators, such as cytokines, during pregnancy have been suggested to contribute to greater susceptibility to gingival inflammation (Lopatin et al., 1980; Miyagi et al., 1993; Lapp et al., 1995).

Cytokines are small proteins released by various types of cells, normally in response to a triggering stimulus. They induce a response from the immune system by bonding to specific receptors. The cytokines secreted by macrophages in response to pathogens are a group of structurally diverse molecules that include IL-1β, IL-6, TNF-α, and the enzyme iNOS.

IL-1β is a cell immune response mediator released as a consequence of microbial components such as lipopolysaccharides interacting with Toll-like receptors. This cytokine increases the recruitment of neutrophils and the expression of adhesion molecules in addition to causing vascular alterations. When produced continuously or excessively, it can cause tissue destruction (Lö et al., 1999). This trait likely explains the more than 12-fold increase in the expression of this gene ($P < 0.01$) in the N-PrD group compared with that in the N-PrND group. This result corroborates the findings of an *in vitro* study by Ejeil et al. (2003), who have demonstrated that gingival tissue cultured for 72 h from sites with moderate to severe inflammation showed higher concentrations of IL-1β than tissue from sites without inflammation. In

contrast, Hirose et al. (2001) have found no significant differences in the expression of *IL-1β* in the gingival tissue of patients with periodontitis compared with that in control tissues.

According to Faizuddin et al. (2003), differences in *IL-1β* expression can be attributed to variation in plaque accumulation or subsequent inflammation due to plaque. Studies suggest that excessive or continuous production of cytokines such as IL-1β can be considered a marker for the clinical severity of periodontitis (Ejeil et al., 2003; Faizuddin et al., 2003). Although the experimental evidence suggests that IL-1β can be considered as such a marker, according to Tobón-Arroyave et al. (2008), this finding may indicate only inherent variations in the local production of IL-1β, because several factors affect the individual immune response (among them, alterations in the function of neutrophils) and the involvement of more pathogenic bacteria can indirectly modulate the increased periodontal degradation observed in periodontitis.

We found no statistically significant difference in *IL-1β* expression in the PrD and PrND groups, which agrees with findings reported by Figuero et al. (2010). One explanation for this result is that the hormones progesterone and estradiol, which remain at high levels during pregnancy, increase the production of prostaglandin E2 (Miyagi et al., 1993) and diminish the production of IL-1β (Morishita et al., 1999).

IL-6, another mediator evaluated, is synthesized by mononuclear phagocytes, vascular endothelium cells, fibroblasts, and other cells in response to microorganisms and other cytokines. The expression level of *IL-6* has been associated with the severity of periodontal disease (Moreira et al., 2007) and age (Mysliwska et al., 1998). In our study, no significant differences in the expression of *IL-6* occurred between the groups. Seeking to understand and relate our results more thoroughly by assessing the possible influence of age, we eliminated the youngest women from the N-PrND and PrND groups and the oldest ones from the N-PrD and PrD groups - the strategy implemented by Hirose et al. (2001) - leaving five women in each group. The mean age of each group changed to 29.2 years for N-PrND, 24.2 for PrND, 29.8 for N-PrD, and 25.4 for PrD. In the new analysis, only *IL-1β* expression was significantly different, with expression in the N-PrD group that was higher than that in the N-PrND group ($P < 0.01$). We observed no significant differences ($P > 0.05$) for the other genes, including *IL-6* (data not shown).

To test the effect of age further, we divided the eight women in the PrD group into two subgroups that included women younger or older than 27 years (mean ages of 24.8 and 32.3 years, respectively). The eight subjects in the N-PrD group were also divided into two subgroups: those younger and those older than 33 years (respective mean ages of 28.8 and 37.3 years). Hirose et al. (2001) have found no significant difference in the expression of *IL-6* between periodontitis patients older and younger than 40 years, but our data showed that *IL-6* expression was 34 times higher in the younger subgroup of non-pregnant women with periodontal disease than in the older subgroup with the same profile ($P < 0.001$; data not shown). The difference in expression between the two subgroups of pregnant women with the disease was not significant ($P > 0.05$), but there was also a tendency for the younger women to show greater *IL-6* expression.

Older patients have been shown to be more susceptible to microbial infections, and this lower resistance has been associated with the incapacity of macrophages to produce certain cytokines in adequate levels when challenged (Beharka et al., 2001). This lack of response could be an explanation for the lower levels of IL-6 in the tissues of individuals with periodontal disease, both among pregnant and non-pregnant women. It may also explain why the younger women in the N-PrD group in our study showed higher levels of *IL-6* expression than that in older subjects.

An *in vitro* study has shown that sex hormones have an inhibiting effect on the secre-

tion of IL-6, and high levels of progesterone during pregnancy affect the development of localized inflammation by reducing the production of IL-6 (Lapp et al., 1995). This relationship may explain the apparent reduction in the expression of *IL-6* in the PrD group compared to that in the PrND group ($P > 0.05$). Additionally, according to Carrillo-de-Albornoz et al. (2012), this pro-inflammatory mediator may not be associated with gingival inflammation.

TNF-α is a local inflammatory response inducer that helps to contain infections, but it also can trigger systemic effects, many of which are harmful. This protein is classified within a group of pro-inflammatory cytokines and has a pleiotropic effect that includes activation of inflammatory leukocytes, modification of vascular permeability, and induction of bone resorption (Assuma et al., 1998). Together with IL-1β, TNF-α is a main inducer of IL-6 (Katz et al., 2001). In the present study, the expression of *TNF-α* in the N-PrND group was higher than that in the N-PrD group ($P < 0.05$), which corroborates results obtained by Bickel et al. (2001), who have observed that the quantity of *TNF-α* mRNA was greater in the gingival tissue of healthy individuals than in gingival tissue of individuals with periodontitis. However, Hirose et al. (2001) have found no significant difference in the expression of this gene in the gingival tissue of patients with and without periodontitis.

Other studies have measured the level of TNF-α in the serum of patients with periodontitis to assess systemic rather than local immune response (Górska et al., 2003; Yamazaki et al., 2005; Chen et al., 2008; Andrukhov et al., 2011). These studies have also presented disparate results. Whereas Górska et al. (2003) and Andrukhov et al. (2011) have found TNF-α in the serum of patients with periodontitis to be significantly higher than that in healthy patients, other studies have found no significant difference between the levels of these groups (Yamazaki et al., 2005; Chen et al., 2008). Few studies have assessed local immune response, and the contradictions have probably been caused by differences in the methods used to detect cytokines and the heterogeneity of the individuals studied with respect to their susceptibility to periodontal disease.

Lappin et al. (2000), Kendall et al. (2000), and Hirose et al. (2001) have observed an increase in the expression of *iNOS* in the tissues of patients with periodontitis compared with that in tissues from healthy individuals. Reher et al. (2007) and Parwani et al. (2012) have conducted similar studies in which they reported a correlation in the levels of nitric oxide (NO) in the saliva of subjects with and without periodontitis. In both studies, the levels of NO in individuals with periodontitis were higher than the levels in those without the disease. Furthermore, higher NO levels were found in subjects presenting severe periodontitis, and a positive correlation between NO levels and probe depth was also observed. The present study found no significant differences in *iNOS* expression among the groups studied.

Rausch-Fan and Matejka (2001) have stated that the formation of dental plaque increases NO production through upregulation of the expression of *iNOS* in gingival cells, which might be induced not only directly by bacterial proliferation but also indirectly by the production of cytokines stimulated by plaque. Macrophages and neutrophils produce a variety of toxic products that help kill encapsulated microorganisms. Among these products is NO, produced by iNOS. NO in large concentrations at a determined site can be cytotoxic, acting against cells infected by fungi, bacteria, and protozoa as well as tumor cells and causing tissue destruction (Kendall et al., 2000). Despite these facts, we found no significant differences in *iNOS* expression, probably because advanced forms of periodontitis were absent among our subjects.

To respond to the conflicting results in the literature, new studies with larger samples are necessary to determine more fully the expression of cytokines in periodontal disease.

Methods of detecting cytokines can also be responsible for these differences. Using real-time PCR, the present study was designed to quantify mRNA levels of the cytokines in gingival tissue instead of in the blood or gingival crevicular fluid because most cytokines in these samples have a short half-life and are rapidly recruited by their circulating receptors. Therefore, the results may inaccurately reflect the concentration of cytokines. The real-time PCR technique was developed to analyze mRNA, enabling verification of gene activation or repression and thus an understanding of its activation route (Giulietti et al., 2001). This procedure assures that the cellular reaction is assessed during the study of the expression of immunological mediators. The technique also allows better insight into many immunological mechanisms and diseases in a fast and relatively automated way (Overbergh et al., 2003).

From the results of this study, we cannot state that the levels of protein present were altered by the immunological mediators studied because post-transcriptional regulation factors might have acted but were not assessed. Therefore, proteomic studies would be an interesting means of validating and better understanding these results.

We were able to verify that no significant difference occurred between the expression of *IL-1β*, *IL-6*, *TNF-α*, and *iNOS* in pregnant women with and without periodontal disease and that in non-pregnant women with and without the disease, suggesting that hormonal changes during pregnancy exert little or no influence on the expression of these genes associated with periodontal disease.

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