Inducible nitric oxide synthase (iNOS) in gingival tissues of chronic periodontitis with and without diabetes: Immunohistochemistry and RT-PCR study

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ABSTRACT

Background: There is few data concerning the pathogenesis and contribution of inducible nitric oxide synthase (iNOS) in the inflammatory reactions of the periodontium in the course of diabetes. This study evaluated the expression of iNOS in the gingival biopsies of periodontitis patients with and without type 2 diabetes.

Methods: 80 subjects were evaluated in four groups: patients with chronic periodontitis and diabetes, patients with chronic periodontitis, periodontally healthy patients with diabetes, and systemically and periodontally healthy control subjects. Gingival biopsies were subjected to immunohistochemistry as well as reverse transcription polymerase chain reaction (RT-PCR) for determination of iNOS.

Results: All diseased gingival tissues had a significant increase in iNOS expression by immunohistochemistry (P < 0.001) compared to controls. There was no significant difference observed between patients with both diabetes and periodontitis and diabetic patients regarding iNOS+ cells. Meanwhile, these two groups had significantly increased iNOS+ cells when compared to periodontitis patients (P < 0.001). There are significantly higher levels of iNOS mRNA expression of all patient groups compared to controls (P < 0.0001). In addition, samples from patients with diabetes and periodontitis showed significantly higher levels of iNOS mRNA expression compared to samples from periodontitis patients and diabetic patients (P < 0.0001) yet, without noting statistically significant differences between the latter two groups.

Conclusions: Although iNOS expression was prominent in the gingiva of patients with diabetes and periodontitis, periodontitis patients and diabetic patients, the higher mRNA for iNOS observed in diabetes and periodontitis may indicate a possible involvement of this mediator in the periodontal destruction of type 2 diabetes.

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1. Introduction

Diabetes mellitus (DM) is a metabolic disorder affecting over 9% of the adult population,1 characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin.2 Hyperglycemia induces non-enzymatic glycation and oxidation of proteins and lipids, resulting in the accumulation of advanced glycation end products (AGEs) in diabetic tissues.3 Oxidative stress is considered to be a common pathogenic factor in diabetes and its complications;4
it reflects increased production of free radicals and/or impaired antioxidant defenses.3,6 Previous studies showed that AGEs produced reactive oxygen species and increased oxidant stress in periodontal tissues when compared to non-diabetic individuals.3,7 The interaction of AGEs with endothelial cells increases oxidative stress, which has been associated with vascular injury.8,9 This enhanced oxidant stress is likely to be involved in the pathogenesis and progression of periodontal disease.4,10,11

Periodontal disease has been termed the sixth complication of diabetes.12 Diabetes does not result in gingivitis or periodontal defects, per se, but it alters the response of the periodontal tissues to local pathogenic factors.11 The relationship between periodontitis and diabetes is bidirectional13 insofar as the presence of one condition tends to promote the other. Wang et al.14 found in an epidemiologic study that the prevalence of periodontal disease was ~10% higher in subjects with diabetes than in those without diabetes. It was shown that individuals with poorly controlled DM had a significantly higher prevalence of severe periodontitis than those without diabetes.15 Periodontal diseases can induce or perpetuate an elevated systemic chronic inflammatory state, as reflected in increased serum C-reactive protein (CRP), interleukin (IL)-6 and -1β and tumour necrosis alpha (TNF-α).16 Elevation of proinflammatory cytokines caused by periodontitis may even predispose to the development of diabetes, and increased levels of inflammatory markers, such as CRP and IL-6, are reported to be significant risk indicators for DM.17

Nitric oxide (NO) is a short-lived bioactive free radical which serves as a messenger molecule for various physiological and pathological processes including strong oxidative activity that contributes to the killing of microorganisms.18,19 NO is synthesized from the amino acid l-arginine by a group of isoenzymes collectively termed nitric oxide synthases (NOS) which exists in 3 distinct isofoms; endothelial (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).20 The constitutive eNOS and nNOS maintain normal physiology and release small amounts of NO for a short period of time following receptor stimulation.21 In contrast, iNOS is induced by a variety of immunologic stimuli22 including bacterial lipopolysaccharides (LPS)23 and proinflammatory cytokines24 contributing to the killing of microorganisms.25 Activation of iNOS leads to the production of large amounts of NO from inflammatory cells and also gingival cells for sustained periods of time.18 As iNOS is expressed almost exclusively under inflammatory condition, this has led to the hypothesis that iNOS promotes the inflammatory response.26

The pathogenesis of chronic periodontitis consists of a cascade of inflammatory and immunological reactions, induced by gram-negative bacteria,27 which have not yet been fully elucidated.28 Bacterial LPS has the ability to trigger host cells, to produce a wide range of proinflammatory cytokines which have been implicated in periodontal disease pathogenesis.29,30 NO has recently received considerable attention as a novel type of mediator.18 NO in periodontal tissue may be part of the non-specific natural defense mechanisms of the oral cavity against pathogenic bacteria or, alternatively, excessive amounts of NO may contribute to local tissue destruction in periodontitis and may have an important role in the pathogenesis of inflammatory periodontal disease. NO synthesis and iNOS activity have been reported to be increased in inflamed periodontal tissues.31–35

Mechanisms by which increased oxidative stress is involved in the diabetic complications are partly known; including activation of transcription factors, AGEs, and protein kinase C. Hyperglycemia modulates the activity of NOS directly or indirectly (through protein kinase C).36 Activity of the NO system in diabetes is somewhat controversial, with studies providing for an increase,37 a decrease,38 or no change.39 Although the literature indicates that in the process of periodontal destruction NO play many important roles, yet there is few data concerning the pathogenic role of iNOS in the inflammatory reactions of the periodontium in the course of diabetes. Accordingly, this study was conducted to examine the localization of iNOS in biopsies from diabetic patients with periodontitis compared to biopsies from patients with diabetes alone, patients with periodontitis alone and healthy individuals, to evaluate whether NO plays a role in the pathogenesis of periodontal disease in type 2 diabetic patients.

2. Materials and methods

This study was conducted at the Oral medicine and Periodontology Department, Faculty of Oral and Dental Medicine, Cairo University, between June 2010 and November 2010. Written and informed consent was obtained after the completion of personal, medical and dental questionnaires.

2.1. Study population

A total of 80 subjects were recruited into the present study. Four study groups (n = 20) were created as follows: patients with DM and chronic periodontitis (CP) (DM + CP group; 9 males and 11 females; mean age 50.1 ± 6.3 years; range 36 to 62 years), otherwise healthy patients with CP (CP group; 9 males and 11 females; mean age, 45.2 ± 8.2 years; range, 35–59 years), periodontally healthy patients with type 2 DM (DM group; 9 males and 11 females; mean age 52.2 ± 6.9 years; range, 37–60 years), and systemically healthy and periodontally healthy control subjects (C group; 9 males and 11 females; mean age 30.4 ± 8.2 years; range, 25–58 years). All subjects were recruited from Oral Medicine and Periodontology Department Faculty of Oral and Dental Medicine, Cairo University.

All patients with diabetes were diagnosed as having DM ≥ 5 years prior to the study, using American Diabetes Association diagnostic criteria,40 all were under the supervision of an endocrinologist and were being treated with stable doses of oral hypoglycemic agents and/or insulin. Patients with diabetes were excluded if they had any known systemic diseases other than diabetes.

The periodontal diagnosis of subjects with chronic periodontitis was established based on clinical and radiographic criteria defined by the 1999 International World Workshop for a Classification of Periodontal Diseases and Conditions.41 Briefly patients with CP had ≥20 teeth with >30% of measured sites with clinical attachment loss >5 mm. They also exhibited alveolar bone loss >50% in at least 2 quadrants. The extent and severity of alveolar bone loss in each patient was estimated
using radiographs. Patients with CP were excluded if they had any known systemic disease that could influence the periodontal status, oral diseases other than CP, or ongoing orthodontic therapy.

Subjects in the control group were systemically and periodontally healthy and had no history or sign of periodontal disease, i.e. probing depths (PDs) <3 mm with no attachment loss, no obvious clinical inflammation and zero plaque and gingival indices.

Subjects were excluded if they had history of systemic or local disease with an influence on the immune system, a history of hepatitis or human immunodeficiency virus infection, immunosuppressive chemotherapy, pregnancy or lactation, requirement for antibiotic prophylaxis, antibiotic therapy within the preceding 3 months or periodontal treatment within 6 months, or <20 teeth. To be included in the study, individuals had to be >18 years of age. Smokers were excluded from this study.

2.2 Glycemic control

The fasting plasma glucose and the HbA1c test were used to monitor the overall glycemic control in patients known to have diabetes. The glycemic control was evaluated by the concentration of glycated HbA1c using high performance liquid chromatography.

2.3 Periodontal examination

Whole-mouth clinical periodontal measurements were recorded at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, disto-lingual, mid-lingual and mesio-lingual) and included plaque index (PI), gingival index (GI), PD and clinical attachment level (CAL). PI was established by measuring the presence or absence of supragingival biofilm with a sweeping movement of the probe around the buccal, mesial, distal, and lingual regions of all teeth. Marginal gingival bleeding was recorded with GI. PD was measured from the free-gingival margin to the base of the periodontal pocket and CAL was measured from the cemento-enamel junction of the tooth to the base of the periodontal pocket. Measurements were rounded to the highest whole millimetre using the Michigan 0 probe with Williams’ markings.

2.4 Gingival biopsy collection and analysis

Gingival biopsies of patients were taken under local anaesthesia with 2% xylocaine adrenaline. The gingival samples included part of the pocket epithelium, connective tissue, and granulation tissue. After washing the samples in sterile 0.15 M saline solution, they were fixed in 10% formalin solution and sent to the department of Histology Faculty of medicine, Cairo University. Analysis was performed on coded samples by one of the authors (S), who was masked with regard to the subjects’ diagnoses until all analysis were finished. After routine paraffin tissue procedures described above, tissues were embedded in paraffin blocks. Then, sections (4–5 μm thick) were obtained from the paraffin blocks. Paraffin sections were stained by haematoxylin and eosin and immunohistochemical staining for inducible nitric oxide synthase (iNOS). Other sections were then subjected to a RT-PCR quantitative analysis of mRNA for iNOS.

Inducible nitric oxide synthase (iNOS) primary antibody is a rabbit polyclonal antibody purchased from Thermo Fisher Scientific USA (cat# RB-9242-R7). Immunostaining with iNOS primary antibody required pretreatment by boiling in 10 mM citrate buffer (cat# AP 9003) pH 6 for antigen retrieval. This was done for 10 min and left to cool in room temperature for 20 min. The reaction is cytoplasmic. Immunostaining was completed by the use of ultravision detection system (cat# TP-015-HD) purchased from lab vision ThermoScientific. Counterstaining was done using Mayer’s haematoxylin (cat# TA-060-MH). Brown staining of the cytoplasm was considered positive for iNOS activity in inflammatory cells.

2.5 Morphometric study

The area percent of positive immunostaining for iNOS (iNOS expression) was measured in 10 non overlapping fields for every specimen at magnification X400 for all groups. Image analysis was done using Leica Qwin 500C image analyzer computer system (England). Number of cells showing positivity for iNOS (iNOS+ cells) was counted at magnification X400 in 10 non overlapping fields (field area 7381.11 μm) in every specimen for all subjects. Results obtained from image analyzer were subjected for statistical analysis.

2.6 RT-PCR for iNOS mRNA

The oligonucleotide PCR primers specific for iNOS were synthesized. The primers for iNOS (490 bp) were: 5’-ATG GAA CAT CCC AAA TAG GA-3’ and (antisense) 5’-GTC GTA GAG GAC CAC TTT GT-3’. The total RNA was extracted from tissues by RNA extraction kit (Qiagene, USA). All the reactions were subjected to different amplification cycles using a programmed thermal cycler under the following conditions: iNOS mRNA: 94 °C for 1 min, 43 °C for 1 min, and 72 °C for 1 min. The PCR fragments were electrophoresed on 1.5% agarose–ethidium bromide gels run at 100 V for 30 min.

2.7 Quantitation of the PCR product

RT-PCR was used to evaluate the expression of iNOS in gingival tissue collected from all studied groups. The PCR products were then quantitated by using a quantitation kit (Promega Corporation, Madison, WI, USA). This method depends on purification of the PCR using Promega Wizard PCR prep DNA purification kit (Promega Corporation, Madison, WI, USA). The mixture for quantitation consisted of DNA quantitation buffer, sodium pyrophosphate, NDPK enzyme solution, T4 DNA polymerase and DNA. All contents were incubated at 37 °C for 10 min. Then, 100 μL of Enliten L/L reagent was added. Immediately, the reaction was read using a luminometer. The same steps were done on DNAs of known concentrations provided by the kit, and a standard curve was performed by plotting the readings of the luminometer against the concentrations. Then, the readings of the amplified PCR products of iNOS after using the luminometer were read from the standard curve and were expressed as pg/gm tissue.
2.8  Statistical analysis

Data were presented as mean and standard deviation (SD) values. Student’s t-test was used to compare between PD and CAL in DM + CP and CP groups. One-way ANOVA test was used to compare between FBG and HbA1c in the four groups. Tukey’s test was used for pair-wise comparisons between the groups when ANOVA test was significant. Kruskal–Wallis test was used to compare between PI, GI, area% of iNOS expression, number of iNOS+ cells and PCR products of iNOS in the four groups. This test is the non-parametric alternative to one-way ANOVA test. Mann–Whitney U-test was used for pair wise comparisons between the groups when Kruskal–Wallis test was significant. The significance level was set at P < 0.05. Statistical analysis was performed with PASW Statistics 18.0 (Predictive Analytics Software) for Windows (SPSS: An IBM Company, Chicago, IL, USA).

3. Results

Patient characteristics and laboratory markers are shown in Table 1.

3.1  Clinical analysis

The mean values for the periodontal parameters are shown in Table 2. PD was statistically significantly higher in patients with DM and CP as well as in CP patients compared to patients with DM and controls (P < 0.001). There was no significant difference for PI, GI and PD between the patients with DM and controls. PI and CAL were significantly higher in patients with DM and CP compared to patients with CP alone (P < 0.001).

3.2  Histological results (haematoxylin and eosin and immunohistochemistry)

Control specimen showed normal architecture of gingiva formed of stratified squamous epithelium with underlying loose connective tissue (Fig. 1A). Sections examined from periodontitis patients revealed massive inflammatory cells within the underlying connective tissue (Fig. 1B). In sections from diabetic patients (Fig. 1C) as well as sections from patients with periodontitis and diabetes (Fig. 1D) the gingival connective tissue showed many dilated congested capillaries adjacent to inflammatory cellular infiltration (Fig. 1C). The immunohistochemically stained control sections lacked immunoreactivity for iNOS (Fig. 2A).

Sections from periodontitis patients revealed numerous inflammatory cells. Few of them expressed positive immunoreactivity for iNOS. Some appeared flattened and were present around blood vessels (Fig. 2B); others were large with pale nuclei (Fig. 2C). Examination of sections from diabetic patients revealed extensive infiltration by inflammatory cells showing cytoplasmic positivity for iNOS immunostaining, some of them were characterized by dark nuclei and irregular out line (Fig. 2D). Positive reaction was also noted in the cytoplasm of flattened cells with tapering ends (Fig. 2E). Sections from diabetic patients with periodontitis revealed almost the same; iNOS immunostaining could be detected in the cytoplasm of the inflammatory cells infiltrating the connective tissue (Fig. 2F).

3.3  Immunohistochemical analysis

The results of the immunohistochemical analysis are summarized in Table 3. The current study demonstrated that gingival tissues from patients in the three test groups had a significantly larger number of infiltrated iNOS+ cells (P < 0.001) and higher% of iNOS expression (P < 0.001) in their gingival tissues compared to controls. However, there were slight but not significant differences between patients with DM and CP, CP patients and patients with DM regarding iNOS expression. On the other hand, patients with DM and CP and DM patients had a significantly larger number of infiltrated iNOS+ cells compared to CP patients alone (P < 0.001). Whereas no statistically significant difference was shown between patients with DM and CP and diabetic patients regarding the number of infiltrated iNOS+ cells.

3.4  iNOS mRNA expression in gingival biopsies

RT-PCR was used to evaluate the expression of iNOS in gingival tissue collected from all patients participating in this study.

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**Table 1 – Laboratory markers of subjects.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Fasting plasma glucose (mg/dl; mean ± SD)</th>
<th>HbA1c (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM + CP</td>
<td>20</td>
<td>142.7 ± 53.66</td>
<td>7.7 ± 0.85</td>
</tr>
<tr>
<td>CP</td>
<td>20</td>
<td>88.8 ± 9.6</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>DM</td>
<td>20</td>
<td>139.6 ± 44.36</td>
<td>7.5 ± 0.66</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>87.6 ± 11.7</td>
<td>5.4 ± 0.5</td>
</tr>
</tbody>
</table>

* Statistically significantly higher than C group (P < 0.001).
† Statistically significantly higher than CP group (P < 0.001).
§ Statistically significantly higher than DM group (P < 0.001).

**Table 2 – Periodontal parameters (mean ± SD) in the study groups.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DM + CP group</th>
<th>CP group</th>
<th>DM group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>2.50 ± 0.51†§</td>
<td>1.35 ± 0.67†§</td>
<td>0.63 ± 0.50</td>
<td>0.47 ± 0.51</td>
</tr>
<tr>
<td>GI</td>
<td>2.0 ± 0.0</td>
<td>1.70 ± 0.47†§</td>
<td>0.58 ± 0.51</td>
<td>0.42 ± 0.51</td>
</tr>
<tr>
<td>PD (mm)</td>
<td>5.4 ± 1.7</td>
<td>4.5 ± 1.1</td>
<td>1.72 ± 0.19</td>
<td>1.67 ± 0.34</td>
</tr>
<tr>
<td>CAL (mm)</td>
<td>8 ± 1.2</td>
<td>4.3 ± 1.1</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Statistically significantly higher than C group (P < 0.001).
† Statistically significantly higher than CP group (P < 0.001).
§ Statistically significantly higher than DM group (P < 0.001).
The RT-PCR product was electrophoresed on agarose gel stained with ethidium bromide. The bands were photographed (Fig. 3). The results of the iNOS mRNA expression in gingival samples of the studied groups are summarized in Fig. 4. This study demonstrated that samples from patients with DM and CP, CP patients and DM patients had significantly higher ($P < 0.0001$) mean levels of iNOS mRNA expression than those from controls ($898.9 \pm 30.8, 540.8 \pm 36.3, 554.8 \pm 28.5$ and $316.6 \pm 8.7$ respectively). In addition, samples from patients with DM and CP showed significantly higher levels of iNOS mRNA expression compared to samples from CP patients or diabetic patients ($P < 0.0001$) yet, there was no statistically significant difference between the latter two groups.

4. Discussion

The strength of the evidence has led some to suggest that periodontitis should be listed among the “classic” complications of diabetes.\textsuperscript{45} Several studies investigated the relationship between diabetes and periodontitis clinically, yet, the outcomes were often controversial.\textsuperscript{15,46} Diagnostic parameters and methodologies are not universally defined which makes comparisons of the available evidence difficult. In subjects with diabetes, the onset and duration of the disease, the level of glycemic control, the type of treatment, and the presence of systemic complications vary.\textsuperscript{47} In this study,

### Table 3 – Data for number of iNOS\textsuperscript{*} cells and area\% of iNOS expression for each group.

<table>
<thead>
<tr>
<th></th>
<th>DM + CP group</th>
<th>CP group</th>
<th>DM group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of iNOS\textsuperscript{*} cells</td>
<td>$10.7 \pm 9.8$\textsuperscript{e}</td>
<td>$5.7 \pm 4.8$</td>
<td>$12.6 \pm 10.5$\textsuperscript{e}</td>
<td>$0.6 \pm 0.5$</td>
</tr>
<tr>
<td>Area% of iNOS expression</td>
<td>$13.5 \pm 8$\textsuperscript{e}</td>
<td>$10.4 \pm 5.4$</td>
<td>$14.7 \pm 5.3$\textsuperscript{e}</td>
<td>$0.6 \pm 0.4$</td>
</tr>
</tbody>
</table>

\textsuperscript{e} Statistically significantly higher than C group ($P < 0.001$).

\textsuperscript{e} Statistically significantly higher than CP group ($P < 0.001$).
patients with DM and CP as well as CP patients showed significantly higher levels of PI, GI and PD compared to patients with DM alone and controls. In agreement with earlier studies the investigation demonstrated that patients with DM and CP had significantly increased CAL compared to patients with CP alone. The findings presented herein were also supported by Goncalves et al. where patients with periodontitis and diabetes showed significantly higher PI compared to otherwise healthy patients with periodontitis. However, Kumar et al. and Pan et al. did

Fig. 2 – (A) Photomicrograph of a section from control subject gingiva showing no immunostaining for iNOS within the stromal cells underlying the gingival epithelium (E). (iNOS immunohistochemical staining with Mayer’s haematoxylin counter stain scale bar = 50 μm). (B) Photomicrographs of sections from gingiva of periodontitis patient showing: few inflammatory cells with positive immunostaining for iNOS (arrow), other cells appear flattened with tapering ends (arrow head). (iNOS immunohistochemical staining with Mayer’s haematoxylin counter stain scale bar = 50 μm). (C) Large cell with large pale nucleus with positive immunostaining for iNOS (arrow). (iNOS immunohistochemical staining with Mayer’s haematoxylin counter stain scale bar = 20 μm). (D) Photomicrographs of sections from gingiva of diabetic patient showing: infiltration of the gingival stroma by many inflammatory cells some of which show positive cytoplasmic immunostaining for iNOS (arrow). (iNOS immunohistochemical staining with Mayer’s haematoxylin counter stain scale bar = 50 μm). (E) Positive cytoplasmic immunostaining for iNOS within some of the inflammatory cells with dark nucleus and irregular outline (arrow head). Also positive immunoreactivity is noted in the cytoplasm of flattened cells with tapering ends (arrow). (iNOS immunohistochemical staining with Mayer’s haematoxylin counter stain scale bar = 20 μm). (F) Photomicrograph of a section from gingiva from diabetic patient with periodontitis showing inflammatory cells with positive cytoplasmic immunostaining for iNOS (arrow) (iNOS immunohistochemical staining with Mayer’s haematoxylin counter stain scale bar = 20 μm).
Fig. 3 – Agarose gel electrophoresis 2% stained with ethidium bromide showing the mRNA of iNOS gene. Lane 1: control case, lane 2: diabetic case, lane 3: periodontitis case and lanes 4 and 5: cases with both periodontitis and diabetes.

Fig. 4 – Mean levels of iNOS mRNA expression in the gingival biopsies of all studied groups.

not find any significant difference for CAL between patients with CP and patients with CP and DM.

NO is probably produced by iNOS and its actions are directed on maintaining the inflammation. NOS activity and NOS mRNA have been detected in human neutrophils. Yet, the detection of NO in tissues is difficult as it is relatively unstable in the presence of oxygen. Accordingly, iNOS activity was thought to be more useful and easier to detect assuming that large amounts of NO are produced by iNOS expressing cells in periodontal tissues leading to periodontal tissue destruction. Mercuriothiolglycine, which is a selective iNOS inhibitor, has been shown to inhibit the cytokine induced production of NO, suggesting that only the iNOS isoform is involved in inflammatory periodontal diseases, and not the other constitutive NOS isoforms; i.e. eNOS or nNOS. Based on the previously mentioned data, this study aimed at investigating the expression of iNOS in gingival biopsies of diabetic patients with and without chronic periodontitis.

In this investigation, patients with DM and CP, CP patients and DM patients showed larger number of infiltrated iNOS+ cells and % of iNOS expression compared to controls. This was further confirmed by the significant increase in mRNA for iNOS in the gingival tissues of these patients as demonstrated by the RT-PCR. These findings are in agreement with earlier reports showing that iNOS expression in gingival tissue obtained from CP patients was higher than in clinically healthy tissue samples. In addition, previous studies demonstrated that iNOS was strongly expressed in periodontal sites with CAL of ≥ 6 mm and that enhancement of NO production via the iNOS pathway in periodontal lesions resulted in the progress of periodontitis. The current study demonstrated the expression of iNOS in the gingival tissues of patients with DM and CP, CP patients as well as diabetic patients with no significant differences noted between them. The target cells for iNOS expression was found to be inflammatory cells probably macrophages characterized by dark nuclei and irregular out line as well as flattened cells with tapering ends likely to be fibroblasts. In ligature-induced periodontitis, iNOS was also shown to be expressed in inflammatory cells, fibroblasts, blood vessels and epithelial cells. Moreover, Lappin et al. demonstrated that iNOS expression level was dependent on the extent of inflammation in the periodontal tissue; which was supported by increased numbers of macrophages. According to the authors, it seems reasonable to assume that the increased levels of NO are produced by iNOS expressing cells during periodontal inflammation.

NO may be an important mediator of bone resorption as iNOS influences osteoclast and osteocyte functions in bone modelling. Previous studies reported that NO synthesis inhibition has been shown to reduce bone resorption in animals. Moreover, NO has been implicated in modulating expression of matrix metalloproteinases (MMPs), and it is also thought to down-regulate the synthesis of tissue inhibitors of MMPs. Consequently, Gülü et al. suggested that increased levels of NO production by macrophages and neutrophils via iNOS enzyme may lead to activation of MMPs and reduction of MMPs inhibitors causing periodontal tissue destruction. Hence, it has been suggested that NO may play a role in the pathogenesis of periodontal disease either directly or indirectly.

The immunohistochemical data presented herein showed that patients with DM and CP along with diabetic patients had a significantly larger number of infiltrated iNOS+ cells compared to patients with CP alone. The explanation of increased risk and severity of periodontitis in diabetes is primarily provided by a number of cellular and molecular alterations taking place in the periodontium as a consequence of sustained hyperglycemia. It has been proposed that periodontal tissues are primed by a hyperinflammatory state and exhibit an exaggerated response to periodontopathic bacteria. In diabetic patients, the interaction of macrophages with AGEs was shown to trigger increased cytokine production, e.g. TNF-α, IL-1β and -6, and prostaglandin E2 leading to greater periodontal tissue destruction. In an in vitro study, Daghiz et al. demonstrated that IL-1β, TNF-α and IFN-γ stimulated the production of iNOS in inflammatory cells and a combination of the three cytokines had a synergistic effect on induction of iNOS. According to Gülü et al., as these cytokines are known to be increased in periodontal diseases, the increased iNOS expression in inflammatory cells in the periodontal tissues of diabetic patients is not so surprising. On the contrary, Pan et al. showed that iNOS+ cells and iNOS expression were higher in gingival tissues of patients with diabetes and periodontitis compared to patients with only diabetes or periodontitis, without noticing any significant difference between the latter two groups. They also found no correlation between the expression of iNOS and inflammatory cells in patients with diabetes and periodontitis. Thus, they concluded that iNOS expression did not seem to have an additional detrimental effect on the course of periodontitis in
patients with diabetes compared to those with periodontitis alone.

On the other hand, this study demonstrated that iNOS was expressed on inflammatory cells in gingival samples from patients with CP and DM. Despite that the number of infiltrated iNOS+ cells was insignificant on comparing patients with DM and CP with diabetic patients yet, iNOS mRNA expression was found to be higher in patients with DM and CP compared to both CP patients and patients with diabetes. This increase in mRNA for iNOS might suggest a possible overproduction at the protein level; however, this cannot be decided by our immunohistochemical staining which might be partially attributable to the insensitivity of the staining technique in immunohistochemistry compared to RT-PCR. Thus, it could be speculated from the results presented in this work that iNOS might be involved in the pathogenesis of periodontitis, but the exact role of NO has yet to be clarified. In addition, the current study provides more evidence to the link between diabetes type 2 and periodontal disease. Further studies comparing and analyzing the expression of iNOS in normal healthy periodontium and gingiva of diabetic patients are warranted.

Funding

None.

Competing interests

None declared.

Ethical approval

The study was ethically approved by the committee of ethical approval from Cairo University.

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