Effect of Non-Surgical Periodontal Therapy On The Expression Pattern Of Vascular Endothelial Growth Factor (VEGF) And Inducible Nitric Oxide Synthase (iNOS) In Gingival Tissues Affected By Chronic Periodontitis

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Abstract: The aim of the study was to evaluate the effect of non-surgical periodontal therapy on the expression of both vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) in gingival tissues affected by chronic periodontitis. Gingival samples (2-3mm) were collected from thirty chronic periodontitis patients before and 4 weeks after non-surgical periodontal therapy, twenty periodontally healthy subjects were included in the study as a control group. All tissue samples were processed using immunohistochemical technique. The results demonstrated expression of iNOS and VEGF in healthy and diseased gingival tissues, there was a statistically significant difference regarding the expression of the two markers in chronic periodontitis before and after periodontal therapy (P<0.05). Also there was a significant difference in the iNOS and VEGF expression between chronic periodontitis patients and the healthy control subjects (P<0.05). There was a significant positive correlation between the two studied biomarkers regarding the immunohistochemical expression in the gingival tissues before and after non-surgical periodontal therapy (P<0.05). In conclusion VEGF and iNOS are continually produced and expressed in healthy and diseased gingival tissues; non-surgical periodontal therapy with antibiotics combination greatly affects the expression patterns of both biomarkers. Further investigations are required to determine the actual changes in the levels and volumes of iNOS and VEGF after periodontal therapy

1. Introduction

VEGF is a homodimeric pro-inflmmatory protein produced by many types of cells including endothelial cells, macrophages, activated T cells and epidermal keratinocytes (Brown et al., 1992 and Robinson & Stringer, 2001). It binds to specific receptors expressed in endothelial cells (Graziani et al., 2006).VEGF is a highly potent angiogenic factor that can regulate and increase microvasculature and vascular permeability (Duyndam et al., 2002 and Artese et al., 2002) it is over-expressed in various human tumors and inflammatory conditions such as periapical granuloma, pulpitis, periodontitis and radicular cysts (Yao et al., 2005, Leonardi et al., 2003 and Metwaly & ElDeeb, 2009).

Nitric oxide (NO) is produced by three different isoforms of NO synthase (NOS): the neural (nNOS) and the endothelial (eNOS) are constitutively expressed, whereas the iNOS can be expressed in response to various cytokines, such as interleukin-1, interferon-γ and tumor necrosis factor-α (Sappayatosok, 2012). Inducible nitric oxide synthase generates a continuous high level of NO in tissues which lasts for a longer period of time. In addition to physiological functions, NO is linked to many inflammatory and neurodegenerative diseases and

cancers (Brennan et al., 2000, Varghese et al., 2011 Chen et al., 2002and Brennan et al., 2000).

Although NO is essential for homeostasis and immunity, its production via the inducible isoform of iNOS has repeatedly been implicated in periodontal disease and its progression. Furthermore, inhibition of nitric oxide synthesis in animal models of both experimental gingivitis and periodontitis has been shown to be beneficial (Harriet and Arrington, 2007).

Lipopolysaccharide and other antigenic substances from putative periodontal pathogens such as A.actinomycetemcomitans, P. gingivalis, P. intermedia, P. nigrescens, and F. nucleatum have been shown to induce iNOS expression and NO production in murine macrophages (Kim et al., 2004,Jo et al., 2006, Sosroseno et al., 2004, Kim et al., 2005 and Kim et al., 2006).

VEGF production has been shown to be induced in gingival fibroblasts by the periodontal pathogens P. gingivalis and A. actinomycetemcomitans, and has been reported to be increased in gingival tissues of periodontal patients and diabetics. It has also been demonstrated that non-selective NOS inhibitors inhibit both VEGF-induced vascular permeability and vascular proliferation while the lack of iNOS activity alone does not (Suthin et al., 2003, Sakallioglu et al.,

2007, Guneri et al., 2004, Unlu et al., 2003, Giannobile et al., 2000 and Ziche et al., 1997).

To the best of our knowledge there are a limited reports regarding the evaluation of the expression of both VEGF and iNOS and their role in local angiogenesis in chronic periodontitis. Therefore, the aim of this study was to investigate the expression of VEGF, iNOS, CD34 in chronic periodontitis before and after non-surgical periodontal therapy and to evaluate their relation to microvessels density (MVD) and degree of inflammation.

2-Subjects and methods

Thirty patients with age range (25y -47y) were selected from the outpatient clinic, College Of Dentistry, Qassim university, The selection criteria was based on the classification of periodontal diseases and conditions by the international workshop for classification of periodontal diseases and conditions in 1999(Armitage,1999). All the selected patients suffered from periodontal diseases having 60% or more of their periodontal sites with moderate to severe chronic periodontitis with a minimum of 3 mm attachment loss. The diagnosis was confirmed by the clinical features and radiographic examination using periapical long cone parallel technique. No history of periodontal therapy for at least 6 months prior to this study, Smoker patients were also excluded from study

Twenty periodontally healthy male & female subjects of same range of age who were going to perform surgical removal for the wisdom tooth. Subjects were selected free from both systemic illness and periodontal diseases (no gingivitis, bleeding on probing at less than 20% of sites and 70% of teeth with probing depth < 3 mm).

All subjects were informed about the nature and objectives of the study and their full signed consent was obtained prior the study. The study complied with the rules set by the International Conference on Harmonisation of Good Clinical Practice Guidelines, and the Declaration of Helsinki, the protocol was reviewed and approved by the Ethical Committee of the Qassim University College of Dentistry.

For each patient the following parameters were recorded: Plaque Index (Pl-I) (Silness and Loe, 1964), Gingival index (GI) (Löe and Silness, 1965), Probing pocket depth (PD), measuring the clinical attachment loss (CLA) and radiographic examination (periapical radiographs using long cone parallel technique were taken for each patient to detect the degree of bone loss). The clinical periodontal parameters were determined for each patient and healthy control subjects at base line and 4 weeks after periodontal therapy for all patients.

Periodontal therapy

Every patient was subjected to oral hygiene instructions and professional tooth cleaning; they also were subjected to full mouth supra-and subgingival scaling, and root planning using ultrasonic scalers and periodontal curettes.   Debridement was completed on three sessions over one week period. The patients were instructed to use chlorhexidine mouth wash (Arab Drug company, Egypt) and antibiotic regimen in the form of a combination of 500 mg Amoxicillin (Arab Drug company, Egypt)and 250 mg metronidazole (Riyadh Pharma company, KSA) 3 times / day for 7 days.

All patients were instructed to follow a maintenance program for mechanical plaque control by using soft toothbrush (Formula system)(ORANG Tua PT ultra prima ,Jakarta ,Indonesia) three times daily with regular tooth paste (Unilever PLC, England) and the use of interdental tooth picks (Jordan as, Oslo, Norway) and interdental brushes (Oral-B) with regular recall visits every 10 days for 4 weeks interval.

Gingival tissue specimens

A small part (2-3mm) from the gingival margin or gingival papillae was cut by sterile blade to be used for analysis, this was performed for every patient before and 4 weeks after periodontal treatment, for the healthy control group part of the discarded tissues during surgical extraction of wisdom teeth was used for analysis.

The tissue specimens were fixed in 10% formalin, routinely processed and embedded in paraffin. Serial sections were cut at 5µm thickness, and one of each set of sections was stained with haematoxylin and eosin (HE). Another set of tissue sections was used for immunohistochemical staining for VEGF, iNOS, and CD34.

Antibodies

A rabbit polyclonal antibody for iNOS (Cat. # RB-9242-P) and mouse monoclonal antibodies to VEGF (clone; VG1, Cat. # MS-1467) (Thermo Fisher Scientific Inc, Fremont, CA, USA).

Mouse monoclonal antibody to CD34 (clone; QBEnd-10, Code # M7165) (Dako Cytomation, Copenhagen, Denmark). The primary antibodies used are summarized in Table 1.

Table 1. The primary antibodies for VEGF, iNOS, and CD34

Antibody against	Species	Immunogen	Clonality/ Clone	Dilution/ Cat #	Source
VEGF	Human	Recombinant VEGF protein	Monoclonal/ VG1	1:200 MS-1467	Thermo Fisher Scientific Inc, CA, USA
iNOS	Rat, Mouse, Human	A synthetic peptides	Polyclonal	1:100 RB-9242-P	Thermo Fisher Scientific Inc, Fremont, CA, USA
CD34	Human	Endothelial cell	Monoclonal/ QBEnd-10	1: 50 M7165	DakoCytomation,Copenhagen, Denmark

Immunohistochemistry

Immunohistochemical staining was performed using a peroxidase labeled streptavidin biotin complex. Five μm thick sections of paraffin-embedded tissues were deparaffinized in xylene and routinely processed through ascending hydrated alcohol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Prior to immunostaining for antigen retrieval, the sections were pretreated with microwave in 10 mM citrate buffer (pH 6.0) for 10-20 min followed by cooling at RT for 20 min and then incubated with 10 % normal goat serum for 30 minutes to block non-specific binding. Rabbit polyclonal antibody for iNOS (dilution 1:100), mouse monoclonal antibodies for VEGF (dilution1:200), and CD34 (dilution 1:50) diluted in phosphate-buffered saline (PBS) were applied directly to the slides and incubated at 4º C overnight.

The sections were treated with secondary antibodies and then detected by using streptavidin biotin conjugates (Vectastain ABC kit, Vector Laboratories, Burlingame, CA).The sections were incubated for 30 minutes at room temperature for both steps. Visualization of the reaction products was developed with 0.02% 3, 3'-diaminobenzidine in 0.05 M Tris-HCl (pH 7.4) containing 0.005% hydrogen peroxide (DAB substrate kit, Vector Lab).The sections were counterstained with hematoxylin. Positive controls of specimens of normal epithelium were used. For negative control studies, the primary antibodies were replaced with normal mouse or rabbit IgGs.

Microvessels Density (MVD) count

Microvessels were recognized by cytoplasmic immunostaining of the endothelial cells of blood vessels with anti- CD34 antibody. MVD assessed according to the method described by Tao X.et al (2007). The entire section was scanned at low magnification in order to identify the most highly vascularized areas of neo-vascularization "hotspots". After five hotspots areas with the highest number of capillaries and small venules were identified, microvessels were counted at high magnification power (×200) and the average count in five fields was calculated.

Analysis of VEGF and iNOS staining

VEGF and iNOS in all cases were recorded according to the staining intensity, the distribution in the cytoplasm, and the percentage of positive keratinocytes. The protein expression was analyzed using a semiquantitative validated scoring method (Hussein, 2007and Salem, 2011).

The mean percentage of positive keratinocytes was determined in five microscopic areas at a magnification of (×400), as the following groups: diffusely positive (3+) when positive cells > 50% of the total cells; moderately positive (2+), positive cells >20% but <50%; weakly positive (1+), positive cells >10% but < 20%; and negative (-), positive cells < 10%.

Statistical analysis

The results were tabulated and statistically analyzed using statistical package for social science (SPSS for Windows, release 15.0; SPSS, Inc., Chicago, IL). Fisher's exact test and Student's t-test were used to obtain statistically significant differences between two groups with $p < 0.05$ being considered statistically significant. Spearman rank correlation was also performed to analyze the correlation between.
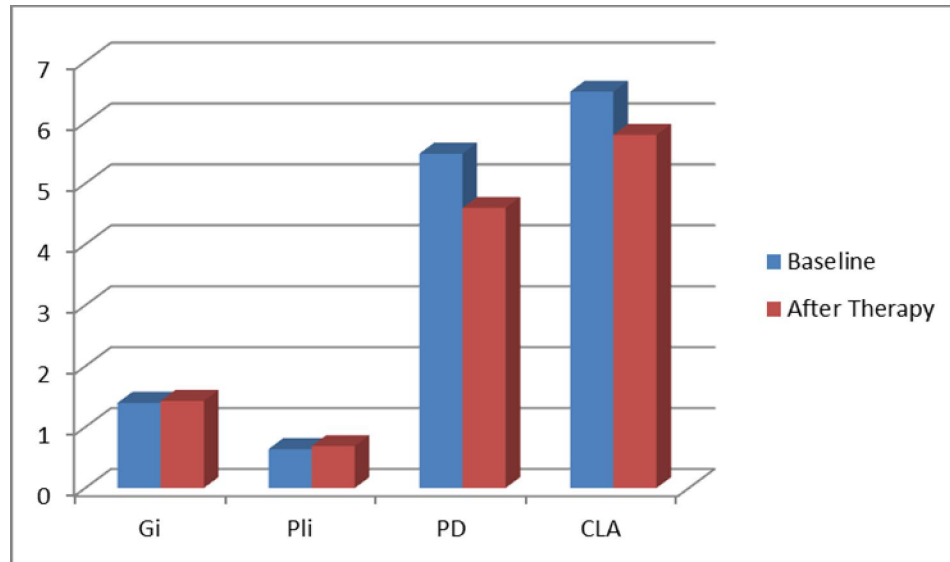
3-Results
Clinical periodontal parameters

Table (2) and figure (1) displays the changes in the mean scores values of clinical periodontal parameters at baseline and 4 weeks of periodontal therapy for chronic periodontitis group.

Table 2. Changes in the mean scores values of clinical parameters at baseline and 4 weeks of periodontal therapy for chronic periodontitis group

	Before therapy X± SD	After therapy X± SD	Mean difference X± SD	t Value	P Value
GI	1.42 ± 0.28	0.68 ± 0.17	0.79 ± 0.17	14.8	<0.001
Pl-I	1.46 ± 0.27	0.66 ± 0.18	0.72 ± 0.13	11.65	<0.001
PD	5.31 ± 0.59	4.30 ± 0.56	0.85± 0.34	4.5	<0.05
CLA	6.43 ± 1.45	5.23 ± 1.37	0.78± 0.24	5.76	<0.05

Figure 1. Changes in the mean scores values of clinical parameters at baseline and 4 weeks of periodontal therapy for chronic periodontitis group

Counting of (MVD), CD34 positive blood vessels in the study group and the healthy control group

The number of anti-CD34 positive micrvessels was counted in the lamina propria and submucosa in 5 fields of 'hot spot' of every gingival specimen of the chronic periodontitis patients and the control groups. The MVD was significantly higher in chronic periodontitis patients samples before periodontal therapy when compared with the control group (P<0.05). There was increase of MVD in the chronic periodontitis patient group at baseline (before non-surgical periodontal therapy) when compared with the MVD of the same patients one month after periodontal therapy (P<0.05).

VEGF expression in healthy control subjects and chronic periodontitis patients before and after periodontal therapy

The expression of VEGF was found as brown cytoplasmic staining in the keratinocytes, the expression was observed in all layers of epithelium including the basal cells but it varied from moderate to strong according to the degree of inflammatory cell infiltrates. The expression

of VEGF was interrupted in some areas with sever periodontal involvement. Positive expression was also observed in small number of stromal fibroblasts, inflammatory cells and endothelial cells of blood vessels (fig.2). The expression staining score for VEGF at baseline were 18 cases (60 %) diffusely positive (+3), 6 cases (20 %) moderately positive (+2), 4 cases (13.34 %) weakly positive (+1) and 2 cases (6.66 %) negative (table3, fig.3). After periodontal therapy the expression staining scores for VEGF were 4 cases (13.34 %) diffusely positive (+3), 6 cases (20 %) moderately positive (+2), 12 cases (40 %) weakly positive (+1) and 8 cases (26.66 %) negative (table3, fig.3).

The expression of iNOS was present as cytoplasmic staining in basal and parabasal cells. It was confined to the middle third of the epithelium. The expression in basal cells appeared as continuous band of strong staining. A large number of stromal fibroblasts, inflammatory cells and endothelial cells showed also positive iNOS expression (Fig. 3). The expression staining score for iNOS before

periodontal therapy were 17 cases (56.6 %) diffusely positive (+3), 6 cases (20 %) moderately positive (+2), 3 cases (10 %) weakly positive (+1) and 4 cases (13.6 %) negative. After periodontal therapy the expression staining scores of iNOS were 5 cases (16.66 %) diffusely positive (+3), 3 cases (10 %) moderately positive (+2), 4 cases (13.34%) weakly positive (+1) and 18 cases (60%) negative (table3, fig.4).
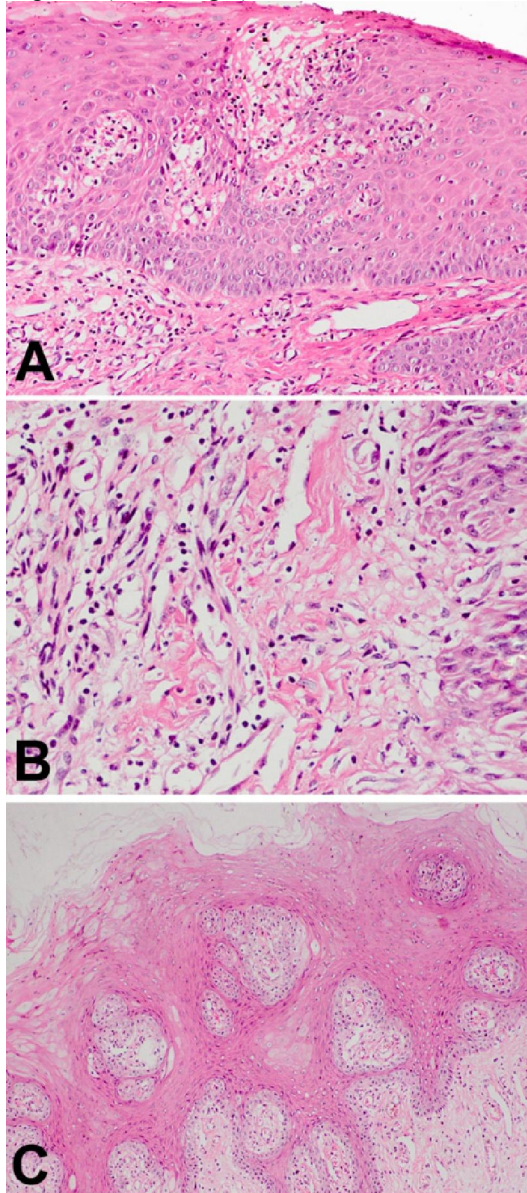

Figure 2. Hematoxylen and Eosin stained sections of gingiva; A. Normal gingiva, B. chronic periodontitis before therapy, C. chronic periodontitis after therapy. A x150, B x200; C x 100.
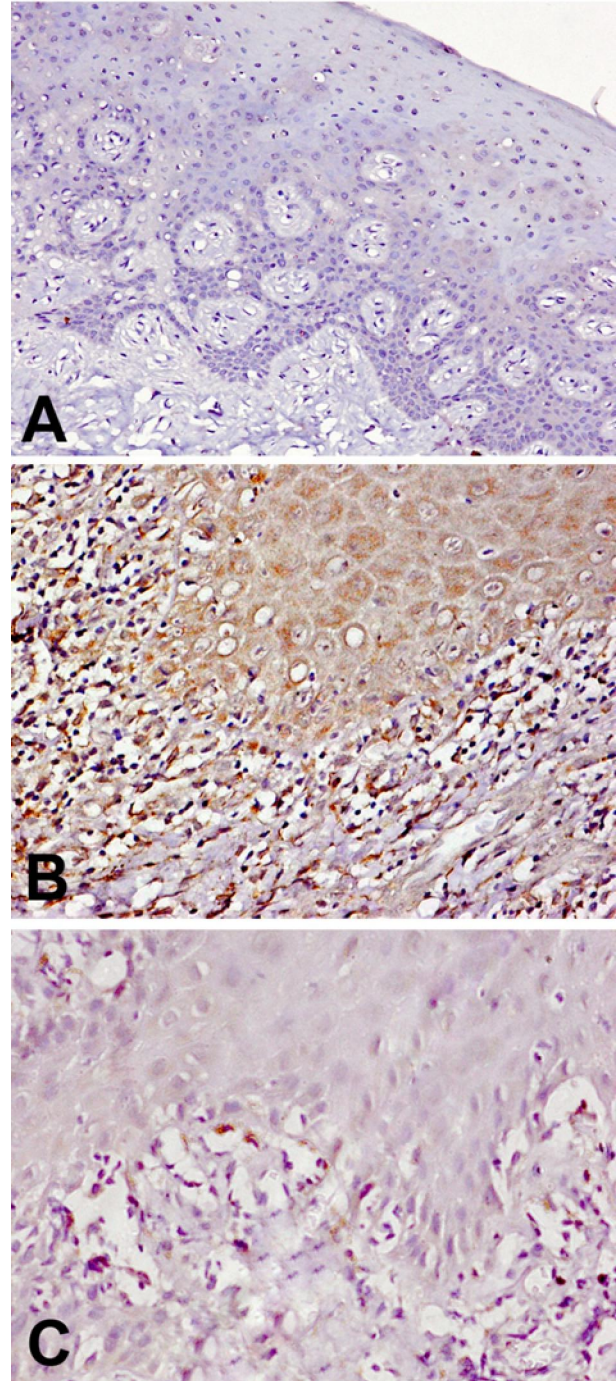

Figure 3. Immunohistochemical staining of VEGF in; A. Normal gingiva, B. chronic periodontitis before periodontal therapy, C. chronic periodontitis 4 weeks after periodontal therapy, Hematoxyline counter stain. A x 100, B, C x 400.
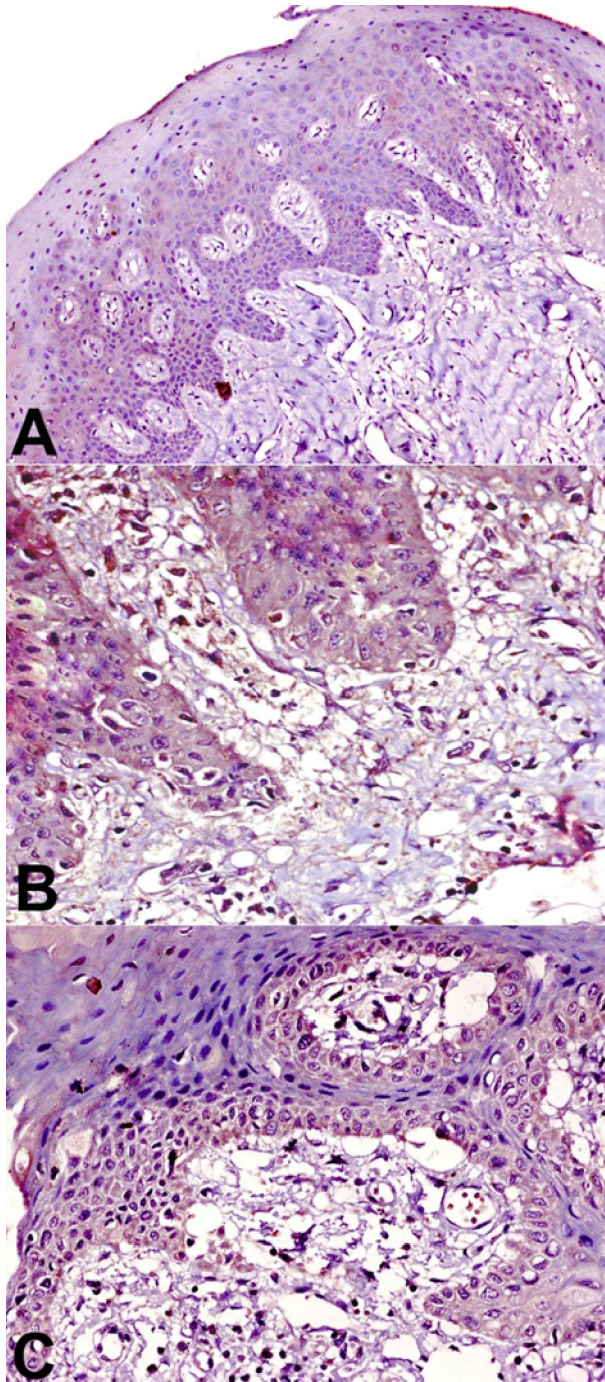
Figure 4. Immunohistochemical staining of iNOS in; A. Normal gingiva, B. chronic periodontitis before periodontal therapy, C. chronic periodontitis 4 weeks after periodontal therapy, Hematoxylen counter stain. A x 100, B x 400, C x 200.

Correlation between expressions of VEGF and iNOS in chronic periodontitis before and after periodontal therapy

Statistically significant positive correlation of VEGF and iNOS expression in chronic periodontitis patients before and after periodontal therapy was demonstrated using Spearman rank-order correlation analysis test (P <0.05) (Table 4).

Correlation between MVD and VEGF expression

The correlation analysis revealed that there were a positive significant correlation between MVD and VEGF expression in chronic periodontitis group before and after periodontal therapy.

4-Discussion

Periodontal disease, encompassing both gingivitis and periodontitis, is a host mediated inflammatory process initiated by oral bacterial insult which may result in significant alterations in the normal structure and/or function of the supporting tissues of the dentition. Although colonization of host tissues by pathogenic organisms is the initiating factor in this disease process, the associated rate of progression and degree of destruction are dependent upon both the virulence of the invading organisms and the magnitude/persistence of the host. Treatment of periodontal diseases has a great deal in common with the treatment of infectious diseases elsewhere in the body by controlling the putative pathogens. The goal of periodontal therapy was the elimination or reduction of periodontal pathogens from the oral cavity and the subgingival area response to this infection (Shiloah et al., 1998).

In the present study the treatment protocol consisted of scaling and root planing in conjunction with antibiotic combination of amoxicillin and metronidazole. The goal of root debridement was to remove microbiologically contaminated cementum and to eliminate or reduce the number of pathogenic bacteria in the periodontal pocket below their disease inducing levels. The rational for the use of systemic antibiotics was to rapidly suppress target microbial species and faster the establishment of a host compatible microflora. Metronidazole has a narrow spectrum and works specifically on anaerobic microorganisms associated with periodontal diseases. Amoxicillin appear very effective against most periodontal pathogens (Walker et al., 1993). Pavicic et al (1994) found that both antibiotics act synergistically on A. actinomycetemcomitans.

Table 3. Expression score of VEGF and iNOS in chronic periodontitis patients before and after periodontal therapy and control group.

| Score | VEGF n (%) | | Control n (%) | iNOS n (%) | | Control n (%) |
	baseline	After therapy		baseline	After therapy	
negative	2(6.66)	8 (26.66%)	17 (85)	4 (13.6)	18(60)	18 (90)
1+	4(13.34)	12 (40)	3(15)	3 (10)	4(13.34)	2 (10)
2+	6(20)	6 (20%)	0	6 (20)	3(10)	0
3+	18(60)	4 (13.34%)	0	17 (56.6)	5(16.66)	0

Table 4. Correlation between expressions of VEGF and iNOS in chronic periodontitis before and after periodontal therapy

| Staining score | Baseline | | | After periodontal therapy | | |
	VEGF expression	iNOS expression	*P* value	VEGF expression	iNOS expression	P Value
negative	2	4		8	18	0.024
1+	4	3	*0.032*	12	4	
2+	6	6		6	3	
3+	18	17		4	5	

P< 0.05

Previous studies have shown an increase in iNOS activity in chronic inflammatory diseases such as arthritis, inflammatory bowel disease, multiple sclerosis, and periodontal disease (Kendall et al., 2001, Singh et al., 2000, Suschek et al., 2004, Pannu & Singh et al., 2006 and Farrell et al., 1992).

The benefits of nitric oxide synthase inhibition in the modulation of the gingival inflammatory response and inhibition of alveolar bone loss in animal models have been repeatedly documented (Di Paola et al., 2004, Leitao et al., 1998 and Lohinai et al., 2005). These findings, however, are in marked contrast to those of the present study which demonstrated a difference in the expression scores of iNOS after periodontal therapy.

It has been demonstrated by several groups that the gingival epithelium expresses iNOS with most activity being localized to the basal keratinocytes and intraepithelial macrophages. Low levels of enzyme expression have even been observed in the tissues of periodontally healthy patients, leading to the presumption that iNOS may be constitutively expressed or continually induced in the gingival epithelium (Kendall et al., 2000 and Gaspirc et al., 2002). This explains the expression patterns of iNOS seen in the present study in the healthy gingiva and in chronic periodontitis patients after periodontal therapy.

Nitric Oxide acts as a feedback inhibitory signal for its own production. (Ralston and Grabowski et al., 2000) Since iNOS induction results in greatly elevated NO production for an extended duration, it would be reasonable to postulate that its induction could result in deactivation and subsequent down regulation of eNOS and nNOS (Di Paola et al., 2004).

This may actually serve to limit angiogenesis and vascular permeability in chronic inflammatory conditions since eNOS has been determined to be the isozyme predominantly responsible for the NO-mediated actions of VEGF, which is a potent factor for the induction of angiogenesis and vascular permeability. (Fukumura et al., 2001 and Lohinai et al., 1998) this explains the positive correlation in the expression of iNOS and VEGF in the gingival tissues before and after periodontal therapy.

VEGF production has been shown to be induced in gingival fibroblasts by the periodontal pathogens P. gingivalis and A. actinomycetemcomitans, and has been reported to be increased in gingival tissues of periodontal patients and diabetics. It has also been demonstrated that non-selective NOS inhibitors inhibit both VEGF-induced vascular permeability and vascular proliferation while the lack of iNOS activity alone does not (Suthin et al., 2003, Sakallioglu et al., 2007, Guneri et al., 2004, Unlu et al., 2003, Giannobile et al., 2000 and Ziche et al., 1997).This is another explanation for uniform expression of iNOS and VEGF at baseline and after periodontal therapy with the significant positive correlation between the two markers.

Conclusion

VEGF and iNOS are continually produced and expressed in healthy and diseased gingival tissues; non-surgical periodontal therapy with antibiotics combination greatly affects the expression patterns of both biomarkers.

Recommendation

Further investigations are required to determine the actual changes in the levels and volumes of iNOS and VEGF after periodontal therapy.

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